



# Lethal and sublethal effects of oil, chemical dispersant, and dispersed oil on the ctenophore *Mnemiopsis leidyi*

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**ABSTRACT:** We established the lethal levels for water-accommodated fractions of Corexit® 9500A chemical dispersant, crude oil (WAF), and dispersed crude oil (CEWAF) for the ctenophore *Mnemiopsis leidyi* at both 15 and 23°C. This gelatinous zooplankton was sensitive to dispersant at both temperatures, as well as to oil solutions, with some increase in toxicity of CEWAF as compared to WAF. Subsequent sublethal assays for routine respiration rate, bioluminescence, and glutathione-s-transferase (GST) activity were conducted on individuals surviving 24 h exposures to test solutions at both 15 and 23°C. GST activity increased significantly in 2.5 and 5 mg l<sup>-1</sup> dispersant solutions at 15°C, suggesting a metabolic detoxification response to the dispersant-containing solutions, but no effect of any solution type on routine respiration rate was observed. Light emission through mechanically stimulated bioluminescence and photocyte lysis decreased with exposure to crude oil WAF and CEWAF at both temperatures and to dispersant exposure at 23°C. Collectively, these results demonstrate that *M. leidyi* exhibits both lethal and sublethal effects from acute crude oil exposure, with an elevation of some sublethal responses upon addition of chemical dispersant. Sublethal effects of oil and dispersants in pelagic species, most notably impairment of luminescence, should be considered when evaluating oil spill response strategies.

**KEY WORDS:** Crude oil · Corexit EC9500A · Zooplankton · Glutathione-s-transferase · Respiration · Bioluminescence

## INTRODUCTION

Accidental offshore oil releases have highlighted the need to better understand oil and chemical dispersant effects on pelagic organisms (Thibodeaux et al. 2011, Barron 2012). In coastal areas, dispersants are often employed to degrade surface slicks in the interest of protecting shorelines, but their use increases exposure of pelagic organisms by allowing oil at the ocean surface to mix into the water column inhabited by these organisms (Reed et al. 2004). Both lethal and sublethal toxicological data are limited for crude oil, dispersed oil, and dispersant itself across a range of ecologically important and abundant

pelagic marine taxa. Zooplankton are important members of pelagic communities, both as grazers on phytoplankton and as food for higher trophic levels (Banse 1995). They are particularly susceptible to oil and dispersant exposure given their limited ability to move away from contaminated areas as compared to nekton; while some of the more mobile zooplankton species exhibit oil avoidance swimming behaviors under laboratory conditions (Seuront 2010), the scales of large spills inevitably result in the widespread exposure of planktonic organisms (Mitra et al. 2012). Toxicological responses of zooplankton to oil and dispersants have largely been examined in crustaceans (e.g. Cowles & Remillard 1983a,b, Hemmer

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et al. 2011, Almeda et al. 2013b, Cohen et al. 2014). Recently, Almeda et al. (2013a) exposed several species of gelatinous zooplankters to crude oil emulsions. Their results suggest that gelatinous zooplankters, particularly coastal ctenophores, have the potential to be vectors of hydrocarbons up pelagic food webs, as these organisms survive oil exposures better than most zooplankton and accumulate high-molecular-weight polycyclic aromatic hydrocarbons (PAHs). Yet, major uncertainties remain concerning lethal effects of dispersed oil on gelatinous zooplankton, as well as oil/dispersant effects on sublethal physiological processes in these organisms.

Ctenophores are important links in marine food webs, serving as both predators of other zooplankton and prey for higher trophic levels including seabirds and turtles (Arai 2005, Cardona et al. 2012). The heavy predation of adult ctenophores on other zooplankton (Granhag et al. 2011), including the larvae of commercially valuable fish and shellfish species, affects not only the populations on which they feed directly but also secondarily on phytoplankton communities (Deason & Smayda 1982). The lobate ctenophore *Mnemiopsis leidyi* in particular has been shown to function as an important predator in coastal ecosystems throughout its native range along the Atlantic coast of North and South America and in the Gulf of Mexico (Kremer 1994, Bayha et al. 2015). Additionally, the clearance rate on autotrophic and heterotrophic microzooplankton by larval and post-larval ctenophores is sufficiently high as to impact the base of coastal food webs during these early life stages (Stoecker et al. 1987). As ctenophore tissue is relatively unprotected and exposed to surrounding seawater, ctenophores are potentially more susceptible to the toxic effects of dispersant and dispersed oil as compared to model fish and crustacean species, given the tendency of chemical dispersants to degrade biological membranes (Abel 1974). Seasonal blooms of *M. leidyi* also create the potential for the exposure of large numbers of animals in the case of a release that coincides with a bloom event (Sullivan et al. 2001). While Almeda et al. (2013a) studied oil-induced mortality and PAH accumulation in *M. leidyi*, data are lacking for exposures to either dispersant or dispersed oil, which are relevant to coastal spills and mitigation scenarios.

Mortality following acute exposure is the most obvious detrimental effect of oil and dispersant, but sublethal toxic effects also have the potential to create lasting ecological consequences (i.e. 'ecological death' sensu Singer et al. 1998). Sublethal effects of hydrocarbons and chemical dispersants on inverte-

brates, including some zooplankton taxa, include altered reproduction and settlement (Singer et al. 1998, Suderman & Marcus 2002, Hjorth & Nielsen 2011, Villanueva et al. 2011, Goodbody-Gringley et al. 2013), respiration rate (Laughlin & Neff 1981, Smith & Hargreaves 1984), growth (Laughlin & Neff 1981), feeding (Cowles & Remillard 1983a), and locomotion (Cowles & Remillard 1983b, Wu et al. 1997, Seuront 2011, Cohen et al. 2014). Sublethal studies of enzyme activity, including glutathione-s-transferases (GSTs), also suggest cellular detoxification pathways in zooplankton can be upregulated with hydrocarbon exposure (Lee 1988, Hansen et al. 2008). In gelatinous zooplankton, sublethal effects studies upon oil/dispersant exposure have been limited to feeding activity and swimming speed (Lee et al. 2012, Almeda et al. 2013a). Effects of oil/dispersant exposure on bioluminescence, a key physiological property of the ctenophore *M. leidyi* and many other pelagic species (reviewed in Haddock et al. 2010), have not been investigated. Ctenophores achieve luminescence by the oxidation of coelenterazine through species-specific, calcium-dependent, oxygen-independent photoproteins located within specialized photocyte cells (Ward & Seliger 1974b, Shimomura 2006). This bioluminescence most likely functions as a predator deterrent, whereby luminescent flashes misdirect or startle a potential predator (Morin 1983, Haddock et al. 2010), and ctenophore luminescence has in turn shaped the predator-avoidance behaviors of their copepod prey (reviewed in Buskey et al. 2012). For *M. leidyi*, the photoprotein (=mnemiopsin) is contained in photocytes localized along meridional canals; luminescence likely propagates via numerous gap junctions after being initiated at peripheral nerve terminals (Anctil 1985). Mnemiopsin activity in *M. leidyi* is potentially susceptible to chemical dispersants, whose membrane disruption properties may interfere with the animal's ability to regulate calcium in the mnemiopsin-containing photocytes.

