The effect of crude oil concentration on hydrocarbon degradation and associated bacterial communities under experimental conditions

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ABSTRACT

Chemical dispersants are an important tool for mitigating the environmental impact of marine oil pollution. Crude oil concentration used in experiments to study the efficacy of dispersants has been shown to impact oil biodegradation rates, which may explain contradictory conclusions between studies. Using high crude oil concentration could be particularly problematic when extrapolating laboratory results to the field, as dispersed oil in the open sea would rapidly dilute to sub-ppm concentrations. This study aimed to quantify the impact of crude oil concentrations ranging from 1 to 1000 mg L^{-1} on biodegradation and microbial community composition in dispersed versus non-dispersed crude oil, Oil biodegradation rates increased linearly from 1 to 100 mg L^{-1} but plateaued at a concentration of 1000 mg L^{-1}. However, with the addition of chemical dispersant, degradation rates were linear across the full range of concentrations, and significantly greater than non-dispersed treatments at 1 mg L^{-1} (81% increase), 10 mg L^{-1} (80%), 100 mg L^{-1} (64%) and 1000 mg L^{-1} (388%). Based on 16S rRNA gene sequence analysis, *Alteromonadaceae* and *Cellulophaga* were more dominant at lower oil concentrations, potentially being inhibited or outcompeted at higher concentrations. Use of dispersant resulted in more rapid changes in community composition. These results highlight the need to consider oil concentration when performing biodegradation experiments and will inform the Net Environment Benefit Analysis (NEBA).

INTRODUCTION

With the rise in transport and production of crude oil, remediation tools to combat marine oil pollution are still needed. Chemical dispersants are valuable for oil-spill response as they disperse oil slicks into small droplets in the water column, enhancing biodegradation (Prince *et al*.,2013; Mu *et al*., 2014), and often prevent oil from reaching sensitive shorelines (Chapman *et al*., 2007; Prince, 2015). However, the efficacy of dispersants has come into question (Kleindienst *et al*., 2015a; Kleindienst *et al*., 2016; Prince *et al*., 2016), and previous studies have documented contrasting results, either showing significant increases in oil biodegradation (Baelum *et al*., 2012; Techtmann *et al*., 2017; Thomas et al., 2021) or inhibition of biodegradation when chemically dispersed (Hamdan and Fulmer, 2011; Kleindienst *et al*., 2015b). These contradictory outcomes are potentially explained by differences in experimental design, notably the oil and/or nutrient concentrations (Techtmann *et al*., 2017; McFarlin and Prince, 2021). The use of water accommodated fractions (WAF) and chemically enhanced water accommodated fractions (CEWAF) in experiments (Kleindienst *et al*., 2015b), may also be unsuitable for biodegradation studies, as they remove most oil droplets and insoluble hydrocarbons, which are a major fraction of dispersed oil that can be biodegraded by microbes (McFarlin and Prince, 2021). Therefore, adding crude oil directly to seawater microcosms, then dispersing with chemical dispersant at the industry standard oil:dispersant ratio (20:1), and directly comparing to non-dispersed floating oil, is a more valid comparison for extrapolation to open water spill scenarios.

Oil concentration also has a significant impact on the biodegradation rates of hydrocarbons and can significantly increase the half-life of various hydrocarbons at higher concentrations, particularly as nutrient limitation becomes more likely, coupled with reduced bioavailability as a consequence of oil forming larger droplets, adhering to the container, or floating to the surface (Prince *et al*, 2017, McFarlin and Prince, 2021). When oil is spilt and

chemically dispersed at sea, its concentration can rapidly decrease to approximately 100 mg L^{-1}, with further dilution occurring over 24 hours to approximately 1-10 mg L^{-1} (Lee *et al*., 2013). Therefore, studies using high concentrations (e.g. 200–2500 mg L^{-1}), may be far less relevant to field scenarios. The present study compared biodegradation of crude oil with and without chemical dispersants over a range of oil concentrations (1, 10, 100 and 1000 mg L^{-1}) to test the effects that oil concentration has on both the biodegradation of hydrocarbons and the composition of bacterial communities over time. It was hypothesised that the presence of dispersants would increase the rate of biodegradation, that biodegradation in oil concentrations over 100 mg L^{-1} would be inhibited both with and without dispersant use, and that the growth of hydrocarbon-degrading bacteria would be enhanced with the use of dispersant.

EXPERIMENTAL DESIGN

***Microcosm Design*:** Surface seawater was sampled from the North Sea (7ºC, 51.7397° N, 1.0221° E on March 3, 2022). Microcosms were created in sterile 40 ml glass vials with PTFE-lined silicon septa, containing 20 ml of seawater (naturally containing 46 µmol L^{-1} nitrate and 1 µmol L^{-1} phosphate), and were sampled over a time series of 0, 1, 3, 6 and 14 days (n = 3). Norwegian Geochemical Standard North Sea Oil (NGS NSO-1, previously weathered at 60ºC over 24 hours) was added at concentrations of 1, 10, 100 or 1000 mg L^{-1} (0.0001% w/v to 0.1% w/v) with or without Slickgone NS dispersant (DASIC International Ltd) at a 20:1 oil:dispersant ratio. Killed controls were set up to determine any abiotic loss of hydrocarbons. Microcosms were incubated in the dark at 12ºC on an orbital shaker at 100 rpm.

