LETHAL AND SUBLETHAL EFFECTS OF OIL AND CHEMICAL DISPERSANT ON THE CTENOPHORE *MNEMIOPSIS LEIDYI*

by

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Contemporary scientists of any age are often said to stand upon the shoulders of giants, and it is inferred that it is only from this vantage point that we are able to see the way forward. This axiom is true only in an epistemological sense, however- the knowledge gained by our progenitors serving as a nucleus for current work. In reality, for every titan there exist legions of smaller figures upon whom we lean every day, on whose shoulders our weight falls rather more squarely, and who must bear the burden standing up. And so, to preface my thesis, I would like to acknowledge the people who have supported me along the road to this milestone.

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The administrative and support staff at the University of Delaware do exceptionally and pleasantly all of those things that no one else wants to do, especially scientists, and without them I would have many times been hopelessly awash in a sea

of forms. Foremost among them for me have been Janis Lopez and Peggy Conlon, though this short list is hardly exhaustive.

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ABSTRACT

Characterization of both the lethal and sublethal effects of oil and chemical dispersants in key species is important for developing an understanding of the impacts of oil spills and their associated mitigation techniques on coastal ecosystems. This study established the lethal levels of COREXIT® 9500A chemical dispersant, MC-252 weathered crude oil (WAF), and MC-252 weathered crude oil dispersed by COREXIT 9500A (CEWAF) for the trophically important ctenophore *Mnemiopsis leidyi* at both 15°C and 23°C. *M. leidyi* was highly sensitive to COREXIT 9500A at both temperatures (15°C: 24 hr LC_{50} = 6.91 mg/L; 23°C: 24 hr LC_{50} = 8.26 mg/L), as well as to oil solutions. There was an apparent increase in toxicity of dispersed oil compared to crude oil by loading concentration (15°C: CEWAF 48 LC_{50} = 6.7 mg/L; WAF 48 LC_{50}= 29.49 mg/L), but this difference was less pronounced when the total petroleum hydrocarbons of the solutions were considered (15°C: CEWAF 48 LC_{50} = 242.07 μg/L; WAF 48 LC_{50} = 143.14 μg/L).

Sublethal assays for glutathione-s-transferase (GST) activity, routine respiration rate, and bioluminescence were conducted on individuals surviving 24 hr exposure to various concentrations of the test solutions, the former at 15°C only and the latter two at both 15°C and 23°C. GST activity increased significantly in low COREXIT 9500A solutions at 15°C, as did weight specific respiration rate in the high dispersant and CEWAF treatments, suggesting a metabolic detoxification response to the dispersant containing solutions. The total light response from mechanically and

chemically stimulated bioluminescence decreased with exposure to the WAF and CEWAF of MC-252 weathered crude oil at both temperatures, and changes in intracellular calcium and photoprotein availability were indicated. This study demonstrates that *M. leidyi* is susceptible to the toxic effects of oil and chemical dispersant upon both lethal and sublethal exposure, with the addition of chemical dispersant resulting in increased toxicity compared to oil alone. The sensitivity of *M. leidyi* to oil and dispersant could have ecosystem wide effects in the event of a coastal spill.

Chapter 1

CONTEXT AND SCOPE OF THE WORK

Oil and Chemical Dispersant in Marine Environments

Oil is a major pollutant of marine ecosystems; its ubiquitous nature in modern society and the extent of efforts to extract, transport, and refine it have inevitably resulted and will continue to result in the discharge of oil and its refined products into the environment. A recent compilation of data estimates the average annual input of petroleum from all sources into waters off of North America to be about 260,000 metric tons; this number increases to 1,300,000 metric tons when marine environments worldwide are considered (NRC 2003). Of the many anthropogenic inputs, catastrophic spills, such as those associated with the extraction of oil (e.g. the 2010 blowout of the Deepwater Horizon rig in the Gulf of Mexico) and its transportation (e.g. the 1989 grounding of the Exxon Valdez tanker in Prince William Sound) garner the majority of the media and public attention. The interim between such large and highly publicized events, however, is punctuated by many smaller spills. The heavily trafficked and refinery laden Delaware Bay has experienced both small and large spills on a relatively periodic basis over the past forty years, the most recent of note being the release of over 97,000 metric tons of Venezuelan Heavy Crude Oil by the tanker Athos I after striking a section of pipe near a refinery in Paulsboro, New Jersey in 2004 (Corbett, 2004).

Despite their prominence, accidental spills only account for a small portion (~12%) of the global input of petroleum into the oceans, and such incidents rank behind other sources, both natural and anthropogenic, such as seeps and discharges associated with the consumption of oil, whose relative contributions to marine inputs are estimated to be 46% and 37%, respectively (NRC 2003). However, while seeps and discharges create relatively constant, low-level environmental hydrocarbon concentrations, spills result in the geographically focused exposure of marine organisms to high levels of hydrocarbons, and thus also elicit a need for the study of the fate of such hydrocarbon inputs in marine environments, as well as of their associated mitigation techniques, in order to determine the effects on organisms and ecosystems. Developing an understanding of these effects is important in assessing the ecological consequences of spills and guiding informed clean-up efforts (Thibodeaux et al., 2011).

Confounding the study of oil spills is the heterogeneous nature of the substance itself. Crude oil is composed of a litany of chemical compounds, including straight chain, branched, cyclic, and both mono- and polycyclic aromatic hydrocarbons (MAHs and PAHs), the latter two classes of which are often implicated as exerting the greatest toxic effect (Almeida et al., 2012). Although also relatively volatile, the solubility of these low molecular weight hydrocarbons means that for at least a short period after a spill they are dissolved in seawater, and are readily bioavailable to marine organisms (NRC 2003). The toxicity of oil to marine organisms in a spill situation is dependent on the specific hydrocarbons present and their relative concentrations, as well as species-specific susceptibility, the duration of exposure, and the physical conditions of the spill (French-McCay, 2002). In the case of a deep water

crude oil release, multiphase compounds emerge, including oil solids, oil liquids, and associated gases, each of which is comprised of many different chemical constituents, and which experience different fates based on their structures and interactions with the physical environment in the weathering process (Reddy et al., 2011). In such an instance, there would be prolonged exposure to low molecular weight, volatile hydrocarbons, such as aliphatic hydrocarbons (alkanes and cycloalkanes) and MAHs and PAHs, versus a shallow water release, in which case these compounds would be quickly lost to the atmosphere and the duration of exposure to them would be brief (on the order of hours). Turbulence is also a factor, as it can lead to the entrainment of oil particles and the downward mixing of surface slicks (Tkalich and Chan, 2002).

The immediate response to an oil spill often includes the use of chemical dispersants, such as those in the COREXIT series (9527, 9524, and 9500), 9500A being the most prevalent during recent events. These complex solutions contain non-ionic and anionic surfactants in either a alcohol, glycol, or hydrocarbon solvent base, and act by breaking large hydrophobic oil particles into smaller oil/dispersant micelles, which sink and are readily dissolvable in the water column (Canevari, 1973). In principle, these dispersants increase the surface area of the oil in order to expedite biodegradation and facilitate weathering processes (Lessard and Demarco, 2000). The body of work on the biodegradation of dispersed oil is ambivalent however, with the rate of degradation apparently more dependent on such factors as the chemical composition of the dispersant and oil involved and the microbial community present, as well as such secondary factors as nutrient concentration, mixing energy, and oil-water ratios, than on the mere presence or absence of dispersant alone (NRC 2005).

Dispersants are usually used on surface spills in order to disperse oil slicks before they reach coastal areas, thus protecting shorelines from oiling. In the open ocean the dilution of dispersed oil is purported to be sufficiently fast as to minimize the duration of exposure of organisms and limit any toxic effects (Lessard and DeMarco, 2000). The decision to use chemical dispersants in shallow coastal environments represents more of a trade-off; their use protects the shoreline and both surface dwelling and surface dependent organisms, such as birds and marine mammals, but increases the risk of exposure of pelagic and benthic organisms to dispersed oil in the water column and sediments (Reed et al., 2004). Chemical dispersion has been shown to increase the load of dissolved hydrocarbons in the water column; monitoring of surface slicks after the Deepwater Horizon spill pre- and post-dispersant application revealed a spike in both total petroleum hydrocarbons and PAHs at 1 meter depth (BenKinney et al., 2011). Whether or not dispersed oil is more toxic to marine life than undispersed crude oil remains relatively unknown however.

Understanding the ecological consequences of dispersant use in the case of oil spills in coastal areas thus necessitates research on the differential toxicity of undispersed and dispersed oil in important and abundant pelagic species. Zooplankton represent a potentially susceptible and trophically key class of organisms. It has been shown that some of the more mobile zooplankton species exhibit oil avoidance swimming behaviors under laboratory conditions (Seuront, 2010), but the scale of large spills, such as the Deepwater Horizon incident, inevitably result in the widespread exposure of organisms (Graham et al., 2010), as was confirmed when PAHs positively identified as coming from the Macondo well were found in mesozooplankton tissues (Mitra et al., 2012).

Toxicity of Oil and Chemical Dispersants

Toxicological studies have demonstrated the lethal effects of various oil constituents on marine invertebrates, focusing mainly on PAHs (see Hylland, 2006 for a review). Often these studies have utilized a single hydrocarbon compound or a few select compounds (e.g. Unger et al., 2008) as opposed to the heterogeneous water-soluble fraction of crude oil. And while these individual compounds can be lethal with acute, high-level exposure and/or high levels of ultraviolet light (Pelletier et al., 1997), their use neglects to consider the partitioning of oil in water and the potential toxic action of multiple compounds (e.g. Barata et al., 2005), either by bioaccumulation or by separate toxicity pathways.

Chemical dispersants are not completely innocuous on their own; their surfactant nature leads to interactions with cell surfaces, both in the case of single-celled organisms and in thin epithelial tissues such as those found in the gills of fish and invertebrates (Abel and Skidmore, 1975; Sandbacka 2000). A study comparing the toxic effects of the eight chemical dispersants listed in the USEPA's National Contingency found COREXIT® 9500A to be only slightly toxic to mysid shrimp, and virtually nontoxic to inland silversides, two commonly studied toxicological model organisms (Hemmer et al., 2011). The MSDS for COREXIT 9500A suggests a wide range of sensitivities for marine invertebrates though, as it lists median lethal concentrations (LC_{50}s) of 32.23 mg/L for mysid shrimp, 20.7 mg/L for *Artemia spp*, and 2 mg/L for the copepod *Acartia tonsa*.

In theory, chemical dispersant is only applied to oil slicks and emulsifications on the water's surface. Therefore, toxicity data for dispersant alone are of limited practical use, except in the context of its comparison to dispersed oil, and the more

relevant research deals with the toxicity of oil alone versus its chemically dispersed analogue. The toxic effects of oil/dispersant mixtures on marine organisms, particularly fish and crustaceans (e.g. Wells et al., 1992; Fucik et al., 1995), have been relatively well studied. Several studies have suggested that dispersed oil is slightly less toxic than oil alone, with some exceptions based on the test species and experimental protocol followed (Singer et al., 1998; Wetzel and Van Fleet, 2001). Other studies, however, have found no significant difference in the toxicity of dispersed and undispersed oil (Long and Holdway, 2002; Wu et al., 2012). Yet another found dispersant and dispersed-oil to be more toxic to mesozooplankton than oil alone (Almeda et al., 2013). Direct comparison of results between studies is difficult however, as disparate exposure regimes and chemical analyses have been used. Further, species may differ drastically in their vulnerability to dispersed oil exposure (e.g. Singer et al., 1998), contributing to this ambivalence.

A few select model organisms appear repeatedly in the toxicological literature (e.g. the mysid *Americamysis bahia* [Hemmer et al., 2010]). While their frequent use allows for certain cross-study comparisons to be made and facilitates the establishment environmentally acceptable levels of pollutants, the data created are of little use in assessing the potential ecological effects of oil spills. These model organisms poorly represent (1) the diversity of organisms at risk of exposure in the case of a spill, (2) those most likely to be susceptible to the toxicants, or (3) those with the potential to manifest ecosystem-wide effects. The toxicological study of ecologically important species is a paramount step in assessing the effects of spills on coastal ecosystems.

Mortality is the most common toxicological endpoint in studies involving oil and dispersant. And while select field studies have noted perennial effects of spills on

zooplankton communities (del Próo et al., 1986), a greater number have observed only minimal mortality and/or ephemeral effects at the community level (Johansson, 1980; Davenport, 1982; Varela, 2006). Sublethal exposure to oil and dispersant still has the potential of affecting the physiology and behavior of organisms, however. Studies of the sublethal effects of hydrocarbons and chemical dispersants have observed changes in the spawning, growth, feeding and locomotion of invertebrates (Wu et al., 1997; Villanueva and Montano, 2011; Seuront 2011; Goodbody-Gringley et al., 2013), but much still remains to be done concerning sublethal effects in marine zooplankton. These sublethal effects vary among taxa, life stages, and also, predictably, with concentrations of oil and dispersant, and are important in understanding the ultimate consequences of a spill for exposed organisms.

This work aims to address the deficiencies in the toxicological data enumerated and discussed above by examining the both lethal and sublethal effects of dispersant, weathered crude oil, and dispersed weathered crude oil exposure on a trophically important gelatinous zooplankter, the ctenophore *Mnemiopsis leidyi*, an abundant coastal/estuarine species which belongs to a poorly studied group of organisms in terms of oil and dispersant toxicity.

Mnemiopsis leidyi

The lobate ctenophore *Mnemiopsis leidyi* (Figure 1.1) is native to the temperate waters of the western Atlantic, with a geographical range extending historically from Cape Cod in the north to South Carolina at its southern extreme (Mayer 1912). Since its description, the range of the species has extended southward

into the Gulf of Mexico, where oil extraction activities are high and it overlaps with another species of the same genus, *Mnemiopsis mccradyi* (Felder and Camp, 2009). In the 1980s *M. leidyi* began to be found in non-native basins, likely transported there via the ballast water of transatlantic ships, and today it can be found in the Black, Caspian, Aegean, North, Mediterranean and Baltic Seas, among other places (Shiganova et al., 2001; Javidpour et al., 2006). The species functions as an important predator in coastal ecosystems throughout both its native and invasive range. The heavy predation of adults on zooplankton (Granhag et al., 2011), including the larvae of commercially valuable fish and shellfish species, affects not only the communities on which they feed but also, secondarily, phytoplankton as a result of reduced grazing pressure (Deason and Smayda, 1981). Additionally, the clearance rate on autotrophic and heterotrophic microplankton by larval and post-larval ctenophores is sufficiently high to impact the base of coastal food webs at these early life stages (Stoecker et al., 1987). *M. leidyi* possesses characteristically bright luminescence (Shimomura, 2006), described in detail in Chapter 3, which may play a role in governing its trophic interactions. The extensive range and trophic importance of *M. leidyi* make it a species of particular interest when studying the effects of coastal oil spills on marine ecosystems worldwide.

Scope of the Study

As no toxicity data were previously available for ctenophores exposed to oil and/or dispersant, mortality curves were first constructed and median lethal concentrations (LC_{50}) values calculated for dispersant, crude oil, and dispersed oil at

two environmentally relevant temperatures, 15°C and 23°C. Once toxicity levels were determined for these solutions at the two temperatures, both general and species-specific measures of sublethal toxicity were employed to further elucidate the effects of oil and dispersant on *M. leidyi*: whole animal routine respiration rates and activity of the detoxification enzyme glutathione-s-transferase were employed as general measures, and bioluminescence was assayed as a sublethal parameter specific to the biology of *M. leidyi*.

This work extends the body of oil and dispersant toxicity research to a previously unstudied organism of trophic importance in coastal ecosystems where oil spills are an environmental concern, and it does so using standardized methodology that should allow for straightforward comparison to other contemporary work. Further, it advances the limited field of more nuanced oil and dispersant toxicology by including sublethal assays and temperature as an experimental factor potentially affecting oil and dispersant toxicity. Studies such as this are important in fully understanding the effects of oil spills and dispersant application in coastal ecosystems, and will provide valuable information to be considered in the decision to use chemical dispersants in the mitigation of spills.

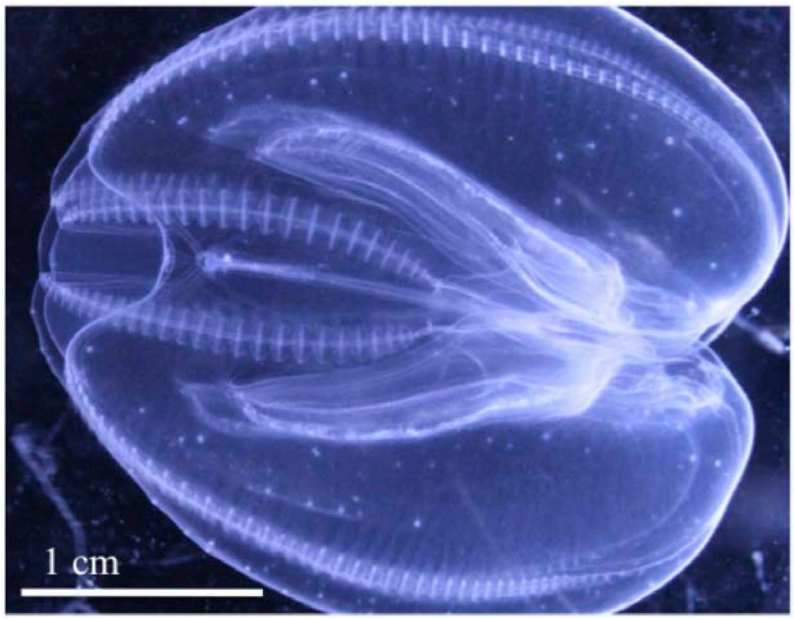

Figure 1.1 ***Mnemiopsis leidyi.*** **Digital photograph of** ***M. leidyi*****, taken under a microscope (7x magnification). The phylum Ctenophora is characterized by the presence of comb rows, used in swimming. In** ***M. leidyi*** **the meridional canals that underlie these comb rows are lined by luminescent cells (photocytes). Scale is shown in the image.**

Chapter 2

MORTALITY

Introduction

Mortality is a standard endpoint in toxicological studies (e.g. ASTM 2007), valuable in and of itself for setting regulatory levels of pollutants, and for identifying sublethal levels of a particular pollutant below which further investigation can be conducted. This study uses the most recently established standard methods to evaluate oil, dispersant, and dispersed oil mortality in an important neritic zooplankter. It will provide data from a gelatinous organism to compare to the sensitivity of common 'model' non-gelatinous test species, and it will also establish sublethal levels for subsequent respiration, detoxification enzyme, and bioluminescence experiments.

