Fall 2016

Variation in Organismic RNA and DNA Content: Analysis and Application to the Assessment of Living Planktonic Biomass

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VARIATION IN ORGANISMIC RNA AND DNA CONTENT: ANALYSIS AND APPLICATION TO THE ASSESSMENT OF LIVING PLANKTONIC BIOMASS

A Thesis

Presented To

The Faculty of Moss Landing Marine Laboratories

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Elizabeth Lam Gagneron

December 2016

The Designated Thesis Committee Approves the Thesis Titled

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APPROVED FOR THE DEPARTMENT OF MARINE SCIENCE

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December 2016

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ABSTRACT

VARIATION IN ORGANISMIC RNA AND DNA CONTENT: ANALYSIS AND APPLICATION TO THE ASSESSMENT OF LIVING PLANKTONIC BIOMASS

by Elizabeth Lam Gagneron

Modern analysis of DNA and RNA nucleic acid sequences has yielded profound changes in our understanding of the genetic biodiversity of planktonic organisms within the microbial food web of aquatic ecosystems. However, the bulk environmental concentrations of DNA and RNA, and their relative ratios, also potentially provide important information on the biomass and metabolic activity of planktonic organisms. Currently, there is a need to quantify the relative living biomass levels of natural water contained in ships' ballast tanks to regulate the spread of aquatic invasive species (AIS) resulting from ballast water discharge practices within the international shipping industry. Ultraviolet (UV) irradiation serves as the most popular form of inactivation treatment through its damaging effects to DNA and thus the reproductive capabilities of aquatic organisms. In this study, the optimization of a fluorometric nucleic acid assay using a handheld fluorometer was investigated. This assay was optimized for use in the field and involved the determination of optimal buffers, extraction time and sample hold times. The RNA, DNA and their ratio measured by this technique were used to assess growth and growth potential in a variety of grow-out experiments. Results showed reductions in nucleic acid concentrations between control and UV-treated samples in both lab and shipboard conditions. This thesis describes the development of a simple method to measure nucleic acids in the field and quantify the effect of UV ballast water treatments.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Nick Welschmeyer, for guiding me through my research, providing full funding, and giving me an opportunity to work in the oceanographic field even before I became a graduate student. I would also like to thank Jason Smith for being so patient with my endless questions and Kenneth Coale for being an amazing teacher and mentor.

I would like to thank my lab mates and the entire Golden Bear Facility team: Julie Kuo, Brian Maurer, Rich Muller, Meredith McPherson, Bill Davidson, April Woods, Jasmine Ruvulcaba, Nilo Alvarado, Jarred Klosinski, and Stephan Bitterwolf. You have all helped me grow as a scientist and progress in my work or in this thesis in some way and I appreciate you taking the time to lend a hand or provide moral support. Our cruises were some of my favorite times in graduate school.

I would like to thank all of my friends and family, especially my parents. I am so thankful for your encouragement and understanding as I have navigated my way through graduate school.

Lastly, I dedicate this completed thesis project to my husband, Rudy. Thank you for always willingly giving your time, for driving all the way down to the lab with me for several weekends in a row and for your unending patience. I could not have done this without you.

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Introduction

Ballast Water and Invasive Species

Commercial shipping is a cornerstone of the worldwide economy. Unfortunately, this essential aspect of global industry has been identified as the primary vector in the spread of aquatic invasive species (Ruiz et al., 1997). The filling and emptying of ballast tanks on commercial ships is an essential operation required to ensure the stability and safe passage of these vessels across the ocean. An unintended consequence is the delivery of microscopic invaders from port to port, which has resulted in a worldwide ecological crisis of invaded marine habitats and ecosystems (Carlton & Geller, 1993). These invaders wreak environmental damage by outcompeting native inhabitants, and economic damage by necessitating costly cleanup, and sometimes even become a public health concern.

Over the past several years, various maritime regulatory bodies, including the International Maritime Organization and the U.S. Coast Guard, have implemented policies that attempt to manage this issue and mitigate the increasing rate of spread of aquatic invasive species (IMO G8, 2008; USCG, 2012). Commercial shipping vessels must now treat their ballast tanks in a way that meets very strict regulatory standards (Table 1). The implementation of these policies has given rise to a booming industry in ballast water management systems. Treatment strategies range from chemical, to heat shock, to ultraviolet (UV) light. UV is a particularly desirable method, due to its lack of chemical byproducts, relatively low energy requirements, and ease-of-use (Tsolaki & Diamadopoulos, 2010). Assessing efficacy of ballast treatments and degree of

compliance with the discharge regulatory standards requires extraordinarily sensitive assays for distinguishing viable from dead organisms particularly within the <50 μm fraction.

Table 1

Maximum allowable discharge concentrations for each size class, as set forth by the International Maritime Organization and United States Coast Guard (IMO G8, 2008; USCG, 2012)

	≥50 μm organisms (Zooplankton)	**10-50 μm organisms (Protists)**	**Indicator Microbes**		
			***V. cholera* (O1 & O139 serotype)**	***E. coli***	***Enterococcus***
Maximum concentration allowable for discharge	<10 live organisms/m^3	<10 live organisms/mL	<1 CFU/100 mL	<250 CFU/100 mL	<100 CFU/100 mL

Indicator microbes are measured in colony forming units (CFU) per 100 milliliters.

Current Methods to Measure "Viable" Biomass

A handful of regulatory compliance methods have been approved to certify the efficacy of ballast water management systems. Ballast water regulatory definitions separate organisms into three broad size classes: ≥50 μm (Zooplankton), 10-50 μm (Protists), and <10 μm, measured through the assay of indicator microbes. The greatest challenge to ballast policy is that each size class is subject to compliance thresholds that are based on numeric concentrations (Table 1). The ≥50 μm organisms are enumerated through the "poke-and-probe" method, wherein a concentrated ballast sample is examined under a stereomicroscope and organisms are prodded until their movement (or lack thereof) denotes their live/dead status. Indicator microbes are assayed using certified, prepackaged kits that estimate colony-forming units (thus, measuring growth reproduction) for *E. coli* and *Enterococci*.

The 10-50 μm size class has been a challenge to consistently and reliably measure. The diversity of this category, consisting mainly of phytoplankton but also some micro-zooplankton and heterotrophic protists, makes ballast compliance especially difficult to evaluate since a method that can indisputably provide a live numeric count does not presently exist (CSLC, 2014). Currently accepted methods include manual counts under an epifluorescence microscope or automated counts using a flow cytometer. Both methods rely on viable stains, the most common of which is fluorescein diacetate (FDA). FDA is a molecule that easily diffuses into and out of cells, and is susceptible to cleavage by esterases – enzymes that are common in all actively metabolizing organisms. When cleaved, the molecule is converted to fluorescein, which gives off a bright green fluorescence that can be easily detected by a fluorometer and even the naked eye. Additionally, both methods (microscopy and cytometry) require extensive training and expensive equipment that is often burdensome to carry aboard ships, especially under time-limited port inspections. In lieu of these techniques, a suite of bulk biomass assays have been explored for their potential to improve convenience and ease-of-use in determining relative viable biomass in ballast water. Chlorophyll *a* solvent extraction is the oceanographic standard for measuring bulk phytoplankton biomass, although it cannot be associated with viability per se. Pulse amplitude modulated (PAM) chlorophyll fluorometry is a whole-cell (non-extraction) bioptical method that specifically quantifies 'physiologically-active' chlorophyll indicative of viable photoautotrophs. Though known as some of the most common and reliable measurements, given sufficient biomass, both

of these chlorophyll-based techniques suffer from the fact that they can only detect photosynthetic biomass, thereby disregarding all heterotrophs.

One method that manages to overcome this issue is measurement of adenosine triphosphate (ATP). ATP measurements have been an oceanographic standard for decades, serving as one of the most common proxies for bulk biomass measurements (Holm-Hansen & Booth, 1966). ATP is the energy currency of all living organisms and is constantly made and destroyed in all living cells. This frequent turnover rate makes it an ideal viability indicator. ATP reacts with the enzyme luciferase to generate light, the intensity of which is proportional to the amount of ATP in a sample. This light can be measured with a luminometer and give a quantitative estimate of ATP. Recently, a study of alternative extraction solutions has found that the cationic surfactant, benzalkonium chloride (BAC), could be used as a much more efficient extractant of the ATP molecules from seawater samples (Kuo, 2015; Welschmeyer and Kuo, 2016). Data collection is currently underway to thoroughly investigate this method for viable planktonic biomass estimates. However, concerns regarding the efficacy of the assay have arisen; some studies have indicated increases in ATP signal immediately after UV irradiation and it is known that some dissolved compounds in environmental samples can interfere with the luminogenic reaction (First & Drake, 2014).

A second technique that attempts to effectively evaluate total viable biomass in the 10-50 μm-organism range is measurement of fluorescein diacetate leakage. As previously mentioned, FDA easily flows into and out of cells and will fluoresce green once cleaved by metabolic activity. Because it diffuses so freely, counting of cells

exhibiting fluorescence must be done within a short time frame; otherwise the fluorescent molecule will quickly "leak" out. The bulk FDA technique capitalizes on this leakage problem by correlating fluorescein production per hour with live cell concentration (Maurer, 2013; Welschmeyer & Maurer 2011).

Unfortunately ballast water treatment system developers, specifically those utilizing UV treatment, are at a particular disadvantage in compliance assessments at the hands of the existing methods, due to the nebulous definition of "viability" (Davey 2011). Currently, regulatory bodies are forced to define viability in terms of what their approved methods can detect. In each of the above cases, including the bulk biomass assays, this simply translates to metabolic activity. However, UV irradiation does not necessarily kill organisms outright but rather sterilizes them by targeting the cell replication machinery. The main target of UV irradiation is deoxyribonucleic acid (DNA), the genetic basis of all living organisms. DNA and its nucleic acid cousin, ribonucleic acid (RNA), both have the highest absorbance coefficients for short wave UV among all other cellular components (Vincent & Neale, 2000). UV's photochemical effects can induce changes in cell morphology, biochemical pathways, and especially genomic damage, which can alter several downstream processes. Most notable is the creation of pyrimidine dimers – lesions caused by the absorption of UV light and the subsequent breakage of the double bond in pyrimidine bases. If the broken bonds are adjacent to another pyrimidine base, two new bonds can form as a tight four-membered ring (Goodsell, 2001). This alteration in DNA disrupts downstream processes such as cell replication and transcription; pyrimidine dimers stall RNA polymerase, the enzyme that transcribes RNA from DNA

(Vincent & Neale, 2000) and a process required to activate any cellular recovery from UV damage.

Perhaps one of the only ways one can be sure of successful sterilization is to conduct full-blown grow-outs of treated ballast water. In the most probable number (MPN) method, samples are organized into matrices of increasing dilution. Positive growth is evaluated through natural chlorophyll fluorescence using the very sensitive capabilities of a fluorometer. By statistical analysis with an MPN calculator, a numerical count of live cells can be determined. Although this appears to be one of the most reliable and overtly clear methods to truly test the reproductive capacity of microscopic organisms, debate continues as to the validity of this test and, critically, the time frame of grow-out assays is too long to be amenable to regulatory compliance testing (Wright & Welschmeyer, 2015). Currently, governing bodies such as the U.S. Coast Guard do not recognize MPN as an approved evaluation method for ballast water treatment system testing. It is possible that the growth conditions provided in the MPN assay are not amenable for some species, presenting the possibility of a false negative result. In addition, the MPN method inherently requires a long wait period so that organisms have ample time to grow. Clearly, the ballast water treatment industry is suffering from a lack of effective and truly reliable methods to evaluate the success of these increasingly important treatment systems.

RNA and DNA as Viable Biomass Indicators

Since UV imparts damage molecularly, there are many potential benefits to applying molecular techniques to the assessment of UV effects. DNA quantitation is a

natural candidate for measuring the extent of UV effects since this is the primary target of damage. However, DNA is also a very robust molecule that can continue to persist even outside the cell and many complications arise when trying to quantify it.

In contrast, RNA is considerably more labile and could potentially prove an effective indicator biomolecule of physiological condition. It has been shown to degrade rapidly in *E. coli* cells starved under a variety of different scenarios (Kaplan, 1975a). RNA also has a natural turnover rate as it is constantly transcribed in viable cells but also destroyed by intracellular and extracellular ribonucleases (RNAses) (Kaplan, 1975b). RNA is transcribed directly from DNA and molecular lesions are known to inhibit this process (Tornaletti, 1999; Vincent & Neale, 2000). Since RNA acts as the necessary mediator between the genetic code and protein synthesis, is has been closely linked to metabolic activity.

Both nucleic acids have been utilized as biomass indicators in aquatic planktonic communities. DNA has typically been the nucleic acid of choice, with Holm-Hansen (1969a) being one of the first to measure with an adaptation of a fluorometric diaminobenzoic acid method. Subsequent analysis of DNA in individual algal cells found that DNA content holds a strong correlation to total organic carbon per cell (Holm-Hansen, 1969b). More recently, researchers have begun to utilize RNA for estimates of living biomass in planktonic samples due to its relation to whole cell metabolism as an intermediate that regulates protein synthesis and enzyme production. Indeed, RNA concentrations were found to have a similar vertical distribution in the water column as protein (Paul & Pichard, 1995).

Though DNA and RNA have been measured independently, many studies have analyzed the nucleic acids together as an RNA:DNA ratio to provide an informative measurement. Ecological studies have utilized the relative abundance of RNA and the RNA:DNA ratio as an indicator of physiological condition or growth rate in fish, copepods, and even some phytoplankton and marine bacterium (Chícharo & Chícharo, 2008; Dortch et al., 1983; Kerkhof et al., 1993). The logic in this relationship stems from the difference in nucleic acid content depending upon the environmental conditions. DNA content is correlated with cell size and its quantity per cell tends to remain relatively stable even during times of environmental stress. In contrast, RNA regulates protein synthesis and is therefore tightly coupled to cell growth and physiology, which responds to changes in the cell's environment (Paul & Pichard, 1995). Such attributes make the RNA:DNA ratio an intriguing metric upon which to evaluate ballast water treatment systems, especially those utilizing UV.

Molecular Methods for Viable Biomass Measurement

Both RNA and DNA have a strong natural UV absorbance at a wavelength of 260 nm. This property allows for quantitation by absorbance measurement on a spectrophotometer (Fleck & Munro, 1966). However, this method severely lacks sensitivity and little spectral discrimination between the two forms of nucleic acids and their composite nucleotides and is easily prone to contaminants (Jones et al., 1998). Traditional methods of more sensitively quantitating nucleic acids relied on ethidium bromide or propidium iodide (Dortch et al., 1983; Smith et al., 1992). However, these molecules are toxic and their red fluorescence can easily suffer from interference by

phytoplankton's natural chlorophyll fluorescence. Such attributes make these methods less-than-ideal for applications such as ballast water treatment system compliance assessment. Over the years, the molecular field has continuously improved upon alternative nucleic acid dyes, which typically bind to the grooves of the molecule's structure or by intercalation. These dyes, known as cyanine dyes, exhibit low intrinsic fluorescence, but yield high fluorescence enhancement upon binding to nucleic acid chains, and high affinity for their target nucleic acid (Spence & Johnson, 2010). Dyes such as SYBRGreen II, PicoGreen, and SytoxGreen have been utilized by phytoplankton researchers to more sensitively quantitate nucleic acids within these organisms (Berdalet et al., 2005b; Veldhuis et al., 1997). However, there remains the challenge of quantitating DNA and RNA individually and separately from each other such that there is no possibility of overlap or interference. Past methods have attempted to solve this by measuring total nucleic acid content and then conducting a secondary measurement after either DNase or RNase treatment to determine individual amounts by subtraction (Berdalet et al., 2005a).

Further advancements in molecular technologies now allow the quantitation of RNA and DNA independently yet from the same sample within a cuvet-based fluorometer. In particular the Qubit 3.0 Fluorometer produced by Life Technologies is a small, portable fluorometer designed with the specific intent of quantitating nucleic acids and proteins. A variety of assay kits manufactured in the Qubit line provide a range of quantitation possibilities relying on a set of fluorescent dyes. These proprietary dyes are purported to bind very specifically to their intended target and will fluoresce orders of

magnitude greater when it has done so. By comparing to a known set of standards, the fluorescence intensity is converted to concentrations of the target molecule through a calculated response factor. These assays are touted to have enormous advantages over the UV absorbance method and earlier dyes, with greater sensitivity, lower detection limits, and the ability to distinguish between RNA and DNA allowing for high specificity. These attributes, combined with its portability and simple mix-and-read format, make the Qubit assay kits an ideal candidate for ballast water treatment testing, especially in attempting to address the false-positive issue for UV systems.

The Qubit dsDNA HS (High Sensitivity) Assay Kit is designed to be highly selective for double stranded DNA over RNA. It is made to detect concentrations in extracts within the range of 10 pg/µL to 100 ng/µL. The assay calls for blue excitation at 502 nm with green emission optimally at 532 nm (Molecular Probes, 2015a). The corresponding RNA analog is the Qubit RNA HS (High Sensitivity) Assay Kit. It is also purported to be highly selective for its target molecule of RNA and is accurate for sample concentrations from 250 pg/µL to 100 ng/µL. Unfortunately, the excitation and emission wavelengths for the RNA HS assay fall into the red spectrum, at 644 nm excitation and 673 nm emission (Molecular Probes, 2015b). This overlaps entirely with the natural red fluorescence of chlorophyll that is common throughout the organisms of interest in this study and in the 10-50 µm size class. Indeed, preliminary attempts to measure RNA concentrations of algal cultures with this assay resulted in high levels of interference from chlorophyll fluorescence that completely masked any fluorescent signal from the RNA-specific dye.

In light of this complication, an alternative but similar assay protocol can be considered for RNA quantitation. The Quant-iT RiboGreen RNA Assay Kit is a precursor to the newer Qubit Assay kits. However, because it is not as selective for RNA as the Qubit HS RNA Assay, using the assay requires the continued use of DNase to remove any potentially interfering DNA molecules. Even so, the RiboGreen dye is designed specifically for RNA detection. In comparison with the Qubit RNA Assay, RiboGreen is said to be more sensitive and most importantly, excites at 500 nm with emission at 525 nm (Molecular Probes, 2008). This places the dye's fluorescence in a non-overlapping spectrum that the hand-held Qubit can still detect.

This thesis project was driven by the following goals:

1. Adapt the aforementioned pre-packaged, commercially available DNA and RNA quantitation assays for planktonic biomass estimates. This includes determination of compatible nucleic acid extractions and method development.
2. Validate the newly developed nucleic acid quantitation method using algal cultures and environmental samples against cell concentrations, cell size and established comparative biomass proxy assays, such as bulk FDA.
3. Investigate the applicability of nucleic acid quantitation for the purpose of measuring ballast water treatment efficacy, especially in comparison to currently used bulk biomass viability assays.

The ultimate goal is to determine whether a new, simpler method of nucleic acid quantitation can be accurately achieved and whether that protocol can provide improved insight on the effect of certain ballast water treatments.

Methods and Materials

Phytoplankton Culture Maintenance

A variety of phytoplankton species were utilized throughout this study. Algal cultures were obtained either from local marine science colleagues or commercially from Carolina Biological (Table 2). Media for the cultures was made by diluting commercially available Guillards F/2 (Sigma-Aldrich) marine enrichment solution 50-fold with ambient Monterey Bay seawater. The media was subsequently filtered through a 0.45 μm filter cartridge with a flow-through filtration unit and collected into a 2 L polycarbonate bottle. The bottle cap was tightened one-quarter turn and media was autoclaved for 20 minutes on liquid setting. After cooling to room temperature, fresh F/2 media was aseptically poured into clean glass culture flasks or tubes. To initiate new algal batch cultures, the newly prepared vessels were inoculated with small volumes of existing culture stocks. Cultures were placed on the north-facing windowsill of the Biological Oceanography lab at Moss Landing Marine Labs to grow in natural light conditions and ambient room temperature (18-22°C).