The present study examined lethal levels of exposure for a mid-Atlantic population of the ctenophore *M. leidyi* at 2 seasonally relevant temperatures upon acute exposure to Corexit 9500A dispersant, crude oil (WAF), and dispersed crude oil (CEWAF) to set exposure levels for sublethal experiments. Subsequent sublethal exposure studies examining parameters that may change over acute exposure time frames determined ctenophore respiration rate and capacity for bioluminescence, as well as activity of the detoxification enzyme GST in ctenophore tissue.

## MATERIALS AND METHODS

### Specimen collection and laboratory acclimation

Ctenophores were collected by 15 to 30 min sets of a plankton net (0.75 m, 335  $\mu\text{m}$  mesh) during daytime flood tides from a stationary davit on a dock in the Broadkill River just inside of Roosevelt Inlet near the mouth of Delaware Bay, USA (38.8167° N, 75.2000° W). Background contamination levels at this site are unknown, but the lower Broadkill River estuary is considered well flushed and an extension of the Delaware Bay throughout most of the tidal cycle (Dewitt & Daiber 1973), and this region of the bay is uncontaminated beyond trace levels for metals, pesticides, polychlorinated biphenyls, tributyltin, and PAHs (Hartwell et al. 2001). Within 1 to 3 h of collection, physically undamaged and actively swimming ctenophores were transferred to individual finger bowls containing ~150 ml of ambient 0.4  $\mu\text{m}$  filtered seawater at a salinity of 33 PSU, hereafter referred to as filtered seawater (FSW). Salinity measured at the collection site with each net set was 29 to 34 PSU. FSW was used to prepare all test solutions and served as an oil/dispersant-free control. Freshly collected ctenophores were acclimated in FSW for 24 h prior to experiments in a dark laboratory incubator. Acclimation temperature matched the experimental temperature, which was either 15°C for ctenophores collected when water temperature was 6.8 to 19.6°C (November to May) or 23°C when water temperature was 18.2 to 25.1°C (June to October). Following acclimation, physically undamaged and actively swimming ctenophores were used in lethal and sublethal assays, described below.

### Test solution preparation

All test solutions were prepared as water-accommodated fractions in FSW according to standard methods developed by the Chemical Response to Oil Spills: Ecological Effects Research Forum (Coelho & Aurand 1997) as revised by Clark et al. (2001). Corexit EC9500A was obtained from Nalco Company (Sugar Land) and was maintained at room temperature in darkness until use. Light Louisiana sweet crude oil was obtained from British Petroleum, was kept in darkness at 4°C until use, and was not weathered. Each test solution was prepared in a 1.8 l volume of FSW brought to a 20% vortex in an aspirator flask on a digital magnetic stir plate, to which the desired volume of dispersant alone (Corexit, 0 to

30 mg dispersant  $\text{l}^{-1}$ ), crude oil alone (WAF, 0 to 150 mg oil  $\text{l}^{-1}$ ), or crude oil and dispersant in a 10:1 ratio (CEWAF, 0 to 30 mg oil  $\text{l}^{-1}$ ) was added by gas-tight syringe. Mixing of stoppered bottles in darkness was maintained for 18 h, followed by 4 to 6 h of settling before drawing test solutions from the bottom of the flasks for immediate use in experiments.

Samples of the test solutions were taken for Corexit, total petroleum hydrocarbon (TPH), and PAH analyses. Corexit concentrations in all dispersant solutions were measured spectrophotometrically by absorbance at 386 nm in a 100 mm pathlength quartz cuvette against a freshly prepared Corexit standard curve. TPHs (*n*-C9 to *n*-C42) were measured in 15°C WAF (1, 10, 50, 150 mg  $\text{l}^{-1}$ ) and CEWAF (1, 10, 50 mg  $\text{l}^{-1}$ ) test solutions at Mote Marine Laboratory (Sarasota, FL, USA) by extraction with modified USEPA Method 3510C, then analyzed by gas chromatography with flame ionization detection using modified USEPA Method 8260 as described in Cohen et al. (2014). PAHs (parent compounds and their alkylated homologues) were measured for 50 mg  $\text{l}^{-1}$  WAF and CEWAF solutions by GC-MS in electron impact selected ion monitoring mode (see Cohen et al. 2014 for detailed methods).

### Mortality experiments to set sublethal exposure levels

For mortality experiments, ctenophores were exposed to test solutions with a single individual in each 125 ml glass bottle devoid of headspace and capped with a Teflon-lined lid. Thirty individuals were tested in FSW and in either 4 or 5 concentrations of each test solution (15 and 23°C, respectively, see methods below for measurement of dissolved oxygen during these experiments). Bottles were held in darkness at the experimental temperature as described for acclimation and then scored for mortality after 24 h. For mortality assessment, animals were observed for 2 min, and those showing any sign of active swimming, defined as ctene beating and/or lobe pulsation, were considered alive; those lacking any of these movements over 2 min were considered dead. For experiments at 15°C, following the mortality scoring, approximately 80% of the solution in bottles containing surviving individuals was replenished with freshly prepared test solutions, and mortality was scored again at the 48 h time point. Similar 48 h experiments were not conducted at 23°C, as preliminary experiments found low survival in control animals during the second 24 h period.

### Respiration rate during acute sublethal exposures

Routine respiration rates were measured for ctenophores in conjunction with the 24 h mortality exposures at 23°C, while 15°C respiration measurements were made on individuals during a separate set of 24 h exposures to accommodate the 48 h mortality experiment at this temperature. Oxygen consumption by individual ctenophores (1 ctenophore per 125 ml bottle) was calculated with the single endpoint method (Ikeda et al. 2000), whereby average oxygen consumption in 125 ml control bottles without ctenophores was subtracted from measurements in bottles containing ctenophores. Two ctenophore-free control bottles were prepared for every 10 bottles with ctenophores. Sample size for each treatment depended on survival in the respective solutions and ranged between 2 and 30 individuals (see 'Results' for details). Dissolved oxygen was measured by a polarographic oxygen microelectrode (MI-730, Microelectrodes) and data acquisition system (PowerLab 26T, AD Instruments) immediately upon opening the bottles at the end of the 24 h exposure. All measurements yielded final oxygen partial pressures greater than the critical partial pressure reported for *Mnemiopsis leidyi* (0.72 kPa, Thuesen et al. 2005), suggesting the animals were capable of oxyregulation throughout the incubation. Following dissolved oxygen measurement, the tissue volume of each ctenophore was measured by its water displacement. Tissue volume was converted to wet weight using an empirically determined relationship (tissue wet weight =  $1.198 + (0.9896 \times \text{tissue volume})$ ,  $r^2 = 0.997$ ,  $F = 3498$ ,  $p < 0.001$ ) to allow for the calculation of weight-specific oxygen consumption rates. The range of calculated wet weights for animals used in the respiration experiments was 1.69 to 9.11 g.  $Q_{10}$  values for weight-specific oxygen consumption rates in each solution type and concentration were calculated from rates at 15 and 23°C according to Hochachka & Somero (2002). Microbes associated with the body surface of ctenophores (e.g. Daniels & Breitbart 2012) introduced into experimental bottles could have contributed to the measured ctenophore respiration rates as calculated above. However, their contribution is considered negligible at lower temperatures (e.g. 15°C, Ikeda et al. 2000) and was found to be minimal (< 3% of ctenophore respiration rates) at 23°C in WAF and CEWAF treatments by measurement of dissolved oxygen during a second 24 h incubation with ctenophore volume replaced by FSW (data not shown).

