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Novel insight into the role of heterotrophic dinoflagellates in the fate of crude oil in the sea

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Although planktonic protozoans are likely to interact with dispersed crude oil after a spill, protozoan-mediated processes affecting crude oil pollution in the sea are still not well known. Here, we present the first evidence of ingestion and defecation of physically or chemically dispersed crude oil droplets (1–86 μm in diameter) by heterotrophic dinoflagellates, major components of marine planktonic food webs. At a crude oil concentration commonly found after an oil spill (1 μL L^{-1}), the heterotrophic dinoflagellates *Noctiluca scintillans* and *Gyrodinium spirale* grew and ingested ~0.37 μg-oil μg-C_{dino}^{-1} d^{-1}, which could represent ~17% to 100% of dispersed oil in surface waters when heterotrophic dinoflagellates are abundant or bloom. Egestion of faecal pellets containing crude oil by heterotrophic dinoflagellates could contribute to the sinking and flux of toxic petroleum hydrocarbons in coastal waters. Our study indicates that crude oil ingestion by heterotrophic dinoflagellates is a noteworthy route by which petroleum enters marine food webs and a previously overlooked biological process influencing the fate of crude oil in the sea after spills.

Crude oil pollution in the sea is a growing environmental problem. The rise in world energy demand during the last decades has resulted in intense exploration, production and transportation of crude oil in the sea, increasing the risk of crude oil spills to marine environments[1,2]. The Deepwater Horizon oil spill in the Gulf of Mexico (2010) is a recent example of the adverse ecological impacts caused by a catastrophic crude oil spill[3]. After a spill, crude oil undergoes a variety of transformations involving physical, chemical, and biological processes that determine the fate of petroleum pollution in the sea[1]. Small crude oil droplets (1–100 μm) generated by wind and waves, natural emulsifiers and/or treatment with chemical dispersants are effectively suspended in the water column after an oil spill[4–8]. These crude oil droplets are frequently in the food size spectra of both micro- and mesozooplankton and may be ingested[9–13]. However, most research on crude oil and zooplankton interactions has been conducted with mesozooplankton and used dissolved petroleum hydrocarbons[14,15], disregarding the potential of microzooplankton, including protozoan zooplankton, to ingest particulate crude oil.

Dinoflagellates are major components of marine plankton and approximately half of living dinoflagellates species (>2000 species) are exclusively heterotrophic[16]. In the last decades, we have learned that heterotrophic dinoflagellates graze heavily on phytoplankton, constitute a substantial part of total microzooplankton biomass, and contribute considerably to the diet of metazooplankton (e.g. copepods and fish larvae)[17–23]. It has also been recently discovered that many species of phototrophic dinoflagellates, which had previously been thought to be exclusively autotrophic, are mixotrophic, suggesting that most dinoflagellates are able to ingest prey[24–25]. Despite the importance of dinoflagellates in marine ecosystems, little is known about the interactions of these planktonic organisms with crude oil. During the Torrey Canyon spill (1967) in the Bay of Biscay[26], blooms of the heterotrophic dinoflagellate *Noctiluca scintillans* were associated with the disappearance of crude oil treated with "craie de Champagne" (French blackboard powdered chalk), leading Cooper (1968) to hypothesize that ingestion of crude oil by these organisms was essential for efficiently eliminating the crude oil[26]. Despite this interesting field observation suggesting heterotrophic dinoflagellates play a key role in the fate of a crude oil spill, ingestion of crude oil by *N. scintillans* has never been proven and the quantitative impact of crude oil ingestion by heterotrophic dinoflagellates has not yet been investigated.

In the present study we investigated ingestion of crude oil droplets by the heterotrophic dinoflagellates *Noctiluca scintillans* and *Gyrodinium spirale*. We used these species as models because of their cosmopolitan distribution, high abundance, and important trophic role in coastal and oceanic waters[17,20,27]. Our specific

objectives were to: 1) determine if heterotrophic dinoflagellates ingest crude oil and whether dispersants or food influences crude oil ingestion, and 2) estimate the potential impact of heterotrophic dinoflagellates on oil spills by quantifying the amount and size spectra of ingested crude oil droplets. For these purposes, we conducted laboratory experiments exposing heterotrophic dinoflagellates to crude oil and dispersant-treated crude oil emulsions with or without the addition of phytoplankton as food during short-term incubations.

Results and Discussion

Ingestion of crude oil by heterotrophic dinoflagellates and influence of dispersants and food. We found that both species of heterotrophic dinoflagellates ingested crude oil droplets after exposure to crude oil emulsions, with or without the addition of phytoplankton as food or dispersants. The presence of crude oil droplets inside the cells was unequivocally confirmed by strong autofluorescence of crude oil under UV light (Fig. 1), where the proportion of cells containing crude oil droplets at the end of the incubation ranged from 28% to 90%, depending on the experimental treatment (Fig. 2 A–B). A significantly higher percentage of *G. spirale* cells contained oil when exposed to crude oil without the presence of food than in the other treatments (ANOVA, $F_{3,4} = 20.971$, $p = .007$, Bonferroni post-hoc test) (Fig. 2B), whereas no significant differences among treatments were observed for *N. scintillans* (ANOVA, $F_{3,4} = 4.978$, $p = .078$) (Fig. 2A).

Previous studies on crude oil ingestion by tunicates[10] and epibentic ciliates[11] found that these organisms only ingest crude oil in the presence of food and/or when oil was chemically dispersed. However, we found that heterotrophic dinoflagellates ingested physically or chemically dispersed crude oil droplets, with or without the presence of food, at a concentration of crude oil commonly found in the water after crude oil spills[5]. Recent laboratory studies also show that copepods, copepod nauplii and barnacle larvae efficiently ingest both chemically and physically dispersed crude oil droplets[12,13]. According to our results, we expect heterotrophic dinoflagellates ingest dispersed crude oil under various conditions after crude oil spills, i.e., with or without the application of dispersants, and in oligotrophic (low food concentration) or eutrophic waters (high food concentrations). But, application of dispersants would enhance the formation of plumes of dispersed crude oil after oil slicks[5], which may foster the ingestion of crude oil droplets by heterotrophic dinoflagellates. In turn, heterotrophic dinoflagellate cells containing crude oil can be prey for consumers, such as crustacean zooplankton and fish larvae[21–23], which would enhance biotransfer of highly toxic, low-solubility petroleum hydrocarbons through marine food webs during crude oil spills.

