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Coral injuries observed at Mesophotic Reefs after the Deepwater Horizon oil discharge

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ARTICLE INFO

Available online 11 June 2015

Keywords:

Mesophotic reefs
Octocoral
Black coral
Coral injuries
Hydrocarbon and dispersant impact
Deepwater Horizon
Gulf of Mexico

ABSTRACT

Pathologies in over 400 octocoral and antipatharian colonies were quantified in the aftermath of the DWH oil discharge. Observations were made in September 2011 at water depths of 65–75 m in the Pinnacle Reef trend area offshore from Mississippi and Alabama, Gulf of Mexico, using a digital macro camera deployed from an ROV to examine the coral populations for injury at two principal sites: Alabama Alps Reef (AAR) and Roughtongue Reef (RTR). Taxa observed to exhibit injury included gorgonian octocorals *Hypnorgia pendula*, *Bebryce* spp., *Thesea nivea*, and *Swiftia exserta*, the antipatharian *Antipathes atlantica*, and the sea whips *Stichopathes* sp., and *Ellisella barbadensis*. The most common type of injury was a biofilm with a clumped or flake-like appearance covering sea-fan branches. Extreme injuries were characterized by bare skeletons, broken and missing branches. Comparing the 2011 results to previous photo surveys of the same study sites between 1997 and 1999, we found significantly more occurrences of injury in 2011 among taxa with growth forms > 0.5 m. We hypothesize that Tropical Storm Bonnie facilitated and accelerated the mixing process of dispersant-treated hydrocarbons into the water column, resulting in harmful contact with coral colonies at mesophotic depths. Analysis of total polycyclic aromatic hydrocarbon (tPAH) concentrations in sediments at AAR and RTR found levels elevated above pre-discharge values, but orders of magnitude below toxicity thresholds established for fauna in estuarine sediments. The tPAH concentrations measured in octocoral and echinoderm tissue samples from AAR and RTR were detectable (mean values ranged from 51 to 345 ppb); however, bioeffect thresholds do not currently exist with which to evaluate the potential harm these levels may cause. Our findings indicate that coral injuries observed in 2011 may have resulted from an acute, isolated event rather than ongoing natural processes.

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1. Introduction

The Pinnacles Reef trend is a region of the Gulf of Mexico that extends from offshore of Mississippi eastward toward the Florida Panhandle; it encompasses numerous rocky outcrops colonized by sessile reef fauna in the 50–150 m depth range (Gittings et al., 1992). High-relief hard grounds on the northeastern Gulf of Mexico shelf are widely understood to have a biogenic origin. They formed when reef organisms were inundated to greater depths because of the rising sea level during the last deglaciation (Continental Shelf Associates and Texas A&M University, 2001). The Pinnacles Reef trend comprises one of four well-known mesophotic coral ecosystem (MCE) sites in the Gulf of Mexico; the others being the Flower Garden Banks and other hard grounds

offshore of Texas (Continental Shelf Associates and Texas A&M University, 2001; Locker et al., 2010), the Florida Middle Ground reef system (Smith et al., 2006), and Pulley Ridge (Locker et al., 2010). Together, they are part of 178,867 km² of substrata that could support MCE in the northern Gulf of Mexico (Continental Shelf Associates and Texas A&M University, 2001).

The Pinnacles Reefs comprise a series of spatially distinct, carbonate features of different sizes and morphologies, which serve as important destinations for commercial and recreational fishing (Dennis and Bright, 1988; Weaver et al., 2002). They host diverse invertebrate and fish fauna. Continental Shelf Associates and Texas A&M University (2001) reported 40 invertebrate taxa for the Pinnacle Reef area offshore of Mississippi–Alabama. The most diverse groups are azooxanthellate octocorals, sponges, antipatharians, and hermatypic corals, all of which also provide structural habitat for invertebrates and fish. Gorgonian octocorals and antipatharian black corals have both sea-fan and sea-whip morphologies: i.e. a single attachment point called a holdfast, from which branches may or may not bifurcate to form a flexible proteinaceous skeleton

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(Bayer, 1961). Large branching colonies have a flabellate shape, along which colonies of polyps are distributed. Corals on the Pinnacles Reefs are predominantly heterotrophic suspension feeders that depend on both plankton and detritus provided by local currents for nutrition (Bayer, 1961). Octocorals in the Pinnacles Reefs have been shown to grow so that their fans are permanently oriented to maximize exposure to local currents (Peccini and MacDonald, 2008).

On April 20th 2010, an explosion on the drilling platform Deepwater Horizon (DWH) and the subsequent well blowout discharged more than 4.1 million barrels of crude oil (McNutt et al., 2012) and over 500,000 T of hydrocarbon gas (Joye et al., 2011) into the water column over a span of 84 days. The ocean surface over the Pinnacle Reefs was covered by oil released by the DWH blowout for approximately 35 days on average depending on location; floating oil received repeated applications of dispersant, including in areas directly above Pinnacles Reefs to the north and northeast of the wellhead (United States Coast Guard, 2011; NOAA, 2013). The combination of surface oil and dispersant application raised concerns that sessile organisms such as octocorals in this vicinity might have suffered injury as a result of exposure to these chemical contaminants. Similar effects have been reported for deep-sea communities of octocorals in the vicinity of the Macondo well, probably as a result of the DWH discharge (White et al., 2012).

The objectives of the present study were to characterize and quantify pathologies observed among octocorals and antipatharian corals on mesophotic reefs situated below the surface oil discharged during the DWH incident and to compare these results with data collected before the oil spill. Sampling efforts were undertaken in September 2010 and September 2011 to obtain sediment and tissue samples (2010 and 2011) and to conduct extensive high-resolution photographic inspections (2011 only). The present study provides a descriptive and quantitative analysis of these data and compares the results to historic data from the

region. Photographic data collected in 1997–1999, which were reanalyzed for this study, provided a quantitative, pre-discharge baseline for the natural frequency of coral injuries in the Pinnacle Reefs habitat.

2. Methods

2.1. Study sites

Between 1997 and 1999, a series of photographic surveys were conducted for the “Mississippi–Alabama Pinnacle Trend Ecosystem Monitoring” (MAPTEM) program, visiting nine MCE sites in total (Continental Shelf Associates and Texas A&M University, 2001). This effort was accomplished using a SeaRover ROV outfitted with a still camera. The frequency of coral injury had not been previously assessed for these data.

Post-discharge assessments of DWH oil impacts were conducted during August 2010 on the RV *Nancy Foster* and September 2011 on the MV *Holiday Chouest*. Over the course of these two post-discharge cruises, a total of six sites were visited (Fig. 1; Table 1): four were within ~100 km distance of the DWH site offshore from Mississippi and Alabama; two presumably unaffected sites were located offshore from Florida. This study focuses on photographs taken during 2011 at two mesophotic sites also surveyed by MAPTEM expeditions: Alabama Alps Reef (AAR) and Roughtongue Reef (RTR), because they provide a good opportunity for before–after comparison of still images. Surveys also took place on ‘secondary sites’ Talus Block (TBR), Yellowtail Reef (YTR), Coral Trees Reef (CTR), and Madison Swanson South Reef (MSSR), but with less effort towards still images. Data on sediment hydrocarbon concentrations presented herein are taken from the 2010 effort, and data on hydrocarbon concentrations in coral tissues are taken from both the 2010 and 2011 efforts. On both cruises, an

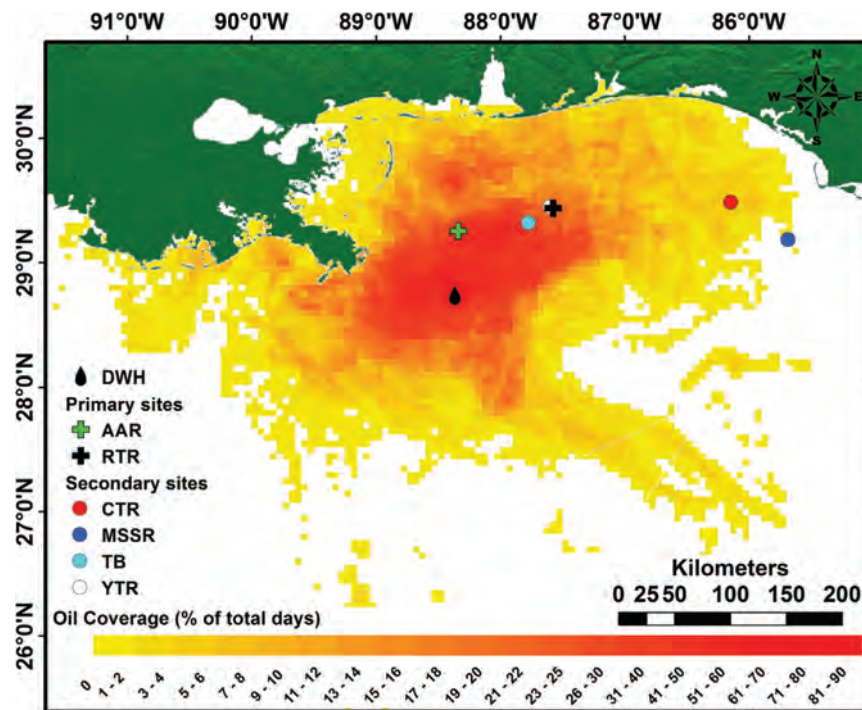


Fig. 1. Map of the location of the study sites in relation of the oil discharge (DWH). Alabama Alps Reef (AAR) and Roughtongue Reef (RTR) are the principal mesophotic reef sites. Additional sites visited during 2010 and/or 2011 included Coral Trees Reef (CTR), Talus Block (TBR), Yellowtail Reef (YTR), and Madison Swanson South Reef (MSSR). Area covered by oil based on normalized observations of oil per unit area ($5 \times 5 \text{ km}^2$ grid) imaged by Satellite Aperture Radar (Lessard and DeMarco, 2000) from García-Pineda et al. (2013).

industrial-class remotely operated vehicle was used to survey the bottom area and collect samples.

2.2. Sample collection

2.2.1. Photography survey

Baseline photographic data from AAR and RTR were collected during the MAPTEM project (Continental Shelf Associates and Texas A&M University, 2001). Photographic surveys were conducted using a ROV outfitted with a still camera illuminated by a 150 W s electronic strobe. Camera and strobe were triggered by shipboard control. The camera and strobe were installed on the front of the ROV looking down at an angle of approximately 70°. The camera covered an area of 0.3 m². The overall location for photographic survey work was determined prior to each cruise using a digital elevation model to focus effort on the reef crest and not the surrounding seabed. A circular area 200 m in diameter was divided into eight equal sectors, each containing 16 randomly chosen points; photographs were then taken to document the sessile community at each of the 16 locations (Fig. 2).

During the 2011 field effort, a digital still camera (AquaSLR) in a deep-sea housing was mounted on a frame that carried two high-output LED lamps; the array was deployed by the ROV manipulator arm. In preparation for photograph collection, the ROV performed a reconnaissance of each site and identified the regions of the two reefs where octocoral colonies were found in noticeable abundance. During reconnaissance, the ROV transited at 0.5–1 m above the bottom in transects across hard ground areas in the respective reefs, while recording position events at 1 min intervals and noting the occurrence of corals (Fig. 2). Generally, sea fans and other octocorals were found concentrated on rocky promontories within the reef crest. Each locality where corals were observed was then revisited to determine presence or absence of injured corals. Coral localities were photographed with the digital camera to document the condition of corals present there. Injured corals were often photographed from several angles to facilitate later evaluation. For detailed photo-documentation, the ROV manipulator arm was used to aim the camera at subjects of interest. The close placement of the camera with abundant lighting produced high resolution images. The camera was also deployed in transit

Table 1
Summary of visited sites during the research expedition in September 2011. Site names in parentheses are abbreviations used in text. Distance indicates proximity to DWH discharge site.

Site	Abrv.	Longitude(Dd)	Latitude(Dd)	Depth(m)	Outcroparea (km ²)	DistanceDWH (km)
Alabama Alps Reef	AAR	−88.33924	29.253668	74	0.276	57
Talus Block	TB	−87.76679	29.320955	130	0.023	87
Yellowtail Reef	YTR	−87.59169	29.450339	64	0.119	109
Roughtongue Reef	RTR	−87.57581	29.439161	66	0.140	109
Coral Tree Reef	CTR	−86.13945	29.486935	88	0.143	231
Madison Swanson Reef	MSSR	−85.67931	29.186576	73	0.402	266

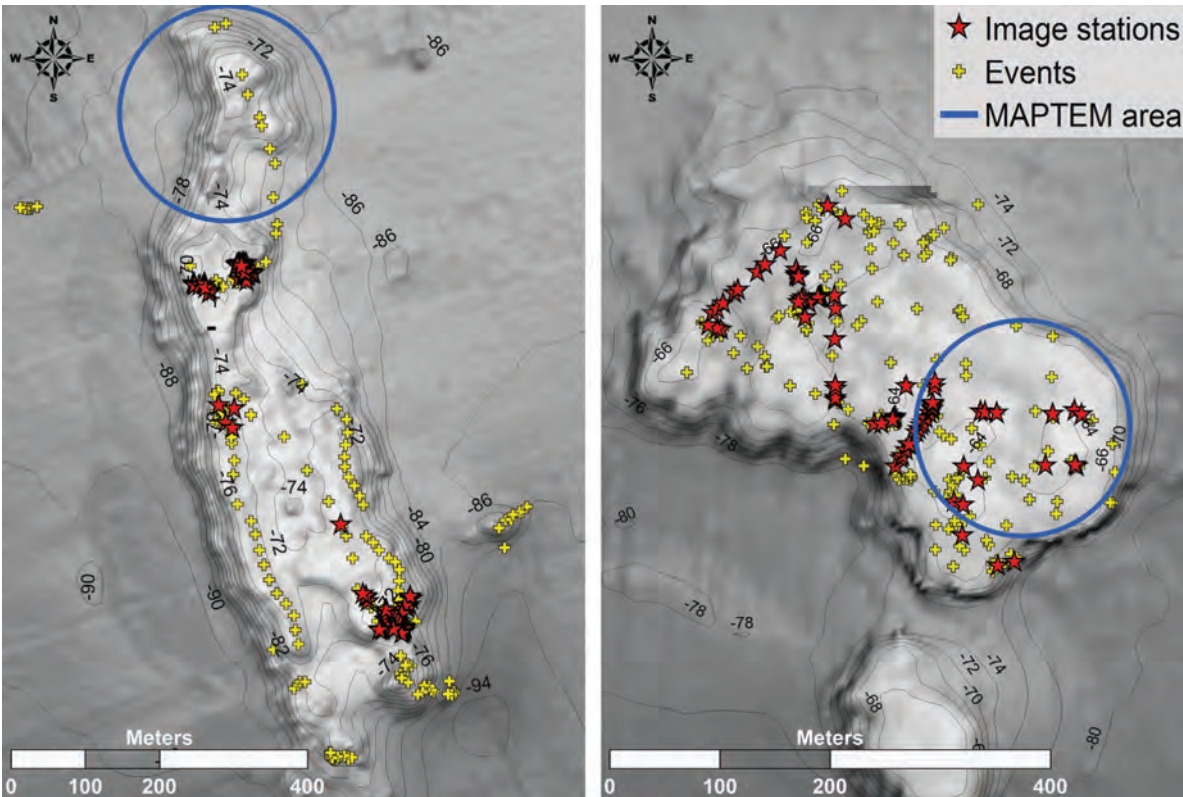


Fig. 2. Photographic documentation of coral injuries. Alabama Alps Reef (left) and Roughtongue Reef (right). Yellow crosses indicate navigation waypoints occupied by the ROV. Each red star represents an image sample analyzed for photo-documentation of coral health. See Table 2 for photograph inventory by site. Blue circles represent the area within which randomly located photographs were collected during the MAPTEM surveys.

mode in conjunction with the video cameras on the ROV to document coral health characteristics over larger areas. The locations of images taken and the dive-tracks of the ROV at AAR and RTR are summarized in Fig. 2.

2.2.2. Coral and sediment collection

Coral tissues for taxonomic identification and chemical analysis were collected using the ROV manipulator arm. Individual colonies were targeted for collection in the video display. Branches were broken off from the main colony, stored temporarily in the ROV collection box, and carried to the surface at the end of each dive. Species diagnoses were achieved using scanning electron microscopy of sclerites, polyps, and branches in conjunction with photographic and taxonomic keys.

Sub-samples for PAH analyses were packed in methanol-rinsed foil and stored frozen at temperature of -20°C . Sediment samples were collected on two occasions by two different sediment sampling devices. In August 2010, sediments were collected from Alabama Alps and Roughtongue Reef using 6.2 cm diameter push-cores. Additionally, in September 2010 sediment samples were collected on a separate cruise using a 0.04 m^2 Young-modified Van Veen benthic grab sampler deployed from the ship deck (Cooksey et al., 2014). Samples for the analysis of contaminants were sub-sampled from composited surface sediment contained in the grab samples (the upper 2–3 cm) and stored frozen.

2.3. Image analysis

Digital images from the 2011 survey and the MAPTEM program were carefully reviewed for indications of injury to corals, including the following pathologies: locally retracted polyps, loose tissue, broken branches, bare skeleton, necrosis, and areas covered by strands of mucus or biofilm overgrowth. We then focused our analysis on those pathologies indicative of polyp mortality, and quantified the extent to which each injured colony was affected. Each coral colony exhibiting biofilm overgrowth, broken branches, bare skeleton, or necrosis was classified according to the extent of injury across the colony into five categories, following the scale proposed by White et al. (2012), where level-0 indicates no evident injury and level-4 is the most extensive (Table 2). Corals exhibiting limited signs of stress, such as retracted polyps or mucus production, were classified as level-1.

A dataset of 185 images from the 2011 survey was analyzed for evidence of coral injuries. Images of poor quality, low resolution, or insufficient luminosity, as well as duplicated images, were discarded from analysis. For baseline comparison, 580 images (those containing corals) were analyzed from MAPTEM dataset (Table 3). For MAPTEM images and the 2011 images, “Image J” software was used to calculate the area of the coral colony and the relative proportion of injury. The nonparametric one-way analysis of variance by ranks test (Kruskal and Wallis, 1952) was used to test for statistical differences comparing the presence of injured corals between the MAPTEM and 2011 studies. Data were grouped by year in eight different categories: a group of all levels of injury (levels 1–4), a group of mild-intermediate injuries (level 1), a

group of intermediate to extreme injuries (levels 2–4) and each 0–4 separately. The dependent variable was the number of injured colonies per photograph, and the independent variable was the sampling year.

2.4. PAH analysis

Tissue samples of benthic invertebrates were analyzed for total polycyclic aromatic hydrocarbon (tPAH) by Alpha Analytical Laboratories in Mansfield, MA. Analyses were conducted pursuant to the Analytical Quality Assurance Plan for Mississippi Canyon 252 (Deepwater Horizon) NRDA Version 3.0 (NOAA, 2011) using the ToxPAH50 methodology. Sediment samples were analyzed for total petroleum hydrocarbon and tPAH, which were measured by Battelle Laboratories using EPA Method 8270-SIM (semi-volatile organic compounds by gas chromatography/mass spectrometry with selective ion monitoring). The tPAH values were calculated using protocols listed in the NOAA PAH-51 method. The tPAH data, as well as additional data on abiotic environmental variables in sediment (including grain size, total organic carbon, latitude–longitude, and water depth) were downloaded from the Environmental Response Management Application (ERMA) Gulf Response website (NOAA, 2013).

3. Results

3.1. Coral injury

Coral samples collected by ROV and identified by morphological techniques included the following taxa: *Hypnorgia pendula*, *Bebruce* spp., *Thesea nivea*, *Swiftia exserta*, *Antipathes atlantica*, *Stichopathes* sp., and *Ellisella barbadensis*. This section will compare the characteristics of injury to these corals at AAR and RTR prior to the DWH discharge with characteristics of injury observed at the same reefs in 2011 after the discharge. The numerical analysis comparing frequency of injury pre- and post-discharge and spatial distribution of post-discharge injury is presented separately.

3.1.1. Pre-discharge conditions

Between 1997 and 1999, the mesophotic corals at AAR and RTR had relatively few cases of injury overall; furthermore, the types of injuries observed differed qualitatively from post-discharge injuries, and injuries occurred in different taxa.

In pre-discharge conditions, the most injured taxon was *Bebruce*, especially in low-growth-form colonies ($< 20\text{ cm}$ above attachment point). *Bebruce* colonies were frequently found covered by a thin layer of fluffy brown sediment. There were no examples of retracted polyps or dead tissue. In some cases, baseline photographs showed a light mucus layer surrounding functional polyps. In the most severe cases, *Bebruce* was covered by sediment, leaving only terminal branches exposed.

Among the taller-growth-form species $> 50\text{ cm}$ above attachment point), such as *H. pendula* and *S. exserta*, broken branches, dead tissue, bare skeleton, and hydroid overgrowth were not observed in pre-discharge images. Injury to coral colonies occurred at mild and intermediate injury levels (levels 1 and 2), characterized by small branch areas that were covered by a thin layer of sediment and mucus, but polyps were functional. The few cases of severe injury in *H. pendula* and *S. exserta* which were observed indicated gross mechanical impact that toppled the colony or buried it under sediment. Some colonies of *T. nivea* had indications of minor stress, such as mucus secretion and branches partly covered by sediment. Injuries to whip corals *Stichopathes* sp., *E. barbadensis*, and the black coral *A. atlantica* were rarely observed. Colonies of *Stichopathes* sp. and *E. barbadensis* were

Table 2
Visual scale of coral injury adapted from White et al. (2012).

Level	Description	Injury area (%)
0	Uninjured	Less than 1
1	Mild	1–10
2	Intermediate	10–50
3	Severe	50–90
4	Extreme	Over 90

Table 3

Summary of images collected and analyzed from primary study sites. Only photos with corals visible were analyzed.

Site	Date	Study ID	Taken	Analyzed
AAR	1997	CSA-TAMU	187	77
	1998	CSA-TAMU	164	71
	1999	CSA-TAMU	188	70
	2011	This study	633	104
RTR	1997	CSA-TAMU	181	139
	1998	CSA-TAMU	171	124
	1999	CSA-TAMU	144	99
	2011	This study	741	81
Total AAR–RTR			2409	765

occasionally found with discolored soft tissue and polyps lacking close to the attachment point (holdfast). Some *A. atlantica* had small patches of green biofilm covering distal branches in the upper portion of the colony or a layer of sediment covering the basal portion of the coral.

3.1.2. Post-discharge condition

Several coral pathologies were observed, and these differed among taxa. In the case of *T. nivea* and *Bebryce* spp., injured branches were typically covered by hydrozoans and/or biofilm; denuded branches were not observed in these species. Patches of healthy and unhealthy polyps, characterized by retracted polyps or areas covered by mucus and biofilm material were observed in some cases in these two taxa when injury only covered less than 10% of the colony (level 1).

Taxa with growth forms taller than approximately 0.5 m, such as *H. pendula*, *S. exserta*, and *T. nivea*, typically displayed the most severe injury levels, while those growing close to the substratum showed fewer severe injuries in the post-discharge study. Among *H. pendula* there was biofilm overgrowth with hydroids or greenish algal material. In severe examples, *H. pendula* colonies lost some or all branches. Broken branches were sometimes observed in a semi-detached state (Fig. 3a). *S. exserta* colonies were commonly observed with intermediate to severe injuries (levels 2–3); extreme injury levels were also seen (level 4). In general, injured corals exhibited sharp gradients between healthy tissues with extended polyps and necrotic tissues with brown or green biofilm overgrowth or skeletal branches denuded of tissue. The living tissue near dead patches of the colony did not show biofilm material or overgrowth with hydroids, but polyps were often retracted (Fig. 3b).

Among the three taller taxa, *H. pendula* was found with exposed skeleton from necrotic loss of soft tissue. The full spectrum of injury was evident in this taxon. The lowest degree of injury (level 1) was characterized by the secretion of mucus and locally retracted polyps. In more pronounced cases of injury, biofilm overgrowth and tissue necrosis were present on soft tissue. Hydroid colonization was visible on necrotic tissue and bare skeleton. Finally (level 4), branches weighted down by hydroids broke apart from the colony (Fig. 4). A similar range was observed in *S. exserta* colonies and, to a lesser degree, in *Stichopathes* sp. colonies. In these specimens, presence of brown biofilm overgrowth was evident along with disintegration of smaller branches. In the case of *T. nivea* and *Bebryce* spp., which are low-growing taxa, mucus secretion and abnormal tissue coloration were common indicators of stress at both study sites. Injuries to *A. atlantica* and *Bebryce* consisted of debris overlain on branches. Epifauna on *Bebryce* in particular made it difficult to assess minor injury levels.

3.2. Coral injury frequency and distribution

Six taxa of octocoral and antipatharian corals were found to have both tissue and skeleton injuries. The following octocoral taxa are sorted according to prevalence of injury: *H. pendula*, which showed the greatest frequency of injured colonies at both sites, comprising 40.3% at AAR and 42.5% at RTR of injured colonies (levels 1–4), followed by *Bebryce* spp. (16.5% and 18.0%), *T. nivea* (15.4% and 13.5%), and *S. exserta* (17.8% and 19.5%); see Tables 4 and 5. Details of species' frequency by injury level are reported in Supplementary Tables 1 and 2. Review of the data shows that *Bebryce* spp., the low-growing species, exhibited an injury frequency that was variable year-by-year during the pre-discharge observations (Table 4).

Coral colonies with intermediate or greater degrees of injury (levels 2–4) were more abundant at AAR and RTR post-discharge (150 cases) than in the pre-discharge surveys. This was the case for *T. nivea* and *S. exserta*. In addition, injured colonies of *H. pendula*, *S. exserta*, and to a lesser degree *Stichopathes* sp. were frequently found with high amounts of hydroids and biofilm growing over bare skeleton, and broken branches were observed on the sea floor. *H. pendula* was the only taxon found in the four different levels of injury at both sites. In contrast, healthy colonies with lower stress indicators (< 10% injury) were more frequent at RTR. Healthier colonies were represented by all coral taxa found at both sites, but mainly by small colonies (< 20 cm) of *T. nivea* and *Bebryce* spp. (Fig. 5). However, a large number of *Bebryce* colonies were observed displaying mild to intermediate injuries in RTR in 1997 and 1999. *Bebryce* is a problematic taxon for assessment because the overgrowth of hydroids and epiphytes occurs in these species with uncertain impact on coral health. In addition, colonies of low profile such as *Bebryce* can be affected by sedimentation and sediment re-suspension to a higher degree than coral species growing above the substrata. Injury quantification for this taxon is therefore presented separately (Fig. 6).

A Kruskal–Wallis test was performed for each treatment, showing significant differences ($p < 0.05$) between post-discharge observations and all pre-discharge years for injury levels 2–4 combined (*Bebryce* spp. excluded). A Friedman “*a posteriori*” test (multiple comparisons between *p*-values and *z*-values) showed that the numbers of intermediate, severe, and extreme injuries (levels 2–4 combined) found in 2011 were significantly higher than the numbers of injuries found in the pre-discharge samples (MAPTEM). (Fig. 7)

3.3. PAH results

Oil was found at detectable levels in invertebrate tissues and sediments from both sites: AAR and RTR (Fig. 8; Table 6). The

sensitivities to total PAHs, dispersants, and mixtures of oil and dispersants have not been assessed for deep-water sea fans, but shallow corals exhibit adverse reactions to low concentrations of

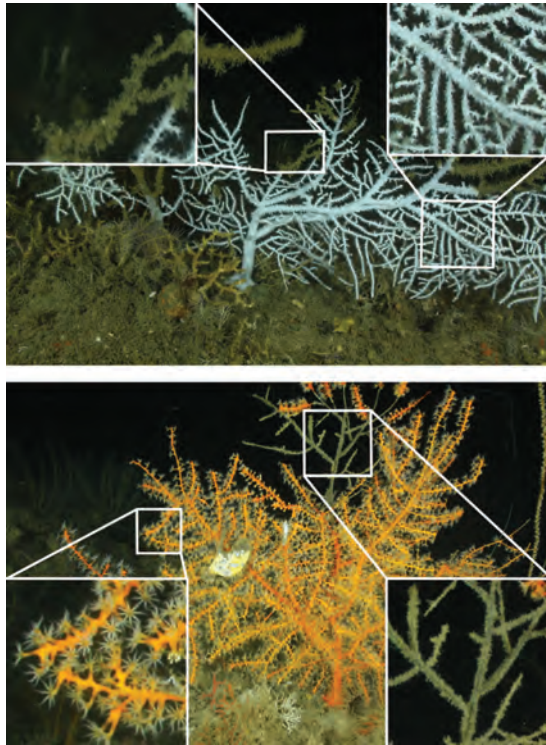


Fig. 3. Healthy an unhealthy tissue observed on *Hypnogorgia pendula* (above) and *Swiftia exserta* (below) colonies. Insets show details of healthy and damaged tissue to distinguish differences in the same colony.

dispersed oil over 3–6 day time periods (National Research Council, 2005). At AAR and RTR, baseline tPAH levels in sediments were available from sampling that pre-dated the DWH discharge (Continental Shelf Associates and Texas A&M University, 2001). The values found post-discharge exceeded baseline values at both sites by a factor of 5 in 2010 and a factor of 2 in 2011. tPAH values from the tissues of invertebrates were relatively high compared to tPAH values from the sediments. The highest tPAH values were found in octocoral tissues at AAR. Sediment PAH values from AAR, closer to the wellhead, also were higher than sediment tPAH values at RTR, which is farther from the wellhead on the eastern side of the northern Gulf of Mexico. The highest post-discharge tPAH concentrations found in sediments (at AAR), however, were

Table 4

Frequency of injured coral taxa (levels 1–4), by taxa, in AAR and RTR study sites. The 1997–1999 columns refer to results from the MAPTEM study, while 2011 is the post-DWH discharge observation.

Species	Site							
	AAR				RTR			
	1997	1998	1999	2011	1997	1998	1999	2011
<i>Hypnogorgia pendula</i>	0	0	0	97	8	0	3	85
<i>Swiftia exserta</i>	1	8	6	43	14	7	20	39
<i>Thesea nivea</i>	0	0	0	37	4	0	0	27
<i>Bebryce</i> spp.	7	34	14	40	107	26	64	36
<i>Antipathes</i> sp.	0	11	2	15	2	0	1	9
<i>Placogorgia</i> sp.	2	0	1	0	25	0	1	0
<i>Ellisella</i> sp.	0	0	2	0	22	16	9	0
<i>Stichopathes</i> sp.	0	0	4	9	1	3	3	4
Total injured (n)	10	53	29	241	183	52	101	200

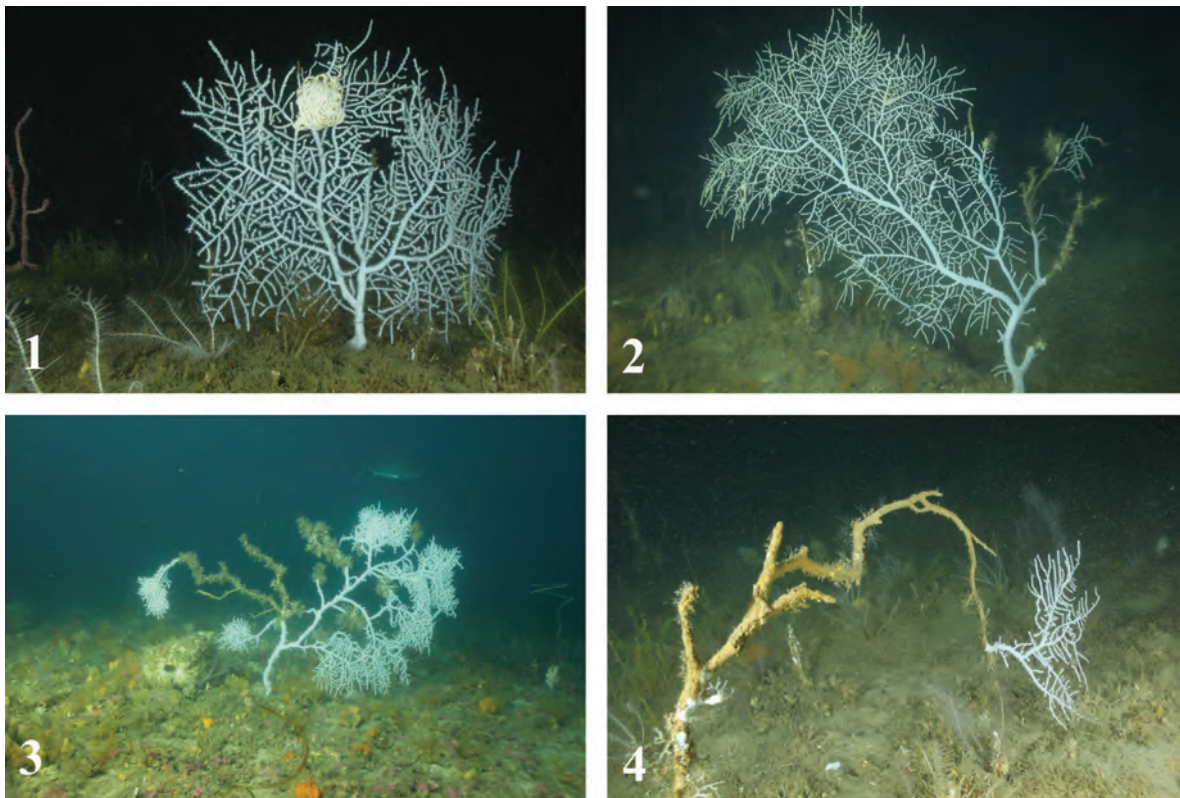


Fig. 4. Injury levels (1–4) observed on *Hypnogorgia pendula* colonies.

25–33% of the lowest values reported within 3 km of DWH (NOAA, 2013).

Table 5
Abundance of tall (> 0.5 m) growth form coral colonies (*Bebryce* spp. excepted) for all injury stages in both study sites.

Injury level		0		1		2		3		4	
Frequency		n	%	n	%	n	%	n	%	n	%
AAR	1997	238	96.0	8	3.2	1	0.4	0	0.0	1	0.4
	1998	190	78.2	35	14.4	17	7.0	1	0.4	0	0.0
	1999	127	81.4	20	12.8	7	4.5	2	1.3	0	0.0
	2011	264	52.3	91	18.0	71	14.1	42	8.3	37	7.3
RTR	1997	405	68.9	104	17.7	60	10.2	14	2.4	5	0.9
	1998	449	89.6	19	3.8	28	5.6	5	1.0	0	0.0
	1999	259	71.9	52	14.4	24	6.7	16	4.4	9	2.5
	2011	358	64.2	98	17.6	60	10.8	9	1.6	33	5.9

4. Discussion

This study found a large number of octocoral and antipatharian corals in September 2011 that exhibited pathologies consistent with injury resulting from acute impact (see for example Chan et al., 2012), but did not observe coral predators, fishing gear, or evidence for sedimentation that would suggest the cause of the injuries. Similar severity and frequency of injuries to corals with growth forms > 0.5 m had not been noted during repeated surveys of the Pinnacle Reefs coral habitats prior to the DWH discharge (Continental Shelf Associates and Texas A&M University, 2001). One objective of the 2001 report was to assess health and condition of hard bottom communities. Our review of the pre-discharge data did find minor (level 1) injuries to a low-growth-form taxa (e.g. *Bebryce* spp.), which might have been caused by regularly occurring processes. However, injuries to the tall-growth-form taxa observed in at the AAR and RTR study sites in

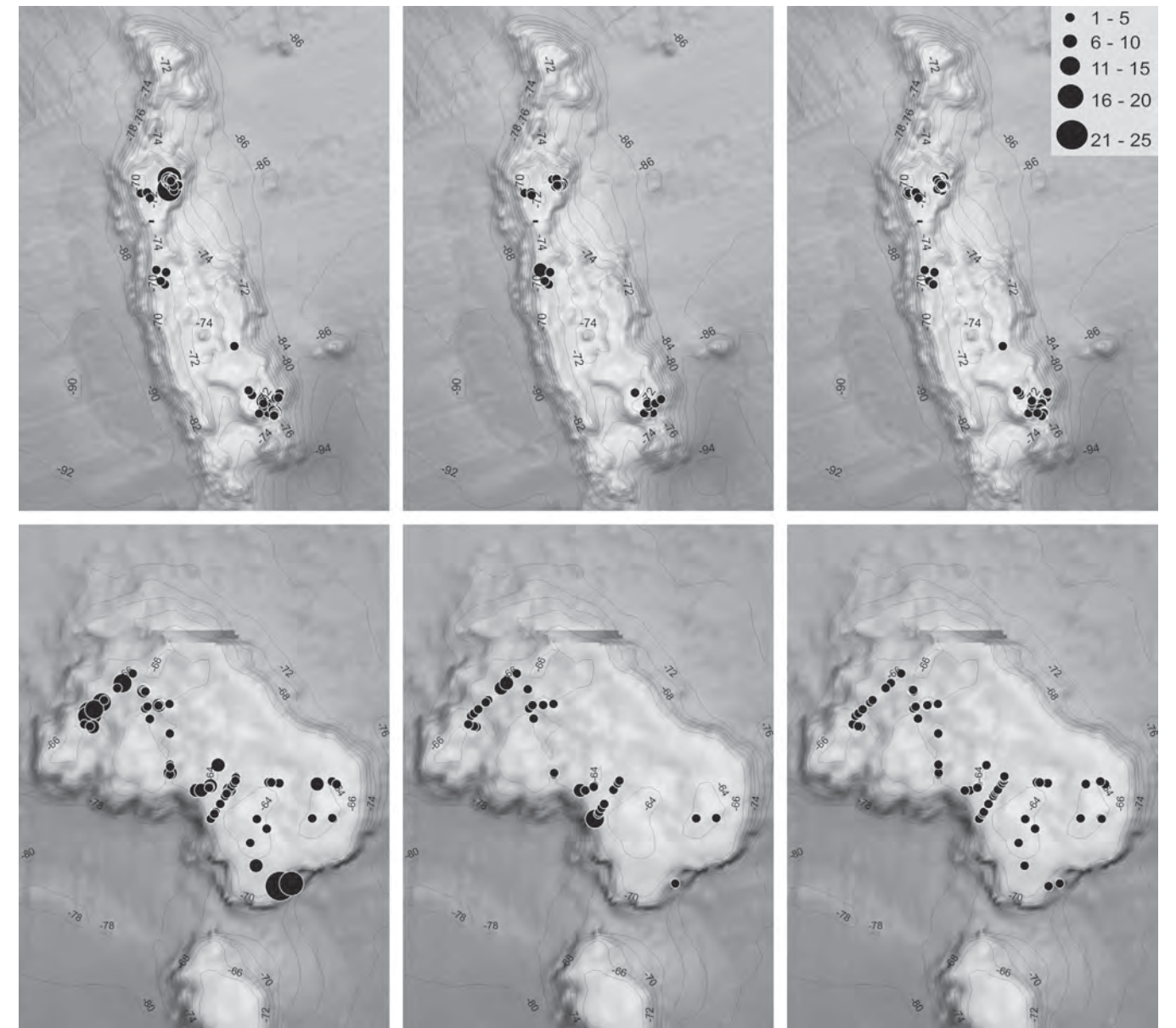


Fig. 5. Distribution of injury levels 0 (left), 1 (center), and 2–4 (right) and coral colony abundances within Alabama Alps Reef (upper panels) and Roughtongue Reef (lower panels). Dot size indicates abundance of injured corals (*Bebryce* spp. excluded).

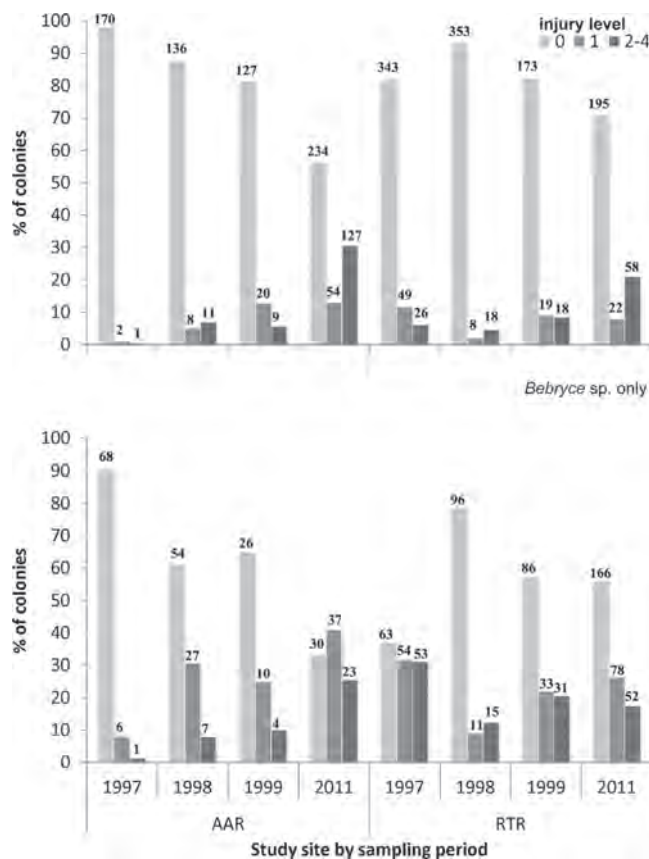


Fig. 6. Damage frequency by injury levels (Table 2) for MAPTEM and 2011 surveys for study sites. Upper chart: all coral colonies taxa except *Beeryce* spp. Lower chart: *Beeryce* spp. alone.

2011 were significantly more frequent and severe than anything reported in 1997, 1998, or 1999 at the same sites.

Pathologies similar to those reported in this study were observed among deep-sea corals thought to have been exposed to crude oil and dispersant-treated oil from the DWH discharge via a deep-water plume (Hsing et al., 2013; Rooker et al., 2012). These authors reported the presence of flocculated biofilm material, mucus secretion, necrosis, bare skeleton, and branches colonized by hydroids on several gorgonians and other coral taxa at a 1370 m coral community in the lease block MC292.

The total numbers of injured colonies documented at AAR and RTR exceeded those reported by White et al. (2012) and Hsing et al. (2013). This reflects the larger size of the shallow reef habitats surveyed and an overall higher abundance of corals in the shallower mesophotic marine setting. According to White et al. (2012), the observed injury to the deep-sea corals was attributed to the DWH oil and gas discharge close to the study sites. This evaluation was corroborated by analysis of biofilm material, which was composed of dead coral tissue and residual hydrocarbon compounds, the biomarker signature of which matched DWH oil. We hypothesize, based on the characteristics and severity of the damage found in coral colonies after the DWH incident and the relative scarcity of coral injury reported in the pre-discharge MAPTEM study, that injury observed among corals in the vicinity of AAR and RTR was caused either directly or indirectly by the DWH oil discharge and/or response activities, which included extensive use of dispersants directly over these affected sites.

Coral injuries attributable to stress from chronic pollution, oil spill incidents, and bioaccumulation of hydrocarbon compounds (PCBs, PAH's and NPEs) have been reported previously by Loya and Rinkevich (1980), Guzman et al. (1994, 1991), Vogt (1995),

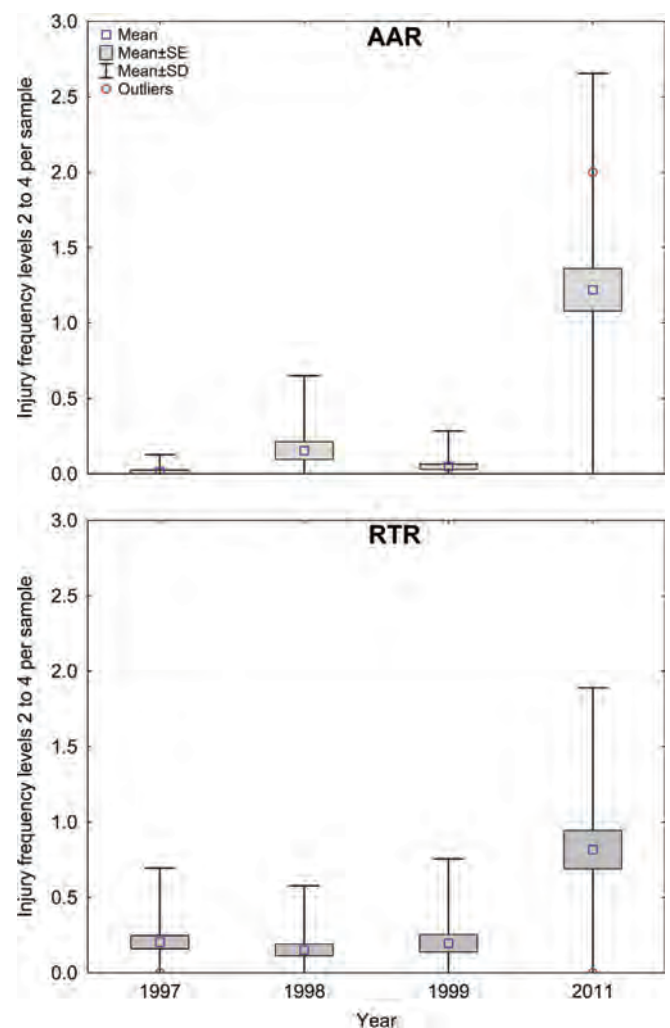


Fig. 7. Box and whisker plots of Kruskal–Wallis test for frequency at Alabama Alps Reef (upper) and Roughtongue Reef (lower) study sites comparing intermediate to extreme injuries for MAPTEM and the 2011 for tall growth forms (*Beeryce* spp. excluded). Plot key as follows: blue square—outliers, bar—greatest value excluding outliers, red diamond—median, upper box limit—quartile 75%, middle line—mean (quartile 50%), and lower box limit—quartile 25%. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Poulsen et al. (2006), Cailleaud et al. (2007). Chan et al. (2012) provide a detailed description of how heavy-metal bioaccumulation produces similar injuries to those reported in the study. McClanahan et al. (2004) propose that necrosis is an extreme response of the soft coral immune system to stresses caused by external factors such as temperature changes, pollution, or diseases. Pollutant intake by corals can result from direct contact and be further exacerbated by feeding behavior that selects floating particles, as has been suggested by Anthony (2000). Mitra et al. (2012) reported evidence that mesozooplankton in the Gulf of Mexico exposed to PAH's related to the DWH blowout eventually entered the marine food chain, transferring PAH's to higher trophic levels. So the literature amply supports pathological responses to stress in this group and indicates possible pathways for exposure.

Other known and documented natural sources of pathology in octocoral colonies include disease, predation, bottom-contact fishing impacts, landslides, and changes to the ocean temperature (i.e. coral bleaching). In shallow Caribbean reefs, fungal infections observed on sea fans are taxon-specific (Toledo-Hernández et al.,

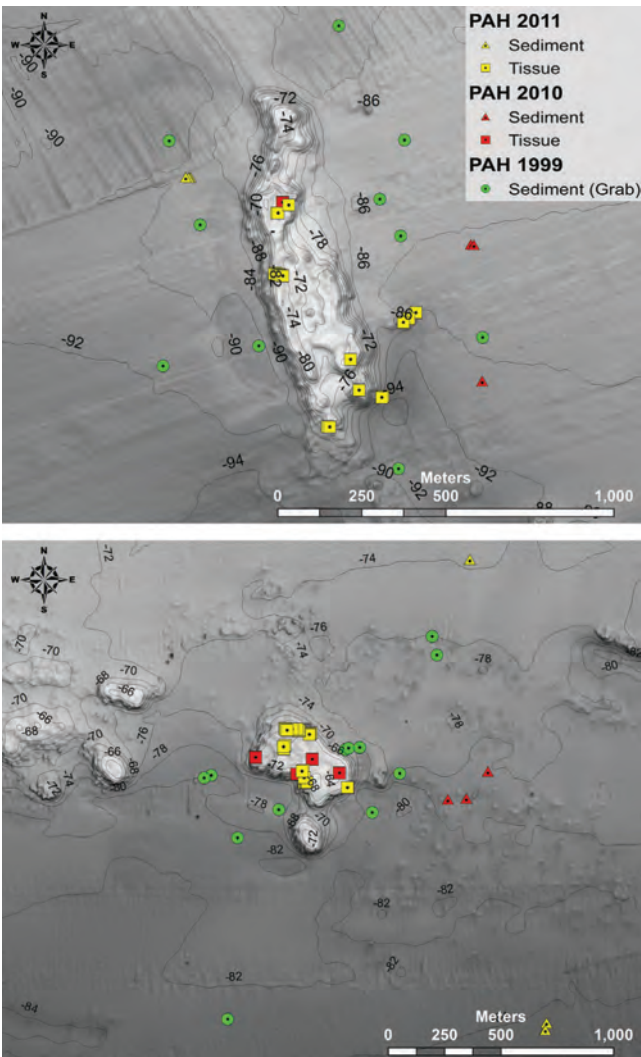


Fig. 8. PAHs from 2000 to 2011. Sediment core and tissue collections for PAH analysis. Location symbols indicate sample type and year. Grab sample locations reported in a 2001 study (Continental Shelf Associates and Texas A&M University, 2001) are plotted for comparison.

Table 6
Total polycyclic aromatic carbon (tPAH) values in parts per billion (PPB) dry weight for invertebrate tissues and sediments collected during surveys in 2010 and 2011 and from baseline samples collected in 2001 (Continental Shelf Associates and Texas A&M University, 2001). Reported as number of samples: mean value (standard deviation).

Sample type	AAR			RTR		
	2000	2010	2011	2000	2010	2011
Sediment						
Grabs	10:26 (n.a.)	1:17.1 (n.a.)	n.a.	12:10 (n.a.)	1:0.86 (n.a.)	n.a.
Push cores	n.a.	3:101 (43.9)	10:41.7 (21.8)	n.a.	4:20.1 (16)	10:20 (10.9)
Tissue						
Cnidaria	n.a.	1:151 (n.a.)	18:345 (791.2)	n.a.	4:179 (252)	27:51 (88)
Porifera	n.a.	n.a.	n.a.	n.a.	1:27 (n.a.)	4:17 (9.6)
Echinodermata ^a	n.a.	8:56 (30.2)	4:32 (34)	n.a.	1:228 (n.a.)	n.a.

^a Basket star.

2008; Zuluaga-Montero et al., 2010); however, in this study we observed similar injuries among several different octocoral and antipatharian taxa. Fishing gear is more likely to impact octocoral taxa indiscriminately than disease. It is possible that recreational fishing lines could inflict sub-lethal abrasions to soft corals that would make the sea fans more vulnerable to infections or predation. However, few of the injuries observed in 2011 or previous MAPTEM ROV imagery showed any evidence of fishing gear.

Predators to deep water octocorals include flamingo tongue snails, gastropods, asteroid sea stars, and butterflyfish. However, few coral predators were observed in either MAPTEM or 2011 surveys. The Pinnacles Reefs may experience periods of high sedimentation associated with the Mississippi outflow, which may also result in injuries similar to what we observed, including exposed, dead skeleton and hydroid growth. However, historical studies of Pinnacles reefs have suggested that MCEs are resistant to adverse impacts from sediment, and may even use sediment as a food source (Continental Shelf Associates and Texas A&M University, 2001).

Prior to the DWH blowout, Peccini and MacDonald (2008) measured soft coral orientation in relation to bottom currents at both AAR and RTR, examining photographs of 365 sea fans at AAR, RTR, and three other sites in the Pinnacle Reef area. They did not report any notable coral injury at that time. This is also consistent with our analysis of the MAPTEM photography collection, of which Sites 1 and 7 correspond to RTR and AAR, respectively. Photographic analysis of coral communities reveals that some injured colonies were present in that period, but the severity and extent of coral injury does not match those reported in the 2011 study. This is corroborated by the Kruskal–Wallis test results, which found statistical differences ($p < 0.05$) between samples, and the Friedman test demonstrated that post-discharge samples (2011) were significantly higher in terms of the numbers of injuries found in all the injury levels in the pre-discharge samples (MAPTEM).

Both sites, AAR and RTR, were situated under layers of floating oil from the DWH oil discharge. Benthic organisms could be exposed to this oil through trophic pathways or by direct contact. Gin et al. (2001) proposed a model of how an oil spill could affect food chain interactions in marine environments. The model assumes that there are two ways that an organism interacts with hydrocarbon compounds: (1) consumption of contaminated prey items and (2) direct incorporation by tissues of dissolved oil from surrounding water or sediments. Measurable concentrations of hydrocarbons in octocoral tissues, more than 1 year after oil had disappeared from the surface waters of the Gulf, were well below the bioeffect concentrations established for estuarine sediments (Long et al., 1995).

One of the palliative measures taken to mitigate the floating oil was the use of dispersants; this could have increased chances of direct contact of oil with coral. Chemical dispersants are surfactants with both lipophilic and hydrophilic properties that serve to break the oil into smaller droplets, increasing surface area for volatilization, dissolution, or degradation (Lessard and DeMarco, 2000). The repeated application of dispersants near and directly over the AAR and RTR sites (Fig. 9) increased the probability of exposure of corals at these sites to oil and/or dispersants by facilitating mixture of floating oil into sub-surface water depths. Dispersant application demonstrates the presence of thick oil layers near the sites, because thicker accumulations of surface oil were specifically targeted by aerial dispersant missions. Moreover, oil dispersants such as Corexit 9500 (Nalco Holding Company), used to respond to the DWH blowout, have deleterious effects in different marine organisms. Goodbody-Gringley et al. (2013) described the effect of different concentrations of Corexit mixed with crude oil on larval behavior of coral *Montastraea faveolata* and *Porites asteroides*. Both coral larvae changed their settlement

behavior. The degree of impact of dispersants depends on its chemical concentrations and which species are affected. In extreme cases, oil and dispersant produce complete failure of settlement and total mortality of coral larvae, especially when they are exposed to high concentration (25 ppm) of Corexit alone. Similar results have been obtained by DeLeo et al. (2016) where colonies of *Paramuricea biscaya*, *Callogorgia americana* and *Leio-pathes glaberrima* exposed to treatments of dispersant alone and oil-dispersant mixtures were harmed more than those exposed to oil alone.

Another mechanism oil spill responders used to decrease the amount of oil on the surface water was burning the thicker layers of oil (Fig. 9). When oil is burned and combusted in ocean environments, large amounts of soot particles (black carbon) are dispersed into broad areas by ocean currents and wind. Perring et al. (2011) reported that 4% ($1.35 \pm 0.72 \times 10^6$ Kg.) of burned oil was released to the atmosphere as black carbon. The particles of black carbon can eventually reach the surface of the water by rain or sedimentation. Weber et al. (2012) suggest that disease and mortality on coral colonies can be attributed to particle sedimentation. Organic-rich material can produce tissue degradation within a day, and inorganic particles produce the same effect after approximately 6 days. White et al. (2012) found dark droplets and particles in biofilm material that was removed from injured deep-water corals close to the DWH site.

An additional event that could have contributed to floating oil reaching the bottom was the passage of Tropical Storm (TS) Bonnie over the Gulf of Mexico between July 22 and July 26. Synthetic Aperture Radar (Lessard and DeMarco) images of the floating oil obtained from NOAA Satellite and Information Service (NESDIS) showed that the floating oil over the mesophotic Pinnacles Reefs almost disappeared from the ocean surface over the Pinnacles on July 26 (Fig. 10). Rain and wind would have accelerated oil and water mixing rates on the ocean surface, removing and breaking the floating oil into small particles, introducing and dissolving it into the water column. Finally, the oil dispersed in the water column would have been mixed downward to the pinnacles because of the large waves generated by TS Bonnie. Such effects would

have been more deleterious to coral health than storms under normal conditions. Continental Shelf Associates and Texas A&M University (2001) reported an increase of surface and bottom (97 cm/s) currents in the Pinnacle Reefs after the passage of hurricanes Earl and George in 1998. However, no occurrence of coral pathologies was noted by researchers who subsequently surveyed gorgonian colonies (e.g. Peccini and MacDonald, 2008). So it is unlikely that coral injuries reported here were caused by the TS Bonnie alone.

Analysis of the TPH and tPAH concentrations in sediments and tissues post-discharge (2010 and 2011) does show a slight increase above pre-discharge levels, but this increase may not in itself be significant for coral health because the concentrations are far below toxic-effect levels for test organisms. Chronic-effect levels of TPH and tPAH for the octocoral taxa have not been developed, nor have effect levels been established for dispersants, so deleterious impact cannot be entirely ruled out. However, the low concentrations suggest that injury did not occur due to exposure from residual pools of hydrocarbons in the reef environment.

5. Conclusions

We conclude that widespread coral injuries observed at two mesophotic communities within the Pinnacles Reefs in the Gulf of Mexico during 2011 were qualitatively different and significantly greater in frequency than anything noted prior to the DWH discharge. A characteristic of this injury was patchy damage affecting portions of the sea fan colony. Anomalous injuries were observed in coral taxa with a growth form > 0.5 m; the species *H. pendula* sustained the most injury at both sites. In more severe levels, biofilm overgrowth covered portions of the colony, and patches of necrotic tissue were observed. The most severe levels of injury were characterized by bare, broken, and missing branches.

The cause of these injuries is hypothesized to be exposure to oil and/or dispersant from the DWH discharge. Proximity of the sites to dispersant applications and, in the case of the western-most site, burning operations increased the risk of harmful impact.

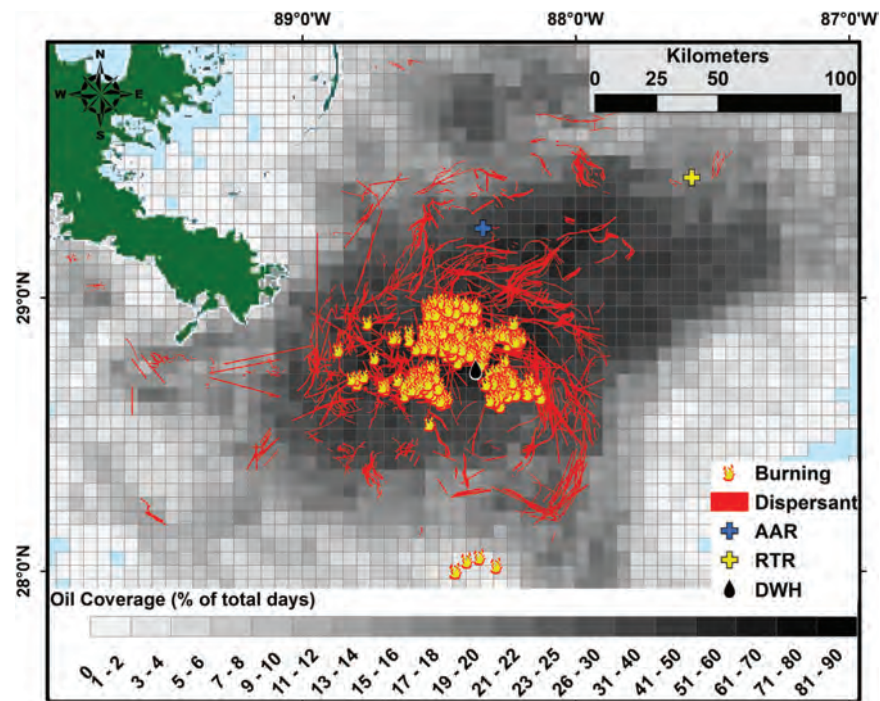


Fig. 9. Map of aerial dispersant application (red lines) and oil burning sites (flames) over floating oil layers discharged by the DWH blowout (NOAA, 2013).

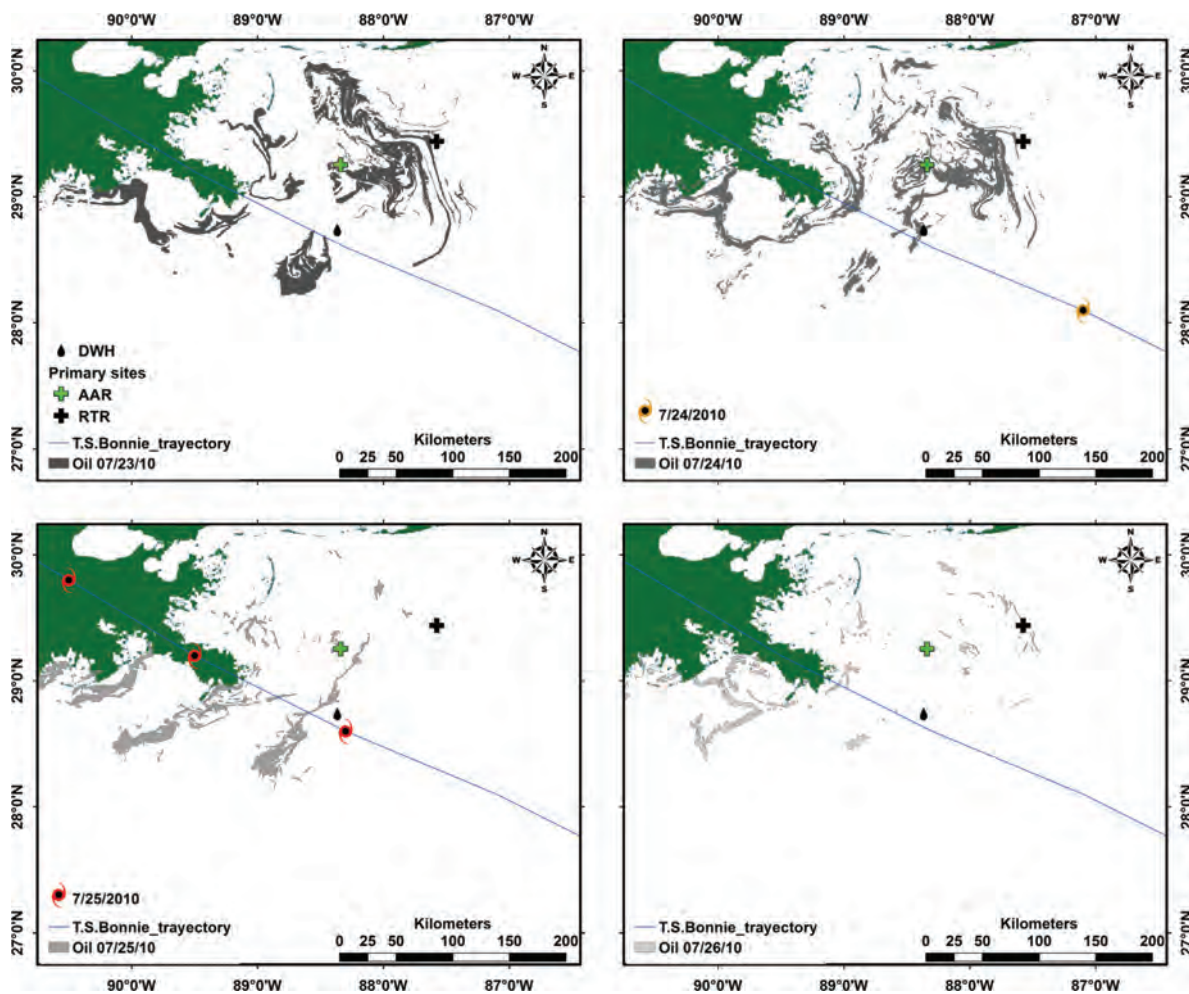


Fig. 10. Areas of floating oil over Gulf of Mexico between July 23 and July 26 of 2010 as interpreted by NOAA NESDIS (NOAA, 2013) and subsequent dissipation after TS Bonnie passed over the region. Blue dotted line represents the complete trajectory of TS Bonnie. Daily location of TS Bonnie is plotted using the hurricane symbol for July 24th (yellow) and July 25th (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Passage of TS Bonnie over the region would have caused the upper water column to mix turbulently downward, which would have exposed corals to material floating near the surface, particularly on promontories of the reefs and for taxa with relatively higher relief above the bottom. Because the concentrations of hydrocarbons in sediments and animal tissues collected in 2011 were well below chronic bio-effect levels, exposure must have occurred as an acute event.

Disclaimer

The scientific results and conclusions, as well as any opinions expressed herein, are those of the author(s) and do not necessarily reflect the views of NOAA or the Department of Commerce. The mention of any commercial product is not meant as an endorsement by the Agency or Department.

Acknowledgments

This work was supported by funding provided as part of the Natural Resource Damage Assessment (NRDA) of the Deepwater Horizon oil spill being conducted in part by the National Oceanic and Atmospheric Administration (NOAA), and by the Bureau of

Ocean Energy Management (BOEM). We gratefully acknowledge the support and dedication of the science party on the 2011's Mesophotic Reef Cruise. We also thank the crew of *Holliday Chouest*, the pilots of the C-Innovation ROV, and the marine technicians of CSA Ocean Sciences Inc., as well to C. Johansen for her valuable comments and suggestions. M. Silva is supported by Fulbright – CONICYT (Comisión Nacional de Investigación Científica y Tecnológica) Scholarship (EEUU and Chile governments) and Latin America-Caribbean (Perring et al.) Scholarship (Florida State University) and by research assistantship support provided by the Deep-C GOMRI consortium.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.dsr2.2015.05.013>.

