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Quantifying the Toxicity of 1-Methylnaphthalene to the Shallow-Water Coral, Porites divaricata, for Use in the Target Lipid Model

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QUANTIFYING THE TOXICITY OF 1-METHYLNAPHTHALENE TO THE SHALLOW-WATER CORAL, *PORITES DIVARICATA*, FOR USE IN THE TARGET LIPID MODEL

By

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ABSTRACT

The proximity of coral reefs to coastal urban areas and shipping lanes predisposes corals to petroleum pollution from multiple sources. Previous research has evaluated petroleum toxicity to coral using a variety of methodologies, including monitoring effects of acute and chronic spills, *in situ* exposures, and *ex situ* exposures with both adult and larval stage corals. Variability in toxicant, bioassay conditions, species and other methodological disparities among studies prevents comprehensive conclusions regarding the toxicity of hydrocarbons to corals. This research evaluated the 48-hour toxicity of 1-methylnaphthalene to *Porites divaricata* using a continuous-flow passive dosing system. The range-finding exposure evaluated the dosing protocol and verified the effectiveness of the passive dosing technique at maintaining exposure concentrations. The full-toxicity exposures resulted in a precise estimate of toxic threshold concentrations for use in the target lipid model. The target lipid model promoted comparisons across different species by calculating the critical target lipid body burden of 355.7 µmol/ g lipid for *P. divaricata*. This indicates a greater resilience to petroleum hydrocarbon exposure compared to other species for which these data are available.

KEY WORDS

hydrocarbon, toxicity, *Porites divaricata*, passive dosing, target lipid model

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INTRODUCTION

As one of the few productive ecosystems that thrive within oligotrophic seas, coral reefs are regarded as diverse and complex marine communities (Loya and Rinkevich 1980, Knap et al. 1983, Ballou et al. 1987b, Haapkylae et al. 2007). Coral reefs are an essential aspect of the geology and ecology of tropical and subtropical oceans. Moreover, coral reefs are vital to the geochemical mass balance of the oceans in regards to fluxes of magnesium, calcium, strontium, and carbonate (Knap et al. 1983). These ecosystems are a major fisheries habitat, protect against coastal erosion, and form the basis for most tropical tourist industries (Knap et al. 1983, Ballou et al. 1987b, Shigenaka 2001). Coral reefs grow in coastal environments that are generally located adjacent to highly populated areas, increasing the possibility for anthropogenic impacts on these ecosystems.

The complex communities associated with coral reefs depend on the structural role provided by hermatypic corals (Ballou et al. 1987b, Shigenaka 2001). Corals provide shelter from predators, substrate for colonization of algae and invertebrates, and are a direct source of nutrients for multiple species whose primary diet consists of coral tissue (Loya and Rinkevich 1980, Shigenaka 2001, Haapkylae et al. 2007). It is widely accepted that many of the world's coral reef ecosystems are in decline, due to an abundance of natural and anthropogenic disturbances.

The diverse and complex nature of coral reefs is often related to physical features such as location, depth, local geography, and topography, and indicates a wide spectrum of disturbances to which corals have adapted over geologic time (Nyström et al. 2000). Disturbances to reefs are increasingly related to human dominance of coastal areas, which has led to increased sediment, nutrients, and other pollutant inputs into the sea. These impacts are amplified by poor land management, and combine to cause increased stress to corals and coral reefs (Knap et al. 1983, Shigenaka 2001). The increased stress on corals may permit diseases caused by infectious or opportunistic microorganisms to spread rapidly across populations and when coupled with increased nutrients, may permit increased predation (i.e., crown-of-thorns starfish) or overgrowth from algae (Shigenaka 2001). Further examples of human-induced disturbances that directly or indirectly impact coral animals and the reefs they construct include over-fishing, destructive fishing

methods, sedimentation due to dredging, drilling activities, physical habitat alteration, and invasive species (Knap et al. 1983, Shigenaka 2001).

Corals are known to be long-lived and slow-growing animals that may take decades to recover from disturbance (Cubit et al. 1987). The more persistent, and often more frequent, occurrence of anthropogenic disturbances on coral reefs leaves little time for recovery. Toxic substances produced by humans often have no natural counterpart, and their release into the marine environment may expose organisms to compounds to which they have adapted poorly, or not at all (Nyström et al. 2000). Damage to the coral animal will likely disrupt associated communities, and has the potential to negatively impact the entire ecosystem (Shigenaka 2001).

Petroleum Inputs and Exposure Scenarios

Crude oil pollution is often considered a primarily anthropogenic contribution to the sea; however, natural seeps are the highest contributors of petroleum hydrocarbons to the marine environment, accounting for 46% of the total worldwide input (NRC 2003). These seeps exist where crude oil migrates directly from oil-bearing rocks through the sediment and into the water column via cracks and faults in the sea bed (Al-Dahash and Mahmoud 2013). They have limited ecological impact, and the constant, slow rate of release over an extended period of time has allowed microbes and benthic organisms to acclimate and even evolve to utilize the petroleum hydrocarbons (NRC 2003). Conversely, the impacts of large and abrupt anthropogenic inputs of petroleum hydrocarbons into relatively pristine waters are of greater concern as potentially affected organisms may lack the adaptive features to use or detoxify the hydrocarbons.

Anthropogenic input of petroleum hydrocarbons can be divided into three main sources. Extraction accounts for 3% of the worldwide total, attributed to offshore production releases from platforms and pipelines, or operational discharges such as loading and cleaning operations, effluents, ballast water, and leakage (NRC 2003). Transportation is the second main source, accounting for 12% of the worldwide input of petroleum hydrocarbons (NRC 2003). This includes tanker accidents and operations, marine terminal and refinery spills, and spills from land-based storage tanks (Dodge et al. 1984, Burns and Knap 1989). The third main source of petroleum hydrocarbon input into the sea is consumption. Consumption of crude oil accounts for 37% of the worldwide total, and 92%

of the anthropogenic load (El-Sikaily et al. 2003, NRC 2003). Terrestrial runoff, recreational vessels, non-tanker accidents, and aircraft dumping are all grouped here, with land-based runoff as the largest contributor of consumption-based crude oil (NRC 2003). Coastal expansion of urban areas has increased this input, placing a significant threat on shallow, fragile coastal ecosystems.

Marine organisms may be exposed to petroleum hydrocarbons in two ways, acutely or chronically (NRC 2003). Acute exposures are typically the result of large, catastrophic spills with immediate short-term effects. The close proximity between tanker routes and many of the world's coral reefs has resulted in significant oil pollution of reefs from tanker accidents in the Persian Gulf, Wake Island, the Florida Keys, and Puerto Rico (Knap et al. 1983). Acute exposure is often related to the proximity to spills originating from refineries, production activities, storage facilities, and offshore platforms (Dodge et al. 1984). Accidental or deliberate release from tankers and pipelines due to war-related incidents has also resulted in acute exposures (Haapkylae et al. 2007, Al-Dahash and Mahmoud 2013). Although these spills generally have a short duration, they have the potential to cause long-term impacts depending on the amount and location of the spill (NRC 2003).

Chronic exposure results from continuous exposure to small amounts of oil over long periods of time (NRC 2003). This typically occurs in close proximity to natural seeps, but anthropogenic sources are also common. Point sources, like leaking pipelines, production discharges, or runoff from land-based facilities contain a strong gradient of high to low oil concentration. Non-point sources, such as atmospheric fallout and terrestrial runoff, also result in chronic exposure, but contain no distinct gradient of concentration. Large spills with acute exposure scenarios may not cause complete mortality, but oil can become trapped in sediments, producing a chronic exposure scenario. Chronic exposures can result in sublethal effects, including altered metabolism, cell structure and function, or enhancement of chromosome mutation. This cascade of biological consequences associated with chronic pollution from frequent smaller spills is often considered to be a larger threat than those associated with acute exposure from tanker accidents (Loya and Rinkevich 1980, Capuzzo 1987). Oil pollution in the sea, whether from anthropogenic or natural sources, chronic or acute, is a major environmental concern (NRC 2003).

Composition and Toxic Mode of Action of Petroleum Hydrocarbons

Crude oil is a complex mixture of several thousand compounds, with each oil containing widely varying amounts of chemicals (NRC 2003, Haapkylae et al. 2007). Hydrocarbons, as saturates, olefins, and aromatics, make up 97% of most petroleum (NRC 2003). Of these compounds, aromatics are usually the most stable and may persist in the environment for long periods of time. Aromatics include at least one benzene ring, with an inverted relationship between abundance and molecular weight. Monocyclic aromatic hydrocarbons (MAH)—benzene, toluene, ethyl-benzene, and xylene (BTEX)—are more volatile and often found in higher proportions than polycyclic aromatics. Polycyclic aromatic hydrocarbons (PAH), or polyaromatics, account for nearly 20% of the total hydrocarbons in crude oil and include compounds that can cause the most serious environmental effects (El-Sikaily et al. 2003, Haapkylae et al. 2007).

The hydrophobic nature of PAHs causes adherence to particulate material in the water column where they can enter the food chain or become deposited in sediments (El-Sikaily et al. 2003). The hydrophobicity of PAHs also means a low aqueous solubility coupled with high lipid solubility, which according to the equilibrium partitioning theory, allows the PAHs to partition across permeable membranes into organismal tissue lipids until equilibrium is reached (NRC 2003). The partitioning of petroleum hydrocarbons into tissues produces a toxic response in the organism that is related to the solubility and bioavailability of specific compounds (Neff and Anderson 1981, Capuzzo 1987, NRC 2003). Bioavailability and solubility of hydrocarbons may be chemically modified through photo-oxidation or other weathering processes (NRC 2003). Since the toxicity of petroleum products is related to the water-soluble fraction (WSF) or water-accommodated fraction (WAF), the relative solubility and persistence of constituent aromatic hydrocarbons results in crude oils with different toxic effects due to the toxicity of the hydrocarbons present (Capuzzo 1987, NRC 2003, Barata et al. 2005, McGrath et al. 2005, Redman et al. 2012, Butler et al. 2013).

Nonionic aromatic hydrocarbons are type 1 narcotic chemicals with similar toxic modes of action (Di Toro et al. 2000, McGrath et al. 2004), and are important contributors to aquatic toxicity (McGrath and Di Toro 2009, Redman et al. 2012). Toxicity depends on the aqueous concentration of constituent hydrocarbons, which controls partitioning into an

organism. Originally it was assumed the chemicals affected the whole organism (McCarty et al. 1991), but is now thought to involve interactions with just the hydrophobic portions of the cellular membranes (Sikkema et al. 1995, Di Toro et al. 2000). Lipophilic hydrocarbons alter membrane structure and function by partitioning into the lipid bilayer, modifying membrane fluidity and permeability (Sikkema et al. 1995, McGrath et al. 2004, de Hoop et al. 2011). The narcotic lethality of hydrocarbons has been linked to the chemical concentration in the target lipid, rather than the whole organism, and is assumed to be independent of species (Di Toro et al. 2000).

The Target Lipid Model

The target lipid model (TLM) assesses aquatic toxicity of nonpolar organic chemicals with a narcotic toxic effect (McGrath et al. 2004), and is based on the assumption that mortality occurs when the concentration in the target lipid reaches a threshold concentration (Di Toro et al. 2000). The TLM estimates this critical target lipid body burden (CTLBB; µmol chemical/ g lipid) using the specific endpoint [i.e., the concentration lethal to 50% of the population: LC_{50} (mmol/L)] and the target lipid–water partition coefficient (K_{LW}), which is defined as the ratio of chemical concentration in the lipid (C_L) to the aqueous concentration (C_W) (Di Toro et al. 2000).

1) $$CTLBB = LC_{50} * K_{LW}$$

2) $$K_{LW} = \frac{C_L}{C_W}$$

Experimental determination of the LC_{50} for a specific narcotic chemical allows calculation of an organism's CTLBB using the TLM.

3) $$\log LC_{50} = \log CTLBB - \log K_{LW}$$

The target lipid model uses K_{LW}, which is calculated using the linear free energy relationship between K_{LW} and the octanol–water partition coefficient (K_{OW}), as octanol has been determined a good surrogate for organism lipid tissues (Di Toro et al. 2000).

4) $$\log K_{LW} = -0.945 * \log K_{OW}$$

It is assumed that the target lipid has the same chemical partitioning property in all organisms, therefore the universal narcosis slope (-0.945) is representative of this ubiquitous mode of action (Di Toro et al. 2000). Combining Equations 3 and 4 results in the TLM.

$$5) \qquad \log LC_{50} = \log CTLBB - 0.945 * \log K_{OW}$$

McGrath and Di Toro (2009) refined the TLM to include a chemical class correction (Δc: MAHs= -0.109, PAHs= -0.352) for hydrocarbons with increased toxicity, and more precise universal narcosis slope (-0.936) determined with updated K_{OW}.

$$6) \qquad \log LC_{50} = \log CTLBB - 0.936 * \log K_{OW} + \Delta c$$

The species-specific CTLBB must be determined in a controlled laboratory experiment by measuring the LC_{50} for a single hydrocarbon with known K_{OW}. The CTLBB is expressed in µmol chemical/g octanol, but because of the relationship between K_{OW} and K_{LW}, the units are assumed to be µmol chemical/g lipid (McGrath et al. 2004). If TLM assumptions are true and partitioning is the same for all species, the CTLBB can be used to estimate the LC_{50} for other type 1 narcotic chemicals with similar toxic modes of action using their respective K_{OW}.

Toxicity of Mixtures

The toxic unit (TU) approach to evaluating mixture toxicity is a means of normalizing the toxicity of different chemicals in a mixture (Di Toro and McGrath 2000). The TU is the ratio of the aqueous concentration (C_w) to the effect concentration (LC_{50}).

$$7) \qquad TU = \frac{C_W}{LC_{50}}$$

Type 1 narcotic chemicals are known to have an additive effect (Capuzzo 1987, Di Toro and McGrath 2000, Barata et al. 2005, Redman et al. 2012, Butler et al. 2013), and combining the toxic effect of all constituents' results in the mixture toxicity.

$$8) \qquad TU_{mixture} = \sum_i TU_i$$

If the combined TU for a chemical mixture is greater than 1, the mixture is toxic at that concentration (Di Toro and McGrath 2000, McGrath and Di Toro 2009). The TLM and the additivity of TUs can be used to predict the toxicity of chemical mixtures.

The TLM assumes the target is lipid, and that concentration is limited by aqueous solubility of the chemical (Di Toro and McGrath 2000, Di Toro et al. 2000, McGrath et al. 2004, McGrath and Di Toro 2009). This cutoff exists because kinetics related to molecular size and solubility prevent accumulation in the organismal lipid at a sufficient level to cause an effect; LC_{50}s are greater than solubility (McGrath et al. 2004). Negri et al. (2016) determined the WAF of natural gas condensate was dominated by BTEX and alkyl

substituted benzenes and naphthalenes, while the parent condensate was composed mainly of n-alkanes and branched alkanes. Similarly, crude oil WAF was also found to contain 82% naphthalene and alkylated derivatives (Mercurio et al. 2004). Endicott crude oil WAF (600 mg/L loading) shows the same dominance of lower molecular weight aromatics compared to the source oil, which had measurable amounts of higher molecular weight polyaromatics (Fig. 1) (Redman and Parkerton 2015).

Multiple other studies have characterized the soluble portion of petrogenic hydrocarbons as being dominated by alkylated PAHs (Neff and Anderson 1981, Hawthorne et al. 2006, Achten and Andersson 2015), which are usually more abundant than parent PAHs (NRC 2003). Alkylation of aromatic hydrocarbons implies increasing lipophilicity, thereby increasing partitioning into the cell and altering toxicity (Achten and Andersson 2015). Hawthorne et al. (2006) found parent and alkylated hydrocarbons to contribute 1% and 99% of the toxicity of crude oil WAF respectively. Therefore, to avoid underestimating toxic effects, estimates of species-specific CTLBBs should be made using alkylated, low molecular weight aromatic hydrocarbons with log K_{ow} <5.5 (McGrath and Di Toro 2009).

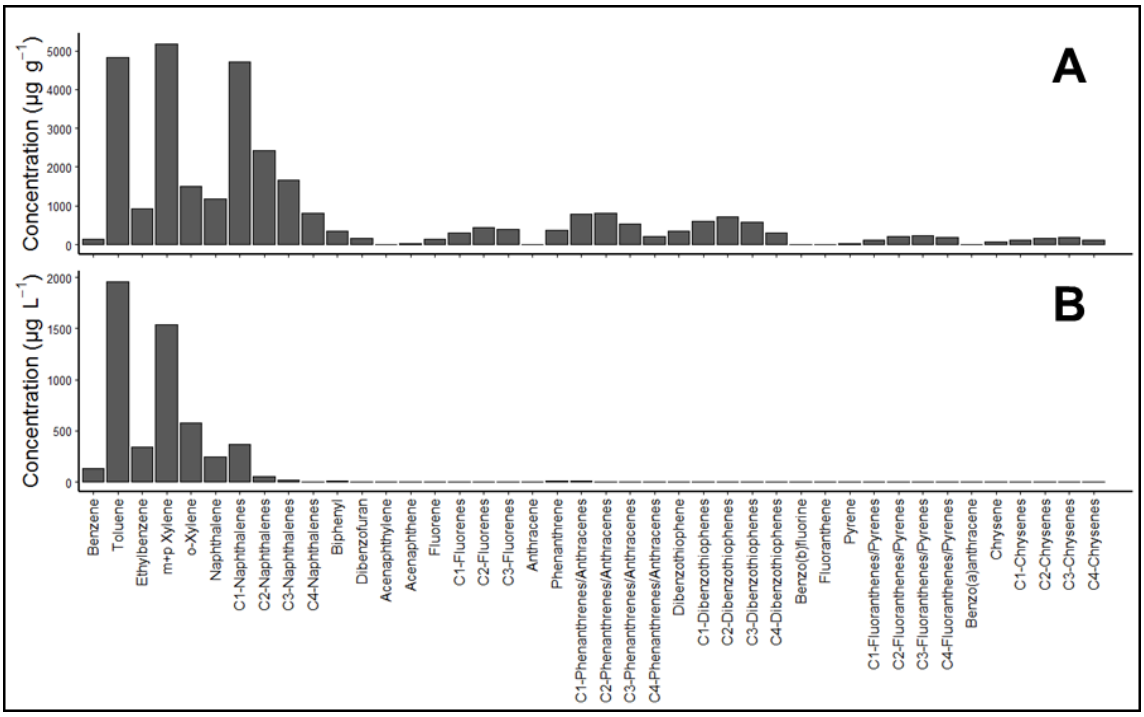

Figure 1. Concentration of aromatic hydrocarbons (C6–C16) present in Endicott Crude Oil (A) and Endicott Crude Oil WAF (B). Adapted from Redman and Parkerton (2015).

Passive Dosing

Determining the LC_{50} for use in the TLM must be completed using a constant concentration throughout the exposure to provide reliable data to generate dose-response curves (McGrath and Di Toro 2009, Butler et al. 2013, Redman and Parkerton 2015). Most petroleum PAHs are sparingly soluble, and obtaining constant exposure concentrations can be challenging due to loss mechanisms (sorption, volatilization, and degradation) (Smith et al. 2010, Butler et al. 2013). Exposure vessels with 10% headspace resulted in a 35–55% evaporation of total PAH over 24 h (Negri et al. 2016), while others saw 64% decline in total PAH over 84 h (Kegler et al. 2015). The passive dosing technique was developed to combat the issue of degradation whereby the chemical is partitioned from a solvent solution into a biocompatible polymer such as polydimethylsiloxane (PDMS) (Butler et al. 2013). A key criterion for successful use of the passive dosing system is to ensure excess mass of hydrocarbon in both the loading solution and PDMS O-ring reservoirs to prevent small amounts of depletion from affecting the target concentrations (Butler 2013). The excessive amount of hydrocarbon loaded into the PDMS O-rings has been proven to produce an accurate and precise constant aqueous concentration for the exposure duration despite potential losses that occur in the test system (Smith et al. 2010, Butler et al. 2013).

HYRDROCARBON TOXICITY TO CORALS

Every oil spill in the marine environment is a unique ecological problem, as potential effects depend on the local physical, chemical, and biological factors that influence the oil (Haapkylae et al. 2007). Weather conditions, seasonal factors, dosage, type of oil, previous exposure to oil, and type of remedial action are a few of the many influential factors determining the toxicity of spilled oil (NRC 2003, Haapkylae et al. 2007). During the past 50 years, multiple studies have attempted to measure the lethal and sublethal effects of oil on corals. Some studies have evaluated community-level effects of an actual oil spill, while others focused on subcellular changes in response to controlled laboratory experiments. The following sections provide a brief summary of the previous research conducted and a more detailed explanation of each study completed is found in Appendix 1.

Incidents resulting in Acute and Chronic Exposure of Petroleum to Corals

Oil spills in the marine environment are a significant ecological problem, but have the potential to provide an invaluable opportunity to assess impacts of oil exposure on the associated organisms. Over the last 50 years, many accidents have exposed benthic organisms to petroleum hydrocarbons. Ecosystem evaluations often overlooked possible damage to corals and other subtidal communities, presumably due to dangerous conditions associated with floating oil and logistical issues during the spill. Understanding the impacts of acute and chronic exposure on corals requires baseline data of the coral community, which is lacking in a majority of areas where spills have occurred. Appendix 1 Table 1 summarizes studies which evaluated the effects of both acute and chronic release of petroleum hydrocarbons on coral individuals, populations, and communities.

Analysis of acute and chronic exposures of coral reefs revealed a variety of effects on coral. No detectable impacts on coral were found after the Gulf War oil spill (Vogt 1995), whereas other spills resulted in major deterioration of the reef community. Community-level changes in response to petroleum pollution include decreases in species' abundance, diversity, and coral cover (Fishelson 1973, Bak 1987, Cubit et al. 1987, Jackson et al. 1989, Guzmán et al. 1991, Guzman et al. 1994). Branching coral species were found to be more sensitive when compared to encrusting or massive species, leading to a decrease in rugosity of the reef (Fishelson 1973, Bak 1987). Multiple acute and chronic exposures

have led to tissue loss and increased coral mortality (Rinkevich and Loya 1977, Cubit et al. 1987, Green et al. 1997, White et al. 2012, Fragoso ados Santos et al. 2015), which have been correlated with hydrocarbon uptake (Burns and Knap 1989). Bioaccumulation of petroleum hydrocarbons from the water column has been reported by multiple studies (Sabourin et al. 2013, Ko et al. 2014), altering cellular physiologic conditions. Increases in protein to lipid ratios have been found (Burns and Knap 1989), as well as altered protein metabolic condition, increased mitochondrial chaperoning, and increased xenobiotic and detoxification responses following both chronic and acute exposures (Downs et al. 2006, Downs et al. 2012). Increases in number of oil-degrading bacteria in the mucus bacterial community were also measured following chronic pollution (Al-Dahash and Mahmoud 2013).

Sublethal effects of corals exposed to petroleum products include increases in bleaching, tissue swelling, mucus production, coral injury, and bacterial infections (Jackson et al. 1989, Guzmán et al. 1991, Guzman et al. 1994, Green et al. 1997, White et al. 2012). Colony size and growth rate were found to decrease following exposure to petroleum pollution (Guzmán et al. 1991, Guzman et al. 1994). Exposure also resulted in immediate polyp retraction and sclerite enlargement in deep sea corals (White et al. 2012). Declines in number of breeding colonies, ova per polyp, and planula larvae released per coral head were established following chronic exposure to Iranian crude oil (Rinkevich and Loya 1977). Along with decreased gonad size, these alterations in reproductive features reduced settlement rates and decreased fecundity (Guzmán and Holst 1993).

Overall, oil spills in close proximity to coral reefs provide a unique opportunity to assess the effects of hydrocarbons on corals *in situ*. Although injurious, it is important to understand how corals react to acute and chronically spilled oil in their natural environment. However, in the absence of pre-spill baseline data, it can be difficult to discern between negative impacts of the oil spill and a decline in coral health linked to some other pre-existing stressor. Most coastal ecosystems are already affected by other anthropogenic stressors, and oil spills in two locations may have drastically different effects due to the compounding stressors present. Differences in type of pollutant spilled, as well as physical conditions at the time of the release also limit comparisons between oil spills.

In-situ Studies Examining Effects of Petroleum Hydrocarbons on Coral

Field experiments exposing coral to oil are a means of limiting differences in type of oil or physical condition, as well as accounting for lack of baseline data in certain areas. Researchers are able to collect data prior to exposure, and choose the precise way in which exposure will take place. Experiments conducted *in situ* are limited, and this review consists of three complete experiments, all conducted before 1990. Appendix 1 Table 2 summarizes the *in situ* experiments examining the effects of oil exposure on corals.

Experiments conducted *in situ* provide a controlled situation to monitor the effects of oil on reef corals, while managing other variables that usually prevent comparability between studies. Researchers have identified a greater affinity for oil in branching species when compared to massive or encrusting forms, with tissue damage occurring if oil adheres to the surface of the coral (Johannes et al. 1972). Exposure to oil resulted in only slight, but not significant reductions in coral cover, with no reduction in growth of individual corals (Ballou et al. 1987a, Ballou et al. 1987b, LeGore et al. 1989, Dodge et al. 1995). On the contrary, exposure to dispersed oil resulted in significant reductions in coral cover and growth, lasting two years, with little evidence of recovery (Ballou et al. 1987a, Ballou et al. 1987b, Dodge et al. 1995). Although initially significant damage occurred, full recovery of all corals in the TROPICS experiment with regard to growth, sclerochronology, and coral cover resulted after 10 years (Dodge et al. 1995, Ward et al. 2003).

Ex situ Laboratory Experiments Examining Effects of Petroleum on Adult Corals

Although the effects of acute and chronic releases of oil and *in situ* field experiments are important for understanding coral's reaction in their natural environment, they are accompanied by certain disadvantages; limited control of physical variables, high costs, and lack of baseline preparedness for evaluation of effects associated with acute exposures. Thus, researchers have employed laboratory experiments to examine effects of petroleum hydrocarbons and dispersant mixtures on corals and their multiple life stages. Using this type of experiment provides the means to control physical variables, concentrations, exposure scenarios, and which toxicant or organism to use, while limiting environmental exposure. Laboratory experiments also provide the opportunity to calculate

precise endpoints, such as the concentration lethal to 50% of the population (LC_{50}), which is often used as a measure of toxicity for chemical compounds.

Of the research conducted on the effects of petroleum hydrocarbons on corals, laboratory experiments are the most numerous. For this reason, controlled laboratory experiments were divided into two groups based on the life stage of the coral tested. The first group consists of research using adult corals, while the second group contains those using any stage of the reproductive cycle. Laboratory experiments evaluating the effects of exposure of petroleum hydrocarbons on adult stage corals are summarized in chronologic order in Appendix 1 Table 3.

Overall, the wide variety of bioassay conditions and exposure durations described in the foregoing experiments have resulted in high variability in the effect petroleum has on adult corals. Following exposure to oil or WAF, levels of mortality were variable, from extreme situations resulting in tissue flaking or rupture, to no significant mortality (Reimer 1975, Elgershuizen and De Kruijf 1976, Cohen et al. 1977, Peters et al. 1981, Wyers et al. 1986, Shafir et al. 2007, DeLeo et al. 2015). Sublethal changes were more common, and were typically related to alterations in polyp behavior. Researchers found abnormal feeding and stimulus reactions, mouth opening with exposed actinopharynx and mesenterial filament extrusion, breakdown of polypal pulsation synchrony, and polyp retraction or extreme elongation (Reimer 1975, Cohen et al. 1977, Ducklow and Mitchell 1979a, Neff and Anderson 1981). There were also increases in mucus bacterial populations, increased symbiont extrusion leading to slight or extensive bleaching, and decreased photosynthetic yield (Reimer 1975, Ducklow and Mitchell 1979a, Neff and Anderson 1981, Mercurio et al. 2004). Bioaccumulation was common in these exposures, which led to changes in normal cellular architecture and shifts in metabolic homeostasis (Peters et al. 1981, Rougee et al. 2006). Most effects of hydrocarbon exposure were temporary, with a return to normal behavior following recovery in clean seawater.

If dispersants or dispersed oil were used in the exposure, the resulting effects were more pronounced when compared to oil alone. Similar sublethal alterations in behavior were examined, including tentacle retraction, inhibition of feeding and tactile response, and nematocyst discharge; in most cases damage was more permanent with poor recovery (Lewis 1971, Elgershuizen and De Kruijf 1976). Tissue rupture followed by increased

mortality was common, with dispersed oil leading to the highest mortality in studies which considered both oil and dispersed oil (Eisler 1975, Shafir et al. 2007, DeLeo et al. 2015). An initial reduction in carbon fixation and incorporation of photosynthetic products was also found following exposure to dispersed oil, when oil alone failed to solicit the same response (Cook and Knap 1983).

The effects of petroleum PAHs or other petroleum products on corals were also evaluated. Phenanthrene was found to significantly reduce calcium deposition following rapid accumulation and slow depuration (Neff and Anderson 1981). Naphthalene was also rapidly accumulated, and the relatively rapid depuration period was consistent with observations in other marine organisms (Solbakken et al. 1983). Benzo(a)pyrene and a 13-PAH mixture led to cellular changes consistent with detoxification of a xenobiotic (Ramos and Garcia 2007, Woo et al. 2014). Gasoline was found to result in significant loss of sclerites and tissue in a gorgonian (White and Strychar 2011).

Ex situ Laboratory Experiments Testing the Effects of Petroleum on Coral Reproduction

It has been previously established that coral gametes and larvae are more sensitive compared to their adult form. This early life-stage sensitivity coupled with the buoyant nature of gametes and larvae increases the potential for oil exposure if a spill occurs during spawning season. Laboratory experiments evaluating the effects of petroleum hydrocarbon exposure on any stage of coral reproduction are summarized in chronological order in Appendix 1 Table 4.

Overall, exposing corals to petroleum pollution during any stage of reproduction significantly alters reproductive output. When oil or oil WAF was used, larvae were prematurely released, the number of female gonads per polyp was reduced, and significant reductions in settlement and metamorphosis occurred (Loya and Rinkevich 1979, Rinkevich and Loya 1979, Te 1991, Kushmaro et al. 1997, Epstein et al. 2000, Goodbody-Gringley et al. 2013, Hartmann et al. 2015, Negri et al. 2016). Multiple studies found delayed settlement, free floating metamorphosis, post-metamorphic deformation and increased mortality of larvae (Te 1991, Kushmaro et al. 1997, Epstein et al. 2000, Goodbody-Gringley et al. 2013). Hydrocarbon exposure also led to significant inhibition of fertilization with unusual embryonic development and disruption of cell membranes (Mercurio et al. 2004).

When dispersants were utilized, researchers found increased mortality, coupled with fertilization, metamorphosis and settlement inhibition (Epstein et al. 2000, Lane and Harrison 2000, Negri and Heyward 2000, Goodbody-Gringley et al. 2013). Major behavioral anomalies and structural deformations of planulae also occurred following dispersed oil exposure (Epstein et al. 2000). Studies that examined effects of both oil and dispersed oil found a greater toxic effect associated with dispersed oil compared to oil alone.

Limitations of Previous Research Examining Hydrocarbon Toxicity to Corals

Coral reefs thrive in coastal waters that are often adjacent to urban centers and major shipping lanes, which predisposes the potential for exposure to petroleum. A necessity to understanding the impacts of oil pollution on coral reefs is to increase our knowledge on the effects to the basic element of a coral reef: the coral animal (Shigenaka 2001). Acute and chronic exposures in history have either lacked quantitative baseline coral community data that are required to assess changes, or neglected to measure hydrocarbon concentrations during the spill. This lack of data prevents the comparison of results between spills, and helps to explain some of the variation in observed effects.

