BIODIESEL AND CRUDE OIL EFFECTS ON FORAGING CAPACITY OF CRAYFISH
ORCONECTUS RUSTICUS

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Previous research suggests that environmental pollutions such as crude oil and other petroleum based fuels inhibit and limit the perception of a chemical stimulus among other toxic effects. Alternative fuels have become more prominent today because of these environmental concerns. Due to the increase in the use of alternative fuels behavioral test are needed to determine whether alternative fuels will affect the ability of organisms to perceive chemical stimuli. To determine the impact of biodiesel on organisms chemically mediated behavior, we acutely exposed crayfish to two different fuel types (biodiesel or crude oil) at two different concentrations. Crayfish were then tested on their ability to appropriately respond to a chemical stimulus within a –maze choice paradigm. Behavior was quantified by measuring the ability of crayfish to find the odor source, the time spent finding an odor source, and other behavioral measures associated with a y-maze choice paradigm. Results indicated that both biodiesel and crude oil impact the ability of a crayfish to perform within this bioassay. However, there were no significant differences between behavioral performances when crayfish were exposed to either fuel source. Thus, biodiesel and crude oil have equal negative effects on chemosensory behavior of crayfish. These findings have shown that biodiesel has the potential to have similar negative ecological impacts as other fuel source toxins.
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BIODIESEL AND CRUDE OIL EFFECTS ON FORAGING CAPACITY OF CRAYFISH, ORCONECTUS RUSTICUS

INTRODUCTION

Freshwater Pollution

With a finite amount of freshwater available, keeping this limited supply uncontaminated has become a priority for humanity (Rogers 2008). However, today’s freshwater systems are being polluted by numerous sources (Trett 1989, Abel 1996, Mason 1996). Adulteration of aquatic ecosystems by different types of pollution is becoming more widespread, especially with the thousands of chemicals in common usage today (Maugh 1978). Some chemicals found throughout environmental systems have been deposited into these systems through interactions by humans. Sources of pollution range from run-off to atmospheric fallout. Contamination from run-off can contain heavy metals (e.g., lead, zinc, copper, and cadmium) and polycyclic aromatic hydrocarbons (PAH). Atmospheric fallout can include PAHs found in oils, and polychlorinated biphenyls (PCBs), which were once used as coolants, and other liquids before being banned because of their possible ability of causing carcinogenesis, mutagenesis or hormonal disruption (Göbel et al. 2006, Blanchard et al. 2007).

Anthropogenic Interactions

Human interactions have been a cause of harmful effects to aquatic ecosystems (Paul and Meyer 2001). These anthropogenic interactions cause changes in the ecosystem through alterations in temperature and chemical composition of the aquatic system due to exposure from pollutants (Sahagian 1998, Paul and Meyer, 2001). Anthropogenic pollutants alter the chemistry of freshwater systems through chronic or acute exposure to the environment (Robotham and Gill 1989). Infiltration of sewage into aquatic ecosystems has been shown to decrease diversity in fish and invertebrate populations (Wright et al. 1995, Thorne et al. 2000, Paul and Meyer, 2001).
Exposure to agricultural products from runoff has been theorized to be a main cause in the decline of amphibian populations (Verrell 2000, Hayes et al. 2002, Rohr et al. 2003). Neotropical fish exposed to diesel fuel exhibited histological damages in the form of lesions on gills and liver (Simonato et al. 2008). King and Wagner (2010) exposed amphibian species to the herbicide Roundup, which produced lethal effects at concentrations lower than allowed by the Environmental Protection Agency (EPA). Exposure to heavy metals and organic pollutants to fish species showed disruption of reproductive, predation avoidance, locomotion ability, and social hierarchy behaviors (Scott and Sloman 2004).

**Pollutant Effects**

Pollutant effects depend mostly on their chemical composition (La Farrè et al. 2008). Herbicides have been known to decrease foraging ability and agonistic behavior of crayfish through alterations in chemosensory abilities (Wolf and Moore 2002, Cook and Moore 2008). Pharmaceuticals such as, fluoxetine and ibuprofen have shown to decrease locomotion and feeding activity in *Gammarus pulex* (freshwater shrimp), while pharmaceutical mixtures (clofibric acid and fluoxetine) cause morphological abnormalities ranging from crinkled carapace, distorted antennae and bent tail spines in *Daphina magna* (water flea) (Flaherty and Dodson 2005, De Lange et al. 2006). Crude oil has also been shown to decrease growth in *Oncorhynchus gorbuscha* (pink salmon), and *Daphina magna* (Geiger and Buikema 1982, Heintz et al. 2000).

