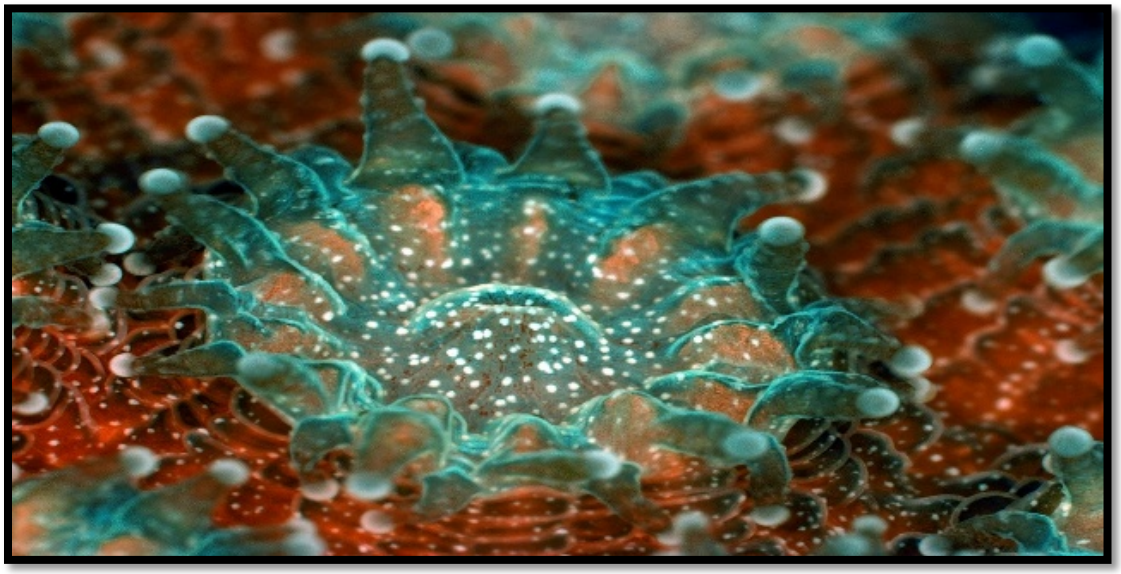
Fate and Effects of Chemical Contaminants Program Review



Volume 4: Key Species & Bioinformatics

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NCCOS/Stressor Detection and Impacts Division

Volumes of the Fate and Effects of Chemical Contaminants Program (F&ECCP) Review

Volume 1: Introduction and F&ECCP Overview

Volume 2: Ecotoxicology

Volume 3 Monitoring and Assessment

Volume 4: Key Species and Bioinformatics

Fate and Effects of Chemical Contaminants Program Review

Volume 4: Key Species and Bioinformatics Branch

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NCCOS's Key Species and Bioinformatics Branch – assessing the ecological impacts of marine stressors through key biological indicator species

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Abstract

The NCCOS Charleston Key Species and Bioinformatics (KSB) branch has four different programmatic areas: Coastal Marine Mammal Assessment (CMMA), Quantitative Ecology (QE), Environmental Genetics (EG), and Coral Health & Disease (CH&D). Among the four KSB programs, our unifying purpose is to assess the impacts of biological, chemical, and physical stressors on the health of the marine ecosystem to support the conservation and management of our nation's coastal resources. The effect of chemical contaminants on the health of individual species, populations, ecosystems or their habitats is therefore one aspect of our research and varies in the degree of emphasis within each program's portfolio. The CMMA program is concerned with anthropogenic impacts to populations of iconic sentinel marine mammals and conducts assessments by quantifying contaminant body burdens in coastal bottlenose dolphins and marine mammals in the southeastern United States. The QE program uses spatial statistics and benthic analyses to assess environmental impacts in areas where the National Oceanic and Atmospheric Administration (NOAA) has primary management responsibility including the National Marine Sanctuaries and the National Estuarine Research Reserves System (NERRS). The EG program uses DNA and expression analyses to identify impacted species/stocks and to characterize cellular expression variability in response to different stressors. The CH&D Program engages in laboratory research using exposure-response studies with different stony coral species and other invertebrates to determine effects of specific stressors including chemical contaminants of concern, multi-stressor effects, and species sensitivity differentials. A schematic of the Key Species and Bioinformatics Branch with the 4 programs including associated personnel is included as Appendix 1.

Introduction

Increased industrialization and coastal development over the last century has resulted in both acute (i.e. spill) and persistent sources of pollution in the coastal environment. Many of the waterways near human populations are contaminated and, while identifying the pollutants is important for classification and mitigation, it is also critical to understand how various pollutants negatively impact life in the marine ecosystem. The objective of our branch is to use key species as indicators to inform us as scientists and our agency as species managers regarding the biological effects of these stressors.

Marine ecosystems are comprised of highly complex communities of animals, plants, and microbes functioning together in an ever-changing environment. To understand how the animal community might be impacted by a particular stressor, we collaborate with a multitude of academic, non-profit, state, federal, and international researchers to characterize the stressor source and to assess the impacts as represented through a number of key species. These species, which are the focus of three of the four KSB Branch programs, include:

1. Stony corals - The Coral Health and Disease (CH&D) Program (Figure 1) is the largest of the four KSB programs with two federal staff, five contract staff and one student providing a wide range of expertise in fields such as molecular and cellular biology, biochemistry, toxicology,

microbiology, pathology, diagnostic assay design and marine aquaculture. Led by Dr. Cheryl Woodley (see her book "Diseases of Coral"), the CH&D Program brings a health perspective, patterned after evidence-based medicine and public health, to scientific investigations that support the NOAA and National Ocean Service (NOS) Coastal Stewardship Mission. The purpose of the CH&D Program is to bring relevant, science-based information to decision-makers and the public to assist them in choosing effective actions to reduce threats to our coral resources and help restore damaged coral reefs.

Corals are keystone species in tropical marine ecosystems where warm water is often nutrient-poor; consequently, these animals are partially reliant on zooxanthellae, symbiotic algae providing them with an additional source of energy, carbon, and nutrients. As the base of the primary coastal food web in tropical seas, corals are critical in the tropical marine ecosystem, but their health is rapidly declining and almost all global reefs are now classified as stressed. Shallow water corals provide habitat for over a million aquatic species, protect land from storm erosion, support tourism, and sustain valuable commercial and recreational fisheries. In 2013, the total annual economic value of U.S. coral reef services was estimated to be over $3.4 billion, with recent worldwide estimates of total economic benefit estimated at $2.7 trillion (https://www.iyor2018.org/ about-coral-reefs/benefits-of-coral-reefs/). Over the last 20 years, the CH&D Program provided scientific data to support management requirements for U.S. tropical near-shore waters. Our work included developing oil toxicity thresholds in Pacific and Atlantic corals (May et al, 2020), assessments of metal contamination in national parks in St. Croix, Virgin Islands (Bayless, 2020), baseline studies in NOAA managed national marine sanctuaries in American Samoa and Florida Keys (Balthis et al., 2018a), and sunscreen toxicity evaluations (Downs et al., 2016; Hazard Quotient Calculations for communities in the Florida Keys and Hawaii).

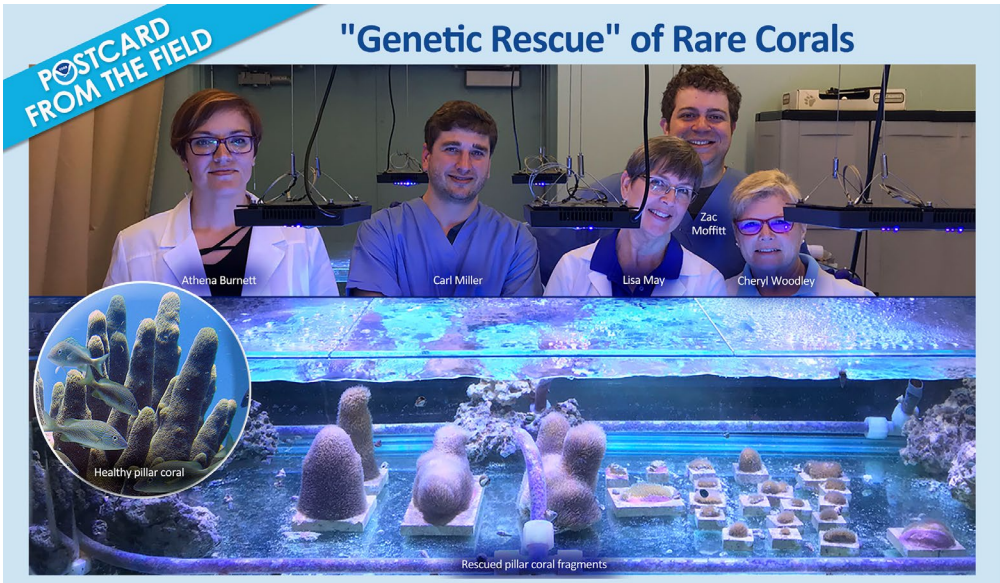

Figure 1 – A recent NOAA "Postcard from the Field" that includes some of the members of the Coral Health and Disease Program next to one of our coral tanks. Missing is Laura Webster (on detail to another NOAA office at the time), Elizabeth Duselis (new post-doctoral fellow), and Ron Kothera (new contractor).

2. Marine Mammals – As the closest marine relatives to humans, marine mammals are an iconic group of megafauna and a critical key species, often considered sentinel species of coastal ocean and human health. Originally supporting primary needs such as coastal health assessments, the NCCOS Coastal Marine Mammal Assessment (CMMA) Program also investigated impacts of harmful algal blooms and industrial runoff from a superfund contamination site in Brunswick, Georgia. The CMMA Program was consolidated in recent years and while small in size (1 federal employee, 1 part-time contractor), the program maintains its prominence in marine mammal research through its many collaborations with universities (College of Charleston (CofC), Coastal Carolina University (CCU), The Citadel, Auburn University, Texas State University, and others), nonprofits (National Marine Mammal Foundation (NMMF), Lowcountry Marine Mammal Network (LMMN), Texas Marine Mammal Network, Mote Laboratories, and others), and other government agencies (South Carolina Department of Natural Resources, National Marine Fisheries Service (NMFS), and others) (Figure 2). Like the CH&D Program, research in marine mammals includes all sources of stressors including biological (bacteria) (McFee, W., under review), physical (tagging research, debris) (Claro et al., 2019) and chemical (Bryan et al, 2017; McCormack et al, 2020). Our contaminant work has included toxicity studies on oil impacts to marine mammals in Barataria Bay, LA (collaboration with the NMMF, NMFS; McDonald et al., 2017), determining metal (vanadium and nickel) loads from oil exposure in dolphin teeth (collaboration with Texas State University, MOTE, and Texas Marine Mammal Stranding Network), determining heavy metal loads in otters and bottlenose dolphins (collaboration with College of Charleston; Bryan et al., 2017), and determining microplastic loads in the dolphin gastrointestinal tract (collaboration with Auburn University, Skidaway Marine Institute, and College of Charleston; Battaglia, 2019; Pfeifer, 2020).

Figure 2 – A stranded pilot whale on Edisto beach in 2019. Pictured include CMMA Program's Tessa Pfeiffer (CSS contractor in the CMMA Program) (front right), Francesca Battaglia (collaborator with Coastal Carolina University and former student with Wayne McFee, Program Manager for the CMMA Program), and 2 volunteers from the South Carolina Marine Mammal Stranding Program (middle).

3. Benthic Invertebrates – Benthic organisms are reliable indicators of environmental stress and are used worldwide for assessment of marine and estuarine sediment condition (Figure 3). Benthic infauna, a highly diverse group of invertebrates, live on or within the upper several centimeters of sediments and are relatively sedentary, and so are exposed to pollutants that may be present. Because of the range of sensitivity of these organisms to chemical contamination or organic over-enrichment – from pollution-sensitive to pollution-tolerant species – the composition of infaunal communities can provide an indication of overall sediment quality with respect to these stressors. Over the past two decades, the former Coastal Ecology (CE) Program conducted place-based assessments of ecological condition to support NOS customers including the Office of National Marine Sanctuaries and the National Estuarine Research Reserve System (NERRS). These studies used combined measures of water quality, sediment contaminants, sediment toxicity, and biological condition to provide overall assessments of environmental status. The Quantitative Ecology (QE) Program emerged from the former CE Program where it played an integral part of these efforts through the development of probabilistic sampling designs and by providing statistical analysis, modeling, and spatial analysis in support of a number of varied initiatives. The QE Program also provides database design, development, and mechanisms for disseminating and communicating results of projects through websites including the National Benthic Inventory (benthic infauna) and Regional Ecological Assessments (water quality and nutrients, sediment contaminants and toxicity, fish and shellfish tissue contaminants). The QE Program participates in the International Oceanographic Data and Information Exchange program (part of IOC-UNESCO) by providing data to the Ocean Biogeographic Information System (OBIS), a global open-access data and information clearing-house on marine biodiversity for science, conservation and sustainable development. In addition to the deep benthos oil toxicity report included in this review, the QE Program has completed a number of ecological condition assessments (which often include a chemical contaminant component) of the soft-bottom habitats at numerous locations including: (1) the Florida Keys National Marine Sanctuary (Balthis et al., 2018a), (2) Florida's Guana Tolomato Matanzas (GTM) National Estuarine Research Reserve (NERR) (Balthis et al., 2017a), and (3) Chesapeake Bay and Southeastern United States (Balthis et al., 2018b).

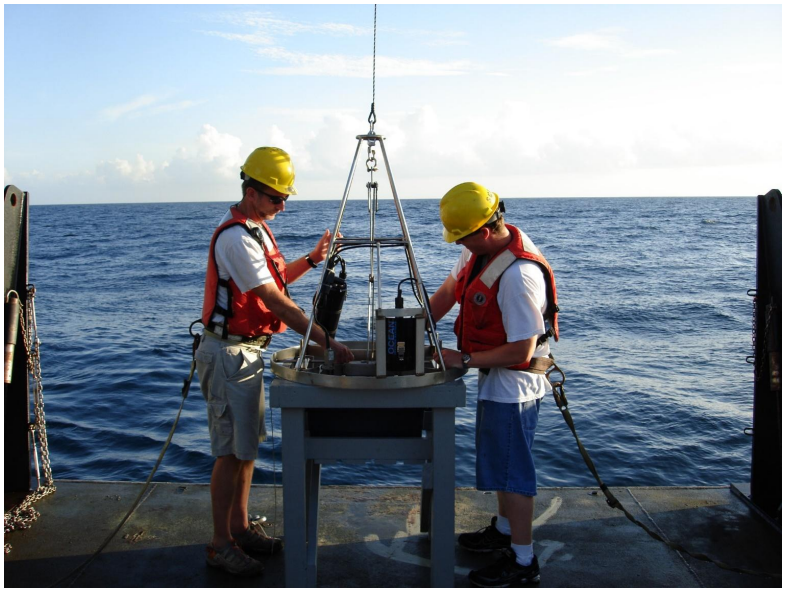

Figure 3 – Benthic sampling device being prepared for deployment off the Florida Keys National Marine Sanctuary. The sediment grab sampler collects the biological and chemical samples necessary for subsequent laboratory analysis.

Key Species and Bioinformatics – Papers included for the Fate and Effects of Chemical Contaminants Review

For this review, the KSB Branch prepared six (6) papers outlining studies that included a chemical contaminant analysis component (see Table 1). Other studies from the Key Species and Bioinformatics Branch that focused predominantly on other metrics such as body health, disease, marsh modeling, etc are not included.

Table 1: KSB papers submitted for the Fate and Effects of Contaminants Review:

Number	Lead Author	Program	Title
1	Greig	Environmental Genetics	Effects of two remediation strategies on the indigenous microbial communities in response to a simulated hydrocarbon release using an experimental mesocosm system
2	Greig	Environmental Genetics	Use of RNA-seq to examine temporal effects on differential gene expression in eastern oyster Crassostrea virginica, exposed to fluoranthene for 48 hours
3	Balthis	Quantitative Ecology	Sediment Quality Benchmarks for Assessing Oil-Related Impacts to the Deep-Sea Benthos
4	May/Webster/ Woodley	Coral Health & Disease	Effect of Louisiana sweet crude oil on a Pacific coral, *Pocillopora damicornis*
5	Woodley	Coral Health & Disease	Investigations into the Effects of Contaminants on Coral and Coral Reef Health
6	McFee	Coastal Marine Mammal Assessment	Chemical Contaminants in Marine Mammals

Methods

The specific chemical methods used within the Key Species and Bioinformatics Branch are discussed in the associated reports for this review (Table 1), whereas our biological and physical contaminant analyses are considered outside the scope and are not included. In general, the methods supporting the chemical analyses conducted using key indicator species or techniques are described for (1) Corals, (2) Marine Mammals, (3) Genetics/Genomics, (4) Benthic Invertebrates, and (5) Statistics and Bioinformatics as outlined below:

1. Corals:

The focus of the CH&D Program includes shallow-water corals in all the tropical waters of the United States and its territories. Many of these corals are protected under the Endangered Species Act (ESA) and their collection is tightly regulated; consequently, our research is heavily dependent on our 1600 square foot coral culture research facility which grows many of the Pacific and Atlantic corals required

Table 2: List of current species cultured by the CH&D Program for health analysis.

Coral and Other Species	Ocean	ESA Status
Coral Species used in Contaminant Research:		
Acropora palmata	Atlantic/Caribbean	Threatened
Acropora cervicornis	Atlantic/Caribbean	Threatened
Pocillopora damicornis	Indo-Pacific	
Orbicella faveolata	Atlantic/Caribbean	Threatened
Other Species for Contaminant Research		
Hawaiian amphipods	Pacific	
Hawaiian brittle stars	Pacific	
Ulva sp. (macroalgae)	Worldwide	
Hawaiian volcano shrimp	Pacific	
sea urchins	Atlantic/Pacific	
Brain Corals for SCTLD Work		
Colpophyllia natans	Atlantic/Caribbean	
Dendrogyra cylindrus	Atlantic/Caribbean	Threatened
Dichocoenia stokesii	Atlantic/Caribbean	
Diploria labyrinthiformis	Atlantic/Caribbean	
Meandrina meandrites	Atlantic/Caribbean	
Montastrea cavernosa	Atlantic/Caribbean	
Orbicella franksi	Atlantic/Caribbean	Threatened
Porites astreoides	Atlantic/Caribbean	
Pseudodiploria clivosa	Atlantic/Caribbean	
Pseudodiploria stringosa	Atlantic/Caribbean	
Siderastrea siderea	Atlantic/Caribbean	
Other cnidarian species as reproductive models		
Cassiopea	Atlantic/Caribbean	
Euphyllia sp.	Pacific	
Nematostella sp.	Atlantic	
Protopalythoa sp.	Atlantic/Caribbean	
Xenia sp.	Indo-Pacific	

for contaminant exposures (Table 2; see summary protocols in Appendix 2). This is an important component of the program, allowing us to forego the need for collecting corals from the wild for scientific experimentation. It also allows us to control growth conditions, particularly important for contaminant exposures. In the Florida Keys reef tract, there are 40 different species of coral with speciation even more apparent in the Pacific; consequently, our studies are focused on key coral species as defined by the experimental need and scientific impact. We maintain a large number of different species of coral (Table 2) including some of the few remaining genotypes in the world for *Dendrogyra*

cylindrus. As corals are non-model organisms, new experimental protocols often need to be developed based on the scientific requirements of an individual study. Following testing and verification, those protocols are then shared through our publications and program's website supporting the Coral Disease and Health Consortium (CDHC) (see https://cdhc.noaa.gov/), a collaboration of over 150 researchers.

2. Marine Mammals:

While our microplastic experimental methods for marine mammals are well documented in Francesca Battaglia's and Tessa Pfeifer's Master's Theses (Battaglia, 2019; Pfiefer, 2020), the collection of marine mammal samples for biological and chemical analyses are supported through the following:

1. South Carolina Marine Mammal Stranding Network: Originally managed by the KSB Branch, the South Carolina Marine Mammal Stranding Network (SCMMSN) is now managed by Coastal Carolina University (CCU) and the Lowcountry Marine Mammal Network (LMMN). They respond to marine mammal strandings in all South Carolina waters to learn more about the species in our waters, to minimize pain and suffering of live-stranded animals, and to protect public safety and health. CCU employees are currently co-located within our laboratory in Charleston and in collaboration with the LMMN, both have access to NCCOS equipment necessary to respond to marine mammal strandings. Samples for chemical and contaminant analyses are collected both in the field and as part of necropsies performed in our Center for Coastal Environmental Health and Biomolecular Research (CCEHBR) building necropsy facility. Samples, many of which have been used for chemical analysis in partnership with collaborators, have been collected from bottlenose dolphins, spotted dolphins, white-sided dolphins, pygmy sperm whales, dwarf sperm whales, pilot whales, river otters, and others.

2. Partnership with the National Marine Mammal Foundation (NMMF): Samples from live animals are also needed. Previously available through dolphin health and assessment surveys conducted by our laboratory, this responsibility has transitioned in recent years to the NMMF. Through a Memorandum of Understanding, the NMMF has access to boats from our laboratory to conduct health assessments of dolphins. In this process, dolphins in Barataria Bay (LA), Brunswick (GA), and Charleston (SC) have been isolated during capture-release operations where samples were collected to support biological and chemical analyses.

3. Dart biopsies and picture identification: Stranded animals provide an invaluable source of samples for analyses, yet the numbers are limited and tissues can degrade with the passing of the animal. As such, protocols for dart biopsies were developed for collecting tissue samples making it possible to investigate contaminant loads in live animals. Due to unique markings on their fins analyzed as part of annual assessments, animals like dolphins can often be individually identified, thus enabling a direct connection between the chemical results from the sample, the individual animal, and the habitat in which they live.

4. Skeletal preparation facility: Bones are used for both element analysis and aging of cetaceans. A dermestid beetle colony has been maintained in our beetle barn for removing tissue from the bones of marine mammals. Carcasses or parts thereof from necropsies are put into incubators where the beetles consume the flesh, leaving the bones. While other methods for tissue removal exist, this is an efficient and cost-effective protocol. In conformance of the National Marine Mammal Protection Act, representative skeletal samples are retained and are predominantly maintained through partnerships with outside organizations including the SC State Museum's Department of Natural History (MOA) and the Charleston Museum.

Samples collected using the protocols above are logged into our Marine Mammal Information System and stored in freezers for histological, biological, physical, and chemical analysis. Samples include muscle, lung, kidney, liver, brain, blubber, skin, stomach, adrenal glands, gonads, thymus, thyroid, pancreas, intestine, lymph nodes, feces, urine, blood, skeletal material, teeth, and lesions. In addition, samples (blubber, liver, and kidney) from fresh animals are also shared with the National Institute of Standards and Technology (NIST) National Marine Mammal Tissue Bank as reference samples. Both of these sources of samples have supported numerous collaborative chemical investigations.

3. Genetics/Genomics:

While the marine genetics and genomics capabilities within NCCOS Charleston used to more considerable, it has been consolidated in recent years both in terms of available equipment and number of people. The expectation though is that our reliance on genetic and genomic techniques will increase in upcoming years. Experimental methods are documented in KSB's two genetics/genomics reports submitted as part of this review (see Table 1) and our analyses are currently supported by a combination of available equipment and nucleic acid sequencing contracts. In house, DNA sequencing is available through an Applied Biosystems 3130xl capillary sequencer and MinION nanopore sequencer. A number of real-time PCR machines are available for DNA amplification and expression analysis. Personnel support in the EG Program includes Dr. Thomas Greig (Program Manager) (Appendix 1), interactions with students from the College of Charleston, and collaborations with other universities.

In addition to the two reports submitted, the EG Program also used genomic techniques as part of a recent collaboration with the NCCOS Marine Spatial Ecology Division to identify deep water coral species that may have been impacted from the Deep Water Horizon oil spill in the Gulf of Mexico (see Figure 4). Genomic analyses are also conducted in the CH&D Program through collaborations with the USGS National Fish Health Laboratory, Florida Department of Fish and Wildlife, and Pennsylvania State University among others to support predominantly health assessments, gene expression, metagenomics and reproductive compatibility of endangered corals.

Figure 4 – Deep-water sea fans (*Swiftia exserta*) on East Flower Garden Bank off the Texas coast. The species is known to have been negatively affected by the Deepwater Horizon oil spill. Credit: NOAA

4. Benthic Invertebrates:

The former Coastal Ecology (CE) Program has been consolidated recently into our Quantitative Ecology (QE) Program. Dr. Len Balthis leads our branch's benthic analyses, coastal mitigation work, and sea-level rise impact modeling through a number of collaborations with the NCCOS Marine Spatial Ecology Division and regional and academic partners. While benthic analysis methods for our deep sea oil work are included in Dr. Bathis's report for this review (Table 1; Balthis et al, 2017b), a schematic summary of the benthos classification protocols as taken from our 2017 NOAA Technical Memorandum describing his work in the Guana Tolomato Matanzas National Estuarine Research Reserve (NERR) is shown in Figure 5. This work highlights and also provides an example of our support for the NERRS network, which includes a series of 27 estuarine reserves co-managed by NOAA and state governments. Our research priority is to conduct an overall assessment of the ecological condition of the investigated sites, part of which includes a chemical contaminant evaluation (Figure 5).

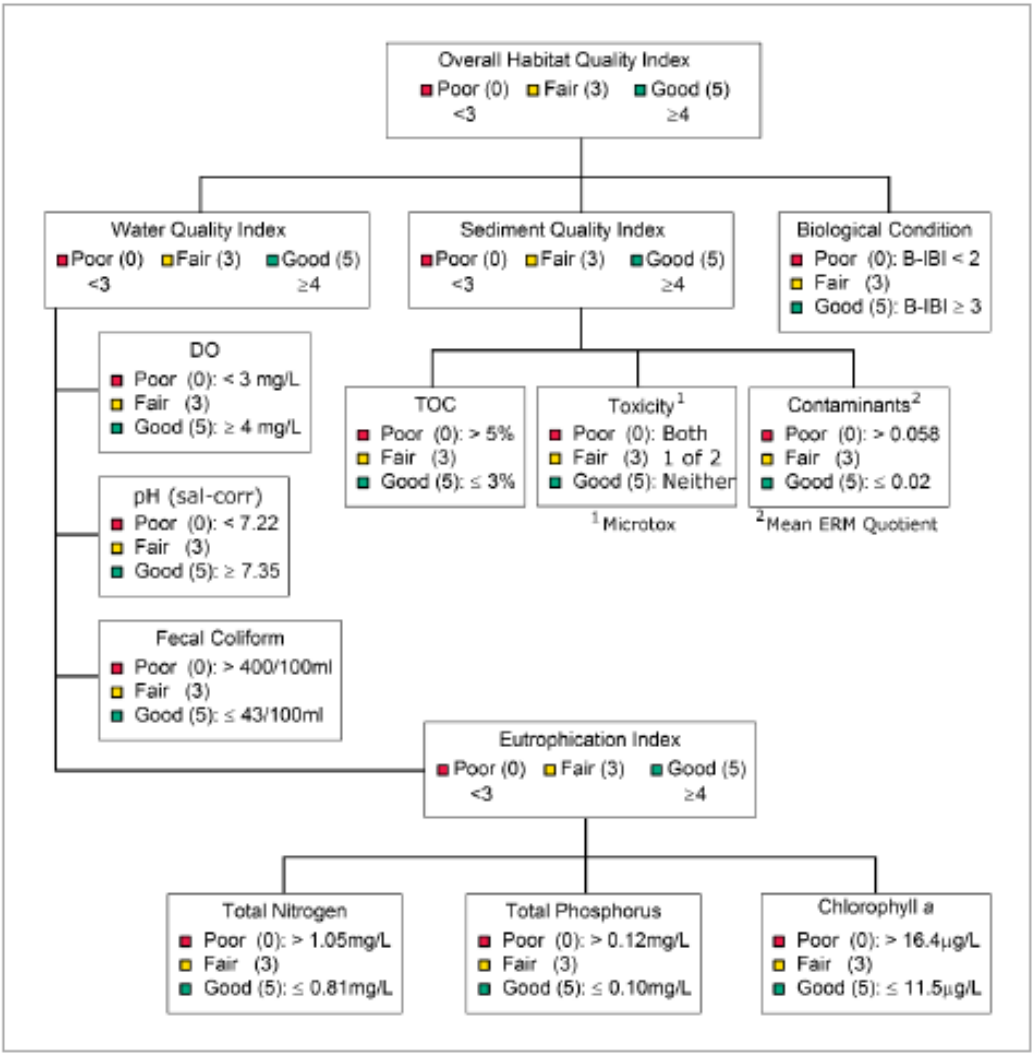

Figure 5. Sampling indicators and scoring criteria used to derive indices of water quality, sediment quality, biological (benthic) condition, and overall habitat quality (modified from Balthis et al., 2015, Van Dolah et al., 2013)

5. Statistics and Bioinformatics:

The bioinformatics and statistics expertise within the Key Species and Bioinformatics Branch is provided predominantly by individual scientists, although we recognize the need to further develop or supplement our current mathematical modeling and computer expertise to support current and future projects. A summary of the statistical and computer methods is described below:

a. Statistical analysis and modeling: A range of parametric, semi-parametric, and nonparametric statistical techniques are used to evaluate potential biological responses to stressors based on measurements derived from field sampling efforts and laboratory experiments. Within the Key Species and Bioinformatics Branch, we have staff who are fluent in using R, SAS, Primer, and MATLAB to develop appropriate models and evaluate statistical significance using a frequentist approach, and efforts are underway to include Bayesian methods in future analyses. We also perform analysis and visualization of spatial data using R and ArcGIS.

b. Bioinformatics – genomics: A number of genomic projects are now underway including those that support chemical contaminants (grass shrimp genome, grass shrimp transcriptomics, quagga mussel transcriptomics). While collaborations exist with the National Institute of Standards and Technology (NIST) co-located here in Charleston, there is a need to continue to develop the bioinformatics skills for the interpretation of -omics data. In addition, computer support is currently limited and we are in consultation with our IT department to gain access to cloud computing resources.

c. Bioinformatics – proteomics: Proteomic analysis offer a novel way to characterize physiological responses to stressors. While currently supporting projects in coral and marine mammal disease, proteomic techniques are also being developed in the KSB Branch (1 post-doctoral fellow, 1 student) through a collaboration with NIST and the College of Charleston. This expertise will support all types of projects in the future including the evaluation of chemical stressors.

d. Computer Programming: Python programs are currently being developed to help automate processes for counting bacteria on plates (as part of the Ames test) and quantitate coral bleaching, a metric of coral and foraminiferan stress (we have projects in both). Additional python scripts were also developed to automate geospatial data processing and publishing of spatial data to mapping applications and story maps on NOAA's GeoPlatform. R scripts were developed for automating data processing tasks and exporting to a standardized format for import into an enterprise Azure SQL database. Finally, custom SQL Server Integration Services packages were developed for importing/updating databases, as well as SQL queries and R scripts for querying the databases.

Background

The purpose of our work is to produce actionable science regarding stressors impacting the marine ecosystem in the coastal waters of the United States. Within the Key Species and Bioinformatics Branch, we focus our work to better understand the impacts of chemical, biological and physical stressors on key species, specifically those that are dramatically impacted and/or provide the best indicators on marine health. Recognizing the critical role the molecular sciences like genomics and proteomics will serve in the future, we continue to develop our expertise through our own novel research and through a host of collaborations with partners.

Partnerships with academic, non-profit, state, federal, and international organizations provide a mechanism that help support the personnel and physical resources needed to meet NOAA missions. Originally a NMFS laboratory, our laboratory's research at the time was a combination of ecosystem sciences research, seafood safety analysis, marine mammal health assessment, and species management including chemical assessments. For example, we published work as early as 1997 on trace element analysis in dolphins. Chemical work continued as the laboratory transitioned to the National Ocean Service in 1999 including many publications such as the collaboration in 2002 with the National Institute of Standards and Technology (NIST) entitled "Persistent Organochlorine Pollutants and Elements Determined in Tissues of Rough-Toothed Dolphins (*Steno bredanensis*) Banked from a Mass Stranding Event" which analyzed the tissue of 15 of the 62 rough-toothed dolphins that stranded near Apalachicola, FL in December of 1997. This study documented persistent organic pollutant (POP) and element concentrations in a pelagic species for which little data of this kind were available. The role of the CMMA and CE (now QE) Programs matured through the mid-2010's collaborating on numerous publications including "Distribution and sources of PCBs (Aroclor 1268) in the Sapelo Island National Estuarine Research Reserve" regarding the impacts of pollutants near a superfund site in Brunswick, Georgia (Wirth et al. 2014). Another paper in 2014 entitled "Health of Common Bottlenose Dolphins (*Tursiops truncatus*) in Barataria Bay, Louisiana, Following the Deepwater Horizon Oil Spill" followed the analysis of chemical contaminants resulting from a natural disaster (Schwacke et al., 2014). In 2016, the size of the CMMA Program decreased significantly and the program adapted by developing strong partnerships with the NMMF, CCU, CofC, and the LMMN. Such work has led to studies of the impacts of microplastics and elemental analysis discussed in the review report presented by Wayne McFee (see Table 1) and the Master's Theses completed within Wayne's program from Francesca Battaglia (Battaglia, 2019) and Tessa Pfiefer (Pfiefer, 2020).

Our genetics program too has adapted. Originally a funded priority at the laboratory, the program was a leader in the development of microarray and other genetic techniques supporting the biomolecular needs throughout the agency. This included marine mammals where a real-time PCR test was developed for the detection of *Brucella* and other pathogen stressors in blowhole swabs from bottlenose dolphins and leptospirosis in California sea lions. While much of the genetics priorities have been consolidated into a single position, the current EG Program is a high priority in recognition of future applications of the molecular techniques and the need for this type of data. The laboratory has undergone a number of reorganizations in recent years including its recent move of the genomics staff (1 federal position) to the Key Species and Bioinformatics Branch last year. We are excited about this transition given our research focus on bioinformatics and my background as a program manager for the NMFS Alaska Fisheries Science Center Genetics Program (5-9 federal employees and 2-8 contractors). Genetics projects in recent years have included: (1) transcriptomics and genomics of grass shrimp which serve as an ecological model organisms for estuarine impacts, (2) metagenomics of bacterial species distribution resulting from the use of oil dispersants, and (3) species identification of deep water corals supporting deep water Horizon oil spill research in NCCOS's Marine Spatial Ecology Division.

The Deepwater Horizon 2010 oil spill was the largest human marine oil spill in history, discharging an estimated 4.9 million barrels of oil into the Gulf of Mexico. Understanding the impacts of natural and anthropogenic disasters has been a high priority for our organization since its inception and this analysis helps the government better understand what happened and assists in developing mitigation strategies in the event of future events. During an oil spill in coastal waters, NOAA's role is to provide scientific support to the U.S. Coast Guard in charge of response operations. Following, we provide the scientific evaluations regarding the effects and ecological consequences that the Coast Guard needs (Figure 6). In support of this role (through NOAA's Office of Response and Restoration) and in addition to work supporting deep water corals (Etnoyer et al., 2016), analysis of dolphins in Barataria Bay, LA (part of collaboration with the NMMF) (McDonald et al, 2017; Smith et al., 2017), and metagenomic analyses investigating the impacts of oil dispersants, the KSB Branch was also involved in two additional ways. First, a 2017 publication from our branch reported the impacts of the spill on the deep-sea benthos. Using a logistic regression analysis to derive sediment quality benchmarks, mathematical models and criteria were developed for assessing biological impacts of this and future oil spills (Table 1, see report submitted by Len Balthis; Balthis et al, 2018a). Second, toxicity thresholds of oil on shallow-water corals were investigated. As mentioned previously, shallow-water corals are keystone species in tropical ecosystems. These animals exist within a complex symbiotic relationship that include species of algae living inside the corals. Through photosynthesis, these algae produce the oxygen and energy necessary to sustain the corals. Impacted by disease, physical stressors like heat, and chemical stressors, corals are in rapid decline throughout the world. Given the endangered status of many of our coral species in both the Atlantic and Pacific and potentially differential impacts from oil exposure, experiments were conducted with both a Pacific branching coral (Table 1, see report from May et al., 2020 manuscript) and Atlantic mounding coral (manuscript in preparation) showing physiological impacts on coral health and wound healing at low oil concentrations.

Figure 6 – Dr. Cheryl Woodley, program lead for the Coral Health and Disease Program, tours personnel from the U.S. Coast Guard through our 1600 sq foot coral culture facility. (2019)

As mentioned previously, a key mantra of the Coral Health and Disease Program is investigation, mitigation and restoration. In 2016, a report was published (Dr. Cheryl Woodley was a co-author) highlighting the negative consequence of oxybenzone on coral, an ingredient in a number of sunscreens. In areas of high swimmer density, the oxybenzone concentrations can reach levels toxic to corals. Analyses completed in collaboration with other agencies found high levels of oxybenzone in certain nearshore areas of Hawaii and Key West where local communities have subsequently limited the sale and use of sunscreens that contain oxybenzone. Infographics have been developed by the Coral Health and Disease Program to help communicate the issue and share ideas on how to protect yourself and marine life (Figure 7). The use of sunscreens with oxybenzone has been discussed in US Senate

committees recently, for which the KSB CH&D Program has provided a synopsis of the current literature and expertise in the last year.

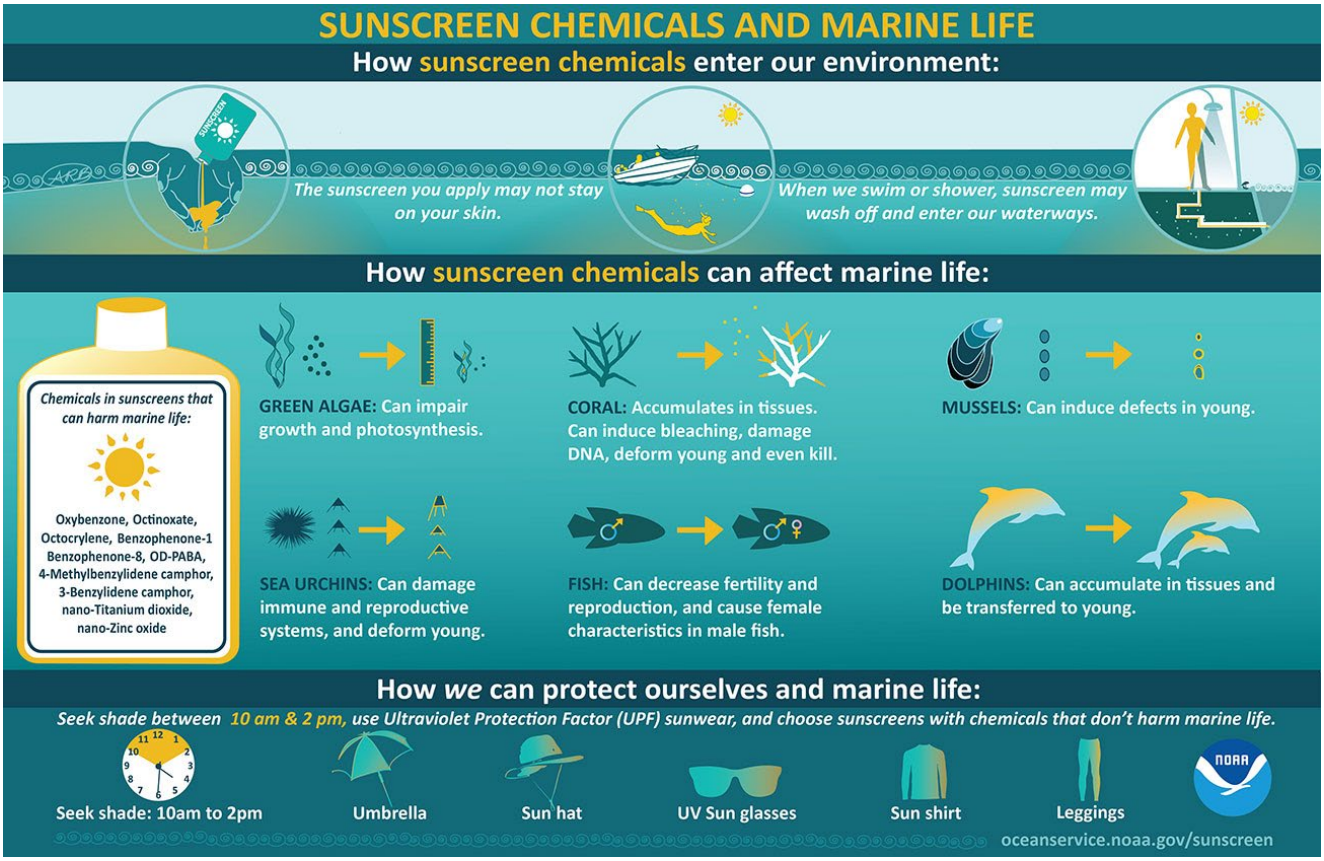

Figure 7 – Sunscreen infographic developed by Athena Burnett from the Coral Health and Disease Program. Taken from: https://oceanservice.noaa.gov/news/sunscreen-corals.html

In addition to specific biological, chemical and physical contaminants, the impacts of nutrients has also been investigated. Total ammonia nitrogen (TAN) is a ubiquitous pollutant in the marine environment. Sources of TAN in near shore waters are both anthropogenic (industrial processes, municipal sewage discharges, agricultural runoff) and natural (bacterial nitrogen fixation, biodegradation of dead plant and animals, fish excrement). The chemical form of ammonia in water consists of two species: ammonium (NH_4^+) and unionized ammonia (NH_3). Unionized ammonia (UAN) is considered the most toxic species and its concentration in surface waters is highly dependent upon pH and temperature. While the toxicity of ammonia is understood, there is little data on the effects of elevated ammonia on marine organisms such as corals. We have evaluated ammonia toxicity (in the form of ammonium chloride) to a Pacific coral (*Acropora formosa*), Atlantic coral larvae (*A. palmata* and *Orbicella faveolata*) and sea urchin embryos (*Lytechinus variegatus* and *Arbacia punctulata*) in acute (48-96 h) and chronic (10 d) dose response tests. Adult *Acropora formosa* tissue regeneration was sensitive to TAN concentrations of 1.3 mg/L and higher (EC_{50} = 0.09 mg/L UAN) in a 10-day test. Fragments exposed to 1.95-2.60 mg/L TAN lost all tissue within 5 days. Viability of coral larvae was impacted at 1.56 mg/L TAN and higher at 29 °C, and toxicity increased with a 2 °C increase in temperature (EC_{50} reduced from 0.07 to 0.05 mg/L UAN). Sea urchin development was less sensitive to ammonia exposure, with embryo development effects noted at 2.1 mg/L TAN (EC_{50} < 0.18 mg/L UAN). This research demonstrates that benthic marine organisms such as corals and sea urchins are particularly

sensitive to elevated ammonia. We predict that elevated seawater temperatures due to climate change will exacerbate negative effects of ammonia exposure in near shore environments.

Over the past 30 years, the health of our shallow water coral ecosystems has decreased significantly, no place more than the Florida Reef Tract. Some stressors implicated in coral decline include biological disease and physical effects like warming and sedimentation, but also include chemical stressors. While there are different classifications of stressors, they are not mutually exclusive and can act synergistically. For example, the controversial dredging of Government Cut in Miami, Florida in 2014/5 increased the amount of sedimentation and stirred chemical contaminants into the water, but also coincided with the onset of stony coral tissue loss disease (potentially a biological stressor) that has ravished 20 of the 40 coral species throughout Florida, driving some species close to extinction. What we do know for certain is that the health of Florida coral reefs is in peril. In 2018, KSB's QE Program published a NOAA Technical Memorandum entitled "Assessment of Ecological Condition and Potential Stressor Impacts in Offshore Areas of Florida Keys National Marine Sanctuary" which included an assessment of the ecological condition of 30 soft-bottom habitat and overlying waters in the Florida Keys National Marine Sanctuary (FKNMS) (Balthis et al, 2018a). Although no contaminants were found in excess of corresponding Effects Range-Median (ERM) values, levels of total polychlorinated biphenyls (PCBs) were measured in excess of the Effects Range-Low (ERL) at one site and biological impacts from uncharacterized stressors were apparent at other sites. Efforts are underway to supplement that study with a shallow-water characterization of the nearby coastal area in future years.

Discussion

The NCCOS Key Species and Bioinformatics branch is currently comprised of 4 seemingly unique programs, each leading and contributing to NOAA's mission in the protection of marine ecosystems. These programs function together as a team using key species in evaluating critical biological responses to chemical, biological and physical stressors. We know that many of the waterways near urbanized areas are contaminated. What we don't know are the impacts of that contamination on the marine and estuarine life there. Because there are so many different species, our results focus on key species that include: (1) iconic apex predators that are closely related to humans (dolphins and other marine mammals), (2) shallow water corals which form the base of all tropical ecosystems, and (3) benthic animals that tend not to migrate and can be severely impacted based on changes to the local environment.

NOAA is the federally designated lead agency responsible for the management of the species and environments mentioned above. As an organization within NOAA, NCCOS and the KSB branch in particular have a unique and critical role identifying stressor impacts and mitigation strategies for managing these species and the marine environment. We are experts in (a) coral health and disease research, (b) biological markers and ecosystem impacts on diversity (we conduct highly collaborative assessments of the benthos in response to environmental stressors), and (c) marine mammals where we are the only federal office in the states of South Carolina and Georgia focused on these iconic species. Over the last 20 years, our laboratory developed and continues to develop the expertise necessary to support the highest quality research into the impacts of all types of stressors. We have adapted over time both in response to urgent scientific needs (i.e. the Deep Water Horizon oil spill) and to fiscal pressure (i.e. our genetics program was mostly disbanded 4 years ago). Then, after adapting, we adapted again as our needs changed; for example, we are now rebuilding our EG Program to take advantage of the scale and technological advances it represents. We recognize that multiplexing capabilities in genomics can provide the most cost effective data sets for investigating biological

resiliency and response to all types of stressors. Over this time, we've shown that we can be nimble and able to adapt to meet the scientific needs of our agency by conducting critical novel research and supporting highly collaborative studies with universities, non-profits, and other governmental organizations.

Figure 8 – A sick dolphin near Charleston, South Carolina. Because these animals remain in the same vicinity year-round (coastal stocks), samples taken from them are valuable for accessing the impacts of local contaminants on our apex predators.

Our single purpose is to produce actionable science in response to the needs of our agency. Some of the responses are more immediate and include collaborations with CCU and LMMN that supported the collection of samples from the pod of pilot whales that stranded on Edisto Beach last year (Figure 2). Some of our responses are more long term such as the investigation of potential pollutants in the benthic environment along the Florida Keys National Marine Sanctuary, an area where corals have decreased over 70% in the last 30 years. These same corals are now under stress from stony coral tissue loss disease. While the causative agent is still not known, this disease has impacted about half of the species in the Keys including the almost complete loss of two species of coral. We have some of the last remaining genotypes of pillar corals growing in our culture facility here in Charleston. Our work has also greatly impacted both the local and national community. For example, Wayne McFee (lead for the CMMA Program) worked with BBC Blue Planet II productions last year to film our research on microplastics in dolphins and also appeared on local Charleston television news twice last year sharing results from their studies on marine mammals (Figure 8). Similarly, Cheryl Woodley (lead of the Coral Health and Disease Program) has responded to congressional committee inquiries regarding sunscreens that contain oxybenzone, a compound Cheryl and others have shown to negatively impact the health of the coral reef. Cheryl has also been interviewed as part of the national news for work in coral research. The Key Species and Bioinformatics Branch includes some of the most pertinent science that our agency can produce. As experts in animal health and disease, we provide the key biological link that connects NCCOS's studies on marine stressor impacts with the management needs of NOAA in the stewardship for all the key species described above.

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Appendix 1: Organizational Structure of the Key Species and Bioinformatics Branch

Key Species and Bioinformatics Branch

Branch Chief: Jeff Guyon, PhD (University of Notre Dame/Harvard Medical School)

Coastal Marine Mammal Assessment (CMMA) Program

Program Lead: Wayne McFee, MS (Northeastern University)

Part-time Contractor: Tessa Pfeiffer, MS (College of Charleston)

Coral Health and Disease (CH&D) Program

Program Lead: Cheryl Woodley, PhD (Medical University of South Carolina)

Federal Staff: Laura Webster, BS (College of Charleston), BS (MUSC)

Contractors: Lisa May, MS (Austin Peay State University)

Carl Miller, BS (Purdue University)

Zac Moffitt, BS (University of South Carolina)

Elizabeth Duselis, PhD (University of Virginia)

Ron Kothera, MS (Clemson University)

Environmental Genetics (EG) Program

Program Lead: Thomas Greig, PhD (University of South Carolina)

Quantitative Ecology (QE) Program

Program Lead: Len Balthis, PhD (Medical University of South Carolina)

Appendix 2: NCCOS Coral Health and Disease Program Aquaculture and Research Support Facility

A commitment to a renewable specimen resource approach

Introduction

With coral populations decreasing at exponential rates globally (Pandolfi *et al.* 2003), there has never been a more compelling need for coral aquaculture facilities to house and produce specimens for scientific research and restoration support. Continuing to remove coral from wild reefs for experiments is ethically debatable and due to increased regulations, it has become more difficult to obtain collection permits. Corals in our holdings have been acquired through multiple channels that minimized the impact on wild populations (e.g. rescuing diseased specimens or specimens in a designated construction/dredge zone, specimens received from other laboratories). Once in culture, there is more control over coral growth parameters, thereby reducing variables that may affect experimental results. Health status and stressor tolerances can vary between wild colonies or genotypes. The NCCOS Coral Health and Disease facility can generate hundreds of healthy asexual fragments from one colony (genotype). These genotypic clones reduce experimental variability. Having these colonies in our facility allows us to produce test specimens as needed, instead of waiting months to obtain proper permits and permissions and spending significant amounts for logistical support to collect wild corals.

The NCCOS Coral Health and Disease Program coral collections are predominantly of Caribbean origin with the primary exception being *Pocillopora damicornis*, a branching IndoPacific model species. *Acropora cervicornis* (ESA listed) and *Orbicella faveolata* (ESA listed) along with *P. damicornis* have been used extensively for most of our experiments. We recently received 10 small *Acropora palmata* (ESA listed) fragments that are being cultured for experimental support. A collection of small fragments of protected (Chapter 68B-42, Florida Administrative Code; FL Keys National Marine Sanctuary, National Park Service) Caribbean mounding brain coral species (*Colpophyllia natans, Dichocoenia stokesii, Diploria labyrinthiformis, Madracis mirabilis, Meandrina meandrites, Montastrea cavernosa, Orbicella franksi, Porites astreoides, Pseudodiploria clivosa, Pseudodiploria stringosa*, and *Siderastrea siderea*) represents highly susceptible species to stony coral tissue loss disease (SCTLD). These are being grown in aquaculture systems for eventual propagation to support experimental research projects that include genome sequencing, coral disease and treatment studies, *ex situ* coral reproduction, bioassay development and contaminant/stressor exposure-response studies. We have 95 *Dendrogyra cylindrus* (ESA listed) rescued fragments representing approximately 80 genotypes which have been designated for spawning and recovery projects.

Acquisition and Permitting Requirements

All collections of Caribbean scleractinian coral require multiple permits that depend on the species and jurisdictional requirements (Table 1). Our culture facility requires a State of South Carolina non-indigenous species permit and a federal permit issued by the Florida Keys National Marine Sanctuary for all specimens originating from the sanctuary in addition to the original collection permits. Similarly, those specimens originating from Biscayne National Park or Dry Tortugas National Park require separate collection and live animal holding permits. We also have holdings from coral nurseries (e.g.,

Coral Restoration Foundation, Nova Southeastern University) and *ex situ* facilities (e.g., Florida Aquarium) that have their own oversight and permitting procedures.

The time required to obtain permits can range from a few weeks to several months, depending on the organizations involved, the number of required permits and the individual reviews. Permits to collect healthy specimens directly from the reef is highly restricted and heavily scrutinized by permitting agencies and wild acquisitions are often limited to samples of opportunity, often associated with removal for construction projects (e.g., dock repairs; or associated with an emergency disease outbreak response). For example, multiple brain coral fragments were acquired for differential analyses associated with SCTLD and for disease intervention studies. On the other hand, diseased *Dendrogyra cylindrus* fragments were successfully treated and rehabilitated for genetic banking, *ex situ* reproduction studies, and eventually to contribute to their restoration in the wild.

Table 1. Examples of collection permits for coral in culture, approval time, and duration.

Jurisdiction	Time consideration	Duration
SC DNR non-indigenous species	Days	Yearly renewal
FL Keys NMS live holdings	2-4 months	5 years
FL Keys NMS collection permits	2-4 months	1 year
State of FL if outside FKNMS	1-2 months	1 year
Biscayne National Park	1-3 months	1 year
Dry Tortugas National Park	1-3 months	1 year
USVI - DPNR	1-3 months	1 year
Salt River Bay NHP&ER	1-3 months	1 year
Buck Island National Coral Reef Monument	1-3 months	1 year
Virgin Islands National Park	1-3 months	1 year
Hawaii Dept. Land & Natural Resources	3-6 months	1 year
Coral Restoration Foundation	1 month	1 year
Florida Aquarium (sexual recruits)	2-3 months	No end date/transfer agreement
International – CITES export & import permits required	2-6 months	per shipment

Life Support System Requirements for Successful Coral Aquaculture

Coral aquaculture requires more complex life support systems (Figures 1 and 2) than those typically designed for other marine invertebrates or finfish. While most marine fish and invertebrates can tolerate larger ranges in commonly tested parameters like temperature and salinity, sudden swings in either can cause stress or death in scleractinian corals. A temperature increase of just two degrees can trigger a coral bleaching response (Hueerkamp *et al.* 2001). Even for coral species that have adapted to areas of the reef that experience more variation in salinity, a sudden change of >10% in salinity can cause reductions in both respiration and photosynthetic rates (Muthiga *et al.* 1987) and efficiency

(Downs *et al.* 2009). In addition to temperature and salinity, other water chemistry parameters must be maintained (via dosing, reactors, and filters) at proper levels for coral to survive, grow and ultimately reproduce (Table 2.)

