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Effects of Deepwater Horizon Oil on the Growth Rates and Pigment Composition of Phytoplankton Isolated From Grand Isle, LA

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EFFECTS OF DEEPWATER HORIZON OIL ON THE GROWTH RATES AND PIGMENT COMPOSITION OF PHYTOPLANKTON ISOLATED FROM GRAND ISLE, LA

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ABSTRACT

This research focused on the effects of un-weathered Macondo crude oil on the growth rates and pigment ratios of phytoplankton isolated from Grand Isle, LA. The experiments involved incubating nutrient-enriched artificial media containing a range of oil concentrations up to 19.2 ppm with small aliquots of coastal water from Grand Isle and measuring the growth rates of the phytoplankton during the subsequent 10–14 days and the pigment ratios of the phytoplankton at the end of log-phase growth to determine whether the crude oil affected the growth rate of the phytoplankton and their composition in terms of pigment ratios. Pigment analysis revealed that the cultures consisted almost entirely of diatoms. Results showed that there was a significant effect on diatom growth rates from the concentration of crude oil, the month, and the interaction between oil concentrations and months. In March, April, and May, growth rates at oil concentrations from 0.1 to 0.6 ppm were about 10% higher than controls. At higher oil concentrations there was a negative correlation between oil concentrations and growth rates. The ratio of fucoxanthin to chlorophyll *a* was positively correlated with oil concentrations in February, March and April, the implication being that the size of the diatom photosynthetic units changed in response to the oil. There was no significant correlation between growth rates and oil concentrations in July and August, nor was there any correlation between pigment ratios and oil concentrations. The July and August phytoplankton grew roughly twice as fast as the March, and April control cultures, although the growth conditions were identical. Analysis with a mathematical model of phytoplankton growth suggested that in the presence of oil, there was a transfer of resources within the March, April, and May phytoplankton, the result being an increase in the size of their photosynthetic units and a

decrease in the number of photosynthetic units in response to the presence of oil. The principal antenna pigment was fucoxanthin. The phytoplankton isolated in July and August, in contrast, appeared to be completely unaffected by the presence of oil up to a concentration of 19.2ppm.

CHAPTER 1. BACKGROUND

1.1 Brief history

Crude oil is produced deep underground under conditions of elevated temperature and pressure as the result of geological and geochemical processes that transform organic matter incorporated into sedimentary rocks (National Research Council 2003, p. 19). Crude oil is composed of dozens of major hydrocarbons compounds and thousands of minor ones, including alkanes, cycloalkanes, aromatics, and polar compounds. Aromatic compounds include at least one benzene ring, which is very stable and thus persistent in the environment. Polycyclic aromatic hydrocarbons (PAHs) contain at least two benzene rings. Most crude oils contain 0.2–7% PAHs. Some related aromatic compounds contain elements such as sulfur and nitrogen. PAHs, including such compounds, are considered to be more toxic than the other compounds in crude oil.

Global crude oil production currently amounts to about 1.3×10^{12} gallons y^{-1}, equivalent to 4.1×10^{9} tons y^{-1}. Crude oil production raised more-or-less exponentially from 1930 to 1975, but since then has averaged $3–4 \times 10^{9}$ tons y^{-1}. Not surprisingly, discharges of oil to the marine environment as a result of human activities have inevitably occurred from time to time from tankers and oil wells.

The record of oil tanker accidents prior to and subsequent to 1990 is provocative (Figure 1.1.1). During the 1970s the oil discharged to the ocean as a result of tanker accidents averaged 317,000 tons y^{-1}. From 2000 to 2009 those same discharges averaged 21,000 tons y^{-1}. The largest tanker-related spill occurred in 1979, when two full supertankers collided with one another during stormy weather off the coast of Tobago in the Caribbean Sea. An estimated 88

million gallons of crude oil were discharged into the ocean from the damaged tankers. The most severe tanker accident in U.S. coastal waters was undoubtedly the Exxon Valdez spill in March 1989. In that case the tanker inexcusably ran aground on Bligh Reef in Prince William Sound, Alaska, and about 11 million gallons of the 55 million gallons of crude oil on board were released into Prince William Sound before the remainder of the cargo could be offloaded. Despite the relatively small amount of oil released as a result of the Exxon Valdez accident, the legal fallout was considerable, and included passage of the Oil Pollution Act of 1990 by the 101st United States Congress.

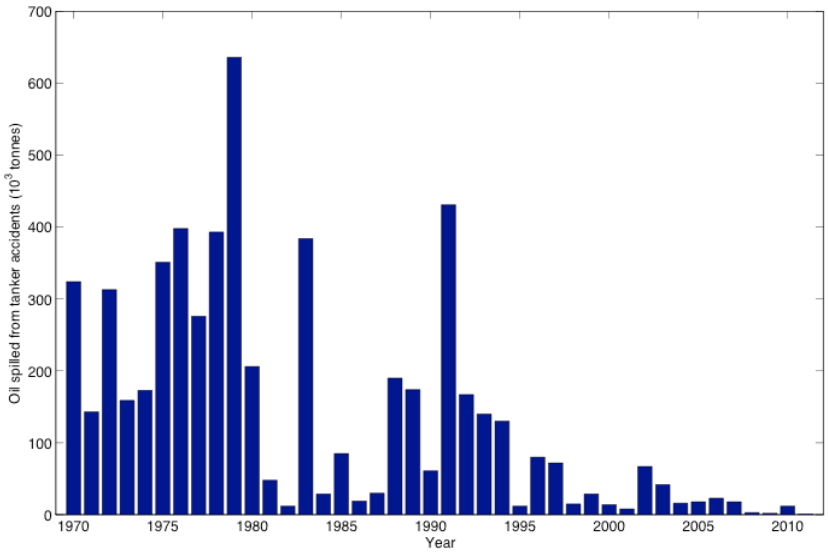

Figure 1.1.1 Quantity of oil spilled into marine waters as a result of oil tanker accidents from 1970 through 2011 (ITOPF, 2011)

Prior to 2010 the most infamous offshore oil platform accident in U.S. waters was undoubtedly the 1969 Santa Barbara oil well blowout, an accident that released an estimated 3.4–4.2 million gallons of crude oil into the Santa Barbara Channel. The spilled oil killed thousands of sea birds in addition to marine mammals such as dolphins, elephant seals, and sea

lions. Public outrage over the spill was among the factors that contributed to the creation of the U.S. Environmental Protection Agency (1970), the National Environmental Policy Act (1970), the Clean Water Act (1972), and ultimately to passage of the 1978 U.S. Port and Tanker Safety Act. At the time, the Santa Barbara oil well blowout was the largest oil spill to ever occur in U.S. waters, and today it still ranks third behind the 1989 Exxon Valdez spill and the 2010 Deepwater Horizon spill.

The 2010 British Petroleum (BP) Deepwater Horizon (BP/DWH) oil spill was the largest accidental marine oil spill of all time (Joye, MacDonald et al. 2011). It lasted 87 days and leaked between 7.9×10^8 and 1.1×10^9 L (210–290 million gallons) of crude oil from the sea floor into the northern Gulf of Mexico (Sammarco, Kolian et al. 2013). The estimated peak flow was 1.6×10^7 L d^{-1} (Reddy, Arey et al. 2012). The leak affected the water column, the benthos, and commercial seafood (Sammarco, Kolian et al. 2013). The economic fallout from the accident has been considerable. As of February 2013 criminal and civil settlements as well as payments to a trust fund had cost British Petroleum in excess of $42 billion. The oil released from the Macondo well contained 0.039% polycyclic aromatic hydrocarbons (Reddy, Arey et al. 2012).

Occasional spills of large amounts of crude oil into the marine environment usually have serious environment consequences and thus receive much public attention. However, oil pollution of the sea also occurs when small amount of crude oil are released over a long time, the result being the chronic exposure of marine organisms to crude oil (National Research Council 2003, p. 23). Sources of chronic exposure include point sources, such as natural seeps and leaking pipelines. In these cases, there is a large gradient from high to low oil

concentrations as a function of distance from the source (National Research Council 2003, p. 28).

Chronic releases from natural and anthropogenic sources (e.g., natural seeps and run-off from land-based sources) are responsible for the majority of petroleum input to both North American waters and the world's oceans (National Research Council 2003, p. 65). A reasonable seep rate for the entire Gulf of Mexico is 140,000 tons per year (ranging from 80,000 to 200,000 tons per year), assuming that the magnitudes of the seeps scale in proportion to the surface area. Based on this information, the annual global oil seepage rate is estimated to be between 200,000 and 2,000,000 tons y^{-1} (National Research Council 2003, p. 192). In recent years, the long-term effects of acute and chronic oil contamination have received increasing attention (Boesch, Butler et al. 1987).

1.2 Environmental effects

After oil enters the ocean through spills or chronic releases, it eventually breaks down or is removed from the marine environment by a series of physical, chemical, and biological processes collectively known as weathering, or is diluted to levels below concern (Sun Peiyan et al. 2007). Physical processes involved in crude oil weathering include evaporation, emulsification, and dissolution; chemical processes involve oxidation. The principal biological process that affects crude oil in the marine environment is microbial oxidation. As crude oil weathers, it may also undergo various transport and transformation processes, including advection and spreading, dispersion and entrainment, sinking and sedimentation, and stranding. These processes lead in some cases to tar ball formation (National Research Council 2003, p. 90). However, oil is potentially toxic from the time it enters the environment until it is removed,

or until its concentration is diluted to insignificance. The solubility of crude oil in water is usually less than 100 ppm (National Research Council 2003, p. 21). The water-soluble fractions of the oil are sometimes the most toxic to aquatic life.

Harm to marine organisms from oil in the ocean falls into two general categories. One is so-called “oiling” and occurs when organisms become coated with oil. Oiling is particularly associated with the high molecular weight components of the oil. Aquatic birds and marine mammals are especially vulnerable to oiling effects if their plumage or pelage respectively becomes matted with oil. As a result they may lose their ability to maintain a proper body temperature and die of hypothermia. Seabirds may literally drown as a result of oiling because the pockets of air within their plumage provide buoyancy, which is lost if their feathers become matted down.

The second general adverse effect is associated with ingestion or uptake of the toxic components of the hydrocarbons in the oil. Crude oil is a complex mixture of chemicals from methane to very large hydrocarbons (>16 carbon atoms) (Bejarano, Chandler et al. 2006).

Toxic effects depend on the oil composition, amounts of spill, spill location, season of the year, the interaction of the introduced petroleum with various processes that subsequently affect the oil, and the sensitivity of the biological community (National Research Council 2003, p. 16). Among the components of crude oil are polycyclic aromatic hydrocarbons (PAHs), which are highly toxic. The more soluble components of the PAHs are rapidly released into the water column after a spill (Gonzalez, Vinas et al. 2006). PAHs remain dissolved in seawater for an extended period of time, even after the insoluble fractions have been removed. The dissolved and refractory PAHs are a direct threat to marine creatures and accumulate in the bodies of

marine organisms (Seymour & Geyer 1992), where they have long-term negative effects on the organisms (Holdway 2002, Peterson, Rice et al. 2003).

1.2.1 Effects on phytoplankton growth

Phytoplankton account for most of the primary production in the ocean and therefore play a crucial role in energy flow and nutrient cycling in marine food chains. The structure and function of marine food webs depends on the organic matter supplied by phytoplankton. Because of the importance of phytoplankton to marine ecology, the effect of oil spills on phytoplankton populations is an important issue. Much previous work has addressed the effects of oil contamination on phytoplankton, both in the laboratory and in the field.

Oil slicks over the sea surface not only limit gas exchange through the air-sea interface, they also reduce light penetration into the water column, and in this way reduce phytoplankton photosynthesis (González, Figueiras et al. 2009). However, the effects of oil on light penetration are generally assumed to be of secondary importance compared to the directly toxic effects of some of the components of the oil on phytoplankton. The toxicities of the different components of crude oil and refined oil products have been compared in numerous studies (D. C. Gordon 1973, Karydis 1979, Liu, Luan et al. 2006). Several investigations have addressed the effect of PAHs on natural phytoplankton assemblages (Kelly, McGuinness et al. 1999, Hjorth, Forbes et al. 2008) or the effects of crude oil (Kusk 1978) and diesel oil on cultured phytoplankton species (Koshikawa, Xu et al. 2007). Differences in the sensitivity of different groups of algae have been reported. The negative effects of the water-accommodated fraction (WAF) of oil have been reported to be greater on coastal phytoplankton than on oceanic phytoplankton (González, Figueiras et al. 2009). The median effective concentrations (EC50) of

crude oil on certain kinds of microalgae have been examined (Liu, Luan et al. 2006, Paixoa, Nascimento et al. 2007, Wang and Zheng 2008). Some scientists have measured algal growth rates as a metric of response to the toxic effects of oil. Growth rates are functions of photosynthesis and respiration rates (D. C. Gordon 1973, Karydis 1979) and can be measured by monitoring cell concentrations (C. Soto 1974, W. M. Dunstan 1975). Some researchers have focused on the distribution of photosynthetic end products, such as proteins, carbohydrates, and lipids (Karydis 1979, Wolfe, Olsen et al. 1999). In other cases, changes in the chlorophyll *a* concentrations (Huang, Jiang et al. 2011) of certain types of microalgae exposed to oil have been measured. Some researchers have investigated in situ changes of phytoplankton chlorophyll and primary production after oil spills in particular areas (Varela, Bode et al. 2006).