Hydrocarbon Degradation Analysis: Hydrocarbons were extracted from the microcosms using previously described methods (Coulon *et al*., 2007). Deuterated alkanes and polyaromatic hydrocarbon (PAH) internal standards were added to the solvent prior to extraction at 0.004, 0.04, 0.4 and 4 µg mL^{-1}, for extraction of the 1, 10, 100 and 1000 mg L^{-1} oil, respectively. Extractions of 1000 mg L^{-1} oil were diluted 10-fold in hexane:DCM 1:1,

whilst 1 and 10 mg L^{-1} samples were concentrated 100 and 10-fold respectively in a TurboVap evaporator (Biotage), standardising all extracts to 100 mg L^{-1} equivalents with final deuterated standard concentrations of 0.4 µg mL^{-1}. Quantification of hydrocarbons (Alkanes C_{10-38}, Pristane and phytane, and a suite of two-six ring PAHs) was performed using a Nexis GC-2030/CMS-TQ8040 NX GC-QqQ-MS/MS (Shimadzu). External standard calibrations were produced with Alkane (C_8-C_{40}) and PAH (QTM PAH Mix) (Sigma) standard mixes over a 1–5 µg mL^{-1} range.

DNA Extraction and qPCR: Microcosms were filtered via 0.22 µm Sterivex filters (Millipore) and DNA extracted using a DNeasy PowerWater Kit (Qiagen), following the manufacturers' instructions. Filtrate was reserved for nutrient analysis. qPCR of the bacterial 16S rRNA gene was performed with primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) (Klindworth *et al*., 2013) on a CFX Opus 384 (Bio-Rad) as previously described (McKew and Smith, 2017).

***Nutrient Analysis*:** NH_4^+, NO_3^- and PO_4^{3-} concentrations were measured as previously described by Thomas *et al*., (2021).

16S rRNA Gene Amplicon Library Preparation and Bioinformatics: Amplicon library preparation for sequencing was performed as previously described (Thomas *et al*., 2021), and sequenced using NovaSeq PE250 strategy at Novogene (Cambridge UK). Sequencing data was processed using the Divisive Amplicon Denoising Algorithm (DADA2) pipeline (Callahan *et al*., 2022). Singleton and doubleton ASVs were discarded, along with sequences below 400 base pairs, containing homopolymers above 8 bp, or assigned to non-target organisms. Taxonomic assignment was conducted using the RDP Classifier (Callahan, 2020; Wang *et al*., 2007) and BlastN (Altschul *et al*., 1990). The bacterial 16S rRNA amplicon library size averaged 114,120 reads.

***Statistical Analysis*:** Prior to diversity and community composition analysis of ASVs, sequence data was rarefied to the lowest read depth (3010). Data was tested for normality (Shapiro-Wilk test) and tested for significance using ANOVAs (normally distributed) or generalised linear models (non-normally distributed).

RESULTS

Total measured alkanes (C_{10}-C_{38}, pristane and phytane) in non-dispersed oil at 1000 mg L^{-1} did not significantly decrease in concentration over 14 days, whereas total Alkanes in non-dispersed oil at 1, 10 and 100 mg L^{-1} did significantly decrease (25-30% degradation; t = 7.3-8.1, $p < 0.05$) (Figure 1). Chemical dispersion significantly enhanced alkane biodegradation at all concentrations of oil (24-71% degradation; t = 7.0-18, $p < 0.05$), over the 14 days, with significantly lower concentrations observed in all dispersed microcosms compared to non-dispersed microcosms (t = 2.8, $p < 0.05$). This was especially noticeable at 1 and 10 mg L^{-1}, where alkanes were significantly more degraded by 41% and 17% respectfully (t = 3.2-6.0, $p < 0.05$). A similar pattern was observed with PAHs, whereby at day 14, a significant decrease in PAH concentration was seen within microcosms treated with non-dispersed oil at concentrations of 1, 10 and 100 mg L^{-1} (31-59% degradation; t = 3.0-11.3, p < 0.053) (Figure 1) but not at 1000 mg L^{-1} (13% degradation, p = ns). The addition of dispersant resulted in a significant reduction in PAH concentration across all oil concentrations (46-70% degradation; t = 5.5-10.2, $p < 0.05$) and by day 14, PAH concentrations were significantly lower within dispersed oil at 1, 100 and 1000 mg L^{-1} when compared to non-dispersed microcosms (t = 2.6-4.5, p < 0.05; 18-33% further degradation). In dispersed microcosms at 1 mg L^{-1} oil, a significant reduction in PAH concentration was seen as early as day 1 when compared to non-dispersed microcosms (t = 2.69, $p < 0.05$). A significantly greater percentage degradation of smaller *n*-Alkanes (C_{11}-C_{20}) occurred within oil concentrations of 1, 10 and 100 mg L^{-1} (dispersed and non-dispersed) when compared to 1000 mg L^{-1} (Figure 2). The branched alkanes

pristane and phytane were significantly more degraded in percentage terms in oil treatments of 1 mg L^{-1} (dispersed and non-dispersed) when compared to treatments of 1000 mg L^{-1}. Even larger *n*-alkanes in the size range, C_{35}-C_{38}, were significantly degraded at an oil concentration of 1 mg L^{-1} (dispersed and non-dispersed) in contrast to higher concentrations where no significant degradation occurred. A targeted analysis of certain PAH analytes was also caried out to compare degradation across a range of different structures and sizes of PAHs.

Naphthalene, fluorene, phenanthrene, anthracene and pyrene were significantly more degraded in percentage terms at 1 mg L^{-1} oil, compared to 1000 mg L^{-1} (dispersed and non-dispersed) (Figure 2). In oil concentrations of 10 mg L^{-1}, naphthalene, fluorene, phenanthrene and pyrene all showed significantly more percentage biodegradation when compared to concentrations of 100 mg L^{-1}, and naphthalenes and fluorenes had significantly more percentage degradation when compared to 1000 mg L^{-1}. The percentage degradation of larger PAHs (4-rings and above) was much lower across all oil concentrations, however at 1 mg L^{-1} these larger PAH compounds were significantly more degraded when compared to 1000 mg L^{-1}. Whilst experiments with higher oil concentrations may show minimal hydrocarbon biodegradation in percentage terms, the actual bulk hydrocarbon content degraded, and rates of hydrocarbon degradation may of course be significantly higher. With an increase in concentration, the degradation rates of dispersed oil increased linearly from 1 – 1000 mg L^{-1}, in contrast to non-dispersed oil where degradation rates were only linear from 1 to 100 mg L^{-1}, and then plateaued at 1000 mg L^{-1} (Figure 3). A similar response was also observed with PAH degradation rates.

