*Via email*

October 18, 2024

Administrator Michael S. Regan
U.S. Environmental Protection Agency
Mail Code 1101A
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Washington, DC 20460
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Dear Administrator Regan,

I am writing on behalf of the Center for Biological Diversity to voice our strong support for the August 2024 petition to remove Corexit dispersants (9500A and 9527A) from your Product Schedule of the National Oil and Hazardous Substances Pollution Contingency Plan. The manufacturer ceased making these deadly dispersants after you strengthened relevant regulations last year, but they remain conditionally listed on your current Product Schedule, which is not set to be updated until December 2025—meaning existing stockpiles can still be used in the United States. Petitioners ALERT Project and Government Accountability Project thoroughly documented severe human health consequences of these toxic chemicals, presenting resounding evidence showing you have both the statutory authority and mandatory duty to take this action today. We join to urge do so, effective immediately.

A growing body of scientific data and information shows severe, widespread, and long-lasting impacts of Corexit 9500A and 9527A to both humans and non-human animals. Indeed, evidence from the BP Deepwater Horizon oil spill disaster and many studies show that instead of lessening environmental impacts of oil spills, these dispersants only exacerbate the harm.

For example, loggerhead sea turtle hatchlings exposed to a combination of oil and Corexit had multiple blood chemistry parameters that were worse than hatchlings exposed only to oil, and researchers noted that hatchlings exposed to a combination of oil and Corexit or Corexit alone "fail[ed] to gain weight."[1] Similar results were found with many other marine animals—from mesozooplankton to sperm whales:

[1] Frasier, K. E., Solsona-Berga, A., Stokes, L., & Hildebrand, J. A. (2020). *Impacts of the deepwater horizon oil spill on marine mammals and sea turtles* (pp. 431–462). Springer International Publishing, https://www.cetus.ucsd.edu/docs/publications/Frasier2020_Chapter_ImpactsDWH.pdf (citing Harms, C. A., McClellan-Green, P., Godfrey, M. H., Christiansen, E. F., Broadhurst, H. J., & Godard-Codding, C. (2014). Clinical pathology effects of crude oil and dispersant on hatchling loggerhead sea turtles (Caretta caretta). *Proceedings of the 45th Annual International Association for Aquatic Animal Medicine, Gold Coast, Australia*, 17–22).

- Researchers found exposure to Corexit collapses the feather plumes of common murres and causes "catastrophic loss of waterproofing"—reducing the birds' buoyancy and ability to swim, fly, feed, and keep warm.[2] With significant concentrations of dispersant-oil combinations, "morbidity and mortality is a likely outcome without human intervention."[3]

- Scientists investigated the effects of oil and dispersant exposure on three deep-sea, cold-water corals that live the Gulf of Mexico, finding, "[a]ll three . . . showed more severe declines in health in response to dispersant alone and the oil–dispersant mixtures than the oil-only treatments," with dispersant and oil/dispersant exposures proving lethal to all three species.[4]

- A study on a fourth deep-water coral had similar results, with coral fragments suffering mortality within 48 hours after exposure to Corexit or a combination of oil and Corexit, while those exposed only to oil were "relatively unaffected."[5] The authors concluded "combinations of oil and dispersants are more toxic to octocorals than exposure to oil alone."[6]

- A study found Corexit 9527 is genotoxic to sperm whale skin cells, causing chromosome damage and abnormalities that negatively impact their ability to survive and successfully reproduce.[7] It also found that both Corexit 9500 and 9527 are cytotoxic to sperm whale cells, which "can lead to fibrosis and impair organ function."[8]

- Scientists found that acute toxicity to *Brachionus manjavacas* (a species of plankton) rose "*up to 52-fold*" when Corexit 9500A and oil are mixed at the ratio used for oil spills.[9] A study on mesozooplankton had similar results, concluding, "[a]t the ratio of dispersant to

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[2] Osborne, O. E., Willie, M. M., & O'Hara, P. D. (2023). The effects of oil spill dispersant use on marine birds: a review of scientific literature and identification of information gaps. *Environmental Reviews*, *31*(2), 243–255, https://cdnsciencepub.com/doi/pdf/10.1139/er-2022-0072 (discussing Whitmer, E.R., Elias, B.A., Harvey, D.J., and Ziccardi, M.H. (2018). An experimental study of the effects of chemically dispersed oil on feather structure and waterproofing in common murres (*Uria aalge*). J. Wildl.Dis. 54(2): 315–328. doi:10.7589/2017-01-016)).

[3] Osborne et al. 2023.

[4] DeLeo, D. M., Ruiz-Ramos, D. V., Baums, I. B., & Cordes, E. E. (2016). Response of deep-water corals to oil and chemical dispersant exposure. *Deep Sea Research Part II: Topical Studies in Oceanography*, *129*, 137–147.

[5] Frometa, J., DeLorenzo, M. E., Pisarski, E. C., & Etnoyer, P. J. (2017). Toxicity of oil and dispersant on the deep water gorgonian octocoral Swiftia exserta, with implications for the effects of the Deepwater Horizon oil spill. *Marine pollution bulletin*, *122*(1–2), 91–99.

[6] *Id*.

[7] Wise, C. F., Wise, J. T., Wise, S. S., Thompson, W. D., Wise Jr, J. P., & Wise Sr, J. P. (2014). Chemical dispersants used in the Gulf of Mexico oil crisis are cytotoxic and genotoxic to sperm whale skin cells. *Aquatic toxicology*, *152*, 335–340.

[8] *Id*.

[9] Rico-Martínez, R., Snell, T. W., & Shearer, T. L. (2013). Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A® to the Brachionus plicatilis species complex (Rotifera). *Environmental Pollution*, *173*, 5–10 (emphasis added).

oil commonly used in the treatment of oil spills (i.e. 1:20), dispersant . . . and dispersant-treated oil were 2.3 and 3.4 times more toxic, respectively, than crude oil alone."[10]

- In looking at the effects of crude oil on capelin embryo development, researchers found impacts "were more pronounced in the presence of dispersant," with higher concentrations being lethal to embryos within only 10 hours of exposure.[11] They also observed "sublethal effects, which can subsequently affect larval survival, . . . at nominal loadings 100 times lower," with impacts "including a reduction in heart rate, abnormal morphology, and induction of the expression of some genes." [12] They warned the use of Corexit could "significantly reduce embryo-larval survival and later recruitment in affected areas."[13]

In short, it is well documented that Corexit causes many serious harms to people and marine life, and the ALERT Project and Government Accountability Project have provided ample evidence showing why the petitioned action is necessary. You are removing these dispersants from your Product Schedule for good reason. It would be a tragedy if existing stockpiles were used in the meantime simply due to a regulatory gap or oversight that is entirely avoidable.

Please take action to remove these unsafe, toxic chemicals from your Product Schedule today, and please consider these comments and our attached references as part of your administrative record for this matter.

Thank you for considering our concerns.

Sincerely,

s/ *Miyoko Sakashita*
Miyoko Sakashita
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cc: Riki Ott, The Alert Project

[10] Almeda, R., Wambaugh, Z., Wang, Z., Hyatt, C., Liu, Z., & Buskey, E. J. (2013). Interactions between zooplankton and crude oil: toxic effects and bioaccumulation of polycyclic aromatic hydrocarbons. *PloS one*, *8*(6), e67212; *see also* Almeda, R., Hyatt, C., & Buskey, E. J. (2014). Toxicity of dispersant Corexit 9500A and crude oil to marine microzooplankton. *Ecotoxicology and environmental safety*, *106*, 76–85 ("Our results indicate that Corexit 9500A is highly toxic to microzooplankton, particularly to small ciliates, and that the combination of dispersant with crude oil significantly increases the toxicity of crude oil to microzooplankton. The negative impact of crude oil and dispersant on microzooplankton may disrupt the transfer of energy from lower to higher trophic levels and change the structure and dynamics of marine planktonic communities.")
[11] Almeda et al. 2014.
[12] *Id*.
[13] *Id*.

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PLOS ONE

# Interactions between Zooplankton and Crude Oil: Toxic Effects and Bioaccumulation of Polycyclic Aromatic Hydrocarbons

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## Abstract

We conducted ship-, shore- and laboratory-based crude oil exposure experiments to investigate (1) the effects of crude oil (Louisiana light sweet oil) on survival and bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) in mesozooplankton communities, (2) the lethal effects of dispersant (Corexit 9500A) and dispersant-treated oil on mesozooplankton, (3) the influence of UVB radiation/sunlight exposure on the toxicity of dispersed crude oil to mesozooplankton, and (4) the role of marine protozoans on the sublethal effects of crude oil and in the bioaccumulation of PAHs in the copepod *Acartia tonsa*. Mortality of mesozooplankton increased with increasing oil concentration following a sigmoid model with a median lethal concentration of 32.4 $\mu l\ L^{-1}$ in 16 h. At the ratio of dispersant to oil commonly used in the treatment of oil spills (i.e. 1:20), dispersant (0.25 $\mu l\ L^{-1}$) and dispersant- treated oil were 2.3 and 3.4 times more toxic, respectively, than crude oil alone (5 $\mu l\ L^{-1}$) to mesozooplankton. UVB radiation increased the lethal effects of dispersed crude oil in mesozooplankton communities by 35%. We observed selective bioaccumulation of five PAHs, fluoranthene, phenanthrene, pyrene, chrysene and benzo[*b*]fluoranthene in both mesozooplankton communities and in the copepod *A. tonsa*. The presence of the protozoan *Oxyrrhis marina* reduced sublethal effects of oil on *A. tonsa* and was related to lower accumulations of PAHs in tissues and fecal pellets, suggesting that protozoa may be important in mitigating the harmful effects of crude oil exposure in copepods and the transfer of PAHs to higher trophic levels. Overall, our results indicate that the negative impact of oil spills on mesozooplankton may be increased by the use of chemical dispersant and UV radiation, but attenuated by crude oil-microbial food webs interactions, and that both mesozooplankton and protozoans may play an important role in fate of PAHs in marine environments.

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## Introduction

Zooplankton play a key role in marine food web dynamics, biogeochemical cycling and fish recruitment [1–3]. However, despite their importance in marine environments, our knowledge of the interactions between zooplankton and anthropogenic pollutants is very limited. There are three main types of interactions between zooplankton and pollutants. First, pollutants can have direct toxic effects on zooplankton, including lethal or sublethal effects [4]. Second, zooplankton are able to influence the physicochemical characteristics of the pollutants in the water column (e.g. by absorption, transformation and elimination) [4–6]. Finally, zooplankton may play an important role in the biomagnification of pollutants up food webs [4,7]. Therefore, understanding the interactions between pollutants and zooplankton is crucial for our understanding of the fate of pollution in the pelagic zone and their impact on marine environments.

Petroleum or crude oil is one of the most common pollutants released into the marine environment. Natural petroleum seeps, extraction, transportation, and consumption are the main sources of crude oil to the sea [8]. Although oil spills represent a small fraction of the total crude oil discharge into the sea, they have strong acute and long-term impacts on marine ecosystems, including effects from physical damages (physical contamination and smothering) and toxicity of their chemical compounds [8]. Recently, the Deepwater Horizon (DWH) oil spill in the Gulf of Mexico has raised concerns about the dramatic environmental and socio-economic impacts caused by oil spills in marine and coastal environments [9–11]. Crude oil is a complex mixture of both hydrocarbons, such as alkanes, cycloalkanes and aromatic hydrocarbons, and non-hydrocarbon compounds. Polycyclic aromatic hydrocarbons (PAHs) are considered to be the most acutely toxic components of crude oil, exerting its toxicity by interfering with membrane fluidity [12]. PAHs are also associated

with potential carcinogenic, teratogenic and mutagenic effects in aquatic animals and humans [13–16]. After an oil spill, small crude oil droplets (1–100 μm in diameter) generated by waves and winds are effectively suspended in the water column [17,18]. Also, plumes of small stable dispersed oil droplets are frequently found in subsurface waters after oil spills are treated with dispersants [19]. These crude oil droplets, which are frequently in the food size spectra of many zooplankters, can easily interact with planktonic organisms. For instance, small crude oil droplets can be ingested by zooplankton (protozoan and metazoans) when they are suspended in the water or attached to phytoplankton [20–26].

Among zooplankton, mesozooplankton (200–2000 μm) occupy a key position in pelagic food webs because of their role in the transfer of matter from primary producers to higher trophic levels [27,28]. Copepods are the dominant group of mesozooplankton in marine environments [27]. Lethal and sublethal effects, including narcosis [29], alterations in feeding [30], development [31], and reproduction [32–34] have been observed in copepods exposed to petroleum hydrocarbons. Effects of petroleum hydrocarbons on mesozooplankton (e.g. copepods) vary widely depending on intrinsic (e.g., species, life stage, size) and extrinsic factors (e.g., oil concentration, exposure time, temperature) [30,35–38]. Field and laboratory studies have also shown that copepods can accumulate PAHs [22,25,39–41]. Most crude oil toxicity tests and PAH bioaccumulation studies on zooplankton have been conducted using the crude oil water soluble fraction (WSF), or certain mixed or individual PAHs. However, since zooplanktons can ingest oil droplets [20,24,25], exposure to dispersed crude oil may promote the uptake of PAHs as compared with experiments using WSF. For example, the concentration of PAHs in fish was higher in fish exposed to dispersed crude oil than when exposed to WSF at the same hydrocarbon concentration [42]. Moreover, toxicity test and PAH bioaccumulation studies have traditionally focused on single species and conducted in the absence of food (starvation) [29,43]. Therefore, experiments with natural mesozooplankton assemblages exposed to suspended crude oil with natural food conditions are required to better estimate the potential accumulation of petroleum hydrocarbons by zooplankton and their toxic effects.

Treatment of oil spills frequently involves the use of dispersants, which are mixtures of surfactants and other soluble compounds. Dispersants promote the removal of an oil slick from the surface waters enhancing the formation of small oil droplets, and therefore increasing their rate of natural dispersion. The first types of dispersants, like those used in the Torrey Canyon (1967) and Sea Empress (1996) oil spills, were highly toxic to marine animals, including fish, bivalves, and crustaceans, according to laboratory studies and field observations [44–48]. New types of dispersants (e.g. Corexit series dispersants, Corexit 9500 and Corexit 9527) are less toxic than the older types and have low to moderate toxicity to most marine animals according to laboratory studies [49,50]. Thus, it has been suggested that the new generation of dispersants and dispersant treated - oil are less toxic than the spilled oil alone [51,52] and that they have minimal deleterious effects on marine life [53]. However, little is known about the effects of this dispersant or dispersant treated oil on copepods or natural mesozooplankton communities, even though they are particularly susceptible to oil/dispersant exposure and they have important roles in marine ecosystems.

Most oil toxicological studies during the last decades have been conducted in the laboratory under artificial, fluorescent light [54]. However, there is increasing evidence that sunlight, mainly UV radiation (UVR), can increase the toxicity of petroleum hydrocarbons to marine organisms [55–58]. Photoenhanced toxicity (i.e., increase in the toxicity in the presence of light) of certain petroleum hydrocarbons has been observed in certain marine organisms [55–57], but information on phototoxicity of crude oil in zooplankton is scarce [59]. Therefore, knowledge of the effects of combined UVR and oil/dispersed oil/dispersant on zooplankton communities is essential for a better understanding of the impact of oil spills in the ocean.

Protozoan microplankton (e.g. ciliates and heterotrophic dinoflagellates) are the major consumers of phytoplankton and are important contributors to the diet of copepods [60]. Protozoans can also ingest oil droplets [21] and oil-contaminated phytoplankton. Bioaccumulation of PAHs in copepods may increase by feeding on oil-contaminated protozoans, but protozoans may also remove oil from the water, reducing the oil available for copepods. Therefore, in natural planktonic communities, the influence of crude oil on copepods may be affected by complex interactions between crude oil and microbial communities, including protozoans. Nevertheless, the potential role of protozoans in the interactions between dispersed crude oil and copepods (e.g. biomagnification or mitigation) has generally been neglected in petroleum toxicological and bioaccumulation studies.

The overall goal of this study was to improve our knowledge of the interactions between crude oil and marine zooplankton. To address this topic we conducted 3 types of experiments: 1) ship-based crude oil exposure experiments with natural mesozooplankton assemblages from the northern Gulf of Mexico, 2) shore-based crude oil and dispersant-treated crude oil exposure experiments with coastal mesozooplankton communities, and 3) laboratory crude oil exposure experiments with the copepod *Acartia tonsa*. *A. tonsa* is a widespread and dominant calanoid copepod species in estuaries and coastal waters, including the Gulf of Mexico. The specific objectives were to (1) determine the effects of short-term crude oil exposure on the survival and bioaccumulation of PAHs in natural mesozooplankton assemblages; (2) assess the lethal effects of dispersant-threated crude oil and dispersant (Corexit EC9500A) on coastal mesozooplankton communities; (3) estimate the influence of UVB radiation/sunlight exposure on the toxicity of dispersed crude oil to mesozooplankton communities; and (4) examine the role of marine protozoans on the sublethal effects (i.e., egg production, egg hatching, and egestion rates) of crude oil and the bioaccumulation of PAHs in the copepod *A. tonsa*. We used *Oxyrrhis marina*, a cosmopolitan heterotrophic dinoflagellate common in many intertidal and coastal habitats, as a model marine protozoan.

## Methodology

### Experimental Organisms

Natural zooplankton assemblages were collected from 3 stations in the northern Gulf of Mexico on the research vessel "Pelican" in May 2012 during a four-day cruise (Fig. 1) and from the Aransas Ship Channel near the University of Texas Marine Science Institute (MSI) in Port Aransas, TX (27°49′39″ N 97°4′20″W). No permission is required for collecting zooplankton within state (Texas) or federal waters in our sampling areas. The University of Texas does not require an Animal Use/Animal Care protocol for invertebrates (only for vertebrates). Our studies did not involve endangered or protected species.

During the cruise, zooplankton samples were obtained by slow-speed plankton tows (10 m $min^{-1}$) using a plankton net (50 cm diameter, 150 μm-mesh) with a 3 L plastic bag as a non-filtering cod end in order to minimize capture stress and physical damage to the organisms. Vertical tows from near the bottom to the surface were conducted at stations A (18 m depth) and B (50 m

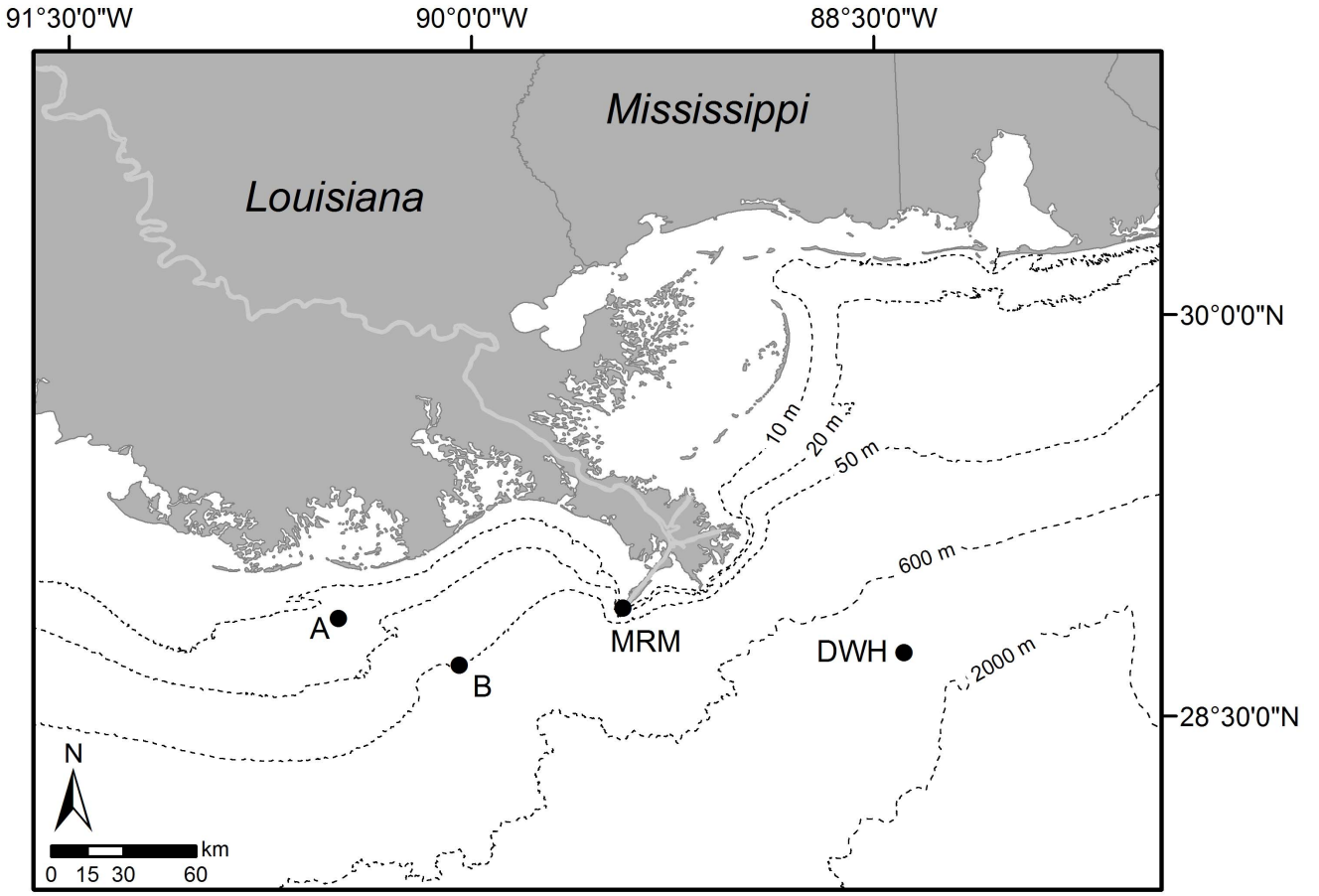


**Figure 1. Map indicating the zooplankton sampling stations during the cruise in the northern Gulf of Mexico: station A (A), station B (B) and Mississippi River Mouth station (MRM).** Stations are located in the area affected by the deepwater horizon (DWH) oil spill on April 2010.
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depth). In the station MRM (Mississippi River Mouth, 6 m depth), zooplankton samples were collected by horizontal tow from surface water. Zooplankton samples from the Aransas Ship Channel were collected from surface waters by tying the plankton net to the MSI pier and allowing it to stream with the tidal current for approximately 5–10 min. The plastic bags were kept in isothermal containers with seawater at in situ temperature until returning to the laboratory. Natural zooplankton assemblages were gently screened through a 2000 μm mesh sieve to remove large zooplankton (e.g. chaetognaths, salps, scyphozoans). Then, the mesozooplankton sample was carefully concentrated with a 150 μm mesh sieve and placed into a glass beaker with 0.2 μm-filtered seawater.

The calanoid copepod *Acartia tonsa* was collected in Aransas Bay (Texas coast) using a similar plankton net as used for the natural zooplankton assemblages. In the laboratory, approximately 100 adults (males and females) were sorted under a stereomicroscope and placed into a beaker with filtered sea water (FSW). To reduce the presence of other planktonic organisms, adult *A. tonsa* were repeatedly transferred through a series of petri dishes with 0.2 μm FSW. Specimens were reared in the laboratory for several weeks in 25 L transparent glass tanks with 1 μm FSW at 25°C under a 12-hour day/night cycle. *A. tonsa* cultures were fed the cryptophyte *Rhodomonas* sp. (equivalent spherical diameter, ESD = 7 μm), which were grown at 24°C in 10 L glass flasks using 'f/2' medium. The heterotrophic dinoflagellate *Oxyrrhis marina* (ESD = 15 μm), was fed with *Rhodomonas* sp. and cultured in 2 L glass beakers at similar temperature and light regime.

## Preparation of Crude Oil Emulsions and Dispersant Treated-oil

In this study, we used a Light Louisiana Sweet Crude Oil and determined the concentration and composition of PAHs in this oil. This crude oil was provided by BP (BP Exploration & Production Inc.) as a surrogate for the Macondo (MC252) crude oil released in the Deepwater Horizon oil spill in the Gulf of Mexico because they are considered to have similar chemical composition and toxicity. Corexit 9500A, one of the Corexit series of oil spill dispersant, was used for the shore-based experiments. The dispersant was provided by NALCO (Nalco/Exxon Energy Chemicals, L.P.) and its chemical composition can be found in the NALCO web page [61].

To prepare crude oil-seawater emulsions (i.e. suspensions of oil droplets in seawater), 0.2 μm filtered seawater was placed in a glass beaker with a magnetic stir bar, which was tightly sealed with aluminum foil to prevent oil absorption on the surface of the bar. Crude oil was added to the seawater using a Hamilton steel plunger microliter syringe and the glass beaker was placed on a magnetic stirrer plate. After covering the beaker with Teflon film, the oil was emulsified by stirring at 900 rpm for 5 min at room temperature (25°C). This stir speed allowed the formation of a

vortex large enough to generate oil droplets in seawater. The formation of oil droplets, most of them between 1–10 μm of diameter, was confirmed using an Imaging Particle Analysis system (FlowCAM). To prepare dispersant threated-oil, we used a ratio of dispersant to oil of 1:20, which is in the range (1:50–1:10) recommended by U.S. EPA [62].

## Experimental Design and Procedures

We conducted ship-based crude oil exposure experiments to investigate the effects of crude oil on survival and bioaccumulation of PAHs in mesozooplankton from the northern Gulf of Mexico. Natural mesozooplankton assemblages (community-based approach) were incubated onboard with natural seawater, which contained emulsified crude oil at a concentration between 10–100 μl $L^{-1}$ (Table 1). Each experiment consisted of three replicates at each crude oil concentration (''experimental bottles'') and three control treatments (no crude oil added, ''control bottles''). Water for these incubations was collected from Niskin bottles from the deep chlorophyll maximum (DCM, surface waters during this cruise) and transferred directly into acid-washed 1 L polycarbonate bottles with silicon tubing using a 3-step filling procedure to ensure homogeneity between replicates. Sea water samples (4 L) from the DCM were filtered through pre-incinerated GF/F filters and frozen (-20°C) for further analysis of polycyclic aromatic hydrocarbons as the background level. Aliquots from the zooplankton concentrate sample were added to the experimental and control bottles. Two additional aliquots were preserved in 4% buffered formaldehyde for later analysis of the initial copepod composition and concentration. After adding emulsified oil to the corresponding experimental bottles, bottles were incubated on deck in a large transparent acrylic container mounted to a plankton wheel with open-circuit seawater from 5-m depth running through it, thus providing exposure to sunlight and *in situ* temperature. The water temperature during the incubations was 25.5°C. After 16 hours of incubation, the contents of each bottle were gently screened through a submerged 150 μm mesh sieve to collect the zooplankton. Zooplankton were then rinsed 2 times with FSW, concentrated and placed in a beaker with 220 ml FSW. One aliquot with at least 20 individuals was placed in Petri dishes filled with 0.2 μm filtered seawater and then, checked for swimming activity and survival after 5 min. After 1 hour of being removed from the crude oil, we checked the copepods again for signs of recovery. One aliquot (20 ml) for the zooplankton concentrate was preserved in 4% buffered formaldehyde for later analysis of the final copepod species composition and abundance. The remaining sample was filtered again using a 150 μm mesh sieve and thoroughly rinsed with surface seawater using a pressure hose to minimize oil droplets that could potentially be attached to the copepods. Then, the rinsed copepod samples were filtered onto pre-combusted (450°C, 6 h) glass-fiber filters (GF/F) and frozen (−20°C) until further hydrocarbon analysis. For the estimation of abundance and species composition of natural mesozooplankton assemblages, one aliquot of at least 100 organisms from each sample was examined under a stereomicroscope.

We conducted two shore-based crude oil exposure experiments (community-based approach). In the first experiment, coastal mesozooplankton communities were incubated in quartz bottles (exposed to the full solar radiation spectrum) with crude oil (5 μl $L^{-1}$), dispersant (0.25 μl $L^{-1}$) and crude oil+dispersant (20:1) for 48 h to determine the lethal effect of dispersant-treated oil and dispersant on mesozooplankton communities. Control and experimental treatments were performed in triplicates. In the second experiment, mesozooplankton communities were incubated in quartz bottles with dispersant treated oil (5 μl $L^{-1}$ oil +0.25 μl $L^{-1}$ dispersant) for 48 h under 3 different light regimes: the full solar radiation spectrum (PAR+UVR), the full spectrum without UVB (i.e., PAR+UVA, covered with Mylar-D foil) and kept in the dark (covered with aluminum foil) to assess the effect of UVR/sunlight in dispersed oil toxicity. Control and experimental treatments were run in duplicates. In both experiments, mesozooplankton communities were incubated with natural seawater collected from surface waters. Experimental procedures used to determine mortality were similar to those described above for the ship-based experiments. Bottles were incubated on the MSI pier in a large open/uncovered transparent acrylic container containing a plankton wheel with open-circuit seawater running through it, thus providing exposure to sunlight and *in situ* temperature. Temperature and light were measured using a YSI® Model

**Table 1.** Initial mesozoplankton concentration (ind. $L^{-1}$) and composition in the crude oil exposure experiments conducted in the northern Gulf of Mexico (Stations A, B and MRM) and in the Aransas Ship Channel (AC1 and AC2).

| *Taxonomic groups/ Category* | *Stations* | | | | |
|---|---|---|---|---|---|
| | **A** | **B** | **MRM** | **AC1** | **AC2** |
| **Calanoid copepods** | | | | | |
| *Acartia tonsa* | 395 | 7 | 901 | 104 | 73 |
| *Paracalanus spp* | 379 | 234 | 0 | 32 | 75 |
| *Parvocalanus crassirostris* | 1 | 0 | 50 | 29 | 8 |
| *Calocalanus spp* | 3 | 95 | 0 | 0 | 0 |
| *Centropages spp* | 74 | 3 | 2 | 0 | 0 |
| *Euchaeta spp* | 0 | 48 | 0 | 4 | 0 |
| *Temora spp* | 11 | 22 | 0 | 32 | 0 |
| Others | 44 | 69 | 0 | 18 | 5 |
| **Cyclopoid copepods** | | | | | |
| *Oithona plumifera* | 0 | 83 | 0 | 0 | 0 |
| *Oithona spp* | 30 | 5 | 442 | 36 | 48 |
| **Poecilostomatoid copepods** | | | | | |
| *Oncaea spp* | 27 | 379 | 12 | 0 | 0 |
| *Corycaeus sp* | 29 | 76 | 0 | 0 | 0 |
| *Farranula sp* | 0 | 12 | 0 | 0 | 0 |
| **Harpacticoid copepods** | | | | | |
| *Euterpina acutifrons* | 23 | 0 | 4 | 4 | 8 |
| *Microsetella sp* | 6 | 17 | 0 | 0 | 0 |
| Others | 0 | 0 | 0 | 0 | 3 |
| **Copepod Nauplii** | 7 | 52 | 10 | 11 | 23 |
| **Other holoplankton** | | | | | |
| *Oikopleura dioica* | 10 | 2 | 0 | 4 | 18 |
| *Mysidacea larvae* | 17 | 5 | 0 | 0 | 0 |
| Others | 11 | 8 | 0 | 0 | 0 |
| **Meroplankton** | | | | | |
| *Polychaeta* | 0 | 0 | 0 | 32 | 10 |
| *Gastropoda* | 0 | 10 | 0 | 0 | 38 |
| *Cirripedia* | 1 | 0 | 8 | 36 | 48 |
| Other larvae | 1 | 2 | 0 | 0 | 8 |
| **Total (ind $L^{-1}$)** | **1069** | **1129** | **1429** | **342** | **365** |

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30 SCT Meter and a LI-COR® LI-250A Light Meter, respectively. In the first experiment, water temperature was 19°C (±3°C) and the measured solar radiation ranged from 48 to 485 µmol photons $m^{-2}$ $s^{-1}$ during the daylight hours. In the second experiment, water temperature was 18°C (±1°C) and the measured solar radiation ranged from 122 to 757 µmol photons $m^{-2}$ $s^{-1}$ during the daylight hours. Survival of mesozooplankton in the different treatments was estimated as describe above for the ship-based experiments.

We conducted laboratory crude oil exposure experiments to evaluate the role of marine protozoans on the sublethal effects of crude oil and the bioaccumulation of PAHs in the copepod *Acartia tonsa*. Adult stages of *A. tonsa* were incubated with crude oil (5 µl $L^{-1}$) in the laboratory for 48h. Two types of incubations experiments were conducted: 1) *A. tonsa* fed with a phytoplankton species, *Rhodomonas* sp. and 2) *A. tonsa* fed with *Rhodomonas* sp. and a protozoan species, *Oxyrrhis marina*. Each experiment included triplicate experimental treatments ("experimental") and 1–2 control treatments ("control"). Adult *A. tonsa* were removed from stock cultures by filtering them through a submerged 150 µm mesh sieve and were concentrated in FSW. Aliquots containing approximately 600 adult copepods were then placed into glass aquariums containing 15 L of FSW and the 2 different food regimes, *Rhodomonas* sp. (50,000 cells $mL^{-1}$) and *Rhodomonas* sp.+*Oxyrrhis marina* (50,000 cells $mL^{-1}$+700 cells $mL^{-1}$, respectively). Next, oil emulsions were added to the corresponding experimental aquariums. To keep the oil droplets suspended in the water, turbulence was created by aeration using 2 glass tubes connected to an air pump. Experimental and control (without oil) treatments were run in duplicate, simultaneously. Incubations were conducted at 25°C under artificial dim light for 48 h. After incubation, two aliquots with at least 25 individuals from each aquarium were placed in Petri dishes filled with 0.2 µm filtered seawater and then checked for swimming activity and survival. Next, all *A. tonsa* adults from each aquarium were separated out from water, which contains their fecal pellets and eggs, using a 150 µm mesh sieve. As with the community-based approach, the samples were thoroughly rinsed with FSW using a pressure sprayer and concentrated in 400 ml of FSW. To separate copepod eggs from fecal pellets, water samples (fraction <150 µm) were screened through a 40 µm mesh sieve, rinsed thoroughly using a pressure sprayer and concentrated in 200 mL of FSW. The separation of eggs from fecal pellets was corroborated under a stereomicroscope. Finally, fecal pellets/debris were filtered using a 20 µm mesh sieve, rinsed and concentrated in 400 mL of FSW. One aliquot (10 or 15 ml) of each type of concentrated sample (copepod, eggs or fecal pellets) was preserved in 1% Lugol's solution for counting. The remaining concentrated samples of the copepod, eggs and fecal pellets were filtered onto pre-combusted (450°C, 6 h) glass-fiber filters (GF/F) and frozen (−20°C) until further hydrocarbon analysis.

## Chemical Analysis

Sixteen priority PAHs defined by the US Environmental Protection Agency (EPA) were analyzed: naphthalene (Nap), acenaphthene (Ace), acenaphthylene (Acy), fluorene (Flu), phenanthrene (Phe), anthracene (An), fluoranthene (Flua), pyrene (Pyr), benzo[*a*]anthracene (BaA), chrysene (Chr), benzo[*b*]fluoranthene (BbF), benzo[*k*,j]fluoranthene (BkF), benzo[*a*]pyrene (BaP), indeno [*1,2,3*]pyrene (InP), dibenzo[*a,h*]anthracene (DBA), and benzo[*ghi*]perylene (BgP). The 16 PAH standards and 3 PAH surrogate standards ($D_{10}$- Acenaphthene (Ace-$D_{10}$), $D_{10}$Phenanthrene (Phe-$D_{10}$), $D_{12}$-Benzo[a] anthracene (BaA-$D_{12}$) were purchased from Sigma. All organic solvents (HPLC grade) were purchased from Fisher Scientific. Sodium sulfate and neutral alumina were baked at 450°C for 4 h. The silica gel was cleaned with dichloromethane (DCM) before using. The neutral alumina and silica gel were activated by heating at 120°C for 12 h. Reagent grade water (5% wt.) was mixed with the neutral alumina for partial deactivation.

Chemical analysis of the crude oil followed the protocol of Liu et al. [63]. Briefly, 100 µL of crude oil was diluted to 1 mL with hexane. The sample was purified with a self-packed chromatographic column with 1g anhydrous sodium sulfate and 8 g silica gel. The column was eluted with 50 mL dichloromethane/hexane (1:4, v/v). The eluted solution was concentrated to 1mL by a rotary evaporator, and preserved in a freezer (−20°C) until analysis by gas chromatography-mass spectrometry (GC/MS). The composition and concentration of PAHs in the Light Louisiana Sweet Crude Oil used in these experiments are shown in Figure 2.

Zooplankton samples were freeze-dried and weighed. Replicate samples were combined to obtain enough biomass for analysis. PAHs in zooplankton samples were extracted by Soxhlet extractors for 24 h, using hexane and DCM (1:1, v/v) as the extraction solution. The solution was concentrated to ca. 2 mL by a rotary evaporator and purified with a chromatographic column packed with 1 g anhydrous sodium sulfate (top), 4 g neutral alumina (middle), and 8 g silica (bottom). The concentrated solution was eluted from the column with 50 mL DCM/hexane (1:4, v/v). The collected solution was concentrated to 0.5 mL and exchanged with hexane by a rotary evaporator. A portion of the solution was used for the PAH analysis. PAHs were analyzed using GC/MS (Shimadzu QP2010 plus) with a RXi-1MS capillary column (20 m×0.18 mm i.d., film thickness 0.18 µm). The injection volume was 1 µL sample with a split ratio of 1/20, and the helium flow was set at 0.8 mL $min^{-1}$. The temperatures of the injector and detector were set at 260°C and 275°C, respectively. The temperature of the column was ramped from 60°C to 240°C at 10°C $min^{-1}$, and increased to 280°C at 4°C $min^{-1}$ and held for 3 min. Selected ion monitoring mode was used to quantify PAHs, which ranged from 126 to 279 a.m.u., and dwell time per ion was 200 ms. The average recovery of surrogate standards for seawater and zooplankton were 93% (n = 12) and 95% (n = 12), respectively. The detection limit of this method is 0.001–0.004 ng/µL.

## Calculations

Mortality, as % of the incubated organisms, was estimated from the number of dead (not swimming after gently touching with a Pasteur pipette tip) individuals at the second visual checking. Narcosis (%) was estimated from the difference in the number of non-swimming individuals at the first checking (which included actual dead and narcotized animals) and the second checking (which included only those copepods that did not recover from toxic effects).

Data on copepod mortality versus crude oil concentration were fitted to the following sigmoid model:

$$M = 100/\left(1 + e^{-(C - LC50/b)}\right) \quad (1)$$

where, M is the copepod mortality (%), $C$ is the crude oil concentration (µl $L^{-1}$), $LC_{50}$ is the median lethal concentration and $b$ is the slope factor.

Egg production rates, fecal pellet production rates and egg hatching of *Acartia tonsa* were evaluated after 48 hours of crude oil exposure. Samples of adult stages, eggs/nauplii and faecal pellets of *A. tonsa* were counted under a stereomicroscope. Egg production was estimated as the total number of eggs and hatched eggs

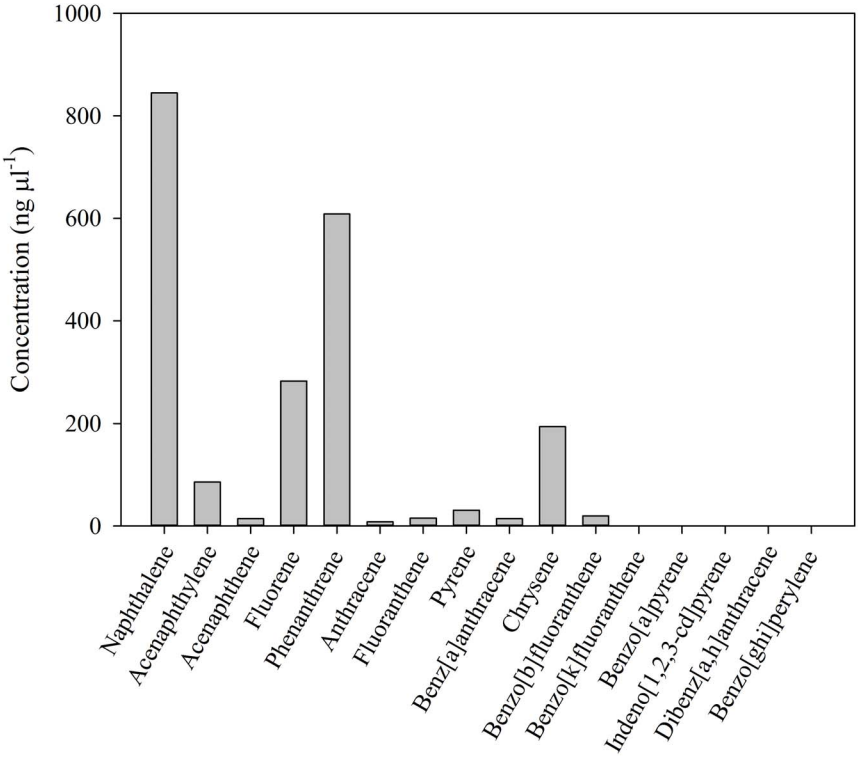


**Figure 2. Concentration of polycyclic aromatic hydrocarbons (PAHs, ng $\mu L^{-1}$) in the crude oil used in the experiments (Louisiana light sweet crude oil).**
doi:10.1371/journal.pone.0067212.g002

(nauplii). Hatching (%) was assessed from number of nauplii in relation to total number of observed eggs and nauplii after incubation time.

Bioaccumulation factor is the ratio of pollutant concentration in an aquatic organism to the water concentration that includes dietary uptake. The bioaccumulation factor (BAF) in the copepods exposed to crude oil was calculated as follows:

$$BAF = [PAH]_{zoo} x1000/[PAH]_{water} \quad (2)$$

where, $[PAH]_{zoo}$ is the concentration of polycyclic aromatic hydrocarbons (PAHs) in exposed copepods after subtracting the concentration of PAHs in the corresponding control treatment, in ng $g^{-1}$ and $[PAH]_{water}$ is the concentration of PAHs in seawater, in ng $L^{-1}$. Biomass was calculated as dry weight (DW). The concentration of PAHs in the water (Table 2) was estimated from the oil added to the containers, using the concentration of PAHs determined in the crude oil (Fig. 2). In our experiments, PAHs in the seawater would have been presented in both dissolved and particulate (oil droplet) forms.

## Results

### Composition of Natural Mesozooplankton Assemblages used in the Experiments

The natural mesozooplankton assemblages from northern Gulf of Mexico (Stations A, B and MRM) used in the experiments were dominated by copepods (96%–99%) (Fig. 3A). Calanoid copepods were the most abundant group of copepods at stations A and MRM, whereas both calanoid and poecilostomatoid copepods were the major components of the copepod community at station B (Fig. 3B). We observed differences in copepod taxonomic composition among stations in the northern Gulf of Mexico (Table 1). Stations C6 and NC had a high diversity of copepod species, whereas at station MRM the copepod community was mainly dominated by the calanoid copepod *Acartia tonsa* and the cyclopoid copepod *Oithona spp* (Table 1). Mesozooplankton communities from the Aransas Ship Channel (AC1, AC2) were also dominated by copepods but meroplanktonic larvae represented ca. 20–30% in abundance (Fig. 3A). The main meroplanktonic larvae were cirripede nauplii, polychaeta larvae, and gastropod veligers. Calanoids (e.g. *Acartia*, *Paracalanus*, *Parvocalanus*, *Temora*) and cyclopoids (*Oithona* spp.) were the main groups of copepods observed in the mesozooplankton communities from the Aransas Ship Channel (Table 1).

### Lethal Effects of Crude Oil on Northern Gulf of Mexico Mesozooplankton Communities

Overall, we observed a significant effect of crude oil on mesozooplankton survival (ANOVA, $F_{6,\ 29} = 181.9$, $p<0.01$; Table 3). Mortality ranged from 12% to 96% depending on crude oil concentrations and station (Table 3). At each station, average mesozooplankton mortality (%) increased as crude oil concentrations increased (Table 3). At station A, massive mesozooplankton mortality (>90%) was observed at crude oil concentrations ≥50 µl $L^{-1}$ after only 16 h (Table 3). By including data from all experiments, the relationship between mesozooplankton mortality (%) and crude oil concentration was well described by the sigmoid model ($r^2 = 0.92$) (Fig. 4). According to the model, the median lethal concentration ($LC_{50}$), i.e. lethal concentration required to kill half the members of a tested population, was 31.4 µl $L^{-1}$ after 16 h (Fig. 4). Narcosis effects

**Table 2.** Concentration of polycyclic aromatic hydrocarbons, PAHs (μg $L^{-1}$), in the water at the different crude oil exposure levels (5–100 μl $L^{-1}$) used in the experiments.

| [crude oil] μl $L^{-1}$ | [crude oil] mg $L^{-1}$ | Nap | Ace | Acy | Flu | Phe | An | Flua | Pyr | BaA | Chr | BbF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *5* | *4.2* | 4.22 | 0.43 | 0.07 | 1.41 | 3.04 | 0.04 | 0.08 | 0.15 | 0.07 | 0.97 | 0.10 |
| *10* | *8.5* | 8.45 | 0.85 | 0.14 | 2.82 | 6.08 | 0.08 | 0.15 | 0.31 | 0.14 | 1.94 | 0.20 |
| *20* | *16.9* | 16.89 | 1.71 | 0.28 | 5.65 | 12.17 | 0.16 | 0.31 | 0.62 | 0.28 | 3.88 | 0.39 |
| *25* | *21.1* | 21.12 | 2.14 | 0.35 | 7.06 | 15.21 | 0.20 | 0.38 | 0.77 | 0.35 | 4.85 | 0.49 |
| *30* | *25.4* | 25.34 | 2.56 | 0.42 | 8.47 | 18.25 | 0.24 | 0.46 | 0.93 | 0.42 | 5.82 | 0.59 |
| *50* | *42.3* | 42.23 | 4.27 | 0.70 | 14.11 | 30.42 | 0.40 | 0.76 | 1.54 | 0.70 | 9.70 | 0.98 |
| *100* | *84.5* | 84.46 | 8.54 | 1.40 | 28.23 | 60.83 | 0.80 | 1.53 | 3.08 | 1.40 | 19.39 | 1.96 |

Concentration of PAHs was estimated from the oil added to the containers using the concentration of PAHs determined in the crude oil (Fig. 2) Crude oil exposure levels are also expressed in mg $L^{-1}$ using a crude oil density of 0.845g/ml. Naphthalene (Nap), acenaphthene (Ace), acenaphthylene (Acy), fluorene (Flu), phenanthrene (Phe), anthracene (An), fluoranthene (Flua), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr), benzo[b]fluoranthene (BbF).

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varied from 1% to 56% depending on the station and crude oil concentration (Table 3). Significant narcotic effects in mesozooplankton communities were observed at station A at crude oil concentration of 10 μl $L^{-1}$ and at station MRM at all crude oil concentrations, where narcosis was higher than 50% at concentrations of 10 and 20 μl $L^{-1}$ (Table 3).

## Lethal Effects of Dispersant and Dispersant-treated Oil on Mesozooplanton Communities

We observed significant differences in mesozooplankton mortality among treatments (ANOVA, $F = 149$, $p<0.01$) (Fig. 5). Mortality in the control treatment was ca. 11%, significantly lower than in the experimental treatments (ANOVA, Tukey test, $F = 149$, $p<0.01$) (Fig. 5). Mortality of mesozooplankton communities exposed to crude oil (5 μl $L^{-1}$) was 21% after 48 h (Fig. 5). Exposure of mesozooplankton communities to the dispersant (0.25 μl $L^{-1}$) caused a mortality of 48% after 48h (Fig. 5). The highest mortality was observed in the dispersant-treated oil treatment, reaching values of 72% after 48 h (Fig. 5). Therefore, dispersant and dispersed-oil were >2.3 and >3.4 times more toxic, respectively, than crude oil alone to coastal mesozooplankton communities (Fig. 5).

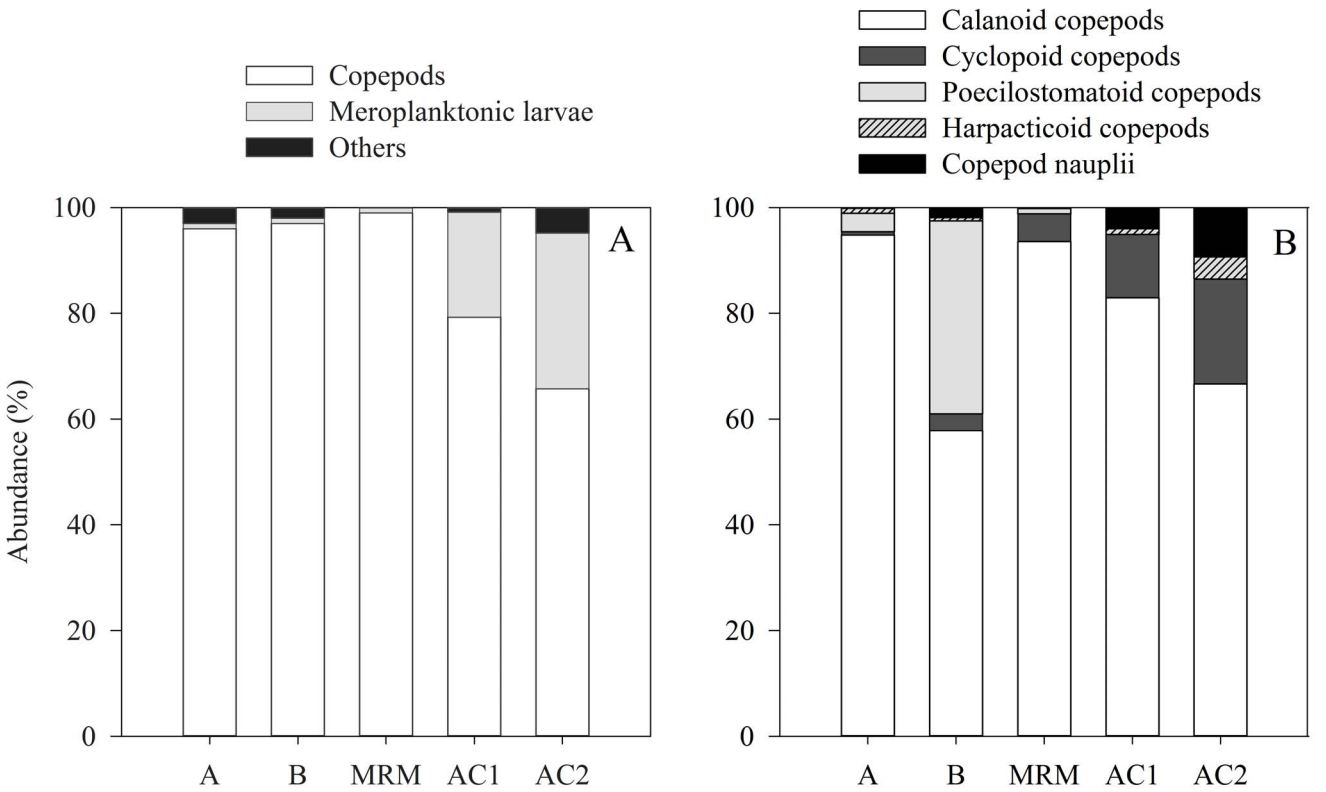


**Figure 3. Composition in abundance (%) of the natural mesozooplankton assemblages used in the experiments.** A: metazooplankton composition. B: copepod composition.

doi:10.1371/journal.pone.0067212.g003

**Table 3.** Mortality and narcosis of natural mesozooplankton communities from the northern Gulf of Mexico (Stations A, B and MRM) after 16 h of crude oil exposure.

| Station | [crude oil] µl $L^{-1}$ | Mortality (%, Avg. ± SE) | Narcosis (%, Avg. ± SE) | n ± SD |
|---|---|---|---|---|
| A | 0 | 14±2 | 7±1 | 25±3 |
| | 25 | 21±3 | 14±2* | 30±5 |
| | 50 | 92±3* | 2±2 | 23±2 |
| | 100 | 96±2* | 1±1 | 24±6 |
| B | 0 | 12±1 | 4±2 | 25±5 |
| | 10 | 16±3 | 7±2 | 27±4 |
| | 20 | 23±1* | 7±4 | 27±4 |
| | 30 | 55±3* | 5±3 | 28±3 |
| MRM | 0 | 13±2 | 10±2 | 30±6 |
| | 10 | 16±2 | 56±4* | 25±1 |
| | 20 | 25±2* | 55±4* | 22±2 |
| | 30 | 44±6* | 35±7* | 25±4 |

The asterisks indicate a significant difference ($P<0.05$) from respective controls.
Avg.: average, SE: standard error.
doi:10.1371/journal.pone.0067212.t003

## Influence of UV Radiation on the Toxicity of Dispersed Crude Oil to Mesozooplankton

Mesozooplankton mortality was higher in experimental (5 µl $L^{-1}$ of oil and 0.25 µl $L^{-1}$ of dispersant) than in control treatments (no oil added) for the three different light regimes (ANOVA, $p<0.01$) (Fig. 6). Mortality was very low (<7%) in all control treatments (Fig. 6). Mesozooplankton mortality was lower in the control treatments without UVB radiation ('Control_-PAR+UVA' and 'Control _dark') than in the control treatment exposed to the full solar radiation spectrum ('Control_PAR+—UVR') (Fig. 6). Mortality of mesozooplankton exposed to dispersant-treated oil with the full solar radiation spectrum ('Exp_PAR+UVR') was 68.6% after 48 hours, significantly higher than with the other light regimes ('Exp_PAR+UVA and 'Ex-

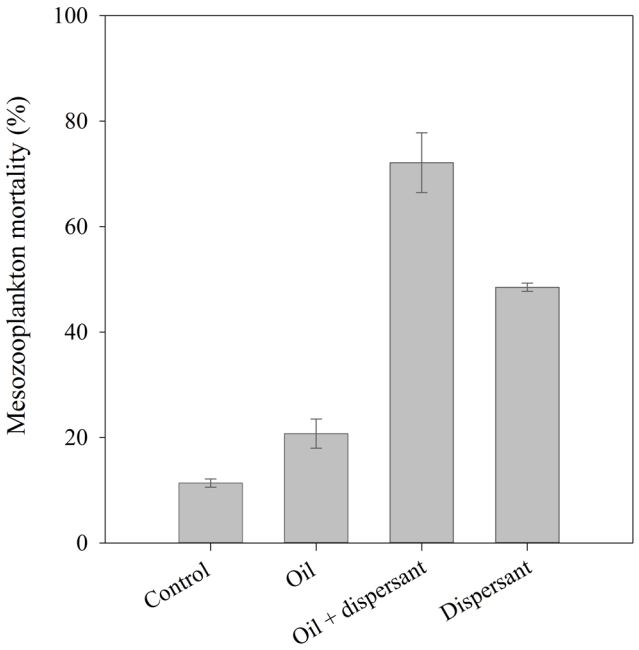


**Figure 5. Lethal effects of crude oil (5 µl $L^{-1}$), dispersant-treated crude oil, and dispersant (0.25 µl $L^{-1}$) on mesozooplanton communities from the Aransas Ship Channel (AC1, Fig. 2) after 48 h incubation (T = 22°C, full solar radiation spectrum).** Error bars represent the standard deviations.
doi:10.1371/journal.pone.0067212.g005

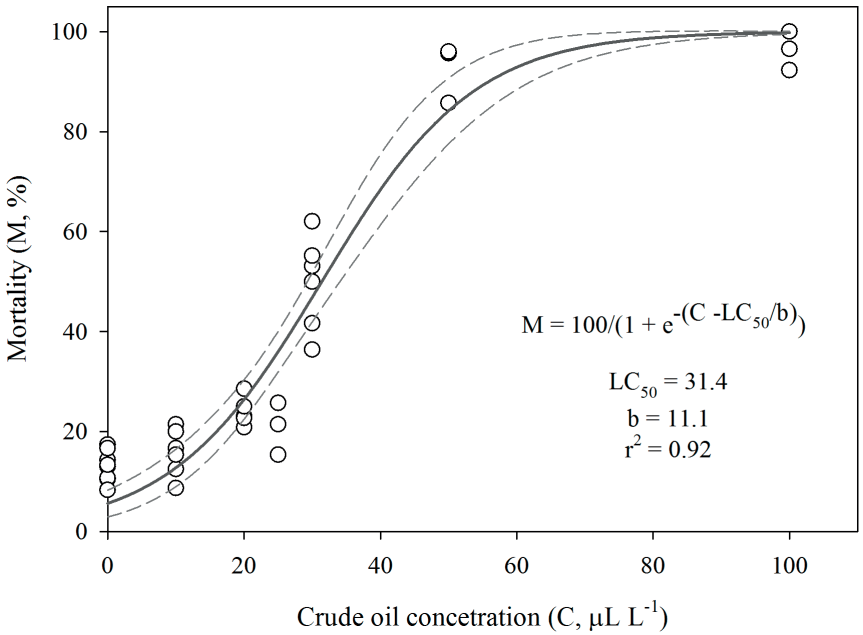


**Figure 4. Relationship between mesozooplankton mortality and crude oil concentration after 16 h of exposure in onboard incubations (25°C, sunlight exposure) conducted in the northern Gulf of Mexico.** Regression line based on Equation (1) (solid line) and 95% confidence intervals (dashed lines).
doi:10.1371/journal.pone.0067212.g004

p_dark') (ANOVA, $F_{2,3} = 17.3$, $p<0.05$) (Fig. 6). Mesozooplankton exposed to dispersant-treated oil without UVB radiation ('Exp_PAR+UVA) and in the dark ('Exp_dark') showed a mortality of 44.8% and 40.7%, respectively, with no significant differences between treatments (ANOVA, $F_{1,2} = 0.5$, $p>0.05$) (Fig. 6). These results indicated that UVA radiation had little influence in the toxicity of crude oil to mesozooplankton, and UVB radiation increased the lethal effects of dispersed crude oil to coastal mesozooplankton communities by 35% (Fig. 6).

## Sublethal Effects of Crude Oil Exposure on Acartia Tonsa

In the laboratory experiments, mortality of *Acartia tonsa* was very low (0%–4%) after 48 hours of exposure (5 µl $L^{-1}$), with no significant differences between experiment and control treatments (ANOVA, $F_{1,8} = 0.3$, $p>0.05$). We did not observe narcotic effects in *Acartia tonsa* in these laboratory experiments. Egg production rates varied from 14–124 eggs $female^{-1}$ $d^{-1}$ depending on the food regime (*Rhodomonas* or *Oxyrrhis/Rhodomonas*) and the treatment (crude oil exposed or non-exposed copepods) (Fig. 7). Egg production rates were >4 times higher when *A. tonsa* was incubated with *Oxyrrhis/Rhodomonas* than when incubated only with *Rhodomonas* (Fig. 7A, 7B). In both food regimes, eggs production rates of *A. tonsa* exposed to crude oil were lower than in non-exposed individuals (Fig. 7A, 7B). The reduction in egg production rates was significantly lower (ANOVA, $F_{1,5} = 13.9$, $p<0.05$) when *A. tonsa* was incubated with *Oxyrrhis/Rhodomonas* than when incubated only with *Rhodomonas* (1.42 and 2.05 times lower, respectively) (Fig. 7A, 7B). Egg hatching after 48 hours ranged from 39% to 59% depending on the food regime and treatment (Fig. 7C, 7D). As observed for egg production rates, egg hatching of *A. tonsa* exposed to crude oil was lower than control treatments for both food regimes (Fig. 7C, 7D). The reduction in egg hatching was significantly lower (ANOVA, $F_{1,5} = 8.8$, $p<0.05$) when *A. tonsa* was incubated with *Oxyrrhis/Rhodomonas* than when incubated with *Rhodomonas* (1.2 and 1.7 times lower, respectively) (Fig. 7C, 7D). Fecal pellets production rates ranged from 39–116 pellets $ind^{-1}$ $d^{-1}$ depending on the food regime and the crude oil treatment (Fig. 7E, 7F). Fecal pellet production rates were >2 times higher in *A. tonsa* incubated with *Oxyrrhis/Rhodomonas* than those incubated only with *Rhodomonas* (Fig. 7E, 7F). Fecal pellet productions rates of individuals not exposed to crude oil were lower than those exposed (Fig. 7E, 7F). However, fecal pellet productions rates showed high variability among replicates, and thus, non-significant differences (ANOVA, $F_{1,4} = 0.6$, $p<0.05$) between treatments were observed in *A. tonsa* incubated with *Oxyrrhis/Rhodomonas* (Fig. 7F).