As a way of addressing this data gap, field and laboratory studies aimed at quantifying the effects of petroleum pollution on corals have been designed to allow researchers the ability to control exposures and compounding stressors. To date, field studies are limited in number, but include research that quantifies actual exposure concentrations and baseline coral community data. Observed effects range from no effect in oiled sites to an initial reduction in coral cover in dispersed oil sites with full recovery in under 10 years (Ballou et al. 1987b, Dodge et al. 1995). Results of the TROPICS experiment indicated that dispersed oil initially led to more severe effects on coral when compared to oil alone, with the difference still present after two years. Other field experiments resulted in no significant difference between oil and dispersed oil. These differences were likely related to differences in oil type, dispersant used, species tested, or the physical conditions during the spills.

Laboratory experiments are designed to limit differences between exposure scenarios as a means of comparing toxicity between different corals and oils. Though lab studies may use environmentally unrealistic concentrations or exposure durations, they are

necessary in order to assess relative species sensitivity and to provide essential information for use in toxicological models. To date, previous laboratory research has examined 34 different species of coral from 23 genera. Members of subclasses Hexacorallia and Octocorallia were used, encompassing shallow, intermediate, and deep-water species. Use of multiple species is beneficial to understanding species sensitivity differences, but makes comparisons of different toxicants challenging.

A key issue in evaluating published toxicity data is whether or not the study has reported measured concentrations in exposure media, as the real utility of toxicity tests is the comparison of threshold concentrations of oil with values measured in the field (Bejarano et al. 2014). This is not limited to lab studies, as there is often a lack of detailed exposure-response data for field and mesocosm studies. Use of nominal concentrations is cautioned because they may either over or underestimate the lethality of a toxicant; all components of oil do not dissolve into water, and an LC_{50} reported as 10 ppm may actually be caused by concentrations closer to 1 ppm (Shigenaka 2001). There are large discrepancies in toxicity estimates between studies reporting measured versus nominal values, and this is particularly important when comparing the toxicity of chemically dispersed to physically dispersed oil. Thus, analytical chemistry is necessary to evaluate the concentration of the exposure medium when conducting laboratory experiments with petroleum products. Nominal concentrations were used in 27 of the 45 laboratory experiments reviewed here. Concentrations were not specified for 4 studies, and 14 experiments used measured concentrations. Of the 14 experiments with measured concentrations, 6 used measured stock solutions serially diluted to treatment concentrations that were not measured. The remaining eight exposure studies using measured concentrations were from four experiments (Peters et al. 1981, Cook and Knap 1983, Dodge et al. 1984, Wyers et al. 1986, Knap 1987, Goodbody-Gringley et al. 2013), providing the most accurate estimate of toxic threshold concentrations, and the best opportunity to compare toxicity among studies.

It is important to not only analyze the actual concentration of the toxicant, but also the composition of toxicant that the organism is exposed to (McGrath and Di Toro 2009, Bejarano et al. 2014). Petroleum is composed of thousands of chemicals, some of which are extremely volatile and may not remain in solution after preparation. One issue with the

use of WAF or WSF is the compositional difference created by the variety of preparation techniques utilized (mixing energy, headspace volume, ratio of oil to dispersant), which have since been standardized (Singer et al. 2000, Aurand and Coelho 2005). Of the 48 laboratory experiments described here, 30 different toxicants were used, comprised of 15 crude oils, 8 refined products, 6 PAHs or PAH mixtures, and an unspecified petroleum toxicant. Although the broad range of toxicants provides a wide view of petroleum toxicity to corals, comparison of effects between studies are cautioned due to the compositional differences between toxicants. No two petroleum products are compositionally the same, and most studies lack quantitative chemical composition analyses, preventing extrapolation of results. Of the studies included in this review, Knap (1987) is the only laboratory study to quantify the composition of the toxicant.

Aside from compositional differences between studies, the route of exposure also influences the toxicity of petroleum products. Petroleum was floated on the surface of the water in 7 experiments, mixed into water in 17 experiments, WAF or WSF created and used in 20 experiments, corals immersed in toxicant or poured over coral in 3 experiments, and one experiment used oil-coated dosing vessels. Corals are benthic organisms as adults and will only be exposed to floating whole oil as gametes or larvae floating on the surface, or if exposed to air during extreme low tides. Exposure routes should focus on the portion of toxicant that is bioavailable to the coral animal, the water-soluble portion.

Bioassay conditions are another factor that may influence toxicity of petroleum hydrocarbons to corals. Static exposure assays were utilized in 35 of the laboratory experiments, while flow-through exposures were conducted for 9 experiments. Eisler (1975) showed an increase in toxicity in static conditions compared to flow-though, indicated by lower LC_{50} values. Similar results were found during another experiment, where static conditions produced mortality, and flow-through conditions produced no mortality (Cohen et al. 1977). These results indicated that corals have increased sensitivity to static conditions when compared to flow-through. These differences in toxicity were likely related to the compounding effects associated with static exposure, which could include decreased oxygen and increased waste products. Aeration was used in seven of the static exposures to prevent depletion of oxygen, but likely resulted in significant increases in evaporation of certain compounds. The type of vessel utilized also has an impact on

toxicity, as open vessels have the potential to allow volatile fractions to escape, whereas closed vessels prevent volatility once equilibrium is reached between the aqueous and gas phases. Te (1991) found a significant increase in mortality associated with a closed vessel, but the open vessel resulted in no mortality.

To increase the potential for comparisons across studies, it is important to follow standardized toxicity testing protocols that have been developed. Future coral toxicological studies should limit the differences in exposure media preparation, exposure scenarios and bioassay conditions. Coupling standardized protocols, such as those established by the Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) (Aurand and Coelho 2005), or Redman and Parkerton (2015), with more descriptive compositional analyses and quantifiable chemistry will increase the comparability of studies and potential to extrapolate results to real world situations.

Although there are a number of studies assessing petroleum toxicity to corals, methodological disparities between studies have prevented comprehensive conclusions regarding the toxicity of hydrocarbons to corals. Every spill event is unique due to the large variation in toxicant, physical, chemical, and biological factors; results from one spill cannot be extrapolated to others with confidence. The same holds true for laboratory studies; differences in study design, toxicant used, and species tested prevent direct comparison of results.

STATEMENT OF RESEARCH

This study evaluated the toxicity of a petroleum hydrocarbon to a representative coral species in a controlled laboratory exposure. Hypotheses tested can be found in Table 1. Determination of the toxicity of a low molecular weight PAH, 1-methylnaphthalene, to *Porites divaricata* was used to calculate the 48-h toxic threshold for use in the TLM. A passive dosing method was employed, utilizing PDMS o-rings loaded with 1-methylnaphthalene (1-MN) in a methanol solvent to maintain constant concentrations throughout the exposure duration (Butler et al. 2013). An initial range-finding test was carried out to determine the appropriate range of concentrations to use in the full-toxicity test and to verify the effectiveness of the passive dosing method. Following the range-finding test, a full-toxicity test was conducted to obtain refined estimates of threshold concentrations as inputs to the TLM. Sublethal effects and lethality were used to determine the concentrations causing a 50% effect on the population (EC_{50}), and the concentration causing 50% mortality (LC_{50}). These values were used in the TLM to calculate the CTLBB in order to compare species sensitivity. Estimates of the toxicity of other narcotic hydrocarbons were predicted based on the CTLBB determined for 1-MN.

Table 1. Hypotheses tested and analytical methods used during this research.

Null Hypotheses	Analytical Method
1-MN has no effect on the gross visual condition of *P. divaricata*.	Semi-quantitative scoring system developed to measure gross physical coral condition.
1-MN has no effect on the photosynthetic efficiency of *P. divaricata* symbionts.	Pulse amplitude modulation (PAM) fluorometry
1-MN has no effect on calcification of *P. divaricata.*	Buoyant wet weight determination
1-MN has no effect on the histological characteristics or cellular architecture of *P. divaricata.*	Semi-quantitative scoring system developed to measure tissue and cellular characteristics
1-MN has no effect on survival of *P. divaricata*	Percent recent mortality measurements

METHODOLOGY

Porites divaricata was collected from a nearshore Florida reef (SAL-15-1685-SRP), returned to the lab, cut into 2-cm fragments (branch tips) and attached to small aragonite tiles using cyanoacrylate gel adhesive. Corals were acclimated in the laboratory in a 1100-liter (L) indoor recirculating seawater system for 6–9 weeks (wk); system temperature was maintained at 25ºC, and water motion was supplied by powerheads and a wave maker. Natural seawater taken from Port Everglades, FL at high tide was used and lighting was provided by LED lights (Photon 32), programmed to mimic sunrise and sunset (photoperiod 12:12). These lights produced a spectrum suited for coral growth without the addition of ultraviolet radiation to avoid phototransformation of toxicant during the exposure. Corals were not fed during the experiments as *P. divaricata* is predominantly autotrophic (Kanwisher and Wainwright 1967).

PDMS O-ring Loading

Stock solutions were prepared by dissolving known amounts of 1-MN (Acros Organics, 97%) in methanol (Fisher Scientific, HPLC Grade) in 500-mL volumetric flasks. Stock solutions of 1-MN in methanol (MeOH) were prepared using the equation:

$$C_{MeOH} = \left[K_{MeOH-PDMS} + \left[\frac{V_{PDMS}}{V_{MeOH}}\right]\right] * \left[K_{PDMS-Water} + \left[\frac{V_{Water}}{V_{PDMS}}\right]\right] * C_{Target}$$

where C_{MeOH} is the concentration of 1-methylnapthalene added to methanol (mg/L); C_{target} is the target concentration in seawater (mg/L); V_{MeOH} is the volume of the methanol dosing solution (mL); V_{PDMS} is the volume of PDMS O-rings (O-Rings West) in the mixing vessel (mL); V_{water} is the volume of seawater in the recirculating flow-through system (mL); $K_{MeOH\text{-}PDMS}$ is the partition coefficient of 1-methylnapthalene between methanol and PDMS (5.012); and $K_{PDMS\text{-}Water}$ is the partition coefficient of 1-methylnapthalene between PDMS and water (954.99) (Butler 2013).

Range-Finding Exposure

The range-finding exposure to 1-MN consisted of five treatments, with three replicate dosing systems per treatment, and five corals per replicate. A seawater control (with O-rings), a methanol control (with O-rings), and three concentrations of 1-MN were tested (nominally 500 µg/L, 5,000 µg/L, and 25,000 µg/L). The seawater control was used

to rule out any effect of the O-rings, and possible effects of the chamber system. The methanol control was used to determine if a solvent effect resulted from loading of the O-rings.

The calculated amount of 1-MN required for each experimental concentration was dissolved in methanol and mixed for 24 h (Table 2). Dosing solutions were transferred to 1000-mL Erlenmeyer flasks containing 114 PDMS O-rings each (38 for each replicate). The mass of each O-ring averaged 1.06 g, giving a total mass of 120.70 g for each loading solution, and 40.23 g in each exposure system. Calculated depletion of 1-MN in both reservoirs was 4.42% in the MeOH loading solution, and 7.41% in the PDMS O-rings. Loading vessels were placed on a shaker table for 72 h for partitioning of 1-MN into the PDMS O-rings for all experiments conducted (Smith et al. 2010, Butler et al. 2013).

Table 2. Calculated and measured amounts of 1-MN dissolved into the 500-mL MeOH O-ring loading solutions to obtain respective treatment concentrations during the range-finding exposure.

Target Concentration	Calculated C_{MeOH}	Range-finding Exp.
500 µg/L	1,282.5 mg	1,286.6 mg
5,000 µg/L	12,825.3 mg	12,829.5 mg
25,000 µg/L	64,126.6 mg	64,125.9 mg

Full-Toxicity Exposures

Six treatments (nominally 1,000 µg/L, 2,000 µg/L, 4,000 µg/L, 8,000 µg/L, 16,000 µg/L) and a seawater control with O-rings were tested in each of the full-toxicity exposures, with five coral fragments in each of the four replicate systems. Calculated amounts of 1-MN for each concentration were added to each volumetric flask of MeOH (Table 3) and mixed for 24 h. Dosing solutions were then transferred to 1000-mL Erlenmeyer flasks containing 152 PDMS O-rings each (38 for each replicate). The mass of each O-ring averaged 1.06 g, giving a total mass of 160.93 g for each loading solution, and 40.23 g in each exposure system. Calculated depletion of 1-MN in both reservoirs was 5.89% in the MeOH loading solution, and 7.36% in the PDMS O-rings. Loading vessels were placed on a shaker table for 72 h for partitioning of 1-MN into the PDMS O-rings for all experiments conducted (Smith et al. 2010, Butler et al. 2013).

Table 3. Calculated and measured amounts of 1-MN dissolved into the 500 mL MeOH O-ring loading solutions to obtain respective treatment concentrations in the dosing system.

Treatment	C_{MeOH}	Experiment #1	Experiment #2
1,000 µg/L	2,584.5 mg	2,584.7 mg	2,587.9 mg
2,000 µg/L	5,168.9 mg	5,170.4 mg	5,176.6 mg
4,000 µg/L	10,337.9 mg	10,345.8 mg	10,343.8 mg
8,000 µg/L	20,675.8 mg	20,679.1 mg	20,679.7 mg
16,000 µg/L	41,351.6 mg	41,352.7 mg	41,353.4 mg

Dosing system

The three exposures completed in this study used the same toxicant preparation and dosing methodology. All exposures were conducted using a continuous-flow recirculating passive dosing system (Fig. 2) in a 48-h constant exposure using chambers similar to those described and employed by the Chemical Response to Oil Spills Ecological Effect Research Forum (CROSERF) (Singer et al. 1993, Aurand and Coelho 2005).

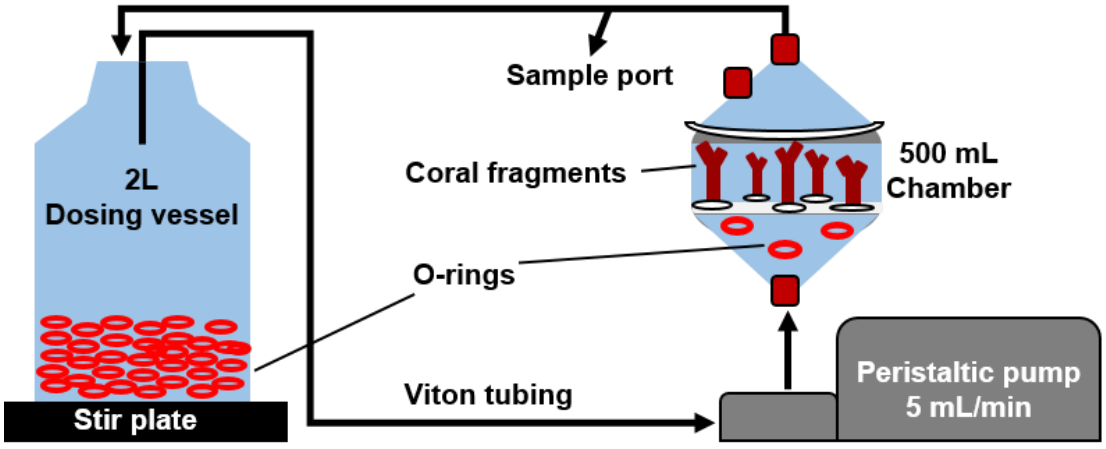

Figure 2. Recirculating-flow exposure system. *Porites divaricata* fragments (5) in a 500-mL glass chamber with 3 O-rings was connected to a multi-channel peristaltic pump by Viton tubing (arrows) with a flow rate of 5 mL/min. Each chamber was supplied by a separate 2-L dosing vessel with 35 O-rings which was stirred vigorously throughout the exposure. Adapted from Renegar et al. (2016).

Seawater for use in the exposures was sourced from the acclimation system, filtered to 1 µm, aerated, and kept at 25°C until use. Treatments were randomly assigned to chambers, which were filled with 500 mL filtered seawater and three loaded O-rings. Corresponding dosing vessels were filled with 2300 mL filtered seawater and 35 loaded O-rings and had <10% headspace to limit volatile loss. Peristaltic pumps were started with dosing vessels stirred vigorously throughout the 16-h equilibration period to ensure targeted concentrations were reached. Following the equilibration period, randomly assigned corals were added to each chamber, and the test was initiated.

Coral fragments, solutions, and equipment were monitored for continuous operation within designated limits throughout the duration of exposure. Following the 48-h exposures, surviving coral fragments were transferred back to the laboratory system and immediately analyzed for photosynthetic efficiency and buoyant wet weight before removing two corals from each chamber for histological analysis. During the 4-wk post-exposure recovery period, coral fragments were maintained under the same conditions as described for pre-exposure with condition of each coral assessed daily for 1 wk, and weekly thereafter, using PAM fluorometry, buoyant wet weight, and visual health metrics as described below.

Water Quality and Chemical Analysis

Water samples for basic water quality [temperature, pH, dissolved oxygen (DO), phosphate (PO_4), ammonia (NH_3), nitrite (NO_2), and nitrate (NO_3)] were collected during laboratory acclimation, and at the start and end of the exposure and analyzed with a HACH DR850 colorimeter and YSI 556 Multiprobe System. Alkalinity was determined by potentiometric titration with a Mettler-Toledo DL22 autotitrator. Samples for 1-MN analysis were collected with no headspace in volatile organic analyte vials (Thermo Scientific) with Teflon-lined caps from the effluent line of each chamber at the beginning (0 h, immediately prior to addition of coral fragments), middle (24 h, range finding only), and end (48 h) of the exposure to verify the stability of the concentration throughout the exposure. Samples were preserved at 4°C and the concentration of 1-MN was quantified using a Horiba Aqualog spectrophotometer after extraction with dichloromethane by The Geochemical and Environmental Research Group at Texas A&M University. Duplicate samples were also collected at each time, and verified for accuracy.

Coral Condition and Mortality

Coral condition was visually assessed by semi-quantitative scores based on the criteria found in Table 4. Individual criteria scores for each coral were averaged to obtain a single coral score at each time. If a criterion was un-scoreable (i.e., tissue swelling after tissue had receded); it was left blank as opposed to a zero to avoid artificially lowering the score at that time point. Coral condition was assessed weekly during the pre-exposure and post-exposure periods. During the exposure, coral condition was assessed hourly for the first 8 hours (h) after exposure initiation, and every 12 h thereafter for the remainder of the 48-h exposure.

Table 4. Criteria for scoring coral condition characteristics. Scores for color, polyps, mucus, and tissue were assigned to each coral.

Range	Diagnostic criteria
0-normal	Color: appears normal Polyps: fully extended or loosely retracted Mucus: normal mucus production Tissue: no tissue swelling, no mesenterial filaments
1-mild	Color: slight lightening of coloration Polyps: retracted and slightly closed Mucus: normal to slightly elevated Tissue: slight coenenchyme swelling and/or polyp distension
2-moderate	Color: moderate lightening of color Polyps: evident polyp retraction with full polyp closure Mucus: moderately elevated mucus production Tissue: moderate coenenchyme swelling and/or polyp distension
3-severe	Color: significant lightening of coloration, bleaching Polyps: polyps tightly retracted and skeletal ridges exposed Mucus: mucus sheets evident Tissue: severe coenenchyme swelling and/or polyp distension

Percent mortality was also visually assessed consistent with established methods of tissue mortality determination in corals (Lirman et al. 2013). As partial coral mortality is possible (Fig. 3), the percent mortality reported is the mean mortality of all corals in each treatment (n=20).

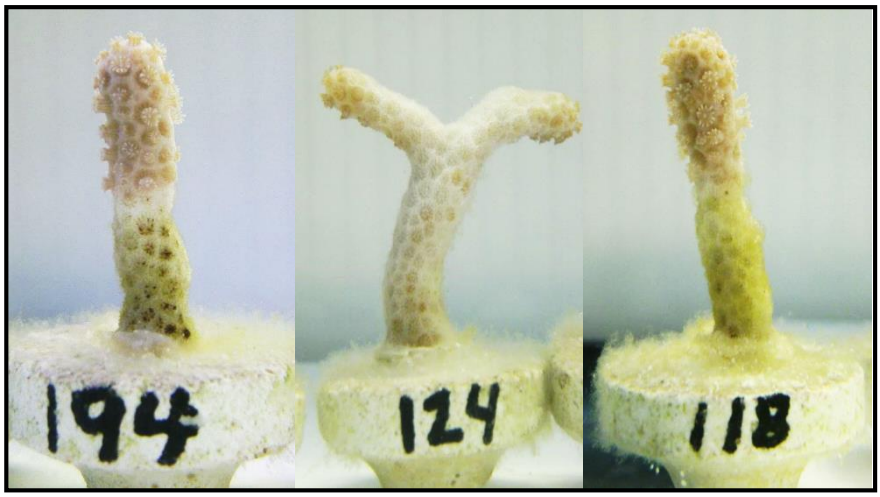

Figure 3. Examples of partial coral mortality following exposure to 1-MN.

Photosynthetic Efficiency

A pulse-amplitude-modulation (PAM) fluorometer (Diving-PAM, Walz, Germany) was utilized to gauge photosynthetic efficiency of symbiotic zooxanthellae weekly during the pre-exposure period, immediately before the exposure period, immediately after the exposure period, daily for 1 wk post-exposure, and weekly thereafter for the remainder of the post-exposure period. PAM fluorometry measured the light adapted effective quantum yield [$(F_m–F)/F_m$ or $\Delta F/F_m$] of the autotrophic endosymbiotic zooxanthellae by applying a saturation pulse of light, and determining yield from the ratio of initial fluorescence (F) to maximum fluorescence (F_m). The following parameters were chosen to determine yield for *P. divaricata*: measuring light intensity = 3, damping = 2, gain = 3, saturation intensity = 7, and saturation width = 0.8. These were determined by a combination of published literature values (Martinez et al. 2007), and parameter adjustment until the saturation curve had the characteristic plateau required for accurate depiction of effective quantum yield (Fig. 4). Lights were kept at an intensity equivalent to 30 minutes post sunrise for the duration of each set of measurements to ensure differences in photosynthetic efficiency were not due to changes in light intensity. Between measurements, the fiber optic light sensor was adjusted between 2 mm and 10 mm to maintain initial fluorescence readings between 350 and 400 units without adjustment of

measurement parameters. Measurements were taken from each side of the branch tip (4 total) to represent the whole coral fragment.

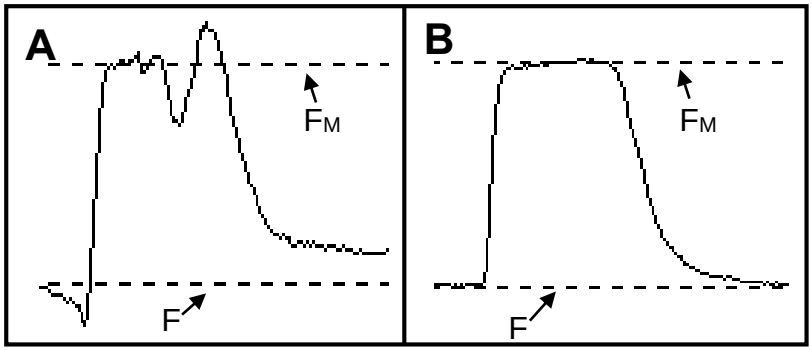

Figure 4. **Saturation curves used to adjust settings on the Diving PAM Fluorometer. A) Representative curve with incorrect settings. B) Representative curve with correct settings. The fluorescence signal starts at initial fluorescence (F), and rises with the initiation of the saturation pulse, until it plateaus at the maximum fluorescence (F_m) and returns to the initial value post saturation pulse.**

Calcification

Calcification of the coral fragments was evaluated using buoyant wet weight (Davies 1989). Buoyant weight is a non-destructive method of measuring growth rates for corals over short time intervals, which removes variability between fragments resulting from tissue thickness and provides weights explicitly related to the mass of the skeleton. Measurements were taken immediately prior to the exposure, immediately following the exposure, after 1 wk of recovery (Full-toxicity Exp. 2 only), and at the end of 4 wk of recovery, to determine if there were long-term effects of the exposure to 1-MN on *P. divaricata* calcification. Growth rates are expressed as percent change per day, and normalized to initial fragment size (Ferrier-Pages et al. 2000).

Histology

Samples for histological analysis were collected at initiation of exposure (10 randomly selected coral fragments), end of exposure (two of the surviving coral fragments per chamber), after 1 wk of post-exposure recovery (one surviving coral fragment per chamber) and at the end of the post-exposure recovery period (the remaining coral fragments). Coral samples were fixed in glutaraldehyde fixative solution [2 mL of 70% glutaraldehyde in 68 mL of cacodylic buffer (2.16 g cacodylic acid in 200 mL of 0.22 µm filtered seawater)]. Samples were maintained at 4°C in the fixative solution for 4–6 days,

and divided into subsets for histological analysis. Histological samples were decalcified after primary fixation in 5% hydrochloric acid (HCl)/ethylenediaminetetraacetic acid (EDTA) in seawater solution, dehydrated in a graded series of ethanol and xylene, and embedded in Paraplast Plus®. Longitudinal and transverse sections (4 µm) were made and mounted on slides. Sections were cleared in xylene and stained with Harris's hematoxylin and eosin (H&E). Stained slides were viewed in an Olympus BX43 light microscope at magnifications ranging from 4–60x and photographed with an Olympus DP21 digital camera for image analysis of cellular structures.

Overall cellular changes were assessed histologically using a semi-quantitative scale, which evaluates general condition of coral and algal cells, epidermal and gastrodermal integrity, and presence of tissue ruptures. Two slides per coral fragment were made, with two longitudinal and two cross sections on each. Analysis of each slide followed a scoring rubric (Appendix 2) which assessed the severity and extent of multiple categories (general cellular condition, zooxanthellae, gastrodermal and epidermal integrity of the surface and basal body walls).

Statistical analysis

All data were tested for normality (Shapiro-Wilk) and homoscedasticity (Bartlett/Levene) and transformed to meet these assumptions where applicable, or nonparametric methods were used. Kruskal-Wallis analysis of variance (ANOVA) (α=0.05) on ranks of untransformed data was used to compare mean coral condition during pre-exposure, exposure, and post-exposure periods, histological changes (full exposure 2) and water quality data between treatments. Post-hoc analysis of non-parametric data (Multiple Comparisons) was used where applicable. One-way ANOVA (α=0.05) was used to compare mean effective quantum yield, histological changes (range-finding exposure), and growth rate among treatments over the pre-exposure and post-exposure periods. Post-hoc analysis (Tukey's Test) was used to determine which treatments were significantly different from others. Threshold concentrations were determined using GraphPad Prism 6 (EC_{50}) and methods established by the EPA (LC_{50}) (USEPA 2002). Statistical tests were performed using the statistical software R (V 3.1.2) and Statistica 13.

RANGE-FINIDING EXPOSURE RESULTS

Hydrocarbon chemistry and water quality

Measured concentrations of 1-MN throughout the exposure period for each treatment are shown in Table 5. Samples were obtained at the start, middle, and end of the exposure to verify consistency of target concentration, resulting in stability over time with minimal loss. Eliminating the controls from analysis of loss/gain resulted in a spread of -7.6% to +11% across all 1-MN treatments.

Significant increases ($p<0.05$) in nutrient levels [PO_4 (0.1 ± 0.04 mg/L), NH_3 (0.11 ± 0.08 mg/L), and NO_2 (0.03 ± 0.003 mg/L)] and a significant decrease in pH (7.59 ± 0.05) were found in the 25,966.7 µg/L treatment chambers due to coral tissue necrosis. No significant differences ($p>0.05$) in pH (7.98 ± 0.02), alkalinity (122.7 ± 7.5 mg/L), PO_4 (0.04 ± 0.02 mg/L), NH_3 (0.03 ± 0.02 mg/L), NO_2 (0.01 ± 0.002 mg/L), NO_3 (0.08 ± 0.02 mg/L) or DO (48.5 ± 3.4%) were found among the seawater control, MeOH control,

Table 5. Measured concentrations (mean ± SD) of 1-methylnaphthalene (µg/L) for each treatment replicate at each time point of the Range-finding exposure.

Treatment	T0	T24	T48	Mean (n=3)	Loss/ Gain (%)	Treatment Mean (n=3)
MeOH Control	10.4	8.9	5.0	8.08±2.80	-52	6.59 ± 1.1
MeOH Control	4.5	9.9	4.4	6.3±3.2	+0.50	
MeOH Control	6.8	6.0	3.5	5.4±1.7	-47	
SW Control	4.0	4.6	2.5	3.7±1.1	-39	6.07 ± 1.7
SW Control	6.8	5.8	9.3	7.3±1.8	+37	
SW Control	8.9	9.7	3.0	7.2±3.6	-66	
500	645.8	584.8	596.3	608.9±32.4	-7.6	643.6 ± 24.6
500	674.8	641.0	674.8	663.5±19.5	0	
500	644.2	671.5	659.5	658.4±13.7	+2.3	
5,000	4,120.6	4,376.1	4,590.7	4,362.5 ± 235.3	+11	5,437.1 ± 779.9
5,000	5,810.2	5,821.1	5,646.7	5,759.3±97.7	-2.8	
5,000	6,142.4	6,109.7	6,316.8	6,189.6±111.3	+2.8	
25,000	25,539.2	25,092.3	24,056.8	24,896.1±760.4	-5.8	25,966.7 ± 757.05
25,000	26,607.4	26,302.2	26,585.6	26,498.4±170.3	-0.08	
25,000	27,577.5	25,811.7	26,127.8	26,505.6±941.6	-5.2	

643.6 µg/L or 5,437.1 µg/L treatments, and no difference in temperature (26.4°C±0.2) was found among all treatments. Dissolved oxygen levels decreased in all exposure systems because chambers were sealed to prevent volatile loss of 1-MN.

Coral Condition

Progressive coral physical response is shown is Figure 5. Overall, corals in both the seawater and methanol control treatments exhibited normal polyp extension, with limited mucus production and no tissue swelling during the 48-h exposure period (Fig. 5A). Corals exposed to the lowest concentration, 643.6 µg/L, displayed mild polyp distension and a qualitative delay in tactile response after 48 h (Fig. 5B). The 5,437.1 µg/L exposed corals had progressive polyp retraction, moderate tissue swelling and mucus production after 24 h (Fig. 5C). The corals exposed to 25,966.7 µg/L exhibited full polyp retraction and abundant mucus production within 6 h of exposure, with 100% mortality occurring after 24 h (Fig. 5D). As no partial mortality was observed, the graphical method was used to calculate an LC_{50} of 12,123 µg/L (USEPA 2002). Utilization of this method prevented calculation of a 95% confidence interval.