### Bioluminescence after acute sublethal exposures

Bioluminescence assays were conducted on the ctenophores used for respiration rate measurements as described above. Samples size for each treatment again depended on survival and ranged between 2 and 30 individuals (see 'Results' for details). Following dissolved oxygen and tissue volume measurements, which lasted ~5 min per individual, the test solutions were discarded, and individuals were returned to their 125 ml bottles in the 25 ml of FSW in which their tissue volume was measured. Individuals were then acclimated in the dark for 30 to 60 min at the appropriate experimental temperature to allow for reversal of any luminescence inhibition by exposure to room lights (e.g. Ward & Seliger 1974b, Schnitzler et al. 2012). Spontaneous luminescence was not observed over this time period. The remainder of the experiment was conducted under dim far-red light in an otherwise light-tight room. After the dark-acclimation period, luminescence of individual ctenophores was elicited mechanically and by photocyte lysis during a 3-phase experiment modified from Ward & Seliger (1974b). Measurements were made within a light-tight chamber consisting of a white stir plate (model AS645, Corning) covered by a white hemispherical plastic cap fitted on the side with a flat spectral response (450 to 950 nm) radiometric sensor head and optometer (models 247 and S471, UDT Instruments), which logged light data at 2 Hz on a laptop computer. The first phase of the bioluminescence assay involved mechanical stimulation of luminescence, that is, the reaction of intracellular calcium with the calcium-dependent mnemiopsin photoprotein found in *M. leidyi* photocytes. To achieve this, a 1 inch magnetic stir bar was placed in the bottle with the animal, light meter logging was initiated, and then after 2.5 s the stir plate was activated for 27.5 s at 400 rpm. This yielded 30 s of light data for this first phase of bioluminescence stimulation. The second phase of the bioluminescence assay again involved mechanical stimulation of luminescence in intact photocytes but, in this case, during homogenization to break down ctenophore tissue and expose the intact photocytes to FSW. To achieve this, the stir bar was removed, and a tissue homogenizer (model PT90/35, Kinematica) was inserted into the bottle through a small port in the top of the hemispherical cap. Recording of light data for 30 s was begun at the onset of homogenization, and light was released by stimulation of the still intact photocytes. The third phase of the bioluminescence assay was immediately begun after the homogenization period and consisted of rapid addition of 25 ml 0.1 M KCl to sea-

water in the bottle (~0.3 M KCl final concentration) via tubing extending through the port, hypotonically lysing the photocytes and releasing mnemiopsin into the FSW. With the homogenizer still running to mix the solution, light data were collected for a third period of 30 s, continuous with the second, during which any residual mnemiopsin from the first 2 phases was free to react with calcium in the FSW. Luminescence was integrated over the 3 phases (=total integrated luminescence) for comparison across treatments.

### GST activity after acute sublethal exposures

GST enzyme activity assays were conducted on tissue from ctenophores that were collected and initially acclimated for 24 h to laboratory conditions in FSW at 15 and 23°C, as described above, then exposed to test solutions for an additional 24 h period. Test solutions included FSW and sublethal concentrations of Corexit (2.5 and 5 mg dispersant l<sup>-1</sup>), WAF (1 and 10 mg oil l<sup>-1</sup>), and CEWAF (1 and 10 mg oil l<sup>-1</sup>) prepared as described for mortality assays. Survival in the exposures was 86% at 15°C and 78% at 23°C. Surviving ctenophores from a given treatment were homogenized in 0.25× Tris/glycine/sodium dodecyl sulfate sample buffer, 4°C and pH 8.3; groups of 3 to 4 individuals were homogenized together to provide sufficient protein for the enzyme assay, with 3 to 5 replicate groups processed at each concentration (see 'Results' for details). Protein was concentrated by transferring 2 ml of the homogenate to a 30 000 NMWL microcentrifuge filter tube (UFC4LTK25, Millipore) and spun for 45 min at 5000 × *g* and 4°C. The retentate containing GST (MW ~45 000) was assayed following the standard method of Habig et al. (1974). Reagents (reduced glutathione and 1,2-dichloro-4-nitrobenzene, Sigma Aldrich G4251 and 138630, respectively) were in molar excess of the GST present in the sample at a final concentration of 0.02 M in 1.5 ml phosphate-buffered saline (PBS), pH 7.8. Following the addition of 100 µl of concentrated sample, absorbance at 340 nm was measured at 30 s intervals over a 5 min period at 23°C (Thermo Evolution 201 UV-visible spectrophotometer). The rate of absorbance increase in a blank reaction mixture—its sample volume replaced by PBS—was subtracted from that of the experimental samples. GST specific activity (µmol ml<sup>-1</sup> min<sup>-1</sup>) was then calculated from the rate of change in absorbance (min<sup>-1</sup>) using the extinction coefficient of the 1-chloro-2,4-dinitrobenzene conjugate at 340 nm (9.6 mM<sup>-1</sup> cm<sup>-1</sup>) and normalized

to total protein in the samples quantified by the method of Bradford (1976) with reagents and BSA standards from Pierce Biotechnology.

### Statistical analysis

To quantify lethal exposure levels, mortality data were used to estimate median lethal concentrations (LC<sub>50</sub>s) and their associated 95% confidence intervals by the trimmed Spearman-Kärber method (Hamilton et al. 1977, 1978). For each sublethal assay (respiration rate, bioluminescence, GST activity), effects of each treatment on the measured parameter were compared to FSW controls within each temperature by Kruskal-Wallis ANOVAs on ranks with treatment concentration as the factor, followed by Dunn's post hoc test ( $\alpha = 0.05$ ). This non-parametric test was selected for all data analyses due to failure of Shapiro-Wilk normality tests in some cases.

## RESULTS

### Mortality experiments to set sublethal exposure levels

Mortality of *Mnemiopsis leidyi* was generally concentration dependent in Corexit, WAF, and CEWAF solutions at both 15 and 23°C, with some variability at Corexit concentrations less than 10 mg l<sup>-1</sup> (Fig. 1). LC<sub>50</sub>s for Corexit were similar at 24 and 48 h for the 15°C exposures, as well as between 15 and 23°C in 24 h exposures based on measured concentrations, although there was a significant difference between the 2 temperatures (based on 95% confidence intervals) when the nominal loadings are considered (Table 1). A difference between 24 and 48 h LC<sub>50</sub>s was observed for CEWAF mortality based on TPH concentration but not on nominal loading concentration (Table 1). Mortality was not sufficiently high in 15°C WAF exposures at 24 h to allow for an LC<sub>50</sub> calculation but increased by 48 h (Table 1).