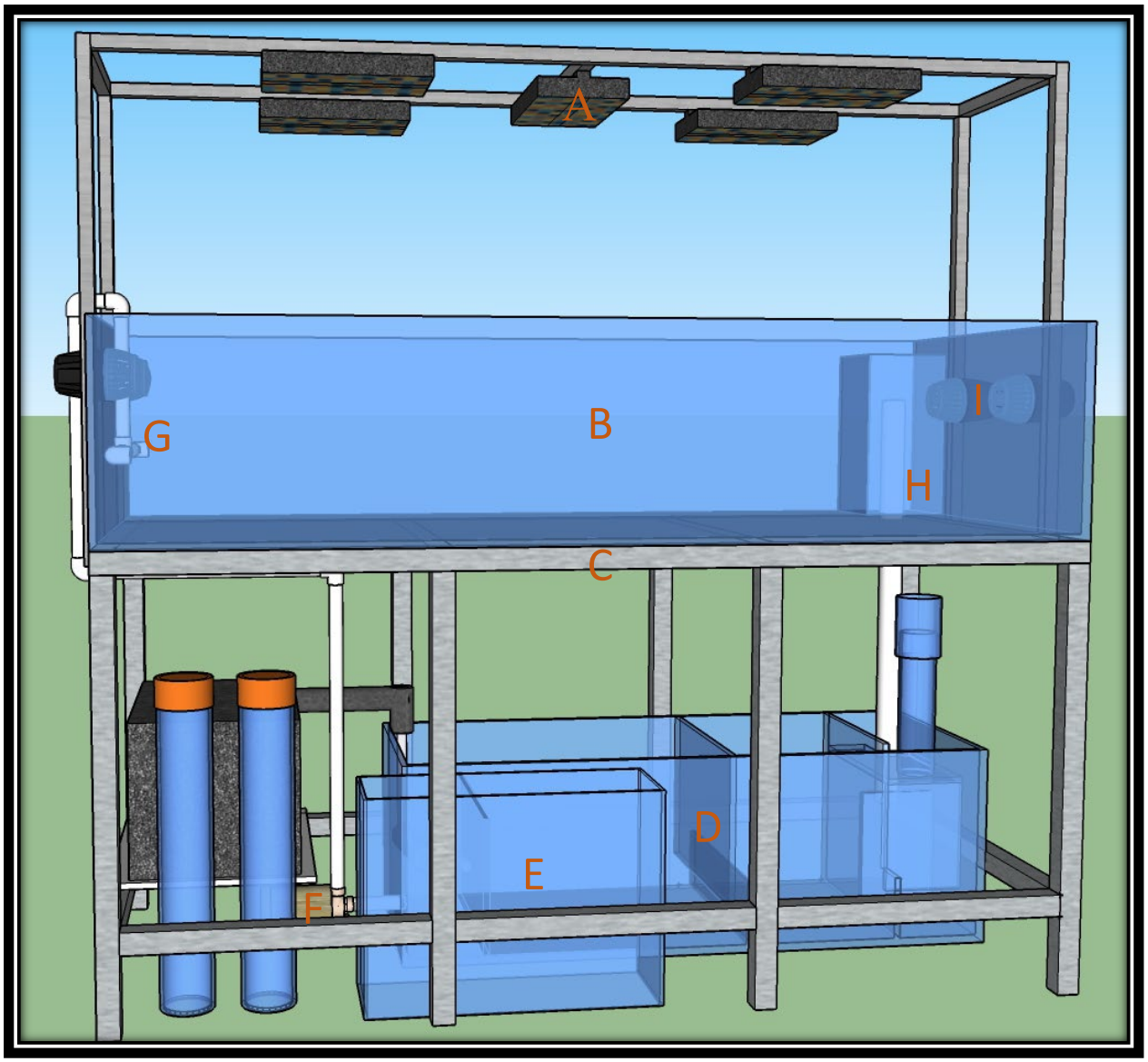

Figure 1. Simplified diagram of essential components of a coral aquaculture system: A. LED lighting, B. large glass culture aquarium, C. sturdy stainless steel support stand, D. glass sump system (see Figure 2 for more detail), E. glass top-off reservoir (adds fresh water to combat evaporation), F. main return pump (pumps water from the sump up to the culture aquarium), G. return plumbing, H. overflow box and drain plumbing (maintains water level in culture aquarium and drains water to the sump system), and I. wavemakers/powerheads (for additional water movement/current)

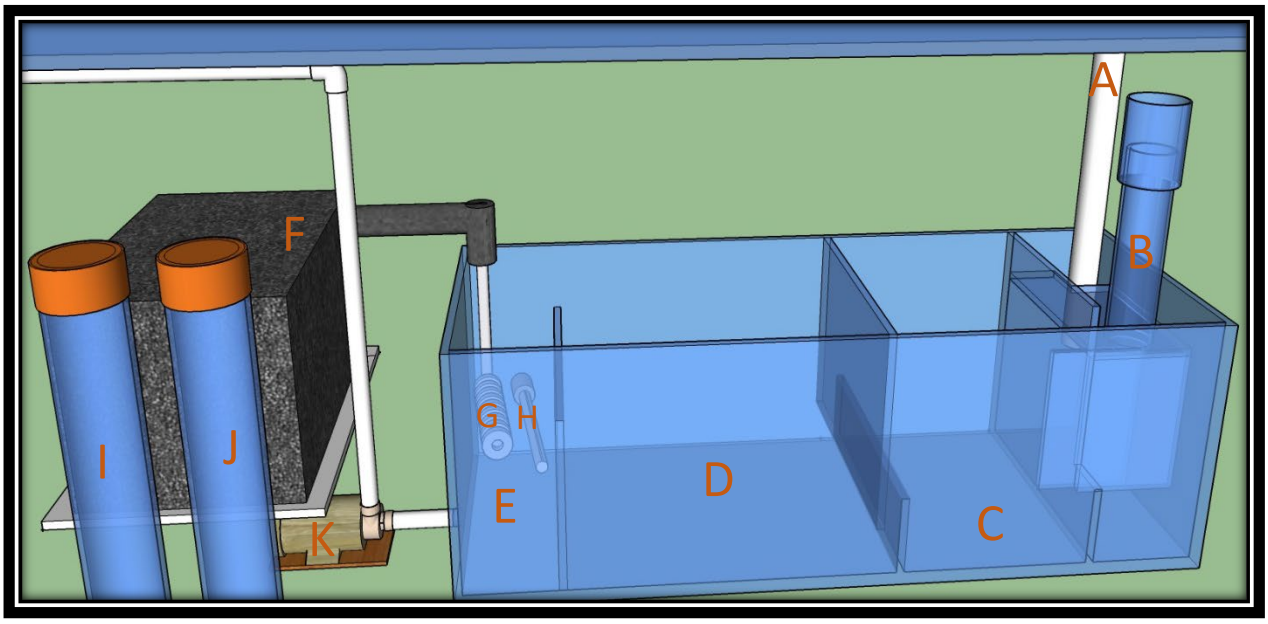

Figure 2. Diagram showing different components of a sump system and associated equipment: A. drain plumbing from culture aquarium, B. protein skimmer (mechanical filtration), C. sump compartment for a deep sand bed (biological filtration), D. sump compartment for liverock and algae culture (biological filtration), E. sump compartment for equipment like top-off censors, water chemistry probes, and temperature regulation equipment (e.g. Chillers (F) and associated coils (G) and heaters (H)), I. calcium reactor, J. media reactor (chemical filtration), and K. return pump

Three modes of filtration (mechanical, biological, and chemical) are generally used in coral aquaculture. We find that mechanical and biological filtration are essential. We occasionally use chemical filtration on systems that house more sensitive species (e.g. *Dendrogyra cylindrus*). Most stony corals prefer very clean (low nutrient and particulate) conditions. Oversized protein skimmers are desired for mechanical removal of organic carbons (TOCs) and other small particulates. Biological filtration in the form of liverock, deep sand beds (DSB), and algal cultures are important for establishing a stable nutrient cycle as well as for actively removing excess nutrients. These modes of filtration along with other equipment (e.g. heater, chiller coils, water parameter monitoring probes) can be kept in a separate tank (generally referred to as a sump). The total water volume of the main system should cycle through the sump at least 5x per hour to provide adequate filtration and temperature control. In addition to this water movement, auxiliary pumps and/or wavemakers are used to create randomized water currents that move the total system volume 10 to more than 30 times per hour depending on the individual requirements of a given species.

Light is required for all coral with obligate symbiotic algae. When natural sunlight is not an option, various types of artificial lighting are used to approximate a light spectrum in the photosynthetically active range (i.e., PAR) of 400-700 nm. Metal halide bulbs were among the first used for coral aquaculture, followed by T-5 and compact fluorescent bulbs. Recently, LED technology with a new appreciation of the photophysiological needs of coral have made several advances making them more adaptable to coral culture. Despite receiving much of their fixed carbon from zooxanthellae, increased growth rates can be achieved from feeding multiple times a week. Best results are achieved with small micron foods with elevated levels of highly unsaturated fatty acids (HUFAs).

Table 2. Optimal ranges for water chemistry parameters in coral aquaculture. Note: ranges can vary by species.

Parameter	Optimal Range
Temperature (°C)	25.5 - 27.5
Salinity (ppt)	35 - 36
pH	8.0 - 8.6
Alkalinity (ppm CaCO3 equivalents)	130 - 180
Ca (mg/L)	400 - 450
Mg (mg/L)	1250 - 1450
Phosphate (mg/L)	< 0.04
Ammonia (mg/L	< 0.02
Nitrite (mg/L)	< 0.02
Nitrate (mg/L)	< 0.02

Optimal ranges for light intensity (PAR) and spectrum, water current strength, and water chemistry vary between species. Hence, monocultures of a given species are ideal for achieving optimal performance. This also prevents aggression between species in the form of allelochemical production and/or sweeper tentacles. If designed properly, monocultures allow adequate space for the continual creation and growth of experimental fragments.

Coral growth rates highly vary from species to species. Typically, branching species grow faster than mounding species with *P. damicornis* exhibiting some of the fastest growth rates (cm/mo) under optimal conditions. Hence its wide use in laboratory experiments. With colonies of this species capable of more than doubling in size in a year, once an adequate stock is achieved, it is relatively easy to keep an ongoing culture to use for several experimental tests per year. For the fastest growing branching species a single medium sized (~150 gallons) aquaculture system may be adequate. *Acropora cervicornis* can grow fast in captivity as well, however it produces fewer branch tips (areas from which test fragments are collected) and requires more culture space. A self-sustaining culture which can be regularly harvested for experiments will take at least two years to grow. Mounding brain coral species like *O. faveolata* grow much slower (cm/yr) and so cultures grown for experiments need to be initiated much earlier and must be much larger. Currently, our facility has enough *O. faveolata* to support all our projects with multiple large-scale (120+ fragments needed) experiments each year; however, it has taken nearly 20 years to grow this mass of tissue from about 10 small thumb-sized colonies acquired from Florida in 2000. Multiple large (200+ gallons) aquaculture systems are designated for this one species to keep adequate stock. Having multiple aquaculture systems for a single species also creates redundancy that prevents total loss should a catastrophic failure occur with critical system equipment. This redundancy is something we would like to establish for each critical species.

NCCOS Coral Health and Disease Aquaculture Advancements

Toxicological work with munition compounds and crude oils in combination with increasing knowledge about leachates from plastics and the potential harms of microplastics (Bejgar et al. 2015; Tetu et al. 2019; Cuhra et al. 2017; Capolupo et al. 2020) have led us to reconsider how corals historically have been aquacultured for experimental purposes (Weir et al. 2014). Most coral nurseries and laboratories still use fiberglass or high-density polyethylene (HDPE) raceways, although some now

prefer to use glass or acrylic aquaria. However, almost all still use pumps, plumbing and filtration equipment manufactured from common plastics such as HDPE, polyvinyl chloride (PVC), low-density polyethylene (LDPE), and acrylic (Polymethyl methacrylate, PMMA) which readily degrade in light and/or seawater. Degradation of these compounds releases halogens, phthalates, heavy metals and a host of other harmful contaminants. In addition, when designing experiments for oil exposure on coral, it seemed counterintuitive to culture them in life support systems built from these petroleum-based materials.

Our coral aquaculture systems have been custom-designed without the use of fiberglass or the commonly used plastics listed above. Raceways are fabricated from glass sheets and assembled with marine grade silicone. All plumbing is constructed from chemically inert and highly stable Teflon thermoplastics (PTFE and PFA) and the wet assembly of all water pumps is constructed of carbon filled Teflon (ETFE). For aquaculture systems used to grow-out coral for other research, unrelated to toxicology, virgin polypropylene (PP) plumbing and PP lined pumps are used as a cost-cutting measure. Virgin PP is free of halogens, phthalates, and heavy metals but cannot be used in cultures or experiments where the material is exposed to significant UV as this will cause it to degrade (i.e., these do not contain UV stabilizers). It is also an incompatible material with petroleum compounds so it is not used to fabricate experimental components used in oil toxicity experiments. Mechanical filtration is achieved by air driven protein skimmers custom designed with only glass and Teflon (PFA) parts and assembled with marine grade silicone. Chemical filters and calcium reactors were also designed and customized with only glass, Teflon, and silicone. The result is a set of coral aquaculture systems that provide a contaminant free grow-out space as a renewable source of test specimens.

Locally sourced Charleston harbor natural seawater does not meet the quality standards for coral toxicology. Therefore, our aquaculture laboratory uses synthetic seawater for all coral culturing. Several years of testing various brands of artificial sea salts has shown that Tropic Marin Classic (Wartenberg, Germany) has out-performed other formulations. Synthetic seawater is made in-house and mixed to 36 ppt in Type-I water. The Type-I water is made from tap water that is first run through a 3-stage deionization filter system and then through a 4-stage Super-Q system (Millipore, Burlington, MA). Water is mixed in 5-gallon glass carboys on stir plates or for non-toxicological work in a 200 gallon polypropylene (PP) reservoir with all virgin PP plumbing and a PP lined mixing pump.

New advances in coral aquaculture such as *ex situ* reproduction, are now possible due to advancements in LED technology. We are in the process of transitioning our lighting to programmable LED units (Radion fixtures, Ecotech Marine, Allentown, PA).These lights provided several advantages which include spectral versatility, long bulb life, energy-savings and less heat (aiding in system temperature control which yields additional energy-savings). Our research goals of optimizing coral health, including growth and reproduction, have made it essential to mimic natural daily, monthly, and seasonal light cycles. LED lighting allows fine adjustments of spectrum (PAR and other physiological important wavelengths) and intensity that can imitate natural conditions experienced at specific locations and depths during sunrise, peak noon sun intensity, and sunset. Lighting controls may be programmed for monthly moon cycles, essential coral spawning cues. In the summer months when most coral spawn, PAR intensity and daylight duration both increase. These seasonal inundations in daylight can also be programmed, however this automation requires additional external control, such as with Apex aquarium controllers (Neptune Systems, Morgan Hill, CA). Although some UV is incorporated into these light fixtures, the LED diodes designated as 'UV' peak at 410 nm. However,

custom versions of Ecotech Marine LED fixtures using diodes emitting UVA (315-400nm) and potentially the UVB (280-315) parts of the spectrum are available. These fixtures also are used to acclimate corals to experimental conditions (e.g. photo-enhanced toxicity, light attenuation, reproduction).

Biosecurity-Biocontainment Considerations

Biosecurity practices prevent foreign agents from entering established coral aquaculture systems. Biocontainment practices prevent the spread of infection or contamination between animals as well as prevent the agent from leaving the laboratory (Pališ et al. 2016). Since our work extends beyond contaminant toxicology, we must consider potential risks from parasitic and pathogenic agents, and also environmental impacts due to release of non-native (and potentially invasive) species. Some species in culture are ultimately designated for restoration projects (*Dendrogyra cylindrus* and potentially other Caribbean brain coral species), thus we must guard against these corals acquiring any foreign biologics during their time in our facility that could be introduced into the restoration site. Strict protocols are adhered to ensure no foreign chemical or biological contaminants are introduced to these aquaculture systems. These protocols have also been adopted to our aquaculture systems that hold coral for experimental support. Examples of NCCOS Coral Health and Disease Program biosecurity and biocontainment practices and procedures are listed below:

• All coral collected from the wild undergoes a minimum of a 30 day quarantine and observation period before being placed in an established aquaculture system. This period can be substantially longer for corals known to have active disease or those that came from a diseased reef location. Corals exposed to disease undergo stringent antibiotic regimens (Miller et al. 2020), however, with the ongoing expansive stony coral tissue loss disease (SCTLD) epidemic, as a safety precaution, a dose of 50 mg/L ampicillin in Tropic Marin artificial seawater (TMASW) is administered for 10 days with 100 % daily renewal to all corals. Exceptions are made for corals designated for disease or other work with which this treatment may interfere (in which case, they are kept in separate systems in separate rooms from systems holding healthy established cultures).

• All liverock is now seeded and cultured in-house from existing stocks to prevent the need of further wild collection. The seeding stocks were collected in 2014 or earlier from the endemic coral regions.

• All beneficial co-cultured organisms (algae, shrimp, crabs, and snails) also are collected from endemic coral regions and undergo a minimum of 2 weeks in quarantine before being added to established aquaculture systems. With the ongoing SCTLD epidemic, it is now standard procedure for all co-cultured organisms to undergo a dosing regimen of 50 mg/L ampicillin in TMASW administered for 10 days with 100 % daily renewal as part of the quarantine process.

• All coral food used in our lab must come from suppliers with established sterilization techniques. Foreign bacteria and even algae can be introduced from non-sterilized foods. We purchase sterilized food from: Polyp Lab (Montreal, Quebec), New Life International, Inc. (Homestead, FL) and Tropical Marine Centre's (Chorleywood, Hertfordshire) Gamma Slice.

• Each aquaculture system has a set of separate arm-length, reusable gloves, pipettes (for drawing water samples). Separate cleaning supplies are assigned to each system to prevent cross-contamination. The gloves also prevent potential introduction of contaminants from laboratory personnel.

• All water chemistry probes are sterilized (typically with 70 % isopropanol) between systems with species of different origin to prevent cross-contamination.

• Glass tops are used to prevent cross-contamination via splatter from tanks in close proximity. They also prevent debris falling into the systems (The amount of debris that accumulates on the glass tops is surprising).

• Waste water is piped to the sewer and decontaminated at the local water treatment plant with sodium hypochlorite. However, waste water from our aquaculture systems is treated with 3 - 5 % bleach (usually 6 % sodium hypochlorite) and left to sit a minimum of 30 minutes before being disposed of down the drain.

Procedures for creating test fragments from parent colonies

Due to differing morphologies, procedures for creating experimental fragments vary by species. Most experiments performed at NCCOS Coral Health and Disease facilities require fragments with 2-6 cm^2 of tissue. In branching species bone cutters are used to cut 2 cm long branch tips (2.5 cm if the top is to be cut for a tissue laceration regeneration study) (Figure 3-A.). Side-branches for species that have them are removed (Figure 3-B.) for fragment size and shape uniformity. Fragments are then glued vertically to small Teflon pegs (Figure 3-C.) and returned to the aquaculture system to heal and grow. Fragments from most branching species will be healed and ready to use in experiments in approximately 1 month. Usually within 2 months, many branching species will start to exhibit significant side-branching and will need to be re-trimmed.

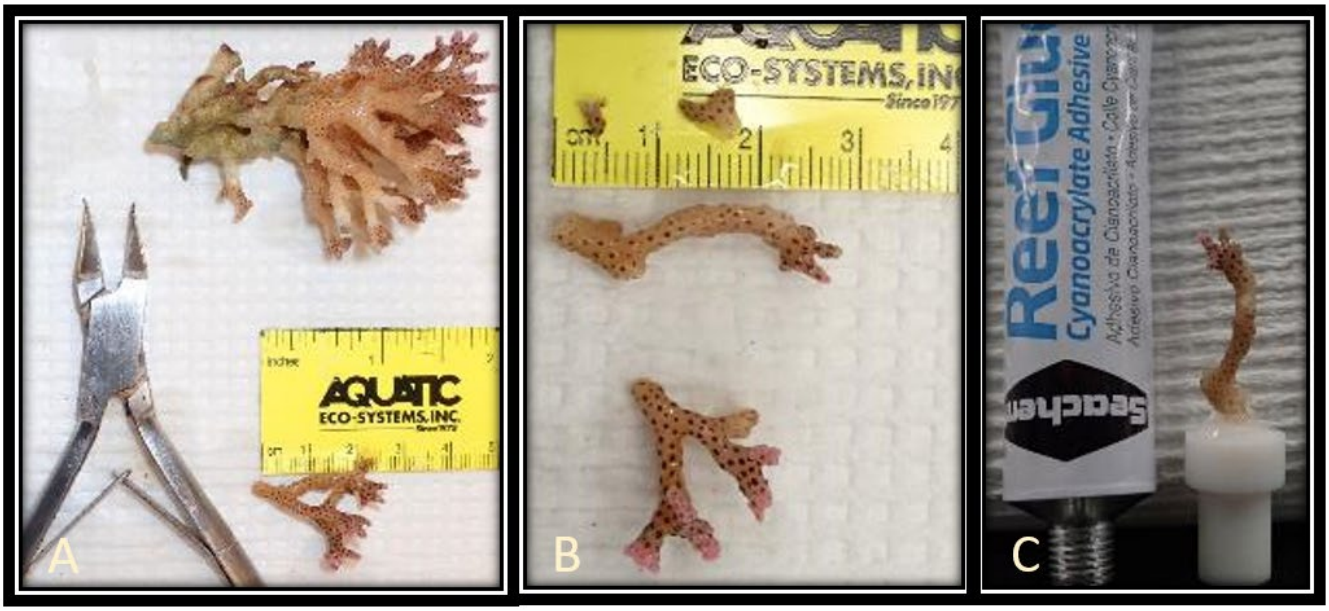

Figure 3. Creating 2.5 cm tall *P. damicornis* test fragments. This involves using bone cutters to cut away small areas of branches at least 2.5 cm tall (Panel A), removing all side branches for fragment uniformity (Panel B) and mounting to a custom Teflon peg with Reef Glue (Panel C) before putting back in aquaculture system for healing and growth.

Creating fragments from brain coral and other mounding species is a more detailed process. We have found that using a Dremmel (Mt. Prospect, IL) rotary tool with a 1.5 inch diamond cutting wheel (Figure 4) makes it possible to cut tissue to desired size (1 cm^2 for *O. faveolata*) while minimizing wasted tissue (when compared to common methods that use a thick tile saw blade). The small diameter and thin width of the diamond cut wheel also allows for more precise cutting. We have refined this fragmentation process with *O. faveolata* to achieve 1 cm^2 experimental fragments which is detailed below (Figures 5-9).

Figure 4. Dremel tool with diamond cut wheel displayed next to an O. faveolata parent colony

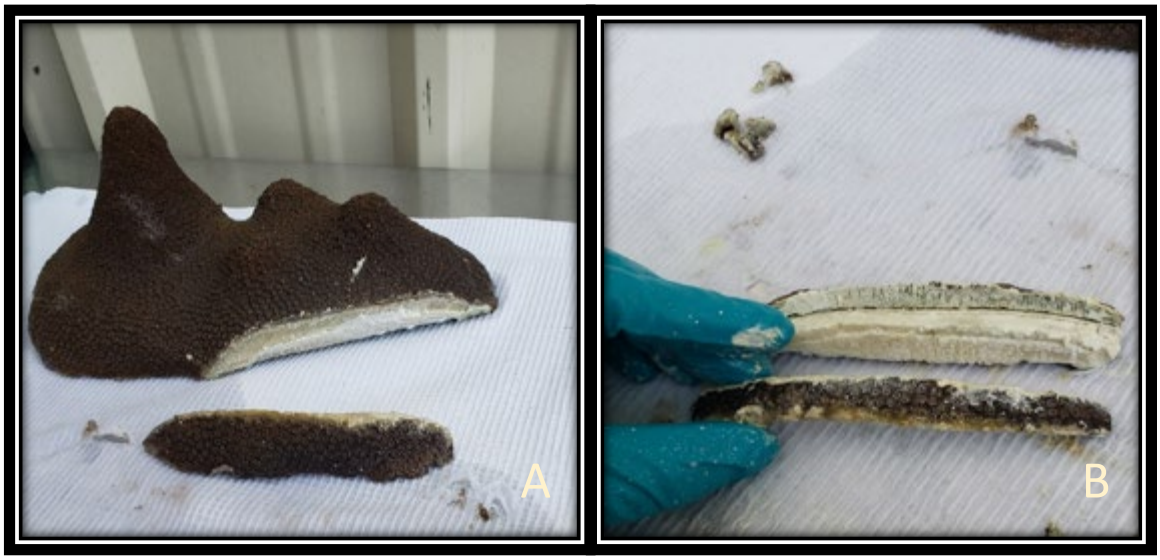

Figure 5. Step 1. Cut parent colony into smaller pieces to work with, preferably in long narrow strips (Panel A). These smaller pieces can then be cut into 1 cm wide strips (Panel B).

Figure 6. Step 2. Take 1 cm wide strip cut in Step 1 and cut into it at 1 cm intervals taking care not to cut all the way through the skeleton (Panel A). The top ~5 mm of skeleton with adjoined tissue can then be cut away from the excess skeleton to create 1 cm^2 fragments (Panel B).

Figure 7. Step 3. Resulting 1 cm^2 fragments will have a lot of skeleton and tissue bound up in mucous from the fragging process (Panel A). A small submersible aquarium pump in a small glass aquaria is used to rinse away all debris from the fragments (Panel B). Failure to perform this step results in large areas of tissue mortality and bleaching on the new fragments.

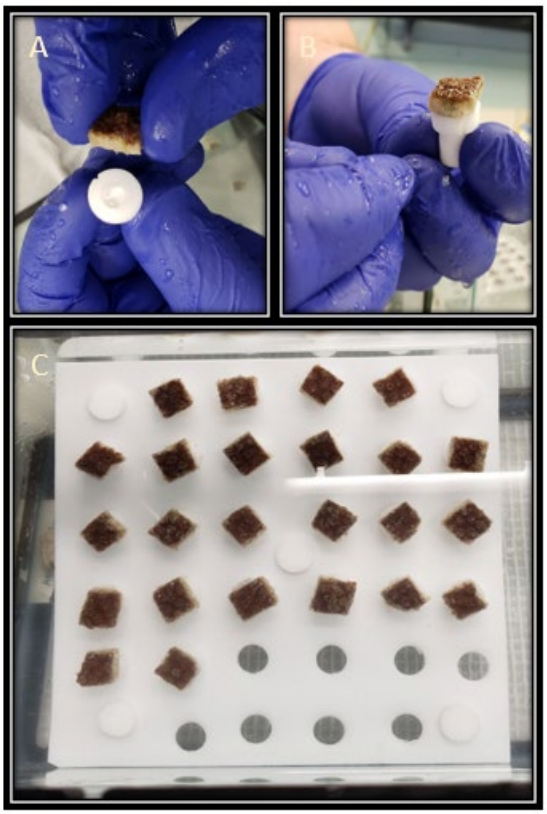

Figure 8. Step 4. Fragments are glued (Seachem Reef Glue, Madison, GA) to custom made Teflon (PTFE) pegs (Panels A and B). Teflon pegs are then placed in custom made Teflon racks made to hold 32 fragments each (Panel C).

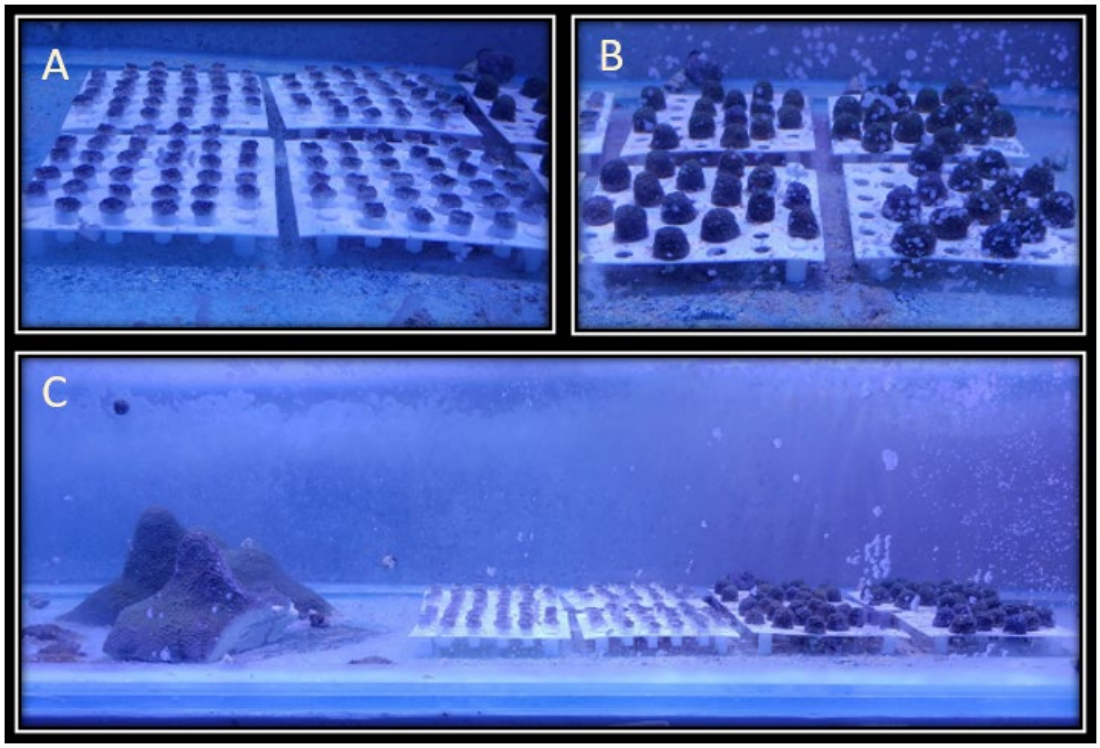

Figure 9. Step 5. Freshly cut fragments (Panel A) are placed back into culture so they can heal and grow until they are ready to be used in 3-6 months (Panel B). Panel C shows (from left to right) a recently cut parent colony, four full racks (128 fragments) of newly cut fragments from the parent, and four racks (not full as some were already used for experiments) of fragments that have been growing for approximately 6 months.

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Effects of two remediation strategies on the indigenous microbial communities in response to a simulated hydrocarbon release using an experimental mesocosm system

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Abstract

Crude oil and processed petroleum products find their way into the environment in a variety of ways that include natural release (via surface or underwater seeps), unintended release (via spills and admixtures), and use (by consumption/production/burning). Mitigation of hydrocarbons released into the environment is a challenging endeavor, and improvements in recovery technologies are needed to enhance restoration efforts. As such, recent advances in nanoparticles science have made these materials candidates in oil recovery applications in aquatic environments. While laboratory studies have yielded impressive results, a paucity of information exists on the ecological safety and efficacy in their use in large-scale remediation applications. In an effort to elucidate potential effects, we examined different crude oil recovery/cleanup strategies and their impact on the microbial community during a simulated hydrocarbon release using an experimental mesocosm system. Treatments included the application of a commercial hydrocarbon bio-remediation agent, Micro-Blaze® (MB), and the use of polyvinylpyrrolidone (PVP)-coated magnetite nanoparticles (NP), individually and in combination. Filtered water samples were taken at 24, 48, 72 and 96 hour time points and their microbial communities evaluated using metagenomics techniques. For all treatments, distinct shifts in microbial community abundances were found over the course of the exposure relative to pre-exposure microbial abundance estimates. Increases in taxonomic groups known to harbor hydrocarbon degrading bacteria were observed in comparisons to native pretreatment experimental inoculum. Major taxonomic phyla present in experimental treatments included Proteobacteria, Bacteroidetes, and Cyanobacteria. Statistical analyses revealed both treatment and UV exposure to be significant factors involved in community change. All treatment grouping had distinct taxonomic shifts compared to oil controls. The addition of nanoparticles had the smallest number of operational taxonomic units showing significant differences using differential abundance analyses, while combined treatment with Micro-Blaze® and nanoparticles had the greatest. Treatments with Micro-Blaze® showed a definite increase in representation of the Bacteroidetes phyla, primarily due to increases in the Flavobacteriaceae, a family reported to contain several chemotrophic and hydrocarbon

degraders. Treatments with coated Fe nanoparticles increased representation of members of the Cyanobacteria phyla with members of the families Oceanospirillaceae and Nostocaceae, showing the most substantial increases. Increases in free Fe may be responsible for these changes. These results document how the use of different remediation agents alters the indigenous microbial community response to a hydrocarbon release in this experimental system.

Introduction

Crude oil is found all over the world, on all continents and in every ocean basin. Nearly half (46%) of the discharge of oil into the world oceans occurs via natural seeps (National Research Council 2003). Use or combustion, including operational release, accounts for roughly 37% and another 12% from transportation spills (National Research Council 2003). While extraction activities account for an estimated 3%, the accidental release around extraction activity can have catastrophic consequences and lead to an environmental disaster, as the wellhead blowout at the Deepwater Horizon (DWH) extraction platform documented.

In the United States, it is estimated that nearly 4 million tons of oil and petroleum products are used daily with close to 20 million tones consumed worldwide. On average there are 100 oil spills in the United States daily. Of these, an estimated 15% occur on navigable waterways and 85% on land or into freshwater. While most of these spills are small in volume, they often garner media attention and public concern. With regard to the marine environment, worldwide release of crude is estimated in excess of 1,300,000 metric tons annually (roughly 380,000,000 gallons) (National Research Council 2003).

Response to spills invokes the deployment of an emergency management plan in an attempt to mitigate damage. Various countermeasures are often employed and include physical containment and recovery through the use of booms and skimming, chemical processing via burning, and the application dispersants. However, these measures are rarely effective in capturing more than a third of the released crude. Evaporation, weathering, dilution, and

biodegradation account for the processing of the remaining material. Biodegradation via bioremediation is considered the most cost-effective and eco-friendly means for mediating spill cleanup. While biodegradation has been considered a minor component of the initial cleanup effort, research has shown that the microbial community change is rapid and significant in processing released hydrocarbons (Hazen et al. 2010, Joye et al. 2014, Kessler et al. 2011, King et al. 2015). Ultimately, for long term breakdown and remineralization of released hydrocarbon, microbial biodegradation is the primary component.

Technologies for the remediation of hydrocarbon release into the environment are continually changing, and recent advances in nanoparticle technologies have made these materials candidates in oil recovery applications. Of these, zero-valent Iron (Fe) nanoparticles have had the most extensive use in remediation of environmental contaminants (Karn et al. 2009). Recently, polyvinylpyrrolidone (PVP)-coated magnetite nanoparticles have been developed as a cost efficient remediation agent, and their efficacy for use on polycyclic aromatic hydrocarbon (oil) in water admixtures has been shown in small scale experimental applications (Mirshahghassemi et al. 2019). However, questions remain regarding the potential impact these materials may have on the environment concerning their fate, transport, and toxicity (Karn et al. 2009).

Therefore, as part of a larger study examining remediation treatments effects under a simulated hydrocarbon release, we investigated changes on the microbial community in self contained experimental mesocosm systems employing different remediation application. Using 16S metagenomics sequencing data, we examine microbial community changes in response to these remediation activities along treatment, temporal, and ultraviolent light exposure scales.

Study Design

In June 2018 , twenty mesocosm systems were setup in an enclosed greenhouse under ambient light and temperature conditions at NCCOS Charleston Laboratory in Charleston, SC according to NOAA Technical Memorandum NOS NCCOS 62 (Pennington et al. 2007). Briefly, each system contained two tanks: a lower tank, which acted as a water reservoir, and an upper tank that

served to house biological test organisms and as the treatment point on entry into the system. Systems mimicked a 6-hour tidal flux via an electric pump used to move water from the lower tank to the upper tanks. Each tank was equipped with a PVC sampling pipe, which allowed for water quality monitoring and water sample collection to be made without interference from floating oil. Filtered seawater, obtained from the Charleston Harbor, Charleston, SC, was added to each system (443 L/system). Salinity of each tank was adjusted to 20 (±1) ppt by adding deionized (DI) water. The indigenous microbial community from this water served as the experimental inoculum.

Study design consisted of three experimental treatments plus an oil spiked control assigned to 5 different light zones. Treatments consisted of 1) oil/dispersant (Micro-Blaze®) (MB) 2) oil/ coated iron nanoparticals (NP) and 3) combined oil/Micro-Blaze®/nanoparticals (NPMB) and 4) oil spiked control (oil). On day one of the exposure, 74 mL of Louisiana Sweet Crude oil was added to the top tank of each mesocosm system creating a surface slick. Remediation agents, nanoparticles and Micro-Blaze®, were added to their respective systems 24 h after oiling. Treatment locations were determined via random assignment prior to the start of the test. For treatments containing the nanoparticles (NP and NPMB), 360 mL of a 20mg/ml the nanoparticle solution was added to the top tank of the mesocosm system. For treatments containing Micro-Blaze® (MB and NPMB), 7.4 mL of Micro-Blaze® was diluted with 239.3 mL of DI water, after which the solution was added to the respective mesocosms.

Three pre-treatment water samples were collected after experimental tanks were filled. After treatment application, filtered water was collected at hourly time points of 24, 48, 72 and 96 hours. Total number of filtered water samples collected across this study was 83 (4 treatments run in replicates of 5 sampled at 4 time points + 3 pre-treatment samples). UV exposure zones were assigned based on previous work measuring *Spartina sp* growth over the course of 12 weeks (Pennington, personal communications).

Methods

DNA extraction

One liter of sample water was filter through 0.23 nm nitrocellulose membranes to collect bacterial samples. Filters were immediately frozen at -80^{o}C and stored. DNA was extracted from sample filters using a Powerwater DNA kit (Qiagen) following the manufacture's protocol. Briefly, filters were placed in 5 ml screw top plastic tubes with polystyrene beads and 1 ml of elution buffer and vortexed horizontally at maximum speed for 5 minutes. Tubes were then centrifuged at <4000 g for 1 minute at room temperature. The resultant supernatant was transferred to a clean 2 ml collection tube and centrifuged at 13000 g for 1 minute at room temperature to pellet debris. Supernatant was transferred a second time to a clean 2 ml collection tube. Two hundred ul of IRS solution was added to collected supernatant, vortexed briefly and allowed to incubate at 4^{o}C for 5 minutes. After incubation, samples were centrifuged at 13000 g for 1 minute and the supernatant transfer to a clean collection tube. To this 650 ul of lysis solution was added and vortexed briefly. Lysates were then loaded onto nitrocellulose filter columns, washed with a series of salt and ethanol solutions and eluted into clean 1.5 collection tube using 100 ul of 10 mM Tris buffer. Sample DNA was quantified using spectrophotometric quantification on a Nanodrop 2000 and stored at -80^{o}C.

16S rRNA bacterial amplification and library construction and sequencing

Amplification profiles and library construction followed the Illumina 16S metagenomic sequencing library preparation protocol. Bacterial 16S rRNA gene sequences targeting the V3V4 region were amplified using the primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 907R 5'-ACTACHVGGGTATCTAATCC-3'). Amplification primers were modified with Illumina overhand adaptor sequences for forward (overhang: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and reverse (5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) primers, respectively. First stage PCR reactions used 15 ng of microbial genomic DNA as template. Reaction volumes of 25

ul consisted of 1 uM of both forward and reverse modified amplification primers and 12.5 ul of 2x KAPA HiFi HotStart ready mix (Takara Biology). Reaction profile consisted of a 3 min denaturation step at 95oC followed by 25 cycles of 95^{o}C for 30 secs, 55^{o}C for 30 secs and 72^{o}C for 30 secs with a final extension step of 72^{o}C for 5 mins and a 4^{o}C hold. Amplification products were visualized using an Agilent 2100 Bioananalyzer to confirm targeted amplicon size and to check for contamination. PCR products of size verified samples were then cleaned using AMPure beads and the cleaned products were used for Index PCR reactions.

Indexing PCR was conducted in 50 ul volumes using 5 ul of both Nextera XT series 1 and 2 primers. Briefly, Nextera primers were arranged in a linear matrix with each sample receiving a unique primer pair for indexing purposes. To this 25 ul of 2x KAPA HiFi Hotstart ReadyMix and 10 ul of PCR grade H_2O was added. Five ul of AMpure cleaned PCR product served as template for the indexing reactions. Thermal cycling profile for indexing reactions followed: initial denaturation at 95^{o}C for 3 mins with 8 cycles at 95^{o}C for 30 secs, 55^{o}C for 30 and 72^{o}C for 30 sec and a final extension at 72^{o}C for 5 mins and a 4^{o}C hold. Index PCR products were cleaned following the AMpure XP bead protocol. Cleaned products were size validated and quantified using an Agilent 2100 Bioanalyzer. Quantification results were used to normalize and pool subsequent indexed libraries following Illumina recommendations. Pooled normalized libraries were then subjected to pair-end sequencing using a Illumina Miseq desktop DNA sequencer at the North Carolina State University Genomics Core Facility (NCSU, GCF Raleigh, NC). The pooled library was sequenced to attain a targeted 100,000 reads per sample. Paired-end reads were post processed, sorted and assigned to their unique index label by the NCSU GCF.

Bioinformatics

Paired-end read data was analyzed using the CLC Genomics Workbench data pipeline using the microbial genomics module (MGM) to preform operational taxonomic unit (OTU) clustering, diversity estimates and statistical analyses. Samples were first trimmed of primers, adapters and low quality score sequences were removed. Next sample reads were merged using

program defaults. Indexed samples were then filtered on minimum number of reads and aligned. Aligned reads were then subject to OTU clustering using the Greengenes reference database. Here, the OTU algorithm reduces sample reads to a collection of representative sequences (clusters) that are 97% similar to other members of the respective cluster. Additionally, chimeric sequences are identified and filtered during the clustering process. Samples were screened for low read count using CLC program defaults. Assigned OTUs were filtered further to remove non-target amplification products. Filtered sample OTU abundances were used for all downstream analysis, which included alpha and beta diversities estimates, and PERMAOVA statistical tests. All statistical analyses were tested for significance based either on a p-value of 0.05 or an adjusted false discovery rate (FDR) of 0.05. Differential abundance analyses incorporated a minimum absolute fold change of 1.5 in addition to false discovery rate (FDR) adjusted p-values for significance.

Results

Total DNA was successfully extracted from nitrocellulose filters for 83 samples (80 experimental samples plus three pretreatment samples). DNA yields ranged from 4.7 ng/ul -147 ng/ul. Targeted variable 16S rRNA region were successfully amplified from all samples, including low yield pre-treatment water samples. Secondary amplification and index PCR library construction was successful in all 83 samples.

A total of 26,361 954 paired-end reads were successfully uploaded for data analysis with 26,358,882 remaining after quality and ambiguity trimming. Sample library size ranged from 4,300-1,355,772 and averaged 277,494. Implementation of low-read cutoffs resulted in the removal of 15 samples. All but the 72 hour sampling time point had sample libraries removed. However, in no case did the removal of low-read libraries leave any treatment or time point without a minimum of three replicates.

Cluster analysis assigned reads to 20 bacterial phyla and a single archaea phyla. For experimental treatments, the dominant bacteria phyla were Proteobacteria (40%)

Bacteroidetes (31%) and Cyanobacteria (16%) (Figure 1-4). These differed from the pretreatment samples which saw Proteobacteria (37%) Bacteroidetes (31%) and Parcubacteria (17%) as the dominant bacteria phyla (not shown). Principal coordinate plots using Bray-Crutis dissimilarity estimates of beta diversity revealed groupings for treatment and UV exposure (Figure 5). These grouping were supported statically in PERMANOVA analyses for treatment and UV factors for both overall and pairwise analyses (Table 1). Whereas time as an analysis factor failed to show significance in pairwise comparisons.

Differential abundance analyses comparing treatments to oil spiked controls identified 1739 operational taxonomic units (OTU) that met the statistical criteria. Of these, 151 were found to be significantly different from the oil control regardless of treatment. The NPMB treatment had the largest number of OTU observed (774) (Figure 6). This was followed by the MB treatment (566) and the NP treatment recording the fewest (399) (Figure 6). The NPMB treatment (382) also had the highest level of unique OTU groups compared to oil spiked control followed by MB (220) and NP (100) treatments. An additional 144 OTUs were significantly different from oil spiked control for both MB and NPMB treatments with NPMB and NP treatments sharing 97 and MB ad NP treatments having 51 (Figure 5) .

All treatments showed a similar pattern in comparison to oil controls in the fold change for the number OTUs meeting statistical cutoffs, where OTUs increase in fold change was higher than a decrease in fold change. As such, fewer identified OTUs occurred in a higher abundance in oil controls than in treatments. For these treatments, after removing low abundance OTUs, MB had the largest ratio in high to low change (158:30) followed by NP (43:26) and finally the NPMB mix at a ratio close to 1:1 (159:136).

Discussion

Data present here show a clear difference in community composition in all three treatment applications. All treatments were found to be statistically different from oil spiked controls in

differential abundance analyses. However, pairwise comparison between NP and NPMB failed to show significance suggesting that the effect of nanoparticles on the community composition may had an overriding effect on both treatments. Regardless, all data suggest that the addition remediation products has an impact on the response of the microbial community to the simulated hydrocarbon release.

Of the three variables examined herein, two (treatment and UV exposure) were shown to be statistical significant in abundance analyses, while time of sampling was not. With regard to treatment, changes in microbial abundance related to the addition of Micro-Blase appear to be centered on members of the Flavobacteriaceae family. In both the MB and combined NPMB treatments, Flavobacteriaceae abundance was at least three times that observed in treatments without Micro-Blaze® . Members of this family have been reported as chemohetrotrophs and known oil degraders (Chaudhary et al. 2019, Chaudhary & Kim 2017, Jurelevicius et al. 2013). The result obtained here suggest that this family of bacteria respond favorably to the MicroBlaze amendments in the presence of hydrocarbons.

Treatment with nanoparticles appeared to effect the Nostocaceae family within the Cyanobacteria phyla. In this case, coated iron nanoparticles both alone (5x in %) and in combination (2x in %) treatments showed a dramatic increase in this family relative to the response observed in oil controls and Micro-Blaze® exposures alone. A similar but less pronounced response was also seen in the Oceanospirillaceae family and suggest that taxa contained within these two groups were responding in a similar fashion to the nanoparticle amendment. While the Oceanospirillaceae family has known to house hydrocarbon degraders (Teramoto et al. 2009, Vila et al. 2010) research in the southern ocean has shown a positive response in this family with Fe amendment (Bertrand et al. 2015). The Nostocaceae family is generally regarded as nitrogen fixers and there is data suggesting that members of this group may also be rate limited by Fe (Larson et al. 2018). This may explain the pronounced response of this family in the NP treatment alone while competition with hydrocarbon degraders (due to nutrient amendment from the addition of the bio-redemiation agent) may have lessened this effect in the combined MBNP treatment (abundance 21.40% versus 9.37% respectively).

Rhodobacteraceae increased dramatically in all experimental samples compared to pretreatment community abundances with the biggest increases seen in NP treatments suggesting that future investigation focusing on this family may prove to be informative. Conversely, members of the Rhodospirillaceae showed a dramatic drop in abundance in all experimental samples in comparisons to pre-treatment levels.

The sample design allowed for the examination of the effect of UV exposure and showed that differences in light had an effect on the community response, regardless of treatment effects. The results suggest that differences in light may be responsible for an additional source of experimental variability, especially when highly sensitive techniques (like metagenomic sequencing) are employed for microbial community structure. While the differences were primarily confined to the extremes of low and high light exposures (ie groups 5 vs 1) they demonstrate the effect that UV exposure has on the dynamics of the microbial community in these experimental systems. Moving forward it is recommended that a greater quantitative assessment of UV intensity be incorporated in future work where microbial community data will be assessed.

An additional concern is patchiness in some OTUs in treatment response. A cursory examination into evenness in identified some OTU groups were the counts from single replicate tanks accounting for the majority of the response for a given treatment. As such, we continue to work through these data to flag those outliers and to identify possible causes. An explanation may simply be the stochastic process in the establishment of the initial microbial community for each experimental tank. All tanks used the same filtered water and metagenomics community analysis failed to reveal differences in three pre-treatment samples collected (suggesting uniformity of the inoculum). However is it likely that each tank undergoes a unique process in the selection of the microbial community as it being established. We expect this variation to decrease under stronger experimental selective pressures. Regardless, further investigation into the causes and the degree to which this variability effects the outcome of other toxicological parameters is warranted.

While a clear shift in the microbial communities in response to treatment were observed, the degradation of hydrocarbons by microbes is a complicated and lengthy process, which involves shifts in the community as exploitable resources are depleted and/or transformed. For example, data for the DWH event showed that upon release, the microbial community's response to the available hydrocarbons was rapid but over the course of the biodegradation process an ecological succession of microorganisms occurred (Hazen *et al..* 2010; Kessler *et al..* 2011; Joye *et al..* 2014; King *et al..* 2015). As such, water chemistry measurements are critical for a complete understanding of the factors driving microbial community change. We are currently awaiting analytic chemistry results on the distribution of PAH compounds for their incorporation into this dataset to provide a more definitive view of the processing effecting community change.

The data presented here clearly document how different remediation agents can impact the microbial community response to an environmental stressor (in this case a hydrocarbon spill). The application of metagenomics allows of an in-depth examination of community succession that standard microbiology analyses lack. Further, combining the metagenomic community assessments with various environmental parameters may allow for a system wide assessment of potential unaccounted sources of experimental variability that may impact results further downstream (ie biomass, survival estimates). Moving forward it is recommended to include metagenomic community analysis in future mesocosm studies.

Data Availability

Data will be made available through the NCBI metagenome Sequence Read Archive.

Acknowledgements

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Table 1. PERMANOVA analysis results for different experimental variables using Bray-Crutis distance.

Variable	Groups	Pseudo-f statistic	p-value
Treatment	MB, NP, NPMB, OIL	4.29778	0.00001
Light	1,2,3,4,5	3.76980	0.00001
Hour	24,48,72,96	0.34753	0.34753

Figures

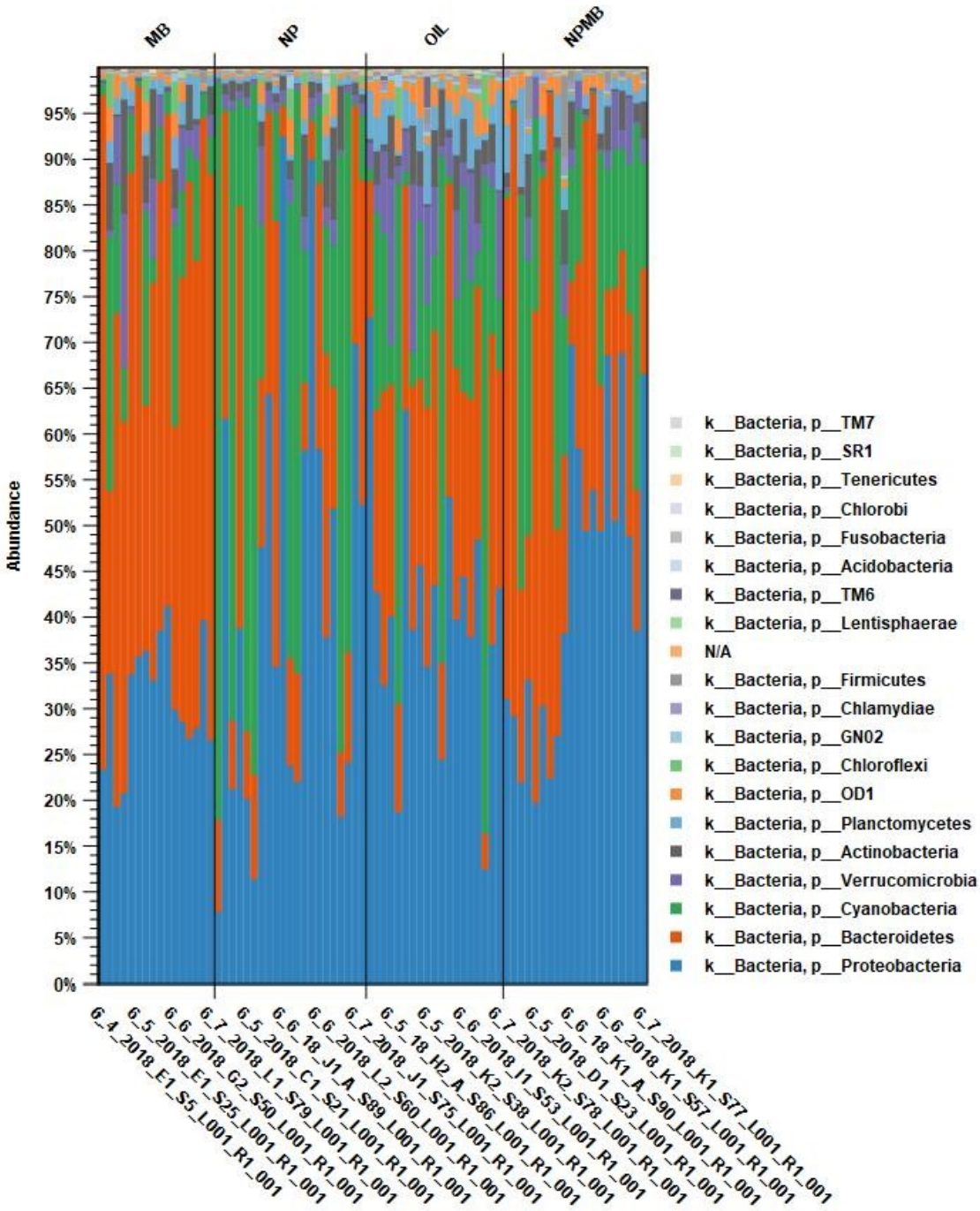
MB
NP
OIL
NPMB
Abundance
95%
90%
85%
80%
75%
70%
65%
60%
55%
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
k__Bacteria, p__TM7
k__Bacteria, p__SR1
k__Bacteria, p__Tenericutes
k__Bacteria, p__Chlorobi
k__Bacteria, p__Fusobacteria
k__Bacteria, p__Acidobacteria
k__Bacteria, p__TM6
k__Bacteria, p__Lentisphaerae
N/A
k__Bacteria, p__Firmicutes
k__Bacteria, p__Chlamydiae
k__Bacteria, p__GN02
k__Bacteria, p__Chloroflexi
k__Bacteria, p__OD1
k__Bacteria, p__Planctomycetes
k__Bacteria, p__Actinobacteria
k__Bacteria, p__Verrucomicrobia
k__Bacteria, p__Cyanobacteria
k__Bacteria, p__Bacteroidetes
k__Bacteria, p__Proteobacteria
6_4_2018_E1_S5_L001_R1_001
6_5_2018_E1_S25_L001_R1_001
6_6_2018_G2_S50_L001_R1_001
6_7_2018_L1_S79_L001_R1_001
6_5_2018_C1_S21_L001_R1_001
6_6_18_J1_A_S89_L001_R1_001
6_6_2018_L2_S60_L001_R1_001
6_7_2018_J1_S75_L001_R1_001
6_5_18_H2_A_S86_L001_R1_001
6_5_2018_K2_S38_L001_R1_001
6_6_2018_I1_S53_L001_R1_001
6_7_2018_K2_S78_L001_R1_001
6_5_2018_D1_S23_L001_R1_001
6_6_18_K1_A_S90_L001_R1_001
6_6_2018_K1_S57_L001_R1_001
6_7_2018_K1_S77_L001_R1_001

Figure 1. Taxonomic diversity and relative abundance of different phyla of Bacteria for all mesocosm exposures grouped based treatment (MB= Micro-Blaze® , NP= nanoparticles, oil= oil control, MBNP= combined Micro-Blaze® /nanoparticles).