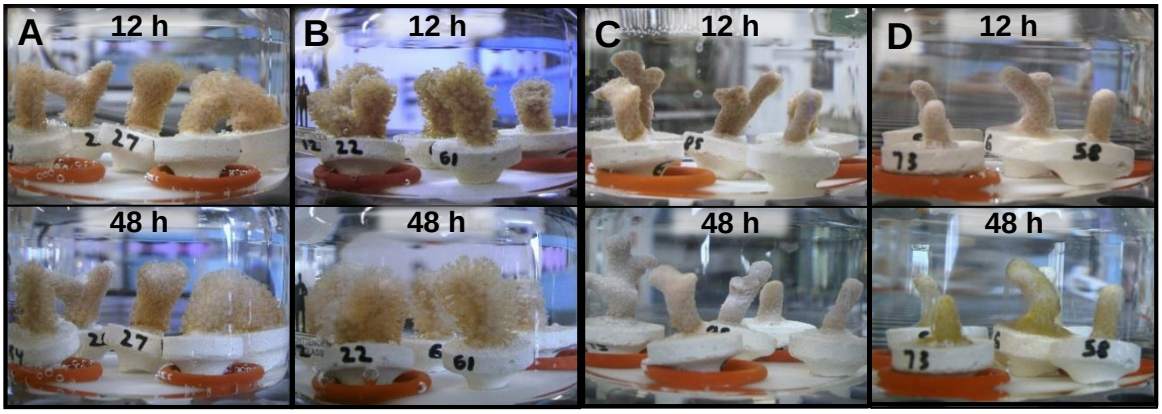

Figure 5. *Porites divaricata.* Coral physical response to 1-methylnaphthalene exposure at 12 and 48 h. A) Control treatment, B) 643.6 µg/L treatment, C) 5,437.1 µg/L treatment, D) 25,966.7 µg/L treatment. Adapted from Renegar et al. (2016)

Comparison of mean coral condition score for each treatment at each interval over the pre-exposure, exposure, and post-exposure periods (Fig. 6) revealed significant treatment effects at all times from 1 h after initiation of exposure to 9 d post-exposure ($p<0.05$). Post-hoc analysis indicated that the 5,437.1 µg/L and 25,966.7 µg/L corals scored significantly higher than 643.6 µg/L and control treatments at the end of the exposure period ($p<0.05$). After one day of recovery, the 643.6 µg/L corals scored similarly to controls ($p>0.05$) while the 5,437.1 µg/L coral scores remained significantly higher than controls ($p<0.05$) until after 1 wk of recovery when scores were no longer different ($p>0.05$). After 9 d of recovery, no treatment effects on coral condition were observed ($p>0.05$). Coral condition scores were used to calculate an EC_{50} of 7,442 µg/L (95% CI: 4,905–11,290 µg/L).

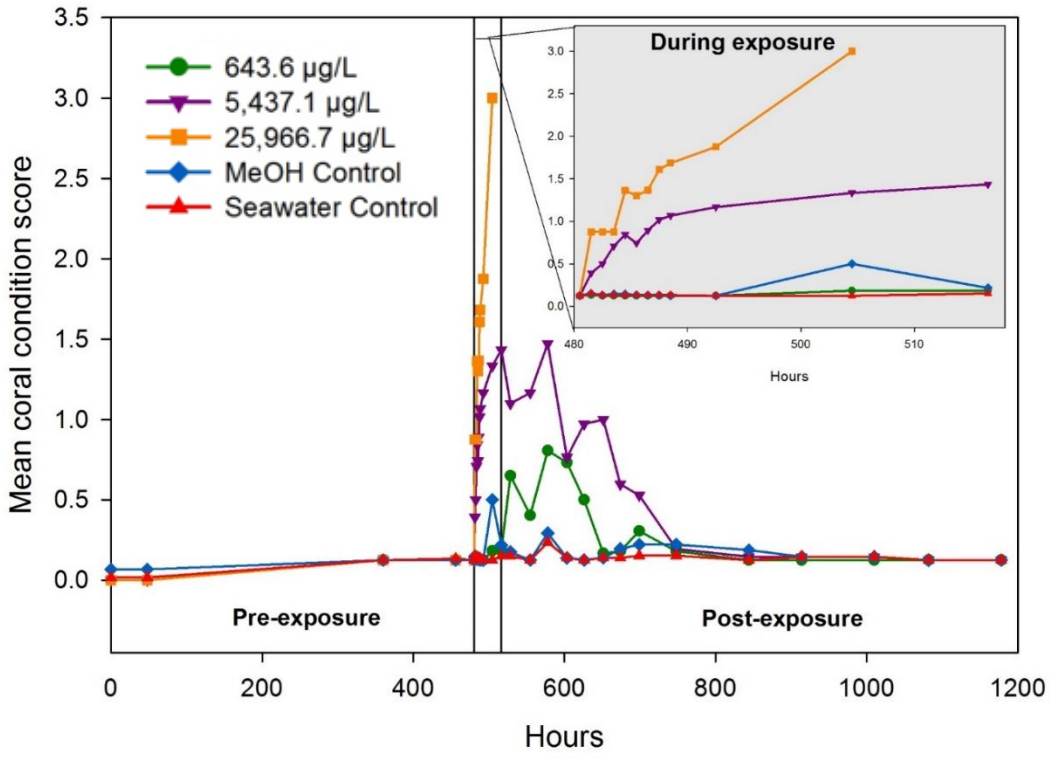

Figure 6. Mean coral condition score for each treatment during pre-exposure, exposure, and post-exposure time periods. Each point represents the treatment mean score of all surviving corals.

Photosynthetic Efficiency

Mean quantum yield was not significantly different among treatments at the end of the pre-exposure and exposure periods, or after 1 wk of recovery ($p>0.05$). However, mean quantum yield of the 5,437.1 µg/L corals was significantly higher than the controls from 1 d to 3 d post-exposure, and higher than the 643.6 µg/L corals from 1 d to 4 d post-exposure ($p<0.05$) (data not shown). After 1 wk of recovery, no significant differences among treatments were found ($p>0.05$).

Cellular and Tissue Changes

Cellular and tissue characteristics were adversely affected by exposure to 1-MN. Significant treatment effects were found in corals fixed immediately following the exposure ($F_{(3,8)}= 21.39$, $p=0.0003$). Post-hoc analysis indicated a significant increase in histological scores of 5,437.1 µg/L corals compared to SW controls, MeOH controls, and 643.6 µg/L corals ($p= 0.00033, 0.0016, 0.0022$ respectively). As concentrations increased, polypal architecture became severely compromised or completely lost, with degeneration of tentacles, hypertrophy of mucocytes, and increases in pigmented granular amoebocyte density at higher concentrations (Fig. 7A, C, E, & G). The coenenchyme also lost normal cellular architecture at higher concentrations; columnar epidermal cells became more squamous and cells fragmented, mucocytes atrophied and lysed, and zooxanthellae density in the gastrodermis decreased (Fig 7B, D, F & H). No significant differences were detected among the SW controls, MeOH controls, or 643.6 µg/L corals at any sampled time ($p>0.05$). After 1 wk of post-exposure recovery, no significant treatment effects were indicated in the surviving corals ($F_{(3,8)}= 2.503$, $p= 0.133$).

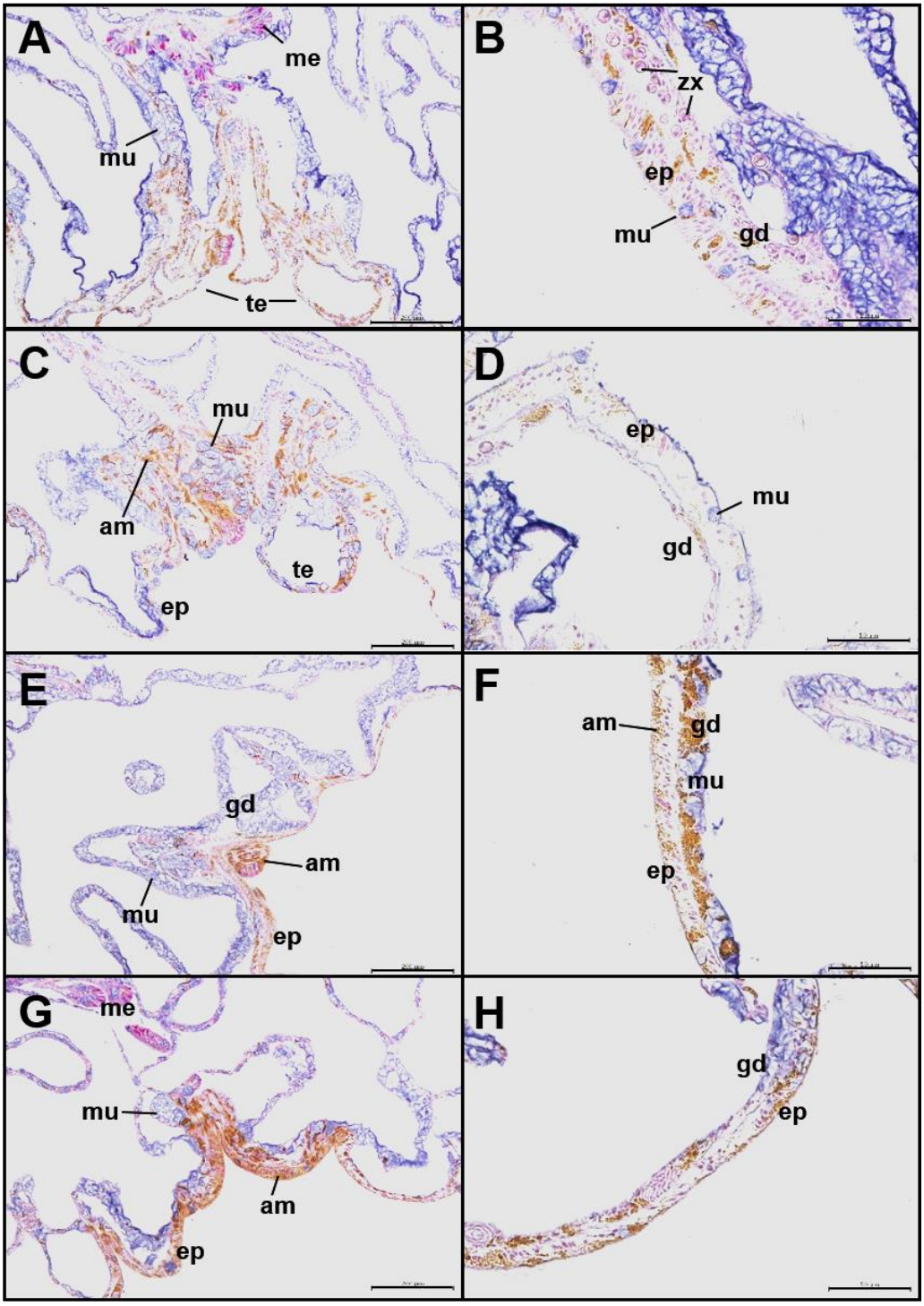

Figure 7. Histological sections of *Porites divaricata.* SW Control polyp (A) and coenenchyme (B); MeOH Control polyp (C) and coenenchyme (D); 643.6 µg/L polyp (E) and coenenchyme (F); and 5,437.1 µg/L polyp (G) and coenenchyme (H). Scale bars are 200 µm for polyp and 50 µm for coenenchyme. me= mesenteries, mu= mucocytes, te= tentacle, zx= zooxanthellae, ep= epidermis, gd= gastrodermis, am= pigmented granular amoebocyte.

Mesenteries were also adversely affected by exposure to 1-MN. Mesenterial architecture was severely compromised and degraded at higher concentrations of 1-MN. The gastrodermal wall and cnidoglandular band of the mesenteries contained hypertrophied mucocytes and pycnotic nuclei in the 5,437.1 µg/L exposed corals, indicating the presence of necrotic cells (Fig. 8).

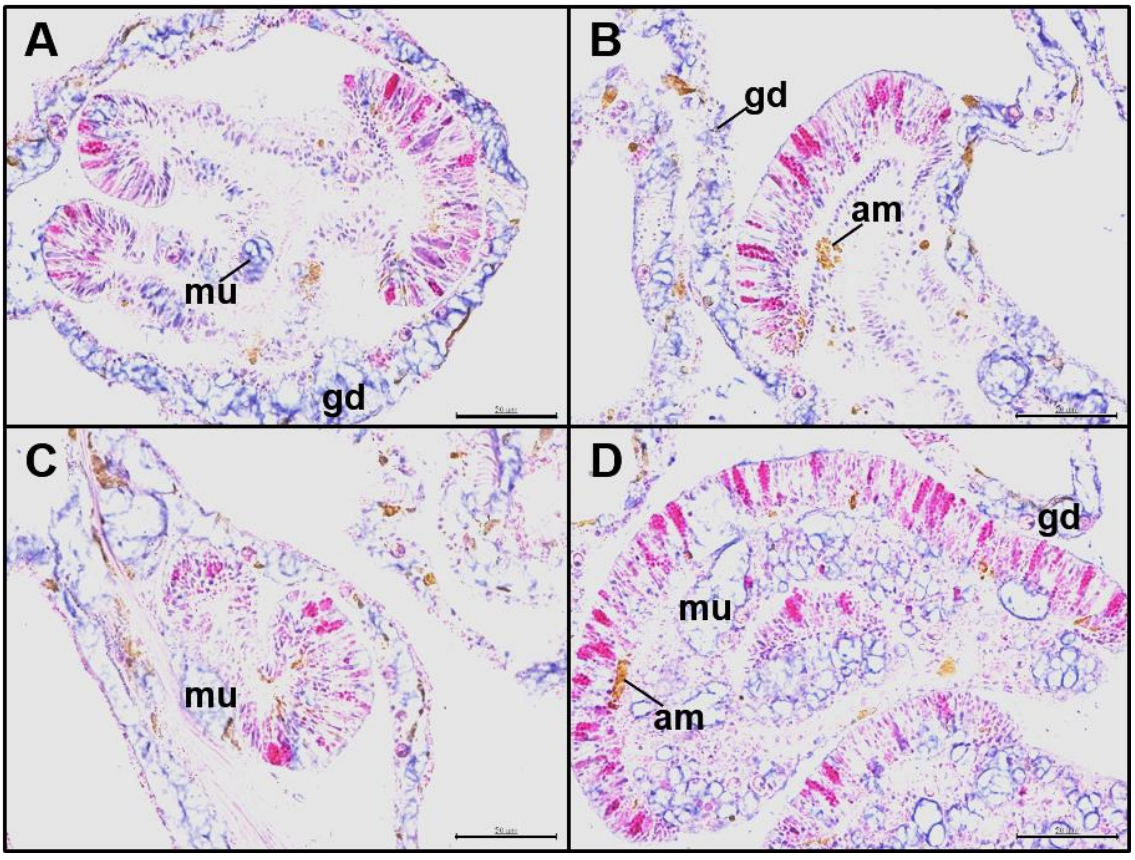

Figure 8. Mesenteries of *Porites divaricata* following the range-finding exposure to 1-MN. SW Control (A), MeOH Control (B), 643.6 µg/L (C), 5,437.1 µg/L (D). Scale bars= 50 µm. gd= gastrodermis, am= granular amoebocyte, mu= mucocytes.

RESULTS FROM EXPERIMENT 1

Hydrocarbon chemistry and water quality

Water quality results from the range-finding study indicated a depletion of dissolved oxygen levels during the exposure. To alleviate this, all dosing vessels were topped off with nitrox gas (34% oxygen), as opposed to normal air.

No significant differences in any tested parameter were found at the beginning of the experiment ($p>0.05$). Treatment effects were present at the end of the exposure ($p<0.05$); post-hoc analysis indicated significant increases in NO_2 and NO_3 in the 16,000 µg/L treatment compared to the 1,000 µg/L treatment ($p=0.044$ and $p=0.043$, respectively). A significant decrease in pH was found in the 16,000 µg/L treatment compared to the seawater control ($p=0.037$) and the 2,000 µg/L treatment ($p=0.032$). A significant decrease in DO was found in the 16,000 µg/L treatment compared to the 2,000 µg/L treatment ($p=0.006$). No significant differences in pH, alkalinity, PO_4, NH_3, NO_2, NO_3 or DO were found among the seawater control, 1,000 µg/L, 2,000 µg/L, 4,000 µg/L, and 8,000 µg/L treatments ($p>0.05$), and no difference in temperature was found among all treatments ($p>0.05$).

Concentration of 1-MN in each treatment was analyzed at the start and end of the exposure (Table 6). The measured concentrations of 1-MN were found to be inconsistent with predicted concentrations, particularly in the higher concentrations tested. Based on coral response observed in the range-finding experiment (i.e., 100% mortality of corals in the "16,000 µg/L" treatment, and partial mortality of corals in the "8,000 µg/L" treatment), it is unlikely that the measured concentrations were accurate. For this reason, the nominal concentrations were used.

Table 6. Measured concentrations (mean ± SD) of 1-MN (µg/L) for each treatment replicate of Exp. 1.

Treatment	0 h	48 h	Mean	%Loss/Gain	Mean
SW Control	0.0	0.0	0.0	-	1.8 ± 1.8
SW Control	0.0	0.0	0.0	-	
SW Control	0.8	6.4	3.6	+661.5	
SW Control	1.0	6.5	1.1	+553.6	
1,000	1,053.2	1,046.4	1,049.8±3.4	-0.6	1,002.4 ± 67.1
1,000	975.4	818.8	897.1±78.3	-16.1	
1,000	1,024.8	1,115.2	1,070.0±45.2	+8.8	
1,000	1,036.8	948.6	992.7± 44.1	-8.5	
2,000	1,925.0	1,882.7	1,903.9±21.1	-2.2	1,853.8 ± 59.6
2,000	1,706.1	1,830.3	1,768.2±62.1	+7.3	
2,000	1,698.6	1,957.8	1,828.2±129.6	+15.3	
2,000	1,881.4	1,948.2	1,914.8±33.4	+3.6	
4,000	3,461.8	3,806.3	3,634.1±172.3	+9.9	3,389.2 ± 595.7
4,000	3,651.3	1,074.3	2,362.8±1,288.5	-70.6	
4,000	3,721.1	3,871.4	3,796.2±75.2	+4.0	
4,000	3,734.7	3,792.7	3,763.7±29.0	+1.6	
8,000	1,766.7	1,952.6	1,859.7±92.9	+10.5	2,723.7 ± 1,612.1
8,000	1,807.5	NA	1,807.5	NA	
8,000	1,747.1	1,679.2	1,713.2±34.0	-3.9	
8,000	5,958.9	5,070.0	5,514.4±444.4	-14.9	
16,000	2,511.7	3,013.8	2,762.8±251.0	+19.9	3,671.3 ± 1,729.9
16,000	2,321.3	2,232.1	2,276.7±44.6	-3.8	
16,000	3,274.4	2,752.5	3,013.5±260.9	-15.9	
16,000	9,779.2	3,485.7	6,632.4±3,146.7	-64.4	

Coral Condition

Coral condition was scored during pre-exposure, exposure, and post-exposure periods using criteria outlined in Table 4. Mean scores for each time interval are shown in Figure 9. Significant treatment effects were found at all intervals from 1 h after initiation of exposure to 19 d post-exposure ($p<0.05$). Multiple comparisons post-hoc analysis found scores from 16,000 µg/L corals to be significantly higher than the control ($p=0.044$) and 1,000 µg/L corals ($p=0.013$) within 1 h after exposure initiation. At the end of the exposure period, the 8,000 µg/L corals scored significantly higher than the control ($p=0.025$) and 1,000 µg/L corals ($p=0.037$); the 16,000 µg/L treatment corals were no longer scored due to complete mortality. The 1,000 µg/L and 2,000 µg/L treated corals were not significantly different from the controls at any point ($p<0.05$). The 8,000 µg/L corals did not recover (i.e., did not score similarly to the controls) for 19 d, after which no treatment effects on coral condition were observed ($p>0.05$).

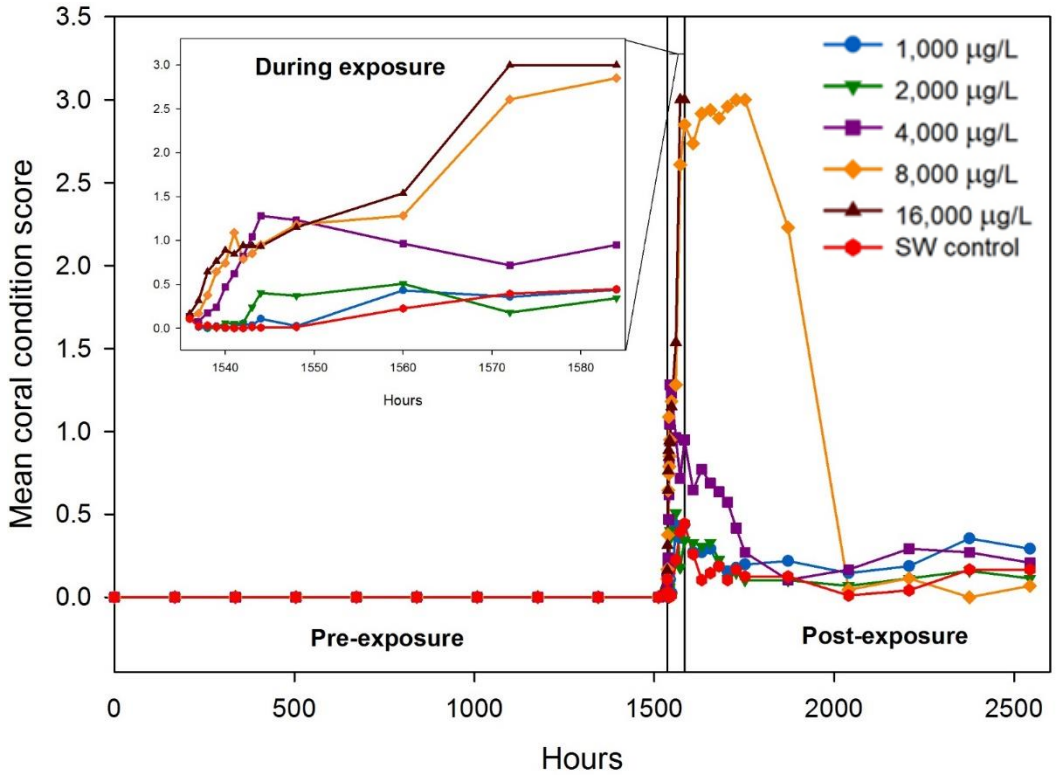

Figure 9. ***Porites divaricata.*** **Mean coral condition scores during pre-exposure, exposure, and post-exposure periods of Exp. 1. Each point represents the treatment mean score of all surviving corals.**

Photosynthetic Efficiency

Mean effective quantum yield (ΔF/Fm) for each treatment over time is shown in Figure 10. A post-exposure decline is observed for all treatments, similar to the range-finding experiment. Mean quantum yield was compared between treatments at each time interval (one-way ANOVA), and significant treatment effects were found at the end of the exposure period (p=0.0001) and after 24 h of recovery (p=0.001). Post-hoc analysis (Tukey's HSD) of the treated corals indicated that photosynthetic efficiency in the 8,000 µg/L treated corals was significantly less compared to all other treatments at the end of the exposure period and after 24 h of recovery ($p<0.05$). After 48 h of recovery, 8,000 µg/L corals were not significantly different than other treatments ($p>0.05$). Corals in the 16,000 µg/L treatment were not measured post exposure due to complete mortality.

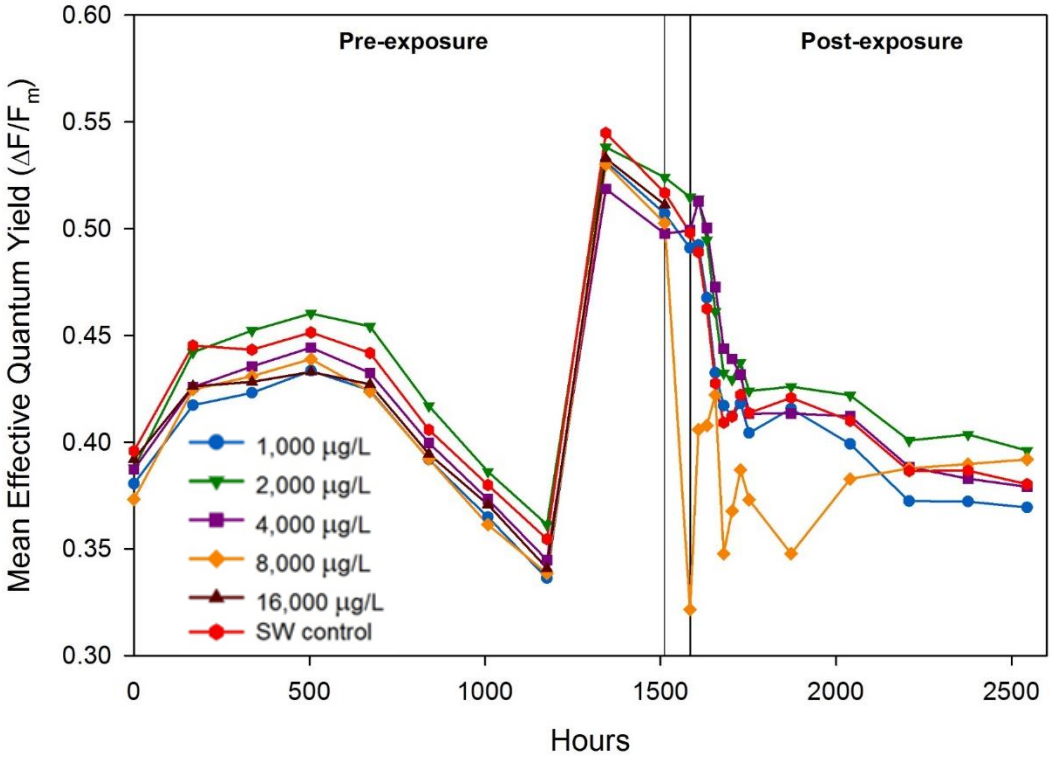

Figure 10. ***Porites divaricata.*** **Mean effective quantum yield ($\Delta F/F_m$) during pre-exposure and post-exposure periods of Exp. 1. The two solid reference lines represent the 48-h exposure to 1-methylnaphthalene.**

Calcification

Change in buoyant wet weight of each coral fragment over each measurement period was normalized to the initial size of each coral fragment, as fragments with larger surface area have the ability to calcify over larger areas (Ferrier-Pages et al. 2000). Mean normalized growth rates expressed as percent per day (% d^{-1}), for each treatment during each exposure period are shown in Figure 11. While no significant treatment effects were found for each period (Kruskal-Wallis, $p>0.05$), a greater decrease in growth rate after the exposure period was observed in the 4,000 µg/L and 8,000 µg/L relative to the other treatments and the control. After the post-exposure period, growth rate in the 8,000 µg/L corals remained lower than the other treatments, although not significantly different.

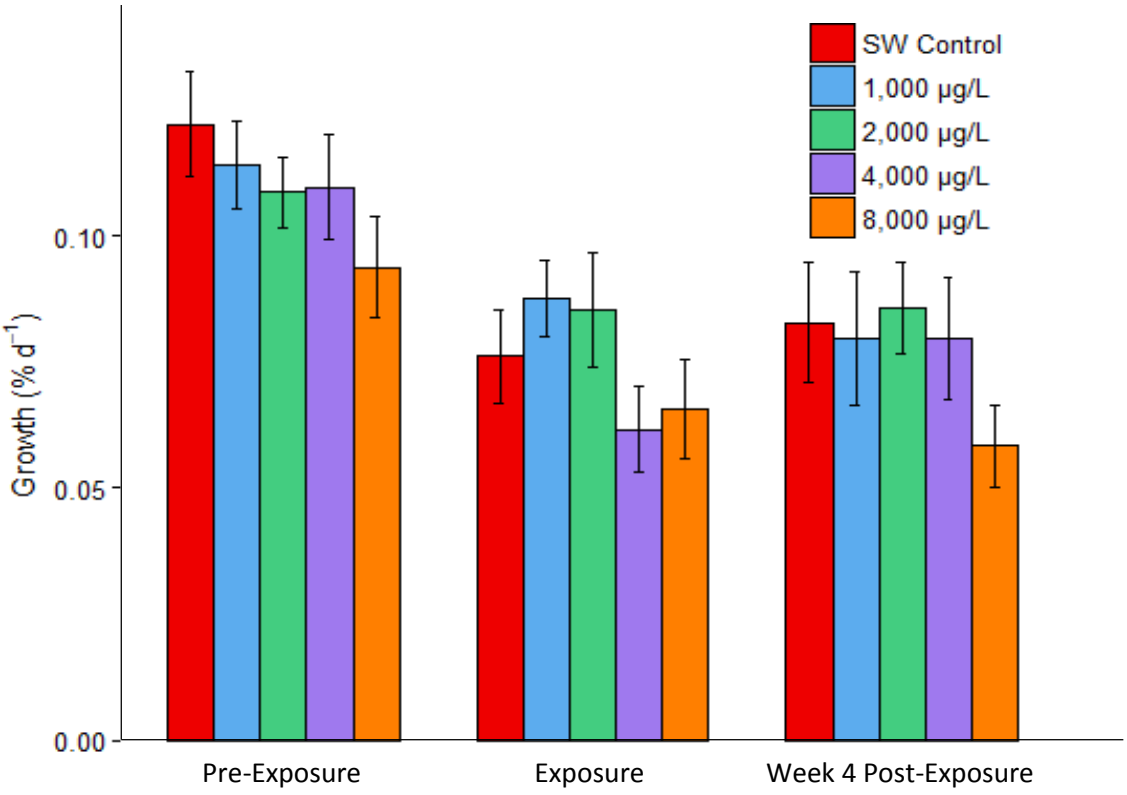

Figure 11. ***Porites divaricata.*** **Mean (±SE) normalized growth rate of each treatment during each time period of Exp. 1, expressed as percent change per day (% d^{-1}).**

Mortality

Each coral fragment was visually assessed for the presence of lesions, and a percent mortality was assigned. Mean mortality percentages for each treatment at each time interval are shown in Figure 12. 100% mortality occurred in the 16,000 µg/L treated corals by the end of the exposure period. Exposure to 8,000 µg/L resulted in partial coral mortality (mean= 16.5%, n=20), although these corals were able to partially recover during the post-exposure period.

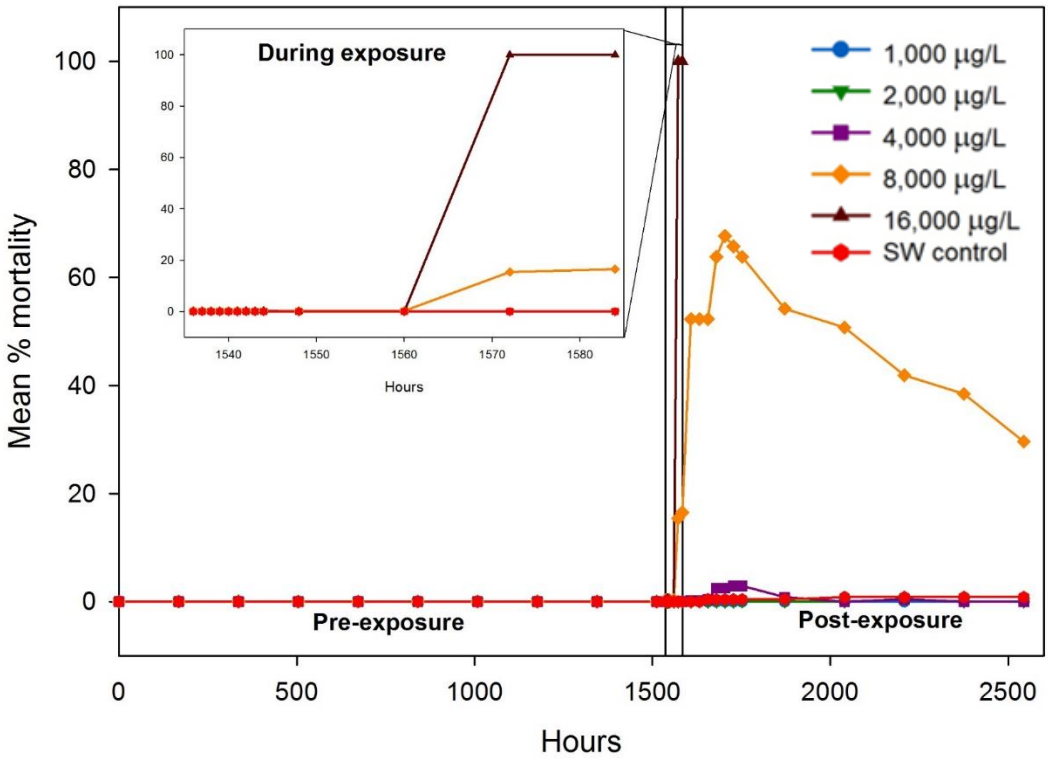

Figure 12. *Porites divaricata.* Mean percent mortality (full/partial) during pre-exposure, exposure, and post-exposure periods of Exp. 1.

