Environmental Pollution 208 (2016) 645–654

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

How much crude oil can zooplankton ingest? Estimating the quantity of dispersed crude oil defecated by planktonic copepods

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ARTICLE INFO

Article history:
Received 11 August 2015
Received in revised form
20 October 2015
Accepted 23 October 2015
Available online 14 November 2015

Keywords:
Crude oil
Zooplankton
Ingestion
Fecal pellets
Oil spills

ABSTRACT

We investigated and quantified defecation rates of crude oil by 3 species of marine planktonic copepods (*Temora turbinata*, *Acartia tonsa*, and *Parvocalanus crassirostris*) and a natural copepod assemblage after exposure to mechanically or chemically dispersed crude oil. Between 88 and 100% of the analyzed fecal pellets from three species of copepods and a natural copepod assemblage exposed for 48 h to physically or chemically dispersed light crude oil contained crude oil droplets. Crude oil droplets inside fecal pellets were smaller (median diameter: 2.4–3.5 μm) than droplets in the physically and chemically dispersed oil emulsions (median diameter: 6.6 and 8.0 μm, respectively). This suggests that copepods can reject large crude oil droplets or that crude oil droplets are broken into smaller oil droplets before or during ingestion. Depending on the species and experimental treatments, crude oil defecation rates ranged from 5.3 to 245 ng-oil copepod^{-1} d^{-1}, which represent a mean weight-specific defecation rate of 0.026 μg-oil μg-C$^{1}_{copepod}$ d^{-1}. Considering a dispersed crude oil concentration commonly found in the water column after oil spills (1 μl L^{-1}) and copepod abundances in high productive coastal areas, copepods may defecate ~1.3–2.6 mg-oil m^{-3} d^{-1}, which would represent ~0.15%–0.30% of the total dispersed oil per day. Our results indicate that ingestion and subsequent defecation of crude oil by planktonic copepods has a small influence on the overall mass of oil spills in the short term, but may be quantitatively important in the flux of oil from surface water to sediments and in the transfer of low-solubility, toxic petroleum hydrocarbons into food webs after crude oil spills in the sea.

1. Introduction

Marine crude oil spills have become a major environmental concern due to growing petroleum industry activities in the sea over the last decades (National Research Council, 2003). When crude oil enters the sea, oil is transformed by physical, chemical, and biological processes ("weathering") that determine the fate and ultimately the impact of petroleum pollution in marine environments (National Research Council, 2003). Among biotic processes affecting marine oil spills, bacterial degradation of petroleum hydrocarbons has been intensively investigated (Atlas, 1984; Das and Chandran, 2011), whereas other plankton-mediated processes have received less attention. For instance, despite the high abundance and key role of zooplankton in marine food webs and biochemical cycles (Banse, 1995; Alcaraz et al., 2010), we still know little about uptake and transformation of dispersed crude oil by zooplankton after oil spills.

After a crude oil spill, zooplankton are exposed to dissolved oil components and dispersed crude oil droplets. Most research on petroleum and zooplankton interactions has been conducted using dissolved petroleum hydrocarbons (Corner et al., 1976; Harris et al., 1977; Berrojalbiz et al., 2009; Jiang et al., 2010, 2012). Several studies have demonstrated that zooplankton may take up dissolved petroleum hydrocarbons by passive mechanisms or consuming contaminated phytoplankton (Corner et al., 1976; Harris et al., 1997; Berrojalbiz et al., 2009). But, there is increasing evidence that zooplankton can also ingest crude oil droplets (Conover, 1971; Mackie et al., 1978; Herbet and Poulet, 1980; Gyllenburg, 1981; Lee et al., 2012; Almeda et al., 2014a, 2014b, 2014c). After marine oil spills, crude oil droplets (1–100 μm) generated by natural mixing (Forrester, 1971; Delvigne and Sweeney, 1988) or/and

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http://dx.doi.org/10.1016/j.envpol.2015.10.041

application of dispersants (Canevari, 1978; Lichtenthaler and Daling, 1985) are frequently in the prey size spectra of zooplankton (Hansen et al., 1984). However, ingestion of crude oil droplets by zooplankton after spills has been frequently neglected in oil spill research and existing crude oil weathering models.

Crude oil droplets have been observed inside zooplankton fecal pellets after oil spills and after exposing zooplankton to crude oil emulsions in the laboratory (Conover, 1971; Lee et al., 2012; Almeda et al., 2014a, 2014b, 2014c). Polycyclic aromatic hydrocarbons (PAHs), one of the most toxic components of crude oil, have also been detected in zooplankton fecal pellets collected from marine sediments (Prahl and Carpenter, 1979; Sleeter and Butler, 1982). However, despite these observations suggesting that ingestion and subsequent defecation of crude oil by zooplankton may be important mechanisms for vertical flux of petroleum pollution, the quantitative role of defecation of crude oil by zooplankton in the fate of crude oil spills is not well known. In fact, direct quantification of crude oil defecation rates by planktonic copepods, the most abundant animals in the sea (Longhurst, 1985; Humes, 1994), has not been experimentally investigated.

In a previous study, we established that the planktonic copepods *Temora turbinata*, *Acartia tonsa*, and *Parvocalanus crassirostris* ingest dispersed crude oil after observing crude oil droplets in the guts of adult and naupliar stages of these species (Almeda et al., 2014a). In this study, we aim to determine defecation rates of dispersed crude oil by adult stages of planktonic copepods, and estimate the quantitative impact of ingestion and defection of dispersed crude oil by copepods to the fate of crude oil after spills. To that end, we quantified the number and volume of crude oil droplets present in fecal pellets after exposing the copepods *T. turbinata*, *A. tonsa*, and *P. crassirostris* (species-level approach) and natural copepod assemblages (community-level approach) to crude oil and dispersant-treated oil emulsions during short-term incubations. Copepod species studied here belong to some of the most representative genera of coastal planktonic copepods (Razouls et al., 2005–2013).

2. Material and methods

2.1. Experimental organisms

Zooplankton were collected from surface waters of the Aransas Ship Channel (Port Aransas, TX) by tying a plankton net (150 μm mesh, 50 cm diameter) to the University of Texas Marine Science Institute pier and allowing it to stream with the tidal current for approximately 5–10 min. Contents of the collection buckets (cod ends) were kept in a cooler containing unfiltered seawater until returning to the laboratory, where samples were aerated.

Calanoid copepods *T. turbinata*, *A. tonsa*, and *P. crassirostris* were identified under a dissecting microscope and sorted from zooplankton samples using borosilicate glass pipettes. Specimens of each species were held in groups (20–50 specimens, depending on the experiments) in small plastic beakers or petri dishes with 0.2 μm-filtered seawater (FSW) until the experiment began (<2 h). To obtain natural copepod assemblages, zooplankton samples were first gently screened through a 2000 μm mesh sieve to remove macrozooplankton and, second, carefully concentrated with a 150 μm mesh sieve. Then, copepod assemblages (150–2000 μm) were placed into a 500 mL glass beaker with 0.2 μm-filtered seawater. When the exposure experiments began, aliquots of this concentrated zooplankton were added to the experimental bottles to obtain the desire copepod concentration (300 ind. L^{-1}).

Copepods were fed with a mixture of cultured phytoplankton species (*Rhodomonas* sp., *Isochrysis galbana*, *Heterocapsa* sp., *Thalassiosira weissflogii*, *Peridinium foliaceum* and *Gyrodinium corsicum*) during the exposure experiments. Phytoplankton cultures were grown in f/2 culture medium prepared with 0.2 μm filtered sterilized natural seawater collected from Aransas Ship Channel. Phytoplankton cultures were held in 250 mL polycarbonate flasks at 20 °C and 34–35 salinity on a 12:12 h light:dark cycle with cool-white fluorescent lights at an irradiance of approximately 25 μmol photons m^{-2} s^{-1}.