**Crude Oil Variation**

Not only do different types of pollutants vary in their chemical composition, there is variation in chemical makeup within a single type of pollutant such as crude oil (Speight 1999). Compositional differences of crude oils are in the differences in amounts of paraffin’s,
naphthenes, alkenes, and aromatics (Gill and Robotham 1989). PAHs are the greatest concern because of their carcinogenic effects (Boffetta et al. 1997; Solomon and Janssen, 2010). Water soluble fractions of crude oil have been known to cause deleterious histological lesions to *Odontesthes argentinens* (marine pejerrey) and the same effect appeared on gills of *Prochilodus lineatus* (characiformes, prochilodontidae) (Simonato et al. 2007; Rodrigues et al. 2010).

The varied chemical composition of oil leads to a multitude of ranges in lethal concentrations to fifty percent of the population (LC50, Franklin and Lloyd 1982). Due to the increase in demand of oil in the around the globe (Cooper 2003), the environment is at increased risk for detrimental effects from the increase in transportation of oil. With the known toxic effects caused by crude oil, fuel sources deemed more environmentally friendly are being sought.

*Biodiesel*

Alternative sources of energy have been investigated for years and more recently biodiesel has come to the forefront of renewable energy. Biodiesel is an alternative diesel fuel processed and made from biological sources such as vegetable oils and animal fats (Ma and Hanna 1999). Biodiesel has begun to receive more attention because of the environmental appeal due to the decreased carbon emissions in comparison to that of crude oil based products (Ma and Hanna 1999, Savvanidou et al. 2010). Compared to crude oil, biodiesel has 90% less unburned hydrocarbons and a 75-90% reduction of noxious PAHs gases all harmful substances that are emitted into the environment (Demirbas 2007). Characteristics of biodiesel fuels demonstrate similarities to petroleum based fuels, making biodiesel an available source to replace standard fuels (Singh and Singh 2010). Biodiesel’s low levels of sulfur and lack of aromatics are the main compositional differences in toxicity between biodiesel and crude oil (von Wedel 1999, Singh
Production of biodiesel fuels comes from many different sources and this leads to differences in the composition of biodiesel (Pinto et al. 2005).

Biodiesel is composed of fatty acids that are chemically transformed into fatty acid methyl esters (FAME) differing from crude oil’s composition of hydrocarbons (Tyson 2001). Composition of biodiesel is different depending upon the feedstock source used for production. With the leading sources of biodiesel feedstock’s being from soybeans, recycled oil, sunflower and palm kernels (Pinto et al. 2005). The diversity between all of these biodiesels is in the composition of the oil (Singh and Singh 2010).

**Biodiesel Toxicity**

Differences in composition of biodiesels cause alterations in the toxicity levels. A study comparing three pure biodiesels or B100, each from a different feedstock (castor, waste cooking and palm oil) found castor oil to be the most toxic to *Echinometra lucunter* (sea urchin) followed by waste cooking oil, and finally palm oil (Leite et al. 2011). Leite et al. (2011) also observed developmental abnormalities in embryonic and larval stages of *Echinometra lucunter* exposed to biodiesels. With biodiesels and crude oils comparable uses as fuels, the increased use and subsequent pollution could lead to toxic effects imposed upon aquatic species (Khan et al. 2007).

**Effects of Biodiesel and Crude Oil**

Pollution of an environmental system either by crude oil or biodiesel is known to cause negative behavioral and physiological effects to animals in those systems (Bucas and Sailot 2002, Rodrigues et al. 2010). Oils used in the production of biodiesel can cause asphyxiation and blockage in the digestive tract of benthic fauna (Bucas and Sailot 2002). A comparative study assessing the acute toxicity (LC50s) of biodiesel, biodiesel blends, and crude oils found biodiesel
to be less toxic than both the biodiesel blends and crude oil to both *Daphnia magna* and *Oncorhynchus mykiss* (rainbow trout) (Khan et al. 2007). Similar results have been shown in studies with *O. mykiss*, where exposure to low sulfur diesel had an LC50 of 160 mg/L compared to 707 mg/L of B100 Canola (Hollebone 2007). Male rats exposed sublingually to three variations of biodiesel and ultra-low sulfur diesel (ULSD) demonstrated that ULSD produced more histopathological and biochemical toxicity compared to the biodiesels (Poon et al. 2009). Decreases in the ability to forage and reproduce were behavioral effects seen after exposure to sub-lethal amounts of crude oil to arctic invertebrates (Percy and Mullin 1977, Bushdosh 1981, Jensen and Carroll 2010). Similar results occurred with *Homarus americanus* (lobsters) when oil was integrated into the aquarium with a food source (Atema and Stein 1974). Delayed physiological changes on growth and survival occurred in *O. gorbuscha* when exposed to crude oil in a concentration of 5.4 ppb as embryos (Heintz et al. 2000). Crude oil can also hamper the immune systems, respiratory ion regulation, and hepatic autoxidation (Hannam et al. 2009; Yuanyuan et al. 2009, Duarte et al. 2010).