EC_{50} and LC_{50}

The 48-h EC_{50} or LC_{50} could not be calculated without valid 1-MN concentrations, as nominal concentrations are not suitable for this calculation. A best estimate is provided in the discussion. Comparison of results from this experiment to the range-finding test suggested the measured hydrocarbon concentrations were incorrect, and were likely closer to the intended nominal values. For example, 5,437.1 µg/L-exposed corals in the range-finding test were severely damaged, but lacked mortality after recovery. In contrast, exposure to 16,000 µg/L (nominal, measured at less than 5,000 µg/L) in this experiment resulted in almost 100% mortality before completion of the 48-h exposure. This, along with the inconsistency between replicates of the same concentration, suggests the hydrocarbon chemistry was incorrect, and the experiment was therefore repeated.

RESULTS FROM EXPERIMENT 2

Hydrocarbon chemistry and water quality

Water quality of each chamber was analyzed for treatment effects on the parameters tested (temperature, pH, dissolved oxygen, alkalinity, phosphate, ammonia, nitrite, and nitrate) at the beginning and end of the experiment. Again, all dosing vessels were topped off with nitrox gas (34% oxygen), as opposed to normal air to limit oxygen depletion.

No significant differences in any tested parameter were found at the beginning of the experiment (Kruskal-Wallis ANOVA, $p>0.05$). Following the exposure, a significant decrease in DO in the 8,615.1 µg/L treatment compared to control ($p=0.001$) and 139.7 µg/L ($p=0.044$) treatments occurred. Alkalinity in the 5,412.5 µg/L and 8,615.1 µg/L treatments was significantly greater than controls after the exposure ($p=0.034$ and 0.001) No significant differences ($p>0.05$) in pH, PO_4, NH_3, NO_2, NO_3 or DO were found among the seawater control, 139.7 µg/L, 1,140.8 µg/L, 2,810.3 µg/L, and 5,412.5 µg/L treatments, and no difference in temperature was found among all treatments ($p>0.05$).

Concentration of 1-MN in each treatment replicate was analyzed at the beginning and end of the exposure. Table 7 summarizes the mean concentration measured for each treatment over the 48-h exposure.

Table 7. Measured concentrations (mean ±SD) of 1-methylnaphthalene (µg/L) for each treatment replicate of Exp. 2.

Treatment	T0 h	T48 h	Mean	Loss/Gain (%)	Treatment Mean
SW Control	0	0	0	0	0
SW Control	0	0	0	0	
SW Control	0	0	0	0	
SW Control	0	0	0	0	
1,000	116.1	108.8	112.5 ± 3.6	-6.3	139.7 ± 17.4
1,000	207.7	87.8	147.7 ± 60	-57.7	
1,000	106.4	171.4	138.9 ± 32.5	+61.0	
1,000	167.2	152.4	159.8 ± 7.4	-8.9	
2,000	1,105.0	1,125	1,115.2 ± 10.2	+1.8	1,140.8 ± 21.1
2,000	1,130.5	1,119	1,124.9 ± 5.6	-1.0	
2,000	1,159.2	1,160	1,159.6 ± 0.4	+0.1	
2,000	1,190.8	1,136	1,163.6 ± 27.2	-4.6	
4,000	3,047.3	2,413	2,730.3 ± 316.9	-20.8	2,810.3 ± 93.5
4,000	2,821.6	2,672	2,746.7 ± 75.0	-5.3	
4,000	2,902.4	2,693	2,797.9 ± 104.6	-7.2	
4,000	3,039.5	2,893	2,966.5 ± 73.0	-4.8	
8,000	5,233.5	5,227	5,230.3 ± 3.2	-0.1	5,412.5 ± 169.6
8,000	5,200.2	5,404	5,302.0 ± 101.8	+3.9	
8,000	5,400.8	5,485	5,443.0 ± 42.3	+1.6	
8,000	5,586.7	5,763	5,674.6 ± 87.9	+3.1	
16,000	8,217.5	8,215	8,216.3 ± 1.2	0.0	8,615.1 ± 277.3
16,000	8,488.0	8,513	8,500.7 ± 12.7	+0.3	
16,000	8,850.8	8,805	8,827.7 ± 23.1	-0.5	
16,000	8,878.0	8,953	8,915.7 ± 37.7	+0.8	

The low variation among treatment replicates suggests accurate measurement of test concentrations, which remained stable throughout the duration of the exposure. All treatments returned concentrations less than calculated target concentrations. This is presumably due to an incorrect partition coefficient from PDMS to water ($K_{PDMS\text{-}Water}$), as the partition coefficient utilized was determined for freshwater (Butler 2013). The means for all treatment replicates were used for overall treatment concentrations.

Coral Condition

Physical coral response is shown in Figure 13. Overall, control corals exhibited normal behavior, with no mucus production and slight polyp retraction and tissue swelling toward the end of the 48-h exposure (Fig. 13A). Corals in the 139.7 µg/L and 1,140.8 µg/L treatments showed an initial response of mild polyp retraction, ending the exposure with moderate tissue swelling, and a qualitative delay in tactile response (Fig. 13B & C

respectively). Corals in the 2,810.3 µg/L treatment exhibited moderate polyp retraction and mild tissue swelling after 12 h, with mild mucus production and moderate lightening of color by the end of the 48-h exposure (Fig. 13D). The 5,412.5 µg/L exposed corals had severe polyp retraction, moderate tissue swelling and mucus production after 12 h, with severe lightening of coloration after 48 h (Fig. 13E). The corals exposed to 8,615.1 µg/L exhibited severe polyp retraction within 2 h of exposure, with severe lightening of coloration (bleaching) and tissue recession and sloughing occurring after 48 h (Fig. 13F).

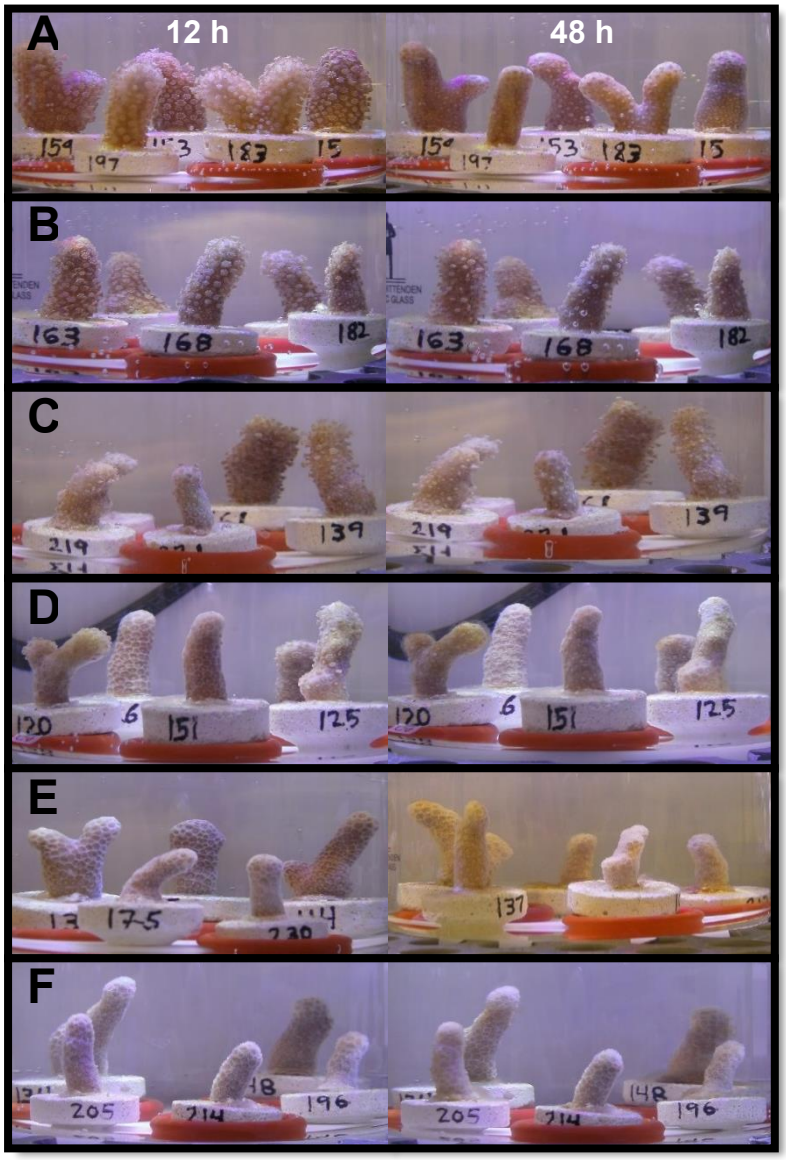

Figure 13. *Porites divaricata.* Physical coral response at 12 h and 48 h of exposure to 1-MN. SW control (A), 139.7 µg/L (B), 1,140.8 µg/L (C), 2,810.3 µg/L (D), 5,412.5 µg/L (E), 8,615.1 µg/L (F).

Coral condition was scored during pre-exposure, exposure, and post-exposure periods using criteria outlined in Table 4, and mean scores for each time interval are shown in Figure 14. Significant treatment effects were found at all intervals from 1 h after initiation of exposure to 1 wk post-exposure (Kruskal-Wallis ANOVA, $p<0.05$). Post-hoc analysis (multiple comparisons) found scores from 8,615.1 µg/L corals to be significantly higher than control corals from 2 h after initiation of exposure ($p=0.0043$) through 1 wk of recovery ($p<0.05$). From 2–36 h of the exposure, 8,615.1 µg/L corals also scored significantly higher than 139.7 µg/L corals ($p<0.05$). The 5.412.0 µg/L treated corals scored significantly higher than controls from 3 h after exposure initiation ($p=0.032$) through the end of the 48-h exposure ($p<0.05$). After one wk of recovery, no treatment effects were determined ($p>0.05$).

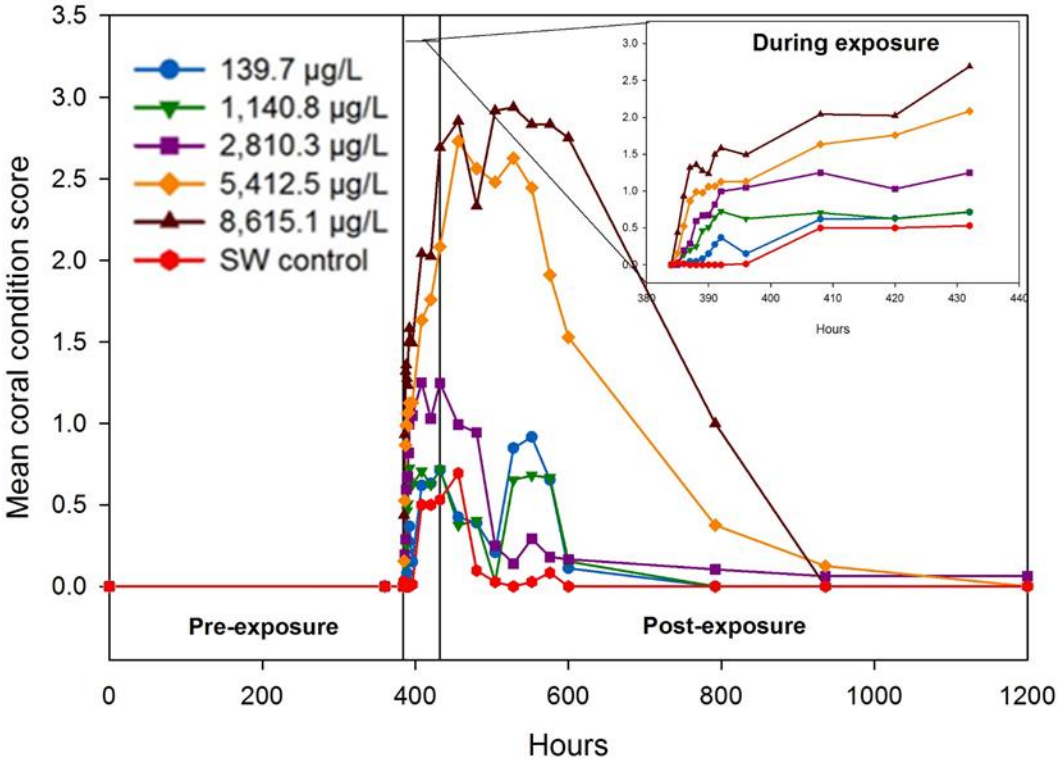

Figure 14. *Porites divaricata.* Mean coral condition scores during pre-exposure, exposure, and post-exposure time periods of Exp. 2. Each point represents the mean treatment score of all surviving corals.

Cellular and Tissue Changes

Exposure to 1-MN negatively affected the cellular and tissue condition of corals. Immediately following the exposure, normal cellular architecture was compromised in the highest treatment, leading to significant treatment effects (p=0.0006). Post-hoc analysis indicated a significant increase in scores for the 8,615.1 µg/L corals compared to both controls and 139.7 µg/L treatments (p=0.0043 for both) (Fig. 15A). The coenenchyme of 8,615.1 µg/L-treated corals incurred abundant fragmentation and lysing immediately following the exposure (Fig 16G). The surface body wall of the 8,615.1 µg/L exposed corals exhibited full thickness ablation; epidermal and gastrodermal cells were missing, and if present, they were lysed and necrotic. Abundance of pigmented granular amoebocytes also increased with increasing concentration, with lysing of these cells occurring at higher concentrations.

The 5,412.5 µg/L-treated corals exhibited many of the same responses as the high concentration, but were not significantly different than controls immediately following the exposure (p= 0.057) (Fig. 15A). The coenenchyme was fragmented and lysed, with necrosis present in most areas (Fig. 16E). Gastrodermis of the basal body wall was fragmented and necrotic, resulting in the gastrovascular cavity being filled with cell debris and mucus. Lower treatments, although not significant, had increased mucous secretion with some atrophy and necrosis of cells (Fig 16C).

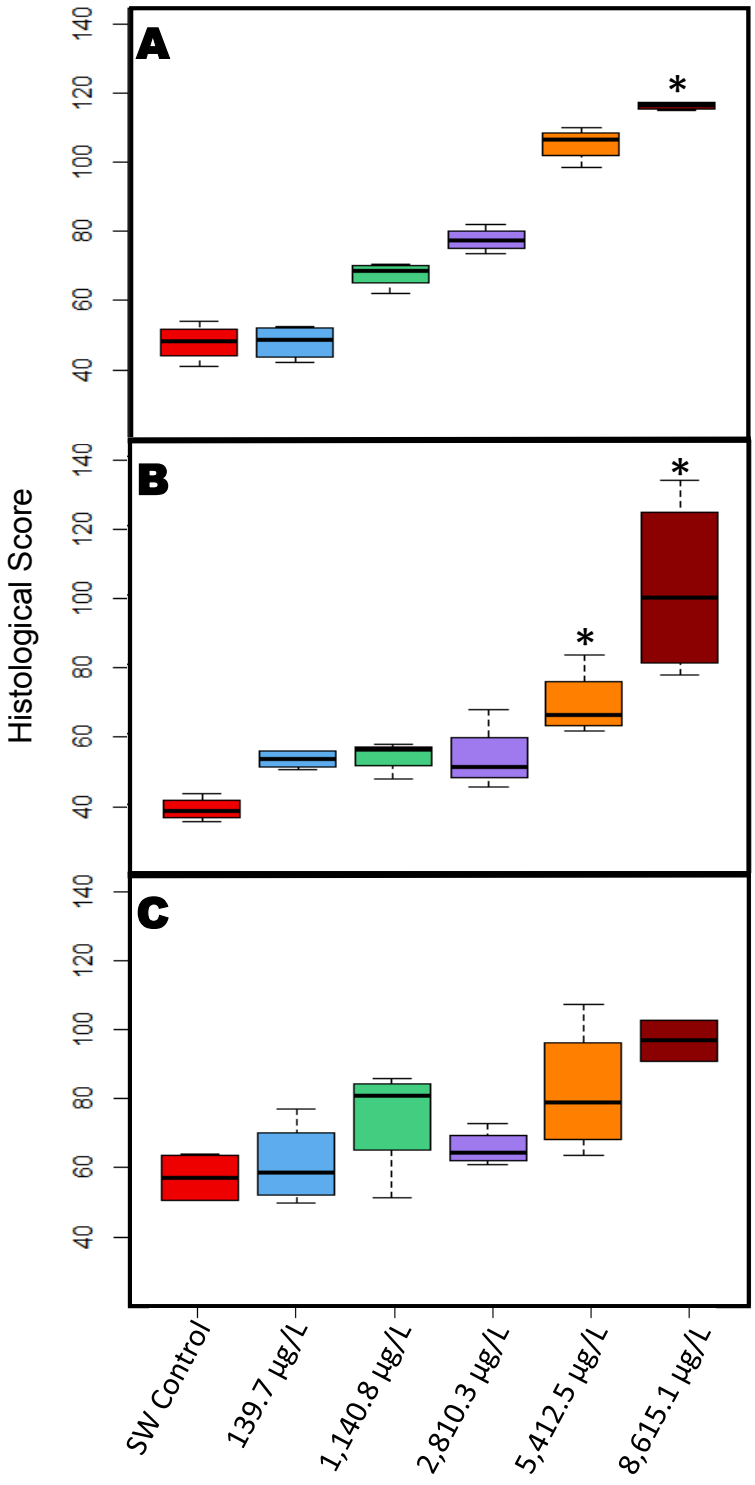

Figure 15. Boxplot of *Porites divaricata* histologic scores by treatment at 48 h (A), 1 wk post-exposure (B), and 4 wk post-exposure (C). * denotes statistical difference from control treatments.

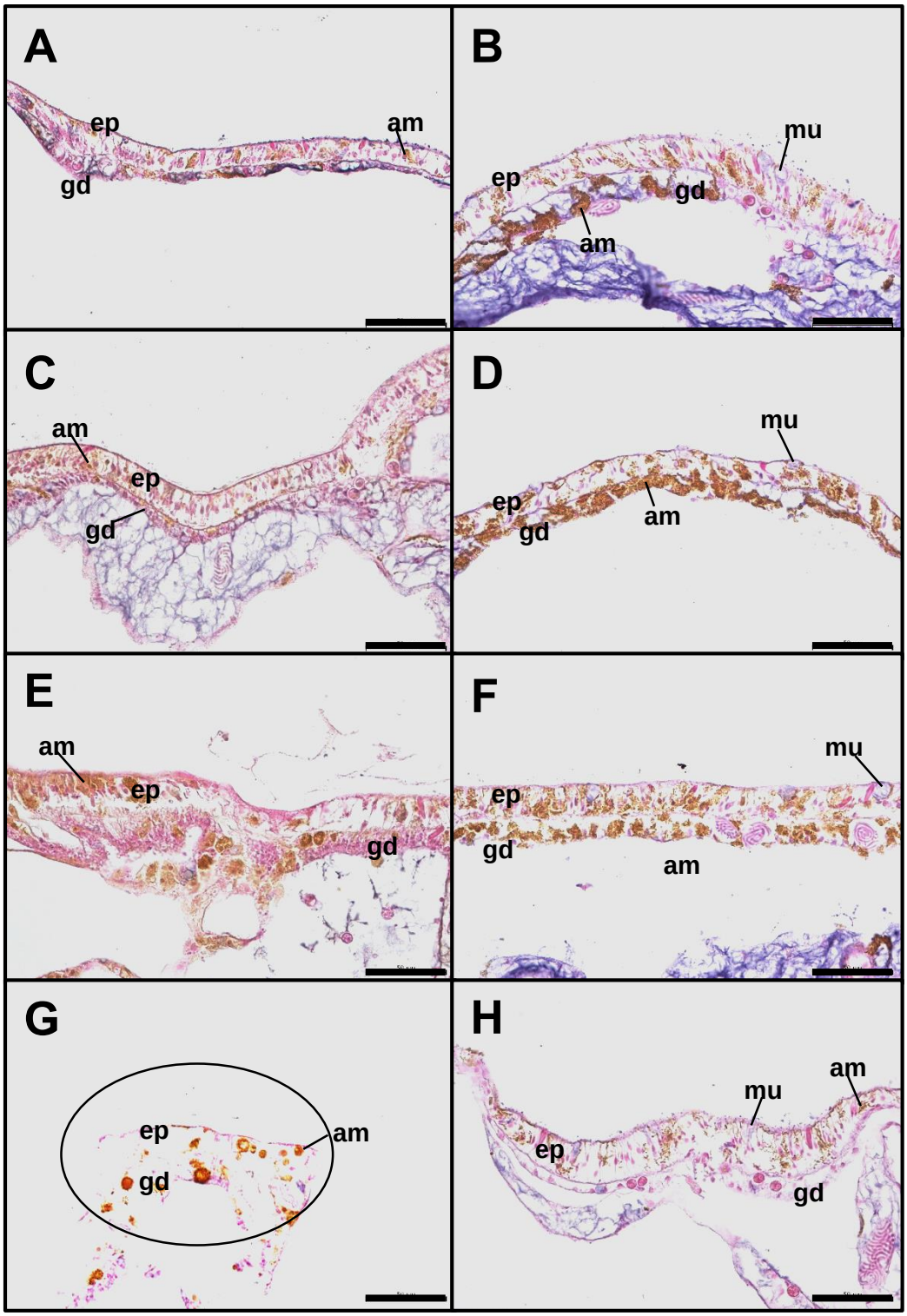

Figure 16. Histological sections of *Porites divaricata* coenenchyme at 40X magnification. SW Control 48 h (A) and 4 wk (B); 2,810.3 µg/L 48 h (C) and 4 wk (D); 5,412.5 µg/L 48 h (E) and 4 wk (F); 8,615.1 µg/L 48 h (G) and 4 wk (H). Circle surrounds area of fragmented and atrophied cells. Scale bars= 50 µm. mu= mucocyte, ep= epidermis, gd= gastrodermis, am= pigmented granular amoebocyte.

Similar to the range-finding exposure, polypal architecture was also compromised at higher concentrations (Fig. 17E and G). Control corals maintained normal polyp structure, with tentacles and the actinopharynx clearly visible (Fig. 17A). As concentrations increased, tentacles degraded and mucocytes in the basal portion of the polyps hypertrophied and eventually lysed (Fig. 17E and G). The 8,615.1 µg/L-exposed corals had no visible signs of polyp structure at the end of the exposure (Ellipse in Fig. 17G).

Treatment effects were also determined after 1 wk post-exposure ($p = 0.0018$) due to the significant differences between 8,615.1 µg/L and control treatments ($p = 0.0011$) and between 5,412.5 µg/L and control treatments ($p = 0.022$) (Fig. 15B). Surface body walls of both treatments exhibited abundant atrophy and fragmentation, but most margins of broken tissue contained acidophilic staining granules, indicating some form of tissue repair (FIG. 18). Again, the lower concentrations which remained statistically similar to controls ($p > 0.05$) exhibited hypertrophied mucocytes in the basal body wall and mesenteries with abundant mucus release.

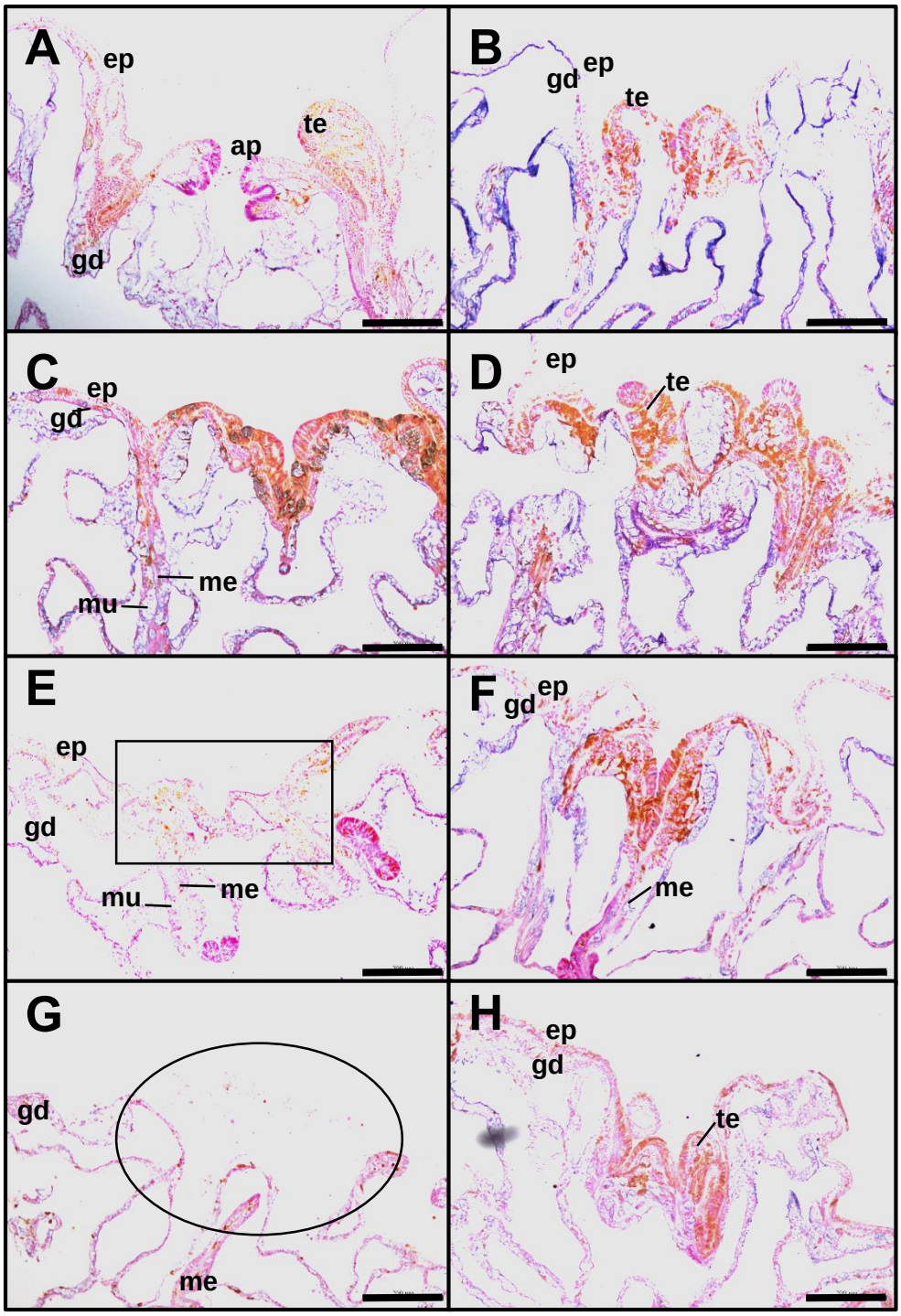

Figure 17. Histological sections of *Porites divaricata* polyps at 10X magnification. SW Control 48 h (A) and 4 wk (B); 2,810.3 µg/L 48 h (C) and 4 wk (D); 5,412.5 µg/L 48 h (E) and 4 wk (F); 8,615.1 µg/L 48 h (G) and 4 wk (H). Rectangle surrounds area of diminishing polyp structure. Ellipse surrounds area of missing polyp structure. Scale bars= 200 µm. mu= mucocytes, me= mesenteries, ep= epidermis, gd= gastrodermis, te= tentacle, ap= actinopharynx.

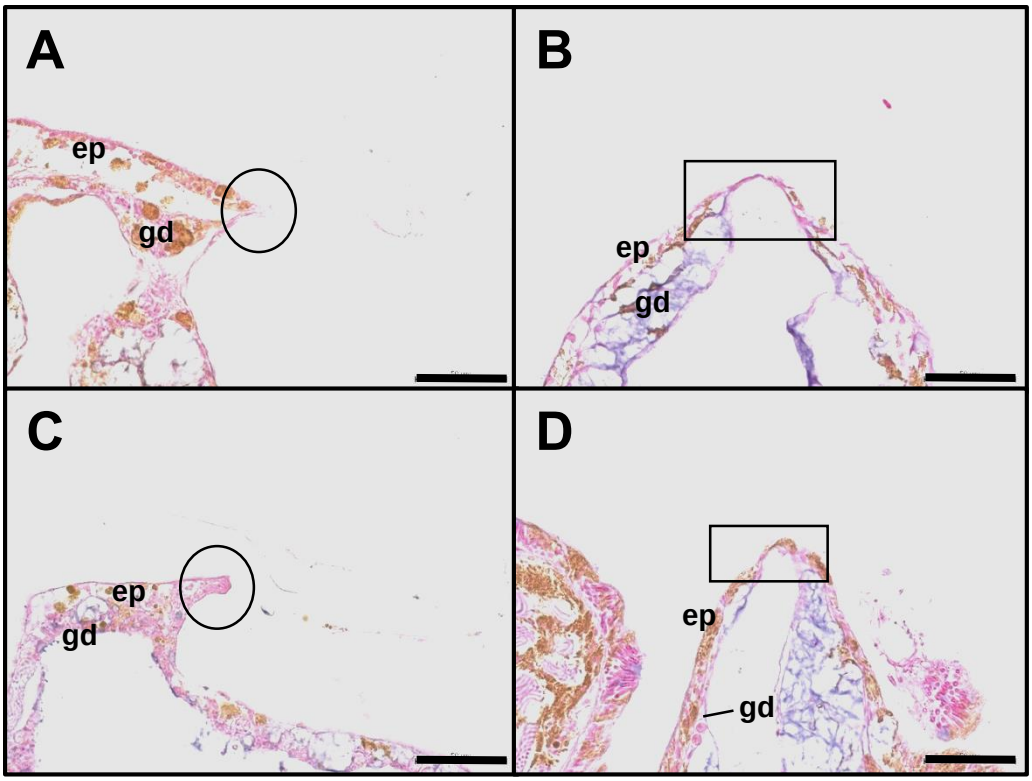

Figure 18. Histological sections of *Porites divaricata* coenenchyme at 40X magnification. 5,412.5 µg/L at 48 h (A) and 4 wk (B); 8,615.1 µg/L at 48 h (C), and 4 wk (D). Ellipses surround acidophilic staining at damaged margin. Rectangle surrounds newly formed epidermis. Scale bars= 50 µm. ep= epidermis, gd= gastrodermis.

Similar to the range-finding exposure, mesenteries were atrophied, necrotic, and infiltrated by mucocytes at higher concentrations (Fig. 19). Lower concentrations contained some mucocytes in mesenteries, but were not as abundant as 5,412.5 µg/L and 8,615.1 µg/L treatments. The mesenteries of the 5,412.5 µg/L and 8,615.1 µg/L treatments contained numerous pycnotic nuclei, with a degenerating and necrotic cnidoglandular band (ellipses of Fig. 19E and F).

