UNIVERSITY OF CALIFORNIA
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A MODIFIED PROTOCOL FOR MONITORING SILICIC ACID UPTAKE IN NATURAL PHYTOPLANKTON ASSEMBLAGES

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Nilo Alvarado

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The Thesis of Nilo Alvarado
Is approved:

_____
Professor Raphael M. Kudela, Chair

_____
Professor Kenneth W. Bruland

_____
Dr. G. Jason Smith

_____
Tyrus Miller
Vice Provost and Dean of Graduate Studies

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Chapter 1: Introduction

The majority of primary productivity in the world's oceans occurs in surface waters where the availability of light and dissolved nutrients stimulate photosynthesis by phytoplankton. It has been proposed that the productivity and growth rates of phytoplankton are limited by the availability of nutrients such as carbon, nitrogen, phosphorus, with a certain subset of the phytoplankton community having an additional need for silicic acid. Diatoms are considered one of the most important contributors to the primary productivity of coastal waters, and require sufficient dissolved silica (Si) in order to form their frustules that provide cell structure. By monitoring the utilization of these nutrients in the environment, we gain insight as to the underlying factors that cause seasonal productivity and can use this information to further our understanding of the ocean.

In order to quantify the amount of Si incorporated into biogenic material over a period of time, the method of using radioisotopes was developed by Groening et al. (1973) who used ^{29}Si to quantify biosilicification in the waters of the Peruvian upwelling zone. The preparation of the ^{29}Si isotope requires a long purification process (Nelson and Gordon 1982), not conducive to shipboard work. These samples were analyzed using solid phase mass spectroscopy, which is a time intensive process. In 1991 Treguer et al. were the first to employ ^{32}Si as a tracer for biogenic silica (BSi) production by the natural phytoplankton

community of the Southern Ocean. Subsequent work on Si kinetics by M.A. Brzezinski began in 1997 when ^{32}Si became commercially available. The samples were analyzed by liquid scintillation counting and gas-flow proportional counting, which alleviates the time it takes for radio isotopic equilibration between parent ^{32}Si and daughter product ^{32}P. Results were comparable (within 30%) to the ^{29}Si measurements of Groening (Brzezinski and Phillips 1997), yet the expense of the ^{32}Si isotope constrains its use for Si kinetics measurements.

PDMPO [2-(4-pyridyl)-5{[4-dimethylaminoethyl-aminocarbamoyl)-methoxy]phenyl}oaxzole] was first used for oceanographic research by Shimizu et al. 2001, who used the fluorophore to measure the amount of biogenic silica produced in a diatom culture study. The PDMPO molecule was first synthesized by Diwu et al. (1999) **(Figure 1)** in order to stain acidic organelles within living eukaryotic cells. Diatom cells contain an acidic organelle called the silica deposition vesicle (SDV), where dissolved silica is polymerized before it is incorporated in new frustule material. The exact mechanism for PDMPO accumulation in the SDV is unknown. However the pK_a of the molecule is estimated at 4.47, which is the pH of many acidic organelles (Diwu et al. 1999), and it is believed that the protonation of the pyridyl and alkyl amino sidechain **(Figure 1)** contributes to the acidophilic nature of the molecule once inside the cell (Diwu et al. 1999). The ring structure of the PDMPO fluorophore has potential to be lipophilic in typical environmental conditions, which could cause

extracellular binding and accumulation on heterotrophic cells producing ectoenzymes. PDMPO has distinctive characteristic fluorescence at different pH **(Figure 2)**.

It was in 2005 that the first field application of PDMPO was published by Leblanc and Hutchins (2005). Here, the authors incubated water from the Delaware Bay in the presence of 0.125 μM PDMPO for a period of 24 hours, filtered the sample, then rinsed the filters with filtered sea water (FSW) in order to remove unbound or excess probe from the exterior of the cells.

We applied the PDMPO method as published by Leblanc and Hutchins (2005) in order to monitor silica utilization by the phytoplankton community at the Santa Cruz Municipal Wharf (SCW). Weekly samples were incubated for 24hours in the presence of 0.125 μM PDMPO from April 2011- February 2012. These samples were analyzed both qualitatively via microscopy and quantitatively via NaOH digestion of the filtered cells. The digested cells were analyzed on a spectrofluorometer for a measure of raw fluorescence units (RFU) from the PDMPO incorporated into the newly polymerized silica during the incubation period. These samples then received further processing to acquire a measurement of the total biogenic silica fraction of the sample via a colorimetric molybdate reaction. Based on the low pH of our assay (pH~3) we chose the excitation/emission wavelengths of 375nm and 530nm respectively for

quantitative analysis, which closely approximate the peak wavelengths at this level of acidity.

The PDMPO probe is valuable for qualitative analysis via UV microscopy. By utilizing the different filter sets of the microscope, it is possible to clearly identify different attributes of the sample. The chlorophyll excitation filter set provides visualization of which cells in the sample have viable chlorophyll and were actively photosynthesizing previous to filtration. The DAPI and long-pass DAPI filter sets are within the excitation/emission spectra of the PDMPO probe and provide a visualization of which diatom cells or which portion of a cell chain have produced new biogenic silica and incorporated PDMPO over the incubation period **(Figure 3)**.

Analysis of the SCW time series data showed abnormalities that became the subject of this thesis. We realized that on certain days where we measured unusually high RFU from the PDMPO probe, there were little to no diatoms present (the dominant siliceous phytoplankton type in Monterey Bay). This realization led us to two principal questions that this thesis will address. The first being what were the species present that could have produced high PDMPO RFU, (which we believe to be indicative of newly polymerized biogenic silica), when no diatoms were present? The second question raised was, can we modify the Leblanc and Hutchins (2005) protocol in order to reduce these seemingly false positive results?

Here we present the time series data from SCW that illustrates the cross reactivity of PDMPO with dinoflagellates in a natural phytoplankton community, as well as the modifications we made to the Leblanc and Hutchins 2005 protocol. We clearly show that using antibiotic rinsing agents removes the exteriorly bound probe from dinoflagellates with significantly less reduction in signal from target diatom species, both in culture and natural settings.

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Chapter 1 Figures

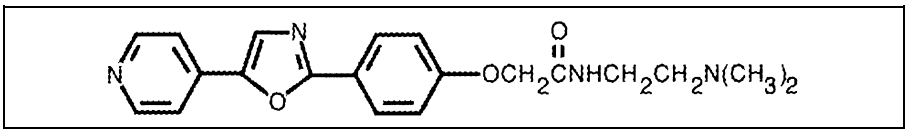

Figure 1. PDMPO molecular structure (Image: Shimizu et al. 2001)

