Phytoplankton Response to South Louisiana Crude Oil Exposure: Determining Impacts at Individual, Community, Toxin Production, Enzymatic-Activity and Gene-Expression Levels

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A Dissertation

Submitted to the Graduate Faculty of the
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Doctor of Philosophy

in

The Department of Oceanography and Coastal Sciences

by

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May 2014
This work is dedicated to the two best women in my life: my lovely wife Melissa and my wonderful mom Firdevs.
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ABSTRACT

The Deepwater Horizon oil spill in the Gulf of Mexico (GoM) raised an important question. What is the ecological impact of the oil, the dispersant, and the dispersed oil to the GoM ecosystem? Significant and varying research efforts have contributed answers to this question. However, to better understand the complete ecological consequences of the spill in the GoM, the impact of the spill at the base of marine food web should be examined. This research aims to understand impact of the spilled oil, South Louisiana crude oil (LSC), the chemical dispersant, Corexit® EC9500A, and the dispersed oil on phytoplankton communities in the GoM at individual, community, toxin-production, enzymatic, and gene-expression levels. At the individual level, phytoplankton size influenced tolerance to crude oil, but taxonomic group seemed to be a more predominant criterion. In general, diatoms showed better tolerance to crude oil than dinoflagellates. Naphthalene and benzo(a)pyrene cannot be solely used as surrogates to assess crude oil toxicity on phytoplankton. Community-level effects were investigated under oligotrophic and eutrophic conditions. Diatoms showed the greatest tolerance to crude oil exposures under every condition that was assessed. Nevertheless, different diatom groups had distinct responses under different nutrient regimes. The amount of nutrients greatly influences phytoplankton response during crude oil exposure. Crude oil also affects toxin production of two ecologically important toxic phytoplankton species of the GoM, Karenia brevis and Prorocentrum minimum. It was revealed that reactive oxygen species are activated in phytoplankton exposed to crude oil. Phytoplankton species also displayed signs of oxidative stress and damage in their lipid structure under crude oil exposure. A gene expression study indicated that crude oil does not cause significant difference in the
expression levels of selected genes between the control group and samples treated with crude oil. This research provides essential data for impact assessment of oil spills and pollution on phytoplankton ecology and bloom dynamics in the GoM. These datasets contribute substantially to existing scientific knowledge about the region and provide baseline information for subsequent research efforts that seek to further understand the impact of oil on the marine planktonic ecosystem in the GoM.
1.1. Crude Oil in Marine Environment

Crude oil is an inherent component of the global ecosystem, which is introduced into aquatic environments by natural and anthropogenic sources, leading to chronic and acute contamination for organisms living within these habitats. The introduction of crude oil into seawater leads to a series of physical, chemical, and biological processes, such as physical transport, dissolution, evaporation, emulsification, oxidation, sedimentation, microbial degradation, and aggregation, which is collectively known as weathering (Hsiao I.C. Stephens 1978, Patin 1999). Weathering begins within seconds after the oil’s first contact with seawater and endures years thereafter. Environmental conditions, the nature of the spilled oil, and the dynamics of oil spills control the progression, duration, and outcomes of these transformations (Patin 1999). As a result, the ecological effects of crude oil are also influenced by the weathering processes. Introduction of crude oil into seawater results in droplet dispersions; colloidal dispersions; oil-in-water emulsion; and single phase, homogeneous mixtures (dissolution) (NRC 2003). It is mainly composed of hydrocarbons (e.g., paraffins, naphthenes, aromatics), asphaltenes, resins, ash, and sulfur (Simanzhenkov and Idem 2003). Crude oil is characterized based on its geographical source. Different geographic locations have their distinctive crude oils. Even though the hydrocarbon compositions in these different oils differ, there are also some similarities between them, such as high concentration of low molecular weight n-paraffins and benzenes and low concentrations of polycyclic aromatic hydrocarbons (PAHs). Relatively lower molecular weight compounds, including PAHs and aliphatic
hydrocarbons, are more soluble; also, their smaller size makes them more bioavailable to organisms via absorption or respiration (American-Petroleum-Institute 1999). Weathering substantially reduces the amount of the lower-log (K_{ow}) chemicals with greater toxic potential, which is attributed to high water solubility, leaving the higher-log (K_{ow}) chemicals with lower toxic potential in water column (Toro et al. 2007). In consequence, weathering significantly reduces the amount of low molecular weight hydrocarbons and, therefore, lessens the apparent toxicity on organisms in marine environment (Lee W. Y. et al. 1978).

The impact of crude oil, a pollutant and toxicant in the marine environment, on marine organisms studied extensively, however, the organisms at the higher trophic level took more attention than the base of marine food web. Phytoplankton are at the base of marine food web and play a key role in the ecology of marine ecosystem and changes in their patterns of distribution and abundance can have significant impact on the entire ecosystem since they are also very important part in regulating the amount of carbon in their system. Phytoplankton under crude oil exposure has been studied; effects have often revealed a mutual relationship. While crude oil can alter water conditions for phytoplankton growth, some phytoplankton groups can alter the crude oil’s composition by degradation of its components. These studies assessed responses both in single phytoplankton species and community structures of phytoplankton. However, less attention has been paid to potential effects at the cellular level and to the modes of action of crude oil hydrocarbons.
1.2. Effects of Crude Oil on Phytoplankton

Phytoplankton, which diverge greatly in physiological properties, vary their response and tolerance to oil toxicants (Liu et al. 2006, Hjorth et al. 2007, Meng et al. 2007, Wang et al. 2008). Geographic location, oceanographic and meteorological conditions, seasonal variations, oil dosage and impact area, and oil types also contribute to the variations observed in the impact of oil on phytoplankton (NRC 2003). The short-term negative effects, such as growth inhibition, on phytoplankton due to toxigenic oil compounds are usually observed at high concentrations. In general, field and laboratory studies on the effects of crude oil on phytoplankton growth have shown that crude oil concentrations up to 1.0 mg/l may stimulate growth, from 1.0 to 100 mg/l may cause slight and severe growth inhibition, and concentrations over 100 mg/l result in severe or complete growth inhibition (Gordon and Prouse 1973, Dunstan et al. 1975, Parsons et al. 1976, Lee R. F. et al. 1977, Hsiao I.C. Stephens 1978, Hsiao Stephen I. C. et al. 1978, Elmgren et al. 1980). Table 1.1 reviews the historical data that present crude oil and its constituents’ effects in terms of EC₅₀ values on individual phytoplankton species. Crude oil constituents’ impact range is larger than crude oil that varied between 1 ppb and 100 ppm (Table 1.1). Evidently PAHs have a highest toxicity potential on phytoplankton. This impact could be observed around 1 ppb level. When phytoplankton mortality occurs with increased crude oil concentration, irrespective of the exposure period, no correlation between toxicity and exposure time has been suggested (Miller et al. 1978, Adekunle et al. 2010).
Table 1.1. Historical data of individual phytoplankton response to crude oil and its constituents in different studies. Responses, where available, are given as EC50 value.

<table>
<thead>
<tr>
<th>Class name</th>
<th>Species</th>
<th>Test substance</th>
<th>EC50 (study duration)</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>Bacillariophyceae</td>
<td>Chaetoceros calcitrans</td>
<td>Diesel oil</td>
<td>37.3 mg/l (96 h)</td>
<td>Bhattacharjee and Fernando 2008</td>
</tr>
<tr>
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<td>1.69 mg/l (72 h)</td>
<td>&quot;</td>
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<td>Phenanthrene</td>
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<tr>
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<td>Phaeodactylum tricornutum</td>
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<td>3.00 mg/l (72 h)</td>
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<tr>
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<td>Toluene</td>
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<td>Pyrene</td>
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<td>Hing et al. 2011</td>
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<tr>
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<td>Siron et al. 1991</td>
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<td>Siron et al. 1991</td>
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<td>Chlorophyceae</td>
<td>Scenedesmus subspicatus</td>
<td>Benzo(a)pyrene</td>
<td>1.48 μg/l (7 d)</td>
<td>Djomo et al. 2004</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>Scenedesmus subspicatus</td>
<td>Pyrene</td>
<td>18.72 μg/l (7 d)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>Scenedesmus subspicatus</td>
<td>Anthracene</td>
<td>1.04 mg/l (7 d)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>Scenedesmus subspicatus</td>
<td>Phenanthrene</td>
<td>50.24 mg/l (7 d)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>Scenedesmus subspicatus</td>
<td>Naphthalene</td>
<td>68.21 mg/l (7 d)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td>Scenedesmus subspicatus</td>
<td>Diesel oil</td>
<td>0.1-10% (12d)</td>
<td>Dennington et al. 1975</td>
</tr>
</tbody>
</table>
(Table 1.1 continued)

<table>
<thead>
<tr>
<th>Class name</th>
<th>Species</th>
<th>Test substance</th>
<th>EC$_{50}$ (study duration)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyceae</td>
<td><em>Selenastrum capricornutum</em></td>
<td>Api No. 2 fuel oil</td>
<td>156 mg/l (7d)</td>
<td>El-Dib et al. 1997</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td><em>Tetraselmis chuiii</em></td>
<td>Gasoline</td>
<td>4.9-96.5 mg/l (96 h)</td>
<td>Paixao et al. 2007</td>
</tr>
<tr>
<td>Chrysophyceae</td>
<td><em>Monochrysis lutheri</em></td>
<td>No. 2 diesel fuel oil</td>
<td>0.09 mg/l (2h)</td>
<td>Vandermeulen et al. 1979</td>
</tr>
<tr>
<td>Chrysophyceae</td>
<td><em>Monochrysis lutheri</em></td>
<td>Kuwait crude oil</td>
<td>8.6 mg/l (2h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chrysophyceae</td>
<td><em>Monochrysis lutheri</em></td>
<td>Amoco Cadiz crude oil</td>
<td>4.4 mg/l (2h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chrysophyceae</td>
<td><em>Monochrysis lutheri</em></td>
<td>Bunker C crude oil</td>
<td>3.3 mg/l (2h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Coscinodiscophyceae</td>
<td><em>Coscinodiscus centralis</em></td>
<td>Nigerian crude oil</td>
<td>&gt; 50 mg/l (24 h)</td>
<td>Adenkule et al. 2010</td>
</tr>
<tr>
<td>Coscinodiscophyceae</td>
<td><em>Thalassiosira pseudonana</em></td>
<td>Fluoranthenne</td>
<td>1031 μg/l (72 h)</td>
<td>Bopp &amp; Lettieri 2007</td>
</tr>
<tr>
<td>Coscinodiscophyceae</td>
<td><em>Thalassiosira pseudonana</em></td>
<td>Pyrene</td>
<td>260.3 μg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Coscinodiscophyceae</td>
<td><em>Thalassiosira pseudonana</em></td>
<td>Benzo(a)pyrene</td>
<td>55.2 μg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Dinophyceae</td>
<td><em>Ceratium trichoceros</em></td>
<td>Nigerian crude oil</td>
<td>&gt; 50 mg/l (24 h)</td>
<td>Adenkule et al. 2010</td>
</tr>
<tr>
<td>Dinophyceae</td>
<td><em>Zooxanthella microadriztica</em></td>
<td>Toluene</td>
<td>35.60 mg/l (72 h)</td>
<td>Jiang et al. 2002</td>
</tr>
<tr>
<td>Dinophyceae</td>
<td><em>Zooxanthella microadriztica</em></td>
<td>Naphthalene</td>
<td>5.29 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Dinophyceae</td>
<td><em>Zooxanthella microadriztica</em></td>
<td>2-methylnaphtelene</td>
<td>2.96 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Dinophyceae</td>
<td><em>Zooxanthella microadriztica</em></td>
<td>Phenanthrene</td>
<td>0.60 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Euglenophyceae</td>
<td><em>Euglena gracilis</em></td>
<td>Diesel oil</td>
<td>&gt;10% (12d)</td>
<td>Dennington et al. 1975</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Cyclotella caspia</em></td>
<td>Fluoranthenne</td>
<td>0.2 mg/l (96 h)</td>
<td>Liu et al. 2006</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Odontella mobiliensis</em></td>
<td>Nigerian crude oil</td>
<td>&gt; 50 mg/l (24 h)</td>
<td>Adenkule et al. 2010</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Skeletonema costatum</em></td>
<td>Toluene</td>
<td>36.70 mg/l (72 h)</td>
<td>Jiang et al. 2002</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Skeletonema costatum</em></td>
<td>Naphthalene</td>
<td>6.53 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Skeletonema costatum</em></td>
<td>2-methylnaphtelene</td>
<td>3.67 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Skeletonema costatum</em></td>
<td>Phenanthrene</td>
<td>0.83 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Skeletonema costatum</em></td>
<td>Phenanthrene</td>
<td>47 μg/l (72 h)</td>
<td>Meng et al. 2007</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Skeletonema costatum</em></td>
<td>Anthracene</td>
<td>39 μg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Skeletonema costatum</em></td>
<td>Fluoranthenne</td>
<td>18 μg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Mediophyceae</td>
<td><em>Skeletonema costatum</em></td>
<td>Pyrene</td>
<td>24 μg/l (72 h)</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
(Table 1.1 continued)

<table>
<thead>
<tr>
<th>Class name</th>
<th>Species</th>
<th>Test substance</th>
<th>EC50 (study duration)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prasinophyceae</td>
<td><em>Platymonas subcordiformis</em></td>
<td>Toluene</td>
<td>114.0 mg/l (72 h)</td>
<td>Jiang et al. 2002</td>
</tr>
<tr>
<td>Prasinophyceae</td>
<td><em>Platymonas subcordiformis</em></td>
<td>Naphthalene</td>
<td>7.30 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Prasinophyceae</td>
<td><em>Platymonas subcordiformis</em></td>
<td>2- methylnaphtelene</td>
<td>3.03 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Prasinophyceae</td>
<td><em>Platymonas subcordiformis</em></td>
<td>Phenanthrene</td>
<td>1.92 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Prymnesiophyceae</td>
<td><em>Isochrysis galbana</em></td>
<td>Diesel oil</td>
<td>&gt; 2.6 mg/l (23 d)</td>
<td>Hing et al. 2011</td>
</tr>
<tr>
<td>Trebouxiophyceae</td>
<td><em>Chlorella salina</em></td>
<td>Diesel oil</td>
<td>&gt; 17.0 mg/l (20 d)</td>
<td>Hing et al. 2011</td>
</tr>
<tr>
<td>Trebouxiophyceae</td>
<td><em>Chlorella spp.</em></td>
<td>Fuel oil (F120)</td>
<td>19.97 mg/l (96h)</td>
<td>Chao et al. 2012</td>
</tr>
<tr>
<td>Trebouxiophyceae</td>
<td><em>Chlorella spp.</em></td>
<td>Fuel oil (F180)</td>
<td>13.63 mg/l (96h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Trebouxiophyceae</td>
<td><em>Chlorella spp.</em></td>
<td>Fuel oil (F1380)</td>
<td>73.15 mg/l (96h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Trebouxiophyceae</td>
<td><em>Chlorella vulgaris</em></td>
<td>Toluene</td>
<td>98.60 mg/l (72 h)</td>
<td>Jiang et al. 2002</td>
</tr>
<tr>
<td>Trebouxiophyceae</td>
<td><em>Chlorella vulgaris</em></td>
<td>Naphthalene</td>
<td>5.59 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Trebouxiophyceae</td>
<td><em>Chlorella vulgaris</em></td>
<td>2- methylnaphtelene</td>
<td>2.75 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
<tr>
<td>Trebouxiophyceae</td>
<td><em>Chlorella vulgaris</em></td>
<td>Phenanthrene</td>
<td>1.11 mg/l (72 h)</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
Table 1.2. Historical data of phytoplankton communities’ response to crude oil in different studies.

<table>
<thead>
<tr>
<th>Crude oil type</th>
<th>Concentration</th>
<th>Duration</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prudhoe Bar</td>
<td>2.0-4.5 mg/l</td>
<td>17 days</td>
<td>Shifting community from diatoms to microflagellates such as haptophytes, chrysophytes and a prasinophyte.</td>
<td>Harrison et al., 1986</td>
</tr>
<tr>
<td>Bunker A</td>
<td>0.22 mg/l</td>
<td>10 days</td>
<td>Suppression of diatom, flagellates predominated</td>
<td>Nomura et al. 2007</td>
</tr>
<tr>
<td>Prestige oil</td>
<td>8.6-23 μg/l chrysene equivalents</td>
<td>5 days</td>
<td>Diatoms were more resistant. Larger diatoms were affected more than the smaller ones. Oceanic phytoplankton were more susceptible to crude oil exposure.</td>
<td>Gonzalez et al. 2009</td>
</tr>
<tr>
<td>Prestige oil</td>
<td>3.5-30 μg/l chrysene equivalents</td>
<td>3 days</td>
<td>Relative abundance of diatoms increased in the community.</td>
<td>Perez et al. 2010</td>
</tr>
<tr>
<td>Lufeng oceanic</td>
<td>0.1-100 mg/l</td>
<td>15 days</td>
<td>≤ 1.21 mg/l promoted the phytoplankton growth and ≥ 2.28 mg/l restrain the growth.</td>
<td>Huang et al. 2011</td>
</tr>
<tr>
<td>South Louisiana + Texas</td>
<td>10-100 μg/l</td>
<td>2 days</td>
<td>Diatoms, chlorophytes and euglenophytes were resistant and prasinophytes did not affected.</td>
<td>Gilde and Pinckney 2012</td>
</tr>
<tr>
<td>Prestige oil</td>
<td>20-60 μg/l chrysene equivalents</td>
<td>8 days</td>
<td>Community dominated by diatoms. Initial compositions of communities determine the degree of response.</td>
<td>Gonzalez et al. 2013</td>
</tr>
</tbody>
</table>
In addition to the inhibition and stimulation of individual phytoplankton growth in the presence of crude oil, community composition changes and succession of certain groups have been studied under controlled ecosystem experiments in field and laboratory experiments (Reviewed in Table 1.2). They have been conducted to predict community level impacts that would affect the structure and function of the natural ecosystem. Relative tolerance of different phytoplankton groups and community shift under the exposure has been given as an outcome based on initial community compositions. An argument was introduced by Gonzales et al. 2013 stated that evolving community composition under crude oil exposure are based on the initial composition of phytoplankton group. Therefore, quite dynamic phytoplankton composition in the field, due to temporal and spatial changes, makes the extrapolation of these studies into real environment more challenging.

Both individual and community level studies claimed certain groups’ higher sensitivity to crude oil. The suppression of diatom growth and the rise in dominancy by flagellates have been observed following oil spills and in laboratory experiments (Dunstan et al. 1975, Parsons et al. 1976, Lee R. F. et al. 1977, Elmgren et al. 1980, Davenport 1982, Harrison et al. 1986). Arguments introduced by Siron et al. (1996) about diatoms are more prone to crude oil due to the presence of their external silica frustule, which is a good absorbent of hydrocarbons; retaining these compounds, of crude oil enables subsequent toxicity or hinders sexual reproduction and auxospore formation in diatoms (Kustenko 1981). Even though many cases of diatom susceptibility to oil have been reported, relative succession of diatoms under crude oil exposure (e.g., Rey et al. 1977, Thomas et al. 1981, Gonzalez et al. 2009, Adekunle et al. 2010, Gilde and
Pinckney 2012) were also reported. One can ask ”Is different phytoplankton groups relative tolerance depend on their taxonomic classification or something else?” It was suggested that cell size can be an answer to answer this question. Gonzales et al. (2009) stated that small diatoms (< 20 μm) were not only more tolerant to oil than bigger diatoms (> 20 μm) but also their growth was stimulated by low concentrations of crude oil. Huang et al. (2010) supported this study by demonstrating relatively smaller sized phytoplankton, *Skeletonema costatum* and *Melosira moniliformis*, to became the dominant species among various sizes and showed more tolerance to oil than bigger sizes of phytoplankton, *Ditylum brightwellii* and *Biddylphia mobiliensis*. It is speculated by Gonzales et al. (2009) that the reason might be indirect trophic interaction. However, a study by Sargain et al. (2007) compared picophytoplankton to nanophytoplankton and observed the tolerance of picophytoplankton was being less tolerant than nanophytoplankton (Sargian P. et al. 2007). Another study (Echeveste et al. 2010), which was investigated cell size dependent toxicity thresholds of polycyclic aromatic hydrocarbons, reported a good correlation between the higher tolerance of phytoplankton with larger sized cell.

Even though it is hard to understand what really govern toxicity of crude oil on phytoplankton, some physical parameters seem to be effective. Temperature is an element that affects the toxicity of crude oil on phytoplankton. Huang et al. (2010) demonstrated that *S. costatum* showed a high tolerance to WAF of crude oil in winter, however; even lower WAF concentrations caused subtle effects on their growth in summer. The reason, the researchers suggested, is that an increase in temperature causes an increase in metabolic rate, leading to greater body absorption of toxicants and,
therefore, further toxicity. Yet, while *S. costatum* had a similar tolerance in a cold water environment in another study (Ostgaard et al. 1984), the same species was shown to be very tolerant in temperate waters (Vargo et al. 1982).

These conflicting observations suggest that temperature alone might not change the toxicity of crude oil to phytoplankton species’ geographic origins (i.e., oceanic or coastal species) can also play a role. Moreover, nutrient regimes in the environment affects the sensitivity of phytoplankton to oil toxicity. Phosphorus-deficient cultures of the diatom *Skeletonema costatum* demonstrated a higher sensitivity to hydrocarbons than nitrogen- or silica-deficient cultures (Karydis 1981). It was considered that increased lipid content of the diatom in nitrogen deficient media might cause a higher dissolution of hydrocarbons through the plasma membrane (Karydis 1981). Even just geographic location by itself for oil spill site can make difference on toxicity of crude oil. Beyond nutrient values, community compositions, and physical conditions of water column, photo-induced toxicity caused by exposure to UV radiation in sunlight can enhance the toxicity of same crude oil differently in different locations (Arfsten et al. 1996). Growing evidence suggests that the real hazards of PAHs (Mekenyan et al. 1994) and crude oil (Sargian Peggy et al. 2005) to aquatic life result from synergistic joint effect of UV radiation and hydrocarbons. The toxicological concern of hydrocarbons has focused on the metabolic activation of these compounds to metabolites that are far more toxic than their parent compounds under enhanced UV radiation (Mekenyan et al., 1994).

In addition to the direct toxic effects of crude oil and its components on phytoplankton, crude oil has indirect effects that can also be detrimental. One example is the formation of oil films (or slicks) on the water surface, which can limit gas exchange
through the air-sea interface (Kinsey 1973) and can reduce light penetration into the water column by up to 90% (Nelson-Smith 1973), limiting phytoplankton photosynthesis.

Toxicity investigations on phytoplankton have been attempted to reveal mode of action of crude oil and dispersants. A gene expression study (Hook and Osborn 2012) demonstrated that crude oil, dispersed oil and the dispersant have a similar mode of action on phytoplankton. Lipophilic oil compounds accumulate in the cell membrane and change its structural and functional properties, including the loss of cell permeability, and other types of irreversible damage at the cell surface (Sikkema et al. 1995). It has been shown that crude oil led to some morphological changes on cells (Tukaj et al. 1998), reduced cell nuclei (Tukaj et al. 1998) and loss of cell mobility (Soto et al. 1975). It interferes with photosynthetic processes and inhibits total primary production of phytoplankton (Miller et al. 1978, Karydis 1979, Bate and Crafford 1985, Harrison et al. 1986, Aksmann and Tukaj 2008, Gonzalez et al. 2009). Alteration of photosynthesis related organelles such as shrinkage of chloroplast (Smith 1968, Tukaj et al. 1998) and pyrenoid (Tukaj et al. 1998), reduction of chlorophyll a content (Tukaj 1987, El-Dib et al. 1997), loss of other pigments (Smith 1968), loss of CO₂ absorption (Koshikawa et al. 2007) were reported. Evidently, the exposure caused to inhibition of nucleic acid synthesis (El-Sheekh et al. 2000) and reduction of protein content (Chen et al. 2008) and DNA and RNA damages and alterations (Bagchi et al. 1998, El-Sheekh et al. 2000, Tang et al. 2002, Parab et al. 2008). Cells under the exposure demonstrated oxidative stress (Tukaj and Aksmann 2007) and interference of anti-oxidant defending system operations (Wolfe et al. 1999, Aksmann and Tukaj 2008) was reported as well.
1.3. Deepwater Horizon Oil Spill

The Deepwater Horizon (DWH) oil spill occurred in the Gulf of Mexico (GoM) between April 20th and July 15th in 2010 and resulted in approximately 4.9 million barrels of South Louisiana sweet crude (LSC) oil spillage (Team 2010). The DWH spill was the largest accidental oil spill in U.S. history, and the impact of this oil within the GoM ecosystem remains to be fully understood. During the oil spill, petroleum hydrocarbons (PHs) mostly formed large, cohesive oil slicks on the surface due to their lower density. To reduce the potential impact of spilled oil to the shoreline and to enhance the degradation of PHs, a large amount of the chemical dispersant, Corexit® EC9500A, was added to mechanical containments such as skimmers or sorbents. The two main concerns over the use of the dispersant were its toxicity and that the dispersed LSC could sink below the surface and become more readily available to marine biota. Complex oceanographic processes have made it difficult to determine the current and future distribution of crude oil throughout the benthos and water column and its persistence in the marine environment. Most importantly, there are no immediate answers to questions concerning short-term and long-term impacts on phytoplankton communities in the path of this disaster. In the light of the aforementioned literature on the relationship between phytoplankton and crude oil and the lack of data on spilled LSC on phytoplankton, it was very crucial to evaluate impacts of LSC, oil dispersed by Corexit® EC9500A, and the dispersant itself on the planktonic communities in the GoM's ecosystem. Because of the distinct characteristics of spilled LSC, its impact on phytoplankton species native to the GoM should be assessed to better understand current changes to the GoM ecosystem and
to predict potential changes in the future. This dissertation work aimed to provide basic data in this regard, and these data can be used to provide a realistic assessment of the spill's impact to the GoM.

1.4. Synopsis of Chapters

The research presented in this dissertation seeks to evaluate the effects of LSC on GoM phytoplankton species. I hypothesize that due to its own characteristics, LSC has distinct impacts on GoM phytoplankton species, and these impacts can only be understood by using LSC and GoM phytoplankton species as principal tools in this dissertation work. In short, the goal of this research is to provide basic data for impact assessment of oil spills on GoM phytoplankton ecology and bloom dynamics by assessing crude oil's impact on phytoplankton at individual, population, toxin production, and subcellular levels.

Chapter 2, the first chapter that covers research, aims to determine threshold concentrations of crude oil, the dispersed oil, and Corexit® EC9500A on individual phytoplankton species to identify relative tolerance of the species and to define whether any correlation exists between the tolerance of different taxonomic groups of phytoplankton and the concentrations of crude oil, the dispersed oil, and Corexit® EC9500A. Also, determination of threshold concentrations is helpful in the experimental setups used in subsequent chapters. Chapter 2 also aims to provide a very detailed analysis of LSC to better understand its components and their bioavailability to phytoplankton under different physical and chemical conditions. In this manner, the dispersed oil mediums were prepared in various ways, such as under high and low energy
mixing, and in different dispersant:oil ratios to evaluate potential field scenarios more realistically.

In Chapter 3, several phytoplankton species were exposed to two PAH compounds, naphthalene and benzo(a)pyrene, to determine the toxicity threshold of these compounds on phytoplankton. The chapter has two main aims: 1) the assessment of toxic risk of each PAH to phytoplankton, and 2) the feasibility of using these toxicity data to predict the overall toxicity of crude oil on phytoplankton. Since PAHs, which are major components of crude oil, are considered to be largely responsible for the toxicity of crude oil to phytoplankton, knowing the toxicity of naphthalene and benzo(a)pyrene might be helpful in the prediction of aquatic toxicity of crude oil.