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EXHIBIT

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Toxicity of Deepwater Horizon Source Oil and the Chemical Dispersant, Corexit® 9500, to Coral Larvae

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Abstract

Acute catastrophic events can cause significant damage to marine environments in a short time period and may have devastating long-term impacts. In April 2010 the BP-operated Deepwater Horizon (DWH) offshore oil rig exploded, releasing an estimated 760 million liters of crude oil into the Gulf of Mexico. This study examines the potential effects of oil spill exposure on coral larvae of the Florida Keys. Larvae of the brooding coral, *Porites astreoides*, and the broadcast spawning coral, *Montastraea faveolata*, were exposed to multiple concentrations of BP Horizon source oil (crude, weathered and WAF), oil in combination with the dispersant Corexit® 9500 (CEWAF), and dispersant alone, and analyzed for behavior, settlement, and survival. Settlement and survival of *P. astreoides* and *M. faveolata* larvae decreased with increasing concentrations of WAF, CEWAF and Corexit® 9500, however the degree of the response varied by species and solution. *P. astreoides* larvae experienced decreased settlement and survival following exposure to 0.62 ppm source oil, while *M. faveolata* larvae were negatively impacted by 0.65, 1.34 and 1.5 ppm, suggesting that *P. astreoides* larvae may be more tolerant to WAF exposure than *M. faveolata* larvae. Exposure to medium and high concentrations of CEWAF (4.28/18.56 and 30.99/35.76 ppm) and dispersant Corexit® 9500 (50 and 100 ppm), significantly decreased larval settlement and survival for both species. Furthermore, exposure to Corexit® 9500 resulted in settlement failure and complete larval mortality after exposure to 50 and 100 ppm for *M. faveolata* and 100 ppm for *P. astreoides*. These results indicate that exposure of coral larvae to oil spill related contaminants, particularly the dispersant Corexit® 9500, has the potential to negatively impact coral settlement and survival, thereby affecting the resilience and recovery of coral reefs following exposure to oil and dispersants.

Citation: Goodbody-Gringley G, Wetzel DL, Gillon D, Pulster E, Miller A, et al. (2013) Toxicity of Deepwater Horizon Source Oil and the Chemical Dispersant, Corexit® 9500, to Coral Larvae. PLoS ONE 8(1): e45574. doi:10.1371/journal.pone.0045574

Editor: Christian R. Voolstra, King Abdullah University of Science and Technology, Saudi Arabia

Received: April 26, 2012; **Accepted:** August 22, 2012; **Published:** January 9, 2013

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Funding: Funding for this project was provided via Protect Our Reefs 2010 Emergency funding to KB Ritchie, NSF (National Science Foundation) REU-1004181 funding to DG, NSF OCE-0926822 funding to Mary Alice Coffroth, and the Dart Foundation to KBR. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The Gulf of Mexico serves as a major source of crude oil for much of the Western hemisphere. It is estimated that over 1.5 million barrels of oil are extracted each day from offshore oil platforms in the Gulf [1], many of which are located within close proximity to the coastline. This intensive extraction and traffic of crude oil has, as seen with the Exxon Valdez and Deepwater Horizon (DWH) spills, the potential to result in large-scale environmental catastrophes with significant environmental impacts.

Remediation of oil spills often involves the application of dispersant chemicals, which can be mixtures of solvents and surface-active agents, along with other compounds. By reducing the interfacial tension between oil and water, dispersants enhance the breakup of an oil slick into small oil droplets that stabilize in the water column. While dispersants do not reduce the amount of oil entering the environment, they affect the fate, transport, and potential effects of an oil spill by altering the oil's physical properties [2].

The decision to use dispersant chemicals poses trade-offs for oil spill responders. While a dispersed surface oil slick is rendered less likely to reach the shore [3], treatment of major oil spills with

dispersant chemicals has been shown to result in significant environmental degradation as a result of increased hydrocarbon dissolution and surfactant toxicity [4]. The effects of dispersed oil on the marine environment are also dependent on the degree of the dispersant's application as well as corresponding weather conditions [5]. While technological advancements have reduced the number of extraction- and transport-related incidents in recent years, the magnitude and potential impact of oil spills, such as the recent DWH spill in the Gulf of Mexico, affirms the fact that oil pollution constitutes a major threat to the marine environment [6,7].

A major percentage of global offshore oil traffic occurs in close proximity to coral reef ecosystems [8]. The sensitivity of many coral species to environmental perturbation, as well as the current decline in reef cover worldwide has prompted a considerable amount of research into the effects of oil pollution on coral reef communities [7,8,9,11]. Studies have shown that exposure of adult coral colonies to crude oil can result in a range of effects including inhibited growth rate, reduced reproductive activity, and tissue loss [10,11]. Evidence also suggests that dispersed oil is significantly more toxic to corals than crude oil alone. Shafir et al. [7] found that in a survivorship assay of *Stylophora pistillata* and *Pocillopora damicornis* nubbins, concentrations of oil-dispersant mixtures above

25% caused 100% mortality to nubbins of both species, while none of the crude oil water-soluble fractions (WSF's) had any significant effect on coral survivorship.

While many field and laboratory experiments have noted significant damage to coral reef communities by oil contamination, the effects of oil exposure on coral reproduction and larval fitness and recruitment have received less attention. Loya and Rinkevich [11] noticed that exposure of *Stylophora pistillata* colonies to crude oil induced immediate mouth opening, followed by the premature release of underdeveloped larvae. Epstein et al. [4] found that exposure of *S. pistillata* planulae to increasing concentrations of dispersed oil resulted in reduced settlement and survivorship over the course of 96 hours. Similarly, Harrison [12] reported a reduction in metamorphosis of *Acropora tenuis* larvae when exposed to dispersed oil.

Coral larvae play a significant role in reef ecology. In scleractinian corals, planula larvae are the result of sexual reproduction, and have the ability to recruit to new substrate and contribute to genetic diversity [13]. Successful settlement of coral larvae involves the sampling of available substrate, followed by adherence and metamorphosis into a competent juvenile polyp [14]. Evidence suggests that larval settlement is strongly influenced by chemical cues, which are believed to stem from naturally occurring biofilms on marine substratum [8,15,16]. Consequently, surfactants alone and in the presence of oil could alter the physical and chemical properties of the ideal biofilm required for settlement or interrupt these sensitive chemical cues. Given the importance of successful larval recruitment in maintaining the reef environment, it is imperative to gain a fundamental understanding of toxicological effects on larval ecology.

Methods for conducting toxicity tests of chemical pollutants such as oil and dispersants have been developed and applied to teleost larvae and other organisms [17,18]. The purpose of the present study was to evaluate effects of exposure to the water accommodated fractions (WAFs) of DWH oil (fresh and weathered), chemically enhanced water accommodated fractions (CEWAFs) of the oil, and Corexit® 9500 dispersant on planula larvae of the scleractinian corals *Porites astreoides* and *Montastraea faveolata* from the Florida Keys. Both species are common on reefs throughout the Caribbean, where *P. astreoides* is an early succession species contributing to reef recovery, and *M. faveolata* is an important reef building species. These species also differ in mode of reproduction; *P. astreoides* is a brooding coral that undergoes internal fertilization and releases semi-mature planula larvae from January to September [19], while *M. faveolata* is a broadcasting coral that spawns gametes in synchrony 1–2 times a year (Aug./Sept.) and requires external fertilization [20]. The longevity and extent of an oil spill in the water column can vary based on weather conditions [21] and microbial interactions [5], and may persist for several days, weeks or years [22]. This study examined the effects of exposure to both fresh and weathered DWH oil and dispersants using short-term assays (≤ 96 -hr) to monitor larval settlement rates, survivorship, and behavioral responses. While the Deepwater Horizon spill did not occur in close proximity to the Florida Keys, it is plausible that oil pollution from similar events could eventually reach these coral reefs via offshore current movement [23], thereby affecting reef health.

Results

Settlement, Behavior, and Survival Assays (Weathered Oil)

Effects of weathered oil exposure on larval settlement and survival. The majority of *Porites astreoides* larval settlement

observed in seawater control dishes and weathered oil applications occurred within the first 24-hrs; no significant difference was observed in settlement success between treatment types within that time period ($p=0.41$; Student's *t*-test) (Fig. 1). Likewise, no significant differences were found in percent larval settlement between treatment and control dishes after 48 hours ($p=0.696$; $F=0.154$; repeated measures ANOVA). After 72-hr, no new settlement occurred by oil-exposed larvae, however, larvae in the control treatment continued to settle throughout the 96-hr observation period (Fig. 1).

Oil-exposed larvae experienced a greater rate of post-settlement mortality than control larvae ($p<0.05$; Student's *t*-test). At 72-hr, mean post-settlement survival of oil-exposed larvae had declined by 42% since the initial observation. At 96-hr, post-settlement survival had declined by almost 80% ($p=0.019$; Student's *t*-test). While post-settlement survival in the control dishes remained relatively constant (survived) throughout the 96-hr observation period, it declined steadily in treatment dishes (Fig. 1).

Effects of weathered oil exposure on larval swimming behavior. Pairwise comparisons (Student's *t*-test) between the mean number of larvae located within each zone (0–6) and the number of larvae in the center of the dish did not differ significantly between oil treatment and control dishes ($p>0.05$), however larvae did show an overall preference for the outermost rim in all treatment dishes ($p<0.001$). On several occasions, larvae were observed contacting the oil mass perhaps as a possible substrate for settlement; neither settlement nor metamorphosis occurred amongst these larvae. Throughout the entire observation period no mortality was observed in any of the seawater control dishes or weathered oil exposure dishes.

Effects of weathered oil exposure on larval survivorship. Mortality of larvae in both acute (24-hr) and prolonged (72-hr) exposure vials was significantly higher than those in control vials ($p=0.0103$; $F=4.039$; repeated measures ANOVA). There was a 2% increase in mortality between the 24 and 48-hr exposures for the oil-exposed larvae, while no mortality was observed in the controls. After 72-hrs, natural mortality was observed in control larvae (4.5%). There was no significant difference between larval mortality associated with acute exposure (24-hr) and prolonged exposure (72-hr) ($p=0.297$; $F=1.238$; repeated measures ANOVA).

Constant Exposure Settlement and Survival Assays

Effects of Water-Accommodated Fractions (WAF). No correlation was found between WAF concentration and *P. astreoides* settlement after 48-hrs (linear regression; $p=0.061$), however, a negative relationship was found among concentration and settlement after 72-hr exposure of *P. astreoides* larvae (linear regression; $p=0.003$) and 48-hr exposure of *M. faveolata* larvae (linear regression; $p<0.0001$), where increasing WAF concentration was associated with decreased larval settlement. Settlement rates of *P. astreoides* larvae differed significantly among concentrations (ANOVA; $p=0.023$; $F=3.301$; Fig. 2). No significant effect on settlement occurred after 48-hr exposure; however, 72-hr exposure to high concentrations of WAF [0.62 ppm] resulted in significantly reduced larval settlement rates of 33% compared to 87% in control treatments (Dunnett's; $\alpha=0.05$; Table 1). Settlement of *M. faveolata* was significantly reduced by exposure to all three WAFs (ANOVA; $p<0.0001$; $F=31.92$; Fig. 2), where 48-hr exposure resulted in 27% mean larval settlement at 0.65 ppm, 16% at 1.34 ppm, and 5% at 1.50 ppm compared to 75% mean settlement in the control treatment (Dunnett's; $\alpha=0.05$).

A negative relationship was found among larval survival and WAF concentration for *P. astreoides* (linear regression; $p=0.008$,

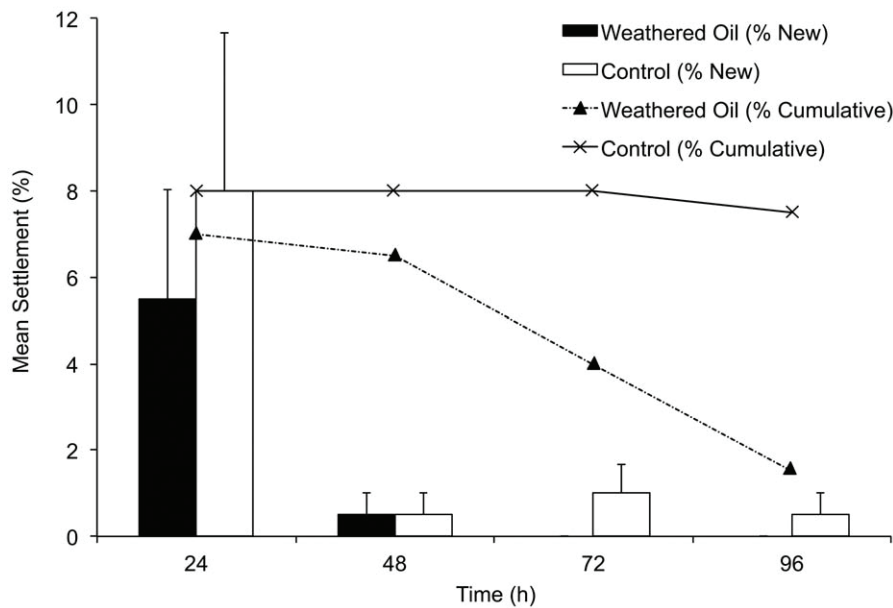


Figure 1. Settlement by larvae exposed to crude oil. Mean percent ($\pm$ SE) new settlement by *P. astreoides* larvae exposed to Louisiana weathered crude oil (solid bars) and a seawater control (open bars) observed at each time point (24, 48, 72 and 96-hr). Mean percent ($\pm$ SE) cumulative settlement by *P. astreoides* larvae after 24, 48, 72 and 96-hr exposure to Louisiana weathered crude oil (dashed line) and a seawater control (solid line).

doi:10.1371/journal.pone.0045574.g001

48-hrs; $p=0.001$, 72-hrs) and *M. faveolata* (linear regression; $p<0.0001$), where increasing WAF concentration was associated with decreased larval survival. Survival of *P. astreoides* larvae varied significantly among WAFs (ANOVA; $p=0.003$; $F=5.40$; Fig. 2). Larval survival was significantly reduced after 48 and 72-hr

exposure to only the highest [0.62 ppm] WAF concentrations [47% survival at 48-hr, 33% at 72-hr] compared to the control [87%] (Dunnett's; $\alpha=0.05$; Table 1). An LC_{50} of 0.51 ppm (95% C.L.s = 0.27–35.19) was determined. Larval survival by *M. faveolata* significantly declined after 48-hr exposure to all three concentra-

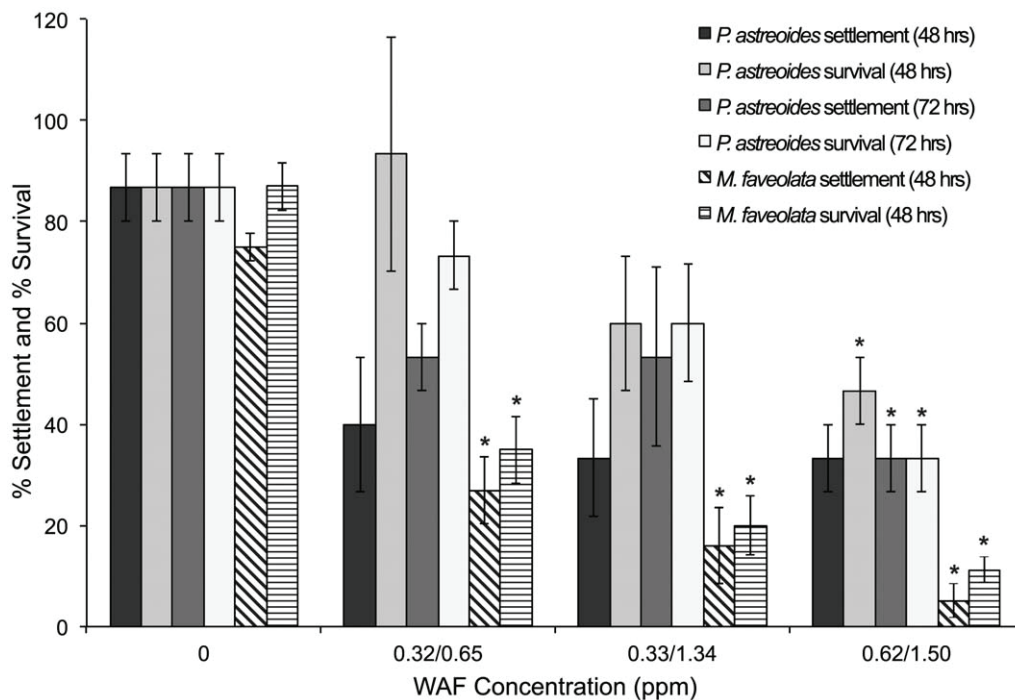


Figure 2. Settlement and survival of larvae exposed to WAF. Mean percent ($\pm$ SE) settlement and survival of *P. astreoides* (solid bars) and *M. faveolata* (striped bars) larvae under constant exposure to DWH crude oil WAF (0, 0.32, 0.33, 0.62 ppm for *P. astreoides*; 0, 0.65, 1.34, 1.50 ppm for *M. faveolata*). Asterisks indicate comparisons that differ significantly from the control (ANOVA/Dunnett's; $\alpha=0.05$).

doi:10.1371/journal.pone.0045574.g002

Table 1. Mean percent of settled, swimming and surviving larvae after 48 and 72-hr constant exposure for *P. astreoides* (#/5 ± S.E.) and 48-hr constant exposure and 96-hr spiked exposure for *M. faveolata* (#/25 ± S.E.; #/5 ± S.E.) to WAF, CEWAF and Corexit® 9500.

	Concentration	% Settled ± S.E.		% Swimming ± S.E.		% Surviving ± S.E.	
<i>P. pastreoides</i>	(ppm)	48 hours	72 Hours	48 hours	72 Hours	48 hours	72 Hours
Control	0	87±7	87±7	0±0	0±0	87±7	87±7
	0.32	40±23	53±7	53±29	20±20	93±23	73±13
WAF	0.33	33±13	53±18	27±18	7±7	60±12	60±12
	0.62	33±7	33±7	13±7	0±0	47±7	33±7
	0.71	67±13	67±13	0±0	0±0	67±13	67±13
CEWAF	4.28	0±0	20±12	27±13	7±7	27±13	27±13
	30.99	0±0	7±7	33±33	0±0	33±33	7±7
	25	40±12	33±7	27±7	33±7	67±7	67±7
Corexit®	50	7±7	13±7	33±33	0±0	40±31	13±7
9500	100	0±0	0±0	0±0	0±0	0±0	0±0
	Concentration	% Settled	% Swimming	% Surviving	Spiked Conc.	% Survival	
<i>M. faveolata</i>	(ppm)	± S.E.	± S.E.	± S.E.	(ppm)	± S.E.	
Control	0	75±3	12±2	87±5	0	73±7	
	0.65	27±7	8±5	35±7	0.49	33±7	
WAF	1.34	16±8	4±2	20±6	0.51	27±7	
	1.5	5±4	5±1	11±3	0.84	7±7	
	14.73	4±2	0±0	4±2	0.86	20±12	
CEWAF	18.56	0±0	0±0	0±0	30.06	7±7	
	35.76	1±1	0±0	1±1	42.08	0±0	
	25	5±4	4±2	9±1	500	20±12	
Corexit®	50	0±0	0±0	0±0	1000	0±0	
9500	100	0±0	0±0	0±0	1500	0±0	

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tions of WAF (ANOVA; $p < 0.0001$; $F = 34.507$; Fig. 2), resulting in mean larval settlement of 35% at 0.65 ppm, 20% at 1.34 ppm, and 11% at 1.50 ppm compared to 87% in the control treatment (Dunnett's; $\alpha = 0.05$); the LC_{50} was 0.50 ppm (95% C.L.s = 0.16–0.70).

Effects of Water-Accommodated Fractions Plus Corexit® 9500 (CEWAF). Linear regressions revealed a negative relationship among settlement and CEWAF concentration for *P. astreoides* ($p = 0.001$, 48-hrs; $p < 0.0001$, 72-hrs) and *M. faveolata* ($p = 0.040$), where increasing CEWAF concentration was associated with decreased larval settlement. *P. astreoides* larval settlement differed significantly after exposure to various concentrations of CEWAFs (ANOVA; $p < 0.0001$; $F = 19.224$; Fig. 3), where exposure to medium and high concentrations resulted in significant reductions after 48 and 72-hr exposure [0 and 20% mean settlement at 4.28 ppm, 0 and 7% at 30.99 ppm] compared to the seawater control [87%] (Dunnett's; $\alpha = 0.05$; Table 1). No effect was found on settlement, however, by the lowest concentrations of CEWAF [0.71 ppm], in which 67% mean settlement occurred after 48 and 72-hrs. Settlement of *M. faveolata* was significantly reduced by exposure to all three CEWAF concentrations (ANOVA; $p < 0.0001$; $F = 176.157$; Fig. 3), where 4% mean settlement occurred at 14.73 ppm, 0% at 18.56 ppm, and 1% at 35.76 ppm compared to 75% in the control treatment (Dunnett's; $\alpha = 0.05$).

No correlation was found between CEWAF concentration and *P. astreoides* survival after 48-hrs (linear regression; $p = 0.068$),

however, a negative relationship was found among concentration and survival after 72-hr exposure (linear regression; $p < 0.0001$). Survival of *P. astreoides* larvae differed significantly after exposure to various concentrations of CEWAF (ANOVA; $p = 0.013$; $F = 3.792$; Fig. 3). No significant effect on survival occurred after 48-hr exposure; however, 72-hr exposure to medium and high concentrations of CEWAF [4.28 ppm, 30.99 ppm] resulted in significantly reduced larval survival rates of 27% and 7% compared to 87% in control treatments (Dunnett's; $\alpha = 0.05$; Table 1). An LC_{50} of 1.84 ppm (95% C.L.s = 0.26–4.72) was determined.

Spearman correlation analyses revealed a negative relationship among *M. faveolata* larval survival and CEWAF concentration ($p = 0.007$), where increasing concentration was associated with decreased larval survival. *M. faveolata* larval survival varied significantly among CEWAFs (Kruskal-Wallis; $p = 0.037$; $df = 3$; Fig. 3), where survival declined significantly after exposure to medium and high concentrations [18.56 ppm, 35.76 ppm] compared to the control treatment [0 ppm], resulting in mean survival of 0 and 1% compared to 87% respectively (Dunn's; $\alpha = 0.05$; Table 1). While survival in low concentrations [14.73 ppm] did not significantly differ from the control, mean larval survival after exposure was only 4%. The LC_{50} for this exposure was 0.28 ppm (no C.L.s were generated).

Effects of Dispersant Corexit® 9500. Linear regression and correlation analyses revealed a negative relationship among

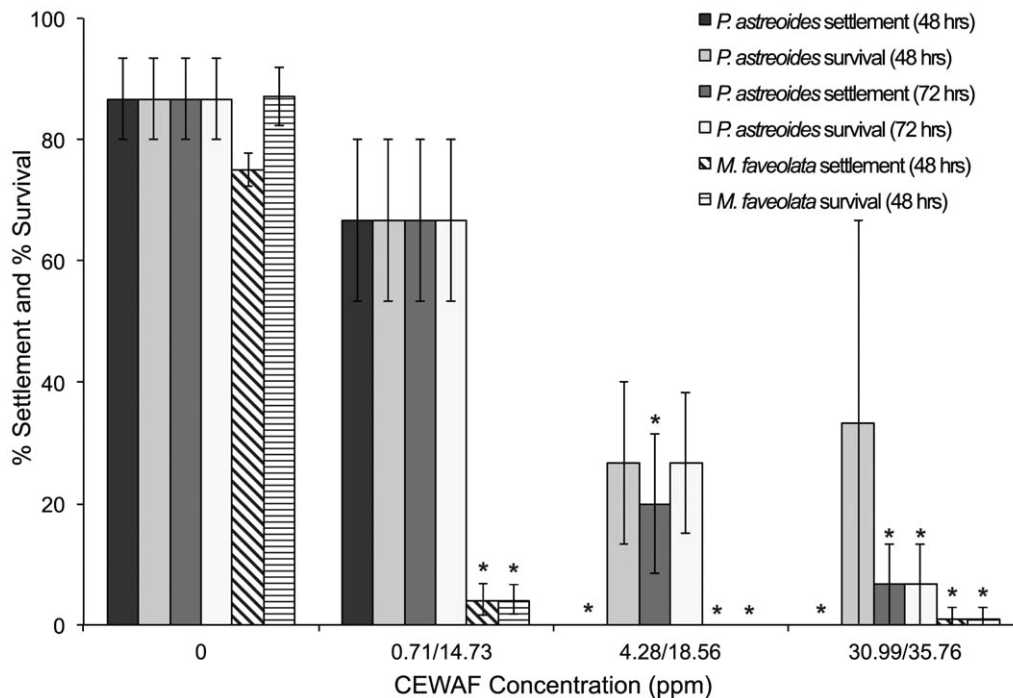


Figure 3. Settlement and survival of larvae exposed to CEWAF. Mean percent ($\pm$ SE) settlement and survival of *P. astreoides* (solid bars) and *M. faveolata* (striped bars) larvae under constant exposure to chemically enhanced crude DWH oil CEWAF (0, 0.71, 4.28, 30.99 ppm for *P. astreoides*; 0, 14.73, 18.56, 35.76 ppm for *M. faveolata*). Asterisks indicate comparisons that differ significantly from the control (ANOVA/Dunnnett's [*P. astreoides* settlement and survival/*M. faveolata* settlement]; Kruskal-Wallis/Dunn's [*M. faveolata* survival]; $\alpha=0.05$). doi:10.1371/journal.pone.0045574.g003

settlement and Corexit® 9500 concentration for *P. astreoides* (linear regression; $p=0.001$, 48-hrs; $p=0.001$, 72-hrs) and *M. faveolata* (Spearman rank; $p<0.0001$), where increasing Corexit® 9500 concentration was associated with decreased larval settlement. Significant differences were found among concentrations for *P. astreoides* larval settlement (ANOVA; $p<0.0001$; $F=29.143$; Fig. 4), where overall settlement was significantly reduced after 48 and 72-hr exposure to all three concentrations of Corexit® 9500 [40 and 33% mean settlement at 25 ppm, 7 and 13% at 50 ppm, 0% at 100 ppm] compared to the control [87% at 0 ppm] (Dunnnett's; $\alpha=0.05$; Table 1). Settlement rates of *M. faveolata* larvae also varied significantly among Corexit® 9500 concentrations (Kruskal-Wallis; $p=0.014$; $df=3$; Fig. 4), where significant reductions occurred in medium and high concentrations [50 ppm, 100 ppm] resulting in 0% mean settlement after 48-hr exposure (Dunn's; $\alpha=0.05$; Table 1). *M. faveolata* settlement was only 5% after exposure to low concentrations of Corexit® 9500 [25 ppm], however, this did not differ significantly from the control (Dunn's; $\alpha=0.05$).

A negative relationship was found between survival rate and Corexit® 9500 concentration for *P. astreoides* (linear regression; $p<0.0001$, 48-hrs; $p<0.0001$, 72-hrs) and *M. faveolata* (Spearman rank; $p<0.0001$), where increasing Corexit® 9500 concentration was associated with decreased larval survival. Survival of *P. astreoides* larvae differed significantly after exposure to various concentrations of Corexit® 9500 dispersant (ANOVA; $p<0.0001$; $F=9.385$; Fig. 4), where exposure to high [100 ppm] concentrations of dispersant resulted in significant declines in survival at 48-hrs, while exposure to medium [50 ppm] and high concentrations [100 ppm] significantly reduced survival at 72-hrs. Exposure to the medium concentrations resulted in 13% mean survival; exposure to high concentrations resulted in complete mortality

(0% survival). Mean *P. astreoides* larval survival was reduced from 87% in control treatments to 67% after exposure to the lowest concentration of Corexit® 9500 [25 ppm]; however, this difference was not significant (Dunnnett's; $\alpha=0.05$). Results of the constant exposure test indicated a 72-hr LC_{50} value of 33.4 ppm (95% C.L.s = 20.11–43.73). Likewise, survival by *M. faveolata* larvae varied significantly between Corexit® 9500 concentrations (Kruskal-Wallis; $p=0.014$; $df=3$; Fig. 4), where survival significantly declined after exposure to medium and high concentrations [50 ppm, 100 ppm] compared to the control (Dunn's; $\alpha=0.05$), resulting in complete mortality (0% survival). While low concentrations [25 ppm] did not significantly affect survival, exposure resulted in mean larval survival of 9%, compared to 87% in control treatments. The 72-hr LC_{50} for this constant exposure was 19.7 ppm (no C.L.s generated).

Spiked Exposure Survival Assays

Correlation analyses of spiked exposure tests revealed a negative relationship among *M. faveolata* larval survival and concentration of WAF, CEWAF and Corexit® 9500 (linear regression, $p<0.0001$; Spearman rank, $p=0.001$; Spearman rank, $p<0.0001$), where increasing concentrations were associated with decreased larval survival. Significant differences in *M. faveolata* survival were found among various WAF concentrations (ANOVA; $p<0.0001$; $F=17.583$; Fig. 5), where survival was significantly reduced by exposure to all three concentrations of WAF [0.49 ppm, 0.51 ppm, 0.84 ppm] compared to the control treatment (Dunnnett's; $\alpha=0.05$; Table 1) resulting in mean larval survival of 33%, 27% and 7% compared to 73% survival in the control, with a 96-hr LC_{50} of 0.45 ppm (no C.L.s were generated). Additionally, larval survival differed significantly among concentrations of CEWAF (Kruskal-Wallis; $p=0.037$; $df=3$; Fig. 5). Survival

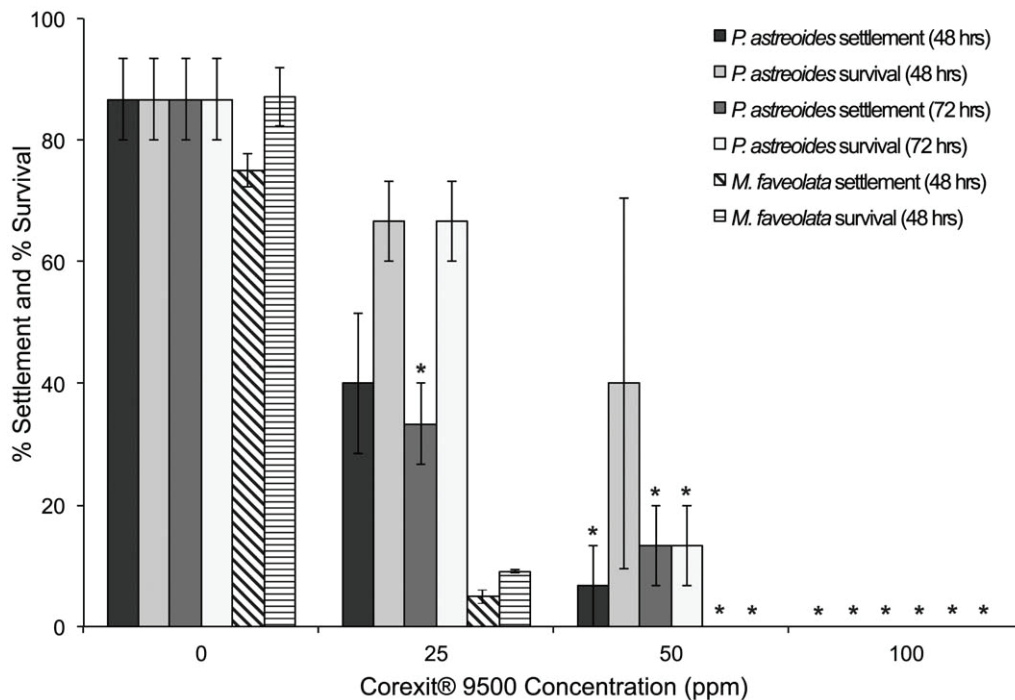


Figure 4. Settlement and survival of larvae exposed to Corexit® 9500. Mean percent (% $\pm$ SE) settlement and survival of *P. astreoides* (solid bars) and *M. faveolata* (striped bars) larvae under constant exposure to dispersant Corexit® 9500 [0, 25, 50, 100 ppm]. Asterisks indicate comparisons that differ significantly from the control (ANOVA/Dunnett's [*P. astreoides* settlement and survival]; Kruskal-Wallis/Dunn's [*M. faveolata* settlement and survival]; $\alpha = 0.05$).

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declined significantly after exposure to medium and high concentrations of CEWAF [30.06 ppm, 42.08 ppm] compared to the control (Dunn's; $\alpha = 0.05$), resulting in 7% mean larval survival in medium concentrations and complete mortality (0% survival) in high concentrations. While *M. faveolata* survival was reduced from 73% in control treatments to 20% in the lowest concentration for spiked CEWAF, this difference was not significant. Results of the spiked exposure test indicated a 96-hr LC₅₀ value of 0.12 ppm (no C.L.s were generated). Survival by *M. faveolata* larvae also varied significantly between Corexit® 9500 concentrations (Kruskal-Wallis; $p = 0.014$; $df = 3$; Fig. 5), where medium and high concentrations of Corexit® 9500 [1000 ppm, 1500 ppm] significantly reduced survival resulting in complete mortality (0% survival) in both concentrations. Although not significant (Dunn's; $\alpha = 0.05$), exposure to low concentrations of Corexit® 9500 [500 ppm] resulted in a mean larval survival of 20%, compared to 73% in control treatments. The spiked exposure resulted in a 96-hr LC₅₀ of 343.8 ppm (95% C.L.s = 146.88–759.37; linear interpolation).

Discussion

Effects of DWH crude oil, weathered oil and Water-Accommodated Fractions (WAF)

Exposure of *P. astreoides* planulae to fresh DWH oil resulted in mortality of experimental larvae within the first 24-hrs of treatment, while mortality in control planulae was not observed until 72-hrs (Fig. 1). Weathered DWH oil exposure also resulted in significant reductions in post-settlement survival after 48-hrs of observation. Moreover, constant and spiked exposure to WAF resulted in decreased larval settlement success and survival for *P. astreoides* and *M. faveolata* with increasing WAF concentration. *P.*

astreoides larvae appear, however, to be more tolerant to WAF exposure than *M. faveolata* larvae as they were only significantly affected after 72-hr exposure to the highest concentrations of WAF [0.62 ppm] and not by lower concentrations [0.32 ppm, 0.33 ppm], whereas *M. faveolata* larvae were negatively affected by all three concentrations in both constant and spiked exposure scenarios [0.65 ppm, 1.34 ppm, 1.50 ppm; 0.49 ppm, 0.51 ppm, 0.84 ppm] (Table 1; Figs. 2 & 5). Chia [24] demonstrated that survival of pelagic larvae after oil-pollution may be related to size; that is, larger larvae are expected to survive longer. In general, planula larvae of brooding species tend to be larger than those of broadcasting species [25]. Tolerance for oil exposure may, therefore, be influenced by the larger size of *P. astreoides* larvae (1 mm) [19] compared to *M. faveolata* larvae (avg. egg size 320 μ m) [20]. This differential tolerance based on size is likely related to variations in respiration rates and available lipid reserves, which are known to scale with size [26,27], as well as the potential inability of planktotrophic larvae to feed and lecithotrophic larvae to photosynthesize in polluted waters.

As oil from a spill interacts with waves, microbes, oxygen, sunlight and other factors, volatile components in the oil gradually evaporate. This process is called 'weathering'. Weathered oil, as a result of loss of its water-soluble elements, is more viscous than fresh crude, and is often dense enough to sink [28]. Results of settlement and survival assays for larvae exposed to weathered DWH oil as well as the constant and spiked exposure experiments presented here indicate that although weathered oil may not be toxic to coral larvae, there exists the potential for weathered oil to detrimentally impact marine organisms suspended in the water column in its proximity.

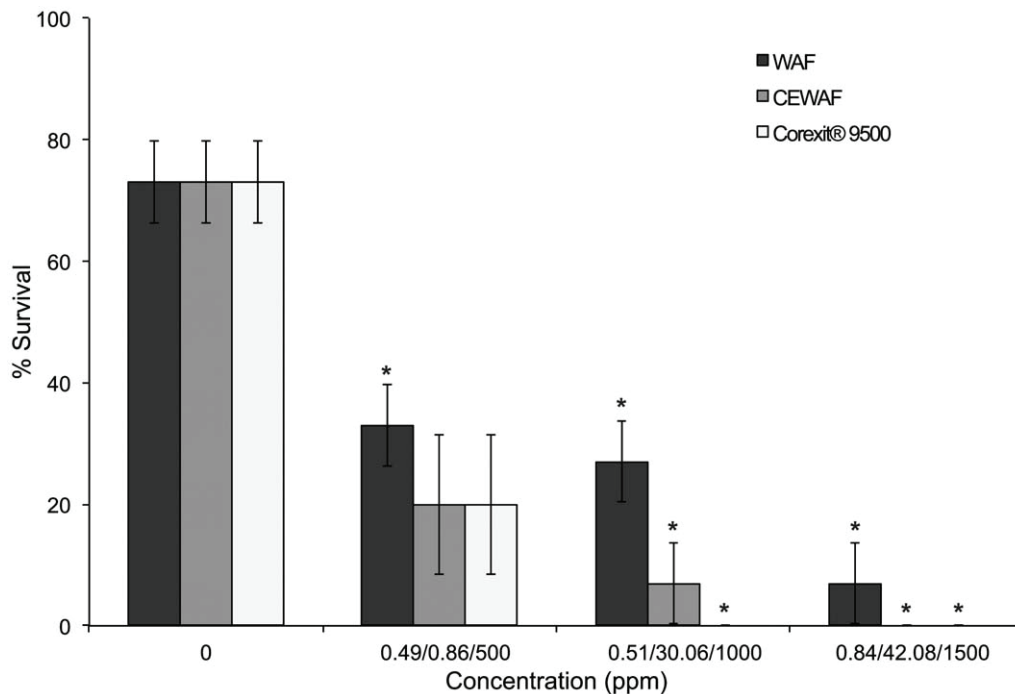


Figure 5. Survival of larvae in spiked exposure assays. Mean percent ($\pm$ SE) survival of *M. faveolata* larvae after 96-hr spiked-declining exposure to WAF (0, 0.49, 0.51, 0.84 ppm), CEWAF (0, 0.86, 30.06, 42.08 ppb), and Corexit® 9500 (0, 500, 1000, 1500 ppm). Asterisks indicate comparisons that differed significantly from the control (ANOVA/Dunnett's [WAF]; Kruskal-Wallis/Dunn's [CEWAF and Corexit® 9500]; $\alpha=0.05$). doi:10.1371/journal.pone.0045574.g005

Effects of Water-Accommodated Fractions Plus Corexit® 9500 (CEWAF)

Short and long-term exposure to medium and high concentrations of CEWAF [4.28 ppm, 30.99 ppm] resulted in significant reductions to both settlement and survival of *P. astreoides* larvae (Table 1; Fig. 3). Likewise, both the survival and settlement of *M. faveolata* larvae were negatively impacted by constant and spiked exposure to all three concentrations of CEWAF [14.73 ppm, 18.56 ppm, 35.76 ppm; 0.86 ppm, 30.06 ppm, 42.08 ppm] (Table 1; Fig. 3 & 5), where constant exposure to medium and high concentrations resulted in complete (0% survival) and near complete (1% survival) mortality. Moreover, near complete mortality (7% survival) occurred after spiked exposure of *M. faveolata* larvae to medium concentrations [30.06 ppm], and complete mortality (0% survival) occurred after spiked exposure to high concentrations of CEWAF [42.08 ppm]. These results indicate a negative relationship among larval settlement and survival with increasing CEWAF concentration and suggest that the application of dispersants to crude oil potentially increases the toxicity of oil exposure for coral larvae. The role of dispersants in mitigating oil spills is to break down the oil slick into stable droplets in the water column, thereby enhancing its rate of dissolution. The addition of dispersants, however, has the potential to compound the toxicological effects on marine organisms by increasing the surface area of oil-water interaction [29]. Likewise, dispersed oil is more evenly distributed throughout the water column and is, therefore, more likely to contact planktonic and benthic larvae, thereby increasing the potential negative impacts.

Effects of dispersant Corexit® 9500

The results of the present study clearly indicate that dispersants are highly toxic to early life stages of coral. Settlement success and survival of *P. astreoides* and *M. faveolata* coral larvae were

significantly reduced after both short and long term constant exposure, as well as spiked exposure, to increasing concentrations of Corexit® 9500 dispersant (Table 1; Fig. 4 & 5). Settlement of *P. astreoides* larvae, in particular, significantly decreased after exposure to all concentrations of dispersant [25 ppm, 50 ppm, 100 ppm], with no settlement occurring at the highest concentrations. Constant exposure to Corexit® 9500 also caused dramatic declines in larval survivorship, resulting in complete larval mortality (100%) after exposure to both medium and high concentrations [50 ppm, 100 ppm] and near complete mortality (9% survival) after exposure to low concentration [25 ppm] for *M. faveolata* larvae (Table 1; Figs. 4 & 5), as well as complete mortality under exposure to high concentrations [100 ppm] for *P. astreoides* larvae (Fig. 4). Similarly, *M. faveolata* larvae experienced complete mortality under spiked exposure to medium and high concentrations of Corexit® 9500 [1000 ppm, 1500 ppm], and only 20% survival under spiked exposure to low concentrations [500 ppm]. Complete mortality for both species after exposure to multiple concentrations of Corexit® 9500 in constant as well as spiked scenarios implies increased toxicity to larvae as compared to WAF and CEWAF. As previously noted, the differential results by species may be related to larval size, and thus larvae produced from broadcasting coral species may be more vulnerable to pollution than those from brooding species. Overall, these findings indicate that exposure of coral larvae to the dispersant Corexit® 9500 is toxic and will result in loss of coral recruitment.

Conclusion

The explosion of the Deepwater Horizon oil rig in April 2010 resulted in the release of over 760 million liters of Louisiana crude oil into the Gulf of Mexico, thus constituting one of the greatest marine disasters in U.S. history [30]. Mitigation of the spill with dispersant chemicals was effective in reducing the magnitude of

the offshore oil slick, however it is plausible that a significant portion of petroleum toxicants have been absorbed into the water column as a result. Much concern has arisen regarding the potential for oil pollution to reach coral reefs, particularly those in the Florida Keys that may be impacted by oil originating in the Gulf of Mexico and arriving via offshore currents.

Coral reefs worldwide have undergone drastic declines in the last several decades [31,32]. This is particularly evident in the Caribbean, where coral cover has been reduced by roughly 80% since 1975 [33]. In the Florida Keys, coral reefs have been affected by anthropogenic and environmental impacts including pollution, overfishing, eutrophication, coastal development, disease, and climate change related bleaching among others. As a result, coral mortality in this region is unsustainably high [34] and substantially increased in 2010, following a cold-water event [35]. Such drivers have caused a dramatic shift in the Florida Keys reef ecosystem from a benthic community dominated by scleractinian corals to one overgrown with macroalgae [36]. With the advent of oil drilling off the coast of Cuba and our limited ability to be able to respond to it, coupled with the current fragile state of coral species in the Florida Keys, it is imperative that the potential impacts of oil pollution on Caribbean reef-building corals be understood at all life-history stages. This study found settlement and survival of *P. astreoides* and *M. faveolata* planulae decreased significantly following exposure to increased concentrations of DWH crude oil, weathered oil, WAF, CEWAF, and dispersant Corexit® 9500, with higher concentrations of CEWAF and Corexit® 9500 resulting in settlement failure and complete larval mortality. The demonstrated effects of pollution by DWH crude oil and the dispersant Corexit® 9500 on *P. astreoides* and *M. faveolata* planulae strongly suggest that the use of dispersants to mitigate oil spills in the vicinity of coral reefs should be avoided.

Materials and Methods

Riser collected Deepwater Horizon (DWH) oil was obtained from British Petroleum (fresh) and weathered oil from oil masses obtained from Florida Wildlife Conservation from the spill site; Corexit® 9500 was obtained directly from the manufacturer (NALCO). Toxicity tests were performed to examine constant and spiked declining exposure to various concentrations of fresh DWH oil water-accommodated fractions (WAFs), chemically enhanced water-accommodated fractions (CEWAFs), and WAFs of Corexit® 9500. Effects studied were settlement and survivorship. Larvae were defined as settled upon observing successful metamorphosis from either free swimming or casually attached planula stage into a firmly attached, radially-symmetric disk shape form with pronounced flattening of the oral-aboral axis [14]. Larval mortality was defined as cessation of movement, followed by tissue degradation and dissolution. As overall guidance, the basic testing protocols of the American Society for Testing and Materials (ASTM) and the Organization for Economic Cooperation and Development (OECD) were followed as far as the routine steps in conducting a toxicity test. The test solution exposure concentrations are included in Table 2. The WAF and CEWAF actual concentrations were verified by gas chromatography-flame ionization detection (GC-FID) total hydrocarbon concentration (THC) analyses.

Larval collections

Two days prior to the new moon in both June and July of 2010, forty and twenty adult colonies of the mustard hill coral *P. astreoides*, approximately 30 cm×30 cm, were collected by hand from the mid-channel reef in the Lower Keys portion of the

Florida Keys National Marine Sanctuary (24°33.6'N×81°30.1'W). Colonies were placed in outdoor flow-through seawater raceways at Mote Marine Laboratory Tropical Research Station in Summerland Key, FL. Each colony was isolated in a plastic container from which water flows into a collection apparatus, consisting of an 800 ml polypropylene beaker with 180 µm nylon mesh secured across the bottom. Larvae of *P. astreoides* float to the surface upon release and therefore overflow into the collecting beakers. Newly released larvae were washed from the collection cylinders into 1 L containers of 0.2 µm filtered seawater and counted. Larvae released from all forty colonies each night were pooled and then separated into glass scintillation vials consisting of 20 ml filtered seawater and maintained at room temperature (24°C) for experimentation.

Collection of gametes from adult *M. faveolata* colonies was done *in situ* using collection tents as described in Sharp et al. [37] in September of 2010 from Cheeca Rocks (24°54.4'N×81°37.6'W). Cross-fertilization was performed immediately after gamete collection at Long Key Marine Laboratory and embryos were maintained in filtered seawater for 5 days. Complete water changes were performed every 6 hours for the first 48 hours and subsequently every 8 hours. Developing larvae were then separated into glass scintillation vials consisting of 20 ml filtered seawater with 25 larvae per vial and maintained at room temperature (24°C) for experimentation.

Experimental Procedure, Weathered Oil

Weathered oil masses were obtained from Florida Wildlife Conservation from the spill site. Acute-exposure tests were performed at room temperature (24°C) to examine the effects of weathered oil exposure on *P. astreoides* planulae. Effects studied were settlement and metamorphosis, swimming behavior, and survivorship (as defined above).

Settlement Assay. Oil effects on larval settlement and metamorphosis were examined in petri dishes filled with 10 mL of ambient seawater. Into each dish was placed a glass microscopy slide that was cured in the natural reef environment for three weeks prior to experiments to form biofilms. Approximately 35 mg of weathered DWH oil was placed in the center of each biofilm slide. Twenty *P. astreoides* planulae were then added to all dishes and subsequently observed daily for settlement, metamorphosis, attachment to dish/slide, and mortality over the course of 96-hrs.

Aversion Assay. Behavioral effects on *P. astreoides* planulae were examined by placing grids of 6 concentric circles oriented ~0.9 cm apart under petri dishes filled with 15 mL of ambient seawater. Uniform amounts of weathered DWH oil, approximately 4 mg, were applied to the center of each treatment dish. Twenty *P. astreoides* larvae were subsequently added to each dish. Larval swimming behavior was assayed to investigate whether larvae would actively avoid the weathered oil, or exhibit any other behavioral abnormalities. Swimming behavior was scored photographically at the following intervals: 10 min, 30 min, 60 min, 6-hr, and subsequent 12-hr intervals for 3 days. Data were analyzed using a repeated measure analysis of variance (ANOVA). Significant comparisons were further analyzed using Student's *t* pairwise comparisons. Scoring was based on location in reference to the center of the dish using a numerical system (1 = center of dish, 6 = outer rim of dish).

Survivorship Assay. To assess exposure-related mortality among *P. astreoides* planulae, approximately 8.5 mg of weathered DWH oil were smeared onto the base of glass scintillation vials (replicated 10 times). Vials were then filled with 15 mL of ambient seawater. Twenty planulae were then added to each vial and left for 24-hr. Larvae and water from 5 of the 10 treatment vials were

Table 2. Actual concentrations (ppm) of Corexit only and total petroleum hydrocarbons in WAF and CEWAF exposure solutions for experiments on *P. astreoides* and *M. faveolata* larval settlement and survival.

Solution	Actual Concentrations		
	<i>P. astreoides</i> Constant Exposure	<i>M. faveolata</i> Constant Exposure	<i>M. faveolata</i> Spiked Exposure
Corexit® 9500	25, 50, 100	25, 50, 100	500, 1000, 1500
WAF	0.32, 0.33, 0.62	0.65, 1.34, 1.50	0.49, 0.51, 0.84
CEWAF	0.71, 4.28, 30.99	14.73, 18.56, 35.76	0.86, 30.06, 42.08

doi:10.1371/journal.pone.0045574.t002

immediately transferred to sterile, oil-free vials to contrast acute exposure with prolonged exposure. Thus, 5 replicates were directly exposed to the smeared oil for 24 hours, while the other 5 replicates were directly exposed for 5 days. Water chemistry done on these vials indicated that there were no detectable levels of total petroleum hydrocarbons (TPHs) or polycyclic aromatic hydrocarbons (PAHs) present post experimentation. The same protocol was followed for control vials. Larval survivorship and metamorphosis were scored every 24 hours for 5 days and analyzed using a repeated measure analysis of variance (ANOVA). Significant comparisons were further analyzed using Student's *t* pairwise comparisons.

Experimental Procedure, Crude Oil & Dispersant

Constant Exposure Procedure. Effects of fresh DWH oil on planula larvae were examined in 250 mL beakers each containing uniform-sized plaster tiles cured in the natural reef environment for 3 weeks to form biofilm on which the larvae could settle. After preparation, the appropriate WAF, CEWAF and dispersant test solutions were added to the containers, followed immediately by addition of the test organisms. Five *P. astreoides* planulae were added to each beaker, with 3 replications per treatment ($n = 3$). Larval settlement and mortality in each beaker was scored as the number of larvae settled or surviving relative to the original number of larvae (5) after 48 and 72-hrs. Data met all assumptions of normality and variance (Lilliefors's test; Levene's test; $\alpha = 0.05$) and were compared among concentrations using linear regression analyses as well as Analysis of Variance (ANOVA). Significant differences were further analyzed using Dunnett's post-hoc comparisons.

The same experimental design was repeated in August 2010 using 25 *M. faveolata* planulae per beaker; settlement and mortality were scored as the number of larvae settled or surviving relative to the original number of larvae (25) after 48-hrs. Data from assays of settlement and survival under exposure to WAF, and settlement under exposure to CEWAF met all assumptions of normality and variance (Lilliefors's test; Levene's test; $\alpha = 0.05$) and were compared among concentrations using linear regression analyses as well as Analysis of Variance (ANOVA). Significant comparisons were further analyzed using Dunnett's post-hoc comparisons. Data from assays of survival under exposure to CEWAF as well as settlement and survival under exposure to Corexit® 9500 did not meet the assumptions of normality and were analyzed using a Spearman rank correlation as well as a nonparametric Kruskal-Wallis test. Significant differences were further analyzed using Dunn's procedure for multiple pairwise comparisons. Unless indicated otherwise, all LC₅₀ values were determined using maximum likelihood probit.

Flow-Through Toxicity Chambers System. A continuous-flow exposure system was developed by Singer et al. [38] and employed by the Chemical Response to Oil Spills Ecological

Effects Research Forum (CROSERF) working group [17] [18] for assessing toxicity of oil and oil dispersants on marine organisms and was used for the spiked exposure experiments. This continuous-flow exposure system provides a useful approach for testing sensitive, early life stages of marine organisms exposed to constant concentrations of toxicants and provides a tool for testing dispersant toxicity under dynamic exposure regimes that are relevant to actual field conditions.

Flow-Through Spiked Exposure Procedure. Exposure chambers (270 ml) were filled with whole (undiluted) test solution, and animals were added to the chambers in random order at the appropriate density, the chambers were sealed and the test commenced with the dilution of all chambers with clean, aerated, filtered seawater at a rate of approximately 2 ml/min. Natural seawater was used for dilution at ambient salinity. Over the duration of the test, the test animals, and equipment were monitored for continuous operation within designated limits.

Three replicates of each of the three concentrations of fresh DWH oil WAF and CEWAF, along with a dispersant only WAF, and seawater control were performed with 5 *M. faveolata* larvae per replicate (Table 1). Larval survivorship was scored after 96 hours. Survival data after exposure to WAF met all assumptions of normality and variance (Lilliefors's test; Levene's test; $\alpha = 0.05$) and were compared among concentrations using linear regression analyses as well as Analysis of Variance (ANOVA). Significant comparisons were further analyzed using Dunnett's post-hoc comparisons. Data from survival assays after exposure to CEWAF and Corexit® 9500 did not meet the assumptions of normality and were analyzed using Spearman rank correlation analyses as well as a nonparametric Kruskal-Wallis test. Significant differences were further analyzed using Dunn's procedure for multiple pairwise comparisons. Unless indicated otherwise, all LC₅₀ values were determined using maximum likelihood probit.

Chemical analyses

WAF and CE-WAF Solutions. Concentrations of the WAF, CEWAF, and dispersant for both spiked declining concentration and constant exposure were selected based in part upon available recommendations from the multi-agency Ecological Risk Assessment (ERA) workshops along with findings of the CROSERF working group [18,39,40]. These exposures of concern were based upon a dispersed oil spill trajectory model supplied by NOAA's Hazardous Materials Response Division (NOAA-HAZMAT) for spill research in Santa Barbara channel and Chesapeake Bay [18,40]. Validation of concentrations of WAF and CEWAF test solutions for the exposure studies were confirmed analytically.

Petroleum hydrocarbons in the test solutions were extracted and analyzed using modified EPA Method 3510C and analyzed for PAHs as above, while THC were analyzed using EPA Method 8260. The results are reported based on integration of the FID signal over the entire hydrocarbon range from n-C9 to n-C42 and

quantified using an internal standard. A performance-based quality-assurance and quality-control program, which includes the parallel analysis of procedural blanks, and matrix spikes were implemented to ensure data of the highest quality. The GC response was monitored every 10 to 12 samples with product check standards. Procedural blanks were checked to confirm they were clear of targeted analytes.

Acknowledgments

We would like to thank Mary Alice Coffroth, Valerie Paul, Laurie Raymundo, Erich Bartels, and Koty Sharp for help with larval collection and rearing, and/or discussions on experimental design and analysis. We

thank Erinn Muller, Cathleen Sullivan and Michaela Stiber for valuable review of the manuscript. Samples were taken under permits FKNMS-2009-031, FKNMS-2010-080, and FKNMS-2010-080-A1 to KB Ritchie. Fresh Deepwater Horizon oil was kindly provided by British Petroleum Company and Corexit® 9500 by Nalco.

Author Contributions

Coordinated funding, permitting and field efforts: KBR. Conceived and designed the experiments: GGG DLW DG KBR. Performed the experiments: GGG DLW DG EP AM KBR. Analyzed the data: GGG DLW DG EP KBR. Contributed reagents/materials/analysis tools: DLW KBR. Wrote the paper: GGG DLW DG EP AM KBR.

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EXHIBIT 127



Comparative sensitivity of the *early life stages* of a coral to heavy fuel oil and UV radiation

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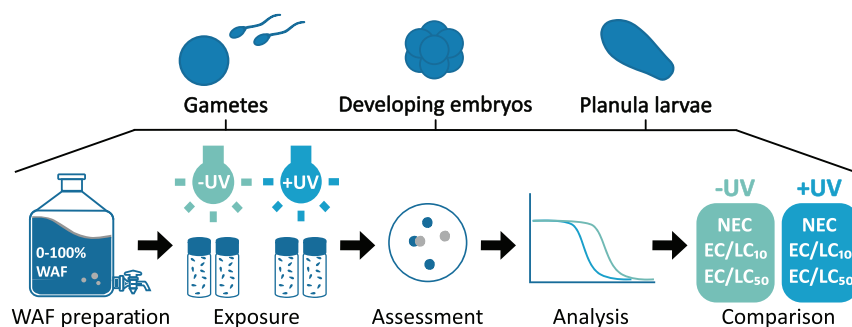
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HIGHLIGHTS

- Oil pollution from shipping remains a significant threat in coral reef environments.
- The effects of heavy fuel oil and UVR on early coral life stages were assessed.
- Heavy fuel oil negatively affects all early life stages of *Acropora millepora*.
- Assessed endpoints among the most sensitive for aquatic organisms tested to date
- UVR significantly increases heavy fuel oil toxicity to early life stages of coral.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 26 January 2021

Received in revised form 17 March 2021

Accepted 18 March 2021

Available online 23 March 2021

Editor: Thomas Kevin V

Keywords:

Petroleum oil
PAH
Pollution
Ultraviolet radiation
Phototoxicity
Coral reefs

ABSTRACT

During an oil spill, shallow, tropical coral reefs are likely to be simultaneously exposed to high intensities of ultraviolet radiation (UVR), which can exacerbate the toxicity of petroleum oils. While successful recruitment of corals is critical for reef recovery following disturbances, the sensitivity of several early life stages of coral to petroleum hydrocarbons has not been investigated, particularly for UVR co-exposure. Here we present the first dataset on the relative sensitivity of three early life stages (gametes, embryos and planula larvae) in a model broadcast spawning coral species, *Acropora millepora*, to the dissolved fraction of a heavy fuel oil (HFO), both in the absence and presence of UVR. All early life stages were negatively impacted by HFO exposure but exhibited distinct sensitivities. Larval metamorphosis was the most sensitive endpoint assessed with a 10% effect concentration of $34 \mu\text{g L}^{-1}$ total aromatic hydrocarbons (TAH) in the absence of UVR. The impact on fertilisation success was highly dependent on sperm density, while the fragmentation of embryos masked embryo mortality. Larval metamorphosis was conclusively the most reliable endpoint for use in risk assessments of the endpoints investigated. Putative critical target lipid body burdens (CTLBBs) were calculated for each life stages, enabling a comparison of their sensitivities against species in the Target Lipid Model (TLM) database. *A. millepora* had a putative CTLBB of $4.4 \mu\text{mol g}^{-1}$ octanol for larval metamorphosis, indicating it is more sensitive than any species currently included in the TLM database. Coexposure to UVR reduced toxicity thresholds by 1.3-fold on average across the investigated life stages and endpoints. This increase in sensitivity in the presence of UVR highlights the need to incorporate UVR co-exposure (where ecologically relevant) when assessing oil toxicity thresholds, otherwise the risks posed by oil spills to shallow coral reefs are likely to be underestimated.

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1. Introduction

Oil pollution is a substantial local threat to coral reefs with large-scale spills posing a potential risk anywhere commercial shipping, oil extraction or oil processing occurs. While large scale spill events are rare, the consequences in reef environments can be catastrophic (Jackson et al., 1989; Guzman et al., 2020) and spill incidents continue to occur in tropical and subtropical seas (Diercks et al., 2010; Storrie, 2011; Sun et al., 2018; The Guardian, 2018; Daley, 2019; Asariotis and Premti, 2020). Coral reefs are facing increasing global threats from warming and acidification of the ocean (Hoegh-Guldberg et al., 2017; Hughes et al., 2018) and reducing local pressures such as pollution is a key management strategy to maximise coral reef resilience as the climate changes (Hughes et al., 2017; MacNeil et al., 2019). The effective management of oil pollution hazards in tropical ecosystems requires an understanding of their potential impacts on reef-building corals (Turner and Renegar, 2017; Nordborg et al., 2020), as the condition of these habitat-forming taxa will have widespread consequences on the ecosystem as a whole (Sorokin, 2013).

Oil pollution is unlikely to be the only stressor faced by a coral reef during a spill event. Other environmental pressures, including temperature, light availability, ultraviolet radiation (UVR), ocean acidification, salinity, nutrients and sediments, can affect the health of corals with several environmental factors commonly affecting reef health simultaneously (Ban et al., 2014; Ellis et al., 2019). Co-exposure to UVR is almost certain during oil spills affecting shallow-water coral reefs, which are mainly found in oligotrophic, tropical (0–30° latitude) waters where UVR exposure is high throughout the year (Nordborg et al., 2020). UVR directly affects coral health and interacts with other environmental factors, including increasing coral bleaching during marine heatwave events (Banaszak and Lesser, 2009). Dissolved polycyclic aromatic hydrocarbons (PAHs) are the primary cause of acute toxic effects observed for pelagic and benthic organisms during spill events (Di Toro et al., 2000; French-McCay, 2002) and UVR increases the toxicity of many PAHs, generally referred to as phototoxicity (Pelletier et al., 1997; Barron, 2017). PAH phototoxicity can occur through two main pathways: photosensitisation or photomodification/photooxidation with the pathway dependent on the molecular structure of the PAH and when UVR exposure occurs (prior to or following uptake of PAHs by an organism) (Barron, 2017). However, a combination of both mechanisms is likely to contribute to the total toxicity observed during a spill on or near tropical coral reefs (Nordborg et al., 2020).

The success of the early life stages of coral is critical for recruitment and recovery of coral reefs following disturbances (Harrison and Wallace, 1990). Yet, only limited information is available that describes which coral life stages may be sensitive to combined oil and UVR exposures (Nordborg et al., 2020). The majority of scleractinian coral species are broadcast spawners, which release gametes into the water column for fertilisation and development (Harrison and Wallace, 1990; Baird et al., 2009). Following gamete release, the lipid rich eggs and embryos of most species will ascend towards the surface and remain there during embryonic and early larval development. The time from fertilisation until larvae reach settlement competency (ability to attach and metamorphose into a sessile primary polyp) varies from 24 h to over one week across described species (Jones et al., 2015; Randall et al., 2020). This time interval spent at, or close to, the surface represents a high-risk period of simultaneous exposure to dissolved PAHs from an oil spill and high UVR intensities during the day. Most studies investigating oil toxicity thresholds for reef-building corals have not included UVR as a co-factor (Turner and Renegar, 2017; Nordborg et al., 2020), and when UVR is considered the exposure methodology varies widely (Nordborg et al., 2020). While laboratory studies have tested the phototoxicity of oils and individual aromatics towards adult coral colonies (Peachey and Crosby, 1995; Guzmán Martínez et al., 2007) and larvae (Peachey and Crosby, 1995; Negri et al., 2016; Nordborg et al., 2018; Overmans et al., 2018), it remains unclear which coral life stages are most sensitive

to oil exposure, in either the presence or absence of UVR (Nordborg et al., 2020). Despite more than four decades of oil toxicity research on corals (Birkeland et al., 1976; Rinkevich and Loya, 1979), no comparative studies of the relative sensitivity between multiple life stages have been published (Turner and Renegar, 2017; Nordborg et al., 2020). Furthermore, the sensitivity of several early life stages of coral, including developing embryos, newly settled recruits and juvenile corals, have not been reported (Turner and Renegar, 2017; Nordborg et al., 2020), preventing their consideration in risk assessments.

To address these issues, the present study aims to: (i) identify the early life stage(s) that are most sensitive to oil exposure in a model coral species; (ii) quantify the impact of UVR co-exposure on toxicity for each life stage assessed; (iii) identify toxicity threshold concentrations applicable in risk assessments for tropical coral reef environments; and (iv) identify ecologically relevant, sub-lethal endpoints that are suitable for use in future coral oil toxicity research. These objectives were achieved by exposing three distinct early life stages of *Acropora millepora* to the water accommodated fractions (WAFs) of a heavy fuel oil (HFO) in the presence and absence of artificial UVR with intensity and spectral qualities representative of conditions occurring on mid-shelf reefs on the central Great Barrier Reef (GBR; Queensland, Australia) (Nordborg et al., 2018).

2. Methods

2.1. Coral collection

Gravid *A. millepora* colonies for all but one assay (the preliminary gamete assay) were collected from nearshore or mid-shelf reefs on the central GBR under Great Barrier Reef Marine Park Authority permits G12/35236.1 and G19.43024. Colonies were collected in the days leading up to the respective October, November or December full moon and transported in shaded plastic flow-through aquaria to the National Sea Simulator at the Australian Institute of Marine Science. On arrival colonies were transferred to flow-through holding tanks and maintained in 1 µm filtered natural seawater (FSW) at 27 °C under 70% shaded natural sunlight until spawning occurred. Refer to Tables S1 and S8 in Supplementary materials for details of parent origin for individual assays.

2.2. Preparation and chemical analysis of treatment solutions

HFO (International Bunker Supplies Pty Ltd, Gladstone, Australia), received as two separate batches (Sep 2016 and Feb 2018), was used to prepare WAFs for each assay as previously described (Negri et al., 2016; Nordborg et al., 2018) in accordance with standardised procedures for the preparation of low energy WAFs (Singer et al., 2000; Barron and Ka'aihue, 2003; Aurand and Coelho, 2005). Briefly, HFO was applied to the surface of 0.5 µm FSW in a solvent-rinsed glass aspirator at a loading of 20 g oil L⁻¹. The mixture was stirred without forming a vortex (180 rpm) for 16–26 h at room temperature (21 ± 1 °C), protected from light. The mixture was allowed to settle for ~30 min and 100% WAF was gently drained via a tap at the bottom of the aspirator. The 100% WAF was diluted using FSW to seven or more treatment concentrations for each assay performed (Barron and Ka'aihue, 2003; Forth et al., 2017). Treatment solutions were used within 24 h unless otherwise specified (Aurand and Coelho, 2005). See also Table S2 for specific details on WAF preparation for each experimental assay.

