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Effects of the 2023 Poole Harbour oil spill on sediment bacterial communities and ecosystem functioning

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ABSTRACT

In March 2023, approximately 27 t of fluid from an oil and gas reservoir (containing approximately 85 % water and 15 % crude oil spilt from a fractured pipeline beneath Ower Bay creek, entering Poole Harbour (Dorset, UK). This event provided a unique opportunity to investigate the impacts of hydrocarbon contamination on microbial communities in-situ in a temperate coastal, shallow, fine sediment environment. Our aims were to quantify hydrocarbon concentrations (via gas chromatography mass spectrometry (GC–MS)) and effects on microbial community structure and functional potential (via metagenomic sequencing) to understand the capacity for microbial biodegradation across the impacted region. Hydrocarbon contamination was localised to the Spill Site (approximately an area of 1500 m^2) at the head of the creek, with minimal impact at the Mid Point (164 m from the Spill Site) and End Point (387 m from the Spill Site) and with no indication of contamination at Brownsea Island located in the heart of the harbour. By October 2023, *n*-alkane and 4–5 ring PAH concentrations had declined to background levels, highlighting the combined effects of the remediation response and natural hydrocarbon biodegradation at the Spill Site. Clear changes in bacterial community structure were observed in the seven months following the spill, with notable hydrocarbon-degrading bacteria i.e. *Anaerolinea, Thiobacillus* and *Dechloromonas* favouring the Spill Site, suggesting a significant increase in anaerobic biodegradation occurred as a result of significant increase in *assA* (anaerobic alkylsuccinate synthase)*, abcA* (anaerobic benzene carboxylase) and *ahyA* (anaerobic alkane hydroxylase) genes. Overall, 24 alkane and aromatic hydrocarbon degradation genes, from both aerobic and anaerobic degradation pathways, were identified from contigs throughout the study site, being present within 48 out of 221 Metagenome-Assembled Genomes (MAGs), highlighting the sites capacity for hydrocarbon biodegradation under both aerobic and anaerobic conditions.

1. Introduction

The amount of oil entering the marine environment globally from pipelines and vessel spills was approximately 7,299,000 kg y^{-1} between 1990 and 1999, with a much lower amount of 588,000 kg y^{-1} between 2010 and 2019 (National Academies of Science, 2022), following improvements in safety standards. However, as the production of crude oil is forecast to grow to above 105 million barrels per day by the end of 2025 (U.S. Energy Information Administration, 2024), the risk of marine oil spills from tankers and pipelines remains. On the 26th of March 2023, approximately 200 barrels (~27 t) of reservoir fluid (comprising 15 % light sweet crude oil (approximately 40.2° API; 0.12 % sulphur contents; viscosity at 40 °C = 4.4 cSt (Supplementary Material: Table S1)) and 85 % water) leaked from a fractured pipeline at Wytch Farm oil field beneath Ower Bay creek, which enters Poole Harbour (Dorset, UK) (PMCC, 2024; Hatch, 2024). The harbour is an important Site of Special

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Scientific Interest (SSSI) and a Special Protection Area (SPA), and contains numerous islands, including Brownsea Island, which forms part of the Purbeck National Nature Reserve and is recognised as an internationally important area for wildlife. A rapid response to the spill was conducted by Oil Spill Response Ltd. (www.osrl.com) and Adler and Allan Environmental Risk Consultancy, in which 653 SCAT (Shoreline Cleanup Assessment Technique) surveys (covering 199 km^2) were performed, along with aerial imagery surveys indicating initial petroleum hydrocarbon contamination throughout Poole Harbour. Absorbent booms were deployed within 24 h of the spill at the Mid Point of the creek and the End Point of the creek at the entry point into Poole Harbour, with ongoing monitoring of the site. In August 2023, a bubble barrier was also implemented to prevent oil that had migrated to the surface of the sediment from being carried out on high tides and contain the oil to the localised spill area. Further remediation from November 2023 to January 2024, involved the removal of sediment and dead plant matter to allow for habitat recovery. From the time of the spill up to February 2024, the multiagency PREMIAM (Pollution Response in Emergencies: Marine Impact Assessment and Monitoring) Monitoring Coordination Cell (PMCC) took action to develop and implement a monitoring plan and issued its final report in March 2024 (PMCC, 2024). Poole harbour is Europe's largest natural harbour and very shallow (average depth = 0.5 m; maximum depth in shipping channels = 7.5 m) with substantial commercial and recreational boating activities as sources of hydrocarbons (Underhill-Day, 2006). Whilst data is limited, sediment polyaromatic hydrocarbons (PAHs) have previously been measured at between 91 and 166 $\mu g\ kg^{-1}$ (Law et al., 1997; Woodhead et al., 1999).

Marine oil pollution can alter sediment microbial community composition, often resulting in decreases in community diversity and selection for hydrocarbon-degrading bacteria (Yakimov et al., 2005; McGenity et al., 2012). This can include increases in the obligate hydrocarbonoclastic bacteria (OHCB), known to use almost exclusively hydrocarbons and their derivatives as a source of carbon and energy (Yakimov et al., 2007), such as alkane-degrading *Alcanivorax* and *Thalassolituus* and polyaromatic hydrocarbon (PAH)-degrading *Cycloclasticus* (Yakimov et al., 1998; McKew et al., 2007a; McKew et al., 2007b; Rubin-Blum et al., 2017). There are also many more metabolically diverse genera that have been evidenced to contain species capable of hydrocarbon degradation, such as *Marinobacter*, *Pseudomonas* and *Colwellia,* resulting in at least 300 bacterial genera that contain some species that can grow on different hydrocarbons as a sole or major carbon source (Prince et al., 2019). Following oil spills, such as the 1989 Exxon Valdez, 1996 Sea Empress (Etkin, 1999), 2017 Agia Zoni II (Parinos et al., 2019; Thomas et al., 2020) and 2018 Sanchi (Chen et al., 2020) spills, there has been an emphasis on understanding oil-induced effects on sediment and seawater microbial community composition and the hydrocarbon biodegradation capabilities of its members (Lamendella et al., 2014). However, a potential limitation of many studies is that they are conducted as ex-situ laboratory experiments (e.g., Alonso-Gutiérrez et al., 2009; Jung et al., 2010; Stauffert et al., 2013; Morais et al., 2016; Engel et al., 2017) due to the difficulties of performing in-situ research (e.g. access, permissions, logistics, cost), so results are often difficult to extrapolate to the field (Head et al., 2006). When oil-contaminated sediments have been studied in-situ, such as after the Agia Zoni Spill, selection for OHCB has been shown, with a higher relative abundance in *Marinobacter hydrocarbonoclasticus, Thalassolituus and Oleiphilus* compared to uncontaminated sites and a legacy of *Alcanivorax* and *Cycloclasticus* many months later, even after hydrocarbon concentrations returned to undetectable levels (Thomas et al., 2020). The microbial response can differ between spills. For example, *Acinetobacter* dominated oiled sediments following the 2017 Ennore spill, representing 52 % of total bacterial community (Neethu et al., 2019). Differences may be attributed to many factors such as the composition of oil, and varying environmental conditions, such as temperature, availability of O_2 (required for aerobic respiration but also the oxidant for oxygenase enzymes that activate hydrocarbons in the initial step of biodegradation), or N and P that often become limiting nutrients following the large influx of carbon from oil (Atlas and Bartha, 1972; Head et al., 2006). The Poole Harbour incident provided a unique opportunity to perform an in-situ investigation on the effects of an oil spill on microbial community structure and functional potential in temperate coastal sediments. The aim of this study was to quantify the extent of petroleum hydrocarbon pollution in real time and its impact on the in-situ sediment bacterial communities, whilst also investigating their capacity for biodegradation via shotgun metagenomic analysis and quantification of genes associated with aerobic and anaerobic hydrocarbon degradation pathways.

2. Methods

2.1. Sample site and sample collection

Surface sediment samples (top 0–5 cm) were collected from Ower Bay creek, located near the Wytch Farm oil field, run by Perenco UK Ltd. (www.perenco.com), which included the Spill Site at the head of the creek, Mid Point (164 m from the Spill Site) and End Point (387 m from the Spill Site) which is the point of entry into Poole Harbour (Fig. 1). At the Mid Point and End Point, sampling took place behind the absorbent booms that were deployed as part of the spill response. The northeastern coast of Brownsea Island was also sampled (Fig. 1), which had evidence of oil sheens from initial surveys. This sample programme commenced on the 14th June, 80 days after the initial spill, followed by subsequent sampling on the 12th July, 30th August and 18th October 2023. Control sites for Brownsea Island (Brownsea Control) and Corfe River (Control Creek) were also sampled for comparison (Fig. 1), as these sites had showed no observational evidence of oiling during initial SCAT surveys. All sediment samples were placed in sterile tubes and immediately snap-frozen (−150 °C on site) using a CryoShipper (MVE Biological Solutions). Samples were collected in triplicate (1–3 m apart) at each site and each time point (Fig. 1C). The Control Creek was not accessible during the first sampling date on the 14th June 2023 whilst awaiting landowner access permission. Additional sediment samples were taken at a single timepoint (18th April 2023, 24 days post-spill) from a range of sites in the wider Harbour area by CEFAS as part of the Premiam Monitoring Coordination Cell post-spill monitoring (Fig. 1A, C) and were frozen at −20 °C for further analysis.

2.2. Hydrocarbon analysis

Sediment samples (2 g) were dried by mixing with 2 g of sodium sulphate, and hydrocarbons were then extracted by adding 12 ml of extraction solvent (Hexane: Dichloromethane 1:1 ratio) and shaking for 24 hours at 1500 rpm at room temperature on a Multi Reax orbital shaker (Heidolph). Deuterated alkanes and polyaromatic hydrocarbon (PAH) internal standards were added to the solvent prior to extraction at 0.04 μg/ml (Supplementary Material: Table S2). Quantification of hydrocarbons was performed using a Nexis GC-2030/CMS-TQ8040 NX GC-QqQ-MS/MS (Shimadzu), targeting *n*-alkanes n-$C_{10-40,}$ and branched alkanes, pristane and phytane, and a selection of two- to six-ring PAHs (Supplementary Material: Table S2) as previously described (Coulon et al., 2007). External standard calibration curves (six points over a range of 0–5 μg/ml) were produced using C8-C40 Alkanes Calibration Standard (Merck), and QTM PAH Mix (Sigma) calibration standard, mixed with an additional range of two- to six-ring PAHs (Supplementary Material: Table S2). A hexane blank was analysed every 25 samples for quality control. For quality control and to ensure accurate quantification, all external standard calibration curves (six points over a range of 0–5 μg/ml) were produced immediately prior to analysing samples, using Certified Reference Materials (CRM), C8-C40 Alkanes Calibration Standard (Merck) and QTM PAH Mix (Sigma) calibration standards, mixed with an additional range of two- to six-ring PAHs (Supplementary