Table 2

Taxonomic listing of algal cultures used in Qubit DNA and RNA quantitation experiments

Phylum	**Class**	**Genus**	**Species**	**Source**
Chlorophyta	Chlorophyceae	Dunaliella	salina	Carolina Biological
Chlorophyta	Chlorophyceae	Haematococcus	droebakansis	Carolina Biological
Chlorophyta	Chlorophyceae	Scenedesmus	quadricauda	Carolina Biological
Chlorophyta	Chlorophyceae	Tetraselmis	sp.	Carolina Biological
Chlorophyta	Zygnemophyceae	Cosmarium	turpinii	Carolina Biological
Dinoflagellata	Dinophyceae	Akashiwo	sanguinea	Kudela Lab, UCSC
Dinoflagellata	Dinophyceae	Amphidinium	carteri	Carolina Biological
Dinoflagellata	Dinophyceae	Gymnodinium	sp.	Carolina Biological
Dinoflagellata	Dinophyceae	Prorocentrum	micans	Kudela Lab, UCSC
Haptophyta	Prymnesiophyceae	Isochrysis	galbana	Carolina Biological
Heterokontophyta	Coscinodiscophyceae	Thalassiosira	weissflogi	Kudela Lab, UCSC
Rhodophyta	Porphyridiophyceae	Porphyridium	sp.	Carolina Biological

UV Irradiation

Samples were subjected to UV irradiation under lab conditions for many experiments in this study. UV irradiation was delivered with a Trojan UV-C collimator to a sample in a glass petri dish or beaker. The sample was held under a black pipe directing semi-collimated UV-C light downward at an intensity of 224 $\mu W/cm^2$ for between 8 and 15 hours while being slowly stirred at ambient room temperature.

Environmental Sample Collection

Offshore Monterey Bay samples were collected for nucleic acid extraction during a routine CANON cruise conducted by the Monterey Bay Aquarium Research Institute aboard the research vessel (R/V) *Rachel Carson*. The cruise took place on October 9th, 2015. A CTD rosette was deployed at predetermined stations (Figure 1) and Niskin bottles were triggered to close and collect samples from a variety of depths between the

ocean surface down to a maximum depth of 1,000 meters. Samples from the Niskin bottles were then transferred into clean 2 L polycarbonate bottles.

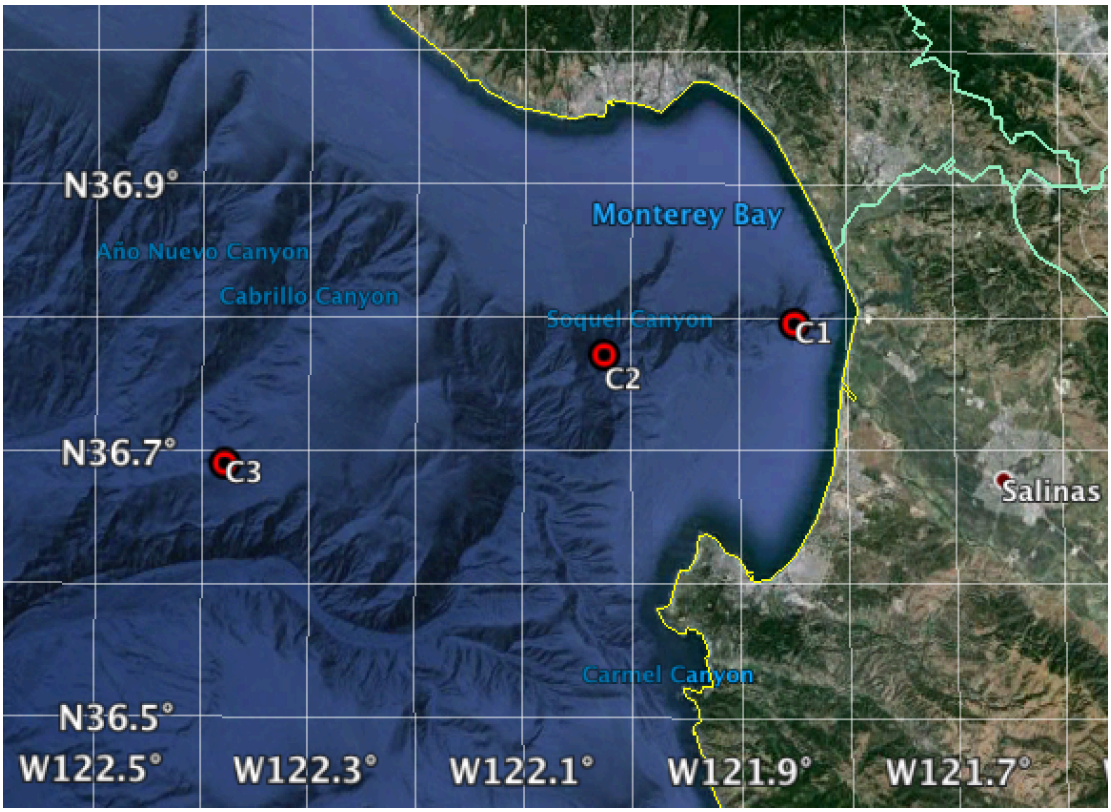


Figure 1. Station locations during the October 9th, 2015 cruise aboard the R/V *Rachel Carson.*

Ballast water samples were collected during ballast water management system (BWMS) Type Approval testing at the Golden Bear Facility onboard the training ship (T/S) *Golden Bear*. Over the course of this study, two different systems were tested: a UV treatment and a chlorine dioxide treatment. During an uptake event, water is pumped from either the ambient Carquinez Strait or from a barge that transported freshwater from the Sacramento River. The water was augmented with a large concentration of batch-grown natural phytoplankton taken from the local water and with cornstarch, test dust and lignin sulfonate (to meet minimum, regulatory "challenge" concentration of particulate

organic carbon (POC), total suspended solids (TSS), and dissolved organic carbon (DOC), respectively) before going through one of the two treatment systems and stored in a ballast tank for up to five days. If the water was UV treated, the sample was then UV treated once more before being discharged overboard. If the water was treated with chlorine dioxide, it was discharged without further treatment after chlorine dioxide levels fell below 0.02 mg/mL. During uptake and discharge events, ballast water was subsampled into clean 22 L or 8 L carboys to be later processed for nucleic acid quantitation, as well as for other corroborative assays.

All samples were concentrated onto 25 mm diameter nylon Millipore filters with a 10 µm pore size, to meet the requirement for the regulated size class of organisms >10 µm. Filters were immediately placed into a 2 mL polyethylene tube and flash frozen in liquid nitrogen. Samples were later stored long-term at -80°C.

Reagents and Nucleic Acid Extraction Procedures

Nucleic acid extractions are often paired with isolation and purification for downstream molecular applications such as PCR or genome sequencing. The reagents and extraction methods tested for this thesis are modifications of commonly used procedures adjusted for compatibility with assays that can be used with the Qubit 3.0 fluorometer for the measurement of bulk total DNA and RNA concentrations. Thus, ability for further purification was not considered and selection of extraction methods was based primarily on consistency of cell lysis and stabilization of nucleic acids.

Guanidinium thiocyanate lysis buffer. Nucleic acid extractions using guanidinium thiocyanate (GITC) lysis buffer were adapted from Harvey et al. (2013,

2014). Harvey's procedure is specifically designed for use with the sandwich hybridization assay (SHA). This assay is based on organism-specific RNA probes that can detect the relative abundance of a certain grouping of species. This extraction was tested with the hope of utilizing both bulk nucleic acid quantitation and SHA to characterize the same sample.

The actual formulation of the guanidinium thiocyanate reagent used in these bulk nucleic acid quantitation experiments was modified from Goffredi, et al. (2006) and is composed of the following:

3 M Guanidinium thiocyanate (Sigma-Aldrich)

50 mM Tris (Sigma)

15 mM EDTA (Sigma)

2% Sodium lauroyl sarcosinate (Sarkosyl) (Sigma-Aldrich)

The final concentration of Tris deviated slightly from 50 mM after the addition of small volumes of 1 M Trizma base (Sigma) to bring the reagent mixture to a final pH of 7.5. The guanidinium thiocyanate reagent was autoclaved in a polycarbonate bottle for 20 minutes on liquid setting and cooled to room temperature before use. Prior to extraction, samples were removed from liquid nitrogen or -80°C storage and 1 mL of GITC reagent was added. The sample was then vortexed for 10 seconds and incubated for 15 minutes at room temperature before being run through the nucleic acid assays.

Tris buffered saline + 0.1% Sarkosyl. Nucleic acid extractions using Tris buffered saline with the addition of 0.1% Sarkosyl (TBS-S) were modified from the protocol by Smith et al. (1992). Though a variety of concentrations for each component

was preliminarily tested, the final formula eventually used for the rest of this study was as follows:

50 mM NaCl (Mallinckrodt Pharmaceuticals)

10 mM Tris-HCl (Sigma)

0.1% Sarkosyl (Sigma-Aldrich)

The final concentration of Tris deviated slightly from 10 mM after the addition of small volumes of 1 M Trizma base (Sigma) to bring the reagent to a final pH of 7.5. The TBS-S was then autoclaved in a polycarbonate bottle for 20 minutes on liquid setting and cooled to room temperature before use. One milliliter of TBS-S was added to samples following their removal from liquid nitrogen or -80°C storage. The sample was then vortexed for 10 seconds and incubated between 15-60 minutes followed by immediate processing through the Qubit nucleic acid assays.

MoBio PowerSoil Kit. Only the extraction portion of the MoBio PowerSoil procedure was performed. Samples were removed from liquid nitrogen and filters were immediately transferred to the provided MoBio sample tubes, which included 750 µL of reagent and bead-beating matrix. The tubes were then placed on a bead-beating attachment to the Life Technologies vortexer and bead-beated for 10 minutes before being processed through the Qubit nucleic acid quantitation assays.

Qiagen DNeasy Animal and Tissue Kit. The Qiagen DNeasy Animal and Tissue Kit had been identified as an efficient method of genomic extraction from phytoplankton (Simonelli, 2009). The primary interest in this kit was the extraction efficiency of Qiagen's proprietary lysis buffer, Buffer ATL. To maintain consistency with other tested

reagents, 1 mL of buffer ATL was added to sample tubes immediately after removal from liquid nitrogen. Samples were then vortexed for 10 seconds and then processed with the Qubit nucleic acid assays approximately 30 minutes after.

Qubit Nucleic Acid Quantitation Assays

The foundation of this work is based on Life Technologies' Qubit 3.0 Fluorometer. Life Technologies developed a line of assays expressly designed to quantitate nucleic acids. The proprietary assay kits all generally use the same mix-and-read method based on a specially designed dye purported to have high sensitivity and specificity for its target nucleic acid. Potential contaminants are supposedly well tolerated by the assays.

The Qubit dsDNA HS Assay Kit was utilized to quantitate double stranded DNA concentrations. The kit includes Qubit dsDNA Reagent and Qubit dsDNA Buffer. A working solution is prepared by creating a 200-fold dilution of the reagent with the buffer. One hundred ninety microliters of working solution and 10 µL of sample-extract were added to a clear 0.5 mL PCR tube. After 2 minutes, the tube is placed in the Qubit fluorometer and the corresponding relative fluorescence units (RFU) were recorded.

The Qubit dsDNA HS Assay Kit is designed to calculate the DNA concentration within the Qubit fluorometer using a pre-programmed assay. The assay provides two standards that allows for an immediate quantitation of the DNA concentration of the sample, as well as within the PCR tube inserted into the Qubit. However, it is important to consider reagent chemistry within the PCR tube, which can have a great influence on the dye's effectiveness. Thus, a standard curve was generated for each extraction reagent

tested by serially diluting the provided 10,000 ng/mL DNA standard thereby producing a matrix-matched correlation between RFU and DNA concentration. In other words, DNA standards were diluted into 100% extraction solution at varying ratios and then run through the assay in order to properly account for the effect the extraction solution may have on the assay reagents.

The analogous RNA quantitation kit is the Qubit HS RNA Assay Kit. However, as mentioned previously, the emission spectrum of the dye designed for this kit falls at 673 nm. This overlaps with the natural emission spectra of chlorophyll *a*, found in all common phytoplankton species. Preliminary attempts to utilize this assay to quantify RNA concentrations from marine algal species proved futile when the chlorophyll *a* signal overpowered any that could be parsed from the Qubit HS RNA dye, measured as fluorescence of extract without any detection reagent added.

In light of this obstacle, an alternative Life Technologies Kit was utilized instead – the Quant-iT RiboGreen RNA Assay Kit (hereafter referred to as, "RiboGreen"). Though not specifically designed for the Qubit, the RiboGreen assay can be easily adapted for use in any fluorometer. Although it does not have as high specificity as the Qubit HS RNA Assay, it does have much greater sensitivity. Most importantly, the RiboGreen assay utilizes a green fluorescent dye with a maximum emission at 525 nm, conveniently falling outside of the realm of any chlorophyll fluorescence interference.

The RiboGreen kit provides concentrated Tris-EDTA (TE) buffer, RiboGreen reagent, and rRNA standard. Instructions for creating a standard curve are provided and a curve was generated prior to each set of samples processed. Because RiboGreen lacks the

high specificity for RNA in comparison with the Qubit line of kits, a DNase treatment was required prior to reading samples on the fluorometer. The exact assay procedure used for this study is as follows. RiboGreen working solution was made by making a 2,000-fold dilution of the provided RiboGreen reagent in 1X TE buffer. Ten microliters of sample was added to a 0.5 mL PCR tube. One microliter of 10X DNase I reaction buffer (containing $MgCl_2$) and 1 µL of DNase I were added and the sample was incubated at 37°C for 90 minutes. Eighty-eight microliters of 1X TE buffer and 100 µL of RiboGreen working solution were added to the PCR tube and the sample was incubated for 3 minutes at room temperature to allow development of fluorescent RNA-based end-products. The sample was then read on the Qubit fluorometer under blue excitation and green emission. Results were compared to an RNA standard curve, which was generated by creating several dilutions of RNA standard in 100% extraction solution and creating a matching sample matrix. Thus, an RNA concentration was computed from the RFU adjusted by the response factor of the linear calibration curve.

Cell Enumeration

Cell enumeration for assay validation and cell-size experiments was conducted using flow cytometry and epifluorescence microscopy. An Attune NxT Flow Cytometer was used to analyze phytoplankton cultures that were less than 50 µm in diameter. Samples were run on the instrument and analyzed with the BL3 fluorescence detector, representing the red fluorescence from chlorophyll-containing cells. Phytoplankton populations formed distinct groupings and the cell/mL concentration was calculated with

the Attune's provided gating software. Algal cultures of larger size were enumerated on an epifluorescence microscope with a Sedgewick Rafter counting chamber.

P-BAC ATP Assay

The phosphoric acid and benzalkonium chloride (P-BAC) ATP assay protocol optimized by Kuo (2015) was used in this study as a comparative method. The basic assay protocol is as follows. A sample is concentrated by gravity filtration onto a 10 μm nylon filter. The filter is immediately placed into 1 mL of P-BAC and incubated at room temperature for 30 to 60 minutes. The sample can either be processed immediately following this incubation or can be frozen for future processing. When a sample is ready to be evaluated, it is first diluted 100-fold with Tricine (pH 7.8) in order to reduce P-BAC's light quenching effects. The sample is then combined with the luciferase enzyme, thereby generating a light reaction that is proportional to the amount of ATP within the sample. By calculation of a standard curve, matrix-matched to account for the P-BAC quench effect and corresponding response factor, the relative luminescence value is converted to concentration of ATP within the sample.

Bulk FDA Assay

The Bulk FDA method, which was developed and optimized by Welschmeyer (2011), Maurer (2013), and Welschmeyer and Maurer (2015) was used in this study as a comparative method. The basic assay protocol is as follows. A sample is concentrated by gravity filtration onto a 10 μm nylon filter. Typically, 100 mL to 500 mL of seawater is sufficient to generate a strong enough signal. The filter is immediately placed in 1 mL of incubation buffer consisting of 500 mM sorbitol stabilized with 20 mM MES-KOH (pH

6.5), hereby referred to as Reagent A. The sample is then inoculated with 4 μL 2.5 mM FDA for a final concentration of 10 μM FDA. The sample is incubated in the dark at room temperature for one hour. During this time, FDA will enter any cells present in the sample. Cells containing metabolically active esterases will convert FDA to fluorescein, which is a compound that fluoresces green. Over time, fluorescein will diffuse out of the cells into the surrounding buffer. After one hour, 200 uL of the sample mixture is transferred to a PCR tube and read on the Qubit 3.0 fluorometer under blue excitation and green emission. The fluorescence is proportional to the amount of FDA produced by metabolically active cells in the sample. By calculation with a standard curve and the fluorometer's response factor, the amount of FDA produced per hour per cell can be determined.

Results

Method Development and Adaptation

Experiments were performed to investigate whether the Qubit 3.0 Fluorometer and its accompanying assays and dyes could be successfully adapted to measure DNA and RNA in planktonic organisms as viable biomass proxies. Several aspects of the methodology and assay procedure were tested before settling on a standardized protocol that would eventually be used throughout this study.

Extraction reagent. The first step in adapting both the DNA and RNA quantitation method for planktonic biomass was to determine the optimal extraction reagent. The ideal reagent would provide high compatibility and low interference with the assay, ease-of-use and high extraction yields for both nucleic acids simultaneously. This would allow a one-to-one comparison of RNA and DNA from the same sample, avoiding the need to conduct separate extractions. The tested buffers were evaluated on the following criteria:

1. Response factor and blank – Reagents can interfere with the nucleic acid dye and fluorometric response. The ideal lysis buffer would provide a high response factor and low reagent and buffer blank, indicating high compatibility with the dyes and the fluorometric assays.
2. Extraction efficiency – Ideally, the extraction buffer would fully lyse the cells and completely extract and protect the nucleic acids that are to be quantitated.
3. Cost

4. Ease-of-use – One of the goals of adapting this assay is to identify a method for determining ballast water compliance that can be easily executed by a regulatory technician.
5. Non-hazardous chemicals with low toxicity to humans for safe shipboard or port control use.

Two homemade extraction buffers, Tris-buffered saline + 0.1% Sarkosyl (TBS-S) and guanidinium thiocyanate (GITC), and two proprietary extraction solutions drawn from commercial kits, MoBio and Qiagen, were evaluated. In order to assess the first criterion, it was necessary to generate a standard reagent matrix identical to that of the samples. A calculated mixture of extraction reagent, standard, and assay working solution was combined to generate standard curves for each mixture (Figures 2 and 3). Using these standard curves, an average response factor (RF) was calculated for each extraction reagent. Blanks were measured by combining only the extraction reagent with the assay working solution without any standard. The relative fluorescence units (RFUs) for the blanks and the average response factors for each extraction reagent tested are displayed in Table 3.