By convention, mortality experiments usually employ 24-96 hr static exposures, the long end of this time range being deemed as a period sufficient for organisms to reach the critical body residue of most chemicals (Sprague, 1970), an alternative endpoint to mortality based on the bioaccumulation of a pollutant. The relatively insoluble and volatile nature of toxic, low molecular weight hydrocarbons means that it might take considerably longer for them to equilibrate between an organism and the test medium however, and shorter static exposures more closely mimic environmental conditions during an oil spill (French-McCay, 2002). Some studies have even employed spiked, flow-through exposures (e.g. Wetzel and Van Fleet, 2001), where the concentration of toxicants is initially high and rapidly declines,

although static exposures are still deemed to be adequate by consensus in the oil toxicology field (Coelho and Aurand, 1998).

The use of model estuarine/marine test organisms, such as mysid shrimp and inland silversides, allows for the comparison of results between studies, and is useful in describing the relative toxicity of different types of oil or chemical dispersant (e.g. Hemmer et al., 2010), as well comparing the toxicity of oil and dispersant to other pollutants. Past these advantages, the continued use of model test species provides little information on the ecological consequences of an oil spill. Marine biota are diverse and likely display a wide range of sensitivity to oil and chemical dispersants, information which is critical to directing oil spill response activities (Tjeerdema et al., 2013). The extension of toxicological work to important and representative species is the next logical step in oil and dispersant research. This work does just that by examining *M. leidyi*, a heretofore unstudied gelatinous organism far removed from model test organisms taxonomically, which also plays a prominent trophic role in coastal ecosystems.

The potentially diverse physical conditions of spills are often ignored by toxicological work. Temperature affects both the solubility of hydrocarbon compounds in crude oil (Di Toro et al., 2007) and the rate of uptake and metabolism of toxic compounds by exposed organisms (Cairns et al., 1975). It is therefore reasonable to believe that temperature may affect the toxicity of oil and chemical dispersant. Korn et al. (1979) observed a decrease in the median lethal concentration of oil constituents and a WAF of crude oil in fish and shrimp with increasing temperature, but the effect was not consistent across all test species. It is also likely that detoxification rates vary between species, as different metabolic pathways could

be involved. Any dispersant effect on oil toxicity may also change with temperature, as the efficacy of COREXIT 9500A has been shown to decrease at lower temperatures (Lindstrom and Braddock 2002). The inclusion of temperature in this study may provide information on differences in toxicity by season, and also by depth, at least insofar as temperature is concerned.

M. leidyi lacks an impermeable integument, an exoskeleton, or any real barrier to its external environment; for this reason it was hypothesized that the species would be substantially more sensitive to the toxic effects of oil and dispersant than model fish and crustacean species, and especially so to dispersant and dispersed oil due to the high membrane activity of chemical dispersants (Abel, 1974). It was also expected that there would be an increase in sensitivity from 15°C to 23°C (i.e. exposure at the warmer temperature would be more toxic), although the magnitude of this increase was difficult to predict given the multiple possible pathways of heightened toxicity (e.g. increased solubility of toxic compounds, increased metabolism, etc.).

Materials and Methods

Mortality upon exposure to oil and/or dispersant was assayed by exposing individual ctenophores to varying concentrations of dispersant solutions, water-accommodated fractions (WAFs) of weathered crude oil, and chemically enhanced water-accommodated fractions (CEWAFs) of weathered crude oil containing dispersant. Mortality was scored at 24 and 48 hr for the 15°C exposures, while higher control mortality at 23°C required mortality to be scored only at 24 hr. The data from

these exposures were used to calculate LC_{50} values for the three solution types for 24 and 48 hr at 15°C, and for 24 hr at 23°C.

All of the seawater used for the experiments, including to acclimate individual ctenophores and to prepare the test solutions, was collected from Indian River Inlet, transported to the Hugh R. Sharp campus of the University of Delaware by tanker truck, and vacuum filtered using GF/F and 0.4 μm-filters to remove bacteria. Ctenophores were collected during daytime rising tides in a 0.75 m, 335 μm mesh plankton net (Sea Gear Corporation) set from a davit at the Pollution Ecology Laboratory dock in Lewes, Delaware, USA, located on the Broadkill River just inside of Roosevelt Inlet. Short net sets (15-30 minutes) resulted in sufficient numbers of physically undamaged individuals for the intended experiments. Within 1-3 hours of collection, individuals captured in the net were transferred to individual finger bowls with approximately 150 mL of 0.4 μm-filtered seawater, and acclimated to the experimental temperature in the dark for 24 hr in a laboratory incubator (Percival E-22L). The experimental temperature was matched as best as possible to the ambient conditions; 15°C experiments were conducted on ctenophores collected when the water temperature in the Broadkill River ranged from 6.8-19.6°C (November-May), and 23°C experiments were conducted when the water temperature ranged from 18.2-25.1°C (June-October). Salinity was not a variable in the experiments; at the sampling site on the Broadkill River it ranged from 29-34 psu, as measured by a refractometer, during the collection periods, and the filtered water used to acclimate ctenophores and prepare solutions was adjusted to 32-33 psu.

Test solutions were prepared in 2 L aspirator flaks according to standard methods outlined by the Chemical Response to Oil Spills: Ecological Effects

Research Forum (CROSERF) (Coelho and Aurand, 1998), and later critically revised (Clark et al., 2001) to result in more uniform treatment of the different solutions. These methods are an established protocol for oil/dispersant solution preparation, and dictate the creation of water accommodated fractions by variable loading of oil and/or dispersant and an 18-hour period of moderate mixing (20-25% vortex) by a magnetic stir bar on a digital stir plate followed by 4-6 hours of settling time. This mixing regime has been demonstrated to be roughly representative of field conditions (Singer et al, 2001). COREXIT 9500A (Nalco) and Mississippi Canyon 252 (MC-252) weathered crude oil (Louisiana Light Sweet Crude; British Petroleum) were used to create the test solutions. The appropriate volume of each was added to the 20-25% vortex of filtered seawater in the aspirator bottles via gas tight syringe. The aspirator bottles were then sealed with rubber stoppers and protected from all light with aluminum foil. Water quality data (temperature, pH, dissolved oxygen [D.O.]) were collected for test solutions prepared at 15°C, but all of the parameters were found to be fairly consistent (temp: 14.9-16.0°C, pH: 6.62-8.09, D.O.: 4.99-7.70 mg/L), as has been observed before (Singer et al., 1998), and the data are not presented here.

Four or five concentrations of each solution type were tested, plus a filtered seawater control. Thirty individuals (3 replicates of 10 ctenophores) were exposed at each concentration, with a few exceptions, replicates at the higher concentrations that resulted in total mortality sacrificed to increase the sample size at lower concentrations. The concentrations were chosen based on published data for other small invertebrates and altered after preliminary experiments to include as best as possible both low and total mortality, as well as points between where mortality increased exponentially. Concentration ranges, by nominal loading, were 0-30 mg/L

for COREXIT 9500A, 0-150 mg/L for the WAF, and 0-30 mg/L for the CEWAF. CEWAFs were prepared by adding COREXIT 9500A in a 1:10 ratio by volume with oil. Control replicates (n=10) were conducted once for each of the three solution types at the two temperatures (COREXIT 9500A, WAF, and CEWAF) and then pooled for a total of thirty individuals at each experimental temperature.

After the acclimation period, ctenophores were re-inspected for any visible signs of injury, and seemingly healthy specimens were placed into individual 125 mL glass bottles with Teflon-lined caps containing either one of the three experimental solutions or, in the case of the control, filtered seawater, and devoid of headspace. After 24 hours in the solution filled bottles, mortality was scored based on the paradigm in Figure 2.1. In the 48-hour experiments, approximately 80% of the solution in bottles containing surviving individuals was replenished with freshly prepared test solutions. Mortality was then scored again at 48 hours. The data from these experiments were used to estimate LC_{50}s, as well as their associated 95% confidence intervals, for the three solutions at the two different experimental temperatures, and for the two time points for the 15°C exposures, by the Trimmed Spearman-Karber method (Hamilton et al., 1977), run in a MS-DOS application. This method corrects for control mortality and smooths the curve before estimating the median lethal concentration (http://sdi.odu.edu/model/lc50.php).

The concentration of COREXIT 9500A in the dispersant only solutions was determined by comparison of ultraviolet-visible spectroscopy absorbance readings to a standard curve constructed at the peak COREXIT 9500A absorbance value of 386 nm (Evolution 201 spectrophotometer, Thermo Scientific). Total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) were measured by Dr. Dana

Wetzel at Mote Marine Laboratory (Sarasota, FL, USA) for a representative subset of WAF and CEWAF solutions prepared at 15°C. For these analyses, 400 mL of each test solution were added to 80 mL dichloromethane in amber TraceClean bottles and stored at 4°C until being shipped on ice to Mote Marine Laboratory for extraction using modified EPA method 3510C. TPH and PAH were then analyzed as described in Wetzel and Van Fleet (2001). TPH was determined by gas chromatograph with a flame ionization detector using modified EPA method 8260. The FID signal was integrated over the entire hydrocarbon range from n-C9 to n-C42 and quantified using an internal standard. PAHs (parent compounds and their alkylated homologues) were determined by gas chromatography/mass spectrometry in electron impact selective ion-monitoring mode. These analyses provided information on the solubility of the oil and dispersant and quantified potential chemical constituents contributing most to toxicity.

Results

Chemical Analysis of COREXIT and Oil Solutions

There was no apparent effect of temperature on the relationship between the nominal loading and measured concentrations of COREXIT 9500A in the test solutions. In both cases, the measured concentration of dispersant increased with increased loading in an approximately linear fashion (Figure 2.2).

The presence of COREXIT 9500A in the CEWAF solutions (1:10, dispersant: oil) resulted in TPH concentrations that were 10-20 fold higher than those in WAF solutions with the same nominal loading of oil over the range of loading concentrations (Figure 2.3).

Despite the 13.6 fold increase in TPH concentration between the 50 mg/L WAF and CEWAF, total PAH concentrations were similar between the two solutions (264.50 and 344.11 μg/L for the 50 mg/L WAF and CEWAF, respectively). The distribution of PAH compounds shifted noticeably with the addition of COREXIT 9500A, however (Figure 2.4). Four classes of PAHs: fluoranthrenes, pyrenes, benzoanthracenes, and chrysenes, which were completely absent from the 50 mg/L WAF, were present in the 50 mg/L CEWAF. There were also more alkyl-substituted hydrocarbon compounds in the CEWAF compared to the WAF of the same loading (alkyl PAH: non-alkyl PAH ratio of 4.62 for the 50 mg/L CEWAF versus 3.43 for the 50 mg/L WAF).

Mortality of Ctenophores in COREXIT 9500A, WAF, and CEWAF Solutions

The mortality data resulted in fairly similar curves for all three of the test solutions, as well as for the two different temperatures. Mortality was concentration dependent for COREXIT 9500A, WAF, and CEWAF at 15°C and 23°C; it was also time dependent in the 15°C exposures (Figures 2.5-2.7). Mortality in the WAF solutions rose sharply at low concentrations at both temperatures (Figure 2.6), but was then slow to reach total mortality, especially at 15°C, compared to the two dispersant containing solutions (Figures 2.5 and 2.7). There was a slight decrease in mortality in the second lowest CEWAF (5 mg/L) prepared at 23°C that mimicked the one observed for COREXIT 9500A at 15°C. The effect of temperature on mortality can be seen in the elevation of individual points for each solution type and arrival at complete mortality at lower concentrations, although elevated mortality is also reflected in the controls.

The LC_{50}s for COREXIT 9500A differed significantly at 24 and 48 hr for the 15°C exposures based on their 95% C.I.s, the longer exposure resulting in increased sensitivity (i.e. lower LC_{50})(Table 2.1). This same difference between time points was observed for CEWAF mortality based on TPH, but not on loading concentration, as the TPH concentration per mg/L of oil loaded increased with oil loading (Figure 2.3). Comparison of 15°C WAF solutions between 24 and 48 hr was unable to be made due to low mortality at the highest concentrations at 24 hr; the trimmed Spearman-Karber method requires the smoothed mortality curve to reach 1 in order for a LC_{50} to be estimated. At 24 hr for the 15°C exposure the CEWAF was at least slightly more toxic than the WAF by the virtue of mortality in the WAFs being too low to allow for the calculation of a LC_{50}. The 48 hr LC_{50} for the CEWAF was lower than that for the WAF by oil loading, although there was no difference between the values calculated based on TPH concentration. Another way to assess the relative toxicities of the WAF and CEWAF solutions is to examine the ratios of their LC_{50}s. The ratio of the 15°C WAF 48 hr LC_{50} to the 15°C CEWAF 48 hr LC_{50} was 2.20 by nominal loading and 0.59 by TPH.

Discussion

These mortality experiments with the ctenophore *M. leidyi* established lethal levels for weathered crude oil and dispersant exposure in a representative species from a potentially sensitive and poorly studied group of organisms, the gelatinous zooplankton. The main questions posed by this aspect of the study were whether dispersed oil is more toxic than oil alone to *M. leidyi*, and if there is an effect of

temperature on the lethality of these pollutants. These data suggest that the answer to the first question, ostensibly, is yes. When only the LC_{50}s calculated by the nominal loading of oil are considered, *M. leidyi* exhibited a greater sensitivity to the CEWAF than the WAF. The relative resilience of the ctenophore to the WAF of weathered crude oil also resulted in slowly increasing mortality that precluded the estimation of a LC_{50} for the 24 hr exposure at 15°C. Mortality in the CEWAF increased much more quickly and approached total mortality at lesser oil loadings than were employed in the WAF.

A completely binary response to the question of which oil-containing solution is more toxic is hardly appropriate however, as the LC_{50} estimates based on TPH suggest the opposite of those based on oil loading, and demand further discussion. The lower oil loading of the CEWAF LC_{50} corresponds to a TPH concentration similar to the one associated with the WAF LC_{50}. This is demonstrated by the ratio of the LC_{50}s of the WAF and CEWAF solutions (2.20 by loading, 0.59 by TPH). The ratio calculated from nominal loading can vary widely based on the oil and dispersant in question, but if the CEWAF actually increases the toxicity of exposure to a given loading of oil, then the value of the ratio calculated from TPH would be expected to be substantially greater than 1 (Wu et al., 2012). Effectively, it takes a greater oil loading to reach the same TPH concentration in the WAF versus the CEWAF. The fact that the CEWAF is more toxic by oil loading, but not by TPH, indicates that the increased toxicity of the CEWAF is due largely to the presence of COREXIT 9500A, to which *M. leidyi* is extremely sensitive, or to its indirect effect in creating oil droplets that ctenophores ingest. High sensitivity of zooplankton species to dispersant-containing solutions has been observed by other studies as well (Almeda et al., 2013).

The similar hydrocarbon concentrations of the WAF and CEWAF solutions at different loadings can also be interpreted in the context of the specific compounds in the solutions. The hydrocarbon solvent base of COREXIT 9500A may be partially responsible for the increase in TPH, but there is also an obvious shift in the relative abundance of PAH compounds with dispersant treatment. The presence of additional PAH classes and the greater relative abundance of more alkylated PAHs in the CEWAF is a typical result of dispersant treatment. Mesocosm experiments have documented the amplification of large, hydrophobic PAHs in the water column with dispersant treatment (Yamada et al, 2003), and these high molecular weight compounds probably also contribute to the increase in TPH in CEWAF versus WAF solutions. Compounds contained in oil/dispersant micelles are generally not considered to be bioavailable, but the size of these micelles (<100 μm (Cannevari, 1978)) is well within the range of prey items ingested by *M. leidyi*, as the species has been shown to feed on microzooplakton in the 20-200 μm range (Sullivan and Gifford, 2004). Additionally, another gelatinous marine invertebrate, the doliolid *Dolioletta gegenbauri*, has been found to ingest dispersed oil droplets indiscriminately (Lee et al., 2012). The specific PAHs that were enriched in this experiment have not been demonstrated to increase toxicity however, and therefore their ingestion might not necessarily lead to heightened sensitivity to the CEWAF solutions. The effect of micelle ingestion might be different in the case of other types of oil and dispersant depending on the compounds incorporated into the micelles.

No effect of temperature was observed on the toxicity of dispersant and dispersed oil in *M. leidyi*, as evidenced by the overlap of the confidence intervals of the LC_{50}s estimated for these two solutions at 15°C and 23°C. This is surprising, as the

separate or combined effects of increased solubility and metabolic rates might have been expected to lead to increases in toxicity. The effect of exposure on metabolic rate will be examined further as a sublethal effect in Chapter 3, and the resulting data may aid in the separation of these two temperature-dependent factors. It was not possible to calculate a 24 hr LC_{50} value for the WAF at 15°C due to low mortality across the range of treatment concentrations, and this precluded a formal temperature comparison for oil alone. Higher mortality in the 23°C WAF exposures suggests that the higher temperature was more toxic, but control mortality was elevated as well at 23°C, obfuscating any direct comparison.