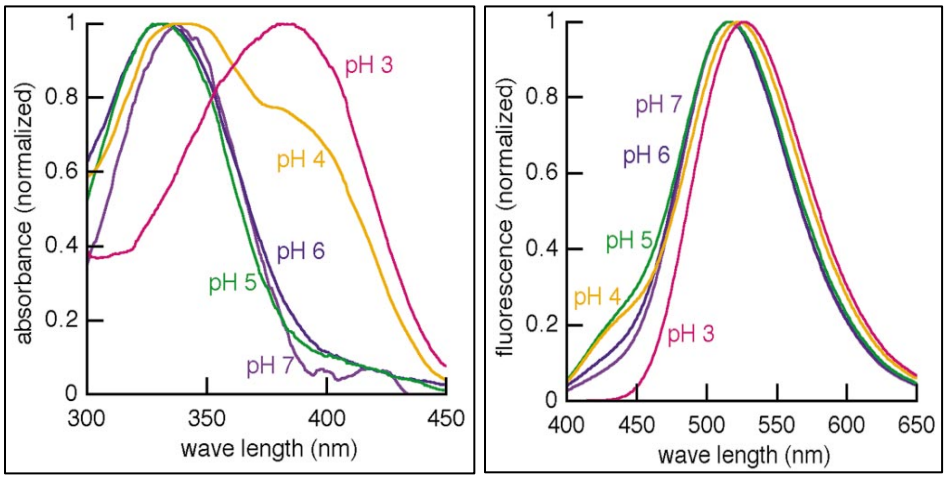

Figure 2. **Left:** absorption/excitation and **Right:** fluorescence/emission spectrum of PDMPO molecule at varying pH. (From Shimizu et al. 2001)

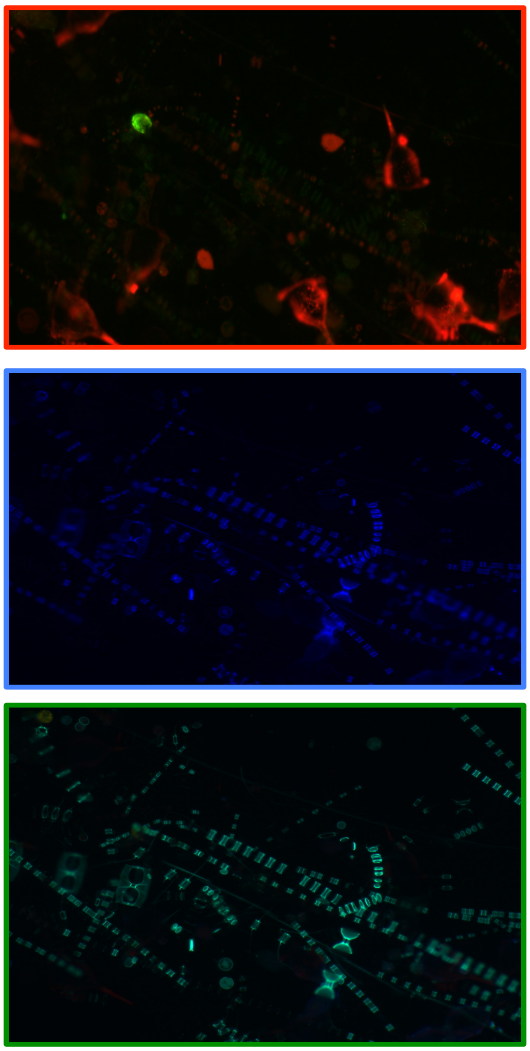

Figure 3. UV microscopic images of the mixed phytoplankton community at the Santa Cruz Wharf. **Top:** Chlorophyll excitation filter set; ex./em. 470/510nm (Red frame). **Middle:** DAPI filter set; ex./em. 350/460nm (Blue frame). **Bottom:** Long-pass DAPI filter set; ex./em. 350/420nm (Green frame).

Chapter 2: *A modified protocol for monitoring silicic acid uptake in natural phytoplankton assemblages*

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Abstract

Diatoms are important contributors to many biogeochemical cycles in aquatic environments due to their relatively large size and rapid growth rates. Amongst the factors that can limit their growth and productivity, the availability of silicic acid ranks high; the lack of this nutrient in the environment can also be the cause of increased toxicity in the diatom genus *Pseudo-nitzschia*. For these reasons, it is desirable to track the uptake and utilization of silicic acid by diatoms. Although stable and radio-labeled isotopes of silica exist, they are not commonly used by marine researchers because of the isotope cost and the complexity involved with analysis. PDMPO [2-(4-pyridyl)-5{[4-dimethylaminoethyl-aminocarbamoyl)-methoxy]phenyl}oaxzole] is a low-cost, commercially available fluorophore for monitoring new silica production by siliceous phytoplankton species. PDMPO has been used by a variety of authors to study natural diatom populations for identifying which parts of a cell or which proportion of the phytoplankton assemblage are actively polymerizing silica. These studies however do not acknowledge possible false positive results due to

exteriorly bound PDMPO on dinoflagellates and other non-siliceous organisms. Here, we use time series data from the Santa Cruz Municipal Wharf (Monterey Bay California) to show the reactivity of PDMPO with non-silicifying species in a natural, mixed phytoplankton community. Lab experiments using the ionophoric antibiotics monensin and nigericin, show that when used as rinsing agents, the extra cellular binding of PDMPO to various dinoflagellate species can be significantly reduced. We feel that this is an important development for the application of the PDMPO probe to natural community Si kinetics studies in order to reduce false positive data when samples have been digested for quantification via spectrofluorometric analysis.

Introduction

The photosynthesis carried out by phytoplankton in the world's oceans generates approximately 70% of the oxygen in the earth's atmosphere. Coastal regions of the ocean often support the highest rates of primary and new production (Ryther 1969), where the upwelling of nutrient rich water into the euphotic zone fuels phytoplankton metabolic activity. Optimum growth rates for diatoms require nutrients at a molar ratio of C:N:P:Si of approximately 106:16:1:18 (Redfield et al.1963, Brzezenski). In most coastally upwelled water, nitrate (NO3-) and silicic acid are found at a molar ratio of approximately 1:1 (Dugdale and Wilkerson 1989) indicating possible co-limitation of diatom

production by one or both of these nutrients. Monterey Bay, California experiences strong coastal upwelling of water that is approximately 20-30% higher in silicic acid relative to nitrate (Brzezinski et al. 1997). This upwelled water can support large spring diatom blooms that can persist into the summer until nitrate levels are depleted. The Santa Cruz Wharf located on the northern end of the Monterey Bay is an ideal location to study silicic acid utilization by the natural phytoplankton assemblage.

Distinguishing between cells that are live and actively depositing silica and those that have died or are in resting phase has typically been done using the radioisotope ^{32}Si. Disadvantages of this method include the scarcity of the isotope, and the necessity for several months exposure to photographic emulsion to make up for the generally small quantities of radio-label used for autoradiography (Trueger et al. 1991), although ^{32}Si can also be used to estimate bulk assimilation similar to ^{14}C and ^{15}N (Brzezinski et al. 1997).

In contrast to ^{32}Si, PDMPO exposure and utilization provides rapid (~4hour) results. The fluorescent probe has both membrane penetrating and acidotrophic characteristics (Diwu et al. 1999, Shimizu et al. 2001) allowing it to enter the cell membrane and accumulate in the low-pH environment of the silica deposition vesicle (SDV). Here, PDMPO binds to polymerizing silicic acid and is codeposited into the cell frustule. Regions of the cell that have deposited PDMPO

exhibit blue or green fluorescence with the use of standard excitation-emission epifluorescence microscopy. New accumulation of BSi can thus be quantified, in contrast to bulk methods such as biogenic silica measurements that include living and dead cells (Leblanc and Hutchins 2005) as well as potential contamination by lithogenic silica.