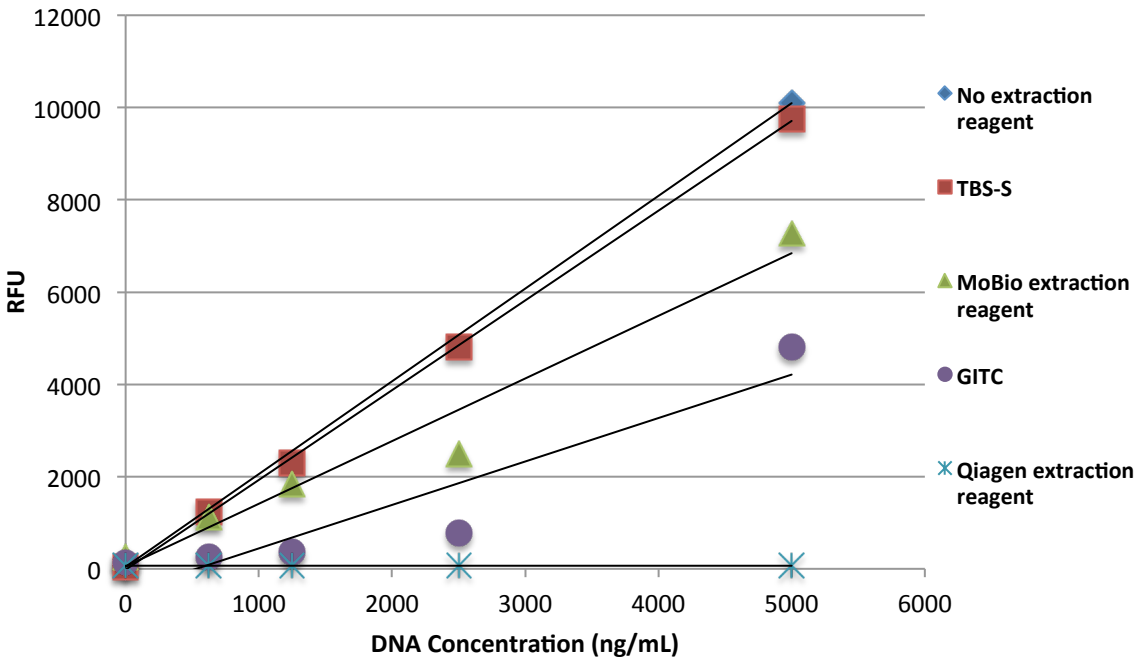


Figure 2. Comparison of four different extraction reagents and their resulting standard curves using the Qubit dsDNA HS assay.

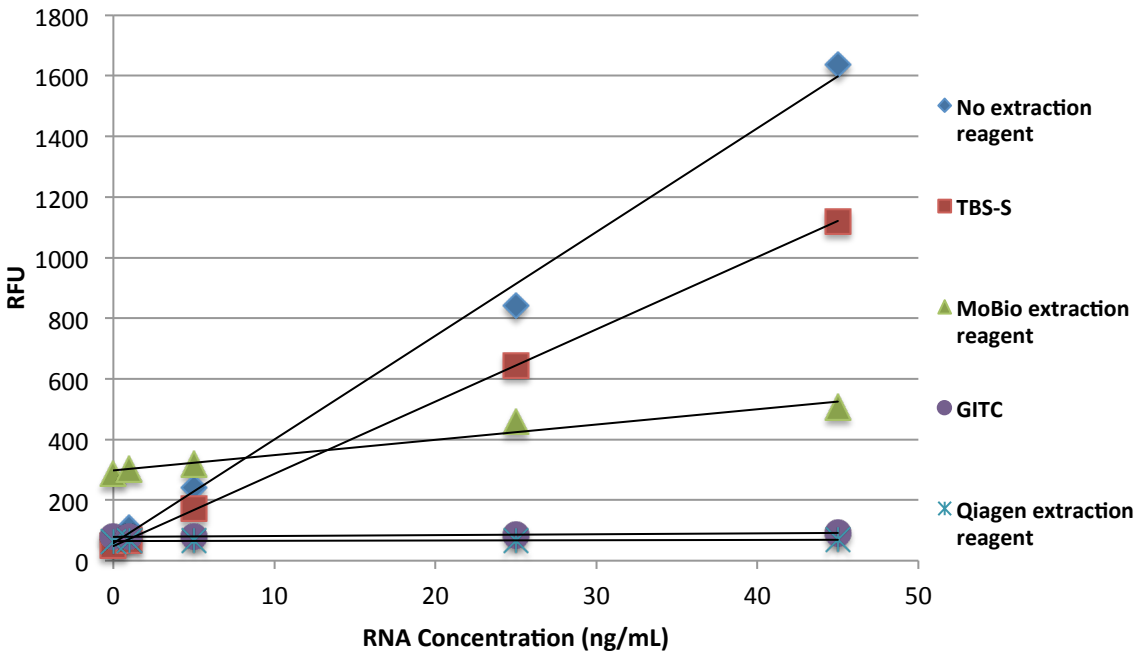


Figure 3. Comparison of four different extraction reagents and their resulting standard curves using the RiboGreen assay.

Table 3

Blanks and average response factors of four extraction reagents tested with the Qubit ds DNA HS assay and the RiboGreen assay

	Qubit dsDNA HS		RiboGreen	
Extraction Reagent	**Blank RFU**	**Avg. Response Factor (RFU/ng DNA•mL^{-1})**	**Blank RFU**	**Avg. Response Factor (RFU/ng RNA•mL^{-1})**
TBS-S	48.95	1.81	53.80	22.20
MoBio	251.70	1.34	242.45	5.47
Qiagen	66.53	0.00	64.17	0.53
GITC	134.65	0.39	78.35	0.59

For both DNA and RNA, the Qiagen Animal and Tissue extraction reagent exhibited poor compatibility with the quantitation assays, having a response factor of nearly zero for DNA and only 0.53 RFU/ng•mL^{-1} for RNA. GITC also exhibited very low response factors for both assays and is relatively toxic at the high concentrations in this formulation, making it less ideal for use in field practice. The MoBio extraction showed a relatively strong response factor for the DNA assay kit but exhibited very high blanks and a less impressive response factor for the RiboGreen assay. Overall, the TBS-S extraction reagent provided the highest response factor along with a very low blank value for both the DNA and RNA quantitation assays.

Since the Qiagen reagent and GITC had very low response factors for both assays, only MoBio and TBS-S were compared for their extraction efficiency. Identical samples from three algal cultures were extracted using each method. Results were normalized to the TBS-S extracted sample and are summarized in Figure 4. The results show that although RNA yields were about 10% greater with the MoBio extraction reagent, DNA yields were 10% lower.

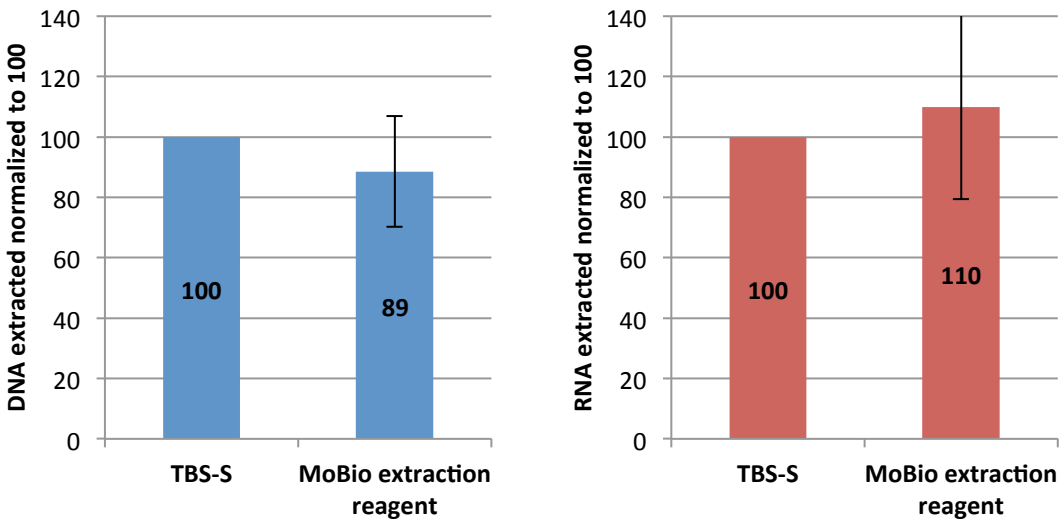


Figure 4. DNA and RNA extraction comparison between TBS-S and MoBio. Error bars represent the standard deviation between multiple samples.

TBS-S was ultimately chosen as the extraction buffer that would be used in both nucleic acid quantitation assays for the rest of this study. It yielded the greatest extraction of DNA and remained within 10% of the optimum for RNA. It by far had the best compatibility with the dyes as evaluated by its low blank and high response factor. Compared to its closest competitor, MoBio, its blank was 5X lower on average and its average response factor was 4X greater. Additionally, the MoBio extraction procedure requires an extra bead-beating step thereby complicating the method. On the other hand, TBS-S extraction only involves the addition of the buffer to the sample and a short vortex, making it much easier to use. These essential characteristics outweighed the difference in error between the two reagents. TBS-S is also ideal due to its low cost and low toxicity.

Long-term liquid nitrogen storage. The use of liquid nitrogen flash freezing and long-term cold storage (-80°C) was evaluated to determine its effects on yields. Two sets

of samples, one originating from Moss Landing Harbor and one from a *Tetraselmis sp.* culture, were flash frozen in liquid nitrogen and then transferred after one day to a -80°C freezer. DNA and RNA measurements from the two sets of samples have been normalized to 100 (Figures 5 and 6). Day 0 samples were extracted and measured for nucleic acids without freezing in liquid nitrogen. It was found that flash freezing not only provides a useful storage for later processing of samples, but it also appears to aid in cell lysis during the freeze-thaw process, thereby increasing yields. The optimum DNA yield was a 1.3-fold increase compared to samples that had no liquid nitrogen storage. Optimum yields for DNA were achieved after one day of flash freezing in liquid nitrogen and extractions remained within 10% of the optimum after seven days. DNA yields continued to remain within 25% of the optimum after thirty-five days of frozen storage. The optimum RNA yield was also a 1.3-fold increase compared to samples that were not frozen in liquid nitrogen. RNA was maximally extracted after five days but yields within 15% of the optimum could be achieved anywhere between three and thirty-five days.

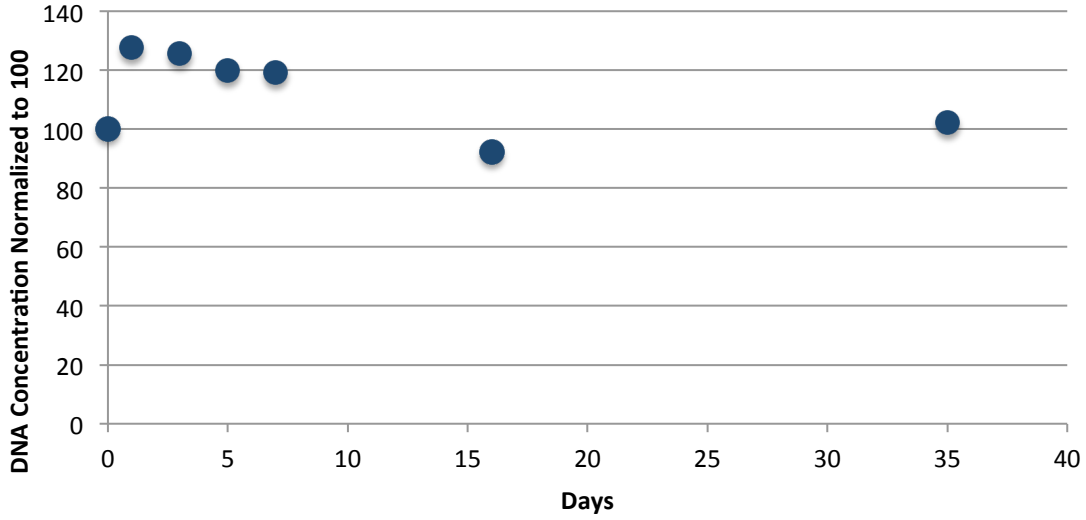


Figure 5. DNA yields over time after flash freezing in liquid nitrogen and long-term cold storage at -80°C.

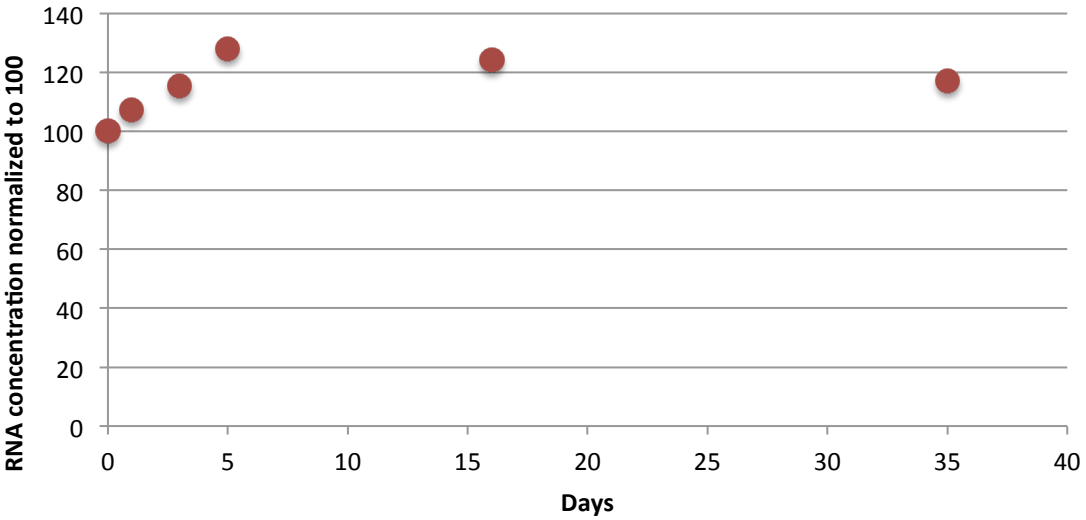


Figure 6. RNA yields over time after flash freezing in liquid nitrogen and long-term cold storage at -80°C.

Extraction time. The ideal sample extraction time was determined by measuring samples over the course of a ninety-minute period. Samples were taken at 5, 10, 20, 40, 60, and 90 minutes using 5 independent extractions of a *Tetraselmis sp.* culture. For DNA, extractions terminated at 10 and 20 minutes yielded results within 20% of the

optimum. Forty-minute extractions proved ideal for DNA. Extraction times beyond 40 minutes would continue to give results within 10% of the optimum. In contrast, RNA extraction times were faster, with the greatest yields occurring within 10 minutes. Extraction times beyond 10 minutes continued to be within 20% of the optimum.

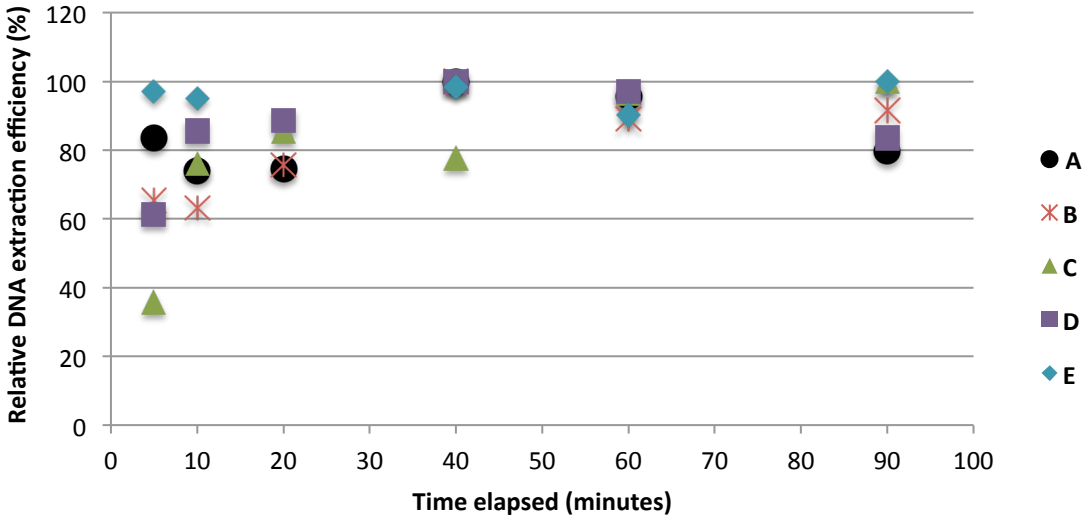


Figure 7. DNA extraction efficiency in TBS-S from five independent samples after various extraction lengths.

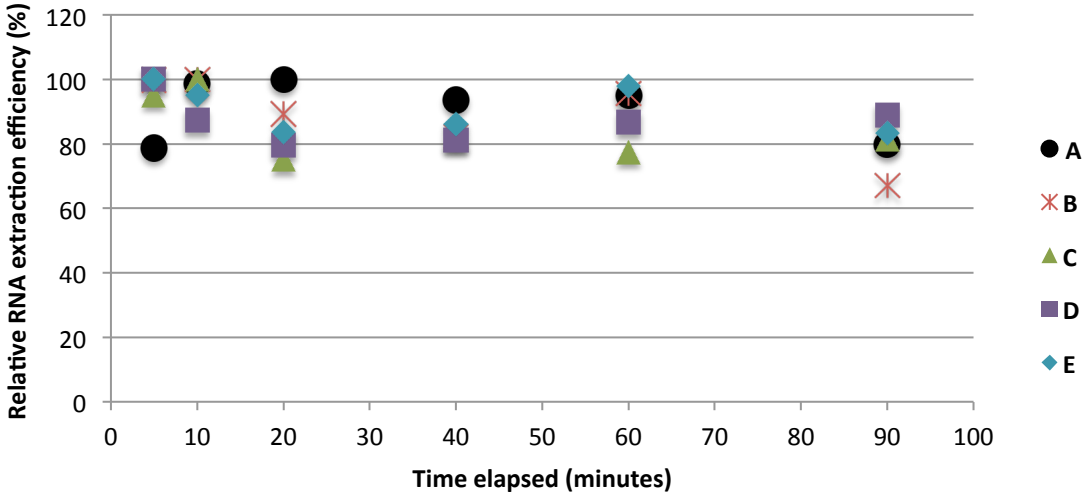


Figure 8. RNA extraction efficiency in TBS-S from five independent samples after various extraction lengths.

Assay Validation

Both the Qubit dsDNA HS and RiboGreen assay methodologies rely on a known volume of sample to be harvested on a filter, extracted by the chosen reagent and subsequently run through the assay. In order for the assay to provide reliable information, the assay response should be proportional to the biomass loaded, indicating equivalent lysis efficiency across that biomass range. In this first, simple experiment, increasing volumes of the same *Tetraselmis sp.* culture were harvested onto GF/F filters and run through the two assays. The results in Figure 9 clearly show the expected linear relationship, where a subsequent greater volume filtered results in a proportional increase in fluorescent signal.