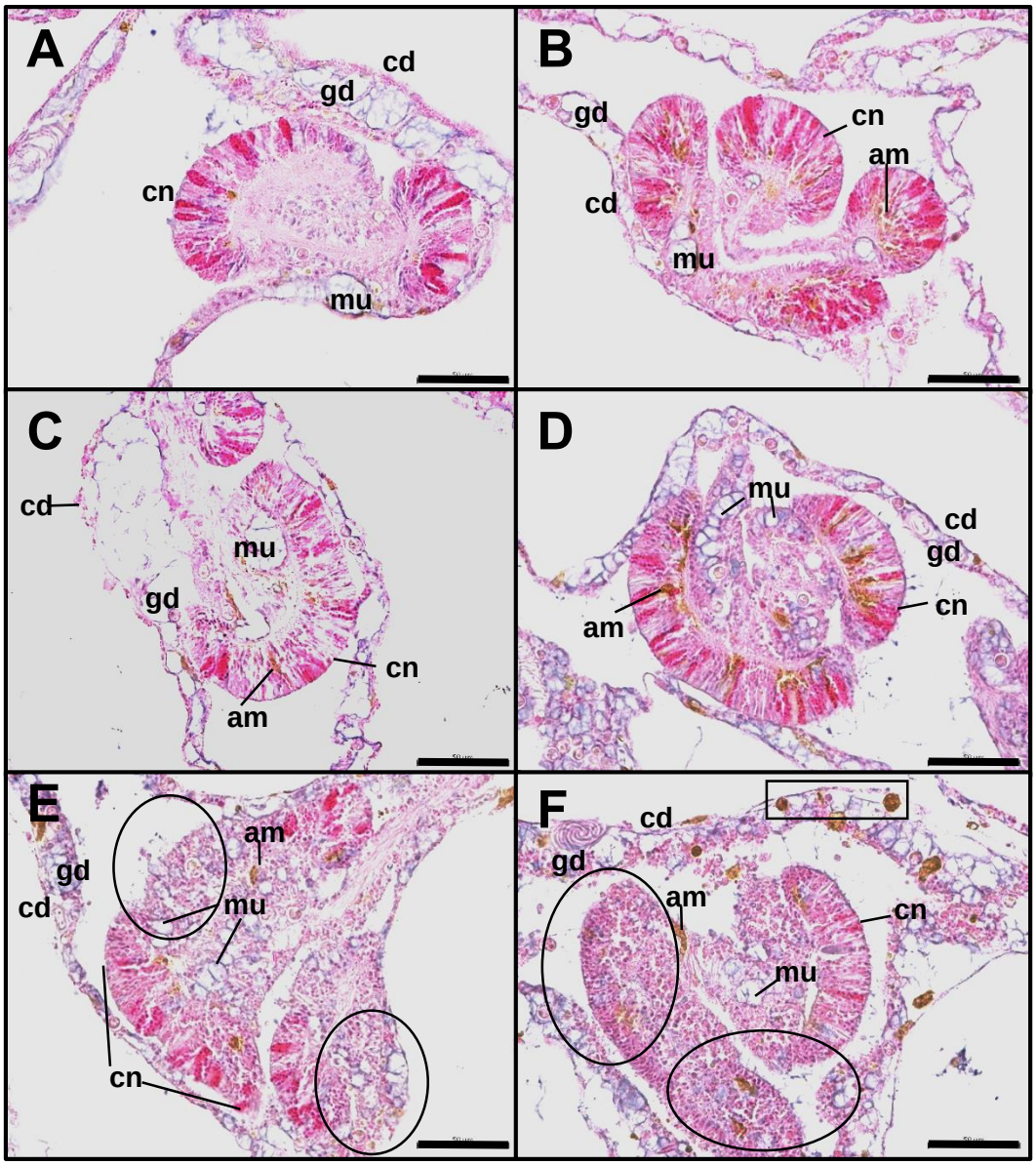

Figure 19. Histological sections of *Porites divaricata* mesenteries at 40X magnification after 48 h. A) SW Control, B) 139.7 µg/L, C) 1,140.8 µg/L D) 2,810.3 µg/L E) 5,412.5 µg/L F) 8,615.1 µg/L. Ellipses surround necrotic cells. Rectangle surrounds necrotic amoebocytes. Scale bars= 50 µm. mu= mucocytes, cn= cnidoglandular band, cd= calicodermis, gd= gastrodermis, am= pigmented granular amoebocyte.

Following 4 wk of post-exposure recovery, no significant treatment effects were determined (p= 0.056) (Fig 15C). Figures 16 and 17 (B, D, F, and H in both) show images at 4 wk of post-exposure recovery. The coenenchyme (Fig 16) of higher treatments contained fragmented cells and atrophied mucocytes, but lacked breaks and areas with no

epidermis. Epidermal cells were becoming more organized and columnar and polypal architecture was beginning to recover normal structure (Fig 17).

Photosynthetic Efficiency

Mean effective quantum yield (ΔF/Fm) for each treatment over time is shown in Figure 20, and illustrates a post-exposure decline for all treatments. Mean quantum yield was compared between treatments at each time interval, with significant treatment effects observable from one to five days post-exposure ($p<0.05$). Post-hoc analysis of the treated corals indicated significantly decreased photosynthetic efficiency in the 8,615.1 µg/L treated corals compared to all other treatments at the end of the exposure period ($p<0.05$), lasting for five days when the corals were no longer measurable due to algae overgrowth or mortality. Photosynthetic yield of the 5,412.5 µg/L treated corals was reduced following the exposure, but only significantly different than the 1,140.8 µg/L treatment immediately after exposure ($p=0.047$).

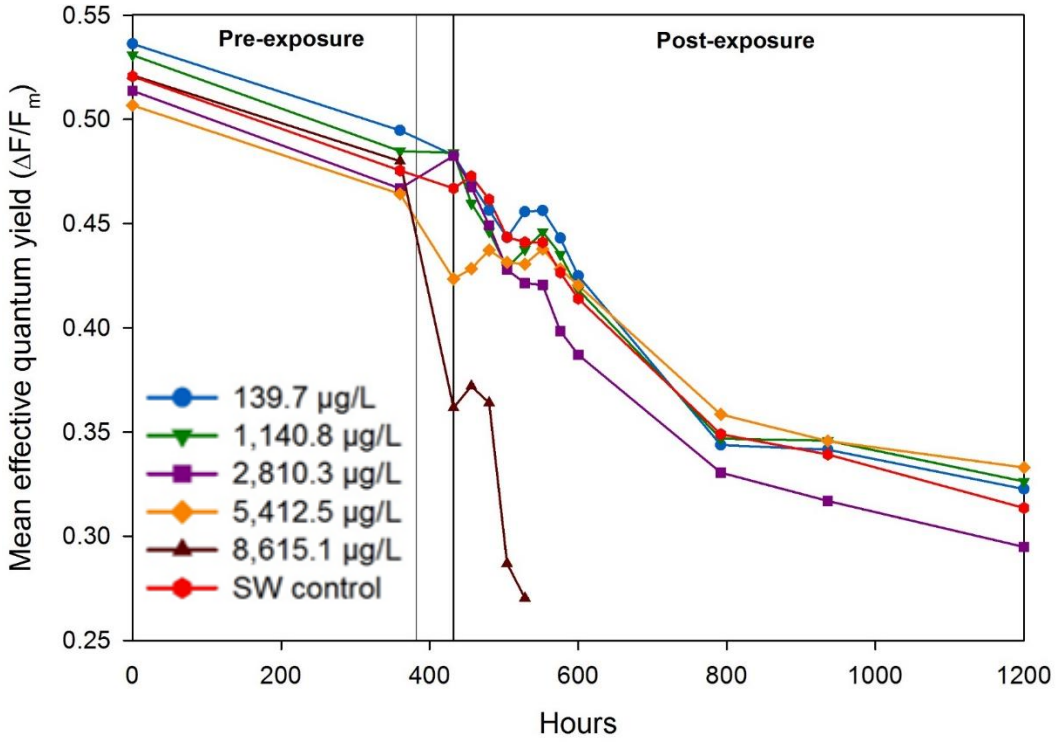

Figure 20. *Porites divaricata.* Mean effective quantum yield ($\Delta F/F_m$) during pre-exposure and post-exposure periods of Exp. 2. The two solid reference lines represent the beginning and end of the 48-h exposure to 1-methylnaphthalene.

Calcification

Mean normalized skeletal growth rates, expressed as percent change per day, for each treatment during each exposure period are shown in Figure 21. Significant treatment effects (one-way ANOVA) were found for the exposure (p=0.023) and 1 wk recovery (p=0.001) time periods. Post-hoc analysis (Tukey's HSD) revealed a significant decrease in growth of the 8,615.1 µg/L-treated corals compared to the 139.7 µg/L-treated corals during the exposure (p=0.028). After 1 wk of recovery, skeletal calcification of the 8,615.1 µg/L-treated corals was significantly less than controls (p=0.001), 139.7 µg/L (p=0.009), and 2,810.3 µg/L (p=0.035) treatments, concurrent with a significant decrease in calcification of the 5,412.5 µg/L corals compared to controls (p=0.007). By the end of recovery (32 d after exposure completion), no significant treatment effects were observable (p>0.05).

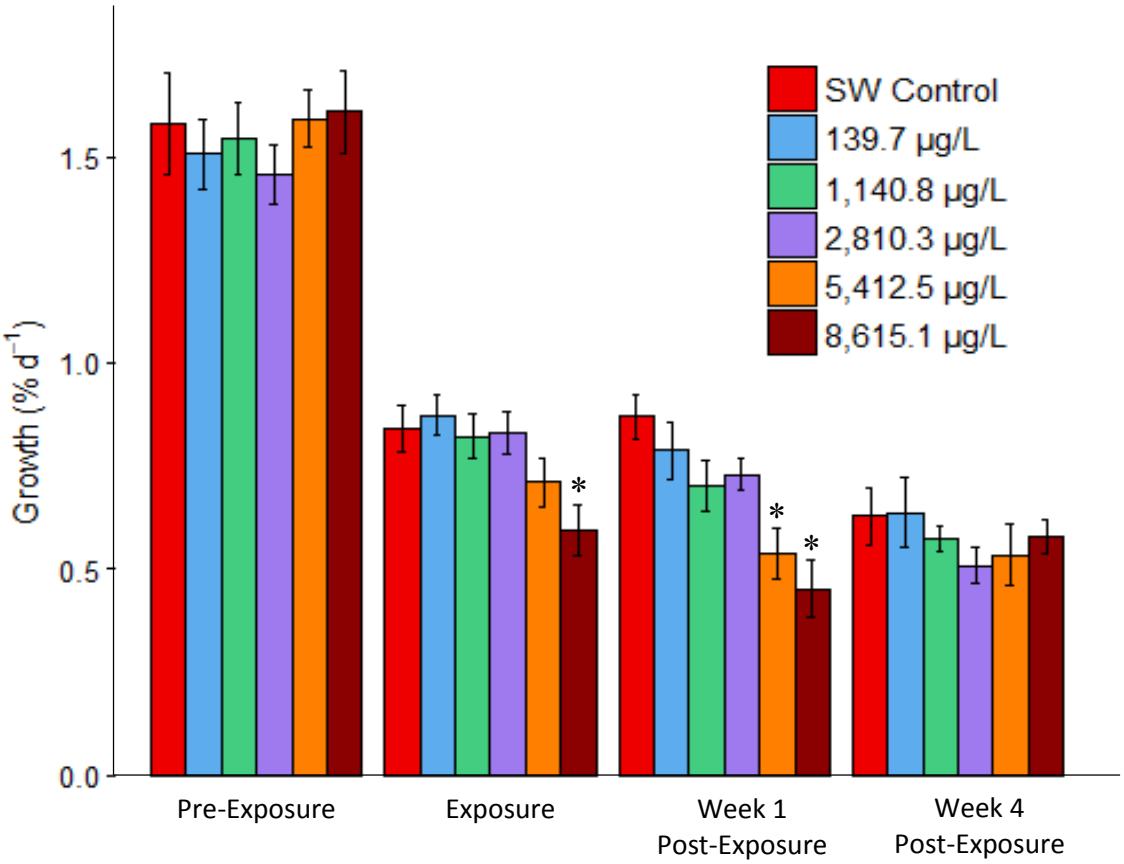

Figure 21. *Porites divaricata.* Mean normalized growth rate of each treatment during each time period of Exp. 2, expressed as percent change per day (% d^{-1})(±SE). (*) denotes significant difference from the control during that time period.

Mortality

Each coral fragment was visually assessed for the presence of lesions, and a percent mortality was assigned. Mean mortality percentages for each treatment at each time interval are shown in Figure 22. After the 48-h exposure, there was 86.7% mortality in the 8,615.1 µg/L-treated corals and 14.6% mortality in the 5,412.5 µg/L-treated corals with little to no recovery during the post-exposure period for both treatments.

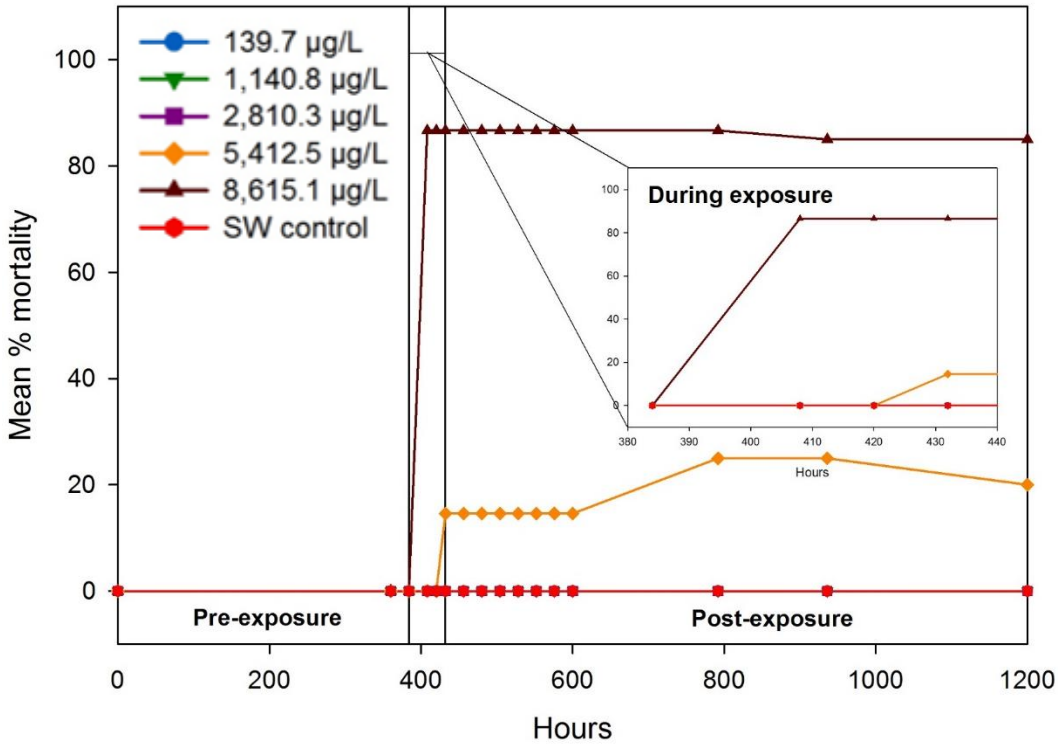

Figure 22. *Porites divaricata.* Mean percent mortality (full/partial) during pre-exposure, exposure, and post-exposure time periods of Exp. 2. Mean values are representative of all coral fragments in each treatment at that time point.

EC_{50} and LC_{50}

Based on the physical changes *P. divaricata* experienced after exposure to 1-MN, the 48-h EC_{50} was calculated using a variable slope, dose-response model in GraphPad Prism 6. A 48-h EC_{50} of 4,543 µg/L (95% CI: 3,071–6,547 µg/L) was determined by plotting mean coral condition scores against the log of the concentration.

Exposure to 1-MN also caused mortality in multiple concentrations after 48 h, providing the data necessary to calculate the concentration lethal to 50% of the population (LC_{50}). According to the United States EPA acute toxicity data analysis guidelines (USEPA 2002), the Spearman-Karber method for determination of LC_{50} was the appropriate test; this technique is utilized when there are concentrations that cause no mortality, at least one concentration that causes partial mortality, and at least one concentration causing 100% mortality. According to this technique, the 48-h LC_{50} for 1-MN to *P. divaricata* is 6,524 µg/L (95% CI: 5,659–7,500 µg/L).

DISCUSSION

Hydrocarbon Chemistry

One purpose of the range-finding exposure was to demonstrate the effectiveness of the passive dosing system at maintaining constant concentrations throughout the exposure duration. Almost all of the aqueous concentrations were in agreement with predicted values, with most concentrations higher than expected. Eliminating background concentrations found in the control treatments, the mean variation between expected values and achieved concentrations was 18.7% at time 0 h, and 15.5% for both time 24 h and 48 h. This variation is likely due to not rinsing the loaded O-rings prior to transferring them to the exposure system. It is assumed some particulate hydrocarbon adhered to the surface of the O-ring and added to the concentration in the seawater dosing system. Analyzing the consistency of concentration throughout the exposure revealed an average fluctuation of 2.2% for all treatments. Although the concentrations were elevated, the ability of the passive dosing system to maintain constant exposure concentrations regardless of loss was verified during the range-finding exposure.

Stability of treatment concentrations is a necessity for determining threshold concentrations in toxicological studies (McGrath et al. 2004, Bejarano et al. 2014). The passive-dosing system was again verified during Experiment (Exp.) 2, with an average fluctuation in 1-MN concentration over time of 1.7%. Failure to achieve target concentrations and inaccurate analytical chemistry results prevented determination of actual exposure concentrations during Exp. 1. Without actual concentrations, calculation of EC_{50} and LC_{50} was irrelevant; actual exposure concentrations were thus estimated for Exp. 1, using measured values from Exp. 2. In order to accomplish this, deviations from expected concentrations in Exp. 2 need examination.

Exp. 2 resulted in concentrations on average 47.4% lower than predicted. The experimental protocol was adjusted to include rinsing the O-rings to avoid transferring particulate hydrocarbon and subsequent increased concentrations as observed in the range-finding exposure. The consistency of treatment concentrations between replicates suggests the decrease in concentration was not an analytical error, but an error in calculation of the amount of 1-MN loaded in the O-rings. The partition coefficient responsible for this error is $K_{PDMS\text{-}Water}$, which represents the partitioning between the PDMS reservoir and seawater,

as it was determined for freshwater (Butler 2013). The partitioning of hydrophobic organic compounds, like 1-MN, depends on environmental factors, especially temperature (T) and salinity (S) (Jonker et al. 2015). Partitioning from the PDMS phase to seawater is reduced in response to decreased temperature and increased salinity. The decreased partitioning from the PDMS reservoir into the seawater is represented by an increase in $K_{PDMS\text{-}Water}$. Jonker et al. (2015) developed a method to calculate condition-specific PDMS-water partition coefficients [$K_{PDMS\text{-}Water}(T,S)$] by correcting values determined under standard conditions: $K_{PDMS\text{-}Water}$ (20°C, 0 ppt). Using this method, it was possible to estimate the partition coefficient for the environmental conditions used in this set of experiments (T=26°C, S=35 ppt).

The value for $\log K_{PDMS\text{-}Water}$ (20°C, 0 ppt) developed under standard conditions initially utilized in calculations was 2.98. After adjustment for environmental conditions using the equation by Jonker et al. (2015), $\log K_{PDMS\text{-}Water}$ (26°C, 35 ppt) was calculated at 3.19. The observed difference in $\log K_{PDMS\text{-}Water}$ is attributed to the increased salinity of seawater as temperature effects were very small. Table 8 shows the relationship between expected and measured concentrations for Exp. 2. The expected concentrations were determined using the amount of 1-MN from Table 2, while accounting for depletion in MeOH (5.89%) and PDMS (7.36%) stages.

Table 8. Expected and measured concentrations (µg/L) of 1-MN during Exp. 2.

Expected concentration using $K_{PDMS\text{-}Water}$(20°C, 0 ppt)	**Expected concentration using $K_{PDMS\text{-}Water}$ (26°C, 35 ppt)**	**Measured mean (±SD) treatment concentration (n=4)**
943	582	139.7 ± 17.4
1,886	1,164	1,140.8 ± 21.1
3,768	2,326	2,810.3 ± 93.5
7,534	4,650	5,412.5 ± 169.6
15,065	9,298	8,615.1 ± 277.3

The measured concentrations for Exp. 2 better aligned with the values calculated with the adjusted $K_{PDMS\text{-}Water}$ (26°C, 35 ppt). Using $K_{PDMS\text{-}Water}$ (20°C, 0 ppt), concentrations obtained varied by 47.4% from those estimated, while the adjusted $K_{PDMS\text{-}Water}$ (26°C, 35 ppt) resulted in a 24.5% average variation from intended concentrations. Although a deviation was still present, possibly due to insufficient equilibration in the

dosing system or inadequate rinsing prior to transfer into the experimental system, the difference was much less than the comparison with concentrations expected with $K_{PDMS\text{-}Water}$ (20°C, 0 ppt). Therefore, the adjusted $K_{PDMS\text{-}Water}$ (26°C, 35 ppt) was used in an effort to provide a better estimate of concentrations obtained in Exp. 1, using the amount of 1-MN in Table 2 and accounting for depletion. The estimated exposure concentrations for Exp. 1 are listed in Table 9, and are used to facilitate comparisons between experiments.

Table 9. Measured and estimated mean (± SD) treatment concentrations (µg/L) during Exp. 1 using $K_{PDMS\text{-}Water}$ (26°C, 35 ppt).

Treatment	Measured Concentration	Estimated Concentration
1,000	1,002.4 ± 67.1	581
2,000	1,853.8 ± 59.6	1,163
4,000	3,389.2 ± 595.7	2,326
8,000	2,723.7 ± 1,612.1	4,650
16,000	3,671.3 ± 1,729.9	9,298

Water Quality

Water quality was consistent across experiments. Elevated levels of nutrients were present in higher 1-MN treatments, likely due to tissue necrosis and mortality. Increases in nutrients examined (PO_4, NH_3, NO_2, NO_3) could be in response to waste products being released from the coral fragments or mortality in bacteria of the holobiont. Mucus from multiple species has been determined to contain high levels of lipids (Ducklow and Mitchell 1979b), and elevated phosphate levels may be linked to increased mucus production or degradation of cell membranes. Phosphate and nitrogenous waste compounds will accumulate over time in a closed system regardless of stress, but it is apparent that more waste was produced by corals and associated bacteria exposed to higher concentrations of 1-MN.

Effects of 1-Methylnaphthalene on Porites divaricata

The range-finding exposure was conducted to verify the utility of the passive dosing system and to determine a suitable range of five concentrations to use during full-toxicity exposures. This was achieved, and the effects of 1-MN on *P. divaricata* will be discussed

based on the response observed during the two full-toxicity experiments (Exp.1 and Exp. 2).

Coral condition. A comparison of coral condition scores between experiments provides evidence that the concentrations obtained in each full-toxicity experiment were similar. Both experiments resulted in treatment effects after only 1 h of exposure due to elevated scores in the high concentrations. Figures 8 and 13 reveal a similar pattern: corals exposed to concentrations above 5,000 µg/L showed an immediate response (polyp retraction and tissue swelling) with delayed recovery, if recovery occurred at all. The seawater controls and the three lowest concentrations in each experiment scored similarly throughout the exposure with only a mild observable effect. It is important to note the full recovery of coral condition scores for surviving fragments in the highest concentration of Exp. 2, which was absent in Exp. 1 due to complete mortality.

Although the toxic concentrations of 1-MN and oil are not comparable between studies, it is possible to compare the sublethal responses displayed by corals. Polyps of *Diploria strigosa* retracted following exposure to higher concentrations of the WAF of crude oil, with extreme tissue contraction and localized tissue rupture (Wyers et al. 1986). Increases in mucus secretion and swelling of tissues of *Manicina areolata* was also found following exposure to No. 2 fuel oil (Peters et al. 1981). These same effects were noted during this study, with higher concentrations above 5,000 µg/L 1-MN resulting in much greater effects.

Photosynthetic efficiency. Significant reduction in mean effective quantum yield ($\Delta F/Fm$) were found for the two highest treatments in both experiments. The second highest treatment concentration recovered to control levels within two days of recovery during Exp. 1 and within one day of recovery in Exp. 2. Corals in the highest concentration were not measured due to complete mortality in Exp. 1, and never recovered to normal levels in Exp. 2. Similar decreases in photosynthetic yield were found when *Acropora microphthalma* was exposed to 190 µg/L mineral derived lubricant (Mercurio et al. (2004).

The presence of pigmented amoebocytes within *P. divaricata* tissue potentially inhibits collection of reliable photosynthetic yield data because of their ability to absorb the fluorescence signal. It seems unlikely that differences among treatments were in response to zooxanthellae health or density, as histologic evaluation did not show any

significant changes to the algal cells. Perhaps photosynthetic efficiency measurements are not a reliable assessment metric for corals which contain these pigmented amoebocytes.

Calcification. Skeletal growth of the corals in each experiment varied slightly. Growth decreased in both experiments at higher concentrations, but significant decreases were only observable in Exp. 2 (Figures 10 and 15). Coral calcification depends on alkalinity, which was significantly higher in the 5,412.5 µg/L and 8,615.1 µg/L treatments compared to controls (Exp. 2), indicating a lack of calcification in these treatments. The aragonite tiles to which coral fragments were affixed in Exp. 2 were weighed and subtracted from the weight of the coral/tile combination at each time point, possibly resulting in higher resolution measurements and greater potential to reveal treatment effects. There was also a lack of 1 wk recovery measurements in Exp. 1. This time period revealed the most significant effects on growth during Exp. 2, which were not observed after 4 wk of recovery. Therefore, it is possible that the delayed effect on growth occurred in the both experiments, but the data were not collected until Exp. 2. Reduced calcification of *Millepora* spp. was found following exposure to 100 and 500 µg/L phenanthrene. Phenanthrene is considered more toxic than 1-MN, which explains the decreased growth rates at concentrations less than 10% of those used in this study.

Cellular Changes. The exposures resulted in significant treatment effects on the cellular and tissue structure of *P. divaricata* following exposure to 1-MN. Corals in control treatments maintained normal cellular architecture, with polyp structure remaining intact and mesenteries presumably healthy. Treatments lower than 5,000 µg/L 1-MN were not significantly different than controls at any time, however, there was a quantifiable effect on mucocytes. At lower concentrations, mucocytes in the polyps were hypertrophied, especially those lining the mesenteries and basal portions of the polyps. As concentrations increased, atrophy became apparent in epidermal mucocytes, and those found within the basal body wall gastrodermis were hypertrophied. Mucus release was abundant on the surface of the coral, as well as within the gastrovascular cavity. Corals produce mucus for a multitude of reasons, including heterotrophic feeding, sediment cleansing, or a defense against desiccation or environmental stressors, and researchers have found that up to 90% of ectodermal cells of some corals are mucocytes (Brown and Bythell 2005).

Crude oil exposure has been linked to increased mucus secretion (Mitchell and Chet 1975), and has caused hypertrophy and hyperplasia of mucous secretory cells (now termed mucocytes) in *Manicina areolata* (Peters et al. 1981). Neff and Anderson (1981) have suggested mucus may bind or absorb pollutants and act as an avenue for release, protecting the underlying coral tissue. This may be particularly true for type 1 narcotic chemicals, which act via nonpolar narcosis. The composition of mucus is temporally variable, as well as variable between species of coral, but has been shown to contain polysaccharides, proteins, and lipids (Brown and Bythell 2005). Exposure to 1-MN during this study may have been mitigated by the release of mucus after it had absorbed the pollutant.

Aside from mucocyte changes, exposure to higher concentrations of 1-MN (>5,000 µg/L) also resulted in surface body wall fragmentation, atrophy, and lysing of cells. The epidermis contained many ruptures over the skeletal ridges of the coenenchyme, presumably due to intense tissue retraction into the polyps, which was a very common response following exposure. Density of melanin-containing granular amoebocytes in the epidermis and polyps also increased, potentially indicating some form of response to cellular damage, as these cells have been previously categorized as part of the wound healing process in *Porites cylindrica* (Palmer et al. 2011).

The two highest treatments, 5,412 µg/L and 8,615 µg/L 1-MN resulted in considerable cellular and tissue damage. Polypal architecture was compromised or completely lost due to degradation of tentacles and epidermal tissues, with the mouth and actinopharynx rarely visible. Polyps contracted very tightly, causing tissue recession off of the skeletal ridges. The margins of tissue loss were dominated by acidophilic-staining cells, which have been suggested as part of the healing process of other corals following recent injury (Renegar 2015). Although not all of the 8,615 µg/L-exposed corals survived, the tissue structure of two fragments, which were severely compromised, showed evidence for recovery. Tissue layers initially lost as a result of the exposure began to reform. After 4 wk of post-exposure recovery, epithelial cells were intact, with polypal architecture returning.

Another noteworthy departure from normal cellular architecture was the effect on the mesenteries of exposed corals. The mesenteries of control corals maintained normal mesentery architecture with well-defined cnidoglandular bands. As concentrations increased, the number of mucocytes was greater in the mesenteries and cnidoglandular

bands, which is consistent with previous research using *M. areolata* (Peters et al. 1981). Accompanying this change was the degeneration of the cnidoglandular band itself. At high concentrations, atrophy was prevalent in the cnidoglandular band, with reductions in number of acidophilic granular gland cells.

Mortality. Mortality also showed similarities between experiments. The seawater controls and three lowest concentrations in both exposures resulted in no mortality throughout the exposure and recovery periods. The two highest concentrations in each experiment differed in percent mortality, but only slightly. The 4,650 µg/L treatment in Exp. 1, and the 5,412 µg/L treatment in Exp. 2 resulted in mortality in both experiments, averaging 16.6% and 14.6%, respectively. The highest concentrations tested, 9,298 µg/L (Exp. 1) and 8,615 µg/L (Exp. 2) resulted in 100% and 86.7% mortality, respectively. If the estimated concentrations for Exp. 1 are accurate, the difference in mortality can be attributed to the difference in concentration corals were exposed to, with the higher concentration resulting in higher mortality. Concentrations used in this research were not environmentally realistic, and release of petroleum into marine environments would typically not result in 1-MN concentrations of this magnitude. However, these elevated concentrations were necessary to obtain mortality at sufficient levels to calculate the threshold concentrations needed as inputs to the TLM.

EC_{50} and LC_{50}. The similarity in results for both experiments is further evidence that the concentrations were similar between the two separate exposures, and provides support for calculation of EC_{50} and LC_{50} for Exp. 1 using the estimated concentrations in Table 9. Using the variable slope dose-response model in GraphPad Prism 6, the EC_{50} for Exp. 1 was calculated at 3,446 µg/L (95% CI: 2.961-3.991 µg/L). This differs from the EC_{50} from Exp. 2 [4,543 µg/L (95% CI: 3,071-6,547 µg/L)] by more than 1,000 µg/L. The difference in EC_{50} may be related to the use of the semi-quantitative coral condition scores or estimated concentrations.

Using the estimated concentrations (Table 9), Exp. 1 resulted in a 48-h LC_{50} of 5,569 µg/L (95% CI: 4,629–6,667 µg/L) (Spearman-Karber). This is similar to the 48-h LC_{50} for Exp. 2, which was calculated at 6,524 µg/L (95% CI: 5,659-7,500 µg/L) using measured concentrations. The difference in LC_{50}s is likely due to the use of estimated concentrations for Exp. 1.

