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Connecting gut microbiome changes with fish health conditions in juvenile Atlantic cod (*Gadus morhua*) exposed to dispersed crude oil

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ABSTRACT

Polycyclic aromatic hydrocarbons found in crude oil can impair fish health following sublethal exposure. However, the dysbiosis of microbial communities within the fish host and influence it has on the toxic response of fish following exposure has been less characterized, particularly in marine species. To better understand the effect of dispersed crude oil (DCO) on juvenile Atlantic cod (*Gadus morhua*) microbiota composition and potential targets of exposure within the gut, fish were exposed to 0.05 ppm DCO for 1, 3, 7, or 28 days and 16 S metagenomic and metatranscriptomic sequencing on the gut and RNA sequencing on intestinal content were conducted. In addition to assessing species composition, richness, and diversity from microbial gut community analysis and transcriptomic profiling, the functional capacity of the microbiome was determined. *Mycoplasma* and *Aliivibrio* were the two most abundant genera after DCO exposure and *Photobacterium* the most abundant genus in controls, after 28 days. Metagenomic profiles were only significantly different between treatments after a 28-day exposure. The top identified pathways were involved in energy and the biosynthesis of carbohydrates, fatty acids, amino acids, and cellular structure. Biological processes following fish transcriptomic profiling shared common pathways with microbial functional annotations such as energy, translation, amide biosynthetic process, and proteolysis. There were 58 differently expressed genes determined from metatranscriptomic profiling after 7 days of exposure. Predicted pathways that were altered included those involved in translation, signal transduction, and Wnt signaling. EIF2 signaling was consistently dysregulated following exposure to DCO, regardless of exposure duration, with impairments in IL-22 signaling and spermine and spermidine biosynthesis in fish after 28 days. Data were consistent with predictions of a potentially reduced immune response related to gastrointestinal disease. Herein, transcriptomic-level responses helped explain the relevance of differences in gut microbial communities in fish following DCO exposure.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are among the most toxic components within crude oil that can contain between 2 and 8 fused benzene rings and occur as either parent, hydroxylated, or alkylated forms. Divided in pyrogenic and petrogenic classes, PAHs are present in marine systems and formed by the incomplete combustion of fossil fuels and from oil drilling and extraction processes, respectively (Manzetti, 2013; Pampanin and Sydnes, 2013). The incidence of large-scale oil spills, such as *Exxon Valdez* and *Deepwater Horizon*, exhibit the highest potential for causing detrimental effects to aquatic organisms, which may have long-term impacts to population health (Beyer et al., 2016; Pasparakis et al., 2019; Peterson et al., 2003; Takeshita et al., 2021). However, the chemical composition of oil differs, and in effect the overall toxicity, due to the extent of weathering following oil spills (Carls and Meador, 2009; Esbaugh et al., 2016). Furthermore, a continuous low concentration input from oil and gas operations, via the discharge of produced water, contributes to PAH contamination in the marine environment (Bakke et al., 2013; Sundt et al., 2012).

PAHs found in crude oil, predominantly 3 to 7 ring PAHs and

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alkylated compounds, are known to induce cytotoxic and teratogenic effects (de Soysa et al., 2012; Hose et al., 1996; Pilcher et al., 2014) and genotoxicity in fish, such as forming DNA and protein adducts following exposure (Pampanin et al., 2017). The uptake of xenobiotics, such as PAHs, can occur through aqueous or dietary routes of exposure, with the gut microbiome potentially coming in direct contact to PAHs from crude oil or as metabolites biotransformed by the liver (Aas et al., 2000; Lu et al., 2015; Pampanin et al., 2017; Pérez et al., 2010). The gut microbiome has important functions related to physiological function itself (Egerton et al., 2018), in addition to forming symbiotic relationships with the fish host aiding in immune function, metabolism, and overall health (Holmes et al., 2011; Pérez et al., 2010; Steinmeyer et al., 2015). However, dysbiosis can result from the exposure of PAHs to marine fish, such as juvenile Atlantic cod (*Gadus morhua*) (Bagi et al., 2018; Walter et al., 2019), although consequences on the fish health are not clear. Nevertheless, gut microbiome changes have been proposed as an environmental stress indicator (Adamovsky et al., 2018; Spilsbury et al., 2022; Walter et al., 2019).

Red seabream (*Pagrus major*) and fathead minnow (*Pimephales promelas*) aqueously exposed to individual PAHs, phenanthrene (Hano et al., 2021) and benzo(a)pyrene (BaP) (DeBofsky et al., 2020a), had significant alterations to gut microbial community structures, determined through metagenomic analysis. Similar responses were noted in fathead minnows dietarily exposed to BaP (DeBofsky et al., 2021, DeBofsky et al., 2022). Environmentally relevant exposure scenarios, such as those seen in wild fish collected from the North Saskatchewan River following the Husky oil spill in 2016, exhibited an altered gut microbial response that was reflective of body PAH compositions (DeBofsky et al., 2020b). Oil and dissolved crude oil (DCO) exposures in marine fish have been shown to exhibit significant alterations to gut microbial community structures (Améndola-Pimenta et al., 2020; Bagi et al., 2018; Bayha et al., 2017; Brown-Peterson et al., 2015; Cerqueda-García et al., 2020; Walter et al., 2019). Atlantic cod is a well-known bioindicator, often used in environmental monitoring studies, with previous studies conducted to assess the impact of PAH exposure to fish health (Aas et al., 2000; Baršiene et al., 2006; Bohne-Kjersem et al., 2009; Enerstvedt et al., 2018; Holth et al., 2017). However, there is very limited information available in Atlantic cod regarding microbiome changes in relation to crude oil exposure.

Although metagenomic sequencing used for the identification of microbial phylogenies by 16 S rRNA analysis has provided a means of understanding how exposure to xenobiotics may influence changes to gut microbial communities, it does not provide information regarding alterations to the metatranscriptome and how dysbiosis may influence transcript profiling differences to the host species' health (Bashiardes et al., 2016; Chi et al., 2021). Metatranscriptomic analysis, the expression of bacterial genes, is a novel assessment alone in toxicological studies in fish, with the complementation of metagenomics and host transcriptomic responses less understood (Bashiardes et al., 2016). The integration of sequencing techniques provides a more informative assessment of what may be driving toxic responses and impact to the host species.

