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MASTS Report: Impact of hydrocarbon contaminated sediments on sediment associated bacterial communities and sponges

INTRODUCTION

It is estimated that more than 1.3 million tons of petroleum enters the seas each year. Of this, 53% originates from multiple anthropogenic sources while the rest comes from natural oil seeps (Blackburn *et al.* 2014). As oil naturally occurs in the marine environment, microorganisms have evolved the capability to use hydrocarbons as a source of energy. This constitutes the main process of hydrocarbon degradation in the marine environment (Harayama *et al.* 2004). A number of bacteria use hydrocarbons almost exclusively as their preferred source of carbon and energy, and are recognised as obligate hydrocarbonoclastic bacteria. These oil-degrading organisms are thought to be present globally at very low abundance. When hydrocarbons are present these taxa may make up to 90% of the total microbial community (Harayama *et al.* 2004, Yakimov *et al.* 2007). Hydrocarbon-degrading bacteria can also produce emulsifiers or surfactants to enhance degradation (Harayama *et al.* 1999) and synthetic dispersants have been developed as oil spill mitigation tools (Greco *et al.* 2006).

Marine sediments cover more than 50% of the planet and are known to have a key role ecosystem processes and in carbon and nutrient cycling (Widdicombe *et al.* 2011). Sediments are also inhabited by diverse microorganism communities (Munn 2011). Initially, it was estimated that marine sediment harboured between 55% and 85% of all the microbial life (Whitman *et al.* 1998) playing an important role in global carbon cycling. However, recent studies (e.g. Kallmeyer *et al.* 2012) have shown lower estimates of microbial life. Differences in microbial abundance in sediment are probably due to the biased sampling efforts towards sediments located along continental shores in former studies (Whitman *et al.* 1998; Parkeset *al.* 2005).

As mentioned above, some microorganisms are able to breakdown hydrocarbons. Oil/petroleum contamination is therefore expected to impact the metabolism and abundance of

natural microbial communities in sediments. However, this process is still poorly understood (Bartha & Atlas, 1987). Studies have shown that petroleum hydrocarbons can enhance (Bunch 1987), reduce (Griffiths *et al.* 1981) or have no effect (Bauer & Capone 1985; Carman *et al.* 1996) on total abundance of sedimentary bacteria; highlighting different responses to hydrocarbons presence (Alexander & Schwarz 1980). Moreover, sediment microbial community diversity have been suggested to be used as a bioindicator and as a tool to assess and develop polluted site studies (Braddock, Lindstrom & Brown, 1995). For this reason, the further study of sediment microbial communities and their resilience to hydrocarbon contamination, is needed.

There is an important interaction between the benthic and pelagic life such as the deposition of non-living organic material to benthic habitats (Smetacek 1985; Graf & Linke 1992), bioresuspension (Graf & Rosenberg 1997), and the release of inorganic nutrients from the sediments (Raffaelli *et al.* 2003). These processes are modulated by factors such as pelagic predation on benthic fauna, ontogenetic changes in the use of the habitat, reproductive cycles, organism migrations (seasonal or daily), nutrient-cycling effects due to processes such as benthic bioturbation and bio-irrigation, and filter-feeding by benthic organisms. Overall, however, the effect of the properties of the sediment on the benthic and pelagic fauna associated to it are still poorly understood.

Known as human carcinogens, hydrocarbon and specifically Poly Aromatic hydrocarbons (PAHs) have also received specific attention as the concentrations of these chemicals can increase during natural crude oil degradation, making them readily bioavailable to marine organisms and leading to oxidative stress, physiological and immunological responses (Nebert *et al.* 2000; Puga *et al.* 2002; Hannam *et al.* 2009; Liu *et al.* 2010; Regoli and Giulani 2014). Moreover, the use of dispersants during a spill at sea is controversial as dispersants themselves can be toxic to marine organisms but also increase the bioavailability of PAHs (Singer *et al.* 1998, Epstein *et al.* 2000, Edwards *et al.* 2003, Ramachandran *et al.* 2004, Couillard *et al.* 2005, Greco *et al.* 2006, Chapman *et al.* 2007).
Furthermore, bacterial degradation of hydrocarbons are known to be slowed down in sediments due to their overall anoxic condition as well as nutriment limitations (Atlas and Hazen 2011 and references therein). Dispersants have also been shown to persist in the marine environment for up to 45 months after their use (White *et al.* 2014). Hydrocarbon and dispersants can therefore present long term impacts on marine organisms once they have entered the sediments.