There was no significant increase in the abundance of the bacterial 16S rRNA gene in treatments with or without dispersant between day 0 and 14 ($p = 0.07$; data not shown). By day 7, there was significant dissimilarity in the community composition between dispersed and non-dispersed microcosms ($F = 2.68$, $p = 0.023$). However by day 14, no difference was

observed and there was no significant dissimilarity in communities associated with different oil concentrations by day 14 (Figure 4).

Colwellia was the most abundant genus in the seawater (approximately 75% of all reads), the relative abundance significantly declined by day 1 in all oiled treatments except at 1000 mg L^{-1} in the non-dispersed microcosms ($F = 24.575$, $p < 0.001$) and seawater controls until day 7 (Figure 5). By day 14, *Pacificibacter*, *Aliiroseovarius* and *Roseovarius* became dominant (approximately 24%, 11% and 9% of the community), significantly increasing in abundance across all treatments ($F = 21.8$-32.4, $p < 0.001$). The relative abundance of 16S rRNA genes from known obligate hydrocarbonoclastic bacteria (a group of marine bacteria often associated with oil pollution that are highly specialist hydrocarbon degraders) (Yakimov et al 2007) varied between replicates, and generally small increases were seen in non-dispersed oil treatments compared with dispersed oil. *Alcanivorax* remained at low relative abundance, increasing (non-significantly) by day 14 in the 10 and 100 mg L^{-1} oil treatments ($F = 0.62$-0.8 p = ns) (Figure 6). *Cycloclasticus* significantly increased in relative abundance over time in non-dispersed treatments of 100 mg L^{-1} concentrations ($Z = 3.3$, $p < 0.001$), from undetectable to 4.9% relative abundance. *Thalassolituus* increased in relative abundance by day 14 in 100 and 1000 mg L^{-1} concentrations ($Z = 0.001$-$.9$, p = ns). *Oleibacter* (sequences assigned to *Oleibacter* in this study, represent ASV sequences highly similar to *Oleibacter marinus* strains, that were recently transferred to the genus *Thalassolitus* under the new name *T. maritimus* (Dong et al. 2022)) increased in relative abundance by day 14 in treatments of 100 mg L^{-1} concentration, at 11.9% of the community ($Z = 3.95$, $p < 0.001$).

At day 7, *Alteromonadaceae* significantly increased in relative abundance in dispersed oil at 1 and 10 mg L^{-1} and dispersant only treatments when compared to 100 and 1000 mg L^{-1} ($z = 2.5$-3, $p < 0.05$) (Figure 7) and by day 14 this family was significantly more abundant in all dispersed oil concentrations ($z = 2.68$-5.01, $p < 0.05$). Unassigned *Oceanospirillaceae*

significantly increased in relative abundance at day 14 ($z = 7.56$, $p < 0.05$) and when oil was dispersed, a significant increase in abundance was observed at oil concentrations of 1, 10 and 100 mg L^{-1} ($z = 19.5$-25.2, $p < 0.05$), with no increase in oil concentrations of 1000 mg L^{-1} and seawater controls. *Cellulophaga* had significantly higher relative abundance by day 7 and 14 in microcosm treated with dispersants at 1 and 10 mg L^{-1} oil ($z = 3.6$-4.0, $p < 0.05$) (Figure 7). *Oceanicola* significantly increased across all oil treatments ($p < 0.05$) and particularly with dispersant addition at 1, 10 and 100 mg L^{-1} oil ($z = 2.7$-3.1, $p < 0.05$). *Sulfitobacter* increased over time across all treatments, however this was not significant.

DISCUSSION AND CONCLUSION

Initial oil concentration had a clear impact on the proportion of oil degraded over 14 days, with a higher percentage of measured alkanes and PAHs being degraded in the lower concentrations of 1 and 10 mg L^{-1} compared to 100 and 1000 mg L^{-1}. However, when considering the mass of hydrocarbons degraded over time, the degradation rates of both alkanes and PAHs increased linearly from concentrations of 1 to 100 mg L^{-1}, in all treatments. There was clear inhibition of the rate of both alkane and PAH degradation at 1000 mg L^{-1} when oil was not dispersed, but the rate increases remained linear when dispersant was added. Observations made by Prince *et al*. (2017) at concentrations 2.5, 25, 250 and 2500 mg L^{-1} indicated a similar inhibition of degradation rates at the concentrations of 2500 mg L^{-1}, suggesting that oil may inhibit degradation rates at concentrations above 1000 mg L^{-1}, or even lower when oil is not dispersed. Therefore, oil concentration must be accounted for when using closed-system degradation experiments, as measurements in open water slicks and dispersed oil plumes would typically be well below these concentrations (Lee *et al*., 2013). The addition of dispersants had an effect at the earliest sampling point of day 1, increasing the biodegradation of alkanes in initial oil concentrations of 1 and 10 mg L^{-1}, and PAHs in treatments of 1 mg L^{-1} in comparison to the non-dispersed floating oil. By day 14, the addition

of dispersant had a positive effect on biodegradation in oil treatments 100 and 1000 mg L^{-1} highlighting the benefits to bacteria when oil is in small droplets with an increased surface area to volume ratio caused by the addition of dispersants and biosurfactants that are produced by hydrocarbon degrading bacteria (McGenity *et al*., 2012; Godfrin *et* al., 2018; McGenity and Laissue, 2023). Prior studies using concentrations of 100-1000 mg L^{-1} found similar results (Baelum *et al*., 2012, Tremblay *et al*., 2017, Thomas *et al*., 2021). Although biodegradation of dispersed oil at concentrations of 1000 mg L^{-1} showed a delayed response compared to lower concentrations, degradation was still significantly enhanced compared to that of non-dispersed oil. This enhancement with dispersant use has also been observed at higher concentrations of 2000 mg L^{-1}, (Zahed *et al*., 2010). However percentage loss of hydrocarbons between dispersed and non-dispersed oil was lower than that of the present study, being 7% (day 45), whereas by day 14 during this study, hydrocarbon loss increased by 41% and 17% when dispersed at 1 and 10 mg L^{-1}, respectively. This may have been due to the higher starting nitrate concentration in the present study. Biodegradation was enhanced by dispersant use to a greater extent at lower oil concentrations of 1 mg L^{-1} than previously observed (Baelum *et al*., 2012).