Although it has been shown that individual PAHs and those present as mixtures, such as in light, medium, and heavy crude oil, can induce dysbiosis in fish, the relation between altered gut microbial communities and response to the host has not been well characterized in marine fish and considered a shortcoming in gut microbiome studies, potentially underrepresenting effects following exposure scenarios. Due to a lack of understanding of what a representatively normal or healthy gut microbiome is, it can be difficult to identify alterations to fish microbiomes. As such, an altered gut does not necessarily mean an impaired host response to xenobiotic exposure (Chi et al., 2021). Thus, if the functional disconcertion of the gut microbial community is not known, the subsequent consequences to the host health are difficult to determine (Chi et al., 2021). Therefore, the objective of this work was to better characterize how DCO influences the gut microbiome of juvenile Atlantic cod and if potential metagenomic shifts relate to metatranscriptomic alterations in bacterial species and the relationship to transcript profiling in the host species, elucidating phylogenetic alterations to changes in transcriptomic profiles.

2. Materials and methods

2.1. Experimental treatment

Juvenile Atlantic cod (mean length = 15.51 ± 1.05 cm; mean weight = 30.30 ± 6.78 g) were sourced from the Centre for Marine Aquaculture of Nofima (Tromsø, Norway) and transported to the International Research Institute of Stavanger (now NORCE) laboratory (Stavanger, Norway). Fish reared in the Nofima facility in Tromsø were fed a commercial fish feed from Skretting (AgloNorse Ekstra 200–900 μm, AgloNorse 900–1200 μm, Amber Neptune 1.5 and 2.0 mm). Fish were placed into 1000 L fiberglass tanks for a two-week acclimation period, as previously described (Enerstvedt et al., 2018). Fish were fed commercial feed (Amber Neptune, Skretting, Norway) during acclimation and experimentation. Feeding was only stopped two days prior to fish sampling to secure bile samples for other subsequent analyses.

A total of 150 fish, 75 control and 75 to be exposed to DCO, were transferred into two separate fiberglass tanks, noted as control or DCO-exposed treatments. Fish were exposed to an environmentally relevant concentration of DCO (0.05 ppm) by a continuous flow system, according to (Sanni et al., 1998), and further described (Enerstvedt et al., 2018) for 1, 3, 7, or 28 days. The oil was distributed to the exposure tanks by automatic flow rate meters (Watson Marlow 520 S) after being diluted in a header tank. The chemical composition of the DCO within the header tank was collected at five time points throughout the exposure period. PAHs were analyzed by Intertek West Lab AS (Tananger, Norway), a certified commercial laboratory, using the ISO 28540:2011 method protocol, as previously conducted (Bagi et al., 2018; Enerstvedt et al., 2018). A brief description of the methods used are provided in the supplementary material. PAH concentrations are reported in Table S1. Salinity (34 ± 1 ppt), temperature (10 ± 1 °C), water flow (6 ± 1 L/min), and photoperiod (8 h light: 16 h dark) were controlled and monitored throughout the study.

A total of 7 control and DCO-exposed fish were taken after a 1- and 3-day treatment and a total of 25 fish from each group were taken after a 7- and 28-day treatment and sedated using 5 mg/L Aquacalm fish sedative (Metomidate HCl) and sacrificed by a sharp blow to the head. The fish intestinal content (i.e., feces and mucosa), commonly defined as gut microbiome content, was gently squeezed out, using sterile equipment, snap frozen in liquid nitrogen, and stored at −80 °C until analysis. The exposure was carried out in compliance with the Norwegian animal welfare act and national regulations for the use of animals in research (Norwegian Animal Research Authority (Forsøksdyrforvatningen tilsyns-og søknadssystem), ID-17737).

2.2. General fish health

Condition index (CI) and hepatosomatic index (HSI) were assessed to determine the general health status of exposed fish (n = 7 for 1 and 3 day-exposure groups, n = 25 for 7 and 28 day-exposure groups).

The CI, also known as Fulton factor, was calculated as:

$$K = \frac{W}{L^3} \times 100$$

where W is the weight (g) and L the length (cm) of the fish (Lambert and Dutil, 1997).

The HSI, considered a measure of energy reserves of the body, was calculated as:

$$HSI = \frac{LW}{W} \times 100$$

where LW and W are the liver weight (g) and total weight (g) of the fish, respectively (Lambert and Dutil, 1997).

2.3. DNA extraction and sequencing

Total DNA was extracted from gut microbiome content (between 180 and 220 mg; n = 4 per treatment at each time point) using the QIAmp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), per manufacturer's instructions. After DNA extraction, samples were sent to the University of California, Riverside genomics core for library preparation and sequencing. TruSeq Nano DNA sample preparation kit was used to prepare the amplicon libraries for 32 samples using the indexed and barcoded V4–V5-primers: 799Fmod3.

(5′-CMGGATTAGATACCCKGG- 3′) and 1115 R (5′-AGGGTTGCGCTCGTTG -3′). A bioanalyzer was used for the quality control of the libraries. DNA quantification was carried out using a Qubit (Invitrogen Qubit 4 Fluorometer). The Illumina MiSeq platform (Illumina, San Diego, CA, USA) (2 × 300 bp paired end) was used to sequence the V4–V5 region of the 16 S rRNA gene for analyzing the microbial diversity. Raw read sequences were deposited to the NCBI Sequence Read Archive (SRA) database (accession: PRJNA980689).

2.4. RNA extraction and sequencing

Total RNA was extracted from 20 mg of gut microbiome content (n = 3–5 per treatment at each time point) using E.Z.N.A.® Total RNA Kit II (Omega Bio-tek, Norcross, GA, USA), per manufacturer's instructions. An Invitrogen Qubit 4 Fluorometer was used to assess the concentration of RNA and RNA integrity (RIN) and purity determined with an Agilent 2100 Bioanalyzer chip. Samples used for downstream library preparation had an RIN >8. Each RNA sample was treated using DNase I (Promega) and then cleaned with RNA Elite beads (MagBio, Gaithersburg, MD, USA), per manufacturer's instructions. Libraries were constructed using the NEB Next Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich MA, USA). Paired-end, 2 × 75 bp sequencing was conducted on an Illumina NextSeq500 high-throughput sequencer at the University of California, Riverside genomics core. Raw read sequences were deposited to the NCBI SRA database (accession: PRJNA980689).