Despite a 13.6-fold increase in TPH concentration between the 50 mg l<sup>-1</sup> WAF and CEWAF, total PAH concentrations were similar in the 2 solutions (265 and 344 µg l<sup>-1</sup>, respectively). The distribution of PAHs was affected by the addition of Corexit (see Appendix 1), as 4 classes of PAHs (fluoranthrenes, pyrenes, benzoanthracenes, and chrysenes) absent from the WAF solution were present in CEWAF. Alkyl-PAHs were also greater in CEWAF than in WAF, with alkyl:non-alkyl ratios of 4.62 and 3.43 for CEWAF and WAF solutions, respectively.

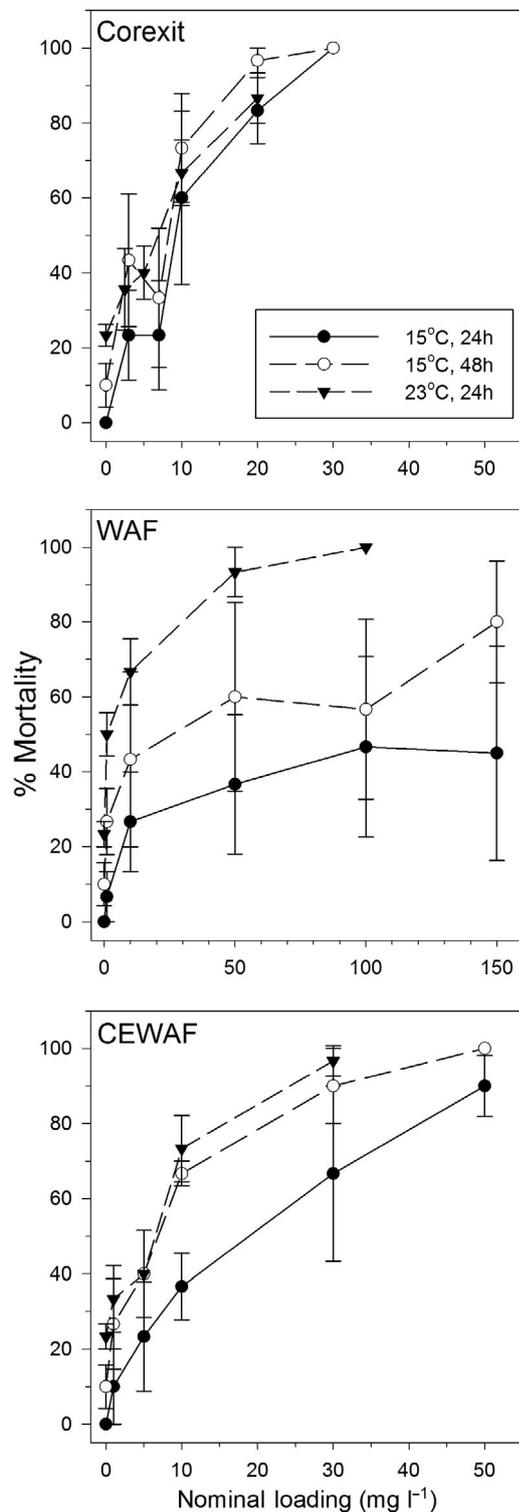


Fig. 1. Mortality of ctenophores exposed to dispersant (Corexit), crude oil (WAF), or dispersed crude oil (CEWAF). Data are mean percentage mortalities ( $\pm$ SE) of ctenophores ( $n = 3$  trials, 10 ctenophores per trial) exposed in individual bottles to test solutions at 15°C (24 and 48 h exposure) and 23°C (24 h exposure). Mortality is plotted as a function of nominal loading of oil (WAF, CEWAF) or dispersant (Corexit)

### Respiration rate during acute sublethal exposures

Wet weight of ctenophores did not differ among the FSW control and all concentrations within each solution type at each temperature (Kruskal-Wallis ANOVAs,  $p > 0.05$  in all cases), allowing for the direct comparison of weight-specific respiration rates among concentrations. Higher concentrations of some solutions (e.g. WAF) showed somewhat elevated mean routine respiration rates relative to FSW controls, but no significant differences were observed for the 24 h exposure periods at either 15 or 23°C ( $p > 0.05$ , Kruskal-Wallis ANOVAs for each solution type) (Fig. 2). Overall,  $Q_{10}$  calculations based on these rates indicated a marginal to moderate temperature effect (Fig. 2). Values did extend below 1.0 at higher Corexit concentrations and intermediate CEWAF concentrations, while  $Q_{10}$ s ranged between 2.4 and 3.6 for WAF exposures (Fig. 2).

### Bioluminescence after acute sublethal exposures

Exposure to Corexit at 15°C for 24 h resulted in no significant differences in total integrated luminescence relative to luminescent emission of ctenophores in the FSW control ( $H = 19.544$ ,  $df = 4$ ,  $p < 0.001$ ; Dunn's test,  $p > 0.05$ ), despite lower mean luminescence values at 10 and 20 mg dispersant l<sup>-1</sup> (Figs. 3 & 4). However, at 23°C, increasing dispersant concentration resulted in lower luminescence, with 10 and 20 mg dispersant l<sup>-1</sup> exposures significantly lower than FSW controls ( $H = 18.201$ ,  $df = 4$ ,  $p = 0.001$ ; Dunn's test,  $p < 0.05$ ) (Figs. 3 & 4). An effect was also observed in oil-containing solutions, as concentration-dependent decreases in luminescence were observed in both WAF and CEWAF solutions at 15°C ( $H = 21.741$ , 14.05;  $df = 4$ ,  $p < 0.001$ ,  $p = 0.03$ ; Dunn's test,  $p < 0.05$ ) and 23°C ( $H = 18.375$ , 17.312;  $df = 4$ ,  $p = 0.001$ ,  $p < 0.001$ ; Dunn's test,  $p < 0.05$ ) (Figs. 3 & 4).

### GST activity after acute sublethal exposures

The effect of 24 h Corexit exposure on GST activity at 15°C was apparent in the sublethal dispersant-only solutions (Fig. 5), with both the 2.5 and 5 mg l<sup>-1</sup> loadings leading to significant increases in GST activity compared to FSW controls ( $H = 23.50$ ,  $df = 6$ ,  $p < 0.001$ ; Dunn's test,  $p < 0.05$ ). WAF and CEWAF exposures both displayed increasing trends in GST activity with increased oil loading, CEWAF to a

Table 1. Median lethal concentrations ( $LC_{50}$ s) of Corexit EC9500A, crude oil (WAF), and dispersed crude oil (CEWAF) solutions for *Mnemiopsis leidyi* estimated using the trimmed Spearman-Kärber method from either the measured concentration of Corexit ( $mg\ l^{-1}$ ) or total petroleum hydrocarbons (TPH,  $\mu g\ l^{-1}$ ), as described in 'Materials and methods', or by the nominal loading concentration. Confidence intervals (95%) are given in parentheses. TPH concentrations were not measured for the oil-containing (WAF and CEWAF) solutions prepared at 23°C, so  $LC_{50}$ s are not presented (dashes). >>trim = mortality too low to allow for  $LC_{50}$  calculation