Bacterial communities need nutrients such as nitrogen (N) and phosphorous (P) to support growth, being fundamental components of biomolecules such as proteins and nucleic acids. Compared to terrestrial environments, open water marine systems are typically N and P limited, especially during a spill event, where the high carbon content of hydrocarbons, significantly raises the C:N:P ratio , and it is well documented that biodegradation of oil is severely inhibited by inorganic nutrient limitations (Vergeynst *et al*., 2018; Imron *et al*., 2020). During this study, initial nutrient concentrations were 50 µM nitrate and 1 µM phosphate (which were the main sources of inorganic N and P in the seawater), and microcosms rapidly became P-limited by day 1 and N-limited by day 14. By day 3 ammonium had become the main inorganic N source, likely released via decomposing organic matter (e.g. from dead algae

and bacteria within the dark-incubated microcosms). This rapidly depleted by day 14. Previously, exponential growth of microbial communities was observed in oil contaminated seawater at concentrations of 30 μM (N) and 1.8 μM (P) and above, whilst growth became limited at 5.5 μM (N) and 0.13 μM (P) (Strynar *et al*., 1999). Here, bacterial abundance was consistent over 14 days. This aligns with previous findings as nutrient concentrations were not high enough to support exponential growth, whilst the community composition changed dramatically (Strynar *et al*., 1999). Significant degradation of hydrocarbons occurred across all treatments by day 7 and only ceased once the microcosms became deficient in inorganic N and P. During this study, even at high concentrations of oil (100, 1000 mg L^{-1}) with the addition of dispersants, nutrient concentrations as low as <5 μM (N) and <0.1 μM (P) were capable of facilitating hydrocarbon biodegradation.

Percentage degradation decreased with an increase in chain length in *n*-alkanes $C_{10\text{-}18}$ at an oil concentration of 1 mg L^{-1}, in which *n*-$C_{10\text{-}12}$ were almost completely degraded (98%, 95% and 88% degradation) clearly showing chain length to have an impact on degradation rates (Setti *et al*., 1995). This percentage degradation of *n*-$C_{10\text{-}18}$ decreased with an increase in oil concentration. There was very little degradation of *n*-$C_{35\text{-}38}$ regardless of the initial oil concentration. It has been well documented that the structural property of individual hydrocarbon constituents, determines the degree and rate of degradation, showing an inverse relationship between degradability and molecular weight, with higher complexity and solubility reducing degradability (Setti *et al*., 1993; Mohanty and Mukherji, 2008). The reduction in degradability of higher weight hydrocarbons may coincide with the limiting nutrient resources. Lower molecular weight n-alkanes (n-$C_{11\text{-}13}$) were significantly more prone to biodegradation than larger n-alkanes and with limiting nutrient resources hydrocarbon-degrading bacteria cannot 'move up' the chain lengths, (as seen at 1 mg L^{-1}) as they run out of N and P resource (Ławniczak *et al*., 2020). This becomes a greater issue in the higher

concentrations as C:N and C:P increases 10-fold with each increase in oil concentration. Therefore, at higher oil concentrations, biodegradation is largely limited to a few of the smaller hydrocarbons as there is a greater mass of the more readily degradable fractions. This was also observed in PAHs, with naphthalene being considerably more degraded regardless of oil concentrations, whilst larger PAHs showed little degradation. Similar results were observed by Bacosa *et al*. (2021) who saw biodegradation of individual alkanes decrease with an increased oil concentration in a nutrient limited environment $N < 1$ μM and $P < 0.6$ μM by day 12.

Colwellia dominated communities, averaging 79% across all treatments. These generally psychrophilic or psychrotolerant bacteria are ubiquitous in cold marine environments, between -6ºC and 19ºC (Bauvois *et* al., 2013). Some *Colwellia* species are capable of hydrocarbon degradation, (Methé *et al*., 2005), although *Colwellia psychrerythraea* (26% of *Colwellia* ASVs) is currently not reported to be a hydrocarbon degrading species (Mason *et al*., 2014). The initial dominance of *Colwellia* spp may be due to the initially nutrient rich, cold waters (7ºC). By day 14, *Pacificibacter*, *Roseovarius* and *Aliiroseovarius* became three dominating genera, coinciding with a decrease in nutrient availability. This may explain the distinct similarities between crude oil treatments and seawater controls, with nutrient availability having the most noticeable impact on community composition (Nõlvak *et al*., 2021). OHCBs *Alcanivorax, Cycloclasticus, Oleibacter* and *Thalassolituus* did not show exponential growth even in the presence of hydrocarbons. Even so, OHCB's were detectable at higher abundances in non – dispersed treatments, notably due to their capacity to produce biosurfactants, increasing hydrocarbon availability naturally (Ron and Rosenberg, 2002). This is in contrast to previous work and real-world spills that saw significant growth in OHCB's (Dubinsky *et al*., 2013; Cappello *et al*., 2007; Thomas *et al*., 2020). However, additional nutrient amendments were added/available in the aforementioned studies influencing microbial growth/succession via biostimulation (Yu *et al*., 2011). Other experiments however, found