WAF samples for chemical analysis were collected for each assay at the start and end of exposures (Barron and Ka'aihue, 2003). Undiluted WAF was collected directly from the aspirator bottle at the start of each experiment. Glass scintillation vials containing undiluted WAF (n = 30) were exposed to the same conditions as the test organisms in the +UVR and –UVR incubators (Nordborg et al., 2018), and pooled WAF was sampled post-exposure. Samples were collected in solvent rinsed, 40 mL volatile organic compound (VOC) vials with

polytetrafluoroethylene (PTFE) septa and 500 mL amber glass bottles with PTFE-lined caps, acidified to pH 2 using 6 M hydrochloric acid and stored at 4 °C. Neat 2018 HFO and WAF samples were shipped to ChemCentre (Perth, Australia) for GC–MS analysis of benzene, toluene, ethylbenzene and xylenes (BTEX; USEPA method 8260), PAHs, including alkyl-substituted PAH (USEPA method 8270), and GC-FID analysis of total recoverable hydrocarbons (TRH; NEPM (2013)) as described previously (Negri et al., 2016; Nordborg et al., 2018). Neat 2016 HFO characterisation was reported in Nordborg et al. (2018). The time-weighted average concentrations of measured total aromatic hydrocarbons (TAH; $\sum$ (BTEX and PAH)) in pre- and post-exposure WAFs, expressed as $\mu\text{g L}^{-1}$, were used for statistical analyses, toxicity modelling and derivation of putative CTLBBs.

2.3. Experimental assays

Three separate experimental assays were performed, each targeting a discrete early life stage or life stage transition of *A. millepora*. Exposure time was individually determined for each life stage to meet the criteria for chronic exposure as defined by the Australian and New Zealand Water Quality Guideline-framework (Warne et al., 2018). For details on treatment concentrations, water quality parameters, incubation temperature, and light conditions for individual assays, refer to Tables S2–S3.

To assess the impact of the available light spectrum on HFO WAF toxicity, assays for gamete, embryo and larval stages were performed under various light treatments: visible light and UVR (+UVR), visible light without UVR (–UVR), or in darkness (Dark) (see Tables 1 and S3 for treatment combinations and light conditions used in individual assays). Glass vials containing treatment solutions and corals were randomly placed within orbital shaker incubators (Thermoline Scientific, Australia), either upside down (gametes) or on their sides (embryos and planula larvae). Both –UVR and +UVR incubators were fitted with photosynthetically active LED lights (Aqualina Blue 450 nm, 10,000 K and 420 nm Actinic LED strips, Aqualina Lighting Australia) and vials were exposed under a 12:12 h constant light:dark cycle ($\sim 50\text{--}80 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$). In addition to visible light, vials in the +UVR treatments were exposed to UVR on a 6:18 h light:dark UVR cycle ($\sim 1.3\text{--}1.6 \text{ mW cm}^{-2}$; $\sim 98\%$ UVA and $\sim 2\%$ UVB) using UVR emitting fluorescent tubes (T8; 18 W Deluxlite BLB and 18 W ReptileOne UVB 5.0). See Table S3 for specific details of light treatments used for individual assays and refer to Nordborg et al. (2018) for spectral profile of UVR fluorescent tubes.

2.4. Fertilisation assay

A. millepora eggs (1 genotype) and sperm (4 or 8 genotypes) were collected, separated and cleaned as per Negri et al. (2011) on spawning nights in 2018 and 2019 from gravid colonies. Sperm were pooled and, depending on the resultant sperm density (determined using a hemocytometer, $n = 6$ counts), diluted using 0.5 μm FSW. Aliquots (0.5 mL) of egg (~ 100 eggs) or sperm solution were pipetted into replicate vials containing 8.5 mL of treatment solution ($n = 6$ per treatment combination and gamete type; $n = 12$ for FSW controls). Gametes were pre-exposed to ≥ 8 treatment concentrations (0–766.4 $\mu\text{g L}^{-1}$ TAH) for 30 min under each light treatment (Dark, –UVR and +UVR), and fertilisation was initiated by gently transferring the sperm-WAF solutions into the corresponding egg vials. The primary assay was conducted at an optimal final sperm density of 1.2×10^6 sperm mL^{-1} (Ricardo et al., 2015). Additionally, a sub-optimal sperm density was used (1.5×10^3 sperm mL^{-1}) to assess if thresholds are sperm-density dependant (dark only; Ricardo et al. (2018)). Exposure continued under each respective light treatment for ~ 3 h post fertilisation (hpf) at 27 °C (0 rpm; Orbital shaker incubator, Thermoline Scientific, Australia), and was terminated when 4-cell embryos were observed in reference FSW samples. Samples were fixed by adding ~ 4 mL of fixative

(10% formaldehyde and 5% Na- β -glycerophosphate in FSW) to the bottom of each replicate vial followed by gentle swirling. Fertilisation success was assessed directly in sealed sample vials by manual counting of unfertilised eggs and cleaved embryos under a dissecting microscope.

2.5. Embryonic and larval development assay

A. millepora embryos at prawn chip/bowl-stage (~ 12 hpf) exhibiting normal morphology were collected from a single mass culture (prepared as per Nordborg et al. (2018)) and gently washed in clean 0.5 μm FSW. Embryos ($\sim 600\text{--}800 \mu\text{m}$ diameter) were gently transferred to glass scintillation vials (10 per vial) in < 1 mL of FSW using wide mouthed, disposable Pasteur pipettes. Treatment solution (20 mL; $\sim 10\%$ headspace) was added to each replicate vial ($n = 6$ per treatment combination, $n = 12$ for FSW controls) and embryos were exposed to 10 treatment concentrations (0–849.8 $\mu\text{g L}^{-1}$ TAH) for 48 h under visible light in the presence (+UVR) or absence (–UVR) of UVR at 27 °C. Gentle shaking (~ 70 rpm; Orbital shaker incubator, Thermoline Scientific, Australia) commenced after ~ 24 h of exposure when embryos had reached the gastrula stage. Vial locations within each incubator were randomized once per 24 h, and survivorship assessed after 48 h, during which surviving embryos had completed development into planula larvae. Living larvae (defined as pigmented and/or swimming) were transferred to fresh 0.5 μm FSW in 6-well plates to recover in the absence of UVR and dissolved aromatics until 9 days post-fertilisation. FSW changes were performed every 48 h and survival reassessed at 2-, 6- and 7-days post-exposure. Additionally, qualitative notes on larval morphology and behaviour (activity level, position within vials or wells, and abnormalities) were recorded during each assessment.

2.6. Larval survival and metamorphosis assay

A. millepora planula larvae (6- or 7-day old) collected from a single larval culture were exposed to 10 concentrations of HFO WAF (0–985 $\mu\text{g L}^{-1}$ TAH) as per Nordborg et al. (2018). Briefly, larvae were transferred to glass scintillation vials ($n = 6$ vials per treatment combination, $n = 12$ vials for FSW controls) and 20 mL of treatment solution was gently added ($\sim 10\%$ headspace). Vials were capped tightly and placed randomly in the +UVR or –UVR orbital shaker incubators (27 °C, 70 rpm) and exposed for 48 h. Vial locations within incubators were randomized once per 24 h. At 48 h, larvae were transferred to 6-well plates in 10 mL of treatment solution, survival assessed, treatment information replaced with an ID code, and settlement induced using 5 or 10 μL crustose coralline algae extract (prepared from *Porolithon onkodes*; Heyward and Negri (1999); Negri et al. (2005)). After ~ 24 h incubation at 27 °C (12:12 h L:D of visible light –UVR), metamorphosis success was assessed blind using a dissecting microscope. Notes were taken regarding larval and recruit morphology, abnormalities, fragmentation, recruit metamorphosis state and the general state of samples (e.g. degradation, presence of lipids, etc.). When present, the number of larval fragments, underdeveloped recruits and abnormal/deformed larvae or recruits was recorded.

Larvae were considered to have undergone metamorphosis if they had changed from planula larva to primary polyp. This included larvae that had formed disc-shaped structures with flattening of the oral-aboral axis and visible septal mesenteries radiating from the central mouth region (Heyward and Negri, 1999), most of which had attached firmly to the well plates. Larvae that had undergone early stage metamorphosis with permanent attachment and flattening but incomplete development of the central mouth region were also included, as these may be capable of continuing to full development after the 24 h incubation period.

2.7. Statistical analysis

Proportional decline in the response for each endpoint was modelled as a function of log concentration of HFO WAF using Bayesian non-linear models through the jagsNEC package (Fisher et al., 2020) in the software R (version 4.0.2; R Core Team (2020)) and the RStudio interface (RStudio Team, 2020). No effect concentrations (NEC; Pires et al. (2002); Fox (2010)), as well as 10% and 50% lethal or effect concentrations (LC/EC₁₀ and LC/EC₅₀), were derived from the median of the model averaged concentration-response curves, using the inbuilt package functions as appropriate, and presented with 95% credible intervals. The model-averaging approach uses weighted-average predictions based on deviance information criterions (DIC) for each candidate model as described by Fisher et al. (2020). Candidate models included commonly used concentration-response relationship models as well as functional NEC threshold models adapted from Fox (2010). All candidate models were initially fit using the binomial distribution. If strong evidence of overdispersion was observed for individual candidate models they were excluded from the final model subset. If all candidate models showed strong evidence of overdispersion, models were re-fitted using a beta distribution unless otherwise indicated. The specific priors, burn-in length and iterations (minimum 20,000) used for each dataset was selected based on data type and model diagnostics (convergence, chain mixing, evidence of autocorrelation, overdispersion and visual inspection of model fit). Where multiple light treatments were tested, differences in threshold values (i.e. NEC, LC/EC₁₀ or LC/EC₅₀) between light treatments were analysed using the inbuilt functions of the jagsNEC package, which follow Labelle et al. (2019). Briefly, the difference between the posteriors for each modelled toxicity threshold concentration was calculated, and the probability (in %) that one light treatment had a larger threshold value than the other was estimated. Additionally, the posterior, median and 95% credible intervals of the threshold values for each comparison were presented graphically. See also Table S4 for full details on distributions and candidate models used for individual datasets and assays. If no, or insufficient, concentration-response was observed for one of the applied light treatments in an experimental assay (based on statistical analysis), no comparisons of NEC or EC/LC₅₀ posteriors were performed. For details on the data quality assessment and exclusion of individual replicates from statistical analysis for each life stage assessed refer to Section S4. Statistical analysis, Supplementary materials.

2.8. Calculation of putative CTLBBs

The most effective method to compare the sensitivity of species or endpoints to aromatic hydrocarbon exposure is to derive species-specific CTLBBs (also referred to as critical body residues; CBR), which are independent of both WAF and neat oil composition (Di Toro et al., 2000; French-McCay, 2002; Redman and Parkerton, 2015). Species-specific CTLBBs for a defined endpoint are derived using the TLM, which is based on the linear relationship between log EC/LC₅₀ and log K_{OW} (octanol-water partitioning coefficient) for non-polar narcotic chemicals, including aromatic hydrocarbons (Di Toro et al., 2000; French-McCay, 2002; McGrath and Di Toro, 2009). Experimental toxicity data for at least three aromatic hydrocarbons of varying K_{OW} are required to derive a definitive CTLBB estimate from the y-intercept of the regression. The CTLBB can then be used to predict the narcotic toxicity of any dissolved aromatic hydrocarbon in terms of EC/LC₅₀ or toxicity units (TUs), where its dissolved concentration is normalised by its predicted toxicity (Di Toro et al., 2000; French-McCay, 2002; Redman et al., 2012). In complex hydrocarbon mixtures, the contribution of individual hydrocarbon TUs to overall toxicity is additive (Di Toro et al., 2007).

As no single aromatic toxicity data are currently available for *A. millepora*, putative CTLBBs were modelled from the experimental EC/LC₅₀ values for each endpoint and the time-averaged concentrations

of individual aromatic hydrocarbons measured in the associated WAF. The TLM was initially parameterised as per McGrath et al. (2018), using a universal slope of $-0.940 \text{ mmol L}^{-1}$, the geometric CTLBB value of $71.1 \mu\text{mol g}^{-1}$ octanol for 79 species in the TLM database, and chemical class corrections for BTEX (-0.025) and PAHs (-0.346) to account for their higher potency compared to baseline narcotic chemicals. Physico-chemical data were sourced from EPI Suite 4.11 (USEPA, 2012). Individual aromatic hydrocarbon TUs were calculated by dividing each measured concentration by the corresponding TLM-predicted EC/LC₅₀ value and the TUs were summed to obtain TU_{mix}. The Excel Goal Seek algorithm was then used to solve for the CTLBB that produced a modelled EC/LC₅₀ (TU_{mix} = 1) equal to the experimentally derived EC/LC₅₀ value.

3. Results

3.1. Chemical analysis

The HFO used to produce WAFs was supplied in two separate batches (2016 and 2018). The embryo and larval assays were performed using the 2016 batch and the 2018 batch was used for the fertilisation assays. The neat oils contained a similar suite of aromatics (Table S5), but the concentration of PAHs in the 2016 HFO ($50,494 \text{ mg kg}^{-1}$) was approximately twice that of the 2018 HFO ($24,150 \text{ mg kg}^{-1}$), primarily due to the 1.9- to 3.0-fold higher concentrations of naphthalene and C1- to C4-alkylnaphthalenes (Table S5). The time-averaged concentrations in the corresponding undiluted WAFs also differed, with the 2016 HFO WAFs containing a higher average TAH concentration than the 2018 HFO WAFs ($839 \mu\text{g L}^{-1}$ ($n = 6$) and $426 \mu\text{g L}^{-1}$ ($n = 3$), respectively; Table S6) and a higher proportion of PAHs (70% of TAH compared to 57%, respectively). This difference is mainly attributed to the 3.6- and 1.8-fold higher concentrations of naphthalene and C1-alkylnaphthalenes, respectively (Table S6). However, across all WAFs, BTEX and the naphthalene series collectively constituted 97–98% of time-weighted TAH concentrations (Table S6). For complete chemical analysis results of WAFs and neat oils refer to Tables S5–S7 and Figs. S1–S2.

3.2. Fertilisation assay

Fertilisation success of *A. millepora* gametes was dependent on sperm density, dissolved TAH concentration and the light treatment applied during HFO exposure. Higher TAH concentrations resulted in decreased fertilisation success in all light treatments (Fig. 1a), and inhibition of fertilisation in the dark occurred at lower TAH concentrations ($21.0 \mu\text{g L}^{-1}$ TAH; NEC) under sperm-limited conditions compared to optimal-sperm conditions ($292 \mu\text{g L}^{-1}$ TAH; NEC) (Fig. 1a, Table 1). However, the magnitude of inhibition was highly dependent on light treatment, with UVR co-exposure decreasing EC₅₀s by greater than 70-fold compared to visible light and dark treatments at optimal-sperm conditions: 5.3, 375 and $>432 \mu\text{g L}^{-1}$ TAH, respectively (Fig. 1b, Table 1).

NEC and 10% effect concentrations followed similar trends with inhibition of fertilisation occurring at very low concentrations (3.7 and $3.5 \mu\text{g L}^{-1}$ TAH, respectively) in the presence of UVR (Table 1). Fertilisation success in FSW controls was strongly dependent on sperm density, averaging 82% at optimal sperm densities ($1.2 \cdot 10^6$ sperm mL^{-1}) and 25% under sperm-limited conditions ($1.5 \cdot 10^3$ sperm mL^{-1}) in the absence of light (Fig. 1a).

3.3. Embryonic and larval development assay

Survival during embryonic development in *A. millepora* was affected by TAH concentration, light treatment and time since fertilisation, with latent effects increasing after the 48 h exposure. At the end of exposure, surviving embryos had completed development into planula larvae, and average survival in controls at 48, 96 and 192 h was 112%, 101% and 96%, respectively (Fig. 2a, c and e). The apparent increase in survival at the

Table 1

Interpolated threshold concentrations for each assay performed and putative CTLBBs. The relative sensitivity of each of the life stages tested is also provided based on a fitted lognormal SSDs of the 79 species CTLBBs in the TLM database (McGrath et al., 2018). NEC and EC/LC_x given as median of the posterior distribution in $\mu\text{g TAH L}^{-1}$ with 95% credible intervals (in brackets). For details of which model subsets were included in the final models, and the distributions used, refer to Table S4 and R scripts.

Life stage	Endpoint	Light regime	NEC ($\mu\text{g TAH L}^{-1}$)	EC/LC ₁₀ ($\mu\text{g TAH L}^{-1}$)	EC/LC ₅₀ ($\mu\text{g TAH L}^{-1}$)	CTLBB _{EC/LC50} ($\mu\text{mol g}^{-1}$ octanol)	Sensitivity rel. to spp. in TLM (%)
Gametes 	Fertilisation ^a ($1.5 \cdot 10^3$ sperm mL^{-1})	Dark	21.0 (8.8–89.2)	30.4 (10.1–99.0)	141 (74.0–341)	8.3 (4.4–20.1)	0.53 (0.05–6.7)
	Fertilisation ($1.2 \cdot 10^6$ sperm mL^{-1})	Dark	292 (209–333)	329 (273–360)	>432	—	—
		–UVR	198 (101–289)	174 (114–236)	375 (342–417)	15.1 (13.8–16.8)	3.3 (2.6–4.3)
		+UVR	3.7 (4.1–4.3)	3.5 (1.6–4.3)	5.3 (4.4–8.6)	—	—
Embryos 	48 h survival	–UVR	>843	>843	>843	—	—
		+UVR	192 (70–304)	354 (240–540)	>850	—	—
	96 h latent survival	–UVR	103 (78.2–140)	111 (70.9–186)	164 (126–208)	9.6 (7.3–12.1)	0.86 (0.34–1.8)
		+UVR	270 (134–406)	268 (170–402)	296 (216–405)	—	—
Planula larvae 	192 h latent survival	–UVR	128 (95.1–172)	131 (98.6–173)	157 (126–195)	9.2 (7.3–11.4)	0.75 (0.34–1.5)
		+UVR	272 (163–406)	267 (0–410)	290 (217–413)	—	—
	Survival	–UVR	373 (356–379)	375 (306–380)	399 (382–481)	21.6 (20.6–26.0)	7.8 (7.0–12)
		+UVR	166 (139–183)	161 (122–194)	328 (267–391)	—	—
	Fragmentation	–UVR	184 (171–189)	186 (175–190)	196 (191–209)	10.6 (10.3–11.3)	1.2 (1.1–1.4)
		+UVR	132 (99–189)	128 (0–186)	139 (102–192)	—	—
Metamorphosis		–UVR	88.3 (75.7–96.9)	33.8 (22.3–48.2)	81.4 (70.9–94.5)	4.4 (3.8–5.1)	0.05 (0.03–0.09)
		+UVR	41.3 (34.3–44.8)	21.2 (14.4–29.8)	63.8 (54.7–74.1)	—	—

— = CTLBB not derived.

^a TRH and BTEX concentrations were estimated, refer to Table S6.

end of the 48 h exposure in some replicates (controls and treatments) resulted from fragmentation of embryos during exposure and subsequent development of the fragments into smaller than normal larvae (i.e. <1 mm length). After transfer to clean FSW the average survival (including normal sized larvae and smaller larvae resulting from embryonic fragmentation) relative to the start of exposure remained high (>80%) for seawater controls, as well as low-mid concentrations (<100 $\mu\text{g L}^{-1}$ TAH) at the 96 and 192 h assessments (Fig. 2c and e).

At the end of the 48 h HFO WAF exposure, impacts on embryonic survival were only observed at high TAH concentrations (>192 $\mu\text{g L}^{-1}$ TAH) in the presence of UVR (Fig. 2a, Table 1). No LC₅₀ could be derived at 48 h but the derived NEC and LC₁₀ were reduced by at least 4.4- and 2.4-fold in the presence of UVR, respectively, indicating phototoxicity (Fig. 2b, Table 1). While limited impacts were observed at 48 h, latent survival (after transfer to FSW) was significantly affected at

substantially lower TAH concentrations for both light treatments. The latent impacts observed on survival were similar at 96 and 192 h (Fig. 2c and e, Table 1), with LC₅₀ estimates at both time points decreasing by at least 2.9- and 5.1-fold in the presence and absence of UVR, respectively, compared to corresponding values at the end of 48 h exposure (Table 1). In contrast to 48 h survival profiles, the latent impacts after 96 and 192 h were greater for larvae exposed to HFO in the absence of UVR, with all threshold values 1.8- to 2.6-fold lower than corresponding estimates in the presence of UVR, respectively (Fig. 2d and f, Table 1).

3.4. Larval survival and metamorphosis assays

Larval survival and metamorphosis success in *A. millepora* were affected by the TAH concentration and light treatment applied during

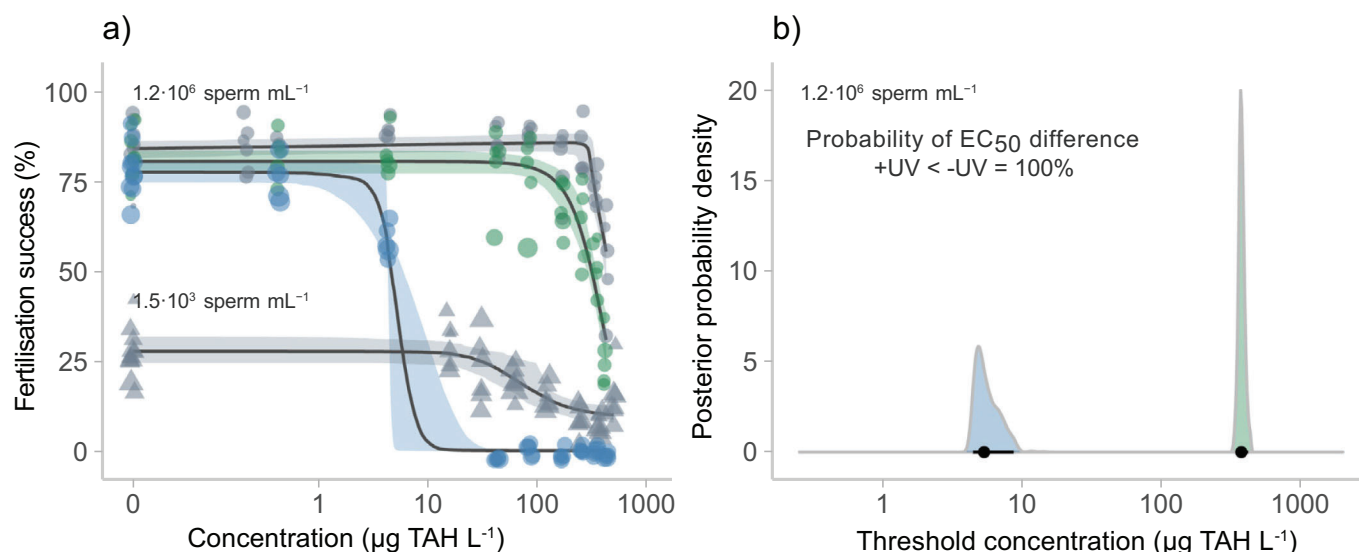


Fig. 1. Fertilisation success of *A. millepora* gametes exposed to HFO WAFs under three different light regimes at two sperm densities (a) and the PPDD for fertilisation success EC50s (b). Gametes exposed to HFO WAF in the absence of light (grey; Dark), under visible light in the absence of UVR (green; –UVR) or under visible light in the presence of UVR (blue; +UVR). For a) the model median (solid line), 95% credible intervals (shaded band) and raw data points are shown for assays performed in 2018 (triangles) and 2019 (circles). Sperm density used in assays indicated above corresponding model median lines. Size of raw data points is indicative of the total number of eggs and embryos assessed for individual replicates. For b) PPDD median (solid dot) and 95% credible intervals (solid line) shown for +UVR and –UVR treatments. For corresponding graphical representations of the NEC model subset refer to Fig. S3. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

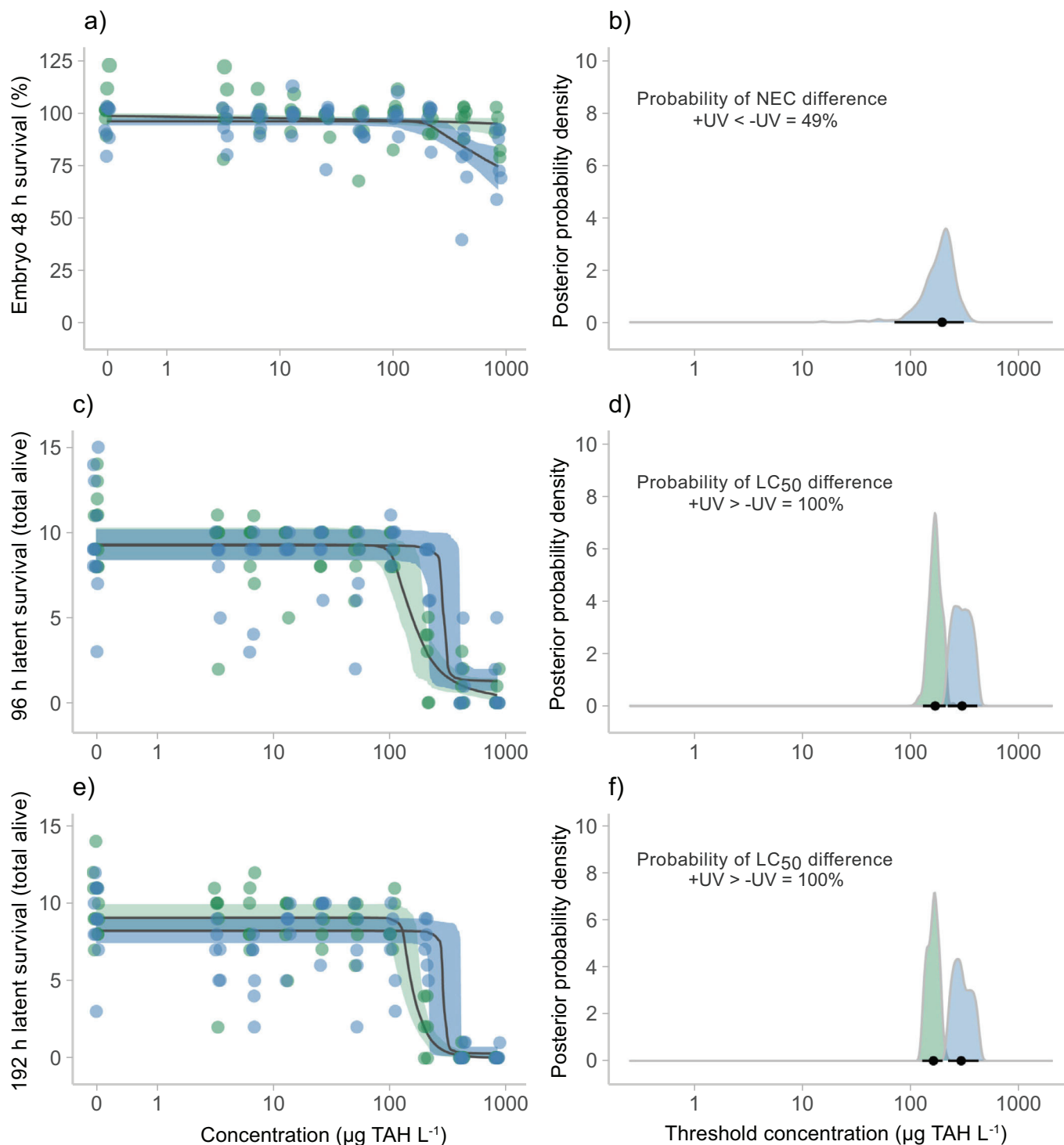


Fig. 2. Direct (a) and latent (c and e) survival of *A. millepora* embryos exposed to HFO WAFs, under visible light in the absence (green; −UVR) or presence (blue; +UVR) of UVR, and the PPDD for threshold concentrations derived for each time point (b, d and f). Model median (solid line), 95% credible intervals (shaded band) and raw data points (circles) are shown for each assessment. PPDD median (solid dot) and 95% credible intervals (solid line) shown for each endpoint. Please note that the comparison of threshold concentration PPDD for 48 h survival (b) refers to the estimated NEC while the corresponding comparisons for 96 h (d) and 192 h (f) refer to the LC₅₀. For corresponding graphical representations of the NEC model subsets for the 96 and 192 h assessments refer to Fig. S4. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

HFO WAF exposure. Average larval survival was high in seawater controls at the end of the 48 h exposure period (96%) and significantly impacted following exposure to increasing TAH concentrations (Fig. 3a). LC₅₀ values were similar across light treatments but approximately 1.2-fold lower in the presence of UVR (98% probability of a difference; Fig. 3b, Table 1). UVR co-exposure also lowered the NEC threshold for larval survival 2.3-fold (100% probability; Fig. S5a–b, Table 1). Replicates

with 100% mortality were only observed at the highest TAH concentrations applied (788 and 760 µg L⁻¹ TAH +UVR and −UVR, respectively; Fig. 3a), and at these concentrations many larvae appeared as white, immobile larva-shaped masses of lysed cells (Fig. 4f).

The occurrence of deformed (Fig. 4d–e) and very small to small (10–600 µm; Fig. 4e) larvae at the end of the 48 h exposure increased with increasing TAH concentration (Fig. 3c, Table 1), sometimes leading

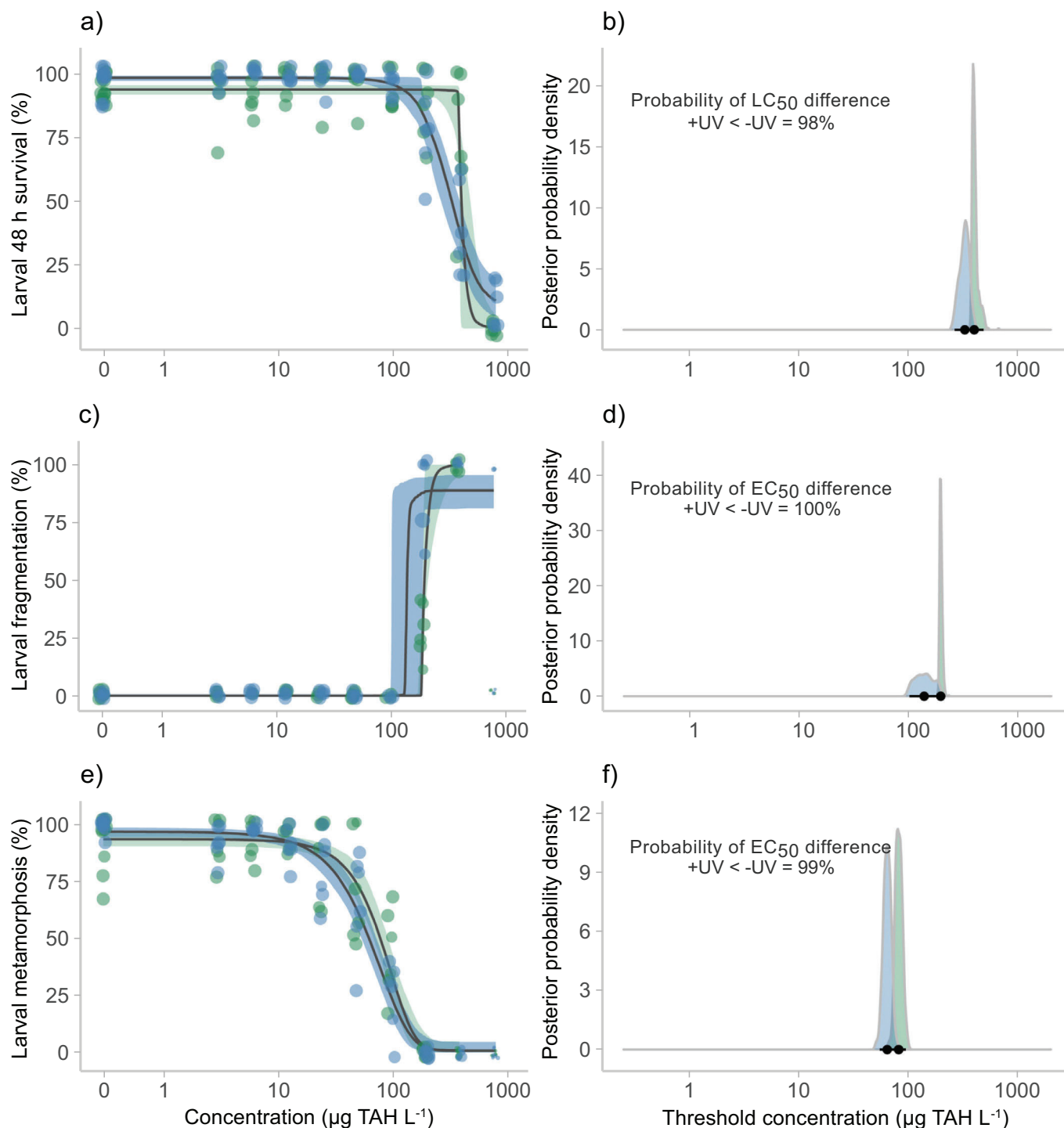


Fig. 3. *A. millepora* larval survival (a), occurrence of small larvae (c) and metamorphosis success (e) following exposure to HFO WAFs under visible light in the absence (green; –UVR) or presence (blue; +UVR) of UVR, and PPDD for derived LC_{50} (b) and EC_{50} (d and f) values. Model median (solid line), 95% credible intervals (shaded band) and raw data points (circles) shown for each assessment. PPDD median (solid dot) and 95% credible intervals (solid line) shown for LC/EC_{50} . Data from larval assay performed in December of 2017. For corresponding graphical representations of the NEC model subset refer to Fig. S4. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

to an increase in the total number of larvae relative to the start of exposure (Fig. 5a). These smaller larvae (Fig. 4e) were determined to be generated via the fragmentation of normal sized, originally healthy larvae (Fig. 4a) that developed abnormalities, became severely deformed (Fig. 4d) and finally fragmented. Deformations observed in the larvae pre-fragmentation included holes, bends, lumps and areas of necrotic tissue, with some larvae noted to have lumps of similar shape and size as the smaller larvae (Fig. 4e). The undersized larvae retained

the ability to swim: from spinning in one place to traversing the entirety of the well. Small larvae occurred at lower concentrations in the presence of UVR resulting in a 1.4-fold decrease in the EC_{50} (100% probability; Fig. 3d). Additional small and very small larvae were observed at the end of the 24 h metamorphosis incubation period relative to the 48 h assessment (Fig. 5b), and for some replicates, all larvae of normal shape and length ($\sim 1000 \mu\text{m}$) had disappeared by the time of metamorphosis assessment (>190 and

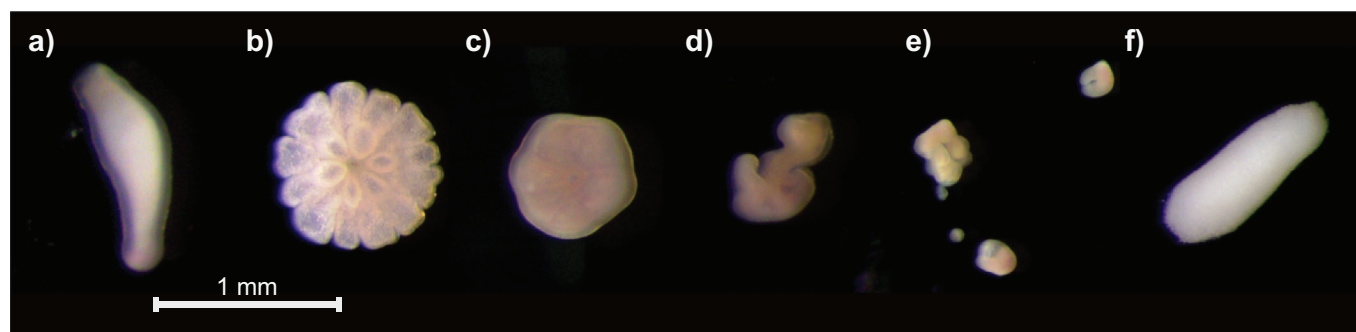


Fig. 4. Examples of normal and abnormal larvae and recruits observed during larval *A. millepora* assay. Morphologies observed included: a) normal sized planula larva (0–100 $\mu\text{g L}^{-1}$ TAH), b) fully metamorphosed recruit (0–100 $\mu\text{g L}^{-1}$ TAH), c) early stage metamorphosed recruit (10–500 $\mu\text{g L}^{-1}$ TAH), d) severely deformed larva undergoing fragmentation (10–500 $\mu\text{g L}^{-1}$ TAH), e) swimming larval fragments and deformed larvae undergoing fragmentation (10–500 $\mu\text{g L}^{-1}$ TAH) and f) larva-shaped mass of dead cells (>350 $\mu\text{g L}^{-1}$ TAH). Treatment concentrations where each morphology was observed shown in brackets. Examples extracted from photographs obtained using a Leica M5S dissecting microscope with a 5.1 Mp camera calibrated using the ToupView software.

370 $\mu\text{g L}^{-1}$ + UVR and – UVR, respectively). Fragmentation occurred at lower TAH concentrations in the presence of UVR (Fig. 3c, Table 1, Fig. S5c–d) but fragments appeared to survive longer in the absence of UVR. Similar observations were made during the preliminary larval experiment performed using larvae originating from Falcon Island (Figs. 5b and S7, Table S13).

Larval metamorphosis success was significantly reduced by exposure to HFO WAF at low TAH concentrations both in the absence and presence of UVR (Figs. 3e and S5e). Metamorphosis was the most sensitive larval endpoint assessed for both light treatments (Fig. 3, Table 1). UVR co-exposure resulted in a 1.3-fold reduction of the metamorphosis EC_{50} (99% probability; Fig. 3e–f) and a 2.1-fold reduction of the NEC (100% probability; Fig. S5e–f). While metamorphosis was still observed at mid-high concentrations (<190 $\mu\text{g L}^{-1}$) recruits were generally underdeveloped or failed to undergo complete metamorphosis (Fig. 4c), with first observations of underdeveloped recruits occurring at low-mid concentrations (>50 $\mu\text{g L}^{-1}$). Small and very small larvae did not generally undergo metamorphosis, but the first phase of metamorphosis, attachment to the substrate, was sometimes observed. No metamorphosis was observed at high TAH concentrations (>200 $\mu\text{g L}^{-1}$) regardless of light treatment (Fig. 3e).

3.5. Putative CTLBB estimation

Putative CTLBBs calculated from the EC_{50} and LC_{50} values (in the absence of UVR) were very low, ranging from 4.4 $\mu\text{mol g}^{-1}$ octanol for *A. millepora* larval metamorphosis to 21.6 $\mu\text{mol g}^{-1}$ octanol for larval survival (Table 1). CTLBBs were not calculated if an EC_{50} or LC_{50} was not reached, or for UVR treatments as the observed toxicity would most likely be a combination of narcosis and phototoxicity. The derived CTLBBs for assessed endpoints all fall within the 10% most sensitive species, when compared to the 79 mostly aquatic species currently included in the TLM database (McGrath et al., 2018). Additionally, the three most sensitive endpoints assessed here (larval metamorphosis success, fertilisation success at low sperm densities and latent embryonic survival) are equally or more sensitive than the most sensitive species in the database (Table 1).

4. Discussion

4.1. Overarching trends/summary

HFO WAF exposure negatively impacted all early life stages of *A. millepora* tested, with UVR co-exposure generally increasing the direct effects of HFO. The order of sensitivity (highest to lowest), based on EC/LC_{50} in the absence of UVR, was: (i) larval metamorphosis; (ii) fertilisation at low sperm densities; (iii) latent embryo survival ≥ 96 h; (iv) larval fragmentation; (v) larval survival; (vi) fertilisation at optimal

sperm densities; and (vii) 48 h embryo survival. The strong latent effects of dissolved aromatics on embryo survival indicate the potential for short exposure assays (without post-exposure monitoring) to underestimate toxicity thresholds, and latent effects should be investigated further in other early life stages. In addition to being the most sensitive early life stage tested, quantification of larval metamorphosis was unaffected by confounding factors, which can affect and/or mask toxicity, including sperm density (fertilisation assay) and fragmentation (embryo and larval assays). Larval metamorphosis was approximately 5-fold more sensitive to HFO WAF than survival, but failure to metamorphose from a larva to a juvenile polyp represents an equally important impact on coral recruitment. Therefore, metamorphosis success is an appropriate toxicity endpoint for deriving toxicity thresholds for oil spill risk assessments. The derivation of putative CTLBB values allowed direct comparison of sensitivity between some endpoints for these early coral life stages and the 79 aquatic species in the TLM narcotic toxicity database, which is used as a basis to model toxicity thresholds of oils from their composition alone (McGrath et al., 2018). Based on the putative CTLBBs, metamorphosis of *A. millepora* larvae is more sensitive than the most sensitive species currently included in the TLM database (*Melanotaenia fluviatilis*, 9 $\mu\text{mol g}^{-1}$ octanol; McGrath et al. (2018)). This further indicates its sensitivity to dissolved aromatics and highlights the value of developing more robust CTLBBs for the early life stages of coral, which can contribute to improving tropical toxicity modelling. Exposure to relatively low UVR intensities increased HFO WAF toxicity between 1.2- (larval LC_{50}) and 94-fold (fertilisation EC_{10} at 10^6 sperm mL^{-1}) across the assessed life stages and endpoints. On average, the end of exposure EC/LC_{50} for embryonic and larval endpoints decreased by 1.3-fold, with 2.0- and 2.6-fold decreases observed for EC/LC_{10} and NEC compared to –UVR treatments. The influence of UVR co-exposure on the toxicity of HFO WAF to all early life stages highlights the need for further studies to quantify phototoxicity across a full range of UVR intensities that are relevant for species living in clear, shallow-water coral reef environments.

4.2. Fertilisation

The sensitivity of fertilisation to dissolved HFO aromatics varied considerably with sperm density and light exposure. Fertilisation success in seawater controls, regardless of light treatment applied, were in line with those previously reported for *A. millepora* in the absence of light (Willis et al., 1997; Ricardo et al., 2015). However, direct comparisons with previous studies on the effects of dissolved aromatics on coral fertilisation are difficult due to differences in methodologies, oil compositions and units of reporting used. Nevertheless, the relative insensitivity of fertilisation success of *A. millepora* gametes exposed to a HFO WAF at optimal-high sperm densities in the dark is consistent with previous reports. The same species exposed to crude oil WAF at 10^6 sperm mL^{-1} ,

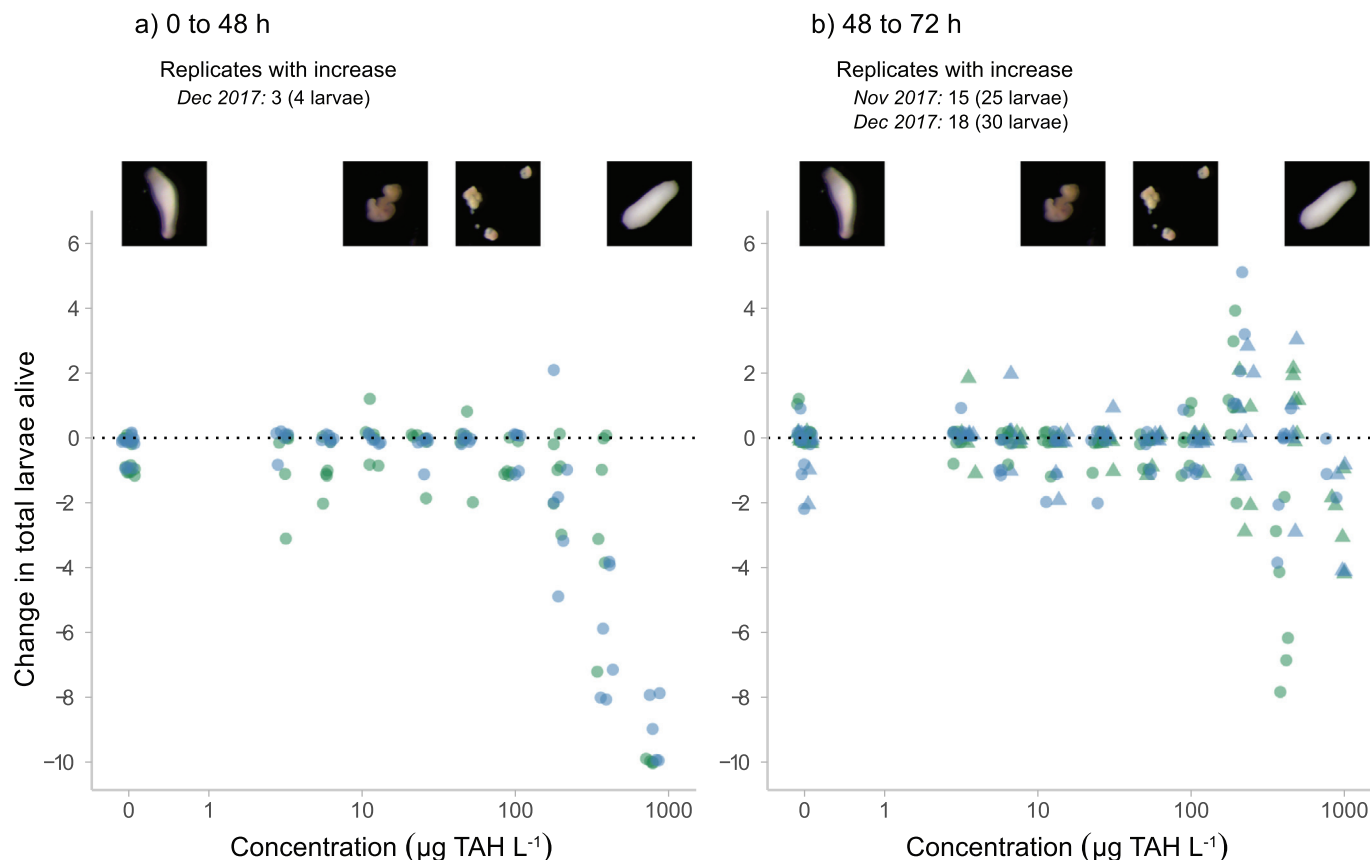


Fig. 5. Change in total number of *A. millepora* larvae and recruits alive between start and end of exposure (a) and between induction of metamorphosis and metamorphosis assessment (b) under visible light in the presence (blue) or absence (green) of ultraviolet radiation. Data from preliminary larval survival and metamorphosis assay performed in November (triangles) and larval survival and metamorphosis assays performed in December (circles) of 2017.

reported no effects at $165 \mu\text{g L}^{-1}$ TRH (determined by UV fluorescence; Negri and Heyward (2000)). However, the same study reported a ~20% reduction in fertilisation for *A. millepora* when exposed to $180 \mu\text{g L}^{-1}$ TRH from produced formation water at the same high sperm densities. Inhibition of fertilisation success in *Acropora microphthalma* (38% reduction) was also observed when exposed to mineral lubricating oil WAF concentrations $\geq 200 \mu\text{g L}^{-1}$ TRH at 10^6 sperm mL^{-1} (Mercurio et al., 2004). While there is mounting evidence that coral fertilisation is relatively insensitive to dissolved aromatics at optimal or high sperm densities, there was >10-fold increase in the sensitivity of *A. millepora* fertilisation (based on NEC/EC_{10S}) to HFO WAF at low sperm densities (10^3 sperm mL^{-1}), making it one of the most sensitive endpoints assessed in the present study.

Fertilisation assays performed on corals and other invertebrates have also shown increased sensitivity to pollutants at sub-optimal sperm densities (Marshall, 2006; Hollows et al., 2007; Ricardo et al., 2018). The current study identified abnormal embryonic development and cell lysis following exposure to dissolved aromatics, supporting previous findings (Harrison, 1994; Harrison, 1999; Mercurio et al., 2004). However, the high dependence of toxicity on sperm density suggests sperm may be more affected than eggs (Ricardo et al., 2018). The sensitivity of fertilisation toxicity thresholds to variations in sperm density raises concerns for their application in risk assessments as sperm densities in situ are not well known. For example, fertilisation success in the field is reported to vary between 0 and 90% in the first 2 h after gamete release (Oliver and Babcock, 1992; Levitan et al., 2004), indicating sub-optimal sperm densities frequently occur. Furthermore, fertilisation success is highly dependent on the environmental conditions (wind, currents, sediments, bleaching, asynchrony) during the spawning event, which affect both sperm densities and the likelihood of encounters with

eggs of conspecifics (Omori et al., 2001; Hollows et al., 2007; Ricardo et al., 2015; Shlesinger and Loya, 2019). Although the sensitivity of coral fertilisation to HFO varies with sperm density, coral gametes concentrated at or near the surface have the potential to be exposed to the highest concentrations of dissolved aromatics following a surface spill (NRC, 2003). Furthermore, the impacts of direct contact between positively buoyant coral eggs and oil droplets or slicks should also be considered. This has not been investigated to date and has the potential to further decrease fertilisation success and thereby recruitment.

4.3. Embryonic development

The present study is the first to demonstrate negative impacts of dissolved aromatics from petroleum products on coral embryos. Previous studies exposing embryos of the mussel *Mytilus galloprovincialis* and the sea urchin *Paracentrotus lividus* to marine fuel oil WAF for 48 h also showed negative impacts, with developmental stage and larval growth EC_{50S} of 82% and 45% WAF, respectively (Bellas et al., 2013). Exposure of coral embryos to other pollutants can also cause embryo mortality; for example, *Acropora tenuis* embryos (12 hpf) exposed to coal dust for 72 h exhibited up to 26% higher mortality than seawater controls (Berry et al., 2017). The potential for latent effects on embryos was also evident for *A. tenuis* (8 hpf) exposed to suspended sediments or elevated nutrients, which showed limited effect on survival at the end of a 28 h exposure, but significantly reduced settlement following recovery in clean seawater (Humanes et al., 2017). Latent oil toxicity-induced mortality has also been observed for other coral life stages including larvae of *Orbicella faveolata* and *Agaricia humilis*, where mortality was only observed during the post-exposure period (Hartmann et al., 2015). Similarly, latent impacts were observed on the growth of *Seriatopora hystrix*, *Seriatopora guttatus*

and *Stylophora pistillata* juveniles exposed to gas condensate WAF for 96 h while larvae or newly settled recruits (Villanueva et al., 2008). These reports support the findings of the present study that latent impacts may outweigh the impacts observed at the end of exposure, and that ignoring latent effects may lead to significant underestimation of toxicity thresholds.

Embryonic survival was affected by two competing processes: (i) mortality from exposure to dissolved aromatics and (ii) embryo fragmentation. This fragmentation caused an apparent increase in embryo numbers in many treatments and resulted in the embryo survival metric representing the “net” outcome of these processes combined. Embryo fragmentation due to physical disturbances has previously been documented for acroporid corals and can produce fully viable larvae in the absence of other stressors (Heyward and Negri, 2012). Embryo fragmentation masked mortality in this assay by increasing apparent numbers of embryos, which resulted in high net survival at 48 h, even at high TAH concentrations. In contrast, latent survival of embryos was significantly affected by exposure to dissolved aromatics with a strong concentration-response observed regardless of light treatment. The latent impacts decreased LC₅₀ values by 2.9 to 5.4-fold compared to the end of exposure (48 h) but did not vary substantially between the two assessment times (96 and 192 h). Latent mortality due to oil toxicity clearly overcame the increased embryo numbers, resulting from fragmentation during exposure, after embryos/larvae were transferred to clean FSW. The smaller larvae resulting from fragmented embryos may also have been more susceptible to the effects of the dissolved aromatics as resources and lipids were split across two or more larvae (Okubo et al., 2017) and the surface-area-to-volume ratio increased (Pelletier et al., 1997). During an oil spill, buoyant embryos are likely to be simultaneously exposed to the oil slick/droplets, the highest dissolved TAH concentrations (NRC, 2003) and turbulence (e.g. from wave action), which also promotes fragmentation (NRC, 2003; Heyward and Negri, 2012). Coral embryos uniquely lack a protective membrane (Heyward and Negri, 2012) and embryos of other invertebrates are therefore unlikely to fragment in the same way. While embryonic development assays are routinely used to assess sensitivity to pollutants for some taxa, and have been suggested as a high-throughput alternative to assays using adults (Capela et al., 2020), the possibility of fragmentation renders net embryonic survival of coral embryos a less reliable endpoint for application in risk assessments.

4.4. Planula larvae

A. millepora larvae were significantly impacted by HFO WAF exposure both in the presence and absence of UVR. Larval metamorphosis was inhibited at the lowest TAH concentrations ($>21.2 \mu\text{g L}^{-1}$ TAH), higher concentrations ($>184 \mu\text{g L}^{-1}$ TAH) also caused larval fragmentation, while larval survival was only affected at the highest TAH concentrations tested ($>373 \mu\text{g L}^{-1}$ TAH).

4.4.1. Metamorphosis

Metamorphosis success was high in seawater controls but decreased with increasing concentration of dissolved hydrocarbons, regardless of light treatment, with no metamorphosis observed at concentrations $>200 \mu\text{g L}^{-1}$ TAH. The sensitivity of metamorphosis success observed here is within range of that previously reported for *A. millepora* larvae exposed to other petroleum products (Negri and Heyward, 2000). *A. millepora* larvae appear to be at least as sensitive to petroleum hydrocarbon exposure as other coral species in the absence of UVR, including *P. astreoides* (Goodbody-Gringley et al., 2013), *A. tenuis* (Negri et al., 2016; Nordborg et al., 2018), *A. humilis* (Hartmann et al., 2015) and *O. faveolata* (Goodbody-Gringley et al., 2013; Hartmann et al., 2015). However, due to differences in the chemical composition of the petroleum products used, and the exposure and analytical methodologies applied, direct quantitative comparisons are of limited significance (Redman and Parkerton, 2015; Hodson et al., 2019).

In addition to inhibition of metamorphosis, partial metamorphosis resulting in underdeveloped recruits also increased at higher TAH concentrations. This partial metamorphosis ranged in severity from incomplete formation of mesenterial septa, characteristic of fully metamorphosed recruits (Heyward and Negri, 1999), to inability to proceed further than attachment to the substrate during the metamorphosis incubation period. Similar observations have previously been made for *Heteroxenia fuscescens* larvae, where attachment but no metamorphosis was observed for larvae exposed to chemically enhanced oil WAF (CEWAF) for 96 h (Epstein et al., 2000). Underdeveloped recruits and delayed settlement was also observed for *A. tenuis* larvae exposed to HFO and diesel WAF (Nordborg et al., 2018). Narcotic toxicity may be responsible for these effects but it has also been suggested that inhibition of metamorphosis may result from the action of dissolved aromatics on specific developmental pathways (Negri et al., 2016). The expression of several stress-related genes has previously been observed in coral larvae (Overmans et al., 2018) and adults (Xiang et al., 2019) exposed to dissolved aromatics, while growth and development-related genes were depressed in soft corals exposed to a mixture of PAHs (Woo et al., 2013). The inclusion of gene expression assays in larvae exposed to dissolved aromatics may clarify the mode of action of aromatics on larval metamorphosis success.

It is unclear whether the *A. millepora* larvae that failed to undergo metamorphosis during the incubation period could recover and potentially attach and complete metamorphosis at a later time, e.g. if transferred to clean seawater. However, Epstein et al. (2000) and Hartmann et al. (2015) both reported that coral larvae exposed to oil WAFs were unable to settle after transfer to clean seawater prior to settlement induction. In this study, the small, fragmented larvae, that were generated at TAH concentrations higher than those that inhibited metamorphosis, may be even less likely to settle than whole larvae as some of the fragments may lack the chemoreceptors required for initiation of settlement (Heyward and Negri, 1999) or genetic regulators of metamorphosis associated with the animal pole (Okubo et al., 2017). Further investigations into the latent impacts of oil exposure on metamorphosis success, the mechanism of action for metamorphosis inhibition, the metamorphosis competency of larval fragments and whether recovery may restore metamorphosis competency are clearly warranted.

4.4.2. Survival and fragmentation of larvae

The sensitivity of coral larval survival appears to vary between species, larval ages and oil pollutants tested, with some larvae experiencing no mortality while others show potentially higher sensitivity than the *A. millepora* larvae assessed here. However, due to inconsistencies in the analytical and exposure methodologies used, as well as a lack of chemical analysis for several previous studies, quantitative comparisons of larval sensitivities are not valid (Redman and Parkerton, 2015; Turner and Renegar, 2017; Hodson et al., 2019). Nevertheless, if the derived threshold concentrations for *A. millepora* are expressed as TRH (e.g. LC₁₀ 1039 and LC₅₀ 1103 $\mu\text{g L}^{-1}$) then *A. millepora* appears to be more sensitive to petroleum hydrocarbons (lower LC₁₀ or LC₅₀) than larvae of *A. tenuis*, *Platygyra sinensis* and *Coelastrea aspera* (previously *Goniastrea*; Lane and Harrison (2000)), *S. pistillata* (Epstein et al., 2000; Villanueva et al., 2008), *Pocillopora damicornis* and *Pocillopora verrucosa* (Villanueva et al., 2008). Larvae of the following species may be more sensitive than *A. millepora*: *O. faveolata* (Goodbody-Gringley et al., 2013; Hartmann et al., 2015), *A. humilis* (Hartmann et al., 2015) and *Porites astreoides* (Goodbody-Gringley et al., 2013).

The observed fragmentation of larvae may have been due to narcotic toxicity, with aromatics concentrating in and disrupting cell membranes. At high concentrations, the structural membranes may have failed altogether, generating fragments, some of which are able to continue swimming. Larval abnormalities and tissue degeneration have been observed for *S. pistillata* larvae exposed to CEWAF of Egyptian oil (Epstein et al., 2000). The authors reported that deformed larvae were observed to release “small spherical bodies, (probably lipid droplets)”

and to lose their normal swimming behaviour, in addition to settlement inhibition (Epstein et al., 2000). Following histological comparisons, the authors concluded that CEWAF exposure had damaged the ectodermal outer layer. In larvae of the soft coral *H. fuscescense*, exposure to the same Egyptian oil CEWAFs also caused deformation of larvae ("ball-like deformed structure") (Epstein et al., 2000). Morphological deformations were also observed in *A. tenuis* larvae exposed to HFO and diesel WAF (Nordborg et al., 2018) as well as those exposed to solutions containing anthracene or phenanthrene (Overmans et al., 2018). It is unclear whether the reports by Epstein et al. (2000) describe the same fragmentation process observed in the present study, in particular as the dispersant may have been the main causative agent, but the spherical/"ball-like" appearance is consistent with the small and very small larvae observed here. While it is unclear whether larval fragments have a comparable viability, or competency for metamorphosis as whole larvae, they retained at least partial ability to swim. As for embryos, the fragmentation of larvae results in an underestimation of the impacts of HFO WAF on larval survival as the number of new, swimming fragmented larvae may be larger than the number of whole larvae and fragments that die during a given period. Additionally, many of the small and very small larvae observed at the end of the 48 h exposure had died and dissolved by the end of the metamorphosis incubation period (at 72 h), making quantification of net larval survival even less reliable. This result further supports the use of larval metamorphosis success as the primary endpoint investigated when testing the toxicity of dissolved aromatics towards coral larvae.

4.5. Phototoxic effects on the early life stages of coral

Co-exposure to UVR affected all early coral life stages and endpoints assessed in this study. Phototoxicity causes damage to cellular structures or genetic material and occurs when dissolved aromatics are exposed to UVR, or short wavelength visible light, through two main pathways: photosensitisation or photomodification/photooxidation. *Photosensitisation* results in production of reactive oxygen species, as photo-excited aromatics decay back to their ground states, while *photomodification* results in the production of more reactive photoproducts or intermediates from parent aromatics, often through oxidation (Barron, 2017).

Co-exposure to light increased the effects of HFO WAF on *A. millepora* fertilisation success, with exposure to UVR resulting in >70-fold reductions of all threshold concentrations (NEC, EC₁₀ and EC₅₀). Interestingly, the presence of visible light alone also led to a reduction of threshold concentrations by 1.2- to 1.9-fold, indicating light in the 400–700 nm range may also play a subtle role in increasing the effects of dissolved aromatics on fertilisation. There was no apparent effect of UVR on fertilisation success in the absence of dissolved aromatics, indicating that the applied UVR intensity may have been too low to cause damage to sperm or inhibit fertilisation (Dahms and Lee, 2010). However, phototoxic effects on fertilisation was apparent at very low TAH concentrations and is likely to primarily impact sperm, which unlike eggs, do not contain UVR-protective mycosporine-like amino acids (Dunlap and Shick, 1998). Nevertheless, broadcast spawning corals such as *A. millepora* generally spawn at night, so phototoxicity would not increase the vulnerability of gametes for this species in situ. However, the fertilisation of some reef-building corals that also spawn during the day (Bouwmeester et al., 2011; Bronstein and Loya, 2011; Schmidt-Roach et al., 2012; Suzuki, 2012) may be vulnerable to phototoxicity.

UVR increased the immediate impacts on embryonic survival (at 48 h) but this increase was small compared to the latent mortality, which occurred after transfer to clean FSW. Indeed, the generation of small embryo and larval fragments during the 48 h exposure confounded the embryo survival data (as described above). Fragmentation rates in the presence of UVR were potentially higher (not possible to

assess), which may have increased the apparent survival, thereby masking toxicity. Further studies should investigate the effect of UVR on embryo fragmentation under a range of turbulence and UVR intensities to increase certainty of the influence of phototoxicity on coral embryos.

A. millepora larval metamorphosis EC₅₀ was 1.3-fold lower in the presence of UVR while the EC₁₀ was reduced by ~1.6-fold. Similar phototoxicity was previously observed for *A. tenuis* larvae (1.6- to 1.9-fold lowering of metamorphosis threshold concentrations) exposed to the same oil using the same exposure methodology (Nordborg et al., 2018). The *A. tenuis* metamorphosis EC₅₀ + UVR (51 µg TAH₃₅ L⁻¹) was lower than that observed for *A. millepora* in the present study, but the EC₅₀ - UVR was lower for *A. millepora* than *A. tenuis* (96 µg TAH₃₅ L⁻¹). While threshold concentrations were similar across the two studies, phototoxicity was more apparent in *A. tenuis* larvae, highlighting the potential for species-specific sensitivities to phototoxicity. Such differences may be due to differences in the protection from UVR by mycosporine-like amino acids (Dunlap and Shick, 1998).

Tropical, shallow-water coral reefs occur in geographic regions experiencing high UVR intensities throughout the year. UVR irradiance at or just below the surface on the central GBR was measured at 5.98–6.28 mW cm⁻² UVA and 0.323 mW cm⁻² UVB (for further details refer to Fig. S9 and Table S14) in the lead up to the annual mass-spawning events in 2017 (generally November–December for mid-shelf reefs on the central GBR; Babcock et al. (1986)). While UVR applied in the present study caused a substantial increase in the direct toxicity of HFO, the total UVR intensity applied was low compared to the potential exposure of buoyant embryos and larvae at the water surface or in clear, shallow water (<22% total UVA and UVB of surface irradiance). Additionally, a lower percentage of the total irradiance from the UVR lights used in the present study consisted of the higher energy, UVB radiation (2% compared to 5%; Tables S3 and S14). Phototoxicity increases markedly with increases in UVR intensity and is dependent on the spectrum (Barron, 2017); hence, the phototoxicity of HFO towards developing coral embryos and coral larvae in the field is likely to be underestimated in the present study. To ensure threshold concentrations for oil toxicity applied in risk assessments are representative of conditions in situ, further research on the impacts of dissolved aromatics to coral larvae under different UVR intensities, and spectral profiles, should be undertaken.

4.6. Putative CTLBB estimates

Comparison of sensitivity to oil exposure across species or endpoints is often required for risk assessments but is confounded by differences in oil composition, solution preparation and exposure methodology. As a result, the direct comparison of threshold concentrations (e.g. µg L⁻¹) is discouraged and alternative methods of comparison should be used (Redman et al., 2012; Redman and Parkerton, 2015). The comparison of species-specific CTLBBs is the most effective method to rank species sensitivity to aromatic hydrocarbon exposure, where low CTLBBs indicate more sensitive species regardless of oil composition (Di Toro et al., 2000; French-McCay, 2002; Redman and Parkerton, 2015).

As expected, the putative CTLBB values for each early life stage followed the same order of sensitivity as the EC₅₀s expressed in µg L⁻¹ TAH (Table 1). However, the derivation of putative CTLBBs allowed the most valid ranking of sensitivity among these life stages as it also accounted for the small differences in WAF composition between assays. CTLBBs were not reported for assays where UVR was applied as the TLM cannot account for the potential contribution of phototoxicity to the observed results, including for the presence of potential photooxidation products in WAFs. The putative CTLBBs derived here indicate that *A. millepora* larval metamorphosis is more sensitive than the 79 species in the TLM database. All assessed endpoints where a CTLBB could be derived were substantially more sensitive than the geometric

mean species sensitivity of the TLM database ($71.1 \mu\text{mol g}^{-1}$ octanol) and within the 10th percentile of most sensitive species currently included (McGrath et al., 2018). The few CTLBB values previously published for corals, including the $51.6 \mu\text{mol g}^{-1}$ octanol definitive CTLBB for adults of the cold-water coral *Lophelia pertusa* (Bytingsvik et al., 2020) and preliminary CTLBBs (based on LC_{50} responses to a single PAH) of $199\text{--}698 \mu\text{mol g}^{-1}$ octanol for three Atlantic scleractinian corals (Renegar and Turner, 2021), are also substantially higher than the putative CTLBBs derived here. The derivation of definitive CTLBBs for metamorphosis in coral larvae, by performing assays using individual aromatic compounds (French-McCay, 2002; Redman et al., 2012), should be prioritised as a valuable contribution to the TLM database as it is currently comprised predominantly of temperate (~90%) and/or freshwater (~60%) species (McGrath et al., 2018). A definitive CTLBB for larval metamorphosis would improve confidence that hazard concentration thresholds (e.g. HC5) calculated using models such as PETROTOX (Redman et al., 2012; Redman et al., 2017) are also protective of these sensitive early life stages of keystone tropical coral reef taxa.

