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Combined Effects Of Corexit Ec 9500A With Secondary Abiotic And Biotic Stressors In The Rotifer Brachionus Plicatilis

Michael Britt Williams
University of Alabama at Birmingham

COMBINED EFFECTS OF COREXIT EC 9500A WITH SECONDARY ABIOTIC AND BIOTIC STRESSORS IN THE ROTIFER *BRACHIONUS PLICATILIS*

by

MICHAEL B. WILLIAMS

STEPHEN A. WATTS, COMMITTEE CHAIR
JULIA M. GOHLKE
MICKIE L. POWELL

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BIOLOGY

ABSTRACT

Following the Deepwater Horizon oil spill the dispersant Corexit EC 9500A (C-9500) was applied to facilitate rapid oil degradation. C-9500 toxicity has been evaluated on a number of marine species; however, many of these studies do not account for environmental conditions that may increase negative outcomes related to dispersant exposure in the wild. We examined lethality and behavioral effects of C-9500 exposure on the model marine zooplankton *Brachionus plicatilis*. We also evaluated interactions of abiotic and biotic stressors that may alter outcomes of exposure. C-9500 exposure at standard husbandry conditions (17.5ppt, 24°C, 200 rotifer*ml^{-1} density) produced a 24 hr median lethal concentration of 107 ppm for cultured *B. plicatilis* by Probit analysis. Rotifers surviving exposure to a low concentration (50 ppm) of C-9500 exhibited increased swimming velocity, suggesting increased metabolic rate and a possible escape response to chemical perturbation. Rotifers surviving exposure to higher concentrations (100 and 150 ppm) exhibited a decreased swimming velocity and a reduced net to gross movement ratio indicative of an altered directional swimming behavior. Significant interaction between C-9500 exposure and increasing temperature was observed resulting

in a decreased upper thermal range of exposed *B. plicatilis* populations. Increasing salinity also interacted with C-9500 exposure resulting in increased mortality at higher salinities. Increased or decreased nutritional availability over the exposure period did not significantly alter mortality of *B. plicatilis* populations at concentrations tested. Results from this study may be useful for predicting possible outcomes on marine zooplankton following dispersant application under diverse natural conditions.

DEDICATION

I dedicate this work to my family. Without their support and love this study would not have been possible.

ACKNOWLEDGEMENTS

I would like to thank my mentor and committee chair Dr. Stephen A. Watts for his instruction and support while carrying out this study. I would also like to thank my other committee members Dr. Mickie L. Powell and Dr. Julia M. Gohlke for advising me. Robert Barry provided assistance with husbandry and training. James C. Taylor has been a great friend during this study and provided insight into writing style and data interpretation. Dr. Kathleen Fischer assisted with writing and presentation of this study. Dr. Robert Angus and Peter Merrill offered statistically advising on this study. I would like to thank the Laboratory of Dr. Charles Amsler for offering use of their space and software in assessing motility. I would like to thank Margaret Amsler and CASIT for helping with technical support. I would like to thank all the members of the laboratory of Dr. Watts for their friendship and service. Funding for this study was provided by British Petroleum through the Gulf Oil Spill Pilot Grant.

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COMBINED EFFECTS OF COREXIT EC 9500A WITH SECONDARY ABIOTIC AND BIOTIC STRESSORS IN THE ROTIFER *BRACHIONUS PLICATILIS*

Michael B. Williams, Mickie L. Powell, and Stephen Watts

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INTRODUCTION

Corexit EC9500A (C-9500) is a chemical dispersant of the Corexit product line produced by Nalco Environmental Solutions LLC of Sugarland Texas. Like many dispersants, C-9500 is a mixture of chemicals designed to allow increased degradation of large hydrocarbon masses such as oil rafts (Nalco, 1995). C-9500 is listed for use as part of the National Contingency Plan (NCP) Product Schedule allowing it to be applied in the Deepwater Horizon oil spill of April 2010 upon approval (EPA, 2013). During the oil spill event 7 million liters of C-9500 and Corexit 9527 (C-9527), also a Nalco product, were applied by surface spraying and subsea pumps (C-9500 only) into Gulf of Mexico waters. This was part of an attempt to reduce the ecological impact of 700 million liters of crude oil released from the Mississippi Canyon Block 252 well (On Scene Coordinator Report DWH, 2011). There is debate in the regulatory community as to whether dispersant application prevents negative ecological outcomes of oil spill events. A number of studies have observed toxic effects of C-9500, mixtures of C-9500 with crude oil, and chemical components of C-9500. Toxicity studies of C-9500 and C-9527 on various marine species indicate survivorship is affected in the absence of crude oil (George-Ares and Clark, 2000). Dioctyl sodium sulfosuccinate, the major anionic surfactant in C-9500, has toxic effects and undergoes bioaccumulation in rainbow trout, *Oncorhynus mykiss* (Goodrich et al., 1990). Additionally, crude oil contains polycyclic aromatic hydrocarbons (PAHs) that have toxic effects on marine organisms at levels found in surface waters following the Deepwater Horizon oil spill (Diercks et al., 2010). The reduced size of crude oil particulates in the dispersant enhanced water accommodated fraction (DEWAF) have been shown to cause more rapid uptake of toxic

PAHs by marine organisms (Ramachandran, 2004). Unfortunately, there is inadequate dispersant toxicity data to make clear predictions of outcomes of application of C-9500 on various ecosystems (Singer et al, 1998).

Many of the current studies on C-9500 toxicity do not account for various conditions that may impact outcomes for populations exposed in the wild. Organisms in the wild are often exposed to variable physical factors that may interact with toxic contaminants. These types of interactions between contaminants and natural stressors can be co-tolerances that are positive (one stressor increases tolerance of the other stressor) or negative (one stressors increases sensitivity to the other stressor) (Vinebrook, 2004). The complex interactions of multiple stressors make development of predictive models challenging, highlighting the need for future toxicity studies to take into account possible positive or negative co-tolerance outcomes. Comparing several toxicity studies of two Corexit dispersants (C-9500 and the previously used C-9527) suggests that physical factors that alter the effectiveness of C-9527 to disperse crude oil may also alter toxicity of the dispersant material. In the grass shrimp, *Palaemonetes pugio*, there were differences between the 96hr LC50s of C-9527 exposures at 17 and 27°C, with the latter showing higher toxicity and being outside the optimal rearing temperature (National Research Council, 1989 and Anderson, 1954). For the medaka, *Oryzia latipes,* a difference can be seen between 24hr C-9527 exposures LC50 when reared in freshwater or seawater (Lessard, 1995). This indicates that C-9527 exposure may disrupt normal functions of homeostasis pathways leading to higher mortality in suboptimal conditions.

Brachionus plicatilis is a representative of keystone primary consumer species that are needed for the flow of carbon and energy to higher trophic level species in

aquatic environments. Decreased survivorship of wild populations of *B. plicatilis* and other zooplankton can have a direct effect on the survival of larger marine species that are unaffected by direct C-9500 exposure (Calbet, 2008). This species has been used to evaluate direct exposure effects, trophic transfer of PAHs, and combinatorial effects following application of oil dispersants (Rico-Martínez, 2013 and Wolfe et al, 2000). In evaluating co-tolerances, *B. plicatilis* has the benefit of being a model with tolerance over a wide range of abiotic and biotic conditions (Epp and Winston, 1977; Yoshinaga, 2000, and Smith et al, 2012). This tolerance of various conditions allows us to use this species as a model to evaluate C-9500 toxicity when zooplankton populations are under stress from increased temperature, increased salinity, increased population density, and decreased nutrient availability.

B. plicatilis has an optimal thermal range of 20 - 28°C as approximated by Q10 evaluations over 2°C intervals (Epp and William, 1980). Survival in high temperature thermal stress (HTTS) requires activation of thermal survival mechanisms by heat shock proteins (HSP). These mechanisms allow for maintenance of the organism's proteome (by targeted protein degradation or chaperone assisted refolding) under exposure to a natural stressor, such as HTTS and ultraviolet radiation (UVR), and anthropogenic stressors (Smith et al., 2012). C-9527 exposure also causes activation of HSP mediated pathways promoting cell survival (Wheelock, 1999). HTTS has been suggested to increase the susceptibility to heavy metals in a temperature dependent manner in zooplankton species (Cairns et al., 1978). Model zooplankton species show decreased body size when exposed to HTTS or toxic concentrations of heavy metals, which is a marker for poor population health (Moore and Folt, 1993). There may be differences in

survival outcomes for cold stressed zooplankton when compared to HTTS under C-9500 exposure.

