AN EVALUATION OF HYDROCARBON DIGESTING MICROBES AND CHEMICAL DISPERSANT ABILITY TO REDUCE LETHAL AND SUBLETHAL EFFECTS OF OIL ON *PALAEMONETES* SPP.

A Thesis

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This thesis meets the standards for scope and quality of
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ABSTRACT

Marine oil spills are a serious concern to government, private, and non-governmental organizations as well as to the public at large. Chemical cleaners and bioremediation techniques can be useful in combating these disasters, but the potential synergistic effects are not well understood. In this research, the toxic effects of mixtures of a dispersant and commercial microbial solution on grass shrimp (*Palaemonetes* spp.) were compared by determining lethal concentrations and mortality over time. The addition of the microbial amendment significantly reduced mortality when they were exposed to an oil/dispersant solution. Increased mixing time of both dispersant and microbial treatments prior to shrimp exposure also increased their survival. There was no significant difference in the mean 48-hour survival rate of shrimp in dispersant and microbial-amended treatments with the same mixing time. The addition of microbial solutions to dispersed oil may produce a net positive effect on survival rates by speeding oil spill remediation and, when feasible, can augment existing techniques.

DEDICATION

This work is dedicated to my wife Heather, son Seth, daughter Rue, and nephew Jonah. Without their support and sacrifices, this would not have been possible.

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I would like to thank my committee chair, Dr. Zimba, and my committee members, Dr. Withers and Tony Wood, for their guidance and support throughout this research.

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TABLE OF CONTENTS

CONTENTS PAGE

ABSTRACT v

DEDICATION vi

ACKNOWLEDGEMENTS vii

TABLE OF CONTENTS viii

LIST OF FIGURES ix

LIST OF TABLES xi

INTRODUCTION 1

MATERIALS AND METHODS 7

- General Setup 7
- LC_{50} Experiments 9
- Mixing Time as a Lethality Factor 9
- Sublethal Effects 10
- Statistical Analysis 12

RESULTS 12

- LC_{50} 12
- Mixing Time as a Factor 15
- PAH Analysis 18
- Sublethal Effects 22

DISCUSSION 26

LITERATURE CITED 35

LIST OF FIGURES

FIGURE PAGE

Figure 1: LC_{50} of amended WAF mixes. Error bars represent ±1 standard deviation units when visible…….13

Figure 2: Corexit and MicroBlaze LC_{50} – dashed lines indicate ±1 standard deviation unit. The LC_{50} curve of MicroBlaze is within the LC_{50} curve of Corexit and Corexit +1σ…….14

Figure 3: LC_{50} of Amendment Mixes. The 1:1 C:MB and 3:1 C:MB treatments overlay each other ……..……...14

Figure 4: Percent survivors of WAF/C and WAF/C/MB for all treatments and observation times. The WAF/C/MB treatment had significantly higher survival compared to the Corexit treatment ($P < 0.05$)...……...…….....15

Figure 5: Percent survivors by mixing time for WAF/C treatment over a 48-hour exposure period post-mixing. Error bars indicate standard error……...……..16

Figure 6: Percent survivors by mixing time for WAF/C/MB treatment over a 48-hour exposure period post-mixing. Error bars indicate standard error……...……..16

Figure 7: Total PAH increases between 0-hour and 36-hour mix time solutions as a percentage of total change. Percentages after the solution name indicate the total percent increase of all PAH concentrations. Pyrene increased by 1 µ/L in the WAF/MB solution and is not shown on the chart……..……..19

Figure 8: Total PAH reductions between 0-hour and 36-hour mix time solutions as a percentage of total change. Percentages after the solution name in the legend indicate the total percent reduction of the top ten decreased PAH concentrations……21

Figure 9: Percent decrease in O_2 by survivor. Full survivorship during baseline was assumed. The second 24 hours W/C T0 treatment had no survivors and was not charted. Error bars indicate standard error. Columns without error bars indicate where standard error could not be calculated.……22

Figure 10: Results of multiple comparison analysis of observed motility using Tukey's range test. Treatments connected by lines are not significantly different (p 0.05)……..23

Figure 11: Average observed motility of all treatments over time. WAF/C 0-hour mix time has the only significantly different results motility when compared to all other treatments…….24

Figure 12: Average number of molts per experiment by treatment. Error bars indicate standard error……25

Figure 13: Average percentage of total lipids by treatment. Error bars indicate standard error……..…26

LIST OF TABLES

TABLE PAGE

Table 1: WAF, Corexit (C), and MicroBlaze (MB) treatments tested, concentrations resulting in LC_{50} values, and standard error of LC_{50} concentrations…….9

Table 2: Mean survival rate by WAF, Corexit (C), and MicroBlaze (MB) treatment and mixing time……...……10

Table 3: Significant differences ($p < 0.05$) in WAF/C treatment. Numbers in cells show observation times with significant differences. Treatments in the leftmost column have a significantly higher survival rate…….……..……17

Table 4: Significant differences ($p < 0.05$) in WAF/C/MB treatment. Numbers in cells show observation times with significant differences……..…….....17

Table 5: Significant differences ($p < 0.05$) in WAF/C treatment. Numbers in cells show observation times with significant differences in survival. Dark gray shading indicates a higher survival rate in WAF/C/MB treatments while light gray shading indicates higher survival rates in WAF/C treatments…..……18

Table 6: Changes in PAH by percentage of PAH compounds..……20

Table 7: Total PAH reductions between 0-hour and 36-hour mix time solutions as a percentage of total change. N/A indicates the change was not one of the ten largest…….……..21

INTRODUCTION

As deep-water drilling techniques advance and oil consumption rates increase worldwide, offshore oil production is predicted to expand by 20% between 2011–2020 (Zhang 2016). As long as oil is either produced offshore or transported over water, marine oil spills are an unavoidable consequence. The most prominent recent example for the public is likely the 2010 Deepwater Horizon (DWH) spill that followed an explosion aboard the Macondo rig and released 3.19 million barrels of crude oil into the Gulf of Mexico (Lin et al. 2016). Despite safety mechanisms and contingencies, the DWH disaster resulted from "a cascade of equipment failures and human errors" (Houck 2010). The spill damaged and destroyed coastal wetlands, reefs in the area, and impacted benthic biota (White et al. 2012; Mendelssohn et al. 2012). Once oil escaped the wellhead, recovery efforts were largely confined to three techniques: mechanical recovery or skimming, in situ burning, and the application of dispersants. Technology used for mechanical recovery has an approximate 20% maximum capture efficiency for spilled oil (Lessard and Demarco 2000) and in situ burning, while effective, has a small window of sea and spill conditions where it is can be used (Mullin and Champ 2003). During the DWH disaster, both skimming and in situ burns were limited by equipment availability, sea conditions, and the characteristics of the oil itself. A National Incident Command report calculated only 2–4% of total oil was skimmed and 5–6% was burned as part of the DWH cleanup (Lehr et al. 2010). Chemical dispersants (i.e. chemicals which emulsify oil), predominately COREXIT® EC9500A henceforth (Corexit), were widely used with almost 4 million liters applied to surface slicks (Lehr et al. 2010) and an additional 2.9 million liters injected into the wellhead itself (Kujawinski 2011). Dispersants are effective under a wider range of conditions increase the biological recovery rate when used after spills (Lessard and

Demarco 2000.) Their application dispersed between 10–29% of total spilled DWH oil (Lehr et al. 2010).

A remediation project is complete when any additional action would do more harm than good. This requires an understanding of the tradeoff between the cleanup method's efficacy and potential impacts. An example would be the toxic effects of dispersants. The decision to use a chemical dispersant on an oil spill requires an understanding of unwanted toxic effects created by using the dispersant versus the impact of an untreated spill.

Dispersants are surfactants that promote emulsion. Their molecules contain a hydrophilic and lipophilic end. The lipophilic end attaches to oil molecules while the hydrophilic end attaches to water. The oil slick can then be effectively broken up by outside forces such as wind and wave action (Fingas 2010). The increased surface area resulting from dispersant use allows the oil to be naturally degraded through processes such as evaporation, photodegradation, or biodegradation (Fingas 2010). However, while dispersal of oil does increase the speed of recovery, it does not reduce the toxicity of the oil itself and may harm some biologic communities more than oil alone (Mendelssohn et al. 2012). The addition of dispersants to an oil spill may cause more harm because increasing the surface-to-volume ratio of oil increases an organism's exposure (National Research Council 1989; Ortmann et al. 2012). Additionally, the use of a chemical dispersant increases the toxicity of a slick due to the release of polycyclic aromatic hydrocarbons (PAHs) into the water column (Fingas 2010). Hemmer (2011) found a mix of Corexit 9500A and south Louisiana sweet crude was less toxic than the dispersant alone, but was more toxic than the petroleum alone to both mysid shrimp (*Americamysis bahia*), and inland silversides, (*Menidia beryllina*). Alternatively, Fuller (2004) found the addition of dispersants resulted in declining exposure toxicity for opossum shrimp (*Mysidopsis beryllina*) in fresh and weathered crude oil. It is likely the reported changes in

toxicity are at least partially dependent on species, exposure time, test protocol, and materials (Singer 1998).

Despite their relative success, the use of dispersants is very restricted. Dispersants were used in Europe in only 18% of spills between 1995 and 2005 due to a combination of legal factors and concern over secondary effects (Chapman et al. 2007). The guidelines for authorization by both the state and federal government limit their use in the United States. The National Oil and Hazardous Substance Pollution Contingency Plan (NCP) guides response to hazardous substance releases, including offshore oil spills, by federal agencies. The NCP also defines the organizational framework of the National Response System (NRS) by national, regional, and local government entities. Each of these entities has either an organization or single individual who may authorize dispersant use under certain circumstances. The Oil Pollution Act of 1990 assigned Regional Response Teams to develop prespill plans which include establishing three zones regarding dispersant use: preauthorized zones where the Federal On-Scene Coordinator (FOSC) may authorize use, case-by-case basis zones which require the FOSC to consult with other agencies, and exclusion zones where dispersants should not be used (API 2010). Oil slicks move at a rate based on surface wind- and depth-averaged current velocities (Al-Rabeh 1994) making unintentional encroachment possible even when restricted zones are strictly enforced. It is important to understand that while commonalities exist across response plans, the detailed instructions regarding use of dispersants can differ drastically. At all organizational levels dispersant use is strictly regulated based on water depth, distance from shore, and the environmental sensitivity of potentially impacted areas, with use outside these criteria approved on a case-by-case basis (National Research Council 2005). Currently, EPA Region 6 (Texas and Louisiana) has not preapproved dispersant application to coastal waters deeper than 10 m and

greater than 5.6 km from shore. Additional restrictions are in place restricting access where birds, marine mammals, and turtles may be present (Federal Region VI Regional Response Team 2001).

