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Morphological and transcriptional effects of crude oil and dispersant exposure on the marine sponge *Cinachyrella alloclada*

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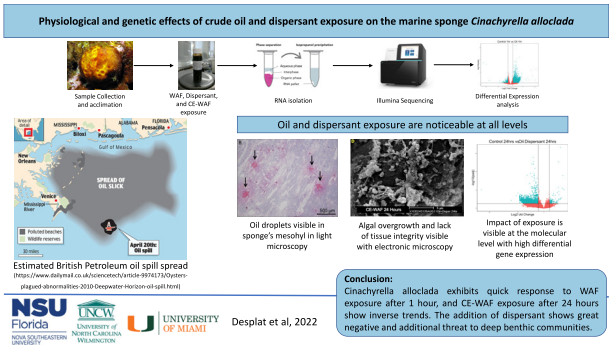
HIGHLIGHTS

- Differential expression is noticeable as early as one hour after exposure.
- *Cinachyrella* can be used as bioindicator species for shallow reefs system in the face of environmental catastophes.
- Better oil spill responses are necessary to preserve deep and shallow marine ecosystems.
- Marine sponges show potential for new laboratory model with relevance to higher taxa studies.

GRAPHICAL ABSTRACT



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ABSTRACT

Marine sponges play important roles in benthic ecosystems. More than providing shelter and food to other species, they help maintain water quality by regulating nitrogen and ammonium levels in the water, and bioaccumulate heavy metals. This system, however, is particularly sensitive to sudden environmental changes including catastrophic pollution event such as oil spills. Hundreds of oil platforms are currently actively extracting oil and gas in the Gulf of Mexico. To test the vulnerability of the benthic ecosystems to oil spills, we utilized the Caribbean reef sponge, *Cinachyrella alloclada*, as a novel experimental indicator. We have exposed organisms to crude oil and oil dispersant for up to 24 h and measured resultant gene expression changes. Our findings indicate that 1-hour exposure to water accommodated fractions (WAF) was enough to elicit massive shifts in gene expression in sponges and host bacterial communities (8052 differentially expressed transcripts) with the up-regulation of stress related pathways, cancer related pathways, and cell integrity pathways. Genes that were upregulated included heat shock proteins, apoptosis, oncogenes (Rab/Ras, Src, CMYC), and several E3 ubiquitin ligases. 24-hour exposure of chemically enhanced WAF (CE-WAF) had the greatest impact to benthic communities, resulting in mostly downregulation of gene expression (4248 differentially expressed transcripts). Gene deregulation from 1-hour treatments follow this decreasing trend of toxicity: **WAF > CE-WAF > D**ispersant, while the 24-hour treatment showed a shift to **CE-WAF > D**ispersant **> WAF** in our experiments. Thus, this study supports the development of *Cinachyrella alloclada* as a research model organism and bioindicator species for Florida reefs and underscores the importance of developing more efficient and safer ways to remove oil in the event of a spill catastrophe.

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1. Introduction

1.1. Environmental riches and threats

The Gulf of Mexico (GoM) is a unique oceanic basin which includes diverse habitats such as saltwater marshes, Flower Garden coral reefs, brine pools, methane seeps, and salt domes. These niches overlie rich reservoirs of crude oil and natural gas. In recent years, the number of oil wells have increased around the globe to match an ever-increasing demand for fossil energy, and the GoM is no exception. As of November 2019, over 1850 rigs were actively extracting crude oil and gas in the gulf (BSEE, 2021). The continued demand for minerals, crude oil and its refined products (Niner et al., 2018; Hughes and Jones, 2020), has driven prospecting to deeper oceanic regions, posing major environmental threats to unique benthic habitats and biodiversity. The GoM reaches down to 4384 m, meaning this race to extraction could put a threat on areas where little about the biology nor the environment is known.

After the Deepwater Horizon (DWH) oil spill in 2010, and its impact on the marine landscape, studying the impact of oil spill related chemicals in the water column became a critical need to understand how ecosystems are impacted by such environmental disasters, and how to mitigate response in such situations. Long-term effects of oil spills may be underestimated at both the temporal (Girard and Fisher, 2018) and spatial level (Berenshtein et al., 2020). For example, the high volatility of several low molecular weight components of crude oil (e.g. benzene, toluene) on the respiratory system of birds and mammals causes severe lung inflammation and breathing problems, in addition to immune system deficiency and reproductive impairment. Additionally, most studies throughout the years have focused on the impact of oil spills on species of commercial interest (Edmunds et al., 2015; Snyder et al., 2019), microbial communities (Bacosa et al., 2018), reef communities (Lewis et al., 2020), and keystone species with biodiversity ramifications such as corals (Turner and Renegar, 2017; DeLeo et al., 2021). Fewer studies have focused on the impact of oil and dispersant on diverse benthic communities (Reuscher et al., 2020; Fisher et al., 2016; Frometa et al., 2017).

Corals can be long lived residents of marine habitats and therefore have been raised as possible indicators of local environmental health (Fisher et al., 2014a, 2014b). A major concern during the Deepwater Horizon oil spill was that crude oil could be swept into the Loop Current and carried thousands of miles to contaminate the reef tract on the east coast of Florida. Though this scenario did not happen during the DWH oil spill (Liu et al., 2011), it prompted development and validation of new oil spill models via complex mesoscale oceanographic features using new datasets to better predict dispersion and circulation (Johnston et al., 2019).

Another concern directly linked to the large amount of crude oil that leaked into the Gulf was the impact on deep sea communities. While efforts at the surface focused on collecting as much crude material as possible, preventing compounds from reaching the shore and preserving ecosystems, many remedial actions were taken at the source of the leak to stop crude oil from reaching the surface. This was effectively accomplished by spraying dispersant directly at the source of the leak at the Macondo well head (National Commission on the BP Deepwater Horizon Oil Spill and Offshore Drilling, 2010). Consequently, a vast amount of crude minerals sank to the bottom of the Gulf (Romero et al., 2015), home to many deep-sea coral reefs and ecosystems only observable through remote deep-sea exploration (White et al., 2012; Fisher et al., 2016). Although more visible beaches and coastal habitats were spared the brunt of the Deepwater Horizon oil spill (DWHOS), the impact to the deep environment are less understood. The DWHOS unveiled that the deep-sea environment and benthic communities should also be considered in mitigation plans during large scale environmental catastrophes (Fisher et al., 2016; Montagna et al., 2013; Sutton et al., 2021). Similarly, detrimental impact to surface fauna and flora must also be adequately addressed and studied (Bretherton et al., 2019; DeMiguel-Jiménez et al., 2021; DeLeo et al., 2021). The long-term effects of crude oil and dispersant in the water column remains less studied as this requires data collection over longer time periods (Stroski et al., 2019). More than a decade has passed since the DWH tragedy, and some studies are starting to show the resilience of organisms across diverse habitats (Barron et al., 2020; Bagby et al., 2017; Reuscher et al., 2020). From the bacterial community changes in the salt marshes of the Louisiana coast (Engel et al., 2017), steady decomposition and concomitant decontamination of sediments around the Gulf of Mexico (Rogers et al., 2019), to recolonization of deep-sea coral communities (White et al., 2012) and other benthic life (Schwing et al., 2020; McClain et al., 2019), a better prognosis of the spill has emerged.

1.2. The sponge model

Marine sponges (Phylum Porifera) are rarely used as experimental models, but their development as such offers several benefits. First, marine sponges appear as common inhabitants of diverse benthic habitats. Second, as one of the earliest emerging metazoan taxa, sponges have a simple, basic physiology with some features that can mirror more complex animals (Riesgo et al., 2014; Feuda et al., 2017; Francis et al., 2017; Pita et al., 2018; Renard et al., 2018). For example, sponges can provide insight into the most basic, fundamental cellular and metabolic processes in response to oil derivatives. Furthermore, sponges contribute to coral reef health through several ecological functions including water filtration, bacterial removal, and recycling of organic matter (Pawlik and McMuurray, 2020; De Goeij et al., 2013, 2017), impacting water quality. More recently, Gokalp et al. (2020) have shown the potential benefits of using sponges in aquaculture, where they improve water quality by transforming dissolved organic matter into particulate organic matter.

Despite their ecological importance in the benthos, sponges have rarely been used to study biological responses to pollutants. Here we sought to determine and document the responses of such an experimental benthic invertebrate species, which could potentially serve as “sentinel” to acute, sublethal doses of oil and dispersant in mesocosms. Our laboratory has identified one globally distributed sponge genus, *Cinachyrella*, which can be easily maintained in aquaculture making it a tractable experimental system for toxicological studies (Cuvelier et al., 2014; Smith, 2013).

1.3. Transcriptional response to pollutant exposure

Using the advances of high throughput sequencing (Fernandez-Valverde et al., 2015; Greer et al., 2019), researchers can identify and catalog a greater number of physiological and genetic signatures that characterize marine organismal “stress”. In the past decade, mRNA-seq data has shown to be a very useful tool to assess the effect of chemical exposure on organisms at the molecular scale (McDermaid et al., 2019; Stark et al., 2019; Han et al., 2014). Using this technique, transcriptional responses of the organism to perturbation can be quickly detected, whereas phenotypic changes usually take more time to manifest.

