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Effects and bioaccumulation in field

D3.13

WP3: Determination of oil and dispersant impacts on biota using effect-based tools and ecological risk assessment

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Executive Summary

In situ oil burning is a response technique of high potential, since it substantially reduces the spilled oil volume and here in particular the low molecular weight components, which are more prone to partitioning into the water phase and thus exert acute toxic effects. Thus, an experimental pilot-scale oil spill was carried out in an enclosed coastal Arctic site in Greenland (in relation with WP4) with the aim of testing the effectiveness and environmental effects of *in situ* oil burning, both on-shore and off-shore. Field caught and transplanted mussels were used for determining the bioaccumulation of contaminants and biological effects (biomarkers). The present report deals with the on-shore *in situ* oil burning field experiment. Due to the extreme conditions of the scenario several logistic hurdles were found and recommendations for future pilot studies in the area have been made based on the achieved experience. Meanwhile, the reliability of the obtained data both on pollutant tissue levels and on biological responses is limited, and the present (preliminary) results had to be interpreted with caution. Tissue-level biomarkers and histopathology stages could not be satisfactorily determined. However, differences in the tissue levels of C10-C25 THCs were recorded, especially at 1 m depth; these can be associated to the presence of diesel-like compounds. In agreement, although oxidative stress biomarkers were not responsive and although a caging effect was also envisaged in lysosomal responses, we obtained evidences of the biological impact of *in situ* oil spill burning, especially at 1 m depth. Lysosomal membrane destabilization and lysosomal shrinking were elicited in mussel digestive cells together with a trend to increase lipofuscin. This is the response profile expected after short term exposure to petroleum hydrocarbons. Therefore a certain cause-effect relationship is seemingly established between oil spill burning onshore, C10-C25 hydrocarbon tissue levels and lysosomal biomarkers of adverse effects.

1. Rationale

Combat of oil spills by alternative response techniques should be performed at water depths and distances to land that will ensure dilution and hence non-toxic effects, as well as avoiding smoke from an *in situ* burn to contaminate inhabited areas or residues to reach the seabed. However, under certain circumstances such as extreme oceanic conditions, including ice infested waters, and especially in sparsely populated areas with difficult logistics, spilled oil could be contained in a closed water body confined by the coastline for mechanical recovery and *in situ* burning, according to the Canadian guidelines (Wegener et al., 2017). *In situ* oil burning is considered a response technique of high potential since it substantially reduces the spilled oil volume including field experiments with high ice concentration. Furthermore, since the burning largely affects volatile components, in particular the low molecular weight PAHs are reduced, which are more prone to partitioning into the water phase and thus exert acute toxic effects.

In this framework, and in relation with WP4 activities, an experimental pilot-scale oil spill in an enclosed coastal Arctic site in Greenland was conducted with the aim of testing the effectiveness and environmental effects of different oil response actions, including the use of two different *in situ* oil burning experiments: on-shore and off-shore. In both experiments, field caught and transplanted mussels were used for determining the bioaccumulation of contaminants, and the biological effects produced after the *in situ* oil burning by means of chemical analysis and biomarker approach analysis, respectively. The present report deals only with the data obtained in the on-shore *in situ* oil burning field experiment.