Population level effects are investigated in Chapter 4. After the DWH oil spill, the oil advanced towards the coastal areas, where high nutrient concentration can be found, as opposed to the location of the spill, which was an area of nutrient-limited conditions. Thus, the oil had impact on oligotrophic and eutrophic conditions. Chapter 4 aims to investigate the changes of phytoplankton composition under both conditions in order to assess the impact of high-nutrient input on the effects of the toxicity of LSC, Corexit® EC9500A, and chemically dispersed oil on phytoplankton and to provide basic data for impact assessment of oil spills and pollution on phytoplankton ecology and bloom dynamics in the GoM.

In Chapters 2 through 4, only non-toxic species of phytoplankton are used to determine the toxicity of LSC, Corexit® EC9500A, and chemically dispersed oil. However, the GoM has very ecologically important toxic phytoplankton species, including *Karenia brevis*, *Prorocentrum minimum*, and *Heterosigma akashiwo*, and their
responses to crude oil are unknown. Chapter 5 aims to (1) show whether any of the three species has a selective advantage due to toxin production when exposed to crude oil compared to non-toxic species studied under the same experimental conditions in Chapter 2, and (2) to determine the vicissitudes of toxin profiles on *K. brevis* and *P. minimum* under different exposure concentrations of LSC.

The last research chapter, Chapter 6, aims to understand the mode of action of the crude oil on phytoplankton at the sub-cellular level. Effects of crude oil on phytoplankton are described in previous chapters and some effects are known from the literature. However, the mechanism by which crude oil causes damage to phytoplankton is still unknown. To understand this mechanism, Chapter 6 investigates 1) changes in chlorophyll *a* content 2) the role of reactive oxygen species and oxidative stress in phytoplankton under crude oil exposure, and 3) the changes at the transcript level of selected genes during crude oil exposure.

The implications of all research chapters are discussed in Chapter 7. Each research chapter of this dissertation work is organized as a scholarly journal format so each chapter has its own introduction, literature review, materials and methods, results, discussion, conclusions, and references. However, the overall abstract, introduction, and conclusions are still provided.

1.5. References


Bate GC, Crafford SD. 1985. Inhibition of phytoplankton photosynthesis by the WSF of used lubricating oil. Mar Pollut Bull 16: 401-404.


Hook SE, Osborn HL. 2012. Comparison of toxicity and transcriptomic profiles in a diatom exposed to oil, dispersants, dispersed oil. Aquatic Toxicology.


Team OSA. 2010. Summary Report for Sub-Sea and Sub-Surface Oil and Dispersant Detection: Sampling and Monitoring.


CHAPTER 2: TOXICITY OF SOUTH LOUISIANA SWEET CRUDE OIL AND THE DISPERSANT COREXIT® EC9500A ON PHYTOPLANKTON SPECIES IN THE GULF OF MEXICO

2.1. Abstract

A controlled laboratory study assessed the toxic effects of water-accommodated fractions (WAFs) of South Louisiana Sweet crude oil (LSC) on five phytoplankton species indigenous to the Gulf of Mexico. Experiments were conducted with individual and combinations of the five phytoplankton species to determine growth inhibitions to eight crude oil concentrations ranging from 461 to 7205 ppb. The composition and concentration of LSC were altered by physical and chemical processes and used to help evaluate LSC toxicity. The impact of LSC exposure on phytoplankton growth varied with the concentration of LSC, species of microalgae, and their community composition. In decreasing order of tolerance, the following species showed a range of sensitivity to low levels of hydrocarbons from stimulation to severe growth inhibition: *Ditylum brightwellii* > *Chaetoceros socialis* > *Pyrocystis lunula* > *Scrippsiella trochoidea* > *Heterocapsa triquetra*. At a concentration of total petroleum hydrocarbons (TPH) <1200 ppb, dinoflagellates showed significantly better tolerance, while diatoms showed a higher tolerance to LSC at higher concentrations of TPH. For both groups, the larger species were more tolerant to LSC than smaller ones. The toxicity potential of LSC seems to be strongly influenced by the concentration of polycyclic aromatic hydrocarbons (PAHs). The addition of the dispersant, Corexit® EC9500A, increased the amount of LSC up to 50 fold in the water column, while the physical enhancement (vigorous mixing of water column) did not significantly increase the amount of TPH concentration in the water.
column. The species response to LSC was also examined in the five-species community. Each phytoplankton species showed considerably less tolerance to LSC in the five-species community compared to their individual responses. This study provides baseline information for subsequent research efforts seeking to understand the impacts of oil on the ecosystem.

2.2. Introduction

The presence of petroleum hydrocarbons (PHs) in marine environments is inevitable. Many different sources contribute to PH input such as natural crude oil seeps, pipeline spills, operational discharges, and platform and tanker accidents. Even though the amount of PHs introduced by tankers and offshore oil platforms accidents are considerably lower than those introduced by natural seeps (Council 2003), the rapid input of high volumes of PHs during these incidents raises serious concerns about the potentially fatal and sub-lethal impacts on marine biota (e.g., Howarth 1991, Fucik et al. 1995, Gilde and Pincney 2012). One example is the catastrophic Deepwater Horizon (DWH) oil spill that occurred in the Gulf of Mexico between April 20th and July 15th in 2010 and resulted in approximately 4.9 million barrels of South Louisiana sweet crude (LSC) oil spillage (Team 2010). During the oil spill, PHs mostly formed large, cohesive oil slicks on the surface due to their lower density. To reduce the potential impact of spilled oil to the shoreline and to enhance the degradation of PHs, a large amount of the chemical dispersant, Corexit® EC9500A, was added to mechanical containments such as skimmers or sorbents. Two main concerns over the use of the dispersant were its toxicity and that the dispersed LSC could sink below the surface and become more readily available to marine biota. There is a dearth of information concerning the impact of LSC,
the dispersed oil by Corexit® EC9500A, and the dispersant itself on the planktonic communities of the Gulf’s ecosystem.

Changes in aquatic ecosystems will likely lead to alterations in both the food webs they support and the composition of the primary producer communities. Phytoplankton species community structure is known to shift in response to changes in environmental conditions such as temperature, nutrient availability, grazing pressure, and increasing contaminant levels. Because phytoplankton constitute the base of the food chain, any negative effects of contaminants on the quantity and quality of primary productivity may affect higher trophic levels, including the ability of these higher trophic levels to recover after exposures.

Phytoplankton are known to respond to oil spills and make excellent indicators of such contaminants on ecosystem productivity (Teal and Howarth 1984, Dunstan et al. 1975, Adekunle et al. 2010, Gonzales et al. 2009, Hook and Osborn 2012). Although a substantial amount of research has established that crude oil is toxic to marine life, relatively little is known about its affect at the base of the marine food web. With respect to contaminants such as PHs, studies show that phytoplankton growth responses vary. Previous studies on the ecological effects of oil spills showed inhibition or enhancement of phytoplankton primary production and changes in phytoplankton population composition that depended on the type of oil and the phytoplankton species (Teal and Howarth 1984, Dunstan et al. 1975, Adekunle et al. 2010, Gonzales et al. 2009, Hook and Osborn 2012). At non-toxic levels of PHs, responses could include enhancement of metabolism due to increased nutrient availability from oil degradation, or stimulation of nitrogen-fixing algae and bacteria. Increased microbial activity in response to oil and the
incorporated increase in nitrogen as a result of their metabolism of hydrocarbons can
further cause increases in phytoplankton biomass (carbon) and contribute to changes in
the overall carbon budget in the system (e.g., Gilde and Pinney 2012).

There is, therefore, a critical need to better understand the effect of PHs on
phytoplankton because this carbon pool comprises a vital link to the higher trophic levels
in terms of food resources as well as integrity of coastal ecosystem stability. In this study
we aimed to examine how LSC with and without the chemical dispersant, Corexit®
EC9500A, affects the phytoplankton community structure and growth in the Gulf of
Mexico.

2.3. Materials and Methods

2.3.1. Preparation of the Test Mediums

Recent studies on fresh and dispersed crude oil toxicity to aquatic organisms have
used both the water-accommodated fraction (WAF, LSC in seawater) and chemically-
enhanced WAF (CEWAF, Corexit® EC9500A–LSC mixtures in seawater) to provide
realistic assessments. We assessed the WAF, CEWAF, and dispersant toxicity using five
phytoplankton species that are both common and abundant in the Gulf of Mexico. These
assays were done with the individual species and as a five-species community.

Enhancement of crude oil in the water column under different physical conditions such as
non-mixing and vigorous mixing was also applied.

Non-weathered LSC was collected by British Petroleum (BP) through a riser vent
pipe from the damaged wellhead of the Deepwater Horizon drilling rig in the Gulf of
Mexico on May 20, 2010, and stored at -4 °C (BP, Ford Collins, CO, USA). The
dispersant, Corexit® EC9500A, was provided by the Department of Oceanography & Coastal Sciences at Louisiana State University, Baton Rouge.

The WAF was prepared according to the method described in The Chemical Response to Oil Spills: Ecological Research Forum (CROSERF) (CROSERF, 2005). The WAF mixtures (Figure 2.1) used in algae toxicology tests were prepared with 0.22 μm filtered and autoclaved Gulf of Mexico seawater (34 to 35 ppt) in 2 L Klimax valved outlet reservoir bottles. Loading of 40 g LSC in 1.6 L seawater is known to result in 20–25% headspace by volume in each bottle. The WAF solutions were prepared at two different conditions: low mixing energy (no vortex), WAF(L), and high mixing energy (vigorous vortex), WAF(H). Replication of these conditions involved creating a seawater sample with an oil film on top that is not disturbed by vortex formation (Figure 2.1). The
WAF(L), which is essentially free of particulates of bulk material, represents a calm seawater surface by which the crude oil slicks enter the water column naturally, without wind and wave action (Figure 2.1A). The stirring rate was adjusted to 160 rpm to prevent micro particulate settlement at the bottom. The WAF(H) (~1100 rpm) represents a rough water surface, where the surface water is vigorously mixed with the layer underneath, forming numerous oil particles of variable size; the vortex in this sample extends from the water surface down to the bottom of the container (Figure 2.1B). The purpose of the WAF(H) preparation is to investigate the amount of hydrocarbons that enter the water column under rough mixing conditions. After 24 hours of mixing, 6 hours of settling time were given for both conditions. Samples from the WAF were withdrawn through a valve located at the bottom of the bottle to avoid disturbing the water/oil interface. Samples for chemical analysis were collected in amber glass jars with Teflon lined caps, allowing no headspace and stored at 4 °C. Serial dilutions (100%, 40%, 16%, and 6.4%) of the water phase from each test medium yielded concentrations ranging from 461 to 7205 ppb PH that were used in the experiments.

The CEWAF was prepared in the same way as the WAF preparation with the exception of mixing energy and the addition of the Corexit® EC9500A (Figure 2.1C). Two different concentrations of Corexit® EC9500A were chosen — 1:20 and 1:100 (dispersant:oil ratios). A moderate mixing energy (~650 rpm) was used to produce chemically dispersed oil, by forming a vortex that was 20–25% of the water’s depth. The solution was mixed for 24 hours and the water phase was collected after a 6-hour equilibrium time. Serial dilutions (100%, 40%, 16%, and 6.4%) of this water phase were
used in the experiments. Other parameters utilized for the two WAF treatments and the CEWAF treatment are summarized in Table 2.1.

Table 2.1. Conditions for preparing test

<table>
<thead>
<tr>
<th></th>
<th>WAF</th>
<th>CEWAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil weathering</td>
<td>Fresh</td>
<td>Fresh</td>
</tr>
<tr>
<td>Oil loading</td>
<td>25 g/L</td>
<td>25 g/L</td>
</tr>
<tr>
<td>Dispersant</td>
<td>None</td>
<td>Corexit® EC9500A</td>
</tr>
<tr>
<td>Mixing energy/time</td>
<td>No vortex for WAF(L) and full</td>
<td>20–25% vortex/24 hrs</td>
</tr>
<tr>
<td></td>
<td>vortex for WAF(H)/24 hrs</td>
<td></td>
</tr>
<tr>
<td>Equilibration time</td>
<td>6 hrs</td>
<td>6 hrs</td>
</tr>
<tr>
<td>Test concentrations</td>
<td>100%, 40%, 16%, 6.4% dilutions of WAF</td>
<td>100%, 40%, 16%, 6.4% dilutions of CEWAF</td>
</tr>
<tr>
<td>Oil exposure regime</td>
<td>Static/open chamber</td>
<td>Static/open chamber</td>
</tr>
<tr>
<td>Analytical chemistry</td>
<td>TPH, alkanes (nC-10 to C-35), PAHs</td>
<td>TPH, alkanes (nC-10 to C-35), PAHs</td>
</tr>
<tr>
<td>Temperature/salinity</td>
<td>25 °C/ 34–35 ppt</td>
<td>25 °C/ 34–35 ppt</td>
</tr>
<tr>
<td>Light intensity</td>
<td>Fluorescent, 85 μE.m².s⁻¹</td>
<td>Fluorescent, 85 μE.m².s⁻¹</td>
</tr>
<tr>
<td>Toxicity end point</td>
<td>Stationary phase of control flasks</td>
<td>Stationary phase of control flasks</td>
</tr>
</tbody>
</table>

2.3.2. Microalgal Cultures

The toxicity of the WAF, CEWAF, and Corexit® EC9500A was assessed using five phytoplankton species that are common and abundant the in Gulf of Mexico:

*Chaetoceros socialis*, *Ditylum brightwellii*, *Heterocapsa triquetra*, *Pyrocystis lunula*, and *Scrippsiella trochoidea*. The initial cultures of phytoplankton were provided by National Center for Culture of Marine Phytoplankton (CCMP), ME, USA. The CCMP numbers and other physiological features are summarized in Table 2.2. The test organisms were acclimated to ambient laboratory conditions prior to use in the experiments. The cultures
were grown at 25 °C and 35 ppt in 0.22 µm filtered and autoclaved natural seawater on a 12:12 hour light:dark cycle with cool-white fluorescent lights at an irradiance of 85 µE.m$^{-2}$.s$^{-1}$ in f/2 or f/2-Si medium.

Table 2.2. General physiological features of phytoplankton used in this study, ± SD

<table>
<thead>
<tr>
<th>Species</th>
<th>CCMP #</th>
<th>Biovolume (µm$^3$)</th>
<th>Growth rate (day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. socialis</em></td>
<td>203</td>
<td>488±103</td>
<td>1.39±0.05</td>
</tr>
<tr>
<td><em>D. brightwellii</em></td>
<td>359</td>
<td>24,594±10,999</td>
<td>0.71±0.04</td>
</tr>
<tr>
<td><em>H. triquetra</em></td>
<td>2981</td>
<td>9,292±4,416</td>
<td>0.92±0.13</td>
</tr>
<tr>
<td><em>P. lunula</em></td>
<td>731</td>
<td>238,511±89,998</td>
<td>0.46±0.06</td>
</tr>
<tr>
<td><em>S. trochoidea</em></td>
<td>3081</td>
<td>9,709±1,280</td>
<td>0.81±0.06</td>
</tr>
</tbody>
</table>

2.3.3. Experimental Design and Conditions

We assessed the toxicity of the WAF, CEWAF, and Corexit® EC9500A using the five phytoplankton species listed above either as individuals or combined. Controlled laboratory microcosm studies were conducted using standard static non-renewal exposure toxicity tests. Growth inhibition was selected as the measure of toxicity for each species. Autoclaved Pyrex flasks (500 mL) were utilized in all experiments, and the experimental exposure medium volume was 350 mL for all conditions. The initial concentrations in cultures were adjusted according to each species’ biovolume so that the initial biomass for all species was the same order of magnitude. Calculations of cell biovolumes and surface areas (n ≥10) were carried out according to geometric models assigned to each species (Sun and Liu 2003, Olenina et al. 2006). These measurements are summarized in Table 2.2.

The WAF exposure experiments were conducted with five phytoplankton species exposed to 4 crude oil concentrations in the WAF (L), and to 4 crude oil concentrations...
in the WAF(H), ranging from 461 to 7205 ppb TPH, to determine individual responses of relative toxicity of LSC. The control flasks contained only the phytoplankton cultures in the growth media. The exposure studies consisted of three replications per treatment. Temperature was monitored continuously; salinity and pH were measured at the beginning of each experiment. Salinity ranged from 34‰ to 35‰, and the pH was about 8.0 for all treatments. The end point for each experiment was determined based on the time when each species reached its stationary phase in control cultures.

The preliminary results were evaluated to determine the LSC concentrations to use in the CEWAF exposure experiment after completion of the WAF exposure experiment. The TPH concentration of 2882 ppb was determined to be the optimal concentration, because this concentration has an intermediate effect on growth inhibition of the phytoplankton. *D. brightwellii* and *S. trochoidea* were selected for these experiments. These two medium-sized species represented each phytoplankton group, diatom and dinoflagellate, respectively, in the experiment. Two types of controls were used in the CEWAF experiments, flasks containing only phytoplankton (without the LSC and Corexit® EC9500A) and flasks containing cultures with Corexit® EC9500A but without the addition of LSC. The amounts of Corexit® EC9500A in the control flasks were calculated from the nominal concentration in the CEWAF flasks and used in control flasks. The preparation of Corexit® EC9500A-containing flasks involved the addition of Corexit® EC9500A and vigorous stirring with a stir bar for 2 min. at high speed (~650 rpm). For both WAF and CEWAF exposure experiments, daily samples were taken for chlorophyll *a* (chl *a*) measurements to construct a growth curve for each treatment.
Samples were also taken for their TPH, alkane, and PAH compositions and concentrations at Day 0 and Day 10.

The individual species were then combined into a 5 species community. The initial population of each species was adjusted by considering the biovolume of each species in an effort to keep the starting biomass similar in all experiments. The 1150 ppb TPH test concentration did not show growth stimulation or excessive inhibitory effects on individual species and was, therefore, selected for the WAF experiments. Changes in the species composition throughout the experiment were assessed by enumeration under an inverted microscope through days 0–14. The chl \( a \) levels and cell counts were monitored daily to track the phytoplankton biomass in the flasks.

2.3.4. Chemical Analysis of the Crude Oil

An average 750 mL of the WAF was placed in a 2 L separatory funnel, and 150 mL dichloromethane (DCM) was added for the first extraction. In addition to DCM, 1 mL of surrogate standard (Absolute Standard, Inc., Hamden, CT, USA) at 20 ppm was added to evaluate extraction efficiencies. The aqueous layer was extracted with additional DCM (2 x 150 mL), and the DCM layers were combined and dried over Na\(_2\)SO\(_4\). The extracts were reduced on a rotary evaporator, yielding a yellow-brown liquid. The reduced extracts were transferred to graduated flasks to reduce them to the desired volumes under nitrogen gas and a water bath in a nitrogen evaporator (N-EVAP 111; Organomation Associates, Inc., MA, USA). A 1-mL sample of the resulted crude was transferred to GC-MS vials, and 10 \( \mu \)L internal standard was added to each vial for GC-MS measurements.
Quantitative analysis of the PHs in the WAF was performed at Day=0 and Day=10 days exposure time, using a combination of external and internal standard methods (EPA 1996). Three different sets of experimental conditions were replicated at different times to evaluate the reproducibility of the method used for the preparation of the test medium. A series of external standard solutions (containing saturated alkanes in the range of nC$_{10}$ through nC$_{35}$ and polycyclic aromatic hydrocarbons [PAHs]) combined with the internal standard (Naphthalene-d8, Acenaphthene-d10, Chrysene-d12, and Perlyene-d12; AccuStandard, New Haven, CT, USA, Lot#: 121004) were used to calculate instrument response factors. Due to the lack of commercially available alkylated PAH homologues; these compounds were quantified by response factors generated by the un-alkylated parent compounds.

Alkanes and PAH measurements were carried out in Agilent Gas Chromatograph (GC) (Santa Clara, CA) equipped with an Agilent 5975 intert XL mass selective detector (MSD) and fitted with an HP-5MS high resolution capillary column (30 m long, 250 μm in diameter, and film thickness of 0.25 μm). Ultra-high purity helium (Air Liquid, Houston, TX, USA) at a constant flow rate of 1 mL min$^{-1}$ was used as the carrier gas. The injection port was set at 250 °C, run in spittless mode, and fitted with a Hewlett-Packard, single-tapered deactivated borosilicate liner. The oven temperature was programmed to 55 °C for 3 min., then raised to 280 °C at a rate of 5 °C min$^{-1}$ held for 3 min. The oven was then heated from 280 °C to 300 °C at a rate of 1.5 °C per min and held at 300 °C for 2 min. The temperature of the MSD interface to MS was set at 280 °C. The MSD was operated in the selective ion-monitoring (SIM) mode for quantifying specific alkanes and PAHs.
Total petroleum hydrocarbon (TPH) analysis was carried out with gas chromatography equipped with a flame ionization detector (GC/FID). The extracts were analyzed within a single batch by gas chromatography, using an HP Agilent 6890 gas chromatograph (Agilent Technologies, www.agilent.com) equipped with a FID detector, an Agilent 7673 autosampler, and a low-bleed Supelco Equity™-5 capillary column (15 m×0.25 mm i.d.).

2.3.5. Determination of Growth Rates and Percent Growth Inhibition

A regression analysis between chl \(a\) content and cell number was determined for each species prior to the experiments. The abundance of each phytoplankton species was estimated by enumerating cells on a Sedgwick–Rafter counting slide (n=3). Samples (10 mL each) were taken daily, filtered through Whatman GF/F filters, and stored at \(-20\)°C until extraction. The filters were then extracted for 24 hours in 90% aqueous acetone at \(-20\)°C, and subsequently analyzed for chl \(a\) using a Turner fluorometer (Parsons et al. 1984). A qualitative assessment of the species in collected water samples were also recorded using a Zeiss Axio Observer-A1 inverted microscope with epifluorescence capability. These chl \(a\) values were converted to algae biomass, and biomass vs. time growth curves were plotted. The common way of calculating specific growth rate from the slope of each exponential growth phase of the growth curve did not work well in this study due to irregularities in exponential growth phases among the different treatment setups, with some having an exponential phase and others having no exponential growth.
Algal growth was instead calculated by using the area under the growth curve, which is equal to total increase in biomass.

\[ A = \frac{(N_1 - N_0) \cdot t_1}{2} + \frac{(N_1 + N_2 - 2N_0) \cdot (t_2 - t_1)}{2} + \ldots + \frac{(N_{n-1} + N_n - 2N_0) \cdot (t_n - t_{n-1})}{2} \]

\[ \mu = \ln \left( \frac{N_{t_2}}{N_{t_1}} \right) / (t_2 - t_1) \]

where \( N_{t_2} \) and \( N_{t_1} \) are cell numbers at time \( t_2 \) and \( t_1 \), respectively.

The inhibition rates of different treatments were calculated according to the following formula: \( I(\%) = (A_c - A_t) / A_c \times 100 \), where \( A_c \) and \( A_t \) are the area under the growth curve of control group and the treatment, respectively.

2.3.6. Statistical Analysis and Calculation of EC\(_{50}\) Values

All statistical analyses requiring comparison of treatments were carried out using SigmaStat 11.2 software (Systat Software, Inc., San Jose, CA, USA). ANOVA and t-test were performed to evaluate significance of individual differences with a probability threshold of 0.05, followed by a post-hoc Tukey test. EC\(_{50}\) (concentration causing 50% inhibition) values were calculated by the relationship between percent growth inhibition and crude oil concentration. The models were fitted to data by nonlinear regression analysis using R (R Development Core Team 2006) with the add-on package “drc” (Ritz and Streibig 2005). In the case of a monotonically decreasing function, a four-parameter, log-logistic model was applied for each species. In the context of hormesis, where growth stimulation is observed, a five-parameter, Brain-Cousens modified log-logistic model (Brain and Cousens 2006) was applied.
2.4. Results

2.4.1. Crude Oil Analysis

All WAF and CEWAF treatments were analyzed with the GC−MS for their TPH, alkanes, and PAH compositions and concentrations at Day 0 (Table 2.3, Figs. 2.2, 2.3, 2.4). The values at Day 0 show that mixing energy increased the amount of TPH, alkanes, and PAHs.

Table 2.3. Total amount of TPHs, alkanes, and PAHs, for four different treatments at Day 0 (mean±SD, n=3).

<table>
<thead>
<tr>
<th></th>
<th>TPH (ppm)</th>
<th>Alkanes (ppb)</th>
<th>PAHs (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAF(L)</td>
<td>7.2±4.9</td>
<td>73.2±0.5</td>
<td>121±0.8</td>
</tr>
<tr>
<td>WAF(H)</td>
<td>8.9±1.2</td>
<td>308±9.0</td>
<td>225±3.1</td>
</tr>
<tr>
<td>CEWAF(1:100)</td>
<td>380±28</td>
<td>5492±82</td>
<td>1100±21</td>
</tr>
<tr>
<td>CEWAF(1:20)</td>
<td>373±16</td>
<td>14419±115</td>
<td>2552±32</td>
</tr>
</tbody>
</table>

Figure 2.2. Percent aliphatic hydrocarbon composition for each exposure treatment. Numbers in parenthesis show the ratio of the amount of crude oil to the dispersant.
in seawater by 1.2, 4.2, and 1.9 fold, respectively. These changes, however, were not
significant when compared to the increases in concentrations in the CEWAF treatments
(Table 2.3). Regardless of the dispersant:oil ratio, addition of the Corexit® EC9500A
increased the TPH concentration approximately 50 fold. Alkanes and PAHs
concentrations increased by approximately 2.5 fold at higher ratio of 1:20 CEWAF.

After the 10-day period, a significant decrease in LSC concentrations was
observed. The decreases in TPH concentrations for all treatments varied from 36% to
49%. Greater declines in the amount of alkanes and PAHs for each treatment were
observed. These declines were up to 81% for alkanes and 92% for PAHs (Table 2.3). As
was expected, the reduction of lighter compounds of alkanes and PAHs was greater than
that of heavier compounds since lighter compounds evaporate more quickly.
Figure 2.4. Aromatic hydrocarbon distribution in the four treatments. C1, C2, C3 and C4 represent different alkylated homologs of the parent compounds. Percent difference represents the fraction changes when the dispersant added.
Alkanes of LSC in the WAF treatment mostly comprised of lower carbon number compounds, and concentration decreased with increasing carbon number (Figure 2.2). Significant compositional changes in alkanes were observed at low C compounds (Figure 2.3). Increasing the mixing energy in the WAF flasks increased the fraction of C_{10} to C_{15} and C_{19} to C_{25} an average of 2.8 times, and reduced the fraction of C_{16} to phytane and C_{26} to C_{30} an average of 41%. The addition of the dispersant increased the fraction of lighter alkane compounds. Even though the amount of total alkanes in the CEWAF (1:20) was three times greater than the amount found in the CEWAF (1:100), the fraction of each compound was almost identical in both treatments. This indicates that the addition of the dispersant increases the fraction of alkanes nC-10 through nC-14, but further additions of the dispersant does not result in more fractional changes, only increases in the total amount of alkanes in the water column.

The composition of the PAHs did not result in significant changes with different mixing energy, but addition of the dispersant significantly changed the fraction of some of the PAH compounds (Table 2.4, Figure 2.4). For both WAF and CEWAF treatments, the predominant PAH was naphthalene, comprising a mean composition of 86.8% and 63.1%, respectively (Figure 2.4). In CEWAF treatments, the concentrations of phenanthrene, pyrene, and chrysene were higher than in the WAF mediums.