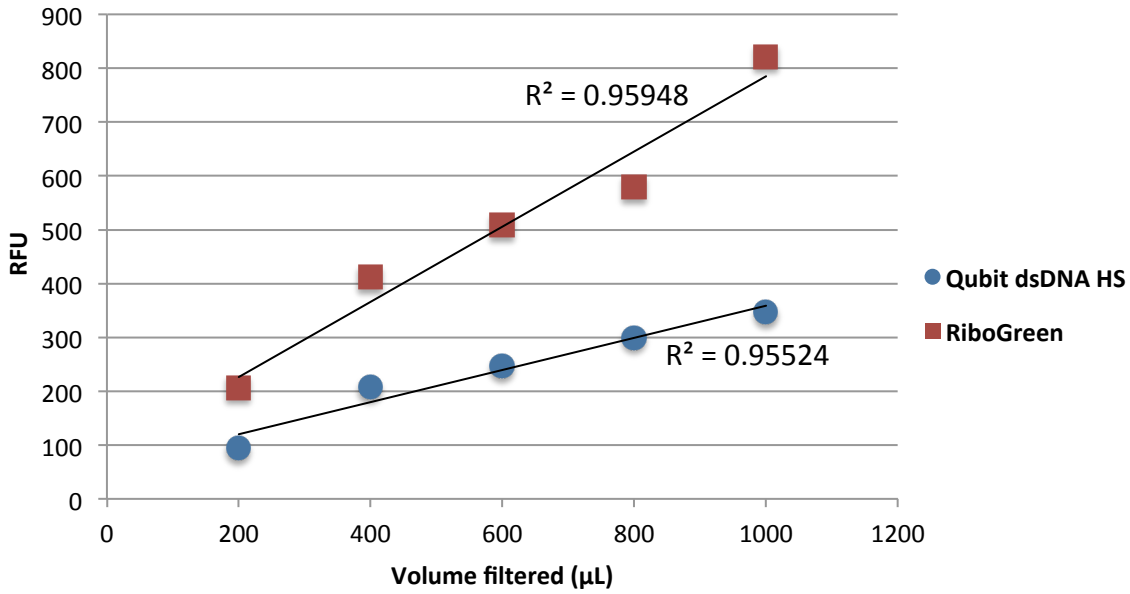
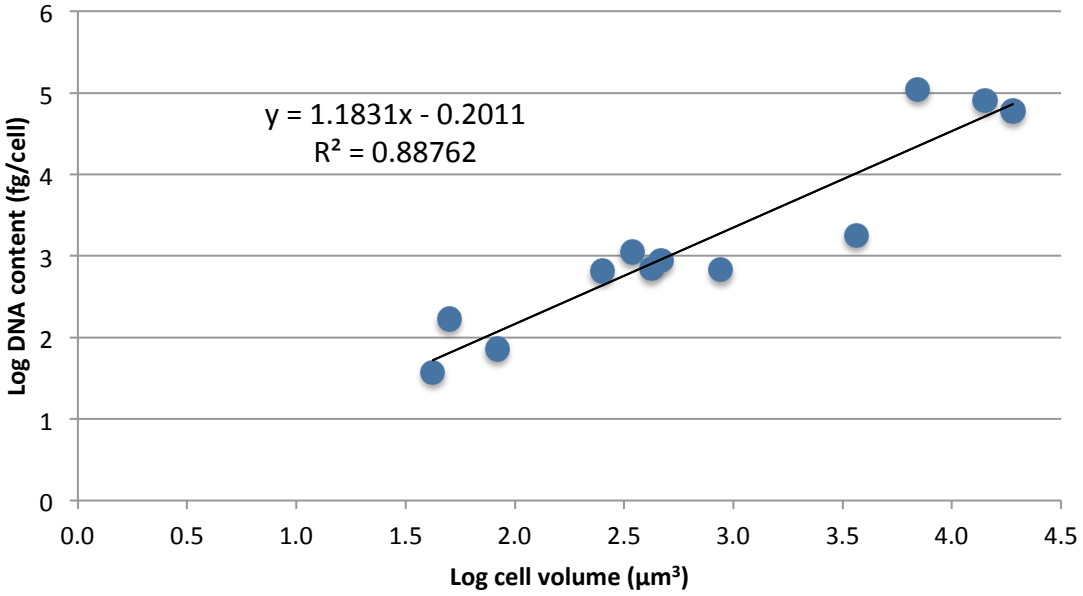


Figure 9. Relative fluorescence units of both DNA and RNA dyes hold a positive linear relationship with volume sampled.

Nucleic acid content of algal cultures varying in size. The validity of both nucleic acid assays was verified by measuring twelve different algal species grown in

monoculture (listed in Table 2). The surveyed species spanned a wide range of cell diameters and volumes. Cells were sized using a Coulter counter and cultures were analyzed on either a flow cytometer or under an epifluorescence microscope in order to obtain cell counts immediately before harvesting onto a GF/F filter. The DNA and RNA content per cell for each species was determined and subsequently correlated with cell volume. The results are illustrated in Figure 10 and shows that both nucleic acids are correlated with cell volume. The resulting linear equations for DNA and RNA allows predictions on nucleic acid content based on cell diameter. Thus, a 50 μm cell would be expected to have approximately 300-fold more DNA and 370-fold more RNA when compared to a 10 μm cell. In contrast, other biomass proxies such as chlorophyll *a* and ATP typically increase according to the analogous volume increase.



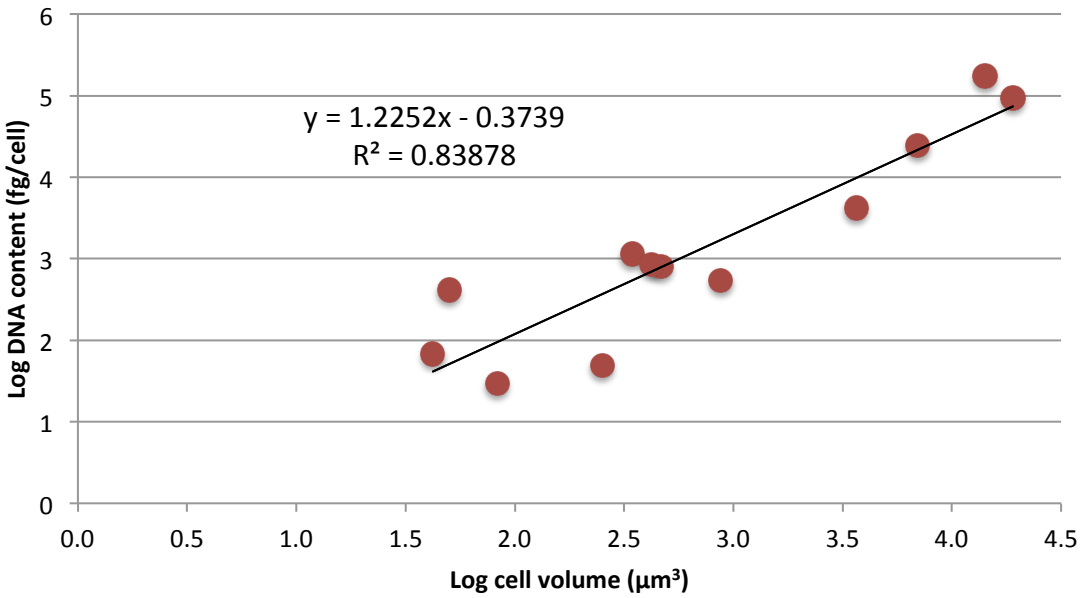


Figure 10. Log of DNA content and log of RNA content holds a positive correlation with log of cell volume in twelve algal species.

A histogram of the RNA:DNA ratios collected during this experiment shows the variety of possible ratios found within cultures that have been randomly sampled during

their growth cycle (Figure 11). Most cultures exhibited RNA:DNA ratios greater than 1, indicating higher levels of RNA production which can be related to positive growth.

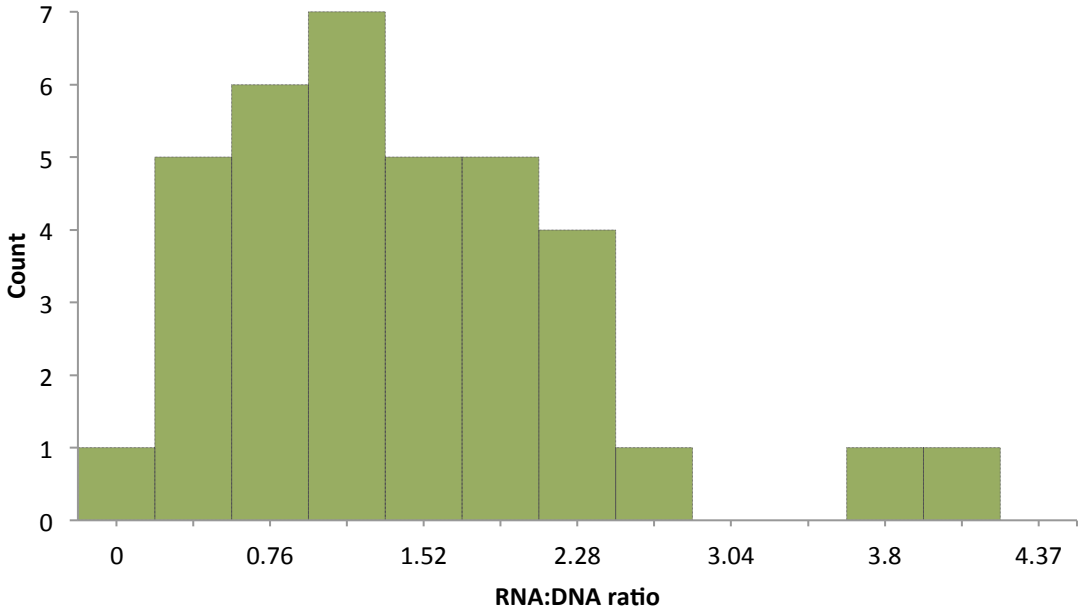


Figure 11. Histogram of RNA:DNA ratios from algal cultures sampled at random time points during their growth.

Relationship between nucleic acid content and growth rate. In order to further validate the relationship between growth and RNA:DNA ratios measured with the Qubit methodology, two independent algal cultures were sampled throughout their growth cycles. One *Tetraselmis sp.* culture and one *T. weiss* culture were monitored over the course of approximately 7 days. Cell counts using a flow cytometer and filtrations for future DNA and RNA quantitation were taken together at chosen time points throughout the growth of each culture. Cell counts, DNA concentration, and RNA concentration over time is displayed for *Tetraselmis sp.* in Figure 12 and for *T. weiss* in Figure 13.

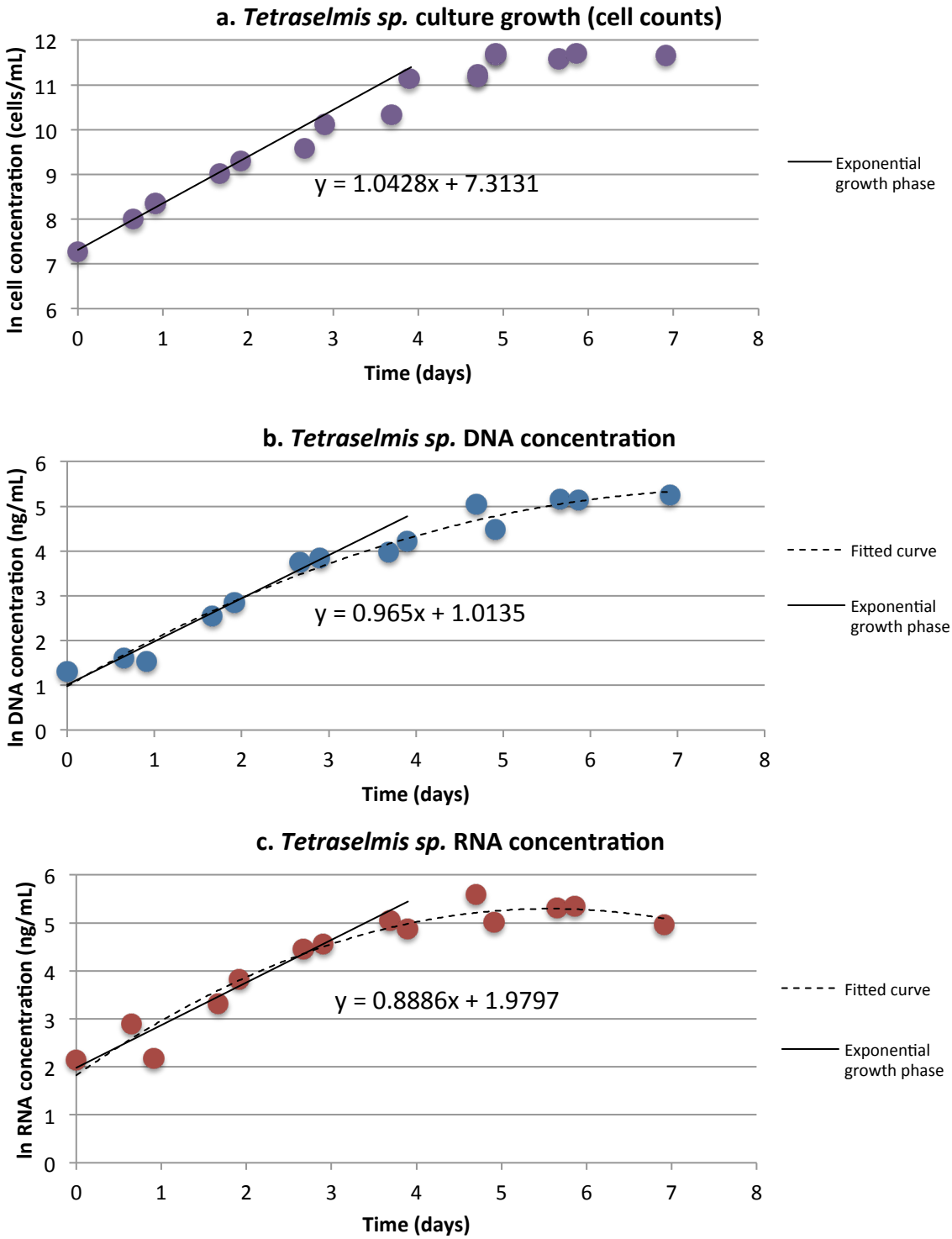


Figure 12. Log of cell counts (a), DNA concentration (b) and RNA concentration (c) of *Tetraselmis sp.* culture growth over the course of 7 days. The displayed equation represents the line fitted to the exponential growth phase.

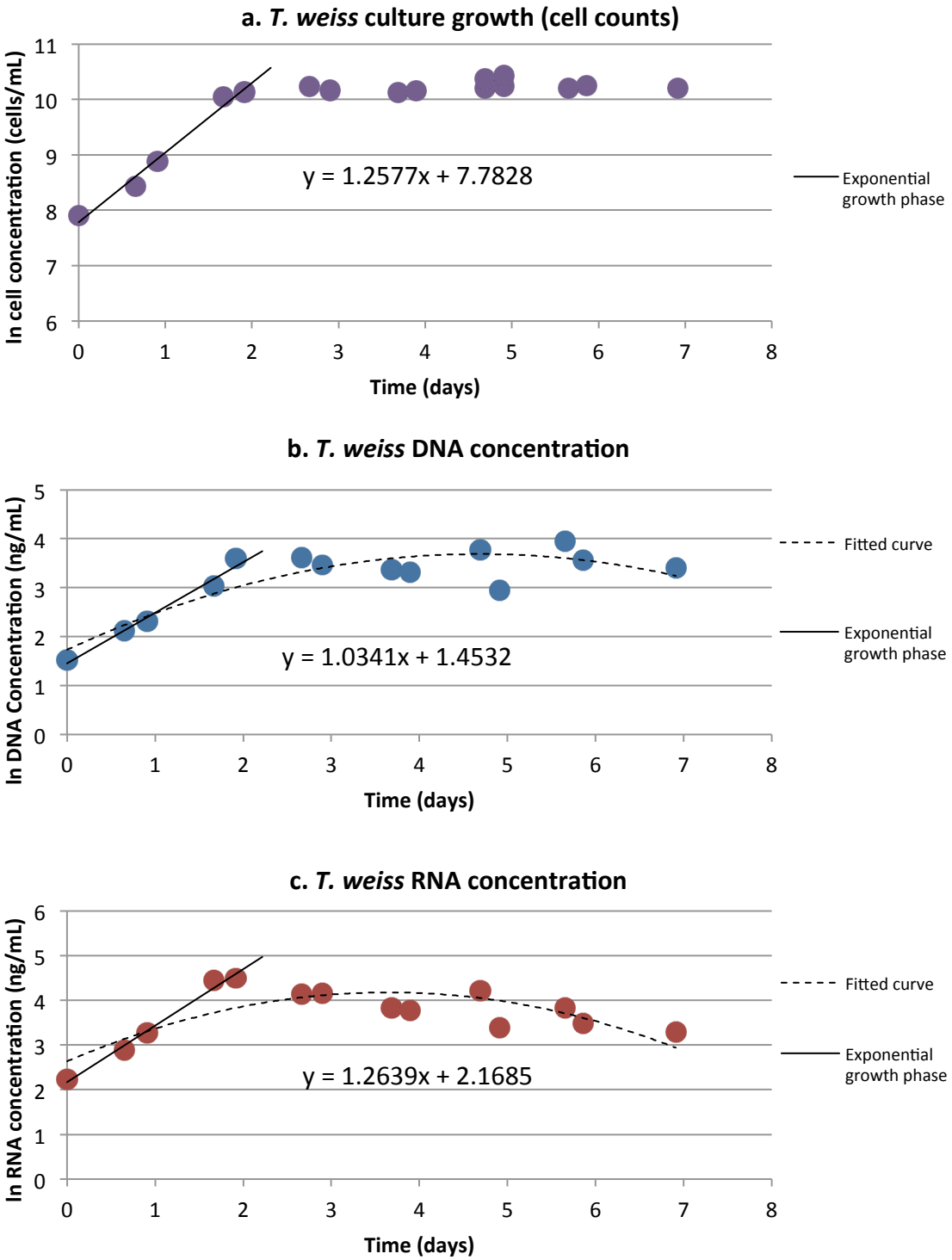


Figure 13. Log of cell counts (a), DNA concentration (b) and RNA concentration (c) of *T. weiss* culture growth over the course of 7 days. The displayed equation represents the line fitted to the exponential growth phase.

Nucleic acid quantities closely followed the pattern of cell growth over time in both cultures. The growth rate of algal cultures typically begins with a lag phase, in which little growth over time is detected, followed by an exponential phase where growth rate rapidly increases and reaches a maximum. The exponential phase is followed by a stationary phase in which growth begins to plateau and eventually discontinues. In the two algal cultures monitored, the lag phase was not captured in the cell counts or nucleic acid quantitation. The first three days in the *Tetraselmis sp.* culture and the first two days in the *T. weiss* culture illustrate the exponential phase of each alga, as depicted by the solid line and corresponding linear equation. The slopes of each equation represent the specific growth rate during exponential phase. In *Tetraselmis sp.*, specific growth rate was 1.04 d^{-1} for cell counts, 0.97 d^{-1} for DNA and 0.89 d^{-1} for RNA. In *T. weiss*, specific growth rate was 1.26 d^{-1} for cell counts, 1.03 d^{-1} for DNA and 1.26 d^{-1} for RNA. Within each species of phytoplankton, the specific growth rates of cell counts, DNA and RNA were all very similar, supporting a positive relationship between nucleic acids and growth.

Polynomial curves were fitted to the cell counts and the raw DNA and RNA concentrations and their respective equations were used to model projected RNA:DNA ratios over the course of each culture's growth (e.g. culture phases that represent nutrient saturated growth and reduced growth due to nutrient depletion and optical self-shading). Note that while the data points displayed on the RNA:DNA ratio versus time figures are the actual RNA:DNA ratios calculated from the raw Qubit data, the overlaid solid curves represent the smoothed, expected values based on the equations of the curves fitted to the

raw DNA and RNA concentrations (Figure 14). The smoothed RNA:DNA ratio overlay illustrates that despite some early noise, the general trend of the raw data follows the predicted trend relatively well.

Actual growth rate for each algal culture was calculated by determining the slope between two adjacent points in the raw cell count data. The growth rates were then related to raw RNA:DNA ratios and their correlations can been seen in Figure 15. Both cultures held a positive correlation between growth rate and RNA:DNA ratio, strongly confirming the findings by Dortch (1983). The two cultures even have remarkably similar slopes, suggesting that the relationship between growth rate and RNA:DNA ratio is similar between the two species.