Growth rates of heterotrophic dinoflagellates ingesting crude oil. Our results corroborate that heterotrophic dinoflagellates have a relatively high tolerance to crude oil and dispersants compared to other microzooplankton[28], despite ingesting crude oil droplets. Specifically, growth rates of heterotrophic dinoflagellates exposed to crude oil or dispersant-treated oil were not significantly different to the controls either in the absence of food (ANOVA, *N. scintillans*: $F_{2,3} = 1.421$, $p = .376$; *G. spirale*: $F_{2,3} = 4.418$, $p = .132$) or with food (ANOVA, *N. scintillans*: $F_{2,3} = 2.023$, $p = .287$; *G. spirale*: $F_{2,3} = 1.375$, $p = .387$) (Fig. 2). In the presence of food, growth rates of heterotrophic dinoflagellates exposed to crude oil or dispersant-treated oil were similar the controls for both species (Fig. 2 C–D). In the absence of food, growth rates in the experimental treatments were also similar to the corresponding controls (Fig. 2 C–D), except for starved *G. spirale* after exposure to dispersant-treated

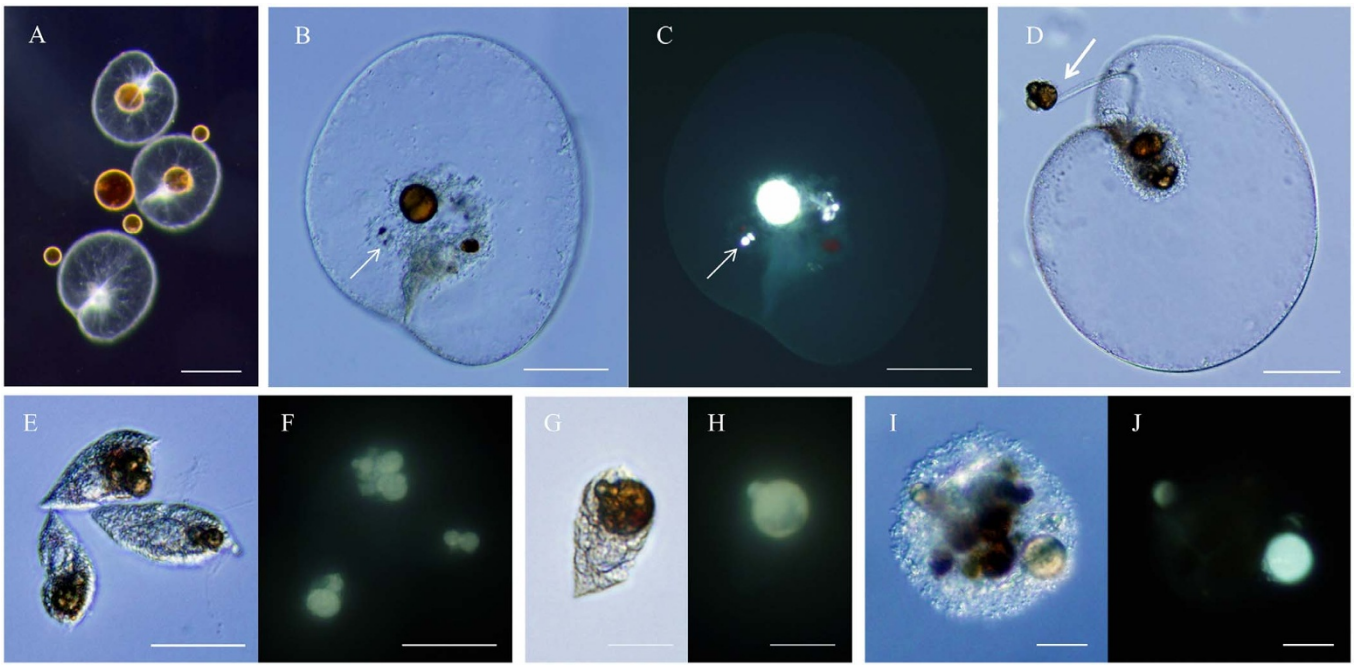

Figure 1 | Ingestion and defecation of dispersed crude oil by heterotrophic dinoflagellates. (A): Microscope image of the heterotrophic dinoflagellate *Noctiluca scintillans* with large crude oil droplets inside the cells. Scale bar = 200 μm. (B–C): *N. scintillans* under bright (B) and UV (C) illumination. Crude oil strongly autofluoresces under UV light (365 nm), which was used to verify crude oil droplets inside cells (C). The red color (C) is autofluorescencing chlorophyll from phytoplankton used as prey. Arrow identifies small crude oil droplets, which were accurately identified and quantified under UV illumination (C), but were similar to other particles under bright field (B). Scale bar = 100 μm. (D): *N. scintillans* with crude oil droplets collected on the tip of the tentacle and inside the cell. Arrow indicates the tentacle of *N. scintillans*. Scale bar = 100 μm. (E–F): The heterotrophic dinoflagellate *Gyrodinium spirale* with crude oil droplets observed under bright (E) and UV (F) illumination. Scale bar = 50 μm. (G–H): *G. spirale* with a large crude oil droplet relative to its cell volume under bright (G) and UV (H) illumination. Scale bar = 25 μm. (I–J): Faecal pellet of *N. scintillans* containing crude oil droplets and other particles observed under bright (I) and UV (J) illumination. Scale bar = 25 μm.