Introduction of crude oil into the environment has been found to inhibit and disrupt chemoreceptive abilities of freshwater and marine species, specifically invertebrates and coral (Blumer et al. 1973, Loya and Rinkevich 1980, Suchanek 1993). Macro-benthic and meiobenthic populations have been directly reduced due to the presence of crude oil (Elmgren et al. 1983; Peterson et al. 2003, Bejarano et al. 2005, Venturini et al. 2008). Hinkle-Conn et al. (1998) found contamination by diesel oil levels of 122 mg PAH kg\(^{-1}\) decreased juvenile spot feeding (*Leiostomus xanthurus* Lacèpède: *Pices*). The complex chemical makeup and possible contamination to the environment with crude or biodiesel have the ability to elicit mortality and behavioral changes, such as decreases in foraging. Problems caused by contamination from crude
oil can have negative influences to a specific species within a system, this than can negatively affect all trophic levels.

Ecological Importance of Crayfish

As consumers, crayfish play the role of omnivores, influencing energy flow through the food web as well as the nutrient cycle of the lake or stream stable (Momot et al. 1978, Momot 1995). Maintenance of these processes are achieved by a crayfish’s ability to decompose dead organic matter and their predation of macroinvertebrates (Covich et al. 1999). As prey, crayfish are a staple food for many aquatic vertebrates especially in earlier life stages (Momot et al. 1978, Momot 1995). Crayfish are considered to be keystone species because, when abundant in numbers, they play a vital role as both consumer and prey (Momot et al. 1978, Momot 1995, Covich et al. 1999). With the importance of crayfish to their ecosystems, the introduction of pollutants such as crude oil or biodiesel, could cause adverse effects to crayfish through changes in aquatic chemistry (Paul and Meyer 2001). Chemosensory abilities of an aquatic species can be negated or hindered when contamination of the aquatic ecosystem occurs (Wolf and Moore 2002, Ward et al. 2008).

Chemoreception

Chemoreception is an intricate part of fitness and survival in crayfish (Zulan
t Schneider et al. 1999, Bergman et al. 2003). Crayfish use chemosensation to orientate themselves to specific chemical cues and signals found in the environment (Moore and Grills 1999, Bergman et al. 2003). Chemical signals relay information used to search for food, determine social status of conspecific, and to locate predators within their environment (Bergman and Moore 2005). If chemical signals from the surrounding environment were to be inhibited or masked by an anthropogenic interaction, important behaviors such as hierarchy establishment,
foraging, and sexual selection could be negatively affected (Mitchell et al. 1972, Bergman and Moore 2005). Crude oil has been shown to mask or retard the ability to differentiate and even slow the ability to distinguish a chemical in the water (Blumer et al. 1973, Atema and Stein 1974). This problem is relevant to crayfish because olfactory organs are directly exposed to their environment. After exposure pollutants damage receptor cells and will impair the crayfish's chemosensory capabilities (Tierney et al. 2010). Decreasing the ability to acquire information through the sensory systems for crayfish would decrease fitness and the ability to survive.

Experiments using crude oil and biodiesel in aquatic systems have mainly consisted of lethal dose experiments. Crude oil research has been more effective in finding physiological and behavioral effects than that of biodiesel. Toxicological studies using behavior as an assay is very limited. Instead the majority of studies use LC50s as the end goal. Leading to the hypothesis of; two fuel sources: biodiesel and crude oil negatively affect foraging.

Crayfish are a good model for studying effects of pollutants on olfaction because information about the environment and its surroundings is acquired through chemical senses (Wolfe and Moore 2002). Given the importance of olfaction, an examination of acute exposures to pollutions are important to test in crayfish. These questions are important to inquire about because of the lack of knowledge biodiesel has upon behavioral and sensory abilities of aquatic species, and how the precise effects of an oil spill in the environment influences behavior in comparison to that of biodiesel.
MATERIALS AND METHODS

Animals and housing

Male crayfish, *Orconectes rusticus*, were collected from tributaries of the Portage River (lat. 41.37° N, long. 83.65° W) by means of a seine net. Individual crayfish were checked for intact antennule and chelae. Crayfish were physically and visually isolated in flow-through tanks in an environmental chamber. Crayfish were measured (mean ± SEM carapace length; 2.93 ± 0.6 cm) and kept on a diurnal light: dark schedule (12: 12 L: D cycle). The animals were not fed during the length of the experiment.