For almost three decades it was initially accepted that *B. plicatilis* was a strict osmo-comformer, attributed to the fact that internal body fluids were iso-osmotic to the surrounding medium in environments with salinity ranging from 2 - 97ppt (Epp and Winston, 1977). However, a recent study suggested ion-regulating strategies with increasing environmental osmotic concentrations in this species. Lowe et al. (2005) suggest that Na^+/K^+ ATPase enzymatic activity measured in the range of 0 – 60ppt salinity shows an increase beginning at intertidal salinities of approximately 25ppt and continued to increase until 60ppt. This study suggests that *B. plicatilis* can shift ion-regulating cellular mechanism from osmo-conforming to osmo-regulating at higher salinities. These different strategies used to maintain homeostasis in different osmotic environments provide a potential difference of susceptibility to environmental pollutants. Supporting this idea is the fact that there is a suggested energy trade off at higher salinities for various zooplankton species including *B. plicatilis*, shown by decreased population growth rate in higher salinities, potentially indicative of stress (Sarma et al., 2006). If so, stress from increased salt concentration may increase susceptibility to C-9500 exposure and result in decreased population survival.

Variation of phytoplankton population density can be caused by agricultural runoff and waste waters that deposit excessive nutrients into the Gulf of Mexico from point and non-point sources (U.S. Fish and Wildlife Services, 2012). Zooplankton population density will increase over a period of “weeks to months” after an increase in phytoplankton population density (Biggs, 2001). Increases to zooplankton population

density can induce crowding responses (or grouping effect) in zooplankton. Crowding response, by the release of crowding signal peptides, will increase the number of haploid eggs that can develop into males and subsequently increase sexual reproduction in the population (Gilbert, 2004). Increased zooplankton population density may also induce stress by decreasing the availability of resources per individual, particularly dissolved oxygen (Park et al., 2001). Alternatively, increased population density may ameliorate the adverse effects of toxicant exposure. Van der Heide et al. (2008) demonstrated the effects of reduced nitrogen on the eelgrass species *Zortera marina* in low and high shoot densities. The authors suggested that increased density of the shoot material in the sea grass beds lowered population susceptibility for high pH species nitrogen toxicity. This reduced susceptibility of high shoot density of *Z.marina* was hypothesized to be caused by growth dilution. Therefore, Van der Heide et al. (2010) proposed new models for nitrogen toxicity that accounts for biomass density and predicts outcomes for sea grass beds following exposure. Population density has not been investigated as a factor in C-9500 toxicity.

Wheelock et al. (2002) exposed *B. plicatilis* populations to C-9527 dispersed crude oil (DO) and water-accommodated fractions (WAF) of crude oil under different nutritional states (8 hr or 24 hr starved). When starved during DO/WAF exposure HSP expression is increased more than exposure to the two stressors independently. In both starved and fed treatments exposed to WAF, HSP expression was higher in 8 hr than in 24 hr exposures suggesting an acclimation response to these stressors over prolonged exposure. Starvation also causes changes to energy utilization and susceptibility to other stressors. *B. plicatilis* populations experiencing periodic short term starvation (21 hr or 23

hr) have an increased life span and reproductive period; however, lifetime fecundity decreased by more than half when compared to populations provided adequate feedings (Yoshinaga, 2000). This suggests an energy tradeoff occurs in periods of starvation stress. Jokela et al. (2005) found that in prolonged starvation of the fresh water clam *Anodonta piscinalis*, infection rates of two common marine parasites (*R. fennica* and *R. campanula*) increased and resulted in increased host mortality. The authors suggest that *A. piscinalis* alters energy partitions during periods of prolonged starvation, resulting in increased susceptibility to parasitic infection.

The aforementioned stressors have a potential to alter the toxicity of C-9500 in zooplankton populations. Our study compares effects of combined stressors (natural and anthropogenic) on *B. plicatilis* populations. We hypothesize that laboratory maintained populations of *B. plicatilis* will be susceptible to increasing concentrations of C-9500. We further hypothesize that exposure to naturally occurring abiotic and biotic stressors will interact to increase mortality in C-9500 exposed populations.

MATERIALS AND METHODS

Maintenance of Stock Populations

A stock population of the rotifer species *Brachionus plicatilis* was established from populations obtained originally from Reed Mariculture Inc. (Campbell, CA). Rotifer stock was maintained under constant aeration and a 12 hr light, 12 hr dark photoperiod at a 10L volume, 17.5ppt salinity, and 25C. Rotifer stock was provided an AM (09:00) and PM (17:00) feeding of a concentrated *Nannochloropsis* alga paste (Reed Mariculture Inc. Campbell, CA) at a concentration of 0.3 $mL*L^{-1}$ and 0.6 $mL*L^{-1}$ respectively. To maintain water quality and exponential growth in the population, prior to each AM feeding 20% of the volume with its associated rotifers was removed and replaced with 2L of 17.5ppt artificial sea water (ASW) (Instant Ocean, United Pet Group, Blacksburg, VA).

Determination of LC 50

A 1L volume of *B. plicatilis* was removed from stock prior to AM feeding and average population density was calculated (n=5 1mL samples were counted and averaged). The separated 1L of rotifers was provided 0.3 mL/L concentrated *Nannochloropsis* and maintained at 24°C until time of exposure. Exposures were always initiated at 15:30 in the afternoon. ASW (17.5ppt) was added to increase volume to reach a final population density of 200 $ind.*mL^{-1}$. This population represents various life stages including juveniles and adults. *B. plicatilis* were exposed in 20mL glass vials at concentrations of C-9500 ranging for 0-300ppm (n=5 vials / treatment concentration) produced by serial dilution and all vials capped with plastic paraffin film. *B. plicatilis*

populations were exposed for a 24 hr period under 12 hr light, 12 hr dark photoperiod in a 24°C incubator. Over the 24 hr exposure period vials received a AM and PM feeding of 0.3 $mL*L^{-1}$ and 0.5 $mL*L^{-1}$, respectively, and an O_2 gas application (bubbled 5 secs directly into each vial using a glass pipet). Following the exposure period vials were shaken and rinsed with ASW and contents emptied onto a glass petri dish. The contents were again mixed with a glass pipet to homogenize the contents and 1ml was removed to assess % alive and % dead. The % alive and % dead was determined for each replicate by observation of the first 100 rotifers to be located in the field of view under light microscope (Nikon SMZ1000, Nikon Inc. Melville, NY). Alive was determined by motility through the water or movement of the mastax or foot, any of which are common indicators of viability (ASTM, 1998). The same experiment was replicated in 3 separate trials and outcomes among trials were averaged for further analysis.

Behavioral Response

In investigating the behavioral response of *B. plicatilis* populations exposed to C-9500 we used the same exposure methodologies used to determine medial lethal concentration with some modifications. *B. plicatilis* populations were exposed in a 22°C incubator to 0, 50, or 100ppm concentrations of C-9500 for 6, 12, or 24 hrs (n=5 vials / treatment concentration at each exposure period). Following exposure periods the vials were remove from the incubator and moved to a 23-24°C ambient temperature and lighting. Five 1ml aliquots from each treatment vial were placed into 3mL of clean ASW of matching temperature and salinity on glass petri dishes (radius of 23mm). A field of view observing 1-12 rotifers was recorded for 5-7 secs at 45 fsp at a resolution of

600x800 pixels by light microscope using a Moticam 2000 microscope camera and Motic Images Plus 2.0 software (Motic North America, British Columbia Canada). Video was analyzed in CellTrak 1.5 motion analysis software (Motion Analysis Co. Santa Rosa, CA) for average swimming velocity ($\mu m*sec^{-1}$) and average net to gross movement (distance from starting location divided by total distance traveled, where a value of 1 would represent perfectly straight line of travel) over the time period.

C-9500 Lethality at Suboptimal Temperature

In investigating C-9500 lethality on *B. plicatilis* populations exposed at different temperatures we used the identical exposure and mortality methodologies used to determine medial lethal concentration with some modifications. *B. plicatili*s populations were exposed to 0, 50, 100, or 150ppm concentrations of C-9500 (n=2 vials / treatment concentration). Exposures were completed in either low (11 - 26°C) or high (24 - 40°C) temperature range maintained by an aluminum block (61x23x8cm) that is heated from one side and cooled from the other to create a linear temperature gradient extending an ca. 15°C range. The same experiment was replicated in 2 trials and outcomes among trials were averaged for further analysis.

C-9500 Lethality at Suboptimal Salinity

In investigating C-9500 lethality on *B. plicatilis* populations exposed to C-9500 at different salinities we used the identical exposure and mortality observation methodologies used to determine median lethal concentration with some modifications. Prior to the experiment the *B. plicatilis* stock was segregated into 4 separate stocks and

acclimated over a 21 day period to salinities of 5, 17.5, 25, or 32ppt by adding ASW of appropriate salinity. Once acclimated and raised to a 10L volume they were maintained as described previously. *B. plicatilis* populations at each of these 4 salinities were exposed in a 22°C incubator to 0, 50, or 100ppm concentrations of C-9500 (n=3 vials / treatment concentration). The same experiment was replicated in 2 trials and outcomes among trials were averaged for further analysis.