			Corexit or TPH conc. ( $mg\ l^{-1}$ or $\mu g\ l^{-1}$ )	Nominal conc. ( $mg\ l^{-1}$ )
Corexit	15°C	24 h	6.9 <sup>a</sup> (5.9–8.2)	9.5 (7.9–11.3)
		48 h	5.4 <sup>a</sup> (5.0–5.9)	8.12 (7.3–9.0)
	23°C	24 h	8.3 <sup>a</sup> (6.3–10.8)	18.9 (15.9–22.4)
WAF	15°C	24 h	>>trim	>>trim
		48 h	143.1 <sup>b</sup> (56.6–362.3)	29.5 (13.8–63.3)
	23°C	24 h	–	4.7 (1.5–14.8)
CEWAF	15°C	24 h	576.6 <sup>b</sup> (380.2–874.7)	13.4 (9.3–19.3)
		48 h	242.1 <sup>b</sup> (153.9–370.8)	6.7 (4.3–10.4)
	23°C	24 h	–	7.7 (5.5–10.8)

<sup>a</sup> $mg\ l^{-1}$ , <sup>b</sup> $\mu g\ l^{-1}$

greater extent than the WAF, but neither was different from the FSW control (Dunn's test,  $p > 0.05$ ). For 23°C exposures, all test solutions resulted in GST activity not significantly different from FSW control levels ( $H = 8.15$ ,  $df = 6$ ,  $p = 0.227$ ).

## DISCUSSION

$LC_{50}$  data for *Mnemiopsis leidyi* exposed for 24 or 48 h to WAFs of crude oil alone at 15 and 23°C suggest this ctenophore has similar acute lethal levels for oil exposure as non-gelatinous zooplankton taxa tested either as individual species (Avila et al. 2010, Goodbody-Gringley et al. 2013, Cohen et al. 2014) or in mixed assemblages (Almeda et al. 2013b) using a range of oil preparation and exposure methods. For example, the coastal copepod *Labidocera aestiva*, tested by Cohen et al. (2014) using similar methods to the present study, had a 24 h  $LC_{50}$  at 23°C ( $5.0\ mg\ l^{-1}$ ) similar to that of *M. leidyi* in this study ( $4.7\ mg\ l^{-1}$ ). Barazandeh et al. (2004) conducted mortality studies with *M. leidyi* in WAF and found  $LC_{50}$ s ( $3.3$  to  $4.6\ ml\ l^{-1}$ ) 3 orders of magnitude greater than those in the present study ( $4.7\ mg\ l^{-1} = 5.6\ \mu l\ oil\ l^{-1}$ ). However, our lower values are consistent with those of Almeda et al. (2013a), who exposed *M. leidyi* to crude oil emulsions, suggesting

that these lower values better represent acute toxicity of crude oil, in various forms, to ctenophores. Our  $LC_{50}$  values follow an expected trend of decrease with increasing temperature (Korn et al. 1979), suggesting increased mortality if exposure occurs in

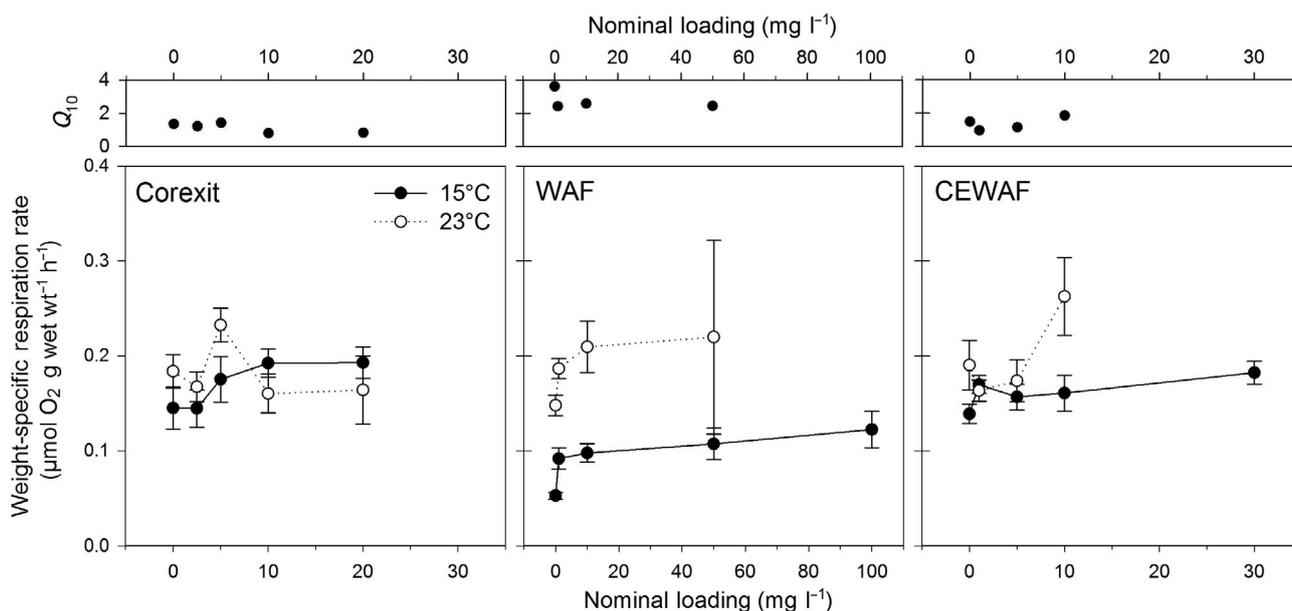


Fig. 2. Routine respiration rates of ctenophores exposed to dispersant (Corexit), crude oil (WAF), or dispersed crude oil (CEWAF). Mean ( $\pm$  SE) weight-specific respiration rates are shown in lower panels. Sample sizes varied depending on mortality at each concentration: Corexit, 15°C ( $n = 13$  to  $30$ ) and 23°C ( $n = 4$  to  $29$ ); WAF, 15°C ( $n = 4$  to  $30$ ) and 23°C ( $n = 2$  to  $29$ ); CEWAF, 15°C ( $n = 13$  to  $30$ ) and 23°C ( $n = 7$  to  $29$ ). Upper panels show  $Q_{10}$ s calculated for nominal loadings where respiration rates at both 15 and 23°C were measured

