THE TOXICITY OF WEATHERED OIL ON AN OIL RESISTANT DIATOM SPECIES

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Toxicity of Weathered Oil on an Oil Resistant Diatom Species

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This project investigated the toxicity of weathered oil on an oil resistant diatom species, *Phaeodactylum tricornutum.* Most oil toxicology studies focus on the use of fresh oil, while phytoplankton that live in the euphotic zone are exposed to photo-oxidized oil. The main research questions focused on the toxicity of weathered oil in comparison to fresh oil and how *P. tricornutum* responds to this form of pollution. These and related questions are of utmost importance when discussing the lasting effects of an oil spill event on primary producers. These questions were explored through batch-culture experiments, where cultures of *P. tricornutum* were exposed to fresh (WAF) and weathered oil (PhotoWAF) in the form of a water accommodated fraction of oil. We found that photo-oxidized oil targets the cell's ability to harvest light and reduces relative electron transport rates after the initial exposure. In addition, the observed negative physiological effects impacted cell growth throughout the experiment. Our data shows that photo-oxidized oil negatively effects the physiology and growth of *P.*

tricornutum, specifically targeting the cell's light harvesting capabilities. Thus, using photo-oxidized oil is an important consideration for oil toxicity studies when simulating real life conditions versus laboratory settings.

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NOMENCLATURE

WAF	Water accommodated fraction of oil
PhotoWAF	Photo-oxidized water accommodated fraction of oil
GoM	Gulf of Mexico
EPS	Exopolymeric substances

CHAPTER I

INTRODUCTION

Oil spills introduce large quantities of crude oil into the environment due to anthropogenic activity. Spills are detrimental to the marine environment and can have lingering effects on marine organisms. Deepwater Horizon was a major oil spill event that occurred in the Gulf of Mexico (GoM) in 2010, killing 11 men, and affecting the U.S. states of Texas, Louisiana, Mississippi, Alabama, and Florida. The spill released ~4.9 million barrels of crude oil into surrounding waters and continues to negatively impact the Gulf coast since (Daly et al. 2016). Oil spills of this size are not victimless; impacts from Deepwater Horizon included shoreline oiling in the five affected states, wildlife oiling, and the presence of PAHs in the water column (Barron, 2012).

Phytoplankton are foundational in the marine food web (Ozhan et al. 2014), so they have a crucial role in determining the health of an ecosystem. Phytoplankton are small but mighty: roughly 50% of primary production globally can be attributed to these organisms (Falkowski and Raven, 2013). When disasters occur, like Deepwater Horizon, it is of utmost importance to assess the damage done to phytoplankton physiology and community dynamics because of potential trophic cascades. Studies focusing on primary producers, the base of the GoM food chain, have concluded that phytoplankton physiology can be negatively affected by the presence of hydrocarbons from the oil spill and added dispersant from the clean up efforts (Bretherton et al. 2019). Previous studies have explored fresh oil toxicity on diatoms (Zhao et al. 2019; Ozhan et al. 2015; Ozhan et al. 2014; Gilde and Pinckney, 2012; Deasi et al. 2010; Pulich et al. 1974), the effect of hydrocarbon photo products on more complex organisms and their byproducts (Sun

et al. 2018; Faksness et al. 2015; Lampi et al. 2009), and dispersed oil toxicity on diatoms (Bretherton et al. 2019; Bretherton et al. 2018; Quigg et al. 2016; Eenennaam et al. 2016; Hook and Osborn, 2012).

P. tricornutum is a diatom found in marine and brackish waters around the world and is widely considered to be a model organism for research pertaining to diatoms (Prestegard et al. 2015). *P. tricornutum* exhibits rapid growth in conditions with varying temperatures, light availability, and media (Lewin et al. 1958). Specifically, *P. tricornutum* utilizes physiological strategies to continue photosynthesis and cell growth at lower energy rates (Quigg et al. 2006). In fresh oil toxicity studies, *P. tricornutum* is regarded as "robust" due to its resistance to oil treatments; parameters such as photo-physiology and growth rates are seen as relatively unaffected (Bretherton et al. 2018). Physiological effects from photo-oxidized oil on *P. tricornutum* has yet to be studied, which is what this project aims to explore.

