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Development and Application of Aquatic Toxicology Studies for the Assessment of Impacts Due to Chemical Stressors Using Non-Standard Indigenous Organisms

Abraham Jeffrey Smith
asmit065@fiu.edu

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

DEVELOPMENT AND APPLICATION OF AQUATIC TOXICOLOGY STUDIES

FOR THE ASSESSMENT OF IMPACTS DUE TO CHEMICAL STRESSORS USING

NON-STANDARD INDIGENOUS ORGANISMS

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by

Abraham Jeffrey Smith

2018

To: Dean Michael R. Heithaus
      College of Arts, Sciences and Education

This dissertation, written by Abraham Jeffrey Smith, and entitled Development and Application of Aquatic Toxicology Studies for the Assessment of Impacts Due to Chemical Stressors Using Non-Standard Indigenous Organisms, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

_____
Gary Rand

_____
Jose Eirin-Lopez

_____
Heather Bracken-Grissom

_____
Kevin Boswell

_____
Michael Heithaus, Major Professor

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The dissertation of Abraham Jeffrey Smith is approved.

_____
Dean Michael R. Heithaus
College of Arts, Sciences and Education

_____
Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2018

DEDICATION

I dedicate this dissertation to my parents. Without their love, patience, support, understanding, and a poke every now and then, I never would have gotten through this. You have always been there for me and encouraged me to push for what I wanted. Well, it is about time isn't it?

And R…thank you for being there for me. Thank you for your kindness, sharing your humor and reminding me to laugh, and listening to all of my stories and graduate school woes. You are the best.

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ABSTRACT OF THE DISSERTATION

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Professor Michael Heithaus, Major Professor

Research in the multidisciplinary science of ecotoxicology is crucial to assess injuries to
ecosystem resources from chemical spills or other stressors used to support
environmental decision-making. Established guidelines recommend the use of non-
standard native species in toxicity investigations. My work focused on the use of native
species for aquatic toxicity assessment to make more relevant conclusions on the
potential for adverse biological effects to occur as a result to single chemical exposures or
exposures to a complex mixture like oil. We apply these studies to investigate petroleum
product impacts from the Deepwater Horizon incident and concerns for metal toxicity in
estuarine environments using a new model organism. Data generated from
comprehensive toxicity testing programs were used in the first probabilistic risk
assessment of Deepwater Horizon oil toxicity highlighting a lack of appropriate data and
representative phyla. Novel toxicity study methods and a stress-response index were
developed and demonstrated sensitivity and success in using the Starlet Anemone in
ecotoxicology studies. Swim performance was used as new method to investigate

sublethal indicators of stress resulting in varied responses from Sheepshead Minnows and Florida Pompano. These studies further our ability for better laboratory-to-field extrapolation and for decision-making. The use of native species and complex mixtures like oil presented novel challenges in conducting aquatic toxicity studies. Special emphasis is placed on the necessity to understand the appropriate laboratory conditions for native species not typically held in the laboratory and maintaining study parameters to obtain quality data for more accurate interpretation and replication.

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DISSERTATION INTRODUCTION

      The science of ecotoxicology arose from the need to understand the impacts of

chemicals on the environment. Rachel Carson's book, *Silent Spring* (1962), brought

initial attention of the public to birds and their sensitivity to the pesticide, DDT because

of its widespread use and potential trophic level effects through food-chain amplification.

Global environmental awareness increased following environmental disasters in the

1970s and early 1980s including the Times Beach, Missouri dioxin catastrophe (1983),

the arsenic poisoning of wells in Bangladesh (1960-1970s), the contamination of Love

Canal by Hooker Chemical and Plastics Company (mid-1970s), and the accidental release

of methyl isocyanate killing thousands of people overnight in Bhopal (1984), India.

These were all important incidents that brought the impacts of chemicals on humans and

the environment to the attention of the public. Heightened environmental awareness led

to the development of the United States Environmental Protection Agency (U.S. EPA)

which is responsible for the protection of human health and the environment.

Ecotoxicology methods quickly became important in U.S. EPA decision-making. For

example, the Federal Insecticide, Fungicide, and Rodenticide Act of 1947 and 1972

(FIFRA), the Clean Air Act of 1970 (CAA), the Clean Water Act of 1972 (CWA), and

the Comprehensive Environmental Response, Compensation, and Liability Act of 1980

(CERCLA) & Superfund Amendments and Reauthorization Act of 1986 (SARA) require

ecotoxicology data for environmental decision-making by the U.S. EPA for regulating

environmental contamination of soil, air, and water.

      The U.S. EPA has established standard guidelines for the testing of chemicals on

aquatic and terrestrial organisms to evaluate safety to these organisms. Similar guidelines

have been developed by organizations such as American Society for Testing and Materials (ASTM), the Organization for Economic Cooperation and Development (OECD), and Environment Canada. These aquatic toxicity guidelines include toxicity test methodologies for organisms living in both the water column and benthic habitats of freshwater, brackish water and marine environments. A common use of these studies is conducting Whole Effluent Toxicity (WET) testing to regulate point source water pollution by determining if a National Pollutant Discharge Elimination System (NPDES) permit holder (e.g., industrial chemical company, waste water treatment plant) is within the imposed state and federal regulations. Another use for these standard toxicity tests includes the preparation of Natural Resource Damage Assessment (NRDA) for NOAA's Damage Assessment, Remediation & Restoration Program (DARRP) following oil spills or releases of hazardous chemicals into the environment to determine injury to native organisms.

The present dissertation research focuses on the use of native species for aquatic toxicity assessment to draw more relevant conclusions on the potential for adverse biological effects to occur as a result of single chemical exposures, or exposures to a complex mixture like oil. The information generated will thus further our ability to extrapolate results to field settings, thereby improving decision-making. Use of native species and complex mixtures like oil presents novel challenges in conducting aquatic toxicology studies because of the challenges in the repeatability of results. Therefore, special emphasis was placed on understanding the appropriate laboratory conditions for native species not typically held in the laboratory and maintaining consistent conditions to obtain quality data for more accurate interpretation and reliable replication.

The history of water pollution biology and aquatic toxicology have a short history compared to other scientific disciplines but the objectives still remain the same: to determine the toxicity and safety of stressors to aquatic organisms and to evaluate the hazard and risks to populations of fresh- and salt-water organisms. The recent Deepwater Horizon (DWH) incident in the Gulf of Mexico with Macondo oils has raised some interesting methodological/procedural issues in the toxicity assessment process for the conduct of aquatic toxicology studies which may be relevant to not only complex mixtures like oils and effluents but also to single chemical stressors. For example,

(1) There are aquatic toxicity standardized acute and chronic laboratory testing methods for single chemicals but not for complex mixtures. (2) Although aquatic toxicity testing is typically conducted with standard laboratory conditions, are they suitable for all chemical stressors? (i.e., standard tests use fluorescent lighting but the presence of PAH components in oils may be sensitized in animal tissue with residues, under natural light/UV conditions in the environment making oils more toxic). For example, the aquatic toxicity assessment process with oils should always include a battery of tests (acute and chronic) under natural UV light conditions.

(3) Are laboratory toxicity test media exposure concentrations for a stressor representative of actual field exposure concentrations for that stressor? (4) Although standard methods suggest the use of native (indigenous) test organisms, typically only standard test organisms are used in aquatic toxicity testing. (i.e., should the community of laboratory investigators that use aquatic toxicity testing as a tool to determine safety in the field begin gathering existing life history information and laboratory acclimation/holding and culturing information relevant to various fresh-and salt-water

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native organisms from different geographic regions with the objective to start incorporating these native species in their programs?)

(5) To evaluate safety of chemical and non-chemical stressors to aquatic organisms, most regulatory agencies throughout the world in decision-making have used four classical biological test endpoints: mortality, growth, development and reproduction. It is indicated that the scientific community should begin evaluating the early "signal" of a "stress response" from exposures to a potential toxic agent (Selye, 1976) which may serve as an early bioindicator (or biomarker) of exposure and/or early adverse effects.

(6) Following from (5) above, sublethal physiological and behavioral test endpoints have been used in aquatic toxicology studies but they have not been used for decision-making. Adverse effects on these endpoints can have serious consequences on the survival of fresh- and salt-water species. Difficulty in establishing a direct link from these endpoints to the classical four (mortality, growth, development, reproduction) has hindered their incorporation into the decision-making process.

In addition, to the above methodological/procedural issues in the toxicity assessment process for petroleum products and other chemical stressors there has neither been a (7) review of the aquatic toxicity database nor (8) an aquatic risk assessment conducted for the recent DWH incident in the Gulf of Mexico with Macondo oils.

This dissertation includes the following chapters which present studies to fill important data needs, interpretations, and conclusions which respond to the eight issues discussed above. All of the chapters contained within this dissertation have the common theme of using non-standard native species in toxicology assessments.

Chapter 1 describes a comprehensive toxicity program conducted at FIU in support of the British Petroleum NRDA process following the DWH incident. Toxicity data are presented in species sensitivity distributions (SSDs) to highlight different exposures and toxicity from a variety of field-collected petroleum products. Toxicity endpoint values are put in perspective using data from a publicly available environmental database containing sample analyses of water collected during the DWH incident.

Chapter 2 contains the first probabilistic risk assessment of aquatic toxicity data generated during the NRDA process of the DWH incident. Data presented in the first chapter are used along with data from other institutions and companies involved in toxicity assessment of DWH petroleum products. Risk is presented in four scenarios with characterization using real-world analytical chemistry data.

Chapter 3 develops new methods for using juvenile Starlet Anemones as a new model organism in ecotoxicology. I present laboratory techniques to culture, induce reproduction, and conduct acute toxicity studies with juvenile Starlet Anemones (*Nematostella vectensis*). The toxicants used here were heavy metals (cadmium and copper) which are contaminants of concern in Florida estuaries that are affected by agricultural lands (the source of metals found in fungicides and molluscicides). The anemone is sensitive to both cadmium and copper and the sensitivity is demonstrated using SSDs from the new anemone toxicity data and data available in the literature.

Chapter 4 contains additional toxicity studies with the Starlet Anemone used to develop a stress-response index. Determining death in cnidarians is notoriously difficult because of their ability to recover from severe stressors (e.g., disease, bleaching, physical damage). A stress-response index is used to categorize anemones using macroscopically

visible characteristics. Death is verified by providing a recovery period (14 days) after an acute exposure (96 hours) to cadmium or copper and determining at which level of the stress index an anemone is not able to recover.

Chapter 5 involves a technique not commonly used in ecotoxicology, swimming performance. Swimming performance has historically been used as a fitness indicator for wild-caught fish in stock assessments. Chapter 5 used swimming performance of Sheepshead Minnows and Florida Pompano as a sublethal indicator of stress. Exposures included petroleum products from the DWH incident and a reference toxicant (KCl). A variety of data normalization techniques are discussed and suggestions for integrating this type of testing into ecotoxicology assessments are provided.

CHAPTER I: THE USE OF NON-STANDARD INDIGENOUS ORGANISMS IN AQUATIC TOXICOLOGY TO INVESTIGATE EFFECTS OF PETROLEUM PRODUCTS RELATED TO THE DEEPWATER HORIZON INCIDENT

ABSTRACT

Established, standard aquatic toxicity test guidelines typically recommend the use of non-standard, native test species in toxicity investigations but they are rarely used. Aquatic toxicity data are summarized here from programs designed to incorporate resident/native species representative of the Gulf of Mexico from the Natural Resource Damage Assessment (NRDA) testing to investigate the effects of different petroleum products (i.e., Macondo oils-MC252; crude oil, weathered oil, dispersed oil, etc.) in water related to the Deepwater Horizon incident. Toxicity data from NRDA sources are also presented in species sensitivity distributions (SSDs) for different oils and related to TPAH concentrations (total polycyclic aromatic hydrocarbons) from field-collected water samples from the DWH incident. The most sensitive toxicity endpoint estimate values, using TPAH were from weathered oils and frequently represented by non-standard native species. These SSDs demonstrate the need for more variety in the use of test organisms to capture a comprehensive picture of adverse effects from petroleum product exposures. This is the first review of an aquatic toxicity database on water exposures to petroleum products from the Deepwater Horizon incident.

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INTRODUCTION

The Deepwater Horizon (DWH) incident, began in April 20, 2010 with a "blowout" of a British Petroleum (BP) Oil Macondo well located (at lease block MC252) ~one-mile deep in the Gulf of Mexico (GoM). The well was discharging oil until day 87 when it was shut down. During this time oil was moving into deep waters, and spreading into mid- and surface waters potentially exposing numerous natural resources to oil (Crone and Tolstoy 2010, OSAT 2011). The GoM is one of the world's most productive and diverse ecosystems encompassing the coastal areas of western Florida, Alabama, Mississippi, Louisiana, and Texas, as well as part of Mexico. It contains environmentally sensitive areas (e.g., coastal wetlands, barrier islands, coral reefs) and provides nursery grounds and habitat for many species including those that support commercial fishing (e.g., oysters, scallops, crab, shrimp, snappers, and other gamefish) and recreational fishing (e.g., groupers, mackerels, snappers, drums, Spotted Sea Trout, Bluefish, Florida Pompano).

Under the Federal Natural Resource Damage Assessment (NRDA), NOAA and the US Fish and Wildlife Service focused on assessing injuries to ecosystem resources from the deep ocean to the coastline of all five states. The efforts entailed assessing the quantity (concentrations) of subsurface oil and its consequences/threat (i.e., toxicity) to biota. Water samples collected by NOAA and other groups (10,800 from the OSAT 2010 report plus additional samples by the BP NRDA effort) during and after the 87 days for analyses. To address the evaluation of the toxicity of the oil-contaminated water to biota, laboratory studies were conducted by various research groups which included academic and non-academic institutions. Historically, standard single-species testing is used and standard methods are followed although federal guidelines clearly suggest the use of native species, similar to those which inhabit the area(s) under consideration, are used for testing. For the aquatic toxicity testing conducted under the NRDA, standard species were used (e.g., Mysid Shrimp, silversides, Sheepshead Minnows, sea urchins, oysters, clams, mussels) and a number of native GoM vertebrate and invertebrate species were used for acute and chronic toxicity testing by academic and non-academic institutions. Toxicity data are presented in numerous manuscripts and documents but there is no review of the database nor an analyses of the relevance of the data to the field exposures that occurred in the GoM.

Here, I (1) present the MC252 (oils collected from GoM) acute toxicity laboratory results conducted at Florida International University (comprehensive aquatic toxicology testing program, Rand and Gardinali 2010), and (2) summarize all the acute toxicity data used in the NRDA (FIU and all other NRDA contractors) and their relevance to GoM exposures. I used species sensitivity distributions (SSDs), which is an assemblage of all

the single-species acute toxicity data for the different MC252 oils (crude, weathered, etc.) to display this data. An SSD is used to predict the 10th centile concentration of the distribution of a specific endpoint data group (i.e., 10th centile of all median lethal concentration endpoint estimates that would correspond with protection of 90% of the species) affecting a proportion of the species in the community of species and its relationship to GoM exposures.

Importantly, there are no standard toxicity test guidelines and there are little to no historical records for these native species requirements for laboratory acclimation/maintenance, holding and testing. Brief details on species-specific culturing and toxicity study protocols developed at FIU are also presented here.

TEST SPECIES

I began oil toxicity testing with standard species including the Mysid Shrimp (*Americamysis bahia*), Inland Silverside (*Menidia menidia*), and the Sheepshead Minnow (*Cyprinodon variegatus*). Toxicity studies continued with non-standard indigenous species including Red Drum (*Sciaenops ocellatus*), Southern Flounder (*Paralithys lethostigma*), Spotted Sea Trout (*Cynoscion nebulosus*), a marine copepod (*Acartia tonsa*), Florida Pompano (*Trachinotus carolinus*), Cobia (*Rachycentron canadum*), Red Porgy (*Pagrus pagrus*), Blue Crab (*Callinectes sapidus*), and the Moon Jellyfish (*Aurelia aurita*). These organisms were chosen on the basis of their availability (from biological supply houses, university aquaculture programs, or state agency culture & stocking programs) and their relevance to the DWH incident in the GoM.

Mysid shrimp (*A. bahia*) and silversides (*Menidia beryllina*, *Menidia menidia*, *Menidia peninsulae*) are two of the three estuarine/marine standard test organisms

included in EPA Whole Effluent Toxicity (WET) acute methods (USEPA 2002a).

Americamysis bahia is one of three sympatric species of *Mysidopsis* shrimp-like

crustaceans generally found in salinities above 15 ppt (parts per thousand), all of which

have been used in toxicity testing (Stuck et al. 1979; Price 1982). Mysid shrimp are easily

cultured in the laboratory in flow-through or recirculating aquarium systems with the

females producing young in brood pouches (Ward 1984; Venables 1987; Lussier et al.

1988). Juvenile *A. bahia*, one to five days of age, were used in acute toxicity testing and

were purchased from Aquatic BioSystems, Inc.

     Menidia beryllina is one of three common silverside fishes that can be found from

Maine to Texas that are important prey for commercially- and recreationally-valued

predators such as Striped Bass, Bluefish, and Spotted Sea Trout (Merriman 1941; Bayliff

1950; DeSylva et al. 1962; Dahlberg 1972; Middaugh 1981). Silversides can be

maintained in laboratory culture systems and spawn year-round (Middaugh and Takita

1983; Middaugh and Hemmer 1984). Juvenile fish, one to 14 days of age, were used in

acute toxicity studies and were obtained from Aquatic BioSystems (ABS) or Aquatic

Research Organisms (ARO).

     The Sheepshead Minnow (*Cyprinodon variegatus*) is a marine standard acute US

EPA WET test species and is the only marine species of Cyprinodontidae (killifish)

(Robins et al. 1980). *Cyprinodon variegatus* is an omnivorous fish found in estuaries

along the Atlantic and Gulf coasts that is an important link in energy transfer from lower

trophic levels and is a forage fish for drum, Bluefish, Spotted Sea Trout, Striped Bass,

and Snook (Gunter 1945; Darnell 1958; Grant 1962; Carter et al. 1973; Sekavec 1974;

Hansen and Parrish 1977). Sheepshead Minnows, one to 14 days of age, were used in acute toxicity testing and were purchased from ABS or ARO.

Red Drum (*Sciaenops ocellatus*) and Spotted Sea Trout (*Cynoscion nebulosus*) are commercially and recreationally important fisheries that have declined to the point of commercial fishery closure (USEPA 1999). Several states along the GoM have developed stocking programs to rebuild populations. Red Drum are found in the GoM between Florida and northern Mexico and up the US Atlantic coast to Massachusetts. During the first three years of life, Red Drum inhabit bays, but move offshore as they mature. Juveniles prefer shorelines, shallow water (< 1.3m) and vegetated areas. Juveniles appear to have a wide salinity tolerance, with similar abundances found from 17 to 35 ppt. Adults migrate from bays to the open water (pelagic) when sexually mature, generally around the age of 3 to 4 yr (Matlock 1990). Spawning occurs in the GOM from mid-August through mid-October near the mouth of bays or passes and shorelines. Eggs generally hatch within 24 hr of spawning and larvae are carried by currents into tidal bays. Red Drum are not known to migrate and tagged fish generally stay within a three-mile distance from their tagging point (Chamberlain et al. 1990). Red Drum are a federally protected game fish, which restricts them from commercial harvest in U.S. federal waters and most state waters; and states regulate size and number of catch in recreational fishing of Red Drum (Chamberlain et al. 1990).

Spotted Sea Trout are a valuable commercial and recreation species that inhabit estuaries for most of their life-cycle. Although found across a wide range of salinities (0-37 ppt), abundance is greatest between 15-35 ppt, while the optimal temperature for larvae ranges from 23-33°C. Reproduction normally begins at 3 to 4 yr of age, and

fecundity increases with age and size of the female. The spherical eggs have been described as both pelagic and demersal, depending on salinity. At 30 ppt, eggs are buoyant, but sink below 25 ppt. Eggs hatch within 20 hr of fertilization, and larvae range from 1.3 to 1.6 mm. Despite their movements in and out of estuaries, *C. nebulosus* are not considered migratory fish. In fact, tagged adults rarely move less than 30 miles from where they were tagged (Johnson and Seaman 1986). Spotted Sea Trout are carnivorous. Larvae and juveniles are planktivorous, while larger juveniles may feed on mysid shrimp. Adults primarily feed on fishes such as anchovies, pinfish, silversides and snapper (Johnson and Seaman 1986).

Fertilized Red Drum and Spotted Sea Trout embryos were obtained from Texas Parks and Wildlife Division where the fishes were cultured in large outdoor ponds for a stocking initiative. They were available from April-October. Embryos were placed into recirculating culture systems, hatched, and raised until appropriate testing age/size.

Southern Flounder (*Paralichthys lethostigma*) is a benthic fish found in estuarine and marine waters of the Atlantic and Gulf coasts from North Carolina to Mexico and is a highly valued recreational and commercial fish. Implementation of fishing quotas in the 1990s has helped manage fish stocks. High demand and wide temperature and salinity tolerances make it an excellent candidate for aquaculture (Watanabe et al. 2001). Spawning was induced through hormone implantation or through manipulation of temperature and lighting cycles with natural spawning (non-injection) producing more and higher quality embryos (Dahlberg 1972; Modde and Ross 1981; Winemiller and Rose 1992; Watanabe et al. 2001).

Red Porgy (*Pagrus pagrus*) is a member of the Sparidae and is found in the Western Atlantic from New York and the northern Gulf of Mexico to Argentina (Robins and Ray 1986). Red Porgy is typically pinkish silver bodied with small blue or yellow spots on each scale sometimes appearing as stripes and a patch of yellow color within the iris of the eye; dorsal, caudal, and pectoral fins are pink (Humann and Deloach 2002). The diet of the Red Porgy consists of crustaceans, fishes, mollusks, polychaetes, and decapods (Manooch 1976; Manooch and Hassler 1978; Bauchot and Hureau 1990) that can be found over rock, rubble, or sand bottoms down to 250 m in depth (Figueiredo 2002). Red Porgy is an important species in Florida west coast commercial reef fisheries. The Red Porgy fishery shifted from primarily recreational to commercial between 1988 and 1991. Red Porgy are protogynous hermaphrodites and may be more susceptible to fishing pressure than a gonochoristic fish such as red snapper if size-selective fishing limits the number of males during spawning due to harvest of larger fish. However, this has not been observed in GoM stocks (Hood and Johnson 1999). Spawning occurs in the winter and spring in the Gulf of Mexico (Manooch 1976; Manooch and Hassler 1978; Nelson 1988; Vassilopoulou and Papaconstantinou 1992; Pajuelo and Lorenzo 1996). As a consequence of the decline of Red Porgy catch over the last two decades, it is being considered an excellent candidate for aquaculture. Benefits include high growth rates and market value and suitability for intensive culture in tanks and cages (Morris et al. 2008).

Southern Flounder and Red Porgy fertilized embryos were obtained from the University of North Carolina Wilmington, hatched and reared at FIU before use in toxicity and photoenhanced toxicity studies.

Copepods are a common food item for developing larval fish and constitute about 80% of zooplankton communities in aquatic ecosystems with *Acartia tonsa* being one of

the most common in coastal communities (Reeve and Walter 1977). The marine calanoid copepod, *Acartia tonsa*, is routinely cultured in the laboratory and releases eggs into the water column making them easy to collect and use in toxicity studies where they have demonstrated sensitivity to a variety of chemicals (Kusk and Petersen 1997; Andersen et al. 1999; Medina et al. 2002; Christoffersen et al. 2003; Medina and Barata 2004). *Acartia tonsa* has been identified as a suitable testing organism in several international standardized protocols (ISO 1999; Medina and Barata 2004; OECD 2004). *Acartia tonsa* stage 4-5, were used in toxicity tests and received from Alga Gen, LLC (Vero Beach, FL) and fed PhycoPure™ CopePOD blend (AlgaGen).

Pompano are a warm water species which range from inshore shallow (coastal) waters to offshore (pelagic) throughout the GoM. Spawning takes place in the GoM and in the summer the young are present in great numbers in the surf. Fish often move offshore in the winter. Pompano is a valued commercial food fish along the Gulf coast. Juvenile and adult pompano tolerate a wide range of environmental conditions, including low levels of dissolved oxygen (down to 4 mg/L) and salinities ranging from 0 to 50 ppt (Main et al. 2007). If acclimated properly, juvenile and adult pompano adjust well to lower salinities; however, in the hatchery phase higher salinities are necessary for buoyancy and survival of the eggs and young larvae (Weirich and Riche 2006; Williams 2008; Riche 2009). Pompano are cold intolerant and show stress at low temperatures, which may restrict their potential for outdoor culture. Mortalities occur at temperatures of 10 to 12°C. Mortalities also occur when there are extreme changes in temperature over a short period of time. The optimal temperatures for juvenile growth appear to range from

25 to 30°C, although juveniles have thrived at temperatures as high as 34°C (Finucane 1969; Tutman et al. 2004; Gothreaux 2008).

Pompano spawning occurs from early spring through October. Reproductive seasonality varies among Atlantic and Gulf of Mexico populations. Those located in the tropical Gulf of Mexico and Caribbean Sea may spawn throughout the year (Fields 1962, Muller et al. 2002)). Seasonal spawning patterns of pompano have been verified by the abundance of small juveniles along exposed, sandy beaches and in the surf zone from late spring through fall. In the Gulf of Mexico, most juvenile recruitment occurs in April and May, with a smaller "wave" of individuals reported in August and September. The actual spawning location for pompano is unknown, but it has been suggested that spawning occurs offshore, where the transport and distribution of pelagic eggs and larvae are influenced by prevailing currents. There are no documented accounts of spawning activity inshore or in estuarine waters. Some studies indicate that there may be more inshore spawning habitats than previously thought (Fields 1962; Bellinger and Avault 1970; Bellinger and Avault 1971; Gilbert and Parsons 1986; Muller et al. 2002).

Cobia are pelagic spawners in warm-temperate to tropical waters and are prevalent in the Northern Gulf of Mexico (Franks et al. 1999; Arendt et al. 2001; Bignami et al. 2013). Cobia tolerate a wide range of temperatures (1.6 and 32.2°C) and salinity (5-44.5 ppt) and are generally found offshore, but they also enter estuaries and mangroves to forage (Sun et al. 2006; Chen et al. 2009). They release buoyant eggs (1.2 mm) into the water which become part of the meroplankton. Eggs float freely with the currents until hatching. The larvae are also planktonic, during their first week until the eyes and mouths develop (Kaiser and Holt 2005; Holt et al. 2007; Faulk and Holt 2008).

Males mature at two years and females at three years. Both sexes have a life span of 15 years or more. Spawning takes place diurnally from April to September in large offshore congregations where females are capable of spawning up to 30 times during the season (Richards 1967; Franks et al. 1999; Williams 2001; van der Velde et al. 2009; Fry and Griffiths 2010).

Cobia migrate north and west along the Gulf coast during the spring and reappear in northern Gulf waters during March and April. Cobia are usually caught in shallow coastal waters, although they are often taken offshore along the Louisiana and Texas coasts in association with oil and gas platforms. Cobia make seasonal migrations along the Atlantic coasts in search of water in their preferred temperature range. Wintering in the Gulf of Mexico, they migrate north as far as Maryland in the summer, passing East Central Florida in March (Shaffer and Nakamura 1989; Pierce et al. 1992; Franks et al. 1999; Hammond 2001). Cobia is sold commercially, and the fish commands a high price. Therefore, Cobia are potentially one of the most important marine fish for future aquaculture production.

Florida Pompano and Cobia fertilized embryos were obtained from Troutlodge Inc. They were hatched and reared at FIU before use in toxicity studies.

Several crabs of the family Portunidae are commercially important species with the Blue Crab, *Callinectes sapidus*, being the most important in the Western Atlantic and the Gulf of Mexico. *Callinectes sapidus* is found from Nova Scotia, Maine and northern Massachusetts to northern Argentina (Williams 1974). Blue Crabs are gonochoristic with clear dimorphism. Males may mate several times while females mate only once while in the soft shell stage after their last molt cycle. Spawning initiates in early spring with the

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onset of warmer temperatures. Hatching occurs 14-17 days post spawn and the zoea are

released into high salinity waters (23-30 ppt) near river mouths, inlets, and coastal areas

(Millikin and Williams 1984). Complete development through zoea stages ranges from

32 to 43 days (Bookhout and Costlow 1975; Bookhout et al. 1976; Bookhout and Monroe

1977; Bookhout et al. 1980). Other species of the same genus are the main competitors

for *C. sapidus* but the Mud Crab, *Panopeus herbstii*, and the Stone Crab, *Menippe*

mercenaria, inhabit similar environments and feed on similar organisms (Williams and

Duke 1979). Predators of Blue Crabs include Black Drum, *Pogonias cromis*; Red Drum,

Sciaenops ocellatus; the American eel, *Anguilla rostrata*; Sandbar Sharks, *Carcharhinus*

plumbeus; Atlantic croakers, *Micropogonias undulatus*; the Oyster Toadfish, *Opsanus*

tau; the White Catfish, *Ictalurus catus*; Spotted Gar, *Lepisosteus oculatus*; and Striped

Bass, *Morone saxatilis* (Millikin and Williams 1984). Larval crabs feed on zooplankton

from 45 to 80 µm in size while juvenile and adult stages feed on molluscs, crustaceans,

and fish (Costlow Jr. and Bookhout 1959; Tagatz 1968). After developing from larvae to

juvenile crabs in high salinity waters, Blue Crabs recruit into lower salinity waters. Once

mating occurs in lower salinity waters females migrate back to higher salinity waters to

release the larvae (Millikin and Williams 1984). Several states prohibit the collection of

gravid females and enforce minimum size limits to manage the fishery for Blue Crabs.

Artificial stocking programs have had mixed results. Declining catches are linked to the

decline of eelgrass beds in Chesapeake Bay demonstrating the importance of eelgrass as a

habitat for Blue Crabs (Millikin and Williams 1984).

The GoM supports one of the most diverse cnidarian populations in the world,

with conservative estimates of over 115 epipelagic species (Phillips 1971; Graham 2001).

As one of the most common, large gelatinous zooplankton species in the world, the Moon Jellyfish (*A. aurita*) is an excellent candidate for an ecotoxicology testing program. *Aurelia aurita* is epipelagic and has a cosmopolitan distribution across the world for netritic waters between 70°N and 40°S (Arai 1997; Lucas 2001). Moon jellyfish are euryhaline (salinities from <10 to 38 ppt) and eurythermal in distribution (Lucas 2001). *Aurelia aurita* possesses a complex lifecycle with a combination of both asexual polyp (fixed to solid substrate) and sexual medusa (free-floating) stages. Populations of *A. aurita* polyps principally attach to suitable substrata in coastal abayments, fjords and estuaries (Lucas 2001), and have been identified offshore in the Northern Gulf of Mexico (Roden et al. 1990). Recent research by Faimali et al. (2014) demonstrated *A. aurita* are a promising new model for ecotoxicological studies. For much of the year, the Moon Jellyfish is prevalent as a polyp which then undergoes a change to a non-feeding transitional stage (strobila). Through a process called strobilation, saucer shaped discs of tissue disconnect from the polyps and become free-floating organisms called ephyra, which is an immature medusae with tentacles needed to feed. In the wild, strobilation occurs in early spring. The ephyrae slowly turn into small medusa, which grow into mature medusa (jellyfish). Mature medusa reproduce sexually creating planulae larvae, which settle and grow into a polyp thus the cycle starts again. Since strobilation is easily induced in the laboratory, we can readily obtain and work with an immature, early life stage of the Moon Jellyfish (ephyra) which is an extremely important stage in the life cycle of an organism for toxicity testing (McKim 1985).

Organism-specific culture systems and techniques were required for several of the testing species. Culture and toxicity study protocols were created specifically for

individual species and will be summarized in the methods section. Initial toxicity studies using native species required several trials and a variety of techniques. Young organisms are extremely delicate and suffer high mortality as a result of handling and contact with solid surfaces. They sometimes require water movement to remain suspended in the testing media. A variety of attempts included: using incubated shaker tables; different shape, size, and material exposure chambers; single organism exposure vs. group exposure chambers; aerated vs. non-aerated containers; closed vs. sealed exposure chambers; suspending embryos/larvae in Imhoff cones with gentle aeration. In addition, using petroleum products is a challenge because of its characteristic adsorption. We limited testing media contact to materials that were least reactive with petroleum products such as borosilicate glass and Teflon.

LABORATORY METHODS

Test Materials/Preparation of Exposure Test Media

The testing materials used in the program come from four field-collected oils relevant to the DWH response and the dispersant applied in the field. These oils included:

1. "Q4000" which was a fresh non-weathered crude source oil collected at the wellhead by the Q4000 Well Intervention vessel sampled on June 19, 2010;

2. "MASS" which was a fresh non-weathered crude source oil collected at the wellhead by the Enterprise Producer and transferred to the oil barge Massachusetts sampled on August 15, 2010;

3. "CTC" also known in the literature as "Slick A" which was a naturally weathered oil collected from the ocean surface by Barge No. CTC02404-04 sampled on July 19, 2010; and

4. "Juniper" also known in the literature as "Slick B" which was a naturally weathered oil collected from the ocean surface by the US Coast Guard Cutter Juniper sampled on July 19, 2010 (Echols et al. 2014; Faksness et al. 2015; Stefansson et al. 2016; Sandoval et al. 2017).

Studies began with an oil called "Q4000", which was the first crude source oil sample collected early on in the incident, but was replaced with "Massachusetts" also known as "MC-252" which was the field source oil collected directly from the wellhead. The oils Q4000 and MASS are least affected by the environment and are considered most representative of the oil that was exiting the wellhead. Weathered oil samples (i.e., oil collected by skimming barges from the ocean's surface exposed to environmental influences) were called "CTC" (also called Slick A in some references) and "Juniper" (also called Slick B in some references) and were considered 64% depleted and 86% depleted, respectively. Depletion (%) was calculated using the percentage of lightweight volatile components that dissipated from the raw crude oil and the presence of 30αβ-hopane. Juniper is considered the most weathered because of an overall PAH depletion of 86% (using the oil biomarker 30αβ-hopane) (Bragg et al. 1994; Prince et al. 1994; Sandoval et al. 2017) and representative of oils that reached the shoreline during the DWH incident. The CTC oil is considered moderately weathered with a PAH depletion of 64% and is representative of floating slicks. The dispersant Corexit® EC9500A (Nalco Inc.) was also used extensively in the field. Therefore, it was used alone and with oil in dispersant mixed exposure treatment media preparations. The dispersant is sometimes referred to as Corexit9500 or CX9500. Echols at al. (2016b) provided thorough details on

the oil characterization including, collection information, test media preparations, corresponding bulk composition and further chemical analysis conducted in this program.

Aquatic toxicity test protocols routinely require the use of reference toxicants (to establish the response of organisms to a known well-established toxicant) and was practiced in our program. Standard reference toxicants included potassium chloride, cadmium chloride, copper sulfate, and sodium dodecyl sulfate and were discussed by Echols et al. (Echols et al. 2015b). Testing media were prepared using filtered (0.45 µm) saltwater consisting of deionized water and a synthetic sea salt (InstantOcean®) mixed to the appropriate salinity for the test species. Echols et al. (Echols et al. 2016b) conducted some studies using filtered natural seawater to investigate a variety of factors that may influence oil toxicity and are included in this summary.

Petroleum products are extremely difficult to work with because they are complex mixtures. To prepare our oil-water media for organismal exposures we used methods developed by a consensus group of academic, industrial and federal agency scientists through the Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) which recommended the use of water accommodated fractions (WAF) (Singer et al. 2000; Barron and Ka'aihue 2003; Aurand and Coelho 2005). Sandoval et al. (Sandoval et al. 2017) provide a thorough examination of oil media preparations to generate a stable testing medium that will provide an environmentally relevant consistent exposure which is required for quality statistical analysis and establishment of toxicity/stress-response relationships.

The WAFs of source and weathered oils were therefore prepared following CROSERF guidelines with minimal modifications (Singer et al. 2000). Aspirator bottles

were filled with water media to maintain a head space of 20% and loaded with the oils at a 1:1000 ratio (1 g oil to 1 L water). Aspirator bottles were sealed and stirred using a magnetic stirring bar and plate for 20h followed by 4h of settling in the dark while at 25 °C. The speed of mixing depended on the desired preparation. A low energy WAF (LEWAF) was mixed at a rate resulting in no visible vortex, while a moderate energy WAF (MEWAF) was mixed at a rate resulting in a vortex of 20-25% of the water depth. Dispersed oil-water mixtures called chemically enhanced WAF (CEWAF) were prepared in an identical manner but with the addition of the dispersant Corexit 9500 to the aspirator bottle at the rate of 1:20 (dispersant to oil). Samples applied in toxicity studies were removed from the bottom of the bottles from the aspirator spigot to prevent taking material floating on the surface. These techniques were applied in all studies published by Echols et al. (Echols et al. 2015a; Echols et al. 2016b).

Test Organisms

Americamysis bahia, *Menidia beryllina*, and *Cyprinodon variegatus* animal husbandry techniques are well known from standard methods (USEPA 2002a). Organisms were held in static holding containers if they were to be used within a few days or recirculating culture systems if they would be maintained for a longer time. All three of these species were fed <24h old *Artemia* hatched from cysts at hatch. Transfers from chambers were done by siphon or gentle pouring and never with a net (contact with a net frequently results in mortality). Plastic transfer pipettes or glass tubes with a source of suction attached were practical for counting and loading organisms into testing chambers.

The native finfish that were used in the FIU testing program are mentioned in other manuscripts (Echols et al. 2015a; Echols et al. 2015b; Echols et al. 2016b) and included *Sciaenops ocellatus*, *Cynoscion nebulosus*, *Paralichthys lethostigma*, *Pagrus pagrus*, *Trachinotus carolinus*, and *Rachycentron canadum*. Upon receipt, embryos or larvae were gently poured out into large flat plastic trays, gently aerated, and gradually acclimated to the system into which they were to be placed by using a dripline. After 4-8 hours of acclimation, they were introduced into the culture systems.

Several iterations of culture systems were used. The first system contained several glass tanks (made from hand-cut and drilled glass panels) arranged on a wire rack and connected with PVC tubing to a sump containing mechanical filtration, biological filtration, UV sterilization, and temperature controls as seen in Fig 1. Overflows in the tanks were created by cutting open sections in 1" PVC pipe for a greater surface area that was covered by a large plastic mesh tube, which was then covered by an appropriate sized nylon mesh to prevent embryos or larval fish from entering the drains. The overflows outside surface mesh size would be adjusted according to what was appropriate for the organism being used at the time. Each tank's return flow was directed in a way that it created a circular flow to avoid physical damage to the larvae. Gentle aeration (no more than a few bubbles per second) was provided by large bubbles from rigid airline tubing inserted to the bottom corner of each tank.

Water quality was monitored during holding and acclimation. Temperature and dissolved oxygen (DO) were measured daily. Salinity was measured at least twice a week. or whenever acclimation involves the alteration of water salinity. Salinity was maintained by an automatic top-off valve (using purified fresh water) or the manual

addition of freshwater to prevent salinity creep due to evaporation. The pH was measured

at least once a week. Dissolved oxygen concentrations were maintained at ≥ 5.0 mg/L.

All laboratory techniques followed good laboratory practices (GLP) as a NELAC

accredited laboratory in the National Environmental Accreditation Program (NELAP).

Many larval fish cannot immediately feed on *Artemia* at hatching and require a

smaller first feed. This first feed was provided in the form of S-type (*Brachionus*

rotundiformis) or L-type (*Brachionus plicatilis*) rotifers cultured in-house using a batch

culture system (size depended on the gape size of the fish being cultured). Rotifers were

harvested multiple times daily and enriched with *Nannochloropsis* (or similar microalgae

blend) and added to the recirculating system to maintain a concentration of 10-30

rotifers/mL. Mechanical filtration was never small enough to remove rotifers so they

would remain in the system until eaten. Concentrated algae was added to the culture

system as well to maintain the quality of the rotifers for feed to the larvae. Once the

mouth gape of larvae was large enough, they were transitioned over a period of several

days (feed type would overlap to allow all larvae to transition) to feeding on <24h old

Artemia nauplii. Once fish were of the desired age/size for testing, they were removed

from the culture systems and used in toxicity studies.

Initial work with *T. carolinus* and *R. canadum* demonstrated the need for higher

DO concentrations in the culture systems. Development of gills in *R. canadum* takes

place around 11-15 days post hatch (DPH) and therefore they respire cutaneously and

require high saturation of oxygen in the water (Benetti et al. 2007). Larger systems were

designed to include gas-exchange columns fabricated from acrylic tubing to create a

counter-current flow of water to aeration over a bio-fill material (Bio-Balls) to strip out

CO$_2$ and increase the DO as much as possible while providing more flow and turnover in the culture tanks as observed in Figure 2. With these larger culture system volumes (over 300 gallons), more rotifers were needed and we built a high-density rotifer culture system that was capable of keeping up with our needs (4-5 million/day or more) and included an automated feeding system and the use of ammonia detoxification (using ChlorAm-X).

The invertebrates in our program possessed uniquely specialized requirements. *Acartia tonsa* was obtained from AlgaGen and maintained in batch cultures until used in toxicity studies. Cultures were maintained in static-renewal containers with regular water renewals and feed provided by a prepared diet of microalgae. There are a variety of microalgal feeds available on the market but we used one that contained *Chaetoceros* and *T-Isochrysus* (from Reed Mariculture).

Blue Crab zoea can be obtained from fertile female crabs and can be tested at a variety of stages including: zoea, megalopae, or early crab stages. However, care must be taken with older crabs because once in the megalopae stage, with functional claws, they are extremely cannibalistic and must be held separately or with sufficient refuge space (Williams and Duke 1979; Zmora et al. 2005). We obtained zoea (stage Z3-Z7) from the University of Maryland Baltimore County shipped overnight. Zoea were maintained in a static or recirculating system and fed a diet of enriched (using AlgaMac) L-type rotifers or *Artemia* (depending on mouth size and feeding ability) for a short time before being used in exposures.

Adult Moon Jellyfish medusa were not practical to use in toxicity studies due to size. To accommodate for the size constraints, we used the smallest medusa available, the ephyra, to remain relevant to what would be present in the GoM. *Aurelia aurita* polyps

(the sessile body form that grows attached to a substrate) were obtained from the Oregon Coast Aquarium and cultured in house. Polyps (scyphopolyp) bases were cleaned and placed onto plastic petri dishes that had been scored with sandpaper to facilitate adherence to the substrate. Once attached, petri dishes were suspended in a culture bowl using monofilament line, aerated with a gentle rate of bubbles from a rigid airline, fed <24h old *Artemia*, with daily water renewals. Strobilation (the act of the scyphopolyp segmenting and generating ephyra) was induced through extreme temperature swings (12-15°C) and/or supplemental iodine (Spangenberg 1967, Berking et al, 2005). Ephyrae were collected during the strobilation period using transfer pipettes and used in toxicity studies.

Testing Protocols

Individual toxicity testing protocols were written specifically for each species used in our program. Protocols were modeled after U.S. EPA standard procedures (Whole Effluent Toxicity testing guidelines) (USEPA 2002a; USEPA 2002b; USEPA 2002c) with modifications where needed for testing with petroleum products or species-specific requirements. Tests were conducted in glass vessels and sealed with Teflon lined lids to limit evaporation and volatile hydrocarbon loss. Studies required a minimum of ten organisms per replicate, four replicates per treatment, and five treatment exposure concentrations plus a control per study. A variable dilution method (Barron and Ka'aihue 2003) was used to prepare the five different treatment exposure concentrations, where a WAF/CEWAF preparation was diluted into a series of concentrations (6.25%, 12.5%, 25%, 50%, and 100%) of the original source (WAF/CEWAF). The diluent water (filtered synthetic seawater) used to prepare the exposure media was used as the untreated control.

Acceptable age/size class range was established for each species and more species-specific details will be discussed in forthcoming manuscripts. Fish used in toxicity studies were of an age/size that they had completely transitioned to feeding solely on *Artemia* nauplii by testing time. For example, Red Drum were tested at 10-28 days of age (all fish included in a study were within 24h range in age); while pompano were tested at 5-17 days of age. Developmental speeds are not the same across species and testing would begin at the completion of transition to brine shrimp feeding to facilitate testing without having to feed rotifers multiple times a day. I observed that sealed containers with high concentrations of rotifers would cause unacceptable drops in DO quickly and prevent achieving the desired study durations. Studies were typically conducted for a duration of 96h (i.e. with some variation) with 16h light: 8h dark photoperiods, salinity ±2 ppt (of species-specific salinity culture requirements), and temperatures of 25±1 °C. Organisms were fed according to their species-specific requirements, mortality was assessed daily, water quality parameters (salinity, dissolved oxygen, pH, and temperature) were collected as required, and study test media were renewed at 48h. Statistical point estimates were calculated for each study using %WAF/CEWAF concentrations and recalculated based on TPAH when analytical chemistry data became available using CETIS® (Tidepool Scientific, Inc.).