By applying high throughput transcriptomics to determine differential gene expression (DGE), it is possible to simultaneously trace the direct impact of crude oil and byproducts as well as dispersants on both sponge physiology, a general marker of reef health, and microbial community, a general marker of regional seawater quality. Characterization of changes in gene transcription following pollutant exposure can highlight which molecular pathways are subject to perturbation to better understand benthic ecosystem response in the face of similar oil spill events and provide insight to better protect such environments. We also posit that novel genes or associated pathways can be discovered using a comparative approach. Here we investigated ex situ the transcriptional response in the marine sponge, *Cinachyrella alloclada*, after exposure to crude oil (Water Accommodated Fraction: WAF), dispersant only and oil-dispersant (Chemically Enhanced Water Accommodated Fraction: CE-WAF) mixtures.

2. Methods

2.1. Sponge collection and aquaculture

Nova Southeastern University Oceanographic Center (NSUOC) SCUBA collection team sampled 29 sponges from the Hollywood's first reef tract,

Florida, USA (coordinates: 26.051425 N; 80.112141 W). All sponges were collected under a standard Florida fishing license (issued by the FL Fish and Wild Commission – myfwc.com). Ambient seawater samples were also collected at the same sponge samples sites. All sponges were checked for viability, based on visual observation of active pumping through open oscules and healthy mesohyl (tissue) coloration (yellow). Dead sponges harbor microbial communities which produce hydrogen sulfides, and this was not evident in any sample. Live sponges were brought back to the lab and acclimated in temperature-controlled integrated closed circulating aquaria culture tanks fabricated by AquaLogic, Inc. (California) at Florida International University. The taxonomy of the sponges was determined by spicule preparations and ultimately via the presence of a Group I mtDNA intron following the guidelines of Schuster et al. (2017).

2.2. Dosing solution preparation and sponge exposure

Working solutions for dosing experiments were prepared following CROSERF guidelines ("Chemical Response to Oil Spills: Ecological Effects Research Forum"), a conclusive report to standardize analytical laboratory procedures in testing the toxicity and environmental effects of dispersants (D) and dispersed oil in oil spill response. These guidelines stated that dispersed oil solutions can be tested using an oil:dispersant ratio of 10:1 (Aurand and Coelho, 2005) and were followed in preparation of the CE-WAF, and the representative dispersant volume was kept constant in preparation of the dispersant-only solution (Smith, 2013). CROSERF guidelines estimated sublethal dosage at 1 ppm when preparing WAF and CE-WAF solutions. In the event of an oil spill, the oil and dispersant concentrations are much higher than 1 ppm. Consequently, in a speculative effort to reproduce the conditions an oil spill could create, WAF and CE-WAF conditions contained oil at a concentration of 200 mg/L.

We obtained crude oil directly from BP (SOB-20100622-084; SOB-20100624-00) and Corexit 9500 from Nalco Holding Company. Three replicate oil dosing experiments (labeled as X1-X3) were performed on a total of 24 sponges based on standard CROSERF protocols. (Table 1).

Approximately 100 L of ambient seawater collected at sponge sample sites was used for preparing three primary treatments: i) water accommodated fractions (WAF) of oil, ii) chemically enhanced (Corexit 9500) WAF or CE-WAFs, and iii) Corexit dispersant only.

Sponges were immediately sacrificed following treatment exposure at time points t = 1-h and t = 24-h and processed as follows: Each sponge was quartered using a 70 % ethanol and flame-sterilized knife. Two pieces were flash frozen in liquid nitrogen (LN_2) and stored at −80 °C for DNA and RNA analyses. The third piece was halved for separate histological processes, transmission and scanning electron microscopy (TEM/SEM) and stored at 4 °C in 2 % glutaraldehyde and sodium cacodylate buffer. The remaining piece was flash frozen in liquid nitrogen (LN_2) and stored at −80 °C and reserved as an archived voucher sample.

2.3. Sponge histology

Sponges do not have true differentiated tissues, but rather a collagen matrix containing differentiated sponge and various microbial cells called a "mesohyl". Sponge explant mesohyl samples were embedded in paraffin wax using an automatic apparatus. The apparatus was used to immerse the sponge in cassettes twice in 80 % ethanol, twice in 95 % ethanol solutions, thrice in 100 % ethanol solution, thrice in 100 % xylene solutions and thrice in paraffin wax solutions for a period of 30 min each. Gradual, increasing percent ethanol solutions were required to prevent an extreme change in hydrophobicity that would otherwise damage the cells. Once the sponge was dehydrated and processed in paraffin, the cassettes were placed in a melted paraffin bath. Samples were taken out of the cassettes and cut at desired locations and placed with the cut side up into a mold, and filled with melted paraffin. The paraffin-embedded sponge blocks were sliced into sections with an Accu-Edge low profile microtome blades (Sakura Finetek). Sponge sections were cut to a width of 10 μm, and mounted on slides. Slides were then placed in an incubator for 12 h at 37 °C. The slides were then dewaxed in xylene and ethanol solutions and air-dried.

Table 1
Crude oil and dispersant treatment groups and time point experimental design.

		Experiment		
Time Point	Sample	X1	X2	X3
t = 1-h	Control	X1C1	X2C1	X3C1
	Oil (WAF)	X1O1	X2O1	X3O1
	Dispersant (D)	X1D1	X2D1	X3D1
	Oil + Dispersant (CE-WAF)	X1OD1	X2OD1	X3OD1
t = 24-h	Control	X1C24	X2C24	X3C24
	Oil (WAF)	X1O24	X2O24	X3O24
	Dispersant (D)	X1D24	X2D24	X3D24
	Oil + Dispersant (CE-WAF)	X1OD24	X2OD24	X3OD24

2.4. Hematoxylin and eosin staining

Mesohyl sections were deparaffinized by washing with xylene thrice followed with 100 % ethanol. Sections were hydrated with decreasing concentrations of ethanol (95 %, 80 %) and distilled water and then stained with Harris hematoxylin for 2 min and washed with distilled water. Destaining was performed with 0.5 % acid alcohol and washed with distilled water. Slides were washed with 0.25 % ammonium hydroxide as mordant and then washed and dehydrated with 70 % ethanol, followed by staining with eosin for 30 s (Avwioro, 2011). Slides were then de-stained with 95 % ethanol and dehydrated with 100 % ethanol followed by three washes of xylene.

2.5. Scanning electron microscopy (SEM)

Sponge samples were dissected and fixed in 2 % glutaraldehyde in sodium cacodylate buffered filtered seawater and fixed overnight. Samples were then immersed in three changes of seawater buffer, post-fixed in 1 % OsO4 in seawater buffer, dehydrated in a graded series of ethanol (20 %, 50 %, 70 %, 95 %, 100 %), and dried overnight after three changes of HMDS. Sponge samples were then placed on SEM stubs covered with carbon adhesive tabs and coated with a 20 nm thick layer of Pd in a plasma sputter coater. Sponge samples were imaged in a Philips XL-30 Field Emission SEM.

2.6. RNA extraction

Frozen mesohyl were homogenized using liquid nitrogen cooled and RNase away (cat# 7000TS1, Thermofisher Scientific) treated pestle and mortar. RNA extractions were performed following a modified phenol:chloroform protocol (Ambion, California), as highlighted by the Porifera Tree of Life (additional LiCl precipitation step at −80 °C overnight). Following extraction, RNA integrity was determined using an Agilent TapeStation 2200 and high sensitivity RNA tapes and reagents (Agilent, Inc. California), quantified using a Qubit 2.0 (Invitrogen, Massachusetts), and purity was checked using a Nanodrop 2000 (Thermofisher Scientific, Massachusetts). Samples with an RNA Integrity Number (RIN) < 6 were re-extracted. RNA with unsatisfactory absorbances ratios were purified using an additional ammonium acetate precipitation and two 75 % ice cold ethanol washes.

2.7. cDNA library preparation and RNA-sequencing

A total of 24 cDNA libraries were generated using the QuantSeq mRNA 3' FWD Library Prep kit from Lexogen, following the manufacturer's protocol. Total RNA input was standardized across samples to 132 ng, which resulted in a total of 14 PCR cycles for final amplification of the libraries. The Lexogen cDNA libraries were then sent to the NSU Genomics Core for sequencing. Final cDNA libraries were qPCR-quantified using KAPA Biosystem's Library Quantification Kit optimized for the Roche LightCycler 480 Instrument II.

24 RNA sequencing libraries were pooled and normalized to 2 nM and denatured according to Illumina's NextSeq System Denature and Dilute Libraries Guide. Final pooled libraries were spiked with 2 % PhiX as an internal control and loaded at a final concentration of 1.6 pM onto the Illumina NextSeq 500 platform. Libraries were sequenced on a 1 × 150 bp single end run using the Illumina NextSeq 500 Mid Output v2.5 Kit (150 cycles, 130 million read flow cell).