To date there have been a variety of experiments using PDMPO to monitor new BSi accumulation in natural diatom communities (LeBlanc and Hutchins 2005, Znachor and Nedoma 2008). However, there is no published data acknowledging the possibility of cross reactivity of PDMPO with dinoflagellates. Silica uptake and polymerization is not known to occur in the dinoflagellate species of Monterey Bay. However, there are a variety of exopolymers produced by dinoflagellates that could potentially bind PDMPO molecules to the exterior of the cell (Alldredge et al. 1998, Dyhrman, 2005) thereby producing false positive labeling.

Here we show that rinsing filtered cells with ionophoric antibiotics such as monensin and nigericin, reduces the exterior fluorescence of PDMPO associated with dinoflagellate cells without significant reduction of labeled diatom signal in both monophyletic and mixed culture species and for natural communities. We also demonstrate that PDMPO can be quantified spectrofluorometrically after digestion (dissolution) of biogenic silica frustules,

providing a more quantitative assay than that typically obtained by visual inspection of individual cells using epifluorescent microscopy.

Materials and Procedures

Sample collection

Starting in April 2011, weekly whole-water samples from the Santa Cruz Municipal Wharf (36 57.48′ N, 122 81.02′ W) were probed with PDMPO. An ongoing time-series from the same site provided macronutrient (NO_3, P, Si, urea, ammonium), chlorophyll, temperature, salinity, and relative abundance index (RAI) of diatoms and dinoflagellates, and other prominent phytoplankton (e.g. silicoflagellates) to the genus or species level. We were therefore able to determine the species present on days where PDMPO fluorescence was abnormally high and RAI of diatoms very low.

Seawater samples were made by integrating 10ft., 5ft. and surface water. Surface collection was via bucket sampling, while the 10 and 5ft. depths were by niskin bottle. Light microscopy observation of net tow samples were made in order to determine adequate sample volumes and to determine relative abundance counts of diatoms and dinoflagellates. Samples were incubated for 24hours in the presence of 0.125uM PDMPO in dark plastic bottles. This seawater was then filtered via low-pressure vacuum onto 25mm, 2um

polycarbonate filters, which were subsequently rinsed with filtered seawater (FSW). The sample filters were frozen at -20°C until the day of processing.

Processing

The frozen filters were brought to room temperature before being placed in 15mL polymethylpentene Nalgene (Cat. No. 3100) centrifuge tubes, to which 4mL of 2N NaOH (Sigma #S-8045) was added. Samples were placed in a 95°C water bath for 30 minutes then immediately placed in an ice bath, neutralized with 1mL of 1N HCl (Fisher#A508SK-212), and vortexed. Filters were then crunched down into the bottom of the tube with a clean spatula and spun down via centrifugation at 6 g for 10 minutes, to separate the lithogenic silica fraction. Approximately 3.5mL of supernatant was then pipetted into a quartz cuvette and read on a Molecular Devices SpectraMax M2e spectrofluorometer. Excitation/emission wavelengths 375nm and 530nm respectively were used, corresponding to peak spectra for the PDMPO probe at low pH (Diwu et al. 1999). Another 1mL of the NaOH digested supernatant was placed in a 50mL polypropylene tube (Corning #430829) for biogenic silica analysis via a colorimetric, ammonium molybdate method (Mullin and Riley 1955).

Microscopy

A 50mL aliquot of the seawater sample was separately incubated for 24hours at a final concentration of 0.125 uM PMPDO, and filtered onto 2uM,

black polycarbonate filters (Poretics #11061) for microscopy. These filters were subsequently mounted onto glass slides as per the LeBlanc and Hutchins (2005) method. Observations were made using a Zeiss Axio Imager (Model A1) and images were made using a Zeiss Axiocam HRc camera with AxioVision Rel. software (Version 4.6). The slides were observed under Chlorophyll (Chroma ex.470/em.510) and DAPI (ex. 350 Chroma-31000v2; em. 460 DAPI C-72145) excitation/ emission wavelengths, as well as a long-pass DAPI (ex. 350 Chroma-11000v3; em. 420 UV C-79999) filter sets.

Antibiotic preparation

Monensin (Sigma# 46468) and nigericin (Fisher# 50-230-6457) antibiotics were each suspended in reagent grade ethanol (Decon Laboratories # 2816), and stored at 4°C until day of processing. A final concentration of 10uM for each antibiotic was made in approximately 15-20mL Mili-Q water and was used to rinse filters in order to remove the exteriorly bound probe.

Assessment

Reducing the signal of non-silica bound PDMPO from the exterior of dinoflagellates

A culture experiment was performed in order to determine if the adherence of the probe to the exterior of dinoflagellates could be diminished by the application of monensin and nigericin, both ionophoric antibiotics. A culture

of *Prorocentrum micans* (Pror MB1206b) was grown in f/2 enriched seawater to a concentration of 8730 cells/mL. 25mL of culture was incubated with 0.125uM PDMPO for 4 hours. The samples were then treated two ways: One set of filters was rinsed with several milliliters of filtered seawater (FSW) as described by LeBlanc et al. (2005). The second set was rinsed with several milliliters each of 10uM nigericin and 10uM monensin ionophores before filtering. 2ml of culture per filter were rinsed and either mounted on glass slides for microscopic observation, or frozen at -20C for quantitative analysis via spectroflourometry. Qualitative analysis via UV microscopy shows the fluorescence of the antibiotic treated samples was greatly reduced at both the 350/460nm **(Figure 1 top row)** and 350/420nm **(Figure 1 bottom row**) excitation/emission spectra. Further analysis of the antibiotic treated samples via spectrofluorometer showed a reduction in signal of approximately 70% vs. the FSW treatment, when read at 375/530 excitation/emission wavelengths respectively **(Table 1).**

The effects of ionophoric antibiotics on silica bound PDMPO in diatoms

A similar experiment was carried out using 8ml of cultured diatom *Thalassiosira pseudonana* (CCMP 1015) at a concentration of 540000 cell/mL. This sample was incubated in the presence of 0.125uM PDMPO for 4hours before being split, filtered and rinsed with either FSW or monensin and nigericin. **Table 1** shows a reduction of PDMPO fluorescence of only 36% for the

antibiotic rinsed filter vs. the FSW treatment, indicating that the ionophoric properties of the antibiotics are more efficient at reducing the fluorescence of externally bound probe on dinoflagellates over diatoms, or alternatively that most of the signal produced by PDMPO in diatoms is from internally bound probe while the PDMPO fluorescence associated with dinoflagellates is loosely attached to the exterior of the cell. The 36% reduction in fluorescence for the diatoms was presumably caused by removal of surface-bound probe that had not been incorporated internally into the cell structure.

