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The Effects of Polyunsaturated Aldehydes on Pelagic Microbial Food Webs in the Chesapeake Bay Area and Atlantic Coastal Waters

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Honors Research Project

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Abstract: Diatoms, one of the most common classes of phytoplankton, are autotrophic plankton, and are the primary food source for many organisms. Diatoms play a key role in nearly all oceanic food webs. Under stressful conditions, diatoms can undergo biological changes that produce cytotoxic secondary metabolites such as polyunsaturated aldehydes (PUA's). This process is a form of allelopathy, utilized by many algae as a defense mechanism against predation, as well as to inhibit competitors. Microzooplankton are an essential part of these aquatic food webs as well, as they are a key herbivore in many oceanic environments. A set of experiments was performed using dissolved PUA (2E,4E-octadienal and 2E,4E-heptadienal), natural phytoplankton and microzooplankton, and the copepod *Acartia tonsa*. This study focused on the effectiveness of PUA's in inhibiting microzooplankton predation on marine phytoplankton, as well as the extent of growth rate reduction of diatom competitors. The results of this study showed significant changes in phytoplankton grazing loss rates for picoplankton and larger phytoplankton in the presence of PUA in 8 of 10 experiments. On the other hand, PUA treatments did not induce significant changes in the phytoplankton growth rates.

1. Introduction

Diatoms contribute between 40 and 45% of the total primary production in oceanic environments (Yool & Tyrrell, 2003). Allelopathy is a common mechanism used by diatoms and other marine phytoplankton, defined as the production of chemicals to inhibit the growth of competitors and deter grazers (Strom, 2008). A specific class of allelopathic chemicals known as oxylipin derivatives are cytotoxic secondary metabolites. Polyunsaturated aldehydes (PUA's) are the best known of these metabolites, with many recent studies shedding light on their effect on other marine organisms (Miralto, et al., 1999). PUA's result from the oxidative cleavage of polyunsaturated fatty acids in many diatom species when in stressful conditions or when the diatom cells sustain damage (Ianora, Miralto, & Romano, 2012). Two primary PUA's that are released by diatoms are (2E,4E)-octadienal (OD) and (2E,4E)-heptadienal (HD),

however, others such as decadienal have negative effects on flagellar activity of starfish sperm cells (Caldwell, Bentley, & Olive, 2004). The full purpose of the PUA's has not been clearly defined, however it is believed that they act to both deter the predation on the diatoms by microzooplankton, and to inhibit the growth of their competitors, such as oceanic cyanobacteria and other species of phytoplankton (Ianora, et al., 2011).

Key diatoms such as *Skeletonema costatum*, *Pseudonitzschia delicatissima*, *Thalassiosira rotula* (Miralto, et al., 1999), and *Skeletonema marinoi* all produce PUA's such as the aforementioned octadienal and heptadienal compounds (Sarno, Kooistra, Medlin, Percopo, & Zingone, 2005). The release of PUA's is linked to cell death in these species, as well as in response to predation as a defense mechanism (Pohnert, 2000; Fontana, et al., 2007). It has been historically thought that the natural predators to diatoms include mesozooplankton (200-2,000µm) such as the copepods (Smetacek, 1999). Little is known regarding the role of allelopathic compounds in pelagic food webs.

Specifically, the role that PUA's have in influencing trophic interactions of microzooplankton with phytoplankton and competing marine bacteria remains unclear. Initial studies show that ambient concentrations of dissolved PUA can have negative effects on natural microzooplankton growth rates when added to whole sea water samlpes (Larentyev, Franzè, Pierson, & Stoecker, 2015). Additionally, PUA's have a collective negative effect on the reproduction rates of mesozooplankton (Miralto, et al., 1999; Fontana, et al., 2007). However, the full extent to which allelopathic chemicals change the dynamic of phytoplankton-bacteria-microzooplankton trophic interaction remains unclear. Obtaining detailed information regarding growth rates of diatoms and their competitirs and grazing rates of microzooplankton within these systems is essential to understanding the intricacies of these oceanic food webs.