5. Conclusion

Heavy fuel oil negatively affected *A. millepora* across all early life stages tested with increased toxicity observed in the presence of UVR. HFO affected early life stages of coral at concentrations as low as 21, 103 and $21 \mu\text{g L}^{-1}$ TAH for fertilisation, embryo survival and larval metamorphosis, respectively (Table 1). These concentrations are below those that have been reported in the field after spills (Diercks et al., 2010; Baum et al., 2016), indicating *A. millepora* recruitment is likely to be impacted if oil spills were to coincide with annual spawning events; both during spawning nights as well as during larval development and settlement in the weeks following spawning. Embryo and larval survival were relatively insensitive to low TAH concentrations and effectively quantifying these endpoints was confounded by fragmentation. While fertilisation could be very sensitive to aromatics, this sensitivity was highly dependent on sperm densities (which are largely unknown during in situ spawning events). In contrast, larval metamorphosis was both sensitive to oil exposure at low concentrations and unaffected by fragmentation. Furthermore, metamorphosis of planula larvae into sessile coral polyps represents a critical lifecycle bottleneck for reef-regeneration following disturbances (Randall et al., 2020) and has been shown to be a robust and replicable assay for the toxicity of dissolved aromatics to coral (Epstein et al., 2000; Negri and Heyward, 2000; Villanueva et al., 2008; Goodbody-Gringley et al., 2013; Hartmann et al., 2015; Negri et al., 2016; Nordborg et al., 2018; Overmans et al., 2018). Therefore, the toxicity thresholds for metamorphosis success are recommended as the most relevant for application in oil spill risk assessments of the early life stages tested in this study.

Coral metamorphosis was also sensitive to dissolved aromatics in comparison to other aquatic species, as demonstrated by its low putative CTLBB. The putative metamorphosis CTLBB places *A. millepora* as the most sensitive species and endpoint assessed to date when compared to the TLM database (Redman et al., 2017; McGrath et al., 2018). Larval metamorphosis is likely to be more sensitive to dissolved aromatics than endpoints for adult corals (Turner and Renegar, 2017; Nordborg et al., 2020), with the only reliable available data being for adults of *Porites divaricata* with an EC_{50} of $7442 \mu\text{g L}^{-1}$ for 1-methylnaphthalene (Renegar et al., 2017). However, further studies to derive definitive CTLBBs for coral larvae, as well as juvenile and adult corals, are required to confirm their relative sensitivity. This would enable the incorporation of these keystone coral reef taxa into TLM databases for toxicity models such as PETROTOX (Redman et al., 2012; McGrath et al., 2018) and OilToxEx (French-McCay, 2002), enabling the application of predictive toxicity modelling for all oils and petroleum products relevant to reef environments.

The marked increase in toxicity of HFO WAF to *A. millepora* embryos in the days following the end of exposure suggests the typically short, traditional ecotoxicity tests performed with aromatics on early life stages may underestimate sensitivity. Further studies are needed to determine if fertilisation, larval metamorphosis and later life stages of coral are also affected at lower TAH concentrations when monitoring extends beyond the end of exposure. Finally, the application of UVR co-exposure is strongly recommended for future investigations of the impacts of dissolved aromatics on early coral life stages. In the present study, impacts increased across all endpoints assessed at end of exposure, supporting existing evidence of phototoxicity across life stages in several other reef-building coral species (Peachey and Crosby, 1995; Guzmán Martínez et al., 2007; Negri et al., 2016; Nordborg et al., 2018; Overmans et al., 2018). Early life stages of marine taxa have long been recognised as the most at risk for phototoxicity but the likelihood of high UVR co-exposure, and therefore the risk of phototoxicity, has generally been deemed low (McDonald and Chapman, 2002; Barron, 2017). As high intensity UVR co-exposure is highly likely during any oil spill in coral reef environments (Nordborg et al., 2020), accounting for phototoxicity will be critical to ensure risk assessments do not underestimate the hazards posed by oil pollution to early coral life stages. Recent risk assessments associated with the Deep Water Horizon spill have also recognised the potential effects of phototoxicity on shallow ecosystems (<20 m depth) and proposed a blanket 10-fold reduction in toxicity thresholds as an interim solution (French-McCay et al., 2018). Further toxicity studies applying relevant UVR intensities are required to refine phototoxic oil toxicity thresholds and ensuring their appropriate application in risk management for coral reefs.

Data statement

Data is available via the Australian Institute of Marine Science data portal (AIMS, 2021) and the scripts used in statistical analysis and visualisation of results are available from GitHub (<https://github.com/MNordborg/Nordborg-et-al.-2021-Early-LS-HFO>).

Funding sources

Funding for the work presented here was provided by the Australian Institute of Marine Science (Australia), King Abdullah University of Science and Technology (Saudi Arabia), the Australian Government Research Training Program Fee Offset and the AIMS@JCU Scholarship program James Cook University (Australia).

CRediT authorship contribution statement

F. Mikaela Nordborg: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Diane L. Brinkman:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Gerard F. Ricardo:** Methodology, Formal analysis, Writing – review & editing. **Susana Agustí:** Conceptualization, Funding acquisition, Writing – review & editing. **Andrew P. Negri:** Conceptualization, Funding acquisition, Methodology, Validation, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors acknowledge the Traditional Owners of the land and sea country where this research was conducted and from which the parent

corals originated, the Wulgurukaba, Bindal and Woppaburra peoples. We pay our respect to their Elders, past, present and emerging, and acknowledge their continuing spiritual connection to their land and sea country. The authors also thank the staff of the National Sea Simulator at the Australian Institute of Marine Science Townsville, QLD, for their technical support and expertise; Florita Flores for her advice regarding the experimental assays; Dr Rebecca Fisher for her advice regarding the statistical analysis; Dr Carly Randall and her team for the collection of parent corals; Tristan Lever, Dr Katarina Damjanovic, Christopher Brunner, Christina Langley, Camille Steel, Edith Strecker, Laura Zeuthen, Roy Borg and Alicia Castle for their time and assistance in performing the experimental work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.146676>.

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EXHIBIT

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Transcriptomic Responses of Deep-Sea Corals Experimentally Exposed to Crude Oil and Dispersant

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OPEN ACCESS

Edited by:

Chiara Romano,
Center for Advanced Studies of
Blanes (CSIC), Spain

Reviewed by:

Rachel Michelle Wright,
Harvard Medical School,
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Michael S. Studivan,
University of Miami, United States

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Specialty section:

This article was submitted to
Deep-Sea Environments and Ecology,
a section of the journal
Frontiers in Marine Science

Received: 05 January 2021

Accepted: 09 March 2021

Published: 08 April 2021

Citation:

DeLeo DM, Glazier A, Herrera S,
Barkman A and Cordes EE (2021)
Transcriptomic Responses
of Deep-Sea Corals Experimentally
Exposed to Crude Oil and Dispersant.
Front. Mar. Sci. 8:649909.
doi: 10.3389/fmars.2021.649909

Resource extraction from the ocean comes with ecosystem-wide risks, including threats to its biota such as the habitat forming corals that support elevated biomass and biodiversity. Despite catastrophic incidents like the *Deepwater Horizon* oil spill (DWHOS) disaster that occurred in 2010, offshore oil and gas drilling continues to occur around the world. Previous work investigating the toxicity of oil and the chemical dispersant used in an attempt to mitigate the effects of the DWHOS revealed that the dispersant elicits a stronger, negative physiological response than oil alone. However, little is known regarding the specific ways in which these anthropogenic pollutants impact organisms at the cellular level. To investigate the impacts of each pollutant and their synergistic effects on corals, we analyzed the transcriptional responses of the deep-sea octocorals *Callogorgia delta* and *Paramuricea* type B3 following 12 h of exposure to oil, dispersant, and mixtures of oil and dispersant. Analyses revealed that the highest levels of significant differential gene expression were found among the treatments containing dispersant, which corresponds to the significant effects observed in physiological experiments. Functional analyses of annotated transcripts further suggest both species- and colony-specific responses to the exposures, likely due to underlying cellular and physiological differences. However, some commonalities were observed among the responses to chemical stress across treatments and species, including immune and cellular stress responses, altered energy metabolism, and oxidative stress, elucidating how corals respond to chemical pollutants. As resource extraction is an ongoing threat, this study demonstrates the importance of considering the varied and diverse responses of biota to anthropogenic disturbances and the implications of introducing chemicals into vulnerable ecosystems like those associated with deep-sea corals.

Keywords: gene expression, oil spill, Deepwater Horizon, octocorals, *Callogorgia*, *Paramuricea*, RNAseq, anthropogenic

INTRODUCTION

Marine ecosystems are increasingly threatened by resource extraction as oil and gas exploration expands offshore. Despite catastrophic incidents like the *Deepwater Horizon* oil spill (DWHOS) disaster which occurred in the Gulf of Mexico (GoM) in 2010, drilling continues in deep waters [>200 meters (m)] worldwide, with active ultra-deep (>1000 m) drilling advancing in the GoM (Fisher et al., 2014b; Cordes et al., 2016) and beyond. Drilling

activities can be detrimental to deep-sea communities in various ways including physical disturbances by mooring anchors and pipelines (Ulfsnes et al., 2013), exposure to toxic drill cuttings, muds, and fluids (Gray et al., 1990; Larsson et al., 2013), and accidental oil spills. In the United States alone there was an oil spill over 160,000 L on average every 1.75 years between 1971 and 2010 (Anderson et al., 2012). Oil and gas extraction in deeper waters also increases the likelihood of accidental spills (Muehlenbachs et al., 2013) like the DWHOS blowout of the Macondo well that occurred at approximately 1500 m depth. This was one of the worst environmental disasters in U.S. history, and the largest accidental oil spill world-wide, releasing approximately 5 million barrels of crude oil into the deep waters of the GoM (McNutt et al., 2012). During active well discharge, approximately 7 million liters of chemical dispersants (Corexit 9500A and 9527; Barron, 2012) were also applied in an attempt to mitigate the impacts of the oil release, both at the sea surface and at depth, without a comprehensive understanding of how these chemicals would affect deep-sea fauna.

Spill impacted deep-sea coral communities were initially found at a depth of 1370 m, approximately 11 km from the Macondo well at a site in the Mississippi Canyon (MC) 294 lease block (White et al., 2012). When first discovered, coral colonies were covered in a brown flocculent material (floc) containing Macondo well oil as well as dispersant constituents (White et al., 2014). Impacted coral species at this site, primarily the deep-sea octocoral *Paramuricea biscaya*, exhibited various phenotypic indicators of impact including abnormal skeletal (sclerite) development, excess mucous production, and tissue death (necrosis), as well as complete colony mortality (White et al., 2012). Corals showing signs of sublethal impacts exhibited subsequent declines in health (i.e., tissue and branch loss) and limited recovery potential (Hsing et al., 2013; Girard and Fisher, 2018). Additional impacted deep-sea coral communities were later identified based on their similarity in appearance to the impacted communities at the original site (Fisher et al., 2014a,b).

A recent study investigating the *in situ* transcriptional response of *P. biscaya* colonies collected in December 2010 at MC294, revealed further impacts at the gene expression level (DeLeo et al., 2018). These included elevated expression of genes associated with oxidative, immune, and metabolic stress responses, as well as wound repair mechanisms (i.e., hyper-melanization). Contrastingly, expression of the polycyclic aromatic hydrocarbon biomarker gene, cytochrome P450 (CYP) was depressed, which may indicate an inhibition of the corals' ability to process xenobiotics at the time of sampling. Further, the study revealed variability among the global expression patterns of the individual spill-impacted colonies (DeLeo et al., 2018).

Because *P. biscaya* occurs in the GoM between 1200 and 2600 m and rarely survives the trip to the surface, it is not amenable to controlled laboratory experiments aimed at testing the toxicity of crude oil and dispersants. A close relative of *P. biscaya*, *Paramuricea* type B3 (Doughty et al., 2014), is also found in the GoM at shallower depths between 800 and 1100 m. This species can survive careful collection and thus serves as the best available model to understand toxicity effects underlying the impacts observed in *P. biscaya*.

Laboratory toxicity tests with *P. type B3*, and with the more distantly related octocoral *Callogorgia delta*, revealed that exposure to dispersant or dispersant/crude oil mixtures is more toxic to these corals than is exposure to crude oil alone (DeLeo et al., 2016). This was particularly evident following exposure to the dissolved, water-accommodated fractions (WAFs) of the dispersants. Phenotypic responses observed in *P. type B3* were similar to those observed in *P. biscaya* at MC294, including excess mucous release, tissue damage, necrosis and mortality (DeLeo et al., 2016). The octocoral *C. delta* however exhibited less severe health declines than *P. type B3*, suggestive of taxon-specific responses.

The objective of this study was to investigate the transcriptional responses of the corals subjected to the laboratory toxicity tests performed by DeLeo et al. (2016) and compare them to the *in situ* transcriptional responses of *P. biscaya* (DeLeo et al., 2018). It is important to investigate the early transcriptional responses to oil, dispersant, and oil/dispersant mixtures in these corals, prior to the onset of visible damage and mortality in order to understand how deep-sea corals respond to, and potentially endure, acute pollution events from marine oil spills. The gene expression patterns of *P. type B3* and *C. delta* were examined after 12 h of exposure to (1) bulk oil and dispersant treatments (heterogeneous solutions, with dissolved and undissolved chemical components), and (2) treatments solely containing the dissolved, or WAF of oil and dispersant. As higher mortality was observed in the WAF exposures during the 96 h toxicity tests, we anticipated a greater degree of differential gene expression in response to the short-term WAF exposures, corresponding to more severely impacted biological processes. Expression profiles were analyzed to investigate the effect of treatment type – oil-only, dispersant-only, and oil/dispersant mixtures – on both coral species. We hypothesized that the corals would have a more pronounced genome-wide response following exposure to dispersant, relative to the crude oil, given the strong physiological response observed in the toxicity tests and the natural occurrence of crude oil in their habitat (Quattrini et al., 2013). Gene expression data were further explored to identify early onset genome-wide impacts underlying the health declines and mortality observed during the full 96 h toxicological assays described by DeLeo et al. (2016). These findings improve our understanding of how resource extraction impacts deep-sea biota, and will aid in the development of diagnostic biomarkers for future spill monitoring efforts.

MATERIALS AND METHODS

Sample Collection and Acclimation

All samples were collected from two sites in the GoM. *P. 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). *C. delta* was 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 et al., 2010). At each site, corals were haphazardly collected with the remotely operated vehicles (ROV) *Global Explorer MK3*

or *Hercules* for experimentation. For each species, five coral colonies were collected alive for the experimental exposures described in DeLeo et al. (2016). Three of the largest colonies were fragmented for use in this study. Samples were collected several meters apart from conspecific colonies to reduce the likelihood of sampling genetic clones. Corals were visually identified using live video from cameras attached to each ROV, before being collected with a manipulator arm and secured in an insulated biobox and/or sealable collection quivers. When possible, branches of large colonies were sampled to avoid whole-colony mortality. Collection of individual branches from large coral colonies produces little or no phenotypic response from the coral colony (EEC unpublished data). Additional coral specimens from the same sites, previously preserved *in situ* on the seafloor using collection quivers filled with RNALater, were used for RNA sequencing. This included fragments from two *P. type B3* (2011, ROV *Schilling UHD*) and four *C. delta* colonies (2010, ROV Jason II).

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

Sublethal Oil and Dispersant Exposures

Partial coral colonies ($n = 3$ genets per species, per exposure series; six genets total) were fragmented into nubbins and exposed to oil and dispersant solutions (Figure 1) prepared according to the protocols described in DeLeo et al. (2016). In brief, corals were exposed to treatments made using either (1) heterogeneous bulk oil, dispersant and oil-dispersant mixtures containing both dissolved and undissolved chemical components, hereafter referred to as the *Bulk-exposure series*, or (2) using only the dissolved or water-accommodated oil (and dispersant) fractions (WAF), referred to as the *WAF-exposure series*. In each set of exposures, fragments were placed in the following treatments: artificial seawater (ASW; control), oil-only, dispersant-only or an oil/dispersant mixture (to represent dispersed oil). ASW was chosen as the control for comparison to the oil and dispersant treatments as all mixtures (Bulk and WAF) were made using sterile ASW (made using Instant Ocean™ at the *in situ* salinity of 35 psu). Coral nubbins were exposed to sublethal concentrations of oil and dispersant for a duration of 12 h and preserved prior to the onset of visible tissue damage. For the *Bulk-exposure series* this corresponds to the “High” concentration of oil and dispersant (~25 ppm) described by DeLeo et al. (2016), and for the *WAF-exposure series* this corresponds to the “Low” concentration of oil (~50 µM) and dispersant (~35.3 mg/L). Nubbins in these concentrations were chosen for sequencing for several reasons: (1) there were clear phenotypic differences observed across treatments during the full 96 h exposures implying the mixtures elicited a physiological response, (2) the concentrations were deemed sublethal as severe stress responses and mortality were not induced across all treatment-types within

those 96 h, and (3) to minimize the differences between the target concentrations of the Bulk and WAF exposure series to facilitate comparisons. After the 12 h exposures, fragments were fixed in RNAlater to preserve changes in gene expression. Samples were then frozen and returned to the lab at Temple University for further processing.

Sample Sequencing and Processing

Total RNA was isolated using a Qiagen RNeasy Kit or a modified Trizol/Qiagen RNeasy protocol (described in Polato et al., 2010; Burge et al., 2013), from both the *in situ* preserved samples and the experimental nubbins sampled after 12 h of exposure. RNA concentrations were determined using a NanoDrop® ND-1000 and RNA integrity was evaluated with gel electrophoresis and an Agilent Bioanalyzer. Library preparations, including a poly-A selection step to target primarily eukaryotic (coral host) RNAs, were performed by the sequencing facilities on high quality (RIN values > 7) RNA samples (Illumina Truseq RNA library kit). *In situ* preserved specimens were sequenced on an Illumina HiSeq2000 at the University of Wisconsin-Madison Biotechnology Center (UWBC, Madison WI, United States) to acquire 150 base-pair (bp) paired-end reads for use in assembling *de novo* reference transcriptomes for downstream analyses. The experimental samples were further multiplexed and sequenced across Illumina HiSeq2500 rapid flow cells at the Fox Chase Cancer Center (FCCC, Philadelphia, PA, United States) sequencing facility to acquire 100 bp single-end reads for use in gene expression analyses, at approximately 20–30 million reads per sample.

Raw reads were quality checked using FastQC v0.11.5 (Andrews, 2010). This output was further used to find overrepresented sequences, which were queried against GenBank using Blastx (e -value: e^{-15}) to determine whether they represented bacterial contamination. Trimmomatic v0.36 (Bolger et al., 2014) was then used to filter for quality, with a Phred score threshold of 30, leading and trailing bases dropped if the score was below 3 (LEADING: 3 TRAILING: 3), and excluding reads shorter than 50 bp (MINLEN:50). Cutadapt v1.15 (Martin, 2011) was used to trim adaptors and cut overrepresented sequences that appeared to be bacterial contamination with an overlap of 10 and a minimum-length of 60.

Transcriptome Assemblies

The filtered and cut paired-end sequences were further processed with Rcorrector (Song and Florea, 2015) using the k-mer counter Jellyfish v2.2.6 (Marcais and Kingsford, 2012), to remove erroneous k-mers. Read pairs deemed unfixable were discarded. Microbial contaminants were further identified and removed using the standard database in Kraken v1.0 (Wood and Salzberg, 2014). Sequences not classified as bacterial, microbial, archaeal, or viral in Kraken were used to generate *de novo* reference assemblies with Trinity v2.4.0 (Grabherr et al., 2011). Transcriptome quality was assessed with Transrate v1.0.3 (Smith-Unna et al., 2016) and the Benchmarking Universal Single-Copy Orthologs (BUSCO v3.0.2) program (Simão et al., 2015; Zdobnov et al., 2017) using the metazoa dataset ($n = 978$

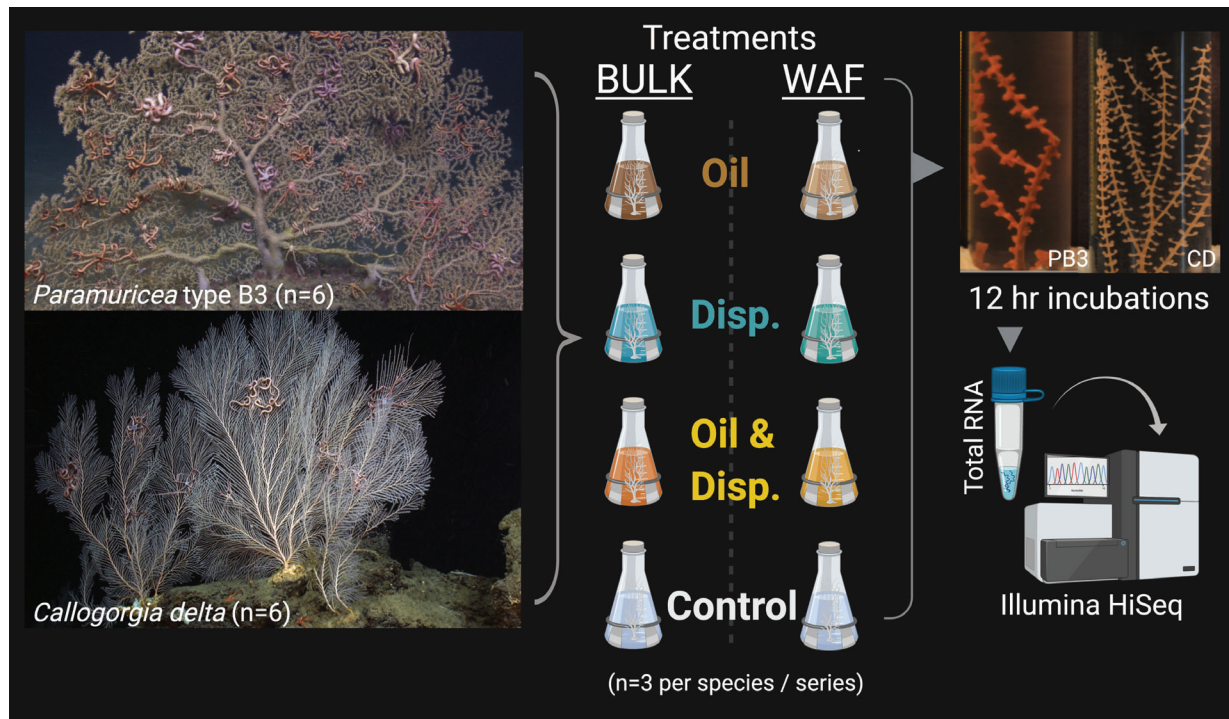


FIGURE 1 | Experimental setup for the deep-sea octocorals *Paramuricea* type B3 (PB3) and *Callogorgia delta* (CD) ($n = 6$ genetis total per species, 3 genetis per exposure series). Corals were exposed to both Bulk (heterogenous) and Water-Accommodated Fractions (WAF) of oil and/or dispersant (center). Coral fragments were maintained at sub-lethal concentrations for 12 h, as determined in DeLeo et al. (2016) (right). For the Bulk exposure series, oil and dispersant concentrations each correspond to ~ 25 ppm. For the WAF exposure series, oil concentrations correspond to ~ 50 μM and dispersant ~ 35.3 mg/L. Control coral fragments were also incubated in the artificial seawater used to make the aforementioned treatments of each exposure series. Fragments were preserved (RNAlater) after 12 h incubations in the treatments, for total RNA extraction and sequencing (1×100 bp). Created with BioRender.com.

orthologs). Assemblies were further annotated via dammit v1.0¹ using the corresponding P-FamA, RFam, OrthoDB, BUSCO, and Uniref90 databases (Scott, 2016). Gene ontology (GO) annotations were also generated using standard parameters (seed ortholog e -value: 0.01; seed ortholog score: 60; min% of query cov.: 20; min% of subject cov.: 0) in eggNOG (Huerta-Cepas et al., 2016) using Github support scripts².

Gene Expression Analyses

Processed reads from *P. type B3* and *C. delta* experimental samples were mapped to their respective reference transcriptomes using Salmon (Patro et al., 2017). The resulting count files were used as input for DESeq2 (Love et al., 2014), using R v3.6.2, to analyze the gene expression patterns of the corals in each exposure series (Bulk or WAF) and treatment. Expression was compared to the corresponding ASW control (of each species) using the tximport package (Soneson et al., 2015), which obtains gene-level expression by summing all transcript-level count estimates corresponding to a given gene. Transcripts were collapsed into genes for analysis at the gene level with a DESeq dataset design of colony + treatment, which was applied discretely to each exposure series. Transcripts were considered over-expressed if they had an adjusted p -value < 0.05

and a log-2 fold change (lfc) > 1 , and under-expressed if they had an adjusted p -value of < 0.05 and a $\text{lfc} < -1$. An adonis (permutational multivariate analysis of variance using distance matrices) test, implemented using the Vegan package in R, was used to analyze the influence of each factor (colony, treatment, and the interaction of colony and treatment) on the variation in gene expression (Oksanen et al., 2013). A Principal Coordinate Analysis (PCoA) was also carried out to visualize how the samples grouped in multivariate space, and whether treatment-type and/or colony genet could explain their distribution.

KEGG Functional Analyses

In order to further understand the influence of each treatment on higher-level functions and biological processes, differentially expressed genes from each treatment were assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper v3.1 tool (Kanehisa et al., 2016). This was done to decipher the molecular interaction networks associated with the genes (KEGG pathway mapping; *reconstruct*), based on all available KEGG Orthology (KO) assignments designated by eggNOG (Huerta-Cepas et al., 2016). These data were compared to investigate cellular processes and pathways that were impacted in both species in the Bulk and WAF exposures and to elucidate species-specific differences in the early (12 h) responses to pollutant introduction.

¹<https://dammit.readthedocs.io>

²<https://github.com/z0on/annotatingTranscriptomes>

In order to compare the initial responses of the experimentally exposed corals to corals impacted by the DWHOS *in situ*, direct comparisons were made between the closely related octocorals *P. type B3* and *P. biscaya*. This was done by comparing the KEGG pathway annotations for the genes differentially expressed in this study to the KO assignments for genes differentially expressed among *P. biscaya* exposed to DWHOS oil and dispersant laden floc *in situ* (DeLeo et al., 2018) via KEGG mapper *reconstruct*. While KEGG pathways are comprised of various components, each with unique KO identifiers, only the overlapping KO identifiers were reported as shared impacts observed for *Paramuricea* spp.

Gene Ontology Analysis

To examine significantly enriched GO categories among the over- and under-expressed genes that correspond to either the biological process, molecular function, or cellular component sub-ontologies, a rank-based gene ontology (GO) analysis with adaptive clustering was performed using the Gene Ontology Mann–Whitney U (GO_MWU) program (Wright et al., 2015). This program utilizes Mann–Whitney U (MWU) tests and a global-ranked list of genes and their associated GO terms to identify enriched GO categories using a continuous measure of significance, $-\log(p\text{-value})$, calculated with DESeq2. Hierarchical clustering of GO categories was based on the number of shared genes (clusterCutHeight = 0.25). Discrete species-specific tests were carried out for the Bulk and WAF exposure series to analyze expression patterns related to all treatments (the factor “treatment” in the DESeq design matrix) as well as each treatment individually. GO terms significantly enriched among the over- ($p\text{-adj} < 0.05$, $\delta\text{rank} > 0$) or under-expressed ($p\text{-adj} < 0.05$, $\delta\text{rank} < 0$) transcripts were reported.

RESULTS

De novo Transcriptome Assemblies

The final *de novo* transcriptome assembly for *C. delta* contained 39,475 transcripts with a contig N50 of 1,467 bases (Table 1). Of the assembled contigs, 94.9% of metazoan orthologs (BUSCOs) were present, indicating a relatively complete reference assembly.

TABLE 1 | Summary statistics for the *de novo* transcriptome assemblies of *Callogorgia delta* and *Paramuricea type B3*.

		<i>C. delta</i>	<i>P. type B3</i>
Total transcripts		39,475	60,989
GC content		0.40	0.39
N50		1,467	1,451
BUSCO	% Complete	94.9	94.5
	% Singletons	85.1	86.0
	% Duplicates	9.8	8.5
	% Fragmented	1.1	1.9
	% Missing	4.00	3.60
Transrate	Score	0.20	0.29
	Optimal Score	0.20	0.32

The final reference transcriptome for *P. type B3* contained 60,989 transcripts with a N50 of 1,451 bases (Table 1). Among these contigs, 94.5% of metazoan BUSCOs were present. The TransRate scores were comparable to the optimal assembly scores, which considers only the “good” transcripts based on a learned cutoff value.

Gene Expression Analyses

The adonis analysis, conducted to investigate the influence of each factor – colony (or genet), treatment, and colony + treatment – on the variation in gene expression, was not significant. However, in both exposure series, “colony” explained the largest proportion of the variation in global gene expression patterns. Further, when analyzing global expression patterns using the PCoA, the samples grouped largely by colony (Figure 2).

Bulk Exposure Series

After 12 h in the Bulk exposure series, the greatest number of significant differentially expressed genes ($\text{adj. } p\text{-value} < 0.05$, $\text{absolute } lfc > 1$) was observed among *P. type B3*, with a total of 140 genes differentially expressed relative to the 66 genes differentially expressed in *C. delta* (Table 2 and Supplementary Table 1). For both species, the highest differential expression response was observed in the Bulk dispersant-only treatment. *P. type B3* had 2.9–34 times as many differentially expressed transcripts in the dispersant-only treatment, relative to the oil/dispersant mixture and oil-only treatments, respectively; *C. delta* had 4.8–6.0 times as many differentially expressed transcripts in the respective treatments. See Supplemental Information for more detailed results on gene expression and functional analyses.

Among the differentially expressed genes in both octocorals, 2 putative genes (annotated as *cartilage matrix protein-like* and *uncharacterized protein*) were shared between *C. delta* and *P. type B3* exposed to the Bulk-dispersant treatment (Supplementary Table 1). No putative genes with annotations were shared among the two species in the Bulk-oil or oil/dispersant mixture treatments.

WAF Exposure Series

After 12 h in the WAF exposure series, the greatest number of significant differentially expressed genes ($\text{adj. } p\text{-value} < 0.05$, $\text{absolute } lfc > 1$) was observed among *C. delta* with a total of 1,681 genes differentially expressed relative to the 133 genes differentially expressed in *P. type B3* (Table 2 and Supplementary Table 2). For *C. delta*, the highest differential expression response was observed in the dispersant-only treatment, though this number was comparable across treatments with at most 1.1 times as many differentially expressed transcripts relative to the oil/dispersant mixture and oil-only treatments. For *P. type B3*, the highest differential expression response was observed in the WAF oil/dispersant mixture treatment, with 1.2–36 times as many differentially expressed transcripts relative to the dispersant-only and oil-only treatments.

Among the differentially expressed genes, one putative gene (annotated as *lactoperoxidase-like*), was shared between *C. delta*

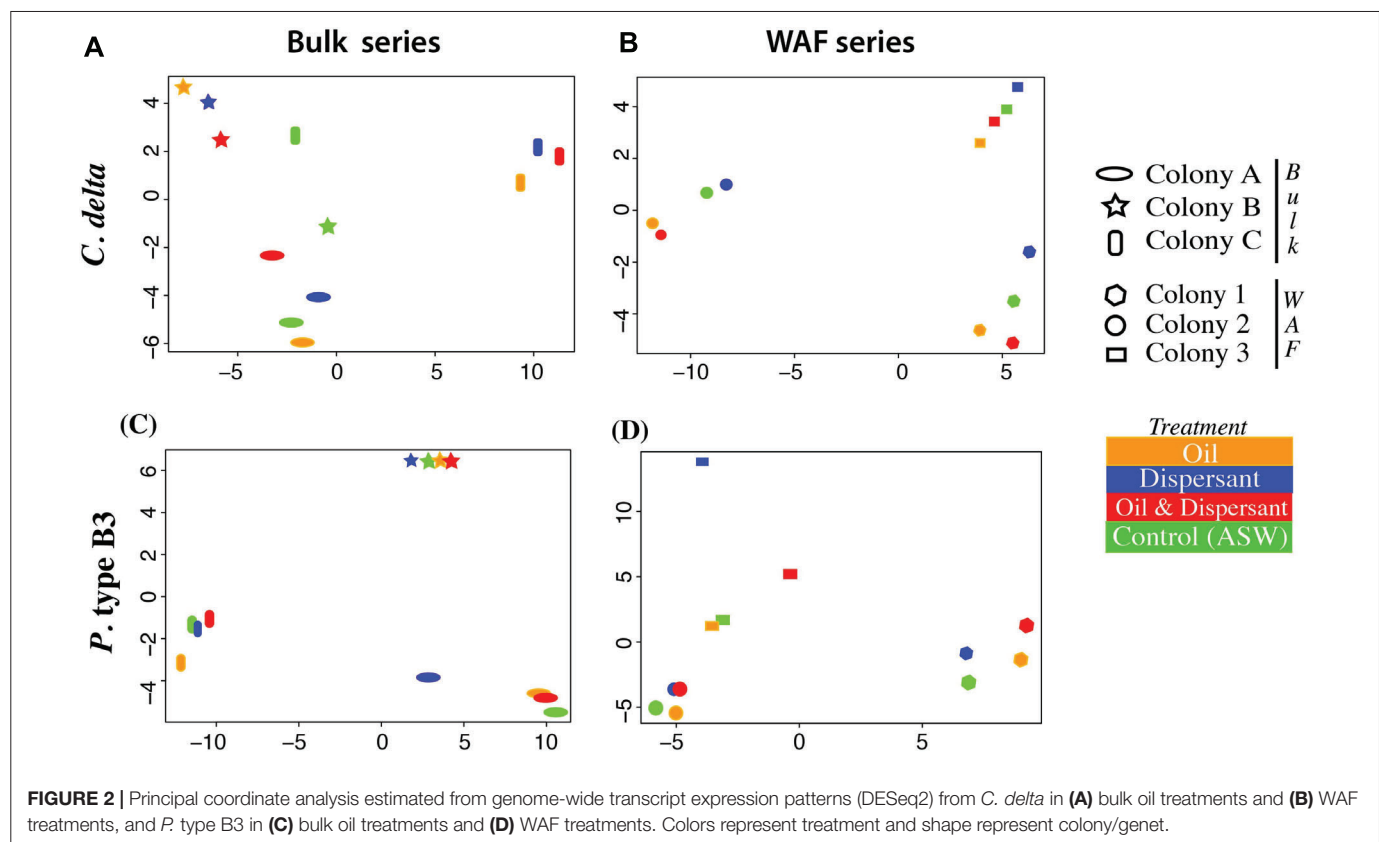


TABLE 2 | Summary of the differentially expressed genes in *C. delta* and *P. type B3* by treatment – oil, dispersant, and oil and dispersant mixtures- relative to control expression (seawater) in both the WAF (dissolved) and Bulk (heterogenous dissolved/undissolved) exposure series.

		Under-expressed	Over-expressed	Fold-change
<i>C. delta- bulk</i>				
Total transcripts with non-zero read counts: 26,306	Oil	0	8	1.2 – 3.1
	Dispersant	20	28	-4.0 – 3.4
	Oil/dispersant	4	6	-7.3 – 4.0
<i>C. delta- WAF</i>				
Total transcripts with non-zero read counts: 25,164	Oil	372	151	-4.1 – 3.1
	Dispersant	196	384	-4.0 – 8.6
	Oil/dispersant	295	283	-3.6 – 4.9
<i>P. type B3- bulk</i>				
Total transcripts with non-zero read counts: 26,850	Oil	1	2	-1.6 – 1.7
	Dispersant	19	83	-1.4 – 2.5
	Oil/dispersant	5	30	-5.5 – 2.9
<i>P. type B3- WAF</i>				
Total transcripts with non-zero read counts: 26,883	Oil	2	0	-5.2 – -4.7
	Dispersant	10	49	-4.7 – 7.1
	Oil/dispersant	52	20	-4.3 – 4.0

Genes were considered significant at an adjusted p -value ≤ 0.05 , under-expressed at a log-2-fold change ≤ -1 , and over-expressed at a log-2-fold change ≥ 1 .

and *P. type B3* exposed to the WAF-oil treatment, with an additional 14 putative genes shared among the two species in the WAF oil/dispersant mixture treatment, including but not limited to: a Slit homolog 1 protein involved in negative chemotaxis, a metalloendopeptidase involved in wound repair pathways, the growth factor bone morphogenetic protein 6 (BMP6) involved

in skeletogenesis and oogenesis, the interferon-induced protein (IFI35) involved in innate immune responses, and the tumor suppressor RNA-binding protein (RBM47) that responds to DNA damage (**Supplementary Table 2**). No putative genes were shared among the WAF dispersant-only treatment after 12 h of exposure.

TABLE 3 | Summary of the KEGG pathway analyses for *Paramuricea* type B3 (PB) and *Callogorgia delta* (Cd) in the Bulk oil and dispersant exposure series.

KO term	BULK exposures					
	Oil		Disp.		Oil/Disp.	
	PB	Cd	PB	Cd	PB	Cd
Metabolism						
<i>Lipid metabolism</i>						
00100 Steroid biosynthesis						
00561 Glycerolipid metabolism						
00564 Glycerophospholipid metabolism						
<i>Xenobiotics biodegradation and metabolism</i>						
00983 Drug metabolism – other enzymes						
Environmental Information Processing						
<i>Signal transduction</i>						
04391 Hippo signaling pathway – fly						
04151 PI3K-Akt signaling pathway			*	*		
<i>Signaling molecules and interaction</i>						
04512 ECM-receptor interaction			*	*		
Cellular Processes						
<i>Cellular community – eukaryotes</i>						
04510 Focal adhesion			*	*	*	
Organismal Systems						
<i>Immune system</i>						
04610 Complement and coagulation cascades			*	*	*	
<i>Digestive system</i>						
04974 Protein digestion and absorption			*	*	*	
04972 Pancreatic secretion						
04975 Fat digestion and absorption						
<i>Nervous system</i>						
04725 Cholinergic synapse						

Processes that are associated with under-expressed (blue) and over-expressed (red) transcripts are highlighted for each species and treatment.

The asterisk (*) denotes transcripts that correspond to the same gene in both species. KEGG analyses were based on KO annotations via eggNOG. Reference **Supplementary Table 3** for more detailed information.

KEGG and GO Functional Analyses

Bulk Exposure Series

KEGG pathway maps revealed that the genes differentially expressed among both coral species in the Bulk dispersant-only treatment were associated with: the immune (complement and coagulation cascades) and digestive (protein digestion and absorption) systems, environmental information processing (intracellular signaling and ECM-receptor interactions), and cellular processes (focal adhesion) (**Table 3** and **Supplementary Table 3**). A response was also elicited in these pathways among *P. type B3* fragments in the bulk oil/dispersant treatment, in addition to several metabolic pathways (e.g., xenobiotic biodegradation and metabolism). There were no KEGG assignments associated with the genes differentially expressed in the Bulk oil-only treatment or for *C. delta* exposed to oil/dispersant.

There were 104 enriched GO terms shared among both octocorals exposed to the Bulk dispersant treatment (**Figure 3**

and **Supplementary Table 4**). Among the shared GO terms were “innate immune responses,” “response to tumor necrosis factor (TNF),” and “regulation of immune effector process,” further revealing elevated immune responses among *C. delta* and *P. type B3* fragments exposed to dispersant. *C. delta* exposed to the oil and oil/dispersant treatments had additional enrichment of unique immunity-related GO terms including “positive regulation of immune system process” and “negative regulation of wound healing” (**Supplementary Table 4**). Shared GO enrichment analyses revealed additional impacts to cellular processes including, but not limited to, elevated “response to oxidative stress” and “regulation of apoptotic signaling pathway” and diminished “DNA repair.” There were no GO terms shared among *C. delta* and *P. type B3* in the Bulk oil-only and oil/dispersant treatments. See **Supplementary Table 4** for additional details on GO enrichment and for details on the differences between species.

WAF Exposure Series

KEGG pathway maps indicated that the gene differentially expressed among both coral species in the WAF oil-only exposures was associated with metabolism (peroxidase) (**Table 4** and **Supplementary Table 5**). Common impacts were also observed in the WAF oil/dispersant treatment, which were further associated with genetic information processing (ribosome), environmental information processing (signal transduction), cellular processes (cell growth and death), and organismal systems (development and regeneration).

Among *C. delta* nubbins in the WAF exposure series, several KEGG pathways were similarly impacted by all three treatments, potentially indicative of a generalized, early (~12 h) response to anthropogenic pollutants. These pathways fell into four main categories: metabolism (carbohydrate metabolism), environmental information processing (signal transduction), cellular processes (cell growth/death and focal adhesion), and organismal systems (including the immune, endocrine and nervous systems, and environmental adaptation) (**Table 4** and **Supplementary Table 5**). KEGG analyses further revealed common impacts to crucial cellular stress response pathways (e.g., NF-kappa B, TNF, and chemokine signaling pathways) among the octocorals exposed to oil (alone or dispersed). Additional sub-lethal impacts were observed among corals exposed to chemical dispersant components (alone or with oil) including elevated calcium signaling and apoptotic processes. See **Supplementary Table 3** for full details on the KEGG analyses and **Supplementary Information** for detailed comparisons between treatments.

There were 10 GO terms shared among *C. delta* and *P. type B3* in the WAF oil treatment, 64 terms shared in the dispersant-only treatment and 435 terms shared among the oil/dispersant mixtures (**Figure 4**). GO terms shared among the corals in treatments containing dispersant (alone or with oil) include “pigment cell differentiation,” “regulation of cytokine production,” “mucosal immune response,” and “regulation of inflammatory response,” which further revealed impairments to immune responses after

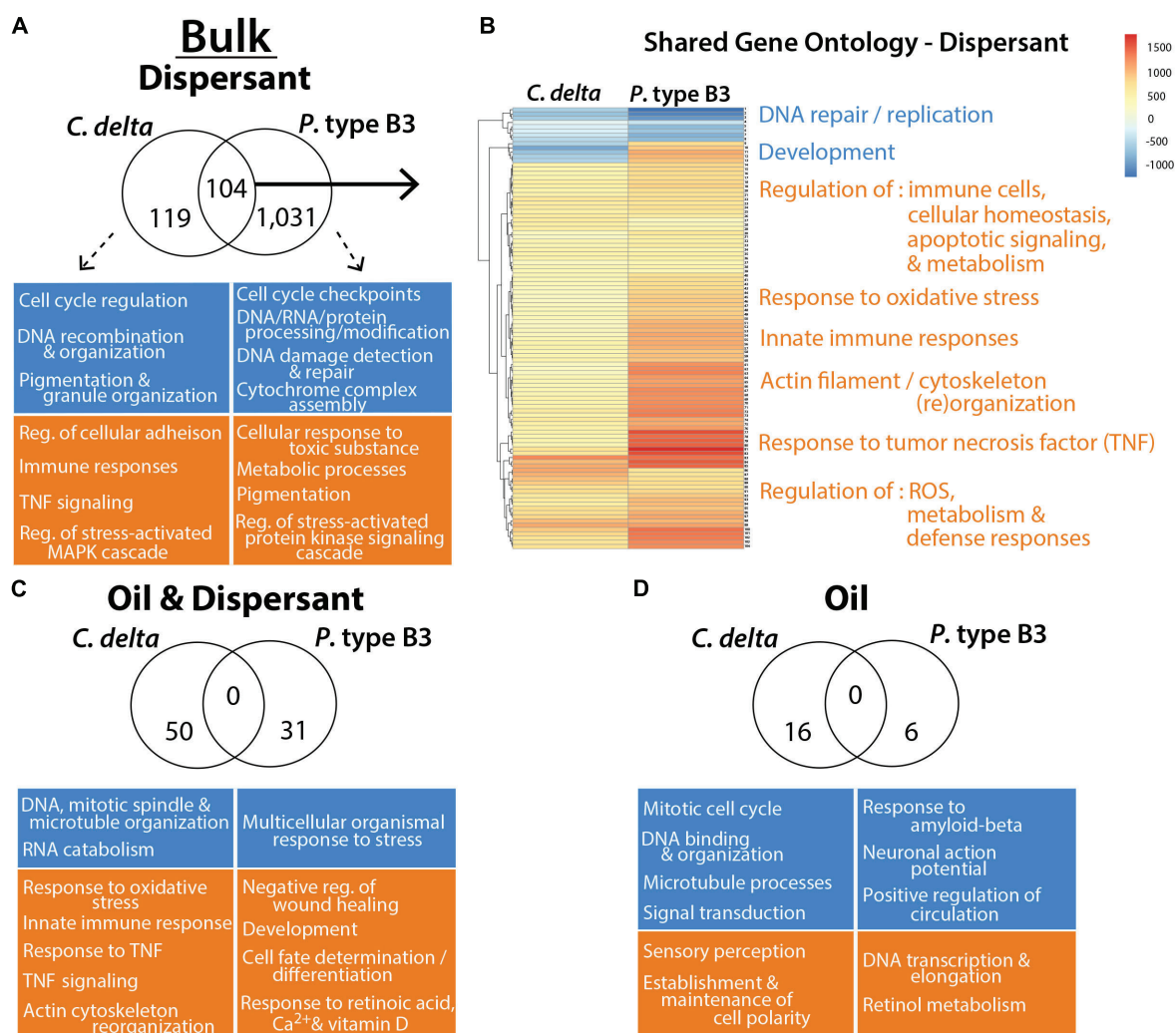


FIGURE 3 | Gene Ontology (GO) terms significantly enriched for over- (orange) or under- (blue) expressed transcripts from *C. delta* and *P. type B3* experimentally exposed to bulk oil and dispersant. Highlighted GO terms represent a snapshot summary of some of the impacted processes/functions either unique to (A,C,D) or shared (B) among species in relation to the dispersant (A,B), oil and dispersant mixture (C) or oil-only (D) treatments. The Venn diagrams represent the total number of shared and unique GO terms for each treatment, with representative GO terms of interest, unique to each species, highlighted in the boxes underneath. (B) The heatmap of the shared GO terms from the dispersant exposure illustrates the corresponding level of expression for each term (row) and compares expression between each coral species (column). Processes (orange/blue) are summarized to highlight impacted biological pathways. More details on GO enrichment can be found in **Supplementary Table 4**, including row-specific heatmap terms (B).

the 12 h exposures. GO enrichment analyses uncovered additional impacts to cellular processes following dispersant exposure including, but not limited to, elevated “ribosomal biogenesis” and “DNA repair/modifications” (Figure 4 and Supplementary Table 4).

Shared Impacts: DWHOS and Experimental

KEGG pathway analyses revealed that both *in situ* impacted *P. biscaya* and *P. type B3*, exposed to the Bulk treatments containing dispersant (alone or dispersed), exhibited similar impacts to environmental information processing pathways, specifically genes associated with signal transduction (i.e., Hippo and PI3K signaling pathways) and ECM-receptor interactions. Common

impacts were also observed among pathways involved in cellular processes including focal adhesion and protein digestion and absorption.

Similar impacts were likewise observed among DWHOS impacted *P. biscaya* and *P. type B3* experimentally exposed to WAFs containing dispersants. This includes impacts to metabolism, translation, protein folding and degradation, environmental information processing pathways (i.e., ABC membrane transporters) and various signal transduction pathways. Common impacts were also observed among pathways involved in crucial cellular processes including endocytosis and actin cytoskeleton regulation, as well as the digestive system (digestive secretions). See **Supplementary Table 6** for more detailed information.

TABLE 4 | Snapshot summary of the KEGG pathway analyses for *Paramuricea* type B3 (PB) and *Callogorgia delta* (Cd) in the WAF oil and dispersant exposure series.

KO term	WAF exposures					
	Oil		Disp.		Oil/Disp.	
	PB	Cd	PB	Cd	PB	Cd
Metabolism						
<i>Metabolic pathways</i>						
01000 Enzymes – Peroxidase PXDN	*	*				
<i>Carbohydrate metabolism</i>						
00010 Glycolysis/Gluconeogenesis						
00620 Pyruvate metabolism						
00630 Glyoxylate and dicarboxylate metabolism		+		+		+
<i>Amino acid metabolism</i>						
00260 Glycine, serine, and threonine metabolism						
00270 Cysteine and methionine metabolism						
00380 Tryptophan metabolism						
00480 Glutathione metabolism						
<i>Xenobiotics biodegradation and metabolism</i>						
00980 Metabolism of xenobiotics by cytochrome P450						
Genetic information processing						
<i>Transcription</i>						
03040 Spliceosome						
<i>Translation</i>						
03010 Ribosome					*	*
03015 mRNA surveillance pathway						
03008 Ribosome biogenesis in eukaryotes						
Environmental information processing						
<i>Signal transduction</i>						
04010 MAPK signaling pathway						
04310 Wnt signaling pathway		-		-		-
04350 TGF-beta signaling pathway		-		-		-
04064 NF-kappa B signaling pathway						
04668 TNF signaling pathway						
04020 Calcium signaling pathway						
<i>Signaling molecules and interaction</i>						
04080 Neuroactive ligand-receptor interaction						
04512 ECM-receptor interaction						
Cellular processes						
<i>Transport and catabolism</i>						
04144 Endocytosis						
04145 Phagosome						
04142 Lysosome						
04140 Autophagy – animal						
04137 Mitophagy – animal					*	*
<i>Cell growth and death</i>						
04110 Cell cycle		+		+		+
04210 Apoptosis					*	*
04216 Ferroptosis						
04217 Necroptosis						
04218 Cellular senescence						
<i>Cellular community – eukaryotes</i>						
04510 Focal adhesion		-		-		-
04520 Adherens junction						
04530 Tight junction						
04540 Gap junction		+		+		+

(Continued)

TABLE 4 | Continued

KO term	WAF exposures					
	Oil		Disp.		Oil/Disp.	
	PB	Cd	PB	Cd	PB	Cd
04550 Sig. pathways reg. pluripotency of stem cells						
<i>Cell motility</i>						
04810 Regulation of actin cytoskeleton						
Organismal Systems						
<i>Immune system</i>						
04610 Complement and coagulation cascades						
04620 Toll-like receptor signaling pathway						
04625 C-type lectin receptor signaling pathway						
04062 Chemokine signaling pathway						
<i>Endocrine system</i>						
04910 Insulin signaling pathway						
04920 Adipocytokine signaling pathway						
04921 Oxytocin signaling pathway						
04928 Parathyroid hormone synthesis/secretion	–			–		–
04916 Melanogenesis	–			–		–
<i>Digestive system</i>						
04970 Salivary secretion						
04974 Protein digestion and absorption						
04975 Fat digestion and absorption						
<i>Nervous system</i>						
04725 Cholinergic synapse	–			–		–
04730 Long-term depression	–			–		–
04722 Neurotrophin signaling pathway	–			–		–
<i>Sensory system</i>						
04742 Taste transduction						
04750 Inflammatory mediator reg. of TRP channels						
<i>Development and regeneration</i>						
04361 Axon regeneration					*	*
04380 Osteoclast differentiation						
<i>Aging</i>						
04211 Longevity regulating pathway	–			–		–
<i>Environmental adaptation</i>						
04713 Circadian entrainment	–			–		–
04714 Thermogenesis	+			+		+

Processes that are associated with under-expressed (blue) and over-expressed (red) transcripts are highlighted for each species and treatment. Processes that were either under-expressed (–) or over-expressed (+) in all three treatments (oil, dispersant, and oil/dispersant mixtures) in a given species are further noted.

The asterisk (*) denotes transcripts that correspond to the same gene in both species. KEGG analyses were based on KO annotations via eggNOG. Reference **Supplementary Tables 3, 5** for more details and impacted pathways.

DISCUSSION

This study provides novel transcriptomic data for two species of deep-sea octocorals, including a close relative of *P. biscaya* – the most severely impacted deep-sea species in the aftermath of the DWHOS (Deepwater Horizon Natural Resource Damage Assessment, 2016). Similar to *in situ* impacted *P. biscaya* (DeLeo et al., 2018), these results highlight the variability among coral responses to anthropogenic pollutant exposure, both inter- and intra-specific, and the utility of expression-level investigations of deep-sea fauna to elucidate sub-lethal impacts. These data add to

the sparse amount of high-throughput sequencing data available for octocorals and deep-sea species, and provide added resources for studying and monitoring coral and deep-sea ecosystems in the face of future anthropogenic impacts and global ocean change.

Past transcriptomic investigations of *P. biscaya* revealed genome-wide impacts following *in situ* exposure to oil and dispersant laden flocc (DeLeo et al., 2018). However, the full extent and duration of the exposure to oil and dispersant during the spill is unknown. There were multiple conceivable routes of exposure as oil and chemical dispersants were released at depth (~1500 m; Hazen et al., 2010) and ultimately persisted

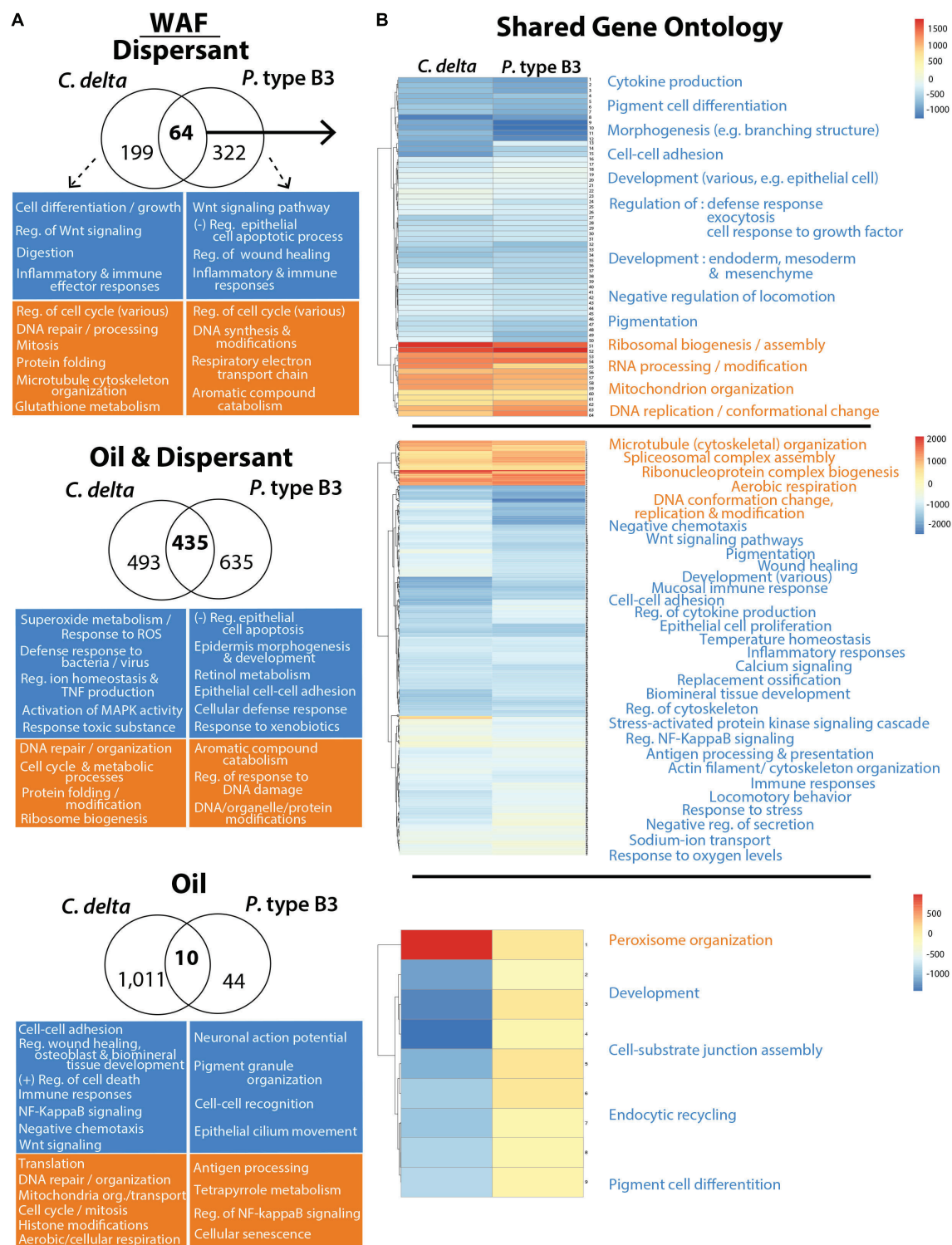


FIGURE 4 | Gene Ontology (GO) terms significantly enriched for over- (orange) or under- (blue) expressed transcripts from *C. delta* and *P. type B3* experimentally exposed to WAF oil and dispersant. Highlighted GO terms represent a snapshot of some of the impacted processes/functions either unique to (A) or shared (B) among species in relation to the dispersant (top), oil and dispersant mixture (middle), or oil-only (bottom) treatments. The Venn diagrams (A) represent the total number of shared and unique GO terms for each species in the different treatments, with representative GO terms of interest, unique to each species, highlighted in the boxes underneath. (B) The heatmaps of the shared GO terms from each treatment illustrates the corresponding level of expression for each term (rows) and compares expression between each coral species (column). Processes (orange/blue) are summarized to highlight various impacted biological pathways. More details on GO enrichment can be found in **Supplementary Table 4**.

as a deep-water oil plume around ~1,100 m (Camilli et al., 2010). These alternative exposure routes may have elicited early onset stress responses following initial pollutant contact, which may not have been detectable among flocc-exposed *P. biscaya* at the time of sampling. The transcriptional investigations of spill-impacted *P. biscaya* colonies by DeLeo et al. (2018) were also limited in terms of biological replication and temporal sampling due to restrictions on site access and sampling in the aftermath of the DWHOS. The experimental transcriptomic profiles presented here elucidate these early onset responses of deep-sea octocorals to oil and dispersants with increased replication, biological controls (genets), and interspecific comparisons. However, the authors note that limitations in obtaining and maintaining live deep-sea corals for laboratory experimentation at the time of sampling, as well as the partial colony sampling done to prevent adverse impacts *in situ* following the DWHOS, also limited the sample size, technical replication, and statistical robustness of this study. Regardless, these data show that the experimental oil and dispersant treatments that ultimately elicited mortality in these coral species during the full 96 h of exposure (DeLeo et al., 2016), particularly in the WAF treatments, also elicited large genome-wide expression changes of crucial stress response genes after just 12 h. The largest number of differentially expressed genes and the highest magnitude of gene expression change was found in treatments containing dispersants, relative to the treatments only containing naturally occurring crude oil. This study improves our understanding of how different species of corals respond to, and cope with, environmental contaminant exposure in the short-term, as well as the variability in these responses.

Common Responses to Pollutants

While gene expression profiles revealed the unique influences of different chemical fractions (Bulk/heterogeneous vs. WAF/dissolved) and treatments (oil-only, dispersant-only, and oil/dispersant mixtures) on the corals, they also revealed mutual responses elicited across various treatments in each exposure series.

Bulk Exposure Series

A common response observed among the bulk treatments, which contain both dissolved and undissolved oil and dispersant chemical constituents, involved altered energy metabolism and oxidative stress responses, both of which were observed among DWHOS impacted *P. biscaya* (DeLeo et al., 2018). Cytochrome c oxidase (CCO) activity, a key enzyme involved in aerobic metabolism (Simon and Robin., 1971) was diminished (*lfc* -1.6, *adj. p-value* 0.02) in *C. delta* exposed to treatments containing dispersant, alone or mixed with (dispersed) oil. As CCO catalyzes the terminal step in aerobic oxidative metabolism, CCO inhibition is suggestive of a hypoxic response among *C. delta* exposed to bulk dispersants. Likewise, CCO is a crucial enzyme regulating cellular energy production (Poyton et al., 1988), further suggesting impacts to tissue energy metabolism and cytotoxicity (Khan et al., 1990) following just 12 hrs of exposure. Conversely, CCO activity was significantly elevated among flocc-exposed *P. biscaya* (*lfc* 1.9, *adj. p-value* 0.005; DeLeo et al., 2018),

and among the liver/gills of fish following longer exposures (24 and 96 h) to WAF-oil, alone or dispersed (Cohen et al., 2001; Mattos et al., 2010). Elevated CCO activity among these animals may be associated with ongoing oxidative stress responses during short-term exposures to oil WAFs, that could ultimately lead to the hypoxic signature observed in our study. It is possible that exposure to bulk oil and dispersant contaminants may cause a relatively rapid hypoxic response in *C. delta* as compared to exposure to dissolved WAF contaminants. Likewise, succinate-cytochrome c oxidoreductase activity (part of the succinate-oxidase complex of the respiratory chain system (Khan et al., 1990), was significantly elevated (*lfc* 1.1, *adj. p-value* 0.02) in *P. type B3*, in addition to genes associated with oxidative stress responses (GO terms). This suggests that the bulk dispersant exposures also induced oxidative stress among *P. type B3* after only 12 h, though to a different degree and possibly in a species-dependent manner.

WAF Exposure Series

The strongest genome-wide response was observed among both species exposed to WAFs, particularly WAF treatments containing dispersants. This suggests the coral species tested here are particularly sensitive to the WAFs of anthropogenic pollutants. The magnitude and severity of this response to WAF treatments containing dispersants was mirrored by the higher rates of health decline observed during the full 96 h exposure series (see DeLeo et al., 2016 for more details). These results support the growing body of evidence that chemical dispersants elicit strong, negative responses from marine invertebrates and can be more harmful than oil alone, as dispersants increase the surface area of oil-water interactions- potentially increasing toxicological impacts and bioavailability (e.g., Chandrasekar et al., 2006; Goodbody-Gringley et al., 2013; DeLeo et al., 2016). However, within the scope of this study, it is also probable that this response was inflated by concentration differences between Bulk and WAF solutions.

Dispersants are also known to impair cell membrane function (Abel, 1974; National Research Council, 1989). Therefore, strong early onset responses to dispersants at the genomic-level were not surprising, as exposure was shown to result in the increased permeability of biological membranes and loss of total membrane function and/or osmoregulating abilities (Benoit et al., 1987; Partearroyo et al., 1990). As genes associated with DNA repair, protein modification and apoptosis (programmed cell death) (Kultz, 2003; Kultz, 2005), were significantly elevated in WAF treatments containing dispersants, this further suggests that even short-term exposures can impact cellular integrity, leading to lasting impacts.

Common genome-wide responses were also observed among WAF treatments containing oil (alone or dispersed). These included an elevated expression of ribosomal proteins which was likewise observed among *in situ* impacted *P. biscaya* (DeLeo et al., 2018), and among shallow-water octocorals following periods of short-term pathogen stress (Burge et al., 2013). It has been suggested that the elevated expression of ribosomal gene products is a generalized response to environmental stressors

(e.g., Burge et al., 2013; DeLeo et al., 2018) and that ribosomal proteins play an important role in regulating metabolism in invertebrates during periods of stress (Travers et al., 2010). It is possible that the elevated expression of these genes following the sub-lethal exposures to oil and dispersant was an attempt to regain cellular homeostasis following stress induced DNA and/or protein damage (Kultz, 2003).

Various structural molecules and ECM components were also inhibited among the corals experimentally exposed to WAF oil treatments. These include fibrillins that confer structural integrity to tissues (Piha-Gossack et al., 2012) and are thought to play a role in stress tolerance, albeit in plants (Singh and McNellis, 2011). Therefore, it is possible that the dissolved, water-accommodated oil and dispersant constituents may both compromise coral tissue integrity to a certain degree.

Additional putative genes involved in wound repair and inflammatory responses (i.e., metalloproteinases and peroxidases; Massova et al., 1998), were inhibited among WAF treatments containing oil (alone or dispersed oil) as well, further suggestive of acute cytotoxicity and cellular damage (i.e., Sterchi et al., 2008). This may be linked to the tissue degradation and health decline observed during the 96 h experimental exposures (DeLeo et al., 2016). Conversely, genes coding for structural/ECM proteins and inflammatory/wound repair pathways (i.e., peroxidase) were significantly elevated in *P. biscaya* exposed to DWHOS floc *in situ* (DeLeo et al., 2018). It is probable that natural hydrodynamic flow *in situ* alters and dampens exposure impacts and enables sessile invertebrates, like corals, to withstand longer exposures and/or combat these stressors at the cellular level. It is also possible that the expression signatures observed in this study reflect the prioritization (elevated expression) of other homeostatic processes as an initial defense response against chemical stressors, which in turn reduced the energetic resources allocated to maintain processes such as tissue integrity and wound-repair in the short-term. The latter scenario is likely the case, as some of the genes under-expressed here (e.g., fibrillins and peroxidase) were over-expressed in the black coral *Leiopathes glaberrima* exposed to identical experimental treatments for 24 h (Ruiz-Ramos et al., 2017). While the functional role of peroxidase is not fully understood among invertebrates, it is believed to contribute to innate immune defenses (Gotenstein et al., 2010), responses to oxidative stress, and programmed cell death (Horikoshi et al., 1999). Based on our results, it is possible that some, if not all, of these processes are initially suppressed during early responses to anthropogenic pollutants to allocate resources to other homeostatic processes.