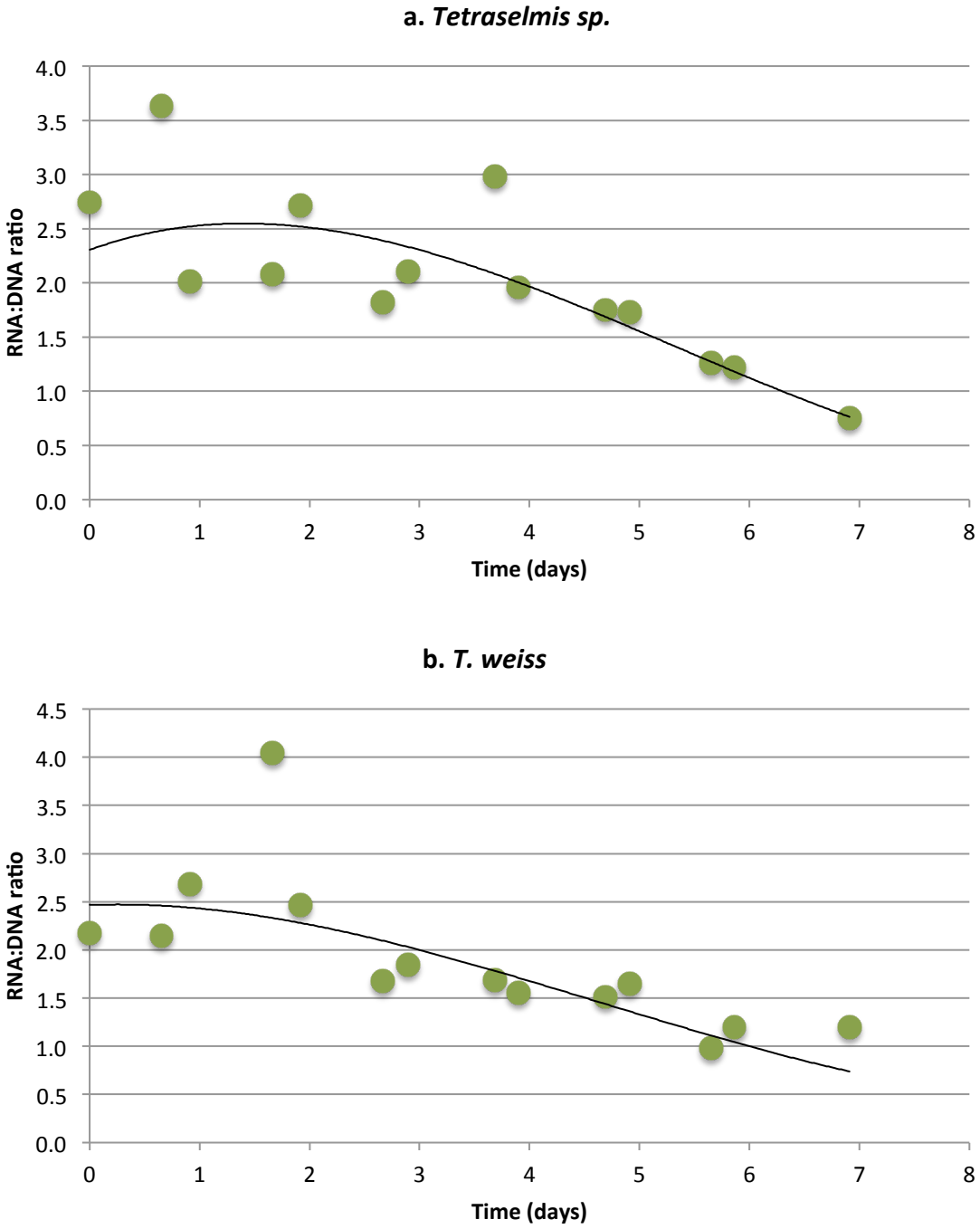


Figure 14. RNA:DNA ratios of the raw RNA and DNA concentration values in *Tetraselmis sp.* (a) and *T. weiss* (b) cultures. The lines represent expected values of the RNA:DNA ratios derived from the equations of the polynomial curves fitted to the raw RNA and DNA concentration data.

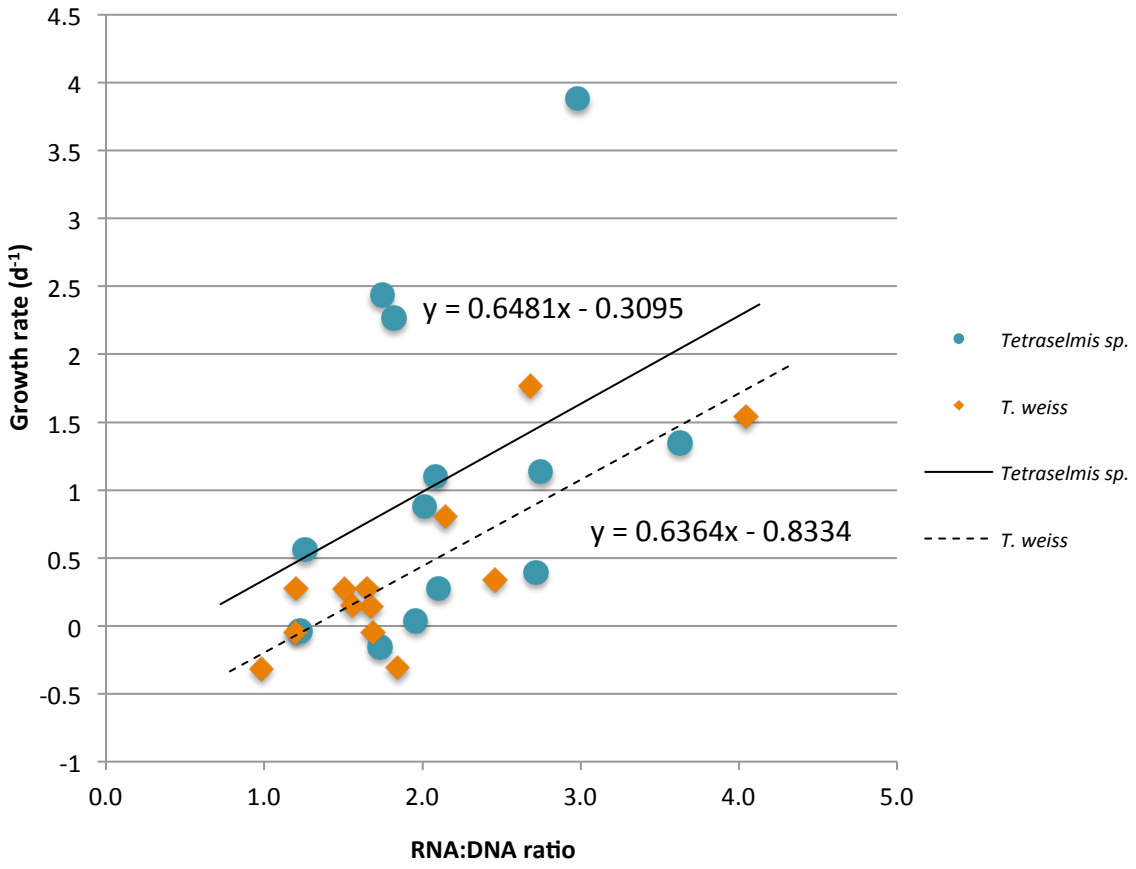


Figure 15. Relationship between actual RNA:DNA ratios and growth rate in *Tetraselmis sp.* and *T. weiss* cultures.

Offshore Monterey Bay nucleic acid concentrations. On October 9th, 2015, three CTD casts were sampled aboard the R/V *Rachel* Carson during a cruise in the Monterey Bay. Measurements of bulk FDA were taken immediately on the ship while samples for nucleic acid quantitation were stored in liquid nitrogen for later processing. Bulk FDA and nucleic acid results for the three stations, including RNA:DNA ratio, are found in Figures 16, 17 and 18. Fluorescein production, nucleic acid concentration and RNA:DNA ratio all exhibited high values in the surface waters, especially above 50 meters. As shown in the grow-out experiments, RNA:DNA ratio is associated with growth rate and higher values in surface waters may indicate higher growth rates of biomass in that region. All metrics drop dramatically after 50 meters depth, in which much of the biomass tapers. The depth profiles illustrate that the nucleic acid quantitation techniques using the hand-held Qubit are accurately reflecting known patterns in biomass and depth, especially when compared with other previously established measurements such as fluorescein production.

The dynamic range of RNA:DNA ratios in these natural samples are much less than what was observed in lab-grown cultures; the greatest ratio observed in these station samples was about 2, whereas algal cultures had ratios exceeding 4. This may imply that phytoplankton in the Monterey Bay are not growing at full capacity, or that some of the cells captured are stressed or dead thereby reducing RNA:DNA ratios.

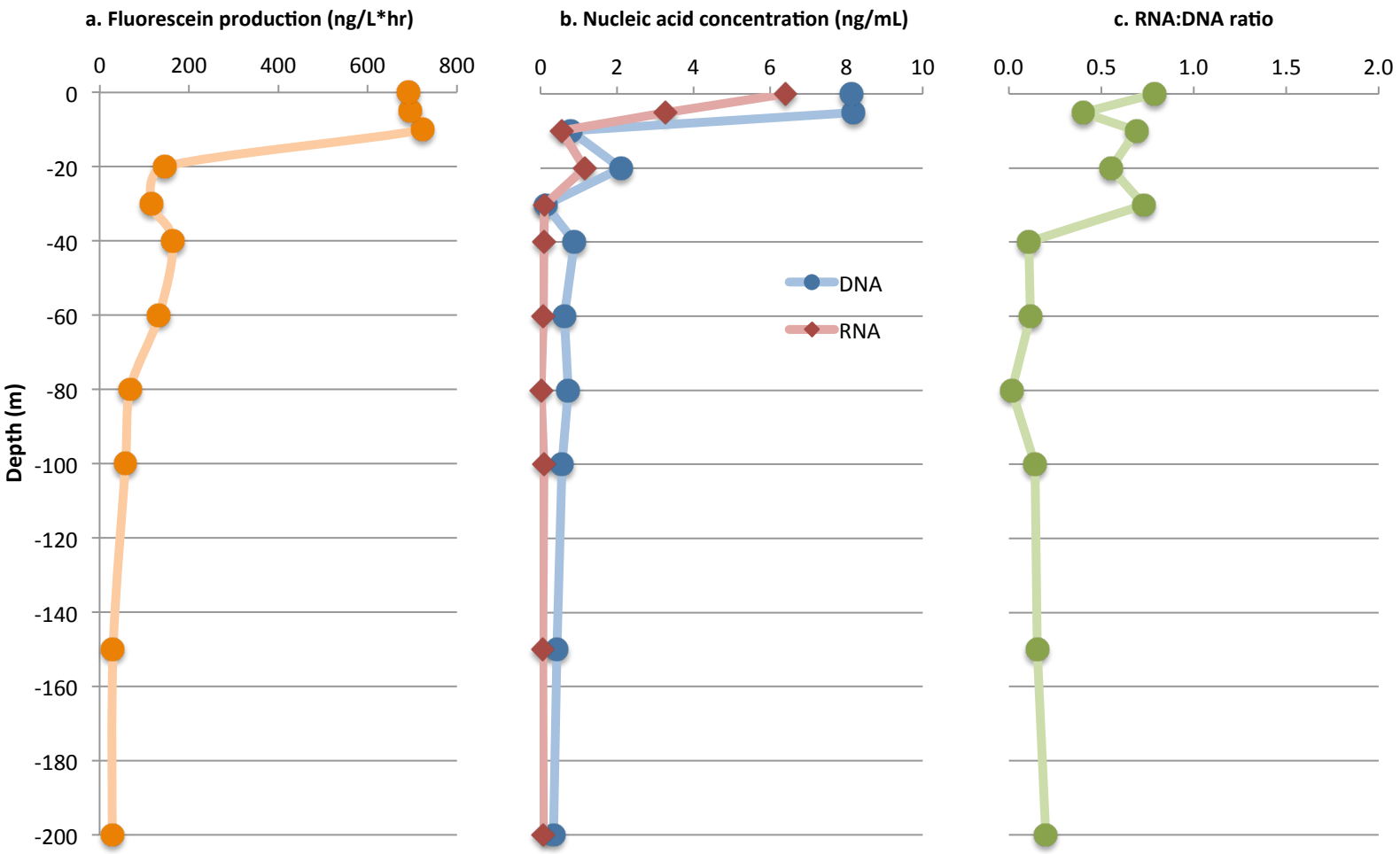


Figure 16. CTD profiles from station C1, including fluorescein production (viable biomass proxy) (a), nucleic acid concentrations (b) and the RNA:DNA ratio (c).

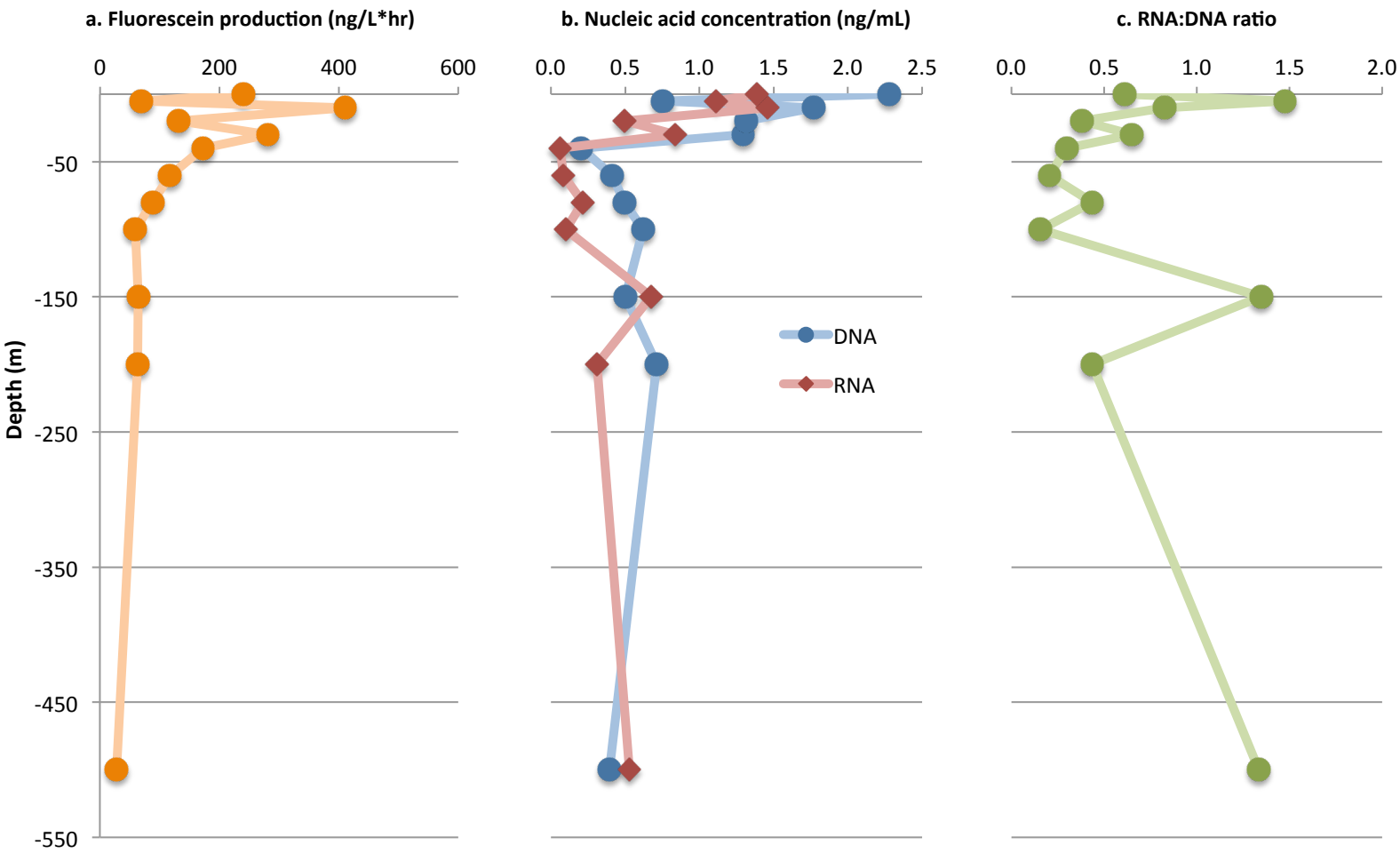


Figure 17. CTD profiles from station C2, including fluorescein production (viable biomass proxy) (a), nucleic acid concentrations (b) and the RNA:DNA ratio (c).

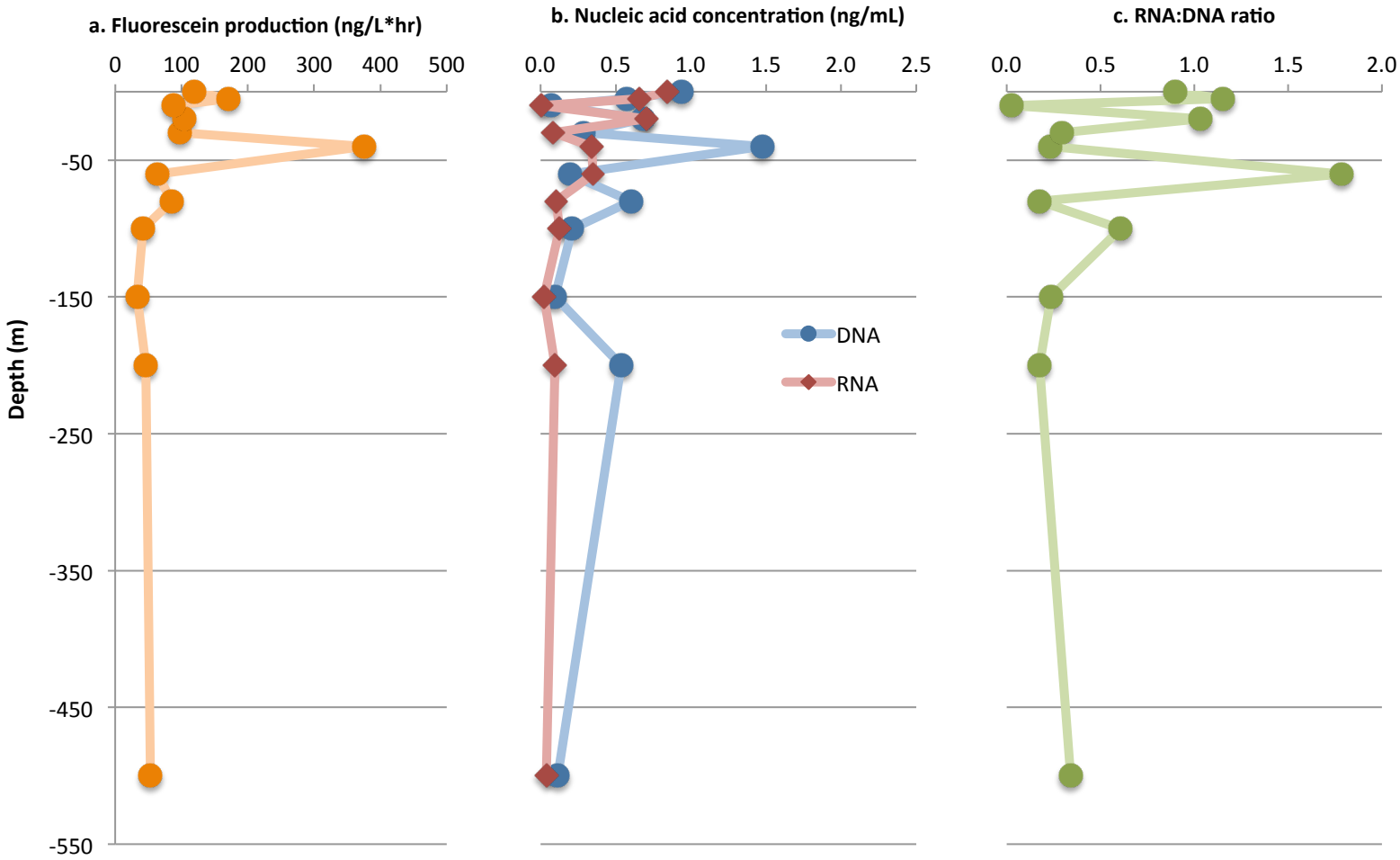


Figure 18. CTD profiles from station C3, including fluorescein production (viable biomass proxy) (a), nucleic acid concentrations (b) and the RNA:DNA ratio (c).

Nucleic Acid Quantitation After UV Irradiation

Proof of concept by alternative kill methods. Samples were subjected to bleach and heat treatment in order to provide a "proof-of-concept" of the reduction in nucleic acids after various kill methods. A strong bleach treatment was achieved by subjecting a culture of *Tetraselmis sp.* to 1,300 ppm sodium hypochlorite (Figure 19). DNA was reduced 23-fold, from 46 ng/mL to 2 ng/mL and RNA was reduced 500-fold from 75 ng/mL to 0.15 ng/mL. Since RNA exhibited a greater change between control and bleach treatment, the RNA:DNA ratio dropped by a factor of 20.5, from 1.64 to 0.08.