This study, originally designed, performed and over seen by Larentyev, Franzè, Pierson, & Stoecker, (results published in 2015), sought to obtain insight into the specific variations in the growth rates of phytoplankton communities, as well as changes in grazing rates of microzooplankton in response to the introduction of PUA's to natural water samples. My involvement in this experiment was centered around the processing of samples by flow cytometry, as well as data acquizition and analysis of samples acquired during the original study. This portion of the study was novel in its findings, but related to the previous study performed in 2015. Specific predictions of this study included: (H1): a reduction in the growth rates of phytoplankton with the addition of PUA; ($H1_0$): no change in growth rates of phytoplankton after addition of PUA; (H2): reduced microzooplankton herbivory of diatoms in the presence of PUA; and ($H2_0$): no change in microzooplankton herbivory of diatoms in the presence of PUA. This study set out to examine these hypotheses using natural marine communities from coastal waters and dissolved PUA treatments to simulate a diatom bloom.

2. Materials and Method

2.1 The Study Sites and Sampling

In April 2013, whole seawater was collected from a tributary to the Chesapeake Bay, the Choptank River, at the Horn Point Laboratory of the University of Maryland Center for Environmental Science (HPL, Cambridge, MD). Samles were collected of the side of the dock. In May 2014, we returned to the Choptank River to gather a second sample and an additional seawater sample from the Chesapeake Bay at Ragged Point. In September, 2014, whole seawater samples were collected from the Virginian Atlantic coast, specifically at the Viginia Institute of Marine Science Eastern Shore Laboratory (ESL), in Wachapreague, Virginia, as well as the Wachapreague Inlet. These locations can be found in Figure 1. Multiple locations were sampled to gain a holistic perspective of oceanic microbial food web interactions. Samples were collected by those who conducted the study published in 2015, then frozen and transported back to the University of Akron for later processing.

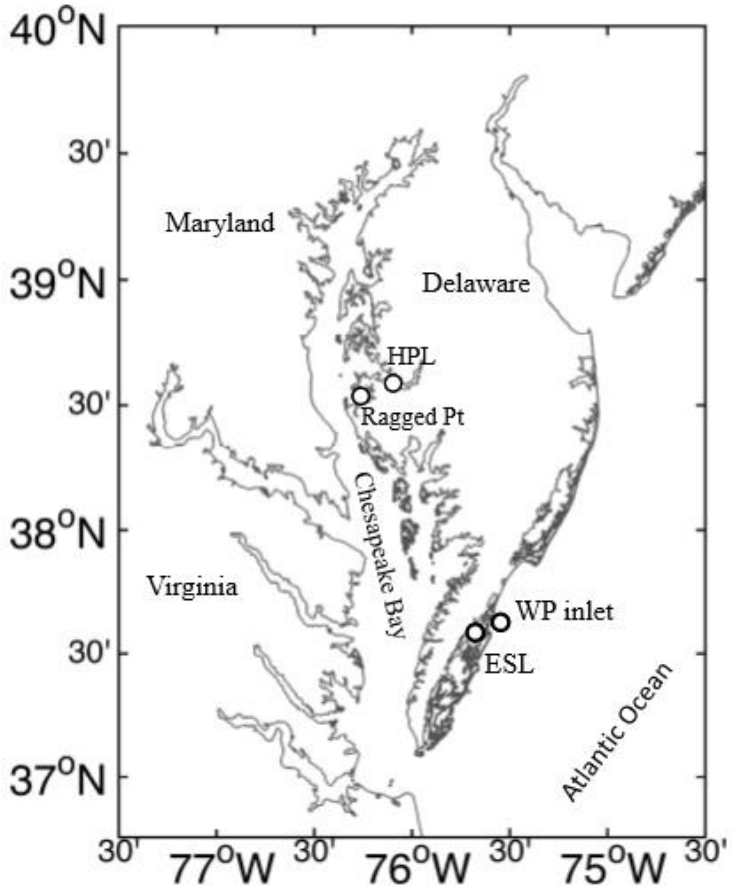

Figure 1. The retrieval sites at the Viginia Institute of Marine Science Eastern Shore Laboratory (ESL), in Wachapreague, Virginia, as well as the Wachapreague Inlet. Source: Larentyev, Franzè, Pierson, & Stoecker, 2015.

