ENRICHED MESOCOSM EXPERIMENTS TO STUDY THE PRODUCTION OF MARINE OIL SNOW IN THE PRESENCE OF BP SURROGATE OIL AND COREXIT 9500A

A Thesis

by

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ABSTRACT

During the Deepwater Horizon (DWH) oil spill, profuse marine snow with associated oil, termed marine oil snow (MOS) was observed but quickly disappeared. This research tested the hypothesis that in water with nutrients and microbes MOS formed in the presence of oil and oil plus dispersant. Four mesocosm experiments were undertaken as part of the ADDOMEx Consortium. Water was collected from near-shore (mesocosom 1, 2 and 4) or off-shore (mesocosm 3) in the Gulf of Mexico. Oil (Macondo surrogate oil) and oil plus dispersant (using Corexit 9500) mixtures known as water accommodated fraction (WAF), chemically enhanced water accommodated fraction (CEWAF) were generated in specially designed 170 L baffled recirculation tanks. WAF and CEWAF were then transferred to 106 L mesocosm tanks for the experiment as well as mesocosm control tanks (sea water only) and 10 times diluted CEWAF (DCEWAF) mesocosm tanks. Concentrated phytoplankton were added to mesocosm experiment 1 and 2. Nutrients were added to mesocosum 3 and 4. Estimated oil equivalents (EOE), Total petroleum hydrocarbons (TPH), including $\underline{n}$-alkanes and pristine and phytane, NO_3^-, NO_2^-, NH_4 and HPO_4 concentrations of mesocosms were measured over time. Exopolyomeric substances formed within 24 hrs in all treatments including the controls. EOE concentrations decreased at similar rates in all treatments. Oil components were removed by

formation and then sedimentation of MOS. Preferential removal of normal alkanes compared to branched alkanes (isoprenoid hydrocarbons) show that biodegradation was also occurring. Study results document that concentrations decreased partially due to sedimentation and biodegradation, although other weathering processes such as evaporation and photo-oxidation may also be responsible for the decrease in hydrocarbons in the mesocosms oil. Correlation between decrease in concentrations of EOE and nutrients indicate growth of microbes is important to MOS formation. The use of mesocosm studies provide a useful tool in understanding the mechanisms of MOS formation and transfer of oil from the water column to sediments.

DEDICATION

A mi padre,

Mi alma, mi fortuna

"To my father,

My soul, my fortune"

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NOMENCLATURE

ADDOMEx: Aggregation and Degradation of Dispersants and Oil by Microbial Exopolymers

BRT: Baffled Recirculation Tanks

CDOM: Dissolved Organic Matter

CEWAF: Chemically Enhanced Water Accommodated oil Fraction

CRM: Certified Reference Material

CROSERF: Chemical Response to Oil Spills Ecological Effects Research Forum

DCEWAF: Diluted Chemically Enhanced Water Accommodated oil Fraction

DCM: dichloromethane

DO: Dissolved Oxygen

DWH: Deepwater Horizon

EOE: Estimated Oil Equivalent

EPS: ExoPolymeric Substances

FGB: Flower Garden Bank

FLH: Fluorescent Line Height

GC/FID: Gas chromatography/Flame Ionization Detector

GERG: Geochemical and Environmental Research Group

HPO_4: Phosphate

LMWH: Low Molecular Weight Hydrocarbons

M1: Mesocosm 1

M2: Mesocosm 2

M3: Mesocosm 3

M4: Mesocosm 4

MC252: Macondo oil

MOS: Marine Oil Snow

Mt: Megatons

N: Nitrogen

NE: North East

NGOM: North Gulf of Mexico

NOAA: National Oceanic and Atmospheric Administration

NO_{3-}: nitrate

NO_{2-}: nitrite

NH_4: ammonia

P: Phosphorous

PAH: Polycyclic Aromatic Hydrocarbons

ppm: parts per million

RCM: total resolved mixtures

rpm: revolutions per minute

SOP: Standard Operating Procedures

T: Time

TEP Transparent ExoPolymeric substances

TIN: Total Inorganic Nitrogen

TIP: Total Inorganic Phosphorous

UCM: Unresolved Complex Mixtures

WAF: Water Accommodated oil Fraction

TPH: total petroleum hydrocarbons

QC: Quality Control

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INTRODUCTION

On April 20, 2010, in the northern Gulf of Mexico, the deep-sea petroleum-drilling rig Deepwater Horizon (DwH), owned by British Petroleum (BP), exploded, and released over the next 87 days, an estimated 3.19 (by a court decree) to 4.1 million barrels of Sweet Louisiana Crude Oil and 205,000 Mt of methane into the water column at a depth of 1500m (Graham et al, 2010; Harlow et al, 2011; Bælum et al, 2012). Both were expelled from the wellhead under considerable pressure, which lead to the formation of small oil-droplets (Socolofsky et al, 2011). Additionally, the depth and high pressure at which the release occurred, along with factors such as the interaction between oil and gas, and the solubility of each component, facilitated the formation of a deep-water oil plume ranging from 900 to 1200 m deep (Camilli et al, 2010; Socolofsky et al, 2011). Some of this oil also reached the seawater surface.

Only about 25% of the spill was successfully removed from the water using immediate response methods such as pumping, skimming, and burning (NOAA, 2017). The other 75% was left to settle or disperse and potentially undergo chemical and/or natural degradation (Graham et al, 2010). It is important to mention that of all interventions, chemical dispersion is considered the most effective (Bælum et al, 2012). Hence, 37,500 barrels of COREXIT 9500 and in lesser amounts COREXIT 9527, were sprayed on the surface of the

ocean and directly into the wellhead at a depth of 1500 m (Bælum et al, 2012). However, little is known about the effects and persistence of this dispersant/oil mixture and the polycyclic aromatic hydrocarbon (PAH) fraction of oil on the marine environment (Diercks et al, 2010; Bælum, 2012). Shortly after the DwH oil spill, profuse flocs of mucus-abundant marine snow with oil droplet inclusions were observed floating on the surface of the impacted region (Passow, 2014). The mucus associated within the marine snow is defined as transparent exopolymeric substances (TEP), and is produced by microbes (Passow, et al, 2012). Less than a month after the event, the marine snow had disappeared from the surface water (Passow, et al, 2012; Ziervogel, et al, 2014). This led to the hypothesis that the marine snow was formed *in situ* in the presence of oil, and eventually sunk into deeper waters (Passow, et al, 2012). If this is the case, this phenomenon could be an important contributor to the removal by sedimentation and degradation of oil.

Considering that marine snow is largely of biological origin, it is possible that a profuse marine snow formation near the spill site was due to a microbial bloom. Throughout the summer during the DwH oil spill, large volumes of nutrient rich fresh water from the Mississippi River were transported into the Northern Gulf of Mexico (Hu et al, 2011; Walsh et al, 2015). During this time, strong northeasterly winds caused this river plume to reach the area of the spill. Hu et al (2011) used MODIS satellite observations from July 2002 to January

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2011 to determine if there had been a significant change in surface phytoplankton biomass in the north eastern Gulf of Mexico before and after the DwH spill. Due to the interference of colored dissolved organic matter (CDOM) with the chlorophyll algorithm measurements, they used fluorescence line height (FLH) as a proxy for phytoplankton biomass over a period of nine years (2002-2011). They compared the patch FLH values from April 22nd to September 30th of 2010 with previous years in order to see if the norther Gulf of Mexico had become "greener" after the DwH spill. They discovered that in early August of 2010 there was a large and continuous patch of approximately 11,100 km^2 of a significant positive anomaly which disappeared by early September of the same year. Hence, they concluded that at that time there was a phytoplankton bloom, which started in early August and disappeared by early September. Based on Government reports, there was no visible surface oil after August 3rd, 2010 (Wade et al, 2011). Therefore, an increase in the sunlight penetration due the disappearance of the surface oil after this date could have triggered the reported phytoplankton bloom that may have been unrelated to the DWH oil spill.

It is well known that for mid latitudes, surface open-ocean waters usually have low nutrients due to the strong thermocline, and therefore plankton populations remain low. During wintertime however, surface water temperature decreases, weakening the thermocline and allowing nutrient rich deep-water to rise to the surface. With temperatures rising and sun incidence increasing

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throughout the spring, phytoplankton communities will bloom and rapidly consume the nutrients that had been previously upwelled. Zooplankton will follow the phytoplankton bloom and graze on it. This last factor plus the depletion of nutrients by the strengthening of the thermocline will make the phytoplankton population crash by the end of the spring. However, the seasonal northeasterly wind patterns in Gulf of Mexico from March to mid-June (Hetland & DiMarco, 2008; Fennel et al, 2011), are likely to have deflected the nutrient rich Mississippi flow east towards area of the spill. It has been suggested that the nutrients introduced by the Mississippi River may have led to enhanced productivity (Hu et al, 2011) and potentially to increased TEP formation (Quigg et al, 2016; Passow & Ziergovel, 2016) and consequently, produced enough marine snow to remove a portion of the DWH surface oil (Passow et al, 2012). Hence, it is imperative to understand the pathways by which oil was weathered (Overton et.al 2016) during the spill. Therefore, the Gulf of Mexico Research Initiative (GOMRI) funded the Aggregation and Degradation of Oil and Dispersants by Microbial Exopolymers (ADDOMEx) consortium as an effort to understand the role of TEP in the aggregation (sedimentation) and degradation of oil. The ADDOMEx consortium has put in place a series of experiments that will potentially explain how the production of TEP by specific phytoplankton and bacteria in the presence of hydrocarbons will simultaneously protect these organisms and contribute to the degradation of oil.

The objective of this consortium is to establish a mechanistic understanding for the interactions of the Macondo surrogate oil and Macondo surrogate oil/dispersant (COREXIT 9500) with TEP under various environmental conditions. It hypothesizes that bacteria and phytoplankton respond to oil and COREXIT 9500 by producing exopolymeric substances, which interact with minerals, organic particles and organisms; and consequently influence the fate, distribution and potential effects of these hydrocarbon. In addition, it proposes that in the presence of oil and/or COREXIT 9500, some members of the microbial community will break down hydrocarbons as a means of obtaining their source of carbon and energy.

This research hypothesizes that the addition of nutrient rich water in WAF and CEWAF could influence microbial activities that lead to the formation of MOS in the surface water. It proposes that some of the surface oil was removed by sedimentation of MOS. In addition the TEP/oil/microbe association enhanced biodegradation of the oil. The ADDOMEx consortium undertook a series of experiments with the objective of explaining the process of TEP formation leading to the sedimentation and degradation of oil.

LITERATURE REVIEW

Marine Snow

In order to understand the bacterial degradation of oil, it is important to define marine snow, its components, and its role in the marine environment. In 1941, Rachel Carson described in her book titled "Under the Sea", the presence of negatively buoyant particles in the ocean as "stupendous snowfall". However, it was not until 1953 that Suzuki and Kato made a broader description of these particles and, in honor of Carson, named them marine snow. Marine snow is composed of all particles, organic and inorganic, larger than 500 μm in diameter, and as it settles, it is one of the most important mechanisms by which surface derived materials reach deep water and the ocean floor (Alldredge & Silver, et al, 1988). It is formed when lysed plankton cells are aggregated by bacteria and detritus suspended in the water (Kato & Suzuki, 1953).

The aggregates forming marine snow serve as microhabitats, which are usually nutrient rich and have complex microbial assemblages (DeLong, et al., 1993). These marine aggregates are composed of two major groups: The first one includes large fecal pellets, zooplanktonic carcasses, and gelatinous phytoplanktonic sheaths, which aggregate once they start to decay. In this category, zooplankton is the major component of marine snow. Some zooplankton groups, such as appendicularians, feed by secreting a sticky

gelatinous "house" that can collect phytoplankton, bacteria, and detritus (Alldredge & Silver, 1988). The second group is smaller in diameter, and includes phytoplankton, especially diatoms, bacteria, small fecal pellets, microaggregates, and inorganic particles (Alldredge & Silver, 1988). They are aggregated by TEP secreted by diatoms and bacteria (DeLong, et al, 1993).

However, the formation process of marine snow occurring during an oil spill is still not clear. To this date, two hypotheses have prevailed because both situations have been observed in multiple experiments. The first one includes the formation of mucus threads that hang from a microbially produced biofilm associated with the surface oil layer. The mucus is made of extracellular polymers, and is mainly produced by oil-degrading bacteria (Passow, et al, 2012). These organisms are thought to produce sufficient amounts of transparent exopolymeric particles (TEP) that will emulsify oil. The latter will allow them to target easily metabolized, soluble compounds, ergo low molecular weight hydrocarbons, which they will uptake. The marine snow produced through this process is extremely sticky and any particle that collides with it will adhere to it. Hence, it will become a rich substrate that can be continuously colonized by bacteria, increasing in biomass and dimensions over time (Passow, et al, 2012; Ziervogel, et al, 2012).