Culture experiment simulating mixed populations of dinoflagellates and diatoms

2 separate cultures of *Thalassiosira pseudonana* and *Prorocentrum micans* were grown to cell densities of 520000 cell/mL and 250 cell/mL respectively, in order to make a synthetic assemblage of diatoms and dinoflagellates. The two cultures were combined at a ratio of 3:1mL *Prorocentrum* to *Thalassiosira*, respectively. Individual replicates were also made at the same cell densities used for the mixed assemblage. These cultures were incubated in the presence of 0.125uM PDMPO for 4 hours. The cultures were then split, concentrated onto filters, and rinsed with either FSW or antibiotics. **Table 1** shows that the antibiotics reduced the fluorescent signal of the dinoflagellate culture by 39% vs. 21% in the diatom culture. The mixed culture showed a reduction of signal by 43%, indicating that much of the extracellular bound probe had been rinsed

from the dinoflagellate cells, and that the remaining signal was primarily associated with the more dense *Thalassiosira* culture

Natural community response to antibiotic rinsing protocol

During the spring diatom bloom of 2012, water from SCW was collected and incubated in the presence of 0.125uM PDMPO for several hours. This water was dominated by diatoms, with little to no dinoflagellates present (determined by microscopy) **(Figure 2 bottom)**. **Table 1** shows that PDMPO fluorescence was diminished by 20% in one experiment, and 8% in another, indicating that using antibiotics in natural populations of diatoms is not detrimental to the signal, consistent with the laboratory results.

Discussion

The PDMPO probe has effectively been used in both field and lab studies to monitor silica utilization by diatom dominated phytoplankton populations. As an example of application of this method, we demonstrate that the probe can potentially be used as an indicator of cellular toxicity in toxigenic strains of the diatom genus *Pseudo-nitzschia*. **Figure 2** shows PDMPO fluorescence (normalized to BSi) relative to dissolved silica and particulate domoic acid (pDA). Here we see elevated PDMPO incorporation corresponding to decreasing dissolved silica concentrations and increasing cellular toxicity during the spring and fall diatom blooms in Monterey Bay **(Figure 3)**. These data are consistent

with a range of laboratory experiments (e.g. Pan et al. 1996) showing that silica depleted media can increase the toxicity of pennate diatom species like *Pseudo-nitzchia australis,* the dominant phytoplankton on the days where pDA levels surpassed 100ng/mL as on June 2 and August 17, 2011 **(Figure 3)**.

Past attempts to link *Pseudo-nitzschia* cell abundance to toxin levels have been met with limited success (Bates et al., 1989, Villac et al. 1993). While the presence of cells is a prerequisite for toxin production, natural populations exhibit widely ranging differences in per-cell toxicity, limiting the utility of cell counts as a predictor of ecological impacts. To test whether PDMPO labeling provides useful insight about toxin production, we analyzed the time period from April 2012- February 2012 **(Figure 3)** when diatoms were dominant (based on RAI) and *Pseudo-nitzschia* was present **(Figure 4)**. This allowed us to analyze the data without adjusting for non-specific labeling of dinoflagellates, which were negligible during this period. Our data show an increase in predictability of pDA compared to using cell abundance alone from R^2=0.39 to 0.62 when the first derivative of the weekly PDMPO signal normalized to chlorophyll (ie. biomass-normalized PDMPO) is used as a predictor of pDA in a multiple linear regression with *Pseudo-nitzschia* abundance, PDMPO labeling, and pDA.

We infer that the change in PDMPO fluorescence is a proxy for the growth rate of the diatom assemblage, and that the predictability of toxicity improves significantly because toxin production generally increases as growth rate decreases in response to the lack of abundant nutrients (Maldonado et al. 2002, Fehling et al. 2004). Thus quantification of the rate of silica uptake provides an important and relatively easy to assess metric for improving the predictability of toxic events in natural populations. While not done in this study, it would also be possible to use probes for *Pseudo-nitzschia* (Scholin et al. 1996, Miller & Scholin 1998) and PDMPO on the same sample, thus providing species-specific proxies for growth rate, potentially improving the relationship between cell number, growth and toxin production.

Our adaptation of a biogenic silica digestion method using NaOH in order to quantify PDMPO accumulation is ideal for making quick and quantifiable measurements compared to traditional methods such as direct counting of cells via microscopy, and is far more cost and time-effective than radioisotope labeling such as with ^{32}Si. As shown by Shimizu et al. (2001), the emission peak of the PDMPO/Si complex is pH dependent which lends an advantage to reading digested samples on a spectrofluorometer with adjustable excitation/emission wavelengths. The use of spectrofluorometry can also account for the intensity of labeled cells, as opposed to the presence/absence signal provided by microscopy.

This measurement can be confounded however, by the presence of dinoflagellates in the phytoplankton community. Time series data from the Santa Cruz wharf shows that on days where there were little to no diatoms in the water **(Figure 2)**, there can be high PDMPO fluorescence due to the abundance of dinoflagellate species, such as *Prorocentrum micans, Akashiwo sanguinea,* and *Noctiluca scintillans* **(Figure 5)**. Externally bound probe on non-silicifying cells can produce signal detectable by both epifluorescent microscopy **(Figure 5)**, and spectrofluorometry of digested samples **(Table 1)**. Dinoflagellates are known to exude a variety of exoploymers that could potentially cause nonspecific binding of the PDMPO probe to the exterior of the cell. For example, the naked dinoflagellate, *Prorocentrum minimum* has been shown to use ectoenzymes such as alkaline phosphatase and leucine aminopeptidase for extracellular cleaving of organic molecules (Dyhrman, 2005). Given the chemistry of the probe binding sites it is likely that these ectoenzymes would bind the probe. While this issue can be rectified by direct microscopic identification of the probed cells, this returns the data to qualitative, time-consuming analysis. Although there is some reduction of signal when siliceous species are rinsed with antibiotics **(Table 1)**, the remaining fluorescence is representative of the newly formed BSi within the frustule. This suggests that whether using microscopy or spectrofluorometry, it is prudent to include an antibiotic wash to minimize non-specific extracellular binding.

As these laboratory experiments have shown, a relatively small number of dinoflagellates relative to diatoms in a sample can alter the quantitative signal produced by silica/ PDMPO complex accumulation. Microscopy has revealed that the exteriorly bound probe is not entirely removed by rinsing filters with FSW alone (Leblanc and Hutchins 2005) and that ionophoric antibiotics should be used for true representation of PDMPO labeled biogenic silica accumulation.

Conclusions

When using the PDMPO probe to study natural populations it is important to couple the quantitative and qualitative aspects of the data via microscopy, even in diatom dominated communities. Visual identification of which species or which proportions of the sample have reacted to the probe is invaluable for making accurate assessments of silica polymerization. This step is critical when the sample is dissolved for spectrophotometric analysis, which provides quantification but loses the species-specific information.

The use of microscopy in conjunction with quantitative measurements is necessary for distinguishing the live fraction of BSi measurements, as well as determining the presence of non-target organisms contributing to the fluorescence signal when the sample has been digested. However, the potential for non-target organisms to produce significant false positive signals in natural communities requires the use of ionophoric rinsing agents for valid

representation of new BSi. This becomes more pertinent with long term data series, where seasonal shifts in the phytoplankton assemblage are prominent in many marine environments. Despite the potential issues introduced with non-diatom binding of PDMPO when using the spectrofluorometric (digestion) method, the benefits in terms of quantification, ease of processing, and ability to generate time-series and directly compare results from different regions outweighs these potential issues, so long as an antibiotic wash step is included.

