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Impacts of Marine Oil Snow on Temperate Shallow-Water Sponge *Halichondria panicea*

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1) Introduction

Sponges (phylum Porifera) constitute key marine organisms in the many aquatic environments they are present in (Maldonado *et al.*, 2015). Sponges perform a wide range of ecological services, including (but not limited to) the recycling of dissolved organic matter to particulate organic matter (de Goeij *et al.,* 2013) as well as participation in the nitrogen, phosphorous and silicate cycles (Maldonado *et al.,* 2012). Furthermore, some of the ecological services provided by sponges are actually performed by the complex microbial community associated with the sponge (Webster *et al.,* 2012). In the deep-sea, sponges can form high-density grounds, which constitute a major deep-sea habitat supporting diverse benthic communities (Hogg *et al.*, 2010). However, the resilience of sponges to human activities such as oil and gas production activities are only just beginning to be investigated. Specifically impacts of oil spills on marine sponges need to be investigated further (Luter *et al.*, 2019; Vad *et al.*, 2018, 2020).

Previous studies exposing sponges to crude oil and dispersants contaminated seawater and sediment have been conducted using the model shallow-water sponge *Halichondria panicea* (Vad *et al.*, 2020, Submitted A, B). These experiments showed that exposure to oil contaminated media led to strong negative impacts in sponge *H. panicea* at physiological and molecular levels and that these impacts were worsened by the addition of dispersants. However, during an oil spill, benthic organisms are not only exposed to contaminated seawater and re-suspended sediments but also to marine oil snow (MOS). MOS is composed of floating mucilaginous organic particles in which oil droplets are embedded and could be consumed by benthic organisms (Duran Suja *et al.,* 2017). The impact of marine oil snow has not previously been investigated in sponges, despite the great risks MOS is known to pose to other marine invertebrates (van Eenennam *et al.,* 2018).

In this study, sponge *H. panicea* was exposed to contaminated seawater and dispersant and MOS for a week to determine the impact of MOS on the sponge physiology, gene expression profile and associated bacterial community. Throughout the experiment (after one, three and five days of exposure), seawater properties were monitored through the measurements of pH, dissolved oxygen and concentration of polycyclic aromatic hydrocarbons. Sponge tissue oxygen concentration was also determined at each time points to determine if the sponges continued its pumping activity. Finally, tissue samples were gathered halfway through the exposure, and meta-transcriptomic sequencing was carried out to identify any changes in gene expression profiles within the sponge and its associated bacterial community.

2) Material and Methods

Sampling

Sponge and seawater samples were collected at Coldingham bay, located 80 kilometres to the south of Edinburgh (55.89°N, 2.13°W). *H. panicea* sponges can easily be found in the bay at low tide and grows in an encrusting yellow to greenish morphotype. Around 20 individuals of *H. panicea* were carefully removed with a scalpel from the rocks and placed into sampling bags filled with freshly collected seawater. Around 50L of surface seawater samples were collected in clean plastic carboys with the returning tide. All samples were stored in insulated containers and quickly returned to University of Edinburgh. Upon arrival, sponges were transferred to recirculating seawater tanks in a control temperature room at 10° C corresponding to the temperature of the seawater at the time of the sample collection. Seawater samples were used immediately in the experiments upon return to the university.

Experimental Design

The flow-through experimental apparatus described in detail in Vad *et al.* (2020) was used in this study. This experimental apparatus was constituted of 15 individual 750 mL glass incubation chambers sealed with a polytetrafluoroethylene lid. An inflow and outflow in the lid allowed seawater to flow through the chamber at a rate of 750 mL/day, thanks to multichannel peristaltic pumps. Individual sponges were placed in the incubation chambers at the beginning of the experiment and left to acclimatise in seawater for 48 h. After the acclimatisation phase, treatment solutions were connected up to the incubation chambers to start the exposure. The treatment conditions (three replicates per treatment) considered in this experiment were: control (filtered seawater), marine snow (MS; filtered seawater with MS added), chemically enhanced accommodated fraction (CEWAF), marine oil snow (MOS; filtered seawater with MOS added) and CEWAF and MOS (CEWAF solution with added MOS).

