Coastal Response Research Center
Dispersant Initiative – Request for Proposals
Grant Funding Application

Project Title: Understanding chronic impacts of chemical dispersant and chemically-dispersed oil on behavior, molting success, and hormone status and of blue crab larvae: inputs for recruitment and population models.

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Priority area: Biological Effects of Dispersants and Dispersed Oil on Surface and Deep Ocean Species

Abstract:

The use of chemical dispersants as a response option following an oil spill has always been controversial, and numerous reports and reviews have highlighted the many data gaps and uncertainties regarding their use (e.g. NRC, 2005). Their goal is to reduce the impacts of oil and provide a net environmental benefit. However, the unprecedented spatial and temporal extent of their use during the 2010 Deepwater Horizon (DWH) incident led to intense media and public outcry, particularly regarding their novel subsurface use directly at the wellhead. A meeting organized by the CRRC in May 2010 concluded that their use was indeed beneficial overall, but it also highlighted data gaps and uncertainties especially regarding the length of time of their use and the need for continued re-evaluations. There are still many unknowns regarding the environmental fate and effects of dispersant and chemically dispersed oil, particularly to non-standard laboratory species and at chronic and sub-lethal levels. The blue crab, a keystone species of the Gulf of Mexico and Atlantic coast, is crucial to both the ecology and economy of both regions. Very little is known regarding the impacts of oil, dispersant and chemically-dispersed oil on any stage of the blue crab life cycle. The timing of the DWH incident coincided with crab spawning, whereby pelagic crab early life stages were exposed to oil and/or chemically-dispersed oil offshore in the photic zone surface waters. Blue crab recruitment, and thence fishery abundance, depends upon larval survival in the coastal zone, which in turn hinges on larval perception, feeding, molting, and swimming abilities. Even at sub-lethal levels (as determined by LC50 methods), oil, dispersant or chemically-dispersed oil may impair these behaviors, resulting in larvae that do not survive until the megalopa/crab transition. These behavioral effects would be therefore functionally lethal, because of the ensuing growth, feeding, recruitment, and ultimate population level consequences.

The proposal described herein seeks to understand the impacts of dispersant, oil and dispersed oil on blue crab larvae at various stages of development. Although we will carry out some preliminary experiments to define acute (for comparison to other species in the literature) and sublethal doses our goal is to focus on the sub-lethal effects that will ultimately result in recruitment failures. These include novel approaches to ascertain physiological (molting success and endocrine disruption) and behavioral perturbations. UMCES has a unique collection of crab hatchery technology and scientific expertise to address the question of how oil, dispersant and dispersed oil affects blue crab larvae using a multiple toolbox of molecular, biochemical, physiological and behavioral endpoints. Findings and methodology of the study proposed by UMCES investigators will have value as tools to assist managers and scientists in rapid risk assessment/decision making for dispersant use during spills. The project will also develop methods adaptable for simple *in situ* and laboratory assessments of the effects of oil, dispersant and dispersed oil on blue crab larval behavior. Interactions with end users at all phases of the project will ensure that protocols and metrics are adapted to be useful in modeling or forecasting the chronic effects of "subacute" levels of chemical dispersant use on larval crab behavior and crab recruitment.

Background and Overview

1. Problem Statement

1.1 The Deepwater Horizon (DWH) incident used unprecedented volumes of dispersant.

The recent Deep Water Horizon incident (April 20th, 2010) has reminded us of the many data gaps and uncertainties regarding the long-term impacts of oil and chemically dispersed oil on ecologically and economically important species. In addition, unique characteristics of this oil leak have added questions given it's depth, volume and duration and unique response options used (i.e. subsea dispersant application). Though the wellhead was capped on July 15th, oil still lingers in sediment. This proposal is designed to fill some of these data gaps regarding the effects of oil, chemical dispersants and chemically dispersed oil on behavior and growth processes that may ultimately impact blue crab populations and fisheries landings.

The limited data on the effects of oil and dispersants on blue crabs, especially during their sensitive larval early life stages illustrates how little is known about the consequences of oil spills and impacts of response options for this keystone species. There are concerns about the possible effects on the blue crab population as a whole and to the food web, including human seafood consumers. The spill occurred during the most **active growth** and **recruitment** period for blue crab. Larvae and early life stages of organisms are often particularly vulnerable to the effects of dispersant, oil and dispersed oil (see NRC, 2005). Small translucent surface pelagic organisms are particularly at risk due to phototoxicity mechanisms (e.g. Barron et al., 2003; NRC, 2005). Polycyclic aromatic hydrocarbons accumulated by these organisms have been shown to be up to 50, 000 times more toxic in surface waters compared to normal laboratory light regimes because of phototoxicity mechanisms (NRC, 2005).

Given the observations of population declines and potential shell disease in the last two years in blue crabs in Louisiana coupled with reports in the media on with mortality in the shipping of soft shell crabs, our proposal seeks to understand the **long-term effects** of chemical **dispersants** and dispersed oil on the **physiological process of growth and molting** and on essential **behaviors** in the blue crab (*Callinectes sapidus).* Hatchery-reared larvae will be exposed to Sweet Louisiana crude oil (MC252) and/or dispersants (using Corexit 9500A) in a laboratory environment using environmentally realistic time and chemically-characterized dose regimes that have been used in numerous previously funded proposals in UMCES laboratories (Mitchelmore, Rowe, Heyes, Chung). Endpoints including survival, growth and photo/geo-tactic **behavior** will be determined to assist in population and recruitment models. We will also measure the level of PAHs in tissues for assessment and correlations of their impact on crab larval behavior. All exposure media (water/sediment) will also be fully chemically characterized.

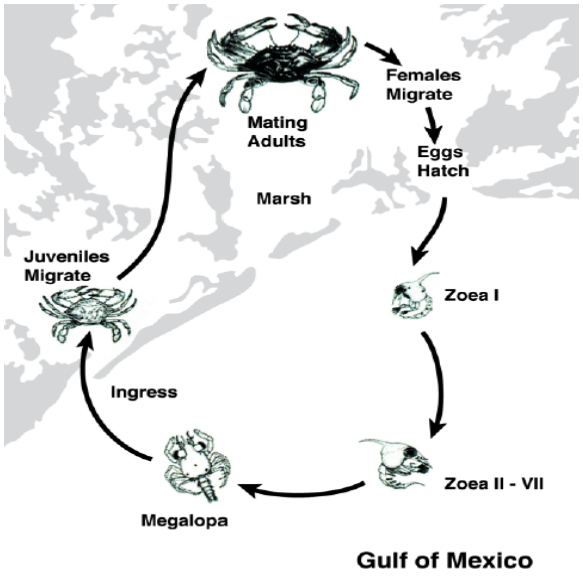

Figure 1: The blue crab life cycle, illustrating the importance of larval stages to recruitment and their location in the Gulf of Mexico..

Oil spill responders and resource managers face a difficult task in determining appropriate strategies for oil spill remediation. Comparing the possible ecological consequences and toxicological impacts for each response option (natural recovery, mechanical recovery and dispersant use) is difficult, firstly, because each oil spill represents a unique situation and secondly, it is often difficult to extrapolate from published research data into field predictions. This is particularly the case when trying to ascertain the possible responses of resident field-located organisms compared to model organisms used in standard laboratory toxicity tests. Final decisions must be made regarding the impact of the ecosystem as a whole and often the protection of one species (or habitat) will be at the cost of another. Ideally, informed decisions on the possibility of long-term consequences would be based on sound scientific data using species of organisms at risk in the environment. The ultimate goal for researchers is to provide data that will aid in reducing the uncertainty associated with oil spill response decisions. The implementation of several of the recommendations made in the 1989 NRC report (NRC, 1989), particularly with respect to the standardization of toxicity test methods, has advanced our understanding upon the risks associated with various response options, especially chemical dispersant use. However, as highlighted in the report from the March 2012 workshop on dispersant use (CRRC, Research Planning Incorporated, 2012), there are unknowns regarding chronic effects of dispersants and the effect of dispersant on aquatic animal behavior. Developing transferable methods and metrics to assess these data gaps would represent substantial progress.

1.2 Choice of species and ecological and economic significance of blue crab.

Information on the long term or chronic effects of dispersant and dispersed oil on early life stages of marine organisms is lacking (NRC, 2005). Larval stages of most species are easily overlooked. Even when they are observed, because larvae are so dispersed and very much at the mercy of large scale environmental or weather factors, large spatial and temporal fluctuations of larvae are the norm. Thus, during a spill event, it is difficult to confidently link decreases in larval abundance to the spill or use of dispersants. We have chosen to focus on the larval (pelagic) stage of blue crab larvae because it is potentially the facet of the blue crab life cycle most vulnerable to oil and dispersants, particularly given the timing of the DWH spill that coincided with the reproductive period of the blue crab (see Figure 1). Blue crab larvae are of crucial importance to the Gulf ecosystem and economy. In the Gulf, the annual dockside value of blue crab exceeds $70 million; in the Chesapeake Bay and Atlantic coast, harvest are worth another $70-80 million. The species is also the foundation of a valuable fishery internationally, such as in Lake Maricaibo, Venezuela, where the fishery coexists with intense oil production. More importantly, blue crab larval abundance largely determines post-larval and juvenile recruitments and subsequent landing.

1.3. Elements of crab life history potentially sensitive to disruption by oil or dispersants

<u>"Sub-lethal" effects may still remove an individual from the population:</u>
One of the primary response options considered after an open ocean spill event is that of chemical dispersion. The purpose of chemical dispersants is to facilitate the movement of the bulk oil into the water column. Chemically dispersed oil is a complex and multi-phased mixture

composed of surfactant-coated oil droplets that can vary from small colloidal to larger particulate sizes, in addition to containing dissolved PAH and surfactant components (dissolved and droplets). Previous work (by others) on oil and dispersants has established the acute endpoints (i.e. 48 to 96 hour LC50s) for many species, including invertebrates (see NRC, 2005 for summary tables). The report from the March 22, 2012 workshop on the ecological effects of dispersants and oil included the recommendation that chronic, low level effects be better investigated. Recommendations specifically mentioned data gaps in the state of knowledge of effects on larval stages of marine organisms (CRRC report: Biological Effects of Dispersants and Dispersed Oil R&D Need Priority 3).

In this context, four of many possible scenarios in which a 'non-lethal' exposure may lead to removal of an individual crab larva from the population are:

1. Endocrine disruption resulting in the inability to molt to the next larval instar, resulting in arrested development and death.
2. Inadequate feeding behavior, resulting in starvation or failure to grow to the next larval instar.
3. Absence or inadequate phototaxis or geotaxis, resulting in sinking of larvae to deeper water in the coastal ocean.
4. Inadequate vertical migration by megalopae in response to tidal or salinity cues, resulting in the inability to enter and settle into estuarine nursery habitat.

Larval behavior: Larval behavior is crucial to recruitment. Recruitment of many species is dependent on behavioral responses of larvae to environmental cues. For example, larval crabs display vertical migrations that allow them to optimize feeding and predator avoidance, as well as swimming behaviors that result in their movement from coastal zone into estuarine waters at the larval-megalopa transition (Epifanio 1988 and Blaxter and Hallers-Tjabbes 1992). Newly hatch zoeae are negatively geotactic and positively phototactic (Sulkin, 1984; Sulkin et al., 1980; Forward, 1990). This assures that zoeae become entrained in the layer of warm, lower saline water flowing from estuaries.

