Akvaplan niva

Rapport Report

Sublethal effects of marine diesel on Arctic scallop fitness.

Development and validation of biomarkers for environmental biomonitoring.

SVALBARD ENVIRONMENTAL PROTECTION FUND

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Rapporttittel / Report title

Sublethal effects of marine diesel on Arctic scallop fitness. Development and validation of new biomarkers for environmental biomonitoring.

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Akvaplan-niva rapport nr / report no
6108 -

Dato / Date
28.02.2014

Antall sider / No. of pages
30 + 0

Distribusjon / Distribution
Offentlig

Oppdragsgiver / Client
Sysselmannen på Svalbard

Oppdragsg. referanse / Client's reference
Oppdragsreferanse

Sammendrag / Summary
Skriv inn sammendrag her

Prosjektleder / Project manager

__Perrine Geraudie_____

Kvalitetskontroll / Quality control

_____Anita Evenset_____

Anita Evenset

INNHOLDSFORTEGNELSE

Abstract

This study investigated the effects of marine diesel in Arctic scallops, in the context of increased shipping activity in the Arctic. For that, antioxidative stress, neurotoxic effect, gonad maturation, gonadal testosterone concentrations and lipid content were studied. Following 7 days of exposure to marine diesel, increased catalase and decreased acethylthiocholine activities were observed in scallops with a concentration-dependent manner. In addition, gonad maturation was significantly inhibited in males from the highest diesel treated group while testosterone levels were decreased in scallops after exposure to low and high diesel concentrations. Finally, no impact of diesel exposure on lipid content was observed in scallops. Overall, this study contributes to the better knowledge of physiological effects of marine diesel in Icelandic scallops.

1 Introduction

Decrease in ice cover in Arctic waters will result in increase of human activities, including shipping activities, offshore oil and gas exploration, tourism and fishery around Svalbard and in the Arctic in general. This can potentially increase the risk of shipping accidents causing oil or fuel spill into the marine environment. Recently, the Government of Norway banned the use of heavy oil in nature reserve on the eastcoast and in the three national parks on the westcoast of Svalbard, allowing only marine diesel as shipping fuel in Arctic waters (Ministry of Environment, 2009). The prohibition of heavy fuel oil was established to prevent its accidental discharge in Arctic environment and also because marine diesel is expected to have less potential to impact the ecosystem. To the author knowledge, only one study has been reported on marine diesel effects on Arctic organisms (Hansen et al., 2013).

Oils, including crude oils, bunker oils and diesel fuel, are submitted to different weathering processes when released in the marine ecosystem such as biodegradation, evaporation, dissolution and dispersion. Unlike heavy oils, which exhibit high viscosity and low dispersion capacity, marine diesel contains more of light polycyclic aromatic hydrocarbon (PAH) compounds allowing them to be dispersed and dissolved quickly in the water column. However, marine diesel could have the potential to harm exposed organisms after a fuel spill due to the toxicity of bioavailable PAHs (Neff et al., 2000).

Benthic organisms live in close association with the substrate and because of their relative stationary nature they cannot avoid exposure to adverse environmental conditions. Marine bivalves have been frequently used for ecotoxicological studies on oil spill effects, mainly due to their capacity to bioaccumulate environmental contaminants as well as to respond to their exposure (Poulton et al., 1998; Muniz et al., 2005, Ocon et al., 2008; Dauvin et al., 2010; Bebianno and Barreira, 2009; Solé et al., 2007). Marine sediments accumulate and retain organic and inorganic contaminants, such as PAHs (Hylland et al., 2005). In addition to their specific genotoxic and carcicogenic effects, some PAHs are known to exhibit (anti)estrogenic, (anti)androgenic activity and can induce harmful effects on the endocrine and/or on the reproductive system of exposed animals (Fertuck et al., 2001, Tian et al., 2013). Reproduction is a key physiological function in population survival and ecosystem health, but few studies have investigated the impact of PAHs on bivalve reproductive function (Gagné et al., 2001, 2003).

Regarding the impact of marine diesel on Arctic organisms, only one study has been undertaken to the author knowledge. Hansen et al., 2013 reported deleterious effects of marine diesel on Arctic zooplankton. No study of marine diesel impact on Arctic benthic species has been carried out. The Icelandic scallop, *Chlamys islandica*, is a clam that is abundant on the (Sub) Arctic sea bottom and has been observed as far North as the Spitsbergen archipelago (Thorarinsdóttir, 1993; Brand, 2006). This epibenthic filter has been suggested as an indicator species for environmental monitoring in the Barents Sea (Nahrgang et al., 2013). It has also been demonstrated that this species is well suited for investigations of biological impacts of dispersed oil (Frantzen et al., in prep; Baussant et al., 2009; Hanmam et al., 2009, 2010).

In our study, we investigated the biological and physiological effects of marine diesel exposure at environmental relevant concentrations in *Chlamys islandica* by combining different biomarkers of PAH exposure, oxidative stress, lipid content, gonad physiology and escape response ability.