2.5. Bioinformatics

2.5.1. 16 S rRNA: QIIME2 and PICRUSt2

16 S rRNA sequences were first subjected to a quality control procedure, using QIIME2 (version 2019.4), and feature table files were created to be used as input for the statistical analysis (Bolyen et al., 2019; Caporaso et al., 2010). Quality scores were assigned and examined. The subsequent steps involved quality filtering, trimming, merging, and removal of chimera. These steps were achieved with dada2 (version 1.8) (Callahan et al., 2016). The process of trimming removed the low quality and the primer sequences (first 35 nucleotides) from all the reads, using the option –p-trim-left 35 for forward and reverse reads in combination with a truncating step, setting the options to –p-trunc-len-f 285 for forward reads and –p-trunc-len-r 210 for reverse reads. This led to the removal of low quality (average Q score <25) nucleotides from the end of each read. The taxonomy was obtained by uploading representative sequences on the RDP classifier website and using the default settings of the RDP Naive Bayesian rRNA Classifier version 2.1. Further processing was done using the R package "Phyloseq" and results were visualized using R package "ggplot2" (McMurdie and Holmes, 2013). PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), retrained for the Silva database, was used to predict pathways for each day of exposure (Douglas et al., 2020; Langille et al., 2013).

2.5.2. Metatranscriptome

The bioBakery (v 3.0.0) workflow was used to analyze RNA reads (McIver et al., 2018). Sequenced reads were run within the KneadData v 0.9.0 1 quality control pipeline, using default parameter settings, to filter reads of human origin and low-quality read bases. HumanN v2.0 was used for functional profiling of the metatranscriptome. Alignment quality and sequence length were combined to produce community totals for each protein family, in addition to species-stratified totals, in RPK (reads per kilobase) units. To account for sequence depth variation across samples, RPK units were additionally normalized to RPKM units (reads per kilobase per million sample reads). Enzyme Commission numbers (ECs) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were used for gene functional characterization, which considered each identification present in ≥50% of the samples (3 replicates).

2.5.3. Fish intestinal content transcriptome

To remove reads containing adapters, low quality reads, and poly-N's from raw data, FastQC (version 0.11.9) (Andrews, 2010) was used. All downstream analyses were based on cleaned, trimmed reads. Paired-end clean reads were mapped to the Atlantic cod reference genome (GCF_902167405.1_gadMor3.0_genomic.gff), using HISAT2 (v2.0.5) software. StringTie (v1.3.3 b) was used to assemble the set of transcript isoforms of each bam file obtained in the mapping step and compare assemblies to the Atlantic cod reference annotation files. FeatureCounts (v1.5.0-p3) was used to count the read numbers mapped of each gene, including known and novel genes. Reads per kilobase of exon model per million mapped reads of each gene was calculated based on gene length and read count. Differential expression analysis between two conditions/groups (three biological replicates per condition) was performed using DESeq2 R package (v1.20.0). The p-values were adjusted following Benjamini-Hochberg's false discovery rate correction (FDR) (Benjamini and Hochberg, 1995). A corrected p-value of 0.05 and |log2 (fold-change)| of 1 was set as the threshold for denoting significant differential gene expression. ClusterProfiler (v3.24.3) was used for functional annotation in Gene Ontology (GO). Significant, differentially expressed genes (DEGs) were uploaded to Ingenuity Pathway Analysis (IPA; Qiagen) to generate predicted canonical, disease and function, and network relationships between treatment groups.

2.6. Statistical analysis

Statistical and graphical analyses were performed using the R software environment (version 4.0.3: RStudio Team, 2021) using ggplot2 (version 3.3.3) for data visualization (Wickham, 2016). Normality and homogeneity of variance were assessed using a Shapiro-Wilk and Levene's test, respectively. IBM SPSS Statistics, version 28 (IBM Corp., Armonk, N.Y., USA) was used to conduct a one-way analysis of variance (ANOVA), followed by a Tukey post-hoc test. A value of $p < 0.05$ was used to denote statistical significance.

3. Results

3.1. PAH composition in DCO and health parameter assessments

The mean PAH composition from the header tank used for exposures was 131.05 ± 9.80 μg/L and the PAH profile was stable throughout the study period (Table S1). There were no significant differences between exposure treatment and duration to HSI or CI in juvenile Atlantic cod ($p > 0.05$; Table 1).

3.2. 16 S rRNA community composition, abundance, and diversity

Raw reads comprised an average of 444,283 reads per sample (Table S2). An average of 16.3% of reads were removed following filtering and trimming, with downstream, clean reads used for

Table 1
Mean (± standard deviation) hepatosomatic index (HSI) and condition index (CI) of juvenile Atlantic cod exposed to dispersed crude oil (DCO) for 1, 3, 7, or 28 days (n = 7 for 1 and 3 day-exposure groups, n = 25 for 7 and 28 day-exposure groups).

Treatment	Exposure duration (days)							
	1		3		7		28	
	HSI	CI	HSI	CI	HSI	CI	HSI	CI
Control	7.77 (0.99)	0.80 (0.08)	8.38 (0.94)	0.87 (0.08)	7.75 (1.40)	0.86 (0.08)	9.17 (0.95)	0.93 (0.08)
DCO	7.85 (0.91)	0.81 (0.08)	8.14 (1.06)	0.80 (0.06)	8.65 (1.18)	0.85 (0.07)	9.27 (1.08)	0.91 (0.09)

bioinformatic analysis, as average quality filtered reads of 99.4% represented read quality. Additionally, chimeric sequences were removed from the dataset prior to downstream analysis, which comprised an average of 1.5% of reads. There was a total of 3438 amplicon sequence variants (ASVs) following data processing, which had a mean length of 270 nucleotides (range between 250 and 413 nucleotides). QIIME2 was used to conduct alpha-diversity analyses following rarefaction, in which operational taxonomic units (OTUs) and sequencing depth were determined. A sequencing depth of 99,181 reads/sample was used and determined to be enough to cover the diversity of sequences present in all samples due to curve plateau (Fig. S1).