## Bioaccumulation of PAHs in Natural Copepod Assemblages Exposed to Crude Oil

The total concentration of PAHs in the crude oil was 2.11 µg $µL^{-1}$ (Fig. 2). Naphthalene, phenanthrene, fluorene, chrysene,

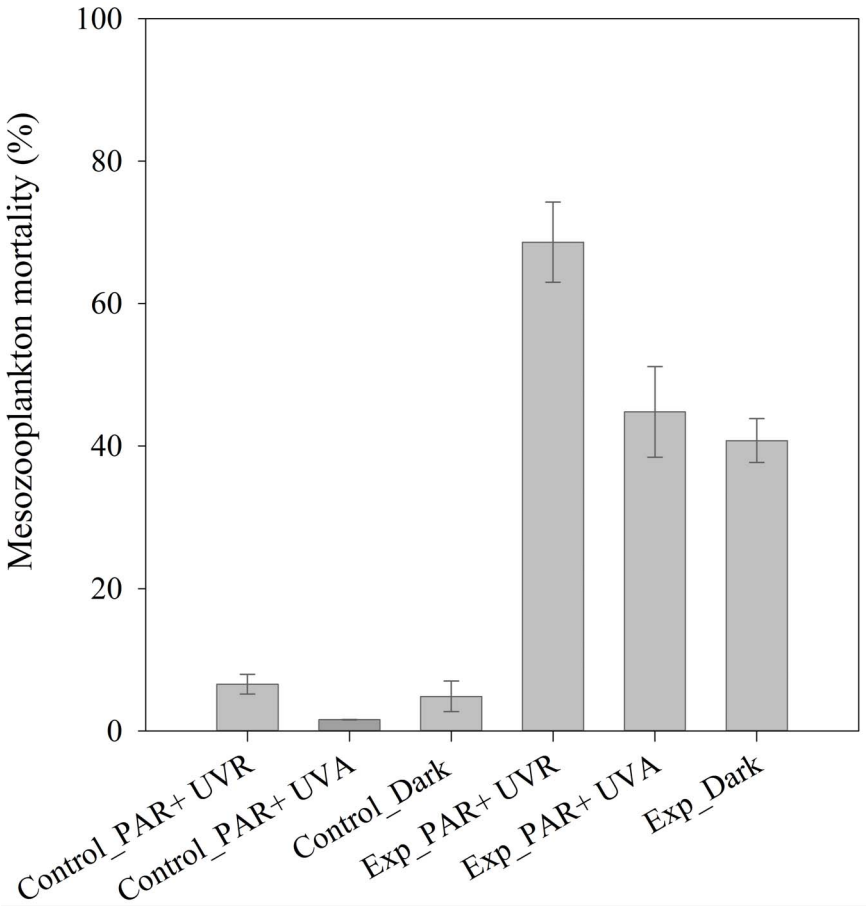


**Figure 6. Lethal effects of dispersant-treated crude oil (5 µl $L^{-1}$) on mesozooplanton communities from the Aransas Channel (AC2) under 3 different light regimes: the full solar radiation spectrum (PAR+UVR), the full spectrum without UVB (i.e., PAR+UVA) and kept in the dark after 48 h (T = 18°C).** Error bars represent the standard deviations.
doi:10.1371/journal.pone.0067212.g006

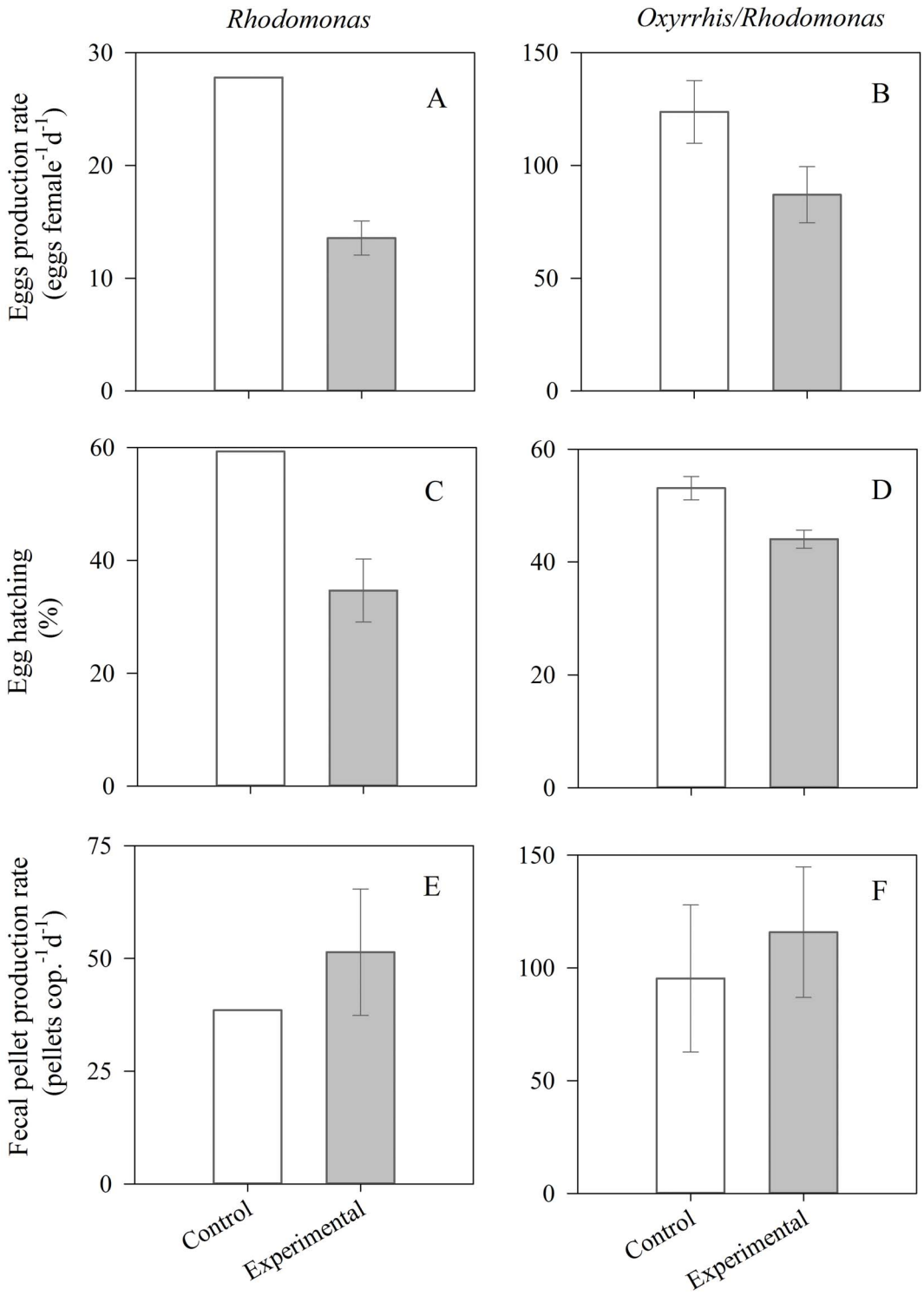


**Figure 7. Effect of crude oil exposure (5 μl $L^{-1}$, 48 h, dim light) on egg production rates, egg hatching and fecal pellet production rates of *Acartia tonsa* feeding on *Rhodomonas* sp. (left column, A, C, E) or *Rhodomonas* sp. plus *Oxyrrhis* marina (right column, B, D, F).** Experimental: oil exposed copepods. Control: non-exposed copepods. Error bars represent the standard deviations.
doi:10.1371/journal.pone.0067212.g007

and acenaphthylene were the most abundant PAHs in the crude oil (Fig. 2). The concentration of PAHs in the water used for the incubation experiments from all stations was undetectable in most cases, except for naphthalene.

Naphthalene, fluoranthene, phenanthrene, pyrene, chrysene and benzo[*b*]fluoranthene were the main PAHs detected in copepods (Fig. 8). Total concentration of PAHs in copepods exposed to crude oil was between 2.5–10 times higher than those not exposed, depending on the station (Fig. 8). Except for

naphthalene (Fig. 8A), the concentration of PAHs in copepods in the control treatment was very low at all stations, ranging from 0 for chrysene and benzo[*b*]fluoranthene (Fig. 8E, 8F) to <30 ng $g^{-1}$ $DW_{zoo}$ for fluoranthene, phenanthrene, pyrene (Fig. 8B, 8C, 8D). The concentrations of fluoranthene, phenanthrene, pyrene, chrysene were significantly higher (ANOVA, $p<0.01$) in copepods exposed to crude oil than in copepods not exposed to crude oil. At stations A and MRM, benzo[*b*]fluoranthene was not found in copepods at low crude oil concentration but was detected in copepods exposed to higher crude oil concentrations (Fig. 8F).

Bioaccumulation factors (BAFs) ranged from 3 to 2570 depending on the type of PAH, the crude oil concentration and the copepod community (Table 4). BAFs for naphthalene and phenanthrene were lower than for the other PAHs (Table 4). The highest bioaccumulation factors (>1000) were for fluoranthene and pyrene in the copepods community from station B at crude oil concentrations of 10 µl $L^{-1}$ (Table 4). At each station, we observed a decrease in BAFs for fluoranthene, phenanthrene, pyrene as crude oil concentrations increased (Table 4). Similarly, the BAFs for these PAHs decreased significantly as copepod mortality increased (Fig. 9 A–C). In contrast, we did not find any clear relationship between BAF of chrysene and Benzo[*b*]fluoranthene and crude oil concentration (Table 4) or copepod mortality (Fig. 9D, 9E).

### Bioaccumulation of PAH in Tissues, Eggs and Fecal Pellets of A. Tonsa Exposed to Crude Oil

As for natural copepod assemblages, naphthalene, fluoranthene, phenanthrene, pyrene, and chrysene were the main PAHs detected in *A. tonsa* (Fig. 10). However, the concentration of PAHs in the control treatments (non-exposed *A. tonsa*) was relatively higher than those of natural copepod assemblages, except for chrysene and benzo[*b*]fluoranthene that were not detected in both experiments (Fig. 8 and 10). Total concentration of PAHs in *A. tonsa* feeding on *Rhodomonas* was 1.4 times higher in exposed than non-exposed copepods (Fig. 10A). All PAHs showed higher concentrations in experimental treatments than in controls except naphthalene (Fig. 10A). In contrast, PAHs in *A. tonsa* incubated with *Oxyrrhis/Rhodomonas* was 1.6 lower in *A. tonsa* exposed to crude oil than in the control treatment (Fig. 10A). The total concentration of PAHs in non-exposed *A. tonsa* incubated with *Oxyrrhis* was similar to those incubated with *Rhodomonas* (664 ng $g^{-1}$ DW). However, the total concentration of PAHs in body tissues of *A. tonsa* incubated with *Oxyrrhis* was >2 times lower than those incubated with *Rhodomomas* (Fig. 10A, 10B). The concentration of all PAHs in body tissues was lower in the experimental treatment with *Oxyrrhis/Rhodomonas* than in that with *Rhodomonas* (Fig. 10A, 10B).

Total concentration of PAHs in fecal pellets of *A. tonsa* incubated with *Rhodomona*s and exposed to crude oil was 2.2 times higher than non-exposed copepods (Fig. 10C). Chrysene and benzo[*b*]-fluoranthene were not found in the controls (Fig. 10C). Concentrations of pyrene and, mainly, chrysene and benzo[*b*]fluoranthene were higher in experimental treatments than those of control treatments (Fig. 10C). Unfortunately, data of the PAH concentration in the control treatment with *Oxyrrhis/Rhodomonas* are not available (Fig. 10D). As for *A. tonsa* tissues, the total concentration of PAHs in fecal pellets from *A. tonsa* incubated with *Oxyrrhis/Rhodomonas* was 2 times lower than those incubated with *Rhodomomas* (Fig. 10C, 10D). The concentration of all PAHs in fecal pellets was lower (1.1–18.3 times depending on the PAH) in the experimental treatment with *Oxyrrhis/Rhodomonas* than in that with *Rhodomonas* (Fig. 10C, 10D).

The total concentration of PAHs in eggs of *A. tonsa* incubated with *Rhodomonas* was quite similar in both the control and experimental treatments (Fig. 10E). In contrast, the total concentration of PAHs in eggs of *A. tonsa* incubated with *Oxyrrhis/Rhodomonas* was 1.3 times higher in the experimental treatment than in the control treatment (Fig. 10F). Although the concentration of chrysene and phenanthrene in eggs was 1.9 and 2.4 times, respectively, higher in the experimental treatment with *Oxyrrhis/Rhodomonas* than in that with *Rhodomonas* (Fig. 10E, 10F), there was not a uniform pattern of increasing or decreasing concentration of PAHs in eggs between experimental treatments (Fig. 10E, 10F), contrary to our observations for copepods and fecal pellets (Fig.10A–D).

Bioaccumulation factors (BAFs) in *Acartia tonsa* tissues ranged from 4 to 1023 depending on the type of PAH and the food regime (Table 5). As for natural copepod assemblages, the highest BAF in the tissues of *A. tonsa* was for fluoranthene and pyrene (Table 5). The highest BAF (>5000) was observed in *A. tonsa* fecal pellets for benzo[*b*]fluoranthene (Table 5). BAF of PAH in eggs did not show any clear relation to the food regime (Table 5). BAF for all PAHs in *A. tonsa* tissues and fecal pellets were lower in those incubated with *Oxyrrhis/Rhodomonas* than those incubated with *Rhodomonas* (Table 5).

## Discussion

### Oil and Dispersant Exposure Levels

The concentration of crude oil in marine environments after oil spills is highly variable, ranging from a few ppb to hundreds of ppm, depending on many different factors, such as temporal and spatial scales, marine topography and hydrodynamics, and the magnitude of the spill accident. The concentrations of crude oil used in these exposure experiments (5–100 µl $L^{-1}$) are equivalent to 4.2 to 84.5 parts per million (ppm). After oil spills, crude oil in the upper few meters of the water column may reach concentrations of 20–40 ppm or higher [64]. The reported crude oil concentrations following the Deepwater Horizon Oil spill ranged from 0.25 parts per billion (ppb) to 0.22 ppm in coastal and estuaries areas [65], between 1–2 ppm in oil plumes at 1 km depth [66] and from 3.1 to 4500 ppm on Florida beaches [67]. Similarly, reported concentration of total polycyclic aromatic hydrocarbons (PAHs) in water samples during the Deepwater Horizon Oil spill ranged from over 100 µg $L^{-1}$ (ppb) near the wellhead to below detection limit in distant waters [68]. Although total PAHs can reach extreme concentrations in seawater, up to 600 µg $L^{-1}$ [69,70] and 10,980 µg $L^{-1}$ [71], total PAHs concentration may frequently range from 1 to 150 µg $L^{-1}$ during oil spills [72–75]. Considering the total concentration of PAHs in the crude oil was 2.1 µg $µL^{-1}$, the concentration of total PAHs used in our experiments would range from approx. 10.2 to 201 µg $L^{-1}$ (ppb). Shore-based and lab experiments were conducted with an oil concentration of 5 µl $L^{-1}$, corresponding to a total PAH concentration of 10.2 µg $L^{-1}$ (10 ppb), which in the range of concentration commonly found in the water column during oil spills [72–75]. Although some crude oil concentrations used in our experiments were in the upper range of observed exposure levels in the field, our studies reflect reasonable/realistic exposure concentrations for mesozooplankton after oil spills, particularly in marine areas close to the oil spill source, upper meters of the water column and coastal waters.

Unfortunately, field measurements of dispersant concentrations in oil spills are scarce, although concentrations up to 13 ppm have been measured in upper surface waters [76]. Also, it generally has been thought that oil dispersant concentrations range from

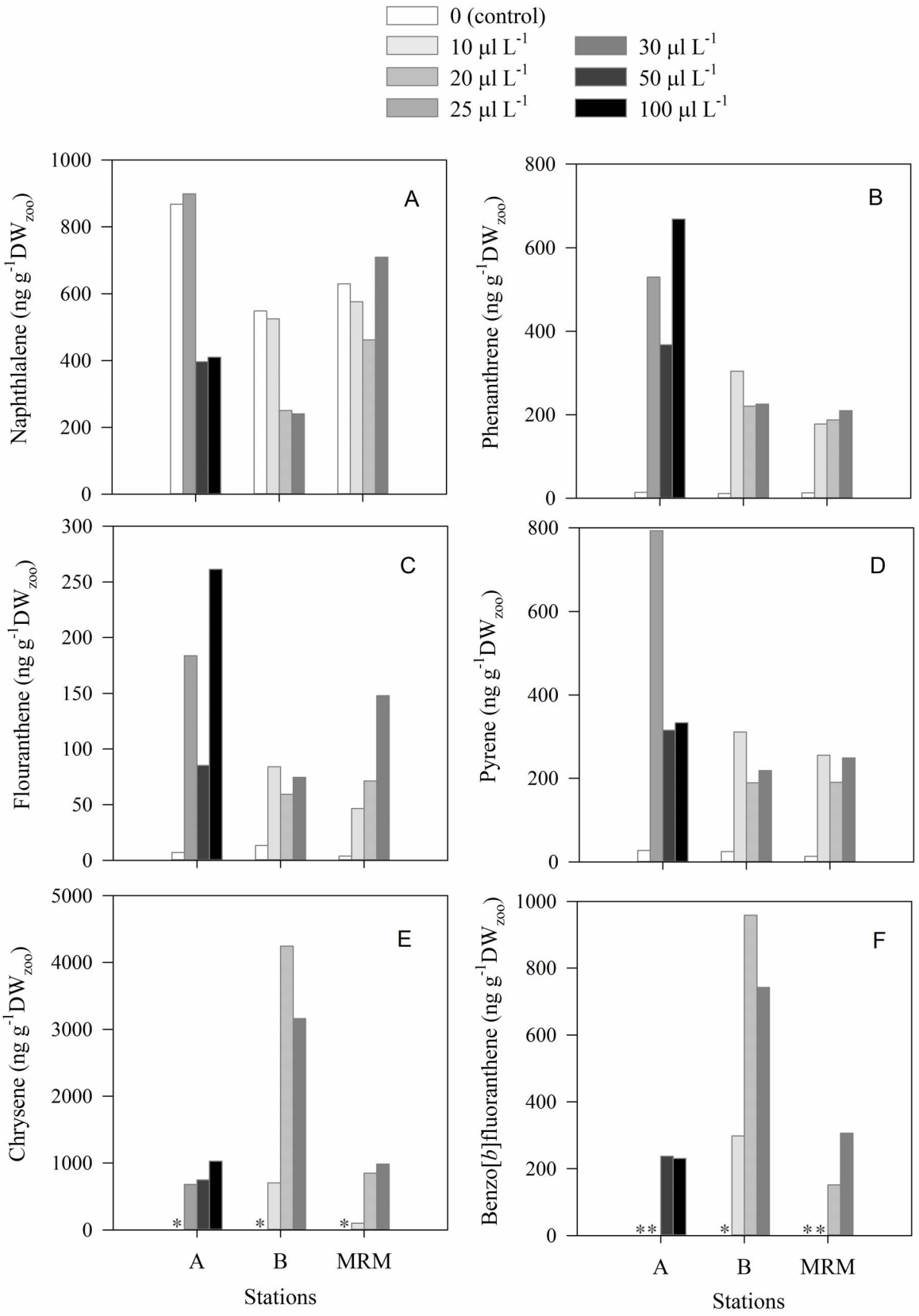


**Figure 8. Concentration of the polycyclic aromatic hydrocarbons detected in natural copepod assemblages after 16 h of exposure to different crude oil concentrations (10–100 µl $L^{-1}$) in the experiments conducted in the North of Gulf Mexico stations (A, B, MRM).** A: naphthalene, B: phenanthrene, C: fluoranthene, D: pyrene, E: chrysene, F: benzo[b]fluorantheneThe asterisks indicate the PAH was not detected.
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**Table 4.** Bioaccumulation factors of PAHs in natural mesozooplankton communities from the northern Gulf of Mexico (Stations A, B and MRM) exposed to different concentrations of crude oil.

| Stations | Oil conc. (μl $L^{-1}$) | Nap | Phe | Flua | Pyr | Chr | BbF |
|---|---|---|---|---|---|---|---|
| A | 25 | 4 | 19 | 186 | 372 | 145 | 606 |
| | 50 | – | 7 | 60 | 107 | 438 | 976 |
| | 100 | – | 4 | 40 | 63 | 163 | 378 |
| B | 10 | – | 89 | 1158 | 2482 | 351 | n.d. |
| | 20 | – | 29 | 256 | 467 | 193 | 604 |
| | 30 | – | 36 | 555 | 330 | 177 | 391 |
| MRM | 10 | – | 27 | 280 | 748 | 52 | n.d. |
| | 20 | – | 14 | 221 | 288 | 219 | 385 |
| | 30 | 3 | 11 | 315 | 254 | 169 | 520 |

Naphthalene (Nap), phenanthrene (Phe), fluoranthene (Flua), pyrene (Pyr), chrysene (Chr), benzo[b]fluoranthene (BbF). The hash symbol indicates that BAF were similar or lower than respective control treatments (non-exposed copepods). *n.d.* = no detected.

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10 ppm to less than 1 ppm after application [77,78]. Therefore, the concentration of dispersant used in our experiments (0.25 μl $L^{-1}$, 0.25 ppm) would be a realistic concentration during the clean-up response to oil spills with dispersants.

## Lethal and Sublethal Effects of Crude Oil in Zooplankton

Our results support previous studies that found zooplankton are especially vulnerable to acute crude oil pollution, showing increased mortality and sublethal alterations of physiological activities, e.g, egg production [79–82]. Direct comparisons among crude oil toxicological studies are difficult due to the variable composition of crude oils and differences in the methodology and experimental conditions (exposure time, temperature, light regime, etc.). Most published studies have been conducted using the crude oil water soluble fraction (WSF), or certain mixed or individual PAHs. However, oil droplet ingestion may be an important entry of oil in zooplankton [22–26,39–41,83]. The exposure to crude oil may promote zooplankton uptake of PAHs as compared with experiments using WSF or single PAHs [42]. In our experiments, PAHs would have been present in both dissolved and particulate (oil droplet) forms. Toxic effects of naphthalene, the most abundant PAH in crude oil, in zooplankton are frequently observed at much higher concentrations compared to crude oil or the WSF exposure experiments [29–30,33,36]. This indicates that other PAHs contained in crude oil, (e.g. fluoranthene, pyrene) are more toxic than naphthalene to copepods [29,36,84]. It is also important to note that weathered oil generally is less toxic than fresh crude oil because of the loss of volatile fractions [81]. In an open system and under marine hydrodynamics, some of the toxic compounds of the crude oil, such as benzene, toluene, ethyl benzene and xylenes (BTEX) and some PAHs, like naphthalene and acenaphthylene, may be lost by evaporation, reducing the potential toxicity of oil after several days. Considering the total concentration of PAHs in the crude oil was 2.1 μg $\mu L^{-1}$, the median lethal concentration (31.6 μl of crude oil $L^{-1}$) observed for mesozooplankton communities after short-term oil exposure corresponded to a total PAH concentration of 63.5 μg $L^{-1}$ This concentration is in the lower range of $LC_{50}$ values commonly reported for copepods exposed to WSF in lab studies after 24 h (from ca. 10 μg $L^{-1}$ to >1000 μg $L^{-1}$) [36–38,85]. Although we did not aim to test the effects of oil on single species, we also observed that small copepod species (e.g. *Oithona*, *Paracalanus*) and copepodites tend to be more sensitive to oil exposure than larger copepods and crustacean larvae, which agrees with other laboratory studies conducted with copepods [38]. Among marine animals, crustaceans are especially sensitive to crude oil exposure [86–87]. In general, according to our results and previous research, marine planktonic copepods seem to be more affected by oil pollution than benthic harpacticoid copepods [31,88–90] and other crustaceans [91–95]. Therefore, planktonic copepods may be used as a target/indicator group for evaluating and monitoring the environmental impact of oil pollution in marine environments.

Narcosis was one of the sublethal effects that we observed in copepods exposed to crude oil, in agreement with other studies [30,36,96]. Narcotic effects in copepods may be associated to both the volatile components of petroleum (BTEX) and the PAHs [29,36] Although narcosis in copepods is reversible after exposure to unpolluted water [36], if it is prolonged, it may reduce feeding and consequently cause death, or may increase the risk of mortality by predation in nature. Alterations in reproduction, feeding and egestion rates have been commonly observed in copepods exposed to specific PAHs [30,97–98]. However, there is a big discrepancy among studies regarding what physiological rates are affected, and the results vary widely depending on the species and oil exposure concentration. Effects of oil on copepod reproduction depend on both the composition and concentration of petroleum hydrocarbons [99–100]. Although in some studies harmful effects to the reproduction of some copepod species has only been found at very high PAH concentrations [33,99], deleterious effects on reproduction success has also been observed in copepods exposed to low concentration of PAHs, including reduced egg production [36,85,101] and reduce/delayed hatching [102–103]. Similarly, effects of oil exposure on fecal pellet production rates depend on the species and exposure levels. Likewise, both reduced [33,85] and unaffected [103] egestion rates have been observed in copepods. Although increased feeding efficiency has been reported in *Calanus finmarchicus* at higher concentrations of naphthalene and WSF oil [104], most studies observed reduced feeding in copepods exposed to high, but sublethal concentrations (>100 μg $L^{-1}$) of WST or naphthalene [30,33,36,97]. However, at lower oil exposure concentrations (<100 μg $L^{-1}$), both reduced [101] and unaffected feeding have been observed in copepods [97,104]. Reduced ingestion and egestion rates have been related to narcosis or sluggish effects disturbing feeding [30]. In our study, we did not find narcosis effects in *Acartia tonsa* with our experimental conditions (5 μL $L^{-1}$, equivalent to total PAH = 10.2 μg $L^{-1}$, dim light), then reduced fecal pellet production rates or feeding due to narcosis would not be expected. A recent study conducted with *A.tonsa* exposed to low concentrations of oil WSF (15.5 μg $L^{-1}$) showed a significant reduction in egg production rates and a delay in eggs hatching time [85] in agreement with our results (Fig. 7). However, in contrast to this published study [85], we did find a significant effect of oil exposure in *A. tonsa* fecal pellet production rates. The decrease in *A. tonsa* egg production observed in our study was not associated to lower ingestion rates, as reflected in the fecal pellets production rates (no significant differences between treatments, Fig. 7). Reduction of egg production not being associated with reducing feeding rates has been reported for other copepod species exposed to oil [88]. Delayed development associated to oil exposure has also been observed in other crustaceans [105–107]. Our results suggest that sublethal oil concentrations may affect the

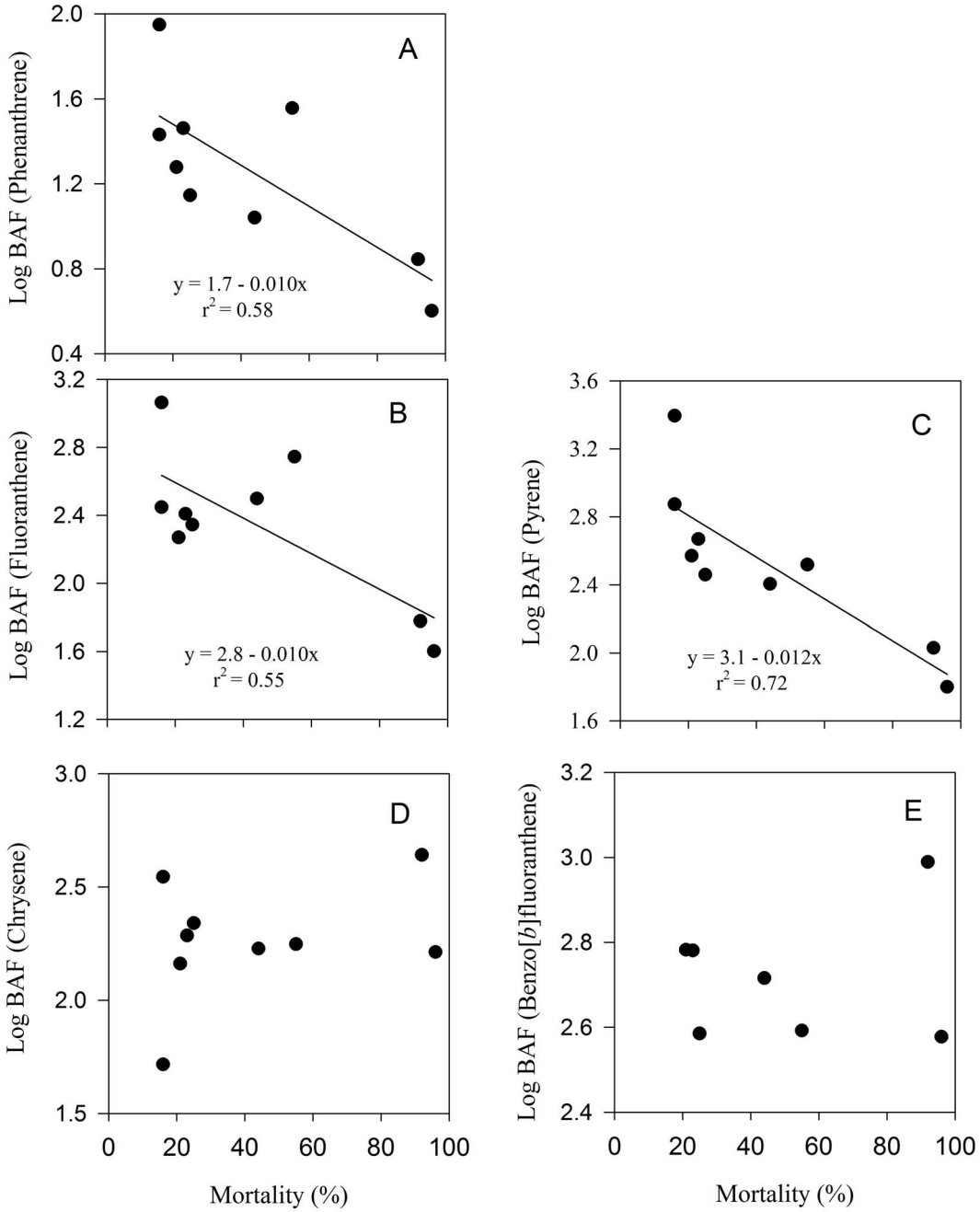


**Figure 9. Relationship between bioaccumulation factors (BAF) and mortality (%) in natural copepod assemblages exposed to crude oil.** A: phenanthrene, B: fluoranthene, C: pyrene, D: chrysene, E: benzo[b]fluoranthene.
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energetics and/or the biochemical processes associated with egg production and embryonic development in copepods. Alterations in the lipid metabolism, including steroid metabolism, may account for energetic and reproduction/developmental anomalies observed in marine crustaceans exposed to petroleum hydrocarbons [107–108].

## Effect of Dispersant and Dispersant Treated Oil

Laboratory studies have found that Corexit dispersants are toxic to marine benthic invertebrates and fishes, particularly eggs and early developmental stages [49,109–110]. The limited previous studies on the effects of Corexit dispersant on marine planktonic copepods showed a $LC_{50}$ of 8–12 ppm for *Pseudocalanus minitus* [111] after 48 h exposure to Corexit 9527, and a $LC_{50}$ of 5.2 ppm for *Eurytemora affinis* after 96 h exposure to Corexit 9500A [112]. Chemical toxicity of dispersant is associated with their chemical components, solvents and surfactants. Surfactants can affect the cellular membranes, increasing membrane permeability and causing membrane lysis in marine organisms [113–114]. Corexit 9500A was the main dispersant type used to clean up the Deepwater Horizon oil spill in the Gulf of Mexico [115]. Although it is assumed that Corexit 9500A is less toxic than previous dispersant types, recent reports found that Corexit 9500A has similar toxicity to other oil dispersants when mixed with South

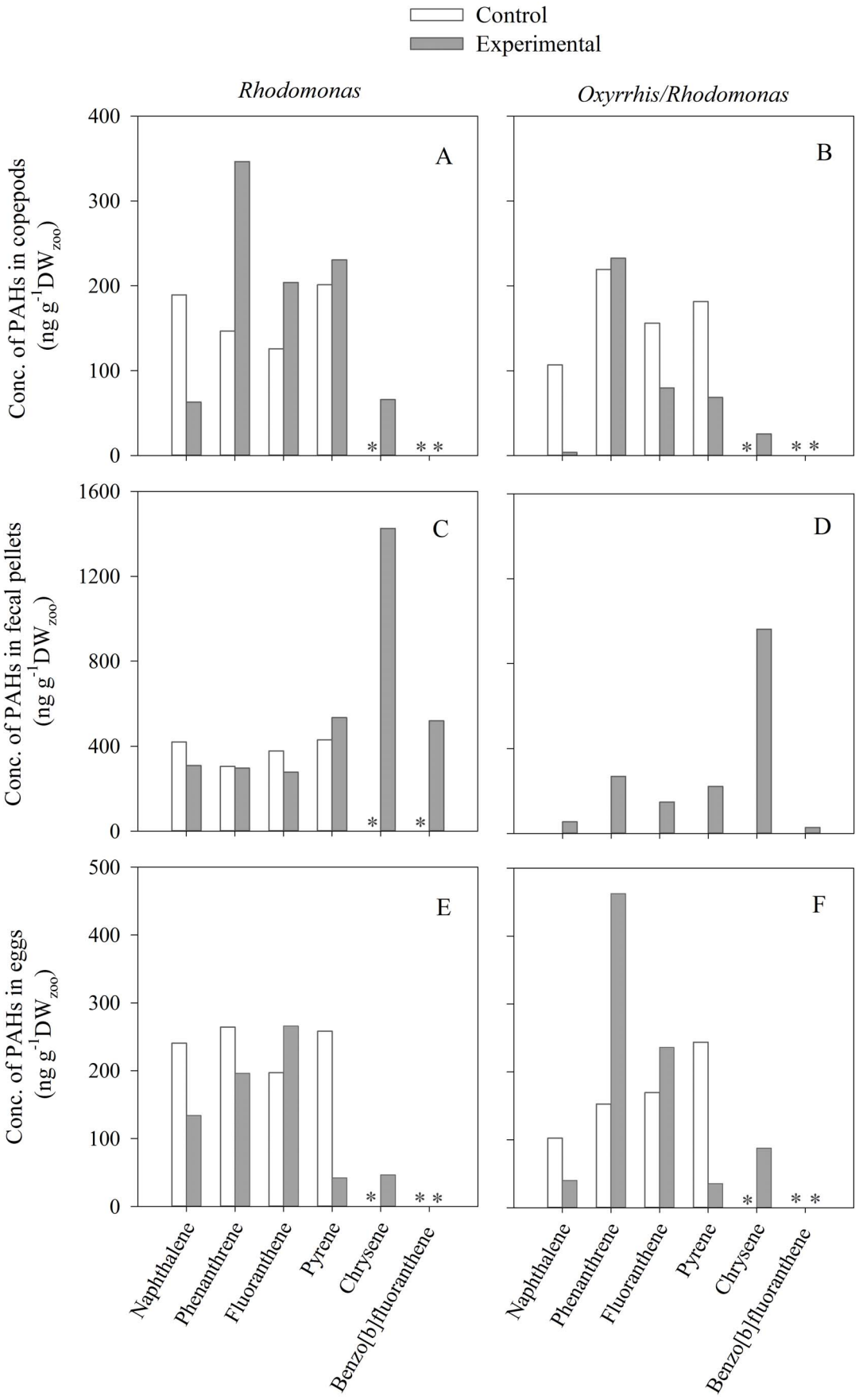
Control
Experimental
Rhodomonas
Oxyrrhis/Rhodomonas
A
B
C
D
E
F
Conc. of PAHs in copepods (ng g-1DWzoo)
Conc. of PAHs in fecal pellets (ng g-1DWzoo)
Conc. of PAHs in eggs (ng g-1DWzoo)
0
100
200
300
400
400
800
1200
1600
100
200
300
400
500
Naphthalene
Phenanthrene
Fluoranthene
Pyrene
Chrysene
Benzo[b]fluoranthene

**Figure 10. Concentration of PAHs in body tissues (A, B), fecal pellets (C, D) and eggs (E, F) of *Acartia tonsa* feeding on *Rhodomonas* sp.(left column) or *Rhodomonas* sp. plus *Oxyrrhis* marina (right column).** Experimental: copepods exposed to oil (5 µl $L^{-1}$). Control: non-exposed copepods. The asterisks indicate the PAH was not detected.
doi:10.1371/journal.pone.0067212.g010

Louisiana sweet crude oil [116]. Furthermore, Corexit 9500A and oil treated with this dispersant are highly toxic to small planktonic organisms, including mollusk embryos [49], fish eggs and larvae [57], coral larvae [117], and rotifers [118]. We found that Corexit 9500A produce nearly 50% mortality in natural mesozooplankton communities at concentrations of 0.25 ppm (Fig. 5), which is more than one order of magnitude lower than lethal concentrations commonly observed in other marine animals exposed to dispersant [109–110,117–118]. This indicates that mesozoplankton communities are highly sensitive to oil dispersant Corexit 9500A.

Several studies have observed the combination of oil and dispersant increased toxicity to marine organisms [57,117–118]. However, studies of the effects of dispersant treated oil on zooplankton communities or copepods are very scarce and sometimes controversial. Linden et al. [119] did not find significant differences in mesozooplankton abundance when exposed to North Sea crude oil and oil treated with Corexit 9550 dispersant. In contrast, Jung et al. [120] observed that zooplankton communities were less affected with crude oil alone than with both crude oil and dispersant, in agreement with our results (Fig. 5). Increased toxicity of dispersant treated oil may be due to additive and/or synergistic effects of oil and dispersant. The dispersant Corexit 9500A may increase the concentration of toxic petroleum hydrocarbons (e.g. PAH) in the water, and consequently, enhance the oil toxicity [121–122]. However, in our experiments we found that the toxicity in the dispersant treated oil (72%) would be caused mainly by additive toxicity of oil (mortality = 21%) and dispersant (mortality = 48%) (Fig. 5).

Given the importance of mesozooplankton in marine food webs and their high sensitivity to dispersant and dispersant treated oil, we highly recommend the use of representative planktonic copepods as a target species to evaluate the impact of oil spill chemical cleanup operations in marine environments.

**Table 5.** Bioaccumulation factor of PAHs in body tissues, fecal pellets and eggs of the copepod *Acartia tonsa* exposed to crude oil (5 µl $L^{-1}$, 48 h, artificial light) with two different food regimes:

| *Type of food* | *A.tonsa sample* | Phe | Flua | Pyr | Chr | BbF |
|---|---|---|---|---|---|---|
| *Rhodomonas* sp. | body tissues | 66 | 1023 | 190 | 68 | *n.d.* |
| | fecal pellets | – | – | 670 | 1471 | 5276 |
| | eggs | – | 902 | – | 48 | *n.d.* |
| *Oxyrrhis marina* *Rhodomonas* sp. | body tissues | 4 | – | – | 27 | *n.d.* |
| | fecal pellets | – | – | – | 992 | 288 |
| | eggs | 102 | 874 | – | 90 | *n.d.* |

(1) *Rhodomonas* sp. and (2) *Rhodomonas* sp plus *Oxyrrhis marina*. Phenanthrene (Phe), fluoranthene (Flua), pyrene (Pyr), chrysene (Chr), benzo[b]fluoranthene (BbF). Dash indicates no bioaccumulation (concentration in experimental treatment was similar or lower than in respective control treatment). *n.d.* = no detected.
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## Bioaccumulation of Polycyclic Aromatic Hydrocarbons in Mesozooplankton

We found that zooplankton can accumulate PAHs when exposed to oil, in agreement with previous studies [20,39,41,43,123–124]. Since we used crude oil emulsions instead of WSF, it is possible that oil droplets could attach to the zooplankton, which has been observed in laboratory and field studies [20]. However, the use of filtration and high pressure washing would substantially remove any attached oil droplets, even though we cannot completely disregard the possibility of attachment of very small oil droplets to zooplankton. The differences in PAH composition between crude oil and contaminated zooplankton (Fig. 2 and Fig. 8), and the PAH concentrations among exposure levels (Fig. 8), support the conclusion that processes other than oil droplet attachment controlled the bioaccumulation observed in our studies. Nevertheless, it is important to note that, in nature, the adhesion of crude oil droplets to zooplankton may be another route of transfer of PAHs up through marine food webs.

The bioaccumulation factors of PAHs reported for zooplankton in oil exposure tests vary widely depending on the species and experimental approach [20,39,41,43,123–124]. Bioaccumulation of a specific pollutant depends on its chemical properties, its bioavailability and the physiology of the organism [125–126]. PAHs are lipophilic and their hydrophobicity increases as their molecular weights increase [127]. Because of their lipophilic nature, PAHs are usually accumulated in the lipids of organisms. This would partly explain the differences in PAH concentration observed in zooplankton from our experiments (Tables 4 and 5) compared with those of Arctic copepods with high lipid contents (BAF>5000) [43].

In our experiments, the PAH bioaccumulation factors (BAF) tend to decrease with increasing oil concentration, indicating that bioaccumulation depends on the exposure levels (Table 4). A decrease in BAF with increasing oil concentration may be related to an increase in mortality due to toxic effects of petroleum hydrocarbons, reducing the bioaccumulation, as we observed in our experiments for some PAH (Fig. 9 A–C). However, an inverse relationship between BAF and pollutant exposure level may also relate to processes or mechanisms, other than passive diffusion, that show saturation kinetics [128]. When uptake and removal of petroleum hydrocarbons is due to passive partitioning alone, BAF of PAHs are associated to their lipophilic properties, i.e., octanol–water partition coefficient, $K_{ow}$, with log BAF increasing linearly as increasing log $K_{ow}$ [125,129]. This pattern has been commonly observed in acute tests conducted with zooplankton exposed to some specific dissolved PAH or WSF [29,41]. We also found BAF tended to be lower for PAH with low $K_{ow}$ (i.e., naphthalene and phenanthrene), than for PAH with higher $K_{ow}$ (i.e. fluoranthrene, pyrene, chrysene, benzo[*b*]fluoranthrene) (Table 4). Since we used crude oil instead of dissolved petroleum hydrocarbons, the deviations from the linear relationship between log BCF and log $K_{ow}$ observed in our studies may be due to the lower availability of more hydrophobic compounds in the water and the ingestion of oil droplets or prey-oil droplet aggregations. It is important to note that BAF would be also inversely related to the capacity of the organisms to depurate (by excretion or egestion) petroleum hydrocarbons [41,83,130–131]. Some copepod species are able to metabolize and rapidly biotransform PAHs [132]. The

metabolism and depuration rates of a specific PAH depend partially on its chemical properties, e.g. molecular-weight [132]. Then, some petroleum hydrocarbons, such as naphthalene, may be excreted rapidly [41,129], whereas other PAHs, such as fluoranthene and pyrene, may remain in zooplankton bodies for extended periods [25,39–40,132–133]. PAHs in zooplankton may be also reduced or eliminated by egg production [41]. Oil droplets or some petroleum hydrocarbons have been found into zooplankton fecal pellets in field and laboratory studies [20,26,134–135]. In the laboratory experiments, we found chrysene and benzo[*b*]fluoranthene, showed low BAF in *Acartia tonsa* despite their high octanol–water partition coefficient, $K_{ow}$ (Table 5). In contrast, we found very high concentrations of these compounds in the fecal pellets (Fig. 10), suggesting chrysene and benzo[b]fluoranthene may be removed from the body via egestion. Field studies found that benzofluoranthenes are frequently accumulated in the marine bottom sediments [136] and Benzo[*b*]fluoranthene was the most abundant PAH in samples of sediments containing mainly copepods feacal pellets [134]. Given their importance in the marine biological fluxes [137–139], zooplankton fecal pellets may play a relevant role in the distribution of petroleum hydrocarbons in the sea.

Copepod eggs are rich in lipids, and therefore may potentially accumulate high concentrations of lipophilic contaminants [140]. Although information on the bioaccumulation of PAHs in zooplankton egg is scarce, accumulation of some specific PAH, i.e. fluoranthrene, has been found in copepods eggs [40]. We found bioaccumulation of some petroleum hydrocarbons, such as phenanthrene, fluoranthene, and chrysene in eggs of *Acartia tonsa* exposed to crude oil (Table 5). However, these results should be considered cautiously due to the high concentration of PAH in the control treatments, except for chrysene (Fig. 10). If PAHs are transferred to the next generation through the eggs (e.g. resting eggs), the persistence of PAH in the planktonic communities would be longer than expected for contaminated copepods with short generation times. More investigation is required to evaluate the importance of oil contaminated copepod eggs in the flux and resilience of PAHs in marine systems.

## Influence of Experimental Conditions (UV Exposure, Food) in Crude Oil Toxicological and Bioaccumulation Studies

Oil toxicity in marine organisms may vary widely depending on environmental variables, including temperature [38], salinity [141], light [54–55], and turbulence [142]. Among the different extrinsic variables affecting oil toxicity, the influence of UV radiation and food on the toxic effects of oil to zooplankton will be discussed in light of our results.

Ultraviolet radiation (UVR) may increase the toxicity of petroleum hydrocarbons (e.g. PAHs) by 2- to 50,000-fold, depending on the aquatic organism and the type of crude oil or petroleum hydrocarbon [55,57,59,124]. PAHs absorb visible and UV radiation, and therefore are particularly susceptible to photoenhanced toxicity [54]. Photoenhanced toxicity of crude oil may be caused by photosensitization (i.e. bioaccumulated petroleum hydrocarbons act as photoreceptors and transfer light energy to other surrounding biological molecules causing cell and tissue damage) and photomodification (i.e. petroleum hydrocarbons are photochemically transformed into more toxic compounds, such as reactive oxygen species or free radicals, capable of damaging cells) [143–144]. Recent studies found that transparent marine organisms, such as fish larvae and embryos [57,145] and planktonic copepods [59,124], are particularly sensitive to the combined effects of oil and UVR exposure. We found a moderate increase in toxicity (35%, Fig. 6) compared to other studies [55,57,59,124]. Unfortunately, we were not able to directly measure the UVR during the incubations due to logistic problems. However, our results indicate that, under natural radiation values, UVB increase the toxicity of dispersed crude oil to mesozooplankton communities, which emphasizes the relevance of considering the photoenhanced toxicity in the evaluation of the potential impact of oil spills. For example, translucent/transparent zooplankton, particularly those adapted to live in the upper layers of the water column (neuston) and in intertidal and shallow coastal areas with elevated UVR would be more sensitive to oil pollution.

Many acute toxicological and bioaccumulation studies with zooplankton have been conducted without food [29,43,146] following standard protocols (ISO 1999). Nevertheless, zooplankton may take up toxic petroleum hydrocarbons directly, through passive uptake (cutaneous absorption), and/or indirectly, through the ingestion of phytoplankton [41,83,131]. The dietary intake of petroleum hydrocarbons is relevant because phytoplankton may accumulate higher concentrations of PAH than zooplankton [41] and BAF of some petroleum hydrocarbons ingested through the diet may be higher than from the dissolved state in seawater [83]. Moreover, some studies have found marine ciliates and pelagic tunicates only ingest oil droplets in presence of phytoplankton [21,26]. Therefore, starvation conditions would represent unrealistic conditions that may bias the food web mediated interactions between oil and zooplankton.

It is important to note the type of prey used in the tests may play an important role in the toxicity of crude oil to zooplankton. Under natural conditions, planktonic communities are composed of many organisms including phytoplankton, protozoan and metazoans. Both phytoplankton and protozoans are part of the metazoans diet (e.g. copepods).The protozoan *Oxyrrhis marina* is a high quality prey for copepods in term of essential lipids and they may enhance the copepod growth and reproduction by trophic upgrading [148]; this would explain the increase in egg production of *Acartia tonsa* with *Oxyrrhis marina* observed in our experiments (Fig. 7). We observed differences in sublethal effects (reduced egg production, delayed hatching) (Fig. 7) and bioaccumulation of PAHs depending on the absence or presence of protozoan *Oxyrrhis marina* in the water (Fig. 10). *O. marina* could remove oil from the water column by both passive uptake of dissolved petroleum hydrocarbons and by ingestion of oil droplets and *Rhodomonas* contaminated with PAHs. This would reduce the oil availability for *Acartia tonsa*, reducing their potential toxicity and bioaccumulation of PAHs, as observed in our study. Unfortunately, there are no available data on the uptake and bioaccumulation of petroleum hydrocarbons by heterotrophic dinoflagellates. Although the abundance of *Oxyrrhis marina* in nature is lower than in our experiments, natural concentrations of heterotrophic flagellates together are commonly higher than in our experiments [149–150]. Note that heterotrophic flagellates may have a higher tolerance to oil pollution than mesozooplankton [151], and the standing stock of protozoan consumed by metazooplankton is very usually very low in nature (<1%) [150,152]. Therefore, this suggests protozoans may play an important role in the toxicity and fate of petroleum in the sea.

Overall, our results emphasize the importance of experimental conditions in the crude oil toxicity tests. More experiments (e.g. mesocosms) mimicking the natural environment (e.g. natural microbial assemblages, sunlight, turbulence, etc.) are required to better understand the effects of oil spills on zooplankton communities and the transfer of petroleum hydrocarbon in marine food webs.

## Ecological Implications of the Interactions between Crude Oil and Zooplankton

Impact of oil spills on planktonic communities depends on many physical, chemical and biological factors, and therefore the effects of oil pollution on zooplankton would vary depending on the circumstances of each spill accident [153]. Overall, given the pivotal role of zooplankton in marine environments, harmful effects of oil in zooplankton communities would strongly affect fish production and benthic invertebrate recruitment. In agreement with other acute toxicological studies, oil pollution has negative short-term impacts on zooplankton, resulting in a significant decrease in zooplankton abundance and biomass, and changes in zooplankton composition after oil spills [154–156]. It has been suggested that copepods may reduce their exposure to oil due to their ability to avoid oily patches [157]. However, even if copepods are able to detect petroleum hydrocarbons [95,158], their capacity to avoid crude oil may be limited due to marine hydrodynamics, which may force zooplankton communities into highly polluted waters masses or coastal areas. The frequent observation of ingested oil droplets in zooplankton collected from the field after oil spills suggests low capability by zooplankton to avoid oil patches under natural hydrodynamic conditions.

During the DWH oil spill, more than 1.7 million gallons of chemical dispersants, mainly Corexit 9500A, were applied at the sea surface and on the seafloor near the wellhead [159]. The use of dispersant in oil spills enhances the formation of small oil droplets, promoting bacterial biodegradation, but at the same time, also increases the potential exposure of oil to pelagic organisms. The application of dispersants may increase the negative impact of oil spills to planktonic communities due to its high toxicity to mesozooplankton as observed in this study. Corexit 9500A is also toxic, more toxic than oil alone, for tintinnid ciliates and dinoflagellates (Almeda et al., unpublished data). Hence, less toxic dispersants are required to reduce their impact on planktonic organisms. Moreover, although it is thought that dispersants are rapidly diluted and degraded in marine environments [49], a recent study [160] found a slow degradation of Corexit 9500A dispersant ingredients in deep waters after the DWH spill. These results accentuate the importance of further studies with key planktonic organisms (e.g., copepods, microzooplankton) from surface and deep waters for a better understanding of the impact of dispersants on planktonic communities and, consequently, a better evaluation of the pros and cons of the application of dispersants in the sea after an oil spill.

Given the capacity of zooplankton to accumulate toxic petroleum hydrocarbons in tissues, fecal pellets and eggs, planktonic communities may play an important role in distribution of toxic petroleum hydrocarbons in marine ecosystems after oil spills [41,131,161]. Since zooplankton are the main food of many marine animals, PAHs may move to higher trophic levels, including pelagic and benthic communities [9]. Sedimentation of fecal pellets produced in the photic zone represents one of the main mechanisms of the vertical flux of particulate organic matter in the ocean [137]. Likewise, fecal pellets may represent part of the diet of coprophagous copepods in the epipelagic zone and an important food source to the deep-sea and the benthos [138–139]. Therefore, zooplankton fecal pellets may also be an important vector in the biological flux of petroleum hydrocarbons in the water column and toward the benthic food web. The accumulation of PAHs in copepods eggs (e.g. resting eggs) would increase resilience of PAH in marine systems. Overall, knowledge on transfer and bioaccumulation of PAH in marine food webs mediated by zooplankton is required to evaluate the fate of oil pollution and their impact in marine environments.

Although negative short term effects of oil pollution to zooplankton are generally accepted, the long term effects of oil pollution and the capacity of recuperation of zooplankton communities are still important questions of debate. Some studies found that zooplankton communities seem to reestablish after several weeks/months after an oil spill, indicating a high capacity for recovery [161 163]. However, marine hydrodynamics and the high natural variability and patchiness in zooplankton abundance may mask the real impacts of oil on zooplankton communities [164]. In open waters, new planktonic communities from unaffected oil areas may be transported to the affected area by the mixing of water masses. However, the recovery of zooplankton communities might not be equally efficient in all ecosystems as it would depend upon the affected area and the planktonic community composition. Zooplankton communities from coasts, estuaries, and enclosed bays with restricted hydrodynamics, would be more susceptible to long term effects than zooplankton communities living in open water with high hydrodynamics, where mixing and dilution may reduce the time and exposure levels. Some reports also suggest that zooplankton may be minimally affected by oil spill pollution over the long term [153,157,165] due to their short generation times and high fecundity. However, the impact of oil may depend of the life history of the specific zooplankter. For instance, some species of calanoid copepods in mid and high latitudes reproduce mainly during specific seasons, producing resting eggs that remain in the sediments until the following year [166]. Similarly, spawning of marine benthic invertebrates in mid and high latitudes shows strong seasonality, with specific peaks of egg and planktonic larvae production. If an oil spill affected these organisms during their reproduction season, reduced egg production and larval survival may affect the recruitment for the following year, and therefore the population dynamics of planktonic and benthic communities. These are just a few examples that highlight the complexity of evaluating the long-term effects of oil spills on zooplankton communities, and their ecological impact in marine environments.

## Main Conclusions

Our experiments indicate zooplankton are especially vulnerable to acute crude oil exposure, showing increased mortality and sublethal alterations of physiological activities (e.g., reduced egg production and delayed hatching). We also found that the chemical dispersant Corexit 9500A was highly toxic to coastal mesozooplankton communities, more toxic than oil alone. Bioaccumulation of certain polycyclic aromatic hydrocarbons (PAHs) was observed in natural mesozooplankton communities, copepods, eggs and fecal pellets exposed to crude oil, suggesting zooplankton may play in important role of the distribution and turnover of petroleum hydrocarbons in marine environments after oil spills.

We found that both environmental (e.g. sunlight radiation) and biological (e.g. microbial community composition) factors affect the interactions between crude oil and mesozooplankton. Natural UVB radiation exposure increased the toxicity of crude oil on mesozooplankton communities. On the other hand, the presence of protozoans in the water reduced the toxic effects of crude oil and the bioaccumulation of PAHs in copepods. These results highlight that further experiments that mimic the natural environment (e.g., mesocosms) are required to accurately evaluate the toxic effects and bioaccumulation of petroleum hydrocarbons in zooplankton.

Overall, our research emphasizes that more knowledge of oil-zooplankton interactions (e.g., zooplankton ingestion of crude oil, transfer of PAHs in food webs as mediated by zooplankton) with

key planktonic organisms (e.g., copepods, meroplankton, microzooplankton) are needed for a better understanding of the impact of oil spills and the fate of petroleum hydrocarbons in marine environments.

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## Author Contributions

Conceived and designed the experiments: RA EJB ZL. Performed the experiments: RA Z. Wambaugh Z. Wang CH. Analyzed the data: RA Z. Wambaugh Z. Wang EJB ZL. Contributed reagents/materials/analysis tools: RA Z. Wambaugh Z. Wang EJB CH ZL. Wrote the paper: RA EJB ZL Z. Wambaugh CH Z. Wang.

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# Toxicity of dispersant Corexit 9500A and crude oil to marine microzooplankton

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ABSTRACT

In 2010, nearly 7 million liters of chemical dispersants, mainly Corexit® 9500A, were released in the Gulf of Mexico to treat the Deepwater Horizon oil spill. However, little is still known about the effects of Corexit 9500A and dispersed crude oil on microzooplankton despite the important roles of these planktonic organisms in marine ecosystems. We conducted laboratory experiments to determine the acute toxicity of Corexit 9500A, and physically and chemically dispersed Louisiana light sweet crude oil to marine microzooplankton (oligotrich ciliates, tintinnids and heterotrophic dinoflagellates). Our results indicate that Corexit 9500A is highly toxic to microzooplankton, particularly to small ciliates, and that the combination of dispersant with crude oil significantly increases the toxicity of crude oil to microzooplankton. The negative impact of crude oil and dispersant on microzooplankton may disrupt the transfer of energy from lower to higher trophic levels and change the structure and dynamics of marine planktonic communities.

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## 1. Introduction

The recent Deepwater Horizon (DWH) Oil spill in the Gulf of Mexico (2010), the world's largest accidental release of crude oil into the ocean in history (National Commission, 2011), has increased public awareness and concerns about environmental impacts of marine crude oil spills. During the last decades, the rising demand for global energy and petroleum products have led to an increase of crude oil pollution in the sea, which is becoming a major environmental problem (Kennish, 1996; National Research Council, 2003; Dalsøren et al., 2007). Petroleum or crude oil pollution in the sea arises from multiple anthropogenic sources, including urban and industrial wastewater, spillages from tanker accidents, and leakages during drilling operations or marine transportation (National Research Council, 2003). Most accidental crude oil spills in the world have occurred in coastal areas and, even though catastrophic oil spills are not the most important source of petroleum introduced into the sea (National Research Council, 2003), the sudden discharge of high concentrations of petroleum in marine environments has harmful effects on marine ecosystems (Kennish, 1996; National Commission, 2011; Barron, 2012; White et al., 2012).

Among the biological components of marine ecosystems, planktonic organisms are particularly vulnerable to crude oil pollution (Walsh, 1978; Graham et al., 2010). More than 90 percent of the biological processes in the ocean are due to planktonic organisms (Hays et al., 2005), and attempts to assess the ecological impact of oil spills in marine environments require a good understanding of the effects of crude oil pollution on planktonic communities. Most of the research on plankton and crude oil interactions has been focused on bacteria, phytoplankton and some large zooplankton (mesozooplankton) (Walsh, 1978, Kuiper and Van den Brink, 1987; Jiang et al., 2010, 2012) whereas other planktonic groups, such as microzooplankton (20–200 μm, Sieburth et al., 1978) have received little attention. The scarce information on the effects of crude oil on microzooplankton contrasts with the fact that microzooplankton are key components of marine plankton communities (Calbet, 2008). Marine microzooplankton, which include many protists, such as ciliates and heterotrophic dinoflagellates, as well as some small metazoans (e.g. copepod nauplii) play a pivotal role in marine food webs as the major consumers of phytoplankton (Calbet, 2008), as important components of the microbial loop (Azam et al., 1983), and as essential contributors to the diet of mesozooplankton and fish larvae (Gifford, 1991; Holt and Holt, 2000; Calbet and Saiz, 2005). Therefore, given the important role of microzooplankton in planktonic communities, knowledge of the interactions between crude oil and microzooplankton is essential for a better understanding of the effects and ecological impact of crude oil spills on marine food webs.

Most crude oil toxicological studies on plankton have been conducted using the water soluble fraction of crude oil, or certain

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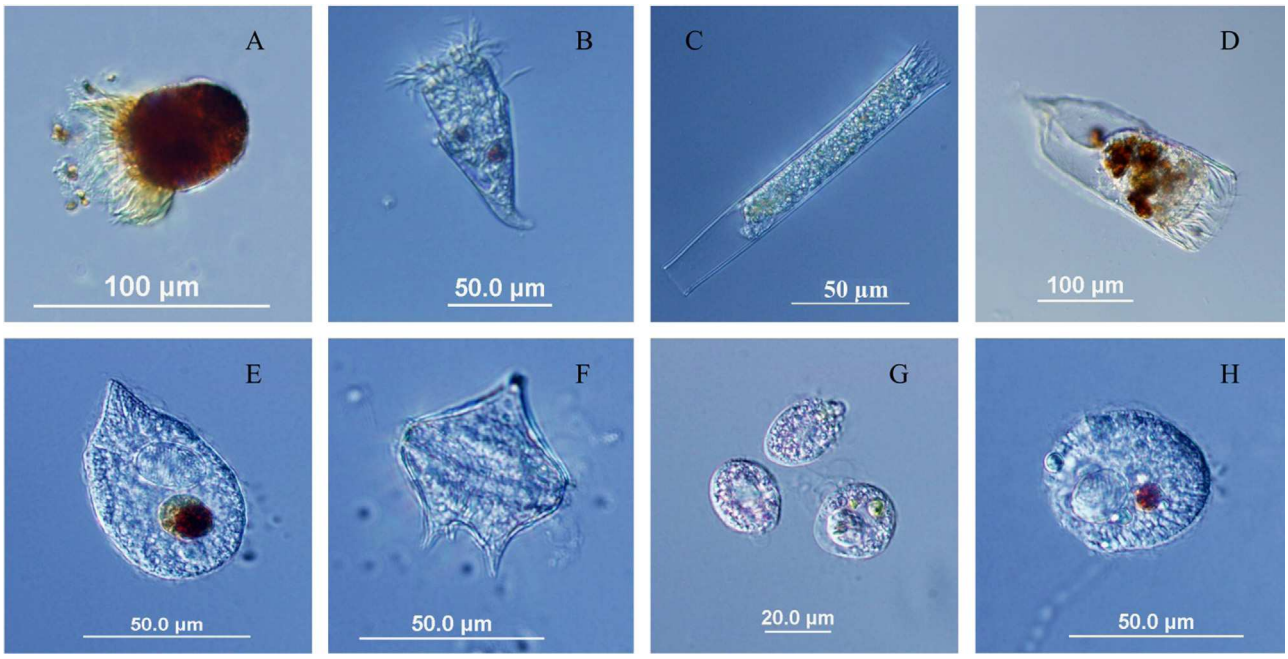


**Fig. 1.** Microscopy images of the protozoa species used in this study to determine the toxicity of crude oil, dispersant and dispersant-treated crude oil on microzooplankton. (A) *Strombidium* sp, (B) *Spirostrombidium* sp, (C) *Eutintinnus pectinis*, (D) *Favella ehrenbergii*, (E) *G. spirale*, (F) *P. divergens*, (G) *O. marina*, (H) *Protoceratium* sp.

mixed or individual dissolved petroleum hydrocarbons (Barata et al., 2005; Calbet et al., 2007; Saiz et al., 2009; Echeveste et al., 2010; Jiang et al., 2010, 2012). However, in the natural environment, after a crude oil spill, petroleum is present in the water column in both dissolved and particulate forms (i.e. crude oil droplets). Plumes of small crude oil droplets generated by wind and waves or by treatment with chemical dispersants (Lichtenthaler and Daling, 1985; Delvigne and Sweeney, 1988; Mukherjee and Wrenn, 2009) are frequently observed after crude oil spills, as occurred in the Deepwater Horizon oil spill in the Gulf of Mexico (Kerr, 2010). These dispersed crude oil droplets are frequently in the food size spectra of many zooplankters and there is evidence some zooplankton groups (Conover, 1971; Mackie et al., 1978; Lee et al., 2012) and epibenthic ciliates (Andrews and Floodgate, 1974; Lanier and Light, 1978) ingest crude oil droplets suspended in the water column. However, little is known about the toxic effects of ingesting dispersed crude oil droplets by marine microzooplankton.