The water column locations and ambient PUA concentrations are shown in Table 1 (Larentyev, Franzè, Pierson, & Stoecker, 2015). Dissolved HD concentrations ranged from 0.004 nM at Ragged Point in the Chesapeake Bay to 0.060 in the Wachapreague Inlet. Particulate levels of HD and OD were both elevated at the Eastern Shore Lab (ESL), relative to the others. Total ambient PUA levels were higher in the Atlantic water sites (0.077 and 0.080 nM in Exp. 4 and 5, respectively) as compared to the Chesapeake Bay sites (0.009 and 0.034 nM in Exp. 2 and 3, respectively).

EXP	Date		Location	Heptadienal		Octadienal	
				Dissolved nM	Particulate nM	Dissolved nM	Particulate nM
1	13-Apr	Chesapeake Bay	Choptank River, HPL	nd	nd	nd	nd
2	14-May		Choptank River, HPL	0.021	0.002	0.011	0.000
3	14-May		Ragged Point, MD	0.004	0.002	0.003	0.000
4	14-Sep	Atlantic waters	Wachapreague, ESL	0.014	0.055	0.004	0.004
5	14-Sep		Wachapreague Inlet	0.060	0.017	0.001	0.002

Table 1. Ambient conditions and HD and OD concentrations from the study site (Larentyev, Franzè, Pierson, & Stoecker, 2015). "nd" denotes no data.

Prior to sample collection, all glassware, plastic containers and tubing were soaked in 10% HCl and rinsed thoroughly with deionized water, followed by filtered seawater. Surface seawater was collected within a submerged 20 L polycarbonate carboy with a 200-µm mesh covering over the opening. The purpose of the mesh covering was to remove any organisms such as naturally-present copepods. All experimentation was performed under dim lighting. Samples that were not being handled were stored in a closed cooler. Samples were transported back to the on-shore lab as soon as possible. Post-incubation screening indicated that our mesh technique was effective in removing unwanted mesozooplankton.

2.2 Experimental Setup

To test the effect of PUA treatments on diatom growth and microzooplankton herbivory, samples with and without PUA additions underwent dilution and incubation on site. Treatments for all experiments included whole seawater (control), whole seawater plus PUA additions, and diluted seawater samples of the same treatments. 5% dilutions were prepared using whole seawater and mixed filtered seawater by means of 0.2 µm volume Pall Science pleated capsules using gravity flow. The growth rates of phytoplankton and microzooplankton grazing rates were determined by 2-point dilution assays (Landry, et al., 2008), where the grazing rate (g) was calculated as the difference in prey growth (µ) in undiluted and highly diluted treatments. The highly diluted sample (here 5% whole seawater and 95% filtered seawater) is meant to simulate growth rates in the absence of predation. All capsules were

rinsed with copious amounts of deionized water prior to use. PUA treatments utilized a methanol solution of 2E,4E-octadienal (OD, W372102, Sigma-Aldrich, Saint Louis, MO, USA) and 2E,4E-heptadienal (HD, 180548, Sigma-Aldrich, Saint Louis, MO, USA) with an OD:HD ratio of 1:10. Final concentrations of OD and HD used for Exp. 1, 4, and 5 samples were equal at 0.5 nM and 5.0 nM, respectively. Exp. 2 and 3 concentrations of OD and HD were 0.2 and 2.0 nM, respectively. These concentrations and ratios were chosen to reproduce the conditions present in coastal waters during PUA-producing diatom blooms (Vidoudez, Casotti, Bastianini, & Pohnert, 2011; Ribalet, et al., 2014).

Triplicates of control and addition treatments were prepared in 0.61 L Nalgene clear polycarbonate bottles. After treatments were added, the bottles were incubated for 24 hours in oyster floats in seawater at the ESL dock to ensure a near-natural environment was maintained. Samples containing marine phyoplankton were collected at initial (t_0) and final (t_{24}, 24 hours) times from the treatment bottles. The triplicates of each treatment were preserved in 0.2 µm formaldehyde (1% final concentration), shock-frozen in liquid N_2, and transported to our Akron laboratory in this condition until thawed for flow cytometry processing. Initial data was acquired using a Partec CyFlow SL flow cytometer, utilizing a 50 mW 488 nm blue solid state laser which allowed for volumetric counting of the sample. The fluorescent properties of the samples were then analyzed via flow cytometry, utilizing a 650 nm long-pass filter to measure red fluorescence (FL3) and a 530/30 nm bandpass filter to measure green fluorescence (FL1). Optical properties of the cultures could be discerned using the forward angle light scatter (FSC) and right angle light scatter (SSC). These allowed for identification of the microbes in our samples by discerning fluorescent properties and relative size. 1 µm fluorescent beads were used as a proxy to differentiate size of sample contents.

