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Abstract

The decrease of ice cover in the Arctic will lead to an increase of ship traffic in the upcoming decades. Consequently, oil pollution is expected. In this context, the goals of this study were to evaluate the biological impact of marine diesel contamination and, on this basis, to determine analytical tools of interest (biomarkers) for future biomonitoring of diesel spills.

Using a seven days contamination protocol, this study investigated biochemical modulations in the digestive gland of the Iceland scallop (*Chlamys islandica*). Incorporation of contaminants was verified assessing hemolymph metabolites. Results showed a response of glutathione-S-transferase to contamination suggesting detoxification processes and the suitability of such a tool for diesel spills biomonitoring. The lack of modulation of superoxide dismutase activity and lipid peroxidation suggests no oxidative stress and the unsuitability of these molecular tools for biomonitoring.

1. Introduction

In Arctic, the decrease of ice cover will permit oil and gas prospecting as well as an increase of ship traffic. This will lead to operational discharges and spills of oil at sea. In order to minimize the impact of this pollution, the government of Norway recently banned the use of heavy fuel oils in parts of the Svalbard territorial waters and permitted only the use of marine diesel (Ministry of the Environment of Norway 2009). This decision is based on the fact that heavier oils are considered to be the more persistent ones (Markarian et al. 1995). However, even though lighter oils, such as marine diesel, tend to be less persistent, they are potentially more toxic to aquatic organisms since their PAHs (polycyclic aromatic hydrocarbons) concentrations are higher (Neff et al. 1979). While some ecotoxicological studies were conducted in temperate (Lotufo 1997; Rodrigues et al. 2010) and tropical (Stirling 1977; Lüchmann et al. 2011) marine organisms to investigate marine diesel toxicity, only one study

to our knowledge investigated the toxicological effects of marine diesel on an Arctic species, the copepod *Calanus glacialis* (Hansen et al. 2013). Thus, taking into account the likelihood of diesel oil spills in the Arctic waters, its potential ecotoxicity and the lack of investigation in this field of research, ecotoxicological studies are needed in order to (i) highlight the biological impact of this emerging environmental threat and (ii) propose valuable biological indicators for future biomonitoring.

Thus, this study aims to assess biomarker modulation in the digestive gland of the Iceland scallop (*C. islandica*) exposed to diesel. This species was chosen since (i) filter-feeding molluscs are considered valuable sentinel species of ecosystems due to their capacity to bioaccumulate and respond to contaminant exposure (Viarengo et al. 1993; Solé et al. 2007); (ii) *C. islandica* is sessile and globally distributed in Arctic waters so that results of this study could be used for several future monitoring programs.

Glutathione-S-transferase (GST) activity was measured, since this enzyme is considered a valuable and sensitive biomarker of hydrocarbons exposure in bivalve molluscs and is consequently commonly used (reviewed in Amiard and Amiard-Triquet, 2008). More specifically, GST has been shown to be a reliable biomarker for marine diesel contamination (Lüchmann et al. 2011).

A biomarker of oxidative stress, lipid peroxidation, has been chosen since it is a suitable biological indicator of hydrocarbon contamination and it reflects a degradation of the functional integrity (Halliwell and Gutteridge 1999). Additionally, activity of the superoxide dismutase (SOD) has been measured considering its important role in antioxidant protection (Valavanidis et al. 2006) as well as the precocity and the sensitivity of this enzyme response to hydrocarbon contamination (Oliveira et al. 2008; Hannam et al. 2010; Sun et al. 2006; Oliva et al. 2011; Milinkovitch et al. 2013a).

Finally, hydrocarbon incorporation was verified by assessing metabolites of hydrocarbon in the hemolymph of scallops.

2. Materials and methods

All Iceland scallops were sampled the third week of April in the fjord of the Norwegian sea close to the city of Tromsø (69° 35.060' N, 18° 55.701' E), by dredging between 10 and 20 m depth (temperature varying between 4 and 6°C). The dredging did not damage the scallops, and all the animals used for the experiment had no sign of exterior diseases or damages. Then, the scallops were acclimatized at the Akvaplan-niva marine laboratory for 6 weeks in tanks supplied with flow through seawater from the fjord (5.14 ± 0.03 °C). Scallops were under natural photoperiod and fed daily with Shellfish Diet 1800 (Reed Mariculture Inc.), a diet made of five microalgae (*Isochrysis, Pavlova, Tetraselmis, Chaetocerous calcitrans, Thalassiosira weissflogii*), suitable for bivalves such as oysters, mussels and scallops. The average length of the shells, at the end of the acclimatization period, was 66.59 ± 0.41 mm (mean ± standard error of the mean) and the average weight was 34.50 ± 5.3 g. The soft tissues weight was 15.80 ± 3.30 g.