Marine sponges are important marine organism, contributing significantly into a wide array of ecosystem services such as the C and N cycling as well as the bentho-pelagic coupling (De Goeij *et al.* 2013; Maldonado 2016). Bioaccumulation of hydrocarbons from the water column by various coastal sponge species through their filtering activities has been well specified (Zahn 1981 and 1983, Glyzina *et al.* 2002, Mahaut *et al.* 2013, Batista *et al.* 2013). It has also been hypothesized that they have access to both dissolved and particulate oil partitions due to their high filtering capacities as well as hydrocarbons within sediments during resuspension periods (Batista *et al.* 2013). No clear experimental work has, however, clearly showed the impact of hydrocarbon contaminated sediments on sponge physiology and metabolism.

The aim of this study is therefore to (1) characterise the changes in bacterial communities associated with sediments when exposed to crude oil and/or dispersant mixtures and (2) determine the impact of sediment contaminated with crude oil and/or dispersant mixtures on sponge physiology and metabolism.

MATERIAL AND METHODS

General overview of the experiment

The experiment conducted in this study can be divided up into two phases: (1) sediment contamination to crude oil and/or dispersant mixtures and (2) sponge exposure to contaminated sediments.

During the first phase, triplicates of sediment samples were exposed to Water Accommodated oil Fraction (WAF i.e. crude oil in seawater mixture), Chemically Enhanced Accommodated oil Fraction (CEWAF i.e crude oil and dispersant in seawater mixture) and dispersant in seawater mixtures (Disp). A control treatment was also used where sediments were kept with uncontaminated seawater.

During the second phase, sediments produced during the first phase were used to expose triplicate sponge samples to control sediments, WAF sediments, CEWAF sediments and Disp sediments.

Sample collection

All samples (sponges and sediments) were collected at low tide at Coldingham bay, south east Scotland (figure 1a). This bay is located about an hour away from Heriot-Watt

University's Edinburgh campus and therefore enable the quick return of all samples to Heriot-Watt University after collection, minimising their degradation.