Table 2.4. Mean distribution of the PAH compounds in four different treatments.*

<table>
<thead>
<tr>
<th>PAHs</th>
<th>WAF(L)</th>
<th>WAF(H)</th>
<th>CEWAF(1:100)</th>
<th>CEWAF(1:20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene (%)</td>
<td>87.7</td>
<td>85.9</td>
<td>62.6</td>
<td>63.5</td>
</tr>
<tr>
<td>Phenanthrene (%)</td>
<td>4.6</td>
<td>5.7</td>
<td>16.2</td>
<td>16.0</td>
</tr>
<tr>
<td>Pyrene (%)</td>
<td>0.5</td>
<td>1.0</td>
<td>4.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Chrysene (%)</td>
<td>0.2</td>
<td>0.9</td>
<td>3.9</td>
<td>3.7</td>
</tr>
<tr>
<td>Others (%)</td>
<td>7.4</td>
<td>7.6</td>
<td>17.1</td>
<td>16.6</td>
</tr>
</tbody>
</table>

* Only the compounds that are significantly different between the WAF and CEWAF are shown.
Specifically, the addition of Corexit® EC9500A caused a 27.4% reduction in naphthalene and increases of 313%, 560%, and 690% in phenanthrene, pyrene, and chrysene concentrations, respectively, for both CEWAF treatments compared to the WAF treatments. These values represent the average changes for all alkylated homologs of the parent compounds. Each of alkylated homologs, however, was diversely altered with the addition of the dispersant (Figure 2.4). For example, while the composition of naphthalene and C1-Naphthalene decreased in the CEWAF, the compositions of C2-, C3- and C4-Naphthalene increased.

2.4.2. Toxicity of WAF

Each phytoplankton species used in the WAF-exposure experiments reached their stationary phases in 8–15 days in the control flasks at varying growth rates (Table 2.2). Percent growth inhibition rates were calculated for each LSC concentration for each species. TPH and PAH concentrations in the WAF treatments were directly correlated to percent growth inhibition, and, therefore, they were plotted against nominal TPH and PAH concentrations (Figs. 2.5 and 2.6). Each phytoplankton species demonstrated a range of sensitivity to LSC, from stimulation to inhibition (Figure 2.5). The degree to which LSC influences phytoplankton growth varied with the concentration of oil and the algae species. Increasing LSC concentrations increased the percent growth inhibition rates for all species (Figures 2.5A, 2.6A). However, at low concentrations, <1200 ppb, dinoflagellates showed significantly (p<0.001) better tolerance to LSC than diatoms (Figures 2.5B, 2.6B). The dinoflagellates reached 90%–100% growth inhibition at 3500 ppb TPH, but the diatoms reached the same range above 7000 ppb TPH (Fig 2.5A).
Figure 2.5. Percent growth inhibition of each phytoplankton species under different concentrations of LSC. Concentration of LSC is given as TPH amount in ppb. The growth curve for each species with TPH concentrations (A) between 0 to 9000 ppb and (B) below 1200 ppb. Negative growth inhibition indicates the stimulation of growth.

Fig 2.6. Percent growth inhibition of each phytoplankton species under different concentrations of LSC. Concentration of LSC is given as PAH amount in ppb. The growth curve for each species with PAH concentrations (A) between 0 to 250 ppb and (B) below 8 ppb. Negative growth inhibition indicates the stimulation of growth.
For *D. brightwellii* and *C. socialis*, flasks containing the lowest TPH concentration (461.1 ppb), showed 15.08% and 16.09% growth inhibition, respectively, compared to in the control flasks. On the other hand, at the same TPH concentration, the three dinoflagellates, *P. lunula, S. trochoidea,* and *H. triquetra*, showed stimulation in growth (Figure 2.5B). Although the difference was not significant (p=0.897), diatoms seemed to be more tolerant than dinoflagellates at higher concentrations (>1200 ppb) of LSC. A sharp change in growth inhibition is observed within a very narrow range (0–2 ppb) for each species when LSC concentration is defined in PAH amounts (Figure 2.6), suggesting high sensitivity to LSC EC$_{50}$ values for each species were also calculated by using both TPH and PAH concentrations (Table 2.5). According to EC$_{50}$ values, the two diatom species, *D. brightwellii* and *C. socialis*, showed higher tolerance to LSC than the three dinoflagellates species, *P. lunula, S. trochoidea,* and *H. triquetra*. The range of EC$_{50}$ values calculated with TPH concentrations was more conservative, with a 2.44 fold difference between the highest and the lowest concentrations, while the EC$_{50}$ values calculated with PAHs had a 6.55 fold difference between the highest and the lowest concentrations.

<table>
<thead>
<tr>
<th>Phytoplankton Species</th>
<th>TPH EC$_{50}$ (ppb)</th>
<th>PAH EC$_{50}$ (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. brightwellii</em></td>
<td>2497.5</td>
<td>48.0</td>
</tr>
<tr>
<td><em>C. socialis</em></td>
<td>1834.6</td>
<td>25.3</td>
</tr>
<tr>
<td><em>P. lunula</em></td>
<td>1751.4</td>
<td>14.0</td>
</tr>
<tr>
<td><em>S. trochoidea</em></td>
<td>1137.7</td>
<td>8.9</td>
</tr>
<tr>
<td><em>H. triquetra</em></td>
<td>1025.3</td>
<td>7.3</td>
</tr>
</tbody>
</table>
Both TPH and PAH EC$_{50}$ values also changed in direct relation to biovolume of phytoplankton within the group. In the diatom group, $D.\ brightwellii$ has much larger biovolume than $C.\ socialis$ and was more resistant to LSC toxicity. Similarly among the dinoflagellates, $P.\ lunula$ is largest in size and showed the greatest tolerance, while $H.\ triquetra$ is the smallest and showed the weakest resistance against LSC toxicity (Table 2.2).

2.4.3. Toxicity of CEWAF

The addition of Corexit® EC9500A increased TPH concentration about 50 fold, so the resulting CEWAF treatments caused 100% mortality in both species exposed during the course of the experiment (Figure 2.7). The highest TPH concentration detected in the dispersed LSC

![Figure 2.7. Percent growth inhibition of each phytoplankton species under different exposures concentrations of CEWAF and Corexit® EC9500A. The left graph shows the response of $S.\ trochoidea$ and the right graph shows the response of $D.\ brightwellii$.](image-url)
treatment (156 ppm) was much higher than the highest concentration used in the WAF exposure experiments (8 ppm). The nominal concentrations of the crude oil and Corexit® EC9500A of each treatment are summarized in Table 2.6. Even though all treatments resulted in complete mortality for both species, some growth of *D. brightwellii* occurred at the beginning of the experiments for all treatments except the treatment with only 500 ppm Corexit® EC9500A. In both CEWAF treatments, *D. brightwellii* growth occurred until day 2; then the cultures crashed.

Table 2.6. The nominal concentrations of Corexit® EC9500A and the crude oil in terms of TPH concentrations of each treatment are summarized. The corresponding % growth inhibitions are given for each treatment.

<table>
<thead>
<tr>
<th></th>
<th>Corexit® EC9500A (ppm)</th>
<th>TPH (ppm)</th>
<th>% Inhibition of Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. brightwellii</em></td>
<td>500</td>
<td>0</td>
<td>101.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
<td>94.9</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>149</td>
<td>100.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>156</td>
<td>99.7</td>
</tr>
<tr>
<td><em>S. trochoidea</em></td>
<td>500</td>
<td>0</td>
<td>106.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
<td>107.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>149</td>
<td>106.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>156</td>
<td>106.1</td>
</tr>
</tbody>
</table>

Growth in flasks containing only 100 ppm Corexit® EC9500A showed much better growth than in flasks containing the CEWAF, but they also crashed after day 7 (Figure 2.8). The treatments with *S. trochoidea* showed complete mortality from the beginning of the experiment. Since 100 ppm Corexit® EC9500A was the lowest concentration used and caused complete mortality on both species at the end of the experiment, it can be concluded that EC50 values of Corexit® EC9500A for both species are <100 ppm.
Figure 2.8. Growth curves of *D. brightwellii* under different concentrations of Corexit® EC9500A and dispersed LSC exposures

2.4.4. Community Response of the Phytoplankton Species

After the assessment of the WAF and the CEWAF exposures on individual phytoplankton species, a five-species community was formed to investigate growth responses within the community compared to the single-species growth responses. In the control flasks, which contained only 5 species with no crude oil, a competitive exclusion was observed over the course of the experiment, resulting in *D. brightwellii* dominancy (Figure 2.9). Comparison of each species growth rate within the community to single-species growth rates showed that *D. brightwellii*’s growth rate enhanced by 35.2% within the community. However, growth rate of the other species reduced drastically in the community in the control flasks (Table 2.7).
Figure 2.9. Growth curve for species in the control flask. The left graph shows all species; the right graph shows all species except *D. brightwellii*.

Table 2.7. Changes of five-species community growth rates in control flasks compared to individual species growth rates.

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth Rate Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. socialis</em></td>
<td>-89.6%</td>
</tr>
<tr>
<td><em>D. brightwellii</em></td>
<td>35.2%</td>
</tr>
<tr>
<td><em>H. triquetra</em></td>
<td>-78.4%</td>
</tr>
<tr>
<td><em>P. lunula</em></td>
<td>-40.3%</td>
</tr>
<tr>
<td><em>S. trochoidea</em></td>
<td>-80.2%</td>
</tr>
</tbody>
</table>

On the other hand, growth in the WAF-exposed flasks showed complete reduction in growth rates of all species (Figure 2.10). With the exception of *C. socialis*, the populations of all species dropped to zero by the end. The population of *C. socialis* did not show any growth, and the population reduced to 75.1% of its initial concentration during the course of experiment.
Figure 2.10. Growth curves for each species in the community after WAF exposure.

The same amount of crude oil in the WAF-exposure experiment involving the single species, *C. socialis*, caused a maximum of 29.5% in growth reduction. These results suggest that single species has more tolerance to crude oil than the collective species in a community.

2.5. Discussion

2.5.1. Chemical Analysis

The results of the chemical analysis demonstrated that physical disturbances, in this case the mixing of the water column, can alter crude oil bioavailability to phytoplankton (Table 2.3; WAF(L) vs. WAF(H)). While there was no significant difference (p>0.1) in the increase of TPH with increased mixing energy, there was a highly significant increase in the concentration of alkanes and PAHs. This most likely resulted from different methods used to prepare each type of test medium. While the preparation of WAF(L) does not allow micro particles to form in the water column,
mixing the water column (>25% vortex) causes the formation of colloidal micro particles (1–70 micron diameter) (CROSERF 2005). This suggests that micro particles can have higher PAH and alkane concentrations than the compounds in their dissolved form, and that wave actions can add these high PAH-containing micro particles into the water column and contribute to the total toxicity. Two different dispersant:oil ratios were also selected as chemical disturbance treatments for this study - 1:20 as an upper limit and 1:100 as a lower limit. Even the addition of the dispersant at the 1:100 ratio caused a remarkable increase in TPH concentration, which was 150–380 times higher than the EC₅₀ concentration values of the phytoplankton species.

Increasing the concentration of dispersant from 1:100 to 1:20 did not cause further change in the TPH concentration, but significant increases in alkane and PAH concentrations were observed (Table 2.3). Therefore, measuring only TPH concentrations can be misleading and should be avoided when evaluating a dispersant’s efficiency. The stability of TPH amounts and increasing amounts of alkanes and PAHs that resulted from the addition of Corexit® EC9500A is most likely explained by a decrease in the fractions of the other constituents in LSC. Because our analysis did not allow for the detection of highly abundant BTEX (benzene, toluene, ethylbenzene and xylene) and other lighter alkane compounds (<C₁₀), their fractional changes were unknown. Future analysis of these compounds would allow us to better understand the mechanism and impact of dispersants on LSC.

The higher amounts of PAHs in the water column is a serious concern because the highly lipophilic nature of PAHs causes longer retention in organisms than alkanes (Anderson et al. 1974, Neff 1979). Unlike PAHs, the WAF of LSC is rich in light
aliphatic, mainly alkanes, which have relatively short life spans and evaporate rapidly (Mackay and Wolkoff 1973). Thus, the effects of alkanes on phytoplankton are short-term compared to the effects of PAHs. Even the reduction (Table 2.3) of TPH concentration did not allow any phytoplankton species to recover over the course of 10-day experiments. This suggests that the initial impact of crude oil on phytoplankton was profound and irreversible at the level studied.

The patchy distribution of LSC after the DWH oil spill made measurements of oil concentration in the Gulf of Mexico difficult. One group (Wade et al. 2011) found LSC concentration ranging from 2 to 442 ppb at approximately 1000 m depth between May 24, 2010, and June 6, 2010. During a similar time frame (May 8 to June 1), NOAA (NOAA 2010) reported surface and subsurface TPH concentrations of LSC in the Gulf of Mexico. The surface concentrations ranged from 1 ppm to 984 ppm. In our study, the TPH concentrations ranged from 7 to 380 ppm, which are within the range observed in the field.

2.5.2. WAF Toxicity

It is essential to understand the role of biotic and abiotic factors that govern the phytoplankton community’s assembly and dynamics because the composition of the phytoplankton community impacts how aquatic ecosystem function, biogeochemical cycling, and global climate change (Litchman and Klausmeier 2008). When both dinoflagellates and diatoms were exposed to varying crude oil levels, three dinoflagellate species showed growth stimulations at low concentrations, while two diatom species did not show any stimulation in their growth (Figs. 5 and 6). Growth stimulation can be explained by the hormesis hypothesis (Stebbing 1982), which suggests that low doses of
toxins or other stressors might activate the repair mechanisms that fix not only the
damage caused by the toxin, but also other types of damage that might have accumulated
before exposure that did not trigger the repair mechanism. The lack of a growth response
from the 2 diatom species can be due to their lack of stimulation by hydrocarbons, or
because stimulation in these species occurs at a concentration that was untested in this
experiment’s setup. The reason remains unknown.

At high concentrations of crude oil, diatoms showed a better tolerance suggesting
that during or right after an oil spill, diatoms can predominate in the area where the oil
spill occurred, but that after the effects of oil lessen, that dinoflagellates can become
dominant. The stimulation in growth can give dinoflagellates a competitive advantage
over diatoms at low hydrocarbon concentrations. This indicates that changes in the field
concentrations of crude oil over time may drive the population shift of phytoplankton.

While the majority of studies in the literature report susceptibility of diatoms to
crude oil, only a few studies (Thomas et al. 1981, Gonzalez et al. 2009, Adekunle et al.
2010, Gilde and Pinckney 2012) show that diatoms had a relatively better tolerance than
other groups. However, none of the studies that we know of demonstrated variations in
the vulnerability of phytoplankton groups under different concentration ranges of
hydrocarbons. Population shifts in the field, particularly between diatoms and
dinoflagellates, may have significant consequences. Different sinking rates of
phytoplankton due their varying cell sizes and interactions can affect the level of
particulate organic matter flux, which would eventually affect microbial food web
stability (Smetacek 1999), such as changes in local nutrient cycling as well as localized
food depletion for both planktonic and benthic ecosystems. A predominance of
dinoflagellates in the field may also increase the presence of toxic species that can introduce additional stress for higher trophic levels.

The growth rates of phytoplankton vary extensively depending on environmental conditions. Even though their growth acclimation, log, and stationary phases show wide variability among species, the majority of the previous studies on toxicant effects on phytoplankton growth lasted ≤4 days, which sometimes prevented the observation of species-specific responses. Our study aimed to observe WAF and CEWAF effects for a longer time period in order to evaluate any possible recovery during exposure. Although some recovery might have been expected due to the loss of volatile fractions, no apparent population recoveries of any culture were observed during the course of the experiments. This shows that toxic compounds in the LSC were persistent and had irreversible impact on microalgae. It is also worth noting that for very short-term toxicity studies of phytoplankton, choosing a test species is extremely important. In our study, extremely slow growth or the very long lag phase profiles (Figure 2.11) of P. lunula might result in a different response if it was evaluated in shorter time frame. If the experiment had been conducted for ≤ 4 days, it would have been inferred that there was no significant difference among the treatments. However, a difference in growth rate only was noticeable after day 9.

When the overall species’ EC\textsubscript{50} values were compared to physiological features of the each phytoplankton species (e.g., biovolume and growing rate), there was no clear correlation between the variables. However, when the species are grouped as either diatoms or dinoflagellates, a relationship became noticeable—the larger the size of the
Figure 2.11. Growth profiles of P. lunula exposed to different concentrations of WAF.

Each group, the greater the EC$_{50}$ values. It shows that larger-sized phytoplankton tend to be more tolerant to crude oil, regardless of their taxa. Because the inverse relationship between species volume and growth rate are well correlated (Figure 2.12), it can be concluded that phytoplankton with high growth rates, have low EC$_{50}$ values. Changes in phytoplankton species composition due to high tolerance of larger cell sizes to crude oil can also result in changes in higher trophic levels. In the case of smaller phytoplankton being replaced by larger species, larger zooplankton species may become dominant, creating a less favorable food source for juvenile fish (Howarth 1991). The preferential
prey diet of zooplankton leads to shifts in their populations. Since grazers such as protozoa, rotifers, and small crustaceans consume small phytoplankton cells more readily than large cells (Graham et al. 2009), their biomass could be affected as well.

2.5.3. CEWAF Toxicity

Even though this paper mainly focuses on crude oil toxicity, the CEWAF was also investigated due to wide field application of dispersants as part of oil spill recovery. In most cases based on oil type, dispersant manufacturers recommend application rates using dispersant to oil ratios between 1:50 and 1:10 (Dispersants 2005). However, during the DWH oil spill cleanup effort, the highest of the dispersant:oil ratios used was 1:20 (Lehr et al. 2010). It is possible that some sprayed dispersants missed the oil or was
deposited on oil layers that were thicker; therefore, we considered this reduction in efficiency when selecting to use a ratio of 1:100 the experiments.

For the CEWAF experiments, two medium-sized phytoplankton, the diatom, *D. brightwellii* and the dinoflagellate *S. trochoidea*, were selected. Although the intention was to compare growth inhibition of CEWAF-exposed phytoplankton to WAF-exposed phytoplankton, the increase in TPH concentration after the addition of Corexit, did not allow for head-to-head comparison. Similar to previous studies that clearly indicate that dispersed oil is more toxic than crude oil and dispersant alone (Hsiao 1978, Bhattacharyya et al. 2003, Couillard et al. 2009), the lowest TPH concentration in the CEWAF was much greater than the highest TPH concentration in WAF. Since each treatment caused complete mortality in both species, Corexit® EC9500A-treated flasks and CEWAF-exposed flasks could not be differentiated in this experiment, and EC_{50} values in the CEWAF and Corexit® EC9500A could not be determined. However, because the applied minimum Corexit® EC9500A concentration (100 ppm) caused mortality on phytoplankton, its EC_{50} value is estimated below 100 ppm. One study showed that Corexit was almost as toxic as the WAF of the oil alone to larval stages of several invertebrates indigenous to the Gulf of Mexico (Fucik et al. 1995).

The dispersant concentrations in the DWH oil spill application scenarios have been estimated to be approximately 30 μg/L (NALCO 2012). At a first glance, this amount seems too low to be toxic in the marine environment, but rather than its direct toxicity to the marine environment being a concern, the major concern about dispersant use should be its potential to introduce large amounts of PAHs into the water column. When the LSC concentration is expressed as TPH and PAH amounts, a strong correlation
between the percent growth inhibition and TPH concentrations was observed, as well as between the percent growth inhibition and PAH concentration (Figure 2.6). Both low- and high-PAH concentration toxicity profiles were almost identical to the TPH toxicity profiles (Figure 2.5). This indicates that the PAHs have a primary role in the LSC toxicity to phytoplankton.

2.5.4. Community Response of the Phytoplankton Species

Natural ecosystems are more complex and variable than laboratory-standardized systems, so effects on individual species tested under laboratory conditions cannot be directly extrapolated to environmental conditions. However, laboratory single-species exposure tests provide useful data for assessing individual species sensitivities when they become dominant during blooms. The goal in using a combination of 5 phytoplankton species was to compare the growth responses of phytoplankton at a higher level of biological organization, which is a more realistic exposure scenario than single-species responses during non-bloom periods. In the control flasks, *D. brightwellii*’s remarkable growth can be explained by several properties that support why large diatom species are dominant in the oceans. *D. brightwellii* can be categorized as a large diatom and a storage-adapted strategy employed by large diatoms can give them a competitive advantage in a fluctuating nutrient environment (Stolte and Riegman 1996). Their disproportionately large vacuoles compared to those of smaller diatoms (Sicko-Goad et al. 1984) can retain sufficiently high concentrations of nitrate and phosphate, such that a cell can undergo several divisions without the need for external macronutrients (Raven 1987). Their significantly higher maximum uptake rates of nutrients (Litchman et al. 2006) is yet
another advantage. In the LSC exposed flasks, the observed reduced tolerance of all species in the community indicates that competition brings extra stress on outcompeted species.

2.6. Conclusions

The effects of physically and chemically enhanced LSC on five phytoplankton species indigenous to the Gulf of Mexico were evaluated. The toxicity of LSC was found to be determined mainly by its TPH and PAH concentrations and not by the concentration of alkanes. PAHs, therefore, have the potential to be a major contributor to LSC toxicity on phytoplankton. Oil that is chemically and physically dispersed can have a completely different impact on phytoplankton species. While the maximum physical enhancement did not cause any significant difference in the amount of LSC in the water column, chemical enhancement caused an extreme dispersion of LSC. Therefore, in the case of an oil spill in the marine environment, the impact of chemical disturbances should be considered more seriously than the effects of physical disturbances. The comparison of the sensitivities of the five individual phytoplankton species revealed that diatoms are more tolerant to LSC than dinoflagellates. The growth stimulation of dinoflagellates at low concentrations (<1200 ppb), however, makes them more likely to survive than diatoms in such conditions. For both groups, the larger species showed greater tolerance than the smaller species. These findings are an indication of the potential impact of LSC has on changes in phytoplankton community structure after a spill. Highly increased amounts of LSC in Corexit® EC9500A-enhanced flasks did not allow us to compare growth inhibition between WAF- and CEWAF-exposed species. Nonetheless, this study revealed that the EC$_{50}$ of Corexit® EC9500A is below 100 ppm for the species used.
Different responses of the species at the community level showed that individual species response to crude oil cannot be the sole indicator of the potential impacts of crude oil in the oceans.

This study contributes to our information on the tolerance of different phytoplankton groups to crude oil and to the assessment of the impacts of the physical and chemical enhancements of crude oil. These types of datasets will also contribute substantially to the existing scientific knowledge in the region and provide baseline information for subsequent research efforts seeking to understand the impacts of oil on the marine ecosystem.

2.7. References


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CHAPTER 3: CAN CRUDE OIL TOXICITY ON PHYTOPLANKTON BE PREDICTED BASED ON TOXICITY DATA ON BENZO(A)PYRENE AND NAPHTHALENE?

3.1. Abstract

PAHs, which are major components of crude oil, are responsible in large part for the toxicity of crude oil to phytoplankton. This study addressed the following question. Can reliable predictions of the aquatic toxicity of crude oil, a multi-component mixture, be described from toxicity data on individual PAH compounds? Naphthalene, the most abundant PAH compound, and benzo(a)pyrene, a highly toxic PAH compound, were selected as model compounds to quantify toxicity of crude oil on two phytoplankton species, *Ditylum brightwellii* and *Heterocapsa triquetra*, by analyzing the effects of different concentrations of these PAHs on growth rate. EC$_{50}$ values suggested that the diatom *D. brightwellii* was more vulnerable to both toxicants than the dinoflagellate *H. triquetra*. However, a previous study, which investigated the impact of crude oil on the same two species, had opposite results. The differences in response from these phytoplankton species to naphthalene and benzo(a)pyrene toxicity compared to their response to crude oil suggest that they may not be solely used as surrogates to assess crude oil toxicity on phytoplankton.

3.2. Introduction

Crude oil is considered to be the most structurally complex and the most damaging pollutants in the ocean. One of the many groups comprising crude oil are polycyclic aromatic hydrocarbons (PAHs), which are biologically toxic and biopersistent compounds produced from both natural and anthropogenic processes (Hylland 2006).
Phytoplankton are not only the major primary producers in the aquatic environment, they are also a key component of global oxygen production and major drivers in the cycling of elements, particularly carbon. Thus, changes in the phytoplankton population may result in changes throughout the rest of the food web, which may have effects on a global scale. Phytoplankton can also play a significant role in the fate of PAHs (Kowalewska 1999, Witt 2002). Chapter 2 showed that toxicity of PAHs on phytoplankton was akin to the toxicity of crude oil overall; therefore, the following question arises. Can reliable predictions of the aquatic toxicity of multi-component mixtures be derived from the toxicity data on individual PAHs compounds? This study attempted to address this question. Two PAH compounds were selected: benzo(a)pyrene and naphthalene. The toxicity of each compound was quantified by analyzing the effect of different concentrations on the growth rate of two different phytoplankton species, *Ditylum brightwellii* and *Heterocapsa triquetra*. The resulting measure of toxicity of each PAH compound could be used as a valuable reference source for determining two things: 1) the assessment of toxic risk of each PAH to phytoplankton, and 2) the feasibility of using these toxicity data to predict the overall toxicity of crude oil on phytoplankton. The phytoplankton species used in this study were also used in the Chapter 2 that quantified crude oil toxicity, allowing for a direct comparison of benzo(a)pyrene and naphthalene toxicity to total crude oil toxicity and to total PAH toxicity.

3.3. Material and Methods

Phytoplankton cultures, diatom *Ditylum brightwellii* (CCMP#: 359) and dinoflagellate *Heterocapsa triquetra* (CCMP#: 2981), were provided by the National Center for Culture of Marine Phytoplankton (CCMP), East Boothbay, ME, USA. The
cultures were grown in f/2 medium (D. brightwellii) or f/2-Si medium (H. triquetra) at 25°C and 35 ppt in 0.22 μm filtered and autoclaved natural seawater. Light was provided by cool-white fluorescent lights with an irradiance of 85 μE.m⁻².s⁻¹ that were kept on a 12:12 h light:dark cycle.

Commercially available benzo(a)pyrene (Sigma-Aldrich, St. Louis, MO, USA) and naphthalene (Sigma-Aldrich) were dissolved in HPLC grade dichloromethane (DCM) (Sigma-Aldrich) to obtain a concentrated stock solution used to prepare different concentrations of the PAHs. The primary stock solutions and DCM were added into each flask for each concentration in triplicate. The concentration of DCM added to the solvent control flasks was equivalent to the concentration of DCM (ca. <1% of the enriched seawater) contained in the highest toxicant concentration treatment. Flasks containing DCM were allowed to evaporate for 24 hours under the fume hood to eliminate any undesired effects of the solvent. When populations of the cultures were in the exponential growth stage, they were dispensed into the flasks. Autoclaved Pyrex flasks (500 mL) with sponge caps were utilized in all experiments, and the experimental exposure medium volume was 380 mL for all conditions.

Controlled laboratory microcosm studies were conducted using standard static non-renewal exposure toxicity tests. Growth inhibition was selected as the measure of toxicity for each species. Based on the literature and on our preliminary study (Chapter 2), the toxic potential of these two compounds on phytoplankton was determined to be highly different. Two phytoplankton species were exposed to six concentrations of benzo(a)pyrene, ranging from 1 to 100 μg L⁻¹, and five concentrations of naphthalene, ranging from 100 to 6,400 μg L⁻¹, to determine individual growth responses of the
cultures. The control flasks contained only the phytoplankton cultures in the growth media. For both species, the DCM control cultures corresponded to the maximum added volume of benzo(a)pyrene and naphthalene stock solution (0.1%, V/V) that was also tested as solvent control. The exposure studies consisted of three replicates per treatment. The inhibition rates of different treatments were calculated according to the following formula: I (%) = (A_c - A_t) / A_c x 100, where A_c and A_t are the areas under the growth curve of control group and the treatment, respectively.