2.8. Differential gene expression (DGE) assessment

Analyses were performed on a Jetstream cloud computing platform. Read quality was assessed with FASTQC (Andrews and Bittencourt, 2010). Reads were then trimmed using cutadapt (Martin, 2011) to remove adapter sequences and trim low-quality bases. Quality filtered reads (Q-score > 25) were aligned to a Trinity assembled reference holo-transcriptome (Desplat et al., 2022) using bowtie2 (Langmead and Salzberg, 2012) with the '*very-sensitive*' parameter. Additional settings were modified to ensure only high confidence alignment to be considered (bowtie2 –end-to-end –very-sensitive –dpad 0 –gbar 99999999 –mp 1,1 –np 1 –score-min L,0,-0.1 –threads 4 –time -x bowtie2_reference_name —U input_file.fastq —S output_name.sam >> output.bowtie.log 2>&1). Reads were quantified at the isoform level using RSEM (Li and Dewey, 2011) and filtered to remove <5 read counts total over <25 % of the sample pool. DGE analysis was performed using DEseq2 (Love et al., 2014). Differential expression was significant when the absolute value of log2 fold change (LFC) was >2 and the value of the adjusted *p*-value (also known as q-value) was <0.05 (|LFC| > 2, p-adj < 0.05). The R package VennDiagram was used to generate the six-dimension plot to visualize transcript expression overlap across the different treatment conditions.

Gene Ontology enrichment was performed using the R package TopGO to generate GO terms for each treatment for the Biological Processes (BP) category with a Fisher's exact test cut-off value of 0.05.

3. Results

3.1. Dosing solutions evaluation

Due to budget limitations we could only analyze a select number of samples. We did not take water samples of the individual tanks with sponges during the experiment, as this would have added another dimension of work which we were not prepared to perform at the time. For example, because collected in the wild, not all sponges were the same size or age and so the water chemistry analyses would not be consistent. The water preparations were done in three different 20 L aspirator bottles (WAF, Dispersant only, and CE-WAF), and thus the same water for each treatment was disbursed to the sponges in sample tanks Also, our focus was on the biology of the test organisms, and not the water chemistry per se. However, a small number of samples (1 samples of each condition: Control (C), Oil (WAF), dispersant (D), oil + dispersant (CE-WAF) from both $t = 0$ h and $t = 24$ h) from the dosing solutions preparations were taken to the NILAP-certified CAChE Nutrient Analysis Core Facility at Florida International University (FIU) for analysis of PAHs concentration. While no extensive water analyses were carried out because of the low sample number, qualitative changes in the water between 0 and 24 h of the water preparations were assessed. These conditions have been documented in more extensive water chemistry studies after the DWHOS by Diercks et al. (2010) and Tidwell et al. (2016).

We have found that dispersant in CE-WAF general makes PAHs linger longer in the water column, compared to oil only WAF. Dispersant also makes higher MW PAFs enter immediately at time 0 h of addition of Deepwater Horizon oil (Fig. 1 in Supplemental File 1; Supplemental File 2). Overall our WAF and CE-WAF oil concentrations were lower or within ranges of previous experiments.

3.2. Sponge histology

To determine the effects of chemical exposure on sponge morphology, treated sponges were stained with hematoxylin-eosin to analyze choanocyte chambers where active pumping begins in the aquiferous system. Abnormal staining patterns were observed in the sponges treated with oil (Fig. 1 B, C) at both time points when compared to the control (X1 C0; Fig. 1 A). These stains label a variety of cell types in the area. The abnormal areas are surrounded by a concentration of spherulous or granular sponge cells (S. Pomponi, pers. comm). This could be due to increased stress linked to sponge immune response. Moreover, the inner regions of the oil treated sponge exhibit disorganized mesohyl compared to its upper layers with intact choanocyte chambers.

One hour treated sponges and 24-hour dispersant only treated sponges did not exhibit abnormally stained structures compared to the control and are not shown here. The choanocyte chambers appeared structurally normal in sponges treated with dispersant only. However, the sponges in experiment X1 treated with only dispersant were difficult to section due to the high concentration of spicules and the position of the section in the paraffin.

Sponges treated with oil only (X1O24; Fig. 1. C and D) and a combination of oil and dispersant after the 24-hour time point (X1 OD24; not shown) were not visually significantly different from each other but were significantly different from control samples. Crude oil can penetrate the sponge wall, despite exposure to dispersant which acts to decrease oil droplet sizes (response.resotration.noaa.gov). Inner regions appear to be disintegrated with the absence of choanocyte chambers. Abnormally stained regions appear scattered in the aquiferous system. Oil droplets were observed in the mesohyl as referenced by the black arrows in Fig. 1. C, D. The presence of oil in the mesohyl could obstruct pumping canals of the sponge and reduce survival rate.

Overall, light microscopy at limited magnification revealed the increase in spherulous cells in sponge mesohyl after 1-hour exposure, however, 24-hour exposure resulted in altered integrity of mesohyl for WAF and CE-WAF samples. Sponge mesohyl changes induced by WAF and CE-WAF exposure could reduce overall pumping ability and negatively impact survival rate.

3.3. SEM results

Electron microscopy of sponge mesohyl revealed no noticeable differences between control and 1-hour exposed sponges. After short exposure, mesohyl appeared mostly viable and integrity was not compromised (Fig. 2). By contrast, 24-hours of exposure showed significant differences between control and treated samples (Fig. 3). Mesohyl integrity was compromised, characterized by the reduction and potential degradation of the mesohyl. In addition, across all treatment groups, 24-hours of exposure resulted in noticeable algal overgrowth in the mesohyl. Finally, all sponges in each treatment group exhibited visible signs of necrosis and major stress following 24-hours of exposure.

3.4. Differential gene expression (DGE) assessment

Illumina NextSeq 500 sequencing of 24 libraries yielded an average of 7.8 M reads/sample. After filtering lowly expressed features (see methods), we identified 31,753 transcripts eligible for genetic profiling. Overall, 14,029 transcripts exhibited significant differential expression (|log2FC| > 2, p-adj < 0.05). A total of 8007 were upregulated, and 6022 were downregulated across all treatments. Supplemental Table 1 summarizes the number of differentially expressed transcripts across all treatments using the full assembly.

3.4.1. Sample to sample variation

Triplicates were run to assess sample to sample variation. Using DESeq2 as the bioinformatics software to characterize differential gene expression allowed for a compensation of variation between replicates of the same conditions. The model fitted by DESeq2 showed some variation on a sample-to-sample basis, but all within reasonable margins. In all treatments, at least two of the three replicates were present in the same cluster as highlighted by the PCA plot and the sample-to-sample distance plot in Supplemental Fig. 2.