2.3 Sample Processing: Flow Cytometer

Samples were removed from the freezer and allowed to thaw. Samples were never allowed to thaw prior to this point, ensuring an accurate representation of their counts at t_o and after incubation, t_{24}. Samples were only handled in dark rooms to ensure we protected the integrity of the fluorescent properties of the chlorophyll, as well as the overall survival of the samples. Fluorescent beads (1 µm) were added to the samples as an internal standard for size. Once, thawed, samples were mixed with 5 µL of beads per 995 µL of sample water (1 mL final total volume). To ensure a homogeneous mixture was prepared, the test tubes containing the samples were well mixed using a VWR Vortex Mixer before being diluted, after each dilution step, and one final time after all dilutions were performed. To ensure that there was no residual contaminant from a previous sample, the flow cytometer was cleaned by running "blanks" of deionized water at high speed through the machine multiple times after each experimental triplet was processed. Clean sheath fluid was regularly prepared in 2-liter batches using 20 mL of Tween 20 2%, 8 mL sodium azimide 4%, and 1972 mL DI water.

2.4 Calculations and Equations

Assuming exponential growth, the rates of prey apparent population growth (μ, day^{-1}) of phytoplankton communities were calculated using the initial (n_0) and final (n_t) counts of prey abundance from the flow cytometry readings, and the incubation time (t, hours) after treatment, as follows:

$$\mu = \frac{\ln(n_t/n_0)}{t}$$

Utilizing the dilution method, grazing rates (g, day^{-1}) were calculated by subtracting the growth rates with predation (100%) of each treatment from the growth rates in the absence of predation (5%) of the same treatment, as follows:

$$g = \mu_{5\%} - \mu_{100\%}$$

In data sets where prey grew slower in diluted samples or not significantly different from the whole seawater treatment, g was not calculated and reported as 0 (Stoecker, Weigel, & Goes, 2014).

Minitab 17 was used to analyze experimental and control data. Dispersion of measured data was acquired via standard deviation within this study.

3. Results

Using flow cytometry scatter and fluorescence patterns, three distinct populations could be discerned (Figure 2). The smallest group (Syn) included *Synechococcus*-like prokaryotic picoplankton cells, the second (Euk <5 μm) consisted of small eukaryotic cells, and the third (Euk >5 μm) –other larger phytoplankton and diatoms. The beads functioned as a standardized point of comparison. These three populations distinguish the diatoms of interest in our study (Euk <5 μm), as well as the relative counts of their competitors in our study.

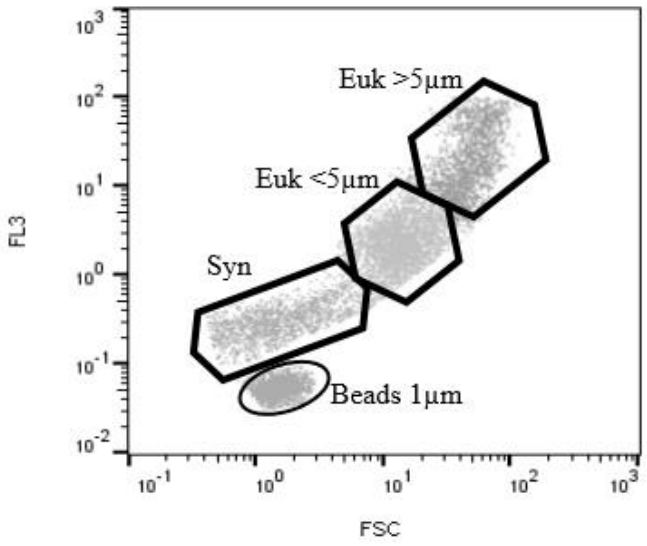

Figure 2. Flow cytometry scatter plots were used to discern three separate microbial populations. Red (FL3) fluorescence was used as a proxy for cellular chlorophyll for microbe identification. FSC shows relative size.