2.2. Preparation of crude oil emulsions

We used Light Louisiana sweet crude oil, which was provided by BP (BP Exploration & Production Inc.), as a surrogate for the Macondo (MC252) crude oil released in the Deepwater Horizon oil spill in the Gulf of Mexico (2010). The chemical dispersant Corexit 9500A (NALCO®/Exxon Energy Chemicals, L.P.), the main type of dispersant used in clean-up operations during the Deepwater Horizon oil spill (National Commission on the BP Deep Ocean Horizon Oil Spill and Offshore Drilling, 2011), was used to prepare dispersant-treated crude oil emulsions.

We prepared 2 types of test media: 1) crude oil emulsions, i.e., suspensions of crude oil droplets in seawater dispersed mechanically without the addition of dispersant, and 2) dispersant-treated crude oil emulsions, i.e., crude oil emulsions in seawater dispersed mechanically and chemically. To prepare crude oil emulsions, 1 L of 0.2 μm filtered seawater was placed in a 2 L glass beaker with a magnetic stir bar, which was tightly sealed with aluminum foil to prevent oil absorption on the surface of the bar. The glass beaker containing the seawater was placed on a magnetic stir plate and stirred at 900 rpm. Then, 1 mL of crude oil was added to the seawater using an automatic pipette with a Pasteur glass pipette as a tip, that was thoroughly washed to remove crude oil that could be attach to the pipette tip. After covering the beaker with aluminum foil, the crude oil was emulsified by keeping the stir rate at 900 rpm for 5 min at room temperature (24 °C). This stirring speed created a vortex, which extended from the bottom of the container to the water surface, forming droplets of crude oil in seawater and keeping the crude oil emulsion homogenous during the mixing. To prepare the dispersant treated-oil emulsions, we used the same methodology used for the preparation of crude oil emulsions, but in this case we added 50 μL of chemical dispersant after adding the crude oil. We used a ratio of dispersant to oil of 1:20, which is recommended by the US Environmental Protection Agency (US Environmental Protection Agency, EPA 1995). Initial crude oil droplet size spectra in the oil emulsions with and without the addition of dispersant were determined using an Imaging Particle Analysis system (FlowSight®). After stirring for 5 min, 1 mL of each test medium was added to the corresponding 1 L experimental bottles to obtain the desired exposure concentration (1 μL L^{-1}). Therefore, nominal concentrations of crude oil alone and dispersant-treated oil emulsions in the experimental treatments would correspond to 1 μL L^{-1}. The concentrations and composition of polycyclic aromatic hydrocarbons (PAHs) in the crude oil used in this study were previously determined by our research group (Almeda et al., 2013a). The nominal total PAH concentration in the experimental treatments would correspond to 2.15 μg L^{-1}.

2.3. Experimental design

Experiments consisted of laboratory incubations of single species of copepods or natural copepod assemblages exposed to crude oil alone or dispersant-treated crude oil ("experimental treatments"), or in absence of crude oil ("control treatments"). *T. turbinata*, *P. crassirostris*, and *A. tonsa* were incubated at densities of 20, 30, or 40 ind. L^{-1}, respectively, for 48 h. Natural copepods assemblages (300 ind. L^{-1}) were incubated for 24 h. Control and

experimental treatments were run in duplicate. Incubations were conducted in 1 L quartz bottles containing 0.2 μm-FSW (S = 34–35) and a mix of cultured phytoplankton as food. The concentration of phytoplankton in the cultures was determined with an inverted microscope (Olympus BX60) using a Sedgewick-Rafter counting chamber. Then, aliquots of phytoplankton culture were added to the incubation bottles to obtain desired target concentrations. *A. tonsa* was fed *Rhodomonas salina* (35,000 cells mL^{-1}) and *Heterocapsa* sp. (5000 cells mL^{-1}), *T. turbinata* with *Rhodomonas* sp. (5000 cells mL^{-1}), *Thalassiosira weissflogii* (2500 cells mL^{-1}) and *Heterocapsa* sp. (2500 cells mL^{-1}), *P. crassirostris* with *Rhodomonas* sp. (15,000 cells mL^{-1}), *T. weissflogii* (3000 cells mL^{-1}) and *Heterocapsa* sp. (1500 mL^{-1}), and natural copepod assemblages with *Rhodomonas* sp. (25,000 cells mL^{-1}), *I. galbana* (5000 cell ml^{-1}), *Heterocapsa* sp. (2000 cells mL^{-1}), *T. weissflogii* (500 cells mL^{-1}) *Peridinum foliaceum* (200 cells mL^{-1}) and *G. corsicum* (100 cells mL^{-1}). These phytoplankton concentrations exceed satiation food levels in terms of carbon biomass (>1 μg C mL^{-1}, Kiørboe and Hirst, 2013; Almeda et al., 2014a) and therefore fecal pellet production should not be limited by food quantity in our experiments.

After adding emulsified crude oil or dispersant-treated oil to the corresponding experimental bottles, bottles were incubated at 24 ± 1 °C with natural light–dark cycles in a Wheaton bench top roller at 2 rpm in the laboratory. After incubating, the contents of each bottle were gently screened through a submerged 150 μm mesh sieve to collect the copepods. Copepods were gently rinsed off the 150 μm mesh sieve and placed in glass dishes filled with 0.2 μm FSW for 5–10 min. We then checked copepod survival and swimming activity by gently touching with a dissecting probe under a stereomicroscope. After determining the mortality of copepods, seawater (<150 μm) containing copepod fecal pellets was concentrated using a 20 μm mesh sieve and placed in 20 mL glass containers. Then, copepod and fecal pellet samples were fixed with glutaraldehyde (2%) and kept at 4 °C until analysis.

2.4. Sample analysis and calculations

To quantify the number of fecal pellets, an aliquot from the fecal pellet sample containing at least 109 fecal pellets (range 109–1218) was fixed with Lugol's (1%) and counted under the stereomicroscope. To determine the presence of crude oil in fecal pellets, aliquots of fecal pellets fixed with glutaraldehyde were placed in Sedgewick-Rafter counting chambers and viewed under an epifluorescence microscope (Olympus BX51) with bright-field and UV illumination. Crude oil droplets in copepod fecal pellets may be difficult to observe with bright light given that the color and morphological characteristics of crude oil droplets are similar to other components, or because fecal pellets can be densely packed. Thus, the presence or absence of crude oil droplets in fecal pellets was verified by exposure to UV light (365 nm), which produces a strong fluorescence for crude oil due to their aromatic hydrocarbon fraction. The crude oil fluorescence under UV illumination was previously identified in crude oil emulsions, i.e., in crude oil droplets suspended in seawater (Almeda et al., 2014c). We also verified that fecal pellets from copepods not exposed to oil (control treatments) did not have any particles with the oil-type fluorescence under UV illumination.