The toxic unit (TU) of a certain toxicant in a mixture is the ratio between the concentration of the compound in the mixture (Z_i) and EC_{50} value of the compound acting individually (EC_{50i}) (Sprague 1970). The sum of toxic units (M) that determines the type of joint action for a specific binary mixture of toxicants is defined by the following equation.

\[ M = \sum TU_i = \frac{Z_i}{EC_{50i}} + \cdots + \frac{Z_j}{EC_{50j}} \]

\( Z_i \) is the concentration of chemical i in the mixture, and \( EC_{50i} \) is the concentration of chemical i at the \( EC_{50} \) level. The additive index (AI) indicates whether the additive effect is synergistic or antagonistic.

\[ AI = \begin{cases} 1/M - 1 & \text{if } M \leq 1, \\ 1 - M & \text{if } M > 1, \end{cases} \]

Synergistic effect

Antagonistic effect

All statistical analyses requiring comparison of treatments were carried out using SigmaStat 12.3 software (Systat Software, Inc., San Jose, CA, USA). ANOVA and t-test were performed to evaluate significance of individual differences with a probability threshold of 0.05, followed by a post-hoc Tukey’s test when required. The 10-day EC_{50}
(concentration causing 50% inhibition on growth) values for phytoplankton were determined by plotting percent growth inhibition values of each treatment against the toxicant concentrations. The models were fitted to data by nonlinear regression analysis using \( R \) \((R\text{ Development Core Team 2006})\) with the add-on package “\textit{drc}” (Ritz and Streibig 2005). In the case of a monotonically decreasing function, a four-parameter, log-logistic model was applied for each species. In the context of hormesis, where growth stimulation is observed at the low dose of the toxicants, a five-parameter, Brain-Cousens modified log-logistic model (Brain and Cousens 2006) was applied. Day 0 and Day 10 samples containing benzo(a)pyrene were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the protocol provided with the test kit (Abraxis, Warminster, PA, USA product code: 530039). Internal standards for benzo(a)pyrene were used provided with the test kit. Linearity of calibration curves for each species resulted in \( R^2 = 0.97 \) and 0.98, for \textit{D. brightwellii} and \textit{H. triquetra}, respectively. In the case of naphthalene analysis, 50 mL of the water samples were placed in a separatory funnel, and 50 mL dichloromethane (DCM) was added for the first extraction. The aqueous layers were extracted with additional DCM \((2 \times 50 \text{ mL})\), and the DCM layers were combined and dried over \( \text{Na}_2\text{SO}_4 \). The extracts were reduced on a rotary evaporator. The reduced extracts were transferred to graduated flasks to reduce them to the desired volumes under nitrogen gas and a water bath in a nitrogen evaporator (N-EVAP 111; Organomation Associates, Inc., MA, USA). A 1 mL sample of the resultant solution was transferred to GC-MS vials, and 10 \( \mu \text{L} \) internal standard (Naphthalene-d8) was added to each vial for GC-MS measurements that were carried out in an Agilent gas chromatograph (GC)
(Santa Clara, CA) equipped with an Agilent 5975 inert XL mass selective detector (MSD). Recoveries of internal standard were above 93.6% for all samples.

3.4. Results and Discussion

The effects of PAHs on marine phytoplankton have been well documented in terms of their toxic effects and mechanisms (Okay et al. 2002, Djomo et al. 2004, Hylland 2006, Bopp and Lettieri 2007, Wang et al. 2008, Othman et al. 2012). These studies demonstrated that PAH toxicity greatly depends on the affected species and on the dose applied. In our study, the results of 6 concentrations of benzo(a)pyrene on the growth rates of *D. brightwellii* and *H. triquetra* after 10 days of exposure are presented in Fig 3.1. The rate of growth inhibition increased with increasing benzo(a)pyrene
concentration for each species. There was no stimulatory effect of the toxicant observed for either species at low concentrations. Instead, there was a progressive decrease in growth rate until cell death occurred at the higher levels of benzo(a)pyrene. At high concentrations (above 4.26 μg L⁻¹), _H. triquetra_ showed greater tolerance to benzo(a)pyrene exposure than _D. brightwellii_, suggesting that the diatom is more vulnerable to benzo(a)pyrene exposure than the dinoflagellate. Similarly, while mortality of _D. brightwellii_ cells was observed after exposure to 20.4 μg L⁻¹ benzo(a)pyrene, a lethal effect was only observed at 48.9 μg L⁻¹ benzo(a)pyrene for _H. triquetra_. The growth of _D. brightwellii_ and _H. triquetra_ was also inhibited by the presence of naphthalene (Fig 3.1). Even though initial cell counts did not differ significantly at day 0, treatments of 1,374 μg L⁻¹ and above caused significant reductions in cell count relative to controls for both species throughout the experiment. While naphthalene concentration of 139 μg L⁻¹ caused no adverse effects on _H. triquetra_, it caused moderate growth inhibition of _D. brightwellii_ (Fig 3.1). The EC₅₀ values (Table 3.1) indicated that

<table>
<thead>
<tr>
<th></th>
<th>EC₅₀</th>
<th>NOEC</th>
<th>LOEC</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(μg L⁻¹)</td>
<td>(μg L⁻¹)</td>
<td>(μg L⁻¹)</td>
</tr>
<tr>
<td><strong>Benzo(a)pyrene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. brightwellii</em></td>
<td>1.13</td>
<td>-</td>
<td>1.20</td>
</tr>
<tr>
<td><em>H. triquetra</em></td>
<td>7.02</td>
<td>-</td>
<td>1.20</td>
</tr>
<tr>
<td><strong>Naphthalene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>D. brightwellii</em></td>
<td>1,011</td>
<td>-</td>
<td>139</td>
</tr>
<tr>
<td><em>H. triquetra</em></td>
<td>1,653</td>
<td>139</td>
<td>1,374</td>
</tr>
</tbody>
</table>
naphthalene had a greater inhibitory effect on *D. brightwellii* than on *H. triquetra*. In addition to EC\(_{50}\) values, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values for *D. brightwellii* and *H. triquetra* are summarized in Table 3.1. While *H. triquetra* has two NOEC values for naphthalene, no other NOEC values were observed in the experiments for other treatments. The LOEC values varied greatly among experiments. While the LOEC value was 1.20 μg L\(^{-1}\) benzo(a)pyrene for both species, it increased to 139 and 1,374 μg L\(^{-1}\) of naphthalene for *D. brightwellii* and *H. triquetra*, respectively. The toxic potential of benzo(a)pyrene and naphthalene, therefore, greatly differed. Benzo(a)pyrene was approximately 1,000-fold more toxic than naphthalene. A notable difference in toxicity between these PAHs has been previously reported by others (Jiang et al. 2002, Djomo et al. 2004, Bopp and Lettieri 2007). Other studies (Hutchinson et al. 1979, Geyer et al. 1981) have suggested that the aqueous solubility of PAHs determines toxic effect. For this reason, the higher octanol-water partition coefficient (K\(_{\text{OW}}\)) of benzo(a)pyrene (log K\(_{\text{OW}}\)= 6.35) compared to that of naphthalene (log K\(_{\text{OW}}\)=3.37) is likely contributing to benzo(a)pyrene’s higher toxicity.

In both exposure scenarios, the diatom *D. brightwellii* was more vulnerable to these toxicants than the dinoflagellate *H. triquetra*. It has been speculated that the silica frustule of diatoms, which absorbs and retains hydrocarbons well, either enables subsequent toxicity (Sargian et al. 2007) and the absorption of the toxicants hinders sexual reproduction and auxospore formation (Kustenko 1981). Our study also verified the diatom’s vulnerability to hydrocarbons under controlled laboratory conditions.
Chapter 2 showed that how *D. brightwellii* displayed much better tolerance to crude oil than *H. triquetra*. The higher tolerance of *D. brightwellii* was also verified with a plot of % growth inhibition against total PAH concentration (Fig 3.2) in the same study.

![Figure 3.2. Growth rate responses of *D. brightwellii* and *H. triquetra* under varying PAH concentrations in South Louisiana crude oil.](image)

The main component of South Louisiana crude oil was naphthalene, which constituted 87.65% of total PAHs with its alkylated homologues (Table 3.2). By itself, the naphthalene fraction was 38.5% and represented 46.6 μg L⁻¹ in the water-accommodated fraction (WAF) of crude oil. With its alkylated homologues, the concentration of naphthalene reached 106 μg L⁻¹ in the WAF crude oil. Addition of the dispersant,
Table 3.2. Concentration and % fraction of naphthalene and its parent compounds and benzo(a)pyrene under water accommodated fraction (WAF) and chemically enhanced water accommodated fraction (CEWAF) of South Louisiana crude oil.

<table>
<thead>
<tr>
<th>Concentration (μg L⁻¹)</th>
<th>% of total PAHs in crude oil</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WAF</td>
<td>CEWAF</td>
<td>WAF</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>87.7%</td>
<td>62.6%</td>
</tr>
<tr>
<td>C1-Naphthalenes</td>
<td>32.4%</td>
<td>17.8%</td>
</tr>
<tr>
<td>C2-Naphthalenes</td>
<td>12.7%</td>
<td>18.7%</td>
</tr>
<tr>
<td>C3-Naphthalenes</td>
<td>3.16%</td>
<td>11.9%</td>
</tr>
<tr>
<td>C4-Naphthalenes</td>
<td>0.87%</td>
<td>4.81%</td>
</tr>
</tbody>
</table>

Corexit EC9500A, increased the concentration of naphthalene to 236 μg L⁻¹ and, with its alkylated homologues, the total naphthalene concentration reached 1,596 μg L⁻¹ in the chemically-enhanced water accommodated fraction (CEWAF) of crude oil. These data suggest that the introduced amount of naphthalene into seawater from crude oil, which is 46.6 μg L⁻¹, is considerably less than the EC₅₀ values found in this experiment. Even the addition of Corexit EC9500A could not bring the naphthalene concentration (236 μg L⁻¹) to the level that can cause a severe impact on growth of the two phytoplankton species.

Similarly, the concentration of benzo(a)pyrene in crude oil was 0.01 μg L⁻¹, and it only increased to 0.65 μg L⁻¹ (Table 3.2) with the addition of Corexit EC9500A. These values are still below the LOEC values of benzo(a)pyrene found in this study.

These data raise the following question. If naphthalene and benzo(a)pyrene concentrations in crude oil are below the toxic threshold for the organisms, then what is the cause of toxicity of PAHs to organisms? One possible answer relates to the cumulative toxicity of crude oil components. In other words, different components of crude oil may act together to produce combined effects that are greater than the effects of
each mixture component applied individually. The calculated additive index value \( M = 0.054 \) for the joint effect of benzo(a)pyrene and naphthalene in crude oil was synergistic in this present study. In addition to demonstrating a toxic threshold, the response of organisms to the relative toxicity of the individual compounds differed between studies. While *D. brightwellii* showed better tolerance to crude oil in our previous study, it was more sensitive to the individual compounds in this study. These results indicate that the toxicity of individual compounds on phytoplankton species cannot solely predict the toxicity of complex mixtures on organisms. In this study, despite the use of the main constituent of PAHs and a compound with high toxicity, neither can be solely used as a surrogate to assess crude oil toxicity on phytoplankton. The Gulf of Mexico offshore, deepwater zone, and nearshore water analyses at various depths by U.S. Coast Guard (Zukunft 2010) between early May 2010 and September 2010 also showed that the concentrations of naphthalene and benzo(a)pyrene were below the EC\(_{50}\) values of these compounds to phytoplankton species that were investigated in this study. Collectively then, there are no data available to suggest that these PAH compounds resulting from the Deepwater Horizon oil spill are above the EC\(_{50}\) values for the phytoplankton species that were investigated in this study.

Temporal and spatial scale changes on both biotic (e.g., predation and competition for resources and/or space) and abiotic (e.g., nutrient status, temperature, and irradiance) conditions make direct extrapolation of the results into the field unreliable. This study mainly focused on head-to-head comparisons of the toxic impact of benzo(a)pyrene and naphthalene with crude oil under the same experimental conditions that were used in the previous chapter. As in the previous study with crude oil, a nutrient-rich environment was
provided for algae growth to eliminate the stress that comes from nutrient limitation and to assess the toxicants’ impacts exclusively. In the natural environment, phytoplankton are mostly under nutrient limitation, which impacts their biomass. In addition to reduced stress due to nutrient limitation, increasing biomass due to increasing nutrient amounts dilutes hydrocarbons and the result is a lower concentration of toxicants in individual organisms in a nutrient rich environment (Skei et al. 2000). Moreover, it is well known that the toxicity of many PAHs is substantially enhanced by ultraviolet (UV) radiation in the natural environment (Landrum et al. 1987, Arfsten et al. 1996). For these reasons, the expected impact of the same amount of PAHs on phytoplankton in the natural environment is greater than what is observed in controlled laboratory settings.

3.5. Conclusions

This study provides new data on the toxicity of benzo(a)pyrene and naphthalene, two critical components of crude oil, to two species of marine phytoplankton. The extent of benzo(a)pyrene’s and naphthalene’s effect on phytoplankton growth varied with the concentration of each compound and with species of phytoplankton. The organisms tested demonstrated a range of sensitivity to different levels of the toxicants that ranged from growth inhibition to mortality. The diatom, D. brightwellii was more susceptible to these hydrocarbons than the dinoflagellate, H. triquetra when the compounds were tested individually. The results also added to our understanding of the complex behavior of crude oil toxicity on phytoplankton. Even though a previous study indicated that PAHs were responsible for the toxicity of crude oil on the phytoplankton, the present study revealed that species sensitivities differed when toxicity tests were conducted with individual PAHs as compared to crude oil. These results indicate that an assessment of
the toxicity of naphthalene and benzo(a)pyrene individually cannot be solely used in understanding the toxic potential of PAHs collectively in crude oil.

3.6. References


CHAPTER 4: DISTINCT RESPONSES OF GULF OF MEXICO PHYTOPLANKTON COMMUNITIES TO CRUDE OIL AND THE DISPERSAT COREXIT® EC9500A UNDER DIFFERENT NUTRIENT REGIMES

4.1. Abstract

This study examines the potential effects of exposure to South Louisiana Sweet crude oil (LSC), Corexit® EC9500A, and dispersed oil on enclosed phytoplankton communities under different nutrient regimes. Three distinct microcosm experiments were conducted for 10 days to assess changes to the structure of natural communities from the Gulf of Mexico as quantified by temporal changes in the biomasses of different phytoplankton groups. Concentration of NO\(_3\), Si and PO\(_4\) were 0.83, 0.99 and 0.09 μM for the unenriched treatments and 14.07, 13.01 and 0.94 μM for the enriched treatments, respectively. Overall, the contaminants LSC and Corexit® EC9500A led to a decrease in the number of sensitive species and an increase in more resistant species. Phytoplankton communities showed more sensitivity to LSC under nutrient-limited conditions. The addition of nutrients to initially nutrient-limited treatments lessened the inhibitory effect of LSC in the short term. Centric diatoms benefited most from this enrichment, but pennate diatoms demonstrated considerably greater tolerance to crude oil at low crude oil concentrations in nutrient-enriched treatments. Dinoflagellates showed relatively higher tolerance in nutrient-limited treatments and high crude oil concentrations. Corexit® EC9500A inputs significantly increased the toxicity of crude oil. Corexit® EC9500A alone had a highly inhibitory effect at 63 ppm on phytoplankton communities. This study highlights the fact that different nutrient regimes play a major role in determining the shifts of the phytoplankton community in response to exposure to different
concentrations of crude oil and dispersant. Determination of the functional equivalence of shifted phytoplankton groups could complement our research and allow for more pertinent extrapolation to real world conditions.

4.2. Introduction

Oil spills are the predominant source of hydrocarbons in aquatic environments. The massive Deep Water Horizon (DWH) oil spill released an estimated 4.16–6.24 million barrels of Louisiana Sweet Crude (LSC) oil into the Gulf of Mexico (GoM) between April 20 and July 15, 2010 (Crone and Tolstoy 2010). To minimize the impact of oil slicks, approximately 1.8 million gallons of commercial dispersants, mainly Corexit® EC9500A, were sprayed on the ocean to disperse the oil. Removal of oil slicks from surface waters reduced the risk of contamination of coastal wetlands, shores, and birds, but dispersing the oil introduced oil into the water column, and thereby increased the risk of contamination of fish and other marine organisms. This possibility is particularly important to the economy of the GoM region, since the area accounts for approximately 25% percent of the nation's seafood landings and about 21% of the total US dockside value for fishery landings (Adams et al. 2004).

Phytoplankton are at the base of the aquatic food web, and as the primary producers, they are a vital source of food to a wide range of species, from microscopic zooplankton to baleen whales. The impact of crude oil has been assessed on phytoplankton under different experimental conditions, including monocultures (e.g., Østgaard et al. 1984, Liu et al. 2006, Parab et al. 2008) and natural communities (e.g., Sargian et al. 2007, González et al. 2009, Huang et al. 2010, Gilde and Pinckney 2012). The outcomes of these studies have indicated that the composition and solubility of crude
oils determines its toxicity. Geographic location, oceanographic and meteorological conditions, seasonal variations, oil dosage, and impact areas also contribute to the impact of oil on phytoplankton. Therefore, evaluation of phytoplankton during episodic marine inputs of LSC is of ecological relevance.

The continental shelf of the northern GoM is physically and biologically dominated by the Mississippi River. The Mississippi River is one of the world's largest rivers in terms of freshwater discharge, and water flowing from it is turbid and rich in nutrients; concentrations of nitrate and silicate at the river mouth can exceed 100 μM (Turner and Rabalais 1991). About two-thirds of the Mississippi River outflow discharges directly onto the outer continental shelf of the northern GoM rather than mixing with coastal waters in an estuary (Strom and Strom 1996). Northern GoM waters are characterized by intense spatial variability and are associated with a large volume of Mississippi river outflow; however, nutrient concentrations decrease in high salinity water with increasing distance from the river and in a seaward direction and can also drop to undetectable concentrations within short distances (Dagg and Breed 2003). Offshore waters of the GoM are mostly stratified and oligotrophic, with the exception of localized coastal upwelling events (Dagg and Whitledge 1991), the loop current, loop current eddies, and the smaller anticyclonic and cyclonic eddies (Jochens and DiMarco 2008), which can enrich nutrients regionally. They can produce a cross-margin flow that pulls shelf waters into the slope of the Gulf, creating conditions favorable for phytoplankton growth (Jochens and DiMarco 2008). These spatial and temporal changes in the Northern GoM contribute highly variable nutrient regimes for phytoplankton. In the case of the DWH oil spill, the LSC extended from offshore to coastal wetlands, an area that includes
almost all variations of salinity and nutrient concentrations that can be measured in the Gulf. It is, therefore, important to assess changes in species composition and species succession in natural phytoplankton communities to crude oil exposure under a range of nutrient concentrations.

Our goal in this study was to investigate the changes of phytoplankton composition under oligotrophic and eutrophic conditions in order to assess the impact of high-nutrient input on the effects of the toxicity of LSC, Corexit® EC9500A, and chemically dispersed oil on phytoplankton and to provide basic data for impact assessment of oil spills and pollution on the phytoplankton ecology and bloom dynamics in the GoM.

4.3. Materials and Methods

4.3.1. Preparation of the Test Media and Experimental Setup

Recent studies of fresh and dispersed crude oil toxicity to aquatic organisms have used both the water-accommodated fraction (WAF; LSC in seawater) and chemically enhanced WAF (CEWAF; Corexit® EC9500A–LSC mixtures in seawater) to provide more realistic assessments. In this study, the toxicities of WAF, CEWAF, and Corexit® EC9500A were assessed by using a native phytoplankton community from the GoM. Five Niskin bottles (2 L) were used to collect surface seawater samples from the GoM (28°50' N, 90°23' W) in the summer of 2012. The station was located on the continental shelf and diagonally about 100 km from the mouth of the Mississippi River off Terrebonne Bay. GoM field samples were filtered through a 100-µm mesh filter to
separate zooplankton, then acclimated to ambient laboratory conditions prior to use in the experiments.

Non-weathered LSC was collected by British Petroleum (BP) through a riser vent pipe from the damaged wellhead of the DWH drilling rig in the GoM on May 20, 2010, and stored at –4 °C (BP, Ford Collins, CO, USA). The dispersant, Corexit® EC9500A, was provided by the Department of Oceanography & Coastal Sciences at Louisiana State University, Baton Rouge.

The WAF was prepared according to the method described in The Chemical Response to Oil Spills: Ecological Research Forum (CROSERF) (CROSERF 2005). The WAF mixtures used in the algae toxicology tests were prepared with 0.22 μm-filtered and autoclaved GoM seawater (salinity: 35) in 2-L Klimax, valved-outlet reservoir bottles. Loading of 40 g LSC in 1.6 L seawater is known to result in 20–25% headspace by volume in each bottle. The WAF solution was prepared by low-energy mixing (no vortex). The stirring rate was adjusted to 160 rpm to prevent micro particulate settlement at the bottom. After 24 hours of mixing, a settling time of 6 hours was applied to both conditions. Samples from the WAF were withdrawn through a valve located at the bottom of the bottle to avoid disturbing the water/oil interface. Samples for chemical analysis were collected in amber glass jars with Teflon-lined caps and no headspace. The samples were stored at 4 °C. Serial dilutions (25% and 50%) of the water phase from each test medium yielded concentrations of 2.5 and 5.2 ppm total petroleum hydrocarbon (TPH), respectively, that were used in the experiments.

The CEWAF was prepared in the same way as the WAF preparation, with the exception of mixing energy and the addition of the Corexit® EC9500A. The
concentration of Corexit® EC9500A was 1:100 (dispersant:oil ratio). A moderate mixing energy (~650 rpm) was used to produce chemically dispersed oil by forming a vortex that was 20–25% of the water’s depth. The solution was mixed for 24 hours, and the water phase was collected after a 6-hour settling time. Serial dilutions of 25% and 50% of this water phase yielded concentrations of 129 and 256 ppm TPH, respectively, which were used in the experiments.

In addition to WAF and CEWAF stock solutions, three additional solutions were used to prepare the test media (Fig 4.1). After the initial 100-µm filtration, the GoM field

![Diagram of stock solutions and treatment compositions](image)

**Figure 4.1.** Summary of stock solutions and treatment compositions used in this study. D: Dispersant (1:100 dispersant:oil ratio was used), (+) = initially nutrient enriched, (–) = unenriched, (–)(+) = initially nutrient limited but nutrient added at Days 3 and 7, (+)(+) = nutrient added initially, and at Days 3 and 7.
sample (FS) was divided into two separate containers. The first half (FS solution) was used without nutrient addition, but the second half (FS(+) solution) was used to prepare enriched seawater by nutrient addition. Diluted nutrients were added from the K culture medium kit (Bigelow laboratory for ocean sciences, ME, USA) that is used for optimum growth of our laboratory cultures. The third solution contained GoM seawater (SW) at the same salinity (35) as the field sample, filtered through 0.22 μm filter, and autoclaved prior to use in dilutions of test media when needed. From these 5 solutions, 14 different test media were prepared (Fig 4.1).

Samples were initially divided into two groups, with and without dispersant. Then, each group was further separated into two parts, with and without nutrient additions. Each sample had the same volume of FS or FS(+) solution, so the concentration of phytoplankton was approximately the same for all flasks at the beginning of the experiment. There were also two additional flasks for each “no dispersant” group, which were labeled WAF25%–(+) and WAF25%(+)(+) and had the same compositions as WAF25%– and WAF25%(+), respectively; however, the nutrients in these two flasks were replenished at Days 3 and 7 to determine any effect of nutrient introduction into the flasks on phytoplankton composition during the course of the experiment. The concentration of the dispersant in the two “with dispersant” groups (D(–) and D(+)) flasks was determined from its nominal concentration in CEWAF, which was 0.25 gL⁻¹. Two control groups containing only phytoplankton communities in nutrient-enriched and non-enriched media were used. Autoclaved Pyrex flasks (500 mL) were utilized in all experiments. Two control groups containing only phytoplankton communities in nutrient-enriched and non-enriched media were used. Autoclaved Pyrex
flasks (500 mL) were utilized in all experiments, and the test medium volume was 350 mL for all conditions. Each treatment was replicated three times. The experimental flasks were kept at 25 °C on a 12:12 hour light:dark cycle with cool-white fluorescent lights at an irradiance of 85 μE·m$^{-2}$·s$^{-1}$ for 10 days. Approximately 10-mL water samples were taken from each flask throughout the experiment to determine daily changes of phytoplankton abundance and composition.

4.3.2. Determination of Phytoplankton Abundance and Composition

A gridded Sedgwick-Rafter slide was used to examine two 1-mL replicates of water from each sample preserved with Lugol’s solution at different magnifications (100–400 ×) on a Zeiss Axios Observer-A1 inverted microscope with epifluorescence capability (Zeiss). In preparation for examination, each archived sample was uniformly mixed by inversion of the sample container for approximately 1 min before the 1-mL subsample was loaded onto a Sedgwick-Rafter slide. The sample was then allowed to settle for 30–45 minutes before examination began. Solitary cells and colonies/chains whose cells were easily differentiated were enumerated as individual cells. When the chamber was too dense to count individual cells, they were diluted. In case of identification difficulties, phytoplankton cells were photographed using AxioVisionLE V4.6.1.0 camera software (Zeiss) for further support of resources. To determine the abundance and composition of the phytoplankton community for each replicate, very abundant species were counted approximately 400 cells. For less abundant groups, whole grids of the chamber were examined. Cells were categorized into the following major groups: cyanobacteria, chlorophytes, centric diatoms, pennate diatoms, dinoflagellates, euglenophytes, cryptophytes, and flagellates. Very abundant, potentially harmful, or
toxin-producing phytoplankton were also identified to at least their genus level, and their abundances were recorded for each replicate. In addition to the composition of the phytoplankton, the size of the plankton was measured by using the microscope’s reticles. Five main size fractions were measured; <2 μm, 2–20 μm, 20–50 μm, 50–100 μm, and >100 μm.

4.3.3. Chemical Analysis of the Crude Oil

TPH analysis was carried out with total scanning fluorescence (TSF) (Aqualog, Horiba Scientific). Standards and samples were assayed at an excitation wavelength of 260 nm and an emission wavelength of 360 nm. At these wavelengths, the instrument provided the maximum intensity that corresponds to total oil equivalents of LSC. Standard solutions were prepared with direct dissolution of LSC in dichloromethane (DCM). The stock solution was diluted to concentrations of 0.5–50 ppm. For unknown samples, 100 mL of the WAF was placed in a 250-mL separatory funnel, and 20 mL of DCM was added for the first extraction. The aqueous layer was extracted with additional DCM (2 × 20 mL), and the DCM layers were combined and dried over Na₂SO₄. The extracts were reduced on a rotary evaporator, yielding a yellow-brown liquid. The reduced extracts were transferred to graduated flasks to reduce them to the desired volumes under nitrogen gas and a water bath in a nitrogen evaporator (N-EVAP 111; Organomation Associates, Inc., MA, USA). A 5-mL sample of the resultant crude was transferred to quartz fluorometer cells (10 mm) for TPH measurements. The samples were diluted enough to prevent quenching effects.
4.3.4. Statistical Analysis

All statistical analyses requiring a comparison of treatments were carried out using SigmaStat 12.3 software (Systat Software, Inc., San Jose, CA, USA). ANOVA and t-tests were performed to evaluate significance of individual differences with a probability threshold of 0.05, followed by a post-hoc Tukey test. Similarity analysis between different treatments at Day 0 was assessed with the Bray-Curtis similarity index by Primer V5 (Primer-E Ltd, Plymouth, UK). The similarity matrices were then subjected to clustering and ordination, using group-average linking and non-metric multidimensional scaling (MDS) analysis techniques, respectively.

4.4. Results

4.4.1. Initial Crude Oil Analyses

The standard curve made by dissolved LSC in DCM provided a well-evident linear correlation between the concentration of LSC and the maximum intensity of the instrument ($R^2=0.97$). Only the concentration range of 1–50 ppm was used in calibration calculations since the concentrations below 1 ppm gave the same level of response as blank samples, and the concentrations above 50 ppm showed a strong quenching effect. Estimates of TPH concentrations in the treatments were determined (Table 4.1) with the instrument set to maximum sensitivity.