Larval crabs are released in the coastal zone (see Figure 1) spend 4 weeks growing and molting into the megalopa stage, which re-enters the estuarine waters upon tidal and salinity cues, then transitioning from a planktonic to a benthic stage. Once settled, megalopae metamorphose into benthic first instar crabs. The ability to respond to stimuli depends on sensory perception and locomotion. Toxic compounds are capable of impairing either of these processes at levels that may not result in acute mortality, but will remove crabs from the ecosystem nevertheless. Low concentrations of hydrocarbons or other chemicals may impair larval perception or the ability to respond by changing swimming behavior. There have been recruitment failures potentially associated with the DHW. For example, the white shrimp harvest in LA is said to be down by 80% in 2011, but the cause is unknown. NY Times 2011, see page 7). The DHW incident is a suspected cause, but no data exist to link the two.

Decreased feeding rate, inability to maintain position in the water column, or failure to perform vertical migrations can all abrogate the ability of larval crabs to persist in the coastal zone or recruit into nursery habitat. Vertical migration depends on stage-specific larval swimming behavior (Ouellet and Allard 2006).

Growth and Molting : Molting is a necessary and periodically reiterated process for somatic

growth and reproduction of the blue crab. A blue crab molts 27-29 times from hatching (size <1 mm) to adult stage (carapace width,~120- 180 mm). The success of each molt cycle is dependent on proper orchestration of a suite of neuropeptide (crustacean hyperglycemic hormone family) hormones and physiological changes. Molting stages (late premolt, ecdysis, and immediate postmolt stages) are vulnerable periods in the life of blue crabs, during which they are susceptible to mortality by predation, hypoxia, and infection. Molting, including hatching, is regulated by an endocrine axis that is negatively affected by environmental perturbations such as heavy metal ions, hypoxia, and polycyclic aromatic hydrocarbons (PAHs) (Zhou 2005). Because the process is an integrator of both internal and external influences, and a necessary part of crab growth and reproduction, it is crucial to understand the effects of crude oil and dispersant on molting. Potential effects include disruption of growth and development and compromised immune status, both of which will contribute to high mortality and population dynamics. Especially crucial, and hard to study with wild larval crabs, is the **behavior** of specific life stages (zoea). Our system at IMET is ideally suited for such studies.

1.4. The state of knowledge of the effects of oil and dispersants on marine crustacea and other invertebrates.

Various organisms have been tested for toxicity using these methods, however, in a particular oil-spill situation many of the local resident organisms will not have undergone these tests to allow decisions makers to accurately assess their unique situation and, therefore, extrapolations are often made using the data obtained from similar test species. Organisms vastly differ in their sensitivity to oil and/or chemical dispersants (see NRC, 2005 for tables summarizing the literature on toxicity test results), which may be a reflection of their basic physiology, life-stage or simply the extent of bioaccumulation of toxic components at the site of toxic action.
The extent of bioaccumulation by an organism will ultimately be related to the concentration and duration of chemical exposure.

The composition or profile of PAHs in oysters has been observed to change with time following oil spills but this change could reflect a change in exposure because of the relative differences in the degradation rates of individual PAHs (Yamada et a;. 2003) and/or differential rates of excretion/metabolism (Soriano et al 2006). The importance of determining exposure and uptake of specific PAHs with respect to toxicity has only recently being highlighted. For example, alklylated PAH can be more toxic than nonalkylated counter parts (Turcotte et al 2011, Fallahtafti et al 2012) and uptake is different in the presence of dispersed versus nondispersed oil (Luna-Acosta et al 2011).

<u>Previous data pertinent to this proposal</u>

J. Sook Chung, conducted an analysis of ecdysone and ecdysone response gene expression, i.e., ecdysone receptors in larvae, and reported that the onset and completion of molting is driven by ecdysteroid during the life cycle of *C. sapidus*. We reported that a peak concentration of ecdysteroids at 230-330 ng/ml in hemolymph is repeated in each molt cycle (Chung, 2010), the level of which is regulated by eyestalk neuropeptides (Chung et al., 2010). The mode of PAH action has not been completely defined yet. In crustaceans, however, it interferes with molting process as reported (Oberdörster et al., 2000a; Oberdörster et al., 2000b; Oberdörster et al., 1999).

Studies by Mitchelmore and colleagues (Mitchelmore and Baker, 2010) on the effects of dispersant on corals using traditional mortality endpoints, revealed that behavioral changes

(polyp pulsing) were evident at much lower dispersant concentrations and that delayed effects and mortality were observed. This concept has been explored as well by Faimali et al (2006), who devised a 'swimming speed endpoint' to complement mortality endpoints. In the example shown in Figure 2, the pesticide methomyl had the same magnitude effect on swimming at less than two orders of magnitude the concentration that it effected mortality. The study by Faimali et al used an accurate and sophisticated video tracking method that is not available to a wide range of research (university) or state laboratories. We will endeavor to adapt simplified methodologies to make this valuable conceptual behavioral endpoint approach more widely accessible.

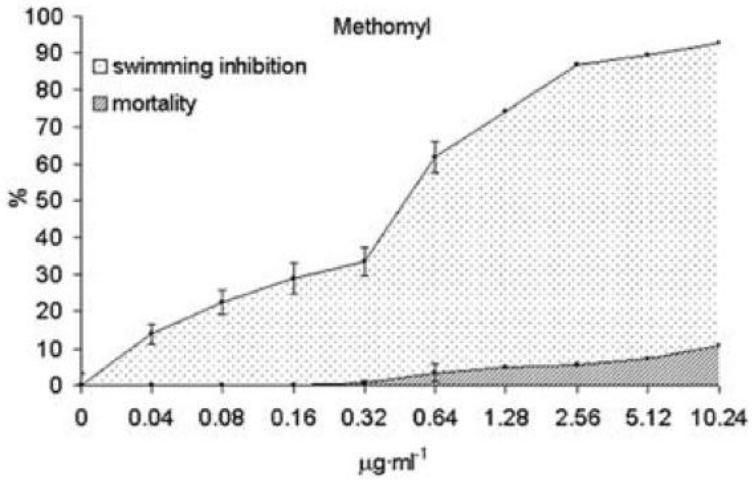

Figure 2: Example, from Faimali et al 2006, of a swimming speed effect that occurs at far lower pollutant levels than acute mortality. In the wild, such swimming perturbations will neverhtless likely result in mortality.

Specific data on the impacts of oil and dispersants in blue crab life stages is lacking. In 2011 a summer undergraduate intern (REU student) carried out some preliminary acute toxicity studies with various blue crab larvae life stages. LC50 levels increased with age, i.e. the lowest LC50 values (i.e. highest acute toxicity) was observed in the lowest zoea stage examined (stage 3-4; LC50 7.7 ppm, Schuchardt, unpublished data). Blue crab megalopa and juvenile blue crab appeared to be much less sensitive with LC50 values 252-1620 ppm).

1.5. Problems with crab and shrimp harvests/health following the DHW

There have been many anecdotal reports of problems with health or abundance of crabs and shrimp in the two years following the DWH incident. These reports have remained in the popular press only, and have not been accompanied by reviewed or grey literature citations. To some degree, the NDRA process and the un-resolved issues of liability may have inhibited the free flow of information about fishery or ecological damage of the DWH.

- Eyeless shrimp- example news report:
(http://www.aljazeera.com/indepth/features/2012/04/201241682318260912.html)
- Shrimp harvests down in 2011 example news report:
(http://www.nytimes.com/2011/10/11/us/gulf-shrimp-are-scarce-this-season.html).
- A drop in the abundance of crabs has also been discussed in the media.

The anecdotal reports that crab and shrimp harvests are off, more than a year after the spill, may indicate that the spill has affected recruitment of these species. This suggests that larval survival has been reduced. Blue crab larvae released in the coastal zone need to survive and grow for 4 weeks before returning to metamorphose into megalopae that settle into lower estuaries.

1.6. Data gaps, and the need for new metrics of the effects of oil and dispersant on blue crab larvae.

Oil and dispersant no doubt persist in the sediment and water of the GoM, albeit at far lower levels than during the spill, and likely below acute mortality endpoints. This does not mean that these levels are not removing crab larvae from the population, effectively killing them by other means.

While we are proposing that blue crab may be an informative model for all crustaceans, we are also aware that toxicity in one system may not be directly transferable to another system. For example, acute effects of xenobiotics on *Daphnia* versus *Gammarius* may vary by three orders of magnitude (Ashaue et al, 2011). This difference directly addresses the question of "why not use artemia?", as that is a species already in the EPA list of test organisms. Most oil spill or related incidents take place in estuaries and open-ocean, rather than in fresh water environments. For this very reason, we strongly believe that marine species are likely more impacted than a freshwater species. More pertinently, the impact of these chemicals depends on life stage- we anticipate that earlier life stage is more sensitive to the changes in environmental conditions. In view of these aspects, we aim to use blue crab larvae at zoeal 1- 3 that are pelagic to examine if/ how chemical dispersants affect the behavior of *Callinectes* larvae.

1.8 The unique complement of technical and intellectual capacity at UMCES:

UMCES/IMET is a world leader in producing blue crab larvae and juveniles year-round, and has the expertise to measure effects of environmental and pharmaceutical components on their imminent physiological and reproductive capacity and growth. UMCES/CBL has a deep history of ecotoxicology research, with the corresponding technical capacity and academic expertise. The research expertise of all UMCES faculty involved, and the capacity to produce larval blue crabs and analyze behavior and chemical constituents are described in section 7 (Roles and Responsibilities) and 8 (Facilities).

2. Hypotheses and Objectives

Callinectes larvae are surface feeders and swim up to the surface of water to feed. We hypothesize that the dispersant and/ dispersed oil affect the surface tension of the water as well as food availability to the larvae. This in turn may have a negative influence on feeding behavior and food consumption of the larvae, resulting in the stunt growth. Our earlier study on the effect of lower salinity on the larval growth shows that the larvae exposed to lower salinity had a significantly longer molt interval and a higher mortality, compared to those kept in normal salinity (Chung et al., 2012).

Very little data exists to determine if low level exposure of crabs to dispersant and dispersed oil will negatively affect blue crab larval behavior. To fill these data gaps we propose the following three main and sub-hypotheses:

H1a: Sub-lethal levels of dispersant and dispersed oil will reduce swimming speed of blue crab larvae in the Z1 to Z8 stages.

H1b: Sub-lethal levels of dispersant and dispersed oil will reduce feeding rates of blue crab larvae in the Z1 to Z8 stages.

H1c: Sub-lethal levels of dispersant and dispersed oil will phototaxis of blue crab larvae in the Z1 to Z8 stages.

H2a: Sub-lethal levels of dispersant and dispersed oil will affect growth and molting of blue crab larvae in the Z1 to Z8 stages.

H2b: Sub-lethal levels of dispersant and dispersed oil will affect endocrine status of blue crab larvae in the Z1 to Z8 stages.

H3: Sub-lethal levels of dispersant and dispersed oil will accumulate to detectable levels in the tissues of blue crab larvae in the Z1 to Z8 stages.