The mortality data from this study can also be used to place *M. leidyi* in the broader context of oil and dispersant toxicological work by comparison against LC_{50} values in the literature. As anticipated, *M. leidyi* demonstrates a greater sensitivity to dispersant than well-studied model organisms; the 24 and 48 hr LC_{50}s for *M. leidyi* for COREXIT 9500A (15°C, 24 hr: 6.91 mg/L= 7.14 μl/L; 15°C, 48 hr: 5.42 mg/L=5.60 μl/L; 23°C, 24 hr: 8.26 mg/ L=8.53 μl/L) were all less than half of the value found for the mysid shrimp *A. bahia* (48 hr: 42 μl/L= 40.7 mg/L), and an order of magnitude lower than the value found for a long exposure of the inland silverside *M. beryllina* (96 hr: 130 μl/L= 125.8 mg/L) by Hemmer et al. (2011). The values for *M. leidyi* were similar to values reported for other zooplankton, such as those for the copepod *Labidocera aestiva* (24 hr: 7.8 mg/L, 48 hr: 4.5 mg/L) found by Cohen, et al. (submitted). The 48 hr LC_{50}s for *A. bahia* in WAF and CEWAF solutions, prepared using COREXIT 9500A and Louisiana Sweet Crude (LSC) oil (2700 μg TPH/mL and 5400 μg TPH/mL, respectively [Hemmer et al., 2011]) were more than an order of magnitude greater than those found for *M. leidyi* (15°C, 48 hr: 143.14 μg TPH/mL).

The sensitivities of *M. leidyi* to oil, dispersant, and dispersed oil established by this experiment were used to inform the examination of effects of sublethal exposure to the solutions presented in Chapter 3. Those experiments use the LC_{50}s presented here to set sublethal levels for each solution type, following exposure to which two general sublethal assays (glutathione-s-transferase activity and respiration rate) and one more ctenophore and *M. leidyi*-specific assay of bioluminescence were conducted.

Treatment	Temperature	Time	TPH (μg/L) or COREXIT (mg/L)	Loading (mg/L)
COREXIT	15°C	24 h	6.91 mg/L (5.85-8.16)	-
		48 h	5.42 mg/L (5.01-5.85)	-
	23°C	24 h	8.26 mg/L (6.30-10.84)	-
WAF	15°C	24 h	>>trim	>>trim
		48 h	143.14 μg/L (56.55-362.3)	29.49 mg/L (13.75-63.25)
	23°C	24 hr	--	4.66 mg/L (1.53-14.8)
CEWAF	15°C	24 h	576.63 μg/L (380.2-874.7)	13.41 mg/L (9.32-19.29)
		48 h	242.07 μg/L (153.9-370.8)	6.7 mg/L (4.33-10.38)
	23°C	24 h	--	7.67 mg/L (5.48-10.75)

Table 2.1 Median lethal concentrations (LC_{50}s) of COREXIT 9500A, WAF, and CEWAF solutions for *Mnemiopsis leidyi.* Mortality was scored at both 24 and 48 hr at 15°C and at 24 hr only at 23°C. LC_{50}s (95% confidence intervals) were calculated using the Trimmed Spearman-Karber Method. TPH concentrations were not measured for the oil-containing (WAF and CEWAF) solutions prepared at 23°C, preventing this calculation from being made. Cases where mortality was too low to allow for the LC_{50} estimation are denoted by ">>trim".

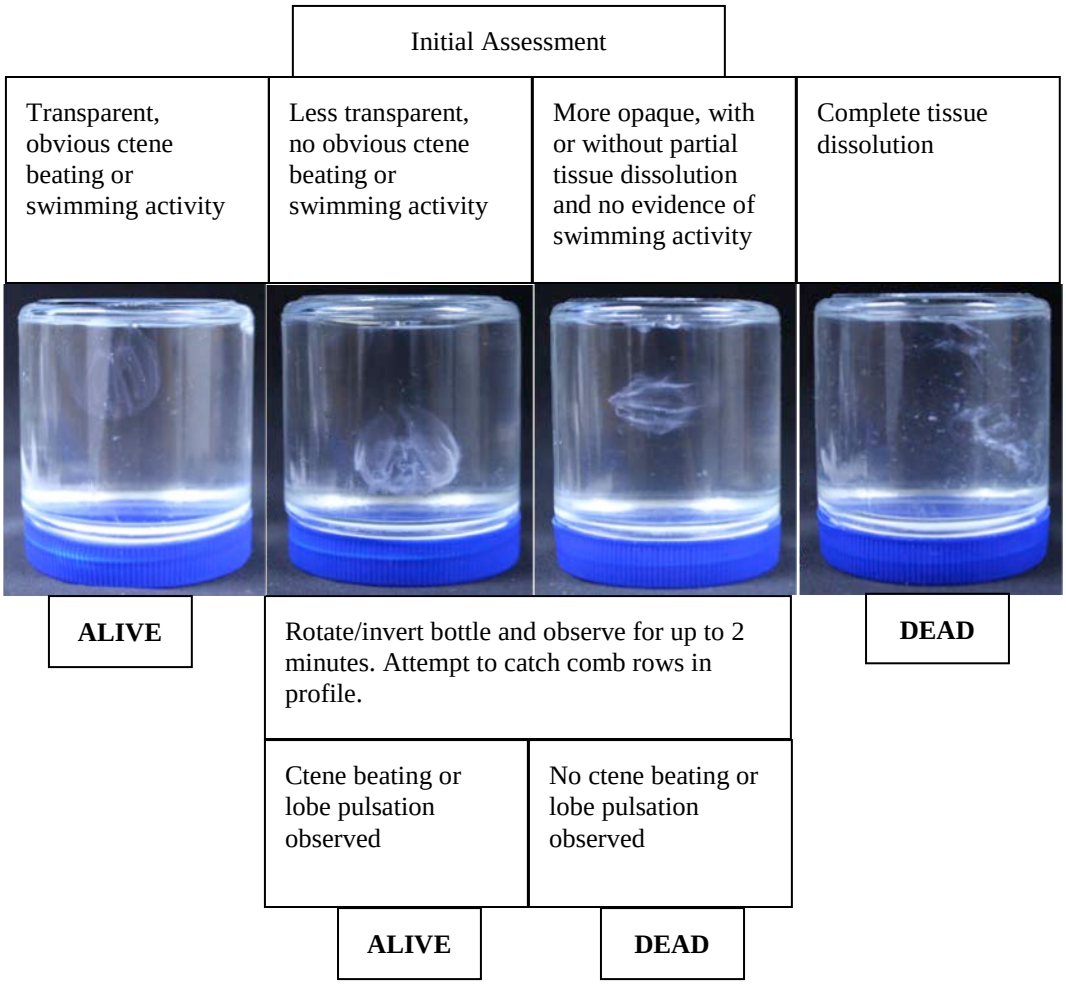

Figure 2.1 Paradigm for assessing mortality in *Mnemiopsis leidyi* following COREXIT 9500A, WAF, and CEWAF exposure. Mortality was judged at 24 hr for the 23°C exposures, and at both 24 and 48 hr for the 15°C exposures.

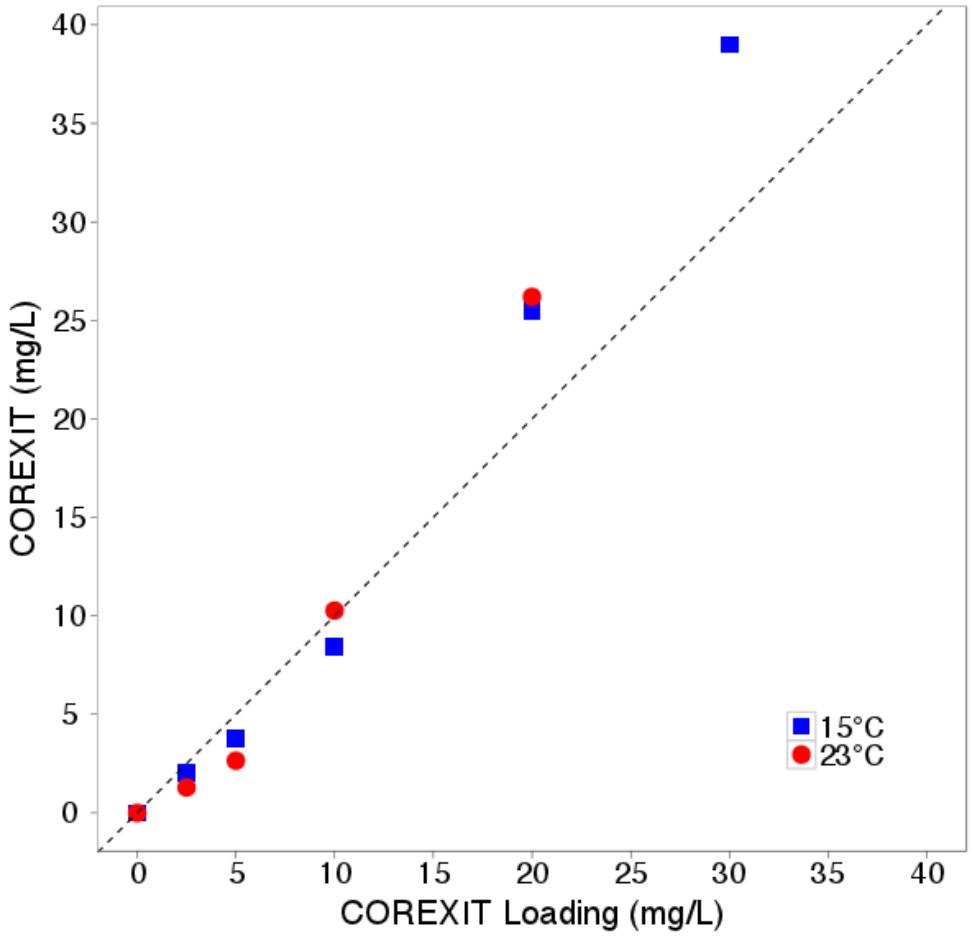

Figure 2.2 Relationship between COREXIT 9500A loading and measured concentrations at 15°C and 23°C. COREXIT 9500A concentration was determined by comparison of ultraviolet-visible spectroscopy absorbance readings to a standard curve constructed at the peak absorbance value of 386 nm. Measurements were made on samples drawn from aspirator bottles after the 18 hr mixing and 4-6 hr settling periods at the time that the solutions were used to start exposures.

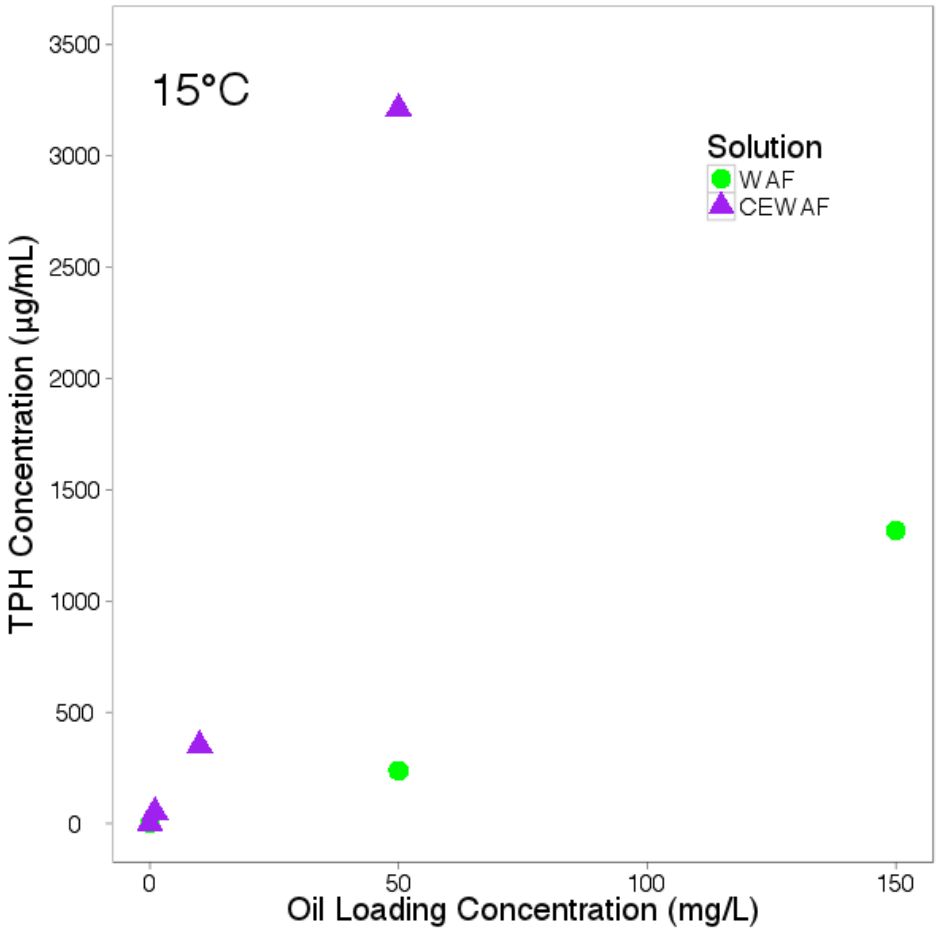

Figure 2.3 Relationship between nominal loading of weathered MC-252 crude oil and TPH concentration for WAF and CEWAF solutions prepared at 15°C. TPH was measured by gas chromatography, CEWAF solutions were dispersed with COREXIT 9500A at 1:10 dispersant to oil ratio by volume. The measured points shown were used to fit a logarithmic curve, from which the TPH concentrations of the remaining loadings were calculated.

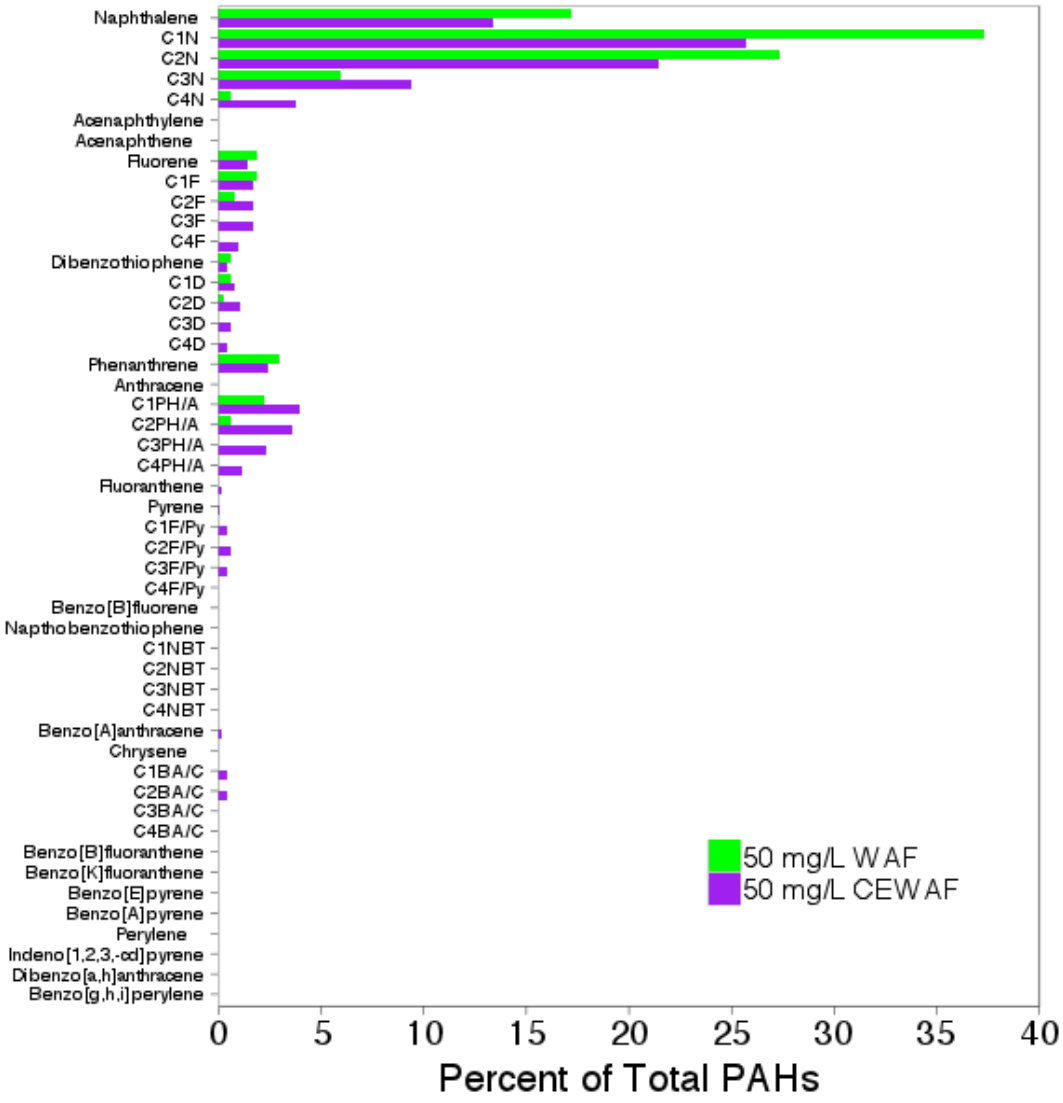

Figure 2.4 Distribution of polycyclic aromatic hydrocarbon (PAH) compounds in a 50 mg/L WAF and 50 mg/L CEWAF of weathered MC-252 crude oil prepared at 15°C. COREXIT 9500A was added in a 1:10 ratio, by volume, in the CEWAF. The abundance of PAH compounds is expressed as a percent of the total concentration of PAHs in each solution.