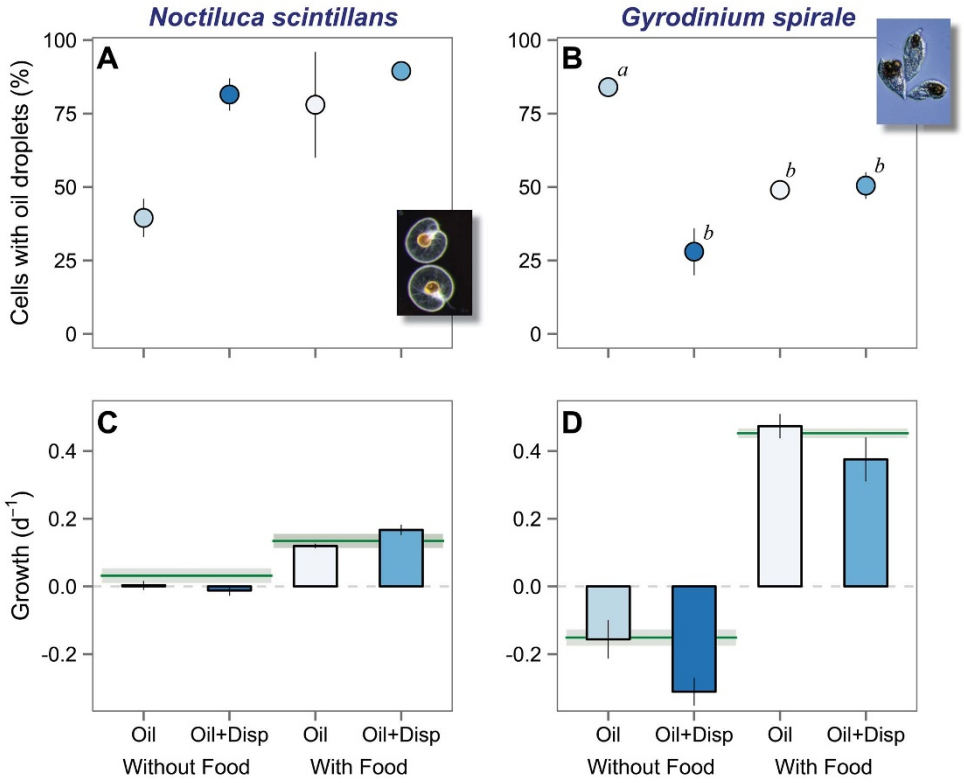

Figure 2 | Number of cells containing oil droplets at the end of the incubation and growth rates of heterotrophic dinoflagellates ingesting crude oil. Percent of Noctiluca scintillans (A) and Gyrodinium spirale (B) cells containing crude oil droplets and population specific growth rates (d^{-1}) of N. scintillans (C) and G. spirale (D) after exposing to crude oil emulsions (Oil) or chemically dispersed crude oil (Oil+Disp) with or without phytoplankton as food. Green lines indicate growth rates in control incubations with no crude oil additions. Error bars and green shadows are the range. The percent of cells of N. scintillans containing oil droplets was not significantly different among treatments (A, ANOVA, $p = .078$). Lower case letters (a, b) indicate different statistical groups in the percent of G. spirale cells containing oil droplets according to the results of pairwise t-test with a Bonferroni correction (B, $p < .05$). There was no significant difference in growth rate between the control and experimental treatments for both species (C–D, ANOVA, $p > .05$).

oil, where growth rates decreased double than in the control, although not significant (Fig. 2 D).

Protozoan zooplankton tolerance to crude oil and dispersants can vary depending on the taxonomic groups/species[28]. In our experiments, starved *G. spirale* tended to be more sensitive to chemically dispersed crude oil exposure than *N. scintillas* (Fig. 2). Although data are limited and sensitivity to crude oil varies among species, small ciliates tend to be more sensitive to crude oil and dispersants than heterotrophic dinoflagellates according to a recent laboratory study[28]. Observation of mixotrophic or heterotrophic dinoflagellates blooms during oil spills[26,29] supports that these protozoans are relatively resistant to crude oil at concentrations found in the water column after oil spills.

Size spectrum of crude oil droplets ingested by heterotrophic dinoflagellates. Crude oil droplets in emulsions were 1–90 μm in diameter, with >95% of droplets being between 1–20 μm (Fig. 3). Median diameters were 8.0 and 6.6 μm for crude oil and dispersant-treated crude oil emulsions, respectively (Fig. 3). *N. scintillans and G. spirale* ingested crude oil droplets with diameters of 1–86 μm and 1–42 μm, respectively, with >99% of droplets being between 1–50 μm for *N. scintillans* and 1–35 μm for *G. spirale*. *N. scintillans* ingested larger oil droplets than *G. spirale* (ANOVA, $F_{1,11} = 19.678$, $p = .001$), where median diameters of ingested oil were 11.4–13.4 μm for *N. scintillans* and 7.6–11.8 μm for *G. spirale*, (Fig. 3). Diameters of ingested oil droplets were not significantly different among treatments for *N. scintillans* (ANOVA, $F_{3,4} = 6.090$, $p = .057$) and only two treatments differed from each other for *G. spirale* (oil without food *versus* dispersant-treated oil with food; ANOVA, $F_{3,4} = 13.782$, $p = .01$, Bonferroni post-hoc test). Both species selected large crude oil droplets consistent with known size-based preferences of natural prey[30]. Our results indicate that the studied heterotrophic dinoflagellates have limited ability to discriminate between appropriate food and crude oil droplets in their prey size spectra.