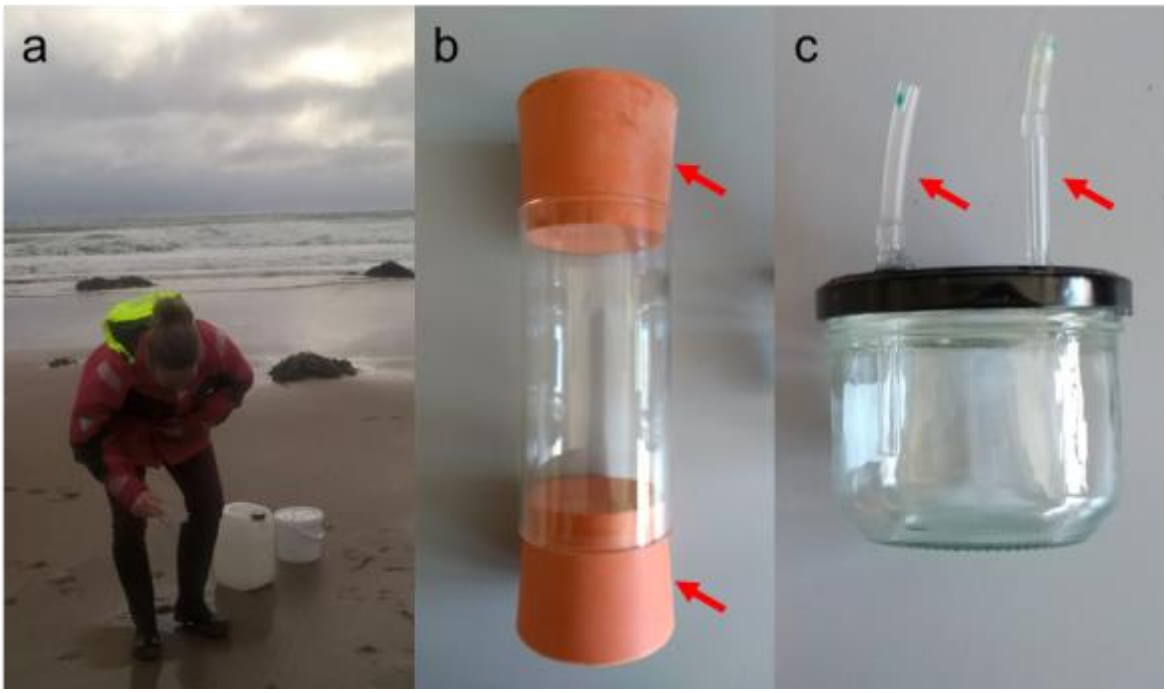

Figure 1 Sampling and incubation chambers used in this study. (a) Sampling of sediments and sponges took place at Coldingham Bay. (b) Glass corer used to sample sediments. The red arrows indicate the rubber bungs used to seal off the samples during collection. (c) Sponge incubation chamber used in this study. The red arrows indicate the inflow and outflow tubes.

Twelve sediment samples were collected using bespoke glass tubes/corers. Each tube of 50 mm in diameter and 100 mm in length were pushed approximately 40mm into the soft sediment. The upper part of the tube was then tightly closed with a rubber bung. The tube could then be removed from the sediment without losing or disturbing the vertical distribution of the samples. A second rubber bung was used to close to second end of the tube in order to transport the sediment samples safely to Heriot-Watt university for the experimental work (figure 1b).

Sample of sponge *Halichondria panicea* were collected from rocks uncovered by the low tide. Samples were carefully scrapped off the rock with a scalpel and placed into a closed container with freshly collected seawater from the bay. Although this sampling method does damage the sponge tissue to remove it from the rock, previous experiments at Heriot-Watt University has shown that *H. panicea* seems to be able to cope with this treatment (personal observation J. Vad).

A 5L seawater sample was also collected at Coldingham bay (refer to as CBSW here after) to be used in the preparation of the experimental treatment solutions.

Experimental set-up for crude oil and/or dispersant contamination of sediments

Upon return to Heriot-Watt University, all samples were placed into a cold-room at 7°C. Sediment samples were kept in the glass corers but the upper rubber rung was removed. A volume of 60.7 mL of CBSW was added to each tube and gentle air mixing was provided for the next 48 hours which allowed for the separate preparation of stock solution for the experimental treatment as described below. The volume of CBSW added was chosen to easily apply the contaminant following Kleindienst *et al.* 2015 proportions as described below.
Stock solution of WAF, CEWAF and Disp were prepared with filtered seawater following Kliendienst *et al.* 2015 and left air mixing for 48hours. Once the mixing period had ended, 17.3 mL of stock solution was added to each tube following the proportions of Kliendienst *et al.* 2015. For the control samples, 17.3 mL of filtered seawater mixed with CDSW was added following the treatment proportions. All corers were then sealed to the best of abilities with Parafilm and kept for another 48hours. Air mixing was still provided during the whole length of the experiment.

At the end of this exposure time, sediments samples were weighted (Wet Weight) and triplicate subsamples were taken of each tube for (1) DAPI staining and bacteria abundance estimation, (2) hydrocarbon analysis and (3) obligate oil degrading bacteria presence analysis. The remaining of the sediments samples were pull together within each treatment and used for the sponge exposure experiment.