The EC_{50} determined in the range-finding experiment was 7,442 µg/L (95% CI: 4,905-11,290 µg/L). The EC_{50}s calculated from subsequent tests were both lower, with narrower 95% confidence intervals [Exp. 1 =3,446 µg/L (95% CI: 2.961-3.991 µg/L); Exp. 2 =4,543 µg/L (95% CI: 3,071-6,547 µg/L)], suggesting a more accurate estimate. The 48-h LC_{50} determined in the range-finding experiment was 12,123 µg/L. This was also higher compared to both full-toxicity experiments [Exp. 1 =5,569 µg/L (95% CI: 4,629–6,667 µg/L); Exp. 2 =6,524 µg/L (95% CI: 5,659-7,500 µg/L)]. Refinement of the test protocols and subsequent decrease in variability of concentrations in Exp. 1 and Exp. 2 resulted in more precise toxicity estimates for *P. divaricata*.

Comparative toxicity

The LC_{50} calculated from Exp. 2 was used to compare 1-MN toxicity for *P. divaricata* to other organisms. NOAA's Office of Response and Restoration has created the Chemical Aquatic Fate and Effects (CAFÉ) database to estimate the fate and effects of multiple chemicals, oils, and dispersants (NOAA/ERD 2015). This tool allows direct comparison of toxicological endpoints across different species. Figure 23 shows the distribution of LC_{50}s for 1-MN and other organisms using the CAFÉ database.

Although this model cannot fit a curve to the data without a minimum of five species, it provides the basis to compare toxicities. With the amount of current available 48-h LC_{50} data on 1-MN, it is apparent that *P. divaricata* ranks similarly, but may be more sensitive than other organisms tested. Other studies have been completed with other forms of naphthalene (parent and other alkylated derivatives), but comparing results and evaluating species sensitivity with those findings is cautioned, as differences in alkylation alter toxicity (Hawthorne et al. 2006, Achten and Andersson 2015). Due to a lack of directly comparable data for the toxicity of 1-MN to coral, estimates of species sensitivity were made using the TLM.

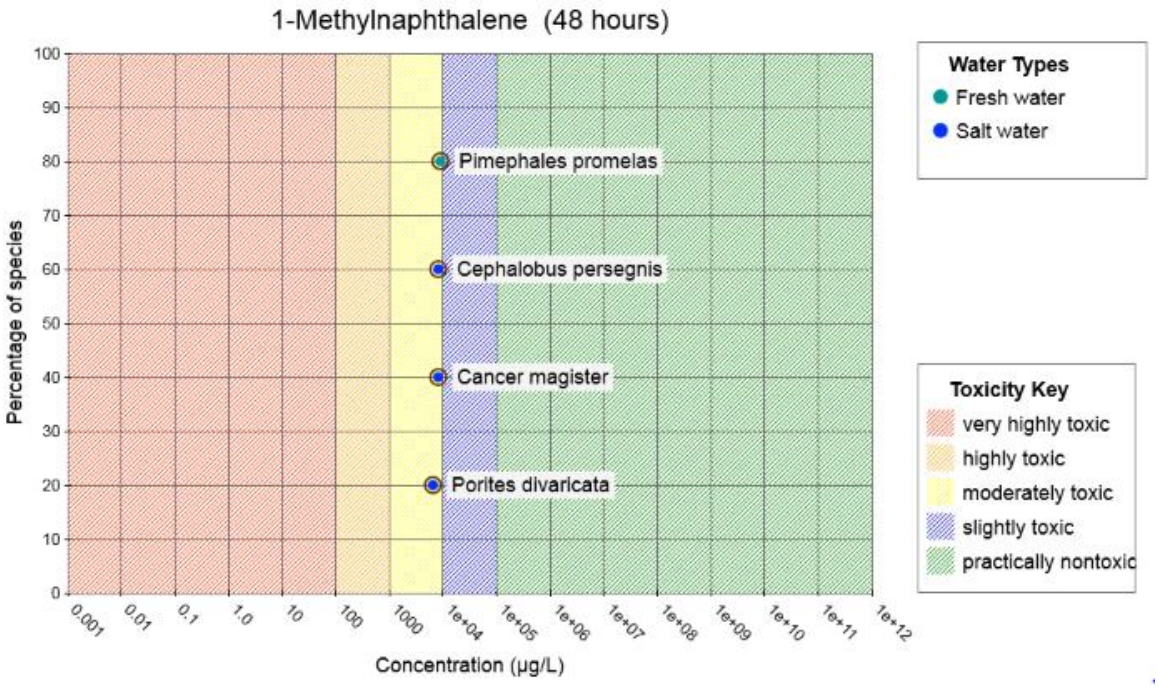

Figure 23. Distribution of LC_{50} values for 1-methylnaphthalene created using NOAAs CAFÉ model.

Application of the Target Lipid Model

Results of this study indicated a 48-h LC_{50} of 6,524 µg/L (95% CI: 5,659-7,500 µg/L) 1-methylnapthalene. The LC_{50} obtained was used to estimate a CTLBB of 355.7 µmol/ g lipid for *P. divaricata* following the TLM. Calculation of CTLBB is similar to a normalization procedure that corrects Type 1 narcotic chemicals with different K_{ow}s (Di Toro et al. 2000), allowing comparisons between species even if different chemicals were used. Due to a lack of comparable studies on the toxic effects of 1-MN, the calculated CTLBB can be used to compare sensitivity of *P. divaricata* to other species for which CTLBBs have been calculated. Table 10 shows a comparison of CTLBBs for saltwater species based on work by McGrath and Di Toro (2009).

Table 10. Comparisons of CTLBB (µmol/g lipid) from McGrath and Di Toro (2009). * = information from this study.

Species	Common Name	Habitat	CTLBB
Oncorhynchus gorbuscha	Pink Salmon	Water Column	24.5
Rhepoxyinus abronius	Amphipod	Infauna	31.2
Mysidopsis bahia	Mysid	Epibenthic	34.3
Eohaustorius estuarius	Amphipod	Infauna	41.4
Leptocheirus plumulosus	Amphipod	Infauna	43.1
Portunus pelagicus	Sand Crab	Epibenthic	53.3
Ampelisca abdita	Amphipod	Infauna	53.8
Palaemonetes pugio	Grass Shrimp	Epibenthic	57.3
Jordanella floridae	American Flagfish	Water Column	67.1
Cyprinodon variegatus	Sheepshead Minnow	Water Column	114
Oithona davisae	Copepod	Epibenthic	142
Meanthes arenaceodentata	Annelid Worm	Infauna	182
Artemia salina nauplii	Brine Shrimp	Water Column	194
Menidia beryllina	Inland Silverside	Water Column	292
*Porites divaricata**	Thin Finger Coral	Benthic	*355.7

From this information, it can be concluded that *P. divaricata* is less sensitive to type 1 narcotic chemical exposure compared to other organisms for which CTLBBs are available. This is possibly linked to the elevated levels of mucous secretion when corals are exposed to xenobiotics. The mucous secretion exhibited by corals may be protective, acting as a physical barrier or avenue of toxicant release (Neff and Anderson 1981). The increased resilience compared to other organisms disagrees with the initial comparisons made using NOAA's CAFÉ database. The organisms included in CAFÉ are not present in the CTLBB comparisons, thus additional data may result in the same order of resilience for the species being compared.

Calculation of the CTLBB also facilitated the prediction of the LC_{50} for *P. divaricata* for other narcotic chemicals found in petroleum using the TLM (Table 11). According to the TLM and calculation of CTLBBs, toxicity of petroleum mixtures is most related to lower molecular weight hydrocarbons, as the predicted LC50s are below the solubility of each chemical in seawater. Fluorene, phenanthrene, and fluoranthene would produce a toxic response, but only at concentrations above solubility. Although above

solubility, the predicted LC_{50} for fluoranthene aligns with an experimentally determined value of 435.1 µg/L for *P. divaricata* (Martinez et al. 2007). The TLM and calculation of the CTLBB for *P. divaricata* facilitated the comparison of toxic thresholds between studies that tested different chemicals, and provides the basis for evaluating the toxicity of complex hydrocarbon mixtures via the toxic unit approach.

Table 11. Predicted LC50s for low molecular weight MAHs and PAHs found in petroleum. Solubility in seawater was determined using the Setschenow Equation. LC_{50}*= predicted LC_{50} using the TLM. $Solubility_{SW}$= solubility at 35 ppt.

Class	Chemical	MW (g/mol)	$Solubility_{SW}$ (µg/L)	LC_{50}* (µg/L)	LC_{50}* <$Solubility_{SW}$
MAH	Benzene	78.11	1,515,221	329,374	Yes
MAH	Toluene	92.14	443,172	133,694	Yes
MAH	*o*-Xylene	106.17	145,082	51,544	Yes
MAH	Ethylbenzene	106.17	125,288	45,291	Yes
MAH	*p*-Xylene	106.17	160,929	42,091	Yes
PAH	Naphthalene	128.19	26,615	18,233	Yes
PAH	1-Methylnaphthalene	142.2	21,698	6,524	Yes
PAH	Fluorene	166.2	1,466	5,531	No
PAH	Phenanthrene	178.23	1,025	1,449	No
PAH	Fluoranthene	202.26	183	445	No

CONCLUSION

These experiments are the first in a series of tests that evaluate the toxicity of petrogenic hydrocarbons to corals. Due to the variety of bioassay conditions, and differences in species or toxicant utilized in previous research, comparisons across studies and extrapolation to actual spill scenarios has been difficult. Providing data as inputs to models that can predict toxicity of any petroleum compound is invaluable, and provides the necessary information spill responders require to act appropriately following an oil spill.

The range-finding exposure was used to refine the initial dosing and monitoring protocol, as well as verifying the effectiveness of the passive dosing technique. The full-toxicity exposures resulted in stable concentrations required for precise estimation of the EC_{50} and LC_{50} of 1-MN to *P. divaricata.* Effects monitored included physical and cellular changes, decreases in growth rate, and altered photosynthetic efficiency. These parameters were used to calculate the threshold concentrations required for the TLM to estimate a CTLBB of 355.7 µmol/g lipid, which indicates a greater resilience to type 1 narcotic chemicals for *P. divaricata* compared to other organisms. Although this is based on results of a single hydrocarbon, future work with other petroleum hydrocarbons will verify the precision of the estimated CTLBB and facilitate further comparisons of species sensitivity across studies.

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APPENDIX 1

The following sections include detailed information of each completed study that evaluated the effects of petroleum hydrocarbons on corals.

Incidents resulting in Acute and Chronic Exposure of Petroleum to Corals

Fishelson (1973) examined chronic exposure of an unspecified oil and phosphate loading following multiple spills from a land-based oil terminal and phosphate loading harbor in the Red Sea. There was an obvious decrease in coral cover, with *Acropora, Seriatopora, and Stylophora* among the most affected genera. Brain corals also decreased in cover, which was coupled with an increase in algal growth, presumably due to phosphate pollution (Fishelson, 1973). Rinkevich and Loya (1977) similarly examined the effects of chronic Iranian crude oil release from multiple spills originating at a terminal in the same area one year later. Examination of *Stylophora pistillata* revealed higher adult coral mortality rates coupled with reproductive alterations. Declines in number of breeding colonies, number of ovaria per polyp, number of planulae per coral head, and lower settlement rates were all significant, which can have lasting effects on the population (Rinkevich and Loya, 1977).

Venezuelan crude oil, refinery waste, and Corexit® dispersant were continuously released into the Caribbean from a refinery in San Nicolas Bay, Aruba from 1923–1985, causing chronic pollution of a nearby fringing reef (Bak, 1987). This type of point source pollution contains a distinct concentration gradient, with decreasing concentration as distance from source increases. A positive correlation was found between coral cover, rugosity and distance from the refinery, with major deterioration of the reef directly in front of- and down-current from the refinery. *Acropora palmata* was "decimated" along the entire study area, while *Orbicella annularis,* and *Agaricia agaricites* were only absent close to the refinery. Abundance of *Diploria strigosa* was highest near the refinery, and less abundant as distance increased. This type of scenario proves valuable when examining community changes in response to chronic oil pollution, and perhaps sheds light on whether or not some corals are more capable of coping with chronic oil pollution.

Perhaps the most studied of all spills impacting coral reefs, the Bahia las Mińas spill released more than 8 million liters of medium weight crude oil from a ruptured storage tank at Refineria Panama on Payardi Island, Panama (Burns and Knap, 1989). Oil leaked

into the Caribbean with roughly 21,000 liters Corexit® 9527 used in the clean-up process. The close proximity of the Smithsonian Tropical Research Institute's marine laboratory at Punta Galeta, provided ample baseline data to which all post-spill changes could be compared. Burns and Knap (1989) found extensive mortality of sub-tidal reef corals, with a positive correlation between hydrocarbon uptake of *Siderastrea siderea* and *Agaricia tenuifolia* tissues and mortality. There was also an increase in protein to lipid ratios at heavily oiled sites (Burns and Knap, 1989). A decrease in cover of *Palythoa caribaeorum* and *Zoanthus sociatus*, as well as complete loss of *Porites* spp. was also observed (Cubit et al., 1987; Jackson et al., 1989). Corals exhibited 22–30%, and 17% mortality in heavily oiled and moderately oiled sites respectively, compared to no mortality in unoiled reefs (Cubit et al., 1987). Abundance of scleractinian corals was reduced by 76%, 56%, and 45% at depths of less than 3 m, 3–6 m, and 9–12 m, respectively. Extensive bleaching, tissue swelling, mucous production, and increased bacterial infections were associated with corals in the oiled sites compared to reference sites and pre-spill data (Guzmán et al., 1991; Jackson et al., 1989). Two years after the spill, coral cover decreased from 28% to 13%, with reductions in colony size, growth rate, and diversity of corals present. More than half of the decrease in cover was due to reductions in *Acropora palmata* and *Orbicella annularis*. Recently dead areas on corals were commonly observed, with most corals showing signs of recent stress, particularly *S. siderea* (Guzmán et al., 1991). Five years after the spill, oil continued to leach from mangrove sediments, prompting evaluation of long-term, chronic effects on *S. siderea*. Gonad size was significantly reduced at the heavily oiled sites, and a decrease in fecundity of corals with recent stress was noted (Guzmán and Holst, 1993). The percentage of injured corals remained significantly higher at oiled sites, coupled with decreased abundance and diversity (Guzman et al., 1994). Growth of *Porites astreoides* and *S. siderea* were both negatively correlated with sediment hydrocarbon concentration, with higher concentration leading to decreased growth in both species. Overall, extensive mortality of sub-tidal reef corals occurred following the Bahia las Mińas spill, with prolonged chronic effects on vital processes lasting well over five years due to continual seepage of oil from mangrove sediments.

During the Gulf War in 1991, a very large amount of unspecified oil was intentionally released into the Persian Gulf. From 1992–1994, corals on the Saudi Arabian

coastline were examined for delayed responses associated with the oil spill (Vogt, 1995). Corals showed no detectable impact from the Gulf War oil spill, and an increase in coral cover was recorded for this time period. The lack of response could possibly be attributed to the history of oil spills in this area, and the possible organismal adaptation to petroleum hydrocarbon exposure. Al-Dahash and Mahmoud (2013) evaluated the coral bacterial community near this same area in southern Kuwait, revealing that the chronic exposure to natural oil seeps and multiple oil spills in the Persian Gulf has led to alterations in the mucous bacterial community of *Acropora clathrata* and *Porites harrisoni*, to favor more oil-degrading bacteria. Thus, due to both continuous release and small pollution events, corals in this area are colonized by oil-utilizing bacteria, which may confer an advantage and may be one of the causes why the Gulf War oil spill had no significant detectable effects on corals.

In 1993, a fishing vessel ran aground at Rose Atoll National Wildlife Refuge, American Samoa, releasing diesel fuel, lube oil, and ammonia onto a pristine oceanic reef (Green et al., 1997). Aside from physical damage to the reef, injury and morality were moderate to high up to 1 km from the wreck site after 6 months. Direct impact of the spilled toxicants to coral communities could not be obtained because of logistic constraints, although the reef structure was compromised for a variety of other reasons; reduction in crustose coralline algae, cyanobacterial blooms, anoxia from organic loading and oxygen reduction all had a negative effect on reef corals.

The M/V Kyowa Violet oil spill in December 2002 in Micronesia released 55,000—80,000 gallons of intermediate fuel oil onto the reef resulting in a large acute exposure situation (Downs et al., 2006). The cellular physiological condition of *Porites lobata* exhibited changes consistent with exposure to a xenobiotic. Differences in protein metabolic condition suggested an increase in mitochondrial protein chaperoning, especially membrane proteins. Alteration in poryphyrin metabolism indicated a major shift in cellular metabolism. Oxidative stress was indicated by elevated levels of catalase and the gene mutY DNA glycosylase (MutY). Significantly elevated levels of Cytochrome P450 (CYTP450) suggested corals were responding to aromatic hydrocarbon exposure. In a related study, cellular physiology of *Pocillopora damicornis* in response to chronic PAH exposure in Guam was also examined (Downs et al., 2012). Although protein metabolic

condition was not significantly altered, other cellular biomarkers were elevated. Mitochondrial chaperoning and protein import increased, concomitant with elevations of oxidative damage and response and increased porphyrin production. These changes, along with increases in xenobiotic and detoxification response biomarkers were consistent with exposure to PAHs.

Corals near Port Aransas on the South Texas coast are also subject to chronic petroleum exposure (Sabourin et al., 2013). The tissues of *Leptogorgia setacea* were determined to contain an average of 811 ppm of unspecified oil. The skeletons of these corals had much greater potential for biodeposition of PAHs compared to coral body tissues. Coral tissue PAH concentrations were consistently higher than surrounding sediment samples, indicating contaminants were accumulated from the water column, as opposed to the sediments. Similarly, PAH concentrations in coral tissues at Kenting Coral Reef, Taiwan were two orders of magnitude higher than in sediments, providing evidence for bioaccumulation from the water column, not surrounding sediments (Ko et al., 2014). Sorption, or feeding on contaminated prey were two methods suggested as pathways of accumulation from the water column. A preferential accumulation of low molecular weight compounds and methylated PAHs was also found (Ko et al. 2014).

Deep water corals were assessed in the northern Gulf of Mexico in response to the Deepwater Horizon (DWH) oil spill in 2010 (White et al., 2012). Of the colonies examined at the study sites, 86% showed signs of negative impact, including excessive mucus production, retracted polyps, tissue loss, and sclerite enlargement. There was also an oily residue, termed "floc", which covered a majority of the coral colonies and contained dead polyp fragments and detached sclerites (White et al., 2012). Silva et al. (2015) examined octocorals and antipatharians on mesophotic reefs from six sites near the DWH platform (four sites within 100 km). *Hypnogorgia pendula, Bebryce* spp., *Thesea nivea, Swiftia exserta, Antipathes atlantica, Tichopathes* sp., and *Ellisella barbadensis* were observed for injuries and samples were taken for hydrocarbon analysis. Following the spill, injuries increased; colonies were covered in mucus, a biofilm material and hydrozoans. Taller growth forms sustained the most severe injuries; loss of branches, necrotic tissue, and complete mortality. Detectable levels of hydrocarbons were also found in coral tissues and surrounding sediments.

Table A 5. Summary of acute and chronic releases of petroleum hydrocarbons with focus on impacts to corals.

Name/ Date of Spill	Oil Type and Amount	Scenario	Coral(s) Examined	Reference
Gulf of Aqaba (Gulf of Eilat), 1973 Red Sea	Unspecified oil and phosphate loading	Multiple oil spills from oil terminal and phosphate loading harbor	Coral cover of all species. *Acropora, Seriatopora,* and *Stylophora* most reduced.	Fishelson 1973
Gulf of Eilat 1974–1975 Red Sea	Iranian crude oil	Chronic pollution by oil terminal with multiple large spills	*Stylophora pistillata* adult and reproduction effects.	Rinkevich and Loya 1977
Aruba 1923–1985 Caribbean Sea	Venezuelan crude oil, refinery waste, and dispersant	Chronic pollution of fringing reef by large refinery	Decline in coral cover of all species, specifically *Orbicella annularis, Agaricia agaricites, Diploria strigosa*, and *Acropora palmata*.	Bak 1987
Bahia las Minas April 27th, 1986 Refineria Panama on Payardi Island. Caribbean coast.	>50,000 barrels (8 million liters) med-weight crude oil (70% Venezuelan crude, 30% Mexican Isthmus crude) with <21,000 L Corexit® 9527	Acute exposure to oil spilled from ruptured storage tank, covering mangroves and seagrasses. Floated over corals.	Mortality and hydrocarbon uptake in *S. siderea* and *A. tenuifolia.*	Burns and Knap 1989
			Palythoa caribaeorum and *Zoanthus sociatus*. *Porites* spp.	Cubit et al. 1987
			Porites, zoanthids, and hydrocorals	Jackson et al. 1989
		Two years post-spill	Sublethal changes, coral cover, and growth rate of *P. asteroides, A. Agaricites, S. siderea, A. cervicornis, O. annularis*	Guzmán, Jackson, and Weil 1991
		Five years post-spill	*Siderastrea siderea* reproduction and fecundity of corals.	Guzmán and Holst 1993
			P.asteroides, *S.siderea, Diploria clivosa,* and *D. strigosa.*	Guzman, Burns, and Jackson 1994
Saudi Arabian Coastline Persian Gulf 1992–1994	Unspecified oil	Delayed response to Gulf War oil spill of 1991.	Corals showed no detectable impact.	Vogt 1995
Rose Atoll National Wildlife Refuge Jin Shiang Fa fishing vessel 10/1/1993	100,000 gallons diesel fuel, 500 gallons lube oil, 2500 pounds ammonia	Physical damage and 6 week release of chemicals.	Reef injury and mortality moderate to high.	Green et al. 1997

Table A1 Continued

Name/ Date of Spill	Oil Type and Amount	Scenario	Coral(s) Examined	Reference
MV Kyowa Violet December 26th, 2002	55,000–80,000 gallons intermediate fuel oil	Intertidal areas coated, fuel floated over reefs.	*Porites lobata* cellular physiological condition consistent with exposure to a xenobiotic of PAH origin.	Downs et al. 2006
Deepwater Horizon,Northern Gulf of Mexico.April - July, 2010	Macondo crude oil	Deep water coral sites examined 3 months after Deepwater Horizon well was capped.	*Paramuricea biscaya, Swiftia pallida, paragorgia regalis, Acanthogorgia aspera,* and *Clavularia rudis* analyzed for impacts associated with DWH spill.	White et al. 2012
Guam, Mariana Islands	Not applicable	Chronic PAH contamination at the port and marina sites	*Pocillopora damicornis.* Cellular biomarkers consistent with xenobiotic response were analyzed.	Downs et al. 2012
Port Aransas, South Texas Coast	Unspecified oil measured at 811 ppm in coral tissue (mean)	Chronic petroleum contamination in the port	*Leptogorgia setacea* tissues examined for PAH concentration	Sabourin et al. 2013
Qaro and Umm Al-Maradim Islands, South Kuwait	Unspecified oil	Chronic exposure to natural oil seeps.	Mucous associated oil degrading bacteria of *Porites compressa* and *Acropora clathrata*	Al-Dahash and Mahmoud 2013
Kenting Coral Reef, Taiwan	Not applicable	Chronic PAH contamination	PAH concentrations in coral tissue higher than sediments. Bioaccumulation of PAHs from water column.	Ko et al. 2014
Deepwater Horizon, Northern Gulf of Mexico. September 2010 and 2011	Macondo crude oil	Mesophotic reefs (4 sites) examined after DWH oil spill and compared to pre-spill data.	*Hypnogorgia pendula. Bebryce spp. Thesea nivea. Swiftia exserta. Antipathes atlantica. Tichopathes sp. Ellisella barbadensis* Increase in number of injured colonies. Mucous and biofilm material covered colonies. Taller growth forms had most severe injuries (necrotic tissue and denuded skeleton)	Silva et al. 2015

In-situ Studies Examining Effects of Hydrocarbons on Coral

The first field experiment exposing coral to oil was completed in 1971 at Eniwetok Atoll, Marshall Islands (Johannes et al., 1972). As coral reefs can be exposed to air during low tide, the authors hypothesized that floating oil may have deleterious effects on the corals during this time. Santa Maria crude oil (SMCO) was poured over corals attached to floating trays to simulate contact while exposed to air at low tide. When oil coated the corals, temperature was elevated by 3 °C. Branching species, such as *Acropora* spp. and *Pocillopora* spp., showed the highest affinity for oil, remaining covered after four weeks. Large-polyped massive corals had the least affinity, presumably due to abundant mucous production and large polyps providing the means to remove the oil droplets. Numerous other corals showed intermediate affinities for the oil droplets. In all cases, tissue damage occurred if oil adhered in patches greater than a few millimeters, while tissues remaining free of oil showed no effect of exposure.

LeGore et al. (1989) utilized containment booms to expose corals in the Arabian Gulf to oil, dispersed oil, and dispersant only for both 24, and 120 hours. The dispersed oil treatment was the only plot to register an increase in hydrocarbon concentration in the water column 15 cm above the coral. Following exposure, there was no significant effect on *Acropora* spp., and growth and colonization appeared unaffected in all exposure plots. However, seasonal bleaching was widespread and occurred in all exposure plots, with the slowest recovery in the dispersed oil plots.

The Tropical Oil Pollution Investigations in Coastal Systems (TROPICS) experiment conducted in 1984 on the Caribbean coast of Panama (Ballou et al., 1987b) is perhaps the most comprehensive field experiment examining effects of oil exposure to tropical marine communities. The TROPICS experiment is one-of-a-kind with respect to the research conducted in the area prior to, and 20+ years following exposure to evaluate long term effects. The researchers intended to simulate a severe but realistic spill scenario, and to establish whether the use of dispersants will reduce or exacerbate the effects of an oil spill on tropical environments (Ballou et al., 1987a). Although this study simulated an oil spill on mangroves, seagrasses, and corals, only the effects on corals will be discussed here.

Immediately following exposure, percent coral cover in the dispersed oil site declined abruptly, and continued to do so for an entire year (Ballou et al., 1987b). Growth of *Porites porites* and *Agaricia tenuifolia* (which dominate the reef community) was significantly reduced by dispersed oil. Contrary to the initial effects observed for dispersed oil, untreated oil caused only a slight but non-significant reduction in cover, with no significant reduction in growth of all species examined (Ballou et al., 1987a; Ballou et al., 1987b). Short term effects of dispersed oil on corals were clear, with coral cover remaining significantly lower for at least two years following exposure, showing little indication of recovery (Dodge et al., 1995). Exposure to oil only treatments did not result in decreased growth and coral cover. By 1994, after ten years of recovery, parameters at all sites were indistinguishable and no significant changes to coral cover, growth, or sclerochronology were found when comparing oil or dispersed oil sites to reference sites. In 2001 and 2002, Ward et al. (2003) revisited the site to compare skeletal density and porosity of corals at each site as a means of addressing long-term recovery. Although no significant differences were determined for any of the treatment sites, analysis of *Porites* spp. revealed increased skeletal porosity and decreased density at the oil only site, which is consistent with elevated growth rates (Ward et al., 2003). These elevated growth rates were likely not related to oil, as the spill was conducted 20 years prior.

Table A 6. Summary of *in situ* experiments assessing the impact of hydrocarbons on corals.

Name/ Location	Oil Type/ Concentration	Scenario	Coral Species	Effects Measured	Reference
Eniwetok Atoll Marshall Islands, 1971	200 mL SMCO (0.6 mm slick)	Oil poured around corals mounted to floating trays partially exposed to air for 1.5 hours	22 species of Indo-Pacific corals	3 °C temperature elevation. Oil adhered most to branching species, least to large-polyped massive species.	Johannes, Maragos, and Coles 1972
TROPICS Experiment, Caribbean coast of Panama November 1984	Prudhoe Bay crude oil (1-4 ppm) Dispersed oil (commercial nonionic glycol ether-based concentrate) averaged 50 ppm. Both declined over time. Measured by UV Fluorometry	Simulated oil spill in mangrove, seagrass, and reef area. Sites were oil only, dispersed oil, and untreated control.	*Porites porites, and Agaricia tenuifolia* dominated. *Orbicella annularis,* and *Acropora cervicornis* also present.	Dispersed Site: % cover declined abruptly. Significant reduction in growth of *P.porites* and *A.tenuifolia*. Untreated Oil Site*:* Slight decrease in coral cover but not significant. No effect on growth rates.	Ballou et al. 1987 & Ballou et al. 1989
				No significant dose response on coverage, growth, or sclerochronology 10 years after dosing.	Dodge et al. 1995
				No significant differences of skeletal porosity and micro-density between sites.	Ward et al. 2003
Arabian Gulf 1989	Arabian light crude oil and Corexit 9527 0.25mm (24 hour) and 0.1mm (120 hour) slicks. Concentration only detectable in dispersed oil site (2.5 ppm)	Oil, dispersed oil (20:1 oil:disp.), and dispersant exposures in floating containment booms	*Acropora* spp.	No significant effect on growth between plots.	LeGore et al. 1989

Ex situ Laboratory Experiments Examining Effects of Hydrocarbons on Adult Corals

Lewis (1971) exposed four Caribbean corals to Barbados crude oil and 'Corexit' dispersant for 24 hours in finger bowls. All species tested were more sensitive to dispersant compared to oil and exhibited tissue rupture, nematocyst discharge, tentacle retraction, and inhibition of feeding/tactile response at all concentrations. These changes were exacerbated in branching species, while encrusting corals showed less effect with a greater ability to recover. Concentrations of both compounds above 100 ppm had harmful effects with incomplete recovery after 24 hours.

Eisler (1975) conducted two experiments with the octocoral *Heteroxenia fuscescens* using two crude oils and ST-5 dispersant, in static and flow-through exposures. Results of the static exposure include LC_{50} values for each of the toxicants, indicating a greater toxicity for the dispersant when compared to either oil alone. The 168-hour flow through exposures solicited a similar response; dispersant only treatments were the most toxic. The highest dispersed oil concentrations in either experiment failed to solicit a mortality response. It should be noted that LC_{50} values from the static exposure were lower (higher toxicity) than those from the flow-through experiment, indicating possible compounding effects associated with static exposures (i.e., oxygen depletion, waste accumulation). Researchers also found bioaccumulation in corals exposed to higher treatments of crude oil, but the bioaccumulated amount was less than 1% natural hydrocarbon content.

Reimer (1975) completed a suite of experiments using four scleractinian corals and marine diesel fuel and bunker oil. Concentrations that corals were exposed to were not specified, as most of the experiments included immersing corals in oil or pouring oil directly onto the corals and monitoring recovery in clean seawater. After 114 days of recovery from a one minute immersion in oil, corals showed varying degrees of mortality, from 0% to 100%. When oil was poured onto corals for 30 minutes, behavioral changes included immediate polyp retraction with no tactile response to stimulus, mouths open with exposed actinopharynx and mesenterial filament extrusion. Additionally, massive expulsion of symbionts occurred with tissue rupture and flaking, causing 70% mortality after 17 days. A 30 second immersion in oil resulted in tissue rupture and flaking, with

extensive bleaching occurring within 5-13 days. Lastly, when 1–4 ml oil was added to finger bowls containing coral, mouths opened and abnormal feeding reactions occurred.

Bak and Elgershuizen (1976) exposed 19 species of coral to five sand-oil combinations to measure rejection efficiency. Researchers found no evidence of adsorption or ingestion of oil, as oiled sediments was removed by ciliary currents and tentacular/ polypal movements, similar to normal sediment removal. Tissue death resulted if sediments remained for two or more days, for both oiled and un-oiled sediments. Oiled sediment failed to induce an obvious increase in mucus secretion compared to that secreted in response to un-oiled sediments.