The use of microbial solutions in bioremediation is classified as either bioaugmentation, the direct application of additional microbes, or biostimulation, adding nutrients that promote growth of the desired bacteria and microbes. The potential use of microbial solutions in oil spill cleanup has been studied since at least the 1940s but research intensified in response to the Exxon Valdez spill in 1989. Those research efforts declined due to mixed results, questionable claims, and loss of public interest (Hoff 1993). The DWH disaster both renewed research efforts and provided a real-world laboratory for development of new microbial remediation techniques. Naturally occurring hydrocarbon consuming microorganisms have been studied and identified in every ocean (Biello 2010; Ron and Rosenberg 2014). These microbes, while specialized, are able to biodegrade even very complex, highly branched hydrocarbons (Nunal 2017; Venosa 1997) degrading them into H_2O and CO_2 (Leahy and Colwell 1990). Bioremediation via hydrocarbon digesting microbes potentially provides an effective, nontoxic, and economical augmentation to existing cleanup methods.

Biodegradation is the end state for the majority of oil spilled in a marine environment (Ron and Rosenberg 2014,) but using microbial solutions as a deployable technique is hampered by real-world limitations. While virtually ubiquitous, biostimulation and bioaugmentation have demonstrated little success outside of laboratory conditions (Biello 2010; Ron and Rosenberg 2014). Variables such as system homogeneity and biological interactions make translation from a laboratory to a field environment difficult leading to very mixed results (Swannell 1996; Venosa 2004). Alternatively, vendor claims and anecdotal results touting the effectiveness of specific products are common but are often lacking controls or a clear methodology (Venosa 2004).

Attempts at genetic modification of existing microbes to increase consumption or the culture of naturally occurring microbes have yielded few positive results (Biello 2010; Briones and Raskin 2003). Actual rates of biodegradation are dependent on the mechanism of degradation, the compounds, and the microbe itself (Kleindienst 2015), as well as a mix of abiotic factors such as pH, temperature, and salinity (Leahy and Colwell 1990). Additionally, growth in seawater is limited by available nitrogen and phosphorous making biostimulation necessary (Rosenberg 1998). Microbes also require direct contact with hydrocarbons for degradation to occur. This requires either specifically adapted adhesion mechanisms or emulsification (Rosenberg 1998), often via the production of bioemulsifiers (Ron and Rosenberg 2002).

The synergistic effects of dispersants and bioremedial solutions are not well studied, but there has been research on the effect of dispersants on naturally occurring microbial communities. Studies have shown dispersants may stimulate or inhibit biodegradation depending on the dispersant used and the microbe. Lindstrom and Braddock (2002) found Corexit EC9500A was preferentially mineralized before oil, which was preferred to dispersed oil. Microbial density increased, possibly indicating the dispersant was being used as a carbon source. Using oxygen depletion as a measure of growth and mineralization studies, Traxler and Bhattacharya (1978) stated Corexit 9527 increased bacterial growth rates and metabolism. Corexit 9500 decreased cell viability in *Acinetobacter venetianus* and *Marinobacter hydrocarbon oclasticus* but had virtually no effect on *Pseudomonas pseudoalcaligenes* at the same dilution (Hamdan and Fulmer 2011). A separate study by Mulkin-Phillips and Stewart (1974) tested the effects of four dispersants on five genera of bacteria and found the dispersants supported growth in all cases. In some cases, the dispersant likely inhibited early growth and caused population shifts. Despite increases in population, the overall effectiveness of the

microbes as potential remediation agents was degraded because they preferentially fed on the dispersants themselves. Kleindienst (2015) found dispersants affected the growth of naturally occurring, oil-degrading microbes found in the DWH plume in both positive and negative ways depending on the species. Additionally, she stated, "it is quite possible that microorganisms stimulated by dispersant addition may outcompete natural hydrocarbon degraders." Replacing, rather than augmenting, the naturally occurring microbes, may reduce any potentials benefits of adding microbial solutions.

The increased surface-area-to-volume ratio of micelles formed by dispersants should allow naturally occurring, hydrocarbon-digesting microbes to consume dispersed oil faster than a large slick, thus reducing the impact of an oil spill on marine organisms. In a previous study, Fern (2015) concluded that by adding microbes to dispersant and oil mixtures, the toxicity of lethal concentration of oil decreased from 36 hours to 12 hours (Fern et al. 2015). While the addition of hydrocarbon digesting microbes can reduce the length of time oil is toxic when it is dispersed through the water column (Ramachandran et al. 2004,) the National Oceanic and Atmospheric Administration's (NOAA) *Guide for Spill Response Planning in Marine Environments* currently states microbe seeding cannot be recommended due to a lack of "information on impacts or effectiveness." The results of this research build on previous studies by further examining the changes in toxicity of oil and dispersants over time on a different sentinel estuarine species.

In this research the effects of the synergistic application of chemical dispersants and hydrocarbon-digesting microbes on grass shrimp was explored with the goal of determining how the toxicity of dispersed oil is reduced over time. Expanding treatment options to improve oil spill remediation and restoration efforts will reduce the initial impact of oil spills and oil residency time, directly benefitting the environment while reducing economic impacts. The

results of this research provide better estimates of the toxicity of dispersant/microbe solutions which could improve the efficient use of human and material resources. If feasible, properly focusing resources would lead to both lower initial impacts from a spill event as well as faster recovery rates, which would ultimately reduce costs.

MATERIALS AND METHODS

General Setup

All glassware was triple washed using Alconox (Alconox Inc., White Plains, NY) detergent followed by tap and deionized water rinses, placed into a 20% HNO_3 solution for 24 hours, triple rinsed with deionized water, and allowed to air dry between experiments. All experiments used freshly prepared 35 ppt salt water using Instant Ocean sea salt (Instant Ocean, Blacksburg, VA).

A 36" x 24" x 24", 150-liter aquarium was used as a holding tank. Approximately one quarter of the water was removed from the aquarium and replaced biweekly. Temperature was maintained at 22 °C ± 1 °C. Air was delivered by an Optima aquarium air pump (Laguna, Mansfield, MA). Two water filters, models Tetra Whisper 10 (Tetra Spectrum Brands, Blacksburg, VA) and TopFin Power 30 (PetSmart, Phoenix, AZ) and an Aquarium Systems protein skimmer (Marineland, Blacksburg, VA) were used to maintain water quality. An automatic timer maintained a 12-hour light:dark cycle.

Grass shrimp (*Palaemonetes* spp.) were selected as the test species. *P. pugio, P. intermedius,* and *P. vulgaris* are all found in the area with *P. pugio* being the most common. Shrimp serve as detritivores, primary consumers, and secondary consumers (Anderson 1985) and are ubiquitous in local seagrasses. While *Palaemonetes* are tolerant to a wide range of biotic factors, studies by NOAA suggest their sensitivity to contaminants combined with their use in multiple ecological studies make them suitable for use as an indicator species (Key et al. 2006).

Palaemonetes are an important constituent of the food web, linking benthic production with planktonic species to higher-level piscivores (Knieb and Stiven 1982).

Shrimp were collected via seine and epibenthic sled at approximately 27°39'15" N, 97°15'39" W, near the John F. Kennedy Memorial Causeway in Corpus Christi, Texas. After collection, samples were held in buckets during transport to the laboratory where any animals with evidence of injury or parasitism were removed. Shrimp were acclimated for at least 48 hours prior to experimentation. Shrimp were fed to satiation using a commercial shrimp food containing 30% protein and 5% fat every other day. Prior to feeding, excess food and waste was removed with a skimming net. Shrimp were all ~2 cm in length.

A Water Accumulated Fraction (WAF) solution of West African light sweet crude oil, obtained from local industry contacts, with an American Petroleum Institute (API) specific gravity 35 was used. West African light sweet crude was chosen due to its availability and similar composition to Louisiana sweet crude oil, API of 35.9, that was spilled during the DWH spill in 2010 (Reddy et al. 2012). WAF is a solution made by mechanically mixing oil and water thereby suspending oil droplets throughout the solution. This is different than Chemically Enhanced WAF (CEWAF), an oil/water solution mixed using chemicals. WAF was prepared using a protocol described by Negri and Heyward (2000). A 1% oil/saltwater solution was mixed on a magnetic stir plate at approximately 75% vortex for two hours. The solution was then allowed to separate in a stoppered 4L media bottle for 24 hours. WAF was removed via a bottom side-arm. Local industry contacts supplied Corexit, the most commonly used dispersant during the DWH spill. Verde Environmental, Inc. supplied the microbial solution MicroBlaze® Emergency Liquid Spill Control (MicroBlaze). A 1:30 WAF/Corexit (C) ratio solution and a 1:10 WAF/MicroBlaze (MB) solution were used per manufacturer instructions.

LC_{50} Experiments

Lethal Concentration 50% (LC_{50}) was determined after a 48-hour exposure period for Corexit, MicroBlaze, mixtures of the two amendments, as well as WAF with and without Corexit and MicroBlaze (Table 1). To determine lethal concentrations, individual shrimp were placed into sealed 125 mL clear glass jars containing 75 mL of each treatment for 48 hours. After 48 hours, percent survival was determined. All experiments were repeated 15 times.

Mixing Time as a Lethality Factor

The second experiment determined the effects of dispersant and MicroBlaze amendments on the toxicity of the WAF treatment over time. The concentration of WAF required to kill 80% of a population (LC_{80}) of *Palaemonetes* spp. was determined using linear regression from the LC_{50} and was the initial concentration used in the second experiment. Treatments were mixed with the manufacturer's suggested ratios of Corexit and MicroBlaze in 300 mL of LC_{80} WAF, resulting in a 1:30 dispersant-to-oil and 1:10 microbial amendment-to-oil solution. The experimental design consisted of twelve exposure treatments in triplicate over 48 hours (Table 2). Sealed test jars were oscillated at 80 rpm on an Innova 2000 platform shaker throughout the experiment to simulate

Table 1 WAF, Corexit (C), and MicroBlaze (MB) treatments tested, concentrations resulting in LC_{50} values, and standard error of LC_{50} concentrations

Treatment	**LC_{50} (% concentration)**	**Standard Error**
C	0.082	7.5097
MB	1.26	0.3558
1:1 C:MB	0.046	7.2669
1:3 C:MB	0.286	1.5336
3:1 C:MB	0.057	6.9895
WAF	31.7	0.02742
WAF/MB	42.8	0.07909
WAF/C	48.57	3.527
WAF/C/MB	34.85	0.03744

tidal action and provide a consistent amount of mixing. Treatment bottles were allowed to mix for 0, 12, 24, and 36 hours prior to addition of treatment animals. Four shrimp were added to each 950 mL clear glass jar containing 300 mL of solution and resealed. The experiment was repeated four times for a total n=12. Observations for mortality occurred every six hours to complete a 48-hour observation period.