Experimental set-up for sponge exposure to crude oil and/or dispersant contaminated sediments

After the first phase of experimentation on sediments, sediments from each treatment were placed in a 4 L conical flasks and 2 L of CBSW was added into the flasks. Rigorous air mixing was provided into each conical flask to allow partial mixing of the sediment and the water. 12 sponge samples were then placed into individual incubation chambers of 250 mL in volume equipped with an inflow at the bottom of the chamber and an outflow at the top of the chamber (Figure 1c). Care was given in selecting sponge samples of similar size between treatment to ensure that the total amount of tissue within each treatment was as similar as possible. The incubation chambers were then connected to 6-channels Color Marine dosing pumps providing a flow of 250 mL per 24 hours from the relevant conical flask and hence relevant treatment. Sponges were left in their incubation chambers for 48 hours.

At the end of this exposure time, respiration and water clearance rates were determined. Consumption of oxygen was measured using an Oxy-4 Presens optode meter over a 60min time period. To determine clearance rate, 2mL of algae solution was added to each chamber and the sponge were left to filter for 120min. Water samples at the beginning and end of the 120min time period were collected and algae cell concentrations were determined. Clearance rates for each sample was calculated as described by de Goeij *et al.* 2008, following the formula below:

$$Clearance\ rate = (\left(\frac{V_{water}}{t}\right)\ln\left(\frac{C_0}{C_t}\right))/V_{sponge}$$

where V_{water} is the volume of water in the chambers, t the time of incubation, C_0 and C_t the initial and final concentration of algae in the chamber and V_{sponge} the volume of sponge tissue.

After respiration and clearance rate measurements were completed, sponge samples were then removed from the incubation chambers and (1) volume of sponge and wet weight was determined for each sample (2) each samples was photographed for visual inspection and (3) tissues samples were collected and preserved in RNAlater for gene expression work.
The sponge samples were then oven-dried for 48 hours at 60°C and dry weight of each sponge was determined.

DAPI staining

To quantify prokaryotic (bacteria and archaea) cell concentrations, we used the DAPI (406-diamidino-2 phenylindole) staining technique. Sub-samples of water and sediments from each treatment and control were fixed with 3.7% formaldehyde and stored at 4C for a maximum of 2 weeks. In the case of the sediment, the sample was sieved with a 32um sieve (to get rid of the sediment) using sterile sea-water (passed through a 0.22um filter) of the same location. For each fixed water sample, 5 ml was filtered (0.22 um) onto gridded (3 mm x 3 mm) polycarbonate filters – this volume was adjusted in order to achieve 10–150 cells per grid. The filters were mounted onto glass slides and the cells stained with DAPI (1 mg/ml) for 20 min and then counted under the Zeiss Axioscope epifluorescence microscope (Carl Zeiss, Germany). A minimum of 10 grids were randomly selected and photographed for counting of cells. The number of cells counted was calculated using the formula:

$$N = \left(\frac{nb}{nSq}\right)Vf\left(\frac{A}{ASQ}\right)$$

where N is the total number of bacteria per mL, nb is the number of bacteria counted, nSq is the number of squares counted, Vf is the volume of sea water filtered, A is the effective filter area, and ASq is the area of one square of the grid.

Hydrocarbon analysis

Sediment subsamples collected for hydrocarbon analysis content were preserved in dichloromethane and will be analysed by GCMS later. Extraction of the hydrocarbon content before GCMS will be done following the UNEP report of 1992.

Gene expression and detection of obligate oil degrading bacteria in sponge and sediment samples

Sediment and sponge subsamples collected for gene expression work or obligate oil degrading bacteria determination were placed into 1.5 mL centrifugation tube and preserved in RNAlater.

Total RNA from the sponge subsamples will be extracted using the Qiagen RNeasy Blood and Tissue extraction kit and kept at -80°C. Primer design is underway to allow for further analysis of these samples. Total DNA will also be extracted from these sponges and sediments subsamples using the Qiagen DNeasy extraction kit and kept at -20°C. Primer design is underway to allow for further analysis of these samples.

Statistical analysis

Analysis of Variance (ANOVA) analysis were carried out to compare numbers of bacteria in sediment and water between treatment as well as clearance and respiration rates between treatment. When the ANOVA was statistically significant, pairwise t test with Bonferroni correction were conducted to determine pairwise difference between treatment. All statistical analysis were performed using R.