Data

Data generated during our study included a variety of standard toxicity point estimates such as: no observed effect concentrations (NOEC); 10% effect concentrations (EC10) where an effect is observed in 10% of the test population; median lethal concentrations (LC50) where mortality is observed in 50% of the test population; 10%

lethal concentrations (LC10) where mortality is observed in 10% of the test population; 25% inhibition concentrations (IC25) where a 25% reduction of a biological parameter (e.g., growth or reproduction) of the test population is observed. The toxicity program at FIU resulted in the generation of well over 250 endpoint values for 10 species. To facilitate the use of these toxicity data, species sensitivity distributions (SSDs) were developed which are practical when using large amounts of toxicity data with different test species (Traas et al. 2002a). When aquatic toxicity data are available for a number of species, the analysis of the distribution of species sensitivities is possible and is represented by a curve. The SSD is therefore an assemblage of single-species toxicity data for a specific chemical or mixture that can be used to predict a hazardous or lethal concentration (HC, LC) or sublethal concentration affecting a proportion (p) of the species in the community represented by the SSD. An SSD is a cumulative distribution function (CDF) of the toxicity of the oil type (crude oil, weathered oil) using the total polycyclic aromatic hydrocarbon (TPAH) concentration (µg/l) as the unit of exposure, SSDs have been used extensively internationally in regulatory guidelines (Posthuma et al. 2002b; Altin et al. 2008; Dyer et al. 2008; Barron et al. 2013). Species sensitivity distributions have had limited use in petroleum aquatic toxicity studies (De Hoop et al. 2011; Barron et al. 2013) although with sizeable diversity of species sensitivity data they can be used to determine community (or habitat)-based benchmarks especially when laboratory studies use field-level exposures. Typically the 10th percentile and/or the 5th percentile of the model-fitted distribution (HC5, HC10, LC5, LC10) is used as the hazardous or lethal concentration and applied in ecological risk assessments (e.g., the hazardous (HCx) or lethal concentration (LCx) to protect 90 or 95 % of the species).

Laboratory SSDs will be discussed as appropriate to specific petroleum field exposure scenarios and related to the GoM TPAH data (field collected exposure levels) (Newman et al. 2000; Aldenberg and Luttik 2002; Posthuma et al. 2002a; Wheeler et al. 2002a; Zwart 2002; Wheeler et al. 2002b; Duboudin et al. 2004a; Duboudin et al. 2004b; Barron et al. 2013).

Analytical Chemistry

Sample collection activities, analytical procedures, and storage and holding times were generally consistent with the Analytical Quality Assurance Plan. Mississippi Canyon 252 (Deepwater Horizon) Natural Resource Damage Assessment. Version 2.2 (NOAA 2010). Quality control (QC) samples (e.g., duplicates, trip blanks, dilution water) were collected during the testing program. Test media samples were collected at test initiation and termination of the acute studies.

One liter (1000 mL) samples were collected immediately following decanting of each 100 and 6.25% WAF/CEWAF into amber glass jars with minimal headspace for parent and alkylated polycyclic aromatic hydrocarbons (PAHs), and saturated hydrocarbons (SHC) analyses. Additional samples (3 x 40 mL) were collected in 40-ml amber vials and preserved with HCL (no headspace) for volatile organic compound (VOC-PIANO) analysis. All samples were labeled and chain-of-custody (COC) forms were completed. Prior to and during shipping to the analytical laboratory, samples were maintained in the dark at 4°C from the time of collection.

Analytical chemistry analysis was conducted at either Battelle or the FIU Environmental Analysis Research Laboratory (EARL). Total petroleum hydrocarbons (TPH) and saturated hydrocarbon compounds (SHC) were analyzed by gas

chromatography-flame ionization (GC-FID; SW-846 Method 8015-modified). Parent and alkylated PAHs and hopane were analyzed by gas chromatography/mass spectrometry in selected ion monitoring (GC/MS-SIM) using modifications of SW-846 Method 8270. Samples for volatile organic compounds (VOCs) were analyzed by purge-and-trap GC/MS (Battelle SOP 5-245, a modification of SW-846 Method 8260). Thorough details of this analysis can be found in a variety of publications (Echols et al. 2014; Echols et al. 2015a; Echols et al. 2016b; Sandoval et al. 2017).

Toxicity and chemistry data went through a rigorous quality assurance and quality control (QA/QC) program. All of the biological and chemistry data presented from this program were subjected to formal data verification and validation prior to use. All laboratory data were reviewed by the laboratory's internal Quality Assurance Unit and external (independent) auditors to assure that each study was performed in accordance with the protocol and laboratory standard operating procedures (SOPs). More detail on the QA/QC program can also be found in the publications from our group mentioned in the previous paragraph.

RESULTS

The review presented here includes a compilation of data from aquatic toxicity studies conducted at the FIU Ecotoxicology & Risk Assessment Laboratory (ERAL), Oregon State University, and from a variety of academic institutions or contractors collaborating in the NOAA NRDA comprehensive toxicity testing program and summarized by Morris et al. (2015). Ours is the first review of the aquatic toxicity laboratory database using SSDs on petroleum generated from the DWH incident that occurred in March-July 2010. Some of the data summarized here has been published

(Echols et al. 2014; Echols et al. 2015a; Morris et al. 2015; Echols et al. 2015b; Echols et al. 2016a; Stefansson et al. 2016; Echols et al. 2016b) by research teams and I am also including some data from FIU that has not been published. It is worth noting that some of the aquatic toxicity studies contained in the database generated data that was not useable in SSDs. For example, many studies using weathered oils resulted in endpoint estimates greater than 100% of the prepared testing media and was reported as OOR (out of range). The highest exposed concentrations are frequently reported and available but are not realistic to use in an SSD. Also, toxicity data from studies conducted outdoors were not used in the SSDs because of the UV-photo-enhanced toxicity of some components of petroleum (e.g. fluoranthene, pyrene, hydroxypyrene) (Little et al. 2000; Cleveland et al. 2000; Bleeker et al. 2002; Bellas et al. 2008).

The SSDs presented here were generated to represent specific oil type and test media preparation scenarios using appropriate and available endpoints. Oils were separated into three categories: crude source oil (MASS & Q4000), moderately weathered oil (CTC/SlickA), and extremely weathered oil (Juniper/SlickB). Preparation scenarios were separated into two categories: mechanically mixed oil-water media (including LEWAF, HEWAF, and Vortex-WAF) and chemically dispersed oil-water mixtures (including CEWAF, Vortex-CEWAF, and HECEWAF).

With the six exposure scenarios, Figs. 3-8 display the SSDs using endpoint estimates from mortality data (LC50s). Following this same format, Figs. 9-14 display acute lethality endpoints at the 10-25% effect range (i.e. LC10s, LC20s, and LC25s). A variety of sublethal endpoints were also grouped together for SSDs. Sublethal endpoints included: larval settlement; growth (total weight, g); fertilization; shell length;

abnormality (e.g., histological or developmental); gill epithelial proliferation; gill telangiectasis; growth (SL, mm); hepatic vacuolization; hepatic vascular congestion; hepatosomatic index; hatching failure and mortality; atrial arrhythmia; atrioventricular angle; ventricular arrhythmia; edema; heart rate; estimated average weight gain (pg.day); neonates per adult. Sublethal endpoints used to calculate point estimates such as EC10s, EC20s, and IC25s were used to generate SSDs displayed in Figs. 15-19. No SSD was generated for sublethal endpoints with chemically dispersed oil-water mixtures using Juniper because no data were available.

Data from the compiled database were filtered using the criteria desired for the SSD comparison. For example, it is not appropriate to include LC50 data in the same SSD as sublethal endpoints. The same principle applies to oils and oil-water mixture preparations. An example of filtering options chosen for Fig. 3 (crude oil exposure using LC50 lethality endpoint estimates) are the following: contaminant = only source oils (MASS and Q4000); exposure = LEWAF, HEWAF, V-WAF (low energy WAF, high energy WAF, vortex-WAF); endpoint = only mortality; UV (ultraviolet light exposure) = none; exposure duration = variable (96h was the most common but it also included 24, 48, 60, and 72h); point estimate calculated = LC50; endpoint values = any not listed as OOR (Out of range) or NA. A similar pattern was followed for the remaining SSDs while altering contaminant, exposure, endpoint collected, and endpoint estimate type.

The 10th centiles and slopes of the curves created in the SSDs are useful for discussion and information from curve fitting is summarized in Table 1. Within all SSD groups, the lowest 10th centiles belong to weathered oils (CTC and Juniper) at 0.135 and 0.557 µg/L TPAH respectively. When considering source oils, the 10th centile is always

lower when Corexit 9500 is present while the opposite is true with weathered oils. The 10th centiles make it appear that weathered oils are more toxic than crude oils with the most sensitive values found in lower percentage lethality effects and sublethal effects data. The greater slopes found in the source oil SSDs indicate a greater chance in the number of species being adversely effected with a small change in concentration for mechanically mixed oil vs. chemically dispersed oil. The opposite is observed in weathered oil toxicity data.

DISCUSSION

The use of SSDs in this chapter rely on the following assumptions:

1. The sample of the species represented in the SSDs is taken from a random selection of the community of concern and are representative for this community;

2. The sensitivities of the species can be described by a distribution (normal, logistic, or nonparametric);

3. Interactions, habitat, importance of keystone/functional groups are not considered in the SSD;

4. Bioavailability, transport, transformation and other fate parameters are not incorporated into the SSDs;

5. Functional endpoints are normally not incorporated into the SSD, community structure is the target of concern;

6. Laboratory sensitivities of a species is approximate to the field sensitivity;

7. Measured concentrations in the form of TPAH, TPH (total petroleum hydrocarbons), THC (total hydrocarbons), or TEM (total extractable material) should be the exposure units used in a petroleum product SSD preparation;

8. Sensitivities are represented by the compound specific toxicity values: median lethal values (LC50), sublethal values (LC10, LC20); and

9. SSDs and resulting toxicity values are based on the quality of data used in making the SSD, like the toxicity data standardization (adhering to criteria for test conditions (Barron et. 2013)

The SSDs generated demonstrate a variety of information and characteristics that should be considered. Not all SSDs from this database have the same species displayed because of variation in data availability. Fish, birds, and mammals are known to be able to metabolize PAHs utilizing a variety of molecular pathways, which are less developed in invertebrates (Tuvikene 1995; Neff 1999; Di-Toro et al. 2000; Fukuyama et al. 2000; Watson et al. 2004; Vrabie et al. 2012; Allan et al. 2012). On the basis of this feature, invertebrates are expected to be the most sensitive organisms displayed on SSDs and this is not always the case since the most sensitive three species in Figure 3 are all fish (Florida Pompano, Cobia, and the anchovy). One reason to use non-standard species is they are more representative of the target study site but they also may potentially be more sensitive than standard species. A non-standard species is not always the most sensitive as can be observed in Figures 4, 10, and 15 where *A. bahia* or *M. beryllina,* two standard test species are the most sensitive on the curve. These SSDs demonstrate the importance of incorporating non-standard native species in aquatic toxicity test programs and highlights species sensitivities.

The slope of SSDs can provide insight into a chemical/mixture's toxicity. The slope of an SSD curve is highly sensitive to the number of species tested and is related to the toxic mode of action of the stressor (Zwart 2002). Steeply sloped SSDs will have

large increases in species response (e.g., mortality) linked with small changes in stressor concentration (Rand et al. 2010). SSDs from studies using complex mixtures frequently have higher slopes than those from individual compounds most likely because of the complex interaction of multiple components of the mixture such as joint additive toxicity (Suter et al. 2002). The slopes of the SSDs based on oil type were similar across all endpoint data. Source oil SSD slope ranged from 5.092-5.482. This steep slope coincides with a more complex mixture due to the presence of lighter volatile components (VOCs) in crude oils. The slopes of the weathered oils were similar to each other and lower. The lower slope correlates with a less complex mixture with a much lower variety in PAHs as a result of environmental exposure resulting in the evaporation, consumption, or modification (through metabolism) of the lighter molecular weight components of oil leaving a higher percentage of heavier molecular weight components present.

To put the SSDs reported ranges in perspective to environmental exposure concentrations, a summary of chemistry samples collected during the DWH is required. A comprehensive environmental chemistry dataset is discussed in the Operational Science Advisory Team (OSAT) assessment (OSAT 2010) and a publicly accessible dataset is available through the NOAA Data Integration Visualization Exploration and Reporting (DIVER) program. The data were compiled by DIVER on August 17, 2017 and included a data summary for chemistry and toxicity test results, calculating total PAHs in water and returned a dataset of 27,762 entries. A few relevant TPAH centiles for GoM TPAHs from the DWH incident include 75th: 0.04 µg/L; 90th: 0.19 µg/L; 95th: 0.63 µg/L; 99th: 8.52 µg/L. Only 61 samples out of 27,762 were >100 µg/L TPAH. The more sensitive species (with the lowest toxicity endpoint values) from the SSDs generated here

came from the sublethal endpoints (EC10-20 and IC25) from mechanically mixed weathered oils. The CTC SSD had 75% of the species at or below the mean stressor intensity of 5.6 µg/L TPAH for *S. ocellatus.* The Juniper SSD had 79% of the species at or below the mean stressor intensity of 6.5 µg/L TPAH for *C. virginica*. These concentrations show that with the current toxicity and field chemistry data available, the potential for exposures at the level of observed effects is rather small. Potential exposure exceeding toxicity levels of concern will be analyzed and discussed further in a risk assessment manuscript (the next chapter).

Forward-use of SSDs have been applied in risk assessment to generate potentially affected fractions (PAF) of organisms while inverse-use has been applied in the establishment of environmental quality criterion (EQC) in Europe and the United States (Traas et al. 2002b). No matter how the SSD is used, the probability of the CDF can be interpreted as the probability that a species included in the curve will be affected, provided the assumptions and criteria of the data within the SSD match the conclusions being made (Posthuma et al. 2002b). This is why care must be taken when choosing data to include in the SSD and how those SSDs are used to avoid over interpretation.

Species sensitivity distributions can be used to calculate point estimates such as protective concentrations (PC) which will protect a certain portion of species, or hazardous concentrations (HC) which is a concentration that will effect a specific proportion of species (van Straalen and Denneman 1989). The most common values include PC95 (concentration that is protective to 95% of the species) and the HC5 or HC10 (hazardous concentrations for 5% or 10% of the species). HCs are typically

estimated based on SSDs generated using NOEC data (Sijm et al. 2002). These concepts will be discussed further in the risk assessment manuscript.

The SSDs generated here clearly demonstrate the need for more data. Even with over 800 lines of data, it only represents five phyla (Arthropoda, Chordata, Cnidaria, Echinodermata, and Mollusca). In the application of SSDs in establishment of safe water quality criteria, acute and chronic data from a variety of phyla (usually ≥ 8) are required and especially more outside of Chordata and Arthropoda (as the most common testing organisms are within these two groups) (Stephen et al. 1985). Using a variety of phyla is to ensure the inclusion of data that is most representative of the whole environment. More toxicity data are being generated and released through programs such as the Gulf of Mexico Research Initiative (GOMRI) and ongoing research at universities and private institutions. These data can be accessed through the Gulf of Mexico Research Initiative Information and Data Cooperative (GRIIDC), NOAA DIVER, and journal publications. As more data becomes available, generation of more inclusive SSDs and the assessment of effects of petroleum products on GoM communities will improve. The logical next step and application of information contained in these SSDs is investigating risk.

As mentioned before, care must be taken in the interpretation and application of SSDs (Traas et al. 2002b). Probabilistic risk assessment relates toxicity data in an SSD to environmental exposures generating joint probability curves (JPCs). JPCs plot the probability of organisms encountering concentrations of the chemical under investigation based on real-world samples. The current compiled data is enough to take a first look at risk to organisms in the GoM from DWH oils. Aquatic risk assessments are categorized into tiers 1-3 based on the available information (Warren-Hicks et al. 2002). A tier 1

screening level risk assessment (worst-case scenario) and tier 2 risk assessment using the

database of toxicity data created here and the database of water samples collected during

the DWH incident will follow in the next chapter.

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Table 1-1. 10th centiles and slopes from SSDs.

Point Estimates (Endpoint)	Oil/Exposure	10th Centile (µg/L TPAH)	SSD Slope	SSD Figure Reference
LC50s (Mortality)	Source	51.13	5.092	1-3
	Source+CX9500	22.38	2.213	1-4
	CTC	2.628	1.418	1-5
	CTC+CX9500	7.525	2.151	1-6
	Juniper	1.282	1.193	1-7
	Juniper+CX9500	5.909	3.220	1-8
LC10s, LC20s, LC25s (Mortality)	Source	38.525	5.482	1-9
	Source+CX9500	9.764	4.013	1-10
	CTC	0.975	1.259	1-11
	CTC+CX9500	3.027	1.774	1-12
	Juniper	0.557	1.189	1-13
	Juniper+CX9500	4.386	3.062	1-14
EC10s, EC20s, & IC25s (Sublethal)	Source	41.415	5.247	1-15
	Source+CX9500	6.023	2.388	1-16
	CTC	0.135	0.822	1-17
	CTC+CX9500	1.143	2.325	1-18
	Juniper	1.066	2.199	1-19

Figure 1-1. Larval fish culture system with individual tanks and nylon mesh covered overflows.

Figure 1-2. Large larval fish culture systems with counter-current air/water flow to strip CO_2, increase DO concentrations, create more flow, and higher turnover rates. Includes particulate filtration (filter socks), protein skimmer, removable chemical filtration (activated carbon), automatic top-off to maintain salinity, and a ¾ horsepower heat pump for temperature control.
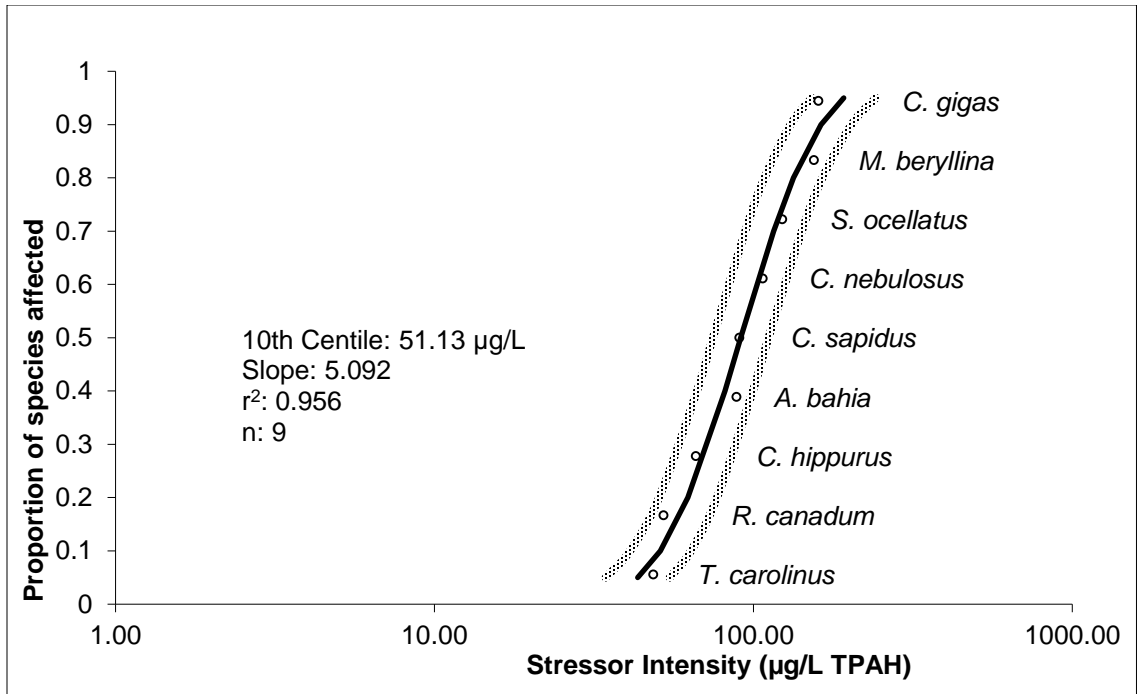
Figure 1-3. Species sensitivity distribution for LC50s using mortality as an endpoint from mechanically mixed oil-water preparations using source oils (MASS and Q4000).
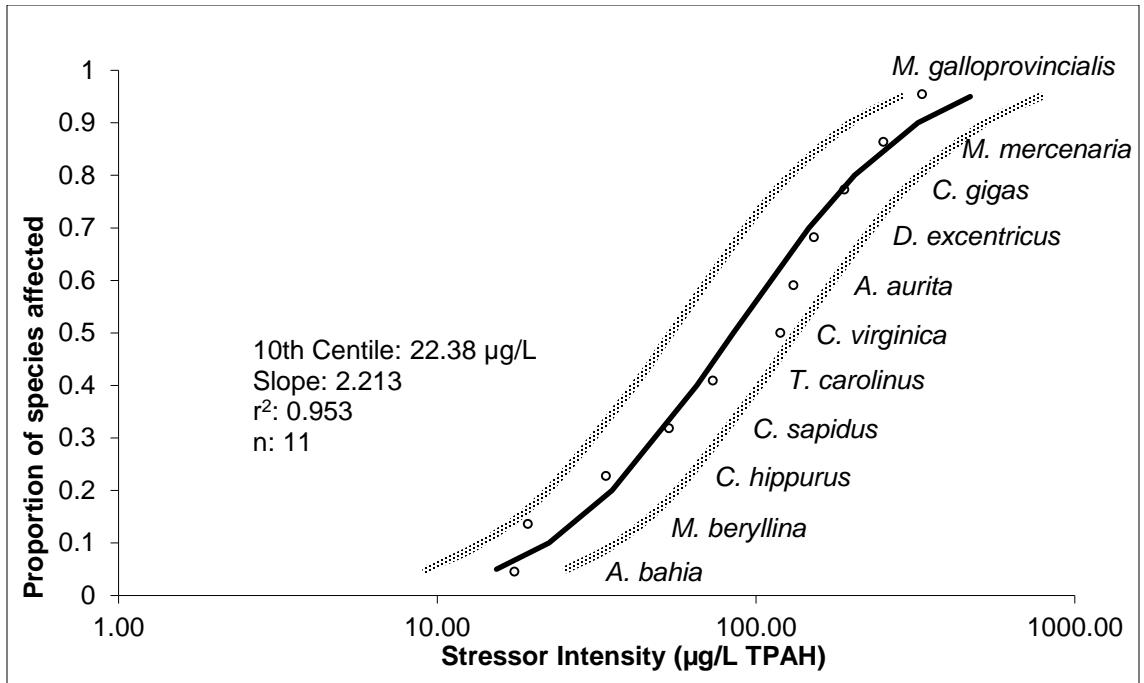
Figure 1-4. Species sensitivity distribution for LC50s using mortality as an endpoint from chemically dispersed oil-water mixtures using source oils (MASS and Q4000 with Corexit9500).
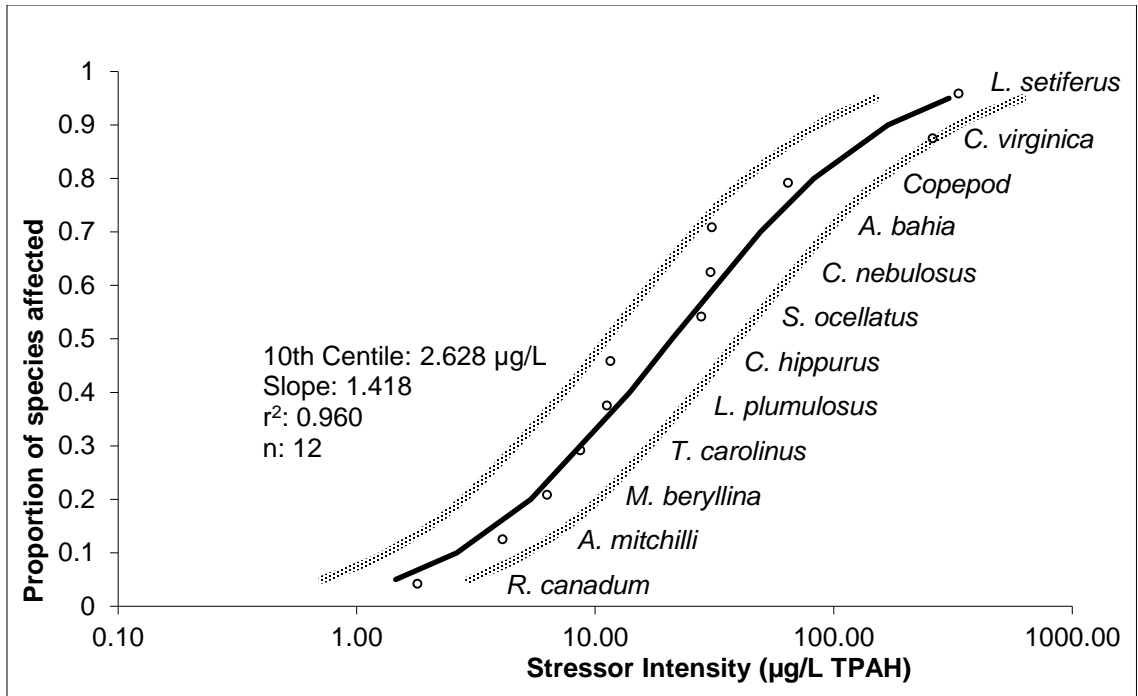
Figure 1-5. Species sensitivity distribution for LC50s using mortality as an endpoint from mechanically mixed oil-water preparations using moderately weathered oil (CTC).
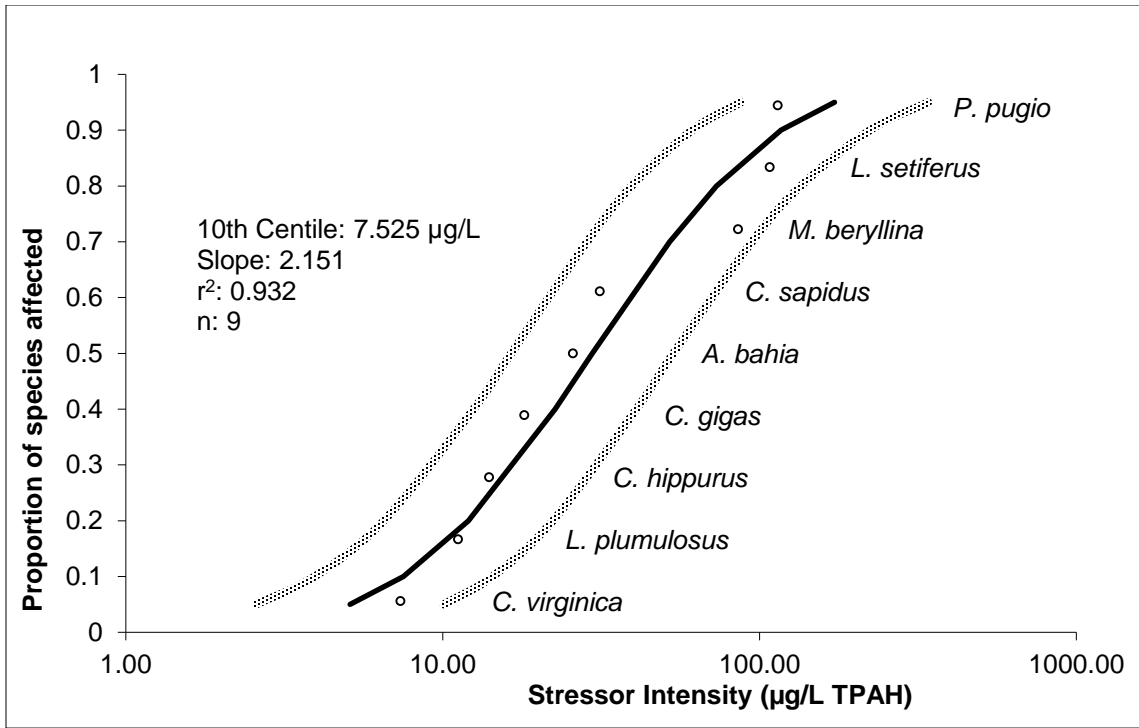
Figure 1-6. Species sensitivity distribution for LC50s using mortality as an endpoint from chemically dispersed oil-water mixtures using moderately weathered oil (CTC and Corexit9500).
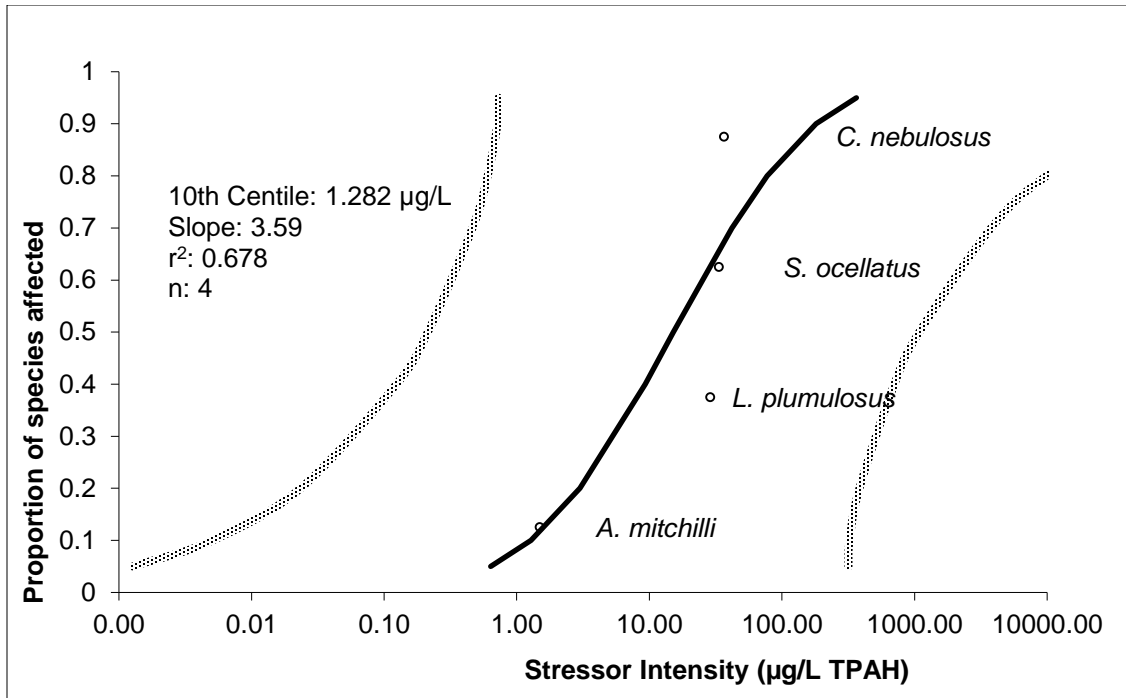
Figure 1-7. Species sensitivity distribution for LC50s using mortality as an endpoint from mechanically mixed oil-water preparations using extremely weathered oil (Juniper). Note: data is limited due to the fact that most studies had LC50 values greater than the highest concentration.
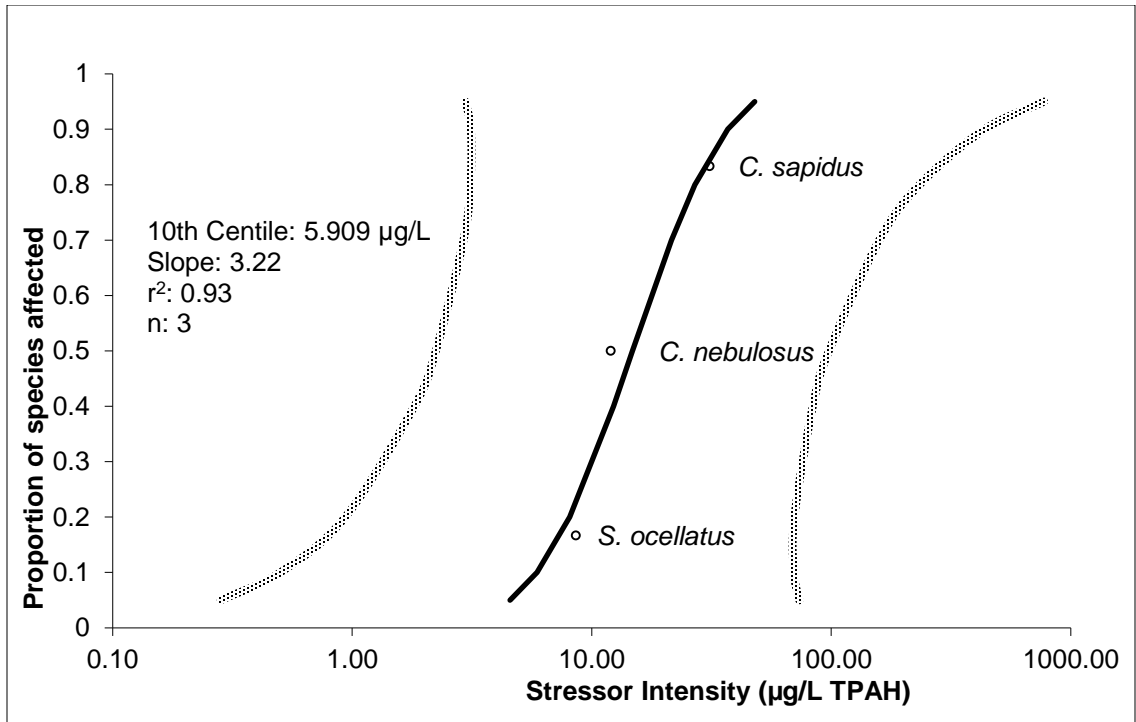
Figure 1-8. Species sensitivity distribution for LC50s using mortality as an endpoint from chemically dispersed oil-water mixtures using extremely weathered oil (Juniper and Corexit9500). Note: Dispersed Juniper is not a common exposure scenario to test due to the fact that Juniper was a weathered oil most likely near shore and therefore most likely already had Corexit9500 applied to it at some point.
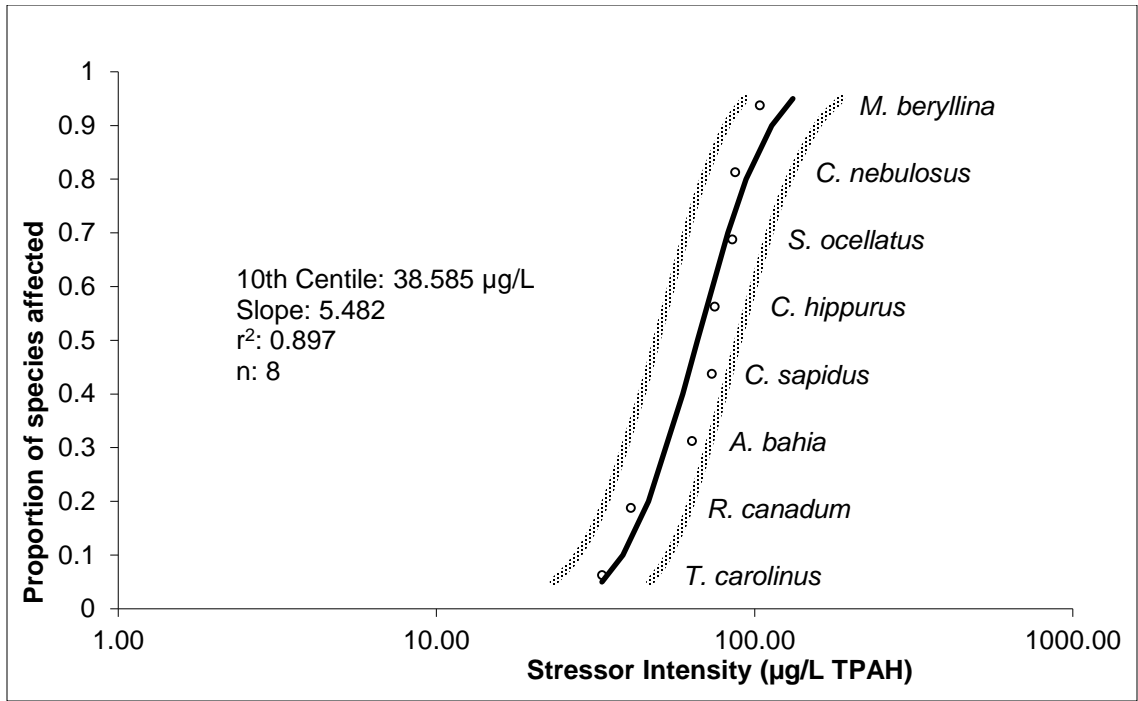
Figure 1-9. Species sensitivity distribution for LC10s and LC20s using mortality as an endpoint from mechanically mixed oil-water preparations using source oils (MASS and Q4000). Note: *C. hippurus* and *R. canadum* are LC20s, the remainder are LC10s.
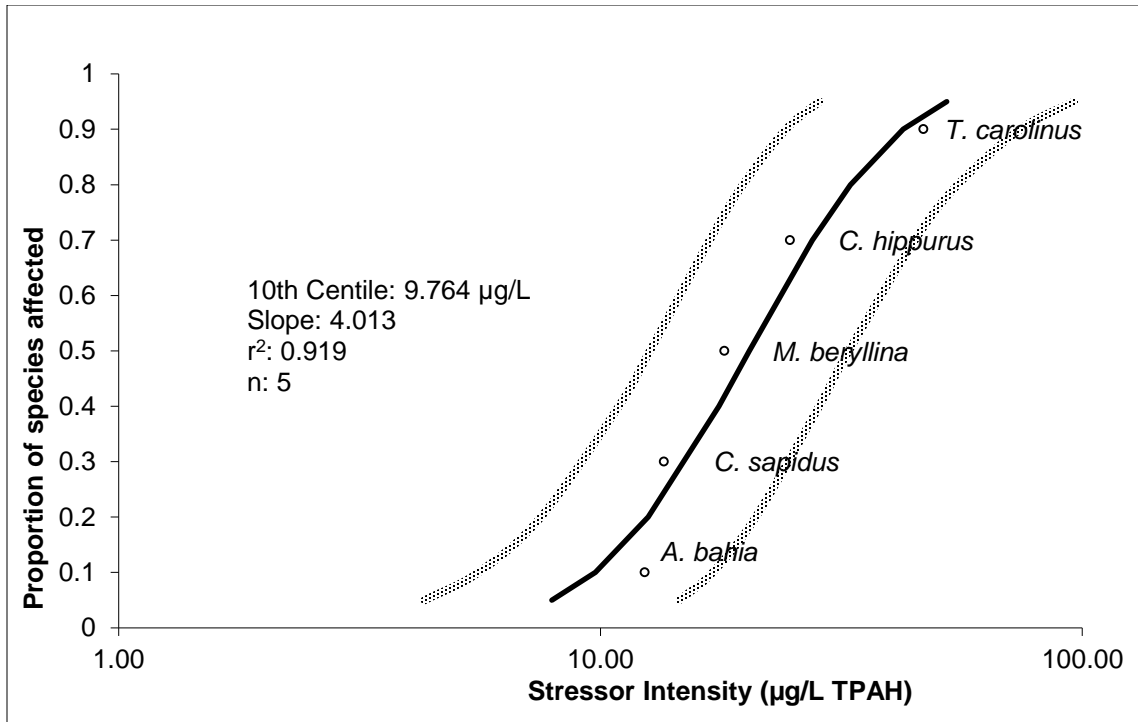
Figure 1-10. Species sensitivity distribution for LC10s and LC20s using mortality as an endpoint from chemically dispersed oil-water mixtures using source oils (MASS and Q4000 with Corexit9500). Note: *C. hippurus* are LC20s remainder are LC10s.
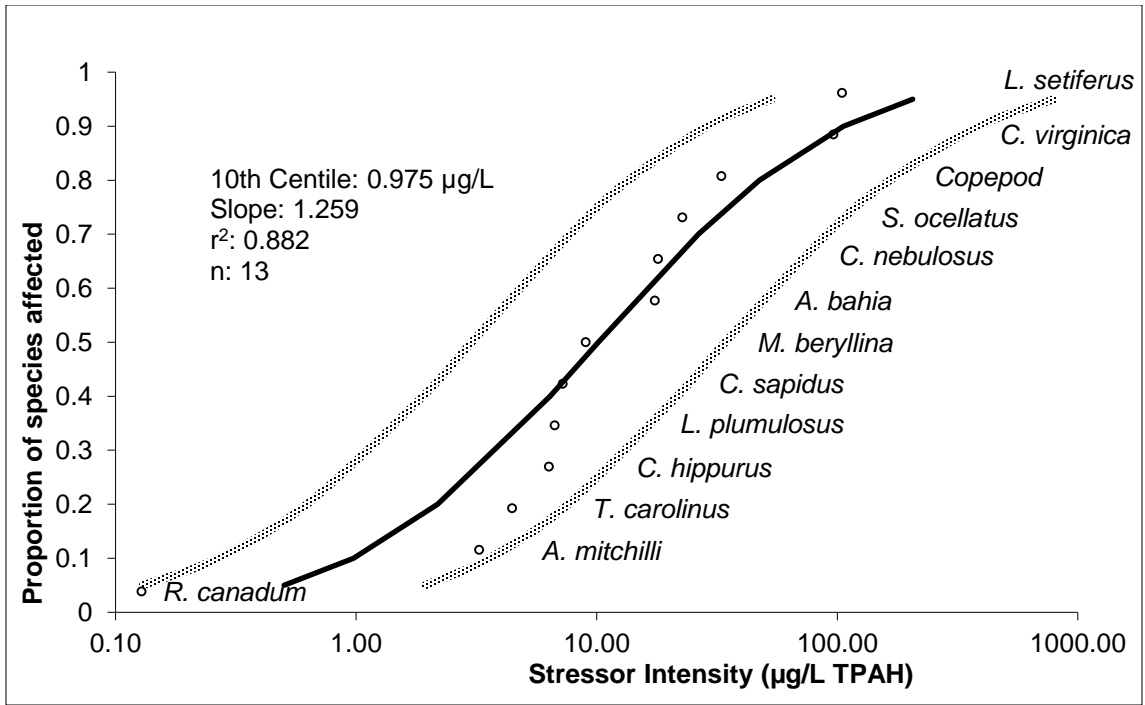
Figure 1-11. Species sensitivity distribution for LC10s and LC20s using mortality as an endpoint from mechanically mixed oil-water preparations using moderately weathered oil (CTC).
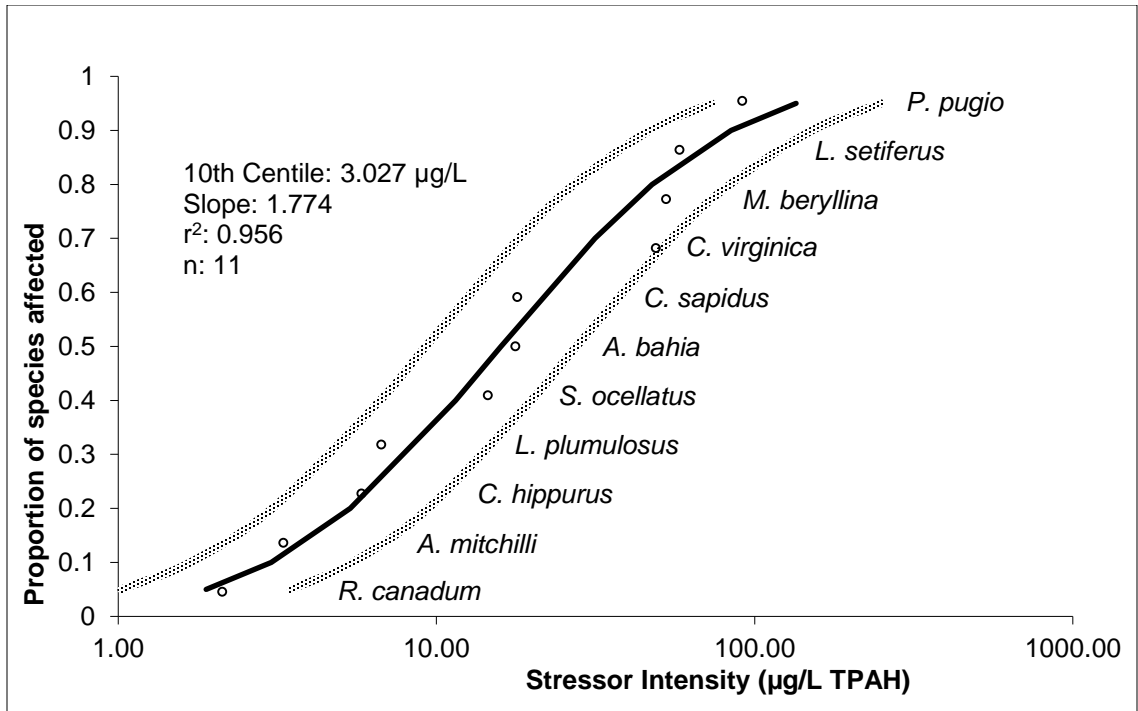
Figure 1-12. Species sensitivity distribution for LC10s and LC20s using mortality as an endpoint from chemically dispersed oil-water mixtures using moderately weathered oil (CTC and Corexit9500).
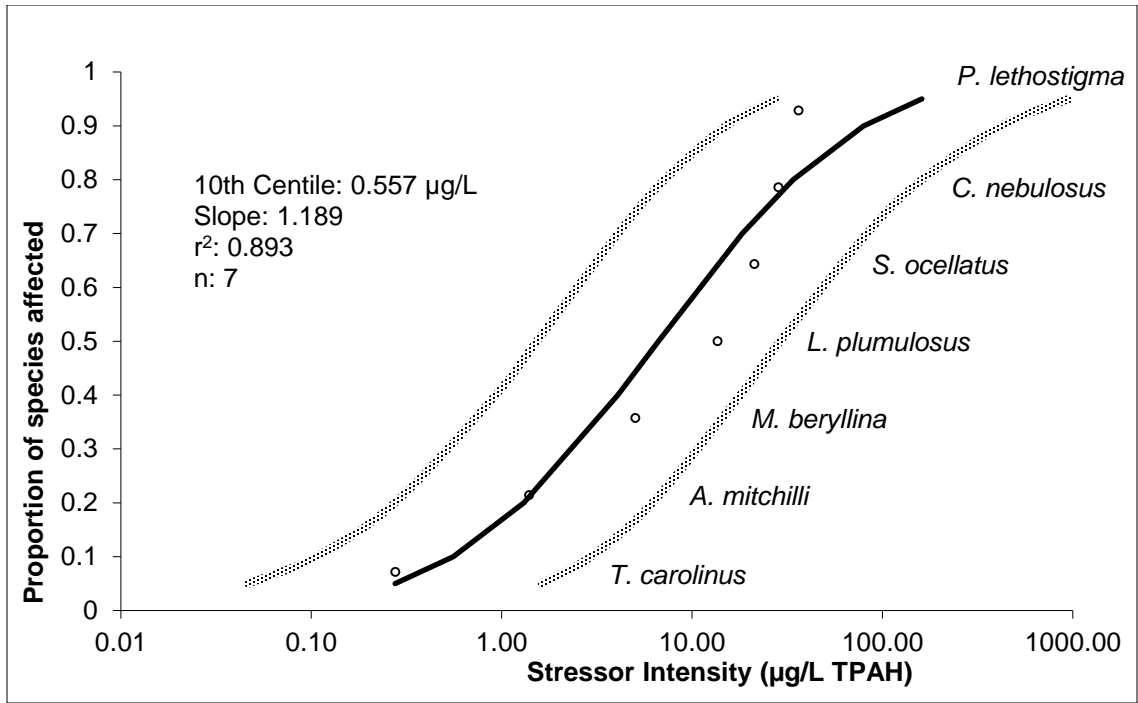
Figure 1-13. Species sensitivity distribution for LC10s and LC20-25s using mortality as an endpoint from mechanically mixed oil-water preparations using extremely weathered oil (Juniper). Note: one value in this dataset (for *M. beryllina*) is an LC25; *T. carolinus* and the other *M. beryllina* were LC10s; the remainder are LC20s.
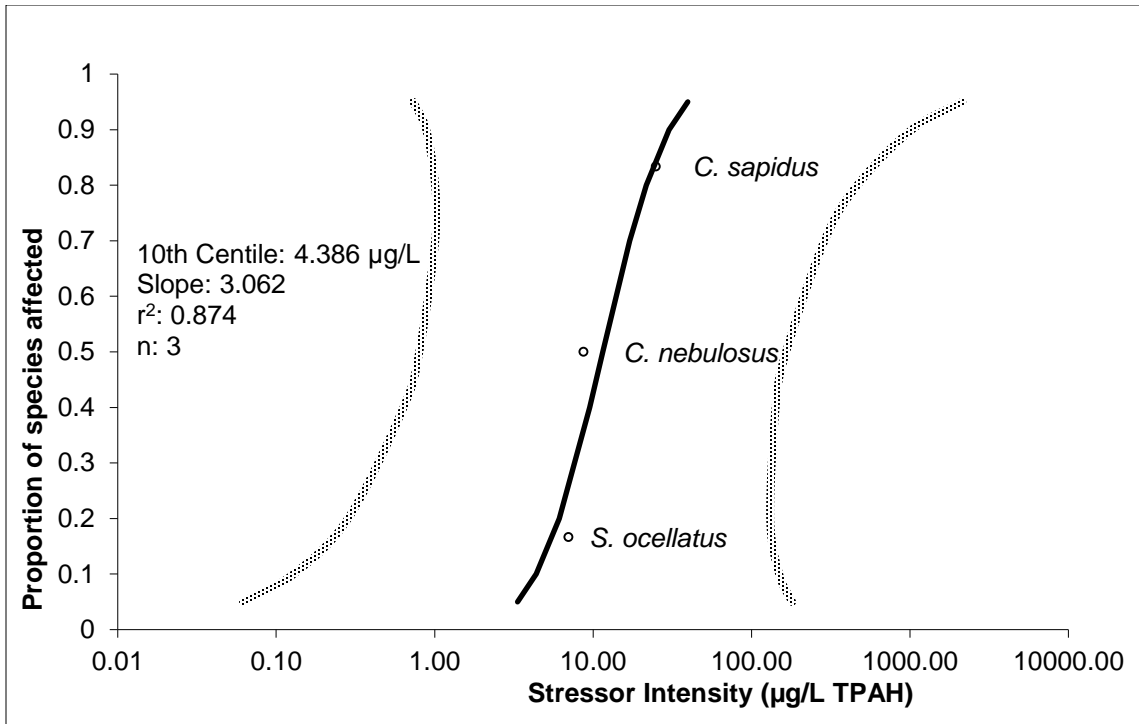
Figure 1-14. Species sensitivity distribution for LC10s and LC20-25s using mortality as an endpoint from chemically dispersed oil-water mixtures using extremely weathered oil (Juniper and Corexit9500). Note: *C. sapidus* data is LC20s at different exposure times (24, 48, 72, 96h) with values ranging from 12.9-50.2 µg/L TPAH.
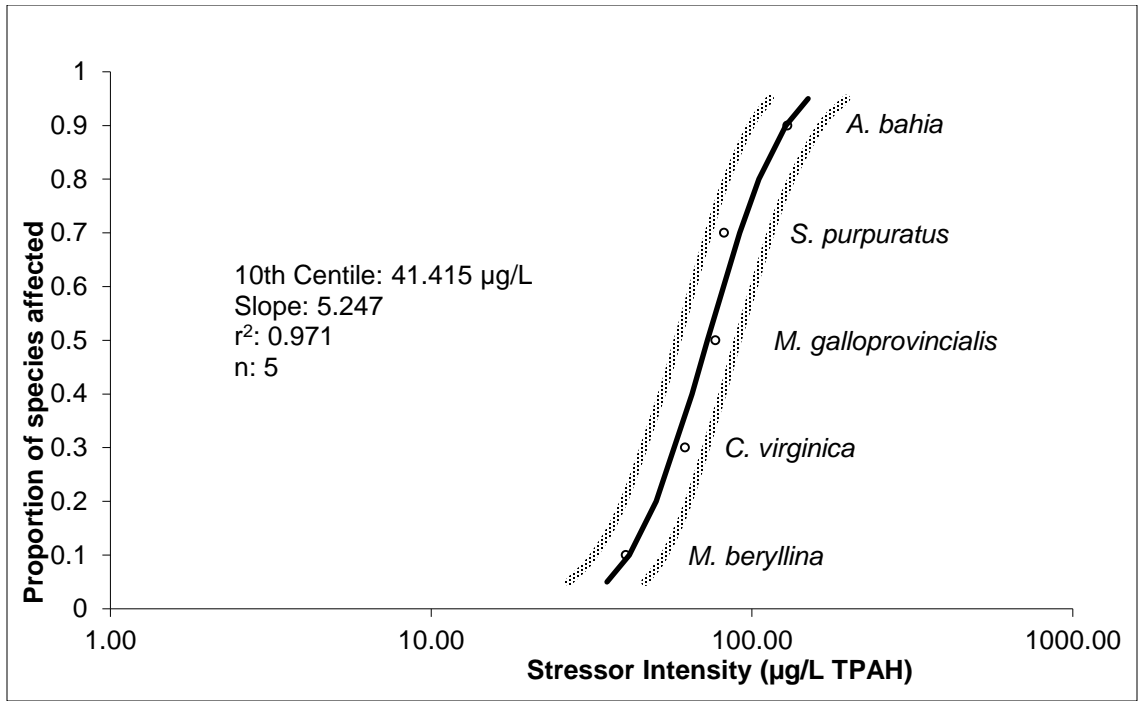
Figure 1-15. Species sensitivity distribution for ECs and ICs using sublethal endpoints from mechanically mixed oil-water preparations using source oils (MASS and Q4000). Available endpoints included growth (total weight, g) and fertilization.
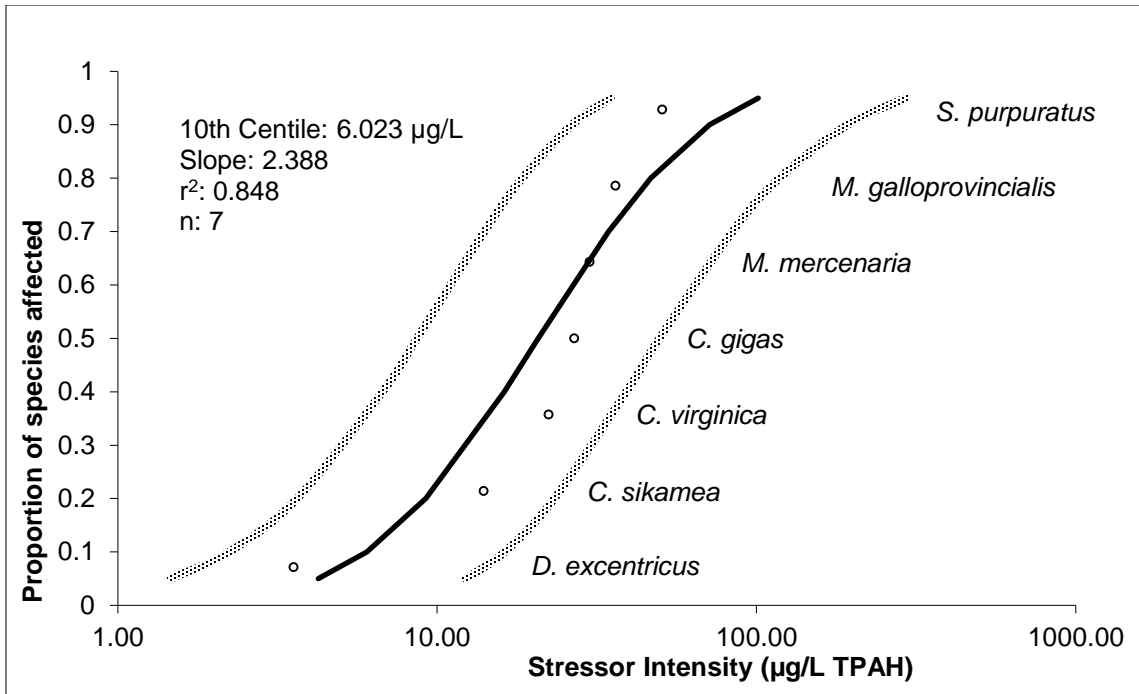
Figure 1-16. Species sensitivity distribution for EC10s using sublethal endpoints from chemically dispersed oil-water mixtures using source oils (MASS and Q4000 with Corexit9500). Available endpoints included fertilization.
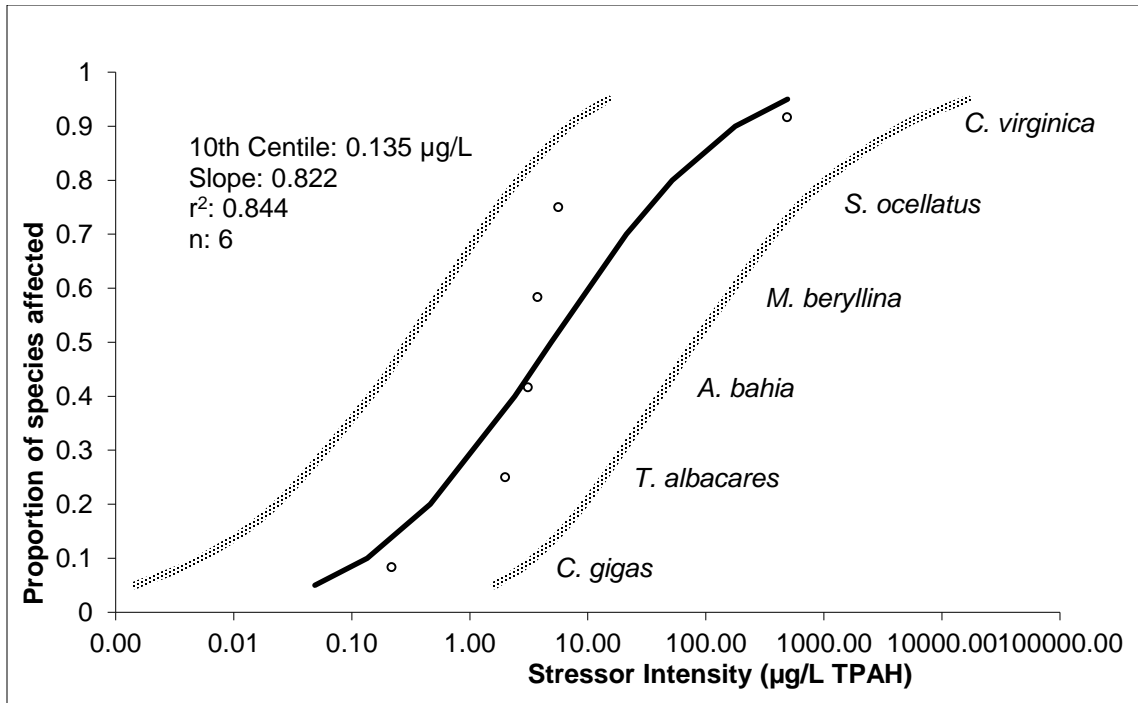
Figure 1-17. Species sensitivity distribution for EC10/20s and IC25s using sublethal endpoints from mechanically mixed oil-water preparations using moderately weathered oil (CTC). Available endpoints included shell length, fertilization, abnormality, growth (total weight, g), arrhythmia, edema, heart rate, atrial arrhythmia, atrioventricular angle, ventricular arrhythmia.
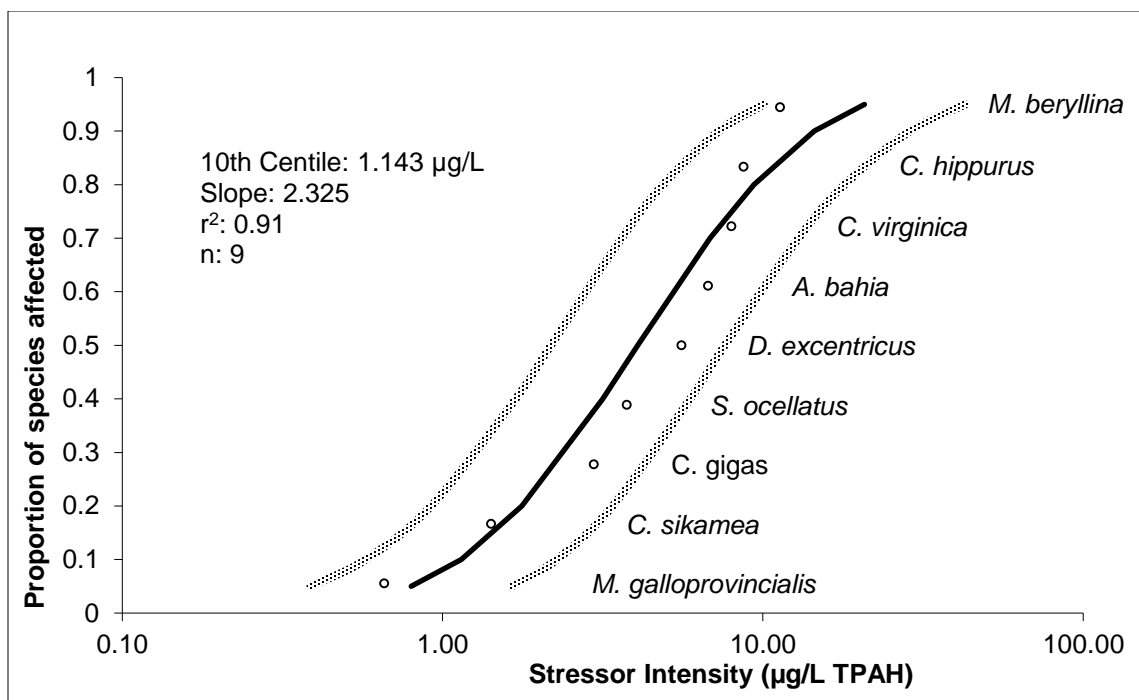
Figure 1-18. Species sensitivity distribution for EC10s and EC20s using sublethal endpoints from chemically dispersed oil-water mixtures using moderately weathered oil (CTC and Corexit9500). Available endpoints included abnormality, development, shell length, growth (total weight, g), fertilization, edema, atrial arrhythmia, atrioventricular angle, ventricular arrhythmia.
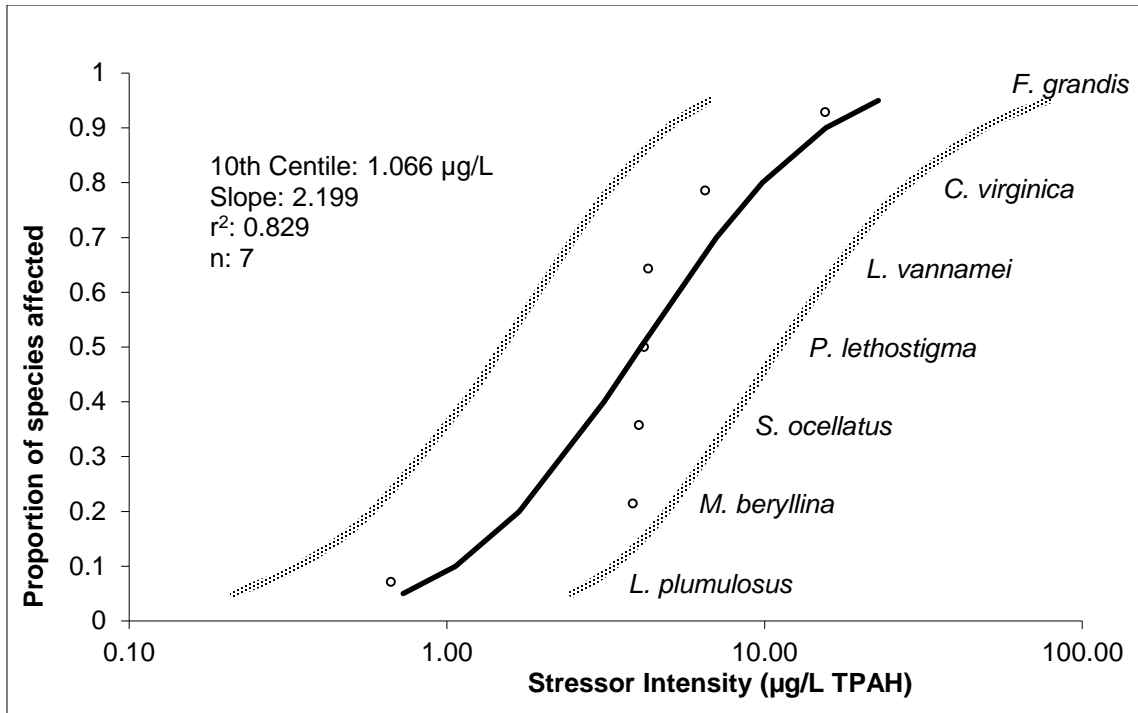
Figure 1-19. Species sensitivity distribution for EC10/20s and IC25s using sublethal endpoints from mechanically mixed oil-water preparations using extremely weathered oil (Juniper). Available endpoints included settlement, growth (total weight, g), gill epithelial proliferation, gill telangiectasis, growth (SL, mm), hepatic vacuolization, hepatic vascular congestion, hepatosomatic index, hatching failure and mortality, atrial arrhythmia, atrioventricular angle, ventricular arrhythmia, estimated average weight gain (pg./day), neonates per adult.