In 2010, nearly 7 million liters of dispersants, mainly Corexit® 9500A, were discharged in the Gulf of Mexico to treat the Deepwater Horizon crude oil spill (TFISG-OBCSET, 2010). This release of large volumes of chemical dispersants, the largest known application of dispersants in the sea in response to a crude oil spill (Wise and Wise, 2011), has also raised new concerns regarding the toxicity and the impact of dispersants and dispersed oil on marine ecosystems. Dispersants, which are mainly composed of solvents and surfactants (i.e. surface active agents), reduce the interfacial tension between crude oil and water, allowing the formation of small crude oil droplets ( $< 100\ \mu m$), and consequently enabling the removal/dilution of crude oil slicks from the surface waters (Canevari, 1978; Lichtenthaler and Daling, 1985; Mukherjee and Wrenn, 2009). New types of dispersants (e.g. Corexit) are less toxic than older types, which caused a devastating impact on marine life as observed in the Torrey Canyon (1967) and Sea Empress (1996) oil spills (Nelson-Smith, 1968; Swedmark et al., 1973). Thus, it has been suggested that the new generation of dispersants and dispersant-treated crude oil are less toxic than crude oil alone (Lewis, 2001; US Environmental Protection Agency, 2010) and that they have minimal deleterious effects on marine life (Lessard and Demarco, 2000). However, studies on the toxicity of Corexit 9500A and chemically dispersed crude oil on planktonic organisms are rare despite increasing evidence that this dispersant is highly toxic (Goodbody-Gringley et al., 2013; Rico-Martinez et al., 2013; Almeda et al., 2013a, Cohen et al., 2014) and increases the toxicity of crude oil to marine zooplankton (Goodbody-Gringley et al., 2013; Rico-Martinez et al., 2013; Almeda et al., 2013a). To our knowledge, there are not any published laboratory studies on the effects of Corexit 9500A and dispersed crude oil on marine microzooplankton despite their important roles in marine systems. Therefore, there is a need for more research into the effects of this type of dispersant on microzooplankton to better evaluate the impact of using chemical dispersants on plankton communities after crude oil spills.

In this study we aimed to estimate the toxicity of physically and chemically dispersed Louisiana light sweet crude oil and dispersant Corexit 9500 on marine microzooplankton. For this purpose, we conducted laboratory exposure experiments to determine the acute effects of crude oil alone, dispersant alone, and dispersant-treated crude oil on the population growth rates of marine oligotrich ciliates (*Strombidium* sp, *Spirostrombidium* sp), tintinnids (*Eutintinnus pectinis*, *Favella ehrenbergii*) and heterotrophic dinoflagellates (*Gyrodinium spirale*, *Protoperidinium divergens*, *Oxyrrhis marina*, *Protoceratium* sp) (Fig. 1). These species belong to some of the most representative genera of marine microzooplankton in the marine environment, including the Gulf of Mexico, which is considered a 'hot spot' for crude oil spills given the intense petroleum industry activity carried out in this area.

## 2. Methodology

The microzooplankton species used in this study are indicated in Table 1. Microzooplankton samples were collected from the Aransas Ship Channel near the University of Texas Marine Science Institute (MSI) in Port Aransas (Texas) except the heterotrophic dinoflagellate, *O. marina*, that was provided by University of Texas Culture Collection of Algae (UTEX). Plankton samples were collected from surface waters by tying a microplankton net (20 µm mesh, 20 cm diameter) to the MSI pier and allowing it to stream with the tidal current for approximately 5 min. The plankton samples were poured into plastic bottles and kept in a cooler until returning to the laboratory. Once in the laboratory, the plankton samples were then screened through a 153 µm mesh sieve to remove large zooplankton. Aliquots of

**Table 1**
Characteristics of the species of protozoan and experimental conditions used in the experiments. Cell volume (Vol.), presence of external skeletal structures, i.e. lorica in ciliates and theca in dinoflagellates (Skel.), initial concentration of protozoan (Conc.), total exposure time ($t$), temperature ($T$), type of food (Food) and initial target food concentration (Food conc.). SD=standard deviation. Rho.=*Rhodomonas* sp, Het.=*Heterocapsa* sp, Per.=*Peridinium foliaceum*.

| Group | Species | Vol. ($10^4$ μm$^3$) | Skel. | Conc. ± SD (cells mL$^{-1}$) | $t$ (h) | $T$ (°C) | Food | Food conc. (cells mL$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Oligotrich ciliates | *Strombidium* sp | 5.63 | No | 5.8 ± 0.4 | 48 | 24.9 | Rho. | 15000 |
| | *Spirostrombidium* sp | 9.09 | No | 4.0 ± 0.2 | 48 | 24.5 | Rho. | 12000 |
| | | | | | | | Het. | 200 |
| Tintinnids | *Eutintinnus pectinis* | 4.10 | Yes | 15.8 ± 1.6 | 48 | 23.5 | Rho. | 7500 |
| | *Favella ehrenbergii* | 56.3 | Yes | 2.5 ± 0.1 | 48 | 25.0 | Rho. | 35000 |
| | | | | | | | Het. | 3000 |
| Heterotrophic dinoflagellates | *Gyrodinium spirale* | 5.82 | No | 21.5 ± 0.8 | 48 | 25.1 | Per. | 50 |
| | | | | 43.1 ± 0.9 | 24 | 24.5 | Per. | 50 |
| | *Protoperidinium divergens* | 5.34 | Yes | 1.6 ± 0.2 | 48 | 24.9 | Per. | 50 |
| | *Oxyrrhis marina* | 0.35 | No | 4050 ± 155 | 24 | 24.5 | Per. | 10000 |
| | *Protoceratium* sp | 4.96 | Yes | 6.3 ± 0.3 | 24 | 24.5 | Per. | 10 |

these plankton samples were then incubated in 1 L polycarbonate bottles and enriched with mixtures of cultured phytoplankton (e.g. *Isochrysis galbana*, *Rhodomonas* sp, *Peridinium foliaceum*). These enrichments were then placed on a bottle roller rotating at 2–4 rpm and were incubated at 24 °C at low light intensities for a period of several days. Enrichments were checked periodically for the growth of ciliates or heterotrophic dinoflagellates. When a species appeared to be growing well, cells were picked individually with a borosilicate glass fine tip Pasteur pipette and placed into 7 mL micro-wells containing sterilized 0.2 μm filtered seawater. Then, a mixture of phytoplankton (*Isochrysis*, *Rhodomononas*, *Cryptomonas*, *Heterocapsa*) for ciliates and *P. foliaceum* for heterotrophic dinoflagelletes were added to the micro-wells as prey for the isolated protozoans. After several days, isolated protozoans species were transferred to 75 mL polystyrene tissue culture flasks and placed on a bottle roller under the conditions described before. Cultures were fed every 3 to 4 d, and transferred into new media at 1 week intervals. Phytoplankton cultures were grown in f/2 culture medium prepared with 0.2 μm filtered sterilized natural seawater collected from the Aransas Ship Channel. Phytoplankton cultures were held in 250 mL polycarbonate flasks at 20 °C and 34–35‰ on a 12:12 h light: dark cycle with cool-white fluorescent lights at an irradiance of approximately 25 μmol photons m$^{-2}$ s$^{-1}$.

Light Louisiana Sweet crude oil was provided by BP (BP Exploration & Production Inc.) as a surrogate for the Macondo (MC252) crude oil released in the Deepwater Horizon (DWH) oil spill in the Gulf of Mexico (2010) because they are considered to have similar chemical composition and toxicity. The concentrations and composition of PAHs in this oil were previously determined by our research group and can be found in Almeda et al. (2013b). We used Corexit® 9500A as the chemical dispersant because it was the main type of dispersant used in the clean-up operations during the DWH oil spill (National Commission, 2011). The dispersant was provided by NALCO (Nalco/Exxon Energy Chemicals, L.P.) and some of its chemical ingredients can be found in the NALCO Environmental Solutions LLC web page (NALCO®, 2010a).

Experiments consisted of 24 and 48 h incubations (Table 1) of single species of protozoans in absence of pollutants ("control treatments") and exposed to 3 types of test media: (1) crude oil emulsions, i.e., suspensions of crude oil droplets in seawater dispersed physically without the addition of dispersant ("crude oil treatment"), (2) dispersant-treated crude oil emulsions i.e., crude oil emulsions in seawater dispersed physically and chemically ("oil+dispersant treatment") and (3) a solution of dispersant alone in seawater ("dispersant treatment"). At the start of the exposure experiments, two to four additional bottles were fixed with 1 percent acidic Lugol's solution to determine the initial cell abundance ("initials"). We used a ratio of dispersant to oil of 1:20 (US Environmental Protection Agency, EPA, 1995) and test media were prepared as described in Almeda et al. (2013a). The concentrations of crude oil and dispersant used in the 48 h experiments were 1, 5, 10 μL L$^{-1}$ and 0.05, 0.25, 0.5 μL L$^{-1}$, respectively. We used a concentration of crude oil and dispersant of 5 and 0.25 μL L$^{-1}$, respectively, in the 24 h exposure experiments. Initially, 24 h experiments at only one concentration were done to explore an appropriate exposure time and concentrations of crude oil and dispersant for additional experiments with heterotrophic dinoflagellates given the lack of published data on this topic. For each species, control and experimental treatments were run in 2–4 replicates. Incubations were conducted in glass bottles (68 mL) containing 0.2 μm filtered seawater and phytoplankton as food (Table 1). In all experiments, seawater salinity was adjusted to 35‰. After adding emulsified crude oil or dispersant to the corresponding experimental bottles, bottles were incubated at 25 °C with ambient laboratory light in a Wheaton bench top roller at 2 rpm in the laboratory. In the case of 24 h incubation experiments with heterotrophic dinoflagellates (Table 1) and the 48 h exposure experiments with the tintinnid *Eutintinnus* (Table 1), the bottles were incubated on the MSI pier in a large acrylic container containing a plankton wheel with open-circuit seawater running. After incubation, the contents of each bottle were poured into 75 mL Falcon cell culture flasks and fixed with 1 percent Lugol's solution. To determine the initial and final concentration of dinoflagellates and ciliates in the different treatments, aliquots of the fixed samples (10–50 mL) were allowed to settle for 24 h in 10–50 mL Utermohl chambers, and then, the whole chamber was counted using an inverted microscope (Olympus BX60) at 100× magnification. For *O. marina* samples, the concentration of cells was determined using a Sedgewick-Rafter counting chamber.

Growth or mortality in each treatment, as percent, was estimated from the number of cells at the beginning and end of the incubation. For each species, significant differences in growth/mortality among treatments (i.e., "control", "crude oil alone", "dispersant" and "dispersant-treated oil") were assessed using three separate one-way analysis of variance (ANOVA), one for each exposure level. When the ANOVA was significant ($p \leq 0.05$), Bonferroni post hoc test was used for pairwise compassion between treatments within each exposure level (SPSS statistics19.0 software).

Data on cell concentration after 48 h incubation in either crude oil, dispersant and dispersant-treated crude oil concentrations were fitted to the following sigmoid/logistic model

$$N = N_0/(1+(C/EC_{50})^b) \quad (1)$$

where, $N$ is the cell concentration, $N_0$ is the cell concentration in absence of crude oil or dispersant (i.e. crude oil or dispersant concentration=0), $C$ is the crude oil/dispersant-treated crude oil/dispersant concentration (μL L$^{-1}$), $EC_{50}$ is the median effect concentration (here defined as the pollutant concentration required to reduce the population by half compared to cell concentrations in the absence of pollutants, $N_0$), and $b$ is the slope factor. The joint action toxicity of crude oil and dispersant was evaluated using the synergistic ratio (SR) (Hewlett and Plackett, 1969) and the concentration-addition (relative toxic units, RTU) (Anderson and Weber, 1975) models.

## 3. Results

The effects of crude oil, dispersant and dispersant-treated crude oil on growth/mortality of microzooplankton varied depending on the species and exposure levels (Fig. 2). Overall, we found a significant difference among the tested pollutants on growth rates of oligotrich ciliates, tintinnids and heterotrophic dinoflagellates (Fig. 2) at all three exposure levels after 48 h of exposure (ANOVA, $p \leq 0.05$), except for *P. divergens* at the lowest exposure concentration (Fig. 2P), when no significant differences were observed (ANOVA, $p=0.089$).

Growth rates of oligotrich ciliates were significantly higher in the controls than all three experimental treatments (ANOVA, Bonferroni post hoc test, $p<0.05$) at all three exposure levels (Fig. 2A–F). For *Strombidium* sp., dispersant had a lower or similar toxicity than crude oil alone at the lower exposure levels (Fig. 2A and B). However, at the highest exposure concentration, dispersant was significantly more toxic than crude oil alone to *Strombidium* sp., causing 100 percent of mortality after 48 h (ANOVA, Bonferroni post hoc test, $p<0.05$) (Fig. 2C). *Spirostrombidium* sp. showed a high mortality after exposure to all three pollutants even at the lowest exposure level (Fig. 2D) with no significant differences between experimental treatments (Fig. 2D and F). In all cases, the combination of crude oil and dispersant caused the

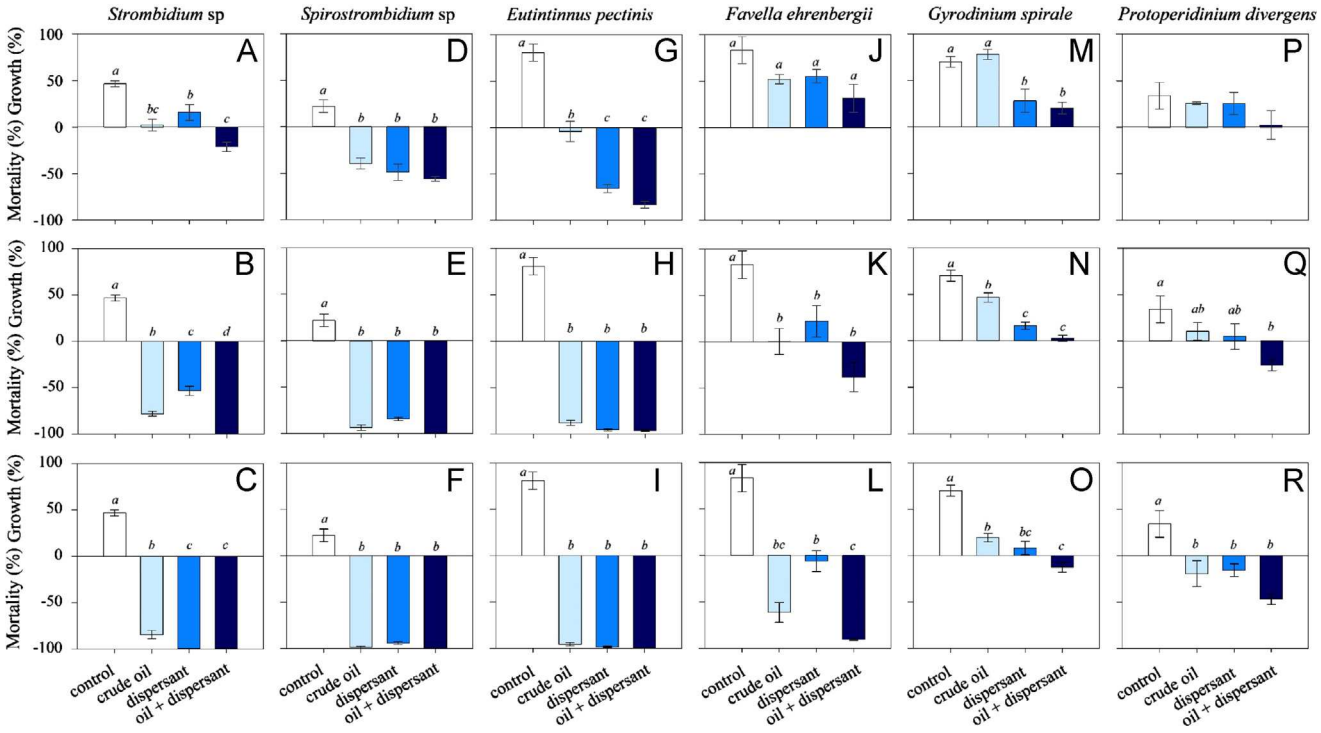


**Fig. 2.** Growth/mortality of the studied species of protozoan (A)–(C) *Strombidium* sp, (D)–(F) *Spirostrombidium* sp, (G)–(I) *E. pectinis* sp, (J)–(L) *F. ehrenbergii*, (M)–(O) *G. spirale*, (P)–(R) *P. divergens*) as percent of the initial cell abundance after 48 h of exposure to 3 different concentrations of crude oil alone, dispersant and dispersant-treated crude oil (oil+dispersant). (A), (D), (G), (J), (M), (P) (first row): crude oil concentration=1 $\mu L\,L^{-1}$, dispersant concentration=0.05 $\mu L\,L^{-1}$, dispersant-treated crude oil=1 $\mu L\,L^{-1}$ of crude oil+0.05 $\mu L\,L^{-1}$ of dispersant. (B), (E), (H), (K), (N), (Q) (second row): crude oil concentration=5 $\mu L\,L^{-1}$, dispersant concentration=0.25 $\mu L\,L^{-1}$, dispersant-treated crude oil=5 $\mu L\,L^{-1}$ of crude oil+0.25 $\mu L\,L^{-1}$ of dispersant. (C), (F), (I), (L), (O), (R) (third row): crude oil concentration=10 $\mu L\,L^{-1}$, dispersant concentration=0.50 $\mu L\,L^{-1}$, dispersant-treated crude oil=10 $\mu L\,L^{-1}$ of crude oil+0.50 $\mu L\,L^{-1}$ of dispersant. Error bars represent the standard deviations. Lowercase letters (*a*, *b*, *c*, *d*) indicated different statistical groups according to the results of post-hoc Bonferroni test. Note that in (J) there was significant difference among treatments according to the one-way ANOVA but no differences between treatments according to the Post-hoc Bonferroni test. Note that in (P) there was no significant difference among treatments according to the one-way ANOVA.

highest mortality to oligotrich ciliates (Fig. 2A–F), although not always statistically significant than the other experimental treatments, particularly at the highest exposure levels (Fig. 2A–F). Mortality in both species of oligotrich ciliates was 100 percent at dispersant-treated crude oil concentrations of 5 and 10 $\mu L\,L^{-1}$ (Fig. 2B, C, E and F). Dispersant-treated crude oil was 1.01 to 1.53 times more toxic, depending on the exposure levels and species, than crude oil alone to oligotrich ciliates (Fig. 2A and F).

Growth rates of tintinnids were significantly higher in the controls than all three experimental treatments (ANOVA, Bonferroni post hoc test, $p < 0.05$) at all three exposure levels (Fig. 2G and L), except for *F. ehrenbergii* at the lowest exposure level (Fig. 2J), where no significant difference between control and experimental treatments was observed. At the lowest exposure level, dispersant was more toxic than crude oil alone to *E. pectinis* (Fig. 2G). At the higher exposure concentration, the tested pollutants caused almost 100 percent mortality to *E. pectinis* after 48 h, suggesting that this species is particularly sensitive to crude oil and dispersant (Fig. 3H and I). *F. ehrenbergii* had a higher tolerance to the tested pollutants, particularly to the dispersant, than *E. pectinis* (Fig. 2G and L). As observed for oligotrich ciliates, the combination of crude oil and dispersant was the most toxic treatment in both species and all exposure levels (Fig. 2G and L), although not always statistically significant than the other experimental treatments (Fig. 2G and L). Dispersant increased the toxicity of crude oil to marine tintinnids by 1.03 to 1.93 times, depending on the exposure levels and the species (Fig. 2G and L).

At the lowest exposure concentration, the growth rates of the heterotrophic dinoflagellate *G. spirale* was slightly higher when exposed to crude oil alone than in the controls after 48 h (Fig. 2M) with no statistically significant differences. Dispersant was significantly more toxic than crude oil alone for *G. spirale* at the lower exposure concentrations after 48 h (ANOVA, Bonferroni post hoc test, $p < 0.05$) (Fig. 2M and N). For *P. divergens*, dispersant showed a similar toxicity than crude oil alone (Fig. 2P and R) with no significant differences at the three exposure levels. In all cases and for both species, the combination of crude oil and dispersant caused the highest reduction in growth rates (Fig. 2M and R). The dispersant-treated crude oil was between 1.49–3.33 times more toxic, depending on exposure levels and species, than crude oil alone to heterotrophic dinoflagellates (Fig. 2M and R).

In the 24 h experiments, significant difference among the tested pollutants on growth rates of heterotrophic dinoflagellates was observed for *O. marina*, *G. spirale* (ANOVA, $p < 0.05$) but not for *Protoceratium* sp. (Fig. 3). According to post hoc Bonferroni test, only *G. spirale* showed significant difference between treatments, with growth rates in the dispersant and dispersant-treated oil treatments being significantly lower than in the controls (Fig. 3B). However, no significant difference in growth rates of *G. spirale* was observed between the crude oil treatment and the control (Fig. 3B).

The relationships between cell concentration and crude oil, dispersant and dispersant-treated crude oil concentration were well described by the sigmoid model for all studied species of marine protozoa (Fig. 4, Table 2). The number of cells decreased with increasing pollutant concentration with differences in the slope depending on the species and treatments (Fig. 4). The decrease in cell concentration as pollutant concentration increased was more notable in oligotrich ciliates and tintinnids than in heterotrophic dinoflagellates (Fig. 4).

According to the sigmoid model, the median effect concentrations, $EC_{50}$ (48 h), ranged from 0.99 to 4.87 $\mu L\,L^{-1}$ for oligotrich

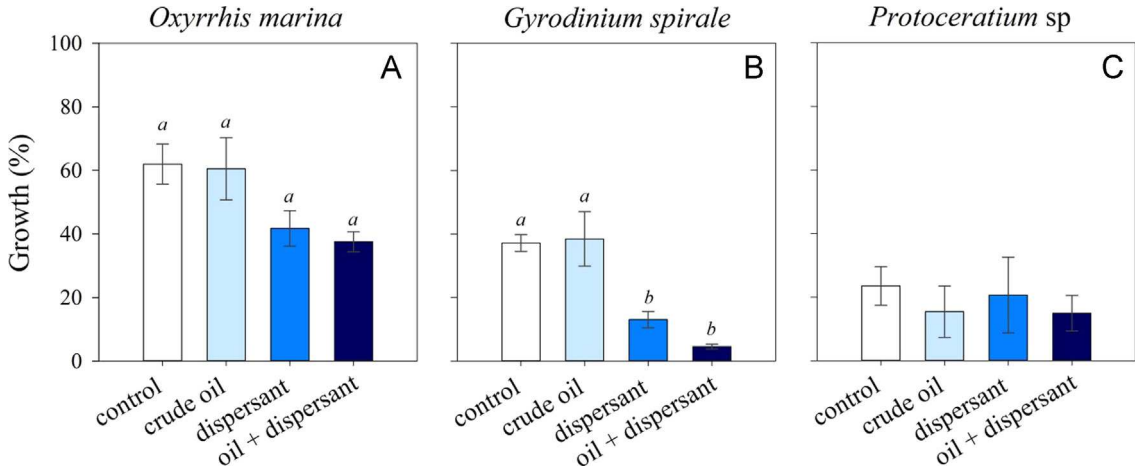


**Fig. 3.** Effects on crude oil alone (5 μL $L^{-1}$), dispersant (0.25 μL $L^{-1}$) and dispersant-treated crude oil (oil+dispersant) on the growth rates (percent) of heterotrophic dinoflagellates after 24 h of exposure. A: *Oxyrrhis marina*, B: *G. spirale*, C: *Protoceratium* sp. Lowercase letters (*a,b*) indicated different statistical groups according to the results of Post-hoc Bonferroni test ($p < 0.05$) Note that in © there was no significant difference among treatments according to the one-way ANOVA, and that in (A) there was significant difference among treatments according to the one-way ANOVA but no differences between treatments according to the Post-hoc Bonferroni test.

ciliates and tintinnids to 13.73–16.42 μL $L^{-1}$ for heterotrophic dinoflagellates when exposed to crude oil alone (Table 2). On average, crude oil alone was between 3.1 and 15.2 times more toxic for ciliates than for heterotrophic dinoflagellates (Table 2). The $EC_{50}$ (48 h) for oligotrich ciliates and tintinnids exposed to dispersant Corexit 9500A ranged from 0.03 to 0.20 μL $L^{-1}$ depending on the species. The tintinnid *F. ehrenbergii* exhibited a higher tolerance to dispersant exposure compared to the other ciliate species (Table 2). The $EC_{50}$ (48 h) of heterotrophic dinoflagellates exposed to dispersant Corexit 9500A varied from 0.76 μL $L^{-1}$ for *G. spirale* to 0.28 for *P. divergens*. On average, the dispersant Corexit 9500A was between 2.6 and 17.3 times more toxic for ciliates than for heterotrophic dinoflagellates (Table 2). $EC_{50}$ (48 h) ranged from 0.85–2.29 μL $L^{-1}$ for oligotrich ciliates and tintinnids to 5.69–13.40 μL $L^{-1}$ for heterotrophic dinoflagellates when exposed to dispersant-treated crude oil (Table 2). On average, dispersant-treated crude oil was between 4 and 64 times more toxic for ciliates than for heterotrophic dinoflagellates (Table 2). The addition of dispersant increased the toxicity of crude oil to all studied species of protozoan, with synergism ratios (SR) and relative toxic units (RTU) ranging from 1.2 to 7.1 and from 1.1 to 6.8, respectively, depending on the species (Table 2).

## 4. Discussion

Our results demonstrate that low concentrations of dispersed crude oil and dispersant Corexit 9500A are highly toxic to marine microzooplankton and consequently, these pollutants may cause important impacts on marine planktonic food webs and natural microbial community structure. After a crude oil spill, the concentration of dispersed crude oil in the water column is highly variable, ranging from more than 200 ppm to less than 1 ppb (McAuliffe et al., 1981; Lichtenthaler and Daling, 1985; Clayton et al., 1993). The median effect concentrations ($EC_{50}$) observed in this study for microzooplankton are in the range of dispersed crude oil concentrations commonly found in the water column after oil spills. For example, after the Deepwater Horizon crude oil spill, concentrations of 1–2 ppm were observed in plumes of dispersed crude oil (Kerr, 2010). Similarly, the $EC_{50}$ of chemical dispersant for microzooplankton are in the lower range of dispersant concentrations estimated in the water column after several field applications (from less than 1 ppm to more than 10 ppm) (Mackay and Hossain, 1982; Bocard et al., 1984; Wells, 1984) and in the same order of magnitude of the dispersant concentration used during the Deepwater Horizon oil spill according to the estimation provided by NALCO Environmental Solutions LLC (~30 ppb, NALCO®, 2010b). Therefore, our results demonstrate that microzooplankton, particularly small ciliates, are adversely affected by crude oil and dispersant pollution under realistic concentrations after catastrophic oil spills. However, in the natural environment, the impact of crude oil spills on plankton will not only depend on crude oil concentrations, but also exposure time and plankton community composition, and other environmental variables such as temperature (Jiang et al., 2012) and UV radiation (Duesterloh et al., 2002; Almeda et al., 2013a). Therefore, our results help to predict the potential acute effects of crude oil pollution and dispersant application on marine planktonic communities, but the impact of a crude oil spill on plankton will depend on the specific circumstances of each accident.

Our current knowledge of the effects of crude oil exposure on marine microzooplankton is very limited and sometimes contradictory. Further, although some crude oil toxicological plankton studies include ciliates, most studies have ignored heterotrophic dinoflagellates. Some studies with natural marine plankton enclosures observed a stimulatory effect of crude oil exposure for certain planktonic ciliates, mainly large tintinnids (Lee et al., 1978; Dahl et al., 1983; Dale, 1987, 1988). In community level experiments, stimulatory effects of crude oil for some protozoans, as commonly observed for bactiverous nanoflagellates (Koshikawa et al., 2007; Jung et al., 2012), may be related to indirect secondary effects, such as increase in the abundance of bacterioplankton or reduced grazing pressure. In contrast, there is increasing evidence that crude oil caused a drastic reduction in the abundance of planktonic ciliates (Skjoldal and Thingstad, 1987; Bak and Nieuwland, 1987; Koshikawa et al., 2007) and heterotrophic dinoflagellates (Koshikawa et al., 2007) in natural marine plankton assemblages, supporting the notion that microzooplankton are negatively affected by crude oil as observed in this study. Direct comparisons among crude oil toxicological studies, however, should be done cautiously due to the different methodology and experimental conditions. Exposure to crude oil often negatively affects phytoplankton growth/photosynthesis activity, but some species are highly tolerant or even stimulated by crude oil pollution (Echeveste et al., 2010; Jiang et al., 2010; Hallare et al., 2011). In our experiments, we observed that the phytoplankton species used as food for the protozoans increased in abundance in the experimental treatments compared to the controls, supporting

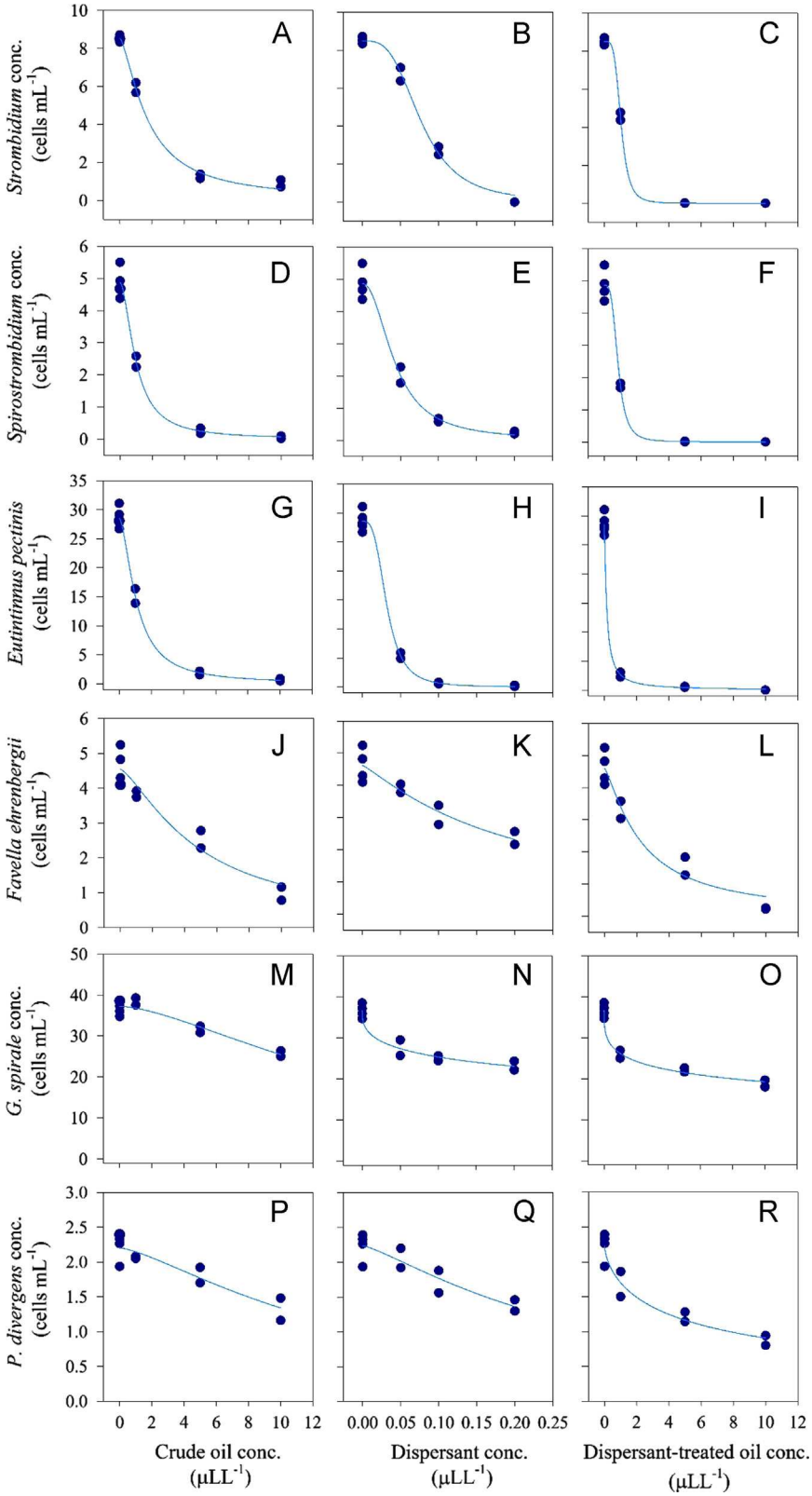


**Fig. 4.** Relationships between cell concentration of the studied protozoa species and crude oil alone, dispersant and dispersant-treated oil concentration after 48 h of exposure. (A)–(C) *Strombidium* sp, (D)–(F) *Spirostrombidium* sp, (G)–(I) *E. pectinis*, (J)–(L) *F. ehrenbergii*, (M)–(O) *G. spirale*, (P)–(R) *P. divergens*. Regression lines based on Eq. (1). Regression model parameters are indicated in Table 2.

the idea that phytoplankton have a higher tolerance to crude oil than microzooplankton and that the increased abundance of phytoplankton after exposure to crude oil may be due to a reduction in grazing pressure by microzooplankton. As we observed for microzooplankton, mesozooplankton (200–2000 μm) are also adversely affected by crude oil according to field and laboratory studies (Lee, 1977a; Johansson et al., 1980; Samain et al., 1980; Guzmán del Próo et al., 1986; Jiang et al., 2012; Almeda et al., 2013a;

**Table 2**
Parameters of the sigmoidal model (Eq. (1)) used to describe the relationships between the cell concentration of the studied protozoa species and either crude oil, dispersant and dispersant-treated crude oil concentrations after 48 h of exposure (Fig. 4). $EC_{50}$: median effect concentration ($\mu L\ L^{-1}$, 48 h), $N_0$: cell concentration in absence of pollutants, $b$: shape factor, $r^2$: correlation coefficient, and SE: standard error. SR: Synergistic ratio = $EC_{50}$ of crude oil alone/$EC_{50}$ of dispersant-treated crude oil. SR = 1.0 indicate no effects of dispersant on crude oil toxicity, whereas values > 1.0 and < 1.0 indicate greater (synergism) and weaker (antagonism) effects. RTU: Relative toxic units = predicted $EC_{50}$/experimentally estimated $EC_{50}$. Predicted $EC_{50}$ value for each species was estimated by summing the $EC_{50}$ values of the single pollutants according to their proportion in the test mixture (1:20). RTU = 1 indicates an additive action, RTU < 1 antagonism and RTU > 1 synergism.

| Species | Crude oil | | | | | |
|---|---|---|---|---|---|---|
| | **$EC_{50}$** ± SE | $N_0$ ± SE | $b$ ± SE | $r^2$ | | |
| *Strombidium* sp | **1.73** ± 0.13 | 8.54 ± 0.18 | 1.48 ± 0.12 | 0.99 | | |
| *Spirostrombidium* sp | **0.99** ± 0.11 | 4.87 ± 0.16 | 1.79 ± 0.56 | 0.97 | | |
| *Eutintinnus pectinis* | **1.07** ± 0.08 | 28.55 ± 0.51 | 1.71 ± 0.29 | 0.99 | | |
| *Favella ehrenbergii* | **4.87** ± 0.93 | 4.54 ± 0.22 | 1.37 ± 0.39 | 0.92 | | |
| *Gyrodinium spirale* | **16.42** ± 3.20 | 37.26 ± 0.74 | 1.55 ± 0.45 | 0.91 | | |
| *Protoperidinium divergens* | **13.73** ± 3.52 | 2.20 ± 0.08 | 1.39 ± 0.60 | 0.88 | | |
| | Dispersant | | | | | |
| | **$EC_{50}$** ± SE | $N_0$ ± SE | $b$ ± SE | $r^2$ | | |
| *Strombidium* sp | **0.08** ± 0.00 | 8.49 ± 0.20 | 3.25 ± 0.33 | 0.99 | | |
| *Spirostrombidium* sp | **0.04** ± 0.01 | 4.87 ± 0.17 | 2.15 ± 0.59 | 0.98 | | |
| *Eutintinnus pectinis* | **0.03** ± 0.01 | 28.55 ± 0.46 | 3.20 ± 1.65 | 0.99 | | |
| *Favella ehrenbergii* | **0.20** ± 0.04 | 4.62 ± 0.20 | 1.14 ± 0.36 | 0.87 | | |
| *Gyrodinium spirale* | **0.76** ± 0.59 | 36.64 ± 0.82 | 0.40 ± 0.16 | 0.94 | | |
| *Protoperidinium divergens* | **0.28** ± 0.08 | 2.24 ± 0.09 | 1.27 ± 0.47 | 0.82 | | |
| | Dispersant-treated crude oil | | | | | |
| | **$EC_{50}$** ± SE | $N_0$ ± SE | $b$ ± SE | $r^2$ | SR | RTU |
| *Strombidium* sp | **1.04** ± 0.06 | 8.54 ± 0.18 | 1.48 ± 0.12 | 0.99 | 1.7 | 1.6 |
| *Spirostrombidium* sp | **0.85** ± 0.56 | 4.87 ± 0.16 | 3.49 ± 13.82 | 0.98 | 1.2 | 1.1 |
| *Eutintinnus pectinis* | **0.15** ± 0.25 | 28.55 ± 0.46 | 1.20 ± 1.01 | 0.99 | 7.1 | 6.8 |
| *Favella ehrenbergii* | **2.29** ± 0.52 | 4.60 ± 0.24 | 1.27 ± 0.28 | 0.95 | 2.1 | 2.0 |
| *Gyrodinium spirale* | **13.40** ± 4.18 | 36.63 ± 0.68 | 0.35 ± 0.07 | 0.97 | 1.2 | 1.2 |
| *Protoperidinium divergens* | **5.69** ± 1.44 | 2.23 ± 0.09 | 0.67 ± 0.17 | 0.93 | 2.4 | 2.3 |

Cohen et al., 2014). Altogether, these findings suggest that crude oil pollution may frequently promote the growth of bactivorous nanoflagellates, but in general negatively affect both micro—and mesozooplankton.

In our experiments, toxic petroleum hydrocarbons would have been present in both dissolved and particulate (oil droplets) forms, whereas most crude oil toxicological studies have been conducted with the water soluble fraction of crude oil (WSF) or individual or mixed dissolved petroleum aromatic hydrocarbons (PAHs) that are considered to be the most toxic fraction of petroleum (Jiang et al. 2010). Considering PAH composition and concentration in the crude oil used in this study (Almeda et al., 2013b), the equivalent median effect concentrations ($EC_{50}$) in terms of PAH concentration for microzooplankton are several orders of magnitude lower than for phytoplankton (Prouse et al., 1976; Echeveste et al., 2010, Jiang et al. 2010) and marine macrofauna (Kennish, 1996; Lee, 1977b), and in the lower range of median lethal concentrations ($LC_{50}$) values commonly reported for mesozooplankton (Barata et al. 2005; Calbet et al., 2007; Saiz et al., 2009; Avila et al., 2010; Jiang et al., 2010, 2012). It is important to note that in this study we used the term median effect concentration ($EC_{50}$) instead of median lethal concentration ($LC_{50}$) because in a few cases, particularly for heterotrophic dinoflagellates, the decrease in cell abundance was due a reduction in population growth instead of mortality. Therefore, we consider the term $EC_{50}$ to be more appropriate for our results and also to emphasize that comparisons with $LC_{50}$ from other studies should be taken cautiously. However, in the cases where the decrease in cell population was due to mortality, the terms $EC_{50}$ and $LC_{50}$ are equivalent.

Besides the high sensitivity of zooplankton compared to other marine organisms, exposure to particulate instead of dissolved petroleum may affect the toxicity of crude oil to marine zooplankton (Lanier and Light, 1978) and the bioaccumulation of toxic PAHs. Most dispersed crude oil droplets are in the food size spectra of zooplankton and therefore crude oil droplets may be directly ingested by zooplankters (Conover, 1971; Lee et al., 2012). In fact, we observed ingestion of crude oil droplets by the planktonic protozoans studied here (Almeda et al., 2014). Previous studies also found that the epibenthic ciliate *Euplotes* ingests crude oil droplets suspended in the water column (Andrews and Floodgate, 1974, Lanier and Light, 1978), but crude oil emulsions were also highly toxic to this organism ($LC_{50,90\ h}$ = 1.7 ppm, Lanier and Light, 1978). The ingestion of particulate crude oil may increase the bioaccumulation of low solubility PAH and affect vital functions (e.g. assimilation of food). Low solubility PAHs are frequently more toxic than more soluble and volatile PAHs (e.g. naphthalene) to zooplankton (Berdugo et al., 1977; Barata et al., 2005). Therefore, the use of particulate rather than dissolved petroleum may represent a more realistic scenario to investigate the interactions between crude oil and zooplankton and to assess the toxicity of crude oil to planktonic communities.

We found that ciliates are more sensitive to crude oil and dispersant than heterotrophic dinoflagellates. In fact, when heterotrophic dinoflagellates were exposed to low concentrations of crude oil alone and short exposure time, the growth of species studied here were not affected when compared with the controls. The reasons underlying this variability in tolerance to crude oil and dispersant between ciliates and heterotrophic dinoflagellates could be related to genetic and physiological differences that confer resistance against environmental chemical pollution, as observed in some phytoplankton species (Wolfe et al., 1999; Romero et al., 2012). It has been suggested that the presence of external skeletal structures may provide some protection against crude oil pollution (Dale, 1988). However, we did not find any direct relationships between the presence of external skeleton, i.e., lorica in ciliates or theca in dinoflagellates (Table 1), and the

resistance to crude oil or dispersant pollution (Table 2). Although, we did find that, among ciliate species, the large tintinnid, *Favella,* showed a higher tolerance to crude oil and dispersant than the smaller ciliates (Table 2). In agreement with this observation, previous studies have found an inverse relationship between the size and the sensitivity to crude oil exposure for phytoplankton (Echeveste et al., 2010) and marine copepods (Jiang et al., 2012). This inverse size-crude oil toxicity relationship may be related to the higher surface to volume ratio of small organisms that may increase uptake of dissolved petroleum hydrocarbons via passive diffusion.

One conclusion of our study is that chemically dispersed crude oil is more toxic than crude oil alone. As demonstrated here, Corexit 9500A dispersant is itself toxic to ciliates and heterotrophic dinoflagellates. The increased toxicity of dispersant-treated crude oil to marine microzooplankton may be associated with both additive and/or synergistic effects of oil and dispersant, and may vary widely depending on the species and exposure levels. One synergistic toxic effect of the combination of crude oil and dispersant is the increase in the dissolution of toxic soluble components of crude oil into the water (Greer et al., 2012; Wu et al., 2012), which may be taken up into the cells through passive mechanisms. Previous studies found that Corexit 9527 and dispersed crude oil have adverse effects on fresh water ciliates (Rogerson and Berger, 1981). Also a recent study found that Corexit 9500A and dispersed oil inhibit the growth of ciliates in natural plankton assemblages (Ortmann et al., 2012). According to the median effect concentrations ($EC_{50}$) for dispersant observed in this study, marine microzooplankton, particularly ciliates, are amongst the most sensitive zooplankton organisms to chemical dispersant (George-Ares and Clark, 2000; Cohen et al., 2014). Therefore, given the important role of microzooplankton in marine food webs and their high sensitivity to chemical dispersants, small planktonic ciliates would be an appropriate target group (bioindicator) to determine the toxicity of chemical dispersants.

The application of chemical dispersant is considered to be an effective technique to clean up marine crude oil spills and to reduce their environmental impacts (Lessard and Demarco, 2000; Lewis, 2001; US Environmental Protection Agency, 2010). Chemical dispersion of crude oil reduces the risk of oil slicks arriving to coastal areas and physical contamination (smothering) on marine vertebrates, and may promote bacterial degradation of petroleum compounds (Churchill et al., 1995; Lessard and Demarco, 2000). However, despite these advantages, the net environmental benefit of dispersant application is still unclear since dispersants increase petroleum hydrocarbon concentrations in water (Greer et al., 2012; Wu et al., 2012; Cohen et al., 2014) and are potentially toxic to marine organisms as observed here for microzooplankton. Our results support other recent studies that demonstrate that Corexit 9500 dispersant is more toxic than previously assumed, particularly to small planktonic organisms (Goodbody-Gringley et al., 2013; Rico-Martinez et al., 2013; Almeda et al., 2013a; Cohen et al., 2014). Therefore, the application of this type of chemical dispersant as a response to crude oil spills may increase the damage to key planktonic organisms, such as larval stages, copepods, microzooplankton, and consequently may not reduce the net environmental impact on marine environments. Among the negative ecological consequences of a harmful impact of chemically dispersed crude oil on microzooplankton are the disruption in the transfer of energy from low to high trophic levels and changes in the microbial planktonic community structure and dynamics. For example, microzooplankton are qualitatively and quantitatively essential prey of copepods and fish larvae and consequently the reduction of these protozoans may affect secondary production in polluted marine areas. In addition, since microzooplankton play a key role in controlling phytoplankton, including toxic marine phytoplankton (Calbet et al., 2003; Rosetta and McManus, 2003; Kamiyama, 1997; Kamiyama et al., 2005), the removal or decrease of microzooplankton due to chemically dispersed crude oil pollution may open 'loopholes ', i.e. disrupting the predator-prey controls that normally function at the level of the microbial loop. This disruption of grazer pressure on toxic phytoplankton may contribute to the initiation of harmful algal blooms (Buskey et al., 1997; Irigoien et al., 2005). More research on the effects of the crude oil and chemical dispersant on planktonic communities, particularly on key, sensitive zooplankton organisms (e.g. microzooplankton, planktonic larval stages, copepods) and their short and long term impacts on marine planktonic communities is required to better evaluate the environmental impact of crude oil spills and the application of chemical dispersant in the sea.

## 5. Main conclusions

This study demonstrates that dispersant Corexit 9500A is highly toxic to marine microzooplankton and that chemically dispersed crude oil is more toxic than crude oil alone to these planktonic organisms. Among microzooplankton, small ciliates are more sensitive to crude oil and dispersant exposure than large tintinnids and heterotrophic dinoflagellates. The negative effects of crude oil and chemically dispersed crude oil on microzooplankton may cause important changes in the natural structure, function and dynamics of planktonic communities.

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# Response of deep-water corals to oil and chemical dispersant exposure

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ABSTRACT

Cold-water corals serve as important foundation species by building complex habitat within deep-sea benthic communities. Little is known about the stress response of these foundation species yet they are increasingly exposed to anthropogenic disturbance as human industrial presence expands further into the deep sea. A recent prominent example is the Deepwater Horizon oil-spill disaster and ensuing clean-up efforts that employed chemical dispersants. This study examined the effects of bulk oil–water mixtures, water-accommodated oil fractions, the dispersant Corexit 9500A®, and the combination of hydrocarbons and dispersants on three species of corals living near the spill site in the Gulf of Mexico between 500 and 1100 m depths: *Paramuricea* type B3, *Callogorgia delta* and *Leiopathes glaberrima*. Following short-term toxicological assays (0–96 h), all three coral species examined showed more severe health declines in response to dispersant alone (2.3–3.4 fold) and the oil–dispersant mixtures (1.1–4.4 fold) than in the oil-only treatments. Higher concentrations of dispersant alone and the oil–dispersant mixtures resulted in more severe health declines. *C. delta* exhibited somewhat less severe health declines than the other two species in response to oil and oil/dispersant mixture treatments, likely related to its increased abundance near natural hydrocarbon seeps. These experiments provide direct evidence for the toxicity of both oil and dispersant on deep-water corals, which should be taken into consideration in the development of strategies for intervention in future oil spills.

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## 1. Introduction

The *Deepwater Horizon* (DWH) oil spill was one of the largest environmental disasters in history, releasing approximately 5 million barrels of crude oil at depth in the Gulf of Mexico (GoM) over a three-month period (Crone and Tolstoy, 2010; Camilli et al., 2011). In addition, nearly 7 million liters of oil dispersants were applied during the ensuing cleanup efforts. Dispersants are chemical emulsifiers that act to increase the rate of oil dispersion thereby increasing the amount of small oil droplets suspended in the water column, reducing oil slicks at the surface. Thus, dispersant applications affect the fate, transport and physical composition of oil. Of the 7 million liters of oil dispersants used, approximately 3 million liters were applied at depth for the first time in history (Barron, 2012), without a comprehensive understanding of how this subsea application might alter the fate of oil and impact benthic ecosystems (National Research Council, 2005).

Petroleum hydrocarbons released under high-pressure undergo a series of interconnected physical and chemical processes that affect their fate and transport in the deep sea (Camilli et al., 2010; Kessler et al., 2011; Reddy et al., 2012). Following the direct injection of disperant (Corexit 9527A and 9500A) to the Macondo well head at a depth of 1544 meters (m) (Hazen et al., 2010), a large oil plume persisted for months centered at approximately 1100 m depth, without substantial biodegradation (Camilli et al., 2010). Oil spewing from the wellhead encountered turbulent mixing and was emulsified as a result of its reduced buoyancy at depth and the application of dispersant (Fodrie and Heck Jr., 2011). Measurements of water-column samples collected from this deep-water plume (defined by Camilli et al., 2010) indicated that a significant portion of water-soluble hydrocarbon components were retained in deep waters, with unknown portions of insoluble hydrocarbons drifting to the sea floor (Reddy et al., 2012). Despite some emulsification of oil throughout the water column, surface waters were still polluted with oil slicks (Fodrie and Heck Jr., 2011). At the surface, some components of the oil were then transformed into aggregations of marine snow (and floc) by coagulation with suspended particulates and planktonic organisms. Although this marine snow disappeared from the surface layers of the GoM within a month, it is likely that it sunk into the deep sea as the oil weathered (Passow et al., 2012).

Recent studies have found both lethal and sub-lethal effects of the DWH blowout on species inhabiting pelagic and coastal environments (Barron, 2012; Silliman et al., 2012; Whitehead et al., 2012; Dubansky et al., 2013; Almeda et al., 2013). Prior studies have shown variable levels of crude oil toxicity on aquatic organisms with some fauna being more susceptible than others (Anderson et al., 1974; Bonsdorff et al.,

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1990; Coull and Chandler, 1992; Stark et al., 2003). Dispersant addition to the oil triggers a transient increase in hydrocarbon concentrations throughout the water-column (Pace et al., 1995), which can then lead to higher, more toxic exposures of dissolved and dispersed oil components upon contact with marine life.

Spill-impacted deep-sea coral communities were first discovered at a depth of approximately 1370 m, 11 km southwest of the Macondo well explosion, at the lease block site Mississippi Canyon (MC) 294 (White et al., 2012). Various species of coral, primarily *Paramuricea biscaya* (Grasshoff, 1977), were found covered with brown flocculent material (floc), exhibiting characteristic signs of stress and mortality, including excess mucus production, sclerite enlargement, and tissue loss. Further analysis of this floc revealed hydrocarbons from the Macondo well were indeed present (White et al., 2012). Whether the damage observed to the corals was induced by sinking oil-filled particulates, dissolved hydrocarbons, dispersants, or a combination of all of these sources is unknown. Subsequently, two additional sites were discovered to contain impacted deep-sea coral communities (Fisher et al., 2014).

Deep-sea corals alter the terrain of the sea floor and produce complex, heterogeneous habitat, which promotes benthic biodiversity (Cordes et al., 2008, 2010). In addition to reef-forming scleractinian corals, which generally occur at upper-slope depths (300–1000 m), octocorals and black corals (antipatharians) form large, tree-like structures from the subtidal to over 3000 m depth. These corals colonize hard substrata, and can form dense fields (Roberts et al., 2006). By increasing the complexity of the seafloor, they provide shelter, feeding areas, and nursery grounds for many fish and invertebrates.

Because deep-sea corals build the foundation for these communities, damage to them can impact biodiversity and ecosystem function (Husebo et al., 2002; Freiwald et al., 2004). Their longevity and slow growth rates make them particularly vulnerable to anthropogenic disturbance (Grigg, 1974; Emiliani et al., 1978; Druffel et al., 1990, 1995; Risk et al., 1998, 2002; Andrews et al., 2002; Adkins et al., 2004; Roark et al., 2009). As crude oil reserves are abundant in the GoM, with 1.5 billion barrels of oil extracted from the sea floor each day (Minerals Management Service, 2009), it is now a critical time for further examination of deep-sea coral response to oil and dispersant exposure.

Here, the effects of oil, dispersant and oil–dispersant mixtures were tested experimentally on three species of deep-sea coral living near the DWH oil spill site in the Gulf of Mexico, including *Paramuricea* type B3 (Doughty et al., 2014), *Callogorgia delta* (Bayer et al., 2014) and *Leiopathes glaberrima* (as re-described in Opresko and Baron-Szabo, 2001). *P. biscaya* was the most common of the corals impacted by the DWH oil spill (White et al., 2012; Fisher et al., 2014), and *Paramuricea* type B3 is the sister species to this coral (Doughty et al., 2014). *Paramuricea* type B3 was chosen because its shallower depth distribution (830–1090 m for *Paramuricea* type B3 vs. 1370–2600 m for *P. biscaya* with one individual collected at 850 m, Doughty et al., 2014) results in higher survivorship shipboard, and to avoid further impact to the relatively small populations of *P. biscaya* that have thus far been discovered. *C. delta* preferentially occupies habitats near natural oil seeps in the deep GoM (Quattrini et al., 2013), suggesting that the species may have evolved a tolerance for hydrocarbon exposure. *L. glaberrima* is slow growing and lives to very old ages, making it one of the oldest skeletal secreting organisms known to date (Roark et al., 2009). Slow growth rates make this species highly sensitive to natural and anthropogenic disturbances.

This study examined the effects of exposure to bulk oil–water mixtures, water-accommodated oil fractions (WAF), dispersants, and mixtures of hydrocarbons and dispersants using short-term toxicological assays ( $\leq$ 96 h) that monitored phenotypic responses and survivorship. Specifically, we tested the hypotheses that oil/dispersant mixtures would be the most toxic to corals, and that *C. delta* would have a higher tolerance for hydrocarbons due to its affinity for natural seep habitats.

## 2. Methodology

### 2.1. Sample collection and acclimatization

All samples were collected from two sites in the GoM. *C. delta* and *L. glaberrima* were collected from the Viosca Knoll (VK) 826 site at a depth of approximately 500 m (29 °09.5′N, 88 °01.0′W; Cordes et al., 2008; Davies and Guinotte, 2011). *Paramuricea* type B3 colonies were collected from a large population of corals at approximately 1050 m depth at Atwater Valley (AT) 357 (27 °58.6′N, 89 °70.4′W; Doughty et al., 2014). At each site, corals were haphazardly collected with the remotely operated vehicles (ROV) Global Explorer MK3 or Hercules.

Samples were taken on multiple dives, with 5–6 colonies of both *C. delta* and *L. glaberrima* collected from VK826, and 5–6 colonies of *Paramuricea* type B3 gathered from AT357. Samples were collected several meters apart from conspecific colonies to reduce the likelihood of sampling clones. Corals were visually identified using live video stream from cameras attached to each ROV, before being collected with a manipulator arm and secured in an insulated “bio” box and or sealable collection quivers. When possible, branches of colonies were sampled to reduce impact.

At the surface, colonies were immediately transferred to containers with filtered seawater of the species-appropriate temperature and salinity (35 psu). *C. delta* and *L. glaberrima* were jointly maintained at approximately 8 °C and later, *Paramuricea* type B3 at 5 °C (the average in situ temperatures at depth) in a temperature-controlled room for the duration of the experiment. Temperature in holding vessels was continuously monitored using temperature probes (Hobo® Data Loggers). Corals were allowed to acclimate for 6–12 h prior to experimentation.

### 2.2. Preparation of bulk-oil treatments

For the bulk-oil experiment three stock solutions were prepared: crude oil (MASS oil collected from the Macondo well during the spill), dispersant (Corexit 9500A), an oil/dispersant mixture, and artificial seawater controls. All solutions were made with sterile artificial seawater (ASW, Instant Ocean™) at 35 psu, the average in situ salinity for both sites. ASW allowed us to accurately maintain desired salinity and temperature for large volumes of water without the potential for introducing contaminants from the ship's seawater system, and to avoid the unreliability of collecting buckets of seawater from over the side in variable sea states. We have used ASW to maintain other cold-water coral species alive in laboratory aquaria for extended periods of time without adverse affects (Lunden et al., 2014).

A stock bulk-oil solution was prepared at a concentration of 250 parts per million (ppm) by adding 50 µL of MASS oil to 199.95 mL ASW. The solution was mixed at room temperature for a 24-h period on an orbital shaker at approximately 500 rpm to achieve highest possible homogeneity. Oil dilutions were prepared from this stock solution. The subsequent oil concentrations were chosen in an attempt to determine the threshold for lethal toxicity, following preliminary toxicity studies on *L. glaberrima*. Dispersant concentrations were the same as the oil concentrations so as to examine the relative toxicity of oil vs. dispersant. The oil/dispersant-mixture stock solution was prepared with an initial targeted concentration of 250 ppm each of crude oil and Corexit 9500A by adding 50 µL of each to 199.90 mL of ASW. The dispersant stock solution was prepared by adding 50 µL Corexit 9500A to 199.95 mL ASW to achieve an initial concentration of 250 ppm. Serial dilutions were

prepared from each of the three stock solutions to produce three target concentrations: 25 ppm (High), 7.9 ppm (Medium) and 0.8 ppm (Low).

All solutions were placed into sterile 50 mL glass vials. These were then incubated at 5 or 8 °C, dependent on species, and mixed continuously at low speeds for 24 h on an orbital shaker table to reduce separation and to encourage even oil distribution. Experiments were conducted between 8 and 27 November 2012 onboard the R/V *Falkor*.

### 2.3. Preparation of treatments using water-accommodated oil fractions (WAF)

For this experiment, stock solutions were prepared using only the water-accommodated oil fractions (WAF). For the WAF oil treatment, a higher oil volume (9.5 mL) of surrogate oil was added to 475 mL of ASW and mixed at high speeds (~350 rpm) in an attempt to produce a 1.2 mM WAF oil solution. The WAF was separated from the insoluble oil layer using a sterile separatory funnel, and used as a stock solution to produce experimental treatments with targeted initial total hydrocarbon concentrations of 250 μM (High), 150 μM (Medium) and 50 μM (Low) WAF. Target concentrations were chosen to find lethal doses, as none of the previous bulk-oil (only) concentrations proved to be lethal. This was done using a standardized WAF protocol (S. Joye, personal communication) and based on the highest concentrations of oil detected during the spill (~300 μM, Joye et al., 2011).

The oil/dispersant mixture treatment was prepared using the same oil volume, with 950 μL of Corexit 9500A added (one-tenth of the oil concentration) to produce a dispersant enhanced WAF (DE–WAF; oil/dispersant treatment), also mixed at high speeds (~350 rpm). As the dispersant concentrations in the bulk-oil exposures were not entirely lethal to *C. delta* in the short term and most of the observed health decline was seen towards the end of the exposures at the highest Corexit 9500A concentration, the range of dispersant concentrations was progressively increased from those used in the previous exposures to attempt to reveal the lethal concentration (LC50). The dispersant stock solution was made by adding 950 μL of Corexit 9500A to 475 mL of ASW, with an initial dispersant concentration of 848 mg/L (mixed at 200–300 rpm). All stock solutions were mixed at room temperature for 48–72 h. Experimental solutions were then made from these two treatments with targeted initial oil concentrations of 250 μM (High), 150 μM (Medium) and 50 μM (Low) and targeted initial total dispersant concentrations of 176.7 mg/L (High), 106.0 mg/L (Medium) and 35.3 mg/L (Low).

All solutions were placed into sterile 50 mL acid-washed glass vials prior to experimentation. There was an anticipated and unavoidable loss of hydrocarbons and dispersant due to the adhesion of hydrophobic components to the dilution containers with each sequential transfer, as well as the chemical and coral–microbial alterations of hydrocarbons and dispersant components over the course of the treatments. Therefore oil and dispersant concentrations are reported as conservative, initial targeted values only, and qualitatively designated as “High” “Medium” and “Low” in the analysis. Experiments were conducted from 23 June 2013 to 3 July 2013 onboard the R/V *Nautilus*.

### 2.4. Fragmentation and exposure experiments

For both bulk-oil and WAF experiments, four to six colonies of each species ($n=3$) were fragmented into similar sized (approximately 3–6 cm tall), genetically identical replicates, or “nubbins” ($n=11$) and placed into the oil, dispersant, oil/dispersant mixture and the control (ASW) treatments. *Paramuricea* type B3 had only three healthy colonies for the bulk-oil exposures. The number of polyps per nubbin varied for each species because of the wide range in polyp sizes and unique branching morphology. Samples were placed in 50 mL pyrex test tubes, mounted on a shaker table in a temperature controlled environment, and aerated every 24 h by bubbling air into the tubes and gently inverting each sample.

Each sample was photographed together with a scale and monitored for signs of stress at four time points (24, 48, 72 and 96 h) during the bioassay. Each experimental nubbin was assigned an overall health rating on a scale ranging from 0 to 5. The percentage of live polyps and tissue-covered skeleton primarily contributed to this rating: dead fragment (score of 0), ⩽50% (score of 1–2), ~50% (score of 3), ⩾50% (score of 4–5), while the other stress responses further differentiated between scores. Ratings were further refined based on the following phenotypic stress responses: percentage of polyp retraction and or inflation, presence and persistence of mucus discharge, dead or darkened tissue, sloughing tissue and exposed skeleton. While polyp mortality, polyp retraction, mucus release, loose tissue, and exposed skeleton were observed in all three species, swollen polyps were only observed in *L. glaberrima*, while darkened tissue was specific to *Paramuricea*. Tissue discoloration and whitening was only observed in *C. delta*. Furthermore, *C. delta* displayed a distinctive polyp coiling, ultimately forming node-like structures that eventually disintegrated, leaving behind exposed skeleton. Samples and treatments were randomized in an attempt to reduce health-scoring bias.

### 2.5. Survival analysis

Health rankings were averaged for replicate coral fragments in each experimental concentration and plotted over time to investigate health decline. This was done discretely for each round of experiments (bulk-oil or WAF), type of treatment (oil, dispersant and oil/dispersant) and species to determine the effect of concentration on fragment health over time. Health differences within the different treatments at the 96-h end-point were tested using a non-parametric Kruskal–Wallis test, and if applicable ($p<0.05$), non-parametric post-hoc, pair-wise comparisons were performed using the Wilcoxon method (using JMP® Pro 10.0.2).

To investigate fragment survival over time, a Kaplan–Meier (K–M) “time to event” survival analysis was performed separately for each experimental series (IBM® SPSS® Statistics v22, Kaplan and Meier, 1958). This test measures the fraction of fragments declining to a health status of 3 or below at each time point and generates a survival curve. To quantify differences amongst the survival curves for a given species and treatment, a Mantel–Cox log-rank test was used to evaluate statistical significance ($\alpha=0.05$); if significant, pair-wise comparisons were made, again using a Mantel–Cox log-rank test.

An additional K–M analysis was performed to compare survival across species in each treatment. Only “event” occurrences contribute to survival estimates; the remaining data becomes censored in the analysis. For this reason the ASW control treatments, in which all fragments maintained health ratings $>3$, were excluded from survival-estimate statistics during species comparisons. A similar percentage of censored cases were present in the oil, dispersant and oil–dispersant treatments for each species, and the pattern of censoring was similar.