A non-metric multidimensional scaling (NMDS) analysis, based on a Bray-Curtis distance matrix, was presented as a scattered matrix distribution for all samples. The NMDS was performed using parameters such as fish weight, length, liver weight, CI, and HSI, and were based on ASV counts. There was not a clear separation between exposure groups based on exposure conditions or time (Fig. S2). An *envfit* analysis noted that ASV counts and Pielou's evenness were significantly correlated ($p = 0.001$ and $p = 0.036$, respectively). To visualize smooth surfaces based on ASV read counts of each sample, *ordisurf* was used (Fig. S3). The NMDS plot revealed that fish from control and exposed groups that were sampled after 7 days were separated from the other samples, forming a cluster (Fig. S4). There were no significant differences in gut microbial diversity (Shannon index) or richness (Pielou's evenness) between DCO-exposed and control groups (Fig. 1).

Taxonomic information and ASV relative abundance were combined and filtered based on confidence values for the classifier. Phylum and order level composition was visualized for ASVs with a taxa confidence of >80%. The phylum level plot shows ASVs with >0.01% relative abundance in at least one sample (667 ASVs). The order and genus level plots were prepared only for the most abundant ASVs (>0.1% relative abundance in at least one sample, 58 ASVs) (Fig. 2). There were no significant differences between DCO-exposed and control groups nor between sampling days.

Vibrionales, *Mycoplasmatales*, *Alteromonadales*, *Rhodobacterales*, and *Pseudomonadales* were the most abundant orders in Atlantic cod gut microbiome content in control and DCO-exposed fish. Between the orders identified, there was a significant difference between *Photobacterium* in the control and DCO-exposed fish following a 3-day exposure. *Vibrionales* was the most prevalent orders in both control and DCO-exposed fish. *Photobacterium*, *Aliivibrio*, and *Mycoplasma* were the most dominant genera. Between the genera identified, there was a significant difference between control and DCO-exposed fish after a 28-day exposure. A principal component analysis (PCA) was conducted on the three most abundant genera (Fig. 3), which explained 99.82% of the variance. *Mycoplasma* and *Aliivibrio* were the two most abundant genera in DCO-exposed treatments, while *Photobacterium* was the most abundant genus in control groups. However, the abundance of genera shifted across time, as *Aliivibrio* was the most abundant genus after a 1 and 3-day exposure, whereas *Mycoplasma* was the most abundant genus following a 7 and 28-day exposure.

A predictive metagenomic tool, PICRUSt2, was used to predict the functional capacity of the microbiome across exposure duration and treatment groups. There was a total of 66 significant pathways identified between control and DCO-exposed treatment groups following a 28-day exposure, although no significant pathways identified in 1-, 3-, or 7-days exposure between control and treatments. The identified pathways were comprised within the following categories: fermentation and energy, degradation/assimilation, and carbohydrate, fatty acid, amino acid/nucleotide/vitamin, and cellular structure biosynthesis (Fig. S5).

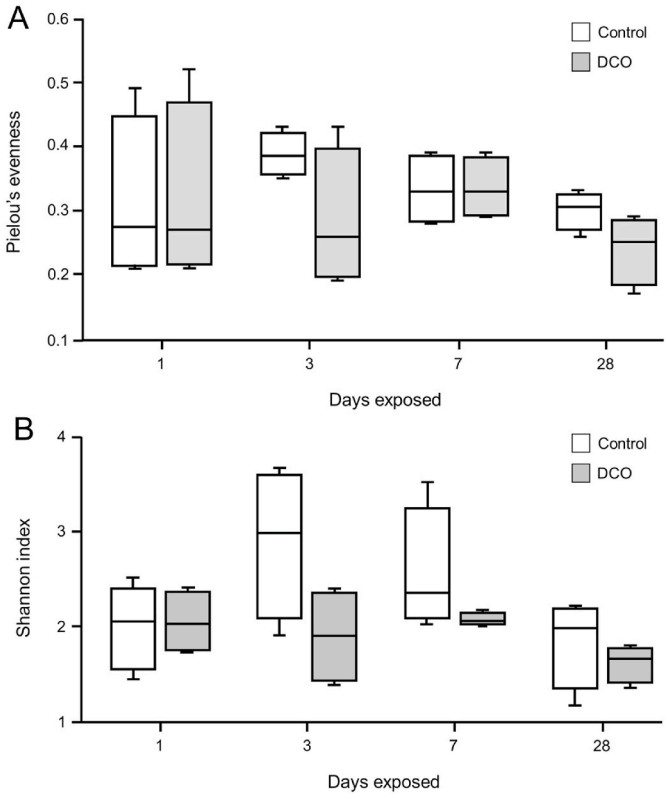

Fig. 1. Boxplots summarizing the diversity indices grouped by sampling time. A) Pielou's evenness and B) Shannon index. DCO = dispersed crude oil. One-way Analysis of Variance (ANOVA), followed by a Tukey post-hoc, (n = 4 per treatment at each time point).

3.3. Metatranscriptome

There was a total of 10,120, 28,618, 29,957, and 16,867 uniquely expressed genes in the guts of Atlantic cod exposed to DCO for 1, 3, 7, and 28 days, respectively (Table S3). Fish exposed for 7 days had 58 DEGs, which were involved in GO pathways related to DNA, RNA, and ATP binding, translation, signal transduction, and Wnt signaling pathway (Table S4). There was one significantly different EC (EC 1.11.1.7) in the 7-day exposed group, which is a heme peroxidase predicted to be involved in metabolic pathways (KEGG: ec01100) and biosynthesis of secondary metabolites (KEGG: ec01110). However, no other treatment group exhibited significantly altered DEGs or ECs, regardless of exposure duration.

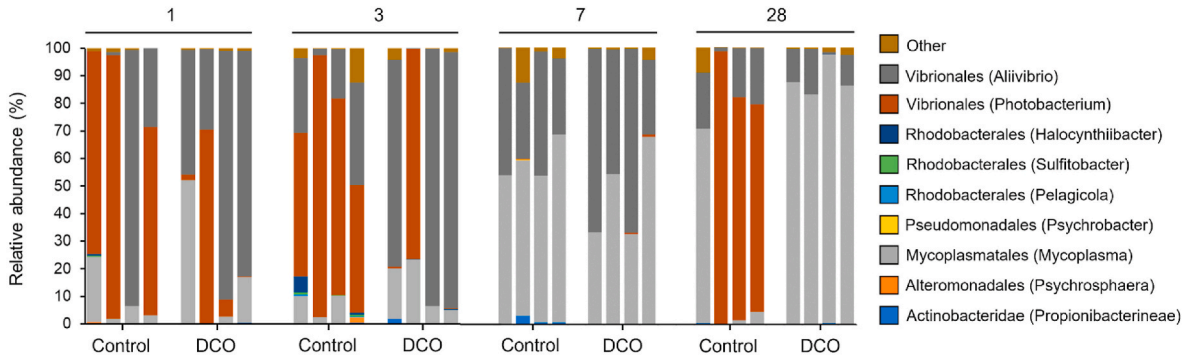

Fig. 2. Effects of dispersed crude oil (DCO) treatment on the orders and genus level of gut microbiome content in juvenile Atlantic cod. Relative abundances are plotted for amplicon sequence variants (ASVs) with >80% classification confidence and relative abundance >0.1% in at least one sample. The legend shows the name of each order and the genus in parentheses. Each relative abundance bar in control and DCO-exposure groups represents an individual fish, with a total of 4 fish per each treatment in fish exposed for either 1, 3, 7, or 28 days ($n = 4$).