CHAPTER II: AN INITIAL PROBABILISTIC RISK ASSESSMENT OF DEEPWATER HORIZON OILS TO AQUATIC ORGANISMS

ABSTRACT

Probabilistic risk assessment is a practical tool for determining risk to organisms in real-world scenarios. To date, a probabilistic risk assessment using laboratory generated toxicity data and environmental conditions from the Deepwater Horizon (DWH) incident in the Gulf of Mexico has not been conducted. We provide the first probabilistic ecological risk assessment of the DWH incident in four scenarios: initial screening, median lethality, low effect lethality, and sublethal toxicity effects using environmental exposure concentrations collected during the Deepwater Horizon Natural Resource Damage Assessment (NRDA). Risk in all scenarios was low; however, despite using large sources of effect data, risk characterization highlighted a severe lack of effect data and a variety of sources of uncertainty. We provide risk characterization using NOAA and BP NRDA comprehensive toxicity program efforts and recommendations for integration of additional techniques into future risk analysis.

Keywords: Probabilistic risk assessment, Deepwater Horizon, oil effects, NRDA, Gulf of Mexico

INTRODUCTION

Ecological risk assessment (ERA) is one of the tools available in the overall management of risk from stressors/contaminants. The risk assessment process provides a way to organize and analyze data, information, assumptions, and uncertainties to consider the potential for adverse effects (USEPA 1998). The discharge of around $4.4 \times 10^6 \pm 20$

% barrels of Mississippi Canyon Block 252 oil (referred to as MS252 or Macondo - MC-252) into the Gulf of Mexico (GoM) during the Deepwater Horizon incident (DWH) prompted a surge in aquatic toxicology investigations to understand effects of the petroleum products on marine biota. A large number of laboratory toxicity studies with aquatic organisms and DWH oil types have been conducted using traditional acute and chronic toxicity testing methods (Hamdan and Fulmer 2011; Hemmer et al. 2011; Goodbody-Gringley et al. 2013; Rico-Martínez et al. 2013; Echols et al. 2014; Echols et al. 2015a; Faksness et al. 2015; Morris et al. 2015; Echols et al. 2016a; Echols et al. 2016b). However, generating laboratory toxicity data alone does not answer the question: What is the quantity of subsurface oil and threat and potential impact to natural (biota) from the DWH oil incident in the Gulf of Mexico? The latter is clearly one of the stated objectives for the DWH incident that is discussed in a special issue of the National Academy of Sciences (Lubchenco et al. 2012). It is recognized, that aside from accidental oil incidents the GoM has been subjected to a host of other point and non-point sources of pollutants which include but are not limited to: nutrients, contaminants from agriculture and natural geological oil seeps (USEPA 1999).

Probabilistic ecological risk assessment (PRA) is a process to synthesize laboratory aquatic toxicity data in light of realistic environmental field exposures in water of contaminants to estimate the probability of risks from actual exposures. On April 4, 2016, the Federal government approved a settlement with BP Exploration and Production including a $5.5 billion civil penalty and $7.1 billion in Natural Resource Damages, which ended all claims under the Oil Pollution Act and the Clean Water Act (Case No.

10-4536 2016). To-date, a PRA with the aquatic toxicity data from the DWH has not been conducted.

Initial deterministic assessments (i.e., comparisons of point estimates) were discussed in the OSAT 2010 report by comparing analytical chemistry and toxicity data generated using field-collected samples to human health benchmarks, aquatic life benchmarks for PAHs, and dispersant benchmarks. Much of the species-specific toxicity data and field exposure data were unavailable for risk analysis at the time of the OSAT 1 report in December, 2010(OSAT 2010; OSAT 2011). Therefore, the objective of the chapter was to conduct the first screening level aquatic risk assessment to quantify the likelihood and extent that adverse effects will occur from surface water exposures to petroleum products (from Macondo oils) in GoM marine systems using the U.S. Environmental Protection Agency (U.S. EPA) Ecological Risk Assessment (ERA) framework. A probabilistic risk approach (PRA) was used in the aquatic ERA, which compares probability distributions of exposure concentrations and species effects data to determine the magnitude of overlap, which is a measure of risk.

METHODS

Ecological risk assessment consists of three main phases: problem formulation, analysis, and risk characterization (USEPA 1998; Suter 2008). Problem formulation identifies the characteristics of the stressor under investigation, the ecosystems potentially at risk, assessment and measurement endpoints, and expected ecological effects. Assessment endpoints are what is at risk and need of protection while measurement endpoints are what will reveal effects on the assessment endpoint(s). Analysis includes the two main parts of risk: environmental field exposures during the

DWH incident and ecological effects (laboratory toxicity studies). Both of these (exposure and effects data) are combined into the last phase, risk characterization, to determine the probability of adverse effects occurring to all species within the dataset because of exposure to petroleum products in the water.

Data compiled to use in the PRA were gathered from several sources but all were generated in the effort to support the Natural Resource Damage Assessment (NRDA) program directed towards the DWH incident. Data were compiled from the NOAA NRDA report (Morris et al. 2015) and the NRDA effort by the Florida International University (FIU) Ecotoxicology & Risk Assessment Laboratory and our partner institution Oregon State University (OSU). Some of the species-specific aquatic toxicity information generated at FIU and OSU is currently available in the literature and contain data on mysid shrimp, silversides, the Moon Jellyfish, Red Drum, Spotted Sea Trout, Florida Pompano, Cobia, Red Porgy, Blue Crab, Eastern Oyster, Pacific Oyster, Mediterranean Mussel, Quahog Clam, Purple Sea Urchin, and Sand Dollar (Echols et al. 2014; Echols et al. 2015a; Echols et al. 2015b; Echols et al. 2016a; Stefansson et al. 2016; Echols et al. 2016b). Environmental chemistry data were collected from one source, the publically accessible dataset available through the NOAA Data Integration Visualization Exploration and Reporting (DIVER) program and will be compared to the OSAT 2010 report.

In the process of compiling large datasets needed for ERA, there are assumptions that have to be made and decisions on inclusion/exclusion of data where appropriate which lead to uncertainties. These uncertainties can come from the following sources: lack of information, human and analytical errors, natural variation, and flawed model

assumptions (Suter 1993). These uncertainties and how they apply to the work here will be addressed in the discussion. Problem formulation is discussed at the beginning of the results and discussion section. The methods for risk analysis and risk characterization used in this PRA follow below.

Risk Analysis (Exposure Data)

Water column exposure data collected during the DWH incident were obtained from the literature. In this assessment, the publicly accessible datasets available on the NOAA DIVER platform were the sources for water exposure data. The NOAA DIVER is a repository of data related to the DWH incident including photographs, telemetry information, field observations, instrument data, and samples results of laboratory analysis on tissue, sediment, oil, and water. This PRA only considers water exposures of total polycyclic aromatic hydrocarbons (TPAH) in water and does not consider other exposures in aquatic systems that could occur from sediment, oil droplets, dietary ingestion, etc.

The NOAA DIVER dataset can provide petroleum chemistry data in a variety of formats but I was interested in TPAH in water (µg/L). Total polycyclic aromatic hydrocarbons can be reported as ToxPAH50 or CROSS_TPAH. The value ToxPAH50 is a sum of 50 common PAHs present in DWH oils detailed in the DWH Analytical Quality Assurance Plan and is used in fingerprinting, toxicity data analyses, and water column modeling to represent PAH concentrations. The variable CROSS_TPAH is a value generated by an algorithm to produce values from incomplete data to approximate ToxPAH50 from across a variety of datasets that may not have followed the NOAA

DWH Analytical Quality Assurance Plan. When using either of these datasets, it is recommended for non-detect values to be set to zero to prevent overestimating TPAH.

Queries were selected from NOAA DIVER following the guided query path: Environmental Data/Chemistry and Bioassay Results/Calculate Total PAHs/Water for ToxPAH50 and CROSS_TPAH. The datasets returned were very similar with 27762 and 27138 data points (individual water sample TPAH concentration), respectively. AS a result of the ToxPAH50 being specifically tailored for toxicity work and being slightly more robust, it was selected for use in the PRA. One benefit of using this database is that it includes a variety of metadata that can be useful in future work such as sample GPS locations and depth parameters. Centiles are easily generated using data in the "Result" column with TPAH values having units in µg/L which are useful for discussion of exposure, toxicity, and risk.

Environmental exposure data is used in risk characterization as a cumulative distribution and assumes that the data available is representative of all possible samples for the duration of the DWH incident (Hall et al. 1998). TPAH concentrations were ranked in ascending order with values below detection limits being assigned values of zero. Although dummy values are required in the ranking process, exposure values at zero are not included in the regression analysis for exposure (Solomon et al. 1996; Hall et al. 1998; Giddings et al. 2000). Exposure analysis included the determination of the 90th centile which is established as an "exposure benchmark" (Solomon et al. 1996). This benchmark value indicates that any sample taken, would have only a ten percent chance of exceeding the 90th centile concentration because the cumulative distribution accurately represents the DWH incident (Giddings et al. 2000).

Risk Analysis (Effects Data)

Acute and chronic effect data were compiled from three sources. The first is the NRDA Comprehensive Testing Program prepared for NOAA including research conducted by 17 different research groups (Morris et al. 2015). The second and third sources are datasets generated by the BP NRDA comprehensive testing program conducted by the Ecotoxicology & Risk Assessment Laboratory at Florida International University and the Ecotoxicology Group at Oregon State University.

The toxicity data from the NOAA NRDA program are located in attachment 2 of the Morris et al. (2015) report. The FIU toxicity data were compiled from information included in the manuscripts mentioned in the introduction chapter but also data that is currently unpublished. The OSU toxicity data was taken from work by Stefansson et al. (2016). All toxicity data were extracted from these sources and compiled into a table containing the following study information: test ID, laboratory, species, life stage, contaminant, exposure, endpoint, UV light percentage, duration of exposure, EC/IC/LC (point estimate type), value (of point estimate), lower 95% confidence interval (CI), upper 95% CI, high concentration, data source location, test organism phylum. This data table is too large to include within this dissertation but will be made available as supplementary information online with the manuscript when published.

Risk Characterization

Risk characterization for the data used in this chapter was divided into four assessments consistent with appropriate groupings of toxicity effect point estimates. The first characterization, PRA-1, used all toxicity effect data combined. The second characterization, PRA-2, included only toxicity data generated from mortality endpoints

that resulted in LC50 (50% effect lethal concentrations) point estimates. The third characterization, PRA-3, included toxicity data from mortality endpoints but was limited to LC10 and LC20 point estimates to represent the more sensitive lethal concentrations (10 and 20% effect lethal concentrations). The fourth characterization, PRA-4, included only toxicity studies with sublethal endpoints resulting in EC10s (10% effect concentrations), EC20s (20% effect concentrations) or IC25s (25% inhibitory effect concentrations). Sublethal endpoints included: larval settlement; growth (total weight, g); fertilization; shell length; histological or developmental abnormality; gill epithelial proliferation; gill telangiectasis; growth (SL, mm); hepatic vacuolization; hepatic vascular congestion; hepatosomatic index; hatching failure and mortality; atrial arrhythmia; atrioventricular angle; ventricular arrhythmia; edema; heart rate; estimated average weight gain (pg.day); neonates per adult.

Each risk characterization was conducted using the steps proposed by Solomon et al. (1996) and include two sets of data: toxicology data and environmental field exposure data. Probability distributions of the individual four groups of toxicity effect data were thus compared to the probability distribution of the water exposure concentrations (in TPAH) from the entire NOAA DIVER water sample database. Toxicity data were transferred into the Probabilistic Risk Assessment Tool (PRAT-1) worksheet and sorted in ascending order (Solomon et al. 2000). Units of data were checked for consistency. Exposure data were also transferred into the worksheet and sorted in ascending order. Note, zero values from environmental concentrations were not be used in the analysis because of the log transformation. However, they need to be present in the dataset because they are used to determine ranks and therefore probability of all values, and

therefore were not discarded. The worksheet was used to conduct a log transformation of the exposure concentrations and a probit transformation of the toxicity data. A linear regression was applied to the toxicity effect and exposure datasets (not including environmental concentrations reporting zero).

The values generated in the regression analysis were used in user-specified exceedence calculations and output includes an exceedence profile or joint probability curve (JPC). JPCs characterize the relationship between the magnitude of effect and the probability of occurrence for that effect (ECOFRAM 1999). Simply, a point on the JPC represents the probability (percentage) that the water exposure concentration exceeds a toxicity endpoint (e.g., LC50) by x% of the species (Solomon et al. 2000) from a species sensitivity distribution (SSD). The curve is the foundation of PRA because it establishes the proportion of species that will be affected and the frequency or percentage of time for such effects based on available exposure observations. The 10th centile of each toxicity lethal and sublethal effects distribution (i.e., for all data combined, for only LC50s, for LC10/LC20/LC25s, and for EC10/EC20/IC25s) were established as the "effects benchmarks" for each scenario. The 10th centile is widely accepted and used to characterize risk (Solomon et al. 1996; Giesy et al. 1999; Versteeg et al. 1999).

Species sensitivity distributions of species-specific toxicity values separated by oils (e.g., crude oil, weathered oil) and exposure media preparation (e.g., high energy, low energy, mechanically mixed, or chemically dispersed) methods were presented in the previous chapter. Species sensitivity distributions representing the data used in the four PRA groupings were generated following the methods described in Chapter 1 and will be presented in the results along with the corresponding JPCs detailing risk characterization.

When multiple endpoint estimates were available for a single species in the generation of an SSD, the values were averaged to provide a "mean stressor intensity" which is used for the remainder of the risk calculations.

RESULTS AND DISCUSSION

Problem Formulation

After 84 days of oil flowing from the wellhead, the DWH well was sealed on 15 July 2010 allowing approximately 4.4 x 10^6 ± 20% barrels of oil to be released into the GoM (Crone and Tolstoy 2010). Oil is a complex mixture of many compounds and makes reporting and comparing toxicity values complicated. Comparing oil toxicity information is exacerbated even more because recommended methods of oil-water mixture exposure preparations are not always followed throughout the literature. Sandoval et al. provide a thorough overview of preparation methods and corresponding chemical characterizations relevant to MC-252 oils used in toxicity studies (Sandoval et al. 2017). We used TPAH (in µg/L) as the exposure parameter in this risk assessment because it is a commonly reported value in oil toxicity data, and it is available in the environmental exposure databases. Other values considered when discussing oil toxicity may include total petroleum hydrocarbons (TPH), total hydrocarbons, (THC), or total extractable material (TEM) but are not interchangeable. All exposure data and toxicity data in this analysis were used with the units µg/L TPAH.

The study site in question includes the greater GoM. The locations sampled in the NRDA programs are discussed in the OSAT 2010 report. The water column environmental chemistry data available from the NOAA DIVER program includes a shape file that can be loaded into GIS software to see all points of sample collection.

MC252 oil is a light south Louisiana sweet crude oil composed of a mixture of thousands of compounds. The oil is relatively high in alkane concentrations and lower in PAH concentrations than other crude oils indicating a propensity for rapid microbial degradation and lower acute toxicity. MC252 also contains volatile organic compounds that are acutely toxic but they evaporate rapidly and thus are typically a hazard with exposure to fresh oil. For a more comprehensive description of a variety of oils including MC252 oil see the book Oil Spill Science and Technology (Fingas 2016).

Environmental risk assessments typically use the long-term viability of an aquatic community as the assessment endpoint. Long-term viability includes the protection of at least 90% of the species in that ecosystem which corresponds to the 10th centile of the SSDs (Eisler 1998). Measurement endpoints include all toxicity values (acute and chronic, lethal and sublethal) from the data sourced in this study. These databases can be built upon for future work when additional data becomes available.

Risk Analysis

The NOAA DIVER water column TPAH exposure dataset included 27762 data points. The values ranged from zero (below detection level) to 152855 µg/L TPAH (which was a sample of crude source oil). To put the dataset in perspective, a TPAH value of 0.01 µg/L was not reached until the 55th centile of all water sample data points. Some important centiles include the 90th: 0.19 µg/L TPAH, the 95th: 0.63 µg/L TPAH, and the 99th: 8.52 µg/L TPAH. Out of the 27762 analyses, only 61 water samples were > 100 µg/L TPAH (only 0.2%). More centiles can be found in Table 2-1 and are very similar to those found using the data in the OSAT 2010 report. Regression of the log-

transformed environmental exposure data in the preparation of JPCs included n=14331 observations since the difference (13431) are zero (~48% below detection level).

The first probabilistic risk assessment (PRA-1) was an overall screening for risk and includes all species and all toxicity effect endpoint estimates (lethal and sublethal at all effect levels calculated) together. The only filter applied to the toxicity data was to exclude values that were not numerical or listed as out of range (OOR). Application of this filter provided 577 toxicity data points (individual endpoint estimate) with information from 28 species representing five phyla (Arthropoda, Chordata, Cnidaria, Echinodermata, Mollusca). The cumulative frequency distribution (SSD) of these toxicity data is presented in Figure 2-1.The most sensitive organism was the tuna, *Thunnus maccoyii*, with a mean stressor intensity of 1.6 µg/L TPAH while the least sensitive organism was the Moon Jellyfish, *Aurelia aurita*, with a mean stressor intensity of 131 µg/L TPAH. The 10th centile for all species sensitivity toxicity data represented in this dataset was 3.55 µg/L TPAH while the 90th centile of environmental exposure was 0.19 µg/L TPAH.

The second probabilistic risk assessment (PRA-2) investigates risk based only on all the median lethal concentration (LC50s) data. Toxicity data was filtered to exclude any non-numerical or OOR values and include only those using the endpoint "mortality" and reporting LC50s. Application of this filter provided 177 toxicity data points with information from 22 species representing five phyla (Arthropoda, Chordata, Cnidaria, Echinodermata, Mollusca). The cumulative frequency distribution (SSD) of these toxicity data is presented in Figure 2-2. The most sensitive organism was the anchovy, *Anchoa mitchili*, with a mean stressor intensity of 2.5 µg/L TPAH while the least sensitive

organism was the Southern Flounder, *Paralichthys lethostigma*, with a mean stressor intensity of 519 µg/L TPAH. The 10th centile for all species sensitivity toxicity data represented in this dataset was 6.44 µg/L TPAH while the 90th centile of environmental exposure was 0.19 µg/L TPAH.

The third probabilistic risk assessment (PRA-3) investigates risk using low percentage lethality point estimates (LC10s, LC20s, and LC25s). Toxicity data was filtered to exclude any non-numerical or OOR values and include only those using the endpoint "mortality" and reporting LC10, LC20 or LC25. Application of this filter provided 196 toxicity data points from 17 species representing three phyla (Arthropoda, Chordata, Mollusca). The cumulative frequency distribution (SSD) of these toxicity data is presented in Figure 2-3. The most sensitive organism was the tuna, *Thunnus albacares*, with a mean stressor intensity of 1.6 µg/L TPAH while the least sensitive organism was the Southern Flounder, *P. lethostigma*, with a mean stressor intensity of 189 µg/L TPAH. The 10th centile for all species sensitivity toxicity data represented in this dataset was 2.55 µg/L TPAH while the 90th centile of environmental exposure was 0.19 µg/L TPAH.

The fourth probabilistic risk assessment (PRA-4) investigates risk using sublethal endpoints which report low percentage effect or inhibition point estimates. Toxicity data was filtered to exclude any non-numerical or OOR values or studies using the endpoint mortality and include only those using sublethal effects reporting EC10, EC20, or IC25. Application of this filter provided 121 toxicity data points from 18 species representing four phyla (Arthropoda, Chordata, Echinodermata, Mollusca). The cumulative frequency distribution (SSD) of these toxicity data is presented in Figure 2-4. The most sensitive organism was the amphipod, *Leptocheirus plumulosus*, with a mean stressor intensity of

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0.67 µg/L TPAH while the least sensitive organism was the urchin, *Strongylocentrotus purpuratus*, with a mean stressor intensity of 55 µg/L TPAH. The 10th centile for all species toxicity data represented in this dataset was 1.56 µg/L TPAH while the 90th centile of environmental exposure was 0.19 µg/L TPAH.

Risk Characterization

Cumulative frequency distributions of exposure and toxicity effect data used in PRA-1 are presented in Figure 2-5. The overlap of these distributions represent risk. The two frequency distributions were combined to create a JPC. The JPC generated in PRA-1 using all available toxicity endpoints is plotted in Figure 2-6. Based on this JPC, ten percent of the species would be affected at concentrations ≤ 2.015 µg/L TPAH but this would be observed only 3.3% of the time. The species at risk below this threshold on the basis of their mean stressor intensity included the southern Bluefin Tuna (*T. maccoyii*). This is relatively low risk but further analysis is warranted to see if grouping appropriately similar toxicity data can highlight what kind of effects are at risk.

Cumulative frequency distributions of exposure and toxicity effect data used in PRA-2 are presented in Figure 2-7. The overlap of these distributions represent risk. The two frequency distributions were combined to create a JPC. The JPC generated in PRA-2 using only acute toxicity median lethality endpoints (mortality; LC50s) is plotted in Figure 2-8. Based on this JPC, ten percent of the species would be affected at concentrations ≤ 3.73 µg/L TPAH but this would be observed only 2.2% of the time. Species at risk below this threshold on the basis of their mean stressor intensity included the anchovy (*A. mitchili*) and the fiddler crab (*Uca* spp.).

Cumulative frequency distributions of exposure and toxicity effect data used in PRA-3 are presented in Figure 2-9. The overlap of these distributions represent risk. The two frequency distributions were combined to create a JPC. The JPC generated in PRA-3 using only low percentage response acute toxicity endpoints (mortality; LC10s, LC20s, and LC25s) is plotted in Figure 2-10. Based on this JPC, ten percent of the species would be affected at concentrations ≤ 1.958 µg/L TPAH but this would be observed only 3.3% of the time. Species at risk below this threshold on the basis of their mean stressor intensity included the tuna (*T. ablacares*) and the fiddler crab (*Uca* spp.).

Cumulative frequency distributions of exposure and toxicity effect data used in PRA-4 are presented in Figure 2-11. The overlap of these distributions represent risk. The two frequency distributions were combined to create a JPC. The JPC generated in PRA-4 using only sublethal toxicity endpoints (any sublethal endpoint reporting EC10, EC20, or IC25) is plotted in Figure 2-12. Based on this JPC, ten percent of the species would be affected at concentrations ≤ 0.799 µg/L TPAH but this would be observed only 5.7% of the time. Species at risk below this threshold on the basis of their mean stressor intensity included the amphipod (*L. plumulosus*).

All four PRAs demonstrated that there was relatively minimal risk on the basis of the effects and water exposure data used. However, this is a conservative approach and further analysis is warranted before making strong conclusions. The 10% or 5% thresholds (HC10 or HC5) are common values used by risk managers to "protect" communities represented in the SSDs (Smrchek and Zeeman 1998; Hall et al. 1999; Schuler et al. 2008; Rand et al. 2010). The species considered at risk below the 10th centile benchmarks in these PRAs included the southern Bluefin tuna (*T. maccoyii*), the

anchovy (*A. mitchili*) the fiddler crab (*Uca* spp.), the yellowfin tuna (*T. ablacares*), and the amphipod (*L. plumulosus*). The southern Bluefin tuna is currently classified as critically endangered on the IUCN Red List of Threatened species making this result from the PRA a cause for concern (Collette et al. 2011). The anchovy is a critically important fish in estuarine and coastal food webs as it links planktonic production to a variety of fisheries (Nizinski and Munroe 2002). Fiddler crabs are important to salt-marsh communities for keeping the marsh clean and helping them grow as a dominant detritovore and are important prey items to herons, egrets, and raccoons (Thomas and Blum 2010). The yellowfin tuna is listed as "near threatened" on the IUCN Red List as stock catch rates are declining (Collette et al. 2011). The amphipod (*L. plumulosus*) is a standard sediment toxicity testing organism that lives in open tubes and is a sensitive indicator species (USEPA 1994; Chapman and Wang 2001). That being said, the data presented here should not be used to discuss protection because no observed effect level (NOEC/NOEL) cumulative distributions are recommended for that analysis (Van Straalen 2002). A critically endangered species and commercially important species being at risk at considerably low concentrations demonstrates the need for more toxicity data to conduct more thorough risk analyses.

Uncertainties

Uncertainties from environmental exposure information are influenced by the quantity and quality of environmental data available. The user has to assume that the sampling program conducted during the DWH incident was as inclusive as possible and accurately and thoroughly describes the exposures present during the incident. Keep in mind that the PRA developed here is only evaluating water exposure risks and it does not

include sediment exposure toxicity scenarios which may be potentially a long-term reservoir source for water exposures, especially in littoral zones where breeding and early juvenile growth in nursery habitats may occur. This PRA considers only water exposures of TPAHs to organisms of the soluble components. There is no consideration given to oil droplet or oil-contaminated dietary exposures to aquatic organisms.

Fate parameters that have considerable impact on exposure, such as physical and chemical properties, dilution, flushing time, stratification, sediment interaction, bioavailability, biotransformation, and bioaccumulation have not been considered in this initial PRA. For example, biodegradation of oil in the environment by marine bacteria is a complex process and influenced by a variety of factors (Beyer et al. 2016). It has been documented that bacterial rates of degradation of MC252 oils were higher than expected in deep GoM waters, which will have an influence on bioavailable components of oil responsible for effects (Hazen et al. 2010). Photochemical degradation and is an important factor involving sunlight (UV) exposure to floating oil influencing toxicity parameters (Neff 1999; Shemer and Linden 2007; Beyer et al. 2016). UV light may facilitate photo-degradation processes but may also cause photoenhanced toxicity to some components of oils. The fate of oil in nearshore habitats is a complex process that is influenced by meteorological factors, coastal erosion, tidal fluctuations, and shoreline movement. This PRA does not incorporate fate variables, which will have considerable influence on toxicity effects. The practice of incorporating fate modelling into risk assessment may help take account for confounding factors (Jager et al. 2006; Ashauer and Escher 2010; Jager 2011; De Laender et al. 2015).

Unfortunately, there is a considerable amount of uncertainty concerning petroleum product effects data. As mentioned by several authors, the literature contains toxicity studies that did not follow recommended guidelines (such as Chemical Response to Oil Spills: Ecological Research Forum [CROSERF]) which present methods for preparing oil-water mixtures for exposures and conducting petroleum product toxicity studies (Coelho et al. 2013; Sandoval et al. 2017). Data from non-standard methods will have toxicity endpoints calculated from chemical exposures that were not properly identified, characterized, or measured. Therefore, for those studies, it is likely that organisms were exposed to unstable test media exposure solutions because they were emulsions and the exposures were not consistent over time. There was most likely significant variability in exposure concentrations, which makes it difficult to define cause-effect relationships and interpretation the data.

Another uncertainty factor is the variety of types of oils and oil-water mixture preparations used for exposure media. Comprehensive toxicity programs contained three groups of oils (source, moderately weathered, and severely weathered) and they were either mixed with water mechanically or chemically dispersed. The environmental exposure sample chemistry dataset is not separated into these categories. Close analysis of each water sample's oil component composition could potentially identify the source or category of exposure (relationship to oil type and presence or absence of dispersant). However, this would take considerable time to modify each the 27762 analyses to indicate what type of exposure it resembles and then link it to corresponding effect data. As a result of the complexity of oil-water mixtures, it is unlikely that acute exposure endpoint estimates (LC50s) derived from TPAHs from (1) a source oil-water mixture

mechanically mixed and (2) a chemically dispersed mixed sample would yield similar point estimates. The inability to separate environmental exposure data is one reason why laboratory exposure data was grouped together in these PRA scenarios.