C-9500 Lethality at Suboptimal Population Density

In investigating C-9500 lethality on *B. plicatilis* populations exposed to C-9500 at different population densities we used the identical exposure and mortality observation methodologies used to determine medial lethal concentration with some modifications. ASW (17.5ppt) was added to increase water volume to reach population densities of 50, 200, and 350 ind.*mL^{-1} at the initial time of exposure. *B. plicatilis* populations at each of these 3 population densities were exposed in a 22°C incubator to 0, 50, or 100ppm concentrations of C-9500 (n=5 vials / treatment concentration). The same experiment was replicated in 2 trials and outcomes were averaged for analysis.

C-9500 Lethality at Sub-Satiating Feeding Regime

In investigating C-9500 lethality on *B. plicatilis* populations exposed to C-9500 with different nutrient availability we used the identical exposure and mortality observation methodologies used to determine medial lethal concentration with some modifications. *B. plicatilis* populations were exposed in a 22°C incubator to 0, 50, or 100ppm concentrations of C-9500 (n=3 vials / treatment). Over the 24 hr exposure period

vials received an AM feeding of 0, 0.15, 0.3, 0.45, or 0.6 $mL*L^{-1}$ and PM feeding of 0, 0.25, 0.5, 0.75, or $1mL*L^{-1}$ respectively. The same experiment was replicated in 2 trials and outcomes among trials were averaged for further analysis.

Statistics

24 hr LC50 was calculated by Probit Analysis. Velocity and net to gross movement were compared by a weighted multi-factorial ANOVA to test for interaction between levels of C-9500 exposure and duration of exposure. Post-hoc of velocity and net to gross movement between groups within the same time period were compared by a weighted one-way ANOVA. Bonferroni correction was applied to compensate for repeated comparisons reducing the level of significance to $p < 0.00263$ for all velocity and net to gross movement statistics (Dunn, 1961). All other comparisons were performed by multi-factorial ANOVA on aligned rank transformed data as described by Wobbrock et al. (2011) to test for interaction between abiotic or biotic secondary stressors and C-9500 exposure. All analyses were computed using IBM SPSS statistical software package ver. 20.

RESULTS

LC50

The 24 hr LC25, LC50, and LC75 for *B. plicatilis* populations exposed to concentrations of C-9500 while maintained at 17.5ppt and 24°C were ca. 78.4 (±5.35), 107.4 (±5.56), and 147 (±9.52) ppm, respectively (Figure 1). This demonstrates that *B. plicatilis* populations cultured in the laboratory are susceptible to increasing concentrations of C-9500 and establishes median lethal concentrations to be used when testing secondary stressors.

Swimming Behavior

Velocity

B. plicatlis populations exposed to 100 or 150ppm concentrations of C-9500 for 6hrs had significantly decreased swimming velocity compared to controls ($p < 0.001$, Figure 2). *B. plicatilis* populations exposed to a 150ppm concentrations of C-9500 for 12 or 24hrs also had significantly decreased swimming velocity compared to controls ($p < 0.001$, Figure 3 and 4). *B. plicatilis* populations exposed to a 50ppm concentration for 24hrs had significantly increased swimming velocity compared to controls ($p < 0.001$, Figure 4). Significant ($p < 0.001$) interaction between levels of C-9500 exposure and duration of exposure on swimming velocity was observed.

Directional Behavior

B. plicatilis populations exposed to C-9500 for 6 hr had no significant difference in directional behavior, as measured by net to gross movement, compared to controls (Figure 5). *B. plicatilis* populations exposed to 100 or 150ppm concentrations of C-9500 for 12 hrs had significantly decreased net to gross movement compared to controls ($p < 0.001$, Figure 6), *B. plicatilis* populations exposed to a 100ppm concentrations of C-9500 for 24hrs also had significantly decreased net to gross movement compared to controls ($p < 0.001$, Figure 7). No significant ($p = 0.021$) interaction between levels of C-9500 exposure and duration of exposure on net to gross movement was observed. Exposure concentration had a significant effect on net to gross movement ($p < 0.001$); however, exposure duration did not have a significant effect ($p = 0.249$).

Combined stressors

Temperature

B. plicatilis populations exposed to C-9500 (0, 50, 100, or 150ppm) at temperatures ranging from 11 to 41°C show a dose-dependent reduction in upper thermal tolerance (Figure 8). As the concentration of C-9500 increases, the populations have increased mortality at all temperatures higher than ca. 24°C compared to control populations. Analysis by multifactorial ANOVA shows significant ($p < 0.001$) interaction between C-9500 exposure and increasing temperature.

Salinity

B. plicatilis populations exposed to C-9500 (0, 50, or 100ppm) at salinity ranging from 5 to 32ppt show a dose-dependent increase in mortality with highest mortalities

occurring at approximately 25ppt (Figure 9). Analysis by multifactorial ANOVA shows significant ($p < 0.001$) interaction between C-9500 exposure and increasing salinity.

Population Density

B. plicatilis populations exposed to C-9500 (0, 50, or 100ppm) at initial population densities of 50, 200, or 350 ind.*ml^{-1} show a dose-dependent increase in mortality with increased mortality at higher population densities (Figure 10). Analysis by multifactorial ANOVA shows significant ($p = 0.007$) interaction between C-9500 exposure and increased population density.

Nutrient Availability

B. plicatilis populations exposed to C-9500 (0, 50, or 100ppm) and provided a rationed feeding of 0 to 2x of the control ration showed increased mortality with increased feeding in both the control and C-9500 exposed populations (Figure 11). Analysis by multifactorial ANOVA shows no significant interaction ($p = 0.765$) between C-9500 exposure and nutrient availability at the levels tested in this study.

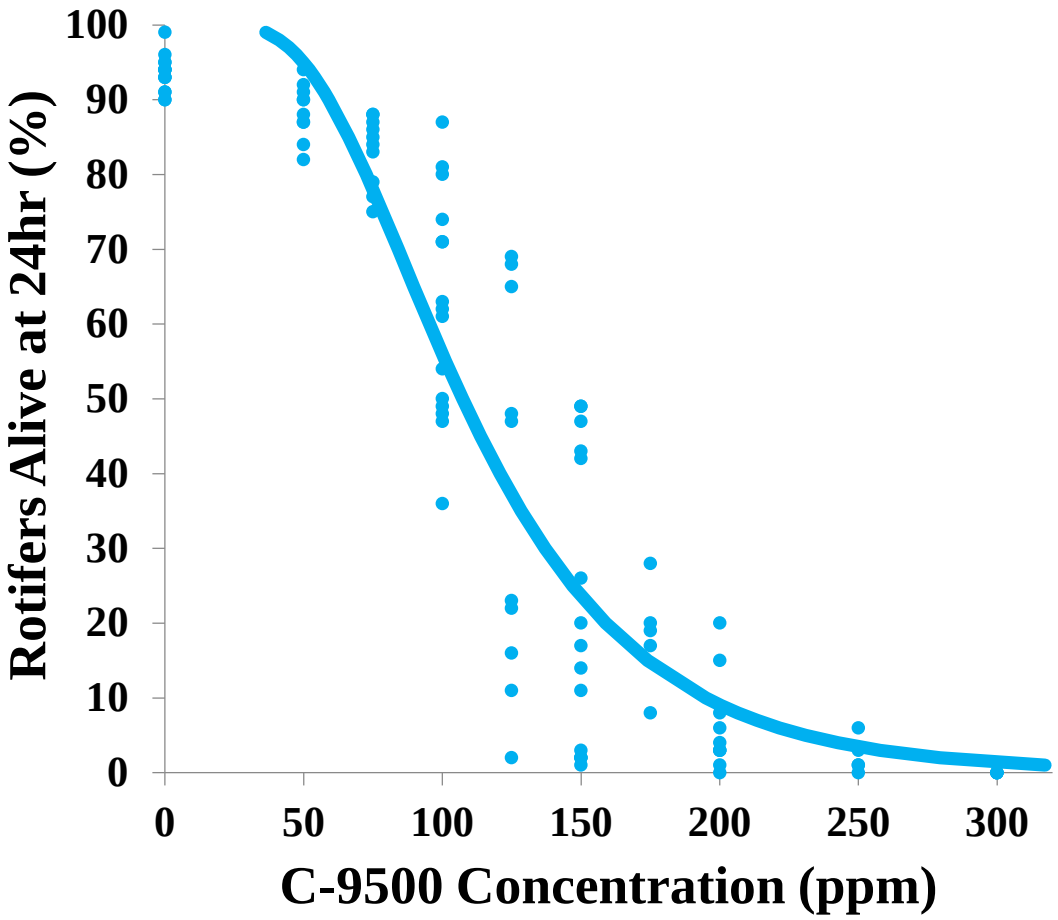

Figure 1: Percent of B. plicatilis alive following a 24hr exposure to C-9500 at 0 to 300ppm (values represent individual trials, line represents Probit fit to dataset used to determine LC50).