The second hypothesis is more physical since it involves a direct formation due to collision and cohesion of particles. The oil components in this particular process will be limited to polar, heavy, persistent hydrocarbons such as asphaltenes and resins (Passow, et al, 2012; Ziervogel, et al, 2012). This is due to their resistance to biodegradation and weathering, which allows them to accumulate in the system (Passow, et al, 2012). Asphaltenes and resins generate a stable emulsion that serves as a coagulation core, and this generates oil aggregates (Ziervogel, et al, 2012). In this case, bacteria will secrete transparent exopolymeric substances (TEP) as a response to the presence of these particles, and incorporate the polar hydrocarbons into the marine snow flocs (Passow, et al, 2012). Despite the significant efforts made by these authors, much of the relationship of the bacteria with the oil and the formation of MOS remains uncertain. These hypotheses have not been tested in a biogeochemical study targeting the fate of single hydrocarbon compounds.

Transparent Exopolymeric Particles

Transparent exopolymeric particless (TEP) are highly sticky, large gels (< 500 μm) formed by polysaccharides (Passow, 2002). They are formed in surface waters, especially during phytoplankton blooms, and particularly by diatoms (Passow, et al, 1994), and bacteria (DeLong, et al, 1993). This process can occur either naturally during their growth cycle and lysis (Alldredge, et al, 1993; Passow, et al, 1994), under high nutrient concentrations or under stressful

conditions such as light deficiency (DeLong, et al, 1993). On a broader scope, the existence of TEP has an important impact in the dynamics of marine snow as a whole. TEP may be used as a food source by grazing zooplankton species, and they can also serve as a substrate and attachment surface for diatoms and bacteria (Passow, et al, 2012).

Experiments done by Passow, et al (1994) compared the number of diatom and bacterial cells associated with TEP, versus free-living cells. In both cases, the percentage of cells associated with TEP was low before the communities bloomed. This is due to the fact that at the start of the experiment (before bloom), cell numbers were too low to produce a significant amount of TEP. Once the cell numbers started increasing, TEP were secreted, and a higher percentage of both diatoms and bacteria were found attached to the exopolymer compared to free-living cells. The maximum number of cells associated with TEP for the two groups was reached in their late bloom, and by the time their population crashed, most cells were attached to the TEP. In the control treatment for this experiment neither the diatoms nor the bacteria bloomed, and hence there was not sufficient TEP to serve as attachment surface. The researchers demonstrated that microbes attached to TEP will increase in number and succeed better than free-living organisms. Some reasons may be that residence on the substrate increases their feeding efficiency and protection from predators (Alldredge, et al, 1993).

Furthermore, as diatoms and bacteria increase in cell number, and subsequently secrete more TEP, the smaller aggregates will tend to coagulate, increasing in diameter (Alldredge, et al, 1993). In 2010, Passow, et al (2012) took MOS samples from the DWH site and measured their size and sinking velocity under a dissecting scope and in a settling column. The excess density of each marine snow particle was calculated from its sinking velocity and its equivalent spherical diameter. There seemed to be a close relationship between the diameter size of the aggregates and their sinking velocity. This indicates that there was not only an increase in size, but also an increase in density, which was most likely due to the incrusted oil droplets and the biodegradation processes that occurred within the aggregates (Passow, et al, 2012).

Bacteria, Marine Snow, and Degradation of Oil

Oil seeps occur naturally on the ocean's floor. For millions of years, an estimated 600,000 tons year^{-1} of oil has entered the ocean from subseafloor seeps (Prince, 2005). Specific annual rates for the Gulf of Mexico suggest that at least 20,000 m^3 yr^{-1} of oil spilled into this basin come from natural seeps (Macdonald et al, 1993). Therefore, many microorganisms, such as bacteria have evolved to obtain their carbon and energy from this source. In 2005, more than 75 genera belonging to the domain Bacteria had been described to be able to grow on petroleum (Prince, 2005). Several authors have demonstrated an

increase in bacterial and diatom density in the presence of oil (Bælum, et al, 2012; Jung, et al, 2010; Passow, et al, 2014). In the latter experiments there was a decrease in oil concentration with time resulting from the combination of mixing, dispersion, and biodegradation by microbes, such as indigenous bacteria residing in the water column (Figure 4) (Lu, et al, 2012). To some extent, microbes' energy metabolism may be enhanced as a result of oil degradation and/or using the oil as a carbon substrate (Bælum, et al, 2012; Jung, et al, 2010), which will thus induce changes in the microbial community (Acosta-González, et al, 2013). In 2010, Bælum, et al (2012), isolated a strain of *Colwelliaceae* to determine its capacity for oil degradation. They incubated it in an oil and dispersant dilution, then transferred it to Marine Broth agar plates, and finally placed it in a liquid medium containing 100 ppm of MC252 oil and 60 ppm COREXIT. After 10 days of incubation, the bacteria had degraded approximately 75% of the oil. This demonstrates the potential importance of bacteria in the degradation of oil.

Around 75 known marine and land bacteria genera, including *Cyanobacteria*, *Proteobacteria*, and others, are capable of growing and feeding on and as a result, degrading hydrocarbons. However, only a few of them have been proven capable of using oil as their sole source of nutrition and energy (Prince, 2005). The bacterial bloom that follows a spill is quickly limited by the shortage of other essential nutrients such as nitrogen, phosphorous, or iron, and

other physiochemical necessities such as dissolved oxygen, which when deficient may generate anaerobic conditions (Magot, 2005; Prince, 2005). The reason for this is that crude oils and refined fuels are relatively uncommon substrates that supply extremely high concentrations of carbon, but none of the other essential nutrients. Therefore, in the event of an oil spill, where the oil covers a large portion of the surface water, biodegradation will usually be limited by the shortage of these nutrients (Prince, 2005). Hence, the planktonic bloom and following formation of marine snow during the DwH spill was unexpected. The relatively high availability of vital nutrients such as N and P followed by an over-abundance of an oil C-source, could likely be the sequence creating such a dramatic microbial bloom in the vicinity of the DwH spill which then led to the profuse formation of marine snow. Following the addition of chemical dispersants such as COREXIT, an oil slick can be broken into small droplets to increase the dilution of the hydrophobic fraction of oil, and make the ambient concentrations of nitrogen and phosphorous sufficient to allow effective biodegradation (Prince, 2005).

Even though the natural and anthropogenic input of crude oil into the sea is substantial, its components are dispersed throughout the water column (MacDonald et al, 1993). Hence, excepting the immediate waters to the oil seeps and spills, the hydrocarbon concentrations in the oceans are quite low. Oil-degrading bacteria are adapted to this pattern (Prince, 2005), and have

extremely low abundances in marine environments (Harayama, et al, 2004; Prince, 2005). Microbial oil degraders are widespread, but they will only be quantitatively dominant in regions where there is a large input of oil (Prince, 2005). Therefore, an oil spill of whatever magnitude may stimulate the growth – up to a 1000 fold increase - of oil degrading organisms, and cause changes in the structure of microbial communities in the contaminated area (Harayama, et al, 2004; Prince, et al, 2005; Jung, et al, 2010).

Not only did the DwH release a large amount of oil into the ocean, it also released substantial amounts of methane. Therefore, the microbial populations in the contaminated water quickly shifted to both a methanotrophes and oil-degrader dominated community. Also, the temperature differences between the surface waters (~20˚C), and deep waters (~4˚C) had a direct effect on the bioavailability of oil and microbial physiology, which thus modifies microbial composition and density (Redmond & Valentine, 2012).

QUESTIONS AND ASSOCIATED HYPOTHESIS

One of the ADDOMEx hypotheses suggests that bacteria and phytoplankton respond to oil and COREXIT 9500 by producing exopolymeric substances, which interact with the oil, minerals, organic particles, and organisms; and consequently affect the fate, distribution and potential impacts of the oil. Additionally, it proposes that in the presence of oil and/or COREXIT 9500, the microbial community will break down hydrocarbons as a means of obtaining its source of carbon and energy.

For this thesis research, I hypothesize that the addition nutrient rich water can promote a microbial bloom in WAF and CEWAF treatments. I propose that CEWAF can facilitate the adhering of a fraction of oil and its components (n-alkanes) to TEP, and thus this oil can be removed from the water column by sedimentation and biodegradation.

In order to test these hypotheses the following questions were addressed in this research:

1) Is MOS formed in WAF, DECWAF and CEWAF treatments?
2) Does MOS and its associated microbes remove oil from the water column by biodegradation and/or sedimentation?

3) Does the addition of nutrients enhance the formation of MOS?

4) Does the concentration and composition of the oil change over time?

5) Does degradation of aliphatics in treatments with COREXIT differ from treatments without dispersant?

6) Does the addition of microbes enhance the formation of MOS?

METHODS

Baffled Recirculation System

The design of the baffled recirculation tanks (BRT) is a modification of Knap, et al (1983). The tanks are 40x40x72 cm, with a total capacity for 170 L however in the case of these experiments they contained 130L. The materials used were non-tempered glass (1/2 in thick) and transparent silicone. Four baffles with two different heights were installed in order to guide the flow of the accommodated fractions of oil and dispersant through the tank. The tanks were previously aged for many seawater cycles with oil and dispersant, depending on
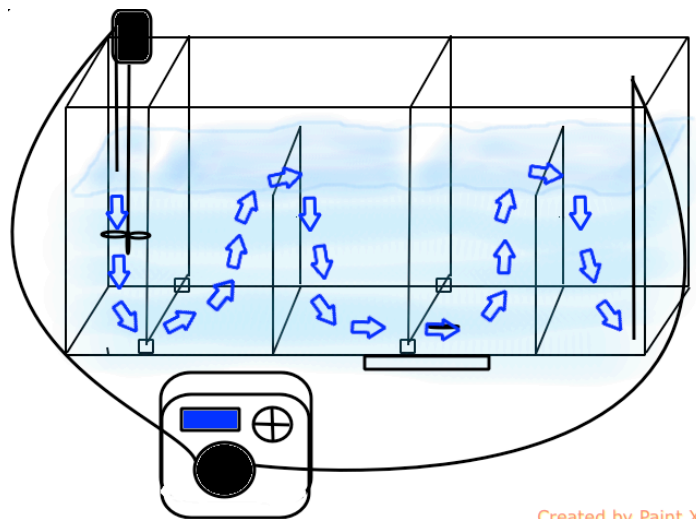
Figure 1. Baffled recirculation system

the purpose of tank, in order to saturate the silicone and prevent absorption of hydrocarbons or contamination of silicone compounds during the actual experiments.

A Masterflex PTFE-Diaphragm Pump with Teflon heads and tubing were used to recirculate the water. The Teflon tubes were connected to two stainless steel tubes for better stability in the system. The inflow was placed in the first chamber (left to right), and the outflow in the last (Figure 1). In addition to the diaphragm pump, mixing was aided with one Thermo Scientific electromagnetic stirrer and one Arrow 1750 electric stirrer.

WAF and CEWAF Generation

The objective of this part of the experiment was to generate reproducible amounts of WAF, CEWAF and DCEWAF at a specific concentration that were later transferred into the mesocosm tanks. The Chemical Response to Oil Spills Ecological Effects Research Forum (CROSERF) has defined water-accommodated fraction (WAF) as "*a laboratory-prepared medium derived from low energy (no vortex) mixing of oil, which is essentially free of particles of bulk material*" (Singer, et al, 2000). In most cases the CROSERF method is used to provide WAF, however our needs were hundreds of liters at a time so we used the BRT. The oil used in this project was the Macondo surrogate oil from the Marlin Platform Dorado, which has a similar specific gravity of 0.86 as the

Louisiana Sweet Crude Oil spilled during the BP incident in 2010. The dispersant used was COREXIT 9500A.

Approximately 120 L of the filtered seawater was transferred to each baffled recirculation tank where the WAF and CEWAF were produced. The BRT physically dispersed Macondo surrogate oil and dispersant (COREXIT 9500) with the flow generated by the PTFE-Diaphragm pump that recirculated the seawater at 250 rpm (or 333 ml min^{-1}); however mesocosm 1 used higher a higher stirring rate. In addition, the electromagnetic stirring plate (only for mesocosm 1 and 2) and the electric stirrer, at rates no higher than 200 rpm to avoid creating a vortex in the water, were used as mixing energy sources. By using low energy mixing, dispersion and emulsification of the oil was easily prevented (Singer, et al, 2000).

Under common production procedures, WAF concentrations in laboratory studies can range from 1 to 20 mg/l (Knap, et al, 1983). When using BRT, concentrations ranged from 0.2 to 1 mg/L for WAF, 2-8 mg/L in the DCEWAF and 20 to 80 mg/L in the CEWAF. However, since the specific gravity of the Macondo surrogate oil is of 0.86, 23 mg/L of the oil were added to the WAF-BRT. In the case of the CEWAF recirculation tank, a premixed 1:20 (1 mL dispersant: 20 ml oil) dilution was added to its corresponding BRT. The oil is added in

excess to the amount of oil required for WAF in the 170L baffled recirculation tank.