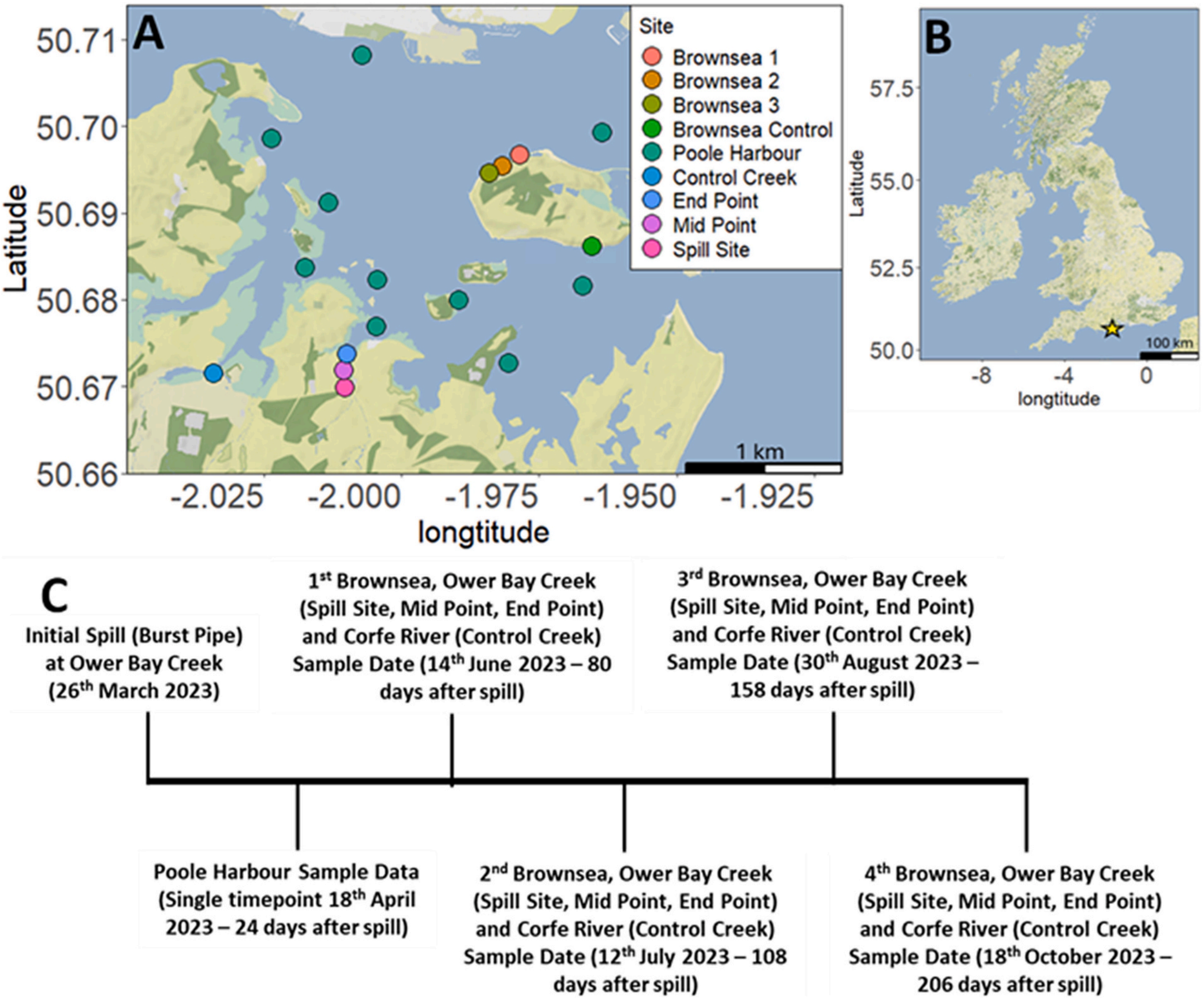

Fig. 1. A. Sample sites across Poole Harbour, Dorset, United Kingdom following the Poole Harbour Oil Spill in March 2023. Harbour samples (represented by green dots) were analysed from a single time point (18th April 2023). All other samples were analysed over a four-month period and included Brownsea Island (Brownsea 1, 2 and 3, Brownsea Control (unoiled)), Ower Bay Creek (End Point, Mid Point and Spill Site) and the Corfe River Control Creek (unoiled). Each circle represents sample location collected in triplicate, on the 14th June, 12th July, 30th August and 18th October 2023. B. Map of the British Isles, the yellow star indicates the location of Poole Harbour. C. Timeline of initial spill and subsequent sample collection. (For interperatation of the references to colour in the figure legend the reader is referred to the web version of this article.)

Material: Table S2). Linearity of all calibration curves were checked and all R2 values were between 0.990 and 0.999. Consistent recovery of the spiked deuterated standards was checked within each sample and calibration checks with the CRMs and procedural blanks were analysed with each sample batch (25 samples) to confirm no instrument drift or contamination from the analytical process. The limit of detection was 0.1 μg kg–1 dry weight sediment for each hydrocarbon compound/class, and the extraction efficiency was measured at 99 %.

2.3. Nutrient analysis of sediments

Sediment samples (10 g) were freeze dried for 24 h in an ALPHA 1–4 LD plus (Christ Freeze Dryers), then 6 g of dried sediment was placed in a 50 ml centrifuge tube with 18 ml of MilliQ ultrapure water and shaken on a Multi Reax orbital shaker (Heidolph) for 15 min at 1000 rpm before centrifugation (30 min at 2875 ×*g*). The supernatant was filtered using a 0.2 μm filter (Millipore) and analysed on a AA3 AutoAnalyzer (Seal) (for nitrate, nitrite, ammonia and phosphate concentrations) and a Dionex ICS-6000 HPIC (Thermo Fisher) for sulphate concentrations.

2.4. DNA extraction and qPCR analysis

DNA was extracted from 250 mg of sediment per sample using the DNeasy PowerSoil Pro Kit (Qiagen), following the manufacturer's instructions. Bacterial 16S rRNA gene abundance was quantified via qPCR with primers 341f: CCTACGGGNGGCWGCAG and 805R: GACTACHVGGGTATCTAATCC (Klindworth et al., 2013) using SensiFAST SYBR No-ROX Kit (Bioline) on a CFX384 Real-Time PCR Detection System (BioRad) using an absolute quantification method against a standard calibration curve (E = 70 %; $R_2 = 0.999$; y-int = 44) of the target gene from 10^2 to 10^9 copies in 20 μl reactions as previously described (McKew and Smith, 2017).

2.5. 16S rRNA gene amplicon library preparation and bioinformatics

Amplicon library sequencing preparation was performed following the 16S Metagenomic Sequencing Library Preparation protocol (Illumina., 2013). Primers used were the same as the qPCR primers for bacterial 16S rRNA amplification, with the addition of Illumina adapter overhang nucleotide sequences (Forward Overhang: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, Reverse Overhang:

5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). PCR products were cleaned using AMPure XP beads (Beckman Coulter). Nextera XT Index Kits v2 (Illumina) were used to add unique combinations of index primers to each PCR product via an 8-cycle PCR, followed by a second clean-up with AMPure XP beads. Gel electrophoresis was performed after each PCR stage for quality control. Quantification of the 16S rRNA gene amplicons was conducted using a FLUOstar Omega microplate reader (BMG LABTECH), following the Quant-iT PicoGreen dsDNA Assay Kit protocol (Thermo Scientific) and then pooled in equimolar concentrations. Final library quantification was then performed using the NEBNext Library Quant Kit for Illumina (New England Biolabs, UK). Sequencing was performed by Novogene (Cambridge, UK) using Illumina NovaSeq PE250.

Sequence data was processed using the Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al., 2022). Singleton ASVs, sequences shorter than 400 bp, sequences containing homopolymers of eight or above bp, and non-target organisms (archaea and chloroplast sequences) were all discarded. Taxonomic assignment was conducted using the Ribosomal Database Project (RDP) Classifier (Callahan, n.d.; Wang et al., 2007) against the RDP Trainset 19. Amplicon library size averaged 526,267 reads per sample. Amplicon sequences have been submitted to the NCBI Sequence Read Archive under the accession number PRJNA1226135.

2.6. Shotgun metagenomic analysis

Shotgun metagenomic sequencing library construction was performed at the Earlham Institute (Norwich Research Park, UK), according to the LITE (Low Input, Transposase Enabled) 2.0 pipeline (Perez-Sepulveda et al., 2021). The DNA library was then sequenced on two lanes of a NovaSeq X Plus PE150, 10B flow cell. Metagenomic sequences have been submitted to the NCBI Sequence Read Archive under the accession number PRJNA1227550.

MetaWRAP (Uritskiy et al., 2018) was used to process raw Illumina sequencing reads. Raw reads were trimmed with Trim-galore v0.4.3 (Krueger, 2015) and any human-derived reads were removed via bmtagger v3.101 (Rotmistrovsky and Agarwala, 2011).

Using the *k*-mer based approach of Kraken 2, metagenomic reads were taxonomically classified (Wood et al., 2019) against a custom Kraken 2 database including complete NCBI RefSeq genomes for bacteria, archaea, fungi, protozoa, plasmids, viruses, human, and a collection of known vectors (UniVec_Core). Bracken (Lu et al., 2017) was then also used to overcome issues with Kraken 2 underestimating the number of reads at lower taxonomic levels. This approach has been shown to provide the most accurate abundance estimations for short reads (Simon et al., 2019; Seppey et al., 2020).

Using SCycDB (Yu et al., 2021), NCycDB (Tu et al., 2019) and MCycDB (Qian et al., 2022) sulphur-cycling, nitrogen-cycling and methane-cycling genes, were identified from metagenomic reads at 100 % sequence identity using DIAMOND v 2.0.4 (Buchfink et al., 2015) with an *E*-value cut-off of 0.0001. Forward and reverse reads were merged and randomly subsampled down to the lowest sample read count.

Co-assembly of the trimmed reads from all samples was then performed with MEGAHIT v1.2.9 (Li et al., 2015). The assembly was formatted to include scaffold length and *k*-mer depth, sorted by length, and contigs shorter than 1000 bp were removed.

Prokka v1.14.6 (Seemann, 2014) was used to functionally annotate contigs. To map processed reads for each sample against the contigs, Bowtie2 was used (Langmead and Salzberg, 2012). The output Sequence Alignment/Map (SAM) file was converted to Binary Alignment Map (BAM) file format and sorted by leftmost alignment coordinate using SAMtools (Li et al., 2009). The MarkDuplicates function in Picard v2.23.3 was used to remove duplicated reads (http://broadinstitute.github.io/picard/). An in-house script was used to search for gene regions within the General Feature Format (GFF) file generated by Prokka. The '*HTSeq*' library htseq-count tool was used to calculate raw counts per gene (Anders et al., 2015). The CANT-HYD (Calgary approach to ANnoTating HYDrocarbon degradation genes) database was then used to extract and characterise functional genes associated with hydrocarbon degradation pathways from the Prokka annotated contigs within all samples, hits with an e-value of $\geq$0.0001 were retained (Khot et al., 2022).

Assembled contigs were binned into three different bin sets within metaWRAP's Binning module (Uritskiy et al., 2018) using MetaBAT (Kang et al., 2015), MetaBAT2 (Kang et al., 2019) and CONCOCT (Alneberg et al., 2014), which were then consolidated into a final bin set using the Bin-refinement module. This resulted in 221 Metagenome-Assembled Genomes (MAGs) estimated to be >50 % complete with <10 % contamination (Uritskiy et al., 2018) (Supplementary Material: Table S5). MAG abundance was quantified using Salmon (Patro et al., 2017). MAGs were taxonomically classified by Taxator-tk (Dröge et al., 2015) against the NCBI nt database (Sayers et al., 2021). To further refine the consensus taxonomy assigned by Taxator-tk, 16S, 5S and 18S rRNA sequences were extracted from MAGs using Barrnap (Seemann, 2015). Extracted sequences were compared to those present in the NCBI nr database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). MAGs were functionally annotated further using Prokka v.1.14.6 (Seemann, 2014) to identify potential functionality. CANT-HYD was again used to determine which MAGs contained hydrocarbon degradation genes, retaining hits with an e-value of $\geq$0.0001 (Khot et al., 2022), highlighting 48 out of the 221 MAGs as containing sequences classified as coding for hydrocarbon degradation gene pathways.