The effects of crude oil on algae depend on the concentration of the oil and the species that have been tested. A slight inhibition of photosynthesis of natural phytoplankton communities has been reported by Gordon and Prouse (1973) at oil concentrations ranging from 0.5 to 60 μg/L. In contrast, a slight stimulation of photosynthesis was reported by Karydis (1979) at crude oil concentrations of 100 μg/L. These latter results are consistent with the results reported by Huang et al. (2011). Concentrations of crude oil less than 1.21 mg/L did not reduce the growth of algae; in fact, growth rates increased.

High concentrations of crude oil, however, seem to have a negative effect on photosynthesis. In the experiments of Karydis (1978), crude oil reduced the photosynthetic rates of *Cyclotella cryptica* by 13% compared to controls at crude oil concentrations of 100 mg/L. The effect of crude oil on *Pavlova lutheri* was even greater. A concentration 10 mg/L decreased the photosynthetic rates by 30%, and the rate was reduced by 80% and 90% at crude

oil concentrations of 100 mg/L and 1.0 g/L, respectively. The response of *Skeletonema costatum* was quite different; only the highest concentration, 1.0 g/L of crude oil, inhibited its growth. In the experiments of Huang et al. (2011), high concentrations (≥2.28 mg/L) of oil greatly inhibited phytoplankton growth, and there was a decrease of the chlorophyll *a* concentration and cell density. Mohammady et al. (2005) examined the influence of the aqueous extract of diesel fuel on the growth of the marine eustigmatophyte *Nannochloropsis* (Monallantus) *salina* Hibberd. The observed increase in the concentrations of the pollutant led to a decrease in growth rates as measured by optical density, the maximum effect being observed (33% of control) in 100% aqueous pollutant.

1.2.2 Effects of oil on phytoplankton species composition

Some studies have concerned the effects of oil pollution on the composition of algal communities, or on specific algal groups, such as diatoms. Effects on community composition depend on many factors, including climatic conditions, oil concentrations, the phytoplankton species present in the water, and water chemistry.

Siron, Pelletier et al. (1995) examined the toxicity of dispersed and dissolved crude oil to micro-algal communities in cold seawater during the winter, with the seawater temperature ranging from –1.5 °C (surface ice cover) to 3 °C. The oil concentrations ranged from < 1 mg L^{-1} (dissolved oil) to 44.6 mg L^{-1} (dispersed oil). The algal species composition was quite different in the treatments versus the control. There was an increase of small microflagellate species and a significant decline in the diversity of centric diatoms. Their results showed that small microflagellates dominated in surface seawater heavily contaminated with oil, and within the diatom community, centrics were replaced by pennates. However, their previous research

(unpublished data) demonstrated that in springtime, only oil concentrations higher than 10 mg L^{-1} inhibited algal growth. An unusual case was the diatom *S. costatum*, the numbers of which were greatly suppressed during the winter experiment, but were less affected than other diatom species in the springtime.

The research of Huang et al. (2011) also showed that the toxic effects of oil on algae varied seasonally. They studied natural phytoplankton collected from Yueqing Bay, China. Their results showed that in all four seasons the biodiversity, richness, and number of species in the samples were all significant functions of the concentrations of the water-accommodated fraction (WAF) of the crude oil ($p< 0.001$). The dominant species changed with the seasons, and different species showed different tolerances to the oil.

CHAPTER 2. THESIS RESEARCH

2.1 Characteristics of the sampling site

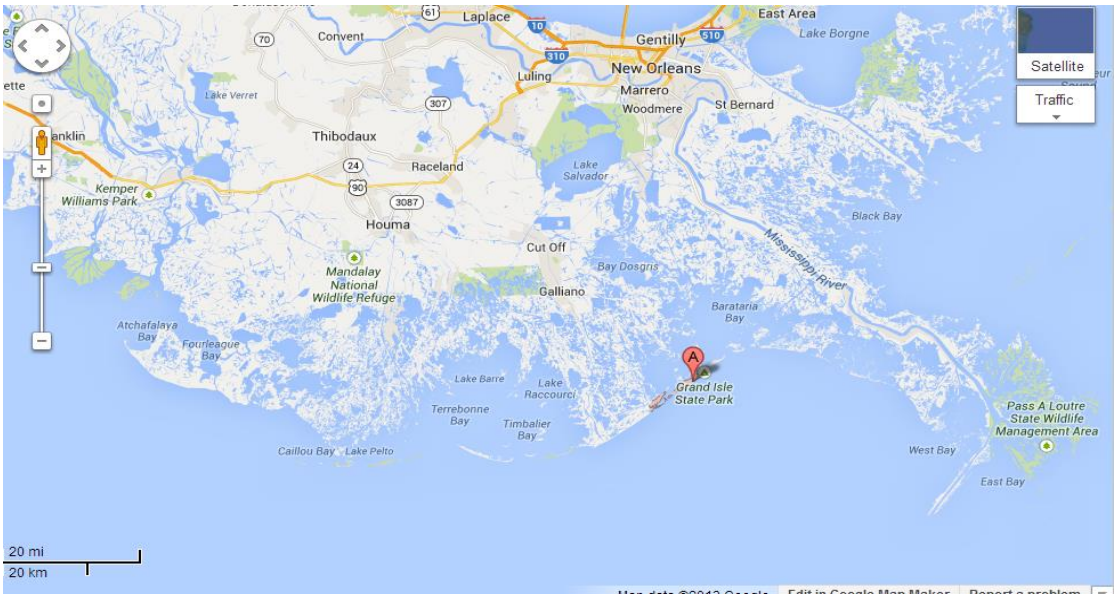

Figure 2.1.1 Map of Louisiana delta and location of Grand Isle (https://maps.google.com/)

The Deepwater Horizon oil spill began on April 20, 2010 as a result of the explosion and subsequent sinking of the Deepwater Horizon oil rig in the Gulf of Mexico. In July 2012 tar balls reached Grand Isle, Louisiana, and the shores of Lake Pontchartrain (Ylan Q. Mui and Fahrenthold 2010). The phytoplankton which was the focus of this study was collected from estuarine waters adjacent to an oyster farm on Grand Isle in Barataria Bay.

Grand Isle is located at 29.2278°N, 90.0122°W. It is a town in Jefferson Parish, Louisiana, located on a barrier island of the same name in the Gulf of Mexico (Figure 2.1.1). The island is at the mouth of Barataria Bay, where the bay merges with the Gulf of Mexico. Grand Isle has been repeatedly impacted by hurricanes throughout its history.

Louisiana Sea Grant Programs Oyster Research station in Grand Isle, Louisiana, served as the sampling site for this study. The oyster farm is located on the edge of an estuary, and

salinity at the sampling site varied between 14 and 22 during this study (Table 2.1.1). The water depth at sampling site, known as Bayou Rigaud, varies seasonally. The bayou is shallower in the winter, when there is less precipitation and runoff. Oyster cages have been assembled in an estuary, where the tide ranges from approximately two to four feet (Tide Predictions, National Oceanic and Atmospheric Association, NOAA).

Table 2.1.1. Temperature and salinity at sampling site

Date	Surface temperature (°C)	Bottom temperature (°C)	Salinity (PSU)
30 January, 2013	18.9	18.9	16.2
27 February, 2013	18.1	16.4	14.5
21 March, 2013	17.4	17.3	21.2
20 July, 2013	28.3	28.1	13.7

Water flowing from the Mississippi is turbid and rich in nutrients. The concentrations of nitrate and silicate at the river mouth can exceed 100 μM (Strom and Strom 1996). Diatoms are an important food species for fish and invertebrates. They require silicon to make their frustules (R. Eugene Turner and Rabalais 1991). The abundance of diatoms in coastal waters impacted by the discharge from the Mississippi is undoubtedly a reflection of the high concentrations of silicate in the river (R. Eugene Turner and Rabalais 1991)

2.2 Growth characteristics

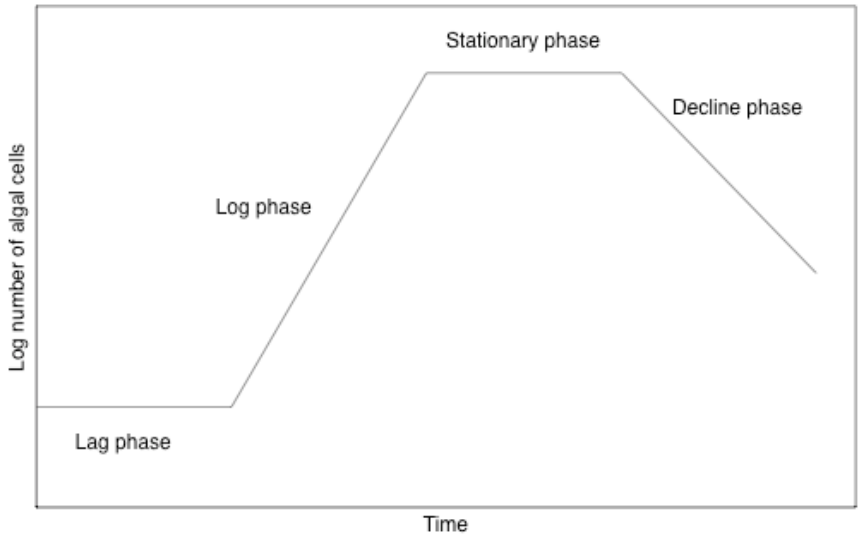

Figure 2.2.1 Phytoplankton growth curve

Generally, there are four phases associated with phytoplankton growth in culture (Figure 2.2.1): lag phase, log phase, stationary phase, and the decline phase (Fogg and Thake 1987). The lag phase of phytoplankton cultures is defined as the period prior to the log growth phase. During the lag phase cell numbers remain nearly constant (Fogg and Thake 1987). Once the phytoplankton become adapted to the conditions in the culture system, they start to produce ATP, DNA, RNA, protein, carbohydrates, lipids, and other essential organic compounds. The log phase (also called the exponential growth phase) is a period characterized by cell doubling at a fixed time interval. Growth during this time is nutrient-saturated and limited only by temperature, irradiance, and the physiological characteristics of the cells. Growth continues at a constant rate. Plotting the natural logarithm of the cell numbers against time produces a straight line. The slope of this line is the specific growth rate of the algae, with units of inverse time (Figure 2.2.2).

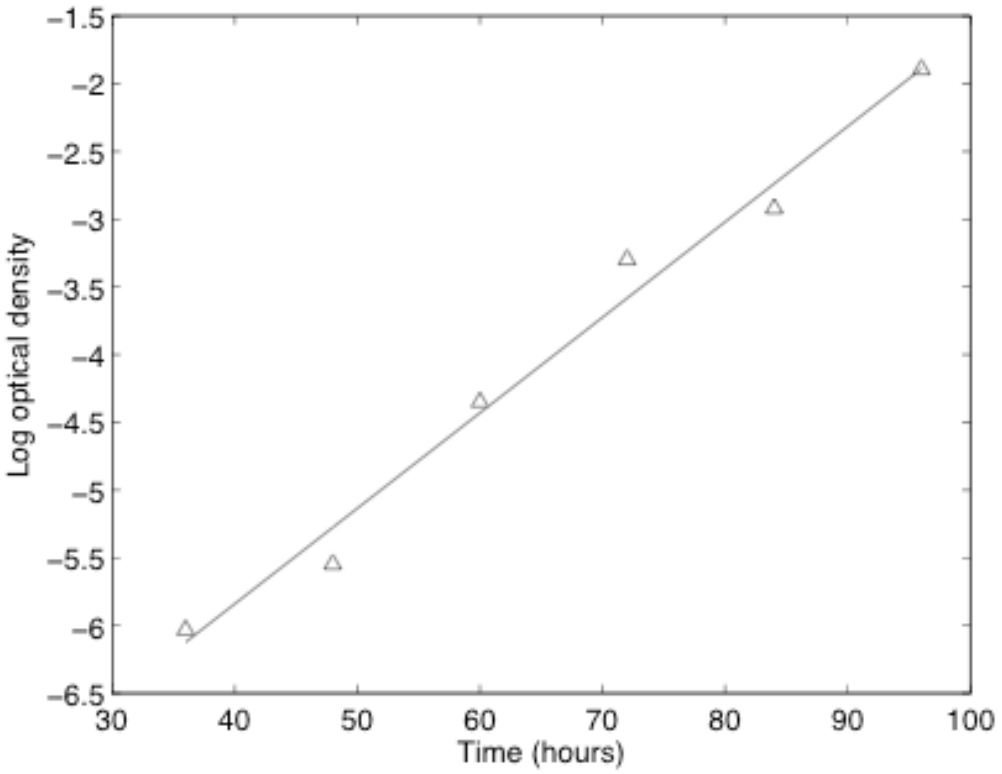

Figure 2.2.2 Example of log-phase growth: sample control from August, 2013.
The solid line was fit to the observations (triangles) by minimizing the residual sum of squares from the log-transformed data (Mulderij, Van Donk et al. 2003).
The growth rate estimated from the least squares line is 0.07 per hour

Mathematically,

$$\frac{dN}{dt} = \mu N \qquad (1)$$

where μ is the specific growth rate of the cells, N is the number of cells or some other measure of biomass (e.g., optical density), and dN/dt is the rate of change of N.