Acetylcholinesterase (AchE) is an enzyme involved in nerve impulse transmission and its inhibition is an established biomarker of neurotoxicity, well known to be sensitive to organophosphate and carbamate pesticides (Fulton and Key, 2001). However, recent studies suggest AChE may also indicate general stress and because of its role in neurotransmission, it is thought to be highly sensitive to biotic and abiotic changes (Lehtonen et al., 2006). Little is known on PAH impacts on AchE inhibition and the involved mechanisms of actions. Antioxidant defense systems comprise antioxidant enzymes like catalase (CAT) and their main function is to eliminate the active oxygen (O_2^-). They have been widely used as biomarkers of contaminant mediated oxidative stress, including PAHs (Lu et al., 2010). Once taken up by organisms, PAHs undergo biotransformation reactions which can stimulate the production of reactive oxygen species (ROS) and produce PAH metabolites (Livingston et al., 1991). PAH metabolites have been quantified in hemolymph, with the aim to evaluate the capacity of scallops to biotransform natural PAH compounds and to estimate internal contamination by marine diesel derived compounds. Lipid content has been measured as a nonspecific response associated with the disruption in the lipid component of cellular membranes, which might reflect exposure and toxicity to pollutants, and alteration of lipid metabolism (Viarengo et al., 2007). As an indication of endocrine disruption and reproductive impairment, the sex steroid, i.e., testosterone, which has a key function in female and male reproduction, has been measured in the gonad tissues in addition to the assessment of gonad maturation (GSI). Moreover, scallops have a characteristic escape response to predators,

reacting by strongly closing or making several adductions of the shells, i.e., jumping or swimming response (Thomas and Gruffydd, 1971, Brand, 1991 for review). Shell closing reaction capacity has been evaluated at the end of the experiment as an indicator of swimming capability and general health, as weak valve closing might reflect narcotic effects.

2 Material and methods

2.1 *Sentinel species*

Figure 1: Icelandic scallops in an exposure tank.

Icelandic scallops, *Chlamys islandica* (60-80 mm shell length) were collected close to Tromsø (69° 35.060' N, 18° 55.701' E) using a triangular dredge 6 weeks prior to the experiment. The scallops were maintained in holding tanks with flow-through seawater at ambient temperature at Akvaplan-niva's marine laboratory facility. Shells were fed *ad libidum* on the microalgae concentrate paste, Nanochloropsis, during the acclimation period, but were kept unfed during the experiment. Moreover, at the end of the experiment the condition index (CI) was calculated derived from Walne and Mann et al. (1995) as CI= $\frac{soft\ tissue\ wet\ weight\ (g)}{shell\ length\ (mm)}$ X100 and the gonado-somatic index (GSI) as GSI= $\frac{gonad\ weigth\ (g)}{soft\ tissue\ wet\ weight\ (g)}$ X100.

2.2 *Chemicals*

Marine diesel was obtained from BP® ship fuel station in Tromsø.

2.3 *Experimental design*

Marine diesel was added in the water (110 L) at the concentrations of 29, 67.5 and 148 ppm, corresponding to the low, medium and high concentrations and then mixed during 2h using a funnel fixed at the surface of the exposure tank and connected to a 12 V water pump according to Milinkovitch et al. (2011; Fig.2). Then 12 adult Icelandic scallops (6 females and 6 males) were randomly distributed into 4 exposure tanks corresponding to the three concentrations of marine diesel, and a control tank filled up with clean seawater. The

experiment was conducted for 7 days, under static conditions, with ambient temperature (5.2 ± 0.6°C), pH daily average (7.85 ± 0.1) and oxygenation (>97 % saturation). At the end of the exposure period, shell closing capacity of the scallops was evaluate after a gentle mechanical stimulation and expressed as escape response, then bivalve were dissected. First, hemolymph (0.4-1 ml) was collected from the striated region of the adductor muscle, using a 21 gauge needle, transferred to a cryotube, snap frozen in liquid nitrogen and stored at -80 °C until further biomarker analysis. The digestive gland, a piece from the adductor muscle and the gonads were sampled, snap frozen in liquid nitrogen and stored at -80 °C until further analysis.

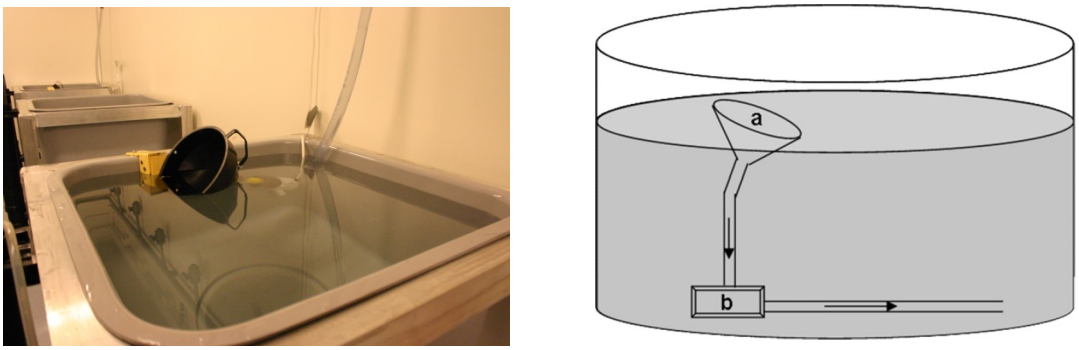

Figure 2: The experimental design consisting of a funnel (a) connected to a water pump (b) in a 110 L seawater tank.