Many GO terms related to cellular adhesion were under-expressed in all treatments (i.e., cell-cell, cell-matrix, and cell-substrate adhesion). Cell adhesion molecules are functionally diverse and are integral in a wide array of cellular processes, such as cell-cell signaling, multicellular tissue development (Gumbiner, 1996), and immune responses (Johansson, 1999; Harjunpää et al., 2019). Reduced expression of cellular adhesion molecules could therefore have wide-reaching implications, and possible associations with impairments to immunity and delayed wound healing (Harjunpää et al., 2019), though

more rigorous investigation is needed to confidently draw these conclusions. Differential expression of genes associated with cellular adhesion were likewise detected among corals following temperature stress (Gates et al., 1992; Traylor-Knowles, 2019) and disease (Daniels et al., 2015; Young et al., 2020). Therefore, cellular adhesion processes appear to be commonly impacted by environmental stressors and could be used in future assessments to gain a snapshot of overall coral health/condition.

Species-Specific Differences

Our data suggest that the octocorals had distinct responses to the Bulk and WAF oil exposures after 12 h, as there was no major overlap among the differentially expressed genes that were annotated- though this may be linked to the limitations of our experimental design. It is possible that there was a delayed or altered reaction to the pollutants in one of the species. From a physiological standpoint, both *C. delta* and *P. type B3* did surprisingly well in both oil-only treatments during the full 96 h exposure series as compared to the treatments containing dispersants (DeLeo et al., 2016). Metabolic depression was primarily observed among the corals exposed to oil in this present study, including genes associated with xenobiotic biodegradation and metabolic pathways (i.e., ABC transporters and GST). Therefore it seems unlikely that these corals were metabolizing oil in the short term as was suggested for *L. glaberrima* (Ruiz-Ramos et al., 2017).

Cytochrome p450 (CYP), which functions in the biotransformation and detoxification of most xenobiotics (Goldstone et al., 2006), was also not significantly differentially expressed among the corals after 12 h. CYPs are required for the efficient elimination of foreign chemicals from the body (Goldstone et al., 2006) and have become a widely used biomarker for pollutant (Devaux et al., 1998; Porte et al., 2001) and oil exposure (Garcia et al., 2012; Zhang et al., 2012; Han et al., 2014). CYPs were significantly elevated in *L. glaberrima* experimentally exposed to oil for 24 h (Ruiz-Ramos et al., 2017) but CYPs, along with ABC transporters and GST, were depressed among *P. biscaya* exposed to oil and dispersant *in situ* (DeLeo et al., 2018), albeit for an unknown duration. Although CYPs were not significantly expressed here, components of this same functional pathway – xenobiotic metabolism by cytochrome P450 – were significantly suppressed in *C. delta* exposed to WAF oil and elevated in *C. delta* exposed to WAF dispersant. This suggests that this pathway is impacted after just 12 h of pollutant contact, but that these impacts are dynamic and may differ depending on the species and/or the duration of exposure.

C. delta and *P. type B3* experimentally exposed to WAF treatments, particularly those containing dispersants, each had a distinct overlap in gene expression with *in situ* impacted *P. biscaya*. Interestingly, this overlap appears to correlate with the treatments that elicited the strongest phenotypic response in the 96 h exposure series (see DeLeo et al., 2016). This is particularly apparent for *C. delta*, which had somewhat less severe rates of health decline in response to longer exposures to these treatments (DeLeo et al., 2016) and the strongest genome-wide response to the WAFs (after 12 h). It is possible

that this is linked to “frontloading” or constitutive expression of genes that respond to oil exposure and/or their increased capacity for gene expression plasticity of environmental stress response (ESR) genes. For shallow-water corals, it has been hypothesized that one or both approaches may impart a certain degree of resilience to environmental stressors (i.e., Barshis et al., 2013; Kenkel and Matz, 2016). As *C. delta* preferentially occupies habitats near natural hydrocarbon seeps (Quattrini et al., 2013), they have likely adapted to low levels of hydrocarbon exposure potentially by employing one of these approaches.

In the case of *C. delta*, plastic expression may be linked to “chemical defense” genes, evolutionarily conserved genes and proteins that are involved in numerous processes including the metabolism and biotransformation of xenobiotic toxins [i.e., polycyclic aromatic hydrocarbons (PAHs), components of oil], antioxidant systems that respond to oxidative stress, as well as protein homeostasis (Reitzel et al., 2008; Lushchak, 2011; Tarrant et al., 2014). Many of the GO terms that were unique to *C. delta* compared to *P. type B3* exposed to oil were associated with cellular stress responses, including the regulation of stress-activated MAPK and protein kinase signaling cascades, and multicellular organismal response to stress, potentially signifying an increased capacity to respond to the stresses of oil exposure. As *C. delta* exhibited somewhat less severe rates of health decline in response to longer exposures to oil (DeLeo et al., 2016), it is possible this species is more resilient to short-term pollutant exposure.

The heightened cellular level response observed for *C. delta*, in terms of the number of genes that were differentially expressed, suggests that short-term dispersant exposure can induce cell damage in as little as 12 h. Expression differences were observed for genes involved in necroptosis (i.e., toll-like receptors), a form of necrosis or inflammatory cell death (reviewed in Dhuriya and Sharma, 2018), and C-type lectin signaling cascades that induce the production of inflammatory cytokines and chemokines (reviewed in Tang et al., 2018). Tumor necrosis factor receptor-associated factors (TRAFs), immune system receptors that elicit immune and inflammatory (e.g., necrosis) responses (Palmer and Traylor-Knowles, 2012), were also significantly differentially expressed. TRAFs were over-expressed in the WAF dispersant-only treatment after 12 h but under-expressed among *C. delta* in the WAF oil/dispersant mixtures, as well as *P. type B3* in WAF-oil. TRAFs were similarly over-expressed in *P. biscaya* exposed to oil and dispersant laden floc (DeLeo et al., 2018), indicating that over-expression may be linked to the dispersant constituents. These results suggest that even short periods of dispersant exposure may influence coral immune pathways and that immune/inflammatory pathway components make good candidates for future spill monitoring efforts.

Unlike *C. delta*, which exhibited a greater number of differentially expressed genes (and a larger average magnitude of gene expression change) when exposed to WAF treatments relative to Bulk treatments, *P. type B3* had a comparable number of differentially expressed genes, albeit a higher magnitude of expression change in the WAF exposures. It is possible that this is linked to a reduced capacity for gene expression plasticity

of important ESR genes among *P. type B3* relative to *C. delta*. Studies of shallow-water corals exposed to thermal stress revealed a higher degree of thermal tolerance and reduced bleaching impacts among populations of corals that exhibited a greater capacity for gene expression plasticity related to changes in environment/condition (Kenkel and Matz, 2016). It is possible that similar interspecific differences in plasticity also exist among deep-sea octocoral species. This would suggest that *C. delta* may be better able to mitigate intracellular damage resulting from anthropogenic stressors, and that *P. type B3* is equally vulnerable to both undissolved (Bulk) and water-accommodated (WAF) components of oil and dispersant, though further investigation is needed to elucidate these differences.

Functional enrichment analyses for *P. type B3* exposed to WAFs revealed that GO terms associated with Wnt signaling were significantly enriched among the under-expressed transcripts. Wnt signaling pathways are integral cascades associated with a myriad of cellular functions including development and cell-cell interactions. Down-regulation of Wnt pathways has also been detected in shallow-water corals in response to high temperature (Polato et al., 2013; Maor-Landaw et al., 2014) and low pH/high temperature conditions (Kaniewska et al., 2015). These signaling pathways could therefore be highly susceptible to environmental stress in corals and warrant further investigation.

CONCLUSION

As offshore drilling continues to increase worldwide, the need to understand species and ecosystem-level impacts has become paramount. Prior to the DWHOS there was no research into how chemical dispersants would react at depth and how prolonged exposure would impact deep-sea biota. As corals are important ecosystem engineers, creating complex structures that provide habitat to diverse suites of organisms, impacts to these species will have significant consequences. This and other studies provide evidence to suggest prolonged dispersant exposure is more harmful than oil exposure alone. Our findings indicate coral fauna have variable responses to anthropogenic environmental stressors, and some species and/or genotypes may be more resilient to environmental stress. These results further highlight the possible impacts of both dissolved and undissolved anthropogenic contaminants to corals, including metabolic and hypoxic consequences and impacts to immune and wound healing processes. Exposure to bulk/heterogenous contaminants appears to cause heightened oxidative stress and metabolic responses, while exposures to dissolved/WAF contaminants appears to elicit stronger impacts to tissue/skeletal development, immune responses and cellular damage/DNA repair. Similar processes were impacted among corals damaged *in situ* by the DWHOS following prolonged floc exposure among other probable routes of contaminant exposure (i.e., deep-water oil plume), highlighting both the early probable inception of this damage and the complex responses of corals to oil and dispersant constituents. This improved understanding of the ways in which oil and dispersant exposure affects corals is integral to improving

our ability to properly manage these ecosystems and respond to future exposure from standard operations and accidental releases.

DATA AVAILABILITY STATEMENT

The RNAseq data presented in this study can be found on NCBI's SRA database [BioProject ID: PRJNA708307].

AUTHOR CONTRIBUTIONS

DD and EC conceived and designed the experiments. DD performed the experiments and lab work with assistance from AB. DD and AG analyzed the data. SH consulted on bioinformatic analyses. DD wrote the manuscript with input from all co-authors. All the authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by a grant awarded to EC from The Gulf of Mexico Research Initiative to support the “Ecosystem Impacts of Oil and Gas in the Gulf” (ECOGIG) research consortium.

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Additional funding for coral collections was provided by (1) the Bureau of Ocean Energy Management (BOEM) and the National Oceanic and Atmospheric Administration (NOAA) contract to EC (M08PC20038) through the “Lophelia II” project administered by TDI Brooks, Inc. and (2) a NSF RAPID grant award to EC (OCE-1045079).

ACKNOWLEDGMENTS

Special thanks to the crews aboard the R/V Falkor and E/V Nautilus and both the ROV Global Explorer and ROV Hercules operators for their assistance with sample collections. Particular thanks to A. Quattrini for assistance with sample collections, J. Lunden for guidance with RNA work, and C. McNicholl, D. Ruiz-Ramos, I. Baums, A. Demopoulos, C. Fisher, and the Cordes lab for support at sea.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.649909/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EXHIBIT

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Chapter 25

Evaluating Impacts of Deep Oil Spills on Oceanic Marine Mammals



Kaitlin E. Frasier

Abstract The *Deepwater Horizon* (DWH) oil spill may be indicative of future large, deep spills that may occur in the coming decades. Given that future deepwater spills are possible, critical considerations include (1) establishing baselines for oceanic marine mammal and populations in at-risk areas, (2) understanding the implications of response choices for oceanic marine mammals, (3) designing studies with adequate coverage for post-spill monitoring, and (4) identifying effective strategies for oceanic marine mammal restoration. In this chapter, we consider these four stages in the context of a series of hypothetical oil spill scenarios, identifying ways that lessons learned from the DWH oil spill and prior events can be applied to future disasters.

Keywords Marine mammal · Sperm whale · Beaked whale · Dolphin · Passive acoustic monitoring (PAM) · Megafauna · Mammal · Odontocete · Bryde's whale · Spotted dolphin · *Stenella* · *Kogia* · Echolocation · Visual survey · Ship strike · Noise · Air gun · HARP · Mississippi Canyon · Green Canyon · Risso's dolphin · Pilot whale · Tag · Aerial survey · Habitat model · Loop Current · AUV · Satellite · Genetic · Monitoring · Dispersant · Hazing · Deterrent · NRDA · Cetacean · Disturbance · NOAA · Stock · Restoration · Mexico · Seismic

25.1 Introduction

The *Deepwater Horizon* (DWH) event differed from previous spills in that it occurred in deep water at an offshore location (1525 m deep, 66 km from the nearest shoreline). As a result it affected offshore marine megafauna in oceanic (>200 m bottom depth) habitats where prior study and monitoring efforts were sparse and infrequent. To characterize the effect of the event on marine mammals, the focus

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turned to coastal impacts and tractable nearshore surrogate species (Trustees 2016), because it was determined to be “unrealistic” to quantify offshore impacts directly. However, bay, sound, and estuary (BSE) bottlenose dolphins (the oceanic marine mammal surrogate) are weak proxies for the diverse, wide-ranging, and deep-diving oceanic species affected by the event. The true impacts of the DWH event on oceanic marine mammals and their offshore habitats may never be fully quantified.

As oil extraction operations deepen and extend into increasingly inaccessible locations, the difficulties of measuring spill impacts will likely increase. Future deep spills may affect deep waters of the Northern Gulf of Mexico (GOM) and Northeastern Atlantic, as well as Arctic waters (Huntington 2009; Cordes et al. 2016). As in the case of the DWH spill, effects of these events on marine mammal populations will likely be challenging to observe. Nonetheless, the ability to characterize the nature and magnitude of the impacts of these events is necessary for response, damage assessment, and restoration activities.

We discuss preparation strategies for future spills in at-risk regions. Based on the lessons from the DWH event, we ask what measures could be taken before, during, and after an offshore spill to characterize oceanic marine mammal populations, incorporate potential effects on marine mammals as a consideration in disaster response decisions, quantitatively evaluate population-level impacts of oil spills, and support population recovery.

25.2 Before a Spill: Establishing Baselines

The lack of precise pre-spill estimates of GOM marine mammal distributions and abundances severely limited efforts to evaluate the impacts of the DWH spill on oceanic megafauna. Measuring baseline marine mammal population sizes and distributions in at-risk areas is clearly a critical part of preparing for future oil spills; however it is rare to have this type of data prior to an event (Bonebrake et al. 2010). Monitoring an area the size of the GOM is expensive and logistically challenging, particularly with the level of readiness required to quantify impacts at an unknown time and location. Furthermore, standard survey methods are unlikely to achieve adequately precise density and abundance estimates or provide the level of spatiotemporal resolution needed to quantify exposure (Taylor et al. 2007). Practical approaches for long-term monitoring of large oceanic marine ecosystems with enough spatiotemporal resolution to quantify impacts of future spills at unknown times and locations on marine mammals have not been demonstrated.

Marine mammals are wide-ranging and capable of transiting long distances over large time scales (e.g., Jochens et al. 2008); therefore any monitoring strategy must account for population mobility and migration within and beyond the study region. Many GOM marine mammal species appear to migrate or shift their distributions seasonally, while others appear to be year-round residents (Hildebrand et al. 2015; Frasier 2015). Tropical and subtropical species may seek different habitat conditions. In the GOM, transient Loop Current features including cold- and warm-core eddies,

as well as the loop itself, have a strong influence on regional oceanographic conditions and likely affect GOM marine mammal distributions (Davis et al. 2002). Interpreting these distributions is further complicated by the fact that surveys are typically limited to the US EEZ, which accounts for only 35% of the GOM ecosystem. Distinguishing between population declines and population shifts is challenging because it is unclear how some species move throughout the GOM.

A combination of in situ monitoring and modeling is likely the most realistic approach for establishing abundance and distribution baselines in large regions of concern. In situ monitoring data can be used to develop habitat models used to interpolate marine mammal distributions between measurements across space and time. In the case of a disaster, models can use observed historical relationships between seasonal and oceanographic drivers and marine mammal encounters (Redfern et al. 2006; Roberts et al. 2016) to estimate exposure at the event location. However, developing robust models requires extensive monitoring effort for species of concern across seasons, habitats, and regional oceanographic variability (Kaschner et al. 2012) and may require integration of multiple observation methods to achieve sufficient predictive power.

25.2.1 In Situ Monitoring Strategies

Visual Surveys

Shipboard line transect surveys with visual observers are the standard method for estimating baseline abundance and describing the distributions of oceanic cetaceans (Davis et al. 1998; Mullin and Hoggard 2000; Fulling et al. 2003; Mullin and Fulling 2003, 2004; Barlow and Forney 2007). This method relies on animal sightings at the sea surface. Visual surveys provide broad spatial coverage of a region at brief snapshots in time (roughly 0.5 hours/10 km transect segment). Some temporal coverage can be obtained if surveys are repeated on a regular schedule; however visual methods are resource intensive, requiring extensive vessel and personnel time; therefore they may not be conducted often enough to provide precise estimates. Visual surveys also rely on fair weather conditions; therefore in the GOM, most visual survey effort has occurred in summer months (Maze-Foley and Mullin 2007; Mullin 2007).

To provide adequate data for training habitat models with broad spatial and temporal predictive capabilities, visual survey methods must cover a large surface area, survey across a variety of oceanographic features, occur in multiple seasons, and develop species-specific sighting rate estimates (Buckland et al. 2007). Given that marine mammals only spend a fraction of their time at the sea surface, visual survey data tend to be sparse (~1 sighting per 50 km of NOAA shipboard pelagic visual survey effort in the GOM, 2002–2014), requiring extensive survey effort to produce robust models. Double-blind visual surveys with two independent visual teams are typically used to accurately estimate sighting probabilities for different species (Palka 2006), as each species has a different probability of seen by observers: The

tall blows of sperm whales or large groups of dolphins are more likely to be sighted than cryptic species such as beaked whales and *Kogia* species. Sighting rates are further influenced by survey platform height; therefore survey vessels are not interchangeable and must be calibrated (e.g., Palka 2006). Recent field studies have also found evidence of vessel avoidance by marine mammals (Cholewiak et al. 2017) which may lead to underestimates of marine mammal densities if not accounted for. In general, the low precision of abundance estimates from large-scale visual surveys prevents estimation of long-term population trends and precludes detection of all but the most catastrophic population-level impacts (Williams et al. 2011; Taylor et al. 2007).

Shipboard visual surveys for oceanic marine mammals were conducted in the GOM prior to the DWH spill (Waring et al. 2009), but due to the expense and limitations of the method, the population size estimates were too imprecise to allow damages to be quantified by comparison with post-spill survey results. Unless Gulf-wide surveys could be conducted frequently across a range of seasons, visual surveys alone would likely remain insufficient for determining the effects of a future spill (Jewell et al. 2012; Taylor et al. 2007). Aerial visual surveys in the GOM typically focus on the expansive continental shelf region (Fulling et al. 2003) and are not used to survey oceanic populations. Unmanned aerial vehicles (UAVs) may become a viable low-cost solution for coastal surveys (Bevan et al. 2016); however the current range and battery life limitations of commercial AUVs limit their use for pelagic monitoring.

Passive Acoustic Monitoring

Static passive acoustic monitoring (PAM) provides an alternative modality for cetacean monitoring; this approach employs acoustic sensors at fixed sites but can provide a nearly continuous record of animal presence at monitored locations (Wiggins and Hildebrand 2007) regardless of time of day or weather. This method relies on underwater detection of species-specific vocalizations; therefore monitored species must be classifiable based on features of their acoustic signals. Passive acoustic monitoring data have been collected in the GOM nearly continuously using fixed seafloor sensors since 2010 (Hildebrand et al. 2015; Hildebrand et al. 2019). The time series from acoustic monitoring sites provides high-resolution temporal coverage; however spatial coverage is limited because sensor locations are fixed, and detection ranges are restricted by the acoustic characteristics of the vocalizations monitored (Frasier et al. 2016; Hildebrand et al. 2015).

PAM tends to result in higher detection rates than visual surveys because they rely on sounds produced during foraging and social behaviors rather than surface sightings. This type of data can provide strong support for habitat modeling efforts (Soldevilla et al. 2011). Because the sensors are generally stationary, they must monitor over long periods (months to years) in order to capture the full range of environmental conditions and variability that visual surveys achieve in part by surveying along transect lines. Despite their stationarity, fixed PAM can monitor across a remarkably wide range of oceanographic conditions due to the dynamic nature of the marine environment (Fig. 25.1).

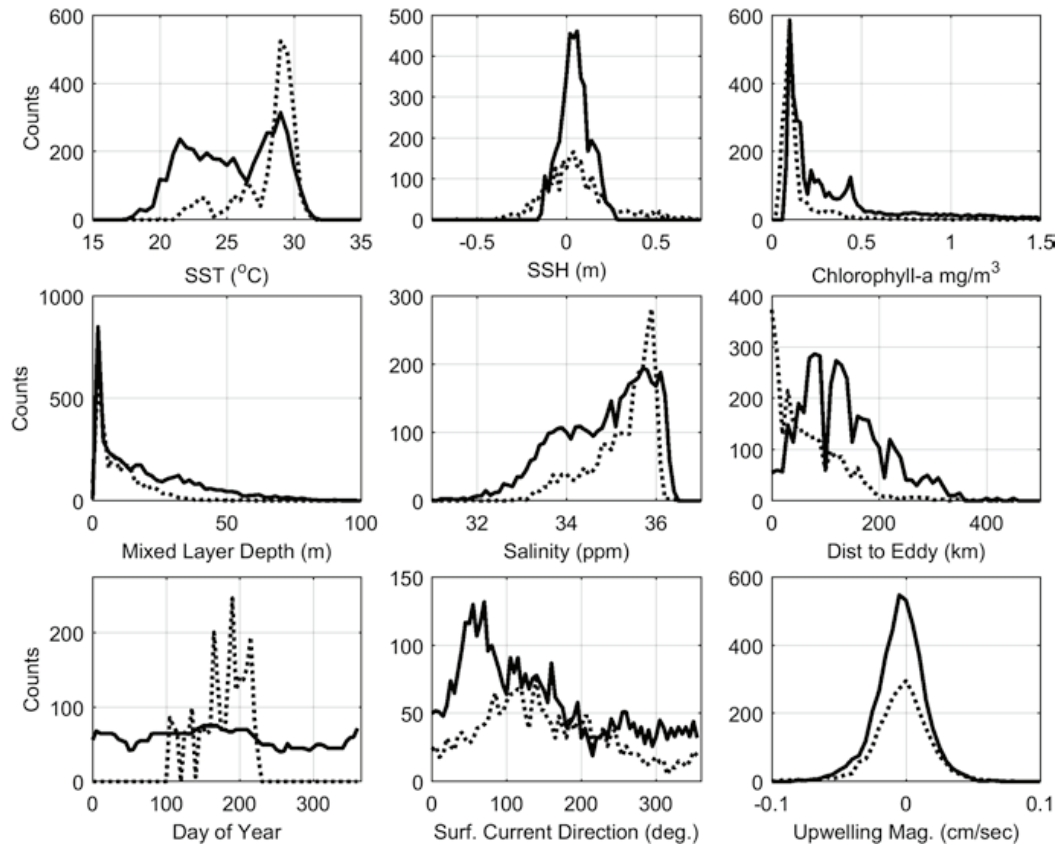


Fig. 25.1 A comparison of environmental parameter distributions observed at static PAM sites (black line) and those traversed by a NOAA visual survey vessel (dashed line). PAM data represents five static sites monitored continuously for 3 years as part of the GOM HARP project. Visual survey data represents five shipboard surveys conducted by NOAA in spring or summer of 2002, 2003, 2009, 2012, and 2014

Mobile passive acoustic sensors can combine some of the advantages of visual surveys (spatial coverage) with the autonomous advantage of PAM (Klinck et al. 2012; Moore et al. 2007; Mellinger et al. 2007). However these sensors come with their own set of challenges that may complicate their use for quantitative density and abundance estimation, including signal distortion and surface noise for near-surface sensor types, variable signal detection probabilities for profiling sensors, self-noise (e.g., electrical noise, onboard pumps, and flow noise), and limited navigational ability in regions with significant currents (Hildebrand et al. 2013). At the time of the DWH event, these systems were not a reliable option for large-scale monitoring, but mobile autonomous PAM may become a viable tool for future monitoring of at-risk regions.

Tagging

Marine mammal tag technology is a rapidly advancing field capable of providing insights to individual animal behaviors, home ranges, and migratory patterns (Jochens et al. 2008). However, insights gained individual animal tracks, and behaviors can be difficult to generalize to a population or species level and can

require significant effort for even a limited sample size. Various tag designs exist, each with different strengths. Long-term implanted tags typically have GPS sensors and remain affixed to the animal for many months (Mate et al. 2007). Most long-term tags are not designed to be recovered; therefore data collection is typically limited to what can be transmitted via satellite, such as location of surface intervals. Satellite tagging studies may be particularly useful for understanding the degree to which populations flow in and out of an area of concern, as in the GOM. For instance, tag data showing seasonal migrations could fill in knowledge gaps related to large-scale distribution shifts, seasonal patterns, and migratory corridors (Costa et al. 2010; Baird et al. 2010). Shorter-term tags designed to be retrieved once they separate from the animal can store more information, such as underwater movement (body rotation, foraging lunges, acoustic recordings, and video footage), but must be recovered to acquire the data (Johnson et al. 2009; Calambokidis et al. 2007). Short-term archival tags are useful for obtaining information on behavior (Soldevilla et al. 2017). Low Impact Minimally Percutaneous Electronic Transmitter (LIMPET) tags are medium-duration option capable of supporting a range of sensors (Baird et al. 2010). Suction cups are another common short-term mounting method, typically remaining attached for a few hours to a few days. Beaked whales and dolphins have been successfully tagged with suction cup and LIMPET tags; however tags are most readily applied to large whales.

Other Methods

Satellite imagery has been proposed as a possible tool for marine mammal population monitoring (Fretwell et al. 2014). This strategy may be a viable option for large whales under favorable conditions (low glare, low Beaufort scale); however the resolution of publicly available satellite imagery is currently too low to detect or identify most marine mammal species. Satellite imagery might be a viable option in the future for studying distributions of large whales (Fretwell et al. 2014), depending on image resolution and the development of methods to account for poor detection conditions and other factors influencing detectability.

Genetic studies can also provide estimates of population sizes (Frankham 1996) and identify possible periods of population expansion or contraction (De Bruyn et al. 2009). These methods use biological samples such as tissue or skin to look at genetic diversity and drift. Genetic approaches have been used to estimate past and current population sizes and to quantify the impacts of historic events such as whaling; however sources of uncertainty including mutation rates, reproductive success, and effects of selection at individual loci can lead to low precision in population size estimates when used in isolation (Harris and Allendorf 1989; Alter et al. 2007). This method is most appropriate for studies over longer time scales but may be used to evaluate long-term effects of historic events when earlier data are not available. Genetic information has been used to identify distinct bottlenose dolphin stocks in the GOM (Sellas et al. 2005), allowing impact assessments to be limited to affected stocks. Similar efforts to delineate stocks for oceanic species could help narrow the focus of future damage assessments.

25.2.2 Complementary Monitoring Data Sources

Given the size of the survey areas, and unknown locations of future disasters, it is unlikely that any of these individual monitoring methods alone can cover space and time well enough to produce the data needed for baseline population size estimates or provide the spatiotemporal resolution required for large-scale disaster preparedness. Habitat models capable of predicting marine mammal density distributions as a function of environmental drivers (Redfern et al. 2006) may provide a mechanism for estimating marine mammal exposure to future events. Habitat models have been developed for the GOM based on visual survey data following the DWH oil spill (Roberts et al. 2016) however they do not currently cover all seasons or achieve high enough confidence to fulfill future damage assessment needs. Since no individual method seems capable of fully censusing mobile and migratory populations, the best approach may involve integrating multiple data sources (Fujioka et al. 2014). In particular, visual surveys and passive acoustics may be able to accomplish the task in combination by leveraging the spatial coverage of one and the temporal coverage of the other.

25.3 During a Spill: Megafauna and Response Efforts

There is no evidence that marine mammals avoid oil (Goodale et al. 1981; Geraci 1990; Vander Zanden et al. 2016; Wilkin et al. 2017); therefore it must be assumed that animals present during an oil spill are injured by the event and that response choices including dispersant use, noise, and vessel activity directly affect marine mammals.

25.3.1 Response Activities

Dispersants

Chemical dispersants have been applied at the sea surface in oil spill responses as early as 1967 (Torrey Canyon spill response; Southward and Southward 1978). The DWH response represented the first use of dispersants at depth, where they were applied directly to the oil outflow (Kujawinski et al. 2011). The use of deep dispersants as part of the DWH response has largely been viewed as a success: Approximately 50% of the spilled oil remained at depth (Joye 2015), never reaching the sea surface where it would have increased slick size, required further cleanup actions, and potentially reached coastlines. Managers have indicated that they would use deep dispersant applications in future response efforts (French-McCay

et al. 2018). However, the trade-offs of deep and surface applications of dispersant approach with respect to implications for pelagic marine organism health are largely unknown. The application of dispersants at depth is thought to increase oil residence times in the water column and increase the influence of subsurface currents on oil transport (Testa et al. 2016). In Frasier et al. (2020) we reviewed the sparse literature on dispersant effects on marine mammal health, which relies on surrogate species and cell cultures. There appears to be little consensus on whether dispersants or dispersed oil are more or less toxic to marine organisms than undispersed oil. Dispersing oil in a deep subsurface plume likely increases routes of exposure for many oceanic marine mammals. Indirect impacts of deep dispersant applications via deposition of large amounts of oil on the seafloor are also a concern (Fisher et al. 2016). Deposited oil has the potential to smother benthic communities and negatively affect pelagic food webs with long-term implications for marine mammal populations.

Although the use of dispersants has been considered a success so far, there is not enough data to conclude that dispersant use results in safer conditions for marine mammal populations. In a future spill scenario, the presence and density of deep-diving marine mammals may need to be considered as a risk factor when weighing the trade-offs of applications of dispersants at depth.

Vessels

Vessel activity was very high in the Mississippi Canyon region during the DWH oil spill response and oil slick cleanup effort. Elevated ship noise, echosounders, and underwater communication signals associated with response activities dominated the acoustic soundscape during the response period. Noise associated with seismic surveys and shipping is generally high in the GOM; therefore distinguishing between the response-associated noise and chronic noise impacts may be challenging. Increased ship traffic raises the risk of marine mammals being struck by vessels (Carrillo and Ritter 2010). Anthropogenic noise has been associated with a wide range of injuries to marine mammal species, ranging from disruption of foraging to possible death (Cox et al. 2006; Tyack 2008). Cleanup activities such as skimming and burning increase the potential for entanglement in deployed gear and reduce air quality and the sea surface for air-breathing marine mammals.

Deterrents

Deterrence or “hazing” strategies aimed at discouraging marine mammal presence in oiled areas do not appear to have been used during the DWH oil spill response but have since been proposed as strategies for future events (NOAA 2014). These strategies use sounds from underwater discharges (“seal bombs”), Oikami pipes, or helicopters to herd or move animals out of affected areas and have the potential to reduce direct exposure during a spill. However, these methods constitute illegal harassment outside of an emergency; therefore they should be viewed with extreme caution and require specific authorization (NMFS 2017).

25.4 After a Spill

25.4.1 *Damage Assessment*

NOAA's natural resource damage assessment (NRDA) process is the primary framework for estimating impacts on marine megafauna following an oil spill. In the DWH case, the injury assessment phase of the NRDA spanned from 2010 to 2015 (Trustees 2016). However the effects on these long-lived species likely play out over a much longer period (Schwacke et al. 2017; Ackleh et al. 2018; Matkin et al. 2008); therefore the full magnitude of the impacts may not be immediately measurable during an NRDA. This leads to a mismatch between the time frame in which damages are assessed (a few years) and the time frame over which the damage may appear (possibly decades).

Models may be necessary to predict the extent of future damage within the time frame of the NRDA. Following the DWH an effort was made to develop life history models to estimate the magnitude of the impacts in terms of "lost cetacean years" (Schwacke et al. 2017) for BSE bottlenose dolphins. These models rely on knowledge of life history traits such as average life span, typical mortality across different age classes, reproductive rates etc., which are difficult to establish for oceanic species (King et al. 2015). Targeted studies to establish these parameters for populations of concern would likely facilitate future damage assessment estimates. Population recovery models may not fully account for cumulative impacts when estimating recovery times (Williams et al. 2016). Even if pre-spill data do exist, some marine mammal populations may not be at their stable or optimal size at the time of an event (e.g., recovering from a prior event or declining due to other impacts), causing models to incorrectly estimate the time to full population recovery. Following the DWH event coordinated efforts began to develop models capable of estimating population-level effects of chronic disturbance (Pirodda et al. 2018) which may be incorporated into future recovery estimation efforts.

As previously discussed, effective short-term damage assessment requires knowledge of the types and numbers of animals impacted by the disaster and a comparison of pre- and post-spill numbers to account for any loss. If habitat models (e.g., Roberts et al. 2016) exist for a region prior to a spill, these could be used to predict the magnitude of exposure based on the location, timing, and oceanographic conditions during the event (Grega et al. 2013). Surveying or monitoring during the event could be conducted to validate the model predictions. In the case of the DWH, the GOM HARP project (a long-term passive acoustic monitoring effort; Hildebrand et al. 2015) began monitoring 19 days after the beginning of the spill, allowing for high temporal resolution monitoring of the wellhead region. NOAA shipboard oceanic marine mammal visual surveys were conducted during June through August and October through November 2010 (SEFSC 2018). Although these were relatively rapid responses, the initial exposure period was not recorded; therefore some immediate effects may have been missed. Preparedness plans for rapid deployment

of monitoring tools following future oil spills could decrease the time lag between event and initial monitoring effort, which could in turn decrease uncertainty around short-term exposure.

25.4.2 Long-Term Monitoring

After a spill, long-term monitoring is necessary to establish trends and assess recovery progress at affected locations. Marine mammal presence varies on fine timescales as animals seek out prey and favorable conditions; follow mobile, ephemeral mesoscale features; and appear to respond to drivers ranging from lunar cycles to human activities (Davis et al. 2002; Simonis et al. 2017; Ellison et al. 2012). The mechanisms that drive oceanic marine mammal spatial distributions and variability are poorly understood, in part because many probable contributing factors such as prey availability and oceanographic conditions at depth are not readily measured on the broad spatial and temporal scales over which monitoring occurs. Indirect drivers such as sea surface conditions, primary productivity, and general ocean conditions, though widely available from satellite data and physical models, typically have only weak explanatory power with respect to oceanic marine mammal occurrence (Forney et al. 2012; Roberts et al. 2016). Unexplained variability in marine mammal distributions complicates interpretation of long-term trends from monitoring data, because short- and long-term population movements and true population size changes are convolved.

Targeted, carefully designed monitoring programs (Taylor et al. 2007; Jewell et al. 2012; Kaschner et al. 2012) are necessary to provide the spatial and temporal coverage required to achieve a level of precision high enough to confidently measure population-level changes on a reasonable time scale (years rather than decades or centuries). Considerations include coverage of the full range of habitats of interest, accounting for possible non-uniform species distributions across the monitored area, surveying across the full range of seasons, and taking measures to reduce inherent uncertainty in parameters such as animal availability for detection, method-specific probability of detection, avoidance or attraction effects, and multipliers used to convert detections into numbers of animals (e.g., cue rates, group size estimates) (Buckland et al. 2007). Although visual surveys are the most common oceanic marine mammal monitoring method, simply increasing the frequency of surveys may not result in more precise population estimates (Jewell et al. 2012). PAM is likely one of the more effective strategies for collecting enough data to resolve long-term trends despite short-term (weeks to months) and inter-annual variability at impacted sites. Where available, identification of impacted stocks can limit the spatial extent of survey effort needed (e.g., BSE bottlenose dolphin stocks in the GOM), but oceanic marine mammal stocks are typically large and poorly defined.

25.4.3 Restoration

Following the DWH oil spill, restoration of large, mobile marine megafauna appeared to be an intractable problem, given the scale of their habitat, the complexity, and length of their life cycles. Direct actions to increase marine mammal populations are not a viable option. However, there appears to be a growing consensus that indirect restoration actions aimed at mitigating the chronic impacts that weaken population resilience (Wright et al. 2011) may support population recovery and reduce harm from possible future events.

We suggest that a potential approach to restoration is addressing the chronic impacts that compromise marine mammal population resilience and reduce their ability to withstand and recover from disasters. Chronic impacts including sublethal stressors can have cumulative effects on survival, reducing reproduction rates, shortening life spans, and increasing sensitivity to disease or unfavorable environmental conditions (Wright et al. 2011). Chronic anthropogenic impacts to marine mammals in the GOM include noise, ship strikes, exposure to pollutants, entanglement, ingestion of debris, bycatch, and reduced prey quality and quantity (see Frasier et al. 2020). In the aftermath of an oil spill, restoration efforts could conceivably consist of identifying, quantifying, and mitigating these threats. For instance, if bycatch is considered a significant stressor in a region of concern, then a restoration strategy might include quantifying the extent of the bycatch issue across fisheries via an observer program, identifying high risk cases, and implementing mitigation strategies (equipment, regulation). Similarly if noise exposure was a concern, then areas of highest exposure could be identified by reviewing species distributions in relation to major shipping corridors and seismic surveys and taking actions to reduce vessel noise (via vessel quieting or speed limits) and move shipping lanes or timing seismic surveys to occur during windows of low expected densities in affected areas. Such efforts to reduce chronic impacts could increase population resilience and indirectly support recovery in the event of future oil spills.

25.5 Putting It into Practice: Alternate Spill Scenarios

Below, we step through three alternate oil spill scenarios to examine possible differences between the impacts of the hypothetical case and the DWH oil spill on oceanic marine mammals. Differences in species exposure are discussed, and implications of these differences for response and damage assessment processes are considered. Given the potential for GOM-wide population connectivity of oceanic marine mammal populations, coupled with how little is known regarding the processes that drive changes in GOM marine mammal densities and distributions, long-term monitoring needs would likely be comparable under all three scenarios, therefore recommendations are only detailed under Scenario 1. Restoration efforts would likely also be comparable; however, we highlight cases where certain species might benefit from targeted management actions.

25.5.1 Scenario 1

In this scenario, a hypothetical spill of similar origin, magnitude, and duration to the DWH event would have occurred during the fall of 2010 (beginning September 1), instead of during spring. Oil release from the well would have occurred over a 90-day period from September through November 2010.

25.5.1.1 Impacts and Damage Assessment

Based on seasonal trends observed in long-term monitoring data collected during the GOM HARP project, the expected presence of Risso's dolphins, mid-frequency (presumed *Stenella* species) dolphins, and *Kogia* spp. would have been lower in the fall scenario than during the spring (Fig. 25.2); therefore potential exposure of these species might have been lower. It is not known where these populations tend to go during winter months, only that occurrence appears to decrease at northern GOM HARP monitoring locations along the continental slope. Some populations may migrate into deeper waters or into the southern GOM during winter months. Sperm whale and low-frequency dolphin (presumed to be primarily short-finned pilot whale) presence are typically somewhat higher in the PAM record during fall at northern monitoring locations; therefore exposure might have been higher for these species. Gervais' beaked whale presence is not strongly seasonal at this site; therefore expected exposure under this scenario would be similar to the spring event. Cuvier's beaked whales are typically only detected in winter at this location; therefore expected exposure would be low but increasing at the very end of the oil spill period.

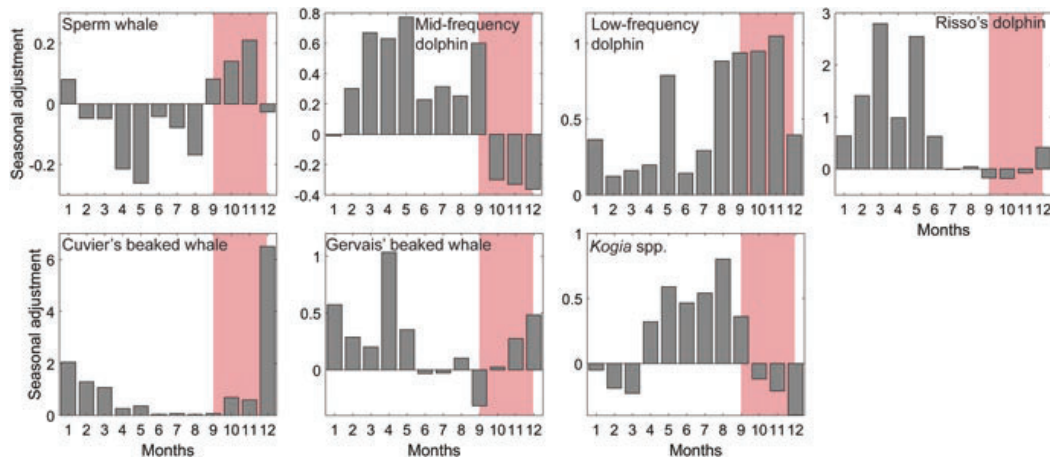


Fig. 25.2 Seasonal patterns in marine mammal presence at a passive acoustic monitoring site in Mississippi Canyon, located approximately 10 km from the DWH wellhead. The vertical axis indicates the factor by which seasonal presence varies relative to mean presence. Higher values indicate stronger seasonality. Pink shading indicates the months of the hypothetical oil spill examined in Scenario 1

Similarly to the DWH case, there would not have been enough survey data to estimate pre-spill population sizes or to develop models capable of estimating the magnitude of marine mammal exposure. Moreover, between 1992 and 2010, NOAA visual surveys were conducted no later than August; therefore there would have been no marine mammal observations for fall months in pre-spill data (Waring et al. 2009). *Stenella* dolphins, particularly pantropical spotted dolphins (*Stenella longirostris*), are the most abundant oceanic marine mammals in the northern GOM based on summer visual survey data (Waring et al. 2015). If *Stenella* dolphins do shift away from the Mississippi Canyon area in fall and winter as suggested by the PAM data, then the overall number of animals directly exposed to oil, dispersants, and response activities would have been significantly lower. The northern GOM appears to be a nursing ground for sperm whales (Jochens et al. 2008); therefore higher exposure might have had larger effects on the population as a whole. A focused effort on estimating sperm whale life history parameters to estimate lost sperm whale years would have been particularly useful under this scenario for quantifying impacts with potential long-lasting, population-level implications.

25.5.1.2 Long-Term Monitoring

Oceanic GOM marine mammal populations are typically classified by NOAA as single oceanic stocks, because no information exists to support more fine-scale structure. The degree to which populations flow between US waters in the north and Mexican waters in the south is unknown, but exchange between the northern and southern GOM is likely. A long-term monitoring strategy for oceanic GOM marine mammals likely needs to cover both US and Mexican waters, monitor year-round, and achieve high enough precision to detect impacts from large-scale events and/or restoration activities. A viable strategy involves the use of static PAM at a combination of permanent and temporary sites in the entire GOM. Temporary sites would be moved periodically across a grid of short-term (<1 year) monitoring locations to provide coverage of the full range of habitats and environmental conditions in the GOM, while long-term sites would be monitored continuously over many years as reference points. Using this type of dataset, habitat models could be produced to interpolate marine mammal density distributions across the entire region such that changes and effects could be evaluated on a gulf-wide scale. Further, impacts of future events could be inferred from modeled density surfaces. Model precision would be dictated by the number of sensors and monitoring locations occupied and the duration of the effort.

25.5.1.3 Restoration

Mississippi Canyon appears to be a hot spot of biological activity in the northern GOM; therefore restoration actions to support biodiversity might be particularly appropriate. The Mississippi River plays a dominant role in shaping offshore

northern GOM ecosystems, by bringing in nutrients that fuel high productivity. Although these nutrients contribute to the creation of a seasonal hypoxic zone on the continental shelf, they also likely form the foundation of the rich food web that appears to sustain high marine mammal presence in the Mississippi Canyon region. One set of management actions to support marine recovery in the region might include minimizing upstream contaminant inputs from agricultural activities. Nitrate from fertilizers is the most abundant and problematic (Rabalais et al. 1996) contaminant found in these riverine inputs, along with pesticides and herbicides (Goolsby and Pereira 1996; Pereira and Hostettler 1993). Recent research also indicates an increase in Mississippi River salinity (Kaushal et al. 2018) which could have impacts on the offshore food web. Pollutants are also derived from oil and gas extraction in the region (Neff 1990; Neff et al. 2011a, b). A second avenue for restoration would include limiting and tightly regulating the activity of increasingly deep drilling rigs which may increase the risks of impacts of future incidents on recovering populations.

25.5.2 Scenario 2

In this scenario the origin of the hypothetical spill would have been at a location along the west Florida shelf (27.0° N, 85.168° W) with oil escaping over a period of 90 days beginning April 20, 2010, and ending July 19, 2010.

25.5.2.1 Impacts and Damage Assessment

This scenario would likely have had greater impacts on beaked whales, which have been recorded at very high densities at a west Florida shelf site relative to other GOM monitoring locations (Hildebrand et al. 2015). However, the PAM site nearest this hypothetical spill location for the GOM HARP project was located further south along the west Florida shelf, and the degree of generalizability of beaked whale habitat preferences based on these observations remains unclear. A PAM study focused on the Mississippi Canyon region (Sidorovskaia et al. 2016) suggested that neighboring sites (50 nm apart) could have quite different beaked whale species compositions. Patchiness in beaked whale distributions may be related to their deep-dive capabilities which could enable them to interact with and take advantage of seafloor features which are not available to shallow-diving species. Applications of deep dispersants might have an outsized impact on beaked whales at this location by increasing oil deposition in their benthic foraging grounds. Beaked whales are also sensitive to anthropogenic noise (particularly echosounders) and might have been repelled or stranded in response to high-frequency anthropogenic noise (communications and echosounders) associated with the response (Weilgart 2007). Densities of Risso's dolphin are also higher in the region but tend to peak in the fall; therefore Risso's might avoid the majority of direct exposure under this scenario.

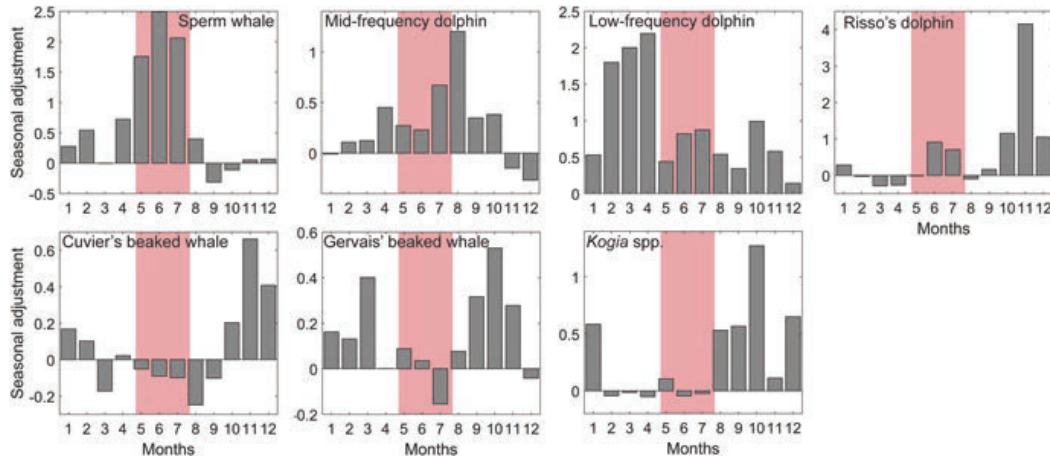


Fig. 25.3 Seasonal patterns in marine mammal presence at a passive acoustic monitoring site on the west Florida shelf. The vertical axis indicates the factor by which seasonal presence varies relative to mean presence. Higher values indicate stronger seasonality. Pink shading indicates the months of the hypothetical oil spill examined in Scenario 2

Direct impacts on female and juvenile GOM sperm whales might also have been reduced because overall sperm whale densities are likely to be lower in this region relative to Mississippi Canyon; however migratory males moving through the area in summer months might have been more strongly affected (Fig. 25.3).

One population of particular concern under this spill scenario is the very small GOM Bryde's whale population (proposed to be listed as endangered; NMFS 2015). GOM Bryde's whale core habitat is located just north of the origin of this hypothetical oil spill. This population appears to be an endemic GOM subspecies and consists of an estimated 33 animals (Hayes et al. 2018). Although the reasons behind its current small population size are largely unknown, Soldevilla et al. (2017) proposed based on a tagged animal that this species may be particularly vulnerable to ship strikes. The tagged individual showed a repetitive behavior of resting at night at the sea surface and foraging near or at the seafloor during the day. These two behaviors, if they are characteristic of the population (subsequent unpublished data suggests that they are), might put the species at high risk of exposure to surface and deposited oil, as well as increased risk being struck by response vessels (Soldevilla et al. 2017). Given the small size of this population, the potential impacts from an oil spill overlapping its core habitat could threaten the long-term survival of GOM Bryde's whales.

As in other scenarios, there would have been limited pre-spill visual survey data for this region of the GOM. Entrainment of oil into the Loop Current and possibly the Gulf Stream would open up the possibility of oil exposure to an even greater number of marine mammal species in the Western Atlantic where marine mammal diversity and densities are fairly high. Marine mammal survey effort in the NE Atlantic (US EEZ) has been more extensive than in the GOM, but habitat models were not available at the time. They have been published since using pre-spill data (Roberts et al. 2016) but lack certainty for many species. Given that beaked whales

might have been particularly affected under this scenario, the ability to quantify impacts to beaked whales in terms of “lost beaked whale years” would be an important tool in estimating the extent of the damage in this scenario. These are particularly cryptic, difficult animals to study; therefore it would be very difficult to establish accurate life history parameters for damage assessment purposes.

25.5.2.2 Restoration

Key restoration actions for Bryde’s whales would likely involve vessel restrictions in their core habitat. Restricting vessel speeds and prohibiting nighttime transits through the area would likely be an effective restoration strategy (Carrillo and Ritter 2010). Beaked whale-oriented restoration efforts might include taking action to minimize acoustic disturbance from echosounders, fish-finders, and sonar. Preliminary research suggests that male sperm whales may transit through this region, but it is unclear where they are coming from. Undertaking tagging efforts to better understand the connectivity between the apparently resident northern GOM population of sperm whales (primarily females and juveniles) with mature males observed in the broader Atlantic might help inform management actions to support recovery of this population. Sperm whales, particularly large males, were highly targeted by the whaling industry; therefore it cannot be assumed that the pre-spill GOM sperm whale numbers reflected a healthy or stable population size.

25.5.3 Scenario 3

In this scenario, an oil spill of similar magnitude, depth, and duration to the DWH event would have occurred in the northwestern GOM (26.66° N, 93.19° W), from April 20 to July 19, 2010.

25.5.3.1 Impacts and Damage Assessment

The oceanography of the northwestern GOM differs significantly from the eastern GOM. Instead of the clearly defined continental slope regions typical of the northeastern GOM, the seafloor in the northwestern GOM gradually deepens from 40 to 2000 meters deep over hundreds of kilometers across a complex network of salt domes and other geological features. Oil and gas infrastructure is more prevalent in the western half of the northern GOM (BOEM 2018), and the Port of Houston, one of the busiest ports in the USA, is associated with high vessel traffic through the region (BOEM 2015). Visual survey and PAM data indicate that overall marine mammal occurrence may be lower in this region, but marine mammal survey effort has also been lower.

The relative influence of differences in habitat, infrastructure, human activity, and marine mammal survey effort on perceived lower marine mammal occurrence

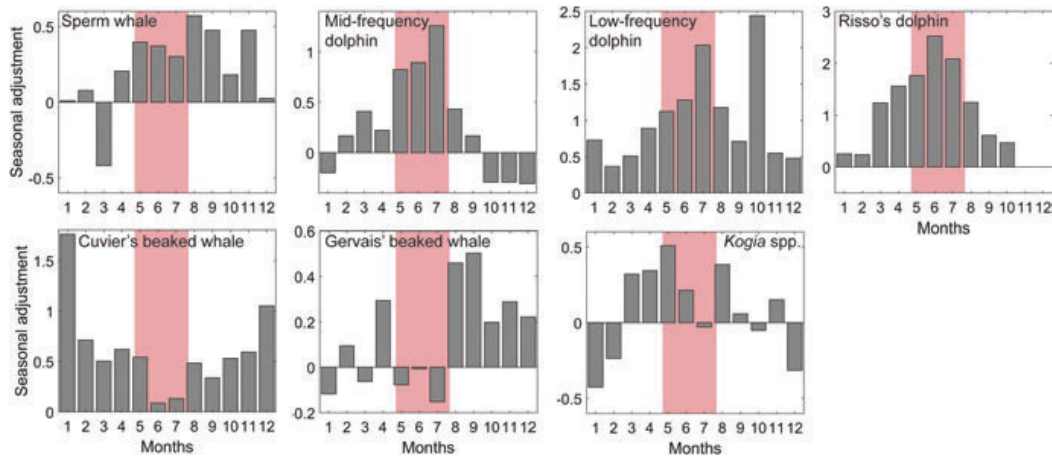


Fig. 25.4 Seasonal patterns in marine mammal presence at a passive acoustic monitoring site in Green Canyon, the western-most year-round monitoring location for toothed whales in the GOM. The vertical axis indicates the factor by which seasonal presence varies relative to mean presence. Higher values indicate stronger seasonality. Pink shading indicates the months of the hypothetical oil spill examined in Scenario 3

in the western GOM is unknown. The westernmost sensor deployed by the GOM HARP project was located near Green Canyon (27.56° N, 91.17° W); however this location was selected as an un-oiled comparison with Mississippi Canyon and does not necessarily represent average western GOM conditions. PAM data from that location indicated somewhat lower occurrence of marine mammals relative to the Mississippi Canyon site. Sperm whales, *Stenella* dolphins, pilot whales, *Kogia* spp., and Risso's dolphins would likely have been directly exposed to oil and response activities (Fig. 25.4). Insufficient monitoring data are available to estimate the extent of the potential exposure. Oil spills of various sizes are not uncommon in the western GOM; therefore it might be particularly difficult to distinguish the effect of one event from impacts from other sources.

25.5.3.2 Restoration

Given the comparatively high risk of future oil spills in the western GOM and the challenges of measuring new impacts in a highly exploited context, a proportional increase in marine mammal monitoring effort relative to the western GOM may be appropriate to establish robust baselines and fill in extensive knowledge gaps. However current marine mammal population sizes and distributions in the western GOM are unlikely to represent historic extents given the clear human footprint on the region. For example, data suggest that the GOM Bryde's whale population's home range, now restricted to the eastern GOM, may have previously extended into the western GOM (Soldevilla et al. 2017).

A particularly common source of disturbance in the western GOM are seismic surveys, in which explosive releases of air are used to produce high amplitude sound waves to map the seafloor and search for oil deposits. Noise generated by these

surveys dominates the low-frequency soundscape in the GOM. Research into the effects of seismic surveys on marine mammals is ongoing; however studies have reported a range of effects including no perceived response, decreased foraging, and displacement (Mate et al. 1994; Miller et al. 2009; Stone and Tasker 2006). Determining seasonal trends in marine mammal abundance and distributions in the GOM might reveal strategies for minimizing spatiotemporal overlap between seismic surveys and critical habitat. Measures taken to quiet container ships could also significantly reduce noise-related stressors on GOM marine mammals (Malakoff 2010).

25.6 Conclusions

Direct measurement of impacts will become more difficult as spills get deeper, further offshore, and in less accessible locations. Robust baselines are needed to measure impacts to oceanic megafauna. A multi-pronged approach to monitoring utilizing visual surveys and passive acoustic monitoring is likely the best method for quantifying injury to and measuring recovery of oceanic marine mammal populations. Marine mammal species are wide-ranging, with long, complex, poorly understood life cycles. Direct restoration of marine mammal populations is unlikely; however management actions aimed at limiting chronic stressors such as ship strikes, pollution, noise, bycatch, entanglement, or actions taken to restore and protect oceanic food webs would likely increase marine mammal population resilience and improve long-term outcomes.

Acknowledgements Collaborators John A. Hildebrand and Alba Solsona Berga from the Scripps Institution of Oceanography contributed substantially to the ideas discussed in this review.

This research was made possible by a grant from The Gulf of Mexico Research Initiative/C-IMAGE I, II, and III. Funding for HARP data collection and analysis was also provided by the Natural Resource Damage Assessment partners (20105138), the US Marine Mammal Commission (20104755/E4061753), the Southeast Fisheries Science Center under the Cooperative Institute for Marine Ecosystems and Climate (NA10OAR4320156) with support through Interagency Agreement #M11PG00041 between the Bureau of Offshore Energy Management, 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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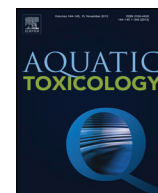
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Chemical dispersants used in the Gulf of Mexico oil crisis are cytotoxic and genotoxic to sperm whale skin cells



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ARTICLE INFO

Article history:

Received 6 March 2014

Received in revised form 12 April 2014

Accepted 16 April 2014

Available online 25 April 2014

Keywords:

Gulf of Mexico

Chemical dispersant

Deepwater Horizon oil spill

Genotoxicity

Corexit

Sperm whale

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 (Bandelet 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% CO₂ 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

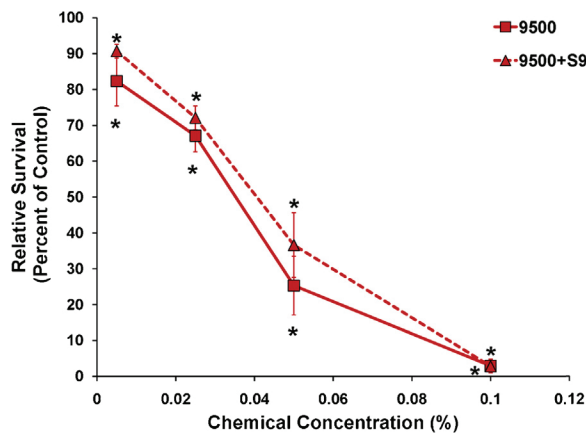


Fig. 1. Corexit 9500 is cytotoxic to sperm whale skin cells. This figure shows that Corexit 9500 was cytotoxic to sperm whale skin cells after a 24 h exposure and S9 mediated metabolism did not alter cytotoxicity (measured as cell survival relative to the control). The overall dose-response is highly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

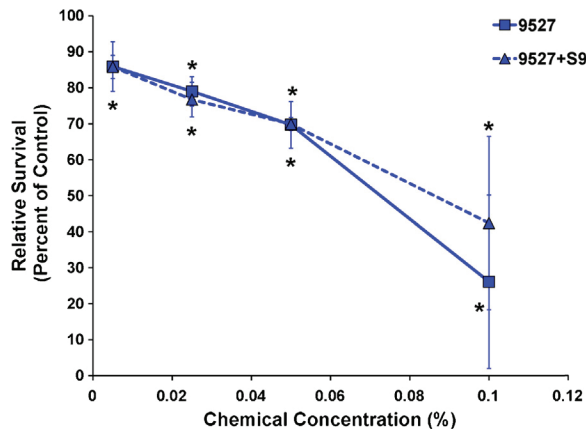


Fig. 2. Corexit 9527 is cytotoxic to sperm whale skin cells. This figure shows Corexit 9527 was cytotoxic to sperm whale skin cells in a concentration dependent manner after a 24 h. Cytotoxicity is measured as cell survival relative to the control. There was no statistical difference observed between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

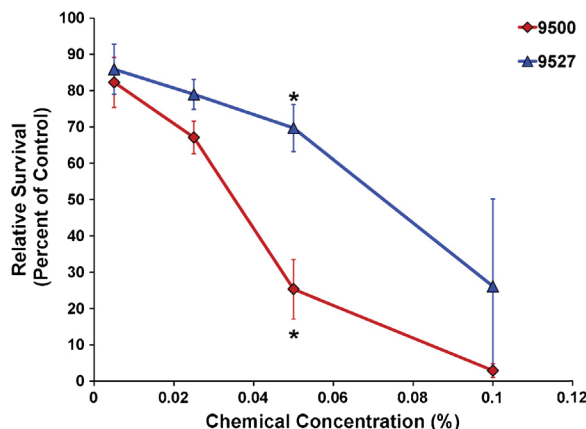


Fig. 3. Corexit 9500 is more cytotoxic than Corexit 9527. This figure compares the cytotoxicity of Corexit 9500 and Corexit 9527 after a 24 h exposure. Sperm whale cells were more sensitive to Corexit 9500 than Corexit 9527 ($p = 0.0078$). Asterisk (*) indicates doses that were significantly different from each other ($p < 0.05$).

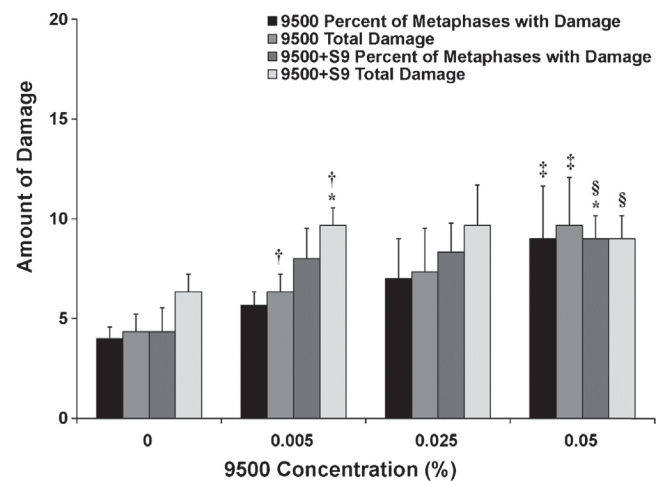


Fig. 4. Corexit 9500 is not strongly genotoxic to sperm whale skin cells. This figure shows Corexit 9500 was not substantially and generally not statistically significantly genotoxic to sperm whale skin cells after a 24 h exposure both with and without S9-mediated metabolism. Data are expressed as the average percent of metaphase with damage and total aberrations in 100 metaphases. Data represent 3 experiments $\pm$ 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 9527 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	Dicentric
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.

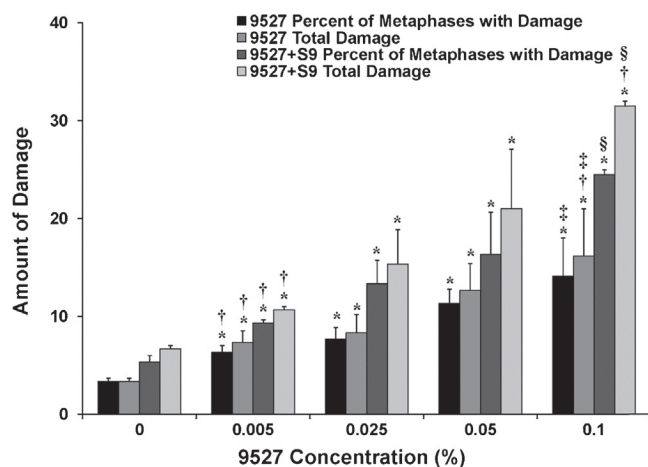


Fig. 5. Corexit 9527 is genotoxic to sperm whale skin cells. This figure shows Corexit 9527 induces a concentration-dependent increase in genotoxicity in sperm whale skin cells after a 24 h exposure. S9 mediated metabolism increases genotoxicity. Data are expressed as the average percent of metaphase 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 $\pm$ 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

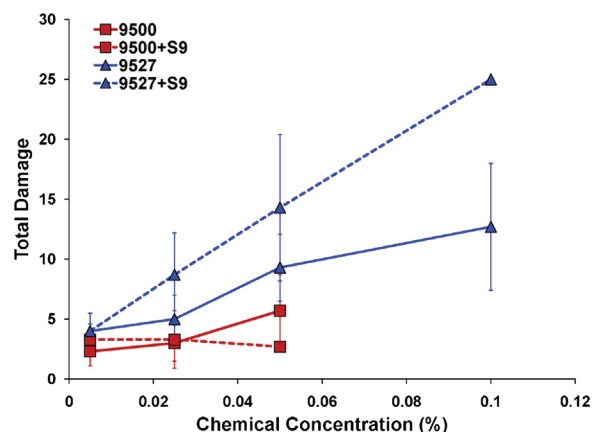


Fig. 6. Corexit 9527 induces more total chromosome damage than Corexit 9500. This figure shows the comparison of the total chromosome damage induced by Corexit 9500 and 9527 with and without S9 fractions after a 24 h exposure. Based on a multiple regression model, there was a significant 3-way interaction 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 (Bandeale 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 (Bandeale 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 than 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-butoxyethanol 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.

Acknowledgements

We would like to thank Louis Falank, Jr. for technical support, Shouping Huang for administrative support and Christy Gianios, Jr. for information technology support. We would also like to thank David Giddings from Nalco for the dispersants. Marine mammal cell line development and use by the Wise Laboratory for Environmental and Genetic Toxicology is performed under NMFS Permit # 16305-00.

This work was supported by the Prince William Sound Regional Citizens Advisory Council (PWSRCAC) and the Maine Center for Toxicology and Environmental Health [955.12.02].

This paper was also developed under GRO Fellowship Assistance Agreement No. [MA-91739301-0] awarded by the U.S. Environmental Protection Agency (EPA). It has not been formally reviewed by EPA. The views expressed in this paper are solely those of the authors, and EPA does not endorse any products or commercial services mentioned in this paper.

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EXHIBIT

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2015

Status of the world's baleen whales

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Status of the world's baleen whales

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ABSTRACT

No global synthesis of the status of baleen whales has been published since the 2008 IUCN Red List assessments. Many populations remain at low numbers from historical commercial whaling, which had ceased for all but a few by 1989. Fishing gear entanglement and ship strikes are the most severe current threats. The acute and long-term effects of anthropogenic noise and the cumulative effects of multiple stressors are of concern but poorly understood. The looming consequences of climate change and ocean acidification remain difficult to characterize. North Atlantic and North Pacific right whales are among the species listed as Endangered. Southern right, bowhead, and gray whales have been assessed as Least Concern but some subpopulations of these species - western North Pacific gray whales, Chile-Peru right whales, and Svalbard/Barents Sea and Sea of Okhotsk bowhead whales - remain at low levels and are either Endangered or Critically Endangered. Eastern North Pacific blue whales have reportedly recovered, but Antarctic blue whales remain at about 1% of pre-exploitation levels. Small isolated subspecies or subpopulations, such as northern Indian Ocean blue whales, Arabian Sea humpback whales, and Mediterranean Sea fin whales are threatened while most subpopulations of sei, Bryde's, and Omura's whales are inadequately monitored and difficult to assess.