By adding aliquots of oil and dispersant, and measuring the oil concentration in the WAF, CEWAF and DCEWAF in a Horiba Scientific Aqualog fluorometer, it was possible to calibrate the addition of oil with its concentration over time. The oil content of water (or water accommodated fraction of oil) was measured every three hours for a period of 24 h. For each measurement, five milliliters of water was extracted from each BRT and diluted in 5ml of dichloromethane (DCM). Approximately 2 ml of the DCM fraction was transferred into cuvettes and analyzed for estimated oil equivalent (EOE) by fluorescence using a Horiba Aqualog spectrofluorometer. Optimum wave-lengths for EOE were λ: 260 nm and λ: 358.29 nm. After 24 hours it was assumed that the oil concentration in the water had reached its maximum, and therefore, the generation process had been completed (Knap et al, 1983).

Sample Collection

During the last week of July and third week of October of 2015, ~1000 L of seawater from the NOAA National Marine Fisheries Laboratory in Galveston. For the third experiment, during the second week of July 2016, the Trident vessel collected ~1500 L of seawater from the coral reef system called "the Flower Garden National Sanctuary" (27° 53.4180'N; 94° 2.2020'W) which is

located ~120 miles off the coast of Galveston (TX). Finally, for the fourth experiment, the same vessel collected 2000 L of sea water off the coast of Texas, near the TGLO (Texas General Land Offices) Texas Automated Buoy System (TABS) buoy R (Figure 2).
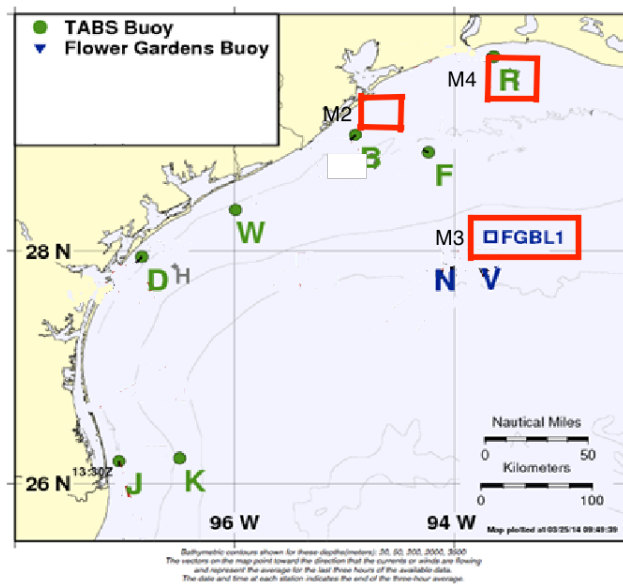
Figure 2. Modified Map of TABS buoys. Sample sites of mesocosm 2, 3 and 4 are marked in red box. M2: mesocosm 2; M3: mesocosm 3; M4: mesocosm 4 (Credit: GERG)

Mesocosm Experiments

During the first experiment setup, four 130 L mesocosm tanks were filled with 81 L of oil only water accommodated fraction (WAF) or oil plus dispersant fraction (CEWAF and DCEWAF). The first tank used as the control was filled

with untreated seawater, the second tank with WAF, the third with DCEWAF, and the last one with CEWAF. For the second, third, and fourth experiments each treatment was done in triplicate, having a total of 12 mesocosm tanks. F/20 media nutrients prepared according to the specifications of Guillard and Ryther (1962) and Guillard (1975) were added at the beginning of mesocosm 3 and 4, Additionally, artificial light was used to simulate 12 h light/12 h dark periods.

Estimated Oil Equivalents

Before each experiment, a calibration curve was generated using a Macondo surrogate standard at five different concentrations and run through the Horiba Scientific Aqualog fluorometer. The fluorometer was used to find the maximum fluorescence and then the concentrations in the samples to be analyzed were calculated. Every 24 h five milliliters were taken out of each treatment and its triplicates, and diluted in 5ml of dichloromethane (DCM). Approximately 2 ml of the DCM fraction of each experiment were transferred into cuvettes and analyzed for EOE by fluorescence using a Horiba Aqualog spectrofluorometer. Optimum wave-lengths for EOE were λ: 260 nm and λ: 358.29 nm. In order to accurately determine the EOE, all samples were compared to the calibration curve.

Alkane Analysis

A Macondo surrogate oil (Marlin oil) standard and samples taken every 24 hours from the control, WAF, DCWAF and CEWAF treatments of mesocosm 3 and 4 were analyzed in a GC/FID. The targeted alkanes were from C10 to C35. The SOP-0008 was followed in order to quantitate the n-alkanes, pristine, phytane, total resolved (RCM) and unresolved complex mixtures (UCM), and total petroleum hydrocarbons (TPH). Samples previously extracted in DCM were analyzed using a high resolution capillary gas chromatograph with flame ionization detector (GC/FID). Each sample and QC extract had an adequate amount of its internal standard. Then the analytical run sequence for the extracts was entered. A calibration check was run between every ten samples.

The individual sample concentrations were determined with the following formula:

$$C = \frac{(A_S \; x \; C_{SU} \; x \; D)}{(A_{SU} \; x \; RF \; x \; W_S)}$$

where:

C $\quad$ = $\quad$ Concentration in sample (ng/g)

A_S $\quad$ = $\quad$ Area of the peak to be measured

A_{SU} $\quad$ = $\quad$ Area of the surrogate standard (deuterated n-C20)

C_{SU} $\quad$ = $\quad$ Amount of surrogate standard added to each extract (ng)

RF   =   Average response factor for an alkane based on the initial calibration

W_S =   The original weight of dried sample extracted (g)

UCM and RCM calculations were made using the average response factor of all n-alkanes, and the sum of all the unresolved peaks minus all the surrogate and internal standard peak areas respectively. The TPH concentration was calculated using the sum of the total UCM and RCM concentrations.

Dissolved Nutrients

In order to conduct a duplicate analysis of each sample, a volume of 30mL was necessary. For the duration of each experiment, 30mL of each treatment were collected as triplicates and filtered under vacuum with a 45 µm Milipore filter and kept frozen until its analysis at Geochemical Environmental Research Group (GERG). All nutrient samples were analyzed with an Astoria Pacifica Auto-Analyzer. The nutrient analysis followed the GERG ARM-SOP-0702. Five standards prepared with specific ranges, a NO_{2-}, NO_{3-}, and a Certified Reference Material (CRM) were run before each sample run. The CRM was also analyzed between each batch of 12 samples with a blank determination. To determine the spike recovery percent, a CRM and a replicate sample were utilized.

Peak heights were analyzed and converted into µmol/l concentrations using the Flow Analyzer Software Package II (FASPACII); which controls, collects and processes data from six digital channels and one analog channel simultaneously from the Astoria Pacifica auto-analyzer. Dissolved inorganic nitrogen was calculated by adding NH_4, NO_2 and NO^3 values. Redfield ratios were also made for each mesocosm. Comparisons between treatments and mesocosms were made, as well as correlations to the other measurements taken.

RESULTS AND DISCUSSION

Transparent Exopolymeric Substances

Transparent exopolymeric substances (TEP) formed within 24 hours in all experiments. The presence of dispersants can lead to an increase in the surface friction and collision among particles, aiding in the formation MOS (Fu et al 2014). Dispersants are known to compress the diffuse layer between particles and promote the increased aggregation rates of particles (Hayworth & Clement, 2012). Particularly, COREXIT 9500A, which consists of nonionic and anionic surfactants, can facilitate the aggregation mechanisms due to the hydrophobic tails and hydrophilic heads (Hayworth & Clement, 2012). In addition, oil and dispersants can enhance the formation of bacterial TEP because they serve as an additional carbon source (Gutierrez et al, 2013). The TEP can also emulsify oil, increasing the bioavailability of hydrocarbons (McGenity, 2014).

Estimated Oil Equivalent

Estimated oil equivalent (EOE) measurements of the WAF, DCEWAF and CEWAF of mesocosms 2, 3 and 4 are provided in Table 1. Measurements from mesocosm 1 were not considered for this analysis as it was a pilot experiment and the variability exceeded confidence intervals. M2, 3 and 4 had slightly different initial concentrations. However, the EOE increase with the addition of oil and oil/dispersant was consistent between the mesocosms (Figure 3).

Table 1. Estimated oil equivalent (EOE) measurements over time of WAF, DCEWAF and CEWAF of mesocosm 2, 3 and 4.

Time (hr)	Mesocosm 2			Mesocosm 3			Mesocosm 4		
	WAF	DCEWAF	CEWAF	WAF	DCEWAF	CEWAF	WAF	DCEWAF	CEWAF
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
0	0.26 (±0.01)	2.74 (±0.47)	41.53 (±3.44)	0.74 (±0.52)	6.17 (±1.34)	39.07 (±0.78)	0.29 (±0.03)	8.13 (±0.97)	81.06 (±20.5)
24	0.09 (±0.01)	1.59 (±0.12)	19.45 (±4.11)	0.43 (±0.22)	5.65 (±0.32)	24.20 (±2.80)	0.14 (±0.04)	5.40 (±0.91)	38.77 (±3.56)
48	0.07 (±0.03)	1.33 (±0.09)	25.78 (±4.58)	0.30 (±0.15)	4.21 (±0.61)	19.63 (±2.53)	0.09 (±0.01)	4.00 (±1.04)	33.17 (±4.64)
72	0.06 (±0.01)	1.03 (±0.09)	17.31 (±5.95)	0.46 (±0.51)	3.20 (±0.81)	12.39 (±1.97)	0.03 (±0.01)	1.84 (±1.12)	19.83 (±1.31)
96	-	-	-	0.07 (±0.05)	2.71 (±0.16)	8.21 (±2.57)	-	-	-

±: standard deviation; -: no data taken at that time point

Nonetheless, EOE decreased with time in all treatments and at a similar rate per hour (Table 2). These rates were calculated from the exponential equations used to measure the decreasing EOE. The percent change per hour in all treatments and mesocosms ranged from -0.9 to 3.2%. At the end of the experiments only 31, 37 and 33% of the oil remained in the WAF, DCEWAF and CEWAF tanks respectively.
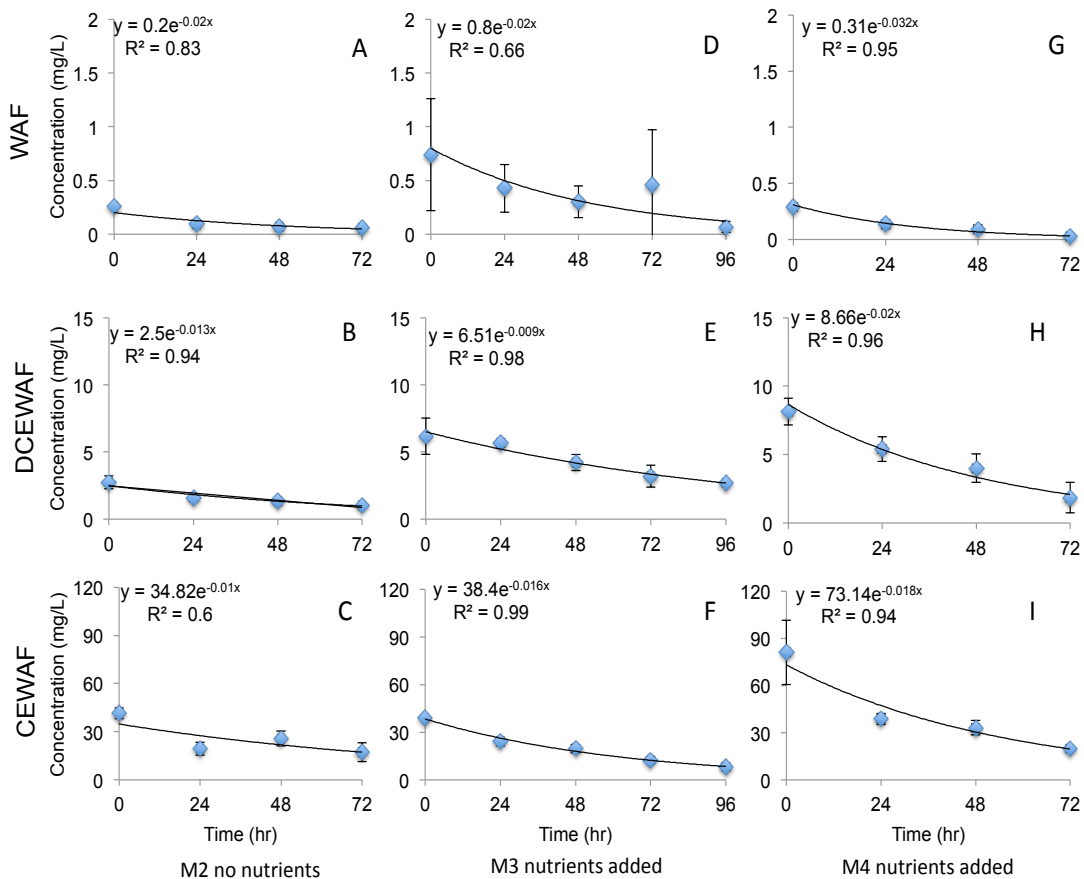
Figure 3. Estimated Oil Equivalence (EOE) of the averaged triplicated of water accommodated fraction (WAF), diluted-chemically-enhanced-water-accomodated-fraction (DCEWAF) and chemically-enhanced-water-accomodated-fraction (CEWAF) of mesocosm 2, 3 and 4.