Not explicitly stated in the hypotheses, but implied in their juxtapositioning, is the possibility that some of the more complex behaviors (phototaxis) may be affected by other effects (swimming speed). For example, phototaxis may be affected by swimming speed, which in turn may be affected by energy levels dependent upon feeding rate. On the other hand, if phototaxis is reduced, but swimming speed is normal, then the defect may be in light perception or ability to respond to light stimuli.
The hypotheses therefore give rise to the following Objectives:
1. Assess the effects of chemical dispersant and chemically-dispersed oil on blue crab larval growth. Specifically measuring:

- molt interval- how frequent larvae molt
- molt increment- how much growth occurs with each molt
- endocrine status- gene expression for peptide hormones regulating growth and molting

2. Assess the effects of chemical dispersant and chemically-dispersed oil on blue crab larval behavior. Specifically measuring:

- Feeding rate
- Swimming speed
- Phototaxis
- Negative gravitaxis

3. Assess the effects of chemical dispersant and chemically-dispersed oil on markers of physiological damage. Specifically measuring:

- DNA damage/genotoxicity
- Oxidative stress markers
- PAH bioaccumulation in pooled animals
- DOSS in pooled animals

3. Methods

3.1. Blue crab hatchery culture and maintenance:

The larvae that will be used for this proposal will be produced in the Aquaculture Research Center, Institute of Marine and Environmental Technology (Baltimore, MD). The blue crab hatchery protocol supported with life food chains has been previously published (Zmora et al., 2005). Extrusion of eggs and hatching process of 1-6 millions larvae of *C. sapidus* are well-synchronized in that these events take place over night, in spite that the growth rate of individual animals derived from a single cohort or brood vary. Using the hatching day of the larvae allow us to minimize the initial variation in life stage of *C. sapidus*. Daily water exchanges and chemical exposures are easily conducted by moving individual zoea from one vessel (24 or 12 well plates) to fresh water/pollutant. At least two batches of larvae will be used for the experiments described below (each set of experiments therefore being conducted at least two

Table 1. Exposure regimes: Environmentally-relevant doses will be determined by consultation with NOAA, state managers and scientists. Target doses will otherwise start at 10% of LC50 as 'high' dose.		
Exp. groups	Description	Doses
Group 1	Control –ASW alone	TBD
2	low dispersant only	TBD
3	low oil only	TBD
4	low oil and dispersant	TBD
5	high dispersant alone	TBD
6	high oil	TBD
7	high oil and dispersant	TBD

times), taking advantage of the obvious benefits of having age-matched zoea that are developing synchronously.

3.2. Preparation and chemical analyses of test solutions:
Before each set of experiments water-accommodated (WAF) and chemically-enhanced water accommodated fractions(CEWAF) will be freshly prepared using the standardized methods for preparing toxicity test solutions essentially as described in Singer *et al*. (2000), with some modifications as suggested in Clark *et al*. (2001) and Baron and Ka'aihue, (2003). A log-series of dilutions of a stock solution (i.e. a 1:1000 oil: water ratio and a 1:10 dispersant: oil ratio) will be used to prepare the exposure solutions for the preliminary acute (and sublethal) range finding tests. These tests (see section 3.4) will be standard invertebrate acute and chronic toxicity tests of 48 hours (acute) and 7-days (chronic) duration. For the definitive tests we will choose a high dilution (i.e. highest level that demonstrated no mortality in the prior 48 hour and 7-day tests) and a low dilution (at least a ten-fold lower value). However, consideration of final doses will take into account field data and laboratory data (if available) and discussion with the CRRC review board and other State and Federal personnel to ensure that our doses chosen are environmentally relevant.

3.3. Exposure experiments:
For our oil /dispersant exposure regimes we will use static, open chamber glass containers for both the acute (48 hours) and chronic (7 day) assays. Organisms will be held in a temperature controlled room (25C) and seawater will be 0.2 micron artificial seawater (30 parts per thousand salinity; so that comparisons with published literature can be maintained). Tests will be standard static acute toxicity test methods (e.g. US EPA 821/R-02-012) as described in Hemmer et al., 2011. A minimum of 6 dilutions of each exposure solution plus control will be used. Following 48 hour exposures LC50 values will be generated. Chronic exposures will also follow standard US EPA toxicity testing methods for invertebrate species

The larvae (Zoeal stage 1, Day 2) two days after hatching will be used for the exposure study as summarized in Table 1. For this, the larvae on the hatching day will be collected using their phototactic ability and transferred to a container holding 200ml of the filtered (0.2 um filter) - artificial sea water (ASW) at 30 ppt for washing. Five or six larvae will be placed gently using a wide pore plastic pipette into wells of 24-well plate, which has already filled with 1 ml of filtered ASW. The larvae will be fed with rotifers and algae as described in Zmora et al (2005). On the day 2, these larvae will be monitored under a dissection microscope and recorded for the mortality. For the exposure study, the exposure solutions will be prepared as described in section 3.3 and 1 ml of each of these solutions will be placed in each of the wells in the 24-well plates. Then, the larvae will be transferred into each well. We have already established this method for screening pharmacological studies using *Callinectes larvae* and found with the overnight-mortality is minimal (<0.1%). Replacing the water from each well often caused a

higher mortality than transferring larvae to another plate. The larvae will be fed once a day and monitored twice a day (morning and late afternoon) as stated above and placed into fresh exposure solutions every 48 hours. *C. sapidus* undergo 7-8 zoeal stages over 14 days, before reaching post-larval stage, megalopae. We will continue our experiment for 2 (acute) to 7-14 days (chronic). During this chronic exposure (14 days), the larvae will undergo molting process at least twice reaching zoeal stage 3, during which we will be able to monitor and record feeding behavior, food consumption, and molting behavior and success, along with mortality. The exuviae and dead larvae will be removed daily.

Table 2. Experimental measurements

	Effect category		
	Growth/molting	**Behaviors**	**Uptake/physiol**
Specific assessments:	* Molt interval * Molt increment * Endocrine genes	* Feeding * Phototaxis * Swimming speed	* PAH * DOSS * GSH * Genotoxicity
Exposure duration:	1-14 days	1-7 days	1-7 days
Larval stages:	Z1-Z4	Z2-Z6	Z2-Z6
Culture scale:	6/12 well plates	0.5–2 liter	4-16 liter
Larvae #/condition:	N=120-150	N=120-150	N=10,000-40,000

3.4 Behavioral assays

Swimming behavior will be assessed after 1, 2 and 7 (and up to 14) days exposure to dispersant/oil mixtures. If dramatic effects are seen with the earliest time points, later assessments will not be needed. Observations from early trials will guide second round experiments (as indicated in the timeline).

Phototaxis: Ability to swim towards light will be measured in a transparent horizontally-oriented elongated chamber (15 cm long x 4 cm wide x 2 cm deep, with 1 cm water depth) that has markings every 1 cm in the long axis. Under far red illumination (using filters described in Forward & Costlow 1974), larvae (10 to 40) will be introduced into the center of the chamber. White light (compact fluorescent or incandescent mini-spot) will be directed towards one end of the chamber for defined intervals, during which time the larvae will be video-recorded (high sensitivity CCD cameras are sensitive to far red) to record their positions. Alternatively, the location of zoea will be captured using still photography, using a flash of illumination with white light- such that zoeae move minimally during exposure. Behavior-stimulating light intensity will be measured using instruments available at IMET (Biospherical QSL-100; Licor LI-250) and intensity adjusted by distance or neutral density filters to avoid intensity that may inhibit phototaxis.

Geotaxis can be measured using a vertically-oriented transparent chamber (15 x 2 x 2 cm). Larvae (20) are introduced to the top of the column (similar to, but smaller than the chamber described by Sulkin et al 1980), which is then capped with a rubber stopper installed without allowing air bubbles. The column is inverted for defined periods of time (10-180 seconds) during which video of larval movement is recorded using far red light. Alternatively, the trial can be conducted in darkness or far red light (using filters, Forward & Costlow 1974), with a brief white light illumination to photograph the positions of larvae at defined time points.

Images and video will be analyzed to produce measurements of mm of travel per second, to allow comparisons to be made between each treatment and control (Table 1).

Swimming speed: using the digital camera and microscope set-up, zoea swimming in the shallow slide chamber (water depth 2-5 mm) under ambient room illumination will be monitored for brief periods of time. Although many research labs record swimming behavior using dedicated particle tracking image software (e.g., Faimali et al. 2006), we will assess whether this level of analysis is needed for our project. The goal is to develop a 'low tech' assay that can be transferred to many labs in a variety of settings to assess the behavior of crab larvae (or other crustacean larvae) suspected of being impacted by xenobiotics in the environment. Swimming behavior will be assessed using an adaptation of the methods of Cronin and Forward (1980). Briefly, in each trial, larvae (10) will be placed on a microscope stage in a shallow (2-5 mm deep, to constrain swimming to the X-Y plane) test chamber under far red illumination. Swimming of larvae will be recorded with a digital video camera for 10-30 seconds. Tracking can be done manually according to the methods of Forward and Costlow (1974). Video analysis will also be conducted using ImageJ with a particle tracking plug-in (Sbalzarini and Koumoutsakos, 2005). Because there can be a relationship between starvation and swimming behavior (Cronin and Forward, 1980) we will look for associations between feeding and swimming behavior.

For feeding behavior: Conducted on larvae that have been exposed to different doses of disp/oil for 1 to 7 days.
Feeding will be quantified by monitoring the uptake of fluorescently-labeled prey. The approach is to label the live rotifer prey (*Brachionus* sp.) with a fluorescent dye (calcein), then feed this to zoea. Rotifers may be directly labeled (see MEPS 2008; Fitzpatrick et al 2011). If direct staining is not intense enough, then it is possible to label the yeast that are fed to rotifers, as in Ayukai (1994). Dr. Schott has prior experience in labeling yeast and protists with calcein and fluorescein (Schott and Vasta 2003). After feeding an excess of rotifers to treated zoea (for 1-4 hours - this may change if so indicated by initial experiments), remove zoea to a small tube, remove all supernatant, grind zoea and measure free calcein fluorescence in a fluorometer (excitation and emission wavelengths of calcein are 495 nm and 516 nm, respectively.), using instruments available at IMET and at CBL). Controls will include larvae fed calcein-free prey and unfed larvae. A calibration curve of known numbers of calcein-stained rotifers will be constructed. Alternatively, we will use a confocal microscope to calculate the intensity of fluorescence in these zoeae. The most crucial way to produce comparable results is to conduct all oil-dispersant treatments in parallel using the same batch of stained prey, so that comparisons can be made directly between treatments.

Biomarkers of oil and dispersant exposure:
Genotoxicity (DNA damage) will be assessed on individual larvae using previously published methods used routinely in the Mitchelmore laboratory, based on the COMET assay as a non-specific marker of the induction of DNA strand breaks (Kinetic Imaging Komet software) (assessed microscopically on isolated cells using eipfluorescence and Image Analysis Software) (e.g. *see Mitchelmore et al., 1998*). The antioxidant responses that will be evaluated are total glutathione (GSH) concentrations (using a DTNB recycling assay), and GSH peroxidase (based on the indirect measurement of GSSG using the t-Butylhydro-peroxide (t-BOOH) assay); these

are standard spectrophotometric assays (many available as commercial kits); a 96 well plate reader can be used to facilitate rapid analyses of the large numbers of samples. Oxidative stress marker assays will be conducted on pooled larvae (0.1 to 0.5 gram total wet weight).