Reproducibility of exposure/response relationships is a foundation of ecotoxicology and critically important to toxicity testing with petroleum products (NRC 1989; Singer et al. 2000; Tsvetnenko and Evans 2002; Barron and Ka'aihue 2003; Gonçalves et al. 2008; Hemmer et al. 2011; Echols et al. 2014). Unfortunately, a large portion of toxicity effect data is reported without demonstrating replication of results. An example of establishing acceptable toxicity endpoint estimate reproducibility can be taken from the laboratory accreditation (e.g., NELAP) process. Ecotoxicology and analytical chemistry testing techniques use intra- and inter-laboratory variation using round robin testing to establish an acceptable variability in toxicity studies to demonstrate the capability for generating quality data (BSAB 1994). A single or a few tests with an organism at one institution conducted by one investigator does not establish a reproducible exposure/response relationship for an exposure/organism scenario with a complex mixture like oil. Generating reproducible studies is difficult especially when working with nonstandard native species but it is necessary to generate quality data. The effect data presented in these PRAs were included from published manuscripts without consideration of sample size.

Exposure duration in toxicity testing is another parameter that causes uncertainty in the effects and risk assessment. For example, over the last 50 years most fish and invertebrate acute lethality studies have been are conducted for 96 hours, however, acute toxicity test results were reported with native species which varied from 12-96 hours. In

addition, bivalve and echinoderm lethality studies were either 48 or 72 hours in duration and also use fertilization success as an endpoint. Different exposure durations and effect endpoints generally should not be mixed in an ERA, but sometimes when data are lacking this is incorporated into the assessment.

The quantity of the effect data is also a limitation in PRA. For example, the present data set contains toxicity information for 14 species of fish. However, an identification guide to fishes of the Gulf of Mexico, Texas, Louisiana, and adjacent waters identify over 550 species of fish (Hoese and More 1998). Species sensitivity distributions generated from single-species studies are assumed to be both representative of the study site and appropriate surrogates for predicting community or population level effects but this has little confirmation (van Leeuwen 1990; Forbes and Forbes 1993). However, Veersteeg et al. showed that mean ecosystem NOEC concentrations were found to adversely affect 10-52% of species from single-species no-effect level data (copper) verifying that the 10th centile effects benchmark is protective for the communities/population of a model ecosystem (Versteeg et al. 1999). The PRA1-4 included 28, 22, 17, and 18 species respectively. With a dataset as large as this is, these species numbers seem low however, it must be pointed out that the data generated in toxicity studies is frequently unusable. For example, toxicity studies using weathered oil (CTC, Slick A, or Juniper, Slick B) frequently result in LC50 values of >100% dilution factor. Although this study provides important information, this value cannot be included in an SSD or risk assessment because it is not a numerical value. This emphasizes the importance of detecting sublethal effects, which will be more likely to return a numerical value that can be incorporated into the risk process.

Another source of uncertainty is not considering the potential of enhanced effects associated with dispersant use in the field. The risk characterization only evaluated TPAHs in water samples (field) without concern for presence/absence of dispersants. Many environmental analyses included detection of indicators for dispersant usage. The two most common indicators included dipropylene glycol n-butyl ether (DPnB) and dioctyl sodium sulfosuccinate (DOSS) (National Research Council (NRC) 2013). The interaction with the dispersant and the oils is important because it facilitates the dissolving of oil components into the water. The dissolving renders them more bioavailabile and some studies have shown that organisms are more sensitive to mixtures including oil and dispersant (Echols et al. 2015a; Echols et al. 2016a).

Further risk characterization should consider the effect endpoints being grouped. Ideally, the effects data of a PRA would include matching acute and chronic data for the species used so that acute to chronic effect ratios can be determined. Not all species in this dataset have both types of data. A more robust dataset will improve the risk assessment. Sediment exposure and sediment toxicity information are available and should be considered for use in the risk process.

CONCLUSIONS AND RECCOMENDATIONS

Risk was briefly discussed in the OSAT (2010) report but only included deterministic human health benchmarks, aquatic life benchmarks for PAHs, and dispersant benchmarks. For example, OSAT found benzene in 20 deep water samples that exceeded conservative cancer risk guidelines recommended by EPA for state water quality criteria. However, there is currently no probabilistic risk assessment for aquatic organisms conducted with toxicity data generated from DWH oils and compared to a

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database of TPAH concentrations from field-collected water samples from during the DWH incident. Our paper provides the first DWH PRA for aquatic organisms using toxicity data compiled from a variety of sources and using the publicly available water chemistry dataset from NOAA DIVER.

All four PRAs resulted in low risk estimates based on TPAH exposures from water samples from the GoM (10% of species would be effected at 0.78-3.73 µg/L TPAH, 2.2-5.7% of the time with 90% of the environmental concentrations being ≤ 0.274 µg/L TPAH). The highest risk was found using sublethal toxicity data but the most and least sensitive organisms were not consistent through all analyses. The risk analysis here is a conservative screening level evaluation of risk. When more information is available and the filtering of data used is more specific to organisms or particular exposure regimes, these risk assessments will change. One advantage to the PRA method is that new data can easily be added to an established dataset to generate an updated risk characterization.

Marine organisms frequently have complicated life histories. For example, adult Blue Crab live and mate in lower salinity waters but release young crab zoea into higher salinity water where they feed on planktonic communities while maturing into juvenile crabs and finally recruiting back into lower salinity waters (Millikin and Williams 1984). With this compicated life cycle, a Blue Crab could have potentially been exposed to a variety of petroleum scenarios. Adults most likely were exposed to more weathered oils in the low salinity estuarine marsh areas while zoea crab had a higher probability of encountering less weathered oils and source oils while suspended in higher salinity

waters in the GoM. We recommend a possible solution to model complex exposure scenarios for Blue Crab and other organisms.

Several groups worked on mapping the plume of oil and dispersed-oil throughout the GoM during the DWH incident (Camilli et al. 2010; Crone and Tolstoy 2010; Hollander et al. 2010; Liu et al. 2011; Zhang et al. 2011). Surveys and sediment samples detail where oil washed up onto the shores and in marsh or mangrove areas (DWH-NRDA 2015; Morris et al. 2015; Beyer et al. 2016; Franco 2016). Oil location mapping along with environmental sample information including GPS and depth locations could be used to generate a 3D model of petroleum component exposures. An organism habitat/distribution map (some are currently available from the United States Geological Survey) could be overlaid on a 3D exposure model to pinpoint exactly where in its movement the Blue Crab (or any other organism) may come into contact with oil plumes, slicks, or washed up oil on beaches/mangrove habitats. This would be a practical tool to use in identifying risk to specific organisms and pinpoint it to life stages, locations, and provide more detailed information to the risk assessor.

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Table 2-1. Examples of TPAH centiles from field samples.

Field Collected Centiles [a]	TPAH (µg/L) [tot=50]
50	(BDL)
55	0.01
70	0.03
75	0.04
80	0.06
90	0.19
95	0.63
96	0.96
97	1.61
98	3.42
99	8.52

[a] ToxPAH50 water analysis data from NOAA DIVER database collected 08/17/2017, n=27762.
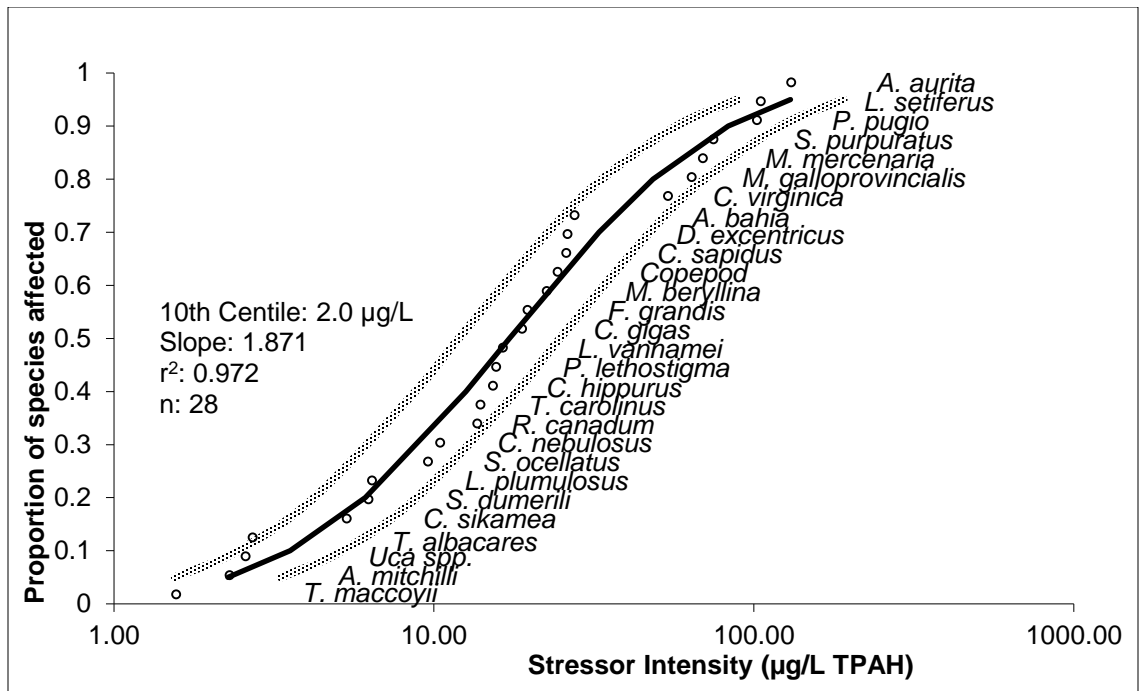
Figure 2-1. Species sensitivity distribution for all toxicity data (lethal and sublethal) used in PRA-1.
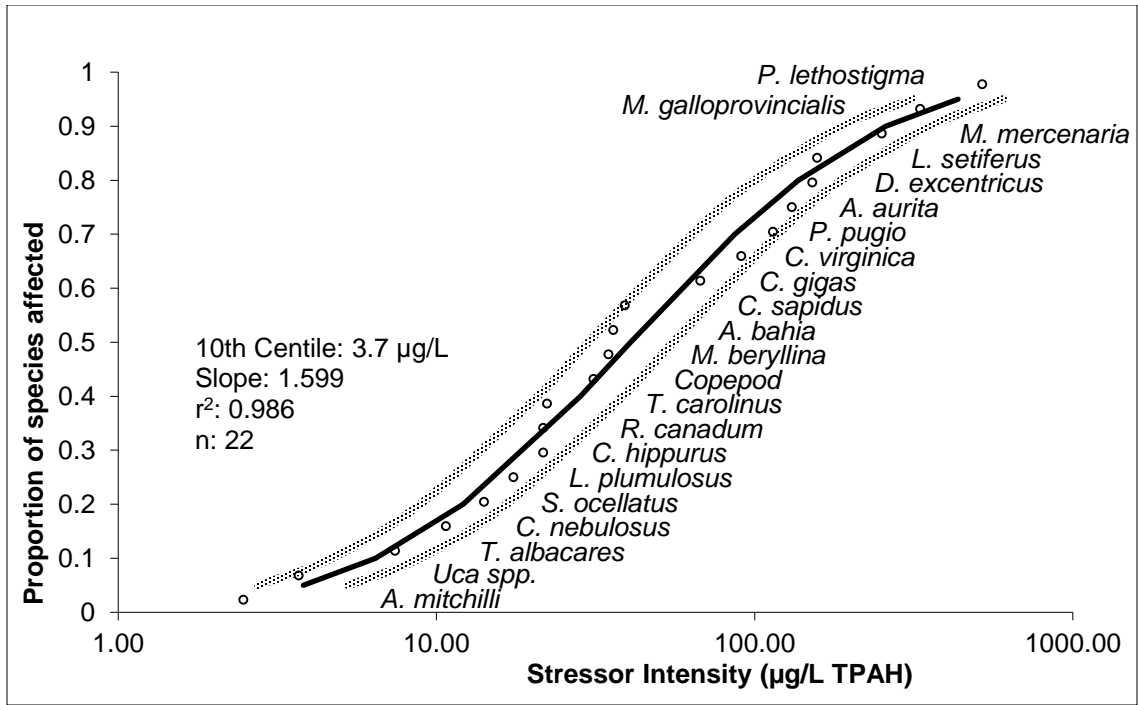
Figure 2-2. Species sensitivity distribution for acute toxicity data reporting LC50s used in PRA-2.
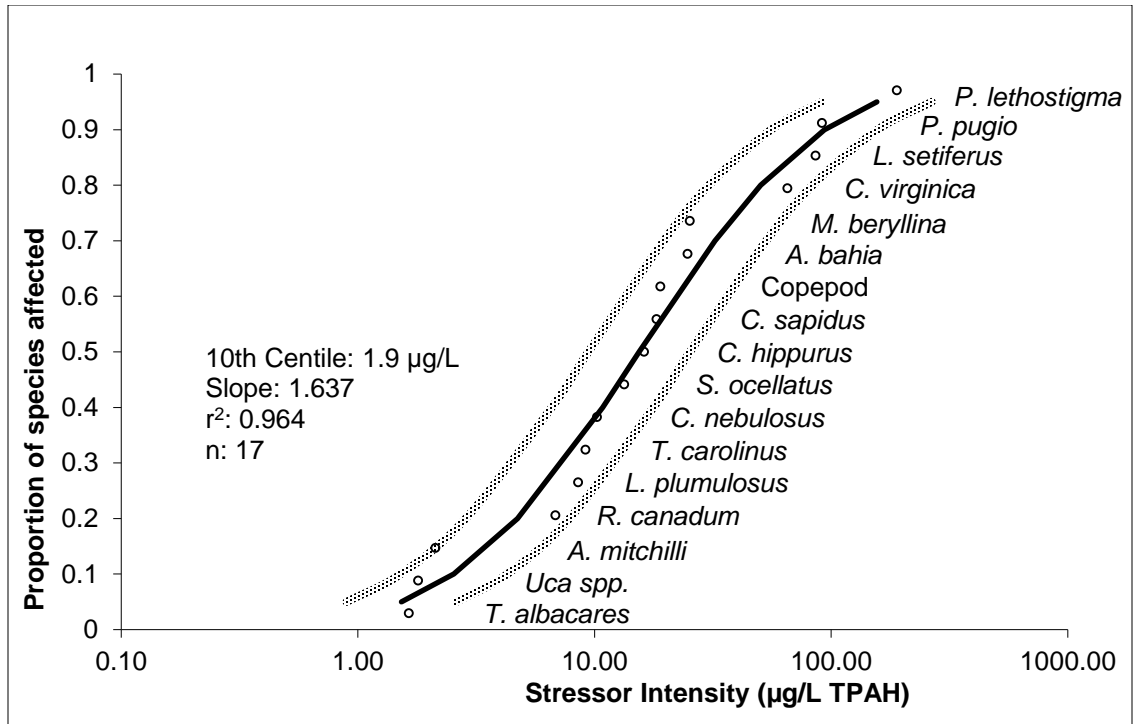
Figure 2-3. Species sensitivity distribution for toxicity lethal data reporting LC10, LC20, or LC25 used in PRA-3.
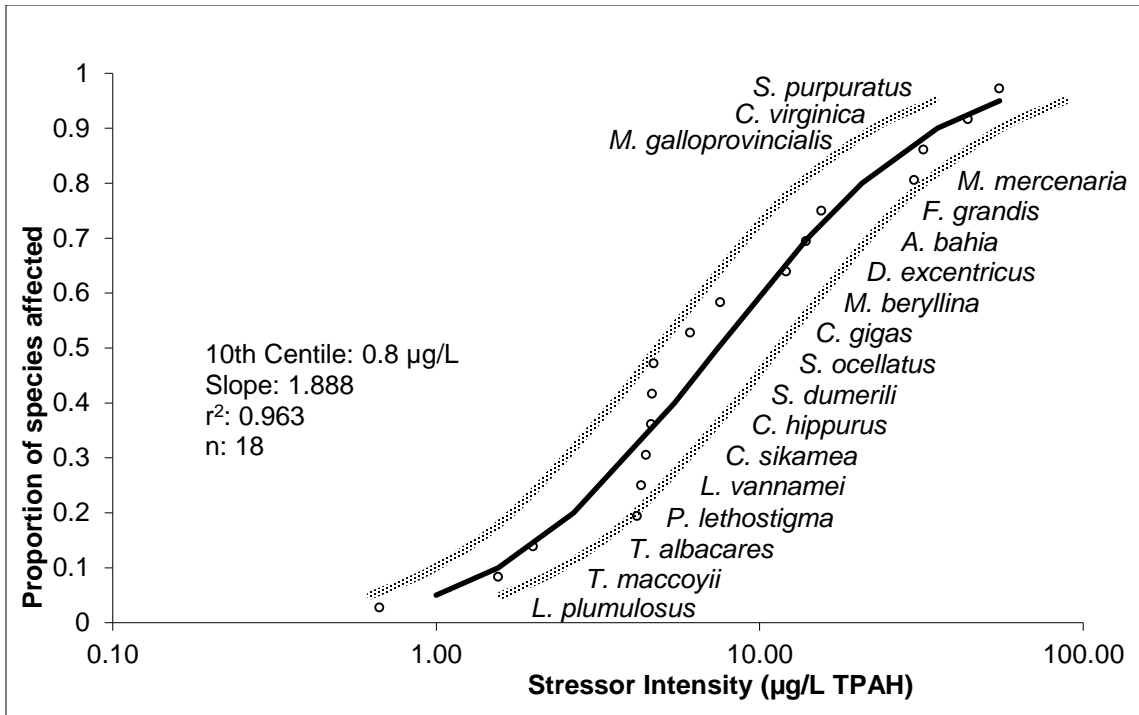
Figure 2-4. Species sensitivity distribution for sublethal toxicity data reporting EC10, EC20, or IC25 used in PRA-4.
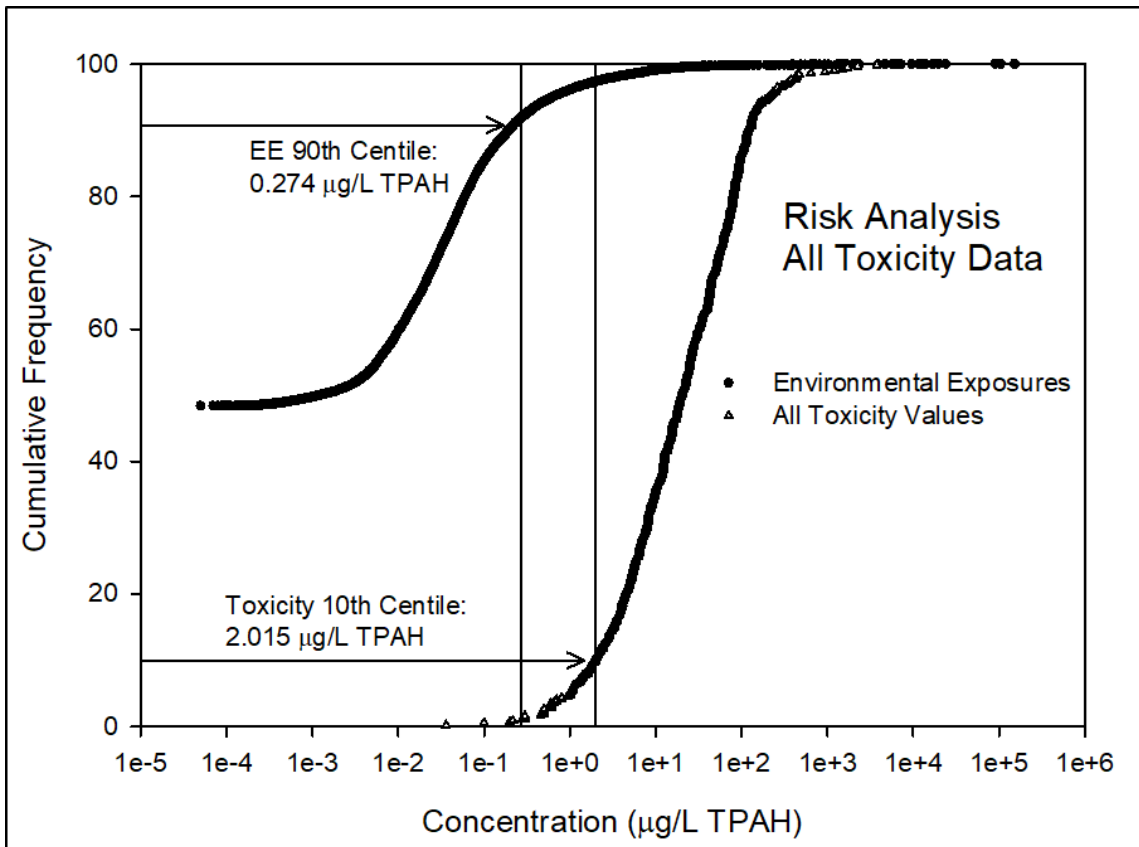
Figure 2-5. Cumulative log-normal distributions of environmental exposures and all toxicity values used in PRA-1.
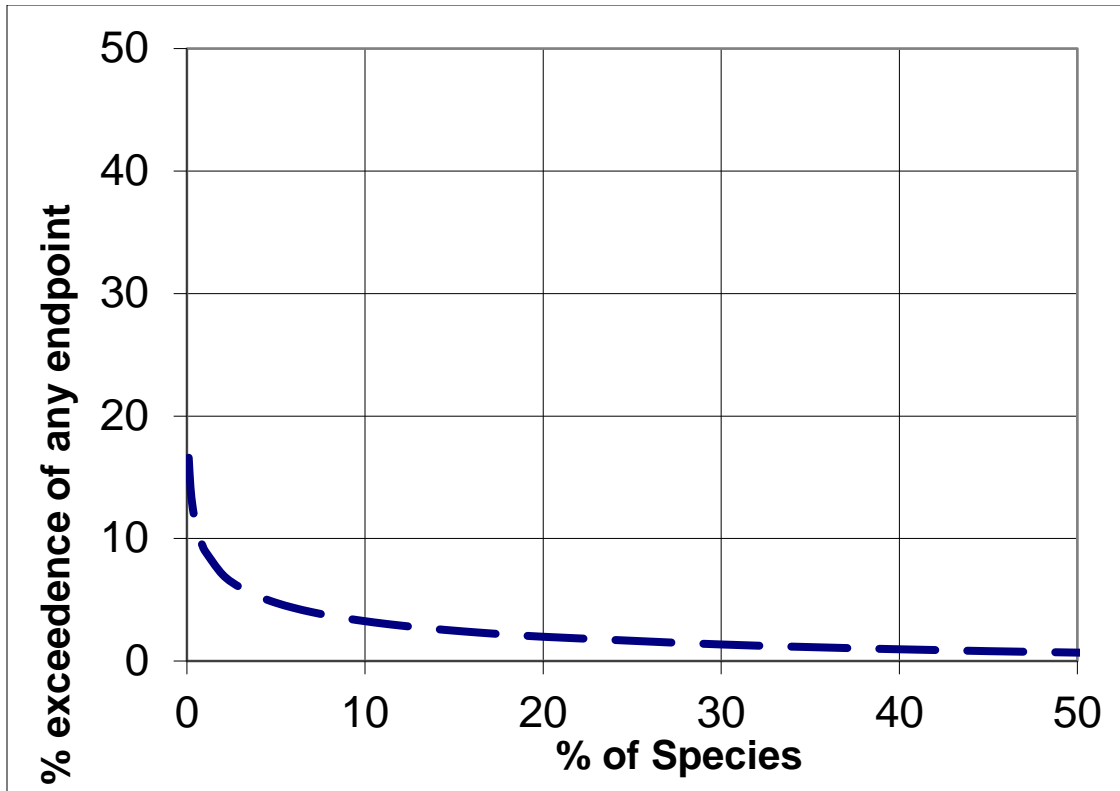
Figure 2-6. Joint probability curve from probabilistic risk assessment-1 using all toxicity information (acute and chronic, lethal and sublethal) for a screening of risk.
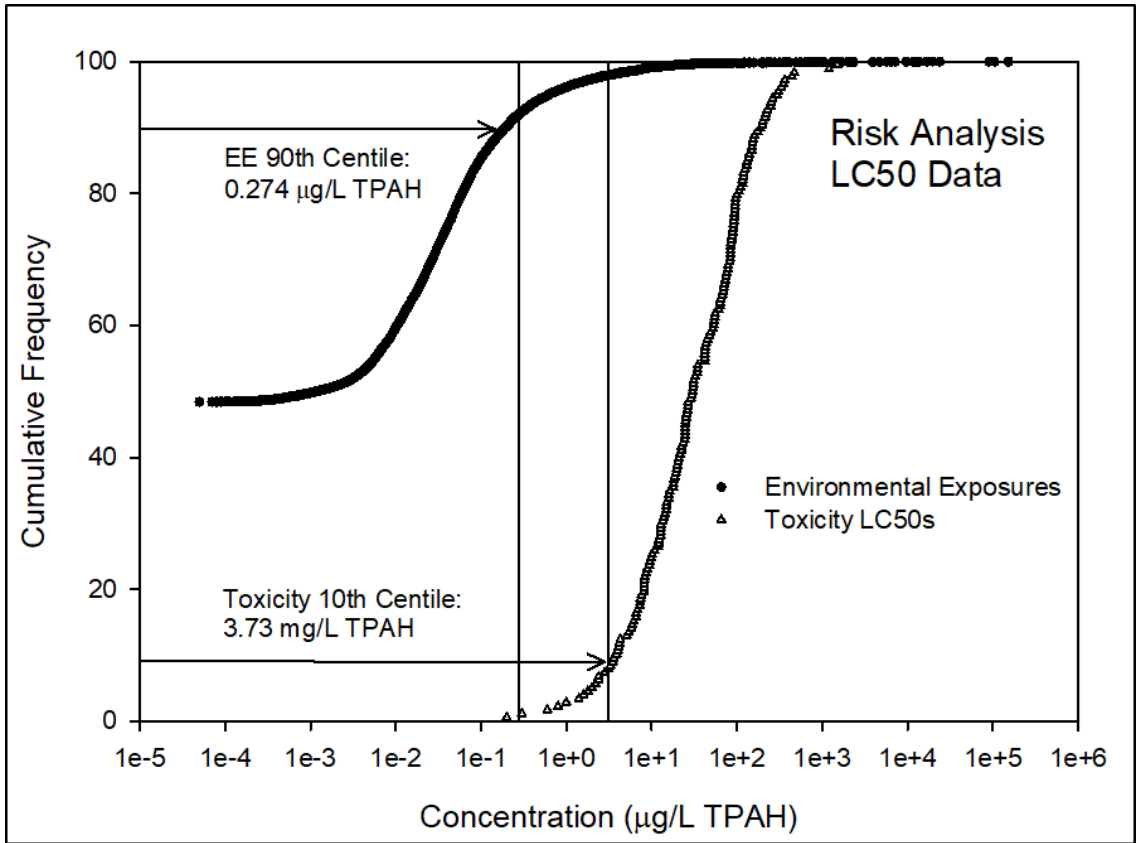
Figure 2-7. Cumulative log-normal distributions of environmental exposures and median lethal concentration toxicity values (LC50s) used in PRA-2.
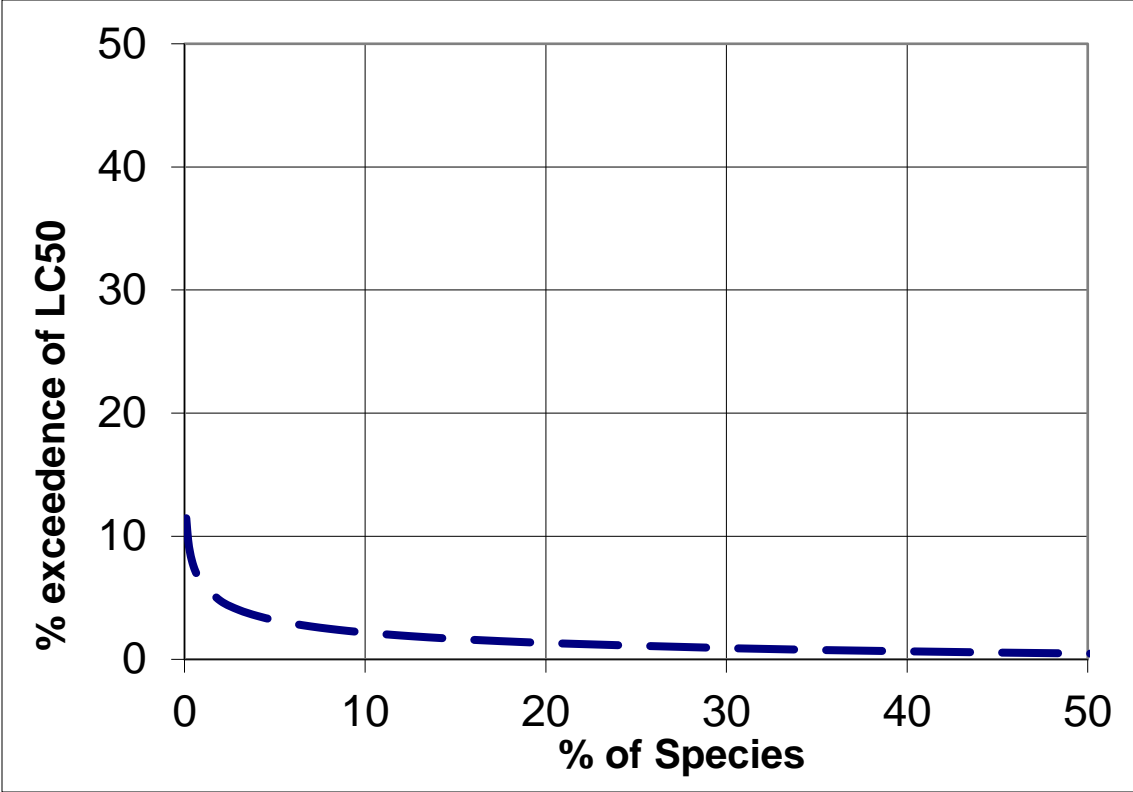
Figure 2-8. Joint probability curve for probabilistic risk assessment-2 using acute median mortality as an endpoint (only LC50s).
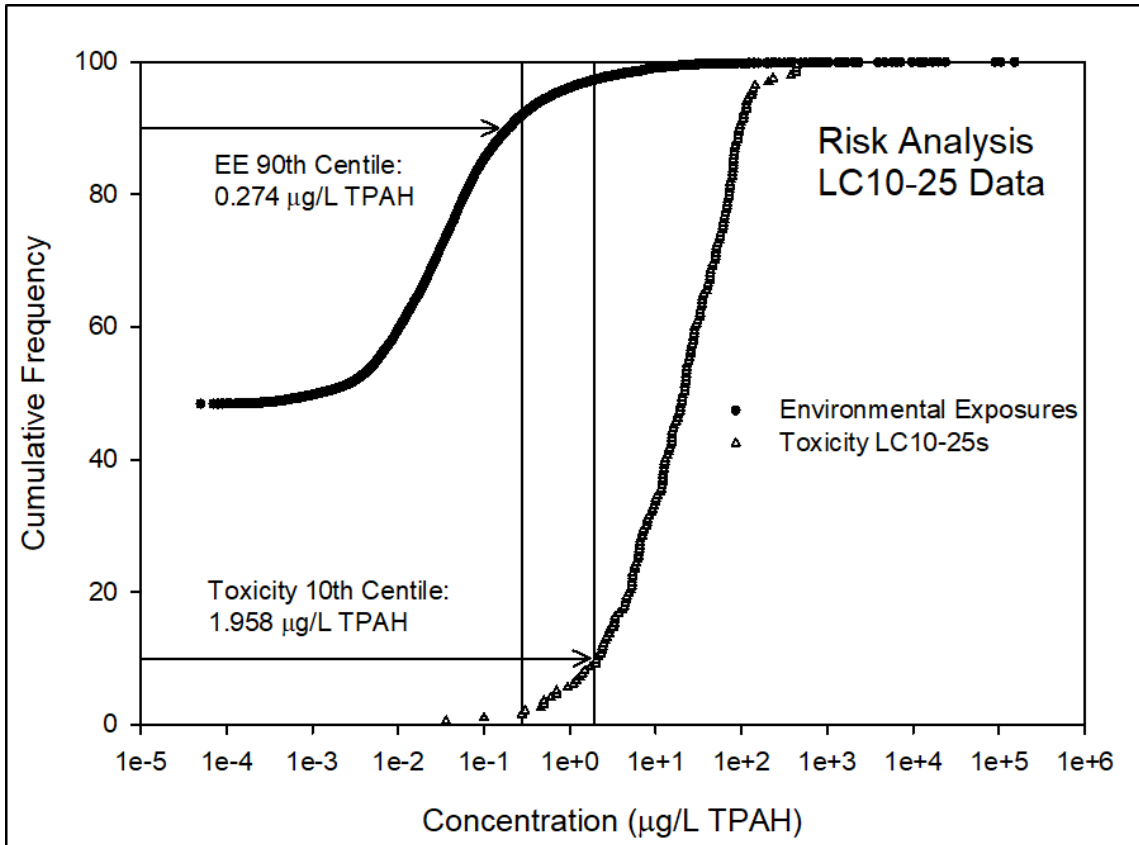
Figure 2-9. Cumulative log-normal distributions of environmental exposures and low percentage lethal response toxicity values (LC10-25s) used in PRA-3.
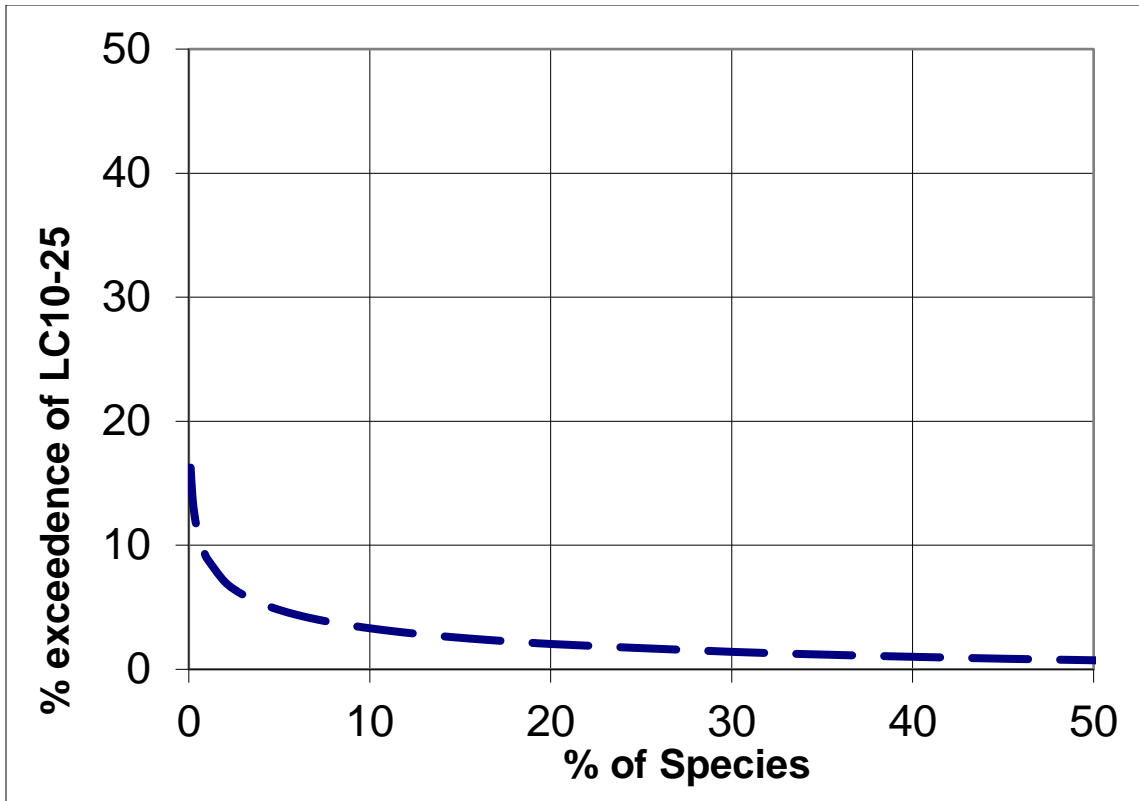
Figure 2-10. Joint probability curve for probabilistic risk assessment-3 using mortality as an endpoint (only LC10, LC20, LC25).
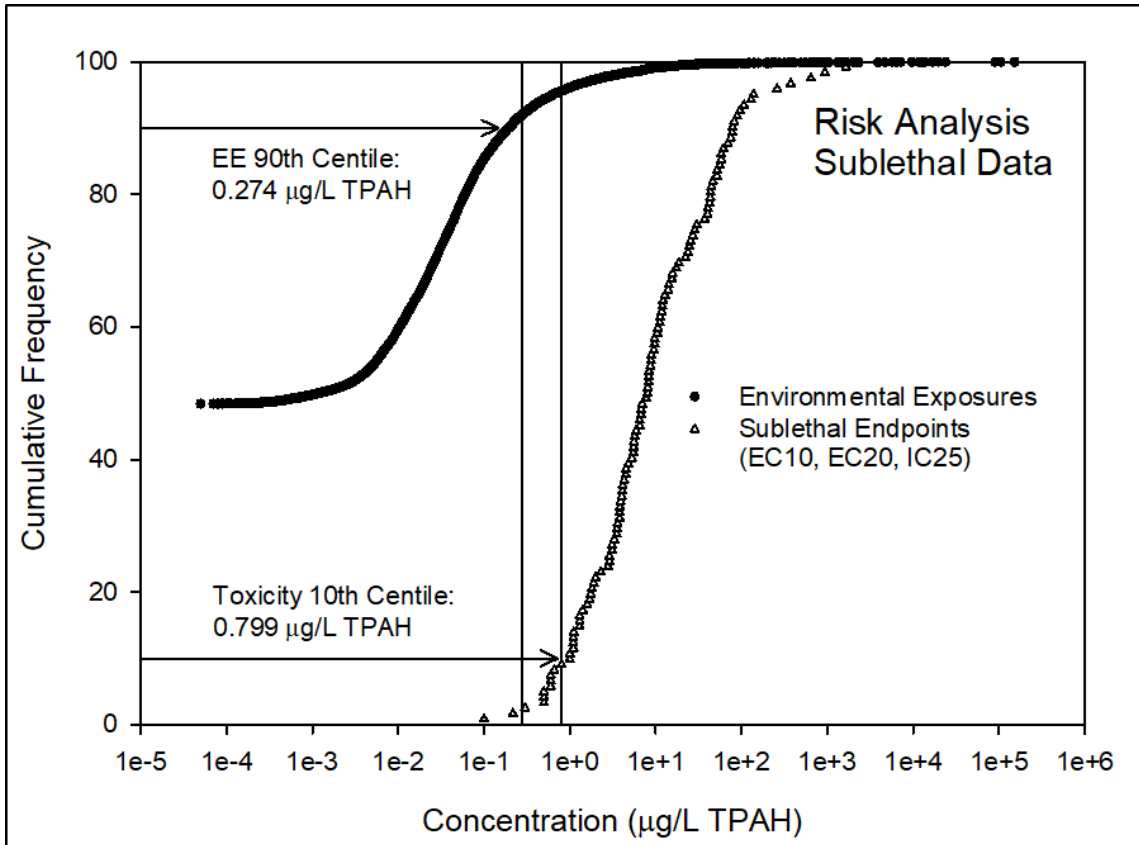
Figure 2-11. Cumulative log-normal distributions of environmental exposures and sublethal endpoint toxicity values (EC10s, EC20s, IC25s)) used in PRA-4.
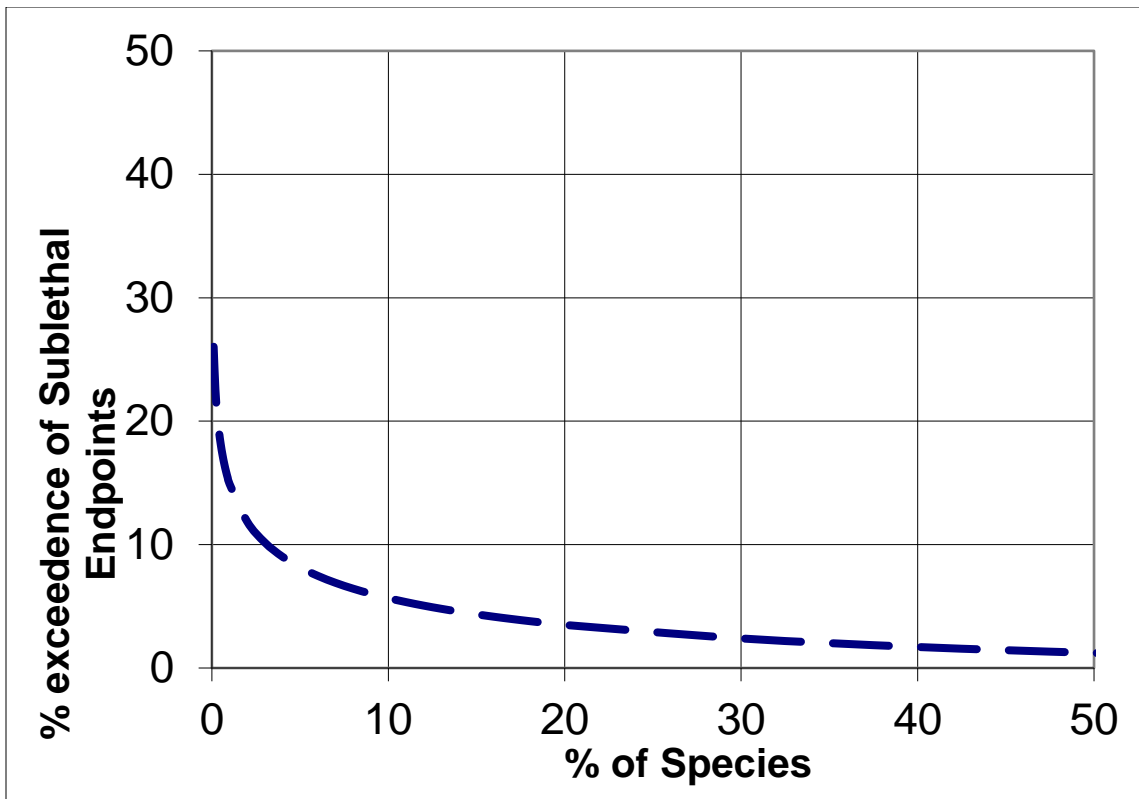
Figure 2-12. Joint probability curve for probabilistic risk assessment-4 using all available sublethal endpoints (EC10s, EC20s, IC25s).

CHAPTER III: LABORATORY CULTURE, REPRODUCTION AND ACUTE TOXICITY STUDIES USING CADMIUM AND COPPER WITH THE STARLET ANEMONE, *NEMATOSTELLA VECTENSIS*.

ABSTRACT

Standard organisms outlined in U.S. Environmental Protection Agency guidelines provide a starting point to understand chemical impacts to organisms in the environment. To fully understand a chemical's impact to the environment, the use of native species is needed; however, the demonstration of successfully reproducible toxicity studies using non-standard aquatic organisms is necessary. Research on effects of chemical stressors in estuarine and marine environments has been far less than those of freshwater. The Starlet Anemone, *Nematostella vectensis*, is an estuarine anemone found in fine sediments of salt marshes of North America and Europe. Animal husbandry, laboratory culture, and induced reproduction with standard laboratory equipment techniques are outlined. Juvenile anemones were used in 96 hour acute toxicity studies exposed to cadmium and copper. Sensitivity to cadmium and copper is demonstrated using species sensitivity distributions where the anemone is below the 10th centile (effects benchmark) for copper. These and other attributes make, *N. vectensis* an excellent candidate for laboratory toxicity studies.

INTRODUCTION

The Starlet Anemone, *Nematostella vectensis*, is a brackish-water cnidarian found upright in fine soft muds where it burrows in salt marsh areas of North America and Europe. Populations have been reported from the East and South coasts of the United

Kingdom; Nova Scotia, Canada; California, Oregon, Washington, Massachusetts, Rhode

Island, New York, Delaware, the Chesapeake Bay, and Florida (Williams 1983).

Nematostella vectensis feeds mainly on small copepods, midge larvae, corixids, and

gastropods (Frank and Bleakney 1978). Reproduction occurs in the summer and autumn

producing non-planktonic ciliated planulae larvae (Frank and Bleakney 1976). Most

larvae settle and metamorphose into a juvenile anemone around seven days of age and by

day ten have started growing tentacles allowing them to feed with the earliest age of

sexual maturity reported at 69 days post fertilization (Hand and Uhlinger 1991; Hand and

Uhlinger 1992). Early embryonic stage development in this species has been well-defined

(Fritzenwanker et al. 2007; Genikhovich and Technau 2009). The uniqueness of *N.*

vectensis makes it amenable for use in aquatic toxicology studies at different stages of the

entire life cycle to determine differences in sensitivities to chemical stressors.

Cnidarians are ecologically important organisms that serve as reef builders and as

predators and prey in planktonic and benthic ecosystems serving as a conduit of energy

from the plankton to the benthos (Sebens 1981; Gili and Coma 1998; Harborne et al.

2006). Several cnidarian species including hydra, anemones, and corals have been used to

demonstrate sensitivity to environmental stressors and changes in water quality (Fleury et

al. 2000; Arkhipchuk et al. 2006; Castillo et al. 2006). In general, changes in benthic

macroinvertebrate communities have been used for over 25 years to demonstrate

deteriorating environmental conditions in aquatic ecosystems including estuaries

(Rosenberg and Resh 1993; Loeb and Spacie 1994; Wildsmith et al. 2011).