Sublethal Effects

Concurrent with evaluating mixing time as a lethality factor, four sublethal effects were also measured; oxygen consumption, motility, molting rate, and total lipids. PAH decomposition rates in WAF, WAF/C, and WAF/MB were analyzed and compared.

Oxygen consumption was measured using a PyroScience FireSting O2 Fiber-Optic Oxygen Meter (FSO2-0x) and Oxygen Probe (OXROB3) (PyroScience GmbH, Aachen, Germany) using FireStingO2 software at 15-minute intervals throughout the mixing time as lethal factor experiments. The probe was inserted in a rubber grommet placed in a hole drilled through the lid of the sample jar. The probe was placed approximately halfway into the headspace of the jars and not in the water to allow probes to be reused without contamination between experiments. Teflon tape was wrapped around the threads of the jar and the

Table 2 Mean survival rate by WAF, Corexit (C), and MicroBlaze (MB) treatment and mixing time.

Treatment	Mixing Time	48-Hour Mean Survival %	Standard Error
WAF/C	0	22.91	1.03
WAF/C	12	39.58	0.75
WAF/C	24	54.17	0.29
WAF/C	36	39.58	0.63
WAF/C/MB	0	22.91	0.48
WAF/C/MB	12	45.83	0.5
WAF/C/MB	24	52.08	1.55
WAF/C/MB	36	43.75	1.89

grommet/probe connection to further seal the jars. O_2 was measured for 24 hours prior to the beginning of the experiment in order to provide a baseline consumption rate. Dead shrimp were not removed during the experiment in order to minimize gas exchange.

Motility of individual shrimp was qualitatively evaluated on a scale of 1–3: 3 = no signs of disorientation; 2 = obvious disorientation, swimming sideways or upside down; 1 = movement was confined to only pleopods or other appendages. Dead animals were scored zero and not included in analysis. Observations were made every six hours concurrent with mortality and molting observations. Molted carapaces were counted at every observation period concurrent with mortality and motility observations.

Samples of WAF, WAF/C, and WAF/MB were analyzed for PAH degradation. A WAF solution was created using methods identical to the "mixing time as lethality factor" experiment. Subsamples of the resulting WAF were then used to create WAF/C and WAF/MB solutions. After mixing the solutions, open jars were oscillated at 80 rpm on an New Brunswick Scientific Innova 2000 Platform Shaker (Eppendorf AG, Hamburg, Germany) and mixed for 0, 12, 24, and 36 hours. Samples were then transferred into Thermo Scientific glass 40 mL amber sample bottles and sealed. Sample bottles (Thermo Fisher Scientific, Waltham, MA) were sealed in ice chests and delivered to Dr. Thomas McDonald at Texas A&M University – College Station for analysis.

PAH samples were extracted with 50 mL 4:1 mixture of n-hexane and dichloromethane (DCM) (Guerin 1999) in a 250 mL separatory funnel. After phase separation, extracts were combined and dried in a water bath at 35 °C under a stream of nitrogen. The dried samples were reconstituted to 2 mL with n-hexane.

The extracts were cleaned using solid phase extraction technique. The response factor of individual PAH to the individual internal standard was measured and calculated at least three times at the beginning, during, and at the end of each batch of GC injection.

The Folch method (Folch 1957) was used to determine total lipids. Multiple whole shrimp from the same experiment and treatment were homogenized to obtain the dry weight needed to perform the extraction. All weights were measured to the nearest 0.1 mg using a Sartorius Talent TE124S Balance (Sartorius AG, Göttingen, Germany).

Statistical Analysis

The open-source statistical package RStudio was used to analyze all data. A binomial linear model was used to determine the LC_{50} of all treatments; LC_{50} values are reported as percentages. ANOVA analyses with post-hoc Tukey range testing was used to examine differences between treatments with different mixing times, changes in the motility of test animals, and oxygen consumption over time. Percentages of remaining PAHs over time were compared observationally only due to a lack of replicates. Both mortality and motility were converted to percentages for analysis.

RESULTS

LC_{50}

The addition of either Corexit or MicroBlaze reduced WAF toxicity (Fig. 1). The addition of MicroBlaze resulted in a 35% decrease in mortality while the addition of Corexit resulted in a 53% decrease. The WAF/C treatment had a standard deviation was 44% greater than the WAF/MB treatment. The WAF/C treatment was less toxic than WAF/MB treatment at concentrations less than 34%. When compared to the WAF/MB treatment, the WAF/C treatment

was less toxic at concentrations less than 34%. The mixture of all three ingredients, WAF/C/MB, reduced the toxicity of the WAF by 10%.

MicroBlaze had a higher calculated LC_{50} than the Corexit, but was within the Corexit's standard error (Fig. 2). In mixed solutions, higher ratios of MicroBlaze resulted in both increased LC_{50} values and reduced standard error. While all mixed treatments reduced toxicity, the 1:3 C:MB treatment was the most effective. Both the 1:1 and 3:1 C:MB treatments also reduced toxicity, but to a lesser degree (Fig. 3). In all testing, an increased concentration of Corexit resulted in a larger standard error.

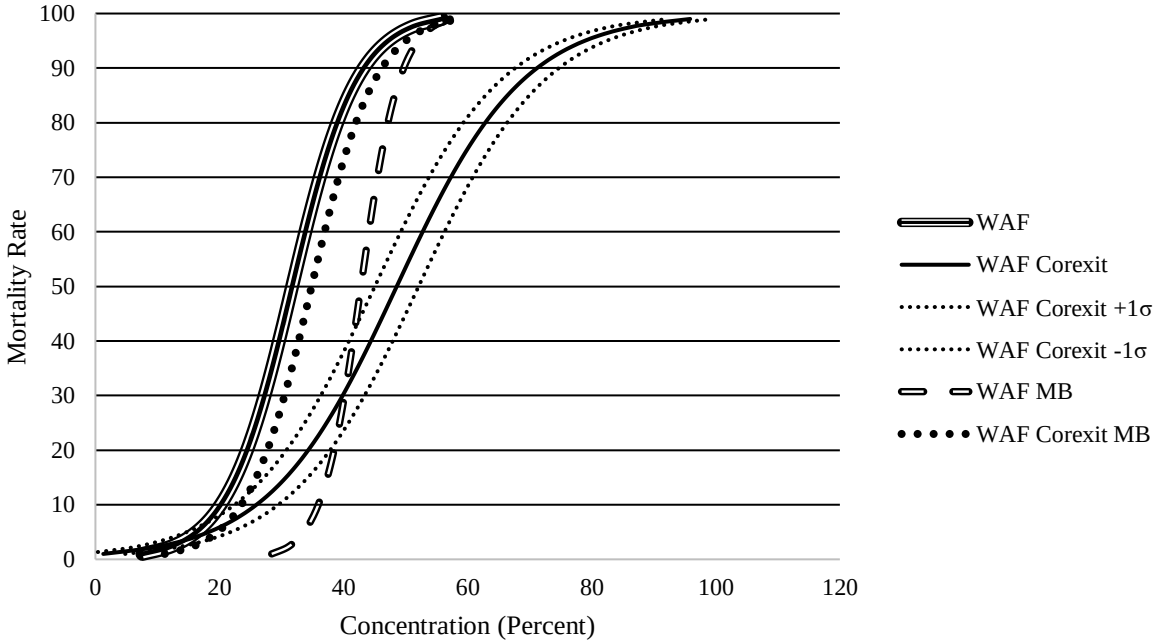

Fig. 1 LC_{50} of amended WAF mixes. Error bars represent ±1 standard deviation units when visible.

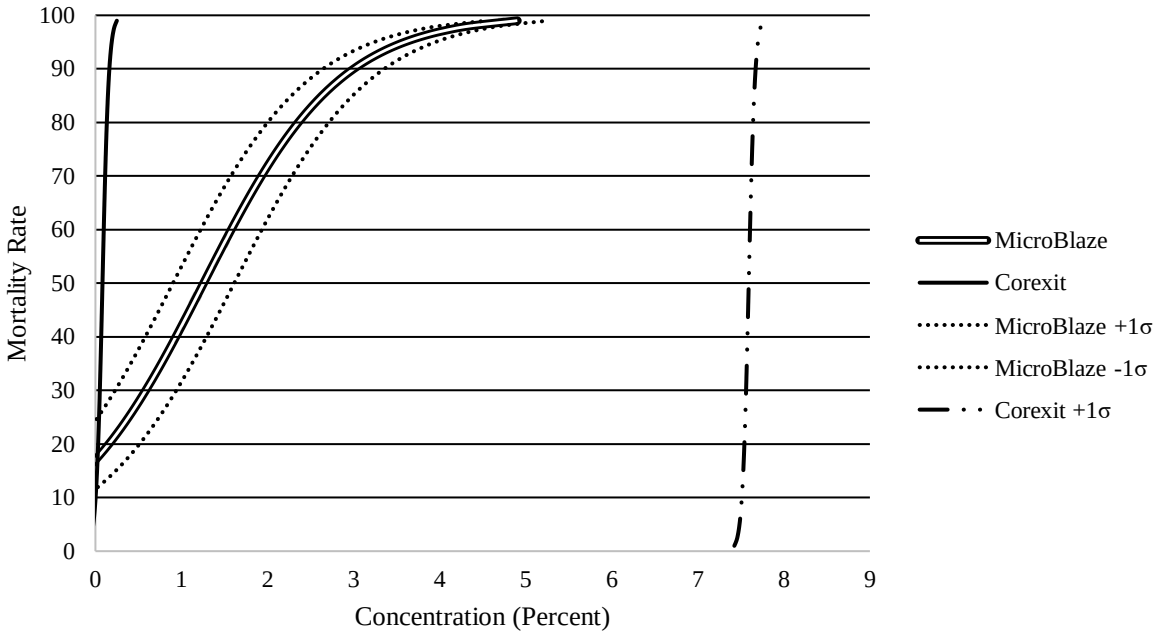

Fig. 2 Corexit and MicroBlaze LC_{50} – dashed lines indicate ±1 standard deviation unit. The LC_{50} curve of MicroBlaze is within the LC_{50} curve of Corexit and Corexit +1σ.