4.4.2. Initial Phytoplankton Abundance, Composition, and Nutrient Levels

The field samples collected from the GoM initially contained many different groups phytoplankton of various sizes; cyanobacteria, chlorophytes, centric and pennate
Table 4.1. Day 0 TPH concentrations of all treatments measured by the spectrofluorometer (n=3).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TPH conc. (ppm) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAF 100%</td>
<td>10.4±1.4</td>
</tr>
<tr>
<td>CEWAF 100%</td>
<td>498.1±15.0</td>
</tr>
<tr>
<td>WAF50%(-)</td>
<td>5.1±0.9</td>
</tr>
<tr>
<td>WAF25%(-)</td>
<td>2.7±0.6</td>
</tr>
<tr>
<td>WAF25%(-)(+)</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>WAF50%(+)</td>
<td>5.2±1.0</td>
</tr>
<tr>
<td>WAF25%(+)</td>
<td>2.5±0.6</td>
</tr>
<tr>
<td>WAF25%(+) (+)</td>
<td>2.7±0.5</td>
</tr>
<tr>
<td>CEWAF50%(-)</td>
<td>251.9±5.8</td>
</tr>
<tr>
<td>CEWAF25%(-)</td>
<td>132.2±3.2</td>
</tr>
<tr>
<td>CEWAF50%(+)</td>
<td>255.8±2.1</td>
</tr>
<tr>
<td>CEWAF25%(+) (+)</td>
<td>128.5±3.1</td>
</tr>
</tbody>
</table>

diatoms, dinoflagellates, euglenophytes, cryptophytes, and flagellates. Approximate total cell abundance at Day 0 was 114 cells mL⁻¹. Initial phytoplankton community composition (Table 4.2) was dominated by centric diatoms, followed by pennate diatoms and dinoflagellates. Even though the majority of the initial phytoplankton composition could be determined, a minor fraction (2%) of cells that were < 2 μm could not be identified under the light microscope. The predominate size fraction of the community was 20–50 μm in size and accounted for about 58% of the cells larger than 2 μm. The relative fraction of 50–100 μm in size was the second most abundant size group accounted for 23% of the cells.

Phytoplankton composition in each treatment was analyzed to find out dissimilarities among treatments. Similarity cluster matrix analysis (Figure 4.2) revealed that the similarity of the treatments was >85% on Day 0. In addition to phytoplankton
Table 4.2. Initial phytoplankton cell abundances (n=28) and species composition in the collected GoM field samples.

<table>
<thead>
<tr>
<th></th>
<th>Cell abundance (ml⁻¹ ± SD)</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria</td>
<td>3.30 ± 1.28</td>
<td><em>Chroococcus minutus, Pseudanabaena</em> spp. <em>Dactylococcopsis</em> sp.</td>
</tr>
<tr>
<td>Chlorophytes</td>
<td>0.43 ± 0.17</td>
<td><em>Kirchneriella lunaris, Tetraselmis</em> sp., <em>Chlamydomonas</em> sp.</td>
</tr>
<tr>
<td>Dinoflagellates</td>
<td>14.91 ± 3.01</td>
<td><em>Prorocentrum</em> spp., <em>Peridinium</em> sp., <em>Gyrodinium fusiforme, Lingulodinium polyedrum</em></td>
</tr>
<tr>
<td>Euglenophytes</td>
<td>0.32 ± 0.18</td>
<td><em>Eutreptia</em> sp.</td>
</tr>
<tr>
<td>Cryptophytes</td>
<td>0.77 ± 0.38</td>
<td><em>Chlamydomonas</em> sp., <em>Cryptomonas</em> sp.</td>
</tr>
<tr>
<td>Centric diatoms</td>
<td>67.90 ± 13.29</td>
<td><em>Chaetoceros</em> spp., <em>Dactyliosolen fragilissimus, Guinardia delicatula, Leptocylindrus danicus, Skeletonema costatum, Odontella mobiliensis, Guinardia striata, Guinardia flaccida</em></td>
</tr>
<tr>
<td>Pennate diatoms</td>
<td>24.50 ± 8.39</td>
<td><em>Pseudo-nitzschia</em> spp., <em>Nitzschia pungens, Nitzschia sicula</em></td>
</tr>
<tr>
<td>Flagellates</td>
<td>0.35 ± 0.13</td>
<td><em>Fibrocapsa japonica</em></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>1.05 ± 0.62</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.2. Similarity cluster matrix of phytoplankton composition and abundance on Day 0 among treatments.
abundance and composition, initial nutrient concentrations were measured for each treatment on Day 0. Concentration of NO$_3$, Si and PO$_4$ were 0.83, 0.99 and 0.09 μM for the unenriched treatments and 14.07, 13.01 and 0.94 μM for the enriched treatments, respectively. The concentrations of nutrients in the nutrient-enriched treatments were higher by at least an order of magnitude than the unenriched treatments.

4.4.3. Unenriched Treatments with No Dispersant

The average initial composition of 4 different treatments (Control 1, WAF50%(−), WAF25%(−) and WAF25%(−)(+)) grown in unenriched media showed that centric diatoms, pennate diatoms, and dinoflagellates made up 95% of the initial population. In control flasks, this composition remained between 93% and 99% throughout the experiment. Growth curves of four different treatments (Figure 4.3) indicated that control groups reached their maximum abundance at Day 5, with their populations decreasing steadily thereafter. As NO$_3$, Si, and PO$_4$ levels dropped to 0.39 μM, 0.11 μM, and 0.04 μM, respectively, at Day 7, the control cultures could not sustain growth afterward. Treatments containing crude oil did not grow as well as the control group (Figure 4.3). WAF50%(−) treatments containing 5.1 ppm crude oil showed the least amount of tolerance to crude oil exposure. Total cell abundance decreased steadily after Day 7 and reached 0 at Day 10. Throughout the experiment, biomass change did not cause remarkable changes in nutrient concentrations in this treatment.

WAF25%(−) and WAF25%(−)(+) treatments, which contained about 2.6 ppm crude oil, grew significantly better than WAF50%(−) treatment ($p<0.05$). During the initial 3 days, the growth of both treatments under exposure to low concentrations of LSC
Figure 4.3. Growth curves and phytoplankton composition of treatments that were grown under a nutrient deficient environment. Cell # values are means (n=3) and error bars represent the standard deviation * = nutrient addition days.

was almost indistinguishable, but the nutrient addition to treatment WAF25%(-)(+) at Day 3 resulted in changes to biomass and composition (Figure 4.3 and 4.4). While in the WAF25%(-) treatment, phytoplankton growth declined severely after Day 3, the phytoplankton continued to grow exponentially for an additional day in the WAF25%(-)
Figure 4.4. Relative abundance of diatoms, dinoflagellates, and other phytoplankton groups in different treatments grown under a nutrient deficient environment. * = nutrient addition days.

(+ treatment; then that population declined severely until Day 7. After the first nutrient addition, a remarkable change in composition was observed in the diatom population. While the abundance of pennate diatoms decreased to about 4% in the nutrient-enriched treatment, the abundance of pennate diatoms remained around 15% in the WAF25%(-) treatment. The reduced number of pennate diatoms resulted in a shift to centric diatoms
in the WAF25%(-)(+) treatment. This result indicates that centric diatoms benefited most from the nutrient additions under LSC exposure. Addition of nutrients to this treatment led to maximum growth differentiation from its nutrient-deficient counterpart. Another round of nutrient additions at Day 7 led to slight recovery of cells for 2 days, after which cell numbers declined again. This suggests that nutrient addition is a way to stimulate growth in the short term, but it does not help in overall survival under LSC exposure. Centric diatoms were the predominant group throughout the 10-day period in all the treatments (Figure 4.4).

4.4.4. Nutrient-Enriched Treatments with No Dispersant

Experiments were also aimed to describe the response to LSC under nutrient-enriched conditions at the same LSC exposure level. Biomass increase of the control flasks in the nutrient-enriched medium was far greater than its non-enriched equivalent (Figure 4.5). Cells grew exponentially until the end of the experiment. Relative abundance of centric diatoms gradually increased from 59% to 99% until Day 10 (Figure 4.6), indicating that centric diatoms become dominant quickly in a nutrient-enriched environment.

Even though the WAF50%(+) treatment, which contained 5.2 ppm LSC, resulted in better growth compared to its unenriched equivalent (WAF50%(-)), growth was highly suppressed with LSC exposure (Figure 4.5). Similar to the control group, the abundance of centric diatoms increased gradually from 65% to 95% throughout the experiment (Figure 4.6). Compared to its unenriched equivalent the tolerance of centric diatoms increased. Dinoflagellates grew considerably better until Day 7, when their population completely crashed. The almost threefold greater growth in overall cell abundance in this
Figure 4.5. Growth curves and phytoplankton composition of treatments grown in a nutrient-enriched environment. Cell # values are means (n=3) and error bars represents the standard deviation *= nutrient addition days.

Treatment compared to its unenriched equivalent demonstrates that nutrient input was beneficial to phytoplankton survival during crude oil exposure. Even though the WAF25%(+) and WAF25%(+)(+) treatments, which contained about 2.6 ppm LSC, showed similar growth profiles, total cell abundance was almost double that in the
Figure 4.6. Relative abundance of diatoms, dinoflagellates, and other phytoplankton groups in different treatments grown in a nutrient-enriched environment. *= nutrient addition days.

unenriched treatments, WAF25%(-) and WAF25%(-)(+), respectively. The only difference was that the WAF25%(+) and WAF25%(+)(+) treatments reached their peak growth point at Day 6 and 5, respectively, as opposed to their unenriched equivalents at Day 3 and 4, respectively. As was the case in the unenriched equivalent, nutrient addition into WAF25%(+)(+) treatment at Day 7 triggered the survival of phytoplankton for an
additional day, even though enough nutrients were provided initially. Phytoplankton composition was similar in both treatments, but it was unlike the rest of the treatments. The abundance of centric diatoms decreased gradually throughout the experiment along with the abundance of dinoflagellates, but the biomass of pennate diatoms, particularly *Pseudo-nitzschia* spp., increased steadily. There was no significant impact of nutrient addition in the WAF25%(+) (+) treatment at Days 3 (*p*=0.342) and 7 (*p*=0.216) on compositional change of the phytoplankton.

4.4.5. Unenriched Treatments with Dispersant

All of the treatments contained the dispersant, Corexit® EC9500A, and the dispersed crude oil survived a maximum of 3 days (Figure 4.7). The amount of Corexit® EC9500A applied, 63 ppm, was highly toxic to phytoplankton species. Corexit® EC9500A led to an increase in the amount of TPH in the dispersed oil that was 50 fold higher compared to treatments with no added dispersant. Both D(−) and D(+) treatments showed similar responses to Corexit® EC9500A exposure regardless of the nutrient amount provided at the beginning of the experiment. Cultures in the D(−) treatment, which was initiated with low nutrient concentrations, completely died after 2 days. During this 3-day period, the abundance of centric diatoms dropped from 63% to 15%, pennate diatoms increased from 26% to 45%, and dinoflagellates increased from 6% to 40%. These results suggest that dinoflagellates and pennate diatoms showed relatively better tolerance to Corexit® EC9500A compared to centric diatoms. Comparison of the D(−) and D(+) treatments in terms of phytoplankton composition demonstrated that
Figure 4.7. Growth curves and abundance of phytoplankton groups that were exposed to the dispersant and dispersed crude oil.

dinoflagellates showed better tolerance to Corexit® EC9500A under nutrient-deficient conditions, and pennate diatoms had the greatest tolerance to Corexit® EC9500A, regardless of the amount of nutrient compared to other groups in the communities. In particular, a pennate diatom, *Pseudo-nitzschia* spp., showed the highest tolerance to
Corexit® EC9500A, since the relative abundance of pennates gradually increased in both control groups from 60% and 80% for D(−) and D(+) treatments, respectively, to 100% at the end of the experiments at Day 3. The treatments containing 50% dispersed WAF of LSC and Corexit® EC9500A (CEWAF50%(−) and CEWAF50%(+) did not show any indication of growth. However, both CEWAF25%(−) and CEWAF25%(+) survived one extra day regardless of their nutrient amount. In both treatments, the relative abundance of dinoflagellates slightly increased during this additional day as a result of a slight decrease in the abundance of centric and pennate diatoms.

Due to their high abundance, the relative abundance of two prominent diatom species, *Chaetoceros* spp. and *Pseudo-nitzschia* spp., in total centric diatoms and pennate diatoms, respectively, was evaluated for both nutrient regimes. In control treatments of nutrient unenriched groups, the relative abundance of *Chaetoceros* spp. increased from 34% to 64% (Figure 4.8) during the first 5-day period and declined to 26% at Day 10. Similarly, the relative abundance of *Pseudo-nitzschia* spp. increased from 60% to 95% during the first 5-day period and then dropped to 50%. This result indicates that the *Chaetoceros* spp. and *Pseudo-nitzschia* spp., among their respective diatom groups, exploited the nutrients until nutrient levels reached a point that no longer supported the community. Even though addition of crude oil caused a crash on *Chaetoceros* spp. first couple days (Figure 4.8), the cells recovered and continued to grow until the end. The relative abundance of the pennate diatom *Pseudo-nitzschia* spp. increased very rapidly in the 25% oil treatments (Figure 4.8), from a 60% margin to a 95% margin, as well as in the control group during the first 4 days. The population then decreased slightly and remained at 85–95% margin. A very steep decline in *Pseudo-nitzschia* spp. (Figure 4.8)
Figure 4.8. Relative abundance (means ± SD) of *Chaetoceros* spp. and *Pseudo-nitzschia* spp. in total centric diatoms and pennate diatoms, respectively, throughout the experiment under a nutrient-deficient environment.

Relative abundance in the control group after Day 4 demonstrates that *Pseudo-nitzschia* spp. showed better tolerance to crude oil than other pennate diatoms under nutrient limited conditions.

*Skeletonema costatum, Dactyliosolen fragilissimus, Leptocylindrus danicus,* and *Guinardia* spp. were other very abundant species of centric diatoms in crude oil-exposed...
treatments. All crude oil-exposed, nutrient-rich cultures showed similar relative abundance changes in *Chaetoceros* spp. and *Pseudo-nitzschia* spp. (Figure 4.9). For *Chaetoceros* spp., there was an increase in relative abundance from a 40–45% margin to a 55% margin for all treatments. It is an indication of these species was negatively affected from the crude oil since control group’s population increase was considerably higher than crude oil treated cultures. In nutrient enriched cultures, regardless of oil exposure, all treatments showed sharp population increase throughout the experiment. It is a suggestion of *Pseudo-nitzschia* spp. showed a better tolerance, compared to other pennate diatoms, to crude oil under nutrient enriched conditions.

It is difficult to determine whether crude oil toxicity is favorable to certain size fractions in this study. The relative abundance of the prominent size fraction, 20–50 μm, remained within the range of 60% throughout the experiment in nutrient unenriched treatments (Figure 4.10) and nutrient enriched treatments (Figure 4.11). The only exception was observed in WAF25%(+) and WAF25%(+)(+) treatments (Figure 4.11) due to remarkable increased abundance of 50–100 μm size pennate diatoms (Figure 4.6) in these treatments.

In summary, centric and pennate diatoms showed relatively better tolerance to LSC than dinoflagellates and the rest of the groups described in this study. In fact, pennate diatoms were more successful under low LSC exposure in nutrient enriched treatments (Figure 4.12). Compared to the control groups, high LSC exposure also increased the relative abundance of pennates. The addition of nutrients either initially or during later days of the experiment enhanced the succession of pennate diatoms.
Figure 4.9. Relative abundance (means ± SD) of *Chaetoceros* spp. and *Pseudo-nitzschia* spp. in total centric diatoms and pennate diatoms, respectively, throughout the experiment under a nutrient-enriched environment.
Figure 4.10. Changes in relative size fraction of the community under different nutrient unenriched treatments. Relative abundance (%) values are means (n=3) and error bars represent the standard deviation.

Figure 4.11. Changes in relative size fraction of the community throughout the experiment under different nutrient enriched treatments. Relative abundance (%) values are means (n=3) and error bars represent the standard deviation.
Centric diatoms showed two distinct progressions under different nutrient environments. While they did relatively better under low LSC exposure in unenriched treatments, they showed remarkably better growth under high LSC exposure in nutrient-enriched treatments. They were negatively affected from low LSC exposure. Dinoflagellates were negatively impacted from LSC exposure under both nutrient conditions. However,
dinoflagellates showed relatively better succession under unenriched conditions. When exposed to the dispersant and the dispersed oil, pennate diatoms had the greatest tolerance, regardless of nutrient conditions compared to other groups in the communities. Centric diatoms showed the lowest tolerance to Corexit® EC9500A. Similar to LSC exposure, dinoflagellates showed relatively better tolerance to Corexit® EC9500A under unenriched conditions.

4.5. Discussion

The time period of the DWH oil spill was in the spring and lasted throughout the summer. The composition of phytoplankton communities with a large number of diatoms in the collected seawater is similar to that seen previously in the GoM during spring (Qian et al. 2003, See et al. 2005, Schaeffer et al. 2012). A high abundance of diatoms in the initial community and closely associated groups of Chaetoceros spp., Skeletonema spp., and Pseudo-nitzschia spp. were also reported (Macintyre et al. 2011) in the GoM. This offered us an opportunity to evaluate the community response to LSC in a more realistic scenario.

Control treatments for both nutrient conditions showed an expected phytoplankton composition shift based on changes in nutrient stoichiometry. Unenriched seawater control flasks were high in diatoms during the first 5 days, likely due to a high ratio of Si:N, but dropping the Si:N ratio from 1 to 0.3 led to an increase in the dinoflagellate population. However, nutrient-enriched seawater control flasks, which were not silicate limited, showed diatom prominence throughout the experiment. Comparison of the control treatments to LSC-exposed treatments suggests that adverse effects of LSC on phytoplankton biomass can occur at concentrations as low as 2.6 ppm.
TPH. Succession of diatoms under LSC exposure is in agreement with previous findings (e.g., Rey et al. 1977, Thomas et al. 1981, Adekunle et al. 2010, Gilde and Pinckney 2012). These studies reported that the relative tolerance of diatoms was higher than other groups studied. Unexpected progression of pennates in nutrient-enriched treatments was observed with treatment WAF25%(-)(+). The addition of nutrients at Day 3 caused a reduction of pennates in this treatment. This may suggest that initial nutrient conditions regulate the succession of pennate diatoms more prominently than with other phytoplankton groups rather than nutrient additions at later time points. Results also suggest that pennate diatoms may show irreversible effects under joint nutrient competition and LSC exposure. The addition of nutrients to WAF25%(-)(+) treatments increased the numbers diatoms. However, utilization of added nutrients after 3 days by diatoms was remarkably high; centric diatoms utilized the nutrients for their growth more efficiently compared to pennates. This may be attributed to the smaller cell sizes of centric diatoms in the community compared to the size of pennates.

Even though LSC exposure in all treatments suppressed phytoplankton growth, significantly greater growth in LSC-exposed treatments in nutrient-enriched samples compared to their unenriched equivalents ($p<0.05$) indicated that nutrient deficiency increased the stress of phytoplankton and led to more inhibitory effects on their growth. This may potentially be explained by nutrient-enriched treatments’ lowering the stress caused by nutrient deficiency and thus helping in their succession. Another possible reason might be related to the fact that additional nutrients can enhance bacterial activities on oil degradation. The presence of oil means the presence of oil-degrading microbes; however, the degradation process typically limits the bioavailability of
nutrients and the terminal electron acceptor oxygen (Leahy and Colwell 1990). Enriched seawater may overcome this issue and benefit phytoplankton succession in such conditions. In addition, the toxic impact of hydrocarbons may be lessened by the addition of nutrients, as the exposure to hydrocarbons are diluted in environments with high populations of phytoplankton (Skei et al. 2000), resulting in a lower concentration of toxicants to individual organism in nutrient-enriched conditions. The two treatments that added nutrients at Days 3 and 7 (WAF25%−(+)(+) and WAF25%+(++)) showed very similar growth profiles. In the WAF25%−(+) treatment, it was thought that later nutrient addition benefited growth, since the phytoplankton were in an unenriched medium, but the addition of nutrients at Days 3 and 7 also facilitated growth in the WAF25%+(++) treatment, despite it being a nutrient-enriched medium initially. Presumably, excess nutrients in enriched treatments helped in the biodegradation of LSC more effectively, and the degraded oil was less detrimental to phytoplankton growth. For this reason, the coupling of bacterial activities with nutrient bioavailability seems a more reasonable explanation for the enhancement of phytoplankton growth with nutrient addition under LSC exposure.

During the DWH oil spill cleanup effort, the highest of the dispersant:oil ratios used was 1:20 (Lehr et al. 2010). Considering that some sprayed dispersant missed the oil or was deposited on oil layers that were thicker, a reduction in efficiency was expected. These conditions were considered when selecting a ratio of 1:100 dispersant:oil in the experiments. Even though the lower end of the dispersant mix was chosen, the amount of the dispersant applied in the experiment was highly toxic to phytoplankton communities. However, the effective concentrations were still lower than other marine organisms. For
example, the toxicity evaluation of Corexit® EC9500A by EPA suggested that LC$_{50}$ values of Corexit® EC9500A is at 25.2 mgL$^{-1}$ and 32.3 mgL$^{-1}$ for menidia and mysidopsis, respectively (Environmental-Protection-Agency 2013). In many studies, use of the dispersant Corexit® EC9500A was in the potentially highly toxic range from 1:20 to 1:150. These studies not only showed that Corexit® EC9500A is toxic by itself to the marine environment (e.g., Singer et al. 1996, George-Ares et al. 2003, Goodbody-Gringley et al. 2013), but that it also increases the toxicity of crude oil (e.g., Bhattacharyya et al. 2003, Goodbody-Gringley et al. 2013, Rico-Martínez et al. 2013), i.e., it shows a synergistic effect. The highly dispersing capacity of Corexit® EC9500A that led to an increase in the crude oil concentration in the water column can explain the more potent crude oil exposure for phytoplankton.

Recent dispersants have been formulated to minimize toxicity to aquatic organisms. For example, the LC$_{50}$ values of dispersants used in the early 1970s ranged from about 5 to 50 mgL$^{-1}$ in rainbow trout in 96-hour exposures. The LC$_{50}$ ranges have increased up to 2,000 mgL$^{-1}$ for dispersants available today, and these dispersants contain a mixture of surfactants and a less toxic solvent (Fingas 2002, Environmental-Protection-Agency 2013). However, these newer formulations can still exert toxic effects on aquatic organisms. Even a 1:100 ratio used in our study showed a detrimental effect on phytoplankton, the recommended 1:10 to 1:50 ratios by the Environmental Protection Agency (EPA) for Corexit application could potentially cause more severe adverse effects on marine organisms. When the higher ratio of dispersant usage was considered in the DWH oil spill case, toxicity associated with use of Corexit® EC9500A may have been markedly underestimated. A point that has remained unknown is whether the
benefits of dispersing the oil by using Corexit® EC9500A are outweighed by the increase in toxicity of the dispersed oil.

In this study, the only remarkable impact of LSC and Corexit® EC9500A on phytoplankton size fraction was observed under low LSC exposure in nutrient-enriched treatments. Predominance of larger sizes towards the end of the experiment can be attributed to increasing relative abundance of *Pseudo-nitzschia* spp. Since size fractions were measured for overall populations instead of for each individual group or species, conclusions concerning whether smaller or larger size cells within the same group or species were negatively impacted in this study were constrained. Previous studies indicated that cell size seems to be a factor that affects the tolerance of phytoplankton to oil, causing shifts in phytoplankton communities. The literature, however, contains contradictory results on this issue. While (González et al. 2009) and (Huang et al. 2010) stated that smaller cell size phytoplankton showed more tolerance to oil than bigger cell sizes, other studies (e.g., Sargian et al. 2007, Echeveste et al. 2010) showed opposite results, with the higher tolerance being observed with larger cells. There is no clear evidence for an association between phytoplankton size and tolerance to oil. Nevertheless, studies have argued that larger phytoplankton have higher tolerance to crude oil due to their smaller surface-to-volume ratios than smaller phytoplankton, which more crude oil penetration on their cell surfaces occur.

It may be worthwhile to bring up the matter of two important and highly abundant phytoplankton species in the GoM; *Chaetoceros* spp. and *Pseudo-nitzschia* spp. Comparison of control groups under two distinct nutrient conditions showed that both diatom species are more successful in unenriched treatments. This succession may be due
to the relatively light silicification of these species, which provides a competitive advantage under Si-limiting conditions (Sommer 1994). While *Pseudo-nitzschia* spp. were not much impacted by LSC exposure under unenriched conditions, their abundance increased with nutrient additions (Figures 4.10 and 4.11). Evidently, *Pseudo-nitzschia* spp. blooms in the GoM have been associated with changes in nutrient stoichiometry in spring (Parsons et al. 2013). Overlapping of a bloom event and an oil spill may cause devastating results in an area, where particularly *Pseudo-nitzschia* spp. dominated, since some *Pseudo-nitzschia* spp. are known to produce the powerful neurotoxin, domoic acid, responsible for both human and marine animal deaths around the world (e.g., Bates et al. 1989, Work et al. 1993, Scholin et al. 2000, Bargu et al. 2011). *Chaetoceros* spp. abundance was reduced with LSC exposure under both nutrient conditions. From a harmful algal bloom perspective, this phenomenon can be fortuitous, since *Chaetoceros* spp. can pierce the gills of fish, causing mechanical damage. Another noteworthy observation in this study related to specific species was the high tolerance of chain-forming diatoms to LSC. Although the mechanism is unknown, their characteristic chains may provide a competitive advantage under crude oil exposure.

The proportion of dissolved Si, N, and P in the nutrient-enriched medium closely approximated the Redfield ratio (Si:N:P = 16:16:1 by atoms) (Redfield 1958). Since coastal eutrophication resulting from increased N and P in runoff from the continents reduced the Si:N and Si:P ratios to nearly the Redfield ratio (Turner and Rabalais, 1991), our nutrient enrichment was reconcilable with the contemporary GoM stoichiometric ratio; however, it was at the high end of the nutrient concentration, where salinities are >32. In the consideration of the progression of the oil spill from offshore to coastal
environments, the used nutrient concentration in this study provides highly realistic assessment for the crude oil toxicity. Salinities >32 are associated with low nutrient concentrations (Lohrenz et al. 1999) and are more closely akin to the unenriched treatments used in this study. Nutrient measurements at the DWH oil spill site (Edwards et al. 2011) during the spill showed that PO₄ (approximately 10 nmol.L⁻¹) levels were even less than the concentration used in this study.

Some factors, including the rapid reproductive rates of phytoplankton, functional equivalence, and possible adaptation to crude oil exposure might lead to an overestimation of the impact of an oil spill to phytoplankton. In addition to crude oil exposure, temporal and spatial variability in nutrient concentrations in the GoM, patchiness of phytoplankton communities, and other environmental factors (e.g., light, temperature, grazing pressure, salinity, physical conditions of the spill location) can lead to changes in phytoplankton composition and make it more difficult to predict environmental consequences. Determining currently unknown recovery rates of different phytoplankton species, the length of exposure impact on recovery rates, potential development of any physiological adaptation to crude oil, and the functional equivalence of shifted phytoplankton groups could complement our research and allow for more pertinent extrapolation to real world conditions. The occurrence of the DWH oil spill during the spring has important ecological relevance to our findings. Due to high cell abundance, spring blooms uptake a large amount of carbon dioxide from the atmosphere and become an important fuel source to sustain marine life during the rest of the year. Therefore, selectivity for certain groups or species may extensively alter the carbon budget in this region and is another unknown in the understanding of the consequences of
oil spills in the GoM. In the light of findings by Gonzales et al. (2009), which show that the final composition of phytoplankton exposed to crude oil is dependent on their initial composition, results of this study could also yield different outcomes based on differences in season or in environmental parameters. Because of the differences in season and time between our water sampling and the DHW oil spill event, these results need to be considered with cautious when evaluating the impact of the DWH oil spill event on the GoM phytoplankton community. Nevertheless, this study clearly highlights the fact that different nutrient regimes play a major role in community shifts of phytoplankton with exposure to different concentrations of crude oil.