EOE concentrations of the controls in mesocosms 2 and 3 were below detection limits; however during mesocosm 4, a larger water sample was extracted and solvent evaporated to provide lower detection limits .The control triplicates had an average initial concentration of only 0.04 mg/L. This is reasonable for coastal waters (Wade et al 2016). Measurements for the water-accommodated-fraction (WAF) in mesocosm 2, 3 and 4 were below 1 mg/L (Figure 3A, D, G). However, in mesocosm 3 (Figure 3D) the standard deviations (shown as error bars) of all time points are considerably higher, indicating elevated variability in the triplicates. Many factors need to be taken into consideration when preparing WAF, including if filtrations and sterilization will be needed, as well as the mixing energy and duration (Singer et al 2000). In addition, previous studies have proven consistent absence of oil droplets, indicating that those concentrations, even if truly dissolved into solution, were

Table 2. EOE percent change per hour in WAF, DCEWAF and CEWAF treatments of M2, M3 and M4

	M2 (%)	M3 (%)	M4 (%)
WAF	-2.0	-2.0	-3.2
DCEWAF	-1.3	-0.9	-2.0
CEWAF	-1.0	-1.6	-1.8

*Negative values indicate the percent loss per hour

highly variable (Sandoval et al, 2017). While other WAF generation procedures, such as the CROSERF technique,have less variable measurements (Singer et al 2000), they fail to produce large enough quantities for mesocosm experiments. The variability in concentration was higher the WAF treatments than in the DCEWAF and CEWAF but it is important to take into consideration the lesser concentrations of oil dissolved in the water, which made it easier to remove/degrade.

All DCEWAF treatments had strong linear relationships with time (R^2 >0.90). The starting EOE concentrations ranged from 2.74 to 8.13 mg/L (Figure 3B, E, H). CEWAF concentrations from the three mesocosms ranged from 39.07 to 81.06 mg/L. This was expected because the introduction of chemical dispersants reduces the surface tension of the oil creating small droplets and increases the concentration of oil (Singer et al 2000; Wang et al, 2016). DCEWAF and CEWAF correlations with time in mesocosm 3 and 4 (Figure 3) had higher R^2 compared to mesocosm 2. Also, the rates of change per hour of mesocosm 3 and 4 (Table 2) were slightly higher than in mesocosm 2. This suggests that the addition of an external source of nutrients may have accelerated the degradation of oil (Coulon et al, 2005). The EOE concentration at time zero in mesocosm 4 was a factor of 2 higher than that of mesocosm 2 and 3. This shows the variability of the process of producing large volumes of WAF and CEWAF.

Alkanes

From the aliphatic fraction, n-alkanes are saturated, straight chain hydrocarbons with single bonds that can be easily biodegraded. This biodegradation is done mainly by oxidation of the terminal carbon atom, hence aerobic conditions are needed (Turner et al, 2014a; Turner et al, 2014b). The composition and abundance of the Macondo surrogate oil used in these
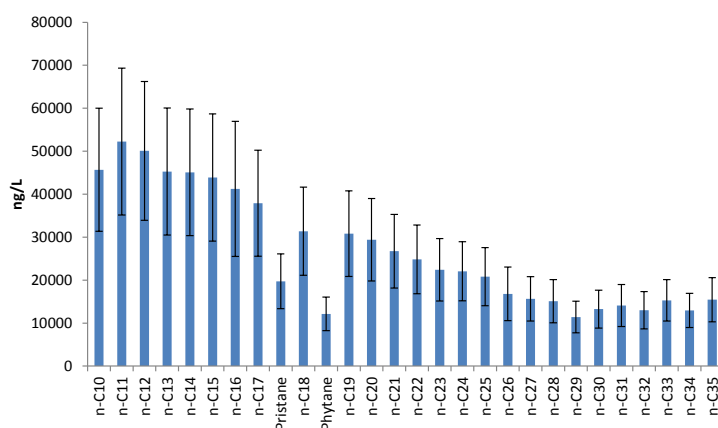
Figure 4. Macondo surrogate oil abundance

experiments was determined in order to have an established fingerprint for oil and oil plus dispersant treatments. The DwH oil and its homologue, the Macondo surrogate oil, have unique qualities. In the first place, as typical light Louisiana crude oils, they are composed of saturated n-alkanes, polycyclic

aromatic hydrocarbons (PAH) and alkylated PAH (Liu et al, 2012). Figure 4 shows higher abundances of the shorter chain alkanes, which coincides with the nature of most light Louisiana crude oils where low molecular weight (LMW) hydrocarbons (C2-C11) contribute more than 50% of the oil (Liu et al, 2012). This composition makes this oil subject to rapid weathering such as evaporation, dissolution, photoxidation and biodegradation (Leahy & Colwell 1990; Ryerson et al, 2011).

The n-alkane analyses were only done for mesocosm 3 and 4 and only the n-alkane compounds from nC10 to nC35 were quantitated to understand their role in biodegradation. In both experiments the n-alkane concentrations varied significantly within treatments, as expected due to the dispersing effect COREXIT has on oil. The concentrations in the control treatments of mesocosm 3 remained extremely low and in some cases below the detection limits (<50 ng/L) of the GC/FID. However, the concentrations of the heavier even alkanes, such as nC24 and nC30, were higher than the rest and increased with time, even after considering the wide variability within samples (Figure 5A). It is noteworthy to mention that this pattern did not happen in the oil itself. N-Alkane profiles with predominant even carbon-number homologs ranging from n-C22 to n-C30, such as the one in mesocosm 3, have been associated with saline and carbonate rich environments (Grimalt & Albaiges, 1987; Aghadadashi et al, 2017).

Figure 5. N-alkane abundances of the Control, WAF, DCEWAF and CEWAF treatments of mesocosm 3. Error bars refer to the standard deviation between triplicates

In the case of the WAF treatments, the concentrations were also low but highly variable within the triplicates (Figure 5B), and its fingerprint pattern matches the Macondo surrogate oil (Figure 4). The n-alkane concentrations were low due to their hydrophobic nature (Liu et al, 2012). Nevertheless, it was possible to observe an overall rate of change of 86%. It is possible that the

concentrations of the lower molecular weight group were low even at time zero because they were already being consumed during the WAF preparation.

The DCEWAF (Figure 5C) and CEWAF (Figure 5D) treatments also match the Macondo surrogate fingerprint (Figure 4), although they are one and two orders of magnitude respectively higher than the WAF. The low-molecular weight (LMW) n-alkanes (<C14) decreased rapidly in the DCEWAF and CEWAF treatments relative to the Macondo surrogate oil, indicating that processes such as evaporation and biodegradation took place. It has been previously reported that a consortium of microorganisms can degrade petroleum components in aerobic marine environments, preferentially medium-chain n-alkanes (C10-C22) (Liu et al, 2012). This event was seen more clearly in the DCEWAF treatments than in the CEWAF. However, due to the higher concentrations of dispersant (Garr et al, 2014) CEWAF concentrations were orders of magnitude larger and it may be taking longer for the oil-degrading bacteria to consume them.

The rate of change for DCEWAF was above 95% in contrast with the CEWAF, which changed only 44%. The latter is not surprising since the concentrations in the CEWAF treatments were at least twice as high as the DCEWAF, therefore biodegradation processes took twice as much time. However, degradation of alkanes is a widespread phenomenon, where diverse prokaryotic and eukaryotic microorganisms easily obtain carbon and
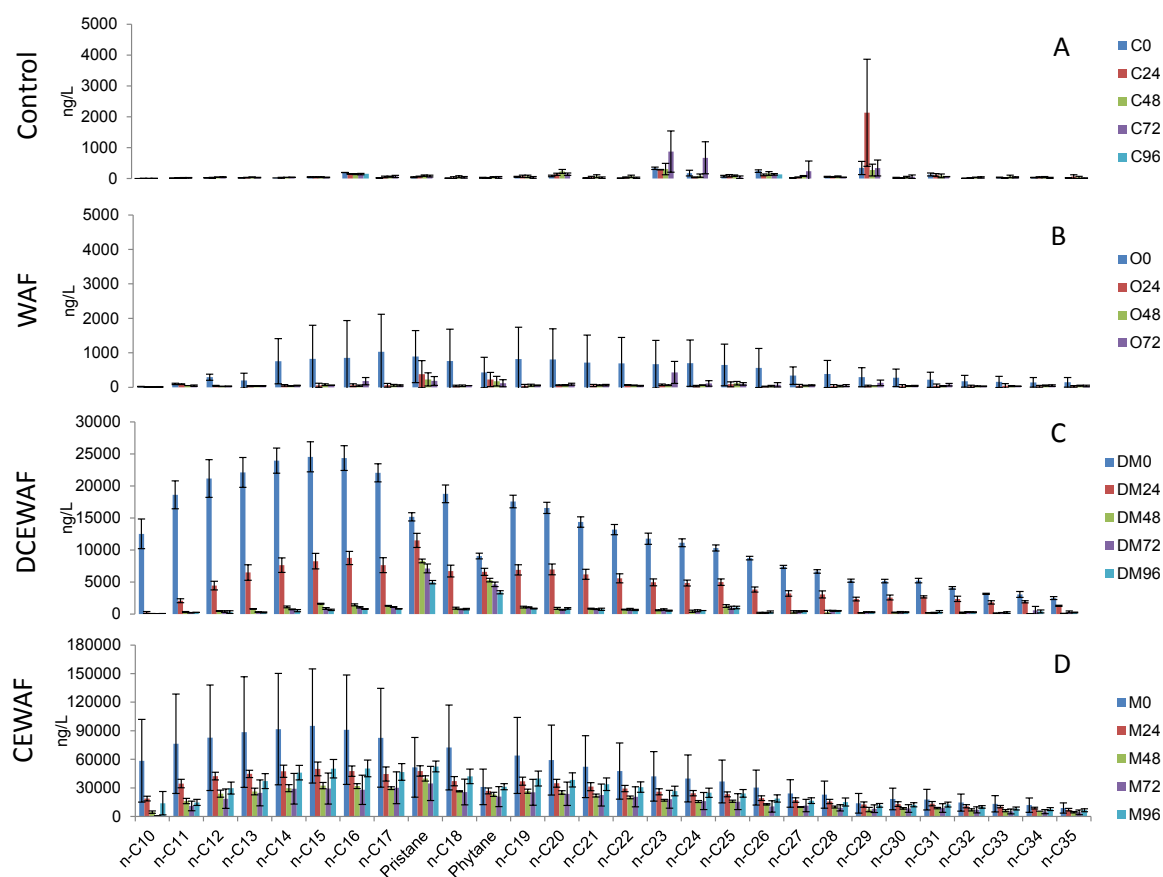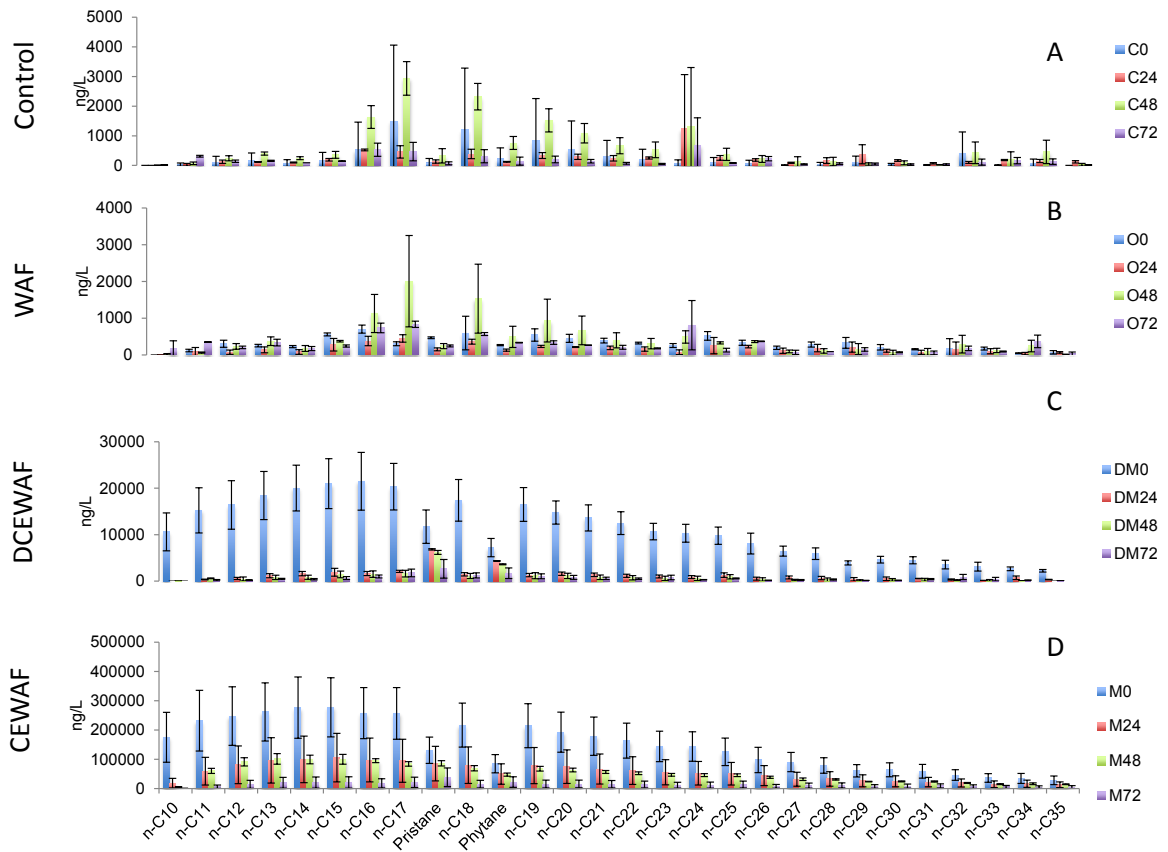
Figure 6. N-alkane abundances of the Control, WAF, DCEWAF and CEWAF treatments of mesocosm 4. Error bars refer to the standard deviation between triplicates