RESULTS AVAILABLE TO DATE & DISCUSSION

General results sediment contamination phase

Overall the sediment contamination phase seemed successful as distinct hydrocarbon and/or dispersant smell could be detected at the end of the experiment from each sediment sample. The vertical layering of the sediments was successfully maintained during the whole phase. Oxygen concentration profile throughout the sediment layers would be interesting to monitor and could be considered as another output in future experiments.

Bacterial abundance estimation in sediments

To assess the free-living prokaryotic community dynamics in the different treatments, dispersant or crude oil, DAPI counts were determined. As shown in Figure 2, prokaryotic cell abundance across all treatments at the end of the experiment was quite low in the untreated control and only reached around 400000 cell/ml. Similarly, low prokaryotic cell abundances were achieved in the Disp and WAF treatments (between 9x 10^5 and 10x 10^5 cells/ml, respectively). Prokaryotic cell abundances in the CEWAF treatment was quite high compared to the other treatments reaching 18x 10^5 cells/ml

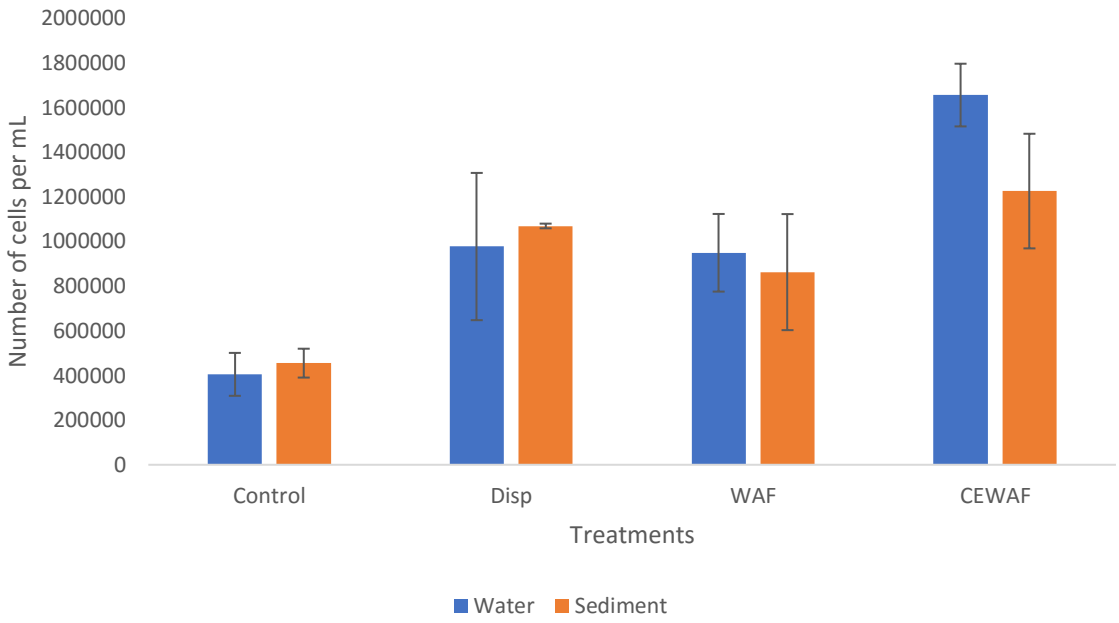

Figure 2 Number of cells per mL in the water column in the different treatments

ANOVA analysis to numbers of bacteria in water and sediments between treatments were conducted. In the water column, a statistical significance could be detected with the ANOVA (table 1) but no pairwise significance difference could be determined.

Table 1 One-Way Analysis of Variance of number of bacteria in water between treatments: Sum of Squares (SS), Mean of Square (MS) F-value (F) and P-value (p)

Source	df	SS	MS	F	p
Between groups	3	$2.363x10^{12}$	$7.877x10^{11}$	12.504	**0.002178**
Within groups	8	$5.040x10^{11}$	$6.299x10^{10}$		
Total	11	$2.867x10^{12}$			

In the sediment, a statistical significance could be detected with the ANOVA (table 2).