Pseudoalteromonas spp to significantly increase in abundance in North Sea mesocosms when oiled, which may have dominated over OHCBs (Chronopoulou *et al*.,2015). During the present study, *Alteromonadaceae* was dominant at day 7 (average 15%), which was quickly succeeded by *Oceanospirillaceae*, by day 14 (average 7%). These two families have been reported to exponential increase in abundance, in the presence of marine oil pollution, namely during the Deepwater Horizon spill (Dubinsky *et al*., 2013; Bacosa *et al*., 2018; Miller *et al*., 2019). The majority of *Alteromonadaceae* (ASV 1) (98%), was made up of *Glaciecola amylolytica* (99.06% identity) (Xiao *et al*., 2019). Another *Glaciecola* Strain (NS 168) (93.91% identity to ASV 1) has been recorded to be capable of hydrocarbon degradation with a preference to alkane degradation (Chronopoulou *et al*., 2015). However, current knowledge of *Glaciecola amylolytica* has yet to address the specifics of their degradation potential. The majority of unassigned *Oceanospirillaceae* (99%), was made up of *Thalassolituus* ASVs (95.6-99.7% identity), suggesting their involvement in aliphatic hydrocarbon degradation between day 7 and 14. *Cellulophaga* increased in abundance at day 7 in oil concentrations of 1 and 10 mg L^{-1} when dispersed. Previously shown to be present around contaminated sites they are prolific producers of biosurfactants, thus facilitating the availability of hydrocarbons within contaminated communities (Chhatre *et al*., 1996; Rizzo *et al*., 2013).

Overall different oil concentrations (1, 10, 100 and 1000 mg L^{-1}) and the addition of dispersants did not have a major impact to bacterial community composition. The subtle differences in communities are likely related to a combination of factors, such as the susceptibility of certain bacteria to increasing hydrocarbon concentrations, nutrient availability stalling successional changes at higher concentrations, and the changing profile of available hydrocarbons. Similar results were noted in Jung *et al*. (2010), in which impacts to heterotrophic bacterial communities were only noted at concentrations over 1000 mg L^{-1} (sublethal levels). This suggests that initial oil concentrations introduced to a system will not

have a major impact to bacterial communities below "sublethal levels". Even so, inhibitory effects were noted in *Alteromonadaceae* and *Cellulophaga* at day 7 and *Oceanospirillaceae* at day 14, which exhibited high relative abundances at concentrations of 1 and 10 mg L^{-1} and no noticeable presence at concentrations of 1000 mg L^{-1} when dispersed. It has previously been shown that the production of intermediate metabolic products during biodegradation in high concentrations can potentially inhibit the growth of hydrocarbon degraders such as *Alcanivorax* and *Polaromonas naphthalenivorans* (Pumphrey and Madsen, 2007; Bacosa *et al*., 2021) with the possibility of inhibiting other genera. *Alteromonadaceae and Oceanospirillaceae* increased in the presence of dispersants. *Alteromonadaceae* being relatively more abundant at day 7 at concentrations of 1 and 10 mg L^{-1}, whereas *Oceanospirillaceae* increased in relative abundance at concentrations 1, 10 and 100 mg L^{-1} by day 14. Previous work has also noted similar patterns in both *Alteromonadaceae* and *Oceanospirillaceae* in relative abundance with dispersant use by day 15 (Bacosa *et al*., 2018). This may also indicate a delayed response in bacterial succession associated with initial oil concentrations, as by day 14, the relative abundance of *Alteromonadaceae* at concentrations of 100 and 1000 mg L^{-1} increased, whilst in contrast they had already increased in abundance at concentrations of 1 and 10 mg L^{-1} by day 7. Microbial community succession has been shown to be influenced by dispersants, as availability of oil increases, the rate of biodegradation increases, thus the rate of succession increases (Baelum *et al*., 2012; Thomas *et al*., 2021). However, initial concentrations of oil within a system may also influence succession due to potential inhibitory effects of higher hydrocarbon concentrations (Bacosa *et al*., 2021).

Higher oil concentrations were shown to inhibit the growth of potential hydrocarbon degrading bacteria, which slowed bacterial succession. This in turn inhibited biodegradation at higher concentrations of oil and the the efficiency of dispersant use on the enhancement of hydrocarbon degradation. Even so significant degradation occurred across all oil

concentrations. This study also highlights that significant degradation is still possible at in situ open water nutrient concentrations, even at higher oil concentrations with the addition of dispersants, although this does become limiting as the system becomes N and P deficient. This is noted as a limitation of this and many closed system studies. Overall initial oil concentrations should be considered when performing closed system biodegradation experiments. Concentrations of 10 mg L^{-1} and lower are recommended to accurately depict concentrations found in situ when oil is dispersed. Future experiments should consider simulating dilution of oil over time to more closely mimic what happens to dispersed oil at sea, where the concentration would continue to dilute over time, with each oil droplet, a hotspot of bacterial hydrocarbon-degrading activity, being exposed to further nutrient enrichment as they move through the water column.