There is potential for future optimization of the use of antibiotic rinsing agents. This could include alterations in the volume and number of rinses applied to the filters before preservation, as well as a thorough analysis of the reactivity of suspended antibiotics over time. The exploration of using imaging software to quantify fluorescent signal from microscopic images would also provide a useful correlation for this methodology.

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Chapter 2 Figures

Date	Target	PDMPO RFU % Difference	Difference in Normalized Fluorescence (RFU/uM BSi)
3/28/12	*Prorocentrum micans*	-70%	15.5
4/4/12	*Thalassiosira pseudonana*	-36%	0.036
4/18/12	SCW; Diatom dominated	-20%	0.86
4/25/12	SCW; Diatom dominated	-8%	0.42
5/1/12	*Prorocentrum micans*	-39%	0.006
5/1/12	*Thalassiosira pseudonana*	-21%	0.019
5/1/12	Combined *P. micans* and *T.pseudonana*	-43%	0.013

Table 1. Laboratory experiments using modified protocol with antibiotic rinse vs. filtered seawater. Shown here are the treatment types and corresponding percent differences in signal between filters rinsed with filtered seawater (FSW) and antibiotics.

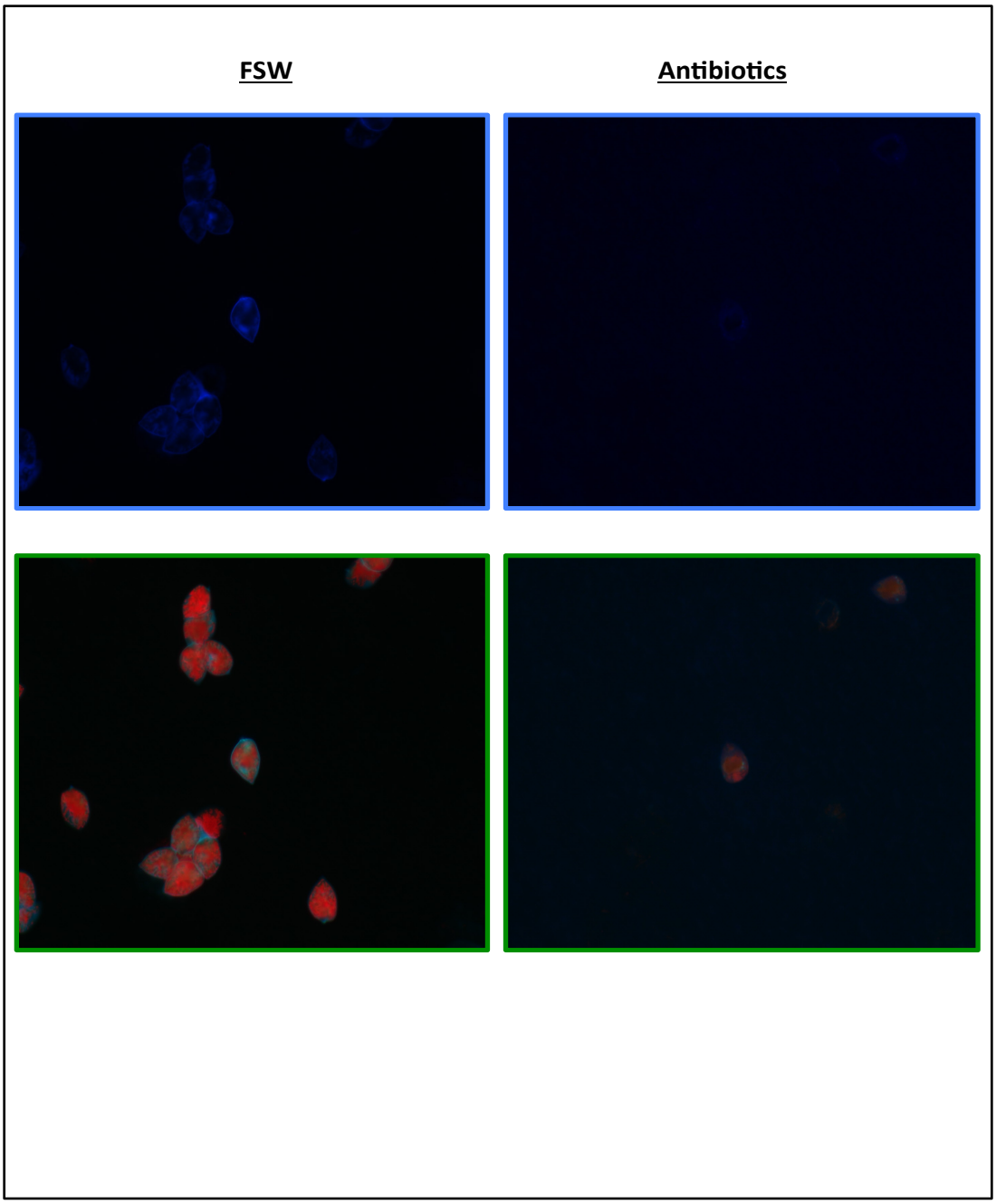

Figure 1. UV microscopic images of *Prorocentrum micans* culture under DAPI (Blue frame, top row), and Long-pass DAPI (Green frame, bottom row) filter sets. **Left column:** filters rinsed with FSW. **Right column:** filters rinsed with monensin and nigericin antibiotics.