Additionally, Cox regressions were performed to quantify the hazard (i.e. a decline in health) associated with (a) treatment (water, oil, dispersant/oil and dispersant), (b) concentration (High, Medium, Low, Zero), and (c) species (*C. delta*, *L. glaberrima*, *Paramuricea* type B3) for the two sets of experiments (bulk-oil and WAF). The “event” in the time-to-event analysis was reaching a health rating of 3 or below (3, 1, 2 or 0), as mortality was not observed in every treatment and concentration during the exposure. The hazard ratios were calculated for each factor with respect

to control treatment (a), the zero concentration (b) and *C. delta* (c), as we had hypothesized this to be the species most likely adapted to oil exposure. Cox regression was performed in IBM® SPSS® Statistics v22.

## 3. Results

### 3.1. Exposure effects on Paramuricea type B3

#### 3.1.1. Oil treatment

Complete fragment mortality was not observed for *Paramuricea* type B3 in the control, bulk-oil or oil–WAF treatments (Figs. 1A and 2A). In examining the effect of concentration on fragment condition at the end of the bulk-oil and WAF exposures, the Kruskal–Wallis test showed no significant differences among the 96-h health ratings across all oil concentrations and controls ($p > 0.05$).

#### 3.1.2. Dispersant treatment

Whole fragment mortality was observed in *Paramuricea* type B3 nubbins exposed to the High dispersant treatment (Fig. 1D). This decline in health originated in the dispersant mixture within 48–72 h, with two of three colonies exhibiting complete fragment mortality at the end of the exposure period. The Kruskal–Wallis test revealed significant differences ($p < 0.05$) in health rankings for *Paramuricea* type B3 at the end of the exposure; pair-wise comparisons revealed significant differences between nubbins in the High dispersant relative to the control samples ($p < 0.05$).

High coral fragment mortality was observed in the dispersant treatment across all concentrations tested in the WAF experiments. One of six *Paramuricea* type B3 replicates died in the Low dispersant solution, with complete mortality observed in four of six replicates in the Medium dispersant treatment by 96 h. At High dispersant concentrations, four of six replicates were dead after only 48 h, with complete mortality of all fragments after 96 h (Fig. 2D). The Kruskal–Wallis test and pair-wise comparisons revealed significantly higher health ratings among the control *Paramuricea* type B3 nubbins relative to all levels of dispersant (Low, Medium and High; $p < 0.005$) as well as in the Low vs. High dispersant concentrations ($p < 0.005$).

#### 3.1.3. Oil/dispersant treatment

Whole fragment mortality was observed in *Paramuricea* type B3 nubbins exposed to the High oil/dispersant treatment (Fig. 1G), with complete mortality in two of three fragments by 96 h. There were significant health differences among concentrations (Kruskal–Wallis, $p < 0.05$), and subsequent pair-wise comparisons revealed significant differences between fragments in the High oil/dispersant relative to the control samples ($p < 0.05$).

During the WAF exposures, complete mortality was observed in the oil/dispersant mixture (DE–WAF), for one of six *Paramuricea* type B3 samples in both the Low and High concentrations (Fig. 2G). The Kruskal–Wallis and post-hoc tests detected significant health differences in fragments exposed to all concentrations of the mixture relative to the controls ($p < 0.05$).

#### 3.1.4. Comparisons between treatments for Paramuricea type B3

For comparisons made between treatments in the bulk-exposure series, the log-rank test revealed significant differences among the K–M survival estimates ($\chi = 7.62$, df = 2, $p = 0.022$); pairwise comparisons (Table 1) indicated these differences were between the oil and oil/dispersant treatments ($p < 0.0167$). The oil/dispersant treatment had the lowest mean estimated survival time of 87.6 h, compared to the overall mean estimate of 90.2 h (Table 2a, Fig. 3). In the WAF exposures there were also significant differences among time-to-event occurrences ($\chi = 57.3$, df = 2, $p < 0.001$), and pair-wise comparisons affirmed significantly different estimates between all treatments. The lowest time-to-event estimate was 82.5 h in dispersant compared to 96 h in oil and an overall average estimate of 90.8 h (Table 2b, Fig. 3).

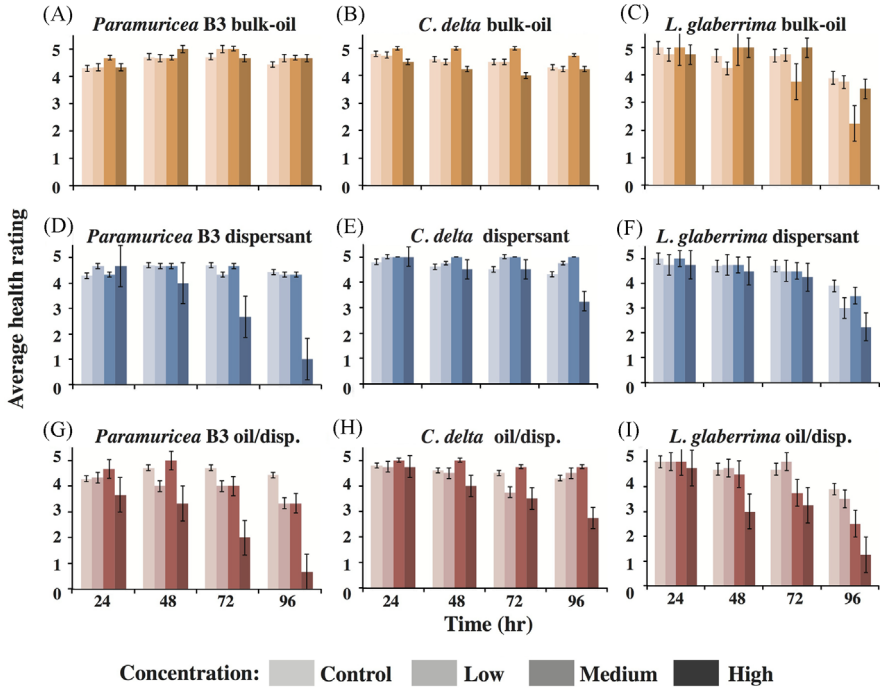


**Fig. 1.** Average health ratings over time for coral fragments exposed to various concentrations of bulk-oil mixtures (yellow/ top row), Corexit 9500A dispersant solutions (blue/ middle row) and oil–dispersant (oil/disp.) combination mixtures (red/ bottom row). Health rating scale 0–5. Bars show standard error.

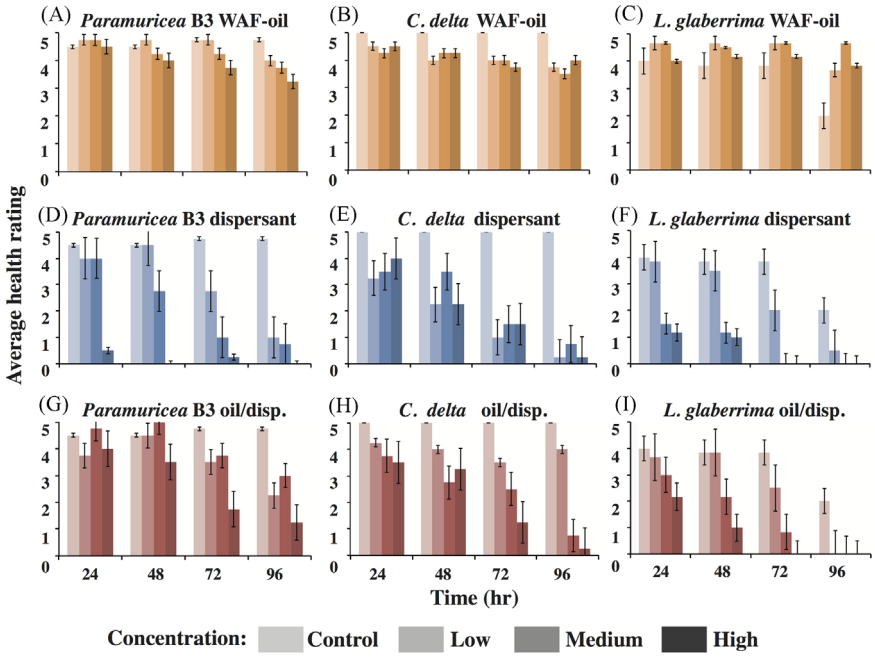


**Fig. 2.** Average health ratings over time for coral fragments exposed to various concentrations of water accommodated oil fractions (yellow/ top row), Corexit 9500A dispersant solutions (blue/ middle row) and water accommodated oil–dispersant (oil/disp.) combination mixtures (red/ bottom row). Health rating scale 0–5. Bars show standard error.

**Table 1**
Pair-wise comparisons of K–M survival estimates in oil, dispersant and oil/dispersant treatments within the bulk-oil and oil–WAF exposure series, using a Mantel–Cox log-rank analysis. Comparisons were done discretely for each of the three coral species: *C. delta*, *Paramuricea* (type) B3 and *L. glaberrima* ($\chi^2$=chi-square, $\alpha$=0.05). The event was a decline in health rating to 3 or below (bulk) or 1 and below (WAF). Bonferroni adjusted *p*-values for each within species comparison are $p < 0.0167$, with values in bold being significant.

| Log-rank (Mantel–Cox) | Oil | | Dispersant | | Oil /dispersant | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | *p*-Val | $\chi^2$ | *p*-Val | $\chi^2$ | *p*-Val |
| **Bulk exposures** | | | | | | |
| *C. delta* | | | | | | |
| Oil | – | – | 1.766 | 0.184 | 0.284 | 0.594 |
| Dispersant | 1.766 | 0.184 | – | – | 3.594 | 0.058 |
| Oil/dispersant | 0.284 | 0.594 | 3.594 | 0.058 | – | – |
| *Paramuricea* B3 | | | | | | |
| Oil | – | – | 3.958 | 0.047 | 10.634 | **0.001** |
| Dispersant | 3.958 | 0.047 | – | – | 2.401 | 0.121 |
| Oil/dispersant | 10.634 | **0.001** | 2.401 | 0.121 | – | – |
| *L. glaberrima* | | | | | | |
| Oil | – | – | 0.152 | 0.696 | 7.364 | **0.007** |
| Dispersant | 0.152 | 0.696 | – | – | 6.919 | **0.009** |
| Oil/dispersant | 7.364 | **0.007** | 6.919 | **0.009** | – | – |
| **WAF exposures** | | | | | | |
| *C. delta* | | | | | | |
| Oil | – | – | 14.127 | **0.000** | 4.788 | 0.029 |
| Dispersant | 14.127 | **0.000** | – | – | 3.651 | 0.056 |
| Oil/dispersant | 4.788 | 0.029 | 3.651 | 0.056 | – | – |
| *Paramuricea* B3 | | | | | | |
| Oil | – | – | 46.594 | **0.000** | 8.695 | **0.003** |
| Dispersant | 46.594 | **0.000** | – | – | 25.770 | **0.000** |
| Oil/dispersant | 8.695 | **0.003** | 25.770 | **0.000** | – | – |
| *L. glaberrima* | | | | | | |
| Oil | – | – | 65.367 | **0.000** | 45.871 | **0.000** |
| Dispersant | 65.367 | **0.000** | – | – | 6.077 | **0.014** |
| Oil/dispersant | 45.871 | **0.000** | 6.077 | **0.014** | – | – |

### 3.2. Exposure effects on *C. delta*

#### 3.2.1. Oil treatment

There was no complete fragment mortality in the control or bulk-oil treatments (Fig. 1B). However, one *C. delta* replicate in the Low oil–WAF died by the end of the exposure (Fig. 2B). The Kruskal–Wallis test showed no significant differences among the 96-h health ratings across all concentrations of bulk and WAF oil ($p > 0.05$).

#### 3.2.2. Dispersant treatment

*C. delta* showed a decline in health in the High dispersant (Fig. 1E), though complete fragment mortality was not observed during the 96 h assay. The Kruskal–Wallis test revealed significant differences ($p < 0.05$) in health rankings, with the High dispersant showing a significantly greater decline in health than the Medium and Low concentrations ($p < 0.05$).

During the WAF exposures, 75% of *C. delta* fragments died in the Low dispersant, 25% in the Medium and 75% in the High dispersant after 96 h (Fig. 2E). Control fragment health was significantly higher relative to all concentrations of dispersant ($p < 0.05$).

#### 3.2.3. Oil/dispersant treatment

Coral fragments also showed a decline in health within the High oil/dispersant treatment (Fig. 1H), but again complete fragment mortality was not observed. Significant differences were detected between nubbins in the High oil/dispersant relative to the control samples ($p < 0.05$).

During the DE–WAF exposures, mortality was observed in one colony in the Medium concentration and three of the four colonies in the High concentration (Fig. 2H). A Kruskal–Wallis test revealed significant health differences among treatments, with the Medium and High DE–WAF treatments significantly lower than the controls ($p < 0.05$), and the High DE–WAF also significantly lower than the Low treatment ($p < 0.05$).

**Table 2**
K–M means for time-to-event estimates for three coral species: *C. delta*, *Paramuricea* type B3 and *L. glaberrima*, in bulk-oil (a) and WAF (b) exposures using a Mantel-Cox Log-rank analysis. The event was a decline in health rating to: a) 3 or below, b) 1 or below.

| Species | Treatment | Survival estimate | Std. error | 95% confidence interval Lower bound | Upper bound |
|---|---|---|---|---|---|
| **(a) Bulk exposure** | | | | | |
| *C. delta* | Bulk-oil | 90.3 | 1.88 | 86.6 | 94.0 |
| | Dispersant | 93.5 | 1.31 | 90.9 | 96.0 |
| | Oil/disp. | 90.5 | 1.77 | 87.1 | 94.0 |
| | Overall | 91.4 | 0.95 | 89.6 | 93.3 |
| *Paramuricea* B3 | Bulk-oil | 91.9 | 2.25 | 87.5 | 96.3 |
| | Dispersant | 91.0 | 2.04 | 87.0 | 95.0 |
| | Oil/disp. | 87.6 | 2.50 | 82.7 | 92.5 |
| | Overall | 90.2 | 1.27 | 87.7 | 92.7 |
| *L. glaberrima* | Bulk-oil | 95.4 | 0.57 | 94.3 | 96.6 |
| | Dispersant | 96.0 | 0.00 | 96.0 | 96.0 |
| | Oil/disp. | 91.0 | 1.71 | 87.7 | 94.4 |
| | Overall | 94.1 | 0.62 | 92.9 | 95.4 |
| Overall | Overall | 92.1 | 0.54 | 91.0 | 93.1 |
| **(b) WAF exposure** | | | | | |
| *C. delta* | Oil WAF | 93.5 | 1.52 | 90.5 | 96.5 |
| | Dispersant | 89.1 | 2.05 | 85.1 | 93.1 |
| | Oil/disp. | 92.3 | 1.60 | 89.1 | 95.4 |
| | Overall | 91.6 | 0.99 | 89.7 | 93.6 |
| *Paramuricea* B3 | Oil WAF | 96.0 | 0.00 | 96.0 | 96.0 |
| | Dispersant | 82.5 | 2.45 | 77.7 | 87.3 |
| | Oil/disp. | 94.5 | 0.88 | 92.8 | 96.2 |
| | Overall | 90.8 | 0.98 | 88.9 | 92.7 |
| *L. glaberrima* | Oil WAF | 96.0 | 0.00 | 96.0 | 96.0 |
| | Dispersant | 77.8 | 2.63 | 72.7 | 83.0 |
| | Oil/Disp. | 86.4 | 2.01 | 82.4 | 90.3 |
| | Overall | 86.4 | 1.23 | 84.0 | 88.8 |
| Overall | Overall | 89.3 | 0.64 | 88.1 | 90.6 |

#### 3.2.4. Comparisons between treatments for C. delta

No significant differences were detected among K–M time-to-event estimates for *C. delta* fragments in all treatments within the bulk-oil series ($\chi=1.72$, df=2, $p=0.422$), with an overall time-to-event (health rating of 3 or less) estimate of 91.4 h (Table 2a, Fig. 3). However, significant differences were detected among treatment estimates in the WAF series ($\chi=12.5$, df=2, $p=0.002$); these differences were between the oil-only and dispersant-only treatments (Table 1). The lowest estimate was 89.1 h in the dispersant treatment relative to the 93.5 h in the oil, and an overall average time-to-event estimate of 91.6 h (Table 2b).

### 3.3. Exposure effects on L. glaberrima

#### 3.3.1. Oil treatment

There was no complete fragment mortality for *L. glaberrima* nubbins in the control, bulk-oil (Fig. 1C) or oil–WAF treatments (Fig. 2C). However, the Kruskal–Wallis test detected significant differences ($p<0.05$) among fragment health ratings in bulk-oil mixtures at 96 h; this difference was due to lower rankings in the Medium oil compared to those in the Low oil ($p<0.05$) and the controls ($p=0.01$), although rankings were similar between the Medium and High oil concentrations.

There was a significant difference among *L. glaberrima* health ratings in the oil–WAF exposure ($p<0.001$); pairwise comparisons revealed that all concentrations of oil had significantly higher health ratings than control fragments ($p\leq0.005$). Ratings in the Medium oil–WAF were also significantly higher than the Low and High ($p<0.05$) oil concentrations.

#### 3.3.2. Dispersant treatment

Whole fragment mortality was not observed for *L. glaberrima* samples, though there was a decline in health within the High dispersant treatment (Fig. 1F). The Kruskal–Wallis test also detected no significant differences among sample health ratings at 96 h ($p>0.05$).

During the WAF exposures, *L. glaberrima* samples in the High and Medium dispersant concentrations were dead by 72 h. By 96 h four of six fragments were also dead in the Low dispersant treatment (Fig. 2F). The Kruskal–Wallis test revealed significantly lower health ratings in all concentrations of dispersant: Low ($p<0.05$), Medium and High ($p<0.005$) relative to controls.

#### 3.3.3. Oil/dispersant treatment

Whole fragment mortality was not observed in the bulk-oil/dispersant mixture (Fig. 1I). The Kruskal–Wallis test and pair-wise comparisons revealed significant health differences between *L. glaberrima* samples in the High and Medium oil/dispersant ($p<0.05$) and between both the High and Medium concentrations relative to the control samples ($p\leq0.01$).

For *L. glaberrima* samples in the DE–WAF, there was complete sample mortality in the High concentration by 72 h, with two of six colonies dead in the Medium DE–WAF (Fig. 2I). Health ratings for nubbins in the control and Low DE–WAF were significantly higher than those in the Medium and High concentrations ($p<0.005$).

#### 3.3.4. Comparisons between treatments for L. glaberrima

The K–M analysis and log rank test revealed significantly different time-to-event estimates for *L. glaberrima* samples ($\chi=7.20$, df=2, $p=0.027$). Pairwise comparisons (Table 1) indicated this difference was between both the oil and dispersant treatments relative to the oil/dispersant mixture, which had the shortest time-to-event estimate of 91.0 h compared to an overall time-to-event estimate of 94.0 h (Table 2a, Fig. 3). Significant differences were also detected among time-to-event estimates in the WAF exposures ($\chi=61.7$, df=2, $p<0.001$) across all treatments. The lowest time-to-event estimate was in the dispersant treatment (77.8 h) with the highest estimate (96 h) in the oil treatment and an overall estimate of 86.4 h (Table 2b, Fig. 3).

### 3.4. Overall comparisons between treatments and concentrations

The Cox regression analysis for the bulk-oil series revealed significant differences ($\chi=57.8$, df=5, $p<0.001$) among rates in health decline (to health-rating 3, ~50% survival) among treatments (control, oil, dispersant and oil/dispersant) and concentrations (zero, low, medium and high). The High concentration significantly increased the hazard of reaching a health rating of 3 or below by 2.5 fold relative to control concentrations, but the Medium concentration did not significantly increase the hazard. Also, relative to controls, samples in the dispersant had an increased hazard risk of 2.3 fold, however the hazard increase in the bulk-oil and oil/dispersant mixture treatments were not significantly different from the control treatment (Table 3).

Similar regression analyses for the WAF exposures also revealed significant differences ($\chi=176.470$, df=7, $p<0.001$) among rates of health decline between treatments and concentrations. Relative to the controls, dispersant significantly increased the hazard of reaching a health rating of 3 or below by 3.4 fold, compared to 4.4 fold in the oil/dispersant treatment; being exposed to oil did not significantly increase the hazard. In addition, the medium treatment concentrations significantly increased the hazard by 1.3 fold relative to the control concentration, whereas the high concentration increased it by 1.6 fold (Table 4).

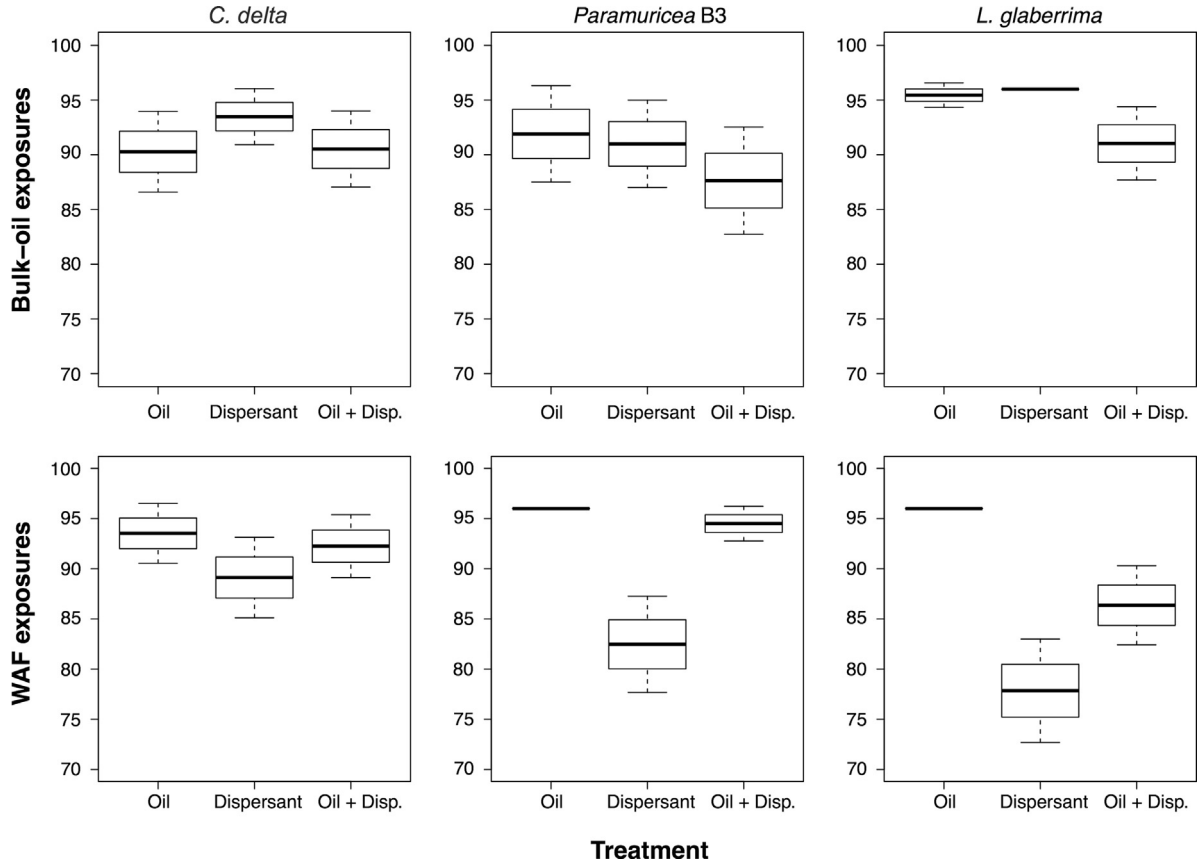


**Fig. 3.** Box-plots showing time-to-event estimates from the Kaplan–Meier survival analysis for coral fragments in three different treatments: oil, dispersant and oil/dispersant. (Top row represents bulk-oil exposures and bottom row represents oil WAF exposures.) The event was a decline in health rating to 3 or below (bulk) and 1 or below (WAF). Box ends represent standard error, line inside the box represents the mean and whiskers represent 95% confidence intervals.

**Table 3**
Log-rank tests of equality on survival distributions for the different levels of concentration in the bulk-oil and WAF exposure series. Significant *p*-values ($p < 0.05$) in bold.

| **Overall log-rank comparisons among concentrations** | | | | |
|---|---|---|---|---|
| Treatment | | Chi-square | df | Sig. |
| **Bulk-oil series** | | | | |
| Control | Log rank (Mantel–Cox) | – | 0 | – |
| Bulk-oil | Log rank (Mantel–Cox) | 0.548 | 2 | 0.760 |
| Dispersant | Log rank (Mantel–Cox) | 15.635 | 2 | **0.000** |
| Oil/dispersant | Log rank (Mantel–Cox) | 21.793 | 2 | **0.000** |
| **WAF–oil series** | | | | |
| Control | Log rank (Mantel–Cox) | – | 0 | – |
| WAF–oil | Log rank (Mantel–Cox) | 1.190 | 2 | 0.552 |
| Dispersant | Log rank (Mantel–Cox) | 33.246 | 2 | **0.000** |
| Oil/dispersant | Log rank (Mantel–Cox) | 20.061 | 2 | **0.000** |

### 3.5. Comparisons between species

Significant differences between K–M survival estimates were detected within the dispersant treatment as well as the control treatment during species comparisons for the bulk-oil series ($p < 0.05$; Table 5). The lowest time-to-event estimate of 90.3 h was for *Paramuricea* type B3 compared to an overall time-to-event estimate of 94.4 h. However, adding species into the Cox regression model did not improve fit, as species' rate of decline comparisons were not significantly different.

In the WAF exposures, these differences ($p < 0.001$) were also detected among species in the oil and oil/dispersant treatments. The lowest time-to-event estimate in oil was for *Paramuricea* type B3 at 68.8 h relative to 75.9 h for *L. glaberrima*, 85.2 h for *C. delta* and an overall time-to-event estimate of 76.3 h. *Paramuricea* type B3 (64.3 h) and *L. glaberrima* (65.3 h) had similarly low time-to-event estimates in the oil/dispersant treatment relative to 93.5 h for *C. delta* and an overall time-to-event estimate of 73.1 h. The Cox regression model also indicated that overall, *L. glaberrima* did significantly worse than *C. delta* by 1.3 fold but decline rates for *Paramuricea* type B3 were not significantly different from those of *C. delta* (Table 4).

## 4. Discussion

All three deep-sea coral species examined showed more severe declines in health in response to dispersant alone and the oil–dispersant mixtures than the oil-only treatments. The experiments reported here are the first ever to investigate the effects of oil and dispersant exposure on live, cold-water corals collected from the deep sea. Impacted corals have been observed at multiple sites in the deep GoM (Fisher et al., 2014), some covered with floc linked to oil from the Macondo well explosion (White et al., 2012). However, the unprecedented application of chemical dispersants in the deep-sea may have contributed to the observed pattern of impact. This exposure series provides crucial insight into the toxicological impacts of oil and dispersant release on three species of long-lived, habitat forming corals.

**Table 4**
Predictor variables in Cox regression analysis and calculated hazard ratios of the odds of reaching health-ratings of interest ($\leq 3$) for the Bulk-oil and WAF–oil exposure series. Significant differences based on Wald test statistics; Low concentration is not present because values are constant or linearly dependent. Hazard ratios were calculated relative to the control water treatment, 0 mg/L concentration and the species *C. delta*, respectively. Significant *p*-values ($p < 0.05$) in bold.

| Variable | Cox regression variables | | | | | |
|---|---|---|---|---|---|---|
| | Level | Wald | df | *p* | Hazard ratio | Standard error |
| **Bulk-oil** | | | | | | |
| Treatment | Treatment | 14.908 | 3 | **0.002** | – | – |
| | Bulk-oil | 0.011 | 1 | 0.918 | 0.963 | 0.355 |
| | Dispersant | 7.067 | 1 | **0.008** | 2.322 | 0.317 |
| | Oil/disp. | 0.123 | 1 | 0.725 | 1.133 | 0.367 |
| Concentration | Concentration | 19.279 | 2 | < **0.001** | – | – |
| | Medium conc. | 0.272 | 1 | 0.602 | 0.833 | 0.350 |
| | High conc. | 10.795 | 1 | **0.001** | 2.500 | 0.279 |
| Species | Species | 0.745 | 2 | 0.689 | – | – |
| | *Paramuricea* B3 | 0.013 | 1 | 0.908 | 1.027 | 0.231 |
| | *L. glaberrima* | 0.645 | 1 | 0.422 | 1.212 | 0.239 |
| **WAF–oil** | | | | | | |
| Treatment | Treatment | 95.263 | 3 | < **0.001** | – | – |
| | WAF–oil | 0.026 | 1 | 0.872 | 0.957 | 0.276 |
| | Dispersant | 27.407 | 1 | < **0.001** | 3.404 | 0.234 |
| | Oil/disp. | 41.745 | 1 | < **0.001** | 4.417 | 0.230 |
| Concentration | Concentration | 10.977 | 2 | **0.004** | – | – |
| | Medium conc. | 3.912 | 1 | **0.048** | 1.342 | 0.149 |
| | High conc. | 10.977 | 1 | **0.001** | 1.608 | 0.143 |
| Species | Species | 15.701 | 2 | < **0.001** | – | – |
| | *Paramuricea* B3 | 1.807 | 1 | 0.179 | 0.817 | 0.137 |
| | *L. glaberrima* | 4.617 | 1 | **0.032** | 1.342 | 0.151 |

**Table 5**
Log-rank tests on equality of survival distributions for all species in the bulk-oil and WAF exposure series. Significant *p*-values ($p < 0.05$) in bold.

| Overall log-rank comparisons among species | | | | |
|---|---|---|---|---|
| **Treatment** | | **Chi-square** | **df** | ***p*-Value** |
| **Bulk-oil series** | | | | |
| Control | Log rank (Mantel–Cox) | 11.222 | 2 | **0.004** |
| Bulk-oil | Log rank (Mantel–Cox) | 2.496 | 2 | 0.287 |
| Dispersant | Log rank (Mantel–Cox) | 6.622 | 2 | **0.036** |
| Oil/dispersant | Log rank (Mantel–Cox) | 5.709 | 2 | 0.058 |
| **WAF–oil series** | | | | |
| Control | Log rank (Mantel–Cox) | 60.353 | 2 | < **0.001** |
| WAF–oil | Log rank (Mantel–Cox) | 7.556 | 2 | 0.023 |
| Dispersant | Log rank (Mantel–Cox) | 2.584 | 2 | 0.275 |
| Oil/dispersant | Log rank (Mantel–Cox) | 22.22 | 2 | < **0.001** |

Regarding the components of the bulk-oil and WAF mixtures, hydrocarbon concentrations are likely an overestimate, given crude oil's variable and complex composition, containing thousands of compounds differing in hydrophobic and hydrophyllic tendencies (Clark Jr. and Brown, 1977; Singer et al., 2000; Di Toro et al., 2007). Dispersants also contain a variety of polar and non-polar surfactants and solvents (Singer et al., 1996). It is highly probable that there was adhesion of oil and dispersant constituents to the mixing flasks used during serial dilutions, as well as to experimental vials. Moreover, loss of water-accomodated oil fractions may have occurred through coalescence and surfacing throughout the exposure period (particularly in the bulk-oil exposure), volatilization during aeration, and/or biodegradation from the microbial communities associated with coral tissues (Couillard et al., 2005). Thus, it is difficult to determine the precise concentrations of oil and dispersant that each coral fragment may encounter at any given time during the course of the experiment but clearly actual exposures were lower than target values, making our results conservative estimates of the effects of oil, dispersant and oil/dispersant mixtures on deep-sea corals. Indeed, similar trends in health decline were observed within each treatment for all three species during four separate experimental trials.

The goal of this experiment was not to reproduce the exact conditions encountered by deep-water corals during the DWH spill, but rather to provide experimental evidence of their sensitivity to various concentrations of oil and dispersant. Reproducing exact conditions encountered by deep-water corals during the DWH spill is challenging because oil, dispersant and seawater mixtures form complex multiphase systems; an organism may then be exposed to many components of the oil and dispersant in various forms (National Research Council, 1989; Langevin et al., 2004). It is also important to note that corals within the vicinity of the DWH may have been exposed to these pollutants for longer than 96 h. Long-term exposures may see additional effects but were not feasible due to the time limitations of experimenting at sea. There is also a low survival rate when transporting deep-sea corals back to laboratory aquaria; *C. delta and L. glaberrima* only survive for approximately 1–3 months, whereas we have had no success keeping *Paramuricea* type B3 or *P. biscaya* alive over the long-term.

All three species of corals did surprisingly well in the oil treatments compared to the dispersant and oil/dispersant treatments (Figs. 1 and 2). In some cases, the corals appeared healthier in both the bulk-oil and oil–WAF treatments relative to the controls (e.g. *C. delta* and *L. glaberrima*, Figs. 1 and 2). Although corals can be negatively impacted when covered by oil particulates or floc (White et al., 2012), it is also possible that corals are deriving some form of nutrition from hydrocarbon components, a process that is likely to be mediated by their associated microbial communities. Anecdotal evidence for this linkage comes from the finding of at least one species of octocoral (*C. delta*) with increased abundances around natural hydrocarbon seeps (Quattrini et al., 2013). Previous studies of shallow-water octocorals also revealed non-selective hydrocarbon uptake of dispersed oil droplets into the gastrovascular cavity of the coral during water uptake (Cohen et al., 1977). Since additional food sources were not supplied during the exposure experiments, and most coral fragments within the oil treatments were frequently observed with a higher degree of polyp extension, similar uptake of dispersed oil components might have occurred.

Although our present study suggests MASS crude oil was not toxic over the range of concentrations tested in these experiments (Figs. 1 and 2), the effect of oil exposure on corals may be dependent on life-history stage. Crude oil (from the Macondo well) exposures of scleratinian coral larvae induced mortality within 24 h, while reducing settlement capabilities and post-settlement survival (Goodbody-Gringley et al., 2013). This suggests an increased vulnerability for coral planulae larvae and juvenile stages, although there was an influence of larval size on exposure tolerance. Other studies have shown premature ejection of planula larvae after exposure to water-soluble fractions of Iranian crude oil (Loya and Rinkevich, 1979) and sub-lethal oil damage to the female reproductive systems of scleratinian corals (Rinkevich and Loya, 1979). Similar sub-lethal impacts may have been imposed on cold-water corals exposed to oil released from the DWH disaster, although these effects may not be manifested for a number of years.

Treatments containing dispersants in both exposure experiments were the most toxic to the corals and induced the highest degree of overall fragment mortality (Figs. 1 and 2). As dispersants tend to increase the surface area of oil–water interactions, they may cause increased toxicological effects to marine organisms (Chandrasekar et al., 2006; Goodbody-Gringley et al., 2013). However, in the WAF exposure series, dispersant-only solutions were

more lethal than the oil/dispersant mixture treatments (as compared to the bulk-oil exposure series), though both treatments resulted in some mortality (Figs. 1 and 2). Toxicity of dispersants is typically attributed to membrane disruption and impairment via surface-active compounds (Abel, 1974; National Research Council, 1989). Exposure results in increased permeability of biological membranes, loss of total membrane function and/or osmoregulation (Benoit et al., 1987; Partearroyo et al., 1990). Although Corexit 9500A was created in an attempt to reduce the toxicity of its predecessors while increasing effectiveness for dispersing more vicous oils, studies have shown that exposure effects are similar to older formulations, Corexit 9527 and 9554 (Singer et al., 1991, 1995, 1996), which are now considered toxic to a variety of marine organisms.

The results from this toxicological assay suggest that dispersant addition during the ensuing cleanup efforts following the DWH spill may have caused more damage to cold-water corals than the initial release of crude oil into the deep sea. Dispersants were toxic at the higher concentrations tested here, and dispersed oil solutions proved to be more toxic than untreated oil solutions (Figs. 1 and 2), as has been found in previous studies (Epstein et al., 2000; Mitchell and Holdway, 2000; Shafir et al., 2007; Bhattacharyya et al., 2003; Milinkovitch et al., 2011; Rico-Martinez et al., 2013). The ability of different types of dispersants to emulsify petroleum hydrocarbon components into the water column as well as the relative toxicity of the dispersants and crude oil, contribute to the overall toxicity of each solution (Epstein et al., 2000). The dispersant and oil/dispersant treatments were lethal to all three species in this study, particularly in the WAF exposure series where dispersant concentrations were higher.

It has been observed in several toxicology studies that dispersant additon increases the total concentration of polycyclic aromatic hydrocarbon (PAH) components in surrounding water (Couillard et al., 2005; Hodson et al., 2007). Specifically, it increases the concentration of less water-soluble high-molecular-weight PAHs, some of which induce enzymatic activity (i.e. cytochrome P4501A) that can metabolize PAHs into toxic forms causing a variety of detrimental effects (Henry et al., 1997; Billiard et al., 1999; Couillard et al., 2005). This could explain the more rapid decline in health for coral fragments exposed to the bulk-oil/dispersant and oil–WAF/dispersant mixtures, where it was likely that a larger proportion of crude oil compounds were made biologically available (Couillard et al., 2005; Schein et al., 2009). Larval exposure experiments on two species of shallow-water scleractinian corals, using BP Horizon source oil and Corexit 9500A, showed a significant decrease in survival and settlement in dispersant solutions and oil–dispersant mixtures, with complete mortality after exposure to 50–100 ppm solutions of dispersant (Goodbody-Gringley et al., 2013). In larvae of hard and soft coral species exposed to dispersants and Egyptian crude oil, all dispersant treatments were more toxic than the oil-only treatments with the highest toxicity observed in oil-dispersed solutions, which also resulted in abnormal development and tissue degeneration (Epstein et al., 2000).

Despite these results, it is unclear whether short-term exposures to oil and dispersant have long-term effects. Following brief (~24 h) exposures to Arabian crude oil or dispersed-oil (with Corexit 9527), there were no significant long-term effects on the yearly in situ skeletal growth of shallow water, hermatypic corals in the genus *Diploria* and *Acropora* (Dodge et al., 1984; LeGore et al., 1989). Though variability in growth rates during that year were not measured, similar experiments using a different scleractinian coral, *Porites furcata*, did reveal reduced growth in exposed fragments relative to controls (Birkeland et al., 1976). This indicates that although short exposure to oil and dispersant may not be lethal to these corals, additional sub-lethal impacts are possible, the extent of which need to be investigated further.

Oil transport to benthic sediments likely occurred through a variety of pathways after the DWH spill, including direct particulate sinking and absorption into marine snow (Passow et al., 2012). Exposure to oil-filled particulates may be more damaging to corals then the dissolved hydrocarbon components when additional stressors are present. As viscous particulates, such as flocs, settle onto benthic communities, the unavoidable exposure imposes many risks (Montagna et al., 2013) including the suffocation of sessile organisms. Floc was likely trapped in the mucous of corals (White et al., 2012) and may have also triggered the excretion of excess mucus in an attempt to remove the debris. This is an energetically costly mechanism, which may lead to reduced health when coupled to additional environmental stressors (Crossland et al., 1980; Riegl and Branch, 1995).

In conclusion, exposure to relatively high concentrations of crude oil does not appear to be as lethal to these species of deep-sea corals as dispersant and mixtures of hydrocarbons and dispersant. However, it is possible that a longer exposure to sub-lethal oil concentrations may cause adverse effects that could not be observed in this short-term toxicological assay. Further examination into the relative effectiveness of different types of dispersants, coupled to examinations of their relative toxicity, is required. To improve future response efforts, alternative methods of oil cleanup are needed, and caution should be used when applying oil dispersants at depth, as it may induce further stress and damage to deep-sea ecosystems.

## Author contributions

Conceived and designed the experiments: DMD, DVR-R, IBB, EEC. Performed the experiments: DMD, DVR-R, IBB. Analyzed the data: DMD, IBB. Wrote the paper: DMD, IBB, EEC.

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# Chapter 26
# Impacts of the Deepwater Horizon Oil Spill on Marine Mammals and Sea Turtles

**Kaitlin E. Frasier, Alba Solsona-Berga, Lesley Stokes, and John A. Hildebrand**

**Abstract** The Gulf of Mexico (GOM) is one of the most diverse ecosystems in the world (Fautin et al. PLoS One 5(8):e11914, 2010). Twenty-one species of marine mammals and five species of sea turtles were routinely identified in the region by the end of the twenty-first century (Waring et al. NOAA Tech Memo NMFS NE 231:361, 2015), a decrease from approximately 39 species prior to intensive exploitation (Darnell RM. The American sea: a natural history of the Gulf of Mexico. Texas A&M University Press, College Station, TX, 2015). Life histories of these megafauna species range from hyperlocal residence patterns of bottlenose dolphins to inter-ocean migrations of leatherback turtles. All species are subject to direct and indirect impacts associated with human activities. These impacts have intensified with major development and extraction efforts since the 1940s. The *Deepwater Horizon* (DWH) oil spill represents a new type of injury to this system: Unlike previous large oil spills, it not only exposed marine megafauna to surface slicks, it also involved an unprecedented release of dispersed oil into deep waters and pelagic habitats, where effects are difficult to observe and quantify. This chapter synthesizes the research conducted following the DWH oil spill to characterize acute and chronic offshore effects on oceanic marine mammals and sea turtles. Marine mammals and sea turtles were exposed to unprecedented amounts of oil and dispersants. Local declines in marine mammal presence observed using passive acoustic monitoring data suggest that the acute and chronic population-level impacts of this exposure were likely high and were underestimated based on coastal observations alone. These population declines may be related to reduced reproductive success as observed in nearshore proxies. Long-term monitoring of oceanic marine mammals is a focus of this chapter because impacts to these populations have not been extensively covered elsewhere. We provide an overview of impacts to sea turtles and

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coastal marine mammals, but other more thorough resources are referenced for in depth reviews of these more widely covered species.

**Keywords** Marine mammal · Sperm whale · Beaked whale · Dolphin · Passive acoustic monitoring · PAM · Sea turtle · Loggerhead · Kemp's ridley · Megafauna · Bottlenose · Barataria Bay · Mammal · Odontocete · Bryde's whale · Spotted dolphin · *Stenella* · *Kogia* · Echolocation · Visual survey · Nesting · Entanglement · Ship strike · Noise · Airgun · UME · Unusual mortality event · Leatherback, Hawksbill · Stranding · Pinniped · HARP · Mississippi Canyon · Green Canyon · *Sargassum* · Green turtle · Trawl · Skimming · Risso's dolphin · Pilot whale · Tag · Aerial survey · Bycatch

## 26.1 Megafauna of the Gulf of Mexico

The Gulf of Mexico (GOM) supports 5 species of sea turtles and at least 21 species of marine mammals including 1 species of baleen whale, 19 species of toothed whales, and 1 species of manatee (Darnell 2015). Gulf marine mammal species fall into several ecological groups: Shallow-dwelling bottlenose dolphins (*Tursiops truncatus*) inhabit coastal waters including bays, sounds, and estuaries, as well as the broad continental shelf regions extending from the coast out to the shelf break. Atlantic spotted dolphins (*Stenella frontalis*) are also commonly found on the continental shelf. The majority of the marine mammal diversity in the GOM is found at or beyond the shelf break, often hundreds of kilometers offshore. Pelagic deep-diving species include sperm whales (*Physeter macrocephalus*), Gervais' and Cuvier's beaked whales (*Mesoplodon densirostris* and *Ziphius cavirostris*), and *Kogia* species, which execute long foraging dives to depths typically exceeding 200 meters. These species feed at depth, primarily on squid and do not typically exhibit diel foraging patterns. At least 13 species of pelagic, shallower diving delphinids (typical diving depths less than 200 m) are also found in the region. These species feed nocturnally on vertically migrating mesopelagic prey in the deep scattering layer. A single baleen whale species, the GOM Bryde's (*Balaenoptera edeni*) whale, is found in the northeastern GOM near Desoto Canyon (Soldevilla et al. 2017).

All marine mammal species currently known to the northern GOM are also found in other oceans; however, little is known about the migration ranges of Gulf populations or the degree to which they mix with populations in the southern GOM and broader Atlantic. The GOM Bryde's whale is thought to be a distinct and isolated subspecies (Rosel and Wilcox 2014). The GOM sperm whale population also appears to be resident in the area (Waring et al. 2009; Jochens et al. 2008). Sperm whale sightings in the GOM often consist of groups of females and juveniles; therefore, the region is thought to serve as a year-round nursing ground for sperm whales. Large solitary males, which are routinely observed in other oceans, are rarely encountered in the GOM, and tag data has shown that males may travel in and out of the Gulf (Jochens et al. 2008).

Leatherback (*Dermochelys coriacea*) and surface-pelagic juvenile loggerhead turtles (*Caretta caretta*) and green (*Chelonia mydas*), Kemp's ridley (*Lepidochelys kempii*), and Atlantic hawksbill (*Eretmochelys imbricata)* sea turtles are found in offshore waters in the GOM, while larger neritic juvenile and adult turtles are found in the continental shelf and nearshore/coastal waters; inshore areas host juvenile and adult Kemp's ridleys, loggerheads, and greens (Wallace et al. 2010, 2017). All five species are listed as endangered or threatened under the Endangered Species Act of 1973. Hatchlings emerge from nesting beaches and disperse into surface-pelagic developmental habitats in convergence zones, using *Sargassum* communities as a foraging resource that affords protection from predation and potential thermal benefits (Bolten 2003, Witherington et al. 2012, Mansfield et al. 2014). Foraging strategies differ by species, with the adult diet of green turtles dominated by seagrasses and algae, loggerheads feeding upon a broad range of pelagic and benthic invertebrates, hawksbills specializing primarily on sponges, Kemp's ridleys feeding mostly on crabs, and leatherbacks depending on cnidarians (see Bjorndal 1997 for a comprehensive review).

Loggerheads and to a lesser extent Kemp's ridleys and green turtles nest on northern GOM beaches in spring and summer months, although the Kemp's ridley's primary nesting beaches are found in the western Gulf in Tamaulipus and Veracruz, Mexico.

## 26.2 A Context of Chronic Impacts

The *Deepwater Horizon* (DWH) oil spill is one chapter in a long history of direct and indirect anthropogenic impacts on marine mammal and sea turtle populations in the GOM. The primary sources of stressors are summarized below.

### *26.2.1 Direct Harvest*

Exploitation of GOM megafauna dates back to the Maya and Aztecs, who intensively harvested sea turtles and manatees (Lange 1971). Impacts were likely mainly limited to coastal zones until the late 1700s when the American whaling industry reached Gulf waters (Darnell 2015). Whalers primarily targeted sperm whales, with pilot whales as secondary targets, and reported that the waters of the mouth of the Mississippi River constituted one of the most profitable whaling grounds (Reeves et al. 2011). Reports of sightings and takes of "finback" whales taken in the region likely refer to the Gulf Bryde's whale, and these reports indicate that the range of this species included most of the Gulf (Reeves et al. 2011), although the current population appears to be restricted to a small region near Desoto Canyon (Soldevilla et al. 2017).

Sea turtles are threatened by direct harvest both in offshore habitats and on nesting beaches, from egg to adult stages. Adult green turtles were intensively harvested for their meat in the 1880s (Valverde and Holzwart 2017) when landings of that species alone are estimated at 4800 to 6000 animals per year, across the GOM and broader Caribbean (Darnell 2015). Adult loggerheads were harvested in Cuba through the mid-1990s (Gavilan 1998). Harvesting of sea turtles became illegal in the United States under the Endangered Species Act of 1973, and illegal poaching at sea is thought to be rare in the US Gulf of Mexico (NMFS 2011). However, active harvest may still occur outside of the US EEZ. An active illegal trade in hawksbill tortoiseshells persists (NOAA 2013). Poaching of sea turtle eggs from nesting sites continues in the United States and neighboring countries (NMFS 2008, 2011).

### *26.2.2 Shipping and Vessel Strikes*

Commercial shipping has been a major industry in the GOM since the 1850s, when the port of New Orleans was the second largest in the country (Darnell 2015). In 2016, GOM ports accounted for nearly 50% of total tonnage transferred through American ports (US Army Corps of Engineers). Both marine mammals and sea turtles are at risk of vessel strikes, and these are likely highly underreported for pelagic species (Williams et al. 2011; Epperly et al. 1996).

### *26.2.3 Anthropogenic Noise*

Offshore human activities also affect megafauna through elevated noise levels and pollution. Oil and gas development took hold in the GOM in 1947 (Darnell 2015), expanding rapidly thereafter. In 2018 over 50 thousand wells and 7 thousand drilling platforms were documented in the GOM (BOEM 2018). Seismic surveys using explosive sound sources (airguns) are used to map subsurface oil and gas deposits. These surveys are nearly continuous in the GOM, and they combine with shipping noise to make average low-frequency ambient noise levels very high in the GOM relative to levels in other ocean regions (Wiggins et al. 2016). Noise is considered a chronic stressor for marine mammals because these species rely on sound to interpret their environment and communicate with one another (e.g., Wright et al. 2007).

### *26.2.4 Debris Entanglement, Ingestion, and Bycatch*

Commercial fishing efforts in the GOM expanded after WWII, adopting novel technologies including purse seines, longlines, trawls, and gillnets, which increased the occurrence of marine mammal and sea turtle entanglement in fishing gear (Lutcavage

et al. 1997), as well bycatch rates and competition for prey species (Darnell 2015). Sea turtles and marine mammals are incidentally caught and killed in trawl, gillnet, hook-and-line, and longline fishing gear, and fishery bycatch is considered the most serious global threat to marine megafauna (Lewison et al. 2004; Wallace et al. 2010, 2013).

Marine mammals and turtles are affected by entanglement in gear and marine debris, with possible effects including injury and drowning (Walker and Coe 1989; Plotkin and Amos 1990). Loggerhead, green, hawksbill, and Kemp's ridley sea turtles take refuge as juveniles in *Sargassum* rafts and are particularly susceptible to entanglement in trash and ingestion of plastics (Witherington et al. 2012).

### *26.2.5 Oil and Gas Development*

Oil and gas development in the GOM is linked to a variety of chronic impacts including occasional small- and large-scale spills (Asl et al. 2016; SERO n.d.), leaking infrastructure, chemical releases related to extraction activities (Neff 1990; Neff et al. 2011a, b), persistence of weathered oil and related compounds in the environment (Van Vleet and Pauly 1987; Botello et al. 1997; Van Vleet et al. 1984), increased vessel activity, and the construction and removal of offshore platforms (Gitschlag et al. 1995). Some amount of natural crude oil seepage also occurs from an estimated 914 natural seep zones in the GOM (MacDonald et al. 2015). Oil from small-scale releases and seeps weathers and spreads in the pelagic ecosystem, accumulating in offshore convergence zones. These zones, which aggregate drifts of *Sargassum* and other macroalgae species, act as vital habitats for surface-pelagic juvenile turtles, putting them at particular risk for exposure to oil accumulating in these zones (Witherington et al. 2012; Bolten 2003).

### *26.2.6 Nesting Beach Impacts*

Turtle populations have susceptibilities related to their reliance on nesting beaches, which are impacted by coastal development, beach erosion, light pollution, dredging, beach re-nourishment programs and armoring, climate change and sea level rise, as well as native and exotic predators (Lutcavage et al. 1997). Current efforts to protect nesting beaches and rescue nests began in some areas as early as the 1950s, and successful nesting beach conservation efforts can result in rapid local population increases (Troëng and Rankin 2005; Hayes 2004).

### *26.2.7 Habitat and Environmental Degradation*

Other major chronic impacts to both marine mammals and turtles include hypoxia in the Mississippi River outflow region, which affects prey quality and density in a previously rich foraging ground for GOM megafauna, as well as harmful algal blooms (Magaña et al. 2003). Direct impacts to turtle habitats including loss of nesting beaches, seagrass beds, and coral reefs, are primarily associated with coastal and continental shelf zones. The same suite of chronic impacts that affect GOM marine mammal and sea turtle health may also have some effect on the health and quality of their prey.

## 26.3 Quantifying Impacts on Pelagic Species/Stages and Habitats

Although occasional reports of various direct impacts to marine mammals and sea turtles including entanglements and ship strikes are reported, they are likely highly underreported (Williams et al. 2011; Epperly et al. 1996) because they occur offshore and may go undetected. Carcasses are unlikely to strand following pelagic deaths, particularly in regions or seasons where higher water temperatures accelerate decomposition (Nero et al. 2013). Equally difficult to quantify are the cumulative effects of chronic impacts such as pollution, noise, and prey depletion, since these effects likely accumulate gradually at sublethal levels over many years.

One possible indicator of chronic stress is the occurrence, intensity, and length of unusual mortality events (UMEs). A UME is an unexpected stranding event that represents a significant die-off in a marine mammal population. From 1990 to 2104, there were 12 UMEs in the GOM (Litz et al. 2014) with recovered carcass counts ranging from 31 to 1141 animals and lasting from 1 to 52 months (Resources NOoP 2018). Coastal bottlenose dolphins are the predominant species in the stranding record, likely because the bodies of this nearshore species are far more likely to reach beaches. The proximate cause of the majority of these UMEs is typically determined to be morbillivirus, biotoxins, and/or cold weather. However, the causes of some events remain unknown, as in the case of the largest, longest-lasting event that occurred from 2012 to 2014 with 1141 recovered carcasses. The occurrence of these events suggests population-level immunodeficiencies (Di Guardo and Mazzariol 2013) or other susceptibility in populations which are already experiencing chronic stress.

Turtle populations also experience mass stranding events such as cold stunning events (Milton and Lutz 2003). In January 2010, unusually cold temperatures resulted in a cold stunning event of unprecedented magnitude involving over 4500 sea turtles (primarily green turtles) in Florida (Avens et al. 2012). As in the case of marine mammals, chronic stress associated with anthropogenic activity may be acting to decrease overall resilience of turtle populations (Lamont et al. 2012).

One particularly observable anthropogenic impact is the loss of historic nesting beaches to coastal development projects. Female sea turtles generally exhibit high site fidelity to particular nesting beaches, and the availability of natural, undeveloped beaches is dwindling. It is unclear how many nesting beaches may have previously existed in the northern GOM. A 2006 study in the Caribbean estimated that 20% of historic (twentieth century) green and hawksbill nesting beaches in the Caribbean had been lost and that 50% of the remaining beaches were visited by fewer than ten nesting females (McClenachan et al. 2006). Beaches in the northern GOM today have minimal sea turtle nesting relative to the Atlantic regions (except for Kemp's ridleys), although previous nesting density is largely unknown (Hildebrand 1982; Valverde and Holzwart 2017). Currently, most nesting beaches in the US GOM are in Florida and Texas (see Valverde and Holzwart 2017 for a thorough account of nesting sites and habitats). However, most Kemp's ridleys found in US waters hatched on Mexican beaches; thus, conservation measures require an international perspective.

The majority of the existing data on population-level effects of oil spills on marine megafauna comes from shallow water spills and their effects on coastal populations. A long-term study of the effects of the Exxon Valdez spill on killer whale populations indicates that two pods suffered acute losses during the event (33% and 41% of their members) and had not returned to pre-spill numbers nearly 20 years later (Matkin et al. 2008). One of the two exposed pods appeared to be headed for extinction at the conclusion of the study (Matkin et al. 2008). Evidence suggests that lipophilic chemical contaminants are often offloaded from mother to calf in marine mammals, including bottlenose dolphins (Irwin 2005) and killer whales (Ylitalo et al. 2001), leading to higher levels of calf mortality.

### *26.3.1 Study Methods: Pre-DWH*

Marine mammal and sea turtle populations in the offshore GOM have historically been quantified in the offshore GOM using shipboard and aerial visual surveys. Aerial continental shelf surveys began in 1979, initially conducted by the USFWS and then by NMFS. Offshore marine mammal surveys were conducted by NMFS in the spring or summer of 1990–1994, 1996–2001, 2003–2004, and 2009 (Waring et al. 2015; Mullin and Fulling 2004; Mullin 2007) (Fig. 26.1). Major survey efforts in the early to mid-1990s (GulfCet study, supported by the Minerals Management Service) focused on the continental slope region (100–2000 m) and recorded both marine mammals and turtles over eight surveys in all seasons (Würsig et al. 2000). Despite these survey efforts, few species had enough sightings to produce robust population size estimates, and none could be analyzed for long-term population trends due to low precision and infrequent assessments. As a result, little baseline information was available on population trends prior to the DWH spill. The only cetaceans with adequate population data were bottlenose dolphins resident in

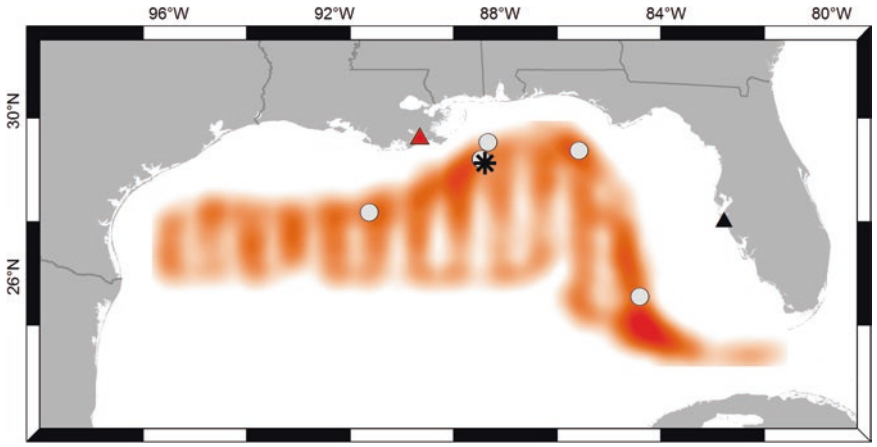


**Fig. 26.1** Oceanic monitoring effort in the GOM. Colormap indicates high visual survey effort (red) to low visual survey effort (white) during deepwater (>200 m target depths) NOAA and NOAA-affiliate shipboard surveys 1992–2014. Gray dots indicate GOM HARP monitoring sites, 2010–2017. Asterisk is site of DWH, and triangles are locations of bottlenose dolphin studies in Barataria Bay (red) and Sarasota Bay (black)

selected bays and estuaries, where high-resolution monitoring was based on mark-recapture analysis (Wells 2014).

Offshore turtle surveys using aerial and shipboard methods have limitations because small turtles are difficult to detect and identify using these methods, although using satellite tags to monitor diving behavior to account for sightability may reduce uncertainty in estimates (Thomas et al. 2010; Seminoff et al. 2014). Beach counts of nesting females and clutch sizes are a reliable census method (Schroeder and Murphy 1999), but this approach only surveys adult females nesting in a given year (nesting cycles differ between species). Satellite systems and drones are now being adopted to survey sea turtles, and satellite tags are being used to track their movement over large distances (Rees et al. 2018).

### *26.3.2 Study Methods: Post-DWH*

In response to the DWH event, numerous additional studies were initiated in the GOM to understand potential impacts from the spill. The longest-term marine mammal study was the GOM High-frequency Acoustic Recording Package (HARP) program based on passive acoustic monitoring of marine mammal sounds (Hildebrand et al. 2017); an 8 + −year broadband passive acoustic monitoring effort was initiated at five locations in the GOM in 2010 (Fig. 26.1). Three deepwater monitoring locations included a site in Mississippi Canyon near the DWH wellhead (site MC), an eastern site at Green Canyon outside of the DWH surface oil footprint (site GC), and a southern site outside of the oil footprint near the Dry Tortugas (site DT).

HARPs were maintained nearly continuously at these representative oil-exposed and oil-unexposed monitoring sites to detect marine mammal sound production as a proxy for animal presence across the region. Marine mammal species and genera were then distinguished in the long-term passive acoustic recordings based on the characteristics of their sounds. Species monitored include sperm whales, Cuvier's and Gervais' beaked whales, Risso's dolphin, and pygmy/dwarf sperm whales (*Kogia* spp.), delphinids in the genus *Stenella* (Atlantic spotted, pantropical spotted, spinner, striped, and Clymene dolphins), and blackfish (primarily short-finned pilot whales).

Bottlenose dolphin health assessments began in 2011 in Barataria Bay, Louisiana (Schwacke et al. 2013). Health metrics from resident bottlenose dolphins in Barataria Bay, which was heavily oiled by the DWH event (Michel et al. 2013), were compared to an unexposed reference population in Sarasota Bay, Florida (Fig. 26.1).

Shorter-term marine mammal studies following the DWH event included low-frequency passive acoustic monitoring for Bryde's whales on the west Florida shelf in 2010 and 2011 (Rice et al. 2014), satellite tagging efforts aimed at understanding sperm whale distributions (Mate unpublished data), and short-term passive acoustic monitoring near the DWH wellhead site (Ackleh et al. 2012). In the absence of long-term data, various population modeling efforts were also undertaken to try to estimate population-level effects and recovery times based on assumed vital rates (Farmer et al. 2018; Ackleh et al. 2018; Schwacke et al. 2017).

In an effort to mitigate the impact of the spill on sea turtle nesting beaches, 25,000 Kemp's ridley and loggerhead eggs were transported from beaches in the GOM to the Atlantic coast in Florida (Inkley et al. 2013). Following the DWH spill, transect searches were conducted in convergence zones within the spill area to rescue oceanic juvenile sea turtles and to document species composition and oiling status (McDonald et al. 2017). Aerial surveys were conducted on the continental shelf throughout the northern GOM to the 200-m isobath between April and September 2010, documenting the distribution and abundance of neritic sea turtles throughout the DWH spill area (Garrison 2015).

Estimates of the probabilities of oil exposure for sea turtles present within the area of the spill were generated from direct observations of surface-pelagic juvenile sea turtles (Stacy 2012) and satellite-derived surface oil distributions (Wallace et al. 2017). Abundance and source populations for impacted turtles were predicted using ocean circulation and particle tracking simulation models, estimating that 321,401 green, loggerhead, and Kemp's ridley turtles were likely within the spill site, originating primarily from Mexico and Costa Rica (Putman et al. 2015).

## 26.4 Acute Effects

We consider acute effects of the DWH oil spill on marine megafauna as those effects that caused immediate harm during the spill and response. This section focuses primarily on effects experienced in oceanic habitats, as coastal impacts have been reviewed extensively elsewhere. Primary acute effects include immediate injury and death from oil and chemical exposure, response activities, and strandings.