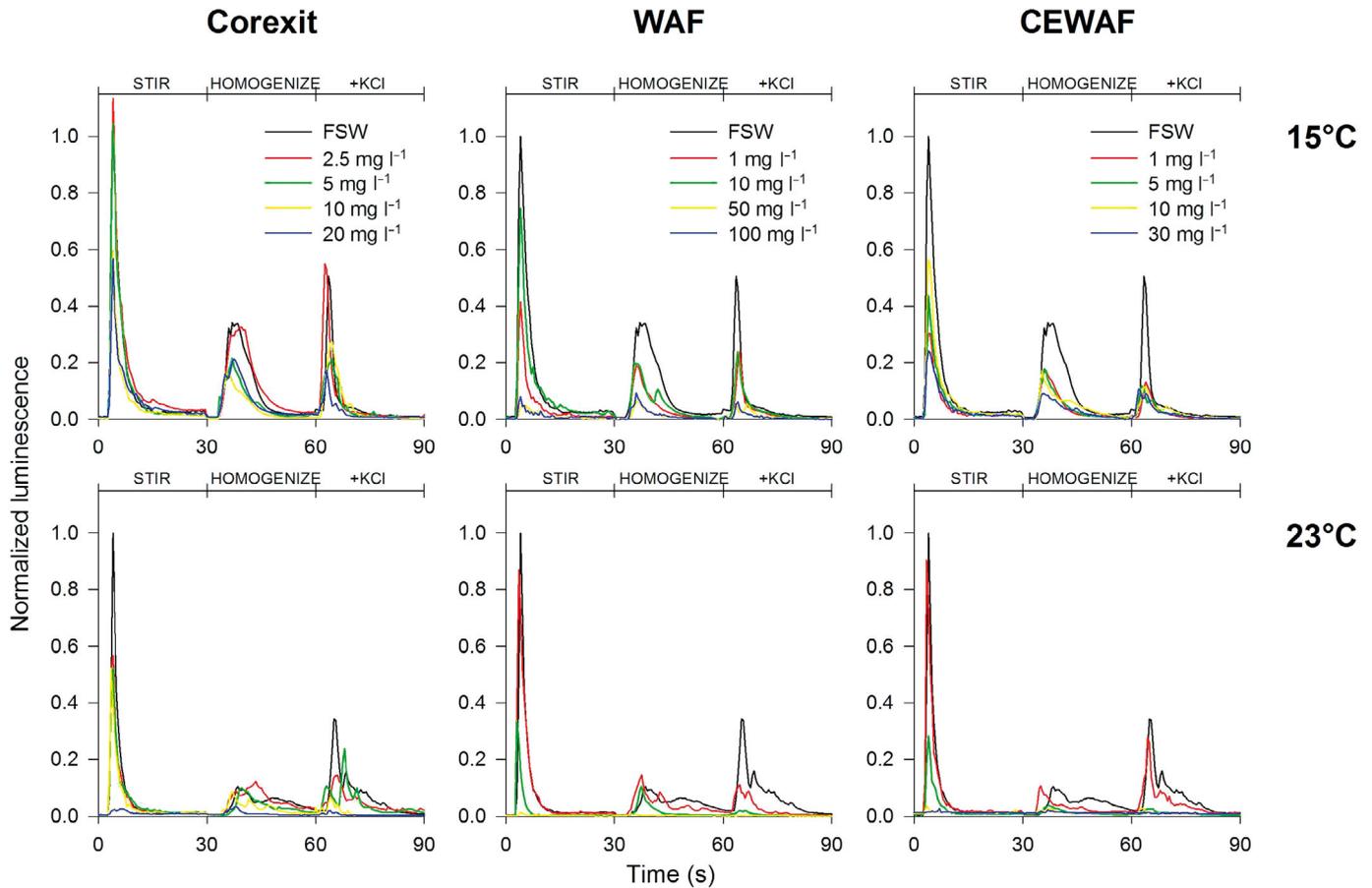


Fig. 3. Bioluminescence profiles of ctenophores exposed to dispersant (Corexit), crude oil (WAF), or dispersed crude oil (CEWAF). Average normalized luminescence in the 3-phase assay is plotted for ctenophores following 24 h exposure to water-accommodated fractions of Corexit, WAF, and CEWAF solutions at 15 and 23°C. Light measured at each time point was normalized to the peak value in the respective controls at each temperature. Sample size varied depending on survival during 24 h exposures prior to luminescence assay: Corexit,  $n = 4$  to 30; WAF,  $n = 2$  to 30; CEWAF,  $n = 7$  to 30

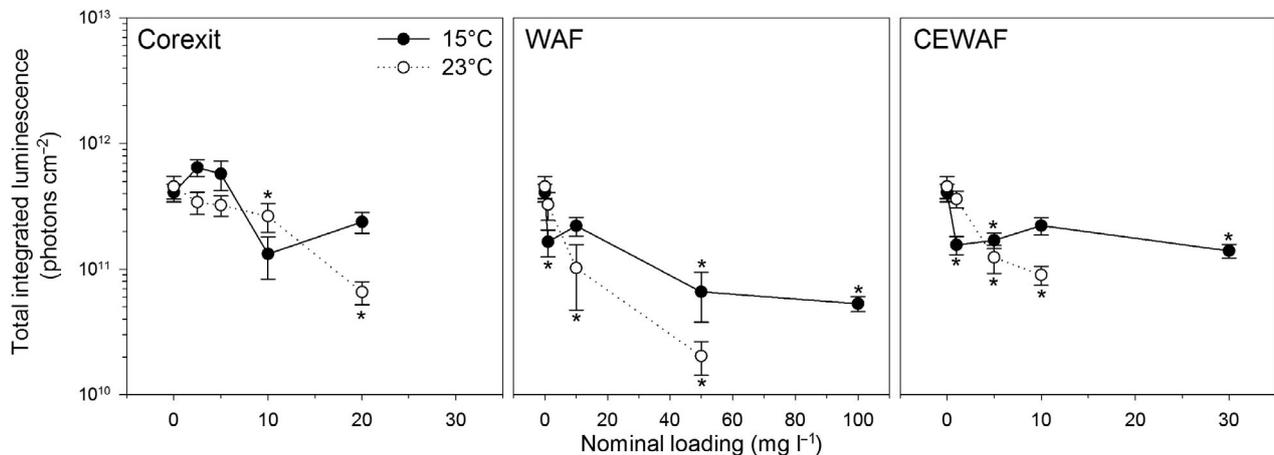


Fig. 4. Total luminescent emissions of ctenophores exposed to dispersant (Corexit), crude oil (WAF), or dispersed crude oil (CEWAF). The integrated luminescence emitted from individual ctenophores (mean  $\pm$  SE) is plotted as a function of test solution nominal concentration. Sample size varied depending on survival during 24 h exposures prior to luminescence assay: Corexit,  $n = 4$  to 30; WAF,  $n = 2$  to 30; CEWAF,  $n = 7$  to 30. Asterisks denote treatments that were significantly different from the filtered seawater control within each temperature

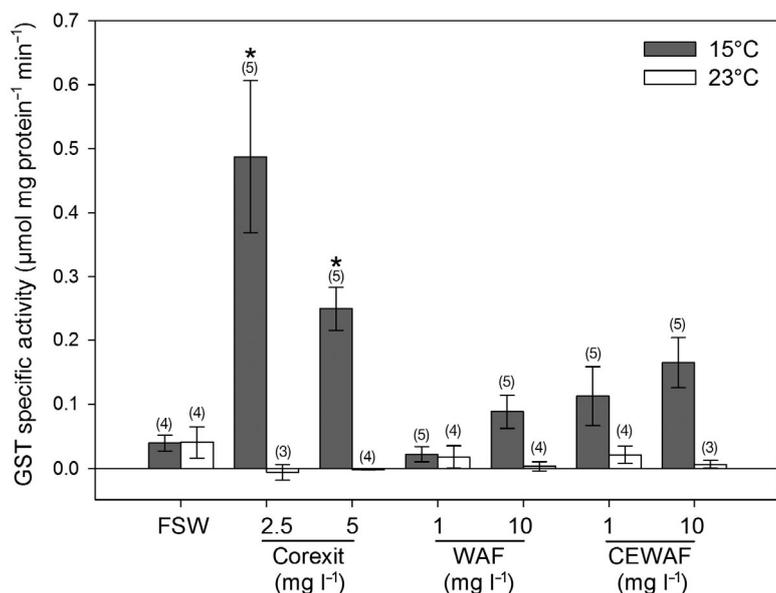


Fig. 5. Glutathione-s-transferase (GST) activity of ctenophores exposed to dispersant (Corexit), crude oil (WAF), or dispersed crude oil (CEWAF). Enzyme activities are means ( $\pm$  SE), with sample sizes in parentheses above each bar. All assays were conducted at 23°C on tissue from ctenophores exposed to the test solutions at either 15 or 23°C for 24 h. Asterisks denote treatments that were significantly different from the filtered seawater (FSW) control within each temperature