Water accommodated fractions and MOS preparation

Chemically enhanced water accommodated fractions (CEWAFs) were produced following the Chemical Response to Oil Spills: Ecological Research Forum (CROSERF) methodology (Aurand and Coelho, 2005) with Schiehallion crude oil (BP) and dispersant Slickgone NS (Dasic International). CEWAF solutions was prepared with 1 L of seawater amended with 1.0 g Schiehallion crude oil and dispersant Slickgone NS (Dasic International) was applied at a volume ratio of 1:10 as advised by the manufacturers. The solutions were then mixed using an oxygen pump for 48 h at 10° C in the dark in clean sterile (acid-washed) 1 L glass bottles. The mixtures were allowed to stand for 1 h and then the aqueous phases (avoiding non-dispersed/solubilized oil or dispersant) were sub-sampled into clean (autoclaved and acid-washed with 5% nitric acid) screw-capped glass tubes with Teflon caps. These CEWAF solutions were stored at 4° C and used within 48 h in the microcosm experiment. Marine snow (MS) and marine oil snow (MOS) aggregates were obtained by rotating three 1 L bottles of seawater and three 1 L bottles CEWAF at 10° C at 50 rpm for 48h. These CEWAF solutions were stored at 4° C and used within 48 h in the microcosm experiment.

Water properties

Water properties were monitored after 1, 3 and 5 days of exposure by sampling water directly from the incubation chambers where each sponge were held and measuring oxygen concentration and pH. A subsample of 100 mL of seawater was also retained to measure concentration of polycyclic aromatic hydrocarbons through GC/MS analysis. Samples were sent to Terra Tek (Birmingham, UK) for total and speciated USEPA PAHs GC/MS analysis (Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[a]Anthracene, Chrysene, Benzo[b]Fluoranthene, Benzo[k]Fluoranthene, Benzo[a]Pyrene, Indeno[1,2,3,c,d]Pyrene, Dibenz[a,h]Anthracene and Benzo [g,h,i]Perylene). Samples were weighed, surrogate spike added (to check recovery), and then extracted in DCM and using ultra-sonication. One millilitre of the extract was then spiked with internal standard in a 2 mL vial, caped and analysed by GC/MS. The GC/MS analysis was performed on an Agilent 6890 GC with 5973 inert MSD detector in SIM mode, on a 30 m x 0.25 mm ID x 0.25 µm df column with 5 m guard column (Restek p/n 13623-124).

Dissolved oxygen concentration VS tissue depth profile

Dissolved oxygen concentration across the sponge tissue was collected after one, three and five days of exposure with a PreSens O2 microneedle mounted on a manual micromanipulator (PreSens Precision Sensing GmbH, Germany). This set-up allows for the measurement of oxygen within the sponge tissue at discrete tissue depth. An oxygen depth profile can be drawn from this data showing the concentration of dissolved oxygen through the sponge tissue.

RNA extraction

Tissue samples from all 15 sponges in this experiment was gathered at the halfway point of the experiment, e.i. after 3 days of exposure. Sponge tissue were rinsed with sterile water before being placed in Eppendorf tubes and kept at -20° C in RNAlater until RNA extractions were performed. Total RNA was extracted from sponge tissue subsamples using Qiagen (UK) RNAeasy® extraction kits following the manufacturer's instruction. Small sections of the sponge subsamples (about 1 mm_3) were homogenised using a MSE SoniPrep (UK) sonicator and lysed using the provided lysing buffer. DNA was removed from the RNA extractions with Qiagen RNase-Free DNase sets. At the end of the protocol, RNA was eluted into 30 µL of DNA/RNA free sterile water. RNA quality and quantity were then assessed by spectrophotometer by NanoDrop™ 2000 (ThermoFisher Scientific, UK). Only RNA samples with a 260/230 and 260/280 ratio of 1.8-2.2 were sent to sequencing.