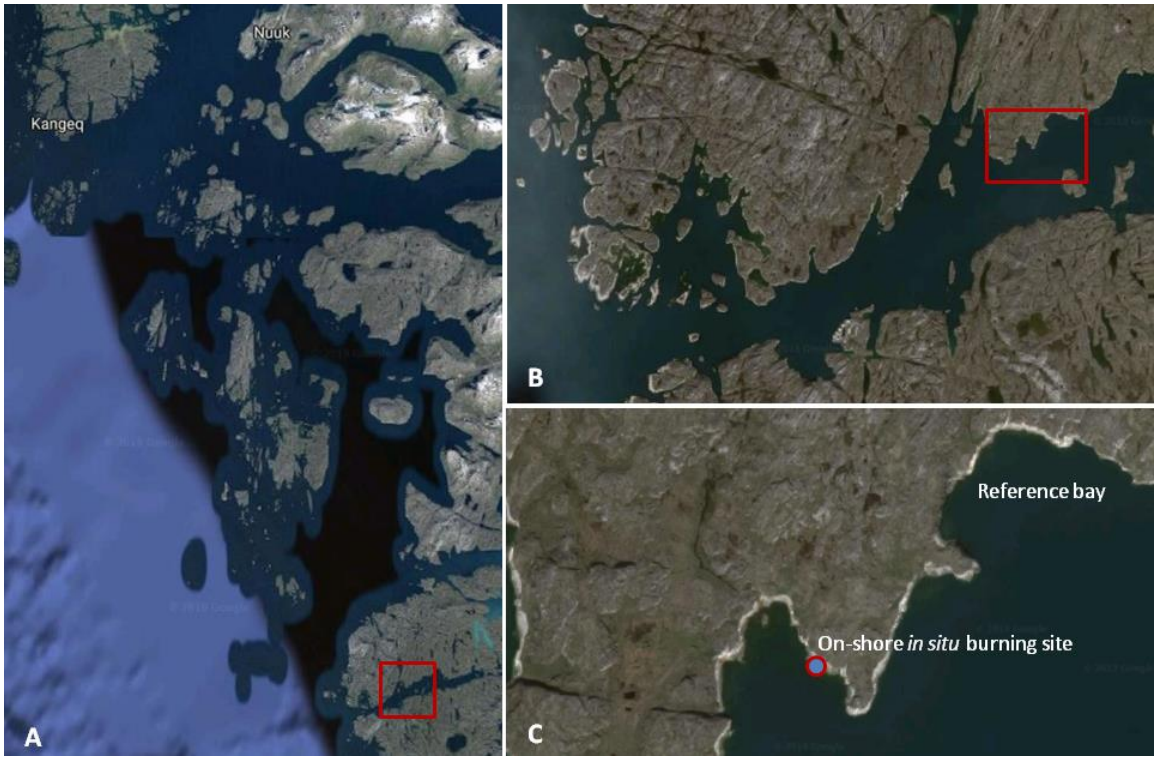

Figure 1. Location of the on-shore *in situ* oil burning experiment in a bay from Faeringehavn, south of Nuuk (A, B) and the bay considered as reference (C). (Source of the images: google maps)

2. On-shore *in situ* burning experiment

2.1 Location

The on-shore *in situ* burning experiment was carried out in a bay (63º 42.1940 N, 51º 27.7180 W) of the vicinity of Faeringehavn, south of Nuuk, Greenland (Figure 1A and B). In addition, an adjacent bay (63º 42.3800 N, 51º27.7180 W) was chosen as reference bay (Figure 1C).

2.2 Experimental set-up

The whole experimental set-up is summarised in a scheme (Figure 3). Intertidal blue mussels (*Mytilus* spp.) were collected at low tide in the shoreline of the reference bay (Figure 1C) the 2nd of July 2017 and caged at 2 different depths (-1m and -4m) under 2 different buoys (RA and RB) in the reference bay. Additionally, mussels from the same reference shoreline where transplanted to the bay where the on-shore *in situ* oil burning experiment (OSB) was going to take place (Figure 1C; 2) and caged at 2 different depths (-1m and -4m) in 4 different lines OSBI, OSBII, OSBIII and OSBIV, settled close to the fire booms (Pyrobooms) to be used for the *in situ* burning of oil at the shoreline (Figure 2).

Figure 2. Image of the bay for the on-shore *in situ* oil burning experiment with the fire booms (footage from the video "Combat of oil spills in Arctic waters - in situ burning experiments, Greenland. Summer 2017. GRACE" https://youtu.be/51ieM7h7ykM)

The on-shore *in situ* oil burning took place the 4th of July. Approximately 800 L of naphtenic North Atlantic crude oil were spilled between the shoreline and the fire booms and the fire was ignited. The fire lasted around 1 h. Caged mussels exposed to the on-shore *in situ* oil burning and from the reference bay were collected 3 days later, the 7th of July, and taken to Nuuk for storage. The mussels were transported in air (dry box) at ambient temperature. Transport lasted between 3 to 4 hours, and once in Nuuk mussels were directly placed in toto (not even valves were opened) a -80 ºC in the freezer.

Further on, some of the collected mussels were taken to AU (Denmark) for chemical analysis while the rest was left in Nuuk stored at -80 ºC for several weeks before they were transported to UPV/EHU and further on to SYKE for biomarker analysis.