### *26.4.1 Oil Exposure: Marine Mammals*

In an oil spill, marine megafauna can be exposed to oil and related compounds through surface slicks when breathing or resting at the air/sea interface (Trustees 2016) and through interaction with subsurface plumes during dives and foraging events. Oil compounds can be taken up through the skin, breathed into the lungs, or ingested with prey (Schwacke et al. 2013). Exposure studies conducted in the 1970s focused on polar species including pinnipeds, sea otters, and polar bears and found a range of effects including eye and skin lesions associated with continued exposure, uptake, distribution and accumulation of oil compounds into body tissues and fat reserves through oil ingestion (Englehardt 1977; Engelhardt 1982), and thermoregulation problems associated with oiling. Marine mammals and turtles were observed in oil-impacted areas during the DWH spill response while the well remained uncapped (Wilkin et al. 2017).

There is limited prior information on the effects of oil spills on marine mammal populations. Early oil spill studies noted that a wide range of marine mammal species including baleen whales, dolphins, and pinnipeds did not appear to avoid oil-contaminated waters (Goodale et al. 1981; Spooner 1967; Geraci 1990a, 1990b) despite the fact that captive bottlenose dolphins could detect and avoid oil in experimental settings (Geraci 1990a). In the case of the DWH oil spill, the acoustic record shows little evidence for near-term avoidance of the wellhead area by marine mammals (see Sect. 26.5).

Marine mammals were exposed to oil at the sea surface but also likely at depth. Deep-diving pelagic species including sperm whales and beaked whales forage at depths of 1000 m or more and likely interacted with the deep plume which formed at approximately 1100 m (Hildebrand et al. 2012). The deep plume formation is largely attributed to dispersant use (Kujawinski et al. 2011) and has not been reported in previous spills; therefore, it likely represents a new route of exposure for deep-diving cetaceans. A large amount of released oil did not reach the surface and likely was eventually deposited on the seafloor (Romero et al. 2017). GOM marine mammal species are not typically benthic foragers; however, bay, sound, and estuary bottlenose dolphins may use benthic hunting tactics (e.g., Lewis and Schroeder 2003; Rossbach and Herzing 1997), which could increase their exposure to deposited oil. In addition, GOM Bryde's whales appear to forage at or near the sea floor

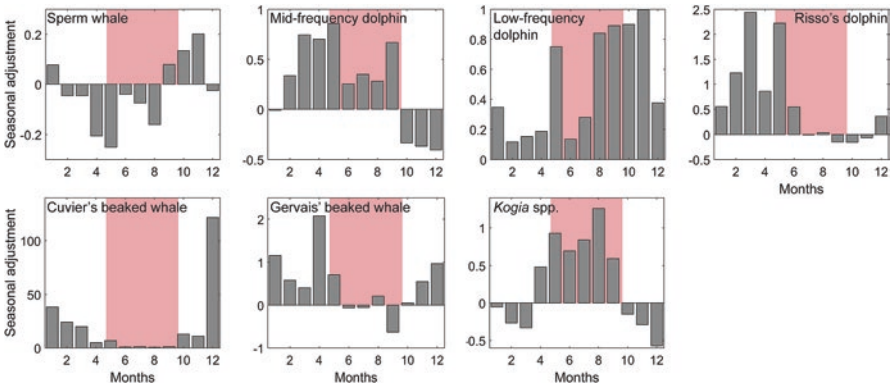


**Fig. 26.2** Seasonal patterns in marine mammal presence at a passive acoustic monitoring site in Mississippi Canyon, located approximately 10 km from the DWH wellhead. The vertical axis indicates the factor by which seasonal presence varies relative to mean presence. Higher values indicate stronger seasonality

(Soldevilla et al. 2017) and, therefore, may also be at risk of exposure to oil deposited in sediments.

Differences in seasonal presence likely played a role in the extent to which marine mammal species were directly exposed to DWH oil and dispersants (Fig. 26.2). Sperm whales, Gervais' beaked whales, *Kogia* species, and mid-frequency dolphins (in the GOM this group primarily consists of species in the *Stenella* genus, categorized based on echolocation click peak frequencies) are found year-round in the region of the oil spill and were likely directly exposed to oil. In particular *Kogia* and mid-frequency delphinid species presence increases in the summer months, increasing the likelihood of exposure to DWH oil and dispersants. Risso's dolphins are seasonally present in spring through summer and therefore likely experienced direct exposure during the first months of the spill but less exposure as the summer progressed. In contrast, Cuvier's beaked whale presence is strongly seasonal near the wellhead with highest occurrence during winter months; therefore, these populations likely experienced minimal direct exposure during the spring spill.

### *26.4.2 Oil Exposure: Sea Turtles*

Potential direct impacts to sea turtles from an oil spill differ depending on the life stage, but all stages are vulnerable to acute toxicity from volatile contaminants, exposure through inhalation and ingestion, physical impairment from heavy oiling, and a variety of physiological and clinicopathological impacts of exposure (see review in Shigenaka 2003). Sea turtles are unlikely to detect oil (Odell and MacMurray 1986), and in experimental conditions they showed no avoidance

behavior (Lutcavage et al. 1995). They are continuously exposed by resurfacing to breathe (Milton et al. 2003), and pelagic juveniles are susceptible to floating tar accumulations in ocean convergence zones due to indiscriminate feeding patterns (Witherington 2002; Lutcavage et al. 1997).

In laboratory studies, juvenile loggerheads were adversely affected by short-term exposures to oil in almost all aspects of physiology (e.g., respiration, diving patterns, energy metabolism, salt gland function, oxygen transport, blood chemistry, and red and white blood cell count) (Lutcavage et al. 1995; Lutz et al. 1986). In sea turtles, oil clings to eyes and nares and causes skin to slough off leaving inflamed soft skin exposed to infection (Lutcavage et al. 1995). Skin lesions and necrosis were observed in leatherback oil exposure studies, and skin returned to normal appearance approximately 1 month after the turtles were removed from oil (Lutcavage et al. 1995). Following the Ixtoc 1 oil spill, necropsied sea turtles were found to have to ingested large amounts of oil, with indications that the ingestion was eventually lethal (Hall et al. 1983). Effects of oil ingestion in loggerheads dying from oil exposure in the Canary Islands include esophageal impaction, necrotizing dermatitis and gastroenteritis, and necrotizing hepatitis (Camacho et al. 2013).

During the DWH spill, live oiled turtles admitted for rehabilitation exhibited abnormalities including relatively severe metabolic and osmoregulatory derangements resulting from a combination of stress, exertion, exhaustion, and dehydration related to oiling, capture and transport (Stacy et al. 2017). Mortalities were examined for evidence of internal exposure to polycyclic aromatic hydrocarbons (PAHs) and dispersant component dioctyl sodium sulfosuccinate (DOSS) (Ylitalo et al. 2017). Visibly oiled turtles had higher concentrations of PAH than unoiled turtles, which may suggest low-level exposure from other sources, and DOSS levels were below the limit of quantitation in almost all samples (Ylitalo et al. 2017).

Transect searches conducted in convergence zones during rescue operations following the DWH spill documented 937 oceanic juvenile Kemp's ridley, green, loggerhead, and hawksbill turtles in the spill area, and 81% of those captured were visibly oiled (McDonald et al. 2017). Based on these observations, turtle density calculations, and spatial extent of the oil, the total number of pelagic-stage sea turtles exposed to DWH oil was estimated at 402,000, with 54,800 of these heavily oiled, although the majority of the dead turtles were believed to be unobserved and therefore unaccounted for in these estimates (McDonald et al. 2017). Researchers estimated an overall mortality of 30% for oceanic turtles within the footprint of the spill in addition to those presumed dead from heavy oiling (Mitchelmore et al. 2017). Dependence on floating *Sargassum* for shelter and food in convergence zones where oil and tar accumulate makes surface-pelagic turtles particularly vulnerable to ingesting oil and tar (Witherington 2002; Witherington et al. 2012). Stranding data indicated that sea turtle stranding rates were at record levels in 2010 and 2011, increasing as much as 5× after the spill (NMFS data).

Kemp's ridley's principal foraging habitat is in the northern GOM (Seney and Landry Jr 2008; Shaver et al. 2013). Stable isotope analyses conducted on nesting Kemp's showed that 51.5% of turtles sampled had evidence of oil exposure (Reich et al. 2017), indicating that the primary foraging grounds in the northern GOM were

contaminated by oil and that Kemp's ridleys continued to forage in these areas after the spill. Loggerhead foraging sites characterized through satellite tracking demonstrated an overlap with the oil spill footprint, with 32% of tracked individuals taking up year-round residence in the northern GOM foraging habitats (Hart et al. 2014). Stable isotope analysis confirmed that loggerheads returned to the oiled area and did not change foraging patterns after the spill, increasing their risk of chronic exposure to oil and dispersants (Vander Zanden et al. 2016).

Declines in reproductive parameters of loggerheads in the northern GOM were reported (Lamont et al. 2012), although the decline could not be linked directly to the DWH spill. Observed declines in nesting may have been partly due to reduced prey availability and therefore an inhibited ability to allocate resources required for nesting. Colder temperatures in 2010 may have delayed or reduced nesting activity or suppressed the ability of turtles to reach breeding condition (Chaloupka et al. 2008; Lauritsen et al. 2017; Weishampel et al. 2010; Hawkes et al. 2007).

### *26.4.3 Response Activities*

Surface skimming and burning of oil slicks during the DWH disaster response may have impacted an unquantified number juvenile turtles living in *Sargassum* (McDonald et al. 2017). Up to 23% of important *Sargassum* habitat was estimated as lost as a result of oil exposure (Trustees 2016).

Response activities related to cleanup efforts, such as mechanical beach cleaning of oiled sand with heavy machinery and the associated disturbance from noise and artificial lighting, impacted sea turtle nesting habitats in the northern GOM (Michel et al. 2013). Enhanced vessel activity and physical barriers (e.g., booms) in nearshore waters may have affected nesting activity as well (Lauritsen et al. 2017). Loggerhead nesting densities in 2010 in northwest Florida were 43.7% lower than expected based on previous data, and an estimated 250 loggerhead nests were lost due to DWH response activities on nesting beaches (Lauritsen et al. 2017).

### *26.4.4 Dispersants*

In addition to being exposed to oil, marine mammals and sea turtles were also exposed to dispersants. Impacts of exposure to dispersants or dispersants in conjunction with oil are not well known, as there are few studies for marine mammals and sea turtles. Since oil itself is generally toxic and can be lethal, dispersants may improve short-term survival of marine megafauna by reducing formation of oil slicks, decreasing the probability of heavy oiling, and accelerating the initial degradation of released oil (Neff 1990).

The ramifications of the unprecedented release of high volumes of dispersant chemicals as part of the DWH spill response are widely unknown. Evidence for

cytotoxicity and genotoxicity of Corexit 9527 and Corexit 9500, the two dispersant chemicals used during the DWH spill, to sperm whale skin cells has been demonstrated in a laboratory setting (Wise et al. 2014). These findings were consistent with cytotoxicity and cell survival studies using Corexit 9500 in human and rat cells (Bandele et al. 2012; Zheng et al. 2014); however, Corexit 9527 was found to be less cytotoxic to whale cells than reported for other species' cells. Cytotoxicity may lead to acute effects, while genotoxicity is expected to lead to delayed effects associated with genetic mutations in somatic and/or germ cells. Mutations in somatic cells from toxic exposure may be associated with cancer in exposed marine mammals (Gauthier et al. 1999), while mutations in germ cells are inherited by offspring.

Effects of dispersants on sea turtles are largely unknown, but dispersants have the potential to interfere with lung function, digestion, and salt gland function (Shigenaka 2003). In an exposure study that investigated the effects of crude oil, dispersant, and a crude oil/dispersant combination on loggerhead hatchlings, significant differences between treatment and nonexposed controls were detected in multiple blood chemistry parameters (Harms et al. 2014). Electrolyte imbalances and hydration challenges were worst in the combined oil/dispersant group, and the failure to gain weight was noted in dispersant and combined exposed hatchlings (Harms et al. 2014). Only one heavily oiled Kemp's ridley showed evidence of DOSS at detectable concentrations (Ylitalo et al. 2017). Recent studies have demonstrated that DOSS degrades more rapidly in surface conditions than under deepwater conditions (Campo et al. 2013; Batchu et al. 2014), suggesting that DOSS exposure was minimized in surface-pelagic turtles (Ylitalo et al. 2017).

### *26.4.5 Mortality Events*

The 2010 marine mammal UME which began prior to the DWH spill complicated measurement of the fatalities from the DWH event itself. It is now thought that the UME was not caused by the spill but was aggravated and potentially prolonged and expanded by the event (Venn-Watson et al. 2015; Antonio et al. 2011). An exponential increase in sea turtle and cetacean mortality was reported beginning 38 days after the initial blowout (Antonio et al. 2011). The relationship between observed strandings and unobserved offshore mortality is difficult to assess, but it has been estimated that strandings accounted for at most 6.2% of the total dead marine mammals in the GOM following the DWH oil spill, depending upon the species (Williams et al. 2011). This study relied on highly uncertain population estimates and mortality rates but strongly suggests that stranded carcass counts are not an adequate means to estimate the total mortality. Similarly, sea turtle carcass stranding rates likely represent a fraction of total at-sea mortality, as carcasses are likely to sink prior to detection (Epperly et al. 1996). Winds, surface currents, and sea temperatures can bias stranding sites with respect to offshore source mortality locations (Nero et al. 2013).

## 26.5 Long-Term Effects

We consider long-term effects of the DWH oil spill on marine megafauna as those occurring after the initial response and cleanup period, extending months to years after the event.

### *26.5.1 Findings of Marine Mammal Passive Acoustic Studies*

The GOM HARP project (Frasier et al. 2017; Hildebrand et al. 2015) provides the only long-term time series documenting marine mammal occurrence in oiled and unoiled oceanic habitats during and after the DWH oil spill. Data collected between 2010 and 2016 are discussed here. Mean weekly presence was calculated for each species (or species group) as the weekly average of time per day in which echolocation clicks were detected (Table 26.1). The seasonal component was removed from the weekly presence time series using a monthly seasonal trend decomposition procedure (Cleveland et al. 1990). Long-term trends in deseasoned mean weekly presence were then estimated for each site and species combination using a Theil-Sen regression (Table 26.2) with 5–95% confidence intervals obtained using a bootstrap method. The median slope across 500 pairs of points selected randomly with replacement within each time series was computed 100 times.

On average across the monitoring period, presence of sperm whales (Fig. 26.3) was substantial at the site adjacent to the wellhead (MC, 36.8% of 5-min time windows detected their presence; Table 26.2), slightly less at site GC (13.8% of time windows), and low at site DT (5.1% of time windows). Long-term trend estimates suggest a slow decline in mean presence of sperm whales at site MC (5 ± 1% annual reduction), between 2010 and 2016, and a greater decline at site GC (8% ±2% annual reduction). A possible slight increase in the presence of sperm whales was found at site DT (5 ± 5% annual increase); however, encounter rates were low and seasonally variable at this southern GOM location.

**Table 26.1** Mean weekly marine mammal presence (as percentage) including [5th, 95th] percentiles at passive acoustic monitoring sites in the GOM HARP study, 2010–2016

| Site | Sperm whale | *Kogia* spp. | Cuvier's BW | Gervais' BW | Risso's dolphin | *Stenella* delphinid | Blackfish delphinid |
|---|---|---|---|---|---|---|---|
| MC | 36.8 [11.6, 64.2] | 0.5 [0.0, 1.4] | 0.1[a] [0, 0.6] | 0.3[a] [0, 1.1] | 1.3 [0, 5.7] | 6.3 [0.5, 16.0] | 0.6 [0, 2.0] |
| GC | 13.8 [0.2, 38.4] | 0.3 [0.0, 0.9] | 0.1 [0, 0.5] | 0.5 [0.0, 1.1] | 0.2 [0, 1.1] | 3.0 [0.2, 9.0] | 0.4 [0, 1.8] |
| DT | 5.1 [0, 19.5] | 0.1 [0.0, 0.2] | 3.6 [0.8, 7.2] | 1.5 [0.3, 3.9] | 4.5 [0, 23.4] | 3.1 [0.0, 9.4] | 0.4 [0, 2.1] |

[a]Indicates subset from 2010 to 2013 was used to calculate the mean

**Table 26.2** Estimated average annual percent change in marine mammal presence including [5th, 95th] confidence intervals at passive acoustic monitoring sites in the GOM HARP study, 2010–2016

| Site | Sperm whale | *Kogia* spp. | Cuvier's BW | Gervais' BW | Risso's dolphin | *Stenella* delphinid | Blackfish delphinid |
|---|---|---|---|---|---|---|---|
| MC | −4.5 [−6.0, −3.3] | 18.8 [12.9, 26.3] | 5.4[a] [2.0, 8.7] | 37.3[a] [24.3, 52.8] | 8.7 [2.1, 19.4] | −1.6 [−3.5, 0.2] | −7.0 [−9.0, −4.6] |
| GC | −8.3 [−10.0, −6.4] | −15.5 [−16.3, −14.8] | 1.0 [−3.0, 6.3] | 4.1 [1.4, 7.3] | −5.0 [−9.1, 0.0] | −10.9 [−12.2, −9.7] | −11.1 [−13.5, −9.3] |
| DT | 5.4 [−0.3, 13.6] | −9.0 [−11.8, −5.4] | −9.7 [−11.0, −8.9] | −8.1 [−9.1, −6.6] | 4.2 [1.4, 8.1] | −13.0 [−14.2, −11.5] | 1.3 [−4.1, 10.2] |

[a]Indicates subset from 2010 to 2013 was used to calculate slope

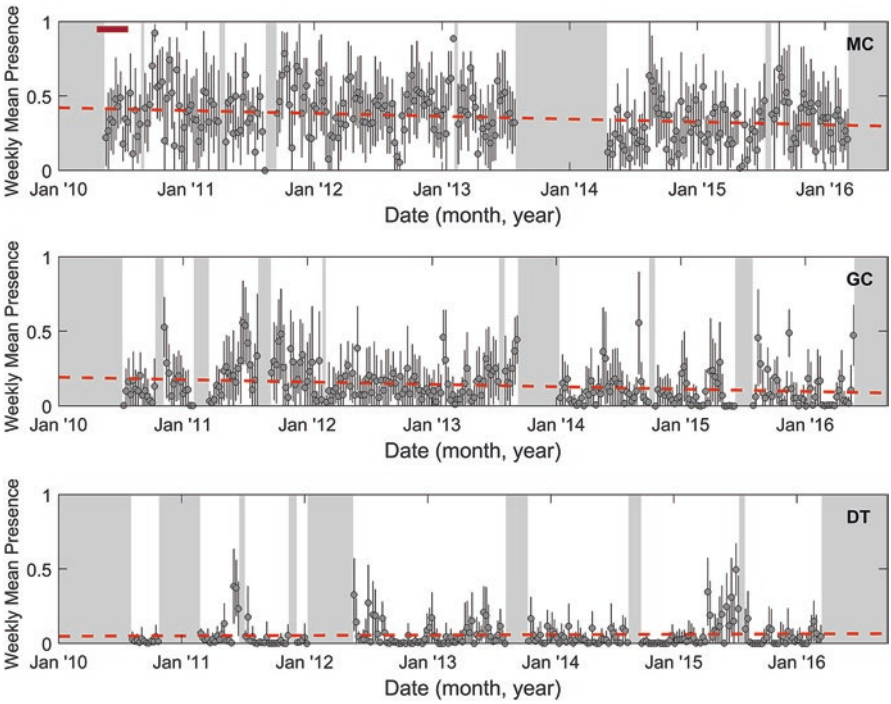


**Fig. 26.3** Sperm whale weekly mean presence (open circles) as fraction of time present at passive acoustic monitoring sites from the GOM HARP study. Error bars indicate standard deviation within each week. Gray rectangles indicate periods without data. Red dashed line indicates estimated trend. Dark red bar on top plot indicates period during which the DWH well remained uncapped

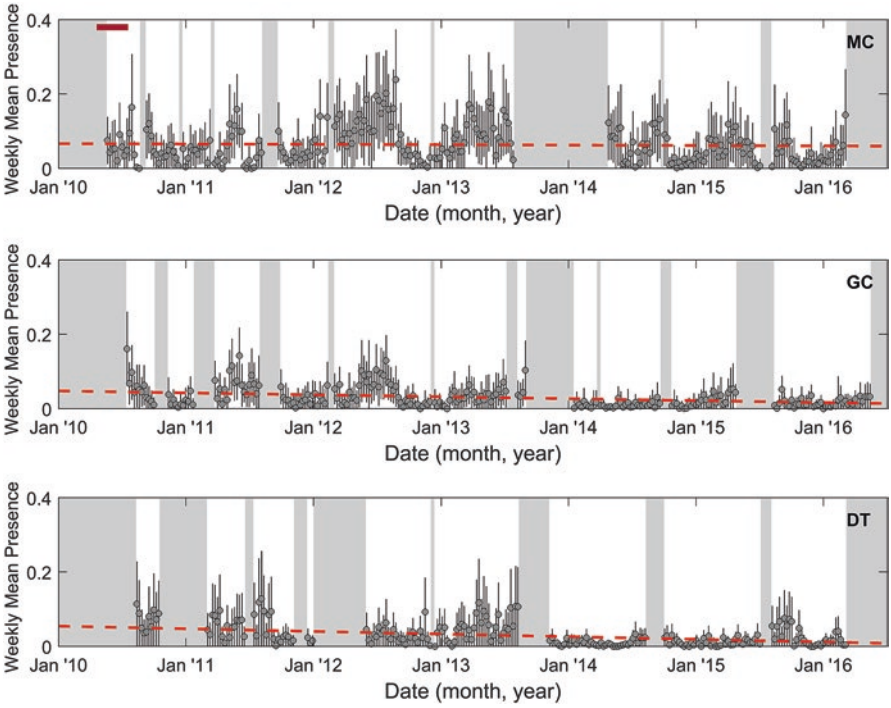


**Fig. 26.4** Weekly mean *Stenella* sp. (mid-frequency delphinid) presence as fraction of time present at passive acoustic monitoring sites from the GOM HARP study. Markings as in Fig. 26.3

*Stenella* species and blackfish (presumably short-finned pilot whales) species were associated with mid- and low-frequency echolocation, respectively. *Stenella* had slightly higher presence at site MC (6.3% for *Stenella*) relative to other sites. Long-term declines in *Stenella* occurrence (Fig. 26.4) were observed at the sites GC (11 ± 1% annual reduction) and DT (13 ± 2% annual reduction) outside of the DWH surface slick footprint, but not at site MC where presence remained nearly constant (2 ± 2% annual reduction). However, relatively higher encounter rates in 2012 may be masking long-term decreases in *Stenella* delphinid presence at site MC. Blackfish presence was low overall (0.4–0.6%), with declines at sites MC and GC (7 ± 2% and 11 ± 2% annual reductions, respectively), but no significant change at site DT (1 ± 6% annual change) (Fig. 26.5). Risso's dolphin presence was low (0.2–4.5%) and strongly seasonal at all sites, and their presence increased slightly at site DT (4 ± 4% annual increase) and more strongly at site MC (9 ± 7% annual increase) (Fig. 26.6). A possible decline in Risso's dolphin presence was found at site GC (5 ± 5% annual reduction) where overall presence was low.

Beaked whale presence was highest at site DT for both Cuvier's (4%) and Gervais' (2%) beaked whale, and both were present year round (Figs. 26.7 and 26.8). Presence of both species declined at site DT (10 ± 1% annual reduction for Cuvier's; 8 ± 1% annual reduction for Gervais'), but remained constant or increased

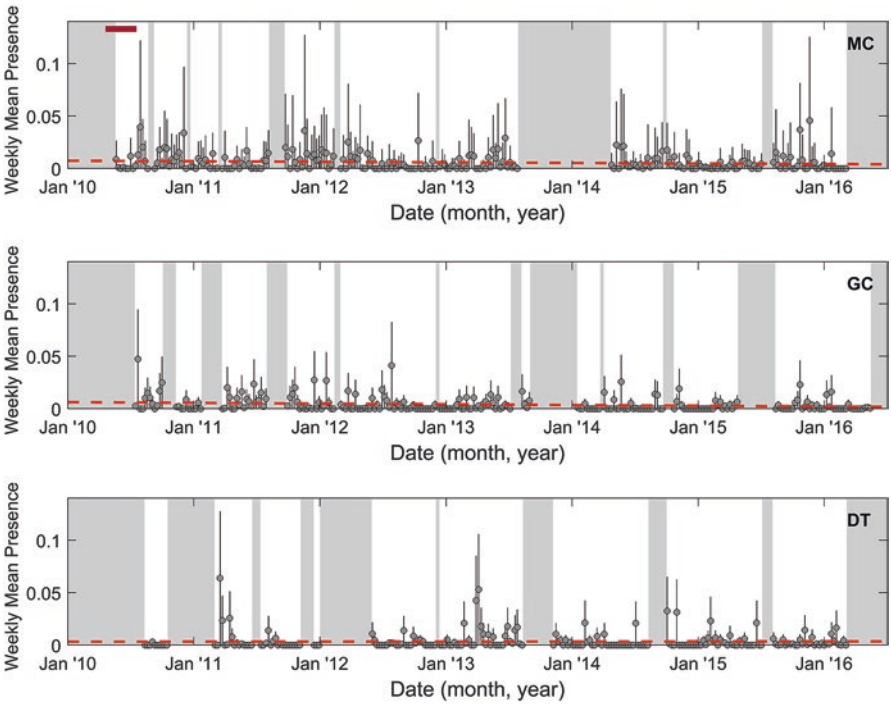


**Fig. 26.5** Weekly mean blackfish (low-frequency echolocation) presence as fraction of time present at passive acoustic monitoring sites from the GOM HARP study. Markings as in Fig. 26.3

at site GC (1 ± 4% annual change for Cuvier's; 4 ± 3% annual increase for Gervais'). An increase in beaked whale presence at site MC is observed; however, analysis for beaked whale presence at this site occurred over a limited date range (2010–2013); therefore, trends are less robust.

*Kogia* spp. presence was relatively high (0.3–0.5% occurrence within a short detection range of <1 km) at site MC and GC (Fig. 26.9). Presence of *Kogia* spp. increased at site MC (19 ± 7% annual increase), but presence at site GC decreased strongly after 2013 resulting in a strongly negative long-term trend in mean presence at this site (15 ± 1% annual decrease). Presence also decreased at site DT (9 ± 3% annual decline), although overall encounter rates at that site were low (0.1%) throughout the monitoring period.

Population movements and declines may be convolved in the trends seen in GOM acoustic monitoring because of the limited number of monitoring locations in the HARP dataset. It is unclear to what degree changes in presence reflect population displacement around the GOM and beyond or rather indicate offshore mortality. Aspects of both processes may be influencing the long-term observed trends.

Population trends may be related to exposure: Based on seasonal trends and encounter rates during the oil spill at site MC, sperm whales, *Stenella*, and *Kogia*

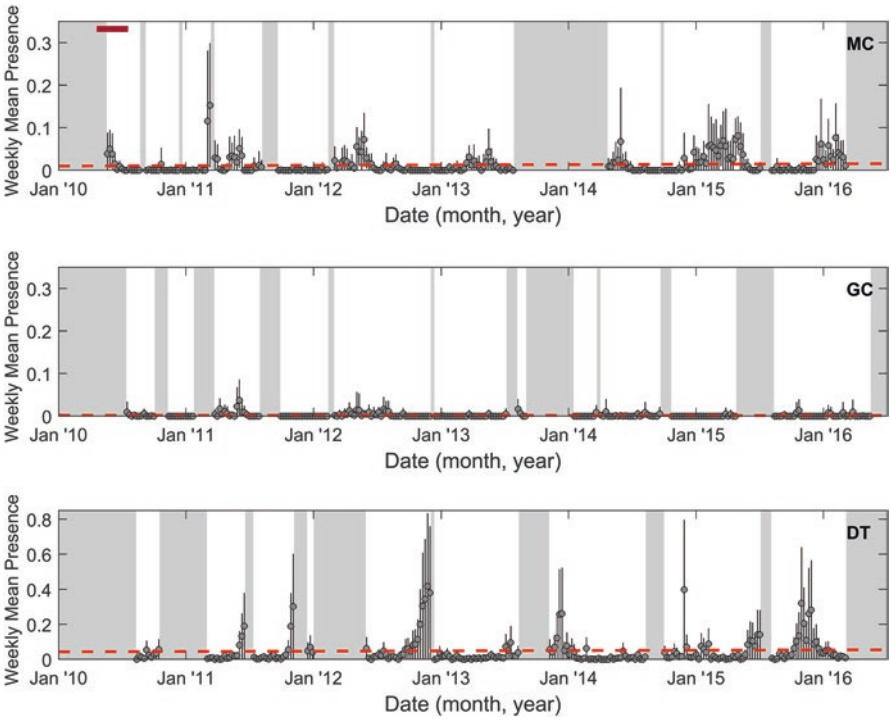


**Fig. 26.6** Weekly mean Risso's dolphin presence as fraction of time present at passive acoustic monitoring sites from the GOM HARP study. Markings as in Fig. 26.3

species are most likely to have interacted with the DWH surface and subsurface footprints for extended periods of time in the spring and summer of 2010. *Stenella* and *Kogia* presence strongly declined at sites GC and DT, *Kogia* presence declined at site MC, and *Stenella* delphinids appear to have declined from 2012 to 2016 following a peak in 2012. Sperm whale presence declined steadily at GC and MC, and while possibly increasing at site DT, this site may not be part of core sperm whale habitat (Jochens et al. 2008) given overall low encounter rates there. Blackfish presence is highly variable at site MC; however, this group appears to have been present during the DWH event based on the acoustic record. Presence of blackfish delphinids has declined at sites MC and GC while remaining approximately constant at site DT. In contrast Risso's dolphins may not have been as strongly exposed to oil in 2010 due to the seasonality of their presence in the northern GOM, and Risso's dolphin encounter rates appear to be increasing at sites MC and DT while decreasing at site GC. Both beaked whale species appear to be declining at site DT, with limited change at site GC. Owing to the seasonality of Cuvier's beaked whales, only the Gervais' beaked whale appears to have been substantially exposed to oil during the DWH spill. The time series for beaked whale presence at site MC may be too short to robustly interpret long-term trends there.

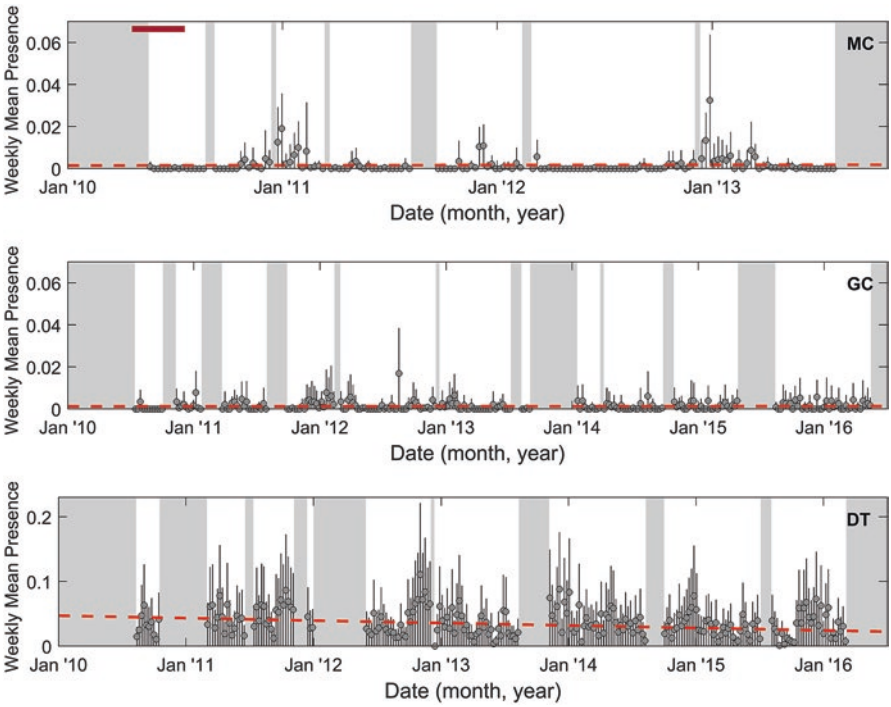


**Fig. 26.7** Weekly mean Cuvier's beaked whale presence as fraction of time present at passive acoustic monitoring sites from the GOM HARP study. Markings as in Fig. 26.3

Population declines in the eastern and southern GOM may be unrelated to the DWH event, since some of the strongest declines are seen at the two sites outside the DWH oil footprint (GC and DT). However, seasonal cycles in the passive acoustic data suggest that these species' distributions shift over time, likely as animals seek out favorable conditions; therefore, many of these pelagic species may not be resident in specific areas throughout the year. The high productivity conditions created by the outflow of the Mississippi River have historically supported higher marine mammal densities than other regions of the GOM (Reeves et al. 2011), and populations may preferentially return to that region. Female sperm whales tagged in the MC region typically had long residence times in the area and appeared to use it as core habitat (Jochens et al. 2008). Declines at other less productive sites may indicate range contraction associated with population-level mortality (Rugh et al. 2010; Worm and Tittensor 2011), or might reflect population shifts in response to other drivers. A broader understanding of migratory patterns on a GOM-wide scale is needed to more confidently interpret site-level trends in the context of the broader GOM ecosystem.

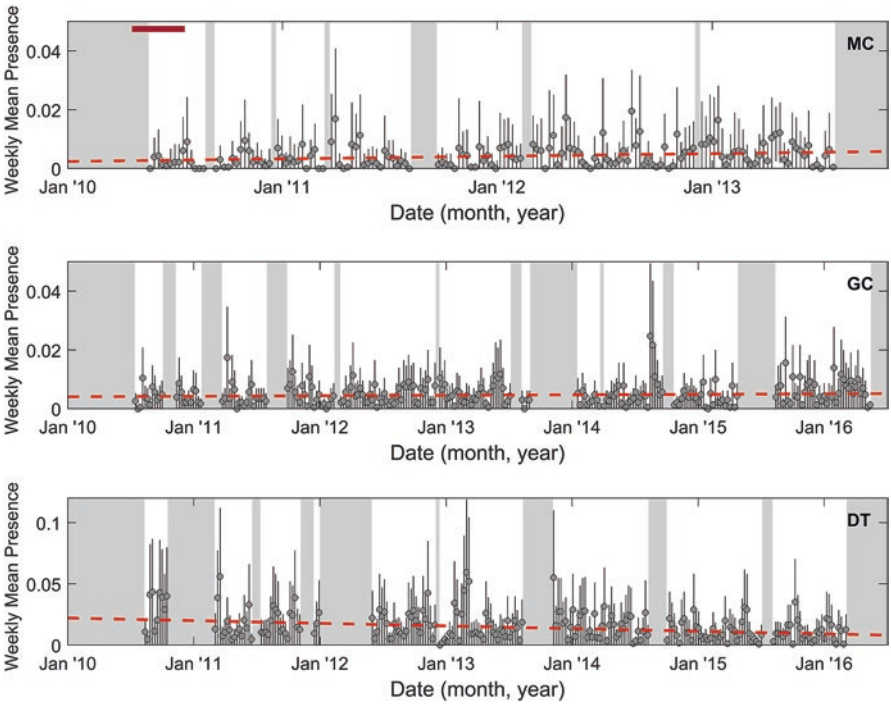


**Fig. 26.8** Weekly mean Gervais' beaked whale presence as fraction of time present at passive acoustic monitoring sites from the GOM HARP study. Markings as in Fig. 26.3

### *26.5.2 Additional Marine Mammal Studies*

Latent effects of exposure have been examined in the case study of the resident Barataria Bay bottlenose dolphin population. Over 5 years after heavy oiling of the bay during the DWH oil spill, successful calving rates were 20% compared to 83% for an unexposed reference population (Lane et al. 2015). It was unclear whether unsuccessful pregnancies were directly caused by oil exposure or were linked indirectly through poor maternal health (Schwacke et al. 2013). Similar reproductive failures occurring in offshore populations (e.g., Farmer et al. 2018) could explain the observed long-term declines in encounter rates at oceanic monitoring locations. Annual survival rates among adults were also lower (86.8%) than in comparable populations (95.1 to 96.2%; Lane et al. 2015). Bottlenose dolphins in Barataria Bay were five times more likely to have moderate to severe lung disease than a reference population (Schwacke et al. 2013).

A study comparing short-term (7–12-day) PAM recordings before and after the DWH spill at a site near the wellhead indicated possible declines in sperm whale occurrence (Ackleh et al. 2012), with an increase of 25 miles from the site. However, due to the high variability in sperm whale presence at fixed monitoring sites on

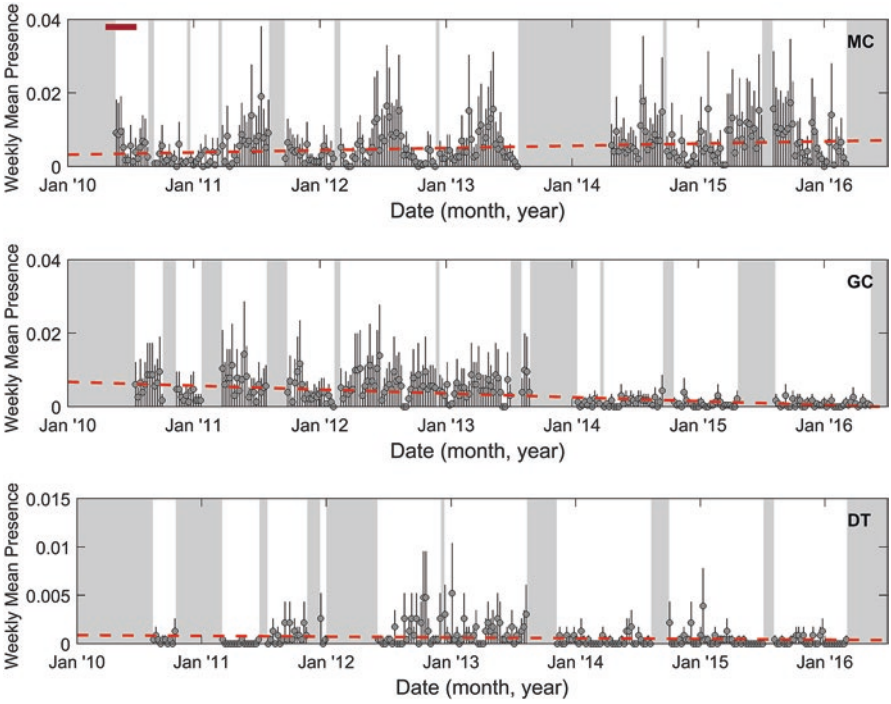


**Fig. 26.9** Weekly mean *Kogia* spp. presence as fraction of time present at passive acoustic monitoring sites from the GOM HARP study. Markings as in Fig. 26.3

weekly timescales (Fig. 26.3), it is not possible to determine whether the difference between the two measurements reflects real change or normal variability.

### *26.5.3 Findings of Sea Turtle Studies*

The long-term effects of oil exposure and the DWH oil spill on sea turtles are not well understood or quantified (Vander Zanden et al. 2016). The number of Kemp's ridley nests in Tamaulipas, Mexico, in 2010 was below predicted levels and has remained below expected levels every nesting season since (Dixon and Heppell 2015), but the reduction has not been definitively attributed to the DWH spill (Caillouet et al. 2016; Caillouet Jr 2014). Some have speculated that the large-scale oiling of *Sargassum* (Hu et al. 2016) and subsequent loss of developmental/foraging habitat for juvenile turtles may have long-term implications for population recovery.

In response to the DWH oil spill, stage-based spatial matrix models have been developed to simulate oil spills to assess the potential impact of oil spills on loggerhead populations, defining oceanic-stage survival followed by fecundity as the

most sensitive parameters for eliciting changes in population growth (Leung et al. 2012). A geospatial assessment of cumulative stressors to evaluate where combined threats and impacts are greatest was conducted on a GOM-wide scale for Kemp's ridleys and loggerheads following the DWH oil spill (Love et al. 2017). This research showed a range of anthropogenic stressors including incidental bycatch in commercial and recreational fisheries and habitat degradation, and it demonstrated that few areas exist in their terrestrial or marine environment without cumulative impacts from multiple stressors (Love et al. 2017).

## 26.6 Remaining Knowledge Gaps

Efforts to assess the comprehensive immediate and long-term effects of the DWH oil spill on pelagic species are limited by a scarcity of pre-disaster baseline data (Bjorndal et al. 2011; Graham et al. 2011; Trustees 2016). Without the ability to compare pre-and post-spill measurements, many potential impacts are unquantifiable. Further, effects on these long-lived species may continue to play out over the coming decades (Schwacke et al. 2017). The assessment of cumulative impacts must be considered on an ecosystem level, as effects are based on direct mortality, degradation of habitat, quality and availability of prey resources, and sublethal impacts such as reduced foraging or reproductive potential (Love et al. 2017). Developing a better understanding of the spatiotemporal overlap of threats with the distribution and abundance of sea turtle populations will guide managers to develop geographically targeted management strategies to mitigate key stressors and restore injured resources (Love et al. 2017).

No comparable long-term data on marine mammal presence were collected in the period prior to the 2010 spill. At best, visual survey data give decadal-scale abundances that cannot be directly applied to understanding the impact of the spill. In addition, the GOM was not a pristine habitat prior to the 2010 spill; therefore, we cannot assume that pre-spill population levels were stable or attribute observed shifts to the DWH event with great confidence. Although a visual marine mammal survey was conducted prior to the spill in 2009 (Waring et al. 2013), it did not provide the kind of spatiotemporal resolution or precise abundance estimates needed to quantify acute impacts. Passive acoustic sensors were deployed 26 days after the initial blowout, so although they did record during the majority of the 152-day spill, recorders were not in place to capture pre-spill levels, and some immediate effects may have been missed. Further, passive acoustic sensors have limited detection ranges (Frasier et al. 2016), and additional research is needed to determine the spatial scale over which the observations from these monitoring locations can be extrapolated. Efforts to estimate chronic effects by any method have necessarily relied on uncertain assumptions regarding pre-spill population sizes, health, and distributions.

Much of the released oil is thought to have been deposited on the seafloor. Little is known about if and how marine megafauna might be interacting with deep water

benthic oil, either directly or via the pelagic food web (see Pulster et al. 2020). Impacts of the spill on mesopelagic and bathypelagic prey availability remains unclear (Fisher et al. 2016). Different prey types likely have differing abilities to metabolize oil-derived compounds. In particular, cephalopods seem less capable of metabolizing polycyclic aromatic hydrocarbons (PAHs) and more likely to bioaccumulate heavy metals than fish (Reijnders et al. 2009). Trace metals are common in crude oil and may further concentrate in weathered oil (Gohlke et al. 2011). Deep-foraging, squid-eating cetaceans including sperm whales and beaked whales may be at higher risk of long-term exposure to oil-related pollutants through their prey. Toxicity of oil and oil-related compounds to marine mammals and sea turtles remains poorly understood.

Lastly, the unknown spatial ranges and movement patterns of most oceanic GOM marine mammal species and sparse habitat use, abundance, and distribution data for sea turtles result in broad uncertainty regarding exposure and long-term impacts of the spill and subsequent environmental pollution on these populations. It remains unclear to what degree observed animals are resident in or systematically return to affected habitats. Without coordinated, international GOM-wide monitoring efforts, it is not possible to determine whether local declines in encounter rates represent population shifts or population decreases.

## 26.7 Conclusion

The majority of research on the effects of oil spills on marine mammals and sea turtles has focused on nearshore species (coastal bottlenose dolphins, killer whales, and pinnipeds), coastal impacts (coastal strandings, sea turtle nesting beaches), and surface oiling. The DWH event was a large-volume oil spill that occurred offshore, with significant subsurface footprint, in poorly understood habitats, and with sparse baseline data. Long-term offshore monitoring suggests ongoing declines in marine mammal presence, which may be related to reduced reproductive success as observed in nearshore proxies. Oceanic species were most heavily and directly impacted by this spill, but discerning the immediate and long-term effects on oceanic populations requires piecing together a patchwork of sparse observations and studies. It is clear however that marine mammals and sea turtles were directly exposed to unprecedented amounts of oil and dispersants and that the acute and chronic population-level impacts of this exposure were likely high and underestimated based on coastal observations.

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Environmental Studies Program and the National Marine Fisheries Service, Southeast Fisheries Science Center. The analyses and opinions expressed are those of the authors and not necessarily those of the funding entities. The data used for this study are archived by the Gulf of Mexico Research Initiative at https://data.gulfresearchinitiative.org/data/R4.x267.180:0011 maintained by the Gulf Research Initiative Information and Data Cooperative.

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# Toxicity of oil and dispersant on the deep water gorgonian octocoral *Swiftia exserta*, with implications for the effects of the Deepwater Horizon oil spill

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ABSTRACT

Benthic surveys of mesophotic reefs in the Gulf of Mexico post Deepwater Horizon (DWH) showed that *Swiftia exserta* octocorals exhibited significantly more injury than in years before the spill. To determine the vulnerability of *S. exserta* to oil and dispersants, 96 h toxicity assays of surrogate DWH oil water-accommodated fractions (WAF), Corexit® 9500 dispersant, and the combination of both (CEWAF) were conducted in the laboratory. Fragment mortality occurred within 48 h for some fragments in the dispersant-alone and oil-dispersant treatments, while the WAF group remained relatively unaffected. The 96 h $LC_{50}$ values were 70.27 mg/L for Corexit-alone and 41.04 mg/L for Corexit in CEWAF. This study provides new information on octocoral sensitivity to toxins, and indicates that combinations of oil and dispersants are more toxic to octocorals than exposure to oil alone. These results have important implications for the assessment of effects of the DWH spill on deep-water organisms.

## 1. Introduction

The Deepwater Horizon (DWH) oil spill was unique compared to historical oil spills, which have almost exclusively occurred at the surface or in shallow depths of nearshore waters, thereby simplifying the fate, transport and exposure pathways of oil to surrounding ecosystems (Peterson et al., 2012). In contrast, the blowout of the Macondo wellhead occurred in deep (1500 m), offshore waters and led to a variety of dispersed phases, including small oil droplets, gas bubbles, insoluble oil-gas mixtures and gas hydrates (Peterson et al., 2012), which led to multiple deep-water plumes (Camilli et al., 2010) and large surface slicks (NOAA, 2016). While half of the oil rose to the sea surface, the rest remained in the deep ocean (McNutt et al., 2012; Bagby et al., 2016).

Efforts to contain the spread of oil included the unprecedented injection of 0.77 million gal of chemical dispersant (Corexit 9527 and Corexit 9500A) directly into the subsurface flow of oil at depth. An additional 1.4 million gal were applied at the surface by airplane and small vessels over the course of two months (Hemmer et al., 2011; Kujawinski et al., 2011; Peterson et al., 2012). It is known that surface dispersants lead to the eventual degradation or dissolution of oil (Kujawinski et al., 2011). Prior to the DWH spill, dispersants had not been applied in deep water; very little is known regarding the efficacy of dispersants applied to oil at depth. Concentrations of dioctyl sodium sulfosuccinate (DOSS), one of the key ingredients of the spill dispersant, were elevated > 1000-fold in surface sediments near the well (White et al., 2014; Bagby et al., 2016). A recent study found that these dispersants persist in subsurface oil-associated environments for up to four years (Kujawinski et al., 2011; White et al., 2014), much longer than previously anticipated.

The Pinnacles Trend Reefs is one of many regions of rocky biogenic structures in the Gulf of Mexico along the outer continental shelf between the Mississippi River and Cape San Blas, Florida that occur in the mesophotic zone (50–150 m; Gittings et al., 1992). These reefs represent drowned remnants of shallow-water coral reefs that formed over 18,000 years ago prior to rises in sea levels (Continental Shelf Associates and Texas A & M University, 2001). Today these reefs host important and diverse ecosystems of invertebrates (Gittings et al., 1992; Continental Shelf Associates, Inc. and Texas A & M University, 2001; Weaver et al., 2002; Lesser et al., 2009) and commercially valuable fish (Dennis and Bright, 1988; Weaver et al., 2002; Silva et al., 2016). Among the dominant invertebrate groups present at the reefs are sessile azooxanthellate, gorgonian sea fans (Continental Shelf Associates, Inc. and Texas A & M University, 2001; Silva et al., 2016). These heterotrophic, suspension feeders rely on the nutritional input from the surface (Sulak et al., 2008) and are therefore especially vulnerable to pollution.

After the DWH event, buoyant oil formed large surface slicks that

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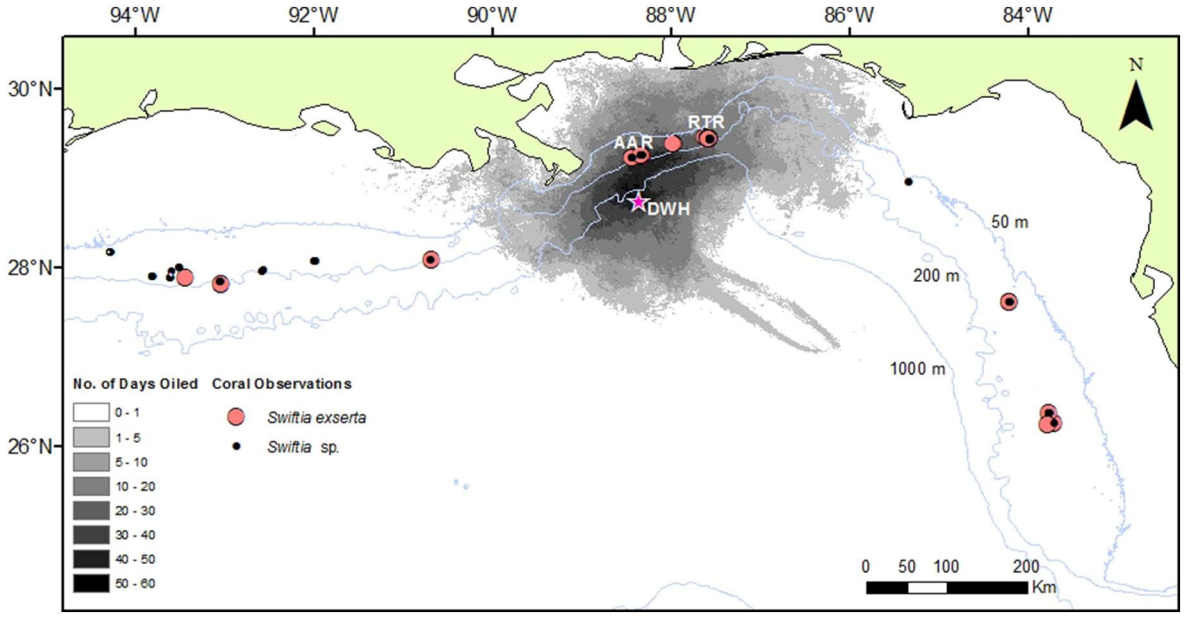


**Fig. 1.** Map of surface oil slick following the DWH spill in the Pinnacles Trend Region, including the Alabama Alps Reef (AAR), and Roughtongue Reef (RTR), two mesophotic reefs located below the slick with documented injuries to *Swiftia exserta* colonies subsequent to the oil spill.

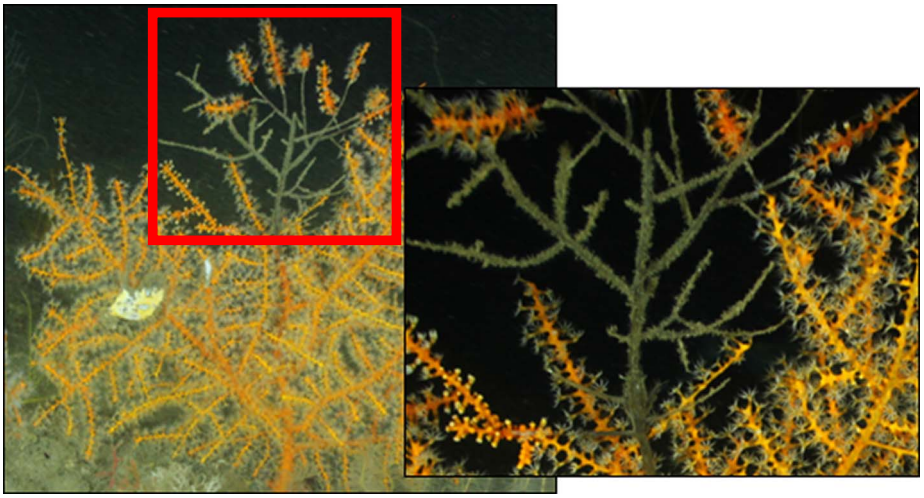

**Fig. 2.** Close-up of unidentified *Swiftia* sp. colony at Alabama Alps Reef, showing large area of dead tissue surrounded by relatively healthy tissue. Polyps also show damage. Image credit: NOAA.

spanned up to 40,000 km$^2$ of the Gulf of Mexico, and dispersed via winds, currents, and tides (NOAA, 2016). Several reefs in the 60–90 m depth range were situated under the oil slick for a period of 24–45 days (Fig. 1; NOAA, 2014; Etnoyer et al., 2016). Post-spill surveys of these reefs showed that large octocoral colonies below the oil slick exhibited significantly more injury than in years before the spill, with 30–50% of large sea fans being injured (Etnoyer et al., 2016; Silva et al., 2016). Other studies in this region found order of magnitude declines in the abundance of demersal reef fish (Sulak and Dixon, 2015), polycyclic aromatic hydrocarbons (PAH) in coral tissues and surrounding sediments (Silva et al., 2016), and traces of DWH oil in sediments and water at nearby reefs (NOAA, 2016).

Isotopic depletion of carbon found in the mesoplankton and other suspended particulate matter suggested that fractions of DWH oil were transported northward in the shallow-water column, and that the oil carbon was transferred at least two trophic levels beyond primary consumers, thereby having substantial effects across the food web (Graham et al., 2010). When oil-derived marine snow becomes part of the pelagic food web, it becomes an important food source for zooplankton, fish and benthic organisms, such as heterotrophic corals (Passow et al., 2012).

Although the presence of oil and dispersant was likely the cause of injury to sea fans in the Pinnacles Trend region, at the time limited information existed on toxicity thresholds for sessile heterotrophic corals, such as gorgonian octocorals. Some studies have shown that oil exposure has detrimental effects on the growth, reproduction, feeding and other behavioral responses of heterotrophic corals (Loya and Rinkevich, 1980; Suchanek, 1993; Epstein et al., 2000; Goodbody-Gringley et al., 2013). In some cases the combination of dispersants and oil has been shown to be more toxic to corals than exposure to oil alone (Nelson-Smith, 1973; Epstein et al., 2000; Goodbody-Gringley et al., 2013; Rico-Martinez et al., 2013; DeLeo et al., 2016).

The primary objective of this study was to examine the acute (96 h) toxicity effects of surrogate DWH oil water-accommodated fractions (WAF), Corexit 9500 dispersant, and the combination of the two, known as a chemically-enhanced WAF (CEWAF), to *Swiftia exserta* (Ellis and Solander, 1786) in the laboratory. *S. exserta*, which occurs throughout the West Atlantic at depths between 10 and 200 m (Goldberg, 2001), is attainable through the aquarium trade. In the Pinnacles Trend Reefs, 40–50% of observed *Swiftia* spp. corals exhibited intermediate to severe injury post-spill, including overgrowth by hydroids, covering by sediment, and broken or bare branches (Fig. 2;

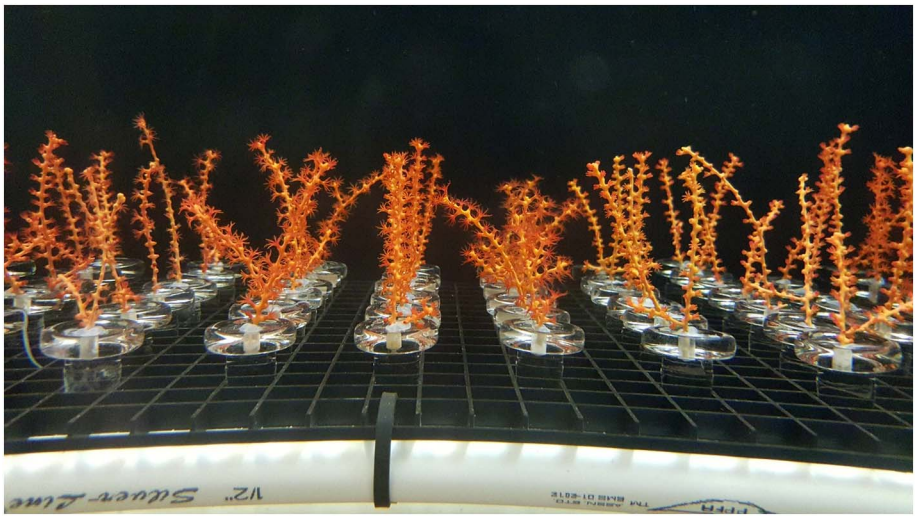

**Fig. 3.** Image of healthy fragments of *Swiftia exserta* in aquaria prior to laboratory toxicity assays.

Etnoyer et al., 2016; Silva et al., 2016).

## 2. Methodology

### 2.1. Coral collection and maintenance

Live *S. exserta* colonies were collected off the southeast coast of Florida at 20–30 m using SCUBA, and shipped to Charleston, SC by Dr. Henry Feddern (Tavernier, FL, USA). Upon receipt, corals were slowly acclimated to seawater [filtered seawater from Charleston Harbor, SC, USA with artificial sea salts to raise salinity (Instant Ocean or Reef Crystals); 36‰; 19 °C] in holding tanks, by replacing 10% of shipping water every 15 min. After approximately 4 h, whole colonies were transferred to a 150-gallon closed-system aquarium composed of a 130-gallon holding tank, small sump, protein skimmer, and small refugium. The refugium contained green macroalgae (*Chaetomorpha* spp.) to filter out nitrates and phosphates. Water quality was maintained using gravel biofiltration and protein skimmers, and continuously monitored. Corals were fed a combination of frozen brine shrimp (Hikari Sales USA, Hayward, CA), rotifers (Hikari Sales USA, Hayward, CA), cyclops copepods (San Francisco Bay Brand, Newark, CA, USA), and microblends of phytoplankton and zooplankton, 3–4 times a week.

### 2.2. Preparation of chemical treatments

#### 2.2.1. Oil water-accommodated fractions (WAFs)

Water-accommodated oil fraction (WAF) and oil-dispersant chemically-enhanced WAF (CEWAF) treatments were prepared using the method of Hemmer et al. (2011) with some modification. Briefly, the full-strength WAF treatment was made in an aspirator bottle with a Teflon stir bar sitting at the bottom. The bottom outlet of the bottle was closed with Tygon tubing and a glass stopper. Seawater (19 L, 36‰) was added to the aspirator bottle and stirred using a magnetic stir plate to create a small vortex. While stirring, 25 g/L of Louisiana Sweet Crude Oil (LSC) was added using a graduated cylinder. The final amount added into the solution was calculated using the difference in weight of the graduated cylinder before and after adding oil.

Stirring speed was then increased to create a vortex reaching approximately 25% of the height of the solution. The solution was stirred for 18 h and allowed to sit for 6 h. The glass stopper was then removed from the Tygon tubing and the solution under the top layer of oil slick was dispensed into a glass collection container. Dilutions of the full-strength solution (100%) were made with seawater, in order to achieve concentrations of 100%, 50%, 16.67%, 5.56%, and 1.85% WAF.

#### 2.2.2. Chemically-enhanced oil water-accommodated fractions (CEWAFs)

The CEWAF treatment was prepared in the same manner as the WAF treatment except for the addition of 1.25 g/L Corexit 9500 in order to achieve a 20:1 oil to dispersant solution. After mixing and stirring, the solution was dispensed into a collection container and dilutions were made with seawater in order to achieve concentrations of 100%, 50%, 16.67%, 5.56%, and 1.85% CEWAF.

#### 2.2.3. Corexit 9500 dispersant-only

To test the dispersant alone, seawater and Corexit 9500 were combined in 1 L glass cylinders to produce five testing concentrations: 100, 50, 25, 12.5, and 6.25 mg/L. The treatments were then poured into 1 L glass beakers.

### 2.3. Toxicity assays

#### 2.3.1. Experimental design and fragmentation

Three short-term (96 h) toxicological assays were conducted in filtered seawater (FSW): (1) crude oil water-accommodated fractions (WAFs), (2) chemical dispersant alone (Corexit 9500A), and (3) the chemically-enhanced WAF (CEWAF). Four *S. exserta* colonies were cut into six smaller fragments of approximately 30–50 polyps and attached with super glue to small glass pegs with a drilled hole in the center (Fig. 3). Within each assay, fragments were placed in one of six groups (control and five experimental treatments). The control group contained seawater alone. Experimental treatments were selected based on preliminary range-finding tests. Each treatment group contained four fragment replicates from four distinct colonies.

#### 2.3.2. Testing conditions

Individual fragments were placed in 1 L glass beakers (one fragment per beaker) containing 1 L of the treatment solution; the beakers were covered with aluminum foil to minimize evaporation. The fragments were kept upright by attaching the glass peg to a small plastic grid set at the bottom of the beaker. Exposures were all static and treatment solutions were not renewed except for the Corexit-alone assay, in which the treatment was renewed every 24 h to maintain dispersant concentrations and allow comparison to $LC_{50}$ values determined for other species using the static-renewal method (DeLorenzo et al., in journal review). Cultures were kept in the dark at 19 °C and 36‰ salinity with aeration.

### 2.4. Health scoring

Coral fragments were photographed and their health was assessed at five time points throughout the assay (0 h, 24 h, 48 h, 72 h and 96 h).

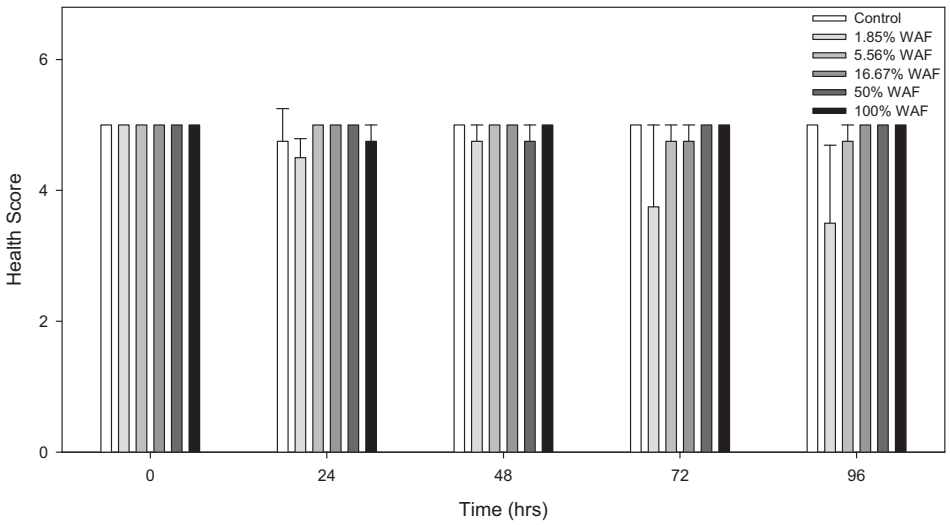


**Fig. 4.** A plot showing results of *Swiftia exserta* exposure to LSC oil water-accommodated fractions (WAF). There was no significant difference among treatments (Kruskal-Wallis $p > 0.05$) at any time point.