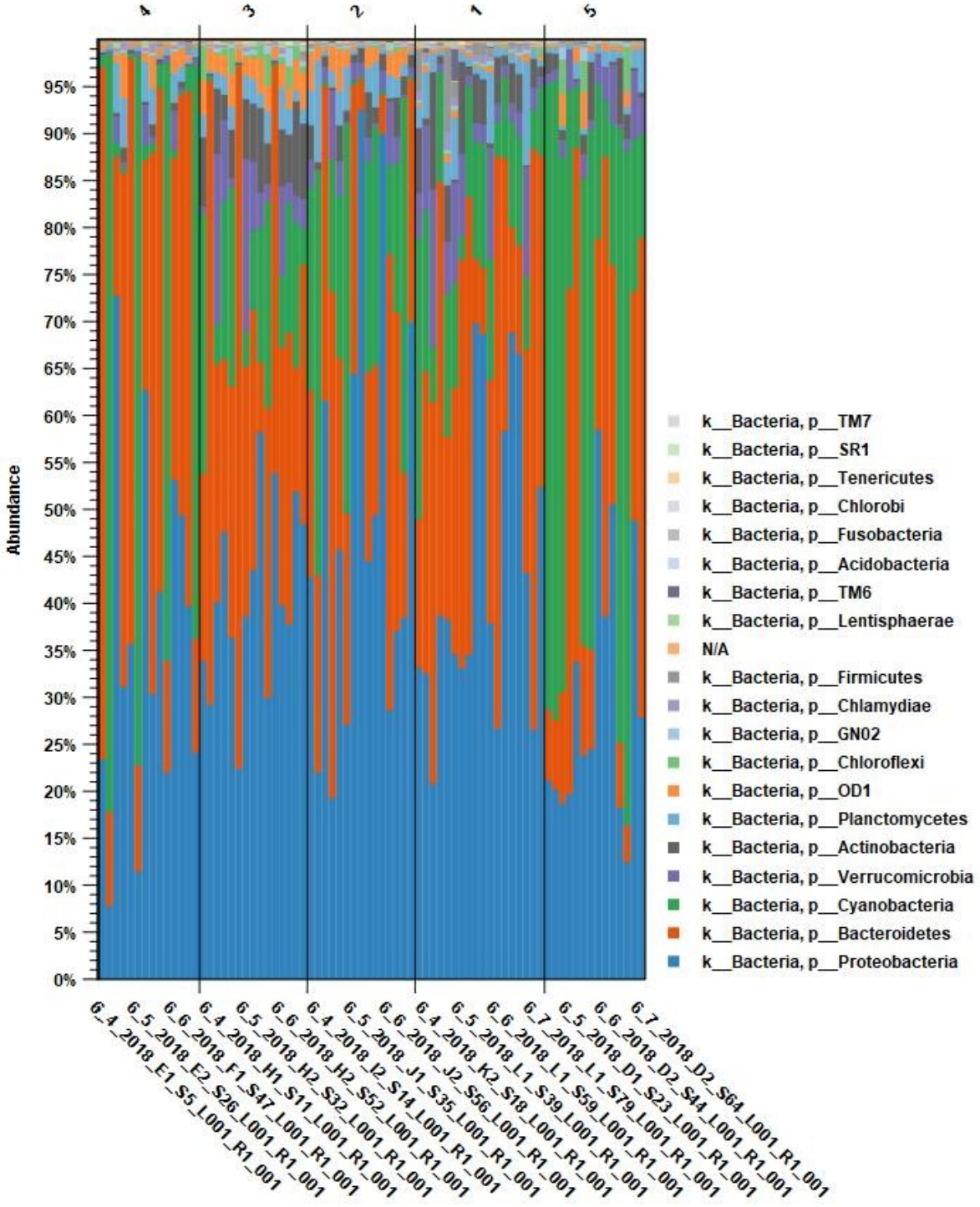

Figure 2. Taxonomic diversity and relative abundance of different phyla of Bacteria for all mesocosm samples grouped on UV exposure (top) ranging from 1 (lowest) to 5 (highest).

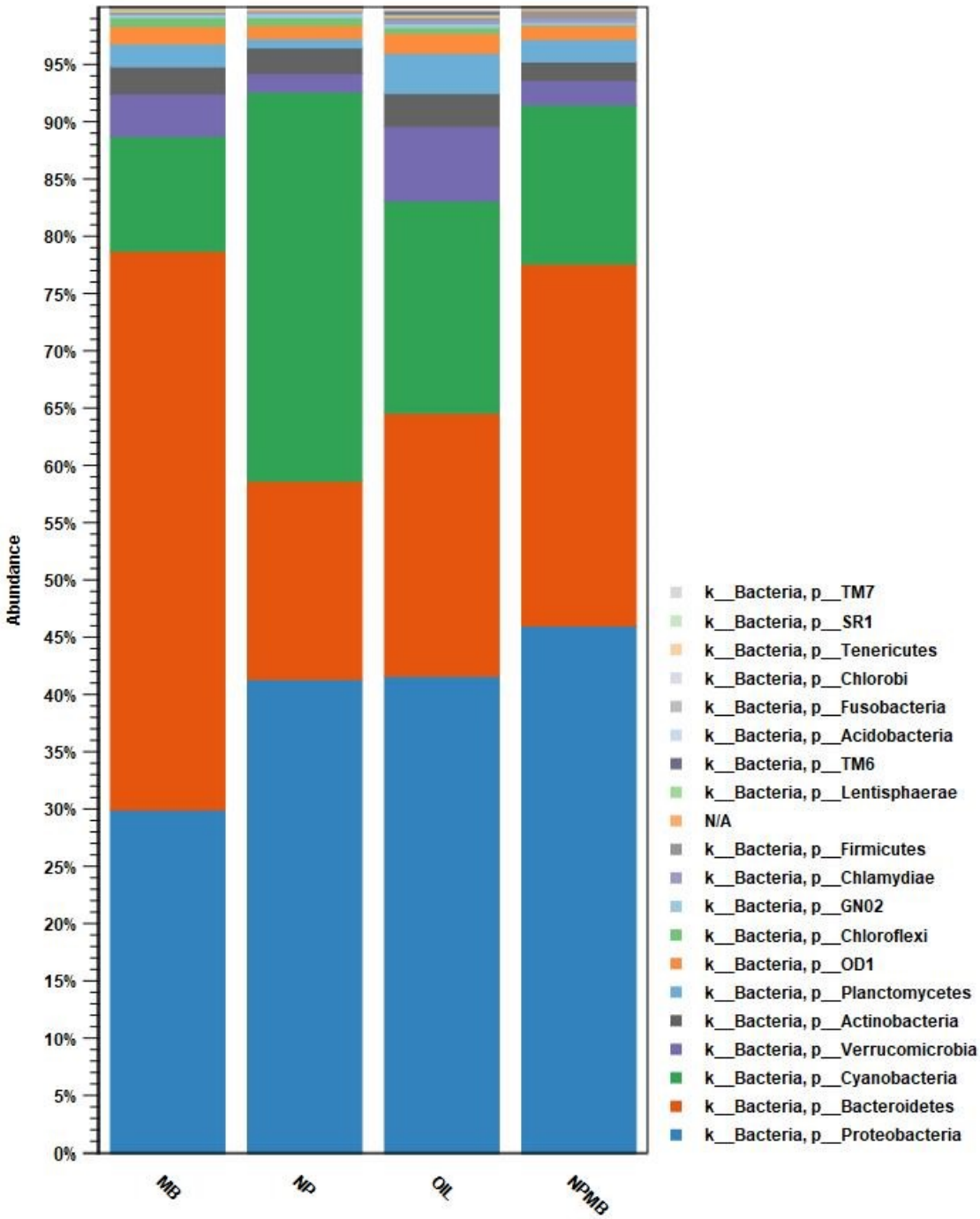

Figure 3 Taxonomic diversity and relative abundance of different phyla of Bacteria for grouped mesocosm samples based treatment (MB= Micro-Blaze® , NP= nanoparticles, oil= oil control, MBNP= combined Micro-Blaze® /nanoparticles).

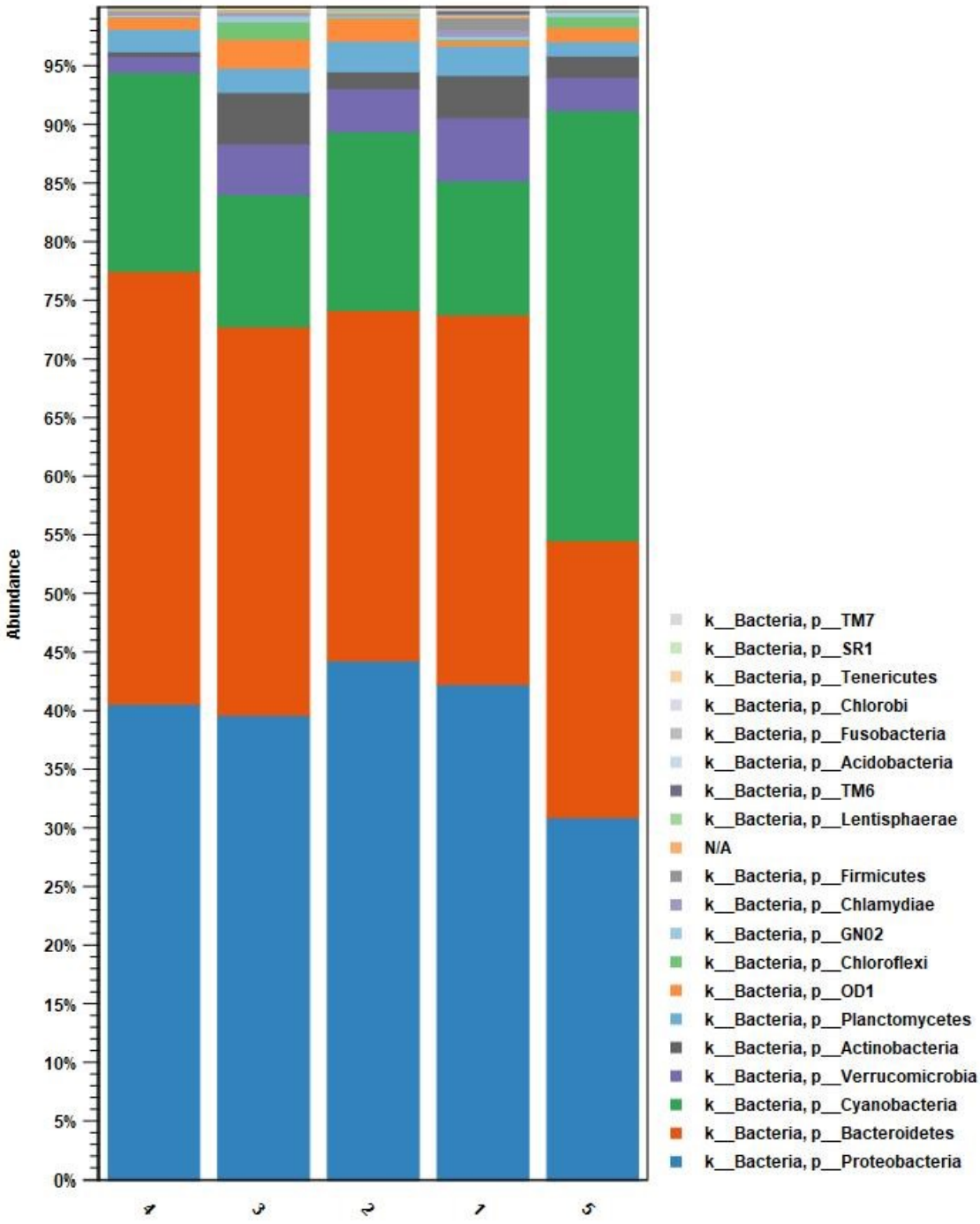

Figure 4. Taxonomic diversity and relative abundance of different phyla of Bacteria for grouped mesocosm samples based on UV exposure ranging from 1 (lowest) to 5 (highest).

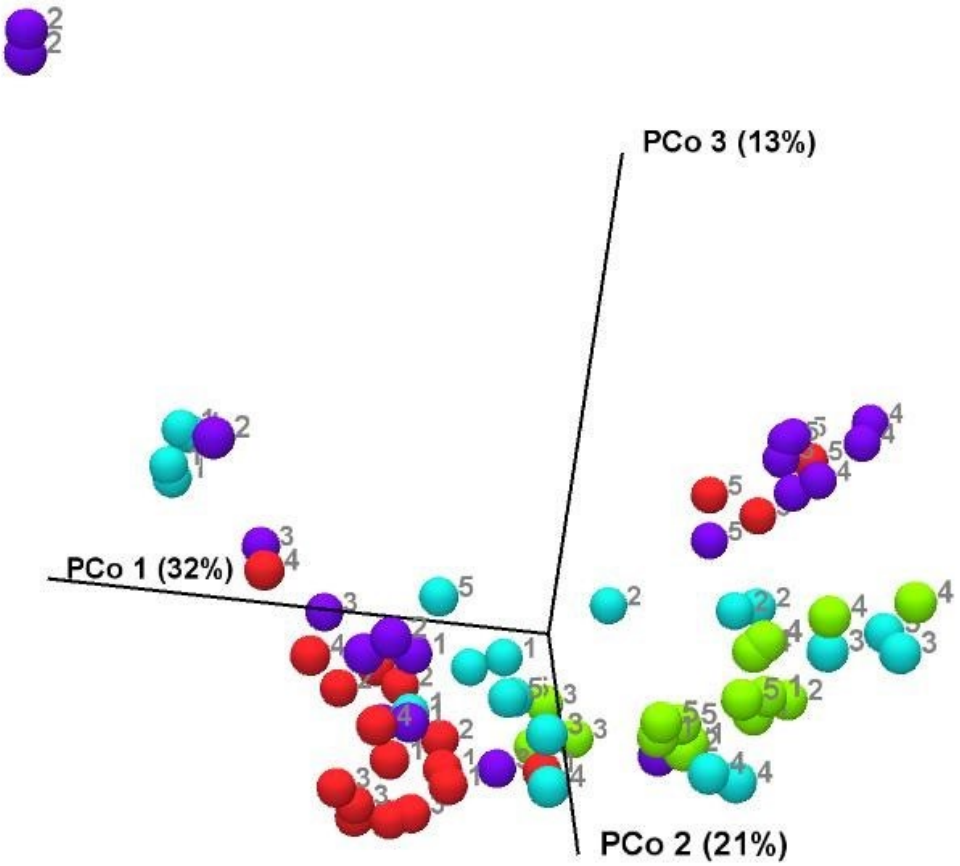

Figure 5. Principal coordinate plot using Bray-Crutis dissimilarity estimates of beta diversity showing treatment groupings (Red= OIL, Green= MB, Purple= NP. Blue = MBNP). Sample data points are labeled with UV expose values ranging from 1 (low) to 5 (high)

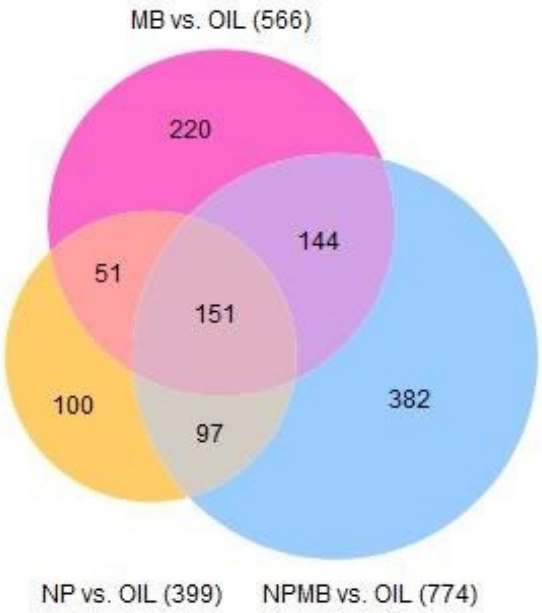

Figure 6. Venn diagram of OTU found to be significantly different from oil controls for their respective treatments in relative abundance.

Appendix

Abundance table of OTU's to family based on experimental treatment. Notable family counts are highlighted in yellow.

Phyla	Family	OIL	NP	NPMB	MB	Pre-Treatment
[Parvarchaeota]		**39**	**36**	**22**	**7**	**1647**
	(blank)	39	36	22	7	1647
[Thermi]		**32**	**10**	**3**	**3**	**2**
	Trueperaceae	32	10	3	3	2
Acidobacteria		**818**	**593**	**932**	**535**	**7**
	Ellin6075	46	36	72	23	0
	PAUC26f	26	25	30	14	0
	(blank)	746	532	830	498	7
Actinobacteria		**34718**	**27691**	**21104**	**31185**	**164**
	AK1AB1_02E	0	0	2	0	0
	Beutenbergiaceae	1	0	2	0	0
	C111	10167	6699	6998	3647	134
	Cellulomonadaceae	6	7	17	0	0
	Corynebacteriaceae	2	2	3	0	2
	Gordoniaceae	7	6	23	9	0
	Intrasporangiaceae	6	2	11	2	0
	JdFBGBact	4	3	5	10	0
	Kineosporiaceae	0	0	8	0	0
	koll13	52	40	62	43	0
	Microbacteriaceae	18039	12737	11502	17355	13
	Micromonosporaceae	3	0	2	1	0

	Microthrixaceae	2	6	11	3	0
	Mycobacteriaceae	3399	2067	1471	3696	7
	Nitriliruptoraceae	46	46	2	2	0
	Nocardiaceae	5	6	10	4	0
	Nocardioidaceae	2	21	15	8	0
	ntu14	1462	1593	227	213	3
	Propionibacteriaceae	7	23	9	5	1
	Solirubrobacteraceae	2	0	0	0	0
	TK06	1	10	1	2	0
	wb1_P06	35	37	58	29	0
	(blank)	1470	4386	665	6156	4
Armatimonadetes		**11**	**5**	**2**	**3**	**0**
	(blank)	11	5	2	3	0
Bacteroidetes		**230019**	**174158**	**329370**	**548176**	**18773**
	[Amoebophilaceae]	148	77	51	64	0
	[Balneolaceae]	3157	3827	655	272	0
	[Weeksellaceae]	3	0	0	0	0
	Bacteroidaceae	3	0	0	6	0
	Chitinophagaceae	988	1131	190	3564	10
	Cryomorphaceae	17921	12939	13244	35909	12
	Cyclobacteriaceae	5	2	9	4	0
	Cytophagaceae	66	105	46	64	1
	Flammeovirgaceae	1646	1082	1393	1663	17
	Flavobacteriaceae	64679	72614	241387	390780	17937
	Marinilabiaceae	6	9	4	2	0

	NS11-12	117	7	16	269	0
	Porphyromonadaceae	0	0	2	2	0
	Prevotellaceae	2	1	0	0	0
	Rhodothermaceae	132	103	140	70	3
	Saprospiraceae	124207	74547	60641	103114	780
	SB-1	17	14	41	16	0
	Sphingobacteriaceae	115	290	68	1	0
	VC21_Bac22	38	7	13	9	0
	(blank)	16769	7403	11470	12367	13
BHI80-139		**20**	**10**	**24**	**17**	**0**
	(blank)	20	10	24	17	0
BRC1		**15**	**15**	**10**	**11**	**4**
	(blank)	15	15	10	11	4
Caldithrix		**45**	**34**	**11**	**49**	**0**
	BA059	39	32	9	47	0
	Caldithrixaceae	6	2	2	2	0
Chlamydiae		**4080**	**1675**	**4756**	**1918**	**1202**
	Chlamydiaceae	489	52	694	414	47
	Criblamydiaceae	214	623	273	188	26
	Parachlamydiaceae	536	164	240	263	346
	Rhabdochlamydiaceae	1075	357	2658	261	656
	Simkaniaceae	100	76	115	103	57
	Waddliaceae	798	121	564	390	43
	(blank)	868	282	212	299	27
Chlorobi		**632**	**387**	**212**	**218**	**2**

	Chlorobiaceae	138	80	64	69	1
	Ignavibacteriaceae	5	0	2	4	0
	lheB3-7	38	9	40	12	0
	(blank)	451	298	106	133	1
Chloroflexi		**5955**	**7781**	**1491**	**10082**	**24**
	A4b	1107	1658	641	928	2
	Anaerolinaceae	2	1	2	0	0
	Caldilineaceae	4426	5728	418	8848	22
	SJA-101	2	2	2	0	0
	(blank)	418	392	428	306	0
Cyanobacteria		**146622**	**298006**	**137482**	**102934**	**3802**
	Cyanobacteriaceae	61	29	106	55	10
	Gomphosphaeriaceae	10267	711	1882	1028	2
	Nostocaceae	44936	210225	101095	10808	52
	Phormidiaceae	31	28	57	25	0
	Pseudanabaenaceae	3893	716	859	3421	3605
	Rivulariaceae	30	17	25	35	24
	Spirulinaceae	193	629	81	340	1
	Synechococcaceae	86575	85296	30967	86812	70
	Xenococcaceae	107	117	210	194	38
	(blank)	529	238	2200	216	0
Deferribacteres		**0**	**0**	**2**	**0**	**0**
	Deferribacteraceae	0	0	2	0	0
Elusimicrobia		**11**	**1**	**0**	**7**	**18**
	(blank)	11	1	0	7	18

Fibrobacteres		**63**	**23**	**19**	**45**	**0**
	B122	0	2	0	0	0
	Fibrobacteraceae	1	3	0	0	0
	(blank)	62	18	19	45	0
Firmicutes		**797**	**708**	**7731**	**328**	**24**
	[Acidaminobacteraceae]	19	54	97	16	0
	[Exiguobacteraceae]	12	345	11	13	0
	[Tissierellaceae]	0	2	0	0	0
	Bacillaceae	103	41	134	73	20
	Caldicoprobacteraceae	430	38	10	4	0
	Christensenellaceae	12	7	20	2	0
	Clostridiaceae	97	80	113	109	0
	Erysipelotrichaceae	6	0	10	4	0
	Gemellaceae	1	1	0	0	0
	Gracilibacteraceae	0	1	13	2	0
	Lachnospiraceae	42	46	66	34	0
	Paenibacillaceae	0	1	54	16	0
	Peptococcaceae	7	1	3	1	0
	Peptostreptococcaceae	0	0	4	0	0
	Planococcaceae	16	13	16	8	0
	Ruminococcaceae	40	56	7161	43	0
	Staphylococcaceae	4	6	6	0	0
	Streptococcaceae	0	4	0	0	0
	Veillonellaceae	0	5	0	0	0
	(blank)	8	7	13	3	4

Fusobacteria		**1256**	**96**	**51**	**16**	**0**
	Fusobacteriaceae	10	13	25	10	0
	(blank)	1246	83	26	6	0
Gemmatimonadetes		**347**	**41**	**63**	**29**	**0**
	Gemmatimonadaceae	4	4	1	2	0
	(blank)	343	37	62	27	0
GN02		**3847**	**4748**	**2326**	**3879**	**1119**
	(blank)	3847	4748	2326	3879	1119
GN04		**2**	**0**	**6**	**1**	**0**
	(blank)	2	0	6	1	0
H-178		**2**	**1**	**3**	**0**	**0**
	(blank)	2	1	3	0	0
Kazan-3B-28		**4**	**3**	**2**	**1**	**0**
	(blank)	4	3	2	1	0
Lentisphaerae		**1882**	**564**	**615**	**2236**	**0**
	Arctic95B-10	1525	479	546	2192	0
	Lentisphaeraceae	303	19	12	23	0
	Victivallaceae	37	31	47	12	0
	(blank)	17	35	10	9	0
NKB19		**83**	**56**	**54**	**59**	**0**
	(blank)	83	56	54	59	0
OD1		**20219**	**13826**	**14702**	**20128**	**10302**
	(blank)	20219	13826	14702	20128	10302
OP11		**2**	**2**	**8**	**3**	**11**
	(blank)	2	2	8	3	11

OP3		**248**	**162**	**90**	**147**	**34**
	kpj58rc	9	11	4	20	0
	(blank)	239	151	86	127	34
OP8		**2**	**0**	**0**	**0**	**0**
	(blank)	2	0	0	0	0
Planctomycetes		**37855**	**10067**	**23196**	**26598**	**115**
	Gemmataceae	17	18	14	6	29
	Isosphaeraceae	2	2	0	1	0
	Phycisphaeraceae	1359	886	738	1781	3
	Pirellulaceae	13855	3304	14686	9512	37
	Planctomycetaceae	2587	1738	2269	2138	14
	(blank)	20035	4119	5489	13160	32
Proteobacteria		**427433**	**422298**	**512148**	**367475**	**22403**
	[Chromatiaceae]	484	181	148	169	0
	[Marinicellaceae]	937	401	862	678	23
	0319-6G20	6	2	1	0	0
	125ds10	4	3	0	1	0
	211ds20	1	3	3	2	0
	Acetobacteraceae	0	0	0	2	0
	Aeromonadaceae	0	0	0	2	0
	Alcaligenaceae	5	1	0	0	0
	Alcanivoracaceae	83	49	97	40	16
	Alteromonadaceae	14889	3171	34827	21689	34
	Anaplasmataceae	2	0	0	0	0
	Aurantimonadaceae	19	14	70	19	6

Bacteriovoracaceae	4467	2665	2063	2954	15
Bdellovibrionaceae	2607	3472	996	234	44
Beijerinckiaceae	3	0	1	0	3
Brucellaceae	0	0	1	3	2
Burkholderiaceae	8	12	1	5	0
Campylobacteraceae	345	282	846	555	0
Cardiobacteriaceae	1	0	2	1	0
Caulobacteraceae	6	2	7	5	0
Chromatiaceae	25	20	30	18	0
Cohaesibacteraceae	60	66	70	37	2
Colwelliaceae	386	177	296	277	8
Comamonadaceae	319	1519	446	1448	4
Coxiellaceae	791	625	324	593	433
Crenotrichaceae	0	2	4	0	0
Cystobacterineae	389	242	94	232	1
Desulfarculaceae	6	7	5	4	0
Desulfobacteraceae	175	197	141	130	1
Desulfobulbaceae	119	58	94	53	0
Desulfovibrionaceae	28	40	32	15	0
Desulfurellaceae	1	1	0	0	0
Desulfuromonadaceae	38	12	33	40	0
Ectothiorhodospiraceae	484	213	150	352	1
Enterobacteriaceae	11	14	6	12	0
Erythrobacteraceae	15613	2829	6959	1913	769
Ferrimonadaceae	10	7	11	15	0

Francisellaceae	242	58	25	107	0
Gallionellaceae	5	2	2	3	0
Geobacteraceae	3	3	12	2	0
Hahellaceae	15	67	74	64	0
Haliangiaceae	35	24	9	23	0
Halomonadaceae	404	155	572	182	10
Helicobacteraceae	132	203	127	77	0
Holosporaceae	2	2	1	2	0
HTCC2089	477	345	1263	487	0
HTCC2188	13620	12091	8477	9402	397
Hyphomicrobiaceae	3512	2354	5340	2990	150
Hyphomonadaceae	8927	12091	34753	10742	258
Idiomarinaceae	9	19	14	9	21
J115	2	1	10	5	0
JTB36	53	49	79	31	0
JTB38	18	6	11	14	3
Kiloniellaceae	52	52	119	44	1
Kordiimonadaceae	310	299	285	204	0
Legionellaceae	281	83	156	109	89
Mariprofundaceae	3	2	1	2	0
Methylococcaceae	1	2	0	2	0
Methylophilaceae	34869	12312	10555	19154	8224
mitochondria	3	2	0	0	0
Moraxellaceae	1	16	109	0	0
Nannocystaceae	36	27	28	36	4

NB1-i	1	1	5	0	0
Neisseriaceae	1	1	0	2	0
Nitrospinaceae	3	0	1	4	0
Oceanospirillaceae	2051	47512	33550	6495	16
Oleiphilaceae	435	267	334	307	175
OM27	54	75	138	254	0
OM60	9512	6682	3912	2657	0
Oxalobacteraceae	3	0	0	1	0
Pelagibacteraceae	43318	16724	2474	27767	25
Pelobacteraceae	2	0	0	0	0
Phyllobacteriaceae	1392	963	3226	2064	64
Piscirickettsiaceae	1551	2321	9467	1808	17
Polyangiaceae	0	3	4	1	0
Procabacteriaceae	4	3	20	5	0
Pseudoalteromonadaceae	5111	7056	22688	15448	2
Pseudomonadaceae	4	7	10	9	1
Psychromonadaceae	25	7	6	15	0
Rhizobiaceae	891	541	39	30	0
Rhodobacteraceae	173260	230834	286417	165067	738
Rhodocyclaceae	0	0	0	0	2
Rhodospirillaceae	16932	10658	4931	9528	8445
Rickettsiaceae	1363	981	1008	744	236
S25_1238	7	24	140	31	0
Saccharospirillaceae	30	19	19	27	0
Salinisphaeraceae	9	14	2	3	0

	Shewanellaceae	30	13	93	11	0
	Sinobacteraceae	16	21	15	13	17
	Sphingomonadaceae	248	268	587	225	8
	SUP05	2	0	2	0	0
	Syntrophaceae	7	2	5	2	0
	Syntrophobacteraceae	40	34	32	43	0
	Thiotrichaceae	169	20	21	15	0
	Vibrionaceae	4666	3888	3948	3436	43
	Xanthobacteraceae	26	3	16	16	0
	Xanthomonadaceae	87	153	32	57	1
	(blank)	60849	36651	28364	56201	2094
SAR406		**10**	**4**	**3**	**0**	**0**
	5bav_B4	2	0	0	0	0
	A714017	1	3	1	0	0
	SHAS460	5	1	2	0	0
	(blank)	2	0	0	0	0
SBR1093		**69**	**24**	**32**	**31**	**19**
	(blank)	69	24	32	31	19
Spirochaetes		**35**	**20**	**28**	**30**	**0**
	Brachyspiraceae	5	3	4	3	0
	Leptospiraceae	7	5	9	10	0
	Sphaerochaetaceae	2	0	0	0	0
	Spirochaetaceae	18	8	10	10	0
	(blank)	3	4	5	7	0
SR1		**132**	**286**	**40**	**306**	**2**

	(blank)	132	286	40	306	2
Tenericutes		**732**	**102**	**62**	**343**	**0**
	Acholeplasmataceae	667	65	0	316	0
	Mycoplasmataceae	22	6	21	9	0
	(blank)	43	31	41	18	0
TM6		**4269**	**513**	**271**	**1937**	**27**
	(blank)	4269	513	271	1937	27
TM7		**147**	**267**	**263**	**131**	**69**
	(blank)	147	267	263	131	69
Verrucomicrobia		**65361**	**16589**	**19843**	**43356**	**48**
	[Cerasicoccaceae]	128	67	23	46	0
	[Chthoniobacteraceae]	389	203	22	428	1
	[Pelagicoccaceae]	238	79	116	88	0
	01D2Z36	1	3	2	0	0
	LD19	38	36	44	30	0
	Opitutaceae	112	54	35	163	0
	Puniceicoccaceae	207	224	93	161	2
	R4-41B	1271	39	32	13	1
	Verrucomicrobiaceae	57984	14607	18729	40328	41
	zEL20	0	0	0	4	0
	(blank)	4993	1277	747	2095	3
WPS-2		**14**	**23**	**38**	**18**	**0**
	(blank)	14	23	38	18	0
WS2		**4**	**2**	**14**	**2**	**0**
	(blank)	4	2	14	2	0

WS3		**35**	**20**	**39**	**37**	**1**
	CV106	3	2	2	6	0
	KSB4	4	0	7	3	0
	(blank)	28	18	30	28	1
WS6		**14**	**19**	**8**	**12**	**3**
	(blank)	14	19	8	12	3
WWE1		**60**	**13**	**10**	**48**	**0**
	MSBL8	60	13	10	48	0
ZB3		**110**	**50**	**85**	**126**	**3**
	(blank)	110	50	85	126	3
(blank)		**1229**	**1302**	**1362**	**1214**	**27**
	(blank)	1229	1302	1362	1214	27

Use of RNA-seq to examine temporal effects on differential gene expression in eastern oyster *Crassostrea virginica*, exposed to fluoranthene for 48 hours

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Abstract

Advances in next generation sequencing technologies have made it possible to examine the transcriptome of organisms at a much greater depth than previously available. Specifically, RNA sequencing (RNA-seq) has allowed for greater accuracy in estimates of gene expression levels, allele specific expression analysis and the examination of differential message splicing. Recently these protocols have been successfully applied to non-model organisms and have provided unique insight into transcriptome wide responses to a variety of environmental stressors. We examined differential gene expression in the eastern oyster, *Crassostrea virginica*, exposed to an anthropogenic stressor (fluoranthene) using RNA-seq in an effort to identify stressor specific genetic responses. Transcript profiles from 40 individual oysters from two time points (10 control and 10 treatment from 24 and 48 hours) were generated using the Illumina platform and compared to identify differentially expressed genes. Bioinformatic analyses were conducted to identify homology of putative stress biomarkers and the metabolic pathways involved. These data serve as an initial investigation as to the utility of this approach in identifying transcript profiles for stressor specific responses in *Crassostrea virginica*.

Introduction

Marine bivalves have been widely employed as a tool for environmental monitoring (Goldberg 1975; Goldberg *et al.* 1978; Tavares *et al.* 1988; Berkman & Nigro 1992; Choi *et al.* 2010). Their post-settlement sedentary life history and filtering of the surrounding water allows them to serve as efficient biosensors for environmental changes as well as repositories of environmental stressors (whether natural or anthropogenic) (Robinson *et al.* 2005; Vaisman *et al.* 2005). Similarly, a variety of oysters species (including the Pacific oyster *Crassostrea gigas* and eastern oyster) have been used extensively as environmental biomonitors for heavy metal and sewage contamination (Soto-Jimenez *et al.* 2001; Vaisman *et al.* 2005; MacFarlane *et al.* 2006; Yap *et al.* 2011). With regard to the *Crassostrea* species; both *C. gigas* and *C. virginica* have been utilized in toxicological assays investigating the effect of a variety of environmental stressors including polycyclic aromatic hydrocarbons (PAHs) (Jeong & Cho 2005, 2007; Kim *et al.* 2007).

PAHs are widely distributed environmental contaminants. Introduction to the marine environment can occur both naturally (seeps) or by anthropogenic means (unintentional releases and the incomplete combustion of carbon fuels). In vertebrate systems, regulatory control of the biotransformation of both PAHs and halogenated aromatic hydrocarbons (HAHs) involves the aryl hydrocarbon receptor (AhR) (Nebert 1994; Schmidt & Bradfield 1996; Nebert *et al.* 2013). As such, the AhR has been shown to control the expression of several members of the cytochrome p450 gene superfamily, including CYP1A1, which are involved in the metabolic processing of aromatic hydrocarbons (Eisen *et al.* 1983; Nebert *et al.* 2013). An invertebrate homologue for AhR has been identified (Butler *et al.* 2001; Butler *et al.* 2004); but only recently has our information regarding this system in invertebrates been expanded (Liu *et al.* 2010). While molecular investigations in the response for *Crassostrea sp* to PAHs have been conducted previously, most have employed either directed techniques targeting specific genes or suppressive subtractive hybridization (SSH) (Boutet *et al.* 2004; Luchmann *et al.* 2011; Luchmann *et al.* 2012). Herein we employ next generation sequencing via

RNA-seq in generating transcript profiles in the eastern oyster (*Crassostrea virginica*) exposed to the PAH compound fluoranthene to examine early exposure (<48 hours) differential gene expression in biological responses.

Experimental Procedure

Sample Collection

Individual wild oysters were collected from commercial holding ponds located at Island Fresh Seafood (32.681120, -80.292758), near Meggett, South Carolina. Ponds are used for rearing commercial brood stock and are flushed daily by tidal influences of the Toogoodoo River. Commercial rearing operations occur between spring and fall during which, spatfall from wild local oyster populations settle out, become attached to support structure and grow over winter. In early spring ponds are cleared of locally recruited oysters in preparation for the upcoming seasons' operations. Our sampling took advantage of this natural recruitment. As such, our sampling were comprised of 2013 summer/fall spatfall and collected in January 2014 prior to removal for the 2014 growing season. Collections were transported to the laboratory in coolers and placed directly into acclimation aquaria upon arrival. Oysters were separated, cleaned of large debris, measured and subdivided into 10 mm size class categories ranging from 40-110 mm in length. These groups were held in 35-L aquaria using carbon filtered seawater at 20 ppt salinity and allowed to acclimate to laboratory conditions for 10-14 days. Acclimation aquaria were housed in an environmental chamber at a constant temperature of 25^{o}C on a 16h-light 8h-dark photoperiod. During acclimation, oyster were fed commercial concentrated algae (Shellfish Diet 1800, Reed Mariculture Inc.) twice daily following manufacturer recommendations.

Individual fluoranthene exposures

Static non-renewal 48 h exposures were conducted individually for both treatments and controls. Forty oysters were randomly assigned to the two treatment groups and held

individually in 1000-mL beakers and exposed to 600 mL of either filtered stock 20 ppt seawater (control) or PAH amended 20 ppt seawater (treatment) at a nominal concentration of 250 µg/L with gentle aeration. Briefly, a targeted working stock solution of fluoranthene at 1000 ng/L was diluted from a commercial stock of 1000 mg/L. Working stock concentrations were verified by GS-MS and were found to be within 2.9% of accuracy of the targeted concentration of 1000 ng/L From this, an exposure stock of 250 ug/L was constructed and aliquoted out to exposure beakers at a volume of 600 mL per 1000 mL beaker. At the start of the exposure, oyster were fed 2 mL of algae stock to measure clearance rates and verify filtering activity over the course of the exposure. Blank exposure chambers for both treatment and controls were set up to serve as feeding controls. Dissolved oxygen, salinity, temperature, pH and water turbidity were measured at 24 h and 48 h time points.

RNA extraction

Gill tissue was excised from individual oysters (n=40, 20 control and 20 treatment; 10 per each time point) at 24 h and 48 h. Extracted tissue was immediately placed into TRIzol reagent (Invitrogen) and homogenized following manufacturer's protocol. Briefly, approximately 0.5 g of extracted tissue was disrupted mechanically for 15 seconds in the presence of one mL of TRIzol reagent using a hand held tissue homogenizer (Pro 200, Pro Scientific). After homogenization, the TRIzol extraction protocol was strictly followed and the resultant RNA pellet resuspended in 200 µL of RNA free water (Ambion). Resuspended RNA was further purified using silica-membrane spin columns (RNAeasy, Qiagen, Inc). Samples were eluted in 60 µL of RNAase free water and immediately frozen at -80^{o}C. Ten µL aliquots of all samples were collected and the quantity of the purified RNA was examined using a Nanodrop 1000 Spectophotometer (Thermo Fisher Scientific, Wilmington, DE) while RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA). Acceptable threshold for RNA quality was set at a RNA integrity number (RIN) of 7 or greater.

RNA sequencing

Illumina sequencing and library preparation was conducted by Genomic Services Laboratory at North Carolina State University using the HiSeq2500 sequencing platform. For each sample, a minimum of 16M reads of 125 bp single end sequencing was generated. Sequence phred quality score were converted to error probabilities and used for base quality calling (limit=0.05).

De Novo Assembly

De novo transcriptome assembly was accomplished using CLC genomics workbench (Qiagen Bioinformatics). A series of initial assembly were conducted adjusting bubble and word size parameters that yielded small differences in the resulting assemblies. As such, subsequent assemblies used system default settings with a minimum contig length of 400 bp. Sample reads were mapped to the de novo assembly using the RNA-seq mapping tool in the CLC genomics workbench suite. The overall trend for all samples was to have approximately 50% of their reads mapped, suggesting that the currently assembly may need additional refinement.

Counts and Differential Gene Expression

Mapped counts were analyzed for differential expression using the empirical analysis of differential gene expression (EDGE) as implemented in the CLC genomics package using RPMK (reads per kilobase million) counts (which yielded a normalized form of a total count calculation). Experimental design for EDGE analysis grouped all treatment and control samples for each time point separately. False discovery rate (FDR) corrected p-values were used for statistical cutoffs (p=0.05). Additional statistical cutoffs were applied (varying p-values cutoffs of 0.01, 0.001 and 0.0001) to examine robustness of statistical significance.

Bioinformatic analysis

Filtered experimental contigs based on read counts and expression values were used as queries against the NCBI non-redundant nucleotide database using the BlastX program as implemented in Blast2GO (Götz *et al.* 2008). Contigs were further screened using InterproScan protein database searches as implemented in Blast2GO. Searches used a 1E-3 e-value cutoff and only the top gene ID was assigned to contigs. From these, gene ontology (GO) annotation analysis was performed using program default settings.

Results

Sequencing and de novo assembly

Contracted RNA sequencing returned on average over 23 million reads per sample and all but three samples reached the targeted depth of 20 million reads. These three samples all had read totals in excess of 19 million. Data files were converted to fastq format for sequence quality trimming which removed residual adaptor sequences and quality sequences. A total of 368 million reads were used for de novo sequence assembly which comprised trimmed sequence data from sixteen experimental samples (four samples per treatment and time). Using these parameters, the de novo assembly of the 368 million reads resulted in a total contig count of 113,395 with an N50 of 860 and an average contig length of 818.

Counts and Differential Gene Expression

Empirical analysis of differential gene expression (EDGE) identified 1251 contigs as being differentially expressed between control and treatment samples at the 24 h time point (Table 1). Adjusting p-value cut offs showed an appreciable reduction of identified tags, with 68% of the identified contigs having p-values ranging between 0.049 and 0.011. Identification of differentially expressed contigs was nearly two orders of magnitude lower for the EDGE analysis from the 48 h samples, with only 13 contigs being identified (Table 1). When applying more stringent statistical cutoffs, a similar

pattern was observed in the 24 h sample with 73% having p-values ranging between 0.049 and 0.011.

Bioinformatic analysis:

24 h sample

A total of 1256 contigs were selected for bioinformatics analyses. Of these, 377 failed to return any homology using online genetic and protein database searches. The remaining, showing some type of homology in either BlastX or Interpro searches, were broken down into three categories relating to molecular function (n=257), biological process (n=230) and cellular component (n=215).

The molecular function category included homology similarity to proteins relating to binding (66%), catalytic activity (52%) and transporter activity (12%). The binding subcategory included ion binding (both metal ion and calcium) and organic cyclic compound binding. Note that subcategory totals are in excess of 100% due to the fact that many of the products are assigned to more than one category (Figure 1)

Products relating to metabolic processes accounted for 70% of the returned homology searches in the biological process category. Other predominately represented subcategories included single organism processes (54%), localization (20%), biological regulation (17%), stimulus response (13%), and transporter activity (12%) (Figure 2).

For the cellular component processes identified, those relating to the cell membrane accounted for over 70% of the overall search. Specifically, subcategories representing processes relating to integral or intrinsic components of the membrane were some of the highest observed (Figure 3)

48 h sample

A total of 13 contigs were identified as being differentially expressed and selected for bioinformatics analyses at the 48 h time point (Table 1). Of these, one was shared between 24 h and 48 h sample time points but homology could not be assigned in any

of the searches (not shown). Of the remaining 12, seven showed homology in either the BlastX or Interporscan searches. Biological process category was represented by three sequences and identified homophilic cell adhesion via plasma membrane adhesion molecules, microtubule-based movement, dephosphorylation, and chitin metabolic processes as potential areas of impact. Three sequences were identified for the molecular functions category and included calcium ion binding, ATP binding, ATPase activity, phosphatase activity, and microtubule motor activity. Similarly, three sequences were identified with GO components relating to cellular components and included integral component of the membrane, extracellular region, collagen trimmer and dynein complex.

A summary of contigs showing homology to major genes associated with stress response are presented in Table 2. Additionally, contigs identified with homology to genes metabolic enzymes known to be involved in phase one and phase two detoxification processes are also included.

Discussion

The data presented in this study show the utility of next generation sequencing approaches in the examination of differential gene expression in a non-model marine organism. Significant differences in gene expression and transcriptome composition between control and treatment groups were observed as were temporal differences. The greatest differences between groups were observed within the first 24 h of exposure. These differences decreased dramatically by 48 h. Both the contigs identified by EDGE analysis and the magnitude of the responses differed with time, with only one contig identified as being differentially expressed at both 24 and 48 h. These data suggest that, under the experimental conditions examined, oysters had initial cellular activation of various stress related genes in response to the PAH stressor which appears to be followed by a rapid acclimation and tempered genetic regulation within 48 h.

Detoxification of lipophilic xenobiotics in eukaryotes is generally divided into four phases. Phase 0 represents the initial uptake of the xenobiotic compound. Phase I involves the processing of the xenobiotic by the addition of polar groups allowing it to be metabolically processed during phase II. The majority of the catabolic activity and processing of the xenobiotic in phase I is accomplished by the cytochrome P450 enzymes (Nebert & Russell 2002). In phase II, metabolic processing of the xenobiotic and its subsequent metabolites continues, facilitated by the addition of polar groups from phase I. Here, transferases, including glutathione S-transferase (GST) and sulfotransferases, become important by conjugating to the added polar groups processing the once lipophilic compounds into hydrophobic derivatives (Walker *et al.* 1996). During phase III, processed conjugates from phase II are removed from the cell by specialized transporters and include glycoproteins and multidrug resistance proteins. During the entire process, reactive oxygen species (ROS) are generated and processed by the cell's antioxidative system (Halliwell & Gutteridge 2007). In this system are various enzymatic and non-enzymatic antioxidants including members of the peroxidase superfamily (Halliwell & Gutteridge 2007).

The changes in gene expression observed over the course of the exposures were comprised of some notable stress related family of genes including cytochrome P450, heat shock 70, and heavy metal binding protein. Phase I enzyme cytochrome P450 was observed to be differentially expressed in the 24 h samples. The three P450 contigs were identified and grouped to two different classes of P450s belonging to the CYP2 family, 12B2 and 2D10. This family is known for the transformation of contaminants in deuterostomes (Nebert & Russell 2002; Kubota *et al.* 2011) and have also been identified in the processing of toxic chemicals in molluscs (Zanette *et al.* 2009; Miao *et al.* 2011). The CYP450 family have been found to be extremely large in *Crassostrea gigas* with over 136 different gene products identified (Zhang *et al.* 2012). While the observed lack of CYP450 diversity in exposed individuals could be attributable to technological biases (i.e. limitation in sequencing depth) it may also be indicative of a true biological signal of a decrease in phase I activity as the exposed individuals processed the initial uptake of the PAHs within the first 24 h. It is notable to

mention that no CYP450 contigs were identified at the 48 h time point suggesting that the initial phase I response was concluded within 48 h of exposure.

Of the recognized phase II genes only glutathione-S transferase was confirmed to be differentially expressed in the 24 h sample. Two contigs having a high degree of homology to GST were identified as being differentially expressed. Of these, one showed strong homology to the GST mu class and the other lacked specificity to be could not categorized beyond the general GST class. The mu designation is significant in that this class of GST has been proposed as a biomarker for hydrocarbon exposure in oysters (Boutet *et al.* 2004). Results for this work would support that designation. There was no indication of differential expression of any phase II gene of interest at 48 h. Similar to the phase I genes examined, this data would suggest that phase II processing of the PAH xenobiotic was concluded sometime between 24 and 48 h from the time of exposure.

While no frequently listed phase III genes, including multidrug resistance (MDR or MDP) or p-glycoprotein genes (MRP-1) were definitely identified in the list of differentially expressed contigs in the 24 h sample, there were a number of contigs showing homology to a uncharacterized threonine-rich GPI-anchored glycoprotein which may serve as a phase III transporter. GPI-anchored proteins have been shown to have a diverse range in function but are thought to be mainly involved as cell receptors and surface adhesion (McConville & Ferguson 1993; Paulick & Bertozzi 2008). Whether or not they could function as a phase III xenobiotic transporter is currently unknown. Like the previously identified phase I and phase II genes none of the aforementioned putative GPI-anchored glycoproteins were identified as being differentially expressed at the 48 h time point.

Many commonly known gene families that are thought to be critically involved in the cellular response to PAH stressors were conspicuously absent, most notably the Aryl hydrocarbon receptor. It has been proposed that the expansion of gene families involved in the response to cellular stress responses was undertaken as an adaptive response (Zhang *et al.* 2012); and recent studies investigating transcriptomic responses in oysters have supported this idea (Eierman & Hare 2014; McDowell *et al.* 2014;

Luchmann *et al.* 2015; Jenny *et al.* 2016). As such, the absence of members of these gene families identified in our group of differentially expressed genes is concerning but given the limited depth of sequencing and the difficulty in mapping between species of oyster (*Crassostrea virginica* and *Crassostrea gigas*) this could be an artifact of the analysis. Deeper sequencing and more robust bioinformatic analyses with expanded data bases may reveal that these genes were present in the analysis but went undetected due to failed homology searches.

In conclusion, a clear signal was present in the data to detect the genetic upregulation of genes involved in the metabolic cellular machinery to process a xenobiotic stressor, in this case a PAH. It appears that the exposed oysters were capable of processing the PAH stressor at the experimental concentration within 48 h; as there was no indication for differential expression of many putative biomarkers for any phase of xenobiotic biotransformation at that time point.

Data Availability

Data are in the process of being uploaded to NBCI short read depository.

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Tables

Table 1. Number for transcripts identified as being differentially expressed at 24 hours and 48 hours for three statistical false discovery rate (FDR) cut-offs (CTL= control; TRT= flouranthene exposed)

	FRD p-value cut off		
	0.05	0.01	0.001
24 hr CTL vs TRT	1251	402	151
48 hr CTL vs TRT	13	4	0

Table 2. Summary of stress related contigs (sequences) identified in BlastX homology searches. Table includes false discovery rate (FDR) adjusted p-vales and fold change between control and fluoranthene exposures. Genbank BlastX top homology hit and associated e-Value are also included

Contig ID	Length	FDR p-value	Fold change	BlastX Homology Description	e-Value
Contig_8781	468	3.49E-02	-1.62	apoptosis-inducing factor 3-like isoform X1	1.26E-82
Contig_19980	605	4.87E-02	-1.56	apoptosis-inducing factor 3-like isoform X1	1.82E-133
Contig_7546	508	2.10E-05	-7.84	Chorion peroxidase	1.30E-82
Contig_12140	479	4.49E-05	-9.20	Chorion peroxidase	3.39E-42
Contig_16285	605	5.63E-05	-5.11	Chorion peroxidase	3.64E-116
Contig_22383	665	4.54E-06	-6.51	Chorion peroxidase	7.36E-78
Contig_2476	441	6.88E-04	-6.91	Chorion peroxidase	5.69E-17
Contig_1180	1904	3.26E-03	-4.28	chorion peroxidase-like	0.00E+00
Contig_8960	980	6.59E-04	2.42	cytochrome P450 27C1-like	1.74E-145
Contig_35598	730	1.44E-04	2.48	cytochrome P450 27C1-like	1.56E-145
Contig_44274	401	6.35E-03	2.66	Cytochrome P450 2C39	1.01E-58
Contig_21725	827	5.59E-04	-3.47	cytochrome P450 2D10-like	1.19E-124
Contig_11248	421	1.35E-03	-2.89	DBH-like monooxygenase 1	4.04E-66
Contig_4182	1231	1.85E-03	-2.91	DBH-like monooxygenase 1	0.00E+00
Contig_3502	929	1.04E-03	-2.89	DBH-like monooxygenase 1	3.44E-07
Contig_956	461	3.40E-02	1.94	DBH-like monooxygenase 2	4.55E-73
Contig_31922	492	2.65E-02	-8.79	dimethylaniline monooxygenase [N-oxide-forming] 2-like	3.16E-82
Contig_6094	1529	3.57E-02	-2.31	dual oxidase-like	0.00E+00
Contig_39836	1511	2.15E-02	-2.34	dual oxidase-like	0.00E+00
Contig_14637	453	3.01E-02	44.54	estradiol 17-beta-dehydrogenase 12-like isoform X1	6.75E-17
Contig_69491	1409	1.08E-02	7.07	FAD-linked oxidoreductase DDB_G0289697-like	7.96E-125
Contig_12798	877	4.87E-02	-2.48	ferric-chelate reductase 1	4.23E-103
Contig_6317	1511	2.84E-02	-1.90	ferric-chelate reductase 1 isoform X1	0.00E+00
Contig_6987	620	6.24E-04	-2.67	glucose dehydrogenase [quinone]-like	1.94E-134

Contig_23999	804	1.96E-03	2.27	glutathione S-transferase 1-1-like isoform X2	1.66E-175
Contig_55237	550	2.17E-02	1.89	glutathione S-transferase-like	1.16E-07
Contig_107877	438	3.40E-02	2.23	Heat shock 70 kDa 12A	7.17E-18
Contig_19319	834	4.63E-02	6.57	heat shock 70 kDa 12A-like	4.87E-14
Contig_98295	1590	7.80E-03	10.15	heat shock 70 kDa 12A-like	9.79E-130
Contig_53994	464	5.38E-03	2.23	heat shock 70 kDa 12B-like	1.06E-69
Contig_8603	720	1.84E-02	3.23	heat shock 70 kDa 12B-like	2.80E-27
Contig_34053	725	3.18E-02	5.34	heat shock 70 kDa 12B-like	5.86E-62
Contig_37360	822	3.13E-02	-2.41	laccase-4-like isoform X2	1.67E-122
Contig_13829	772	1.77E-04	-3.20	L-ascorbate oxidase	4.19E-119
Contig_11291	473	3.98E-04	-3.27	L-ascorbate oxidase	1.92E-63
Contig_25197	848	3.37E-05	-4.34	L-ascorbate oxidase	5.48E-32
Contig_5724	592	2.77E-02	-1.59	peptidyl-glycine alpha-amidating monooxygenase A-like isoform	4.58E-104
Contig_9757	1196	1.14E-02	-2.17	Peroxidasin	0.00E+00
Contig_3087	1058	9.26E-03	-1.59	peroxidasin-like isoform X1	1.19E-161
Contig_5576	563	4.68E-02	-1.49	peroxidasin-like isoform X2	4.76E-106
Contig_22500	744	1.85E-02	-1.61	peroxidasin-like isoform X2	1.91E-166
Contig_6669	2470	2.17E-02	-2.10	Protein roadkill	0.00E+00
Contig_86199	624	4.13E-02	7.43	sulfite mitochondrial	4.21E-62
Contig_17974	902	1.47E-02	-2.24	Thyroid peroxidase	2.07E-137
Contig_37074	575	5.99E-03	-15.63	tyrosinase 1 isoform X2	5.70E-69
Contig_5914	2142	8.91E-03	-2.25	tyrosinase tyr-1	0.00E+00
Contig_8693	679	3.75E-02	-15.30	tyrosinase tyr-1	7.00E-86
Contig_8694	1824	6.24E-04	-6.58	tyrosinase tyr-1	0.00E+00
Contig_677	2003	1.44E-04	-3.15	tyrosinase tyr-1	0.00E+00
Contig_4283	1312	3.26E-03	-3.90	tyrosinase tyr-3	0.00E+00
Contig_5744	936	1.08E-02	-3.98	tyrosinase tyr-3	1.33E-151
Contig_9357	1118	3.00E-03	-2.71	tyrosinase tyr-3	0.00E+00
Contig_13443	1122	4.03E-03	-2.68	tyrosinase tyr-3	0.00E+00
Contig_19204	1627	4.20E-02	-3.78	tyrosinase tyr-3	0.00E+00
Contig_42478	630	3.69E-02	-3.38	tyrosinase tyr-3	2.58E-57

Figures

Figure 1. Descriptive organizational pathway for differentially expressed genes at 24 hours based on gene ontology for molecular function. Note: Subcategory totals are in excess of 100% as many of the products are assigned to more than one category.