2.4 *Total petroleum hydrocarbon measurement*

The concentration of TPH, i.e., dissolved hydrocarbons and oil droplets in the water, was determined in triplicate for each exposure tank. About 20 ml of water were collected from the middle of the tank at the beginning of the experiment (D0), after 4 days (D4), and at the end of the experiment (D7) and directly fixed with 10% of dichloromethane before being stored at 4°C until further analyses. Water samples were extracted three times with 10 ml dichloromethane. The organic phases were collected in a glass bottle and filtered on anhydrous sulphate and were scanned synchronously at a fixed wavelength difference of 42 nm from 200 to 400 nm in a quartz cuvette (UV-Vis spectrophometer, Perkin-Elmer, Deutschland) as described by Fusey and Oudot (1976). A peak area was recorded at 215 nm excitation. Results, expressed in $mg.l^{-1}$, were calculated using a standard curve of marine diesel in the range of 5 to 100 $mg.l^{-1}$.

2.5 *Escape response capacity*

At the end of the exposure, the escape response capacity of each scallop was evaluated. Clams were taken out of the water and their capacity to close naturally or after a gentle stimulation

was recorded. A stimulus score from 0 to 3 was attributed to each scallops with the specific following characteristics, 3 was given to animal exhibiting rapid and strong closing response, 2, intermediate response, 1 slow and weak closing, and 0 for scallops not able to close themselves 1 minute after stimulation.

2.6 *PAH metabolites in hemolymph of scallops*

PAH metabolites were analyzed in hemolymph samples diluted (1:2 to 1.5) in distilled water using a Perkin Elmer LS55 spectrofluorometer. A scan of arbitrary fluorescence intensity from 200 to 500 nm excitation wavelengths was performed in triplicates with a delta lambda interval of 42 nm (Aas et al., 2000). A clear peak was found at 225 nm wavelength excitation. This corresponds to naphthalene type metabolites (Aas et al., 2003).

2.7 *Oxidative stress biomarker, i.e., catalase activity*

Catalase (CAT) activity was measured in triplicates on the digestive gland cytosolic fraction. Briefly, tissues were homogenized (1:5, W/v) in phosphate buffer (50 mM, pH 7) using a potter Elvehjem, and then centrifuged (15 000g, 4°C, 15 min). Supernatants were used as enzymatic extracts for the assay and for protein quantification assessed according to Bradford (1976). Results are expressed in $nmol.min^{-1}.mg^{-1}$ of total cytosolic protein. The decrease in absorbance was recorded at 240 nm ($\varepsilon=40M^{-1}.cm^{-1}$) using 600 mM H_2O_2 as substrate (Claiborne, 1985) on the cuvette spectrophotometer LAMBDA 35.

2.8 *Neurotoxic effect, i.e., inhibition of the acetylcholinesterase (AchE) activity*

Muscle samples of *C. Islandica* were homogenized in 1:5 ice cold phosphate buffer (100 mM, pH: 7.4) containing 0.1% triton and Tris (0.1 M) with a precellys 24 dual homogenizer (Bertin technologies) using 2ml hard tissue homgenising tubes (CK28 ceramic beads) by running the program twice (2x 20s, 5500 rpm). Between the two runs, samples were put on ice for a few minutes. Then homogenates were centrifuged for 15 min (9000g, 4 °C). The supernatant was collected and used as enzyme extract for the assay.

The AchE enzymatic activity was determined in triplicates according to the colorimetric method initially developed by Ellman et al. (1961) modified for microtiter plates. Briefly, 15 µl of DTNB was added to 225 µl of sample diluted 1:100 in phosphate buffer, and then the reaction was started with the addition of 7.5 µl of acetylthiocholine (0.1 M). Absorption of the

2-nitro-5- thiobenzoate anion, formed from the reaction, was recorded at 405 nm every 60 s for 10 min (at 20 °C) using a Perkin-Elmer spectrofluorometer microplate reader.

Absorption kinetics were calculated in a linear range, then converted to nanomoles per minute according to the molar extinction coefficient of DTNB ($\varepsilon = 1.36\times10^4 \text{l.mol}^{-1}.\text{cm}^{-1}$).

2.9 *Lipid content*

Lipid content was measured on the muscle sample homogenates prepared as described in the previous section.

200 µl of muscle homogenates were mixed with 500 µl chloroform, 500 µl methanol and 250 µl distilled water, then vigorously vortexed before centrifugation (5 min, 10000g, 4 °C). A 100 µl sample of the chloroform phase was then pipetted into glass reagent tubes, 500 µl of H_2SO_4 (97%) was added, and then samples and standards were charred for 15 min at 200°C. Samples were diluted 1:6 in distilled water and 3 replicates were read spectrophotometrically at 340 nm. Lipid concentrations (mg.ml^{-1}) were determined against a glyceryl tripalmitate standard curve (0–0.5 mg.ml^{-1}).

2.10 *Testosterone concentration in gonad tissues*

Gonad tissues were homogenized in cold methanol-water (4:1) in a proportion of 2:1 (v:w) using a precellys 24 dual homogenizer (Bertin technologies) using 2 ml hard tissue homogenising tubes (CK28 ceramic beads) by running the program twice (2x 20s, 5500 rpm). Between the two runs, samples were put on ice for a few minutes. Then homogenates were centrifuged (12000 rpm, 10 min, 4°C), supernatants were transferred to new tubes and the pellets were homogenized one more time following the same procedure than previously. The second supernatant was pooled to the first one and evaporated in a 45°C water bath until total dehydration (approx. 24 h). 600 µl of EIA buffer was then added to the dry extract, slightly vortexed and kept as the steroid extracts at -80°C until the steroid analysis.