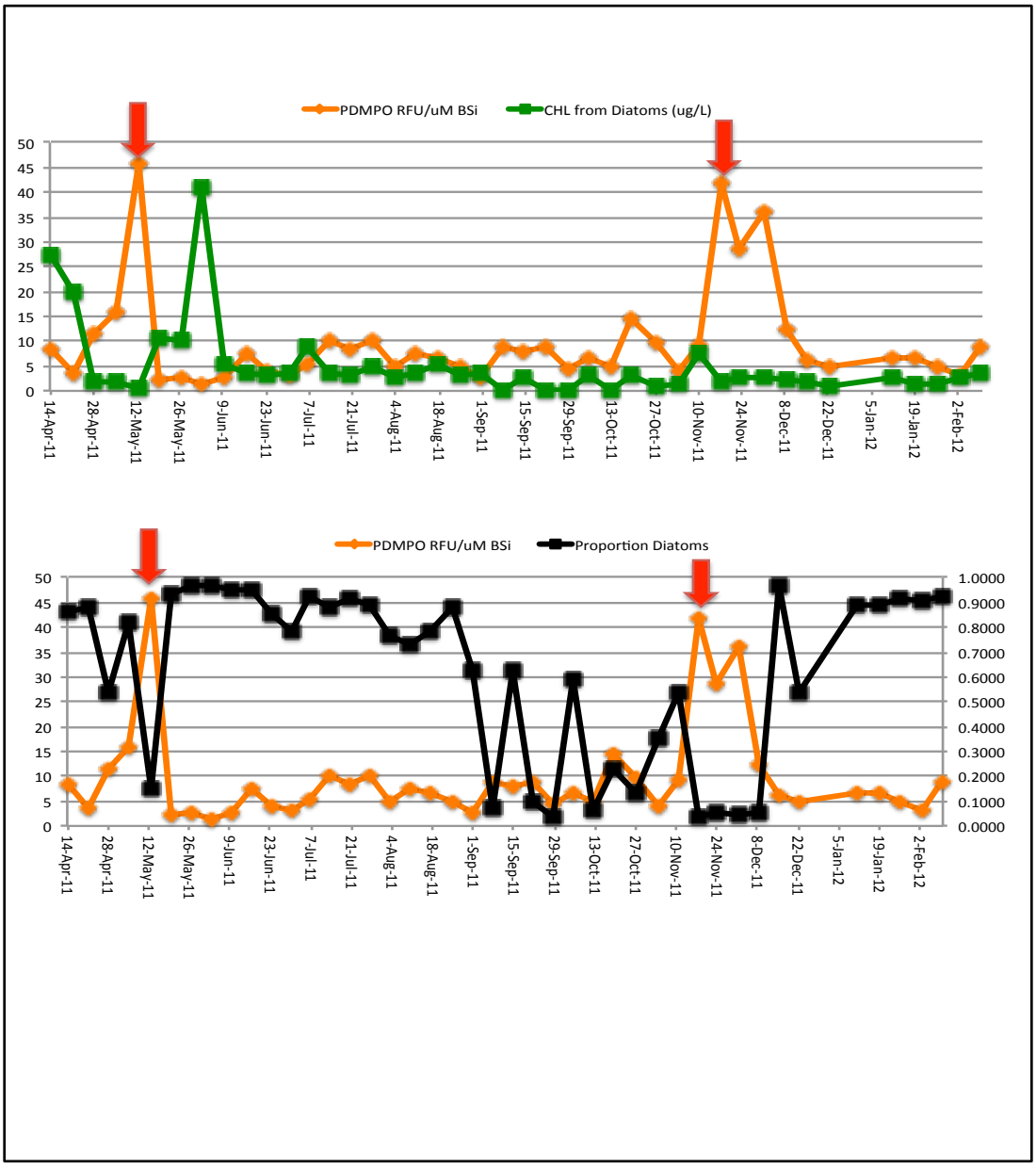

Figure 2. Top: Time series data showing PDMPO fluorescence normalized to biogenic silica and the amount of chlorophyll produced by diatoms. **Bottom:** Normalized PDMPO and the diatom proportion of the phytoplankton community at the Santa Cruz Municipal Wharf. Arrows indicate high fluorescence values on May 12 and November 11 2011, when the relative abundance of diatoms are 15 and ~4% respectively indicating false positive results from dinoflagellates.

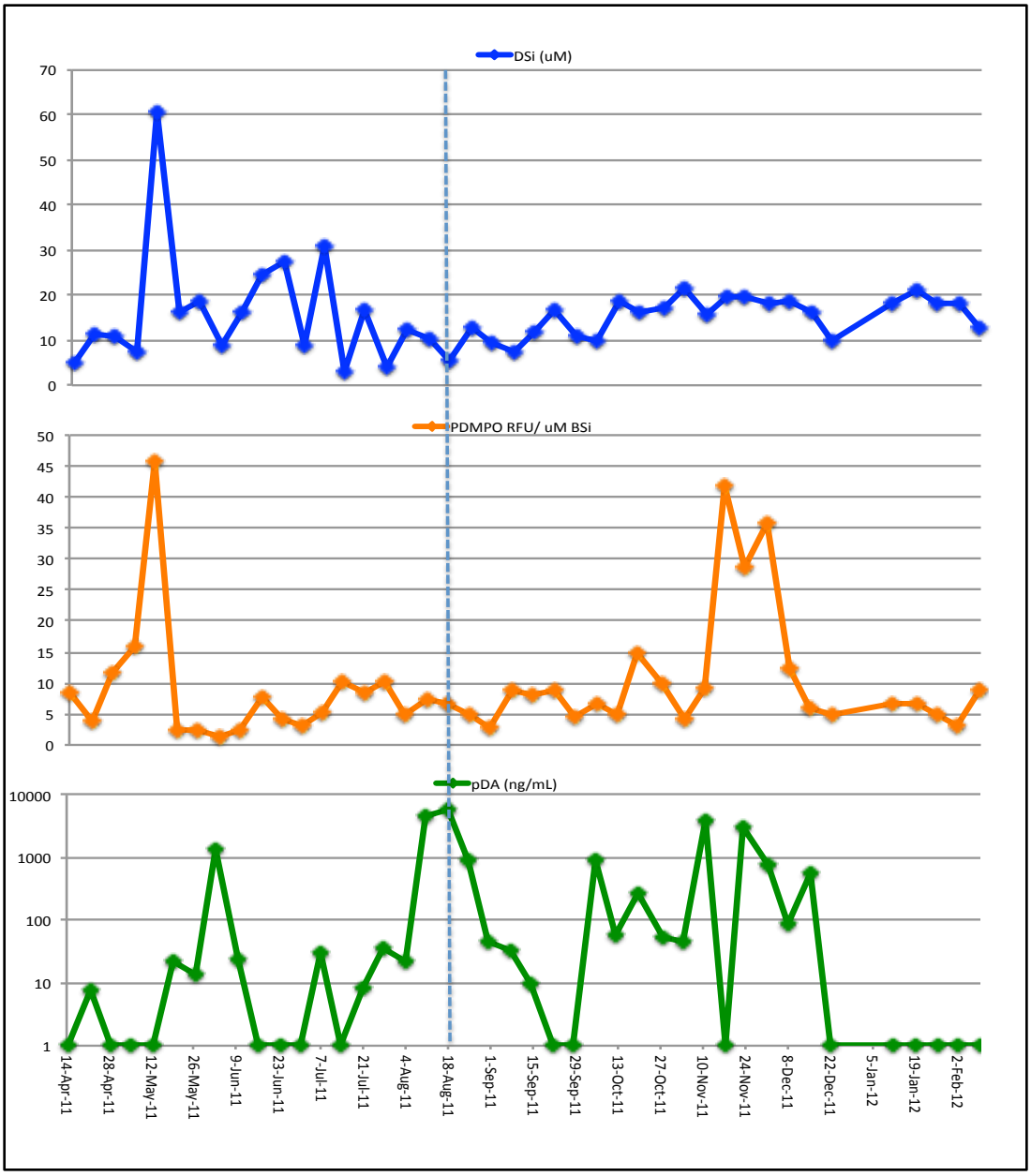

Figure 3. Time series data showing dissolved silica (DSi) **(Top)**, PDMPO fluorescence normalized to biogenic silica (RFU/BSi) **(Middle)** and particulate domoic acid (pDA) **(Bottom)**. Note: pDA values correspond to a logarithmic scale. Blue dashed line shows high cellular domoic acid content correlating to low dissolved silica and corresponding low PDMPO activity.