2.7. Statistical analysis

ASV sequence data was rarefied to the lowest read depth (30,405 bp). Diversity indices (Richness, Shannon Diversity and Pielou's Evenness) and community dissimilarity analysis were conducted using the vegan package (Oksanen et al., 2024), which included nMDS (non-metric multidimensional scaling) using the Bray-Curtis dissimilarity index (Bray and Curtis, 1957) with the EnvFit function to fit environmental variables as vectors onto the ordination, calculated via the squared correlation coefficient as a goodness-of-fit statistic (arrow length indicates the strength of the relationship between the environmental factor and the community differences). Significant differences in the community composition were determined via PERMANOVA (Permutational Multivariate Analysis of Variance) (Anderson, 2014). A SIMPER (Similarity Percentage) analysis (Clarke, 1993) within the vegan package was used to identify genera contributing most to Brays-Curtis dissimilarity between communities and differences in functional gene abundance between sample sites. The relative abundance of these identified genera and functional genes were further presented. Data was further analysed using multivariate generalised linear modelling through R (R Core Team, 2022).

3. Results

3.1. The spill impact on hydrocarbon concentrations across poole harbour

All measured alkanes (*n*-alkanes, pristane and phytane) (Supplementary Material: Table S3) and parent and alkylated PAHs (Supplementary Material: Table S4) within sediment samples followed a broadly similar pattern (Figs. 2 and 3), being significantly higher in the Spill Site compared to all other locations in June (80 days after the spill) and increasing to a maximum in late August (158 days after the spill), before returning to background levels by mid-October (206 days after the spill). Specifically, *n*-alkane concentrations were approximately 8–12-fold higher at the Spill Site compared to all other sites in June, July and August ($Z = 3.41–9.67$, $p < 0.01$) (Fig. 2A, B, C). The branched alkanes, pristane and phytane (Fig. 2D) followed a very similar pattern to the *n*-

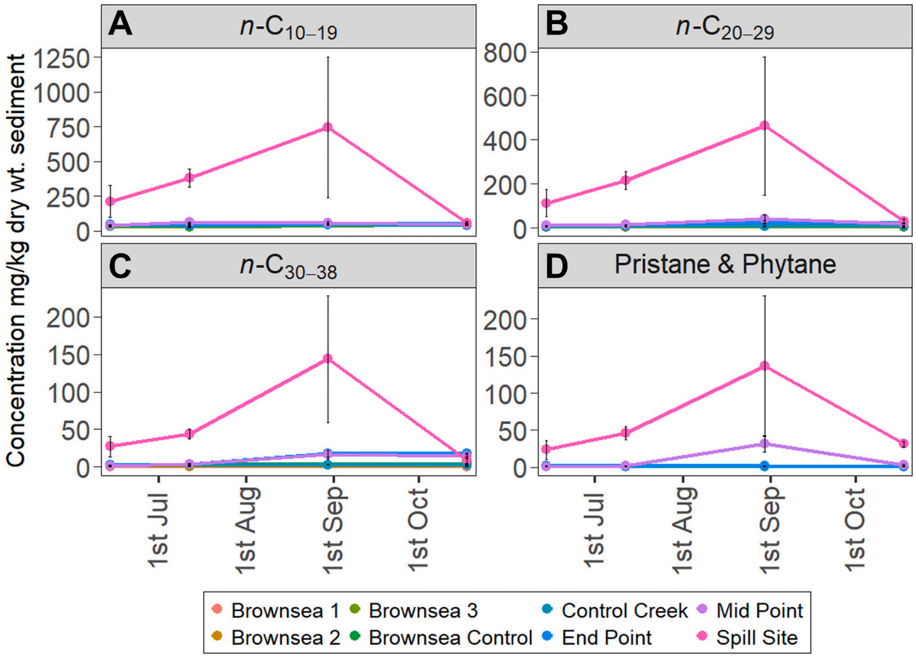

Fig. 2. Alkane (A. n-$C_{10–19}$, B. n-$C_{20–29}$, C. n-$C_{30–38}$ and D. pristane and phytane) concentrations (mg/kg dry wt. sediment) from surface sediments (means +/− s.e; n = 3) across Brownsea Island (Brownsea 1, 2 and 3, Brownsea Control (unoiled)) and Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipeline spill on 26th March 2023.

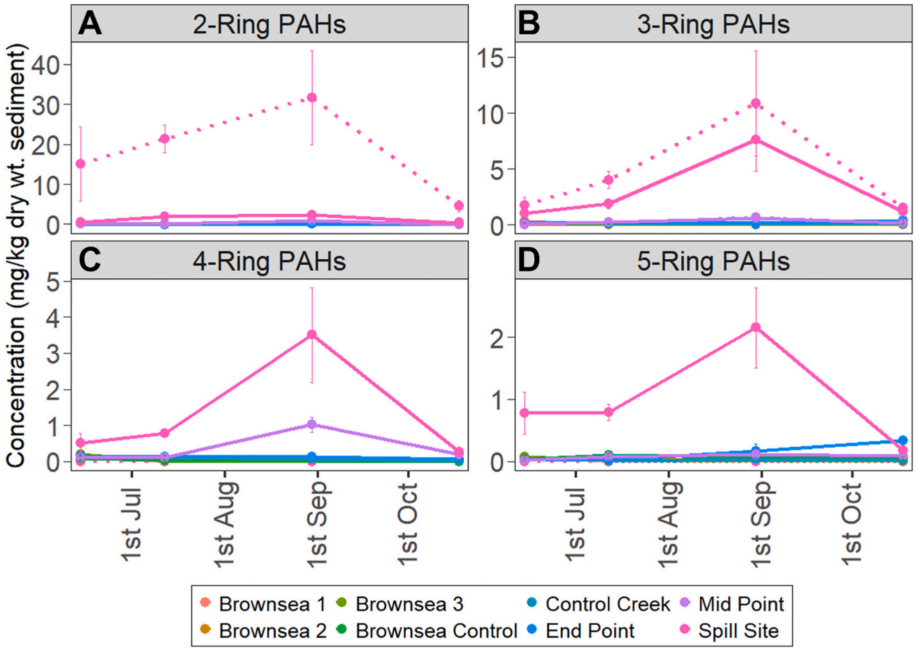

Fig. 3. Polyaromatic hydrocarbon (PAH) concentration (mg/kg dry wt. sediment), grouped via number of benzene rings, A. 2-Ring PAHs, B. 3-Ring PAHs, C. 4-Ring, D. 5-Ring PAHs, (inlcudes Parent (Soild line) and subsituted derivatives (Dotted Line); see Supplementary Material: Table S2 for full list of targeted compounds), from surface sediment samples (means +/− s.e; n = 3) across Brownsea Island (Borwnsea 1, 2 and 3, Brownsea Control (unoiled)) and Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipeline spill on 26th March 2023.

alkanes at the Spill Site ($Z = 2.57–3.41$, $p < 0.05$), although concentrations still remained significantly 8-fold higher than at all other sites at the final sampling time in October. Similarly, 2- to 5-ringed PAHs were also approximately 8–14-fold higher in concentration at the Spill Site compared to all other sites in June, July and August ($Z = 2.78–12.26$, $p < 0.05$) (Fig. 3A, B, C, D) before declining significantly by October ($Z = -2.95$ to -5.12, $p < 0.01$). The 2- and 3-ring PAHs were in much higher concentrations than the 4- to 5-ringed PAHs, and these larger PAHs had returned to background levels found across all other sites including the non-oiled control sites by October. In August, 4-ringed PAHs were also significantly higher by approximately 10-fold at the Mid Point site compared to Brownsea Island, the End Point and control site ($Z = 3.495$, $p < 0.001$). n-alkane concentrations and Brownsea Island showed no elevated levels at any time in comparison to the control site.

Concentration of 6-ringed PAHs were below the limit of detection at all sites, with no detectable change in concentration over time throughout the study site.

To distinguish the source of PAHs between the proportion of low molecular weight and alkylated PAHs to total PAHs (Law et al., 1999) was calculated. The index confirmed that the PAHs at the Spill Site were petrogenic, whilst the much lower levels of PAHs at all other sites were predicted to be of pyrogenic origin (Supplementary Material: Fig. S1). Due to analytical constraints other calculations such as the pyrogenic index (Wang et al., 1999) were unable to be calculated. Carbon Preference Index (ratio of odd:even *n*-alkanes used to help determine anthropogenic vs biogenic sources of alkanes, with high CPI suggesting biogenic origin as many cyanobacteria, aquatic and terrestrial plants produce odd chain length *n*-alkanes; Bray and Evans, 1961) calculations were also performed, confirming that hydrocarbons within the spill site in all months were attributed to crude oil contamination, with low CPI and broad range of both odd and even *n*-alkanes, together with the branched alkanes pristine and phytane, and an unresolved complex mixture (UCM) typical of crude oils. CPI also indicated that the small increase in hydrocarbon concentration found in august at the Mid Point