Meta-transcriptomics sequencing

RNA extraction from all fifteen samples were sent to Edinburgh Genomics for Illumina library preparation with RiboZero treatment and with one round of prokarotic and eukaryotic rRNA depletions, followed by NovaSeq 100PE sequencing. Paired-end reads were run to 100 base pairs to yield at least 750 M + 750 M reads. Analysis of the sequencing data was based on the method described in Gonzalez *et al.* (2018). Upon reception of the sequencing data, Illumina adaptors were trimmed, and the quality of the reads was checked using Fastqc (version 0.11.5; Andrews, 2015). Reads under a quality Phred score of 30 were removed. A meta-transcriptome assembly was then constructed with Megahit (version 1.2.9; Li *et al.*, 2016) using the University of Edinburgh Compute and Data Facility (Edinburgh Compute and Data Facility web site, last accessed: 16/07/2020). Annotation of the meta-assembly was achieved through diamond blastx searches(version 0.9.32; Buchfink *et al*.; 2015) against the *nr* database using an e-value cut-off of 1e-5. Binning of the meta-transcriptome assembly was performed using MEGAN6 Community Edition (version 6.19.4; Huson *et al.*, 2016) to separate the sponge transcriptome from the bacterial meta-transcriptome. To assess sponge assembly completeness, BUSCO was run against the eukaryote_odb9 and metazoan_odb9 datasets (Kenny et al., 2018; Seppey et al., 2019). Transcripts were then quantified by RSEM (Li and Dewey, 2011) and a non-metric multidimensional scaling (nMDS) ordination analysis of normalised expression levels from each sample was performed. Differential expression analysis was conducted on the sponge transcriptome and bacterial meta transcriptome separately with the package EdgeR in R (version 3.24.3; Robinson et al., 2010). Finally, a gene ontology (GO) enrichment analysis was conducted using the package TopGO in R (version 2.34.00; Alexa and Rahnenfuhrer, 2016).

3) Preliminary Results and Discussion

Gross observations

In the control bottles containing only seawater, MS aggregates formed after 4 days. They appear white and of flocculent texture. They stayed quite small through the length of the experiment and were characterised by an amorphous shape never larger than 5mm. In the CEWAF bottles, formation of MOS was observed within 4 days. In all bottles the MOS particles appeared brownish, round and of flocculent texture**.** At the beginning of the experiment, aggregates were small (<3 mm in diameter) and exhibiting amorphous definition. Small oil droplets could be observed within the amorphous matrix of the MOS aggregates, with the naked eye. This confirms the fact that the oil droplets were associated to MS, creating MOS. The MOS aggregates got bigger as the experiment continued reaching almost 1.5 cm in diameter.

In the CEWAF and CEWAF+MOS treatments, sponge changed in colour and darkened after 4 days of exposure. In the CEWAF+MOS treatment, all sponges died and turned fully black after 5 days of exposure. This stage was reached in the CEWAF treatment at 7 days of exposure. Signs of distress (darker coloration) were also observed in the MOS treatment for two of the samples although the sponges appeared to survive the treatment. No external signs of stress were observed in the control and MS treatments.

Water properties

pH and oxygen concentration changed rapidly in the MOS, CEWAF and CEWAF+MOS treatments. Within the first day of exposure, pH dropped from an average of 8.1 in the control and MS treatments to 7.9, 7.5 and 7.7 in the MOS, CEWAF and CEWAF+MOS treatments respectively (**Fig. 1A**). Within the first day of exposure, dissolved oxygen concentration decreased from 106.0 µmol/L and 95.7 µmol/L in the control and MS treatments to 87.5 µmol/L and 73.5 µmol/L in the CEWAF and CEWAF+MOS treatments respectively (**Fig. 1B**). The oxygen concentration in MOS treatment stayed within control levels and reached 106.8 µmol/L (**Fig. 1B**). Changes in pH and oxygen concentration seen are most probably due to the microbial degradation of oil in all the hydrocarbon treatments and is consistent with measurement taken in the field during an oil spill (Valentine *et al.,* 2010).

Polycyclic aromatic hydrocarbons were detected throughout the experiment in the CEWAF and CEWAF+MOS treatments. No hydrocarbons were found above detection limit in the control, MS and MOS treatments (**Fig. 1C**). Specifically, naphthalene, fluorene and phenanthrene were detected in the CEWAF and CEWAF+MOS treatments (**Fig. 1C**). The Σ_{16}PAH concentration reached a maximum of 1.03×10^4 ng/µL in the CEWAF treatment and of 2.19×10^4 ng/µL in the CEWAF+MOS treatment (**Fig. 1C**). The hydrocarbon concentrations found in this study are consistent the CROSERF report (Aurand and Coelho, 2005) but several order of magnitude lower than measurements made in the field during oil spills (Diercks *et al.,* 2010). No hydrocarbon was detected in the MOS treatment probably because

of the very low amount of MOS added to this treatment. This highlights the sensitivity of sponges to the effect of MOS as even a very low exposure can lead to negative biological impacts (see sections below).