During the stationary phase, the growth rate slows and eventually comes to zero when some environmental factor, such as the concentration of an essential nutrient, becomes limiting. The graph of cell numbers versus time becomes a horizontal line. The death phase can be quite irregular. Cells run out of nutrients and begin to die. For analysis of the effects of oil on

the growth of algae, the stationary and death phases would be irrelevant, because during these phases algal growth is affected by factors other than oil (Mulderij, Van Donk et al. 2003).

My laboratory experiments were usually terminated shortly after the cells entered the stationary phase. A visual analysis of my data suggested that my experiments included the lag-phase, the log phase, and in some cultures the algae entered stationary phase. The duration of the lag phase and the growth rate during log phase growth were the foci of my research.

There are several ways to measure phytoplankton growth rates, including measuring photosynthetic oxygen production (Kohler 1998) and monitoring cell densities (Litchman 1998) or optical densities (Koji Suzuki 1998, Mohammady, Chen et al. 2005, Jiang, Yoshida et al. 2012). I monitored optical densities (ODs) and used the slope of a regression line fit to the log-transformed ODs to estimate the growth rates (Fig. 2.2.2). Algal biomass was monitored twice daily by using a spectrophotometer (UV-2501PC UVeVIS, Shimadzu) to measure the extent to which the culture attenuated monochromatic light with a wavelength of 750 nm. Because algal pigments do not absorb light at 750 nm, the attenuation was due entirely to scattering of the light beam by the algal cells. Fresh medium was used for blank measurements.

I could not estimate precisely the time when the algae entered log phase growth because measurements were taken at discrete time intervals. Instead I used an analysis of covariance (ANCOVA) to calculate the duration of the lag phase relative to the control cultures. If the growth rates were not significantly different, I fit parallel lines to the log OD datasets and then calculated the difference of the intercepts of the parallel regression lines. The relative lag time was determined from the difference between the intercept for the treatment groups versus the control (no oil) intercept.

2.3 Growth characteristics determined from optical density measurements

Optical density (OD) was measured as a metric of phytoplankton biomass, and the rate of change of the logarithm of the OD was used to calculate the growth rate of phytoplankton. An increase of algal cell numbers is often used as a measure of algal growth, but OD rather than the concentration of cells has frequently been used as a direct measure of growth (Joanna M. Kain 1958). The OD is measured at a wavelength (750 nm), where there is no absorption of light by algal pigments, and the attenuation of a beam of monochromatic light is due entirely to light scattering, which is a function of the concentration and size of particles in the water.

I measured the OD with a spectrophotometer (Figure 2.3.1). I "zeroed" the spectrophotometer with a sample of water that contained no particles, such as distilled water or filtered artificial seawater. If there were cells in the medium, the light scattered by the cells did not reach the photoelectric cell, and the electric signal was weaker than was the case for the reference sample (Widdel 2007). The spectrophotometer reported the attenuation of light as if the light had been absorbed, but at 750 nm all light attenuation is due to scattering, not absorption.

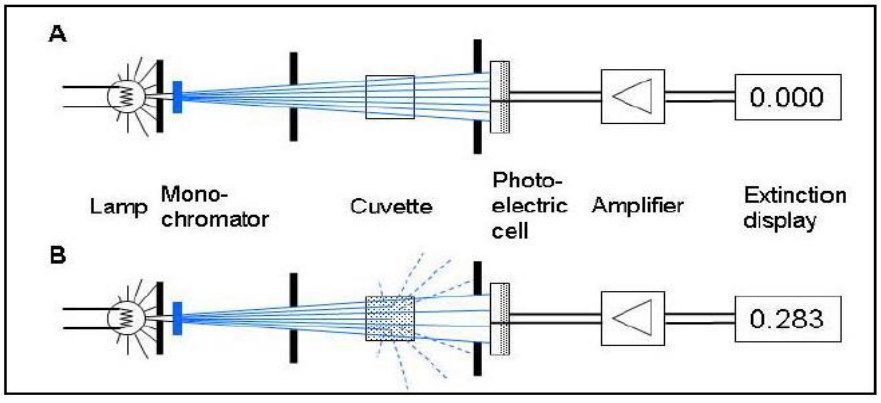

Figure 2.3.1 Measurement of OD with a spectrophotometer

Some practical considerations include the following (Widdel 2007):

Photosynthetic pigments absorb at wavelengths between roughly 400 and 700 nm (Figure 2.3.2). Because I wanted a metric of cell concentration unaffected by the degree of pigmentation of the cells, I made my measurements at 750 nm, where there is no absorption by photosynthetic pigments. The optical density measured at this wavelength is referred to as the OD_{750}.

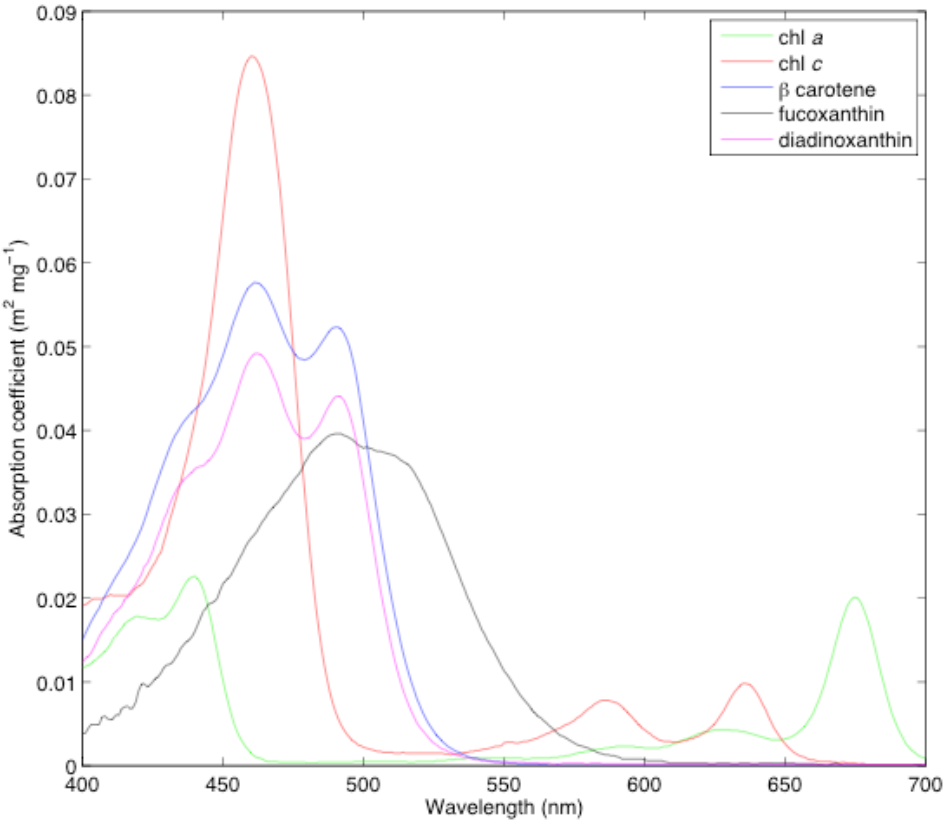

Figure 2.3.2 Absorption spectra of the pigments found in diatom cells.

Any light absorption caused by the medium has to be subtracted to guarantee that only the OD associated with the phytoplankton cells is measured. The spectrophotometer was zeroed by initially filling a sample tube with a medium containing no particles, such as distilled

water or filtered artificial seawater, inserting the spectrophotometer probe in the tube, and then zeroing the instrument. The result, when a subsequent reading is taken, is an extinction of 0.000 on the instrument display. Typically the noise level is ±0.0005. Any subsequently measured extinction greater than 0.000 is due to scattering of light by the algal cells. From time to time during a given set of measurements I verified that the zero-point had not changed and/or readjusted the zero if necessary.

The proportionality between OD and cell density exists only for OD≤0.4 (Figure 2.3.3) because OD above a certain value (usually about 0.4) is no longer directly proportional to the cell density. Samples needed to be diluted if the OD of the undiluted sample was greater than 0.4 (Widdel 2007).

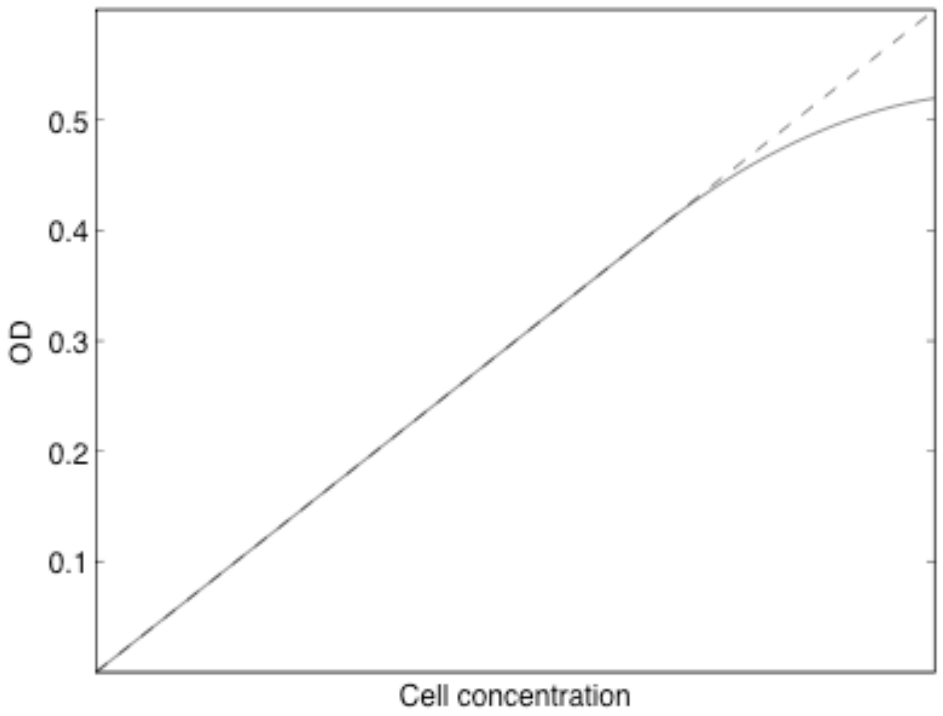

Figure 2.3.3 OD above 0.4 deviates from being proportional to cell concentration.

2.4 Community composition determined from diagnostic pigments

In my thesis research, I used diagnostic pigments to study changes in phytoplankton community composition. The alternative would have been microscopic analysis. However, because many marine phytoplankton cells are too small to be accurately identified under a microscope, use of diagnostic pigments would be a better way to assess the impact of oil on the composition of the phytoplankton community. Diagnostic pigments can be used to assign phytoplankton to major taxonomic groups. A more detailed analysis (e.g., centric versus pennate diatoms) would require microscopic analysis.

Chlorophyll *a* is the major light-harvesting pigment in all microalgae, with the exception of Prochlorococcus, whose light-harvesting complex consists primarily of divinyl derivatives of chlorophyll *a* and chlorophyll *b*. Absorption of light by photosynthetic pigments initiates the process of converting light energy into chemical energy through photosynthesis. Because chlorophyll *a* is found in almost all microalgae, it is routinely used as a measure of the total biomass of phytoplankton (Koji Suzuki 1998). In addition to chlorophyll *a*, there are numerous accessory pigments distributed among the various algal groups. Jeffrey, Wright et al. (1999) have reported that there are 56 pigments in a total of 32 phytoplankton species. Because many of these pigments are found in more than one algal group, most are not, per se, diagnostic for any particular group of algae. However, certain pigments or combinations of pigments are unique to particular algal groups: peridinin is diagnostic for Dinophyceae, prasinoxanthin is diagnostic for Prasinophyceae, 19′-butanoyloxyfucoxanthin is diagnostic for Pelagophyceae, 19′-hexanoyloxyfucoxanthin is diagnostic for Prymnesiophyceae, and divinyl chlorophyll *a* is diagnostic for Prochlorophyceae (Table 2.4.1). Many diagnostic pigments are found in more

than one group of algae. For example, fucoxanthin is found in Bacilliophyceae (diatoms), Chrysophyceae, Pelagophyceae, and Prymnesiophyceae. The absence of a pigment found in multiple groups of algae can be used to rule out the presence of all of the groups of algae that contain that pigment. Obviously, it is therefore necessary to consider the suite of pigments rather than a single marker pigment when using pigments to determine the composition of the phytoplankton community (Deng Chunmei 2010).

In the absence of oil pollution, the composition of the algal community may vary naturally in time and space (Hutchinson 1967) as a function of both biotic and abiotic factors. The abiotic factors include, inter alia, solar radiation (quality and quantity), temperature, photoperiod, turbulence, and nutrient concentrations. Changes in these conditions can affect both the distribution of pigments within a group of algae and the composition of the algal community (Moreno, Marrero et al. 2012). Biotic factors include interspecies competition and zooplankton grazing (Strom and Strom 1996).