The Atlantic water sites showed higher growth rates (μ) in all three groups compared to the Chesapeake Bay sites (Figure 3 a-c). Syn populations averaged a growth of 0.15 ± 0.09 d^{-1} in Exp. 1-3 and 1.2 ± 0.28 d^{-1} in Exp. 4 and 5 in control samples. Euk <5 μm and Euk >5 μm also showed lower growth rates in the first three experimental sites ($0.18 \leq \mu \leq 0.61$ d^{-1}) compared to the final two sites ($1.17 \leq \mu \leq 1.68$ d^{-1}). PUA treatments did not show any significant changes in growth rate for any of the three groups (t-test; $p > 0.05$).

Control experiments showed microzooplankton grazing rates (g) on Syn ranging from 0.25 ± 0.04 d^{-1} and 1.49 ± 0.07 d^{-1}, excluding Exp. 3 which showed no grazing (Figure 3 d). Grazing of Euk <5 μm did not occur in the Chesapeake Bay experiments, but did show significant grazing occurring at the Atlantic waters sites (0.30 ± 0.12 d^{-1}, Figure 3 d). Euk >5 μm grazing rates ranged from 0.07 ± 0.03 d^{-1} in Exp. 3 to 0.73 ± 0.00 d^{-1} in Exp. 1. No significant grazing on Euk >5 μm in Exp. 2 (Figure 3 f). PUA treatments had varying effects on the three cytometric groups. Grazing of Syn was significantly higher in the presence of PUA (t-test; $p < 0.05$) in four of five experiments (Figure 3 d). No difference between Euk <5 μm grazing in PUA treatment and control samples was distinguished (Figure 3 e). However, Euk > 5 μm showed reduced or even inhibited grazing in the presence of PUA (Figure 3 f). This is in stark contrast to PUA's effect on Syn grazing.