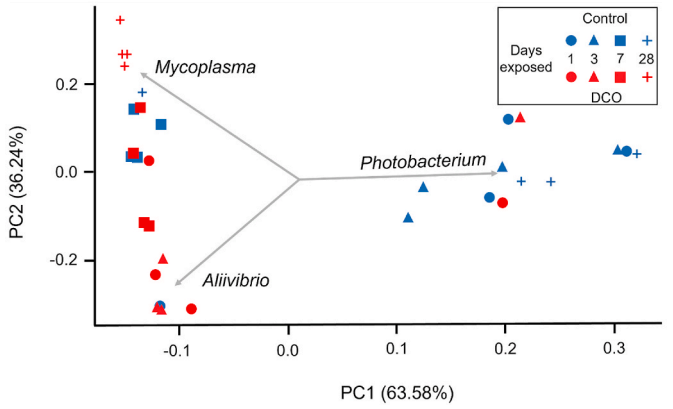

Fig. 3. Principal component analysis. Sampling times are represented by different symbols and exposure conditions denoted as blue (control) or red (dispersed crude oil (DCO)).

3.4. Fish intestinal content transcriptome

A total of 3,850,602,180 mapped reads were generated from juvenile Atlantic cod intestinal content samples and following adapter trimming there were 2,027,094,960 assembled bases (~63.3 million/replicate) used for transcriptome assembly (Table S5). More than 95% of the reads were aligned to the Atlantic cod transcriptome, which indicates a representative assembly of the transcriptome. A total of 9698, 16,280, 12,375, and 10,640 DEGs were detected after a 1, 3, 7, and 28-day oil exposure, respectively (Fig. S6).

3.4.1. Day 1 exposure

The top significant GO biological processes in juvenile Atlantic cod exposed to DCO for 1 day were involved in the generation of precursor metabolites and energy, cellular respiration, energy derivation, the electronic transport chain, and oxidation-reduction processes (Fig. 4A). Oxidative phosphorylation, mitochondrial dysfunction, sirtuin signaling pathway, neutrophil extracellular trap signaling pathway, and glucocorticoid receptor signaling were among the top 10 predicted canonical pathways (Fig. 5A; Table S6). The top five molecular and cellular functions were cell-to-cell signaling and interaction, carbohydrate metabolism, cell morphology, cellular assembly and organization, and

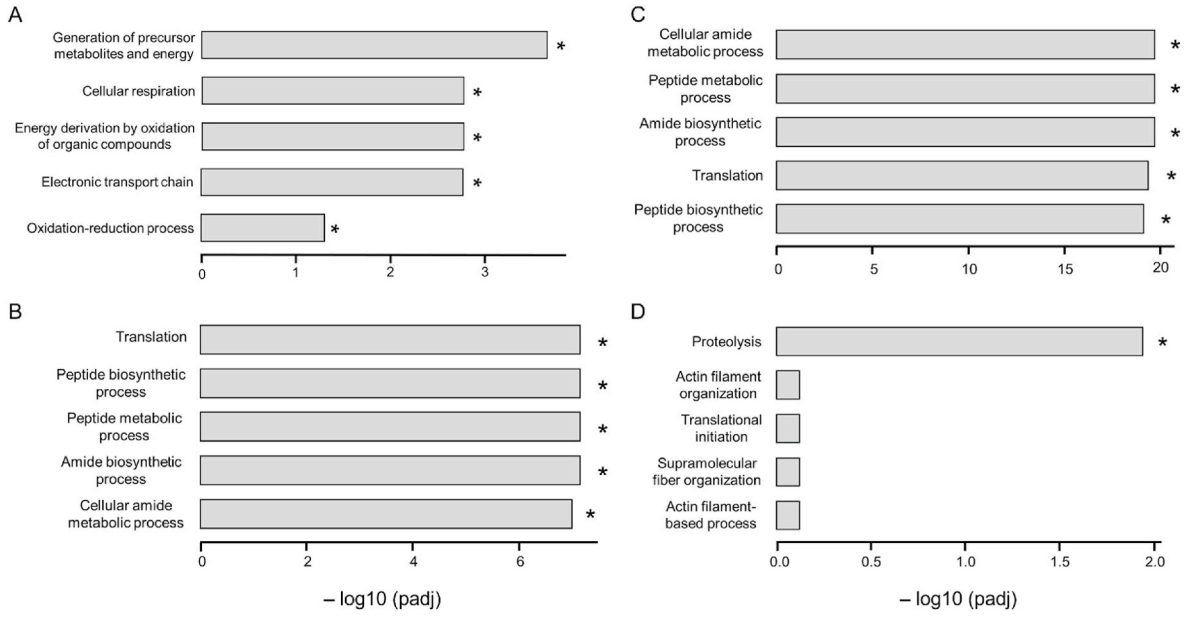

Fig. 4. The top five gene ontology (GO) biological processes in juvenile Atlantic cod exposed to dispersed crude oil (DCO) for A) 1, B) 3, C) 7, and D) 28 days. Asterisks denote significant differences between control and DCO-exposed groups (p-adjusted <0.05).