Health scores were based on methods developed by DeLeo et al. (2016) and modified for *S. exserta* based on prior observations while caring for the whole live colonies. Health scores (0–5) were assigned to each fragment based primarily on the percentage of live polyps and tissue remaining as follows: 4 or 5 = fragments with > 50% live polyps and tissue, 3 = ~50% live polyps and tissue, 1–2 ≤ 50% live polyps and tissue, and a score of zero was given to dead fragments with no remaining live polyps or tissue. Other signs of stress, which further refined scores of 1 or 2 and 4 or 5, were polyp retraction, mucus production, tissue sloughing, tissue discoloration and necrotic tissue.

### 2.5. Analytical chemistry

#### 2.5.1. Hydrocarbons

Water samples for all treatments (composite of all replicates) from the WAF and CEWAF assays were collected immediately after dosing (time = 0 h) and analyzed for total extractable hydrocarbons (TEH) and total PAH using methods from Reddy and Quinn (1999) and modified by DeLorenzo et al. (in journal review). Briefly, samples were acidified to pH 2, extracted via liquid/liquid extraction with dichloromethane and hexane, followed by clean-up with silica solid phase extraction. Samples were analyzed using gas chromatography mass spectrometry (GC/MS-Agilent 6890/5973N). The GC/MS contained a DB17ms analytical column (60 m × 0.25 mm × 0.25 μm) and was operated in selected ion monitoring mode.

#### 2.5.2. DOSS (dioctyl sodium sulfosuccinate)

Samples from the CEWAFs and Corexit-only treatments were analyzed for the concentration of DOSS, one of the key ingredients of the dispersant. After the initial 24 h of the dispersant-only test, water samples were again collected for DOSS analysis, to quantify change in concentration, if any, within the first 24 h of the assay. Methods for the DOSS extraction followed those of Flurer et al. (2010) with a few modifications. Water samples were first diluted to calibration ranges. Samples were extracted using QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe; AOAC Method 2007.01) and then filtered, concentrated, and analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS-Agilent 1100 HPLC/API 4000 Mass Spectrometer, equipped with electrospray ionization, operated in negative mode) on a C18 analytical column (2.5 μm, 2.1 mm × 50 mm).

### 2.6. Statistical analyses

Health scores for each of the three assays were averaged for each treatment (control and experimental) at each of the five exposure times. To test for differences from the control at 96 h, Kruskal-Wallis tests were performed (JMP 12.1.0, SAS Institute, Cary, NC, USA) followed by Wilcoxon pairwise comparisons when results were significant ($p < 0.05$).

Where mortality was observed at the end of the 96 h assay, nominal and measured (using chemistry values at $t = 0$) median lethal toxicity concentrations ($LC_{50}$) and the concentrations to cause 10% lethality ($LC_{10}$) were calculated using SAS Probit analysis (PROC PROBIT, SAS 9.4, SAS Institute, Cary, NC, USA). For the Corexit-only and CEWAF tests, $LC_{50}$ of Corexit was also calculated using measured DOSS chemistry values (17% DOSS in Corexit 9500, determined by mass).

## 3. Results

### 3.1. Exposure effects

#### 3.1.1. Oil water-accommodated fractions (WAFs)

In the oil WAF assay, there was only one unexpected case of fragment death. This was observed in the 1.85% WAF group after 72 h, and this fragment was likely stressed before the experiment. However, even when that observation was included in statistical analyses, there were no significant differences ($p > 0.05$) among treatments at any time point (Fig. 4). After 96 h, all fragments exposed to 100% WAF had their polyps fully retracted, and all fragments, minus the one in 1.85% WAF, had a health score of 4 or higher. After 96 h, some fragments in experimental groups looked healthier than some in the control group (Fig. 5).

#### 3.1.2. Corexit 9500 dispersant

Severe health declines (health score ≤ 3) were first observed after 24 h in the 50 and 100 mg/L treatments (Fig. 6). These corals exhibited tissue sloughing or loss but polyps kept their red color (Fig. 7). Complete mortality (100% of all replicates in a group), was observed after 48 h in the fragments exposed to 100 mg/L Corexit. After 96 h, partial mortality (1 of 4 fragments) was observed in the group exposed to 50 mg/L Corexit, but fragments that were exposed to doses < 50 mg/L all retained a health score ≥ 4. There was a significant effect of treatment at all times except at $t = 0$ (Kruskal-Wallis $p$-values < 0.005). After 96 h, the 50 and 100 mg/L treatments were significantly different from the control (Wilcoxon $p$-values = 0.0256 and 0.0177, respectively).

The nominal-based $LC_{50}$ at 96 h (calculated using a Probit model of the log-transformed concentration data, distribution = Gompertz) was 51.17 mg/L Corexit (95% CI = 48.59, 53.90). The nominal $LC_{10}$ was 48.69 mg/L Corexit (95% CI = 46.23, 51.29). The measured $LC_{50}$

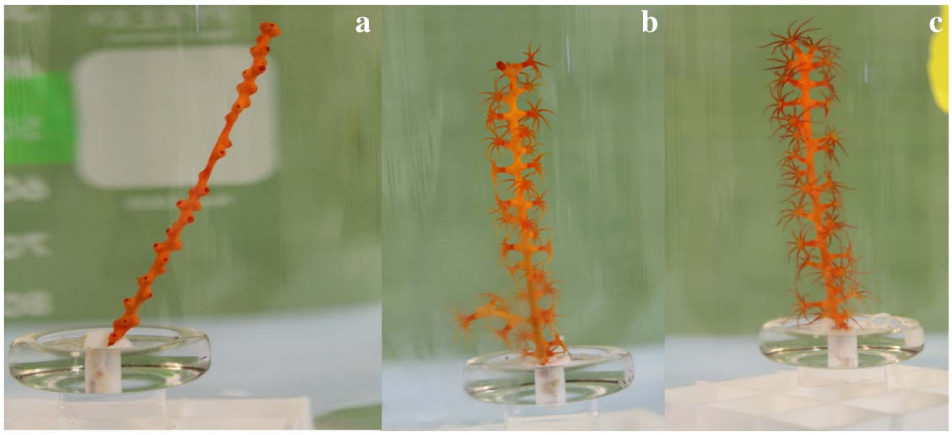


**Fig. 5.** Images of *Swifta exserta* octocoral fragments in a) control group after 96 h duration, b) exposed to 1.85% water-accommodated fraction (WAF) for 96 h, and c) exposed to 16.67% WAF for 96 h. There were no significant differences among the treatments, other than retraction of polyps.

(Probit model, no transformation, distribution = Normal) was 70.27 mg/L Corexit. The measured $LC_{10}$ was 64.23 mg/L Corexit. 95% confidence intervals could not be calculated at 96 h due to lack of partial mortality.

### 3.1.3. Chemically-enhanced WAF

Partial mortality was observed within 48 h, while complete mortality (all fragments) was observed within 72 h in both the 50 and 100% treatments (Figs. 8–9). Control fragments remained healthy (health score ≥ 4), albeit showing minimal signs of stress (polyp retraction) after 96 h (Fig. 8). Significant treatment effects were observed in the CEWAF exposure at all times except $t = 0$ (Kruskal-Wallis $p < 0.005$). After 96 h, the 50 and 100% CEWAF treatments were significantly different from the control (Wilcoxon $p$-values = 0.0131 for both treatments).

The nominal-based $LC_{50}$ at 96 h (Probit model, log transformation, distribution = Gompertz) was 45.58% CEWAF and the $LC_{10}$ was 43.79% CEWAF (Table 1). The measured $LC_{50}$ of TEHs (calculated using the Gompertz log model) was 45.86 mg/L and the $LC_{10}$ was 44.31 mg/L the (Table 2). The measured $LC_{50}$ of Corexit in CEWAF (Probit model, log transformation, distribution = Gompertz) was 41.04 and the $LC_{10}$ was 40.28 mg/L Corexit in CEWAF (Table 2). 95% confidence intervals were not estimable.

### 3.2. Measured chemistry concentrations

The total $PAH_{50}$ and TEH concentrations measured in the full-strength (100%) CEWAF were 1.7 and 132 mg/L, respectively (Table 3). These concentrations were significantly higher than those measured in the WAF (total $PAH_{50}$ = 0.28 mg/L; TEH was below detection limit). DOSS values and subsequent calculated Corexit concentrations for the CEWAF and Corexit-alone tests are shown in Tables 4 and 5, respectively. Measured concentrations of Corexit in the CEWAFs were, on average, 116% of the nominal concentrations (Table 4).

Measured Corexit concentrations in the Corexit-alone treatments (at $t = 0$), were 128% of the nominal, on average (Table 5). Additionally, DOSS concentration, which is only one component of Corexit (17% in Corexit), decreased minimally after the first 24 h of exposure for the 6.25, 12.5, and 100 mg/L doses, but increased in the doses of 25 and 50 mg/L Corexit (Table 5). These minimal decreases in DOSS may be due to chemical adherence to the container or adsorption or uptake by the coral fragment, while increases could be a result of further dissolution in the seawater within the first 24 h of the dosing.

## 4. Discussion

This study found that exposure of chemically dispersed oil to *Swiftia exserta* octocorals was more detrimental to coral health than exposure to non-dispersed water-accommodated oil fractions. Mortality occurred quickly in both the dispersant-alone and chemically-enhanced WAF treatments, over the course of 96 h for most of the fragments. Mortality (health score = 0) was observed after 96 h for 63% of the fragments exposed to high doses (50 and 100 mg/L) of Corexit 9500 and complete mortality was observed in fragments exposed to the two higher concentrations of CEWAF (50 and 100%). These data support the hypothesis that mesophotic octocorals are vulnerable to chemical dispersants

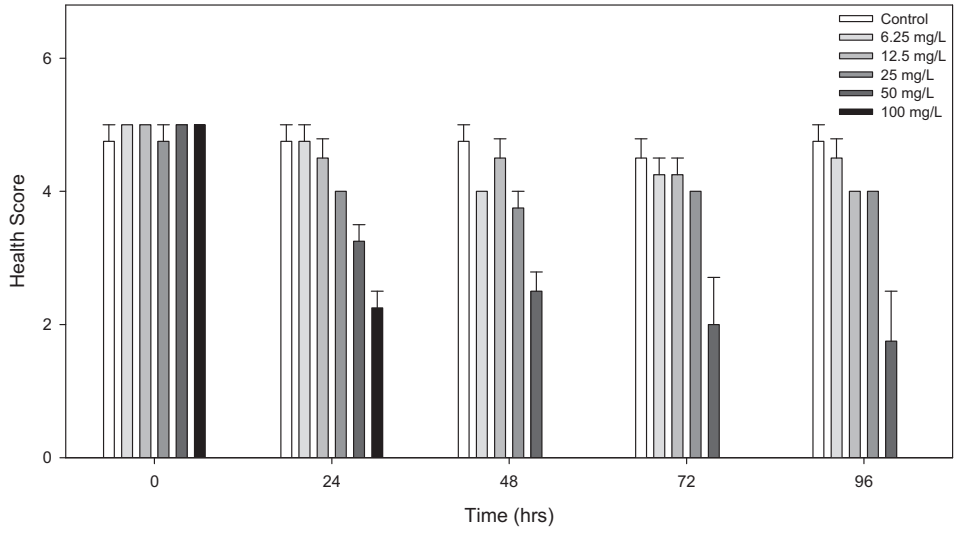


**Fig. 6.** A plot showing results of *Swiftia exserta* exposures to Corexit 9500 dispersant. Severe health declines occurred in the two high-dose groups after 24 h.

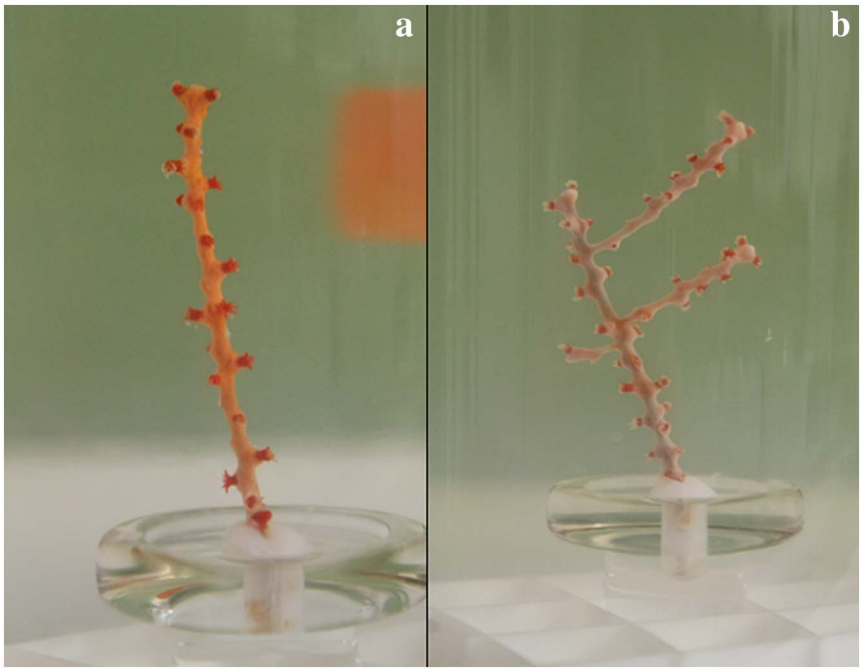


**Fig. 7.** Images of *Swiftia exserta* fragments exposed to a) 50 mg/L Corexit for 24 h, and b) 100 mg/L Corexit for 24 h.

and chemically dispersed oil at high concentrations over a short time period of just a few days.

These results are consistent with other studies that show that chemically dispersed oil exposures are more toxic than oil-alone treatments (Rico-Martinez et al., 2013; Goodbody-Gringley et al., 2013; DeLeo et al., 2016). The study is also consistent with others in that the dispersant-alone (measured $LC_{50}$ = 70.27 mg/L Corexit) was slightly less toxic than the oil-dispersant mixture (measured $LC_{50}$ = 41.04 mg/L Corexit). This result is expected due to the chemical dispersant's ability to increase the amount of oil that mixes into the water column, thereby increasing the hydrocarbon concentration (National Research Council, 2005).

This study is the first to examine the effects of oil and dispersants on a gorgonian octocoral from the mesophotic depth range (50–150 m). Previous studies have shown effects of deep-sea octocorals (White et al., 2014; DeLeo et al., 2016). This is also the first to calculate median lethal toxicity values for LSC oil and Corexit 9500 for any deep water coral species. The nominal 96 h $LC_{50}$ for Corexit 9500 alone was approximately 51.17 mg/L, which is comparable to that of juvenile clams and mysids (32.80 and 43.40 mg/L Corexit, respectively (DeLorenzo et al., in journal review). Octocoral colonies of *S. exserta* from our study were more sensitive to Corexit-only exposures than seven out of the twelve species of benthic invertebrates and life stage combinations tested by DeLorenzo et al. (in journal review). The 96 h $LC_{50}$ for the Corexit-CEWAF based on measured TEH concentrations (45.86 mg/L TEH) was lower than several species of different life stages that were exposed to similar Corexit-LSC oil CEWAF preparations (DeLorenzo et al., in journal review). *S. exserta* fragments were more sensitive than embryo-larval and adult fish, adult snails, juvenile clams, juvenile polychaetes, and embryo, larval, and adult grass shrimp to Corexit-CEWAFs.

At high concentrations of CEWAF or Corexit-alone mixtures, the

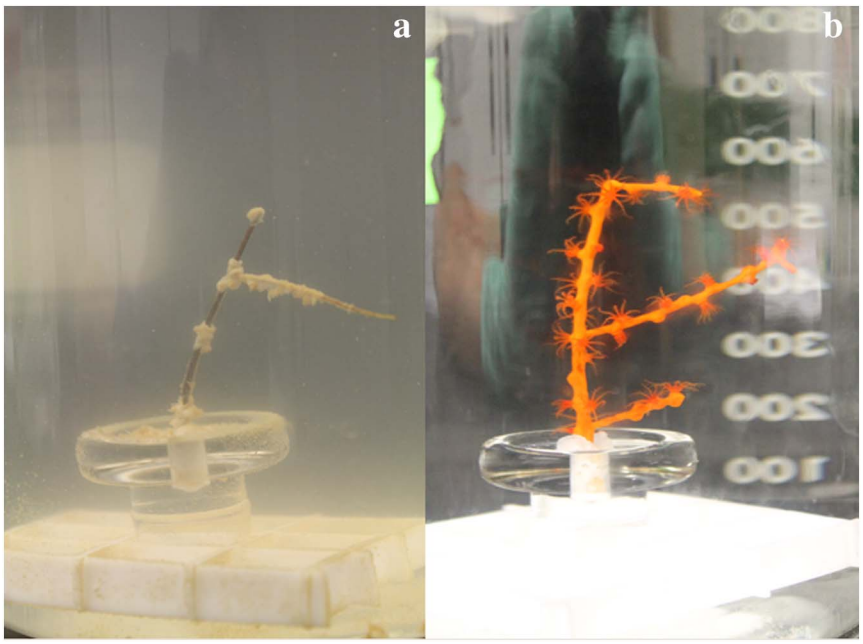


**Fig. 8.** Images of *Swiftia exserta* fragments a) exposed to 50% chemically-enhanced WAF (CEWAF) for 48 h, and b) in control group after 96 h.

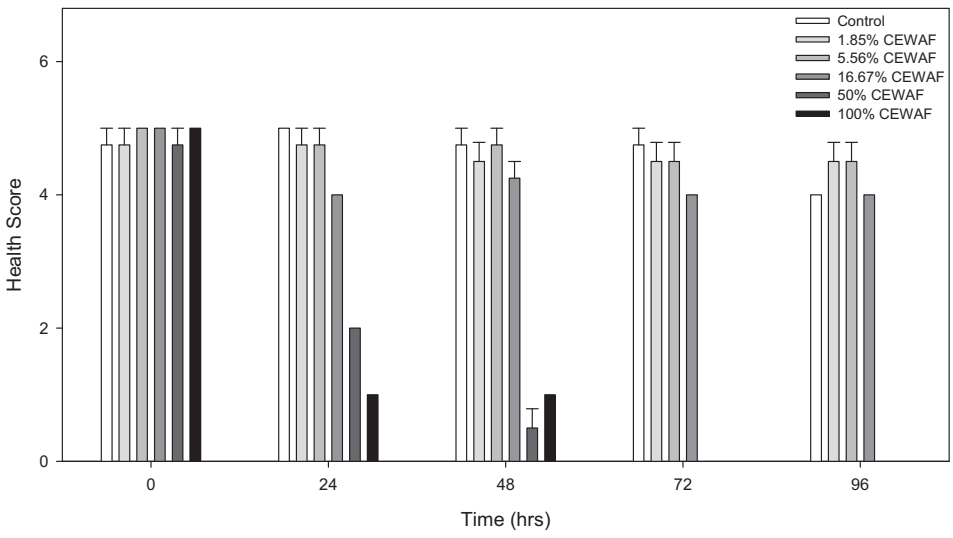


**Fig. 9.** A plot showing results of *Swiftia exserta* exposures to chemically-enhanced WAF (CEWAF). Severe health declines occurred in the two high-dose groups after 24 h.

**Table 1**
Nominal-based toxicity values of *Swiftia exserta* octocorals after 96 h exposure to Corexit 9500 alone and in chemically-enhanced water-accommodated oil fraction (CEWAF). Confidence intervals for CEWAF treatments could not be calculated due to lack of partial mortality.

| Test | Nominal | | | | Model used | |
|---|---|---|---|---|---|---|
| | $LC_{50}$ | 95% CI | $LC_{10}$ | 95% CI | Distribution | Data transformation |
| Corexit-only (mg/L) | 51.17 | 48.59–53.90 | 48.69 | 46.23–51.29 | Gompertz | Log |
| CEWAF (%) | 45.58 | – | 43.79 | – | Gompertz | Log |

**Table 2**
Measured Corexit and total extractable hydrocarbon (TEH) toxicity values (using concentrations at $t = 0$) of *Swiftia exserta* octocorals after 96 h exposure to Corexit 9500 alone and in chemically-enhanced water-accommodated oil fraction (CEWAF). Confidence intervals could not be calculated due to lack of partial mortality.

| Test | Measured | | | | Model used | |
|---|---|---|---|---|---|---|
| | $LC_{50}$ | 95% CI | $LC_{10}$ | 95% CI | Distribution | Data transformation |
| Corexit-only (mg/L) | 70.27 | – | 64.23 | – | Normal | None |
| CEWAF TEH (mg/L) | 45.86 | – | 44.31 | – | Gompertz | Log |
| CEWAF Corexit (mg/L) | 41.04 | – | 40.28 | – | Gompertz | Log |

very thin coenenchymal tissue of *S. exserta* (Goldberg, 2001) breaks down, leaving the sclerites exposed. Eventually, with the help of water flow, these sclerites disassociate from the central axis, leaving bare skeleton. Necrosis is proposed to be an extreme immune response to severe stress from temperature, pollutants, or disease (McClanahan et al., 2004; Silva et al., 2016).

These fine-scale effects were not consistent with the in situ trajectory of degradation to *Swiftia* spp. sea fans reported by Silva et al. (2016). Injuries reported for octocorals at mesophotic sites below the DWH oil slick were overgrowth by hydroids, covering by sediment, broken or bare branches (Etnoyer et al., 2016; Silva et al., 2016). The corals in our experiments were not fed and were isolated in beakers, so conditions were not conducive to flocculent material or overgrowth. Furthermore, the effect of oil and dispersants to these benthic organisms in a natural environment is possibly exacerbated by feeding on the oil-derived marine snow that rapidly sank to the sea floor (Daly et al., 2016; Passow, 2016).

It is important to recognize that the concentrations at which

**Table 3**
Measured total polycyclic aromatic hydrocarbon ($PAH_{50}$) and total extractable hydrocarbon (TEH) concentrations oil water-accommodated fraction (WAF) and chemically-enhanced water-accommodated fraction (CEWAF) at $t = 0$.

| Nominal (%) | Total $PAH_{50}$ (mg/L) | TEH (mg/L) |
|---|---|---|
| WAF | | |
| Control | 0.001 | Less than MDL[a] |
| 1.85 | 0.004 | Less than MDL[a] |
| 5.56 | 0.015 | Less than MDL[a] |
| 16.67 | 0.044 | Less than MDL[a] |
| 50 | 0.123 | Less than MDL[a] |
| 100 | 0.280 | Less than MDL[a] |
| CEWAF | | |
| Control | 0.000 | Less than MDL[a] |
| 1.85 | 0.056 | 2.64 |
| 5.56 | 0.123 | 6.75 |
| 16.67 | 0.151 | 19.6 |
| 50 | 0.868 | 49.6 |
| 100 | 1.70 | 132 |

[a] MDL = measured detectable limit (0.25 mg/L).

**Table 4**
Measured dioctyl sodium sulfosuccinate (DOSS) and Corexit 9500 concentrations in CEWAF treatments at $t = 0$.

| Nominal | Measured ($t = 0$) | | |
|---|---|---|---|
| CEWAF (mg/L) | DOSS (mg/L) | Corexit (mg/L) | % nominal |
| Control | Less than MDL[a] | Less than MDL[a] | 100 |
| 1.85 | 0.305 | 1.8 | 97.0 |
| 5.56 | 1.56 | 9.18 | 165 |
| 16.67 | 4.47 | 26.3 | 158 |
| 50 | 7.28 | 42.8 | 85.7 |
| 100 | 15.4 | 90.6 | 90.6 |

[a] MDL = measured detectable limit (0.01 mg/L).

**Table 5**
Measured dioctyl sodium sulfosuccinate (DOSS) and Corexit concentrations in dispersant-only treatments at $t = 0$ and $t = 24$ h.

| Nominal | Measured (mg/L) | | | | | |
|---|---|---|---|---|---|---|
| Corexit-alone (mg/L) | DOSS ($t = 0$) | DOSS ($t = 24$ h) | Corexit ($t = 0$) | Corexit ($t = 24$ h) | % nominal ($t = 0$) | % nominal ($t = 24$ h) |
| Control | Less than MDL[a] | Less than MDL[a] | NA | NA | 100 | 100 |
| 6.25 | 1.45 | 1.30 | 8.52 | 7.65 | 136 | 122 |
| 12.5 | 1.55 | 1.31 | 9.13 | 7.71 | 73.0 | 61.7 |
| 25 | 6.33 | 6.55 | 37.3 | 38.5 | 149 | 154 |
| 50 | 11.4 | 17.0 | 67.1 | 100 | 134 | 200 |
| 100 | 30.8 | 28.9 | 181 | 170 | 181 | 170 |

[a] MDL = measured detectable limit (0.01 mg/L).

complete mortality occurred at 96 h in the experimental setting was higher than would be expected to occur over a large area of the seafloor. Yet, it is extremely difficult to estimate the fate of oil and dispersants after the DWH spill. A study by Silva et al. (2016) detected non-toxic levels of tPAHs (mean = 51–345 ppb) in the tissues of octocorals ($n = 50$) from the Pinnacles Trend. These tissue levels of hydrocarbons are similar to the aqueous exposure concentrations measured in our study (i.e., 280 ppb in the 100% WAF, 868 ppb in the 50% CEWAF). Although no mortality was observed in *S. exserta* corals exposed to 280 ppb of tPAHs alone, the dispersed oil solutions yielded approximately seven times the level of hydrocarbons in solution. We hypothesize that coral toxicity in the CEWAFs may be due to a combination of Corexit and higher bioavailability of PAHs.

Due to palpable limitations of the experimental setting, our study did not exactly replicate the conditions of the DWH oil spill. Many factors, like wind and waves, contributed to the mixture of LSC oil and Corexit throughout the water column of the Gulf of Mexico and those are difficult to simulate in the laboratory. Additionally, our experiments focused on quantifying the effects of short-term exposures of toxins, and it is likely that mortalities would have been higher had the experiments continued for a longer duration. The DWH oil slick affected mesophotic reefs sites in the Gulf of Mexico over a period of several weeks in 2010 (Etnoyer et al., 2016), while this study was relatively brief, conducted over a 96 h period.

The short-term assay was conducted to understand the toxicity thresholds to *S. exserta*, which had not been previously examined. The oil and dispersant concentrations tested in our study are comparable to those of other studies focusing on other aquatic or marine species (Hemmer et al., 2011; Goodbody-Gringley et al., 2013; DeLeo et al., 2016; DeLorenzo et al., in journal review).

This study is the first to quantify vulnerability of deep water gorgonian octocorals to chemical contaminants, and as such provides important information to inform management in the event of a future oil spill. With the rise of oil and gas production throughout the Gulf of Mexico, potential impacts to deep-water ecosystems, such as future spills, will remain a looming threat. Responding to such threats requires a better understanding of the sensitivity of contaminants to affected species, and we hope that our study will provide important information to improve management efforts of these important, yet vastly undersurveyed deep-water ecosystems.

## Author contributions

JF conducted the experiments, collected the data, analyzed and interpreted the data, and drafted the article. MED designed the work, interpreted the data, and critically revised the article. ECP analyzed chemistry samples and critically revised the article. PJE conceived the experiments, assisted with data collection, and critically revised the article.

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# Clinical Pathology Effects of Crude Oil and Dispersant on Hatchling Loggerhead Sea Turtles (*Caretta caretta*)

**IAAAM 2014**

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Abstract

For 84 days following the 20 April 2010 explosion of the **Deepwater Horizon** MC252 offshore oil drilling rig, an estimated 200 million gallons (4.9 million barrels, 780,000 $m^3$) of crude oil were released into the Gulf of Mexico, with 1.8 million gallons (7,000 $m^3$) of surface and subsurface chemical dispersants applied in an attempt to mitigate shoreline impact.[6] Of the approximately 456 visibly oiled and 80 not visibly oiled live sea turtles rescued, nearly 90% were successfully rehabilitated and released.[5] Physiologic and pathologic effects expected from crude oil exposure used to guide treatment of sea turtles came from extrapolation of studies in other species[7] and from a single loggerhead sea turtle (*Caretta caretta*) study.[4] While invaluable starting points, inherent limitations to extrapolation,[7] and small sample size (5 controls and 5 exposed turtles) with changes in controls that trended with those of exposed turtles[4] limit utility for clinical guidance and for investigating oil spill impacts. Pending litigation prevents release of clinical data from the MC252 sea turtle response until cases are resolved. Effects of dispersants were not included in the previous sea turtle oil exposure study[4] and cannot be effectively isolated in the eventual analysis of field data from the MC252 incident.

An ongoing terminal pivotal temperature incubation study utilizing eggs salvaged from doomed loggerhead sea turtle nests provided an opportunity for a separate add-on exposure study to investigate the effects of crude oil, dispersant, and a crude oil/dispersant combination in sea turtle hatchlings. Clinical pathology findings are presented here. Investigations in toxicology assays,[3] histopathology, and NMR metabolomics are in progress. Eggs in the pivotal temperature study were incubated at 27.4–31.0°C, and hatchlings were randomly assigned to control, oil, dispersant, and oil/dispersant exposures for 1 d or 4 d. Exposures were begun after a 3 d post-hatching period simulating nest emergence. Turtles were placed in individual glass basins containing aged seawater and exposed to oil (Gulf Coast - Mixed Crude Oil Sweet, CAS #8002-05-9, 0.833 mL/L) and/or dispersant (Corexit 9500A, 0.083 mL/L), replicating concentrations encountered during oil spills and subsequent response. Turtles were weighed and measured before and after exposures. Blood was collected into lithium-heparin tubes immediately following euthanasia. Packed cell volumes were determined by centrifugation and plasma chemistry panels were acquired with an in-house tabletop biochemical analyzer (Abaxis VetScan, Avian/Reptilian Profile Plus rotor) starting within 5 min of blood collection. Statistically significant differences between treatments and their respective nonexposed controls were detected for PCV, AST, uric acid, glucose, calcium, phosphorus, total protein, albumin, globulin, potassium and sodium (all except for calcium were increased where differences were present). The principal dyscrasias reflected osmolar, electrolyte and hydration challenges that were worst in combined oil/dispersant exposures at 4 d. Clinical pathology findings were supported by a failure to gain weight (associated with normal hatchling hydration in seawater)[1] in dispersant and combination exposed hatchlings. These findings indicate potential hazards to consider when deploying

dispersants in an oil spill response.

## Acknowledgements

The authors recognize the controversial nature of an investigation such as this[2] and deliberated before proceeding. In the context of eggs from doomed nests being used in an ongoing terminal study, and the paucity of toxicology data for sea turtles exposed to crude oil and dispersants, we opted to maximize use of these turtles destined for euthanasia in order to generate freely accessible data to be available for the next oil spill affecting sea turtles. Support for the pivotal temperature study came from a NOAA Section 6 Research Grant (NA10NMF4720035). Support for the add-on exposure study came from the state of North Carolina. No support for the exposure study was received from the Natural Resource Damage Assessment (NRDA) process, the National Oceanic and Atmospheric Administration (NOAA), British Petroleum (BP) or any of its affiliates, or from any other source constrained by the legal process surrounding the **Deepwater Horizon** MC252 oil spill. Work was conducted under endangered species permit 13ST50 from the North Carolina Wildlife Resources Commission, with federal authority delegated from the U.S. Fish and Wildlife Service, and with approval of the North Carolina State University Institutional Animal Care and Use Committee (11-078-O, 11-103-O). We thank B. Phillips and E. McCarthy for technical assistance, and J. Griffitt for providing crude oil.

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# The effects of oil spill dispersant use on marine birds: a review of scientific literature and identification of information gaps

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## Abstract

Dispersants, a class of chemical spill-treating agents used to treat oil spills, are commonly used globally as an alternative response measure. Applying dispersants to an oil slick, shortly after the spill has occurred, can protect shoreline environments and sea surface-dwelling animals, such as some marine bird species, limiting individuals or local populations from the consequences of coming into contact with large quantities of oil. However, this benefit comes with the cost of increasing oil exposure risk to marine biota that spend time in the water column. It is generally believed that the benefits of dispersant use outweigh the costs under most circumstances. However, it is rarely acknowledged that the use of dispersants may have negative impacts on marine biota at the individual or local population level, including marine birds. In Canada, Corexit EC9500A, a regulated dispersant, is being proposed for expanded use beyond treating spills from an offshore oil and gas facility. To understand what the potential impacts from dispersant use are to marine birds, we conducted a literature review to identify the direct and indirect effects of their use. We also provide oil spill responders with a Pathway of Effects (POE) conceptual model, a tool for understanding the interactions between dispersants, marine birds, and their environment to support a holistic consideration as part of the oil spill response decision-making process. Fundamental uncertainties remain, however, and if left unaccounted for in the decision-making process, they may compromise the appropriateness of spill response approaches and outcomes. We recommend that oil spill responders incorporate the known benefits and costs of dispersant use on marine birds into a decision-making framework such as a Net Environmental Benefit Analysis (NEBA) and with consideration of the POE concept models provided. These recommendations are particularly relevant where a decision-making framework such as NEBA is becoming a more standardized component of the response process. Additionally, greater investment in lab and field-based research, and field observations through monitoring, is required to address existing decision-making uncertainties and provide information gap closure.

**Key words:** dispersant, Corexit, net benefits analysis, Pathway of Effects, exposure risk

## Introduction

With the increase in global oil demand and a rise in oil transportation (Chang et al. 2014; IEA 2022), the potential for a large oil spill event is a persistent potential reality. In Canada, the volume of marine pollution spills has varied over the past decade; however, the frequency of spill incidents, particularly in coastal environments, has shown a marked increase (ECCC 2021). For Canadian shorelines impacted by spill incidents, 70% of spills are sourced from marine vessels (Feng et al. 2021). In Canada and elsewhere, it is well documented that oil can be lethal to marine organisms, having extensive and lasting consequences on individuals, populations, and ecosystems (e.g., Irons et al. 2000; Peterson et al. 2003; Lincoln et al. 2020). Oil pollution is estimated to impact upwards of hundreds of thousands of marine birds in Canada each year (Government of Canada 2017). Marine birds can be especially sensitive to the effects of oil exposure with some species demonstrating slow population recovery times (Albers 1984; Wiese et al. 2004; O'Hara and Morandin 2010; Esler et al. 2018).

Natural attenuation of an oil spill, a no-intervention response method, in some circumstances may not adequately protect ecosystems from the harmful effects of oil, thus necessitating intervention to minimize the environmental effects of spilled oil (Pequin et al. 2022). The primary approach in responding to an oil spill is to mechanically remove the oil from the marine environment, however, this is not always feasible nor entirely effective (NRC 2005; DFO 2021; Transport Canada 2022). Under certain conditions, it may be advantageous to integrate an alternative response measure, which

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include spill-treating agents, in situ burning, translocations, and decanting, to enhance the dispersal or removal of oil or to protect high priority habitat, wildlife, and other ecologically, economically, or culturally valuable resources. Spill-treating agents include several classes of chemical and biological treating agents such as dispersants, herding agents, solidifiers, surface washing agents, demulsifiers, recovery agents, gelling agents, biodegradation enhancers, sinking agents, and elasticity modifiers. They are used to reduce overall adverse impacts of an oil spill and each class achieves this in a different way (Walker et al. 1999; Brown et al. 2011).

Dispersants, the focus of this review, are a mixture of solvents and surfactants. They can be applied to an oil slick to redistribute the oil from the water's surface into the water column by reducing the interfacial tension between oil and water, which enhances the natural process of oil dispersion (NRC 2005). The advantage of dispersants stems from their ability to redirect oil from stranding along shorelines and presumably, reducing the effect of oil exposure for sensitive surface-dwelling animals such as marine birds (Prince 2015). Dispersants can be applied rapidly over large areas and under a range of environmental conditions (Transport Canada 2022). There are costs, however, associated with transferring oil from one part of the marine environment (e.g., the water's surface) to another (e.g., the water column). Decision-making frameworks such as Net Environmental Benefit Analysis (NEBA) attempt to account for these trade-offs using a systematic approach for evaluating the benefits and costs associated with their use, considering impacts to individual, local, and regional populations as appropriate (IPIECA 2015). Ultimately, the objective is to make an informed decision on which oil spill response method(s) optimize response success while minimizing impacts to the environment and sensitive receptors (IPIECA 2015).

A broadly defined literature search using two commonly used search engines (Google Scholar and Web of Science) returned complementary (i.e., few repeats between the two search engines) lists of papers using the following search terms: ("marine bird" OR seabird) AND (dispers OR "dispersed oil"). Each identified paper was scrutinized resulting in 9 relevant papers from Google Scholar (from a total of 47 papers identified) and 14 papers from Web of Science (from a total of 50) linking direct effects on marine birds from dispersant use. Interestingly, several important earlier experimental papers were not identified in either search engine (Albers 1979; Peakall et al. 1981; Albers and Gay 1982 for example). Most existing data on effects of dispersant use on marine birds come from papers published in the 1970s and early 1980s, producing mixed results in terms of dispersant impacts. Since then, little has been published until the 2010s, with the notable exception of Jensen and Ekker (1991). Data from three recent papers (Duerr et al. 2011; Fiorello et al. 2016; Whitmer et al. 2018) indicate concerns or caveats that should be considered when deciding whether or not to deploy dispersants. Many of the remaining papers identified by the two search engines were policy oriented or model based, with the explicit yet often unsubstantiated assumption that dispersant use is beneficial to marine birds. It is difficult to identify when this largely untested assumption became a rationale for dispersant use in the literature. Indeed, as an example of how accepted this assumption is, minimizing oil spill impacts on marine birds as a principal reason for the use of a dispersant was introduced in the abstract of a recent paper, with no further discussion in the text or citations to support this claim (Zhu et al. 2022).

In 1989, the US National Research Council (hereafter, NRC) called for more research on the effects of dispersant use on marine birds as there was no conclusive evidence at the time that dispersants caused less harm (NRC 1989). With minimal progress made in the intervening years (within the US and globally), the NRC reiterated its recommendations in 2005 to increase research efforts on the effects of dispersants to marine birds (NRC 2005). Since then, research efforts have focused elsewhere, and extensive information gaps remain (Whitmer et al. 2018; NASEM 2019). This review identifies the known and potential impacts of dispersant use to marine birds, on an individual physiological and toxicological level as well as the local population and integrated ecosystem level. While we integrate information from a variety of international sources, this review is presented in the context of the Canadian marine oil spill response regime, placing emphasis on the associated species and habitats. To this end, we outline limits in the general understanding of outcomes of dispersant use on marine birds. This includes identification of important information gaps, particularly conditions and climates representative of Canada's coastal ecosystems. Notwithstanding, information provided herein may support the assessment of dispersant use on marine birds in other regions, including crossover applicability in freshwater environments.

In the Canadian context, we focus on Corexit® EC9500A (formally Corexit® 9500). It is the only dispersant regulated within Canada and is limited for use in offshore spills in Atlantic Canada, as defined by applicable offshore petroleum legislation (Government of Canada 2016). Legislative amendments are being proposed to enable the expansion of Corexit® EC9500A use for other designated sectors including marine traffic (Transport Canada 2022). Given the proposed legislative amendments in Canada, we present some timely recommendations to support informed assessment of dispersant use, and on handling the uncertainty that exists around their potential impacts on marine birds. We also focus on the use of NEBA decision-making framework, an approach being developed for use in Canada and one that is used in other countries such as the United States, Australia, the European Union, and the United Kingdom. Thus, this review has a Canadian context, yet is broadly applicable to other international jurisdictions. As future amendments may include legislative requirements on how to determine a net environmental benefit for oil response measures (Transport Canada 2022), we propose Pathway of Effects (POE) conceptual models as a beneficial tool for inclusion in the NEBA process to assess quickly the impacts of dispersant use to marine birds.

## Pathway of Effects models

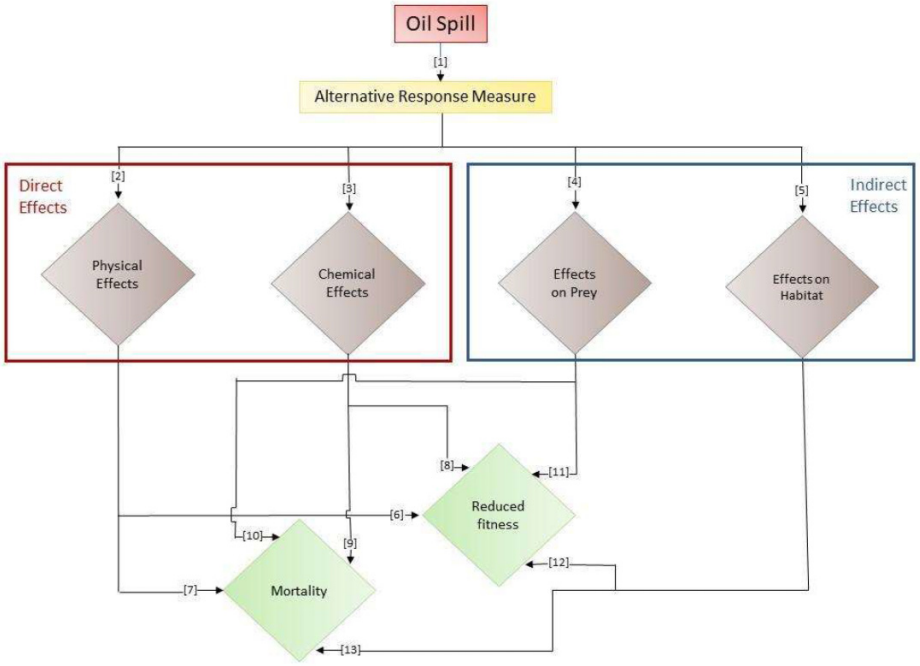
The potential effects anthropogenic activities have on a receptor organism and its habitat can be described using POE

**Table 1.** Numbered linkages between pathway components in Fig. 1 and their associated supporting evidence in-text descriptions.

| Pathways of Effects | Evidence description |
|---|---|
| ARM [1]—Physical effects [2]—Reduced fitness [6] or Mortality [7] | See "Physical effects" section |
| ARM [1]—Chemical effects [3]—Reduced fitness [8] or Mortality [9] | See "Toxicological effects" section |
| ARM [1]—Effects on prey [4]—Reduced fitness [10] or Mortality [11] | See "Toxicological effects on prey" section |
| ARM [1]—Effects on habitat [5]—Reduced fitness [12] or Mortality [13] | See "Harmful algal blooms" and "Habitat effects and persistence" sections |

**Note:** Alternative response measure (ARM), in this case, applies only to dispersants.

**Fig. 1.** Pathway of Effects conceptual model of the direct and indirect alternative response measure (ARM) exposure pathways for marine birds.




conceptual models. They illustrate the mechanisms by which potential stressors may act on an organism through direct and indirect effects (Hannah et al. 2020). POE conceptual models for oil exposure on wildlife have been discussed and used elsewhere (Henkel et al. 2012; Hannah et al. 2020). They are a useful starting point in understanding how dispersants may affect wildlife when they are included as a spill-treating option. We propose a generic POE for marine birds that illustrates the pathways through which chemically dispersed oil may affect marine birds (Table 1 and Fig. 1). It should be noted, however, that depending on the circumstances of a spill POEs may differ depending on the nature of dispersant use in combination with life-history strategies of implicated marine bird species (Fig. 2).

## Direct effects

### Physical effects

Physical effects from contamination refers to a change in the physical structure or function of parts of a bird that result in a measurable effect. The impacts to marine birds from oiling include the loss of waterproofing, buoyancy, and insulative properties of feathers, leading to hypothermia, starvation, and/or drowning of the animal (Jenssen 1994; King et al. 2021). The water-repellent properties of marine bird plumage depend on the interaction between water surface tension and the microstructure of feathers, which prevent water from penetrating their plumage (Stephenson 1997). This waterproofing mechanism also traps air within the plumage, which provides buoyancy and thermal insulation (Jenssen and Ekker 1991). Dispersants and dispersant–oil mixtures reduce the surface tension of water thereby allowing water to penetrate plumage (Jenssen and Ekker 1991; Jenssen 1994). Marine birds will preen in an attempt to restore the physical structure and waterproofing function of oil-contaminated feathers by realigning hooks and barbules in the feathers (Stephenson 1997; O'Hara and Morandin 2010). However, exposure to chemically dispersed oil has been found to result in similar disruption to maintaining feather integrity (Duerr et al. 2011; Whitmer et al. 2018).

Whitmer et al. (2018) conducted a study to evaluate the effects of dispersants and crude oil on the waterproofing of live common murres (*Uria aalge*) during a simulated dive through

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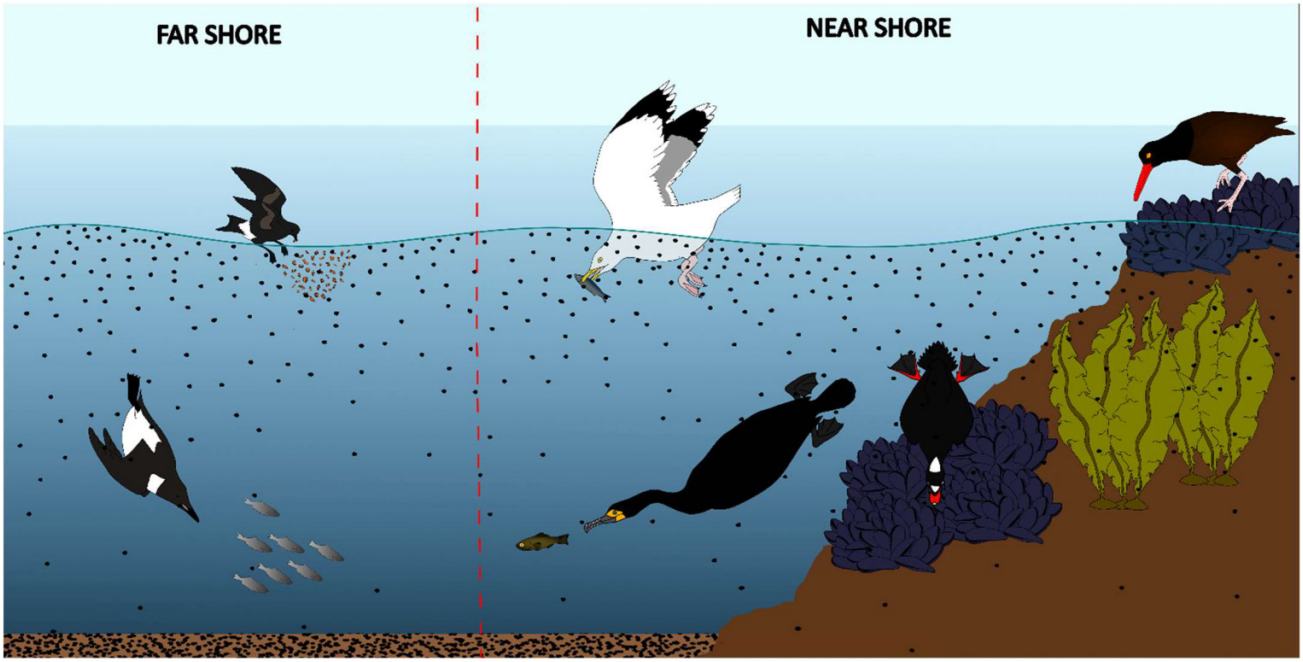
**Fig. 2.** General habitat, foraging guilds, and direct or indirect exposure pathways for marine birds.




contaminated water. Treatments of increasing contaminant concentrations included a control, dispersant only, and a mixture of Corexit® EC9500A and Prudhoe Bay Crude Oil (PBCO) at an industry standard ratio of 1:20. The authors used contaminant concentrations based on scientific literature that identified representative concentrations found in the upper 10 m of the water column shortly after an oil spill. The treatment with the highest concentration of dispersant alone simulated exposure to dispersant application from a vessel or airplane.

Immediately after treatment exposure, catastrophic loss of waterproofing occurred. Loss of waterproofing also occurred at the medium and low concentrations of dispersant only, but the effects were less severe. These results indicate that exposure to high concentrations of dispersant (e.g., being accidentally sprayed during dispersant application) or significant dispersant–oil concentrations, morbidity and mortality is a likely outcome without human intervention. Whitmer et al. (2018) also showed over a 2 day experimental period, that dispersant does not change the effects oil has on plumage waterproofing, nor are birds more likely to regain waterproofing after exposure. Therefore, for diving birds, the impacts of exposure to a surface slick could be comparable to the impacts of exposure to chemically dispersed oil within the water column (Fig. 2).

In a related study, Duerr et al. (2011) conducted experiments on the structural changes to common murre feathers when exposed to crude oil, Corexit® 9500, and a mixture of the two. They found that dispersants affected the geometry and orderliness of feather structures that affected the waterproofing characteristics by collapsing the feather plume. Given this, the authors found that a pursuit diving marine bird would likely lose insulation and buoyancy once contamination occurred, and subsequent swimming through uncontaminated waters would not be sufficient to remove the contamination. To restore plumage aeration, preening would be necessary. In a study by Lambert et al. (1982), mallards (*Anas platyrhynchos*) were placed in a cold chamber following exposure to oil, dispersant (Corexit® 9527), or dispersant–oil mixtures (30:1 ratio) for 1 h. The ducks exposed to the oil and mixture in this study showed a similar and significant increase in basal metabolic rate and loss of plumage waterproofing. Ducks exposed to dispersant only did not show an increase in basal metabolic rate but did show loss of buoyancy and plumage waterlogging.

Additional research has further demonstrated differences in response between species. Jenssen and Ekker (1991) found that Statfjord A crude oil and dispersant–oil mixture (with Finasol OSR-5 or Finasol OSR-12) reduced plumage waterproofing in common eiders (*Somateria mollissima*) and mallards, leading to heat loss and a subsequent increase in heat production. Effects on insulation for eiders occurred at a much smaller volume of chemically dispersed oil than oil alone; surfactants may be the ingredient responsible for the increased effects by allowing water to pass through the plumage (Jenssen and Ekker 1991; Stephenson 1997). Common eiders were more vulnerable to dispersant–oil mixtures compared to mallards, potentially due to differences in feather structure (Jenssen and Ekker 1991). After plumage became contaminated, preening spread the contamination throughout the plumage, thereby increasing the loss of waterproofing and subsequently enhancing thermoregulatory impairment compared to the immediate effects of contamination (Jenssen and Ekker 1991). However, these studies are species-specific and lab-based, therefore do not account for the wide variability of factors (e.g., species, season, marine

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habitat, and contaminant properties) that could affect a bird's sensitivity to chemically dispersed oil exposure.

## Toxicological effects

Early studies have shown that the acute physiological effects of oil to marine birds do not change significantly with the use of dispersants (for a review see Peakall et al. 1987). Most of these studies used PBCO and Corexit® 9527 in their experiments (Peakall et al. 1987). Stroski et al. (2019) indicate that Corexit® EC9500, which is regulated in Canada, uses a solvent that is less toxic to marine organisms than its predecessor Corexit® 9527 and has become more commonly used. The basis for testing the toxicity of Corexit® EC9500 remains unclear. As such, more research is needed using Corexit® EC9500A on a wider variety of oils, especially regarding its long term, sublethal, and delayed toxicological effects (Wise and Wise 2011; NRC 2005).

### Toxic effects of Corexit® 9527

Previous reviews have discussed at greater length the relevant findings on the toxicological effects of Corexit® 9527 from studies conducted in the 1970s and 1980s (Peakall et al. 1987; Michel et al. 1992). The following is a summary of these relevant findings, followed by current studies not included in Peakall et al. (1987) and Michel et al. (1992).

In embryotoxicity lab experiments, Albers (1979) found that a higher ratio of dispersant to oil had greater embryotoxic effects than oil alone, while Albers and Gay (1982) found that dispersants had no effect and dispersant–oil mixtures had the same effects as oil alone.

In a field study, Butler et al. (1988) found that Leach's storm petrels (*Oceanodroma leucorhoa*) internally and externally exposed to oil or dispersant–oil mixture, demonstrated decreased hatching success and chick survival. Chick growth and survival also decreased more severely when one adult parent was exposed to the dispersant–oil mixture. No long-term effects to reproductive success were observed. In thermoregulation experiments, Eastin and Rattner (1982) found that ingestion of a dispersant and oil combined had fewer toxic effects than oil, and the ingestion of dispersant alone had very little effect. They concluded that the effects of ingesting low levels of dispersant or dispersants with oil is unlikely to harm chicks over a short period. In a similar study, Peakall et al. (1985) found no difference between the effects in weight loss between oil and a dispersant–oil mixture when ingested by herring gulls (*Larus argentutus*). Significant weight loss did not occur for ingestion of dispersant alone. Conversely, they found that external application of the dispersant–oil mixture caused impaired insulation and subsequent weight loss, but oil alone did not.

In immunotoxicity experiments, Rocke et al. (1984) found that ingestion of oil and a dispersant–oil mixture lowered the bacterial resistance in mallards when using Bunker C fuel oil but not South Louisiana crude oil. In endocrine toxicity experiments, Peakall et al. (1982) found that ingestion of oil and a dispersant–oil mixture by herring gull nestlings had similar effects on their growth and organ weights, and that dispersant alone caused minimal effects. In a similar lab and field experiment, Peakall et al. (1981) found that found that dispersant alone caused an increase in corticosterone in gulls on day two of the experiment (for that day only), and the effects of dispersant–oil mixture was similar to that of oil alone.

### Toxic effects of Corexit® EC9500A

More recent studies have used Corexit® EC9500A on embryotoxicity, ocular toxicity, immunotoxicity experiments. In an embryotoxicity study by Wooten et al. (2012), exposure to Corexit® EC9500A alone resulted in decreased hatching success with increased dispersant dosage. Finch et al. (2012) compared the embryotoxic effects of weathered crude oil from the Gulf of Mexico, Corexit® 9500, and dispersant–oil mixture at 50:1 and 10:1 oil to dispersant ratios. The authors found that Corexit® EC9500A decreased the toxicity of weathered crude oil to mallard embryos at the 10:1 ratio but increased it at the 50:1 ratio. In an ocular toxicity study, Fiorello et al. (2016) conducted experiments on the ophthalmic effects dispersants and dispersant–oil mixture have on marine birds. Common murres were exposed to Corexit® EC9500A and PBCO, and their intraocular pressure and tear production was measured before and after the exposure. Development of conjunctivitis was associated with exposure to dispersant and a dispersant–oil mixture, both products are known to be eye irritants. Corneal ulcers were also associated with dispersant exposure but only when exposed to a high concentration of oil. Untreated corneal ulcers can lead to vision impairment or loss, and thus negatively affect marine bird foraging ability and survival. In an immunotoxicity study, Finch et al. (2012) found that when a dispersant–oil mixture (weathered crude oil collected from the Gulf of Mexico and Corexit® 9500, at a 50:1 ratio), applied to mallard eggs and hatchlings, had lower spleen weights compared to the crude oil treatment. The spleen, an immune organ, is associated with immune responses in wildlife. These results indicate that the dispersants used, at the ratios recommended by the manufacturers, may have affected nesting marine bird hatchlings during the Deepwater Horizon spill.

## Exposure risk

The toxicological and physical effects of dispersant alone were also investigated with varying results. Several studies have shown that dispersants alone can cause physical and physiological harm to birds (e.g., Wooten et al. 2012; Fiorello et al. 2016; Whitmer et al. 2018), while others have shown negligible impacts (e.g., Albers and Gay 1982; Eastin and Rattner 1982; Peakall et al. 1982). The application of dispersants to an oil spill may result in a certain amount of unused dispersant partitioning back into the water or through missing the target during application (Peakall et al. 1987). The environmental fate of this unused dispersant following application and its effects on marine birds under real-world environmental conditions are needed to understand whether exposure to dispersant alone adds an extra element of risk to that which exists from chemically dispersed oil. Consequently, it is the exposure risk of dispersants and chemically

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dispersed oil compared to oil alone that is crucial in understanding their relative harm to marine birds (Peakall et al. 1987; NRC 2005; NASEM 2019).

Experimental results have shown that exposure to chemically dispersed oil causes similar toxic effects to oil alone (Albers and Gay 1982; Peakall et al. 1982; Peakall et al. 1987). Similarly, the physical effects of chemically dispersed oil to plumage waterproofing are similar to that of oil alone (Lambert et al. 1982; Duerr et al. 2011; Whitmer et al. 2018), and some research has shown it can be more harmful than oil alone (Jenssen and Ekker 1991). Physical exposure risk to chemically dispersed oil is threshold dependent; if a bird moves through contaminated water and the feathers come in contact with a certain volume of chemically dispersed oil or dispersant (i.e., a threshold amount of contamination is reached), then a lethal outcome will occur (Whitmer et al. 2018).

Understanding whether the consequences of physical exposure to realistic concentrations of chemically dispersed oil and unused dispersant after an oil spill event may be harmful to a bird hinges on estimating its adsorption to feather structures while moving through a realistic volume of contaminated water (Jenssen and Ekker 1991). Although the adsorption of chemically dispersed oil was not quantified, Whitmer et al. (2018) has shown that chemically dispersed oil and dispersant alone, at realistic concentrations, can be lethal for diving murres which spend more time in subsurface waters than other birds. This, among other research (e.g., Jenssen 1994; Stephenson 1997), challenges the assumptions Peakall et al. (1987) made based on their hypothetical scenario for the adsorption of chemically dispersed oil, which concluded that there is no significant adsorption of chemically dispersed oil to plumage underwater.

In addition to quantifying the adsorption of chemically dispersed oil to estimate exposure risk, it is important to understand how this will change under variable concentrations, weathering, or until the concentration has reached a threshold below which it is no longer a threat regardless of how many dives or how long a bird spends underwater in a contaminated area. This is likely to vary by marine bird foraging guild and species (Peakall et al. 1987; Stephenson 1997; Fig. 2). Seasonality also influences bird foraging behaviour, where an increase in the proportion of time spent diving may be seasonally influenced by energetic demands or resource availability (Burke and Montevecchi 2018). Additionally, factors such as spill and environmental conditions are likely to affect the persistence of a harmful concentration of chemically dispersed oil. Understanding the period for which a chemically dispersed oil spill is estimated to maintain a harmful concentration will help decision makers estimate its relative exposure risk compared to an untreated spill, which among other considerations should be incorporated in NEBA.

## Indirect effects

In addition to the direct effects of chemically treated oil, indirect effects on marine birds are also critical in understanding the full scope of impacts of dispersant use to marine ecosystems as a whole (Velando et al. 2005; Quigg et al. 2021).

### Toxicological effects on prey

Toxicity of dispersant–oil mixtures are dependent on several factors (e.g., effectiveness of dispersants, concentration, oil type, weathering of oil, etc.) (NRC 2005; Lee et al. 2016). Modern dispersants are reported to have relatively low levels of toxicity to aquatic organisms compared to earlier formulations (NRC 2005; NASEM 2019; Stroski et al. 2019). Some studies indicate that dispersants alone are toxic to aquatic organisms (Almeda et al. 2013; DeLeo et al. 2016; Echols et al. 2016; Ruiz-Ramos et al. 2017; reviewed by Stroski et al. 2019), or that dispersant–oil mixtures are as toxic as oil (Hemmer et al. 2011; NASEM 2019). In some cases, dispersant–oil mixtures can be more toxic than oil alone (Almeda et al. 2013, 2014; Laramore et al. 2014; Vignier et al. 2015; DeLeo et al. 2016; Echols et al. 2016; Ruiz-Ramos et al. 2017; Johann et al. 2020). Fingas (2008) found in his review that most research concluded that chemically dispersed oil was more toxic than physically dispersed oil.

While effects continue in modern formulations, acute and sublethal severity varies by species (Fingas 2008, 2017; Stroski et al. 2019). The acute toxicity thresholds of dispersants are well documented for many marine taxa, including cnidarians, crustaceans, molluscs, fish, bacteria, seagrass, algae, and polychaete worms (e.g., George-Ares and Clark 2000; Stroski et al. 2019). Chemically dispersed oil can be toxic to marine biota that are present near the water surface immediately following dispersant application; however, due to the rapid dilution and biodegradation of chemically dispersed oil to subtoxicity levels, toxic effects may be short term (Prince 2015; Fig. 2). Notwithstanding, other research has found that dispersants and chemically dispersed oil inhibit the biodegradation of oil by altering the microbial community responsible for oil biodegradation (Kleindienst et al. 2015; Rahsepar et al. 2016). Additionally, Hickl and Juarez (2022) found that dispersants may enhance or inhibit oil biodegradation depending on the oil-degrading bacteria concentration present in the environment. Diel vertical migration of organisms into contaminated waters can further increase the redistribution of contaminants into deeper waters and subsequent exposure of deep-pelagic organisms (Sutton et al. 2020).

Polycyclic aromatic hydrocarbons (PAHs) are widely known to have lethal and sublethal effects on fish (such as embryo abnormalities, early-stage mortality, DNA damage, lesions, swimming impairment, and reproductive failure) (Honda and Suzuki 2020; Sutton et al. 2020). Dispersion (natural or chemical) of oil increases the concentration of PAHs by an estimated 5–50 times (reviewed in Fingas 2017), leading to increased bioavailability and uptake of PAHs in fish (Ramachandran et al. 2004; Schien et al. 2009; Esteban-Sanchez et al. 2021). Chronic exposures of oil-contaminated sediments and suspended organic matter by affiliated species (e.g., bivalves, fish, sea ducks, sea otter (*Enhydra lutris*)) continued long after the *Exxon Valdez* oil spill and delayed recovery in some taxa for years to decades (reviewed in Peterson et al. 2003 and Esler et al. 2018). In Canada, affiliated species such as blue

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mussels (*Mytilus* sp.) and fish such as sand lance (*Ammodytes* sp.) are important prey to many marine bird species. Given the enhanced toxicity of chemically dispersed oil-contaminated sediments, the risk of exposure to sediment-affiliated species has unknown implications for marine birds.