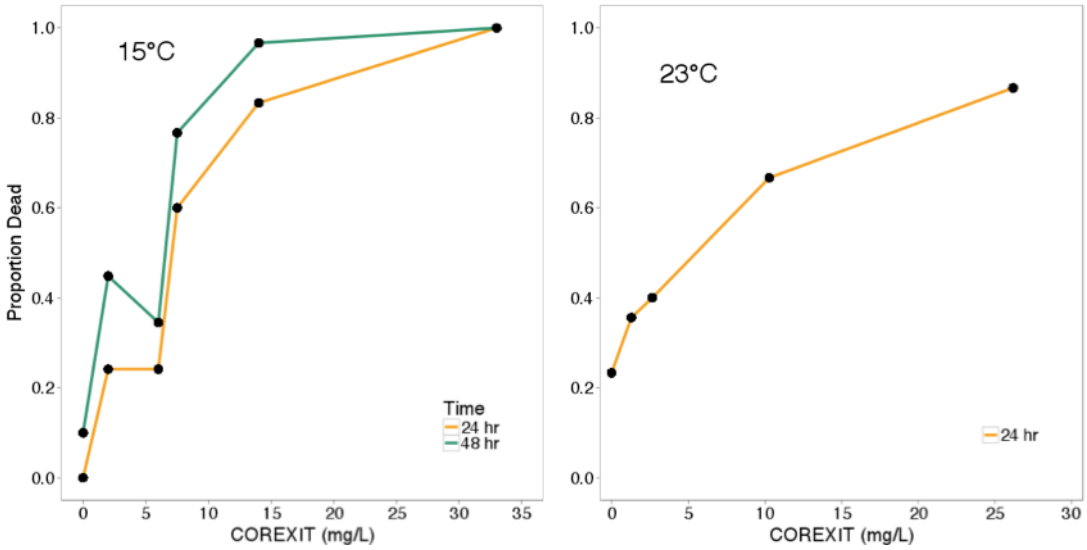

Figure 2.5 Mortality curves for *M. leidyi* exposed to COREXIT 9500A for 24 and 48 hr at 15°C, and for 24 hr at 23°C. Mortality is expressed as the proportion of dead individuals in the total number exposed. 15°C: n= 30, 29, 29, 30, 20; 23°C: n= 30 for all solutions.

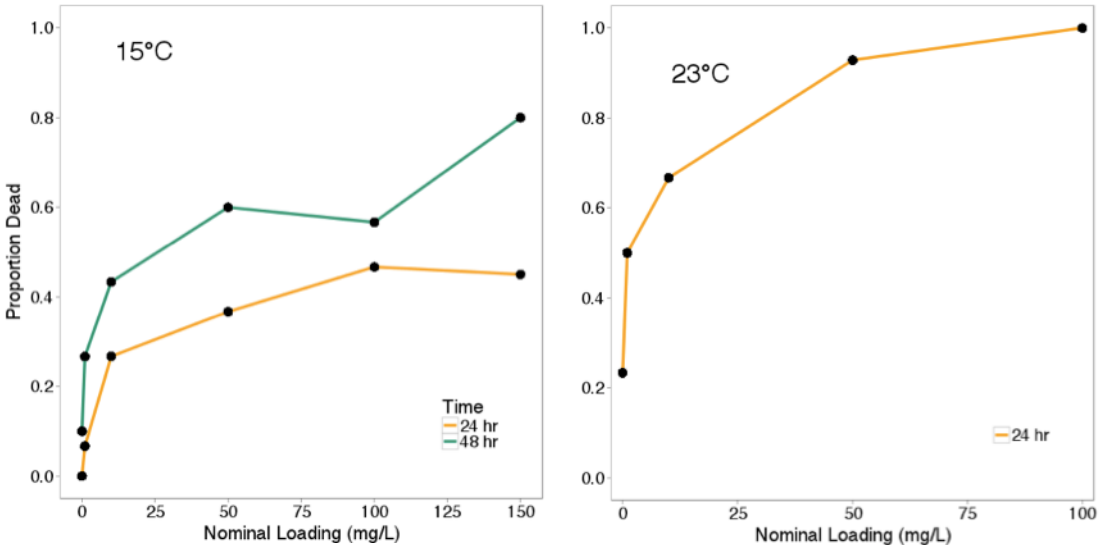

Figure 2.6 Mortality curves for *M. leidyi* exposed to weathered MC-252 crude oil WAF solutions for 24 and 48 hr at 15°C, and for 24 hr at 23°C. Mortality is expressed as the proportion of dead individuals in the total number exposed. 15°C: n= 30, 30, 30, 30, 30, 20; 23°C: n= 30, 30, 30, 28, 20.

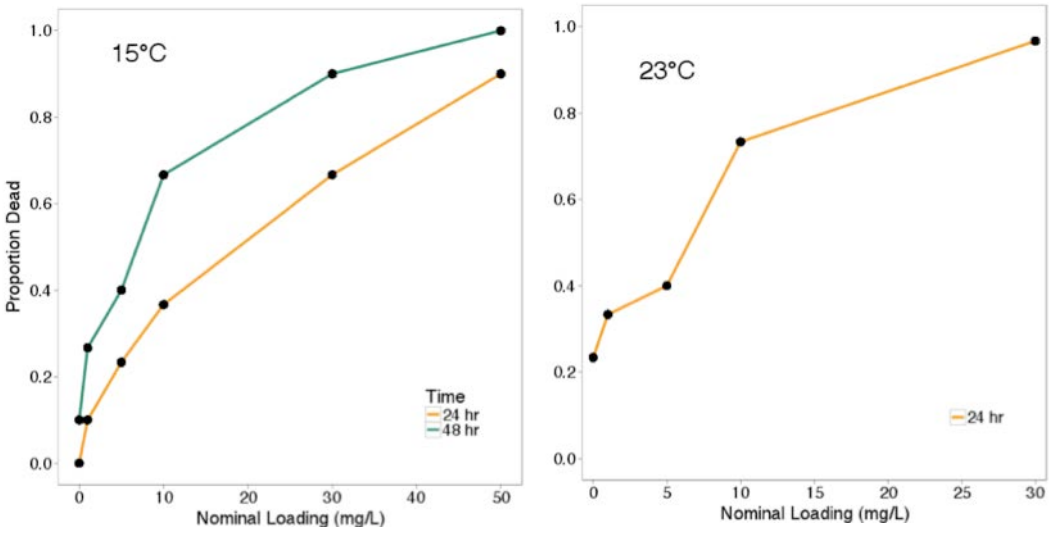

Figure 2.7 Mortality curves for *M. leidyi* exposed to weathered MC-252 crude oil CEWAF solutions for 24 and 48 hr at 15°C, and for 24 hr at 23°C. The oil was dispersed with COREXIT 9500A (1:10 dispersant to oil by volume). Mortality is expressed as the proportion of dead individuals in the total number exposed. 15°C: n= 30, 30, 30, 30, 30, 20; 23°C: n= 30 for all solutions.

Chapter 3

SUBLETHAL EFFECTS

Introduction

Below the lethal levels of the dispersant COREXIT 9500A, weathered light sweet crude oil (MC-252), and dispersed oil for the ctenophore *M. leidyi*, established by the preceding mortality experiments, there still exists the possibility of sublethal physiological effects on the molecular, cellular and organismal levels. These effects may have consequences on the population, community, and ecosystem levels. Such sublethal effects have been noted in other abundant marine zooplankton. For example, sublethal exposure of the copepod *Acartia tonsa* to crude oil resulted in significantly decreased egg production (Almeda et al., 2013), and in a similar study on meiobenthic copepod *Amphiascus* tenuiremis, the development of nauplii and copepodites was delayed by exposure to high level WAFs of crude oil (Bejarano et al., 2006). As with mortality, sublethal data for oil/dispersant exposure in gelatinous zooplankton are absent from the toxicological literature, and this study will serve an important purpose by characterizing sublethal effects in the trophically important species *M. leidyi*.

Glutathione-s-transferases (GSTs) have long been known to be important detoxification enzymes for their role in mercapturic acid formation (Booth et al., 1960). GSTs are a class of enzymes that initiate the detoxification of potentially alkylating agents by catalyzing the conjugation of xenobiotic compounds to the thiol group of glutathione, effectively neutralizing their electrophilic sites and rendering

them more water-soluble. Glutathione conjugates are metabolized further until they reach the final product, mercapturic acids, which are excreted (Habig et al., 1974). Work has been done to establish the activity of these widely distributed detoxification enzymes as an ecotoxicological model parameter in marine invertebrates (e.g. Raisuddin et al., 2007), and other work has shown GST activity in response to hydrocarbon pollutants (Lee 1988). GST activity has yet to be recorded in ctenophores, although the genome of *M. leidyi* has been sequenced and shown possess the genes that code for the enzymes (NIH).

Whole animal respiration rate has been employed by other toxicological studies as a sublethal parameter (e.g. Hook and Fisher, 2009) because it serves as a proxy for aerobic metabolic rate, which can be affected by toxins, potentially increasing as detoxification pathways are upregulated (Hansen et al., 2008). Select crustacean zooplankton species (i.e. some copepods and portunid crab megalopae) have been shown to display increased respiration rates upon COREXIT 9500A exposure, concomitant with other sublethal effects (Cohen et al., submitted). Measuring routine respiration rates over the exposure period provides information about the metabolic activity of individuals. Respiration of *M. leidyi* is temperature sensitive (Kremer, 1977), and an increase in respiration rate with temperature is expected, toxic exposure notwithstanding, but differences between the solutions types and concentrations within each solution may provide insight into the relative metabolic stress associated with the different test solutions.

While GSTs are nearly ubiquitous among animals, bioluminescence is a much more species-specific trait. While it is common among pelagic organisms in the open ocean, bioluminescence is much scarcer in coastal waters, where the light environment

is more heterogeneous and less stable (Morin 1983). Ctenophores are one of the relatively few abundant neritic luminescent organisms. Almost all known ctenophore species are bioluminescent (Haddock and Case, 1995), and the phylum contains some of the most brightly luminous organisms in the sea. The production of light by marine organisms may serve functions as varied as controlling predator-prey relationships, providing camouflage by counter-illumination, or aiding in mate identification and selection (Haddock et al., 2010). It has been suggested that the primary function of neritic luminescence, which is often characterized by a quick flash upon contact stimulation, as displayed in *M.leidyi*, is predator avoidance, with the secondary functions such as intraspecific signaling and mate attraction also being possible (Morin 1983). However, definitive studies on functional aspects of luminescence are very limited (e.g. Rivers and Morin, 2013).

The mechanisms that underlie biologically produced light are as numerous and varied as the organisms capable of producing it, although all operate on the basic principle of oxidizing a substrate, or luciferin, with an enzyme, either a luciferase or photoprotein, to create an excited state in the light-emitting molecule. Ctenophores achieve luminescence with the oxidation of coelenterazine by species-specific, calcium-dependent, and oxygen-independent photoproteins (Shimomura 2006). The photoprotein mnemiopsin (from *Mnemiopsis* spp) was first characterized by Ward and Seliger (1974), and demonstrates *in vivo* emission spectra with a maximum at 488 nm. Mnemiopsin is reversibly inhibited by light exposure in live ctenophores, or with the addition of the appropriate reducing agent (Ward and Seliger 1974).

The calcium dependency of mnemiopsin represents a potential susceptibility to toxins, especially to chemical dispersants, whose membrane disruption properties may

interfere with the animal's ability to concentrate calcium in the photocytes where mnemiopsin is localized. If dispersant exposure increases the permeability of photocytes to calcium, an increase in *in vivo* luminescence may be observed. The extra energy required during periods of toxin-induced metabolic stress might also interfere with the energetics of calcium transport or mnemiopsin reduction, thereby affecting luminescence. If *M. leidyi* uses the glutathione-s-transferase pathway to detoxify weathered crude oil or chemical dispersant, there should be an increase in enzyme activity in the treatments relative to the controls, which may also be accompanied by an increase in aerobic metabolic rate, depending on the magnitude of the response. The effect of temperature on these sublethal parameters, respiration rate in particular, was expected to be representative of Q_{10}s typical for metabolic processes.

Materials and Methods

Glutathione-s-transferase Activity

GST activity assays were conducted on separate exposures of ctenophores to the treatment solutions from those described for mortality (Chapter 2), respiration rate (this Chapter), and bioluminescence (this Chapter). This assay was only conducted for the following subset of sublethal solutions prepared at 15°C in the same manner as described in Chapter 2: a filtered seawater control; 2.5 and 5 mg/L COREXIT 9500A; 1 and 10 mg/L WAF; and 1 and 10 mg/L CEWAF. After the 24 hr exposure period, groups of 3-4 ctenophores exposed to a given test solution were homogenized together in cold (4°C) 0.25x Tris/Glycine/SDS sample buffer (pH 8.3), and placed on ice. At least four replicate groups of 3-4 individuals were tested from each concentration of

the solutions. From the resulting homogenate, 2 mL was transferred to a 30,000 NMWL microcentrifuge filter tube (Millipore UFC4LTK25), and spun at 5,000 rcf and 4°C for 45 minutes to increase the concentration of GST (MW= 45,000) in each the sample. The retentate was removed from the tubes and utilized in the GST assay following the standard methods of Habig (1974). This colorimetric method involves the combination of equal molar fractions of the substrate, glutathione (Sigma Aldrich G4251**)**, with 1,2-Dichloro-4-nitrobenzene (Sigma Aldrich 138630**)** in a PBS buffer solution (pH 7.8), with both reactants being in molar excess of the glutathione-s-transferase present in the sample. In this case, reactants were at a final concentration of 0.02*M* in 1.5 mL PBS, with 100μL of the concentrated sample added. Homogenate from the digestive gland of *Littorina littorea*, which exhibits exceptionally high GST activity (Lee, 1988), was prepared in the same manner as the samples and used as a positive control at a dilution below that required to saturate the reaction. The presence of the GST enzyme in a sample results in the conjugation of glutathione to the thiol group of the 1,2-Dichloro-4-nitrobenzene, which results in a colormetric change in the test solution at 340 nm. This change was recorded spectrophotometrically at 30 second intervals over a 5 minute period (Evolution 201 spectrophotometer, Thermo Scientific). The rate of color change of a blank reaction mixture, its sample volume replaced by PBS, was subtracted from that of the sample mixtures to obtain GST activity, which was then normalized to total protein in the sample, measured via a Coomassie Protein Assay (Thermo Scientific). GST activity of exposed ctenophores was compared amongst treatments via a Kruskal Wallis One-Way Analysis of Variance on Ranks, with Dunn's Test used to compare the treatments to the control.

Respiration Rate

Routine respiration rates were measured for ctenophores at both temperatures after 24 hr exposure. The 15°C assays involved a separate set of exposures (due to 48 hr mortality experiments), while the 23°C assays were conducted in conjunction with the 24 hr exposures used for mortality. Dissolved oxygen measurements were taken immediately upon opening the bottles after the 24 hr exposure period. These measurements were made with a miniature polargraphic oxygen electrode (MI-730, Microelectrodes, Inc.) with negligible oxygen consumption and no requirement for stirring, connected to a laptop computer via a data acquisition system (PowerLab 26T analog-digital converter with LabChart v.7 software, AD Instruments). Oxygen consumption by individual ctenophores was calculated by the single end-point method (Ikeda et al., 2000), whereby oxygen consumption in control bottles without animals was subtracted from the observations in the chambers containing animals. Generally, 2 control bottles were prepared for every 10 individuals exposed at a given concentration of a solution. Following the dissolved oxygen measurement, the tissue volume of individual ctenophores was measured by its displacement in a 100 mL graduated cylinder containing 25 mL of filtered seawater. Tissue volume was converted to wet weight using an empirically determined relationship (wet weight= 1.198 + (0.99 x tissue volume), R^2=0.997, F=3498, d.f.=1, $p<0.001$) to allow for the calculation of weight specific oxygen consumption rates. Wet weight did not vary significantly between concentrations within the solution types at the two temperatures (Kruskal Wallis One-Way Analysis of Variance on Ranks, $p>0.05$ in all cases), allowing for the direct comparison of weight specific respiration rates between

concentrations by a Kruskal Wallis One-Way Analysis of Variance on Ranks, with Dunn's Test used to compare the treatments to the control.

While the water used to prepare test solutions had been filtered to remove microbes, the body surface of ctenophores is hardly sterile, and likely contributed microbes to the test bottles (Daniels and Breitbart, 2012). Accordingly, the portion of respiration attributable to microbes must be measured in order to accurately assess the respiration of ctenophores in the test solutions. Microbial respiration was measured in a subset of WAF and CEWAF concentrations at 23°C in conjunction with the 24 hr exposures for respiration described above. Microbial respiration in the 15°C exposures was presumed to be negligible (Ikeda et al., 2000). After removal of an individual from a bottle, its tissue volume was replaced with filtered seawater and the bottle resealed (Ikeda et al., 2000). After a subsequent 24 hr period dissolved oxygen was measured again, and the respiration rate for this second 24 hr period was expressed as a percent of the first 24 hr period when a ctenophore was in the test bottle. The microbial contribution to the measured respiration rate was compared amongst treatments using a Kruskal Wallis One-Way Analysis of Variance, and treatments were compared to the control using Dunn's test.

Bioluminescence

As with the respiration rate experiments, bioluminescence assays were conducted on ctenophores exposed for 24 hr, via a separate set of exposures at 15°C, and in conjunction with mortality experiments at 23°C. The bioluminescence of individual ctenophores was assessed in the same 125 mL glass bottles used for the exposures. The dissolved oxygen and tissue volume measurements took approximately

5 minutes, after which the test solutions were discarded and individuals were returned to the bottles in the 25 mL of filtered seawater in which their tissue volume was measured. Individuals were then acclimated in the dark for 30-60 minutes at the appropriate experimental temperature to allow for reversal of any light-inhibition of their luminescence (Ward and Seliger, 1974). The remainder of the experiment was conducted in near darkness within a light-tight room, with the aid of light from a dim far-red headlamp. After the dark-acclimation period, the luminescence of individual ctenophores was elicited mechanically and chemically using the setup pictured in Figure 3.1, which was housed in a light-tight enclosure. A short section of capped white PVC pipe, centered on top of a white stir plate (Corning model AS645), functioned as an integrating sphere. Through a 3 cm hole in its side, the pipe accommodated a radiometrically calibrated light sensor head (model 247, UDT Instruments) coupled to a detector (S471 optometer, UDT Instruments). The detector was set to measure irradiance at the 490 nm energy calibration point, and light data were logged at 2 Hz via a laptop computer (*S471 Demo* software).