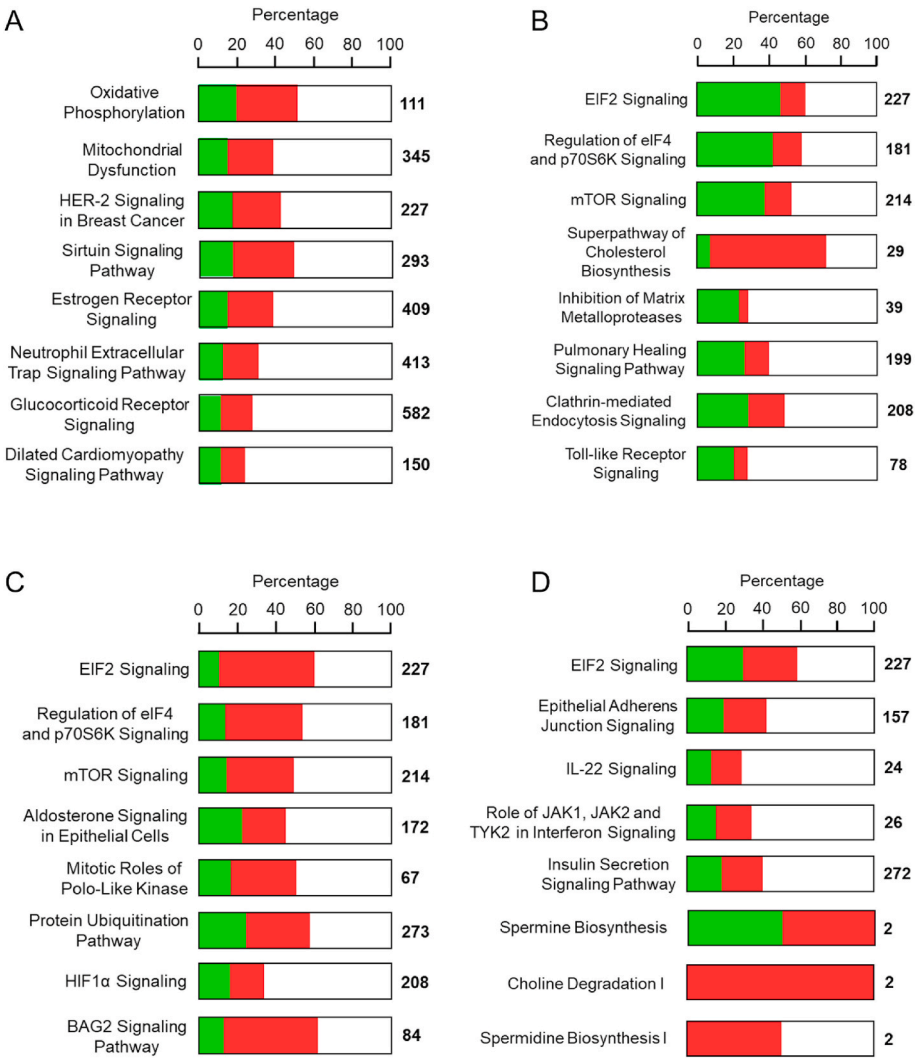

Fig. 5. The top predicted canonical pathways in Ingenuity Pathway Analysis (IPA) in juvenile Atlantic cod exposed to dispersed crude oil for A) 1, B) 3, C) 7, or D) 28 days.

cell death and survival (Table S7). Developmental disorder, hereditary disorder, and metabolic disease was the top altered network, with a score of 22 and 7 target DEGs involved (Fig. S7).

3.4.2. Day 3 exposure

The top significant GO biological processes in juvenile Atlantic cod exposed to DCO for 3 days were involved in translation and peptide biosynthetic, peptide metabolic, amide biosynthetic, and cellular amide metabolic processes (Fig. 4B). EIF2 signaling, regulation of eIF4 and p70S6K signaling, mTOR signaling, superpathway of cholesterol biosynthesis, and inhibition of matrix metalloproteases were the top five predicted canonical pathways (Fig. 5B; Table S6). Cell-to-cell signaling and interaction, cellular assembly and organization, cell death and survival, cell morphology, and cellular movement were the top five molecular and cellular functions (Table S7). Connective tissue disorders, developmental disorder, and hereditary disorder was the top altered network with a score of 34 and 15 target DEGs involved (Fig. S8).

3.4.3. Day 7 exposure

The top significant GO biological processes in juvenile Atlantic cod exposed to DCO for 7 days were involved in cellular amide metabolic process, peptide metabolic process, amide biosynthetic process, translation, and peptide biosynthetic process (Fig. 4C). EIF2 signaling, regulation of eIF4 and p70S6k signaling, mTOR signaling, HIF1α, and immunogenic cell death signaling pathway were among the top 10 predicted canonical pathways (Fig. 5C; Table S6). The top five molecular and cellular functions were cell death and survival, protein synthesis, post-translational modification, protein folding, and RNA post-transcriptional modification (Table S7). Cellular assembly and organization, post-translational modification, and protein folding was a top altered network with a score of 61 and 27 target DEGs involved (Fig. S9).

3.4.4. Day 28 exposure

The significant GO biological process in juvenile Atlantic cod exposed to DCO for 28 days was proteolysis (Fig. 4D). EIF2 signaling, epithelial adherens junction signaling, IL-22 signaling, macrophage classical activation signaling pathway, and ATM signaling pathway were among the top 10 predicted canonical pathways (Fig. 5D; Table S6). Cell morphology, cellular assembly and organization, cellular function and maintenance, cell death and survival, and cellular movement were the top five molecular and cellular functions (Table S7). The top two altered networks were involved in cancer, cell death and survival, and organismal injury and abnormalities with a score of 70 and 29 target DEGs involved (Fig. S10A), and cell death and survival, gastrointestinal disease, and hepatic system disease with a score of 37 and 18 target DEGs involved (Fig. S10B), respectively.

4. Discussion

Metagenomic, 16 S rRNA sequencing was conducted on gut microbiome content of juvenile Atlantic cod following a 1, 3, 7, and 28-day exposure to DCO. Gut microbial community composition, abundance, and diversity were assessed and functional annotations predicted based on metagenomic and metatranscriptomic profiles. Transcriptomic profiling was subsequently assessed in intestinal content of DCO-exposed Atlantic cod with the use of *in silico* software to predict downstream pathways involved in disease and functions based on differential gene expression differences between DCO exposure treatments. There were similarities in the functional capacity of the microbiome and biological processes in samples, which were predominantly reflective of two genera represented in Atlantic cod guts following 28 days of DCO exposure, *Mycoplasma* and *Photobacterium*, although alterations to the metatranscriptome did not exhibit significant expression differences based on DCO exposure durations.