Crude oil droplet capture and ingestion mechanisms of heterotrophic dinoflagellates. Crude oil ingestion was consistent with known feeding behaviour of the studied species. *N. scintillans* is an interception feeder that captures prey in a clump of mucus secreted on the tip of the tentacle[31]. Crude oil droplets seem to be collected by this mechanism since we observed the adhesion of crude oil droplets on the tentacle tip of *N. scintillas* (Fig. 1D). *G. spirale* ingest prey by direct engulfment and have the ability to ingest relatively large prey considering their size[32–34]. We observed that *G. spirale* ingested large oil droplets in relation to the cell size (Fig. 1 G–H). These different feeding behaviours likely explain the differences in the number of oil droplets consumed per cell between species (Fig. 4 A–B), which was significantly higher for *N. scintillans* than for *G. spirale* (ANOVA, $F_{1,675} = 414.667$, $p < .001$).

Crude oil ingestion rates and potential impact of heterotrophic dinoflagellates on oil spills. At a crude oil concentration of 1 $\mu L\,L^{-1}$, *N. scintillans* and *G. spirale* ingested 7.3–55.1 $\times 10^3$ μm^3 and 0.9–2.3 $\times 10^3$ μm^3 of crude oil per cell, respectively, depending on the experimental treatments (Fig. 4 C–D). The volume of oil ingested by individual *N. scintillans* cells was significantly greater than by individual *G. spirale* cells (ANOVA, $F_{1,675} = 463.057$, and $p < .001$). The mean weight-specific ingestion rates of crude oil were

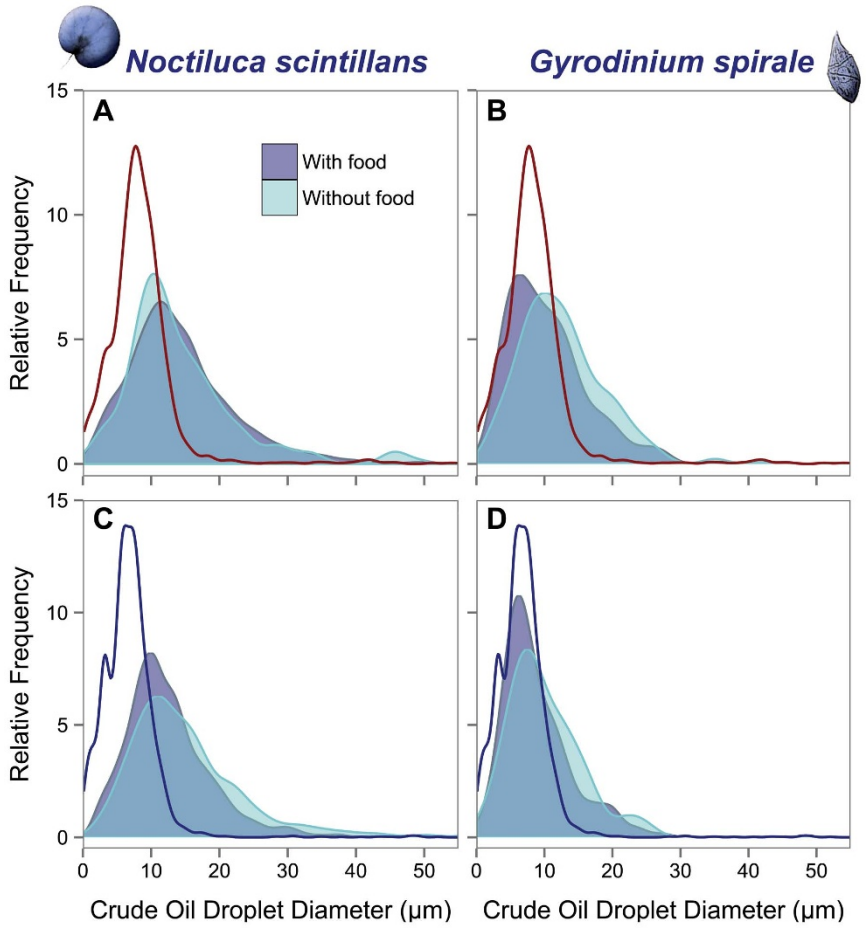

Figure 3 | Relative frequency of crude oil droplet size ingested by heterotrophic dinoflagellates and in crude oil emulsions. Diameter of crude oil droplets ingested by Noctiluca scintillans (left panel) and Gyrodinium spirale (right panel) incubated without chemical dispersants (A–B) or with chemical dispersants (C–D), with (shaded dark blue) or without phytoplankton as food (shaded light blue). Red and blue lines are the size of droplets in crude oil emulsions added to experimental bottles without dispersants (A–B) and with dispersants (C–D), respectively. In all cases the relative frequency data were fit to a density function. Actual range of droplet size was 1–100 µm, but only 0–50 µm droplets are shown as they include 99% of the oil droplets.

similar for both species, 0.35 and 0.39 µg-oil µg-C_{dino}^{-1} d^1 for *G. spirale* and *N. scintillans*, respectively (Table 1).

After a marine crude oil spill, crude oil concentration in the water column may vary from a few ppb to hundreds of ppm, depending on temporal and spatial scales, turbulence and mixing energy caused by wind, waves and currents, and if dispersants are applied[4–8,35–38]. During the first hours, crude oil in surface waters near the oil spill source or oil slicks may reach concentrations of 20–200 ppm[35–38]. At these oil concentrations, heterotrophic dinoflagellates would be negatively affected according to the median effect concentrations (EC_{50}) for crude oil observed for heterotrophic dinoflagellates[26]. After several hours to days, crude oil is dispersed in the sea reaching concentration usually ≤2 ppm[4–8,33–36]. For example, crude oil concentrations following the Deepwater Horizon Oil spill ranged from <0.25 ppb to 0.22 ppm in coastal and estuaries areas[37] to 1–2 ppm in dispersed crude oil plumes at 1 km depth[8]. At these concentrations, survival and growth of heterotrophic dinoflagellates would be unaffected or slightly affected by acute exposure to crude oil, and ingestion of crude oil droplets would be expected.