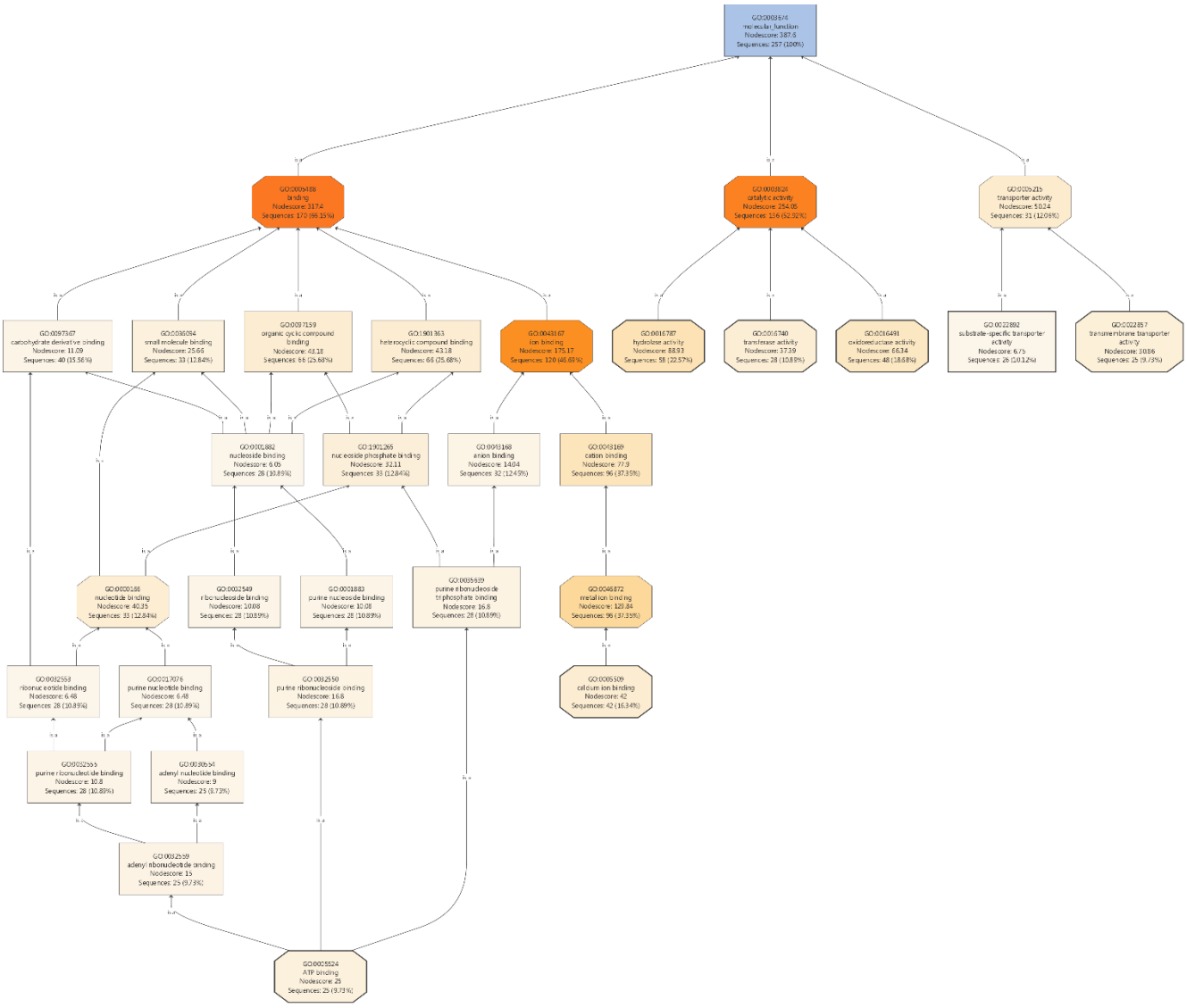


Figure 2. Descriptive organizational pathway for differentially expressed genes at 24 hours based on gene ontology for biological process. Note: Subcategory totals are in excess of 100% as many of the products are assigned to more than one category.

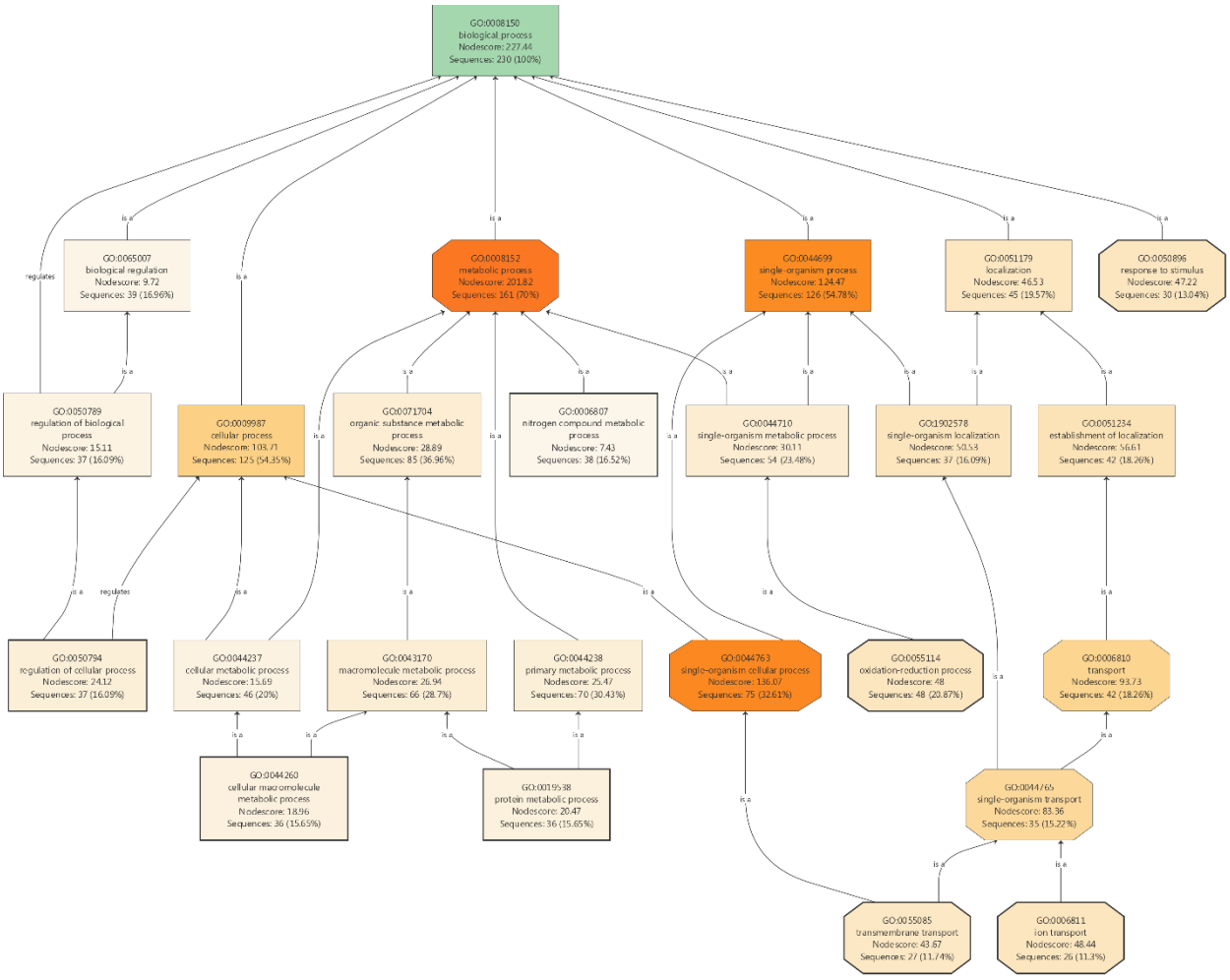
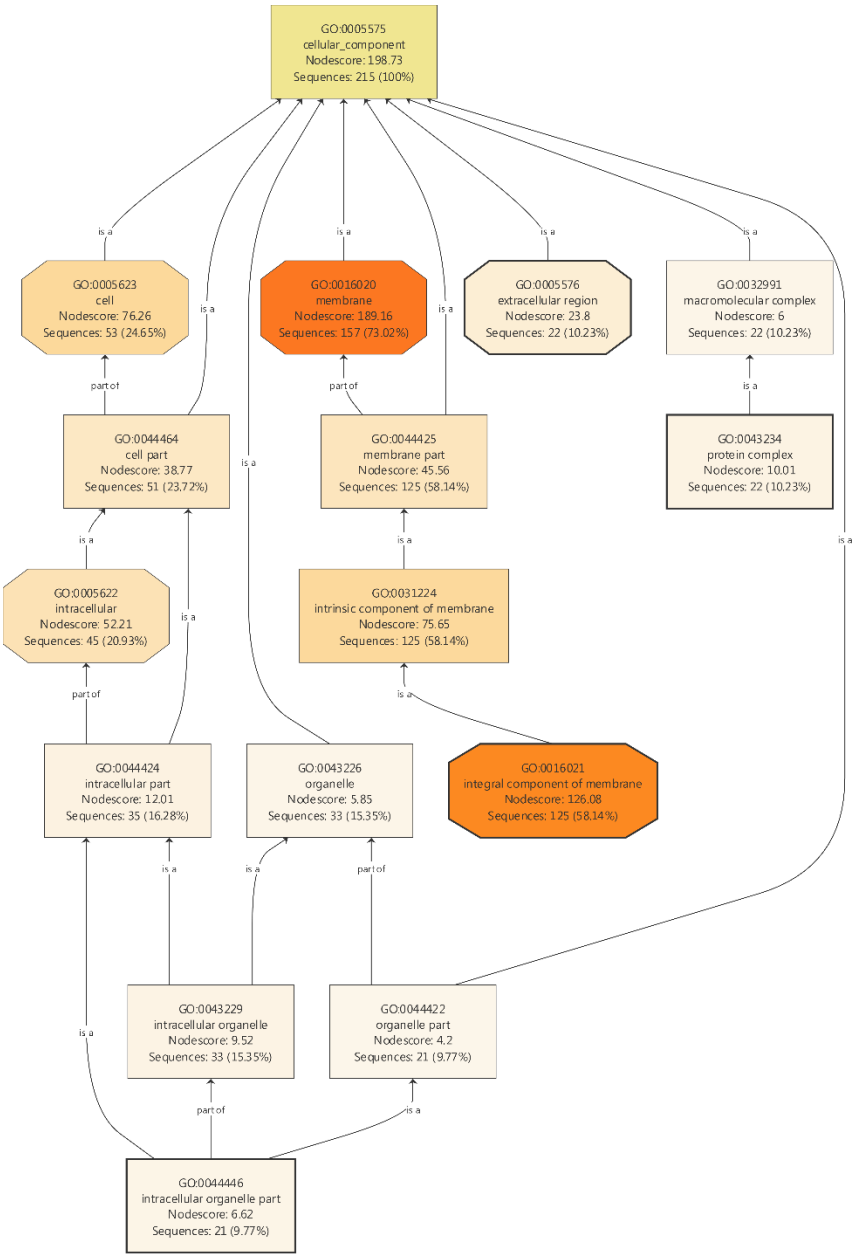


Figure 3. Descriptive organizational pathway for differentially expressed genes at 24 hours based on gene ontology for cellular components. Note: Subcategory totals are in excess of 100% as many of the products are assigned to more than one category.



Sediment Quality Benchmarks for Assessing Oil-Related Impacts to the Deep-Sea Benthos

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Abstract

Paired sediment contaminant and benthic infaunal data from prior studies following the 2010 Deepwater Horizon (DWH) oil spill in the Gulf of Mexico were analyzed using logistic regression models (LRMs) to derive sediment quality benchmarks for assessing risks of oil-related impacts to the deep-sea benthos. Sediment total polycyclic aromatic hydrocarbon (PAH) and total petroleum hydrocarbon (TPH) concentrations were used as measures of oil exposure. Taxonomic richness (average number of taxa/sample) was selected as the primary benthic response variable. Data are from 37 stations (1300 – 1700 m water depth) in fine-grained sediments (92% - 99% silt-clay) sampled within 200 km of the DWH wellhead (most within 40 km) in 2010 and 32 stations sampled in 2011 (29 of which were common to both years). Results suggest the likelihood of impacts to benthic macrofauna and meiofauna communities is low (<20%) at TPH concentrations of less than 606 mg kg^{-1} (ppm dry weight) and 700 mg kg^{-1} respectively, high (>80%) at concentrations greater than 2144 mg kg^{-1} and 2359 mg kg^{-1} respectively, and intermediate at concentrations in between. For total PAHs, the probability of impacts is low (<20%) at concentrations of less than 4.0 mg kg^{-1} (ppm) for both macrofauna and meiofauna, high (>80%) at concentrations greater than 24 mg kg^{-1} and 25 mg kg^{-1} for macrofauna and meiofauna, respectively, and intermediate at concentrations in between. Although numerical sediment quality guidelines (SQGs) are available for total PAHs and other chemical contaminants based on bioeffect data for shallower estuarine, marine, and freshwater biota, to our knowledge, none have been developed for measures of total oil (e.g., TPH) or specifically for deep-sea benthic applications. The benchmarks presented herein provide valuable screening tools for evaluating the biological significance of observed oil concentrations in similar deep-sea sediments following future spills and as potential restoration targets to aid in managing recovery.

Introduction

The Deepwater Horizon (DWH) blowout occurred on April 20, 2010 at a water depth of 1525 meters, in Mississippi Canyon Block 252 in the northern Gulf of Mexico (hereafter the Gulf), releasing an estimated 134 million gallons (i.e., 507 million liters) of oil (US v. BP et al. 2015, DWH/NRDA Trustees 2016) over the following three months. A study was conducted under the direction of the DWH Natural Resource

Damage Assessment (NRDA), Deepwater Benthic Communities Technical Working Group (NRDA Deep Benthic TWG), for the purpose of assessing potential impacts of the spill on sediments and resident benthic fauna in deep-water (> 200 meters) areas of the Gulf. Results of that study, based on data from samples collected in September-October 2010 on two DWH Response cruises (RV Gyre and RV Ocean Veritas), showed a footprint of impacts to benthic macro- and meiofauna over a 172 km^2 area (Montagna et al. 2013 a,b; Baguley et al. 2015). The most severe impacts, including reductions in abundance and diversity, occurred within 3 km of the wellhead, covering an area approximately 24 km^2. Moderate impacts were observed up to 17 km to the southwest and 8.5 km to the northeast of the wellhead, covering an additional 148 km^2. Adverse benthic effects were strongly correlated with concentrations of oil as well as proximity to the wellhead. Results of a follow-up survey conducted in May-June 2011 showed some signs of recovery (particularly for the meiofauna) though there was also evidence of persistent, statistically significant impacts to both macrofauna and meiofauna communities one year after the spill (Montagna et al. 2016).

In the present paper, we use paired contaminant and benthic infaunal data from sediment samples collected in 2010 and 2011 from the DWH benthic studies (Montagna et al. 2013 a,b; Baguley et al. 2015; Montagna et al. 2016) to derive sediment-quality benchmarks for assessing risks of oil-related impacts to the deep-sea benthos. The term risk here refers to the likelihood of an adverse ecological event occurring based on its observed incidence in the sample population (sensu Hyland et al. 2003). Total Polycyclic Aromatic Hydrocarbons (PAHs) and Total Petroleum Hydrocarbons (TPH) levels measured in sediments were used as measures of oil exposure. Numerical sediment quality guidelines (SQGs) have been developed in the past as interpretive tools for assessing the biological significance of a variety of chemical contaminants in sediments in estuarine/marine (e.g., Long et al. 1995, 1998; MacDonald et al. 1996) and freshwater systems (e.g., MacDonald et al. 2000, Ingersoll et al. 2001). However, while there are SQGs for total PAHs, there are none for measures of total oil (e.g., TPH). Moreover, the estuarine/marine SQGs were developed using effects data associated with shallow-water organisms; thus, to our knowledge, there are none that have been developed specifically for deep-sea benthic marine systems.

Methods

Methods for the collection and processing of samples that generated the benthic and contaminant data used in the present study are described in full detail elsewhere (OSAT 2010; Montagna et al. 2013 a,b; Baguley et al. 2015; Montagna et al. 2016; DWH/NRDA Trustees 2016). Briefly, (1) data are from 37 deep-water stations (1300 - 1700 m water depth) sampled within 200 km of the DWH wellhead (most within 40 km) in September-October 2010 on two DWH Response cruises (RV Gyre and RV Ocean Veritas) and from 32 stations sampled on a follow-up NRDA cruise in May – June 2011 on the MV Sarah Bordelon (including 29 stations common to both years); (2) benthic (macrofauna and meiofauna) and sediment contaminant chemistry samples were collected synoptically at each station using an OSIL multi-corer (12-core system, OSIL 2012); (3) macrofauna from each of three cores (10-cm inner diameter) per each multi-corer deployment were sieved on a 0.3-mm mesh screen and identified to family level; (4) meiofauna from a single sub-core (5.5-cm inner diameter) per each multi-corer deployment were sieved on a 0.042-mm mesh screen and identified to the lowest possible taxonomic

level (generally order or higher); (5) data on TPH and total PAH concentrations in the upper 3 cm of sediment, measured by DWH Response and NRDA contract analytical laboratories, were downloaded from the DWH/NRDA DIVER Explorer website (https://dwhdiver.orr.noaa.gov); (6) concentrations of TPH (dry weight), including both aromatic and aliphatic fractions, were measured using EPA Method 8015 (non-halogenated organics by gas chromatography; (7) concentrations of PAHs (dry weight) were measured using EPA Method 8270-SIM (semi-volatile organic compounds by gas chromatography/mass spectrometry with selective ion monitoring); and (8) to be consistent with the related study by Montagna et al. (2013), total PAHs were calculated as the sum of individually measured PAHs using the NOAA total PAH-44 list from the above DWH/NRDA DIVER Explorer website as a guide for which analytes to include (although values for all 44 were not available for some samples). Quality Assurance/Quality Control (QA/QC) procedures for chemical analysis of sediments included blanks, internal standards, and standard reference materials to ensure analytical precision and accuracy.

Logistic-regression models (LRMs) were used to relate incidence of impaired benthic condition to concentrations of chemical contaminants (TPH and total PAHs) and to derive corresponding benchmark values for assessing low to high risks of oil-related impacts to the benthos. Taxonomic richness (average number of taxa/sample) was selected as the primary benthic response variable. Multi-metric benthic indices for assessing impaired versus non-impaired benthic condition have been developed for various estuarine and near-coastal applications (review by Diaz et al. 2004). However, to our knowledge, no such index has been developed for the Gulf of Mexico deep-sea benthos. In the absence of such an index, a station was classified as having impaired benthic condition if its benthic richness value was below the corresponding 25th percentile of all values for the overall population of stations. The 25th percentile was selected to define the lower range because: 1) it represents an objective and widely-used distributional property of a dataset, and 2) selection of a smaller percentile (e.g., 5th or 10th) would not have produced an adequate number of data points in the lower range needed for statistical analysis.

The LRMs used here relate the probability of an event (i.e., impaired benthic condition) to an explanatory variable (i.e., contaminant concentration) as:

$$\text{logit}(\pi) = \log\left(\frac{\pi}{(1-\pi)}\right) = \alpha + \beta x\,,$$

where π = Prob(Y=1 | x) is the response probability to be modeled, α is the intercept parameter, and β is the slope parameter. As an initial analysis step, stations were assigned a binary classification of impaired (event = 1) or non-impaired (event = 0). Solving the above equation for π, the estimated probability of an event is given by

$$\hat{\pi} = \frac{e^{\alpha+\beta x}}{(1+e^{\alpha+\beta x})}\,.$$

Paired benthic and contaminant data from the 37 stations sampled in 2010 were used to develop a LRM for each benthic (macrofauna, meiofauna) versus contaminant (TPH, total PAH) combination, calibrate the models, and test their discriminatory power.

Calibration refers to the agreement between observed outcomes and model predictions. For example, if a model predicts a 20% probability of impaired benthos, the observed frequency of impairment should be 20 out of 100 stations with such a prediction. LMRs in the present study were calibrated using the Hosmer-Lemeshow goodness-of-fit test (Hosmer et al. 2013), which calculates observed event rates in subgroups of the model population. Resulting graphical plots of agreement between observed versus predicted incidence of impacts are presented for each benthic-contaminant combination along with corresponding chi-square values as measures of calibration success. Plots having points close to the diagonal (45°) line and with more spread between subgroups indicate better calibration.

Accurate predictions discriminate between sites with and without the outcome (Steyerberg et al. 2010). Discrimination refers to the ability to distinguish high-risk sites from low-risk sites and is commonly quantified by a measure of concordance, i.e. the c statistic. For binary outcomes, c is identical to the area under the receiver operating characteristic (ROC) curve, or "AUC" hereafter, in plots of true-positive versus false-positive rates; c varies between 0.5 and 1.0 for sensible models (the higher the better) (Harrell et al. 1996, Steyerberg et al. 2001). Perfect prediction corresponds to an AUC equal to 1, while an AUC of 0.5 corresponds to a model with no discriminatory power.

External validation of an LRM can be accomplished by applying model coefficients obtained using the development (or "training") dataset to a set of data that were not used in developing the model. Various measures of calibration and discrimination can be calculated to assess how well the original models and corresponding coefficients fit the new data. In the present study, additional data collected from 32 stations sampled in 2011 were used to validate the models developed with 2010 data.

LRMs derived from the combined 2010 and 2011 datasets were used to derive sediment quality benchmarks for TPH and total PAH concentrations associated with selected probabilities of impaired benthic condition (0.2 = low, 0.5 = moderate, 0.8 = high).

Results

Model Development

Measures of macro- and meiofaunal taxonomic richness (S) were inversely related to concentrations of TPH and total PAH (Figure 1). Number of benthic taxa also tended to be lower at sites within close proximity (< ~ 1 km) to the DWH wellhead, since contaminant concentrations typically were higher at these locations. The associations between taxonomic richness and contaminant concentrations were significant in all cases ($p < 0.001$), based on a likelihood ratio test comparing a null (intercept-only) model to a generalized additive model (GAM) with a smooth term for the predictor (TPH or PAH). Linear regressions of taxonomic richness on log-transformed contaminant concentrations also gave significant ($p < 0.001$) results.

As stated above, impaired benthic condition was defined by using the lower quartile (25th percentile) of taxonomic richness values for both macro- and meiofauna. Resulting cut points were 14.7 and 14.2 taxa per sample for TPH and total PAH respectively in the case of macrofauna, and were 7.0 taxa per sample for meiofauna with respect to both TPH and total PAH (Table 1). Based on these cut points, stations

were classified as either non-impaired (Event = 0) or impaired (Event = 1). As illustrated in Figure 2, stations with high taxonomic richness (> 25th percentile, i.e., Event = 0) had relatively low concentrations of TPH and PAH, while the impaired (Event = 1) group was characterized by levels roughly an order of magnitude higher.

In each of the four macrofauna or meiofauna versus TPH or total PAH combinations, a likelihood-ratio test indicated a significant influence of sediment contamination on in-situ benthic condition (Table 2, $p < 0.001$). The estimated probabilities (fitted line) and observed proportions in equal-interval categories (points) fit the data well in most cases (Figure 3).

Model Calibration

Plots of agreement between the observed versus predicted incidence of degraded benthic condition, based on the Hosmer-Lemeshow goodness-of-fit test, indicate adequate model calibration for each of the benthic-contaminant combinations (Figure 4). While there is good calibration in each case, the closer the data points are to the 45° diagonal and more widely spread they are across subgroups, the stronger the calibration. Non-significant chi-square values, ranging from 0.1151 – 0.6758 for the four benthic-contaminant combinations, does not provide evidence for lack of fit of the models (Table 3).

Model Discriminatory Power

Predictive ability of the models for each of the four benthic-contaminant combinations ranged from good (AUC of 0.815) to excellent (AUC of 0.94) compared to a reference curve (AUC = 0.50, i.e. no discrimination; Table 4). AUC values correspond to the area under the ROC plots of true-positive versus false-positive rates constructed for each of the four models (Figure 5). Results of chi-square tests for both macro- and meiofauna (P values of 0.0048 or less in all cases, Table 4) also indicate that TPH and PAH are good discriminators of impaired benthic condition, as defined in this application.

Model Validation

External validation of the LRMs developed above was accomplished by applying model coefficients obtained using the development (2010) dataset to the data collected in 2011. In this investigation, the external validation set also represents a temporal validation, since the validation data were collected in 2011, while the training data were collected in 2010. There was not a statistically significant difference in log-transformed TPH or PAH concentrations between the two time periods (Welch's two-sample t-test: p=0.87 in both cases). Figure 6 lists various measures of calibration and discrimination and displays the logistic calibration curve originally proposed by Cox (1958). Cox's approach uses logistic regression to test the agreement between a series of hypothesized probabilities and a binary outcome variable (Miller et al. 1991). The resulting model coefficients indicate predictive ability of the models: the predictions (π_i) are equal to the true probabilities when $\alpha = 0$ and $\beta = 1$ (these values are indicated by 'Intercept' and 'Slope' for α and β respectively in Figure 6). The parameter α measures calibration, where the predictive probabilities π_i are too low when $\alpha > 0$ and too high if $\alpha < 0$. The refinement parameter β indicates that the π_is do not vary enough if $|\beta| > 1$; that they vary too much if $0 < |\beta| < 1$; that they are in the right general direction if $\beta > 0$; and that they are in the wrong direction if $\beta < 0$

(Miller et al. 1993). High values (near 1) for Somer's rank correlation between predicted probabilities and binary outcomes (D_{xy}) and the concordance statistic c (equal to the AUC as noted above) suggest good ability to discriminate between impaired and non-impaired sites.

Measures of discrimination indicate that the models developed using 2010 data performed well in classifying sites as impaired versus non-impaired when applied to the 2011 validation dataset (Figure 6). In particular, high values for both Somer's rank correlation D_{xy} (0.818 – 0.951) and the concordance statistic c (0.909 – 0.976) indicate good ability to discriminate between impaired and non-impaired sites and thus positive validation of the models for each of the four benthic-contaminant combinations. Also, in the case of TPH, the model is fairly well calibrated (i.e., close agreement between predictions and observed outcomes), although there was a slight tendency to over- (Intercept < 0) or under- (Intercept > 0) estimate probabilities for macrofauna and meiofauna respectively. By comparison, LMRs for total PAHs show less agreement between predictions and observed outcomes (slope values deviating further from 0) and thus poorer calibration. Still, as for TPH, the above-mentioned D_{xy} and c statistics for total PAHs suggest good ability to discriminate impaired versus non-impaired condition in the 2011 data and thus validation of the original PAH models. Tighter calibration and other refinements to these models, especially the ones for total PAHs, are advised through the addition of new data as they become available.

Sediment Quality Benchmarks

In the previous validation section, model coefficients were applied to the 2011 data to assess transportability of the LRMs that were fit to the training (2010) dataset. Here we report concentrations of TPH and total PAHs corresponding to predicted probabilities of impaired benthic condition, based on LMRs fit to the 2010 data alone as well as both data sets combined (2010 + 2011).

Plots of estimated probabilities and confidence limits over the measured range of contaminant values showed consistent results for the pooled 2010 + 2011 data, compared to 2010 data alone, for both macrofauna and meiofauna (Figure 7). The shapes of the curves are very similar. Contaminant concentrations corresponding to specific probabilities of impaired benthic condition – 0.2 (low), 0.5 (intermediate), and 0.8 (high) – are listed in Table 5 along with estimated confidence limits on the individual predictions. In addition to the similarity of the probability curves, high discriminatory power of the original models developed with 2010 data persisted when applied to the 2011 validation dataset, as noted in the above section. Given these results, values in Table 5 corresponding to the combined 2010 + 2011 data are suggested for use as sediment-quality benchmarks in subsequent applications.

Based on these benchmarks, the percentages of samples with oil concentrations in the low-risk range (< 0.2 probability of impairment) and having a non-impaired benthos (correct negatives) are high, ranging from 92% to 94% among the four benthic-contaminant combinations, and percentages of samples with oil concentrations in the high-risk range (> 0.8 probability of impairment) accompanied by an impaired benthos (correct positives) are also reasonably high, ranging from 75% to 87.5% (Figure 7, Table 6). Conversely, percentages of samples with oil concentrations in the low-risk range and an impaired benthos (false negatives) are low, ranging from 6% to 8%, and percentages of samples with oil

concentrations in the high-risk zone and a non-impaired benthos (false positives) are also low to moderately low, ranging from 12.5% to 25%.

Discussion

This work provides an empirical framework for evaluating risks of impaired benthic condition (reduced taxa richness) in Gulf of Mexico deep-sea sediments in relation to increasing concentrations of two oil-related indicators (TPH and total PAHs). Results suggest that the likelihood of impairment to benthic macrofauna and meiofauna communities should be low (< 20%) at TPH concentrations of < 606 mg kg^{-1} (ppm) and 700 mg kg^{-1} respectively, relatively high (> 80%) at concentrations > 2144 mg kg^{-1} and 2359 mg kg^{-1} respectively, and intermediate at concentrations in between. For total PAH, likelihood of impairment should be low (< 20%) at sediment concentrations < 4.0 mg kg^{-1} (ppm) for both macrofauna and meiofauna, high (> 80%) at concentrations > 24 mg kg^{-1} and 25 mg kg^{-1} for macrofauna and meiofauna respectively, and intermediate at concentrations in between. Tests for goodness-of-fit and the discriminatory ability of the LRMs indicated that TPH and total PAH concentrations in sediment discriminate well between healthy versus impaired benthic condition (as defined here) for both macro- and meiofauna. Predictive ability of the various models developed with 2010 data, based on the c-statistic, ranged from good (0.815) to excellent (0.94). Results of chi-square tests also showed good discriminatory power with P-values of 0.0048 or less in all cases. High discriminatory power of the original models developed with 2010 data persisted when applied to the 2011 validation dataset.

While the ability to predict risks of benthic impacts across oil-concentration ranges is reasonable, it is not free of uncertainty. As depicted in Figure 7, it is possible that some stations in the low risk zone (low levels of TPH or total PAHs) will have impaired benthic communities with low taxa richness (false negatives) and that some stations in the high risk zone will have non-impaired ones (false positives). Thus for any given oil concentration selected as a benchmark (e.g., along the X-axis in Figure 7), the corresponding probability of observing an impaired benthos (along the Y–axis) may actually lie within a range of values of the 90% confidence intervals. Predictive ability of these benchmarks in future applications is subject to such variability, although as noted above actual % false negatives corresponding to the lower-risk 0.20 cutpoints for the present 2010 + 2011 data are low (6% to 8%) and false-positive percentages corresponding to the higher-risk 0.80 cutpoints are also low to moderately low (12.5% to 25%) (Figure 7, Table 6).

There are a variety of factors that can contribute to such variability and data uncertainty. For example, other pollutants or natural controlling factors such as depth and grain-size that are known sources of benthic variation could cause false negatives or false positives in the data set (Word et al. 2005). However, there were efforts in the present study to account for as many of these confounding factors as possible. Namely, (1) all stations are from muddy sediment sites with high silt-clay content (92-99%), thus minimal grain-size variation; (2) all stations are within the same general deep-sea oceanographic regime (northeastern Gulf of Mexico slope waters), and thus have minimal salinity or biogeographic variation; (3) all benthic samples were collected and processed using the same methods, thus eliminating potential effects due to differences in sampling size or sieve size; and (4) all samples used in

the present analysis fall within a limited depth range of 1300 – 1700 m, thus minimizing depth-related spatial variations.

In addition to the above efforts, potentially toxic contaminants other than oil – such as the metals Pb, Zn, and Cr – known to occur at elevated levels on the seafloor in association with offshore drilling operations (Neff et al. 1987, Neff 2005) were at low background levels (Montagna et al. 2013b) and below corresponding sediment quality bioeffect guidelines (Long et al. 1995, 1998). Although dispersants were applied during DWH Response operations, including subsurface injections of Corexit 9500 at the wellhead (DWH/NRDA Trustees 2016), levels in sediments in the present study area were generally low and not a likely source of observed benthic impacts. Concentrations of dioctyl sulfosuccinate ("DOSS," a principal component of Corexit 9500), measured in the upper 1 cm of sediment in 96 replicate samples from our 32 stations in 2011, ranged with one exception from 0 ppb (< detection limits) to 84 ppb, 35% of which were below detection limits (DIVER Explorer website).

The association between impaired benthic condition and presence of oil as the likely source of stress in the present study is also supported by results of sediment toxicity assays (Krasnec et al. 2015) which showed toxic effects on several benthic species in sediments from sites near the DWH wellhead at oil concentrations in the ranges given here. Moreover, the total PAH benchmarks presented here (< 4 mg kg^{-1} for low-risk bioeffect range for both macrofauna and meiofauna, > 24 and 25 mg kg^{-1} for high-risk range for macrofauna and meiofauna respectively) are consistent with total PAH Sediment Quality Guideline (SQG) values developed for shallower-water estuarine/marine benthic fauna, i.e. 4,002 ppb (~4 mg kg^{-1}) and 44,792 ppb (~ 44.8 mg kg^{-1}) for lower-threshold Effect-Range-Low (ERL) and upper-threshold Effect-Range-Median (ERM) values respectively (Long et al. 1995, 1998). However, note that while in the same order of magnitude, the upper-range benchmarks for total PAHs presented here are about half the corresponding ERM value, suggesting the potential greater sensitivity of these deep-sea fauna to such stressors. The difference could also be due to varying numbers of individual PAH analytes included in their respective calculations.

A fairly limited sample size may also have contributed to data uncertainty in the present study. Given that statistical power is dependent upon sample size (Cohen 1988), more stations should have helped to support a more robust data set. However, while additional stations from deeper and shallower water depths were sampled during various DWH Response and NRDA cruises, it was necessary to focus the present analysis on a reduced subset of stations within the narrower depth range noted above (1300 – 1700 m) in order to minimize the influence of this potential confounding factor. Tighter calibration and other refinements to the LMR models presented here should be possible through the addition of new data as they become available and help to improve predictive ability.

There are other potential factors that can contribute to data variability and uncertainty but could not be controlled in the present study (see similar discussions by Hyland et al. 2003, Hyland et al. 2005). For example, an impaired benthic community might occur in sediments with low oil levels (false negatives) due to potential effects from: (1) unmeasured chemical stressors other than the ones mentioned above (e.g., high pore-water ammonia, sulfide); or (2) other biological and physical sources of disturbance (e.g., sediment deposition and erosion due to bottom currents; predation and other biological interactions).

In contrast, possible explanations for a non-impaired benthic community occurring in sediments with high oil exposure levels (false positives) include: (1) presence of contaminants at high concentrations but not in bioavailable forms; (2) small-scale (within sample) spatial variability of the oil; or (3) the fauna were not exposed to oil toxicity due to various avoidance or tolerance mechanisms (e.g., tubes, shells, burrows, mucous coatings, ability to metabolize oil compounds).

An additional important caveat is a reminder that not all oil spills are the same. Different types of crude and refined oil can vary widely in their physical, chemical, and toxicological properties (e.g., reviews by NAS 1975; NRC 1985, 2003, Farrington 2013). Similarly, the same oil once spilled can undergo significant changes due to the interaction of various physical, chemical, and biological weathering processes (Neff et al. 2000). For example, as oil weathers, the more soluble lower-molecular-weight components tend to decline affecting its toxic potential (Di Toro et al. 2007, Carls and Meador 2009). Thus it is possible that two sediment samples having equal concentrations of TPH or total PAH will show different degrees of biological impacts due to differences in the relative composition and toxicities of the individual components. Furthermore, although levels of dispersants measured in sediments in the present study area were generally low, recent research suggests that the use of dispersants may increase the toxicity of spilled oil due to increased exposure to and bioavailability of the dispersed oil components (Delorenzo et al. 2012). Predictive ability of the present benchmarks in practice may be affected by such variability given that they pertain to measures of oil comprising multiple components interacting with a variety of environmental factors.

Burton (2002), in a review of the use of SQGs, also notes a number of data uncertainties and limitations that should be recognized by the user to avoid their potential misapplication. These include potentially high false-negative and false-positive predictions due to many different confounding factors such as the influence of temporal and spatial variability, lack of applicability in some habitats (e.g., dynamic or larger-grained sediments), alterations of sediment properties due to sample collection and processing, and the influence of other co-occurring stressors that are often present as contaminant mixtures in field-collected samples. Moreover, as with other empirical SQGs derived from correlative data on contaminant levels and biotic response, the benchmarks presented here do not establish cause and effect. Therefore, they should be used only as screening tools – not definitive criteria or standards for setting enforceable regulations – and only in conjunction with other components of broader weight-of-evidence assessment approaches, including in-situ measures of habitat characteristics, hydrodynamics, stressor levels and toxicity, and biological condition (Burton 2002).

While it is important to recognize these limitations, the present work provides a reasonable quantitative framework for evaluating risks of observing impaired benthic communities (reduced taxa richness) in Gulf of Mexico deep-sea sediments in relation to increasing oil concentration (total PAHs and TPH). Although SQGs are available for total PAHs and other chemical contaminants based on bioeffect data for shallower estuarine and marine biota (Long et al. 1995, 1998), to our knowledge none have been developed for measures of total oil (e.g., TPH) or specifically for deep-sea benthic applications. The availability of synoptically measured benthic infaunal health and oil-contaminant data, from both impaired and non-impaired sites following the DWH oil spill, provided a unique opportunity to help fill this void. It is anticipated that the resulting benchmarks, when used appropriately as suggested above,

will serve as valuable screening tools for evaluating the biological significance of observed oil concentrations in Gulf of Mexico deep-sea sediments and as potential restoration targets to aid in determining recovery. They also should be appropriate for use in comparable deep-sea habitats beyond the Gulf of Mexico (e.g., similar slope depths, fine-grained sediments, cold water temperatures). Future research should include efforts to reduce the above kinds of uncertainties and to develop similar indicators for application in other deep-sea geographic regions and spill scenarios, given our continuing global reliance on offshore oil exploration, development, and transportation and thus a potential demand to meet additional environmental assessment needs over broader areas.

Data Availability

The data used in this study are available for download through NOAA's Data Integration, Visualization, Exploration, and Reporting (DIVER) data warehouse and query tool. The data are publicly available through the NOAA repository for environmental data related to the Deepwater Horizon Natural Resource Damage Assessment and can be accessed through the DIVER website located at https://www.diver.orr.noaa.gov/home

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Supporting Documents

Link to journal publication: https://setac.onlinelibrary.wiley.com/doi/full/10.1002/ieam.1898

Tables

Table 1. Lower 25th-percentile values of taxonomic richness (average # taxa/sample, S) used as cut points to define impaired benthic condition (Event=1) vs. non-impaired condition (Event=0).

Contaminant*	S (Macrofauna)	S (Meiofauna)
TPH	14.7	7
Total PAH	14.2	7

* Note: Data for total PAHs were missing at two stations, thus n = 35 for total PAHs and n = 37 for TPH. This resulted in slightly different 25th-percentile cut points for TPH-macrofaunal (14.7) and PAH-macrofaunal (14.2) comparisons. Meiofaunal cut points were the same for TPH and total PAHs.

Table 2. Summary of Logistic Regression Modeling (LRM) results.

Model	Fauna	Modeled probability $Pr(Y_i = 1 \mid x_i)$	LR test result
$logit(\pi_i) = \alpha + \beta \cdot TPH_i$	Macrofauna	$\pi_i = Pr(S_i < 14.7 \mid TPH_i)$	$p < 0.0001$
	Meiofauna	$\pi_i = Pr(S_i < 7 \mid TPH_i)$	$p = 0.0002$
$logit(\pi_i) = \alpha + \beta \cdot PAH_i$	Macrofauna	$\pi_i = Pr(S_i < 14.2 \mid PAH_i)$	$p < 0.0001$
	Meiofauna	$\pi_i = Pr(S_i < 7 \mid PAH_i)$	$p = 0.0004$

Table 3. Results of Hosmer-Lemeshow goodness-of-fit test. Non-significant results indicate adequate fit of the model.

Contaminant	Parameter	Chi-square	DF	Pr > Chi-Square
TPH	# of macrofauna taxa (S)	4.8704	7	0.6758
	# of meiofauna taxa (S)	8.2846	7	0.3082
Total PAH	# of macrofauna taxa (S)	11.5838	7	0.1151
	# of meiofauna taxa (S)	6.2097	7	0.5155

Table 4. Table 4. Results of chi-square tests for significance of differences between the various model AUCs and a reference curve (with AUC = 0.50, i.e., no discrimination). A significant test result indicates a model with good discriminatory power.

Comparison	AUC	Chi-square	P-value	Good discriminator
# of macrofauna taxa (S) vs. TPH	0.8148	7.955	0.0048	Yes
# of meiofauna taxa (S) vs. TPH	0.9397	141.9395	<0.0001	Yes
# of macrofauna taxa (S) vs. Total PAH	0.885	27.2086	<0.0001	Yes
# of meiofauna taxa (S) vs. Total PAH	0.9028	43.8573	<0.0001	Yes

Table 5. Contaminant concentrations (mg kg^{-1} dry weight) associated with predicted probabilities of impaired benthic condition, based on # of taxa (macrofauna and meiofauna).

Benthic parameter	Contaminant	Year(s)	Concentration (mg kg^{-1} dry weight)	Pred. Prob. [90% C.L.]
# of macrofauna taxa	TPH	2010	555	0.20 [0.10, 0.37]
		2010 & 2011	606	0.20 [0.12, 0.32]
		2010	1380	0.50 [0.24, 0.76]
		2010 & 2011	1375	0.50 [0.28, 0.72]
		2010	2204	0.80 [0.39, 0.96]
		2010 & 2011	2144	0.80 [0.46, 0.95]
	PAH	2010	5	0.20 [0.09, 0.39]
		2010 & 2011	4	0.20 [0.12, 0.31]
		2010	12	0.50 [0.23, 0.77]
		2010 & 2011	14	0.50 [0.30, 0.70]
		2010	18	0.80 [0.40, 0.96]
		2010 & 2011	24	0.80 [0.48, 0.95]
# of meiofauna taxa	TPH	2010	914	0.20 [0.09, 0.37]
		2010 & 2011	700	0.20 [0.11 0.33]
		2010	1991	0.50 [0.23, 0.77]
		2010 & 2011	1529	0.50 [0.28, 0.72]
		2010	3068	0.80 [0.38, 0.96]
		2010 & 2011	2359	0.80 [0.47, 0.95]
	PAH	2010	7	0.20 [0.10, 0.37]
		2010 & 2011	4	0.20 [0.12, 0.31]
		2010	15	0.50 [0.24, 0.76]
		2010 & 2011	14	0.50 [0.30, 0.70]
		2010	24	0.80 [0.40, 0.96]
		2010 & 2011	25	0.80 [0.47, 0.95]

Table 6. Percent (%) false positives and negatives and % correct positives and negatives corresponding to proposed 0.20 and 0.80 cutpoints for predicted probabilities of an impaired benthos (based on combined 2010 + 2011 data). Data points used to calculate these percentages are also reflected in Figure 7 of the manuscript.

LRM Combination	Risk zone	# of samples	Classification efficiency
TPH vs Macrofauna	Low (< 606 ppm)	49	Not degraded (correct negative) = 94%
			Degraded (false negative) = 6%
	High (> 2144 ppm)	8	Degraded (correct positive) = 87.5%
			Not degraded (false positive) = 12.5%
TPH vs Meiofauana	Low (< 700 ppm)	51	Not degraded (correct negative) = 92%
			Degraded (false negative) = 8%
	High (> 2359 ppm)	8	Degraded (correct positive) = 87.5%
			Not degraded (false positive) = 12.5%
Total PAH vs Macrofauna	Low (< 4 ppm)	49	Not degraded (correct negative) = 94%
			Degraded (false negative) = 6%
	High (> 24 ppm)	4	Degraded (correct positive) = 75%
			Not degraded (false positive) = 25%
Total PAH vs Meiofauna	Low (< 4 ppm)	49	Not degraded (correct negative) = 94%
			Degraded (false negative) = 6%
	High (> 25 ppm)	4	Degraded (correct positive) = 75%
			Not degraded (false positive) = 25%

Figures

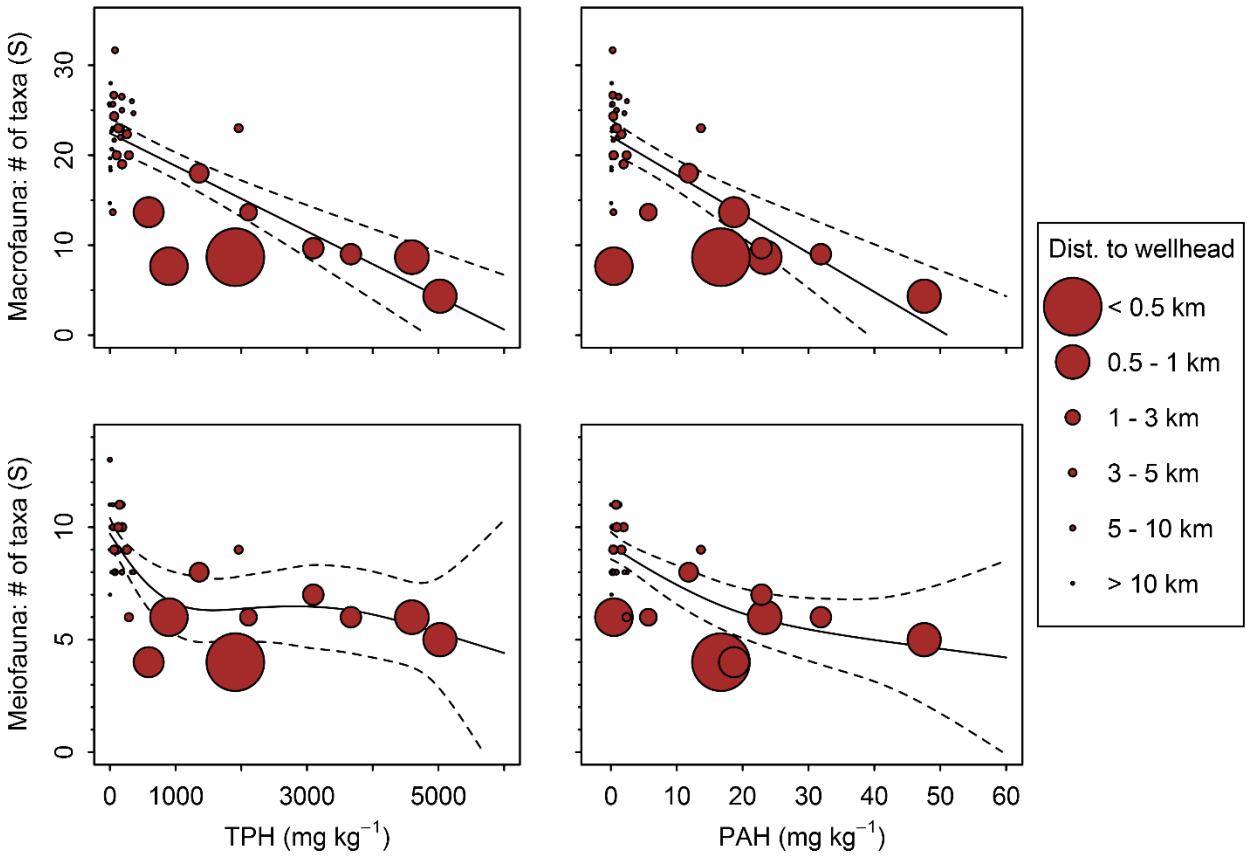

Figure 1. Measured TPH and total PAH concentrations in relation to macro- and meiofaunal taxa richness. Symbols are scaled to be inversely proportional to distance from the wellhead: the largest symbols indicate sites in closest proximity to the wellhead, while smaller symbols indicate sites farther away. Lines represent fitted values and confidence intervals from a generalized additive model (GAM) using regression splines to fit the response as a smooth function of the predictor (TPH or PAH).

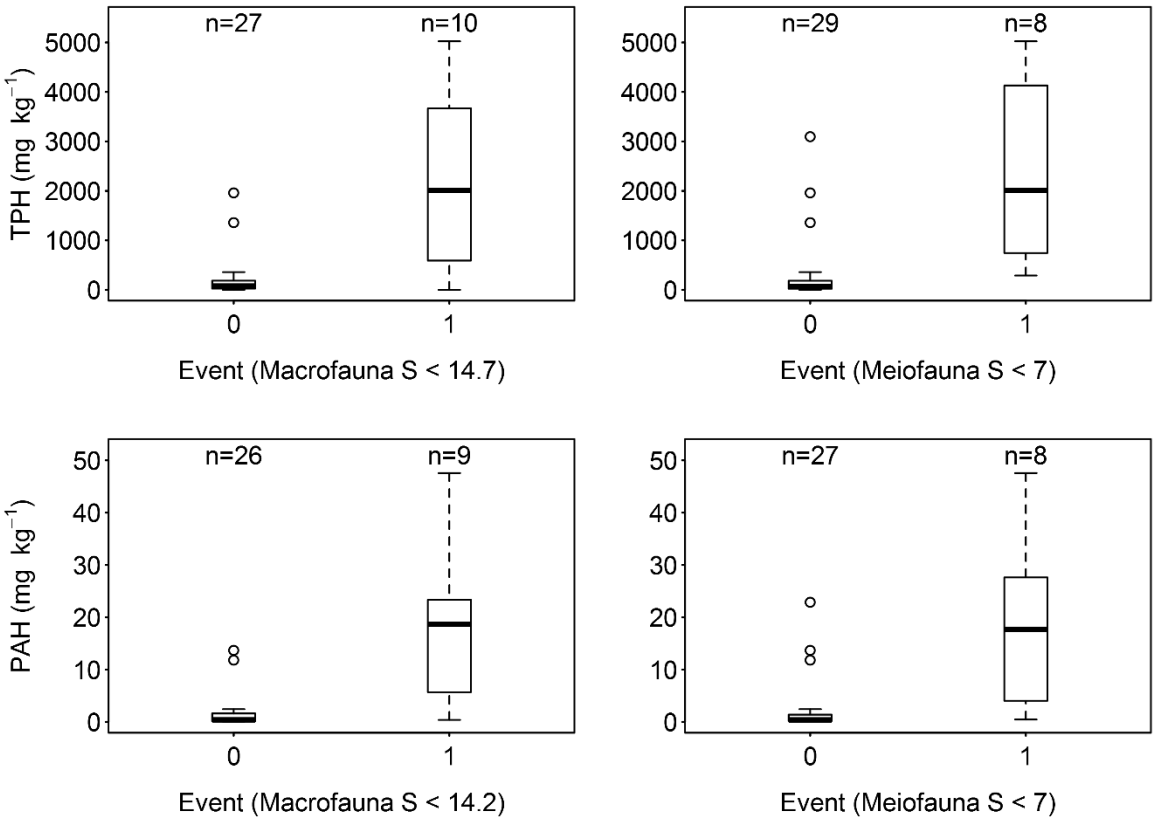

Figure 2. Box-and-whisker plot comparisons of TPH (n=37) and total PAH (n=35) concentrations in impaired vs. non-impaired benthic categories for stations sampled in 2010.

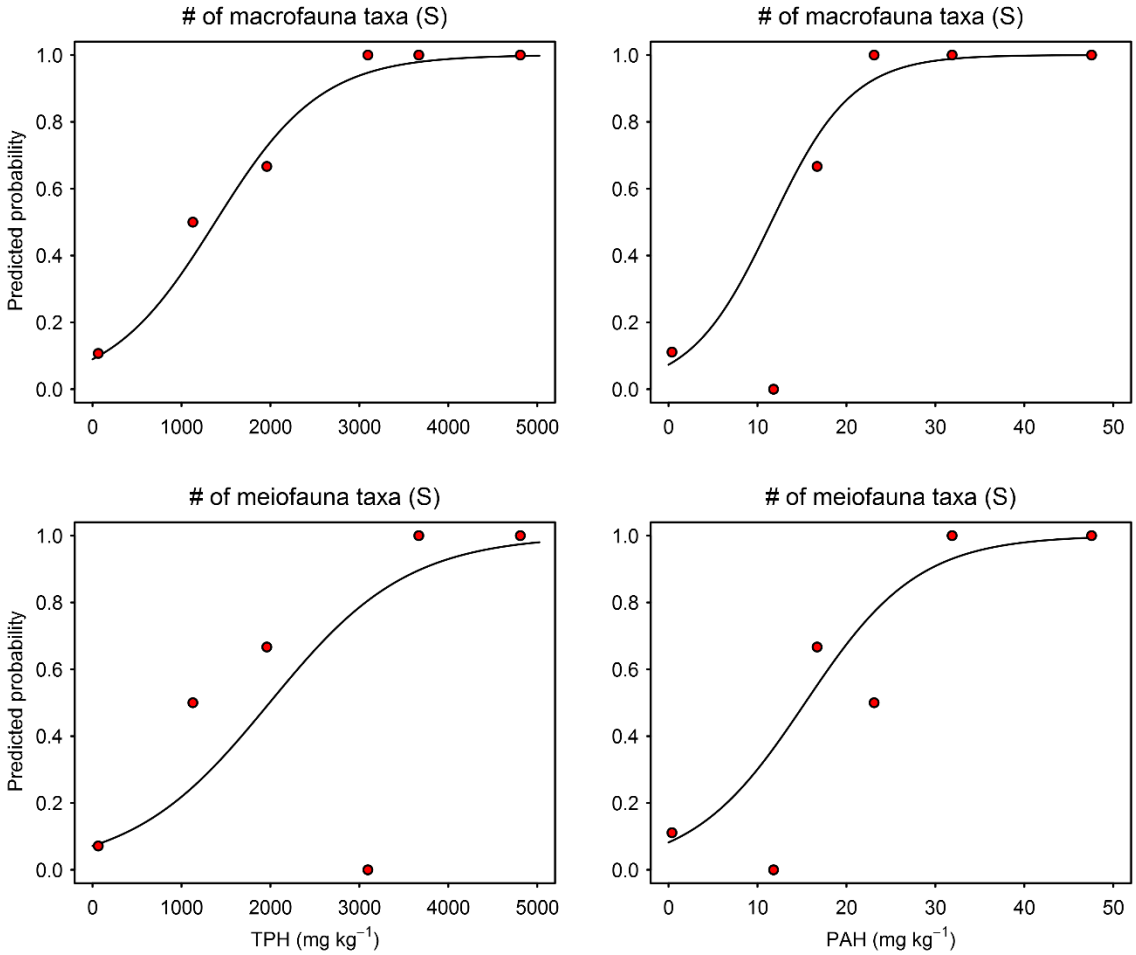

Figure 3. Plots of the estimated probabilities (fitted line) and observed proportions in equal-interval categories (points) for each of the four LRMs estimated for stations sampled in 2010.