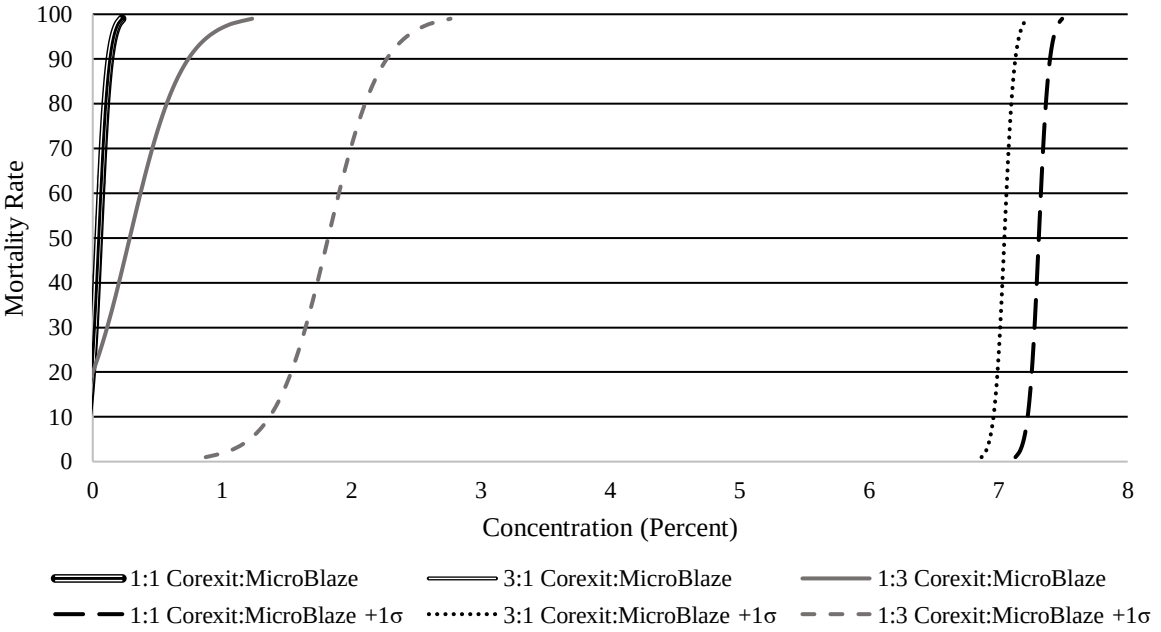

Fig. 3 LC_{50} of Amendment Mixes. The 1:1 C:MB and 3:1 C:MB treatments overlay each other.

Mixing Time as a Factor

When examined across all treatments, there was a statistically significant ($p < 0.05$) improvement in survival rates in WAF/C/MB treatments when compared to WAF/C treatments (Fig. 4). The amount of mixing time also had a significant ($p < 0.05$) effect on survival in both the WAF/C and WAF/C/MB treatments. Increased mixing time resulted in higher survival rates over 0-hour mixed treatments, but a 24-hour mixing time had the greatest effect. The 24-hour mixed WAF/C and WAF/C/MB treatments had a 54% and 52% 48-hour mean survival rate respectively compared to the 0-hour mixed WAF/C and WAF/C/MB treatments which both resulted in a 23% mean survival rate (Table 2). A general pattern of survival held over the course of the experiment. The highest survival rates in both the WAF/C and WAF/C/MB were the 24, 36, and 12-hour mixing time treatments (Fig. 5, Fig. 6).

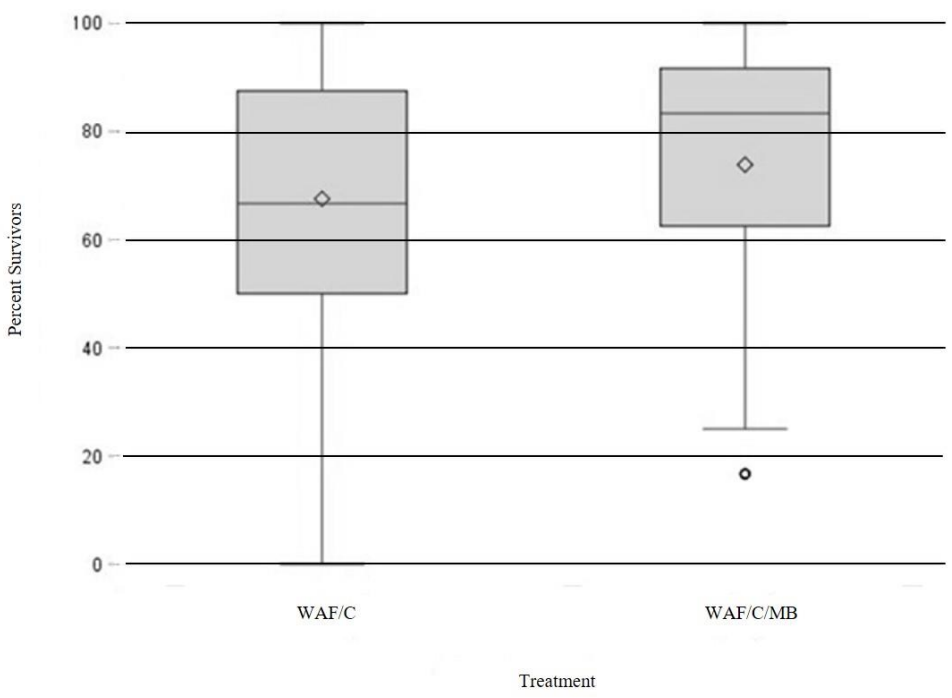

Fig. 4 Percent survival of WAF/C and WAF/C/MB for all treatments and observation times. The WAF/C/MB treatment had significantly higher survival compared to the Corexit treatment ($P < 0.05$).

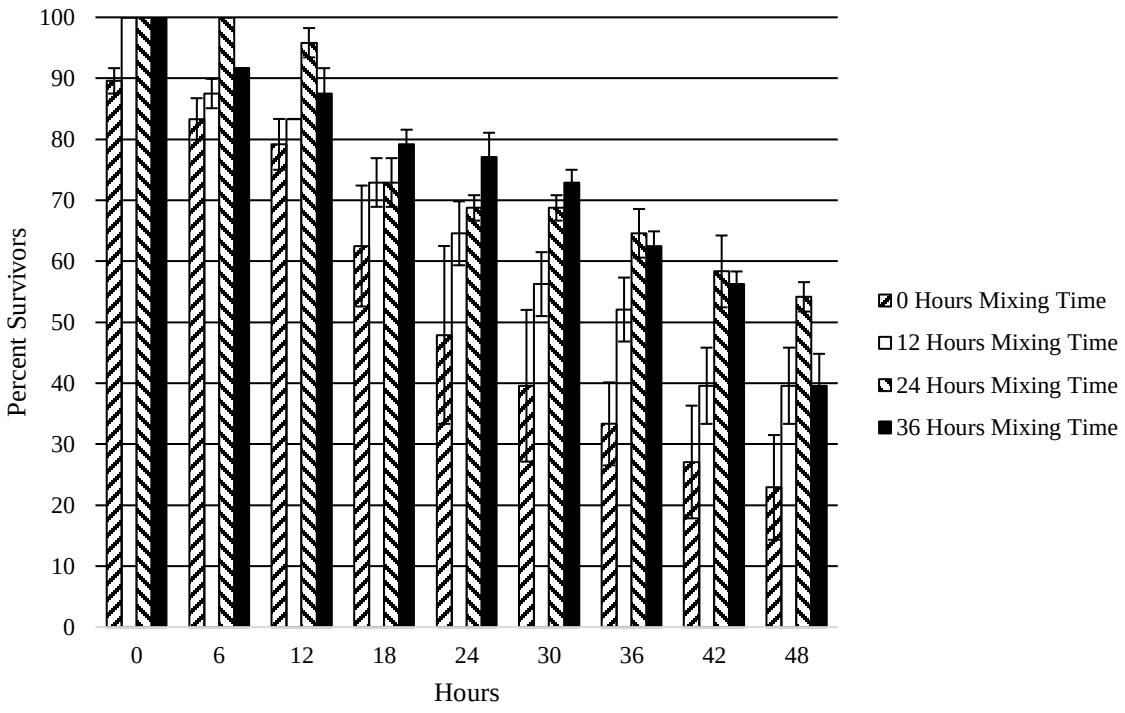

Fig. 5 Percent survival by mixing time for WAF/C treatment over a 48-hour exposure period post-mixing. Error bars indicate standard error.

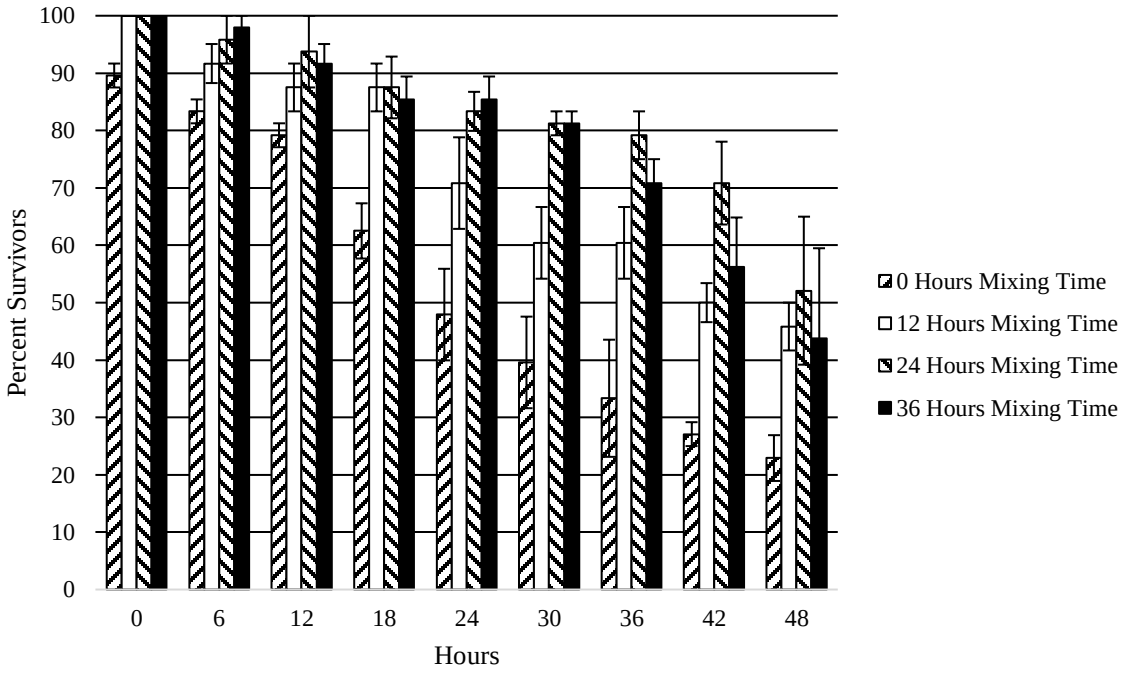

Fig. 6 Percent survival by mixing time for WAF/C/MB treatment over a 48-hour exposure period post-mixing. Error bars indicate standard error.