Figure 3. Image of the on-shore *in situ* burning of naphtenic North Atlantic crude oil after the fire was ignited. (footage from the video "Combat of oil spills in Arctic waters - in situ burning experiments, Greenland. Summer 2017. GRACE" https://youtu.be/51ieM7h7ykM)

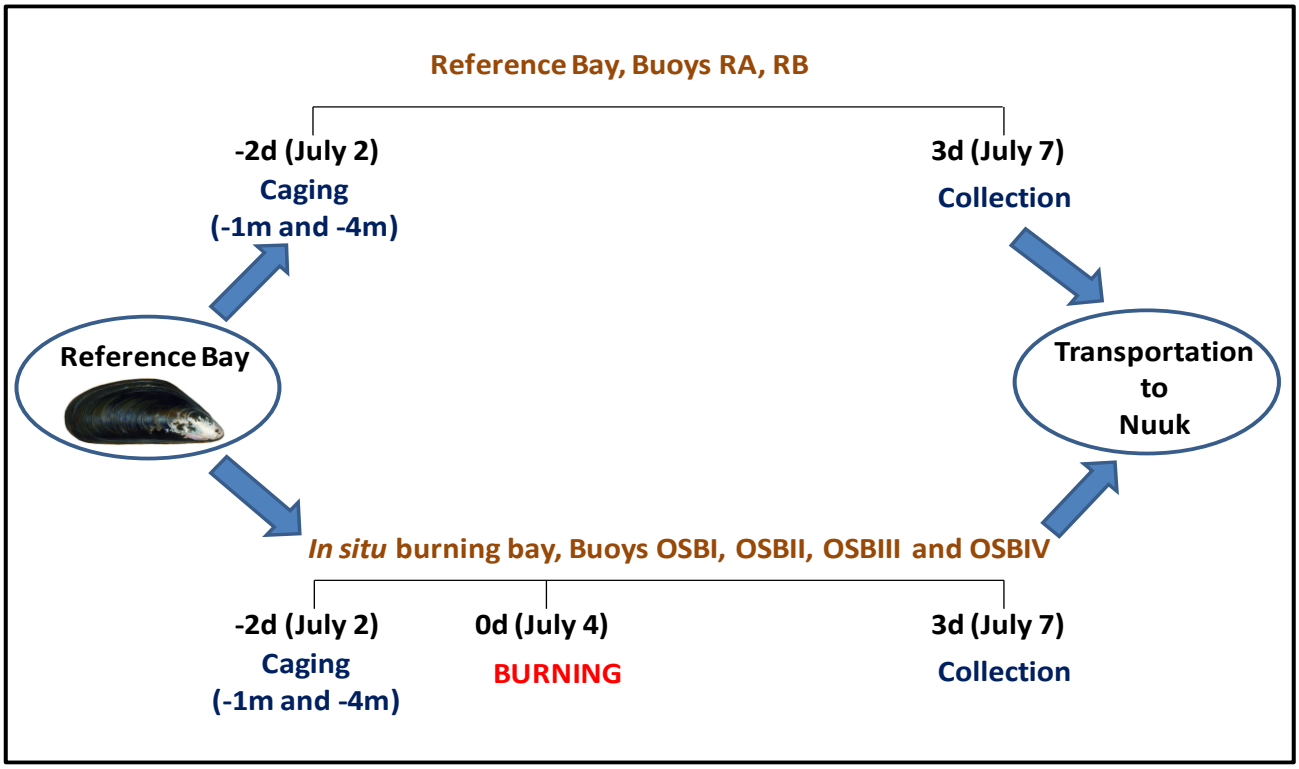

Figure 4. Scheme summarising the set-up of the on-shore *in situ* burning of the naphtenic north Atlantic crude oil.

2.3 Sample transportation and dissection

Working in Greenland involves having several limitations when it comes to logistic issues. In this sense, the lack of dry-ice and liquid nitrogen was a major hurdle for the transportation of samples at the required temperature (at least of -80 ºC to secure fully reliable biomarker analysis). After different consultations with different specialized logistic companies they all agreed in the difficulties of collecting the samples and transporting them in the required conditions mostly due to the lack of partner companies in the area and the limited flight connections with Nuuk. This led us to conclude that the only way of collecting and bringing the samples was travelling to Nuuk with a dry-shipper with enough autonomy so as to travel, collect the samples and return without compromising the temperature of the samples. The disadvantage of having autonomy in a dry-shipper is that the space availability is compromised. Consequently, we were forced to select the samples to be transported and required for the intended biological analysis (NOTE: together with samples from the on-shore burning experiment also samples from off-shore *in situ* oil burning experiment had to be transported). Thus, a meticulous selection of samples from the on-shore *in situ* oil burning experiment had to be made (Table 1).