Experimental Design

Five different experimental conditions were created to examine the impact of two fuel exposures on foraging ability of crayfish. Behavioral foraging tests were performed at hour 48, 96, and 144. Experimental conditions consisted of:

A. Control; exposed to artificial pond water; N = 10
B. Low concentration of biodiesel; 32.65 ppm in artificial pond water (vol/vol); N = 10
C. High concentration of biodiesel; 326.5 ppm in artificial pond water (vol/vol); N = 10
D. Low concentration of crude oil; 32.65 ppm in artificial pond water (vol/vol); N = 10
E. High concentration of crude oil; 326.5 ppm in artificial pond water (vol/vol); N = 10

Each exposure treatment lasted 168 days consisting of a single acclimation day. Acclimation was followed by 96 consecutive hours of exposure to the fuel oil and finally a 48 day recovery period (Fig 1). The high concentrations were chosen by averaging the LC50’s of twenty-five oils found in a fisheries technical report (Franklin and Lloyd, 1982). In addition, the four concentrations were chosen to mimic the concentrations found within an oil spill (Singer et al. 1991, 1998, Pace 1995).
**Fuel Sources**

Two different types of fuel were used in this experiment: crude oil and biodiesel. Sweet Crude Oil (Husky Energy; Lima, Ohio) was used due to the refinery's proximity to the local crayfish’s natural ecosystem. Sweet crude oil composition consisted of twelve main compounds, pentane (0.975-1.73%), isopentane (0.716-1.39%), cyclopentane (0.935-1.63%), n-hexane (1.13-2.19%), heptane (1.15-1.79), methycyclohexane (1.35-2.39%), octane (1.37-2.39%), nonane (1.37-2.19%), benzene (0.199-0.259%), xylenes (1.15-1.63%), ethylbenzene (0.179-0.458%) and PAH (<9.95%; MSDS Crude Oil, Sweet). The Biodiesel (Peter Cremer North America; Cincinnati, Ohio) used was pure biodiesel (B100) or “neat” fuel (Demirbas, 2007). The biodiesel bought from Peter Cremer was made through the transesterification of rapeseed to form a rapeseed methyl ester (RME, MSDS Peter Cremer 2002).

**Experimental Exposure**

Crayfish were exposed to fuel sources in separate exposure tanks with one animal per aquarium. The aquaria were fitted with gravel filters, and gravel sediment to simulate an aquatic environment. Crayfish were placed within the exposure tank for an acclimation period of one day. The respective treatment concentration of fuel oil (high or low; biodiesel or crude) was added by discharge of the fuel upon the surface of the water in each aquarium (Atema and Stein 1974). While the behavioral trials were being run (at 48-h, 96-h and 144-h) the exposure aquaria were emptied and the experimental setup was implemented again to the same treatment amount to counter the potential effect of oil evaporation decreasing the exposure concentration (Stiver and Mackay 1984, Fig 1). After the behavioral trial, crayfish were re-exposed directly into the same concentration without an acclimation period to simulate prolonged exposures. After the fifth day of the experiment the aquarium was emptied and
cleaned but instead of recontaminating the aquarium with fuel, the aquarium was filled with clean water to allow for a two day recovery phase, with a subsequent behavioral trial run on the seventh day.

**Behavioral tests**

Foraging choice tests occurred within a constructed y-maze (Adams et al. 2005). The y-maze consisted of a test arena (tank = 77.5 x 42 x 18 cm, arm = 56 x 21.5 x 18 cm l: w: d) with two reservoir tanks (25 x 14 x 14 cm) that were used to house the stimuli (see Fig 2). Stimuli flowed from the reservoir tanks through 0.95 cm (ID) Tygon®. Two in-line flowmeters (Monostat Riteflow #4) controlled flow at a constant rate (112 ± 0.5 ml/min). Trials with fluorescent dye were run prior to testing to confirm flow from each holding tank equal when traveling from the reservoir through the arms of the maze. Speed of the outflow water was controlled by five outflow pipes with valves located 6 cm above the bottom of the maze, clamps were used to start and stop flow before, after and during trials in the y-maze. Once the trial started, the clamps were loosened to allow water to flow out.