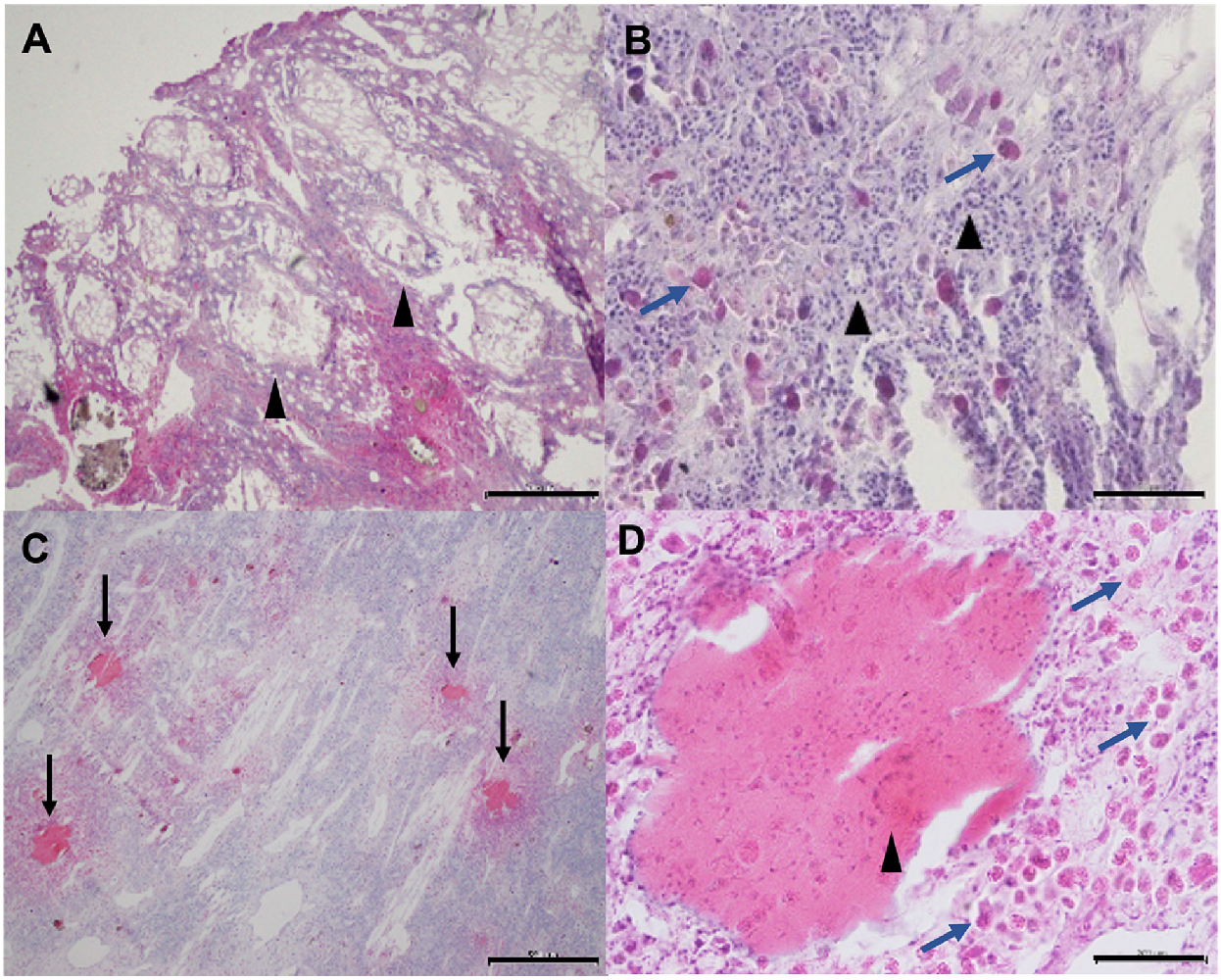

Fig. 1. Light micrographs of sponge mesohyl histology reveal the presence of oil droplets and potential effects on sponge physiology. A) Light micrograph shows the untreated X1 control sample. Upward triangles point to intact choanocyte chambers. Scale bar = 500 um. B) Another untreated control CO sample shows intact choanocyte chambers indicated by arrows. Diagonal blue arrows point to spherulous cells. Scale bar = 50 um. C) WAF 24. Downward arrows point to likely oil droplets which appear pink. Scale bar = 500 um. D) Close up of a single oil droplet in WAF 24 sample that appears to cover a partially intact choanocyte chamber (indicated by the arrow). Diagonal blue arrows point to spherulous cells, which have increased in number. Scale bar = 200 um. In both treatments WAF and CE-WAF (not shown), oil droplets were clearly visible within the sponge tissue (mesohyl), potentially indicating non-sufficient dispersant properties of the chemical dispersant used to break down the oil. Both treatments resulted in broken down aquiferous systems of the sponge, potentially indicating reduced pumping ability by the organism. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4.2. One hour WAF, dispersant, and CE-WAF exposure

1-hour of exposure to oil only (WAF), triggered a significant genetic response with 7714 upregulated transcripts and 562 downregulated transcripts (Fig. 4, first row, left side). Overall, 1293 transcripts matched *Amphimedon queenslandica* (annotated by DIAMOND BLASTX). A total of 1756 transcripts were identified as gene products with no predicted ortholog in other organisms. In addition, 2380 novel predicted transcripts were differentially expressed. Among the differentially expressed transcripts, 3 heat shock proteins were detected, along with 38 E3 ubiquitin ligases, 9 initiation factors, 6 cell death and apoptosis related protein, and 87 oncogenes and tumor suppressor genes (including Rab/Ras, CMYC, and Src), all related to the sponge host. Along with these cell regulating proteins, cytochrome P450, a protein known as a major metabolic activator of polycyclic aromatic hydrocarbons (PAHs, Matsuo et al., 2006), was observed differentially expressed in the WAF treated samples. Cytochrome P450 was found to be differentially expressed in both the host and its bacterial communities and always upregulated, showing that the entire organism was affected by the treatment. GO enrichment analysis of 1-hour WAF treated samples revealed regulation of apoptotic signaling pathway (GO:2001233) and I-kappaB kinase/NF-kappaB signaling (GO:0007249) as two of the most upregulated biological processes. Upregulation of these biological pathways confirms that crude oil acts as a stimulus of sponge gene expression and induces a stress response. On the other hand, several macromolecule pathways and cell signaling, or communication pathways were actively repressed.

After 1-hour of dispersant exposure, 10 differentially expressed transcripts were observed; 9 upregulated and 1 downregulated (Fig. 4, second row, left side). Transcripts identified include 8 predicted novel transcripts, 1 hypothetical protein, and 1 annotated gene identified as 30S ribosomal protein S7. None of the identified transcripts belong to the sponge host. These results indicate a limited sponge transcriptional response following short time dispersant exposure. Only 3 biological pathways were associated with the significantly upregulated genes: DNA mediated transposition, DNA recombination, and transposition (GO:0006313, GO:0006310, and GO:0032196, respectively). Dispersant may act as a driver to induce genotypic changes in symbiotic communities of the sponge host.

One hour exposure to oil:dispersant (CE-WAF) triggered a slightly increased response than dispersant only, albeit minimal. A total of 32 transcripts were significantly differentially expressed with 4 transcripts identified as *A. queenslandica*, 10 novel predicted transcripts, and 6 transcripts with uncharacterized protein function (Fig. 4, third row, left side). Differential expression testing showed 31 transcripts upregulated and 1

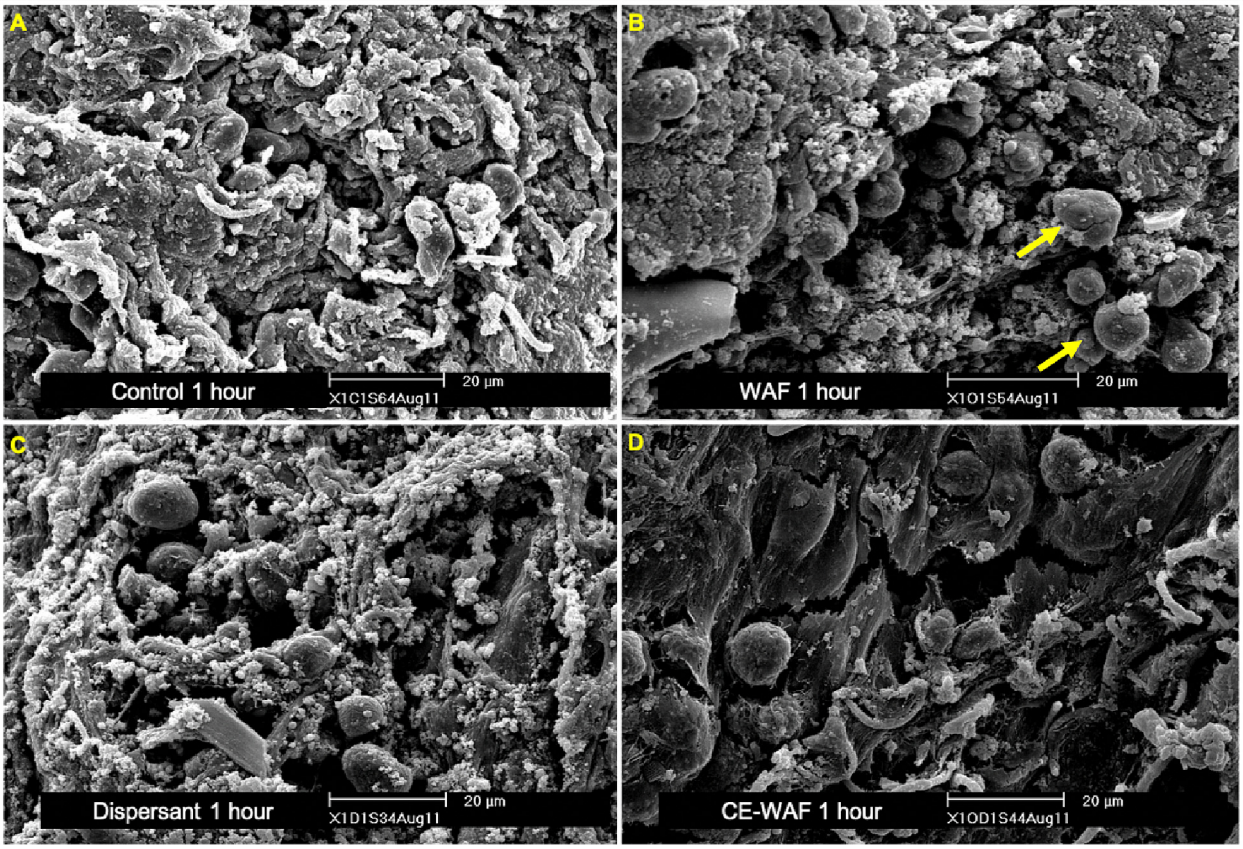

Fig. 2. Scanning Electron Microscopy (SEM) images show changes in mesohyl over time. Comparisons are made after 1-hour control (Panel A), oil treated (WAF; Panel B), dispersant (D; Panel C), oil:dispersant (CE-WAF; Panel D) sponges. Scale bar is in microns and independent to each graph. At 1-hour of exposure, mesohyl from treated specimens looked similar to the control: intact, healthy compact cells and mesohyl indicating sponges appeared healthy, except more spherulous cells marked by the diagonal yellow arrows appear in WAF (B,), similar to Fig. 1B . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

downregulated transcript. Catalase (hydrogen peroxide processing) and tubulin (cytoskeleton structural protein) were observed differentially expressed in 1-hour oil:dispersant treatment. After enrichment of gene ontology, 92 biological processes were positively affected. Among those, several pathways related to stress responses were observed significantly affected: response to stress (GO:0006950, regulation of response to stimulus (GO:0048583), and response to oxidative stress (GO:0006979) along with several apoptosis and cell death regulating pathways: regulation of cell death (GO:0010941), regulation of programmed cell death (GO:0043067), and regulation of apoptotic process (GO:0042981). Induction of stress-induced biological pathways demonstrates the harmful effects of oil:dispersant mixtures on benthic organisms and host communities.