Key words: status, cetaceans, baleen whales, blue whales, whaling, ship strike, entanglement, bycatch, anthropogenic noise, climate change, ocean acidification, endangered species, IUCN, Red List.

Baleen whales (Mysticeti, or mysticetes) and their ocean habitat (Gattuso *et al.* 2015) are under stress throughout the world, yet there has been no up-to-date synthesis on the status² of this group since Clapham *et al.* (1999). Baleen whales have acquired iconic status in the conservation discourse, for their “charisma” (size, appearance, behavior, *etc.*), their global distribution, and especially the fact that several of the species and populations were nearly extirpated by commercial whaling, which continued on a massive scale until the early 1970s. It is generally assumed

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²Here status is considered to mean the degree to which a species or population is at risk of extinction. In other contexts status may refer to the size or general health of a population relative to some management standard (Barlow and Reeves 2009).

by nonspecialists that all whales are endangered. Actually, the level of endangerment of different species and populations is quite variable. The identification of species, subspecies, and subpopulations³ (or what are often called “stocks” of whales within the Scientific Committee of the International Whaling Commission [IWC]; Allen 1980, Donovan 1991) is itself a major scientific task. Once the “units of conservation concern” (Taylor 2005) have been identified, the next scientific imperative is to determine their status. Key indicators of status are population size (abundance), rates of removals, and rates of population growth or decline. As part of analyses to determine status, consideration is given to the nature and severity of threats, which can apply at either the individual or population level. In considering anthropogenic factors that cause harm of some kind, from temporary disturbance all the way to death, managers have to evaluate whether the impacts on individuals are sufficient, in the aggregate, to affect parameters of the population as a whole, *e.g.*, birth rate, survival rates, recruitment rate, longevity).

In this paper we provide the first synthesis on the status of the world’s baleen whales and the threats they face since Clapham *et al.* (1999).

METHODS

We used as a starting point the assessment information provided for the International Union for Conservation of Nature’s (IUCN) Red List of Threatened Species (Red List) on the status of, and the threats faced by, all baleen whale species as well as some subspecies and subpopulations. The documentation behind the 2008 Red List assessments, in most instances coming from a 2007 workshop of the IUCN Species Survival Commission’s Cetacean Specialist Group, is available on the Red List website (<http://redlist.org>).⁴

A major underpinning of the Red List assessments and of this current synthesis is the work of the IWC’s Scientific Committee.⁵ In addition to reviewing much of the primary literature cited in the Red List analyses, we conducted an in-depth review of IWC reports and publications, as well as of new literature available (through May 2015), with the goal of producing a comprehensive, up-to-date synthesis of what is presently known about the status of baleen whales and the conservation threats to individual populations. Sometimes the IUCN assessments are more recent or detailed than the IWC assessments and *vice versa*. Therefore the terminology (subpopulation or stock) we use may vary as we reflect the most current information. Also, it is important to note that for some species, *e.g.*, blue (*Balaenoptera musculus*), fin (*Balaenoptera physalus*), and sei whales (*Balaenoptera borealis*), the IUCN Red List classifica-

³In the present context, the term “subpopulation” follows Red List usage to mean a geographically or otherwise distinct group with little demographic or genetic exchange with other groups (typically one successful migrant individual per year or less) (IUCN 2012).

⁴The Red List documentation is cited herein following Red List convention, *i.e.*, under the authorship of the assessor(s) (Reilly *et al.* in most cases; Stephen Reilly chaired the working group dealing with baleen whales at the La Jolla, California, workshop) and with the “publication” year given in most cases as 2008 (according to the citation as recommended on the relevant page of the Red List website).

⁵The International Convention for the Regulation of Whaling exists for the explicit purpose of managing the exploitation and assuring the conservation of whales, including all of the baleen whales, most of which have been subjected to prolonged commercial whaling. The Scientific Committee is expected to provide authoritative advice on the status of whale stocks which is then used by the Commission in making management decisions.

tion of Endangered is misleading because the global, range-wide assessments were dominated by the massive removals by historical commercial whaling, mainly in the Southern Hemisphere. Not all regional populations of those species are endangered. Where new information was available on taxonomy (through May 2015), we updated the Red List documentation in that respect (generally following the Society for Marine Mammalogy's Committee on Taxonomy (cited herein as Committee on Taxonomy 2015; see <http://www.marinemammalscience.org>).

The currently recognized species of baleen whales are the blue whale, fin whale, humpback whale (*Megaptera novaeangliae*), sei whale, Bryde's whale (*Balaenoptera edeni*), Omura's whale (*Balaenoptera omurai*), common minke whale (*Balaenoptera acutorostrata*), Antarctic minke whale (*Balaenoptera bonaerensis*), bowhead whale (*Balaena mysticetus*), North Atlantic right whale (*Eubalaena glacialis*), North Pacific right whale (*Eubalaena japonica*), southern right whale (*Eubalaena australis*), pygmy right whale (*Caperea marginata*), and gray whale (*Eschrichtius robustus*). In addition, the currently recognized subspecies of baleen whales are the North Atlantic minke whale (*B. acutorostrata acutorostrata*), North Pacific minke whale (*B. a. scammoni*), dwarf minke whale (*B. a.* unnamed subsp.), northern sei whale (*B. borealis borealis*), southern sei whale (*B. b. schlegellii*), offshore Bryde's whale (*B. edeni brydei*), Eden's whale (*B. e. edeni*), northern blue whale (*B. musculus musculus*), Antarctic blue whale (*B. m. intermedia*), northern Indian Ocean blue whale (*B. m. indica*), pygmy blue whale (*B. m. breviceauda*), Chilean blue whale (*B. m.* unnamed subsp.), pygmy fin whale (*B. physalus patachonica*), northern fin whale (*B. p. physalus*), southern fin whale (*B. p. quoyi*), southern humpback whale (*M. novaeangliae australis*), North Pacific humpback whale (*M. n. kuzira*), and North Atlantic humpback whale (*M. n. novaeangliae*).

THREATS

Clapham *et al.* (1999) provided an overall review of the threats to baleen whales. They identified bycatch (entanglement or entrapment in fishing gear) and ship strikes as the primary threats at the population level and concluded that those threats were most significant for populations already at critically low numbers. We found that 15 yr later, these same threats remain among the most serious. In addition, commercial whaling in the form of research whaling or whaling under objection to the commercial whaling moratorium continues by Japan, Norway, and Iceland. An updated review of threats to baleen whales must consider, in addition to those identified by Clapham *et al.* (1999) (entanglement/entrapment, ship strike, whaling, pollution, disease, habitat degradation from oil spills), the cumulative impacts of anthropogenic noise and other stressors and the short- and long-term effects of climate change and ocean acidification on marine ecosystems.

Acute or Lethal Threats

Whaling has an immediate effect on populations through the direct removal of individuals. Two other threats—mortality in fishing gear and ship strikes—result in the deaths of individuals and their outright, immediate loss to the population. Indeed, the IWC Scientific Committee's Working Group on Estimation of Bycatch and other Human-induced Mortality recognizes this equivalence: the group's remit is to estimate these removals “so that such mortality can be subtracted from any catch limits that might be calculated using the RMP [Revised Management Procedure]” (IWC 2012b:221). Despite uncertainties as to their causes, we also include discussion

of unusual mortality events in this section because of their possible consequences for populations.

Historical and Current Whaling

Whaling has been a major threat to most baleen whale populations in the past, and the legacy of commercial exploitation remains as a prominent force in determining their current status. Many populations are showing signs of recovery; others remain depleted; some are at very low numbers and may be vulnerable to extirpation by stochastic processes or catastrophic events (Clapham *et al.* 1999).

North Atlantic right whales and Svalbard/Barents Sea bowhead whales remain endangered from whaling that ceased a century or more ago, but several other depleted populations were the target of illegal Soviet whaling as recently as the 1970s (Ivashchenko and Clapham 2014). Significant information continues to be brought to light and published on the scale of Soviet illegal hunts in the Arabian Sea (Mikhalev 1996, 1997, 2000), South Atlantic (Tormosov *et al.* 1998), Antarctic (Yablokov 1994, Zemsky and Sazhinov 1994, Clapham and Ivashchenko 2009, Ivashchenko *et al.* 2011), and North Pacific (Brownell *et al.* 2001; Ivashchenko and Clapham 2012; Ivashchenko *et al.* 2013, 2014), which reduced populations of southern right whales, humpback whales, pygmy blue whales (*B. m. breviceauda*), North Pacific right whales, and Sea of Okhotsk bowhead whales as well as sperm whales (*Physeter macrocephalus*) in many parts of the world's oceans.

Although ongoing whaling (commercial whaling by Norway and Iceland under an objection to the moratorium and "research" or "scientific" whaling by Japan and Iceland) is a subject of controversy and public debate, it is not an *active* threat to most baleen whale species and subspecies (Clapham *et al.* 1999). Current levels of take in most of these hunts are near or below levels determined to be sustainable under the IWC's Revised Management Procedure (RMP) or by Iceland and Norway on the basis of similar algorithms. An exception is the taking of J-stock common minke whales by Japanese research whaling, bycatch in fishing gear in Japan and the Republic of Korea (Korea), and some illegal whaling by Korea (Song *et al.* 2010, MacMillan and Han 2011). Also, the sei whales in the North Pacific subject to research whaling have not recently been assessed (both in terms of numbers and population structure) by the IWC. The levels of noncommercial ("aboriginal subsistence") whaling (Reeves 2002) on gray whales in Russia, bowhead whales in Russia and the United States, bowhead, humpback, fin, and common minke whales in Greenland (Denmark), and humpback whales in St. Vincent and the Grenadines are based on IWC assessments and are generally considered sustainable (IWC 2012a, <http://iwc.int/aboriginal>).

Entanglement/entrapment in Fishing Gear

Incidental mortality (as well as morbidity) in fishing gear (hereafter "bycatch") is a ubiquitous problem for cetaceans in coastal and continental shelf waters and to a lesser extent in offshore areas (Read *et al.* 2006, Reeves *et al.* 2013). In some cases, bycatch has species- or population-level impacts. Gill net and trap/pot fisheries that use vertical lines to mark gear are responsible for much of the baleen whale bycatch (Reeves *et al.* 2013) although large-mesh shark control nets are a particular problem in South Africa and Australia (Meÿer *et al.* 2011). The susceptibility of species differs according to their size or habits and to the types of fishing gear used within their range. Bycatch has known population-level consequences for North Atlantic right

whales (Johnson *et al.* 2007, Knowlton *et al.* 2012) and the western North Pacific subpopulation of gray whales (Weller *et al.* 2002, 2008) and it has been inferred to be the primary threat to the Arabian Sea subpopulation of humpback whales (Minton *et al.* 2011). Other baleen whale populations potentially threatened by bycatch, but for which data are very limited, include North Pacific right whales and the southeastern Pacific (Chile-Peru) subpopulation of southern right whales. Humpback whales are frequently entangled in fishing gear in the North Pacific and North Atlantic but U.S. stock assessments indicate that mortality levels from such entanglement are not high enough to threaten the humpback whale populations in U.S. EEZ waters, *i.e.*, they are below the potential biological removal or PBR level (Allen and Angliss 2011, Waring *et al.* 2011).⁶ Because of their small size, nearshore and continental shelf distribution, and fish diet, minke whales are especially vulnerable to gillnet entanglement (Reeves *et al.* 2013).

Ship Strike

The threat of ship strikes is certain to increase as commercial ship traffic increases around the world (Clapham *et al.* 1999, Laist *et al.* 2001, Jensen and Silber 2003, McKenna *et al.* 2012a, Redfern *et al.* 2013, Monnahan *et al.* 2014b). Recently, Tournaire (2014) has shown that commercial ship traffic increased by a factor of four between 1992 and 2012 with maximums in the Indian Ocean and China seas. Ship strikes are of particular concern where the ranges (*e.g.*, feeding areas, calving areas, migratory routes) of threatened populations overlap with major shipping routes, entrances to major ports, or other areas of intense vessel traffic such as offshore industrial sites or high-speed ferry routes (Clapham *et al.* 1999, Kraus *et al.* 2005, Corbett and Winebrake 2007, Neilson *et al.* 2012).

The best-documented example of ship strikes having a significant impact on a baleen whale species is the North Atlantic right whale. In spite of measures to reduce the incidence of ship strikes along the east coasts of both Canada and the United States (Knowlton and Brown 2007), the average reported mortality and serious injury of right whales due to ship strikes from 2005 through 2009 was 1.6 whales per year—double the PBR level (Waring *et al.* 2011). In 2003 the traffic separation scheme in the lower Bay of Fundy (Canada) was modified to avoid an area with recurrent large concentrations of right whales in summer and fall (Knowlton and Brown 2007) and in 2006 the International Maritime Organization also approved a modification of the shipping lanes into Boston harbor to avoid an area of right whale concentration on Stellwagen Bank (Marine Mammal Commission [MMC] 2006). In December 2008 the U.S. National Marine Fisheries Service implemented a 5 yr rule requiring that within areas where North Atlantic right whales are likely to occur, large ships restrict their speed to 10 knots (MMC 2010) and preliminary analyses indicated it was effective (Laist *et al.* 2014). The rule was extended indefinitely in 2013.

The risk of ship strikes is affected by economic and climatic conditions (Corbett and Winebrake 2007, McKenna *et al.* 2012b). For example, it is expected that the dramatic, ongoing decline of sea ice in the Arctic will lead to more ship traffic in areas currently used intensively by baleen whales. Of particular concern are shipping and migratory chokepoints such as the Bering Strait, which connects the Bering and

⁶Potential biological removal (PBR) is the product of minimum population size, one-half the maximum net productivity rate, and a “recovery” factor ranging from 0.1 to 1 (MMPA Sec. 3. 16 U.S.C. 1362; Wade and Angliss 1997).

Chukchi seas (Reeves *et al.* 2012), and Unimak Pass, Alaska, and other straits through the Aleutian Islands used by both ships and whales to move from the North Pacific into and out of the Bering Sea.

Following a spate of fatal ship strikes on blue whales in the eastern North Pacific in 2007, voluntary measures were put in place to reduce the ship strike risk in California waters, especially the Santa Barbara Channel, an area of heavy traffic for Los Angeles and Long Beach harbors and seasonally high blue whale density, and off the port of San Francisco (Carretta *et al.* 2013, Redfern *et al.* 2013). McKenna *et al.* (2012a) found little compliance with voluntary speed-reduction measures in the Santa Barbara Channel, suggesting the need for alternative context-specific strategies to achieve speed reductions. However, Monnahan *et al.* (2014b) concluded that ship strikes have not had population-level effects on blue whales in the eastern North Pacific. Other subspecies and subpopulations known or suspected to be affected by ship strikes include pygmy blue whales, which frequent the busy shipping lanes off the southern coast of Sri Lanka (IWC 2012b), and Arabian Sea humpback whales, which are also exposed to intensive ship traffic in parts of their range. In neither case, however, has mortality been monitored sufficiently to assess the severity of the ship strike threat.

Health

Large-scale mortality events involving baleen whales are rare (Gulland and Hall 2007, Coughran *et al.* 2013, Rowntree *et al.* 2013). Biotoxins were implicated in two large events off New England in 1987 and 2003. The first involved 14 humpback whales that died in Cape Cod Bay after eating Atlantic mackerel (*Scomber scombrus*) containing saxitoxin, a dinoflagellate neurotoxin responsible for paralytic shellfish poisoning in humans (Geraci *et al.* 1989). The second, in Maine, involved the deaths of 16 humpback whales, one fin whale, one common minke whale, one pilot whale (*Globicephala* sp.), and two whales unidentified to species. While the cause of this second event was not determined, two kinds of biotoxin—saxitoxin and domoic acid—were detected in a few of the humpback whales examined (Gulland and Hall 2007). A large die-off of cetaceans of unknown cause was recorded in 1995 in the upper Gulf of California, Mexico. This event included hundreds of long-beaked common dolphins, *Delphinus capensis*, and at least eight baleen whales of three species (Vidal 1996). Forty-six humpback whales, 44% of them calves of the year, stranded on Western Australia beaches in 2009. While many animals appeared to be underweight, it proved impossible to determine cause of death (Coughran *et al.* 2013). Thirteen humpback whales stranded on Brazilian beaches in 2010 (Moura *et al.* 2012).

Starvation was considered a possible cause of a widespread gray whale die-off in 1999–2001, in which at least 651 deaths were confirmed, not all of which could be linked directly to poor body condition or starvation. The proximate cause of death differed for the few animals conclusively necropsied and it was hypothesized that parasite infestations, biotoxins, or infections finally killed animals that had been made vulnerable by malnutrition (Gulland *et al.* 2005). This event added substance to the ongoing discussion of whether eastern Pacific gray whales had reached or exceeded the environmental carrying capacity and it more generally prompted analyses of how environmental variability affects baleen whale life history and mortality patterns (Moore *et al.* 2001, Perryman *et al.* 2002).

Some of the best tools and baseline health information on baleen whales have come from studies of North Atlantic right whales (Rolland *et al.* 2007, 2012). Analyses of fecal samples have permitted evaluation of stress hormone levels (see below). Such analyses also confirmed the presence of biotoxins and potentially harmful parasites such as *Giardia* and *Cryptosporidium* in live right whales, yet none of these, or other disease processes, were determined to be the primary cause of death for any of the 39 North Atlantic right whales necropsied between 1975 and 2005. These whales died from blunt or sharp trauma from vessel strikes ($n = 19$), fishing gear entanglement ($n = 5$), or undetermined causes including neonatal complications ($n = 15$) (Moore *et al.* 2007).

Photo-identification research on some baleen whale populations has led to investigations of skin lesions (Hamilton and Marx 2005, Brownell *et al.* 2007, Van Bressem *et al.* 2014). An analysis of two types of lesions (white and blister) on North Atlantic right whales found that these were sublethal conditions that might somehow impact or reflect overall health. Large “swath lesions” (which are white) were found to be correlated with entanglement, thin body condition, and known or suspected mortality of most of the affected whales (Hamilton and Marx 2005). There was some evidence, although not conclusive, that white lesions are the result of a contagious agent. Fifty-two of 68 individual blue whales observed off Isla Grande de Chiloe, Chile, had raised or blister-like skin lesions and at least one whale had extensive tattoo-like lesions (Brownell *et al.* 2007).

Since 2003, southern right whales, mostly calves, have been dying in unusually large numbers in the calving/nursery grounds around Península Valdés, Argentina (Uhart *et al.* 2008, 2009; Rowntree *et al.* 2013). So far the cause(s) of these deaths has not been determined, but three hypotheses have been put forward to explain the die-off (IWC 2011). The mortality of calves may be a consequence of: (1) the poor nutritional state of the mothers; (2) exposure to algal or bacterial biotoxins in (a) the feeding ground resulting in *in utero* exposure of the calf or (b) in the calving/nursery ground; and (3) infectious disease (viral, bacterial, protozoal, *etc.*). More recently, attention has focused on a fourth possibility: that repeated attacks on calves by kelp gulls (*Larus dominicanus*) are leading to stress, dehydration, and shock as a result of gaping peck wounds (Rowntree *et al.* 2013, Thomas *et al.* 2013). Other factors such as contaminants, predation by killer whales (*Orcinus orca*), disturbance from whale watching, fishery interactions, and ship strikes were ruled out or judged to be unlikely major contributors to these die-offs (IWC 2011).

SUBLETHAL OR CHRONIC THREATS

Disturbance from Human Activities

It has long been recognized that baleen whales, like other wild animals, can be disturbed by certain kinds of human activity. Because of their reliance on sound as a primary sensory modality, noise-generating activities are of particular concern (Richardson *et al.* 1995, Nowacek *et al.* 2007, Southall *et al.* 2007, Moore *et al.* 2012a). Beginning in the 1970s, scientists drew the attention of management agencies to the risks posed by underwater noise to small, recovering populations of baleen whales. For example, salt barge traffic was believed responsible for preventing gray whales from using Guerrero Negro Lagoon in Baja California, Mexico, for a period during the 1950s and 1960s (Gard 1974), and there was much discussion of the possibility that “harassment” by whale-watching boats was jeopardizing recovery of east-

ern Pacific gray whales (Reeves 1977). Similar concerns were expressed regarding humpback whales in Hawaii (Norris and Reeves 1978, but also see Tyack 1989). Off-shore oil and gas exploration in the U.S. and Canadian Arctic, beginning in the 1970s, brought the possibility of disturbance or auditory injury of bowhead whales by noise from seismic exploration, vessel traffic, and other industrial activity, a concern that now extends throughout the world's oceans and applies to all species of baleen whales (Richardson *et al.* 1995). Since the mid-1990s, there has been much concern about the impact of oil and gas development on the small population of gray whales that feeds in summer and fall off the northeastern coast of Sakhalin Island, Russia (Weller *et al.* 2002, Reeves *et al.* 2005).

The disturbance of whales by underwater noise (including masking of their vocalizations, impairment of their listening capabilities, and physiological stress) has lately become a much more salient issue for several reasons. First, efforts to monitor and measure ambient and anthropogenic underwater noise have increased and been enhanced by technologies that allow for greater geographical coverage, more extensive and sustained sampling, and more effective identification and localization of the animals. Second, commercial vessel traffic and offshore industrial, military, and extractive activities are increasing, especially near populated coastlines. Even near-pristine areas such as in parts of the Arctic, where seasonal sea ice cover is diminishing (see below), are experiencing a demand-driven rush to exploit previously inaccessible oil and gas and other mineral resources and an expansion in ship traffic (Reeves *et al.* 2014).

Much of the concern over acute sound impacts and the potential for death or injury of cetaceans has focused on active sources such as mid-frequency sonar used by military vessels and airguns used to conduct geophysical (seismic) surveys. Unusual mortality events associated with the use of mid-frequency sonar have primarily involved toothed cetaceans (odontocetes), and especially beaked whales (Cox *et al.* 2006, Nowacek *et al.* 2007, Hooker *et al.* 2012). Although two stranded common minke whales were involved in one of these incidents (Balcomb and Claridge 2001), the focus of concern over military sonar has been on beaked whales because they have been affected disproportionately (Moore and Barlow 2013).

Noise from seismic surveys is of greater concern for baleen whales. Although their hearing sensitivities are poorly known, their vocalizations tend to reside in the same low frequencies as the loud pulses produced by airguns, leading to concern over the potential to alter or disrupt behavior and even cause acoustic trauma—that is, “ear injury caused by a sudden and intense acoustic stimulus that causes a degree of permanent or temporary hearing loss” (Gedamke *et al.* 2011:496). A series of studies has shown behavioral changes in response to seismic noise, most notably in bowhead whales and gray whales (reviewed in Richardson *et al.* 1995, Gailey *et al.* 2007) and in humpback whales (McCauley *et al.* 2000), with the animals avoiding close approaches by operating airgun arrays and changing their surfacing, respiration, and dive behavior. Mitigation measures are often imposed by regulatory agencies (Nowacek *et al.* 2013). For example, delay or cessation of seismic operations is generally required when whales are observed close enough to the sound source to experience injury, *i.e.*, within “safety zones” calculated on the basis of the distance from the sound source at which received levels could lead to either temporary or permanent threshold shifts in the hearing of exposed animals (Southall *et al.* 2007). Also, “ramp up” and other procedures thought to encourage or allow whales to move away from disturbing sound sources are often included in the mitigation of noise impacts. There are likely other anthropogenic noise sources that affect baleen whales. For example,

Risch *et al.* (2012) demonstrated that the low frequency pulses from an experimental sounder used to image fish shoals over a 100 km diameter area affected the singing of humpback whales roughly 200 km away.

Considerable effort has been made in recent years to understand the masking effects of elevated ambient sound levels on the ability of baleen whales to detect and project natural sounds (Clark *et al.* 2009, Ellison *et al.* 2011, Hatch *et al.* 2012, Moore *et al.* 2012a). Hatch *et al.* (2012) found that background noise levels from ship traffic in the Stellwagen Bank National Marine Sanctuary off Massachusetts had increased so much by 2008 that the contact-calling right whales in the area would have “lost 63% of the communication opportunities available to them in the mid 20th century” (Hatch *et al.* 2012:990). With the close passage of commercial vessels, the loss of “communication space” increased by as much as 67%. This loss of communication space and corresponding masking of other acoustic cues may affect intraspecific communication related to foraging, mother-young interactions, migration, and mate selection, as well as prey and predator detection and navigation (Hatch *et al.* 2012, Moore *et al.* 2012a). Rolland *et al.* (2012) concluded from measurements of stress-related fecal hormone metabolites (glucocorticoids) that exposure to low-frequency noise may be associated with chronic stress in North Atlantic right whales.

In addition to commercial shipping, seismic surveys, discussed earlier with regard to acute impacts, contribute to the increase in ambient sound levels in the ocean (Hildebrand 2009, Moore *et al.* 2012b). Gedamke⁷ found that sounds from distant seismic survey activity increased background noise to levels that would reduce the potential for blue and fin whales (at 28 Hz and across a 20–30 Hz bandwidth) to communicate by 29%–40%.

In summary, concern has often been expressed in recent years about the largely unknown long-term and cumulative impacts of anthropogenic noise on cetaceans (as well as other marine life) (King *et al.* 2015). In our view, the noise-related issues that critically need additional study are (1) the nature and consequences of stress from noise on individual whales; (2) the role of noise in causing displacement from important habitat, *e.g.*, reduced time to feed; and (3) the masking of salient acoustic signals used by baleen whales for communication.

Pollution

Although a few studies of odontocetes and pinnipeds have demonstrated a causal relationship between contaminant exposure and impaired reproduction or immunosuppression (see Reijnders *et al.* 2009 for review), it is generally believed that because baleen whales tend to feed at lower trophic levels, they are less prone to uptake and bioaccumulation of pollutants (O’Shea and Brownell 1994, Weisbrod *et al.* 2000, Elfes *et al.* 2010). Tissue concentrations of organochlorines are lower in baleen whales than in toothed whales, a relationship that has been demonstrated both generally and in comparisons between those that inhabit the same ecosystem (Gauthier *et al.* 1997; Aguilar *et al.* 1999, 2002; Reijnders *et al.* 2009). Exposure to oil may cause respiratory problems in cetaceans (Venn-Watson *et al.* 2015), fouling of the baleen, and impacts on prey (Geraci and St. Aubin 1988). Long-term exposure to polycyclic aromatic hydrocarbons (PAHs) has been shown to lead to cancers in toothed whales (Martineau *et al.* 2002), but the consequences of such exposure for baleen whales are

⁷Personal communication from Jason Gedamke, Ocean Acoustics Program, Office of Science and Technology, NOAA Fisheries, 1315 East-West Highway Silver Spring, MD 20910, 15 May 2015.

not known (Angell *et al.* 2004, Pomilla *et al.* 2004). Biomarkers of exposure and biological response to PAHs in cetacean skin indicate that, unlike for contaminants that bioaccumulate, levels of these byproducts of fossil fuel production and consumption are comparable in baleen and toothed whales (Angell *et al.* 2004). There is also evidence that some populations living closer to industrial activity have higher levels of PAHs than more remote ones (Angell *et al.* 2004, Kraus and Rolland 2007).

Climate Change and Ocean Acidification

The potential direct and indirect effects of climate change on marine mammals, including baleen whales, have been widely discussed over the last decade (Learmonth *et al.* 2006, Simmonds and Isaac 2007, Moore and Huntington 2008, Tynan and Russell 2008, MacLeod 2009, Simmonds and Elliot 2009, IWC 2010, Kovacs *et al.* 2011a, Gattuso *et al.* 2015).

Climate change-induced reductions in seasonal sea-ice and warming temperatures have spurred increasing industrial interest in the Arctic, thereby increasing the anthropogenic threats to Arctic cetaceans from ship strikes, pollution, and disturbance from underwater noise (Reeves *et al.* 2014). With regard to the direct effects of climate change on baleen whales, however, the prognosis may vary both within and across species. Bowhead whales, for example, do not have much possibility of northward range expansion in the face of warming ocean temperatures (Kovacs *et al.* 2011a). On the other hand, while they are adapted to life in the ice, bowheads are also able to travel and feed independently of it. Despite the decline of sea-ice in the Chukchi and Beaufort seas over the past 20 yr, bowhead numbers have increased at about 3.7% per year (despite subsistence hunting) (Givens *et al.* 2013), their distribution and migration timing have not changed markedly (although they appear to arrive near Barrow earlier in the spring and to linger longer in the northeastern Chukchi Sea and western Beaufort Sea in the summer), and observations suggest that the seasonal opening of ice-free areas has improved feeding conditions for these whales (Moore 2009, Ashjian *et al.* 2010). Examination of body condition relative to available foraging habitat provides confirmation that more open water in the Beaufort Sea in the summer has resulted in healthier bowheads (George *et al.* 2005). Nonetheless, it remains to be seen whether, and to what degree, the changed trophic dynamics and ecosystem structuring under the new seasonally ice-free conditions and the shift to a more strongly pelagic ecosystem in the Arctic will affect bowhead populations over the long-term.

Another factor to consider in the Arctic is the arrival and more regular presence of seasonally migrant species that are responding to climate change by moving into higher latitudes. This will bring not only the potential for competition with bowheads, but also more predation by killer whales, exposure to novel pathogens, and impacts of invasive species on prey (Moore and Gulland 2014). The influx of new species will follow, and potentially influence, changes in trophic dynamics in the Arctic due to warmer water temperatures and reduced sea ice. Gray, fin, common minke, humpback, and killer whales have already expanded their ranges northward and into Arctic waters (Kovacs *et al.* 2011a, Clarke *et al.* 2013). Such expansion does not necessarily represent a net benefit to such populations, at least in the short term. For example, the access of gray whales to summer feeding grounds in the Bering Sea and north of the Bering Strait continues to be limited by the timing of spring ice retreat at the southern limit of sea ice in the Bering Sea. In years of late ice retreat, the whales appear unable to compensate for lost feeding time and suffer reduced calf production

and increased calf mortality (Perryman *et al.* 2002). Also, the timing of arrival in the southern portion of the southward gray whale migration was later in 1998/1999 in response to El Niño events, with corresponding reports of more calf births than normal north of the calving lagoons in Baja California, Mexico (Moore 2009). The expansion of range will not be only latitudinal. Reduced sea ice also may allow bowhead whale populations to mix more frequently across the Arctic than they have since the Little Ice Age from the 15th to the 19th century (Heide-Jørgensen *et al.* 2011, Alter *et al.* 2012).

There is also ample uncertainty with regard to the impacts of climate change on whales in the Southern Hemisphere. One analysis used climate models from the Fourth Intergovernmental Panel on Climate Change (IPCC) and information on distribution and feeding ecology to assess the impacts of a 2°C global warming on Southern Ocean cetaceans (Tynan and Russell 2008). Among projected changes were a decrease in the extent of sea ice and ice-edge habitat along the Antarctic continent and a southerly shift and shrinking of ocean fronts (*e.g.*, the Antarctic Convergence) which may in turn affect the availability of krill (Reid and Croxall 2001, Fraser and Hoffman 2003, Trathan *et al.* 2003). Tynan and Russell (2008) predicted that Antarctic minke whales, which forage along the Antarctic ice edges, will lose 5%–30% of their ice-associated habitat with this temperature increase. Migratory cetaceans will have to travel farther south (3°–5° of latitude) to reach the ocean fronts where they forage.

Some baleen whale populations are thought to be especially vulnerable because of cul-de-sac geography (Simmonds and Isaac 2007). For example, bowhead whales in the Sea of Okhotsk will likely be unable to shift northward to reach cold waters as ocean temperatures warm. Fin whales in the Mediterranean and resident fin whales in the Gulf of California may be limited by the enclosed geography of the seas they inhabit (Vidal *et al.* 1993, Panigada and Notarolo di Sciara 2012). The Arabian Sea subpopulation of humpback whales and the northern Indian Ocean blue whale subspecies (*B. m. indica*) also appear to be “trapped.” Both breed 6 mo out of phase with conspecific populations to the south and appear to be restricted to northwestern portions of the Indian Ocean. They follow a Northern Hemisphere breeding cycle and feed in the northern Indian Ocean during the austral summer, at the time when Southern Hemisphere humpback and blue whale populations to the south are feeding at the southern end of their annual migration in Antarctic waters (Mikhalev 1996, 1997; Minton *et al.* 2011). Individuals in these two northern Indian Ocean populations do not have the option of moving north in the face of warming temperatures; they are blocked from doing so by the continental mass of Asia. The notion that they could simply shift southward to find suitable habitat may ignore the drivers of prey abundance for these populations, which are likely the Northern Hemisphere monsoons that create regional upwellings and productive prey patches (de Vos *et al.* 2014). Changes in the timing and intensity of the monsoons may be more relevant to these and other Indian Ocean populations than changes in north-south seasonal temperature gradients. Global monsoon patterns are poorly understood and complex. Recent research on the substantial intensification of the Northern Hemisphere Summer Monsoon (which runs contrary to large-scale climate model predictions) from the 1970s to the present shows strong El Niño influences, overlain by multidecadal oscillations in the Atlantic, further influenced by “hemispherical asymmetrical global warming” (Wang *et al.* 2013). The net effect of climate change on a marine population in a cul-de-sac situation is not simple to predict.

The habitat of some species or subspecies judged to be tropical (*i.e.*, warm water-limited), such as Bryde’s whales and dwarf minke whales, could expand, and the pop-

ulations of some cosmopolitan species could be largely unaffected by climate change (MacLeod 2009). Recent extralimital records of baleen whales, such as gray whales in the Mediterranean Sea (Scheinin *et al.* 2011) and Namibia⁸ and Bryde's whales in southern Californian waters (Kerosky *et al.* 2012), suggest that whales are responding to changing ocean conditions.

Baleen whales are likely to be negatively affected by ocean acidification even though, given the present state of scientific understanding, we can do little more than speculate on the mechanisms and levels of impact. A wide range of marine organisms, from planktonic coccolithophores and pteropods and other mollusks, to echinoderms, corals and coralline algae, build shells from calcium carbonate and are therefore at direct risk from ocean acidification (Doney *et al.* 2009). Changes in the survival and distribution of these organisms are expected to have major impacts on trophic dynamics and ecosystem processes over the next century (Fabry *et al.* 2008, 2009). These impacts are expected to be felt first in the Arctic and Antarctic seas which, due to cold water temperatures and other factors, have naturally low calcium carbonate concentrations (Orr *et al.* 2005, Fabry *et al.* 2009) and are predicted to become undersaturated with respect to aragonite by the end of this century. The effects of ocean acidification are anticipated to be both widespread and variable (Doney 2010). One major concern is that the biogeochemical, ecological and economic consequences of losing pteropods in the Arctic and Antarctic could have significant impacts on predators such as zooplankton, fish, seabirds, or cetaceans (Comeau *et al.* 2012, Mathis *et al.* 2015). The calcifying prey organisms of the northern Bering Sea that support higher trophic predators such as diving sea ducks, bearded seals (*Erignathus barbatus*), walrus, (*Odobenus rosmarus*) and gray whales may be at early risk from ocean acidification (Doney *et al.* 2009). There is little basis to evaluate the ability of such predators to modify their diets or otherwise respond to changes in availability of their primary prey (Fabry *et al.* 2009).

We conclude that as oceans warm and oceanic and meteorological conditions change, cul-de-sac or range-limitation conditions will come to represent major challenges for some populations. It must be acknowledged, however, that the baleen whales are capable of moving extremely large distances in short times to find shifting food resources and then return to distant breeding grounds. Their annual and seasonal movements already reflect an ability (whether acquired through selective forces over different climatic periods or learned within a few generations) to respond to considerable environmental variability. Reported range expansions (Kovacs *et al.* 2011a, Clarke *et al.* 2013) and temporal and geographical overlap of previously discrete populations across the Arctic Ocean (Heide-Jørgensen *et al.* 2011, Alter *et al.* 2012) indicate some potential for adaptive responses in the face of climate change (Moore and Huntington 2008). Whether this potential extends to responding to the consequences of ocean acidification is an open question.

CONSERVATION STATUS

Overview of Red List Status

Many of the “great whales” (meaning all baleen whales plus the sperm whale) were severely depleted in all oceans by commercial whaling, which ended

⁸<http://namibiandolphinproject.blogspot.com/2013/05/a-rare-and-mysterious-visitor-in-walvis.html>.

(almost but not quite entirely) in 1986 with implementation of the IWC's global "moratorium" on commercial whaling. The legacy of commercial whaling continues to exert a strong influence on how the conservation status of species and populations is assessed. For example, blue, fin, sei, and North Pacific right whales are classified on the IUCN Red List as Endangered based primarily on their global declines from whaling. The other Endangered baleen whale species, the North Atlantic right whale, was also nearly annihilated by whaling, which began in the northeastern Atlantic in the 11th century or earlier and continued into the 20th century. This species' recovery has been slowed considerably by ship strikes and entanglements in fishing gear so its current status is influenced not only by the decline caused by whaling but also by these ongoing threats. A blue whale subspecies, the Antarctic blue whale (*B. m. intermedia*), and a North Pacific right whale subpopulation (eastern) are listed as Critically Endangered because their numbers were driven to very low levels by commercial whaling and remain there. In recent years Antarctic blue whales have been increasing at about 8.2% per year but their total abundance in 1998 was still only about 1% of what it was before whaling.

Several baleen whale populations have been recovering from the depletion caused by commercial whaling. The bowhead whale, southern right whale, common minke whale, humpback whale, and gray whale are classified as Least Concern, indicating that at the species level they do not meet any of the criteria for threatened status. In each of these cases, increased numbers across the total range have been sufficient to push the species as a whole above the threshold for threatened listing. Nevertheless, some subpopulations of these Least Concern species—western Pacific gray whales, Arabian Sea humpback whales, Oceania humpback whales, Peru-Chile right whales, and Svalbard/Barents Sea and Sea of Okhotsk bowhead whales—remain at low levels and are listed separately as either Endangered or Critically Endangered. The common minke whale was heavily exploited in parts of its range during the second half of the 20th century but was, overall, less severely depleted by commercial whaling than the larger, more individually valuable baleen whales.

Four species of baleen whales, the Bryde's whale, Omura's whale, pygmy right whale, and Antarctic minke whale, are listed as Data Deficient, meaning available information is inadequate for a conclusive assessment of status. Despite having the same assigned status, there are major differences among these species with regard to the type of information that is lacking. In the case of the Bryde's whale, the problem resides mainly in systematics and taxonomy and therefore how to define units for assessment not only at the species level but also at the population level and how to allocate the large historical catches in some areas (*e.g.*, the North Pacific) when estimating population trends. Omura's whale was described and named in 2003 and very little is known about its distribution, ecology, and basic biology, but it is generally believed that this species was never heavily exploited. The pygmy right whale, the smallest baleen whale, is restricted to the Southern Hemisphere. It was rarely the target of whalers and little is known about it. Antarctic minke whales are still classified as Data Deficient although, as explained later, some progress toward resolving the relevant uncertainty has been made since 2008.

Species-level assessments of the baleen whales are problematic. All species listed as Least Concern have subpopulations that are at risk. Some species assigned to a threatened category (Endangered or Vulnerable) include subpopulations that are nonthreatened. Assigning a single "global" status to what are very widespread (in some cases cosmopolitan) meta-populations tends to disguise the highly variable status of local,

Table 1. Red List status of baleen whale species and those subspecies and subpopulations that have been assessed.

Taxa or conservation units	Critically Endangered	Endangered	Vulnerable	Least Concern	Data Deficient	Unassessed
Species	0	5	0	5	4	0
Subspecies	1	0	0	0	1	12
Subpopulations	4	3	1	1	0	uncertain

regional, and even basin-scale populations (subpopulations in Red List terms) (IUCN 2012). While there is interest in making species-level comparisons when evaluating global biodiversity trends for terrestrial and marine mammals (Schipper *et al.* 2008, Kovacs *et al.* 2011b), such comparisons can break down at the subspecies and subpopulation levels. In the case of baleen whales, the universe of such units is indeterminate; in other words, it is not feasible at present to establish how many subspecies and subpopulations there are. The number of identified subpopulations increases steadily as genetic and other research reveals more about population structure. Also, only a relatively small subset of those units that are recognized has been assessed for the Red List. The Cetacean Specialist Group has taken a selective, hierarchical approach when setting priorities for assessment, based primarily on indications of conservation concern from its expert members, and this has meant that most of the assessed subspecies and subpopulations are ones already suspected of being threatened. In other words, many subpopulations (if not also subspecies) that would be considered non-threatened have never been assessed; only one subpopulation (Bering-Chukchi-Beaufort Sea bowhead whales) has been assessed as Least Concern and one subspecies (pygmy blue whale) as Data Deficient (Table 1). Finally, not all subspecies are sufficiently well described to be considered appropriate units of concern for conservation. In contrast, subpopulations (or in IWC terms, stocks) are recognized as such because they are geographically or demographically distinct and are *perforce* units to conserve (Taylor 2005).

Current Status of Species, Subspecies, and Subpopulations

In the course of this review, we considered information on all baleen whale species, subspecies, and subpopulations that had been assessed for the IUCN Red List, subspecies that had not been assessed but were recognized by the Committee on Taxonomy (2015),⁹ and stocks recognized by the IWC Scientific Committee (Table S1).

Blue, fin, and sei whales were decimated throughout their ranges by commercial whaling, and their populations have followed broadly similar trajectories. We provide a detailed treatment of blue whales here to illustrate the impact of commercial whaling on them and several other species as the whaling effort moved from region to region. This section also shows some of the complexities of identifying and defining stocks and the difficulty of determining abundance levels and population parameters

⁹Current at October 2014; see http://www.marinemammalscience.org/index.php?option=com_content&view=article&id=714&Itemid=340.

for baleen whales even with recently developed tools in genetics, satellite tracking, statistical analysis, and acoustic monitoring.

Blue Whale

Since they provided the largest yield per unit of hunting effort, blue whales were the most valuable and thus were among the first whales to be depleted by modern commercial whaling, which began in Norway in the 1860s and had spread to all oceans, including the Antarctic, by the early 20th century. Although blue whales have been legally protected by the IWC for over half a century, beginning in 1955 in the North Atlantic (although Denmark and Iceland continued to take blue whales there under formal objection to the protection provision until 1960) (Committee for Whaling Statistics 1962), 1966 in the North Pacific, and 1965 in the Antarctic, the depletion in most of the range was severe, and evidence of population increase has been spotty and often equivocal. Also, illegal or pirate hunting of blue whales continued into the early 1970s, mainly by the Soviet Union (Ivashchenko *et al.* 2011) but also by other countries operating without regard to IWC regulations, and this further depleted or at least stalled the recovery of some populations and depleted most of the Southern Hemisphere pygmy blue whale populations (Branch *et al.* 2007a).

In the North Atlantic, at least 11,000 blue whales were killed between the late 19th century and 1960 (Jonsgård 1977). Despite full protection since 1960, the North Atlantic population does not appear to have increased to anywhere near its former size. Abundance estimates for the Gulf of St. Lawrence are around 400 whales based on photo-identification studies (Ramp *et al.* 2006). Estimates for the central North Atlantic, which includes the waters around Iceland, East Greenland, Jan Mayen, the Færoes, and the British Isles, have ranged between 222 (95% CI 115–440) in 1987 and 979 (95% CI 137–2,542) in 1995 (Pike *et al.* 2009). Observations of blue whales off Norway and especially northern Norway where substantial numbers were taken in the late 19th and early 20th centuries (Jonsgård 1977) are now very rare (Christensen *et al.* 1992).

In the North Pacific, blue whales were hunted extensively in the 20th century with 9,773 reported taken between 1905 and 1971. In addition, a significant proportion of the whales killed but not assigned to species between 1900 and 1936 were blue whales (Ohsumi and Wada 1972, Reilly *et al.* 2008a, Monnahan *et al.* 2014a). At least two blue whale subpopulations are extant in the North Pacific (Stafford *et al.* 2001, Gilpatrick and Perryman 2008, Monnahan *et al.* 2014a). Blue whales in the far west, including off Japan, appear to have been extirpated as there have been no reports of kills, sightings, or other evidence there for over 50 yr (Clapham *et al.* 2008). A recent abundance estimate for the eastern North Pacific is 2,497 (CV = 0.24) (Calambokidis *et al.* 2009) and it has been suggested that blue whales in this region are approaching pre-exploitation numbers (Monnahan *et al.* 2014b). No subpopulations in the North Pacific have been assessed for the IUCN Red List. The greatest threat identified for individual blue whales in the eastern North Pacific is ship strikes although there is evidence suggesting that the current rate of ship strikes does not have a population-level impact (Monnahan *et al.* 2014b). There is also growing concern about the ever increasing amount of anthropogenic noise in their environment, especially their feeding grounds along the southern California coast (McKenna 2012a).

The Antarctic blue whale subspecies is redlisted as Critically Endangered (Reilly *et al.* 2008b). In an assessment of Antarctic blue whales, the 1905 population (before

the start of commercial whaling) was estimated at 239,000 (202,000–311,000) whales compared with a 1996 estimate of 1,700 (860–2,900) (Branch *et al.* 2004). A newer estimate of this subspecies is 2,280 (1,160–4,500) in 1998 (Branch 2007), which is still less than 1% of the prewhaling abundance. If commercial exploitation of krill in the Antarctic expands in the future, it could have a strong effect on Antarctic blue whales, as could any major change in krill availability due to climate change (Reid and Croxall 2001, Fraser and Hoffman 2003, Trathan *et al.* 2003).

Another subspecies, the pygmy blue whale, is considered Data Deficient (Cetacean Specialist Group 1996a).¹⁰ This subspecies (*B. m. breviceauda*) was described from a specimen taken southwest of the Prince Edward Islands in the southwestern Indian Ocean (Ichihara 1966) and other specimens of “pygmy” like blue whales have since been reported from various locations in the Southern Hemisphere and the Arabian Sea. Pygmy blue whales were exploited starting in the late 1950s to the late 1960s when almost 13,000 individuals were killed (Branch *et al.* 2004).

LeDuc *et al.* (2007) showed that the populations of pygmy blue whales in at least two regions (Western Australia and Chile–Peru) are as different from each other as either is from the Antarctic blue whale. Branch *et al.* (2007b) proposed, and the Committee on Taxonomy (2015) accepted, that the blue whales off Chile and probably Peru constitute an unnamed subspecies. Thus, the suite of populations referred to as pygmy blue whales, differentiated on the basis of genetic profiles and call types, may consist of separate subspecies or subpopulations, each with a different history of exploitation and depletion (Brownell *et al.* 2015). Regardless of their taxonomic rank, these populations should be assessed individually.

Off Western Australia, whales genetically identified as pygmy blue whales are best known from Geographe Bay and Perth Canyon in the west (Attard *et al.* 2012) to Bass Strait in the east (Gill *et al.* 2011). Blue whales off the west coast of Australia migrate north into the Banda Sea, around Timor, Indonesia (Double *et al.* 2014). Their abundance has been estimated as 662–1,559 based on passive acoustics (McCauley and Jenner 2010) and 712–1,754 based on photographic mark-recapture (Jenner *et al.* 2008). Most of the exploitation of this group of blue whales was by Soviet pelagic operations in the 1960s (Mikhalev 1996). Chittleborough reported that “pygmy blue whales have been captured along the western coast of Australia in recent years,” including a single whale landed at the whaling station of Carnarvaron, Western Australia, in May 1959 (Chittleborough in Ichihara 1966:82). The population has not been assessed separately for the Red List, at least partly because the Soviet catches of pygmy blue whales have not yet been allocated to subpopulations (also see Zemsky and Sazhinov 1994). Moreover, the population structure of pygmy blue whales in the Indian Ocean is poorly known. Based on call types, there is likely more than one Indian Ocean subpopulation in addition to the one in the Arabian Sea, at least one in the west and one in the east (Stafford *et al.* 2011).

Almost 3,000 blue whales were taken off Chile from 1926 to 1971, more than a third of them in the 1960s (Aguayo L. 1974). Williams *et al.* (2011) estimated 303 (CI 95% 176–625) blue whales off Chile in December 1997, and Galletti Vernazzani *et al.* (2012) identified 363 different blue whales in this region between 2004 and 2010. The main feeding area for these whales is the nearshore waters off the north-

¹⁰The pygmy blue whale subspecies was assessed as Data Deficient in 1996. Based on a more recent species-level assessment, which included pygmy blue whales, the blue whale is currently listed as Endangered (Reilly *et al.* 2008a).

west coast of Isla Grande de Chiloe (Galletti Vernazzani *et al.* 2012). Ship strikes are a concern for this subpopulation because of the increasing presence in the region of both cargo and cruise ships (Brownell *et al.* 2009, 2014). Recently, a match (both photographic and genetic) was made for a female sampled first near the Galápagos and then in the Gulf of Corcovado, Chile, just south of Chiloe (Torres-Florez *et al.* 2015). This is the first link between the feeding area and the likely the breeding area for this population.

No blue whale calls have been recorded in the South Atlantic, but a few specimens of pygmy blue whales, amongst a predominance of Antarctic blue whales, were taken at South Georgia and logged as “myrbjønners”—described as a “distinct race” of small blue whales with a large quantity of pale spots on the dorsal surface (Mackintosh 1942, Fraser in Ichihara 1966). In the eastern South Atlantic, most of the blue whales taken off the west coast of Africa (Congo, Angola and Namibia, and western South Africa) were Antarctic blue whales; however, based on their length frequencies, a small portion (3.9%) may have been pygmy blue whales (Branch *et al.* 2008 and see Bannister and Grindley 1966).

Blue whales have been documented almost year-round in New Zealand waters through sightings and acoustic recordings (Miller *et al.* 2014; Torres *et al.* 2014; Olson *et al.*, in press). Pygmy blue whales (New Zealand, Call Type 3) are known from both eastern and western coasts of the North Island and the South Island as well as from the South Taranaki Bight (Torres 2013; Torres *et al.* 2014; Olson *et al.*, in press). Feeding behavior has been observed in the Hauraki Gulf and off the eastern and western coasts of the South Island (Olson *et al.*, in press). Limited Soviet catches were made in the 1960s around the North Island and the northern half of the South Island (Mikhalev 2000). A ship strike has been reported off the coast of Auckland (Torres 2013). Also, there has been significant growth in the offshore oil and gas exploration around New Zealand, including frequent and extensive seismic surveys and drilling of test wells (Torres 2013, Torres *et al.* 2014). On the Australian coast of the Tasman Sea blue whale strandings and sightings are rare (Anonymous 1954). Blue whales in this region have not been assessed.

Blue whale sightings around New Caledonia and the Solomon Islands region of the Coral Sea are rare. Ohsumi and Shigemune (1993) reported that 21 groups (41 individuals) were observed near the Solomon Islands in August 1957 and Frank and Ferris (2011) recorded blue whale vocalizations in the Solomon Sea in 1999. However, no blue whales were observed in the region during cruises in 1993 and 1994 (Shimada and Pastene 1995, Goto *et al.* 1995). Blue whale records on the Coral Sea coast of Australia are rare. Illegal Soviet pelagic whaling is known to have occurred around New Zealand during the 1960s and would have depleted the blue whales there, but it is not known if the whales near the Solomons are connected to those in New Zealand. If so, this could explain their absence around the Solomon Islands in the 1960s (IWC 1996).

The blue whales, likely pygmy blue whales, that feed over the Madagascar Plateau in the western Indian Ocean are another enigma. Best *et al.* (2003) estimated 424 pygmy blue whales south of Madagascar from a survey in December 1996. The range of this population is much larger than the area surveyed and therefore that number should not be considered a population estimate. It is likely that the Madagascar whales are part of the population hunted in the western Indian Ocean during the late 1950s and 1960s by both Japanese and Soviet pelagic whaling operations, mainly around the Prince Edward Islands and east to 55°E (Ichihara 1966, Mikhalev 1996).

The northern Indian Ocean (Arabian Sea) blue whale (*B. m. indica*) has not been assessed as no current abundance estimate is available (Anderson *et al.* 2012). During four seasons (1963–1966), illegal Soviet pelagic whaling operations killed 1,294 blue whales off the Seychelles and Maldives, in the Gulf of Aden, and south to the west coast of India and Sri Lanka (Mikhalev 1996). Recent reports of fatal ship strikes on blue whales in the shipping lanes off southern Sri Lanka highlight the importance of assessing this subspecies (De Vos *et al.* 2013). Due to its limited range, heavy past exploitation, and ongoing ship strikes, this may be the most at-risk blue whale population.

Fin Whale

Fin whales were first targeted in the North Atlantic in the 1870s and more than 72,000 were reportedly taken there between 1900 and 1999 (Rocha *et al.* 2014). More than 725,000 and 74,000 were reported as having been killed in the Southern Hemisphere and North Pacific, respectively, between 1905 and 1976 (Rocha *et al.* 2014). As explained in detail by Rocha *et al.* (2014), catch records of fin and sei whales are confounded by the fact that, at times, the Soviet Union overreported catches of fin whales to cover up illegal catches of other species and to make oil production figures consistent in reports to authorities. The Red List assessment used an estimate of 53,000 fin whales in the North Atlantic in 2000, 17,000 in the North Pacific in 1975, and somewhat more than 15,000 in the Southern Hemisphere in 1983 to determine that the global population had declined by more than 70% over the preceding three generations (1929–2007) (Reilly *et al.* 2008c). Fin whales may be increasing in most areas given that they are now protected from commercial whaling in all of their range except off Iceland. Trend data indicate that they are increasing in the North Pacific (Zerbini *et al.* 2006, Moore and Barlow 2011).

The Mediterranean subpopulation, which is redlisted as Vulnerable, is genetically differentiated from fin whales elsewhere in the North Atlantic (Panigada and Notarbartolo di Sciara 2012, also see Castellote *et al.* 2012) and estimated to be in excess of 3,500 animals (Forcada *et al.* 1996). The cumulative effects of a variety of threats in this semienclosed basin (entanglement, anthropogenic noise, ship strikes, pollution) are thought to be inhibiting recruitment and adding to mortality in the Mediterranean subpopulation. Two other areas where fin whales merit assessment at the subpopulation level are the Gulf of California (Mexico) and East China Sea (Reilly *et al.* 2008c). The latter population was heavily exploited by commercial whaling before 1960.

Sei Whale

Hunting of sei whales started in the North Atlantic in the late 1800s off Norway and it continued there until the 1950s and off Iceland mainly after the 1950s and until 1989. They were also hunted off Nova Scotia, Newfoundland, the Shetlands, Hebrides, Færoes, and Spain from land stations. In the North Pacific, sei whales were being taken in northern Japanese waters by 1910 (Andrews 1916) (some of the whales reported were Bryde's whales; Omura 1977) and off California in the 1920s (Clapham *et al.* 1997). With the depletion of blue whales and then fin whales, both pelagic and land station operations started to concentrate on sei whales in the Southern Ocean and North Pacific. Large catches of sei whales (>1,000 per season) were made during pelagic operations in the Southern Hemisphere, beginning in the 1959/1960 season and reaching a peak in the 1964/1965 season when 17,721 were killed (Horwood 1987). The peak catch in the South Atlantic at South Georgia land stations

was 1,183 whales during the 1949/1950 season (Horwood 1987). Populations world-wide were seriously depleted by the mid-1970s. With limited or no survey effort for sei whales in either the Southern Hemisphere or North Pacific, there is no basis to determine whether, or to what extent, populations in either ocean have increased since the end of commercial whaling. The Red List assessment used estimates of 12,000 sei whales in the North Atlantic in 1989, 8,600 in the North Pacific in 1974, and 11,000 in the Southern Hemisphere in 1979 to determine that the global population had declined by more than 70% over the last three generations (1930–2007), thus warranting an Endangered status for the species (Reilly *et al.* 2008*d*).

In 2015 the IWC Scientific Committee accepted a 2010–2012 abundance estimate of 29,632 (CV = 0.242; 95% CI 18,576–47,267) for sei whales in the North Pacific (IWC, in press). However, there is no current agreement on the stock structure of sei whales in the North Pacific. From 2004 through 2013, 100 sei whales were taken per year in Japan's western North Pacific research whaling program called JARPN II (Pastene *et al.* 2009). Starting in 2014, Japan reduced the offshore component of JARPN II from 100 to 90 sei whales (IWC 2015:65).

Bryde's Whale

Catch histories for sei and Bryde's whales are confused because the literature and IWC records lumped them as a single species in early years and catch records in the western North Pacific were only recently segregated by species. Bryde's whales are redlisted as Data Deficient (Reilly *et al.* 2008*e*). From the early 1970s and until fairly recently, they were considered to comprise a single species, *Balaenoptera edeni*, but increasingly *B. edeni* has been used for the small coastal form found in the western Pacific and Indian oceans and *B. brydei* for the larger, more oceanic form found in temperate and tropical waters of the Atlantic, Pacific, and Indian Oceans. LeDuc and Dizon (2002) suggested that these two forms be considered full species. However, Kato and Perrin (2009) noted that the differences between them are closer to what are now considered to be subspecies. A recent genetic analysis of specimens from Oman, the Maldives, Bangladesh, Java (Indonesia), and the northwestern Pacific identified two subspecies: *B. edeni brydei* and *B. edeni edeni* corresponding to the two forms mentioned above (Kershaw *et al.* 2013). The same study distinguished "distinct population units" (which should be considered units for conservation) within each of the subspecies. Similarly, Rosel and Wilcox (2014) found that the small population resident year-round in the northeastern Gulf of Mexico is genetically distinct from other members of the Bryde's whale complex examined to date.

There is a long history of shore-based whaling for Bryde's whales in Japan going back to at least 1910 (Omura 1977) and probably to the start of modern commercial whaling in Japan around 1900. In the North Pacific, Soviet and Japanese factory ships took large numbers of Bryde's whales in the mid 1970s after all the other larger baleen whales had been depleted (IWC 1997, Danner *et al.* 2006). Substantial numbers (848) were taken by illegal pelagic whaling operations in the Arabian Sea during a few years in the 1960s (Mikhalev 2000). In the Southern Hemisphere, mainly between the 1950s and 1970s, large numbers of Bryde's whales were taken by shore-based whaling operations in places such as Iquique and Valparaíso, Chile (Aguayo L. 1974); Paita, Peru (Valdivia *et al.* 1981); Costinha and mainly Cabo Frio, Brazil (Williamson 1975); Cape Lopez, Gabon (Budker 1951); and Durban and Saldanha Bay (Donkergat), South Africa (Olsen 1913, Best 1977). Also in the 1970s, Japan started "research whaling" (under special permits issued by the national government

for 3 yr) in tropical portions of the western Pacific and eastern Indian oceans where they took about 450 whales (Ohsumi 1980). Now research whaling is limited to the northwestern Pacific (50/yr). Bryde's whales are also subject to bycatch and ship strikes in much of their range. As with other species, it is important to manage human activities on the basis of distinct, demographically independent populations such as those provisionally identified in the Maldives, Java, and northwestern Pacific and in the northern Indian Ocean and coastal waters of Japan (Kershaw *et al.* 2013).

Omura's Whale

Omura's whale appears to be restricted to warm-temperate and tropical Indo-Pacific waters on both sides of the equator but the vast majority of records are from the Northern Hemisphere. It is one of the least known baleen whales (Sasaki *et al.* 2006, Yamada 2009); a new population was recently discovered off the northwestern coast of Madagascar (Cerchio *et al.* 2015). The full range of the Omura's whale has yet to be determined, but is thought to be smaller than that of any other baleen whale. Few catch records are available, in part because of the past problems with species identification. The numbers of Omura's whales killed by Japanese operations, including during research whaling in the Indo-Pacific (Wada *et al.* 2003) and possibly by small-type whaling in southwestern Japan, are believed to have been low, but even small levels of removals could have affected small, localized populations. This species is red-listed as Data Deficient (Reilly *et al.* 2008f).

Common Minke Whale

Until the late 1990s, only one species of minke whale was recognized. Most of the scientific literature prior to that time used the name *B. acutorostrata* for all minke whales including Antarctic minke whales. Since 2000, the IWC has recognized the Antarctic minke whale (*B. bonaerensis*) as a separate species (Rice 1998, Brownell *et al.* 2000). In addition, Best (1985) and Arnold *et al.* (1987) identified and described a dwarf form of minke whale in the Southern Hemisphere on the basis of morphology and coloration. In the early 1990s the dwarf minke whale of the Southern Hemisphere was assigned to *B. acutorostrata* based on genetic analyses (Pastene *et al.* 1994), but these dwarf minke whales are not the same as the Northern Hemisphere form of the species and therefore are considered to represent an unnamed and undescribed subspecies (Committee on Taxonomy 2015). Because of their wide distribution in the Southern Hemisphere, dwarf minke whales likely occur as multiple subpopulations and as such they need to be managed and assessed separately. The minke whales in the North Pacific are usually referred to as *B. a. scammoni*, but this subspecies is based on a fossil and more research is needed to understand and describe the populations or subspecies that occur in the North Pacific.

In the North Pacific, common minke whales are best known from the Sea of Okhotsk, Sea of Japan, off the Pacific coast of Japan, the Yellow Sea and the East China Sea. Those in the Sea of Okhotsk and off northern Japan number about 25,000 (CV = 0.316) (Buckland *et al.* 1992). The minke whales within this region are divided by the IWC into two units, O (Okhotsk) stock and J (Japan) stock. The J-stock is found mainly in the East China Sea, Yellow Sea, and Sea of Japan and the O stock is found in the Sea of Okhotsk and off the Pacific coast of Japan. The 25,000 estimate refers mainly to the O-stock.

The autumn-breeding J-stock, centered mainly in coastal waters of the Sea of Japan and along the Pacific coast of Japan, is of the greatest immediate conservation concern. No abundance estimate is available over the entire range of this stock, but various regional surveys indicate a total of around 4,500 animals (Miyashita and Okamura 2011). Small numbers are taken in Japanese research whaling operations and hundreds are taken each year as bycatch in fishing gear off South Korea, Japan, China, and perhaps North Korea. It is important to regard official catch reports critically as genetic analyses indicate that the number of animals actually taken in Japan and South Korea is considerably higher than reported (Baker *et al.* 2007). In addition, each year some level of illegal whaling from J-stock is reported to the IWC by South Korea. The IWC classifies the J-stock as a “Protection Stock” because of its depleted status (IWC 2013c:176), and Red List documentation refers to it as a “distinct subpopulation” (Reilly *et al.* 2008g). However, it has not been assessed for the Red List; therefore assessment of this stock should be a high priority.

All minke whales in the central and eastern North Pacific are currently called the “Remainder” stock by the IWC, but this large region is poorly studied. Minke whales occur in low numbers in tropical waters around the Hawaiian Islands from about November to March. There is no abundance estimate for this region. In the eastern North Pacific, minke whales range from the Bering Sea south to Baja California, Mexico, but they do not appear to be abundant, with only about 2,000 in the central and southeastern Bering Sea (Moore *et al.* 2002) and another 1,000 along the west coast of North America (Forney 2007).

Common minke whales are widespread and abundant in the North Atlantic, numbering perhaps 180,000 (Reilly *et al.* 2008g). Aboriginal subsistence whaling for this species off East and West Greenland (Denmark) is managed under the IWC and commercial whaling by Norway and Iceland is carried out under objection to the IWC commercial moratorium.

The dwarf minke whale was never hunted commercially (Kato and Fujise (2000), but at least a few individuals were taken in the large commercial hunt for Antarctic minke whales from 1971 to 1986. Sixteen dwarf minke whales were reported as taken in Japan’s “scientific” whaling between the 1987/1988 and the 1992/1993 Antarctic whaling seasons (Nishiwaki *et al.* 2005) and all but one of these were killed in latitudes between 62°S and 55°S, the northern limit of Japan’s Antarctic research whaling operations.

Antarctic Minke Whale

The stock structure of Antarctic minke whales is poorly understood, but the aggregate abundance of the species is in the hundreds of thousands. Factory-ship whaling in the Antarctic turned to minke whales in 1971 and nearly 100,000 have been taken there since then. An additional 14,000 were taken at a Brazilian land station (Costinha) from 1964 to 1985 (Reilly *et al.* 2008b). Factory-ship whaling has continued at a lower level since 1987/1988 under special permits issued by Japan, with catches concentrated in certain areas (*e.g.*, IWC Antarctic Areas III, IV, and V). Analyses of two survey programs (CPII 1985/86–1990/91, CPIII 1992/93–2003/04) have sought to determine whether a difference in estimates—around 720,000 (95% CI 512,000–1,012,000) in CPII *vs.* 515,000 (95% CI 361,000–733,000) in CPIII (IWC 2013b)—indicates a real decline in abundance or reflects a problem with survey methodology (IWC 2013b). In 2012 the IWC Scientific Committee concluded that although it could agree on the estimates of abundance “within the areas surveyed in CPII and

CPIII,” it could not reach a conclusion on “whether (and if so to what extent) these numbers indicate a real decline ... between the two periods of the two surveys” (IWC 2013b:29). Further, the committee was “unable to exclude the possibility of a real decline in minke whale abundance between CPII and CPIII” (IWC 2013b:29). Williams *et al.* (2014) discussed the difficulties of detecting abundance trends from open-water surveys in Antarctic waters.

Humpback Whale

The humpback whale is redlisted as Least Concern because of its strong recovery in many parts of its global range (Reilly *et al.* 2008i, Fleming and Jackson 2011), with aggregate numbers currently estimated at 110,000+. This figure is derived from the IWC estimates, by stock, totaling more than 80,000 for the Southern Hemisphere (Table 2; seven stocks, including the Arabian Sea), 10,290–13,390 for the North Atlantic in 1993 (Stevick *et al.* 2003 and see Ryan *et al.* 2014; one stock), and 21,063 for the North Pacific in 2006 (Barlow *et al.* 2011; basin-wide estimate from multiple feeding and breeding areas). Humpback whales continue to be hunted regularly only in Greenland and Bequia (St. Vincent and the Grenadines); in both areas the removals are reported to the IWC and subject to catch limits determined by the IWC Scientific Committee.