The experimental device is a static water system adapted from Milinkovitch et al. (2011a) and already used in several other studies investigating dispersed oil (e.g. Claireaux et al. 2013; Milinkovitch et al. 2013a, 2013b). It is made of two 120-L tanks stocked in a 5 °C temperature-controlled room. Each tank is composed of a funnel (at the water surface) connected to a 12-V water pump (on the floor of the tank). The marine diesel is constantly drawn up through the funnel into the pump so that it is maintained homogenously as droplets in the water column, *i.e.* physically dispersed, and no coalescence of oil slick at the water surface is possible.

Marine diesel (obtained from a BP® ship fuel station in Tromsø) was added to 110 L of filtered seawater in the contaminated tank (described above) to obtain a nominal concentration of 29 ppm. In parallel, a similar 120-L control (C) tank with 110 L of uncontaminated seawater was set up. Hydrocarbons concentration matches with values observed *in situ* in the water column during some oil spills: for instance, from 1 to 100 mg/L of total petroleum hydrocarbons were measured in coastal waters around Shetland during the Braer oil spill, as reported by Lunel (1995). After addition of contaminants, 10 scallops with a 50% sex ratio (sexed according to the colour of the gonads) were randomly distributed into each tank (*i.e.* each treatment) and exposed for 7 days without being fed. Physico-chemical parameters were measured daily for the 2 tanks and remained constant during all the experiment: temperature was 5.2 ± 0.08°C, pH was 7.85 ± 0.012 and dissolved oxygen was higher than 97 %.

At the end of the exposure period, the hemolymph was sampled and the digestive glands were removed and stored at -80°C.

Hemolymph was diluted (1/16 or 1/40, depending on the metabolites concentration) in absolute ethanol in quartz cuvettes. Fixed wavelength fluorescence (FF) was then measured for control and contaminated organisms on a spectrofluorimeter. Excitation-emission wavelength pair 290:335 was employed to detect naphthalene-derived metabolites (Aas et al. 2000). The FF values are expressed as arbitrary units of fluorescence and provide an estimation of the relative concentration of metabolites between control and the contaminated condition.

For all biochemical analysis, ten digestive glands were considered for control organisms as well as for exposed organisms. They were homogenised in ice-cold phosphate buffer (100 mM, pH 7.2, 1100 mOsm) containing ethylenediaminetetraacetic acid (EDTA) free protease inhibitors (Halt protease inhibitor cocktail, Thermo Scientific). The homogenates were

centrifuged at 12500 g at 4 °C for 15 min and the post-mitochondrial fractions (PMF) were used for assays.

Total protein concentrations were determined using the method of Bradford (1976) with a BioRad assay kit (500-0002) containing bovine serum albumin as a standard and Brilliant Blue G 250 as a reactant. The reaction was measured spectrophotometrically at 570 nm.

The GST activity was measured using a commercially available kit (GST assay kit, Sigma-Aldrich) adapted for this study (*i.e.* samples were diluted to 1/20 in kit sample buffer and analyzed in triplicates). The chromogenic substrate 1-Chloro-2,4-dinitrobenzene (CNDB), conjugated with the reduced glutathione by the GST, was measured at 340 nm. GST is expressed in U/mg of protein.

Lipid peroxidation was evaluated via malondialdehyde (MDA) concentration using a commercially available MDA assay kit (Oxis International). The method was based on the reaction of a chromogenic reagent (n-methyl-2-phenylindole) with MDA measured at 586 nm (Gérard-Monnier et al. 1998). The results are presented in nmol of MDA/mg of protein.

The SOD activity was measured using the assay developed by Paoletti et al. (1986) adapted for use in microplate. The assay involves EDTA, $MnCl_2$ and mercaptoethanol and measures the decrease of nicotinamide adenine dinucleotide (NADH) oxidation. The results were presented in U of SOD/mg of protein.

The statistical analysis was carried out using Statistica software. Equality of variance (homoscedasticity) was demonstrated for FF, SOD, GST activities as well as MDA content using a Levene test. Consequently, for all variables, Student tests were conducted to highlight significant differences (for $P_{value}<0.05$) due to the contamination. The results were expressed as mean ± standard error of the means.