Images of fecal pellets (40–100) with both bright-field and UV illumination were captured with a digital camera attached to the microscope. Fecal pellet volume (μm^3) was calculated considering an ellipsoid shaped pellet and using the lengths of the major and minor axes measured from bright-field images by image analysis (ImageJ). The number and volume of oil droplets inside the fecal pellets were determined using ImageJ software (NIH, version 10.2) with images taken under UV illumination. For each fecal pellet, we combined the images of oil droplets taken at different planes into a composite. Composite images were filtered by applying the Laplacian of Gaussian operator (ImageJ plugin: FeatureJ Laplacian developed by Erik Meijering) and then converted to binary images using an automatic threshold that best reflected each individual image, generally Huang (Huang and Wang, 1995) or MaxEntropy (Kapur et al., 1985) method. Adjacent oil droplets were automatically separated using watershed segmentation. The final binary image was inspected against the original to ensure that each oil droplet was represented and analyzed. Volume of an oil droplet was calculated using lengths of the major and minor axes as determined by ImageJ, where volume = $4/3\pi$ x (major axis/2) x (minor axis/2)^2. When oil was densely packed, individual oil droplets could not be discerned. In these cases, the number of oil droplets per fecal pellets is underestimated, but the total volume of oil inside these fecal pellets would not be affected.

Mean volume of crude oil per fecal pellet was converted to mass using a crude oil density of 0.84 g cm^{-3} (Fingas, 2015). Crude oil defecation rates (ng-oil copepod^{-1} d^{-1}) in each treatment were calculated considering the mean crude oil content per fecal pellet and fecal pellet production rates estimated in our experiments. To determine mean length of the studied copepods, digital pictures of 40–48 copepods from each experiment were taken with a camera attached to a stereomicroscope and copepod prosome length was measured using image analysis (ImageJ software). To calculate weight-specific defecation rates (μg-oil μg-$C^{-1}_{copepod}$ d^{-1}), carbon content of copepods was estimated using the length to biomass equations described in Ara (2001) for *T. turbinata* and *P. crassirostris*, Berggren et al. (1988) for *A. tonsa*, and Uye (1982) (total Copepoda equation) for the natural copepod assemblage.

For statistical analyses, we conducted one way analysis of variance (ANOVA) to determine significant differences among treatments within a species and among species within an experimental treatment using the mean or median values of the two replicates per treatment. Only in those cases when no significant difference was observed between experimental treatments for all studied species, both experimental treatments were used to determine significant differences among species. Bonferroni post hoc tests were used for pairwise comparison. A statistically significance level (α) of 0.05 was considered. Statistical analyses were performed with SPSS statistics 19.0 software.

3. Results

Copepods represented 97% of the total mesozooplankton abundance in the natural zooplankton assemblages used in our experiments. Cirripede nauplii were present in the natural mesozooplankton assemblage but only contributed 3% of abundance. Copepod composition was dominated by calanoids (96.9%, mainly *Centropages* sp., *P. crassirostris*, *Labidocera aestiva*, *Acartia* sp., *T. turbinata*), whereas cyclopoid (*Oithona* spp., 2.9%) and poecilostomatoid copepods (*Corycaeus* sp. 0.2%) were scarce. Mean mortality of single copepod species and natural copepod assemblage varied from 1% to 23% depending on experimental treatments (Table 1). Copepod mortality in control treatments was low (1–5%) (Table 1). Mean mortality was significantly higher when copepods were exposed to dispersant-treated oil than in the controls for *A. tonsa* (ANOVA, $F_{2,3}$ = 29.9, p = .010; Bonferroni, p = .013) and *P. crassirostris* (ANOVA, $F_{2,3}$ = 15.38, p = .026; Bonferroni, p = .036), whereas no significant differences were observed among treatments for *T. turbinata* (ANOVA, $F_{2,3}$ = 1.3, p = .385) or between experimental treatments for natural copepod assemblages (ANOVA, $F_{1,2}$ = 2.16, p = .279) (Table 1). Mean fecal pellet production rates ranged from 9 to 113 pellets copepod^{-1} d^{-1}, depending

Table 1
Production rates of fecal pellets containing crude oil by the planktonic copepods *Temora turbinata*, *Acartia tonsa*, *Parvocalaus crassirostris* and natural copepod assemblages after exposure to physically dispersed crude oil (Crude oil) and dispersant-treated crude oil (Oil + Disp.). Exposure time was 48 h for single copepod species and 24 for natural copepod assemblages. Values correspond to mean ± standard deviation. PL = prosome length, W = carbon biomass, M = mortality, FPPR = fecal pellet production rates, Oil-DR = crude oil defecation rate (ng-oil copepod^{-1} d^{-1}), Oil-SDR = weight specific crude oil defecation rate (μg-oil μg-$C^{-1}_{copepod}$ d^{-1}). Asterisk indicates significantly different than the corresponding controls ($p < .05$).

Organisms	PL (μm)	W (μg C)	Treatment	M (%)	FPPR (pellets cop^{-1} d^{-1})	Oil content per FP (ng-oil pellet^{-1})	Oil-DR (ng-oil cop^{-1} d^{-1})	Oil-SDR (μg-oil μg-$C^{-1}_{copepod}$ d^{-1})
Temora turbinata	729 ± 66	3.88 ± 0.02	Control	3 ± 4	127 ± 5			
			Crude oil	3 ± 4	113 ± 5*	1.09 ± 0.26	122 ± 24	0.031 ± 0.006
			Oil + Disp	8 ± 4	91 ± 2*	2.68 ± 0.41	245 ± 32	0.063 ± 0.008
Acartia tonsa	648 ± 29	1.80 ± 0.00	Control	1 ± 2	48 ± 3			
			Crude oil	11 ± 1*	42 ± 4	1.26 ± 0.08	53 ± 6	0.030 ± 0.004
			Oil + Disp	20 ± 4*	29 ± 3*	0.58 ± 0.04	17 ± 3	0.010 ± 0.001
Parvocalanus crassirostris	421 ± 59	0.54 ± 0.00	Control	5 ± 2	15 ± 1			
			Crude oil	12 ± 2	10 ± 0.2*	0.32 ± 0.20	6 ± 4	0.011 ± 0.007
			Oil + Disp	23 ± 5*	9 ± 1*	0.32 ± 0.01	5 ± 0.3	0.010 ± 0.001
Natural copepod assemblage	598 ± 138	1.43 ± 0.02	Control	n.d	n.d.			
			Crude oil	1 ± 2	57 ± 4	0.69 ± 0.08	39 ± 3	0.027 ± 0.002
			Oil + Disp	5 ± 3	39 ± 2	0.86 ± 0.15	33 ± 3	0.023 ± 0.002

on the species and experimental treatments (Table 1). Mean fecal pellet production rates were significantly lower in dispersant-treated oil treatment than in the corresponding control of *T. turbinata* (ANOVA, $F_{2,3} = 36.7$, $p = .008$; Bonferroni, $p = .047$), *A. tonsa* (ANOVA, $F_{2,3} = 23.58$, $p = .015$; Bonferroni, $p = .020$) and *P. crassirostris* (ANOVA, $F_{2,3} = 13.0$, $p = .033$; Bonferroni, $p = .049$). No significant differences were observed between oil alone and dispersant-treated oil treatments for the three species (Bonferroni, $p > .05$), but mean fecal pellet production rates of the natural copepod assemblage were significantly lower when exposed to dispersant-treated oil than crude oil alone (ANOVA, $F_{1,2} = 38.1$, $p = .025$).