In the natural environment, oil spilled will become photo-oxidized as it moves through the euphotic zone (Radović et al. 2014). Studies have shown that photo-oxidized oil differs from its "original source form" in that there is a disappearance of saturated hydrocarbons (Hall et al. 2013). This project aims to fill the gap in the knowledge between fresh oil toxicity and photo-oxidized oil toxicity on *P. tricornutum* along with their physiological responses to these conditions.

CHAPTER II

METHODS

Experimental Design

A 7-day batch culture bottle experiment was conducted following procedures in Bretherton et al. (2018). *P. tricornutum* UTEX 646 was purchased from the Culture Collection of Algae at the University of Texas at Austin (Bretherton et al. 2018). f/2 growth media was prepared according to Guillard and Ryther (1962) and Guillard (1975). The seawater was collected from the NOAA National Marine Fisheries Laboratory (Galveston, TX). *P. tricornutum* was grown at 19˚C on a 12:12 light dark cycle with a light intensity of ~60 µmol photons m^{-2} s^{-1}.

Macondo surrogate oil (4 mL), provided by BP Oil Company, was photo-oxidized with natural sunlight in quartz round bottom flasks for 3 days. Unphoto-oxidized Macondo surrogate oil (0.4 mL L^{-1}) was added to 7 L of sterile f/2 growth media and stirred in the dark overnight. Similarly, 0.4 mL L^{-1} photo-oxidized Macondo surrogate oil was added to 7 L of sterile f/2 growth media and stirred in the dark overnight. This procedure is considered the standard method of making a water accommodated fraction of oil (WAF) (Singer, 2001). f/2 growth media was prepared according to Bretherton et al. 2018. The WAF solutions were filtered through a 20 µm mesh filter to make sure that there were not any large oil droplets present in experiment bottles. The bottle experiment was comprised of 1 L bottles that included an aliquot of *P. tricornutum* and either WAF or photo-oxidized WAF (PhotoWAF). Using this procedure, the estimated oil equivalent concentrations in the WAF and PhotoWAF treatments is expected to be in the range 0.5-2 mg L^{-1} (Sammarco et al. 2013).

P. tricornutum cultures were added to the f/2 growth media (Control), WAF, and PhotoWAF bottles with an average cell density of 1.69 x 10^6 cells mL^{-1}. Samples were taken at the 9 am each day for the extent of the experiment and were analyzed to determine culture growth and photo-physiology.

Culture growth

P. tricornutum culture growth was monitored daily. Cell counts were conducted using microscopy with a Neubauer hemocytometer. For each treatment, cells were counted within the hemocytometer grid system composed of 8 large grids each containing 16 squares.

Chlorophyll *a* fluorescence was also used as a culture growth parameter. Samples (5 mL) were dark-acclimated for 15 minutes and fluorescence was measured using a 10AU Turner Fluorometer. Chlorophyll *a* concentration was then determined using a chlorophyll standard curve as described in Kamalanathan et al. (2019).

Photo-physiology

Photo-physiology effects were determined with 10 mL samples that were dark-acclimated for 15 minutes. The Pulse Amplitude Modulated fluorescence machine (PhytoPAM) operated by a Phyto-Win program (version 2.13), measured relative electron transport rate ($rETR_{max}$) and light harvesting efficiency (α) parameters. Settings for the PAM fluorometer were set according to Bretherton et al. (2019): 8 light steps were used to perform rapid light curves (16, 32, 64, 164, 264, 364, 464, 564, 664, 764 µmol photons m^{-2} s^{-1}). The Fluorescence Induction and Relaxation fluorometer machine (FIRe, Satlantic) measured the maximum PSII quantum yield (F_v/F_m) and Quinone A redox rate (Q_A). Settings for the FIRe fluorometer were set according to Bretherton et al. (2019): 80 µs ST flashes with a 60 µs relaxation phase and 40 iterations per sample to reduce noise.

Statistics

Two factor ANOVA tests with a p value of 0.05 were conducted to determine effects from the three treatments across day 1 through day 7 on culture growth and photo-physiology. Single factor ANOVA tests with a p value of 0.05 were conducted to determine effects from the three treatments on culture growth and photo-physiology looking at specific days if results were not significant across day 1 through day 7. The tests were completed using the Data Analysis package in Microsoft Excel (version 15.17).