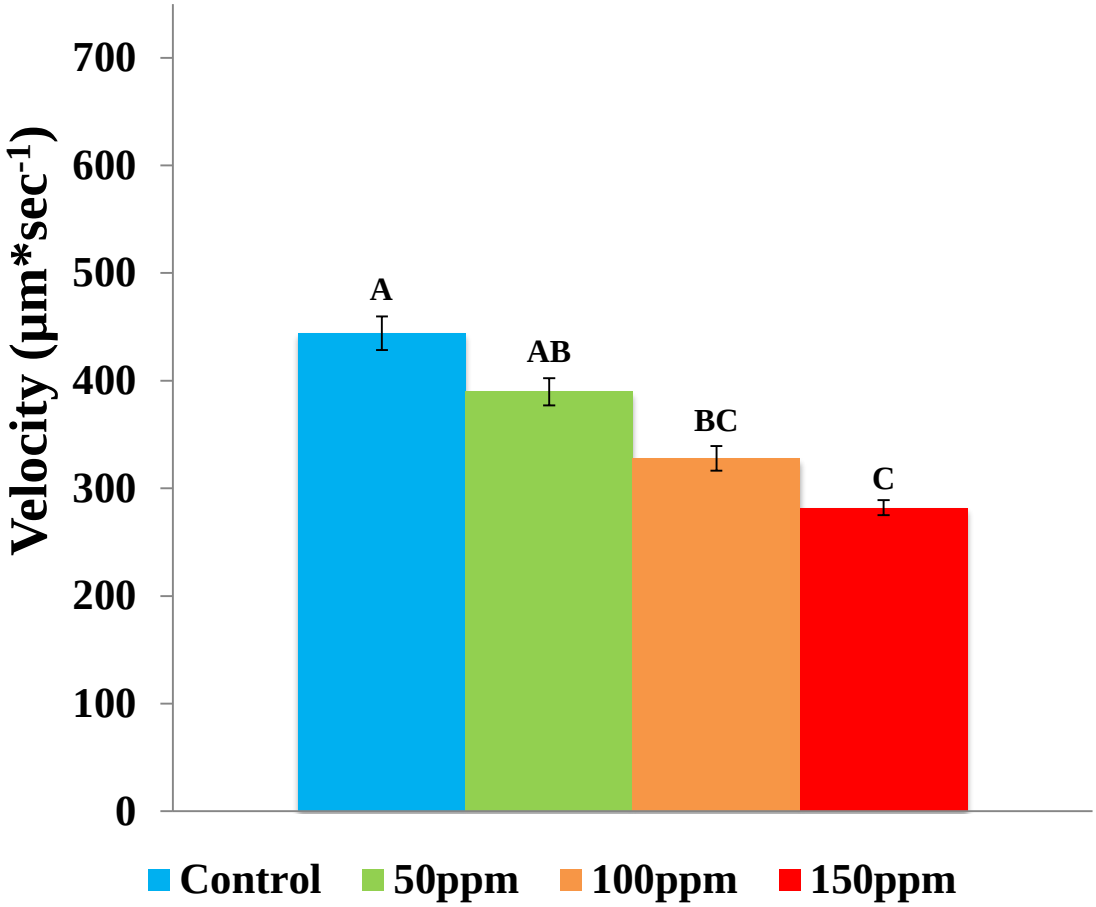

Figure 2: Swimming velocity of *B. plicatilis* populations following a 6hr exposure to C-9500 concentrations of 0, 50, 100, or 150ppm (values represent means, error bars are standard error of the mean). Bars with the same letter are not significantly different.

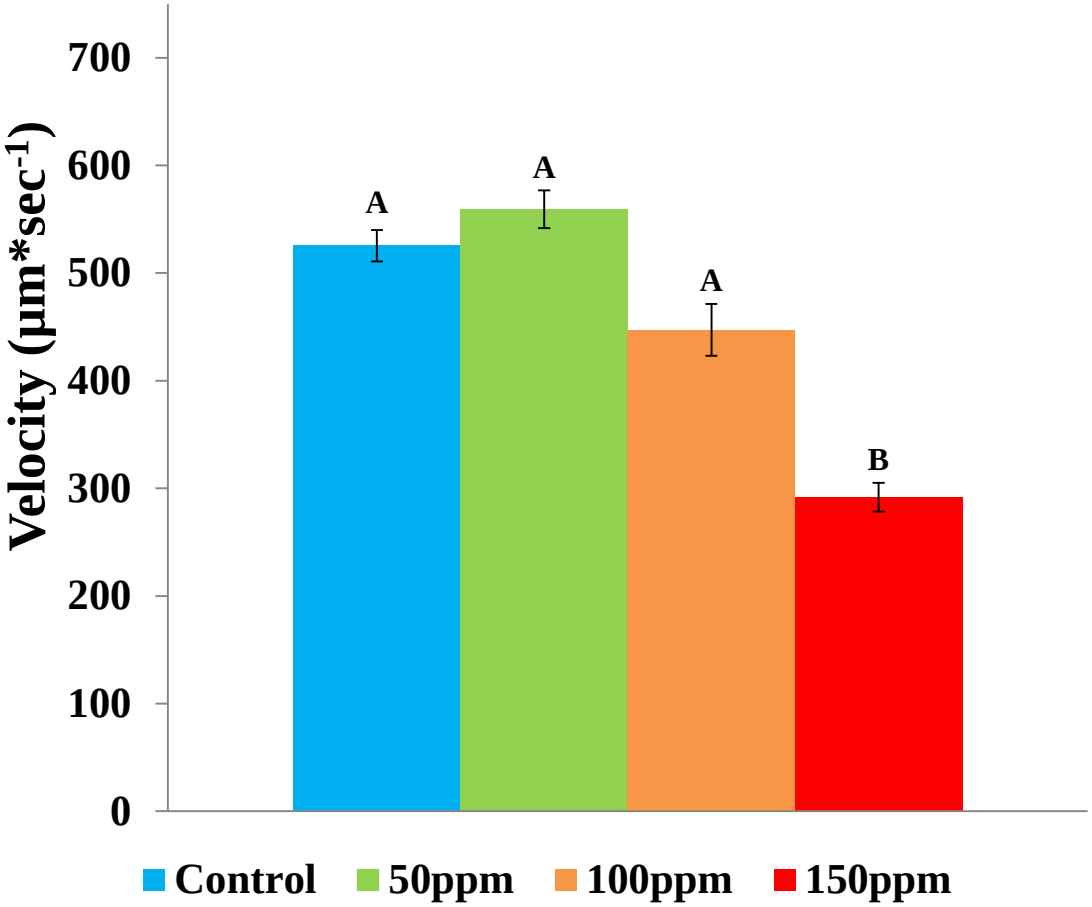

Figure 3: Swimming velocity of *B. plicatilis* populations following a 12hr exposure to C-9500 concentrations of 0, 50, 100, or 150ppm (bars represent means, error bars are standard error of the mean). Bars with the same letter are not significantly different.