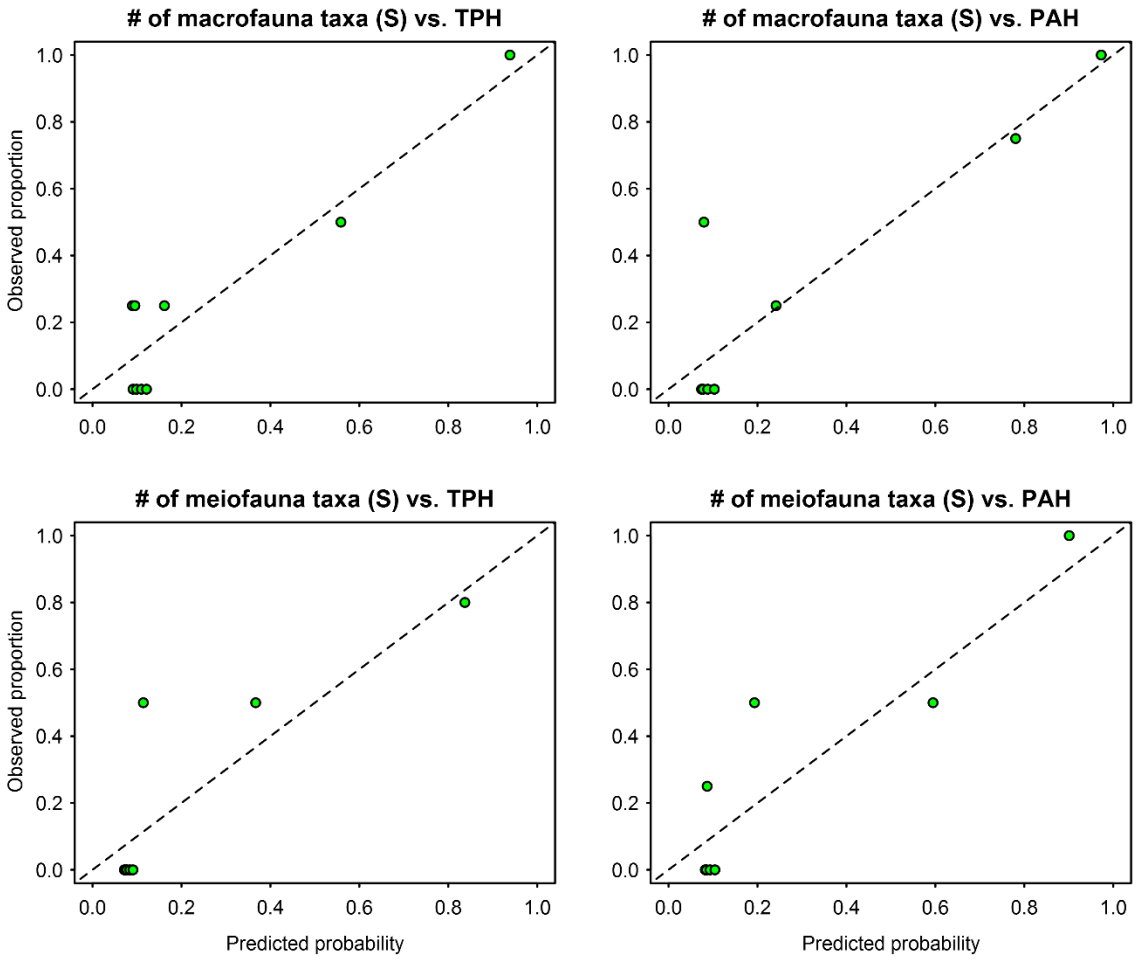

Figure 4. Plots of agreement between predicted vs. observed incidence of impaired benthic condition for stations sampled in 2010.

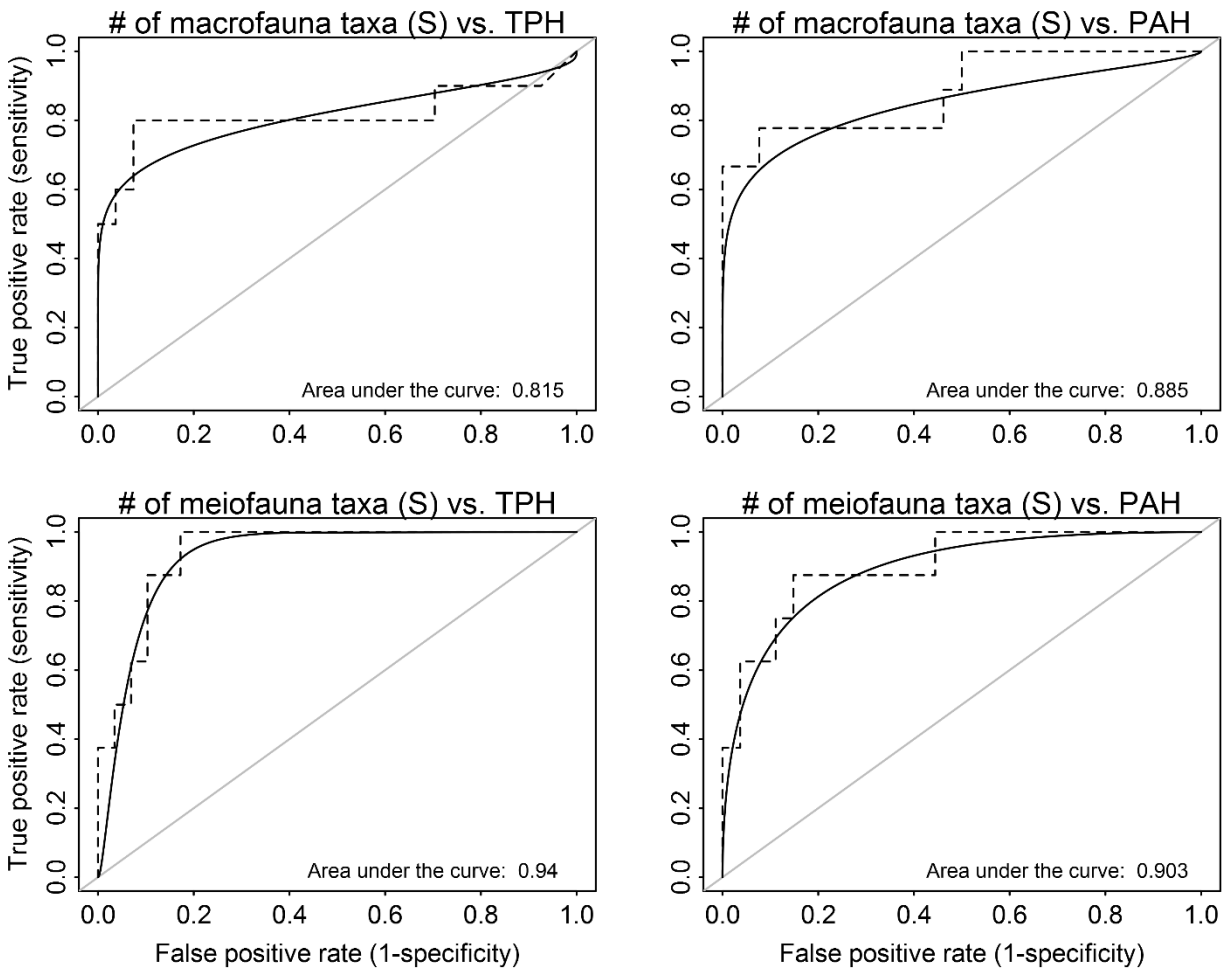

Figure 5. Graphical depiction of discriminatory power for the various Logistic Regression Models (LRMs).

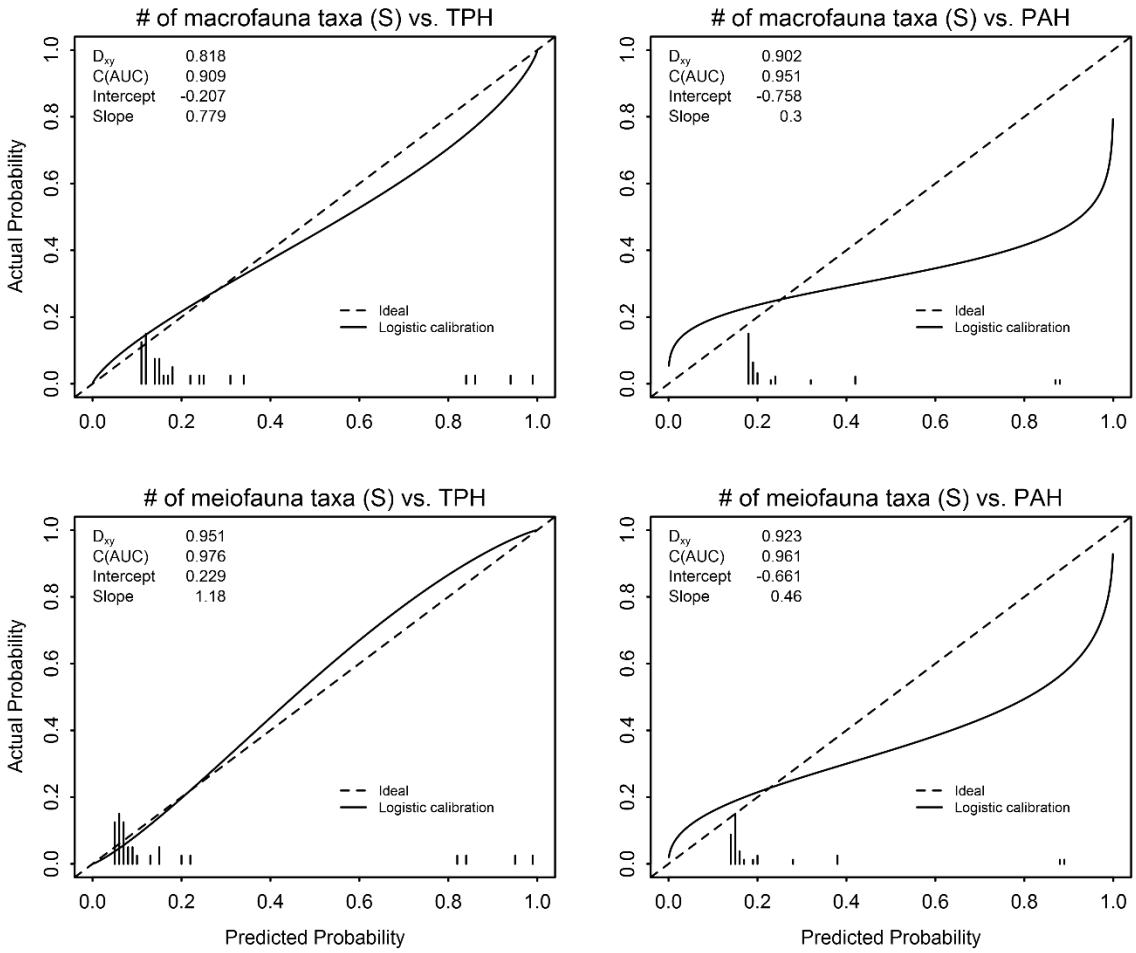

Figure 6. Calibration curves and measures of model discrimination (Dxy, C(AUC)) and refinement (logistic calibration intercept and slope) obtained by applying LRM coefficients developed from 2010 (“training”) dataset to 2011 (“validation”) data. The rug plot along the x-axis provides a histogram showing the distribution of predicted probabilities.

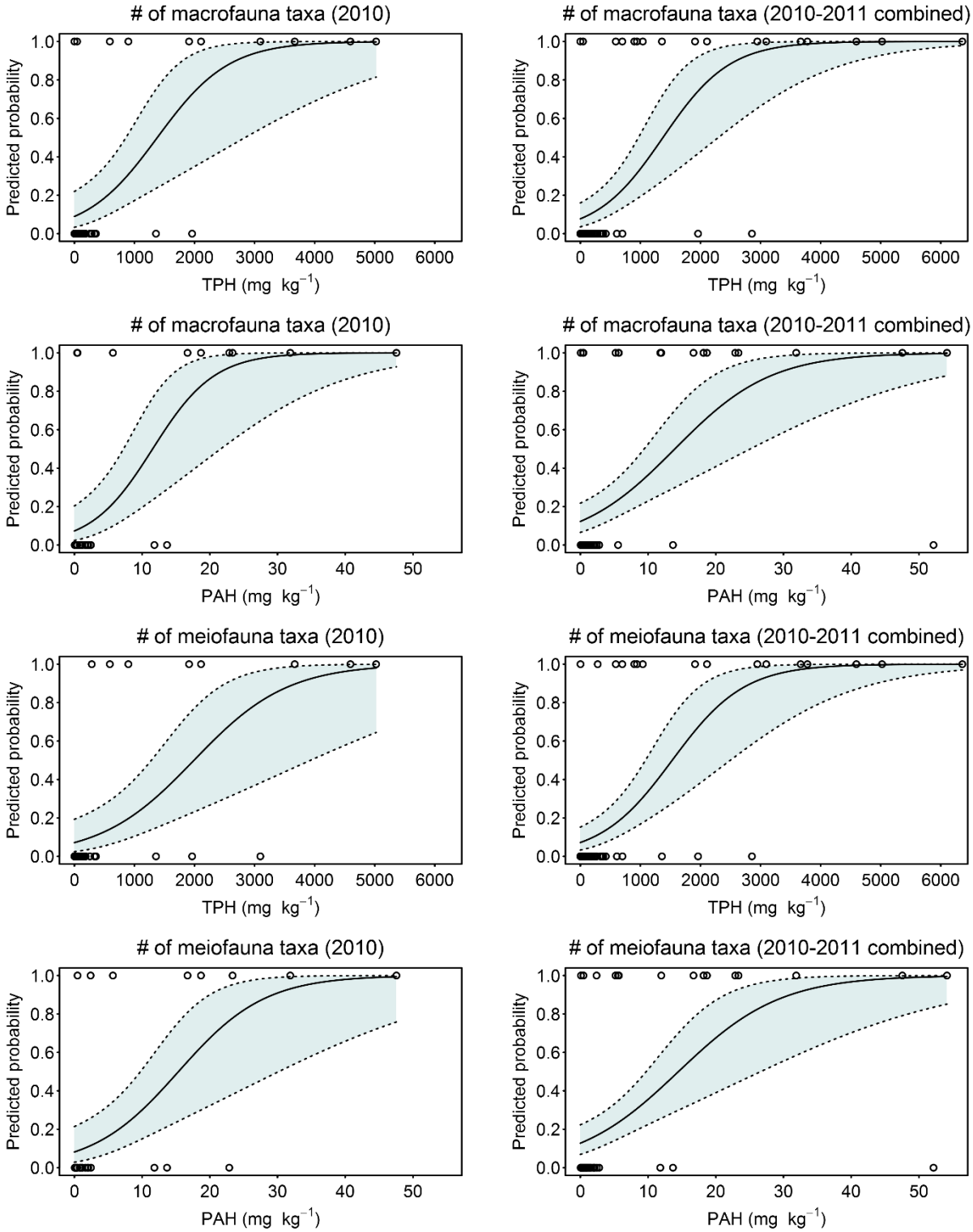

Figure 7. Predictions of the probability of impaired benthic condition (based on # of macrofauna or meiofauna taxa) modeled as a response to contaminant concentrations (TPH or total PAHs) for stations sampled in 2010 only and 2010-2011 combined.

Effect of Louisiana Sweet Crude Oil on a Pacific Coral, *Pocillopora damicornis*

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Abstract

Recent oil spill responses (e.g., Deepwater Horizon) have accentuated the need for crude oil ecotoxicological threshold data for shallow water corals to assist in natural resource damage assessments. We determined the toxicity of a mechanically-agitated oil-seawater mixture of a sweet crude oil on a branched stony coral, *Pocillopora damicornis*. We report the results of two experiments: a 96 h static renewal exposure experiment and a “pulse-chase” experiment (short-term exposures followed by recovery). Five endpoints were used to determine ecotoxicological values: 1) symbiont chlorophyll fluorescence, 2) a tissue regeneration assay and a visual health metric comprising: 3) tissue integrity, 4) tissue color, and 5) polyp behavior. The sum of 50 polycyclic aromatic hydrocarbons (tPAH50) was used as a proxy for oil exposure. For the 96 h dose response experiment, maximum quantum yield (Fv/Fm) of the dinoflagellate symbionts was less affected by crude oil (EC_{50} = 913 µg/L tPAH50) than the effective quantum yield (EQY, EC_{50} = 428 µg/L tPAH50). In the health assessment, polyp behavior (EC_{50} = 27 µg/L tPAH50) was more sensitive than tissue integrity (EC_{50} = 806 µg/L tPAH50) or tissue color (EC_{50} = 926 µg/L tPAH50). Tissue regeneration was a particularly sensitive toxicity measurement (EC_{50} = 10 µg/L tPAH50). Recovery of chlorophyll *a* fluorescence levels following 6-24 h oil exposures was observed in a few hours (Fv/Fm) to several days (EQY) following transfer into fresh seawater. Polyp behavior was the driver for decreased health scores. Wounded and intact fragments exposed to short-term oil treatments were particularly sensitive, demonstrating significant delays in tissue regeneration. These data, when combined with ecotoxicological values for other coral reef species, will contribute to the development of species sensitivity models.

Keywords: coral; crude oil; high-energy water-accommodated fraction (HEWAF); *Pocillopora damicornis*; pulse amplitude modulated fluorometry; tissue regeneration assay

Abbreviations: artificial seawater, ASW; confidence interval, C.I.; Deepwater Horizon, DWH; effective quantum yield, EQY; high-energy water-accommodated fraction, HEWAF; maximum quantum yield, Fv/Fm; parts per thousand, ppt; pulse amplitude modulated, PAM; photosystem II, PSII; photosynthetically active radiation, PAR; polycyclic aromatic hydrocarbons, PAH; toluidine blue O, TBO; sum of 50 polycyclic aromatic hydrocarbons, tPAH50

Introduction

Scleractinian coral species are exposed to oil from both chronic input into shallow marine waters and from acute exposures (e.g., spills) (National Research Council, 2003). Annually, approximately 390 million gallons of petroleum products enter the world's oceans, with 54 % coming from human-derived activities such as extraction, transportation and consumption of oil products (National Research Council, 2003). Recent studies indicate that chronic hydrocarbon exposure (e.g., natural seeps, land-based runoff) may impact both the coral animal and its dinoflagellate symbionts (Downs et al. 2012; Jafarabadi et al. 2018). Highly polluted areas such as the Persian Gulf (Sinaei and Mashinchian 2014) and Jakarta Bay, Indonesia (Baum et al. 2016) have chronic PAH levels between 10-385 µg/L (Σ16 and Σ15 PAHs, respectively). Most reported levels of chronic oil input to coral reef waters have been in the ng/L (parts per trillion) range, however (Cheng et al. 2010; Jafarabadi et al. 2018; Zhou et al. 2000). By contrast, the sudden exposure of corals to large oil spill events (e.g., Deep Water Horizon (DWH)), can have catastrophic short-term effects, as the capacity to acclimatize quickly to the petroleum hydrocarbon load is limited, and cellular metabolic processes can be overwhelmed (Downs et al. 2006).

Evaluations of coral physiological condition in response to xenobiotics have included assessing color change from the loss of the dinoflagellate symbionts (zooxanthellae), i.e., coral 'bleaching' (Brown 2000; Douglas 2003; Jones 1997), physiological changes such as polyp retraction (Reimer 1975; Renegar et al. 2016; Wyers et al.1986) and estimating percent tissue loss during disease events or exposure to stressors (Reimer 1975; Renegar et al. 2016; Thompson et al.1980). Stony corals acquire an estimated 90 % of the fixed carbon required for growth from zooxanthellae (Muscatine and Porter 1977); therefore, it is important to evaluate possible oil effects on the coral symbionts. Measuring algal photosynthetic quantum yield under dark- and light-adapted conditions using a pulse amplitude modulating (PAM) fluorometer provides a method to evaluate chlorophyll *a* fluorescence, providing insight into carbon fixation and photosystem II (PSII) damage (Ralph et al. 2005). Coral condition also has been evaluated by monitoring tissue repair of experimentally induced wounds, as slower regeneration rates are associated with increased exposure to stressors (Fisher et al. 2007; Kramarsky-Winter and Loya 2000; Meesters and Bak 1993; Moses and Hallock 2016; Rodriguez-Villalobos et al. 2016; Traylor-Knowles 2016). Since coral reefs are often in close proximity to sources of oil such as marinas, drilling rigs, tanker routes and shoreline oil refineries, it is important to understand how petroleum spill exposures impact these critical ecosystems. Oil spills are dependent upon ocean currents, tides and wind. These forces may move the slick away within a few hours, or hold the oil in place for days to weeks. While decades of research have documented negative impacts of crude oil exposure on stony coral species, there has been little evidence linking crude oil effects using hydrocarbon chemistry measurements (Negri et al. 2016; Turner and Renegar 2017).

Pocillopora damicornis (P. damicornis, Linnaeus 1758), an Indo-Pacific branching stony coral species, was used to determine oil exposure effects (Hoeksema et al. 2014). It is found in areas with oil-related activities (e.g., shipping lanes, oil rigs) and its relatively

fast growth rate makes it particularly suitable for use in tissue regeneration assessments (Rodriguez-Villalobos et al. 2016). Previous studies of petroleum hydrocarbon effects on *P. damicornis* indicate that this species is relatively sensitive to exposure compared to other corals (e.g., *Porites*, *Montipora*, *Fungia*), with documented effects on both the coral animal and the algal symbiont (Johannes et al. 1972; Peachey and Crosby 1995; Reimer 1975; Rougee et al. 2006; Te 1992; Villanueva et al. 2011).

Our study focuses on short-term effects of oil spills in coral reef environments on adult *P. damicornis* fragments. We have coupled analytical chemistry measurements of tPAH50 as a proxy for oil exposure with behavioral and physiological endpoints for coral and associated dinoflagellate symbionts to determine ecotoxicological concentration values of acute oil exposures using a 96 h static renewal dose-response experiment. The effect of shorter-term (6-24 h) oil exposure scenarios was tested for *P. damicornis* by varying exposure duration of coral to a single high-energy water-accommodated fraction (HEWAF) oil load (62.5 mg/L) over three separate short exposure durations (6, 12, and 24 h) and including an additional 96 h exposure for comparison. In addition, recovery responses in fresh artificial seawater were evaluated by following exposed fragment responses for up to 184 h post treatment. We hypothesize that acute crude oil exposures will result in 1) health effects (polyp retraction, bleaching, tissue loss), 2) a reduction in symbiont chlorophyll *a* fluorescence, and 3) reduced wound healing ability of wounded coral fragments. We expect that recovery of the measured health parameters may be delayed once the stressor is removed.

Experimental Procedures

Chemicals

Solvents (pesticide-free acetone, isopropanol, and pesticide-free hexane), Liquinox detergent, hydrochloric acid, sodium sulfate, toluidine blue O and dimethyl sulfoxide were from Thermo Fisher Scientific (Waltham, MA). Dichloromethane and GF/F paper were from VWR International (Radnor, PA). Louisiana sweet crude oil (Mississippi Canyon Block 252) was a gift from the NOAA Office of Response and Restoration and was stored at 4 °C until use. Internal polycyclic aromatic hydrocarbon (PAH) standards used in the quantitative analysis of PAHs were from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA).

Equipment Preparation

Precleaned glassware was used for all coral exposures and HEWAF preparation and storage. Straight-sided, wide mouth glass jars (500 mL) were used for coral acclimation and dosing (VWR, Radnor, PA, part #89093-982, cleaning protocol A, level PC). Amber glass bottles for the analysis of semivolatiles and pesticides (group 2) were used for HEWAF storage and all dilutions (I-Chem Certified, Chase Scientific Glass, Rockwood, TN). All vessels were rinsed with Tropic Marin artificial seawater (ASW, Tropic Marin Sea Salts, Wartenburg, Germany) prior to use. New glassware, Teflon and metal utensils were cleaned according to Chapman et al. (1995). Oiled glassware and Teflon-ware reused in generating HEWAFs were cleaned according to the method of Forth (2017).

Coral specimen preparation

Pocillopora damicornis colonies were originally purchased from Pacific East Aquaculture (Mardela Springs, MD), subsequently propagated, and held under permit (#NI17-0401) from South Carolina Department of Natural Resources at the NOAA Coral Culture Facility in Charleston, SC. Eight weeks prior to experiment initiation, colonies were fragmented to generate approximately 120 small (~2.0 cm height) nubbins. Coral nubbins for the tissue regeneration experiment were 3.0 cm in height, so that when cut, they would meet the target (2.0 cm) height. Each nubbin was attached with cyanoacrylate gel (e.g., superglue gel) to a custom-made Teflon mounting peg. Mounted nubbins were cultured in a glass and Teflon aquarium system (26.0 +/- 0.5 °C) containing artificial seawater (ASW) (36 ppt) on a 10 h:14 h light:dark cycle at least 4 weeks to allow recovery from fragmenting. Lighting was provided by two 4 X 39 W T-5 HO light fixtures, each with two AquaSun (UV Lighting Co., Avon, OH) and two Blue Plus (ATI, Denver, CO) 54 W bulbs. The photosynthetically active radiation (PAR) at coral depth ranged from 68–77 μmol m^{-2} s^{-1}. Spectral analysis (JAZ spectrometer, Ocean Optics, Largo, FL) of the lighting system showed three major peaks at 430-440, 540-550 and 610-620 nm. Corals were fed a mixture of Bio-Pure® frozen brine shrimp nauplii, rotifers, and cyclopods (Hakari, Hayward, CA), Grow Fry Starter (New Life Spectrum® , Homestead, FL) and Reef-Roids (Polyp Lab, Lenexa, KS) three times per week until oil exposure initiation. Accumulated algal biofilms were manually debrided from the Teflon pegs 2-3 days before experimental acclimation to prevent interference.

Coral acclimation

Pocillopora damicornis fragments were acclimated in an environmentally controlled room (26 °C) for 72 h prior to experiment initiation. Coral nubbins on Teflon pegs were placed in custom-made Teflon jar stands and then transferred to clean 500 mL jars. Each was filled with 400 mL ASW (36.0 +/- 0.5 ppt, 26 °C) during the acclimation period to accommodate the 3.0 cm tissue regeneration nubbins. A Teflon air line (attached to house air line and vinyl tubing via a quick-release valve) was placed in each jar and the bubble rate was adjusted (~1-2/s). Lighting and seawater temperature were as described for coral culture conditions. Water changes (100 %) were performed every 12 h. Salinity and temperature on fresh and spent treatment solutions were monitored at each water change.

Crude oil high-energy water-accommodated fraction (HEWAF)

The HEWAF was generated with Louisiana sweet crude oil (Macondo source oil collected during the Mississippi Canyon 252 oil spill response) and ASW using a Waring™ CB commercial blender according to the methods of Forth (2017). This method generates an oil suspension containing both dissolved hydrocarbon fractions and small oil droplets (Redman 2015). Briefly, 3.75 L of ASW (36 ppt, 26.0 °C) and 3.75 g of crude oil (1 g/L) were added to the blender under reduced (red) lighting and agitated on low speed for 30 s. The oil-seawater suspension immediately was transferred to a 2.0 L separatory funnel and allowed to separate for 1 h (26 °C). The bottom layer of the unfiltered HEWAF (1.5 L total volume) was collected into a clean, rinsed (ASW) 4 L amber glass bottle and used immediately to make the required dilutions (two-fold series, 3.9-250.0 mg/L, nominal oil concentrations). The sum of 50

individual polycyclic aromatic hydrocarbons and alkylated homolog groups (tPAH50) was used as a surrogate for crude oil exposure. The tPAH50 results are presented graphically as the geometric mean of fresh and 12 h spent treatment concentrations (see Analytical Chemistry section). The effect concentrations were calculated from chemical analysis of the fresh (time 0) treatments.

HEWAF 96 h Exposure

Corals were exposed to HEWAF dilutions (3.9-250 mg/L oil load) in a 96 h static renewal experiment. Treatments were changed every 12 h to keep salinity in check. This 12 h cycle also mimics tidal ebb and flow in the marine environment, which could act to move spilled oil back and forth over a reef. Following the acclimation period and at the onset of the dark period, Teflon stands with coral were transferred with clean Teflon-coated metal tongs to dosing jars containing 350 mL of freshly prepared treatment solution (n = 4 replicates). Jars remained open for the duration of the experiment. To reduce placement effects, jars were organized randomly under light fixtures in a temperature-controlled environmental room. One jar with no coral at the highest nominal HEWAF concentration (250 mg/L) was included in the dosing experiment to monitor water quality. Temperature and salinity were measured every 12 h on fresh and spent treatment solutions. Daily pH measurements were made on spent treatment solutions using a Thermo Orion 5-Star multimeter. Samples (400 µL) for ammonia quantification (sodium salicylate method) were removed from each vessel once daily (12 h spent treatment) and stored at -20 °C until further analysis (Bower and Holm-Hansen, 1980). Water changes (100 %) occurred every 12 h, with newly made HEWAF in clean dosing vessels. Water samples were collected for chemical analysis at experiment initiation (T0, fresh ASW and fresh HEWAF dilutions). Spent treatment solutions from pooled replicates were also analyzed for tPAH50 at 12 h and 96 h.

Pulse-Chase Experiment

A pulse-chase experiment was designed to determine effects of acute short-term exposures of coral to oil and to evaluate coral recovery following varying exposure durations. A single HEWAF oil load (62.5 mg/L nominal oil concentration) was selected for this experiment based on effects observed during the 96 h dose-response experiment and reported levels of PAHs documented following oil spills (DIVER, 2017). The five treatment regimens (n = 4 replicates) included: an ASW control, 6 h, 12 h, 24 h and 96 h HEWAF exposures (pulse) followed by a recovery period (chase) of 7-11 days in ASW. Coral nubbins were acclimated as above (single replicate for each endpoint per dosing jar), then exposed to the single oil load across treatment times. Coral fragments were transferred to fresh treatment solutions in clean jars every 12 h. Fragments in the 6 h and 12 h treatments received a single pulse of oil treatment, while the 24 h and 96 h treatments were subjected to repeated pulses (2 and 8, respectively). Samples for chemical analysis were collected: fresh control ASW (T0 and 84 h, n = 2) fresh HEWAF (T0, 12 h, 24 h and 84 h, n = 8), spent ASW (6 h, 12 h, 24 h and 96 h, n = 4) and spent HEWAF (6 h, 12 h, 24 h and 96 h, n = 4). Each treatment jar contained two intact 2.0 cm fragments (for health score and photosynthetic quantum yield endpoints) and two 3.0 cm nubbins for tissue regeneration. One tissue regeneration fragment per treatment replicate was used to determine oil effects on wounded coral fragments (cut prior to oil exposure). A second tissue regeneration fragment was exposed to oil intact, cut at the

beginning of the recovery period and evaluated when fragments reached the approximate percent tissue regeneration of control fragments. A comparison between control and exposed fragments was used to estimate approximate recovery times following oil exposure. Temperature and salinity were measured on fresh and spent treatment solutions every 12 h, and pH and ammonia were measured every 24 h (during light cycle).

PAM Fluorometry

One *P. damicornis* coral nubbin per treatment replicate was used to determine dark-adapted maximum quantum yield (Fv/Fm) and light-adapted effective quantum yield (EQY) of dinoflagellate symbionts using an Imaging PAM M-series chlorophyll fluorometer (MAXI version, Walz GmbH, Effeltrich, Germany). Each coral fragment was analyzed 2 h before the end of the dark cycle (Fv/Fm) and 2 h before lights went off (EQY). Experimental time points for the 96 h dose-response experiment were -14 h, 34 h and 82 h (dark adaption) and -4, 44 and 92 h (light adaption). Experimental time points for the pulse-chase experiment were -14, 10, 34, 58, 82, 106, 130, 154 and 250 h (dark adaption) and -4, 20, 44, 68, 92, 116, 140, 164 and 260 h (light adaption). Replicate fragments were evaluated as previously reported (Ralph et al. 2005). Data were collected on three areas of interest placed on a flat surface in the center of a given fragment and averages for each replicate fragment were calculated.

Health Assessment

One 2.0 cm coral fragment in each dosing jar was examined visually and scored daily (middle of light period) for changes in any of the three parameters associated with health condition (Table 1). The scoring criteria were refined to distinguish gradations of change in tissue integrity, tissue color and polyp behavior (modified from De Leo et al. 2016). Care was taken to avoid disturbing the coral during the assessment (e.g., bumping jars), so that maximum polyp extension could be determined.

Tissue Regeneration Assay

Immediately prior to experiment initiation, 3.0 cm fragments of *P. damicornis* were cropped using clean, stainless steel bone cutters and leaving a flat apical surface. Corals exposed to oil intact during the pulse-chase experiment were cropped immediately following oil exposure (along with control fragments). Each cut fragment (~2.0 cm) was placed into a dosing jar filled with ASW and a bright field image was taken of the cut surface next to a Teflon centimeter rule using a MVX10 research macro zoom microscope with a 0.63x objective (Olympus, Melville, NY) and equipped with a DP71 digital camera (Olympus, Center Valley, PA). Since new tissue growth is translucent (lacks symbionts), coral tissue was stained prior to imaging at experiment termination. Coral fragments were removed from the treatment solution, rinsed in ASW and placed in a jar filled with ASW approximately 1 cm below the cut surface of the coral fragment. The translucent new tissue growth was dyed with a vital stain prior to imaging. Toluidine blue O (TBO, 1 % in DMSO) was diluted to 0.1 % in ASW. Approximately 30 µL of the dilute TBO was placed onto the cut surface of the coral and incubated at room temperature for 3 min. Stain was reapplied as needed (i.e., if it drained away from the coral surface). Fragments were rinsed in ASW and imaged as previously described. Image analysis was performed using Adobe Photoshop CC 2017.

Pixel units were calibrated to the centimeter ruler in each image. Total area of bare skeleton was recorded for each fragment at time 0 and experiment termination. Percent tissue regeneration was determined from the difference in skeletal area between the two values.

Analytical Chemistry

The tPAH50 concentrations were determined for fresh and spent treatment solutions (as detailed above for each experiment) and used as a surrogate for crude oil exposure. Water samples (130-1000 mL) were collected in solvent rinsed, pre-acidified (0.2-1.5 mL 18 % HCl), amber bottles and stored at 4°C until analysis. Samples were spiked with internal standards (18 deuterated polycyclic aromatic hydrocarbons) and extracted using a liquid/liquid extraction adapted from methods detailed in Reddy and Quinn (1999). Samples were passed through silica solid phase extraction (SPE) cartridges and eluted from the cartridge with hexane and dichloromethane. A recovery standard, d_{14}-p-terphenyl, was added prior to GC/MS analysis to measure internal standard recovery. Samples were analyzed on an Agilent 6890/5973N GC/MS with split/splitless injector and a DB17ms 60m x 0.25 mm x 0.25 µm analytical column. All analytes had a coefficient of determination (r^2) greater than or equal to 0.995. Data analyses were performed using MSD Chemstation software. A procedural blank and a PAH reagent spike sample were included in all sample extraction batches (n = 8).

Statistical Analyses

Fresh (time 0) and the geometric mean of fresh and 12 h spent tPAH50 measurements were used for statistical analyses of each endpoint. All analyses were performed using SAS v9.4 (SAS Institute Inc., Cary, NC, USA). The response variables for the dose-response experiment (health metric scores, percent tissue regeneration, and PAM fluorometry measurements) met the assumptions for parametric statistics. The model residuals followed the normal distribution and the residual variances were homogeneous. A single factor ANOVA (PROC GLM) was performed on each variable using the ASW treatment as the experimental control. A Dunnett's test for multiple comparisons versus control was performed post-hoc to determine significant differences between treatment groups and control. Alpha was set to 0.05 for all statistical tests. To determine the effective concentration to cause a 50% effect (EC_{50}) in the aforementioned response variables, a three-parameter normal probability (fitted probit) equation was constructed (PROC NLIN METHOD=GAUSS) using the PROBNORM function.

For the pulse-chase experiment, tissue regeneration and PAM fluorometry measurements were analyzed using a two-factor ANOVA with a test for interaction. The two factors were pulse duration (6 h, 12 h, 24 h, and 96 h) and treatment (control or dosed). The model residuals followed the normal distribution and the residual variances were homogeneous. Finding no significant interaction, a test for simple effects was performed at each level of pulse duration to examine if control treatments were significantly different from dosed treatments. Alpha was set to 0.05 for all statistical tests. The health score data from the pulse-chase experiment were analyzed using the non-parametric Kruskall-Wallis test followed by non-parametric multiple comparison tests for treatments versus a control (Zar 1999).

Results

Water quality

Temperature and salinity levels remained within normal growth parameters for the course of each experiment (26.0 +/- 0.5 °C, 36 +/- 1 ppt). Total ammonia nitrogen (12 h spent treatment sample) ranged from 0.00-0.05 for the initial dose response experiment and 0.00-0.16 mg/L for the pulse-chase experiment. The pH values for the 96 h exposure were between 8.12 and 8.42 and for the pulse-chase experiment ranged from 8.17-8.59.

Analytical chemistry

Crude oil high-energy WAF mixtures consisted of both dissolved PAHs and droplet oil. Chemical analysis of tPAH50 concentrations in HEWAFs followed expected trends, based on degree of stock dilution. Analysis of extracted Louisiana sweet crude oil HEWAF at time zero (T0, 250 mg/L nominal oil load) resulted in 3971 µg/L tPAH50 with roughly 50% reduction in each subsequently lower treatment dilution. After 12 h in the dosing jar, HEWAF tPAH50 concentrations were reduced by at least 88 % in all treatments. For example, the 7.8 mg/L oil loading treatment tPAH50 was measured in the fresh treatment solution as 62 µg/L and decreased to 8 µg/L tPAH50 after 12 h under experimental conditions.

Chemical analysis of the pulse-chase experimental treatment also exhibited ~86 % reduction of tPAH50 concentration over 12 h. Initial (fresh, load 62.5 mg/L) average tPAH50 concentrations in eight HEWAF preparations was 503 µg/L (percent relative standard deviation ~8 %). The 6 h spent treatment solution retained 131 µg/L tPAH50. Repeated spent treatment analysis after 12 h resulted in average tPAH50 concentrations ranging from 64 to 79 µg/L. Spent media tPAH50 concentration was reduced by approximately 74 % after 6 h vs 86 % after 12 h.

Effect of 96 h HEWAF concentrations on coral and symbionts

HEWAF impacts to symbiont chlorophyll a *fluorescence*

Following a static 12 h renewal exposure of *P. damicornis* fragments to dilutions of HEWAF (12-945 µg/L tPAH50, geometric mean of fresh and 12 h spent treatment), oil-exposed symbiont Fv/Fm and EQY were decreased significantly compared to controls ($p<0.05$, Figure 1). A reduction in dark-adapted chlorophyll *a* fluorescence was observed with an 82 h exposure (Panel A). Decreased fluorescence was noted for EQY vs Fv/Fm (Panel B). Table 2 shows the effect concentrations for the tPAH50 exposures. At 82 h the oil exposure resulted in an Fv/Fm EC_{50} value of 913 µg/L tPAH50 (95% C.I. = 862-968), reported as the geometric mean of T0 and 12 h spent treatment. The EC_{50} value for EQY at 92 h was 428 µg/L tPAH50 (95% C.I. = 375-488). The EC_{50} values calculated using the time zero (fresh) treatment chemistry data were 3804 and 1640 µg/L tPAH50 for Fv/Fm and EQY, respectively (Table 2).

General coral health effects of HEWAF concentration exposure

Pocillopora damicornis fragments exposed to increasing concentrations of HEWAF for 96 h resulted in significantly decreased ($p<0.001$) health rubric scores at 89 h (Figure 2). Tissue loss was first observed for three fragments at 65 h (245 and 456 µg/L tPAH50, geometric mean). Tissue loss continued for the three highest treatments until experiment termination. Tissue loss was most severe for the 945 µg/L tPAH50 treatment, with three of four fragments losing 50-75 % tissue by 89 h (Figure 2, Panel A). Coral fragments started bleaching by 31 h for the 22-945 µg/L tPAH50 treatments, but at 89 h was significant only for the 945 µg/L tPAH50 treatment (Figure 2, Panel B). A decline in health was driven by polyp behavior (reduced 3-4 points within the first 17 h of exposure), with treatments of 127 µg/L tPAH50 and higher significantly different from the control at 89 h (Figure 2, Panel C). Some polyp recovery was noted in the 12 µg/L tPAH50 treatment at 41 h post treatment initiation. The EC_{50} values for tissue integrity were 806 µg/L tPAH50 (geometric mean) and 3296 µg/L tPAH50 (fresh treatment), while tissue color resulted in EC_{50} values of 926 (geometric mean) and 3280 (fresh treatment) µg/L tPAH50, respectively (Table 2). Polyp behavior was the most sensitive endpoint in the visual health assessment, with EC_{50} values ranging from 27 (geometric mean) to 84 (fresh) µg/L tPAH50 (Table 2).

Inhibition of coral tissue regeneration by crude oil HEWAF concentrations
The tissue regeneration assay was most sensitive to crude oil HEWAF exposure for *P. damicornis*. Tissue regeneration was inhibited in the 96 h dose response experiment at all tested concentrations (Figure 3, Panel A, $p<0.005$). Figure 4 shows representative *P. damicornis* coral fragments at time zero (after initial cut) and at 96 h (with TBO tissue staining). Treatments of 12-127 µg/L tPAH50 (geometric mean of fresh and 12 h spent treatments) had little to no tissue regeneration compared to the control. Increasing tissue loss was observed for fragments subjected to 245-945 µg/L tPAH50. The tissue regeneration EC_{10} values ranged from 3 (geometric mean) to 7 (fresh) µg/L tPAH50 and EC_{50} values were 10 (geometric mean) and 29 (fresh) µg/L tPAH50 (Table 2).

Pulse-Chase Experiment: Effects of oil exposure duration

Symbiont chlorophyll a fluorescence effects
Shorter durations of a single HEWAF dose (266 µg/L HEWAF tPAH50 concentration, 6 h geometric mean; 189 µg/L HEWAF tPAH50 concentration, 12 h geometric mean) significantly affected ($p<0.05$) symbiont chlorophyll *a* fluorescence (Fv/Fm and EQY, Figure 5). Maximum quantum yield (Fv/Fm) impacts were variable at the 10 h time point with significant reduction for the 12 h and 96 h exposure durations (Figure 5, Panel A). The 12 h exposure fragments fully recovered within 24 h. The Fv/Fm of the 96 h HEWAF exposure required 34 h to recover to the level of the controls following placement in fresh ASW. The EQY for 6, 12, 24 and 96 h HEWAF exposures was significantly decreased at the 20 h time point (Figure 5, Panel B). Complete EQY recovery for the 6, 12 and 24 h HEWAF exposures occurred by 92 h (68-86 h) after placement in fresh ASW. Recovery time for *P. damicornis* symbionts with significant impacts to EQY after a 96 h HEWAF exposure was between (68-164 h post-treatment).

Coral health score effects

Decreased health effects were observed for *P. damicornis* fragments exposed to crude oil HEWAF. Tissue integrity and tissue color were not affected by the short-term (6-24 h) oil exposures; however, immediate polyp retraction was observed in response to all pulse (exposure) durations compared to the no pulse control (Figure 6). There was little (<5 %) to no tissue loss for coral fragments in the 6, 12 or 24 h oil treatments and these treatment durations did not result in significant effects when compared to the no treatment control (Figure 6, Panels A, D). Coral fragments in the 96 h oil treatment exhibited a loss of tissue integrity after 89 h of exposure and this tissue loss increased with time. Coral tissue for all oil treatment durations partially bleached over time; the 96 h treatment duration resulted in significant differences ($p<0.05$) from the control between 65 and 185 h (Figure 6, Panels B, E). *Pocillopora damicornis* fragments in the control also showed a very slight loss of color over time, especially after 185 h. Low initial polyp behavior scores at – 7 h (before treatment started) were due to disturbing fragments during photography (Figure 6, Panel C). Control fragments returned to full extension at 17 h. Polyps exhibited normal behavior (fully extended) for the 6, 12 and 24 h HEWAF following placement in fresh ASW; the normal response was delayed for 24-53 h (Figure 6, Panel). Fragments exposed to oil for 96 h did not fully extend polyps in the recovery period (up to 233 h). This resulted in a significant difference ($p<0.05$) for the 96 h oil exposure when compared to the no pulse control after 65 h HEWAF treatment (Figure 6, Panel F).

Impacts to tissue regeneration

Pocillopora damicornis fragments cut prior to exposures and subjected to short-term HEWAF exposures (189 µg/L, 12 h geometric mean; 266 µg/L, 6 h geometric mean tPAH50) showed significantly ($p<0.0001$) decreased tissue regeneration after 72 h, with tissue loss noted for the longest exposure (Figure 7, Panel A). Following placement in fresh seawater, delayed tissue regeneration times were observed for all HEWAF treatments compared to the controls at 72 h (approximately 50 % tissue regeneration). The 6 h oil treatment resulted in a 48 h lag in recovery, the 12 h treatment had a 72 h lag and the 24 h treatment resulted in 84 h delayed recovery. The cut coral fragments in the 96 h exposure did not recover to the level of the 72 h controls by the end of the experiment (168 h post treatment). Significant impacts ($p<0.0001$) to oil-exposed intact coral fragments were also noted (Figure 7, Panel B). Intact fragments cut following oil exposure and placed in fresh seawater resulted in significantly slowed tissue regeneration 72-84 h post treatment compared to controls cut at the same time.

Discussion

Oil exploration, production and transport often intersect spatially with shallow coral reef ecosystems throughout the tropics and sub-tropics, presenting numerous threats of exposure through spills, groundings or wellhead blowouts, as in DWH. This is the first study to demonstrate ecotoxicological effects of acute crude oil HEWAF exposures on the adult life stage of an ecologically important shallow water coral species, *P. damicornis,* using the surrogate, tPAH50. Total PAH50 has been used as a proxy for oil toxicity to other marine organisms (Esbaugh et al. 2016; Incardona et al. 2014; Stieglitz

et al. 2016), providing a platform to compare toxicity between species and contaminant type that are suitable for oil spill-related ecological risk assessments and natural resource damage assessments. We emphasize that our experiments were performed under laboratory light conditions (no ultraviolet light, UV). As it has been demonstrated that UV light enhances toxicity of hydrocarbon compounds for marine organisms (Alloy et al. 2016; Finch et al. 2017; Sweet et al. 2017), the coral toxicity data we report are likely conservative values.

Ecotoxicological Potential of Louisiana sweet crude oil to coral

Oil effects on symbiont photosynthesis

We observed that symbiont Fv/Fm was impacted by crude oil exposure (EC_{10} = 330 µg/L tPAH50, geometric mean) and this was further reduced to 176 µg/L tPAH50 following *P. damicornis* exposure during the light period. The reported effect concentration values for *P. damicornis* symbionts were 1-2 orders of magnitude higher than for tissue regeneration, indicating that PAHs are less toxic to the algal symbiont than to the coral animal. Chlorophyll *a* fluorescence measurements are valuable indicators of the state of PSII in plants. Exposure to light naturally damages PSII reactions centers (photoinhibition) and the dark cycle provides time for photosynthetic reaction centers in the chloroplast to repair damage normally resulting from exposure to sunlight. Photoinhibition is exacerbated when light is combined with various other environmental stressors, yielding an increase in damaged PSII reaction centers that cannot be repaired during the dark cycle (Nishiyama et al. 2001; Ralph 2000; Takahashi and Murata 2005). Since symbiotic zooxanthellae have been reported to contribute as much as 90 % of the fixed carbon required for growth of scleractinian corals (Muscatine and Porter 1977), impacts to PSII also have severe impacts on coral nutrition, and in turn on growth and other physiological processes (e.g., reproduction, defense, wound repair, etc.).

Oil exposure is known to impact photosynthetic output in several ways. Physical barriers created by the oil can block sunlight required for photosynthesis or decrease gas exchange (Baker 1970). Reductions in chlorophyll content have been observed in response to oil exposure of corals (Baruah et al. 2014; Jafarabadi et al. 2018; Olaranont et al. 2018). In addition, Jafarabadi et al. (2018) have shown that PAHs can penetrate both coral tissues and zooxanthellae membranes. Since the chlorophyll-peridinin complexes of many autotrophic dinoflagellates incorporate both hydrophilic and lipophilic proteins, cellular penetration of PAH compounds could act to rupture the photosynthetic assembly, with pigments subsequently degraded.

Similar to our study, decreases in Fv/Fm from oil exposure have been reported for *Acropora formosa* (Mercurio et al. 2004) and *Porites divaricata* (Guzman Martinez et al. 2007) symbionts with effective concentrations of hydrocarbon in the ppb (µg/L) range. However *Pocillopora verrucosa* exposure to a diesel fuel WAF (415 µg/L total PAH, geometric mean, *our calculation*) resulted in no significant effect on symbiont Fv/Fm in a static 84-h test (Kegler et al. 2015). Additionally, Renegar et al. (2016) found no effect on Fv/Fm following a 48-h exposure of *P. divaricata* to a single PAH compound, 1-methylnaphthelene in a continuous-flow system (640-25,832 µg/L). While experimental

design (static, continuous flow, or static renewal test), exposure duration, lighting and the use of different PAH mixtures may have played a role in the contrasting results, it is possible that variable rates of PSII electron transport among zooxanthellae species are also a factor (Cantin et al. 2009). Additionally, variable optical properties of corals (fluorescent proteins, chromoproteins) can confound PAM-based fluorescence measurements (Mayfield et al. 2014; Wangpraseurt et al. 2019). We observed significant tissue color loss for *P. damicornis* in the visual health score only at the highest HEWAF treatment (945 µg/L tPAH50), but significant decreases in Fv/Fm at much lower concentrations (to 48 µg/L tPAH50, Figure 1). While it is possible that symbionts could be dead or dying within the coral tissues, reduction in Fv/Fm for oil-exposed corals also may be due to PAH effects on the photosynthetic machinery. Further research to understand mechanisms of coral symbiont photosynthetic responses to crude oil exposure is yet to be elucidated.

Oil effects on polyp behavior and tissue integrity

Pocillopora damicornis exhibited severe polyp retraction in response to crude oil HEWAF exposure (EC_{10} = 3 µg/L tPAH50, geometric mean). Corals can respond in a limited number of ways to xenobiotic exposure. Retracting polyps in response to irritants such as crude oil closes off the gastrodermal cavity, possibly delaying or limiting effects of toxic compounds on the gastrodermal cells not protected by a mucus layer. Since coral species such as *P. damicornis* extend polyp tentacles as a normal heterotrophic feeding behavior, sustained polyp retraction can reduce feeding activity, and potentially nutrient intake. When the concentration of a contaminant reaches a threshold that overcomes these natural defense mechanisms, biochemical and cellular processes break down, which can result in tissue degradation.

We also observed that *P. damicornis* lost tissue in response to crude oil HEWAF exposure (EC_{10} = 202 µg/L tPAH50. While corals can recover from partial tissue losses (Kramarsky-Winter and Loya 2000), even small tissue lesions can have a negative impact on colony health by increasing chances of disease by opportunistic microorganisms (Page and Willis 2008). In the wild, tissue integrity changes likely affect feeding activity, increase susceptibility to disease or encourage algal overgrowth, and/or decrease photoprotective mechanisms due to chromoprotein or fluorescent protein loss (Lamb et al. 2014; Lirman 2001; Salih et al. 2000).

Results from this study and others indicate that corals elicit an innate protective response to oil exposure (Bak and Elgershuizen 1976; De Leo et al. 2016; Elgershuizen and De Kruijf 1976; Frometa et al. 2017; Lewis 1971; Reimer 1975; Renegar et al. 2016; White et al. 2012; Wyers et al. 1986). We observed significant negative gross health effects for *P. damicornis* in an acute 96 h static renewal test. Renegar et al. (2016) recently reported a health score EC_{50} value of 7442 µg/L tPAH50 for *P. divaricata* exposure using a single PAH analyte, 1-methylnaphthalene. We can make no direct comparison with our study due to differences in health metric reporting, PAH composition and exposure duration, however. *Porites divaricata and P. damicornis* are both ‘weedy’ coral species, often found in disturbed environments or when reef species complexity is low (Darling et al. 2012; Newman et al. 2015; Smith et al. 2013). Increased relative sensitivity of *P. damicornis* compared to other corals has been

demonstrated for both natural and anthropogenic stressors, however (Reimer 1975, Te et al. 1998; Stimson et al. 2002; Downs et al. 2016). Our method of static renewal (vs continuous flow) also may have contributed to increased toxicity of crude oil to *P. damicornis*. As the 12 h static renewal mimics tidal ebb and flow in shallow marine waters, it suggests that the action of tidal forces to move oil slicks back and forth over a coral reef may increase detrimental effects of oil exposure to stony corals.

Oil effect on tissue regeneration

Tissue regeneration in *P. damicornis* was significantly affected in a 96 h HEWAF exposure at all tested doses (as low as 12 µg/L tPAH50), demonstrating that it is a particularly sensitive quantitative assay for coral toxicology evaluations. Corals are susceptible to wounds from a variety of natural (e.g., fish bites, storms, disease) and anthropogenic (e.g., boat groundings, diver interactions, pollution) causes and regeneration of tissue over bare skeleton requires significant energy input. Wound healing has been used previously as a field assessment tool to gauge coral condition (Dustan et al. 2008; Fisher et al. 2007; Moses and Hallock 2016). Coral colonies that cannot undergo lesion repair are susceptible to infection, infiltration by boring organisms or algal overgrowth, often leading to poor reproductive output, slowed growth and death (Meesters and Bak 1993; Rinkevich and Loya 1979). Kramarsky-Winter and Loya (2000) first demonstrated how tissue repair is dependent upon intrinsic (size, reproductive state) and extrinsic (season, water temperature) factors by comparing field and laboratory wound healing rates. We have shown that this assay is amenable to laboratory toxicology studies with fast-growing, branching corals, which are easily fragmented. Slower-growing corals, such as *Orbicella, Dendrogyra,* or *Diploria* spp., may not be easily wounded, nor exhibit enough tissue regrowth to be utilized in shorter timeframe toxicological studies using this assay, however.

While the precise mechanism of oil toxicity is not well understood, we know that corals can bioaccumulate hydrocarbons (Burns and Knap 1989; Jafarabadi et al. 2018; Ko et al. 2014; Peters et al. 1981; Sabourin et al. 2013) and uptake of these hydrophobic compounds can result in disruption of cellular biochemical and physiological processes. Exposure to oil is linked with a decrease in lipid biosynthesis (Cook and Knap 1983) and disruption of cellular protein production (Rougee et al. 2006), thus inhibiting cell membrane biosynthesis. Peters et al. (1981) observed that crude oil exposure impaired development of reproductive tissues and resulted in atrophied muscle bundles, further evidence that hydrocarbon exposure negatively impacts coral tissue growth. Combined with a possible reduction in fixed carbon availability or energy reserves from photosynthetic processes, we would expect oil-exposed corals to have greatly decreased capacity for tissue regeneration compared to unexposed corals.

Time-dependent toxicity of Louisiana sweet crude oil to coral

To examine the effect of exposure duration, coral fragments were challenged with a single concentration HEWAF load over three short time periods (6-24 h), representing possible scenarios during an oil spill. We also included one longer exposure of 96 h for comparison to the 96 h dose-response experiment. Corals in the 6 h treatment duration were exposed in total darkness, representing the most conservative estimate of oil effects. We selected a median oil load (62.5 mg/L) from the 96 h dose-response

experiment, resulting in environmentally relevant tPAH50 concentrations. PAH loss is relatively rapid due to volatilization or degradation, thus at 6 h, the tPAH50 geometric mean of fresh and spent treatment solutions was higher than at 12 h. For example, our calculated tPAH50 dose for the 6 h crude oil exposure was 266 µg/L, while the 12 h duration resulted in an exposure of 189 µg/L tPAH50. Treatments longer than 12 h were dosed repeatedly (static renewal) at 189 µg/L tPAH50.

We found that 12 h and 96 h durations of HEWAF exposure significantly affected photosynthetic quantum yield in *P. damicornis* symbionts. We observed no significant effect to chlorophyll fluorescence with either the 6 h or 24 h oil exposure. Variable numbers of symbionts in each coral fragment or varying amounts of chlorophyll *a* in each algal cell may explain these results, especially if 189 µg/L tPAH50 is at the lower limit for chlorophyll fluorescence effects. Additionally, the timing of the 6 h oil exposure may have resulted in symbiont recovery prior to the PAM fluorometry evaluation, particularly since this treatment was not exposed to light and oil, concurrently.