CHAPTER III

RESULTS

Culture growth

Over the course of the 7-day bottle experiment, cell growth increased exponentially in all three treatments (Figure 1). The Control treatment had the most cells mL^{-1} on day 4 and day 7 in comparison to WAF and PhotoWAF treatments. Growth was inhibited in WAF and PhotoWAF treatments, with PhotoWAF containing the least cells mL^{-1} on day 4 and day 7. The negative effects seen in WAF and PhotoWAF treatments compared to Control were significant (Two Factor ANOVA, p value = 0.01).

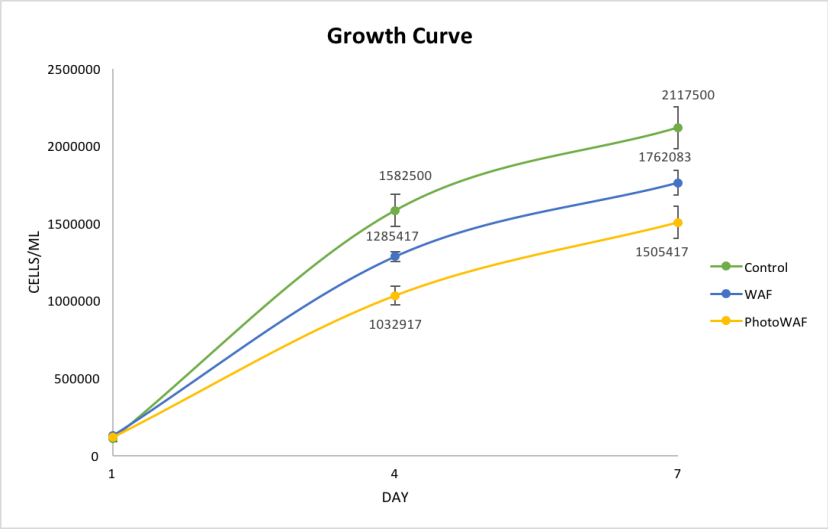

Figure 1. Average growth curve (± standard error) of *P. tricornutum* for Control, WAF, and PhotoWAF (n=3).

Average chlorophyll *a* fluorescence, another parameter for cell growth, followed similar patterns to the growth curve. The chlorophyll *a* trend mirrors that for cell counts for the Control and WAF treatments (Figure 2) but it deviates on day 7, as the PhotoWAF treatment exceeds the

measurements for both Control and WAF treatments (Figure 2). Fluorescence measurements increase throughout day 7 for all three treatments, with a noticeable increase seen in PhotoWAF. There was no significant difference between treatments on day 1, but a significant difference between treatments was seen on day 4 (Single Factor ANOVA, p value = 09.48 x 10^{-9}) and day 7 (Single Factor ANOVA, p value = 0.01).

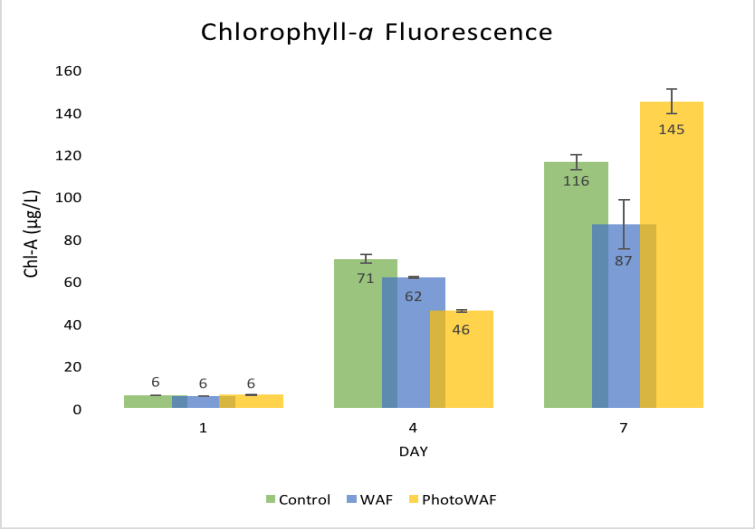

Figure 2. Average chlorophyll *a* fluorescence (± standard error) of *P. tricornutum* for Control, WAF, and PhotoWAF (n=3).

Photo-physiology

The relative electron transport rate was reduced in WAF and PhotoWAF treatments after initial exposure on day 1 compared to the Control (Figure 3). The PhotoWAF treatment exhibited the slowest relative electron transport rate, with an average rate of 82 µmol electrons m^2 s^{-1} on day 1. Although WAF values decrease through day 7 and PhotoWAF values increase and then decrease through day 7, the distinct decline of relative electron transport rates in oil treatments after initial exposure on day 1 is key. There was no significant difference between treatments on day 7, but a significant difference between treatments was seen on day 1 (Single Factor ANOVA, p value = 4.41 x 10^{-5}) and day 4 (Single Factor ANOVA, p value = 0.02).