The survival rate of the same treatment with different mixing times was examined for significant differences in survival rates (Table 3, Table 5). Among WAF/C treatments, survival rates in 24- and 36-hour mixing time treatments was greater than in 0-hour mixing time treatments ($p < 0.05$). WAF/C/MB treatments had a similar trend with greater survival rates in 12-, 24-, and 36-hour mixing times had higher survival rates when compared to 0-hour mixing times ($p < 0.05$). Higher survival rates were significant starting at the 24-hour observation period for the 24- and 36-hour mixing time treatments and the 36-hour observation time for the 12-hour mixing time treatment. These higher survival rates held until the 42- and 48-hour observation periods.

The survival rate of the WAF/C treatment was also compared to the survival rate of the WAF/C/MB treatment for different mixing times (Table 5). There was no significant difference in the mean 48-hour survival rate of WAF/C and WAF/C/MB treatments with the same mixing time (Fig. 5, Fig. 6). With the exception of the 36-hour mixing time treatment, survival in WAF/C/MB treatments was higher in WAF/C treatments of the same mixing time in at least one

Table 3 Significant differences ($p < 0.05$) in WAF/C treatment. Numbers in cells show observation times with significant differences.

Treatment	WAF/C T0
WAF/C T0	
WAF/C T12	
WAF/C T24	6, 12, 30, 36, 42, 48
WAF/C T36	6, 18, 24, 30, 36, 42

Table 4 Significant differences ($p < 0.05$) in WAF/C/MB treatment. Numbers in cells show observation times with significant differences.

Treatment	WAF/C/MB T0	WAF/C/MB T12
WAF/C/MB T0		
WAF/C/MB T12	36, 42, 48	
WAF/C/MB T24	24, 30, 36, 42, 48	30, 36, 42
WAF/C/MB T36	24, 30, 36, 42	

observation period. All WAF/C/MB mixing times resulted in significantly higher survival rates than the WAF/C 0-hour mixing time treatment. The WAF/C/MB 24- and 36-hour mixing time treatments showed a higher survival rate than the WAF/C 12-hour mixing time treatment beginning at the 30-hour observation period. The WAF/C 24- and 36-hour mixing time treatments had a higher survival rate than the WAF/C/MB 0-hour mixing time treatment beginning at the 18- and 24-hour observation periods respectively.

PAH Analysis

Despite being derived from the same source solution, the starting total PAH concentrations differed by as much as a 49.42% difference: 538.396 µ/L Corexit/WAF, 417.231 µ/L MicroBlaze/WAF, and 325.044 µ/L WAF. Effects were examined as changes in both percentages and actual concentrations. The WAF/MB solution resulted in the largest reduction of total PAH concentrations. Total PAH concentration was reduced by 57.20% in the WAF/MB solution, 56.14% in the WAF solution, and 52.21% in the WAF/C solution. Of 36 target compounds with observable changes in concentration, the WAF solution showed the greatest reduction as a percentage in 17 cases, followed by WAF/C in 12 cases, and WAF/MB in 6 cases. Seven PAH compounds increased in concentration between the 0-hour and 36-hour mixing time

Table 5 Significant differences ($p < 0.05$) in WAF/C treatment. Numbers in cells show observation times with significant differences in survival. Dark gray shading indicates a higher survival rate in WAF/C/MB treatments while light gray shading indicates higher survival rates in WAF/C treatments.

Treatment	WAF/C T0	WAF/C T12	WAF/C T24	WAF/C T36
WAF/C/MB T0	6, 12		18, 30, 36, 42, 48	24, 30, 36, 42
WAF/C/MB T12	6, 24, 30, 36, 42	18		
WAF/C/MB T24	6, 12, 18, 24, 30, 36, 42, 48	30, 36, 42	18, 24, 36	
WAF/C/MB T36	6, 12, 24, 30, 36, 42	30, 36		

solutions (Fig. 8). Carbazole increased by 122.61% in the WAF solution, 152.69% in the WAF/MB solution, and 546.10% in the WAF/C solution. The WAF/MB solution increased the concentrations of C3-phenanthrenes/anthracenes by 21.42%, C2-dibenzothiophenes by 116.73%, and C3-dibenzothiophenes by 2.14% (Table 6) The effect of increases in some PAHs on overall changes in concentration was enough to impact the final percentages. The highest combined increase was approximately 0.91% of the total final PAH concentration in the WAF/MB solution compared to 0.14% of the total final PAH concentration in the WAF/C solution.

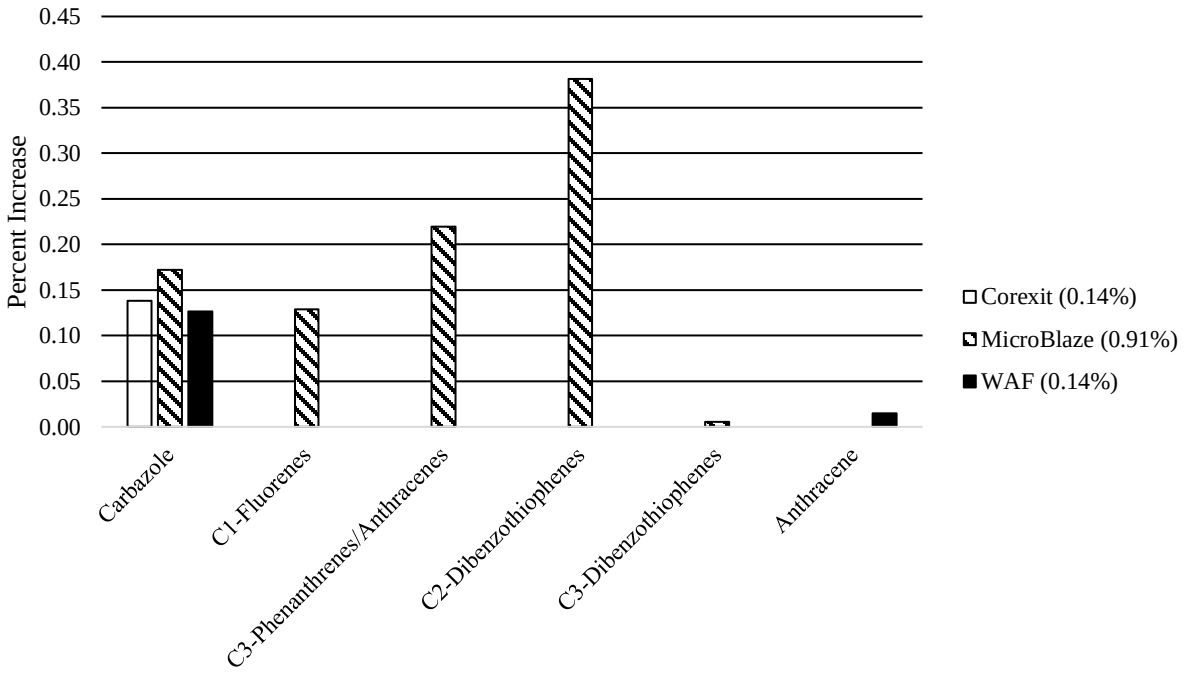

Fig. 7. Total PAH increases between 0-hour and 36-hour mix time solutions as a percentage of total change. Percentages after the solution name indicate the total percent increase of all PAH concentrations. Pyrene increased by 1 µ/L in the WAF/MB solution and is not shown on the chart.

Table 6 Changes in PAH by percentage of PAH compounds.

PAH	WAF/C	WAF/MB	WAF	PAH	WAF/C	WAF/MB	WAF
Total PAHs	**52.21%**	**57.20%**	**56.14%**	C3-Fluorenes	19.57%	14.87%	0.00%
cis/trans Decalin	35.81%	22.97%	34.94%	Carbazole	-546.10%	-152.69%	-122.61%
C1-Decalins	44.11%	99.45%	61.57%	Anthracene	76.06%	0.00%	-42.37%
C2-Decalins	40.97%	99.60%	55.14%	Phenanthrene	13.58%	18.88%	35.33%
C3-Decalins	68.09%	99.77%	55.56%	C1-Phenanthrenes /Anthracenes	11.83%	0.32%	45.12%
C4-Decalins	48.86%	99.91%	60.19%	C2-Phenanthrenes /Anthracenes	21.25%	8.68%	27.59%
Naphthalene	79.34%	60.21%	67.84%	C3-Phenanthrenes /Anthracenes	98.97%	-21.42%	39.94%
C1-Naphthalenes	49.43%	46.24%	55.83%	C4-Phenanthrenes /Anthracenes	93.59%	4.87%	42.14%
C2-Naphthalenes	38.81%	38.69%	49.99%	Dibenzothiophene	2.84%	10.28%	20.67%
C3-Naphthalenes	16.33%	24.65%	50.42%	C1-Dibenzothiophenes	3.93%	5.18%	27.80%
C4-Naphthalenes	26.92%	13.43%	47.82%	C2-Dibenzothiophenes	96.49%	-116.73%	95.70%
Biphenyl	55.57%	38.60%	37.05%	C3-Dibenzothiophenes	95.44%	-2.14%	94.95%
Acenaphthylene	94.56%	0.00%	42.67%	Fluoranthene	76.82%	73.10%	18.02%
Acenaphthene	93.86%	0.00%	61.03%	Pyrene	39.16%	-4.34%	43.31%
Dibenzofuran	30.22%	29.11%	40.04%	C1-Fluoranthenes /Pyrenes	18.65%	4.47%	81.95%
Fluorene	24.21	21.76%	35.61%	C2-Fluoranthenes /Pyrenes	4.59%	11.17%	77.42%
C1-Fluorenes	3.76	-10.54%	22.51%	C3-Fluoranthenes /Pyrenes	2.82%	4.19%	73.53%
C2-Fluorenes	1.57	21.96%	0.00%	C4-Fluoranthenes /Pyrenes	13.71%	50.44%	59.98%

In all cases, eight of the ten highest total reductions in PAH from the 0-hour to 36-hour mix time solutions included C1-C4 decalins, naphthalene, and C1-C3 naphthalenes. In terms of total reductions in PAH concentration, the top ten reduced compounds amounted to over 90% of the total reduced compounds. (Fig. 9, Table 7).