According to the data logger of the dry-shipper, the mussel samples were transported at -153 ºC and reached to the Plentzia Marine Station in perfect conditions for biomarker analysis.

Table 1. The samples available from the on-shore *in situ* oil burning experiment for the biomarker analysis.

Reference area 2 buoys (A and B)		**On-shore *in situ* burning** 2 buoys (OSBI and OSBIV)	
RA	**no. samples**	**OSBI**	**no. samples**
1m depth	10	1m depth	7
4m depth	9	4m depth	13
RB		**OSBIV**	
1m depth	10	1m depth	8
4m depth	8	4m depth	9

Mussel dissection was carried out with special care avoiding breaking the cold chain in order to maximize sample availability and quality. It has to be mentioned that during dissection the general occurrence of thick layers of ice inside the mussel was observed .

As far as the digestive gland is concerned, a tissue core (small biopsy: approx. 8-12 mm^3) was obtained from each single mussel in order to prepare a set of tissue arrays to analyse lysosomal biomarkers (histochemistry). The remaining part of the digestive gland was divided in two portions.

One of them was excised for oxidative stress biomarkers and processed and transported to SYKE for this purpose. The second portion was left inside a cross-section of the mussel containing different organs including mantle, gills and foot tissue for the analysis of tissue level biomarkers and histological (e.g., gonad) and histopathological examination (essentially gills and digestive tract). A portion of the gills was also left out of this cross-section and processed and transported for oxidative stress biomarker analysis at SYKE. Digestive gland and gill samples dissected for SYKE were transported to Finland in a dry-shipper.

3. Battery of biomarkers

The selected biomarkers are commonly employed for biological effect assessment in marine pollution monitoring (Brooks et al., 2011; Garmendia et al., 2011; ICES, 2013; Turja et al., 2013, 2014; Lehtonen et al., 2016). The battery of biomarkers included catalase (CAT), glutathione reductase (GR) and glutathione (GST) enzymatic activities, lysosomal responses and tissue-level biomarkers. Gamete maturation was also to be used as supporting parameter.

CAT is an essential antioxidant defense used as biomarkers of oxidative stress; GST catalyzes the conjugation of reduced glutathione with xenobiotics or oxidized cellular components, and GR replenishes the glutathione substrate. These oxidative stress biomarkers have been regularly used in biomarker-based pollution impact assessment in the North Sea and the Baltic sea (Brooks et al., 2011; Turja et al., 2013; 2014; Lehtonen et al., 2016). Lysosomal responses to pollutants in mussel digestive cells are widely used as effect biomarkers (Izagirre & Marigómez, 2009; Brooks et al., 2011; Garmendia et al., 2011; Marigómez et al., 2013). Lysosomal enlargement (augmented volume density: Vv_{LYS}) has been reported in response to pollutant exposure, and lysosomal membrane destabilization (reduced labilization period: LP) is recommended by OSPAR as a core biomarker for marine pollution monitoring programmes (OSPAR, 2103) . Intracellular accumulation of neutral lipids (augmented volume density; Vv_{NL}) has been related to exposure to various stress sources including, e.g., PAHs and other organic chemicals (Cancio et al., 1999; Marigómez and Baybay-Villacorta, 2003; Marigómez et al., 2013). Likewise, changes in cell type composition in the digestive gland epithelium (e.g., increase in volume density of basophilic cells: Vv_{BAS}), atrophy of the digestive epithelium (augmented lumen-to-epithelium ratio: MLR/MET), inflammatory responses, and loss of digestive gland histological integrity (augmented connective-to-diverticula ratio: CTD) have been reported to occur in response to pollutant exposure (Brooks et al., 2011; Marigómez et al., 2013).

Therefore, this battery of biomarkers was conceived as a realistic tool (under the particular conditions and restrains of a field experiment in Nuuk) in order to investigate the impact of *in situ* burning of an oil spill on shore in Arctic conditions.

4. Preliminary Results and Discussion

4. 1. Chemical analysis

The chemical analysis of the blue mussels caged in the reference bay and after on-shore *in situ* oil burning were performed by AU as a part of WP4. They were kindly provided for discussion herein. For this purpose, the results are summarized in terms of total hydrocarbons in Figure 5. Basically, it seems that only differences in the tissue levels of C10-C25 THCs can be observed, which can be associated to the presence of diesel-like compounds, especially at 1 m depth.