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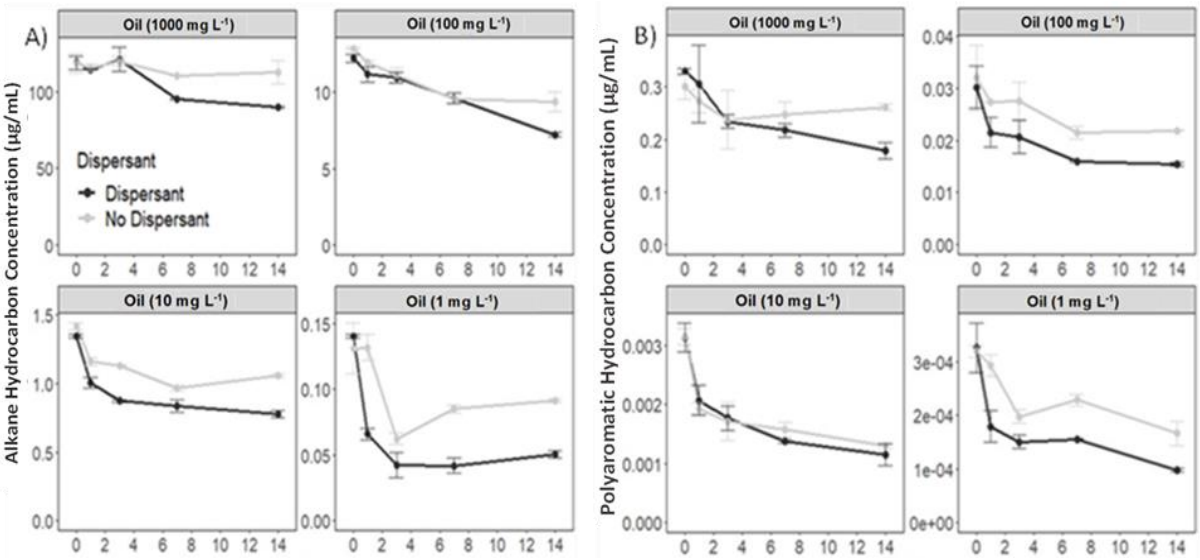

Figure 1 (A) Total alkane and (B) polyaromatic hydrocarbon concentration collected from 20 ml microcosms of North Sea seawater collected of the Mersea Coast, Essex, UK, exposed to Norwegian Geochemical Standard North Sea Oil at a range of concentrations (1 – 1000 mg L^{-1}) (NGS NSO-1), with and without the presence of chemical dispersants (Slickgone NS), (± SE, n = 3).

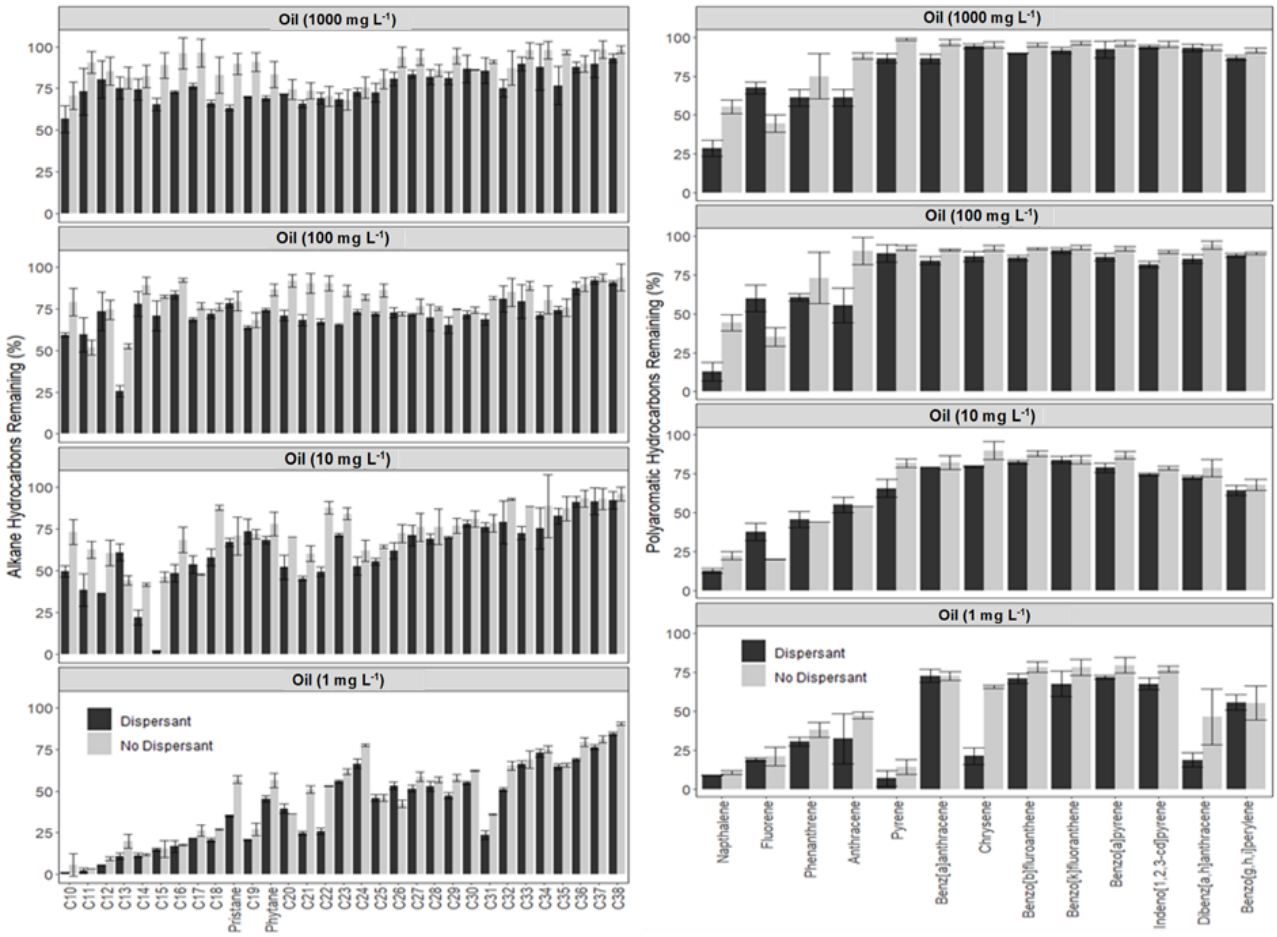

Figure 2: Percentage remaining alkane hydrocarbon and polyaromatic hydrocarbon compounds within 20 ml North Sea seawater microcosms, with and without chemical dispersants (Slickgone NS) at varying Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) concentrations (1– 1000 mg L^{-1}) by day 14 (± SE, n = 3).