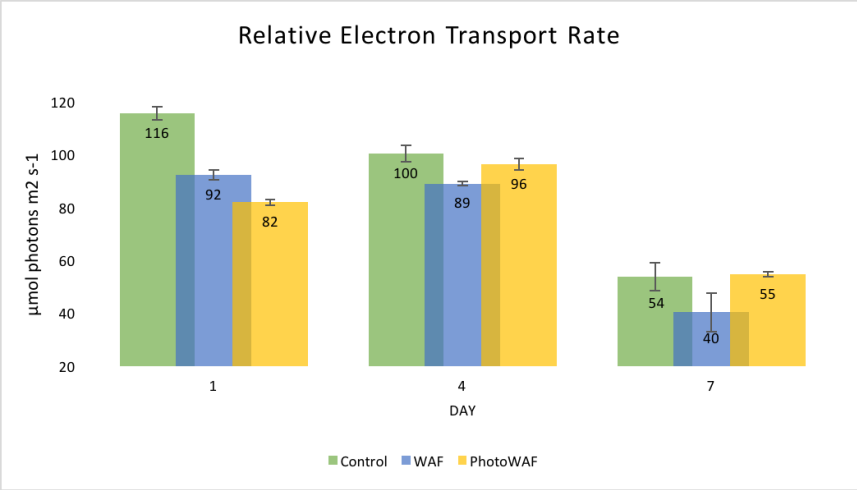

Figure 3. Average relative electron transport rate (± standard error) of *P. tricornutum* for Control, WAF, and PhotoWAF (n=3).

Quinone A redox rate did not exhibit major differences between treatments, with the exception of PhotoWAF on day 7 (Figure 4). Day 1 readings did not exhibit the same pattern as day 1 of relative electron transport rate, which would indicate effects of initial exposure to oil. There was no significant difference between treatments on day 1, day 4, and day 7 (Two Factor ANOVA, p value > 0.05).

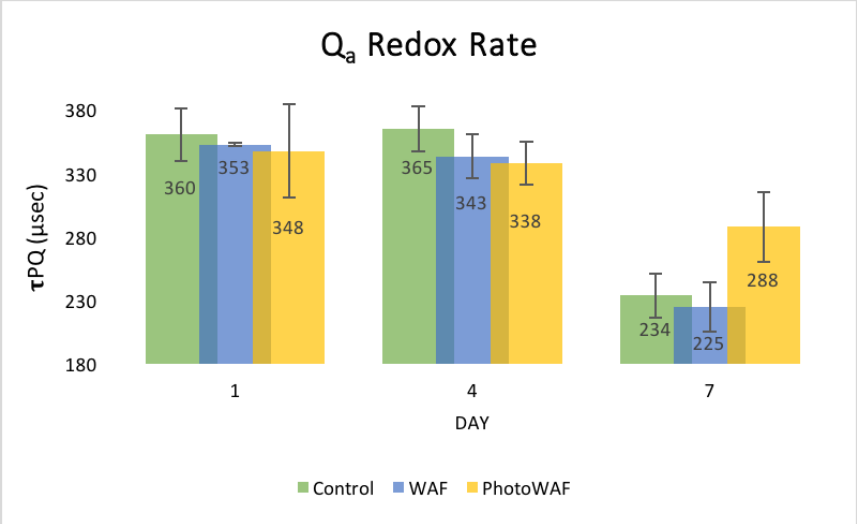

Figure 4. Average Quinone A (Qa) red-ox rates (± standard error) of *P. tricornutum* for Control, WAF, and PhotoWAF (n=3).

Light harvesting efficiency was negatively affected in WAF and PhotoWAF treatments after initial exposure on day 1 (Figure 5). The PhotoWAF treatment exhibited the least efficient light harvesting, with a measurement of 0.59 µmol electrons µmol photons^{-1}. Although WAF values increase through day 7 and PhotoWAF values increase and then decrease through day 7, the distinct decline of light harvesting efficiency in oil treatments after initial exposure on day 1 is key. There was no significant difference between treatments on day 4 or day 7, but a significant difference between treatments was seen on day 1 (Single Factor ANOVA, p value = 0.02).