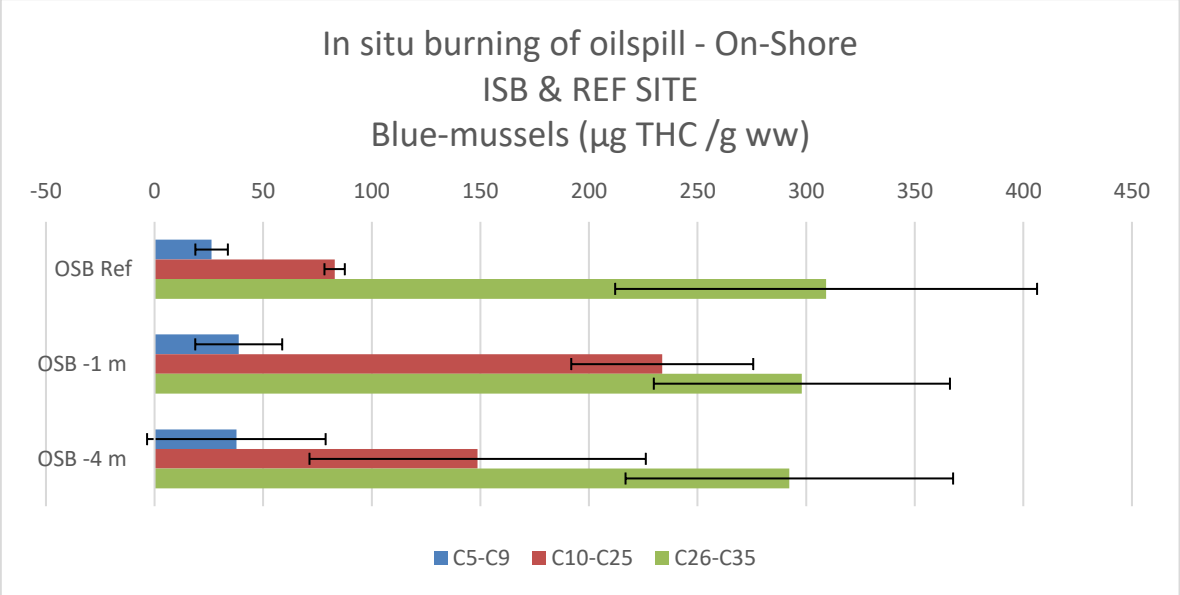

Figure 5. Plot showing the total hydrocarbon levels measured in mussel caged in the reference site and in the on-shore *in situ* burning bay after the experiment (by courtesy of K Gustavson, Aarhus Univ, GRACE WP4).

4. 2. Tissue level biomarkers and histopathology

It was not technically possible to perform a reliable analysis, as the integrity of the digestive gland tissue was critically compromised (Figure 6A). Thus, general stress biomarkers such as basophilic cell volume density, epithelial thinning and connective to digestive tissue ratio could not be calculated, and the histopathological examination of the digestive gland was unfeasible. Likewise, the histological integrity of the gills (Figure 6B) and mantle (Figure 6C) was affected as well. These damages were seemingly due to the volume changes and further ice-crystals formation in the tissues as mussels were frozen *in toto* with water in the shell cavity. This way of processing might also have consequences for other biomarker calculations and therefore their results must be cautiously interpreted.

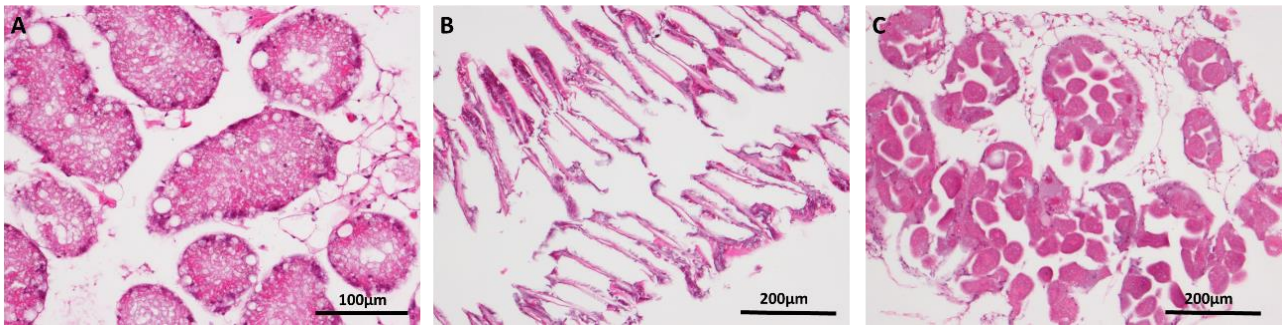

Figure 6. Micrographs of the histological preparation of mussels from the on-shore *in situ* burning experiments. A: detailed view of the digestive gland, where heavily vacuolated digestive alveoli can be observed. B: detailed view of the gills with severely damaged gill lamellae. C: detailed view of the gonad tissue in the mantle with structurally compromised oocytes.