The Endangered Arabian Sea subpopulation of humpback whales is geographically, demographically, and genetically discrete (Minton *et al.* 2008, 2011; Rosenbaum *et al.* 2009; Pomilla *et al.* 2014). Its year-round residency in a subtropical region makes it unique among humpback whale populations. Unlike the Southern Hemisphere humpback whales to the south, the Arabian Sea whales are on a Northern Hemisphere breeding cycle (Mikhalev 1997, Minton *et al.* 2011). After being nearly extirpated by illegal Soviet whaling in the 1960s (238 were killed in 1966 alone) (Mikhalev 1997, 2000), population recovery has been hampered by the continuing problem of entanglement in fishing gear and likely also ship strikes (Minton *et al.* 2008, 2011). Based on mark-recapture estimates of fewer than 100 whales along the coast of Oman, Minton *et al.* (2008, 2011) concluded that there were fewer than 250 mature individuals in the entire Arabian Sea.

The Oceania subpopulation of humpback whales (breeding stocks E and F, combined; see Table 2) is redlisted as Endangered because at the time of the most recent formal Red List assessment it was thought to have declined by more than 70% within the preceding three generations (*i.e.*, since 1942) (Jackson *et al.* 2006, Childerhouse *et al.* 2008). In the light of further assessment work by the IWC Scientific Committee, it has become clear that a new assessment is needed. The links between whales migrating past eastern Australia and those migrating past New Zealand and/or New Caledonia as well as their connections with the whales that occur near Tonga and French Polynesia require re-examination (Valsecchi *et al.* 2010, Steel *et al.* 2014). The Oceania subpopulation was estimated to number 4,300 whales in 2005 (Constantine *et al.* 2012). It has been suggested that the rarity of whales in some formerly populous breeding habitats in Oceania (*e.g.*, Fiji) does not necessarily represent a failure to recover, but could instead indicate a redistribution of animals as they seek out more populous mating grounds. This might also explain “above the maximum plausible” rates of increase observed in other places (*e.g.*, eastern Australia) (Clapham and Zerbini 2015).

In part because of the increasing numbers of whales and in part because of concomitant increases in vessel traffic and changes in fishing activity in their habitat,

Table 2. Humpback whale stocks and abundance estimates. (A detailed breakdown for North Pacific subpopulations is in Table S1).

Breeding stock	Area	Estimate	Year of estimate	Citation
Breeding stock A, Western Atlantic Ocean	Brazil	6,300 (95% CI = 4,300–8,600)	2005	Andriolo <i>et al.</i> (2010)
Breeding stock B, Eastern Atlantic	Gabon (substock B1)	6,800 (95% CI = 4,350–10,400)	2005	Collins <i>et al.</i> (2010)
	West South Africa (substock B2)	300 (95% CI = 200–400)	2001	Barendse <i>et al.</i> (2011)
Breeding stock C, Western Indian Ocean	Mozambique (substock C1)	6,000 (95% CI = 4,400–8,400)	2003	Findlay <i>et al.</i> (2011)
	Madagascar (substock C3)	7,500 (95% CI = 2,100–12,700)	2004	Cerchio <i>et al.</i> (2009)
Breeding stock D, Eastern Indian Ocean	Western Australia	28,800 (95% CI = 23,700–40,100)	2008	Hedley <i>et al.</i> (2011)
Breeding stocks E and F, Western/Central South Pacific	Eastern Australia (substock E1)	14,500 (95% CI = 12,700–16,500)	2010	Noad <i>et al.</i> (2011)
Breeding stock G, Eastern South Pacific	Ecuador	6,500 (95% CI = 4,300–9,900)	2006	Felix <i>et al.</i> (2011)
Arabian Sea		80 (95% CI = 60–110)	2004	Minton <i>et al.</i> (2011)
North Atlantic		11,570 (95% CI = 10,290–13,390)	1992/1993	Stevick <i>et al.</i> (2003)
North Pacific		21,063 (95% CI = 20,099–23,552)	2006	Barlow <i>et al.</i> (2011)

ship strikes and entanglements of humpbacks are occurring with greater frequency in many areas, *e.g.*, the western North Atlantic (Robbins *et al.* 2007; Robbins 2008, 2009); Ecuador (Félix *et al.* 2011); the Arabian Sea (Minton *et al.* 2011). It is difficult to separate, measure, and assess the factors most responsible for the high rates of mortality and injury, but there is reason for concern that in some regions at least, human activities are limiting or even preventing population recovery (Fleming and Jackson 2011). Offshore oil and gas development is intensive and expanding in many of the breeding and feeding areas of humpback whales, such as off the west coast of Africa, and this could be a significant source of disturbance to the animals in such areas (Rosenbaum *et al.* 2014).

Besides those in the Arabian Sea, the humpback whales in greatest need of assessment are in the western North Pacific (west of 180°) (Brownell *et al.* 2010, Baker *et al.* 2013) and eastern North Atlantic (IWC 2002). In both areas, numbers and densities are thought to be far below historical levels and we lack sufficient information to evaluate trends. In addition, judging by information in annual progress reports to the IWC, the number of humpback whales that die in fishing gear in Japan appears to be increasing.

In the eastern North Atlantic, there is evidence of substantial whaling for humpback whales around the Cape Verde Islands during the 19th century (Reeves *et al.* 2002). Recent observations suggest that the subpopulation breeding there is far too small to account for the relatively large numbers observed in the central and eastern North Atlantic feeding grounds off Iceland and Norway (Ryan *et al.* 2013, 2014). There has been considerable discussion within the IWC Scientific Committee concerning genetic evidence of at least one additional breeding area (*i.e.*, in addition to the well-known area in the northern Antilles off Hispaniola and Puerto Rico) for humpback whales that feed in the north-central and northeastern North Atlantic. One of these appears to be the Cape Verde Islands but recent evidence suggests a tendency for the whales wintering in the southeastern Caribbean (*e.g.*, around Guadeloupe and Trinidad) to also migrate to feeding grounds (Iceland-Norway) in the northeastern North Atlantic (Stevick *et al.* 2015).

Bowhead Whale

The IWC recognizes four stocks of bowhead whales. The Bering-Chukchi-Beaufort subpopulation has increased steadily over the last 30 yr (3.7% with 95% CI 2.8%–4.7% per year) with a 2011 abundance estimate of 16,892 individuals (95% CI: 15,704–18,928) (Givens *et al.* 2013). The 2002 abundance estimate for the Eastern Canada-West Greenland subpopulation was 6,344 (95% CI 3,119–12,906) (IWC 2009:23), but there is no IUCN assessment for this subpopulation. The species as a whole and the Bering-Chukchi-Beaufort Sea subpopulation are redlisted as Least Concern, but two subpopulations are still considered threatened by IUCN (Reilly *et al.* 2012*a, b, c*; Cetacean Specialist Group 1996*b*).

The Critically Endangered Svalbard-Barents Sea (Spitsbergen) subpopulation was probably the largest of the bowhead whale subpopulations immediately before commercial whaling began in the northeastern Atlantic in 1611. There has been no whaling on this subpopulation for over a century, and it has had little exposure to threats from fishing, shipping, and industrial activity. There is no current estimate of population size (hypothesized to be in the range of 50–120) and no clear or convincing evidence of substantial growth (Reilly *et al.* 2012*c*). Visual and acoustic observations over the past decade do suggest, however, that this subpopulation persists and may

be increasing (Moore *et al.* 2012b, IWC 2014). Boertmann *et al.* (2015) reported a corrected abundance estimate of 102 whales (95% CI 32–329) from an August 2009 aerial survey of the Northeast Water Polynya off the northeastern coast of Greenland.

The Endangered Sea of Okhotsk subpopulation is thought to number only in the low hundreds compared to at least a few thousand prewhaling, and there is no clear evidence on trend (Reilly *et al.* 2012b). The subpopulation was subjected to intensive commercial whaling in the mid 1800s and at least sporadic whaling until 1913 (Ivashchenko and Clapham 2010). In addition, at least 145 were killed illegally by Soviet whalers in 1967 and 1968 (Ivashchenko and Clapham 2012). No additional deliberate kills are known, but at least one whale from this subpopulation is known to have died from entanglement in fishing gear (Brownell 1999). Oil and gas exploration activity is expanding rapidly in the Sea of Okhotsk and this brings risks of various kinds to bowheads (*e.g.*, habitat modification and degradation, noise disturbance, ship strikes).

As mentioned earlier, hunting of this species in Russia, Alaska (United States), and Greenland (Denmark) is managed within the IWC's aboriginal subsistence whaling framework. The hunting of bowhead whales became legal in Arctic Canada in 1991 and in Greenland in 2008. Although Canada is not a member of the IWC, it has agreed to report catches annually, thus enabling the IWC Scientific Committee to incorporate those catches in assessments and catch limits for the Eastern Canada–West Greenland stock as they apply to Greenland, which is a member. Aboriginal whaling is not currently viewed as a population-level threat to this species anywhere in its range.

North Atlantic Right Whale

North Atlantic right whales are functionally extinct in European waters from whaling which started 1,000 yr ago, and the most recent substantial whaling was in the first third of the 20th century on both sides of the North Atlantic (Reeves *et al.* 2007). Most, but not all (Brown *et al.* 2007), of the few European sightings in recent decades have been of individuals previously photo-identified off the U.S. and Canada (Jacobsen *et al.* 2004, Hamilton *et al.* 2007, 2009; Silva *et al.* 2012). The subpopulation in the western North Atlantic is considered Endangered but has been increasing since 2000. At the time of the last Red List assessment (Reilly *et al.* 2008j), the total population (all ages) was believed to number only 300–350, all of them in the western North Atlantic, and there was serious concern about the high rate of ongoing human-caused mortality from ship strikes and entanglement. The total estimated population size (all ages) in 2012 was somewhat more than 500 individuals (Pettis 2013) with an annual increase of 2.8% in the minimum number of live whales between 1990 and 2012.

North Pacific Right Whale

North Pacific right whales have not recovered after their extreme depletion by commercial whaling from 1835 through the 1840s (Josephson *et al.* 2008, Scarff 2001), continued hunting pressure throughout the rest of the 19th century and into the early 20th century, and illegal Soviet whaling on the small remnant population(s) in the 1960s (Brownell *et al.* 2001, Ivashchenko and Clapham 2012, Ivashchenko *et al.* 2013). The last commercial catch was by China in 1977 in the Yellow Sea (Brownell *et al.* 2001). There are thought to be two subpopulations, the eastern,

described mostly from feeding areas in the Bering Sea and offshore in the Gulf of Alaska, and the western, feeding mainly in the Sea of Okhotsk and along the northern Kuriles and off Kamchatka. The species is redlisted as Endangered and the subpopulation in the eastern North Pacific as Critically Endangered because of its very small size and consequent vulnerability to extinction (Reilly *et al.* 2008*k*, *l*). The most recent accounting indicates that 765 right whales were taken by illegal Soviet whaling in the North Pacific¹¹ and this is thought to have removed the bulk of the population in the eastern North Pacific where they are now rarely seen. Genetic and photo-ID mark-recapture analyses each suggest there are only about 30 animals left in the eastern subpopulation, mainly observed in the southeastern Bering Sea and with a male bias to the population (Wade *et al.* 2011, LeDuc *et al.* 2012). There is very little current information on the species in the Gulf of Alaska. There is concern that increased ship traffic through Unimak Pass and in the Bering Sea will put eastern North Pacific right whales at greater risk of ship strikes.

The situation is better in the west, where data obtained during Japanese sighting surveys for minke whales in 1989, 1990, and 1992 led to an abundance estimate of 922 right whales in the Sea of Okhotsk (95% CI: 404–2,108) (Miyashita and Kato 1998, IWC 2001:26). However, the Red List documentation concluded that given the very wide confidence intervals and the lack of clear evidence of any recent increase in numbers, the lower end of the range of that abundance estimate (*i.e.*, about 400) should be used for assessment (Reilly *et al.* 2008*k*). Surveys for large whales in offshore waters east of Hokkaido (Japan) and the Kuril Islands from 1994 to 2013 resulted in 55 sightings of right whales (77 individuals) including ten female/calf pairs (Matsuoka *et al.* 2014), 10 sightings of right whales in five groups were reported in 2012 surveys southeast of the Kamchatka Peninsula and off the Kuril Islands (Sekiguchi *et al.* 2014) and there were 19 sightings of 31 whales in the Russian EEZ (mainly around the northern Kuril Islands, the southern Kamchatka Peninsula and the Commander Islands) between 2003 and 2014 (Ovsyanikova *et al.* 2015). Whales from this subpopulation are occasionally killed incidentally in coastal fishing operations around Japan and Russia (Burdin *et al.* 2004).¹² A highly publicized right whale entanglement in and subsequent escape from mussel mariculture gear occurred in South Korea in February 2015 (Kim *et al.* 2015). All data collected since 1992 in the western North Pacific, especially the Sea of Okhotsk, need to be analyzed for a new abundance estimate so that an assessment can be completed for this subpopulation.

Southern Right Whale

Southern right whales were hunted throughout the Southern Hemisphere by commercial whalers in the 18th and 19th centuries; as few as 400 individuals may have remained in the 1920s by which time the whaling had mostly ended (IWC 2001:24, IWC 2013*a*). Population growth occurred during the first half of the 20th century, but was set back between 1951/1952 and 1970/1971 when Soviet whaling expeditions killed over 3,300 southern right whales illegally (Tormosov *et al.* 1998). Over the past four decades, southern right whales in well-studied subpopulations off southern Africa, in the southwestern Atlantic (off Argentina and Brazil), and off south-central and southwestern Australia have shown relatively high rates of increase (IWC

¹¹Yulia Vladimirovna Ivashchenko, 2363 16th Avenue S, Seattle, WA 98144, 11 February 2015.

¹²Japan Progress Reports to the IWC, <https://iwc.int/scprogress>.

2013a). The heavily exploited subpopulation around the Auckland Islands (New Zealand) has also increased since whaling stopped about 1970 (Carroll *et al.* 2011). The 1997 global estimate of southern right whales was about 7,500 (IWC 2001:23) and in 2009 it was about 13,600 (IWC 2013a). Although the species is now listed as Least Concern (Reilly *et al.* 2008m), at least one subpopulation is still in serious trouble. The Chile-Peru subpopulation in the southeastern Pacific is thought to include fewer than 50 mature individuals and thus is redlisted as Critically Endangered (Reilly *et al.* 2008n). The main concerns for this subpopulation are entanglement in fishing gear and coastal development (Galletti Vernazzani *et al.* 2014). Other subpopulations that have been at least tentatively identified as meriting consideration for threatened status are those off mainland New Zealand, although recent records indicate increasing occupancy of these waters by females with calves originally identified in the subantarctic calving grounds at the Auckland and Campbell Islands (Carroll *et al.* 2014), in the central South Atlantic around Tristan da Cunha, and around Crozet and Kerguelen Islands in the central Indian Ocean (IWC 2013a).

Pygmy Right Whale

The pygmy right whale is the smallest baleen whale with a maximum body length of 6.34 m in females and 6.1 m in males (Budylenko *et al.* 1973), compared to the 7 m length of a *newborn* Antarctic blue whale (Mackintosh and Wheeler 1929). It is also one of the least known species. Its phylogenetic relationships to other baleen whales are enigmatic and it has long been assigned to a monotypic family, Neobalaenidae. However, a recent analysis of morphological characters and molecular data concluded that the species possesses a number of synapomorphies with members of the fossil family Cetotheriidae (Fordyce and Marx 2012). Pygmy right whales are known from cold-temperate waters of the Southern Hemisphere, mainly between 19°S (in the Benguela Current) and 52°S (Kemper 2009), but most strandings and sightings have been in South Africa, New Zealand, and Australia (Kemper 2002). They apparently feed primarily on calanoid copepods (Budylenko *et al.* 1973) and their range overlaps that of sei whales, which also feed mainly on copepods. The species has never been a significant target of commercial whaling. All of what is known about these whales comes from stranded specimens, a few individuals killed during commercial whaling, and at-sea observations. No estimates of abundance are available. The pygmy right whale is classified as Data Deficient on the IUCN Red List (Reilly *et al.* 2008o).

Gray Whale

When assessed as a species, the gray whale is in the Least Concern category (Reilly *et al.* 2008p). Gray whales occurred in the North Atlantic until the late 1600s or early 1700s and perhaps somewhat longer (Mead and Mitchell 1984), but they are now extinct in this ocean basin. In the western and eastern North Pacific, gray whales were hunted commercially from the middle of the 19th century through the early 20th century, by which time both subpopulations had become seriously depleted. Following limited protection from commercial whaling in 1937 and full protection in 1946, the eastern North Pacific subpopulation recovered to a large degree. Estimates of abundance from the southbound migration for the period 2006/2007 to 2010/2011 ranged from 17,820 to 21,210 (IWC 2014:25) and were broadly consistent with previous estimates of between 15,000 and 22,000 (Laake *et al.* 2009, Punt and Wade 2010). This subpopulation may be large enough that the present-day car-

rying capacity is exceeded in years when delayed ice retreat or stochastic environmental processes reduce foraging opportunities in its subarctic and Arctic feeding grounds (Moore *et al.* 2003, Perryman *et al.* 2002, Reilly *et al.* 2008*p*). By contrast, the western subpopulation is redlisted as Critically Endangered (Reilly *et al.* 2008*q*) and has shown little sign of recovery from near-extirpation by commercial whaling which lasted until 1966 in Korea (Brownell and Chun 1977).

Subsistence whaling on the eastern stock of gray whales, which was widespread and at least locally intensive historically (Reeves 2002), continues off Chukotka, Russia, where under the current IWC catch limit 744 whales can be taken over the years 2013 through 2018 with no more than 140 to be taken in any one year (IWC 2014). Under a Russia-U.S. bilateral agreement, most of that quota is allocated to Russia. From 2004 to 2013 an average of 126 (111–143) whales was taken per year in this monitored hunt.¹³ The possibility of a resumed legal hunt of gray whales by the Makah Tribe of Washington State is the subject of ongoing negotiations within the U.S. government (MMC 2014). A major point of concern is how to prevent whales from the Sakhalin feeding group (see below) or the Pacific Coast Feeding Group (PCFG) (again, see below) from being struck by the Makah whalers if and when their hunting resumes.

A small subpopulation of gray whales, numbering about 150, that feeds during the summer and autumn in Russian waters off northeastern Sakhalin Island and southeastern Kamchatka is redlisted as Critically Endangered (Reilly *et al.* 2008*q*). Extreme concern in recent years has centered on the possibility that oil and gas development at Sakhalin could degrade these whales' foraging habitat and cause direct disturbance or even physical harm to them. Deaths of gray whales in recent years in fishing gear around Japan and in Chinese waters have unquestionably slowed, or possibly entirely prevented, the return of gray whales to portions of the species' natural range that are now largely unoccupied. Such mortality demonstrates the vulnerability of the animals that hold the potential for restoring a regular gray whale migration along the coasts of eastern Asia. It was recently discovered that some of the gray whales found off Sakhalin in summer migrate eastward and southward to the wintering areas used by eastern gray whales (Mate *et al.* 2011, Weller *et al.* 2013*a*, IWC 2014). This would seem to indicate that the number of individuals using the species' historical migration route(s) and wintering area(s) along the Asian mainland is even smaller than previously thought. Continued intensive efforts are needed to protect (and further assess) gray whales in the western North Pacific.

The PCFG consists of a small group of whales that forages primarily between southeastern Alaska and northern California, *i.e.*, in an ecosystem distinct from that used by the larger migratory population that feeds mainly in the Bering and Chukchi Seas (Weller *et al.* 2013*b*). Photo-identification and genetic studies indicate that although the PCFG whales interbreed with the other eastern gray whales, they may still comprise a demographically distinct stock (Calambokidis *et al.* 2010, Frasier *et al.* 2011, Lang *et al.* 2011).

SUBSPECIES AND SUBPOPULATIONS NOT YET ASSESSED

In this review, we have identified a number of baleen whale subspecies and subpopulations of conservation concern that have yet to be assessed for the IUCN Red List

¹³http://iwc.int/table_aboriginal.

or that may need to be changed from Data Deficient to some other Red List status. In our view, the following subspecies and subpopulations should be high priorities for IUCN Red List assessment as new data become available:

1. Small populations of “pygmy” type blue whales in numerous parts of the Southern Hemisphere need much more study of population structure, abundance, and trends; and, once sufficient information is available, Red List assessment.
2. The northern Indian Ocean blue whale (Arabian Sea) subspecies, heavily exploited by illegal Soviet whaling in the 1960s, is now of particular concern because of ongoing mortality from ship strikes and the likelihood of entanglement in fishing gear. The lack of information on current numbers is a significant impediment to assessment, but these whales otherwise rank high among global priorities for baleen whale conservation.
3. Gulf of California fin whales are considered discrete from other eastern North Pacific animals and, as occupants of a geographical cul-de-sac, they may be vulnerable to the impacts of climate change on their prey. East China Sea fin whales have long been recognized as a separate population but they have not been assessed by the IWC since they were intensively hunted by Japan up to the 1960s. Current threats are unknown but the most likely is ship strike as this subpopulation inhabits a region with extremely heavy vessel traffic, which is expected to grow rapidly as a result of changes in world trade patterns.
4. The resident subpopulation of Bryde’s whale recently described in the northeastern Gulf of Mexico may be subject to the impacts of intensive oil and gas development and there is concern regarding ship strikes within their limited range.
5. The J-stock of common minke whales in the western North Pacific has long been recognized as a management stock by the IWC. Because it has been studied extensively, this subpopulation’s distinctiveness, discreteness, and geographical distribution are reasonably well known. The combined impacts of past commercial whaling, “scientific” whaling, “bycatch,” and illegal whaling on J-stock minke whales has resulted in their well-documented depletion.
6. Humpback whales in the North Atlantic Ocean constitute one of the most thoroughly studied basin-wide populations of baleen whales. However, the very small subpopulation in the eastern portion of the basin (migrating between the Cape Verde Islands in the winter and Iceland and Norway in the summer) has yet to be assessed. Any entanglements or ship strikes could have a negative impact on this population. Also, the possibility of a third North Atlantic breeding area in the southeastern Caribbean requires more investigation.
7. Humpback whales in the western North Pacific were heavily exploited by Japan until the 1960s. This subpopulation is one of the smallest in the North Pacific and is therefore of particular concern because of ongoing entanglement in fishing gear and possibly ship strikes in one of the world’s fastest-growing marine traffic areas.
8. The central South Atlantic (around Tristan da Cunha) and southwestern and central Indian Ocean subpopulations of southern right whales are small, remote, and little known.
9. The gray whale, certainly one of the most intensively studied baleen whale species, is considered Least Concern at the species level while the western subpopulation is listed as Critically Endangered. The IWC Scientific Committee is carrying out an in-depth investigation of the population structure and conservation status of

North Pacific gray whales (IWC 2014). It will be important for the results of that investigation to be used in a Red List reassessment of gray whales in the western North Pacific as soon as feasible.

Based on the above list, we believe it should be possible to move forward without delay to assess the following subpopulations: J-stock common minke whales, Gulf of Mexico Bryde's whales, and western North Pacific humpback whales.

SUMMARY AND CONCLUSIONS

Among the baleen whales, the right whales remain the group of greatest conservation concern at the species level. Both the North Atlantic and North Pacific right whales are endangered. In the eastern North Atlantic, right whales were effectively extirpated (by whaling) by the early 20th century, and the principal ongoing threats to right whales in the western North Atlantic are immediate and well-documented: entanglement and ship strike. In the North Pacific, there are probably still hundreds of right whales on the western side but only a few tens on the eastern side, where the prognosis for recovery is bleak. North Pacific right whales presumably face the same threats as their North Atlantic congeners (entanglement and ship strike) although it is not certain to what degree. Further research is needed to understand more about their distribution, movements, numbers, and areas where they are most vulnerable to these and other threats (*e.g.*, future offshore oil and gas development and increased northern ship traffic).

While southern right whales are showing strong evidence of recovery in much of their former range, their status in several areas is still of great concern. The very small size of the Chile-Peru subpopulation puts it at high risk from entanglement and ship strike. Very few right whales are present today around the New Zealand mainland, but at least there are encouraging signs that animals from the Auckland Islands are moving in to reestablish the species in that area. Emergent problems, such as the growing attacks by kelp gulls on right whales in Argentina due to increases in the local human population and fishing (Thomas *et al.* 2013), are reminders that the process of recovery can be impeded by unpredictable and unlikely developments even for a population that has been growing steadily for more than 40 years.

Among the rorquals, Mediterranean fin whales have been identified for concern because of the cumulative impacts of human activities in that semienclosed basin. Similar concerns, plus those related to oil and gas development, are likely to apply to the very small population of Bryde's whales in the northern Gulf of Mexico (Rosel and Wilcox 2014). Antarctic blue whales were reduced to such a degree (less than 1%) by commercial whaling in the 20th century that their recovery, even in the absence of whaling, has been very slow. Blue whales have been effectively extirpated from east Asian waters (off Japan) where they were present in good numbers before commercial whaling started about 1900. Other blue whale populations (*e.g.*, northern Indian Ocean (Arabian Sea) and Chile-Peru) are being affected by ship strikes but the population-level consequences of the mortality remain difficult to assess.

Despite encouraging resurgences in most of their range, humpback whales are still not secure in a few areas. In particular, the small population in the Arabian Sea breeds out of phase with more southerly populations and is at considerable risk from entanglement in fishing gear and perhaps ship strikes. Numerous other humpback populations experience substantial mortality from ship strikes and entanglement. In many such cases the population is still increasing, but in other cases the population-level

consequences are uncertain, similar to the situation for the blue whale populations mentioned above. As whale numbers grow, the incidence of ship strikes and entanglements is likely to increase even if vessel traffic and fishing activity in their habitat remains unchanged. It is difficult to separate, measure, and assess the factors most responsible for the high rates of mortality and injury, but there is reason for concern that in some regions at least, human activities are limiting or even preventing population recovery. In this regard, one area of concern is the western North Pacific humpback subpopulation which is subject to bycatch and inhabits a region with high vessel traffic.

In addition to the longstanding threat of entanglement in fishing gear (Clapham *et al.* 1999), the problem of ship strikes has come to be recognized as the other clearest and most direct threat to small baleen whale populations. It will get worse as international commerce continues to expand and as maritime traffic moves into areas formerly difficult or impossible to navigate because of sea ice. Biologically rich straits such as the Bering Strait and the passes through the Aleutian Islands, other places where major shipping routes come near shore, such as the southern tip of Sri Lanka, the waters off China and South Africa, and heavily used ports such as those on the eastern, Gulf of Mexico, and western seaboard of North America, are conflict zones where the ship strike menace is particularly in evidence.

Oil and gas development activities in the Arctic represent an emerging threat to bowhead whales, especially in their feeding range. This is especially worrisome for the small populations in the Sea of Okhotsk and around Svalbard-East Greenland and in the Barents Sea. Oil and gas development on the Sakhalin shelf is of great concern for the small population of gray whales that relies on this area as a summer feeding ground. It is difficult to see how gray whales will be able to expand their effective range in the coastal waters of China, Korea, and Japan, where they were all but extirpated by whaling in the 20th century, unless their feeding habitat in the Sea of Okhotsk and off southern Kamchatka is maintained in a healthy condition and the threats of entanglement and ship-strike are rigorously managed along their migration route(s) and at their wintering grounds.

Chemical contaminants are a general, longstanding concern, but they are not known to be having population-level impacts on baleen whales. This could, however, simply reflect the difficulty of documenting and measuring such impacts. The actual long-term effects of increasing anthropogenic noise are unknown, but in addition to the need to manage the acute impacts of noise, there is growing concern about the potentially serious impacts of chronic stress from noise exposure (Rolland *et al.* 2012), the masking of salient acoustic signals (Clark *et al.* 2009), and the possible displacement of animals from critical habitat (Moore and Huntington 2008). Efforts to assess the cumulative effects of the range of anthropogenic and natural stressors on marine mammals are challenging and require long-term commitment (National Research Council 2005, Schick *et al.* 2013, King *et al.* 2015).

Climate change is already affecting aspects of baleen whale ecology and phenology. For example, bowhead whale populations formerly separated by sea ice in the summer have begun to mingle more frequently in the Northwest Passage and species such as gray, humpback, and fin whales are increasingly observed in high Arctic waters. Annual changes in the distribution of rare animals such as North Atlantic right whales are often hypothesized to be related to changing climatic or oceanic conditions but we often lack the data to draw firm conclusions. Similarly, there are serious concerns that cul-de-sac populations such as the humpback whales and blue whales in the Arabian Sea, bowhead whales in the Sea of Okhotsk, and fin whales in the

Table 3. Baleen whales: species, subspecies, and populations of greatest concern based on this review.

Species/subspecies/subpopulation	Current situation including Red List status	Primary conservation requirements
Bowhead whale (Sea of Okhotsk subpopulation)	Endangered: population size thought to be in the low to mid 100s, only known immediate lethal threat is entanglement	Document distribution and abundance; implement measures to mitigate disturbance from oil/gas activity including seismic surveys
Bowhead whale (Svalbard-Barents Sea subpopulation)	Critically Endangered: population size unknown but few sightings, no immediate lethal threats known	Document distribution and abundance; monitor potential for increasing disturbance from oil/gas activity including seismic surveys
Southern right whale (Chile-Peru/SE Pacific subpopulation)	Critically Endangered: current population likely <50, most immediate lethal threat entanglement and they are at risk of ship strikes in much of their range	Characterize and mitigate bycatch risk (from coastal artisanal and commercial fisheries) and risk of ship strikes (ship traffic is heavy in some parts of range)
North Atlantic right whale	Endangered: total population ~500, extirpated from eastern portion of range, mortality and injury from entanglements and ship strikes well documented in United States and Canada	Continued and strengthened measures to reduce ship strike and entanglement risks
North Pacific right whale (western subpopulation)	Not assessed separately but species is Endangered: hundreds in the Sea of Okhotsk and Kamchatka/Kuriles portion of range but still far below level of precommercial whaling	Document distribution and abundance; evaluate risk of ship strikes, entanglements, and disturbance from oil/gas activity including seismic surveys
North Pacific right whale (eastern subpopulation)	Critically Endangered: current population estimated at around 30, considerable risk of ship strikes suspected but not well documented	Document year-round distribution and evaluate risk of ship strikes from increased ship traffic and entanglements
Common minke whale (western North Pacific "J-Stock")	Not assessed: Autumn-breeding stock centered in Sea of Japan, numbers in thousands, substantial "bycatch" (100s/yr) in Japan, Korea, and China (but not reported)	Characterize and mitigate "bycatch" risk in Japan, Korea, and China; eliminate illegal whaling

(Continued)

Table 3. (Continued)

Species/subspecies/subpopulation	Current situation including Red List status	Primary conservation requirements
Antarctic blue whale	Critically Endangered: 1997 population (2,280) around 1% of pre-exploitation level (239,000), but increasing, no immediate lethal threats known	Document abundance and distribution; continue protection from whaling
Pygmy blue whale populations (e.g., East South Africa, Madagascar to Kerguelen, South Atlantic, Chile-Peru, New Zealand, and Southwest Pacific: New Caledonia, and Solomon Islands)	Subspecies Data Deficient and all subpopulations not assessed: all presumed to be less than 1,000.	Document subpopulation characteristics and threats, especially entanglement, ship strikes and oil and gas development
Northern Indian Ocean blue whale	Not assessed: small distinct population mainly in northern Indian Ocean between Gulf of Aden and South Asian subcontinent, breeds 6 mo out of phase with populations in the Southern Hemisphere	Document abundance and year-round distribution; implement measures required to reduce ship strike risk
Fin whale (Mediterranean subpopulation)	Vulnerable: population size <5,000 mature individuals, declining	Mitigate risks from ship strike, entanglement, ship and other noise
Bryde's whale (northeastern Gulf of Mexico subpopulation)	Not assessed: small distinct subpopulation resident year-round in the northeastern Gulf of Mexico	Document subpopulation characteristics and threats and mitigate risks from intensive oil and gas development and potential ship strikes
Humpback whale (Arabian Sea subpopulation)	Endangered: abundance estimate in Oman 82 (95% CI = 60–111), breeds 6 mo out of phase with populations to south	Document abundance and year-round distribution; implement measures to reduce entanglement (and ship strike) risk
Gray whale (western subpopulation)	Critically Endangered: gray whales at Sakhalin Island comprise a discrete subpopulation of about 150 animals or less; at least part of this subpopulation migrates annually to west coast of North America including Mexican breeding lagoons	Continue and strengthen measures to mitigate disturbance from oil and gas activity, ship and other noise, and reduce bycatch risk in set nets in Japan and China

Mediterranean Sea, East China Sea, and Gulf of California are habitat-limited and will suffer as ocean temperatures rise and their prey base is altered.

The population-level consequences of ongoing environmental changes stemming from climate change and ocean acidification, *e.g.*, in ecosystem structure, prey availability, and access to seasonally occupied habitat, remain uncertain and difficult to characterize. Intensive research, including the development and application of new methods, is needed to gain a better understanding of those consequences. At the same time, there is a pressing need for more “baseline” data on the historical and present-day ranges, seasonal movements, and habitat requirements of baleen whales against which to assess impacts and decide where mitigation is most warranted. The large differences among countries in their resources available to support scientific assessment and management action, even when it comes to tackling the most basic threats of entanglement and ship strike, mean that regional or broader international cooperation is needed. Such cooperation may be especially urgent when endemic or highly localized populations are under threat.

Future conservation of baleen whales must focus at the population level to be meaningful. This requires a much better understanding of the population structure and abundance of all species so that we can better assess and manage the population-level impacts of the threats they face. Such research is bound to add to the list of species, subspecies, and subpopulations of greatest concern (Table 3).

ACKNOWLEDGMENTS

We thank the Marine Mammal Commission for supporting this review. In particular, we thank former Executive Director Tim Ragen for initiating and championing the idea of such a global synthesis and current Executive Director Rebecca Lent for supporting its conclusion. We thank Daryl Boness, Frances Gulland, Michael Tillman, and David Laist for their reviews of earlier versions. William Perrin, Brian Smith, and Mark Richardson provided guidance at the outset of this effort. We thank Phil Clapham, Peter Corkeron, and other reviewers whose comments greatly improved the quality and content of the manuscript.

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Received: 22 January 2015

Accepted: 18 September 2015

SUPPORTING INFORMATION

The following supporting information is available for this article online at <http://onlinelibrary.wiley.com/doi/10.1111/mms.12281/supinfo>.

Table S1. Baleen whales species, subspecies, and subpopulations: Current situation, primary and potential, or emerging threats.

Table S1. Baleen whales species, subspecies, and subpopulations: Current situation, primary and potential, or emerging threats.

Cetacean Species/subspecies/sub-population	Red List or other classification	Current situation	Primary threats	Potential, low-level, or long-term threats
Blue whale (<i>Balaenoptera musculus</i>)	EN	HCW including illegal Soviet whaling in the mid-20th century; cosmopolitan; Red List assessment suggests 2007 global population was in the range of 10,000 to 25,000 corresponding to about 3%–11% of the 1911 population size	Ship strikes in some areas, ship and other noise	Some populations inhabiting waters with high levels of human activity are at risk from entanglement and noise disturbance; climate change-induced shifts in krill availability vary greatly by region
Blue whale (<i>B. m. musculus</i>)	SMM	HCW; combined abundance for North Pacific and North Atlantic is no more than about 5,000		Ship strikes and disturbance from vessel traffic, including ship and other noise; entanglement; climate change-induced shifts in krill availability vary greatly by region
Blue whale (<i>B. m. musculus</i>) North Atlantic stock	IWC	HCW; subsumed by SMM under <i>B. m. musculus</i> . Several localized feeding stocks or concentrations (e.g., Gulf of St. Lawrence, Norwegian, Icelandic, and Shetland/Hebrides/Ireland grounds) with song common to entire basin (McDonald <i>et al.</i> 2006)		Ship strikes and disturbance from vessel traffic, including ship and other noise (e.g., Gulf of St. Lawrence); entanglement; climate change-induced shifts in krill availability vary greatly by region
Blue whale (<i>B. m. musculus</i>) North Pacific stock	IWC	HCW including illegal Soviet whaling in the mid-20th century; subsumed by SMM under <i>B. m. musculus</i> . Up to five subpopulations suggested (i) southern Japan (which appears to have been virtually extirpated by whaling); (ii) Kamchatka; (iii) Aleutian Islands (the central stock, which may winter in deep water north of Hawaii); (iv) eastern Gulf of Alaska; and (v) California/Mexico		Ship strikes and disturbance from vessel traffic, including ship and other noise (e.g., Port of Los Angeles); entanglement; climate change-induced shifts in krill availability vary greatly by region
Antarctic blue whale (<i>B. m. intermedia</i>)	CR	HCW including illegal Soviet whaling in the mid-20th century; 1997 population (2,280) around 1% of pre-exploitation level (239,000), but increasing; management by IWC Antarctic Management Areas recognizes the likely existence of multiple stocks although song and tagging data indicate wide mixing across these areas (Branch <i>et al.</i> 2007, , Le Duc <i>et al.</i> 2007); winter distribution poorly known		Potential prey depletion by large-scale krill fishery; climate change-induced shifts in krill availability vary greatly by region
Pygmy blue whale (<i>B. m. brevicauda</i>)	DD	HCW including illegal Soviet whaling in the mid-20th century; multiple stocks centered in sub-Antarctic (e.g., East South	None known	Entanglement; ship strikes, ship and other noise; climate

		Africa, Madagascar to Kerguelen, South Atlantic, Chile-Peru [see below], New Zealand, and southwest Pacific–New Caledonia and Solomon Islands [Brownell <i>et al.</i> 2015])		change-induced shifts in krill availability vary greatly by region
Northern Indian Ocean blue whale (<i>B. m. indica</i>)	SMM	HCW including illegal Soviet whaling in the mid-20th century (>1,200); small distinct population mainly in the northern Indian Ocean between the Gulf of Aden and the South Asian subcontinent (Sri Lanka); breeds 6 mo out of phase with Antarctic blue whales	Ship strikes	Entanglement; ship and other noise; climate change-induced shifts in krill availability vary greatly by region
Blue whale (<i>B. m. unnamed</i>) Chilean	IWC	HCW; 2010 abundance estimate 900; distinct population of intermediate size and genetics between Antarctic and pygmy subspecies found off Chile and Peru; does not migrate to Antarctic		Ship strikes; entanglement; ship and other noise; climate change-induced shifts in krill availability vary greatly by region
Fin whale (<i>Balaenoptera physalus</i>)	EN	HCW including illegal Soviet whaling in the mid-20th century; Red List assessment model suggests global population declined from 400,000 to 100,000 over the period 1929–2007; aboriginal subsistence whaling in Greenland, annual strike limit of 16. Substantial catches by Iceland under special permit in 1986–1989 and commercial whaling under IWC reservation by Iceland (7 taken in 2007, 125 in 2010, 148 in 2011, and 134 taken in 2013). Several subspecies and subpopulations (<i>e.g.</i> , Gulf of California, East China Sea) need further conservation attention		Ship strikes; entanglement; ship and other noise; climate change-induced shifts in prey availability vary greatly by region
Fin whale (Mediterranean subpopulation)	VU	Vulnerable listing based on total population of less than 5,000 mature individuals and declining	Ship strikes, entanglement, ship and other noise	See species level
Northern fin whale (<i>B. p. physalus</i>)	SMM	HCW including illegal Soviet whaling in the North Pacific during the mid-20th century (Pacific stocks only)	See species level	See species level
Northern fin whale (<i>B. p. physalus</i>) North Atlantic stocks	IWC	HCW; subsumed by SMM under <i>B. p. physalus</i> ; abundance in central and eastern North Atlantic was about 22,000 in 2007 (IWC 2014:101). IWC recognizes 7 stocks (Nova Scotia, Newfoundland-Labrador, West Greenland, East Greenland-Iceland, North Norway, West Norway-Faroe Islands, British Isles-Spain and Portugal) [with some mixing between them]; aboriginal subsistence whaling in Greenland and substantial commercial and special permit catches by Iceland	See species level	See species level
Northern fin whale (<i>B. p. physalus</i>) North Pacific stocks	IWC	HCW including illegal Soviet whaling in the mid-20th century; subsumed by SMM under <i>B. p. physalus</i> ; North Pacific stocks have not recently been assessed. IWC recognizes 2 stocks (East China Sea, North Pacific). Single North Pacific stock unlikely	See species level	See species level
Southern fin whale (<i>B. p. quoyi</i>)	SMM	HCW including illegal Soviet whaling in the mid-20th century; although most recent estimate in Red List documentation is around 15,000 for 1983, numbers are likely in the tens of thousands; still far below prewhaling abundance; management by	See species level	See species level

Pygmy fin whale (<i>B. p. patachonica</i>)	SMM	IWC Antarctic Management Areas recognizes the likely existence of multiple stocks HCW in Peru and Chile; recently described; distribution uncertain but thought to be primarily in low latitudes of eastern South Pacific	See species level	See species level
Humpback whale (<i>Megaptera novaeangliae</i>)	LC	HCW including illegal Soviet whaling in the mid-20th century. Global trend of increasing numbers and return to historic range, but concerns remain about subpopulations in Arabian Sea, western North Pacific, and Oceania; no global population estimate, but partial estimates total more than 110,000. Subsistence whaling in Greenland under IWC annual strike limit of 9 for 2010–2012; St. Vincent and the Grenadines 5 yr catch limit of 20 (total) for 2008–2012		Entanglement; ship and other noise; disturbance from oil/gas; ship strikes; climate change-induced shifts in prey availability vary greatly by region
Humpback whale North Atlantic stock (<i>M. n. novaeangliae</i>)	IWC, SMM	HCW; 5 principal feeding aggregations (Gulf of Maine, Eastern Canada, West Greenland, Iceland, Norway); all known feeding grounds represented in West Indies breeding ground. Feeding location of animals wintering near Cape Verde Islands (northwest Africa) uncertain although photo-id match recorded with Iceland. Connection between Southeast Caribbean breeding ground and Iceland–Norway feeding ground. 1992–1993 abundance <i>ca.</i> 10,290–13,390 is old and considered an underestimate. IWC Protected Stock; Aboriginal subsistence whaling in Greenland under IWC annual strike limit of 9 for 2010–2012 and in St. Vincent and the Grenadines under 5 yr IWC take limit of 20 (total) for 2008–2012	See species level	See species level
Humpback whale North Pacific stock (<i>M. n. kuzira</i>)	IWC, SMM	HCW including illegal Soviet whaling in the mid-20th century; IWC Protected Stock since 1966		See species level
Humpback whale North Pacific Central America	Baker <i>et al.</i> 2013, Fleming and Jackson, 2011	HCW including illegal Soviet whaling in the mid-20th century; abundance <i>ca.</i> 500 (Barlow <i>et al.</i> 2011)		See species level
Humpback whale North Pacific Mexico	Baker <i>et al.</i> 2013, Fleming and Jackson, 2011	HCW including illegal Soviet whaling in the mid-20th century; abundance 6,000–7,000 (Barlow <i>et al.</i> 2011)		See species level
Humpback whale North Pacific Hawaii	Baker <i>et al.</i> 2013, Fleming and Jackson, 2011	HCW including illegal Soviet whaling in the mid-20th century; abundance near 10,000 (Barlow <i>et al.</i> 2011)		See species level
Humpback whale North Pacific Okinawa/Philippines	Baker <i>et al.</i> 2013, Fleming and Jackson, 2011	HCW including illegal Soviet whaling in the mid-20th century; combined abundance of North Pacific Okinawa/Philippines and “second west Pacific” <i>ca.</i> 1,000 (Barlow <i>et al.</i> 2011)		See species level

Humpback whale North Pacific “second west Pacific Discrete population segment”	Baker <i>et al.</i> 2013, Fleming and Jackson, 2011	HCW including illegal Soviet whaling in the mid-20th century; combined abundance of North Pacific, Okinawa/Philippines and “second west Pacific” <i>ca.</i> 1,000 (Barlow <i>et al.</i> 2011)	See species level
Southern Hemisphere humpback whale (<i>M. n. australis</i>)	SMM	HCW including illegal Soviet whaling in the mid-20th century	
Humpback whale (Arabian Sea subpopulation)	EN	HCW including illegal Soviet whaling in the mid-20th century; unlike most humpback whale populations, no increase has been detected since illegal whaling ended in the 1960s; a 2008 photographic mark-recapture abundance estimate in Oman was 82 (95% CI = 60–111); breeds 6 mo out of phase with populations to the south	Ship strikes; ship and other noise; disturbance from oil/gas activity; oil spills; climate change-induced shifts in prey availability
Humpback whale (Oceania subpopulation)	EN	HCW including illegal Soviet whaling in the mid-20th century; redlisted subpopulation consists of IWC breeding stocks E and F. Basis for Endangered listing is estimated decline of >70% over last 3 generations (Jackson <i>et al.</i> 2006)	Entanglement; ship and other noise
Humpback whale Southern Hemisphere Breeding Stock A – Brazil	IWC	HCW including illegal Soviet whaling in the mid-20th century; 2005 abundance <i>ca.</i> 6,800	
Humpback whale Southern Hemisphere Breeding Stock B – West Africa	IWC	HCW including illegal Soviet whaling in the mid-20th century; substock B1 (Gabon) 2005 abundance <i>ca.</i> 6,800; substock B2 (W. South Africa) 2001 abundance <i>ca.</i> 300	
Humpback whale Southern Hemisphere Breeding Stock C – East Africa	IWC	HCW including illegal Soviet whaling in the mid-20th century; substock C1 (Mozambique) 2003 abundance <i>ca.</i> 6,000; substock C3 (Madagascar) 2004 abundance <i>ca.</i> 7,500	
Humpback whale Southern Hemisphere Breeding Stock D – Western Australia	IWC	HCW including illegal Soviet whaling in the mid-20th century; 2008 abundance <i>ca.</i> 28,800	
Humpback whale Southern Hemisphere Breeding Stock E – Eastern Australia	IWC	HCW including illegal Soviet whaling in the mid-20th century; substock E1 (east Australia) 2010 abundance <i>ca.</i> 14,500;	
Humpback whale Southern Hemisphere “Oceania” Breeding Stock	IWC	HCW including illegal Soviet whaling in the mid-20th century; combined abundance of substocks E2 (New Caledonia), E3 (Tonga), and F (French Polynesia) abundance <i>ca.</i> 4,300 in 2005	
Humpback whale Southern Hemisphere	IWC	HCW including illegal Soviet whaling in the mid-20th century; 2006 abundance <i>ca.</i> 6,500	

Breeding Stock G – Ecuador					
Sei whale (<i>Balaenoptera borealis</i>)	EN	HCW; Red List assessment model suggests global numbers declined from about 135,000 to 30,000 over the period 1937–2007. Whaling by Japan in Western North Pacific under special permit (100/yr) and smaller numbers in the North Atlantic by Iceland (1986–1989)			Baleen disease in eastern North Pacific; ship strikes; loss of sea-ice; climate change-induced shifts in prey availability vary greatly by region
Northern sei whale (<i>B. b. borealis</i>)	SMM	See species level; HCW; multiple stocks recognized by IWC: 3 in the North Atlantic and 2 in the North Pacific			Baleen disease in eastern North Pacific; ship strikes
Northern sei whale (<i>B. b. borealis</i>) North Atlantic stocks	IWC	Subsumed by SMM under <i>B. b. borealis</i> . IWC recognizes 3 stocks (Nova Scotia, Iceland-Denmark Strait, Eastern Atlantic); whaling by Iceland under special permit in 1986–1989			
Northern sei whale (<i>B. b. borealis</i>) North Pacific stocks	IWC	Subsumed by SMM under <i>B. b. borealis</i> . IWC recognizes 2 stocks (Eastern and Western); whaling by Japan in Western North Pacific under special permit (100/yr)			See species level
Southern sei whale (<i>B. b. schlegellii</i>)	SMM	HCW; See species level; management by IWC Antarctic Management Areas recognizes the likely existence of multiple stocks			See species level
Bryde's whale (<i>Balaenoptera edeni</i>)	DD	HCW in limited areas and whaling by Japan under special permits in various areas starting in the 1970s; some regional abundance estimates exist but global numbers and trends unknown; whaling by Japan in North Pacific under special permit (50/yr); management by IWC recognizes the likely existence of 7 southern hemisphere stocks			Ship strikes; entanglement; disturbance from oil/gas activity including seismic surveys in various parts of their range; climate change-induced shifts in prey availability vary greatly by region
Bryde's whale (<i>B. e. edeni</i>) (smaller coastal form)	SMM	HCW off southern Japan likely included this subspecies; limited to tropical and warm temperate waters in Indian Ocean and western Pacific			See species level
Bryde's whale (<i>B. e. brydei</i>) (larger oceanic form)	SMM	HCW including illegal Soviet whaling in the mid-20th century; ongoing whaling by Japan in the western North Pacific under special permit (50/yr); see species level			See species level
Omura's whale (<i>Balaenoptera omurai</i>)	DD	Historically taken in artisanal hunt in Bohol Sea (Philippines) and by Japan under special permit (for Bryde's whales) in Solomon Sea and off Java in 1976–1977; HCW off southern Japan could have included this species; this and the pygmy right whale are the least known baleen whale species; only known to occur in the tropical and warm temperate Indo-Pacific			Entanglement (entrapment in set nets in Japan); ship and other noise
Common minke whale (<i>Balaenoptera acutorostrata</i>)	LC	HCW; no estimate of global population size but aggregate numbers are ≥200,000; regulated whaling in a few areas			Entanglement; climate change-induced shifts in prey availability vary greatly by region
North Atlantic minke whale	SMM	HCW; widespread and abundant, numbers well over 100,000. Aboriginal/subsistence catches off of East and West Greenland			Entanglement; climate change-induced shifts in prey

<i>(B. a. acutorostrata)</i>		managed under IWC. Commercial coastal whaling by Norway and Iceland under objection to moratorium			availability vary greatly by region
North Pacific minke whale <i>(B. a. scammoni)</i>	SMM	HCW; no basin-wide abundance estimate but at least low tens of thousands. Catches by Japan in western North Pacific under special permits		Entanglement (substantial “entanglement” in both Japan and South Korea (100s/yr) and likely in China); illegal whaling in South Korea	Climate change-induced shifts in prey availability vary greatly by region
North Pacific minke whale (Sea of Japan-Yellow Sea-East China Sea stock or “J-Stock”)	IWC	HCW; autumn-breeding stock centered in Sea of Japan; numbers in thousands; IWC classifies as “protection stock” because of its depleted condition		Entanglement (substantial “entanglement” (100s/yr). Some of the entanglement in Japan and all entanglement in South Korea and probably China includes a significant proportion of J-stock whales)	Climate change-induced shifts in prey availability vary greatly by region
Dwarf minke whale <i>(B. a. unnamed subsp.)</i>	SMM	HCW; no estimate of abundance; patchy in various locations in the Southern Hemisphere range, but best known in Great Barrier Reef			Disturbance by tourism; climate change-induced shifts in prey availability vary greatly by region
Antarctic minke whale <i>(Balaenoptera bonaerensis)</i>	DD	HCW (1971–1986); stock structure poorly understood, takes concentrated in certain areas (e.g., IWC Antarctic Areas III, IV, V) total numbers in the hundreds of thousands. Factory ship whaling continues (since 1987/1988 under special permit issued by Japan)			Loss of sea-ice and climate change-induced shifts in prey availability vary greatly by region
Bowhead whale <i>(Balaena mysticetus)</i>	LC	HCW; global population is increasing, due primarily to the increases in the relatively large Bering-Chukchi-Beaufort and Eastern Canada-West Greenland subpopulations; ongoing subsistence hunts are managed and considered sustainable		Disturbance from oil/gas activity including seismic surveys	Ship strikes; entanglement; ship and other noise; oil spills; loss of sea-ice and climate change-induced shifts in prey availability vary greatly by region
Bowhead whale (Bering-Chukchi-Beaufort Sea subpopulation)	LC	HCW; subpopulation is increasing and may be approaching precommercial whaling level (ca. 17,000 in 2011, increasing at 3.5%/yr); ongoing subsistence hunts in Alaska and Russia are managed and considered sustainable		Disturbance from oil/gas activity including seismic surveys	Ship strikes; entanglement; ship and other noise; oil spills; loss of sea-ice and climate change-induced shifts in prey availability vary greatly by region
Bowhead whale (Okhotsk Sea subpopulation)	EN	HCW including illegal Soviet whaling in the mid-20th century but not hunted since then; population size thought to be in the low 100s		Disturbance from oil/gas activity including seismic surveys	Ship strikes; entanglement; ship and other noise; oil spills; loss of sea-ice and climate change-induced shifts in prey availability vary greatly by region
Bowhead whale (Svalbard-Barents Sea subpopulation)	CR	HCW; historically the largest subpopulation but few sightings			Ship strikes; entanglement; ship and other noise; oil spills; loss of sea-ice and climate

					change-induced shifts in prey availability vary greatly by region
Bowhead whale (Eastern Canada–West Greenland subpopulation)	NA	HCW; provisional estimate of 7,000 for part of range in early 2000s; ongoing subsistence hunts in Greenland and Canada are managed and considered sustainable		Disturbance from oil/gas activity including seismic surveys	Ship strikes; entanglement; ship and other noise; oil spills; loss of sea-ice and climate change-induced shifts in prey availability vary greatly by region
North Atlantic right whale (<i>Eubalaena glacialis</i>)	EN	HCW; current population of close to 500 (all ages) off the coast of N. America, considered well below precommercial whaling level		Ongoing mortality from entanglement and ship strikes	Disturbance from oil/gas activity including seismic surveys; ship and other noise; climate change-induced shifts in prey availability vary greatly by region
North Atlantic right whale (Eastern North Atlantic Stock)	IWC	HCW; functionally extinct			
North Atlantic right whale (Western North Atlantic Stock)	IWC	See species level		See species level	See species level
North Pacific right whale (<i>Eubalaena japonica</i>)	EN	HCW including illegal Soviet whaling in the mid-20th century; current population is far below precommercial whaling level			Entanglement; ship strikes; disturbance from oil/gas activity including seismic surveys; ship and other noise; climate change-induced shifts in prey availability vary greatly by region
North Pacific right whale (Western subpopulation)	NA	HCW including illegal Soviet whaling in the mid-20th century; subpopulation is far below precommercial whaling level; at least 400 in the Okhotsk Sea portion of range			Entanglement; ship strikes; ship and other noise; reduced prey availability related to climate change
North Pacific right whale (Eastern subpopulation)	CR	HCW including illegal Soviet whaling in the mid-20th century; the few animals observed today are often solitary and scattered, except for a small recurring aggregation in southeastern Bering Sea; current population is about 30			Entanglement; ship strikes; disturbance from oil/gas activity including seismic surveys; ship and other noise; climate change-induced shifts in prey availability vary greatly by region
Southern right whale (<i>Eubalaena australis</i>)	LC	HCW including illegal Soviet whaling in the mid-20th century (except for Chile-Peru subpopulation); following severe depletion by whaling, several subpopulations are increasing (IWC recognizes multiple stocks, all of which are thought be increasing except Chile-Peru/SE Pacific)			Entanglement; ship strikes; climate change-induced shifts in krill availability

Southern right whale (Chile-Peru/SE Pacific subpopulation)	CR	HCW (but no Soviet whaling in the mid-20th century); paucity of sightings over past 50 yr indicates current population size (number mature) is probably <50.	Entanglement (high density of coastal artisanal and commercial fisheries); ship strikes (heavy ship traffic in some parts of range)	See species level
Southern right whale Southwest Atlantic	IWC	HCW including illegal Soviet whaling in the mid-20th century mainly in the western South Atlantic; increasing; Calving grounds Argentina and Brazil		See species level; significant unexplained calf mortality at Peninsula Valdés, Argentina
Southern right whale South Central Atlantic	IWC	HCW including illegal Soviet whaling in the mid-20th century; Tristan da Cunha		See species level
Southern right whale Southern Africa	IWC	HCW including illegal Soviet whaling in the mid-20th century; increasing; South Africa, Namibia, Mozambique/Madagascar		See species level
Southern right whale Southwest Pacific	IWC	HCW including illegal Soviet whaling in the mid-20th century; Sub-Antarctic New Zealand, mainland New Zealand		See species level
Southern right whale Australia	IWC	HCW including illegal Soviet whaling in the mid-20th century; increasing in south central and western Australia, low numbers in eastern Australia		See species level
Pygmy right whale (<i>Caperea marginata</i>)	DD	This and Omura's whale are the least known baleen whale species; no estimates of abundance, found only in Southern Hemisphere		Climate change-induced shifts in prey availability vary greatly by region
Gray whale (<i>Eschrichtius robustus</i>)	LC	HCW (19th and 20th centuries); eastern subpopulation has made a strong recovery from past exploitation; ongoing aboriginal subsistence hunting (nearly all in Russia) is managed and considered sustainable with 5 yr IWC catch limit of 620		Entanglement; ship strikes; ship and other noise; disturbance from oil and gas activity; climate change-induced shifts in prey availability vary greatly by region
Gray whale (Eastern North Pacific stock)	IWC	See species level	See species level	See species level
Gray whale (western subpopulation)	CR	HCW; until recently gray whales at Sakhalin Island thought to comprise discrete subpopulation of about 150 animals; recent satellite tagging, photo-identification, and genetic matching indicate a more complex situation as at least part of this subpopulation migrates annually to west coast of North America including Mexican breeding lagoons	Disturbance from oil and gas activity, ship and other noise; entanglement in Japan (5 whales known to have died in fishing gear along Pacific coast of Japan from 2005 to 2007; in net and/or trap fisheries) and China	Ship strikes

Note: Under Red List classification, CR = Critically Endangered, EN = Endangered, VU = Vulnerable, NT = Near Threatened, LC = Least Concern, DD = Data Deficient, and NA = not assessed for the Red List but identified in Red List documentation as a potential conservation unit. SMM = identified in SMM Taxonomy List but NA for the Red List, HCW = Historical commercial whaling (see text and note: illegal Soviet whaling took place from 1948 to 1979 in the North Pacific and from 1946 to 1986 in the Southern Hemisphere, especially Antarctica; Ivaschenko and Clapham 2014). IWC = recognized as a stock or management unit by the Scientific Committee of the International Whaling Commission but NA for the Red List. Primary threats = threats known to have population level consequences or the potential to have such consequences if unmanaged.

EXHIBIT

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Quantifying risk of whale–vessel collisions across space, time, and management policies

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Citation: Crum, N., T. Gowan, A. Krzystan, and J. Martin. 2019. Quantifying risk of whale–vessel collisions across space, time, and management policies. *Ecosphere* 10(4):e02713. 10.1002/ecs2.2713

Abstract. Transportation industries can negatively impact wildlife populations, including through increased risk of mortality. To mitigate this risk successfully, managers and conservationists must estimate risk across space, time, and alternative management policies. Evaluating this risk at fine spatial and temporal scales can be challenging, especially in systems where wildlife–vehicle collisions are rare or imperfectly detected. The sizes and behaviors of wildlife and vehicles influence collision risk, as well as how much they co-occur in space and time. We applied a modeling framework based on encounter theory to quantify the risk of lethal collisions between endangered North Atlantic right whales and vessels. Using Automatic Identification System vessel traffic data and spatially explicit estimates of right whale abundance that account for imperfect detection, we modeled risk at fine spatiotemporal scales before and after implementation of a vessel speed rule in the southeastern United States. The expected seasonal mortality rates of right whales decreased by 22% on average after the speed rule was implemented, indicating that the rule is effective at reducing lethal collisions. The rule's effect on risk was greatest where right whales were abundant and vessel traffic was heavy, and its effect varied considerably across time and space. Our framework is spatiotemporally flexible, process-oriented, computationally efficient and accounts for uncertainty, making it an ideal approach for evaluating many wildlife management policies, including those regarding collisions between wildlife and vehicles and cases in which wildlife may encounter other dangerous features such as wind farms, seismic surveys, or fishing gear.

Key words: encounter theory; *Eubalaena glacialis*; North Atlantic right whale; spatial modeling; speed restrictions; speed zones; whale–vessel collision; wildlife–vehicle collision.

Received 13 November 2018; revised 4 March 2019; accepted 15 March 2019. Corresponding Editor: Hunter S. Lenihan.

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INTRODUCTION

The transportation industry creates many problems for wildlife, including altered movement patterns and home ranges, decreased reproductive success and gene flow, and increased mortality through wildlife–vehicle collisions (Trombulak and Frissel 2000). Traffic from human transportation has the most negative population-level impacts on wildlife species that move long distances, have low reproductive

rates, and do not avoid transportation networks or vehicles (Fahrig and Rytwinski 2009). These problems have been studied extensively across many taxa in terrestrial systems (e.g., road ecology; Forman and Alexander 1998, Trombulak and Frissel 2000). In marine systems, they have received less attention, mostly related to collisions between vessels and cetaceans (Laist et al. 2001, Van Waerbeek et al. 2007), sirenians (Calle-son and Frohlich 2007, Hodgson and Marsh 2007), and sea turtles (Hazel et al. 2007).

Estimating the risk of wildlife–vehicle collisions across space and time is necessary to develop effective conservation policies.

Studies that examined the risk of whale–vessel collisions have taken many approaches to quantify the problem. These include estimating the co-occurrence of whales and vessels (Fonnesbeck et al. 2008, Vanderlaan et al. 2008, 2009, Nichol et al. 2017), simulating whale and vessel movements and collisions (van der Hoop et al. 2012b), comparing the number of detected whale mortalities due to vessel collisions at a regional scale before and after management activities (Laist et al. 2014, van der Hoop et al. 2015), and analyzing the relationship between vessel speed and the probability of lethal injury to whales from reported collisions (Vanderlaan and Taggart 2007, Conn and Silber 2013). Each of these approaches has limitations that prevent researchers from fully accounting for processes that affect the risk of whale–vessel collisions. For instance, metrics of whale–vessel co-occurrence do not account for the influence of vessel size and speed and whale behavior on the risk of a collision occurring. Simulating the movements of individual vessels and whales is computationally expensive, making it cumbersome to run enough simulations to account for uncertainty in model parameters and processes. Estimates of risk based on the number of detected whale mortalities that are determined to be due to a vessel collision may be confounded by temporal or spatial variation in the probability of detecting a whale carcass or the probability of assigning its cause of death to a collision. Finally, evaluating the risk of collisions at a regional scale over time ignores any changes in whale abundance and vessel traffic at fine spatial scales, which could dramatically alter risk.

We extend a modeling framework based on encounter rate theory for estimating the risk of vessel collisions that addresses the aforementioned limitations (Martin et al. 2016). The framework is process-based, decomposing collision risk into its underlying elements, and estimates the encounter rate between whales and vessels (i.e., the rate at which their paths intersect in two-dimensional space and time, where space can be restricted to two dimensions because vessels only travel along the water’s surface). This encounter rate is determined by the area that

vessels and whales share; the abundance, speed, and size of vessels and whales in the area; and the distance traveled by vessels (Martin et al. 2016). We extend this framework to estimate whether an encounter results in a collision and whether a collision results in a death by accounting for the probability that a whale is at the water’s surface during an encounter, the probability that a vessel and a whale avoid each other during an encounter, and the probability that a whale dies when a collision occurs. We demonstrate the utility of this framework by quantifying the risk of lethal collisions between endangered North Atlantic right whales (*Eubalaena glacialis*, referred to henceforth as right whales) and vessels in the southeastern United States before and after the implementation of a vessel speed restriction rule. Our analyses are based on an extensive data set of Automatic Identification System (AIS) vessel traffic data and spatially explicit estimates of right whale abundance, which account for imperfect detection of right whales.

Collisions with vessels are a leading cause of mortality for right whales (Moore et al. 2004, van der Hoop et al. 2012a, Henry et al. 2017). Right whales die from collisions with vessels more often, per capita, than any other large whale species (Vanderlaan and Taggart 2007). Moreover, right whales are among the most endangered species of large whales (Kraus et al. 2005), with a recent estimated population size of 458 and a declining population trajectory (Pace et al. 2017).

The distribution and life history of right whales expose them to anthropogenic threats (Moore et al. 2004, Campbell-Malone et al. 2008, Knowlton et al. 2012). Right whales are migratory, with individuals summering in feeding grounds in coastal waters off the northeastern United States and eastern Canada, and some individuals, including calving females, wintering in the coastal waters of the southeastern United States, their only known calving grounds (Firestone et al. 2008, Brilliant et al. 2015). Throughout these regions, right whales co-occur with busy shipping and fishing industries. These industries expose right whales to vessel collisions and entanglement in fishing gear, the causes of most recorded right whale deaths (Moore et al. 2004, Campbell-Malone et al. 2008, Knowlton et al. 2012).