3.4.3. Twenty-four hours WAF, dispersant, CE-WAF exposure

Following 24-hour of exposure to oil (WAF), more downregulated genes were identified than upregulated compared to the 1-hour samples. A total of 46 transcripts were significantly upregulated, and 226 transcripts were significantly downregulated (Fig. 4, first row, right side). Among differentially expressed transcripts, only 1 transcript exhibited orthology to *A. queenslandica,* the plant cadmium resistance factor 2 (PCR2). Sixty-two transcripts were identified as novel predicted transcripts, and 146 transcripts were found to be transcripts with uncharacterized protein function. Interestingly, cytochrome P450 expression was not measurable at the 24-hour time point. Additionally, in contrast to 1-hour WAF samples, HSPs, apoptosis related proteins, TSGs and oncogenes, and E3 ubiquitins were not differentially expressed, suggesting that the initial stress response to oil had surmounted and the sponges had adapted. The reduction of pathways related to stress response and stimulus was reflected in GO enrichment which demonstrated downregulation of these pathways: response to stress (GO:0006950), response to external stimulus (GO:0009605), regulation of response to stress (GO:0080134), and negative regulation of response to stimulus (GO:0048585). In addition, immune system related pathways: immune response (GO:0006955), innate immune response (GO:0045087), regulation of innate immune response (GO:0045088), negative regulation of immune response (GO:0050777), and negative regulation of innate immune response (GO:0045824) were also suppressed after 24-hours of exposure.

Exposure to dispersant only for 24-hours resulted in increased gene expression as compared to the 1-hour treatment characterized by primarily downregulated expression. Significant upregulation was observed for 52 transcripts, and 270 transcripts were significantly downregulated (Fig. 4, second row, right side). Overall, this pool of differentially expressed transcripts was made of 13 sponge orthologs, 109 novel predicted transcripts, and 95 transcripts with uncharacterized protein function. Upregulation of actin filament polymerization and deployment: actin filament polymerization (GO:0030041), regulation of actin polymerization or depolymerization (GO:0008064), regulation of actin filament length (GO:0030832), regulation of actin filament polymerization (GO:0030833), and positive regulation of actin filament polymerization (GO:0030838) was induced by 24-hour of exposure to dispersant only. In contrast, downregulation of actin filament bundle organization, assembly, and network formation pathways, along with pathways related to actin cytoskeleton organization (GO:0051017, GO:0061572, GO:0051639, GO:2000249) was observed.

Exposure to CE-WAF for 24-hours significantly increased the expression of 155 transcripts while 4962 transcripts were significantly downregulated (Fig. 4, third row, right side). In total, 35 transcripts shared homology with

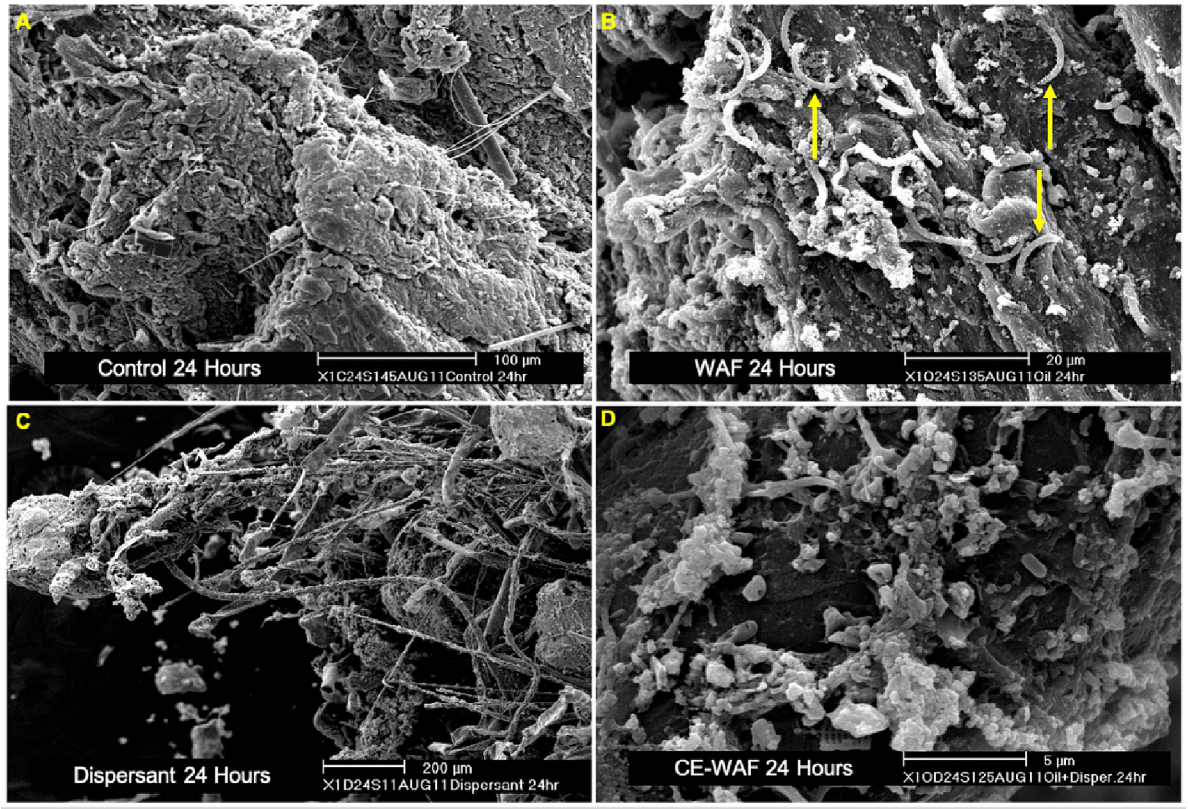

Fig. 3. SEM images depict ultrastructural effects of treatments after 24-hour between the control (Panel A), oil treated (WAF; Panel B), dispersant (Dispersant; Panel C), and oil:dispersant (CE-WAF; Panel D). Scale bar is in microns and independent to each graph. In contrast to the 24-hour control and 1-hour treatments (Fig. 2), oil and dispersant-treated mesohyl appear disorganized and reduced cohesion. Yellow vertical arrows point to sigmaspire spicules which have a semi-circular shape, appear more prevalent in WAF samples, and which could reflect retraction of the mesohyl. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A. queenslandica genes, 669 were novel predicted transcripts, and 1920 were non-protein coding transcripts. Interestingly, cytochrome P450, tubulin, and TGFBR1 (potential tumor suppressor gene) were differentially expressed in the CE-WAF 24-hour treatment. Furthermore, in contrast to our observations at 1-hour of exposure, no cell death or apoptosis related proteins were observed to be differentially expressed. However, GO enrichment analyses revealed an increase of multiple pathways involved in regulation and response to stimulus and organic substances (GO:0048583, GO:0010033, GO:0070887, GO:0071310, GO:0048584). In addition, pathways involved in cell motility, migration, and differentiation were also up-regulated, indicating a clear biological response to oil exposure. Further, impact of these organic compounds at the cellular level is denoted by GO enrichment in pathways involved in cell function and localization (GO:0090130, GO:0090132, GO:0045595, GO:0048870, GO:0016477). Conversely, pathways controlling translation, DNA replication, and protein refolding (GO:0006412, GO:0006260, GO:0042026) were negatively impacted along with pathways mediating catabolism of aromatic and organic cyclic compounds (GO:0019439, GO:1901361). Suppression of xenobiotic response indicates reduced ability of the host and symbionts to actively metabolize toxic oil compounds and reveals detrimental impact to vital cell processes and survival.

One hour WAF exposed samples and 24-hour dispersant and CE-WAF exposed samples showed opposing expression trends. The Venn diagram shown in Fig. 5, aimed to characterize any transcripts that might be expressed in several conditions, regardless of trend up or down regulation. The largest number of differentially expressed transcripts shared between two conditions was 1457 transcripts, overlapping WAF 1-hour treated samples and CE-WAF 24-hour exposed samples. All other treatment conditions showed minimal overlap, mostly due to the very low number of significantly expressed transcripts in the 1-hour dispersant and CE-WAF conditions. The large number of differentially expressed transcripts in the 1-hour WAF versus 24-hour CE-WAF samples, combined with the shift in expression profile trend, indicated an acclimation response by the sponge to mitigate harmful effects of cytotoxic agents.