In the measurement of bioluminescence in an individual ctenophore, the first phase of mechanical stimulation utilized a 1-inch magnetic stirbar placed in the bottle with the animal. Stimulation at this point led to *in vivo* luminescence from the reaction of calcium and mnemiopsin within the photocytes (Ward and Seliger, 1974). The stirplate was set at an intermediate speed (~400 rpm), switched on 2.5 s after the start of data recording, and left on for 27.5 s, yielding a total of 30 s of light data in the first stimulation period. At this time the stirplate was switched off, ending the mechanical stimulation of the whole animal, and the stirbar removed by a magnetic retriever, with caution taken not to disturb the ctenophore. A tissue homogenizer (Kinematica model

PT90/35) was then set in place through a 3 cm port in the top of the PVC pipe cap, and the recording of light data was begun again, with a 2.5 s lag time before the homogenizer was switched on at about half speed. During the next 27.5 s the animal was homogenized, and light was released *in vivo* by intense stimulation of the still intact photocytes. At the end of this 30 s period, 25 mL of 0.1 *M* KCl was added to the 125 mL bottle via rubber tubing (0.5 cm diameter) connected through the 3 cm top port to a funnel outside of the light-tight enclosure, hypotonically lysing the cells. Light data were then collected for a third period of 30 s, continuous with the second, during which any residual mnemiopsin from the first two periods was free to react with calcium in the seawater.

For the purposes of the analyses described below, the first two 30 s periods of mechanical stimulation (i.e. stirring and homogenization) elicited what was considered *in vivo* luminescence, and the last period, when photocytes were lysed, *in vitro* luminescence. The total light response was integrated over all three periods and compared between concentrations within each solution using a Kruskal-Wallis One Way Analysis of Variance on Ranks, with Dunn's Test being used to compare treatments to the control. The ratio of *in vitro* to *in vivo* luminescence was calculated as a proxy for the 'luminescent potential' of individual ctenophores, which provides an estimation of the relative concentrations of calcium and mnemiopsin within the photocytes. A higher luminescent potential index is indicative of reduced intracellular calcium relative to mnemiopsin (and hence a larger *in vitro* reaction with calcium in seawater), and a low luminescent potential index indicates the opposite, that is a higher concentration of intracellular calcium relative to mnemiopsin, and hence a greater portion of the total light is released *in vivo*. Luminescent potential was

compared between concentrations within each solution type in the same manner as was described for the total integrated response (Kruskal Wallis One-Way Analysis of Variance on Ranks, Dunn's post-hoc Test).

The photoprotein reaction kinetics were also examined. The decay curve from the *in vivo* reaction of mnemiopsin with calcium is first order (Ward and Seliger, 1974), or exponential. Plotted on a logarithmic scale this first order decay curve becomes linear, and the rate of luminescent decay, represented by the slope of the line, can be compared amongst groups. In this study, the 5 s period following the peak response during the first mechanical stimulation phase (stirring) of each individual was normalized to its respective peak value, and regression lines were fitted by treatment. The slope of the resulting regression lines were compared between concentrations within solution type and temperature with ANCOVAs.

Results

Glutathione-s-transferase Activity

The effect of COREXIT 9500A on GST activity was apparent in the low dispersant-only solutions (Figure 3.2), with both the 2.5 mg/L and 5 mg/L loadings leading to significant increases in activity compared to the control ($H=23.501$, $d.f.=6$, $p< 0.001$, Dunn's Test $p<0.05$). The WAF and CEWAF both displayed increasing trends in activity with increased oil loading, the CEWAF to a greater extent than the WAF, but neither were different from the control.

Respiration Rate

Routine respiration rate for the 24 hr period at 15°C generally increased with COREXIT 9500A concentration (Figure 3.3), becoming significantly greater than the controls at 20 mg/L. ($H= 12.738$, d.f.=4, $p=0.013$, Dunn's Test, $p< 0.05$). At 23°C the rates were much more constant, with no significant differences compared to the controls ($H=8.365$, d.f.=4, $p=0.071$), although there was a elevation in respiration rate at the 5 mg/L loading. The effect of temperature on respiration rate diminished with increasing COREXIT 9500A concentration, the increase in respiration at 15°C leading to a decreasing trend in the Q_{10} values (Table 3.1).

In the WAF exposures there was no significant effect of oil loading at either of the two experimental temperatures (15°C: $H=8.263$, d.f.=4, $p=0.082$; 23°C: $H=4.896$, d.f.=3, $p=0.180$). There was a trend, however, of decreased respiration rates relative to the controls at the intermediate concentrations of the 15°C exposures. There was also a notable increase in respiration rate at 23°C for the 10 mg/L loading (Figure 3.4). The Q_{10} values appear to have been dictated more strongly by the decrease seen at 15°C, as they were greater than 2 for all of the concentrations (Table 3.1).

In CEWAF, respiration rates at 15°C generally increased with oil loading, only the highest concentration (30 mg/L) resulting in significantly increased respiration compared to the control (15°C: $H=13.983$, d.f.=4, $p=0.007$, Dunn's Test, $p<0.05$). While a similar general increase in respiration rate with oil loading was observed at 23°C, the effect was non-significant ($H=3.514$, d.f.=3, $p=0.319$) (Figure 3.5). The rate of the increase in respiration rates at 23°C seemed to outpace that at 15°C, however, as the Q_{10} values grow with increasing oil loading (Table 3.1).

Microbial respiration in the WAF and CEWAF solutions, measured at 23°C as the change in dissolved oxygen in individual bottles relative to the ctenophore-free controls for a second 24 hr period following the exposure and removal of ctenophores, revealed no statistically significant difference between treatments and the controls ($H= 8.382$, $d.f.=6$, $p=0.211$). This measurement demonstrated the relatively small contribution of microbial respiration to the 24 hr rates measured for ctenophores, as for all of the solutions tested the average contribution was less than 3%, and the interquartile range of the median contained 0% (median= -1.8, IQR= -2.92 - 0.362)(Figure 3.6).

Bioluminescence

The average luminescent responses of ctenophores exposed to the various concentrations of the three solution types are plotted in Figure 3.7, which is intended to aid in the interpretation of subsequent, more quantitative results by illustrating the data in the context of the experimental design and analysis. General trends in the light response can be inferred by reference to this figure and used to understand both the total integrated light response and the calculated luminescent potential index.

Exposure to COREXIT 9500A at 15°C resulted in an apparent increase in total integrated luminescence at the two lower concentrations (2.5 and 5 mg/L), but then a rather precipitous drop at the higher two (Figure 3.8). None of these changes in luminescence were significantly different from the controls ($H=19.648$, $d.f.=4$, $p<0.001$), as the small p value was due to differences between the two lower and two higher concentrations. This same pattern was not observed at 23°C, where luminescence decreased steadily, though non-significantly with increasing COREXIT

9500A concentration (H=8.075, d.f.=4, p=0.089). The effect of COREXIT 9500A concentration on luminescent potential at 15°C was non-significant (H=2.77, d.f.=4, p=0.596). At 23°C luminescent potential tended to decrease with dispersant loading, and the value for 10 mg/L was lower than the control (H=10.685, d.f.=4, p=0.03, Dunn's Test, p <0.05)(Figure 3.9). There was no significant effect of COREXIT 9500A concentration on the slope of the first order decay curve at 15°C (F= 0.5667, d.f.=4, p=0.6881) or 23°C (F=0.2558, d.f.=3, p=0.8545)(Figure 3.10).

In the WAF solutions at 15°C the total integrated light response was significantly diminished at 1 mg/L, 50 mg/L, and 100 mg/L versus the control (H=21.741, d.f.=4, p<0.001, Dunn's Test, p<0.05). The integrated values for the 23°C exposure also decreased with oil loading, with significant drops at 10, and 50 mg/L (H=14.05, d.f.=3, p=0.03, Dunn's Test, p<0.05)(Figure 3.11). At 15°C there was a curious increase in luminescent potential at 50 mg/L, which led to a significant p value due to its difference from the other concentrations, although it was not significantly greater than the control value (H=10.715, d.f.=4, p=0.03). The effect of oil loading on luminescent potential at 23°C was nearly significant, with higher concentrations decreasing relative to the control (H=7.753, d.f.=4, p=0.051)(Figure 3.12).The effect of oil loading on luminescent decay in the WAF solutions was non-significant at both 15°C(F=1.137, 4 d.f., p= 0.3515) and 23°C(F= 0.0304, 2 d.f., p=0.97)(Figure 3.13).

The integrated light response decreased significantly versus the control at all CEWAF concentrations tested at 15°C with the exception of the 10 mg/L treatment, which was slightly higher than the values for the other loadings (H=18.375, d.f.=4, p=0.001, Dunn's Test, p<0.05). At 23°C there were decreased integrated responses at 5 and 10 mg/L when the single observation at 30 mg/L loading was excluded from the

analysis (H=17.312, d.f.=3, p<0.001, Dunn's test, p<0.05)(Figure 3.14). There was no significant effect of loading concentration of CEWAFs on luminescent potential versus the controls at 15°C (H=0.412, d.f.=4, p=0.981), however at 23°C the 1 mg/L treatment decreased relative to the control (H=9.8, d.f.=3, p=0.024, Dunn's test, p<0.005)(Figure 3.15). As in the other solution types, luminescent decay did not change with CEWAF exposure (15°C: F=0.470, d.f.=4, p=0.757; 23°C: F= 0.178, d.f.=2, p=0.838)(Figure 3.16).

Discussion

Sublethal toxic effects on the metabolism and bioluminescent emissions of *M. leidyi* were discovered in response to oil and chemical dispersant exposure. Routine respiration rates, measured as a proxy for aerobic metabolic rate, were in the same range in the control and treatment solutions as those found by Anninsky et al. (2005) for *M. leidyi* from the Black Sea under starvation conditions, which were the same conditions of the present study. Exposure to the highest concentrations of COREXIT 9500A and CEWAF prepared at 15°C increased respiration rates, which is suggestive of some degree of metabolic stress associated with exposure to these solutions. Similar increases in oxygen consumption rates elicited by dispersant exposure have been noted in other invertebrates, including the copepod *Pontella meadii* and megalopae of the blue crab *Callinectes sapidus* (Cohen et al., submitted). Exposure to dispersed oil has also been shown to lead to increased respiration in the snail *Littorina littorea* (Hargrave and Newcombe, 1973). The fact that these increases occurred in the

dispersant-containing solutions but not in the WAF implicates COREXIT 9500A as being responsible for triggering the response.

The upregulation of detoxification pathways is likely a contributing factor in the observed increase in aerobic metabolism of *M. leidyi*, as the GST activity data also displayed the dispersant effect at the two lowest COREXIT 9500A concentrations (2.5 mg/L and 5 mg/L), and in the generally increasing trend in activity with increasing CEWAF concentration. The volume of COREXIT 9500A loaded into the 10 mg/L CEWAF solution approaches that of the low dispersant only solutions, suggesting that the increased GST activity resulting from exposure to this solution is likely due in part to the presence of the chemical dispersant. Although the control levels of GST activity for *M. leidyi* (0.039 μmol min^{-1} mg $protein^{-1}$) were lower than those found in some other marine invertebrates (*Carcinus maenas*: 0.368 μmol min^{-1} mg $protein^{-1}$; *Littorina littorea*: 0.748 μmol min^{-1} mg $protein^{-1}$ [Lee, 1988]), they were similar to those reported for mussels (*Mytilus edulis*: 0.053 μmol min^{-1} mg $protein^{-1}$ [Lee, 1988]), and more than double those found in coral colonies (*Favia fragum*: 0.015 μmol min^{-1} mg $protein^{-1}$ [Gassman and Kennedy, 1992]). The 6-12 fold increase in GST activity with COREXIT exposure is on the same order of the response of the sea urchin *Hemientrotus pulcherrimus* with exposure to dispersant and CEWAFs of diesel oil (Lv and Xiong, 2010), although in the case of the urchin it took multiple days to induce such a response, as opposed to the 24 hr exposure utilized by this study. The pronounced effect of COREXIT 9500A on GST activity in *M. leidyi* suggests that this species actively detoxifies one or more of the compounds in the chemical dispersant mixture via this common pathway. At the same time, the data would seem to indicate that weathered crude oil is not detoxified via the GST pathway in *M. leidyi*, as it is not

in copepods (Hansen et al., 2011), at least not weathered crude MC-252 oil at the low concentrations tested.

At 23°C whole animal respiration rates were unaffected by exposure to any of the three solutions, although they were generally higher than those for the same concentrations at 15°C. The relationship between temperature and respiration varied between solution types, although this can largely be traced to trends in the respiration data at 15°C. The WAF had the highest Q_{10} values, ostensibly due to the slight but consistent depression of respiration of ctenophores exposed to those solutions at 15°C. A similar effect of WAF exposure on respiration rates has been observed previously for *C. sapidus* megalopae (Cohen et al., submitted). In CEWA, Q_{10} values tended to increase with increasing concentration, as respiration rates at 23°C increased more relative to those at 15°C. The exact opposite trend in Q_{10} values was observed for COREXIT 9500A, as elevated respiration rates at 15°C were paralleled by fairly constant rates at 23°C. This resulted in apparent heat coma, indicated by Q_{10} values less than 1. However this could merely be the result of the detoxification response that occurred at the lower experimental temperature, and complementary GST activity experiments at 23°C would help to determine if the differences in respiration can be accounted for in part by differences in the detoxification response. Microbial respiration in the experimental chambers, measured at 23°C, was minimal compared to that of the ctenophores, and did not vary between treatments, eliminating the possibility of its contribution to the observed results. The design of this experiment did not control for any increases in activity related to attempted avoidance of the toxicants which might have occurred in the treatment solutions (Seuront, 2010), but such an effect would have been expected to be consistent across temperatures.

It was thought that the calcium-dependent luminescence of *M. leidyi* might decrease upon toxic exposure, as dispersant containing solutions are known to disrupt membranes (Abel 1974). The observations of this study support that initial hypothesis. The total amount of light emitted by individual ctenophores decreased almost universally with increasing toxicant concentrations. This effect was most notable at 15°C in WAF and CEWAF solutions. The two lowest COREXIT 9500A concentrations at 15°C represent the sole exceptions to this trend, as they displayed increased light output, albeit not significantly greater than the control. It was these same concentrations that resulted in increased GST activity, and there might be some connection between the two observations, although the physiological basis for such a connection is not clear. The experimental design was such that all available mnemiopsin should have been reacted by the end of the third period with the excess calcium in seawater. Since light output is independent of body size and dependent solely on mnemiopsin concentration (Ward and Seliger 1974), the reasonable explanation for this observation is that ctenophores exposed to higher concentrations of the toxins possessed diminished cellular stores of the photoprotein after 24 hr incubation. This could be due to increased degradation or loss of the protein, or to a reduction in its synthesis. Cellular energy budgets change with sublethal toxic exposure (Smolders et al, 2009), and mnemiopsin and bioluminescence might become peripheral to more essential cellular compounds and functions (e.g. repair and detoxification pathways, such as GST) under times of duress.

Bioluminescence in *M. leidyi* requires both the photoprotein mnemiopsin and calcium as an intracellular co-factor; changes in the luminescent potential index reveal how this intracellular calcium, vital for *in vivo* luminescence, is affected by toxic

exposure. High luminescence potential indicates an excess of mnemiopsin relative to intracellular calcium, and low luminescence potential indicates an excess of intracellular calcium, which might alternatively be described as a paucity of mnemiopsin in the photocytes. At 23°C, there was a generally decreasing trend in luminescent potential, with a significant decrease at 10 mg/L COREXIT 9500A. This drop signifies a greater amount of light produced *in vivo* (within the photocytes) relative to light produced *in vitro* (by the reaction with calcium in the seawater). This would be explained by an increase in intracellular calcium with toxicant exposure. The concentration of calcium outside of animal cells is typically on the order of 10,000 times greater than the concentration inside of cells (Evans, 2009). Minor disruption of the integrity of photocyte membranes could allow for continued cellular function while calcium from the extracellular fluid (and in the case of homogenization, from seawater) diffuses down its electrochemical gradient into the cell and reacts with mnemiopsin, leaving a smaller residual pool for *in vitro* luminescence post lysis. At 15°C the luminescent potential did not change in any of the treatments relative to the control, indicating that intracellular calcium supply did not change. The trends in intracellular calcium concentrations did not manifest themselves in a significant way in the luminescent decay curves, possibly due to the fact that intracellular calcium is not yet limiting at this point in the assay, as evidenced by the second mechanical stimulation peak. In contrast to the GST activity and respiration data, bioluminescence appears to have been affected similarly by exposure to COREXIT 9500A, WAF, and CEWAF solutions.

The numerous sublethal toxic effects of acute oil and chemical dispersant exposure on *M. leidyi* observed by this study demonstrate the potential of oil spill

events to affect important members of coastal marine ecosystems, even at low concentrations that do not lead to direct mortality. Such effects should be considered across the diversity of biota when assessing the impact of an oil spill in coastal environments.