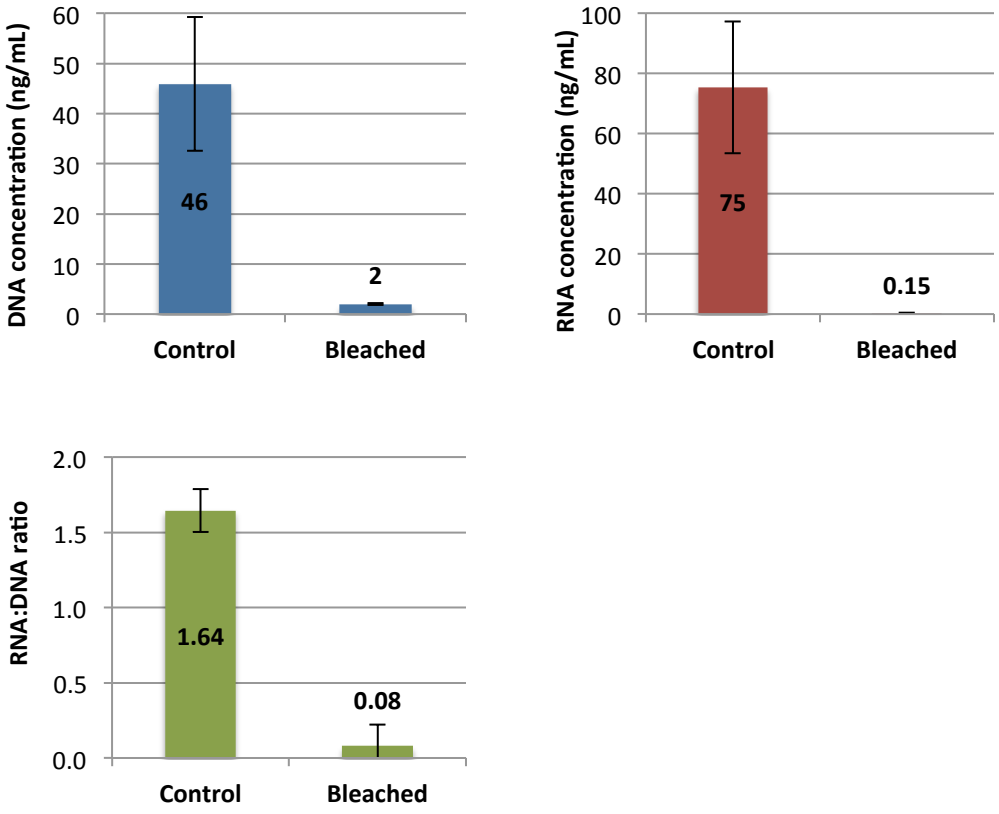


Figure 19. DNA and RNA concentration and RNA:DNA ratio after a *Tetraselmis sp.* culture was treated with 10% bleach. Error bars represent the standard deviation between replicate samples.

For heat treatment, three individual algal cultures were placed in a heat bath at 72°C for 30-45 minutes. While DNA amongst the three cultures showed a 3 to 4-fold drop, RNA exhibited a greater decrease, ranging from 10 to 18-fold. Because the decrease in RNA was greater throughout all three species, the RNA:DNA ratio dropped in all cases, ranging from a 3.4 to 5-fold reduction. Both kill methods confirmed the decrease in overall nucleic acid content, as well as a decrease in the RNA:DNA ratio in algal cells exposed to sterilization treatments.

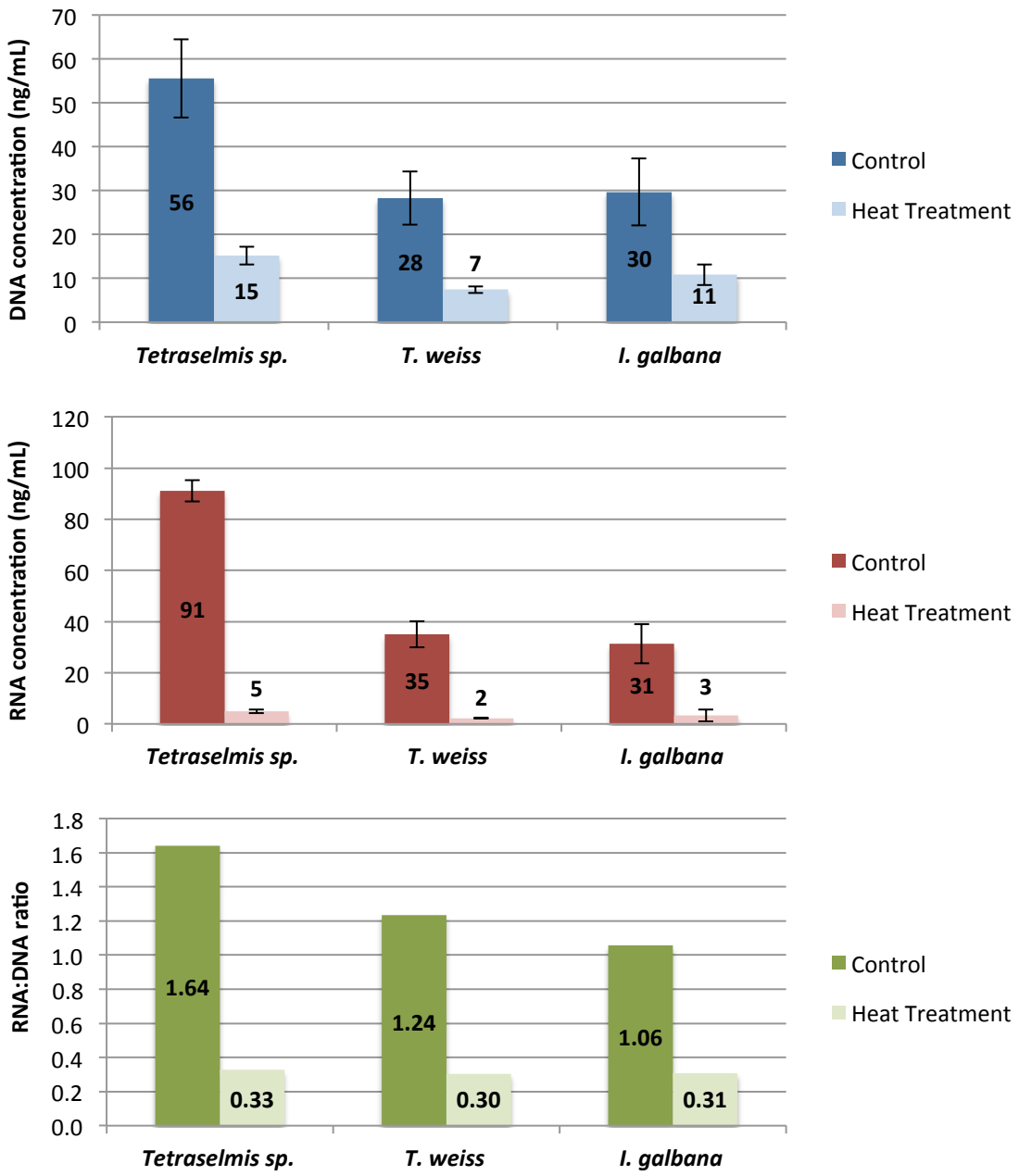


Figure 20. DNA and RNA concentrations and RNA:DNA ratios of three algal cultures after heat treatment. Error bars represent the standard deviation between replicate samples.

Lab UV irradiated algal cultures. Three cultures, *Tetraselmis sp., T. weiss, and I. galbana* were UV irradiated in triplicate under a Trojan UV-C collimator, generating a total of nine distinct UV trials. Samples were irradiated between 8 and 15 hours while

being stirred, resulting in an effective UV dosage between 3,500 to 6,600 mJ/cm^2. In experiments using *Tetraselmis suecica* Olsen et al. (2016) concluded that UV dosages between 200-400 mJ/cm^2 should be sufficient to permanently inactivate cells. Thus, the UV dose given here is effectively an order of magnitude greater.

After irradiation, control and treatment samples were taken in triplicate for nucleic acid quantitation and bulk FDA measurements. Nucleic acid results were variable between cultures (Figures 21, 22 and 23). Raw DNA and RNA concentrations consistently dropped after UV irradiation in all three cultures. However, whereas *Tetraselmis sp.* exhibited a decrease in RNA:DNA ratios, *T. weiss* had mixed results and *I. galbana* had increased RNA:DNA ratios after irradiation. *Tetraselmis sp.* showed a 3.8-fold average drop in RNA and a 2.7-fold average drop in DNA. Decreases in RNA were greater than DNA in each of the *Tetraselmis* treatments, resulting in an average 1.7-fold decrease in the RNA:DNA ratio. *I. galbana* showed reverse results, with an average 2-fold decrease in RNA and a 2.9-fold decrease in DNA. All *I. galbana* samples had greater RNA:DNA ratios after UV treatment, with an average 1.5-fold increase. *T. weiss* showed an average 3.6-fold drop in RNA and 4.7-fold drop in DNA. Although the average change in the RNA:DNA ratio was a 1.4-fold increase, one *T. weiss* trial exhibited a slight decrease in the RNA:DNA ratio, from 0.9 to 0.8.

On the other hand, bulk FDA consistently exhibited large reductions in fluorescein production per hour across all three species (Figure 24). *Tetraselmis sp.* experienced the greatest decrease with an average 312-fold drop. *T. weiss* and *I. galbana* exhibited reductions ranging from 30 to 86-fold.

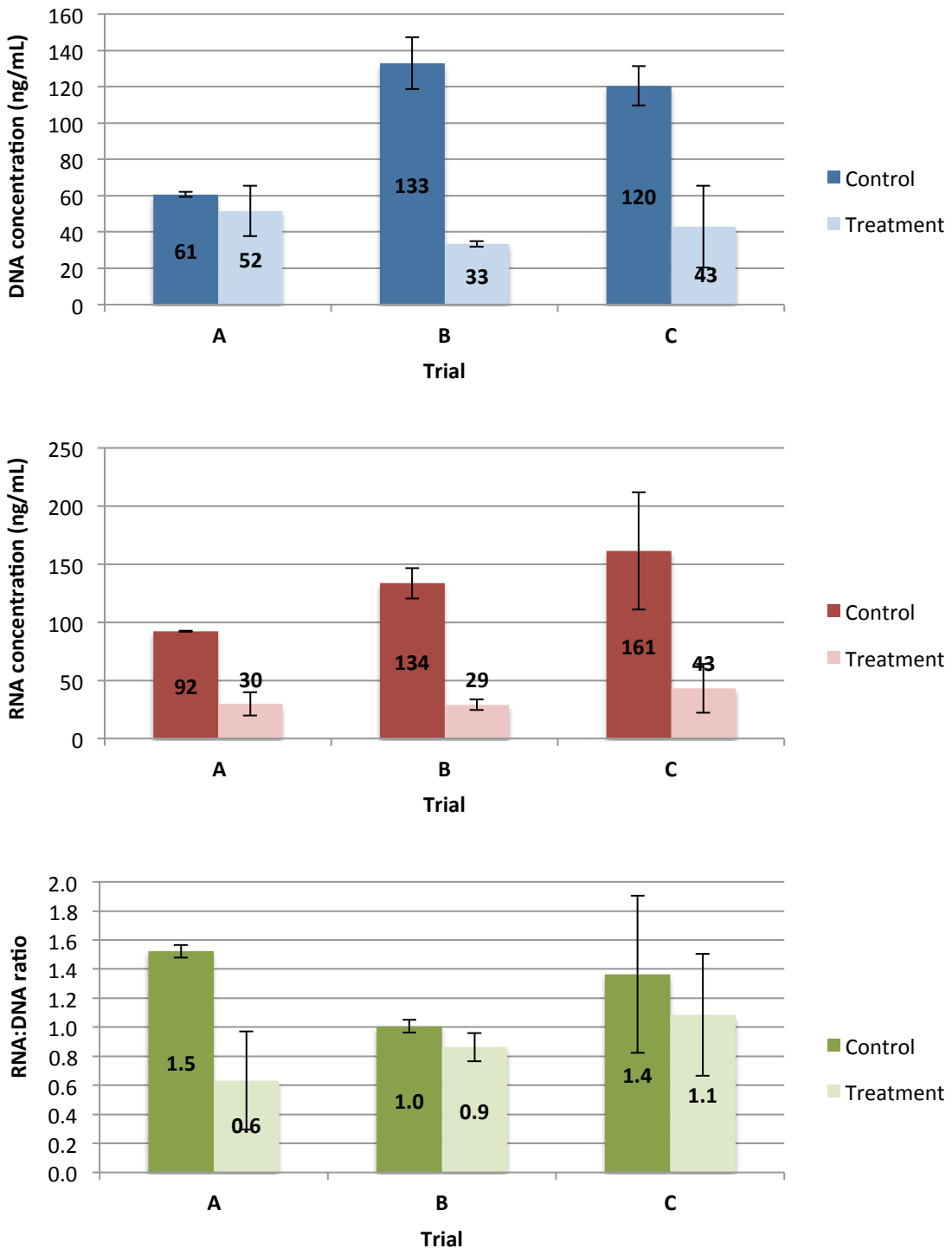


Figure 21. Control versus UV treated concentrations of DNA and RNA and the RNA:DNA ratios in three individual *Tetraselmis sp.* cultures. Error bars represent the standard deviation between replicate samples.

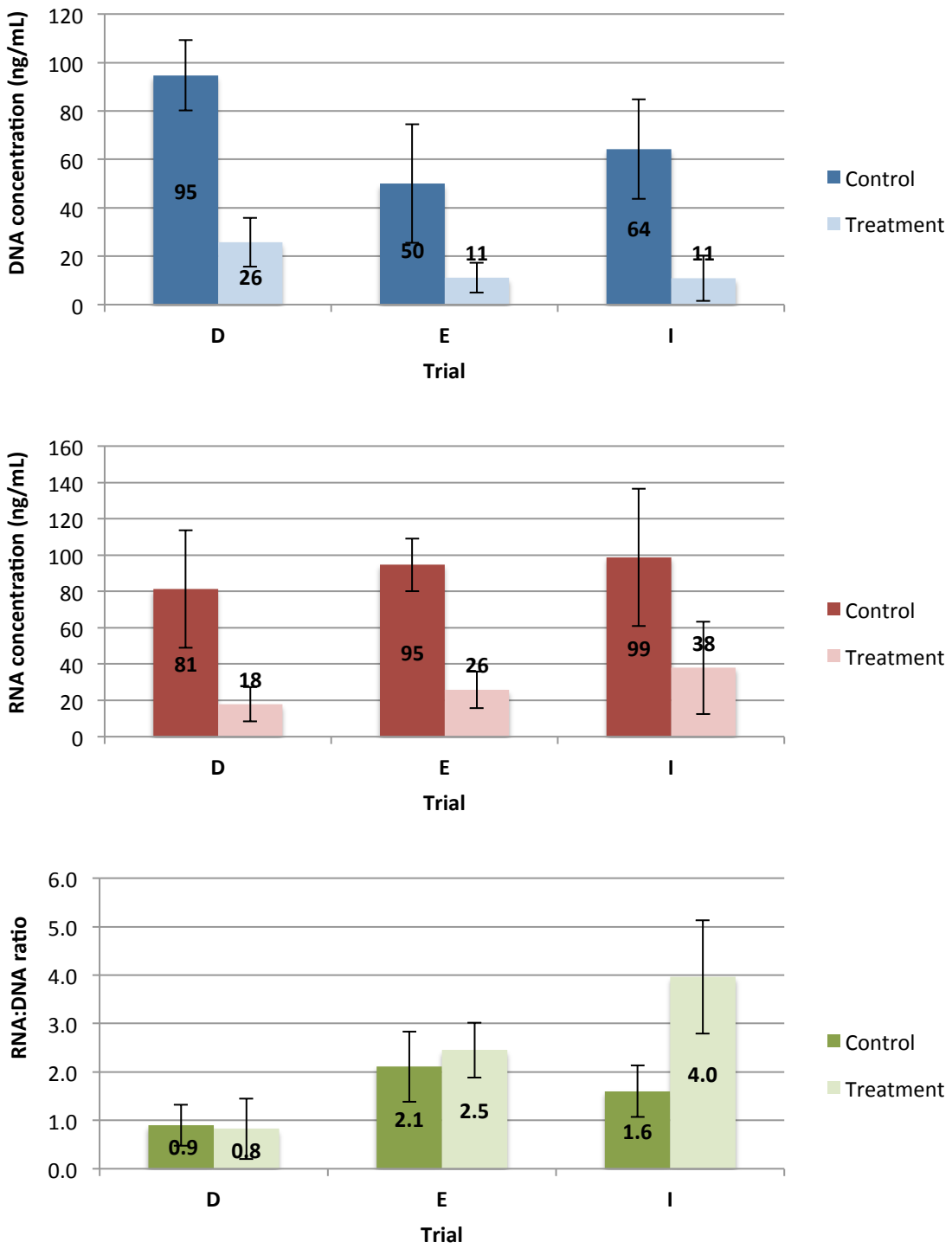


Figure 22. Control versus UV treated concentrations of DNA and RNA and the RNA:DNA ratios in three individual *T. weiss* cultures. Error bars represent the standard deviation between replicate samples.

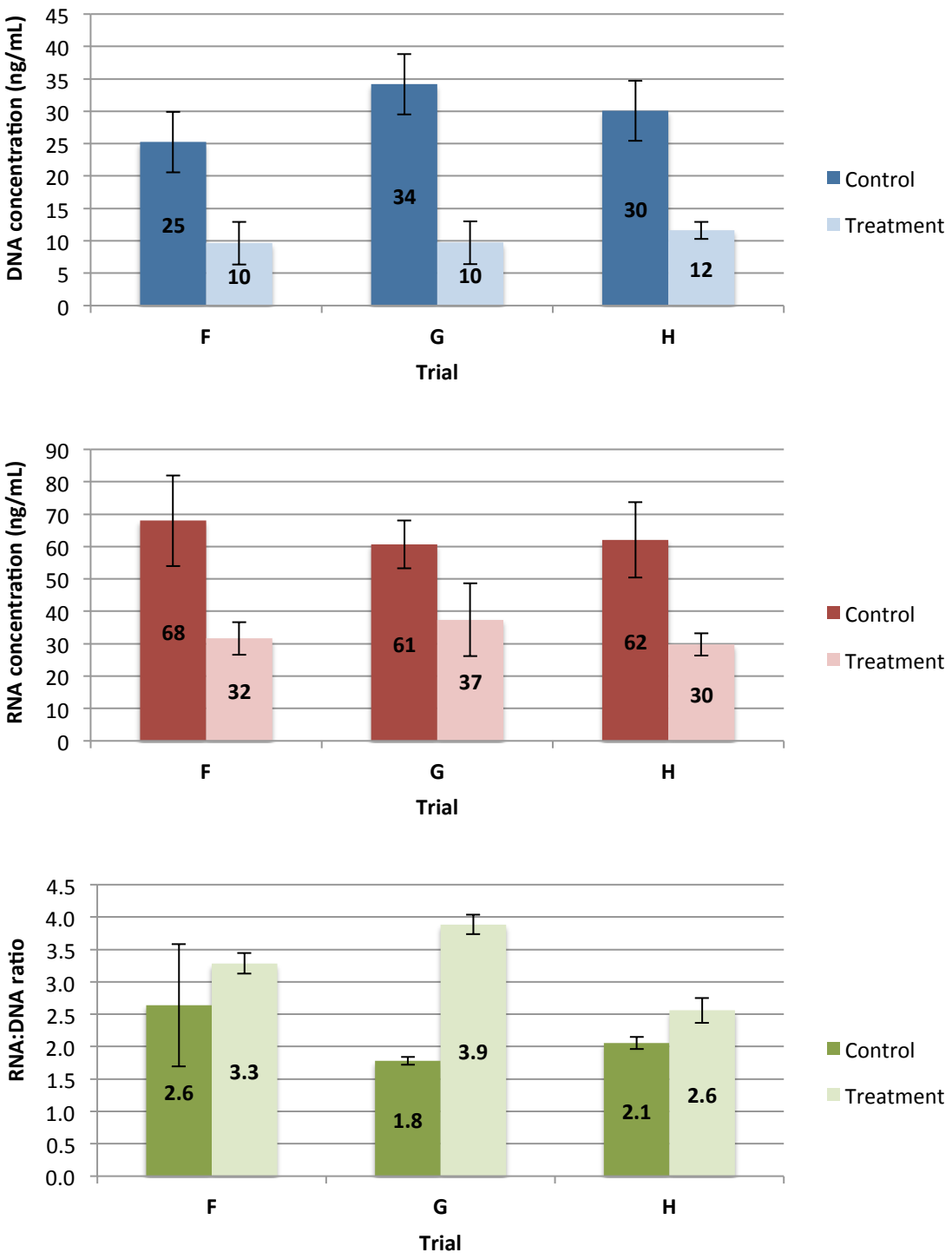


Figure 23. Control versus UV treated concentrations of DNA and RNA and the RNA:DNA ratios in three individual *I. galbana* cultures. Error bars represent the standard deviation between replicate samples.