Changes in the composition of the phytoplankton community are typically quantified in terms of the ratios of the concentrations of specific diagnostic pigments (Fishwick, Aiken et al. 2006, Aiken, Hardman-Mountford et al. 2008, Aiken, Pradhan et al. 2009). Knowledge of the ratio of a marker pigment to chlorophyll *a* in a group of algae combined with the concentration of the marker pigment allows one to calculate the contribution of that group of algae to the total phytoplankton biomass in terms of chlorophyll *a*. Such information on multiple groups of algae can be used to elucidate the structure and richness of the whole algal community (Deng Chunmei 2010).

Table 2.4.1. Pigments characteristic of major classes of algae. References include Stauber and Jeffrey (1988), Vernet and Lorenzen (1987), Jeffrey (1974), Guillard et al. (1985), Withers et al. (1981), Foss et al. (1984), and Moreno et al. (2012).

Algal group	Pigment biomarker
Prochlorophyceae	divinyl chlorophylls *a* and *b*, chlorophyll *b*, zeaxanthin, α-Carotene, phycoerythrin
Cyanophyceae	zeaxanthin, β-Carotene, phycoerythrin, phycocyanin, allophycocyanin
Bacillariophyceae	chlorophyll *c*, fucoxanthin, diadinoxanthin, diatoxanthin, β-Carotene
Prymnesiophyceae	chlorophyll *c*, 19'-hexanoyloxyfucoxanthin, fucoxanthin, diadinoxanthin, diatoxanthin, β-Carotene
Pelagophyceae	chlorophyll *c*, 19'-butonoyloxyfucoxanthin, diatoxanthin, diadinoxanthin, fucoxanthin, β-Carotene
Cryptophyceae	chlorophyll *c*, alloxanthin, phycoerythrin or phycocyanin, crocoxanthin, monadoxanthin, α-Carotene,
Dinophyceae	chlorophyll *c*, peridinin, dinoxanthin, diadinoxanthin, diatoxanthin, β-Carotene
Prasinophyceae	chlorophyll *b*, prasinoxanthin, zeaxanthin, neoxanthin, violaxanthin, α-Carotene, β-Carotene
Chlorophyceae	chlorophyll *b*, lutein, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, α-Carotene, β-Carotene

In some cases measurements of pigment ratios can also be used to elucidate physiological changes in groups of algae. For instance, an important indicator in the case of diatoms is the ratio of diadinoxanthin and diatoxanthin to chlorophyll *a*, or the ratio of diatoxanthin to the sum of diadinoxanthin plus diatoxanthin. Diadinoxanthin and diatoxanthin

are not directly involved in the photosynthetic process, but instead function as photoprotective pigments (Fujiki and Taguchi 2001). Part of the light energy absorbed by pigments is used in photosynthesis, and the rest is emitted as fluorescence or dissipated as heat (Fujiki and Taguchi 2001). If algae cannot utilize the energy absorbed by pigments, the excess energy could lead to damage of intracellular components or metabolic processes. Some carotenoids can play a photoprotective role by quenching the redundant energy (Demmigadams 1990, Cogdell, Howard et al. 2000). The xanthophyll cycle involves the enzymatic removal of epoxy groups from xanthophylls (e. g., diadinoxanthin) to create de-epoxidised xanthophylls (e. g. diatoxanthin). In higher plants there are three carotenoid pigments active in the xanthophyll cycle: violaxanthin, antheraxanthin, and zeaxanthin. However, in diatoms and dinoflagellates the xanthophyll cycle consists of the pigment diadinoxanthin, which is transformed into diatoxanthin (diatoms) or dinoxanthin (dinoflagellates) when the light intensity becomes stressful (Jeffrey 1997).

Most research concerned with pigment ratios has concerned changes of ratios as a function of abiotic conditions such as irradiance/depth. Fujiki and Taguchi (2001), for example, examined relationships between light absorption and xanthophyll cycle pigments in three species of marine diatoms grown at six irradiances from 90 to 750 μmol quanta m^{-2} s^{-1}. Riegman and Kraay (2001) reported decreasing ratios of diadinoxanthin and diadinoxanthin to chlorophyll *a* with increasing depth, a pattern that was correlated with the attenuation of light with increasing depth. In diatoms, the ratio of fucoxanthin to chlorophyll *a* tends to increase with depth in most cases, whereas the ratio of diadinoxanthin to chlorophyll *a* decreases with depth (Descy, Sarmento et al. 2009).

2.5 Sampling and incubation methodology

2.5.1 General procedures

Seawater was collected from Grand Isle, LA every month from February 2013 to August 2013. The water was passed through 35-micron mesh netting to eliminate large zooplankton grazers. The water samples were returned to the laboratory, and small aliquots were added to 300-mL clear glass BOD (biochemical oxygen demand) bottles containing artificial seawater medium, nutrient salts, and various concentrations of Deepwater Horizon oil.

The growth medium consisted of artificial seawater (Instant Ocean) enriched with f/2 nutrients (Guillard and Ryther 1962, Guillard 1975). The medium was then added to the BOD bottles, and an aliquot (~2 mL) of the original seawater sample collected from Grand Isle, LA was added to each bottle. The experimental design included triplicate control cultures with no oil and triplicate treatments to which oil was added. The oil added to each bottle was provided by Dr. Edward Overton; it was a sample of un-weathered Macondo crude oil. The Macondo crude oil composition has been reported by Reddy et al. (2012). Macondo crude oil has an American Petroleum Institute gravity of 37 (Federal Interagency Solutions Group 2010), and its solubility in seawater at 25 °C should therefore be greater than 55 parts per million (ppm) (Hamam, Hamoda et al. 1988). Water-accommodated fractions (WAFs) were prepared with oil concentrations of 0.1 ppm, 0.3 ppm, 0.6 ppm, 1.2 ppm, 2.4 ppm, 4.8 ppm, 9.6 ppm, and 19.2 ppm (see section 2.4.3). The bottles containing WAF were then incubated in front of a light bank for several days at a temperature of 19–20 °C.

The algal cultures were grown on a 12:12 light: dark (L:D) cycle with irradiance provided by daylight fluorescent lamps at an intensity of 300 µmol photons m^{-2} s^{-1} (average), which is

adequate to saturate phytoplankton photosynthetic rates (Laws and Bannister 1980, Harding, Prezelin et al. 1982, Harrison and Platt 1986). The light intensity was measured with a QSL-2100 quantum scalar laboratory radiometer. The L:D cycle was timed so that the lights came on at 8 am and went off at 8 pm. I agitated the bottles three times a day to make sure everything was well mixed and that the algae did not adhere to the bottom of the bottle. I monitored the cell growth in the bottles twice daily if possible by measuring the optical density (OD) at 750 nm using Cary model 50 UV-Visible Spectrophotometer and Cary WinUV software. The signal averaging time was 3 seconds. The instrument was first zeroed with filtered artificial seawater. I took an aliquot of 10 mL from a BOD bottle and put it in a glass tube. Then a sample reading was taken. I recorded the optical density of each sample three times.

Growth rates were determined from the slope of a least squares line fit to the natural logarithm of the OD values versus time. The incubations were stopped when the measurements indicated that the algae had entered stationary phase. The algal biomass was quantified in terms of the concentration of chl *a*. I followed the protocol in section 2.5.5 for the pigment analyses.

2.5.2 f/2 medium

The medium f/2 is a widely used enriched seawater medium designed for growing marine algae. I prepared each liter of f/2 medium by adding 35 grams of Instant Ocean seawater salts to 950 mL of distilled water. The medium was then amended with the inorganic nutrients, trace metals, and vitamins at the concentrations indicated in Tables 2.5.2.1-2.5.2.3. The final volume was then brought to 1 liter with distilled water.

Table 2.5.2.1 Composition of f/2 medium

Component	Stock solution	Quantity	Molar concentration in final medium
$NaNO_3$	75 g/L dH_2O	1 mL	8.82 μM
$NaH_2PO_4 \cdot H_2O$	5 g/L dH_2O	1 mL	36.2 μM
$Na_2SiO_3 \cdot 9H_20$	30 g/L dH_2O	1 mL	10.6 μM
Trace metal solution	(see recipe below)	1 mL	--
Vitamin solution	(see recipe below)	0.5mL	--

Table 2.5.2.2 Trace metal solution for f/2 medium

Component	Primary stock solution	Quantity	Molar concentration in final medium
$FeCl_3 \cdot 6H_20$	--	3.15 g	11.7 μM
$Na_2EDTA \cdot 2H_2O$	--	4.36 g	11.7 μM
$CuSO_4 \cdot 5H_2O$	9.8 g/L dH_2O	1 mL	39.3 nM
$Na_2MoO_4 \cdot 2H_2O$	6.3 g/L dH_2O	1 mL	26.0 nM
$ZnSO_4 \cdot 7H_2O$	22.0 g/L dH_2O	1 mL	76.5 nM
$CoCl_2 \cdot 6H_2O$	10.0 g/L dH_2O	1 mL	42.0 nM
$MnCl_2 \cdot 4H_2O$	180.g/L dH_2O	1 mL	0.91 μM

Table 2.5.2.3 Vitamin mix for f/2 medium

Component	Primary stock solution	Quantity	Molar concentration in final medium
Thiamine HCl (vit. B_1)	--	200 mg	0.296 μM
Biotin (vit. H)	0.1 g/L dH_2O	10 mL	2.05 nM
Cyanocobalamin (vit. B_{12})	1.0 g/L dH_2O	1 mL	0.369 nM

2.5.3 Preparing untreated oil water-accommodated fractions (WAFs)

Preparation of a water-accommodated fraction (WAF) followed preparation of the f/2 medium. WAF is defined as a laboratory-prepared medium derived from low-energy (no vortex)

mixing of a poorly soluble test material (e.g., an oil or petroleum product) that is essentially free of particles (Aurand and Coelho 1996, Coelho and Aurand 1997). Although the solubility of Macondo crude oil was expected to exceed 55 ppm (vide supra), the various components of crude oil differ greatly in terms of their solubility, and as noted by the Federal Interagency Solutions Group (2010), "n-alkanes up to maximum n-C8-9 may have a potential to dissolve, but larger alkanes have very limited solubility potentials. However, the mono-aromatic benzene compounds. . . C0-C3 napthalenes, and the C0-C1-alkylated 3-ring PAHs can be stripped out of oil droplets because of their water solubility." The basic procedure for preparing a WAF is to layer a known amount of oil onto a mass of still water of known volume (in this case, the f/2 medium). The oil-and-water are then mixed slowly (about 200 rpm; no vortex) for a sufficient duration to achieve stability (24 hours).

The WAF was prepared in two-liter glass reagent bottles. The headspace-to-vessel ratio was maintained at about 20%. Magnetic stirrers were used to mix the oil and f/2 medium because the stirrers can be used with essentially any size vessel with little or no modification. A lower-energy method, in which no vortex results, is preferred, because the resultant WAFs are more replicable (Blenkinsopp, Boileau et al. 1996). Vessels were sealed immediately after loading to minimize volatilization and were mixed in the dark at 20 °C.

Another important aspect of the reproducibility of WAFs is achieving a "functional equilibrium" (Aurand, Coelho et al. 2001) within the preparation vessel so that the liquid phase can be considered "saturated" in terms of the available soluble compounds. Generally, a mixing time of 24–48 hours is sufficient to achieve equilibration.

After 24 hours, the liquid phase (the WAF) was withdrawn from underneath the film of oil at the surface, care being taken not to disturb the oil. The process was accomplished by using a siphon tube. WAFs should be used immediately to minimize the effects of bacterial activity during storage (Aurand, Coelho et al. 2001).

2.5.4 Relationship between optical density and cell counts

To determine the relationship between OD and cell counts, I counted the number of cells in the cultures on a Beckman Coulter model Z1 particle counter. The sample was shaken vigorously to break up the colonies. Blank counts (filtered artificial seawater) were subtracted from all counts. I found the OD to be directly proportional to the cell concentration up to 2500 cells mL^{-1}.

2.5.5 Pigment analysis

At the end of log-phase growth, samples of WAFs from each bottle (mixture of f/2 medium, crude oil, and seawater) were collected and filtered onto 25-mm-diameter Whatman GF/F glass-fiber filters at a vacuum pressure of 160 mm Hg. The filters were folded twice and wrapped in aluminum foil to exclude air. The filters were then stored in a freezer at –15 °C. Subsequently the filters were sent on dry ice via FedEx to the University of Hawaii. High performance liquid chromatography (HPLC) pigment analyses were carried out at the University of Hawaii at the Center for Marine Microbial Ecology and Diversity (CMMED), following the method described by Bidigare et al. (2005).

CHAPTER 3. RESULTS

3.1 Oil effects on growth characteristics

I estimated phytoplankton growth rates for the months of March, April, May, July, and August. I excluded the results from February because the OD readings were badly scattered, perhaps because I was still becoming familiar with the use of the spectrophotometer. I analyzed the data with a two-way analysis of variance in which oil concentrations and months were the independent variables. Growth rate was the dependent variable. I did not perform an experiment at an oil concentration of 19.2 ppm in July. Because a two-way ANOVA cannot accommodate missing data, I could either exclude the month of July or run the two-way ANOVA for all oil concentrations, or I could exclude the results from 19.2 ppm and run the two-way ANOVA for all months. I decided to use both options. In both cases, the two-way ANOVA revealed that the concentration of oil ($p < 0.0001$), the month ($p < 0.0001$), and the interaction between oil and month ($p < 0.0001$) significantly affected the phytoplankton growth rates.