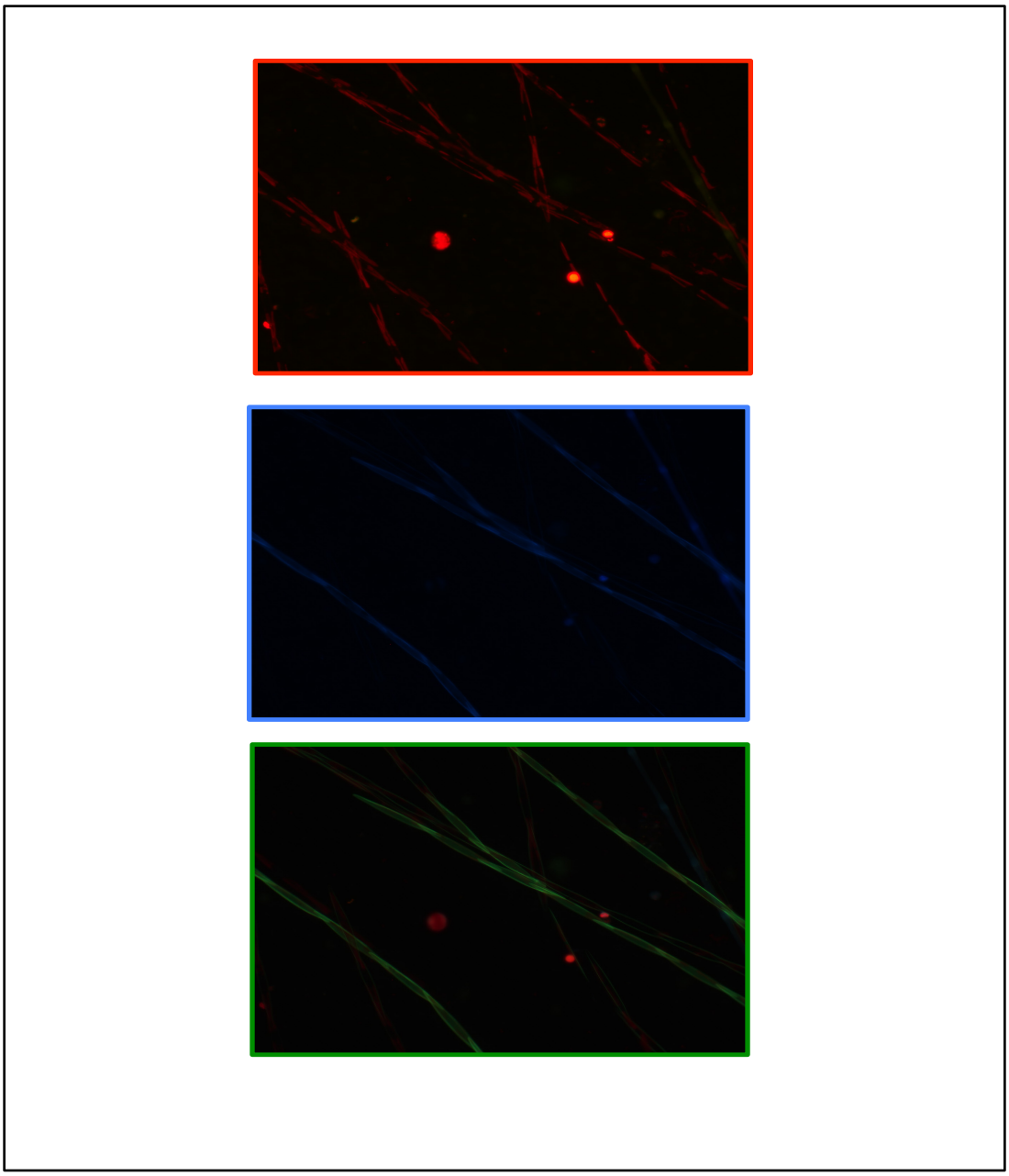

Figure 4. UV microscopic image from SCW showing *Pseudo-nitzschia australis* under: Chlorophyll excitation **(Top),** PDMPO fluorescence under DAPI **(Middle),** and Long-pass DAPI filter sets **(Bottom)**.

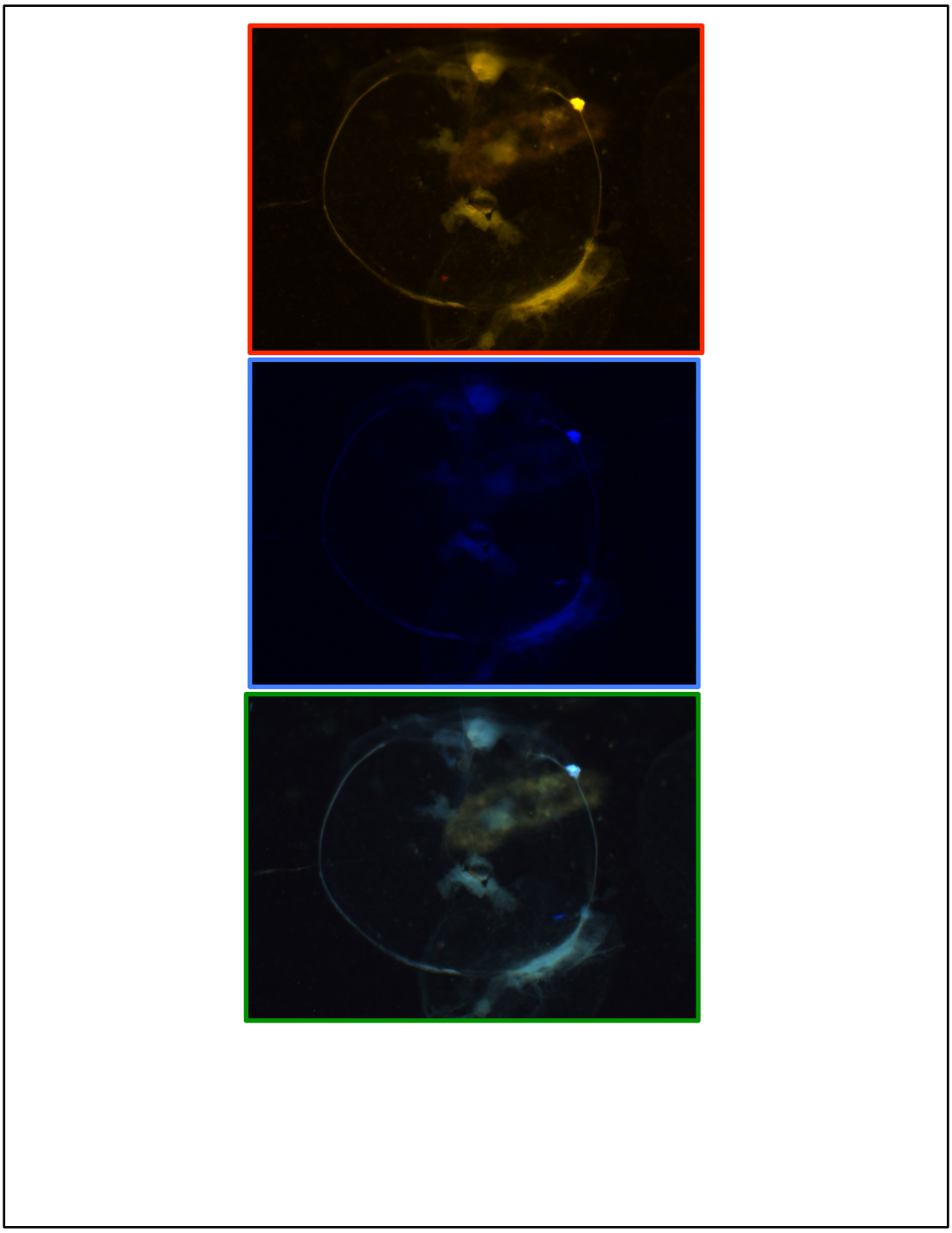

Figure 5. UV microscopy images of *Noctiluca scintillans* showing emission patterns indicative of the silica-PDMPO complex fluorescence from SCW on May 12 2011. Colored frames indicate different filter sets as in **Figure 4.**