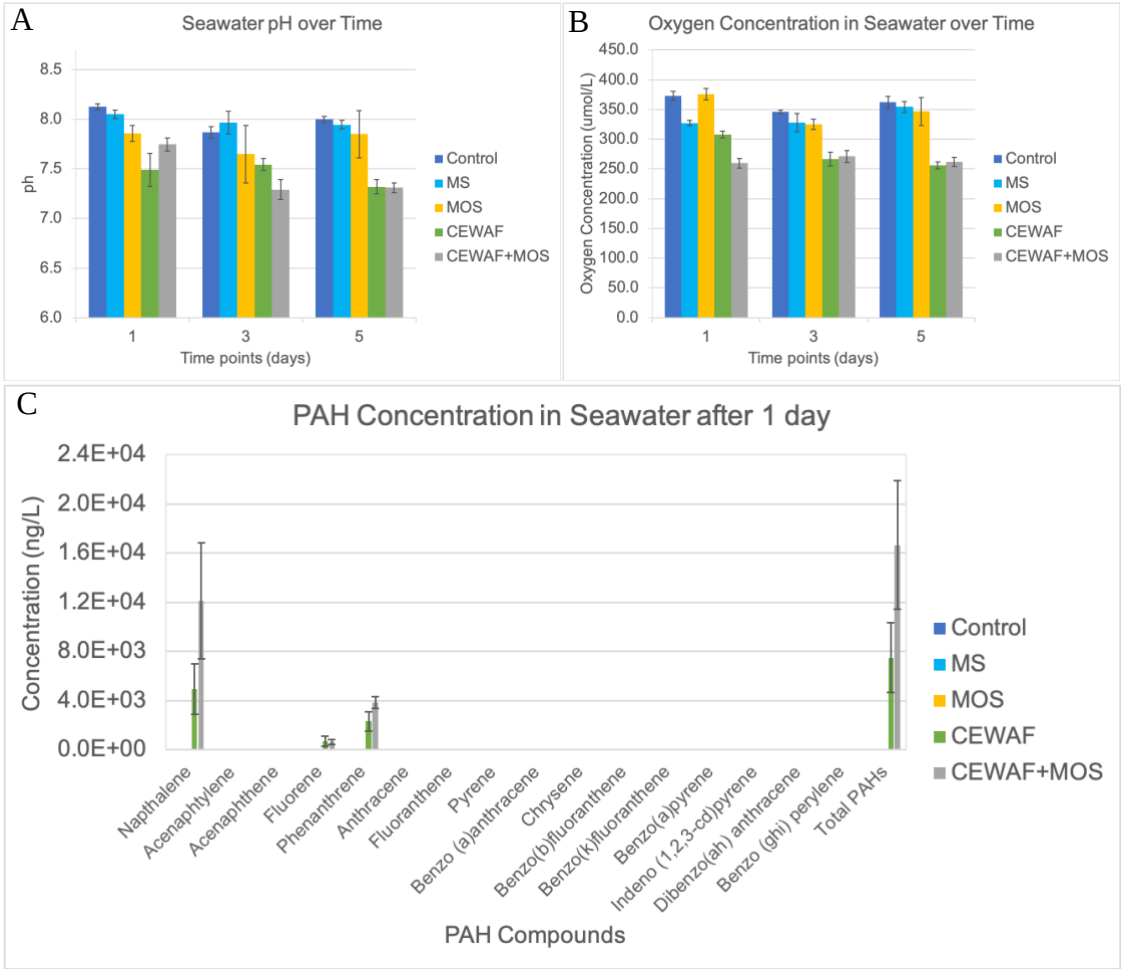

Figure 1 Seawater properties across treatments. (A) pH in each treatment across sampling time. (B) Dissolved oxygen concentration in each treatment across sampling points. (C) Polycyclic aromatic hydrocarbon (PAH) concentration in seawater after one day of exposure.

Sponge Tissue Oxygen concentration

Oxygen tissue concentration differed significantly between treatments. In the control treatment, oxygen tissue concentration decreased with tissue depth until it stabilised at ~58 µmol/L from a tissue depth of 400 µm (**Fig. 2**). The MS treatment followed a similar trend, but the plateau was significantly higher (~88 µmol/L) and reached at a shallower tissue depth of 300 µm (**Fig. 2**). In all the hydrocarbons treatments (MOS, CEWAF and CEWAF+MOS), oxygen tissue concentration dropped with tissue depth until it reached 0 µmol/L at a depth of 800 µm (**Fig. 2**). The pattern seen in the tissue oxygen concentration data is demonstrating that the sponge pumping activity stops when exposed to hydrocarbons (MOS, CEWAF or CEWAF+MOS). This is consistent with previous studies on *H. panicea* exposed to hydrocarbon contaminated seawater and sediments (Vad *et al*., 2020 and Submitted A). The elevated oxygen plateau observed in the MS treatment is most likely due to a

higher pumping rate measured in sponges exposed to MS. This would suggest that sponges are actively feeding on the added MS.