To understand why these effects were significant, I calculated the average growth rate at each oil concentration and the average growth rate for each month. Then I subtracted the average of all the growth rates from these values. The results (Figure 3.1.1) showed that growth rates were above average at oil concentrations of 0.6 ppm or less and were below average (with one exception) at oil concentrations of 1.2 ppm or greater. Growth rates were below average in March and April and above average in May and August. Thus both oil concentrations and months were judged to have a significant effect on growth rates. Furthermore, the effect of a given oil concentration depended on the month. Low oil concentrations, for example, resulted in higher growth rates in May and August than in March and April, and high oil

concentrations resulted in lower growth rates in March, April and May than in July and August (Table 3.1.1).

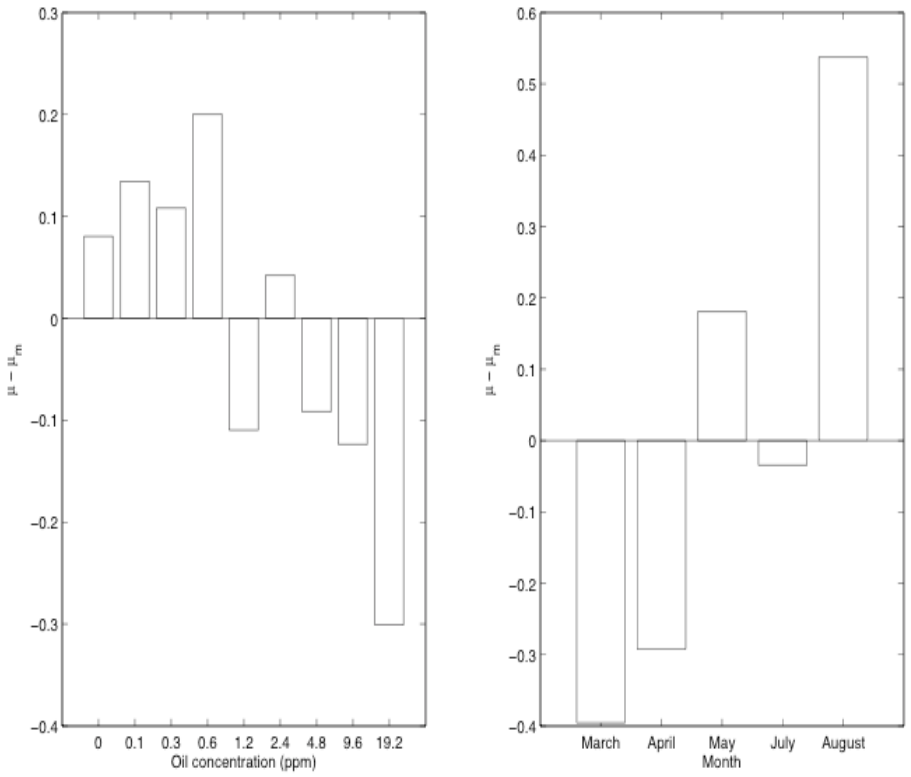

Figure 3.1.1 Growth rates relative to the overall average growth rate as a function of oil concentration (left) and month of the year (right)

Because I wanted to focus on the effects of oil on the growth rates, I assigned the growth rates to cells, each cell consisting of a specific month and oil concentration. I then normalized the growth rates in each cell by dividing each of the replicate growth rates in the cell by the average control growth rate for that month (Table 3.1.1, Figure 3.1.2). I reasoned that there were undoubtedly differences in the phytoplankton communities that I sampled from February through August, and that those differences might be reflected by differences in growth rates that had nothing to do with the effects of the oil. By dividing all calculated growth

rates by the average growth rate of the control cultures for that month, I hoped to filter out, insofar as possible, differences associated with the composition of the phytoplankton community so that I could focus my analysis on oil effects. The Spearman rank correlation coefficient (–0.90) between oil concentration and normalized growth rate (Figure 3.1.2) was significant at $p = 0.0046$.

Table 3.1.1 Phytoplankton growth rates (d^{-1}) in different concentrations of WAF.

Values in red font are rates as % of the control growth rates

	0	0.1ppm	0.3ppm	0.6ppm	1.2ppm	2.4ppm	4.8ppm	9.6ppm	19.2ppm
March	0.93	0.93	0.95	1.64	0.80	0.43	0.55	0.60	0.37
	100%	100%	102%	176%	86%	46%	59%	64%	39%
April	0.96	1.37	1.24	1.12	0.92	1.14	0.63	0.50	0.25
	100%	143%	130%	117%	96%	119%	66%	52%	26%
May	1.57	1.50	1.51	1.66	1.34	1.38	1.12	1.00	1.31
	100%	96%	96%	106%	85%	88%	71%	64%	83%
July	1.18	1.06	1.18	0.87	0.94	1.25	1.43	1.38	
	100%	90%	100%	74%	80%	106%	121%	117%	
August	1.74	1.79	1.64	1.69	1.43	1.99	1.79	1.88	1.65
	100%	103%	95%	98%	82%	115%	103%	108%	95%

When the normalized growth rates were examined on a monthly basis, two patterns were apparent. During the months of March, April, and May, growth rates at oil concentrations of 0.6 ppm or less were comparable to or roughly 10% higher than control growth rates, with the exception of one anomalously high growth rate at 0.6 ppm during March. At higher oil concentrations there was a negative correlation between oil concentrations and growth rates (Figure 3.1.3).

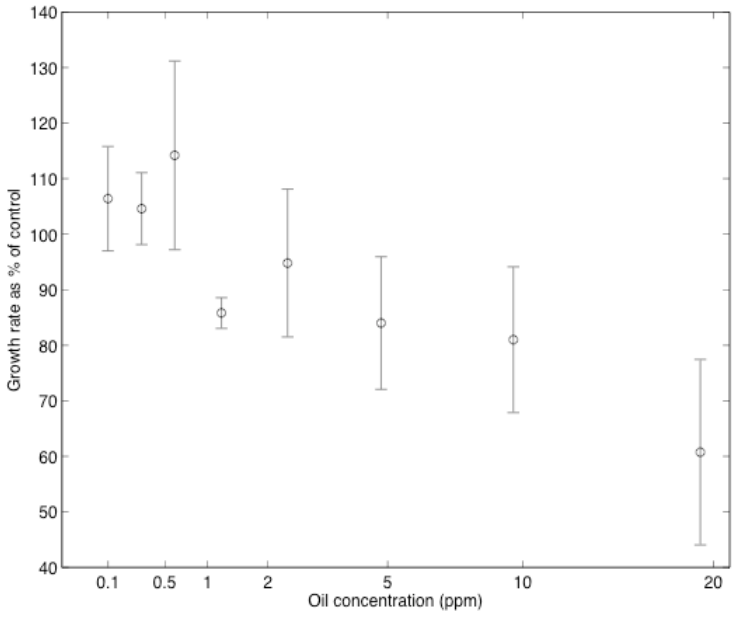

Figure 3.1.2 Phytoplankton growth rates as a percent of control growth rates versus oil concentrations from 0.1 to 19.2 ppm. Error bars are standard deviations of the mean values at each oil concentration

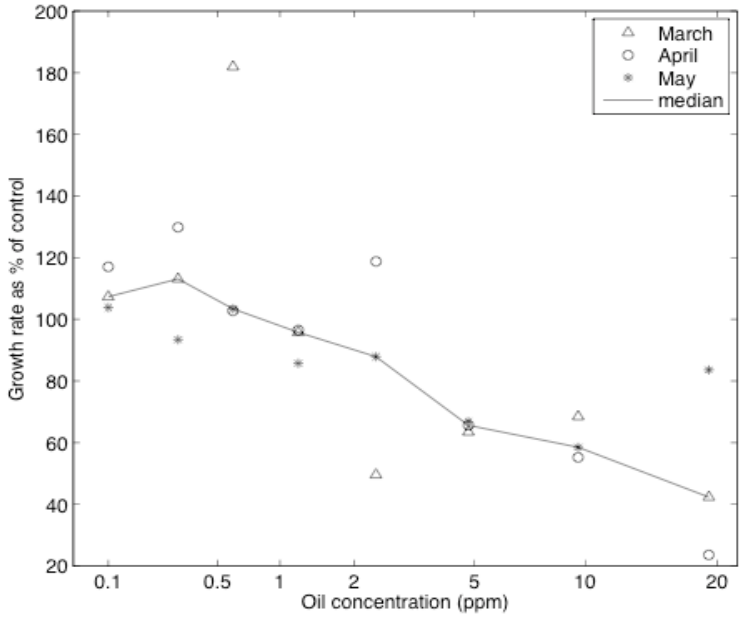

Figure 3.1.3 Normalized growth rates during March, April, and May versus oil concentrations

During July and August growth rates at oil concentrations of 1.2 ppm or less were comparable to or at most about 20% lower than control growth rates and at higher oil concentrations were roughly 5% higher than control growth rates (Figure 3.1.4). In contrast to the results from March, April, and May, there was very little correlation between oil concentrations and normalized growth rates during July and August.

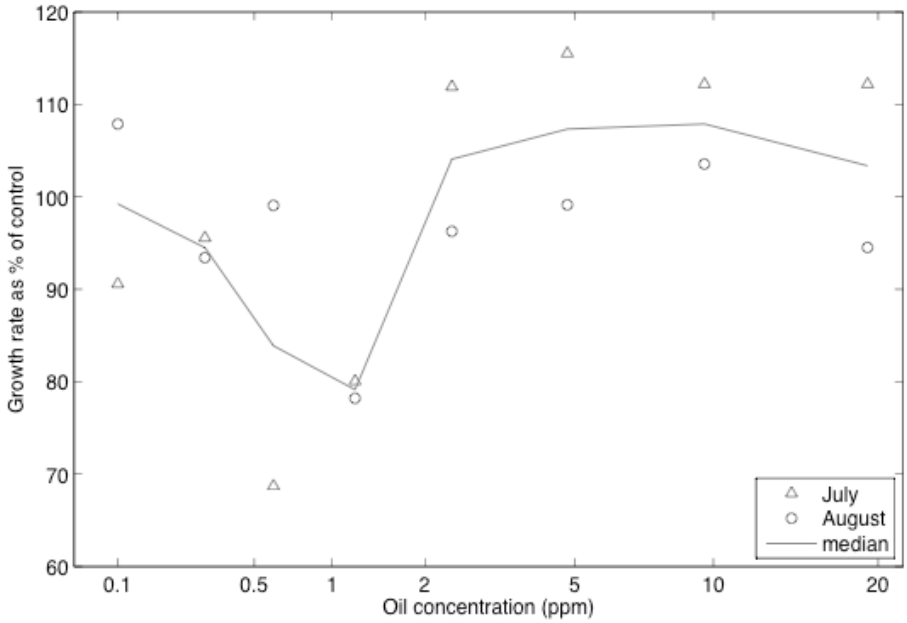

Figure 3.1.4 Normalized growth rates during July and August versus oil concentrations

I also looked for lag time differences when the growth rates were similar by drawing parallel lines through the logarithm of OD versus time using an analysis of covariance program (ANCOVA). The results showed that the crude oil affected the duration of the lag time. Within the context of the ANCOVA, this was apparent from the fact that the “elevations” of the parallel lines were significantly different. For instance, during the experiments in May, the 0.1, 0.3, and 0.6 ppm treatments entered log phase earlier than the controls, the differences in lag times

being 45–56 hours. The 2.4, 4.8, and 9.6 ppm treatments, however, entered log phase later than the controls, the difference in lag times being 3–11 hours. However, there were a small number of data in each dataset, and no consistent correlation between lag time and oil concentration was apparent.

3.2 Oil effects on pigment ratios

The only pigments consistently found in all samples, including the initial samples and control samples, were chlorophyll *a* (CHLA), fucoxanthin (FUCO), chlorophyll *c* (CHLC), β-carotene (β-CARO), diadinoxanthin (DDX) and diatoxanthin (DTX). The implication is that in most cases the phytoplankton community consisted entirely of diatoms (Bacillariophyceae). However, all of the samples from February 2013 also contained zeaxanthin, and the initial samples and control samples from March 2013 also contained zeaxanthin. Most of the samples that contained zeaxanthin contained no chlorophyll *b*, the implication being that the zeaxanthin was associated with Cyanophyceae . Three samples contained both chlorophyll *b* and lutein, the implication being that Chlorophyceae were present in those samples. The initial samples in February and March both contained alloxanthin and α-carotene, the implication being that Cryptophyceae were present.

Unlike the growth rate measurements, pigment ratios were measured in February, March, May, July and August. The CHLC/CHLA ratio was remarkably constant (0.0996 ± 0.0200) (Figure 3.2.1) and was not significantly correlated with oil concentrations ($p = 0.7614$). The CHLC/CHLA ratio ranged from 0.091 to 0.117, and the Pearson correlation coefficient between the CHLC/CHLA ratio and oil was –0.1111. Likewise, the β-CARO/CHLA ratio was remarkably constant (0.0188 ± 0.0058) (Figure 3.2.2) and was not significantly correlated with oil

concentrations (p = 0.9195). The associated Pearson correlation coefficient between the β-CARO/CHLA ratio and oil concentration was 0.056.