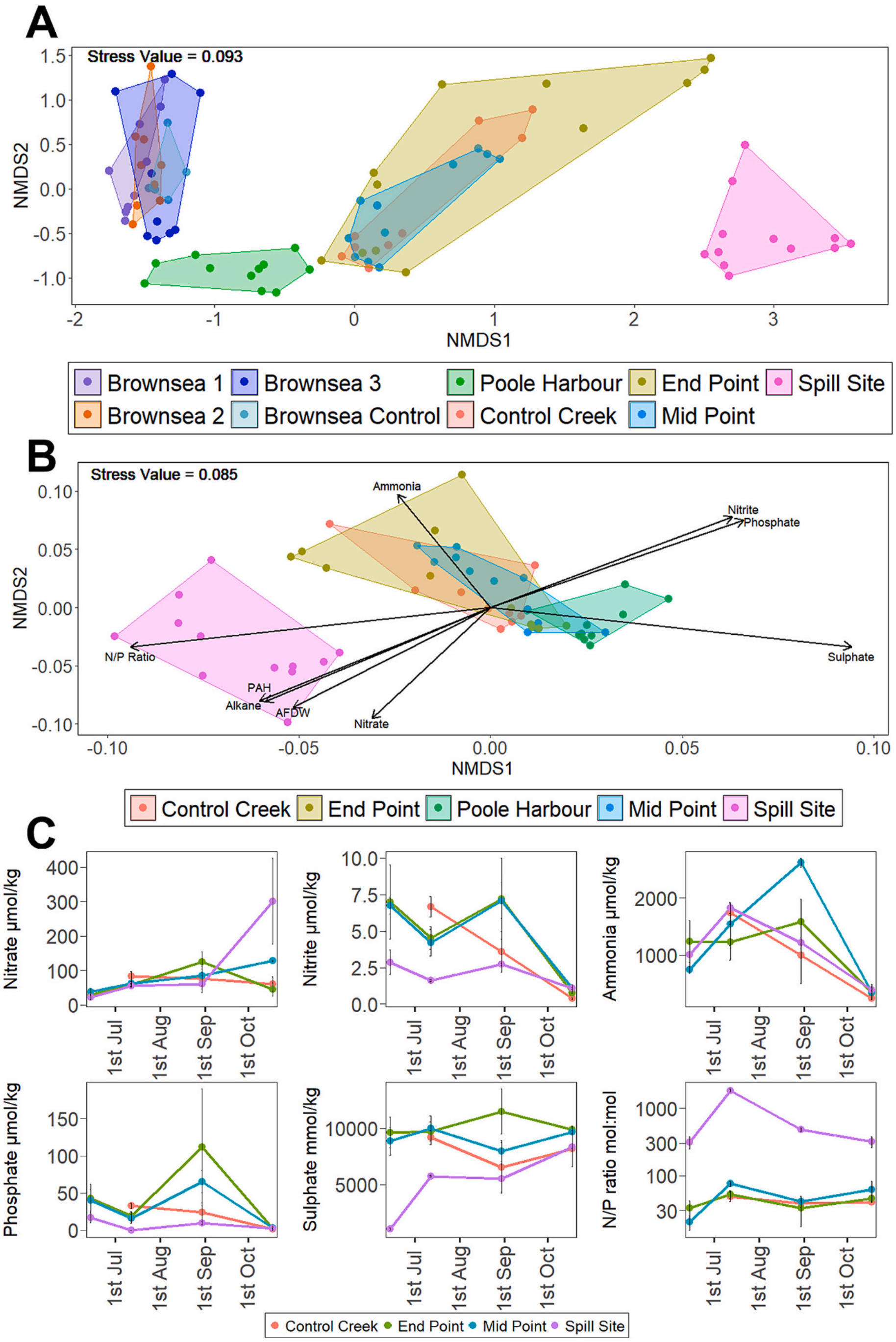

Fig. 4. nMDS (non-metric multidimensional scaling) ordination using Bray-Curtis dissimilarity, based on A. ASV (amplicon sequence variants) of bacterial 16S rRNA genes, and B. metagenomic sequence reads classified using the *k*-mer Kraken 2 approach paired with Bracken, including nutrient and hydrocarbon vectors and C. nutrient concentrations (µmol/kg) and molar N:P ratio between surface sediment samples (means +/− s.e; *n* = 3) across Brownsea Island (Brownsea 1, 2 and 3, Brownsea Control (unoiled)) and Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipline spill on 26th March 2023.

was also attributed to crude oil contamination, however at the End Point this slight increase in *n*-alkanes in August and October was more attributed to the presence of biogenic organic matter, with a significant increase in large odd chain length *n*-alkanes, particularly *n*-C_{25}, *n*-C_{27}, *n*-C_{29} and *n*-C_{31} associated with terrestrial plant origin (Supplementary Material: Fig. S2).

3.2. *The impact of hydrocarbon pollution on microbial communities*

In June, bacterial 16S rRNA gene abundance (16S rRNA gene copies/g dry wt. sediment) was significantly lower, by approximately an order of magnitude, at the Spill Site compared to the Mid Point, End Point and Control Creek ($Z = 2.94–3.38$, $p < 0.01$). However, from July onward gene abundance had increased at the Spill Site and was no longer significantly different. Compared to the muddy fine sediments of the Ower Bay creek and Control Creek sites, bacterial 16S rRNA genes were significantly less abundant by approximately two orders of magnitude within the sandy coarse sediment of the Brownsea Island sites ($Z = -4.39$ to -2.00, $p < 0.05$) but there were no significant differences between any of the Brownsea Island sites including the control site (Supplementary Material: Fig. S3).

16S rRNA amplicon sequencing showed that the bacterial community composition was significantly dissimilar between Brownsea Island, the harbour and creek sites (PERMANOVA, $F = 6.24–16.28$, $p < 0.001$) (Fig. 4A). Within the creek sites, a significant dissimilarity in community composition was observed between the Spill Site and Control Creek ($F = 8.53–11.24$, $p < 0.001$), whereas in the Mid Point and End Point sites, the composition was highly similar to that of the Control Creek. Across

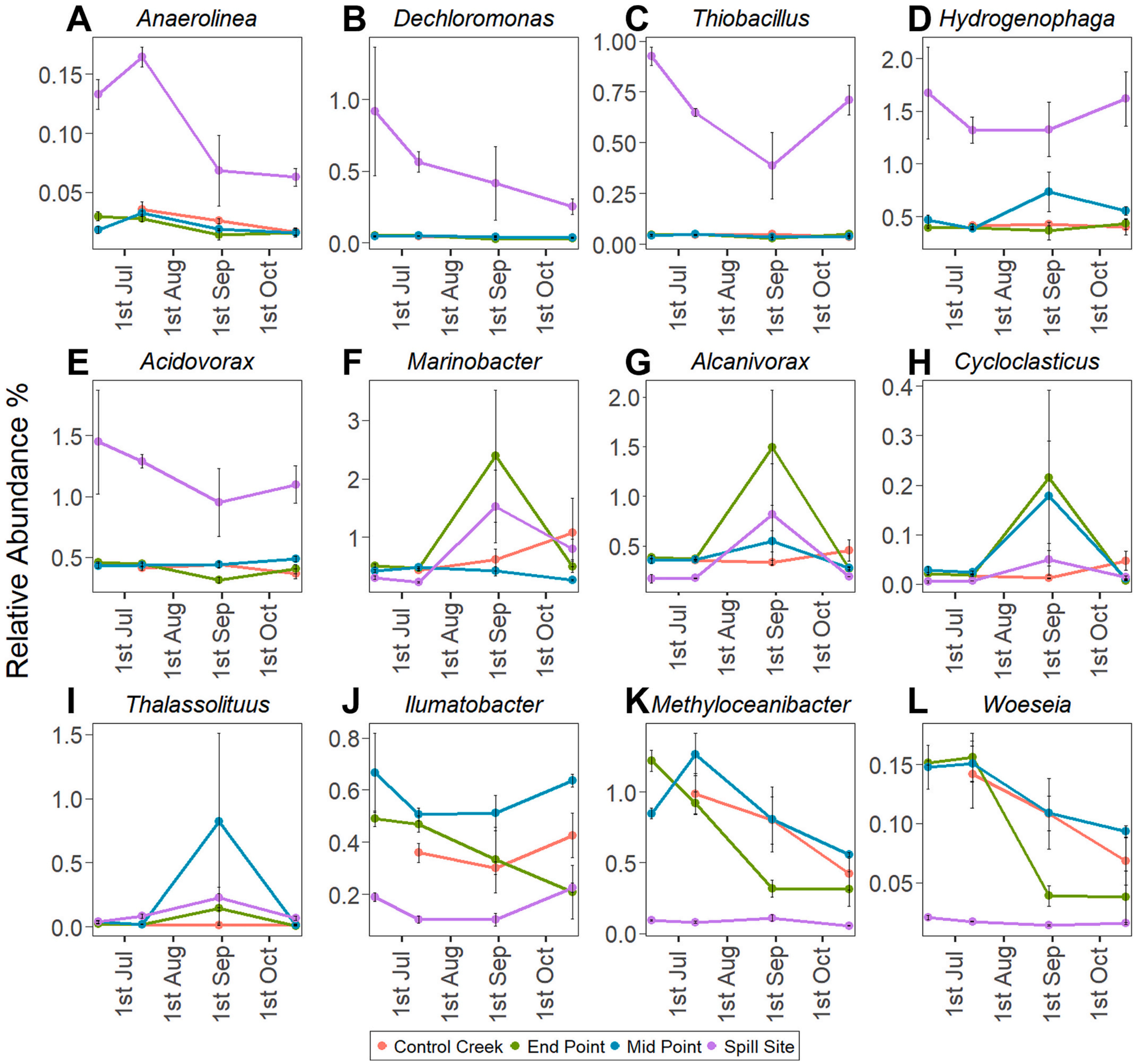

Fig. 5. Relative abundance (%) of genus showing differential abundance between sites based on SIMPER analysis within communities, based on metagenomic bacterial sequence reads classified using the *k*-mer Kraken 2 approach paired with Bracken from surface sediment samples (means +/− s.e; $n = 3$) across Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipline spill on 26th March 2023.

all time points the Spill Site bacterial community composition was significantly dissimilar to all other sites (F = 4.98–6.46, $p < 0.005$).

ASV richness was significantly higher within the creek sites in comparison to Brownsea Island within June and July ($t = -4.26$ to -3.22, $p < 0.05$), with a similar trend being observed with the Shannon diversity index. However, Pielou's evenness did not significantly change throughout the study period (Supplementary Material: Fig. S4). By the 30th August ASV richness at the spill site had declined significantly compared to the Control Creek ($t = -3.26$ to -2.84, $p < 0.05$), however the Shannon diversity index and Pielou's evenness remained similar throughout.

As both the hydrocarbon analaysis and bacterial 16S rRNA amplicon analysis showed no impact of the oil spill on sediment hydrocarbon concentrations or community composition at any of the Brownsea Island sites, we used metagenomic data (encompassing all taxa and both functional and phylogenetic genes) to further investigate the Ower Bay creek sites (Spill Site, Mid Point and End Point), Control Creek and Poole Harbour samples. Community composition based on Kraken2 metagenomic taxonomic classifications was also significantly dissimilar between the Spill Site and all other creek sites and the surrounding Poole Harbour region (F = 2.31–3.33, $p < 0.05$) (Fig. 4B). EnvFit analysis indicated that higher hydrocarbon and organic carbon (AFDW) concentrations (Supplementary Material: Fig. S5), and lower sulphate and phosphate (resulting in a much higher N:P ratio) concentrations were key predictors in community structure changes at the Spill Site (Fig. 4B, C).

SIMPER analysis of bacterial metagenomic reads taxonomically assigned to genus level, revealed that *Anaerolinea* (0.1 % contribution), *Dechloromonas* (0.5 % contribution), *Thiobacillus* (0.8 % contribution), *Hydrogenophaga* (1.5 % contribution), *Acidovorax* (1.1 % contribution), *Ilumatobacter* (0.7 % contribution), *Methyloceanibacter* (1.1 % contribution), Woeseia (0.2 % contribution) and *Marinobacter* (0.5 % contribution) were contributing most significantly to Brays-Curtis dissimilarity between the Spill Site and all other sites ($P < 0.05$). *Anaerolinea* reads were in significantly higher relative abundance at the Spill Site from June to Oct, being approximatly five-fold more abundant compared to all other sites at its peak in June ($t = -5.34$ to -3.01, $p < 0.001$) (Fig. 5A). Both *Dechloromonas* and *Thiobacillus* reads were also significantly more abundant (approximately five- to 10-fold greater) at the Spill Site compared to all other sites, as were *Hydrogenophaga* and *Acidovorax* (approximately two-fold greater) ($z = -7.71$ to -3.78, $p < 0.001$) (Fig. 5B, C, D, E). Other taxa increased in relative abundance much later after the spill. For example, *Marinobacter* reads were significantly higher at the Spill Site (three-fold more abundant) and End Point site (five-fold more abundant) compared to the Control Creek and Mid Point site in August ($z = -2.14$ to -1.72, $p < 0.05$) (Fig. 5F). Similarly, reads assigned to *Alcanivorax*, *Cycloclasticus* and *Thalassolituus* (known Obligate Hydrocarbonoclastic Bacteria (OHCB)) were all significantly higher in relative abundance at or near the Spill Site in later months (($t = 2.63$–4.08, $p < 0.05$) (Fig. 5G, H, I). *Alcanivorax* reads peaked significantly (relative to control site) in August at the Spill Site and End Point site ($z = -2.04$, $p < 0.05$), whilst *Cycloclasticus* reads were significantly higher also at the End Point, Mid Point and Spill Site in August, and *Thalassolituus* reads were significantly higher compared to the control site in August (20-fold at the End Point and Mid Point and 5-fold at the Spill Site; $z = -2.77$, $p < 0.01$). Numerous other genera became significantly less abundant at the Spill Site. For example, the relative abundance of *Ilumatobacter, Methyloceanibacter* and *Woeseia* were all significantly lower (2.47–9.55, $p < 0.05$) at the Spill Site compared to all other sites, for most of the study period (Fig. 5J, K, L).