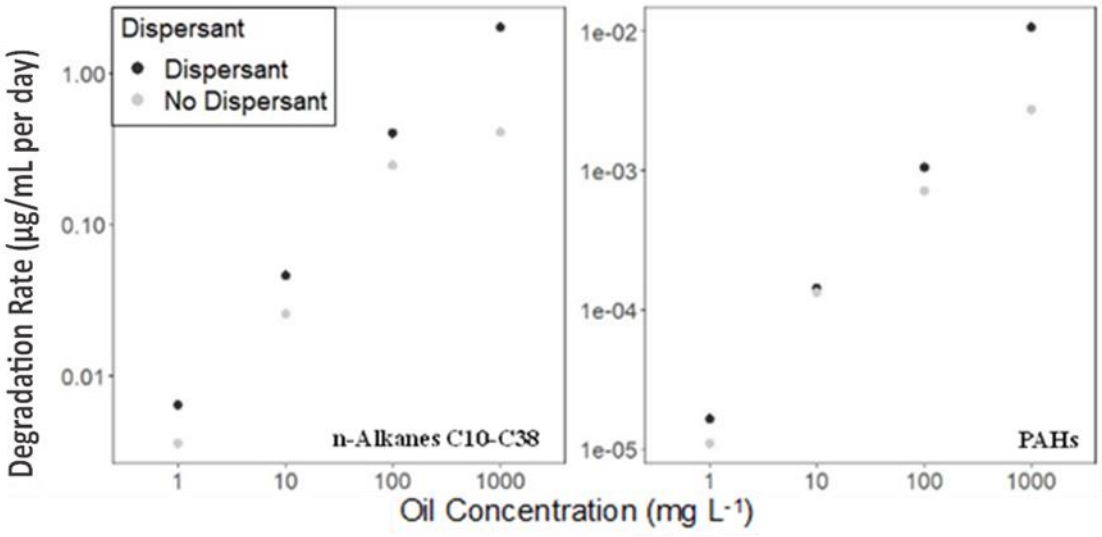

Figure 3: Alkane and polyaromatic hydrocarbon degradation rates in natural North Sea Seawater over 14 days across a range of Norwegian Geochemical Standard North Sea Oil (NGS NSO-1) concentrations (1 – 1000 mg L^{-1}) with and without chemical dispersant (Slickgone NS) treatment (± SE, n = 3).

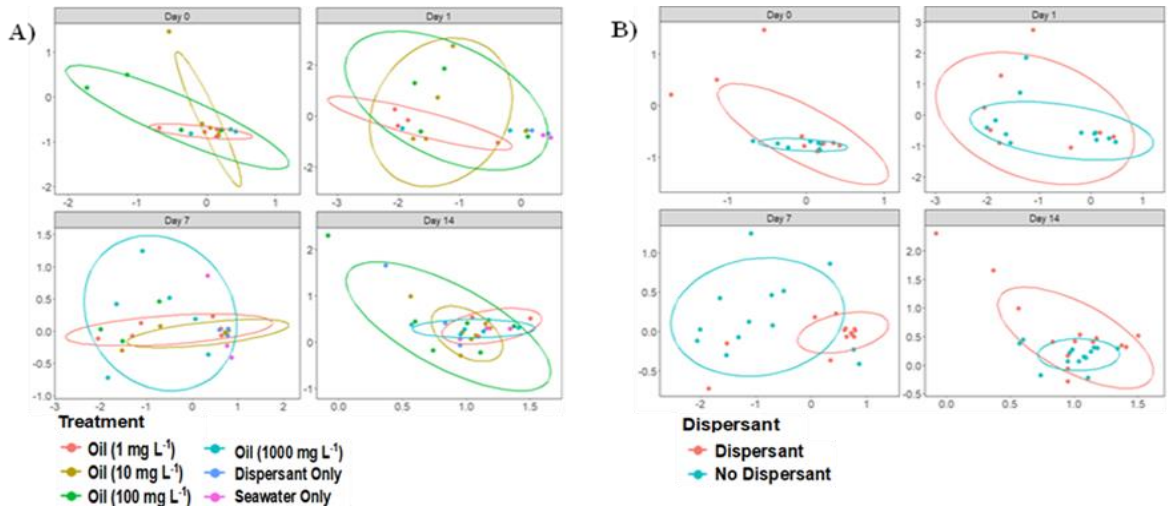

Figure 4 nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity, based on ASV (amplicon sequence variants) of bacterial 16S rRNA genes at varying concentrations of Norwegian Geochemical Standard North Sea Oil (1 - 1000 mg L^{-1}) with and without dispersants (Slickgone NS) over a period of 14 days, within microcosms using seawater samples collected off the coast of Mersea Island, Essex, UK. A) Community dissimilarity between treatments, B) Community dissimilarity between dispersant use (± SE, n = 3).

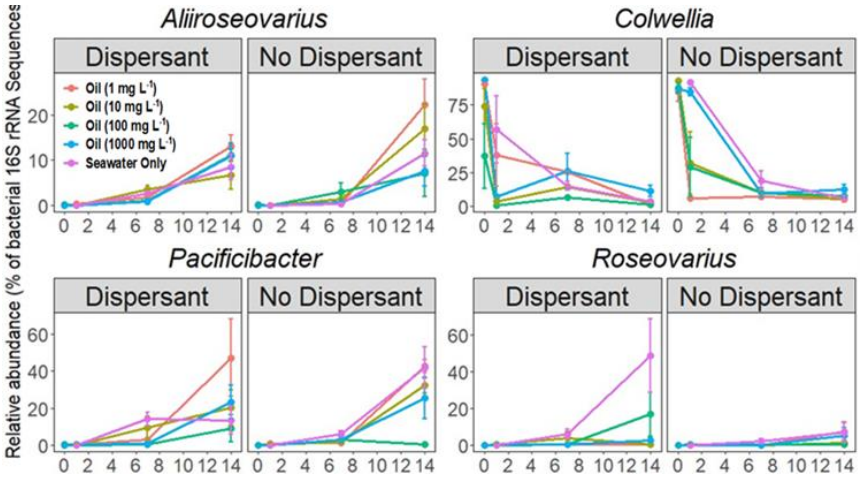

Figure 5 Highest relative abundance genera associated with marine oil pollution at varying concentrations of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