The gonadal concentration of testosterone (T) was determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits (Cayman Chemical Company, Ann Arbor, Michigan, USA). Fifty microliters of each diluted sample (1:5) was assessed in duplicate in 96 well microtiter precoated plate containing a standard curve of testosterone (3-250 pg.ml^{-1} T). Detection limit of the assay was 6 pg.ml^{-1}.

2.11 *Vtg and estradiol receptor gene characterization and molecular cloning*

Muscle, digestive gland and gonad samples of scallops (around 30 mg) were homogenized using a Qiagen TissueLyser Ⅱ (Qiagen, Hilden, Germany). Total RNA was extracted using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany). This kit includes an initial step of genomic DNA removal, and a No-RT test on a selection of samples showed that this was effective. Quantification of RNA was done by NanoDrop® ND-2000 UV-Vis Spectrophotometer (NanoDrop Tehnologies, ThermoScientific, Wilmington, USA). To prevent any potential genomic DNA contamination, the total RNA was purified with TURBO DNA-free® kit (Ambion Applied Biosystems). Only RNAs with an A260/280 ratio between 1.9 and 2.1 were used for cDNA synthesis.

Reverse transcription of total RNA was performed using iScript^{TM} Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories 2000 Alfred Nobel Drive, Hercules, CA 94547) with 2 µg of RNA per 20 µl cDNA reaction according to the manufacturer's instructions.

Specific primer sets were used for real-time PCR (RT-PCR) and were designed using sequences from other scallop species, *Chlamys nobilis* and *Chlamys farreri* (Zhang et al., 2012; Zheng et al., 2012). The tissue distribution of the vtg and estradiol receptor (ER) genes (gene of interest) and β-actin (internal control gene) was analysed by RT-PCR using the comparative C_T method, on Bio-Rad CFX connect Real-Time PCR System. The cDNA corresponding to 60 ng RNA was amplified for 40 cycles in a 20 µl PCR mix (Sso Advanced SYBR® Green super Mix, Bio-Rad Laboratories 2000 Alfred Nobel Drive, Hercules, CA 94547 Fast City, CA 94404) containing a final concentration of 500 nM of each primer (Primer sequences listed in table 1, annexe). Cycling conditions were: 50 ˚C for 10 min, 95 ˚C for 5 min, 40 cycles at 95 ˚C for 10 sec, and 60 ˚C for 30 sec including melt curve analysis. Duplicate PCR analyses were performed on each cDNA sample, the absence of genomic DNA was confirmed by performing a no reverse transcriptase (NoRT) control for randomly selected RNA samples, and absence of contaminations was assessed by including a no template control (NTC) in every run.

2.12 *Statistics*

All results are expressed as means ± 95% confidence interval (CI). Normal distribution of data was controlled using a Shapiro–Wilk's W test ($p \leq 0.05$). Homogeneity of variance was assessed using Levene's test ($p>0.05$). Comparisons between treatment groups were then made using a one-way analysis of variance (ANOVA) followed by multiple independent group's comparison (Tukey test, parametric analysis, $p \leq 0.05$). When data sets were not normally distributed, they were analyzed using a non-parametric test of Kruskall–Wallis ANOVA, followed by a Mann–Withney U test ($p \leq 0.05$). Significant differences among groups are indicated by different letters on the graphs.

3 Results

3.1 *Mortality*

No mortality was observed in the control group, while 1 dead scallop was found in both low and medium diesel exposed groups at the end of the 7 day's experiment. In the highest diesel treated group, 3 scallops were dead at the end of the exposure.

3.2 Total petroleum hydrocarbon (TPH) seawater concentrations

TPH concentrations in seawater samples indicated that the measured values were close to the nominal ones at D0 for the highest concentrations (149 and 189 µg.ml^{-1} for nominal and measured respectively, Fig. 3). For the medium and low diesel treatment groups, TPH levels were about 2 times higher than the nominal ones. However, the relative increase of TPH concentrations from low to medium concentrations was similar for nominal and measured values indicating that this method can give a good estimation of diesel exposure in the water. After 4 days of exposure (D4), the TPH concentrations decreased to less than 40 µg.ml^{-1} in all diesel treatment tanks.

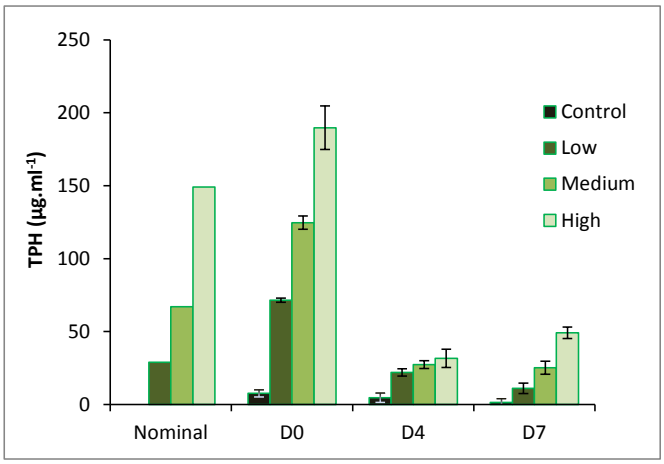

Figure 3: Total petroleum hydrocarbon seawater concentrations measured in the exposure tanks at the beginning of the experiment (D0), after 4 days (D4) and at the end of the exposure (D7).