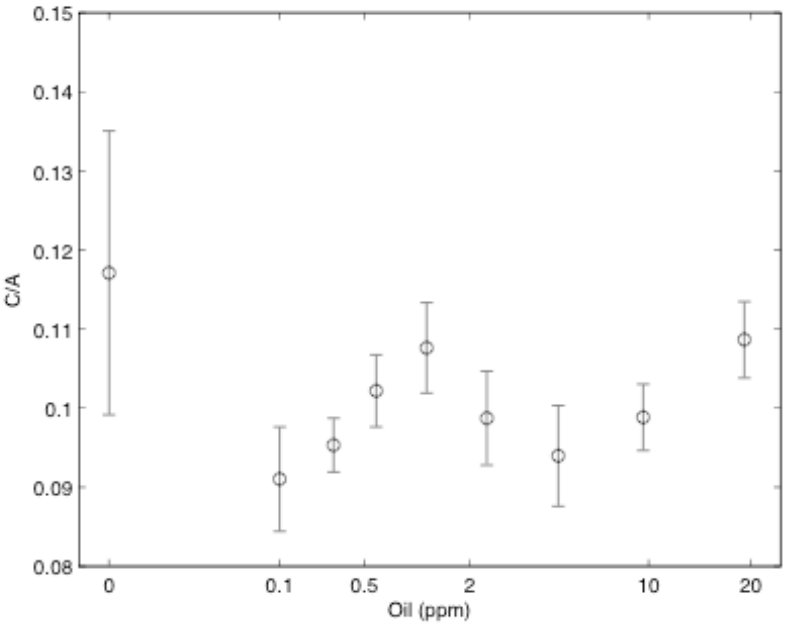

Figure 3.2.1 Ratio of CHLC to CHLA

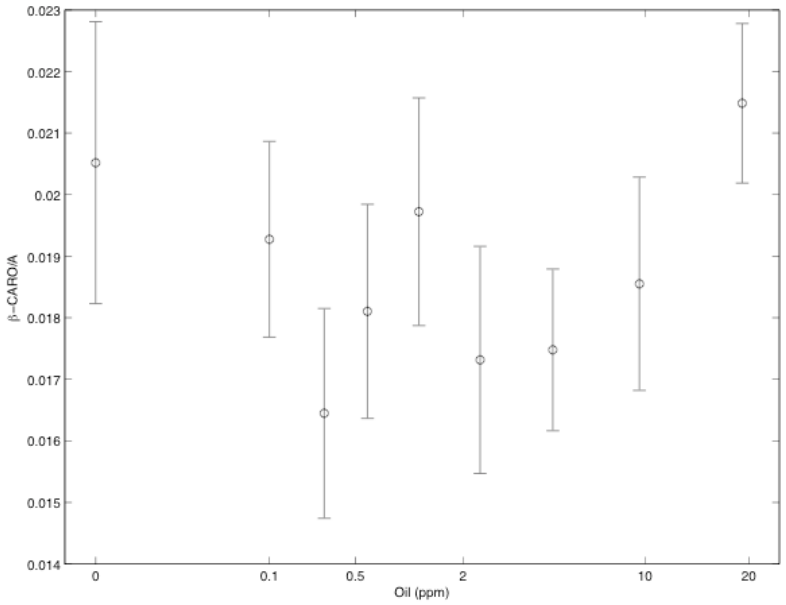

Figure 3.2.2 Ratio of β-CARO to CHLA

However, results for the FUCO/CHLA ratio were different. In the first three months (February, March and April), the FUCO/CHLA ratios were positively correlated with oil concentrations (p = 0.0059), the Kendall correlation coefficient being 0.7222 (Figure 3.2.3). In July and August there was no significant correlation between the FUCO/CHLA ratios and oil concentrations (p = 0.7614), the Kendall correlation coefficient being –0.1111 (Figure 3.2.3). I used an ANCOVA to determine whether the slopes of regression lines fit to the data in Figs. 3.2.3 were different. The ANCOVA showed that the slopes were significantly different (p < 0.001).

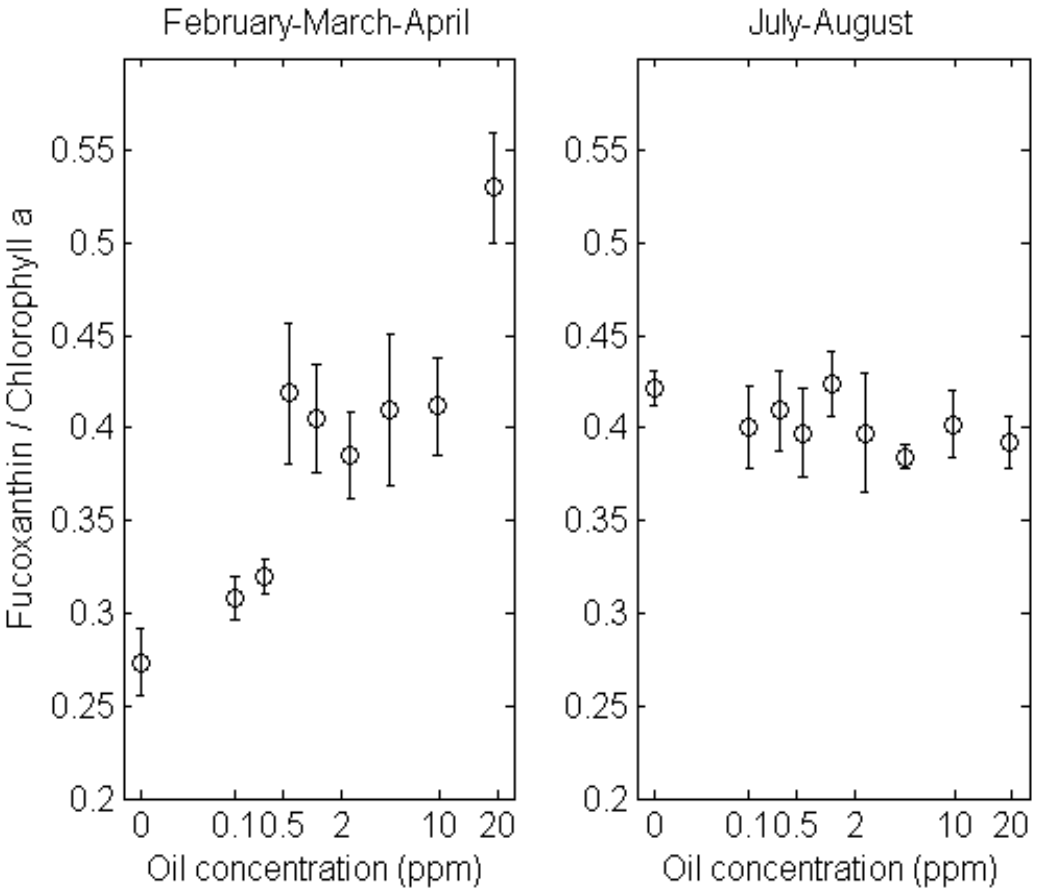

Figure 3.2.3 Ratio of FUCO/CHLA

Coincidentally, the growth rate bioassays showed that phytoplankton growth rates were significantly correlated with oil concentrations in March, April and May, but that there was no significant correlation between growth rates and oil concentrations in July and August. The pattern of significance was the same for the FUCO/CHLA ratios, but the sign of the correlation was opposite to the sign of the growth rate correlation.

In February, March and April, the (DDX+DTX)/CHLA ratio was negatively correlated with oil concentration (p = 0.0446), the Kendall correlation coefficient being –0.5556. In July and August, the (DDX+DTX)/CHLA ratio was more-or-less constant (p = 0.9195), the Kendall correlation coefficient being 0.0556. In this case an ANCOVA revealed no significant difference in the slopes of regression lines fit to the two datasets (p = 0.1461) (Figure 3.2.4).

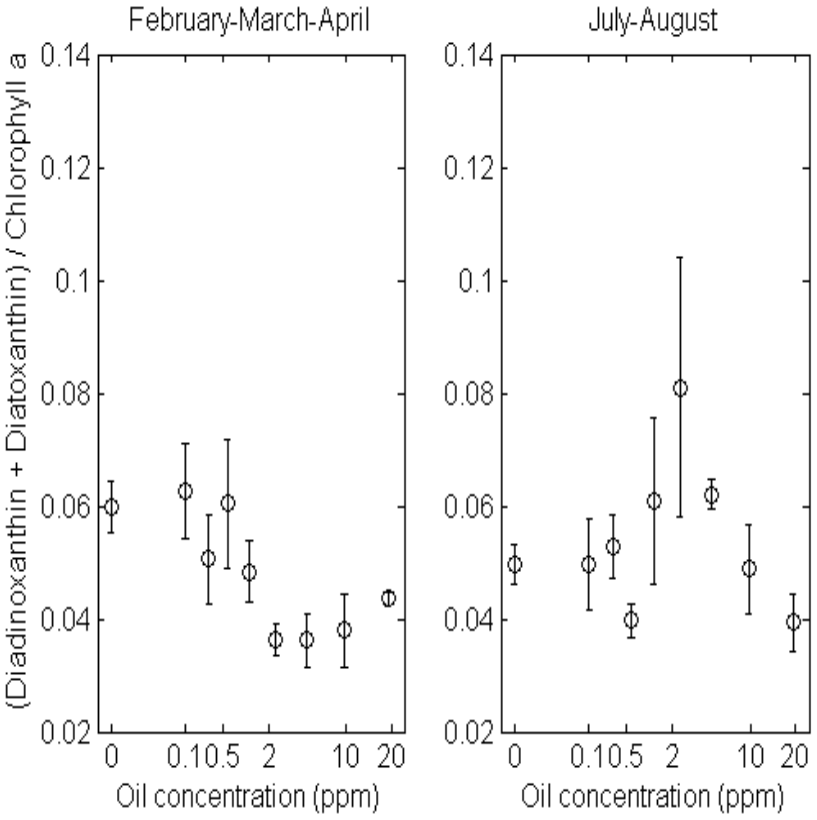

Figure 3.2.4 Ratio of (DDX+DTX)/CHLA

CHAPTER 4. DISCUSSION

4.1 Exponential growth rates

The pattern apparent in Figure 3.1.2 is generally consistent with results from previous research and current understanding of the effects of oil on phytoplankton. High concentrations of crude oil significantly reduced the growth rates of the phytoplankton compared to the control cultures. Inhibition effects are directly associated with the toxicity of hydrocarbons in the crude oil (WANG, TANG et al. 2002, Fleeger, Carman et al. 2003).

Current research indicates that the toxicity of oil pollution stress to phytoplankton results from the activity of oxygen free radicals, which attack the DNA, protein, biological membranes, and chloroplast of a cell, the final result being the death of the cell (Singh and Kumar 1991, Zhao 1998). Superoxide dismutase (SOD) is an enzyme that removes free radicals in vivo (Winston and Digiulio 1991). The concentration of oxygen free radicals in phytoplankton increases after exposure to crude oil. Low concentrations of these free radicals interfere with SOD production inside the cells. The SODs in turn remove the reactive oxygen and thereby protect the cells. Higher concentrations of toxic substances, such as crude oil or petroleum products, lower the activity of SODs, the result being the accumulation of active oxygen free radicals in the phytoplankton cells and damage to the cells (WANG, TANG et al. 2002).

However, low concentrations of crude oil, roughly 0.1–0.6 ppm, enhanced the growth rate of the phytoplankton in my experiments. The production of beneficial effects at low doses by something that produces harmful biological effects at moderate to high doses is called hormesis (Stebbing 1982). Although the hormesis model of dose-response is not widely accepted, and the biochemical mechanisms are still not well understood (Kaiser 2003, Axelrod,

Burns et al. 2004), there have been many studies of hormesis, both in the laboratory and in situ (Karydis 1979, You, Xuexi et al. 2002, Nayar, Goh et al. 2005, Yijun, Quanzhen et al. 2011). For example, Chakraborty et al(2010) examined the stress and toxicity of transition metals (Co, Ni, Cu and Zn) on freshwater phytoplankton grown in metal-contaminated water. The results showed that the phytoplankton biomass and the marker pigment concentrations did not change significantly under low levels of exposure (1×10^{-9} to 1×10^{-8} M). At concentrations of 1×10^{-7} M, four different metals all acted as nutrients and increased the biomass. A combination of 1×10^{-6} M Cu combined with biotic ligands showed the greatest toxicity. Compared to Ni and Co, Cu and Zn were found be lethal at higher concentration for both green algae and cyanobacteria.

Several phenomena observed by other researchers can help to explain why low concentrations of crude oil promote the growth of phytoplankton. In the experiments of Wang You et al. (2002), two species of marine microalgae were exposed to low concentrations of anthracene (1.5–6 μg/L). Results indicated that the amounts of chlorophyll *a* and carotenoids were increased by 61% and 39%, respectively in one species, and by 15% and 50% respectively in the second species compared to control cultures. Amounts of protein were also enhanced. Higher cell quotas of chlorophyll *a* and protein would be expected to enhance the light and dark reactions of photosynthesis, respectively, and thereby promote algal growth.

Although oxygen free radicals would be expected to harm cells, Dypbukt et al. (1994) have argued that they could also promote cell growth to some extent at low levels of exposure. The experiments of Wang (2002) have shown that exposure to low concentrations of anthracene enhances the cell quota of SOD, which could keep the damaging effects of oxygen

free radicals at an acceptable level and then promote cell growth (Dypbukt, Ankarcrona et al. 1994) .