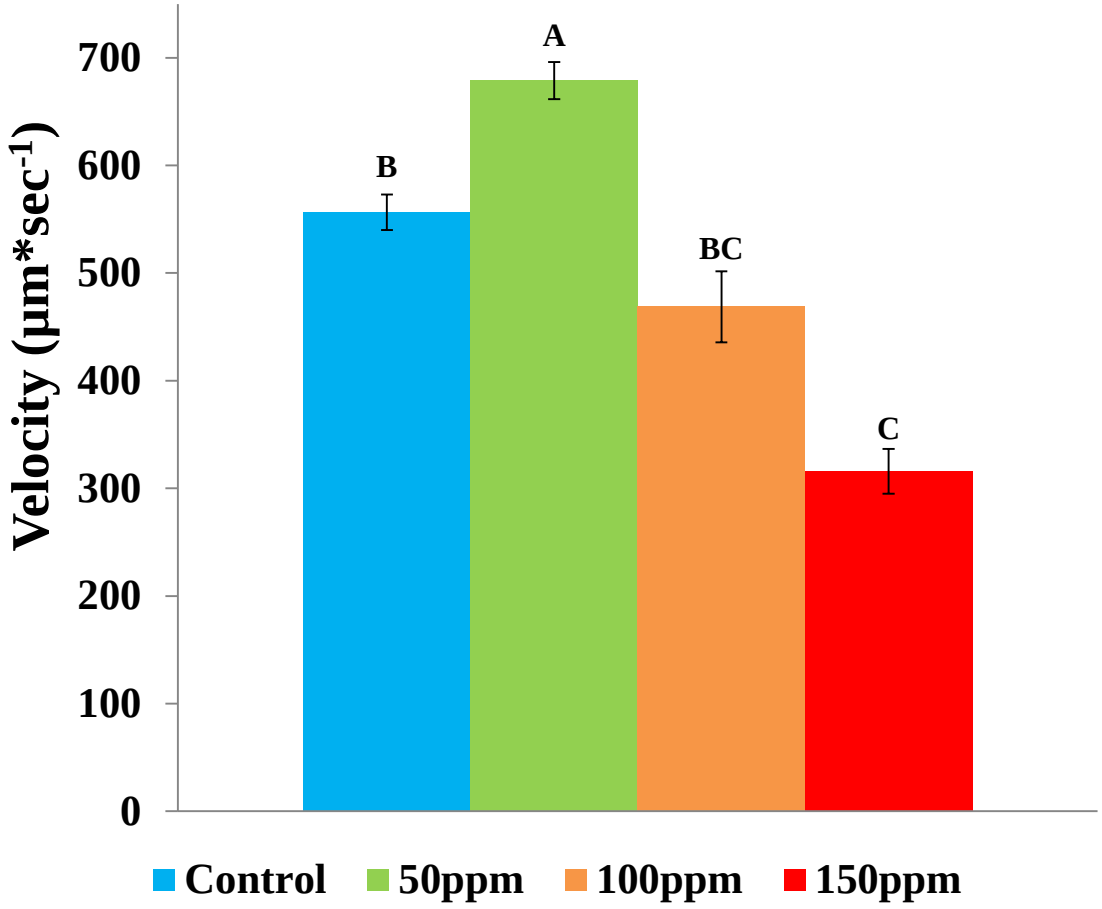

Figure 4: Swimming velocity of *B. plicatilis* populations following a 24hr exposure to C-9500 concentrations of 0, 50, 100, or 150ppm (bars represent means, error bars are standard error of the mean). Bars with the same letter are not significantly different.

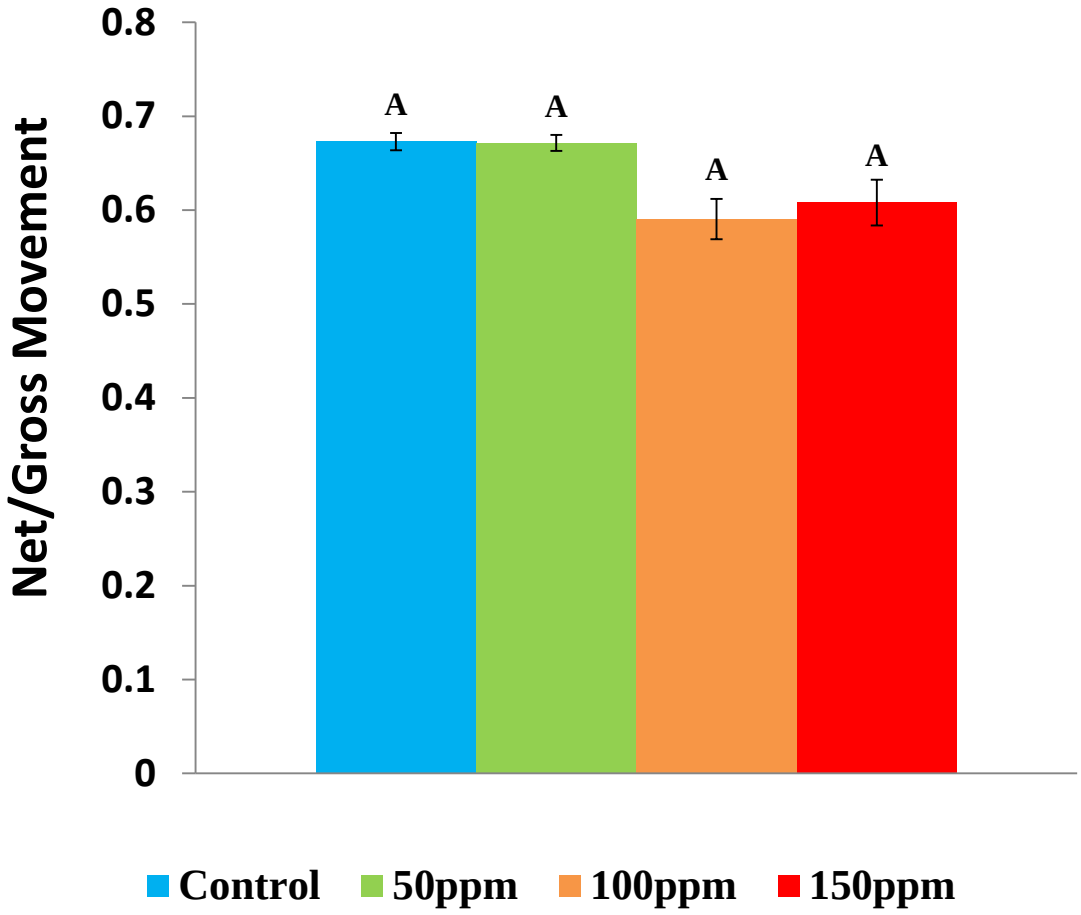

Figure 5: Net to gross movement of *B. plicatilis* populations following a 6hr exposure to C-9500 concentrations of 0, 50, 100, or 150ppm (bars represent means, error bars are standard error of the mean). Bars with the same letter are not significantly different.

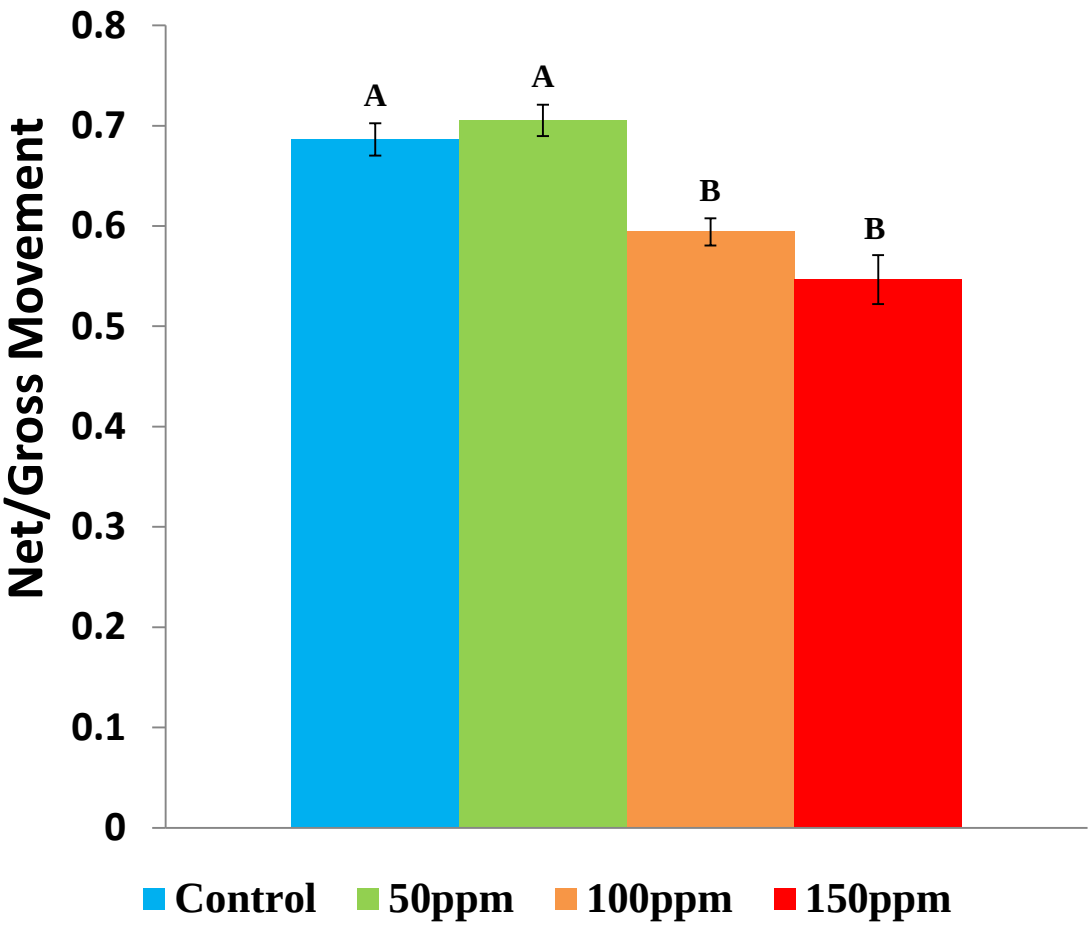

Figure 6: Net to gross movement of *B. plicatilis* populations following a 12hr exposure to C-9500 concentrations of 0, 50, 100, or 150ppm (bars represent means, error bars are standard error of the mean). Bars with the same letter are not significantly different.