energy (Wentzel et al, 2007). As reference, it is important to point out the differences in the degradation patterns of Pristane and Phytane in these two treatments. These compounds were degraded slowly but at a constant rate in the CEWAF treatments, which is characteristic of hypoxic environments (Koopmans et al, 1999). In contrast, in the DCEWAF tanks these two compounds remained unchanged within experimental variability. This could

possibly be a reflection of the lower concentrations of hydrocarbons in the DCEWAF solution. In this treatment, the bulk of the straight-chained alkanes was consumed within the first 24 hr, forcing the bacteria to consume the more complex branched n-alkanes. In the case of the CEWAF, the concentrations were so high that the preferred straight-chained compounds never were completely consumed.

The composition of the control (Figure 6A) and WAF (Figure 6B) treatments from mesocosm 4 differed considerably from mesocosm 3 (Figure 5). The concentrations from nC10-nC15 were extremely low or below detection limits. It is possible that these alkanes were consumed during the WAF preparation. However, neither treatment matches those of the MC252 fingerprint (Figure 4),and even-number alkanes from group C14-C24 were predominant. These distributions are typical of coastal oxygenated systems and could point to a biological origin (Grimalt and Albaiges, 1987). The increase of phytane in both treatments corroborates this assumption since bacteria can consume the OH group from phytol, a common compound present in chlorophyll, transforming it into phytane (Grossi et al, 1998; Rontani & Bonin, 2011).

In the DCEWAF (Figure 6C) treatments of mesocosm 4, the concentrations of all compounds decreased 95% from their initial concentration. Coulon et al (2005) reports similar losses and suggests that nutrient fertilization

was a key factor in the degradation of oil. The concentrations of the heavy molecular weight groups were lower than the low molecular weight, which is expected due to the increasing hydrophobicity with the length of the alkanes. As a consequence of the hypoxic levels in the treatments (Rontani & Bonin, 2011), the degradation of the branched alkanes was slower than the straight-chained alkanes. However, the fact that there was constant degradation of Pristane and Phytane although slow, speaks of the speed the straight-chained alkanes were degraded.

The rate of change of the n-alkanes in the CEWAF treatment (Figure 6D) was 91%. The data contrasts with the results reported by Pi et al (2017), where after 30 days only 43% of the oil was removed. In the CEWAF, the remaining alkanes were considerably higher than those in the DCEWAF treatment; however it is important to take into consideration the larger amounts of dispersant in this treatment. It then appears necessary to increase the length of future experiments to measure the further evolution of biodegradation. Similarly with the DCEWAF treatments, hypoxia could have accelerated the degradation of the Pristane and Phytane (Rontani & Bonin, 2011).

In order to see which n-alkane group was being degraded first, it is important to normalize them to a compound resistant to biodegradation. Historically, hopane has been a widely used compound for this purpose (Prince

Figure 7. N-alkanes of control, WAF, DCEWAF and CEWAF of mesocosm 3 normalized to their respective total petroleum hydrocarbon (TPH) concentration

et al,1994). However, recent research has shown that hopane is in fact subject to biodegradation. Therefore, n-alkanes in each treatment were normalized to their respective total petroleum hydrocarbon (TPH) at time zero. The normalized n-alkanes of the control treatment in mesocosm 3 (Figure 7A) showed a predominance of the higher molecular weight compounds. In the case of the WAF treatment (Figure 7B), most alkanes were consumed within 24 hours,

except for the branched n-alkanes. The fingerprint of these n-alkanes indicates a biological origin (Grimalt & Albaiges, 1987) and possibly low doses of the Macondo surrogate oil used in the experiment. The DCEWAF treatment of mesocosm 3 (Figure 7C) showed a predominant degradation of n-C10 within 24 hr. On the other hand, the bulk degradation of the n-C11-nC16 and the n-C19-nC22 groups occurred after 48h. These results are in agreement with those reported by Coulon et al (2005) who state that the degree of degradation of short chain n-alkanes, was higher than the ones with longer chains, independently of the initial level of contamination. It was apparent that the longer, heavier straight-chain alkanes were consumed after the lighter compounds were depleted. Pristane and Phytane remained unaltered for the first 24 h and decreased slightly by hour 72 and 96. There was an unexpected and abrupt decrease of both branched alkanes by hour 48, the reason for which remains unknown; however analytical errors should not be discarded. There was preferential consumption in the CEWAF treatment (Figure 7D) of the shorter n-C10, followed by the n-C11 to n-C16 group. However, after 48 hours the concentrations of the latter group changed minimally. Similar results were reported by Pi et al (2017), which they attribute to toxic effects of COREXIT 9500A on bacterial populations. Nonetheless, it is likely that since the hydrocarbon concentration was so elevated, the duration of the experiments was insufficient to observe significant changes in the n-alkane abundance.

Biodegradation rates depend mostly on the composition, weathering and concentration of oil. However, factors such as temperature, oxygen, and nutrients also have strong roles in the degradation of oil. A reason for this is that the initial steps in the catabolism of aliphatic, cyclic and aromatic hydrocarbons by bacteria involve oxidation (Leahy & Colwell, 1990; Wetzel et al 2007).
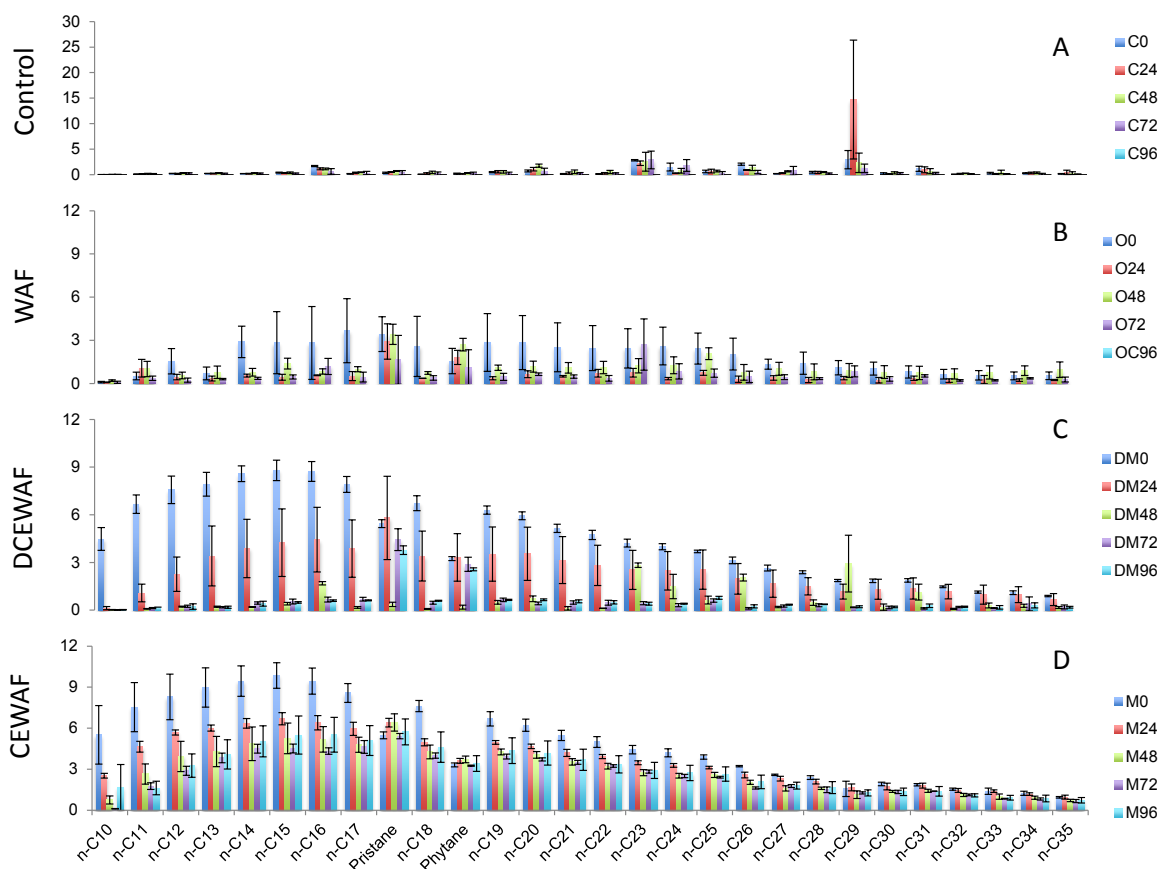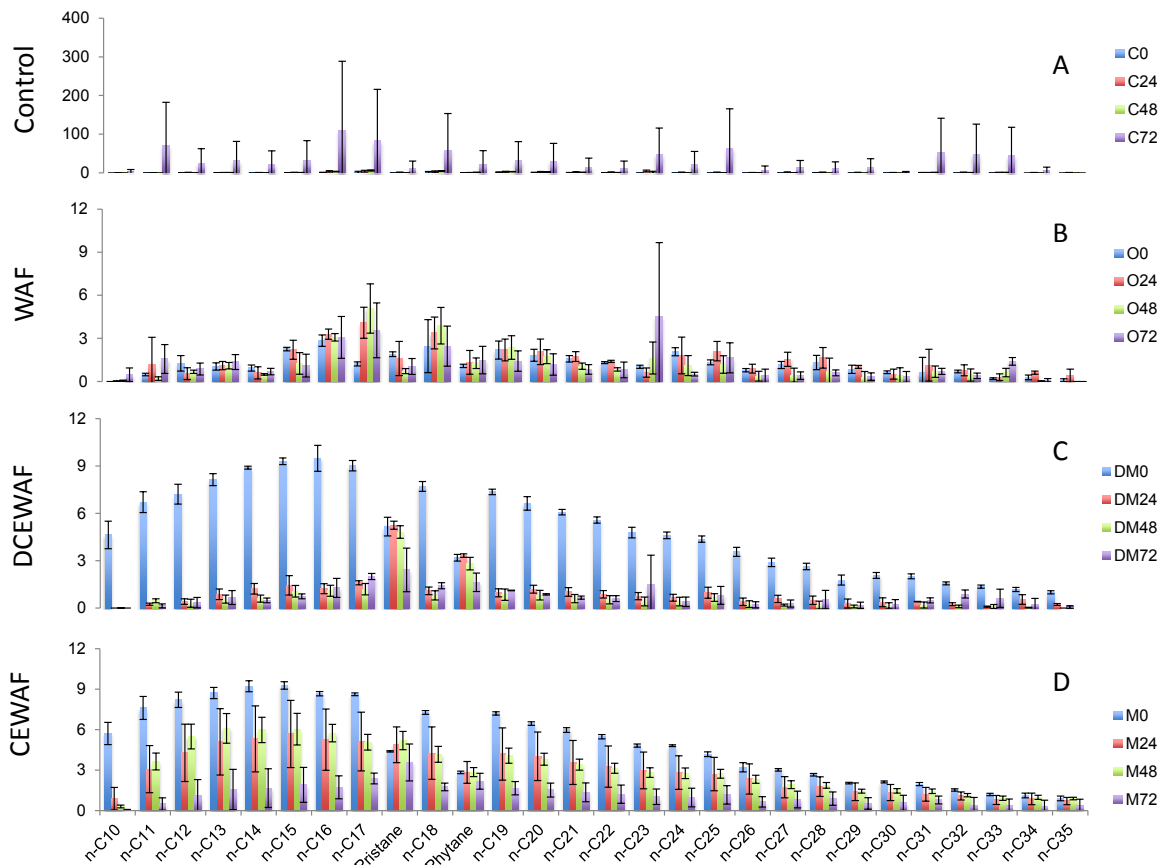

Figure 8. N-alkanes of control, WAF, DCEWAF and CEWAF of mesocosm 4 normalized to their respective total petroleum hydrocarbons (TPH) concentration

Experiments measuring bioremediation rates in fertilized experiments showed a complete removal of the resolved n-alkanes (Roling et al 2002) over longer timescales, usually ranging from 30 to 90 days (Singh et al, 2014). In addition, toxic effects on the microbial community from the high concentrations of COREXIT 9500A (Pi et al, 2017) should not be discarded. Therefore, toxicity tests should be included in future work.