Before each behavioral test, the y-maze and reservoirs were rinsed for 10 minutes with hot and distilled water and subsequently filled with clean artificial pond water. The food stimulus was randomly assigned using a random numbers table to one of the two reservoirs (Fig 2), with the stimulus being held down by a weighted metal mesh bag. Crayfish were rinsed before each trial by placing them in artificial pond water and then placed and held at the end of the Y-maze for a 15 minute acclimation period by a Plexiglas wall. After this period of time, flow was started and crayfish were allowed to orient for a total of 10 minutes. Trials were recorded using a digital camera (Panasonic HDC-H250) which was mounted above the maze.
**Stimuli**

Food stimulus (fish gelatin) was made from a recipe used with success in previous research (Moore and Grills 1999, Keller et al. 2001). Fish gelatin was prepared by mixing 53 g of sardines, 28 g of unflavored gelatin (Kroger brand), and 0.7 L of boiling water in a high speed blender. This mixture was placed into a baking pan (34.0 x 21.5 cm) to solidify and subsequently cut in 2 cm x 2 cm x 1 cm blocks for use.

**Data and Statistical Analysis**

Behavioral measures quantified included time spent in odor arm, time at food nozzle, time to locate stimulus nozzle source, and stimulus nozzle choice. Time spent in odor arm was defined when the crayfish's entire body crossed a line drawn perpendicular from the end of the y-maze middle arm to the side of the tank. Arm choice was taken as the initial arm entered and a choice was quantified when the entire carapace of the crayfish crossed a line drawn perpendicular from the end of the y-maze middle arm to the side of the tank. Time at nozzle was quantified when crayfish approached and came into contact with the odor source nozzle. The time spent at source was quantified until the crayfish left the source. Each time the crayfish left the source timing was stopped with the possibility of the crayfish returning to the stimulus. The summation of total encounter of time at source was used. Similarly, time to locate odor source was calculated from the start of the trial until the crayfish either came in contact with the odor nozzle or the trial ended. Orientation parameters were statistically analyzed using a-two way repeated measures ANOVA with time point and exposures as the two factors. Differences between the groups (time to find stimulus, time in stimulus arm, and time at stimulus) were obtained using a Fisher LSD post-hoc comparison test (STATISTICA INK. Version 9). Success
rates of correct arm choice as well as ability to locate odor source were analyzed using a modified Chi Square test ($\chi^2$).
RESULTS

Overall Results

Analysis of the overall results comparing the five different exposures (crude oil, biodiesel, and control) indicated an overall significant treatment effect due to length (time) of fuel exposure (two-way repeated measures ANOVA; F\(_{(3,010, 0.05)}\) = 42.0 \(p < 0.05\)). In particular, crayfish exhibited different behaviors when exposed to a fuel source as compared to control treatments. A Tukey-LSD post-hoc analysis revealed differences in behavioral measures across fuel treatment and days. The factors which proved to be significant to the findings were time point of the exposure as well as concentration and time point. No significance was found for a comparison of concentrations (F\(_{(1,447, 0.05)}\) = 88.00, \(p > 0.05\)) between concentrations and time (F\(_{(0.978, 0.05)}\) = 128.9, \(p > 0.05\)). Over time, the two different fuels proved to significantly affect the crayfish’s ability to forage negatively.

TIME TO FIND STIMULUS

Differences Due to Concentration

The results comparing biodiesel concentrations to control treatments showed a significant decrease in the crayfish’s ability to find and locate the source (LSD post-hoc test \(p < 0.05\)). This significant increase in time to find the source was observed on the recovery day (144-h, Fig 3). A similar increase in time to locate the food source was exhibited in 32.65 ppm of crude oil (LSD post-hoc test \(p < 0.05\), Fig 1). Differences were not observed when comparing crayfish exposed to 326.5 ppm of crude oil to the control animals. A significant increase in time to find and locate the stimulus source was found between 32.65 ppm of biodiesel and 326.5 ppm crude oil (LSD post-hoc test \(p < 0.05\), Fig 1). No significant change in time to locate the food
source was observed between the specific concentrations of biodiesel and crude oil (LSD post-hoc test \( p > 0.05 \)).

**Differences Due to Time**

Crayfish took significantly longer to find the odor source when exposed to fuel oils (LSD post-hoc test \( p < 0.05 \), Fig 1) than control. Time to locate the odor source increased both in the high and low concentrations for biodiesel and crude oil in comparison to control trials at the 144-h period of the experimental set up. Comparing the exposures 326.5 ppm crude oil and 32.65 ppm biodiesel, crayfish exposed to the biodiesel showed significant decrease in ability to locate the food source (LSD post-hoc test \( p < 0.05 \)). No significant difference was exhibited for time to locate odor stimulus within each concentration due to length of exposure time (LSD post-hoc test \( p > 0.05 \)). The overall significance indicates that exposures to both biodiesel and crude oil negatively affect crayfish’s ability to find and locate a food source.