Bioaccumulation of PAH and dispersant:
The requirement for relatively large biomass for PAH and DOSS assays necessitates that large numbers of pooled larvae be exposed for these measurements. For each dispersant/oil dosage used in the behavior and growth studies, a separate but equivalent exposure will be conducted on a culture of 10,000-40,000 zoeae (biomass of ~0.5 to 1 gram, depending on larval instar stage). Larvae exposed for 1-7 days will be collected on a 200 micron sieve, rinsed with clean artificial seawater, and stored in -80 ˚C.

PAH's and alkylated PAHs will be quantified in water stocks and tissues. Water samples will be extracted following the methods of Reddy and Quinn (1999) and tissue samples using dichloromethane-hexane and an automated solvent extractor (ASE Dionex) followed by clean up and concentration. The four-step identification approach described in Wang et al. (2007) will be used to identify and quantify PAHs and alkylated PAHs by GC-MS. Total PAHs in water will be quantified as done by Reddy and Quinn (60). Multipoint calibration curves, response factors calculated from internal standards, and NIST SRMs will be used to validate our measurements. The focus will be on the 53 PAHs (including 29 parent PAHs, 11 monomethyl-, 9 dimethyl- and 1 trimethyl-substituted PAHs, dibenzothiophene and two methylated dibenzothiophenes) associated with the study oil but we will characterize the samples as completely as possible. The GC-MS method provides a balance between being cost effective and providing required detailed information. The method will allow early degradation products to be tracked and determine TPH with reasonable accuracy. Water samples will be tested for dispersant concentrations using LC MS-MS targeting dioctylsulfosuccinate (DOSS) and analyzed at the Columbia Analytical Sciences (CAS) laboratory (centers in Columbia MD and Chambersburg PA)..

3.5 Growth and endocrine assays
Exposure period: 14 days.

For growth and molting behavior: First instar zoea (Z1) will be collected, allowed to equilibrate 24 hrs, then exposed to disp/oil through the period of time normally required for two molts, from Z1-Z2 and Z2-Z3 as described above in the section 3.3 and Table 2. In each treatment, a total of 120-150 larvae will be used. During this period, the larvae will be monitored daily and recorded for mortality and food consumption. The expected first molt from Z1 to 2 occurs days 5- 7 and the 2nd molt of Z2 to 3, in days 10-12. At the end of 14 days' exposure, the larvae will be retrieved from each well, washed in filtered ASW, and collected into 1.5 ml tubes for ecdysone and expression analyses. Both of these assays have been established in Chung laboratory (Chung, 2010; Chung et al., 2011).

Growth and molting experiment will be conducted at least 2 times, with the batches of zoeae produced by different females.

3.6. Chemical analysis:
PAH's and alkylated PAHs will be quantified in water stocks and pooled zoea (n=>100). Water samples will be extracted following the methods of Reddy and Quinn (1999) and tissue samples

using dichloromethane-hexane and an automated solvent extractor (ASE Dionex) followed by clean up and concentration. The four-step identification approach described in Wang et al. (2007) will be used to identify and quantify PAHs and alkylated PAHs by GC-MS. Total PAHs in water will be quantified as done by Reddy and Quinn (60). Multipoint calibration curves, response factors calculated from internal standards, and NIST SRMs will be used to validate our measurements. The focus will be on the 53 PAHs (including 29 parent PAHs, 11 monomethyl-, 9 dimethyl- and 1 trimethyl-substituted PAHs, dibenzothiophene and two methylated dibenzothiophenes) associated with the study oil but we will characterize the samples as completely as possible. The GC-MS method provides a balance between being cost effective and providing required detailed information. The method will allow early degradation products to be tracked and determine TPH with reasonable accuracy. Water samples will be tested for dispersant concentrations using LC MS-MS targeting dioctylsulfosuccinate (DOSS) and analyzed at the Columbia Analytical Sciences (CAS) laboratory (centers in Columbia MD and Chambersburg PA).

3.7 Statistical analyses:

The experiments are designed for analysis by single factor analysis of variance (ANOVA) followed by Tukey's pairwise comparisons to compare measured responses among treatments. Un-paired *2*-tailed students *t*-tests will be conducted under a Type I error rate of $\alpha = 0.05$, and significance of treatment differences will be judged based upon a sequential Bonferroni adjustment of this error rate. Prior to statistical analyses, data will be tested for normality and homoscedasticity. In the event that these assumptions are not met, transformations will be employed or nonparametric methods will be used.

4. Innovation

This proposed research would be the first study of its kind to examine the toxicity of the Corexit 9500 dispersant and oil on the important keystone species, the blue crab. Despite the importance of this species ecologically and economically only one prior study (Fucik et al., 1995) has investigated the acute toxicity of Corexit 9527 on these species. No data is available on the toxicity of Corexit 9500 or on the acute or chronic impacts of oil, Corexit 9500 or chemically-dispersed oil on these species. We will combine state of the art chemical and biological measures to assess the impact of oil and/or dispersant and furthermore provide novel data on the behavioral impacts to blue crab larvae, which may be transferable to other crustacean and invertebrate larval species. . Our multiple metrics for sublethal impacts will include an in depth detailed study of both growth/molting effects, endocrine gene expression effects, behavioral impacts which will all feed into estimations for population and recruitment models. Furthermore, it will be the first study to define the average body burden of PAH and DOSS in larval crabs exposed to acute and chronic long-term dispersant/oil exposure solutions.
One eventual outcome of the proposed research is the development of a behavioral bioassay to assess whether field –caught larvae are showing impacts of oil or dispersants.
Results of this study may be applicable to other crustacea, including commercially and ecologically valuable blue crab, stone crab, and shrimp species, as well as copepods, the foundation of much of the arctic food web. Given the increasing exploration and production of oil in the arctic, threats to copepods are potentially significant.

Benefits and impacts of the proposed study: The outcome of our project will contribute robust

information on **chronic long term** effects of oil and dispersants exposure on crustacean **growth**, reproduction, and health, as well as **behavior** that is critical to successful recruitment into the estuary. The blue crab, as an experimentally tractable model organism, can be used to gain insight into the responses of other crustaceans in the Gulf of Mexico, including numerous shrimp and crab species. Data will be made available to other researchers who are currently conducting field studies of the impacts of the Gulf oil spill on the blue crab and shrimp fisheries.

5. Transferability

Our approach uses a potentially sensitive stage of an economically and ecologically important species in the Gulf and Atlantic coastal waters. The metrics developed in this project should be adaptable by many labs, in diverse settings, to assess the threat of dispersants, oil or other pollutants to crab larvae and recruitment. It is intended to be sufficiently detailed to provide reliable information, but not so sophisticated or specialized as to preclude its use by other users. For example, it may be possible to use these methods with field-exposed larvae to assess whether they have been affected by a spill. Our work will establish a framework on which future studies could expand and hopefully aid in future refinements of the current standard toxicity tests for oil and dispersants. One specific area of interest is Chukchi sea, which has been approved for oil exploration and may see drilling begin this summer. These waters are home to populations of snow crab (Paul et al, 1997), a species whose landings are valued in the tens of millions of dollars.

6. End Product

The output of the project will be an evaluation of chronic long term exposure to dispersants/oil on crustacean larval behavior. Measurements will including:

- Larval growth, molt interval, and endocrine status,
- Larval behavior, including feeding, swimming, and tactic responses,
- Physiological damage, as genotoxicity and oxidative stress markers ,
- Bioaccumilation of PAH and DOSS in pooled animals.

Findings and methodology of the proposed study will be valuable as tools to assist managers and scientists in rapid risk assessment/decision-making for dispersant use during spills. The project will also develop methods adaptable to simple *in situ* and laboratory assessments of the effects of dispersant and dispersed oil on blue crab larval behavior.

Findings and methodology will be shared with end-users through professional conferences, and experimental design will be improved ahead of time by direct contact with managers and crab population modelers.

7. Roles and Responsibilities

PI: Eric Schott: The Schott lab has an interest in non-predator mortality of blue crab. Dr. Schott will coordinate, oversee and monitor performance of all components of the project. He will be specifically responsible for conducting or overseeing behavioral assays of swimming speed, phototaxis, and feeding behaviors.

Co-PI Carys Mitchelmore: Mitchelmore laboratory expertise relates to the effects of oil and chemically dispersed oil on sensitive species, including with respect to route of oil exposure i.e. dissolved versus droplet fractions. Dr. Mitchelmore will oversee the assessment of genotoxic and oxidative stress effects.

Co-PI: J. Sook Chung: The Chung lab is an international leader in crustacean endocrinology. Dr. Chung will oversee the larval production for the exposure experiments, estimating hormonal status together with molt interval and rate, food consumption, and data analyses
Co-PI: Andrew Heyes: Heyes will oversee the collection and analysis of samples for PAH's and dispersant. He will also provide interpretation the results in support the projects goals.
The PI and all co-PIs will be responsible for producing peer-reviewed papers reporting the studies. Dr. Schott and Dr. Mitchelmore will facilitate and ensure adequate communications with end-users.

Expertise of key personnel: Dr. Schott has expertise in blue crab health and disease, with an interest in non-predation mortality of blue crab. Dr. Mitchelmore has extensive experience working with oil/dispersants in invertebrate taxa using Corexit 9500, was co-author on the NRC report (2005) and recently gave 5 House/Senate testimonies on the impacts of dispersants/dispersed oil. Dr. Chung is an international leader in crustacean endocrinology, with specific expertise in blue crab growth and molting regulation.

8. Facilities:
New equipment: To complement the facilities and equipment listed below, this proposal includes the request for funds to acquire a dedicated microscope, high-sensitivity digital SLR camera/video, and desktop computer to conduct crab larvae swimming and growth assessments.
UMCES-IMET:
IMET's Aquaculture Research Center (ARC) facility: Facilities include a 15,000 square foot, state-of-the-art facility marine recirculating aquaculture center with light, temperature and water quality controls. It has a functioning blue crab hatchery including ovigerous females, larval rearing, megalopae, and crabs, and facilities for culturing food chain organisms such as Artemia, rotifers, and algae. At a given time, 300 large juvenile crabs and many thousands of larval crabs can be held in compartmentalized tanks. ARC also has dedicated rooms for pathogen and hazardous material studies, and quarantine.
Schott lab and **Chung labs** (500 and 1,045 sq ft respectively) are equipped for carrying out all the physiological, biochemical, endocrinological and molecular work proposed in the present plan. All the equipment (PCRs, computers and essential software, etc) required for cDNA cloning, expression and mRNA assays, a full range of electrophoresis and blotting manifolds equipment, a microtiter plate reader (Molecular Device M5 including lanthanide and fluorescence) and washers (for ELISA), HPLC (Agilent) with a radiochemical detector are available in my laboratory. All the necessary equipment for other biochemical and isotope studies are available: centrifuges, and β and γ counters.
IMET has shared- use equipment for low, fast and high-pressure liquid chromatography (Waters Alliance 2695). In addition, a shared cryostat, microtome, and all other equipment necessary for automated processing of tissues for histological work are available at our facility. Additional molecular and microbiological equipment is available in the IMET BioAnalytical Services lab, a fee-for-service core facility.