As a result of the large geographic range of *N. vectensis*, it could be used as an

indicator of ecosystem health. In fact, because they are sessile, until conditions are

physically unstable, they may be valuable to monitor and assess site-specific chemical changes *in situ* for acute as well as for long-term chronic exposures. Because benthic macroinvertebrates can be diverse with varying sensitivities, toxicity data for this organism would also be important in the ecological risk assessment process (USEPA 1998). The Starlet Anemone has been developed as a model organism for use in developmental, molecular, and evolutionary ecology (Darling et al. 2005; Putnam et al. 2007). This anemone has been praised for its exceptional value in studying embryogenesis and larval development, convenience in laboratory procedures, and easy access to natural populations (Darling et al. 2005). The unique cnidarian life cycle and the ability to culture the anemone throughout the entire life cycle provides the opportunity to look at toxicological impacts on a variety of growth patterns and morphological transitional processes. Another benefit is the availability of a draft genome accessible online (Putnam et al. 2007) with multiple genetic resources from other fields of research (Gilmore et al. 2013; Tarrant et al. 2014). As a result of the ease of culturing and its large distribution, *N. vectensis* is considered an excellent candidate for acute and chronic aquatic toxicity studies to determine the biological effects of pollutants (Darling et al. 2005; Harter and Matthews 2005). The anemone has application for sediment and water-only exposure toxicity studies. As a member of salt marsh communities, *N. vectensis* is frequently influenced by anthropogenic impacts and would be useful in assessing impacts to estuarine ecosystems. Harter and Matthews (2005) conducted preliminary work on the feasibility of using *N. vectensis* for acute and chronic toxicity studies with cadmium chloride. In Harter and Matthews' (2005) acute studies (96 h) using 3–7 month old *N. vectensis* mean lethal concentration (LC50) values were

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comparable to sensitive marine and estuarine amphipods. Chronic biological endpoints included anemone weight and daily egg counts. Increased cadmium exposure was linked to a decrease in anemone weight while causing increased reproductive frequency and inhibited total egg production.

The use of anemones, especially *N. vectensis*, may be relevant in evaluating the biological impact of materials like metals considering that metals not only dissolve in water but they also reside bound in the surficial sediment of different benthic habitats and produce adverse effects (De Groot et al. 1976; Santschi et al. 1984). Metals are thus common contaminants of aquatic habitats, but many are also found in background concentrations in sediments and water and when they reach a threshold concentration they will produce effects to the organisms exposed in the benthic community. In south Florida, arsenic, cadmium, chromium, copper, lead, mercury, nickel, and zinc have been found in terrestrial and aquatic ecosystems including the Miami River and Biscayne Bay at higher concentrations than can be explained by natural processes (Schropp et al. 1990). Furthermore, copper is prevalent in soil, and fresh and salt water systems in south Florida adjacent to citrus agriculture and it represents potential risks to aquatic receptors as a result of its use as an algaecide/fungicide (Schuler et al. 2008).

Our study was conducted to investigate the use of *N. vectensis* in acute toxicity exposure studies using classical standard reference toxicants ($CdCl_2$, $CuSO_4$, $CuCl_2$) which contain copper and cadmium to determine suitable methods for protocols to conduct 48 to 96 hour toxicity studies. The use of species sensitivity distributions (SSDs), a method used in aquatic risk assessment, is also used to compare and contrast acute toxicity sensitivity results of other estuarine species exposed to these metals. I present

these findings to demonstrate the ease of use of this organism in a laboratory setting, recommend methods for conducting acute toxicity studies, begin to establish toxicant ranges of standard acute toxicity endpoints, introduce this organism to the toxicological community, and promote future use of this organism in toxicological studies.

METHODS

Nematostella vectensis is a member of the family Edwarsiidae which contains several sea anemones. The anatomy is similar to other anemones which are diploblastic and do not contain organs or organ systems (Daly 2002). An understanding of the anatomy of *N. vectensis* is necessary to discuss impacts of stressors observed in these studies on the anemone. Macro-scale anatomy that is useful for discussion is presented in Figure 3-1.

Nematostella vectensis can reproduce in a variety of methods giving it a unique life history. Regeneration, asexual reproduction, and sexual reproduction are all capabilities of the Starlet Anemone. These life history characteristics are presented in Figure 3-2.

Culturing Methods

In the present paper I am presenting the toxicity information from the use of juvenile anemones but also discussing the methods required to maintain and induce spawning in mixed-sex adult cultures. *Nematostella vectensis* is tolerant to a large range of salinities: 8.96–51.54 ppt, and temperatures: -1–28°C (Williams 1983) which is a beneficial attribute for conducting aquatic toxicity studies since variations in salinity and temperature will not directly affect the physiology of this species. Culturing is an important aspect of conducting toxicity studies. Standard toxicity study species are

available from a variety of sources around the world but access to native species is limited and may be difficult to obtain depending on the time of year and volume of use. Maintaining laboratory cultures provides a greater assurance of healthy, quality test organisms. The ease of culturing *N. vectensis* is discussed below.

All anemones used in the laboratory were all cultured in filtered (0.45µm) natural seawater diluted with filtered freshwater to a salinity of 13 ppt. Laboratory cultures were initiated with 10 adults obtained from the cultures of Woods Hole Oceanographic Institute (A. Tarrant, A. Reitzel) in October of 2010. The culture was supplemented with additional adults in November of 2012. Little space and expense is required to maintain these anemones. They can survive, grow, and reproduce using natural or artificial seawater at high densities with weekly water renewals and semi-weekly feedings (Hand and Uhlinger 1992; Hand and Uhlinger 1994). *Nematostella vectensis* will reproduce sexually and asexually and undergo a complete reproductive-developmental cycle under laboratory conditions. Bidirectional regeneration has also been observed. The ability to observe these traits provides a variety of biological endpoints that can be measured in the laboratory to be used as indicators of adverse effects at different parts of the life cycle of *N. vectensis*. Regulating the temperature, lighting, and feeding schedule can be used to induce weekly spawning of mature anemones (Hand and Uhlinger 1992; Fritzenwanker and Technau 2002). The following daily routine is based on (Hand and Uhlinger 1992) and personal communications with Ann Tarrant and Adam Reitzel and has proven successful in generating reliable weekly spawning of sexually mature anemones.

Adult anemones of mixed sex were held in covered glass culture bowls (200x80mm) in an incubator or on the laboratory benchtop with varying temperature and

lighting cycle. Culture dishes should be covered unless indicated to reduce evaporation that would result in fluctuations in the salinity of the holding water. Table 3-1 contains the daily routine that was conducted and that successfully induced weekly spawning from these mixed cultures.

The husbandry routine of collected egg masses, embryos, and very young anemones differs slightly from adult cultures. Collected egg masses were held in crystallizing dishes (100x50 mm). Acceptable water quality parameters are identical to adult anemones. It typically took nine to ten days from the excretion of the egg masses for the larvae to emerge from the egg mass as ciliated planulae, metamorphose into juvenile anemones, and develop tentacles large enough to begin eating. At nine to ten days of age, anemones should be fed <24h old *Artemia* nauplii. Excess *Artemia* should be removed after two hours of feeding. Once anemones began eating, they were separated into two size classes; <0.5 cm at full extension and >0.5 cm at full extension to facilitate culture cleaning and separation into sizes appropriate for the testing desired. Overfeeding of smaller anemone cultures can easily lead to fouling of the water and drastic drops in dissolved oxygen. The smaller class anemones held in crystalizing dishes (100x50 mm) were labeled with a lot number which was the date the egg mass was collected and was practical to facilitate the use of aged and smaller anemones for other experiments. Once anemones were over 0.5 cm, they could be grouped into mixed lot containers if the initial lot did not contain enough individuals for the testing desired. Juvenile anemone cultures required more frequent feedings and water renewals than adult cultures so the routines are slightly different. The following culture routine outlined in Table 3-2 describes this process while temperature and lighting cycles were identical to the adult anemones

described previously. Observation demonstrated that small anemone cultures left on a laboratory benchtop at ambient temperature and lighting did not appear to influence the growth or development vs those that were cycled similar to the adult cultures.

Aquatic Toxicity Study Methods

Acute toxicity study methods typically require an exposure of organisms for 24, 48, or 96 hours of duration to determine the relative LC50 (acute lethal concentration to 50% of the test population) for the toxicant in question (USEPA 2002). The assessment of conditional changes in study organisms can be recorded to serve as premonitory signs of stress (Selye 1976). Stress in this application is at the individual level of ecological organization, and is a short-term conditional response to a non-optimal environment or to a state which exceeds the adaptive responses of an animal beyond the normal range (Brett 1958; Selye 1976). Stress assessment, by itself, may provide early signs of disturbance of normal functioning and is not typically used in traditional laboratory aquatic toxicity studies but will be mentioned to describe observed responses during these studies (Rand and Petrocelli 1985; Rand 1995).

Acute toxicity studies can be conducted for 48 or 96 hour exposures with juvenile or adult anemones in 125 mL clear glass test chambers with polytetrafluoroethylene lined screw cap lids containing 100 mL of test solution volume. Initial age of test organisms in standard toxicity studies may vary from less than a week to several months depending on the type of study to be conducted and the objective. One objective for these studies was to select a size class of anemone that is appropriate to use in an acute toxicity method.

Natural or artificial seawater (filtered at a minimum of 0.45 µm) at 13 ± 2 ppt salinity should be used as dilution water for all studies when anemones are cultured at the

same salinity. Studies should be static-renewal exposures where the water exposure media will be periodically replaced (typically at 48 hours if study is longer than 48 hours). Test chambers should be held in a temperature and light controlled incubator at a temperature of $25 \pm 1°C$ with a photoperiod of 16 hours of light and 8 hours of dark with an intensity of 10-20 $\mu Em^{-2}s^{-1}$. If no previous toxicity data are available for the toxicant in question, preliminary range-finding studies should be conducted first to determine and narrow the range of exposure concentrations for a final full-scale definitive study (Rand 1995). Preliminary studies can be conducted with single or duplicate test chambers per concentration to reduce the number of organisms used if needed. A range-finding study is recommended as it ensures better success in the definitive study and can use smaller groups of organisms.

For the determination of a definitive LC50 or EC50 (median effect concentration; if not using mortality, the endpoint may be some sort of stress condition variable that can be quantified) the appropriate number of test organisms should be exposed to at least five treatment concentrations of test material in a geometric progression with untreated controls. Below is described the methods for acute toxicity testing with *N. vectensis* and the reference toxicants copper chloride, copper sulfate, and cadmium chloride. Metals (cadmium and copper) were chosen as the reference toxicants because they are regularly used as reference toxicants when conducting invertebrate toxicity studies (USEPA 1994).

In the present study, the range-finding studies consisted of three replicates per treatment concentration with five organisms per replicate while definitive studies consisted of four replicates per treatment concentration with five organisms per replicate. Anemones were not fed during the studies. Test water media was not renewed in

preliminary screening studies, however, if it is known that the test material degrades rapidly, periodic water media renewals should be conducted or a flow-through system should be used to maintain exposure concentrations. For definitive acute studies, water media was renewed at 48 hours since metal exposures in water without sediment are stable. At a minimum, the following water quality parameters should be recorded: temperature, dissolved oxygen, and pH in each treatment daily; and salinity in each treatment at initiation, renewal, and termination. Survival (or mortality per treatment) and any other general conditional observations (this will be discussed in further detail in a forthcoming manuscript) should be recorded daily at set time intervals. Death is the common criterion for acute studies and the criteria for death in these studies were anemones that exhibited extremely shrunken columns, severe tissue loss, loss of tentacles, exposed mesenteries, and lack of response from stimuli such as gentle prodding.

General observations included changes in shape or condition of the anemone body column or tentacles (refer to anatomy in Fig. 3-1). The column of the anemone is composed of three parts from the basal end to the oral end and includes the physa, scapus, and the capitulum (Williams 1975). The physa and scapus are located basally and make up the major portion of the column and are relatively distinguishable. An unstressed anemone considered "normal" possessed a column that was completely expanded, the tissues were clear with a slight pink tint, the mesenteries were visible, and both the physa and scapus areas were distinguishable. A moderately impacted anemone would have a shortened/shrunken column, darkening of the tissues, distinguishable mesenteries still visible, and the physa was expanded enough to see through. A severely impacted

anemone would have a completely shrunken column with opaque tissues that prevented the mesenteries from being visible, and differentiation between the physa and scapus portions of the column was not possible. Tissue sloughing along the column was also observed in some severely impacted anemones which would result in curly portions of mesenteries being exposed and visible.

The anterior end of the column is invertible, which allows the anemone to withdraw the tentacles into its body cavity. Tentacles of a normal healthy anemone would be smooth, elongated, and clear, with a smooth taper to the tips. A moderately impacted individual would have limp, shortened, or retracted tentacles and sometimes would exhibit clubbed ends. A severely impacted individual would have stubby and/or bloated tentacles that were extremely shortened and sometimes disintegrate completely or disconnect from the anemone.

Observations of the stress level of anemones were used to assist in determining mortality. Anemones determined to be severely impacted were considered dead according to the previously mentioned criteria. The endpoint of number of organisms surviving was used to calculate each study's LC50 (median lethal concentration-concentration at which 50% of the test population is killed in a given time) which is a standard acute toxicity endpoint value. Statistical analysis was conducted using CETIS (Tidepool Scientific, v.1.8.4.30) to determine the corresponding LC50 for each study. These LC50 values were used in the preparation of the SSDs which will be discussed later.

Using the above information, we provide a summary of recommended study

conditions and criteria for acceptable studies in Table 3-3 in a format similar to those

found in U.S. EPA test guidelines (USEPA 2002).

Analytical Chemistry Methods

Water samples were collected for metals and total organic carbon analysis.

Samples were collected at the initiation of a selected study for each toxicant used

(cadmium chloride, copper sulfate, and copper chloride). Samples for metal analysis were

acidified with nitric acid to a pH <2 and immediately chilled for shipment. Analysis of

metals in solution was conducted by inductively coupled plasma mass spectrometry (ICP-

MS) at Clemson University Agricultural Service Laboratory. Samples for total organic

carbon (TOC) analysis were immediately chilled for shipment. The TOC analysis was

conducted by the Southeast Environmental Research Center Laboratory at FIU following

a modification of EPA method 415.1.

Species Sensitivity Distribution Methods

Species sensitivity distributions were generated using data collected during these

studies and from an online database. The search function on the U.S. EPA ECOTOX

database was used for compiling acute toxicity data for studies with cadmium and copper

toxicity, using mortality as the endpoint for crustaceans, fish, other invertebrates,

molluscs, worms, and included standard U.S. EPA test species. The search resulted in

687 records for cadmium and 6615 for copper. These data were filtered to include only

mortality data from 96h studies. The World Register of Marine Species (WoRMS 2015)

was then used for each species to verify the environment in which they could be found.

The results were then filtered by only including organisms that were listed as being found

in brackish environments similar to *N. vectensis*. The parsing narrowed the data to 95

records containing 23 species for cadmium and 136 records containing 53 species for

copper. These data were then used to generate the SSDs contained in Figures 3-3 and 3-4

to help illustrate the sensitivity of the Starlet Anemone to cadmium and copper in relation

to other organisms that could be found in the same natural environment. Species

sensitivity distributions were prepared using the U. S. EPA CADDIS SSD Generator

(USEPA 2012). The data from the toxicity studies conducted in this paper presented in

the SSDs were the LC50 values calculated based on the measured metal concentration

recovery. The 10th centile (effects benchmark) of the SSD was used as a means of

comparing sensitivities (Schuler et al. 2008).

RESULTS

   Toxicity Study Results

       Toxicity study results using cadmium chloride, copper sulfate, and copper

chloride are included in Tables 3-3, 3-4, and 3-5 respectively. LC50 values of 2.11 and

1.78 mg/L $CdCl_2$ reported by Harter and Matthews (Harter and Matthews 2005) were

used to determine initial concentrations of cadmium chloride used in range-finding

studies. Range finding studies numbers one and two resulted in LC50 values of 371.4 and

209.3 µg/L Cd. After studies one and two were conducted, a different study setup was

considered and conducted as a size class range finding study. The size-class study was

used to determine if there was a differential response associated with anemone size. Sizes

were assigned using column length at full extension. The three classes included small

anemones which were less than 0.5 cm, medium anemones which were 0.5–1.5 cm, and

large anemones which were greater than 1.5 cm. Observations from this screening

indicated that there may a differential response depending on size. Therefore, studies 7, 8, and 9 were planned and conducted. Study 7 used anemones from the large size class (>1.5 cm), study 8 used anemones from the medium size class (0.5–1.5 cm), while study 9 used anemones from the small size class (<0.5 cm). The corresponding LC50 values were 264.5, 218.8, 146.3 µg/L Cd respectively. The 95% confidence intervals (CI) demonstrate that the LC50 values from large and medium size classes were statistically similar while the smallest size class was statistically different from both larger sized anemones. Further studies were conducted with anemones from the medium size class including studies 3, 4, 5, 6, and 10. The small size class LC50 from study 9 was similar to the values from studies 3, 4, and 5; all of which used anemones from the medium size class. Macroscopic observations with anemones less than 0.5 cm in column length are difficult, even while using magnifying glasses. Difficulty making observations led to the decision to use anemones of the medium size class for any future acute studies. Analysis of the eight 96 h definitive studies using cadmium chloride resulted in a mean LC50 value of 200.9 ± 53.92 µg/L Cd based on measured cadmium recovery. Details of acute studies using cadmium chloride including LC50 values and 95% confidence intervals are presented in Table 3-4. The coefficient of variation (CV) is used as a measurement of variability within studies and the eight definitive studies (non-range-finding) had a CV of 0.268.

The LC50 values found in the literature were used to determine a range of concentrations of copper sulfate that would be appropriate and were used in study number 11 as a range finding study for copper using *N. vectensis*. Details of acute studies using copper sulfate including LC50 values and confidence intervals are presented in

Table 3-5. Analysis of six definitive 96 hour acute toxicity studies (study numbers 12-17 found in Table 3-5) resulted in a mean LC50 value of 35.9 ± 12.33 µg/L Cu based on analytical recovery. The CV between the six full studies with copper sulfate was 0.343.

Details of acute studies using copper chloride including LC50 values and confidence intervals are presented in Table 3-6. Analysis of three definitive 96 hour acute toxicity studies (study numbers 19- 21 found in Table 3-6) resulted in a mean LC50 value of 42.7 ± 4.62 µg/L Cu based on nominal concentrations. The CV between the three full studies was 0.108.

Species Sensitivity Distribution Results

The SSDs generated using the mean LC50 concentration values from these studies and from the U.S. EPA ECOTOX database are presented in Figures 3-3 and 3-4. The cadmium SSD had a 10th centile of 65.7 µg/L Cd with the mysid shrimp (*Americamysis bahia*) being the most sensitive and below this benchmark. The copper SSD had a 10th centile of 50.3 µg/L Cu with several species including mudskippers, amphipods, copepods, and *N. vectensis* below this benchmark (*P. waltoni*, *Corophium* sp. *A. tonsa*, *A. abdita*, and *A. bato*).

DISCUSSION

Estuaries are dynamic systems which makes understanding and discussing toxicity of chemicals to organisms in these ecosystems difficult. Fluctuations in salinity, pH, temperature, dissolved oxygen, redox potential, and sediment characteristics all influence the interactions of chemicals in estuaries (Chapman and Wang 2001). Chapman and Wang discuss the importance of integrating fluctuating abiotic factors that will certainly affect chemical behavior and therefore toxicity to organisms in estuarine

environmental assessments. There are few single-species toxicity studies using test organisms that allow for a range of salinity and temperature and the need for this flexibility is apparent. *N. vectensis* can fill this void. The Starlet Anemone has a wide salinity and temperature tolerance and also provides the flexibility to be used in water or sediment toxicity studies. The studies contained within this paper were used to explore the ability to conduct acute toxicity studies similar to standard toxicity testing methods with *N. vectensis* using standard reference toxicants. The information provides details on how the studies were conducted, makes recommendations for how other laboratories would be able to duplicate and expand the use of *N. vectensis* in ecotoxicology.

Metals are a common contaminant in estuarine sediments and have been detected in several locations in Florida. Metals of concern include Zn, Cu, Cr, Cd, Hg, Pb, and Ni (Schropp et al. 1990; Alexander et al. 1993; Caccia et al. 2003; Carnahan et al. 2008). In addition, cadmium and copper are commonly used as reference toxicants in toxicity studies particularly with sediment or sediment-associated organisms. Cadmium and copper were used with *N. vectensis* to determine their sensitivity and demonstrate their use in acute toxicity testing.

Cadmium typically enters the environment through various anthropogenic sources including zinc refining, coal combustion, mine waste, electroplating processes, iron and steel production, pigments, fertilizers, and pesticides (Pickering and Gast 1972; Hutton 1983). Although a rare element that is a nutrient for plants, it is toxic to aquatic organisms at low concentrations. Once cadmium enters a water system, it is typically found as free divalent cadmium in freshwaters with low organic carbon and cadmium

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chloride complexes in saltwaters (USEPA 2001). This makes cadmium chloride an appropriate reference toxicant in these studies with *N. vectensis*.

Copper is used in agricultural and home use as an algaecide, herbicide, bactericide, and fungicide in various forms. Copper is also used to control freshwater snails and macro-invertebrates. Copper is introduced into the environment through waste discharges, industrial discharges, leaching of antifouling paints and marine preservatives, and precipitation of atmospheric fallout from mining and industrial activities (Eisler 1998; USEPA 2008). Once copper enters the environment, it ultimately dissociates into the cupric ion which is an active component of concern to environmental pollution. Copper is toxic to aquatic organisms. Binding of copper to gill membranes causing damage and interfering with osmoregulatory activities is the main cause of copper toxicity to fish and aquatic invertebrates (USEPA 2008). *N. vectensis* is found in estuarine habitats where there is a high likelihood of the presence of copper in the water and sediment. For this reason, the sensitivity of *N. vectensis* to copper using the most common forms of copper used including copper sulfate and copper chloride was examined.

After preliminary toxicity range-finding studies were conducted, the need to determine the appropriate size of an anemone to use in an acute toxicity study arose. It was demonstrated that a small anemone, <0.5 cm in column length, is not large enough to easily distinguish changes in tentacle condition, body shape, color, and the resulting stress level macroscopically. Observations on small anemones require the use of a dissecting microscope and increase the time required to conduct the study which is not conducive for an acute toxicity study. The size class studies (range-finding and 7-9)

showed that although there was some variability in LC50 values, further use of the medium size class provided reproducible results. The reproducible endpoints led to the conclusion that the anemones in the medium size class (0.5–1.5 cm) would be best for future acute toxicity studies.

There was variability in all reference toxicant studies as is evidenced in Tables 3-3, 3-4, and 3-5. The coefficient of variation (CV) which is the standard deviation of the endpoint divided by the mean value was calculated for each toxicant used to put this variability in perspective. The CV for definitive studies conducted with cadmium chloride was 0.268, copper sulfate was 0.352, and copper chloride was 0.122. The Biomonitoring Science Advisory Board (BSAB) from Washington State Department of Ecology suggested criteria for acceptable intralaboratory variability with reference toxicity studies in aquatic toxicity testing. They proposed a CV of less than 0.35 is considered excellent, while 0.35–0.60 is good, and 0.61–0.85 is acceptable (BSAB 1994). With a sample size of eight studies using cadmium chloride, the resulting CV value of 0.268 is considered in the excellent range. Studies using copper also resulted in CV values that are considered excellent (0.35 and 0.122). Although the sample sizes are currently small, the low CV values suggest the ability to use juvenile *N. vectensis* in water-only acute toxicity studies with cadmium and copper as reference toxicants and also to generate reproducible LC50 values.

In order to understand *N. vectensis* sensitivity to cadmium and copper in relation to other organisms, a species sensitivity distribution (SSD) was prepared for each metal. SSDs are a practical way to visually represent the sensitivity of a chemical to a variety of species (Posthuma et al. 2002). Twenty seven percent of the taxa (6 species including 55

observations out of 24 species including 96 observations) in the cadmium SSD were at or below the LC50 value for *N. vectensis* as illustrated in Figure 3-3. 10% and 12% of the taxa (5 species including 10 observations out of 54 species including 138 observations) in the copper SSD were at or below the LC50 values for *N. vectensis,* respectively as illustrated in Figure 3-4. The presence in the lower portions of the SSDs demonstrates that *N. vectensis* is sensitive to cadmium and copper when compared to other organisms living in estuarine habitats.

Fluctuating environmental factors, which is common spatially and temporally in estuarine ecosystems, can influence toxicity of metals to aquatic organisms. Salinity, temperature, organic carbon load of water and/or sediment, and the presence of iron and manganese hydroxides in sediment have all been demonstrated to influence the toxicity of metals to a variety of organisms including Blue Crab, Fiddler Crabs, mysid shrimp, copepods, and the Mummichog (O'Hara 1973; Voyer 1975; Nimmo et al. 1978; Frank and Robertson 1979; Gentile et al. 1982; Toudal and Riisgard 1987; Du Laing et al. 2009). These examples indicate that environmental conditions effects on metal toxicity are likely species-specific which makes it impractical to apply historical water quality parameter bioavailability or biogeochemical models to metal toxicity in estuarine environments. *N. vectensis'* wide tolerance to varying salinity and temperature provide a unique opportunity to investigate the variability of toxicity of contaminants to an estuarine organism due to fluctuating environmental conditions.

Information gathered from many studies and the generation of SSDs similar to the ones above with a greater taxonomic variety are used to develop the national recommended water quality criteria. Ambient water quality criteria establishes Criteria

Maximum Concentrations (CMC) [relevant to acute exposures] and Criterion Continuous Concentrations (CCC) [relevant to chronic exposures]. The current CMC is 40 µg/L and the CCC is 8.8 µg/L for cadmium. The current CMC is 4.8 µg/L and the CCC is 3.1 µg/L for copper. While the results from the studies presented in this paper do not implicate the need to revise any national water quality criterion, it does warrant further suggestion for the incorporation of *N. vectensis* into estuarine environmental assessments and a larger variety of ecotoxicological studies.

One difficulty regularly observed when working with Cnidaria is the determination of death. Whether working with corals, jellyfish, or anemones, the exact time of ecological death is difficult to obtain. The initial screening studies using cadmium with *N. vectensis* provided the opportunity to train the investigators how to observe stress responses with the anemone. The first response was typically limp tentacles and/or a shrunken body column. After several studies, the investigators were curious if anemones that were extremely stressed and on the verge of death could recover. Some standard ecotoxicological studies incorporate recovery into the endpoint observations. Recovery was observed in several studies and these observations were used to create a stress-response index of macroscopically observable morphological changes. This and the influence of these observations on the toxicity studies will be discussed in a future manuscript.

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Table 3-1. Culture routine for induced spawning of mixed sex cultures.

Day	Tasks/Holding Conditions
1	Morning: Remove from incubator: Warm up to room temp; Feed mussel ovary Two hours later: Remove uneaten food Afternoon: Back into incubator at 28 °C, covered with lights on
2	Morning: Place cultures on lab benchtop, uncovered Afternoon: Remove fertilized egg masses; heavy feeding of *Artemia* nauplii; cover; leave on benchtop under ambient lighting at room temp (22-25 °C)
3	Water renewal with diluted seawater at 13 ppt and 25 °C and culture container cleaning
4	Morning: light feeding of *Artemia* nauplii Afternoon: Covered culture container into incubator at 16-18 °C with no light
5-7	16-18 °C; Covered; No light

Table 3-2. Culture routines for embryo, larval, and smaller size class anemones.

Frequency	Task
2-3x/week	Feeding with <24h old *Artemia* nauplii; uneaten food removed after 2 hours
2-3x/week	Water renewals with diluted seawater at 13 ppt and 25 °C and culture container cleaning
1/week	Culture container changing
Holding conditions: room temperature; 13 ppt salinity; ambient lighting	

Table 3-3: Summary of recommended study conditions and acceptability criteria for the Starlet Anemone, *Nematostella vectensis*, water-only acute toxicity tests.

1.	Test type:	Static-renewal, water only exposure, salinity ± 1ppt from desired
2.	Test duration:	48, 96 h
3.	Temperature:	25 ± 1°C (Other temperatures are acceptable when necessary)
4.	Light quality:	Ambient laboratory lighting
5.	Light intensity:	10-20 $\mu E/m^2/s$ (50-100 ft-c)
6.	Photoperiod:	16 h light, 8 h dark
7.	Test chamber size:	125 mL (minimum)
8.	Test solution volume:	100 mL (minimum)
9.	Renewal of test solutions:	85-90% at 48 h
10.	Age of test organisms:	0.5-1.5 cm column length (about 3 months)
11.	No. organisms per rest chamber:	5 minimum
12.	No. replicate chambers per concentration:	4 minimum (2 minimum for screening)
13.	No. organisms per concentration:	20 minimum (10 minimum for screening)
14.	Feeding regime:	*Artemia* nauplii should be made available a minimum of 2 hours prior to test initiation and renewal if applicable.
15.	Test chamber cleaning:	Any debris will be removed during the 48 h renewal
16.	Test solution aeration:	None unless DO concentration falls below 4.0 mg/L
17.	Dilution water:	13 ± 2 ppt salinity; diluted natural or artificial seawater (minimum 0.45 µm filtered)
18.	Test concentrations:	Minimum of 5 concentrations and a control
19.	Dilution series:	≥0.5 dilution series; variable loading when not appropriate
20.	Endpoint:	Mortality, or stress index indicator number of 4 or 5
21.	Sample volume required:	Minimum 1 L
22.	Test acceptability criterion:	90% or greater survival in controls

Table 3-4. Summary of *Nematostella vectensis* acute studies with cadmium chloride (µg/L).

Study Number: Description	Organism Size	LC50[†]	95% CI
1: 96h Acute Range-Finding	>0.7 cm	371.4	311.5-442.8
2: 96h Acute Range-Finding	>0.7 cm	209.3	168.2-260.4
3: 96h Acute Definitive	1.0 cm	156.8	137.8-178.6
4: 96h Acute Definitive	1-1.5 cm	151.5	131.1-175.1
5: 96h Acute Definitive	0.5-1.5 cm	156.8	142.9-172.1
6: 96h Acute Definitive	0.5-1.5 cm	273.1	248.8-299.7
7: 96h Acute Definitive	>1.5 cm	264.5	228.4-306.4
8: 96h Acute Definitive	0.5-1.5 cm	218.8	183.4-261.0
9: 96h Acute Definitive	<0.5 cm	146.3	125.3-170.9
10: 96h Acute Definitive	0.5-1.5 cm	239.2	207.9-275.3

[†] The median lethal concentration (LC50) as well as the corresponding confidence limits is reported in µg/L Cd. Values based on analytical recovery.

Table 3-5. Summary of *Nematostella vectensis* acute studies with copper sulfate (µg/L).

Study Number: Description	Organism Size	LC50[†]	95% CI
11: 96h Acute Range-Finding	1.0 cm	72.8	57.2-92.7
12: 96h Acute Definitive	1.0 cm	39.6	34.4-45.5
13: 96h Acute Definitive	0.5-1.5 cm	18.6	14.0-24.5
14: 96h Acute Definitive	0.5-1.5 cm	41.9	35.3-49.7
15: 96h Acute Definitive	0.5-1.5 cm	23.0	18.3-27.8
16: 96h Acute Definitive	0.5-1.5 cm	42.8	35.4-51.8
17: 96h Acute Definitive	0.5-1.5 cm	49.9	42.9-58.0

[†] The medain lethal concentration (LC50) as well as the corresponding confidence limits is reported in µg/L Cu. Values based on analytical recovery.

Table 3-6. Summary of *Nematostella* vectensis acute studies with copper chloride (µg/L).

Study Number: Description	Organism Size	LC50[†]	95% CI
18: 96h Acute Range-Finding	0.5-1.5 cm	56.1	45.6-69.0
19: 96h Acute Definitive	0.5-1.5 cm	41.8	37.5-46.5
20: 96h Acute Definitive	0.5-1.5 cm	47.7	41.0-55.5
21: 96h Acute Definitive	0.5-1.5 cm	38.6	34.3-43.4

[†] The median lethal concentration (LC50) as well as the corresponding confidence limits is reported in µg/L Cu. Values based on analytical recovery.
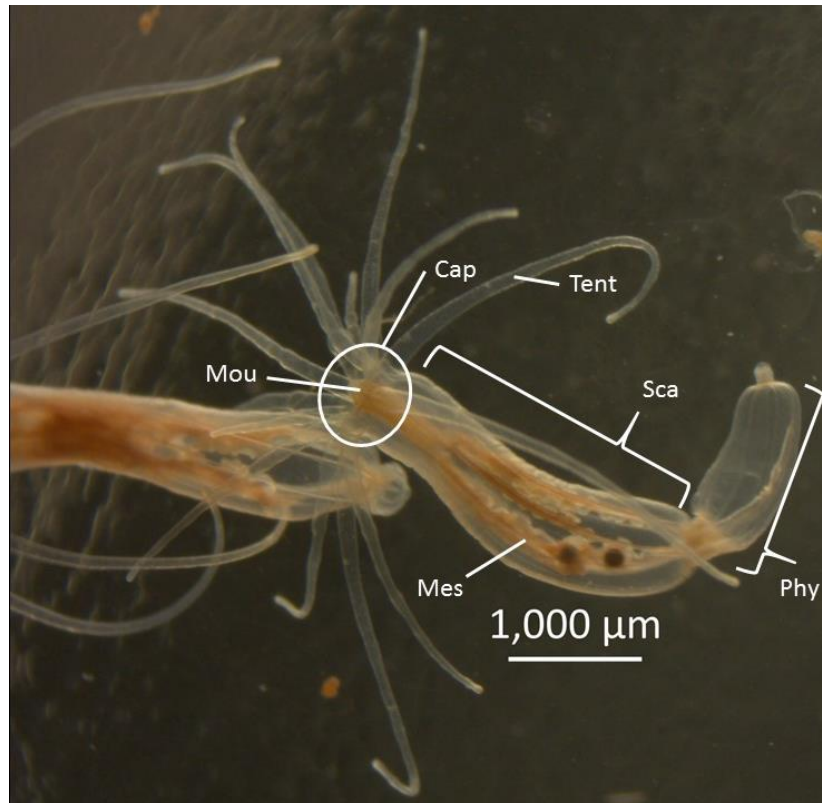
Figure 3-1. Anatomy of *Nematostella vectensis*. Mou: Mouth; Cap: Capitulum; Tent: Tentacle; Sca: Scapus; Mes: Mesentery; Phy: Physa.
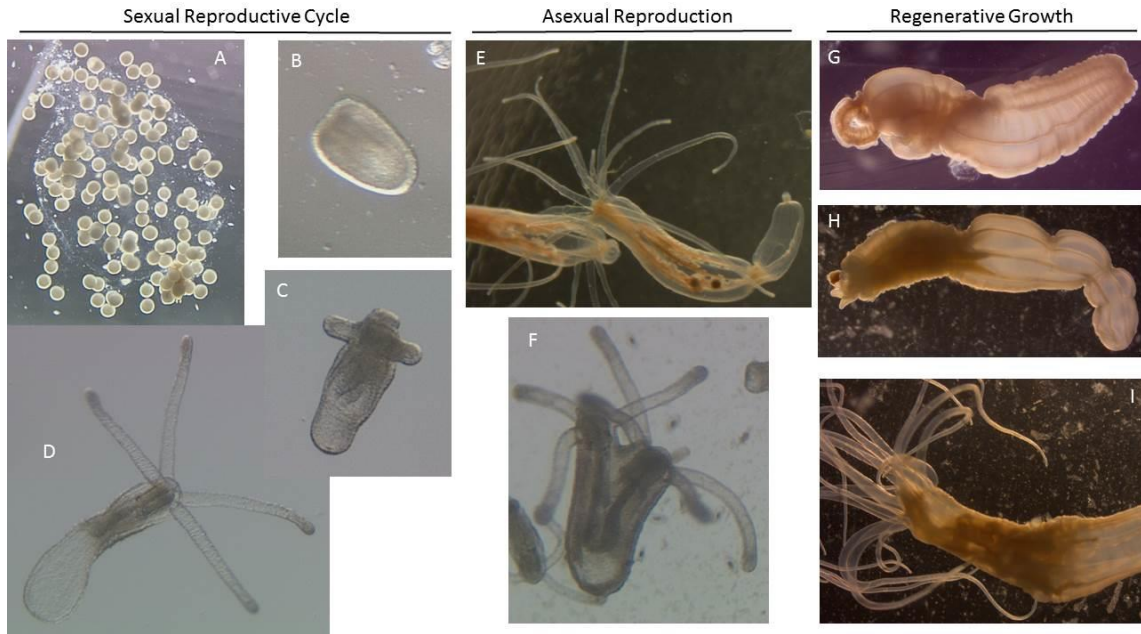
Figure 3-2. Life cycle of *Nematostella vectensis*. Sexual reproduction produces an egg mass (A) excreted by females in a gelatinous mass. Eggs develop into free-swimming planula larvae (B) within 36-48 hours post-fertilization. As larvae settle, tentacle buds (C) form within about 5 days as it is transitioning into a polyp. A juvenile polyp (D) contains a ring of four tentacles and may begin feeding as early as 7 days of age. Mature anemones (E) may also reproduce asexually, primarily through transverse fission. Although other forms of asexual reproduction (budding) were observed in juvenile anemones (F) as young as 14 days of age while conducting studies for this manuscript. Images (G-I) demonstrate regenerative growth of the oral side of an anemone that was cut in half.
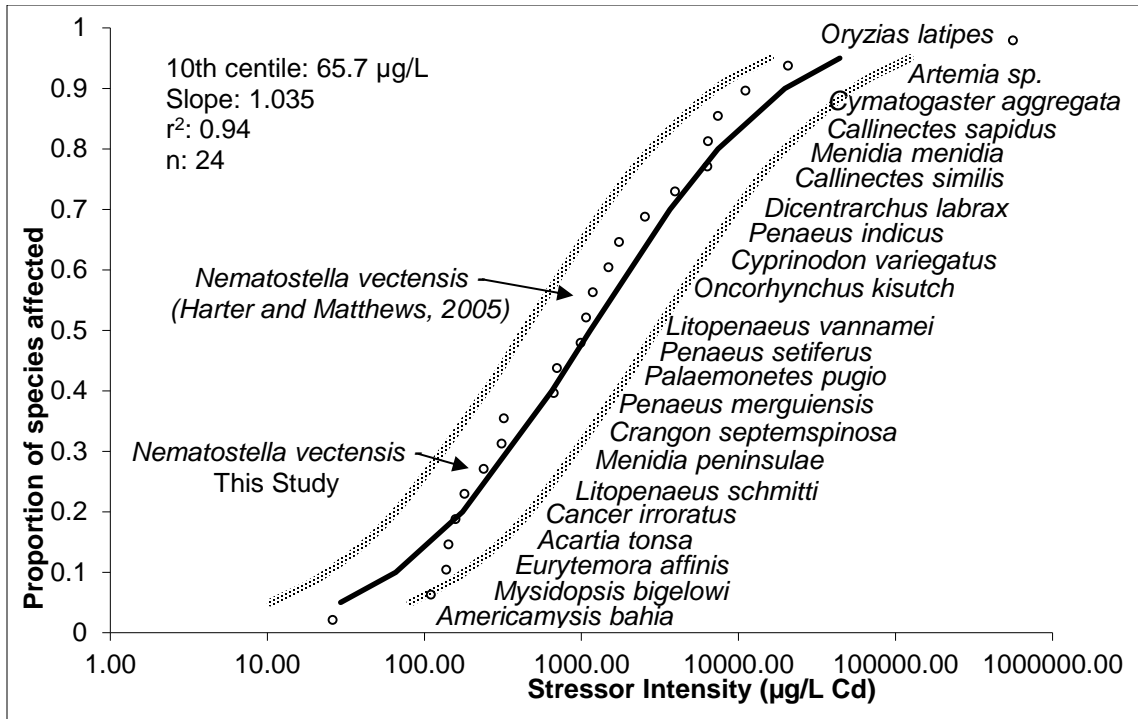
Figure 3-3. Species sensitivity distribution (SSD) of 96 hour LC50s for estuarine organisms exposed to cadmium. Toxicity data obtained from U.S. EPA's ECOTOX database.
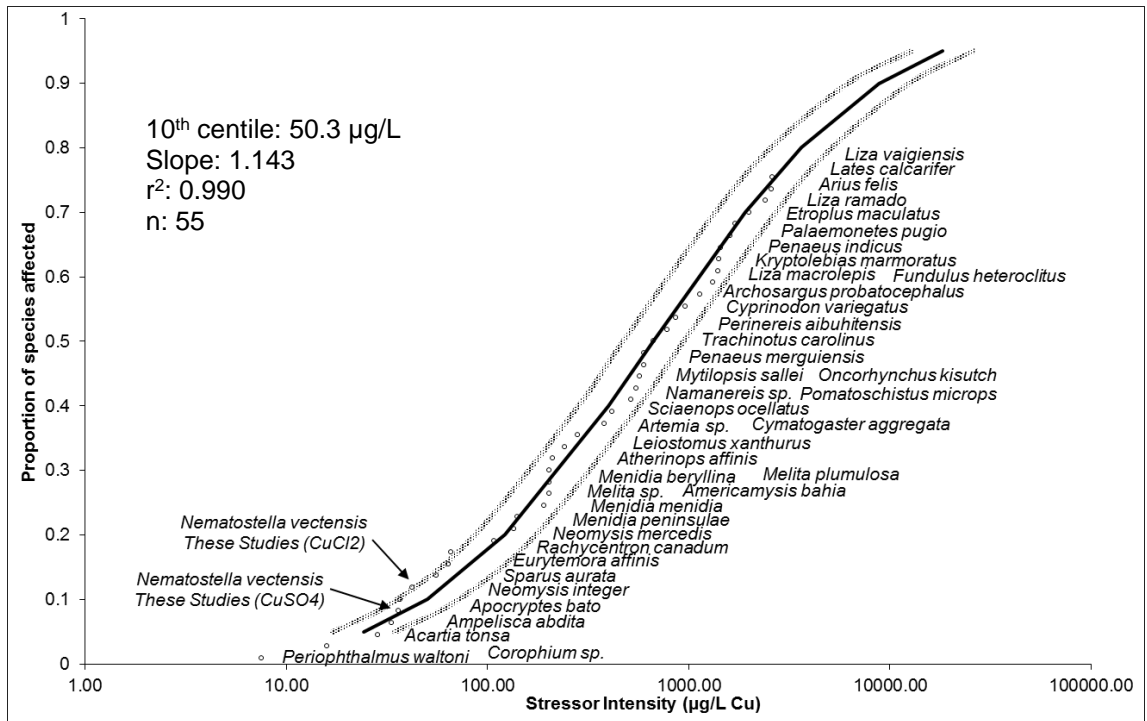
Figure 3-4. Species sensitivity distribution (SSD) of 96 hour LC50s for estuarine organisms exposed to copper. Toxicity data obtained from U.S. EPA's ECOTOX database.

CHAPTER IV: DEVELOPMENT OF A STRESS-RESPONSE INDEX IN MARINE TOXICITY STUDIES: USE OF THE MODEL CNIDARIAN, *NEMATOSTELLA VECTENSIS*, FOLLOWING RECOVERY PERIOD OF ACUTE EXPOSURES TO CADMIUM AND COPPER

ABSTRACT

To assess biological impact following an accidental chemical spill, aquatic toxicity test method guidelines recommend the use and evaluation of native resident species, representative of the contaminated study area. During method development of acute toxicity studies using the Starlet Anemone, *Nematostella vectensis*, definitive determination of mortality proved difficult because of the cnidarian's ability to recover from stressors. However, macroscopic observations of effects on anemones collected during the acute toxicity exposures to cadmium and copper followed by a recovery period were used to create a stress-response index. The index was applied to determine mortality and proved effective in generating reproducible endpoint estimates. The use of the stress index and it's its application using the Starlet Anemone in ecotoxicology investigations is presented.

Keywords: stress response index, *Nematostella vectensis*, native resident species, recovery, acute toxicity

INTRODUCTION

Traditional marine toxicity studies are based on guidelines from the United States Environmental Protection Agency (US EPA) Whole Effluent Toxicity (WET), American Society for Testing and Materials International (ASTM International), or Organization

for Economic Co-operation and Development (OECD) which rely on classical quantifiable biological endpoints such as mortality, development, growth, and reproduction. Toxicity testing guidelines recommend the use of native resident species representative of the area of study in question but this practice is rarely used because of our cursory knowledge of their basic requirements for optimum survival (e.g., water quality, diet) and the difficulty of acclimating and holding non-standard test species in the laboratory [1,2]. Using a new test organism also frequently involves the development of new culture protocols including animal husbandry techniques to facilitate spawning and requires considerable time investment and trial/error. However, ecological risk assessments typically highlight the lack of diversity in toxicity data needed across a wide range of phyla to fully understand the biological effects, range of sensitivities and potential risks of stressor exposures [3–10]. As part of new method development, an acute stress-response index test is presented here with the Starlet Anemone, *Nematostella vectensis*, (a model organism used extensively in evolutionary and developmental biology) which can serve as a sublethal indicator of mortality. In addition, the stressor-index test includes a phase after stressor exposure in clean untreated water to evaluate anemone recovery, a phase that is rarely evaluated in aquatic toxicity studies.

METHODS AND MATERIALS

In testing cnidarians, it can be difficult to determine mortality, with confidence. Immobility and loss of equilibrium for aquatic organisms has been used as an endpoint for invertebrates [11]. Furthermore, a variety of marine invertebrates have displayed the ability to regenerate damaged tissues including jellyfish, sponges, corals, ctenophores, clams, polychaetes, seastars, brittle stars, and sea anemones [12–18]. The Moon Jellyfish,

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Aurelia aurita, may self-repair radial symmetry from physical damage through

reorganization [19]. Goh found inhibition of larval settlement and survival from

exposures as low as 1 mg/L nickel [20].

Marine invertebrates have been used in stress/response studies because of their

sensitivity to metals, pesticides, and environmental conditions [21–27]. However, in these

studies, it is also frequently observed that cnidarians recover from a variety of stressor

exposures. Long-term exposures to chlordane as low as 1 ug/L produced sublethal effects

(depressed photosynthesis, respiration, and P/R, bleaching, induction of GST, and death

for 50% of the corals) in the corals *Porites divaricata* and *Montastrea faveolata* while the

anemone *Aiptasia pallida* was tolerant to these level of exposures [28]. Corals frequently

bleach (expel symbiotic zooxanthellae) in response to elevated temperatures as observed

in laboratory and field conditions throughout the Caribbean and more recently on the

Great Barrier Reef [29–33]. The coral genus, *Agaracia*, has congeners that differentially

express heat shock proteins (HSC70/HSP90) and have different abilities to tolerate and

recover from extreme heat swings resulting in dramatic changes in coral species

ambulance patterns [34].