The results of pigment analyses revealed that the phytoplankton that grew in my experiments consisted almost entirely of diatoms. Diatoms can tolerate crude oil to some extent. The diatom frustule tends to adsorb and retain hydrocarbons, because silica is a very good adsorbent of hydrocarbons. Thus a diatom may be able to restrict entry of hydrocarbons into the cell at low oil concentrations and thereby tolerate crude oil to some extent (Rosen and Middleton 1955, Karydis 1979).

Another important consideration is oil degradation. In Yijun et al.'s 15-day experiment (Yijun, Quanzhen et al. 2011), seven groups of WAF were used at concentrations from 0.20–9.56 mg/L. Crude oil degraded quickly in the first seven days, at which time the oil concentrations in the seven groups were about the same. This result was apparent in all four seasons. Some bacteria are able to degrade some of the components of crude oil. Nayar et al. (2005), for example, have shown that bacteria respond positively to diesel fuel, because it represents a source of organic carbon, and the toxic effects of the oil reduce grazing pressure and competition with less tolerant microorganisms for nutrients. Phytoplankton can also contribute to the degradation of crude oil. Semple et al. (1999) have shown that in natural waters and wastewater eukaryotic algae are capable of biotransforming and biodegrading aromatic hydrocarbons, for example by transforming naphthalene and benzo[*a*]pryene to produce hydroxylated intermediates. Such transformations help to eliminate these pollutants from the ecosystem. Similar results have been reported by Juhasz and Naidu (2000). Many genera of algae, such as Chlorella, Navicula, and Nitzschia, have the ability to degrade low-

molecular-weight PAHs. In addition, some mixotrophic algae have the ability to degrade long-chain hydrocarbons into short-chain carbon sources that can be assimilated by the phytoplankton. The result is promotion of the growth of the algae (Ohwada, Nishimura et al. 2003).

Although the concentration of crude oil clearly affected the growth of the algae in my experiments, the dose-response relationship differed between months (Figure 3.1.3, 3.1.4). The variable response of the phytoplankton to crude oil in different months (Rosen and Middleton 1955) presumably reflects the fact that the composition of the community structure is different in different seasons (Yijun, Quanzhen et al. 2011). My pigment analyses showed that the ratio of fucoxanthin to chlorophyll *a* changed in February, March and April. These changes could mean that the composition of the diatom community changed. The tolerance of diatoms to crude oil is almost certainly not the same for all species. My experiments showed that in July and August the growth rates of the diatoms were little affected by the oil. The implication is that the diatom community in the summer was more tolerant to the oil. Huang's experiment (2011) indicated that during the summer the phytoplankton were less tolerant to oil than in the spring. My results showed the opposite.

However, one thing that needs to be considered is that my bioassays were carried out in the laboratory under ideal conditions (abundant nutrients and light). It is possible that the diatoms would have been less tolerant to the oil had they been grown under less ideal conditions. Therefore my experiments might underestimate the real effects of crude oil on natural phytoplankton communities.

4.2 Pigment ratios

From the functional perspective, the abundance of photosynthetic pigments, especially photosynthetic carotenoids (such as fucoxanthin), is considered a good indicator of high productivity waters; whereas the abundance of photoprotective carotenoids (such as diadinoxanthin and diatoxanthin) has been related to ecosystems with low productivity (Barlow, Aiken et al. 2002). The ratio between the main photoprotective pigments and chlorophyll a is used as an indicator of the response of the phytoplankton community to changing environments.

The only detectable pigments included were chlorophyll c (CHLC), fucoxanthin (FUCO), diatoxanthin (DTX), diadinoxanthin (DDX) and β-carotene (β-CARO). Fucoxanthin is found only in diatoms, prymnesiophytes, pelagophytes, and chrysophytes. However, 19'-hexanoyloxyfucoxanthin, 19'-butonoyloxyfucoxanthin, and violaxanthin were not discovered, which means there were no prymnesiophytes, pelagophnytes, nor chrysophytes present. In addition, the pigments alloxanthin, peridinin, prasinoxanthin, and lutein were seldom detected. The implication is that cryptophytes, dinoflagellates, prasinophytes, and chlorophytes were in most cases not part of the phytoplankton community. Thus the algae were in all cases dominated by diatoms.

The ratios of CHLC to CHLA varied little with oil concentrations (p = 0.6413). In fact, Stauber and Jeffrey (1988) examined the photosynthetic pigments of 51 species (71 isolates) of tropical and sub-tropical diatoms from 13 out of 22 families. The results showed that chlorophyll c2 was present in all the diatoms used. In 88% of the diatoms tested, chlorophyll c1

and c2 appeared together. Thus the presence of both chlorophyll c1 and c2 cannot be considered as the universal characteristic of the diatom class.

β-CARO is thought to be a photoprotective pigments in chloropytes (Loeblich 1982), but plays a less significant role than diadinoxanthin and diatoxanthin in diatoms (Brown 1988). This is consistent with my experiments results (p = 0.8571). The ratio of *β*-CARO to CHLA remained constant and less than 0.021 for the algae at all the different concentrations of oil.

The FUCO to CHLA ratio was significantly correlated with oil concentration (p = 0.0127) in the first three months and ranged from 0.176 to 0.566. This result may indicate that the diatom community structure changed. In Fujiki and Taguchi's experiments (2001), with an irradiance of 330 umol quanta $m^{-2} s^{-1}$, three species of diatoms had different FUCO-to-CHLA ratios. In *Phaeodactylum tricornutum*, *Chaetoceros Gracilis*, and *Thalassiosira Weissflogii*, the ratios were 0.814, 0.454, and 0.452, respectively.

Various reports have indicated that diatoms usually show a greater sensitivity to dispersed oil when compared to other groups (Parsons, Harrison et al. 1984, Harrison, Cochlan et al. 1986), although there are always some special cases. For instance, Dahl et al. (1983) reported that the diatom *S. costatum* was very oil sensitive, while Vargo et al (1982) found that it was among the most oil-tolerant of the phytoplankton from a more temperate area. In Siron et al's experiment (1995), the microalgal community recovered when the oil concentration dropped below 1 mg L^{-1}. Only a few oil-adapted species grew again, including the diatom *Chaetoceros septentrionalis*. Haiso's report (1978) also indicated that *C. septentrionalis* is one of the most oil-tolerant diatoms at low seawater temperatures.

In fact, diatoms are highly sensitive to environmental changes and are among the bio-indicators of aquatic ecosystem health (LUAN Zhuo 2010, DING Teng-da 2012). Different species of diatoms have different adaptability to changes in water quality. While water quality changes the diatom species composition changes. Some species become more abundant; others die or are replaced by new diatom species. Currently, a widely used method of monitoring water pollution is use of diatom indicator organisms (Battegazzore M 2004). The fact that oil spills affect the diatom community structure has been examined.

Some examples have shown that diatom species change following an oil spill. In November 2005, a series of explosions occurred in one petrochemical plant in Jilin City, China. The blasts created an 80 km long toxic slick in the Songhua River, made up predominantly of benzene and nitrobenzene (BBC News, 2005). Research from December 2005 to September 2006 revealed the appearance of some diatom species characteristic of eutrophic conditions, such as *Melosira granulata*, *Surirella ovate*, and *Gomphonema angustatum*.Besides. One tolerant species, *Hantzschia amphioxys* var. major Grun, was much more abundant after the oil spill than before.

The pigment ratio of DDX and DTX to CHLA is a measure of the pool size of photoprotective pigments (Fujiki and Taguchi 2001). One of the most important factors affecting this ratio is irradiance. Fujiki and Taguchi's experiments (2001) showed that the ratios of DDX plus DTX to CHLA of all diatom species tested increased with increasing irradiance ($p < 0.05$), from 300 μmol m^{-2} s^{-1} to 750 μmol m^{-2} s^{-1}. Sakshaug et al(1991) also showed the diatoms *Thalassiosira nordenskioeldii* and *Chaetoceros furcellatus* contained twice as much DDX plus DTX relative to CHLA in high light (400 umol m^{-2} s^{-1}) than in low light (200 μmol m^{-2} s^{-1}). In

my experiments, the ratio of DDX and DTX to CHLA significantly decreased with the increasing oil concentrations (p = 0.0446). A possible explanation would be that the presence of the crude oil in the water reduced the amount of light that reached the cells. The oil may also have directly adhered to the phytoplankton and thereby reduced the amount of light that the phytoplankton received. When crude oil is chemically dispersed, a reduction of irradiance may occur due to the presence of a fine suspension of oil droplets. This reduction of irradiance may also contribute to the inhibitory effects on photosynthetic activity (SIRON, PELLETIER et al. 1995).

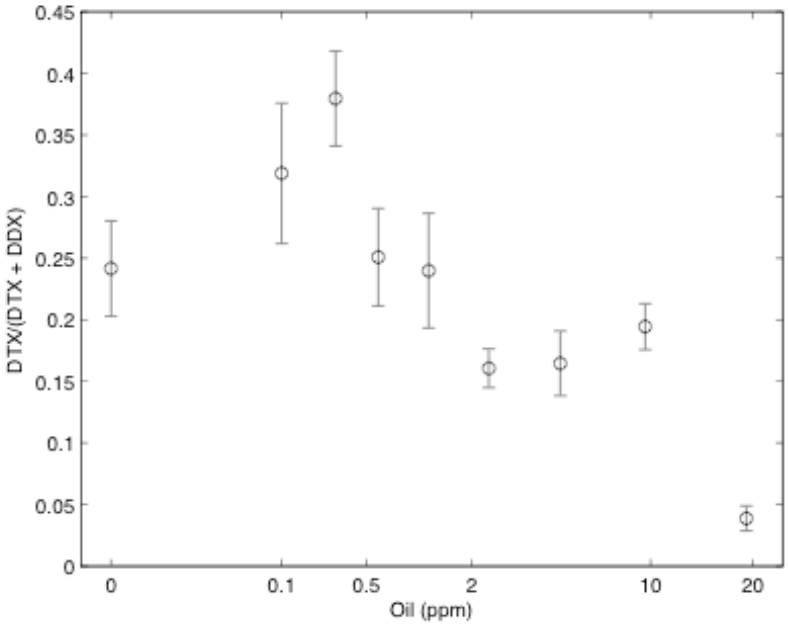

Figure 4.2.1 Ratio of DTX to DTX plus DDX in February, March and April

The pigment ratio of DTX to DDX plus DTX is a metric of the activity of the xanthophyll cycle (Fujiki and Taguchi 2001). As the oil concentration varied from 0 to 19.2 ppm in February, March, and April, that ratio significantly decreased (p = 0.0247) (Figure 4.2.1). In the xanthophyll cycle of diatoms, DDX is transformed into DTX to quench redundant energy. The

implication is that crude oil can suppress the xanthophyll cycle, and at high oil concentrations, diatoms would be more vulnerable to the stressful effects of bright light.

4.3 Synthesis and conclusions

4.3.1 Light absorption by pigments

To explore the implications of the changes I observed in the growth rates and pigment ratios of the phytoplankton, I first needed to know what fraction of visible light (400–700 nm radiation) was being absorbed by the various pigments. To make that calculation, I needed to know the in vivo absorption spectra of the various pigments. I was able to find graphs of the absorption spectra in a publication by Fujiki and Taguchi (2001), and the digitized data were kindly provided by Dr. Taguchi.

Fujiki and Taguchi (2001) generated their data from the absorption spectra of pure pigments standards, which were then wavelength-shifted to match their in vivo absorption peaks and shoulders using methods described by Bidigare et al. (1990).

The absorption coefficient is a measure of how strongly a pigment absorbs light at a given wavelength. The chlorophyll-specific absorption coefficient of phytoplankton is very important in estimating the amounts of light absorbed by the phytoplankton in bio-optical models of marine primary production (Koji Suzuki, Motoaki Kishino et al. 1998). Learning something about the absorption coefficient is helpful in understanding the effect of the crude oil on the growth rates of the phytoplankton.

To determine the fraction of visible light absorbed by each of these pigments, I multiplied the absorption spectrum of each pigment by the concentration of the pigment in the cultures at the end of log-phase growth and integrated the result from 400 to 700 nm. This

calculation implicitly assumes that visible light is truly white light, i.e., light with the same intensity at all wavelengths between 400 and 700 nm. I then divided the absorption by each pigment by the total absorption by all pigments to determine what fraction of visible light was being absorbed by each of the pigments.

Table 4.3.1 Fraction of visible light absorbed by each pigment as a function of oil concentration.

Oil	control	0.1PPM	0.3PPM	0.6PPM	1.2PPM	2.4PPM	4.8PPM	9.6PPM	19.2PPM
FUCO	0.2914	0.3273	0.343	0.3964	0.3898	0.388	0.4066	0.4071	0.4512
DDX+DTX	0.0644	0.0674	0.0548	0.0579	0.0471	0.0371	0.0363	0.038	0.0375
CHLC	0.1534	0.1171	0.1165	0.1216	0.1163	0.1163	0.1056	0.1043	0.1161
β-CARO	0.0257	0.0241	0.0179	0.0211	0.0217	0.0192	0.0186	0.0192	0.0238
CHLA	0.4651	0.4641	0.4678	0.4132	0.4199	0.4394	0.4329	0.4314	0.3714

Table 4.3.1 and figures 4.3.1.1 and 4.3.1.2 show the fraction of visible light absorbed by fucoxanthin and chlorophyll *a* as a function of oil concentration. For the cultures isolated in February, March, and April, the contribution of chlorophyll *a* to the total absorption varied from 47% in the control cultures to 37% in the cultures grown in 19.2 ppm oil. In the same cultures, the contribution of fucoxanthin to the total absorption varied from 29% in the control cultures to 45% in the cultures grown in 19.2 ppm oil. It is apparent from these two figures that chlorophyll *a* and fucoxanthin accounted for about 80% of the absorption of visible light by the cultures in all cases.