Chapter 3: Conclusions

In summary, past usage of PDMPO to monitor silica utilization in a natural, mixed phytoplankton community failed to acknowledge potentially false positive results from the extracellular binding of the probe to non-siliceous species. The method of using PDMPO to monitor silica utilization by diatoms was initially published in 2001 by Shimizu et al., yet we see that over the last decade there have been few published papers using this technique. We believe that our adaptation of the method by means of using the antibiotic rinse when studying mixed assemblage phytoplankton populations will alleviate some of the issues other researchers have had using the PDMPO probe in natural settings. We illustrated the utility of our modified protocol by showing a side-by-side comparison of RFU from PDMPO accumulation via our method with that previously published for both the qualitative and quantitative assays.

It should be noted that the exact nature of the interaction between the PDMPO molecule and the ionophoric antibiotics is not known at this moment. It could be theorized however that the negative charge of the alcohol groups the antibiotics **(Figures 1 and 2)** would have at the pH of seawater could help disrupt the charge of the lipid bilayer of the cell wall, allowing the exteriorly bound probe to release and be rinsed away. It was also suggested by my committee member Jason Smith, that it could simply be the ethanol that the antibiotics are suspended in that is the active component of the rinse solution

responsible for removing the PDMPO from the outside of the dinoflagellate cells. This would be a good first step for future research, as the cost of reagent grade ethanol is significantly less than that of the monensin and nigericin antibiotics, and this would simplify the protocol. There is also potential for future optimization of the use of antibiotic rinsing agents, which could include alterations in the volume and number of rinses applied to the filters before preservation, as well as a thorough analysis of the reactivity of suspended antibiotics over time. The exploration of using imaging software to quantify the fluorescent signal from microscopic images would also provide a useful accompaniment for this methodology.

Our research has shown that when using PDMPO in mixed assemblage phytoplankton communities, the use of antibiotic rinses is necessary for a discrete measurement of biogenic silica production. Qualitative assessment via UV microscopy should also accompany any quantitative measurements in order to determine the presence and abundance of non-silicifying species such as dinoflagellates, which could potentially alter the signal from the PDMPO labeled biogenic silica in the sample.

Chapter 3 References

Shimizu, K., Del Amo, Y., Brzezinski, M.A., Stucky, G.D., and D.E. Morse. 2001. A novel fluorescent silica tracer for biological silicification studies. *Chemistry & Biology* 8: 1051-1060

Chapter 3 Figures

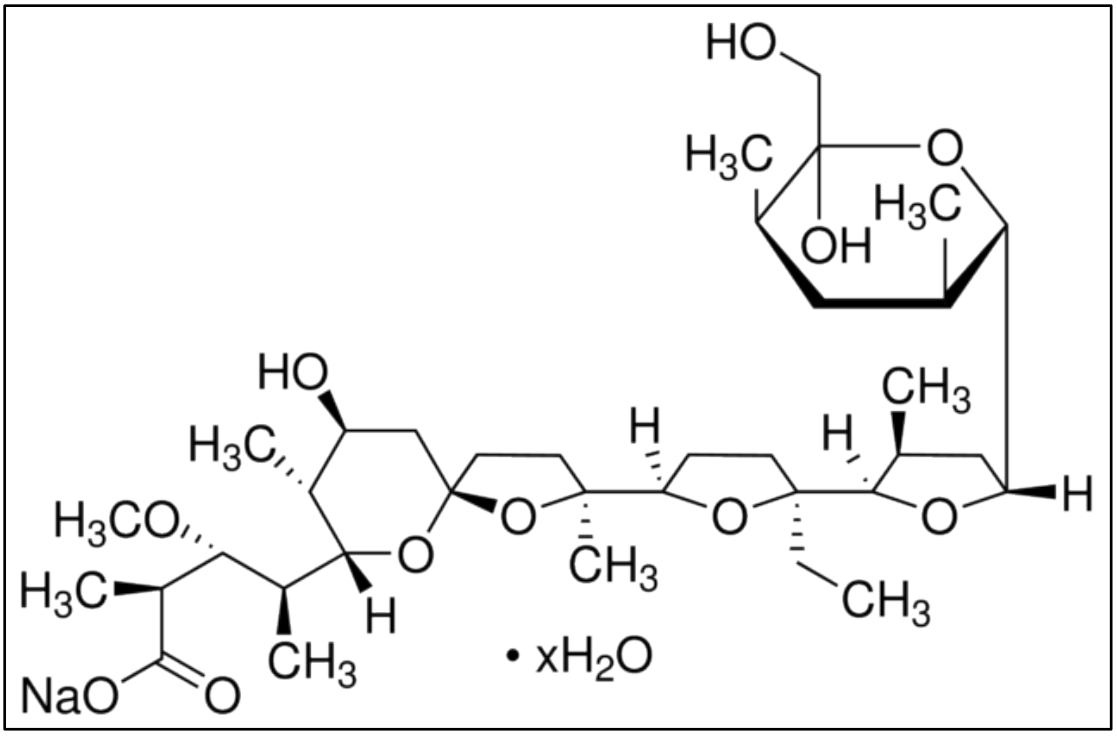

Figure 1: Molecular structure of Monensin antibiotic
Image: ©Sigma-Aldrich
<http://www.sigmaaldrich.com/catalog/product/sigma/m5273?lang=en&region=US>

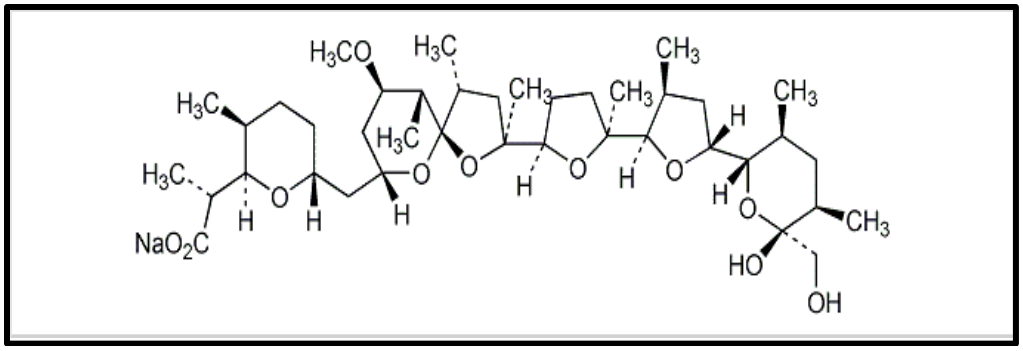

Figure 2: Molecular structure of Nigericin antibiotic
Image:© Merck KGaA
<http://www.emdmillipore.com/life-science-research/nigericin-sodium-salt>