4.6. Conclusions

In conclusion, distinct responses of phytoplankton communities to LSC, the dispersant Corexit® EC9500A, and the dispersed oil were observed under different nutrient regimes. Diatoms showed the greatest tolerance to LSC exposures under every condition that was assessed. Nevertheless, different diatom groups also had distinct responses under such conditions. Pennate diatoms showed greater tolerance to LSC under nutrient-enriched conditions. Centric diatoms demonstrated higher tolerance to LSC under unenriched conditions. Dinoflagellates and the other phytoplankton species were the most sensitive group to the conditions that were tested in this study. Initial nutrient enrichment lessened the inhibitory effect of the crude oil toxicity and stimulated the growth of all phytoplankton groups. Nutrient addition, in later days, was helpful to the recovery in growth of some algal cells on a short-term basis, but was not as effective as the initial responses in nutrient-enriched treatments. Evidently, the effects of crude oil toxicity and of nutrient deficiency jointly inhibit phytoplankton growth.
4.7. References


5.1. Abstract

Crude oil, a pollutant and toxicant in the marine environment, is introduced into an aquatic system by natural seeps and anthropogenic sources. Although a substantial amount of research has established that oil is toxic to marine life, relatively little is known about the responses to these compounds at the base of the marine food web, particularly, the tolerability and changes to the toxin profiles of harmful toxic algal species. The degree of crude oil influence on sympatric Karenia brevis, Prorocentrum minimum, and Heterosigma akashiwo was investigated. Growths varied with exposure concentrations and with species of phytoplankton. Comparison of their tolerability to that of non-toxic species as measured by EC₅₀ values showed that the toxin production potential of harmful the algal species does not provide a selective advantage. Investigated toxin profiles for Karenia brevis and Prorocentrum minimum demonstrated an increase in toxin productivity at the lowest crude oil concentration (0.66 mg.L⁻¹) tested in this study. The higher crude oil concentrations led to significant growth inhibition and a decrease in toxin production. Findings from this study could provide a basis for the assessment of shellfish bed closures due to high risk of increased toxin potential of the species. The findings may also be helpful in evaluating prey-grazing interactions in the Gulf of Mexico marine ecosystem.
5.2. Introduction

Oil spills, which are ubiquitous in the marine environment, can result in serious pollution, affecting marine plants and animals, the fishing industry, and tourism. The Deepwater Horizon (DWH) oil spill occurred on April 20th, 2010, in the Gulf of Mexico (GoM) and was worst environmental disaster in the United States and ranks as the largest accidental oil spill. It released between 4.16–6.24 million barrels of South Louisiana Sweet Crude (LSC) oil into the GoM until July 15th, 2010 (Crone and Tolstoy 2010). The ecological effects of oil spills like the DWH have been the subject of substantial laboratory and field research involving many species of marine organisms. Phytoplankton are a critical component to the functioning of ecosystems due to their generous oxygen production, carbon sequestration, and their base position in the aquatic food web. Despite their importance, adequate concern about the ecological impact of a crude oil spill to phytoplankton has been lacking compared to the same concerns about larger marine organisms. The effect of crude oil on phytoplankton has only been broadly studied and in a wide range of ecosystems. Studies conducted in different parts of the world on the effects of oil spills have indicated both inhibition and enhancement of primary production by phytoplankton and changes in population composition depending on exposure to the type and concentration of oil (Teal and Howarth 1984, Dunstan et al. 1975, Adekunle et al. 2010, Gonzales et al. 2009, Hook and Osborn 2012). However, there is a dearth of information concerning the impact of LSC on GoM phytoplankton species. (Paul et al. 2013) showed that 34% of water collected in the Northern GoM in August 2010 was toxic to phytoplankton. Chapter 2 demonstrated relative growth responses of GoM phytoplankton to physically- and chemically-dispersed LSC, revealing that individual
diatom species showed greater tolerance to LSC than dinoflagellate species. Chapter 4 examined the distinct responses of GoM phytoplankton communities to crude oil under different nutrient regimes. It was shown that nutrient levels affect the tolerance of phytoplankton to LSC. To our knowledge, no additional reports observing a direct impact of crude oil on GoM phytoplankton species have been published.

The GoM is host to many different phytoplankton species that contribute to the primary productivity in the Gulf (Rabalais et al. 1996, Schaeffer et al. 2012a). However, 14 of them have been identified as potential harmful species (Schaeffer et al. 2012a). Blooms of these potentially harmful species pose environmental or public-health threats and are thus referred to as harmful algal blooms (HABs). HABs have adverse effects in the marine environment, either due to their production of toxins or to the alteration of food web dynamics as a result of biomass accumulation, which may result in oxygen deficiency in surrounding water mass, diminution of photosynthesis by shading underneath water column or mechanical irritation in fishes. Particularly toxin producing phytoplankton blooms carry a higher threat for environment since impacts of toxic phytoplankton species include mass mortalities of fish and shellfish; illness and death of marine mammals, seabirds, and other animals; and human illness and death from toxic seafood or from toxin exposure through water inhalation or other contact. Increasing frequency of the harmful blooms over the past two decades (Lohrenz et al. 1990, Turner and Rabalais 1994, Rabalais et al. 1996) due to nutrient outflow from the Mississippi River and increasing oil activities in the GoM raise the change of toxic phytoplankton species exposure to crude oil in this region. Crude oil impact on commonly found phytoplankton species have been studied in this region (Chapter 2), yet there is no study
specifically targeting responses of toxic phytoplankton species to crude oil exposure. The impact of toxin producing phytoplankton species in the GoM is especially of interest, since the region accounts for approximately 25% percent of the nation's seafood corridors and about 21% of the total US dockside value for fishery landings (Adams et al. 2004).

HAB species mostly elicit their effects through the production of a suite of potent phycotoxins. Karenia brevis, Pseudo-nitzschia spp., Alexandrium spp., Amphidinium spp., Gymnodinium sp., Lingulodinium spp., Prorocentrum spp., and Heterosigma spp. are known to produce toxins in the GoM (Dortch et al. 1999). Blooms of the toxic dinoflagellate, K. brevis (formerly Gymnodinium breve and known to produce the toxin called “brevetoxins”) has certain importance because it occurs on a nearly annual basis along the Florida coast during late summer or early autumn and can persist from less than a month to more than a year (Tester and Steidinger 1997). K. brevis blooms can cover areas from 10 km² to 1000 km² (Steidinger 2009). These blooms have a devastating impact on fish and marine mammal mortalities and human illness and result in large economic losses (Steidinger 2009).

Toxin production by cells varies among different strain of the same species (e.g., (Errera et al. 2010, Hagstrom et al. 2011). Previous studies exhibited that environmental parameters also contribute to the variation of toxin levels produced in cells. For example, nitrogen limitation (Hardison D Ransom et al. 2012), phosphate limitation (Hardison Donnie Ransom et al. 2013), and salinity (Brown et al. 2006) lead to changes in toxin production by K. brevis; salinity, temperature, and light (Morton et al. 1994) affected toxin production by P. minimum, and nutrient levels (Hagstrom et al. 2011) and copper exposure (Ladizinsky 2003) influenced toxin production of Pseudo-nitzschia spp.
As realized with the DWH oil spill, the GoM region is prone to oil spills due to the high oil activities that occur there. The broad distribution area of oil during the DWH oil spill reached until the coast of Louisiana and Florida raised a critical question. What would the impact of crude oil be on ecologically important toxic HAB species in the GoM? The study aimed to better understand the varying production of toxins by crude oil on toxic phytoplankton, as they are critical to the coastal ecosystem and are linked to higher trophic levels as food resources. Crude oil was evaluated as another environmental parameter could possibly impact phytoplankton toxin production. The most significant HAB species in the GoM, *K. brevis*, and two other toxic HAB species, *P. minimum* and *H. akashiwo*, detected in very high abundance shortly after DWH oil spill (Unpublished data) were chosen to (1) show whether any of the three species has a selective advantage due to toxin production when exposed to LSC compared to non-toxic species previously studied under the same experimental conditions, and (2) to determine the vicissitudes of toxin profiles on *K. brevis* and *P. minimum* under different exposure concentration of LSC.

5.3. Materials and Methods

5.3.1. Microalgal Cultures

The impact of crude oil exposure was assessed using three harmful/toxic phytoplankton species that are common and abundant in the northern GoM: dinoflagellates *Karenia brevis* (CCMP#: 2281) and *Prorocentrum minimum* (CCMP#: 2812), and a raphidophyte *Heterosigma akashiwo* (CCMP#: 2815). The initial cultures of these phytoplankton were provided by National Center for Culture of Marine
Phytoplankton (CCMP), ME, USA. The test organisms were acclimated to ambient laboratory conditions prior to use in the experiments. The cultures were grown in f/2–Si medium (Guillard 1975) at 25 °C and 35 practical salinity units (psu) in 0.22 μm filtered and autoclaved natural seawater. The light source was cool-white fluorescent lights with an irradiance of 85 μE.m\(^{-2}\).s\(^{-1}\) that were kept on a 12:12 hour light:dark cycle.

5.3.2. Preparation of the Test Mediums and Experimental Setup

The water-accommodated fraction (WAF; LSC in seawater) of crude oil was used in this study to provide a more realistic assessment. Non-weathered LSC was collected by British Petroleum (BP) through a riser vent pipe from the damaged wellhead of the DWH drilling rig in the GoM on May 20, 2010, and stored at -4 °C (BP, Ford Collins, CO, USA).

The WAF was prepared according to the method described in *The Chemical Response to Oil Spills: Ecological Research Forum* (CROSERF) (CROSERF 2005) with minor modifications. The WAF mixtures used in algae toxicology tests were prepared with 0.22 μm filtered and autoclaved GoM seawater (salinity: 35) in 2 L Klimax valved outlet reservoir bottles. Loading of 40 g LSC in 1.6 L seawater is known to result in 20–25% headspace by volume in each bottle. The WAF solution was prepared by low mixing energy (no vortex). The stirring rate was adjusted to 160 rpm to prevent micro particulate settlement at the bottom. After 24 hours of mixing, a settling time of 6 hours was applied. Samples from the WAF were withdrawn through a valve located at the bottom of the bottle to avoid distorting the water/oil interface. Samples for chemical analysis were
collected in amber glass jars with Teflon lined caps, allowing no headspace and stored at 4 °C. Serial dilutions (100%, 40%, 16%, and 6.4%) of the water phase were prepared for each test medium and were used in the experiments.

The WAF exposure experiments were conducted with 3 phytoplankton species exposed to 4 crude oil concentrations and control flasks to determine individual responses of relative toxicity of LSC. The control flasks contained only the phytoplankton cultures in the growth media. There were two steps in the experiment; determination of growth curves and sampling for toxin measurements. The phytoplankton cultures were first exposed to the WAF of LSC to determine their growth curves. Samplings of toxin measurements were then conducted under the same conditions. Since different growth phases can result in different toxin productions in phytoplankton, sampling time points for toxin production were performed successfully. The end point for the growth curve experiment was 2 days after each species reached its stationary phase in control flasks. The growth curves were determined by daily quantification of chlorophyll \( a \) concentration. These chlorophyll \( a \) values were converted to cell count, and cell count vs. time growth curves were plotted. Algal growth was calculated by using the area under the growth curve, which is equal to the total increase in biomass.

\[
A = \frac{(N_1 - N_0) \cdot t_1}{2} + \frac{(N_1 + N_2 - 2N_0) \cdot (t_2 - t_1)}{2} + \ldots + \frac{(N_{n-1} + N_n - 2N_0) \cdot (t_n - t_{n-1})}{2}
\]

\[
\mu = \ln \left( \frac{N_{t_2}}{N_{t_1}} \right) / (t_2 - t_1)
\]

where \( N_{t_2} \) and \( N_{t_1} \) are cell numbers at time \( t_2 \) and \( t_1 \), respectively.

The inhibition rates of different treatments were calculated according to the following formula: \( I(\%) = \frac{(A_c - A_t)}{A_c} \times 100 \), where \( A_c \) and \( A_t \) is the area under the growth curve of control group and the treatment, respectively.
Upon determination of growth curves of the cultures under such conditions, the same experimental setup was performed to obtain water and algal samples for toxin measurements. Cell counting and chlorophyll \( a \) measurements were performed for conformation and comparison of cell growth for these two experimental setups. For both steps, the flasks were capped with foam lid that allowed air exchange but prevented particle exchange from the air. The samples were kept in a \(-20 \, ^\circ C\) freezer until analysis. The exposure studies consisted of three replications per treatment. Temperature was monitored continuously; salinity and pH were measured at the beginning of each experiment. Salinity was 35 and the pH was about 8.0 for all treatments.

5.3.3. Chemical Analysis of the Crude Oil

Total petroleum hydrocarbon (TPH) analysis was performed with total scanning fluorescence (TSF) (Aqualog, Horiba Scientific). Standards and samples were measured at an excitation wavelength of 260 nm and an emission wavelength of 360 nm where the instrument provided the maximum intensity that corresponds to total oil equivalents of LSC. Estimates of TPH concentration of the treatments were based on the instrument’s maximum intensity. Standard solutions were prepared with direct dissolution of LSC in dichloromethane (DCM). The standard curve provided a well correlation \((y=990.86x-16.579 \, R^2=0.99607)\) between the concentration of LSC and the maximum intensity of the instrument. The stock solution was diluted to concentrations of 0.05–20 ppm. For unknown samples, 100 mL of the WAF was placed in a 250 mL separatory funnel, and 20 mL DCM was added to the first extraction. The aqueous layer was extracted with additional DCM \((2\times20 \, mL)\), and the DCM layers were combined and dried over \( \text{Na}_2\text{SO}_4 \). Then, the extracts were reduced on a rotary evaporator. The reduced extracts were
transferred to graduated flasks to reduce them to the desired volumes under nitrogen gas and a water bath in a nitrogen evaporator (N-EVAP 111; Organomation Associates, Inc., MA, USA). A 5-mL sample of the resulted crude was transferred to quartz fluorometer cells (10 mm) for TPH measurements.

5.3.4. Extraction and Measurement of Toxins

Toxin measurements aimed to analyze toxin concentrations from phytoplankton at different life phases. Because the growth phase for each species was reached at different days, samples for toxin measurements were taken at days 1, 4, 7, 10, 13 and 16 and days 1, 4, 6, 8, 10 and 12 for *K. brevis* and *P. minimum*, respectively. The brevetoxin and okadaic acid enzyme-linked immunosorbent assays (ELISA) kits were purchased from Abraxis (Warminster, PA) to determine the toxin quota within the cells versus that in external media. The concentration of brevetoxin and okadaic acid in the media was determined by removing an aliquot of culture media based on the culture’s concentration, which was then filtered on GF/F filter under a very gentle vacuum to avoid cell lysis. Algal cells on the filter were used to determine intracellular toxin production per cell, and the filtrate was used to quantify the extracellular toxin concentration for each treatment.

The brevetoxin concentration in *Karenia brevis* cells was determined by extraction of the cells on the filters, following a modified method by (Roth et al. 2007). Filters were inserted in 15 mL glass centrifuge tubes and placed in the -20 °C freezer until the extraction. Four mL of 100% methanol (MeOH) was added in each centrifuge tube and sonicated (5 watt) for 10 minutes. Then, they were centrifuged for 10 minutes at 4400 rpm to remove as much cell debris as possible, and the supernatant was decanted. The remaining algal cell residue was washed with MeOH in duplicate and centrifuged.
All supernatants were pooled, and the final volume was brought up to 12 mL by addition of MeOH. Samples from the combined MeOH extract were diluted 10 fold for measurement of brevetoxin concentrations in all extracted cells by competitive ELISA (Naar et al. 2002).

In order to concentrate water samples for brevetoxin analysis, a 40 mL water sample was placed in each centrifuge tube, and 4 mL of ethyl acetate (EtOAc) was added. After 1 min. of vortex, samples were centrifuged for 5 min. at 4000 rpm. The EtOAc layer was collected, reduced to dryness in a 40 ºC water bath, and resuspended in 400 μL of 90% MeOH. The resulting solution was diluted 25 fold (from 20 μL to 500 μL) for ELISA analysis. For both brevetoxin analyses, spiking for the determination of extraction efficiency were conducted by standard PbTx-3 (Abraxis, Warminster, PA). Extraction efficiencies were 78.9%±8.8% and 80.7%±6.6% for intracellular and extracellular toxin analysis, respectively. Data reported for the samples were not adjusted for percent recovery. Results are expressed as PbTx-3 equivalents and reflect the overall concentration of brevetoxins present in the samples.

The okadaic acid concentration in *Prorocentrum minimum* cells was determined by extraction of the cells on the filters, following a modified method by (Rao et al. 1993). Filters were inserted in 15 mL glass centrifuge tubes and placed in the -20 ºC freezer until the extraction. Four mL of 100% methanol (MeOH) was added in each centrifuge tube and sonicated (5 watt) for 10 minutes. Then, they were centrifuged for 10 minutes at 3400 rpm to remove as much cell debris as possible, and the supernatant was decanted. The remaining algal cell residue was washed with 80% MeOH in duplicate and centrifuged. All supernatants were combined, and the final volume was brought up to 12...
mL by addition of 80% MeOH. Samples from the combined MeOH extract were diluted 10 fold for measurement of okadaic acid concentrations in all extracted cells by competitive ELISA method.

In order to concentrate water samples for extracellular okadaic acid analysis, a 40 mL water sample was placed in each centrifuge tube, and 4 mL of HPLC grade EtOAc was added. After 1 min. of vortex, samples were centrifuged for 5 min. at 4000 rpm. The EtOAc layer was collected, reduced to dryness in a 40 °C water bath, and resuspended in 400 μL of 80% MeOH. The resulting solution was diluted 25 fold (from 20 μL to 500 μL) for ELISA analysis. For both okadaic acid analyses, spiking for the determination of extraction efficiency were conducted by standard okadaic acid (Abraxis, Warminster, PA). Extraction efficiencies were 86.2%±12.2% and 80.3%±15.9% for intracellular and extracellular toxin analysis, respectively. Data reported for the samples were not adjusted for percent recovery.

5.3.5. Statistical Analysis

All statistical analyses requiring comparison of treatments were carried out using SigmaStat 12.3 software (Systat Software, Inc., San Jose, CA, USA). ANOVA (followed by a post-hoc Tukey test if required) and t-test were performed to evaluate significance of different treatment’s and individual differences with a probability threshold of 0.05. EC$_{50}$ (concentration causing 50% inhibition on growth) values for phytoplankton were determined by plotting percent growth inhibition values of each treatment against the toxicant concentrations. The models were fitted to data by nonlinear regression analysis using R (R Development Core Team 2006) with the add-on package “drc” (Ritz and Streibig 2005). In the case of a monotonically decreasing function, a four-parameter, log-
logistic model was applied for each species. In the context of hormesis, where growth stimulation is observed at the low dose of the toxicants, a five-parameter, Brain–Cousens modified log-logistic model (Brain and Cousens 2006) was applied.

5.4. Results

5.4.1. Crude Oil Analysis

Crude oil concentrations were measured by the TSF method showed that the non-diluted (100% WAF) crude oil solution contains 10.13±0.54 mg.L\(^{-1}\) TPH. The other three concentrations that were used in the experimental flasks as an exposure media were measured as 0.66±0.03, 1.63±0.09, and 4.39±0.24 mg.L\(^{-1}\) TPH equivalent.

5.4.2. Phytoplankton Growth Rate Under Crude Oil Exposure

Crude oil exposure inhibited the population growth of \textit{K. brevis}, \textit{P. minimum}, and \textit{H. akashiwo} compared to control groups but, at the lowest concentration, stimulated the population growth of \textit{P. minimum} (Figure 5.1). Measured effective concentrations (EC\(_{50}\)) that cause 50% growth inhibition were 1.06 mg.L\(^{-1}\), 2.79 mg.L\(^{-1}\), and 2.75 mg.L\(^{-1}\) for \textit{K. brevis}, \textit{P. minimum} and \textit{H. akashiwo}, respectively. The lowest crude oil concentration (0.66 mg.L\(^{-1}\)) caused a non-significant (\(p=0.1\)) growth inhibition on \textit{K. brevis}, whereas the concentration above 1.63 mg.L\(^{-1}\) showed a highly inhibitory effect. Growth inhibition for \textit{P. minimum} was very gradual with increasing crude oil concentrations and growth stimulation (\(p<0.05\)) was observed only at 0.66 mg.L\(^{-1}\) crude oil concentration. The growth inhibition for \textit{H. akashiwo} was different from the others as there was a highly significant (\(p<0.01\)) inhibitory effect between two consecutive treatments (1.63 mg.L\(^{-1}\) and 4.39 mg.L\(^{-1}\)) (Figure 5.1).
Figure 5.1. Growth curves of *K. brevis*, *P. minimum*, and *H. akashiwo* under different concentrations of LSC. Crude oil concentrations are given as total petroleum hydrocarbons (TPH).
5.4.3. Toxin Production of *K. brevis* and *P. minimum*

The effect of the crude oil on brevetoxin production for each treatment is shown in Figure 5.2. Brevetoxin levels increased gradually throughout the experiment in the control treatment without crude oil. This suggests that *K. brevis* produced more toxin in the stationary phase compared to exponential and lag phases of the culture’s natural growth.

Extracellular brevetoxin amounts showed a similar trend for the control treatment. Leaching of the toxin out of the cells increased with increasing cellular toxin production. A significant (*p*<0.05) brevetoxin production increase was observed at 0.66 mg.L\(^{-1}\) crude oil treatment. While intracellular toxin production throughout the experiment was 3.8 to 7.5 fold higher, the extracellular toxin concentrations were at most 2.3 fold higher compared to the control group at this concentration. The other treatments containing 1.63 and 4.39 mg.L\(^{-1}\) crude oil caused reduction of intracellular toxin production on *K. brevis*. Parallel to the reduction of toxin production, extracellular brevetoxin concentration declined drastically compared to the control group.

Okadaic acid production from *P. minimum* was also affected by crude oil exposures (Figure 5.3). Comparable to brevetoxin production response, 0.66 mg.L\(^{-1}\) caused significant (*p*<0.05) increase on intracellular and extracellular toxin production of *P. minimum*. Increase in intracellular and extracellular okadaic acid concentrations were insignificant (*p*<0.05) for 1.63 mg.L\(^{-1}\) crude oil treatment compared to the control group.
Figure 5.2. Concentration of (A) intracellular brevetoxin (PbTx-3 equivalents) extracted from *K. brevis* cells and (B) extracellular brevetoxin extracted from filtrates after the filtration of samples.

throughout the experiment. The treatment containing 4.39 mg.L\(^{-1}\) crude oil caused reduction of okadaic acid production (*p*<0.05) after day 6, and extracellular okadaic acid concentration was lower than the other treatments (Figure 5.3).
Figure 5.3. Concentration of (A) intracellular okadaic acid extracted from *P. minimum* cells and (B) extracellular okadaic acid extracted from filtrates after the filtration of samples. The * indicates that samples contained excessive okadaic acid that is above the detection limit of the ELISA plates.

For both toxins, there was a good correlation between intracellular toxin production per cell and extracellular toxin concentration for control groups for both species (Figs. 5.4 and 5.5). However plots, which indicate the ratios of intracellular toxin...
Figure 5.4. Change in the ratios of intracellular brevetoxin concentration per cell to extracellular brevetoxin concentration during the experiments for all treatments.

Figure 5.5. Change in the ratios of intracellular okadaic acid concentration per cell to extracellular okadaic acid concentration during the experiments for all treatments.
production per cell to extracellular toxin concentration, demonstrated that the retention of brevetoxin and okadaic acid within cells vs. secretion into the external medium is not well correlated among all treatments. For brevetoxin and okadaic acid, the common trend was increasing extracellular brevetoxin concentration with elevated toxin production per cell, however, in some cases differences between these toxin responses were observed. The treatment with 4.39 mg.L\(^{-1}\) crude oil showed a similar correlation to the control groups for intracellular okadaic acid production per cell and extracellular toxin concentration. Another difference between brevetoxin and okadaic acid was the ratios of toxins produced by cells to secreted amounts. These ratios varied between 28 and 1166 for brevetoxin and 0.4 to 7.0 for okadaic acid (Figs. 5.4 and 5.5). Even though the toxin production was similar between these two species, the amount of toxin secreted into external media from the cells differed considerably.

5.5. Discussion

Nutrient rich f/2 media was used throughout the course of the experiments to prevent stress due to nutrient limitations. Our previous study confirmed that f/2 media can support longer-term population growth without limitation of any nutrients. The study compared the EC\(_{50}\) values of non-toxic species grown under identical conditions in Chapter 2. Results indicated that EC\(_{50}\) values (which varied from 1.06 to 2.79 mg.L\(^{-1}\)) of toxic species are not significantly below those of non-toxic species (which varied from 1.03 to 2.50 mg.L\(^{-1}\)), suggesting that the toxin production does not provide a competitive advantage against crude oil toxicity.
5.5.1. Brevetoxin Production

Brevetoxin concentrations and profiles obtained from the control flasks in this study were similar to previous observations of brevetoxin profiles (Backer et al. 2005, Pierce RH et al. 2005, Brown et al. 2006, Pierce Richard et al. 2008, Tester et al. 2008, Hardison D Ransom et al. 2012), which indicate that the cultures were not under stress. The PbTx-3, which, along with PbTx-1 and PbTx-2, is one of the most abundant brevetoxin congeners measured in whole water samples collected during a *K. brevis* bloom (Pierce RH et al. 2005), was used to report overall brevetoxin concentration in this study. It is well known that wide clonal variability of the toxin fractions of *K. brevis* (Baden and Tomas 1988, Loret et al. 2002, Errera et al. 2010) makes the precise estimation of total brevetoxins concentration from its one of the congeners. During the experiment, careful attention was paid to avoid cell lysis by physical means to allow for only natural excretion of brevetoxin into the external medium. The increase in extracellular brevetoxin concentrations throughout the experiment was consistent with the bloom age that increases the formation of PbTx-3 from PbTx-2 congener (Roszell et al. 1988, Brown et al. 2006, Pierce Richard et al. 2008, Tester et al. 2008).

Even though the relationship between hydrocarbons and *K. brevis* is not known, the reduction of toxin production in the presence of high concentrations of the crude oil might be attributed to the interference of toxin production mechanisms of *K. brevis*. Brevetoxins are polyketides synthesized by the polyketide synthase (PKS) pathway (Wright Jeffrey LC and Cembella 1998). The biosynthesis of polyketides share remarkable similarities with fatty acid biosynthesis (Khosla et al. 1999, Jenke-Kodama et al. 2005). Therefore, it is proposed that the similarities of these two pathways causes a
disruption in brevetoxin production, as crude oil can disrupt the biosynthesis mechanisms required for a glycolipids and lipid pigments (Morales-Loo and Goutx 1990). On the other hand hydrocarbon toxicity can cause damage to and alterations of DNA and RNA (Bagchi et al. 1998, El-Sheekh et al. 2000, Tang et al. 2002, Parab et al. 2008), reduction in cell nuclei (Tukaj et al. 1998), and loss of CO$_2$ absorption (Koshikawa et al. 2007), all of which could directly or indirectly disrupt the PKS pathway.