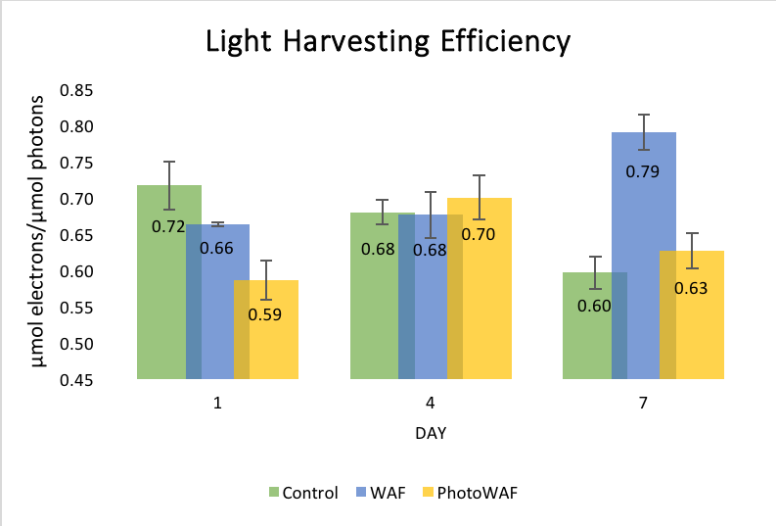

Figure 5. Average light harvesting efficiency (± standard error) of *P. tricornutum* for Control, WAF, and PhotoWAF (n=3).

Average maximum PSII quantum yield, F_v/F_m, did not exhibit major differences from day 1 through day 7, even though the averages for all three treatments on day 7 declined compared to day 1 and day 4 averages. There was no significant difference between treatments on day 1, day 4, and day 7 (Two Factor ANOVA, p value > 0.05).

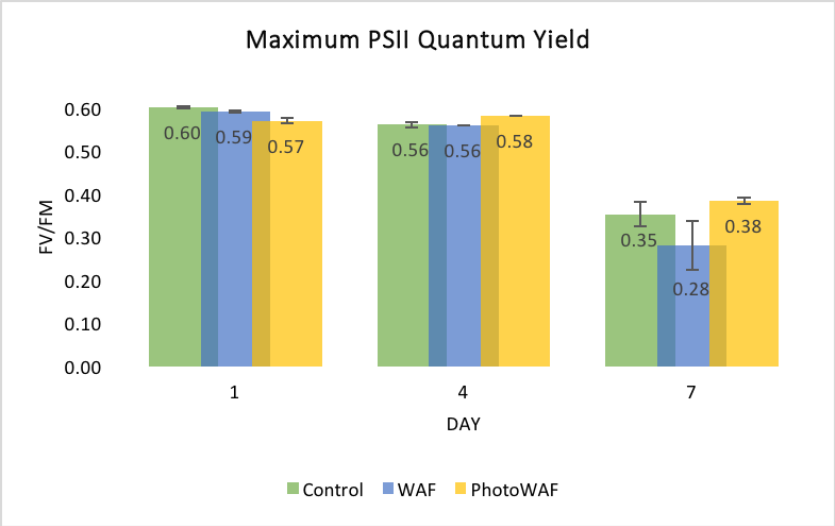

Figure 6. Average maximum PSII quantum yield (± standard error) of *P. tricornutum* for Control, WAF, and PhotoWAF (n=3).

CHAPTER IV

DISCUSSION

Culture growth

The negatively impacted growth curve (Figure 1) seen in both oil treatments in comparison to the Control treatment, especially in photo-oxidized conditions, reaffirms the expectation for *P. tricornutum* to exhibit negative physiological effects when exposed to photo-oxidized oil. The negative effects can be seen through day 7 of the experiment after initial exposure on day 1. Similarly, the negatively impacted chlorophyll *a* fluorescence measurements are seen in WAF and PhotoWAF treatments, with the exception of the PhotoWAF reading on day 7. The chlorophyll *a* fluorescence measurement in Control and WAF treatments mirror the trend seen in Bretherton et al (2018), with the WAF treatments having lower readings compared to Control. Determinations in regards to *P. tricornutum* recovery after oil has been removed from the water column cannot be confirmed in light of incomplete oil analysis data, however, previous studies show that estimated oil equivalent measurements decline over time (Bretherton et al. 2018).