The normalized values of the control treatment in mesocosm 4 show an increase in all alkanes by hour 72 (Figure 8A); however this may be due to the high variability between samples and to their low concentrations of alkanes. The WAF treatments (Figure 8B) showed a decrease with time of all alkane groups; yet a preference for any particular n-alkane group is not visible. It is possible that the absence of surfactants in the mixture impeded the availability of these compounds for microbial degradation. The bulk consumption of all the straight-chain n-alkanes in the DCEWAF treatments of mesocosm 4 (Figure 8C) occurred within the first 24 hours. Both branched alkanes appear to have taken longer to degrade due to their resistance to microbial feeding (Atlas, 1981; Balba, et al, 1998). Similar to the CEWAF treatment of mesocosm 3 (Figure 7D), most straight-chain n-alkanes of mesocosm 4 (Figure 8D) were consumed within 48 hours from the beginning of the experiment. It is likely that microorganisms present in these treatments took longer than in the DCEWAF to consume these compounds due to their elevated concentration.

The n-C17:Pristane and n-C18:Phytane ratios are well known indicators of biodegradation and evaporation patterns in a system (Liu et al, 2012; Singh et al, 2014; Turner et al, 2014). The reason behind this is that both n-C17 and n-C18 are straight-chain alkanes, while Pristane and Phytane are branched. Most oil-degrading bacteria prefer using the straight-chain alkanes as their primary source of C because they are easier to break down (Turner et al, 2014). Hence, a decreasing rate of any of these ratios indicates biodegradation. The ratio given for the MC252, depending on the study consulted, ranges between 1.8-2.0 (Wade et al, 2011; Liu et al, 2012; Singh et al, 2014); therefore it was expected

Table 3. n-C17/Pristane ratios of WAF, DCEWAF and CEWAS of mesocosm 3 and 4

Time (hr)	Mesocosm 3			Mesocosm 4		
	WAF	DCEWAF	CEWAF	WAF	DCEWAF	CEWAF
0	1.0 (±0.2)	1.7 (±0.01)	1.7 (±0.02)	0.6 (±0.1)	1.8 (±0.2)	2.0 (±0.0)
24	0.2 (±0.2)	1.7 (±0.06)	1.7 (±0.01)	3.2 (±1.5)	0.3 (±0.0)	1.0 (±0.2)
48	0.3 (±0.1)	1.6 (±0.05)	1.7 (±0.07)	8.3 (±4.5)	0.3 (±0.1)	1.0 (±0.0)
72	0.3 (±0.08)	1.5 (±0.03)	1.7 (±0.01)	3.4 (±0.0)	0.6 (±0.0)	0.6 (±0.2)
96	0.3 (±0.01)	1.4 (±0.05)	1.7 (±0.01)	-	-	

*± values indicate standard deviations between the triplicates of each treatment.

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to find a similar ratio in these treatments. The Macondo surrogate chromatogram (Figure 4) shown in this study matched other fingerprints reported by the aforementioned studies, and additionally it had a n-C17:Pristane ratio of 1.9. Analytical mistakes have been discarded; therefore it is safe to conclude that the Macondo surrogate oil fingerprint reported in this study is accordance with the other reports.

In the case of the WAF treatments of mesocosm 4 (Table 3), the ratios were highly variable with time and had elevated standard deviations. It is important to consider that their total oil concentrations were low or below detection limits, and the alkane origin could have partially been biological (Wentzel et al, 2007). After considering the low abundance of n-alkanes in these treatments, it was not surprising to find such an erratic pattern. On the other hand, the decreasing ratios of the DCEWAF treatments of mesocosms 3 and 4 were clear evidence of biodegradation (Liu et al, 2012). Their initial ratios were below the one reported for the Macondo surrogate oil which suggests that during the accommodated fractions preparation some of the alkanes could have been biodegraded. The n-C17/Pristane ratio showed a much faster decrease with time in mesocosm 4 than in mesocosm 4, which could indicate stronger microbial activity in these experiments.

CEWAF treatments of mesocosm 3 and 4 had opposite patterns. CEWAF in mesocosm 3 presented no apparent changes through time (Table 3). This apparent lack of change in these ratios may indicate that the loss of the individual alkanes was happening at a similar rate (Turner et al, 2014b). This data was in agreement with the TPH-normalized alkane distribution (Figure 7D; Figure 8D). The latter suggests that high concentrations of COREXIT 9500A and oil could have inhibited to some extent biological degradation (Pi et al, 2017). A few other possibilities could explain this event; the first being that the n-alkanes, due to their hydrophobicity, precipitated out of solution. The second possibility is that the oil was strongly associated with the marine snow and was being removed from the water column. A combination or all of these reasons could have been occurring in the CEWAF treatments. However, little can be proven due to the difficulty of measuring marine oil snow (MOS). In contrast, CEWAF ratios in the coastal water experiment (mesocosm 4) show a clear decrease with time that implies constant biodegradation throughout the experiment (Turner et al, 2014b) and reflects the different bacterial communities represented in the two mesocosms.

The TPH-normalized n-alkane distribution suggested that there could have been some degradation of the branched alkanes. Therefore, a correlation between Pristane and Phytane was made to determine the veracity of this

statement. The degradation of alkanes was expected to result in an increasing

Pr: Ph ratio due to the hypoxic conditions (Koopmans et al, 1999), and the less

Table 4. Pristane/Phytane ratio of WAF, DCEWAF and CEWAF of mesocosm 3 and 4

Time	Mesocosm 3			Mesocosm 4		
(hr)	WAF	DCEWAF	CEWAF	WAF	DCEWAF	CEWAF
0	2.4 (±0.5)	1.7 (±0.01)	1.7 (±0.02)	1.8 (±0.05)	1.6 (±0.08)	1.5 (±0.03)
24	1.6 (±0.2)	1.7 (±0.06)	1.7 (±0.01)	1.4 (±0.16)	1.6 (±0.02)	1.7 (±0.04)
48	1.3 (±0.005)	1.6 (±0.05)	1.7 (±0.07)	0.6 (±0.46)	1.7 (±0.1)	1.8 (±0.05)
72	1.7 (±0.2)	1.5 (±0.03)	1.7 (±0.01)	0.7 (±0.09)	1.4 (±0.5)	1.7 (±0.2)
96	1.6 (±0.2)	1.5 (±0.05)	1.7 (±0.01)	-	-	

*± values indicate standard deviations between the triplicates of each treatment.

recalcitrant nature of LMW compounds, which makes them degrade at a faster

rate than the heavier ones (Turner et al, 2014b). On the other hand, the absence

of change indicates a slow degradation rate (Turner et al, 2014b).

The WAF and DCEWAF treatments (Table 4) of both mesocosms showed

a low decrease of the Pristane to Phytane ratio over time, which indicates that

since the straight-chain alkanes were consumed so rapidly, the microbial community was forced to consume the branched group (Koopmans et al, 1999; Turner et al, 2014b). The CEWAF treatments remained unchanged, which agrees with the previous data and indicates slower degradation rates (Turner et al, 2014b). In addition, the elevated concentration of the straight-chain alkanes deterred organisms from consuming Pristane or Phytane.

Finally, comparisons between the estimated oil equivalence (EOE), the total petroleum hydrocarbons (TPH), total resolved, total unresolved complex mixture (UCM), and total alkanes were made in order to see their concentration differences (Table 5; Table 6). The gross chemical composition of crude oil varies greatly; however light crude oils usually contain a large proportion of light components with nearly half of resolved peaks in which <C16 hydrocarbons account for about 70% of TPHs (Yang et al, 2015). The total resolved fraction in both mesocosms and in all treatments accounted for less than 50% of the TPHs, yet as mentioned before, as a typical light Louisiana crude oil, more than half of the total resolved peaks were LMW hydrocarbons. Given the fact that aromatics comprise only 13.3% in weathered light Louisiana crude oil (Wang et al, 2003), it was surprising to find that EOE values were consistently higher than the rest of the other oil components at time zero and throughout the length of the experiments in all treatments. This could indicate a preferential dissolution of hydrocarbons, meaning that hydrophobic compounds such as the n-alkanes do

Table 5. Chemical composition of the oil and oil/dispersant in WAF, DCEWAF and CEWAF of mesocosm 3.

Mesocosm 3

Time (hr)	Treatment	Total Resolved (ug/L)	Total TPH (ug/L)	Total UCM (ug/L)	Total Alkanes (ug/L)	EOE (ug/L)
0	WAF	75	233	158	2	739
24	WAF	17	111	94	4	427
48	WAF	5	62	57	2	301
72	WAF	45	143	97	3	460
96	WAF	16	85	67	0	67
0	DCEWAF	598	2790	2192	14	6170
24	DCEWAF	235	2297	2062	2	5653
48	DCEWAF	33	911	878	2	4213
72	DCEWAF	137	1621	1484	2	3198
96	DCEWAF	87	1320	1233	2	2710
0	CEWAF	2889	9366	6477	358	39067
24	CEWAF	1453	7454	6001	136	24200
48	CEWAF	1535	6268	4733	29	19630
72	CEWAF	1412	6421	5009	25	12386
96	CEWAF	1770	9282	7511	21	8212

not tend to dissolve in water (Barron et al, 1999). This proves that in nature the dissolved fraction of oil is different from oil itself.

Table 6. Chemical composition of the oil and oil/dispersant in WAF, DCEWAF and CEWAF of mesocosm 4

Mesocosm 4

Time		Total Resolved	Total TPH	Total UCM	Total Alkanes	EOE
(hr)	Treatment	(ug/L)	(ug/L)	(ug/L)	(ug/L)	(ug/L)
0	WAF	115	249	134	8	290
24	WAF	40	118	77	5	136
48	WAF	69	366	296	11	92
72	WAF	63	282	219	8	26
0	DCEWAF	721	2250	1529	313	8134
24	DCEWAF	201	1303	1103	35	5403
48	DCEWAF	121	1292	1171	26	4003
72	DCEWAF	95	907	812	17	1843
0	CEWAF	10056	29726	19670	4174	81060
24	CEWAF	4198	15855	11657	1551	38767
48	CEWAF	4124	16688	12563	1456	33167
72	CEWAF	1334	8808	7474	367	19833

Nutrients

Availability of inorganic nutrients, particularly nitrogen and phosphorous, has been proven to be an important factor in the degradation of hydrocarbons in marine environments (Singh et al 2014). Therefore, an unenhanced (no additional nutrients added) mesocosm experiment (mesocosm 2) was performed to contrast the enhanced (nutrient media added) coastal and open ocean experiments. Unsurprisingly, initial nutrient concentrations of mesocosm 2 were significantly lower than mesocosms 3 and 4, and the evolution of the experiments were likewise significantly different because the first had only ambient constituents, while the other two were spiked with the f/20 media nutrients.