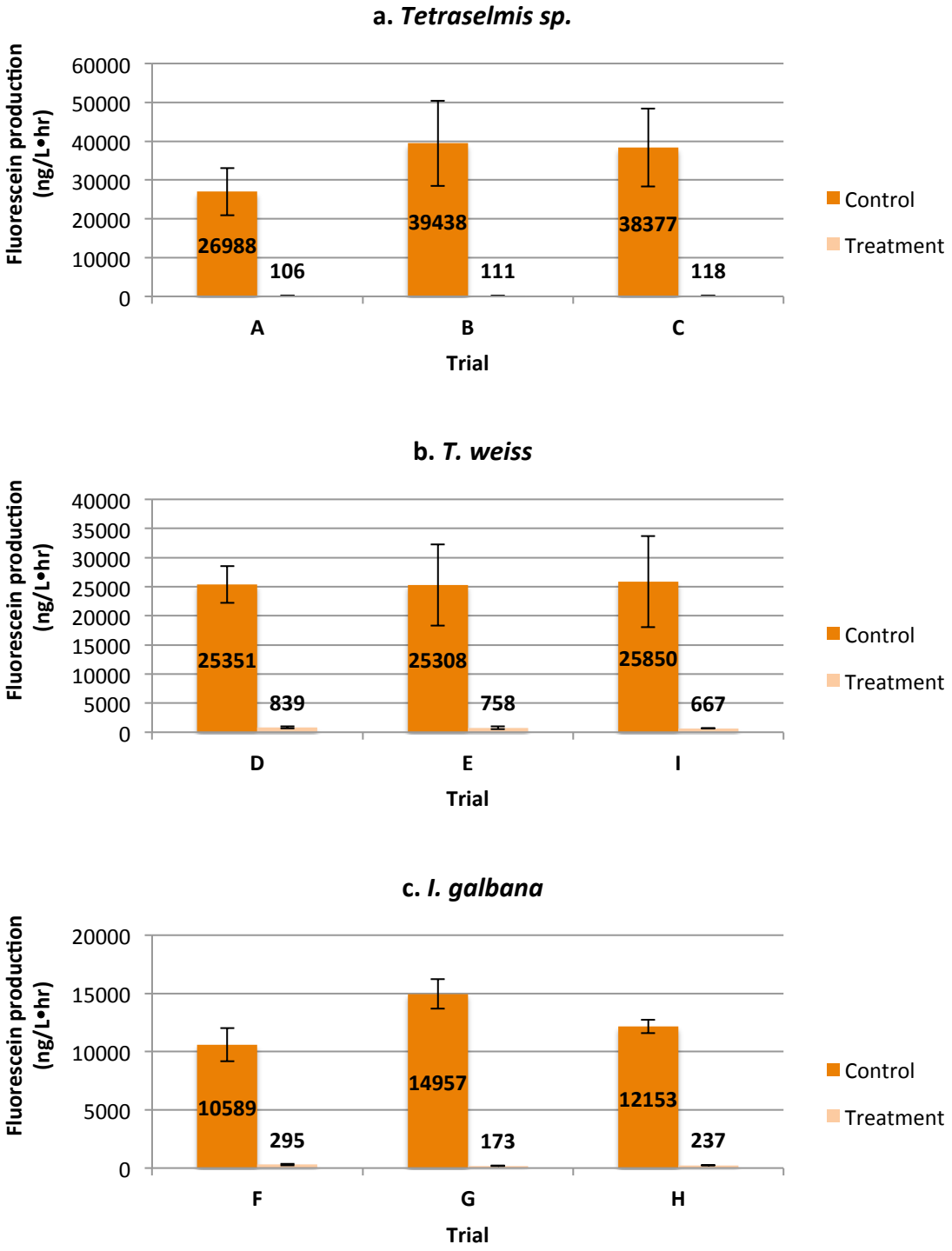


Figure 24. Control versus UV treated comparisons of fluorescein production in three algal cultures: *Tetraselmis sp.* (a), *T. weiss* (b), and *I. galbana* (c). Error bars represent the standard deviation between replicate samples.

***Tetraselmis sp.* grow-out with UV irradiation.** A culture of *Tetraselmis sp.* was monitored throughout its growth cycle. After 3.75 days, when the cell count had reached approximately 18,000 cells/mL, 225 mL of the culture was placed in a glass beaker and held under the Trojan UV-C collimator for 9 hours resulting in an average UV dose of approximately 1,000 mJ/cm^2. The control and UV treated samples were simultaneously monitored for the next 8 days. Comparison of live cell counts, DNA concentration, RNA concentration and RNA:DNA ratio can be seen in Figures 25 and 26.

Cell counts were based off of flow cytometric analysis in which populations of cells were enumerated within a fixed gate measuring forward scatter and green fluorescence. Algal samples were first inoculated with fluorescein diacetate (FDA) and allowed to incubate for 10 minutes. Metabolically active cells would cleave FDA and thereafter exhibit green fluorescence due to the presence of fluorescein. Thus, live counts depicted in Figure 25 represent actively metabolizing cells.

UV treatment had a clear adverse affect on nucleic acid content. While DNA in the control culture continued to increase before reaching a plateau, DNA in the irradiated culture stagnated and stayed constant for the remainder of the time series. RNA in the UV treatment showed a gradual decline while the control, as with DNA, continued to rise before reaching a maximum steady state. These two metrics combined created an obvious and immediate drop in the RNA:DNA ratio of the treated culture, which held at an average of 0.18. Meanwhile, the control culture had an average RNA:DNA ratio of 1.81, ten times higher than that of the irradiated culture, for the next two days until it reached its stationary phase where its growth rate and RNA:DNA ratio began to decrease. Even

after reaching this phase, the control maintained a 3.6-fold higher RNA:DNA ratio than the treated culture during the last days of monitored growth.

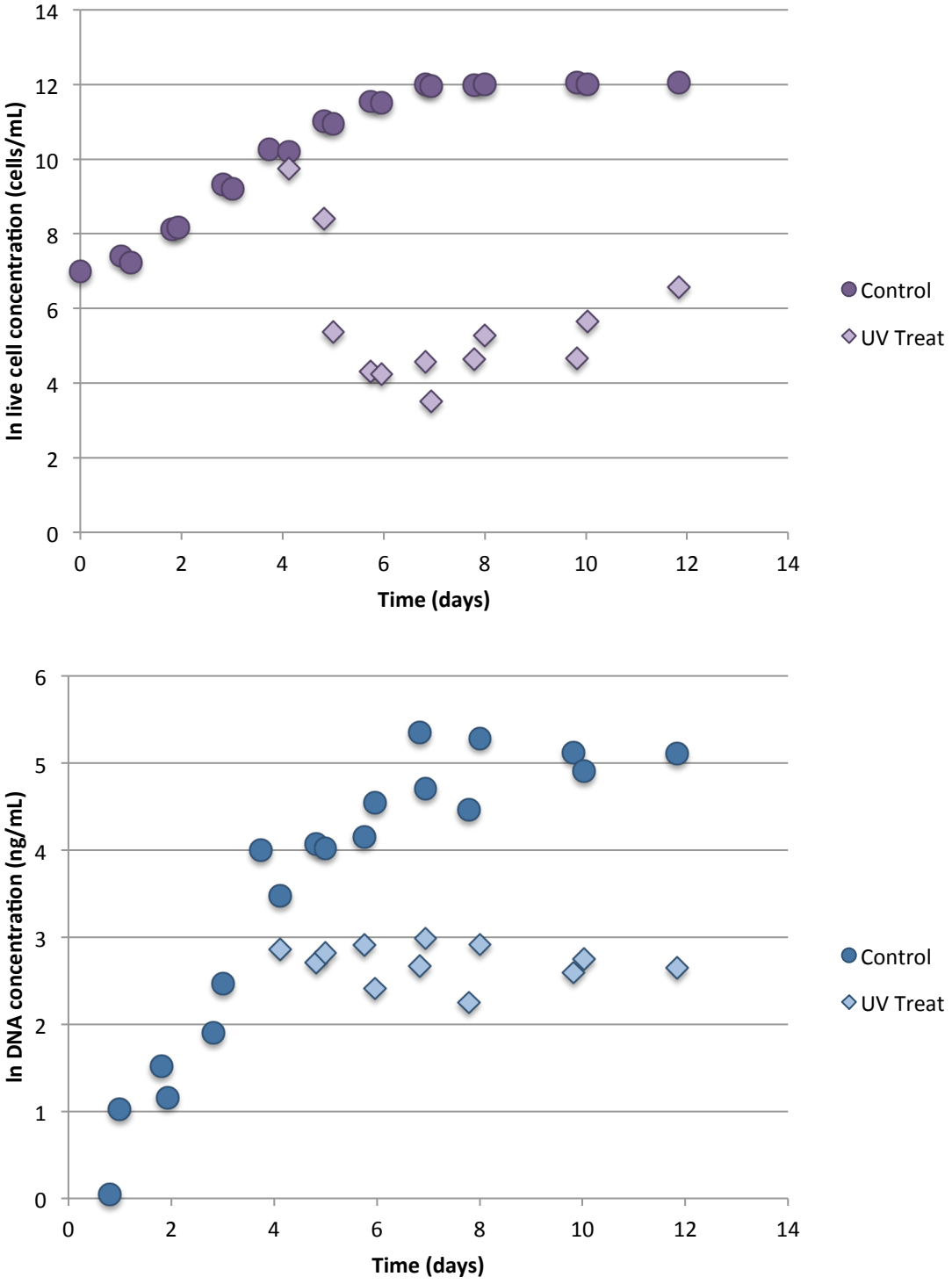


Figure 25. Log of cell counts and log of DNA concentrations over time in the control and UV treated *Tetraselmis sp.* culture.

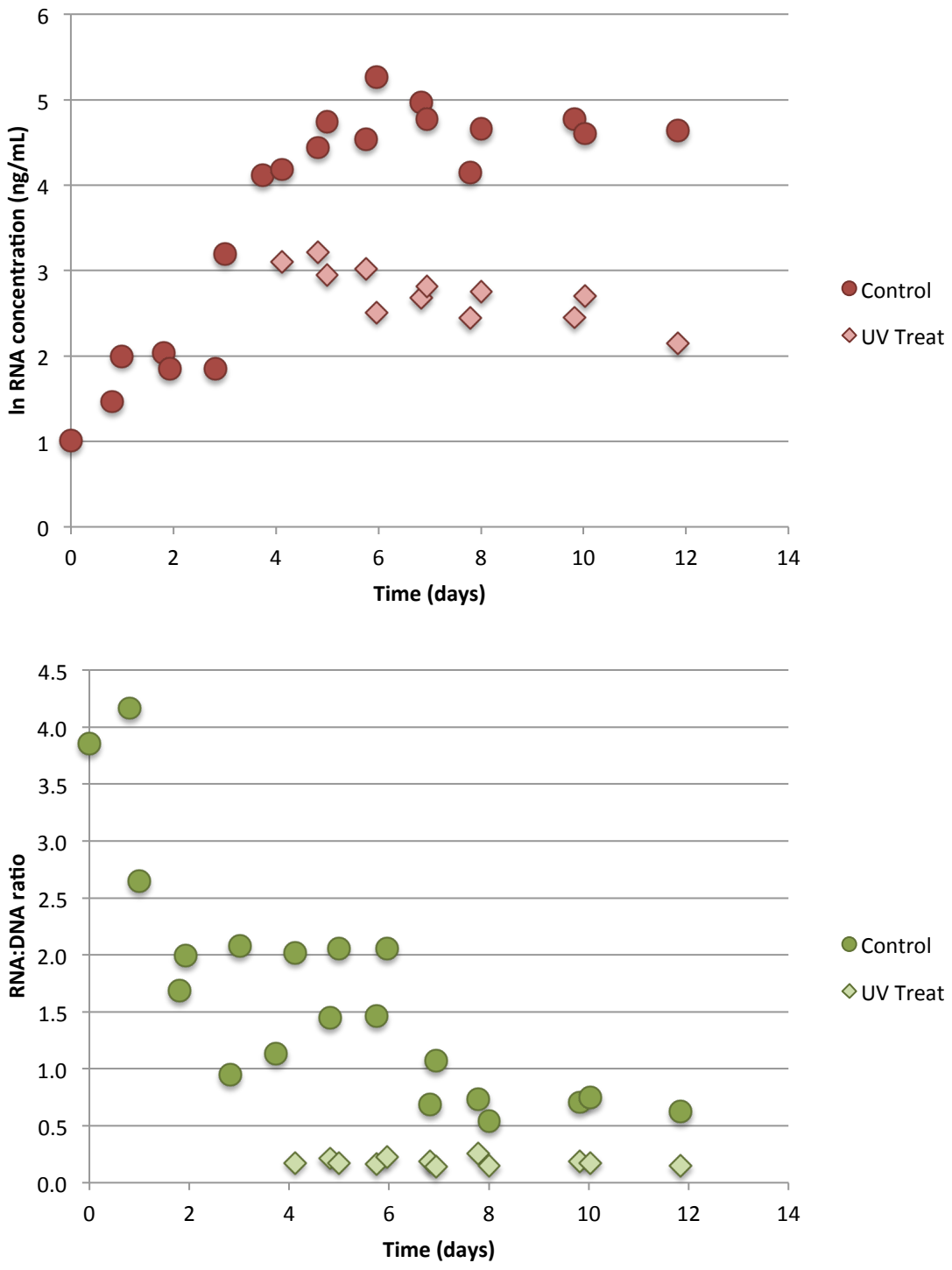


Figure 26. Log of RNA concentrations and RNA:DNA ratios over time in the control and UV treated *Tetraselmis sp.* culture.

Field Ballast Samples

Two different commercial ballast water management systems (BWMS) were tested at the Golden Bear Facility during the data collection phase of this study. The first utilized chlorine dioxide (ClO_2) as the treatment method while the second used a combination of filters and UV irradiation. Samples from these tests were collected in triplicate on 10 μm nylon filters and processed for nucleic acid quantitation, bulk FDA (measuring enzyme activity through fluorescein production) and P-BAC ATP measurements. Treatments are paired with control samples that are collected and stored in the same way, but untreated. Samples from four unique ClO_2 treatments and six unique UV treatments, along with their controls, were analyzed. Figures 27 and 28 depict representative subsamples from each respective treatment method.

When attempting to evaluate the effectiveness of BWMS, the magnitude of change from a control versus a treated sample is a clear and meaningful indication. Figures 29 and 30 show aggregated calculations for the degree of change in the five metrics analyzed from each treatment method. The numbers in the table specifically represent the ratio of the treatment measurement to the control measurement. Thus, numbers less than one represent a decrease after treatment (colored blue), whereas numbers greater than one represent an increase (colored red).

The most glaring result from this figure is that RNA:DNA ratio appears to be a poor indicator of treatment, regardless of the treatment method. With the ClO_2 method, relatively consistent decreases in DNA, RNA, enzyme activity, and ATP were observed in the majority of the treatments. DNA and ATP shared the same average magnitude of

decrease after treatment and appeared to be the best metrics to evaluate ClO_2 efficacy. Meanwhile, RNA concentration proved highly variable following UV treatment and actually increased in half of the cases. Of the five metrics, DNA and ATP again displayed the greatest average reduction. Surprisingly, of the new nucleic acid measurements considered, DNA was the molecule that most consistently decreased following UV treatment.

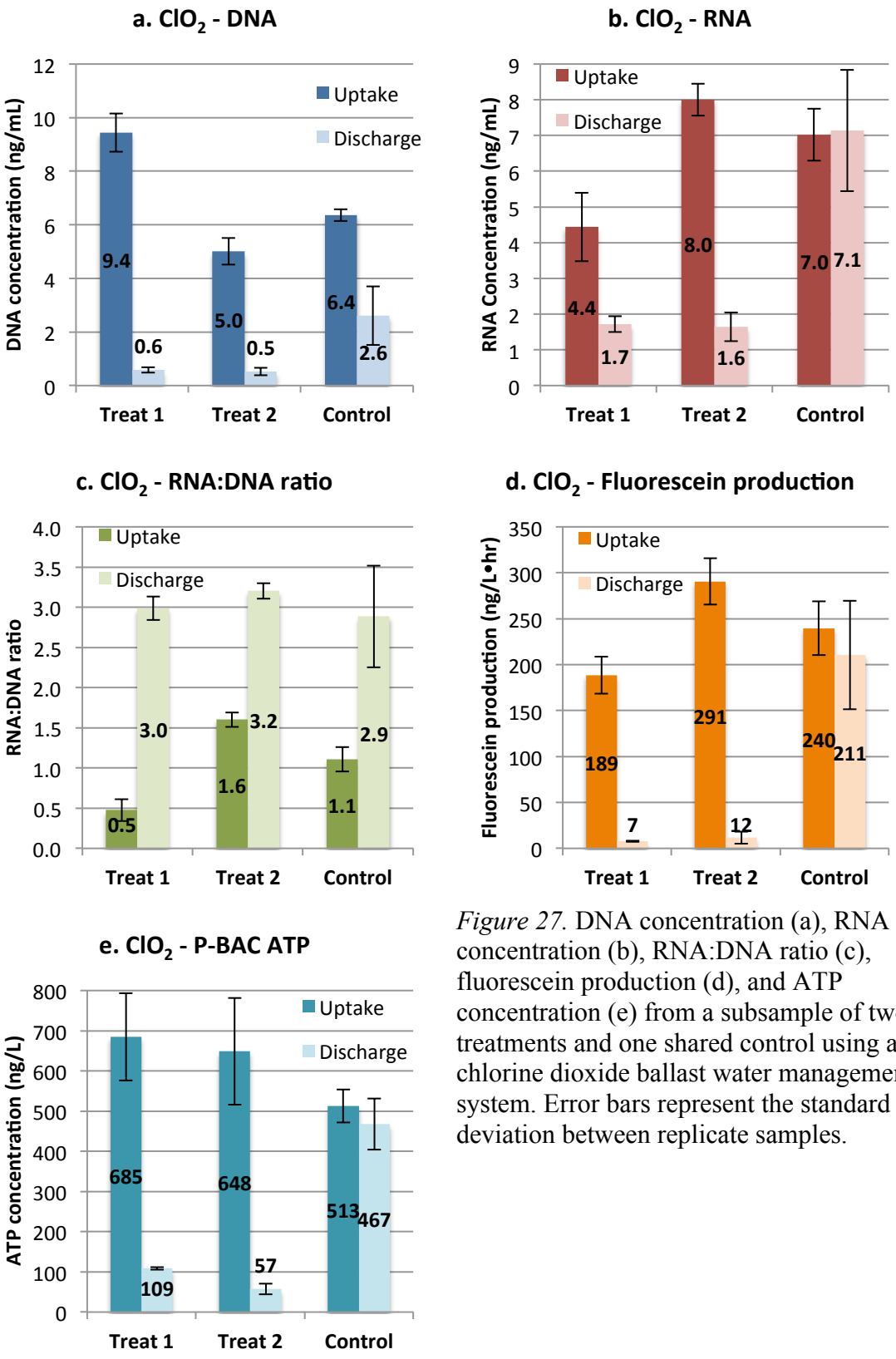


Figure 27. DNA concentration (a), RNA concentration (b), RNA:DNA ratio (c), fluorescein production (d), and ATP concentration (e) from a subsample of two treatments and one shared control using a chlorine dioxide ballast water management system. Error bars represent the standard deviation between replicate samples.