Beyond facilitating PAH uptake, nonlethal dispersant effects to fish and other prey species include impaired sperm fertilizing ability (Beirão et al. 2018), oxidative stress, gene expression, embryo mortality and abnormalities, and impaired immune system and liver function (reviewed in Stroski et al. 2019). Even at low-level exposure, early life stages of many marine taxa are very sensitive (Kinner 2020; NRC 2005; Stroski et al. 2019). Cold-water species, such as those found in Canada, might have similar sensitivity to oil constituents as temperate species effects may take longer to exhibit (NASEM 2019).

Despite the ongoing establishment of toxicity thresholds, study organisms used for dispersant toxicity testing may not adequately represent local native species, or adequately evaluate long-term effects (DFO 2021). Although lab-based studies provide information on individual (and usually acute) effects, few studies have looked at how exposure may affect populations through reduced fitness, reproduction, and recruitment from long-term and sublethal effects which affect population viability (Vikebø et al. 2015; NASEM 2019). Given this, Stroski et al. (2019)suggests that other response alternatives to dispersants may be advisable for spills that occur within especially sensitive or productive habitats for prey (e.g., spawning locations).

Following large oil spills, marine birds can show delayed recovery. These are due in part to reductions in prey availability resulting from oil-contaminated sediment, changes in prey behaviour, cascades of indirect effects, or direct toxicity effects to prey (see, for example, Golet et al. 2002; Peterson et al. 2003; Velando et al. 2005; Irons et al. 2000; Moreno et al. 2013). Prey-related recovery limitations may be further compounded when accounting for acute and sublethal effects resulting from the introduction of dispersants in the water column and ocean floor (Fig. 2).

## Harmful algal blooms

Harmful algal blooms (HABs) have been documented after events in which oil spills have been treated with dispersants (e.g., Smayda 1997; Ozhan and Bargu 2014; Liu et al. 2021). The disrupting effects of oil and dispersants on algal and bacterial communities and available nutrients may promote the formation of HABs (Almeda et al. 2018; Park et al. 2020; Kamalanathan et al. 2021). Surveillance of HABs and research on how dispersant use affects their formation is limited within cold-water marine environments typical of Canada's oceans. Given that many toxin producing species occur in these environments (Pućko et al. 2019), more research will help to determine the extent to which dispersant use may promote HABs.

The life-history strategies of many marine birds make them vulnerable to toxic effects of HABs (Gibble and Hoover 2018). Ingestion can cause central nervous system impairment, mortality, disorientation, and morbidity in birds (Fritz et al. 1992; Work et al. 1993; Bargu et al. 2012; Ayala et al. 2013; Shearn-Bochsler et al. 2014). Plumage fouling caused by *Akashiwo sanguinea* causes loss of waterproofing, hypothermia, illness, and death (NRC 2005; Jessup et al. 2009).

HAB-induced marine bird mass mortality events are well documented for many species (e.g., Coulson et al. 1968; Jessup et al. 2009; Phillips et al. 2011; Ayala et al. 2013; Jones et al. 2017; Van Hemmert et al. 2021). In Canada, the Canadian Wildlife Service maintains an incidental archive of avian mortalities attributed to algae blooms, although the resulting effects from dispersant-associated HABs are not well known.

## Habitat effects and persistence

There is a need to consider the potential effects of dispersant application on marine bird habitat, particularly when applied near areas of important seasonal aggregations (e.g., breeding and foraging habitats) or within ecologically significant boundaries (e.g., critical habitat, important bird areas; Fig. 2). Additionally, how currents and winds may transport chemically dispersed oil relative to these locations will be important considerations in an NEBA.

Despite the difficulty of discerning the impacts to habitats and tracking the fate of dispersants, researchers have found that dispersants reduce biodiversity resistance and resilience, and increase oiled sedimentation, which reduces the rate of biodegradation (Khelifa et al. 2008; Passow and Lee 2022; Zerebecki et al. 2022). Biodegradation of dispersants is also limited when dispersant-treated oil becomes weathered, mixing with sand, which protects it from dissolution and biodegradation (White et al. 2014). Conversely, older research from the 1980s has shown that dispersants reduce the incorporation and persistence of oil within sediments (reviewed in NASEM 2019).

Experimental results show that low temperatures, such as those found in the Canadian marine environment, slow the degradation process of dispersant surfactants (Campo et al. 2013; reviewed in Péquin 2022; however see McFarlin et al. 2018). In addition to experimental findings, field observations have revealed that dispersant components such as dioctyl sodium sulfosuccinate (a surfactant in many dispersants and major component of Corexit) may persist for long periods of time, likely due to their chemical stability (Farahani and Zheng 2022). Post-application, dispersants have been found in deep water after 64 days, in deep-sea coral after 6 months, and on beached oil–dispersant–sand patties after 4 years (Kujawinski et al. 2011; White et al. 2014; McDaniel et al. 2015). This indicates that dispersant removal from the environment is likely circumstantial. Claims that the long-term effects of dispersant use are negligible or unlikely, based on the premise that dispersant dilutes and degrades rapidly, should be considered with caution until further research is undertaken. More information is needed to better understand the impacts of dispersants on marine bird habitats, especially for conditions and climates representative of colder coastal ecosystems.

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### Bioaccumulation of contaminants

The uptake and bioaccumulation of PAHs have been documented for various marine taxa, as has PAH metabolism and elimination (Eisler 1987; Honda and Suzuki 2020). The rate of the bioaccumulation is generally found to be greater for vertebrates than marine invertebrates but still relatively less studied among marine birds (reviewed in Meador et al. 1995). Experimental evidence suggests that an increased uptake, biotransfer, and bioaccumulation of highly toxic, low-soluble PAHs may occur in marine food webs begin with the ingestion of chemically dispersed crude oil by low-trophic organisms such as zooplankton (Almeda et al. 2013; Almeda et al. 2014). These organisms are typically consumed in large quantities by higher trophic levels, including marine birds, facilitating the biotransfer and bioaccumulation of PAHs (Buskey et al. 2016).

PAH bioaccumulation potential is also understood to be higher in sedentary filter-feeding organisms (Honda and Suzuki 2020). Gao et al. (2019) demonstrated that the interaction of suspended sediment with dispersant promotes the sinking of oil thereby facilitating entrainment in sediments, the content of PAHs increasing with increasing dispersants-to-oil ratio. Few studies available (e.g., Falk-Petersen et al. 2007; Nørregaard et al. 2015) indicate that PAH metabolism may be inhibited in organisms exposed to dispersant–oil mixtures to some degree, potentially facilitating trophic bioaccumulation. How chemically dispersed oil affects interaction with sediments and organic matter, or the variability in bioaccumulation and biotransfer of PAHs between taxa is an essential component to understand related trophic impacts to marine birds.

## Integration with NEBA

### The NEBA framework

Net Environmental Benefits Analysis is one of several existing decision-making frameworks used by various international jurisdictions for evaluating oil spill response alternatives. It is used to determine the best operational response before and during an oil spill to minimize the overall impacts to people, resources, and the environment (IPIECA 2015). The establishment of a national framework for enhancing oil spill response in Canada is being developed, and a possible framework has been made available for feedback (Transport Canada 2022). The process used in this framework, like NEBA, evaluates whether there is a net environmental benefit across potential response measures as a means to inform decision-making during an oil spill. The steps involved in the NEBA process are (1) compile and evaluate data and information to identify an exposure scenario and potential response options, and to understand the potential impacts of that spill scenario on receptors, (2) predict the outcomes of the potential response options for the given scenario, (3) balance trade-offs by weighing a range of ecological, socioeconomic, or cultural benefits and drawbacks resulting from each feasible response option, and (4) decide which option will minimize the impacts for a given spill scenario (Interspill 2012; IPIECA 2015).

Since the window of opportunity for dispersant application following a spill can be short, the appropriateness of application should be considered in the NEBA process and be informed by current understanding of the potential impacts on various receptor organisms. When the decision to use dispersants has been supported by a NEBA, their use has the potential to mitigate some of the impacts of an oil spill as a primary or integrated response (DFO 2021). It is recognized, however, that while NEBAs aim to reduce the impacts of an oil spill there are no response options, dispersant use included, that are entirely effective or completely without risk (Passow and Lee 2022).

### Assessing the trade-offs

Evaluating the trade-offs associated with dispersant use is a complex and difficult task faced by oil spill responders (NRC 2005). The assumption that the use of dispersants will reduce oil exposure to marine receptors, or that oil–dispersant mixtures are less harmful than oil alone, is often a key deciding factor and important consideration when balancing the trade-offs in the decision process on whether to use dispersants for an oil spill scenario (NRC 2005).

Spilled oil can have long-term impacts on marine bird populations and their recovery can take years if recovery is possible (Cairns and Elliot 1987; Peterson et al. 2003; Esler et al. 2018). Uncertainty remains over the acute and long-term effects of dispersant use to marine birds and to what extent they may reduce the impacts of oil to marine bird populations or habitats or lessen their recovery times. An additional consideration is that marine bird species may vary in the degree of impact and rate of recovery they experience from an oil spill event. This reinforces the notion that oil spill response decision-making frameworks should incorporate site- and spill-specific considerations to evaluate the benefits and costs and acknowledge the trade-offs associated with any given response option. This includes accounting for factors like seasonality, conservation status, as well as the ecological considerations of indirect impacts to prey and habitat.

Where trade-offs are concerned, no decision is likely to satisfy all response partners and interest groups, as protection goals and priorities will differ. Given this, decisions should be made transparently and with reference to supporting information (Grote et al. 2018). This is especially true for trade-offs regarding marine bird protection as they are often identified as a high priority. Mitigating the effects of an oil spill on marine birds, however, is just one component of a framework balancing myriad factors and other valued resources that may have contradictory net benefits. Additionally, it is difficult to weigh the importance of each component considered within this framework. This in turn emphasizes the need to quantify as precisely as possible the benefits and costs of dispersant use when considering the mitigation of oil spill impacts on a particular resource. As such, an important part of these formalized decision-making frameworks, beyond transparency, is a structure that facilitates discussion and the identification of knowledge gaps that informs science and policy.

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## Information gaps and uncertainties

Despite what is known about how dispersants interact directly with marine birds, their prey, and their habitats, many outstanding information gaps influence our ability to predict outcomes and make informed recommendations on their use. Briefly summarized, these include

- the direct and indirect effects and impacts to marine birds from dispersant use (especially Corexit® 9500), and their severity relative to untreated oil.
- the long-term, chronic, sublethal, and delayed effects to marine birds, their prey, and habitats from dispersant use and persistence in the environment.
- how exposure to untreated oil and chemically dispersed oil differs between foraging guilds of marine birds.
- whether, and to what extent, the results of lab-based studies involving birds or other biota are representative of marine birds under natural conditions.
- unintended and unforeseen interactions between marine biota, oil, and dispersants that may include HABs, or bioaccumulation and other trophic and population dynamics.
- whether reduction of an oil slick at the surface from dispersant use will result in a proportional reduction in lethal and/or sublethal effects (Albers and Gay 1982).

## Recommendations

Bridging current information gaps and reducing the uncertainty we incorporate into the decision-making process for oil spill response will minimize, as much as possible, the effects and impacts to marine birds. While using conservative assumptions to account for these uncertainties is our best option, we recommend the following approach in addressing these uncertainties.

- Routinely incorporate considerations for marine birds, habitats, and prey into NEBA (or similar) decision-making processes, to evaluate merits of dispersant use based on both the benefits and the costs. This should include POE models, species and foraging guild-specific knowledge as appropriate, integrating best-available science and information with local knowledge.
- Where uncertainties and information gaps exist in an NEBA (or similar) decision-making process, greater transparency is needed in communicating these shortcomings. Documenting decisions, including how uncertainties may affect outcomes. Paired with marine bird monitoring strategies, this will help to address incident-specific information gaps and inform future decision-making processes.

To address information gaps associated with the impacts of dispersant use we offer several recommendations to improve informed, science-based decision-making on dispersant treatment strategies and improve predictions on outcomes to marine birds.

- Continue preliminary research by Peakall et al. (1987) in investigating the extent to which different marine bird foraging guilds are susceptible to chemically dispersed oil exposure relative to an untreated oil spill, at different dispersant efficiencies.
- Incorporate targeted or opportunistic field studies and experiments into controlled spill/dispersant application scenarios and incident response to assess the response of marine bird species to chemically dispersed oil.
- Investigate the relevance of laboratory studies on marine birds (e.g., toxicity testing and mechanical interactions of dispersants) to real-world oil spill scenarios.
- Continue research into improved dispersant formulations, or alternatives, that are less harmful to marine birds, prey, and habitats (e.g., see Dannreuther et al. 2021 for a review of emergent dispersant technologies; Omarova et al. 2018; Guo et al. 2019; Kurita-Oyamada et al. 2020; Farahani and Zheng 2022).
- Advance research on the efficacy of dispersant application at varying volumes and concentrations as well as under a range of conditions, particularly representative of conditions and climates of northern coastal ecosystems, to determine the fate and persistence of dispersants.
- Continue to compile and share information and lessons learned on the relative impacts and sensitivities of marine bird species in Canada to chemically dispersed oil.

## Conclusion

Protecting marine birds from the effects of spilled oil is a priority given their documented direct and indirect sensitivities to hydrocarbon pollution, which includes both impacts to prey and habitats. The goal of protecting marine birds from oil spills through dispersant application is clear; however, our scientific understanding of how dispersants and chemically dispersed oil affect marine birds directly and indirectly is still in its infancy. The complexity of how natural processes and interspecific interactions influence on hydrocarbon fate, bioaccumulation, biodegradation, persistence, and recovery factors may be altered by dispersants is only beginning to be explored. Our understanding is further impeded by the need to disentangle the effects of oil, dispersant–oil mixtures, and environmental variability.

Given current knowledge about dispersants, their use is not without the risk of affecting marine birds directly or indirectly. Almost two decades have lapsed since the NRC's (2005) recommendations, yet there has been limited advancement in our understanding of the net benefits of dispersant use. This includes how they compare to an untreated oil spill under a variety of environmental conditions and among marine bird species. To better account for the relative value of the benefits and costs of dispersant use to marine birds, we have offered several recommendations to improve decision-making outcomes, which focus on the direct and indirect effects of dispersant use to marine birds, their prey and habitat. Concurrently, it is important to prioritize the development of less toxic oil spill response measures that have the potential to replace current dispersant formulations. The expanded use of dispersants necessitates increased responsibility in addressing outstanding information gaps and uncertainties on various receptor organisms, marine birds being one. Greater

transparency is needed in communicating our uncertainties and greater effort is needed to reduce them.

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### Data availability

There are no research data associated with this review.

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The authors declare there are no competing interests.

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# Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A® to the *Brachionus plicatilis* species complex (Rotifera)

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ABSTRACT

Using the marine rotifer *Brachionus plicatilis* acute toxicity tests, we estimated the toxicity of Corexit 9500A®, propylene glycol, and Macondo oil. Ratios of 1:10, 1:50 and 1:130 for Corexit 9500A®:Macondo oil mixture represent: maximum exposure concentrations, recommended ratios for deploying Corexit (1:10–1:50), 1:130 the actual dispersant:oil ratio used in the Deep Water Horizon spill. Corexit 9500A® and oil are similar in their toxicity. However, when Corexit 9500A® and oil are mixed, toxicity to *B. manjavacas* increases up to 52-fold. Extrapolating these results to the oil released by the Macondo well, suggests underestimation of increased toxicity from Corexit application. We found small differences in sensitivity among species of the *B. plicatilis* species complex, likely reflecting phylogenetic similarity. Just 2.6% of the water-accommodated fraction of oil inhibited rotifer cyst hatching by 50%, an ecologically significant result because rotifer cyst in sediments are critical resources for the recolonization of populations each Spring.

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## 1. Introduction

The April 2010 oil spill in the Gulf of Mexico discharged 4.9 million barrels of crude oil from the Macondo well (OSAT/NOAA report, 2010). One of the first responses was to apply more than 1 million gallons of the oil dispersants Corexit 9527A® and Corexit 9500A® to the sea surface, and more than 770 thousand gallons to the sub-sea (On Scene Coordinator Report DWH, 2011). This large scale application of oil dispersants, motivated us to examine the effects of the dispersants on toxicity, especially given the limited toxicity information that is available (Judson et al., 2010).

Although oil dispersants are preapproved for this use and their deployment is widespread, there are doubts in the regulatory community about the efficacy of dispersants to ameliorate the biological impacts of oil spills because of the poor understanding of oil dispersant toxicity (Singer et al., 1998). Rigorous toxicological comparison of untreated and dispersant-treated oil is complicated by the fact that when oil, seawater, and dispersants are mixed, a complex multiphase system results. In this complex system, aquatic organisms can be exposed to many toxicants, in many forms, which can have several modes of action (National Research Council, 1989). Moreover, chemical dispersion of oil can yield: (1) dissolved petroleum hydrocarbons; (2) dissolved dispersant surfactants; (3) mixed droplets of bulk oil and surfactants (often in micellar form); and (4) nonmicellar, particulate bulk oil (Singer et al., 1998).

A second important issue for determining the effects of dispersants, is the separate and combined toxicity of the dispersant and the crude oil droplets. Toxicity became an important issue in the late 1960s and early 1970s when application of toxic products resulted in substantial loss of sea life (Fingas, 2002). Since that time, dispersants have been formulated to minimize toxicity to aquatic organisms. For example, the LC50 values of dispersants used in the early 1970s ranged from about 5 to 50 mg/L to the rainbow trout in 96 h exposures. In contrast, LC50s for dispersants available today vary from 200 to 500 mg/L and contain a mixture of surfactants and a less toxic solvent (Fingas, 2002). Nonetheless, use of oil dispersants remains a controversial countermeasure to minimize the impact of oil spills. Their ecological effects depend on whether oil dispersion increases or decreases exposure of aquatic species to toxic components of oil (Ramachandran et al., 2004). Ramachandran et al. (2004) evaluated whether fish exposure increased to polycyclic aromatic hydrocarbon (PAH) in dispersed oil relative to equivalent amounts of the water-accommodated fraction (WAF). They used fish cytochrome P4501A gene (CYP1A) induction in trout exposed to the dispersant Corexit 9500A, WAFs, and the chemically enhanced WAF dispersant of three crude oils. They concluded that Corexit 9500A® was not an inducer of CYP1A and it did not appear to affect the permeability of the gill surface to known inducers such as

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**Table 1**
Characteristics of the five strains of the *Brachionus plicatilis* species complex used in this work.

| Description of strain | Abbreviation | Location of original collection | GenBank accession number of coxI gene sequence |
|---|---|---|---|
| *Brachionus manjavacas* | MAN | Sea of Azov, Russia | AY785194 |
| *Brachionus plicatilis sensu stricto* | TOK | Tokyo, Japan | AY785175 |
| *Brachionus rotundiformis* | HAW | Hawaii, USA | HM024708 |
| *Brachionus* sp. | VER | Alvarado Lagoon, off the coast of Veracruz, Gulf of Mexico | JX644944 |

β-napthoflavone. Therefore, the use of oil dispersants will not increase the exposure of fish to hydrocarbons in crude oil.

The EPA required BP p.l.c. to use the *Brachionus plicatilis* acute toxicity test to assess the toxicity of oil dispersant mixtures in the Gulf of Mexico (U.S. EPA subsurface dispersant directive to BP, 2010). The species *B. plicatilis* has long been used in ecotoxicology to assess toxicity in marine waters (American Society for Testing Materials, 1998; Anon., 1998). It is one of the few cost-effective marine toxicity tests that can be replicated hundreds of times in a few days. *Brachionus plicatilis* was thought to be one species, and therefore only a single *Brachionus* marine species has been mostly used in toxicity tests, although at least 15 are believed to exist (Suatoni et al., 2006). Some of these may be more sensitive to toxicants or have other properties that make them more useful in toxicity assessments of marine waters. In light of the recent environmental catastrophe in the Gulf, it seemed prudent to systematically explore the full range of biodiversity of *Brachionus* species to identify the most sensitive species for marine toxicity assessment.

Therefore, the goals of our investigation are: 1) to study the effect of crude oil, Corexit 9500A® oil dispersant and its water-accommodated fractions on five *B. plicatilis* species complex lineages whose phylogenetic signature can be investigated and correlated with sensitivity to these toxicants, 2) assess the effects of a crude oil and Corexit 9500A® mixture at concentrations that are environmentally relevant.

## 2. Materials and methods

### 2.1. Sampling, resting egg hatching, and culturing

Geographical strains of marine *Brachionus* sp. were collected from 5 localities from several parts of the world (Table 1). The Veracruz strain is unable to produce cysts (at least under laboratory conditions) and therefore the culture was started from parthenogenetic females. Instant Ocean™ was use to prepare reconstituted seawater. Resting eggs of the other four strains were hatched in 15 psu reconstituted seawater approximately 15 cm below 40 W white fluorescent light bulbs. Rotifer were cultured in 3 mL in wells of a 9-well plastic plate filled with 15 psu reconstituted seawater, and the green alga *Tetraselmis suecica*.

### 2.2. Acute toxicity tests

We used the *B. plicatilis* acute toxicity test protocol described in Standard Methods (Anon., 1998) and in the American Society for Testing Materials (ASTM) protocol (ASTM, 1998). It is now understood that the species *B. manjavacas* is the species originally used to develop that protocol according to genetic analysis (Fontaneto et al., 2007). Instead of using neonates hatched from cysts (diapausing eggs) as described in protocol, we used neonates hatched from parthenogenetic eggs that were less than 24-h old. Toxicity tests with *Brachionus manjavacas* neonates hatched from cysts were also conducted to conform to the original Standard Methods and ASTM protocols and to compare results with neonates hatched from parthenogenetic eggs. A total of five independent replicates each consisting of 10 rotifer per well were conducted to obtain the Median Lethal Concentration (LC50) values for each treatment. The protocol for preparation of oil–water-accommodated-fractions (WAF) and enhanced water-accommodated-fractions (CEWAF) solutions for toxicity testing followed the recommendations of Singer et al. (2000). We stirred Macondo sweet crude oil with Instant Ocean® artificial seawater at 15 psu for 8-h with a magnetic stirrer to obtain the WAF's. LC50 values for crude oil, Corexit 9500A®, propylene glycol, which is a major component of Corexit 9500A® (Nalco Energy Services, 2012) and the Macondo oil fractions were calculated using probit models (Díaz et al., 2004).

### 2.3. Acute toxicity tests with Corexit 9500A:Macondo oil mix

Clark et al. (2001) suggest a 1:10 maximum exposure concentration for the Corexit 9500A®:oil mix. In contrast, the U.S. EPA (1995) recommended a 1:50 ratio. Therefore, we tested 1:10, 1:50 and 1:130 Corexit 9500A:Macondo oil ratios. This was accomplished by 8-h stirring of both the oil and the dispersant as previously described for preparing of WAF's. A different experiment consisted of adding 0.01% Corexit 9500A® (the 24-h NOEC value for Corexit with *B. manjavacas*) to a different set of Macondo oil concentrations to investigate synergistic effects during oil dispersion without stirring for 8-h. In this experiment the Corexit 9500A®:oil ratios were variable for each concentration ranging from 1:25 to 1: 500. Toxicity tests were done as described above. Five independent replicates each consisting of 10 rotifer per well were conducted to obtain the LC50 values for each treatment.

### 2.4. Reproductive and cyst hatching inhibition end-points

Reproductive tests were performed on neonates born from parthenogenetic *B. manjavacas* females according to the Standard Methods protocol (Anon., 1998). Twelve replicate neonates (five neonate rotifers per well), were exposed for 48 and 72-h to sublethal concentrations of Corexit 9500A® [$1 \times 10^{-6}$–0.001% (v/v)], Macondo oil [0.25–5% oil (v/v)], and propylene glycol [0.1–5% (v/v)] in 1 mL volumes in a 24-well plate with $1 \times 10^5$ cells/mL of *Tetraselmis suecica*. The 24-well plates were then placed in a bioclimatic chamber under continuous light at a temperature of 25 °C for 48 and 72-h. At the end of these incubation periods, we counted the number of individuals in each well and calculated *r* (the instantaneous growth rate).

Cyst hatching inhibition assays consisted in hydrating dry *B. manjavacas* cysts for three hours, then, exposing them to same sublethal concentrations as above of Corexit 9500A®, Macondo oil WAF's, propylene glycol, or a Corexit 9500A®:Macondo oil mix for 24 or 48-h periods under fluorescent light. The number of hatching and non-hatching cysts was recorded, compared to controls in which no oil mixtures were added, in twelve replicates performed in three different dates. Each replicate consisted of ten cysts.

### 2.5. Statistical analysis and interpretation of data

We performed a one-way analysis of variance (ANOVA) with three independent treatments (each with four replicates) to compare five toxicant concentrations against the negative control and Dunnett's test to determine significant differences between the means of each toxicant concentration versus the no toxicity control. This allowed determination of the NOEC (no observed effect concentration) and the LOEC (lowest observed effect concentration). The EC50 values (the concentration where a 50% reduction in either the *r*-value or cyst hatching percentage, was observed) were calculated by linear regression of the different toxicant concentrations and the *r* values or cyst hatching percentages.

### 2.6. DNA sequencing

Genetic analyses using the cytochrome *c* oxidase subunit 1 (COI) gene were conducted to verify the species of each rotifer isolate used in these experiments. To minimize algal contamination, rotifers were incubated in 15 psu artificial seawater for 30 min to allow the rotifer guts to clear digested algal material. Genomic DNA was extracted from fresh rotifer tissue (500–1000 rotifers) using the DNeasy Tissue Extraction Kit (Qiagen). A 713 nucleotide region of the COI gene (Palumbi, 1996) was amplified via the polymerase chain reaction (PCR) using either universal COI primers LCOI1490: GGTCAACAAATCATAAAGATATTGG and HCOI2198: TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al., 1994), or (VER strain only) degenerate COI primers modified from Folmer et al. (1994), dgLCO: GGTCAACAAATCATAAAGAYATYGG and dgHCO TAAACTTCAGGGTGACCAAARAAYCA (Meyer et al., 2005). Amplifications were performed in 10 μl volume solutions with 10–50 ng genomic DNA, 1 unit *Taq* DNA polymerase and a final concentration of 0.2 mM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2.5 mM $MgCl_2$, and 0.2 mM of each primer. Thermal cycling protocol conditions consisted of a denaturing step of 2 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 47 °C for 90 s and 72 °C for 90 s on an Eppendorf MasterCycler. PCR products from TOK and HAW strains were directly sequenced in both directions (Nevada Genomics Center, University of Nevada, Reno). PCR products from the VER strain were cloned using TOPO TA Cloning Kit (Invitrogen) due to amplification with degenerate primers prior to sequencing. All sequences were manually edited in BioEdit vers 7.0.5.3 (Hall, 1999) and aligned using ClustalW (Larkin et al., 2007). Similarity to other *Brachionus* species was determined in a BLAST search (Altschul et al., 1990) of sequences deposited in the NCBI GenBank nucleotide database (www.ncbi.nlm.nih.gov).

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**Table 2**
Comparison of lethal toxicity sensitivity among species of the *Brachionus plicatilis* species complex.

| Species/strain | 24-h LC50 | LC50 95% confidence limits | NOEC | LOEC |
|---|---|---|---|---|
| **Propylene glycol (mg/L)** | | | | |
| *B. manjavacas* from cyst | 39.41 | 30.32–51.23 | 5.15 | 10.31 |
| *B. manjavacas* parthenogenetic | 26.50 | 19.32–36.32 | 25.90 | 51.80 |
| *B. plicatilis s.s.* Tokyo strain | 39.15 | 24.04–63.75 | 51.54 | 77.31 |
| *B. rotundiformis* Haw strain | 26.56 | 18.40–38.34 | 25.77 | 51.54 |
| *Brachionus* sp. from Veracruz | 31.47 | 19.05–51.32 | 10.31 | 25.77 |
| **Corexit 9500A® (mg/L)** | | | | |
| *B. manjavacas* from cyst | 14.25 | 12.52–16.20 | 4.75 | 9.49 |
| *B. manjavacas* parthenogenetic | 10.39 | 8.69–12.42 | 9.49 | 14.24 |
| *B. plicatilis s.s.* Tokyo strain | 0.447 | 0.253–0.791 | <0.474 | 0.474 |
| *B. rotundiformis* Haw strain | 1.75 | 0.98–3.12 | 0.47 | 0.95 |
| *Brachionus* from Veracruz | 4.30 | 3.38–5.48 | 1.19 | 3.56 |
| **Macondo sweet crude oil % water-accommodated fractions** | | | | |
| *B. manjavacas* from cyst | 11.02 | 9.04–13.45 | 5.0 | 7.5 |
| *B. manjavacas* parthenogenetic | 5.43 | 3.98–7.42 | 7.5 | 10 |
| *B. plicatilis s.s.* Tokyo strain | 2.47 | 1.74–3.51 | 0.5 | 1.0 |
| *B. rotundiformis* Haw strain | 11.02 | 9.04–13.44 | 5.0 | 7.5 |
| *Brachionus* sp. from Veracruz | 19.33 | 14.65–25.49 | 10.0 | 12.5 |

### 2.7. Genetic analysis

To determine the relationship of the VER strain to other *Brachionus* species (Fig. 4), phylogenetic analysis of rotifers was assessed through neighbor-joining analysis of COI nucleotide sequences using PAUP* vers 4.0b10 (Swofford, 2002). Bootstrapping confidence values were determined over 1000 iterations. *B. calyciflorus* (GQ466414) was included as the outgroup based on this species' relationship to other brachionids in previous studies (Gómez et al., 2002; Suatoni et al., 2006).

## 3. Results

### 3.1. Acute toxicity test comparisons among species of the *Brachionus plicatilis* species complex

Acute toxicity of propylene glycol among the tested species of the *B. plicatilis* species complex ranged from LC50 = 26.50 mg/L *B. manjavacas* parthenogenetic to 39.4 mg/L for *B. manjavacas* hatched from cysts (Table 1). However, by comparing the 95% confidence limits, there were no significant differences among species in their acute toxicity response to propylene glycol.

The LC50s for Macondo oil acute toxicity ranged from 2.47 for *B. plicatilis sensu stricto* TOK strain to LC50 = 19.3 mg/L (*Brachionus* sp. VER strain). The decreasing sensitivities to Macondo oil were as follows: *B. plicatilis sensu stricto* > *B. manjavacas* parthenogenetic > *B. rotundiformis* = *B. manjavacas* from cysts = *Brachionus* sp. VER strain ($p < 0.05$ in all cases) (Table 2). The linear regression of the exposure concentration/response curve for Macondo oil for *B. manjavacas* hatched from cyst is shown in Fig. 1A.

For Corexit 9500A®, LC50s ranged from 0.447 for *B. plicatilis sensu stricto* TOK strain to 14.2 mg/L for *B. manjavacas* hatched from cysts (Fig. 1B). The decreasing Corexit 9500A® sensitivities were as follows: *B. plicatilis sensu stricto* > *B. rotundiformis* > *Brachionus* sp. VER strain > *B. manjavacas* parthenogenetic > *B. manjavacas* from cysts ($p < 0.05$ in all cases) (Table 2).

Acute toxicity of Macondo oil and Corexit 9500A® is similar in range (less than one order of magnitude) for most of the strains tested. However, Corexit 9500A® is consistently more toxic than propylene glycol. In the case of *B. manjavacas* there are no significant differences in the Corexit toxicity between females hatched from parthenogenetic eggs and females hatched from cysts. Likewise, there were few significant differences between parthenogenetic egg hatchlings and females hatched from cysts for Macondo oil ($p < 0.05$) (Table 2).

### 3.2. Reproductive and cyst hatching inhibition end-points

Reproductive tests were about 4-fold more sensitive than acute toxicity tests (Tables 2 and 3). For example, *B. manjavacas* hatched from cysts had a LC50 of 11.02% Macondo oil as compared to 2.55% EC50 for the reproductive test and the cyst hatching test. Both end-points follow similar dynamics, although for different time periods (Fig. 2). No significant differences ($p < 0.05$) were found when comparing the 48-h and 72-h EC50s of the reproductive test for both propylene glycol and Macondo oil (Table 3).

### 3.3. Synergistic effect of Corexit 9500A® and Macondo oil

Acute toxicity tests with 1:10, 1:50 Corexit 9500A®:Macondo oil mixtures resulted in 47–52-fold increases in toxicity (lower LC50 values in Table 4 and Fig. 3A and B). Similarly, addition of the 24-h NOEC concentration of Corexit 9500A® to Macondo oil increased toxicity by 27-fold (Fig. 3C). However, the 1:130 Corexit 9500A®:Macondo oil ratio mixture produced no increase in toxicity

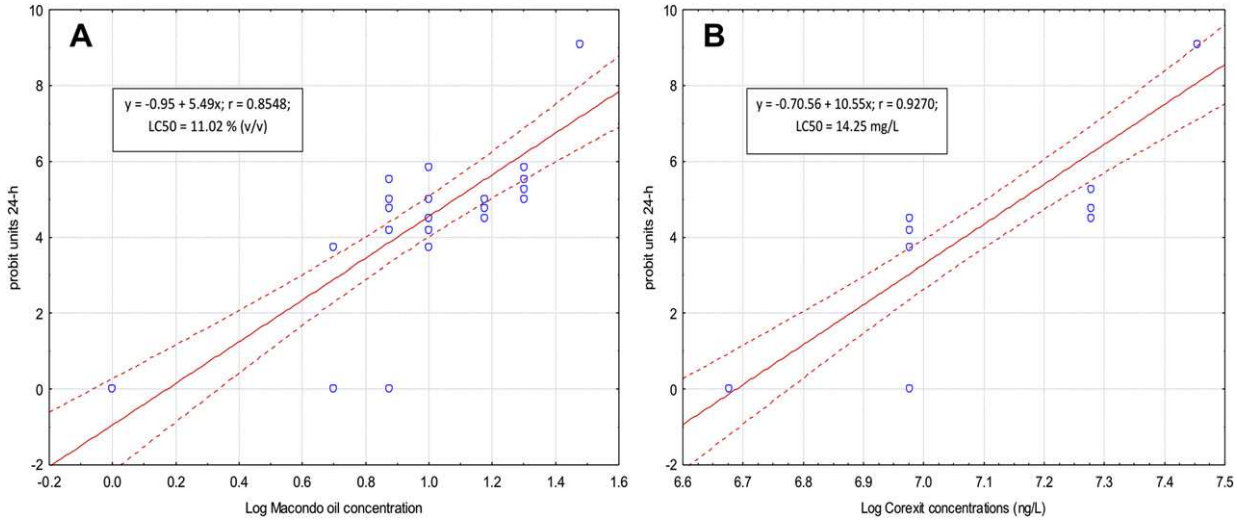


**Fig. 1.** Linear regression graphs of the results of the acute toxicity 24-h tests of *Brachionus manjavacas* neonates hatched from cysts exposed to: A) Macondo sweet crude oil. B) Corexit 9500A®. $N = 5$ for both treatments. Lines along the regression represent the 95% confidence intervals.

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**Table 3**
Comparison of toxicant sensitivity among marine *Brachionus* species/strains. Reproductive and hatching inhibition end-points.

| Species/strain | EC50 | EC50 95% confidence limits | NOEC | LOEC |
|---|---|---|---|---|
| **Propylene glycol (mg/L)** | | | | |
| *Reproductive test* | | | | |
| *B. manjavacas* parthenogenetic | 16.68 (48-h) | 13.39–20.05 | 1.03 | 5.15 |
| *B. manjavacas* parthenogenetic | 19.03 (72-h) | 14.24–20.80 | 5.15 | 10.31 |
| *Cyst hatching inhibition test* (24-h) | | | | |
| *B. manjavacas* | 1.4194 | 1.2028–1.6359 | 1.2885 | 2.0616 |
| **Macondo sweet crude oil % water-accommodated fractions** | | | | |
| *Reproductive test* | | | | |
| *B. manjavacas* parthenogenetic | 2.50 (48-h) | 2.03–2.97 | 1.0 | 2.5 |
| *B. manjavacas* parthenogenetic | 2.57 (72-h) | 2.05–3.10 | 1.0 | 2.5 |
| Cyst hatching inhibition test (24-h) | | | | |
| *B. manjavacas* | 2.55 | 1.70–3.39 | 1.0 | 2.5 |

(data not shown). The slope of the curve is steeper in Fig. 3A (1:10 ratio) and is reduced in Fig. 3B (1:50 ratio) and 3C (the NOEC-Corexit 9500A® experiment), as expected based on the toxicity of the 1:10 ratio > 1:50 ratio > NOEC-Corexit 9500A® treatments.

### 3.4. DNA sequencing and phylogeny analysis

COI sequences from MAN were confirmed to belong to *B. manjavacas* from GenBank (AY785194), TOK and HAW strains were consistent with sequences previously obtained from these strains and provided in GenBank (AY785175 and HM024708, respectively), confirming the TOK strain was *B. plicatilis sensu stricto* and the HAW strain was *B. rotundiformis* (Suatoni et al., 2006; Smith et al., 2011). The COI sequence for the VER strain was not previously represented in GenBank, but was most similar (*e*-value 3*e*-146 with 99% similarity) to two members (*B. ibericus s.s.* and *Brachionus* sp. Cayman clone) of the SM clade from the *B. plicatilis* complex (Gómez et al., 2002; Suatoni et al., 2006). The VER strain COI sequence was deposited in GenBank (JX644944). The COI phylogeny indicates a close relationship between the VER strain and a *B. ibericus* strain (okgu), supporting the hypothesis that the VER strain is a member of the SM clade from the *B. plicatilis* complex (Fig. 4).

**Table 4**
Synergistic effects of Corexit 9500A® when mix with Macondo oil ($n = 12$) with *Brachionus manjavacas* neonate hatchlings (less than 24-h old). $N = 5$.

| Experiment description | LC50/95% CL | NOEC | LOEC | Increase in toxicity[a] |
|---|---|---|---|---|
| 1:10 Corexit/Macondo oil Ratio (8-h stir) | 0.21/0.17–0.27 | 0.05 | 0.10 | 52.48-fold |
| 1.50 Corexit/Macondo oil Ratio (8-h stir) | 0.23/0.19–0.28 | 0.05 | 0.10 | 47.91-fold |
| NOEC-Corexit/Macondo oil Ratio (no stir) | 0.40/0.27–0.59 | <0.25 | 0.25 | 27.55-fold |

[a] Compare with *B. manjavacas* from cyst 24-h LC50 from Table 1.

## 4. Discussion

The 1:10, 1:50 and 1:130 ratios for the Corexit 9500A®:Macondo oil ratio mixture were chosen because they represent maximum exposure concentrations (Clark et al., 2001), or the recommended 1:10 to 1:50 ratios for Corexit application (U.S. EPA, 1995). The 1:130 ratio is the dispersant:oil mix actually used in the Deep Water Horizon spill: 4.9 millions of barrels of crude oil released into the Gulf of Mexico (OSAT/NOAA report, 2010), and about two million gallons of oil dispersant (mainly Corexit) applied to the Gulf of Mexico (On Scene Coordinator Report Deep Water Horizon, 2011). Corexit 9500A® and oil are more or less equivalent in toxicity (Fig. 1). However, when Corexit 9500A® and oil are mixed, our results show that Corexit 9500A®:Macondo oil at the recommended ratios increases acute toxicity up to 52-fold to *B. manjavacas*. Recall that this rotifer was endorsed by the EPA for this oil spill (U.S. EPA, Dispersant Monitoring and Assessment Final Directive for Subsurface Dispersant Application, 2010). Even if Corexit 9500A® was not mixed with oil for 8-h by stirring, its application increased toxicity of the dispersant:oil mixture by 27.6-fold. If we extrapolate the results of our experiments to the oil released by the Macondo well in the Gulf of Mexico, then the increase in toxicity by using Corexit, may have been markedly underestimated. What remains to be determined is whether the benefits of dispersing the oil by using Corexit 9500A® are outweighted by the substantial increase in toxicity of the oil: Corexit 9500A® mixture.

Surprisingly, there are few articles in the mainstream scientific literature recording synergistic effects of oil and oil dispersants resulting in toxicity increments (Mitchell and Holdway, 2000). Shafir et al. (2007) found that the dispersed oil and oil dispersants were more toxic to hard and soft coral species than crude oil, and

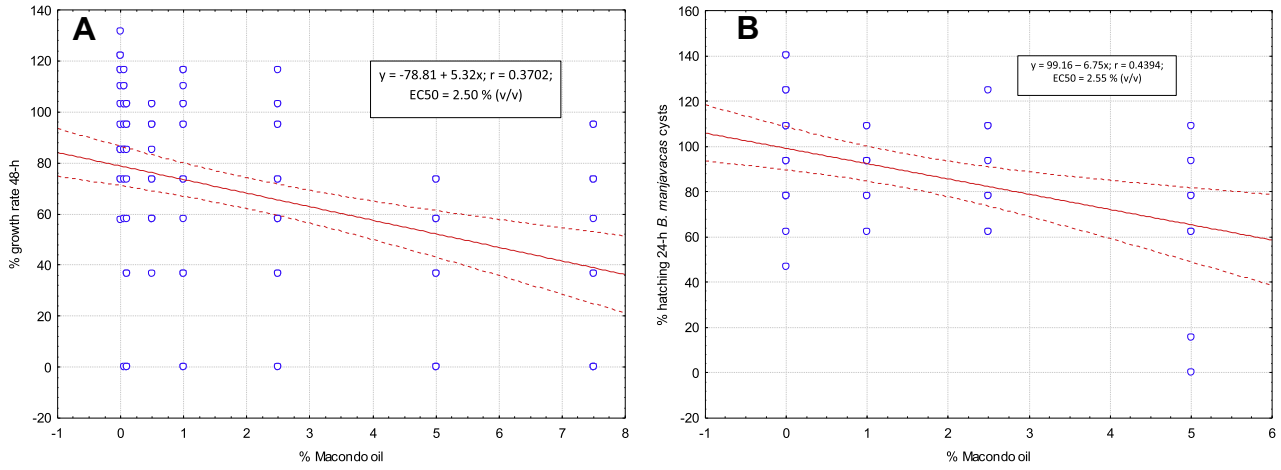


**Fig. 2.** Linear regression graphs of the results of the sublethal toxicity tests of *Brachionus manjavacas*. A) Reproductive test with neonates hatched from parthenogenetic eggs exposed 48-h to Macondo sweet crude oil. B) Cyst hatching inhibition 24-h exposure experiment.

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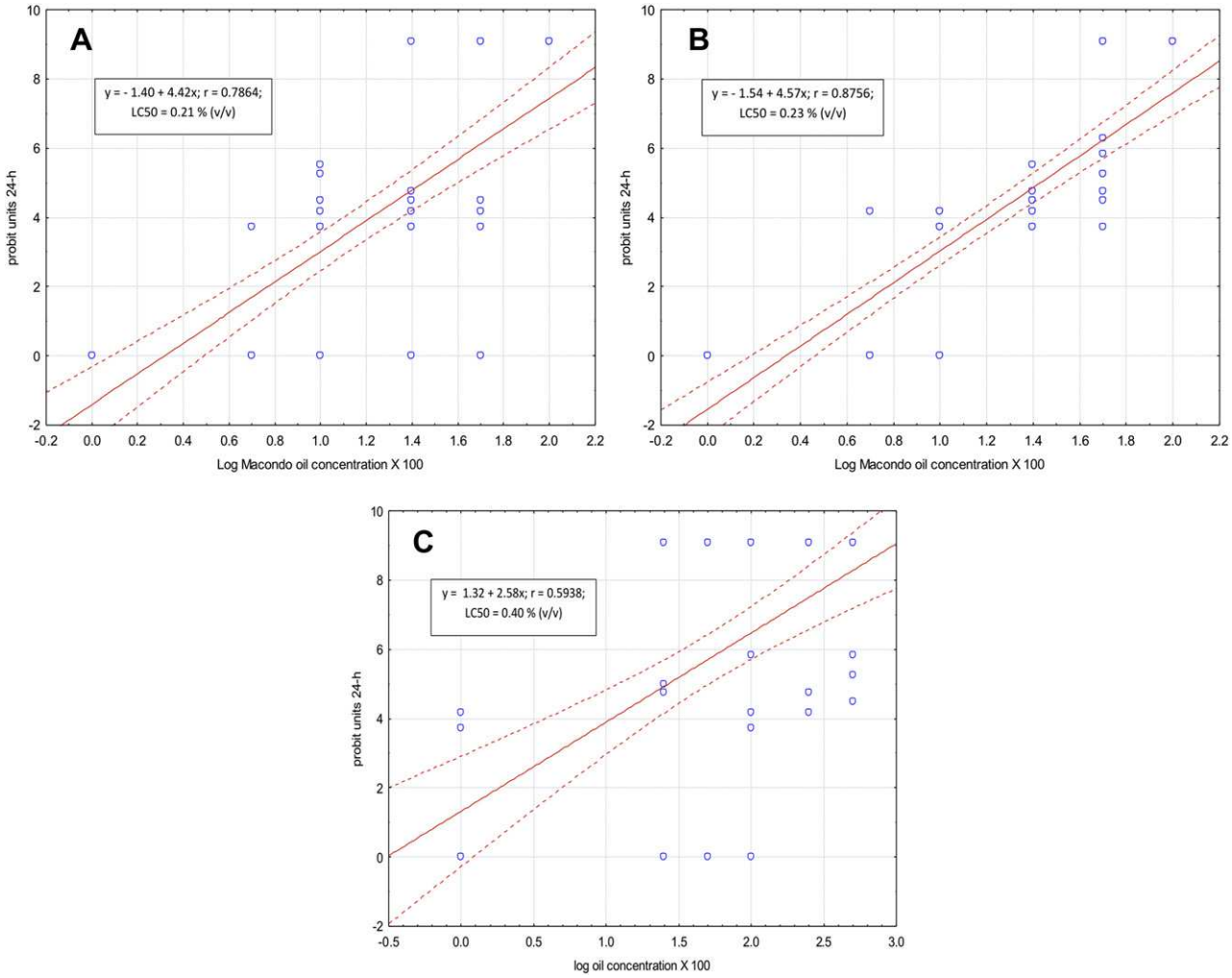


**Fig. 3.** Linear regression graphs of the results of the Corexit 9500A®:Macondo oil mixture experiments of *B. manjavacas* neonates hatched from cysts. A) 1:10 ratio with 8-h stirring prior to exposure. B) 1:50 ratio with 8-h stirring prior to exposure. C) Corexit 9500A®-NOEC added (0.01%) without 8-h stirring prior to exposure.

they recommend based on their findings, that no oil dispersant should be used near a coral reef. Milinkovitch et al. (2011) showed that juvenile, thin-lipped gray mullets (*Liza ramada*) exposed to oil dispersed chemically bioconcentrate more polycyclic aromatic hydrocarbons (PAH), and have higher mortality than mullets exposed to crude oil or oil that has been mechanically dispersed. Bhattacharyya et al. (2003) concluded that oil dispersants enhanced South Louisiana crude oil toxicity in microcosms containing three freshwater organisms. Hemmer et al. (2011) found that Corexit 9500A® has similar toxicity to other oil dispersants when mixed with South Louisiana sweet crude oil. Greer et al. (2012) performed wave tank experiments that demonstrated that Corexit 9500A® increase the toxicity of petroleum to herring embryos by increasing the amount of petroleum hydrocarbons in the water column. Wu et al. (2012) found that Corexit 9500A® enhanced toxicity by 30–360 times in terms of percentage of v/v because dispersion by Corexit 9500A® accelerated partitioning of hydrocarbons making them more bioavailable to rainbow trout embryos.

An interesting result is that the *Brachionus* sp. VER strain from the Gulf of Mexico was the most tolerant to Macondo oil (LC50 = 19.33% in Table 2). Perhaps, *Brachionus* sp. VER strain adapted to petroleum from natural seeps in the Gulf of Mexico. However, this explanation deserves further investigation.

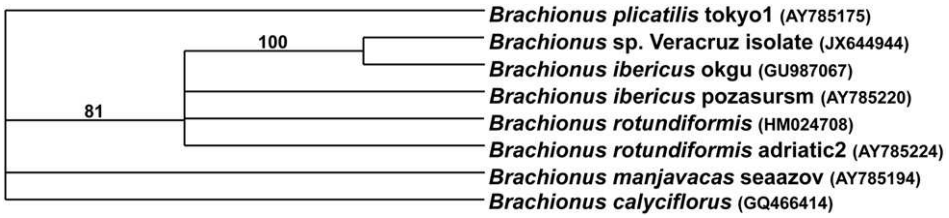


**Fig. 4.** Phylogenetic reconstruction of *Brachionus* rotifers using neighbor-joining analysis based on the cytochrome *c* oxidase subunit 1 gene. *B. calyciflorus* was included as the outgroup. Numbers on branches indicate support (≥70%) after 1000 bootstrap replicates. GenBank accession numbers are provided after species names.

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We confirmed the identity of each of the four *Brachionus* species used in the toxicant sensitivity experiments through genetic analysis of the mitochondrial COI gene (Fig. 4), and allows confirmation that differences in sensitivity among species of the *B. plicatilis* species complex are minor for the three toxicants tested, mostly within one order of magnitude. This likely reflects the phylogenetic closeness of these sibling species (Table 2). This may be important since the RotoxkitM™ (Microbiosystems. http://www.microbiotests.be), the commercial toxicity kit suggested by EPA to monitor Macondo oil toxicity (U.S. EPA, 2010), contains cysts of *Brachionus manjavacas* although it is labeled as *Brachionus plicatilis*. This is because the kit was developed when *Brachionus plicatilis* was recognized as a single species rather than a species complex. Further studies by several authors (see Segers, 1995; Ciros-Pérez et al., 2001; Suatoni et al., 2006; Fontaneto et al., 2007) have shown that the original lineage used to produce the RotoxkitM™ cysts is indeed *Brachionus manjavacas*, based upon the genetic signature of the COI sequence, and is well recognized today.

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# AN EXPERIMENTAL STUDY OF THE EFFECTS OF CHEMICALLY DISPERSED OIL ON FEATHER STRUCTURE AND WATERPROOFING IN COMMON MURRES (*URIA AALGE*)

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ABSTRACT: Following an oil spill in the marine environment, chemical dispersants, which increase oil droplet formation and distribution into the water column, are assumed to provide a net benefit to seabirds by reducing the risk of exposure to oil on the water surface. However, few data are available regarding acute, external impacts of exposure to dispersed oil. We evaluated the effects of known concentrations of dispersant and crude oil in artificial seawater on live Common Murres (*Uria aalge*). Waterproofing and microscopic feather geometry were evaluated over time and compared to pre-exposure values. Birds exposed to a high concentration of dispersant experienced an immediate, life-threatening loss of waterproofing and buoyancy, both of which resolved within 2 d. Birds exposed to oil, or a dispersant and oil mixture, experienced dose-dependent waterproofing impairment without resolution over 2 d. Alterations in feather geometry were observed in oil-exposed or dispersant- and oil-exposed birds and were associated with increased odds of waterproofing impairment compared to control birds. At a given contaminant concentration, there were no significant differences in waterproofing between oil-exposed and dispersant- and oil-exposed birds. We found that acute, external effects of oil and dispersed oil exposure are comparable and dose-dependent. Our results also indicate that a zero-risk assumption should not be used when seabirds are present within the dispersant application zone.

*Key words:* Common Murre, Corexit 9500A®, crude oil, dispersant, feather structure, oil spill, seabird, waterproofing.

## INTRODUCTION

Many seabirds, including the Common Murre (*Uria aalge*), are exquisitely sensitive to external oil contamination due to their unique reliance on plumage for thermoregulation and buoyancy. Structural properties of feathers establish a water-resistant barrier between the body and the environment, trapping an insulating layer of air against the skin (Jessup and Leighton 1996; Albers 2003). Oil exposure acutely disrupts the plumage barrier in a dose-dependent manner (Hartung 1967; Jenssen and Ekker 1988), allowing water to penetrate to the skin and resulting in loss of insulation and buoyancy, often to lethal effect (Leighton 1991; Newman et al. 2000).

Surface oil slicks present a high exposure risk for seabirds (French-McCay 2004). Spill response measures that reduce surface oil, such as chemical dispersion, have the potential to decrease exposure risk and thereby reduce morbidity and mortality (Peakall et al. 1987; National Resource Council [NRC] 2005). Chemical dispersants are typically applied to the water surface of an oil slick from a boat or airplane. Their detergent-like action increases oil droplet formation and promotes entrainment into the water column. This reduces surface oil, increases availability of petroleum to water-borne bacteria for biodegradation, and decreases shoreline habitat contamination (French-McCay 2004). Therefore, appropriate dispersant use is often considered to provide a net environmental benefit when compared to allowing oil to remain at the surface or to come ashore (Pond et al. 2000; Addassi et al. 2005; McCay and Graham 2014).

Although use of dispersant has a theoretic net benefit to seabirds, there are few data with



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which to evaluate risks. Lambert et al. (1982) and Jensen and Ecker (1991) documented increased basal metabolic rate and increased heat loss in birds experimentally exposed to oil and dispersant mixtures compared to controls; however, effects on plumage structure, differences in survival between oil and dispersant exposure, and change in effects over time were not explored. The lack of information was highlighted in a 1989 report by the NRC (1989), which called for research into the effects of dispersant and dispersed oil on water repellency of seabirds in realistic exposure conditions. In 2005, the NRC reiterated that the available data were insufficient to evaluate impacts of dispersant on seabirds and recommended additional study (NRC 2005). This knowledge gap remains today (Coastal Response Research Center 2017).

To assess the potential acute effects of oil and dispersant on seabirds, we ran a pilot study that examined impacts of a dispersant, Prudhoe Bay crude oil, or dispersant-treated oil on Common Murre feathers (Duerr et al. 2011). Exposure to dispersant alone, and a dispersant and Prudhoe Bay crude oil mixture, resulted in grossly decreased water repellency, altered microscopic feather geometry, and increased crystalline debris as compared to controls. However, limitations of that study precluded confident extrapolation of its results to effects in live birds. Therefore, we designed a multifactorial study to build on these preliminary data to evaluate the effects of known concentrations of dispersant alone and a dispersant and Prudhoe Bay crude oil mixture on feather geometry and whole-body waterproofing in a live seabird exposed in a single, simulated dive through a plume of contaminated water.

## MATERIALS AND METHODS

All procedures were conducted under the University of California Davis Institutional Animal Care and Use Committee Protocol no. 17350. Collection and release were conducted under US Fish and Wildlife Service Scientific Collecting Permit MB-101637-0 and in collaboration with the California Department of Fish and Wildlife. Methods were briefly described in Fiorello et al. (2016).

### Capture and husbandry

In December 2013, 40 Common Murre were captured on Monterey Bay, California, US (36°57′38″N, 122°0′7″W) using the Whitworth et al. (1997) technique. Birds were housed indoors in ambient temperatures (15.5–18.3 C) in freshwater pools (diameter 3.0–3.6 m, depth 1–1.3 m). Facility constraints prohibited the use of salt water except in exposure pools. All birds received night smelt (*Spirinchus starski*) ad libitum and were force-fed four to six fish once daily. Force-feeding was discontinued on study day 9 (2 d prior to initial waterproofing evaluation) and reinstituted on days 14 and 17. Itraconazole (Sporanox Oral Suspension, Amerisource Bergan, Chesterbrook, Pennsylvania, USA) was administered orally daily at 20 mg/kg body weight for prevention of aspergillosis. Sodium chloride tablets (Consolidated Midland Corporation, Brewster, New York, USA) were administered orally every other day to mitigate physiologic effects of freshwater housing (Frankfurter et al. 2012). Vitamin supplements (Seatabs, Pacific Research Laboratories, San Diego, California, USA) were administered orally every other day.

### Pre-exposure assessment

Initially, birds received physical examinations and evaluation of complete blood counts and plasma chemistry panels by a veterinarian, and individuals with abnormalities were excluded. Each resultant healthy bird was randomly assigned to a control or treatment groups (Tables 1, 2). Age class was estimated from plumage and supraorbital ridge prominence in a modification of Nevins and Carter (2003). The following baseline data were collected 2 d prior to exposure: body weight and pectoral muscle mass (normal, decreased, severely decreased; scored by palpation of pectoral muscle contour and keel prominence); attitude (alert, quiet, depressed, nonresponsive); hydration status (slight, moderate, or severe dehydration; scored by mucous membrane moisture and eyelid skin turgor); and plumage condition (scored by a single observer as poor, fair, good, or excellent by presence of broken, stripped, worn, or absent contour feathers). Waterproofing status (reflected by depth of wetness) was evaluated by visual and manual inspection and categorized as the estimated percent of body surface area superficially wet (SW; presence of water-logged feathers on the exterior plumage overlying dry skin) or wet-to-skin (WTS; regions of wet skin with or without overlying wet plumage). Categories were selected

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TABLE 1. Treatment groups, sample sizes, contaminant loading doses (mL/L) in the water, and measured contaminant concentrations (ppm) used for experimental exposure of Common Murres (*Uria aalge*) to oil (Prudhoe Bay Crude oil), dispersant (Corexit 9500A), and dispersed oil in artificial seawater.[a]

| Treatment group | *n* | Prudhoe Bay crude oil | | | Corexit 9500A | | |
|---|---|---|---|---|---|---|---|
| | | Loading dose mL/L | TPH concentration (μg/mL) | | Loading dose mL/L | Concentration (μg/mL) | |
| | | | Tank 1 | Tank 2 | | Tank 1 | Tank 2 |
| Control | 5 | 0 | 0.71 | 2.18 | 0 | RL | RL |
| DISP-L | 6 | 0 | RL | RL | 0.01 | 12.1 | 5.6 |
| DISP-M | 5 | 0 | RL | RL | 0.1 | 96 | 75 |
| DISP-H | 5 | 0 | RL | RL | 1.0 | 918 | 971 |
| OIL-L | 4 | 0.2 | 99 | 134 | 0 | RL | RL |
| OIL-M | 3 | 2.0 | 1,066 | — | 0 | RL | — |
| MIX-L | 5 | 0.2 | 88 | 99 | 0.01 | 6.3 | 8.4 |
| MIX-M | 3 | 2.0 | 1,128 | — | 0.1 | 78 | — |

[a] TPH = total petroleum hydrocarbon; RL = below reporting limit; DISP-L = low concentration dispersant; DISP-M = medium concentration dispersant; DISP-H = high concentration dispersant; OIL-L = low concentration oil; OIL-M = medium concentration oil; MIX-L = low concentration dispersed oil; MIX-M = medium concentration dispersed oil; — = a second exposure tank was not used due to small sample size in that group.

to identify birds that were completely unaffected (0%), mildly affected (1–25%), or moderately to severely affected (26–100%) by waterproofing loss, with the physiologic interpretation that greater than 25% of body surface area represents significant impairment which requires intervention for recovery (Stephenson 1997). Two feathers were plucked from the central-most portion of the ventrum for later comparison with postexposure changes. Additional behavioral and physiologic data were collected as part of this study, but will be analyzed separately as they do not directly relate to waterproofing and feather structural abnormalities.

### Exposure

The control group was exposed to artificial seawater while treatment groups were exposed to increasing concentrations of the dispersant Corexit (Corexit® 9500, Ecolab, St. Paul, Michigan, USA) alone (DISP) or in combination (MIX) with Prudhoe Bay crude oil (OIL) in artificial seawater (Table 1), with an industry-standard dispersant-to-oil ratio of 1:20 (Lewis and Aurand 1997; International Tanker Owners Pollution Federation 2014). Treatments were classified as low (L), medium (M), or high (H) according to DISP or OIL level (Table 1). To confirm exposure doses, total petroleum hydrocarbon and Corexit concen-

TABLE 2. Study timeline and data collected during experimental exposure of Common Murres (*Uria aalge*) to dispersant, oil, and dispersed oil in artificial seawater.[a]

| Study day | Event | Physical exam | Waterproofing | Feather sample |
|---|---|---|---|---|
| 1–6 | Capture | ✓ | — | — |
| 11 | Examination, baseline data collection | ✓ | ✓ | — |
| 13 | Pre-exposure evaluation | — | ✓ | ✓ |
| 13 | Postexposure evaluation | — | ✓ | ✓ |
| 14 | Day 1 postexposure evaluation | — | ✓ | — |
| 15 | Day 2 postexposure evaluation | ✓ | ✓ | ✓ |
| 16 | Cleaning | — | — | ✓ |
| 17 | Postcleaning evaluation | — | ✓ | — |
| 21–24 | Examination and release | ✓ | ✓ | — |

[a] ✓ = data collected; — = data not collected.

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tration were analyzed in water samples by the Petroleum Chemistry Lab (California Department of Fish and Wildlife, Sacramento, California, USA) using gas chromatography-mass spectrometry and standardized methodology in accordance with US Environmental Protection Agency Method 8015 (US Environmental Protection Agency 2014). Contaminant concentrations were representative of potential exposures in the upper 10 m of the water column shortly after a surface release (e.g., Kim et al. 2013) and within the reported total petroleum hydrocarbon ranges after the Deepwater Horizon spill (Sammarco et al. 2013). The DISP-H treatment was selected to model exposure of a seabird in the direct path of aerial or vessel-based dispersant application.

Two exposure tanks (308 L volume, 96 cm diameter, 45 cm deep) were filled with fresh water and Instant Ocean (Aquarium Sea Salt Mixture, Blacksburg, Virginia, USA) to 3.5% salinity. A circular current involving the entire depth of the water column was established using a 57 L/min aquarium pump. Contaminants were added via the pump intake line and circulated in the tank for 90 s; this interval was selected from pilot testing to allow full mixing of oil through the water column but minimize formation of a surface slick. Water samples were collected from the pump intake line immediately prior to bird exposure. Each exposure pool accommodated up to three birds simultaneously, so treatment groups were split into two groups for exposure in separate pools. Two to three birds in each group were placed simultaneously into exposure tanks and encouraged to dive by waving hands at the water surface. Birds were hand-captured starting at 75 s and held submerged to the neck until simultaneous removal at the 90-s mark. A 90-s exposure was selected to approximate a single dive (60 s; Ainley et al. 1990) with an additional 30 s to account for surfacing multiple times during the exposure dive. After exposure, waterproofing was evaluated and two feathers were plucked from the ventrum. Birds were then placed in a 600-L freshwater rinse pool for 60 min to simulate movement away from the plume and into uncontaminated water. A haul-out platform was introduced into the rinse pool if it appeared that birds were struggling to stay afloat and would not survive without assistance.

### Postexposure assessment

After exposure, birds were housed by treatment group in pens custom-designed for out-of-water seabird housing (Oiled Wildlife Care Network 2014). On days 1 and 2 after exposure, each group was placed in a freshwater pool for a 45-min evaluation period. A haul-out was provided at minute five and removed at minute 40, and birds were removed at minute 45. Waterproofing was assessed directly after removal. Two feathers were plucked from the ventrum after the day 2 evaluation.