warmer months, but in general these  $LC_{50}$ s are higher than concentrations ctenophores would likely experience during spills. While oil concentrations during spills can exceed  $20 \mu\text{l l}^{-1}$  (McAuliffe et al. 1981), during the recent Deepwater Horizon spill in the Gulf of Mexico, oil in estuarine/coastal waters was thought to be less than  $0.2 \mu\text{l l}^{-1}$  (Whitehead et al. 2012), well below lethal levels for ctenophores at both temperatures in our study.

The toxicity of dispersed crude oil relative to crude oil alone (i.e. CEWAF vs. WAF in the present study) is an open question for zooplankton (Wise & Wise 2011). Some work (e.g. Almeda et al. 2013b) reports that chemical dispersant added to crude oil increases toxicity, while others (e.g. Cohen et al. 2014) have found dispersant addition to only weakly alter crude oil toxicity during acute exposures. Such differences may be species dependent rather than a function of differences in study design (Singer et al. 1998). In the case of *M. leidyi*, when  $LC_{50}$ s calculated by the nominal loading of oil are considered, CEWAF is more toxic than WAF, as mortality was higher in CEWAF at lower loadings than in WAF. The addition of Corexit in CEWAF solutions appears to elevate the toxicity of oil solutions, at least in these acute laboratory exposures conducted in darkness, representing a scenario where photoenhanced toxicity (e.g. Almeda et al.

2013b) and dilution cannot occur. Corexit likely contributed to the elevated TPH observed in CEWAF, both from hydrocarbons in the dispersant solution and by dissolving the oil into micelles within the water-soluble fraction that were then extracted during TPH measurement (Reed et al. 2004). Our PAH data provide evidence for the formation of micelles in dispersed oil solutions, with a greater contribution of less soluble 3- and 4-ring PAHs in CEWAF as compared to WAF, along with a greater proportion of alkylated PAHs, as has been observed previously and correlated with mortality and detoxification responses in larval fish (Couillard et al. 2005). While the compounds in micelles are generally not considered bioavailable, the size of crude oil micelles ( $<100 \mu\text{m}$ , Canevari 1978) is within the range of prey ingested by *M. leidyi* (20 to 200  $\mu\text{m}$ , Sullivan & Gifford 2004). Thus, oil micelles may be bioavailable to ctenophores and could explain enhanced CEWAF toxicity. The ingestion of small oil droplets has been reported for the doliolid *Dolioletta gegenbauri* (Lee et al. 2012). Additionally, *M. leidyi* bioaccumulates PAHs,

particularly those of higher molecular weight, likely due in part to dietary uptake of oil droplets and/or contaminated prey (Almeda et al. 2013a).

Indirect effects of dispersant on ctenophore mortality through micelle formation in CEWAF discussed above are likely more relevant than exposure to dispersant alone in an actual spill response scenario where dispersants are applied directly to an oil release. However, knowledge of dispersant toxicity is useful, as these compounds are regulated in part based on their toxicity to several estuarine model species, which may or may not be representative of gelatinous marine species. Indeed, the dispersant Corexit EC9500A in the present study was more toxic to *M. leidyi* than to a model estuarine crustacean, the mysid *Americamysis bahia* (48 h  $LC_{50} = 38 \text{ mg l}^{-1}$ , Hemmer et al. 2011). Mortality studies with Corexit and other common coastal zooplankton such as the copepods *Acartia tonsa* (Avila et al. 2010) and *L. aestiva* (Cohen et al. 2014) reported  $LC_{50}$ s similar to those found for *M. leidyi*. However, the observed  $LC_{50}$ s in these studies are higher than dispersant concentrations likely to be encountered in the water column during a spill response scenario (Lewis & Aurand 1997). Both dispersant and oil compounds could have contributed to the elevated mortality in CEWAF discussed above, which is consistent with

other studies on mesozooplankton (e.g. Almeda et al. 2013b). However, the low concentrations of Corexit in the CEWAF solutions relative to its  $LC_{50}$  suggest that indirect mechanisms of dispersant, such as enhancing bioavailability of hydrocarbons in consumable micelles, play a more important role in toxicity for *M. leidyi*.

The sublethal assays with *M. leidyi* in the present study (GST activity, respiration rate, and bioluminescence) suggest that negative effects of exposure to Corexit EC9500A, WAF, and CEWAF occur, but these effects depend on both the assay itself and the exposure temperature. Activity of the detoxification enzyme GST, which increases solubility of potentially alkylating xenobiotics, is an established biomarker for hydrocarbon and surfactant exposure in marine invertebrates (e.g. Lee 1988, McLoughlin et al. 2000, Raisuddin et al. 2007). Upregulation of GST transcription has been observed in marine copepods after only 12 h of acute static exposure to crude oil WAF (Hansen et al. 2011). In the present study, GST activity for *M. leidyi* in FSW controls ( $0.039 \mu\text{mol mg protein}^{-1} \text{min}^{-1}$ ) is at the low end of that reported for a range of marine invertebrates ( $0.015$  to  $0.748 \mu\text{mol mg protein}^{-1} \text{min}^{-1}$ , e.g. Lee 1988, Gassman & Kennedy 1992). No significant effect of WAF or CEWAF on GST activity was observed in *M. leidyi* whole body homogenates at the tested exposure levels and durations, despite some suggestion of a concentration-dependent increase, particularly in CEWAF at  $15^\circ\text{C}$ . The overall low GST activity and minimal induction by hydrocarbons is consistent with Lee (1975), who suggested ctenophores have limited capacity for depurating petroleum hydrocarbons, and supports Almeda et al. (2013a), who posited that *M. leidyi* may be a vector for transfer of hydrocarbons in marine food webs. Interestingly, Corexit solutions did have elevated GST activity relative to FSW controls at  $15^\circ\text{C}$ . The volume of Corexit loaded into the  $10 \text{ mg l}^{-1}$  CEWAF solution (i.e.  $2.1 \mu\text{l}$ ) was nearly half that of the  $2.5 \text{ mg l}^{-1}$  Corexit solution (i.e.  $5 \mu\text{l}$ ); any GST activity in CEWAF exposures may have resulted more from a detoxification response to Corexit rather than to oil. The lack of oil/dispersant induction of GST activity in  $23^\circ\text{C}$  exposures as compared to  $15^\circ\text{C}$  is striking and may be due to the underlying physiological condition of ctenophores used in winter/spring ( $15^\circ\text{C}$ ) and summer/fall ( $23^\circ\text{C}$ ) experiments. Protein-normalized GST activities were the same for both temperatures in FSW; therefore, the difference between these 2 seasons/temperatures is not apparent in the constitutive activity level of the enzyme, as has been reported for other invertebrates (e.g. Power &