Enhanced brevetoxin production at low concentrations of crude oil may be attributed to two bacterial activities: (1) by-products of bacterial degradation of the crude oil could stimulate brevetoxin production, and (2) the allelopathic relationship between algae and bacteria could lead to an increase in toxin production. Goutx et al. (1984) reported that sugars, lipids, and amino acids are the main by-products of petroleum degradation by bacteria. The impact of these by-products on algal growth was investigated, and both stimulation and inhibition of growth was reported based on the concentration of the by-products (Goutx et al. 1984). A study by Souto et al. (2001), *Prorocentrum lima* cultivated in K medium enriched with selected amino acids resulted in a considerable increase in toxin production, which even had effects on toxin profiles. *P. lima* produces okadaic acid, which, like brevetoxin, is also a polyketide that is produced via the PKS pathway (Needham et al. 1994, Wright JLC et al. 1996). Another study (Shimizu 1993) indicated that the amino acid [3,4-$^{13}$C$_2$] leucine is incorporated in the PKS pathway and metabolized to 3-$^{13}$C-acetoacetate and 2-$^{13}$C acetate during *K. brevis* biosynthesis of brevetoxins. Both brevetoxin and okadaic acid production share the same mechanism even they were produced by different phytoplankton genus. In addition to amino acids, sugars, and lipids, other metabolic by-products of microbial degradation
could potentially affect their production. The aforementioned bacterial interaction may only exist at low concentrations of crude oil, as the bacteria and phytoplankton should coexist for there to be a symbiotic and/or mutualistic relationship. Therefore, at crude oil concentrations that are above the threshold for phytoplankton survival, such a relationship is not possible. Although the implication of brevetoxin as the causative agent in *Karenia brevis* allelopathy is controversial, it is possible that enhanced brevetoxin production is responsible for the suppression of bacteria, compete for same resources in the presence of the crude oil.

5.5.2. Okadaic Acid Production

Stimulation of *P. minimum* growth was in agreement with a previous study (Morales-Loo and Goutx 1990) that involved the exposure of low concentrations of the crude oil. Moreover, 0.66 mg.L\(^{-1}\) crude oil led to toxin profiles that are very similar to those observed for *K. brevis* under the same treatment. The similarities in toxin profiles and in the mechanisms for toxin production between *K. brevis* and *P. minimum* suggest that hydrocarbons affect okadaic production in similar way as they do brevetoxin production, and the same assertions on the possible cause of these effects discussed above could be applied to *P. minimum*. The lack of data on bacterial activities and missing analysis of biodegradation by-products do not allow for a full interpretation of the toxin profile data for *P. minimum* and is a limitation of this study. The high ratios of intracellular toxin to extracellular toxin were generally observed at the early stages of bloom; however, as the bloom aged, toxins were secreted at a higher rate into the external medium, which is in agreement with previous findings (Hardison D Ransom et al. 2012).
Our study also indicated that crude oil affects increase in extracellular toxin secretion in such a way that the dissolved toxin would be more available to its surrounding areas.

5.5.3. Environmental Implications

This study shows that hydrocarbons not only impact toxin production but also growth rate of *K. brevis*. When *K. brevis*’ niche is considered, cell number and brevetoxin concentration are equally important. The findings of this study may be useful in a scenario where *K. brevis* is present in an area where there is oil as the result of a spill or from oil transport. Crude oil concentrations at or above 4.39 mg. L⁻¹ drastically suppressed the growth of *K. brevis* and toxin production either decreased or did not increase considerably; therefore, the expected negative impact of brevetoxin would be less during an oil spill than the impact of the toxin in uncontaminated waters. However, slightly suppressed growth of *K. brevis* at the concentration of 0.66 mg.L⁻¹ could potentially increase the impact *K. brevis* ascribe to highly increased toxin production. The measured increase of brevetoxin production at this concentration was 3.3–6.3 times higher than the controls.

Increased levels of brevetoxins only add to the negative impacts the accumulation of hydrocarbons cause to species that graze on *K. brevis* and, ultimately, to higher trophic levels within the impacted area (Jiang et al. 2010, Almeda et al. 2013). The affect can be advantageous to *K. brevis* since the populations of organisms that feed on it are reduced (Cohen et al. 2007, Hong et al. 2012, Waggett et al. 2012). Furthermore, the reduced grazing pressure can allow *K. brevis* to increase biomass, creating a potentially greater negative impact.
Amounts of brevetoxins produced by *K. brevis* vary depending on different strains of the algae (Baden and Tomas 1988, Backer et al. 2005, Pierce RH et al. 2005, Tester et al. 2008, Errera et al. 2010, Hardison D Ransom et al. 2012). In addition to genetically varied toxin production, other natural environmental factors such as salinity (Brown et al. 2006), nitrogen limitation (Hardison D Ransom et al. 2012), and phosphate limitation (Hardison Donnie Ransom et al. 2013), could contribute to this variation. Even though massive blooms of *K. brevis* form on an almost annual basis, it is routinely present at low levels throughout the GoM (Geesey and Tester 1993). It is known that decisions on shellfish bed closures depend upon cell concentrations of *K. brevis* rather than the amount produced toxin (Heil David C 2009). In order to avoid underestimating the negative impact of *K. brevis*, more efficient regulations for shellfish bed closures can be developed by considering the amount of toxin production per cell, especially under stressed environmental conditions.

*P. minimum*, another bloom-forming planktonic mixotrophic dinoflagellate with known occurrences in the GoM (Dortch et al. 1999, Licea et al. 2002, Licea et al. 2004, Schaeffer et al. 2012b), is recognized for the production of okadaic acid (Harper 2005). However, the production of okadaic acid from *P. minimum* and the resulting implication of the toxin has not been clearly documented in the Northern GoM. It has, however, a large potential threat to the GoM due to increasing coastal eutrophication (Justić et al. 2005), since a strong relationship appears to exist between nutrient enhancement and blooms of this species (Heil Cynthia A et al. 2005). Similar to *K. brevis* species, *P. minimum*’s toxin production enhanced under 0.66 mg.L\(^{-1}\) crude oil exposure, and its growth significantly (*p* <0.05) increased. This combination of growth and toxin
production enhancement could lead to more adverse consequences in the GoM. Also like *K. brevis*, the measured increase of the combined effects at 0.66 mg.L$^{-1}$ crude oil was 3.1–6.5 times higher than the controls. Similar to *P. minimum*, *H. akashiwo*, which has not taken enough attention in the GoM, is another emerging species for the Gulf and their large bloom was observed after the oil spill (W. Morrison at LUMCON, pers. comm.) in summer 2010. The lack of data on *P. minimum*’s okadaic acid production and *H. akashiwo*’s toxin production effects on the GoM’s ecosystem limits possible interpretations of our findings for these species.

5.6. Conclusions

This study reveals the fate of harmful algal species under the crude oil contamination and attempts to explain possible mechanisms. It has been clearly shown that crude oil impacts *K. brevis*, *P. minimum*, and *H. akashiwo* growth in both a positive and negative direction way. This study’s findings also warrant further investigation of microbial degradation of crude oil and the impact of its by-products on phytoplankton.

5.7. References


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CHAPTER 6: INDUCTION OF REACTIVE OXYGEN SPECIES IN MARINE PHYTOPLANKTON UNDER CRUDE OIL EXPOSURE

6.1. Abstract

Exposure of phytoplankton to crude oil can elicit a number of stress-responses, but the mechanisms that drive these responses are unclear. South Louisiana crude oil was selected to investigate its effects on population growth, cellular chlorophyll $a$ (Chl $a$) content, antioxidative defense, lipid peroxidation, and changes in transcript levels of several possible stress-responsive genes of the marine diatom, *Ditylum brightwellii*, and the dinoflagellate, *Heterocapsa triquetra*, in laboratory-based microcosm experiments. The microalgae were exposed to crude oil for up to 96 hours, and Chl $a$ content, superoxide dismutase (SOD), the glutathione pool (GSH and GSSG), and lipid peroxidation content were analyzed. The cell growth of both phytoplankton species was inhibited with increasing crude oil concentrations. Crude oil exposure did not affect Chl $a$ content significantly in cells. SOD activities showed similar responses in both species, being enhanced at 4- and 8-mg/L crude oil exposure. Only *H. triquetra* demonstrated enhanced activity in GSSG pool and lipid peroxidation at 8-mg/L crude oil exposure, suggesting that phytoplankton species have distinct physiological responses and tolerance levels to crude oil exposure. This study indicated the activation of reactive oxygen species (ROS) in phytoplankton under crude oil exposure; however, the progressive damage in cells is still unknown. Thus, ROS-related damage in nucleic acid, lipids, proteins, and DNA, due to crude oil exposure could be a worthwhile subject of study to better understand crude oil toxicity at the base of the food web.
6.2. Introduction

Natural and anthropogenic sources introduce crude oil into aquatic environments, which can lead to chronic and acute contamination for organisms living within these habitats. Toxic effects of crude oil on phytoplankton have been studied extensively and revealed that phytoplankton, which diverge greatly in physiological properties, vary their response and tolerance to oil toxicants (Liu et al. 2006, Hjorth et al. 2007, Meng et al. 2007, Wang et al. 2008). Crude oil has been shown to interfere with photosynthetic processes and respiratory mechanisms and inhibit total primary production of phytoplankton (Miller et al. 1978, Karydis 1979, Bate and Crafford 1985, Harrison et al. 1986, Aksmann and Tukaj 2008, Gonzalez J. et al. 2009). Lipophilic oil compounds accumulate in the cell membrane and change its structural and functional properties, including the loss of cell permeability, and cause other types of irreversible damage at the cell surface (Sikkema et al. 1995). Furthermore, toxicity studies have demonstrated that hydrocarbons can cause loss of cell mobility (Soto et al. 1975), DNA damage (Bagchi et al. 1998, Tang et al. 2002), prevention of nutrient and CO₂ absorption (Koshikawa et al. 2007), inhibition of nucleic acid and protein synthesis (Chen et al. 2008), chloroplast shrinkage, and loss of pigments in phytoplankton (Smith J.E. 1968). Many of these processes involve reactive oxygen species (ROS) (Torres et al. 2008, Lushchak 2011). ROS are produced directly by the excitation of O₂ and the subsequent formation of singlet oxygen or by the transfer of one, two, or three electrons to O₂, which results in the formation of superoxide radicals (O₂⁻•), hydrogen peroxide (H₂O₂), or hydroxyl radical (HO’) (Baker and Orlandi 1995). It is important to consider that ROS are the natural byproducts of a number of essential metabolic pathways, including photosynthesis and
respiration, signaling molecules during cell differentiation, cell cycle progression, and in response to extracellular stimuli.

The proliferation of ROS is mediated by antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and nonenzymatic antioxidants, such as glutathione, vitamin E, ascorbate, β-carotene, and urate (Foyer et al. 1997, Noctor and Foyer 1998, de Zwart et al. 1999). The enzymes catalyze the removal of ROS by scavenging and reducing them to less reactive molecules such as oxygen or water. Oxidative stress is a physiological stage characterized by a net increase in ROS that results from an insufficient scavenging capacity of the antioxidant defenses (Baker and Orlandi 1995, Mittler 2002). Thus, when the production of ROS exceeds the scavenging capacity of the antioxidant defenses, extensive oxidative damage to membrane lipids (Gutteridge 1995), DNA (Beckman and Ames 1997), and proteins (Berlett and Stadtman 1997) and peroxidation of lipoproteins (Esterbauer H and Ramos 1996) takes place.

To date, no study has shown the formation of ROS under crude oil exposure in order to better understand crude oil toxicity mechanisms on phytoplankton. Previous investigations in the literature have revealed the formation of ROS in phytoplankton subject to polycyclic aromatic hydrocarbons (PAHs) such as phenanthrenequinone, anthraquinone (Tukaj and Aksmann 2007), and fluoranthene (Wang and Zheng 2008) as well as the interference of anti-oxidant defending system operations after anthracene exposure (Aksmann and Tukaj 2008). The interactions between these hydrocarbon compounds with ROS support our hypothesis that crude oil, which contains thousands of different hydrocarbons, causes oxidative stress and damage in phytoplankton.
Antioxidant enzymes represent a primary mechanism to control concentrations of ROS in all organisms. The first ROS formed is the $O_2^{-}$, SOD, which acts as a first line of defense in the enzymatic ROS scavenging system by dismutating $O_2^{-}$ to $H_2O_2$ (Bowler et al. 1992) to prevent oxidation of biomolecules and production of $HO^*$, one of the most reactive oxygen species known to chemistry (Gregory and Fridovich 1973). In addition to this duty, SOD is also the only enzyme capable of process the dismutation. Therefore, SOD holds a key position within the antioxidant network. Efficient destruction of $O_2^{-}$ and $H_2O_2$ requires the action of several antioxidant enzymes acting in synchrony and the reduction of $H_2O_2$ to $O_2$ and $H_2O$ is catalyzed by either the CAT or the GPx enzyme system. Glutathione, which is a part of the GPx enzyme system, has several physiological roles, including the detoxification of ROS in chloroplasts, where it acts as an intermediate in the detoxification of free radicals and peroxides (Meister 1988). The reduced form of glutathione, GSH, is a tripeptide that exists interchangeably with its oxidized form, GSSG, in which two glutathione molecules are linked via a disulfide bond. GSH can be oxidized to GSSG by some ROS, such as $H_2O_2$. In active oxygen elimination, GSH is partly oxidized to GSSG, and the glutathione redox state (GRS) = $[GSH/(GSH+0.5GSSG)]$ is a useful indicator of oxidative stress (Agrawal 1992, Zenlinski et al. 1999). A failure of the antioxidant defense system to prevent efficient $O_2^{-}$ and $H_2O_2$ proliferation may also result in a variety of oxyradical-induced perturbations, including lipid peroxidation. It is also a widely recognized consequence of oxyradical production (Winston and Di Giulio 1991).

It is possible that other cellular processes may respond to disruptions in cellular equilibrium during crude oil exposure. In addition to ROS-induced stress, quantification
of gene expression may indicate which biological pathways are affected by contamination in the target organisms. Phytoplankton have been previously used to detect alterations at the transcript level due to environmental stressors such as PAHs (Bopp and Lettieri 2007), light (Schroda et al. 1999), phycotoxin (Yang et al. 2010), and herbicides (Qian et al. 2008).

In the current study, we assessed the cellular and subcellular level responses of two marine phytoplankton species, *Ditylum brightwellii* and *Heterocapsa triquetra*, exposed to elevated concentrations of crude oil above and below their EC$_{50}$ values. The present work was therefore focused to investigate 1) changes in cellular chlorophyll *a* (Chl *a*) content 2) the role of ROS and oxidative stress in phytoplankton under crude oil exposure, and 3) the changes at the transcript level of selected genes (Table 6.1) during crude oil exposure.

6.3. Materials and Methods

6.3.1. General Experimental Setup and Microalgal Cultures

Controlled laboratory microcosm studies were conducted using standard static non-renewal exposure toxicity tests. Control flasks and crude oil contaminated treatments (3 different concentrations) were set up. All flasks were started with approximately 8×10$^3$ cell/mL *D. brightwellii* and 12×10$^3$ cell/mL *H. triquetra* cell concentrations. This scenario represents their late exponential phase cell density under normal conditions. Autoclaved Pyrex flasks (500 mL) were utilized in all experiments (n=6 for all treatments), and the experimental exposure medium volume was 400 mL for all conditions. Phytoplankton cultures, *D. brightwellii* (CCMP#: 359) and *H. triquetra*
Table 6.1. Details on genes analyzed by quantitative RT-PCR.

**D. brightwellii**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
<th>Cellular process involved</th>
<th>GenBank ref.</th>
<th>Amplificon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock protein 70</td>
<td>Hsp70</td>
<td>F-primer</td>
<td>CATGGTAGGAGGCAGCTGAAA CAAAACATAAAGTTTCCGCCTTGAT</td>
<td>Stress Response</td>
<td>AFO84296.1</td>
<td>98</td>
</tr>
<tr>
<td>Heat Shock Protein 90</td>
<td>Hsp90</td>
<td>F-primer</td>
<td>ACGAGCCAGCCACTTTTTTCT TGGTCTGCTCATCCTCATCA</td>
<td>Stress Response</td>
<td>AFO84300.1</td>
<td>90</td>
</tr>
<tr>
<td>Nitrate Transporter</td>
<td>NRT2</td>
<td>F-primer</td>
<td>TGCTGCCATTGCTCTATTT TCGTCTCGTGTAGCTTGCTCA</td>
<td>Transporter</td>
<td>ABP01753.1</td>
<td>86</td>
</tr>
<tr>
<td>Photosystem II CP43</td>
<td>psbC</td>
<td>F-primer</td>
<td>TCCAACTGGTCCAGAACAGCAT TGGACCTCTGTGCTGATGAA</td>
<td>Photosynthesis</td>
<td>AGN91125.1</td>
<td>94</td>
</tr>
<tr>
<td>Actin</td>
<td>act.</td>
<td>F-primer</td>
<td>CCCTGGAATCACTGAGCGTA TGGGGCGACATCTTTAACCT</td>
<td>Cellular Structure</td>
<td>AFO84294.1</td>
<td>79</td>
</tr>
</tbody>
</table>

**H. triquetra**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
<th>Cellular process involved</th>
<th>GenBank number</th>
<th>Amplificon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast ascorbate peroxidase</td>
<td>cAPX</td>
<td>F-primer</td>
<td>CAAGAACTGGCTGACCTTCG GGGTACAGCAGGGAGTGGTC</td>
<td>Stress Response</td>
<td>AAW79294.1</td>
<td>81</td>
</tr>
<tr>
<td>Heat Shock Protein 90</td>
<td>Hsp90</td>
<td>F-primer</td>
<td>GGAGGAGATGAAACGGGAGT GCCTGAGATGACCTTCTCCA</td>
<td>Stress Response</td>
<td>AAR27541.1</td>
<td>84</td>
</tr>
<tr>
<td>Actin</td>
<td>act.</td>
<td>F-primer</td>
<td>CCCTCCACCATGAAAGATCAA GTGCTGAGGGAGGAGGAT</td>
<td>Cellular Structure</td>
<td>ABV00890.1</td>
<td>89</td>
</tr>
</tbody>
</table>
(CCMP#: 2981), were provided by the National Center for Culture of Marine Phytoplankton (CCMP), ME, USA. The cultures were grown in f/2 medium (D. *brightwellii*) and f/2-Si medium (*H. triquetra*) at 25 °C and salinity: 35 in 0.22 μm filtered and autoclaved natural seawater. The light source was cool-white fluorescent lights with an irradiance of 85 μE.m⁻²s⁻¹ kept on a 12:12 hour light:dark cycle. Daily 60 mL of samples were collected and stored at -20 °C for enzyme analysis. Additional 10–15 mL samples were also taken at each time point for Chl *a* measurements and cell counts performed under an inverted microscope. The abundance of each phytoplankton species was estimated by enumerating cells on a Sedgwick–Rafter counting slide (n=3).

6.3.2. Preparation of the Test Media

Recent studies on both fresh and dispersed crude oil toxicity to aquatic organisms have used the water-accommodated fraction (WAF) to provide realistic assessments. We assessed the WAF of two phytoplankton species that are common and abundant in the Gulf of Mexico. Non-weathered LSC was collected by British Petroleum (BP) through a riser vent pipe from the damaged wellhead of the Deepwater Horizon drilling rig in the Gulf of Mexico on May 20, 2010, and stored at -4 °C (BP, Ford Collins, CO, USA). The WAF was prepared according to the method described in *The Chemical Response to Oil Spills: Ecological Research Forum (CROSERF 2005)*. The WAF mixtures used in algae toxicology tests were prepared with 0.22 μm filtered and autoclaved Gulf of Mexico seawater in 2 L Klimax valved outlet reservoir bottles. Loading of 40 g LSC in 1.6 L seawater is known to result in 20–25% headspace by volume in each bottle. The WAF solutions were prepared at low mixing energy (no vortex). Replication of these conditions involved creating a seawater sample with an oil film on top that is not disturbed by vortex
formation. The stirring rate was adjusted to 160 rpm to prevent micro particulate settlement. After 24 hours, the samples were settled for 6 hours. Samples from the WAF were withdrawn through a valve located at the bottom of the bottle to avoid disturbing the water/oil interface. Samples for chemical analysis were collected in amber glass jars with Teflon lined caps, allowing no headspace and stored at 4 °C. Serial dilutions (10%, 40%, and 80%) of the water phase from each test medium yielded concentrations ranging from 1 to 8-mg/L total petroleum hydrocarbon (TPH) used in the experiments.

6.3.3. Chemical Analysis of the Crude Oil

TPH analysis was carried out with total scanning fluorescence (TSF) (Aqualog, Horiba Scientific), which measured standards and samples at an excitation wavelength of 260 nm and an emission wavelength of 360 nm. Standard solutions were prepared with direct dissolution of LSC in dichloromethane (DCM). The stock solution was diluted to concentrations of 1–20 mg/L. For unknown samples, 100 mL of the WAF was placed in a 250 mL separatory funnel, and 20 mL DCM was added to the first extraction. The aqueous layer was extracted with additional DCM (2x20 mL), and the DCM layers were combined and dried over Na₂SO₄. The extracts were reduced on a rotary evaporator, yielding a pale yellow liquid. The reduced extracts were transferred to graduated flasks under nitrogen gas and a water bath in a nitrogen evaporator (N-EVAP 111; Organomation Associates, Inc., MA, USA) to reduce them to the desired volumes. A 5 mL sample of the resulting crude was transferred to quartz fluorometer cells (10 mm) for TPH measurements. The samples were diluted to prevent quenching effects.
6.3.4. Chlorophyll a Content

Comparison of Chl $a$ contents of crude oil treated phytoplankton to control groups was performed. Samples (10 mL each) were taken daily from each flask, filtered through Whatman GF/F filters, and stored at -20 ºC until extraction. The filters were then extracted for 24 hours in 90% aqueous acetone at -20 ºC, and subsequently analyzed for Chl $a$ using a Turner fluorometer (Parsons et al. 1984). In addition to Chl $a$ content, each phytoplankton species was enumerated on a Sedgwick–Rafter counting slide (n=3). The cellular Chl $a$ content was detected for each cell for the control and crude oil treated samples.

6.3.5. Analysis and Extraction of Enzymes

SOD activity was chosen as an enzymatic antioxidant index and the glutathione pool, containing both GSH and GSSG activities, was chosen as a non-enzymatic antioxidant response to assess ROS activity of the phytoplankton exposed to crude oil. The role of lipid peroxidation in crude oil toxicity was also examined as an oxidative injury index by measuring hydroperoxide concentrations. SOD, glutathione, and lipid hydroperoxide enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cayman Chemical (Ann Arbor, MI) to analyze the concentrations within the cells.

For, superoxide dismutase, approximately $2 \times 10^5$ the phytoplankton cells were collected by centrifugation. The cell pellets were sonicated in 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. Cell extracts were
centrifuged at 2000×g for 5 min. Supernatants were removed for the assay. One unit of SOD activity (U) was defined as the enzyme dosage used for exhibiting 50% dismutation of the superoxide radical.

For glutathione extraction, approximately 2×10^5 the phytoplankton cells were collected by centrifugation. The cell pellets were sonicated in 2 mL of cold phosphate buffer, pH 7.0, containing 1 mM EDTA and were centrifuged at 10000×g for 15 min. Supernatants were deproteinated by adding an equal volume of the MPA reagent (Dissolve 5 g of metaphosphoric acid in 50 mL water) to each sample and mixing by vortexing. The mixture was incubated at room temperature for 5 min and centrifuged at 2500×g for 3 min. The supernatant was carefully collected without disturbing the precipitate. Fifty µL of TEAM Reagent (4M solution of triethanolamine in water) per mL of the supernatant was added, and the mixture was vortexed immediately for assaying. This method measured the total glutathione (GSH+GSSG). Quantification of only GSSG was accomplished by first derivatizing GSH with 2-vinylpyridine. The addition of 10 µL of 2-vinylpyridine solution (1 M of 2-vinylpyridine in ethanol) per mL of the sample was followed by vortexing and incubation at room temperature for 1 hour for GSSG determination. GRS was expressed in sulphur atoms and calculated according to the GSH/(GSH+0.5GSSG) equation.

Prior to lipid hydroperoxide extraction of cells, about 100 mL each of chloroform and methanol was deoxygenated by bubbling nitrogen through the solvents for at least 30 min. The deoxygenated chloroform was stored on ice for extraction of the samples. Approximately 2×10^5 phytoplankton cells were collected by centrifugation. The cell pellets were sonicated in HPLC-grade water. Known volumes of sample were transferred
to glass test tubes. An equal volume of Extract R saturated methanol (weighing 100 mg of solid Extract R provided with the assay kit into a test tube, then adding 15 mL methanol) was added to each tube and vortexed. One mL of cold chloroform was added to each tube and mixed thoroughly by vortexing before centrifugation at 1500×g for 5 min. The bottom chloroform layer was collected by carefully inserting a Pasteur pipette along the side of the test tube and transferred to a new test tube on ice prior to assaying.

6.3.6. Total RNA Isolation and Reverse Transcription

About 80 mL of three replicates of *D. brightwellii* and *H. triquetra* samples, which were exposed to crude oil for 12 and 24 hours at 4 mg/L crude oil concentration, and control groups were collected for RNA extraction. Samples were centrifuged at 15,000 rpm for 10 min to collect cell pellets. The pellets were used for RNA extraction, which was performed using the Qiagen RNeasy® Mini Kit (50) according to the manufacturer's protocol. Each 2 μg of RNA was treated with RQ1 RNase-free DNase I (Promega) according to the manufacturer's instructions. DNase-treated RNA was transcribed to cDNA using an oligodT primer by the SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturer’s instructions.

6.3.7. Quantitative PCR

Quantitative PCR was performed with an Applied Biosystems ViiA7 real-time PCR system using SYBR Green detection chemistry (Applied Biosystems) and gene-specific primers. Amplification reactions were performed with 10 μL of Master Mix (Applied Biosystems), 0.5 μM each primer, and 4 μL of 1:10 diluted cDNA in a final volume of 20 μL. Samples were loaded in triplicate on 96-well optical reaction plates
prior to real-time PCR. The reaction conditions were as follows: 1 min at 95 °C, followed by 40 cycles with 15 s at 95 °C and 1 min at 60 °C. Amplicon melting temperatures were determined to ensure product specificity. Transcript abundance data were evaluated using Q-Gene (Muller et al., 2002), which takes into account the amplification efficiencies of target and reference genes. Actin was used as an internal control, and the relative expression levels of the genes were computed by the $2^{ΔΔCT}$ method of relative quantification (Livak and Schmittgen 2001).

**6.3.8. Statistical Analysis**

Three replicates, unless otherwise stated, were used in each experiment, and all data were expressed as mean values (95% CI). The figure for the end point was run as the mean ± SD. Results from different treatments were compared statistically using SigmaStat 12.3 software (Systat Software, Inc., San Jose, CA, USA). ANOVA and t-test were performed to evaluate significance of individual differences with a probability threshold of 0.05, followed by a post-hoc Tukey test.

**6.4. Results**

Crude oil had an inhibitory effect on the growth of both *D. brightwellii* and *H. triquetra* (Figure 6.1). The results in cell density obtained over 4 days of experiments demonstrated that both species had similar responses to crude oil exposure at 1- and 4-mg/L crude oil concentrations. At a concentration of 1-mg/L, neither species showed significant differences in cell density compared to the control groups. Also, exposure to 4-mg/L crude oil moderately inhibited growth of both species. However, the response at 8-mg/L crude oil exposure was significantly different between the two species. While 8-
mg/L crude oil exposure caused complete growth inhibition on *H. triquetra*, *D. brightwellii* showed only moderate growth inhibition at this concentration (Figure 6.1).

There was no significant difference in cellular Chl *a* content between the groups exposed to crude oil and the control groups (ANOVA: n=6; *p*=0.264 and *p*=0.657 for *D. brightwellii* and *H. triquetra*, respectively). Although Chl *a* content was not induced by the addition of crude oil in this study, a number of parameters indicated that cell stress was induced upon addition of the crude oil. SOD activity was analyzed as a parameter of...
antioxidant response. For both species, SOD activity increased with increasing crude oil concentrations (Figure 6.2). However, at 4-mg/L for both species, SOD activities showed a tendency to drop to baseline towards the end of the experiment. At 8-mg/L, SOD activities increased significantly compared to the control groups for both species at 12 hours, then slightly dropped and maintained their level throughout the experiment.