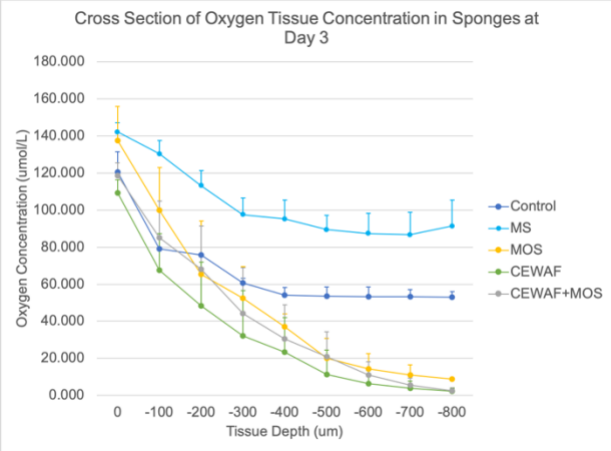

Figure 2 Dissolved Oxygen concentration across the sponge tissue.

Meta-transcriptomics

The meta-transcriptomic sequencing yielded a large amount of sequencing information and an average of 53 million reads per sample. The megahit meta-assembly consisted of 557,483 contigs of which 69% could be identified against the *nr* database (**table 1**). Amongst the contigs with ID, 11% were identified as belonging to the Eukaryotic domain and therefore to the sponge transcriptome while 70% were identified as Bacterial contigs and hence binned into the symbiotic meta-assembly (**table 1**). A BUSCO analysis revealed that the sponge assembly was highly complete against both eukaryotic (91.5% complete BUSCOs, 8.5% fragmented BUSCOs and 0.0% missing BUSCOs) and metazoan (85.4% complete BUSCOs, 9.3% fragmented BUSCOs and 5.3% missing BUSCOs) databases, demonstrating the high quality of the sequencing analysis performed in this study.

Table 1 Overview of megahit meta-assembly

	NUMBER OF CONTIGS
META-ASSEMBLY	557,483
WITH BLAST ID	383,883
SPONGE ASSEMBLY (EUKARYOTIC CONTIGS)	43,782
SYMBIONT META-ASSEMBLY (BACTERIAL CONTIGS)	269,037

Quantification of reads aligned to the meta-assembly revealed significant differences in gene expression pattern across samples. CEWAF and CEWAFMOS samples display close expression profiles which are significantly different from control samples (**Fig. 4**). Interestingly, MOS and MS samples display closer expression profiles (**Fig. 4**) and further comparison between control and MS samples should be carried out. Differential expression analysis is currently underway to identify down- and up-regulated genes amongst both the sponge host transcriptome as well as the bacterial symbiont meta-transcriptome.

4) Conclusion

The preliminary results of this study demonstrate that sponge *H. panicea* is unable to survive above a seven-day exposure to marine oil snow and contaminated seawater. In light of these results and taking into consideration previous studies, it is recommended that the use of dispersant in sponge-dense areas should be avoided. The metatranscriptomic dataset is expected to further reveal the impact of MOS and MS on the *H. panicea* holobiont. A manuscript for submission to a high impact paper is in preparation summarising the finding of this study.

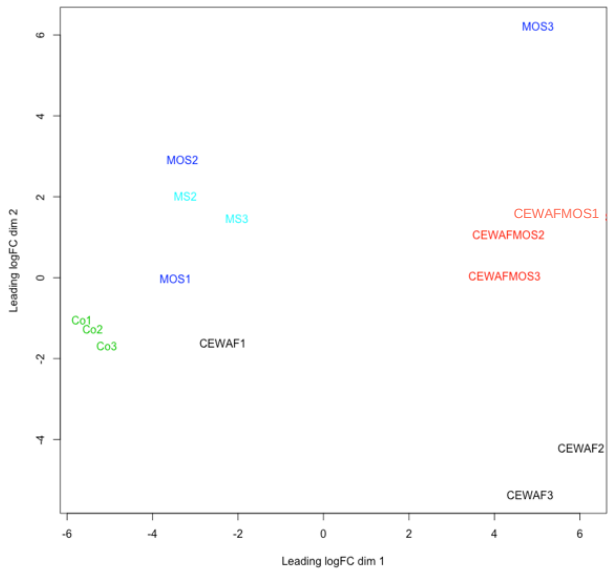

Figure 3 nMDS of holobiont normalised expression matrix.

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