3.3 Biological measurements

Scallops used for the experiment had similar shell length (total mean equals to 6.74 ± 0.43 cm; Fig. 1A, annex) with no significant difference between the treatment groups. Soft tissue mass were taken and scallops exposed to the medium oil concentration had significantly lower

weight than control scallops, while other groups had similar weight, ranging from 10 to 17 g (Fig. 1A, annex). Similar results were obtained for the condition index (CI), showing significantly lower values in scallops from the medium group compared to the control one (Fig. 4). Escape response capacity was evaluated by recording the valve closing capacity which could give an indication on indirect effects of diesel exposure on scallop survival ability and swimming capacity. All animals from the control group exhibited high escape response capability, expressed as a stimulus score of 3, meaning that they were all highly reactive to external mechanical stimulus. Scallops exposed to marine diesel exhibited altered escape response with a proportional dose effect pattern. Most of the animals from the high diesel exposure group did not show any clap closure reaction and were slow to close their valves. Furthermore, some of them were not able to close totally their valves one minute after the stimulus.

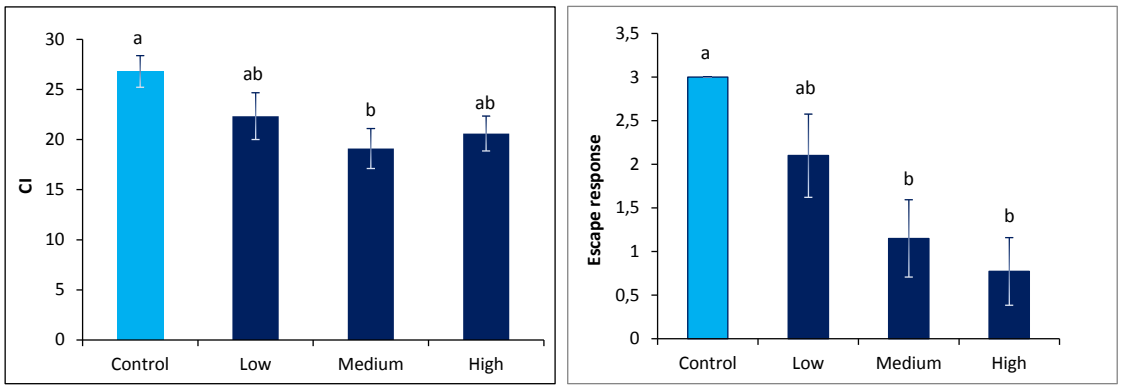

Figure 4: Condition index and escape response measured in scallops of both sexes.

The gonad maturation, expressed as the gonado somatic index (GSI), ranged from 20 to 23% of the body mass. No significant impact of diesel exposure was observed when female and male data were analyzed together (data not shown). However, when examined separately a sex dependent pattern was observed (Fig. 5).

An inhibition of the GSI, was observed in males from the highest diesel concentration group, with a significant decrease of 32% compared to control values ($p=0.03$; Fig. 5). On the contrary, GSI values seemed to be increased in females after diesel exposure, but no significant difference was observed.

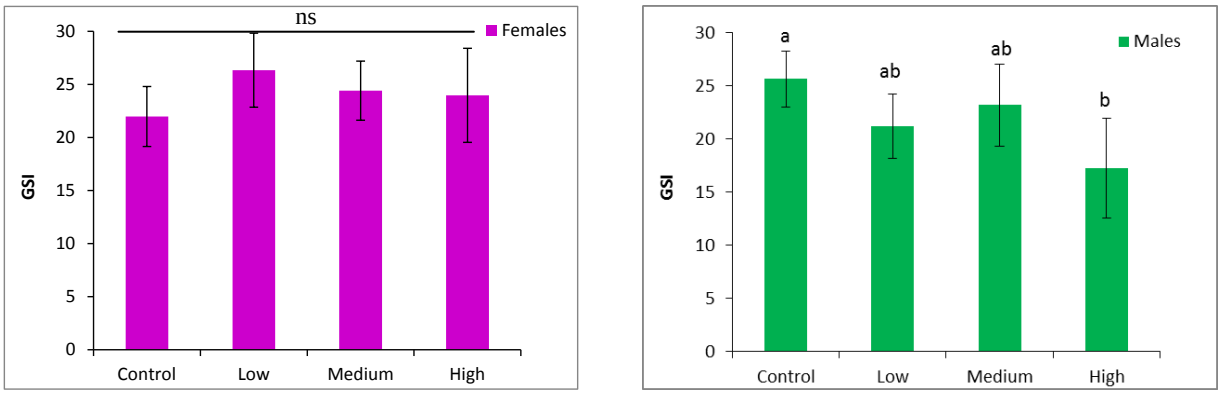

Figure 5: Gonado-somatic index (GSI) in male and female scallops.

3.4 PAH metabolites in the hemolymph

With regards to the levels of PAH metabolites found in hemolymph, medium diesel exposed scallops exhibited significantly higher values compared to control ones (p=0.04), while low and high exposed groups had similar levels than the control group (Fig. 6). No significant difference between diesel exposed groups was observed. The fluorescence peak detected at 225 nm excitation corresponds to the naphthalene type metabolites, as previously structurally identified by Aas et al. (2000).

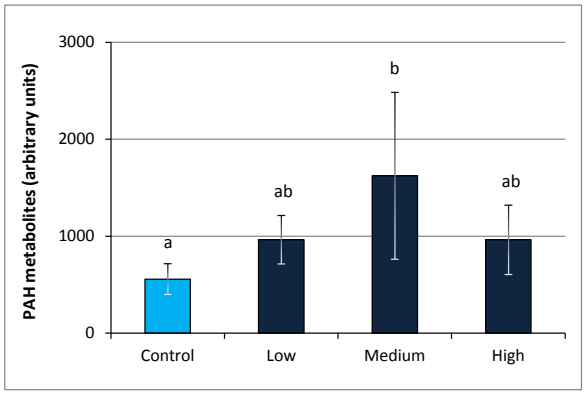

Figure 6: PAH metabolites measured by fluorospectrometer in hemolymph of scallops.