Recovery of chlorophyll *a* fluorescence was observed within 24 h after placement in fresh ASW for the fragments exposed to oil for 12 h and coral fragments in the 96 h exposure recovered after 34 h in ASW, demonstrating resilience of *P. damicornis* algal symbionts to shorter-term oil exposures. Similar quick recoveries of photosynthetic effects have been observed with placement in fresh seawater following short-term (8-48 h) hydrocarbon exposures (Cook and Knap 1983; Jones et al. 2003). Cook and Knap (1983) postulated that had damage occurred to cellular membranes, it would take much longer to recover and proposed that the most likely explanation of the decrease in photosynthetic output was due to interference with enzymatic processes. They did not measure chlorophyll or chlorophyll fluorescence, however, and admitted that the loss and resynthesis of chlorophyll *a* could be another likely explanation. Jones et al. (2003) observed reduced chlorophyll fluorescence (Fv/Fm) from freshly-isolated symbionts (1 x 10^7 cells/mL) exposed to metal- and hydrocarbon-contaminated water and gradual recovery was observed for the algal cells following placement in fresh seawater. The results from our experiments and these works support a theory of oil destruction of photosynthetic pigments. Further studies should incorporate chlorophyll measurements and enumeration of algal symbionts to provide a more definitive answer to this question.

Pocillopora damicornis tissue integrity and tissue color were not significantly affected by 6-24 h oil treatments. Timing of the health data collection (2:00 pm daily, mid-light cycle) did not coincide with HEWAF exposures for the 6, 12 and 24 h treatments, which may have resulted in underreported effects. Additionally, reduction in tissue color scores for all *P. damicornis* fragments over time may have resulted in the observed ‘recovery’ of the 96 h treatment duration after 209 h (Figure 6, Panels B, E).

Polyp behavior was significantly affected by oil exposure for all treatment durations and was the principal driver of decreased health scores. This was not unexpected, since polyp retraction has been reported following coral exposure to various types of hydrocarbons (DeLeo, et al. 2016; Reimer 1975; Renegar et al. 2016; Ruis-Ramos et al. 2017). Recovery for the 6-24 h treatments was within 53 h (Figure 6, Panel C). Coral fragments subjected to the 96 h HEWAF exposure exhibited severe polyp retraction at

89 h, mirroring effects noted in the 96 h dose-response experiment, and polyp retraction continued throughout the recovery period. As mentioned above, bioaccumulation of hydrocarbons has been reported for oil-exposed corals (Burns and Knap 1989; Jafarabadi et al. 2018; Ko et al. 2014, Peters et al. 1981; Sabourin et al. 2013) and hydrocarbons can remain in coral tissues weeks after placement in clean seawater (Peters et al. 1981). The continued polyp retraction for the longest oil exposure duration may result from PAH accumulation in the lipophilic coral polyp tissues. Polyp behavior provided a key visual clue to decreasing coral health and this health metric took days to recover to the level of the controls once the stressor was removed.

The ability to regenerate tissue over wounded areas was inhibited following 6 h of crude oil exposure (266 µg/L tPAH50) to pre-wounded *P. damicornis* fragments, demonstrating that significant health impacts do occur with short-term oil exposures. The lower tPAH50 dose (189 µg/L tPAH50) represented in the 12-24 h crude oil exposures also impeded *P. damicornis* coral tissue regeneration. Recoveries for all short-term exposures were delayed for 48-84 h after transferring fragments to fresh ASW. Complete recovery did not occur for *P. damicornis* following 7 days in fresh ASW for the fragments treated with crude oil HEWAF for 96 h. This result is not unprecedented. Johannes et al. (1972) observed complete tissue breakdown for several coral species exposed directly to crude oil for 1.5 h, with no signs of regeneration on the oil-affected parts up to 4 weeks later. It is not clear if a longer recovery time would result in total recovery for these fragments, or eventual death would occur. While there has been a recent report of polyp rejuvenescence in presumably totally ‘dead’ corals following a bleaching event (Kersting and Linares 2019), significant coral tissue loss from oil exposure may be more difficult to repair, since bioaccumulated oil may continue to alter normal cellular processes long after the environmental exposure has ended.

Significantly decreased tissue regeneration was observed for intact fragments exposed to moderate tPAH50 levels and wounded in the recovery phase, indicating that oil is not simply affecting abraded tissue. This is further evidence that crude oil has effects at the cellular or subcellular level as detailed previously. The implications of oil damage to corals are lowered resistance to disease or predation and possible colony death, with increased negative impacts predicted for corals with lesions prior to exposure.

Conclusion

Following the DWH oil spill, measured tPAH50 concentrations in the 0-10 m depth (shallow water coral habitat) ranged from undetectable to over 100 µg/L, with the highest concentration observed in surface waters (0-1 m), 10-20 miles northwest of the wellhead (Boehm et al. 2016). Crude oil was released from the site for nearly three months, significantly damaging natural resources across many species extending hundreds of kilometers from the release site. Total PAH50 concentrations in our study were between 12 and 945 µg/L, with coral and zooxanthellae effects well within reported environmental levels for this event. The toxic responses of *P. damicornis* to the 96 h oil exposure are in the same range (low ppb) found for other marine organisms such as fish, shrimp and echinoderms (Esbaugh et al. 2016; Hemmer et al. 2011; Incardona et al. 2014; Neff et al. 2000; Stieglitz et al. 2016). We emphasize that care should be taken when comparing studies, since the varying analyte composition in different oil types

may result in altered toxicity profiles. This is important especially when using the data in risk assessments. We note that our study results likely underestimate oil effects due to the omission of UV and the nature of the HEWAF (containing small droplets, which may not be bioavailable). Our work shows that short exposure times with moderate tPAH50 levels can negatively affect *P. damicornis* and its symbionts, providing evidence that both acute and longer-term (chronic) PAH exposures in the ppb range can impair coral health. Finally, we demonstrated that coral tissue regeneration is a very sensitive indicator of petroleum hydrocarbon exposure. With appropriate controls, wound healing could be used in field assessments to determine impacts of oil exposure to corals.

We have limited our initial investigation to one species and one life stage and have used standardized laboratory settings (e.g., artificial light without UV, artificial seawater, etc). Photo-enhanced oil toxicity has been reported for several species of marine organisms (Alloy et al. 2016; Alloy et al. 2015; Boese et al. 1998; Newsted and Giesy, 1987; Oris and Giesy Jr, 1987; Sweet et al. 2017) and coral larvae (Negri et al. 2016; Nordborg et al. 2018), however. We would expect that under field conditions (e.g., UV light) that oil toxicity thresholds for *P. damicornis* would be even lower than those reported in this work. Further studies should include additional species representing other distinctive morphologies, environmentally relevant levels of UV, as well as gametes or larvae, as early life stages often exhibit higher sensitivity to xenobiotic stressors.

Continuing Research

Rationale: Coral taxa vary in many of their life history characteristics (i.e., size, morphology, physiology, growth rates, reproduction, genetics, symbiont type) and requirements of their environment. Shape and size of a coral species influences how they interact with their environment and as well as their biological and ecological processes (Zawada et al. 2019). The work presented above in this briefing report used *Pocillorpora damicornis*, which is finely branched (corymbose) with a bushy-shaped colony growth form. Since the objective of our work is to provide toxicity thresholds for use in natural resource damage assessments, it is important to include other species to evaluate whether or not responses are consistent across species. We selected a slow-growing, massive species, *Orbicella faveolata*, with dome-shaped mounds, commonly called the mountainous star coral, native to the Atlantic, Caribbean and Gulf of Mexico. An added benefit for NRDA is that *O. faveolata* is listed as threatened under the U. S. Endangered Species Act (Federal Register 2014).

We have conducted similarly designed experiments as described for *P. damicornis.* . Due to the growth form of *O. faveolata,* this set of experiments required significant technique development to create a wound that was reproducible across replicates. Ultimately reproducible wounds were created with a 2 mm diamond drill bit using a rotary tool (Dremel) affixed to its drill press accessory. Data analyses and manuscript preparation for these experiments are ongoing.

Preliminary results with *Orbicella faveolata*

96 h Dose Response Exposure

1. *Orbicella faveolata* were more resistant to Louisiana sweet crude oil:seawater HEWAFs than *P. damicornis* across the same tested concentration range.
2. Maximum quantum yield (Fv/Fm) for *O. faveolata* was affected by oil exposure only at the highest tested concentration (874 µg/L tPAH50).
3. Tissue regeneration was significantly reduced at exposures of 203 µg/L tPAH50and higher.
4. Fragments exposed to the highest concentration (874 µg/L tPAH50) did not regenerate tissue and some replicates lost tissue.
5. Oil-exposed *O.faveolata* healed more slowly than the control but was faster than *P. damicornis*.
6. The health score declined with increasing oil concentration, largely due to polyp retraction, but not to the same extent as *P. damicornis*.

Pulse-chase Exposures

1. *O. faveolata* fragments were less affected by oil than the *P. damicornis* fragments.
2. Tissue regeneration was mildly affected by oil exposure and recovery was observed.
3. Health scores were similar to those of *P. damicornis*. Polyp behavior was the principal driver of decreased health scores.

Data Availability

Data are published in May, L.A., Burnett, A.R., Miller, C.V., Pisarski, E., Webster, L.F., Moffitt, Z.J., Pennington, P., Wirth, E., Baker, G., Ricker, R., Woodley, C.M. 2020. Effect of Louisiana sweet crude oil on a Pacific Coral, *Pocillopora damicornis*. *Aquatic Toxicology*. 222: 105454. https://doi.org/10.1016/j.aquatox.2020.105454

Pocillopora damicornis oil exposure data has been submitted to the NOAA Office of Response and Restoration for incorporation into the Chemical Aquatic Fate and Effects database https://cafe.orr.noaa.gov/

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Supporting Documents

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Table 1. Coral health score rubric. Maximum cumulative score for a healthy coral is 15: five points for color, five points for tissue integrity and five points for polyp extension.

Score	Polyp Behavior	Color	Tissue loss (%)
5	All fully extended	Normal, 100% color	0
4	Fully extended, few withdrawn	25% color loss	1-25
3	Polyps extended 50%	Pale, 50% color loss	26-50
2	Polyps extended 10%	75% color loss	51-75
1	No polyps extended	Bleached, with pale areas	76-99
0	Polyp bailout	Totally bleached	100

Table 2. Reported effect concentrations for the 96 h HEWAF dose-response exposure using fresh (time 0) treatments or the geometric mean (Geomean) of fresh and 12 h spent treatment solutions (units = µg/L tPAH50). C. I. = 95 % confidence interval, Fv/Fm = maximum quantum yield, EQY = effective quantum yield, Tissue reg. = tissue regeneration, Tissue integ. = tissue integrity, Polyp retract. = polyp retraction.

Endpoint	Fresh EC_{10} (C. I.)	Fresh EC_{50} (C. I.)	Geomean EC_{10} (C. I.)	Geomean EC_{50} (C. I.)
Fv/Fm (82 h)	**1193** (1051-1355)	**3804** (3575-4049)	**330** (292-372)	**913** (862-968)
EQY (92 h)	**632** (472-847)	**1640** (1429-1882)	**176** (13-234)	**428** (375-488)
Tissue reg. (96 h)	**7** (1-52)	**29** (15-57)	**3** (0.4-16)	**10** (6-19)
Tissue integ. (89 h)	**711** (431-1174)	**3296** (2595-4187)	**202** (128-319)	**806** (649-1000)
Tissue color (89 h)	**111** (15-853)	**3820** (1741-8385)	**36** (5-234)	**926** (450-1907)
Polyp retract. (89 h)	**8** (2-23)	**84** (50-139)	**3** (1-9)	**27** (17-43)

Figures and Figure Legends

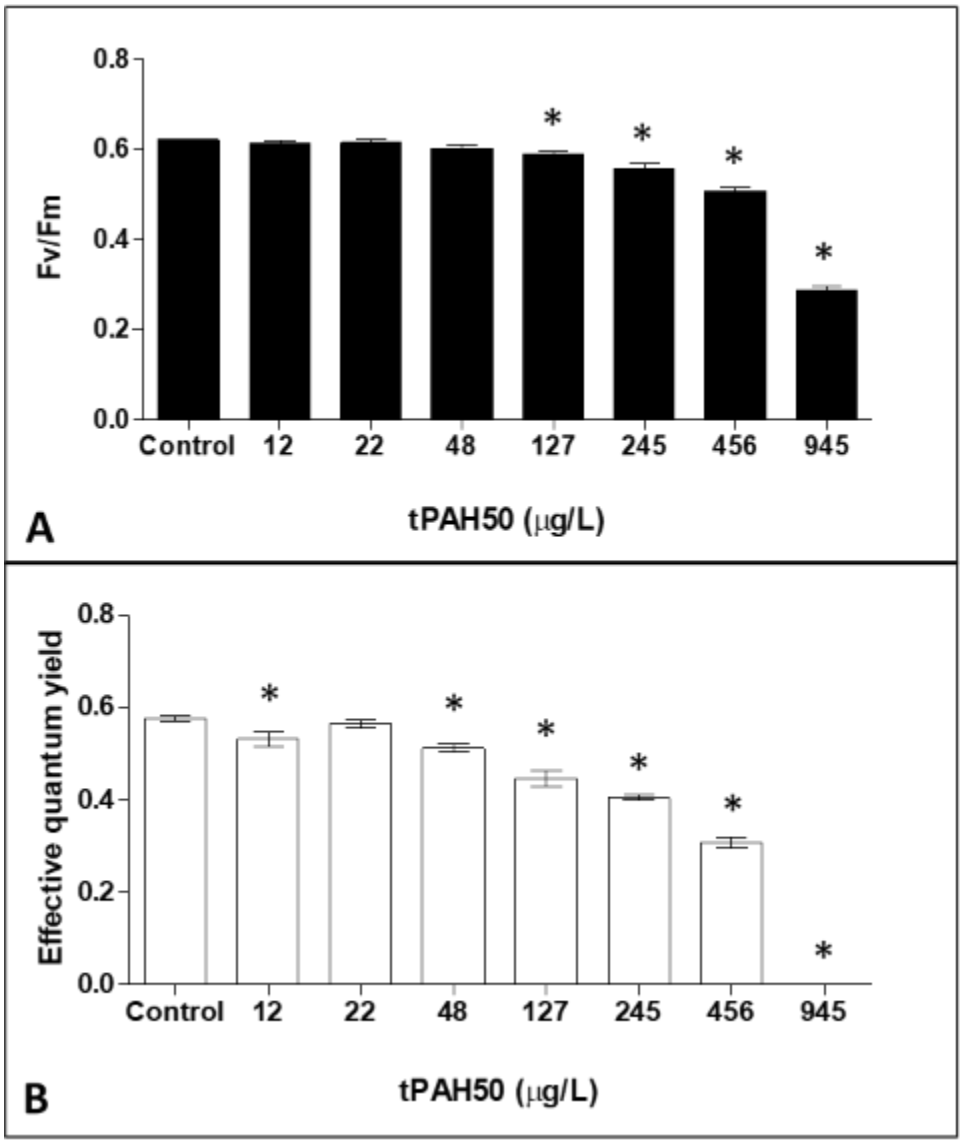

Figure 1. *Pocillipora damicornis* symbiont chlorophyll *a* fluorescence measurements following crude oil exposure. Panel A: dark-adapted maximum quantum yield after 82 h; Panel B: light-adapted effective quantum yield after 92 h. Total PAH50 (tPAH50) is reported as the sum of 50 polycyclic aromatic hydrocarbons. Maximum quantum yield (Fv/Fm, panel A) was reduced significantly (indicated by *) for treatments of 127 µg/L tPAH50 and higher after 82 h of exposure ($p<0.05$). Effective quantum yield (EQY, panel B) was significantly lower ($p<0.05$) following exposure with 12 µg/L tPAH50 for 92 h. Effective quantum yield was zero for the 945 µg/L tPAH50 treatment. Total PAH50 values are reported as the geometric mean of fresh and spent treatment solution (refreshed every 12 h).

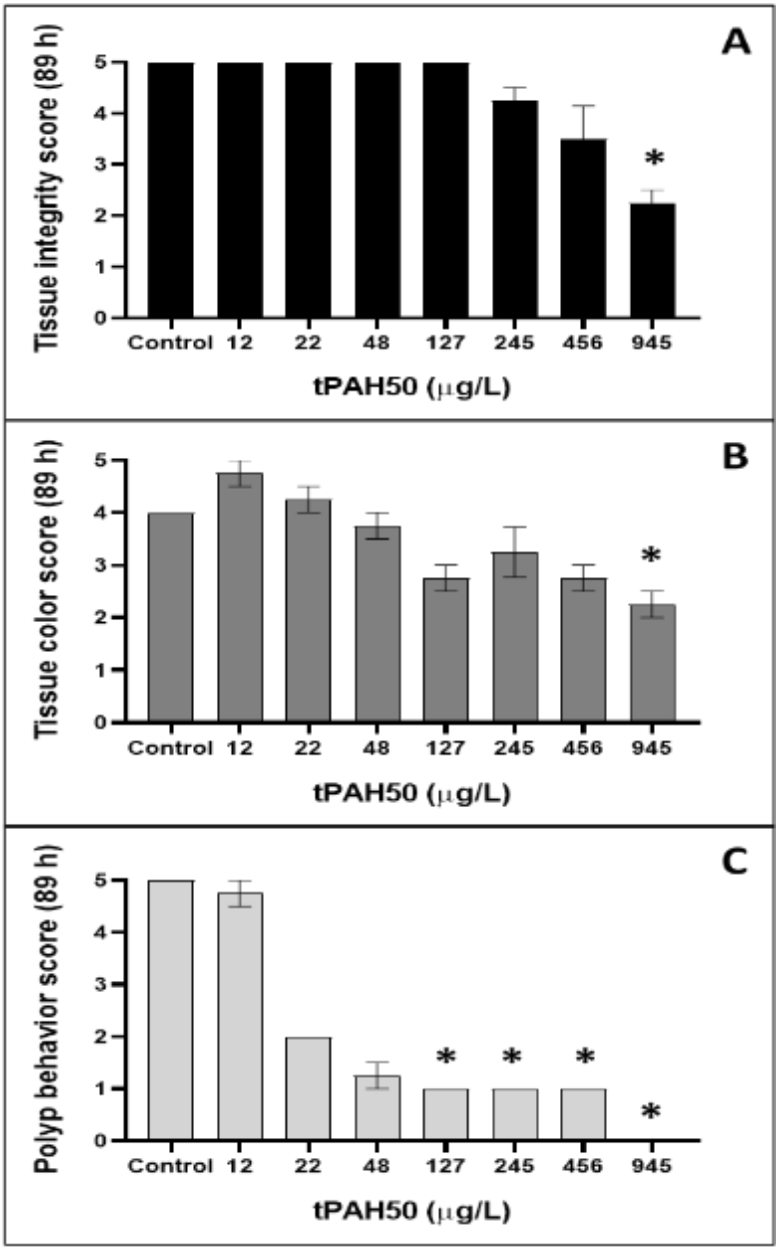

Figure 2. *Pocillopora damicornis* health status effects after 96 h HEWAF exposure. Oil treatments are reported as the sum of 50 polycyclic aromatic hydrocarbons (tPAH50). Panel A: tissue integrity metric, Panel B: tissue color metric and Panel C: polyp behavior metric. Treatments marked with an asterisk (*) were significantly different from the artificial seawater control at $p<0.001$. Concentrations of tPAH50 are reported as the geometric mean of fresh and spent HEWAF treatment, which was refreshed every 12 h.

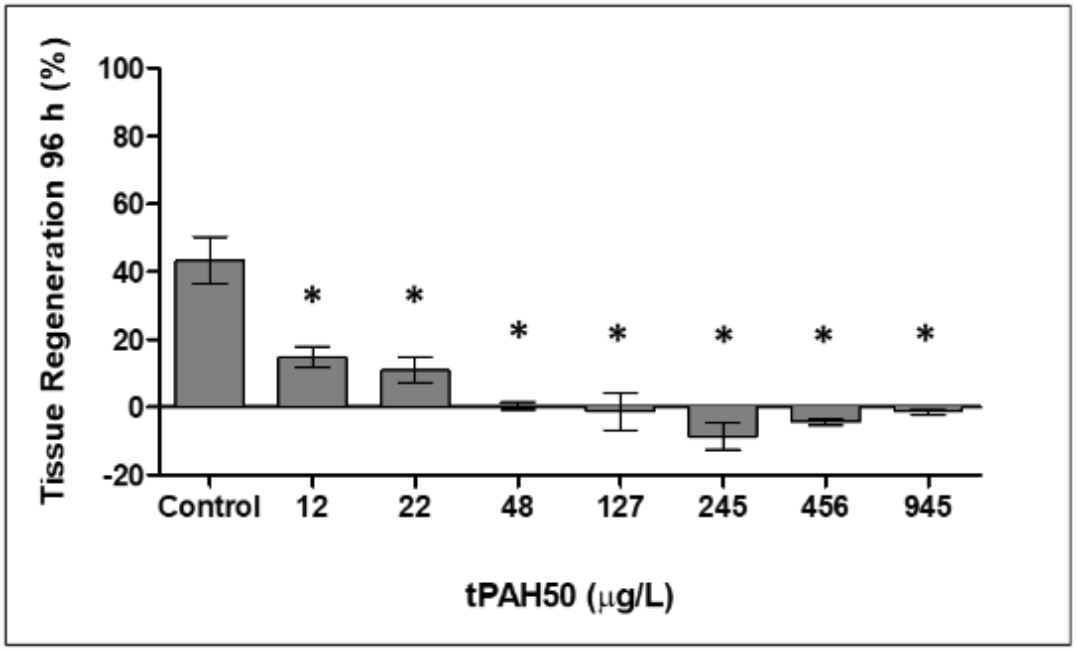

Figure 3. Percent tissue regeneration for *Pocillopora damicornis* following 96 h HEWAF exposure. Oil dose is reported as the sum of 50 polycyclic aromatic hydrocarbons (tPAH50). Treatments designated with an asterisk (*) were significantly different from the control ($p<0.005$). Total PAH50 values are reported the geometric mean of fresh and spent treatment solution (refreshed every 12 h).

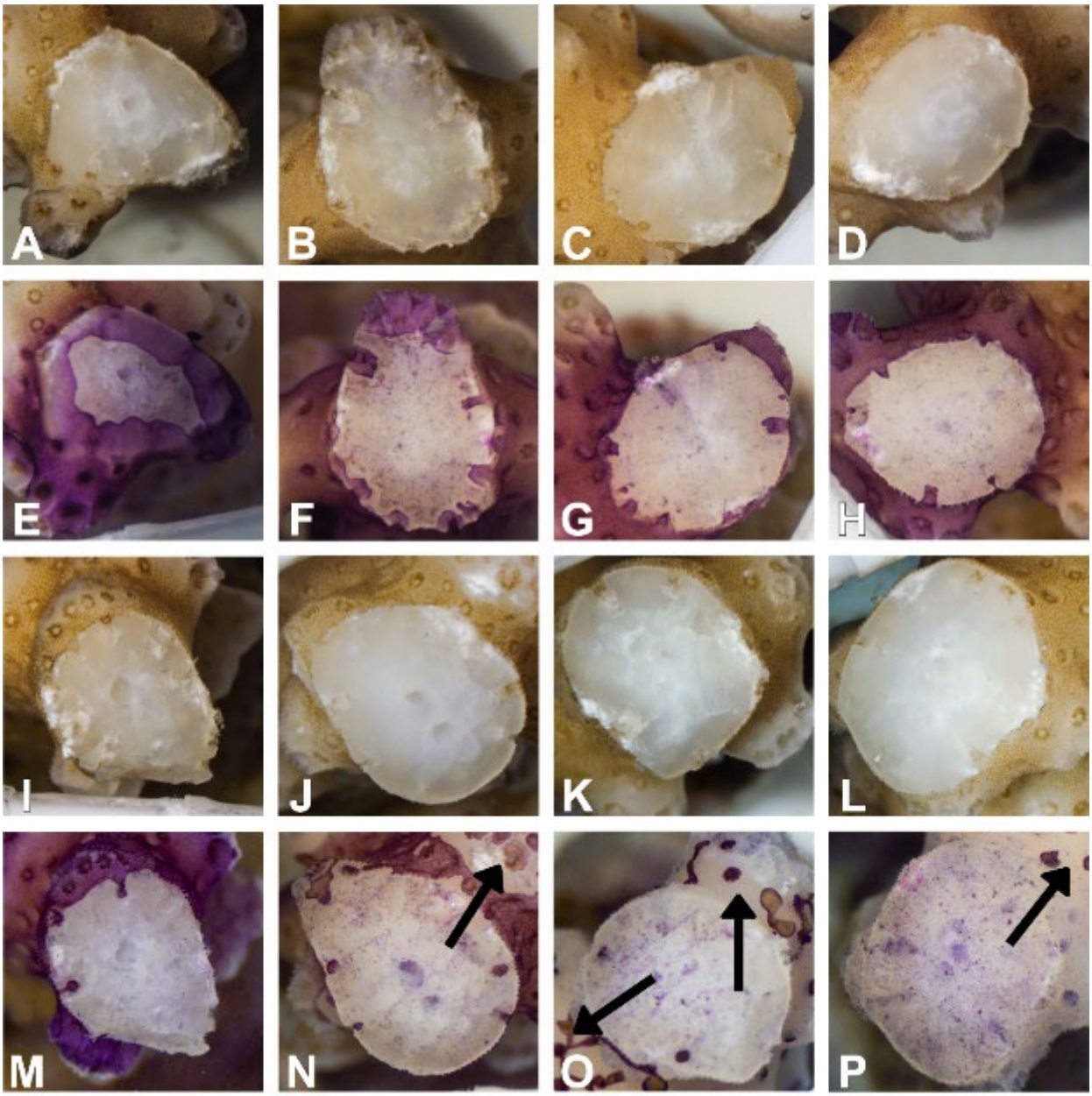

Figure 4. *Pocillopora damicornis* tissue regeneration images. Images are shown at time 0 following initial cut (Panels A-D and I-L) and after 96 h treatment, stained with toluidine blue O (Panels E-H and M-P). Panels A and E = ASW control, panels B and F = 12 µg/L tPAH50, panels C and G = 22 µg/L tPAH50, panels D and H = 48 µg/L tPAH50, panels I and M = 127 µg/L tPAH50, panels J and N = 245 µg/L tPAH50, panels K and O = 456 µg/L tPAH50 and panels L and P = 945 µg/L tPAH50**.** Areas of tissue loss for treatments at and above 245 µg/L tPAH50 are indicated by arrows in panels N, O and P. Total PAH50 values are reported as the geometric mean of fresh and spent treatment solution (refreshed every 12 h).

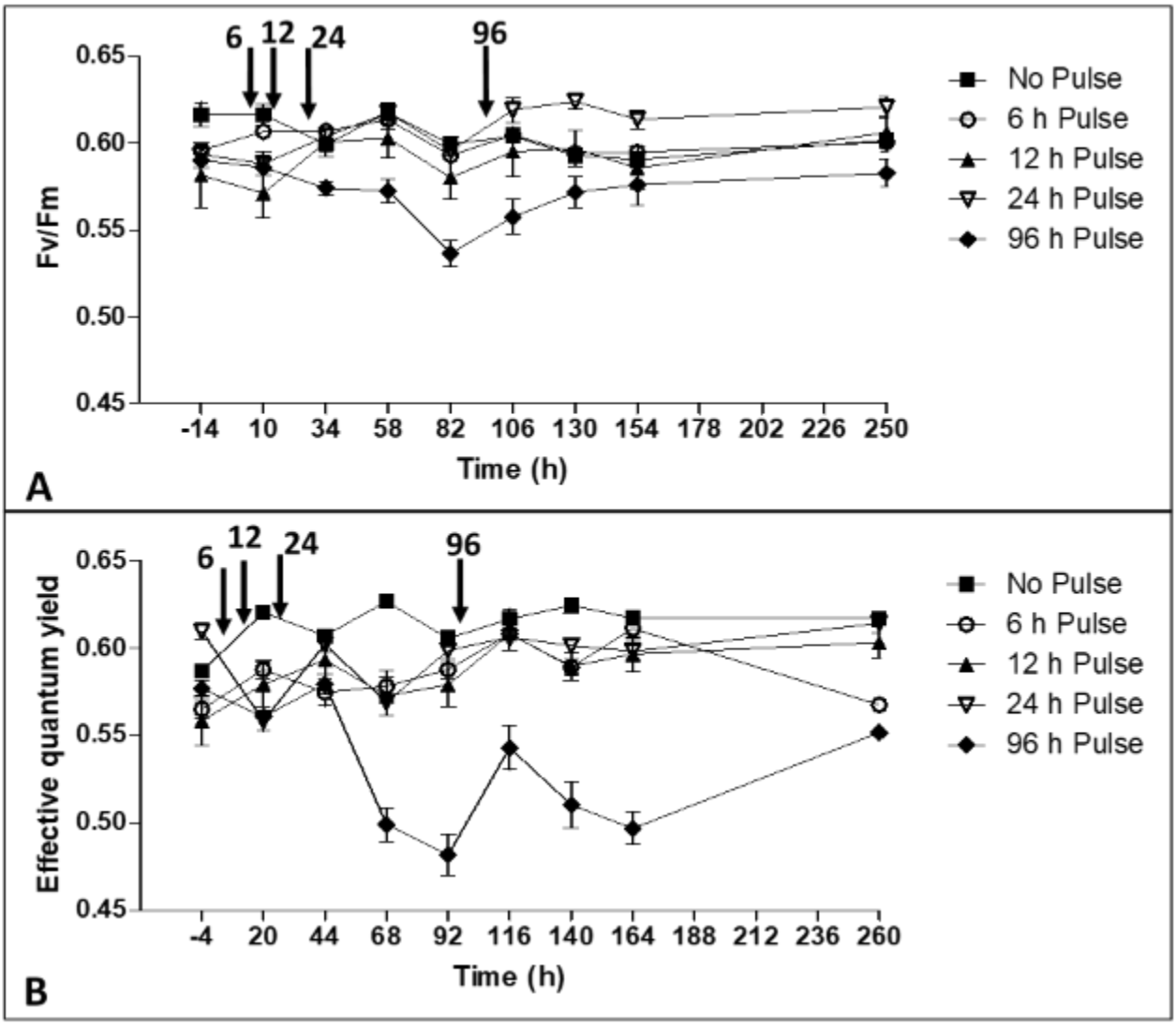

Figure 5. Chlorophyll *a* fluorescence measurements of *Pocillopora damicornis* algal symbionts following short-term crude oil exposure. The HEWAF exposure durations of 6, 12, 24 and 96 h (indicated by arrows) contained 266 µg/L tPAH50 (6 h duration) or 189 µg/L tPAH50 (12, 24 or 96 h duration) (geometric mean of fresh and spent HEWAF treatment). Maximum quantum yield (Fv/Fm, evaluated two hours before the light cycle began) was reduced significantly ($p<0.05$) for the 12 h and 96 h pulse durations at the 10-h time point (Panel A). No significance was observed for Fv/Fm in the 6 h and 24 h pulse durations. Fragments exposed to a 12 h pulse recovered Fv/Fm within 24 h after treatment ended and fragments exposed for 96 h recovered to the level of the controls within 34 h post treatment (130 h time point). Effective quantum yield (EQY, measured two hours before light cycle ended) for all oil pulse durations was impacted significantly compared to controls at the 20 h time point (Panel B, $p<0.05$). The EQY for 6, 12 and 24 h pulse exposures recovered to the level of the controls by 92 h. The 96 h pulse duration was significantly different from the control up to 68 h post treatment and recovered by the end of the experiment (260 h).

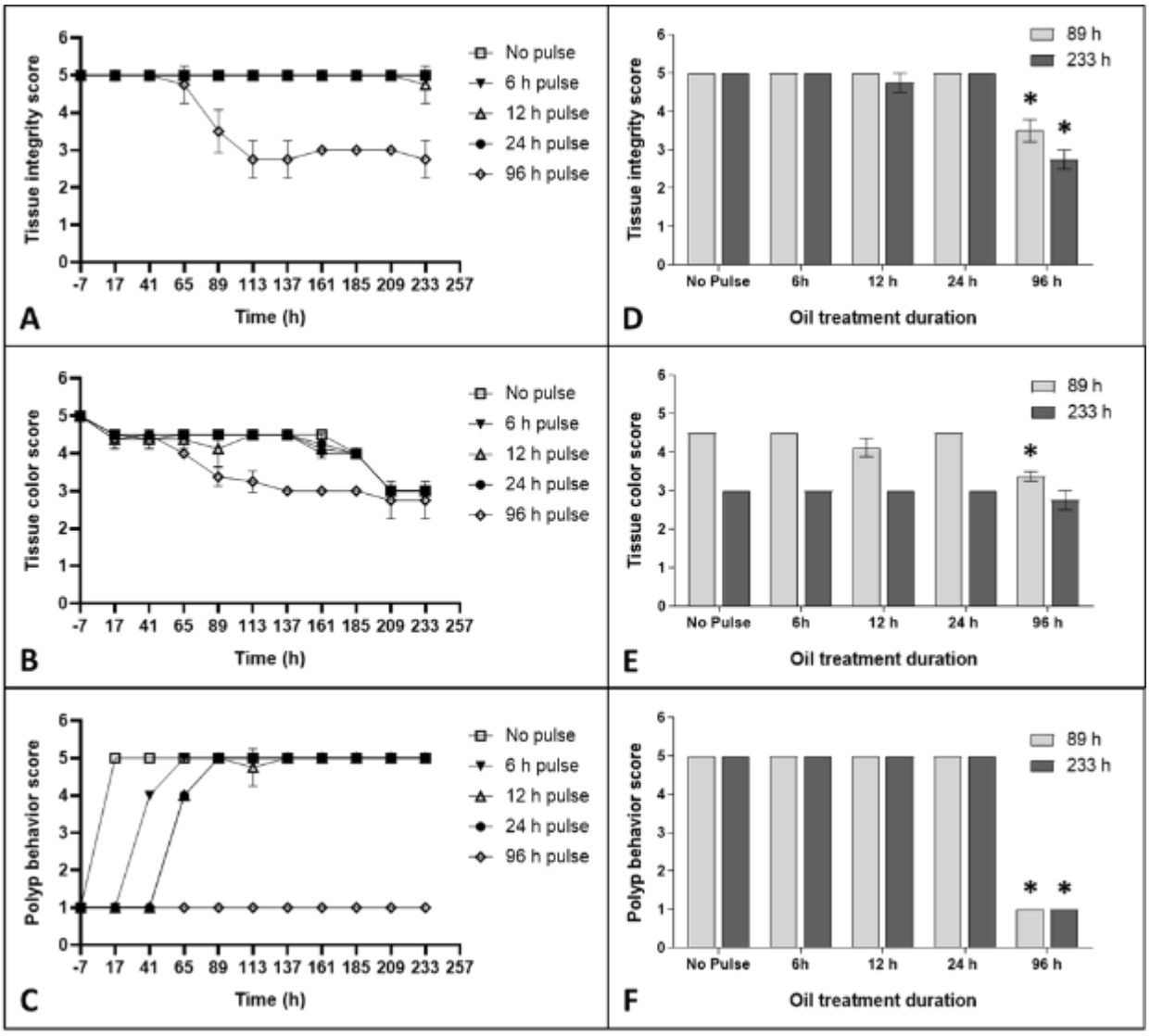

Figure 6. *Pocillopora damicornis* health status scores following short-term HEWAF pulse durations (189-266 µg/L tPAH50, refreshed every 12 h). Coral fragments were evaluated at 2 pm daily. Initial evaluation at -7 h included polyp retraction for all treatments following image analysis. Tissue integrity (Panels A and D) and tissue color (Panels B and E) were not affected by short-term (6-24 h) oil exposures. Coral fragments exposed to HEWAF for 96 h lost tissue and did not recover to the level of the controls after one week in fresh artificial seawater (Panel D, * = significant at $p<0.05$). Tissue color for the 96 h treatment duration was significantly reduced (indicated by *, $p<0.05$) from 65 to 185 h (Panel E). All treatments (including controls) had reduced tissue color scores after 185 h. Polyp behavior was affected significantly by all treatment durations (Panels C and F). Recovery for the 6 h (266 µg/L tPAH50), 12 h and 24 h (189 µg/L tPAH50) exposures occurred within 53 h after treatment ceased (Panel C). The 96 h pulse (189 µg/L tPAH50) exposure had significantly reduced (indicated by *, $p<0.05$) polyp behavior at 89 h and at 137 h post treatment (233 h) time point (Panel F). Total PAH50 is the geometric mean of fresh and spent (6 or 12 h) HEWAF solutions.

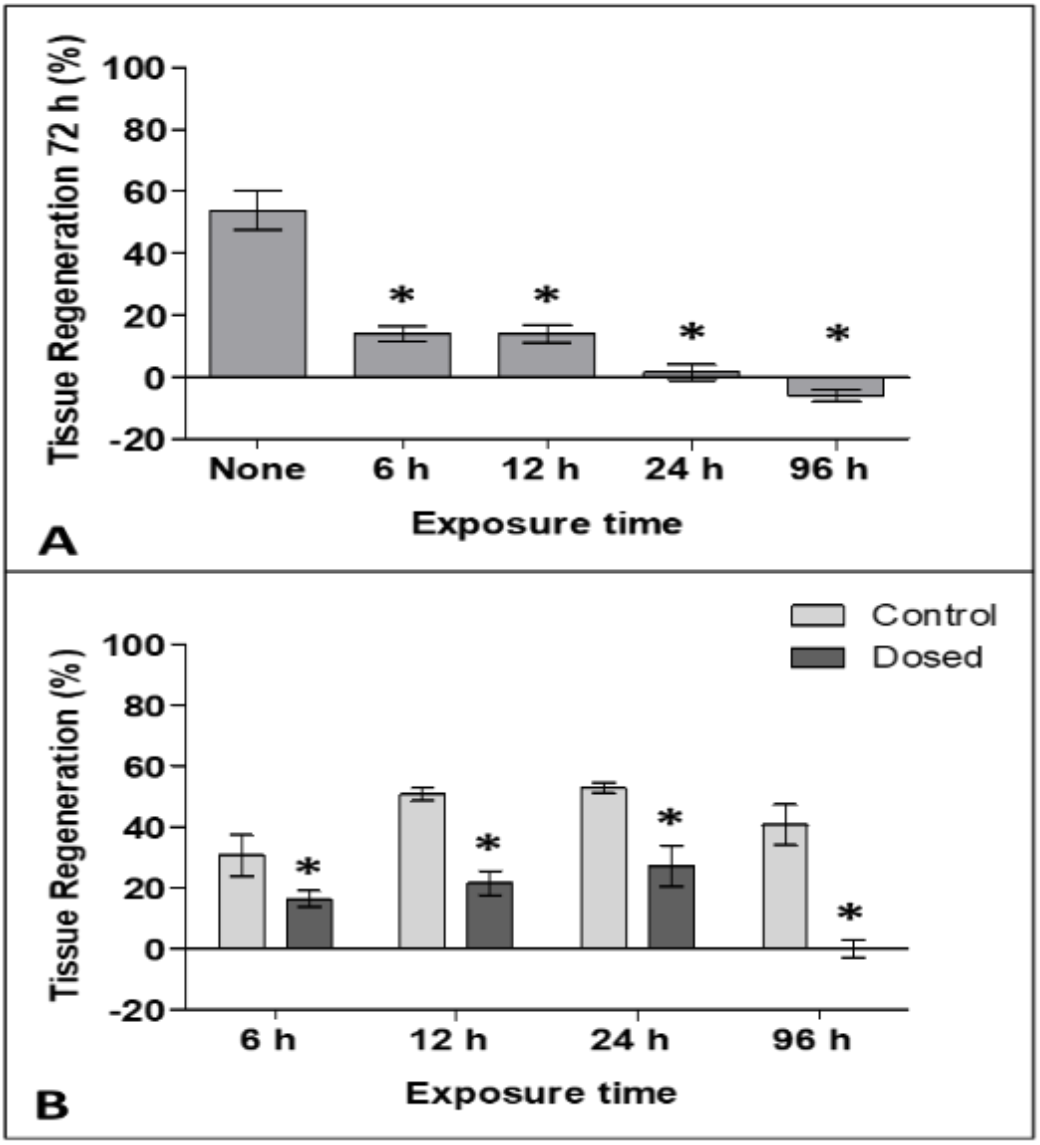

Figure 7. Percent tissue regeneration for *Pocillopora damicornis* fragments after short-term HEWAF exposures (189-266 µg/L tPAH50, refreshed every 12 h). Panel A: tissue regeneration for cut fragments exposed to HEWAF. At 72 h, all treatments had significantly reduced (*) tissue regeneration when compared to the artificial seawater control ($p<0.0001$). Fragments exposed for 96 h did not recover to the level of the controls by experiment termination (168 h post treatment). Panel B: Results of intact fragments exposed to HEWAF (cut and placed in fresh artificial seawater). Dosed and control treatments for each exposure time were imaged when controls for each treatment duration reached 30-50 % tissue regeneration (72-84 h post exposure). All dosed fragments exhibited significantly reduced ($p<0.05$) tissue regeneration when compared to the controls (indicated by *).

Investigations into the Effects of Contaminants on Coral and Coral Reef Health

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ABSTRACT

Corals and coral reef conservation, recovery, restoration and resilience are priorities for NOAA, NOS and our nation because of their social and economic value, the ecological services they provide, and as foci of biodiversity. The NCCOS Coral Health and Disease Program (CHDP) operates within the Key Species and Bioinformatics Branch with three major objectives: Strategic Research, Innovation and Discovery, and Capacity Building. Strategic Research encompasses our laboratory and field investigations that are mission oriented but issue driven by specific science needs. Our Innovation and Discovery efforts build on experience, skills and insights to construct new assays, adapt technologies for novel uses, and fabricate new systems that allow us to investigate issues from new or different vantage points to develop a more complete picture of the resource problem, its causes, and help devise innovative and effective solutions. We work to build capacity among students, stakeholders and resource managers by providing training, technology transfer, working with community groups to address reef-related issues, communicating science for public awareness or by offering educational opportunities. Our science addresses stressors and impacts from the perspective of an organism's health, akin to a medical diagnostic approach, rather than from the perspective of a particular contaminant or other type of stressor. We use this approach to discover underlying causes of impairments to the habitat or organism, for devising threat-reduction strategies, and protecting and restoring vulnerable coral reefs.

Program Goal
To bring relevant, science-based information to decision-makers and the public to assist them in selecting effective actions for reducing threats to our marine and coastal resources and helping to restore damaged ones.

INTRODUCTION

The NCCOS Coral Health and Disease Program focuses on characterizing the health of coastal resources and habitats, primarily in coral reef ecosystems, to aid in the recovery and protection of marine resources and their habitats. Therefore, our research focus is the health of the coral and understanding factors that impair their normal functioning. Coral reefs are prominent among the world's failing ecosystems. Catastrophic declines of more than 90 % in some areas (Jackson et al. 2014) have been attributed to local stressors such as overfishing, habitat degradation and pollution (Burke et al. 2011). Predictions indicate that all coral species will be threatened or endangered by 2050, without intervention (Burke et al. 2011). Our research investigations seek to uncover potential causes of impairments in coral reef resources that could be biological, physical or chemical in nature, and then work with resource managers and local NGOs to devise options for threat-reduction, recovery and protection of the resource.

The broad expertise (e.g., biochemistry, molecular and cellular biology (plant and animal), pathobiology, microbiology, assay development and saltwater aquaculture) and versatility of CHDP staff enable trans-disciplinary research studies with multiple coral species as well as other marine invertebrates and algae. Laboratory studies characterize the acute effects of chemical, physical and biological stressors, singularly or in combination. Threshold effects are determined from endpoints that range from gross observations (e.g., mortality, color, behavior) to sub-clinical biochemical, molecular, cellular and physiological changes. Since we work with non-model organisms, our bioassays have required adapting, modifying and validating from methods used for other species to determine nominal response levels and assay performance in our target species. Where possible our studies use standard methods (e.g., ASTM, OECD and US EPA). Techniques and methods used in the program are described in the Experimental Procedures and Reference sections that follow.

The CHDP staff also conducts field operations to address specific science needs of coastal managers in particular locations. Field missions commonly occur in managed or protected areas, such as the National Marine Sanctuaries or National Parks, but also in state or territorial jurisdictional priority-areas and internationally. Field operations are carried out in close collaboration with resource managers to formulate the nature of their problem and establish parameters of the study. In most cases, field missions are executed jointly, leveraging local expertise and logistics support (e.g., divers and boats). The particular issue drives a project, which can involve investigations that are site-specific assessments using toxicity bioassays and toxicity identification evaluation (TIE) protocols to characterize the quality of the water and sediments. When feasible, contaminant chemistry is used to assist in identifying candidates involved with an impairment. Histology and other molecular and cellular diagnostic assays are used to evaluate the condition of the receptor species and assist in diagnosing possible causes of anomalies. CHDP staff conducts diagnostic assays in-house, while staff in the Ecotoxicology branch or external collaborators assists with our environmental analytical chemistry needs.

EXPERIMENTAL PROCEDURES

A. Aquaculture of Research Specimens as a Renewable Resource

1. Background

The NCCOS Coral Health and Disease Program maintains a separate aquaculture space used explicitly to propagate research specimens as a renewable resource. Caribbean coral cannot be collected from the wild except under extensive permitting review and requires state and federal permits. Permitting agencies further scrutinize our Endangered Species Act (ESA)-listed coral holdings because the threatened status of these species also reflects exceedingly low demographics. Wild collections therefore incur further take from extant populations.

Corals are symbiotic, long-lived, slow growing species that have narrow tolerance limits in the wild that need to be replicated in captive culture. Each species can vary in their optimal requirements (or tolerances) for lighting (spectrum and quanta), temperature, water chemistry (nutrients, salinity), water circulation, food, and spatial densities. The aquaculture support for coral research is a long-term commitment that requires specialized skills and knowledge. We have four priority coral species supporting contaminant-related research: *Pocillopora damicornis, Orbicella faveolata, Acropora palmata* and *Acropora cervicornis.* The latter three are ESA-listed species. The facility cultures 15-20 other species of coral, invertebrates and macroalgae to support other aspects of the CHDP's research portfolio.

Advantages of cultivating coral research specimens:

- Ability to use ESA species in contaminant toxicity tests and other research can assist in recovery of the species.
- Optimized health conditions can be achieved by controlled water chemistry, lighting and water motion within custom life-support systems (as opposed to pulling specimens from compromised environments e.g., the FL Keys).
- Cultivating specimens under optimized conditions, serves to reduce signal-to-noise responses in sensitive cellular physiological and biomarker-type assays, thus aiding in improving assay sensitivity.
- Asexual propagation creates genetically identical clones, which reduces experimental variability that is commonly encountered when using wild specimens.
- Captive culture conditions provides naïve specimens.
- Technology now exists to create *ex situ* conditions for full-term gametogenesis in broadcast spawning corals.
- Early life-history and controlled laboratory experiments to investigate the effects of contaminants on coral reproduction are more feasible with ability for *ex situ* reproduction, allowing manipulation of exposures during the 8-9 months required for gametogenesis.

Caveats to consider when cultivating coral research specimens:

- Use of single genetic clones may not reflect responses of other genotypes within a species.
- Coral are non-model species and present challenges for standardizing test procedures and developing relevant test endpoints.
- Diagnostic assays require optimization for each new coral species to account for differences in nominal levels of response and verification of physiological mechanism.
- Interruption of facility utilities (electricity, water, sewer) can quickly compromise coral life-support systems leading to loss.

2. Coral life-support system

The systems housing grow-out of experimental specimens have been converted to glass and Teflon construction (from plastics, PVC and fiberglass) to reduce chronic low-level leachates that can affect sensitive response endpoints. The recirculating systems (ranging from 40-400 gallons) use artificial seawater (Tropic Marin) which required extensive testing of various sources and formulations of artificial salts during optimization of the systems. Large systems have refugia with deep sand beds and seeded liverock for biological filtration. Temperature is maintained at 26 ± 1 °C within a temperature-controlled room with fine adjustments made with in-water 300 W submersible glass heaters and ¼ HP chillers with titanium drop-in coils. System lighting is being converted to LED light fixtures (Radion XR30 G5 Pro) programmable to coral specific quanta and spectrum (from metal halide and compact fluorescent lighting) to accurately reflect lunar and seasonal cues. Depending on species irradiance ranges from 150-500 µmol photons m^{-2} s^{-1} with a 10 h:14 h light:dark cycle. Water circulation is driven by a Teflon (ETFE) lined pump (Iwaki) and Teflon (PTFE, PFA) piping. Calcium, alkalinity and trace element levels are regulated with custom-made glass calcium reactors via a manual drip system. Supports for coral nubbins are custom-made in-house from PTFE. Despite having photosynthetic symbionts, coral health is optimized with multiple heterotrophic feedings per week using small micron foods with elevated levels of highly unsaturated fatty acids.

3. Other research animals

Adult sea urchins for toxicity testing (i.e., embryo development or fertilization) are purchased commercially at this time. During an acclimation and depuration period, they are held in 40-gallon glass

aquaria filled with Tropic Marin artificial seawater (TMASW). The ability to aquaculture sea urchins for a readily available resource of gravid animals is a current limitation due to staffing and space limitations.

B. Field Sampling Techniques

1. Equipment Preparation

Cleaning of all glassware, Teflon sample vials, Teflon centrifuge tubes, and syringes is according to Chapman et al. (1995) including acid baths and final rinses with pesticide-free acetone. Similar methods are used for laboratory and field preparations. This video link: https://cdhc.noaa.gov/education/field_health.aspx demonstrates the step-by-step procedures used to perform water and sediment collections techniques for contaminant analysis in a uniform manner.

2. Seawater collection

- Bacterial water quality as an indicator of sewage contamination.

Prior to water collection sample bottles (1L) are sterilized by autoclaving or in the field with isopropyl alcohol, leaving residual alcohol in the bottles. At each site, sample handlers donned disposable nitrile gloves and while facing the current, rinse the bottle three times prior to sample collection to remove residual isopropyl alcohol. The bottles of natural seawater (three per site) are placed in an ambient cooler filled with seawater for the trip to the laboratory (no more than 2 h).

- Personal care product chemistry

To collect seawater samples for organic sunscreens and other personal care products, samplers (washed free of personal care products), don nitrile gloves prior to entering the water. Pre-cleaned amber glass bottles (1 L) are filled with seawater by placing the jar arm-length below the surface, opening the jar to fill and capping underwater. Samples are then acidified to pH 3 with 6N HCl. Within 6 h the 1 L of seawater is pre-filtered using a Buchner funnel and GF/F filter paper. The filtrate is then extracted using Oasis HLB 6cc, 500mg (Waters) cartridges. The columns are stored frozen (-40°C) until analyzed.

3. Sediment collection

- Porewater toxicity testing

Sediment samplers, free of personal care products, don nitrile gloves prior to entering the water. Sediment samples (~150 g) are collected from each site under permit by snorkelers or SCUBA divers from near-shore waters using the modified syringe method. A visual demonstration video of the method can be found at: https://cdhc.noaa.gov/education/field_health.aspx and described in Downs et al. (2011). All samples are placed on ice until extraction, within 4 h. Porewater (interstitial water in the sediment) is aspirated from sediment samples using a glass pipet and placed in a clean 30 mL Teflon centrifuge tube. Samples are centrifuged at 1200 x g for 20 min and clarified supernatant is transferred to a clean Teflon jars and archived at -80 °C until assay initiation.

- Metals and trace element analysis

The first few centimeters of sediment are collected using acid washed 50 mL polypropylene tubes. The tubes are immediately placed into a cooler on ice and then frozen at -20 °C until transferred to a -80 °C freezer until analysis.

4. Coral biopsies

Biopsies of coral tissue (1.5 cm^2) are collected by SCUBA divers using a stainless steel hammer and chisel or a punch biopsy technique with a leather punch. Biopsy tools are pre-cleaned with isopropanol and

ultrapure water (resistivity = 18 MΩ·cm; TOC < 5 ppb). Clean nitrile gloves are worn by the diver and a clean chisel or leather punch is used for each colony to prevent cross-contamination. Tissue biopsies can be used for chemical analysis (metals or organic body burden), histopathology, measurements of carbon and nitrogen stable isotopes (to evaluate exposure to terrestrial versus or oceanic sources), and to determine healing rates for site assessments. A video demonstration of the biopsy techniques is found at https://cdhc.noaa.gov/education/coral_assay.aspx.

C. Test methods for field assessments

1. Water Quality Methods

- Ammonia (total ammonia nitrogen, TAN) concentrations are verified for each experiment with 400 µL of fresh treatment solution using a colorimetric microplate assay based on a commercial (Red Sea, Houston, TX) kit. Ammonia standards for the assay were generated using 100 mg/L ammonia standard (Hach) in a two-fold dilution series (0.13-8.0 mg/L) in 35 ppt artificial seawater (ASW, Pro-Reef Sea Salt, Tropic Marin). Unionized ammonia is calculated using the equation from Bower and Bidwell (1978) and the measured ammonia nitrogen, temperature, salinity and pH of the sample.

- Dissolved oxygen and pH are measured using a Thermo Orion 5-Star multimeter.

- Salinity is determined using a saltwater refractometer, either manual or digital.

- Nitrite nitrogen concentration is verified with the USEPA diazotization method (#8507) using a microplate format for the Hach nitrite nitrogen assay kit.

- Total inorganic phosphate concentrations are determined by the ascorbic acid method using a Hanna Checker adapted to microplate format. Total phosphorus is calculated as a percentage of total inorganic phosphate (32.62%).

2. Sea urchin Toxicity Tests

Porewater toxicity testing is conducted following EPA standard method for sea urchin fertilization (EPA 2002, 2012) and the echinoid embryo toxicity assay (ASTM 2012). The methods of Carr and Chapman (1992) and Carr et al. (1996) are used to score embryo developmental anomalies. Water chemistry parameters are measured as described above to identify conditions that would confound the assay and amended where possible to bring them within the test tolerance.

We have modified the standard method of scoring normal and abnormal embryos by additionally scoring four categories of development: normal, underdeveloped, malformed and arrested development. This addition provides possible insights into how the developmental program is being affected and insights into the possible type(s) of contaminants or stressors that may be involved. Because sediment porewater samples are from coral reef environments, the procedures opted to use sea urchins associated with these habitats (e.g., *Lytechinus variegatus, Tripneustes gratilla*).

3. Bacterial Water Quality as Sewage Indicator

Enumeration of enterococci bacteria in marine waters is an EPA standard method as an indication of fecal pollution related sewage input. This parameter is used to determine if sewage-related

contaminants may be affecting the site(s) under investigation. EPA Method 1600 (2002) used in our studies is a membrane filter procedure that provides a direct count of the bacteria in a water sample by using a selective media (mEI) and incubating the filters at a selective temperature (41 ± 0.5 °C).

4. Bacterial Reverse Mutation Test (Ames test)

The Bacterial Reverse Mutation test (aka: Ames test) is conducted following the Organization for Economic Co-operation and Development (OECD) Guideline 471 (1997). The test is widely used as an initial screen to identify and characterize the mutagenicity of chemicals and drugs in basic research and as part of the safety evaluation for industrial products by regulatory agencies. We use a commercial source (Molecular Toxicology Inc.) of bacterial strains and reagents for this assay to screen chemicals. We are currently involved in developing a method for testing marine waters and sediment porewaters.