When abundant, *N. scintillans* and *G. spirale* type-dinoflagellates (gymnodinoids) could ingest between 17%–28% of dispersed crude oil droplets in surface waters daily, considering a crude oil concentration commonly found in surface waters after a spill (1 µL L^{-1}, 0.84 ppm) and the oil ingestion rates calculated in our experiments (Table 1). Even when heterotrophic dinoflagellate abundance is low and daily crude oil ingestion rates are low (Table 1), the total amount of crude oil ingested by heterotrophic dinoflagellates can become quantitatively important after several days. The impact of crude oil ingestion by heterotrophic dinoflagellates could be particularly relevant during intense blooms (>17%–100%, Table 1), where high abundances of dinoflagellates can extend from several to hundreds of kilometres[39]. *N. scintillans* is one of the most common bloom-forming dinoflagellate species, producing frequent and recurrent blooms in coastal areas around the world[27]. According to our estimates, an intense bloom of *N. scintillans* has the potential to ingest, in one day, most of the dispersed crude oil droplets in the area covered by the bloom when oil concentrations are ~1–2 ppm. Gymnodinoid heterotrophic dinoflagellates (e.g. *G. spirale*, *Gymnodinium* spp) can become very abundant (>130000 cells L^{-1}) and even dominate the planktonic particle volume during phytoplankton spring blooms in some coastal areas[40]. In addition, mixotrophic dinoflagellates, which frequently bloom in coastal areas[25], can potentially ingest dispersed crude oil during oil spills. Therefore, given the high abundance of phagotrophic dinoflagellates in marine environments[16–18] and their higher tolerance to crude oil and dispersant compared to other microzooplankton[28], crude oil ingestion by dinoflagellates could be a quantitatively important process affecting the fate of oil spills in marine environments. Crude oil ingestion by dinoflagellates, however, will not only depend on crude oil concentration and dinoflagellate abund-

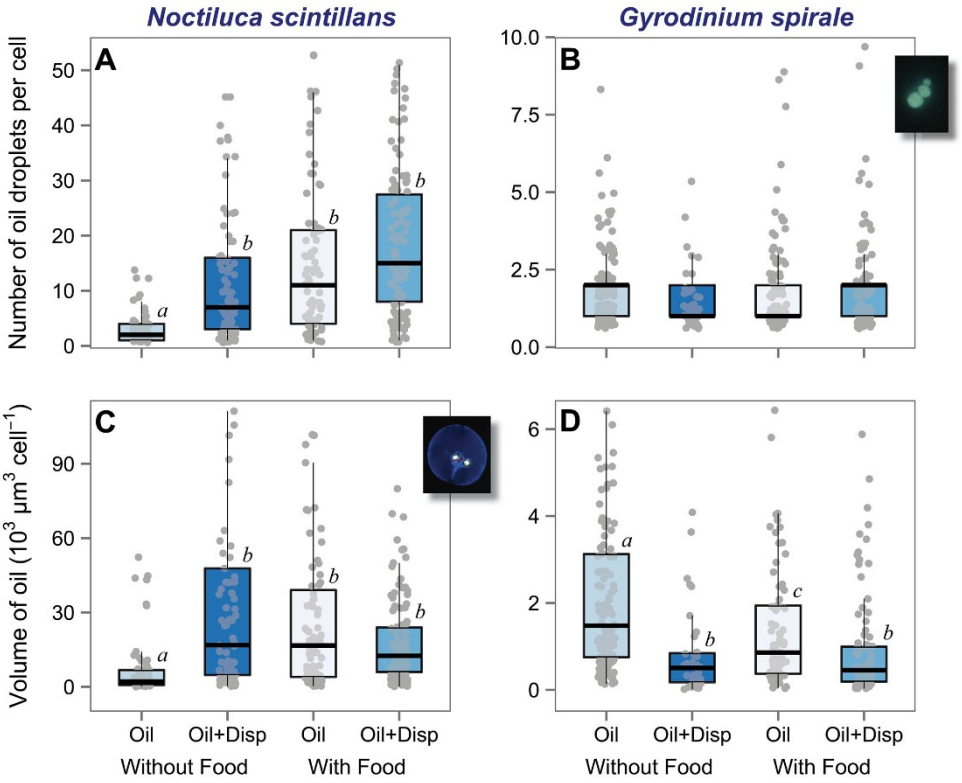

Figure 4 | Quantification of the number of oil droplets and amount of oil ingested by heterotrophic dinoflagellates. Number of oil droplets ingested per cell (A–B), and total volume of crude oil ingested per cell (C–D) for *Noctiluca scintillans* (left panel) and *Gyrodinium spirale* (right panel) incubated with crude oil emulsions (Oil) or chemically dispersed crude oil (Oil+Disp), with or without phytoplankton for food. Horizontal black bar shows the median, boxes encompass the interquartile range, and whiskers are 1.5 times the interquartile range. Lower case letters (*a*, *b*, *c*) indicated different statistical groups according to the results of pairwise t-test with a Bonferroni correction ($p < .05$). Note that there was no significant difference in the number of oil droplets in *G. spirale* (B, ANOVA, $p > .05$).

ance but also on multiple other factors, such as temperature, exposure time, crude oil type, marine hydrodynamics, and dispersion of crude oil. Further, given the differences in sensitivity to crude oil among protozoan species[28], the impact of ingestion of dispersed crude oil by planktonic protozoans would also depend on the community composition. Therefore, the quantitative importance of crude oil ingestion by protozoan zooplankton would vary depending on the specific circumstances of each oil spill.