Table 2 One-Way Analysis of Variance of number of bacteria in water between treatments: Sum of Squares (SS), Mean of Square (MS) F-value (F) and P-value (p)

Source	df	SS	MS	F	p
Between groups	3	$1.003x10^{12}$	$3.343x10^{11}$	6.491	**0.01563**
Within groups	8	$4.134x10^{11}$	$5.167x10^{10}$		
Total	11	$1.416x10^{12}$			

Furthermore, a statistical difference between control numbers and CEWAF could also be detected (table 3).

Table 3 Bonferroni corrected P-value for pairwise comparison t-test of numbers of bacteria in sediments between treatments. Bold font highlights significant results.

	Control	WAF	Disp	CEWAF
Control	-	-	-	-
WAF	0.356	-	-	-
Disp	0.064	1	-	-
CEWAF	**0.019**	0.57	1	-

General results sponge exposure phase

All sponge samples used in this experiment seemed, overall, to be coping well with the collection and treatments. All sponges survived the experiment and no visual differences between samples exposed to the 4 treatment conditions could be determined.

Respiration rate

Respiration rate for *H. panicea* samples at the end of the experiment ranged between 0.155 and 0.288 µmol of O_2 per hour per cm^{-3} of dry tissue (Figure 3). These results are in accordance with previous measurements made on *H. panicea* (data J. Vad) and slightly lower than observed for tropical encrusting sponges (De Goeij *et al.* 2008).

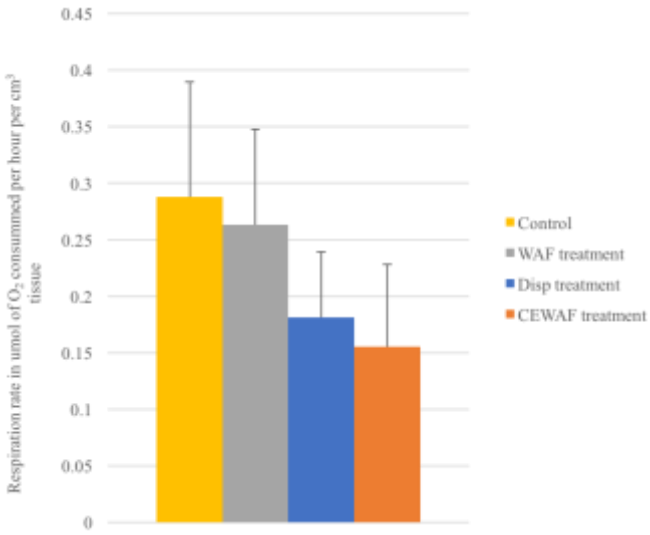

Figure 3 Respiration rate (µmol of O_2 consumed per hour per cm^3 of tissue) between treatment

No significant differences in respiration rate between treatment was detected here (table 4). However, a decreasing tendency with sponges in the CEWAF treatment displaying the lowest average respiration rate was observed (figure 3).

Table 4 One-Way Analysis of Variance of respiration rates between treatments: Sum of Squares (SS), Mean of Square (MS) F-value (F) and P-value (p)

Source	df	SS	MS	F	p
Between groups	3	0.036504	0.0121681	1.8668	0.2135
Within groups	8	0.052145	0.0065181		
Total	11	0.088649			

The lack of significant difference in the result is mainly due to the high inter-individual variability in respiration rate. This has been previously observed for *H. panicea* in previous experiment (data J. Vad) and for other species in previous work (Schuster 2015).

Clearance rate

Clearance rate for *H. panicea* samples at the end of the experiment ranged between 0.0435 to 0.318 cm^3 of water cleared per min per cm^3 of tissue (Figure 4).