Crude oil droplets were detected inside fecal pellets of the three studied copepods species and natural copepod assemblages after exposure to dispersant-treated oil or crude oil emulsions (Fig. 1). The presence of crude oil droplets in copepod fecal pellets was unambiguously verified by the observation of strong fluorescence of crude oil under UV illumination (Fig. 1). Approximately 88–100% of the analyzed fecal pellets in studied copepods contained crude oil droplets (Fig. 2A). We did not observe significant differences in

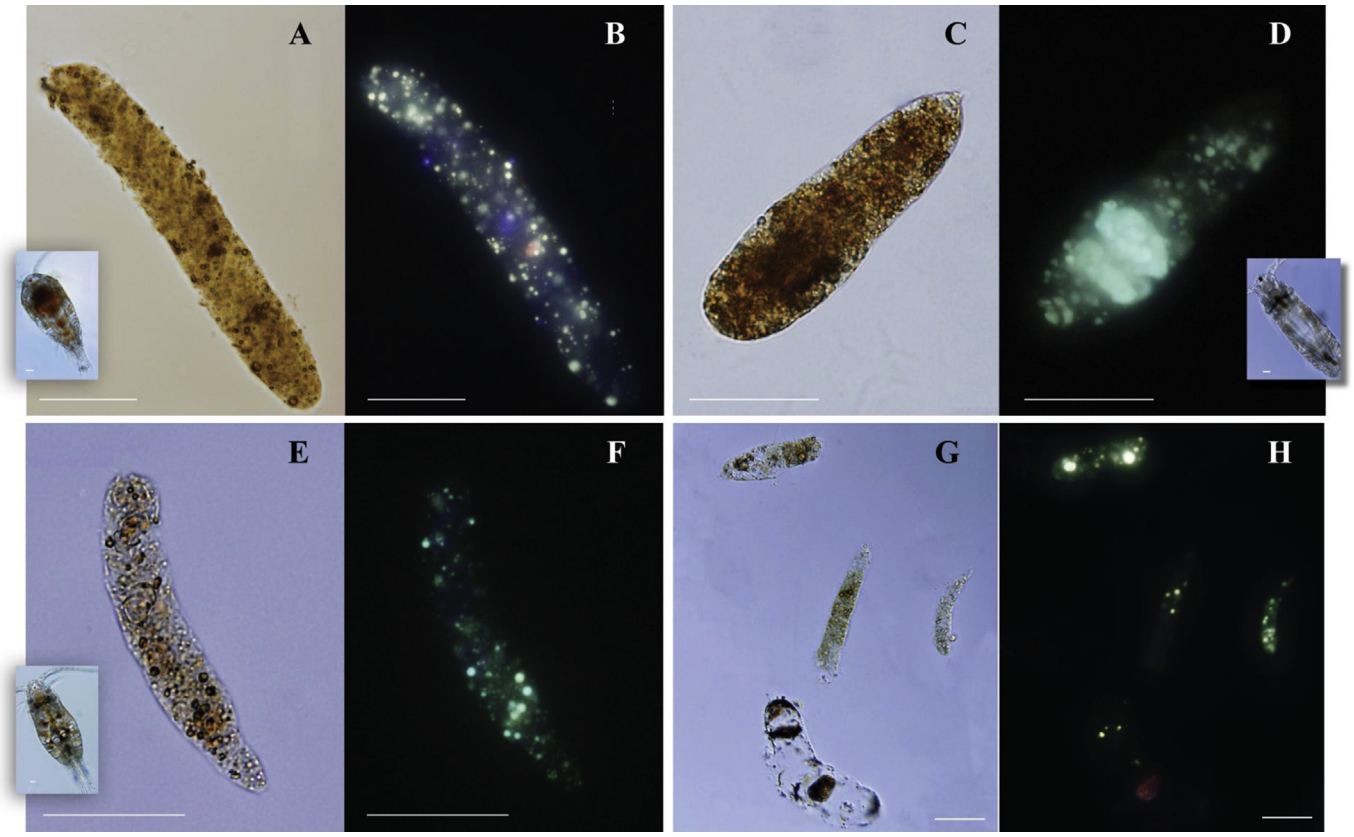

Fig. 1. Microscope images of copepod fecal pellets containing crude oil droplets. Crude oil-contaminated fecal pellets of the copepods *Acartia tonsa* (**A**–**B**), *Temora turbinata* (**C**–**D**), *Parvocalanus crassirostris* (**E**–**F**) and natural copepod assemblages (**G**–**H**) observed under bright (**A**, **C**, **E**, **G**) and UV illumination (**B**, **D**, **F**, **H**). Crude oil strongly autofluoresces under UV light (**B**, **D**, **F**, **H**), which was used to verify and quantify the number and volume of crude oil droplets inside the fecal pellets. Scale bar = 50 μm.

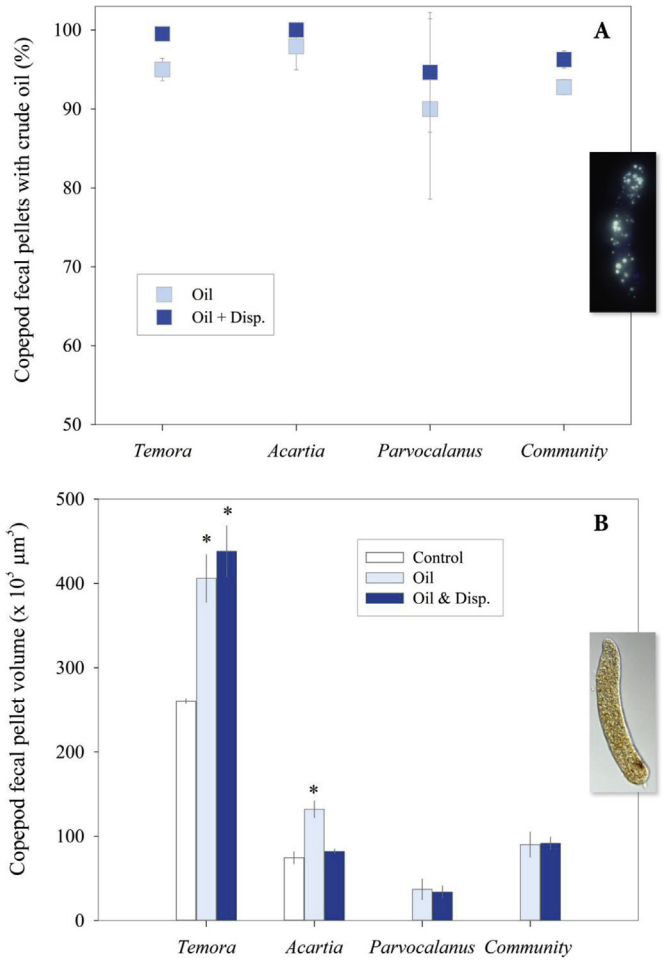

Fig. 2. Percent of fecal pellets containing crude oil droplets (**A**) and mean fecal pellet volume (**B**) after exposing copepods *Temora turbinata*, *Acartia tonsa*, *Parvocalanus. crassirostris* and natural copepod assemblages (Community) to crude oil (Oil) or dispersant treated oil (Oil + Disp) emulsions. Error bars indicate standard deviation. Note that volume of fecal pellet in control treatments was only determined for *A. tonsa* and *T. turbinata*. Asterisk indicates significantly different than the corresponding controls ($p < .05$).

the percent of fecal pellets containing crude oil droplets between experimental treatments within a species (ANOVA, $p > .05$) or among species when exposed to crude oil (ANOVA, $F_{3,4} = 1.01$, $p = .474$) or dispersant treated oil (ANOVA, $F_{3,4} = 0.910$, $p = .511$) (Fig. 2A). Mean volume of fecal pellets containing crude oil droplets varied from ~29,000 to 460,000 µm^3 depending on species and treatment (Fig. 2B). Mean volume of *T. turbinata* fecal pellets (µm^3) was ~1.6 times significantly larger when exposed to crude oil or dispersant-treated oil than in the controls (ANOVA, $F_{2,3} = 31.2$, $p = .010$, Bonferroni, $p < .05$) (Fig. 2B). *A. tonsa* produced ~1.8 times significantly larger fecal pellets when exposed to crude oil than in the other treatments (ANOVA, $F_{2,3} = 38.1$, $p = .007$, Bonferroni, $p < .05$) (Fig. 2B).