Solution	Loading Concentration	Q_{10}
FSW	0 mg/L	1.35
COREXIT 9500A	2.5 mg/L	1.37
	5 mg/L	1.50
	10 mg/L	0.76
	20 mg/L	0.59
WAF	1 mg/L	2.12
	10 mg/L	2.604
	50 mg/L	2.00
	100 mg/L	N/A
CEWAF	1 mg/L	1.06
	5 mg/L	1.41
	10 mg/L	1.75
	30 mg/L	N/A

Table 3.1 Q_{10} values for average 24 hr respiration rates of ctenophores exposed to COREXIT 9500A and WAFs and CEWAFs of MC-252 weathered crude oil for 24 hr at 15°C and 23°C. N/A represents concentrations where observations were lacking due to high mortality, preventing the subsequent calculation of a Q_{10} value.

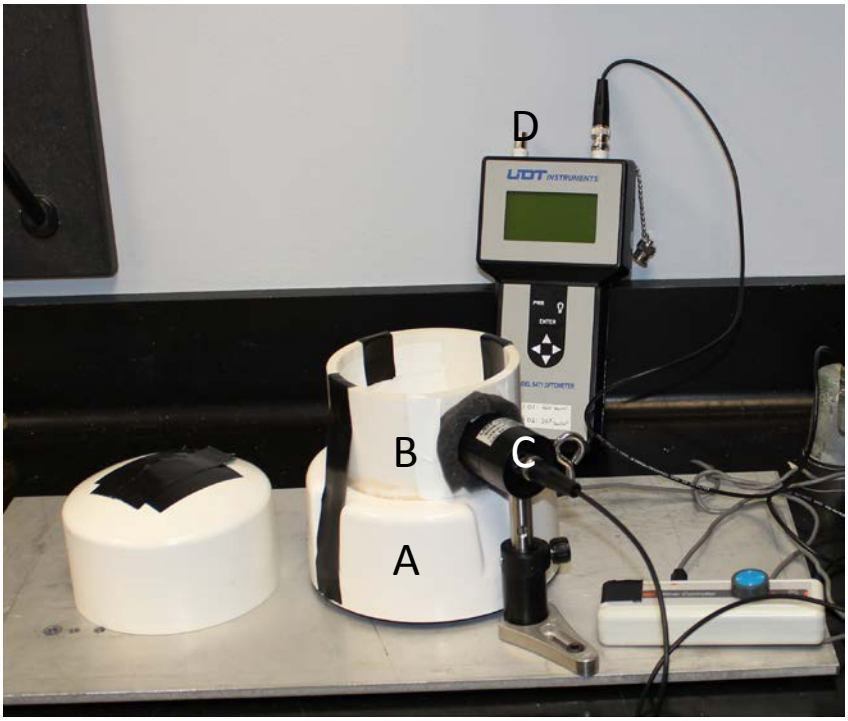

Figure 3.1 Experimental setup for measuring bioluminescence in *M. leidyi*. Shown are the stirplate (A), PVC pipe chamber (B), sensor head (C), and UDT Instruments optometer (D). The tissue homogenizer was mounted to a ring stand and lowered through a 3 cm in the cap. The funnel was mounted to a separate ring stand and run along the length of the homogenizer, secured by zip ties. The entire apparatus, exclusive of the funnel and its ring stand, was contained within a light tight enclosure.

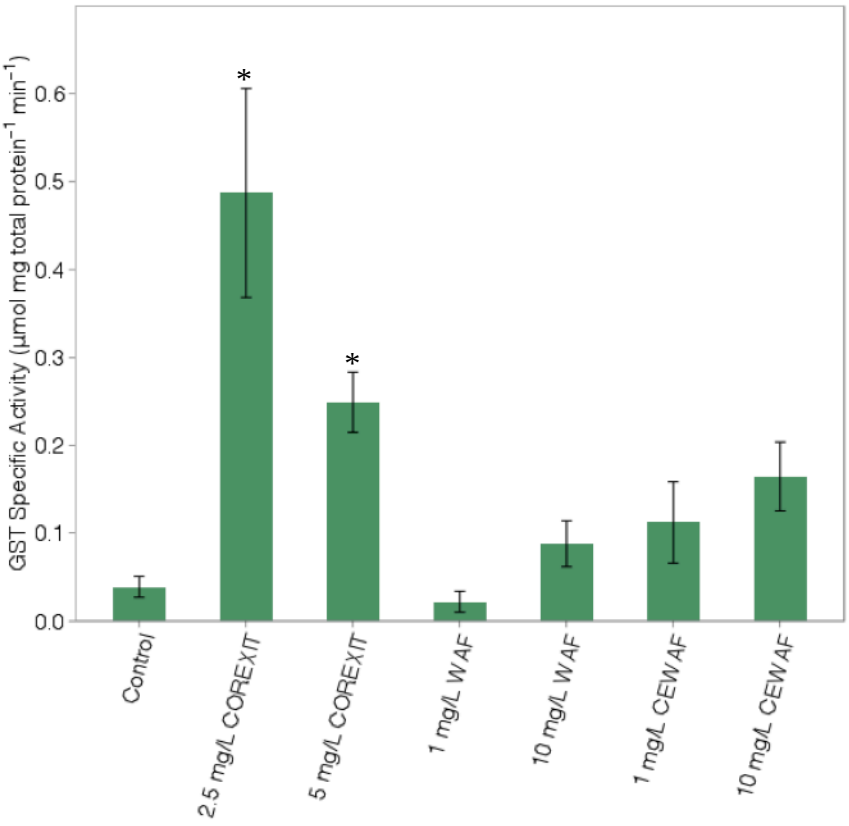

Figure 3.2 Average glutathione-s-transferase activity (± SE) of ctenophores exposed to COREXIT 9500A, WAF, and CEWAF solutions for 24 hr at 15°C. Activity was measured at 30-second intervals over a 5 min period. Groups of 3-4 individuals were combined for the assay, and 4-5 replicates of these groups were tested for the control and each of the treatments (n= 16 (Control), 16 (2.5 m g/L COREXIT), 17 (5 mg/L COREXIT), 16 (1 mg/L WAF), 16 (10 mg/L WAF), 18 (1 mg/L CEWAF), 17 (10 mg/L CEWAF). Treatments marked * are significantly different (p <0.05) from the control.

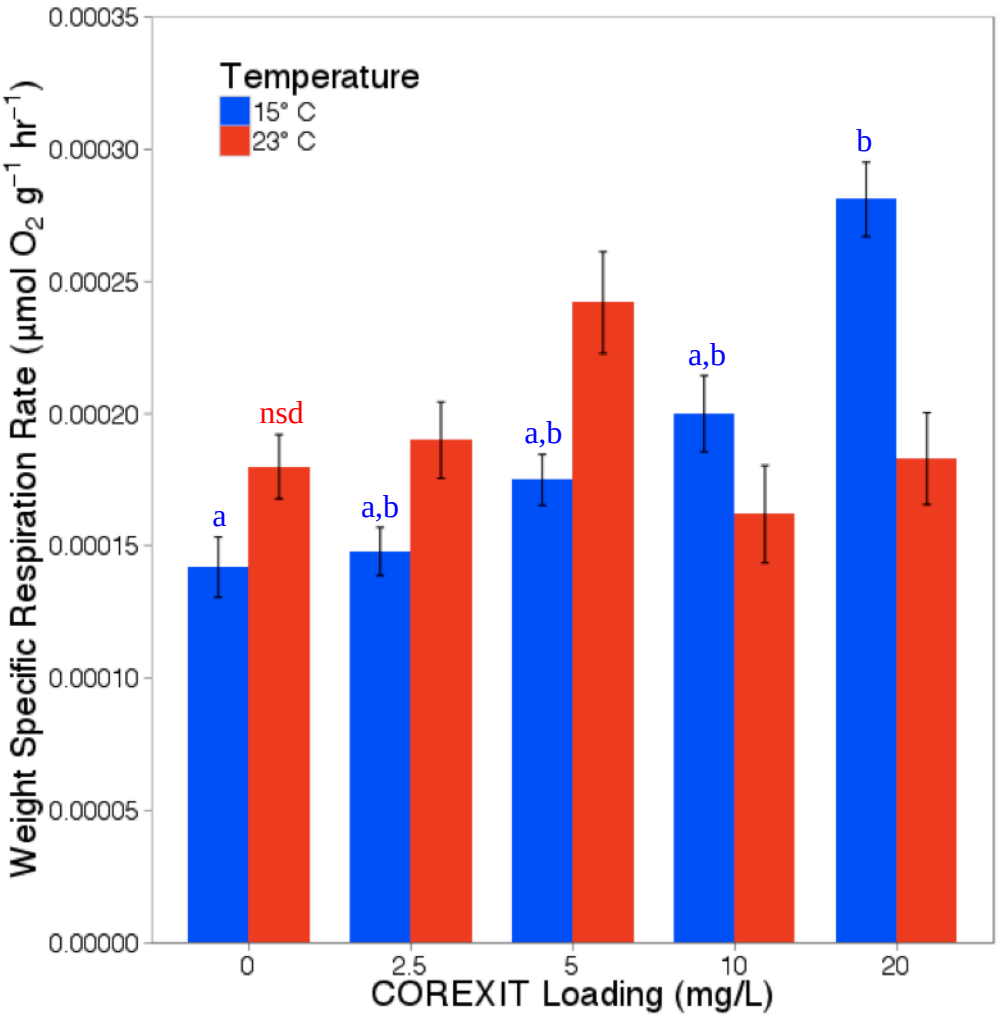

Figure 3.3 Average weight specific respiration rates (± SE) for ctenophores exposed to COREXIT 9500A solutions for 24 hr at 15°C and 23°C. 15°C: n= 30, 22, 24, 16, 13; 23°C: n= 29, 19, 10, 7, 4. Differences in lower case letters (a,b,c…) denote statistically significant differences ($p<0.05$) between treatments.

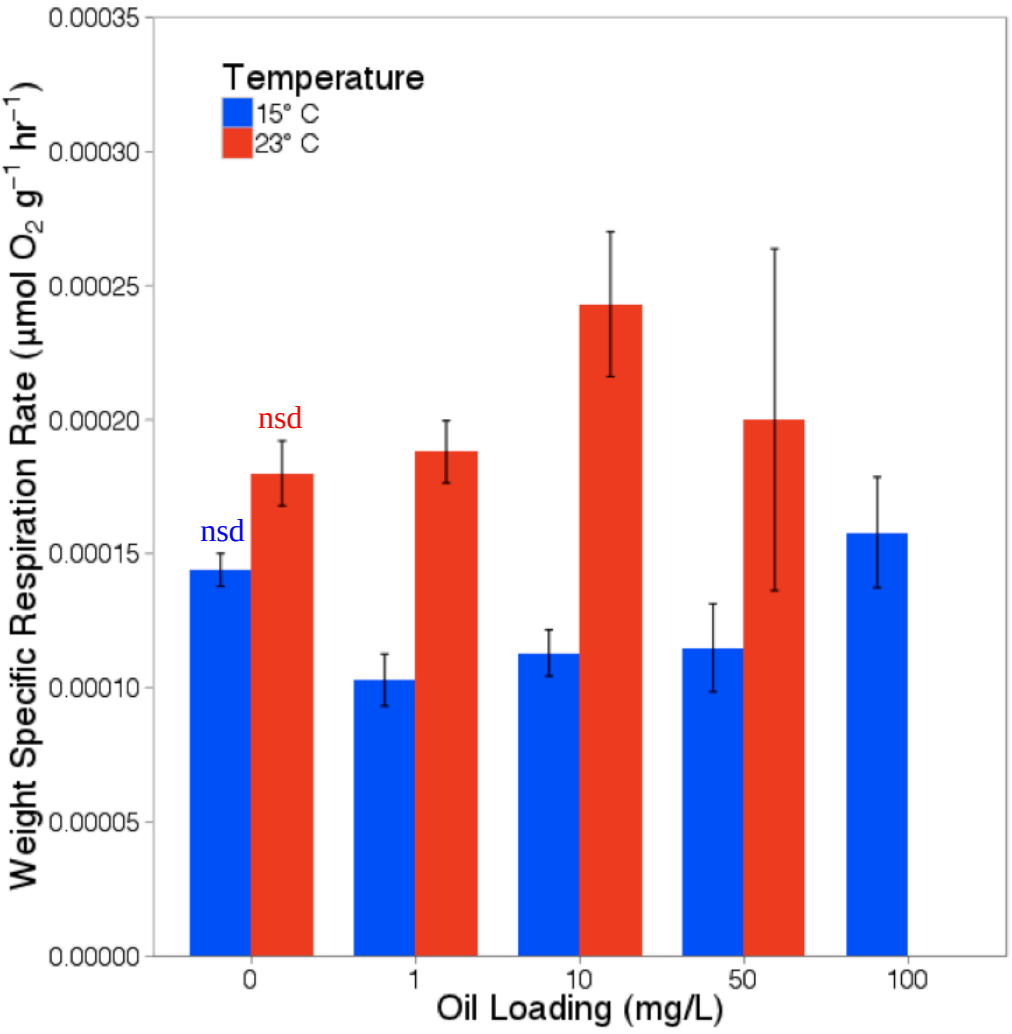

Figure 3.4 Average weight specific respiration rates (± SE) for ctenophores exposed to WAFs of weathered MC-252 crude oil for 24 hr at 15°C and 23°C. 15°C: n= 30, 21, 15, 6, 5; 23°C: n= 29, 10, 15, 2. Differences in lower case letters (a,b,c…) denote statistically significant differences ($p<0.05$) between treatments.

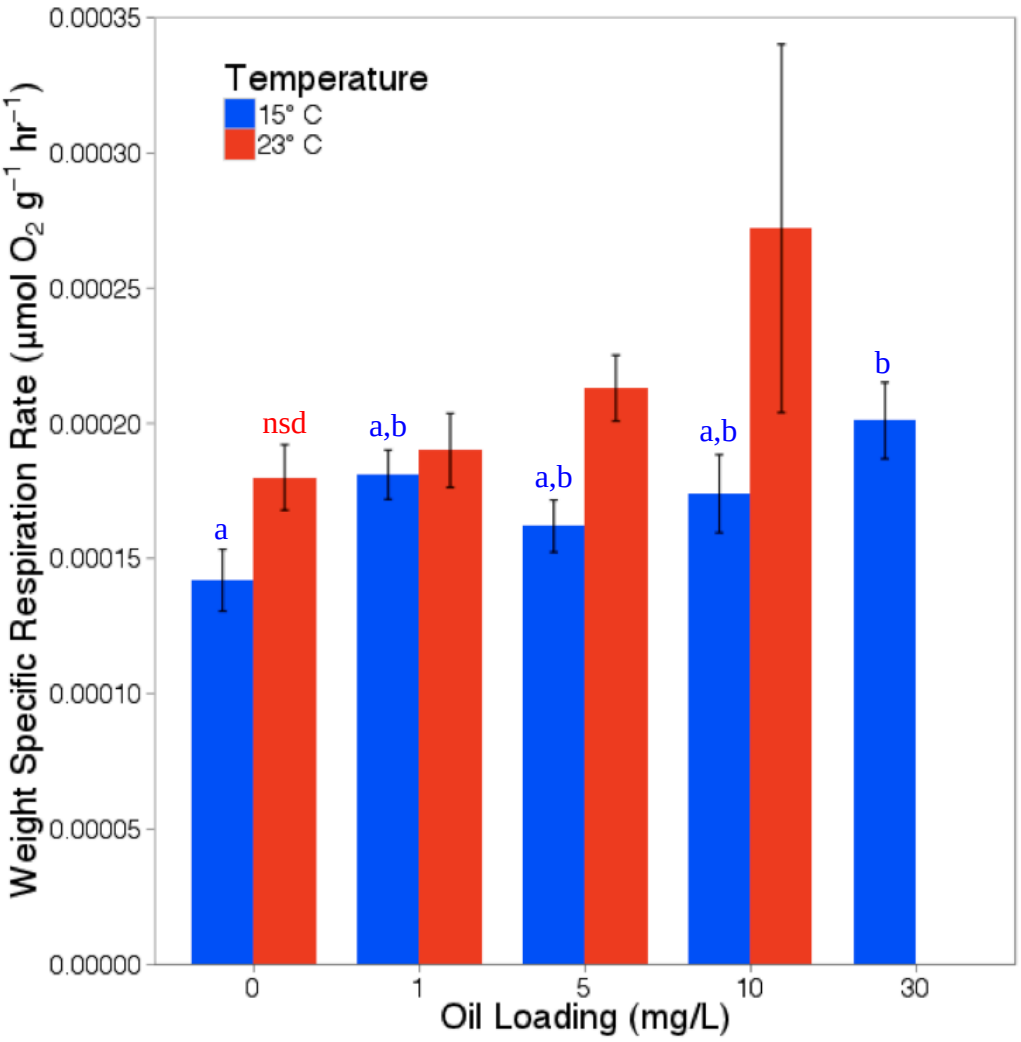

Figure 3.5 Average weight specific respiration rates (± SE) for ctenophores exposed to CEWAFs of weathered MC-252 crude oil, dispersed with COREXIT 9500A for 24 hr at 15°C and 23°C. Dispersant was added in a 1:10 ratio with oil, by volume. 15°C: n= 30, 24, 18, 13, 18; 23°C: n= 29, 20, 17, 7, 0. Differences in lower case letters (a,b,c…) denote statistically significant differences ($p<0.05$) between treatments.