The National Oceanic and Atmospheric Administration's (NOAA) National Marine Fisheries Service has taken steps to address the threat of vessel collisions to right whales. In the southeastern United States, NOAA implemented voluntary speed restrictions of 12 and 10 kt (22.2 and 18.5 km/h) in 2004 and 2005, respectively, and established recommended shipping lanes in 2006 to reroute vessel traffic and minimize co-occurrence with right whales (Fonnesbeck et al. 2008, Lagueux et al. 2011). Compliance with the voluntary speed restrictions was low, 9–24% during years the voluntary restriction was implemented, and NOAA implemented a mandatory vessel speed restriction rule of 10 kt (18.5 km/h) starting in December 2008 (NOAA 2008; Lagueux et al. 2011). The speed restriction rule established seasonal management areas (SMAs) along the U.S. Eastern Seaboard. Under the rule, at times of the year when SMAs are active, vessels ≥ 65 ft (19.8 m) long, excluding military and other government vessels, are required to travel at ≤ 10 kt (18.5 km/h). These slower vessel speeds are thought to increase the ability of whales and vessel operators to avoid each other and to reduce the severity of injury and the likelihood of death when a collision does occur (Calleson and Frohlich 2007, Vanderlaan and Taggart 2007, Conn and Silber 2013, Calleson 2014, Rycyk et al. 2018).

Using our modeling framework, we estimate the expected number of deaths and mortality rates of right whales due to vessel collisions in the southeastern U.S. (SEUS) SMA during the winters from 2006–2007 through 2010–2011 while accounting for uncertainty in the framework's parameters. Additionally, we evaluated the effectiveness of the SEUS SMA in reducing the risk of lethal vessel collisions by comparing the expected number of right whale deaths and mortality rates in and adjacent to the SEUS SMA under two hypothetical scenarios, in which vessels traveled at speeds typical of the winters either before or after the implementation of the speed restriction rule in December 2008.

METHODS

Study area

We examined the risk to right whales from vessel traffic within and just east of NOAA's SEUS

SMA (Fig 1). The SEUS SMA covers ~ 9100 km², extending from 29.75° N to 31.45° N and from the Atlantic coastlines of Florida and Georgia to 80.86° W (NOAA 2008). The SEUS SMA is active between 15 November and 15 April each year. Our study area extended 12 nm east of the SEUS SMA to 80.60°W, covering an additional ~ 4700 km² (Fig. 1). The SEUS SMA is adjacent to three shipping ports: Brunswick, Georgia; Fernandina Beach, Florida; and Jacksonville, Florida.

Model framework

We evaluated the risk posed to right whales by vessels subject to the NOAA speed restriction rule. We overlaid our study area with a grid of 30.87 km² (5.556×5.556 km) grid cells and evaluated risk across this grid for each semimonth (days 1–15 and 16–end) of the winter months (December–March) from 2006–2007 to 2010–2011. We did not evaluate risk during the last semimonth of November, the first semimonth of April, or any winter since 2010–2011, though the speed restriction rule was in effect, because estimates of right whale abundance in the study area during those times were not available. We evaluated risk using the framework described by Martin et al. (2016), which estimates the encounter rate, $\lambda_{i,t}$, at grid cell i , during semimonth t , between whales and vessels in two-dimensional space as:

$$\lambda_{i,t} = \frac{2r_{c,i,t}d_{i,t}N_{i,t}}{Sv_{b,i,t}} \int_{v_m} I(v_m, v_{b,i,t})f_v(v_m)dv_m \quad (1)$$

here, the encounter rate is directly proportional to the critical distance of encounter, r_c , which is based on the size of right whales and vessels; the distance transited by vessels, d ; and the abundance of right whales, N , in a grid cell during a semimonth. The encounter rate is inversely proportional to the surface area, S , of the study region (grid cell in this case) and to vessel speed, v_b . The encounter rate is also influenced by the relative velocity of a right whale with respect to a vessel, $I(v_m, v_b)$, and the distribution of right whale speed, $f_v(v_m)$. See Martin et al. (2016) for a thorough explanation of this framework. We then estimated the expected number of collisions and deaths in each grid cell by incorporating the probability that a right whale is at the water's

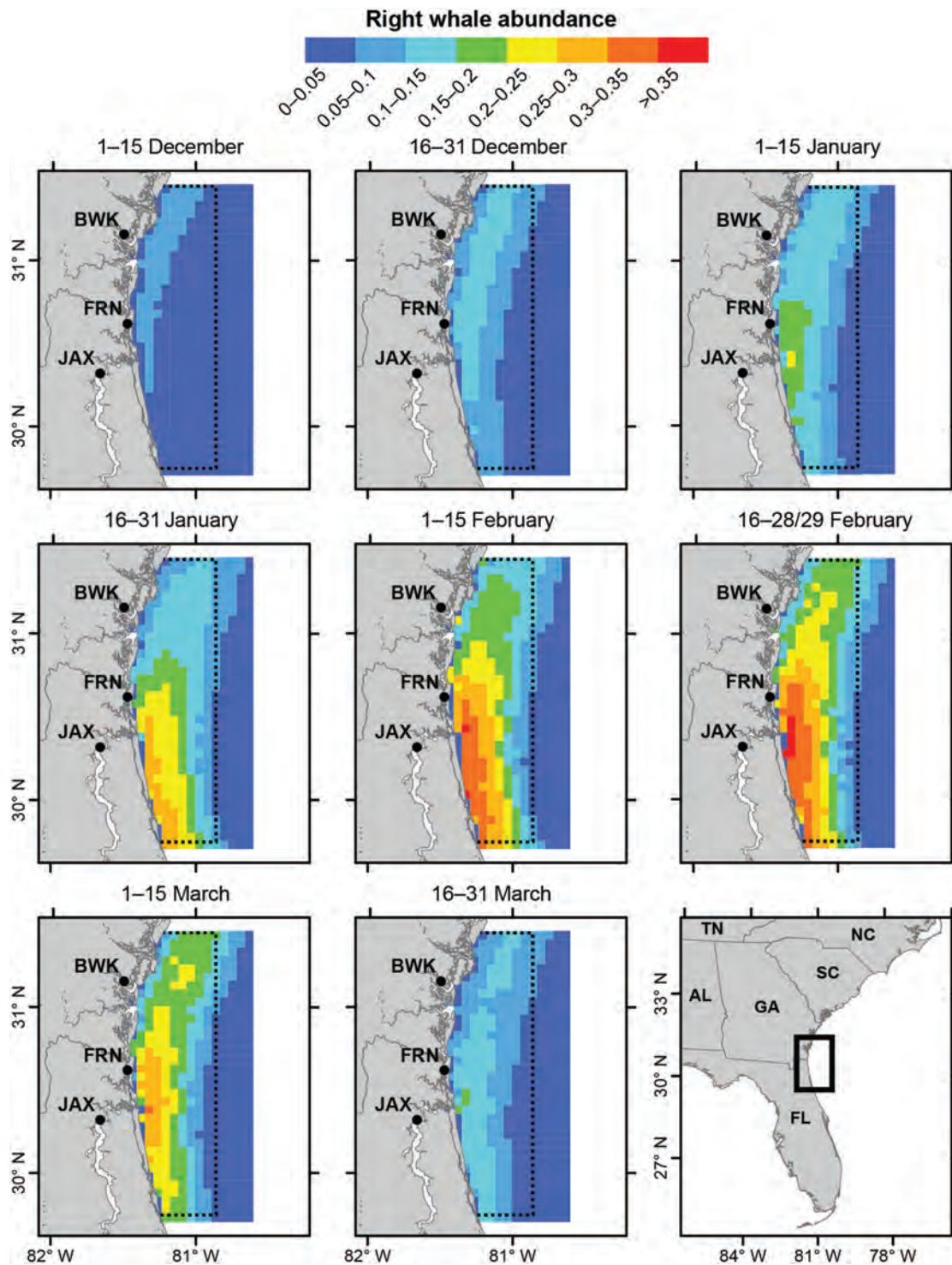


Fig. 1. Mean abundance of North Atlantic right whales in the southeastern United States during the winters from December 2006 through March 2011. The southeastern U.S. right whale seasonal management area is outlined by a black dashed line. The ports Brunswick, Georgia (BWK), Fernandina Beach, Florida (FRN), and Jacksonville, Florida (JAX), are indicated by black circles.

surface during an encounter, $p_{\text{surfacing}}$, and the probability of death given vessel speed during a collision, $p_{\text{death|vb}}$. These parameters were informed by available data and relevant literature, described below.

Whale abundance

We used results from Gowan and Ortega-Ortiz (2014) and Krzystan et al. (2018) to estimate right whale abundance, N , across our study area during each semimonth, the temporal scale we adopted. Both studies analyzed right whale sightings from aerial surveys and were conducted across a similar spatial extent in the southeastern United States and at a semimonthly temporal scale. Gowan and Ortega-Ortiz (2014) estimated spatial patterns of relative abundance (i.e., the expected number of sightings per grid cell) of right whales in relation to environmental covariates using a hurdle model (Dorazio et al. 2013), which derives relative abundance from estimates of occurrence probability, o , and expected count, c . They estimated relative abundance at a resolution of 30.87 km², which we adopted as the spatial resolution of our risk analyses. Krzystan et al. (2018) estimated the detection probabilities of five demographic groups (male, calving female, non-calving female, juvenile, and unknown) of right whales in the southeastern United States from December of 2004 to March of 2011 using mark-recapture data. We estimated regional abundance of right whales across the southeastern United States in each semimonth by summing the number of right whales observed in each demographic group during each semimonth corrected for their respective detection probability from Krzystan et al. (2018). Then, we derived our estimates of abundance by scaling the spatially explicit estimates of relative abundance from Gowan and Ortega-Ortiz (2014) so that they summed to the regional abundance estimates for each semimonth (Fig. 1).

Vessel traffic

We collected data regarding transit distance, d , and speed, v_b , of vessels ≥ 65 ft long using the AIS, which tracks vessels' movements through VHF radio transmissions, starting in 2006. The International Maritime Organization requires that ships of ≥ 300 gross tons traveling internationally, cargo ships of ≥ 500 gross tons, and all passenger ships carry AIS transceivers. We used

data collected by AIS receivers stationed in Brunswick, Georgia (operated by the Florida Fish and Wildlife Conservation Commission), and Jacksonville, Florida (operated by Jacksonville Marine Transportation Exchange). Dynamic data, including a vessel's location and timestamp, were recorded from AIS transmissions at 1-min intervals. Static ship data, which vessel operators report and which include a vessel's identity, ship type, length, and width, were recorded every 6 min. We reconstructed a vessel's path by connecting consecutive locations, and we calculated its speed from the time and distance between consecutive locations. If consecutive transmissions from a vessel were over one hour apart, a new path was started for that vessel. Transmissions were omitted if consecutive transmissions indicated that a vessel traveled at greater than 35 kt (65 km/h) or traveled over 65 km. Military and other government vessels and vessels < 65 ft long were excluded because they were not subject to the speed restriction rule. Because we were not interested in the risk posed by individual vessels, we aggregated vessel transit distance within each grid cell and semimonth by ship type. This allowed us to assess the risk due to different ship types over time and space.

Right whale and vessel sizes

We calculated the critical distance of encounter, r_c , using the disk method ($r_c = \text{radius}_{\text{whale}} + \text{radius}_{\text{vessel}}$), which represents whales and vessels as circles with radii of $\sqrt{(\text{length} \times \text{width}) / \pi}$ (Martin et al. 2016). We assumed that right whales had a length of 14 m and a width of 3.5 m (Fortune et al. 2012, Miller et al. 2012). Because we were only interested in risk posed by different ship types, we averaged vessel length and width for each ship type, in each grid cell, during each semimonth, weighted by each vessel's distance traveled. Vessels with unreported length or width data, which constituted 1.6% of vessels included, were assigned average values of length and width from other vessels of the same ship type during the same winter for the purpose of these calculations.

Right whale and vessel speeds

We assumed that the swimming speed of right whales, v_m , was 0.39 m/s, the average swimming

speed of right whales observed in the southeastern United States (Hain et al. 2013). We evaluated three hypothetical scenarios related to vessel speed, v_b . In each scenario, vessel speed varied by grid cell and ship type. The speed of vessels of a given ship type in a grid cell was the average speed of vessels, weighted by distance traveled, of the same ship type in the same grid cell. Scenarios differed by the period of time from which average vessel speeds were calculated. Scenario 1 was used to estimate the true risk of lethal vessel collisions. Therefore, we averaged vessel speeds for each semimonth, meaning that our model accounted for variation in vessel speed across time, space, and ship types. Scenarios 2 and 3 estimated what risk would have been if the entire study area was not or was subject to the speed restriction rule for all five winters of our study, respectively. We evaluated the effectiveness of the speed restriction rule within the current SEUS SMA and of extending the eastern boundary of the SEUS SMA 12 nm east based on the differences between scenarios 2 and 3. In scenario 2, we used vessel speed averaged from the two winters before the speed restriction rule was implemented (December 2006–March 2008). In scenario 3, we used vessel speed averaged from the three winters after the speed restriction rule was implemented (December 2008–March 2011). Additionally, in scenario 3, vessel speeds east of the SEUS SMA were assigned the average vessel speed from the three eastern-most columns of grid cells within the SEUS SMA. Therefore, vessel speed was constant across time for each ship type and grid cell combination in scenarios 2 and 3, reflecting the average vessel speed before and after the speed restriction rule was implemented, respectively. By estimating risk using AIS data, we accounted for vessel operator compliance with the speed restriction rule without having to make assumptions regarding compliance rate.

Simulation analysis

We accounted for uncertainty in our estimates of whale abundance and in the collision process using Monte Carlo simulation. We used 10,000 Monte Carlo simulations to estimate the expected number of encounters, collisions, and deaths in each grid cell during each semimonth for each ship type. First, we simulated abundance, N , of right whales across the study area to

account for the uncertainty in Gowan and Ortega-Ortiz's (2014) estimates of relative abundance. We simulated abundance from normal distributions based on the mean and standard error of the estimated probability of occurrence, o , and expected count, c , on the link scale (logit and natural log, respectively) for each grid cell, i , during each semimonth, t . Simulated values, o and c , were back-transformed and multiplied together to obtain estimates of relative abundance. We rescaled the simulated relative abundance estimates to sum to estimates of regional right whale abundance for each semimonth in the southeastern United States based on Krzystan et al. (2018); cells within our study area were retained for subsequent analysis.

$$\begin{aligned} o_{i,t} &\sim \text{Normal}(\mu_{\text{occurrence},i,t}, \sigma_{\text{occurrence},i,t}) \\ c_{i,t} &\sim \text{Normal}(\mu_{\text{count},i,t}, \sigma_{\text{count},i,t}) \\ N_{\text{relative abundance},i,t} &= \frac{1}{1 + e^{-o_{i,t}}} * e^{c_{i,t}} \\ N_{\text{abundance},i,t} &= \frac{N_{\text{relative abundance},i,t} * N_{\text{regional abundance},t}}{\sum_{i=1}^I N_{\text{relative abundance},i,t}} \end{aligned} \quad (2)$$

Using Eq. 1, we calculated encounter rates between right whales and vessels under each of the three speed scenarios during each semimonth for each of the simulated grids. We then simulated the expected number of encounters in each grid cell during each semimonth using a Poisson distribution with a mean equal to the encounter rate.

$$N_{\text{encounters},i,t} \sim \text{Poisson}(\lambda_{i,t}) \quad (3)$$

Next, we simulated the expected number of collisions based on the simulated number of encounters. We assumed that if a right whale was at the surface during an encounter, it would be struck by the vessel. We used a beta distribution based on data obtained by Hain et al. (1999) to simulate the probability that a right whale is at the surface during an encounter. Then, we simulated the number of collisions under each speed scenario for each grid cell during each semimonth using a binomial distribution with the number of draws equal to the number of encounters and with the probability that a collision occurs equal to the simulated probability that a right whale is at the surface.

$$p_{\text{surfacing}} \sim \text{Beta}(\alpha = 2.38, \beta = 1.58)$$

$$N_{\text{collisions},i,t} \sim \text{Binomial}(N_{\text{encounters},i,t}, p_{\text{surfacing}}) \quad (4)$$

This approach could be extended by incorporating the probability that a right whale and vessel avoid each other and the probability that a right whale collides with a vessel's draft below the water's surface during an encounter. Due to a lack of data to inform these parameters, we omitted these extensions from our approach (but see Appendix S2). Therefore, our model's expectations should be considered a relative metric of collision risk.

Finally, we simulated the expected number of right whale deaths based on the simulated number of collisions. Conn and Silber (2013) and Vanderlaan and Taggart (2007) used a logistic regression to estimate the relationship between vessel speed and the probability of death given a collision for large whales. We simulated the slope and intercept of this relationship on the logit scale from normal distributions using estimates from Conn and Silber (2013). Simulated values were back-transformed to obtain probabilities of death given vessel speed. We then simulated the number of deaths under each speed scenario for each grid cell during each semimonth using a binomial distribution with the number of draws equal to the simulated number of collisions and the probability of a death equal to the simulated probability of death given vessel speed.

$$a \sim \text{Normal}(\mu = 0.217, \sigma = 0.058);$$

$$b \sim \text{Normal}(\mu = -1.905, \sigma = 0.821)$$

$$p_{\text{death}|v_b} = \frac{1}{1 + e^{-(a(v_b)+b)}}$$

$$N_{\text{deaths},i,t} \sim \text{Binomial}(N_{\text{collisions},i,t}, p_{\text{death}|v_b}) \quad (5)$$

Because whale abundance differed between years, we calculated per capita mortality rates (number of deaths divided by abundance; hereafter referred to as mortality rates) to assess the speed restriction rule's effectiveness.

We summarized the mean mortality rate and the number of encounters, collisions, and deaths and their 95% confidence intervals across all simulations over the entire study area, for each semimonth and year of our study period. Additionally, we summarized the mortality rate and

the number of encounters, collisions, and deaths across all simulations within each grid cell for each semimonth and year of our study period.

RESULTS

Mean abundance of right whales within the SEUS SMA peaked during the last semimonth of February in each winter except 2009–2010, when abundance peaked in the first semimonth of March. Peak semimonthly abundance ranged from 39.8 right whales (2006–2007) to 94.8 right whales (2008–2009). Abundance was lowest in the first semimonth of December of each year except 2010–2011, when abundance was lowest in the last semimonth of March. Minimum semimonthly abundance ranged from 1.7 right whales (2010–2011) to 16.9 right whales (2008–2009). The majority of right whale abundance (94–96.5%) during the study occurred in grid cells within or overlapping the SEUS SMA (Fig. 1).

Most of the vessel traffic (77–91% of transit distance each winter) recorded by AIS receivers within our study area was of vessels subject to the speed restriction rule. The total distance that these vessels transited within a grid cell during a semimonth varied across the study area, ranging from 0 to 3024.2 km. Vessel transit distance was greatest in grid cells adjacent to Jacksonville and was relatively high in cells adjacent to Brunswick and in Jacksonville's shipping lanes (Appendix S1: Fig. S1). Transit distance was greater in grid cells within and overlapping the SEUS SMA (yearly mean = 127,016.8 km, standard deviation [SD] = 7,981.5 km) than in grid cells east of the SEUS SMA (yearly mean = 14,337.1 km, SD = 3391.2 km). We recorded no traffic of vessels subject to the speed restriction rule in an average of 39% of grid cells during each semimonth. In the two winters before the speed restriction rule was implemented, 27.7% (2006–2007) and 39.3% (2007–2008) of the distance transited by vessels that would be subject to the rule were under 10 kt (18.5 km/h), respectively. In the three winters following implementation of the speed restriction rule, 59.5% (2008–2009), 66.8% (2009–2010), and 71.3% (2010–2011) of the distance transited by vessels subject to the rule was under 10 kt (18.5 km/h). In grid cells within and overlapping the SEUS

SMA, vessels traveled at an average of 11.89 kt (22.02 km/h; SD = 3.55 kt [6.57 km/h]) before and 9.27 kt (17.17 km/h; SD = 2.07 kt [3.83 km/h]) after implementation of the rule. In grid cells east of the SEUS SMA, vessels traveled at an average of 15.20 kt (28.15 km/h; SD = 2.92 kt [5.41 km/h]) before and 15.01 kt (27.80 km/h; SD = 3.03 kt [5.61 km/h]) after implementation of the rule (Appendix S1: Fig. S2).

In scenario 1, in which we approximated the actual risk of lethal vessel collisions, we estimated the greatest number of right whale deaths from vessel collisions occurred in the winter of 2008–2009 and the highest right whale mortality rate in the winter of 2006–2007. The smallest number of right whale deaths occurred in the winter of 2010–2011 and the lowest mortality rate in the winter of 2008–2009 (Table 1). Risk of lethal vessel collisions was highest in the shipping lanes east of Jacksonville, Florida, and to a lesser extent in the shipping lanes east of Brunswick, Georgia, where vessel traffic was heaviest (Fig. 2). Less than one encounter, collision, and death per winter occurred in our study area east of the SEUS SMA.

Cargo ships, which comprised 62% of the transit distance recorded by our AIS receivers, accounted for 69–78% of expected right whale deaths each year, while tanker ships (8% of total transit distance) accounted for 8–11%. Tugs (14% of total transit distance), dredges (8% of total transit distance), and passenger ships (5% of total transit distance) each accounted for 2–10% of expected right whale deaths each year. Expected mortality rates of right whales due to collisions with cargo and tanker ships were 14–32% lower in winters following implementation of the speed restriction rule. Expected mortality rates of right

whales due to collisions with other common ship types were not consistently lower following implementation of the speed restriction rule (Appendix S1: Figs. S3–S7). Expected mortality rates of right whales due to collisions with all vessels decreased by an average of 22% in the winters following implementation of the speed restriction rule (Table 1).

An average of 2.05 fewer right whale deaths, a 17% decrease, caused by vessel collisions occurred each winter when vessels within the SEUS SMA traveled at speeds as if they were subject to the speed restriction rule, scenario 3, in comparison with scenario 2, in which vessels traveled at speeds as if they were not subject to the speed restriction rule. Differences in risk of lethal vessel collisions between scenarios 2 and 3 were greatest in the shipping lanes east of Jacksonville, Florida, and to a lesser extent the shipping lanes east of Brunswick, Georgia (Fig. 3). These scenarios were similar in risk of lethal vessel collisions east of the SEUS SMA, with 0.04 fewer right whale deaths occurring in this area each winter when vessels traveled at speeds as if they were subject to the speed restriction rule. Fewer deaths of right whales occurred in each semimonth, especially from late January through early March, when vessels traveled at speeds as if they were, rather than were not, subject to the speed restriction rule (Fig. 4).

DISCUSSION

Our model framework allowed us to quantify the relative risk of lethal vessel collisions to right whales before and after implementation of NOAA's speed restriction rule. The rule was effective in reducing risk; according to our

Table 1. Expected number of encounters and collisions between vessels and right whales and deaths and mortality rates of right whales (95% confidence intervals in parentheses) due to vessel collisions in and adjacent to the southeastern U.S. seasonal management area under scenario 1, our closest approximation to reality.

Year	Encounters	Collisions	Deaths	Mortality rate
2006–2007	23.37 (14–34)	14.05 (7–23)	9.02 (2–18)	0.044 (0.010–0.089)
2007–2008	36.20 (25–50)	21.72 (13–32)	13.12 (3–25)	0.043 (0.010–0.081)
2008–2009	42.55 (30–58)	25.51 (16–37)	13.59 (3–28)	0.033 (0.007–0.067)
2009–2010	40.26 (28–55)	24.13 (15–36)	12.80 (2–26)	0.035 (0.005–0.071)
2010–2011	24.99 (16–37)	15.00 (8–24)	7.77 (1–18)	0.034 (0.004–0.079)

Notes: A speed restriction rule that required vessels ≥ 65 ft long to travel at <10 kt (18.5 km/h) was implemented during the winters of 2008–2009 through 2010–2011.

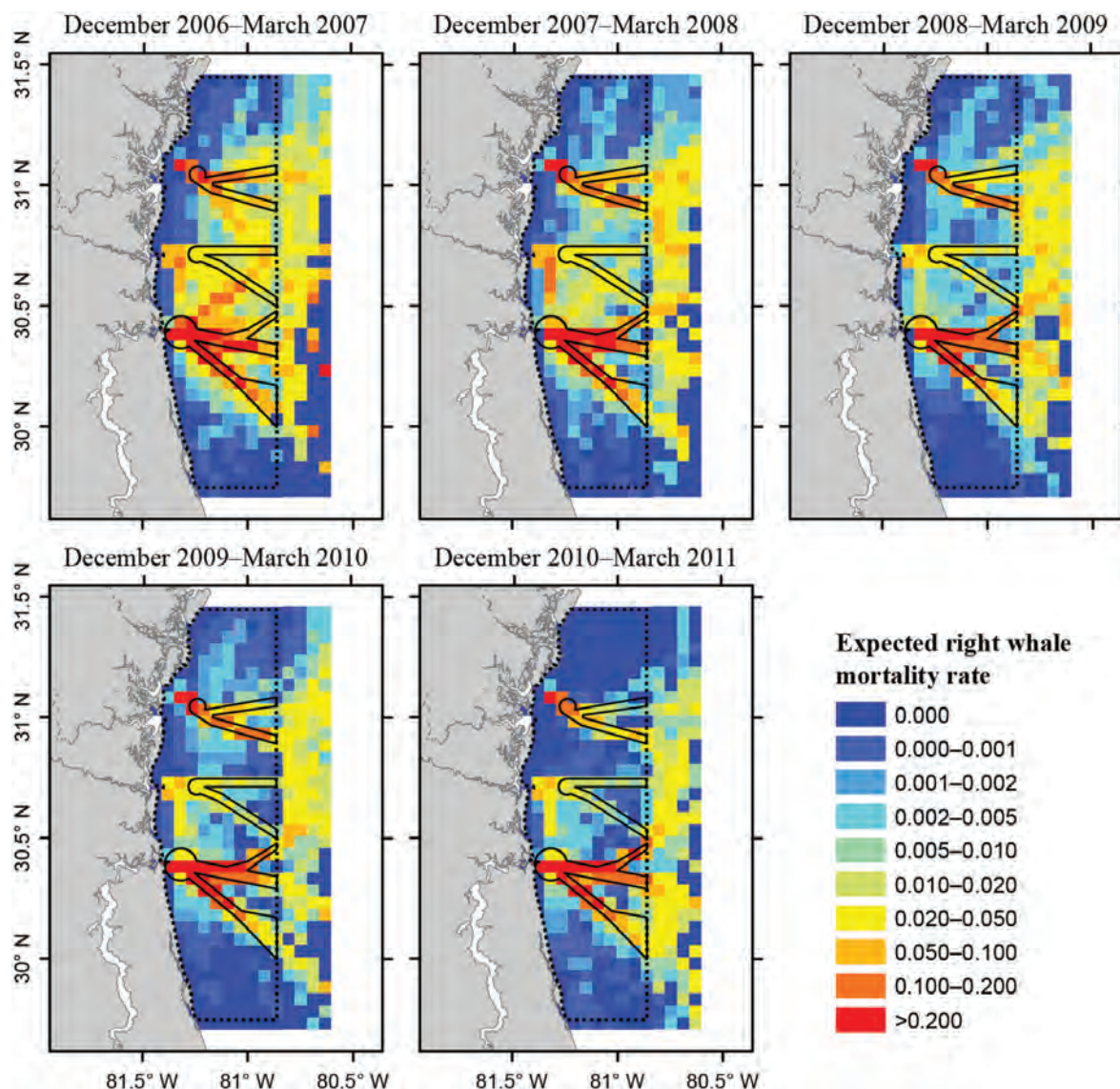


Fig. 2. Mean expected right whale mortality rates due to vessel collisions during each winter from 2006–2007 through 2010–2011 within the southeastern U.S. right whale seasonal management area (black dashed outline) according to scenario 1. Vessel speed restrictions were in effect in the seasonal management area during the 2008–2009 through 2010–2011 winters. Recommended shipping lanes are outlined in black.

model, right whale mortality rates declined 22% on average in the three winters following implementation, and 17% fewer right whale deaths occurred in scenario 3, where vessels traveled at speeds as if they were subject to the speed restriction rule, in comparison with scenario 2, where they were not subject to the rule. However, this reduction in relative risk varied across space and time.

The speed restriction rule was most effective at reducing risk of lethal vessel collisions at times when and in areas where right whales were abundant and vessel traffic was heavy and fast. These times and locations showed the greatest differences in the number of lethal collisions between scenarios 2 and 3 (Figs. 3, 4). The difference in risk between scenarios 2 and 3 (17%), which had identical input parameters except for

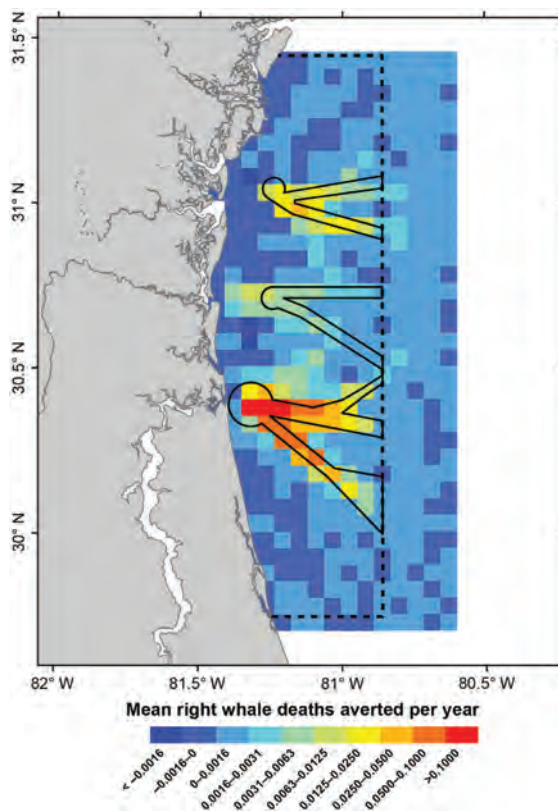


Fig. 3. Expected number of North Atlantic right whale deaths caused by vessel collisions averted under the speed restriction rule. The southeastern U.S. right whale seasonal management area (SEUS SMA) has a dashed black outline, and recommended shipping lanes are outlined in solid black. We calculated the number of deaths averted as the difference between two scenarios, (1) vessels traveled at the average speed from when the speed restriction rule was not in effect (scenario 2) and (2) vessels traveled at the average speed from when the speed rule was in effect, unless they were outside of the SEUS SMA, in which case they traveled at the average vessel speed from the three eastern-most columns of cells within the SEUS SMA when the speed rule was in effect (scenario 3).

vessel speed, estimated a smaller reduction in risk than the difference between the two years before and three years after the speed restriction rule was implemented according to scenario 1 (22%). This suggests that the reduction in risk following the implementation of the speed restriction rule may not be entirely attributable to slower vessel speeds. Total vessel transit

distances each winter were similar before and after the rule was implemented (Appendix S1: Table S1), but increased compliance with recommended shipping lanes may have contributed to the reduction in risk (Fig. 2; Fonnesbeck et al. 2008). Additionally, the rule primarily reduced the risk of lethal collisions from cargo ships and tankers. These two ship types accounted for the majority of AIS vessel traffic in the SEUS SMA each winter (Appendix S1: Table S1), traveled faster than other ship types, such as tugs and dredges, before the speed restriction rule was implemented (Appendix S1: Table S2), and commonly used the recommended shipping lanes after they were established (Appendix S1: Figs. S3–S7).

Our model expected low risk of lethal vessel collisions east of the SEUS SMA and nearly no difference in risk between scenarios with and without the speed restriction rule in place (Fig. 3). Risk of lethal vessel collisions was very low east of the SEUS SMA because there was less vessel traffic and relatively low predicted abundance of right whales. With low initial risk, reducing vessel speeds hardly altered the expected number of lethal collisions. Conversely, our model expected the highest risk and greatest reduction of risk in and around shipping lanes inside the SEUS SMA, where vessel traffic is heaviest. Similarly, risk and the effectiveness of the speed restriction rule were greatest between late December and late February, when predicted right whale abundance was highest, compared to early December and late March. Because the risk of lethal collisions is sensitive to abundance, changes in right whale abundance and distribution could alter the number of right whale deaths that the speed restriction rule averts, that is, the difference in the number of right whale deaths expected if the rule were or were not implemented. For instance, since 2012 the number of observed right whales in the SEUS SMA has been lower than during our study period, which ended in 2011 (Gowan et al. 2019), likely leading to fewer lethal collisions being averted due to the speed restriction rule. However, even during years of low abundance in the SEUS, the speed restriction rule remains important, because the survival of pregnant and nursing females and their calves is key to the species' recovery (Fujisawa and Caswell 2001). Additionally, decreased

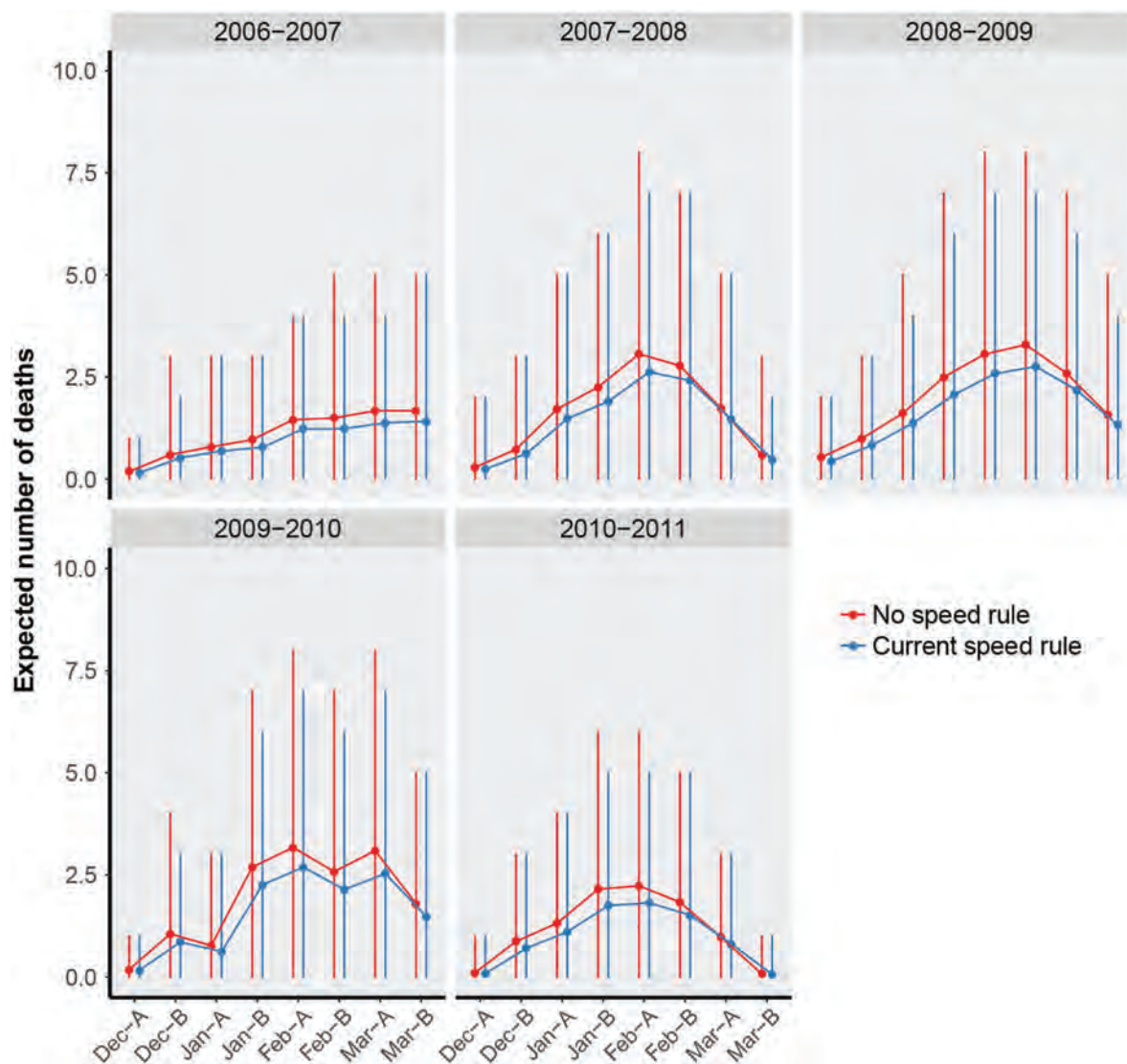


Fig. 4. Expected number of deaths, and associated 95% confidence intervals, of North Atlantic right whales due to collisions with vessels either subject to (scenario 3, in blue) or not subject to (scenario 2, in red) the speed restriction rule inside the southeastern U.S. right whale seasonal management area during each semimonth of the winters from 2006–2007 through 2010–2011.

observations of right whales in the SEUS SMA in recent years suggest that many right whales may be wintering elsewhere (Gowan et al. 2019). If this trend continues, it may be worth investigating where these right whales are wintering and how speed restriction rules would alter risk at those locations.

According to our model, 11 right whales on average were expected to have been struck and killed by vessels each winter in our study area

between December 2006 and March 2011. In contrast, only one right whale death attributable to a vessel collision was documented within our study area during that time (Laist et al. 2014). Many factors can help explain this discrepancy, including that not every right whale death is detected, and the cause, location, and time of detected deaths cannot always be determined (Epperly et al. 1996, Williams et al. 2011). Therefore, the number of observed right whale deaths

due to vessel collisions typically underestimates the actual number of deaths. Nevertheless, our model likely overestimated the risk of lethal vessel collisions due to our modeling choices. We did not account for avoidance of collisions by either right whales or vessel operators, which may have positively biased our expectations of risk (Appendix S2). Although there is a lack of evidence suggesting right whales avoid oncoming vessels (Nowacek et al. 2004), limited avoidance behavior has been documented in other large whale species (McKenna et al. 2015). Additionally, our approximation of the critical distance of encounter may have been too large. By using the disk method to represent vessels, we accounted for their length and width in the critical distance of encounter. However, since a vessel travels in the direction perpendicular to the axis of its width, it might be more reasonable to use only a vessel's width in calculating the critical distance of encounter. Martin et al. (2016) found that this method reduced risk estimates as much as 30% compared to the disk method. Our model also considered the process of lethal vessel collisions as sampling with replacement, meaning that a simulation could result in more deaths than there were whales. This was an infrequent occurrence (happening in fewer than 1% of simulations within an average grid cell during a semi-month) but may have contributed to an overestimation of risk, particularly in areas of high traffic. This issue could be addressed by modeling the process of lethal vessel collisions as sampling without replacement or using an individual-based simulation approach that removes a right whale from the population following a lethal vessel collision as van der Hoop et al. (2012b) did. Finally, when estimating risk of lethal vessel collisions inside of the SEUS SMA, we included all grid cells that overlapped the SEUS SMA. A small subset of these grid cells did not fall entirely within the SEUS SMA, and therefore, our estimates of risk inside the SEUS SMA included risk that, in reality, would be outside of the SEUS SMA.

Conversely, we modeled risk only from vessels that were subject to the speed rule and were recorded by our AIS receivers and risk to right whales that were at the water's surface. So, there was unmodeled risk from vessels <65 ft long and military and other government vessels, which are

not subject to the speed restriction rule, and from vessels whose AIS transmissions were not received because their signal was too weak, or they were out of range of our receivers, for example. Additionally, our model underestimates risk for whales that are not at the surface but still no deeper than a vessel's draft or hydrodynamic effects (Silber et al. 2010). This could be incorporated into our model if information was available regarding vessels' drafts (often reported through AIS), the region's bathymetry, and the diving profiles of right whales in the southeastern United States.

Although we found that the speed restriction rule reduced the risk of lethal vessel collisions, we did not find as large of an effect as have other evaluations of the rule, such as Conn and Silber (2013). They estimated a reduction in the mortality rate of right whales due to vessel collisions of 80–90%. They measured the effect of vessel speed on a process that combined the encounter rate and avoidance rate, whereas our model considered these as separate processes. Our model would expect the rule to be as effective as Conn and Silber (2013) did if right whales were four to eight times more likely to avoid collisions with encountered vessels traveling at post-rule speeds than at pre-rule speeds (Appendix S2: Figure S2). McKenna et al. (2015) predicted that blue whales exhibiting avoidance behavior are more susceptible to collisions with faster vessels. If right whales exhibit similar avoidance behavior, then, depending on when they react to oncoming vessels and how this varies with vessel speed, the speed restriction rule would be more effective than our model expects currently. Additionally, compliance with the speed restriction rule increased throughout and after our study period (Silber et al. 2014). With all other factors equal, this would indicate that the rule may be more effective now than during the three years over which we evaluated it, highlighting the importance of considering compliance during policy development (Silber et al. 2014).

Our framework is a step forward from other metrics of right whale–vessel collision risk. We accounted for several factors that had not been collectively considered by other studies, namely the effects of vessel size, speed, and transit distance and right whale abundance and behavior on encounter and collision rates. Additionally, the framework's computational efficiency and our use

of Monte Carlo simulations allowed us to account for parametric uncertainty and the stochastic nature of processes leading to a lethal vessel collision for which other studies have not fully accounted (van der Hoop et al. 2012b, Conn and Silber 2013, Rockwood et al. 2017). Still, our study only provides a relative metric of risk, as have other studies, because we did not fully account for potentially important processes, including avoidance, or parameters, including traffic from vessels excluded from the speed restriction rule. Moreover, this framework could be advanced in multiple ways, including developing an integrated population model that incorporates carcass recovery data to improve understanding of mortality and carcass recovery processes; optimizing the timing and location of management activities based on managers' valuations of risk and the cost of such activities to shipping and boating communities (Udell et al. 2018); and producing coastwide estimates of risk (Rockwood et al. 2017).

Our modeling framework facilitated a thorough evaluation of how NOAA's speed restriction rule affects the risk of vessels colliding with and killing right whales in the SEUS SMA. This framework accounts for factors that are key to the collision process and were generally overlooked by earlier evaluations of the speed restriction rule and whale–vessel collisions; is spatiotemporally flexible, accommodating any spatial or temporal scales for which abundance and traffic data are available; and is computationally efficient, expediting sensitivity analyses, model updates, and the ability to account for parametric and process uncertainty. With such attributes, this framework can be used to help managers evaluate risk associated with shipping industry trends and prospective policies. Additionally, the framework can be useful to managers in other fields, including those who must evaluate policies related to the timing and locations of fishing activities that pose entanglement risk to marine wildlife; military activities, such as sonar testing, and seismic surveys that may impact marine mammals; transportation planning that may affect wildlife–vehicle collision risk; and the location of wind farms, which present collision risk to wildlife (Martin et al. 2016, Udell et al. 2018). Using this framework, we provided a better understanding of where and when right whales are at risk of being killed by vessel

collisions in the SEUS SMA. Future work can build upon this understanding and make practical use of the framework by using methods of systematic conservation planning to optimize the location and timing of SMAs.

ACKNOWLEDGMENTS

This study was supported by the National Sea Grant College Program of the USA and Department of Commerce's National Oceanic and Atmospheric Administration (NOAA; grant nos. NA14OAR4170108 and NA16NMF4720319). We thank Stephanie Cain for managing and processing the AIS databases used in this study. We thank Jacksonville Marine Transportation Exchange for providing us with access to their AIS receiver's data stream. We thank the right whale observers for collecting data regarding right whale distribution and abundance. We thank reviewers Leslie Ward, Shannon Whaley, and Colin Shea for their insightful comments. Any use of trade, product, or firm names in this article is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Future Directions in Research on Bryde's Whales

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Edited by:

Rob Harcourt,
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Specialty section:

This article was submitted to
Marine Megafauna,
a section of the journal
Frontiers in Marine Science

Received: 06 July 2018

Accepted: 28 August 2018

Published: 18 September 2018

Citation:

Constantine R, Iwata T, Nieukirk SL
and Penry GS (2018) Future
Directions in Research on Bryde's
Whales. *Front. Mar. Sci.* 5:333.
doi: 10.3389/fmars.2018.00333

One of the lesser known species of baleen whales, the Bryde's whale, also known as Eden's whale (*Balaenoptera edeni edeni* and *B. edeni brydei*), although hunted as part of a North Pacific Japanese research programme¹, was not heavily exploited by commercial whaling and remains a data deficient species. Their taxonomic status is not fully resolved and they are often mistaken for other species leading to uncertainty about their true distribution, behavior and conservation status. Some populations are critically endangered, whilst others are small but have high genetic diversity suggesting wider connectivity. The species' unpredictable coastal and offshore global distribution throughout warm-temperate waters has led to populations with unknown genetic variation, and facing different threats. Few areas are well-studied, but each study reveals often contrasting movement patterns, foraging strategies, and vocal repertoires; there are considerable knowledge gaps for Bryde's whales. There are few Bryde's populations with abundance estimates but they typically number in the mid- to high-hundreds of individuals, with other populations small, <100 mature individuals, and exposed to high levels of anthropogenic impacts. Future research should focus on understanding the diversity within and between populations. Here, we suggest an integrative, comparative approach toward future work on Bryde's whales, including acoustic monitoring, trophic interactions, telemetry tools, understanding their novel behaviors, and resolving their species status. This will inform conservation management of this unusual species of whale vulnerable to anthropogenic impacts.

Keywords: Bryde's whale, Eden's whale, *Balaenoptera edeni*, taxonomy, acoustics, foraging behavior, movement ecology, conservation

BRYDE'S WHALE TAXONOMY—UNTANGLING THE GLOBAL CONFUSION

Bryde's whales, also called Eden's whale, are currently classified as a single species, *Balaenoptera edeni* (Committee on Taxonomy, 2017). After much debate, two provisional subspecies were recently recognized, *B. edeni edeni* and *B. edeni brydei*, referring to the small, coastal form and larger, oceanic form respectively (Kershaw et al., 2013; Rosel and Wilcox, 2014). However, when combined with ecological and morphological data, there is strong evidence to suggest the two forms could be separated at the species level and perhaps even disconnected from their coastal and oceanic descriptors that can lead to incorrect species assignment [e.g., the New Zealand coastal population is *B. e. brydei*, the offshore form (Wiseman, 2008)].

¹ iwc.int/total-catches

Taxonomic clarity within the Bryde's whale group is hampered by the lack of a type specimen and accurate description of *B. brydei*, and verification of the genetic identity of the *B. edeni* holotype (Anderson, 1879). The realization that Olsen's description of *B. brydei* was incorrect resulted from the discovery of two ecotypes off South Africa (Best, 1977). Olsen's description of *B. brydei* included features from both the South African inshore and offshore forms (Best, 1977, 2001; Kanda et al., 2007; Yamada et al., 2008). These two forms were hunted concurrently in the early 1900's when the existence of the species was not yet known and they were reported as sei whales (*Balaenoptera borealis*) due to their similar morphology. Little did we know that multiple forms of this new large whale species would subsequently be discovered, resulting in the current taxonomic tangle.

Several regional studies have been conducted on Bryde's whale populations from different geographic regions resulting in a plethora of suggestions regarding the genetic identity, phylogenetic position, and proposed nomenclature of the populations in those areas (Yoshida and Kato, 1999; Wada et al., 2003; Sasaki et al., 2006; Kanda et al., 2007; Kershaw et al., 2013; Rosel and Wilcox, 2014; Luksenburg et al., 2015; Penry et al., 2018). For example, Wada et al. (2003) found that the number of nucleotide differences in the complete mitochondrial DNA control region (bp 901) between *B. edeni* (coastal Japan) and *B. brydei* (pelagic North Pacific) was greater than that between *B. brydei* and the sei whale. They also separated *B. edeni* from the sei-Bryde's group. Sasaki et al. (2006) supported this differentiation using complete mtDNA sequences and short interspersed nuclear elements insertion patterns. It is becoming increasingly apparent that Eden's whale and Bryde's whale are likely to be two separate species, but with sub-species differentiation; as seen off South Africa (Penry et al., 2016) and the Gulf of Mexico (Rosel and Wilcox, 2014). The taxonomic confusion may act against the interests of protecting vulnerable, isolated populations, perhaps most urgently in the Gulf of Mexico (Rosel and Wilcox, 2014; Soldevilla et al., 2017; Corkeron and Kraus, 2018).

The molecular markers, analytical techniques and sample sizes from the studies mentioned above vary and are not necessarily comparable, however the results present strong cases for the identification of discrete genetic units. Recommendations on the level at which these units should be recognized, their respective nomenclature, and phylogenetic position within the Balaeopteridae, conclude the majority of these studies. Regardless of the strength of these studies, the suggestions made cannot be adopted until such time as the type specimen for *B. brydei* has been described, and all available molecular data on Bryde's whales are included in a global analysis. To do this it is necessary to consolidate the available genetic material and supporting information on ecology for each region and population. A first step would be to establish a working group of key geneticists, taxonomists and Bryde's whale researchers to identify and consolidate all available molecular and morphometric knowledge to date, then agree upon a standardized approach to analyzing and interpreting the results. The establishment of an IUCN Specialist Group and/or an

International Whaling Commission (IWC) working group to resolve the status, threats and conservation actions for these whales would be a valuable step forward. This would enable a pathway forward for Bryde's whale research and management throughout their range.

BRYDE'S WHALE IDENTITY IN A SEA OF NOISE

Passive acoustic monitoring (PAM) has become a powerful tool in understanding the movements and distribution of cryptic but vocal species. This is especially true for highly mobile marine animals such as cetaceans, where acoustic monitoring has provided much needed information on the migratory movements of endangered species such as blue whales (*Balaenoptera musculus*; Stafford et al., 2001). However, effective use of this technology requires thorough knowledge of the vocal repertoire of the species of interest and visual confirmation of the species producing the sounds.

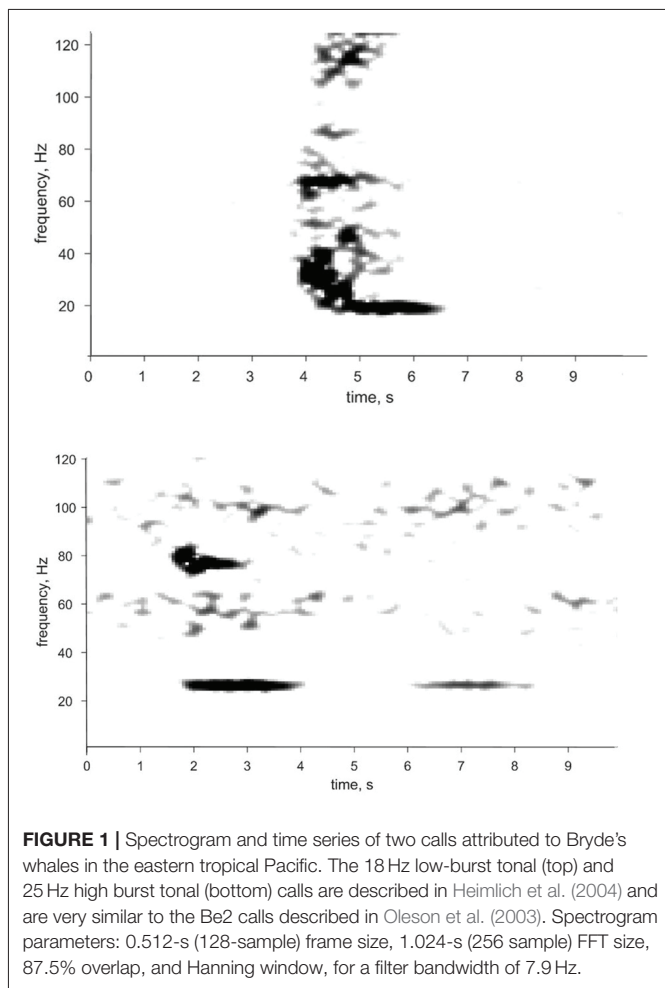
Worldwide Bryde's whale vocalizations are very low-frequency, like other baleen whales, but are also quite short (<5 s long) making them difficult to identify in acoustic data. Bryde's whales produce a variety of sounds, including <100 Hz tonal calls, often with harmonics or overtones, frequency modulated (FM) downsweeps (<1,000 Hz), and amplitude modulated sounds (Figure 1). Calls are typically lower amplitude than other baleen whales, but when sounds are produced in long sequences they are more identifiable in acoustic data archives (SLN pers. obs.). Although there are similarities among Bryde's whale calls around the world, there appear to be regional call differences. When combined with genetic data, these regional differences may be useful in identifying stock or population boundaries (Mellinger and Barlow, 2003).

Information on the vocal repertoire of Bryde's whales has been expanding but is still limited. Studies linking vocalizations to *in situ* observations of Bryde's whales include Cummings et al. (1986) and Vilorio-Gómora et al. (2015), Edds et al. (1993) in the Gulf of California and in the Gulf of Mexico, Oleson et al. (2003) in the northwest Pacific (off Japan), in the Eastern Tropical Pacific (ETP) and the southern Caribbean, Širović et al. (2014) in the Gulf of Mexico and Figueiredo and Simão (2014) from southeast Brazil. Others have used these confirmed Bryde's whale sounds as a powerful reference for identifying vocalizations in archival acoustic data from moored hydrophones (e.g., Heimlich et al., 2004; Rice et al., 2014; Širović et al., 2014; Putland et al., 2018), while Kerosky et al. (2012) used passive acoustic data to document a range expansion of Bryde's whales in the Southern California Bight. More recently, Bryde's whale movements were remotely tracked through a cabled observatory off Hawai'i (Helble et al., 2016).

The tools used to collect passive acoustic data are diverse, constantly improving and are chosen based on the research question. Animal-borne passive acoustic tags, including the D-tag² and the Acousonde³ are usually attached via suction

²<http://www.whoi.edu/website/marine-mammal-behavior-lab/dtag>

³<http://www.acousonde.com/index.html>



cups and provide a means of making short-term (hours—days), detailed recordings of the vocalizations of the animal wearing the tag, as well as those nearby. This provides a definitive link between a species and the recorded vocalization, information on the acoustic repertoire of the animal and the behavioral context of those vocalizations. Limpet style acoustic tags have an embedded attachment and can collect very basic acoustic data for periods of days to weeks. Sonobuoys can passively record sounds produced by whales vocalizing within range of the buoy; when buoys are directional, acoustic data can be coupled with boat-based visual observations to confirm which whale is vocalizing *in situ* (e.g., Širović et al., 2014). Autonomous moored recorders are routinely deployed in nearshore and remote oceans of the world, providing a continuous record of the soundscape around the deployment area for long periods spanning months to years.

To move forward in the conservation of this species, we need to define and refine the Bryde's whale acoustic repertoire around the world and collect long-term acoustic datasets to identify movements within ocean basins. This is best accomplished by collecting passive acoustic data with multiple tools and technology. Based on previous studies, acoustic data should be collected at a sample rate of at least 2,000 Hz; using a sample rate of ~16 kHz could potentially answer lingering questions

regarding unidentified calls recorded in places such as the Marianas Trench (Nieukirk et al., 2016) that are potentially produced by Bryde's whales. In an area of interest, an array of 2+ moored autonomous hydrophones could be positioned on- and off-shore to collect year-long datasets to answer movement and stock questions. Simultaneously, to confirm the calls collected via moored hydrophones are indeed from Bryde's whales, *in situ* acoustic data could be collected, during a time of year when vocalizations are likely, via sonobuoys, underwater gliders, and animal-borne tags and could provide information on the behavior associated with vocalizations (feeding and breeding). Eventually, tags could be used to collect information on calling rates so that acoustic data can possibly be used to estimate the density of vocalizing Bryde's whales, the degree of acoustic masking from anthropogenic noise, and co-occurrence with sympatric species. With a species like the Bryde's whale that has, to date, been difficult to study, passive acoustic data are a vital component in understanding migratory movements and population boundaries of this species.

MOVEMENT

Bryde's whales do not undertake the long-range seasonal migrations typically associated with most other baleen whales, but they may travel widely throughout ocean basins as they move through tropical and warm-temperate waters (Kato and Perrin, 2018). It is widely assumed that there are inshore and offshore species of Bryde's whales but their movement patterns are almost certainly more complex both within and between populations. In some areas with low abundance estimates there is high genetic diversity suggesting wider connectivity with whales from other regions, and/or dispersal by the surveyed population (Wiseman, 2008; Tezanos-Pinto et al., 2017). Most Bryde's whale populations found away from the easily accessible near-coastal waters remain un-surveyed with little or no knowledge of their connectivity or genetic diversity.

Longer migratory movements of whales of approximately 2,000–3,500 km in distance have been reported off the west coast of South Africa (Best, 2001). Differences in residency patterns of whales suggest there are migratory movements at several sites, but the distances remain unknown (Alves et al., 2010; Penry et al., 2011) and may cover only short distances of hundreds of kilometers (Wiseman, 2008; Lodi et al., 2015). This may be in response to prey movements, as found off Brazil, Venezuela and the Gulf of California (Notarbartolo di Sciara, 1983; Tershy, 1992; Zerbini et al., 1997). To date, there are no studies using satellite telemetry data to reveal long-range movements of Bryde's whales. Tags with short prongs, such as LIMPET tags should be used as Bryde's have a thin blubber layer (see use on fin whales (*Balaenoptera physalus*) Panigada et al., 2017). Satellite telemetry studies would be particularly effective in oceanic regions where there are inshore and offshore whale populations [e.g., South Africa (Best, 2001)], but also where there are small, but genetically diverse populations [e.g., Hauraki Gulf, New Zealand (Tezanos-Pinto et al., 2017)] to determine connectivity.

Studies on different populations have used short-term suction cup tags (Alves et al., 2010; Soldevilla et al., 2017; Izadi et al., 2018). These have revealed vertical movements including shallow (Izadi et al., 2018), mid-water (Soldevilla et al., 2017), and deep-dive patterns (Alves et al., 2010), reflective of the environment they live in and highlighting the whales' ability to exploit a variety of surface and deeper water prey (Kato and Perrin, 2018). A range of oceanographic, physical and biological variables influence Bryde's whale movements but there is no clear pattern across populations (Corkeron et al., 2011; Weir et al., 2012; Soldevilla et al., 2017; Tardin et al., 2017). A multi-disciplinary, comparative approach to determine oceanography, prey movements and availability, triggers that drive whale movements and predictions under change scenarios would enable us to better understand whether Bryde's are the ultimate flexible baleen whale. We have improved capabilities to understand the trophic ecology of open ocean organisms through tools such as stable isotopes, fatty acids, radio isotopes, and isoscape models which can reveal spatial and foraging niche shifts over space and time (e.g., Newsome et al., 2010; Quillfeldt et al., 2010; Eisenmann et al., 2017). Determining the processes that influence whale movements, their interaction with the environment and how they are affected by change is an area of future importance.

With no large-scale connectivity studies to date, our current, limited understanding of the variability in site fidelity suggest long-range satellite tagging would be valuable. There are indications that some Bryde's populations are expanding their local range, perhaps in response to prey shifts [e.g., Gulf of California (Kerosky et al., 2012)]. Similar small-scale shifts have been observed in response to La Niña events in New Zealand (RC unpub. data), suggesting a reduction in sightings in areas where whales are typically observed. Bryde's have a preferred thermal range (Kato and Perrin, 2018) that may influence future movements and distribution patterns as ocean temperature increases; a recent phenomenon observed in other cetaceans (see review in MacLeod, 2009).

PLASTICITY IN FORAGING BEHAVIORS

Balaenopteridae including Bryde's whales employ a foraging strategy, lunge feeding, characterized by the engulfment of a large volume of water at high speed and subsequent filtering with the mouth closed (Goldbogen et al., 2017). Bryde's whales commonly use lunge feeding behaviors throughout their range (e.g., Miyazaki and Wada, 1978; Best et al., 1984; Tershy, 1992; Anderson, 2005; Steiner et al., 2008; Alves et al., 2010; Penry et al., 2011; Lodi et al., 2015; Iwata et al., 2017). They have a broad diet of pelagic and mesopelagic fishes, squids, krill, and other zooplankton which varies by location (Olsen, 1913; Notarbartolo di Sciarra, 1983; Best et al., 1984; Tershy, 1992; Best, 2001; Anderson, 2005; Murase et al., 2007; Gonçalves et al., 2015; Lodi et al., 2015; Iwata et al., 2017; Izadi, 2018; Kato and Perrin, 2018). Bryde's whales are efficient and adaptable predators, adopting behaviors in relation to prey species, feeding grounds and oceanographic environment.

Bryde's whales have diel patterns in foraging behavior. In the Gulf of California, whales fed more often at dawn and dusk when

fish schools are less likely to detect predators (Tershy, 1992). In Madeira whale dive patterns of up to 250 m depth appear to mirror the diel vertical migration of zooplankton (Alves et al., 2010). In the shallow waters of the Hauraki Gulf, whales showed foraging behavior during the day and rest during the night-time (Izadi et al., 2018). In the Gulf of Thailand, they perform passive tread-water feeding (Iwata et al., 2017). This foraging strategy takes advantage of the behavior of the prey whereby the hypoxic environment in the upper Gulf of Thailand limits fish to the water surface where there is some oxygenated water. The whales tread water with their mouth open wide at the sea surface as fish spill from the surface into the whales' mouths. In New Zealand, Bryde's whales perform head-slaps to aggregate zooplankton prey, but the same individuals will switch strategies and lunge at speed from underneath fish schools (Izadi, 2018). Lunges also occur at depth (e.g., Alves et al., 2010).

Bryde's whales have a wide array of novel behaviors to catch prey, perhaps more than any other species of baleen whale. They forage at the sea-surface and at depth, during day-time and night-time, and feed on pelagic and mesopelagic prey. Behaviors are often specific to an area with many never observed in Bryde's whales in any another area, nor by any other species of baleen whale. These specializations may leave them vulnerable if a preferred prey is over-fished or affected by environmental shifts, requiring the whales to relocate to find other prey or rapidly develop a new foraging strategy. Bryde's whales have characteristics of income breeders, feeding regularly rather than relying on stored reserves, so determining their energetic requirements needs consideration if protecting important prey or feeding areas. Ascertaining how the novel foraging behaviors are developed and transmitted, especially between mothers and calves and between wider ranging populations of whales would change our traditional thoughts about baleen whales.

THREATS AND CONSERVATION

Bryde's whales are listed as Least Concern by the IUCN (Cooke and Brownell, 2018), but the Gulf of Mexico population is listed as Critically Endangered. Bryde's whales face similar threats to other baleen whales with entanglement, ship strike and prey depletion already reported. Entanglement in a variety of fishing gear such as rock lobster and octopus fisheries in South Africa (Penry et al., 2016) long-line interactions in the endangered Gulf of Mexico population (Soldevilla et al., 2017) and mussel aquaculture lines in New Zealand (Constantine et al., 2015) are of concern, in particular where these fisheries are expanding and/or poorly managed. These need to be managed to avoid population decline, especially as the few known abundance estimates are in the mid- to high-hundreds of individuals (Best et al., 1984; Urbán and Flores, 1996; Carretta et al., 2015), or small with <100 mature individuals (Cherdsukjai et al., 2015; Rosel et al., 2016).

As with many baleen whale populations, they are vulnerable to ship strike, especially inshore populations. Bryde's whales are less frequently represented in the ship strike statistics than other Balaenopterids (Laist et al., 2001) but in some areas, ship strike was the primary cause of whale mortality (e.g., New Zealand, Constantine et al., 2015). As with other species, the threat may be mitigated through re-routing traffic or reducing vessel speed,

as implemented in the Hauraki Gulf, New Zealand. As shipping traffic increases, coastal populations of Bryde's whales are most vulnerable. Future establishment of new ports, oil and gas operations, or shipping routes need to consider whale presence to mitigate the mortality risk. Areas of high shipping traffic also mask the low frequency communications of whales through ship noise. Slowing ships can reduce their acoustic footprint, lessening another potential impact (Putland et al., 2017).

Bryde's whales were not extensively hunted during the commercial whaling era, but up to 50 whales were killed per year until 2016 as part of the Japanese whale research programme⁴. There may be other areas, possibly throughout the south-east Asian region where Bryde's whales are hunted opportunistically but these may also consist of Omura's whales (*Balaenoptera omurai*). Overall, hunting currently poses a low risk to whale populations but resumption of the Japanese programme should be monitored carefully.

With recent genetic studies revealing sub-species status for the South African and Gulf of Mexico populations, immediate action is needed to reduce and mitigate all anthropogenic mortality to ensure their future viability. In general, compared to many baleen whales, we know little about the Bryde's whales so a precautionary approach to managing threats is recommended as it is likely that there are isolated populations in other locations.

CONCLUSIONS

Whilst broadly distributed throughout warm-temperate waters, Bryde's whales have high variability in their distribution, foraging

⁴iwc.int/total-catches

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behavior, movement patterns and threats. With their flexible and diverse foraging strategies and broad prey preferences, Bryde's whales may be one of the more mobile baleen whales as our oceans change. As recent research has revealed the genetic isolation of some populations, we cannot assume that all Bryde's whale populations are secure. Future work should focus on defining who Bryde's and Eden's whales are, what defines populations and ascertain the real breadth of behavioral plasticity exhibited by the species. We suggest that the IUCN and IWC establish working groups to resolve the taxonomic and threat status to known populations, so we can manage Bryde's whales to avoid future conservation crises, as currently occurring in the Gulf of Mexico (Corkeron and Kraus, 2018). Given the prevalence of offshore populations, we recommend passive acoustic monitoring as a useful tool in determining stock status, possibly for abundance and distribution, and to assess potential levels of anthropogenic acoustic threats. As the environment changes, a multi-disciplinary, comparative approach will enable an ocean-wide assessment of shifts in habitat use, trophic interactions, distribution, and overall status of this lesser known baleen whale.

AUTHOR CONTRIBUTIONS

RC coordinated this work. All authors (RC, TI, SLN, and GSP) contributed to writing and editing the work.

ACKNOWLEDGMENTS

We thank our many colleagues for thoughtful conversations over the years about whales and their oceanic realm. This work is PMEL contribution #4826 (SLN).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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