Size of crude oil droplets in emulsions used in the experiments ranged from 1 to 90 µm in diameter, with >95% of droplets being between 1 and 20 µm (Fig. 3). Median diameters were 8.0 and 6.6 µm for crude oil and dispersant-treated crude oil emulsions, respectively (Fig. 3). The size of the crude oil droplets defecated by the copepods ranged from below 1 µm–36 µm, with 99% of droplets <10 µm (Fig. 3). Median diameters of crude oil droplets inside copepod fecal pellets ranged from 2.4 to 3.5 µm and there was no significant difference in the median diameter of oil droplets inside fecal pellets between experimental treatments in any of the studied species/natural assemblages (ANOVA, $p > .05$) (Fig. 3). Considering both experimental treatments together for each species, median diameters of crude oil droplets inside copepod fecal pellets were significantly larger for *T. turbinata* and natural copepods assemblages than for *A. tonsa* and *P. crassirostris* (ANOVA, $F_{3,12} = 75.9$, $p < .001$, Bonferroni, $p < .05$) (Fig. 3). The median size of crude oil droplets defecated by copepods was lower than the median size in the crude oil emulsions (Fig. 3).

The median number of crude oil droplets per fecal pellet ranged from 18 to 77 depending on species and experimental treatments (Fig. 4A). The median number of crude oil droplets in *T. turbinata* fecal pellets was significantly higher when exposed to dispersant-treated oil than crude oil emulsions (ANOVA, $F_{1,2} = 256.0$, $p = .004$) whereas there was no significant difference in the number of oil droplets between treatments in fecal pellets of the other species or natural copepod assemblages (ANOVA, $p > .05$) (Fig. 4A). We did not find significant differences in the number of crude oil droplets per fecal pellet among species when exposed to crude oil alone (ANOVA, $F_{3,4} = 5.80$, $p = .061$) or dispersant treated oil (ANOVA, $F_{3,4} = 4.99$, $p = .077$) (Fig. 4A). Median total volume of crude oil inside of fecal pellets varied from 240 to 2239 µm^3 pellet^{-1}, depending on the species and experimental treatments (Fig. 4B). The volume of oil in fecal pellets of *T. turbinata* was significantly higher when exposed to dispersant treated oil than to crude oil emulsions (ANOVA, $F_{1,2} = 162.47$, $p = .006$), whereas *A. tonsa* showed higher volume of oil per fecal pellet when exposed to crude oil emulsions (ANOVA, $F_{1,2} = 24.78$, $p = .038$). The median volume of oil per fecal pellet was not significantly different between experimental treatments for *P. crassirostris* (ANOVA, $F_{1,2} = 0.023$, $p = .893$) and natural community assemblages (ANOVA, $F_{1,2} = 1.001$, $p = .422$). Comparing among species, *T. turbinata* produced fecal pellets with a volume of crude oil significant higher than the other species/natural copepods assemblages when exposed to dispersant-treated oil (ANOVA, $F_{3,4} = 71.32$, $p = .001$, Bonferroni, $p < 0.05$).

Crude oil defecation rates by copepods ranged from 5.3 to 245 ng-oil copepod^{-1} d^{-1}, considering the mean crude oil content per fecal pellet and copepod fecal pellet production rates found in the experimental treatments (Table 1). Crude oil defection rates of *T. turbinata* were significantly higher when exposed to dispersant-treated oil than to crude oil emulsions (ANOVA, $F_{1,2} = 18.92$, $p = .042$). In contrast, oil defection rates by *A. tonsa* were significantly lower when exposed to dispersant-treated oil than to crude oil emulsions (ANOVA, $F_{1,2} = 53.16$, $p = .018$) (Table 1). Defecation rates of crude oil were not significantly different between experimental treatments for *P. crassirostris* (ANOVA, $F_{1,2} = 0.08$, $p = .804$) and natural copepod assemblages (ANOVA, $F_{1,2} = 4.181$, $p = .178$) (Table 1). *T. turbinata* showed crude oil defecation significantly higher than the other species when exposed to crude oil (ANOVA, $F_{3,4} = 30.70$, $p = .003$, Bonferroni, $p < .05$) and dispersant treated oil (ANOVA, $F_{3,4} = 98.29$, $p < .001$, Bonferroni, $p < .05$) (Table 1). Crude oil defection rates increased with increasing copepod body biomass (Fig. 5). Depending on species and treatments, weight-specific crude oil defecation rates ranged from 0.010 to 0.063 µg-oil µg-$C_{copepod}$ d^{-1}, with a mean rate of 0.026 µg-oil µg-$C_{copepod}$ d^{-1} (Table 1).

4. Discussion

Ingestion of crude oil droplets by planktonic copepods was first noticed in several field and laboratory studies in the 1970s and early 1980s (Conover, 1971; Mackie et al., 1978; Hebert and Poulet, 1980; Gyllenburg, 1981) Despite these early observations, ingestion of crude oil droplets by zooplankton has been neglected in most oil

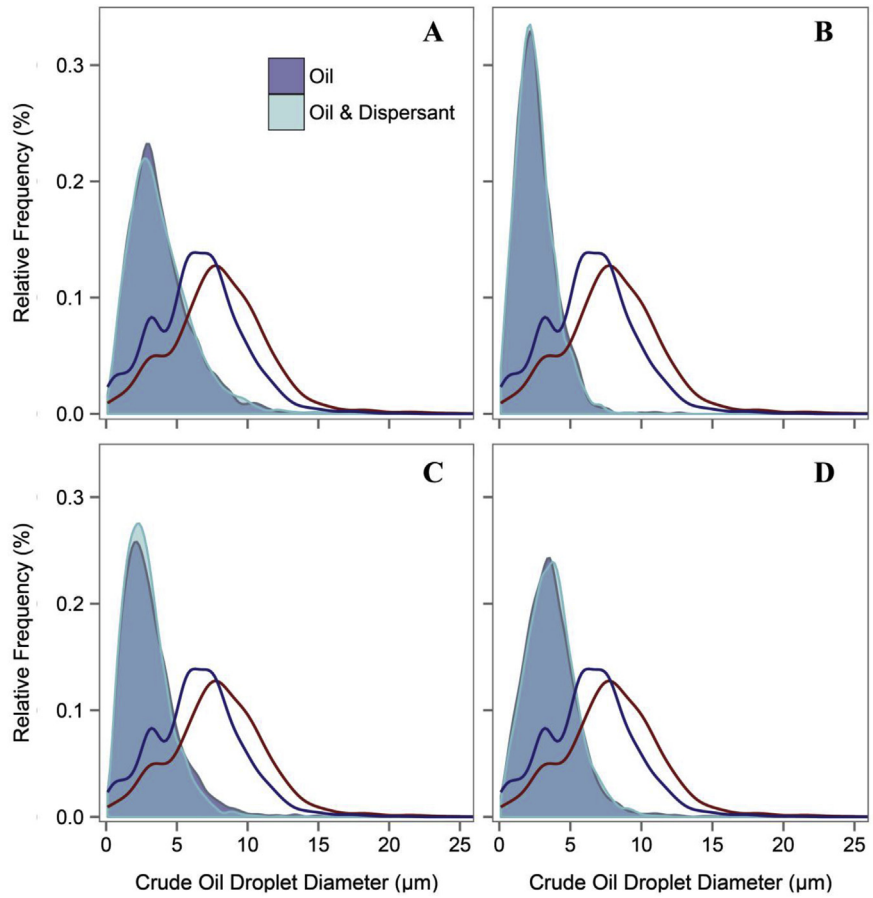

Fig. 3. Relative frequency of crude oil droplet size ingested by copepods and in crude oil emulsions. Diameter of crude oil droplets defecated by *Temora turbinata* (**A**), *Acartia tonsa* (**B**), *Parvocalanus crassirostris* (**C**) and natural copepod assemblages (**D**) exposed to crude oil (shaded dark blue) or dispersant treated oil (shaded light blue) emulsions. Red and blue lines are the size of droplets in crude oil emulsions added to experimental bottles without dispersants and with dispersants, respectively. In all cases the relative frequency data were fit to a density function using R software. Note that there is an overlap in the diameter of ingested oil droplets between crude oil (shaded dark blue) or dispersant treated oil (shaded light blue) treatments.