In the case of the cultures isolated in February, March, and April, the fraction of visible light absorbed by fucoxanthin was positively correlated with oil concentration (Spearman rank correlation coefficient = 0.93, p = 0.0007). There was no analogous behavior in the case of the

other antenna pigments—chlorophyll *c* and β-carotene, but these accounted for only a minor fraction of the light absorption.

In the case of the July-August cultures, there was no correlation between oil concentrations and growth rate, and there was no evidence of changes in pigment ratios or in the fraction of light absorbed by the various pigments. The pigment ratios in the July-August cultures were very similar to the pigment ratios in the February-March-April cultures at oil concentrations of 0.6–9.6 ppm.

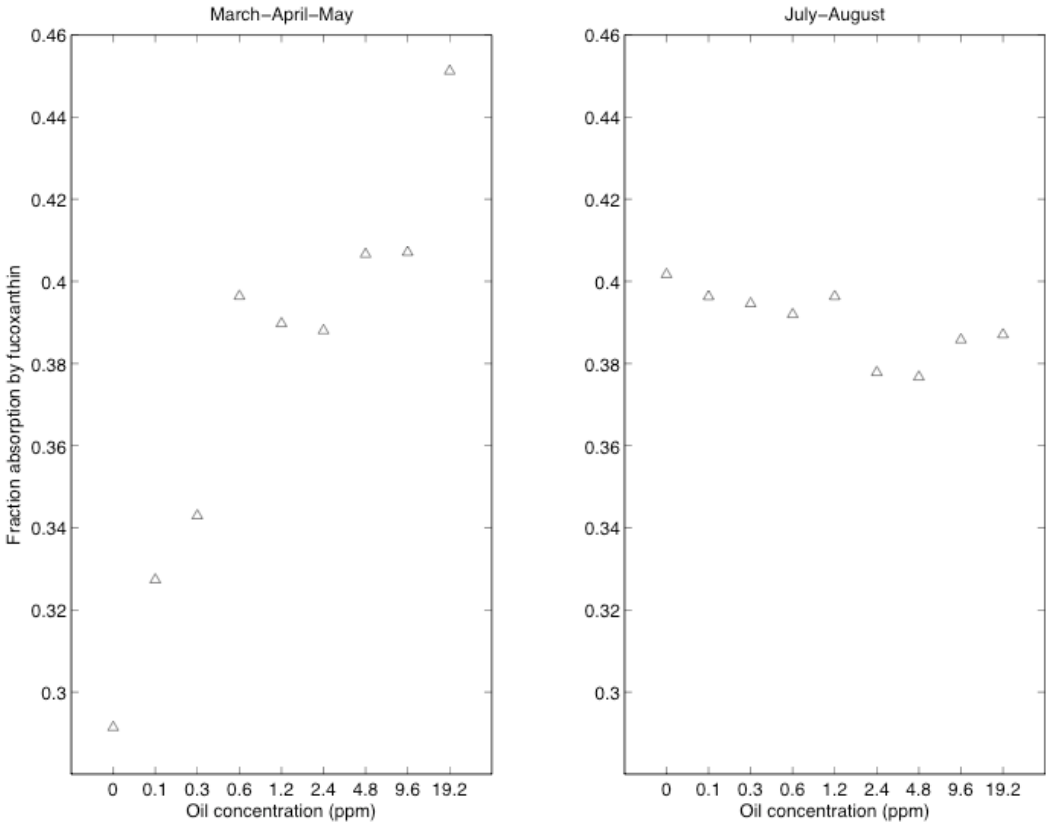

Figure 4.3.1.1 Fraction of visible (400–700 nm) light absorbed by fucoxanthin as a function of oil concentration for the cultures isolated in February, March and April (left) and July-August (right)

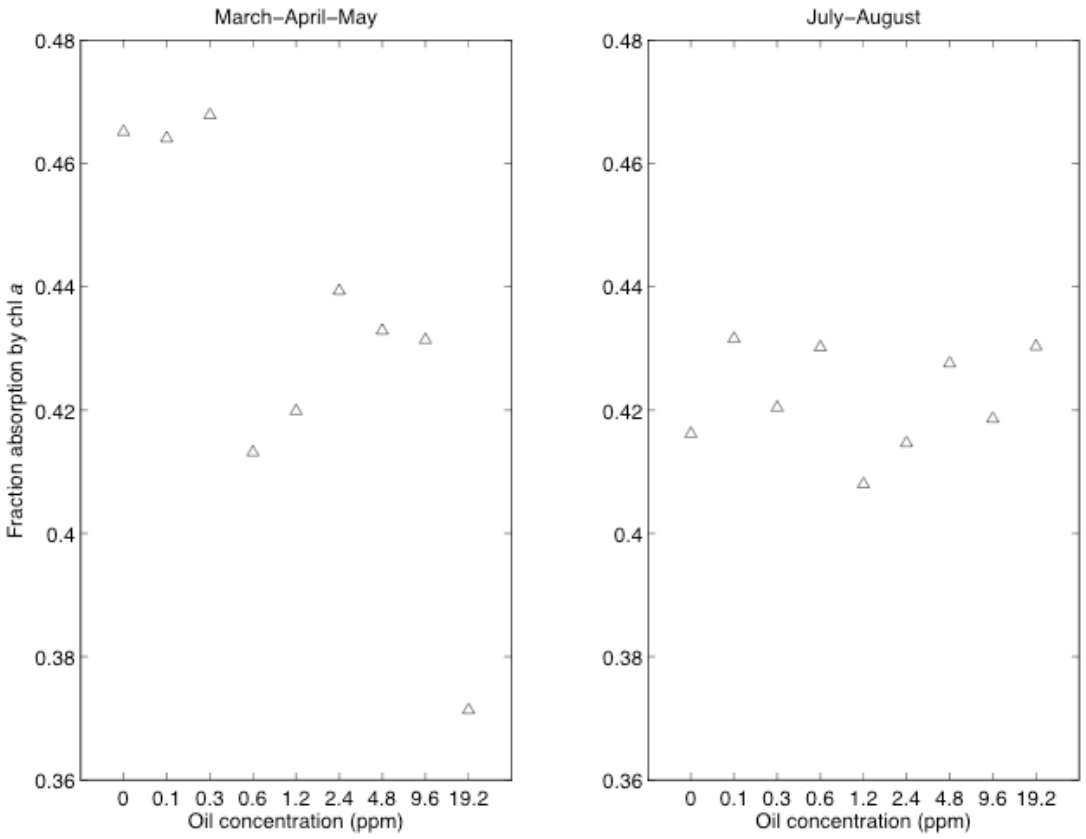

Figure 4.3.1.2 Fraction of visible (400–700 nm) light absorbed by chlorophyll *a* as a function of oil concentration for the cultures isolated in February, March and April (left) and July-August (right)

4.3.2 Phytoplankton growth model

The effect of the oil on phytoplankton growth rates and pigment ratios can be rationalized qualitatively with the use of the growth rate model originally developed by Shuter (1979) and later modified by Laws and Chalup (1990). According to the model, the carbon (C) in a phytoplankton cell is partitioned into four functional units:

$$C = R + P + S + E \quad (1)$$

Where C = carbon per cell, g cell^{-1}

R= Carbon allocated to storage, g C cell^{-1}

P= Carbon associated with the light reactions of photosynthesis, g $cell^{-1}$

S= Carbon allocated to structure, g C $cell^{-1}$

E= Carbon associated with the dark reactions of photosynthesis, g $cell^{-1}$

The model assumes that the cell allocates carbon to these four functional units in a way that maximizes growth rate under a given set of environmental conditions, specifically irradiance, temperature, and nutrient availability. Under nutrient-saturated conditions, maximization of growth rate implies that no carbon is allocated to storage. The percentage of carbon allocated to structure is assumed to be constant. Therefore, under nutrient-saturated conditions, only the percentages of carbon allocated to the light and dark reactions of photosynthesis vary. It is furthermore necessary that the rates of the light and dark reactions be balanced. This condition implies (Laws and Chalup 1990) that

$$f_p PI = K_e E \qquad (2)$$

Where f_p is the gross rate of photosynthesis per unit P per unit light intensity;

I is the incident irradiance; and

K_e is the gross rate of photosynthesis per unit E.

When the cells are stressed by the presence of oil, it is reasonable to assume that some resources are devoted to mitigating the effect of that stress. For example, cytochrome P450 monooxygenases mediate the oxidation of many toxic organic compounds. Wang et al. (2002) have noted that the protein content of phytoplankton cells is enhanced when the cells are exposed to anthracene. The up-regulated proteins are stress proteins, which are induced under a variety of stressful conditions. To the extent that resources in E are diverted toward

catabolizing toxic hydrocarbons, the result would be a decrease in K_e and hence a decrease in the growth rate of the cells.

Under nutrient-saturated conditions, the net growth rate, μ, of the cells is given by the equation (Laws and Chalup 1990):

$$\mu = \frac{K_e f_p (1 - r_g)(1 - S) I}{f_p I + K_e} - \frac{r_o}{C} \qquad (3)$$

where μ is the nutrient-saturated growth rate;

r_o is the basal respiration rate;

r_g is the growth-rate-dependent component of the respiration rate;

I is the irradiance, mol quanta $m^{-2}d^{-1}$.

Given the fact that f_pPI must equal K_eE, it is straightforward to show that under nutrient-saturated conditions the first term on the right-hand side of Eq. 3 equals $\frac{K_e E(1 - r_g)(1 - S)}{C - S}$. If K_e decreases as a result of diversion of resources in E to the catabolism of hydrocarbons, then the product f_pP must also decrease to maintain balanced growth. This can be achieved by reducing the number of photosynthetic units in P and allocating those resources to E until balanced growth is restored, with f_p remaining constant. An alternative scenario is that the phytoplankton community adjusts to the stress associated with the oil by shifting some resources from P to E and simultaneously increasing f_p in a way that maintains a balance between the light and dark reactions of photosynthesis (Eq. 2). Increasing f_p would allow more resources to be transferred from P to E and hence minimize the reduction in growth rate.

It is customary to assume that the size of P is proportional to the chlorophyll *a* content of the cell, which in turn is assumed to be proportional to the number of photosynthetic units

in the cell (Falkowski and Owens 1980). A photosynthetic unit is the smallest assemblage of molecules that can carry out photosynthetic O_2 production (Hangarter) . An increase in f_p could be achieved by increasing the size, but not the number, of photosynthetic units. An increase in the size of the photosynthetic units would imply an increase in the number of antenna pigments, i.e., an increase in the number of accessory pigments that absorb light and transfer the absorbed energy to chlorophyll *a* (Hangarter). In the case of diatoms, one of the most important antenna pigments is fucoxanthin (Papagiannakis, van Stokkum et al. 2005). My results (Fig. 1–2) show that fucoxanthin absorbed almost as much light as chlorophyll *a*, and fucoxanthin and chlorophyll *a* together absorbed 75–85% of the light absorbed by the cultures. It is therefore plausible that the contribution of fucoxanthin to total absorption increased in response to the stress associated with the oil.

4.3.3 Conclusion

The apparent change in the size of the photosynthetic units of the February-March-April cultures as a function of oil concentration could reflect either acclimation or adaptation. If the species composition of the phytoplankton community was similar at all oil concentrations, then the observed changes in pigment ratios can be attributed to acclimation. Although the cultures were in all cases dominated by diatoms, it is possible that the species composition of the diatom community changed systematically as a function of oil concentration, in which case the changes in pigment ratios might be attributed to adaption rather than acclimation. Without information on the species composition of the diatom communities in the cultures, it is impossible to say whether my observations reflect acclimation, adaption, or some combination thereof.

The July-August cultures behaved very differently from the February-March-April cultures. There was no effect of oil concentrations on either pigment ratios or growth rates. Another noteworthy difference between the July-August and March-April-May cultures is the fact that in the absence of oil the former grew almost twice as fast (average growth rate = 1.43 $\pm$ 0.11 d^{-1}) as the latter (average growth rate = 0.78 $\pm$ 0.07 d^{-1}). Because the growth conditions were identical, this observation suggests that the diatom communities in July-August and February-March-April were very different, and that the former were better able to tolerate exposure to oil than the latter.

During my experiments, I kept the experimental conditions the same, including the temperature and salinity. But actually they were both different from the temperature (18.1°C-28.3°C) and salinity (13.7PSU-21.2PSU) of the field samples. Besides, the real environmental conditions vary in different months.

I think next time we would use laboratory experimental conditions that are the same as the field conditions. For example, I could collect specific amounts of sea water, then add oil directly into the sea water in the lab, and control the lab temperature to be the same as the field temperature. If possible, we could do this experiment in the field.

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