4.1. Fish health

The concentrations of DCO used in this study did not exhibit mortality in juvenile Atlantic cod, regardless of exposure duration. Furthermore, these concentrations did not induce significant alterations to HSI or CI in any treatment. Atlantic cod exposed to between 0.01 and 0.10 mg/L DCO for 28 days did not have significant alterations in HSI or CI (Bagi et al., 2018). Atlantic cod exposed to up to 2.35 μg/L DCO for 15 days did not exhibit a significant difference in HSI or CI, either (Tollefsen et al., 2011). Similarly, Gulf toadfish (*Opsanus beta*) exposed up to 2.82 μg/L $tPAH_{50}$ for 7 days did not exhibit a significant difference in HSI compared to the control group (Alloy et al., 2021). It is apparent that the environmentally relevant concentrations of PAH mixtures comprised within DCO used in this study did not cause overt toxicity. To determine whether sublethal impacts might occur, genomic analyses with subsequent predictions of organism impairment were performed.

4.2. Metagenomics

Neither gut microbial diversity nor richness were significantly different in DCO-exposed juvenile Atlantic cod compared to the controls. *Vibrionales* and *Mycoplasmatales* were among the most dominant orders represented in all treatment groups. The most dominant genera were composed of *Photobacterium*, *Aliivibrio*, and *Mycoplasma* across all samples. These findings are similar to those reported in marine fish (Egerton et al., 2018) that determined *Vibrionales* bacteria comprised a majority (70%) of *Vibrio* and *Photobacterium* genera (Sullam et al., 2012) and 50% *Photobacterium* in untreated, natural populations of Atlantic cod obtained in the Oslo fjord, Norway (Star et al., 2013). Additionally, the bacterial composition of predominately *Photobacterium* identified in this study were reflective of recently wild caught Atlantic cod in Norway (Bagi et al., 2018; Walter et al., 2019). There was an overall shift in genera abundance over time, with *Aliivibrio* being the most abundant genus after 1 and 3 days of DCO exposure and *Mycoplasma* the most abundant after both 7 and 28 days. In Atlantic cod exposed to DCO for 28 days, *Mycoplasma* and *Aliivibrio* were the most representative genera. *Aliivibrio* was among the most predominant genera in the gut of Atlantic salmon (*Salmo salar*) that were determined to be unhealthy (Bozzi et al., 2021), exhibiting severe enteritis (Wang et al., 2018).

The control group was predominantly composed of *Photobacterium*. *Photobacterium* has been reported to aid digestion in fish (Ray et al., 2012) and exhibit an antagonistic relationship with bacterial pathogens found in Atlantic cod, likely contributing to a protective immune function (Caipang et al., 2010). The predominance of *Photobacterium* in the control group shared a similar relationship as red snapper (*Lutjanus campechanus*), which had a significantly higher abundance of *Photobacterium* in seawater controls relative to those exposed to chemically enhanced water accommodated fraction containing Corexit 9500 and *Deepwater Horizon* crude oil for 28 days (Tarnecki et al., 2022).

Regarding the occurrence of *Mycoplasma*, previous studies have reported that all strains are considered to be either pathogenic or parasitic (Fraser et al., 1995; Razin et al., 1998). However, a phylotype that resides in salmonid guts (Holben et al., 2002) has recently been proposed to have co-diversified within the gut of Atlantic salmon and provided adaptive traits to microbial populations that include the metabolism of B vitamins, biosynthesis of essential amino acids, and aiding in defense mechanisms (Rasmussen et al., 2023), acting as a commensal gut species (Llewellyn et al., 2016). Additionally, a correlation between *Mycoplasma* species and weight of Atlantic salmon was observed, with an increased abundance of *Mycoplasma* indicative of fish health (Bozzi et al., 2021). The dominance of *Mycoplasma* in both the control and DCO-exposed groups following 7 days of treatment may be due to both commensal and pathogenic species being represented, reflecting treatment, although this is speculative and additional identification would be needed to determine this relationship. The shift in abundance profiles to *Mycoplasma* species following 28 days of exposure between the DCO-exposed and control groups may be reflective of this relationship, although the consequence of higher abundance has not been characterized in Atlantic cod.

4.3. Metagenomic functional annotations

Relating abundance values with functional annotations can provide interpretations of the significance of alterations in microbiome communities and what role microbiome changes may have in fish health. The top identified functional pathways altered in the intestinal content of Atlantic cod exposed to DCO for 28 days were involved in energy and carbohydrate, fatty acid, amino acid/nucleotide/vitamin, and cellular structure biosynthesis. Similarly, the most representative bacterial genus in Atlantic cod collected along the Norwegian coast was *Photobacterium* (Le Doujet et al., 2019) and the top gut microbiome functional profiles based on genomic assessment were involved in pathways related to carbohydrates, amino acids and derivatives, metabolism of protein, and vitamins (Le Doujet et al., 2019).

As discussed above, *Photobacterium* has been reported to play a large role in protective immune function and aiding in digestion (Caipang et al., 2010; Egerton et al., 2018; MacDonald et al., 1986; Ray et al., 2012; Riiser et al., 2019), with a strong association of *Photobacterium* with the mucosal layer in the intestine, relative to the gut (Riiser et al., 2018). The functional classification of metabolites of the turbot (*Scophthalmus maximus*) gut, which *Photobacterium* was the second most abundant genera, are also involved with energy, carbohydrate and protein metabolism, amino acids and derivatives, cofactors, vitamins, prosthetic groups, pigments, and metabolism of RNA which is consistent with a beneficial relationship between the bacteria and host (Xing et al., 2013).

Regarding the potential relationships between *Mycoplasma* and sublethal toxic response to the host, adult zebrafish exposed to 5 ppm phenanthrene for 24 days had a significant increase in *Mycoplasma* in the intestines, which was suggested to reduce fish immunity, as several targeted immune-specific genes were dysregulated following phenanthrene treatment (Xu et al., 2021). Consistent with a detrimental relationship with the host, there was a significant increase in *Mycoplasma* in the guts of adult zebrafish exposed to 1000 μg/L imazalil for 21 days, with targeted genes involved in glycolysis, nucleotide, lipid, and amino acid metabolism dysregulated following the same exposure, suggesting the involvement of *Mycoplasma* in disrupting gut function (Jin et al., 2017). Additionally, adult zebrafish exposed to 0.5 μg/L enrofloxacin for 4 weeks had a significant increase in the abundance of *Mycoplasma* in the gut, which was associated with reduced immune function (Guo et al., 2020).