Using the CANT-HYD database, genes known to be involved in aerobic or anaerobic alkane and aromatic hydrocarbon degradation were identified from the metagenomic contigs. The abundance counts (Z-score normalised) for each sampling site have been presented and analysed (Fig. 6). *assA, assB* (genes coding for: anaerobic alkylsuccinate synthase A and B subunit)*, abcA1, abcA2* (anaerobic benzene carboxylase) and *ndoA* (aerobic naphthalene dioxygenase A subunit), were on average approximately 119 %, 187 %, 117 %, 185 % and 75 %

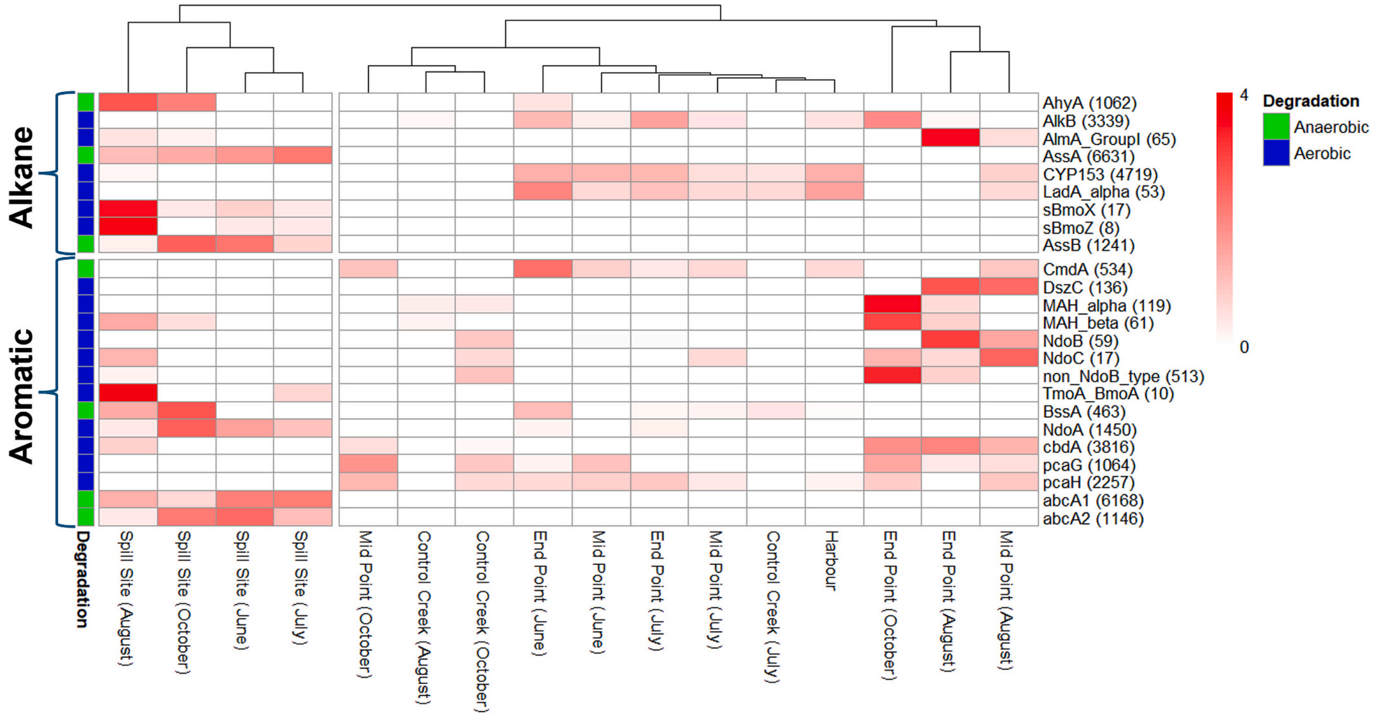

Fig. 6. Heatmap of functional gene hits (total gene abundance count in brackets) using Hidden Markov Model (HMM) coding for proteins associated with aerobic and anaerobic hydrocarbon degradation pathways based on sequences (normalised by row using z-score normalisation) classified from Contigs, using the CANT-HYD database, from surface sediment samples ($n = 3$) across Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipline spill on 26th March 2023.

respectively more abundant at the Spill Site compared to all other sites ($z = -4.95$ to -2.03, $p < 0.05$). *sBmoX, Z* (aerobic butane monooxygenase) and *tmoA_bmoA* (aerobic toluene/benzene monooxygenase) genes were only present at the Spill Site throughout the study period, being on average greater in abundance in August compared to all other times points by 108 %, 143 % and 200 % respectively ($t = 2.13$–3.03, $p < 0.05$). In August and October *ahyA* (anaerobic alkane hydroxylase A subunit) and *bssA* (anaerobic benzylsuccinate synthase A subunit) were approximately 200 % and 165 % more abundant at the Spill Site respectively, compared to all other sites ($z = 3.05$–3.67, $p < 0.01$). *cmdA* (anaerobic aromatic dehydrogenase A subunit), CYP153 (cytochrome P450 aerobic alkane hydroxylase), *ladA_Alpha* (aerobic long-chain alkane monooxygenase), *alkB* (aerobic alkane hydroxylase) and *pcaG, H* (aerobic aromatic protocatechuate dioxygenase) were all approximately 145 % – 291 % more abundant within the Mid Point, End Point and Control Creek compared to the Spill Site ($t = 2.16$–3.89, $p < 0.05$). Dependent on the hydrocarbon degrading genes present and their abundance, the Spill Site samples clustered together via Euclidian distance, being highly similar to one another and dissimilar to all other sites.

In order to analyse the capacity for hydrocarbon degradation within individual genomes, MAGs (Metagenome-Assembled Genomes) were reconstructed from assembled contigs and their abundance was quantified for each sampling site (Fig. 7). Clustering via Euclidian distance reaffirmed much of the findings from the taxonomic classification of metagenomic reads following similar patterns presented in Fig. 5, with notable bins 143 (Anaerolineales), 99 (*Dechloromonas*) and 84 (*Thiobacillus*) being highly abundant at the Spill Site and absent at other sites ($t = -7.78$–15.86, $p < 0.001$), whilst bin 170 (*Woeseia*) and 196 (*Ilumatobacter*) were predominantly abundant across the rest of the creek sites ($t = -6.47$ to -2.22, $p < 0.05$). The CANT-HYD database was further used to identify hydrocarbon degradation genes within the MAGs to explore the contribution these MAGs have towards oil degradation potential (Supplementary Material Fig. S8). Anaerobic hydrocarbon degradation potential within the Spill Site was shown to be considerable. This was confirmed by the presence of multiple anaerobic hydrocarbon degradation genes, including *assA* and *bssA* within multiple MAGs that were detected only at the Spill Site (associated with bin 143 (Anaerolineales), 33 (*Desulfosarcina*), 148 (*Desulfobacter*), 138 (*Desulfomonile*) and 136 (Proteobacteria), as well as *abcA1/2* (associated with bin.29 (Betaproteobacteria), 84 (*Thiobacillus*), 162 (*Caldilinea*) and 11 (Methanomicrobiaceae)) and *ahyA* (associated with bin. 11 (Methanomicrobiaceae), 143 (Anaerolineales), 148 (*Desulfobacter*), 136 (Proteobacteria), 53 (*Porphyrobacter*), 206 (Bacteroidetes), 162 (*Caldilinea*), 29 (Betaproteobacteria), 164 (*Geobacillus*), 99 (*Dechloromonas*), 84 (*Thiobacillus*), 138 (*Desulfomonile*), 33 (*Desulfosarcina*)). At the Mid Point, End Point and Control Creek sites, further observations indicated a high potential of hydrocarbon degradation through both aerobic degradation pathways and anaerobic degradation pathways highlighted particularly by bins 102 (*Pseudohordobacter*), 113 (*Lamiaceae*), 134 (Myxoccocales) and 16 (*Spongiibacter*).

3.3. Assessment of biogeochemical cycle genes throughout Ower Bay Creek

The effect of the oil spill on the abundance of biogeochemical cycling genes associated with the nitrogen, sulphur, and methane cycle was determined using the NCycDB, SCycDB and MCycDB databases. No significant change over time or between sites was observed based on total gene count within each cycle (Supplementary Material: Fig. S8). However, nitrogen cycling functional genes *nifD* (nitrogenase molybdenum-iron protein alpha chain) and *nifK* (nitrogenase molybdenum-iron protein beta chain), genes associated with nitrogen fixation were significantly more abundant at the Spill Site compared to all other sites ($t = 3.22$–3.93, $p < 0.001$) (Fig. 8), whilst no change in the *nifH* gene were observed ($t = 3.22$–3.93, $p < 0.001$). *nirK and S* (nitrite reductase) genes, associated with denitrification were also shown to be less abundant at the Spill Site compared to all other sites ($t = -2.43$, $p < 0.05$).

The sulphur cycling gene *asrB* (anaerobic sulphite reductase), was found to be more abundant at the Spill Site by approximately 55.5 % ($t = -3.21$, $p < 0.05$) in June (Fig. 9), reducing in abundance by 44.4 % over time between June and October but still remained higher than at all other sites. *asrA* which codes for the alpha subunit of the same enzyme complex was however not significantly different between any of the sites. *cysA, cysU* (sulphate/thiosulphate transporter) genes, associated with the uptake of sulphate/thiosulphate, were approximately 28 and 24 % more abundant at the Spill Site compared to all other sites (-3.23 to -2.84, $p < 0.05$), whilst *cysC, I, J* (sulphite reductase) and *dsrA, B* (dissimilatory sulphite reductase) genes did not differ significantly between sites.

Within the methane cycle, *mbhJ* (membrane-bound hydrogenase) genes, were approximately 40 % more abundant at the Spill Site compared to other sites (-5.43, $p < 0.05$) (Fig. 10). *mcrA* and *B* (methyl-coenzyme M reductase) genes were both approximately 80 % more abundant at the Spill Site compared to all other sites (-2.54 to -3.23, $p < 0.05$) and were greater in abundance by approximately 667 %. *mvhA* (F_{420}-non-reducing hydrogenase), associated with the central methanogenesis pathway was approximately 44 % more abundant at the Spill Site compared to other sites in June and July ($t = -5.47$ to -2.74, $p < 0.01$) but declined by August to similar abundances at all other sites. *acdA* (acetate-CoA ligase (ADP-forming)) and *acs* (acetyl-CoA synthetase) genes, associated with acetoclastic methanogenesis, were shown to be 33.3 % and 28.6 % less abundant at the Spill Site compared to all other sites ($t = 2.70$–5.29, $p < 0.05$).