Therefore, an aquatic toxicity test relying on traditional assessment of mortality at

the end of an acute 96 hour stressor exposure may not accurately represent the

environmental impact of a stressor on *N. vectensis* if the cnidarian has the ability to

recover in the period after a short-term exposure. The establishment of a confident

decision of mortality is needed to generate reproducible toxicity endpoint values.

Cessation of movement (or moribundity) is frequently used as an indicator of mortality in

ecotoxicology studies and is practical with fish and sometimes cnidarians (e.g. jellyfish

ephyrae bell pulsing) but is not applicable to *N. vectensis* [35]. In addition, organisms which appear dead, may recover when placed in clean control media water.

To determine the exposure concentration (or level of chemical stress) an anemone is able to withstand and not be able to recover (mortality), we included a recovery phase (time period: 14 days) after acute chemical exposures (96 hours) to cadmium and copper (separately). Observations collected during acute exposures and the recovery period were compiled to create a quantifiable multi-level stress-response index correlated to the intensity of effects observed. The index is presented here to demonstrate its application resulting in the ability to confidently identify mortality of *N. vectensis* and generate reproducible toxicity endpoints.

Nematostella vectensis is an estuarine anemone that is easily cultured in the laboratory with standard equipment. Anemone cultures can be maintained in glass finger bowls or crystallizing dishes on the laboratory benchtop and in incubators to control temperature, lighting, and feeding cycles. The animal husbandry tasks involved in the culture protocol may be modified depending on the goal, inducing spawning or culture maintenance. Details on these protocols can be found in the previous section of this chapter and are based on those proposed by Hand and Uhlinger [36–39].

Spawning was induced and embryo masses were collected and maintained in 13 ppt diluted seawater until study initiation. Earlier work and personal observations found that test organisms should be selected for studies based on size for better observation of effects macroscopically. Anemones used in the recovery studies were around 3 months of age (84–91 days) and were 0.5 to 1.5 cm in length.

Toxicity studies presented here were conducted following the methods/protocol

discussed in the previous chapter (see Table 3-3. with study conditions summary).

Exposure chambers held 5 organisms per replicate, with 4 replicates per treatment

concentration and five treatment concentrations. Anemones were exposed to five

treatment concentrations of cadmium (25.9-414 µg/L [measured]) or copper (1.7-445

µg/L [measured]) for 96h. Treatment dilutions were prepared from stock solutions of

cadmium chloride, copper sulfate, or copper chloride, with water renewals at 48h ($\geq$90%

of treatment solution volume), including controls followed by a 14day recovery phase in

untreated water. Studies were conducted at a water temp of 25 ± 1 °C, DO >4.0 mg/L,

and a photoperiod of 16h L: 8h D. Observations were documented in toxicity studies to

compile macroscopically identifiable characteristics to create a numerical stress-response

index which could be assigned to anemones based on their condition. Observations

included changes in color, transparency vs opaque tissue, visible morphological

characteristics of mesenteries and tentacles, and condition and shape of the main body

column (physa and scapus).

After 96 hours of acute exposure, final water quality parameters (i.e, temperature,

DO, pH) and stress-response index designations, anemones were removed from testing

chambers, rinsed in control water, and isolated by test treatment and stress-response

index characterization. Recovery was monitored in plastic 400 mL beakers filled with

estuarine water at 13 ppt in an incubator at 25°C with a photoperiod of 16 hours light and

8 hours dark (identical to testing conditions). The anemones were allowed to recover for

2 days without interference (no feeding or renewal). On days 3, 5, 7, 10, and 12 post-

exposure, anemones were fed 0.1 to 0.3 ml of concentrated <24-hour-old brine shrimp

and uneaten food was removed after an appropriate length of time for feeding (minimum of 2 hours). Recovery holding chambers were renewed with clean control water on days 3, 6, and 13. Stress-response index characterization was monitored throughout the recovery duration with a final stress-response characterization on recovery day 14.

Observations documented leading to the development of the stress-response index were collected in three categories. The first was the body column of the anemone which included the scapus (middle of body column) and physa (basal end of the anemone body column) (refer to anatomy Fig. 3-1 in the previous chapter). Observations included: the length of the column and any visible retraction; the condition of mesenteries, size, shape, and transparency of the physa. The second category was the anemone tentacles. Observations included: extension or retraction of tentacles, even taper of tentacle towards the tip, clubbed tips, limp or shortened tentacles, or short bloated tentacles; inverted tentacles. The last category was anemone color. Observations included: tissues clear and transparent; basal end clear and transparent; mesenteries pink/orange and clearly visible though scapus wall; darkened tissues; mesenteries not visible because of opaque tissue; completely opaque. Additional observations included anemone ability to anchor to the bottom of the holding container, response to stimuli (optical: light table being turned on/off, physical: gentle water movement from transfer pipette), tissue disintegration, anemone lacking structural integrity, inverted body where mesenteries are exposed. Observations were compiled over the studies presented in this paper and those discussed in the previous section and used to create the stress-response index.

Standard toxicity endpoints included a: no-observable-effect concentration (NOEC), lethal-effect concentration at the 10% level (LC10), and median lethal

concentration value (LC50). Endpoints were calculated for each study following the US EPA methods for the determination of point estimates from definitive multi-concentration acute toxicity tests [41]. Statistical estimates were determined using CETIS® statistical software version 1.8.4.30 (Tidepool Scientific, Inc.). Anemone mortality was designated based on exhibiting characteristics of index categories 4 or 5 of the stress-response index classification and will be discussed further later. Endpoint values (NOEC, LC10, LC50) were calculated using data from acute study termination (after the 96h exposure) and after the 14d recovery period. F-tests were used to determine whether variances of the endpoint value groups were equal followed by differences between the pre- and post-recovery means tested using two sample t-tests. Recovery of anemones was tracked throughout the 14d post-exposure time period to determine which anemones recovered. Data was compiled and tabulated to determine percentages of stress-response index groups that either improved in condition (moved from high index # to low) or worsened in condition (moved into a higher index # or remained in a high index #).

RESULTS

The stress-response index is designed as a six point scale (condition numbers zero through five) which ranges from a normal healthy anemone appearance and behavior (zero) to a severely impacted anemone (five). Table 4-1 contains the stress-response index numbers and the associated conditions assigned to each category including observations of the body column, tentacles, and tissue color. Corresponding images of anemones representing each stress-response index category are presented in Figure 4-1.

Common ecotoxicological endpoint point estimates including the NOEC, LC10, and LC50s were calculated at acute study termination (after 96 hours of exposure) and

after the 14d recovery period and are summarized in Table 4-2. Studies using cadmium chloride had a pre-recovery mean 96h NOEC of 138.7 ± 60.9 µg/L Cd and a post-recovery mean NOEC of 173.1 ± 60.99 µg/L Cd; a pre-recovery mean 96h LC10 of 161.1 ± 40.2 µg/L Cd and a post-recovery mean LC10 of 127.3 ± 1.0 µg/L Cd; a pre-recovery 96h mean LC50 of 243.7 ± 27.4 µg/L Cd and a post-recovery mean LC50 of 227.5 ± 5.0 µg/L Cd. Studies using copper sulfate had a pre-recovery mean 96h NOEC of 30.6 ± 21.7 µg/L Cu and a post-recovery mean NOEC of 28.2 ± 20.8 µg/L Cu; a pre-recovery mean 96h LC10 of 22.5 ± 7.9 µg/L Cu and a post-recovery mean LC10 of 23.1 ± 7.9 µg/L Cu; a pre-recovery 96h mean LC50 of 39.4 ± 11.5 µg/L Cu and a post-recovery mean LC50 of 46.4 ± 14.7 µg/L Cu. Studies using copper chloride had a pre-recovery mean 96h NOEC of 30.6 ± 12.1 µg/L Cu and a post-recovery mean NOEC of 23.2 ± 1.6 µg/L Cu; a pre-recovery mean 96h LC10 of 26.2 ± 0.9 µg/L Cu and a post-recovery mean LC10 of 26.1 ± 8.4 µg/L Cu; a pre-recovery 96h mean LC50 of 42.7 ± 4.6 µg/L Cu and a post-recovery mean LC50 of 42.2 ± 14.0 µg/L Cu.

Following F-tests to determine equal/unequal variance, all t-tests between point estimates from each toxicant group between pre-recovery and post-recovery found no differences ($\alpha=0.05$, all p>0.05). Designating anemones exhibiting characteristics of stress-response index categories 4/5 as dead, resulted in endpoint estimates that were statistically identical even given potential time to recover. These results indicate that the 4/5 stress index mortality category is successful to use to assign mortality when using *N. vectensis*.

During the recovery time period, there was a significant portion of anemones that were able to recover (corresponding with a decrease in assigned index number)

demonstrating the common cnidarian ability to recover from damaging stressors. Table 4-3 includes a summary of the 14d recovery period. Of the anemones assigned to index numbers zero through three (n=784) at acute exposure termination (T=96h), 86% of them were able to recover to an index number of zero or one (no to minimal effect) while 3.6% worsened (increased to index of 4 or 5). Of the anemones assigned to index numbers 4 or 5 (n=395) at acute exposure termination, 5.1% were able to recover to an index number of zero or one while 92% remained in index 4 or 5.

DISCUSSION

Documentation of observations of effects in anemones used in acute exposure studies (those discussed here along with the studies in the previous section) were compiled to create the stress-response index. The progression of the index classes were grouped so that numbers zero and one demonstrate either no or minimal transient effects, numbers two to three indicate moderate but reversible morphological changes resulting from the stressor, and numbers four to five are effects that will lead to death of the organism (unable to recover). The index was created with numerical categories paired with description categories for two reasons. The first was to be able to track the progression of anemones during the recovery period. The second was to investigate the use of the index as a sub-lethal indicator of stress. This index allows for the determination of a quantifiable stress level. Monitoring this level during a recovery period determined if these effects were transient, and at what level an anemone would not be able to recover from this stress. Recovery is not part of most standard toxicity studies (i.e., except in bioconcentration/depuration studies) but may be necessary to use when working with invertebrates that have the ability to recover from sublethal effects or low concentrations.

The application of this index confidently identified mortality in a cnidarian as the endpoint. However, the effects observed in the other index categories could be used to quantify sublethal endpoints. This approach would be practical when working with exposures from contaminants that an organism is able to metabolize. Figs. 4-1A and 4-1B illustrates anemones with index of zero and one with minimal effects. A healthy or normal anemone (index number zero and one) will generally have an inflated body column with transparent tissues and easily identifiable internal components such as the mesenteries (practical anemone anatomy mentioned here can be found in Fig. 3-1 of the previous section on acute toxicity). Tentacles are clear with no deformation and a smooth taper to the tips. There were a few instances when an anemone may retract its tentacles into the capitulum (portion of the body column closest to the mouth) and the body would retain the characteristics of index zero and one. Typically, if an anemone exhibiting this tentacle retraction was left without disturbance, the tentacles would extend and appear normal after about 5 to 10 minutes. The color of the tissue of the gastrovascular cavity may vary depending on the food source. The examples of anemones pictured here in Fig. 4-1 were fed a diet of <24h old *Artemia* nauplii which is why they have a pink color to their tissue. Figs. 4-1C and 4-1D illustrates anemones with index two and three with moderate effects. The column of the anemone generally shortens as the index number increases. The transparency of the tissues decrease and turn more opaque yet the mesenteries should still be distinguishable though the basal end of the anemone at the moderate index numbers. The tentacles start to develop deformations including clubbed tips, limp or shortened, bloating at the base, or severe shortening (similar to observations found in stressed hydra) [42,43]. Figs. 4-1E and 4-1F illustrate anemones in index four

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and five with severe effects (which will lead to death). The column of the anemone is extremely shortened and so much so that the tissues are opaque and mesenteries and other internal structures are not distinguishable. The basal end of the anemone (physa) is no longer inflated. Tentacles exhibit severe deformation including bloating, appearing stubby, even sometimes falling off the anemone body. The most severe index condition (five) included severe disintegration of the ectodermal tissue layers of the body column which led to exposed mesenteries (easily distinguishable by their wavy ribbon-like appearance). Some anemones would invert their bodies and expose their mesenteries through their mouth. Index number five anemones were unable to anchor themselves and would disconnect from the chamber bottom after a gentle current of water (e.g. from the squeeze of the bulb of a plastic transfer pipette). Healthy anemones typically secrete mucus to anchor themselves to the bottom of the testing chamber. Any anemone listed in index five would usually end up as a ball of indistinguishable cells with no morphological characteristics of an anemone and would disintegrate rather quickly.

Tracking the stress-response index number of 1179 anemones after acute exposure to cadmium or copper demonstrated the ability of *N. vectensis* to recover after negative effects are observed, similar to other marine invertebrates. Eighty-six percent of the anemones assigned to indexes zero to three after acute exposure were able to end at index number to zero or one after the 14d recovery period while only 3.6% increased in index number. Ninety-two percent of the anemones within index four and five after acute exposure remained in index four and five (or disintegrated) after the 14d recovery while only 5.1% were able to recover to index zero and one. This recovery data indicates that the proposed demarcation between zero through three and four to five index values is an

appropriate point for determining anemone mortality. This finding is further confirmed by comparing the point estimates calculated at acute study termination and recovery termination.

Table 4-2 lists all NOEC, LC10, and LC50s from pre- and post-recovery and they appear to be similar at both time points. Two-sample t-tests detected no difference in the mean values (tested in groups by endpoint and toxicant used: NOEC, LC10, LC50; and CdCl$_2$, CuSO$_4$, and CuCl$_2$). With no detectible difference in the point estimates pre- or post-recovery, this confirms success of applying the stress-response index to confidently determine mortality. The inability to recover indicates that the effects observed in stress-response index categories 4/5 are not transient and there is no need to include a recovery period after an acute toxicity exposure using *N. vectensis* if the stress-response index is applied to determine mortality when using these metals. However, this may not apply to other toxicants and would need to be tested.

Species sensitivity distributions (SSDs) are frequently used to display toxicity data from multiple phyla to see which taxa is sensitive to the chemical in question and have been used in establishing water quality criteria and in ecological risk assessment [7,10,44,45]. SSDs prepared from toxicity data of estuarine organisms collected from the U.S. EPA ECOTOX database previously showed that *N. vectensis* is sensitive to cadmium and copper [40]. With 26% of the species at or below an LC50 value of 239 µg/L Cd and 10% of the species at or below the LC50 value of 38.59 µg/L Cu, *N. vectensis* should be considered a candidate when investigating toxicity in estuarine systems where the anemone would be found because of its sensitivity to metals.

The limited animal husbandry requirements, ease of inducing spawning, and reliability of this stress-response index provides techniques to easily use *N. vectensis* as a native, non-standard organism in toxicity investigations. *Nematostalla vectensis* is an excellent model organism to use in estuarine or marine (water only or sediment) ecotoxicology studies and has great potential for the integration of molecular and omic techniques [46–49]. In addition, the availability of data on embryological development, genetic pathways, epigenetics, and the annotated genome, as well as tools including gene knockdown, *in situ* hybridization, stable transgenic lines, and CRISPR/Cas9-mediated genome editing add to the value of using *N. vectensis* as a native, non-standard organism in future aquatic ecotoxicology investigations.

The next step in using *N. vectensis* in other ecotoxicology studies would include determining if this stress-response index can be applicable to other toxicants. This index currently has only been demonstrated effective with cadmium and copper as a toxicant. The anemone may react differently to pharmaceuticals or personal care products, pesticides, petroleum products or other metals depending on lipid solubility. Further testing with a variety of toxicants and a larger age/size range of anemones (young anemones with their first set of tentacles, or possibly in early embryonic developmental stages) could further demonstrate the potential of the stress-response index.

ACKNOWLEDGEMENTS

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Table 4-1. Stress-response index categories used to characterize effects observed during acute exposures to cadmium or copper.

Stress Index #	Conditions
0	Column: fully extended with mesenteries clearly visible/distinguishable, clear Tentacles: fully extended, even taper to tip with smooth surface Color: Tentacles clear, basal end clear, mesenteries pink/orange
1	Column: slight retraction (20-40%) Tentacles: shortened or retracted (no deformation) Color: Same as 0
2	Column: shortened (40-70%) Tentacles: mild deformation (clubbing at tips or limp/shortened) Color: clear with all internal parts visible
3	Column: shrunk but with mesenteries partially distinguishable and inflated basal end Tentacles: moderate deformation (bloating at base, severe shortening) Color: darker, slightly opaque, internal parts not clear
4	Column: shrunk, mesenteries not distinguishable, basal end indistinguishable Tentacles: severe deformation (bloating or stubby) Color: opaque
5	Severe disintegration, no structural integrity, body inversion with mesenteries exposed, tissue sloughing, no response to stimuli (physical or light), opaque color, no anchoring

Table 4-2. Summary of endpoint estimates after acute toxicity test exposure of 96 hours and after 14 day recovery period.

Study #	Toxicant	Pre-Recovery Endpoints[a]			Post-Recovery Endpoints[a]		
		NOEC	LC10	LC50	NOEC	LC10	LC50
Nv15	$CdCl_2$	103.5	207.0	273.1	103.5	126.1	229.6
Nv16		103.5	144.3	218.8	207.0	127.7	221.8
Nv17		209.0	132.1	239.2	209.0	127.9	231.2
Nv6	$CuSO_4$	60.6	20.9	41.9	NC[b]	NC[b]	64.7
Nv8		8.7	12.2	23.0	8.7	14.6	29.9
Nv10		26.0	26.8	42.8	26.0	24.4	40.9
Nv14		27.0	30.2	49.9	50.0	30.2	49.9
Nv19	$CuCl_2$	22.2	25.2	41.8	22.2	25.2	41.8
Nv20		44.5	26.5	47.7	22.2	34.9	56.5
Nv21		25.0	27.0	38.6	25.0	18.1	28.4

Notes:
[a] Units = µg/L (Analytical Recovery - Total Cd or Cu)
[b] NC: Not Calculated

Table 4-3. Summary of anemone stress-response index designation from study exposure termination (after 96 hour exposure) and after 14 day recovery period.

Stress-Response Index #	Pre-Recovery # of anemones in Index @ T96	Post-Recovery			
		Anemones with Index # 0/1	% from T96	Anemones with Index # 4/5	% from T96
0	417	417	100.0	0	0.0
1	52	51	98.1	0	0.0
2	150	124	82.7	0	0.0
3	165	83	50.3	28	17.0
4	137	19	13.9	107	78.1
5	258	1	0.4	256	99.2
	Total n=1179				
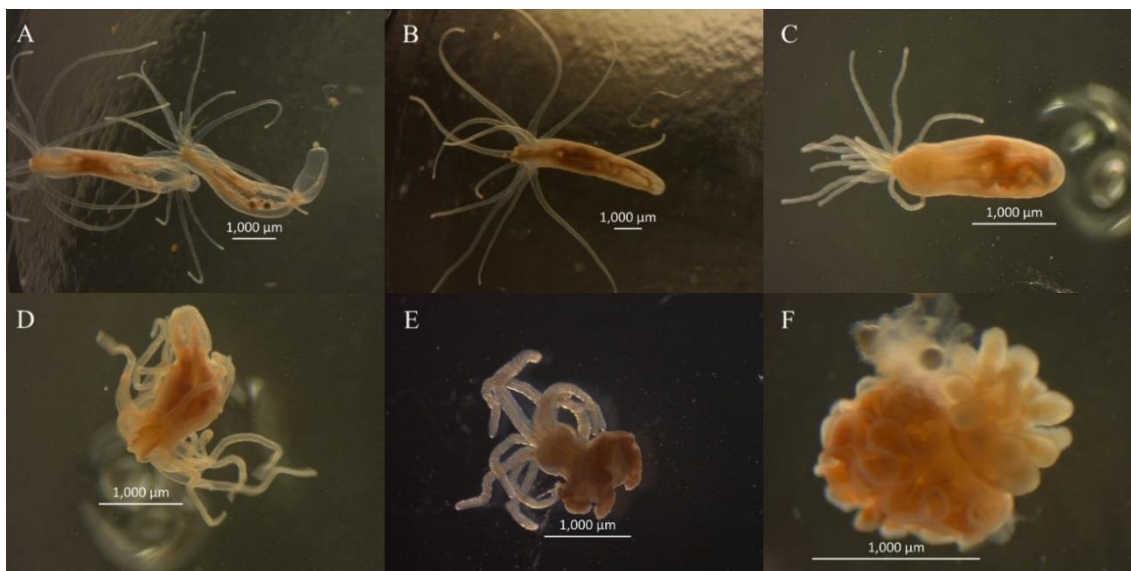
Fig. 4-1. Images of Starlet Anemones used in cadmium and copper acute exposure studies exhibiting characteristics of all stress-response index numbers. A-Index #0: no negative effects, healthy anemone. B-Index #1: minimal effects with slight column retraction. C-Index #2: moderate effects with partially shrunken column and deformed (clubbed) tentacles. D-Index #3: moderate effects with shrunken column and shortened deformed (limp & clubbed) tentacles. E-Index #4: severe effects with deformed tentacles, tissue loss along body column, severely shrunken column. F-Index #5: severe effects with complete loss of ectodermal tissue along body column resulting in exposed mesenteries, stubbed tentacles, opaque tissue, and loss of anchoring.

CHAPTER V: SUB-LETHAL EFFECTS OF DEEPWATER HORIZON OIL ON THE SWIMMING PERFORMANCE OF SHEEPSHEAD MINNOW, *CYPRINODON VARIEGATUS*, AND FLORIDA POMPANO, *TRACHINOTUS CAROLINUS*

ABSTRACT

Swimming ability has been used in the past as an indicator of performance or fitness in fish and is integral to traits like preference/avoidance responses, attraction to food and/or mates, and other social behaviors. We explore the technique of using swimming performance trials as a way to detect sublethal effects in an ecotoxicology assessment of a potential stressor. Sheepshead Minnows and Florida Pompano were exposed to a variety of oil/water mixtures and a reference toxicant for 24 hours and then placed into a swim respirometer to determine individual fish critical swimming speeds and oxygen consumption rates. Crude, weathered, and dispersed oil/water mixtures were prepared with samples collected during the Deepwater Horizon incident and were used at concentrations expected to demonstrate sub-lethal effects. Depressed and elevated responses to petroleum exposures from controls were observed, but were not consistent across both species. Sub-lethal swimming performance endpoints have excellent potential to be incorporated into ecotoxicology studies as they are sensitive at low concentrations of exposure and relevant to ecological impacts in the environment. We share the successes and failures of the trials and offer our insight and recommendations for future applications of this technique in ecotoxicology.

INTRODUCTION

Swimming ability is frequently used to determine the performance or fitness of individual fish or fish stocks (Brett 1964, Jones 1971, Farlinger and Beamish 1977, Hammer 1995, McDonald et al. 1998). Swimming speed and rates of metabolism can be used as fitness descriptors for fish and to demonstrate the influence of potential stressors such as temperature, oxygen, salinity, or chemical exposure (Jones 1971, Brocksen and Bailey 1973, Hammer 1995, Farrell et al. 1998, Jain et al. 1998, Plaut 2001, Massé et al. 2013a, Berli et al. 2014, Klinger et al. 2015). We investigate using swim performance study endpoints as sub-lethal indicators of stress using petroleum products and a reference toxicant. The aim is to present results and recommendations for future applications of this technique in ecotoxicology.

The sustained and increasing demand of oil and petrochemical products has resulted in a constant search and exploration of new deposits, especially offshore. These activities contribute to the release of petroleum products into the world's oceans. Charter (Charter et al. 1973) estimated the annual oil influx to the ocean to be 3 million tons. Thirty years later, this number has decreased to 1.3 million tons (NRC 2003), but the risk of major blowouts has increased with the oil exploration moving into deeper waters (Jernelv 2010). The Deepwater Horizon (DWH) incident beginning on April 20, 2010 resulted in an estimated discharge of 4.4×10^6 ±20% barrels of Macondo (MC-252) oil over 84 days (Crone and Tolstoy 2010).

A large portion of laboratory toxicity studies with aquatic organisms and MC-252 oil types have been based on traditional acute and chronic toxicity test methodology assessing classical acute and chronic testing endpoints (Hamdan and Fulmer 2011,

Hemmer et al. 2011, Goodbody-Gringley et al. 2013, Rico-Martínez et al. 2013, Echols et al. 2014, Faksness et al. 2015, Stefansson et al. 2016). When following standard ecotoxicology protocols based on methods such as those from American Society for Testing and Materials (ASTM), American Public Health Association (APHA), Organization for Economic Co-operation and Development (OECD), the studies generally used for safety assessment in regulatory actions and policy decision-making commonly use standard biological endpoints (e.g., mortality, growth, development, and reproduction) (USEPA 1975, 1994, 2002a, 2002b, 2002c). Acute toxicity studies can begin to explain the impacts of chemicals on organisms but they are limited because they are typically based on lethality. Sub-lethal indicators of stress are also useful in assessing impacts of chemicals and more specifically physiological and behavioral changes are frequently the most sensitive indicators of stress (Warner 1967, Little and Finger 1990, Gerhardt 2007). In addition, in the aquatic environment there is more likelihood that an organism is exposed to sublethal concentrations than acutely toxic levels unless they are within the immediate vicinity of a chemical spill. Sublethal effects fall into three categories: biochemical and physiological, behavioral, and histological (Sheehan et al. 1984). Behavioral responses are a combination of biochemical and physiological functions and are thus sensitive indicators of sublethal effect endpoints (Rand 1995).

A swimming performance study can generate a variety of quantitative endpoint values (e.g., critical swimming speed, sustained swimming speed, aerobic scope) which can be used as an indicator of fitness for an individual organism (Tierney and Farrell 2004, Tierney 2011a). Swimming performance studies monitor the ability of fish to swim at induced current speeds and the corresponding oxygen consumption over time while

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increasing the velocity of water over several steps. It is similar to a stress test in humans. The obtained data can provide MO_{2max} (maximum rate of oxygen consumption), MO_{2min} (minimum rate of oxygen consumption), total O_2 consumption, aerobic scope (absolute or fractional difference between minimum and maximum oxygen consumption), and U_{crit} (critical sustained or endurance swimming speed).

Research suggests that polycyclic aromatic hydrocarbons (PAHs), a major component of crude oils, are cardiotoxic (Incardona et al. 2004, 2009, 2011, Hicken et al. 2011, Brette et al. 2014) which can ultimately compromise the swimming performance of fish (Kennedy and Farrell 2006, Hicken et al. 2011, Milinkovitch et al. 2012, Mager et al. 2014). Exposures to PAHs have been shown to produce a dilation of the cardiac chambers in fish larvae (Incardona et al. 2004, 2011) and impair cardiac excitation-contraction coupling (Brette et al. 2014). We investigated the effects of short term sublethal exposure concentrations to petroleum products and a standard reference toxicant on swimming performance trial endpoints.

Effects of PAHs on swimming performance have been shown in a limited number of species (e.g. zebrafish, salmonids, and herring). The assessment of cardiac function and swimming performance/capacity has received some attention relevant to MC-252 after the DWH incident. De Soysa et al. showed that MC-252 exposures dramatically reduced touch sensitivity and impaired swimming behavior in zebrafish (de Soysa et al. 2012). Recently, Incardona et al. showed effects on heart development using bluefin tuna (*Thunnus thynnus*), yellowfin tuna (*Thunnus albacares*) and amberjack (*Seriola dumerili*) (Incardona et al. 2014). Swim tunnels were used with chub mackerel (*Scomber japonicas*) and detected an elevated routine energetic demand due to oil exposure

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(Klinger et al. 2015). Furthermore, Mager et al. showed effects on critical swimming velocities from exposure of embryos and juvenile mahi-mahi (*Coryphaena hippurus*) exposed to water accommodated fractions (WAFs) at 1.2 ± 0.6 µg/L and 30 ± 7 µg/L TPAH$_{50}$ respectively (Mager et al. 2014).

Swimming performance is important to individuals and populations since it can influence preference/avoidance responses, attraction to food and/or mates, and other social behaviors. Although rarely extrapolated, it is regularly assumed that changes in swimming performance ultimately can influence an organism's fitness, and therefore its contribution to the population and ecosystem (Hammer 1995, Plaut 2001). An impairment of swim performance could be linked to an underlying biophysical or physiological issue such as impairment to the cardiovascular system, liver function, or an inefficient metabolism of fat stores. Swim performance endpoints are also more practical than attraction/avoidance and locomotion tracking due to the ability to integrate physiological parameters. Swimming performance endpoints are commonly used in academic work but rarely in regulatory policy decisions but we believe they have potential and should be given consideration. Effects of short-term exposures to crude and weathered oil, a crude oil and dispersant mixture, dispersant-only, and a reference toxicant were investigated in this study using a series of swim performance studies with the Sheepshead Minnow, *Cyprinodon variegatus*, a standard reference species classically used in aquatic toxicology, and Florida Pompano, *Trachinotus carolinus*, a GoM native species. The goal of this study is to explore the idea of using swimming performance trials as a way of detecting sub-lethal effects.

The studies presented here were conducted at Florida International University (FIU) under the independent Natural Resource Damage Assessment (NRDA) Toxicology Program, and were based in part on the proposal: Aquatic Toxicity Test Program: Evaluate the Potential Acute and Chronic Toxicity of the MC-252 Crude Oil and Dispersant (Corexit 9500) Individually, and in Combination as well as the Weathered Dispersed Oil to Estuarine and Marine Organisms (Rand and Gardinali 2010).

METHODS

A swim performance protocol was prepared for each fish species studied which resembled the organization of the standard testing protocols used by the U.S. Environmental Protection Agency for aquatic toxicity testing. Each study underwent auditing through the laboratory's internal Quality Assurance Unit and from an external (independent) auditor to assure that each study was performed in accordance with the protocol and laboratory standard operating procedures (SOPs) for each phase of each study which ranged from test protocol development, through the in-life portion of testing including data documentation, statistical evaluation/calculations and generation of results.

Swim performance studies were conducted in Brett-style respirometer swim tunnels (Loligo systems, Tjele, Denmark, www.loligosystems.com). Studies included the use of Sheepshead Minnow, *Cyprinodon variegatus*, and Florida Pompano, *Trachinotus carolinus* to evaluate responses as a result of exposures to fresh and weathered oils, dispersant, and oil/dispersant mixtures of field-collected Mississippi Canyon Block 252 oils (Location of Deepwater Horizon Oil Incident). A reference toxicant (potassium chloride) typically used in acute aquatic toxicity testing was also used.

Test Organisms

Cyprinodon variegatus ranging from 0.5–4 g and 2–6 cm used in swim performance trials, were obtained from a commercial supplier (Aquatic Bio Systems Inc.) as embryos and maintained in-house in 50L glass tanks prior to experimentation. *T. carolinus* ranging from 1.1–9.7 g and 4.0–8.6 cm used in swim performance trials, were obtained from Troutlodge Marine Farms as embryos and held in 400 L fiberglass tanks prior to experimentation. Recirculating holding filtration systems for both species provided filtered natural seawater at 25±1 °C and salinities of 30±2 ppt (*C. variegatus*) and 36±2 ppt (*T. carolinus*) which were maintained in-house prior to experimentation at the Florida International University Ecotoxicology and Risk Assessment Laboratory (North Miami, Florida).

Animals were fed commercial flake or pellet food *ad libitum* during the holding period but were fasted during exposure (the 24 h immediately prior to the swimming performance trial), to avoid any potentially confounding effects of feeding on respiratory metabolism and swimming performance (Alsop and Wood 1997). All animal husbandry and testing protocols were conducted following the Florida International University's Institutional Animal Care and Use Committee approval.

A minimum of ten individual fish of each species were selected at random and used per treatment including controls.

Exposure Treatments

Oil sample exposure treatments were prepared using field-collected Macondo oil samples collected during the DWH incident. These samples were part of the collection of oils applicable to toxicity testing with the DWH NRDA program. More detail of these

oils can be found in the Echols et al publication discussing test variables affecting acute oil toxicity (Echols et al. 2016). Petroleum products and reference toxicants used to prepare exposure treatments included the following:

- Massachusetts, MC252 (MASS); unweathered crude source oil [Collected 26 July 2010 from the subsea containment system positioned directly over the well]

- Barge CTC02404 (Referred to as CTC or SlickA); weathered oil [Collected 29 July 2010 from a surface skimming barge]

- Corexit 9500; dispersant

- KCl (Potassium chloride); common reference toxicant used in standardized aquatic toxicity studies

Six treatment scenarios were performed for each species which included exposures to untreated control water (or diluent); a standard reference toxicant (KCl); the water accommodated fraction (WAF) of crude source oil (also known as MASS or MC-252); the WAF of weathered oil (also known as CTC or SlickA); the chemically enhanced water accommodated fraction (CEWAF) of crude oil and dispersant (MASS and Corexit 9500); and a dispersant-only WAF (Corexit 9500 only). All exposures of fish to test media were performed in 13.3 L chambers containing 8 L of the testing solution along with a small powerhead to generate gentle water movement to assist in maintaining dissolved oxygen levels. Test media was prepared using 0.45 µm filtered artificial salt water using an appropriate amount of Instant Ocean® salt to obtain the desired salinity. Treatment exposures were conducted for each individual fish separately for a duration of 24h prior to the swimming performance trial.

Exposure media preparation methods were based on previous techniques described in the literature specific to oil-water mixtures, e.g., *Chemical Response to Oil Spills: Ecological Effects Research Forum* (CROSERF) from 1994-2000 as discussed in Singer et al. (Singer et al. 2000, 2001a, 2001b), Neff (Neff 1999), Rhoton et al. (Rhoton et al. 2001), and the report *Critical Evaluation of CROSERF Test Methods for Oil Dispersant Toxicity Testing under Subarctic Conditions* (Barron and Ka'aihue 2003). The non-vortex WAF method described in these documents was used in all oil/water or oil and dispersant and water preparations to ameliorate the production of oil droplets in the exposure media and to generate a more stable and accurate measurement of the water soluble and bioavailable components from the mixture. A thorough discussion on oil/water mixture preparations specific to DWH oils can be found in the recent Sandoval et al. publication (Sandoval et al. 2017).

Crude oil (MASS) was extracted from the sample container using an air-tight glass syringe (weighed pre- and post-) to accurately determine the amount dispensed. The weathered oil (SlickA), due to its viscosity, was removed from the sample containers with stainless steel spatulas and placed into pre-weighed aluminum weigh pans. Contents of the pans were then scraped into the WAF solutions and then pans were post-weighed to determine the amount dispensed. Dispersant (Corexit 9500) was also measured using air-tight glass syringes.

WAFs, CEWAFs, and dispersant only WAFs were prepared in glass aspirator bottles with a bottom dispensing port. 1.8 L of 0.45 µm filtered artificial saltwater was added to a 2.0 L aspirator bottle containing a magnetic stir bar. Oil samples were added to the water at a loading rate of 1 g of oil per liter of water. After weighed test material was

added, the aspirator bottles were sealed with Duraseal® laboratory film. Sealed bottles were placed on individual stir plates (Corning PC-610D digital stir plates) and spun at 110 rpm. Mixing occurred in the dark in a temperature-controlled incubator at 25±1°C for 20 hours, and the oil-water mixture was then allowed to settle for four hours before decanting. All exposures were started on the day the prepared WAF solution completed mixing. To prepare a CEWAF, the identical procedure was followed except the dispersant (Corexit 9500) was added to the prepared WAF once it was on the stir plate at a loading rate of 1:20, dispersant: oil. The stir plate speed was increased to 120 rpm to ensure oil dispersion (but still maintaining no vortex). Dispersant-only mixtures were prepared by adding Corexit 9500 to saltwater at the identical ratio of a CEWAF but without any oil present.

Two factors were taken into consideration for choosing the concentrations of each exposure media treatment for each species in the tests conducted in this program because I was evaluating sublethal effects and therefore first assumed that behavioral and physiological changes from sublethal exposures are more sensitive indicators of environmental changes than extreme endpoints such as mortality or reproduction (Gerhardt 2007). In fact, behavioral effects in fish are typically observed at exposure concentrations that are orders of magnitude below those observed to produce mortality (LC50s) in standard toxicity tests (Beitinger, 1990; Little and Finger, 1990). Little and Finger showed that concentrations ranging from 0.1 to 5.0 % of an acute LC50 were commonly reported to induce changes in swimming behavior (Little and Finger 1990). Gerhardt also found behavioral effects 10 to 100 times more sensitive than mortality

(Gerhardt 2007). The exposure durations producing these effects were from hours to months for a diverse group of contaminants.

Second, exposure concentrations in this program also needed to be comparable to TPAH surface water concentrations to which organisms in the GOM were potentially exposed to during the DWH incident. Table 3 contains the 90th, 75th, 50th, and 25th centiles of TPAH concentrations from field-collected water samples in the GOM during the DWH incident (OSAT, 2010). As a source of comparison to the 90th centile (0.2102 µg/L $TPAH_{44}$), the ranges of TPAH concentrations from prepared exposure test media (100%, no dilution) from previous experience were estimated to be: MASS (crude) WAF: 80-100µg/L; CTC/SlickA (weathered oil) WAF: 12-15µg/L; and MASS CEWAF: 1000-1200µg/L.

Based on the above two factors we chose an exposure level of one tenth of the mean acute LC50 values obtained from previous toxicity studies conducted at FIU (unpublished) as previous work has indicated that swimming behavioral changes should be observed at this level. Exposure concentrations for each species with these test substances was calculated (Table 2) and compared to the database of field-collected samples (Table 1). These calculated exposure concentrations were found to be above the 90th centile of field-collected samples from the GOM and within a range believed to produce behavioral effects. The rationale behind the use of the 90th centile exposure concentration is that it has been used as an "exposure benchmark" in aquatic ecological risk assessments (Solomon et al. 1996, 2000, Giddings et al. 2005). The 90th centile exposure estimate assumes that 90% of the exposure samples will be below that benchmark if it comes from an exposure distribution which is unbiased and which

accurately represents the exposure concentrations found for a location during a given time period (Giddings et al. 2005). The 90th centile exposure concentration in the environment is located at the upper portion of the distribution and would be expected to be encountered less frequently (e.g., 10% of the time) and would represent episodic or pulsed exposures. The 1/10th LC50 values used to choose the dilution factors, the predicted TPAH, and the mean analytical TPAH detected for each treatment are shown in Table 2.

Swimming Performance

FIU Ecotoxicology & Risk Assessment Laboratory Protocol 218 (Assessment of swimming performance and exercise metabolism in fish following a 24-h exposure) was developed to provide guidelines for the swimming performance trials. A 5-L Brett-type swimming tunnel (Loligo Systems) was used for the swimming performance trials where the fish was contained within a portion of the chamber that maintains a laminar flow of water at a desired speed. The swimming tunnel was thermo-regulated at 25±1 °C using a recirculating water bath connected to sump with a submersible heater. Oxygen consumption was measured with an optical dissolved oxygen (DO) meter (Witrox®, Loligo System). Flow speeds were calibrated using a flow meter (Miniair®20) as recommended by the manufacturer. All the test parameters including the swimming speeds were controlled by a computer through the software Autoresp™. Oxygen levels were maintained at ≥4 mg/L.

Fish were fasted during the 24h exposure period prior the swimming trials. Individual fish were netted at random from the holding tank, measured and weighted, and placed into the exposure chamber containing a small powerhead to generate gentle water

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movement to assist in maintaining oxygen levels. After a 24 h exposure, animals were

netted from the bucket and placed into the swimming chamber which was surrounded by

black curtains to limit any external influence. Fish were allowed to acclimate to the

tunnel without flow until no signs of stress were observed and then for an additional 30

minutes minimum at a speed of 10 cm/s (Peake et al., 1997 proposed 30 min). The

acclimation process was monitored with cameras mounted near the tunnels and more time

was added if any sign of stress was observed. Each critical swimming speed test was

initiated at 15 cm/s and was performed as a ramp-U_{crit} test which allowed for changes in

step duration (Jain et al., 1997). Step duration and speed intervals were adjusted specific

to each species as illustrated in Figure 5-1 and were based on preliminary swim trials.

The speed was increased to approximately 75% of the expected U_{crit} for the particular fish

species in small steps of 5 min duration, after which step length was increased to 15 min,

for a goal of a total of approximately 10-13 steps (Jain et al., 1997; Plaut, 2001; Tierney,

2011). Step speed and duration were designed based on the swimming capabilities of the

species obtained in previous screening studies with the respective species and our

untreated control data.

If necessary, fish were motivated to not rest at the back of the chamber by using a

reversal of water flow and/or light stimuli. If the fish was unwilling to swim at the initial

steps of a trial, the test was considered invalid. The test was ended for individual fish

when they were unwilling to move off of the rear gate after two consecutive reverse flow

stimuli attempts. Fatigued fish were euthanized using buffered MS-222 (0.5 mg/L

tricaine methanesulfonate; Syndel).

Critical swimming speed (U_{crit}) was calculated using Equation 1 (Bret, 1974):

$$(1)\ U_{crit} = U_f + U_s \times \left(\frac{t_f}{t_s}\right)$$

U_f = water velocity of the last fully completed step;

U_s = increase of water velocity for the step prior to fatigue;

t_f = length of time the fish swam in the last step before fatigue;

t_s = full duration of the last complete step.

Dissolved oxygen concentrations recorded during the test were used to calculate oxygen consumption per time unit per weight unit (MO_2). MO_2 maximum and minimum were calculated as well as the aerobic scope. Aerobic scope was obtained both as absolute and fractional difference between minimum and maximum oxygen consumption (MO_2max - MO_2min or MO_2max /MO_2min).

    Chemistry

Sample collection activities, analytical procedures, and storage and holding times were consistent with the *Analytical Quality Assurance Plan. Mississippi Canyon 252 (Deepwater Horizon) Natural Resource Damage Assessment.* Version 2.2 (NOAA 2010). Quality control (QC) samples (e.g., duplicates, trip blanks, dilution water) were collected during the testing program. Samples for analytical chemistry analysis of exposure media (diluted WAFs) were collected at the initiation and termination of exposures.

One liter (1000 mL) samples were collected immediately following the preparation of the exposure solutions into amber glass jars with minimal headspace for parent and alkylated polycyclic aromatic hydrocarbons (PAHs), and saturated hydrocarbon (SHC) analyses. Additional samples (3 x 40 mL) were collected in 40-ml amber vials and preserved with HCL (no headspace) for volatile organic compound (VOA-PIANO) analysis. Additional

samples (2 x 24 mL) preserved with 30% acetonitrile were collected for treatments where

dispersant was used for DOSS analysis. All samples were labeled and chain-of-custody

(COC) forms were completed. Prior to and during shipping to the analytical laboratory

Battelle Duxbury Operations in Duxbury, Massachusetts (via overnight courier) or the

Environmental Analysis Research Laboratory (EARL), FIU in North Miami, FL samples

were maintained in the dark at 4°C from the time of collection.

To prepare exposure media for treatments in these studies, a variable dilution method was

used in which a single 1:1000 oil-to-water ratio WAF, CEWAF, or dispersant only WAF

solution was used as the source to prepare the desired dilution of test media exposures

expressed as volume percentages of the initial WAF for a test using laboratory control

water (0.45 µm filtered artificial seawater) as the diluent. All samples were collected in

certified, new glass containers and refrigerated immediately at or below 4°C during

storage and transit to the analytical laboratory. Samples were shipped overnight with full

COC documentation.

Samples of each treatment prepared used for these studies were analyzed at FIU-EARL

(North Miami, FL) or Battelle (Duxbury, MA). Each sample was extracted with

methylene chloride and analyzed for trace semi-volatile hydrocarbon analyses, polycyclic

aromatic hydrocarbons (parent PAHs and alkyl PAHs or APAHs; Table 5-3) and

saturated hydrocarbons (SHC; Table 5-4) at FIU-ERAL. Samples were also analyzed for

volatile organic compounds (Table 5-5) at Battelle. Exposure media containing dispersant

(Corexit 9500) were also analyzed for the individual dispersant marker DOSS at FIU-

EARL. Analytical chemistry results for the test solutions are summarized in Table 5-6.

Data Analysis

Mean and standard deviations were determined for all endpoints collected for each treatment group of each species. One-way analysis of variance (ANOVA) was used to compare treatment groups to the control. Significant difference from the control treatments and grouping assignments was determined using Dunnett's test and is indicated in box-plots of the endpoints using letters. Further investigation and data visualization included data transformations, flow dynamic indices (Reynolds numbers), and standardization using body condition indices. The latter will be explained in the discussion.

RESULTS

Analytical chemistry analysis verified that the exposure scenarios were within range of the predicted TPAH as demonstrated in Table 5-2. A summary of analytical chemistry analysis data from swimming performance study exposures is presented in Table 5-6. Measured TPAH is reported as TPAH(50) which is the sum of the 50 PAHs contained in Table 5-3. Additional information and discussion on analytical chemistry, the oils used, and preparation techniques can be found in the Echols et al. and Sandoval et al. manuscripts (Echols et al. 2015, 2016, Sandoval et al. 2017).