Defecation of crude oil by heterotrophic dinoflagellates and their implications in the flux and fate of petroleum pollution in the sea. In investigating the fate of ingested crude oil droplets, we observed that, in <10 h, ~45% of *N. scintillans* cells that had contained crude

Table 1 | Crude oil ingestion rates by the heterotrophic dinoflagellates Noctiluca scintillans and Gyrodinium spirale, and their potential impact on a dispersed crude oil spill in the sea. IR_{oil} and IR^{s}_{oil} are respectively the crude oil ingestion rates per cell and the crude oil weight-specific ingestion rates determined in the laboratory. IR^{p}_{oil} is the estimated crude oil ingestion rates by the heterotrophic dinoflagellate population. "Impact" of crude oil ingestion was calculated as the percentage of the total dispersed crude oil in surface waters ingested in one day, considering a crude oil concentrate of 1 µL L^{-1} and abundances of heterotrophic dinoflagellates in different conditions. [a]Abundance considering total gymnodinoid heterotrophic dinoflagellates

Species	IR_{oil} (ng oil $cell^{-1}$ d^{-1})	IR^{s}_{oil} (µg oil µgC_{dino}^{-1} d^{-1})	Condition	Abundance (cells L^{-1})	IR^{p}_{oil} (mg oil L^{-1} d^{-1})	Impact (%)
Noctiluca scintillans	Range: **6.1–46.3**	Range: **0.14–0.63**	Extreme bloom	$>10^6$	>23.3	**100**
			Intense Bloom	10^4–10^5	0.23–2.32	**28–100**
	Mean: **23.3**	Mean: **0. 39**	Seasons/areas of high abundance	10^3–10^4	0.023–0.23	**2.8–28**
			Seasons/areas of low abundance	<10–10^3	<0.0002–0.023	<**0.03–2.8**
Gyrodinium spirale	Range: **0.8–1.9**	Range: **0.20–0.58**	Intense Bloom	$>10^{5a}$	>0.14	>**17**
			Seasons/areas of high productivity	10^4–10^{5a}	0.02–0.14	**1.7–17**
	Mean: **1.2**	Mean: **0.35**	Season/areas of medium productivity	10^3–10^{4a}	0.001–0.02	**0.2–1.7**
			Seasons/areas of low productivity	$<10^{3a}$	<0.001	<**0.2**

oil droplets no longer did. Crude oil droplets previously identified inside the cells were observed in faecal pellets (Fig. 1 H–J). Faecal pellets of *N. scintillans* were oval or spherical with a mean diameter of 82 μm (range: 60–113 μm) (Fig. 1 H–J). The volume of crude oil initially observed inside the cells was similar to the volume of oil found later inside the faecal pellets, indicating that heterotrophic dinoflagellates eliminated most of the insoluble fraction of crude oil through egestion. However, it is unknown how the chemical composition of crude oil is modified after being ingested and defecated by heterotrophic dinoflagellates. There is some evidence of selective accumulation of toxic petroleum hydrocarbons in copepod faecal pellets[41,42], but more research is required to understand biochemical partitioning of crude oil after being ingested by both proto- and metazooplankton.

In our laboratory experiments, we observed that faecal pellets of heterotrophic dinoflagellates containing oil droplets settled in the base of the counting chambers. This indicated that dinoflagellate faecal pellets containing crude oil are negatively buoyant particles. Thus, although it is unknown how the presence of oil can affect sinking rates, we expect that dinoflagellate crude oil-contaminated faecal pellets sink in the water column after crude oil spills. Even though the majority of protozoan faecal pellets are presumably recycled in the epipelagic waters[43], crude oil droplets compacted into faecal material would settle more efficiently than suspended crude oil droplets. Particularly, faecal pellets produced by *N. scintillans* (60–113 μm) are larger than typical protozoan faecal pellets, "minipellets" (3–50 μm)[43], and consequently, *N. scintillans* faecal pellets would sink faster than smaller protozoan faecal pellets. Considering the mean volume of *N. scintillans* faecal pellets observed in this study ($\sim 2.16 \times 10^5$ μm^3), these faecal pellets would sink at a rate of ~ 38 m d^{-1}, according to natural sinking rates determined from pellet volume/sinking rate relationship found for small copepod faecal pellets[44]. Therefore, our results support the previous notion that ingestion and defecation of crude oil by *N. scintillans* blooms were the main mechanisms for efficient removal of dispersed crude oil in surface waters observed during the Torrey Canyon spill in the Bay of Biscay[26].

Dinoflagellate faecal pellets containing crude oil droplets can be consumed by detritivorous and coprophagous zooplankton[45], promoting the biological flux of toxic petroleum hydrocarbons in marine food webs. Most field studies on zooplankton faecal pellets have been focused on metazoans, and comparatively little is known about protozoan faeces[43]. Several studies found that small faecal pellets produced by planktonic protozoans are ubiquitous and abundant in sediment traps and water samples[46–51]. Although the vertical flux of protozoan faecal pellets is poorly understood, there is evidence that protozoan faecal pellets settle and can reach the bottom/benthos in coastal areas, contributing to carbon flux[46–51]. For instance, large numbers of protozoan faecal pellets produced in the euphotic zone were found in mesopelagic and bathypelagic waters (2 km depth) in the eastern tropical Pacific[46]. Several studies in temperate and polar waters reported that minipellets, including faecal pellets produced by dinoflagellates, were very abundant in sediment traps (50–100 m depth)[47,51] and that ca. 28% of minipellets produced in the water column were found in the sediments[47]. Since small faecal pellets ($<10^5$ μm^3) are commonly an important constituent of marine snow[52], the aggregation of dinoflagellate crude oil-contaminated faecal pellets to other particles forming marine snow could enhance the vertical flux of petroleum pollution to the benthos. Therefore, although individual protozoan pellets sink slowly and may be mostly recycled or repackaged by microbial degradation and coprophagy in epipelagic waters[43], protozoan faecal pellets containing crude oil may contribute to the vertical flux petroleum pollution to the benthos in shallow coastal zones. More research is needed to determine the fate of zooplankton crude oil-contaminated faecal pellets in the sea, and their role in the flux of petroleum pollution in marine environments.

Main conclusions. Overall, our study highlights that ingestion of crude oil by heterotrophic dinoflagellates is a significant path by which crude oil pollution enters marine food webs and an important mechanism affecting the fate of dispersed crude oil in the sea after oil spills. Our results emphasize the need to understand and quantify crude oil ingestion and defecation by zooplankton, both protozoans and metazoans, to determine the fate and impact of petroleum pollution in marine environments.