Carys L. Mitchelmore and Andrew Heyes: Facilities at the Chesapeake Biological Laboratory include one main laboratory (approx. 400sq. ft.) and 2 ancillary laboratories (approx. 250 sq. ft. each), which are well equipped for biochemical, cellular and molecular biological investigations. In addition 650 ft2 of temperature controlled wet laboratory space currently houses the anemone

cultures. A common-use temperature controlled room (15˚C) is also available for exposure experiments. The major pieces of equipment in the laboratory, which will be used in this research includes; an array of PCR machines (ABI 9700, Stratagene Robocycler, MJ Research PCR), a real-time PCR machine (ABI 7000) and a DNA sequencer (ABI 3700), an epifluorescent microscope (Olympus BX-50) with digital camera and computer equipped with a graphics monitor and Kinetic Imaging (Komet 4.0) analysis software (for the COMET assay), a digital imaging system (Alpha Inotech FluorChem 8800), BioRad and Owl gel apparatuses and power supplies for SDS-page, polyacrylamide and agarose chromatography, a Molecular Dynamics 96-well spectrophotometer and fluorometer (cytochrome P450 measures), incubator, shaking incubator, ice machine, autoclaves, two chemical fume hoods, -80°C freezer, various

Facilities and instruments for chemical analysis are present in the Organics Analytical Laboratory (OAL) at CBL. Available instruments include an Agilent 6890-5973 GC-MS, an Agilent 7890-5975 GC-MS and Agilent 6890 GC-ECD, Thermo LTQ-Orbitrap. The lab has the standard equipment for sample extraction and concentration including a Dionex Automated Solvent Extractor (ASE 300).

9. Dissemination

Results of the studies will be disseminated through publication in the peer-reviewed literature, presentation at national/international meetings (including the International Oil Spill Conference and SETAC), and via the construction of a web site dedicated to this project (for which funds have been earmarked in the budget. This site will also include background material and links to relevant entities or research on oil spill-related research and larval behavior and recruitment. Findings will be presented to the CRRC, including at annual NOAA/UNH and other pertinent oil-spill community workshops.

As indicated by the letters of support from Dr. Ogburn (Smithsonian Env Research Center), Dr. North (Horn Point Lab, UMCES), and Dr. Ryan Gandy (Florida Fish and Wildlife Research Institute), we have identified several end-users of the information to be generated by this project. Additionally, we have relationships with managers in multiple states (DE, MD, FL, MA, GA, MS) who also have an interest in the blue crab fishery. To our mutual benefit, we will conduct meetings with named parties and others at the beginning of the funding period to solicit input on possible adaptations to our approach that would better serve their needs.

Dr. Schott or Mitchelmore will travel to meet with managers in the Gulf to discuss our findings and learn from them how to best help them use the results of this study. We will benefit from the contacts provided by Ryan Gandy at FWRI, who has an active program in assessing the health of blue crab and stone crab stocks along the Florida Gulf coast. It will be in the interests of both managers and us to follow this brief 1 year study with applications to other agencies for funds to focus on one or more aspects of the findings.

Literature Cited

Ashauer, R., Hintermeister, A., Potthoff, E., Escher, B.I. 2011. Acute toxicity of organic chemicals to Gammarus pulex correlates with sensitivity of Daphnia magna across most modes of action. *Aquatic Toxicology*. 103: 38-45

Ayukai, T. 1994. Ingestion of ultraplankton by the planktonic larvae of the crown-of-thorns starfish, Acanthaster planci. *Biol. Bull.* 186: 90-100

Biggers, W.J., Laufer, H. 2004. Identifcation of juvenile hormone-active alkylphenols in the lobster Homarus americanus and in marine sediments. *Biol. Bull.* 206, 13–24

Blaxter, J.H.S., Ten Hallers-Tjabbes, C.C. 1992. The effect of pollutants on sensory systems and behaviour of aquatic animals. *Aquatic Ecology.* 26: 43-58

Chung, J. S., et al. 2012. Cloning of aquaporin-1 of the blue crab, Callinectes sapidus: its expression during the larval development in hyposalinity. *Aquatic Biosystems*. under revision.

Chung, J.S. 2010. Hemolymph ecdysteroids during the last three molt cycles of the blue crab, Callinectes sapidus: quantitative and qualitative analyses and regulation. *Arch. Insect Biochem. Physiol.* 73: 1-13

Chung, J.S., et al., 2010. Crustacean hyperglycemic hormone (CHH) neuropeptides family: function, titer, and binding to target tissues. *Gen. Comp. Endocrinol.* 166: 447-454.

Cronin, T.W., Forward Jr., R.B. 1980. The effects of starvation on phototaxis and swimming of larvae of the crab Rhithropanopeus harrisii. *Biol. Bull.* 158: 283-294

Epifanio, C.E., 1988. Dispersal strategies of two species of swimming crab on the continental shelf adjacent to Delaware Bay. *Marine Ecology Progress Series.* 49: 243-248

Faimali, M., Garaventa, F., Piazza, V., Greco, G., Corrà, C., Magillo, F., Pittore, M., Giacco, E., Gallus L., Falugi, C., et al. 2006. Swimming speed alteration of larvae of Balanus amphitrite as a behavioural end-point for laboratory toxicological bioassays. *Marine Biology*. 149: 87-96

Fallahtafti, S., Rantanen, T., Brown, R.S., Snieckus, V., Hodson, P.V. 2012. Toxicity of hydroxylated alkyl phenanthrenes to the early life stages of the Japanese Medaka (Oryzias latipes). *Aquat. Tox.* 106-107:56-64

Fitzpatrick, M.P., Jeffs, A.G., and Dunphy, B.J. 2011. Efficacy of calcein as a chemical marker of green-lipped mussel (Perna canaliculus) larvae and its potential use for tracking larval dispersal. *Aquaculture Research.* doi: 10.1111/j.1365-2109.2011.03034.x

Forward, R.B., Costlow, J.D. 1974. The ontogeny of phototaxis by larvae of the crab Rhithropanopeus harrisii. *Marine Biology.* 26: 27-33

Forward, R.B., Frankel Jr., D.A.Z., and Rittschof, D. 1994. Molting of megalopae from the blue crab Callinectes sapidus: Effects of offshore and estuarine cues. *Marine Ecology Progress Series* 113: 55-59

Garaventa, F., Gambardella, C., Di Fino, A., Pittore, M., and Faimali, M. 2010. Swimming speed alteration of Artemia sp. and Brachionus plicatilis as a sub-lethal behavioural end-point for ecotoxicological surveys. *Ecotoxicology*. 19: 512-519, DOI: 10.1007/s10646-010-0461-8

López-Duarte, P., Tankersley, R. 2007. Circatidal swimming behavior of brachyuran crab zoea larvae: implications for ebb-tide transport. *Marine Biology*, 151: 2037-2051

Luna-Acossta, A., Kanan, R., Le Floch, S., Huet, V., Pineau, P., Bustamante, P., Thomas-Guyan, H. 2011. Enhanced Immunological and detoxification responses in Pacific oysters, Crassostrea gigas, exposed to chemically dispersed oil. *Water Resear*. 45: 4103-4118

Marine Environmental Protection Committee. 2008. HARMFUL AQUATIC ORGANISMS IN BALLAST WATER: A technical protocol for uniform analysis of organisms ≥ 50 micrometres in ballast water to verify compliance with the discharge standards in the Ballast Water Management Convention. Submitted by Friends of the Earth International. http://www.amtcc.com/imosite/meetings/IMOMeeting2008/MEPC58/MEPC%2058-INF.23.pdf

McCahon, C.P., Pascoe, D.,1988 Use of Gammarus pulex (L.) in safety evaluation tests: Culture and selection of a sensitive life stage. *Ecotoxicology and Environmental Safety*. 15: 245–252

Meijering, E., Dzyubachyk, O., Smal, I., 2012. Methods for Cell and Particle Tracking. *Methods in Enzymology*, Vol. 504, Ch. 9: 183-200

North, E.W., Schlag, Z., Hood, R.R., Li, M., Zhong, L., Gross T., Kennedy, V.S. 2008. Vertical swimming behavior influences the dispersal of simulated oyster larvae in a coupled particle-tracking and hydrodynamic model of Chesapeake Bay. *Mar. Ecol. Prog. Ser.* 359: 99-115

Oberdörster, E., et al. 2000a. Long-term pyrene exposure of grass shrimp, Palaemonetes pugio, affects molting and reproduction of exposed males and offspring of exposed females. *Environ. Health Perspectives*. 108: 641-646

Oberdörster, E., et al. 2000b. PAH effects on grass shrimp, Palaeomonetes pugio: interactions with the ecdysteroid system. *Mar. Environ. Res.* 50: 194-195

Oberdörster, E., et al. 1999. Interaction of PAHs and PCBs with ecdysone-dependent gene expression and cell proliferation. *Toxicol. App. Pharmacol*. 160: 101-108

Ouellet, P., Allard, J.P. 2006. Vertical distribution and behaviour of shrimp Pandalus borealis larval stages in thermally stratified water columns: laboratory experiment and field observations. *Fisheries Oceanography*, 15: 373–389

Paul, J.M., Paul, A.J., Barber, W.E. 1997. Reproductive biology and distribution of the snow crab from the northeastern Chukchi Sea. *Am. Fish Soc. Symp*. 19: 287-294

Reddy, C.M., Quinn, J.G. 1999. GC-MS analysis of total petroleum hydrocarbons and polycyclic aromatic hydrocarbons in seawater samples after the North Cape oil spill. *Marine Poll. Bul.* 38: 126-135

Sbalzarini, F., Koumoutsakos, P. 2005. Feature point tracking and trajectory analysis for video imaging in cell biology. *J. Struct. Biol.* 151(2): 182-195

Schott, E.J., Vasta, G.R. 2003. A superoxide dismutase gene from the protistan parasite Perkinsus marinus is genetically and biochemically functional in Saccharomyces cerevisiae. *Molec. Bioch. Parasitol.*, 126: 81-92

Soriano, J.A., Viñas, L., Franco, M.A., González J.J., Ortiz, L., Bayona, J.M., Albaigés, J. 2006. Spatial and temporal trends of petroleum hydrocarbons in wild mussels from the Galician coast (NW Spain) affected by the Prestige oil spill. *Sci. Tot. Environ.* 370: 80-90

Sulkin, S.D.W.Van Heukelem, Kelly, P., Van Heukelem, L. 1980. The behavioral basis of larval recruitment in the crab Callinectes sapidus Rathbun: A laboratory investigation of ontogenetic changes in geotaxis and barokinesis. *Biol. Bull*. 159: 402-417

Research Planning Incorporated. "The Future of Dispersant Use in Oil Spill Response Initiative March 22, 2012". Coastal Response Research Center. National Oceanic and Atmospheric Administration

Turcotte, D., Akhtar, P., Bowerman, M., Kiparissis, Y., Brown, R., Hodson, P.V. 2011. Measuring the toxicity of Alkyl alkyl phenanthrenes to the early life stages of the Japanese Medaka (Oryzias latipes) using partition controlled delivery. *Environ. Tox. Chem*. 30:487-495

Wang, Z., Li, K., Lambert, P., Yang, C. 2007. Identification, characterization and quantitation of pyrogenic polycyclic aromatic hydrocarbons and other organic compounds in tire fire products. *J. Chrom. A.* 1139: 14-26.