petroleum pollution studies over the last three decades. Recent laboratory studies have demonstrated that, not only copepods ingest crude oil droplets, but other important groups of zooplankton, such as pelagic tunicates (Lee et al., 2012), copepod nauplii (Almeda et al., 2014a), meroplanktonic larvae (Almeda et al., 2014b) and heterotrophic dinoflagellates (Almeda et al., 2014c) can also ingest dispersed crude oil. Although a previous study found that only crude oil droplets formed with dispersants are stable and can be ingested by gelatinous zooplankton (Lee et al., 2012), our results indicated that low concentrations of mechanically dispersed small crude oil droplets were stable in seawater and were ingested by copepods. Thus, copepods could ingest dispersed crude oil after oil spills with or without the addition of chemical dispersants, with ingestion and defecation rates varying depending on copepod species. Although defecation of crude oil was higher in some copepods species when exposed to oil dispersed only mechanically, application of dispersants in the field would increase the formation of small crude oil droplets after oil slicks compared to natural mixing/dispersion, which would foster the ingestion of oil by some zooplankton after spills. Therefore, a negative consequence of applying dispersant to treat oil spills, besides its own toxicity, is that dispersants could promote ingestion of oil droplets by zooplankton and consequently the entry of toxic petroleum compounds into marine food webs.

Toxic effects and ingestion of dispersed crude oil by zooplankton depend on crude oil concentration and exposure time. After a marine oil spill, concentrations of dispersed crude oil in the water column may range from a few ppb to hundreds of ppm, depending on mixing energy caused by wind, waves and currents, and whether dispersants are applied (Forrester,1971; Canevari, 1978; McAuliffe et al., 1981; Lichtenthaler and Daling, 1985; Delvigne and Sweeney, 1988; Clayton et al., 1993) During the first hours, crude oil near the oil spill source and oil slicks may reach concentrations of 20–100 ppm in surface waters (McAuliffe et al., 1981; Clayton et al., 1993). These high concentrations of crude oil are commonly lethal for planktonic copepods, although sensitivity to crude oil exposure varies among copepod species (Jiang et al., 2010, 2012). For instance, median lethal concentration of dispersed crude oil for natural copepod assemblages from the Gulf of Mexico was estimated to be 32 µl L^{-1} (~27 ppm) after 16 h of exposure to dispersed crude oil (Almeda et al., 2013b). After several hours to days, crude oil is dispersed, dropping in concentration to $\leq$1 ppm (Canevari, 1978; Lichtenthaler and Daling, 1985; Delvigne and Sweeney, 1988). At these lower crude oil concentrations, most planktonic copepods can tolerate acute crude oil exposure and ingest crude oil droplets (Spooner and Corkett, 1979; Herbet and Poulet, 1980; Almeda et al., 2014a). But, planktonic copepods can suffer sublethal effects, such as reduced feeding activity (Herbet and Poulet, 1980; Spooner and Corkett, 1979; Cowles and Remillard, 1983; Hansen et al., 2012) and consequently decreased fecal pellets production rates (Spooner and Corkett, 1979; Almeda et al., 2014a,b,c,d). Dispersant-treated crude oil was more toxic than

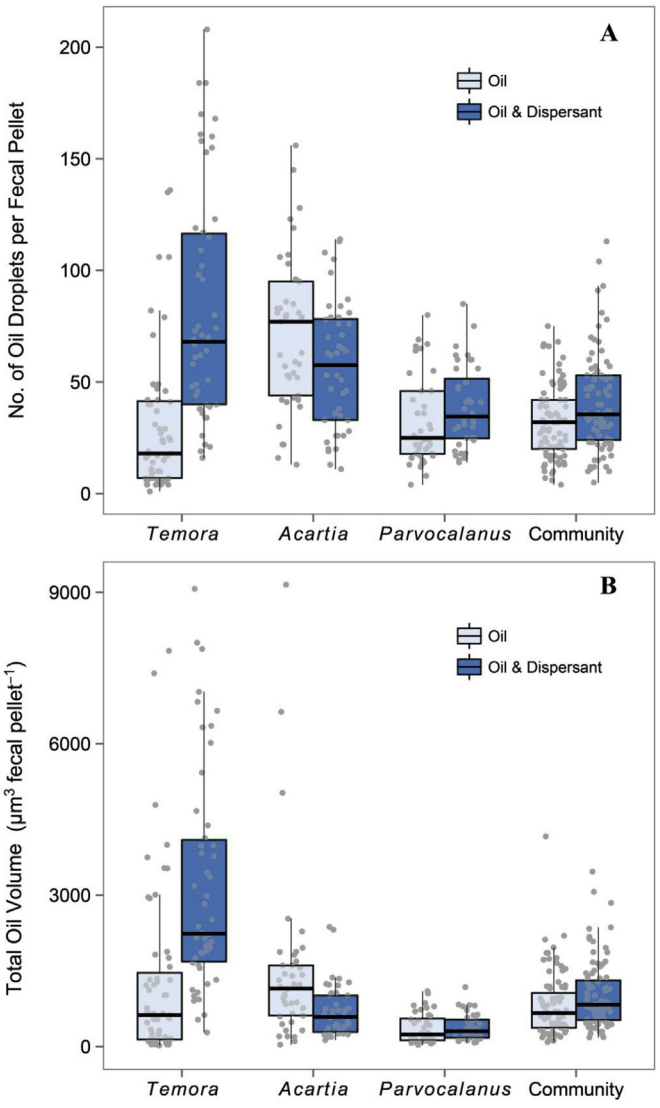

Fig. 4. Number of crude oil droplets per fecal pellet (**A**) and total crude oil volume per fecal pellet (**B**) defecated by copepods *Temora turbinata*, *Acartia tonsa*, *Parvocalanus crassirostris* and natural copepod assemblages (Community) that were incubated with crude oil emulsions (Oil) or chemically dispersed crude oil (Oil + Disp). Grey dots are individual data points (jittered), horizontal black bar shows the median, boxes encompass the interquartile range, and whiskers are 1.5 times the interquartile range.