4.4. Metatranscriptomic and transcriptomic profiling

There was a total of 58 DEGs in Atlantic cod exposed to DCO for 7 days involved in translation, signal transduction, and Wnt signaling. However, there was only one EC, a heme peroxidase, downstream, that was predicted to be involved in metabolic pathways and the biosynthesis of secondary metabolites. No other treatment, regardless of exposure duration had significantly altered DEGs. Different bacteria can exhibit the same gene expression changes, so shifts in the microbial community may not necessarily follow a shift in overall gene expression profiles (Moya and Ferrer, 2016; Nichols and Davenport, 2021). Therefore, community structure may not be reflective of overall microbiome function. Additionally, there are currently limitations in the ability to annotate transcript changes due to a lack of suitable bioinformatic data, which can limit the capacity to discover and evaluate metatranscriptional functional changes (Aguiar-Pulido et al., 2016; Shakya et al., 2019).

There were significant alterations to bacterial functional annotations seen in Atlantic cod exposed to DCO for 28 days and the transcriptomic response of intestinal content exhibited pathways that were similar to predicted microbiome annotations across treatment groups as a whole, which were irrespective of exposure duration. The top predicted GO biological processes were involved in energy, translation, amide biosynthetic process, and proteolysis. This suggests that transcriptomic responses may be more sensitive and reflective of progressive changes in microbial communities over time. Dysbiosis in gut microbiota can provide a prediction of functional annotations that may influence fish health, but the information provided is somewhat limited as to how the fish responds to such alterations.

Therefore, the use of *in silico* software to generate integrative networks of dysregulated transcripts into potential disease and function pathways provides a means of assessing how altered microbial communities may influence a sublethal toxic response to the host. Functional annotations of bacterial communities were not significantly different in Atlantic cod exposed to DCO for 28 days. However, in Atlantic cod, significant alterations in transcripts were observed even after an exposure for 1 day. Alterations in EIF2 and mTOR signaling were among the top canonical pathways predicted among DCO-exposed Atlantic cod across treatments. IL-22 signaling was the third most enriched canonical pathway in fish exposed for 28 days, driven by an increased expression of *signal transducer and activator of transcription 3* (*stat3*) and *tyrosine kinase 2* (*tyk2*). IL-22 has been shown to be among the first cytokines that initiate an immune response in fish (Costa et al., 2013), as well as serve as a mediator in inflammation, tissue regeneration, and as a protective role against pathogens (Arshad et al., 2020).

It has also been reported that IL-22 is a top mediator in the relationship between the host and microbiota, influencing pathogen resistance (Schreiber et al., 2015). It was recently found that an induction of IL-22 is prolonged through an activation of both the AhR and NF-κB signaling pathways (Ishihara et al., 2022). As bacterial pathogen exposure can induce EIF2 signaling, which is required for the inhibition of NF-κB activation, alterations to EIF2 signaling has a higher potential for bacterial invasion (Shrestha et al., 2012). This suggests that the altered EIF2 signaling in Atlantic cod following DCO exposure may induce a greater toxic response over time, as those treated for 28 days had altered IL-22 signaling, potentially inducing a greater immune response that was suggested with an increase in *Mycoplasma*.

Furthermore, in Atlantic cod exposed to DCO for 28 days, alterations in spermine and spermidine biosynthesis were also predicted. Both polyamines are known to exhibit protective functions for DNA against reactive oxygen species (Rider et al., 2007), which serve as free radical scavengers (Ha et al., 1998), as well as supporting gut immunity by regulating T-cell differentiation (Carriche et al., 2021). Similarly, there were altered levels of spermine and spermidine in Atlantic killifish (*Fundulus heteroclitus*) exposed to PAHs from the Elizabeth River (Redfern et al., 2021). Reduced levels of spermidine have been linked with colitis and dysbiosis of the microbiome (Gobert et al., 2022), which was consistent with predictions of gastrointestinal disease as the top predicted network in 28 day exposed Atlantic cod. This suggests that spermine and spermidine could serve as sensitive targets of chronic oil exposure in the gut.

5. Conclusions

Juvenile Atlantic cod exposed to DCO for between 1 and 28 days had a shift in microbial community composition, reflecting an increase in *Mycoplasma* in DCO-exposed fish and *Photobacterium* in controls after 28 days. The functional profile of the gut microbiota was significantly different between treatments after 28 days of exposure, which were characterized by energy and carbohydrate, fatty acid, and amino acid biosynthesis. Metagenomic annotations were comparable to predictive gene ontology biological processes in DCO-exposed Atlantic cod, although there was an overall lack of change in metatranscriptomic responses. Transcriptomic profiling of Atlantic cod intestinal content noted significant alterations as early as 1 day following oil treatment, with similar canonical pathways shared across exposure duration, including EIF2 signaling. Immune function, driven by altered IL-22 signaling and spermine and spermidine biosynthesis, were the top canonical pathways in 28-day DCO-exposed Atlantic cod, which was further related to predicted gastrointestinal disease. As a normal or healthy gut microbiome is still not well defined (Chi et al., 2021), it is of increasing importance to incorporate not only multiomic approaches, such as metagenomic and RNA sequencing, but several functional annotation techniques to better characterize the influence of alterations in gut microbial content to the host species, gaining further insights into potential, downstream diseases and functions that may impair fish health following crude oil exposure. As PAH exposure profiles may further influence gut microbial composition in fish, it is possible that oil-specific microbiome changes could indicate a light or heavy oil PAH profile. However, further study is needed to support this proposed relationship.

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Ethical treatment of animals

This research was carried out in compliance with the Norwegian animal welfare act and national regulations for the use of animals in research (Norwegian Animal Research Authority (Forsøksdyrforvatningen tilsyns-og søknadssystem), ID-17737).

CRediT authorship contribution statement

Jason T. Magnuson: Formal analysis, Visualization, Writing - original draft. **Giovanna Monticelli:** Formal analysis, Visualization, Investigation, Writing-review & editing. **Daniel Schlenk:** Conceptualization, Resources, Writing-review & editing. **Joseph H. Bisesi:** Resources, Investigation, Writing-review & editing. **Daniela M. Pampanin:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing-review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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