Methods

Experimental organisms. The heterotrophic dinoflagellates *Noctiluca scintillans* and *Gyrodinium spirale* were isolated from plankton samples collected from the Aransas Ship Channel near the University of Texas Marine Science Institute (MSI) in Port Aransas (Texas, USA). Microplankton samples were collected from surface waters by tying a microplankton net (20 μm mesh, 20 cm diameter) to the MSI pier and allowing it to stream with the tidal current for approximately 5 min. The plankton samples were poured into plastic bottles and kept in a cooler until returning to the laboratory. To isolate *G. spirale*, aliquots of the microplankton samples were then incubated in 1 L polycarbonate bottles and enriched with mixtures of cultured phytoplankton (e.g. *Isochrysis galbana*, *Rhodomonas* sp, *Peridinium foliaceum*). These enrichments were placed on a bottle roller rotating at 2–4 rpm and were incubated at 24°C at low light intensities for several days. Enrichments were checked periodically for the growth of *G. spirale*. When *G. spirale* appeared to be growing well, cells were picked individually with a borosilicate glass fine tip Pasteur pipette and placed into 7 ml micro-wells containing sterilized 0.2 μm filtered seawater. Then, the autotrophic dinoflagellate *P. foliaceum* was added to the micro-wells as prey for *G. spirale*. After several days, isolated protozoan species were transferred to 75 ml polystyrene tissue culture flasks and placed on a bottle roller under the conditions described above. To isolate *N. scintillans*, aliquots of the microplankton samples were examined under a dissecting microscope and *N. scintillans* cells were identified and gently picked individually with a borosilicate glass pipette. Isolated cells were repeatedly rinsed by transferring them through a series of Petri dishes filled with autoclaved, 1 μm filtered sea water (FSW). Then, *N. scintillans* cells were transferred to 75 ml polystyrene tissue culture flasks containing sterilized 0.2 μm FSW and the autotrophic dinoflagellates *Gymnodinium dorsum, P. foliaceum* and *Heterocapsa* sp as food. *N. scintillans* culture flasks were incubated at 20°C and a salinity of 34–35 on a 12 : 12 hour light : dark cycle at low light intensities. After several days, cultures of both species of heterotrophic dinoflagellate were transferred to 250 mL polycarbonate flasks, fed every 3 d, and transferred into new media at 1 week intervals. Phytoplankton cultures were grown in f/2 culture medium prepared with sterilized 0.2 μm FSW collected from the Aransas Ship Channel. Phytoplankton cultures were held in 250 mL polycarbonate flasks at 20°C and a salinity of 34–35 on a 12 : 12 hour light : dark cycle with cool-white fluorescent lights at an irradiance of approximately 25 μmol photons m^{-2} s^{-1}.

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 concentrations and composition of polycyclic aromatic hydrocarbons in this type of crude oil were previously determined by our research group[53]. The chemical dispersant Corexit 9500A, the main type of dispersant used in the clean-up operations during the Deepwater Horizon oil spill[3], was used to prepare dispersant-treated crude oil emulsions. Corexit 9500A was provided by NALCO® (Nalco/Exxon Energy Chemicals, L.P.) and some of its chemical components can be found in the NALCO Environmental Solutions LLC web page[54].

We prepared 2 types of test media: 1) crude oil emulsions, i.e., suspensions of crude oil droplets in seawater dispersed physically without the addition of dispersant, 2) dispersant-treated crude oil emulsions i.e., crude oil emulsions in seawater dispersed physically and chemically. To prepare crude oil-seawater emulsions, 0.2 μm filtered seawater was placed in a 1 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 stirrer 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 the 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 caused the formation of 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. We used a ratio of dispersant to oil of 1 : 20, which is in the range recommended by the U.S. Environmental Protection Agency, EPA[55]. After stirring for 5 min, aliquots of each test medium were added to the corresponding experimental bottles to obtain the desired exposure concentration (1 μL L^{-1}). 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®).

Experimental design. We conducted two types of experiments, first we investigated if the heterotrophic dinoflagellate *G. spirale* and *N. scintillans* ingest crude oil droplets

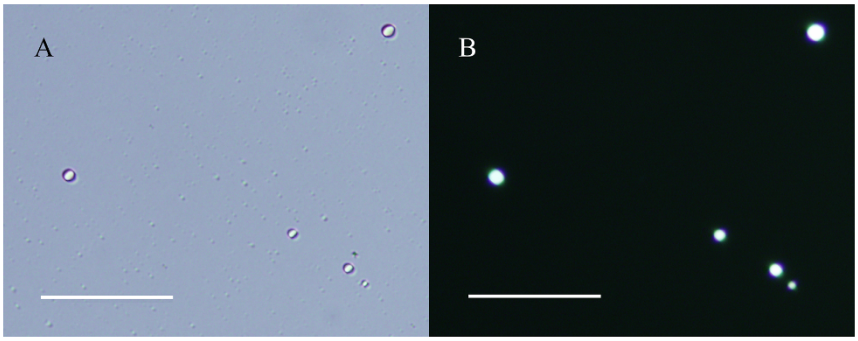

Figure 5 | Crude oil autoflorescence under UV light. Microscope images of light Louisiana sweet crude oil droplets suspended in sea water under bright (A) and UV (B) illumination. Crude oil strongly autofluoresces under UV light (365 nm) (B). Scale bar = 50 μm.

and whether dispersants or food influences crude oil ingestion, and second we examined the fate of the crude oil droplets after being ingested by *N. scintillans*.