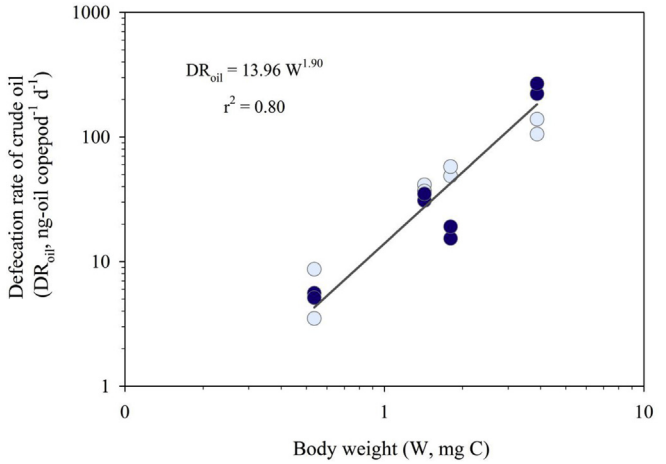

Fig. 5. Relationship between mean crude oil defecation rates by planktonic copepods (ng-oil copepod^{-1} d^{-1}) and mean copepod body weight (µg-C) after exposing copepods to crude oil (light blue dots) and dispersant-treated oil (dark blue dots) emulsions. The continuous line corresponds to the allometric function fitted to the data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dispersed crude oil alone to the studied copepod species, causing greater reductions in fecal pellet production rates, mainly due to the toxicity of dispersants (Almeda et al., 2014a). At an nominal oil concentration of 1 µL L^{-1} (~0.84 ppm), we found not only reduced fecal pellet production rates, but also different volumes of fecal pellets egested by copepods after ingesting crude oil droplets, suggesting that oil ingestion could affect gut transit, and probably assimilation efficiency. Effects of crude oil would lessen as oil concentration decreases, until reaching a concentration, which may vary depending on the species, with no apparent effects on copepods (e.g. 0.1 ppm for *Calanus firmanchicus*, Hansen et al., 2012).

Similar to previous studies (e.g., Hebert and Poulet, 1980) we found that the size of crude oil droplets produced by mechanical mixing in the laboratory (1–90 µm), with or without the addition of dispersants, was similar to the oil droplet size range generated by natural mixing or/and application of dispersants after marine oil spills (1–100 µm) (Forrester, 1971; Delvigne and Sweeney, 1988; Canevari, 1978; Lichtenthaler and Daling, 1985). Some filter-feeding zooplankton, such as doliolid tunicates, do not select prey and feed on prey that are just large enough to be retained on their feeding filters (Deibel and Paffenhöfer, 1988). Therefore, doliolids likely cannot avoid ingesting crude oil droplets in the size spectra of their prey. The studied copepods are feeding current feeders that feed on prey ranging from ~2 to >200 µm, with an optimal prey size >15 µm (Hansen et al., 1984; Berggren et al., 1988; Calbet et al., 2000; Kwang-Hyeon et al., 2014; Kiørboe, 2011). However, we found that most crude oil droplets inside copepod fecal pellets were in the lower end of the prey size range (~2–4 µm). The studied copepods can perceive and capture individual prey arriving in the feeding current (Kiørboe, 2011), and even chemically discriminate among prey depending on toxicity (Huntley et al., 1986). However, there is a lower size limit for detecting and discriminating individual prey in feeding-current copepods (Kiørboe, 2011). This threshold is frequently between 5 and 10 µm depending on the species (Price et al., 1983; Kiørboe, 2011; Gonçalves et al., 2014). Therefore, the difference in size between the oil droplets found in the water column and in the fecal pellets may be explained by the ability of the studied copepods to reject crude oil droplets larger that ~5 µm, but not smaller oil droplets, which were ingested. High-speed video observations of copepods exposed to different sized crude oil droplets would help confirm this hypothesis. An alternative hypothesis to explain the difference in size between oil droplets in the water column and in fecal pellets could be that oil droplets are mechanically broken up into smaller droplets by copepod swimming activity before/during ingestion or during gut transit after ingestion.

Little is still known about how crude oil droplets are chemically modified after being ingested and defecated by copepods or how ingestion of oil droplets affects bioaccumulation of toxic petroleum hydrocarbons in copepods. Berrojalbiz et al. (2009) showed that the copepod *Paracartia grani* eliminated dissolved polycyclic aromatic hydrocarbons (PAHs) bioconcentrated from water through metabolism and passive excretion, whereas PAHs bioaccumulated from phytoplankton food (dietary uptake) were removed via fecal pellets. Further, a recent study found that bacteria inside copepod fecal pellets have the capacity to degrade crude oil (Størdal et al., 2015). There is evidence that petroleum hydrocarbons with low solubility

accumulate in copepod and copepod fecal pellets after exposure to dispersed crude oil (Prahl and Carpenter, 1979; Sleeter and Butler, 1982; Almeda et al., 2013b). Therefore, ingestion of oil droplets may be an important mechanism in the uptake of oil compounds that have low water solubility and remain in crude oil droplets after spills (Redman et al., 2012). Highly soluble PAHs, such as naphthalene, may be excreted rapidly by copepods (Berrojalbiz et al., 2009) whereas PAHs with a lower solubility tend to remain in zooplankton bodies for longer periods (Harris et al., 1977; Gyllenburg, 1981; Cailleaud et al., 2009; Mitra et al., 2012). Low solubility polycyclic PAHs are considered the most harmful components of crude oil, with potential carcinogenic, teratogenic and mutagenic effects to marine animals and humans (De Flora et al., 1991). Copepods containing petroleum compounds, such as crude oil droplets in their gut or accumulated in their tissues can be ingested by other consumers, promoting the biotransfer of toxins up the food web after oil spills. For instance, jellyfish and ctenophores bioaccumulated low solubility PAHs, such as chrysene, pyrene, and benzo[*b*]fluoranthene, when fed copepods exposed to dispersed crude oil (Almeda et al., 2013a). Copepods are the main food of many pelagic and benthic animals, therefore, ingestion of micro-oil droplets by copepods may be a significant pathway in the biological flux and transfer of highly toxic petroleum hydrocarbons through marine food webs after oil spills.

Zooplankton egest most of the insoluble fraction of ingested crude oil droplets via fecal pellets (Conover, 1971; Lee et al., 2012; Almeda et al., 2014c). Crude oil-contaminated fecal pellets can be recycled in the water column by microbial degradation and detritivory/coprophagy, or exported and deposited on the seafloor (Turner, 2002; Small et al., 1979; Gonzalez and Smetacek, 1994; Møller et al., 2011; Dagg et al., 2003). Bacterial biodegradation of crude oil inside fecal pellets could be increased relative to degradation of crude oil suspended in the water, promoting the removal of oil from the sea (Størdal et al., 2015). Ingestion of fecal pellets containing crude oil droplets by detritivorous or coprophagous zooplankton would foster the biological flux of toxic petroleum hydrocarbons in marine food webs. It is unknown how the presence of oil can affect sinking rates of copepod fecal pellets but oil droplets compacted into fecal material would sink more efficiently than suspended crude oil droplets. Dispersed crude and refined oils droplets, even after weathering, do not tend to sink nor accumulate in sediments because they are less dense than seawater. In contrast, copepod fecal pellets are denser than seawater (mean density = 1.23 g cm^{-3}, Komar et al., 1981) and tend to sink, particularly when they are ballasted with opal (from diatoms), calcium carbonate (from many species of planktonic algae and protists), or sediment particles. Fecal pellet sinking rates vary widely depending on pellet volume, contents, and density, and water currents/turbulence (Small et al., 1979; Plough et al., 2008; Wiedman et al., 2014). Although the vertical flux of fecal pellets is highly variable (Turner, 2002; Small et al., 1979; Møller et al., 2011; Dagg et al., 2003), field studies found PAHs in sediments that contained mainly copepod fecal pellets (Prahl and Carpenter, 1979). Thus, our results and those of previous studies indicate that zooplankton fecal pellets may be a major vector in the vertical flux of petroleum pollution to the seafloor and in the biological flux of these pollutants to benthic food webs after oil spills. The impact of oil-containing fecal pellets to the benthos may be particularly relevant in coastal areas where/when large copepods are very abundant (e.g. high latitudes, Thibault et al., 1999) and the water column is shallow.