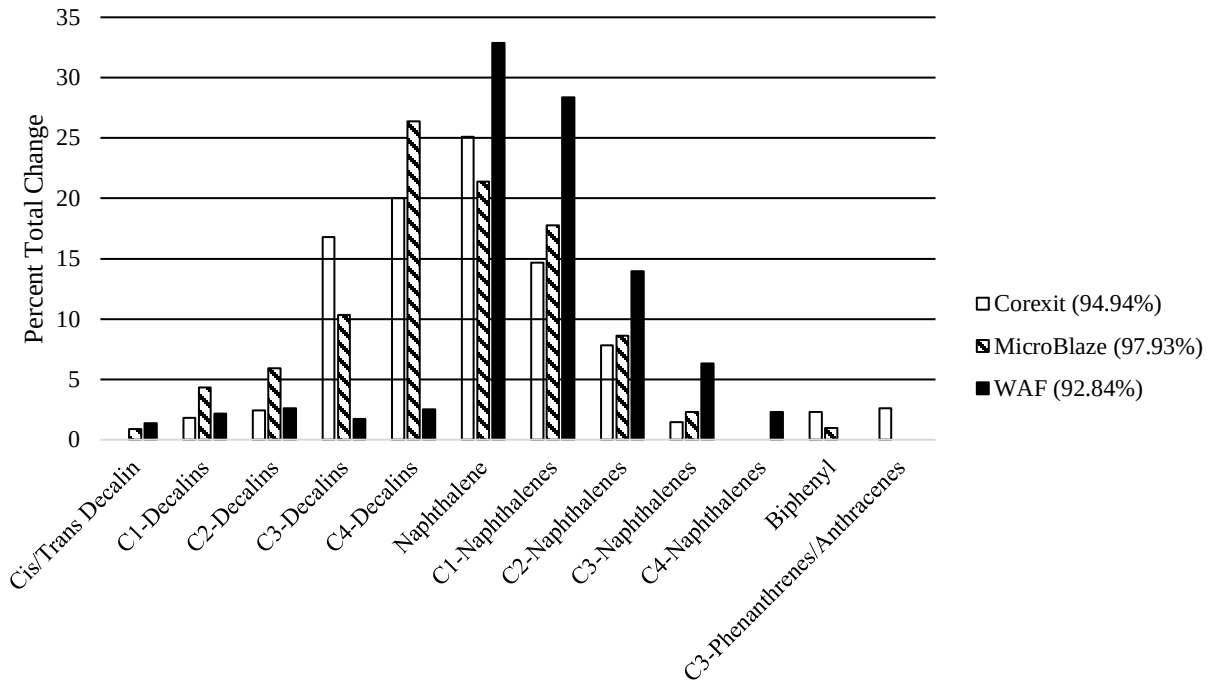

Fig. 8 Total PAH reductions between 0-hour and 36-hour mix time solutions as a percentage of total change. Percentages after the solution name in the legend indicate the total percent reduction of the top ten compounds that decreased PAH concentrations.

Table 7 Total PAH reductions between 0-hour and 36-hour mix time solutions as a percentage of total change. N/A indicates the change was not one of the ten largest.

Compound	**WAF/C**	**WAF/MB**	**WAF**
Cis/Trans Decalin	N/A	0.87%	1.35%
C1-Decalins	1.80%	4.33%	2.17%
C2-Decalins	2.43%	5.90%	2.60%
C3-Decalins	16.78%	10.33%	1.73%
C4-Decalins	20.03%	26.38%	2.51%
Naphthalene	25.10%	21.38%	32.87%
C1-Naphthalenes	14.65%	17.74%	28.36%
C2-Naphthalenes	7.83%	8.59%	13.96%
C3-Naphthalenes	1.43%	2.29%	6.33%
C4-Naphthalenes	N/A	N/A	2.31%
Biphenyl	2.30%	0.98%	N/A
C3-Phenanthrenes/Anthracenes	2.59%	N/A	N/A

Sublethal Effects

There was an observable increase in oxygen consumption during the experiment when compared to baseline measurements (Fig. 10). Average oxygen consumption per animal during the 24-hour pretreatment baseline ranged from 0.12–1.44% compared to 0.70–3.73% during the first 24 hours of the experiment and from 0.88–10.17% during the second 24 hours of the experiment. In all cases, the last 24 hours of the experiment resulted in the greatest percent decrease in oxygen. With the exception of the 12-hour mixing time treatments, the W/C treatments showed a greater increase in oxygen consumption than W/C/MB treatments. No consistent rapid decrease in consumption was observed at any point. Due to experimental errors a meaningful statistical comparison was not feasible.

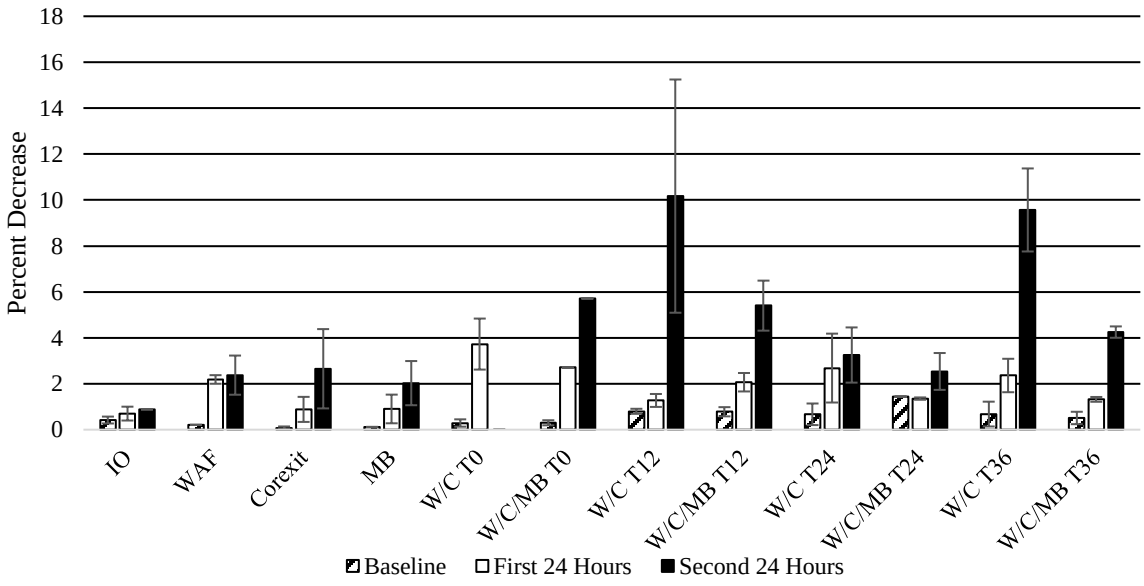

Fig. 9 Percent decrease in oxygen by survivor. The second 24 hours W/C T0 treatment had no survivors and was not included. Error bars indicate standard error. Columns without error bars indicate standard error could not be calculated.

The WAF/C 0-hour mixing time treatment resulted in statistically significant ($p < 0.05$) decrease in the mean observed motility versus all other treatments. The Corexit treatment resulted in a significantly higher mean observed motility than either the WAF/C or WAF/C/MB 0-hour mixing time treatments (Fig. 11). Across treatments, observed motility was extremely consistent until the final two observations of the WAF/C 0-hour mixing time treatment; 69.17% for the 42-hour observation period and 69.44% for the 48-hour observation period. The next lowest average motility was 87.73% for the WAF treatment 36-hour observation period (Fig. 12). There was no correlation between mixing time and motility.

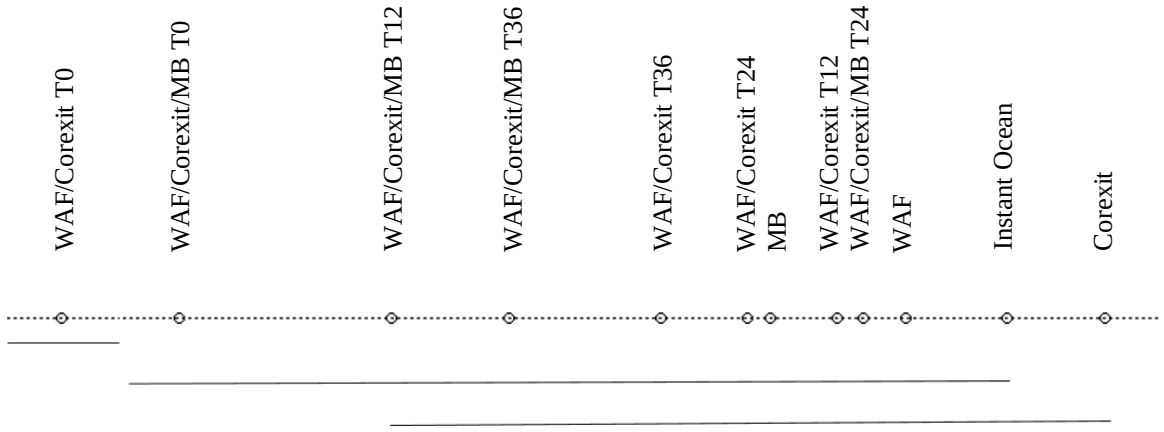

Fig. 10 Results of multiple comparison analysis of observed motility using Tukey's range test. Treatments connected by lines are not significantly different ($p < 0.05$).