4. Discussion

4.1. Hydrocarbon concentrations after initial spill and during clean up

During the first 80 days of the spill (before the initial sample collection) substantial removal of oiled water and sediment had been conducted, which included the recovery of 417,000 l of oil and oily water from around the loss point by the 19th April 2023 (Hatch, 2024). Individual hydrocarbon concentrations in the sediment during this recovery phase remain unknown but visual evidence indicated some oil had continued to migrate through the sediment with small pockets of oil visible on the surface. Despite the significant removal of oil, as of June 2023 (80 days after initial spill), concentrations of alkanes and PAHs, were highest at the Spill Site, whilst the rest of Poole Harbour and all other sites were similar to control sites, being largely unimpacted. This was to be expected as according to response reports (Hatch, 2024), by April 2023 (one month after the initial spill) no further contamination was found outside of the localised Spill Site due to rapid containment by deployment of booms. As Poole Harbour is historically a busy recreational and fisheries port, hydrocarbons would accumulate in sediments from both anthropogenic and natural sources (Woodhead et al., 1999; May and Humphreys, 2005; Underhill-Day, 2006) which would explain the concentrations of hydrocarbons measured outside of the immediate Spill Site, and the PAHs were further highlighted as pyrogenic in origin, meaning they were unlikely to be associated with the pipeline spill. During the investigation, hydrocarbon concentrations outside of the localised Spill Site stayed consistently lower than at the Spill Site, indicating the effectiveness of absorbent booms as a physical barrier in reducing hydrocarbon contamination to the wider harbour area (Dave and Ghaly, 2011; Prendergast and Gschwend, 2014).

By the end of August, alkane and PAH concentrations peaked at the Spill Site. This increase in hydrocarbon concentration may be due to subsurface crude oil trapped in the sediment from the initial spill migrating to the sediment surface. In mid August, there was also some mobilisation of oil sheens caused by high tides (Hatch, 2024), which may have potentially caused the small increases in hydrocarbon concentration (particularly larger n-alkanes, pristane, phytane and 3- and 4-

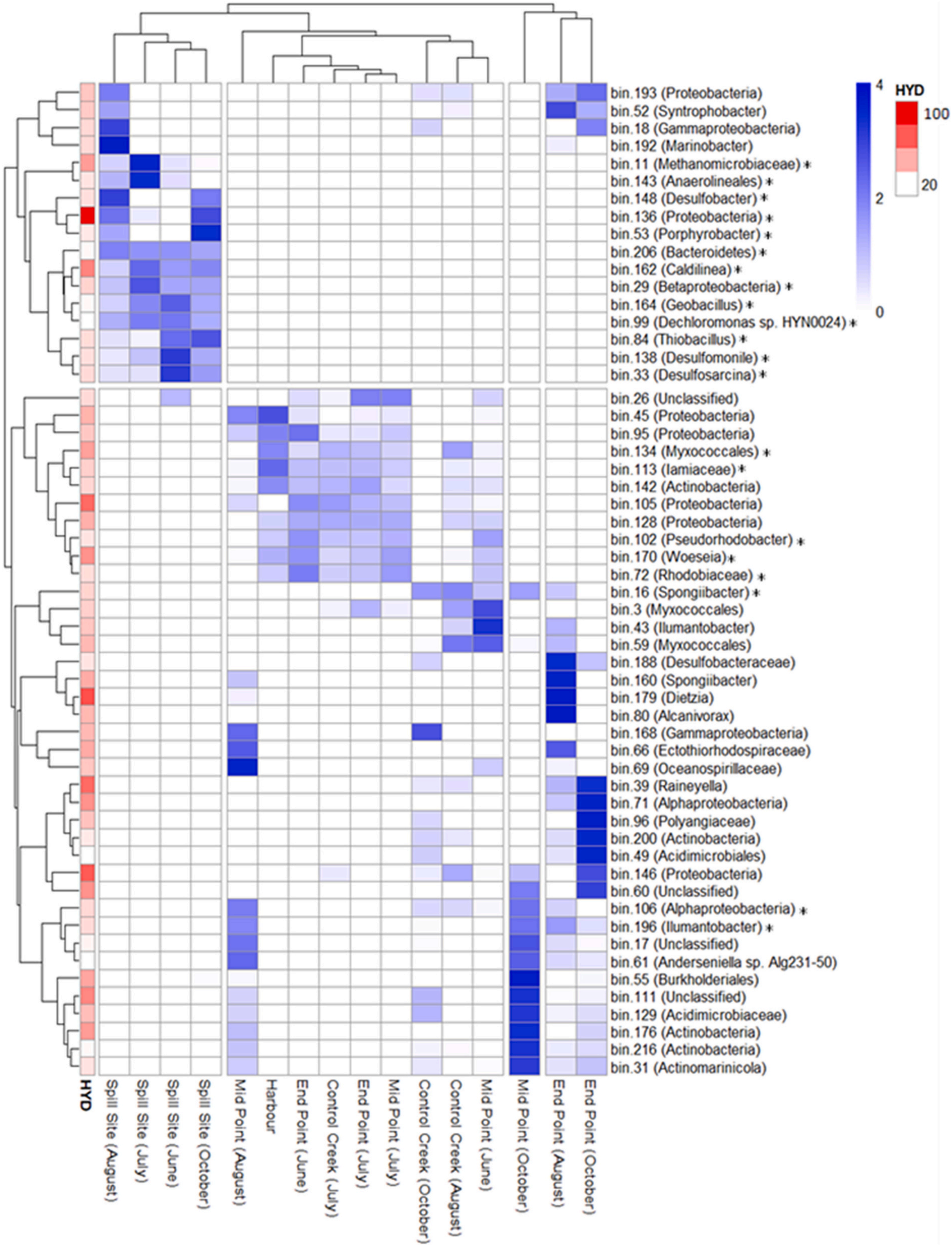

Fig. 7. Heatmap of MAGs (Metagenome-Assembled Genome's) count (blue shading = MAG copies per million reads, normalised by row using z-score normalisation) from surface sediment samples ($n = 3$) across Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipline spill on 26th March 2023. MAGs were taxonomically classified by Taxator-Tk against the NCBI nt database. The number of functional genes associated with hydrocarbon degradation are shown within each MAG (red shading), based on matches against the CANT-HYD database. HYD = Count of hydrocarbon degrading genes within each MAG. *highest abundant MAG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

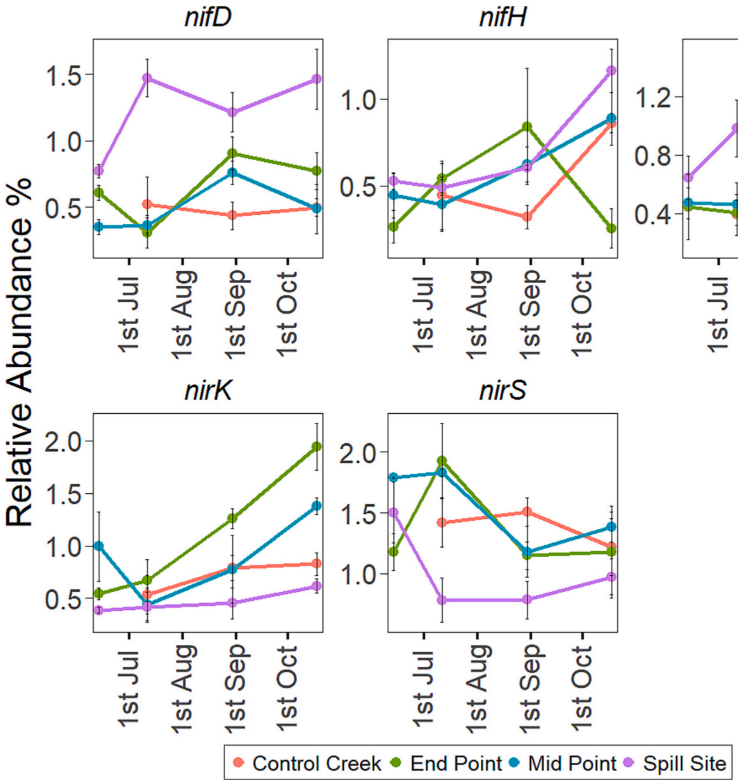

Fig. 8. Relative abundance (%) of nitrogen cycling genes causing dissimilarity within bacterial communities (Based off SIMPER analysis) based on shotgun metagenomic sequencing reads taxonomically classified using NCycDB sediment samples (means +/− s.e; $n = 3$) across Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipline spill on 26th March 2023.

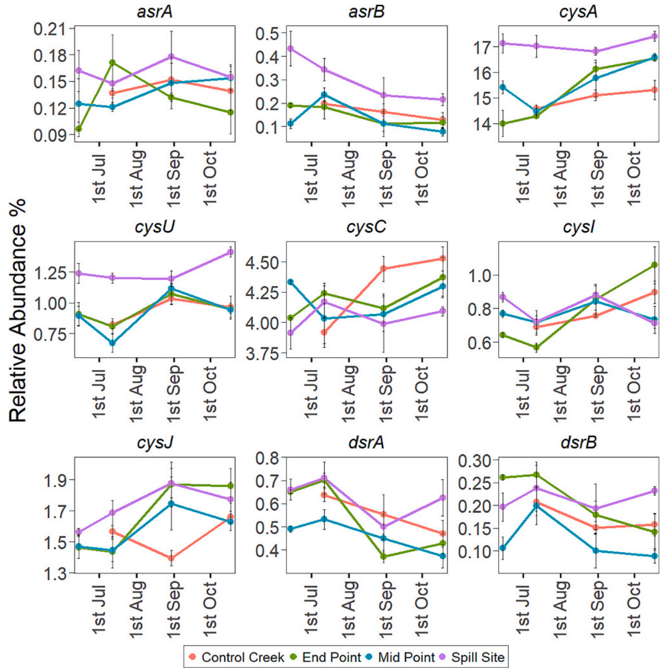

Fig. 9. Relative abundance (%) of sulphur cycling genes causing dissimilarity within bacterial communities (Based off SIMPER analysis) based on shotgun metagenomic sequencing reads taxonomically classified using SCycDB sediment samples (means +/− s.e; $n = 3$) across Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipline spill on 26th March 2023.

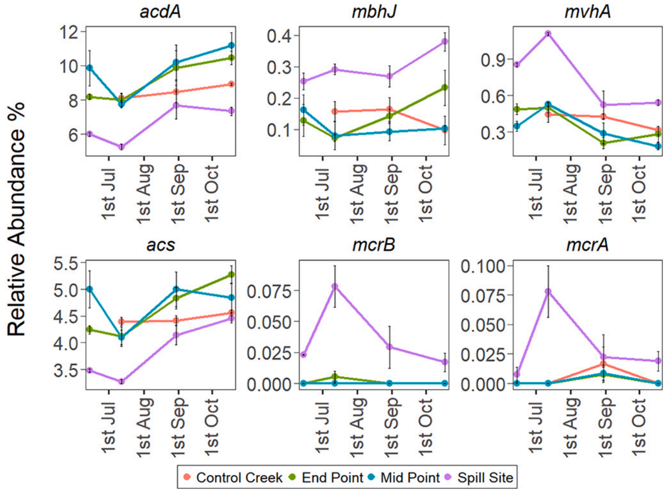

Fig. 10. Relative abundance (%) of methane cycling genes causing dissimilarity within bacterial communities (Based off SIMPER analysis) based on shotgun metagenomic sequencing reads taxonomically classified using MCycDB sediment samples (means +/− s.e; $n = 3$) across Ower Bay Creek (End Point, Mid Point and Spill Site) and Corfe River Control Creek (unoiled), Dorset, UK on the 14th June, 12th July, 30th August and 18th October 2023, following the Poole Harbour oil pipline spill on 26th March 2023.

ring PAHs) that occurred in the adjacent Mid Point zone of the creek. CPI ratios further confirmed this to be the case being CPI < 1 indicating an anthropogenic crude oil origin. An increase in larger odd chain length *n*-alkanes associated with plant material was also observed at the End Point at the same time, with CPI calculations at this site were CPI > 1, suggesting the boom and the Mid Point was effective in stopping mobilised oil travelling further up to the End Point. Further response via the deployment of a bubble barrier as an extra safeguard was implemented to avoid further mobilisation of hydrocarbons during high tides (Feng and Zhang, 2023; Hatch, 2024). By October 2023, there was a substantial decline in the concentration of alkanes n-C_{10-38} and PAH's at the Spill Site, returning to similar concentrations found across control sites and the surrounding Poole Harbour area. The biodegradation of hydrocarbons via bacteria has been well documented in prior studies and is most likely one of the main processes causing this significant decline (McGenity et al., 2012; Liu et al., 2020). Larger branched alkanes such as pristane and phytane, along with methylated 2, 3-ringed PAH's persisted within the Spill Site due to their higher molecular weight and complexity reducing their degradability (Setti et al., 1995; Mohanty and Mukherji, 2008). Over time concentrations of these larger complex hydrocarbons may further reduce to similar levels of the surrounding area (Prince and Lessard, 2004) although in this case, significant amounts of sediment were removed after our final sampling (October 2023) and the site is completely remediated and recovered as observed in March 2025 (Supplementary Material: Fig. S9).