**Overall Summary**

Overall biodiesel and crude oil negatively affected foraging of crayfish. A comparison between crayfish exposed to biodiesel and crude oil showed a significant difference with exposure to biodiesel increasing the amount of time to find the stimulus. Length (days) of exposure appeared have more of an effect upon ability to find the stimulus source than concentration (LSD post-hoc \( p < 0.05 \)). The results demonstrate there are similarities between the two fuel sources in determining crayfish’s ability to locate an odor source when exposed to these two fuel sources.
TIME IN STIMULUS ARM

*Differences Due to Concentration and Time*

No significant differences were observed with respect to the amount of time spent in the stimulus arm (LSD post-hoc p > 0.05, Fig 5). Time spent in the stimulus arm also exhibited no significant differences due to a specific concentration of fuel (LSD post-hoc p > 0.05).

TIME AT ODOR SOURCE

*Differences Due to Concentration and Time*

Time at the stimulus source exhibited no significant difference overall (LSD post-hoc p > 0.05, Fig 4). Length of time spent at the stimulus source displayed no significant difference (LSD post-hoc p > 0.05). No significant statistical difference was observed upon time at stimulus source due to a specific fuel concentration (LSD post-hoc p > 0.05). These results demonstrated that crayfish exposed to both fuel types had similar behavior due to time and concentration on ability of the crayfish to stay at the stimulus source.

Correct Arm Choice, Correct Location of Stimulus

A χ² test was used to determine if there were any significant differences between correct arm choices as well as the animals’ ability to correctly locate the stimulus source. Overall, the crayfish’s ability to distinguish a correct arm choice was not found to be significant between the four exposure groups. Recovery days exhibited a significant difference or all trials compared to 326.5 ppm crude oil exposure (χ² = 23.6, Fig 6). A significant difference was also observed at the 48-h treatment period between control and the high exposure of biodiesel (χ² = 21.9). A comparison between high concentration of biodiesel and crude oil revealed a significant difference for both the 48-h and 144-h trial periods (χ² = 21.9, χ² = 40.2).
Crayfish’s ability to correctly locate the stimulus source had a significant difference overall ($\chi^2 = 24.9$, Fig 7). Significance was observed when comparing the recovery (144-h) trial period for control exposures to all of the fuel exposures. Day five of 32.65 ppm biodiesel exhibited a significant difference in ability to both crude oil exposures ($\chi^2 = 21.5$). These findings exhibit that crude oil and biodiesel both similarly affect crayfish’s ability to locate a food source.
DISCUSSION

**Biodiesel and Crude Oil Effect Alter Crayfish Behavior**

Both fuel sources biodiesel and crude oil had similar effects in alteration of the crayfish chemosensory ability to locate a food source during foraging trials in comparison to the control crayfish. Animals exposed to crude oil and biodiesel both displayed impairment in their ability to respond to a food odor. These results were evident in all of the concentrations, from the 326.5 ppm and 32.65 ppm of both biodiesel and crude oil. Exposure to the two fuel sources caused impairment and delay in being able to locate a food odor source. Similar results were found in studies exposing *Homarus americanus* (American lobster) to crude oil (Atema and Stein, 1974, Atema et al. 1982). Our results are novel due to the lack of toxicological studies using alternative fuels. Statistical analysis showed that when exposed to either biodiesel or crude oil crayfish ability to locate a stimulus source location was impaired.

**Possible neurological effects**

It is possible that exposure to fuel sources negatively impact a crayfish’s chemosensory system. Jensen and Carroll (2010) found inhibited feeding behavior to the *C. finmarchicus* when exposed to a high treatment of the water soluble fraction of crude oil (7.0 µg l⁻¹). Results using *Homarus americanus* (lobster) indicated that crude oil causes a noxious odor which possibly suppresses appetite as well as chemical excitability through neurotoxicity (Atema and Stein 1974). Atema et al. (1982) found negative chemosensory effects in *Homarus americanus* (lobster), when exposed to crude oil levels as low as 1 ppm. Oils used in production of biodiesel have caused a decrease in oxygen levels in the water column causing mass death of numerous species from avian to crustacean (Bucas and Saliot 2002). Findings from our study indicate that
chemosensory abilities of crayfish are negatively impacted, but the underlying mechanism of that impact is unknown.