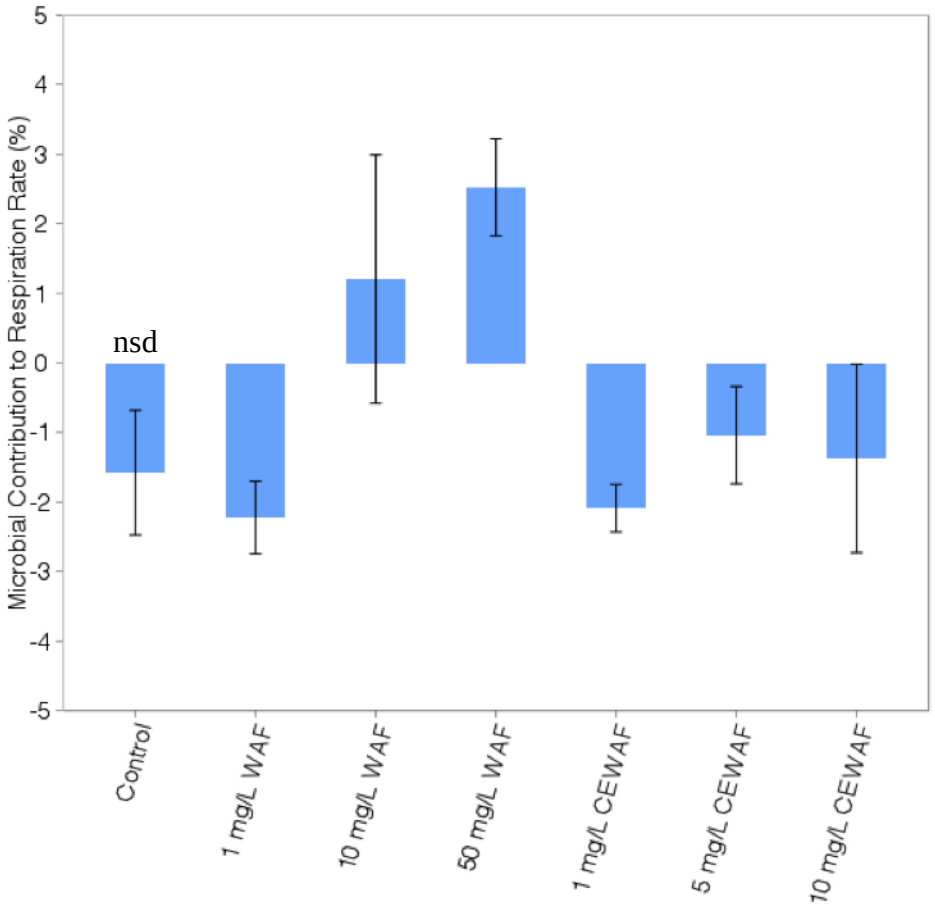

Figure 3.6 Average microbial contribution (± SE) to 24 hr ctenophore respiration rates in filtered seawater, WAF, and CEWAF solutions at 23°C. Bottles were resealed and oxygen measured again after a second 24 hr period. The microbial contribution is expressed as respiration during the second period as a percent of the first. n= 6, 15, 10, 2, 12, 10, 4. nsd= no significant differences between treatments and the control.

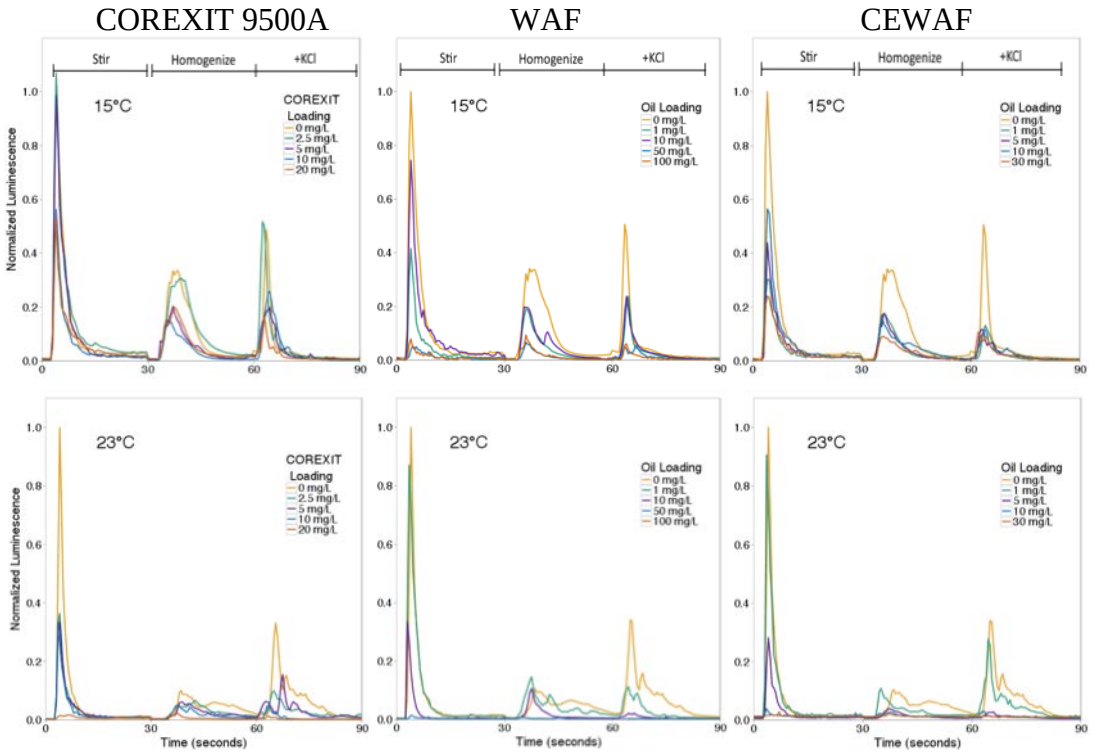

Figure 3.7 Average luminescent response of ctenophores to mechanical and chemical stimulation after 24 hr exposure to COREXIT 9500A, WAF, and CEWAF solutions at 15°C and 23°C. Luminescence was elicited mechanically by stirring and homogenization (first two peaks), as well as chemically by hypoptonic lysis in 01*M* KCl (third peak), and normalized to the peak value in the respective controls at each temperature. Sample sizes can be found in the individual integrated luminescence graphs below.

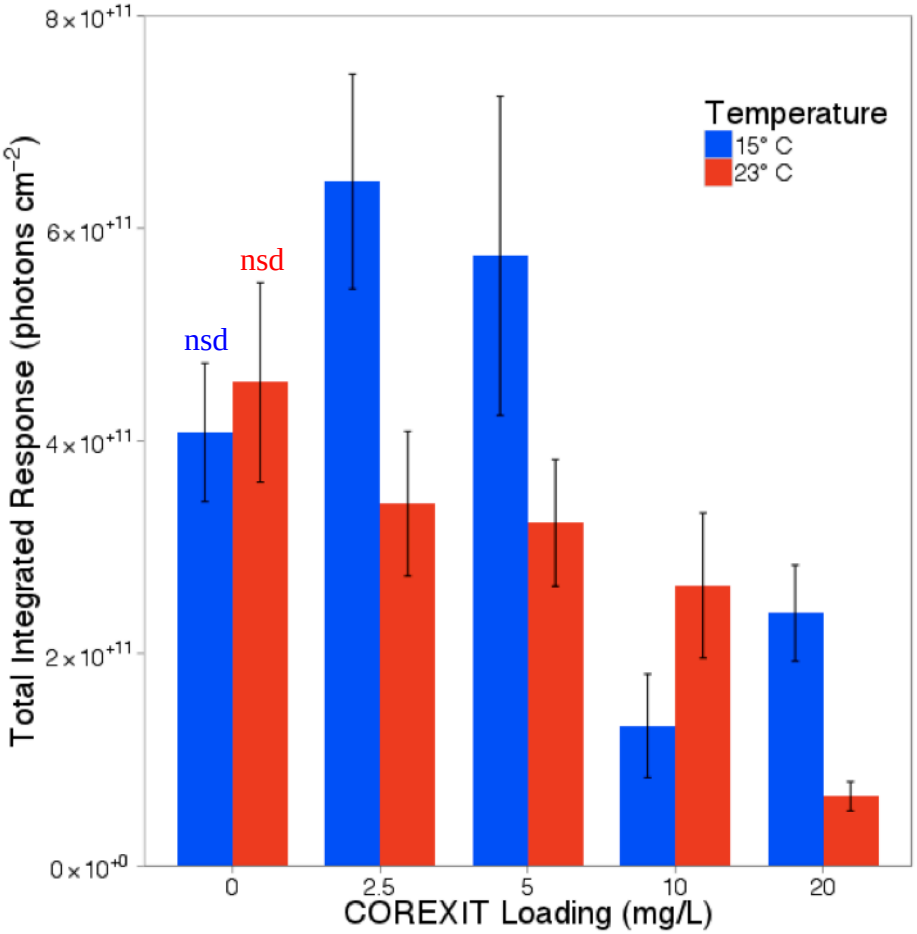

Figure 3.8 Average total integrated luminescent response (±SE) of ctenophores exposed to COREXIT 9500A solutions for 24 hr at 15°C and 23°C. The luminescent response was integrated over all three periods. 15°C: n= 30, 22, 24, 16, 13; 23°C: n= 29, 19, 10, 7, 4. nsd= no significant differences between treatments and the control.

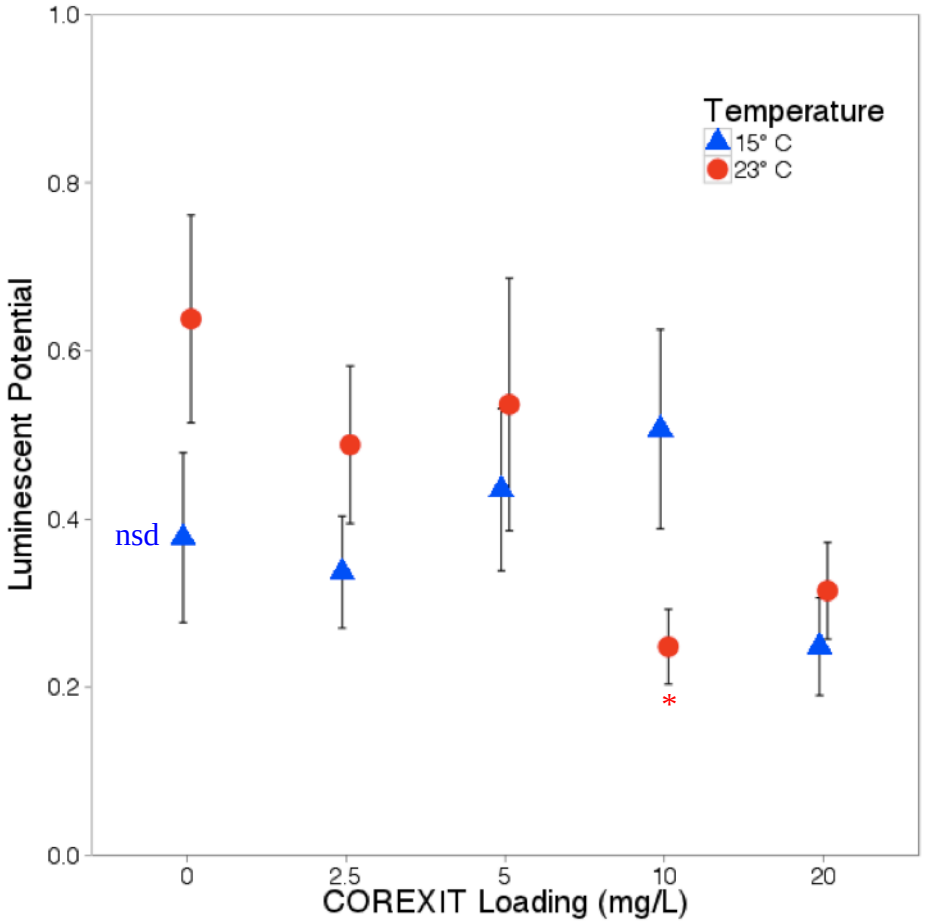

Figure 3.9 Average luminescent potential (in vitro: in vivo luminescence) (±SE) of ctenophores exposed to COREXIT 9500A solutions for 24 hr at 15°C and 23°C. 15°C: n= 30, 22, 24, 16, 13; 23°C: n= 29, 19, 10, 7, 4. Treatments marked * are significantly different (p <0.05) from the control. nsd= no significant differences between treatments and the control.

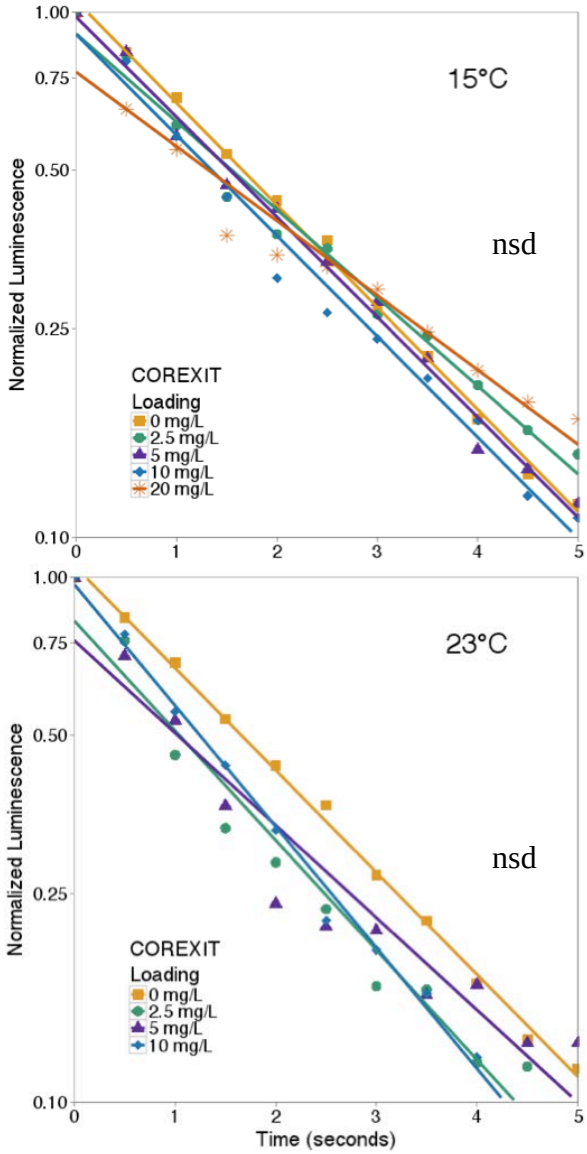

Figure 3.10 Average luminescent decay for the 5-second period following peak luminescence by mechanical stimulation with a stirbar for ctenophores exposed to COREXIT 9500A solutions for 24 hr at 15°C and 23°C. 15°C: n= 30, 22, 24, 16, 13; 23°C: n= 29, 19, 10, 7, 4. nsd= no significant differences between treatments and the control.

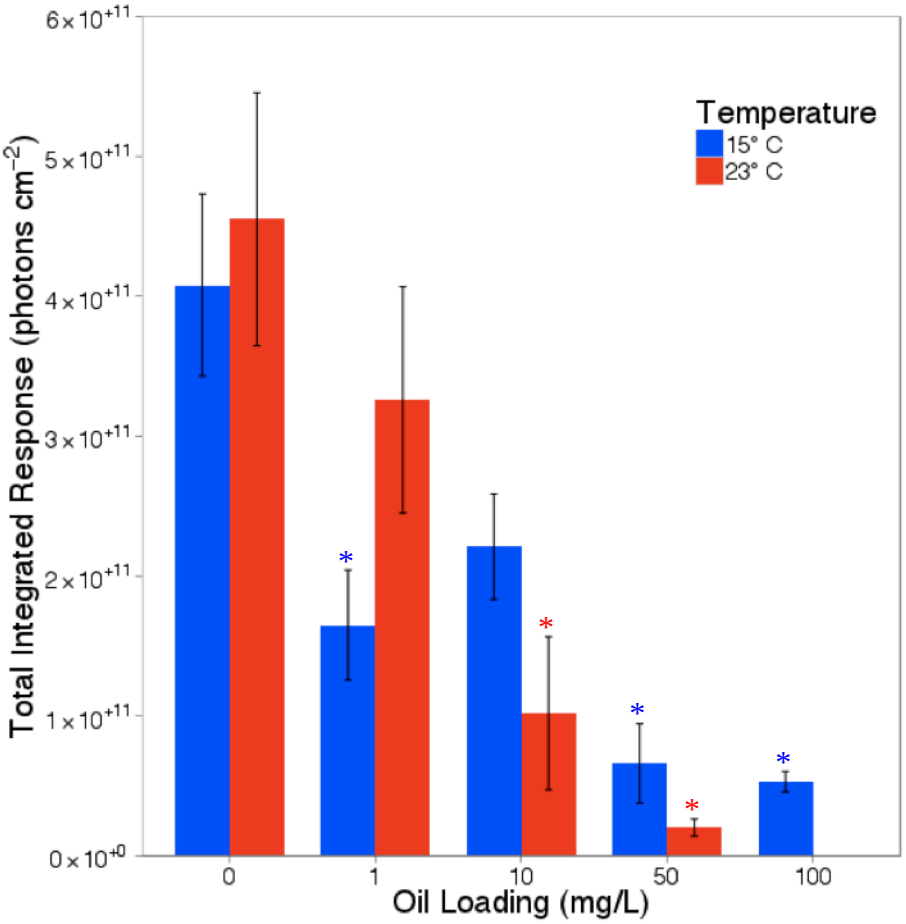

Figure 3.11 Average total integrated luminescent response (±SE) of ctenophores exposed to weathered MC-252 crude oil WAF solutions for 24 hr at 15°C and 23°C. The luminescent response was integrated over all three periods. 15°C: n= 30, 21, 15, 6, 5; 23°C: n= 29, 10, 15, 2, 0. Treatments marked * are significantly different (p <0.05) from the control.