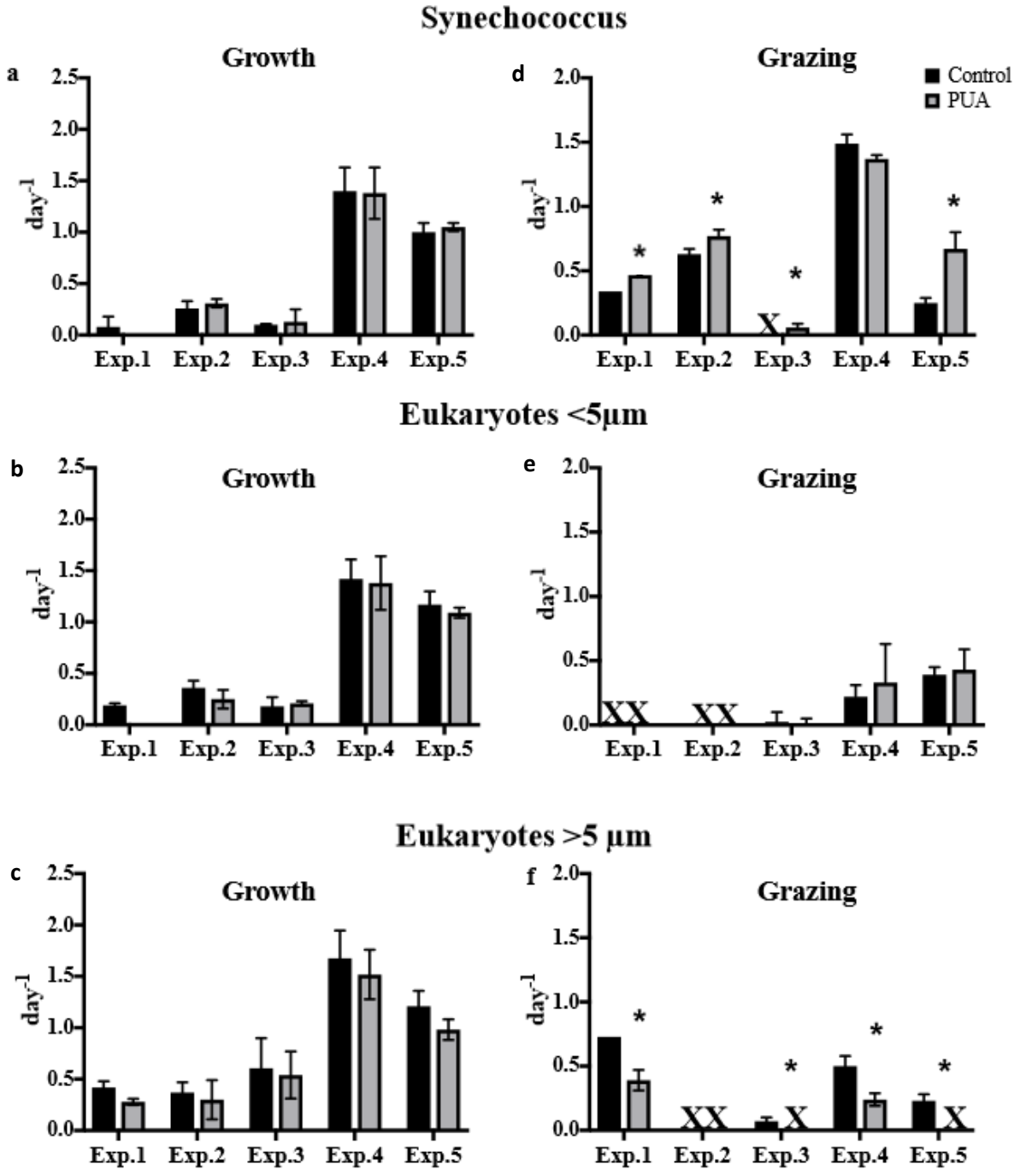

Figure 3. Growth (a,b,c) and grazing loss (d,e,f) rates of picoplankton and phytoplankton populations, as depicted in Figure 2. Asterisks denote significant changes from the control (t-test; $p < 0.05$). X denotes values less than 0. N = 3.

4. Conclusions

Natural production of heptadienal and octadienal from the diatom *Skeletonema marinoi* have shown maximum yields of 9.8 fmol cell^{-1}, with an average yield of 7.5 fmol cell^{-1} (Ribalet, et al., 2007; Ribalet, Berges, Ianora, & Casotti, 2007). PUA concentrations used in this experiment resemble diatom cell counts of 2.7 and 6.7 x 10^6 diatom cells L^{-1} in Exp. 2, 3, and 1, 4, 5 respectively. PUA-producing diatom counts in natural coastal waters can reach 10^7 cell L^{-1} (Casotti, et al., 2005; Ribalet, et al., 2014). The concentrations of PUA used in this experiment are within natural limites, as dissolved PUA concentrations during *S. marinoi* blooms in the northern Adriatic Sea have been shown to range from 2.53 to 28.3 nM (Vidoudez, Casotti, Bastianini, & Pohnert, 2011; Ribalet, et al., 2014).

Growth rates across all sizes of marine phytoplankton showed no change in the presence of dissolved PUA. H1, a reduction in the growth rates of phytoplankton with the addition of PUA, is hence rejected secondary to these findings, supporting $H1_0$. In contrast, microzooplankton herbivory was significantly altered in the presence of allelopathic chemicals. Grazing rates of larger eukaryotes (Euk >5 µm), mostly diatoms, displayed a drastically different pattern from those of *Synechococcus*-like picoplankton. These results suggest that predation can be diverted to populations other than cytotoxin-producing species, although the presence of PUA does not affect all diatom competitors equally (Figure 3). This finding supports the idea that cytotoxic secondary metabolites deter microzooplankton predation on diatoms and other larger phytoplankton, thus supporting H2, reduced microzooplankton herbivory of diatoms in the presence of PUA, and leading me to reject $H2_0$.

In conclusion, these results suggest that the effects of dissolved PUA in oceanic aquatic communities have complex effects on diatom defenses. The apparent transferal of microzooplankton herbivory from diatoms to their competitors in the presence of allelopathic chemicals attests to the complexity of pelagic microbial food webs. The conditions established in this experiment showed that PUA-producing diatoms benefit significantly by their evolved defense mechanism. At peak diatom blooms, increases in PUA concentration should allow for an overall increase in primary production, as well as an increase in silicon and carbon vertical transport which may be otherwise lost in the oceanic elemental sink had their bloom not succeeded.

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