3.5 Catalase (CAT) activity in scallop digestive gland

No significant difference in CAT activities was observed between males and female scallops so pooled data from both sexes are presented in the graph (Fig.7). Control scallops exhibited CAT activity with a mean about 52 ± 21 mmol.mg^{-1} prot, and similar levels were measured in bivalves exposed to the low diesel concentration (32 ± 11 mmol.mg^{-1} prot, Fig. 7). In scallops exposed to medium and high diesel concentrations, CAT activities were higher than control ones showing a 1.5 and 2.3 fold increase respectively. However, only scallops from the highest diesel group had CAT activities that were significantly different from the control

group (121 ± 37 nmol.mg^{-1} prot, p<0.01). Moreover, no significant difference was found between medium and high group CAT activities (p=0.13).

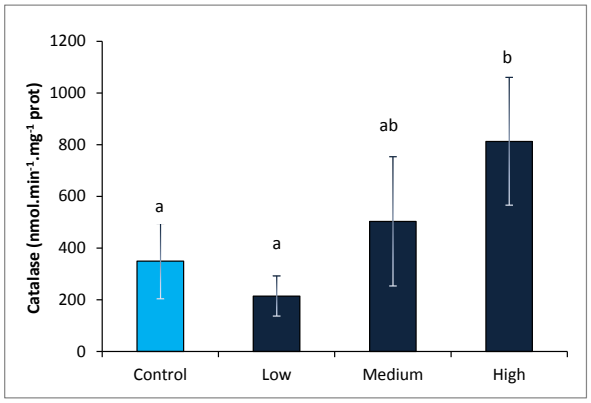

Figure 7: Catalase activity (mmol. min^{-1}. mg^{-1} prot) in the digestive gland of *Chlamys islandica*.

3.6 Acetylcholinesterase activities (AchE) measured in the adductor muscle of *Chlamys islandica*

Average AchE activities in muscle tissue of control scallops were 5.34 ± 2.8 nmol.min^{-1}.mg^{-1} prot. After 7 days of exposure to marine diesel, the AchE activities decreased to values ranging from 1.49 ± 0.9 to 3.6 ± 2.2 nmol.min^{-1}.mg^{-1} prot. However, only scallops exposed to the medium diesel treatment displayed values that were significantly lower than the control group, showing a 72% inhibition of control activities (p=0.043).

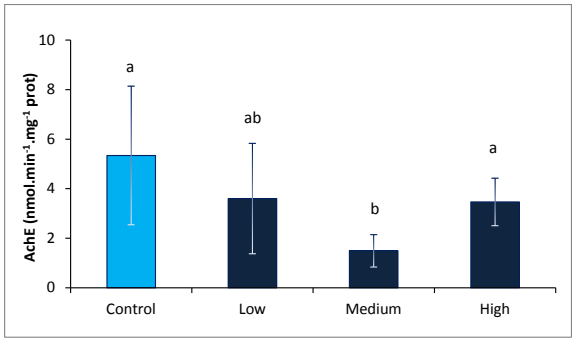

Figure 8: AchE activities in muscles of *Chlamys islandica*.

3.7 Lipid content in muscle tissue of the scallops.

The lipid contents measured in the muscles of *C. Islandica* ranged from 2.5 ± 0.45 to 3.1 ± 0.41 mg.ml^{-1} and a different pattern was observed in females compared to males.

In females, lipid concentrations seemed to be increased in scallops from diesel treated groups but were not significantly different. An alternative pattern was observed in males, as marine diesel exposed scallops exhibited lower lipid content values than controls, but no significant differences were found.

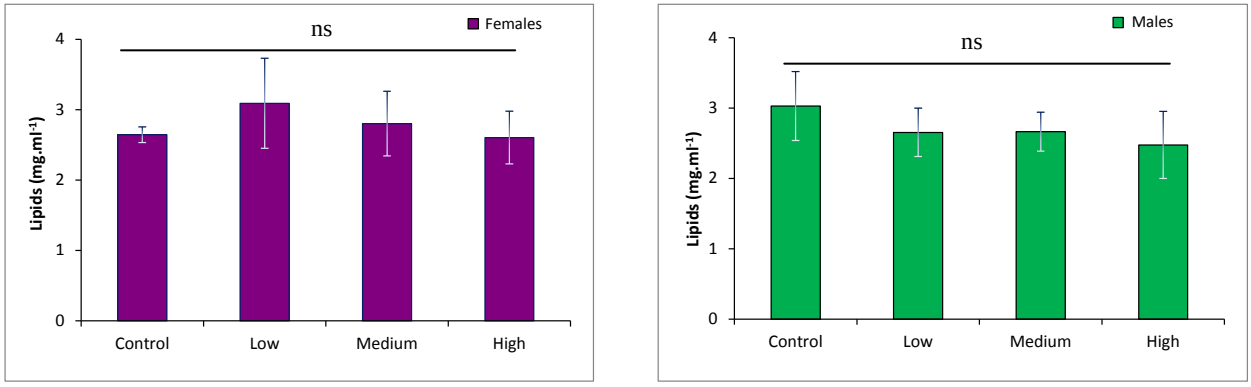

Figure 9: Lipid contents measured in the muscles of female and male scallops.