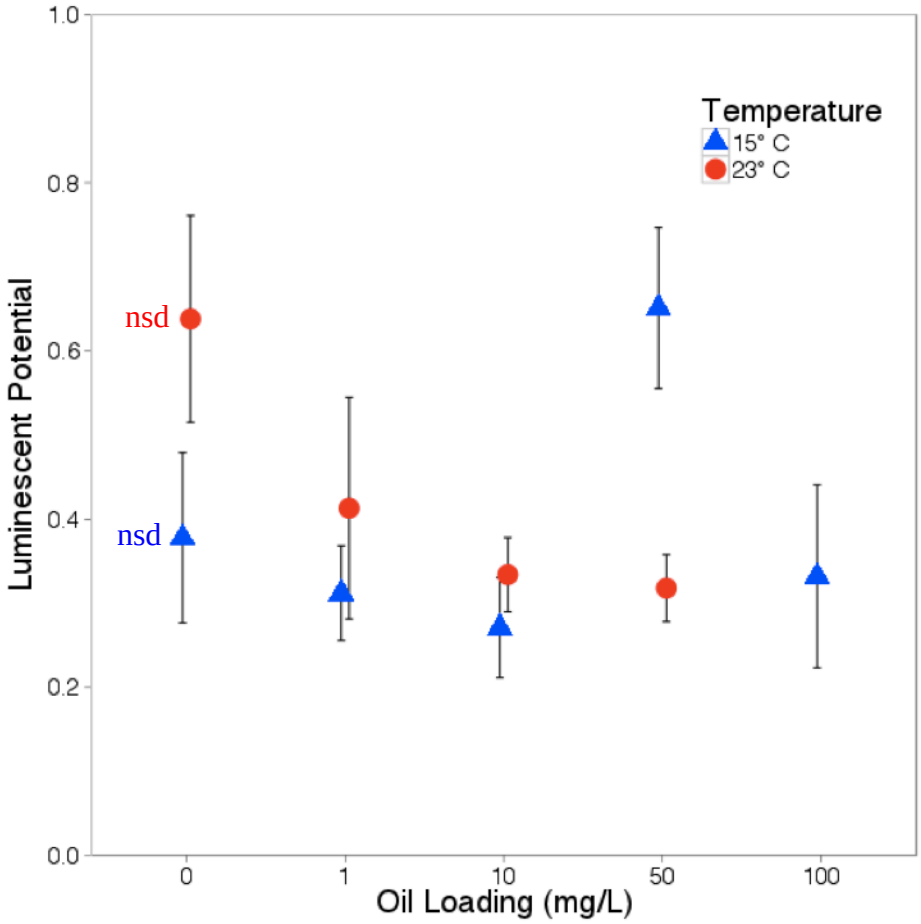

Figure 3.12 Average luminescent potential (in vitro: in vivo luminescence) (±SE) of ctenophores exposed to weathered MC-252 crude oil WAF solutions for 24 hr at 15°C and 23°C. 15°C: n= 30, 21, 15, 6, 5; 23°C: n= 29, 10, 15, 2, 0. nsd= no significant differences between treatments and the control.

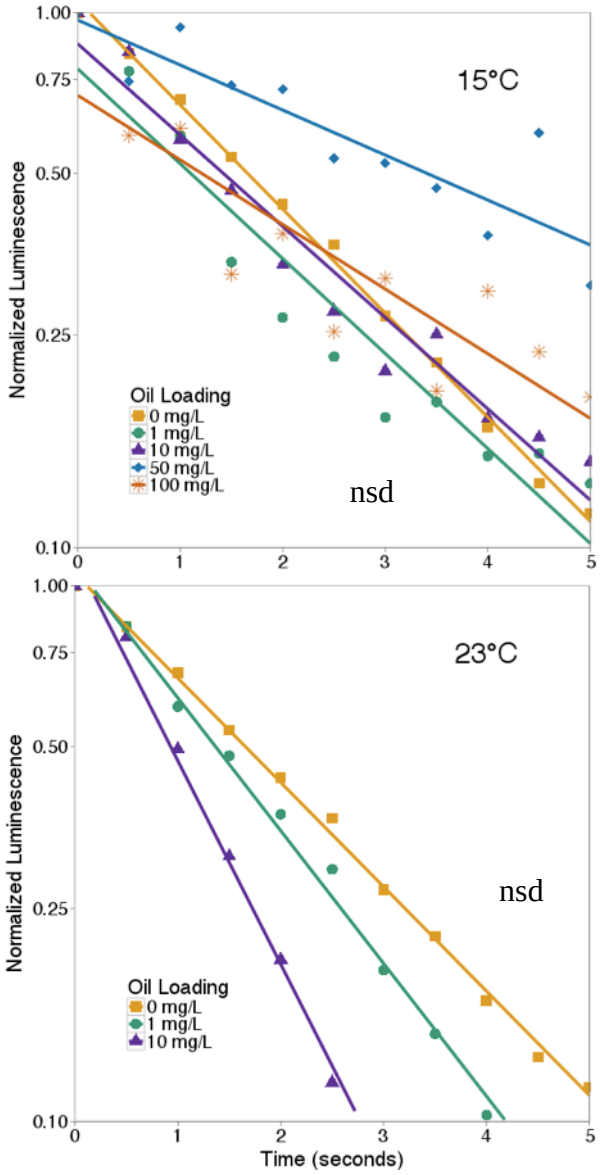

Figure 3.13 Average luminescent decay for the 5-second period following peak luminescence by mechanical stimulation with a stirbar for ctenophores exposed to weathered MC-252 crude oil WAF solutions for 24 hr at 15°C and 23°C. 15°C: n= 30, 21, 15, 6; 23°C: n= 29, 10, 15, 2. nsd= no significant differences between treatments and the control.

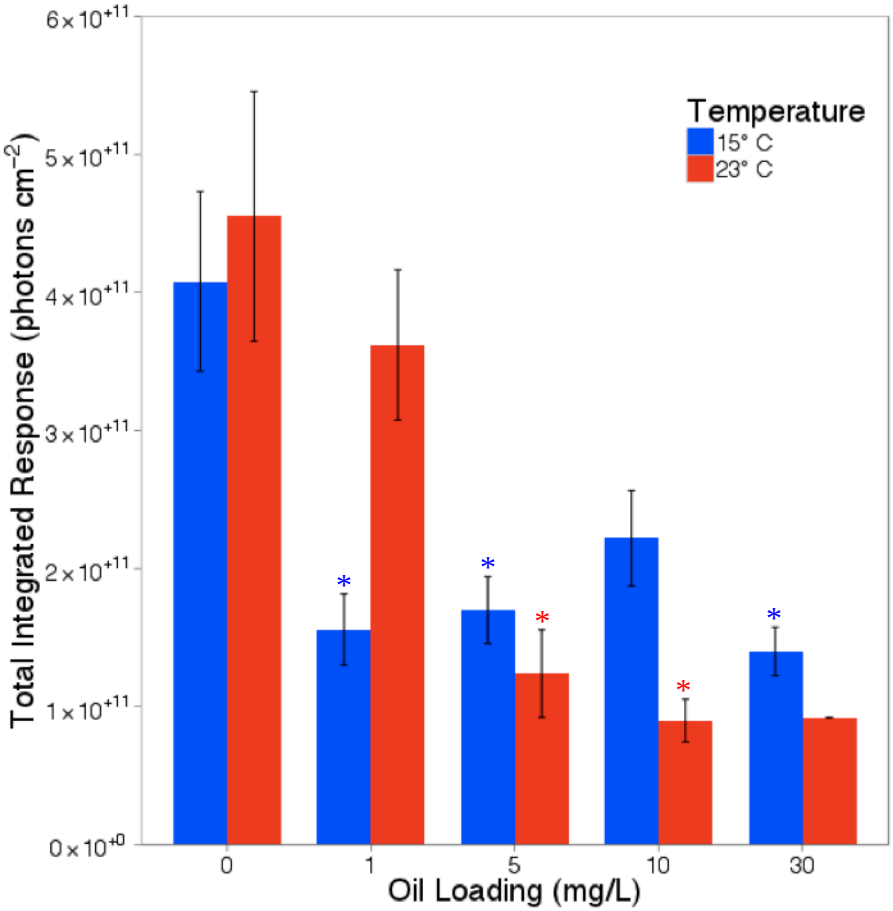

Figure 3.14 Average total integrated luminescent response (±SE) of ctenophores exposed to weathered MC-252 crude oil CEWAF solutions, dispersed with COREXIT 9500A, for 24 hr at 15°C and 23°C. The luminescent response was integrated over all three periods. 15°C: n= 30, 24, 18, 13, 18; 23°C: n= 29, 20, 17, 7, 1. Treatments marked * are significantly different (p <0.05) from the control.

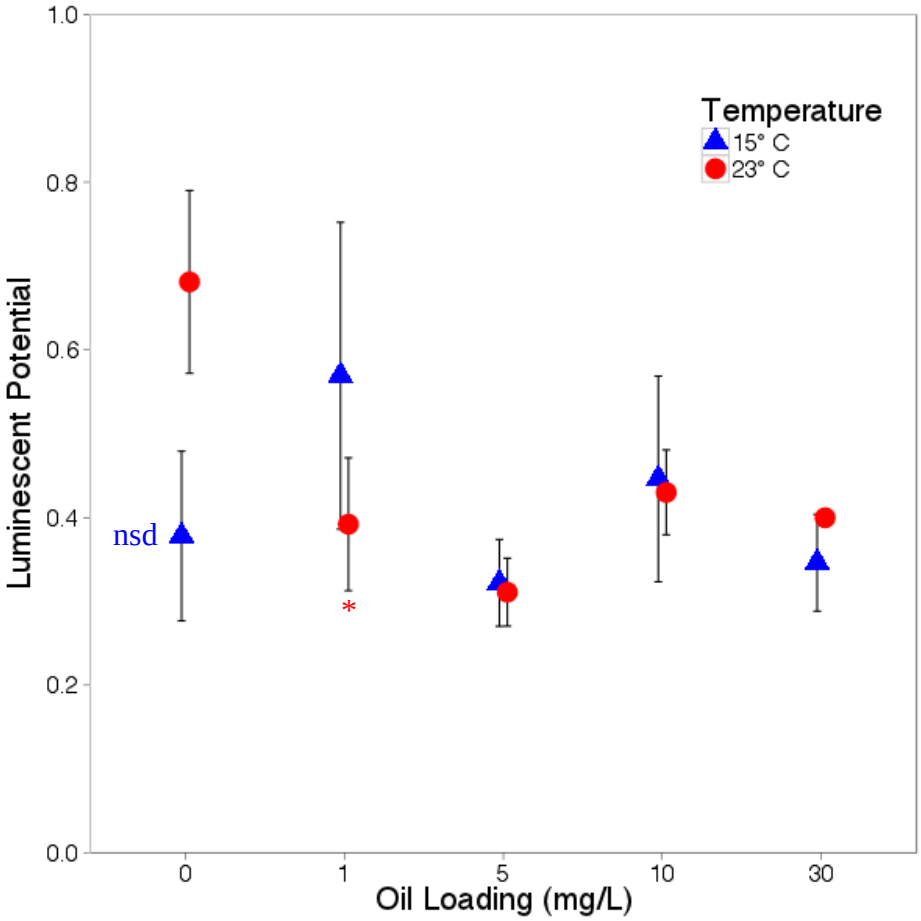

Figure 3.15 Average luminescent potential (in vitro: in vivo luminescence) (±SE) of ctenophores exposed to weathered MC-252 crude oil CEWAF solutions, dispersed with COREXIT 9500A, for 24 hr at 15°C and 23°C. 15°C: n= 30, 24, 18, 13, 18; 23°C: n= 29, 20, 17, 7, 1. Treatments marked * are significantly different (p <0.05) from the control. nsd= no significant differences between treatments and the control.

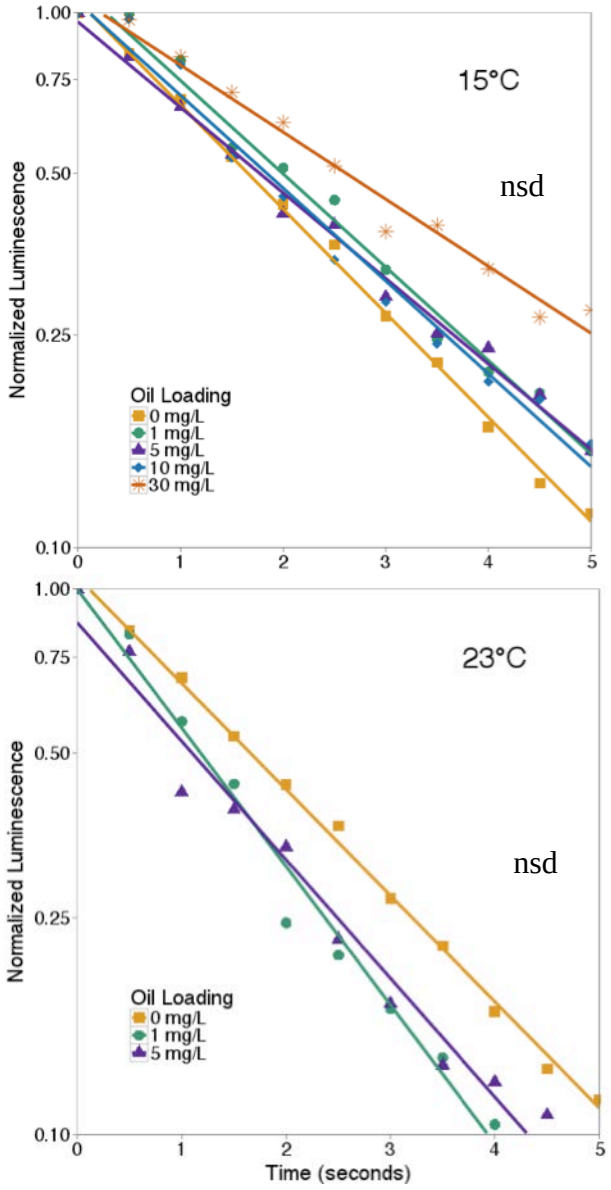

Figure 3.16 Average luminescent decay for the 5-second period following peak luminescence by mechanical stimulation with a stirbar for ctenophores exposed to weathered MC-252 crude oil CEWAF solutions, dispersed with COREXIT 9500A for 24 hr at 15°C and 23°C. 15°C: n= 30, 24, 18, 13, 18; 23°C: n= 29, 20, 17, 7. nsd= no significant differences between treatments and the control.

Chapter 4

CONCLUSIONS AND POTENTIAL IMPACTS

From an ecotoxicological perspective, the successful execution of GST activity assays in the ctenophore *M. leidyi* and the further development of bioluminescence as a measure of sublethal toxicity both represent novelties of this work. GSTs are broadly distributed amongst marine organisms and are used to detoxify a wide range of xenobiotic compounds (Blanchette et al., 2007), but their activity has not been previously measured in ctenophores. By establishing that ctenophores upregulate this pathway in response to chemical dispersant exposure, this study allows for the direct comparison of the toxic effects of oil and chemical dispersant on *M. leidyi* to other organisms and/or toxins, although it remains to be established which compounds in COREXIT 9500A (and CEWAF) induced this detoxification response.

Bioluminescence has been increasingly utilized as an endpoint in aquatic toxicology studies with the bacterium *Vibrio fisheri* to assess the relative effects of acute toxic exposure (Munkittrick et al., 1991), including some examining the effects of hydrocarbons (e.g. Ricking et al., 2002). This EPA standard, commercially available Microtox® assay (http://www.epa.gov/greatlakes/arcs/EPA-905-B94-002/B94002-ch4.html) has been shown to be fairly sensitive to chemical dispersant exposure (George-Ares et al., 1999), but the development of bioluminescence as a sublethal toxic parameter in a marine zooplankter is a new approach to understanding the effects of toxins in the marine environment. Decreased bioluminescence in ctenophores exposed to oil and dispersant in coastal ecosystems could have effects on

its trophic interactions, as bioluminescence is often closely tied to feeding ecology (Haddock, 2007). A better understanding of the function(s) of luminescence in ctenophores would allow for the more straightforward extrapolation of the results of this study. It has been found that some copepods demonstrate a photophobic response that might aid them in avoiding predation by luminescent species (Buskey et al., 1987). If this is indeed the case, decreased luminescence could lead to increased predation on species possessing this behavioral trait after an oil spill. If, as Morin (1983) suggests, coastal luminescence functions primarily to startle potential predators, then a decrease in luminescence following oil or dispersant exposure could lead to increased predation on ctenophores by fish and other predators. The potential ecological effects of similar sublethal toxicity in deep environments impacted by spills, where there are both ctenophores and cnidarians that rely on calcium-dependent photoproteins (Haddock and Case, 1999) could be amplified given the paramount importance of bioluminescence in governing trophic interactions in the deep ocean (Haddock et al., 2010; Johnsen et al., 2012). The development of functional bioluminescence assays in deep sea taxa would increase the power for interpreting these results, and others like them.

As stated elsewhere above, this study extends the toxicological understanding of oil and chemical dispersants to a new class of highly sensitive and ecologically important organisms. It demonstrated the greater relative toxicity of dispersed oil compared over oil alone to *M. leidyi* in terms of both direct mortality and sublethal metabolic effects, a difference that was most likely due to the high sensitivity of the species to COREXIT 9500A. Hopefully more studies like this will follow that select species with the aim of understanding the ecological impact of spills and their

mitigation and which target the sublethal effects of environmentally relevant exposure regimes. Laboratory exposures, such as those conducted here, or at sea (Almeda et al., 2013; Cohen et al., submitted) provide the potential to help understand oil spill impacts, but are almost purely esoteric when left uncoupled from extensive field studies that survey zooplankton populations and communities with the aim of identifying post spill trends. Such studies are often quickly forthcoming after a spill (e.g. Davenport, 1982; Varela, 2006), but the data that they collect are difficult to interpret unless there is sufficient baseline data for the region to allow seasonal and interannual variability to be accounted for (e.g. Batten et al, 1998). An effort should be made then to collect and make publicly available baseline data on zooplankton abundance and distribution in areas sensitive to oil spills in order to facilitate the assessment of spill impacts. As we go to greater and greater lengths (and depths) to sate the demand for petroleum, accidental spills are sure to follow, and our understanding of the ecological impact of spills and their remediation ought to grow apace with such efforts.

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