Dissolved inorganic nitrogen

Dissolved inorganic nitrogen (DIN) is the sum of nitrate, nitrite and ammonium. The DIN concentrations in the coastal unenriched experiment (mesocosm 2) were lower than the other two experiments because mesocosm 2 only contained ambient nutrients (Figure 9), yet these concentrations were not environmentally low. The percent of change per hour was calculated by using the exponential rate, and they indicate a higher consumption of DIN in mesocosm 2 than in the other two experiments (Table 7). However it is important to take into consideration that changes over time in M3 and M4 may
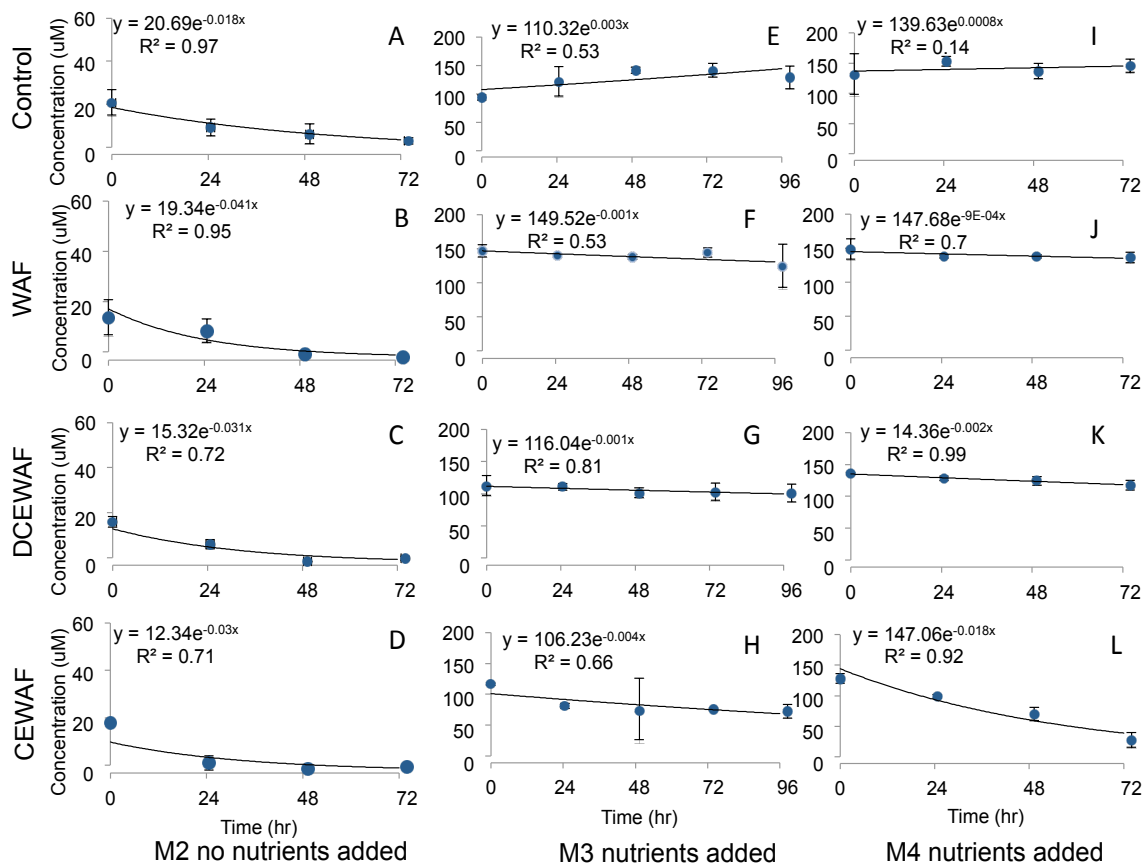
Figure 9. Dissolved inorganic nitrogen (DIN) of the averaged triplicates of water – accommodated-fraction (WAF), diluted-chemically-enhanced-water-accommodated-fraction (DCEWAF) and chemically-enhanced-water-accommodated-fraction (CEWAF) of mesocosm 2, 3 and 4

not be as obvious due to their high DIN concentrations. The trend of the DIN to be increasing with time in the control treatments of mesocosm 3 and 4 (Table 7) may only be a reflection of the marked standard deviations in the triplicates. In the open ocean experiment (mesocosm 3) the decrease of DIN was low (Table 7) and it did not appear to have a well-defined linear relationship with time

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Table 7. Rate of change per hpur of DIN concentrations in control, WAF, DCEWAF and CEWAF treatments.

Dissolved inorganic nitrogen

	M2 (%)	M3 (%)	M4 (%)
Control	-1.8	0.3*	0.08*
WAF	-4.1	-0.1	-0.09
DCEWAF	-3.1	-0.1	-0.2
CEWAF	-4.9	-0.4	-1.8

*Positive values indicate an increase in the DIN concentrations with time

(Figure 9E, F, G, H). The coastal enriched experiment (mesocosm 4) also had a low decrease of DIN in the control, WAF and DCEWAF (Table 7) treatments. However, the CEWAF treatment (Table 7) had a percent loss per hour of 1.8%; therefore by the last time point it lost 73% of its initial concentration.

Total inorganic phosphorous

Phosphate (PO_4^{3-}) in all treatments of mesocosm 2 remained unchanged throughout the experiment and at concentrations below 1 uM. Although with higher concentrations (~10 uM), all treatments in mesocosm 3 and 4 had also an extremely low rate of change per hour with the exception of the CEWAF treatments (Table 8). The latter (Figure 10) showed a decrease of almost half

their initiaconcentration. This demonstrates the enhancement of PO_4^{3-} uptake in oil and dispersant mixtures (Ptanik et al, 2010).

Table 8. Rate of change per hour of PO_4^{3-} concentrations in control, WAF, DCEWAF and CEWAF treatments

| | Phosphate | | |
	Mesocosm 2 (%)	Mesocosm 3 (%)	Mesocosm 4 (%)
Control	-0.04	-0.003	-0.01
WAF	-0.06	-0.02	-0.003
DCEWAF	-0.05	-0.1	-0.3
CEWAF	-0.02	-0.5	-0.8

N:P ratio

As previously stated, mesocosm 2 was coastal water with only ambient nutrients. The ratios within each treatment vary significantly because nutrients were probably beginning to be utilized during the WAF making procedure. Only the DCEWAF treatment (Figure 11C) had an estimated threshold N:P ratio similar to the Redfield ratio at time zero. In any case, the N:P ratio decreased with time in all treatments (Figure 11)., which indicated a N limitation as frequently found in the Gulf of Mexico during the summer (Fennel et al, 2011; Quigg et al, 2011).
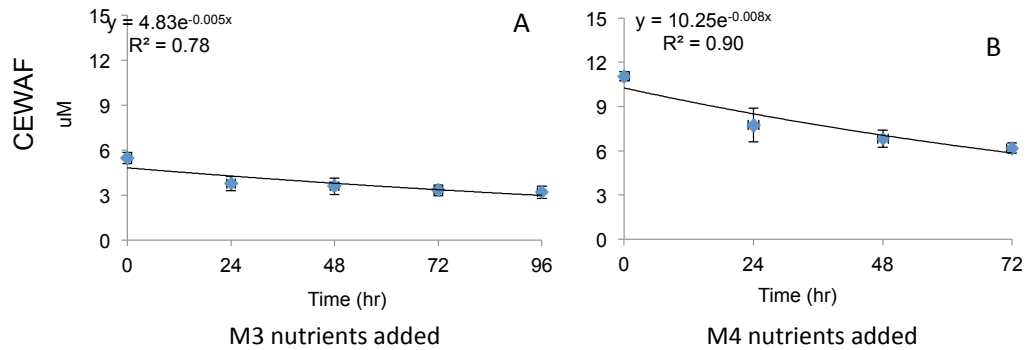
Figure 10. Phosphate concentration in CEWAF treatments of mesocosm 3 and 4. Concentration is given in uM.

In mesocosm 2, WAF (Figure 11B) and CEWAF (Figure 11D) had high N:P ratios. This could also indicate several events. The first being that the phytoplankton and bacterial communities could have been different from the other two tanks and have different N:P requirements (Redfield, 1958; Ptacnik et al, 2010). Also, there could have been a large scale die off of the phytoplankton community during transport of the water from the sampling site to the TAMUG facilities or during WAF preparation, and the subsequent oxidation of this new organic matter by bacteria that would then form dissolved inorganic nitrate. An apparently stable 16:1 N:P ratio in mesocosm 3 and 4 was observed for the WAF and DCEWAF treatments, which indicates these nutrients were never limited. On the other hand, the N:P ratio of the CEWAF of mesocosm 4 decreased with time, which indicates N limitation.
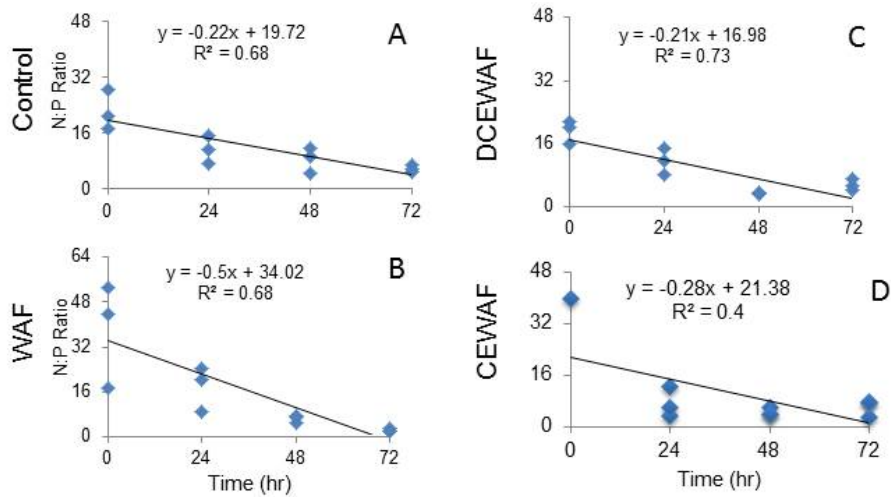
Figure 11. N:P ratios over time of control, WAF, DCEWAF and CEWAF from mesocosm 2

Oil correlations with DIN and phosphate

The correlations between the dissolved inorganic nitrogen (DIN) with the estimated oil equivalent (EOE) were very high. In mesocosm 2, all treatments had an R^2 value above 0.80 (Figure 12A, B, C), while in mesocosm 3 the highest correlation was in the WAF treatment (Figure 12D) and its R^2 value decreased with the addition of dispersants. This implies that oil degradation was enhaced by the addition of nutrients (Singh et al, 2014). In contrast, in mesocosm 4 the highest correlations were found in the DCEWAF (Figure 12H) and CEWAF (Figure 12I) treatments. The consumption of ambient nutrients was in agreement

with the degradation of oil (Figure 12A, B, C). On the other hand, opposite trends in the coastal and open-ocean enhanced experiments suggest that both environments have different microbial communities that react in unique ways to the presence of oil. In fertilized coastal environments, microbial communities reacted positively to high concentrations of hydrocarbons and chemical

Figure 12. Estimated oil equivalent (EOE) and dissolved inorganic nitrogen (DIN) correlations of control, WAF, DCEWAF and CEWAF from mesocosm 2, 3 and 4.