Crayfish have shown the ability to distinguish between multiple chemical stimuli or odors when presented simultaneously (Tomba et al. 2001). Diminished sensory capabilities can lead to failure to distinguish multiple odors which, in turn, can have negative ecological and behavioral impacts such as a decrease in foraging ability, an inability to establish a hierarchy, or interference in mating decisions. Given the critical nature of chemical signals for crayfish ecology, any impairment of the chemical sensory system could have large impacts for crayfish populations.

**Effects on Crayfish and Aquatic Ecosystems**

Crayfish are keystone species for many aquatic habitats. Crayfish have two main roles in aquatic ecosystems. First, crayfish as consumers influence carbon flow through the food web by grazing on macrophytes, shredding detritus, and preying upon macroinvertebrates (Momot et al. 1978, Momot 1995, Covich et al. 1999). Secondly, as prey items, crayfish are the staple foods for many aquatic vertebrates (Momot et al. 1978, Momot 1995). Crayfish use chemical stimulus to detect and react to the surroundings is an integral aspect for crayfish survival.

Chemical signals are used by crayfish in the detection of a food stimulus’s (Moore and Grills, 1999), establishment of social hierarchies (Zulandt Schneider et al. 1999), and predation avoidance (Zulandt Schneider et al. 2000). When exposed to biodiesel and crude oil, crayfish exhibited a decreased ability to locate a chemical stimulus (food odor). If there is a decrease in the ability to find and locate a food source, a decrease in crayfish fitness may occur. Inhibiting the ability to detect and respond to a chemical signal will leave crayfish susceptible to predation.
Factors of this nature can affect overall survival of the animal due to increased risk of predation and starvation.

A decrease in chemosensation due to a pollutant in the environment elicits an increase time spent foraging (Wolf and Moore 2002). When crayfish are exposed to the fuel sources biodiesel and crude oil more time was spent searching and locating an odor. Increased time foraging increases the risk of predation and decreases time spent doing other behaviors (e.g., mating, dominance maintenance). An increase in exposure and possible subsequent predation of crayfish has implications on other aquatic species and the ecosystem.

A decrease in the size of a population of a keystone species has the ability to cause direct and indirect effects upon an ecosystem. Creed and Reed (2002) have shown that crayfish play a role in stream dynamics not only as a stream engineer but also affecting invertebrate taxa through predation. The removal of these dynamics could cause an increase in both detritus and crayfish predation could occur. An overabundance of detritus has been shown to decrease plant biomass (Knapp and Seastedt 1986). Additional effects include increased in crayfish prey densities further altering ecosystem dynamics (Hanson et al. 1990, Lodge et al. 1994). Crayfish negatively affect both biomass and invertebrate species in both lentic and lotic systems (Lodge et al. 1994). With a decrease in ability to respond to an alarm singles, crayfish are more susceptible to predation by smallmouth bass (*Micropetrus dolomieui*, Stein 1977). The increase in predation has the potential to decrease crayfish population and open more breeding sites and cause more competition for food in the predator species.

Investigating sub-lethal effects of a pollutant on crayfish behavior is critical in understanding potential changes in aquatic ecosystems. The impact of crude oil mode on organisms has been studied numerous times in the past (Bowyer et al. 1994, Simonato et al. 1994).
2008). Sublethal effects biodiesel has on behavior is missing from the literature. Recent studies have started to investigate the potential toxicity of biodiesel (Hollebone et al. 2007, Khan et al. 2007, Poon et al. 2009). These studies have begun to look at the specific actions biodiesel might have upon a species and there is potential that biodiesels may be as detrimental to aquatic life as crude oil.
CONCLUSION

Chemical stimuli are involved in the behavioral repertoire of crayfish including the location of food, detection of predators, and establishment of social hierarchies. With impaired chemosensory abilities, survival rates of crayfish would decrease. This study has shown that sub-lethal effects on the chemosensory ability of crayfish can occur with exposure to environmental pollutants.

Exposure to biodiesel and crude oil is clearly shown to compromise the ability to detect a food stimulus (Figs 2 and 7). Crayfish normally orient toward a food stimulus in a fairly linear path which reduces orienting time (Moore and Grills 1999). Observations during this study showed increased time to locate a food source even after crayfish were removed from exposure treatments, which would increase foraging times and increase exposure to predation risk. These results indicate a possible latency effect of pollution upon chemosensory impairment. With impairment occurring after the recovery period, the chances for a residual effect due to the exposure fuel sources is present.