4. 3. Oxidative stress biomarkers

In summer, in Baltic mussels CAT values are around 50 µmol min^{-1} mg-$protein^{-1}$ in digestive gland and 14 in gills; GR_{gills} is 19.0 µmol min^{-1} mg-$protein^{-1}$; and GST_{dg} and GST_{gills} are in the range of 134-232 µmol min^{-1} mg-$protein^{-1}$ (Marigómez et al., 2018). Presently, the enzyme activity values obtained are of similar magnitude and remained unchanged between the reference bay and after the on-shore *in situ* oil burning (Fig. 7). Likewise, identical enzyme activities were recorded at both depths. Elevated concentrations of PAHs in spring and early summer have been associated with elevated CAT, GR and GST in mussels caged in the Baltic and in the North Sea (Brook et al., 2011; Turja et al., 2013; 2014; Lehtonen et al., 2016). It seems therefore that oil burning under the present conditions did not exert any oxidative stress in mussels. However, it cannot be disregarded that confounding factors can be masking biological responses or their measurement.

On the one hand, intertidal mussels were collected from the reference bay and caged both at the reference and the experimental bays only for 1 day, and therefore they were already subjected to circatidal and feeding cyclic rhythms or could be reacting to an "eventual" prolonged immersion. Whichever the case, this would be causing marked "normal" physiological responses that could be compromising the responsiveness of these enzymes or simply act as confounding factors. On the other hand, as above mentioned, sample processing (3-4 hr transportation and *in toto* freezing) could have affected cellular integrity and enzyme activity, thus adding "noise" to the signal of these biomarkers (Blanco-Rayon et al., 2019).

It could also be concluded that *in situ* burning did not cause biological effects. However, this seems to be less likely because the tissue levels of diesel-like HCs (C10-C25) seem to be higher in mussels caged in the vicinity of the oil burning site than in the reference site and higher in those kept at 1 m depth than in those kept at 4 m depth; thus, differences in biological responses could

be expected. Moreover, some differences in lysosomal biomarkers were recorded between reference and exposed mussels (see below), and these resembled the profile of tissue C10-C25 levels.