4. Discussion

The Deepwater Horizon (DWH) oil spill (DWHOS) in the Gulf of Mexico represents one of the most lethal, human-mediated accidents in US history, with over 134 million US gallons of crude oil released (National Oceanic and Atmospheric Administration, 2020). Consequently, characterization of the wide-ranging effects of crude oil and its derivatives continue in attempts to better understand detrimental impacts to the wide diversity of aquatic organisms. For example, Venn-Watson et al. (2015) have documented the negative effect of the Deepwater Horizon oil spill on adrenal glands and respiratory systems of bottlenose dolphins, while Lane et al. (2015) showed the adverse effect of oil spills on reproduction status and survival. Han et al. (2014) have demonstrated the effect of crude oil on the development of the copepod *Tigriopus japonicus*; development was delayed and molting was interrupted when the intertidal species was exposed to oil. In fur bearing animals, such as otters and polar bears (U.S. Fish &amp and Wildlife Service, 2004), crude oil impairs waterproofing, making them vulnerable to hypothermia, and leading to death by drowning. Oiling also harms various submerged and pelagic shellfish, plants, phytoplankton, and marine microorganisms. Corals (DeLeo et al., 2021), shellfish (DeMiguel-Jiménez et al., 2021), fishes (Incardona et al., 2014), and other micro-organisms (microbes, phyto/zoo-plankton) are affected across multiple biological stages of development; such impacts include stunted

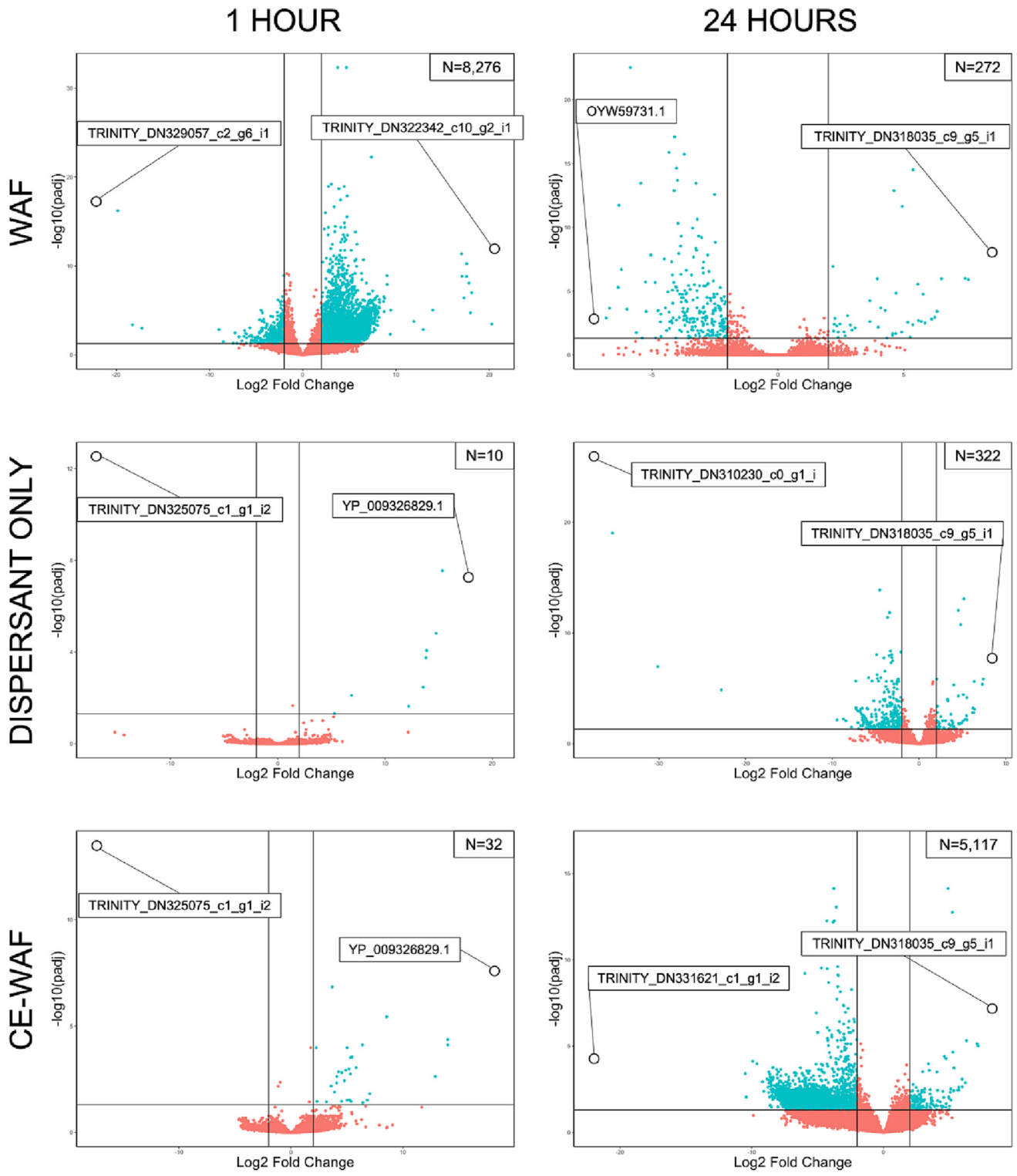

Fig. 4. Volcano plots representing differential gene expression of each treatment compared to the respective time point control. Each point on the plot represents a transcribed gene sequence that may be affected by the three treatments. Left column represents 1-hour treated samples and 24-hour treated samples are located in the right column. The first row depicts WAF (oil only) treated samples. Second row shows dispersant only treated samples, and the third row display oil:dispersant (CE-WAF) treated samples. Scale is independent to each graph. Turquoise indicates significant up or down differential expression (padj <0.05 and |L2FC| > 2), and red denotes non-significant differential expression. The vertical line represents |L2FC| > 2, and horizontal lines represent padj = 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth and reproductive impairment. In addition, studies have revealed differential expression of several genes highly impacted by oil spills such as CYP1A, GCHFR, or NUPR1 in Gulf fish such as *Fundulus grandis* (Dubansky et al., 2014; Greer et al., 2019). Jung et al. (2017) reported similar results in both olive flounder and spotted seabass embryos. It has also been reported in oil spill studies that zooplankton ingest and bioaccumulate sublethal doses of crude oil, ultimately introducing oil in the food chain (Bretherton et al., 2019).

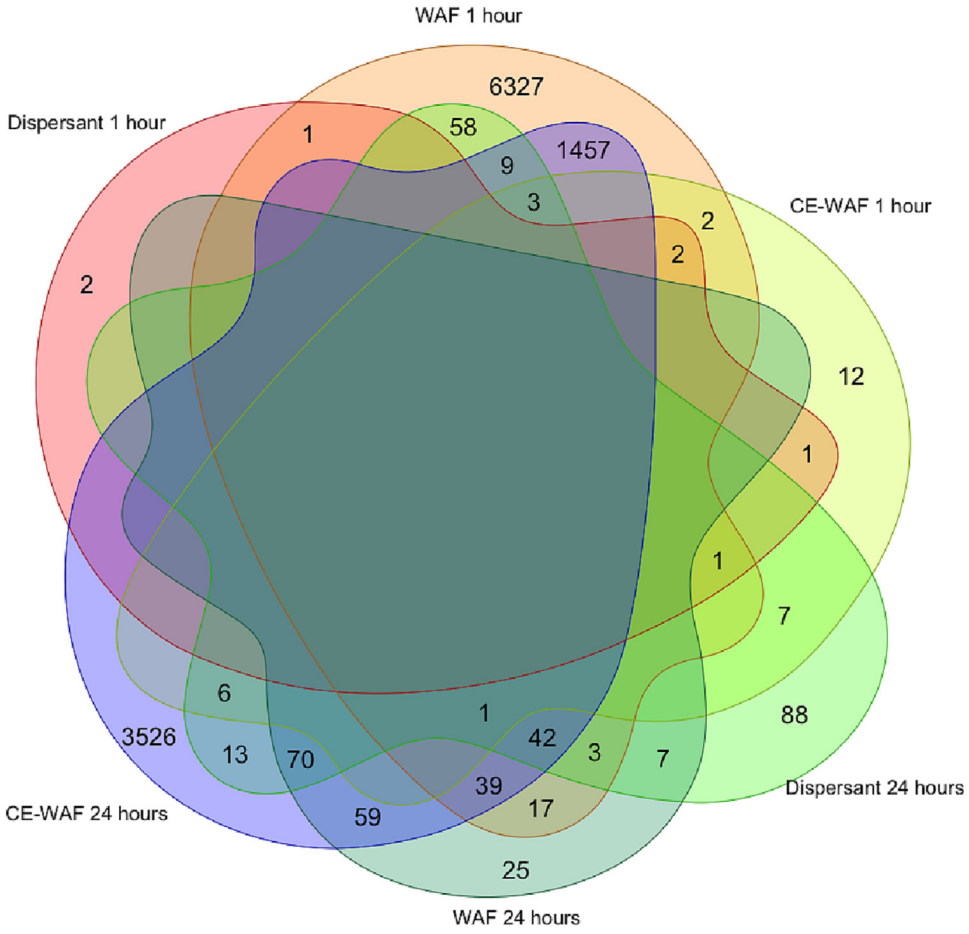

Fig. 5. Six dimension Venn diagram representing the total number of differentially expressed genes (includes both up and downregulated genes) across the different treatments. From the top, clockwise, orange, light green, medium green, dark green, blue, and red, represent respectively WAF 1-hour (C1O1), CE-WAF 1-hour (C1OD1), dispersant only 24-hours (C24D24), WAF 24-hours (C24O24), CE-WAF 24-hours (C24D24), and dispersant only 1-hour (C1D1). Each group represents differential expression calculated by DESeq2 (Padj < 0.05 and |log2 FC| > 2) across the 3 replicates for each treatment condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1. Sponge responses to WAF, dispersant and CE-WAF

Sponges were not the most dominant invertebrate phylum at the Macondo wellhead, site of the DWHOS. However, sponges can compose large proportions of benthic fauna of many reef ecosystems and other marine habitats worldwide (Bell et al., 2018; McMurray et al., 2010). Evaluating shifts in the functional differential gene expression of marine sponges will allow us to better understand the overall effects of hydrocarbon loading in the water column that result from oil spills. Our findings indicate that the sponge *Cinachyrella alloclada*, perhaps like many other benthic organisms, is particularly sensitive to oil after short periods of time (<1-hour) and very responsive to oil and dispersant mixtures after 24-hours of exposure. Our results also urge the need to act fast in the face of such environmental catastrophes as this study highlighted numerous biological pathways responsible for the viability of the organism. Finally, our findings provide evidence that this species is a tractable indicator system for pollution exposure studies in the benthic environment.