The four endpoints collected from swimming performance studies included: U_{crit} in centimeters per second, U_{crit} in body lengths per second, aerobic scope as a fraction (MO_{2max}/MO_{2min}), and aerobic scope as a difference ($MO_{2max}-MO_{2min}$). *C. variegatus* U_{crit} in distance ranged from 30.6-40.5 cm/s with the highest from the control treatment while U_{crit} in body length ranged from 5.4-8.5 bl/s with the highest from the KCl treatment. Aerobic scope of *C. variegatus* ranged from 1.37-2.22 (fraction) and 0.24-0.84 (difference) with the highest for both from the control treatment. *T. carolinus* U_{crit} in

distance ranged from 52.4-62.0 cm/s while U_{crit} in body length ranged from 8.17-12.4 bl/s with the highest for both from the crude oil (MASS) WAF treatment. Aerobic scope of *T. carolinus* ranged from 1.42-1.90 (fraction) and 0.39-0.78 (difference) with the highest for both in the crude oil (MASS) WAF treatment. Means and standard deviations of these endpoints for all treatments with *C. variegatus* are included in Table 5-7 and *T. carolinus* in Table 5-9. The results of ANOVA tests are found in Table 5-8 and 5-10 and include the p-value associated with each statistical test. Figures 5-2 through 5-5 and 5-6 through 5-9 are boxplots of the four endpoints across all treatments collected during *C. variegatus* and *T. carolinus* swim trials. Median and quartiles are represented by a line within a box while the treatment means are represented by a cross within a circle and an asterisk indicates outliers while significant differences from the control treatment group determined by Dunnett's test is indicated with letter groupings.

All petroleum treatments with *C. variegatus* demonstrated significantly lower U_{crit} values (cm/s and bl/s) than the control and reference toxicant (KCL) treatments (Figures 5-2 and 5-3). All oil treatments and the reference toxicant treatment using *C. variegatus* demonstrated significantly lower aerobic scope (fraction and difference) than the control treatment (Figures 5-4 and 5-5).

U_{crit} values in cm/s of *T. carolinus* swim trials showed no difference from the control treatment, however when using body length/s, the weathered oil (SlickA/CTC) and crude oil (MASS) treatments demonstrated elevated U_{crit} values (Figures 5-6 and 5-7). The aerobic scope (fraction) of *T. carolinus* swim trials was higher in the dispersed crude oil (MASS CEWAF) than the control treatment while the aerobic scope

(difference) of the crude oil (MASS WAF) and the dispersed crude oil (MASS CEWAF) treatments were higher than the control treatment (Figures 5-8 and 5-9).

The reference toxicant treatment of *C. variegatus* along with all oil treatments had lower aerobic scope than the control (Figures 5-4 and 5-5) whereas the crude oil CEWAF and WAF treatments of *T. carolinus* had elevated aerobic scope (Figures 5-8 and 5-9)

DISCUSSION

The results from the trials are presented first, followed by variables that may influence fish swimming ability and the implications of how that affects swimming data, theory and application of swimming ability data, followed by recommendations and comments for future work. Upon initial observation when looking at the critical swimming speed endpoint in cm/second, it appears that all petroleum treated *C. variegatus* (treatments 3-6) had depressed swimming ability (Figure 5-2) while there was no change in any of the *T. carolinus* treatments (Figure 5-6). However, when looking at critical swimming speed in body lengths/second the same result is observed in *C. variegatus* (Figure 5-3) but the *T. carolinus* treatments with weathered and unweathered oils (CTC and MASS) demonstrated elevated swimming abilities (Figure 5-7). This is an example of how the presentation of swimming data may affect initial conclusions. Ideally, the goal is to establish a direct link between an exposure level and a reduced or elevated organismal response.

Swim performance is considered a fitness parameter because of its potential link to predator-prey interactions, attraction-avoidance responses, locating food, and reproductive behaviors. Changes in swimming performance endpoints could thus be considered indicators of stress for evaluating sublethal effects which could potentially

lead to death making them critically important to the fitness of those individuals. Swimming performance studies provide an opportunity to collect data on the physical condition of an aquatic organism and link that to physiological stress at a much lower concentration than standard aquatic toxicity studies. This kind of information is useful in the determination of early signs of stress in organisms. The determinations of concentrations of chemicals that induce sub-lethal physiological effects are equally important as they can cause effects that could have impacts at the individual and population level.

The data demonstrate that sublethal exposures to toxicants (at one tenth of lethal concentrations) can provide detectible responses in critical swimming speeds and oxygen consumption rates. Although that response may not always be the inhibition of swimming ability as observed in the elevated responses from *T. carolinus* (Figures 5-6 through 5-9). During these experimental trials, it was determined that a variety of variables are linked with swimming ability and some are difficult to control.

Restrictions on age or size are frequently used to facilitate the reproducibility of aquatic toxicity studies. This will also play an important role in swimming performance studies. Our experience with these two species has confirmed that fish length and mass have considerable influence on swimming ability as shown when plotting fish length or mass versus U_{crit} (Figures 5-10 and 5-11). Two fish of the same length but differing masses would expectedly have different swimming abilities. If one is older than the other, it has had more time to build up more muscle and/or fat which will have an influence on the swimming ability. This demonstrates the need to have swimming ability data for a wide range of size classes to be able to determine with certainty any effects of exposures.

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Previous studies have shown that fish have different profiles of swimming ability during times of morphological changes in developing from larval to juvenile and into adult stages (Fisher et al. 2000, Bellwood and Fisher 2001). Most fish have an increasing trend in swimming ability during early development from hatch to early larval stages (at less than 20 mm of length, but with differing slopes) followed by a decreasing trend in swimming ability with increasing length or mass when looking at body lengths per second swimming ability. Declining trend is visible from these studies (Figures 5-10B, 5-10D, 5-11B, 5-11D and Figures 1 and 2 from Bellwood and Fisher, 2001). Ideally, it would be advantageous to work with a hatchery or an aquaculture facility which would provide access to all the stages of a fish species throughout its development to be able to create a species-specific standard expected swimming ability profile for an untreated fish. This could then be used to determine appropriate stages of a fish's development where swimming ability is relatively stable and potential effects of exposures would be easier to elucidate.

Swimming speed (U_{crit}) in cm/s is a common and valuable endpoint used to measure individual fish fitness but it is difficult to use for comparisons between fish of different length, mass, or age as is evidenced in Figure 5-10A, 5-10C, 5-11A, and 5-11C. Previous work has demonstrated a variety of ways to compare swimming speeds using data transformations or normalization attempts using body condition factors (McDonald et al. 1998, Peig and Green 2009, Ralph et al. 2012, Massé et al. 2013b). Some experimental designs looks at pre- and post-exposure swimming performance for comparisons and would not require any data transformation, but if the study design has a control treatment group and all trials are meant to be novel experiences for each fish,

transformation may need to be considered (Hymel et al. 2002). The simplest is to use a body length per second transformation. Plotting fish size vs swimming ability may demonstrate differences in treatments but it is not always clear (see Figure 5-10B, 5-10D, 5-11B, 5-11D). Other examples of transformations include log transformation of length and speed, age vs log speed, and length vs Reynolds number, or body condition factors (Little and Finger 1990, Fisher et al. 2000, Stead et al. 2005, Luís and Guilhermino 2012, Massé et al. 2013b).

Log transformations can sometimes result in relatively linear relationships between length or age and swimming speed and the slopes of regression lines from treatment groups may be used as a comparison tool between treatments or species and help visualize difference between treatments (Fisher et al. 2000). Linear regressions from plotting log(length) vs log($U_{crit(bl/s)}$) provide lines with differing slopes but poor correlation coefficients (Figures 5-13 and 5-15). Regressions from log(length) vs log($U_{crit(cm/s)}$) returned similar results and are not practical to use for statistical comparisons with the current data (Figures 5-12 and 5-14). However, a more robust data set may be needed to demonstrate this technique. This illustrates the need for a full swimming profile of the species used to know how the swimming ability changes over the full range of lengths and masses from larval to juvenile to adult stages and with varying body conditions.

Reynolds number is a unit-less value commonly used in fluid dynamics, which is an index of the hydrodynamic conditions governing flow around an object (Webb and Weihs 1986, Wilson and Franklin 2000, Bellwood and Fisher 2001). This index is an appropriate value to use when describing fish swimming abilities due to the integration of

water quality parameters and fish size along with swimming velocity. Fuiman and Batty demonstrated using Reynolds number based on a larval length parameter using Equation 2 when describing effects of temperature on the swimming ability of larval Atlantic herring (Fuiman and Batty 1997).

$$(2)\ Re_L = \rho U L \mu^{-1}$$

Re_L = Reynolds number based on a length parameter;

ρ = density of water;

U = swimming speed;

L = length parameter (usually total length of fish but other length parameters have been used);

μ^{-1} = dynamic viscosity of water

Webb and Weihs demonstrated using Reynolds number also based on a length parameter using Equation 3 while describing functional locomotor morphology of early life stage fishes (Webb and Weihs 1986).

$$(3)\ R = U \times \frac{L}{\nu}$$

R = Reynolds number based on some characteristic linear dimension, often indicated by a subscript;

U = velocity;

L = Length;

ν = kinematic viscosity

Formulas provided by El-Dessouky and Ettouney allow for the calculation of density, kinematic and dynamic viscosity of water based on a known salinity and

temperature (El-Dessouky and Ettouney 2002). The units of these viscosities are different and care must be taken to be sure the calculation is done correctly so that the velocity and length parameters cancel out so the Reynolds number is unit-less. For example, using Equation 3, seawater at 36 ppt and 25°C will have a kinematic viscosity of 0.938×10^{-6} m^2/s. A Florida Pompano 7.79 cm in length with an U_{crit} of 90 cm/s would have a Reynolds number of 74744. A Sheepshead Minnow tested at 30 ppt and 25°C (kinematic viscosity=0.930×10^{-6} m^2/s) with a length of 4.5 cm and an U_{crit} of 37 cm/s would have a Reynolds number of 17903.

Calculating the Reynolds number based on a fish length is commonly used to understand how larval or fish body shape interacts with flow to determine drag but could potentially be used as a way to normalize swimming performance data (Webb and Weihs 1986). Something to keep in mind is that not all fish have the same body shapes or swimming patterns. For example, Florida Pompano are slender fish and exhibit a Carangiform mode of swimming where majority of thrust is generated in the posterior third of the fish with a narrow caudal peduncle and high aspect forked caudal fin which increases thrust efficiency (Honebrink 2000). On the other hand, Sheepshead Minnows are a bulkier tube shaped fish and frequently hover by undulating their pectoral and caudal fins (similar to the Rajiform and Diodontiform swimming mode) and only using the full force of their paddle-shaped caudal fin in a Subcarangiform mode for quick bursts of speed when necessary (personal unrecorded observation) (Sfakiotakis et al. 1999). Swimming behavior, body shape, location of fins, body proportions, embryonic size, and other factors all influence locomotor activities and therefore swimming ability and Reynolds numbers (Webb and Weihs 1986, Fuiman and Batty 1997, Fisher et al. 2000,

Wilson and Franklin 2000, Bellwood and Fisher 2001, McDonald and Grunbaum 2010).

This should be taken into consideration when determining which body size parameter/s to use to normalize the data or to calculate a Reynolds number. For an example, Figures 5-16 and 5-17 demonstrate using Reynolds number to normalize swimming ability and show the typical increasing trend of Reynolds number with fish length. However, it is difficult to visualize differences in swimming abilities between treatments of *T. carolinus* (Figure 5-16) yet the lower swimming speeds from the petroleum treatments in *C. variegatus* is evident (Figure 5-17).

Body condition, condition factor, or a condition index is commonly used in ecology, aquaculture, and fishery sciences as a non-destructive method to assess the fitness of a fish or fish stocks (Bolger and Connolly 1989, McDonald et al. 1998, Tierney and Farrell 2004, Peig and Green 2009, Weirich et al. 2009, Marit and Weber 2012, Ralph et al. 2012). This is another parameter that may be used to standardize swimming speeds. Condition factors (K) have been calculated in many ways but one of the most common is Equation 4 using mass and length (McDonald et al. 1998, Weirich et al. 2009). While another example found in Equation 5 is frequently used with swim performance studies when fork length is available (Tierney and Farrell 2004).

$$(4)\ K = 100 \times \left(\frac{W}{L^3}\right)$$

W = organism mass in g

L = total length in cm

$$(5)\ K_{FL} = \frac{(W \times 10^5)}{FL^3}$$

W = organism mass in kg

FL = fork length in cm

Using total length, we provide an example of using body condition K (Equation 4) as a data transformation with U_{crit}. Data transformation and plots of body condition vs swimming abilities can be visualized in Figures 5-18 and 5-19. This type of transformation provides a clear separation of the treatment groups as seen with the elevated swim performance of the CTC WAF (green diamonds) and MASS WAF (blue triangles) exposure groups in the Florida Pompano (Figure 5-18B) or the depressed swim performance of the petroleum exposure groups in Sheepshead Minnows (Figure 5-19).

Linking swimming performance directly to organism traits considered ecologically relevant is a difficult task. Buckham demonstrated that ocean acidification (increased pCO_2) affected larval swimming abilities in *Ostrea lurida* but not *Crassostrea gigas* (Buckham 2015). The implications of this include impacts on larval transport, connectivity and success of later developmental stages. Plaut's review comments on correlations between routine activity, metabolic rates, and body size to U_{crit} but emphasizes that this data indirectly suggests ecological relevancy of U_{crit} but does not make direct connections to reproductive success or survival (Plaut 2001). Tierney and Farrell investigated physical abnormalities linked to reduced swim speed and recovery ratio and only the most severe abnormalities showed a correlation (Tierney and Farrell 2004). Due to the difficulty and longevity of low concentration non-lethal studies to be able to achieve those direct connections, the use of probabilistic models integrating a variety of fitness parameters and stressors may be a practical tool to investigate.

One of the purposes of this work was to help understand how the use of swimming performance could be incorporated into toxicity studies. Experimental design

of a swim performance study is paramount. This experience demonstrated that preliminary trails are crucial for determining an appropriate experimental setup for each fish species. An estimated U_{crit} value for an untreated fish is required to be able to plan the appropriate step duration and speed increases for that species. The "typical" or normal performance of the fish species will dictate these parameters. Looking at the steps of a ramp-U_{crit} swim performance study for both *C. variegatus* and *T. carolinus* (Figure 5-1) show, that one test design is not appropriate for the other species. Preliminary trials will provide experience to the individuals handling the fish and data necessary to design a proper swim test for the species in order to get the desired 10-13 steps before fish exhaustion (Jain et al. 1997, Plaut 2001, Tierney 2011a).

The examples of data transformation and attempts to normalize swim performance data demonstrate the need for previous knowledge before designing a study from an ecotoxicogical point of view. Acute lethality information on the toxicant in question is necessary to know appropriate exposure ranges. Prior work should include developing a swimming profile from the embryonic stage through larval development and into juvenile stages for the species in question to determine the appropriate age/sizes for a study with treatment groups where the variability can be decreased as much as possible to facilitate the comparison between groups to detect effects. It has been shown that a considerable number of variables can influence swimming abilities from fin size and lengths, higher temperatures in development resulting in increased swimming ability, while lower temperature resulted in a larger larva at metamorphosis with poorer swimming abilities (Wilson and Franklin 2000, Green and Fisher 2004, Sfakianakis et al. 2011). Establishing a swimming ability profile will remove these factors from

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confounding effects between the treatment groups. In the examples provided, using body lengths per second appears a better demonstration of swimming ability as is evidenced in Figure 5-10B and 5-10D. Also, the use of a body condition transformation performed the best for visually separating treatment groups as shown in Figures 5-18B and 5-19B.

Fish personalities also play a role in the ability to have successful trials (Toms et al. 2010, Tierney 2011b). Behavior is a trait of an organism that contributes to its fitness (Biro and Stamps 2008, Dingemanse et al. 2010, Langerhans and Reznick 2010). Some fish simply do not desire to swim. Even with encouraging stimuli, they may continue to rest on the back grate of the chamber and not swim. Some fish would even position their body in a way that they could rest their caudal fin on the grate while orientating their body into the flow of water and lean on the side of the chamber so they did not have to expend the effort to swim. It has been suggested to use mechanical or electrical motivation using an electric grate at the back of the chamber to encourage the fish to swim in the center of the chamber and stay away from the grate (Tierney 2011, Ralph et al. 2012). For the trials presented here, individuals demonstrating excessive non-swimming behavior were not included in any analysis.

Several questions arose while analyzing our swim performance data that would be interesting to investigate in the future. How does a fish's "normal" behavior or life history traits influence swimming ability and more importantly a chemical's effect on that ability? For example, Sheepshead Minnows are relatively sedentary while living in marsh/glade areas and rarely exert themselves while Florida Pompano are known to be extremely active while living in the surf zone. How will these activities and metabolism capabilities influence how a chemical (in our case petroleum products) interacts with an

organism during a short-term exposure and exercise? If so, how long does a short-term exposure impact the fish? Fish are known to metabolize PAHs through a variety of enzymatic actions and excrete toxic metabolites (Tuvikene 1995) but how quickly can these fish metabolize the PAHs in their bodies and does this influence the chemicals effect on the organism's swimming ability immediately following an exposure? Why does this data appear to demonstrate decreases in the endpoints collected for Sheepshead Minnows while Florida Pompano appear to be elevated? A larger range of exposure concentrations and organism sizes would be needed to determine if this trend continues and/or intensifies throughout the swimming profile and increasing PAH concentrations. Swimming ability is comprised of a mix of locomotor abilities and is just one component of several also including fish body design, life history traits, behavior, and others, all contributing to an organism's fitness (Langerhans and Reznick 2010).

Another aspect of organism fitness is the ability to recover from a stressor or exhaustion. Several studies have proposed back to back swimming performance tests separated by a short recovery period as an indicator of stressors to fish health (Farrell et al. 1998, Jain et al. 1998, Massé et al. 2013a). This is a more intensive test of the physiological condition of the fish and provides more information than individual U_{crit} values. Repeat swim performance trials produce recovery ratios and normalized recovery ratios which can relate treated fish to the control group. This information could also be applied to predator/prey interaction scenarios. The Jain et al. study showed that the recovery ratio endpoints are a more sensitive endpoint than initial U_{crit} values when working with stockeye salmon exposed to dehydroabietic acid (Jain et al. 1998). The next

step in using data gathered from swimming performance studies is linking changes in swim performance to ecological level impacts.

In summary, sub-lethal swimming performance endpoints have excellent potential to be incorporated into ecotoxicology studies as they are sensitive at low concentrations of exposure and relevant to ecological impacts. Prior knowledge of swimming performance of the species in question is absolutely necessary to establish an appropriate testing method/protocol for that organism. Establishing a swimming profile throughout the development of the fish is a necessity to determine the appropriate stage for testing. A variety of normalization or data transformation techniques should be attempted to appropriately visualize the data and to determine differences between treatment groups. Our trials demonstrated it was possible to detect changes in swim performance endpoints at one tenth of lethal concentrations. However, ,the species used had conflicting responses with a depression in endpoint values in Sheepshead Minnows while some treatment groups from the Florida Pompano exhibited elevated endpoints. Testing a broader range of concentrations may help understand these effects. We recommend future swimming performance work apply the repeat swim performance trial technique (for potential recovery and to provide more physiological data). This addition of another trial will create a more robust data set and better statistical comparison. Consideration should be given to longer-term exposures and multiple swim trials throughout the life span of organisms to attempt to bridge the knowledge gap between transient effects and physiological endpoints and the ecological implications of those changes. This longer-term data would provide a much clearer indication of lasting effects on the organismal fitness and its connection to population level and ecological impacts.

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Table 5-1. Examples of TPAH from field samples.

Field Collected Centiles [a]	TPAH (µg/L) [tot=44]
90th	0.2102
75th	0.0435
50th	BDL (0.00597)
25th	ND

[a] Data from field sample database (OSAT, 2010)

Table 5-2. Treatment concentration dilution decision process.

Organism Exposure Scenario	1/10th of mean LC50 (%WAF)[a]	Chosen Dilution (%WAF)	Predicted TPAH (µg/L)	Measured TPAH (µg/L)[b]
T. carolinus MASS WAF	3.05	3	3	7.22
T. carolinus SlickA WAF	5.23	5	0.75	0.682
T. carolinus MASS CEWAF	6.1	3[c]	30	23.8
T. carolinus DISP Only WAF	3.45	3.50	NA	0.0177
C. variegatus MASS WAF	>10	10	10	26.25
C. variegatus SlickA WAF	>10	10	1.5	1.64
C. variegatus MASS CEWAF	NA[d]	10	100	115
C. variegatus DISP Only WAF	NA[d]	10	NA	0.0123

[a] Data from acute toxicity studies previously conducted at FIU (unpublished data)
[b] Treatment mean calculated from analytical data for each exposure scenario
[c] 3% chosen for MASS CEWAF exposure to be comparable to MASS WAF exposure as a CEWAF is expected to have higher concentration of PAHs and using a higher percentage dilution of CEWAF vs WAF product is not logical.
[d] Data not available.

Table 5-3. Summary of PAH (Parent and Alkyl Homologs) and Related Compounds Evaluated for the FIU Swim Performance Studies

Polycyclic Aromatic Hydrocarbons		
1,3-Dimethylnaphthalene	Biphenyl	C3-Phenanthrenes/Anthracenes
1-Methylnaphthalene	C1-Chrysenes	C4-Chrysenes
1-Methylphenanthrene	C1-Dibenzothiophenes	C4-Naphthalenes
2,3,5-Trimethylnaphthalene	C1-Fluoranthenes/Pyrenes	C4-Phenanthrenes/Anthracenes
2,6-Dimethylnaphthalene	C1-Fluorenes	Carbazole
2-Methylnaphthalene	C1-Phenanthrenes/Anthracenes	Chrysene
Acenaphthene	C2-Chrysenes	Dibenz[a,h]anthracene
Acenaphthylene	C2-Dibenzothiophenes	Dibenzothiophene
Anthracene	C2-Fluorenes	Fluoranthene
Benz[a]anthracene	C2-Naphthalenes	Fluorene
Benzo[a]fluoranthene	C2-Phenanthrenes/Anthracenes	Indeno[1,2,3-cd]pyrene
Benzo[a]pyrene	C3-Chrysenes	Naphthalene
Benzo[e]pyrene	C3-Dibenzothiophenes	Perylene
Benzo[g,h,i]perylene	C3-Fluorenes	Phenanthrene
Benzo[k]fluoranthene	C3-Naphthalenes	Pyrene

Table 5-4. Summary of Saturated Hydrocarbon Compounds Evaluated for the FIU Swim
Performance Studies

Saturated Hydrocarbon Compounds		
n-Decane	n-Hexadecane	n-Tetracosane
n-Docosane	n-Hexatriacontane	n-Tetradecane
n-Dodecane	n-Nonacosane	n-Tetratriacontane
n-Dotriacontane	n-Nonadecane	n-Triacontane
n-Eicosane	n-Nonane	n-Tricosane
n-Heneicosane	n-Octacosane	n-Tridecane
n-Hentriacontane	n-Octadecane	n-Tritriacontane
n-Heptacosane	n-Pentacosane	n-Undecane
n-Heptadecane	n-Pentadecane	Phytane
n-Hexacosane	n-Pentatriacontane	Pristane

Table 5-5. Summary of VOC Analyses for the FIU Swim Performance Studies. (BTEX indicated in bold)

Volatile Organic Compounds			
1,1,3-trimethylpentane	2,2-dimethylpentane	4-isopropyltoluene	n-C6
1,2,4,5-Tetramethylbenzene	2,3,4-trimethylpentane	**Benzene**	n-C8
1,2,4-Trimethylbenzene	2,3-dimethylbutane	Benzothiophene	n-C9
1,2-Diethylbenzene	2,3-dimethylhexane	Benzene, 1-Methyl-4-Propyl-	n-C10
1,2-Dimethyl-4-Ethylbenzene	2,3-dimethylpentane	Benzene, 1-methyl-2-(1-methylethyl)-	n-C11
1,3,5-trimethylbenzene	2,4-dimethylpentane	cis-2-Pentene	n-C12
1,3-Dimethyl-2-Ethylbenzene	2,5-Dimethylhexane	Cyclohexane	n-Pentylbenzene
1,3-dimethyl-4-ethylbenzene	2-Ethylthiophene	Cyclopentane	Octene-1
1,3-Dimethyl-5-Ethylbenzene	2-Methyl-1-Butene	**Ethylbenzene**	**o-XYLENE**
1,4-dimethyl-2-ethylbenzene	2-Methyl-1-Hexene	Heptane	Pentane, 2,3,3-trimethyl
1-Decene	2-methylheptane	Hydrindene	Propylbenzene
1-Ethyl-2,3-Dimethylbenzene	2-methylhexane	Isopentane	**p-XYLENE**
1-Hexene	2-Methylpentane	Isopropylbenzene	sec-Butylbenzene
1-Methyl-2-ethylbenzene	2-Methylthiophene	m-cymene	STYRENE
1-Methyl-2-n-Propylbenzene	3-ethylhexane	Methylcyclohexane	tert-Butylbenzene
1-Methyl-3-ethylbenzene	3-methylheptane	Methylcyclopentane	Thiophene
1-methyl-3-n-propylbenzene	3-methylhexane	**m-XYLENE**	**Toluene**
1-Nonene	3-Methylpentane	Naphthalene	trans-2-Pentene
1-Pentene	3-Methylthiophene	n-Butylbenzene	
2,2,4-Ttimethylpentane	4-Ethyltoluene	n-C5	

Table 5-6. Summary of analytical chemistry mean values for study treatment exposure scenarios.

Organism Exposure Scenario	Measured TPAH (µg/L)[a]	Measured DOSS (µg/L)[a]
T. carolinus MASS WAF	7.22	NA[b]
T. carolinus SlickA WAF	0.682	NA[b]
T. carolinus MASS CEWAF	23.8	194
T. carolinus CX9500 Only	0.0177	220
C. variegatus MASS WAF	26.25	NA[b]
C. variegatus SlickA WAF	1.64	NA[b]
C. variegatus MASS CEWAF	115	846
C. variegatus CX9500 Only	0.0123	645.2

[a] Treatment mean calculated from analytical data for each exposure scenario. TPAH=sum of polycyclic aromatic hydrocarbon concentrations (n=50) from SVOC GC/MS-SIM analysis.
[b] Not Applicable

Table 5-7. Statistical summary for *C. variegatus* swimming performance study endpoints.

Endpoint:	U_{crit} (cm/s)		U_{crit} (bl/s)		Aerobic Scope (Fraction)		Aerobic Scope (Difference)	
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	40.5	3.8	8.1	0.8	2.22	0.63	0.84	0.43
KCl	40.3	6.9	8.5	1.3	1.37	0.19	0.46	0.21
SlickA WAF	25.9	2.8	5.4	0.8	1.49	0.25	0.24	0.09
MASS WAF	29.1	6.6	5.7	1.4	1.52	0.30	0.25	0.18
MASS CEWAF	30.7	5.7	6.5	1.0	1.45	0.24	0.27	0.09
CX9500 Only	30.6	4.7	6.8	0.9	1.51	0.36	0.28	0.15

Table 5-8. Statistical analysis of endpoints from *C. variegatus* swimming performance studies.

Endpoint:	U_{crit} (cm/s)		U_{crit} (bl/s)		Aerobic Scope (Fraction)		Aerobic Scope (Difference)	
Treatment	Different from CTL?	p-value	Different from CTL?	p-value	Different from CTL?	p-value	Different from CTL?	p-value
Control	NA	NA	NA	NA	NA	NA	NA	NA
KCl	No	0.918	No	0.473	Yes	0.000	Yes	0.000
SlickA WAF	Yes	0.000	Yes	0.000	Yes	0.000	Yes	0.000
MASS WAF	Yes	0.000	Yes	0.000	Yes	0.000	Yes	0.000
MASS CEWAF	Yes	0.001	Yes	0.002	Yes	0.000	Yes	0.000
CX9500 Only	Yes	0.000	Yes	0.009	Yes	0.000	Yes	0.000

Table 5-9. Statistical summary for *T. carolinus* swimming performance study endpoints.

Endpoint:	U_{crit} (cm/s)		U_{crit} (bl/s)		Aerobic Scope (Fraction)		Aerobic Scope (Difference)	
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	55.9	14.7	8.17	1.9	1.42	0.23	0.39	0.12
KCl	53.4	9.7	9.9	3.8	1.69	0.33	0.64	0.31
SlickA WAF	56.3	6.1	11.6	1.2	1.51	0.23	0.69	0.27
MASS WAF	62.0	18.5	12.4	3.2	1.66	0.34	0.77	0.26
MASS CEWAF	52.4	7.9	9.5	1.5	1.90	0.62	0.78	0.34
CX9500 Only	56.2	9.2	9.7	1.5	1.75	0.23	0.71	0.18

Table 5-10. Statistical analysis of endpoints from *T. carolinus* swimming performance studies.

Endpoint:	U_{crit} (cm/s)		U_{crit} (bl/s)		Aerobic Scope (Fraction)		Aerobic Scope (Difference)	
Treatment	Different from CTL?	p-value	Different from CTL?	p-value	Different from CTL?	p-value	Different from CTL?	p-value
Control	NA	NA	NA	NA	NA	NA	NA	NA
KCl	No	0.955	No	0.220	No	0.077	No	0.051
SlickA WAF	No	0.752	Yes	0.000	No	0.382	Yes	0.009
MASS WAF	No	0.314	Yes	0.002	No	0.107	Yes	0.002
MASS CEWAF	No	0.798	No	0.086	Yes	0.049	Yes	0.007
CX9500 Only	No	0.701	No	0.069	Yes	0.007	Yes	0.001
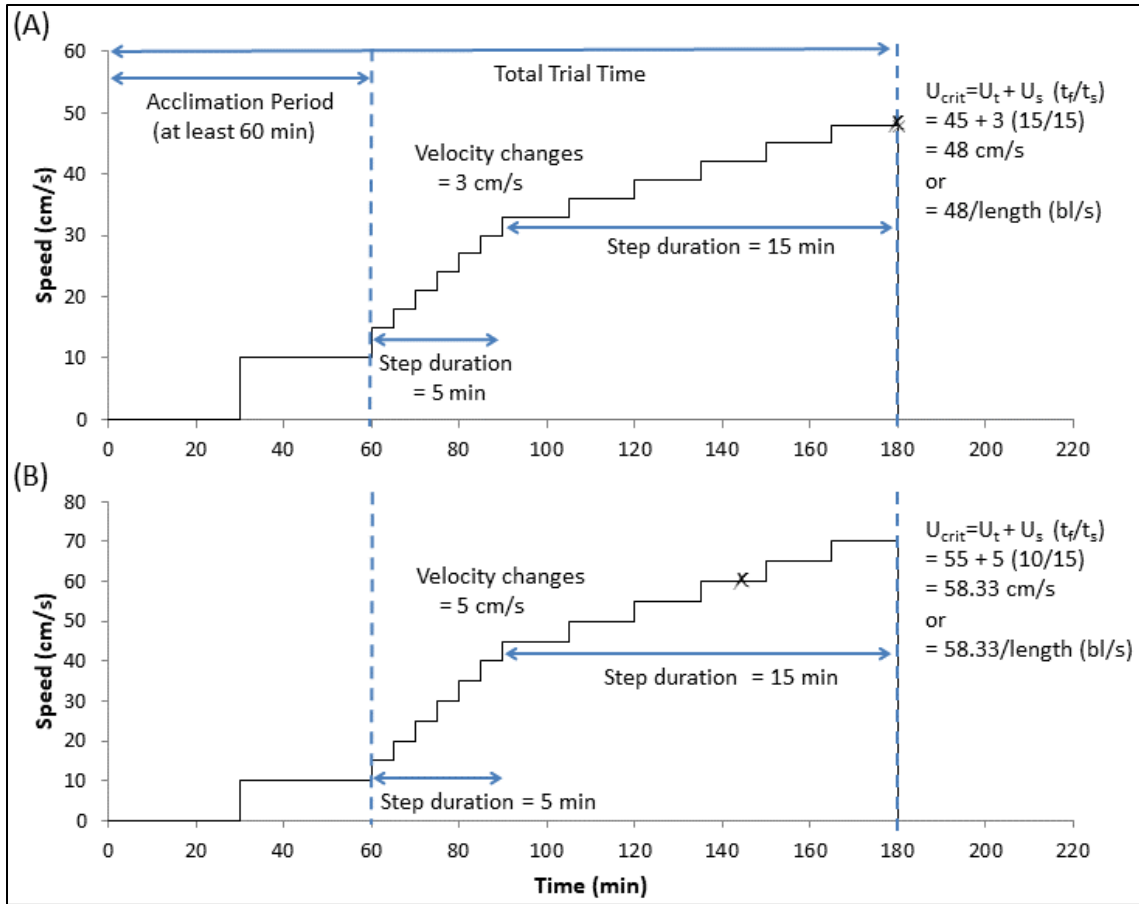
Figure 5-1. Description of the ramp-U$_{crit}$ methodology used for the swimming performance studies with (A) Sheepshead Minnow (*Cyprinodon variegatus*) and (B) Florida Pompano (*Trachinotus carolinus*). An "X" on the stepped line indicates where the fish was unable to continue swimming. A hypothetic example of U$_{crit}$ calculation is presented for each case.
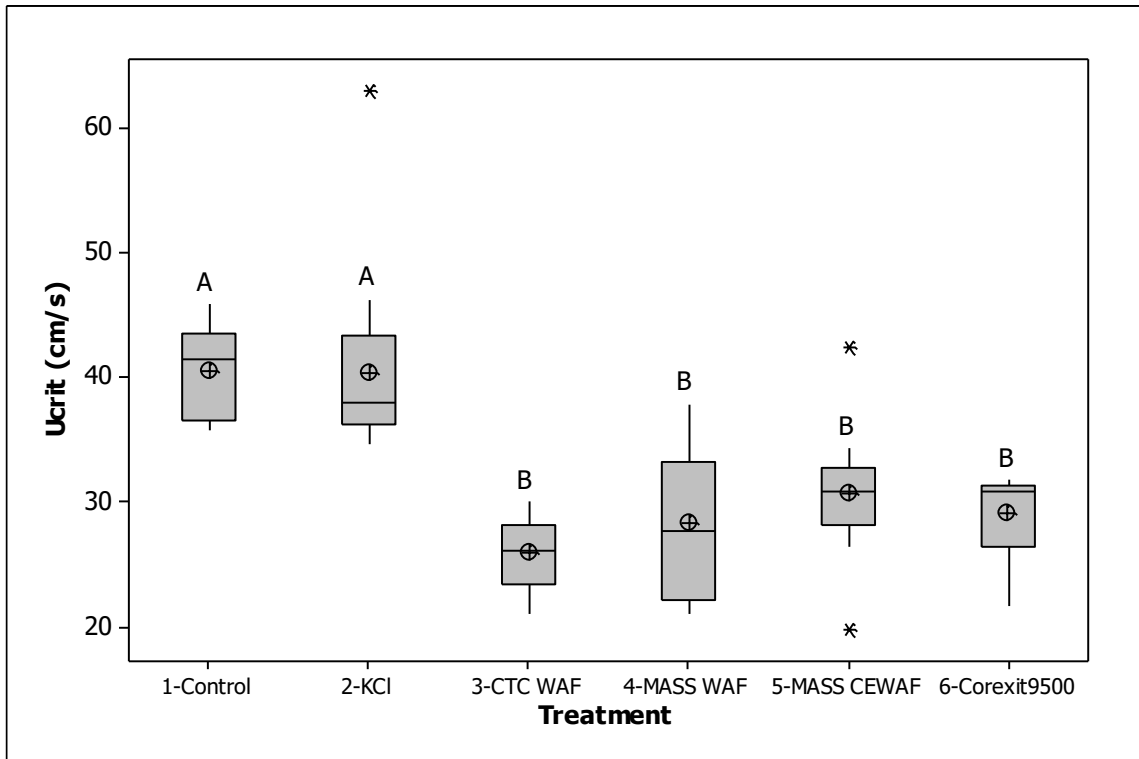
Figure 5-2. U$_{crit}$ in cm/s for swim performance trials with *Cyprinodon variegatus*.
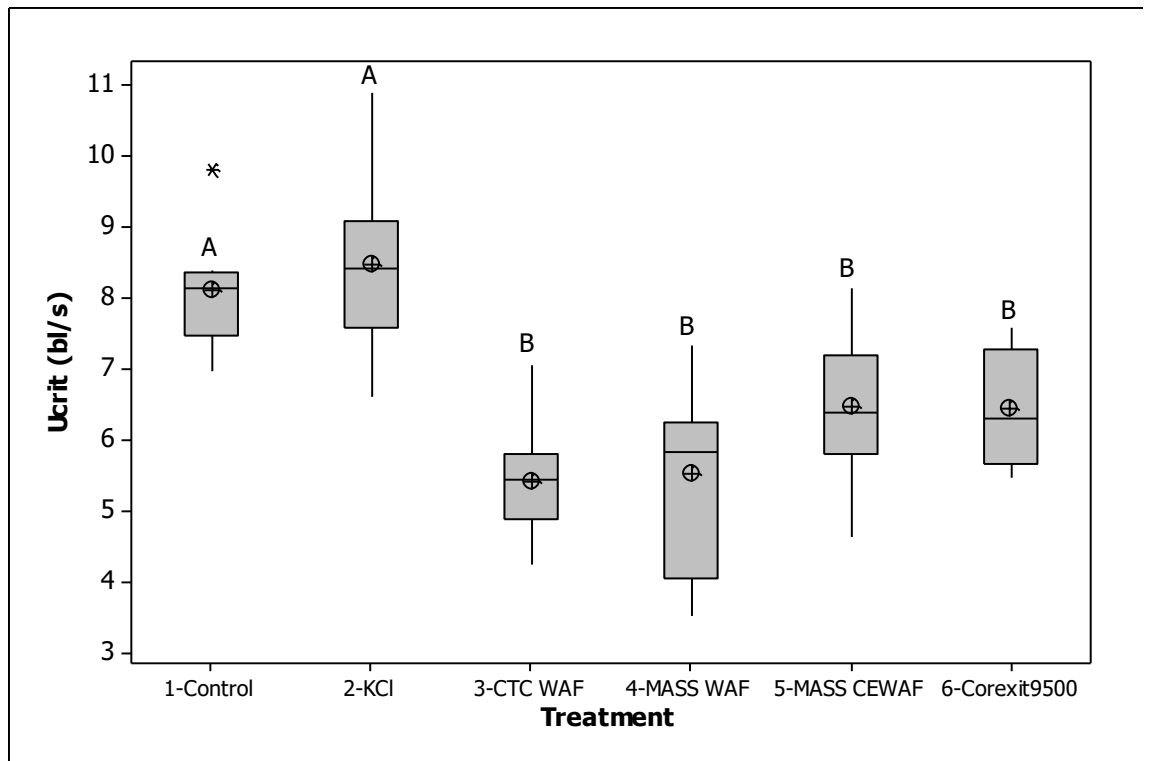
Figure 5-3. U$_{crit}$ in body lenth/s for swim performance trials with *Cyprinodon variegatus*.
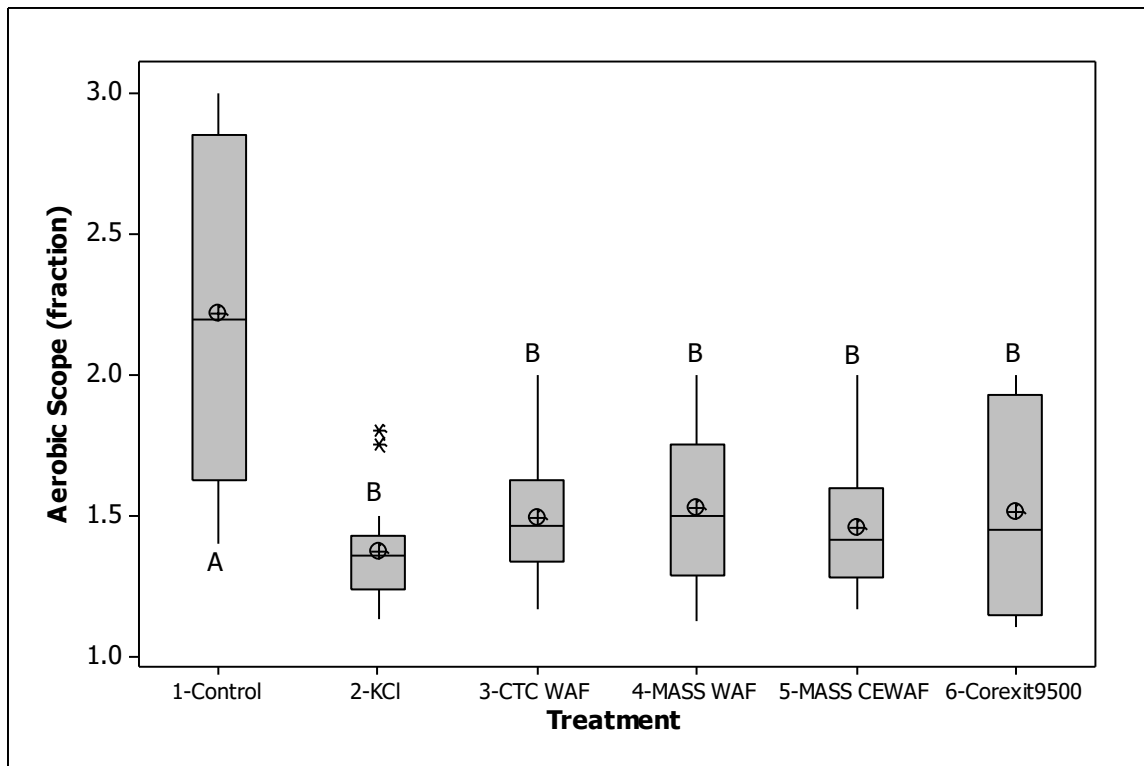
Figure 5-4. Aerobic scope (fraction) for swim performance trials with *Cyprinodon*
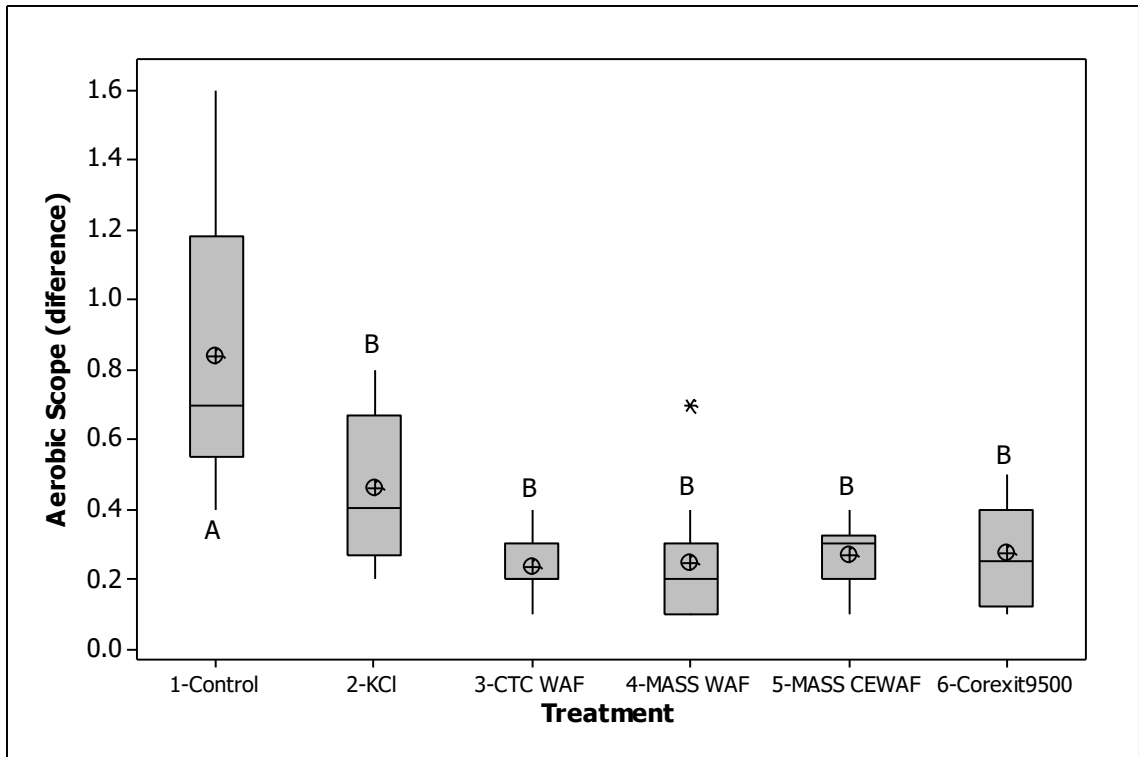
variegatus.