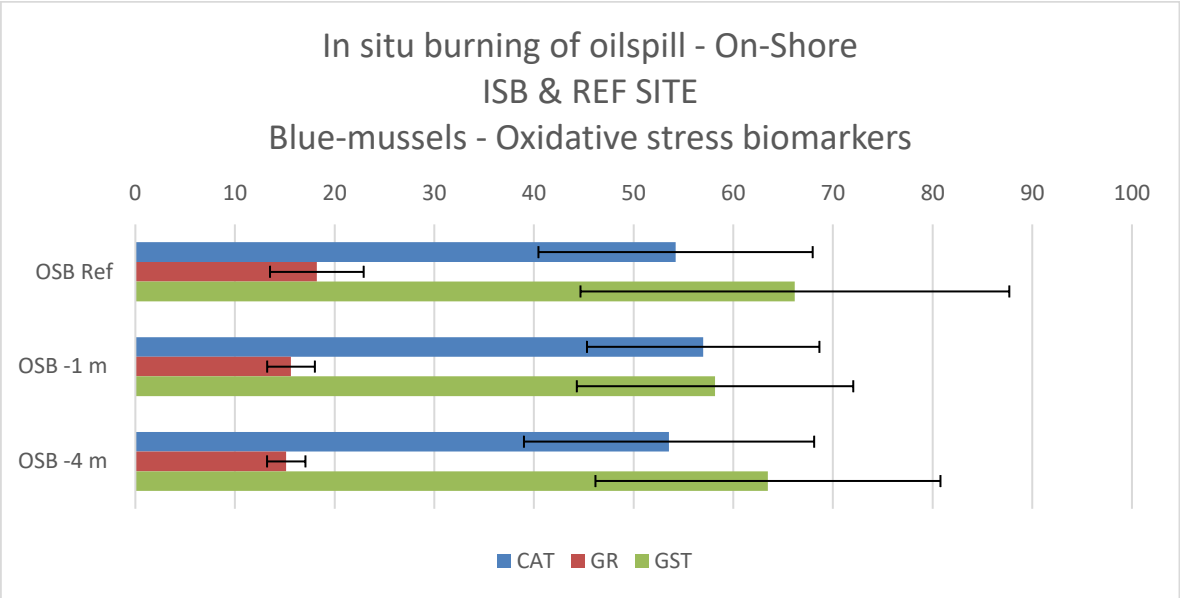

Figure 7. Plot showing the oxidative stress biomarkers (enzyme activities) measured after the experiment in the digestive gland of mussel caged at the reference site and in the on-shore *in situ* burning bay . CAT, catalase; GR, glutathione reductase; GST, glutathione- S-transferase. Units: µmol min^{-1} mg-$protein^{-1}$.

4. 4. Lysosomal biomarkers

Preliminary observations (Lekube et al., 2019) revealed that in Arctic mussels (Trømso) in summer 2017 the regional values of lysosomal biomarkers were 15 min for LP, ~0.0050 μm^3 / μm^3 for Vv_{LYS} ~0.0050 μm^3 / μm^3 , ~0.0970 μm^3 / μm^3 for Vv_{LPF} ~0.0970 μm^3 / μm^3 and ~0.1100 μm^3 / μm^3 for Vv_{NL}. Presently, in mussels from the reference site caged *in situ* LP values were slightly lower and Vv_{LYS} values slightly higher than those putative regional values (Figure 8), which could be attributed to the season (early summer *vs*. late summer), local characteristics and to the effect of caging. Indeed, the effect of caging has been shown to cause decrease in LP values and an increase in Vv_{LYS} values in mussels (Brooks et al., 2011; Marigómez et al., 2013; Lehtonen et al, 2016). Moreover, as discussed above , also the way in which the samples were processed may have influenced lysosomal biomarkers (Blanco-Rayón et al., 2019). On the other hand, the levels of lipofuscins (Vv_{LPF}) and neutral lipids (Vv_{NL}) were lower than expected. Since the opposite could be expected after caging (Brooks et al., 2011; Marigómez et al., 2013) these differences might be attributed to either differences between localities or between seasons. The latter option is very likely to be relevant: in temperate mussels it has been reported (Cancio et al., 1999; Garmendia et al., 2010) that the intracellular levels of neutral lipids and lipofuscins markedly increase since early

summer (on shore *in situ* oil burning experiment; present report) and late summer (field study to establish regional values; Lekube et al., 2019).

Upon exposure to *in situ* burning of the oil spill on shore Vv_{NL} remained unchanged. In contrast, other lysosomal responses were apparently elicited in mussels in comparison to mussels caged at the reference site. Lysosomal responsiveness in digestive cells of mussels is governed by a combination of the interaction between chemicals and cellular membranes and by the progression of intracellular digestion (Izagirre et al., 2008; 2009); basically, exposure to chemicals is effective after 4 h, the duration of a digestion cycle when this is impaired, as shown in mussels exposed to oil WAF (Izagirre et al., 2009). Thus, although the degree of responsiveness was most likely attenuated for the reasons aforementioned, unlike for other biomarkers we succeeded in recording evidences of changes in lysosomal biomarkers.