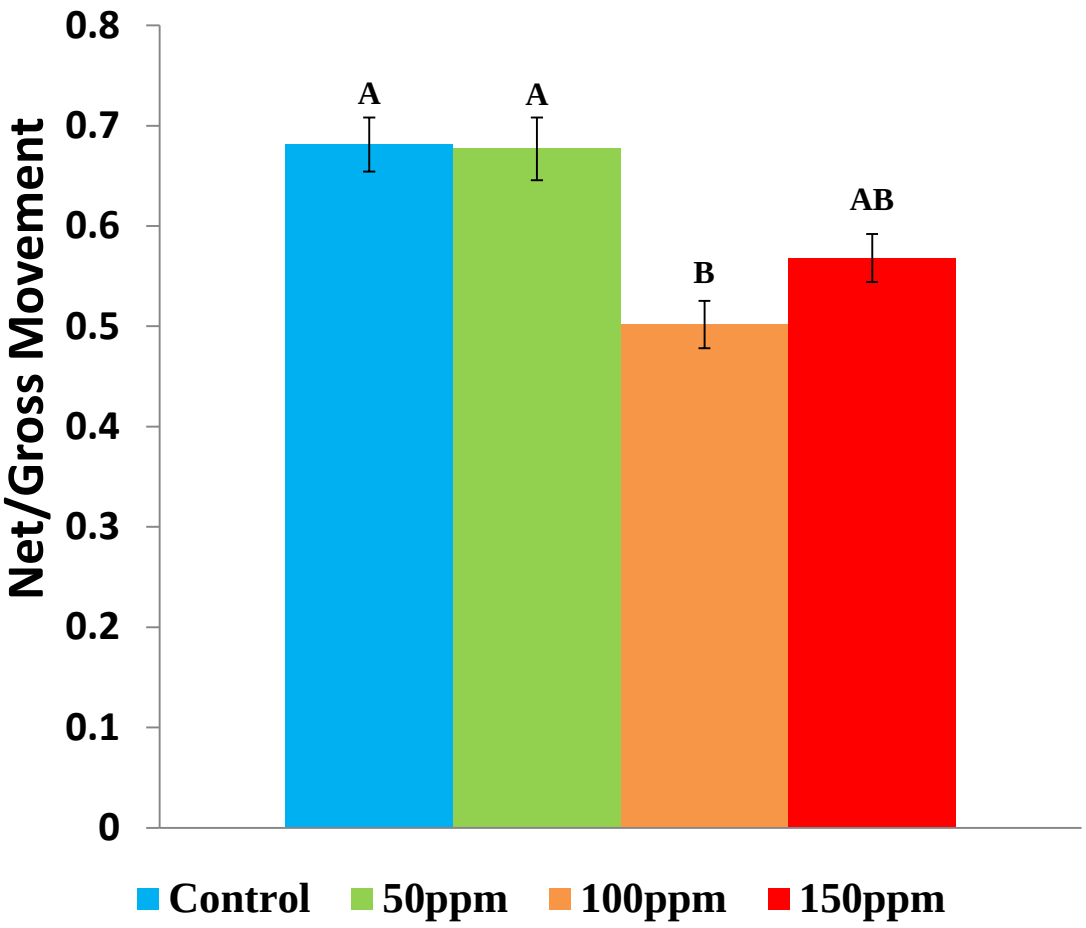

Figure 7: Net to gross movement of *B. plicatilis* populations following a 24hr exposure to C-9500 concentrations of 0, 50, 100, or 150ppm (bars represent means, error bars are standard error of the mean). Bars with the same letter are not significantly different.

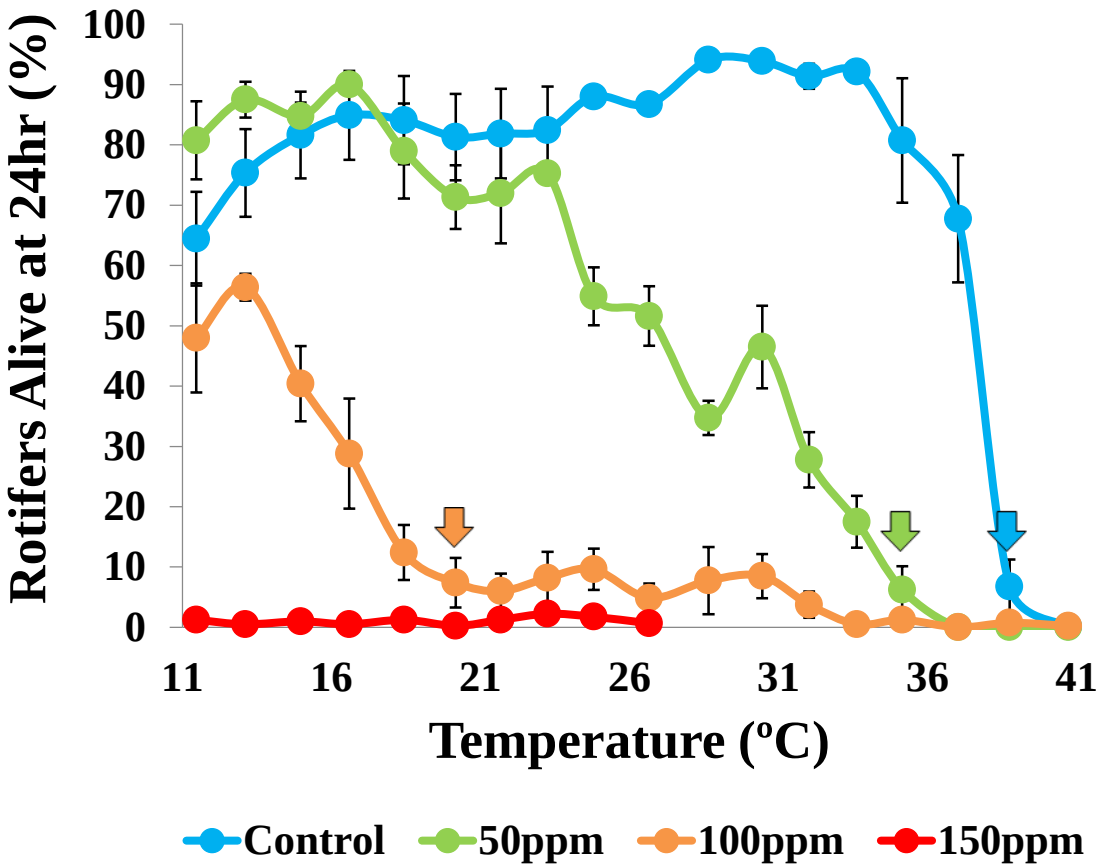

Figure 8: Percent of *B. plicatilis* alive following a 24hr exposure to C-9500 at concentrations of 0, 50,100, or 150ppm at temperatures ranging from 11 to 41°C (values represent mean of 2 trials, error bars are standard error of the mean). Arrows indicate the temperature where mean alive rotifer after 24hrs was less than 10% at each concentration.

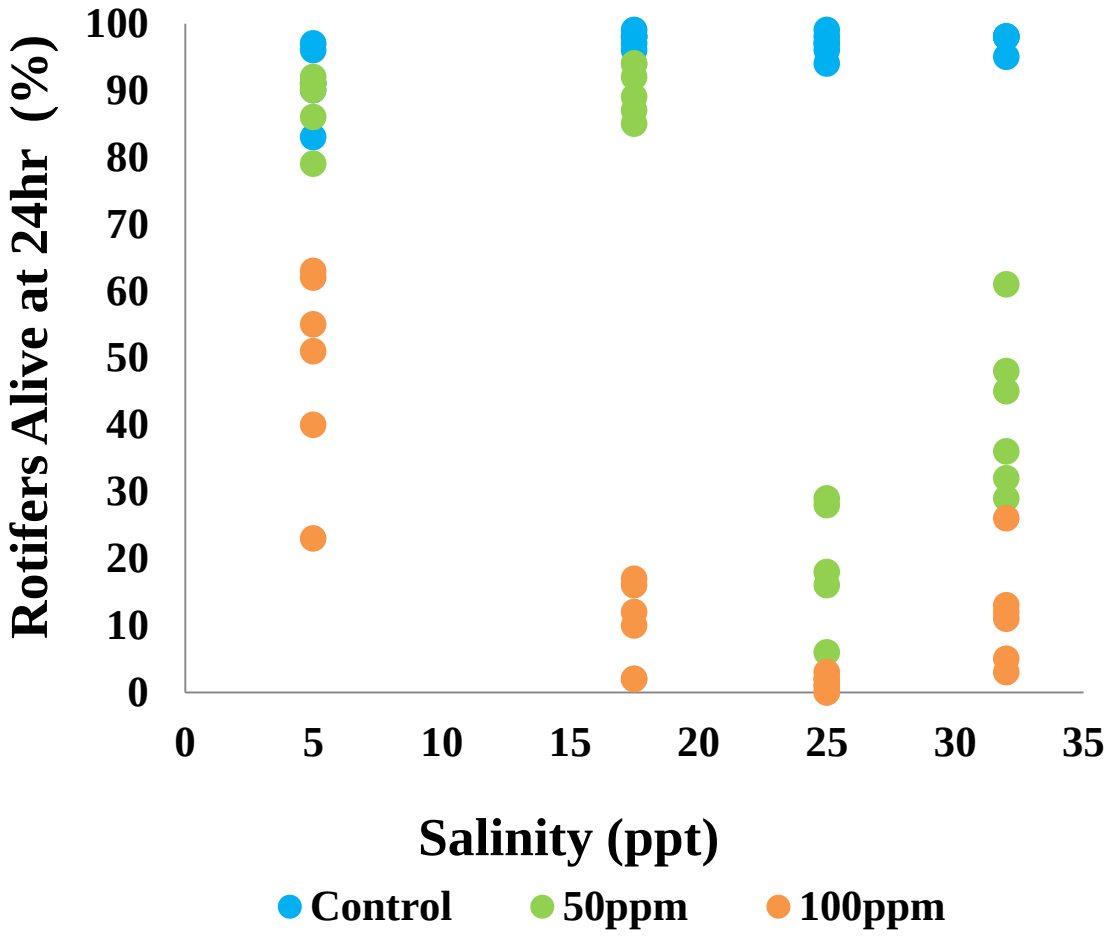

Figure 9: Percent of *B. plicatilis* alive following a 24hr exposure to C-9500 at concentrations of 0, 50 or 100ppm at salinities ranging from 5 to 32ppt (values represent individual trials).