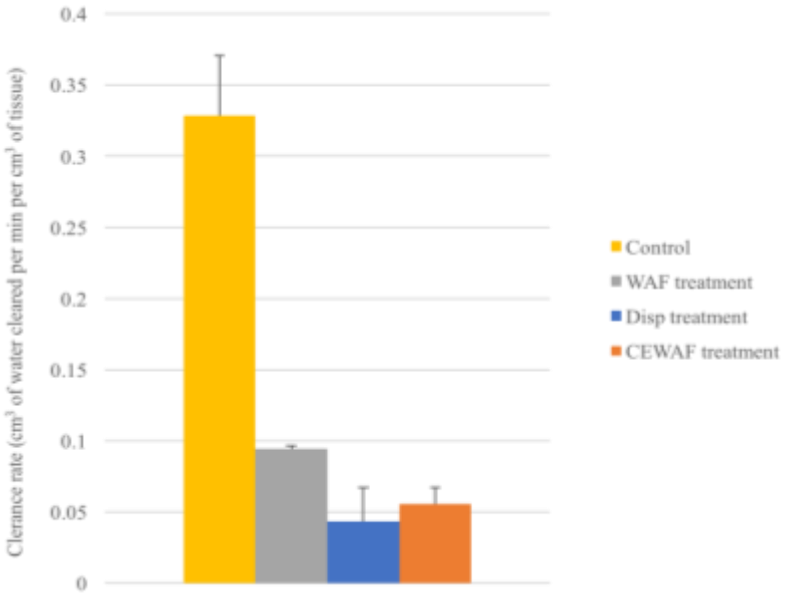

Figure 4 Clearance rate (cm3 of water cleared per cm3 of tissue per min) between treatments

These results are in accordance with previous measurements made on *H. panicea* (data J. Vad) but slightly lower than the clearance rate observed for tropical encrusting sponges (De Goeij *et al.* 2008).

The ANOVA analysis showed a significant difference between treatments (Table 5).

Table 5 One-Way Analysis of Variance of clearance rates between treatments: Sum of Squares (SS), Mean of Square (MS) F-value (F) and P-value (p)

Source	df	SS	MS	F	p
Between groups	3	0.160999	0.053666	87.544	**$1.85x10^{-6}$**
Within groups	8	0.004904	0.000613		
Total	11	0.165903			

Pairwise t-test determined statistically significant difference between the control conditions and all three treatments but no statistical differences between the three treatments (table 6).

Table 6 Bonferroni corrected P-value for pairwise comparison t-test of clearance rates between treatments. Bold font highlights significant results.

	Control	WAF	Disp	CEWAF
Control	-	-	-	-
WAF	**$5.2x10^{-6}$**	-	-	-
Disp	**$3.7x10^{-6}$**	1	-	-
CEWAF	**$1.7x10^{-5}$**	0.54	0.21	-

These results are in accordance with previous studies showing that sponges can stop their filtering activities when environmental conditions changes (Tompkins-Macdonald and Leys, 2008; Bannister *et al.* 2012; Kutti *et al.* 2016). Moreover, sponges exposed to 'unnatural' sediments such as drill cuttings charged with barite and bentonite also halted their filtering behaviour (Kutti *et al.* 2016). This is the first study to the authors knowledge which shows a similar behaviour in sponges exposed to crude oil and/or dispersant contaminated sediments.

CONCLUSION

The CEWAF treatment simulated the application of a UK-approved dispersant (Superdispersant-25) – approach that is often used as a bioremediation strategy for combatting marine oil spills –to investigate the microbial response in coastal Scottish waters. Interestingly, other studies such as Kleindients et al. (2015) and Duran Suja et al. (2017) also measured higher cell abundances in the CEWAF treatments compared to in the WAF treatments. Taken collectively, these results suggest that the presence of dispersant stimulate microbial growth in coastal surface waters and sediments when contaminated with crude oil. Whether any microbial group was able to degrade and grow on the dispersant used in this study (Superdispersant-25) remains to be investigated.

In this study, *H. panicea* sponge appear able to cope with exposure to oil and dispersant contaminated sediments by altering its filtering behaviours. Here we used a short exposure time but long time effects on sponges as hydrocarbons and dispersants degrade very slowly (scale of years) in sediments could be anticipated. Further experiments to determine the effect of long-term exposure of contaminated sediments on sponges would be required to investigate this question fully. Some results from this experiment are not available yet to analysis but an output in the form of a published scientific paper is expected once all the results and analysis are available.

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