Yamada, M., Takada, H., Toyoda, K., Yoshida, A., Shibata, A., Nomura, H., Wada, M., Nishimura. M., Okamoto, K., Ohwada, K. 2003. Study on the fate of petroleum-derived polycyclic aromatic hydrocarbons (PAHs) and the effect of chemical dispersant using an enclosed ecosystem, mesocosm. Mar. Pollut. Bull. 47:105-113

Zmora, O., Findiesen, A., Stubblefield, J., Frenkel, V., and Zohar, Y. 2005. Large-scale juvenile production of the blue crab Callinectes sapidus. *Aquaculture.* 244: 129-139

Zmora, O., et al. 2005. Large-scale juvenile production of the blue crab Callinectes sapidus. *Aquaculture*. 244: 129-139

Zohar, Y., Hines, A.H., Zmora, O., Johnson, E.G., Lipcius, R.N., Seitz, R.D., Eggleston, D.B., Place, A.R., Schott, E.J., Stubblefield, J.D., Chung, J.S. 2008. The Chesapeake Bay blue crab (Callinectes sapidus): a multidisciplinary approach to responsible stock replenishment. *Reviews in Fisheries Science*. 16: 25-35

Timeline:
Schott et al. Chronic impacts of chemically dispersed oil on blue crab larvae

Activity	Proposed timeline 2013											
	J	F	M	A	M	J	J	A	S	O	N	D
Procurement, systems set-up	x	x										
Meet with Gulf coast state manager -end user objectives	x											
Dispersant/oil preparations		x	x									
Zoea exposures1: feeding and swimming studies				x	x							
*First progress report due				x								
Zoea exposures1: growth/molting studies					x	x						
Dispersant uptake and physiology studies					x	x						
Data analysis- experimental design refinements						x						
Zoea exposures2: feeding and swimming studies						x	x	x				
*Second progress report due							x					
Zoea exposures2: growth/molting studies- endocrine gene expression								x	x			
Dispersant uptake and physiology studies								x	x			
Data analysis- PIs meetings- develop outreach materials for web										x	x	
*Third progress report due, review findings with state manager(s)										x	x	
*Final progress report due, presentations, manuscript preparation												x

COMPONENTS OF THE PUBLISHED FACILITIES & ADMINISTRATIVE COST RATE

INSTITUTION: UNIVERSITY OF MARYLAND CENTER FOR ENVIRONMENTAL SCIENCE

FY COVERED BY RATE: FYE 06/30/09 - 06/30/12

	ORGANIZED RESEARCH		
	On-Camp FY 09	On-Camp FY 10 - 12	Off-Camp FY 09 - 12
RATE COMPONENTS:			
Facilities Group:			
Building Depreciation	4.0	4.5	
Equipment Depreciation	3.0	3.5	
Interest	0.7	0.7	
Operations & Maintenance	14.5	15.0	
Library	0.8	0.8	
Administrative Group:	26.0	26.0	26.0
F&A Rate	49.0	50.5	26.0

CONCURRENCE:

Donald J Boesch

(Signature)

Donald Boesch, Ph.D.

(Name)

President

(Title)

(Date)

CRRC scope of work supplement

For the Project

Understanding chronic impacts of chemical dispersant and chemically-dispersed oil on behavior, molting success, and hormone status and of blue crab larvae: inputs for recruitment and population models

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November 1, 2012

Funded by The Coastal Response Research Center (December, 2012 – November, 2013)

A**ssessment of particulate and soluble oil and oil constituents in exposure water for crab larvae experiments**

The set of experiments outlined in the main proposal address the following:

1. What are the effects of sub-lethal exposure to oil/dispersant on larval growth and molting?
2. What are the effects of sub-lethal exposure to oil/dispersant on larval feeding, swimming and phototaxis?
3. What are the effects of sub-lethal exposure to oil/dispersant on bioaccumulation, oxidative stress and genotoxicity?

If sublethal exposure to oil and/or dispersant affects growth, endocrine status, or behavior, or if bioaccumulation occurs, it will be important to know whether the effects are associated with oil droplets and/or dissolved oil components (i.e. PAHs). Exposure to oil constituents, via various routes of exposure, are controlled by the type, form and physical/chemical characteristics of the oil and the specific organism and life stage exposed. Oil droplets (including fine colloidal sizes) may impact an organism by simple physical coating and/or oil particle adherence to sensitive (e.g. respiratory) surfaces. Furthermore, these adhered particles may result in enhanced uptake due to interactions with dermal surfaces. Oil droplets may be mistaken for food particles and ingested, resulting in enhanced PAH bioavailability and internalization of PAHs or these oil droplets may simply interfere with normal feeding processes. Dissolved oil components (i.e. PAHs) may be directly absorbed through surfaces such as gills, the alimentary canal or even transdermally. It is beyond the scope of this project to investigate every possible route of exposure (alimentary, respiratory, transdermal), but a preliminary characterization of the physical form of oil and chemically dispersed oil is possible using methodology and expertise within the research team.

The gape size of zoeal stages z1-z3 larval blue crabs is not reported in the literature, but the feed that they ingest in the hatchery includes phytoplankton (e.g. *Tetraselmis*, size 5-10 micron) to small zooplankton (e.g. rotifers, size 130-180 microns). Therefore, it is important to know how much oil or dispersed oil falls into the ~5-200 micron range.

Table 1. Exposure regimes: Environmentally-relevant doses will be determined by consultation with NOAA, state managers and scientists. Target doses will otherwise start at 10% of LC50 as 'high' dose. Dispersant: oil ratios will be 1:20.

Experiment groups	**Description**	**Doses**
1	Control –ASW alone	TBD
2	low dispersant only	TBD
3	low oil only	TBD
4	low oil and dispersant	TBD
5	high dispersant alone	TBD
6	high oil	TBD
7	high oil and dispersant	TBD

To begin to assess oil partitioning, each of the 7 exposure combinations used in the study (Table 1) will be analyzed by one or two additional methods (see below). Analysis will include the physical separation of oil droplets from the soluble phase, and quantification of PAHs in the two

fractions using the methods detailed in Sections 3.4 and 3.6 (page 11) of the proposal. We propose to fully analyze our doses using the methods outlined below to ascertain the percentage of the oil that is in the dissolved versus particulate phases and furthermore to characterize the specific size range(s) of the oil droplets.

The two methods that we will employ to assess the partitioning of the oil between the droplet and dissolved phase are physical separation using glass fiber filters (GF/F) and direct particle counting using LISST analysis, which is routinely used in the field as part of the SMART program (Lee et al. 2010). Following GC/MS analysis, filtration provides a measure of the fractionation of PAHs between phases while particle analyses give a quantitative analysis of the volume of oil in the droplet phase and an assessment as to the size of droplets. These additional analyses will aid the assessment of what route(s) of exposure we involved for the crab larvae i.e. ingestion, absorption and/or adsorption. Our laboratory has expertise in the use of GF/F filtration, and LISST applications (including a trained technician in field and laboratory based application of the LISST with oil solutions; Dr. Steve Suttles (see CV enclosed)).

One alternate method for estimation of dissolved PAH in the water column is the use of passive samplers. We feel that the use of this method, and the attendant need for controls, standardization, and additions to the experimental plan, would divert effort from the main purpose of this study: assessment of biological metrics of growth, behavior, and stress markers.

Additional Specific Methodology:

1. Filtration.

Glass fiber/filtration (GF/F) has been successful for the most part in preparing dissolved versus total (dissolved and oil droplets) WAF and CEWAF preparations, particularly when prepared at the lower loadings (0.5g/l oil) (Jim Payne, pers com; Payne and Driskell, 2003). Khelifa, et al. (2008) reported that at the optimal Corexit 9500 DOR of 1:20, the smallest dispersed oil particles were 2 microns, which will be captured by the GF/F method, although this will be tested when we analyze our filtrates with the LISST and coulter counters.

We will employ standard 0.7 micron GF/F filtration to separate particulate and non-particulate phases. Previous work in the Mitchelmore lab using this method has shown that some colloidal (i.e. PAHs in excess of their maximal solubility predicted) do pass through the filters using stock solutions of chemically dispersed oil fractions. However, in lower dilutions the ratio of retained/pass through PAH was not reduced. Therefore, we propose to filter a diluted sample, with the exact dilution to be determined in a preliminary chemical-only test exposure, to optimize this procedure. In conjunction, we will also employ particle characterization methods (see below) to provide an independent estimate of mass balance between soluble and particulate forms.

Chemical (PAH) analysis of pre/post filter fractions, plus analysis of unfiltered exposure material (7 conditions, Table 1) will indicate whether there is differential partitioning of some PAHs to each phase. These values will be compared to PAHs detected in larvae after exposure (Section 3.6 of proposal).

2. Particle counting: LISST (droplet size distribution) analyses

We will measure oil droplet (particle) size distribution in the test solutions using LISST instrumentation (LISST-100x and sample chamber). We have extensive experience in using this equipment for particle size distribution analyses (sediment and oil particles) and in writing the Matlab codes for specific applications (see the listed experience by Dr. Suttles (see CV). Our equipment is the same as routinely used in the filed (e.g. during the Deepwater Horizon sill) i.e. by the use of the LISST-100x and the small sample cell chamber. The use of the small chamber will allow us to reduce our sample size volume of the test solutions to be more in line with those volumes prepared for the outlined blue crab toxicity tests. The LISST-100X by Sequoia Scientific measures size distribution of particles (or droplets) using a collimated laser diode and a specially constructed annular ring detector. Smallangle scattering from suspended particles (or droplets) is sensed at 32 specific log-spaced angle 7 ranges. Scattering at 32 angles is the primary information that is recorded. This primary measurement is mathematically inverted to get the size distribution, and also scaled to obtain the volume scattering function (VSF). The size distribution is presented as volume concentration (micro-l/l) in each of 32 log-spaced size bins. Optical transmission, water depth and temperature are recorded as supporting measurements. Other statistical parameters can be obtained from the results, such as median particle size (D50), cumulative distribution curves, and others. The instrument proposed for use on this project is a LISST-100X (Type-C), which measures particles or droplets in the 2.5 – 500 micron range. The instrument may be deployed in situ or used in the laboratory as a bench top device using the flow-through chamber mentioned above. For additional information see Sequoia Scientific's website; (http://www.sequoiasci.com/products/part_LISST_100.aspx).

LISST data will also be compared with direct epifluorescence microscopy observations. This is a qualitative assessment only to verify particle sizes. We will not assess to the level as outlined in Khelifa et al, but will examine droplets of each mixture using 40x and 100x objectives on a Zeiss epifluorescence microscope, available in at IMET.

References

Khelifa, A., Fingas, M., and C. Brown. 2008. CRRC final report: Effects of Dispersants on Oil-SPM Aggregation and Fate in US Coastal Waters.
http://www.crrc.unh.edu/final/khelifafinal2008/

Hatzianestis, I., and Sklivagou, E. 2002. Dissolved and Suspended Polycyclic Aromatic Hydrocarbons (PAH) in the North Aegean Sea. *Mediterranean Marine Science.* Vol. 3: 89-98

Lee, K., Li, Z., and H. Niu – Centre for Offshore Oil, Gas and Energy Research, Fisheries and Oceans Canada Appendix 7 "Bench Top" LISST Particle Size Analysis. Oil Budget Calculator Deepwater Horizon TECHNICAL DOCUMENTATION November 2010 A Report by: The Federal Interagency Solutions Group, Oil Budget Calculator Science and Engineering Team.