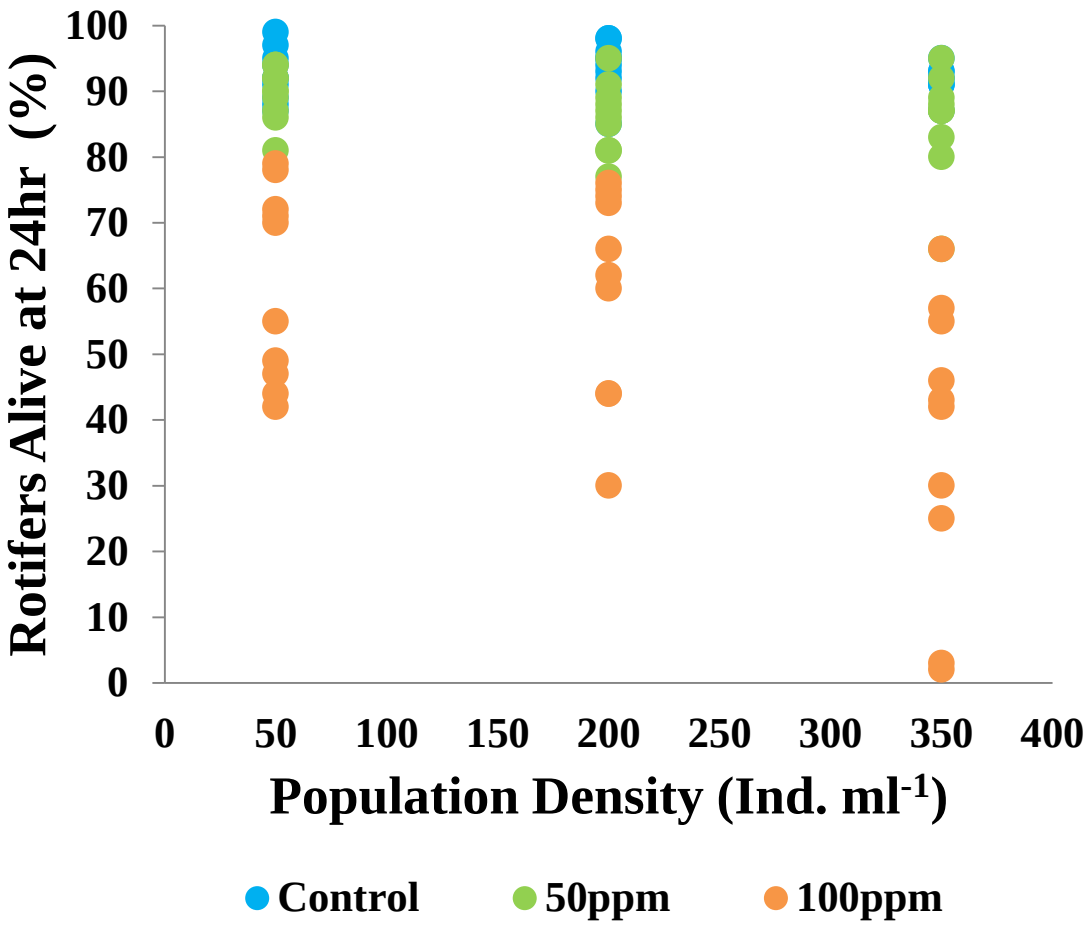

Figure 10: Percent of *B. plicatilis* alive following a 24hr exposure to C-9500 at concentrations of 0, 50 or 100ppm at population densities ranging from 50 to 350 ind.*ml^{-1} (values represent individual trials).

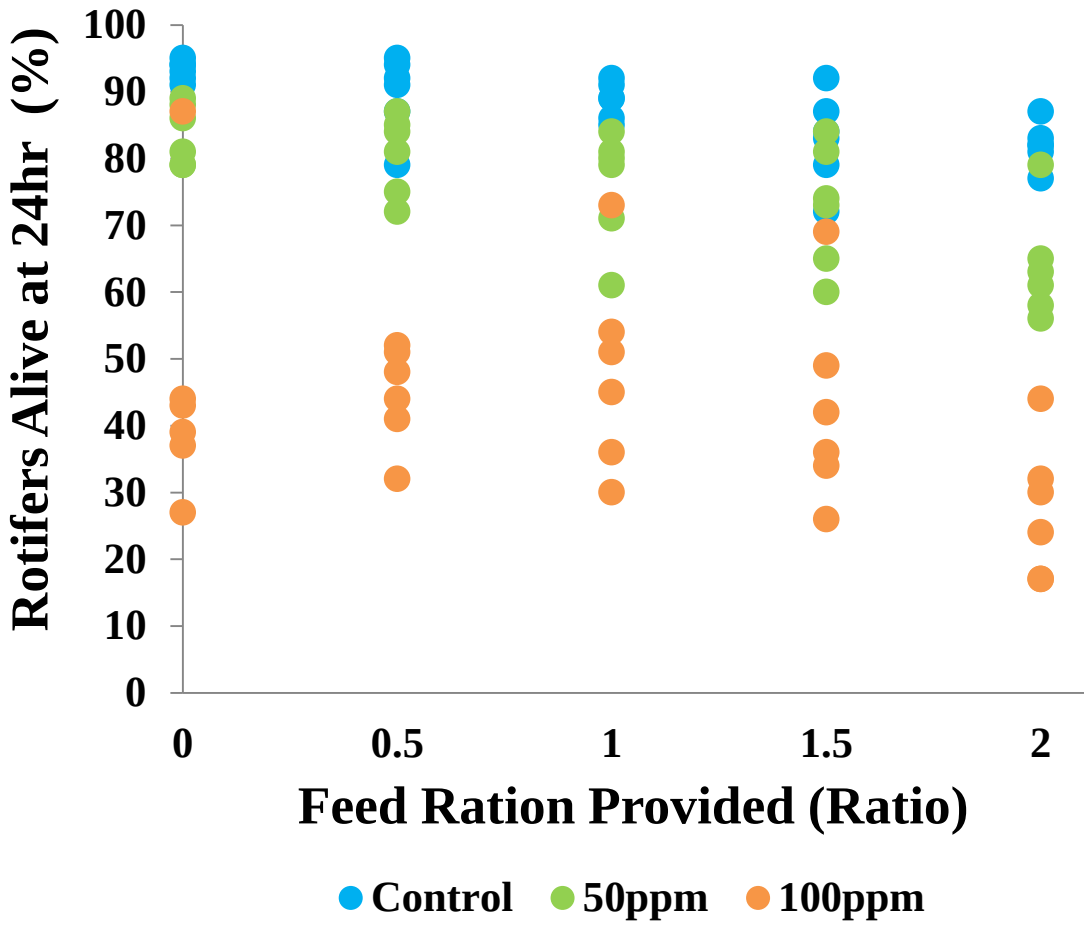

Figure 11: Percent of *B. plicatilis* alive following a 24hr exposure to C-9500 at concentrations of 0, 50 or100ppm provided a rationed feeding of 0 to 2x of the control ration (values represent individual trials).

DISCUSSION

The median lethal concentration calculated in our experiment was higher than what was reported in recent studies on outcomes of C-9500 exposure for *B. plicatilis* (Rico-Martínez et al., 2013). A differential mortality response can be attributed to differing experimental design and lifestages. In these experiments we exposed populations of rotifers containing individuals representing all life stages, from juveniles to reproductively-active adults. This is in contrast to methodologies in which zooplankton would be exposed at a single life stage at very low density (ASTM, 1998). We hypothesize that different life stages could differ in susceptibility to C-9500; however, a mixed stage population would most likely be similar to population structure found in natural populations.