3.8 Gonadal testosterone concentrations

Testosterone concentrations were measured in gonadal tissues of scallops and results are expressed as pooled values for females and males. T concentrations were about 24 $pg.ml^{-1}$ in control scallops while diesel exposed individuals exhibited testosterone levels from 40 to 55 $pg.ml^{-1}$ and increased proportionally with diesel treatment. Scallops exposed to the low and high diesel concentrations had respectively 1.7 and 2.4 times significantly higher testosterone levels compared to control values. However, no significant difference was found between the different diesel contaminated groups.

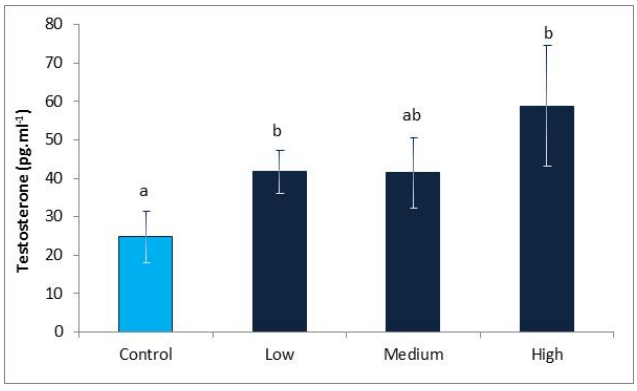

Figure 10: Testosterone concentrations in gonadal tissues of Chlamys islandica.

3.9 Vtg gene characterization and molecular cloning

The two primers used from *Chlamys farreri* and *Chlamys nobilis* (Annex, Tab.1) were not conserved enough to allow the molecular characterization of vtg and ER gene expression in *Chlamys islandica*. The cDNA extraction was showing good quality in all three tissues tested, and higher cDNA quantities were found from digestive gland and gonad samples compared to muscles (data not shown).

4 Discussion

In this study, we investigated for the first time the effects of marine diesel on Icelandic scallops, *Chlamys islandica*. As no previous study has been conducted on the specific target tissues of marine diesel on Arctic bivalves, different biomarker analysis have been undertaken to provide a general overview of the effects on the whole physiological and biological functions of scallops. Marine diesel was chosen as a contaminant due to its relevance as diesel fuel being the most commonly used boat fuel in the world (Kennish, 1992). Besides, as heavy fuel oil has been forbidden around the Svalbard archipelago most ships in the area use diesel as fuel. This will increase the risk for Arctic species to be exposed to marine diesel leak and spill in the future.

TPH seawater concentrations measured in the exposure tanks were relatively close to the nominal levels, especially for the highest group and indicated that a quick and drastic decrease of PAH concentrations appeared after 4 days of exposure, from 62 to 84% compared to D0 values for medium and high group respectively. Moreover, the PAH metabolites measured in the hemolymph of the scallops indicated that they were able to metabolize PAHs to hydroxylated metabolites, probably corresponding to naphthalene type metabolites (2 ring compounds; Aas et al., 2000). Furthermore, it has been suggested that bivalves bioaccumulate more-soluble PAHs than the less-soluble and heavier PAH compounds (Neff, 2002). This suggests that scallops could be particularly sensitive to light fuel such as marine diesel. Interestingly, scallops exposed to the medium marine diesel treatment had the highest internal PAH metabolite levels while low and high groups exhibited intermediate concentrations. The fact that scallops from the high diesel exposed group did not exhibit the highest PAH-metabolite levels could be explained by the fact that higher mortality (25%), lower closing valve reaction and decreased CI were observed in these scallops. These results indicated that scallops from the highest diesel exposed group could have some altered metabolism or physiological functions, which could lead to reduced filtration rate, bioaccumulation and biotransformation processes. This can explain the lower capacity to accumulate PAHs, and then to biotransform them to hydroxylated metabolites.

Scallops exposed to marine diesel had decreased soft tissue mass and CI, but did not exhibit altered lipid levels. Marine polar organisms generally have higher lipid contents than temperate organisms, and lipids play a key role in these extreme environments to face low

temperature and starvation period (Zheng et al., 2010). Moreover, inhibition of AchE activity and decreased of CI were maximum in scallops exposed to the medium concentration of diesel (69 ppm) and this is in concordance with the PAH metabolite levels measured for this group indicating highest internal PAH concentrations in scallops from the medium group.

The gonad maturation, expressed as GSI, was 30% lower in male scallops from the high diesel treated group than in the control one. This indicated that marine diesel might impact the gonad development in males maybe due to the endocrine disrupting properties of some PAH compounds found in the diesel mixture. Furthermore, exposure to marine diesel induced a significant increase in gonadal T levels in scallops from low and high treatment groups. Lavado et al. (2006) reported that mussels exposed to North Sea crude oil had significant higher free E2 concentrations in peripheral tissues but no effects on T levels. It has been suggested that PAHs and alkylphenols found in diesel and crude oil can exhibit endocrine disrupting properties and impair reproductive function in vertebrates and invertebrates (Lavado et al., 2006; Aarab et al., 2004; Meier et al., 2002). Moreover, increases in T production due to exposure to alkylphenols, as 4-tert-octylphenol, has been described in vertebrates (Murono et al., 1999, 2001). However, the increase of gonadal T concentrations in diesel exposed scallops was not correlated to the GSI inhibition in males. Additional reproductive and endocrine disrupting biomarkers could be studied to validate the endocrine effects of diesel. In particular, gonad histology would allow the identification of maturation stages of the scallops and to identify a delay in gametogenesis or any particular histopathologies (Miao et al., 2009).