Elgershuizen and De Kruijf (1976) exposed *Madracis mirabilis* to four types of oil and Shell LTX dispersant in 500-mL beakers by either floating the oil on the surface or creating toxicant-seawater mixtures. No mortality was observed for floating oil treatments, thus LC_{50} values were greater than the highest concentration tested. Oil and seawater mixtures were more toxic when compared to floating oil, but effects were temporary and LC_{50} could not be calculated. Exposure to dispersant only solicited more permanent effects with poor recovery. Dispersed oil was the most toxic of the compounds examined, with LC_{50} values 10–50 times lower than oil-water mixtures. Authors suggest the increased water soluble fraction of oil and the dispersants effect on membrane permeability as the culprit for increased toxicity.

Cohen et al. (1977) used Iranian crude oil and *H. fuscescens* in both static and flow-through exposures. Corals were more sensitive to static conditions, showing breakdown of pulsation synchrony and decreased ability to respond to mechanical stimulus with increased oil concentration. Static test concentrations were below LC_{50} values for 24 and 48 hours (LC_{50} > 30 mL/L), while 72 hour LC_{50} was calculated at 17 mL/L. The flow-through exposure lead to a decrease in pulsation synchrony in corals closer to the surface of the depth divided tank, but no mortality was observed in any of the colonies. The authors concluded that corals are more sensitive to oil pollution under static conditions, which are less representative of observed environmental characteristics, and that acute exposure to comparatively high concentrations of crude oil is relatively non-toxic to *H.* fuscescens, but adverse effects will emerge over extended exposure durations.

Ducklow and Mitchell (1979) also used *H. fuscescens* and Iranian crude oil, but monitored coral health and changes to the mucous bacterial population following a five-day exposure to floating oil. Polyp pulsation was initially impaired, with polyps closing, and remaining so until the exposure was completed. Polyps elongated and lost their ability to stand upright, collapsing and extending towards the bottom of the tank. Mucous bacteria populations significantly increased following exposure to oil. All effects measured were temporary, and returned to normal following the exposure.

Neff and Anderson (1981) exposed five species of corals to South Louisiana crude oil, no. 2 fuel oil, and phenanthrene for 72 hours then incubated them with radio labeled calcium chloride to measure calcification. Following exposure to crude oil WSF, *Millepora* spp. showed no significant differences in calcification, while calcification in *Madracis decactis* significantly increased in response to the same exposure. In response to the exposure to no. 2 fuel oil WSF, calcium deposition in all corals was variable. Calcification was significantly reduced in *Oculina diffusa*, while *Millepora* spp. and *Favia fragum* both showed no effect from exposure to no. 2 fuel oil. Calcification in *M. decactis* and *Orbicella annularis* increased with increasing no. 2 fuel oil concentration. Polyp extension in all corals exposed to no. 2 fuel oil was reduced during the exposure, with some corals showing slight bleaching. *Millepora* spp. was also exposed to phenanthrene, a PAH found in crude oil and other refined products, resulting in high variability in calcification rate following exposure, with only high concentrations causing a significant reduction. Based on the results of this study, authors concluded that coral calcification after oil exposure is variable, with some species more susceptible than others.

Peters et al. (1981) examined histopathological effects of hydrocarbon uptake during a three-month exposure to the WAF of no. 2 fuel oil on *Manicina areolata*. Hydrocarbon uptake was detected after 2 and 6 weeks exposure in high and low concentrations respectively, causing extensive cellular changes, although no mortality occurred throughout the exposure. Effects included increases in mucous secretory cell activity, with proliferation and hypertrophy of epidermal cells and mesenteries, and mesogleal swelling. Mucocytes also appeared in tips of mesenterial filaments, where they are not normally found. After 12 weeks of exposure, mucocytes atrophied, and degeneration and loss of symbionts in the gastrodermis and mesenteries was noted.

Swelling, nematocyst fragmentation, and loss of granular gland cells in mesenterial filaments was also apparent by the end of the exposure. Authors concluded that long term chronic exposure to petroleum hydrocarbons has the ability to initiate cellular degeneration and atrophy of coral tissue even at low concentrations, because these hydrocarbons partition into the cells, disrupting vital biosynthetic processes of both coral and symbiont cells.

Cook and Knap (1983) measured carbon fixation and incorporation of photosynthetic products in *Diploria strigosa* following exposure to the WAF of Arabian light crude oil and Corexit® 9527. Exposure to oil or dispersant alone resulted in no effect on carbon fixation or incorporation of photosynthetic products at any time. Exposure to dispersed oil led to an initial 85% reduction in carbon fixation after 1–3 hours, which recovered after 3–5 hours. The same pattern occurred for incorporation of photosynthetic products; an initial reduction, followed by recovery within 3–24 hours. Authors concluded that dispersed oil has a much greater effect on photosynthesis in *D. strigosa,* although the ability to rapidly recover suggests the effects are temporary.

Solbakken et al. (1983a) measured accumulation and depuration of petroleum PAHs naphthalene and phenanthrene in 19 coral species following a 24-hour incubation. Corals rapidly accumulated the lipid-soluble xenobiotics used, with uptake being a function of specific compound and coral species. Naphthalene was most efficiently depurated by day 10 of recovery, while significant levels of phenanthrene were still detectable within 21–37 days post exposure. This pattern of naphthalene being removed much faster relative to phenanthrene has been reported for other marine organisms (Solbakken et al., 1983b) with the depuration periods for both compounds being comparatively slower for other subtropical marine organisms.

Multiple experiments exposing *Diploria strigosa* to Arabian light crude oil and chemically dispersed oil for 6–24 hours, with a one year recovery period, were completed in the 1980's (Dodge et al., 1984; Knap, 1987; Wyers et al., 1986). Dodge et al. (1984) examined skeletal growth characteristics of *Diploria strigosa* following the exposure and found no significant differences between any of the treatments in regard to upward growth or new endotheca length. When comparing the ratio of new fossa: old fossa, only 2 ppm chemically enhanced water accommodated fraction (CEWAF) and 12–19 ppm oil WAF

treatments showed a significant decrease compared to controls. Although the trend hinted that dispersed oil has a negative effect on skeletal growth, the lack of significant differences between treatments was attributed to the high variability within and between coral colonies. Wyers et al. (1986) observed the external appearance of coral colonies for changes in survival, behavior characteristics, and morphological changes during the same experiment. No mortality occurred at any of the concentrations utilized, and no significant differences in characteristics between any treatments were determined. However, CEWAF and WAF of oil alone did lead to adverse effects at concentrations near 20 ppm. Mesenterial filament extrusion, extreme tissue contraction, tentacle retraction, and localized tissue rupture were common in these corals following the onset of exposure, but returned to pre-exposure conditions within four days during the winter, and 24 hours during the summer. Authors concluded that there were no significant differences in coral behavior when comparing WAF and CEWAF, and the observed effects seemed unlikely to impair coral viability in the long term. Knap (1987) measured the hydrocarbon uptake in *D. strigosa* during this experiment, and found evidence for accumulation of the entire molecular weight range of Arabian light crude oil regardless of concentration or whether the oil was physically or chemically dispersed. Physically dispersed oil droplets were also found to adhere to coral mucus with much more affinity than chemically dispersed oil droplets.

Thorhaug et al. (1989) completed a 10-hour experiment using three scleractinian corals, fresh and weathered Venezuelan crude oil, and 11 different chemical dispersants in order to rank them according to their toxicity. Some of the dispersed oil treatments led to 100% mortality, while others resulted in less the 50% mortality, allowing dispersants to be ranked as high, medium, and low toxicity. Conco K, OFC D609, Corexit 9527, Kemarine, ADP 7, and Janosolv were among the most toxic, while the less toxic dispersants included Elastosol, Cold Clean, and Finasol.

Mercurio et al. (2004) used *Acropora formosa* in 48-hour exposures to WAF of mineral derived lubricant (MDL) to assess hydrocarbon exposure impacts on photosynthesis, disruption of symbiosis, and mortality in adult corals. Significant differences were found for mortality, symbiont density, and photosynthetic yield at 48 hours when exposed to 190 µg/L MDL. The corals first exhibited lightening and bleaching of the tips of branches, followed by mortality. Decreases in photosynthetic yield and

symbiont density were more sensitive indicators of pollution stress when compared to mortality.

Rougee et al. (2006) exposed *Pocillopora damicornis* to WAF of Intermediate Fuel Oil (IFO) 180 to assess the potential for a shift in cellular homeostasis from petroleum pollution. Significant changes in cellular biomarkers involved in cellular response and protection, manipulation, and excretion of toxicants were observed. A significant xenobiotic response, specifically CYTP450, was generated above 1 g/L, which indicated a reaction to PAH exposure. Significant elevation of glutathione-*S*-transferase (GST-pi) also indicated a detoxification response. Shifts in porphyrin metabolism were also apparent, which was likely the result of PAH interactions in the cell. Oxidative damage response was significantly elevated in 1 g/L treatments, and when coupled with elevated levels of MutY, suggested DNA repair was occurring. Elevated levels of heat shock protein 70 (Hsp-70) also indicated a shift in protein metabolic condition. Based on the changes to these cellular biomarkers, the authors concluded that exposure to IFO180 WAF leads to stress and a shift from metabolic homeostasis in the organism.

Martinez et al. (2007) examined the ultraviolet radiation (UVR) enhanced toxicity of fluoranthene to *Porites divaricata.* When combined, fluoranthene and ecologically relevant levels of UVR led to decreased photosynthetic efficiency and bleaching, with mortality occurring within 3–6 days. Corals exposed to fluoranthene in the absence of UVR showed initial decreases in measured parameters, but returned to normal levels within 4 days. The effects of PAH exposure on *P. divaricata* were significantly increased in the presence of UVR.

Ramos and Garcia (2007) examined changes in the mixed function oxygenase system (MFO) in *Orbicella faveolata* exposed to the PAH benzo(a)pyrene. The main component of MFO is CYTP450, which is responsible for biotransformation of a variety of compounds like PAHs. An increase in CYTP450 occurred in colonies exposed to benzo(a)pyrene, as well as increased in enzymatic activity of antioxidant complexes, demonstrating antioxidant defense to a xenobiotic. The MFO activity of *O. faveolata* indicated a short term activation of detoxifying response, at levels consistent with mollusks, echinoderms, and annelids.

Shafir et al. (2007) exposed *Stylophora pistillata* and *Pocillopora damicornis* to the WSF of Egyptian crude oil, CEWAF, and six common dispersants in a 24-hour exposure. Corals exposed to all concentrations of oil WSF had 100% survivorship, with no impact on lateral growth throughout the exposure or recovery phase. Corals exposed to dispersant only had 100% mortality in all concentrations above 10% stock, with most corals surviving below 10% stock dispersant levels. Dispersed oil treatments led to 100% mortality above 10% stock, with significant mortality in 4 of the 6 dispersants at 10%. Sublethal levels of dispersed oil (<10%) led to delayed tissue development and growth in both corals examined. The authors concluded that corals are more susceptible to dispersants and dispersed oil when compared to oil only.

White and Strychar (2011) exposed the gorgonian *Leptogorgia virgulata* to gasoline for 168-hours. After 48 hours, corals showed no visible signs of impact. After 120 hours of exposure, significant loss of tissue and sclerites occurred. Authors also noted that the bases of the coral seemed more resilient compared to the tips.

Woo et al. (2014) subjected the soft coral *Scleronephthya gracillimum* to a mixture of 13 petroleum PAHs in equal proportion for 24 hours to assess gene expression. Genes involved with oxidative stress were upregulated. Many signaling pathways associated with protein kinase activation were altered, as well as downregulation of certain growth inhibitors which may result in carcinogenesis or tumorigenesis. Induction of cellular redox stress conditions resulted from exposure, suggesting a defense mechanism was initiated. Polymerases involved in DNA repair were repressed, suggesting the cells had little ability to repair damaged DNA. Fertilization and other developmental processes, as well as intracellular protein processing were also altered.

DeLeo et al. (2015) assessed the effects of Macondo crude oil and Corexit® 9500 on three deep sea coral species in a suite of experiments using both mixtures and WAFs. Mixtures utilized the entire prepared solution without separation, while WAFs only used the aqueous phase of the toxicant following separation. Oil-only treatments (oil-seawater mixtures and WAF) had either no or very low mortality, with very few significant differences in health rating compared to controls. Health ratings of dispersant treatments (dispersant-seawater mixtures and dispersant-only WAF) were all significantly lower than controls and oil treatments. Dispersant-only treatments also showed increased mortality,

with dispersant-only WAF producing higher mortality in all corals compared to dispersant-seawater mixtures. The treatments causing the highest mortality, as well as the most significant changes to health ratings contained CEWAF or dispersant only. It is also apparent that CEWAF solutions were more toxic compared to treatments containing oil/dispersant mixtures. The authors concluded that dispersants, in mixtures or WAFs, were more toxic compared to untreated oil, and dispersant use during the Macondo spill may have caused more damage to cold water corals than the initial release of oil.

Kegler et al. (2015) examined *Pocillopora verrucosa* following an 84 hour exposure to diesel. Concentrations declined by almost 64% over the exposure duration (0.69 – 0.25 mg/L TPAH). There were no significant effects on dark respiration rates or net photosynthesis. Photosynthetic yield was also unaffected by exposure to diesel.

Renegar et al. (2016) assessed the effects of the petroleum PAH 1-methylnaphthalene on *Porites divaricata* in a 48-hour continuous flow recirculating passive dosing system. Hydrocarbon was partitioned into polydimethylsiloxane (PDMS) O-rings to maintain constant concentrations throughout the exposure. Concentrations were measured at the beginning, middle, and end of the 48-hour exposure and showed little loss due to volatilization or degradation. Physical coral response, photosynthetic efficiency, mortality, and histologic cellular changes were used to quantify the coral response. Corals exposed to 5,427 µg/L exhibited progressive polyp retraction and moderate tissue swelling and mucus production, with no mortality occurring throughout the exposure. The 25,832 µg/L 1-methylnaphthalene exposed corals exhibited full polyp retraction with substantial mucus production within 6 h, and 100% mortality within 24 h. These two treatments scored significantly higher than controls and lower concentrations following the exposure. Histologically, corals exposed to 640 µg/L showed increased mucus production, while 5,427 µg/L corals had significantly less mucus area, presumably due to exhaustion of mucous production capacity. The sublethal changes were used to calculate an EC_{50} of 6,695 µg/L, while mortality data was used to calculate an LC_{50} of 12,123 µg/L.

Table A 3. Summary of *ex situ* experiments assessing the impact of petroleum hydrocarbons on adult corals.

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Porites porites, Agaricia agaricites, Favia fragum, Madracis asperula	General crude oil of Barbados with 'Corexit' dispersant	Oil only and dispersant only: 0, 10, 50, 100, 200, 500, 1000 ppm Nominal	Static 24-hour exposure to floating toxicant in finger bowls	Behavioral and health changes at all concentrations. Branching species more affected than encrusting species. Dispersant more harmful than oil. Both pollutants have harmful effect at 100–500 ppm with incomplete recovery after 24 hours.	Lewis 1971
Heteroxenia fuscescens	Iranian crude oil Sinai crude oil ST-5 dispersant	Oils only: 3 and 10 mL/L. Dispersant only: 0.001-0.100 mL/L Dispersed oil: 0.110 mL/L Nominal	Static exposure to floating toxicant in 3-L aerated jars	Crude oil alone (Iranian) LC50 = 12 mL/L Crude oil alone (Sinai) LC50 = 30 mL/L ST-5 dispersant alone LC50 = 0.018 mL/L Oil-dispersant LC50 >0.110 mL/L	Eisler 1975
			168-hour Continuous flow with floating toxicant in 1,500-L tanks	Crude oil alone (both types) LC50 >10 mL/L ST-5 dispersant alone LC50 = 0.052 mL/L Oil-dispersant mixture LC50 >0.110 mL/L Bioaccumulation in corals exposed to 10 mL/L	
Pocillopora damicornis, Pavona gigantean, Psammocora stellata, Porites furcata	Marine diesel and bunker oil	Not specified	Immersion in both oils for 1 minute. Rinsed, with recovery in clean seawater.	P. damicornis, 100% mortality after 20 days. After 114 days: *P. stellate,* no change P. gigantean, Bunker 100%, Diesel 12% mortality P. furcata, Bunker 100%, Diesel 23% mortality	Reimer 1975
			Oil poured over corals without water for 30 minutes. Rinsed, with recovery in clean seawater.	Polyp retraction, no tactile response to stimuli, exposed actinopharynx with mouth open and mesenteries extruded. Massive extrusion of symbionts. Tissue rupture and flaking off with 70% mortality after 17 days.	
			Immersion for 30 seconds, rinsed, with recovery in clean seawater.	Tissue rupture and flaking initially following exposure. Extensive bleaching within 5–13 days after exposure.	
			Static exposure to 1–4 mL oil in finger bowls	Mouth opening with abnormal feeding reactions. *Porites furcata* most exaggerated.	

Table A3 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
19 coral species	Nigerian, Forcados, Tia Juana Pesado crude oils. Forcados long and Langomar short residues	Not applicable	Sand-oil combinations were placed on corals and rejection efficiency was measured	No evidence of adsorption, no ingestion of oil droplets. If sediment not removed by two days, death of underlying tissue resulted (regardless of oil presence). Rejection was the same for oiled or non-oiled sediment.	Bak and Elgershuizen 1976
Madracis mirabilis	Nigerian, Forcados, Tia Juana Pesado crude oils and Forcados long residue. And Shell LTX dispersant	10, 50, 100, 500, 1000, 5000, 10000 ppm Nominal	24-hour static exposure to floating oil in 500-mL beaker	No mortality observed LC50 >10^5 ppm	Elgershuizen and De Kruijf 1976
			24-hour static exposure to oil and SW mixtures in 500-mL beaker	Low mortality, high recovery. More toxic than floating oil, but damage was temporary. LC50 >10^5 ppm	
			24-hour static exposure to dispersant and SW mixtures in 500-mL beaker	More toxic, with poor recovery and damage more permanent.	
			24-hour static exposure to dispersed oil mixtures in 500-mL beaker	LC50: Between 10 and 50 times lower for dispersant-oil mixtures than for oil water mixtures. Dispersed oil more toxic than oil or dispersant only.	
Heteroxenia fuscescens	Iranian crude oil	1, 3, 10, 30 mL/L Nominal	96-hour static dosing of floating oil in 3-L jars	Decrease in pulsation synchrony. Decreased ability to respond to mechanical stimulus. 24- and 48-hour LC50 >30 mL/L oil. 72-hour LC50= 17 mL/L oil	Cohen et al. 1977
		10 mL/L Nominal	168-hour continuous flow with floating oil in 1500-L depth divided tank	No mortality observed. Decrease in pulsation synchrony the closer to surface.	

Table A3 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Heteroxenia fuscescens	Iranian crude oil	5000 ppm Nominal	Oil floated on surface of 10 liter flow through tank for 5 days with no surface agitation.	Significant increase in mucus bacterial populations. Polyp pulsation impaired, closed on 4th day of exposure, remained closed. Polyps elongated and lost turgor, collapsing and extending toward bottom of tank.	Ducklow and Mitchell 1979
***Madracis decactis, Oculina diffusa,Orbicella annularis,Favia fragum,Millepora* spp.**	South Louisiana crude oil, no. 2 fuel oil, or phenanthrene	WSF crude oil 0, 10, 20, 30 % dilution of stockMeasured but nominal after dilution.	72-hour static exposure to 1-liter WSF solution in 20-cm finger bowls.Corals then incubated with radio labeled calcium chloride for 6 hours in naturally lit clean seawater. *O. diffusa* incubated with radio labeled calcium chloride for 3-6 hours.	*Millepora* spp.: no significant differences in calcium deposition.*M. decactis:* rate of calcium deposition significantly increased with increasing WSF of crude oil.	Neff and Anderson 1981
		WSF no. 2 fuel oil dilution of stock 0, 10, 20, 30 % Measured but nominal after dilution.		*O. diffusa:* Calcium deposition significantly reduced compared to controls. Polyp extension less pronounced in exposed corals. *Millepora* spp. and *F. fragum*: No significant differences in deposition. *M. decactis* and *O. annularis:* Calcium deposition increased. Polyp retraction with slight bleaching during exposure.	
		25, 100, 500 µg/L phenanthrene	96-hr exposure to 1-liter solution in 20-cm finger bowls.	*Millepora* spp. had high variability in deposition rate. 100 and 500 µg/L significantly less than controls.	
Manicina areolata	No. 2 fuel oil	Daily fluctuations. Averaged 0.15 ppm (high) and 0.07 ppm (low) Measured	3-month chronic flow through exposure to WAF	Hydrocarbon uptake occurred. No mortality during 3-month exposure.	Peters et al. 1981
				Histological changes to coral tissue: Increase in mucous secretory cell activity. Epidermal and mesentery cell proliferation and hypertrophy. Mesogleal swelling. Mucocytes atrophy, degeneration/ loss of symbionts in gastrodermis and mesenteries, mesenterial filament swelling, fragmentation of nematocysts, degeneration/ loss of granular gland cells.	

Table A3 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Diploria strigosa	Arabian light crude oil and Corexit 9527	Averaged 18–20 ppm Measured	8-hour exposure to WAF of oil, dispersant, or dispersed oil (20:1 oil:disp) in continuous flow system	Oil or dispersant alone: No effect on carbon fixation or incorporation of photosynthetic products at any time. Dispersed oil: Initially reduced carbon fixation and incorporation of photosynthetic products in all corals but recovered quickly	Cook and Knap 1983
19 Bermuda coral species	Petroleum PAHs: Naphthalene and phenanthrene	Averaged 0.016 ppm naphthalene, and 0.003 ppm phenanthrene Measured	24-hour static incubation followed by 37-day flow through recovery	Accumulation and depuration of lipid-soluble xenobiotics. Efficient depuration of naphthalene within 10 days. Phenanthrene still present within 21–37 days post exposure.	Solbakken et al. 1983
Diploria strigosa	Arabian light crude oil and Corexit 9527 or BP 1100WD	Oil WAF, dispersant WAF, and dispersed oil WAF Ranged from 1–50 ppm Measured	6- to 24-hour exposure in flow through aquaria. Recovery for one month, then placed back onto reef for 1-year.	No significant difference between treatments for upward growth or new endotheca length. New fossa/old fossa: 2 ppm treatments significantly lower for both dispersed oil combinations. 12–19 ppm dispersed oil and oil only significantly lower compared to controls.	Dodge et al. 1984
				No mortality occurred. No significant differences between oil alone, or dispersed oil treatments. Little adverse effects at 1-5 ppm all treatments. 20 ppm leads to mesenterial filament extrusion, extreme tissue contraction, tentacle retraction, and localized tissue rupture.	Wyers et al. 1986
			6- or 24-hour exposure to oil and dispersed oil in continuous flow aquaria.	Uptake of entire molecular weight range of oil regardless of physically/chemically dispersed, or concentration. Adherence to tissue of physically dispersed oil, absent in chemically dispersed oil.	Knap 1987
Porites, Orbicella annularis, Acropora palmata	Fresh and 24-hour aged Venezuelan crude oil and 11 dispersants	125 mL fresh oil with 12.5mL dispersant or 75 mL aged oil with 7.5 mL dispersant. Nominal	10-hour static exposure with dispersant poured onto floating oil in 100-L aerated aquaria	Dispersants utilized were ranked based on toxicity. 100% mortality observed in 18 of 55 treatments. <50% mortality observed in 27 of 55 treatments.	Thorhaug et al. 1989

Table A3 continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Acropora microphthalma	Mineral derived lubricant (MDL) and Vegetable derived lubricant (VDL)	100% MDL WAF: 380 µg/L 100% VDL WAF: 590 µg/L Measured but nominal after dilution	48-hour static exposure to dilutions of stock WAF in 1-L aerated glass beakers.	MDL 190 µg/L THC resulted in significant increases in mortality, and decreases in symbiont density, and photosynthetic YIELD at 48 hours. Sublethal changes more sensitive indicators of pollution stress.	Mercurio et al. 2004
Pocillopora damicornis	IFO 180 marine fuel oil	0.25, 1, and 4 g oil/L SW variable loading Nominal	24-hour static exposure to WAF in 1-L aerated glass beakers	Xenobiotic response showed reaction to the PAHs as well as BTEX at concentrations above 1 g/L Detoxification response increased. Significant shifts in Porphyrin metabolism, oxidative damage response, and protein metabolic condition indicate a shift in metabolic homeostasis.	Rougee et al. 2006
Porites divaricata	UVR enhanced fluoranthene	12, 30, and 60 µg/L Nominal	4.5-hour static exposure in 3-L glass aquaria with/without UVR	Fluoranthene +UVR: decreased photosynthetic efficiency leading to bleaching and mortality within 3–6 days. LC50 of 31.4 µg/L Non-UVR exposed corals decreased initially but returned to normal levels with 4 days.	Martinez et al. 2007
Orbicella faveolata	Petroleum PAH, benzo(a)pyrene	0.01 and 0.1 ppm	24 and 72-hour static exposure in covered 3.5-L glass aquaria with aeration	Cytochrome P450 concentration greater in colonies exposed to highest concentration of contaminant. Induction of antioxidant enzyme complexes (catalase, and superoxide dismutase) indicate a response of antioxidant defense.	Ramos and Garcia 2007
Stylophora pistillata and Pocillopora damicornis	Egyptian crude oil and 6 dispersants	Oil WSF 5 mL/L Nominal Diluted to 100, 75, 50, 25, 10, 0%	24-hour static exposure to toxicant with 7- or 50-day recovery in clean SW	No impact of any concentration on survivorship. No delayed mortality in 50-day recovery with no impact on lateral growth of either coral.	Shafir, Van Rijn, and Rinkevich 2007
		Dispersed oil, 5.5 ml/L Nominal Diluted to 100, 75, 50, 25, 10, 5, 1, 0.5, 0%		100% mortality in dispersed oil fractions above 10% (except Slickgone). Significant mortality in 4 of the 10% dispersed oil fractions. 5% and 10% dispersed oil fractions showed delayed tissue development and growth in both corals.	
		Dispersant-only 0.5 mL/L Diluted to 100, 75, 50, 25, 10, 5, 1, 0.5, 0%		100% mortality in dispersant treatments above 10%. 97–100% mortality in 3 10% dispersant treatments.	

Table A3 continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Leptogorgia virgulata	gasoline	10, 50, and 100 ppm Nominal	168-hour static exposure in 1-L glass containers with aeration	No signs of impact for first 48 hours. No tissue sloughing for first 120 hours. Loss of tissue and sclerites occurred after 120 hours.	White and Strychar 2011
Scleronephthya gracillimum	EPA 525 PAH Mix-B, containing 13 petroleum PAHs in equal proportion	100 µg/L solution (solution contains 0.5 µg/L of each PAH)	24 hour exposure to PAH mix in glass tanks	20 genes upregulated and 17 genes downregulated. Involved in oxidative stress, protein kinase activation, and defense mechanisms. Transcription polymerases repressed. Fertilization and developmental processes altered. Abnormal intracellular protein processing due to repressed ubiquitin-protein ligase.	Woo et al. 2014
Paramuricea* B3, *Callogorgia delta*,*Leiopathes glaberrima	Macondo crude oil Corexit 9500	Bulk oil stock diluted to 25, 7.9, 0.8 ppm Nominal	96 hour exposure in 50 mL Pyrex test tubes on shaker table with aeration every 24 hours	No complete mortality. *L. glaberrima*: Significant decline in health rating for med and high corals.	DeLeo et al. 2015
		Dispersant stock diluted to 25, 7.9, 0.8 ppm Nominal		*Paramuricea* B3: Mortality in high treatment within 48-72 hours. Health rating significantly different in high treatment. *C. delta*: High treatment significantly lower health rating.	
		Oil and dispersant mixture diluted to 25, 7.9, 0.8 ppm Nominal		*Paramuricea* B3: Mortality and significantly lower health rating in high treatment. *C. delta* and *L. glaberrima*: Health rating significantly lower in higher treatments compared to controls.	
		Oil WAF: Stock diluted to 50, 150, and 250 µM Nominal		Mortality only in one fragment of C. delta. *Paramuricea* B3 and *C. delta*: No significant health differences. *L. glaberrima*: All treatments had significantly lower health ratings.	
		CEWAF: 1:10 dispersant to oil Stock diluted to 50, 150, and 250 µM Nominal		*Paramuricea* B3: Mortality in few fragments of low and high treatments. Health rating significantly lower for all treatments compared to control. *C. delta*: Medium and high treatments led to incomplete mortality and significant health rating decline. *L. glaberrima*: Partial and complete mortality in medium and high treatments with significantly lower health rating	

Table A3 continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Paramuricea* B3, *Callogorgia delta*,*Leiopathes glaberrima	Macondo crude oil Corexit 9500	Dispersant WAF: Stock 848 mg/L Diluted to 35.3, 106, 176.7 mg/L Nominal	96 hour exposure in 50 mL Pyrex test tubes on shaker table with aeration every 24 hours	*Paramuricea* B3 and *C. delta*: High mortality and significantly lower health rating in all treatments. *L. glaberrima*: High and medium treatments led to complete mortality, partial mortality in low treatment. Significant decline in medium and high treatment health ratings.	DeLeo et al. 2015
Pocillopora verrucosa	Diesel	490 mL WAF in 600 L seawater. Measured at 0.69 mg/L TPAH (start) and 0.25 mg/L TPAH (end)	84 hour exposure in 600 L tanks	No significant effects of diesel WAF on dark respiration rates and net photosynthesis. Photosynthetic Yield also not affected.	Kegler et al. 2015

Laboratory Experiments Testing the Effects of Hydrocarbons on Coral Reproduction

Loya and Rinkevich (1979) examined acute effects of Iranian crude oil on *Stylophora pistillata* during reproduction. Following exposure to the WSF of crude oil, corals immediately opened mouths and prematurely shed larvae at quantities significantly higher than controls. Although spawning events usually take place at night, this induced larval shedding had no connection to time of day. This forced spawning can increase predation pressure and lead to an extended period of development in the water column before settlement can occur. The authors concluded that the release of buoyant larvae in combination with floating oil is the most severe problem associated with hydrocarbon pollution.

Rinkevich and Loya (1979) assessed chronic effects of a weekly addition of floating Iranian crude oil on Stylophora *pistillata* for 2–6 months during gametogenesis. Researchers found a significant decrease in average number of female gonads per polyp following exposure.

Te (1991) monitored metamorphosis, settlement, and calcification of *Pocillopora damicornis* planula following exposure to a mixture of gasoline and motor oil for 15 days in both open and closed vessels. No mortality was observed during the open vessel exposure, with normal metamorphosis, settlement, and calcification at concentrations less than 50 ppm; higher concentrations significantly inhibited settlement. The closed vessel exposure led to 100% mortality in the 100 ppm treatment, with settlement significantly reduced in treatments greater than or equal to 20 ppm. The effects of benzene, with or without settlement plates, was also examined. No significant effects were found when settlement plates were absent, although settlement was inhibited at 1 ppm. When settlement plates were present, variability was high, but there was a significant treatment effect on settlement. Differences in the water soluble fractions of the test compounds in seawater prevented clear correlations between treatment and settlement rates, and authors do not list post hoc test results to distinguish where treatment effects were present.

Kushmaro et al. (1997) examined the effects of Israel crude oil on *Heteroxenia fuscescens* planula following a three-day exposure to floating oil or vessels coated in oil. Significant decreases in metamorphosis and variable mortality occurred in response to increasing concentrations of surface applied oil. Metamorphosis was inhibited by 50% in

vessels coated with oil at 0.1 ppm. Free floating metamorphosis and post metamorphosis deformation occurred at the higher oil loadings. The authors concluded that inhibition of metamorphosis and mortality were significantly dependent upon crude oil concentration, and settlement was less frequent on oil covered surfaces.