3. Results and discussion

By investigating the modulation of several chosen biomarkers in *C. islandica*, the goals of this study were to evaluate the biological impact of the marine diesel and, on this basis, to validate new analytical tools of interest for future biomonitoring of diesel spills in the Arctic.

Hydrocarbon concentration is expressed as nominal concentration and was not measured in the water column, so that any decrease of hydrocarbon concentration during the exposure period (already observed and reported in Milinkovitch et al., 2011a, b, c; 2012; 2013a, b) cannot be measured. However, the relative concentration of metabolites (estimated through FF measurement) insures the incorporation of hydrocarbons contaminants by the organisms since the relative concentration of biliary metabolites was significantly higher in the hemolymph of scallops exposed to marine diesel until the last day of exposure (when compared to control, **Figure 1A**).

Regarding GST activity (**Figure 1B**), results showed values of 0.526 ± 0.021 U/mg of protein for control scallops. This is in accordance with a recent study conducted by Nahrgang et al. (2013), who showed that the mean of the GST activity (measured at the same season in the digestive gland of *C. islandica*) was 0.5 U/mg of protein. In our study, the activity of the enzyme was significantly higher in contaminated organisms (compared to uncontaminated ones), which is in accordance with two studies conducted by Pan et al. (2005, 2009) who showed an increase of GST activity in the digestive glands of *Pectinidae* contaminated with PAHs. The induction of GST activity by environmentally relevant concentrations of marine diesel showed the sensitivity of this biomarker. Thus, when measured in the digestive glands of *C. islandica*, GST activity appears to be a suitable tool, sensitively predicting the detoxification response to marine diesel.

In order to evaluate the degradation of the health status due to marine diesel exposure, oxidative stress was evaluated through lipid peroxidation (**Figure 1C**). The MDA content was 1.439 ± 0.083 nmol/mg of protein in the digestive glands of scallops exposed to

uncontaminated water. This order of magnitude is in accordance with a recent study conducted by Liu et al. (2014) who assessed MDA contents ranging from 0.9 to 2.2 nmol/mg of protein in the digestive glands of uncontaminated and hydrocarbon contaminated scallops *Chlamys farreri*. Although marine diesel contamination seemed to induce an increase of lipid peroxidation, statistical analyses did not indicate significant differences due to these contaminations ($P = 0.13$). These results are in accordance with two recent studies: (i) a study conducted by Lüchmann et al. (2011), which did not highlight lipid peroxidation in digestive glands of *Crassostrea brasiliana* exposed to water accommodation fractions of marine diesel for 96h, and (ii) a study conducted by Hannam et al. (2010) that showed no modulation of MDA contents in the haemocytes of *C. islandica* after an 1 week exposure to crude oil. On another hand, our results disagree with previous studies that showed an increase of MDA in the digestive glands of *C. farreri* exposed to PAHs (Pan et al. 2005, 2009). This discrepancy could be due to the fact that, in contrast to our study, these previous studies exposed *Pectinidae* to high concentrations of a single PAH such as benzo(k)fluoranthene or benzo[a]pyrene (Pan et al. 2005 and 2009, respectively).

In parallel, regarding the antioxidant system, our study investigated the activity of the superoxide dismutase (SOD) (**Figure 1D**). In control scallops, this activity was 32.68 ± 4.00 U/mg of protein which agrees with a previous study conducted in the digestive glands of *Chlamys varia* (Milinkovitch et al. 2015) since these authors reported activity values of 29.4 U/mg of protein. Regarding the effects of marine diesel contamination, our results did not reveal modulation of SOD activity.

The absence of antioxidant enzyme reactivity (SOD) as well as of lipid peroxidation (MDA content) could be due to the fact that no increase of ROS was induced by the contamination. Probably the efficiency of upstream detoxification systems (such as GST) may have

eliminated most of the hydrocarbons incorporated and consequently no ROS were produced downstream, leading to no increase of SOD activity and MDA content.

The lack of significance regarding MDA content and SOD activity seems to indicate that these biomarkers cannot be considered valuable analytical tools for biomonitoring diesel spills. However, such a conclusion should be cautious since this lack of significance could also be due to a decrease of hydrocarbons exposure in the last days of experiment: a lower level of hydrocarbons exposure could have (i) permitted a recovery process leading to the absence of lipid peroxidation; (ii) induced no increase of SOD activity.