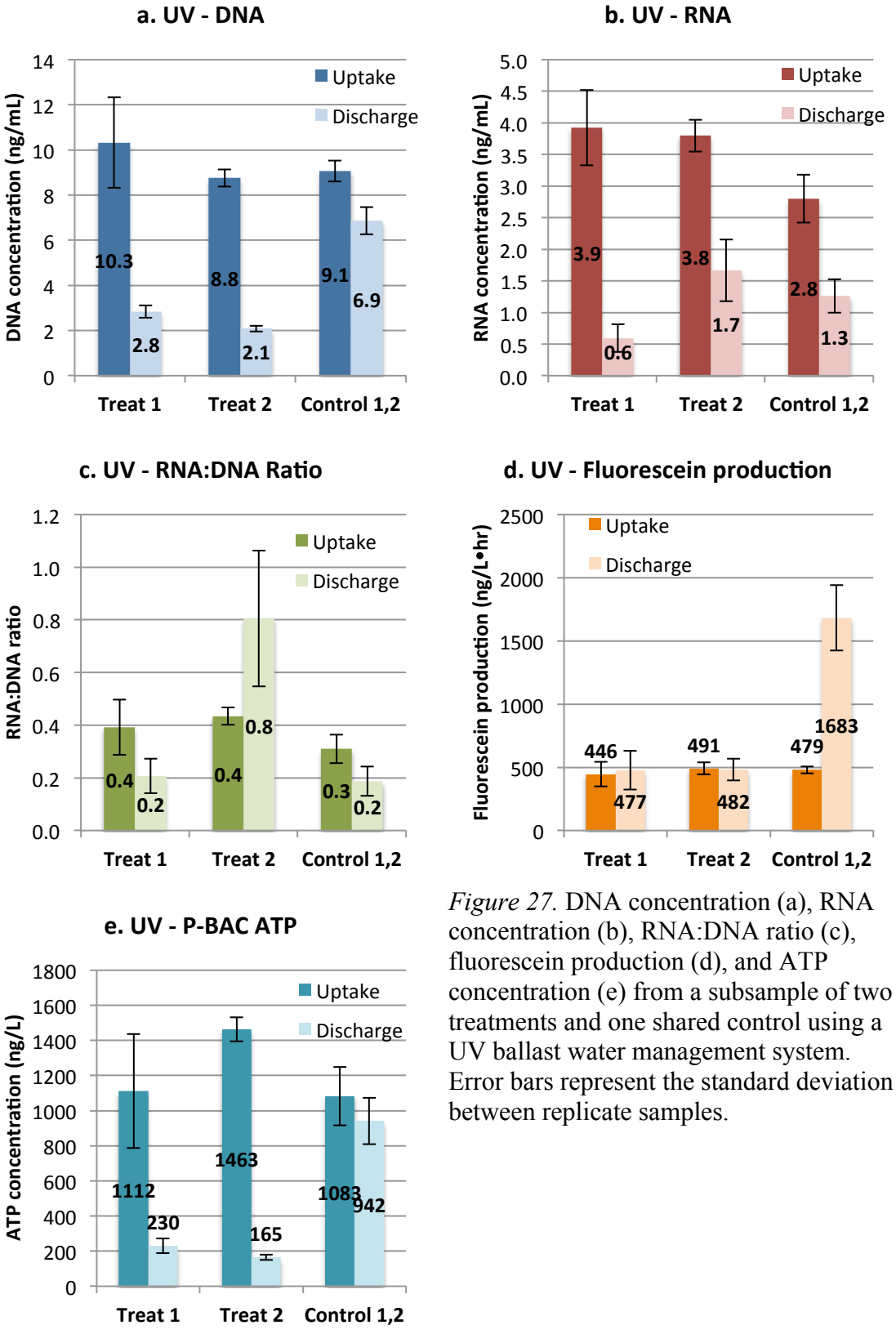


Figure 27. DNA concentration (a), RNA concentration (b), RNA:DNA ratio (c), fluorescein production (d), and ATP concentration (e) from a subsample of two treatments and one shared control using a UV ballast water management system. Error bars represent the standard deviation between replicate samples.

Sample ID	DNA	RNA	RNA:DNA Ratio	Enzyme activity	ATP
ClO_2-1	0.06	0.39	6.27	0.04	0.16
ClO_2-2	0.10	0.21	2.00	0.04	0.09
ClO_2-3	0.15	0.37	5.71	0.47	0.06
ClO_2-4	0.06	0.39	6.91	0.05	0.03
Averages	0.09	0.34	5.22	0.15	0.09

Figure 28. The magnitude of change between control and treatment samples in DNA concentration, RNA concentration, RNA:DNA ratio, enzyme activity (bulk FDA), and ATP after chlorine dioxide treatment during field ballast tests aboard the T/S *Golden Bear*.

Sample ID	DNA	RNA	RNA:DNA Ratio	Enzyme activity	ATP
UV-1	0.27	0.15	0.53	1.07	0.21
UV-2	0.24	0.44	1.85	0.98	0.11
UV-3	0.10	1.68	16.78	0.05	0.23
UV-4	0.12	1.48	12.50	0.07	0.22
UV-5	0.28	4.02	14.71	0.15	0.30
UV-6	0.12	0.77	9.55	0.01	0.06
Averages	0.19	1.42	9.32	0.39	0.19

Figure 30. The magnitude of change between control and treatment samples in DNA concentration, RNA concentration, RNA:DNA ratio, enzyme activity (bulk FDA), and ATP after UV treatment during ballast tests aboard the T/S *Golden Bear*.

Discussion

There is a critical need for robust planktonic biomass assays that can be carried out on convenient, portable devices, especially in the context of ballast water treatment efficacy testing and regulation. Time, complexity, and accuracy are just a handful of the factors that must be considered when looking for ideal compliance monitoring methods. Meanwhile, the fields of human and cell biology have accelerated the rate of advancement in molecular techniques, including the specificity and sensitivity of target nucleic acid detection. The primary goal of this thesis was to assess the latest molecular technology for its applicability within the framework of oceanographic and ballast water regulation requirements. The work completed here can be considered a preliminary investigation into the possibilities of rapid nucleic acid quantitation and other molecular methodologies that have potential in this field.

Nucleic Acid Quantitation Method: Successes and Areas of Improvement

The Qubit 3.0 bench top fluorometer and its corresponding assays are major methodological components of this study. Two nucleic acid assays were adapted and tested for applicability in phytoplankton and microbial oceanographic work specifically with this handy and portable device. Both assays benefit from having long-term storage capabilities, allowing batch processing and preservation for later analysis. The first, the Qubit dsDNA HS Assay, was quite successfully adapted to oceanographic work and resulted in a rapid, easy-to-use method that could specifically quantitate double stranded DNA. The analogous Qubit RNA HS Assay, however, utilized a dye that overlapped entirely with the natural fluorescence of chlorophyll, thereby precluding its utility in this

field of work. In order to work around this, RiboGreen was utilized instead and a protocol for the dye was adapted for use in the handheld Qubit fluorometer. Unlike the dye provided in the Qubit RNA HS Assay, RiboGreen lacks the high specificity for RNA and requires extra steps in order to ensure no DNA interference, e.g. elimination of DNA by means of DNase treatment. One advantage of the RiboGreen assay is its cost effectiveness – one RiboGreen kit can analyze 20,000 samples whereas the same cost applied towards a Qubit kit would only cover 1,000. Regardless, the field of phytoplankton biology would greatly benefit from improved kits and dyes that do not overlap with the chlorophyll fluorescence in the red end of the spectrum.

Perhaps one of the greatest areas for potential improvement is the extraction reagent. Because the primary purpose of this work was to investigate the utility of quantitating nucleic acids, much time could not be dedicated to fully optimizing finer details of the method. It was particularly difficult to settle on an extraction reagent, as there were trade offs between extraction efficiency and assay compatibility. Guanidinium thiocyanate was a highly desirable candidate for use in the assay. Not only is it efficient at lysing cells but it also protects nucleic acids by denaturing RNase and DNase enzymes. However, guanidinium thiocyanate suffered similar complications as that of the P-BAC ATP assay. Where P-BAC quenches the luminescent signal and must thereby be diluted with Tricine, guanidinium thiocyanate similarly interferes with the fluorescent dye signal in both the DNA and RNA assays. Tris-buffered saline with the addition of a mild detergent was eventually chosen as the reagent to be used throughout this study, primarily for its high compatibility with the assay dyes. Though it provided satisfactory results for

the purposes of this study, it is likely that a better extraction reagent exists and there certainly warrants further investigation to determine whether different formulations or perhaps even dilution steps could yield higher extraction efficiencies. Due to the relatively mild nature of TBS-S, it is also possible that certain planktonic species may be more resistant to extraction using this reagent.

Another area with potential for improvement is the sensitivity of the assays. For algal cultures, sensitivity is a nonissue. Healthy and concentrated cultures consistently provide an assay fluorescence signal an order of magnitude above the blank with as little as 5 milliliters filtered and any culture samples with low concentrations can receive a signal boost via increased volume filtrations. This type of signal boost, however, is more difficult to enact with environmental samples where biomass concentrations are significantly lower than cultures. Additionally, sediment and other debris make large volume filtrations much more complicated. Sensitivity in environmental samples may also be affected by particle interference that could hinder binding of the dye or quench the fluorescent signal.

To fully illustrate this sensitivity issue, a signal-to-blank comparison of relative fluorescence units (RFUs) in the nucleic acid quantitation assays was conducted on the cruise samples collected between 30 and 500 feet deep. Five hundred thirty-five milliliters of seawater was filtered for all of these samples. Ninety-two percent of the DNA assay signal-to-blank ratios were less than 10. Thus, sample signals were rarely 10 times higher than the blank. The RNA assay displayed even worse sensitivity, with all signal-to-blank ratios less than 10 and 44% less than 2. Nearly half of the RNA sample

signals were less than twice the size of the blank. For comparison, the analogous bulk FDA signals were at least one order of magnitude higher than the blank, with some reaching more than two orders of magnitude.

Investigation into a better extraction reagent is one route to improve the sensitivity. Another is to further concentrate and isolate the nucleic acids from the rest of the sample to reduce interference from other particles. This was initially considered and tested. However solid phase purification of nucleic acids requires different columns and reagents for DNA and RNA with concomitant differential loss, confounding the interpretation of RNA:DNA indexes. Companies such as Molecular Probes are constantly developing new, improved kits and assays and if such companies continue on this trajectory, dyes with increased sensitivity will hopefully be realized in the future.

Despite these areas of improvement, the method was confirmed successful in its ability to quantify nucleic acids and maintained consistency with other measurements. In culture growth experiments, cell counts were strongly correlated with DNA and RNA concentrations. In depth profiles of environmental samples gathered offshore, nucleic acid values followed that of other reliable measurements such as fluorescein production. Reliable data and valuable information can be successfully garnered by this technique.

Nucleic Acid Concentrations, Their Ratio and Growth Rate in Algal Cultures

DNA quantification per cell in twelve individual algal species confirmed Holm-Hansen's (1969b) results correlating DNA content with cell size (Figure 10). RNA content per cell was also positively correlated with cell volume, further confirming expectations of increased nucleic acids in larger cells. These data point to the potential

for estimates of cell enumeration based on nucleic acid concentrations. The variability in RNA:DNA ratios in these same algal cultures (Figure 11) demonstrates the dynamic range of possible ratios among phytoplankton species. The highest RNA:DNA ratio found in this sampling was 27 times greater than the lowest ratio measured. Relating RNA content with DNA in form of an RNA:DNA ratio may suggest other physiological aspects of a culture, especially its growth rate, and may thereby provide an indicator of health.

Flow-cytometric cell counts coupled with the corresponding RNA and DNA concentrations illustrated a strong relationship between growth rate and RNA:DNA ratio (Figure 15). Because both DNA and RNA exhibited obvious patterns of increase and plateau that matched that of cell counts, curves could be easily fitted to both nucleic acid metrics. The equations generated from the curves were used together to create a smoothed model for expected RNA:DNA ratios over time of the culture's growth. Although the raw, actual RNA:DNA ratios appear somewhat noisy at initial glance, an overlay with the smoothed model of the average RNA:DNA ratio implies that the data does in fact follow the expected trend. RNA:DNA ratios start high and peak early as the culture rapidly grows in its early stages. The ratios then begin to gradually decline as the culture reaches stationary phase and growth rate begins to decline.

Past studies have indicated that growth rate in cultured algae should be linearly related to the RNA:DNA ratio. Growth in the current study was measured by flow cytometric counts of individual cells. Slopes calculated between each adjacent cell count data point allowed for growth rate calculations for each period between measurements.

This data paired with RNA:DNA ratios calculated from raw nucleic acid measurements illustrated a positive linear relationship that strongly resembled the findings by Dortch (Figure 15).

The histogram in Figure 11 provides a general range of expected RNA:DNA ratios in algal cultures sampled at a random point in their growth cycle. Among species, the range of RNA:DNA ratios is approximately 27X. The data generated in the grow-out experiments provides insight into the RNA:DNA ratios that can be found within algal species throughout different stages of their life cycle (Figures 14 and 15). Between the lowest and highest measured growth, a range of about 5X is observed. Although this range is not particularly large, especially in comparison to other biomass proxy assays, the difference increases in subsequent tests where cells are treated.

Using Nucleic Acids to Measure Viable Biomass After UV Treatment

DNA has been shown to be a robust and persistent molecule, especially in comparison with RNA, which though thought to be more abundant in healthy cells, has a much shorter turnover rate and more potential opportunities to degrade with the high prevalence of RNases. It was hypothesized that following ballast treatment, DNA levels would remain relatively the same and RNA would experience a large drop in concentration. This was mostly confirmed in the bleach and heat treatment trials. Although both RNA and DNA experienced large drops in concentration following treatment, DNA experienced a less dramatic drop than RNA. This resulted in consistent reductions in the RNA:DNA ratio following treatment.

In contrast, UV treatment trials with algal cultures showed variable results. The effective UV dose for the single time-point trials fell between 3,500 to 6,600 mJ/cm^2, which is 8 to 16 times greater than the recommended dose suggested by Olsen et al. (2016) for permanent inactivation. Although RNA and DNA concentrations alone experienced decreases in all cases after treatment, the RNA:DNA ratio was highly variable, and sometimes greatly increased. DNA deviated from the initial hypothesis and displayed much higher susceptibility to UV treatment than expected. UV irradiation affects cells by damaging DNA and creating pyrimidine dimers; it does not necessarily destroy DNA or the cells themselves. However, the Qubit dsDNA HS assay utilizes a proprietary dye and it is highly possible that pyrimidine dimers on the DNA strands negatively affect the binding and activation of this probe. This may explain the larger decreases in DNA concentration after UV treatment in comparison to the other kill methods.

Destruction of RNAses by UV irradiation could be another possible culprit for the smaller than expected reductions of RNA. Sterilization against RNAses is known to be very difficult and typically UV irradiation would not be considered enough to fully prevent RNAse contamination. However, RNAse inactivation within these irradiated samples might explain the persistence of RNA. It would also be consistent with the large drops between control and treatment in the bulk FDA measurements, which specifically measures esterase activity.

Additionally, variability in the RNA pools may again be a factor in the inconsistency of post-treatment RNA:DNA ratios. Although samples chosen for UV

experiments were first measured using a pulse amplitude fluorometer to ensure a robust culture was being treated, this metric does not necessarily take into account growth rate. It is possible that algal cultures plucked for UV experiments were irradiated during stages where growth rates were simply not that high. Indeed, the culture that did display consistent reductions in RNA:DNA ratio was *Tetraselmis sp.* which over the course of this study exhibited a longer growth phase than other cultures.

It is important to note that measurements during all of these UV trials were taken at only a single time point. Meanwhile, the time series monitoring a control and UV treated algal culture over the course of 12 days beautifully illustrates a stark contrast between the two samples in every metric measured, even RNA:DNA ratio (Figures 25 and 26). The effective UV dose was approximately 1,000 mJ/cm^2; less than what the cultures in the previous single time point experiment experienced but apparently more than enough to illustrate a dramatic contrast over time between treatment and control. The changes induced by UV irradiation are emphasized as the control culture continues to grow and the treated subsample completely stagnates. Though cytograms indicated that physical particles were still present in the sample, the immense reduction in DNA and RNA concentrations and the significant drop in RNA:DNA ratios points to a culture that is no longer viable. Additionally, the dynamic range of RNA:DNA ratios is greater in the UV irradiated grow-out than the simple case of observing algal culture growth. The RNA:DNA ratio of the "healthiest" set of cells in this experiment is 30X greater than the UV treated "sickest" cells. This particular experiment supports the potential benefits of using nucleic acid quantitation for the purposes of evaluating UV treatments.

Field Ballast and the "Best" Indicator of Successful Treatment

One of the goals of this work was to evaluate the use of nucleic acid quantitation for ballast water treatment efficacy testing, especially in comparison to existing methods. It is important to reiterate that field ballast samples are subjected to several augmentation techniques, some of which are currently under scrutiny for creating unusual results such as enhanced bacterial growth. Though in theory, bacteria should flow through a 10 μm filter, it is possible for bacteria to colonize particles that could still be captured. Thus, augmentation may have affected results by generating compromised data, especially for treatment discharge. With this caveat in mind, the following discussion is an analysis of the data collected.

In contrast to what was expected, RNA:DNA ratio was a very poor metric overall to evaluate the effectiveness of a treatment. In all field ballast cases (both UV and chlorine dioxide treatments) RNA:DNA ratios of treatment samples were higher than control samples. This could be due to the factors discussed earlier with regards to variability in available RNA pools. In chlorine dioxide treatments, all four metrics evaluated – DNA, RNA, enzyme activity, and ATP – showed decreases between control and treatment. Considering the magnitude of decrease for each measurement, DNA and ATP appeared the most sensitive to this type of treatment, follow by bulk FDA and RNA respectively.

For UV treatments, DNA and ATP once again displayed the greatest reductions and most consistent results. Indeed, of the new nucleic acid metrics, DNA concentration is the only measurement to have consistently decreased after every single treatment,

regardless of treatment method. RNA on the other hand, performed quite poorly and actually increased in half of the UV treated samples. In order to find a potentially reliable nucleic acid metric for evaluating UV systems, further pursuit of DNA concentration alone appears worthwhile. Because there are still many unknown ways that UV affects these biomass proxies, determining UV efficacy remains complex. Overall, these results simply seem to confirm that UV treatments are notoriously difficult to reliably evaluate.

Conclusion

A rapid, simple nucleic acid quantitation assay using a handheld fluorometer was successfully adapted for oceanographic biomass. Though the method itself bears plenty of room for improvement, measurements using this method established a clear relationship between growth rate and RNA:DNA ratios in algal cultures. Because this method is user-friendly and samples can be stored for long periods of time, there is a great deal of potential for this nucleic acid quantitation to enrich further studies on algal physiology, growth and health. Although environmental samples could benefit from improved sensitivity, data still showed strong correlations with other established assays and areas of known biomass in depth profiles. Thus, nucleic acid quantitation using the Qubit 3.0 fluorometer has been proven to be effective in studies involving both algal cultures and environmental samples. Though the RNA:DNA ratio was hypothesized to be a potentially useful indicator of ballast water treatment success, especially in systems using UV, it proved ineffective. Changes in nucleic acid concentration on their own were comparable to enzyme activity and ATP measurements for both treatment types. DNA concentration especially presented itself as a potentially useful measurement as it consistently exhibited decreased concentrations following treatment. This work provides a jumping off point for future considerations in molecular techniques. Especially as technologies such as sandwich hybridization or next generation sequencing become easier to access, it is increasingly worthwhile to investigate the usefulness of these methods and their applicability to both the further study of living biomass in aquatic environments and in the ballast water management field.

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