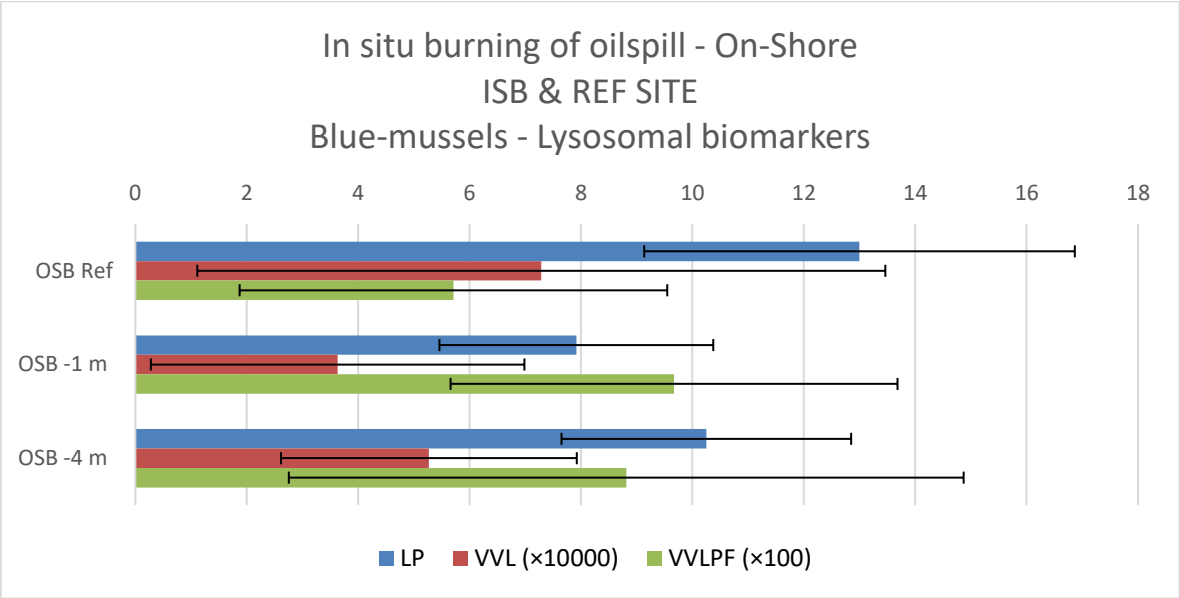

Figure 8. Plot showing the lysosomal biomarkers (histochemical tests) measured after the experiment in digestive gland of mussel caged at the reference site and in the on-shore *in situ* burning bay. LP, lysosomal membrane labilization period (min); VVL (× 10000), volume density of digestive cell lysosomes (Vv_{LYS}; μm^3 LYS/ μm^3 DC) multiplied by 10^4 for scaling the representation; VVLPF (× 100), volume density of digestive cell lipofuscins ($Vv_{LPF;}$ μm^3 LPF/ μm^3 DC) multiplied by 10^2 for scaling the representation.

Lysosomal membrane destabilization (reduced LP; Figure 6) and lysosomal shrinking (reduced Vv_{LYS}, Figure 6) were elicited in mussels exposed to oil spill burning on shore, more markedly in mussels caged at 1 m depth than in those caged at 4 m. Likewise, a trend to increase lipofuscins (Vv_{LPF}) was also observed, irrespective of the depth in this case. This response profile (lysosomal size reduction and membrane destabilization, and slight lipofuscin accumulation) corresponds to the one that could be expected after short term exposure to petroleum hydrocarbons (Marigómez & Baybay-Villacorta, 2003; Izagirre & Marigómez, 2009), and resembles the same trends that has

been envisaged in the tissue levels of C10-C25 HCs. This is a challenging initial point to establish a cause-effect relationship.

5. Concluding Remarks

For future studies, logistics needs to be improved in order to get fully reliable data both on pollutant tissue levels and on biological responses to burnt oil exposure *in situ*. First, the biology of the caged mussels needs to be considered for the experimental design: use subtidal mussels for subtidal caging (best option) or extend the acclimatization for at least beyond 2 weeks (which is not easy from the logistic point of view and most likely not the best solution from the biological point of view; intertidal and subtidal mussels respond differently) and/or collect in parallel intertidal feral mussels both in the reference and the experimental sites. Second, samples must be dissected *in situ* (or at least taken to the lab under proper conditions, depending on the endpoint) for further on processing for either biomarker determination (properly frozen or fixed) or depuration before chemical analysis. Finally, best available practices including a person in charge (and sufficient budget) must be secured for sample transportation without breaking the cold chain.

Consequently, all the present results had to be interpreted with caution and the tissue-level biomarkers, gills and digestive gland histopathology, and gamete developmental stages could not be properly determined. Nevertheless, differences in the tissue levels of C10-C25 THCs were recorded, especially at 1 m depth. These can be associated to the presence of diesel-like compounds. In agreement, although oxidative stress biomarkers were not responsive and although a caging effect was also envisaged in lysosomal responses, we obtained evidences of the biological impact of *in situ* oil spill burning, especially at 1 m depth. Lysosomal membrane destabilization and lysosomal shrinking were elicited together with a trend to increase lipofuscin, which corresponds to the response profile expected after short-term exposure to petroleum hydrocarbons. This is a challenging initial point to establish a cause-effect relationship.

Acknowledgements. The chemical analysis of the blue mussels caged in the reference bay and after on-shore in situ oil burning were performed by AU as a part of GRACE WP4. The results and Figure 1 were kindly provided by Kim Gustavson, Janne Fritt-Rasmussen and Susse Wegeberg (Aarhus University, GRACE WP4) for discussion herein.

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