Swimming of aquatic species meets the needed characteristics of a behavioral response for toxicant studies such as ecological relevance and defined and practical measurements (Garaventa, 2010). While investigating toxicant lethality we observed rotifers that survived exposure to high concentrations of C-9500 swimming at a decreased velocity or in different patterns (including whirling patterns). We observed differences in swim velocity and directional behavior (net to gross movement) between controls and rotifers surviving C-9500 exposure. Both swim velocity and directional behavior of C-9500 exposed populations of *B. plicatilis* were concentration-dependent, and increasing concentrations of the dispersant generally reduced swimming velocity and altered swimming behavior. Interestingly, rotifers exposed to low concentrations of C-9500 increased swimming velocity compared to control populations. This increased velocity

may be indicative of an increased metabolic rate or a behavioral response of rotifers actively trying to escape contaminated areas. This overcompensation effect of swimming velocity has been observed in other toxicity studies utilizing *B. plicatilis* as a model when concentrations less than the median lethal concentration were applied (Garaventa et al., 2010). Further investigation of zooplankton ability to respond to contaminants, to mount an escape response, and to remove themselves from contaminated areas is warranted.

B. plicatilis population exposed to 100 and 150ppm (concentrations at and above our calculated median lethal concentration) showed both a decrease in swimming velocity and an altered directional pattern of swimming behavior. At 6 hrs of exposure we observed decreased swimming velocity in rotifers surviving exposure to these concentrations. At 12 and 24 hrs of exposure we observed a recovery in rotifer populations exposed to 100ppm, wherein the velocity was not significantly different from control populations. Swimming velocity was decreased in all time periods for rotifers exposed to 150ppm. Rotifers surviving 150ppm exposure did not recover normal swimming velocity in the time frame of this study. We hypothesize that these changes in swimming behavior are indicative of stress, and could affect the response of the rotifers to their environment. Food intake and avoidance of predators or perturbations are dependent on swimming ability (Charoy 1995 and Gilbert 1985) Swimming deficiencies can be a detriment to the survival and health of zooplankton populations, whose loss would indirectly impact higher trophic level planktivores. Decreased ability to move towards phytoplankton or obtain nutrients in the environment will alter nutritional state of zooplankton and reduce nutrient transfer among trophic levels. In addition, the lack of directional swimming in survivors of both 100 and 150ppm exposures suggests that there

is an increasing pattern of random movement. It is possible that C-9500 is affecting neuromuscular coordination. We hypothesize that C-9500 at these exposure levels may reduce feeding potential of the rotifers and therefore reduce even further nutrient delivery to larval fishes and marine invertebrates that feed on these species (McQueen, 1986). We hypothesize that differences seen in swimming velocity and directional behavior may be caused by altered population structure following C-9500 exposure, assuming differential mortality of various life stages. However, if a change in population structure were the sole reason for the differences in swimming parameters, we would expect to see decreased variation over the exposure time at the same exposure concentrations. Instead, variation remains unchanged and the mean of these parameters change location over the exposure time.

Common abiotic stressors (temperature and salinity) affected, through interactions with C-9500 exposure, the viability of *B. plicatilis*. Temperature was a significant factor in the outcome of C-9500 exposures in our rotifer populations. We suggest the combined effects of increased temperature and exposure to C-9500 may decrease the ability of the HSP pathway to respond to these combined stressors (Smith et al., 2012 and Wheelock et al., 2002), leading to dose-dependent mortalities increasing with increasing temperatures. Alternatively, an increase in temperature may increase oil dispersing efficiency on C-9500 as seen in C-9527 (Fingas et al., 1991). This would increase the ability of C-9500 to disrupt lipid structures in rotifers (e.g. cell membranes) and would mean the synergistic effect observed is more an effect of the dispersant chemical properties and not biological. In any case, the decrease in upper lethal limits with C-9500 exposure suggests that application temperature will affect mortality outcomes and population structure in natural

populations. Application of C-9500 when seasonal temperatures are cool might reduce the level of mortality in zooplankton populations, and reduce the impact of C-9500 application on this important trophic level.

In investigating the combined effect of thermal stress and C-9500, population mortality at 24°C was higher than mortality seen in our examination of the median lethal concentration. These experiments were conducted separately at similar temperatures, but vials were held in different environments that differed in other parameters (eg., an open incubator with full light vs an aluminum block with partially obstructed vials held under low light). This difference in light exposure or another unidentified factor may account for this altered mortality when compared to our LC50 experiments at the same temperature. These differences do not change the interpretation of outcomes related to temperature and concentration-dependent effects of C-9500 on *B. plicatilis* (Epp and William, 1980).

Mortality was influenced by the interaction between salinity and C-9500 concentration. However, the highest mortality in both trials and all concentrations tested occured at a salinity of 25ppt. We hypothesize that different ion-regulating mechanisms utilized at these salinities contributed to this effect. Rotifer populations at 25ppt may be inducing mechanisms related to osmoregulation (Lowe et al., 2004). We suggest that the transition to ion regulating mechanisms contributes to increased mortality (increase in susceptibility) in zooplankton populations. In field populations this would be most relevant in coastal estuarine regions subject to periodic fresh water intrusion from runoff. However, in the present study evaluation was made on populations that had been

acclimated to their respective salinity. It is not known if *B. plicatilis* would be affected when salinity is changed acutely during exposure to C-9500 in a similar manner.

In addition to abiotic stressors, biotic stressors (density and nutrient availability) affected mortality in *B. plicatilis.* However, only density interacted with concentrations of C-9500 to influence mortality of *B. plicatilis* populations. In our study, rotifers held at higher densities exhibited increased mortality at the highest concentration of C-9500 (100ppm) tested. Either grouping effects or crowding responses may be secondary stressors leading to increased mortality of C-9500-exposed rotifer populations. The altered energy utilization and population interactions related to higher densities may increase toxicant susceptibility. Oxygen in all stages of the experiment was adequate to maintain high viability of control groups; however; oxygen requirements of zooplankton under toxicant exposure or combined stressors are not known. Australian Bass (*Macquaria novemaculeata*) show increase oxygen consumption when exposed to oil dispersed by C-9527 concomitant with an increased expression of metabolic enzymes (Cohen, 2001). In the present study, increased metabolic rate (indirectly suggested by higher swimming velocities at low C-9500 concentrations) for zooplankton populations exposed to C-9500 may be resulting in higher mortality at high densities. C-9500 was applied during the Deepwater Horizon oil spill in an area reported previously to have high seasonal variation in phytoplankton population densities (Müller-Karger et al., 1991). In marine environments increased population density does not typically result in oxygen depletion sufficient to act as a potential stressor; however, C-9500 was applied close to an annual hypoxic zone that occurs near the coast of Louisiana which could be exacerbated by the presence of crude oil (NOAA, 2010). The low levels of dissolved

oxygen that characterize hypoxic zones make them uninhabitable for high densities of zooplankton. Our results suggest that these factors may act synergistically to lower *B. plicatilis* survival if dispersant is applied in these areas. The role of oxygen availability and C-9500 exposure requires further study.

Nutrient availability did not affect the response of *B. plicatilis* to C-9500 exposure. Nutrient availability did have an effect on *B. plicatilis* mortality, but this was independent of C-9500 exposure, and decreased viability was associated with high nutrient availability rather than low availability or short term starvation. Increased concentrations of feed (previously frozen *Nannochloropsis*) may have increased excretion rates (water fouling) or reduced oxygen availability via biological oxygen demand in both control and exposed rotifer populations. Treatments that were not provided a feeding ration during exposure were proffered a final feeding 7hrs prior to the start of the experiment, thus, these animals were proffered no new food ration for the 31hrs (7 +24 hrs) prior to mortality assessments. Although food deprivation could potentially act as a stressor, viability in the non-fed treatments was equivalent to those fed half or whole rations. A reduced nutrition state, as suggested by our other experiments, will not result in higher mortality for zooplankton populations already undergoing exposure. Chronic food deprivation was not evaluated and could affect outcomes related to C-9500 exposure. Further research is needed to investigate the trophic transfer of nutrients following toxic contaminates and long term ecosystem effects.

This study indicates that interactions between C-9500 and environmental factors increase negative outcomes for zooplankton populations. We also demonstrated

differential behavior in *B. plicatilis* surviving exposure that is reliant on the exposure concentration, and these changes may have ecological implications. We suggest that differential abiotic and biotic factors produced by geographic and seasonal variation may impact ecosystem stability following dispersant application. Additionally, in areas where environmental factors can have extreme fluctuations, such as coastal estuaries, zooplankton populations may experience many of these factors concomitant with C-9500 exposure, leading to diverse and unpredictable interactions. Abiotic and biotic conditions, and how organisms maintain themselves against those possibly stressful conditions, should be considered prior to use of oil dispersants as bioremediation tools. Data from this study may be used in management considerations for the future application of dispersants under diverse conditions.

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