Figure 5-5. Aerobic scope (difference) for swim performance trials with *Cyprinodon*

variegatus.
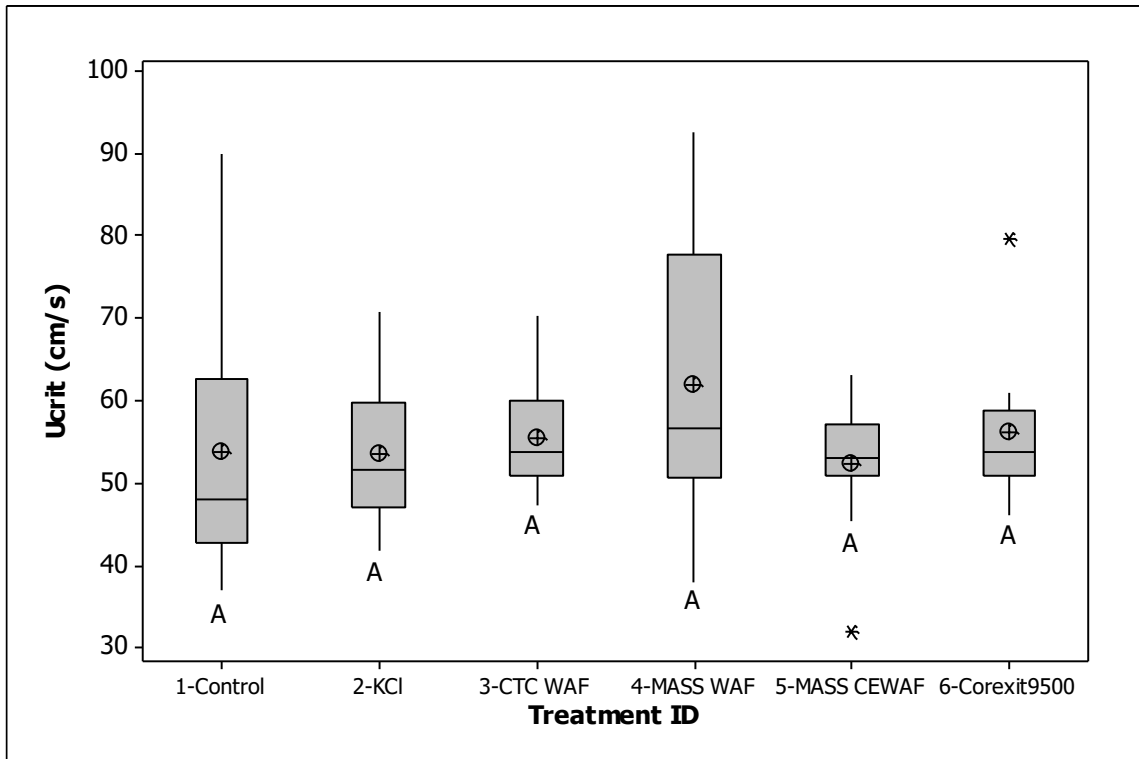
Figure 5-6. U$_{crit}$ in cm/s for swim performance trials with *Trachinotus carolinus*.
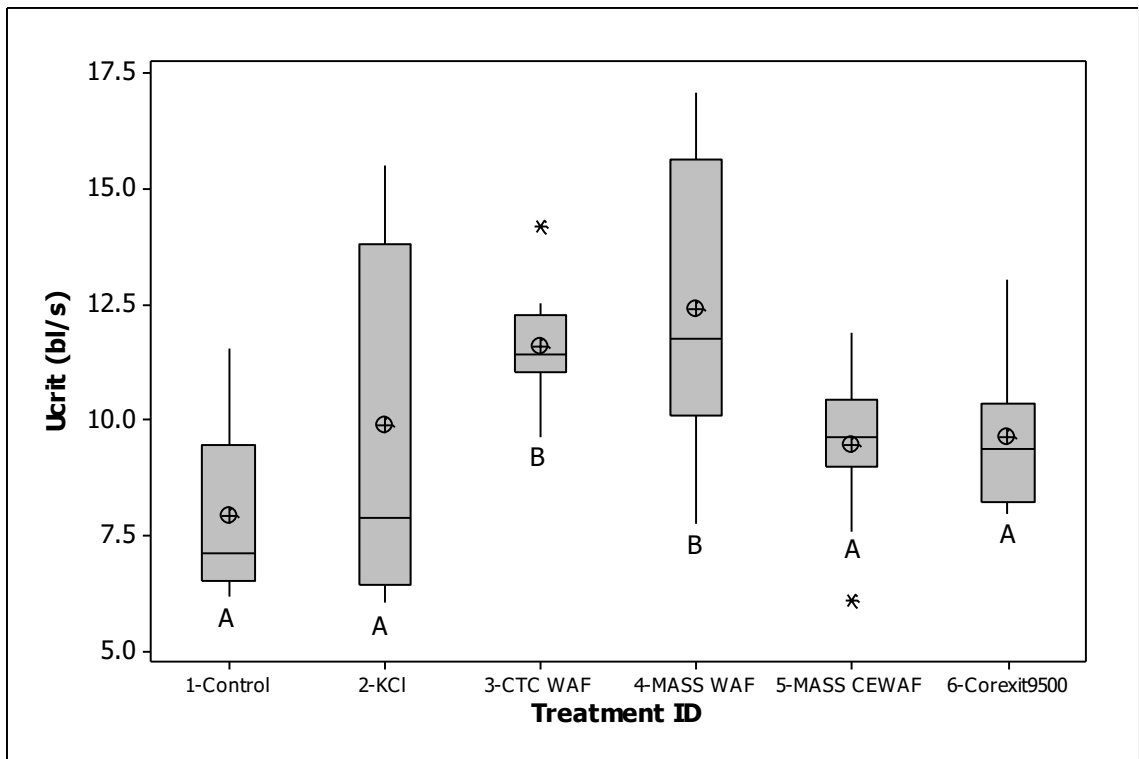
Figure 5-7. U$_{crit}$ in body lenth/s for swim performance trials with *Trachinotus carolinus*.
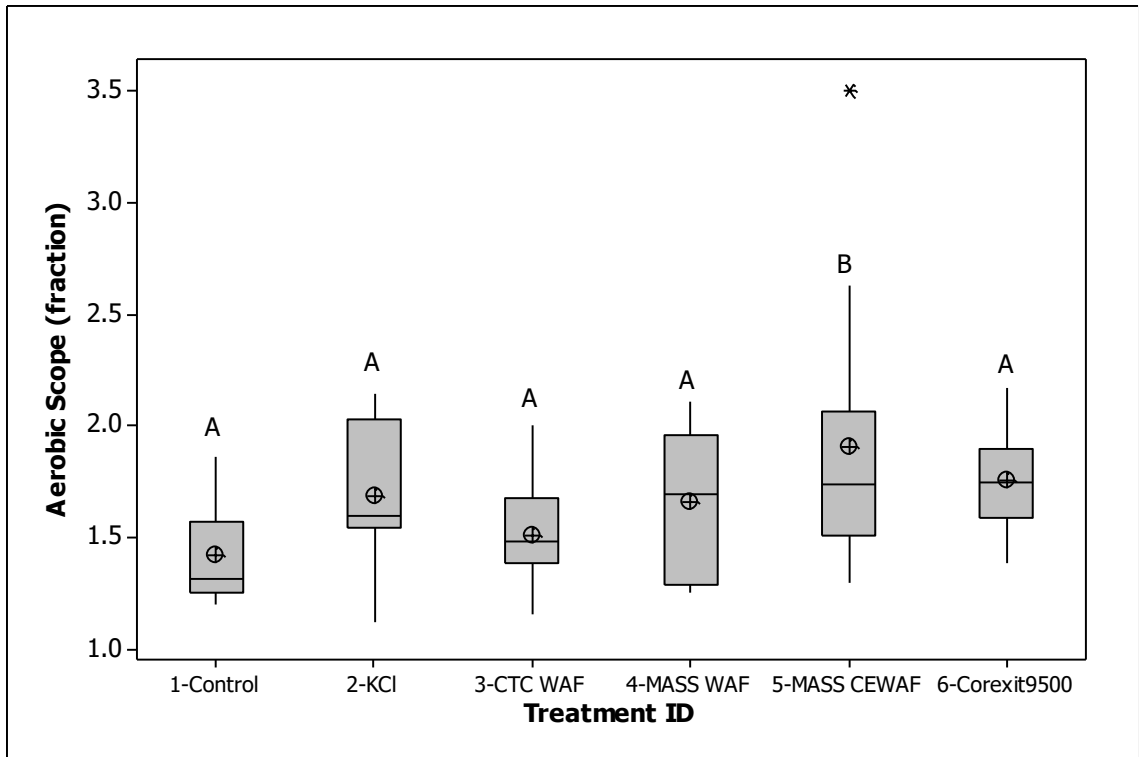
Figure 5-8. Aerobic scope (fraction) for swim performance trials with *Trachinotus carolinus*.
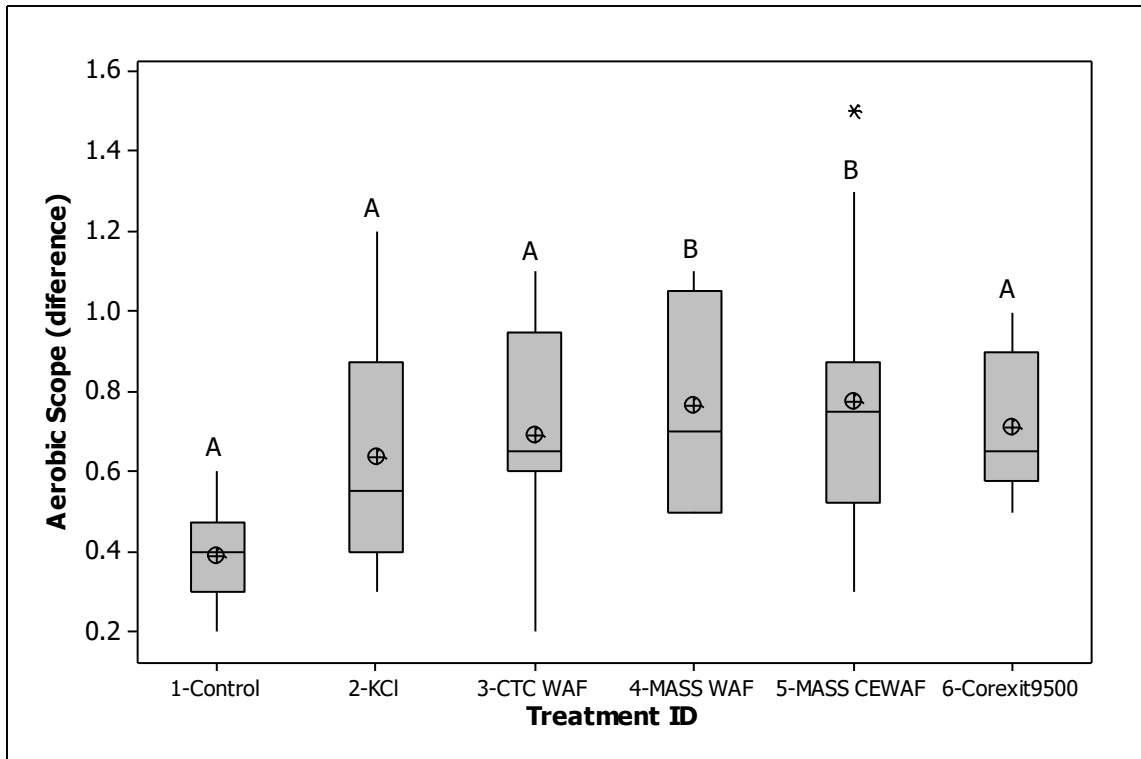
Figure 5-9. Aerobic scope (difference) for swim performance trials with *Trachinotus carolinus*.
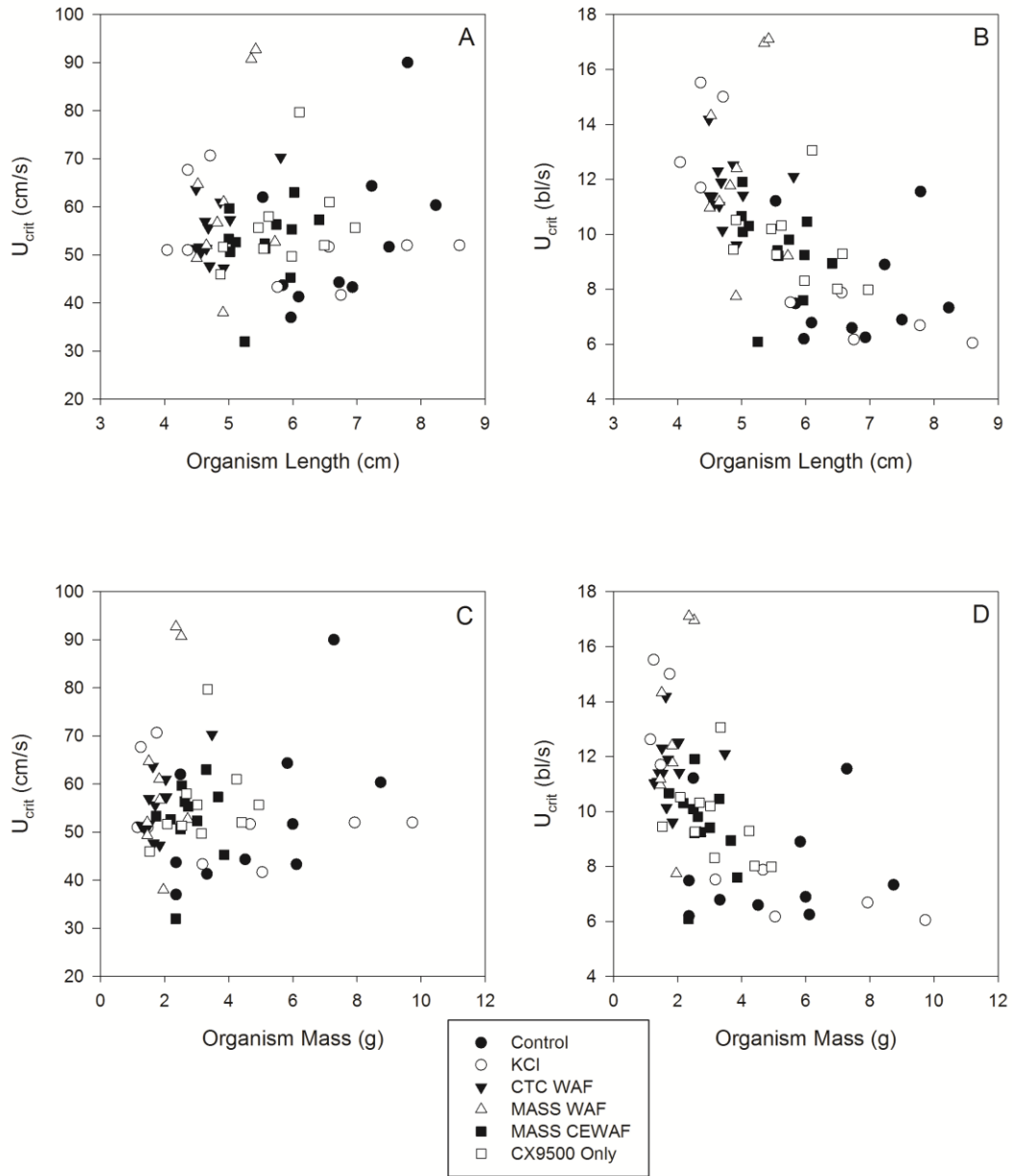
Figure 5-10. Florida Pompano, *Trachinotus carolinus*, size versus swimming ability plots. A&B use length for the size parameter while C&D use mass. A&C provide swimming ability in cm/second while B&C use body length/second.
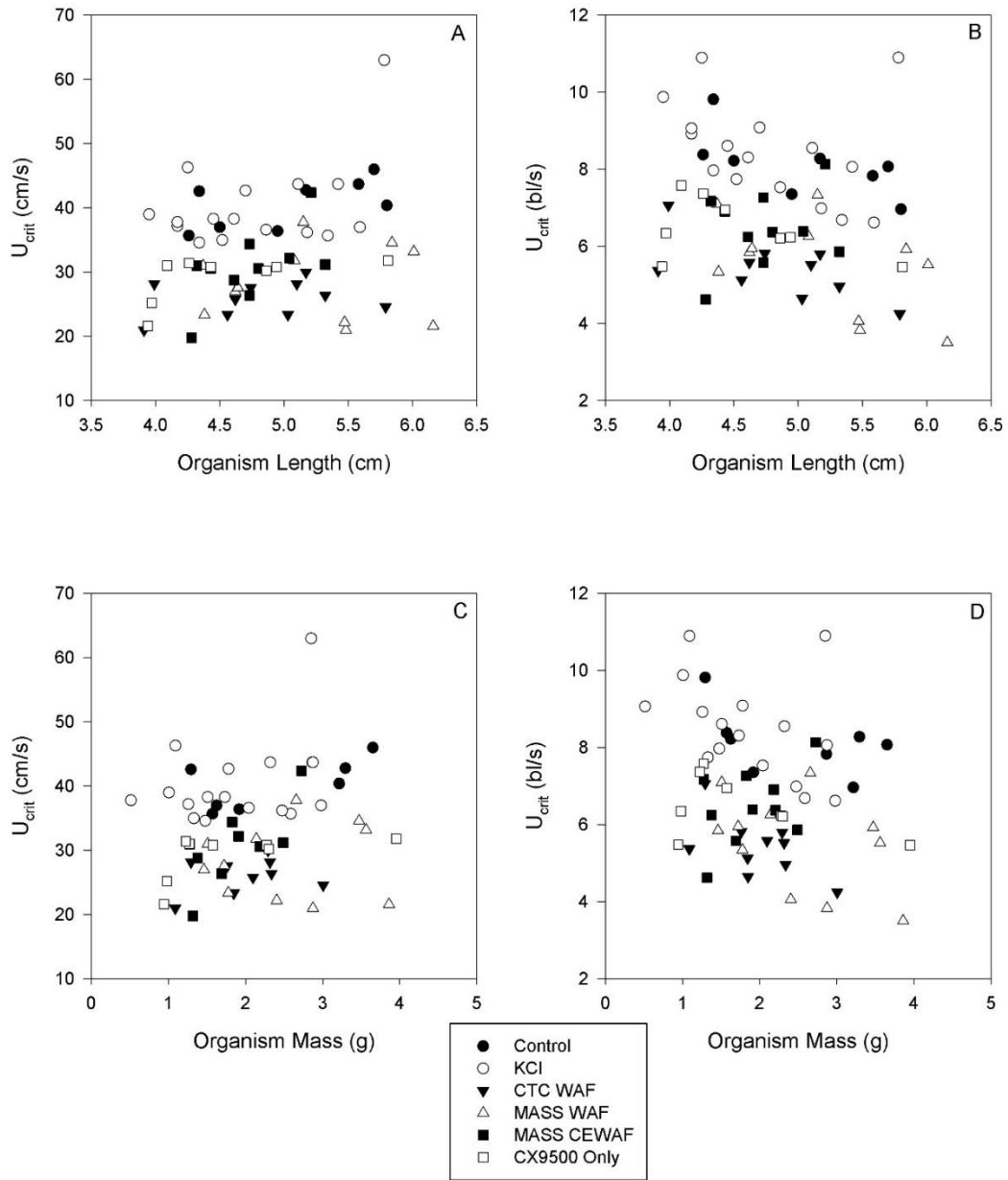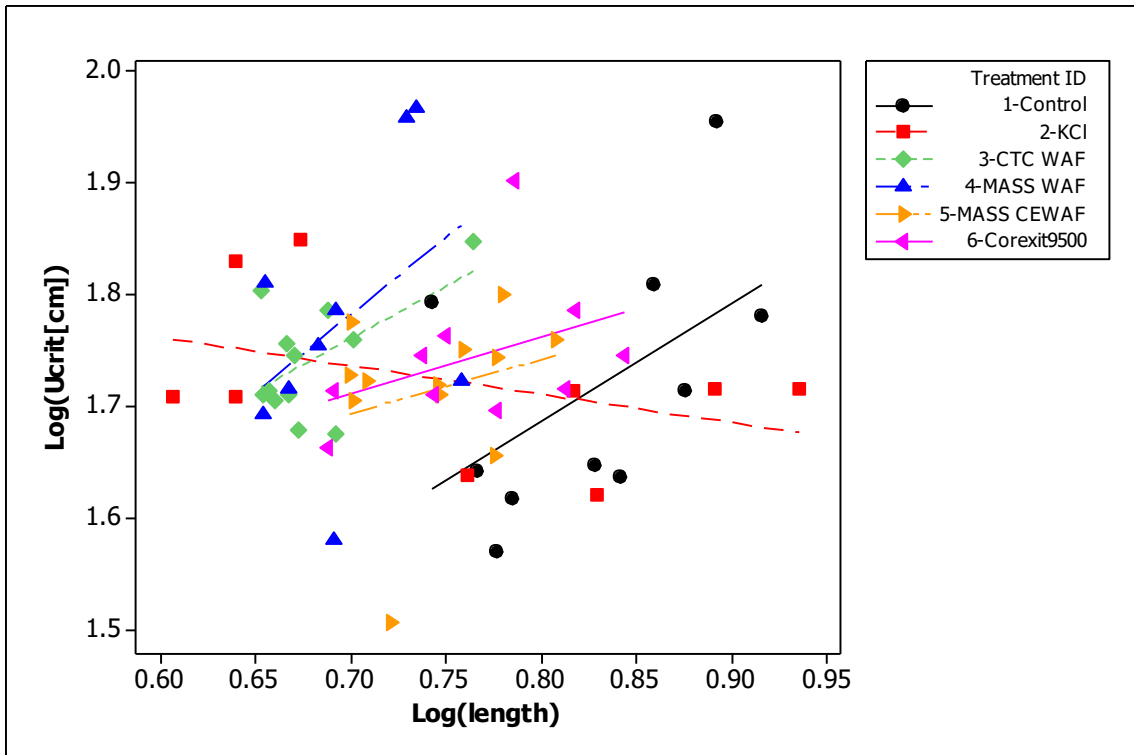
Figure 5-11. Sheepshead Minnow, *Cyprinodon variegatus*, size versus swimming ability plots. A&B use length for the size parameter while C&D use mass. A&C provide swimming ability in cm/second while B&C use body length/second
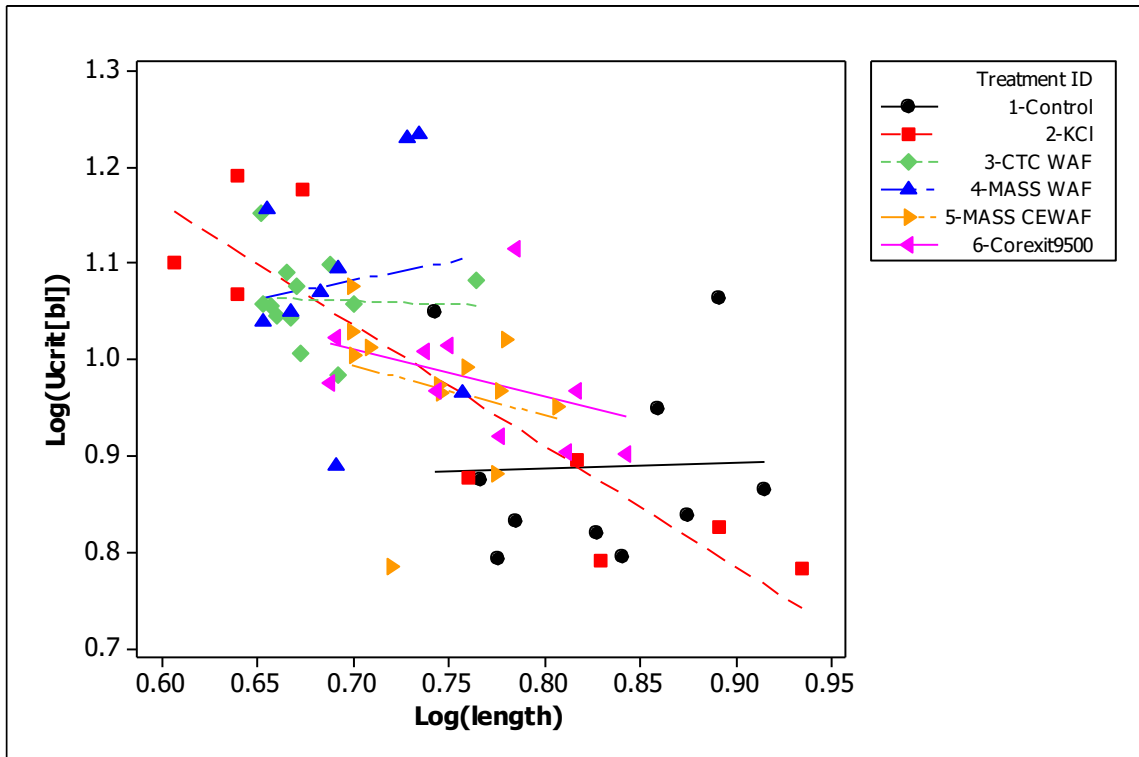
Figure 5-12. Florida Pompano, size vs swimming ability transformation plot: log(length) vs log(U$_{crit}$(cm/s)) with regression lines for treatment groups.

Figure 5-13. Florida Pompano, size vs swimming ability transformation plot: log(length) vs log(U_{crit}(bl/s)) with regression lines for treatment groups.
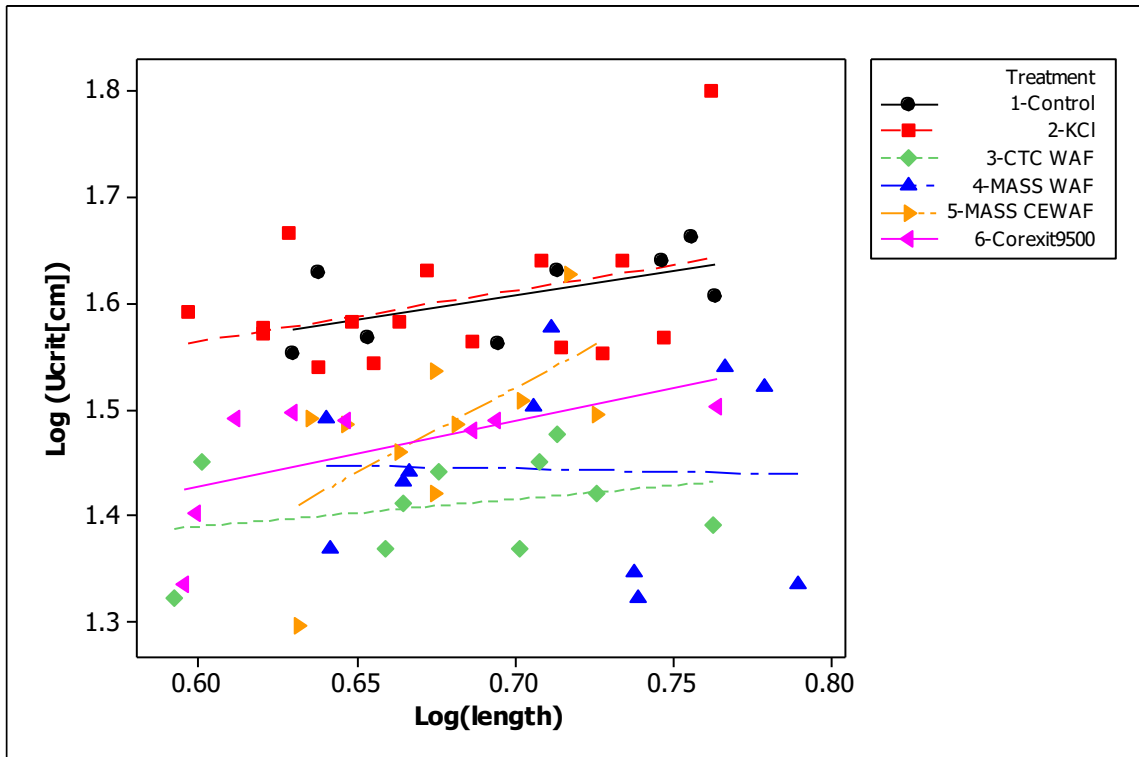
Figure 5-14. Sheepshead Minnow, size vs swimming ability transformation plot: log(length) vs log(U_{crit}(cm/s)) with regression lines for treatment groups.
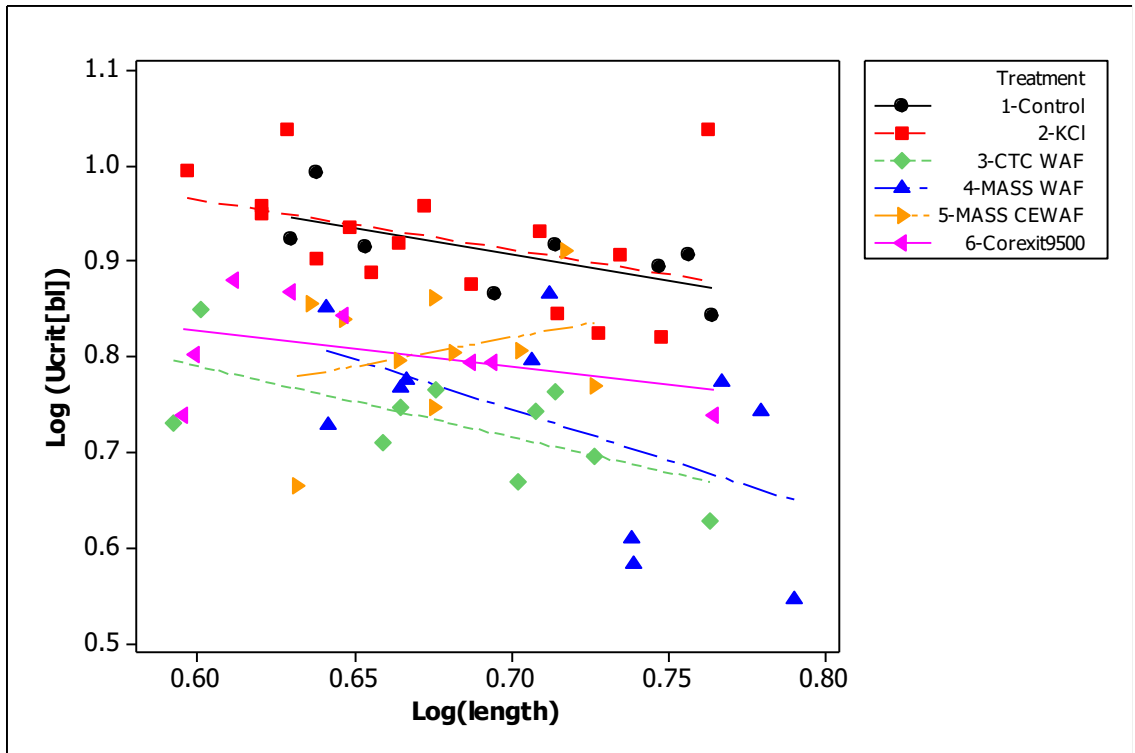
Figure 5-15. Sheepshead Minnow, size vs swimming ability transformation plot: log(length) vs log(U$_{crit}$(bl/s)) with regression lines for treatment groups.
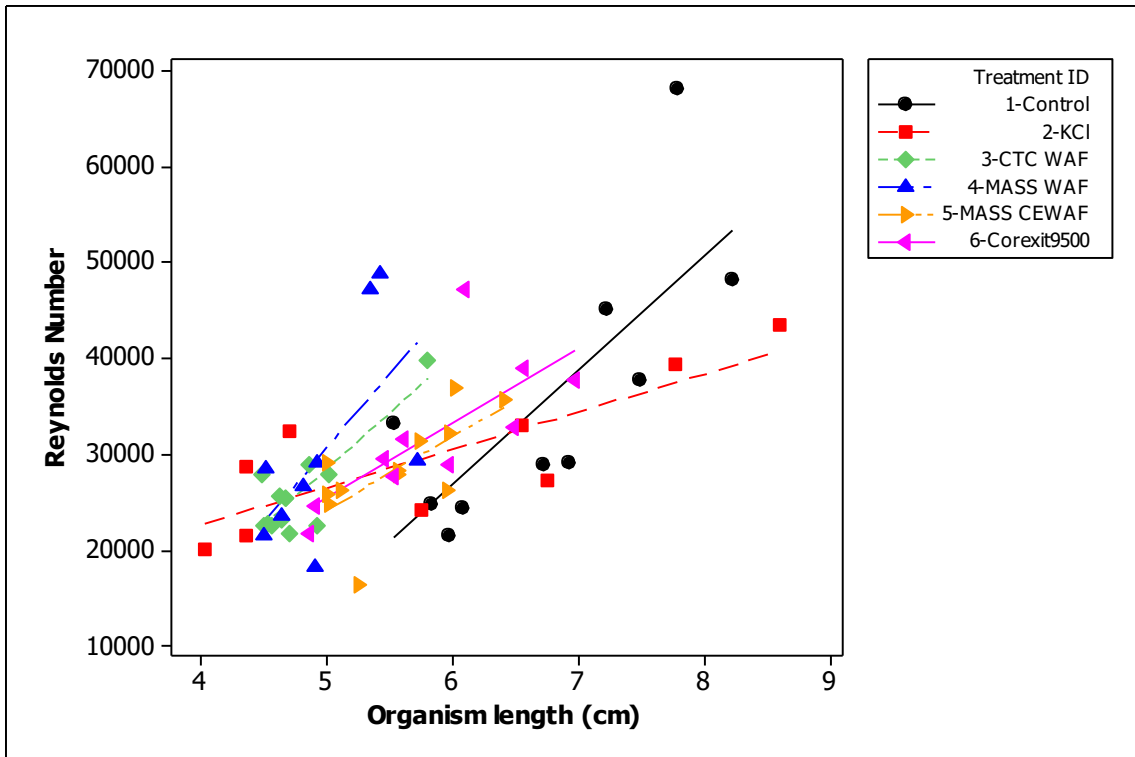
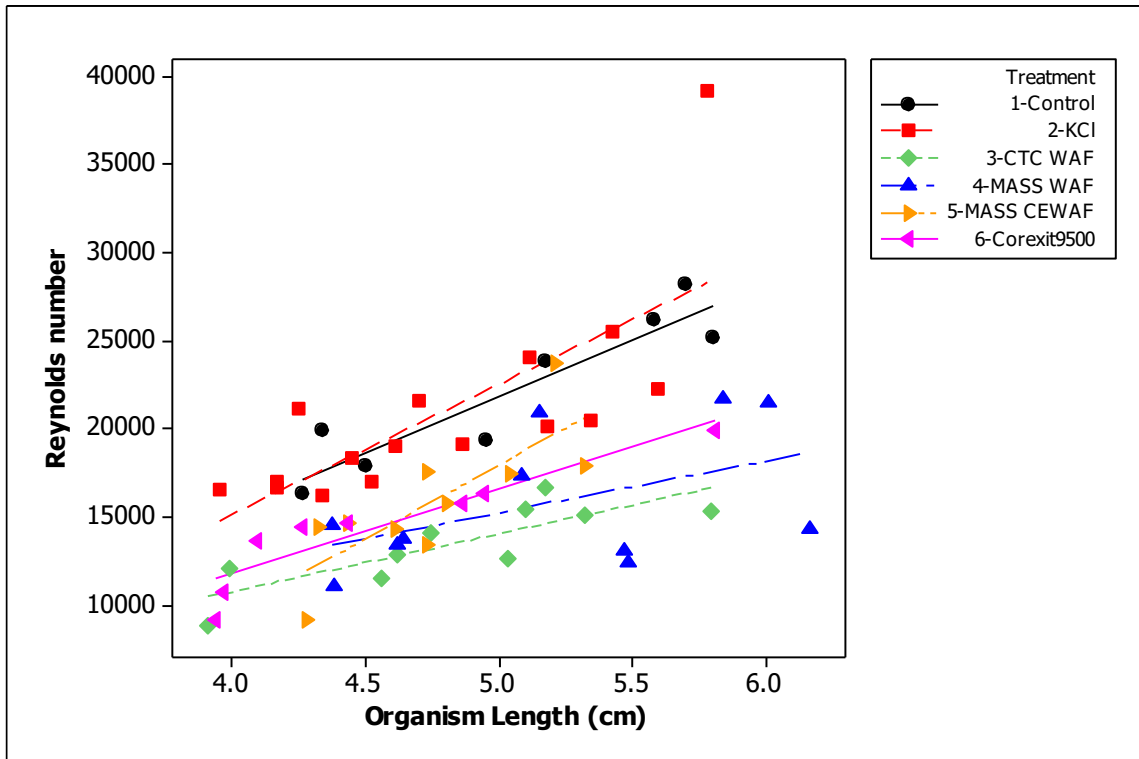Figure 5-16. Florida Pompano, size vs swimming ability transformation plot: length vs Reynolds number

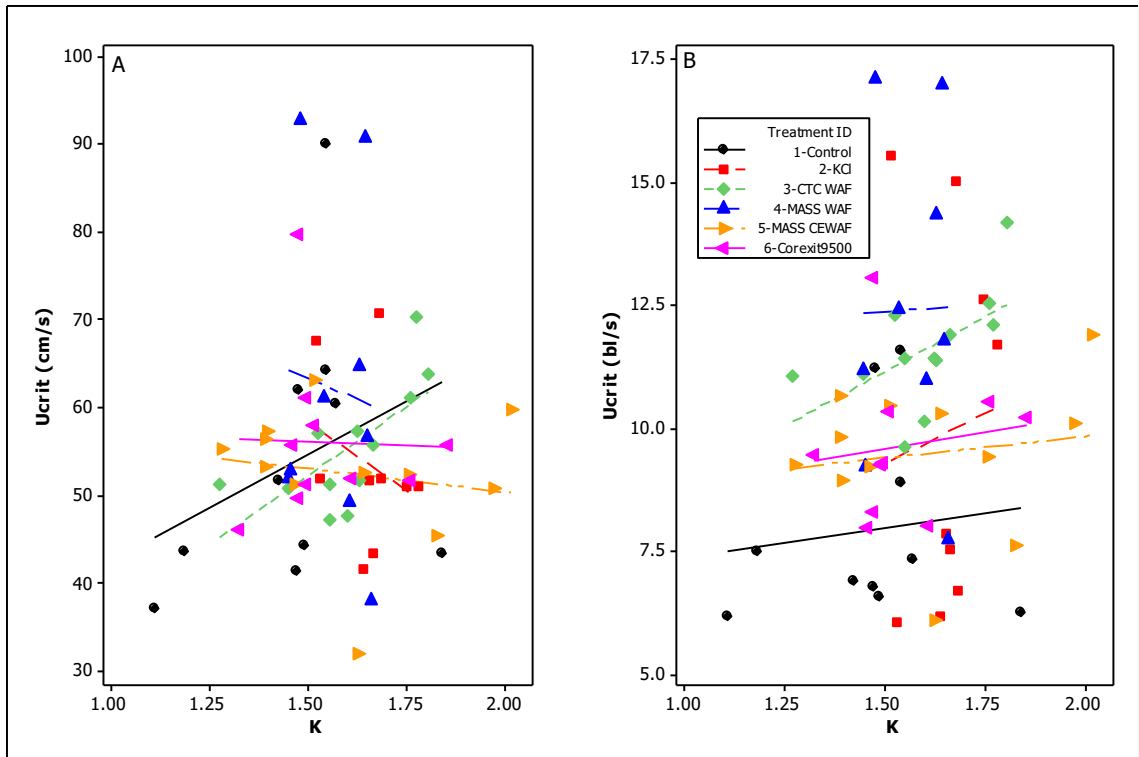Figure 5-17. Sheepshead Minnow, size vs swimming ability transformation plot: length vs Reynolds number

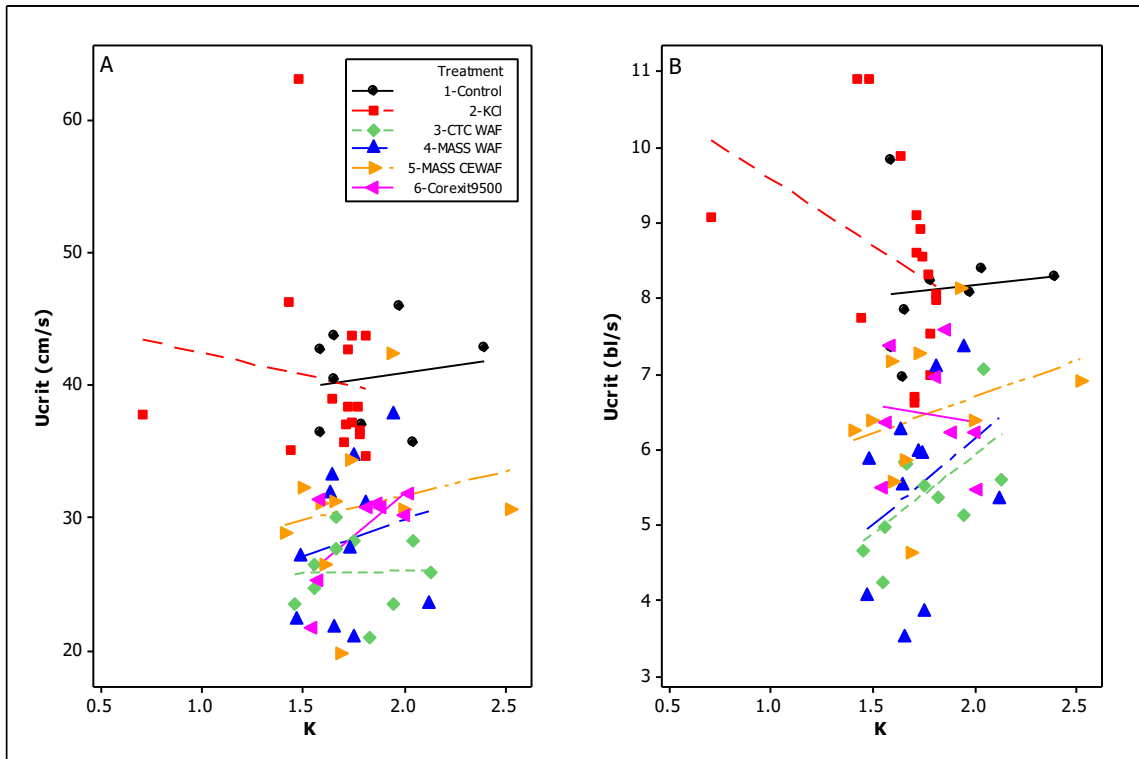Figure 5-18. Florida Pompano data transformation plots: body condition (K) vs swimming ability.

Figure 5-19. Sheepshead Minnow data transformation plot: body condition (K) vs swimming ability.

DISSERTATION CONCLUSION

All chapters of this dissertation focus on the use of non-standard indigenous organisms in aquatic toxicology. Chapters one, two, and five are associated with research conducted in the comprehensive toxicity program conducted at the FIU Ecotoxicology & Risk Assessment Laboratory to support the BP NRDA effort. Chapters three and four focus on new methods to use a new model organism in aquatic toxicology.

There was a considerable amount of toxicity data generated for the DWH NRDA. The SSDs show that the major marine phyla are extremely underrepresented with only five included in the dataset. SSDs used in establishing safe water quality criteria require representative data from a diverse collection of phyla with a minimum of eight. This ensures the inclusion of data that will be representative of the whole environment studied. This analysis highlights the need for more toxicity data to truly understand impacts of petroleum presence in the GoM during the DWH incident. The next logical step with this data was to conduct a risk assessment.

Four probabilistic risk assessments were conducted using the data presented in the first chapter. Environmental chemistry data from an extensive sampling program was used to characterize risk in these four scenarios. Risk was found to be low with 10% of species being effected at 0.78-3.73 µg/L TPAH, 2.2-5.7% of the time with 95% of the environmental concentrations being <0.992 µg/L TPAH. The highest risk was found with sublethal effect data as expected. Although risk was low, it is important to note that a risk assessment is only as good as the data contained within. The lack of toxicity data is apparent in the risk assessments as it was in the first chapter, However, this provides a first look at risk associated with the DWH incident.

The Starlet Anemone proved to be an excellent aquatic toxicity study organism. Laboratory culture techniques and testing guidelines were created and verified that others should be able to follow. Toxicity endpoints proved to be reproducible using cadmium and copper as a toxicant. The anemone mean effect values from acute toxicity were below the 10th centile benchmark from a copper toxicity SSD. This highlights the practicality in using the anemone when investigating metal toxicity in estuarine or marine environments. Determining mortality in the initial acute studies was difficult which lead to the creation of the stress-response index. Integrating a recovery period after acute exposures demonstrated the ability to use this index to confidently identify mortality and generate reproducible endpoint values. This index may be useful when conducting studies with other cnidarians to identify levels of stress but will need verified using a variety of other toxicants.

Swimming performance proved to be a sensitive sub-lethal indicator of exposure to petroleum and reference toxicants. Concentrations at one tenth of median lethality values (LC50s) induced elevated or depressed swimming ability. The interesting thing is that the effects were not consistent across both species. Elevated swimming ability was observed in some treatments of Florida Pompano while depressed ability was observed in some treatments of Sheepshead Minnows. A difficult component of this technique is determining the best way to normalize the data due to different swim speed units and fish sizes and ages. Ideally, a swimming profile would be developed for a fish species throughout the stages of development based on a body condition factor that incorporates size and weight parameters which could be used for comparison. This technique could be

incorporated into aquatic toxicology studies as a sensitive sub-lethal stressor indicator but requires a considerable amount of up-front work.

All of the work presented here highlights difficulty using non-standard organisms in aquatic toxicology but also the necessity of generating toxicity data from these organisms. Water quality criteria is established based on the information available to protect the organisms that have toxicity information. Quite frequently, non-standard species are more sensitive than standard testing species. It is important that the recommendations in toxicity guidelines are followed and non-standard species are included in ecotoxicology assessments. I hope this can serve as a reference for others wanting to use a new testing organism and help guide their efforts.

VITA

ABRAHAM JEFFREY SMITH

Born, Charleston, West Virginia

- PhD Candidate; Biology Department, Florida International University, 2018

  Development and Application of Aquatic Toxicology Studies for the Assessment of Impacts Due to Chemical Stressors Using Non-Standard Indigenous Organisms.

- MSc Marine Biology, Nova Southeastern University Oceanographic Center, 2013

  Thesis Title: Tissue Loss Syndromes in *Acropora cervicornis* off Broward County, Florida: Transmissibility, Rates of Skeletal Extension and Tissue Loss.

- BS Marine Biology, University of North Carolina at Wilmington, 2002

- BA General Music, University of North Carolina at Wilmington, 2002

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Smith, AJ and GM Rand (2017) *Nematostella vectensis* in Ecotoxicology and Risk Assessment. Hydra-Meeting International Workshop: The diversification of early emerging metazoans: a window into bilaterian origins? Tutzing, Germany. Poster Presentation.

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Smith, AJ, GD Miller, GM Rand (2016) Using the Starlet Anemone, *Nematostella vectensis*, for the assessment of effects and recovery in acute studies: development of a stress-response index. 7th SETAC World Congress, SETAC North America 37th Annual Meeting. Orlando, FL. Platform Oral Presentation in Fate and Effects of Metals: Marine Issues.

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Smith, AJ, GD Miller, BS Echols, GM Rand (2014) Laboratory culture, reproduction, and toxicity studies with cadmium and copper using the Starlet Anemone, *Nematostella vectensis*. SETAC North America 35th Annual Meeting. Vancouver, BC. Oral Presentation in Fate and Effects of Metals: Marine Concerns.

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