These residual effects can have negative consequences for important ecological behaviors. Cook and Moore (2008) exposed crayfish to non-lethal levels of the herbicide metolachlor causing a decreased tendency to initiate and win fight encounters. Predator prey interactions have also been observed to be negatively affected due to non-lethal exposures to toxins. Bundschuh et al. (2011) exposed *Asellus aquaticus* and *Dendrocoelum lacteum* to the fungicide tebuconazole causing a decrease in predation of *D. lacteum* by *A. aquaticus*. Several other studies have found that sublethal effects inhibit behaviors integral to fitness (Atema and Stein 1974, Atema et al. 1984). These studies exhibited a decreased ability for lobsters to locate a food source. A similar decrease was found in this present study. Changes in behavior occurred
after the 144-h period despite the recovery period. In nature sublethal exposures have demonstrated the ability to cause detrimental effects upon the fitness of a species. Foraging, a chemically mediated process was altered in crayfish due to sublethal exposures to biodiesel and crude oil.

The use of a chemical stimulus is essential for crayfish survival. When the ability to detect a chemical stimulus is inhibited, crayfish have a decreased chance to locate food or avoid a predator. The inability to detect a chemical stimulus could alter social structure as well as fight dynamics (Bergman and Moore 2005). Finally, evidence has been found that social interactions in rainbow trout (*Oncorhynchus mykiss*; Sloman et al. 2003) were altered when fish were exposed to sub-lethal concentrations of pollutants. The present study shows how exposure to biodiesel or crude oil alters response to chemical stimuli. Evidence from this study and from previous research has shown that several chemically mediated behaviors are significantly impaired by environmental pollutants.

Findings from this study demonstrate that fuel sources alter behaviors essential to crayfish fitness. After exposure to biodiesel or crude oil results showed a sub lethal sensory impairment to crayfish’s ability to react to a chemical stimulus (food source). The known toxic effects of crude oil were observed in this study as well as the novel findings that demonstrate behavioral alterations in crayfish exposed to an eco-safe fuel sources.
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Figures:

Figure 1: Experimental length and exposure treatment schedule.
**Figure 2.** Y-maze design. Flow is from top to bottom in the diagram. Water flowed from the reservoirs through the flowmeters, and into either arm. Water exited through 6 holes at the base of the Y-maze. Crayfish began the experiment in the downstream portion of the Y-maze.
Figure 3: Mean (± SEM) of time to find stimulus source for crayfish in control, 32.65 ppm crude oil, 326.5 ppm crude oil, 32.65 ppm biodiesel, and 326.5 ppm biodiesel. Columns with same letter are significant different. Significant difference can be seen observed between exposures (except 326.5 ppm crude oil on day seven (recovery, LSD post-hoc p < 0.05). A significant difference between 326.5 ppm crude oil and 32.65 ppm biodiesel was observed on day seven (LSD post-hoc p < 0.05).
**Figure 4:** Mean (± SEM) of time at odor source for crayfish in control (black), 32.65 ppm crude oil (gray left slanted lines), 326.5 ppm crude oil (gray increased left slanted lines), 32.65 ppm biodiesel (horizontal lines), and 326.5 ppm biodiesel (increased density of horizontal lines). There were no significant differences (LSD post-hoc test, p > 0.05).
**Figure 5:** Mean (± SEM) of time in stimulus arm for crayfish in control (black), 32.65 ppm crude oil (gray left slanted lines), 326.5 ppm crude oil (gray increased left slanted lines), 32.65 ppm biodiesel (horizontal lines), and 326.5 ppm biodiesel (increased density of horizontal lines). There were no significant differences (LSD post-hoc test, p > 0.05).
Figure 6: Percentage success in correct stimulus arm choice. Unmarked columns were not statistically significantly. Day seven had significance when comparing all exposure concentrations to 326.5 ppm crude oil ($\chi^2$ test, p < 0.05). Significance differences were observed when comparing control to 326.5 ppm biodiesel on day five ($\chi^2$ test, p < 0.05), as well when comparing 326.5 ppm biodiesel to 326.5 ppm crude oil on day seven ($\chi^2$ test, p < 0.05).
Figure 7: Percentage success in locating the food odor. Unmarked columns are not statistically significant. Day seven had significance when comparing all exposure concentrations to the control ($\chi^2$ test, $p < 0.05$). Significance differences were observed when comparing 32.65 ppm biodiesel to both 326.5 ppm crude oil and 32.65 ppm crude oil on ($\chi^2$ test, $p < 0.05$).