![Graph showing SOD activity over time for D. brightwellii and H. triquetra under different concentrations of crude oil exposure.](image)

Figure 6.2. Superoxide dismutase (SOD) activity in *D. brightwellii* and *H. triquetra* under different concentrations of the crude oil exposure. Data points are averages of replicate samples (n=4) ± SD.
Figure 6.3 shows that total glutathione pools were significantly ($p<0.05$) altered by exposure to 8 mg/L of crude oil in both species. In addition to the highest concentration (8-mg/L), 4-mg/L crude oil also induced the total glutathione pool significantly ($p<0.05$) in *H. triquetra*.

Similar to the total glutathione pool, the GSSG pool was enhanced ($p<0.05$) by 4- and 8-mg/L crude oil exposure compared to amounts for the control and 1-mg/L treatments for both species (Figure 6.4). Overall, *H. triquetra* demonstrated a higher
sensitivity to crude oil exposure ($p<0.05$) in terms of total glutathione and GSSG activity than *D. brightwellii*. Total glutathione and GSSG levels at 8-mg/L for *D. brightwellii*

Figure 6.4. Oxidized glutathione (GSSG) levels in *D. brightwellii* and *H. triquetra* under different concentrations of the crude oil exposure. Data points are averages of replicate samples (n=4) ± SD.

showed a tendency to return to the same level as the control group after 48 hours exposure. The increasing SOD activities and total glutathione and GSSG levels indicate that active oxygen production was stimulated by the addition of high crude oil concentration. Hydroperoxide concentrations in *D. brightwellii* did not change significantly by crude oil exposure; however, exposure of *H. triquetra* to 8-mg/L crude
oil led to a significant ($p<0.05$) increase in hydroperoxide (Figure 6.5). This hydroperoxide stimulation in *H. triquetra* suggests that lipid peroxidation could also be enhanced by crude oil exposure in this phytoplankton.

Figure 6.5. Hydroperoxide concentration changes in *D. brightwellii* and *H. triquetra* under different concentrations of crude oil exposure. Data points are averages of replicate samples (n=4) ± SD.

Observing changes in transcript levels for appropriate genes can indicate early stress responses. Quantitative reverse-transcription PCR (qRT-PCR) was used to measure
the transcript abundance of genes encoding heat shock proteins, ascorbate peroxidase, a photosystem II component, and a nitrate transporter. Comparisons of transcript abundance did not indicate any significantly different up- or down-regulation in the genes examined compared to controls under 4-mg/L crude oil exposure (Figure 6.6).

![Figure 6.6](image_url)

Figure 6.6. Transcript abundance for select genes in *D. brightwellii* and *H. triquetra* in response to crude oil. Relative transcript abundance is shown for samples prepared from phytoplankton cultures exposed to 4-mg/L crude oil for 0, 12, or 24 hours. The values represent transcript abundance of genes normalized to control samples. Actin was used as a reference gene. Error bars indicate the standard error of biological samples performed in triplicate (total n=6).

6.5. Discussion

In this study, growth response of *D. brightwellii* and *H. triquetra* over 4 days of exposure to crude oil varied remarkably at the highest concentration (8-mg/L) of crude oil. Previous studies indicated that crude oil tolerance could vary significantly between different phytoplankton species (e.g., Harrison et al. 1986, NRC, 2005, Huang et al. 2010,
Gonzalez Jose et al. 2013). Chapter 2 clearly shows that at a longer period, the growth responses of these two species were highly different when exposed to the crude oil. During a 10-day period, while *D. brightwellii* showed high tolerance to the crude oil, *H. triquetra* was dramatically affected. The increase of crude oil concentration was not accompanied by a change of the Chl *a* content in cells of *D. brightwellii* and *H. triquetra*. Previously, some studies (Tukaj 1987, Koshikawa et al. 2007, Sargian et al. 2007, Aksmann and Tukaj 2008) reported changes in chlorophyll pigments as a target for crude oil toxicity in phytoplankton. While Koshikawa et al. (2007) and Aksmann and Tukaj (2008) reported that there was no significant effect of crude oil in Chl *a* content; Tukaj (1987) and Sargian et al. (2007) observed some changes in Chl *a* content of phytoplankton under crude oil exposure. Sargian et al. (2007) used ultraviolet-B radiation with crude oil exposure so the measured impact of the two was synergistic. It is difficult to deduce what the sole impact of crude oil to cellular Chl *a* content may be, since the authors reported that ultraviolet-B radiation alone causes significant changes in Chl *a* content. In the study by Tukaj (1987), there was no reported quantitative crude oil concentration that could provide a basis for comparison to our conditions. It is possible that the crude oil concentrations used were quite different than those we used in our study.

A very similar response of SOD activities between *D. brightwellii* and *H. triquetra* suggests that these two phytoplankton species were initially impacted by crude oil in a similar way. Activity values for SOD were of the same order of magnitude reported in previous studies (Rijstenbil Jan 2001, Wang and Zheng 2008) despite the use of different xenobiotic stressors. Since this study is the only study that reports the
oxidative stress enzyme responses of phytoplankton subject to crude oil exposure, it does not allow for the comparison of enzyme activity levels under crude oil exposure reported in previous literature. Observation of the increased SOD activities only at 12 hours suggests that O$_2^\cdot$ production took place at an early stage of the exposure, and then detoxification continued throughout the experiment, particularly for the 4-mg/L crude oil exposure. Since the growth of *D. brightwellii* continued at a reduced rate at 4- and 8-mg/L crude oil exposure, it suggests that resulting oxidative stress by ROS could not reach the threshold value for irreversible damage to the cells. While growth rates for *D. brightwellii* seemed to be similar at 4- and 8-mg/L crude oil exposure, SOD activities showed highly distinctive responses that suggest that *D. brightwellii* could be a bioindicator organism to indicate oxidative stress due to crude oil exposure. The effect on *H. triquetra*’s growth response was clearly discernable after 48 hours at 4- and 8-mg/L crude oil exposure, and SOD activity levels were distinctive after that time point as well. Reduced O$_2^\cdot$ levels after 48 hours may have caused the continued growth at 4-mg/L crude oil exposure for this species. Increasing activity of antioxidant enzymes can be expected to reduce oxidative stress to algal cells. However, highly elevated O$_2^\cdot$ levels at 8-mg/L crude oil exposure ceased growth of this species and suggests that the threshold level for irreversible damage for *H. triquetra* was reached.

In both algal species, the activity of SOD, which converts O$_2^\cdot$ into H$_2$O$_2$, increased with increasing concentration of crude oil. However, the increase of SOD alone cannot ease the burden of ROS in the cells. Resulting H$_2$O$_2$ due to dismutation of O$_2^\cdot$ is a strong oxidant that rapidly oxidizes thiol groups and accumulates in excess within organelles such as chloroplasts, where photosynthesis depends on thiol-regulated
enzymes, causing potential harm to cells (Noctor and Foyer 1998). Even though H$_2$O$_2$ has more oxidizing power than superoxide, it is biologically less toxic (Miwa et al. 2008). Yet, it must be sequestered by the action of other enzymes such as peroxidases (PODs) and/or CAT enzymes (Cirulis et al. 2013). In addition to these enzymes, GPx, which is one of the major reductants for some of the peroxidase enzymes, takes an active role in detoxification. GPx enzymes catalyze the reduction of H$_2$O$_2$ to water and molecular oxygen using cellular glutathione as the reducing agent (Kühn and Borchert 2002). The profile of total glutathione (GSH+GSSG) and GSSG levels showed similarities to SOD activities. Both 4- and 8-mg/L crude oil exposures caused highly significant enhancement of GSH+GSSG and GSSG levels. At this point, it is essential to discuss which part of our glutathione pool data should be used as an indicator of oxidative stress in cells, because the link between glutathione pool alteration and oxidative stress has evolved over time. Previous studies have proposed that rates of glutathione synthesis increase in response to increased H$_2$O$_2$ levels (Smith IK et al. 1984, Smith Ivan K 1985). But it is well known that in healthy cells, more than 90% of the total glutathione pool is in its reduced form and that the GSH:GSSG ratio is typically high, greater than 10:1 (Stegeman et al. 1992, Mittler et al. 2004). The existence of effective feedback mechanisms for the maintenance of GSH levels in response to contaminant-induced effects may mean that GSH levels alone are not useful as biomarkers of oxidative stress (Stegeman et al. 1992). GSH can be regenerated from GSSG by the action of glutathione reductase (GR). A key characteristic of the cellular GSH pool is its high reduction state due to GR, which is constitutively active and inducible upon oxidative stress (Jozefczak et al. 2012). Thus, under stress conditions, oxidation of GSH to GSSG would decrease GSH levels (Smith IK et al. 1984).
and, subsequently, the level of GSSG increases (Noctor and Foyer 1998). The measurement of elevated GSSG levels, however, suggests that the hepatic GSH:GSSG ratio may be a potential biomarker for oxidative stress (Van der Oost et al. 2003, Ballatori et al. 2009). However, in consideration of excess GSSG in the cell since GR may partially reduce GSSG to GSH during active oxygen production, in more recent years \( \text{GRS} = \frac{\text{GSH}}{\text{GSH} + 0.5 \times \text{GSSG}} \), is therefore commonly used as biochemical measure of oxidative stress (Zenlinski et al. 1999, Rijstenbil Jan 2001, Schafer and Buettner 2001, Rijstenbil JW 2002). At this point, neither GSH+GSSG nor individual GSSG and GSH contents may be used as a proxy for the presence of oxidative stress in cells in this study. To indicate the cells’ health, the fraction of GSH in the total glutathione pool was calculated. The results varied between 85% and 95%, except for \( H. \ triquetra \) exposed to 8-mg/L crude oil, which had values that varied between 65% and 84%. GRS values (Figure 6.7) also shows that only a significant reduction was observed for \( H. \ triquetra \) at 8-mg/L crude oil exposure. It was the only treatment that showed no growth in this study. Chapter 2 showed that \( H. \ triquetra \), at 8-mg/L crude oil exposure, showed complete growth inhibition, and all cells died in a 10-day period of crude oil exposure. Thus, GRS values could potentially be used as a stress indicator for cells that were seriously affected by crude oil exposure and had experienced irreversible damage. Unlike \( D. \ brightwellii \) (enhanced SOD activity; steady GRS levels), a reduced level of GRS values for \( H. \ triquetra \) at 8-mg/L crude oil exposure at the end of the experimental period suggested that \( H. \ triquetra \) cells were not able to adapt to the crude oil exposure conditions.
Figure 6.7. Glutathione redox state, GRS=GSH/(GSH + 0.5GSSG) values in D. brightwellii and H. triquetra under different concentrations of crude oil exposure. Data points are averages of replicate samples (n=4) ± SD.

Lipid peroxidation is often used as an indicator of the effect of ROS-generated oxidative damage (Lushchak 2011). It usually occurs when above-threshold ROS level are reached. Commonly, measuring end products from the degradation of polyunsaturated fatty acid hydroperoxides, such as malonic dialdehyde (MDA) and 4-hydroxy nonenal (4-HNE), are the most widely used assays to monitor lipid peroxidation (Janero et al. 1990, Esterbauer Hermann et al. 1991, Pedrajas et al. 1995, Roméo et al. 2003). However, in this study, hydroperoxides were directly measured using the ferric thiocyanate assay.
(Mihaljevic et al. 1996), which relies on the measurement of ferric ions generated during the reaction of ferrous ion with hydroperoxides. Even though the kit manufacturer claims that ferric ions present in seawater samples are not a source of error, any such errors are easily circumvented by performing the assay in chloroform. The interaction of other trace metals in seawater with these processes is still unknown. The increase in hydroperoxide levels at the 8-mg/L crude oil treatment for *H. triquetra* suggests that antioxidant enzymes induced by the crude oil may not be able to completely eliminate ROS within a short period of time and may cause further damage to pigments, proteins (e.g. Rubisco), nucleic acids, lipid membrane damage, and cell lysis.

Monitoring changes in transcript abundance may allow for the detection of early and/or sensitive stress responses. In the current study, a number of genes were selected as being potentially associated with physiological stress factors. Several genes failed to be reliably detected by the qRT-PCR experiments (data not shown). This could be due to low expression levels in the samples examined or sub-optimal reaction conditions. The genes that could be accurately tested include those that encode heat shock proteins (*Hsp70* and *Hsp90*), an ascorbate reductase (*APX*), a photosystem II component (*psbC*), and a nitrate transporter (*NRT*). The comparison of the relative expression of these genes to those of the control groups provided circumstantial evidence that these genes may not be specifically impacted by crude oil exposure in *D. brightwellii* and *H. triquetra*. Even though heat shock proteins have potential as biomarkers of exposure to environmental contaminants on phytoplankton (Torres et al. 2008), the concentration and duration of crude oil exposure used in this study did not induce heat shock protein genes significantly. For some of the genes examined, relatively high variations in transcript
abundance between replicates were observed. The reason may be due to the culture conditions, since collection of non-homogeneous cultures for RNA extraction was possible. Even if cultures used for the experiments were kept under the same conditions, rapid reproduction rates of the phytoplankton may have caused maturity levels for individuals to be reached at different time points, allowing for different sensitivities to the crude oil exposure. The lack of data in the literature about gene expression of phytoplankton under crude oil exposure limits the comparison of our data to any previous study. The closest study was conducted by Bopp and Lettieri (2007), and indicates that PAHs (pyrene, fluoranthene, and benzo[a]pyrene) strongly influenced only select genes involved in fatty acid metabolism and silica shell formation in phytoplankton. Interestingly, the genes involved in photosynthesis were not influenced by PAHs either.

This study provides the first line of evidence that enhanced SOD, glutathione pool activity, and hydroperoxide content are indicative of the ability of biologically active crude oil to promote ROS production, oxidative stress, and lipid peroxidation in phytoplankton. Particularly, distinct responses of these two phytoplankton species in terms of glutathione pool activities and lipid peroxidation suggests that each species has different tolerance levels to crude oil, as confirmed by the different growth responses at high crude oil concentrations. Even though some literature on subcellular response of microalgae to PAH contamination exists (Aksmann and Tukaj 2008, Chen et al. 2008, Wang and Zheng 2008), little attention has been given to subcellular investigations of crude oil toxicity on phytoplankton, making it difficult to link our data to any other damage potentially initiated by ROS, such as damage to nucleic acids, lipids, proteins, and DNA. The exact mechanism of toxicity whereby crude oil inhibited phytoplankton
population growth is unknown. Thus, further investigation of damage in cells as the result of oxidative stress is needed to complement the current study.

6.6. Conclusions

This study shows that antioxidant enzymes provide protection to phytoplankton species only if crude oil concentration is low. The data suggests that Chl a content in cells is not affected by crude oil exposure. The results indicate that high crude oil exposure induces oxidative stress in phytoplankton species. Since SOD is an O$_2^-$ scavenger, and glutathione plays an important role in H$_2$O$_2$ detoxification, observed changes suggest prolonged and increased levels of ROS production when cells are subject to a 4-day crude oil exposure. The antioxidative defense system was strongly activated, mainly through the activation of SOD. *D. brightwellii* and *H. triquetra* showed distinct responses to crude oil exposure. Evidently, *D. brightwellii* more effectively eliminates excess ROS through its first line of antioxidative defense mechanism than *H. triquetra*, which further serves as a sign of oxidative stress under the crude oil exposure. The gene expression study indicated that though such enzymes activities were triggered at 4-mg/L crude oil exposure, the same concentration of crude oil did not cause significant difference in expression levels of the selected genes between the control group and samples treated with crude oil.

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CHAPTER 7: SUMMARY AND SYNTHESIS

7.1. Overall Summary and Implications

With this dissertation work, we have aimed to find answers to many questions concerning the role and effects of crude oil on coastal ecosystems and on phytoplankton at individual, population, toxin-production, and sub-cellular levels. Though there is a long road ahead in determining the greater effects of crude oil, our research has revealed several important points that will contribute to this understanding. They are summarized as follows.

Before discussing the effects of crude oil on phytoplankton, it is useful to understand how crude oil behaves in the marine environment under different conditions such as physical and chemical perturbations. The detailed crude oil analysis in described in Chapter 2 demonstrates how a lot of surface oil could enter the water column and become bioavailable to phytoplankton. Physical mixing of the water column does not significantly change the amount of TPH; however, it does cause a significant increase in specific groups of compounds, such as alkanes and PAHs, in the water column. This suggests that the groups of compounds that comprise crude oil behave differently under physical mixing of water column. While dissolution and dispersion of some groups increase, others had the opposite reaction. The addition of the dispersant (at 1:100 and 1:20 dispersant:oil ratios), Corexit® EC9500A, greatly increases crude oil concentrations (ca. 50 fold), and these increases are similar regardless of the dispersant:oil ratio, suggesting that a 1:100 ratio is as efficient as a 1:20 ratio to disperse the oil load (25 g/L) used in this study. Since it is evident that the addition of the dispersant causes a much
larger deposit of oil into the water column than any physical mixing potentially does, in
the event of an oil spill in the marine environment, the impact of chemical disturbances
should be more seriously considered than the effects of physical disturbances. As the
bioavailability of oil to phytoplankton is increased, these effects question whether or not
the use of dispersants in the field is the best choice.

The effects of physically and chemically enhanced crude oil on individual species
were also determined. Comparison of the sensitivities of five individual phytoplankton
species revealed that diatoms are more tolerant to crude oil than dinoflagellates. The
growth stimulation of dinoflagellates at low crude oil concentrations (<1200 ppb TPH),
however, makes them more likely to survive than diatoms in such conditions. This
indicates that concentrations of crude oil not only determine the degree of toxicity on
phytoplankton, but also determine which group can better survive. Larger species of
diatoms and dinoflagellates showed greater tolerance than the smaller species used in this
study. The concept of phytoplankton size influencing tolerance to crude oil has been
discussed previously in the literature. The findings presented in Chapter 2 indicate that
while size does matter, the taxonomic group seems to be a more predominant criterion in
the prediction of phytoplankton tolerance to crude oil. Though our experiments revealed
that Corexit® EC9500A increases the amount of crude oil and that phytoplankton have
very low tolerance to the dispersant, the data did not allow EC₅₀ values of Corexit®
EC9500A for each species to be precisely predicted. Instead, the EC₅₀ value of Corexit®
EC9500A was found to be <100 ppm for all species studied.

Highly altered responses of phytoplankton species in a five-species community
compared to the response of monocultures observed in Chapter 2 underline the necessity
of impact assessment of crude oil at the population level. Overall, the findings presented in Chapter 2 contribute substantially to understanding the nature of crude oil and the dispersant, the concentration ranges of crude oil that are toxic to phytoplankton groups, and the differences in responses at the individual and community levels.

Chapter 2 indicates that PAHs have a primary role in the crude oil toxicity to phytoplankton. The following question arose. Can reliable predictions of the aquatic toxicity of crude oil, a multi-component mixture, be derived from toxicity data on individual PAH compounds? Naphthalene, the most abundant PAH compound, and benzo(a)pyrene, a highly toxic PAH compound, were selected in Chapter 3 as model compounds to quantify toxicity of crude oil on two phytoplankton species, *Ditylum brightwellii* and *Heterocapsa triquetra*, by analyzing the effects of different concentrations of these PAHs on growth rate. EC$_{50}$ values suggest that the diatom *D. brightwellii* is more vulnerable to both toxicants than the dinoflagellate *H. triquetra*. However, as seen in Chapter 2, the diatom *D. brightwellii* is less vulnerable to crude oil than the dinoflagellate *H. triquetra*. This suggest that naphthalene and benzo(a)pyrene may not be solely used as surrogates to assess crude oil toxicity on phytoplankton. In general, toxicity data from laboratory tests with single, pure chemicals provide essential input to scientific assessments of chemical risks to aquatic ecosystems. However, as shown in this study, the behavior of chemicals in a mixture may not correspond to data on the pure compounds. Besides the fact that directly testing all of the potential combinations of crude oil components is not feasible, we are confronted with the task of deriving valid predictions of multiple mixture toxicity based on data on two
toxic individual compounds. Therefore, a great deal of attention must be paid to combined exposure when assessing risk characterization of mixtures at individual, population, and ecosystem levels.

In Chapter 4, population-level effects of crude oil were investigated under oligotrophic and eutrophic conditions in order to assess the impact of high-nutrient input on the toxic effects of crude oil, Corexit® EC9500A, and chemically dispersed oil on phytoplankton composition. Diatoms show the greatest tolerance to crude oil exposure under every condition that was assessed. Succession of diatoms was also observed in Chapter 2, at individual-level responses, so the expectancy of an increase in diatom population during and post the Deepwater Horizon oil spill in the GoM have been raised. Different diatom groups also have distinct responses under different nutrient regimes. While pennate diatoms show greater tolerance to crude oil under nutrient-enriched conditions, centric diatoms demonstrate higher tolerance to crude oil under unenriched conditions. This collectively suggests an increase in the pennate diatom population after the spill in coastal GoM areas, where high nutrient concentrations are observed. Similarly, centric diatom dominancy can be expected in oligotrophic GoM waters during and post oil spill. Crude oil toxicity and nutrient deficiency jointly inhibit phytoplankton growth due to the synergistic effect of stress from toxicity and high competition for very limited nutrient resources. When phytoplankton are exposed to crude oil under nutrient rich conditions, they show greater tolerance compared to exposure to crude oil in conditions that are initially nutrient limited and have nutrients added later. This suggests that crude oil toxicity cause damage to phytoplankton at the early stages of exposure when nutrients are not available to be properly utilized.
In Chapter 5, crude oil’s impact on the ecologically important GoM toxic phytoplankton species, *Karenia brevis*, *Prorocentrum minimum*, and *Heterosigma akashiwo*, is addressed. Comparison of their tolerability to that of non-toxic species, as measured by EC50 values in Chapter 2, shows that the toxin production potential of harmful the algal species does not provide a selective advantage. This indicates that crude oil by itself does not promote community shifts from non-toxic species to toxic species in the GoM. Toxin production profiles of *Karenia brevis* and *Prorocentrum minimum* are altered with varying crude oil concentrations, thus, making the determination of crude oil concentrations during harmful algal blooms in the GoM during and post oil spills important in getting a better impact assessment, especially the assessment of shellfish bed closures due to a high risk of increased toxin production.

Upon completion of impact assessments at individual, population, and toxin production levels, impacts were evaluated on chlorophyll *a* content, antioxidative defense system, lipid peroxidation, and changes in the transcript level of the genes of the marine phytoplankton in Chapter 6. The activation of reactive oxygen species in phytoplankton exposed to crude oil was shown. Although oxidative stress under crude oil exposure was present, whether there is progressive damage in cells is still unknown. The gene expression study indicates that crude oil does not cause a significant difference between the control group and samples treated with crude oil in expression levels of the selected genes. These findings partially reveal subcellular activities of phytoplankton under crude oil exposure and contribute to scientific literature on how crude oil works in phytoplankton. Overall, this dissertation work provides data on the influence of LSC on phytoplankton at individual, population, toxin production and subcellular levels. Even
though the main research focus was crude oil impact, the impact of chemical dispersant, Corexit® EC9500A, and of the dispersed oil were studied. This research provides essential data for impact assessment of oil spills and pollution on phytoplankton ecology and bloom dynamics in the GoM. These datasets contribute substantially to existing scientific knowledge in the region and provide baseline information for subsequent research efforts that seek to further elucidate the impact of oil on the marine planktonic ecosystem in the GoM.

7.2. Current Limitations and Future Prospects

Phytoplankton populations typically have rapid regeneration rates and usually show patchy distribution. Thus, even after a 100% mortality resulting at the site of a spill, it would be difficult to demonstrate the significance of such effects on other locations unless the patchy distribution is well understood. Populations may be conveyed rapidly to adjacent unaffected waters. Therefore, the phytoplankton population can be expected to rebound to pre-spill levels rapidly once the contamination levels have lessened. It is highly essential to determine recovery rates of phytoplankton that are already exposed to crude oil in the marine environment. Which concentrations of crude cause irreversible effects, and which concentrations cause inhibitory effects from which phytoplankton can easily recover once the contamination levels are lessened are two main questions that warrant attention.

Maintaining a known history of environmental conditions and of the phytoplankton being studied is a critical aspect of hazard assessment. Research groups collect phytoplankton data to establish baseline conditions and examine the dynamics of the phytoplankton community over seasonal, annual, and decadal time scales, and future
perturbations to pelagic ecosystems can be assessed if/when such impacts occur. Even in a chronically polluted aquatic system, the spatial and temporal variability in plankton often makes the detection of subtle effects in the field extremely difficult, if not impossible. Phytoplankton populations can be altered quickly on small temporal and spatial scales; thus, it can be difficult to predict how a phytoplankton community as a whole will respond to an oil spill over time (and space). Many environmental factors affect phytoplankton composition and the degree of crude oil bioavailability to phytoplankton. The weathering of crude oil can affect its toxicity, and the presence of a dispersant can make the oil more readily available to phytoplankton. Furthermore, physical processes in water column also affect crude oil distribution and bioavailability. Phytoplankton composition in the marine environment are affected by many other factors such as temperature, salinity, light, nutrient regimes, and grazing. This makes it difficult to discount the importance of environmental dynamics as a factor of crude oil toxicity to phytoplankton. A large-scale modeling study that takes environmental dynamics into account would compliment this dissertation work.

Phytoplankton are not isolated from other organisms in the marine environment, so such an impact by crude oil cannot be overlooked in the presence of zooplankton and bacterial communities. Due to the close coupling among these organisms, the impact of crude oil should also be assessed on bacterial and zooplankton population. Crude oil biodegradation by bacteria and the resulting byproducts are not fully understood. The extent of the impact of these byproducts on phytoplankton needs attention as well.
Similarly, the response of zooplankton communities to crude oil should be well documented to better understand potential grazing pressure on phytoplankton under crude oil exposure.

Last but not least, while there have been many laboratory-based studies examining the toxicity of crude oil and its various components (in the presence/absence of dispersants and under varying environmental conditions), much work remains to be done in terms of field-based (in-situ) studies to better understand how phytoplankton will respond to an oil spill and how to assess its subsequent impacts on the community. Inconsistent analytical measurements and biological methods have created a diverse pool of data that does not easily allow for cross comparison. Standardization of oil toxicity tests in the aquatic environment and laboratory studies is required in order to obtain reproducible data and to eliminate significantly different test results, inconsistent analytical measurements, and inconsistent preparations of the exposure medium.
APPENDIX

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Koray Özhan was born in Istanbul, Turkey, and he grew up in Mersin, a lovely city on the Mediterranean coast. He earned a Bachelor of Science degree in chemistry education in 2003 from Middle East Technical University, one of the top universities in Turkey. Koray then moved to the United States and worked as a chemist at a pharmaceutical company in Danbury, CT, where he investigated and developed new methods and technologies for drug product advancement for diabetics and performed many different instrumental analyses on a variety of proteins and organic compounds. He also pursued an advanced degree in chemistry in Southern Connecticut State University in 2008. His research focused on the synthesis and characterization of novel molecules as ligands for the preparation of metal organic framework materials. Koray was awarded the most outstanding graduate student in the chemistry department in 2010 for his efforts.

A goal to have an academic position in a department related to environmental science formed. For his doctoral research at Louisiana State University (LSU), Koray has focused on impacts of Deepwater Horizon oil spill on the Gulf of Mexico phytoplankton species. He was awarded a dissertation-year fellowship, an achievement granted to only 12 graduate students at LSU each year, to support his studies during the final academic year. Furthermore, he was selected the 2013 awardee of “Dr. Theodore Ford Scholarship,” a highly distinguished award from the Department of Oceanography and Coastal Sciences at LSU.