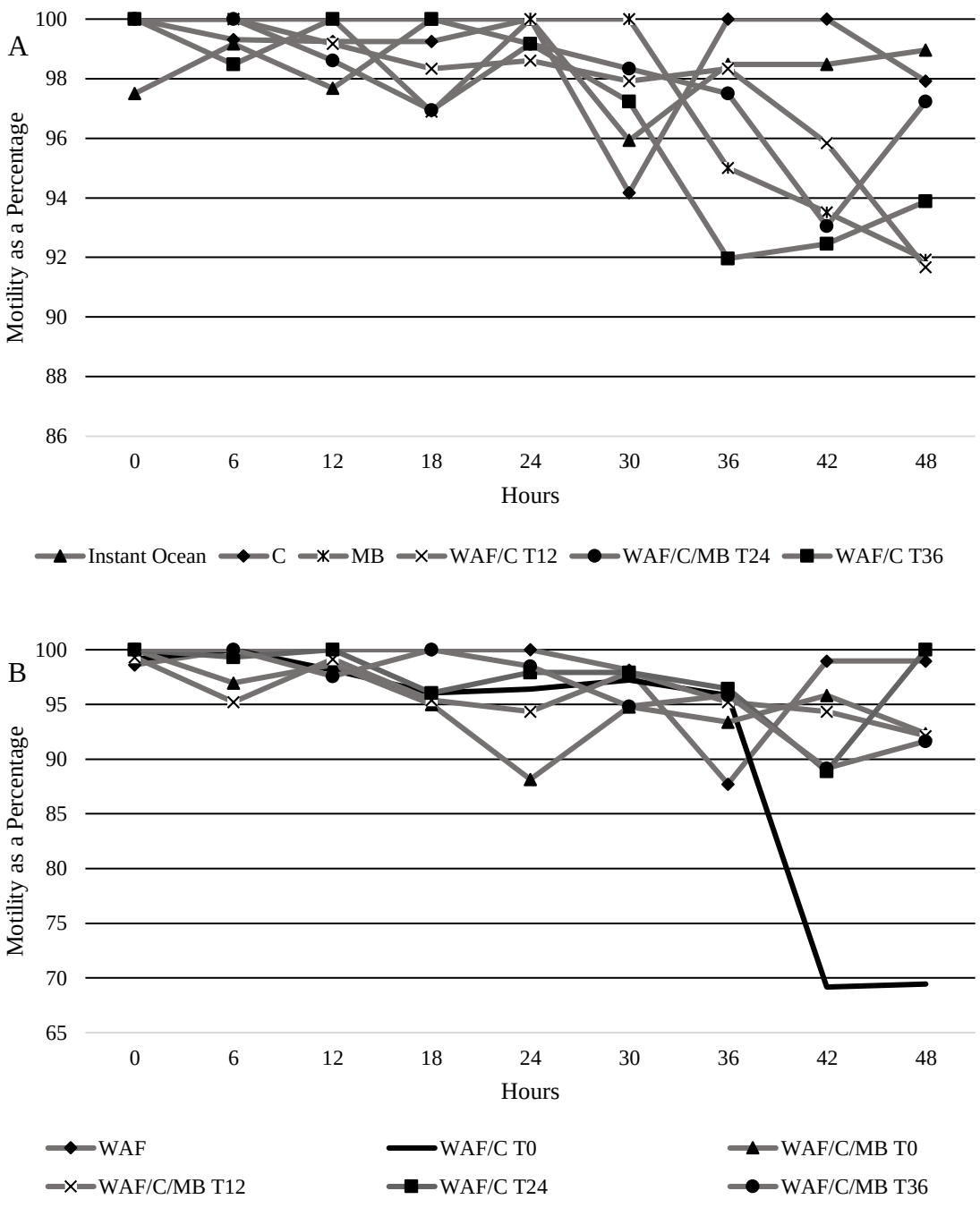

Fig. 11 Average observed motility of A) treatments with less variation and B) treatments with greater variation over time. WAF/C 0-hour mix time has the only significantly different motility when compared to all other treatments.

There is no statistically significant ($p < 0.05$) difference in the number of molts over the course of the experiments by either treatment or mixing time. The number of molts ranged from 0 to 4 with an overall average of 1.60 molts per experiment (Fig. 13).

Neither treatment nor mixing time resulted in a statistically significant ($p < 0.05$) difference in the total lipids. Average total lipids ranged from 3.84% - 8.44% with an overall average of 6.21% (Fig. 14).

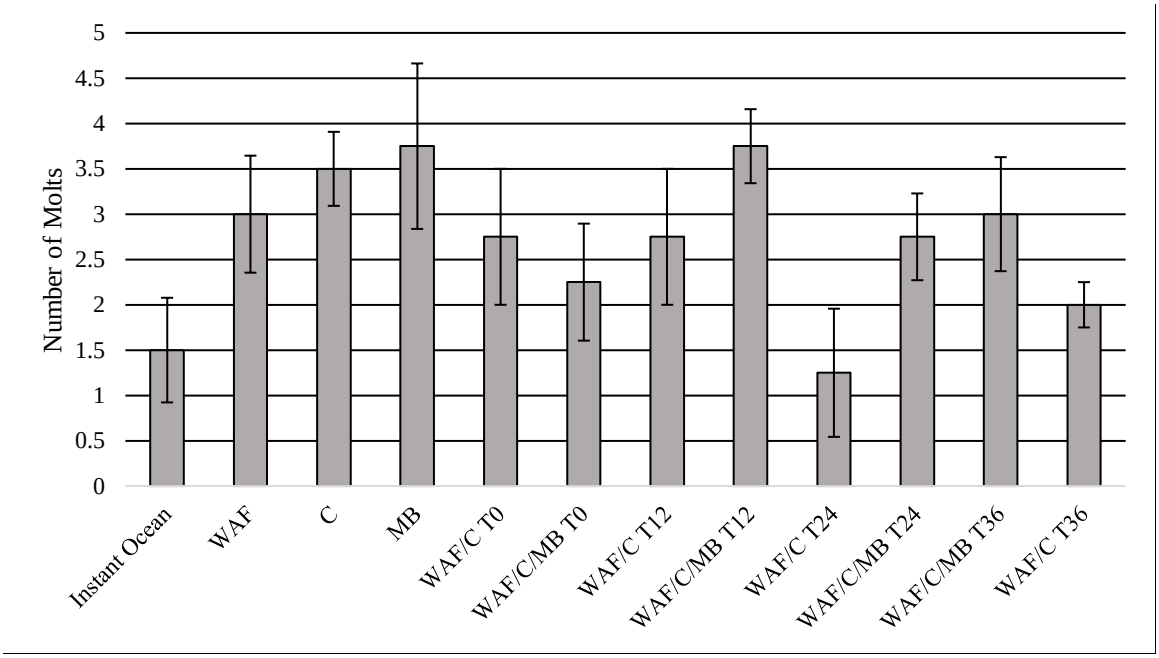

Fig. 12 Average number of molts per experiment by treatment. Error bars indicate standard error.

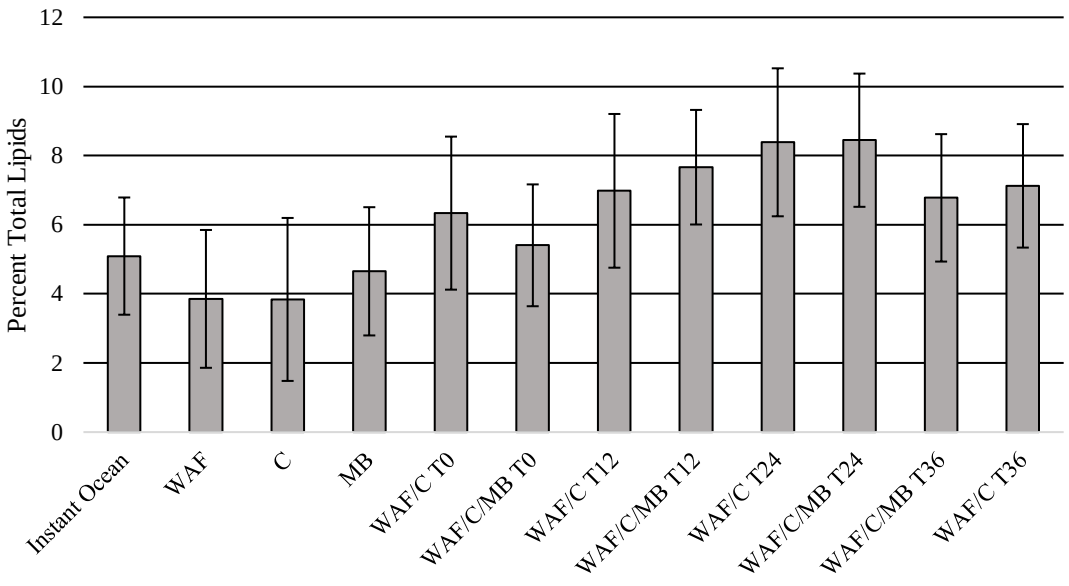

Fig. 13 Average percentage of total lipids by treatment. Error bars indicate standard error.

DISCUSSION

The results of the LC_{50} experiment show both MicroBlaze and Corexit reduce the toxicity of WAF, but using the two products are together is less effective. The LC_{50} of the amendments by themselves and at different ratios indicate a greater concentration of Corexit results in a more toxic solution. However, both the 3:1 C:MB and 1:1 C:MB treatments were more toxic than the Corexit by itself. This would suggest that the MicroBlaze is either reducing the effectiveness of the Corexit on oil, increasing the dispersal of oil compared to the 1:1 C:MB treatment, or producing toxic compounds from altered degradation pathways. It is possible the mixture of both produces a synergistic effect simultaneously reducing the effectiveness of Corexit or inhibiting microbial efficiency. Depending on the microbes used in MicroBlaze, the product may degrade dispersant or dispersants may have toxic effects, killing microbes thus reducing biodegradation rates.

A combined analysis of all treatments showed the addition of MicroBlaze results in a significantly higher survival rate ($p < 0.05$). Combined observations identified a time window needed for either dispersants or microbial treatments to reduce WAF toxicity. While the microbial

amendment may have a greater immediate effect, prolonged mixing with dispersant was as effective in reducing toxicity. There was a correlation between increased mixing time and lower mortality in both WAF/C and WAF/C/MB treatments. The toxicity of WAF/C was decreased in treatments with a 24 hour or greater mixing time. WAF/C/MB showed a similar reduction only after the 24-hour sampling time. WAF/C/MB treatments at all mixing times had a lower mortality rate than the WAF/C treatment with no mixing time. WAF/C treatments with 24- and 36-hour mixing times resulted in a higher survival rate when compared to either the WAF/C/MB 0-hour mixing time treatment. Reduced PAH concentrations were detected over time in all analyzed solutions. It is highly likely the reduced mortality in WAF/C/MB solutions was a direct result of hydrocarbon digesting microbes biodegrading dispersed oil.

The lack of statistically significant differences in mean survival between WAF/C and WAF/C/MB treatments with the same mixing times has two potential explanations. First, this may indicate both treatments reach a saturation point where the WAF solution will not be further degraded under the conditions of the experiment. It is also possible the experimental design is partially responsible for shrimp mortality at the end of the experiment. While there was no decrease in survival in the control treatments, the increased oxygen consumption in the control indicates additional stress attributable to the experiment itself. That increased stress may have increased mortality in the WAF/C and WAF/C/MB treatments. Standard errors were greater at higher concentrations of Corexit. Because Corexit samples were obtained through industry contacts, it is possible age or storage conditions may have affected the dispersant composition and efficiency.

The 48-hour LC_{50} of Corexit from this experiment (820 ppm) was approximately 5-30 times higher than those reported for other species. For example, the reported 96-hour LC_{50} for green hydra (*Hydra viridissima*) is 160 ppm (Mitchell and Holdway 2000), 48-hour LC_{50} for

opossum shrimp is 32.23 ppm (Environmental Protection Agency 1995), or the 96-hour LC_{50} for *Daphnia magna* is reported as 25 ppm (Brown et al. 1989). MicroBlaze is generally considered non-toxic and an LC_{50} could not be found in either scientific or grey literature. Due to the variety of methods, oil compositions, and ratios used in the preparation of WAF in previous research, comparisons between species are difficult.