### Cleaning, conditioning, and release

On day 3 after exposure, birds were cleaned, rinsed, and dried in a standardized manner (Oiled Wildlife Care Network 2014). The 2-d interval from exposure to cleaning was selected to allow documentation of effects over time without compromising ability to rehabilitate and release study subjects. Two feathers were plucked from the ventrum after cleaning. The day after cleaning, each group was placed in a freshwater pool for a final 45-min evaluation period followed by waterproofing assessment. Conditioning for release was initiated the following day. Birds were released in Monterey Bay after meeting preestablished criteria (Oiled Wildlife Care Network 2014) or approval by a veterinarian.

### Analysis

Collected feathers were suspended by the calamus and air-dried. Each rachis was cut to produce two samples (a 1-cm section centered on midpoint of the rachis and the distal tip) and mounted on glass slides with coverslips secured by Cytoseal (ThermoFisher Scientific, Waltham, Massachusetts, USA) at the margin. Two images were collected from opposite sides of the rachis at 100× magnification, and images were evaluated in QCapture Pro 7 software (QImaging, Surrey, British Columbia, Canada). Three measures were evaluated at three locations (Fig. 1): distance (measurement between barbs at 200 μm from the rachis), angle (measurement between rachis and barb), and clumping (ratio of number of barbules arising from a 0.5-cm section of barb 200 μm from the rachis and the number of clumps formed from those same barbules; modified from O'Hara and Morandin 2010). Angle and distance measures were evaluated in both the center and tip sections while clumping was only evaluated in the tip due to feather morphology. Analyses were performed separately for the tip and center sections due to differing levels of gross contamination and morphologic change.

Differences in distribution of morphologic and physiologic characteristics (e.g., age class, plumage condition) within and between exposure groups were assessed using Kruskal-Wallis H tests (KW) and one-way analysis of variance. The KW was used to investigate for differences in distribution of waterproofing scores across groups at each time period, and post hoc pairwise comparisons were performed with Dunn's (1964) procedure and Bonferroni correction for multiple

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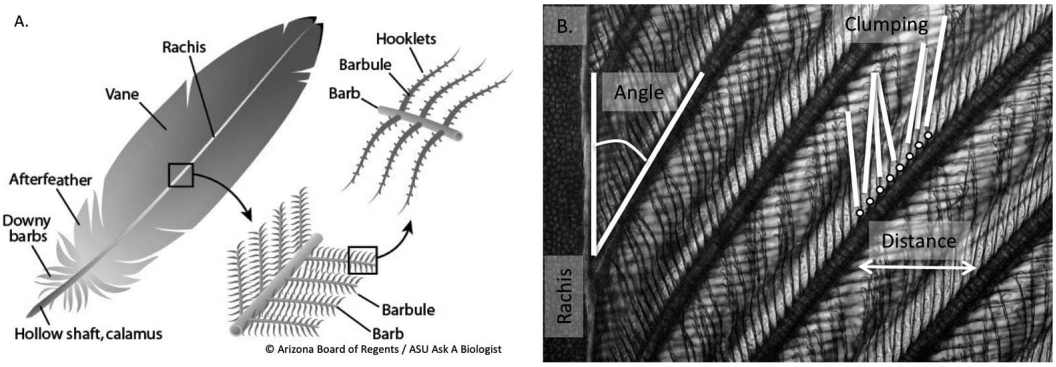


FIGURE 1. (A) Schematic representation of feather structure and (B) light microscopy image of a Common Murre (*Uria aalge*) feather at 100× light magnification. The light microscopy image (B) illustrates the measures used for quantification feather structural change after exposure of live Common Murres to oil (Prudhoe Bay Crude oil), dispersant (Corexit 9500A), and dispersed oil in artificial seawater. Angle is measured between the central rachis and a barb. Distance is measured between adjacent barbs 200 μm from the rachis. Clumping is the ratio of number of barbules arising from a 0.5-cm section of barb 200 μm from the rachis and the number of clumps formed from those same barbules. Schematic used with permission of Arizona Board of Regents, ASU School of Life Sciences, "Ask a Biologist" (https://askabiologist.asu.edu).

comparisons. Repeated measure random effects (mixed effects) models were used to evaluate differences in feather measures between treatments and control. Fixed effects for treatment, time period, and the treatment by time period interaction were included in the models, in addition to random effects for bird and feathers nested within birds, to account for the multiple measurements on each feather from each bird. Distance and clumping were log transformed to meet underlying homoscedasticity assumptions of the models. Post hoc comparisons were performed between exposure groups and control at each time point as well as between the three groups at the medium contaminant level. Feather characteristics (distance, angle, clumping) were averaged across feathers at each time point and evaluated as predictors of wetness. Generalized estimating equation approaches for repeated measures ordinal data, in the context of multinomial logistic regression with a cumulative logit link function, were used to assess how feather characteristics were associated with wetness and the difference by groups. Model building began with single feather characteristics, and variables with a *P*-value less than 0.1 were considered together in a joint model (including interactions between feather characteristics). Analyses were conducted in SAS (SAS Institute 2011) and SPSS (IBM 2013), with an alpha level of 0.05.

## RESULTS

Of 40 birds captured, four were excluded from the study and transferred to rehabilitative care due to chronic disease ($n$=3) or gross plumage oil contamination ($n$=1). Of the remaining 36 birds, four mortalities (three deaths and one euthanasia) occurred between the exposure and cleaning phases including birds from OIL-M ($n$=2), DISP-H ($n$=1), and MIX-M ($n$=1). Gross necropsy and histopathology revealed multiple abnormalities in each bird including bacterial pneumonia ($n$=3), air sacculitis ($n$=2), suspected viral bronchitis ($n$=2), and coccidial enteritis ($n$=1).

Of 36 birds enrolled in the study and observed during the pre-exposure exam, plumage condition was excellent ($n$=35) or good ($n$=1), with no gross evidence of molt or plumage contamination. There were no significant differences in distribution of age class (KW, $P$=0.663), plumage condition (KW, $P$=0.348), body condition (KW, $P$=0.663), attitude (KW, $P$=1.0), or hydration status (KW, $P$=0.817) across treatment groups or in mean body weight (analysis of variance, $F_{7,28}$=0.680, $P$=0.688). Similarly, the distribution of waterproofing scores was not significantly different between treatment groups before exposure (KW, SW $P$=0.143, WTS $P$=1.0).

Distribution of waterproofing scores (Table 3) was significantly different across treatment groups immediately after and on days 1 and 2

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TABLE 3. Waterproofing of Common Murre (*Uria aalge*) plumage as evaluated after exposure to oil (Prudhoe Bay Crude oil), dispersant (Corexit 9500A), and dispersed oil in artificial seawater. Data are presented for each exposure group from five time points: immediately before exposure, immediately after exposure, after 45-min in-water evaluation periods at days 1 and 2 after exposure, and after cleaning. Values are given for the proportion of each treatment group with percent of body surface area (0%, 1–25%, or 26–100%) superficially wet (SW) or wet-to-skin (WTS).

| | | Proportion of each treatment group with percent of body surface area SW or WTS at time points before and after exposure | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Before exposure | | | | Immediately after exposure | | | | Day 1 after exposure | | | | Day 2 after exposure | | | | After cleaning | | | |
| Treatment group[a] | Measure[b] | *n* | 0% | 1–25% | 26–100% | *n* | 0% | 1–25% | 26–100% | *n* | 0% | 1–25% | 26–100% | *n* | 0% | 1–25% | 26–100% | *n* | 0% | 1–25% | 26–100% |
| Control | SW | 5 | 0 | 1 | 0 | 5 | 0 | 0.8 | 0.2 | 5 | 0.2 | 0.8 | 0 | 5 | 0.2 | 0.8 | 0 | 5 | 0 | 1 | 0 |
| | WTS | | 1 | 0 | 0 | | 1 | 0 | 0 | | 0.2 | 0.8 | 0 | | 0.6 | 0.4 | 0 | | 0.4 | 0.4 | 0.2 |
| DISP-L | SW | 6 | 0 | 1 | 0 | 6 | 0 | 0.83 | 0.17 | 6 | 0.2 | 0.6 | 0.2 | 6 | 0.17 | 0.5 | 0.33 | 6 | 0.5 | 0.5 | 0 |
| | WTS | | 1 | 0 | 0 | | 0.17 | 0.83 | 0 | | 1 | 0 | 0 | | 0.5 | 0.5 | 0 | | 0 | 0.5 | 0.5 |
| DISP-M | SW | 5 | 0 | 0.4 | 0.6 | 5 | 0 | 0 | 1 | 5 | 0.4 | 0.6 | 0 | 5 | 0.4 | 0.6 | 0 | 5 | 0.4 | 0.6 | 0 |
| | WTS | | 1 | 0 | 0 | | 0.25 | 0.75 | 0 | | 1 | 0 | 0 | | 1 | 0 | 0 | | 0.2 | 0.4 | 0.4 |
| DISP-H | SW | 5 | 0 | 0.6 | 0.4 | 5 | 0 | 0 | 1 | 5 | 0.2 | 0.2 | 0.6 | 4 | 0.25 | 0.75 | 0 | 4 | 0.25 | 0.75 | 0 |
| | WTS | | 1 | 0 | 0 | | 0 | 0 | 1 | | 0.2 | 0.4 | 0.4 | | 0.5 | 0.5 | 0 | | 0.75 | 0.25 | 0 |
| OIL-L | SW | 4 | 0 | 1 | 0 | 4 | 0 | 0 | 1 | 4 | 0.25 | 0 | 0.75 | 4 | 0 | 0.25 | 0.75 | 4 | 0.25 | 0.5 | 0.25 |
| | WTS | | 1 | 0 | 0 | | 0 | 1 | 0 | | 0.5 | 0.5 | 0 | | 0 | 1 | 0 | | 0.5 | 0.25 | 0.25 |
| OIL-M | SW | 3 | 0 | 1 | 0 | 3 | 0 | 0 | 1 | 3 | 0 | 0 | 1 | 3 | 0 | 0 | 1 | 2 | 0 | 0.5 | 0.5 |
| | WTS | | 1 | 0 | 0 | | 0 | 0.33 | 0.67 | | 0 | 0 | 1 | | 0 | 0 | 1 | | 0 | 1 | 0 |
| MIX-L | SW | 5 | 0.2 | 0.6 | 0.2 | 5 | 0 | 0 | 1 | 5 | 0 | 0 | 1 | 5 | 0 | 0.2 | 0.8 | 4 | 0.5 | 0.25 | 0.25 |
| | WTS | | 1 | 0 | 0 | | 0 | 1 | 0 | | 0 | 0.8 | 0.2 | | 0 | 1 | 0 | | 0.25 | 0.25 | 0.5 |
| MIX-M | SW | 3 | 0 | 1 | 0 | 3 | 0 | 0 | 1 | 3 | 0 | 0 | 1 | 3 | 0 | 0 | 1 | 3 | 0 | 0.33 | 0.67 |
| | WTS | | 1 | 0 | 0 | | 0 | 0.67 | 0.33 | | 0 | 0 | 1 | | 0 | 0 | 1 | | 0 | 0.33 | 0.67 |

[a] DISP-L = low concentration dispersant; DISP-M = medium concentration dispersant; DISP-H = high concentration dispersant; OIL-L = low concentration oil; OIL-M = medium concentration oil; MIX-L = low concentration dispersed oil; MIX-M = medium concentration dispersed oil.

[b] SW = presence of water-logged feathers on the exterior plumage overlying dry skin; WTS = regions of wet skin with or without overlying wet plumage.

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TABLE 4. Results of a Kruskal-Wallis H test (KW) for differences in distribution of plumage waterproofing scores of Common Murres (*Uria aalge*) exposed to varying levels of oil (Prudhoe Bay Crude oil), dispersant (Corexit 9500A), and dispersed oil in artificial seawater. Plumage waterproofing was evaluated by estimating percent of body surface area superficially wet (SW) and wet-to-skin (WTS). At each time point after exposure, the distribution of waterproofing scores across treatment groups was compared to the scores of the control group at that time point and compared to the scores of all birds before exposure. Distribution of waterproofing scores across-treatment groups was significantly different from the control group immediately after exposure and on days 1 and 2 after exposure. Distribution of scores was not significantly different from control after cleaning. For all time points after exposure, distribution of scores was significantly different from the pooled scores of all birds before exposure.

| | | *P*-values for KW of distribution of waterproofing scores across treatment groups | | | |
|---|---|---|---|---|---|
| Comparison group | Waterproofing measure[a] | Immediately after exposure | Day 1 after exposure | Day 2 after exposure | After cleaning |
| Control group at each time point | SW | <0.001 | 0.006 | 0.003 | 0.084 |
| | WTS | <0.001 | <0.001 | 0.001 | 0.278 |
| All birds before exposure | SW | <0.001 | <0.001 | <0.001 | 0.004 |
| | WTS | <0.001 | <0.001 | <0.001 | <0.001 |

[a] SW = presence of water-logged feathers on the exterior plumage overlying dry skin; WTS = regions of wet skin with or without overlying wet plumage.

after exposure, both when compared to control and to pooled pre-exposure scores for all birds (KW, all $P<0.05$; Table 4). Most striking of these differences was a catastrophic loss of waterproofing in DISP-H, which was evident immediately after exposure and lessened over the subsequent 2 d (Figs. 2C, 3C). We observed negative effects on waterproofing from exposure to lower dispersant concentrations, but they were not as severe as in DISP-H (Figs. 2, 3). Waterproofing scores of all oil-exposed groups (OIL or MIX) worsened after exposure in a dose-dependent manner and did not resolve over time (Figs. 2, 3). There were no significant differences in distribution of scores between OIL and MIX groups at the same contamination level at each time period (KW, all $P=1.000$). The distribution of waterproofing scores was not significantly different across treatment groups after cleaning when compared to control (KW $P=0.278$; Tables 3, 4).

The log transformed distance in the tip section of feathers differed across groups over time. Values for each group were not different from control before exposure, apart from DISP-L, which had a smaller distance on average ($P=0.037$; Fig. 4). The magnitude of change in distance from pre-exposure to each time point after exposure varied significantly compared to the control ($P<0.001$; Fig. 4). Immediately after exposure, there was a significantly greater decrease in distance in OIL-L ($P=0.003$), OIL-M ($P<0.001$), MIX-L ($P=0.028$), and MIX-M ($P=0.002$) compared to control. At day 2 after exposure, there was a significantly greater decrease in distance in OIL-L ($P=0.043$), OIL-M ($P<0.001$), and MIX-M ($P<0.001$) compared to control. After cleaning, there were no significant differences from the control in the magnitude of the change in distance from pre-exposure to postcleaning. There were no significant differences between groups in distance at the center section of feathers.

In a similar fashion, clumping in the tip section of feathers varied significantly between exposure groups over time ($P<0.001$; Fig. 5). Before exposure, values for each treatment group were not different from

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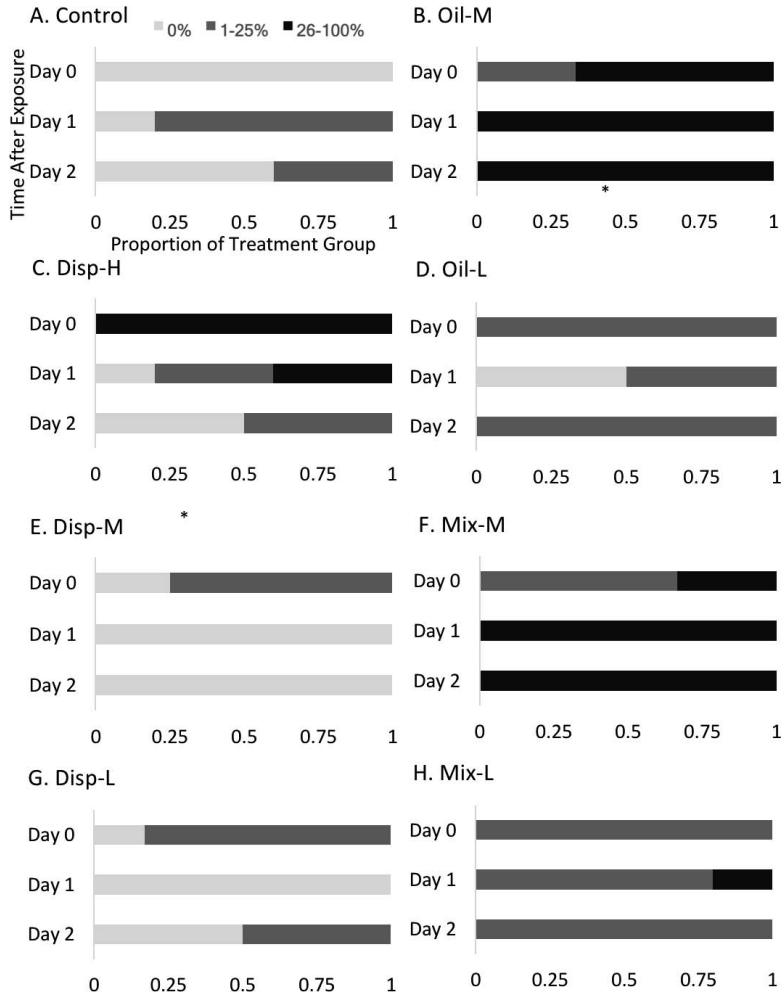


FIGURE 2. Waterproofing of Common Murre (*Uria aalge*) plumage after exposure to oil (Prudhoe Bay Crude oil), dispersant (Corexit 9500A), and dispersed oil in artificial seawater. Data are presented from three times: immediately after exposure and after 45-min in-water evaluation periods 1 and 2 d after exposure. Plumage waterproofing was quantified by estimating percent of body surface area wet-to-skin. An asterisk indicates significant difference in distribution of waterproofing scores from control at that time point (Kruskal-Wallis H test with post hoc pairwise comparisons using Dunn's [1964] procedure and a Bonferroni correction for multiple comparisons, alpha level 0.05). Oil-M=medium concentration oil; Disp-H=high concentration dispersant; Oil-L=low concentration oil; Disp-M=medium concentration dispersant; Mix-M=medium concentration dispersed oil; Disp-L=low concentration dispersant; Mix-L=low concentration dispersed oil.

control with the exception of a lower score in MIX-M ($P$=0.004). In the control, clumping was increased at day 2 after exposure ($P$<0.001) and after cleaning ($P$<0.001) compared to before exposure. For the majority of treatment groups, clumping increased from before to immediately after exposure and decreased from day 2 after exposure to after cleaning (Fig. 5). Immediately after exposure, the magnitude of increase in clumping from pre-exposure was significantly greater compared to control for MIX-M ($P$<0.001), OIL-M ($P$<0.001), and OIL-L ($P$=0.016) immediately after exposure. On day 2 after exposure, MIX-M and OIL-M had a further significant increase in clumping

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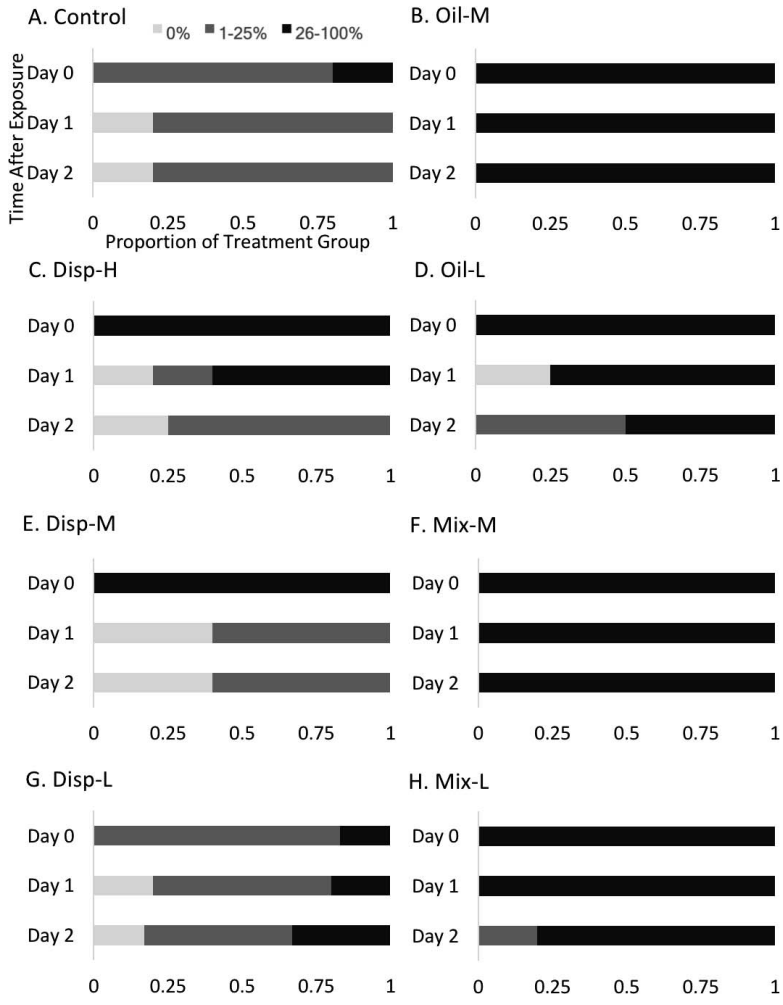


FIGURE 3. Waterproofing of Common Murre (*Uria aalge*) plumage after exposure to oil (Prudhoe Bay Crude oil), dispersant (Corexit 9500A), and dispersed oil in artificial seawater. Plumage waterproofing was quantified by estimating percent of body surface area superficially wet. Data are presented from three times: immediately after exposure and after 45-min in-water evaluation periods 1 and 2 d after exposure. In parts C, E, F, and H, on Day 0 there was a significant difference in distribution of waterproofing scores compared to control (Kruskal-Wallis H test with post hoc pairwise comparisons using Dunn's [1964] procedure and a Bonferroni correction for multiple comparisons, alpha level 0.05). Oil-M=medium concentration oil; Disp-H=high concentration dispersant; Oil-L=low concentration oil; Disp-M=medium concentration dispersant; Mix-M=medium concentration dispersed oil; Disp-L=low concentration dispersant; Mix-L=low concentration dispersed oil.

compared to control ($P<0.001$) while changes in clumping for other groups were similar to control. After cleaning, there were no significant differences between any exposure group and control.

To further elucidate the impacts of oil versus dispersant on clumping, differences for OIL-M, DISP-M, and MIX-M were compared. Immediately after exposure, clumping in both OIL-M and MIX-M was significantly higher than in DISP-M ($P<0.001$). Clumping in OIL-M was also significantly higher than in MIX-M ($P=0.022$). On day 2 after exposure, clumping in OIL-M and MIX-M was still significantly higher than in DISP-M ($P<0.001$), but there was no significant difference in clumping between OIL-M and MIX-M.

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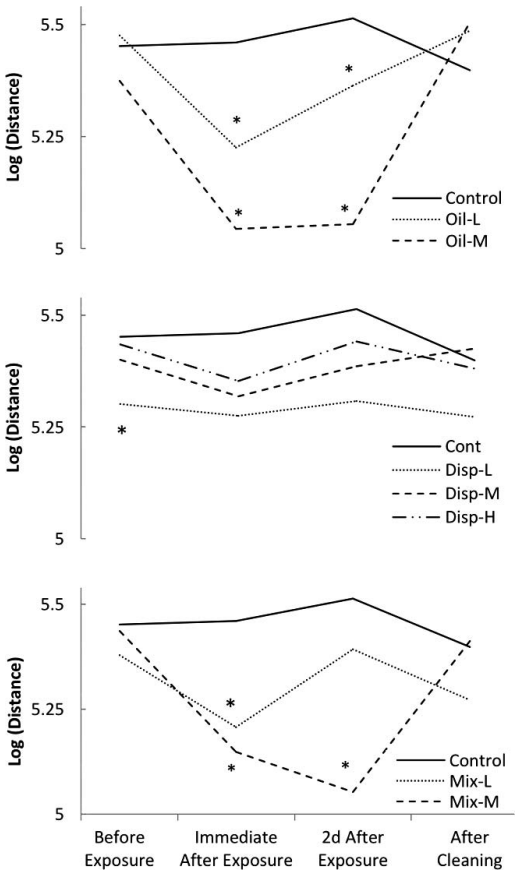


FIGURE 4. Microscopic structure of feathers collected from Common Murres (*Uria aalge*) after exposure to oil (Prudhoe Bay Crude oil), dispersant (Corexit 9500A), and dispersed oil in artificial seawater. Structure was quantified by measuring the distance between adjacent barbules at the distal tip of each feather. The log transformed data are presented as estimated from a fitted mixed-effects model from four times: before exposure, immediately after exposure, after a 45-min in-water evaluation period 2 d after exposure, and after cleaning. An asterisk indicates the magnitude of change in tip distance from pre-exposure through that time point is significantly different from the control (alpha level 0.05). Oil-L=low concentration oil; Oil-M=medium concentration oil; Cont=control; Disp-L=low concentration dispersant; Disp-M=medium concentration dispersant; Disp-H=high concentration dispersant; Mix-L=low concentration dispersed oil; Mix-M=medium concentration dispersed oil.

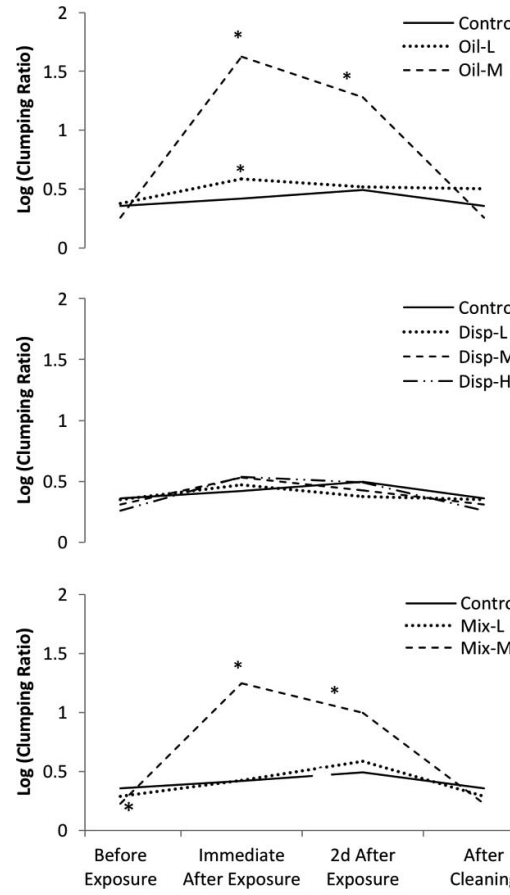


FIGURE 5. Microscopic structure of feathers collected from Common Murres (*Uria aalge*) after exposure to oil (Prudhoe Bay Crude oil), dispersant (Corexit 9500A), and dispersed oil in artificial seawater. Structure was quantified from the ratio of barbules to barbule-clumps along a 0.5-cm section of feather barb at 200 μm from the rachis in the central section of each feather. The log transformed data are presented as estimated from a fitted mixed-effects model from four time points: before exposure, immediately after exposure, after a 45-min in-water evaluation period 2 d after exposure, and after cleaning. Higher values indicate greater clumping of barbules. An asterisk indicates significant difference from control at that time point (alpha level 0.05). Oil-L=low concentration oil; Oil-M=medium concentration oil; Disp-L=low concentration dispersant; Disp-M=medium concentration dispersant; Disp-H=high concentration dispersant; Mix-L=low concentration dispersed oil; Mix-M=medium concentration dispersed oil.

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To evaluate associations of feather structural changes with qualitative waterproofing scores, univariate and multivariate models were designed incorporating distance, clumping, time, and exposure. In the simple models including only one feather measure, smaller mean distance ($P$=0.025) and higher mean clumping ($P$=0.010) in the tip section were the only measures that had a marginal or significant association with increased SW. In the multivariate model including all factors, there were significant differences in SW by time period ($P<0.001$) and by clumping ($P$=0.047). Compared to before exposure, all groups had increased odds of higher SW scores immediately after exposure ($P<0.001$) and all groups had decreased odds of higher SW scores after cleaning ($P$=0.048). Smaller mean tip distance ($P$=0.039) and higher mean clumping ($P$=0.029) were associated with greater odds of higher WTS scores in univariate models. In the multivariate assessment, there was a significant interaction between clumping and time period ($P$=0.031). On day 2 after exposure, an increase in clumping was associated with greater odds of high WTS score ($P$=0.004) while an increase in clumping was marginally significantly associated with lower odds of a high WTS score after cleaning ($P$=0.050).

## DISCUSSION

Results demonstrated that seabird waterproofing is negatively affected in a similar, dose-dependent manner by both crude oil and chemically dispersed crude oil. Dispersant alone also has negative waterproofing effects, with catastrophic consequences at high concentrations. Impacts of dispersant improved with the time birds spent out of water whereas the impacts of oil and dispersed oil did not improve over time. Before exposure, measures of demographics, plumage quality, waterproofing, and feather structure were largely comparable across treatment groups. Therefore, results reflect effects of treatment rather than of previous condition.

The control group exhibited mild impairment of waterproofing over the course of the study, likely due to the effects of handling and of housing out of water. This established a baseline from which treatment impacts at each time point could be compared. However, minor petroleum product contamination present in control tanks (likely originating from pilot testing) may have slightly contributed to baseline waterproofing impairment.

Birds exposed to oil were affected in a dose-dependent fashion across all measures throughout the duration of the study. Immediately after exposure, OIL-L and OIL-M had decreased distance and increased clumping in feather tips relative to control, indicating collapse of normal architecture. Both decreased distance and increased clumping were associated with increased SW and WTS, indicating these structural changes may affect plumage waterproofing. On day 2 after exposure, OIL-L and OIL-M had persistent but slightly improved decrease in distance at feather tips and OIL-M had continued significant clumping relative to control. There was no evidence of recovery from oil-associated feather structural change and waterproofing impairment 2 days after exposure, suggesting that recovery from contamination without human intervention is unlikely.

Effects of dipsersant-treated oil were similar to those of oil alone. Groups exposed to dispersant and oil had decreased distance at feather tips immediately after exposure relative to control, with this decrease persisting in MIX-M on day 2. The MIX-M feathers also had increased clumping relative to control immediately after and on day 2 after exposure. There was a nonsignificant trend of increased SW and WTS scores in MIX-L and MIX-M. These findings suggest that chemical dispersant does not notably alter the impact of oil exposure on waterproofing, nor does it improve the likelihood of recovering functional waterproofing after exposure, and thus findings are comparable to those few in the literature. Lambert et al. (1982) measured the basal metabolic rate of adult mallards (*Anas platyrhynchos*) experimentally exposed to

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OIL, DISP, or MIX and found basal metabolic rates of oil- and oil and dispersant-exposed mallards did not differ, but both increased significantly relative to controls.

Three phenomena were observed in birds exposed to dispersant only. First, observed impacts of DISP-H exposure were immediate and life-threatening. The SW and WTS scores increased significantly after exposure, and birds experienced complete loss of buoyancy; intervention in the rinse pool was deemed necessary to prevent drowning. Lambert et al. (1982) described similar findings in mallards exposed to Corexit 9527 alone. Second, loss of waterproofing in dispersant-exposed groups was distinctly improved after 1 day. Third, dispersant-only exposure did not impact distance or clumping, indicating that, in contrast to oil, the observed impacts to waterproofing do not arise from feather structural change. This finding held true for all time points, including immediately after exposure, prior to the rinse pool.

Collectively, these results indicate that accidental exposure of birds to pure, high-concentration dispersant, such as during aerial or boat-based application, may result in high morbidity and mortality. However, affected birds that are off water for at least 1 day (e.g., that make landfall or are collected from the water) may survive to recover functional waterproofing without further intervention. Feather structural changes that appear to be associated with waterproofing loss after oil exposure are not found after dispersant exposure alone. Several hypotheses are available to explain these findings. Lambert et al. (1982) hypothesized that loss of waterproofing in dispersant-exposed birds was due to infiltration of a hydrophilic surfactant component of dispersant into the plumage. Stephenson and Andrews (1997) measured water penetration due to reduced surface tension (resulting in penetration of water through gaps between feather barbs and barbules) in a variety of waterbird species and estimated that ducks and geese would experience total loss of waterproofing if exposed to surface tension 50–55% lower than normal. In our study, the immediate waterproofing impact may have been due to evaporation of a volatile component of the dispersant over the interval between exposure and evaluation the following day or due to rinsing of the water-soluble dispersant off the feathers during the 60-min rinse period in clean water after exposure. Further work is required to investigate these hypotheses.

Overall, our results suggest that chemical dispersants such as Corexit can have immediate external impacts on seabirds, with possible life-threatening consequences. Further, this study shows that oil-dispersant mixes have similar waterproofing impacts to oil alone; therefore, exposure within a water column could have comparable impacts to that seen swimming through a surface slick. It is important to note that the impact of dispersed oil might vary based on the dispersant to oil ratio. The 1:20 ratio used here reflects US industry recommendations; the actual ratio applied to a spill and that encountered by a bird at sea may be both spatially and temporally variable (Bejarano et al. 2013). Therefore, in net environmental benefit analyses, a "zero risk" assumption associated with dispersant application should not be used when seabirds are present. However, it is clearly understood and acknowledged that surface oiling constitutes a great risk to seabirds in a spill, and effective chemical dispersion of a surface slick (resulting in distribution of oil into the water column) can lead to decreasing the overall concentration of oil to which a given bird might be exposed. These advantages and disadvantages must be weighed carefully when faced with chemical dispersant use in seabird habitats. Additionally, further work is necessary to elucidate broader impacts of dispersed oil on seabirds, including data on effects of internal and chronic exposure and the role of surface tension and volatile components on waterproofing.

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# Chemical dispersants used in the Gulf of Mexico oil crisis are cytotoxic and genotoxic to sperm whale skin cells

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ABSTRACT

The 2010 Deepwater Horizon oil rig explosion in the Gulf of Mexico drew attention to the need for toxicological studies of chemical dispersants. We are still learning the effects these spills had on wildlife. Little is known about the toxicity of these substances in marine mammals. The objective of this study was to determine the toxicity of the two dispersants (Corexit 9500 and 9527). Corexit 9500 and 9527 were both cytotoxic to sperm whale skin fibroblasts. Corexit 9527 was less cytotoxic than 9500. S9 mediated metabolism did not alter cytotoxicity of either dispersant. Both dispersants were genotoxic to sperm whale skin fibroblasts; S9 mediated metabolism increased Corexit 9527 genotoxicity.

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## 1. Introduction

In 2010, the Deepwater Horizon oil rig exploded unleashing uncontrolled amounts of crude oil into the Gulf of Mexico. To combat the spill, unprecedented amounts of chemical dispersants were used. It is estimated that about 8 million liters (converted from gallons to liters) of dispersants were applied beneath the ocean surface at the depth of the well head leak as well as on the surface (Kujawinski et al., 2011). These agents were applied despite the fact that little was known about their potential toxicological impact to marine mammals; a shortcoming that continues to exist as, in general, most of the toxicology studies that are available are limited to LC50-type acute lethality endpoints in non-mammalian species. Thus, there is wide concern about the potential toxicity of these dispersants for both human and wildlife health.

Two types of dispersants, Corexit 9500 and Corexit 9527, were used to combat the oil spill. Limited data suggests these agents can be toxic, but experimental outcomes were generally limited to lethality. For example, a recent review summarized the available data for the toxicity of the two dispersants and found LC50s for Corexit 9500 in crustaceans, mollusks and fish, and for Corexit 9527 in daphnia, anemones, coral, crustaceans, mollusks, starfish, fish and birds (Wise and Wise, 2011). Remarkably, only one study considered effects in a mammal and reported impacts on body weights and intestinal flora in rats (George et al., as cited in Wise and Wise, 2011). More recently, the impact of dispersants on mammalian model systems have been considered in two human cell culture models. Corexit 9500 and Corexit 9527 induced cytotoxicity and oxidative stress in human HepG2/C3A hepatocytes (Bandele et al., 2012) and induced cytotoxicity in the immortalized human bronchial cell line, BEAS-2B (Shi et al., 2013). These data suggest a significant concern for dispersant toxicity and indicate the need for further study.

One major concern in the Gulf of Mexico is the potential impact of these dispersants on marine mammals. In particular, there are two resident populations of large whale species in the Gulf of Mexico: Sperm whales (*Physeter macrocephalus*) with a population of about 1600 individuals and Bryde's whales (*Balaenoptera edeni*) with a population of about 15 individuals (Waring et al.,

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2009, 2010). The loss or impairment of just a few reproductively productive animals in these small populations could result in their ultimate loss from the Gulf. Of note, the sperm whales are known to reside in the areas most affected by the spill (Waring et al., 2009, 2010). Indeed, anecdotal reports indicate that these whales were often observed by workers on the Deepwater Horizon rig and the National Oceanic and Atmospheric Administration (NOAA) and Unified Command received numerous reports of sperm whales swimming in oiled water after the spill (Warren, 2010). Thus, it is highly likely that these animals were exposed to the dispersants. We were unable to locate any data concerning the toxicity of these dispersants in any marine mammals or marine mammal cell cultures. Therefore, to address this concern, we investigated the cytotoxic and genotoxic effects of Corexit 9500 and Corexit 9527 in cultured sperm whale skin cells.

## 2. Materials and methods

### 2.1. Materials

All plasticware was purchased from BD Falcon. Dulbecco's Phosphate-Buffered Saline (DPBS), Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F-12) and Glutagro supplement were purchased from Corning. Nicotinamide adenine dinucleotide phosphate (NADPH) solutions were purchased from BD Gentest. S9 and fractions were purchased from Celsis In Vitro Technologies. Potassium chloride, demecolcine and sodium chromate were purchased from Sigma/Aldrich. Crystal violet, methanol and acetic acid were purchased from JT Baker. Microscope slides were purchased from Thermo scientific. Giemsa stain was manufactured by Rica Chemical Co. Gurr's buffer, trypsin, penicillin-streptomycin and sodium pyruvate was purchased from GIBCO Invitrogen Corporation. Cosmic calf serum was purchased from Hyclone.

### 2.2. Cell culture

Primary skin fibroblast cells were obtained from a free ranging sperm whale biopsy that was taken prior to the Deepwater Horizon oil spill as previously described (Wise et al., 2011). Cells were cultured in DMEM/F-12 (50:50 mixture of Modified Eagle's Medium and Ham's F-12) supplemented with 15% Cosmic calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM sodium pyruvate. Cells were maintained in a humidified incubator with 5% $CO_2$ at 33 °C (estimated whale body temperature). Cells were allowed to grow to near confluence as a monolayer. They were fed three times a week and expanded weekly (Wise et al., 2011).

### 2.3. Chemical and S9 fraction preparation

The dispersants used (Corexit EC9500A and Corexit EC9527A) were generously provided by the Nalco Holding Company. Treatment dilutions were prepared under dark conditions using the 100% stock solution and sterile water. Sodium chromate was dissolved in water and was filter sterilized. This was used as a positive control for all experiments.

Although the metabolism of the dispersants is unknown, we also considered phase 1 metabolism to determine if there is a difference in toxicity between the metabolite and the parent compound. S9 fractions were used to induce phase 1 metabolism because fibroblast cells often do not express cytochrome P450 enzymes. S9 fractions were added at the time of treatment, and made using 1× Tris buffer, NADPH regenerating system solution A (451220), NADPH regenerating system solution B (451200), and liver S9 fractions. These were prepared and applied at the time of chemical treatment.

### 2.4. Cytotoxicity

We used a clonogenic assay based on our published methods to determine the cytotoxicity of each dispersant (Wise et al., 2011). Briefly, cells were seeded into two 6 well tissue culture plates and allowed 48 h to resume normal log phase growth. Then they were treated with either Corexit 9500 or Corexit 9527 for 24 h. All treatment doses were done with and without S9 fractions. After the treatment time cells were reseeded into gelatin coated 100 mm tissue culture dishes at colony forming density. Once adequate cell colonies formed (~2 weeks) dishes were rinsed twice with 1× phosphate-buffered saline (PBS) then fixed in methanol for 20 min. After the methanol was removed the dishes were stained with crystal violet stain for 30 min. Dishes were analyzed for number of cell colonies then averaged per dose and divided by the average number of colonies in the negative control.

### 2.5. Clastogenicity

We used a chromosomal aberration assay to determine the clastogenicity of each dispersant, based on our published methods (Wise et al., 2011). Briefly, cells were seeded into 100 mm tissue culture dishes for 48 h. Then they were treated with either Corexit 9500 or Corexit 9527 for 24 h. All treatment doses were done with and without S9 fractions. Five hours prior to the end of the treatment period cells were arrested in metaphase using 0.1 g/ml demecolcine. After the full 24 h treatment period, cells were resuspended in a potassium chloride hypotonic solution (KCl) for 17 min then fixed with 3:1 methanol:acetic acid. After two changes of fixative, cells were dropped onto microscope slides and stained with 5% Giemsa stain in Gurr's Buffer. Slides were analyzed for chromosome aberrations in 100 metaphases per treatment concentration according to our published methods (Wise et al., 2011).

### 2.6. Statistics

The statistical difference between values for cytotoxicity and genotoxicity were evaluated using *t*-test and multiple regression analysis with dispersant, concentration, and S9 as the three independent variables. No adjustment was made for multiple comparisons, because all of the comparisons were made of a priori substantive interest.

## 3. Results

### 3.1. Corexit 9500 and 9527 Cytotoxicity in Sperm Whale Skin Cells

Corexit 9500 induced a concentration dependent increase in cytotoxicity to sperm whale skin cells (Fig. 1). Concentrations of 0.005, 0.025, 0.05 and 0.1% 9500 induced 82, 67, 25 and 3% relative survival, respectively. S9 mediated metabolism did not significantly alter the cytotoxicity of Corexit 9500 (Fig. 1). Concentrations of 0.005, 0.025, 0.05 and 0.1% 9500 with S9 fractions induced 91, 72, 37 and 3% relative survival, respectively.

Corexit 9527 also induced a concentration dependent increase in cytotoxicity (Fig. 2). Concentrations of 0.005, 0.025, 0.05 and 0.1% 9527 induced 86, 79, 70 and 26% relative survival, respectively. S9 mediated metabolism resulted in a similar dose response (Fig. 2). Concentrations of 0.005, 0.025, 0.05 and 0.1% 9527 with S9 fractions induced 86, 77, 70 and 42% relative survival, respectively.

Comparison of Corexit 9500 and Corexit 9527 parent compounds shows that 9500 is more toxic than 9527 (Fig. 3). For

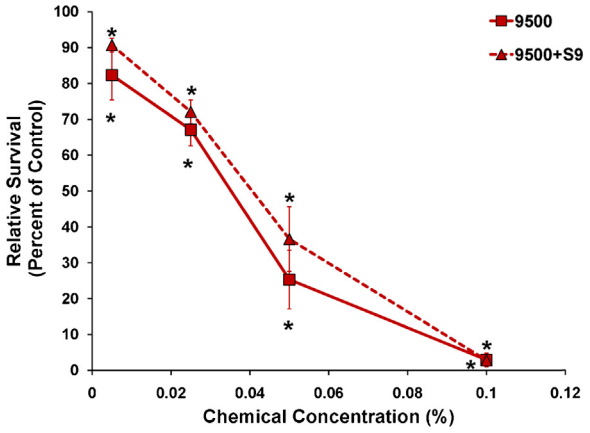


**Fig. 1.** Corexit 9500 is cytotoxic to sperm whale skin cells. This figure shows that Corexit 9500 was cytotoxic to sperm whale skin cells after a 24 h exposure and S9 mediated metabolism did not alter cytotoxicity (measured as cell survival relative to the control). The overall dose-response is highly significant ($p < 0.0001$). There was no statistical difference observed between 9500 and 9500+S9 ($p = 0.19$). Data represent 3–4 experiments ± the standard error of the mean. Asterisk (*) indicates doses that are significantly different from control ($p < 0.05$).

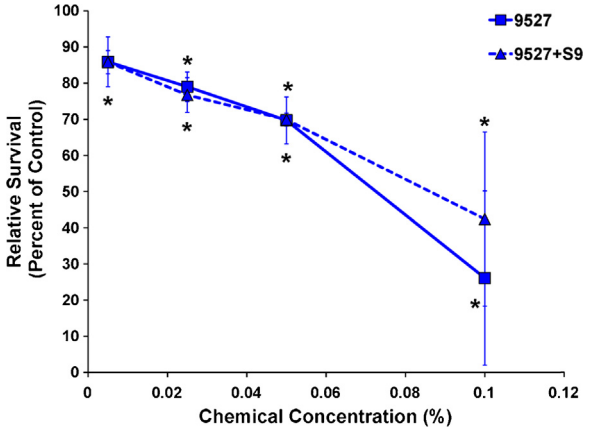


**Fig. 2.** Corexit 9527 is cytotoxic to sperm whale skin cells. This figure shows Corexit 9527 was cytotoxic to sperm whale skin cells in a concentration dependent manner after a 24 h. Cytotoxicity is measured as cell survival relative to the control. There was no statistical difference observed between 9527 and 9527+S9 ($p > 0.05$). Data represent 3 experiments ± the standard error of the mean. Asterisk (*) indicates doses that are statistically significant from control ($p < 0.05$).

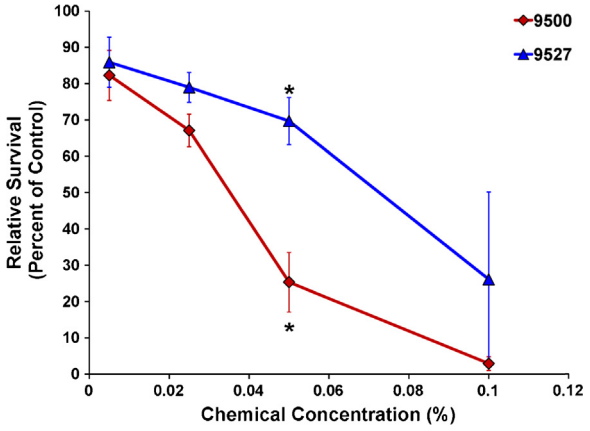


**Fig. 3.** Corexit 9500 is more cytotoxic than Corexit 9527. This figure compares the cytotoxicity of Corexit 9500 and Corexit 9527 after a 24 h exposure. Sperm whale cells were more sensitive to Corexit 9500 than Corexit 9527 ($p = 0078$). Asterisk (*) indicates doses that were significantly different from each other ($p < 0.05$).

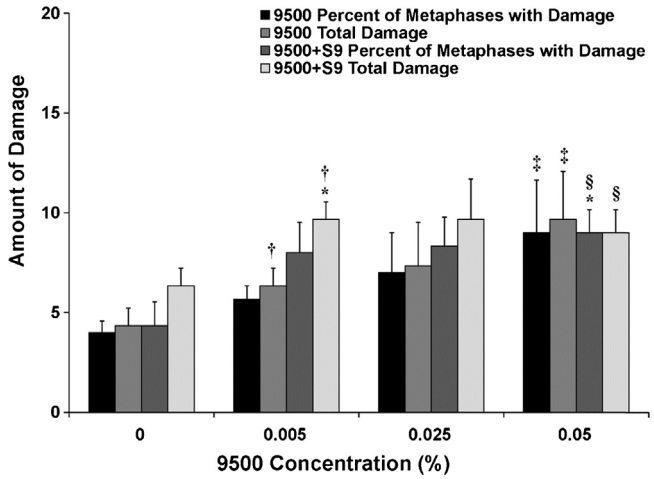


**Fig. 4.** Corexit 9500 is not strongly genotoxic to sperm whale skin cells. This figure shows Corexit 9500 was not substantially and generally not statistically significantly genotoxic to sperm whale skin cells after a 24 h exposure both with and without S9-mediated metabolism. Data are expressed as the average percent of metaphase with damage and total aberrations in 100 metaphases. Data represent 3 experiments ± the standard error of the mean. Symbol (†) indicates that 9500 is statistically significant from 9500 + S9 ($p < 0.05$). Asterisk (*) indicates doses that are significantly different from control ($p < 0.05$). Symbol (‡) indicates that in one experiment only 68 metaphases could be scored. Symbol (§) indicates that in one experiment only 86 metaphases could be scored.

example, there is a 3-fold increase in toxicity at 0.05% concentration ($p = 0.0002$).

### 3.2. Corexit 9500 and 9527 clastogenicity in sperm whale skin cells

Corexit 9500 induced a minimal increase in genotoxicity in sperm whale skin cells (Fig. 4). S9 mediated metabolism had no effect on the genotoxicity of Corexit 9500 (Fig. 4). Specifically, concentrations of 0.005, 0.025 and 0.05% 9500 damaged 1.7, 3 and 5.3% of metaphases and induced 2.3, 3 and 5.7 total aberrations per 100 metaphases, respectively (minus the control levels). S9 mediated metabolism damaged 3.7, 4 and 4.7% of metaphases and induced 3.3, 3.3 and 2.7 total aberrations per 100 metaphases, respectively (minus the control levels).

By contrast, Corexit 9527 induced a concentration-dependent increase in genotoxicity after 24 h exposure in sperm whale cells (Fig. 5). Specifically, concentrations of 0.005, 0.025, 0.05 and 0.1% 9527 damaged 3, 4.3, 8 and 10.6% of metaphases and induced 4, 5, 9.3 and 12.7 total aberrations per 100 metaphases, respectively (minus the control values). S9 mediated metabolism increased this effect damaging chromosomes in 4, 8, 11 and 19.5% of metaphases and induced 4, 8.7, 14.3 and 25 total aberrations per 100 metaphases, respectively (Fig. 5; minus the control values). The spectrum of chromosome aberrations for both compounds consisted of mostly chromatid lesions (Table 1).

Comparing of Corexit 9500 and 9527 shows that 9257 is more genotoxic than 9500 (Fig. 6). Corexit 9527 with S9 fractions was the most genotoxic condition, inducing the most total chromosome damage. Corexit 9527 had a higher amount of isochromatid lesions than 9500. Corexit 9500 had a higher amount of dicentric chromosomes and double minutes. The double minutes only occurred in the S9 treated cells. There were few chromatid exchanges in both compounds. Double minutes, dicentrics and chromatid exchanges were not present in any of the controls (Table 1).

## 4. Discussion

In the aftermath of the Deepwater Horizon explosion, two dispersants Corexit 9500 and Corexit 9527 were sprayed on oil at the

**Table 1**
Spectrum of chromosome aberrations.[a].

| Concentration | Chromatid lesions | Isochromatid lesions | Chromatid exchanges | Rings | Double minutes | Acentric fragments | Dicentrics |
|---|---|---|---|---|---|---|---|
| Corexit 9500 | | | | | | | |
| 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.005 | 5 | 0 | 1 | 0 | 0 | 0 | 0 |
| 0.025 | 2 | 0 | 0 | 0 | 0 | 0 | 1 |
| 0.05 | 11 | 1 | 0 | 0 | 0 | 0 | 1 |
| 0 + S9 | 1 | 0 | 0 | 0 | 4 | 0 | 1 |
| 0.005 + S9 | 6 | 0 | 0 | 0 | 1 | 0 | 1 |
| 0.025 + S9 | 5 | 0 | 0 | 0 | 1 | 0 | 0 |
| 0.05 + S9 | 8 | 1 | 0 | 0 | 0 | 0 | 0 |
| Corexit 9527 | | | | | | | |
| 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.005 | 2 | 6 | 0 | 0 | 0 | 0 | 0 |
| 0.025 | 12 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.05 | 17 | 0 | 1 | 0 | 0 | 0 | 0 |
| 0 + S9 | 3 | 4 | 0 | 0 | 0 | 0 | 0 |
| 0.005 + S9 | 10 | 1 | 0 | 0 | 0 | 0 | 0 |
| 0.025 + S9 | 20 | 2 | 0 | 0 | 0 | 0 | 0 |
| 0.05 + S9 | 31 | 0 | 0 | 0 | 0 | 0 | 0 |

surface of the Gulf of Mexico and injected into the wellhead in an effort to reduce the impact of the crude oil as quickly as possible. Little is known about the toxicity and environmental fate of these chemicals and there is significant concern about their potential long term health effects. Our data are the first assessment of Corexit 9500 and Corexit 9257 toxicity in a marine mammal model system and the first data to evaluate the ability of these agents to induce chromosome damage in any species. Our data show that Corexit 9527 is indeed genotoxic to sperm whale skin cells. Such an outcome raises concern about the impact of this agent on reproduction, development and potentially carcinogenesis in marine mammals and in other species.

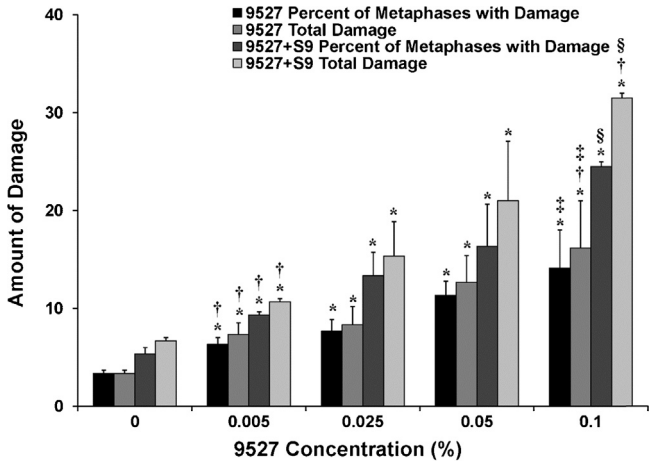


**Fig. 5.** Corexit 9527 is genotoxic to sperm whale skin cells. This figure shows Corexit 9527 induces a concentration-dependent increase in genotoxicity in sperm whale skin cells after a 24 h exposure. S9 mediated metabolism increases genotoxicity. Data are expressed as the average percent of metaphase with damage and total aberrations in 100 metaphases. The effect for S9 increased with concentration ($p = 0.0005$ for interaction based on percent damage and $p = 0.0003$ for interaction based on total damage). The effect for concentration without S9-mediated metabolism was significant for both percent damage ($p = 0.002$) and for total damage ($p = 0.011$). When evaluated in a multivariate regression and at a concentration of 0.05%, the effect for S9 was significant for both percent damage and total damage ($p < 0.0001$). Data represent 3 experiments ± the standard error of the mean. 0.1% 9500 with and without S9 was not done for the third experiment because at these concentrations not enough metaphases could be obtained due to cell cycle arrest. Asterisk (*) indicates doses that were significantly different from control ($p < 0.05$). Symbol (†) indicates that 9527 was significantly different from 9527 + S9 ($p < 0.05$). Symbol (‡) indicates that in one experiment only 88 could be scored. Symbol (§) indicates that in one experiment only 54 metaphases could be scored respectively.

Our data showed that Corexit 9527 induced an increase in metaphases with chromosome damage and an increase in the total numbers of aberrations in a concentration-dependent manner in sperm whale skin cells. If genotoxicity occurs during essential stages of reproduction or embryogenesis it could cause loss of the offspring or affect individual calf development (El-Makawy et al., 2006; Keshava and Ong, 1999; Nayak et al., 1989). If these outcomes were to affect the ability of an affected individual to survive or reproduce successfully then there could be detrimental effects to the population which is small in number and endangered.

Our genotoxicity results differ from the only other previous report to consider the genotoxic effects of dispersants in mammals. Specifically, the other study reported that, in Fischer 344 rats, a 5 week exposure to Corexit 9527 did not induce hepatic DNA adducts (George et al., 2001). Such an outcome is not inconsistent with our findings, it is simply different. DNA adducts, while a lesion on the DNA, and a genotoxic event, are not necessarily related to chromosomal aberrations. In other words, while an adduct may lead to a chromosome aberration, there are other mechanisms to cause aberrations. Thus, it is reasonable for a chemical mixture like Corexit

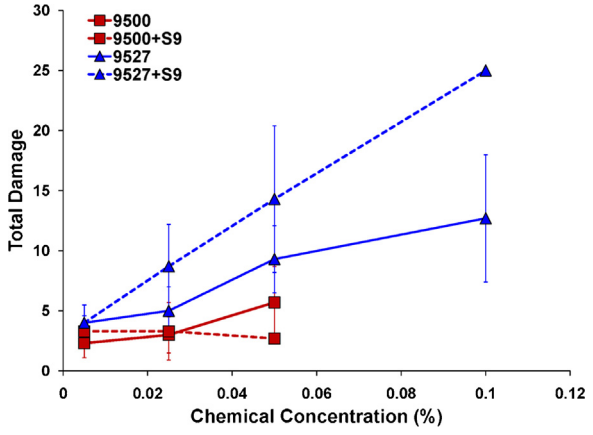


**Fig. 6.** Corexit 9527 induces more total chromosome damage than Corexit 9500. This figure shows the comparison of the total chromosome damage induced by Corexit 9500 and 9527 with and without S9 fractions after a 24 h exposure. Based on a multiple regression model, there was a significant 3-way interaction involving dispersant, concentration and S9 ($p = 0.023$). When evaluated at a concentration of 0.05 percent, Corexit 9527 was significantly more genotoxic than 9500 with S9 fractions ($p < 0.001$) but not without S9 fractions ($p = 0.33$). Data represent the aberrations in 100 metaphases shown in Figs. 4 and 5 minus their respective negative control levels.

9527 to induce aberrations and not induce adducts. By contrast, Corexit 9500 was only genotoxic at the highest dose 0.05%. At this dose we were unable to score 100 metaphases due to cell cycle arrest for each experiment, so it is unclear if there is a genotoxic effect or another cellular process occurring to arrest the cells. We could not find any literature regarding the genotoxicity of Corexit 9500.

We also found that both Corexit compounds were cytotoxic to sperm whale cells. Extensive cytotoxicity can lead to fibrosis and impair organ function. There are no published reports of Corexit cytotoxicity in whale cells, but our Corexit 9500 cytotoxicity results are consistent with other studies of Corexit 9500 in human cells. Specifically, Corexit cytotoxicity has only been measured in three other studies (Bandele et al., 2012; Shi et al., 2013; Zheng et al., 2014). One study considered bronchial airway cells (BEAS-2B) and used the MTT assay to determine cell viability (Shi et al., 2013). They found Corexit 9500 doses of 0.02 and 0.03% (reported in the study as 200 and 300 ppm) induced 50% and 90% cell loss. These outcomes are similar to our findings, using a clonogenic assay, that 0.025% Corexit 9500 induced 67% cell survival.

The second study measured Corexit 9500 cytotoxicity in human HepG2 hepatocytes using Hoechst 33258 fluorescence (Bandele et al., 2012). In that system, Corexit 9500 doses of 0.02 and 0.03% (inferred from the figure presenting the data) resulted in relative cell viability of 80 and 50%, respectively. Our finding that Corexit 9500 induced 67% cell survival fits right in between those previous outcomes.

The third study used the MTT assay to determine cell viability in 5 different established mammalian cell lines exposed to Corexit 9500, 1 mouse, 1 rat and 3 human cell lines. After being treated with 0.02% Corexit 9500 for 48 h they found a range of about 35–80% cell viability. These data are consistent with the 67% cell survival in sperm whale skin cells that we reported.

Our Corexit 9527 cytotoxicity data was similar to one, but differed from another of these two human cell studies. We found 0.025% Corexit 9527 was not particularly cytotoxic, resulting in 79% relative cell survival. Similarly, 0.02 and 0.03% Corexit 9527 induced 90 and 40% relative cell viability in human HepG2/C3A cells. However, in BEAS-2B cells, 0.02 and 0.03% Corexit 9527 resulted in no cell survival. The underlying explanation for why the sperm whale cells were more resistant then BEAS-2B cells to Corexit 9527 is uncertain, especially when the results for 9500 were similar. The most likely explanation, albeit untested is that 9527 has the additional ingredient of 2-butoxyethanol (Wise and Wise, 2011), thus, the whale cells may be responding differently to this chemical.

One of the concerns raised in both scientific and public circles was to determine which Corexit dispersants was the more toxic of the two. Our data shows the challenge in answering that question in a meaningful way. We found that Corexit 9500 is more cytotoxic than Corexit 9527; however, Corexit 9527 is more genotoxic than 9500. Thus, the answer would be different depending on which outcome is considered. It is curious that 9527 is more genotoxic and less cytotoxic. The underlying mechanisms that explain this difference is uncertain. It might be due to the 2-butoxyethanol in the 9527, or, perhaps, one or more metabolite of 2-butoxyethanol inducing chromosome aberrations while inhibiting cell death pathways. However, studies in rodent and human cells show that 2-butoxyethanol does not induce chromosomal aberrations specifically, and is generally negative in other genotoxic assays (Elliott and Ashby, 1997; NTP, 2000). Of course, it is possible that whale cells may simply respond differently to 2-butoxyethanol than rodent and human cells. Alternatively, it may represent some unanticipated interaction of 2-butoxyehthanol with another Corexit ingredient.

Of course an important, but difficult to address question is to determine how our laboratory exposures relate to actual exposures in the Gulf. There is no known accurate method to measure the amount of whale exposure to dispersants. In the Gulf of Mexico Deepwater Horizon oil spill a final ratio of about 1:63 dispersant to oil was used, i.e. a final concentration of about 1.6% (calculated based on a total applied dispersant amount of approximately 8,000,000 L, reported in Kujawinski et al., 2011 and a total released crude oil amount of 500,000,000 L of crude oil data from Crone and Tolstoy as cited in Joung and Shiller, 2013). Corexit dispersants were sprayed aerially and injected at depth. While some whales may have avoided any exposure, given the locations of the oil and the spraying and the whales, it is highly likely some whales were indeed exposed to dispersants. Thus, the spectrum of dispersant concentrations the Gulf whales might have encountered would have ranged from very high (i.e. 100%) if the whale was directly exposed to the dispersant spray or stream as it entered the gulf; to moderate (i.e. 1–50%) if the whale was exposed as the dispersant became mixed with oil and water; to something much lower (i.e. <0.1%) if the whale was exposed after the dispersant mixture dispersed through the water column or food sources. Given these scenarios and the final ration of dispersant applied, our doses of 0.005, 0.025, 0.05 and 0.1% certainly seem plausible.

In sum, our data show both Corexit 9500 and 9527 are cytotoxic and Corexit 9527 is genotoxic to sperm whale skin cells. Recent reports show that cetaceans in the Gulf of Mexico are suffering from reproductive, respiratory and other health issues in the aftermath of this crisis (Schwacke et al., 2014). Given the DNA damaging potential of the chemicals used in the crisis, care should be taken to monitor the populations for further long term health effects.

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