In conclusion, although this study clearly exposes the effects of marine diesel on GST activity, no effect was observed for oxidative stress and antioxidant response (MDA content and SOD activity, respectively). Altogether, these results are in accordance with the literature since GST, commonly claimed as a sensitive biomarker of detoxification, was modulated while MDA, a biomarker of damages claimed as less sensitive, was not modulated (see Amiard and Amiard-Triquet 2008 for an extensive review). Further studies, investigating several levels of biological organisation (sub-individual, individual and population levels) and including several species of the ecosystems, are required in order to evaluate the ecological impact of marine diesel pollution upon Arctic ecosystems and to set up analytical tools of interest for diesel spill monitoring.

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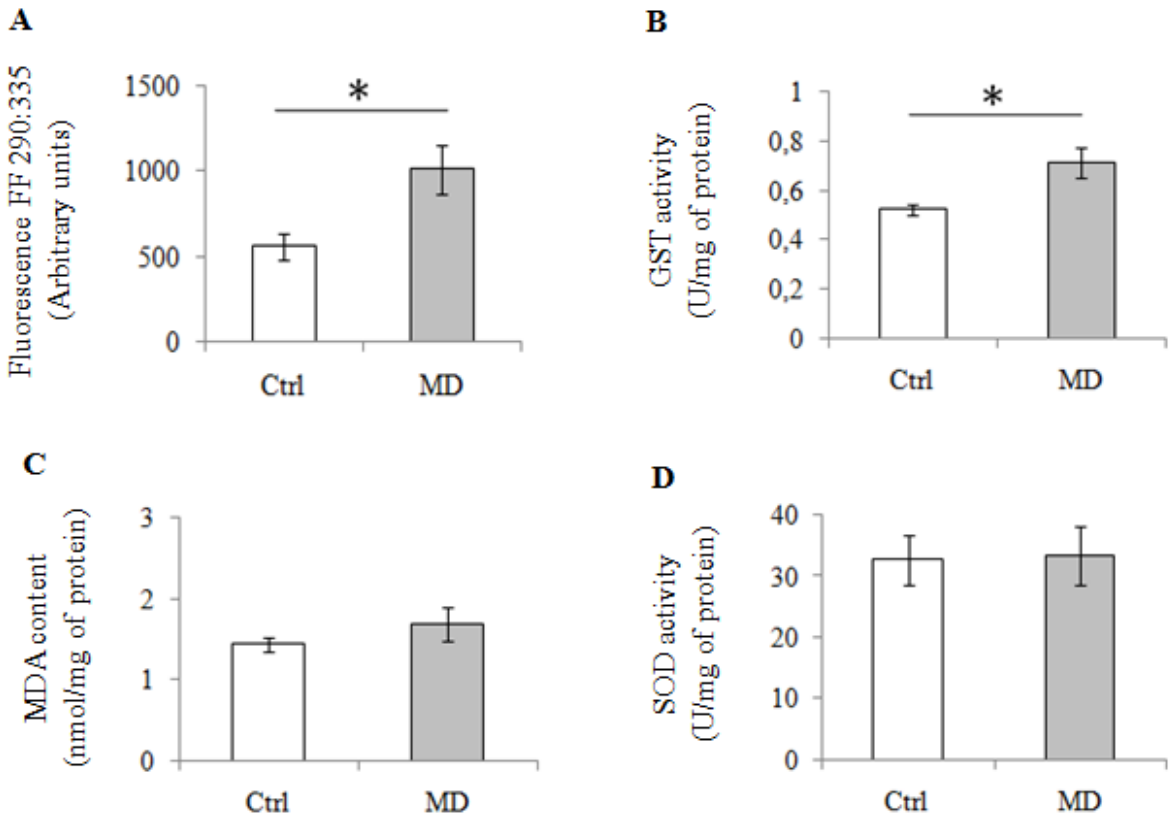

Figure 1: Biomarkers assessed after 7 days of exposure to Control solution (**Ctrl**) and marine diesel (**MD**) in *Chlamys islandica*. **A**: Fixed wavelength fluorescence (FF 290:335) of hemolymph. **B**: Glutathione-S-Transferase (GST) activity in the PMF of digestive glands. **C**: MDA contents in the PMF of digestive glands. **D**: Superoxide Dismutase (SOD) activity in the PMF of digestive glands. Values represent mean ± standard error (n=10 per treatment). * indicates a ***significant difference,*** where $P < 0.05$.