Sheehan 1996), but rather the induction of higher GST activity with dispersant exposure. Respiration rates, which in FSW were similar to those reported for *M. leidyi* previously (e.g. Anninsky et al. 2005, Thuesen et al. 2005), were not significantly elevated in any test solution, as might be expected with an increase in aerobic metabolic demand from upregulation of detoxification pathways (Hatlen et al. 2009, Rodrigues et al. 2013) or from attempted avoidance of the toxicants (Seuront 2010). As respiration rate experiments employed single endpoint measurements and were terminated after 24 h, when ctenophores were then assayed for bioluminescence, it is not known whether alterations in respiration rate and/or GST activity (in oil treatments and/or in  $23^\circ\text{C}$  exposures) occurred but only lasted a short time or if they would occur with longer exposure durations. Previous examination of oil and PAH exposure on zooplankton metabolism suggests that while sublethal effects may be observed, they are often limited or transient (Laughlin & Neff 1981, Samain et al. 1981, Depledge 1984, Smith & Hargreaves 1984, Gilfillan et al. 1986).

Little is known about specific effects of oil and dispersant on bioluminescent emissions in marine organisms, which themselves are varied in mechanism and function (Haddock et al. 2010). Exposure to crude oil, dispersant, and dispersed crude oil has been shown to decrease luminescence in bacteria and dinoflagellates in commercially available toxicity assays (Paul et al. 2013). Our results are consistent with this finding, as the total amount of light emitted by individual ctenophores (total integrated luminescence) decreased with increasing oil and dispersant concentration. Luminescent output in *M. leidyi* is dependent on mnemiopsin concentration and is independent of body size (Ward & Seliger 1974a). Accordingly, total integrated luminescence as measured here represents the cumulative response of all mnemiopsin present in *M. leidyi*, as we first stimulated the mnemiopsin photoreaction in intact photocytes and then in lysed photocytes, where all unreacted mnemiopsin was free to react with calcium in FSW. The distribution of light production among these phases, and temporal dynamics within these phases, was temperature dependent in FSW controls (Fig. 3), which fits with previous reports of a  $22$  to  $26^\circ\text{C}$  optimum for luminescence emission in *M. leidyi* (Olga & Yuriy 2012). Total integrated luminescence measurements suggest that ctenophores exposed to higher concentrations of test solutions, particularly those containing oil and those at the  $23^\circ\text{C}$  exposure temperature, either possessed diminished cellular stores of the mnemiopsin photoprotein after 24 h incubations or experienced alterations in the re-

activity of mnemiopsin with oil/dispersant exposure. If the former, it is unknown whether this was due to a reduction in mnemiopsin through protein degradation, loss through membrane damage, or a reduction in mnemiopsin synthesis as toxic exposure resulted in prioritizing other cellular functions such as repair and detoxification pathways (e.g. Camus et al. 2003, Smolders et al. 2003, Olsvik et al. 2012). If mnemiopsin activity was affected, there may have been a reduction in binding or light production efficiency, either through direct toxic effects or indirectly (e.g. through photocyte pH changes, Jafarian et al. 2011). Irrespective of mechanism, these data suggest ctenophore bioluminescence can be impacted by acute exposure to oil and chemical dispersant, and these effects may be greatest if such exposure occurs during warmer periods of the year when ctenophores are generally more abundant (Sullivan et al. 2001) and higher mortality occurs for a given oil concentration. Subsequent work is needed to determine if a reduction in light emission from such exposures would result in functional impairment of ctenophore bioluminescence in a natural predator-prey context (e.g. predator deterrence) and to what extent the pelagic bioluminescent community is more broadly affected by widespread oil releases and spill response efforts.

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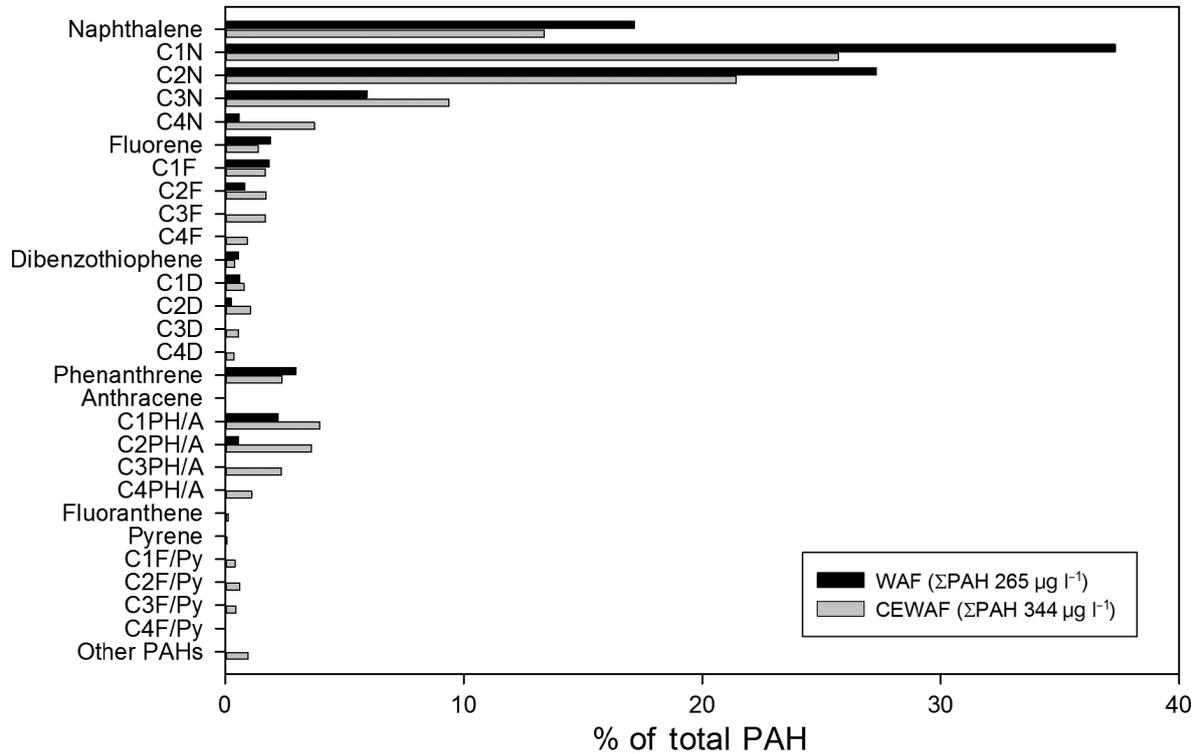
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**Appendix.** Polycyclic aromatic hydrocarbons (PAHs) in crude oil (WAF) and dispersed crude oil (CEWAF) solutions. Classes of PAHs, represented as parent compounds and their alkylated homologues (C1–C4), measured in freshly prepared 50 mg l<sup>-1</sup> WAF and CEWAF. 'Other PAHs' refers to the sum of the remaining PAHs that are USEPA priority pollutants and their alkylated derivatives



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