surfactants. DIN consumption was better correlated to oil degradation in coastal environments (mesocosm 4) than in open-ocean (mesocosm 3). Within the coastal experiments, the DCEWAF treatments had the best correlations. Increased concentrations of dispersants decreased the linear relationship of EOE with DIN. Perhaps high concentrations of dispersants affect the consumption of nitrogen.
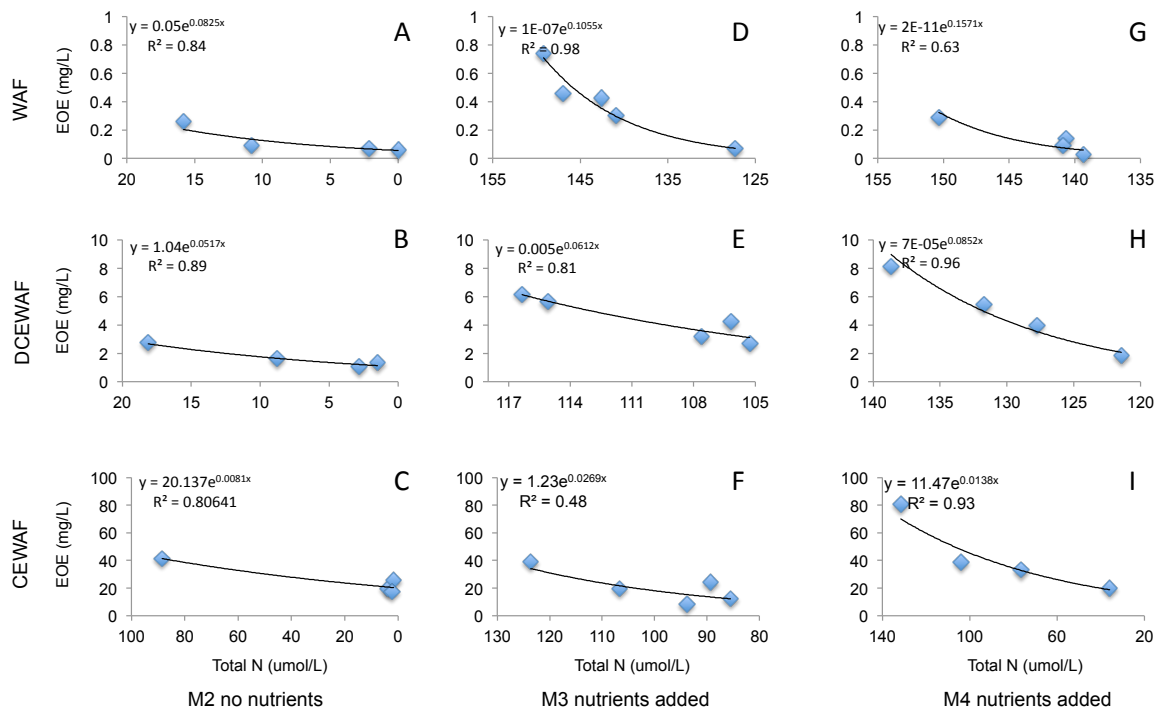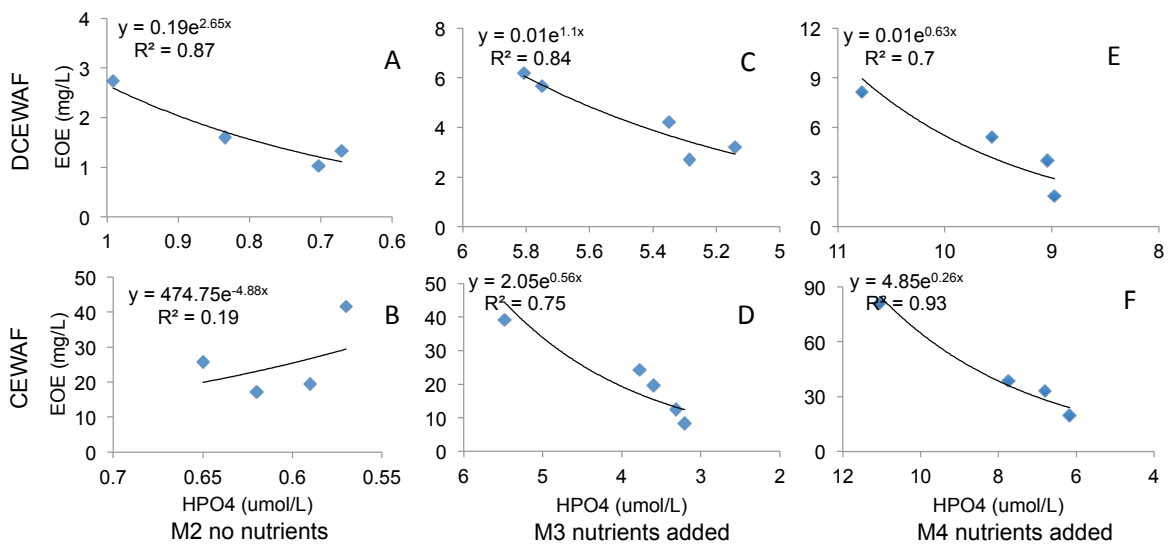

Figure 13. Estimated oil equivalent (EOE) and phosphate correlations of control, WAF, DCEWAF and CEWAF from mesocosm 2, 3 and 4.

PO_4^{3-} and EOE had higher linear relationships with the addition of dispersant (Figure 13). In mesocosm 2, the R^2 value in DCEWAF (Figure 13A)

was similar to the other two mesocosms. However, in the CEWAF treatment (Figure 13B) there was no relationship between the EOE and the consumption of HPO_4. In mesocosm 3 (Figure 13C) and 4 (Figure 13E), the R^2 values of the DCEWAF treatments were also statistically. On the other hand, this linear relationship was even stronger in the CEWAF treatments (Figure 13F, I). Of these two treatments, the coastal enriched (mesocosm 4) treatment that had the highest correlations. Phosphate is now a commonly used nutrient in bioremediation activities because it is thought to stimulate biodegradation of hydrocarbons (Siciliano et al, 2016). In fact, it has been suggested that most degradation measurements are phosphate adsorption dependent (Siciliano et al, 2016). Provided that nitrate and oxygen are not exhausted, phosphate has been proven to have strong stimulating effects on aerobic and denitrifying rates of oil degradation (Ponsin et al, 2014).

Nitrate, nitrite and ammonia

In order to understand the DIN uptake and the geochemical processes occurring in the systems, each of its components was analyzed separately. The primary source of nitrogen, i.e. nitrate, in all treatments of mesocosm 2 was lower than mesocosms 3 and 4 because they were not enhanced with the f/20 media nutrients. The initial concentration of NO_3- in M2 for control, WAF and DCEWAF was ~17.75 uM (±3.11), however their loss rates differed greatly between each other (Table 9). On the other hand, CEWAF had an average

concentration at time zero of 87.18 uM with a standard deviation that ranged

from 19 to 166 uM. After 24 hours, the nitrate concentration in CEWAF

plummeted to an average concentration of 2.58 uM and stabilized at that range

until the end of the experiment. Nitrate concentrations showed little change in

Table 9. Percent of change per hour of NO_3-, NO_2- and NH_4 in control, WAF, DCEWAF and CEWAF of mesocosm 2, 3 and 4

	Mesocosm 2 (%)				Mesocosm 3 (%)				Mesocosm 4 (%)			
	C	O	DM	M	C	O	DM	M	C	O	DM	M
NO3-	-2.2	-4.1	-4.6	-6.4	-0.3	-0.1	-0.1	-1.0	-0.08	-0.03	-0.2	-2.5
NO2-	NA	NA	NA	NA	0.6	0.4	0.04	5.02	1.2	1.4	0.003	3.1
NH4	0.5	-5.7	0.6	0.02	0.4	-0.1	-0.2	-0.1	NA	NA	NA	NA

C= control, O= WAF, DM= DCEWAF, M= CEWAF. NA= no data or below detection limits.
Negative values refer to loss of concentration per hour.

the control, WAF and DCEWAF triplicates of mesocosms 3 and 4, however the

loss of the nutrient occurred at a faster rate in M3 (Table 9). In the case of the

CEWAF treatments, both M3 and M4 showed a rapid loss of the nutrient with

time (-1% and -2.5% per hour respectively), yet NO_3- was never exhausted

(Figure 14). QA/QC values in the three mesocosms passed inspection; therefore

instrumental error was discarded and the variability that was presented was attributed to environmental factors in each tank.

All nitrite (NO_{2-}) concentrations remained below 1 uM, except for the CEWAF treatments of mesocosms 3 and 4. During mesocosm 3, NO_{2-} increased at a rate of ~5% per hour, reaching a maximum concentration of 33.03 uM (Figure 14A). The standard error was not significant at any time point. On the other hand, in mesocosm 4, nitrite increased at a slightly lower rate (3.1% per hour); however the concentration in each triplicate varied considerably at every time point (Figure 14B). Lastly, ammonia (NH_4) measurements were taken only for mesocosm 2 and 3. In all treatments of both mesocosms 2 and 3, except CEWAF of mesocosm 3, the NH_4 concentrations remained below 6 uM and over all unchanged. The CEWAF treatment had an abnormally high pulse of NH_4 at 48 hours in its "C" triplicate tank (72.55 uM) (Figure 14).

In aerobic conditions, the dominant form of nitrogen is nitrate, while nitrite and ammonia remain low or absent (Francis et al, 2007). This was the overall case for the control, WAF and DCEWAF in all mesocosms. However in the CEWAF treatments of mescosms 3 and 4, nitrate decreased rapidly while nitrite conversely increased. As stated before, hypoxic conditions were reported in the CEWAF experiments (Kamalanathan, in prep). Theoretically, under redox conditions there is an initial rapid consumption of oxygen followed by a complete

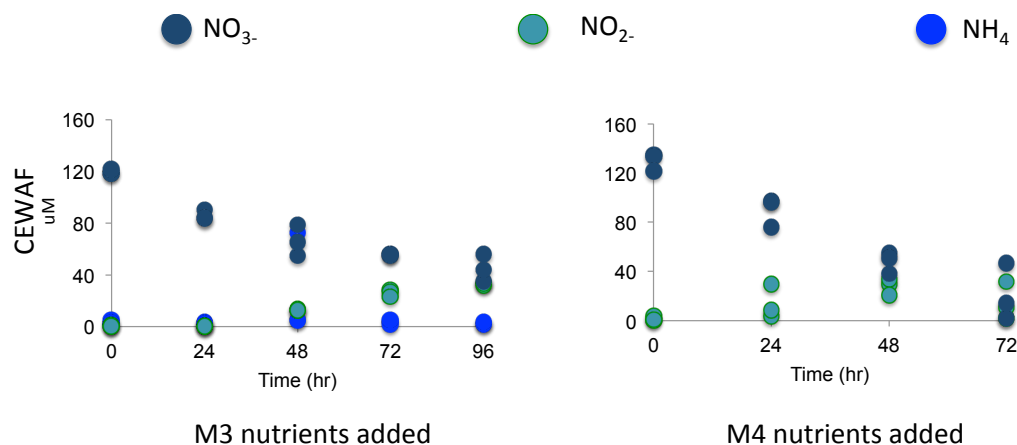nitrate reduction (Ponsin et al 2014), where the nutrient is transformed into nitrite (denitrification).

Figure 14. Nitrate, nitrite and ammonia concentration of chemically-enhanced-water-accommodated-fraction (CEWAF) of mesocosm 3 and 4 over time. Concentration is given in uM.

Facultative heterotrophic bacteria utilize nitrate as a substitute terminal electron acceptor in oxygen limiting environments and convert it into N_{2-} (Leahy & Colwell, 1990). Nitrate can also be reduced to ammonia, however can only be performed by specific organisms. Additionally, oil can inhibit the penetration of dissolved oxygen, and labile hydrocarbons can stimulate the consumption of dissolved oxygen by bacteria. The presented experiments were only mixed during the preparation of the accommodated oil fractions (WAF and CEWAF); therefore it is likely that with time they became oxygen limited. Since it is mostly

heterotrophic bacteria that perform denitrification, it was suggested that this group of organisms predominated in the CEWAF treatments of mesocosms 3 and 4. However, some authors (Shi & Yu, 2014; Pietroski et al, 2015; Pi et al, 2017) report that dispersants inhibit afore mentioned process.

On the other hand, Ribeiro et al (2016) found that denitrification rates were stimulated under diverse crude oil treatments. These authors found a 30-fold increase of nitrite in those treatments enriched with crude oil. It is important to mention that most of these studies have focused on the impacts of oil and dispersant in sediments where the geochemical conditions are extremely different from those in surface water. The basic difference between these two environments is that oxygen has never been a limiting element in the upper layers of the water column (Leahy & Colwell, 1990). One study, however, found an increase in the heterotrophic populations in fresh water experiments enriched with dispersants (Dutka & Kwan, 1984). The authors did not measure oxygen and nutrient consumption, but the dominance of heterotrophic populations could have perhaps been triggered by hypoxic conditions such as the ones thought to be present in the current experiments. Lastly, the enhancement with high concentrations of bioavailable nutrients could have caused the CEWAF treatments to become eutrophic with cascading consequences such as oxygen deficits (Paerl et al, 1990; Ptanik et al, 2010; Singh et al 2014).

CONCLUSIONS

The baffled recirculation system has proven to be an efficient technique for the generation of large quantities of WAF and CEWAF. The variability seen in the WAF, DCEWAF and CEWAF treatments was a reflection of the high hydrophobicity of the oil and the difficulty to generate a stable mixture. However, a variety of factors could have affected the homogeneity in the mixtures. First, it is likely that oil droplets were associated with the marine snow (MOS), and could have contributed to misleading readings. Second, weathering processes such as sedimentation, evaporation, biodegradation and evaporation could have occurred at different rates within the triplicates and treatments. Third, the results presented in this study make it apparent that the coastal and open-ocean communities are different from each other, and as such the decay of oil and changes in availability of nutrients occur at different rates. This last statement could also explain the variability in the nutrient measurements. Similarly, biodegradation of the n-alkanes by the different bacterial populations may have been species specific.

Oil decreased with time in all treatments; however, it occurred at a faster rate in nutrient enhanced water. This underlines the importance of nutrients in supporting the biology and hence a greater degradation of oil in these

mesocosms. . In nutrient enhanced experiments, it was the dispersant treatments that had the highest rate of loss.

Contrary to other studies (Shi & Yu, 2014; Pietroski et al, 2015; Pi et al, 2017), oil plus COREXIT 9500A appeared to enhance consumption of DIN and HPO4 in these experiments. It is still unknown why denitrification occurred only in the CEWAF treatments and this will be investigated further by ADDOMEx. It is likely that lack of mixing thus leading to low oxygen concentrations played a role.

The results reported in this study suggest that small additions of dispersant increase the biodegradation rates of the n-alkanes. Coastal water and open-ocean experiments had different biodegradation rates in the CEWAF treatments, which implies that their respective microbial communities react differently to COREXIT 9500A. However, for further studies to corroborate these results should be undertaken.

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