Following exposure to WAF for only 1-hour, 6,327 differentially expressed genes were observed. By contrast, 1-hour exposure to dispersant and CE-WAF mixtures elicited minimal measurable response, highlighting a limitation of our study data for comparison. Longer exposure times (24-hours) to dispersant only and CE-WAF resulted in a noticeable change in gene expression, with CE-WAF mixtures to a greater extent. Gene expression data, combined with phenotypic changes characterized by degraded aquiferous systems, presence of oil droplets in the mesohyl captured by light microscopy, and algal overgrowth after 24-hours of exposure as seen via SEM, may indicate hydrocarbon and dispersant toxicity to sponges and other benthic organisms following oil spill events.

4.1.1. WAF-related genetic response

Among differentially expressed transcripts, we detected galectin, *Rab/Ras* related proteins, Heat Shock Proteins (HSP), and other transcripts consistent with previous preliminary findings (Smith, 2013), while also increasing the number of replicates to improve robustness. For example, HSPs have been previously shown to be over-expressed in organisms under stress (Santoro, 2000), as supported in our data. Many proteins in this group function as chaperones, acting as controllers to ensure the correct folding of other proteins or the refolding of damaged proteins due to cellular stress. HSPs have various functions in an organism, ranging from simple management of proper protein conformation under non-stressful conditions, to potential implication in cancer cell apoptosis (Salamanca et al., 2014). HSPs also have been found to be part of the “chemical defensome”, genes that code for proteins that are chemical stressors, of several organisms (Goldstone, 2008; Goldstone et al., 2006; Shinzato et al., 2012) and are found virtually in all living organisms from bacteria to humans. The presence of HSPs among genes differentially expressed in this study indicates a significant stress response by the sponge in the face of oil exposure.

Ubiquitins and translational initiation factors (eIF2, eIF3, eIF4, eIF5) were also differentially expressed in treated WAF samples. These proteins are important to organismal survival as they regulate cell replication and

proliferation. Both types of proteins affect several essential biological processes, such as endocytic trafficking, inflammation, translation, DNA repair, or apoptosis (Miranda and Sorkin, 2007; Teixeira and Reed, 2013). Upregulation of these two transcription factors promotes cell proliferation, thereby regulating apoptosis. In this study, we demonstrate that hydrocarbon and chemical surfactant exposure may alter the cell cycle distribution of sponges, ultimately affecting survival and mortality rates.

Another major class of proteins differentially expressed in our treated samples were related to cell death and apoptotic pathways. Upregulation of these proteins indicates an increase in cell damage and concomitant decrease in sponge cell viability and survivability under xenobiotic stress. However, because initiation factors and ubiquitination both play a complex role in both cell proliferation and apoptosis, seeing both protein types expressed hand in hand is not unusual (Lee et al., 2005).

The evidence of upregulated apoptotic genes is also supported by the microscopic data showing an increase in spherulous sponge cells (Figs. 1B, 2B). These cells have been documented to increase with apoptosis during sponge regeneration after wound healing (Ereskovsky et al., 2020). Several cancer related genes, also known as oncogenes and antioncogenes (tumor suppressor genes (TSGs)) were found differentially over expressed in our dataset. TSGs occur in sponges, but these animals do not develop tumors, as they have no true tissue. In sponges, TSG roles are unclear, but as in multicellular organisms, a balanced expression between oncogenes and TSGs proteins is crucial for an organism's survival (Chow, 2010; Cetkovic et al., 2018). *Ras* genes code for proteins that transduce extracellular signals which in turn regulate cell cycles, survival and differentiation. Similarly, *Rab* products belong to the Ras family of proteins, which function as membrane GTPases that control intercellular trafficking and membrane repair. These important cellular functions are corroborated by the deep phylogenetic origins and homologies of the Rab/Ras family (Stenmark and Olkkonen, 2001).

Consequently, differential expression of genes that control and regulate vital cell proliferation processes are typically part of complex networks, and thus differential expression will cascade and reverberate (Davidson and Levin, 2005; Kumar et al., 2013). The change in transcript expression levels following sublethal exposure to hydrocarbons highlights the importance regulatory proteins play in enabling adaptation to environmental stressors. Nonetheless, the existence of proto-oncogenes and primary metabolic pathways has been found in other sponges species and more complex taxa across other studies (Riesgo et al., 2014; Kenny et al., 2020), supporting homology. The ancient linkages provide functional context and implications for clinical research.

4.1.2. Dispersant (D) effect

Short term dispersant exposure has demonstrated relatively mild effects on benthic organisms. However, dispersants act as detergents to homogenize and miniaturize oil droplets, permeating the entire water column, potentially harming benthic communities over longer periods of exposure. In our study, no HSPs, cell death or apoptotic related genes were observed differentially expressed after either 1 or 24-hours. Luter et al. (2019) have shown differential expression of these genes on sponge larvae after 2-hours of exposure to PAHs and dispersant, however at a lower concentration than this study. Indeed, our oil and dispersant concentration were 200 and 100-fold higher respectively. However, Luter and colleagues exposed larvae and not fully grown specimens. Juvenile organisms tend to be much more sensitive to incremental environment changes and using too high concentrations of chemicals most likely resulted in the death of the animals. Hence the use of lower concentration was crucial in order to fully study the ecological impact of exposure to oil and dispersant mixtures. Interestingly, when performing Gene Ontology enrichment on the 24-hour dispersant treated samples, overrepresentation of pathways involved in cell integrity were reported. This highlights the negative effects of dispersant solely on cell structures and the potential inability of the host organism to maintain proper cell matrices. However, while the use of dispersant alone is rare, such scenarios can happen, specifically when dispersant is not applied at the surface and thus does not fulfill its primary purpose. In such cases, dispersant ends up mixing within the water column, exposing marine organisms to unadulterated dispersant (NRC, 2013). As such, dispersant only conditions and effects should not be ignored in oil exposure studies or oil spill mitigation plans. Furthermore, future studies should consider longer exposure times as the harmful effects of dispersants may compound over time, and 24-hours of exposure may not capture the full scope of dispersant effect on gene expression.

4.1.3. Oil:Dispersant (CE-WAF) effects

Finally, oil:dispersant (CE-WAF) exposure, as with dispersant, increased transcriptional response over time. After 1-hour of exposure, 36 transcripts were differentially expressed. Similar to dispersant, this indicates that short term exposure would likely not detrimentally affect benthic organisms. Chemically dispersed oil would need to time to reach the benthic zone and interfere with biological functions of benthic habitats. After 24-hours of exposure, 5,117 transcripts were significantly changed. Furthermore, a shift from predominantly upregulated expression in 1-hour WAF treated samples to a primarily downregulated trend after 24-hours exposure of CE-WAF was observed. It is possible that the addition of chemical dispersant induced changes in oil properties to cause shifts in gene expression patterns. Additionally, longer exposure times (> 24-hours) may be needed for CE-WAF treated samples to reach similar numbers of differentially expressed transcripts as WAF treated samples. Overall, longer exposure times (24-hours) resulted in greater numbers of differentially expressed transcripts. Chemical dispersant breaks down crude oil into smaller particles to allow them to sink in the water column. With such chemicals in the water column, benthic organisms may be exposed for longer periods of time than surface or mid water column organisms (Reuscher et al., 2020). Furthermore, benthic organisms probably face a bigger challenge when in presence of CE-WAF mixtures as dispersant seems to affect the ability of the organism to process organic compounds as suggested by GO enrichment analyses. Consequently, new protocols should be explored to make oil dispersion and elimination faster and more effective for both benthic ecosystems and pelagic organisms.

4.2. Strengths and limitations of our methods

Overall, the methods we have applied add more information to Poriferan biology and their response to PAHs and surfactants. The strong response by *Cinachyrella alloclada* at the gene expression level after 1-hour of exposure is reflected with over expressed transcripts (8052 differentially expressed transcripts). This indicates that oil can elicit large genetic and cellular responses quickly. This may be attributed to our solution preparation which exceeded CROSERF guidelines in dosing experiments, since it was not clear what fractions could induce physiological changes of sponges to oil spill chemicals. The decrease in gene expression after 24-hours of treatment with a predominant downregulation of transcripts is most likely due to a chemical concentration equilibrium being reached between the water and the air layer in the aquaculture tanks. However, these results still remain abstractions until they are verified through laboratory and empirical evidence.