D. Laboratory toxicity testing for effects characterization

1. Adult coral

- Laceration regeneration assay

The premise of the laceration regeneration assay (aka: tissue regeneration or wound healing) is that regeneration rates is an integration of a number of physiological processes that are influenced by environmental stressors (physical, chemical or biological) and as such provides a useful indicator of adverse conditions. Technically the assay depends on photogrammetry of measurements from digital photomacrographs of lesions at varying time points and calculating either percentage of healing compared to a control or calculating linear wound healing rates according to Gorin et al. (1996).

This is a non-standard assay modified for laboratory testing based on a body of fieldwork summarized by Moses and Hallock (2016). Considerable technical development was required to optimize the assay for laboratory use. This included empirically determining for each test species: how to create wounds of appropriate and reproducible shapes and sizes; determining healing rates; photography set-up and settings; and appropriate vital stains to provide contrast for photographing transparent new tissue growth. This has proven to be one of our most sensitive bioassays. The field version of this assay and image analysis procedures are available in these two videos: https://cdhc.noaa.gov/education/coral_assay.aspx.

- PAM fluorometry

Shallow-water hard corals have obligate photosynthetic algal symbionts that respond to unfavorable conditions (e.g., chemical pollution, temperature stress, salinity extremes). Pulse-Amplitude Modulated fluorometry (PAM fluorometry) is a non-invasive technique for monitoring and assessing the photophysiological state (i.e., health) of the coral's algal symbiont (zooxanthellae) by measuring chlorophyll-a fluorescence as a proxy. We use a Walz Imaging PAM fluorometer to measure photochemical efficiency of photosystem II (PSII) in light- and dark-adapted states of the photosynthetic process in coral nubbins and algal cell cultures. Measurements of PSII provide an indication of the ability of PSII to process electrons in this vital physiological process.
A full explanation of the theory and practice of using PAM fluorometry in coral health assessments is provided by Ralph et al. (2016).

- Visual health assessment

Close visual inspections of test organisms is a critical first step in effects characterization. For coral, there are limited characters to score. Berzins et al. (2007) first established visual criteria for use in a

veterinary health certification process for the state of Florida. This was to support a growing coral aquaculture community. The assessment relied on two endpoints: condition (dead to no tissue loss) and color using an ordinal scale. We have modified this approach based on our experience culturing each of our test species into a three-parameter scoring rubric. The scoring criteria were refined to distinguish gradations of change in tissue integrity, tissue color and polyp behavior by May et al. (2020).

- Histology

Histology allows the examination of the microscopic anatomy (i.e., structure) of cells and tissue slices (4-6 µm thick) of fixed and paraffin embedded tissues that are visualized with either contrast or specialty stains. A differential from normal structure indicates likely dysfunctions. For example, histological sections of coral tissue can be examined to evaluate the number and condition of ova and spermaries to assess reproductive pathologies. We use light microscopy to determine if cellular pathologies exist for field assessments or are induced during laboratory exposure experiments.

2. Coral larvae

The ability to conduct reproductive and early life history exposure-response experiments is technically challenging and logistically demanding. Ideally spawning could be controlled *ex-situ*, however only one laboratory in the US, the Florida Aquarium, has successfully created *ex-situ* conditions resulting in sexual reproduction of Caribbean coral in captivity. Although this is a future objective, coral larval research is limited to wild-caught gametes. This requires extensive coordination and collaboration with field teams in Florida that can provide boats, sampling gear and SCUBA divers skilled in collecting gametes. During 2-4 nights after the full moon once or twice a year in August or September, colonies of a given species synchronously release gametes. The three species used in our research, *Acropora palmata*, *A. cervicornis* and *Orbicella faveolata* are simultaneously hermaphroditic broadcast-spawners. Ova and sperm are packaged into bundles that are released from gravid polyps. The bundles are positively buoyant allowing their capture in specially designed nets. Bundles are allowed to break, releasing ova and sperm that are subsequently either separated and washed or batch fertilized. In addition to colonies unable to self-fertilize, these species require genotypes that are sexually compatible for successful fertilization. Once gametes from compatible genotypes are available, assays to determine impacts of particular compounds or substances on fertilization efficiency can begin. Unlike the sea urchin fertilization test described previously, coral gamete fertilization can only be determined during the first few cell divisions that occur within 2-6 h. Embryos are fixed with either formalin or glutaraldehyde and scored for percent fertilization in the control vs treated samples. Larval exposures frequently use batch-fertilized (i.e., multiple genotypes mixed) larvae that are usually 24 h post fertilization, noting that such batch fertilization specimens do not contain synchronized embryonic stages at exposure initiation. Percent mortality is determined by placing defined numbers of larvae into exposure treatments and counting survivors (larvae will disintegrate once dead) at pre-determined exposure times. To increase timing and counting efficiency larvae are diluted to approximately 20-40 larvae per 50 µL and photographed, then the photomicrograph is used to enumerate larvae at assay initiation. Percent mortality is calculated from the difference between the initial number of larvae and the number surviving at assay termination. Treatment significance is based on comparisons with control samples.

3. Eukaryotic Cell-based assays

- Sperm cells

We primarily use cytotoxicity (cell death) assays when conducting stressor-effects experiments with sea urchin or coral sperm. Depending on the cell size, counting can be done manually, using a cell-counting

chamber (e.g., hemocytometer, Sedgwick-Rafter) or automated (e.g., cellometer). Live-dead fixable stains have been used successfully with sperm cells to discriminate between live and dead cells.

- Cultured coral symbiotic dinoflagellate algae

The CHDP maintains an ex-situ collection of algal cultures representing major species groupings of coral symbionts. These cell cultures are used in toxicity testing to determine effects on the algal component. Cell proliferation and cytotoxicity assays follow methods of Burtscher et al. (2016). Using an imaging pulse-amplitude modulated (PAM) fluorometer photophysiological impacts of stressors can be detected and measured in algal cell cultures (Ralph et al. 2016).

4. Biochemical-based assays https://cdhc.noaa.gov/education/protocols.aspx

Biochemical assays have the advantage of determining sublethal effects of various stressors on coral species. It is at these molecular and biochemical levels that direct effects of stressors (e.g, contaminants, pollutants, environmental factors etc) can induce structural and functional changes that can interpolate through hierarchical biological responses to effects that are manifested at organismal, population, community or ecosystem levels, ultimately resulting in significant ecological impacts. Measurement of impairments at these lower levels can provide early warnings and be diagnostic or prognostic indicators of injury or dysfunction of targeted receptor species. Often biochemical-based assays are more sensitive, less costly and less time-consuming to conduct compared with traditional toxicological exposure assays with whole animals.

- DNA damage- DNA damage assays (DNA AP site, Comet, Surveyor nuclease assays) evaluate insults to nuclear material from xenobiotic exposures. The CHDP has several molecular biologists on staff, with experience in adapting traditional human-based assays for coral diagnostic evaluations.
  - DNA AP-site: This assay identifies a site in the DNA strand that is missing a nucleotide base (apurinic/apyrimidinic site). The method utilizes an aldehyde reactive probe tagged with a biotin residue. The sites can then be quantified by incubating samples with a streptavidin-conjugated enzyme such as horseradish peroxidase as a reporter.

  - Comet assay: Single cell gel electrophoresis, or Comet assay, is a method used to quantify total DNA strand breaks within a cell. Individual cells (e.g., coral sperm) are immobilized within an agarose layer on a slide. Cell membranes are lysed with an alkaline solution and subjected to an electric field. The negatively-charged DNA migrates toward the positive electrode, with smaller DNA fragments moving more quickly through the agarose matrix. Cells that have more DNA strand breaks will form a ‘comet tail’ away from the cell nucleus. Samples are quantified based on the migrated distance from the nucleoid body.

  - Surveyor nuclease assay: Originally isolated from celery, the cel nuclease family of enzymes (e.g., Surveyor® nuclease) will cleave double-stranded DNA in small areas of mismatch (insertions or deletions). This activity is used to determine the number of aberrations within a defined segment of DNA, when comparing one sample with another (e.g., mutant vs wild type). An unknown sample PCR product of a defined gene region is hybridized with that from a reference sample. The resulting hybrid strand is incubated with the enzyme and the resulting products evaluated by electrophoresis. Samples with areas of mismatch with the reference will have multiple bands. Sequence analysis can be used to further determine the mutation type.

- Protein-based assays can provide a snapshot of cellular metabolism that provide insights in to cellular functional roles such as xenobiotic responses, porphyrin metabolism, and protein chaperoning and degradation. The CHDP staff has a wide range of expertise in cellular biochemistry, including protein isolation, Western blot and enzyme-linked immunosorbent assays (ELISA) for a variety of marine species.

    - Porphyrin metabolism: Macrocyclic compounds of the heme biosynthetic pathway and ubiquitous in nature, forming the basic structure of hemoproteins, e.g., chlorophyll, hemoglobin; cytochromes and catalase. Xenobiotics (e.g., pesticides, PCBs, PAHs) can inhibit enzymes in this pathway causing accumulation of different porphyrin species depending on which steps in the biochemical pathway are affected, thus providing cellular diagnostic information (Marks 1985).

    - Functional Proteomics: This will ultimately form the foundation for further diagnostic assay development. Integrating this MS-based approach for investigating biomarkers of effect and exposure is a new area of research for the CHD program in 2020.

E. Contaminant Quantification – Exposure Characterization

Analytical chemistry for CHDP projects is routinely conducted by the Ecotoxicology Branch's chemistry program.

1. Persistent Organic Pollutants

Marine sediments are extracted for a suite of persistent organic pollutants; polychlorinated biphenyls (PCBs), organochlorine pesticides (OCs), brominated flame retardants (PBDEs), and polycyclic aromatic hydrocarbons (PAHs). Sample extracts are spiked with recovery standards prior to instrumental analysis. An Agilent gas chromatograph mass spectrometer (6890/5973) is used for PCBs, OC pesticides, PDBEs, and PAHs analyses. Various analytical columns are used to separate the different analyte classes. All analytes are quantified using MSD Chemstation software. A 10-point calibration curve is run with each analyte class and calibration curves achieve r^2 values greater than or equal to 0.99.

2. Personal Care Products

Reef water samples extracted onto solid phase extraction (SPE) cartridges (HLB or C-18) are eluted from the SPE cartridges with 1:1 methanol/acetone, concentrated and spiked with a recovery standard. An Agilent 1100 high performance liquid chromatograph (HPLC) coupled to an Sciex API 4000 triple quadrupole mass spectrometer is used for sample analysis. Analytes are detected using electrospray ionization in positive mode with two transitions per analyte for identification and confirmation. A seven-point calibration curve is included with the samples and sample quantitation is performed using Analyst software. All analytes are expected to have an r^2 value greater than or equal to 0.99.

3. Metals analysis

Metal analysis of reef sediment and coral skeleton was part of a larger field investigation in St. Croix, USVI. Metals analysis was conducted by Ms. Amanda Bayless as part of her MS thesis in collaboration with NIST. The methods and results of this work is included in her thesis, for which a link is provided in this briefing book.

RESULTS

To highlight how contaminant research is incorporated into the goals and objectives of the Coral Health and Disease Program, we provide vignettes from three investigations undertaken in the past three years, one field investigation and two laboratory-based investigations.

INVESTIGATION 1: Field investigation
Phase I Porewater Toxicity Testing of Sediment from 25 Near-Shore Sites in St. Croix, USVI

MANAGEMENT ISSUE:
Caribbean reef-building corals have experienced catastrophic declines > 97% in many parts of their range. Consequently, seven species (including *Acropora palmata*) are listed as threatened under the U.S. Endangered Species Act. Elkhorn coral (*A. palmata*) was once a dominant reef-building species, providing extensive fringing and barrier reef structures that afford critical shoreline. Reproductive failure in *A. palmata* is a major impediment to the recovery of this species and its delisting from ESA. Until causal factors can be determined and the threats managed, reproductive failure will remain an impediment to *A. palmata* recovery.

CLIENTS: NOAA's Coral Reef Conservation Program, NOAA NMFS SERO Protected Resources, USVI Department of Planning and Natural Resources, National Park Service Salt River Bay National Historic Park and Ecological Preserve, St. Croix, National Park Service Buck Island Reef National Monument, St. Croix

ABSTRACT
Pollution is one of the primary threats to coral reefs with accompanying toxicities that can vary locally from one site to another. *Acropora palmata* populations have undergone massive declines across the Caribbean, and as a result have been listed under the Endangered Species Act. A survey of *A. palmata* reproductive condition across the U.S. Caribbean pointed to populations in St. Croix, U.S Virgin Islands as being highly impacted. An investigation into possible causes began by surveying 25 near-shore sites (including MPAs) around St. Croix to evaluate the toxicity potential. Toxicity of these sites was assessed using sediment porewater in the sea urchin (*Lytechinus variegatus*) embryo development toxicity bioassay. Six of the 25 sites tested positive for toxicity, including four sites in national parks. A subsequent toxicity-reduction assay (Phase I TIE) was used to characterize the nature of the observed toxicity in the original samples by fractionating the porewater using C-18 solid phase extraction. Results of the toxicity reduction assays indicated the toxicants were primarily non-polar or moderately polar compounds. This survey provides a foundation for a more targeted environmental chemical investigation into the toxicity 'hot-spots' that were identified.

BACKGROUND & RATIONALE
Reproductive failure in corals has been linked to a number of natural and anthropogenic stressors (Kramarsky-Winter 2016). Natural stressors such as physical damage (Rinkevich 1996; Kramarsky-Winter and Loya 2000), physiochemical changes such as high water temperature anomalies (Levitan et al. 2014), salinity (Downs et al. 2009), pH (Albright 2011) and disease (Boyer and Colley 2010) are significant threats to reefs, but are difficult to manage on a local and regional scale. More tractable are stressors that are anthropogenic such as sedimentation (Speare et al. 2020), nutrification (Loya et al. 2004) and a wide range of pollutants and contaminants, including endocrine disrupting chemicals (Richmond et al. 2018).

We learned from prior work that visually intact and 'apparently healthy' *Acropora palmata* colonies could in fact carry mild to severe reproductive pathologies to the point of reproductive sterility. The extent of elkhorn sterility was investigated in 2013, when we conducted a U.S. Caribbean-wide survey to assess the reproductive condition of *A. palmata*. Populations from three U.S. jurisdictions covering 34 sites were sampled: Florida (8 sites), Puerto Rico (9 sites) and the U.S. Virgin Islands (17 sites). Ten colonies per site were biopsied two to three weeks prior to their expected spawning date in mid-August. The resulting 327 biopsies were examined for reproductive effort and pathologies. Out of the 34 sites surveyed, *A. palmata* from Salt River Bay National Historic Park and Ecological Preserve (SARI) in St. Croix, USVI had the lowest reproductive effort of all areas surveyed---only three colonies of 40 showed any evidence of reproductive tissues. In contrast, at Buck Island Reef National Monument (BUIS) in St. Croix, only one site exhibited poor reproductive effort (10 %; 9 of 10 sampled colonies contained no gonadal tissue) while the remaining four sites (40 colonies) exhibited 60-80 % reproductive effort.

Sterility and the lack of coral recruitment are key indicators that populations are unsustainable and will eventually disappear. The results of the survey assessment, against a backdrop of 2013 being a good spawning year for *A. palmata* and other Caribbean coral (Miller *et al.* 2016) were alarming. This and the fact that these highly imperiled corals were located in a national historical park and ecological preserve and a NOAA priority site, prompted a multi-year study to investigate possible sources and causes of damage to *A. palmata* in the near-shore reefs of St. Croix, USVI.

As the first step of an environmental investigation into the potential causes of reproductive failure in *Acropora palmata* corals in the waters of St. Croix, a survey of 25 near shore sites (including marine protected areas; MPAs) was conducted during June 2015 to evaluate the toxicity potential. Toxicity of these sites was assessed using sediment porewater in the sea urchin (*Lytechinus variegatus*) embryo development toxicity bioassay. A subsequent toxicity-reduction effort was also initiated to characterize the observed toxicity in the original samples (preliminary phase I Toxicity Identification Evaluation (TIE)) after an initial fractionation using solid phase extraction technology. In addition, sediment interstitial water (porewater) was evaluated for salinity, pH, dissolved oxygen, total ammonia nitrogen (TAN), nitrite, inorganic phosphate and total phosphorus (TP) prior to the bioassay.

METHODS SPECIFIC TO THIS PROJECT

Methods and procedures for equipment preparation, sediment collection, extraction of sediment porewater, water quality analysis, and the sea urchin embryo development toxicity assay were conducted as described in the Experimental Procedures section. The following details are specific to this project.

Site Selection

NOAA Coral Reef Conservation Program (CRCP) jurisdictional liaison, staff from the USVI Department of Planning and Natural Resources, and National Park Service biologists assisted in identifying 25 priority collection sites that included representative reef sites, receiving waters from industrial sites, urban watersheds and public recreational areas (Figure 1.1).

Sample Collection

Sediment samples were collected by snorkelers from near-shore waters using the modified syringe method, filling two Teflon bags at each site (approx. 250 g). A water column sample collected on the eastern end of Buck Island for use as a reference (unimpacted) sample.

Water Quality Analysis

- Total ammonia nitrogen (TAN)
- pH
- Dissolved oxygen
- Salinity
- Nitrite nitrogen concentration was verified with the USEPA diazotization method (#8507) using a microplate format for the Hach nitrite nitrogen assay kit.
- Total inorganic phosphate concentrations were determined by the ascorbic acid method using a Hanna Checker adapted to microplate format. Total phosphorus was calculated as a percentage of total inorganic phosphate (32.62%).

Sea Urchin Embryo Development Toxicity Assay

Gravid sea urchins (*Lytechinus variegatus*) were acquired commercially from the Florida Keys. Lighting was on a 12h:12h light:dark cycle. Urchins were fed a rotating diet of organic carrots, organic spinach, and seaweed (Julian Sprung's Sea Veggies®) three times per week.

Phase I Toxicity Identification and Evaluation (TIE)

A toxicity reduction assay was conducted with 8 sediment porewater samples demonstrating toxicity in the sea urchin development toxicity test. Three BUIS sites (Underwater Trail, Scuba Mooring# 2 and West Beach), three SARI sites (Judith's Fancy, Site 3 and Sugar Bay), Great Pond Bay and Pelican Cove Beach were included. A HyperSep™ C18 column (Thermo Fisher Scientific) was used for sample fractionation (binding nonpolar to moderately polar organic compounds from aquatic matrices). Columns (1 mL bed volume) were charged with 1 mL pesticide-free methanol and rinsed with 1 mL Type 1 water as per the manufacturer's instructions. One milliliter of the porewater sample (previously adjusted for appropriate salinity) was used to rinse the column before applying the remaining sample for collection using a vacuum manifold (~1 drop/s). Salinity and pH for filtered samples were verified prior to beginning the sea urchin development assay. Sea urchin embryos (*L. variegatus*) were exposed to the column eluates (3 mL volume) as detailed above. Artificial seawater and SDS were used as assay controls.

Statistical analyses

Data (expressed as percent normal development for each replicate) were arcsine square root transformed (Zar 1999) prior to other analyses. After transformation, the data met the assumptions for the normal distribution and that the residual variances were homogeneous. A two-sample t-test was used to compare the response of the artificial seawater (TM ASW) negative control to the positive control. A single factor ANOVA (PROC GLM) was performed on urchin development using the TM ASW negative control as the experimental control. A Dunnett's test for multiple comparisons versus controls was performed post-hoc to determine significant differences between treatment groups and control. A repeated measures ANOVA (PROC MIXED method=ML) was used to analyze a subset of the aforementioned samples before and after filtration through C18 SPE media. Sample SITE was the Between-Subject factor and FILTER (pre- or post-C18) was the Within-Subject factor. Post-hoc Contrasts where performed to compare pre-filtered samples versus post-filtered samples by SITE. Alpha was set to 0.05 for all statistical tests. Power for all tests was >0.9. All analyses used SAS v9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS & DISCUSSION

The results of water quality measurements and sediment porewater toxicity testing of samples were obtained during June 2015, and represent one sampling time point during the dry season (low tourist visitation). Samples taken during a wet season and/or high tourist visitation will likely provide different results and further insights into the causes of impacts to St. Croix USVI reefs.

Water Quality Analysis

Water quality measurements were conducted on sediment porewater from 25 sites in the near shore waters of St. Croix, USVI (Figure 1.1A; Table 1.1). Reference water collected at BUIS Site 3 had expected salinity, pH, dissolved oxygen and contained no detectable ammonia or phosphate. The pH and dissolved oxygen for each sample were measured to ensure these parameters were in normal range for sea urchin toxicity tests. Salinity adjustments with Type 1 water (Milli-Q) was required for only two samples, Great Salt Pond Outflow (98 % final concentration porewater) and Salt River Bioluminescent Pond (95 % final concentration porewater).

Nitrogen and phosphorus are essential elements for most living organisms; however, high levels of these nutrients are often indicators of land-based sources of pollution. Sources of these pollutants can include agricultural or urban run-off, sewage, industrial discharges, combustion of fuels and sedimentation (Carpenter et al. 1998). These nutrient increases lead to processes referred to as eutrophication that are detrimental to the structure and function of ecosystems (Paytan & McLaughlin 2007). Tests for levels of various forms of nitrogen (TAN, UAN, nitrite) (Table 1.1) showed only one porewater sample from the Great Salt Pond Outflow that contained abnormally high levels (246.1 µg/L) of unionized ammonia (the most toxic fraction). This level exceeds the EC50 (174 µg/L) for *Arbacia punctulata* sea urchin embryos during development. In addition, this level of unionized ammonia also exceeds the EC50 (73.58 µg/L) for developing *Acropora palmata* larvae (our unpublished data). Together these data indicate a heightened

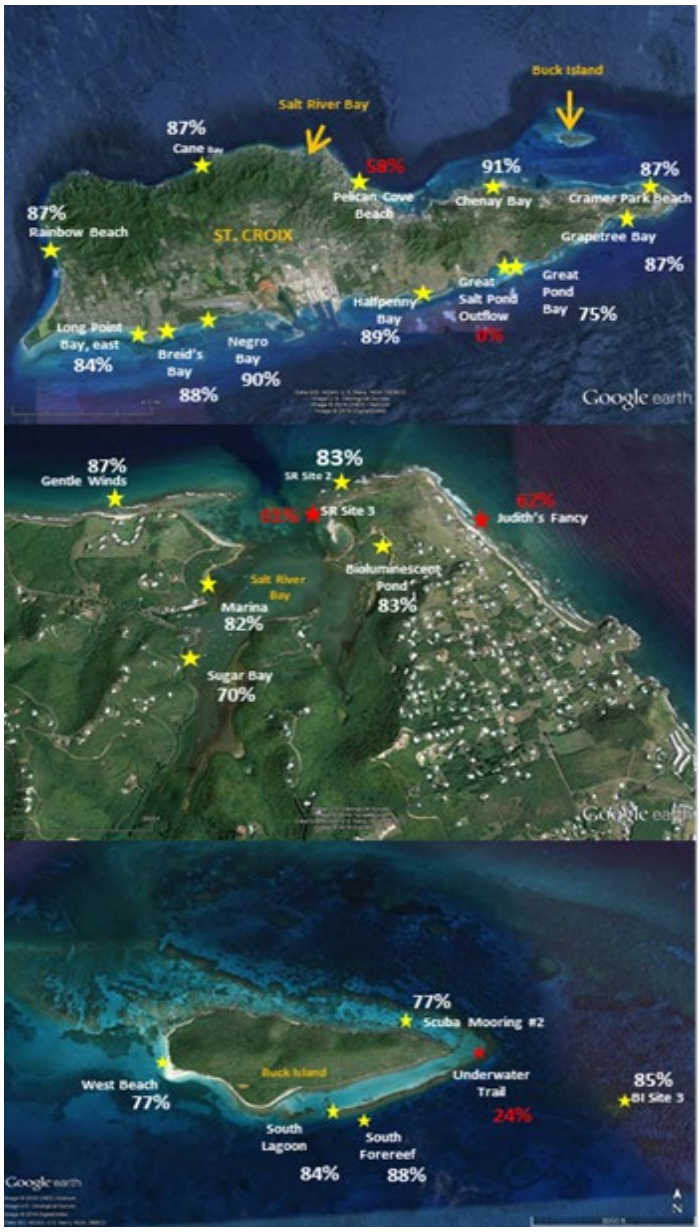

Figure 1.1. A. Map of St. Croix USVI showing results of sea urchin embryo development toxicity testing of sediment porewater from 25 sites in near shore waters. B. Map of Salt River Bay, St. Croix, USVI showing toxicity results. C. Map of Buck Island off shore of St. Croix, USVI showing toxicity results. Sampling sites (yellow stars) with percent normal sea urchin embryos observed. Values in red indicate sites with percent normal embryos significantly different from artificial seawater control.

threat posed to developing sea urchin and coral embryos at this location due to ammonia toxicity.

Inorganic phosphate (as sodium phosphate) treatment at concentrations above 0.8 mg/L has been shown to cause arrested and abnormal embryonic development in *L. variegatus* embryos (Bottger and McClintock 2001). For coral, low-level inorganic phosphate exposure (0.09-0.50 mg/L) has been linked to weaker skeleton structure in acroporid corals (Dunn 2011). Recently, Tovar-Sanchez (2013) demonstrated that sunscreens are a source of phosphates and ammonia in coastal waters. Twelve of the 25 samples contained levels of total phosphorus that exceeded the recommended limit for Class A, B, or C waters (USVI Integrated Water Quality Report 2010) (Table 1.1). Our findings indicate a significant threat potential from elevated phosphate levels in multiple locations of suitable sea urchin and coral habitat and should be considered a potential contributor to reproductive impairment in marine life.

Table 1.1 Nutrient load in reference, control and porewater samples. ND= not detected

Sample	Ammonia-nitrogen (mg/L)	Unionized ammonia[1] (µg/L)	Nitrite-nitrogen (mg/L)	Inorganic phosphate (mg/L)	Total phosphorus[2] (µg/L)
Buck Is. Scuba Mooring #2	0.815	26.1	0.004	1.125	**367.0**
Buck Is. Site 3	ND	0.0	0.007	ND	0.0
Buck Is. South Forereef	0.285	9.8	0.005	0.333	**108.6**
Buck Is. South Lagoon	0.110	3.9	0.008	0.031	10.1
Buck Is. Underwater Trail	0.404	16.2	0.004	0.572	**186.6**
Buck Is. West Beach	0.052	1.9	0.012	0.069	22.5
Breid's Bay	0.164	5.7	0.004	0.106	34.6
Buck Is. Reference Water	ND	0.0	0.012	ND	0.0
Cane Bay	0.216	7.1	0.003	0.044	14.4
Chenay Bay	0.551	17.3	0.005	0.119	38.8
Cramer Beach Park	0.151	5.7	0.008	1.716	**559.8**
Grapetree Bay	0.008	0.4	0.003	0.044	14.4
Great Pond Bay	0.170	7.3	0.004	0.232	**75.7**
Great Salt Pond Outflow	4.521	**246.1**	0.006	0.308	**100.5**
Halfpenny Bay	0.184	7.2	0.007	0.169	**55.1**
Long Point Bay, east	0.137	5.2	0.006	ND	0.0
Negro Bay	0.436	17.8	0.006	0.069	22.5

Pelican Cove Beach	ND	0.0	0.009	ND	0.0
Rainbow Beach	ND	0.0	0.003	ND	0.0
Salt River @ Gentle Winds	ND	0.0	0.005	ND	0.0
Salt River Bioluminescent Pond	0.241	9.2	0.003	0.157	**51.2**
Salt River Marina	0.656	28.7	0.013	0.371	**121.0**
Salt River Judith's Fancy	0.056	2.6	0.010	0.031	10.1
Salt River Site 2	0.081	4.2	0.008	0.157	**51.2**
Salt River Site 3	ND	0.0	0.007	0.245	**79.9**
Salt River Sugar Bay	0.261	11.7	0.005	0.308	**100.5**
Artificial Seawater (ASW)	ND	0.0	0.004	ND	0.0
4 mg/L SDS in ASW	ND	0.0	0.003	ND	0.0

1 Unionized ammonia EC50 for *Arbacia punctulata* sea urchins = 174 µg/L. Toxic level is in bolded red numerals.
2 Recommended total phosphorus for Class A, B and C waters is <50 µg/L. Samples with values above the water quality standard maximum are in bolded red numerals.

Sea Urchin Embryo Development Toxicity Assay

Green sea urchins (*Lytechinus variegatus*) are common from North Carolina throughout the Caribbean Sea to Brazil and are found in near-shore sea grass beds, rocky reef areas, and sandy or hard bottoms, as such, they provide a good model species for bioassays of samples from tropical and sub-tropical locations. The sea urchin embryo development assay was used to evaluate the effects of the sediment porewater samples and control waters. Seven of the 25 samples showed positive toxicity with impacts to normal embryo development that were significantly different from the negative control ($p < 0.05$ (#), $p < 0.0005$ (+) and $p < 0.0001$ (*); Figure 1.2; all other samples showed no toxicity in this bioassay. The artificial seawater control exposure (Figure 1.3A) resulted in less than 3% of embryos with retarded growth and less than 4% with malformations. Increased malformations such as missing appendages (Figure 1.3B-D) were observed at Pelican Cove Beach (average = 35% of total) (Figure 1.1A) and three Salt River sites (Judith's Fancy, 27%; Site 3, 31%; Sugar Bay, 22%) (Figure 1.1B). High percentages of embryos at retarded developmental stages were observed at the Great Salt Pond Outflow (96%) (Figures 1.1A & 1.3E) and Buck Island Underwater Trail (69%) (Figures 1.1C & 1.3F). The overall retarded development observed in the bioassay using sediment porewaters from the Great Salt Pond Outflow (Figures 1.1A & 1.3E) is likely due to high total ammonia nitrogen in the sample water. This type of developmental pathology is consistent with that observed in the sea urchin, *Heliocidaris tuberculate*, when exposed to ammonium chloride (Byrne et al. 2008) resulting in an EC50 of 1.3 mg/L TAN for this species. Although the origin of the toxicity observed in these assays has not been elucidated for these sites, scoring the different developmental anomalies has the potential of providing insight into the mechanisms of toxicity or the developmental programs being affected (Figure 1.3).

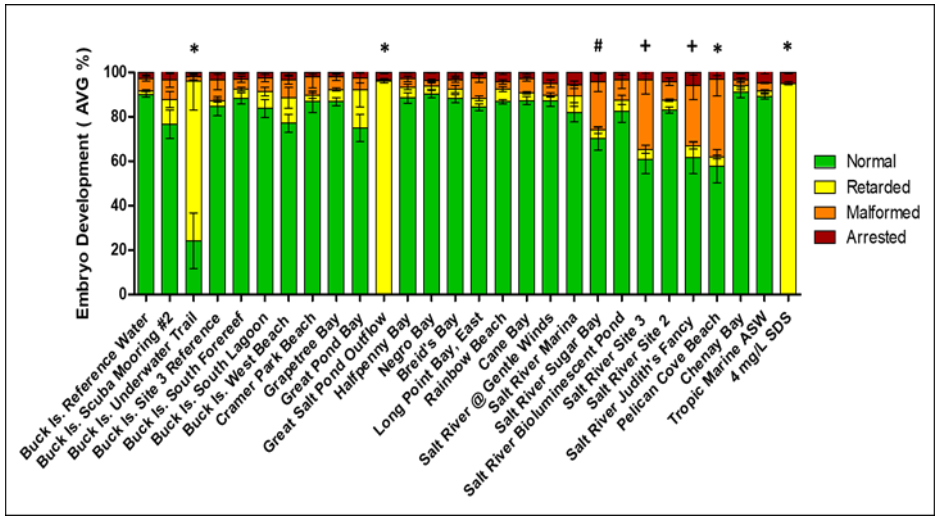

Figure 1.2 Percent normal (green), retarded (yellow), malformed (orange) and arrested (red) development for *L. variegatus* embryos in sample sediment porewaters and reference water from St. Croix, USVI (n=4). TM ASW = Tropic Marin artificial seawater (negative control), SDS = 4 mg/L sodium dodecyl sulfate in TM ASW (positive control). Treatments with significant differences in normal embryos as compared to the artificial seawater control are designated: $p < 0.05$ (#), $p < 0.0005$ (+) and $p < 0.0001$ (*).

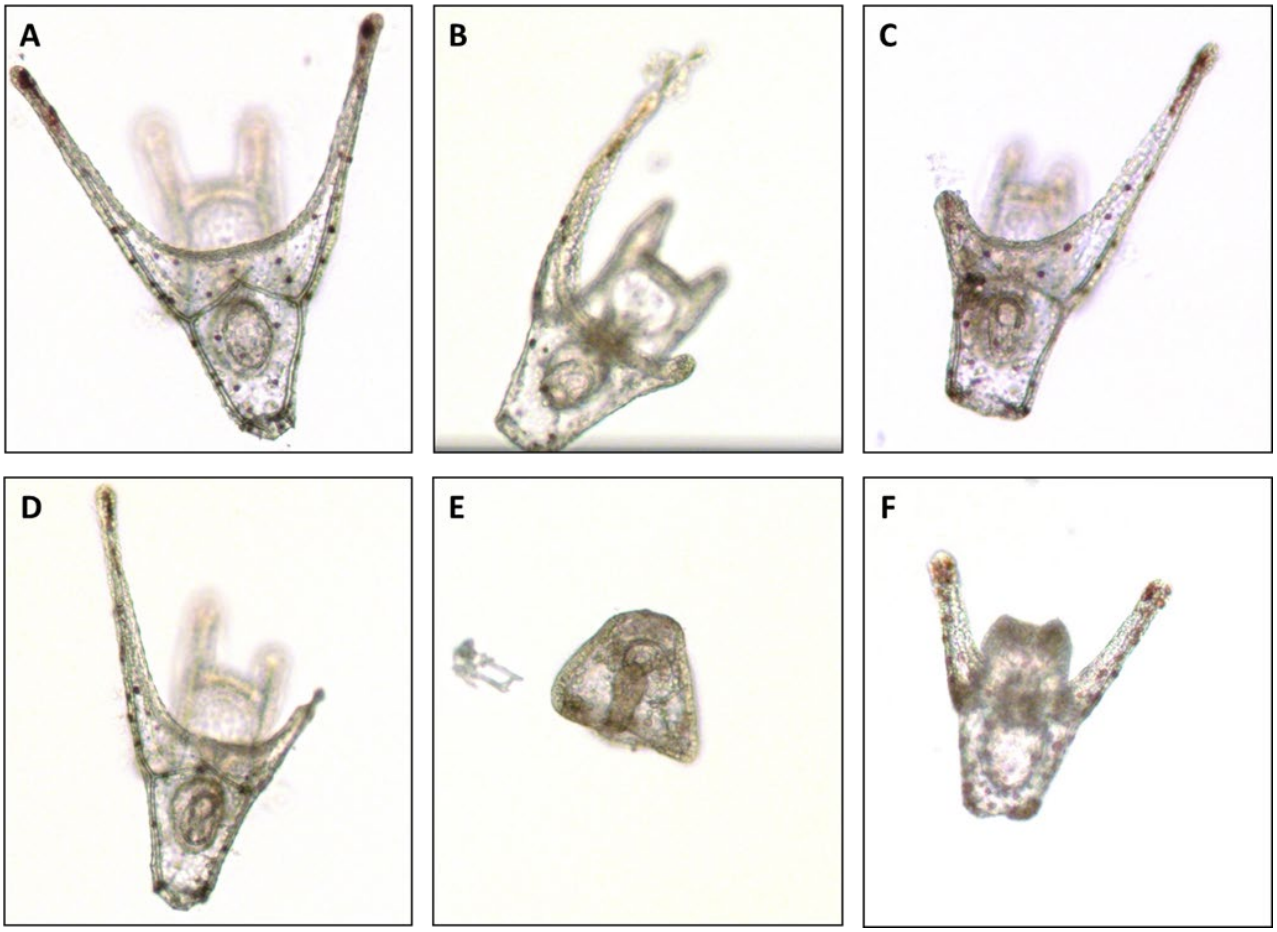

Figure 1.3. Examples of *Lytechinus variegatus* 48 h embryo development following St. Croix, USVI sediment porewater treatment. Panel A: artificial seawater control (normal development), Panel B: Pelican Cove Beach (malformed arm), Panel C: Salt River Judith's Fancy (malformed arm), Panel D: Salt River Site 3 (malformed arm), Panel E: Great Salt Pond Outflow (retarded development, prism stage), Panel F: Buck Island Underwater Trail (retarded development, early pluteus stage). Increased malformations such as missing appendages were observed at Pelican Cove Beach and both Salt River sites. Increased numbers of embryos at retarded developmental stages were observed at the Great Salt Pond Outflow and at Buck Island Underwater Trail. All other treatments were not significantly different from the negative control. Magnification = 100X.

Phase I Toxicity Identification Evaluation with C18 Column

Phase I toxicity identification evaluations were conducted on eight of the samples showing toxicity ranging from 0% normal development to 76.4% in the original toxicity bioassay (Figures 1.1 & 1.2). The toxicity reduction test was performed by filtering another aliquot of the sediment porewater over a C-18 SPE column. The C-18 SPE filtrate was subjected to another embryo development test. Column treatment of the sample porewater improved sea urchin embryo development outcome for all treatments, however three post-filtered porewaters were significantly different from the pre-filtered samples (BUIS Underwater Trail, Judith's Fancy and Salt River Site 3) (Figure 1.4; Table 1.2). C-18 SPE columns bind nonpolar or moderately-polar compounds which likely contribute to sea urchin embryo toxicity at Buck Island Underwater Trail, Great Pond Bay, Pelican Cove Beach, and the three Salt River sites. At the BUIS Underwater Trail, these chemicals constitute the majority of the contaminants and may include hydrocarbons (i.e., boat motor operations), antifoulants and/or personal care products (e.g., certain sunscreens) from recreational swimmers and boaters. Non-targeted analytical chemistry to identify the toxicants binding the C-18 SPE column was not available.

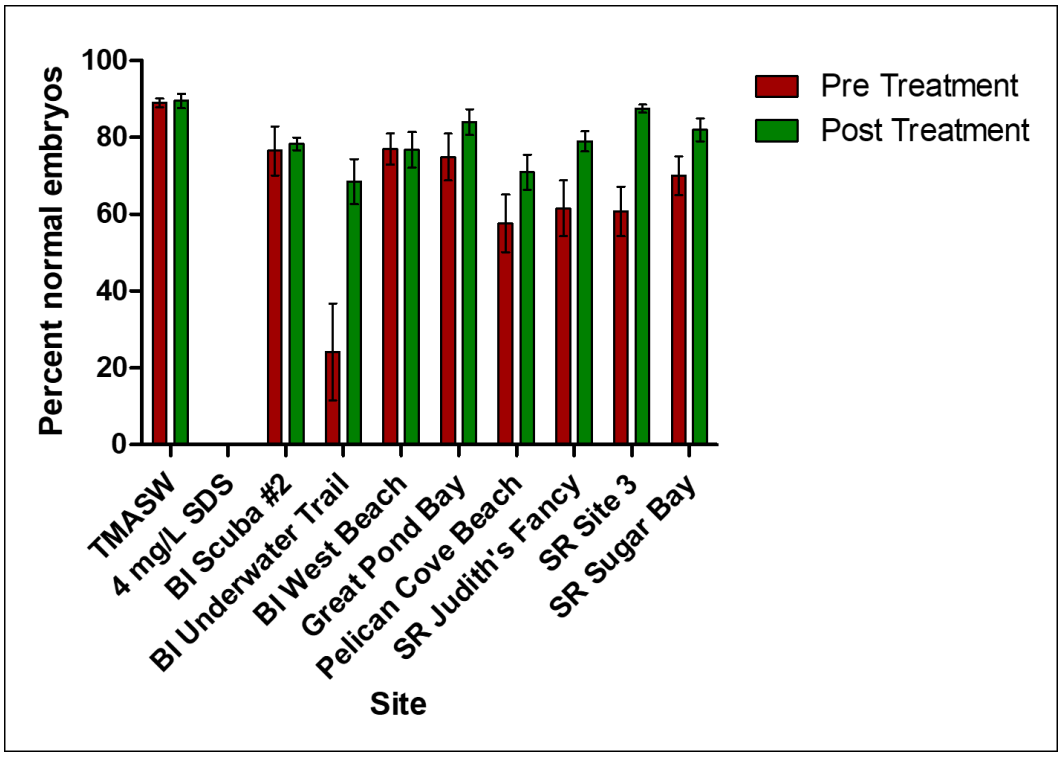

Figure 1.4. Results of Phase I TIE using a C18 column. Pretreatment percent normal embryos (red bars) and post-treatment percent normal embryos (green bars) indicate toxicity reduction at each site with column purification. Post treatment samples indicated with an asterisk (*) were significantly improved over the pretreatment. BUIS Underwater Trail $p<0.00001$; SARI Judith's Fancy, $p<0.05$; SR Site #3, $p<0.001$.

Table 1.2. Results of Phase I TIE - Toxicity Reduction

SITE	% normal pre-treatment	% normal post-treatment
Buck Is. Scuba #2	76.4	78.3
Buck Is Underwater Trail	**26.1**	**68.5***
Buck Is West Beach	77.0	76.8
Great Pond Bay	74.9	84.0
Pelican Cover Beach	57.7	70.9
Salt River Judith's Fancy	**61.9**	**79.0***
Salt River Site 3	**61.1**	**87.5***
Salt River Sugar Bay	70.4	82.0

CONCLUSIONS

Water quality

- Sediment porewater from the Great Salt Pond Outflow site contained a level of unionized ammonia that is toxic to *L. variegatus* and coral (*Acropora palmata* larvae).
- Sediment porewater from 12 sampling sites had levels of total phosphorus above the recommended limit for Class A, B and C marine waters in the USVI. However, the phosphorus loads of water column samples is unknown. Effects of phosphorus on marine organisms and its sources needs further study.

Sea Urchin Toxicity

- Six sites showed sea urchin embryo toxicity in the sediment porewater analysis (Great Salt Pond Outflow, Pelican Cove Beach, BUIS Underwater Trail, SARI Site 3, SARI Sugar Bay and SARI Judith's Fancy). As mentioned previously, the toxicity associated with the Great Salt Pond Outflow can be attributed to the toxic level of unionized ammonia.
- The results of the Phase I toxicity identification evaluation indicate that most of the toxicity at the BUIS Underwater Trail, SARI Judith's Fancy and SARI Site 3 is due to non-polar or moderately polar compounds. This may include hydrocarbons, anti-foulants, detergents, and personal care products. The toxicity may be a cause of the reproductive failure in *Acropora palmata*, noted in our 2013 study.
- Toxicity in sediment porewaters from Pelican Cove Beach and SARI Sugar Bay is partially due to non-polar and moderately polar compounds, however further investigation is needed to determine what other toxicants may be contributing to degraded water quality.

EPILOGUE

This survey of St. Croix for toxicity 'hot-spots' was instrumental funding for indepth investigations into possible causes of reproductive failure in ESA coral in SARI and BUIS. In 2017, investigations showed the *A. palmata* reproductive failure was persistent and evidence of sewage input from fecal indicator bacteria. A significant portion of the investigation formed the Master's Thesis for Ms. Amanda Bayless and focused on assessing the heavy metals and trace elements at multiple reef and embayment sites and using the sea urchin fertilization and embryo development toxicity assays in conjunction with the chemistry of sediments, coral skeleton and coral tissues. In 2019, we were able to re-visit these sites in the rainy season. One of the first efforts for a pseudo-non-targeted chemistry approach was taken by Dr. Ed Wirth's chemistry group that tested water samples from SARI and BUIS for personal care products and a suite of pharmaceuticals and sunscreen UV filters. These preliminary results identified several candidate contaminants that have been characterized as endocrine-disrupting chemicals and targets for laboratory testing for their possible involvement in the reproductive failure of the ESA coral, *Acropora palmata*.

ACKNOWLEDGMENTS

We acknowledge funding support for this work (CRCP Project 1133) from the Coral Reef Conservation Program to CMW. We especially thank Benjamin Wilson of Tobago Cays Marine Park, St. Vincent and the Grenadines; Zandy Hillis-Starr and Clayton Pollock of the NPS, St. Croix for field support. We especially thank Dr. Paul Pennington (NCCOS, Charleston SC) for conducting statistical analyses.

Supporting Documents

May, LA and Woodley CM (2016) Phase I Porewater Toxicity Testing of Sediment from 25 Near-Shore Sites in St. Croix, USVI. Final Report CRCP Project 1133. NCCOS Charleston, SC 29412 https://repository.library.noaa.gov/view/noaa/12912

INVESTIGATION 2: Determining a Protective Turbidity Criterion for ESA-listed Coral (*ongoing project*)

MANAGEMENT ISSUE: Consultations under the Essential Fish Habitat (EFH) provisions of the Magnuson-Stevens Fishery Conservation and Management Act and Section 7 of the Endangered Species Act (ESA) are required to be completed by NOAA's National Marine Fisheries Services when federal agencies propose to authorize, fund, or carry out activities that may adversely affect EFH, ESA listed corals, or their designated critical habitat. While the State of Florida has a regulatory standard for turbidity in Florida waters, that standard has not been demonstrated as effective to avoid impacts to corals, which are designated EFH and include seven species also listed as threatened under the ESA. Having a science-based standard protective of corals from turbidity is needed for inclusion in consultations for coastal development activities that result in generating turbidity, including dredging projects, to help ensure such activities can move forward in the public interest without damage to corals.

CLIENTS: NOAA National Marine Fisheries Service Habitat Conservation Division and Protected Resources Division, NOAA Coral Reef Conservation Program, Florida Department of Environmental Protection

ABSTRACT

Dredging, coastal construction, cruise and shipping terminals, and beach renourishment are among many of the anthropogenic activities that contribute to increased sedimentation and turbidity in near-shore coral reef ecosystems. Such activities require state and federal permits with accompanying environmental impact statements and regular monitoring of the activity to ensure compliance with Water Quality Standards. Current water quality standards were established from toxicity studies that did not include corals. This study aims to fill data gaps by determining toxicity thresholds for two ESA-listed corals, *Orbicella faveolata* and *Acropora cervicornis.* These species represent boulder and branching growth forms, respectively because we hypothesize these growth forms are important in determining how these corals interact and respond to suspended sediment stress as measured by turbidity. Our initial investigations exposed *Orbicella faveolata* nubbins to three sediment loads (50, 100, 150 mg/L) to approximate the Florida turbidity standard of 29 NTU above background and lower turbidity conditions. Using the laceration regeneration assay, healing rates of an incision that removed a single polyp on the apical side of the fragment were calculated as an effect parameter. No differences among the sediment loads were detected, but all three were significantly slower in wound healing than the no-sediment control. A second set of experiments examined the effects of a single sediment load (100 mg/L) for 48 h exposure (pulse), followed by 11 days in fresh artificial seawater (chase) versus 13 days of continuous exposure. The linear healing rates of the lacerations in the 48 h pulse-chase experiment did not show a significant difference. The 13-day continuous exposure, however, did show a significant reduction in the amount of new tissue generated over the wound compared to the controls.

BACKGROUND & RATIONALE

Dredging in ports along the Florida coast is necessary for ship ingress and egress; however, negative impacts to benthic fauna can occur from increased turbidity or sediment accumulation. Elevated turbidity and increased sedimentation from dredging near coral reefs may result in adverse health effects due to reduced light levels, or potential toxicity and tissue loss with surface accumulation of

sediments, particularly if the sediment harbors significant amounts of pollutants (metals, organohalide compounds, etc.) (Erftemeijer et al., 2012a, review). While there have been many scientific reports on the effects of turbidity on corals, there are few controlled laboratory studies linking discreet health effects from increased turbidity as measured in nephelometric turbidity units, NTU (Fourney and Figueiredo, 2017; Telesnicki and Goldberg, 1995).

For shallow water corals, increased seawater turbidity is observed to have gross physiological effects (reduced growth, inhibited larval development) (Bak, 1978; Erftemeijer et al., 2012b; Kendall et al., 1985) and/or alterations in symbiont photosynthetic output (symbiont loss and decreased photosynthesis or energy production rates) (Dallmeyer et al., 1982; Telesnicki and Goldberg, 1995). The algal symbionts of stony corals are a significant source of fixed carbon for the animal (Muscatine and Porter, 1977). This fixed carbon is used for growth, and a significant portion is incorporated into the coral's protective surface mucopolysaccharide layer (Davies, 1984). In response to irritants such as dredge sediments, corals produce excess mucus, which acts to clear accumulated debris from the epidermis (Lasker, 1980; Stafford-Smith and Ormond, 1992); this can be a large net energy loss for the animal, however. Significant sediment accumulation on coral colonies may overwhelm this innate protective process, resulting in anoxic conditions, with eventual tissue necrosis and possible death. Tissue regeneration has been used historically as an effective field management tool to evaluate coral health *in situ,* with slower regeneration rates associated with increased exposure to stressors (Dustan et al., 2008; Fisher et al., 2007; Kramarsky-Winter and Loya, 2000; Meesters and Bak, 1993; Moses and Hallock, 2016; Rodriguez-Villalobos et al., 2016; Traylor-Knowles, 2016). Recently we have demonstrated its sensitivity as a tool to determine ecotoxicological effects to an oil-exposed Pacific coral (May et al., 2020). Additionally, the sea urchin embryo development toxicity assay is a standard method routinely used to gauge toxicity of sediment interstitial waters to early life stages (ASTM, 1995; Chapman et al., 1995).

The project objectives are to:

1. optimize a laboratory turbidity dosing system for coral fragments,
2. evaluate turbidity effects on tissue regeneration for two ESA shallow water coral species a mounding growth form, *Orbicella faveolata* and branching morphology, *Acropora cervicornis* using sediment samples from the Port of Miami, FL, and
3. determine port sediment toxicity levels using a standard EPA test protocol for an early life stage marine species

METHODS SPECIFIC TO THIS PROJECT

Objective 1: Optimization of experimental turbidity dosing system

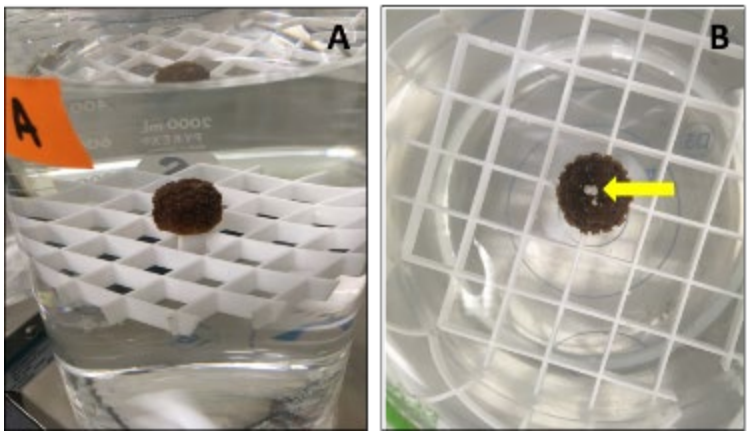

Figure 2.1 Side (panel A) and top (panel B) views of *Orbicella faveolata* fragment in the treatment vessel. Coral is supported by an egg crate louver, cut to fit snugly in the beaker. Wound created for the tissue regeneration assay is indicated with a yellow arrow (panel B, shown with accumulated sediment during exposure).

Several reports document the effect of turbid conditions on shallow water coral species; however, there are no cost-effective, standardized laboratory methods to test turbidity effects on benthic marine organisms. Thus, we performed extensive system validation tests to address this knowledge gap. Port of Miami sediment samples were collected at 20 discreet sites in April 2016, by the NOAA National Marine Fisheries Service, and held at room temperature for mineralogy and stable isotope analysis (Swart, 2016). Following analysis, remaining samples were transferred to NOAA National Centers for Coastal Ocean Science, Charleston Laboratory, for use in coral turbidity challenge experiments. Sample subsets were pooled and mixed thoroughly by hand for the preliminary tests. Subsequently the optimal sediment mixture was homogenized for the coral and sea urchin challenge experiments by cryomilling for 30 min and drying for 72 h (105 °C).

Ten turbidity tests (with and without coral) were performed over 11 months to determine the optimal method for the coral exposures. The method resulting in the most stable and easily-replicated turbidity mixture included using defined weights of dry, cryomilled sediment, continuous mixing using a stir plate-Teflon stir bar combination at 300 rpm, and supporting the coral (on Teflon peg) on an egg crate louver without silicone supports in a 2 L glass beaker (Figure 2.1).

Objective 2a: *Orbicella faveolata* turbidity challenge experiments

Laceration regeneration tests with Orbicella faveolata

Previous toxicity assays in our laboratory had utilized branching coral species, however it was recognized that species with differing growth forms also should be tested, particularly as responses to various toxicants and stressors may vary. Chisels and leather punches have been used to wound large bouldering corals in the field, however when small chisels and punches were tested on the *O. faveolata* nubbins, the small coral fragments shattered. Our goal was to find a method that would result in uniform wound sizes, complete tissue removal at a defined depth, and have >50 % tissue regeneration within 96 h. We tested various drilling, scraping and chiseling techniques for wounding small *O. faveolata* coral fragments and monitored recovery for up to four weeks. The wounds created by scraping and chiseling were difficult to measure, since tissue was not removed evenly. Additionally, we could not create lacerations of uniform sizes across replicates easily. We found that removing a single polyp with a rotary drilling tool created uniform wound sizes (Figure 2.2). The drilling process resulted in a tissue and skeleton slurry, which caused the remaining live tissue to bleach, if not removed. Therefore, the slurry was rinsed from the live tissue with a gentle stream of ASW. This process resulted in *O.*

faveolata nubbins with uniform wounds (~3 mm dia x 2 mm deep), and which healed ~60 % within four days. The conclusions of these experiments are reflected in the *O. faveolata* tissue regeneration analysis methods detailed below.

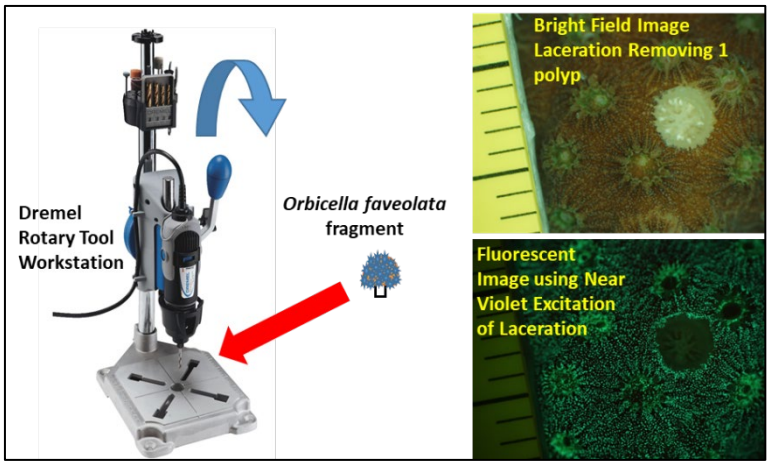

Figure 2.2. Illustration of technique developed to create uniform lacerations in corals with mounding or boulder growth forms. The technique uses a Dremel rotary tool secured in a drill press accessory and equipped with a 2.0 mm diamond hole-saw bit.