The first type of experiments consisted of short term incubations (24–48 h) of a single species of heterotrophic dinoflagellate incubated with emulsions of crude oil alone (1 μl L^{-1}), with dispersant-treated crude oil (1 μl L^{-1}) or in absence of crude oil (control treatments) with or without the addition of phytoplankton as food. For the treatments without food, cultures of *N. scintillans* and *G. spirale* were not fed 3 days before the experiments began to ensure that all food was depleted in the cultures. The absence of food was verified by checking the cultures before the experiments. Aliquots of each culture for *G. spirale* or single cells for *N. scintillans* were taken from the stock cultures and added to the incubation bottles. Incubations were conducted in glass bottles containing 0.2 μm filtered seawater (salinity = 35). For treatments that included food, suspensions of the phytoplankton *Gymnodinium dorsum* (750 cells mL^{-1}) and *Heterocapsa* sp (700 cells mL^{-1}) were added to bottles for *N scintillans* and *Peridinium foliaceum* (60 cells mL^{-1}) for *G. spirale*. The initial cell densities (cells mL^{-1}) were 2.2 for *N. scintillans*, 6 for *G. spirale* in the treatment without food, and 23 for *G. spirale* in the treatment with food. Control and experimental treatments were run in duplicates. After adding emulsified crude oil or dispersant-treated oil to the corresponding experimental bottles, bottles were incubated at 23°C with natural light-dark cycles in a Wheaton bench top roller at 2 rpm in the laboratory. At the end of the incubation, all samples were placed in plastic bottles, fixed with glutaraldehyde (2%) and kept at 4°C until analysis.

In the second type of experiments, *N. scintillans* (n = 68, density = 1 cell mL^{-1}) were exposed to physically dispersed crude oil (1 μl L^{-1}) in presence of food (*P. foliaceum*, 700 cells mL^{-1}) for ca. 24 h. After the exposure time, images of 24 cells containing crude oil droplets were captured with a digital camera attached to the microscope. Then, these cells were individually isolated, transferred to glass plate wells with FSW and food and incubated overnight at 21°C. After ca. 8–10 h, we checked for the presence/absence and volume of oil droplets previously identified inside each cell. When oil was no longer found in the cells, we looked in the water/faecal pellets.

Sample analysis and calculations. To determine the initial and final concentration of *G. spirale* cells in the different treatments, aliquots of the fixed samples (10–50 mL) were allowed to settle for 24 h in 10–50 mL Utermöhl chambers, and then, the whole chamber was counted using an inverted microscope (Olympus BX60) at 100× magnification. In the case of *N. scintillans*, all initial and final fixed samples were poured in a glass bowl and the number of cells counted under a stereomicroscope. To examine the presence of crude oil inside the heterotrophic dinoflagellates, cells from each treatment (n = 54–136) were placed in glass chambers and observed under an epifluorescence microscope (Olympus BX51) with bright-field and UV illumination. The presence or absence of crude oil droplets in each cell was verified by the exposure to UV light (365 nm). Crude oil produces a strong fluorescence due to the presence of aromatic hydrocarbons (Fig. 5).

Images of each cell with both bright-field and UV illumination were captured with a digital camera attached to the microscope. The number and volume of oil droplets inside the heterotrophic dinoflagellate cells were determined using ImageJ software (NIH, version 10.2) with images taken under UV illumination. 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 the triangle algorithm for automatic thresholding[56]. 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 the length of the major and minor axes as determined by ImageJ, where volume = $4/3\pi \times$ (major axis/2) $\times$ (minor axis/2)^2. When oil was densely packed in *N. scintillans*, individual oil droplets could not be discerned. In these cases, the number of oil droplets per cell is underestimated, but the total volume of oil ingested in these cells would not be affected. Statistical analyses were done with the general linear model (i.e., ANOVA) to determine differences among treatments within and between species where $\alpha = .05$. When residuals did not meet the assumptions of homoscedasticity, independence or normality, data was log transformed. A pairwise t-test with a Bonferroni correction was used to determine which treatments were statistically different from the others. Statistical analyses were done using R statistical software.

The mean volume of crude oil ingested per cell in each treatment was converted to mass using an oil density of 0.84 g cm^{-3}. Crude oil ingestion rates (ng oil $cell^{-1}$ d^{-1}) in each treatment were calculated considering that the amount oil found inside the heterotrophic dinoflagellate cells at the end of the incubation were ingested in 24 h. This may represent an underestimation of crude oil ingestion rates by heterotrophic dinoflagellates since we observed oil egestion rates for *N. scintillans* in <12 h. To calculate weight-specific ingestion rates (μg oil μgC_{dinos}^{-1} d^{-1}), carbon content of the heterotrophic dinoflagellates was estimated using a conversion factor of 1.98 fg-C μm^{-3} for *N. scintillans*[57] and the equation pg-C $cell^{-1} = 0.760 \times volume^{0.819}$ for *G. spirale*[58]. Cell volume (μm^{-3}) was calculated considering an ellipsoid shaped cell and using the lengths of the major and minor axes measured from bright-field images by image analysis (ImageJ). The amount of oil ingested by heterotrophic dinoflagellate population (mg-oil L^{-1} d^{-1}) was estimated using the mean ingestion rates determined in the laboratory and the abundance of cells observed in natural conditions under different situations. The range in abundance of *N. scintillans* was stated according to cell concentrations from different world regions, including bloom events[17,59–65]. Abundance of *G.spirale*-type dinoflagellates was estimated considering total cell concentration of gymnodinoid dinoflagellates found in different areas/seasons[17,20,40,66–69]. The "impact" (0–100%) of crude oil ingestion by heterotrophic dinoflagellate population on an oil spill was calculated as the percentage of the total dispersed crude oil in surface waters ingested in one day, considering a crude oil concentration of 1 μL L^{-1} (~0.84 ppm = 0.84 mg L^{-1}).

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Author contributions

All the authors conceived and designed the experiments. Experiments and analyses were conducted by R.A. and T.L.C. R.A. and T.L.C. prepared the manuscript. All of the authors discussed the results and revised the manuscript extensively.

Additional information

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