Moreover, physiological responses may depend on the relative abundances of specific oil-related compounds in the environment at the time of sampling (Turner and Renegar, 2017). It is well known that crude oil hydrocarbon profiles are complex and can vary according to source (geography, well location, depth) and temporal change due to natural weathering, degradation, and exposure to dispersants (Olson et al., 2017; Romero et al., 2017). Indeed, while crude oil is mostly immiscible, some dissolution does occur for a small semi-soluble fraction as a function of the surface area of the oil-water interface. This study did not separate crude oil into their specific components; instead, the physiological effects of whole crude oil from the Macondo wellhead was studied. Turner and Renegar (2017) provided a comprehensive review of the effects of crude oil toxicants on marine organisms, specifically corals. Many different organic compounds compose crude oil (Reddy et al., 2012). Most of these compounds are 2 or 3 ringed PAHs, such as naphthalene (2 rings),

anthracene and phenanthrene (3 rings), that are very volatile (Overton et al., 2016). Oil and dispersant exposures display stark effects during large spills and extreme oiling scenarios, in which exposure far exceeds sublethal levels. Across many taxa, such as coral, ctenophores, oysters, the strongest response in the organism happens in which the dispersant is present (Peiffer and Cohen, 2015; Gardiner et al., 2013; Jones et al., 2017; DeLeo et al., 2021). The differentially expressed transcripts at 24-hours and imaging results from light and SEM micrographs could then be the consequence of volatility and the remains of the initial dosing and short-term exposure (before evaporation of volatile compounds). This may be supported by the upregulation of HSPs, TSGs, oncogenes, ubiquitins, CP450 at the 1-hour time point, while at 24-hours, select gene response was absent. Results suggest that the sponges faced a stressful environmental change early in the exposure study, which we tried to mimic but likely adapted to conditions as time progressed. However, the negative impact on the sponge immune system, as evidenced by the GO enrichment analyses, indicate a decreased resistance to external stressors after only 24-hours of exposure. Longer exposure under initial, volatile conditions may confirm these postulations.

The identification of specific upregulated genes remains a function of the existing sequence databases available for reference and annotation, which remains sparse for many invertebrates (Dunn and Ryan, 2015). For what is considered one of the earliest emerging taxa, besides taxonomic identification relevant sequences, the phylum Porifera remains rather poor in terms of genomics resources. To this date, only two full genomes are publicly available, namely *Amphimedon queenslandica*, a great barrier reef sponge (Srivastava et al., 2010; Fernandez-Valverde et al., 2015) and *Ephydatia muelleri*, a freshwater sponge (Kenny et al., 2020). With such limited genomic resources, studies that focus on any aspect of the characterization of individuals at the molecular level is rendered challenging.

4.3. The utility in developing more invertebrates model systems

Disastrous oil spills are prone to happen more often with an increasing worldwide demand for oil, and accidents will introduce a wide array of chemicals into the aquatic environment. Therefore, a need exists to determine precisely how marine organisms will likely respond to petroleum-based compounds, and invertebrates can fulfill knowledge gaps. For example, filter feeding oysters have provided insight into oil spill responses (Jenny et al., 2016; López-Landavery et al., 2019). The most active KEGG biological pathways following exposure of *Crassostrea virginica* to 50–200 μg/L of super-light and light crude oil mixtures for two weeks, identified as metabolism (23.93 %), ubiquitin-mediated proteolysis (Genetic information processing, 11.49 %) and the PI3K-Akt signaling pathway (Environmental information processing, 6.51 %)(López-Landavery et al., 2019; Sandrini-Neto et al., 2016).

The DGE results of a recent study with two deep sea corals, *Callogorgia delta* and *Paramuricea* type B3 (DeLeo et al., 2021), provide an interesting contrast to our sponge results. The coral methodologies, with respect to WAF, CE-WAF, and D sublethal concentrations, were similar to our study. After 12 h of exposure, the deep-sea corals exhibited differential expression of GO pathways similar to our findings. GO pathways such as “response to oxidative stress”, “regulation of apoptotic signaling pathway”, or “innate immune responses” were among the observed differentially expressed pathways in both taxa. However, even though DeLeo et al. (2021) used deep sea corals, only sea level (not deep sea) pressure parameters were applied. Additionally, a different number of genes appear affected between the two different phyla. These differences may be explained by the difference in symbiont composition and abundance. *Cinachyrella alloclada* has a high microbial abundance embedded in its mesohyl. Corals tends to be highly symbiotic but with fewer symbionts species within their tissues. Hence, the number of differentially expressed transcripts at the holo-transcriptome level may vary greatly from one organism to the other. Duration of exposure, as well as concentration of mixtures, were different between the oyster and coral studies, and although both studies used sublethal levels of oil and dispersant, our sponge study used lower levels of chemicals, potentially indicating a higher sensitivity of sponges to those mixtures.

Our study used the sponge *Cinachyrella alloclada,* a commonly found genus on Florida reefs, with relatives present around the globe to assess the gene expression level changes when exposed to suspected high doses of WAF, dispersant only, and CE-WAF mixtures. Results show immediate genetic response after 1-h exposure to oil only and an increasing effect on expression over time (24-h end point) for the dispersant and CE-WAF treatments. The effect of such treatments demonstrates an impact to several pathways linked to stress response, immune response, cell structure and organization, and cell fate, including clinically relevant pathways linked to tumorigenesis. These results are consistent with the oyster studies described above and confirm previous pioneering studies of sponges measuring differential expression of genes involved in immunity, thermal stress, and symbiotic interactions (Steindler et al., 2007; Pita et al., 2018; Guzman and Conaco, 2016).

4.3.1. Sponge physiology and function in response to oil and dispersant

Marine sponges are thought to be one of the oldest and earliest animal taxa to have evolved (Littlewood, 2017). Nonetheless, the dosing experiments described in this study showed evidence that oil droplets, even if not dispersed, could affect the most basic sponge physiological process - pumping ability and water circulation. The presence of oil droplets could clog choanocyte pumping channels of the sponge, resulting in impaired feeding ability. This would directly impact organism survival and recovery rate. Our more detailed analysis using scanning electron microscopy, however, revealed that sponge mesohyl was markedly affected. The SEM micrographs provided phenotypic evidence of mesohyl disintegration and supported genotypic changes due to WAF, dispersant, and CE-WAF dosing following a 24-hour time point study. The cumulative effects of stress can be reflected in the larger number of differentially expressed genes (n = 5117) after 24-hours exposure to CE-WAF as compared to 1-hour treatments (Fig. 4). Linking the molecular DGE data with these micrographs provides strong evidence of the detrimental effects of these chemicals. In an actual in situ oil spill, exposure may last for months, thus sponges and their mesohyl may experience severe chronic damage, eventually leading to organismal death. In order to confirm or infirm this speculation, future work could focus on long-term exposure (months long treatments), in parallel of assessing recovery of exposed sponges by returning specimens in clean water tanks and observe physiological recovery over time.

However, studies have shown that oscula density as well as sponge size is affected by the depths at which the animals are located (Gokalp et al., 2020; Morganti et al., 2021). Oscula size and abundance are directly linked to depth at which sponges are located, which in turn results in different pumping rate. These differences can result in different exposure to contaminants when comparing deep water sponges and shallow ecosystems sponges. In our case study, the live sponges were collected on the first reef system of the Florida reef tract. This reef stands at a max depth of 10 m (Banks et al., 2008) hence the difference in environment, especially pressure, between the wild and acclimated conditions shouldn't differ enough to cause significant difference in genetic response to pollutant exposure. At this point, we do not have data for deep water sponges. Also, many deep water sponges are harder, more siliceous and do not easily show active pumping. Thus, their oscula diameters may not vary as widely as more shallow sponges.

5. Conclusions

Exposure of sponges to sublethal doses of WAF have shown rapid cellular and genetic responses in *Cinachyrella alloclada*, resembling apoptosis, after as little as 1-hour of treatment. Extended exposure of up to 24-hours elicits gene level expression decreases and a shift towards a predominantly downregulated biological system. CE-WAF and dispersant only (D) exposure resulted in a more prominent response after longer exposures (24-hour time point), with a dominant trend of downregulation, indicating that over time, effects of CE-WAF and dispersant only mixtures may have more serious negative impacts to benthic organisms. Overall, while the 1-hour exposure profiles followed the WAF>CE-WAF>D toxicity pattern, it shifted to CE-WAF>D>WAF after 24-hour of exposure.

Additionally, even with sublethal dosing, our study highlighted the importance of removing the oil as early as possible to minimize physiological and environmental impact to marine organisms, whether pelagic or benthic. In consequence, it is essential to find better, faster and more efficient ways to clean up spills in order to maximize ecosystem survival and recovery.

Finally, the results presented here add an important dataset describing Porifera as a novel research model. The sensitivity of sponges and the resistance to harsh environmental changes provide supporting evidence to the establishment of the marine sponge *Cinachyrella alloclada* as a sensitive bioindicator species of water quality for shallow reef systems in the face of environmental disasters.

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CRediT authorship contribution statement

Yvain Desplat: Nucleic Acid Isolation, Library Generation, Data Analysis & Curation, Writing – Original Draft, Editing

Jacob F. Warner: Data analysis, Writing – Review & Editing , Supervision

Emily J. Blake: Illumina Sequencing, Writing – Review & Editing

Nidhi Vijayan: Data Generation (light microscopy), Writing – Review & Editing

Marie Cuvelier: Specimen Collection, Methodology and Experimental Design

Patricia Blackwelder: Data Generation (scanning electron microscopy), Writing – Review & Editing

Jose V. Lopez: Lab Head, Funding Acquisition, Investigation, Supervision, Writing – Review and Editing,

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Data availability

Raw sequence files generated for this study are available in the NCBI database under the BioProject PRJNA663558.

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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