"Final chronic values" (FCVs) were developed by Di Toro (2000) using LC_{50} data for 33 marine species, including orfe (*Leuciscus idus melanotus*) and Japanese rice fish (*Oryzias latipes*), to predict consolidated threshold toxicity levels. These values were based on critical body residue to develop PAH water standards. Decalin has a low toxicity when compared to other PAHs with a 48-hour LC_{50} of 4300 µ/L for orfe and 1840 µ/L for Japanese rice fish (Hazardous Substances Data Bank). Comparatively, the FCV of naphthalene is 320 µ/L (CCME 2010). Two PAHs with the greatest reductions in WAF/C and WAF/MB solutions as a percentage are anthracene (FCV 36 µ/L) and fluoranthene (FCV 12 µ/L) (CCME 2010). Given their relatively high toxicity and high reductions in concentration, it seems reasonable to suggest anthracene and fluoranthene could be responsible for higher mortality in WAF treatments.

The decrease in concentration in decalin is partially due to its rapid volatilization, estimated at 3.5 hours when modeled for flowing water (Hazardous Substances Data Bank). However, the WAF/MB and WAF/C solutions showed a reduction of C3 and C4-Decalins as much as ten times that of the WAF solution. C1–C4-decalins were almost completely eliminated within 36 hours (Table 9). Few studies have examined the microbial transformation of decalin, but Kirkwood (2008) found mixed bacterial cultures oxidized decalin into 2-decahydronaphthol and 2-decalone in liquid. Those findings correspond to the decalin concentration reductions found in this study. While some PAH compounds decreased in the WAF solution at a higher percentage, the vast

majority were either in small concentrations, had a relatively low toxicity, or both. Due to the specializations of hydrocarbon-digesting bacteria (Harayama 1999; Ron and Rosenberg 2014) and the unknown composition of MicroBlaze it is impossible to completely attribute specific degradations to the mixture.

Percent changes in PAH concentration, and the specific PAHs which were most reduced, were fairly similar across solutions. The naphthalenes and cis/trans decahydronaphthalene (decalin) had the greatest reduction in WAF, while C1, C2, and C4 decalins had the greatest reduction in WAF/MB. C3 decalins are the only compounds had the greatest reduction in WAF/C. Anthracene and fluoranthene concentrations were also much lower in the WAF/C and WAF/MB solutions when compared to WAF.

Increases in some PAH compounds were likely the result of the breakdown of other PAHs. In all treatments carbazole concentrations increased. Carbazole and anthracene are co-products derived from "anthracene oil" a liquid complex combination of PAHs composed primarily of carbazole, anthracene, and phenantherene (Franck and Stadelhofer 1988; European Chemicals Agency 2017). In the WAF solution anthracene decreased from 80.2 µ/L to 19 µ/L between the 0- and 24-hour mixing time solutions then increased again to 61 µ/L in the 36-hour mixing time WAF/C solution. Anthracene decreased in the WAF/MB solution but was only present in trace amounts in any sample. All other PAHs that increased in concentration are present in anthracene oil (Franck and Stadelhofer 1988).

Generally, stress responses such as increased metabolism and movement result in higher energy demand which would be reflected by an increased respiration rate. The specific response to oil pollution is dependent on the hydrocarbon composition, amount of exposure, abiotic factors, test species, sex, and life cycle stage. Depending on these factors, oxygen consumption can

decrease (Vandermeulen 1980), increase (Baden 1982), or even do both over the length of an exposure (Vargo 1981). In one experiment, the oxygen consumption of the common littoral crab (*Carcinus maenas*) increased upon exposure to both crude oil and dispersant mixtures, but respiration rates returned to baseline levels within hours (Depledge 1984). In that experiment, the highest respiration rates were reported after exposure to dispersant and CEWAF. Oxygen consumption by microbes often increases after exposure to oil and dispersants. While studying microbial activity near the surface of the DWH, one study reported microbial respiration rates inside the oil slick five times greater than those in nearby "pristine conditions" (Edwards et al. 2011). A second study found increased respiration in *Pseudomonas aeruginosa* after six hours of exposure to oil, dispersants, and with the greatest increases caused by dispersant and CEWAF mixtures (Al-Hadrami 1996).

The increased respiration during the experiment compared to the baseline assessment indicates a severe stress reaction. Animals in the Instant Ocean control increased their oxygen consumption over time, but the change was minor when compared to other treatments indicating the additives were likely responsible for the majority of increased respiration. All baseline treatments consisted solely of an instant ocean control, but the initial decrease in oxygen during baseline measurements varied widely. The main factor in oxygen consumption was length of exposure. With the exception of the W/C/MB 24-hour mixing time treatment, the percent decrease in oxygen is higher in the first 24 hours of the experiment than in the control and in all cases the second 24 hours of the experiment had higher oxygen consumption than the first 24 hours of the experiment. Based on the results of the mortality experiment, it is reasonable to expect shorter mixing times would result in higher oxygen consumption, but oxygen appears to be largely independent of mixing times. The WAF/C 12- and 36-hour mixing times had the highest overall

oxygen consumption. Oxygen consumption between the WAF/C/MB 0-, 12-, and 36-hour mixing times were very similar and the 24-hour mixing time solution was the lowest. To test this, we would need to compare recovery of animals by moving all treatments to fresh saline water and assess oxygen use. Despite the likelihood that additional microbes would increase oxygen use, the addition of MicroBlaze appears to reduce oxygen consumption. This is likely due to a decreased stress reaction from the test animals.

It is unlikely that the 36-hour mixing time treatments had a greater oxygen consumption than the 24-hour mixing time treatments due to increased stress caused by differences in PAH concentrations. If a high toxicity PAH is responsible for increased respiration with longer mixing times, an exponential or logarithmic change in one or more PAH concentrations would be expected in the 36-hour PAH analysis assuming toxicity is not a cumulative response. Increases and decreases in concentrations were linear in all solutions in which case the 24-hour mixing time solution should have a higher oxygen consumption than the 12-hour mixing time. It is possible there is a synergistic effect which increases the concentration of a high-toxicity PAH when mixing WAF, Corexit, and MicroBlaze, but further studies would be required to confirm the possibility.

Inconsistent respiration results may be due to multiple factors. The oxygen dataset for the third experiment was corrupted and unusable due to technical issues limiting available data to three replications. In multiple cases, the jars were not completely sealed resulting in gas exchange. Because of the gas exchange, final oxygen concentrations were greater than initial readings. The resulting data were not usable. Shrimp were not weighed either before or after the experiments. Weighing before the experiments was not feasible due to concerns about the impact of handling. Weighing after the experiment was also not possible. Dead animals could not be removed during

the experiments because opening the jars would allow gas exchange, and dead shrimp were immediately cannibalized.

Various motility observations are commonly studied as behavioral responses to sublethal toxic exposures. These changes may exhibit themselves as either an effect of toxins themselves or as adaptive responses attempting to mitigate those effects and can be extremely sensitive indicators of the health of an organism (Gerhardt 2007). Studies have shown that WAF altered Atlantic rock crab (*Cancer irrotatus*) larval responses to light and pressure within 12 hours (Bigford 1977), Corexit 9500A reduced motility of bay barnacle (*Amphibalanus improvises*) larva (Almeda 2014), and both have been shown to reduce the swimming activity of pink shrimp (*Farfantepenaeus duorarum*) at sublethal concentrations over a 48-hour exposure (Laramore 2016).

Given previous results in similar studies, significant differences in motility across treatments was expected after sublethal exposures. The WAF/C 0-hour mixing time treatment had one of the lowest survival rates which should correlate to lower motility. However, the differences in motility between treatments were not apparent until the final two observation periods. The aim of this metric was to isolate a sublethal behavioral change rather than reinforce mortality observations. During the experiment, motility of individuals was less for approximately 1–2 observation periods before the animals died. Since analysis excluded the dead animals, the lowest scores were effectively removed increasing the overall average score per treatment. It is likely the reduced motility would have been followed by the death of at least one shrimp, in turn raising the observed motility.

Molting rates in *Palaemonetes* spp. are affected by environmental conditions such as salinity, temperature, and oxygen availability, and can be as short as 6.4 days (Vernberg and Piyatiratitivorakul 1998) or as long as 37 days (Brinton and Curran 2015). Molting rates of

crustaceans after PAH exposure have been documented to increase and decrease in different studies. Pyrene has been shown to delay molting in male *Palaemonetes pugio* during a six-week exposure (Oberdörster 2000). WAF also delayed molting in Chesapeake blue crab (*Callinectes sapidus*) following a 48-hour exposure (Giltz and Taylor 2017). Conversely, exposure to WAF and Corexit 9500A solutions resulted in faster molting rates in pink shrimp after a 72 to 96-hour exposure (Laramore 2016). The different responses are potentially attributable to a pollutant affecting hormone production which would delay molts (Oberdörster 2000) or as a stress response intended to shed pollutants adhering to the carapace (Laramore 2016) which would increase the molting rate.

The lack of a significant difference in molting rates in the study may be due to multiple factors. There was no control for the last molt of the test animals, and given the potential time between molts, molting may have been biologically impossible. The 48-hour exposure time is relatively short, and molting was only monitored over the course of the experiment. Changes in molting frequency could have potentially been shown had monitoring continued post-experiment.

Crustacean hyperglycemic hormone (CHH) plays a key role in wide array of biological processes in crustacea including lipid metabolism (Santos 1997) and glucose levels which may result in a stress response to environmental pollutants (Lorenzon 2005). Kim (2013) reported exposure to WAF caused variation in the transcript level of the TJ-CCH gene of the intertidal copepod, (*Tigriopus japonicas*) depending on the time and level of exposure. Hansen (2011) found greater lipid content correlated to reduced mortality in the copepod *Calanus* spp. when exposed to WAF.

No statistical difference in initial lipid levels indicates the shrimp were in similarly healthy states at the beginning of the experiment and variation of lipid content in individual shrimp was

not responsible for difference in mortality levels by treatment. It is likely the levels of exposure were not sufficient to produce a hormonal response or the time of exposure was insufficient to change the shrimp's composition.

Fern (2014) used acute toxicity tests of the same ingredients in a laboratory setting and this study used one dispersant and one microbial solution for all treatments. External factors such as temperature, salinity, and UV exposure known to affect the treatments used in the study (Pace et al. 1995; Garrett et al.1998,) but were controlled in this study. Currently, 22 dispersants and 40 bioremediation agents, all proprietary mixtures, have been submitted to EPA's NCP product schedule list for consideration as remediation tools (Environmental Protection Agency 2017). These other dispersants and bioremediation products likely contain their own unique proprietary components making broad generalizations based on the results of this study possible, but specific results would be limited to the products tested.

The addition of microbial amendments to chemically dispersed oil will result in a higher survival rate of marine organisms exposed to marine oil spills. While the addition of MicroBlaze did not lead to significant reductions in mortality in all treatments, the likelihood of enhanced animal survival, shorter recovery times, and reduced economic impact of oil spills warrants its use.

LITERATURE CITED

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