Epstein et al. (2000) evaluated effects of the WSF of Egyptian crude oil and five dispersants on survivorship and settlement of *Stylophora pistillata* and *Heteroxenia fuscescens* planula in 2–96 hour bioassays. No mortality was observed in *S. pistillata* after exposure to oil WSF; although settlement was delayed, swimming behavior and settled polyp morphology remained unaltered. Dispersant WSF was only toxic to *S. pistillata* at higher concentrations, with settlement less than controls but similar to oil WSF. Planula morphology was deformed at all concentrations except the lowest tested. All dispersant only treatments at all concentrations exhibited detrimental effects to planula, exceeding oil WSF impact. Following exposure to dispersed oil WSF, *S. pistillata* planula showed complete mortality in all treatments greater than 10%, except those using Petrotech dispersant. Settlement was inhibited in all treatments, and major behavioral anomalies and structural deformations resulted. In *H. fuscescens*, all dispersed oil WSF concentrations led to high toxicity with no settlement, and caused major behavioral anomalies and increased structural deformations. It was apparent that dispersed oil had a marked increase in toxicity when compared to oil or dispersant alone, with higher mortality rates, no settlement, and significant alterations in behavior and morphology of both species.

Negri and Heyward (2000) assessed fertilization and metamorphosis of *Acropora millepora* following a four-hour exposure to Wandoo platform heavy crude oil and Corexit® EC9527A dispersant mixtures. Crude oil WAF failed to inhibit fertilization up to 0.165 ppm total hydrocarbon content (THC), while metamorphosis was significantly inhibited at 0.0824 ppm THC, and completely inhibited at 0.165 ppm THC. Fertilization and metamorphosis were both significantly inhibited at 0.225 ppm THC when exposed to 1% v/v dispersed oil, with full inhibition occurring at 0.325 ppm and 1.13 ppm THC for fertilization and metamorphosis respectively. Following exposure to dispersed oil at 10% v/v dispersant/ oil, fertilization and metamorphosis were both significantly inhibited at 0.0325 ppm THC. When using dispersant only, fertilization and metamorphosis were inhibited between 5 and 10 ppm THC. The authors concluded that both crude oil and

dispersant contribute to observed toxicity, and the dispersed oil concentration that significantly inhibited metamorphosis was equal to that which significantly inhibited fertilization. It was also apparent that fertilization success was more sensitive to CEWAF when compared to oil WAF. This could be attributed to the elevated levels of hydrocarbons in the water column following dispersant application.

Lane and Harrison (2000) exposed planula of three scleractinian corals to WAF of Fuel oil 467 and dispersed oil mixtures, using Ardrox 6120, for up to 96 hours. Oil WAF exposure increased mortality in *Acropora tenuis*, but variability in results limited significance. *Goniastrea aspera* mortality was significantly increased by oil WAF exposure, while *Platygyra sinensis* was least sensitive to oil WAF, with no significant toxic effects at any concentration. Exposure to dispersant only yielded LC_{50} values greater than oil WAF for all three coral species. Although toxic, dispersant only was less toxic to the three corals tested when compared to oil. Dispersed oil WAF however, led to a significant increase in mortality compared to controls, with an increasing toxic effect over time. All species tested were most sensitive to dispersed oil, indicated by a drastic decrease in LC_{50} values. The mortality response to dispersed oil WAF was rapid, with high levels of mortality at less than 5 ppm after only 6–12 hours of onset of exposure. The authors concluded that mortality increased as chemical dispersion raised the concentration and spatial extent of hydrocarbons in the water.

Mercurio et al. (2004) exposed gametes of *Acropora microphthalma* to mineral derived oil WAF in culture plates for four hours to monitor effects of exposure on fertilization. Mineral derived oil was significantly more toxic when compared to vegetable derived oil, with fertilization significantly inhibited at 200 µg/L. When fertilization was successful, embryonic development was unusual, and outer cell membranes were disrupted; these changes reduce chance of survival and settlement.

Villanueva et al. (2008) exposed planula of five coral species to WAF of Malampaya natural gas condensate for 96 hours to monitor survivorship, metamorphosis, and post-settlement growth. Mortality was increased for two of the five coral species in response to exposure, but LC_{50} values were higher than any concentration tested, with the other three species incurring no mortality. Metamorphosis was delayed or impeded in four of the species tested, with the concentration leading to inhibition of metamorphosis in 50%

of the population (MIC_{50}) calculated between 25 and >100% WAF. Post-settlement growth was reduced in three species, with two of the species experiencing no significant reduction in growth. Both *Seriatopora* and *Stylophora* spp. were more effected by increasing WAF concentrations than the *Pocillopora* species tested.

Villanueva et al. (2011) examined the effects of Malampaya natural gas condensate on gametogenesis and embryogenesis of *Pocillopora damicornis* following a 24 hour exposure. Coral fragments exposed to higher WAF concentrations incurred heavy mortality but gametogenesis was unaffected, as all corals planulated in high numbers when polyps were alive. The increase in mortality caused a reduction in number of reproducing polyps, which impairs reproductive output directly. Following exposure during late embryogenesis, concentrations of 50% and greater WAF led to dose dependent larval abortion immediately after onset of exposure. If the exposure occurred early in embryogenesis, larvae released were significantly smaller and metamorphic competency was reduced, while exposure later in embryogenesis led to fully developed planula with 100% metamorphosis. It is apparent that time of exposure during the reproductive cycle plays a major role in the effects corals will incur.

Goodbody-Gringley et al. (2013) completed a suite of experiments measuring the effects of weathered and fresh Macondo crude oil on *Porites astreoides* and *Orbicella faveolata* planula. The effects of weathered oil on swimming behavior, settlement, and mortality of *P. astreoides* were examined for up to 120 hours. Weathered oil (267 mg/L) resulted in no significant changes to behavior or mortality when compared to controls; however, larvae which contacted oil failed to settle or metamorphose. Weathered oil (567 mg/L) led to a significant increase in mortality after 24 and 72 hours. For the first 48 hours, no differences in settlement were observed, but after 72 hours, no new settlement in the oil treatments occurred while controls continued to settle. Post-settlement survivorship also decreased following exposure to 3,500 mg/L weathered oil. The effects of WAF, CEWAF, and dispersant only on *P. astreoides* were also examined using fresh Macondo crude oil and Corexit® 9500. Exposure to WAF solicited no effect on settlement after 48 hours for all concentrations, but a significant response occurred after 72 hours, where increased WAF concentration led to decreased settlement. After 48 hours, survival was significantly reduced, leading to a 48-hour LC_{50} of 0.51 ppm. Exposure to increased concentrations of

CEWAF reduced settlement after 48 hours and reduced survival after 72 hours in the 4.28 mg/L and 30.99 mg/L treatments. The 72-hour LC_{50} of CEWAF for *P. astreoides* was calculated at 1.84 ppm. The same pattern occurred for dispersant only treatments, whereas increased concentrations led to decreased settlement and survival after 72 hours, leading to a 72-hour LC_{50} of 33.4 ppm.

Orbicella faveolata larvae were exposed to WAF, CEWAF, and dispersant only in two experiments; a 48-hour constant exposure, and a 96-hour spiked exposure. The 48-hour constant exposure to WAF revealed a negative relationship between WAF and settlement; increased WAF led to decreased settlement. Larval settlement and survival were both significantly reduced at all concentrations, producing a 48-hour LC_{50} of 0.50 ppm. Similarly, exposure to increasing CEWAF concentrations led to decreased settlement and survival, and the 48-hour LC_{50} for CEWAF was calculated at 0.28 ppm. Exposure to dispersant only also resulted in reduced settlement and survival as concentration of dispersant increased, producing a 48-hour LC_{50} of 19.7 ppm. Survival was monitored during the 96-hour spiked exposure, and was significantly reduced in all treatments. The 96-hour LC_{50} for WAF, CEWAF, and dispersant only were calculated at 0.45 ppm, 0.12 ppm, and 343.8 ppm respectively.

This study allowed direct comparison of effects associated with weathered and fresh crude oil on *P. astreoides.* Mortality occurred within the first 24 hours of exposure to fresh WAF, compared to 48 hours of exposure to weathered oil. This suggests fresh oil is more toxic than weathered oil, which could be linked to the presence of the more toxic, volatile components in fresh oil. It was also apparent that increased WAF resulted in decreased settlement and survival for both corals examined, although *P. astreoides* was more tolerant. Increased concentrations of CEWAF also resulted in decreased settlement and survival for both coral species, and higher concentrations of dispersed oil resulted in settlement failure and complete mortality. The authors concluded that the application of dispersants potentially increases the toxicity of oil exposure.

Hartmann et al. (2015) exposed *Agaricia humilis* and *Orbicella faveolata* larvae to seawater from a site that was polluted by Venezuelan light crude oil from a land-based facility on the southern coast of the Caribbean island Curacao. Survival of *O. faveolata* decreased by 10% after 6 days exposure, with an 85% reduction in settlement. After 10

days of recovery, survival was reduced to 75% of control values. Survival of *A. humilis* was unaffected by exposure, but a 40% reduction in settlement occurred. Corals were also exposed to the WAF of the same crude oil in six-day static assays. Survival was initially unaffected, but declined after 10 days in clean seawater for both *O. faveolata* and *A. humilis*. Settlement was also reduced in both corals following the exposure to WAF.

Negri et al. (2016) assessed the effects of the WAF of natural gas condensate, and four single aromatic petroleum hydrocarbons on settlement of *Acropora tenuis* larva. Larvae were exposed to dilutions of WAF for 24 hours and settlement was assessed following an 18 hour period in culture plates with addition of crustose coralline algae extract to initiate settlement and metamorphosis. Composition and concentration of 100% WAF solutions were analytically verified in order to analyze the contribution of each constituent hydrocarbon by applying the toxic unit approach. Larvae exposed to <100 µg/L TPAH exhibited normal settlement and metamorphosis with development becoming increasingly inhibited at higher concentrations, producing an IC_{50} of 339 µg/L TPAH. Abnormal development and partial metamorphosis without attachment occurred in 54% of the larvae exposed to 5,600 µg/L TPAH. Larvae experiencing concentrations >3,900 µg/L TPAH exhibited abnormal development of polyps with no recovery following isolation in clean seawater for 48 hours. The single hydrocarbons tested were ranked based on toxicity to *A. tenuis* larvae (naphthalene> xylene> toluene> benzene). The IC_{50}s generated for each aromatic hydrocarbon were used to predict the toxicity of 100% WAF, resulting in 0.85 toxic units. This value proved to be 39 fold less toxic than the measured toxicity of the natural gas WAF, suggesting early developmental stages of corals are impeded by an additional non-additive effect of petroleum hydrocarbons.

Table A 4. Summary of *ex situ* experiments assessing the impact of petroleum hydrocarbons on coral reproduction.

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Stylophora pistillata	Iranian crude oil	0.1, 1, 2.5, 5 mL WSF/L seawater Nominal	WSF of oil added to jars in static exposure	Immediate mouth opening and extensive symbiont extrusion. Larvae prematurely shed, and took significantly longer to develop to settlement stage.	Loya and Rinkevich 1979
Stylophora pistillata	Iranian crude oil	Weekly addition of 3 mL/L WSF for 2–6 months Nominal	Oil floated on surface of 1500-L tanks for 24 hours per week	Significant decline in average number of female gonads per polyp. 80% polluted corals died after 6 months.	Rinkevich and Loya 1979
Pocillopora damicornis	gasoline:motor oil (50:1 ratio)	5, 10, 50, 100 ppm Nominal Diluted from stock	15-day static exposure in open 50-mL petri dishes	No mortality observed. Planulae metamorphosed, settled, and calcified in control and 50 ppm, no settlement response in others. Significant treatment effect on settlement.	Te 1991
		1, 5, 20, 100 ppm Nominal Diluted from stock	15-day static exposure in 200-mL closed glass bottles	Corallite formation after 3 days exposure in most treatments. 100% mortality in 100 ppm after 2 days. Significant treatment effect on settlement.	
	Benzene	1, 5, 20, 100 ppm Nominal Diluted from stock	10-day static exposure in 200-mL closed glass bottles w/out settling plates	No mortality observed. No settlement in 1 ppm but no significant treatment effect.	
			6-day static exposure in 200-mL closed glass bottles with settling plates	More corallite formation compared to without plate treatment. Significant treatment effect on settlement, but variability was high.	

Table A4 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Heteroxenia fuscescens	Israel crude oil	1– 5000 ppm Nominal	3-day static exposure to floating oil in 24 well culture plates.	Significant decreased in ability to metamorphose with increasing oil concentration. 50% at 10 ppm Variable mortality: 67% mortality at 500 ppm, 33% mortality at 1000 ppm.	Kushmaro et al. 1997
		0.1–5000 ppm Nominal	3-day static exposure to oil coated vessels	Significant metamorphosis inhibition at concentrations as low as 0.1 ppm (50%). Inhibition of metamorphosis and mortality significantly dependent upon crude oil concentration. Increased free floating metamorphosis and post metamorphosis deformation of polyps.	
Stylophora pistillata* and *Heteroxenia fuscescens	Egyptian crude oil and 5 dispersants: Inipol IP-90 Petrotech PTI-25 Biosolve Bioreico R-93 Emulgal C-100	5 mL/L oil Stock diluted to 0.1, 1, 10, 50, 100% Nominal	2–96 hour static bioassays in 5-mL culture dishes containing WSF of test solutions.	*S. pistillata*: No mortality observed. Reduced/delayed settlement in WSFs. No alterations in settled polyp morphology or larval swimming behavior.	Epstein, Bak, and Rinkevich 2000
		0.5 mL dispersant added to 5 mL/L oil Stock diluted to 0.1, 1, 10, 50, 100% Nominal		*S. pistillata:* Complete mortality at concentrations >10% after 96 hour (except Petrotech). No successful settlement and major behavioral anomalies and structural deformations at all concentrations. H. fuscescens: High toxicity of all; no settlement, with major behavioral anomalies and structural deformations at all concentrations.	
		0.5 mL/L dispersant Stock diluted to 0.1, 1, 10, 50, 100% Nominal		*S. pistillata:* No mortality observed in 0.1 and 1 % treatments, all other concentrations highly toxic, except Petrotech. All dispersants significantly reduced settlement compared to controls, but not significantly different than WSF. Planulae morphology deformed at all concentrations except 0.1%.	

Table A4 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Acropora millepora	Wandoo platform heavy crude oil and Corexit EC9527A	Oil WAF stock (1:99 oil: seawater) 1.65 ppm THC Measured but nominal after dilution.	Static bioassays carried out in static 6-well culture plates for 4 hours	Failed to inhibit fertilization up to 0.165 ppm THC Metamorphosis significantly inhibited at 0.0824 ppm THC, completely inhibited at 0.165 ppm THC	Negri and Heyward 2000
		1% v/v dispersant/oil stock 22.5 ppm THC Measured but nominal after dilution.		Fertilization significantly inhibited at 0.225 ppm THC, completely inhibited at 0.325 ppm THC Metamorphosis significantly inhibited at 0.225 ppm THC, completely inhibited at 1.13 ppm THC	
		10% v/v dispersant/oil stock 32.5 ppm THC Measured but nominal after dilution.		Fertilization inhibited at 0.0325 ppm THC Metamorphosis inhibited at 0.0325 ppm THC.	
		Dispersant-only stock		Fertilization inhibited at 10 ppm dispersant. Metamorphosis significantly inhibited between 5 and 10 ppm THC	
Acropora tenuis, Platygyra sinensis, Goniastrea aspera	Bunker Fuel Oil 467 and Ardrox 6120 dispersant	Oil WAF stock: 1:10 oil:SW. Measured but nominal after dilution.	Static exposure in 21-mL sealed static glass vials and placed on reef	Increased mortality of *A. tenuis* but not significant. 48-hour LC50 for 3 day old planulae: 6.1 ppm 96-hour LC50 for 3 day old planulae: 5.9 ppm 96-hour LC50 for 11 day old planulae: 3.38 ppm Significantly increased mortality of *G. aspera* with higher concentration, but too low for LC50. *P. sinensis* least sensitive, with no evidence of significant toxic effects to any concentrations.	Lane and Harrison 2000
		Dispersed oil stock (20 mL oil+1.5 mL dispersant) 750 ppm Dispersed oil WAF Nominal		Significantly increased mortality in all species with increased toxic effect over time. *A. tenuis*: 3-day old 96-hour LC50: 0.6 ppm 11-day old 96-hour LC50: 0.7 ppm *G. aspera*: 24-hour LC50: 3.5 ppm, 96-hour LC50: 0.8 ppm *P. sinensis*: 12-hour LC50: 8.2 ppm, 96-hour LC50: 1.5 ppm	

Table A4 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Acropora tenuis, Platygyra sinensis, Goniastrea aspera	Bunker Fuel Oil 467 and Ardrox 6120 dispersant	Dispersant only stock at 750 ppm Nominal	Static exposure in 21-mL sealed static glass vials and placed on reef	*A. tenuis:* 12-hour LC50 for 3-day old planulae: 32.8 ppm 96-hour LC50 for 3-day old planulae: 25.4 ppm 12-hour LC50 for 11-day old planulae: 35 ppm 96-hour LC50 for 11-day old planulae: 8.3 ppm *G. aspera:* 24-hour LC50: 49.2 ppm, 96-hour LC50: 12.5 ppm *P. sinensis:*12-hour LC50: 35 ppm, 96-hour LC50: 23.7 ppm	Lane and Harrison 2000
Acropora microphthalma	Mineral derived (MDL) and Vegetable derived (VDL)	Stock WAF (9:1 SW:oil) Measured 100% MDL: 400 µg/L 100% VDL: 600 µg/L Nominal after dilution	Gametes exposed to dilutions of stock WAF in static 6-well cell culture plates for 4 hours	Fertilization inhibited at 50% MDL (53% fertilization). 7.5% fertilization in 100% MDL. 26% fertilization in 100% VDL MDL significantly more toxic than VDL Inhibition accompanied with unusual embryonic development and disruption of outer cell membrane.	Mercurio et al. 2004
Seriatopora hystrix, Seriatopora guttatus, Stylophora pistillata, Pocillopora verrucos, Pocillopora damicornis	Malampaya natural gas condensate	10 mL/L stock WAF prepared and diluted to 1, 10, 50, 100% Nominal	96-hour static exposure to WAF in glass culture dishes	Survivorship: Increased mortality for *S. hystrix* and *S. guttatus*. No mortality of other larvae. Metamorphosis: *Seriatopora* and *Stylophora* spp. delayed or impeded metamorphosis. MIC50s: *S. hystrix*- 60% WAF, *S. guttata*- 25% WAF, *S. pistillata*- >100% WAF. *P. verrucosa* had decreased metamorphosis, with complete inhibition at 50 and 100% WAF. Post-metamorphosis growth: Decreased growth rates for *S. hystrix*, *S. guttata*, and *S. pistillata*. No significant differences in growth for *P. damicornis* or *P. verrucosa*.	Villanueva et al. 2008
Pocillopora damicornis	Malampaya natural gas condensate	10 mL/L stock WAF prepared and diluted to 10, 25, 50, 75, 100% Nominal	24-hour static exposure to WAF in 300 mL glass jar, returned to reef and larval release monitored.	Increased mortality when exposed to higher WAF. Gametogenesis: No sublethal reproductive effects, all corals planulated in high numbers. Embryogenesis: 50% and greater WAF led to dose dependent larval abortion. Exposed early in embryogensis, significantly smaller larvae, with lower metamorphic competency. Exposed late in embryogenesis, planula fully developed and metamorphosis is 100%.	Villanueva et al. 2011

Table A4 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Porites asteroides	Weathered Macondo crude oil	4 mg weathered oil in 15 mL seawater (267 ppm)	Static exposure in petri dishes	No significant difference in behavior or mortality. Larvae contacting oil never settled or metamorphosed.	Goodbody-Gringley et al. 2013
		8.5 mg weathered oil in 15 mL seawater (567 ppm)	24–120 hour static exposure in glass scintillation vials	Mortality significantly higher in 24 and 72-hour exposed larvae when compared to controls.	
		35 mg weathered oil in 10 mL seawater (3,500 ppm)	Static exposure in petri dishes	No differences in settlement after 48 hours in all treatments. After 72 hours, no new settlement in oil exposed larvae, while controls continued to settle. Increased mortality in oil exposed larvae. Post-settlement mortality greater than controls.	
	Macondo crude oil and Corexit 9500	WAF at 0.32, 0.33, 0.62 ppm Measured	48–72 hour static exposure in 250-mL beakers with cured plaster tiles	No correlation between WAF concentration and settlement after 48 hours. Significant dose response after 72 hours, increased WAF decreased settlement. Survival significantly reduced 48-hour LC50= 0.51 ppm	
		CEWAF at 0.71, 4.28, 30.99 ppm Measured		Increased CEWAF, decreased settlement. 48 hours: medium and high both 0% settlement. Survival significantly reduced. 72-hour LC50= 1.84 ppm	
		Dispersant only at 25, 50, 100 ppm		Increased dispersant, decreased settlement. Survival significantly reduced. 72 hour LC50= 33.4 ppm	

Table 4 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Orbicella faveolata	Macondo crude oil and Corexit 9500	WAF at 0.65, 1.34, 1.5 ppm Measured	48 hour static exposure in 250 mL beakers with cured plaster tiles	Negative relationship between WAF and settlement (increase WAF, decrease settlement). Settlement and survival significantly reduced at all WAF concentrations LC50= 0.50 ppm	Goodbody-Gringley et al. 2013
		CEWAF at 14.73, 18.56, 35.76 ppm Measured		Settlement significantly reduced at all concentrations (<4%, 75% in controls). Survival significantly reduced at all concentrations (<4% in all, compared to 87% in controls). LC50= 0.28 ppm	
		Dispersant only at 25, 50, 100 ppm Measured		Settlement significantly reduced at all concentrations (<5%, 75% in controls). Survival significantly reduced in all concentrations. LC50= 19.7 ppm	
		WAF at 0.49, 0.51, 0.84 ppm Measured	96-hour flow-through, spiked exposure in 270 mL exposure chambers	Survival significantly reduced in all concentrations 96 hour LC50= 0.45 ppm	
		CEWAF at 0.86, 30.06, 42.08 ppm Measured		Survival significantly reduced in all concentrations 96 hour LC50= 0.12 ppm	
		Dispersant only at 500, 1000, 1500 ppm Measured		Survival significantly reduced in all concentrations 96 hour LC50= 343.8 ppm	
Agaricia humilis	18 day post oil spill seawater Venezuelan light crude oil	145 µg/L total mineral oil	6 day static exposure in 15 mL scintillation vials	Survival unaffected by exposure. Settlement declined by 40% compared to non-oil contaminated seawater.	Hartman et al. 2015
	WAF of Venezuelan light crude oil	100% WAF Measured 550 µg/L Nominal after dilution		Survival unaffected by exposure but reduced after 10 days post exposure. Settlement declined after exposure.	

Table A4 Continued

Coral Species	Oil Type	Concentration	Assay Condition	Effects Measured	Reference
Orbicella faveolata	21 day post oil spill seawater. Venezuelan light crude oil	135 µg/L total mineral oil	6 day static exposure in 15 mL scintillation vials	10% reduction in survival compared to non-oiled seawater. After 10 days recovery, survival was 25% lower than non-oiled seawater. Settlement reduced by 85% in oil contaminated seawater	Hartman et al. 2015
	WAF of Venezuelan light crude oil	100% WAF Measured 550 µg/L Nominal after dilution		Survival unaffected by exposure but declined after 10 days post exposure. Settlement declined after exposure.	
Acropora tenuis	Natural Gas Condensate (similar to Type I light crude oil)	100% WAF Measured =12,877 µg/L TPAH (12,720 µg/L BTEX; 157 µg/L PAHs) Nominal after dilution	24-hour static exposure to WAF in 7 mL glass vials. With 18-hour metamorphosis assessment in 12 mL culture plates	<100 µg/L TPAH = normal settlement and metamorphosis. Increasingly inhibited development at higher concentrations. IC10 = 103 µg/L TPAH IC50 = 339 µg/L TPAH Concentrations above 3,900 µg/L TPAH led to abnormal development of polyps, partial metamorphosis without attachment, and had no recovery after isolation in clean seawater.	Negri et al. 2016
	Benzene	100% WAF Measured at 630,000–465,608 µg/L averaged 547,804 µg/L Nominal after dilution		IC50 = 80,351 µg/L (70,661 – 91,369 µg/L) IC10 = 31,087 µg/L (23,508 – 41,109 µg/L) NOEC = 34,000 µg/L	
	Toluene	100% WAF Measured at 140,000–100,000 µg/L averaged 120,000 µg/L Nominal after dilution		IC50 = 15,559 µg/L (13,054 – 18,543 µg/L) IC10 = 8,282 µg/L (5,416 – 12,666 µg/L) NOEC = 15,000 µg/L	
Acropora tenuis	Xylene	100% WAF Measured at 36,000–41,000 µg/L averaged 38,500 µg/L Nominal after dilution	24-hour static exposure to WAF in 7 mL glass vials. With 18-hour metamorphosis assessment in 12 mL culture plates	IC50 = 3,939 µg/L (3,423 – 4,532 µg/L) IC10 = 2,160µg/L (1,576 – 2,960 µg/L) NOEC = 4,800 µg/L	
	Naphthalene	100% WAF Measured at 15,000 µg/L Nominal after dilution		IC50 = 2,077 µg/L (1,694 – 2,458 µg/L) IC10 = 1,285 µg/L (797 – 2,070 µg/L) NOEC = 1,900 µg/L	

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APPENDIX 2

This appendix includes the semi-quantitative scale for scoring histology tissue sections to evaluate general condition of coral and algal cells, epidermal and gastrodermal integrity, and presence of tissue ruptures. This scoring system assessed the severity and extent of multiple categories (general cellular condition, zooxanthellae, gastrodermal and epidermal integrity of the surface and basal body walls).

Table A5. Histology Scoring Rubric

Parameter	0 Normal	1 Minimal	2 Mild	3 Moderate	4 Marked	5 Severe
General Condition 10X magnification	Similar to controls. Intact epithelia and mesoglea, highly cellular with well-developed polyp and coenenchyme architecture.	Similar to controls. Epithelia and mesoglea not as thick, with slight atrophy of epidermal mucocytes.	Atrophy of epidermal mucocytes, intact epithelia and mesoglea not atrophied. Mesentery and filament architecture normal	Hypertrophy of epidermal mucocytes, minimal atrophy of epithelia and mesoglea. Damage to epithelia present. Polypal architecture compromised.	Polypal architecture lost, moderate atrophy of epithelia and mesoglea. Degenerating architecture of mesentery and filaments.	Severe atrophy of epithelia and mesoglea vacuolation of mesogleal pleats. Necrosis and degeneration of mesenterial filaments
Zooxanthellae 40X magnification	Gastrodermal cells packed with well stained symbionts in surface body wall and tentacles.	Similar to controls. Slightly fewer and scattered symbionts in gastrodermis of surface body wall and tentacles.	Layer of well stained symbionts but less abundant than controls.	Fewer symbionts in gastrodermis, which is mildly atrophied. Most still stain appropriately, but some degenerating.	Even fewer symbionts in gastrodermis of surface body wall and tentacle. Some loss of acidophilic staining	No symbionts present in gastrodermis of colony. (Bleached)
Epidermal Architecture: SBW	Short columnar cells, uniform distribution and not taller than ciliated supporting cells, pale mucus.	Slightly atrophied, attenuated over septal ridges. Minimal lysing.	Many cells atrophied, severe attenuation over septal ridges. Moderate lysing	Moderate lysing and fragmentation. Cells more squamous.	Abundant fragmentation and lysing.	Epidermis severely atrophied to at least half normal thickness or more.
Ruptures: SBW	Epithelia and mesoglea intact	One or few beaks in epithelia	One-quarter of epithelial area has structural gaps, mucus discharge.	One-half of epithelial area has structural gaps, mucus discharge.	Three-quarters of epithelial area has structural gaps, mucus discharge.	Entire surface body wall dissociated, extensive mucus release. Full thickness ablation evident.

Table A5 Continued

Parameter	0 Normal	1 Minimal	2 Mild	3 Moderate	4 Marked	5 Severe
Mucocytes: SBW	Similar to controls, pale staining mucus.	Slight atrophy of mucocytes. Frothy mucus. With some release	Atrophy of mucocytes. Abundant release of mucus.	Uneven appearance of mucocytes, some atrophied, some reduced in size and secretion, darker staining mucus.	Some epidermal foci lack mucocytes entirely, darker staining and stringy mucus.	Loss of mucocytes.
Granular Amoebocytes in SBW	None present.	One to a few or almost all dead.	One quarter of area contain these cells or three quarters dead.	Half of the area contain these cells or half dead.	Three- quarters of the area contain these cells or one-quarter dead.	Epidermis and gastrodermis heavily infiltrated by these cells, none dead.
Surface Body Wall Necrosis	No necrosis or apoptosis.	Pycnotic nuclei rare.	Necrosis in about one-quarter epidermis area.	Necrosis in about one-half epidermis area.	Necrosis in about three-quarters epidermis area.	Full thickness ablation evident.
Gastrodermal Architecture: SBW	Gastrodermis of surface body wall intact.	One or few areas of gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released	One-quarter of the area of gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released	One-half of the area of gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released	Three-quarters of the area of gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released	All gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released
Gastrodermal Architecture: BBW	Gastrodermis of basal body wall intact.	One or few areas of gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released	One-quarter of the area of gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released	One-half of the area of gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released	Three-quarters of the area of gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released	All gastrodermal cells lysed, mucus filling gastrovascular canals/ cavity. Granular gland cells, nematocysts and zooxanthellae released

Table A5 Continued

Parameter	0 Normal	1 Minimal	2 Mild	3 Moderate	4 Marked	5 Severe
Mucocytes: BBW	Similar to controls, pale staining mucus.	Slight atrophy of mucocytes. Frothy mucus with some release.	Atrophy of mucocytes. Abundant release of mucus.	Mucocytes present with no staining.	Mucocytes lysed and degraded.	Loss of mucocytes.
Granular Amoebocytes in Mesenteries and BBW	None present or all dead.	One to a few or almost all dead.	One quarter of area contain these cells or three quarters dead.	Half of the area contain these cells or half dead.	Three- quarters of the area contain these cells or one-quarter dead.	Epidermis and gastrodermis heavily infiltrated by these cells, none dead.
Basal Body Wall Necrosis	No necrosis or apoptosis present	Pycnotic nuclei rare	Necrosis in about one-quarter gastrodermis area.	Necrosis in about one-half gastrodermis area.	Necrosis in about three-quarters gastrodermis area.	Complete necrosis of gastrodermis
Calicodermis	Squamous to low columnar cells, fine acidophilic granules of organic matrix proteins present.	Slight atrophy of calicodermis with fewer acidophilic granules	More variable in thinning of calicodermis, fewer acidophilic granules, and more areas affected.	Squamous calicodermis, fewer acidophilic granules, lysing in some areas	Squamous calicodermis, necrotic or lysing, no acidophilic granules	Loss of calicoblasts along mesoglea, necrotic or lysing.

Gonad presence= 0 (none) or 1 (present)

Extent of changes:

1. Focal

2. Multifocal

3. Diffuse

Severity*Extent = parameter score. Sum each corals parameter scores to get the coral's **Specific Condition Score.**