Payne, J.R and Driskell, W.B. 2003. The importance of distinguishing dissolved- versus oil-droplet phases in assessing the fate, transport, and toxic effects of marine oil pollution. Pp. 771-778. In Proceedings of the 2003 International Oil Spill Conference, Vancouver Canada, American Petroleum Institute. Washington, D.C.

Expertise of the Investigators (see CVs for further details):

Carys L. Mitchelmore: co-P.I. Mitchelmore has been investigating the impacts and effects of oil spills in both field and laboratory settings for over 15 years. She was co-author of the NRC (2005) dispersant report and has recently given 5 congressional testimonies to various House and Senate committees (May-July, 2010) concerning the fate and effects of dispersants and dispersed oil (pdfs are posted on the www.umces.edu website) and was an invitee at the CRRC NOAA dispersant working group (Baton Rouge, May 2010). She has also been an invited participant for many other NOAA working groups/workshops on dispersants and PAH toxicity. Previously funded research has included investigating the exposure routes (dissolved/particulate oil) for chemically dispersed oil (using Corexit 9500A) in sensitive species such as corals and reptiles (joint with PI Rowe).

Andrew Heyes: co-PI Heyes is an environmental chemist who direct the Organics Analytical Laboratory at Chesapeake Biological Laboratory. He has extensive experience in characterizing fate and transport of organic and inorganic contaminants in large scale experimental and natural systems.

Steve Suttles: Suttles has been using LISST particle size analyzers for 10 years. He is familiar with all aspects of the instruments operation and analysis of the data it collects. While working as Dr. Lawrence Sanford's (UMCES-HPL) Senior Technician/Engineer, S. Suttles has collected and analyzed numerous data sets using the LISST; including mapping particle size distributions in the Upper Chesapeake Bay, using LISST with video system to quantify in situ floc sizes and settling speeds, and conducted laboratory experiments measuring suspended particle clearing rates in laboratory mesocosm. In 2010 S. Suttles participated in 2 cruises in the Gulf of Mexico using a LISST-Deep (and ABS) to attempt to measure and track sub-surface oil plumes from the Deep Water Horizon Oil Spill. As part of that work S. Suttles was responsible for integrating the LISST-Deep instrument on to a deep tow-fish for real-time data collection. S. Suttles has developed custom processing scripts in MatLab (The Math Works, Inc.) to process LISST data and to produce graphical results.

Quality Assurance Plan

For the Project:

Understanding chronic impacts of chemical dispersant and chemically-dispersed oil on behavior, molting success, and hormone status and of blue crab larvae: inputs for recruitment and population models

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October 15, 2012

Funded by The Coastal Response Research Center (December, 2012 – November, 2013)

Quality Assurance Plan

1. Hatchery-reared crab larvae- production protocols.

Larvae will be produced in the Aquaculture Research Center, Institute of Marine and Environmental Technology (Baltimore, MD) according to optimized, standardized, protocols (Zmora et al., 2005). The hatchery is capable of producing 8-16 broods per year, using wild broodstock collected at least twice yearly and held in optimal conditions for egg extrusion. All broodstock are quarantined for health inspection and disease screening before moving to the hatchery. Hatching of *C. sapidus* larvae is well-synchronized in that these events take place overnight. Using day 1 larvae allows us to minimize the initial variation in life stage of *C. sapidus*. At least two batches of larvae will be used for the experiments described below (each set of experiments therefore being conducted at least two times), taking advantage of the obvious benefits of having age-matched zoea that are developing synchronously. Though unlikely to be an issue, a sample of the feed (rotifer/algae/yeast mixture) for z1-z3 larvae will be saved for analysis of PAH contaminants.

2. Preparation and chemical analyses of test solutions. See also Table 1.

Before each set of experiments water-accommodated (WAF) and chemically-enhanced water accommodated fractions (CEWAF) will be freshly prepared using standardized methods for preparing toxicity test solutions essentially as described in Singer et al. (2000), with some modifications as suggested in Clark et al. (2001) and Baron and Ka'aihue, (2003). All containers and measuring syringes/pipettes will be ultra-cleaned glassware (solvent rinsed using acetone, hexane and DCM). Aspirator bottles will have attached plastic piping (for removal of the test solutions) that has also been solvent rinsed before each use). For each test solution make-up a control of ASW only will also be prepared using the same equipment and methods to assess for any laboratory contamination in blank samples. Volumes of artificial seawater will be measured using certified volumetric flasks. The oil will be added to the ASW by weight (i.e. g oil / L ASW) using clean glass pipette and weighing before and after addition of oil. Dispersant will also be added on a weight basis using a glass syringe and the amount added also calculated using a weighing balance. The weighing balance will be calibrated before each test to assure the accuracy of the weights. All solutions will be prepared in a fume hood, aspirator bottle openings

covered with solvent rinsed foil and Teflon taped to seal and the whole bottle covered in foil to prevent any photolysis reactions. At regular intervals aspirator bottles will be monitored to ensure that the vortex speed is driving mixing to the appropriate depth. When tapping off the solutions (after the set settlement periods) the first 200mls will not be collected. The remaining test solution (as it will be graded in the aspirator bottle in concentration after settling i.e. lower at the bottom, higher at the top etc) will be collected into another glass container, except for the last few 100 ml (so that the slick / top-layers are not collected). This will ensure that a homogenous starting stock solution is prepared. This stock will be immediately used and processed and sub-sampled for PAH and/or DOSS analyses.

A log-series of dilutions of a stock solution (i.e. a 1:1000 oil: water ratio and a 1:10 dispersant: oil ratio) will be used to prepare the exposure solutions for the preliminary acute (and sublethal) range finding tests. As effectiveness of dispersion (mechanical and chemical) varies from batch to batch PAH analyses will be carried out on each stock solution prepared. Ideally, each dilution should also be characterized but this is out of the scope of the budget for this proposal.

3. Exposure experiments.

Exposures will take place in artificial seawater used for rearing larvae, at the same salinity and temperature as rearing conditions (expected to be 30 psu and 22-24˚C).
Tests will be standard static acute toxicity test methods (e.g. US EPA 821/R-02-012) as described in Hemmer et al., 2011. A minimum of 6 dilutions of each exposure solution plus control will be used. Following 48 hour exposures LC50 values will be generated. Chronic exposures will also follow standard US EPA toxicity testing methods for invertebrate species. Standard QA/QC test procedures will be followed for the toxicity tests. These include, water quality monitoring e.g. dissolved oxygen, ammonia, temperature, pH, salinity. Any water quality values reporting above or below set guidelines will void the entire test. Furthermore, both negative and positive controls will be used alongside the toxicity tests to ensure that larval batches are consistent and that there are no other issues impacting the toxicity tests. All toxicity tests will follow the standard QA/QC guidelines, such as, controls will show <90% survival, mortality less than this in controls will also void the whole test.

3a. Assessment of PAH and DOSS in crab larvae.

The aim of the chemical analysis is to confirm the level of potential exposure by quantifying PAHs in the dosing stock solutions, as some loss of PAHs is likely to occur during preparation, and assess exposure by measuring uptake of PAHs in the crab larvae.

3a.1 Sample Extraction and Clean-up

For all samples, deuterated internal standards, used for analyte quantitation, will be added before extraction.

For stock water samples – liquid-liquid extractions will be performed in separatory funnels with dichloromethane with solvent finally exchanged to hexane after clean up. With heavy oil SPE columns are often employed, but experience indicates this won't be required. After extraction, samples will be concentrated to 100 ul and transferred into GC vials. A recovery standard will be added before the sample is reduced to final volume for analysis by GC-MS. This recovery standard will be used to measure the recovery of the internal standards used in quantitation.

Tissue samples (filter-collected pooled crab larvae), will be mixed with anhydrous Na_2SO_4 (previously Soxhlet extracted with hexane and dried) to eliminate water. The mixture will be transferred into a mortar and ground with a pestle along with 100 µL of the internal standard solution. The dried sample placed in a stainless steel cell containing deactivated alumina and extracted using the ASE 300 System (Dionex, Sunnyvale, CA). Instrumental settings suggested by the instrument's manufacturer include a temperature of 100 °C and a static time of 10 min (2 cycles) at a pressure of 1500 psi. The solvent used is dichloromethane with a flush volume of 60% and a purge time of 100 s. The final extract is in the range of 100 ml when using a cell size of 66 mL. The sample is concentrated to 1-2 mL by rotary evaporation and transferred to GC vials.

3a.2 Sample Analysis

Table 1: List of PAHs expected to be measured.	
Naphthalene	Dibenzothiophene
2 methylnaphthalene	4 methyldibenzothiophene
1 methylnaphthalene	2 methyldibenzothiophene
1,3 dimethylnaphthalene	Fluoranthene
1,6 dimethylnaphthalene	2 methylfluoranthene
1,4 dimethylnaphthalene	Pyrene
1,5 dimethylnaphthalene	1 methylpyrene
1,2 dimethylnaphthalene	Benzo(e)pyrene
1,8 dimethylnaphthalene	Benzo(a)pyrene
2,6 dimethylnaphthalene	4,5 methylenephenanthrene
2,3 dimethylnaphthalene	Benzo(a)anthracene
2,3,5 trimethylnaphthalene	Chrysene
Biphenyl	6 methylchrysene
Acenaphthylene	4 methylchrysene
Acenaphthene	Benzo(b)fluoranthene
Fluorene	Benzo(k)fluoranthene
1 methylfluorenePhenanthrene	Benzo(a)pyrene
1 methylphenanthrene	Indeno(1,2,3-c,d)pyrene
2 methylphenanthrene	Dibenz(a,h)anthracene
3,6 dimethylphenanthrene	Dibenz(a,c)anthracene
Anthracene	Perylene
1 methylanthracene	Benzo(g,h,i)perylene
2 methylanthracene	Anthanthrene
9 methylanthracene	3-methylcholanthrene
9,10 dimethylanthracene	Naphthacene
2,3 dimethylanthracene	Coronene

All extracts will be analyzed on an Agilent 7890A GC coupled to an Agilent 5975c MS detector. The instrumental detection limits for the GC-MS analyses are on the order of ca. 10-100 pg per component on column. The PAHs to be analyzed are listed in Table 1. The PAHs will

be analyzed in selected ion monitoring (SIM) on a generic 30. The column is a 5% phenylmethyl capillary column (DB-5) of 25m length, 0.2 mm internal diameter, and 0.33 mm stationary phase film thickness (J&W Scientific). Phenol and its alkylated homologues will be analyzed in separate SIM runs on the same column.

3b. Monitoring Performance

Quality assurance/quality control procedures will include the processing of laboratory blanks (1 in 6) and spiked solvent samples (1 in 10 each), and the analysis of certified reference materials NIST 2779 oil and NIST Oyster tissue 2974a. We will use a set of isotope-labeled standards added upon extraction, and be used to quantify the concentrations in the samples via the isotope-dilution method. Calibration standards will be chosen to bracket the sample concentrations of compounds in question, and will be used to derive response factors for the analytes relative to the appropriate isotope-labeled internal standards. Identification of analytes will be based on retention time and isotopically labeled internal standards.