4.2. Spill impact on sediment bacterial communities

In June 2023 (80 days post-spill), the bacterial 16S rRNA gene abundance was observed to be lowest at the Spill Site. Notably, abundance showed no significant change across all other sites. By July 2023, the bacterial 16S rRNA gene abundance had increased to similar levels found across the rest of the Ower Bay creek area. In this instance, the increased concentration of hydrocarbons in the system may have caused a depletion in oxygen due to an increased oxygen demand via hydrocarbon-degrading bacteria (Shin et al., 2000), accounting for the initial drop in bacterial abundance, as well as some bacterial taxa being negatively impacted by oil toxicity, or being outcompeted by hydrocarbon-degrading taxa for nutrients such as N and P (Sikkema et al., 1995; Head et al., 2006). By July 2023, the increase in bacterial abundance to levels similar to the Control Creek, Mid Point and End Point could be attributed to an increase in bacteria better suited to a more anoxic and hydrocarbon contaminated systems, which was clearly observed with the increase in anaerobic bacterial genera and hydrocarbon degradation genes associated with anaerobic degradation pathways found at the Spill Site. Similar switches to anaerobic bacteria has previously been observed in Guo et al. (2022), across an increasingly hypoxic region of the Bohai Sea. Bacterial abundance was observed to be much lower within the coarse Brownsea Island sediments, compared to the fine sediment of Ower Bay Creek. The different physical and biological properties of these sediments, e.g. sands vs muds respectively, would likely be the main driver to account for this difference rather than any differences in hydrocarbon contamination (Szava-Kovats, 2008; Xu et al., 2021).

Throughout this study *Dechloromonas*, *Anaerolinea*, *Thiobacillus*, *Acidovorax* and *Hydrogenophaga*, were the most prevalent genera at the Spill Site. Outside the Spill Site, *Woeseia* and *Methyloceanibacter* were most prevalent, initially indicating an impact on these genera from hydrocarbon contamination, further altering community structure (Yakimov et al., 2005; McGenity et al., 2012). *Dechloromonas,* which was only detected at the Spill Site, has been highlighted as a potential anaerobic hydrocarbon degrader, notably in the degradation of PAHs (Salinero et al., 2009; Yan et al., 2017; Zhang et al., 2021) and benzene under nitrate and sulphate reducing conditions (Chakraborty et al., 2005). During this study, *Dechloromonas* was shown to have the potential of both anaerobic alkane and aromatic degradation via the *abcA* (anaerobic benzene carboxylase)*,* *cmdA* (anaerobic aromatic dehydrogenase A) and *ahyA* gene (anaerobic alkane hydroxylase A) pathways, in which sulphate/sulphite may be the primary electron acceptor during fumarate addition aromatic biodegradation (Zhang et al., 2010). The presence of dissimilatory sulphite reductase encoding genes such as *dsr* and *asr* throughout the creek along with the large reduction in sulphate further suggests the use of sulphate as an electron acceptor supporting anaerobic biodegradation at the Spill Site. *Anaerolinea* was observed to be more abundant at the Spill Site and has been shown to thrive under anaerobic conditions. Recent studies have suggested *Anaerolinea* may be an important sulphate reducer with genes associated with both assimilatory and dissimilatory sulphate reduction being present in numerous MAGs (Payne et al., 2024) but may also have a role to play in anaerobic alkane degradation via *assA*-like genes that were observed within MAGs taxonomically classified as Anaerolineales. This was further emphasised in the current study within MAG bin.143 (Anaerolineales) in which *sat/cysC* (putative bifunctional *SAT/APS* kinase) *cysNC* (*APS kinase* and *adenylyltransferase*) and *nrnA* (*oligoribonuclease* and *PAP phosphatase*), genes associated with assimilatory sulphate reduction were identified. According to prior studies, *assA* genes have not currently been identified in MAGs assigned to Anaerolineales, however it has been heavily suggested that they do likely contain these genes, encoding for alkylsuccinate synthase allowing for the initial activation for long-chain n-alkane biodegradation (Liang et al., 2016; Wu et al., 2024). Further classification highlighted these *assA*-like genes as glycl radical proteins (Backman et al., 2017) associated with *Anaerolinea* (89 % sequence identity), being a part of the *Anaerolineaceae* family that has the potential for anaerobic alkane degradation.

Thiobacillus has been previously documented to be present within hydrocarbon polluted areas (Kellermann and Griebler, 2009; Tian et al., 2017), which was observed during the present study to be highly abundant at the Spill Site, in contrast to all other sites where its abundance was very low. *Thiobacillus* itself is a sulphur oxidiser, capable of oxidising elemental sulphur, hydrogen sulphide and thiosulphate to sulphate (Sublette and Sylvester, 1987; Zhi-Hui et al., 2010). Notably this tends to occur in aerobic conditions, however, it also occurs anaerobically, in which thiosulphate is split by rhodanese to sulphite and elemental sulphur, which is further oxidised to sulphate (Schedel and Trüper, 1980). The increase of *Thiobacillus* at the Spill Site suggests *Thiobacillus* to be the dominant sulphur oxidiser involved in hydrocarbon degradation. The presence of sulphur oxidation genes *sqr* (*sulfide-quionone*), *glpE* (*thiosulfate sulfurtransferase*) and *soeABC* (*Sulphite dehydrogenase*) within MAG bin.84 (*Thiobacillus*) that was abundant within the Spill Site further highlights this functionality. It's adaptability to both oxic and anoxic conditions, and both alkane and aromatic hydrocarbon degradation, is highlighted by the wide range of aerobic and anaerobic hydrocarbon degradation genes identified within the *Thiobacillus* MAGs (e.g. *abcA*, *ahyA*, *alkB*, *ndoB* and *almA*) (Supplementary Material: Fig. S7). Outside of the Spill Site, sequencing indicated that Woeseia, another sulphur oxidiser (highlighted by the presence of sulphur oxidation genes *soxA* ((*L-cysteine S-thiosulfotransferase subunit*) and *fccA* (*cytochrome subunit* of *sulfide dehydrogenase*) (within MAG bin.170 (Woeseia)), similarly capable of anaerobic sulphur oxidation (Buongiorno et al., 2020; Hoffmann et al., 2020) was prevalent throughout all other creek sites, with much lower relative abundance at the Spill Site. Therefore, it is suggested that *Woeseia* may act as a potential biomarker for system health. Prior studies have also suggested other potential biomarkers of system health, such as *Thioprofundum,* which has previously been shown to decline in hydrocarbon contaminated sediments (Godoy-Lozano et al., 2018; Suárez-Moo et al., 2020), as well as a broader range of bacteria such as nitrifiers that have been shown to be highly sensitive to hydrocarbon toxicity (Urakawa et al., 2019). Particularly, this highlights the importance of understanding genera that are either absent or reduce in abundance at oil Spill Sites, as well as those that increased due to their involvement in hydrocarbon degradation, in understanding the full impacts of hydrocarbon contaminants on microbial community structure and function. Both *Woeseia* and *Thioballicus* are shown to play similar roles within the sulphur cycle, therefore, due to this functional redundancy, oil pollution does not necessarily negatively impact sulphur oxidation, but does however impact the community composition, by selecting for sulphur oxidising bacteria with the capacity to deal with the presence of hydrocarbon contamination and changing environmental conditions (Badmadashiev et al., 2023).

Sequences assigned to aerobic *Ilumatobacter* (Matsumoto et al., 2009) and *Methyloceanibacter* were much lower in abundance at the Spill Site, suggesting they may potentially be outcompeted by anaerobes, such as *Dechloromonas* and *Anaerolinea* (Achenbach et al., 2001). Prior studies have highlighted the presence of *Ilumatobacter* within marine polluted sites, suggesting a potential for aerobic alkane and aromatic degradation (Ellis et al., 2022; Ashade et al., 2024). However, little is known about what hydrocarbons some *Ilumatobacter* species may successfully grow on. Even so, as seen in the present study with the presence of alkane degradation pathway genes *CYP153*, *ladB* and *almA* and aromatic degradation pathway gene *ndoB* within MAGs taxonomically classified as *Ilumatobacter*, their possible capacity for aerobic hydrocarbon degradation has been further highlighted. Interestingly, the putative anaerobic alkane pathway gene *ahyA* was also found within *Ilumatobacter* MAGs suggesting the possibility for alkane degradation under anaerobic conditions. However, prior studies have shown this genus to be outcompeted by Anaerolineaceae under anaerobic conditions, with the environment selecting for anaerobes (Zhao et al., 2023; Gao et al., 2024). Thus, in the context of this study *Ilumatobacter* may have been outcompeted by *Anaerolinea, Dechloromonas and Thiobacillus* at the Spill Site as dominant hydrocarbon degrading anaerobes. The lower relative abundance of another aerobe, *Methyloceanibacter,* may further indicate the anoxic nature of the Spill Site compared to other sites with the creek (Takeuchi et al., 2014). By October a legacy of some taxa associated with hydrocarbon degradation persisted, even when hydrocarbon concentrations had dropped considerably to background levels found throughout Poole Harbour. This phenomenon has also been observed after other oil spills, such as the Agia Zoni II spill, when Alcanivorax and Cycloclasticus persisted in sediments for several months after the spill and after combined biodegradation and physical clean up response had removed the hydrocarbon contamination (Thomas et al., 2020). However, the Ecological Index of Hydrocarbon Exposure (Lozada et al., 2014) suggests that between August and October, there was a considerable decrease overall the relative abundance of taxa known to be capable of hydrocarbon degradations (Supplementary Material: Fig. S6), coinciding with the decrease in hydrocarbon concentrations.