Quantitative importance of crude oil ingestion and defecation by zooplankton to the fate of crude oil after an oil spill would vary depending on copepod abundance. Biomass of copepods in the marine environment is spatially and temporarily variable, ranging from <1 to 250 mg C m^{-3} (O'Brien, 2005). Given this copepod biomass range and the mean crude oil weight-specific defecation rate calculated in our experiments (0.026 μg-oil μg-C $^{-1}$ d^{-1}), we estimated that the amount of oil that copepods could ingest and defecate ranges from <0.03 to 6.4 mg-oil m^{-3} d^{-1}. Considering a dispersed crude oil concentration commonly found in surface waters after a spill (1 μL L^{-1}, ~840 mg m^{-3}), and maximum biomass of copepods frequently observed in eutrophic coastal waters (50–100 mg-C m^{-3}, Raabe et al., 1997; Poulet et al., 1996; Wu et al., 2014), the daily amount of oil ingested and defected by copepods would represent ~0.15%–0.30% of the total dispersed crude oil. Therefore, our results indicate that ingestion and defecation of crude oil by planktonic copepods would have a minor effect on the overall mass of spilled crude oil in the short term. However, some oil droplets in the fecal pellets may have been overlaid and not observed in the images, and therefore, we likely underestimated the total amount of oil per fecal pellet. Although ingestion and defecation of crude oil by copepods has a small effect on the overall mass of spilled oil in the short term, it substantially affects the distribution of oil hydrocarbons in the marine environment, facilitating bioaccumulation of petroleum hydrocarbons by scavengers and sedimentation of oil to the seafloor via fecal pellets.

Ingestion and egestion of crude oil by zooplankton would depend not only on copepod abundance but also on copepod community composition. In our study, survival of small to medium sized copepods *A. tonsa* and *P. crassirostris* were more negatively affected by dispersed crude oil than the larger copepod *T. turbinata*, probably because of the inverse relationship between body size and crude oil toxicity observed in copepods (Jiang et al., 2012). The differences in crude oil specific defecation rates observed among copepods is likely related to differences in clearance rates and sensitivity to crude oil and dispersant among species. Mesozooplankton communities dominated by large copepods (e.g *Calanus*, *Temora*) or other large planktonic crustacean (euphausiids), which have a higher tolerance to crude oil exposure (Hebert and Poulet, 1980; Jiang et al., 2012; Hansen et al., 2012) and likely higher crude oil defecation rates than small copepods, would have a higher quantitative impact on oil spills, but only after several weeks. Since copepod survival is affected by crude oil exposure time, experiments that expose copepods to low oil concentrations over a longer duration are needed to better evaluate the impact of crude oil ingestion by copepods after oil spills. In a recent study, a modeling approach estimated that the amount of dispersed crude oil ingested by the large copepod *Calanus finmarchicus* would be low during the first days of an oil spill (Nepstad et al., 2015) in agreement with our estimations for the copepod species studied here. However, ingestion of crude oil by *C. finmarchicus* may be 1–40% of the total oil mass after 20 days, with the lower values of that range being more probable in an actual spill situation (Nepstad et al., 2015).

Weight-specific egestion rates of crude oil by the studied copepods were one order of magnitude lower than specific ingestion rates of heterotrophic dinoflagellates (Almeda et al., 2014c). Ingestion rates of crude oil by heterotrophic dinoflagellates may represent ~17%–100% of spilled dispersed oil in surface waters when heterotrophic dinoflagellates are abundant or bloom (Almeda et al., 2014c). Heterotrophic dinoflagellates can reach higher biomass in nature than copepods and have higher tolerance to crude oil compared to other microzooplankton and copepods (Almeda et al., 2014c, 2014d). Also, heterotrophic dinoflagellates ingest larger oil droplets than the studied copepods did (Almeda et al., 2014c), probably because calanoid copepods were able to avoid ingesting large crude oil droplets. Thus, differences in abundance, tolerance to crude oil and size of ingested oil droplets between copepods and heterotrophic dinoflagellates can explain

the difference in the potential and quantitative impact of these planktonic organisms after an oil spill.

Laboratory exposure experiments under controlled conditions are an essential tool to investigate the interactions between crude oil and zooplankton, but direct extrapolation to the field should be considered carefully. In nature, ingestion and defecation of crude oil by copepods after oil spills will depend on multiple environmental factors (e.g., temperature, sunlight, turbulence) which may affect the toxicity of crude oil and/or feeding rates of copepods (Duesterloh et al., 2003; Saiz et al., 2003; Jiang et al., 2012). The rate at which copepods encounter crude oil droplets may be higher in bottle incubations than in the field, where currents, tides, and turbulence would affect the encounter rates between oil droplets and copepods. Also, in the open ocean, some copepods vertically migrate, spending part of the day at depth, which would reduce their exposure to spilled oil that is mainly present in surface waters. Food quantity and quality will also affect production rates and characteristics (e.g. size, density, morphology) of fecal pellets of copepods (Dagg and Walser, 1986; Butler and Dam, 1994; Feinberg and Dam, 1998) and consequently, crude oil defecation rates and vertical flux of fecal pellets after oil spills. In our experiments, we used high food concentrations to ensure a representative number of fecal pellets containing oil droplets for image analysis. Since fecal pellets production rates depends on food availability, crude oil defecation rates estimated in this study will be expected only during oil spills in coastal waters during productive seasons. For example, *in situ* copepod fecal production rates in productive waters range from 2 to 50 pellets copepod^{-1} d^{-1}, with maximum values observed during the spring bloom (Urban-Rich et al., 1999; Wexels Riser et al., 2001; Møller et al., 2011). Also fecal pellets production rates of *A. tonsa* under simulated phytoplankton bloom conditions may reach values of 100 pellets copepod^{-1} d^{-1} (Butler and Dam, 1994). In contrast, crude oil defecation rates by copepods would be comparatively lower during oil spills in oligotrophic waters. Therefore, the quantitative impact of crude oil ingestion and defecation by copepods on the fate of crude oil will depend on the specific circumstances of each spill.

Although the quantity of ingested and defecated crude oil may widely vary depending on zooplankton groups and environmental conditions, the impact of crude oil ingestion and defecation from the entire zooplankton community to the fate of oil spills can be significant under certain situations. Long-term mesocosms experiments of zooplankton exposed to low, sublethal concentrations of dispersed crude oil are required to accurately evaluate the quantitative importance of crude oil ingestion by copepods and other zooplankton (e.g. protozoans) after several weeks following an oil spill. More research is also needed to determine the role of zooplankton fecal pellets containing crude oil droplets in the transfer of petroleum pollution through marine food webs after crude oil spills. Overall, our results emphasize that ingestion and defecation of dispersed crude oil by zooplankton should be considered in oil spill research and weathering models to better estimate the fate and impact of crude oil spills on marine systems.

Acknowledgments

We thank T. Villarreal for use of his microscope, camera and Imaging Particle Analysis system (FlowSight®), and C. Hyatt for help with the phytoplankton cultures. This research was made possible by a Grant from BP/The Gulf of Mexico Research Initiative through the University of Texas Marine Science Institute (DROPPS Consortium: 'Dispersion Research on Oil: Physics and Plankton Studies'. The Centre for Ocean Life is a VKR Center of Excellence funded by the Villum Foundation. The work was further supported by a grant from the Danish Council for Independent Research to RA.

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