3c. PAH Personnel

The two lead technicians in the Organics Analytical Laboratory have 35 years combined experience in measuring PAHs and have recently been involved in quantifying PAHs in select crude oil. The lab also has experience working with the dispersants.

3d. Dispersant

Water samples will be tested for dispersant concentrations using LC MS-MS targeting dioctylsulfosuccinate (DOSS) and analyzed at the Columbia Analytical Sciences (CAS) laboratory (centers in Columbia MD and Chambersburg PA). External QA will take the form of a blind replicate.

4. Behavioral assays data collection and storage.

Swimming assays will be conducted in triplicate for each condition tested. For example, at a given concentration of dispersant, three trials with fresh larvae will be video-recorded. A minimum of 20 larvae per trial will be recorded and measured (x 3 trials per batch, two batches

=120). Each set of exposures will have an accompanying control trial. Swimming rates will be immediately assessed and the mean and standard deviation calculated. If the s.d. of control swimming rate trials is greater than 20%, additional trials will be conducted for each condition. i.e., raising the N to 4 or 5 instead of N=3. Video and still photographs of swimming speed and phototaxis trials will be saved in a standard format, copied, and archived as raw data that can be independently re-analyzed. Video and photographs for each trial will include imaging a glass 2" x 2" calibration grid or a stage micrometer (10mm/ 100 Divisions) at the magnification used for behavior assays. Data collection for all trials will entail recording microscope settings before and after conducing the behavior trials. Light intensity for phototaxis will be recorded at each trial using a Biospherical QSL-100 (range 10^{14} to 3 x 10^{17} quanta cm-2 sec-1); Licor LI-250, range of 199-19999 $\mu E/m^2/s$). Useful literature references for design, data collection and replicates are Faimli et al.(2006), Forward et al.(1984) and Sulkin et al.(1980). We will use an incandescent light source similar to those in Sulkin et al.(1980) or Forward et al.(1984), with a bandpass filter to omit heat and light above 725 nm. All behavioral assays will be conducted with killed larvae as controls (N=3). This will permit detection (and subtraction) of convection or other currents in the behavior chambers.

Image processing will be conducted using ImageJ (http://rsbweb.nih.gov/ij/index.html) and a manual particle tracking plug-in (Fabrice Cordeliéres, Institut Curie; http://rsbweb.nih.gov/ij/plugins/track/track.html).

5. Growth and endocrine assays- quantitative PCR.

The larval culture and incubation for the exposure study proposed here has been established in Chung laboratory as described in Chung et al. (2012): molt interval and growth rate. We intend to measure the molt increment by measuring protein and total RNA estimation. We have a good base line for the amount of total RNA/ individual larvae at different larval stages. We have shown that individual larval growth can be monitored and examined at molecular levels. Hormones controlling the growth by molting process have been characterized and measured in Chung laboratory (Chung et al., 2010; Chung et al., 2008; Chung et al., 2005; Chung et al., 1999). To minimize the variation of larval quality due to genetic variation, the larvae obtained from a single female will be used for proposed study. However, the experiment will be repeated at least three times (total), as reported in Chung et al. (2012). Quantitative real

time PCR assessment of gene expression will conform to MIQE standards as outlined in Bustin et al. (2009).

6. Sublethal DNA damage and oxidative stress biomarkers.

Genotoxicity (DNA damage) will be assessed on individual larvae using previously published methods used routinely in the Mitchelmore laboratory, based on the COMET assay as a nonspecific marker of the induction of DNA strand breaks (Kinetic Imaging Komet software) (assessed microscopically on isolated cells using epifluorescence and Image Analysis Software) (e.g. see Mitchelmore et al., 1998). To assess the reproducibility and performance of the assay for each test larval batch some control larval cells will be processed as negative and positive (i.e. exposed to hydrogen peroxide) controls.

The antioxidant responses that will be evaluated are total glutathione (GSH) concentrations (using a DTNB recycling assay), and GSH peroxidase (based on the indirect measurement of GSSG using the t-Butylhydro-peroxide (t-BOOH) assay); these are standard spectrophotometric assays (many available as commercial kits); a 96 well plate reader can be used to facilitate rapid analyses of the large numbers of samples. Oxidative stress marker assays will be conducted on pooled larvae (0.1 to 0.5 gram total wet weight). Standard calibrations curves (negative and positive controls) are prepared for each assay for each test batch of larvae.

References

Barron, M.G. and Ka'aihue, L. 2003. Critical evaluation of CROSERF test methods for oil dispersant toxicity testing under subarctic conditions. *Marine Poll. Bull*. 46:1191-1199.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry.* 55:611–622.

Chung J.S., Maurer L., Bratcher M., Pitula J.S., Ogburn M.B., 2012. Cloning of aquaporin-1 of the blue crab, *Callinectes sapidus*: its expression during the larval development in hyposalinity. *Aquatic Biosystems*, 8:21.

Chung J.S. 2010. Hemolymph ecdysteroids during the last three molt cycles of the blue crab, *Callinectes sapidus*: quantitative and qualitative analyses and regulation. *Arch Insect Biochem Physiol.* 73:1-13.

Chung J.S., Zmora N. 2008. Functional studies of crustacean hyperglycemic hormone (CHHs) of the blue crab, *Callinectes sapidus*- the expression and release of CHH in eyestalk and pericardial organ in response to environmental stress. *FEBS J*. 275:693-704.

Chung J.S., Webster S.G. 2005. Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *Carcinus maenas. Endocrinology*. 146:5545-5551.

Chung J.S., Dircksen H., Webster S.G. 1999. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. *Proc Nat Acad Sci* USA. 96:13013-13107.

Clark, J.R., Bragin, G.E., Febbo, E.J., and Letinski, D.J. 2001. Toxicity of physically and chemically dispersed oils under continuous and environmentally realistic exposure conditions: applicability to dispersant use decisions in spill response planning. *In:* Proceedings, 2001 International oil Spill Conference. American Petroleum Institute, Washington DC, pp. 1249-1255.

Cronin T. W. and Forward, Jr., R. B. 1980. The Effects of Starvation on Phototaxis and Swimming of Larvae of the Crab *Rhithropanopeus harrisii*. *Biological Bulletin.* 158: 283-294.

Faimali, M., Garaventa, F., Piazza, V., Greco, G., Corrà, C., Magillo, F., Pittore, M., Giacco, E., L. Gallus and C. Falugi, et al. 2006. Swimming speed alteration of larvae of *Balanus amphitrite* as a behavioural end-point for laboratory toxicological bioassays. *Marine Biology*. 149:87-96.

Hemmer M.J., Barron M.G., Greene R.M. 2011. Comparative toxicity of eight oil dispersants, Louisiana sweet crude oil (LSC), and chemically dispersed LSC to two aquatic test species. *Environ Toxicol Chem*. 30:2244-52.

Mitchelmore, C. L., and Hyatt, S. 2004. Assessing DNA damage in cnidarians using the Comet assay. *Mar Environ Res*. 58:707-11.

Mitchelmore, C. L., and Chipman, J. K. 1998. DNA strand breakage in aquatic organisms and the potential value of the Comet assay in environmental monitoring. *Mutation Research* 399: 135-147.

Singer, M.M., Aurand, D., Bragins, G.E., Clark, J.R., Coelho, G.M., Sowby, M.L. and Tjeerdema, R.S. 2000. Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. *Mar. Pollut. Bull.* 40:1007-1016.

Sulkin, W. Van Heukelem, P. Kelly and L. Van Heukelem., S.D. 1980. The Behavioral Basis of Larval Recruitment in the Crab *Callinectes sapidus* Rathbun: A Laboratory Investigation of Ontogenetic Changes in Geotaxis and Barokinesis. *Biological Bulletin.* 159: 402-417.

Zmora, O., Findiesen, A., Stubblefield, J., Frenkel, V., and Zohar, Y. 2005. Large-scale juvenile production of the blue crab *Callinectes sapidus. Aquaculture* 244: 129-139.

Table 1: Minimum analytical quality control criteria

Sample Type	Minimum Frequency	Acceptance Criteria
I. Chemical analyses of exposure media / tissues - TPH (CBL analytical services)		
Performance Evaluation	Initial	Relative standard deviation of multiple TPH analyses within 20% of the mean. Analysis of spiked samples between 80-120% analytical recovery
Instrument Calibration	Initial/weekly	Calibration curve based upon at least 5 measurements. Correlation coefficient >0.99 for all analytes.
Continuing Calibrations	As needed	Calibration curve based upon at least 5 measurements. Correlation coefficient >0.99 for all analytes.
Reference Material	Every 20 samples	As there are no NIST SRM for the TPH technique, we will rely on intercalibrations with other laboratories
Method Blank	Every 10 samples	<10% of mean concentrations in exposure samples
Sample replication	Every 20 samples	Method precision within 20%
Internal Standards	Every sample.	See below
Inter-laboratory Comparisons	Six month intervals	NIST criteria.
II. Detailed chemical analyses (e.g. 53 PAH's) of exposure media / tissues (CBL analytical services)		
Performance Evaluation	Initial	Overall method performance determined by matrix spike recoveries. All analytes > 70% recovered.

Calibration by NIST standards	Initial/daily as needed	Gas chromatographic calibration against authentic PAH and alkane standards traceable to NIST. Multiple point calibrations of mass spectrometer. Calibration within performance specifications of instrument, linear within range of analyte mass in samples
Continuing Calibrations	See above	As above, with single point calibrations daily
Reference Material	5% of analyzed samples	NIST SRM 2974a (Organics in Mussel Tissue) NIST 2779 oil. All analytes quantified within 75-125% of certified values.
Method Blank	5% of analyzed samples	Deionized water (aqueous) and sodium sulfate (biota) blanks. Each analyte present at <10% the level in the lowest concentrated sample.
Sample replication	5% of analyzed samples	Precision within 25% for all analytes above 3x MDL
Surrogates	All samples	*o*-terphenyl and perdeuterated PAHs used as surrogates in each sample. Method recoveries >70% for each surrogate.
Inter-laboratory Comparisons	Semi-annually	NIST Interlaboratory comparisons within acceptable limits.
III. Biological measurements		
Gene expression analyses by quantitative PCR.		

Performance evaluation	Each sample run in triplicate	SD not to exceed ±15% on any individual sample.
Real time thermocycler calibration	Quartlerly, by Bioanalytical Lab staff	Spectral calibration plate used for each reporter dye as per manufacturers instructions (ABI).
Internal standard/ correction	Each sample	Gene of interest corrected for actual RNA concentration using housekeeping gene actin. SD between sample triplicate not to exceed ±15%.
Method blank	Each sample	RT and no RT reaction controls and no template, no primer and no dye blank controls. No response expected (>38 cycle number) using blanks and no RT controls.
DNA damage (Comet assay)		Mitchelmore and Hyatt, 2004.
Performance evaluation	Each sample run on triplicate slides. n=50 cells scored per slide	SD for slide averages not to exceed ±15%.
Calibration / standard	Each set of samples a positive control (H_2O_2) dose response series (including control no dose) will be used using human leucocytes.	SD between runs for positive control not to exceed ±15%.