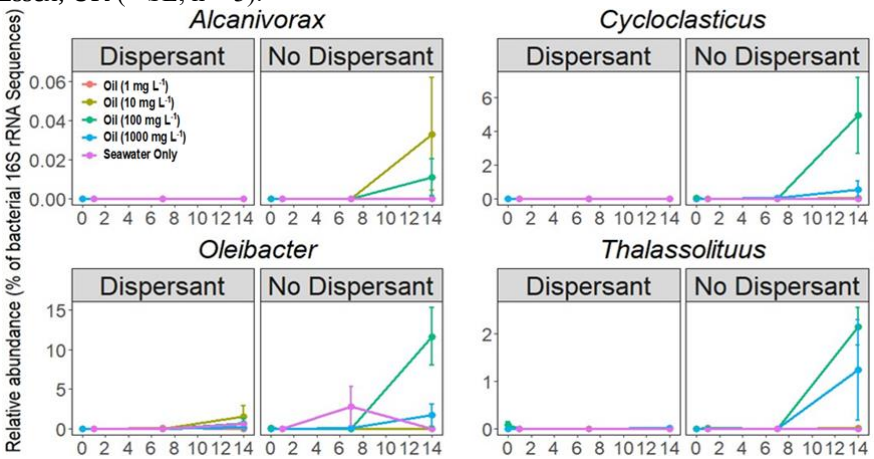

Figure 6 Relative abundance of genera that are known to include hydrocarbon degraders after the addition to seawater of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without the presence of chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

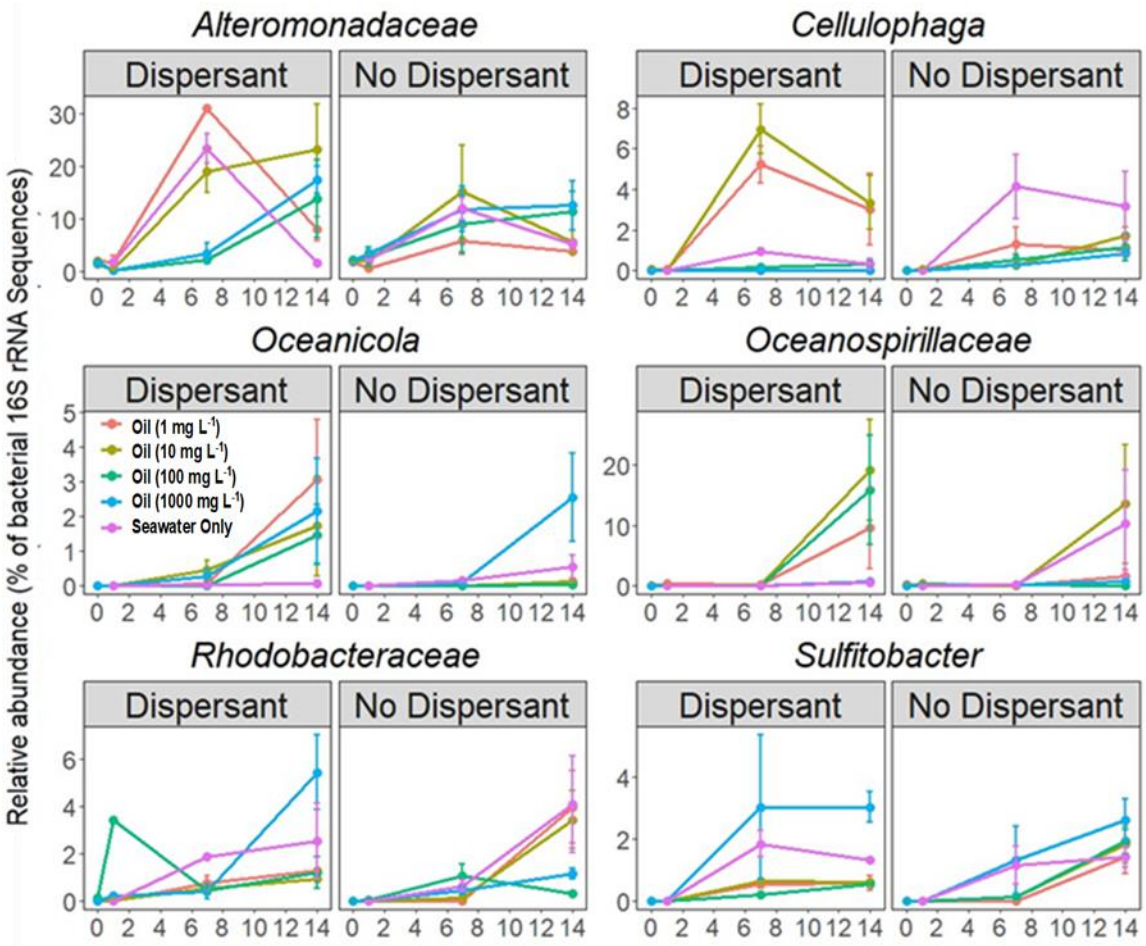

Figure 7 Relative abundance of other notable genera at varying oil concentrations of Norwegian Geochemical Standard North Sea Oil (1 – 1000 mg L^{-1}), with and without the presence of chemical dispersant (Slickgone NS). Collected over a 14-day period from microcosms containing natural seawater collected off the Mersea Coast, Essex, UK (± SE, n = 3).

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