Photo-physiology

The relative electron transport rate exhibited reduced rates in oil treatments, especially in PhotoWAF. This reaffirms the expectation for *P. tricornutum* to exhibit negative physiological effects when exposed to photo-oxidized oil. The rate disparity can be clearly seen in day 1 measurements, highlighting the differential behavior after initial exposure. Specifically, the relative electron transport rate measured the maximum relative rate of electron transport between PSII and PSI. Two parameters that are important facets of the electron transport chain, Quinone

A redox rates and light harvesting efficiency, were measured to further pinpoint the source of the reduced relative electron transport rate. The Quinone A redox rate measured the rate electron transfer through the means of reduction and oxidation and light harvesting efficiency measured the cell's ability to capture light and use photons for photosynthesis. Between Quinone A redox rates and light harvesting efficiency, light harvesting efficiency, a process upstream of Quinone A reduction-oxidation, followed the same disparity pattern as the relative electron transport rate and the Quinone A redox rate exhibited insignificant differences between all three treatments on day 1. The negative effects seen in light harvesting efficiency indicated the reduced relative electron transport rate was due to an alteration of the light harvesting capabilities of *P. tricornutum.* Photosynthetic efficiency, which can be described by F_v/F_m, did not significantly change in response to oil exposure between all three treatments. The lack of significant change of *P. tricornutum* photosynthetic efficiency (F_v/F_m) and day 1 Quinone A redox rates (Q_A) mirrors results seen in Bretherton et al. (2018). The lower relative electron transport rates seen in WAF treatments compared to Control treatments on day 1 mirror results seen in Bretherton et al. (2019).

Of the three treatments, PhotoWAF exhibits negative physiological effects on *P. tricornutum*, specifically inhibiting the diatom's ability to proliferate and to harvest light. Harvesting light is vital for diatom survival and is being targeted by the toxic properties of the photo-oxidized oil. When diatoms are hindered in their ability to produce energy via photosynthesis, growth and proliferation will be hindered as a result. Although previous literature suggests that *P. tricornutum* is oil resistant, this data shows negative physiological effects in the presence of photo-oxidized oil. Therefore, future experiments should utilize photo-oxidized oil to represent real world effects on phytoplankton growth and physiology. This is an

area that has been poorly explored but has important implications to observations of diatoms exposed to oil spill events in the natural environment versus lab settings.

CHAPTER V

ANTICIPATED OUTCOMES

Due to unforeseen circumstances surrounding COVID-19 viral spread during the Spring 2020 semester, a complete dataset was unavailable at the time of submission of this thesis. Oil analysis and exopolymeric substances (EPS) were not measured before university closings. Both of these analyses would have produced findings that could add a piece of information to be related to the central research topic: the toxicity of weathered oil in comparison to fresh oil and how *P. tricornutum* responds to this form of pollution.

Oil analysis would have investigated the chemical change in fresh oil versus photo-oxidized oil. Based on negative physiological effects already seen in photo-physiology data, this chemical reaction could be a key component in understanding the increased negative effects seen in the PhotoWAF treatment versus the WAF treatment on *P. tricornutum.* Based on oil analysis data from Kalamanathan et al. (2019), oil degraded over the course of the 96-hour experiment and specifically observed lower total alkane and PAH content on the last day compared to the starting time point. WAF and PhotoWAF treatments would be expected to exhibit the same patterns, possibly with an even lower reading of total alkane and PAH content in the PhotoWAF treatment.

Exopolymeric substances (EPS) are molecules secreted by phytoplankton, and other microbes, naturally in response to stressors in the environment (Quigg et al. 2016). These molecules are key components in determining the fate of oil in the water column after oil spill events, ultimately aiding in the carbon cycle. Largely, EPS is composed of polysaccharides and proteins. The protein/polysaccharide ratio can vary based on environmental conditions and have been associated with characteristics such as hydrophobicity (Xu et al. 2011). These features have

implications in regard to the ability to degrade or sink oil in the water column. EPS data would have given insight into the diatom's ability to mitigate oil toxicity through the means of EPS composition and production. Based on EPS data from Xu et al. (2019), WAF is expected to produce more EPS than Control. With PhotoWAF treatments exhibiting more negative effects than WAF, PhotoWAF treatments are expected to produce more EPS than both WAF and Control.

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