96 h dose response experiment

Port of Miami sediment loads (50, 100 and 150 mg/L) were chosen based on preliminary tests to target peak turbidity measurements of approximately 30, 20 and 5 nephelometric turbidity units (NTU). The appropriate amount of dried sediment homogenate was added to beakers filled with 2 L of ASW while stirring with a 2-inch Teflon stir bar (300 rpm). Control beakers contained no added sediment. Sediment was mixed for 15 min prior to placing coral fragments in the treatment vessels (Figure 2.1). Beakers were covered with 1/8-inch glass plates to keep salinity in check. Lighting was provided by a single Ecotech Radion LED source (PAR = 133-168 µmol photons m^{-2} s^{-1}) on a 10 h:14 h light: dark cycle, equivalent to aquaculture conditions. Turbidity and temperature measurements were collected every 4 h during the experiment beginning at 10:30 am, except for the 2:30 am time point. Turbidity was measured using a Hach model 2100P turbidimeter, calibrated with Hach StablCal® formazine standards once daily. Salinity and pH were measured every 24 h. Total ammonia nitrogen remained below 0.07 mg/L in preliminary 96 h tests, so was not measured during the experiment.

Acute short-term turbidity exposure and 13-day continuous exposure

A pulse-chase experiment was devised to determine effects of an acute short-term (48 h) exposure of coral to turbidity (100 mg/L sediment load) and to evaluate coral recovery following the exposure (up to 7 days). In addition, turbidity effects (100 mg/L sediment) on *O. faveolata* tissue regeneration over 13 days also was measured. Experimental parameters (water quality, lighting, etc.) were as detailed for the 96 h dose response experiment. Treatment changes (fresh ASW) for the acute exposure occurred at 48 h for treatment and controls and then every 96 h. Treatment water changes for the 13-day continuous exposure occurred every 96 h following treatment initiation. Tissue re-growth was measured with daily imaging beginning at 24 h for the 13-day experiment and at 48 h for the pulse-chase experiment as described.

Coral tissue regeneration analysis

Prior to experiment initiation, *O. faveolata* fragments (1 cm x 1 cm x 1cm) were removed from the culture aquarium and placed in a Dremel workstation, approximately 2 cm below a Dremel rotary tool fitted with a 2.0 mm diamond hole saw bit (Lasco Diamond Products, Los Angeles, CA). Coral support pegs were manually held in place as the rotating bit was lowered to the coral surface over the center of a polyp (Figure 2.2). Nubbins were wounded to a depth of approximately 2 mm (entire polyp was removed). Tissue slurry was removed by gentle application of an ASW stream using a 1000 µL

micropipettor. The coral was placed in a 500 mL clean glass jar filled with ASW and bright field and fluorescent (near violet) images were taken of the cut surface next to a centimeter rule using an Olympus MVX10 research macro zoom microscope with a 0.63x objective using an Olympus DP71 digital camera. Near violet excites the green fluorescent protein in *O. faveolata* tissues, allowing for accurate measurement of tissue re-growth. At each experimental time point, coral fragments were gently agitated in ASW to remove accumulated sediment and imaged again as described above.

Skeletal area for each coral image was determined using ImageJ macro from the NOAA/NCCOS Coral Disease and Health Consortium website (https://cdhc.noaa.gov/education/coral_assay.aspx) with modifications to the code to reflect the use of 3 mm as the standard reference size (vs 50 mm). Pixel units were calibrated to the centimeter rule in each image. Total area of bare skeleton and wound perimeter values were recorded for each fragment at time 0 and each experimental time point. Percent tissue regeneration was determined from the difference in skeletal area between the time 0 and the experimental time points. Linear healing per day was calculated using the area and perimeter of the wound in Equation 2.1 (modified from Gorin et al. 1996).

Equation 2.1: $(A_{t-1} - A_t)/\text{mean}(P_{t-1}, P_t)$
Where: A_t = skeletal area at given experimental time point
A_{t-1} = skeletal area at previous time point
P_t = wound perimeter at given experimental time point
P_{t-1} = wound perimeter at previous time point

Objective 2b. *Acropora cervicornis* turbidity challenge experiments **(*ongoing research*)**

Objective 3. Determining sediment toxicity to sea urchin embryos **(*ongoing research*)**

RESULTS (to date)

Orbicella faveolata **turbidity challenge experiments**

Effects of 96 h dose response turbidity exposure on Orbicella faveolata tissue regeneration

Water quality parameters remained within acceptable parameters for the course of the experiment. Results of the turbidity 96 h dose response challenge for *O. faveolata* tissue regeneration are presented in Figure 2.3 and 2.4. Turbidity from Port of Miami sediment homogenate significantly impacted coral tissue regeneration at 50, 100 and 150 mg/L sediment loads (Turbidity ranges = 1-4, 7-20 and 16-30 NTU). Percent regeneration was significantly lower for all three treatments compared to the control.

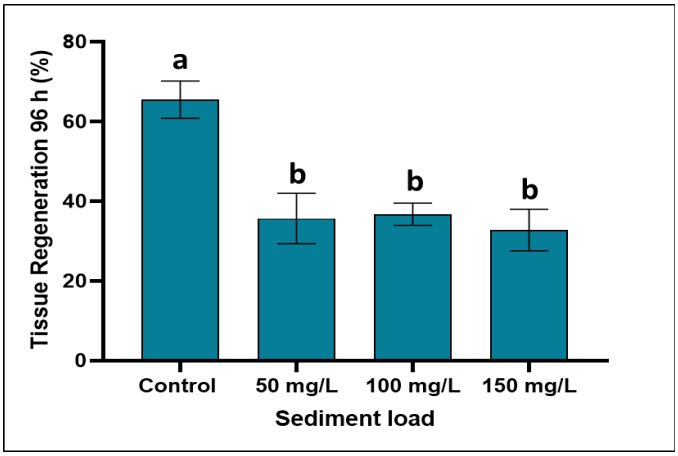

Figure 2.3. Percent tissue regeneration for *Orbicella faveolata* following 96 h exposure to Port of Miami sediment homogenate at three concentrations. All treatments had significant reductions in tissue growth (ANOVA, p=0.0016), indicated in letter designations. Average peak turbidity for each sediment load was 4 NTU (50 mg/L), 20 NTU (100 mg/L) and 30 NTU (150 mg/L).

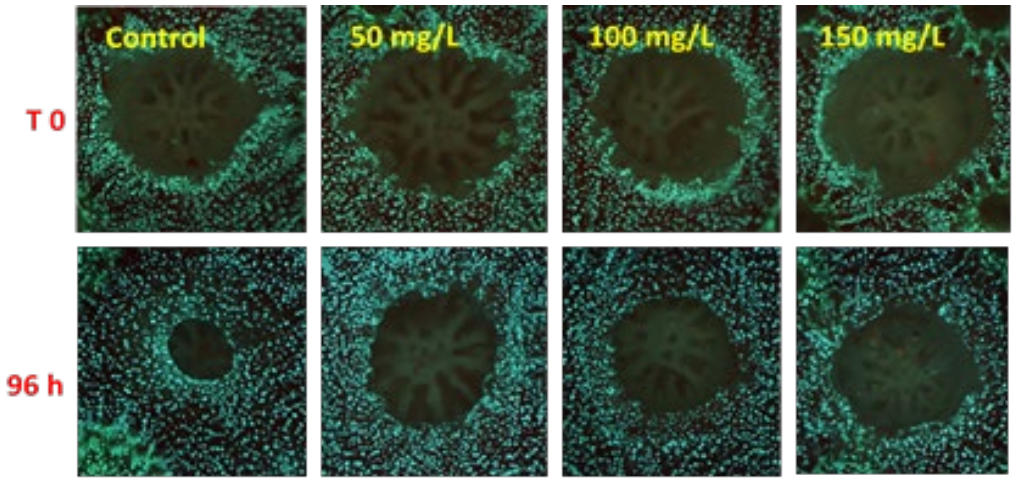

Figure 2.4. Photomicrographs of the laceration regeneration assay results for a 96 h dose-response exposure of *Orbicella faveolata* to 50, 100 and 150 mg/L of Port of Miami homogenized sediment dredge materials. Percent of new tissue growth was significantly different from the no-sediment control, but not different from each other.

Effects of acute, short-term and 13-day turbidity exposures on Orbicella faveolata tissue regeneration

A linear mixed-effects model fit to results of the 48-h pulse-chase experiment did not detect any significant effect of turbidity on tissue regeneration and linear healing rates at any time point (Figure 2.5). Results of the 13-day turbidity exposure for *O. faveolata* are presented in Figure 2.6. While differences in tissue regeneration between treatment and control were observed, the effect of treatment varied by time point. Accelerated linear healing rate between day 1 and day 2 was noted despite continued sediment exposure. The results of a nonlinear mixed-effects model incorporating a three-parameter logistic function to model the change in percent regeneration over time found the only parameter to vary significantly between treatment and control to be xmid (which represents the x value at the inflection point of the curve, ~50 %), with control xmid at 3.1 d versus 5.5 d for treated samples (Figure 2.7).

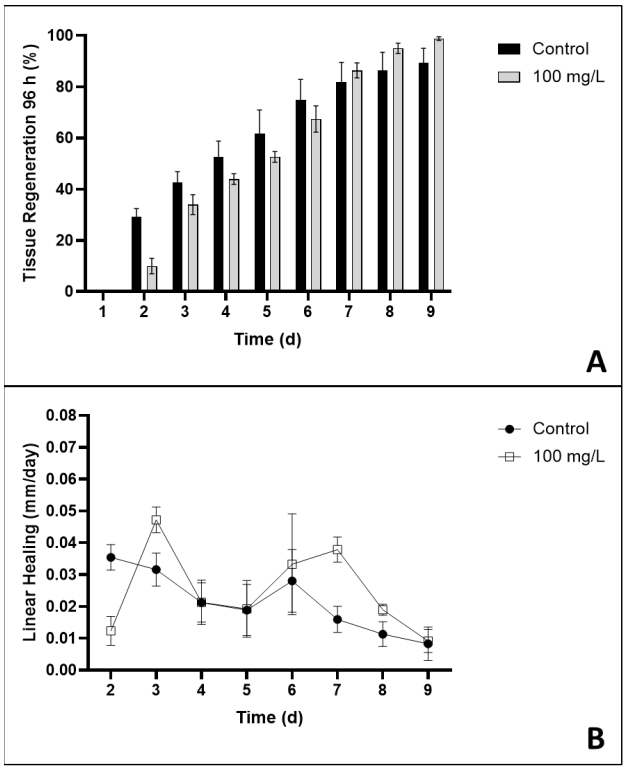

Figure 2.5. Results of the 48 h pulse-chase experiment with *Orbicella faveolata.* Panel A = percent tissue regeneration and panel B = linear healing (mm/day). No significant differences were observed between the 30 NTU (100 mg/L sediment) treatment and control using a linear mixed-effects model.

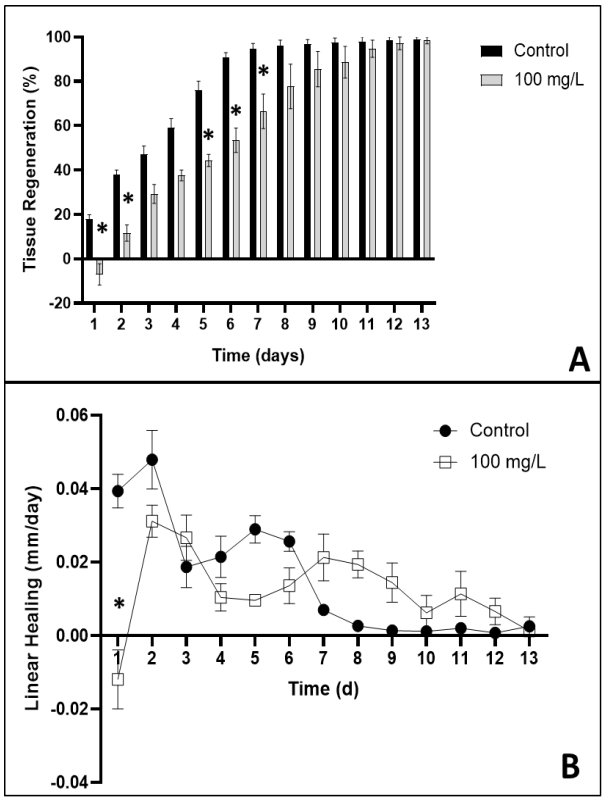

Figure 2.6. Results of the 13-day turbidity exposure with *Orbicella faveolata*. Panel A = percent tissue regeneration and panel B = linear healing (mm/day). Differences between the 30 NTU (100 mg/L) treatment and control were determined using a linear mixed-effects model. Percent tissue regeneration was significantly different on days 1, 2, 5, 6 and 7. Linear healing rate was significantly different on the first day. (Asterisks denote significance, where $p < 0.05$.) Accelerated healing for treated coral fragments was observed in the presence of sediment between days 1 and 2.

PRELIMINARY CONCLUSIONS

96 h Dose-Response Experiment:

- Port of Miami sediment homogenate inhibited *O. faveolata* tissue regeneration at all tested concentrations, with effects noted for peak turbidity as low as 4 NTU (range = 1-4 NTU) over 96 h.

- The results of this experiment indicate that the current turbidity criterion for the State of Florida is not protective for this ESA coral species, since turbidity due to dredging activities can increase turbidity more than 4 NTU over background and may last much longer than 96 h.

48 h Pulse-Chase Experiment:

- A sediment load of 100 mg/L Port of Miami cryomilled homogenate had no effect on percent tissue regeneration or linear healing rate in *O. faveolata* after 48 h exposure.

- The linear healing rate (mm/day) was variable (not significant) over time for both controls and treated fragments, but increases seem to coincide with water changes.

13-day Sediment Exposure:

- A continuous (13 day) treatment of 100 mg/L sediment homogenate resulted in a significant reduction in tissue regeneration for the treated fragments compared to the controls.

- Treated fragments appeared to compensate for the initial sediment impact by accelerating healing rates between 24 and 48 h of exposure.

- Variable linear healing rates over time were observed again, with treated fragments responding more slowly to the fresh treatment solution (taking days to increase tissue regeneration compared to controls).

- The healing delay for treated samples was 2.4 days, as calculated using a nonlinear mixed-effects model.

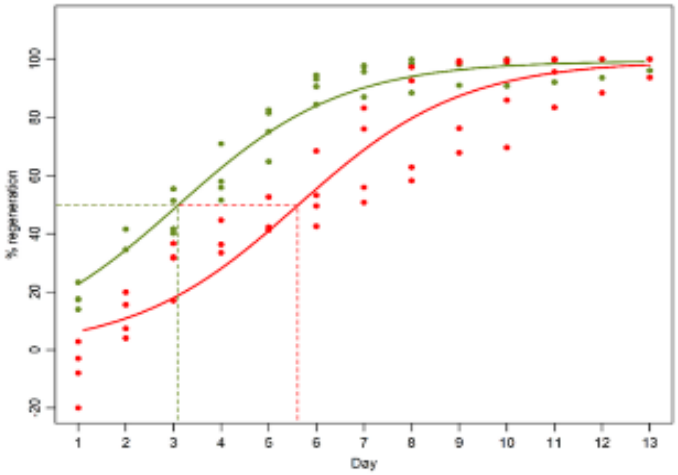

Figure 2.7. Tissue regeneration results with the 13-day turbidity exposure using the nonlinear mixed-effects model, with percent regeneration over time modeled using a three-parameter logistic function. Based on likelihood ratio tests, the only parameter to vary significantly ($p < 0.05$) between treatment and controls was xmid (representing the x value at the inflection point of the curve), which was at 3.1 days for the controls and 5.5 days for coral fragments exposed to Port of Miami dredge sediment.

Data availability

This is a newly initiated ongoing project. This report reflects only three of the initial experiments that have been conducted in this project as examples of our research question, rationale, experimental design and data analysis. Frequent project updates are held with all clients via teleconference. Following completion of all experiments, it is anticipated that the results will be published in a peer-reviewed manuscript.

INVESTIGATION 3: Nutrients as a contaminant
Is the EPA Water Quality Criterion for TAN Protective for Coral Reef Organisms?

MANAGEMENT ISSUE: Coral reef ecosystems are undergoing catastrophic decline globally. In U.S. waters 22 species are listed as threatened under the Endangered Species Act; three additional species are listed as endangered. Anthropogenically derived nutrient pollution (largely due to nitrogen pollution) is dramatically affecting global nitrogen cycling and oligotrophic coral reef ecosystems. Ammonia is a common toxicant associated with nitrogen pollution. With a paucity of ammonia toxicity data for coral and coral reef organisms, there is insufficient information to derive water quality criteria or water quality standards that are protective of coral reef resources.

CLIENTS: Coral Reef Conservation Program, NMFS SERO Protected Species, FL Department of Environmental Protection, FL Keys National Marine Sanctuary, Biscayne National Park

ABSTRACT
Ammonia (NH_3) is one of the most toxic constituents of nitrogen pollution in aquatic environments. The National Water Quality Criteria (WQC) for ammonia in saltwater, set by EPA in 1989, is based on studies with crustaceans, bivalve mollusks and fishes. The chronic criterion specifies a 4-day average concentration of 0.035 mg/L unionized ammonia (UAN) not to be exceeded more than once every 3 years, the acute criterion specifies a 1-h average concentration of 0.223 mg/L UAN not to be exceeded more than once every 3 years. With recent catastrophic declines of shallow-water coral reefs, the threat-potential of ammonia toxicity for recently ESA-listed corals is unknown. Acute dose-response toxicity tests (48 or 96 h) were conducted for ammonia with coral nubbins (*Acropora formosa*), coral planula larvae (*Acropora palmata*), and two sea urchin species (*Arbacia punctulata, Lytechinus variegatus*). The effect of temperature (29 °C and 31 °C) on ammonia toxicity with *A. palmata* larvae also was tested. Our results indicate that the existing national acute WQC is unlikely to be protective for either species of sea urchin under the acute criterion, but the chronic WQC is likely to be protective. In this study, both the adult coral nubbins and coral planula larvae would be marginally protected at the chronic WQC levels, but not at the acute national WQC. Furthermore, we have shown that a 2 degree increase in temperature during embryo development results in a 35 % decrease in larval the LC_{50} values. In the context of these data, review of current water quality criteria and water quality standards that are established in local jurisdictions that are protective of coral reef resources is encouraged.

INTRODUCTION
Nitrogen and phosphorus are essential elements for most living organisms; however, high levels of these nutrients are often indicators of land-based sources of pollution. Sources of these pollutants can include agricultural or urban run-off, sewage, industrial discharges, combustion of fuels and sedimentation (Carpenter et al. 1998). These nutrient increases lead to processes referred to as eutrophication that are detrimental to the structure and function of ecosystems (Paytan & McLaughlin 2007).

Nutrient loading in tropical waters can have detrimental effects upon a variety of marine organisms. The total ammonia as nitrogen (TAN) is an important measure of nutrient loading resulting from decaying organic matter, agricultural runoff, or sewage input. Understanding the effects of TAN in near shore waters is a critical tool for coral reef recovery efforts. We have determined the effects of TAN on sea urchins and coral at various life stages using ammonium chloride exposures.

Ammonia is highly soluble in water and can be highly toxic to organisms. In the ocean, it exists in equilibrium between ionized ammonium nitrogen (NH_4^+-N) and unionized ammonia nitrogen (NH_3-N), and the ratio of each is dependent upon salinity, pH and temperature. The molecular weight of ammonium chloride is 53.49 g/mol. Since there is only one nitrogen molecule in the compound that is associated with ammonia (mass = 14.01), the TAN of ammonium chloride (as a percent of the compound) can be calculated (Equation 1):

Equation 3.1: TAN NH_4Cl = mass of nitrogen associated with ammonia/formula weight of compound = 14.01/53.49 = 0.26

Therefore, the amount of TAN in an ammonium chloride solution can be determined by multiplying the NH_4Cl concentration by 0.26. The toxic unionized ammonia (UAN) fraction is calculated based upon TAN, pH, temperature and salinity (Equation 3.2, Bower and Bidwell 1978):

Equation 3.2: % un-ionized ammonia = 100/[1 + antilog (pK_a^s + (T) – pH)],

where TAN is the measured total ammonia-nitrogen concentration, pKa^s is the overall acid hydrolysis constant of ammonium ions in seawater, T is the water temperature and pH is the measured pH of the water. This equation also may be used to model predicted climate change scenarios for known TAN, temperature, pH and salinity. The toxicity of a given TAN will change with increases in ocean temperature or decreases in ocean pH.

In this series of laboratory studies, we performed toxicity tests to develop acute toxicity thresholds of ammonia for coral and sea urchin species. Acute dose-response toxicity tests (48 or 96 h) were conducted with coral nubbins (*Acropora formosa*), coral planula larvae (*Acropora palmata*), and two sea urchin species (*Arbacia punctulata, Lytechinus variegatus*) in artificial seawater amended with ammonium chloride. The effect of temperature (29 °C and 31 °C) on ammonia toxicity with *A. palmata* larvae also was tested.

METHODS SPECIFIC TO THIS PROJECT

The experiments for this project used methods described in the earlier Experimental Procedures section of this document. Procedures followed preparation and set-up protocols, dose-response exposures for adult coral nubbins, coral embryos, sea urchin fertilization and sea urchin embryo development toxicity testing. Endpoints included the micro-plate adapted colorimetric ammonia assay for exposure determination, laceration regeneration assay, morphological scoring or urchin developmental abnormalities using our more detailed scoring rubric and cell enumerations using a compound microscope and either hematocytometers or Sedgwick-Rafter cell counting chambers.

Specimens
Adult coral nubbins: *Acropora formosa*
Coral larvae: *Acropora palmata*
Sea urchins: *Arbacia punctulata* and *Lytechinus variegatus*

Contaminant
Ammonium chloride (8 g/L; 1000X in Type I water) stocks were made in artificial seawater filtered (0.2 µm). An 8-point dilution series (0.2, 0.6, 0.9, 1.2, 1.5, 1.8, 2.0 mg/L TAN) was prepared in ASW (30 ppt for *A. punctulata*) and 5 mL was placed into each 20 mL glass vial for the sea urchin tests or 200 mL was placed into Berzelius beakers (300 mL) for coral nubbins. Positive controls were SDS solutions (8 mg/L) prepared in ASW. Treatment solutions were brought to experimental temperatures prior to initiating the experiment.

Adult coral
Acropora formosa is an IndoPacific branching coral that was selected on the basis of having a strong green fluorescent protein (GFP) signal in newly formed tissue, allowing for accurate measurement of new tissue progression for the tissue regeneration assay. Fragments (2.5 cm) were generated from adult colony branches immediately prior to experiment initiation. Each treatment vessel was aerated individually with a custom-made manifold and Teflon tubing, and airflow adjusted with individual gang valves. All beakers were held in a waterbath using a Ranco temperature controller (26 °C) connected to a 100 W heater and water circulated with two Rio400 aquarium pumps. Culture conditions (lighting, salinity, pH and temperature) were replicated for the experiment.

The laceration regeneration assay was initiated by creating uniform wounds using cleaned stainless steel bone cutters to remove ~0.5 cm of the apical fragment in cross-section. Imaging was using a MVX10 fluorescent macro zoom microscope equipped with a triple cube fluorescence filter (DAPI/FITC/TRITC with Em: 450±10 nm, 520±10 nm, and 595±10 nm) and recorded with an Olympus Model DP71 digital camera. Coral fragments underwent a dose-response exposure to an ammonia treatment dose range 0.0-10.0 mg/L NH_4Cl (n=3). Treatment solutions were renewed daily after imaging for 10 d. Image analysis was as previously described (https://cdhc.noaa.gov/education/coral_assay.aspx).

Coral larvae
Acropora palmata gametes were collected on August 21, 2016 at Elbow Reef, Key Largo, Florida. Coral larvae were generated from batch genotype (Orange, Blue, Green) crosses in the laboratory, approximately 2 h post-spawning. Larvae (~12 h old) were transferred into 5 mL control or ammonia treatment solutions (35 ppt) with a glass Pasteur pipet (10-12 larvae/vial), enumerated with 4X magnification, and placed in a temperature-controlled incubator under ambient lighting. Larvae were

transferred into fresh treatment solutions and enumerated after 24 h. The experiment was terminated at 48 h with a zinc-formalin fixative and final numbers of larvae were recorded.

Sea urchin embryos

Two species were used in these experiments, *Lytechinus variegatus* and *Arbacia punctulata* to evaluate species sensitivities. Both were commercially purchased and held in culture as previously described. The sea urchin embryo development and fertilization toxicity tests were performed as previously described.

Statistical analyses

One-way analysis of variance analyses (ANOVAs) were performed on 48 h coral larvae, 4-day adult coral, and 48 h sea urchin embryo data. Dunnett's multiple comparison post tests were used to determine significant differences between treatments and the control for each experiment. Dose response curves with 95 % confidence intervals were generated using log-transformed data. All statistical analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, www.graphpad.com).

RESULTS and DISCUSSION

Ammonia effects on adult coral tissue regeneration

Coral nubbins of *A. formosa* with an open wound that exposed a cross section of the skeleton on one end (Figure 3.1A) were subjected to an ammonium chloride dose-dependent assay (ammonium chloride solutions in Tropic Marin Pro Reef artificial seawater, 0.5-10 mg/L dosing range) The laceration regeneration assay was used as an indicator of an integrated physiological response. Seawater temperature was maintained at 26.0 ±0.5 °C and the pH range was 8.3-8.4 for the experiment duration. Percent tissue growth over the initial laceration was calculated over a 10-day period using natural tissue-containing green fluorescence protein (Figure 3.1) to visualize new tissue accretion. Results of the ammonium chloride exposures at 96 h for all treatments are presented in Figures 3.2A. Tissue regeneration was negatively affected at 1.3 mg/L TAN and higher ($p<0.01$). Because of the mortality observed at by day 10, the median effective concentration (EC_{50}) was calculated with 96 h data. The EC_{50}

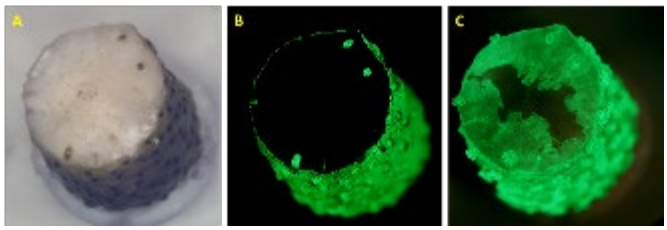

Figure 3.1 *Acropora formosa* nubbin with tip excised on Day 1 to reveal bare skeleton under bright field illumination (A) and fluorescence (B). Nubbin tissue regeneration after 10 days in control artificial seawater is shown in panel C.

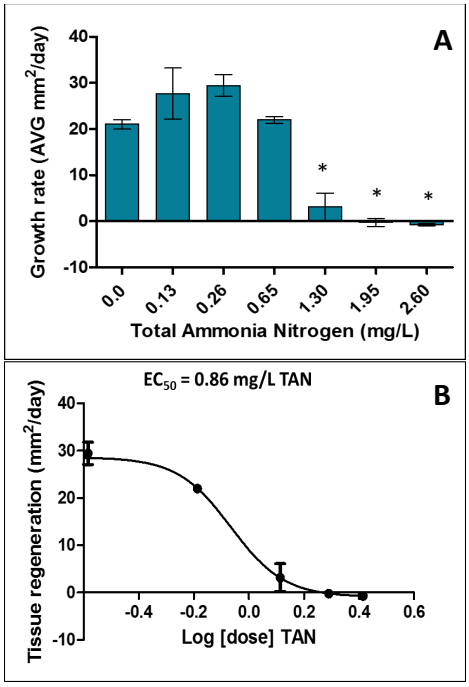

Figure 3.2 Tissue regeneration 96 h response of adult *Acropora formosa* to ammonium chloride treatment. Panel A= Average tissue regeneration rate for each ammonium chloride treatment after 96 h of exposure in artificial seawater at 26 °C. Total ammonia nitrogen was verified for each dose using a colorimetric assay. Treatments marked with an asterisk (*) were significantly different from control ($p<0.01$). Panel B= Dose-response curve for *A. formosa*. The median effective concentration (EC_{50}) was 0.86 mg/L total ammonia nitrogen (95% confidence interval = 0.65-1.14) was calculated based on the area of tissue growth.

value for *A. formosa* tissue regeneration was 0.86 mg/L total ammonia nitrogen (95% confidence interval = 0.65-1.14).

Ammonia toxicity to *Acropora palmata* larvae

Mortality was observed in *A. palmata* planula with higher ammonia concentration that was temperature dependent. The LC_{50} was reduced by nearly 35 % with a two-degree increase in temperature. Following a 48 h exposure, the percent mortality for each treatment regime was graphed (Figure 3.3A). The 50% lethal concentration (LC_{50}) of TAN at each temperature calculated from the dose response assay are presented in Figures 3.3 B & C. Larval mortality was significantly increased compared with controls with 1.56 mg/L TAN at 29 °C and with 1.04 mg/L TAN at 31 °C. Temperature significantly lowered susceptibility of *A. palmata* larvae to ammonia, with the LC_{50} reduced from 0.89 to 0.58 mg/L TAN with the 2.0 °C temperature increase.

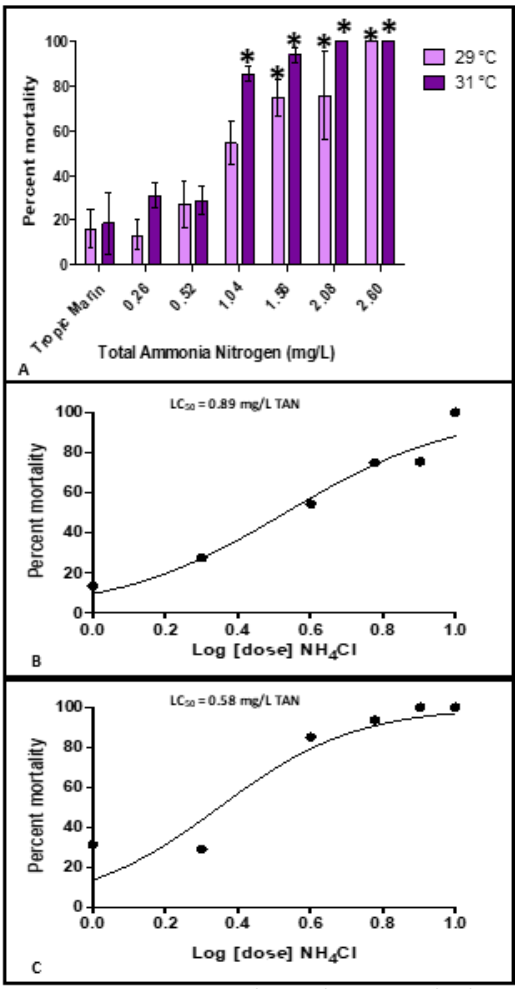

Figure 3.3 *Acropora palmata* larvae 48 h dose-response to ammonium chloride exposure. Panel A. *Acropora palmata* larvae survival at two temperatures. Increased temperature resulted in increased mortality at lower ammonia treatment concentrations. Treatments marked with an asterisk (*) were significantly different from the artificial seawater control at $p<0.05$. Panel B = 29 °C, Panel C = 31°C. Increased seawater temperature lowered the concentration required for 50% lethality from 0.89 mg/L (95% confidence interval = 0.67-1.18) to 0.58 mg/L (95% confidence interval = 0.37-0.91) total ammonia nitrogen.

Embryonic and early life stages of many species are more sensitive than adults to a wide variety of pollutants. Harrison and Ward (2001) demonstrated that even small elevations in ammonium and phosphate concentrations, singularly or in combination, can affect or block fertilization and embryo development in *Acropor longicyathus* and *Goniastrea aspera.* Cox and Ward (2002) suggest that broadcast-spawning corals with azooxanthelate ova and embryos appear more sensitive to nutrient enriched waters than planulating corals, many of which do carry maternal zooxanthellae.

Humanes et al. (2016) have recently demonstrated toxicity resulting from the combined effects of temperature and nutrient load to *Acropora tenuis* early life stages. In that study, nutrient enrichments were prepared by adding inorganic and organic nutrients derived from organic matter and plankton to filtered sea water and measuring carbon, nitrogen and phosphorus loads of low, medium and high treatment concentrations at varying temperatures (27-32 °C). While it was shown that increased temperature and nutrient load decreased fertilization success and increased developmental abnormalities, nutrients alone increased maximum photosynthetic efficiency and survivorship of juveniles (tested ammonia concentrations ranged from 0.009-0.270 mg/L TAN). Our results showed that ammonia concentrations in this range were not significantly different from the control for *A. palmata* larvae, but that higher, environmentally relevant concentrations could have significant impacts to coral larvae survivorship.

We also demonstrated that after 48 h incubation, temperature alone at 29 or 31 °C had no significant effect

on control coral planula survival (Figure 3.3). Bassim and Sammarco (2003) showed however, that mortality of *Diploria strigosa* planula increased with increasing temperature in less than 24 h with differences plateauing at approximately 75 h post fertilization. The differences may be attributed to species sensitivities.

Effects of total ammonia nitrogen on *Arbacia punctulata* fertilization

Carr (2006) reported that the fertilization endpoint was an insensitive measure of ammonia toxicity to *Arbacia punctulata.* Similarly, we found *A. punctulata* fertilization was unaffected by TAN concentrations as high as 2.1 mg/L. Fertilization of *A. punctulata* was not affected by ammonia at the tested concentrations tested (0.12- 2.11 mg/L) (data not shown). Fertilization trials were not conducted with *Lytechinus variegatus*.

Effects of total ammonia nitrogen on sea urchin embryo development

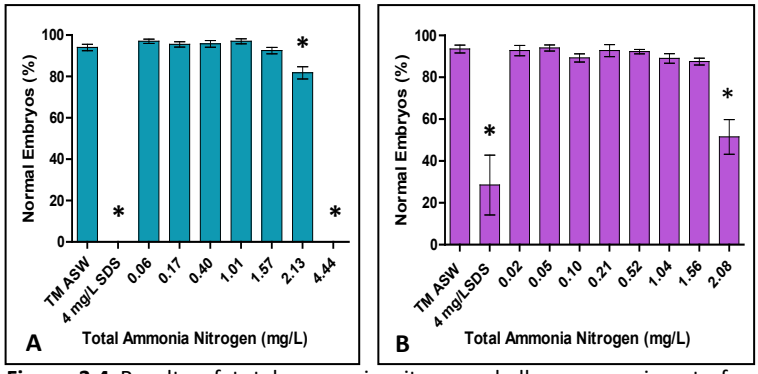

Figure 3.4 Results of total ammonia nitrogen challenge experiments for *Lytechinus variegatus* (panel A) and *Arbacia punctulata* (panel B). The 2.08 mg/L TAN treatment for *L.* variegatus was significantly different from the negative control (Tropic Marin artificial seawater, TMASW, p<0.0001). Embryo development for *A. punctulata* was significantly different from the control in treatments of 2.13 mg/L TAN and higher. Sodium dodecyl sulfate (SDS, 4 mg/L) was used as the positive control in both experiments.

Ammonia toxicity to sea urchin embryos has been demonstrated for several species including *Strongylocentrotus purpuratus*, *Heliocidaris tuberculata* and *Arbacia punctulata* (Greenstein, 1995; Byrne 2008, Carr 2006). In our tests, *Lytechinus variegatus* and *A. punctulata* embryos showed sensitivity to TAN concentrations above 2.1 mg/L (toxicity threshold for UAN ~140 µg/L). The results of the sea urchin embryo development toxicity assays for *L. variegatus* and *A. punctulata* are shown in Figure 3.4. The 2.08 mg/L TAN treatment for *L. variegatus* was significantly different from the negative control (p<0.0001). Embryo development for *A. punctulata* was significantly impacted by treatments of 2.13 mg/L TAN and higher (p<0.001).

Table 3.1 Summary of Lethal and Effect Concentrations of Unionized Ammonia Toxicity Testing

Species	Test duration	Endpoint	Temp (°C)	TAN tested range (mg/L)	UAN tested range (mg/L)	UAN EC50/LC50 (mg/L)	NOEC (mg/L TAN)	LOEC (mg/L TAN)
Arbacia punctulata	48 h	embryo development	20	0.10-4.16	0.0-0.267	0.174	1.56	2.08
Arbacia punctulata	0.5 h	fertilization	20	0.21-2.08	0.007-0.121	ND	2.08	>2.08
Lytechinus variegatus	48 h	embryo development	23	0.21-2.08	0.005-0.149	ND	1.87	2.08
Acropora formosa	10 d	tissue regeneration	26	0.13-2.60	0.008-0.302	0.095	1.30	1.95
Acropora palmata	48 h	larvae mortality	29	0.26-2.60	0.021-0.219	0.074	1.04	1.56
Acropora palmata	48 h	larvae mortality	31	0.26-2.60	0.024-0.249	0.055	0.52	1.04

Lethal and effect concentrations of unionized ammonia on corals and sea urchins

The calculated LC_{50} and EC_{50} values for the ammonia exposure experiments are presented in Table 3.1. The results indicate that the existing national WQC (US EPA 1989) is unlikely to be protective for either sea urchin species under the acute criterion, but the chronic WQC would be protective. Both the adult coral nubbins (EC_{50}= 0.095 mg/L UAN) and coral planula larvae median lethal concentration values (LC_{50} = 0.074 mg/L UAN at 29 °C; 0.055 mg/L UAN at 31 °C) are slightly above the chronic national WQC (0.035 mg/L UAN). In contrast, the toxicity thresholds are well below the acute national WQC (0.223 mg/L). Furthermore, our data show that a 2 degree increase in temperature during embryo development has a 35 % decrease in larval the LC_{50} values. With the threatened ESA status of *A. palmata*, whether the potential loss of 50 % of larvae, as reflected in the LC_{50}, is an acceptable loss is a management decision when setting Water Quality Standards. In the context of these data, review of current water quality criteria and water quality standards that are established in local jurisdictions that are protective of coral reef resources is encouraged.

Data Availability

These experiments are in progress, data are available on request.

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Chemical Contaminants in Marine Mammals

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Key Species and Bioinformatics Branch

Stressor, Detection and Impacts Division

September 2020

Historical Perspective

The NOAA/NOS/NCCOS Charleston laboratory has a long history of chemical contaminant research in marine mammals, particularly with coastal bottlenose dolphins (*Tursiops truncatus*). Originally part of NOAA's National Marine Fisheries Service, the Charleston laboratory developed its key species research program in the late 1980s providing scientific support for marine mammals to better understand the impacts of contaminants, provide support for law enforcement, and conduct local dolphin health assessments.

Contaminant research into the impacts of persistent organic pollutants (POPs) started in 1987-88 in response to a bottlenose dolphin unusual mortality event (UME) which resulted in the deaths of nearly 800 dolphins from New Jersey to northern Florida. Dolphin contaminant studies in the Gulf of Mexico followed two additional UME events in 1992 and 1994. Expertise developed from these initial studies proved invaluable in supporting subsequent NOAA federal investigations throughout the 1990s assessing the impacts of contaminant releases on bottlenose dolphin mortality and morbidity. Federal investigations we supported included (1) the multi-species UME in Virginia, North Carolina, South Carolina, and Georgia in 2009 (stranding investigation), (2) the bottlenose dolphin UME in South Carolina in 2011 (stranding investigation), (3) the US East Coast bottlenose dolphin UME from 2013-2015 (stranding investigation), (4) the NRDA, GOMRI, and CARMMHA Deepwater Horizon Oil spill investigations in Barataria Bay, LA bottlenose dolphins from 2010-2017 (yearly capture-release health assessments), and (5) the NRDA LCP-Brunswick in 2009 (capture-release health assessment).

Juvenile bottlenose dolphin dead stranded in the Stono River, Charleston, SC

In addition to contaminant research, the NCCOS Charleston Laboratory also led local NOAA dolphin health assessments. Critical species in Southeastern waters, local dolphins are a mixture of estuarine stocks that remain in Charleston year-round and coastal migrating stocks. Our estuarine population have developed unique strand feeding patterns that are seen in only a

few places in the world. Starting in 1995, the Charleston Laboratory began assessing the health of our local dolphin populations using capture-release methods which included studies focusing on chemical contaminant levels in free-swimming animals. Health assessments continued in collaboration with other studies conducted in Beaufort, NC and Cape May, NJ. By the early 2000s, the marine mammal community was beginning to move away from using stranded animals for contaminant studies, particularly with research involving POPs. In response, NCCOS Charleston developed protocols incorporating dart biopsies collecting blubber and skin samples for contaminant studies. In 2003, the lab began a collaboration with the Harbor Branch Oceanographic Institute (HBOI) to better understand health and contaminant loads of dolphins in two locations, the Indian River Lagoon (IRL), FL and in Charleston, SC through the Health and Environmental Risk Assessment (HERA) program. One capture-release study per year, per site was conducted from 2003-2005, 2007, 2009. Once the HERA program was finished, HBOI continued to perform health assessments yearly in the IRL with the assistance of NCCOS staff, but not in Charleston. The last health assessment in Charleston was conducted in 2013, again with the main focus being on POPs.

Dolphin Capture-Release Health Assessment in Charleston, SC

While the need and importance of the work has been well recognized, resources available to the Marine Mammal Program have changed in recent years, most notably the decrease in the number of available staff (10 staff in 2016 to 1.5 in 2020). This has required us to refocus in recent years to develop a large number of highly successful partnerships investigating disease

and health in marine mammals including the heavy metal and microplastic research presented as part of this review.

Tissue Sample Collection

As stated above, long before October 2016, NCCOS Charleston has been collecting marine mammal tissues from both dead stranded cetaceans and pinnipeds (ie, seals), live captured bottlenose dolphins, and from remote biopsy darts. Approximately 5-50 g of liver and kidney tissues were collected from dead stranded marine mammals according to standard necropsy protocols (Geraci and Lounsbury 2005; NCCOS Coastal Marine Mammal Assessments Program [CMMAP] SOP) and stored at -80°C until analysis for trace elements. Blubber samples were further collected from dead stranded marine mammals according to standard necropsy protocols (~50 g) and stored as above before testing for POPs. Tissue samples were only collected from fresh dead animals (termed condition code 2; condition code 1 is an alive animal, code 3 is moderately decomposed, code 4 is advanced decomposition, and code 5 is skeletal).

Necropsy of false killer whales at NCCOS CCEHBR necropsy lab

This practice has continued to the present day. Since 2016, stomach samples have been collected for microplastic analysis and method development. Blubber samples from capture-release health assessments and remote biopsy were collected according to established protocols (Fair et al.

2006) for POP analyses. Likewise, blood samples were collected aseptically according to established protocols for trace elements and other blood chemistry analyses.

Due to reduction in staff within the CMMAP, we have leveraged personnel resources through MOA's with Coastal Carolina University (CCU) who administers the South Carolina Marine Mammal Stranding Network under a Stranding Agreement (SA) with the National Marine Fisheries Service, and the Lowcountry Marine Mammal Network (LMMN) a non-profit organization with a SA with NMFS and who specializes in outreach and education. The collaboration with CCU has been in effect since 2009 and LMMN since 2018. CCU employs a marine mammal technician that is stationed at NCCOS Charleston to respond to marine mammal strandings and assist in the collection of the samples for contaminant studies as described above.

Archiving

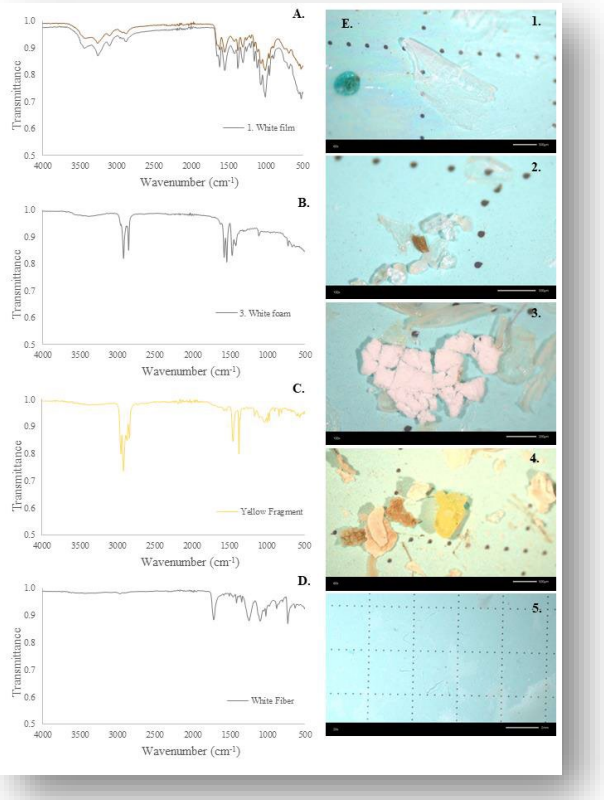
The CMMAP has an extensive list of tissue samples that are archived in -80°C ultralow freezers for chemical contaminant studies. The list includes greater than 3000 tissue samples including: blubber, liver, kidney, skin, muscle, whole blood, serum, and plasma. Greater than 300 of these samples have been collected since 2016. Further, the CMMAP has an extensive list of bone and teeth, which can be used for heavy metal analysis, archived currently at the South Carolina State Museum in Columbia, SC through an MOA. Currently, there are skeletal remains of >1500 individual animals archived at the museum, most of them bottlenose dolphins. All samples are tracked in the NCCOS Marine Mammal Information System, a web-based platform developed between 2017-2019, to replace the outdated Access-based database. The general public will soon be able to access portions of this database and can request samples for contaminant studies if needed.

The CMMAP also contributes marine mammal blubber, liver, and kidney samples of only fresh dead animals to the National Institute of Standards and Technology's (NIST) National Marine Mammal Tissue Bank, located at the Hollings Marine Laboratory (HML) in Charleston, SC. From 1998-2020, CMMAP has contributed 360 tissues from 128 individual marine mammals and 16 different species. The CMMAP also contributed a whole liver of a pygmy sperm whale (*Kogia breviceps*) to NIST for heavy metals reference material. Subsamples from this liver reference material has been used for interlaboratory comparisons and numerous research studies over the years (R. Pugh, NIST, personal communication).

Projects Supported

Since 2016, samples collected through the CMMAP for chemical contaminant studies have been utilized mainly for heavy metals analysis and microplastic research (Bryan et al. 2017; Battaglia 2019; Pfeifer 2020). Furthermore, NCCOS contract staff in the former Protected Areas and Resources Branch, now the Key Species Branch, were still conducting some chemical contaminant studies between 2016 and 2019 before their transition to the National Marine Mammal Foundation (NMMF).

For CMMAP, chemical contaminants research has focused primarily on planning and developing microplastic research in coastal dolphin species in a phased approach with the implementation of studies through graduate students at the College of Charleston Grice Marine Biology Program. The first phase (Battaglia 2019) was to develop the methodology to extract microplastics from the stomachs and intestines of stranded bottlenose dolphins and begin to quantify the different types of microplastics present (eg., fibers, fragments, etc.).



Microplastics from gut of bottlenose dolphins and associated FTIR analysis

Phase 2 (Pfeifer 2020) expanded the research on different species of dolphins in various regions of the US, quantified microplastics in these different species, and compared to regional sediment research on microplastics that was primarily conducted by the National Park Service (NPS). COVID19 has derailed the next phase to examine the chemical composition of microplastics found in dolphins where funding was secured from the Marine Debris Program in collaboration with Auburn University and Skidaway Institute of Oceanography in Georgia. Further, CMMAP has been supplying ovary tissue to the University of South Carolina to begin work on the exposure of dolphin reproductive tissue to phthalates in a microfluidic chamber. This study aims to show a direct cause and effect relationship of phthalates as endocrine disruptors in negatively affecting the female reproductive system.

In 2018, CMMAP led a multi-institutional team for planning and developing a proposal on heavy metals analysis of tissues from coastal Northern river otters and bottlenose dolphins following large scale precipitation events over a 10 year period in the Gulf of Mexico for a RESTORE Science solicitation. The proposal made it to the final round of selection but ultimately was not selected for funding. A similar, smaller scale pilot study would have been completed by May 2020, had COVID19 not suspended operations. This pilot study aims to analyze tissues from dead stranded bottlenose dolphins and dead road killed or trapped, or live-caught coastal river otters in SC, for heavy metals contamination.

At present, the CMMAP is collaborating with Texas State University to determine if chemical oil signatures can be found in the growth layers of dolphin teeth. Because teeth contain a permanent record of trace elements, it is hypothesized that trace elements in relatively high concentration in crude oil, such as vanadium and/or nickel, may spike in a growth layer when environmentally exposed. Teeth from stranded dolphins prior, during, and after the *Deepwater Horizon* oil spill will be used to determine if oil exposure can be determined. Two different methods are being used to detect the metals signatures using SEM EDS and LA- MS.

Numerous samples collected from stranded marine mammals have been used for collaborative projects performed outside the agency. For instance, a Medical University of South Carolina (MUSC) PhD candidate and NIST employee requested heart tissue samples from pygmy sperm whales stranded in SC to investigate the role of selenium on the progression of cardiomyopathy, a condition with very high (>50%) prevalence in stranded animals (Bryan et al. 2017). Further, NIST has used blubber samples collected from the program to develop rapid and reliable steroid hormone profiles using LC-MS/MS techniques (Boggs et al. 2017) and for a cortisol-cortisone metabolism study (Galligan et al. 2018), both of which are relevant in understanding the pathways and effects of POPs as endocrine disruptors in dolphins.

Prior to moving over to the NMMF, employees of PAR completed work that had been done in St. Andrews Bay, Florida, specifically on the POP contaminant burdens from dart biopsies of dolphins, photo-identification, and tracking (Balmer et al. 2019). This work, funded by the USN and NMFS OPR, analyzed contaminants of dolphins in close proximity to the Tyndall Air Force Base and added to the knowledge of range of these dolphins, who could have been exposed to the DWH oil spill.

Value of Work

Marine mammals are considered sentinels of our coastal oceans (Bossart 2011), providing a barometer of contaminant burdens in the environment that may negatively impact marine mammal population and human health. These animals are long-lived, feed at the top of the food chain, concentrate contaminants in a unique fatty blubber layer, and many populations are coastal residents with relatively small home ranges that share the same coastal areas and food resources that humans do. Baseline contaminant data has shown that marine mammal populations that inhabit areas of high human populations with high industry and agriculture processes have high tissue contaminant burdens (Aguilar et al. 2002; O'Shea and Tanabe 2003;

Houde et al. 2005; Fair et al. 2010). As such, marine mammals are relevant as important key species and sentinels because of their widespread coastal distribution and as apex predators for biomonitoring spatial and temporal trends in contaminants that could be harmful to coastal human populations. These contaminants, or persistent organic pollutants, are widely distributed and because of their lipophilic nature in fats of humans and marine mammals, can be mobilized throughout the body to cause adverse health effects that have been linked to increases in infectious disease, immunosuppression, reproductive impairment, and endocrine disruption, among others (Bossart 2011).

NCCOS Charleston was at the forefront of chemical contaminant research in marine mammals since the early 1990s. The work has played an important role in determining causes of marine mammal UME's, provided insight into the role oil contaminants (PAHs) played in the health and reproduction of dolphins following the DWH oil spill (Schwacke et al. 2017; Smith et al. 2017, Colegrove et al. 2016; Balmer et al. 2019), and detailed the exposure of dolphins and humans to PCBs, mercury, and PAHs in Brunswick, GA responsible for reproductive problems for dolphins inhabiting that area. All of this work supports NOAA, NOS, and NCCOS missions to protect and conserve our natural resources and to maintain healthy coastal ecosystems.

The use of marine mammal tissues from carcasses stranded on beaches has allowed for the archive of tissues for retrospective contaminant analyses and the contribution of marine mammal tissue to the National Marine Mammal Tissue Bank at NIST, including Standard Reference Material (whale liver) that is continuously being used by researchers for various contaminant studies around the US.

Current and ongoing projects including heavy metals in coastal river otters and bottlenose dolphins, microplastics in marine mammals, and the use of dolphin teeth to serve as a proxy of exposure to oil trace elements all use opportunistic sampling methods of dead stranded marine mammals. The heavy metals research on two coastal apex predators (river otters and dolphins), both of which are considered sentinel species, will substantially increase our knowledge of heavy metal burden in these species as well as in our coastal environment. Very little information is available on coastal river otters and the effects heavy metals may have on reproductive or neurological health. Using a multi-species approach to assessing coastal environmental health will be valuable to resource managers and state environmental agencies in coastal areas where humans compete for food resources.

College of Charleston graduate student Jenna Klingsick holds a river otter prior to necropsy.

Likewise, the role microplastics have on the health of marine mammals is largely unknown even though phthalates contained in microplastics are known endocrine disruptors. Our research on cetaceans was the first such research in North America for bottlenose dolphins, harbor porpoises, and the long-beaked common dolphin. These data are important for ORR's Marine Debris Program and is a strategic goal in the Wildlife and Habitat Interactions section of the 2019 Southeast Marine Debris Action Plan. Further, microplastic data generated from these projects have been uploaded into the NESDIS/NCEI archived database and will be important information for NCEI's proposed global database on microplastics in the environment and in wildlife (Scott Cross. Personal communication, NCEI). ORR also has interest in the timing of exposure of chemical contaminants from oil spills to marine wildlife that has been a subject of the DWH Natural Resource Damage Assessment (NRDA). Our research, in collaboration with Texas State University, to use dolphin teeth to determine oil signatures has met with positive responses from ORR and could corroborate when animals are exposed to a spill.

Due to our small staff size, we've developed close collaborations with the College of Charleston's Grice Marine Biology and Environmental Sciences Graduate Programs, the Medical University of South Carolina, and Texas State University to complete our mission in regards to chemical contaminants. Since 2016 graduate students have performed two projects related to microplastic exposure in dolphins, blubber analysis, heavy metals analysis in river otters and dolphins, and oil signature preliminary analysis in dolphin teeth. As a result, the message of what we do has been able to be distributed among a wide audience including presentations at their representative universities, at the Society of Environmental Toxicology and Chemistry (SETAC)-Toronto, at the Southeast Atlantic and Mid-Atlantic Marine Mammal Symposium (SEAMAMMS), the Gulf of Mexico Research Initiative (GOMRI) meetings, and at

internal venues such as the Bioeffects Workshop. Publications from the Program related to chemical contaminants since 2016 can be found below.

Our research and activities have also generated much public interest locally, nationally, and internationally. At minimum, four or five local news reports per year are provided to newspapers and television stations in the State on causes of stranding events. As high profile species, public interest in the role of contaminants in the deaths of stranding individuals is high. This information is provided to local schools, nature organizations, and fishermen through invited presentations or booths at outdoor science events. The microplastic research in collaboration with the College of Charleston was featured in the BBC Blue Planet II documentary series on plastics in the oceans in 2018. This generated much interest both locally and nationally. Facts from what we've learned through contaminant research on marine mammals over the years has even been a source of information for two recently published children's book series: Seaside Sanctuary: Orca in open water (Berne 2019) and Seaside Sanctuary: A dolphin named Star (Berne 2019). Further, our past research involving dolphin capture-release in the southeastern US has generated numerous media events and coverage both locally and nationally, particularly those that investigated contaminants from the *Deepwater Horizon* oil spill and the effects on dolphin health.

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