4.3. *The capacity for hydrocarbon degradation within the saltmarsh creek environments*

CANT-HYD identified 24 hydrocarbon degrading genes with 17 being associated with aerobic degradation and seven being associated anaerobic degradation throughout the study sites (Khot et al., 2022). The Spill Site was observed to be dominated by anaerobic hydrocarbon degrading genes, whilst all other sites outside of the Spill Site were dominated by various aerobic HYDs. This highlights a high capacity for hydrocarbon degradation and the capacity to change from aerobic to anaerobic hydrocarbon degradation under anoxic conditions. Notably, *assA, B, abcA1, 2* and *bssA* were the most prevalent anaerobic HYD genes at the Spill Site. *assA and B* are involved in anaerobic alkane degradation, catalysing the initial steps via fumarate addition (Callaghan et al., 2010). Analysis of MAGs highlighted the presence of *assA*-like genes, notably in bin.143 (Anaerolineales)*,* bin.148 *(Desulfobacter) and* bin.136 (Proteobacteria). Further exploration of the *assA* genes within bin.136 (Proteobacteria), identified them as glycl radical proteins associated with Desulfobulbaceae (90.26 Identity). Outside of the Spill Site at the Mid Point and End Point aerobic degradation genes were present in August and November, notably *almA* and *cmdA* targeting long chain alkanes potentially attributed to the plant matter within these sites (Supplementary Material: Fig. S2). The presence of other degradation genes within the Mid Point and End Point such as *ndoA, B* may be attributed to so minor mobilisation of hydrocarbon throughout the system during August, however the gene count during this sampling period was much lower at these site compared to the Spill Site.

Genes *abcA and bssA* have previously been shown to be associated with PAH degradation, by the direct carboxylation of benzene and toluene via fumarate addition (Luo et al., 2014; Bacosa et al., 2018; Toth et al., 2021; van Leeuwen et al., 2022). Notably, MAGs including bin.99 (*Dechloromonas*), bin.84 (*Thiobacillus*) and bin.11 (Methanomicrobiaceae) contained putative *abcA*-like genes that were identified as coding for UbiD family decarboxylases (93.79–98.63 % identity), highlighting potential involvement in anaerobic aromatic degradation at the Spill Site (Kim et al., 2003; Chakraborty et al., 2005). The UbiD enzyme family is however highly widespread, with considerable diversity, with many of them catalysing aromatic decarboxylation, suggesting this may be a possibility that should be considered for further investigation (Marshall et al., 2021). Methanomicrobiaceae *abcA* genes were shown to be similar to that of *abcA*-like genes within *Methanoregula* (97.92 % identity), indicating the potential for direct anaerobic aromatic degradation within methanogens. Even so it must be highlighted, that the sequences classified as *abcA,* within bin.11 *(Methanomicrobiaceae)* was based off homology to only two available putative *abcA Clostridia* bacterium sequences (Abu Laban et al., 2010) within the CANT-HYD database, with a 21.95 % identity, thus the potential for degradation cannot be directly confirmed.

By August an uplift in the presence of aerobic degradation genes *ndoA*, *ndoC*, CYP153 P450, *sBmoX*, *Z* and *tmoA/bmoA* was observed. *ndoA* and *ndoC* have been shown to be associated with aerobic PAH degradation via the hydroxylated gentian acid pathway, specifically being upregulated in the presence of naphthalene, playing a significant role in initiating the degradation of lower-molecular-weight PAHs (Ribeiro et al., 2018; Li et al., 2023). Cytochrome P450 CYP153 alkane hydroxylases catalyse the hydroxylation of aliphatic and alicyclic alkanes (van Beilen et al., 2006; Funhoff et al., 2007). During monitoring and clean-up of the site some further mobilisation of hydrocarbons occurred due to high tides during the August sampling time period, potentially mobilising more oil to the oxygenated surface which may explain the increase in aerobic hydrocarbon degradation genes at this time. Overall, these communities highlight high hydrocarbon degrading potential within the creek system under both oxic and anoxic conditions and a high level of adaptability within the community, rapidly selecting for genera capable of aerobic and anaerobic hydrocarbon degradation.

4.4. *Effects of the oil spill on the biogeochemical functionality of Ower Bay Creek*

Through the analysis of sediment nutrient concentrations and functional genes associated with the nitrogen-cycle, sulphur-cycle, and methane-cycle, any impact of the oil spill on macronutrient cycling was addressed. At the Spill Site specifically, a greater abundance of *nifD and nifK,* genes associated with nitrogen fixation were present compared to other creek sites. This process converts atmospheric dinitrogen gas (N_2) to NH_3, potentially contributing to NH_3 input at the Spill Site (Zehr and Capone, 2020), but it is also worth noting that the further inputs of NH_3 at the site may also come from the ammonification of biomass (e.g. reed material killed off during the initial spill event). Prior studies have shown that various hydrocarbonoclastic bacteria (82 potential species) may also be diazotrophic, thus may explain the uplift of genes associated with nitrogen fixation within the Spill Site (Taketani et al., 2009; Dashti et al., 2015). Although differences in the gene abundance of *nifD,K* and *nirK,S* were observed between the Spill Site and the rest of the creek, nitrate, nitrite and ammonia concentrations remained fairly consistent throughout the study. As nitrate was not limiting, the observable decline in the relative abundance of denitrification genes (*nirK* and *nirS*), may be attributed to the relative increase in sulphate reducing bacteria that became dominant at the Spill Site, as sulphate reduction is favoured in highly reduced sediments and significant reductions in denitrification capacity have often been observed in heavily oiled saltmarsh sediments (Levine et al., 2017; Tatariw et al., 2018). At the Spill Site, the system became increasingly phosphate limited compared to the other creek sites, notably, due to the heavy demand from hydrocarbon degraders following the influx of oil as a rich carbon source (Engelhardt, 1985).

During the study sulphate concentrations were observed to be significantly considerably lower at the Spill Site compared to all other sites in June and July, suggesting an increased level of sulphate reduction compared to all other sites. As previously stated, *Anaerolinea* reads were more abundant at the Spill Site, notably due their capacity for anaerobic hydrocarbon degradation, but also their potential importance in sulphate reduction as observed in MAG bin.143 (Anaerolineales) (Sherry et al., 2013). This along with the presence of the *cysA and cysU* genes, that are subunits of the sulphate transport system and the *asrB* (anaerobic sulphate reductase) and *dsrA, B* (dissimilatory sulphate reductase) genes (Laudenbach and Grossman, 1991; Anantharaman et al., 2017; Kawano et al., 2018), indicates a potential for significant sulphate reduction, notably at the Spill Site. Due to the increase in sulphate reduction it can be suggested an increase in the production of hydrogen sulphide is released into the environment (Muyzer and Stams, 2008). Hydrogen sulphide has been shown to cause various negative impacts, including oxidative damage to RNA and DNA in invertebrates and reduced bacterial growth (Reis et al., 1992; Joyner-Matos et al., 2010). This would suggest during the initial spill and potential increase in hydrogen sulphide to the system, the surrounding area may suffer many of these negative impacts. However sulphate concentrations at the spill increased back to similar levels seen throughout the creek by August relating with the decrease in *Anaerolinea* reads, suggesting rapid recovery of the system, which may partly be attributed to increases in sulphide oxidisers such as *Thiobacillus* that was significantly more abundant at the Spill Site and capable of hydrogen sulphide oxidation to sulphate (Cline and Richards, 1969; Sublette and Sylvester, 1987).

The *mcrA, mcrB and mbhJ* genes involved in the central methanogenic pathway (Qian et al., 2022) were in higher abundance at the Spill Site suggesting an increase in methanogenesis. Notably, bin.11 (Methanomicrobiaceae) (with 95–98 % identity to Methanoregula) was highlighted as a prominent archaeal taxa within the spill site only, and it was undetected at all other sites (Fig. 7), accounting for the increase in abundance of the methanogenic gene pathways. It has previously been suggested that within sites of crude oil contamination, enhanced methanogenesis can take place, via hydrogenotrophic methanogenesis, in which Yang et al. (2018) observed an 8 % increase from 35 % to 43 % in the proportion of hydrogenotrophic methanogens within the oil contaminated soils, highlighting hydrogenotrophic methanogenesis to be significantly more dominant that that of acetoclastic methanogenesis in contaminated sites. This was also observed during the current study as outside of the Spill Site a larger abundance of *acdA* and *acs* genes were present being associated with acetoclastic methanogenesis (Qian et al., 2022), suggesting a switch from hydrogenotrophic methanogenesis to acetoclastic methanogenesis. Further comparisons of metagenomic reads classified as *mcr* via the NCBI nr database using the Basic Local Alignment Search Tool (BLAST), confirmed many of these genes to be associated with a variety of methanogenic archaea, notably *Methanosarcina* (94.5–100 % identity) and *Methanoregula* (95–98 % identity) (Alvarado et al., 2014).

In summary anaerobic processes appeared to dominate the Spill Site between 80 and 158 days after the initial spill, evidenced particularly by increases in abundance of anaerobic hydrocarbon degradation genes *assA, bssA, ahyA* and *abcA*, along with an increase in sulphate reduction coupled to anaerobic hydrocarbon degradation and methanogenesis related genes. By August (158 days after the spill) there was a partial shift to aerobic processes, evidenced by an increase in aerobic hydrocarbon degradation genes such as *ndoA, ndoC, CYP153 P450, sBmoX, Z* and *tmoA/bmoA* and decrease in relative abundance of genes related to methanogenesis. By October (206 days after the spill) both aerobic and anaerobic hydrocarbon degradation genes remained abundant, indicating both aerobic and anaerobic processes were occurring within the Spill Site. Outside of the Spill Site, aerobic processes are shown to be dominant throughout the *study* period, attributed to the high abundance of aerobic hydrocarbon degradation genes, comparative to the Spill Site.

5. Conclusion

By October 2023, 7 months after the spill, the majority of hydrocarbons within the immediate Spill Site had decreased back to the background levels, measured at the outer areas of the spill creek, Brownsea Island and both control sites, highlighting the effectiveness of the combined effects of natural attenuation by aerobic and anaerobic bacteria and the response effort, and further site visits in March 2025 showed complete restoration of the Spill Site (Supplementary Material: Fig. S9). Throughout the spill creek, the presence of multiple functional genes associated with both alkane and PAH degradation indicates the high capacity for both aerobic and anaerobic biodegradation, also highlighting a variety of genera capable of aerobic/anaerobic hydrocarbon degradation and potential candidates as biomarkers for healthy vs. polluted systems.

Overall, this study highlights the potential of coastal microbial communities to naturally attenuate hydrocarbon pollution when coupled with effective remediation response and furthers our understanding of hydrocarbon/bacterial interactions and the degradation pathways highlighted within this system. Further research should be conducted to understand the succession of hydrocarbon degrading functional genes expressed during a spill event and their potential as biomarkers for hydrocarbon exposure (Reid et al., 2020). Most post spill monitoring of microbial communities, is still typically carried out only by 16S rRNA sequence analysis, which significantly limits predictions of an environment's capacity to attenuate hydrocarbon pollution, or the wider effects on other ecosystem processes, but metagenomic sequencing coupled with specific metagenomic tools such as CANT-HYD (Khot et al., 2022) and HADEG (Rojas-Vargas et al., 2023) have much greater predictive power and should be more routinely used as tools with the aim of informing the Net Environment Benefit Analysis (NEBA) and post oil spill monitoring (Kirby et al., 2018).

CRediT authorship contribution statement

Jake A. Smallbone: Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Benjamin H. Gregson:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Terry J. McGenity:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Robert D. Holland:** Writing – review & editing, Supervision. **Corinne Whitby:** Writing – review & editing, Funding acquisition, Conceptualization. **Tom C. Cameron:** Writing – review & editing, Funding acquisition, Conceptualization. **Jon Chamberlain:** Resources, Conceptualization. **Louis G. Clift:** Resources. **Clare Hynes:** Resources. **Boyd A. McKew:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2025.118904.

Data availability

Data will be made available on request.

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