The results for oxidative stress system showed that CAT-activities were significantly induced in high diesel treated scallops compared to control ones. Although scallops from the medium diesel treatment group had increased CAT-activities compared to controls, the difference was not statistically significant. Similarly to our results, Baussant et al. (2009) showed that CAT-activities in *C. Islandica* were significantly increased after one month of exposure to a PAH mixture (0.2 to 3.8 $\mu g.L^{-1}$ sum PAHs). However, the CAT-activities in their study ranged from 15 to 35 $nmol.min^{-1}.mg^{-1}$ prot while our results indicated CAT-activities from 10 to 50 times higher. The differences can be explained by tissue specific variations, as Baussant et al. (2009) did not explicitly indicate from which tissue the CAT activity was measured. Exposure to marine diesel seems to induce oxidative stress in scallops exposed to high diesel concentrations.

Regarding the neurotoxic effects, AchE activities were strongly inhibited in scallops from the three marine diesel exposed groups. The most neurotoxic response was found in scallops

exposed to the medium concentration of marine diesel, with a 73% inhibition of AchE activity compared to control levels. Our results suggest that AChE activity appears as a potential biomarker of PAH exposure in Icelandic scallops, although the mechanism of this inhibition is unknown. Similar neurotoxic effects may therefore be expected to occur in the central and peripheral nervous system. This may lead to impacted muscular and nervous processes such as filtration rate or reduced swimming capacity, which is crucial for escape reaction to predator and environmental stimulation.

The results from the valve closing capacity clearly demonstrated that scallops exposed to marine diesel showed a strong inhibition of their capability to close or clap their valves after being mechanically stimulated. As valvometry is a key process in bivalve to filtrate, breath, and swim to escape environmental stressors, impacted closing capability could strongly decrease the general health of scallops, by negatively impacting metabolism and other crucial biological function (Tremblay et al., 2006). This suggests that in the field scallops exposed to diesel would be less capable to escape predators and react to environmental stressors. Maximum effects for valve closing impairment were found in scallops exposed to medium diesel concentrations and can be related to PAH metabolites indicating than even if the seawater diesel concentrations were higher in the high group, internal PAH concentrations appeared to be maximal in the medium scallop group.

The molecular cloning and characterization of vtg and ER genes in *Chlamys islandica* require the design of degenerated primers as existing primers from similar species, i.e, from the same Chlamys gender, did not allow their isolation. Further investigation needs to be done with degenerated primers to characterize the genes of interest in Icelandic scallop tissues and study the interactions of marine diesel compounds with vtg and ER.

This study demonstrated some deleterious effects of marine diesel exposure on Arctic scallops with a dose response pattern and with a sex specific impact for some of the biomarkers investigated. This highlights the importance to consider both sexes when investigating ecotoxicological studies.

5 Conclusions

This study investigated for the first time marine diesel effects on Arctic scallop physiology. The results indicated that exposure to environmentally relevant concentrations of marine diesel can lead to neurotoxic effects, associated to a drastic decrease in escape capability, which is crucial for survival of organisms in the field. Furthermore, evidence of marine diesel impacts on gonad physiology and reproduction in scallops indicated the occurrence of endocrine disrupting compounds in marine diesel. As the reproductive system is a key physiological process to maintain population and ensure healthy next generations, monitoring reproductive function in Arctic organism is crucial as an indicator of ecosystem health. Given the fact that shipping activities are expected to increase in Arctic and around Svalbard archipelago, these results give preliminary results on evidence of some deleterious effects of marine diesel on Icelandic scallops.

6 Acknowledgement

This project has been funded by the Svalbard Environmental Protection Fund.

7 References

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Annexes

Valid entries for Scale = 0.025, 0.05, 0.2, 1, 10, or 15
Valid entries for Purification = DST, RP1, PAGE, or HPLC

SIGMA Life Science

Oligo Name	Sequence 5' to 3' (include modification codes if applicable)	Scale (µmole)	Purification	Comments (optional, may delay order processing)
Cfactin-Q-F	**TTCTTGGGAATGGAATCTGC**	0.025	DST	
Cfactin-Q-R	**TCTGCGATACCTGGGAACAT**	0.025	DST	
CfER-Q-F	**GTTCCTTCGTCCACAACATCC**	0.025	DST	
CfER-Q-R	**GAAAGCCTTACAGCCCTCACA**	0.025	DST	
CfVtg-Q-F	**GCGTGAACCGACCATCCT**	0.025	DST	
CfVtg-Q-R	**GTTGCGACACTGCCTCCC**	0.025	DST	
Cnactin-F	CAAACAGCAGCCTCCTCGTCAT	0.025	DST	
Cnactin-R	CTGGGCACCTGAACCTTTCGTT	0.025	DST	
CnRV-F	AAAGGCTGATATGAAAGACCGAC	0.025	DST	
CnRV-R	GCGTTGGTGGATTTTGTGAC	0.025	DST	

Table 1: Different primers used for the characterization of vitellogenin gene in Chlamys islandica. Actin has been used as house-keeping genes, and two different specie's primers, i.e., Chlamys farreri and the oyster Crassos giganstrea were used.

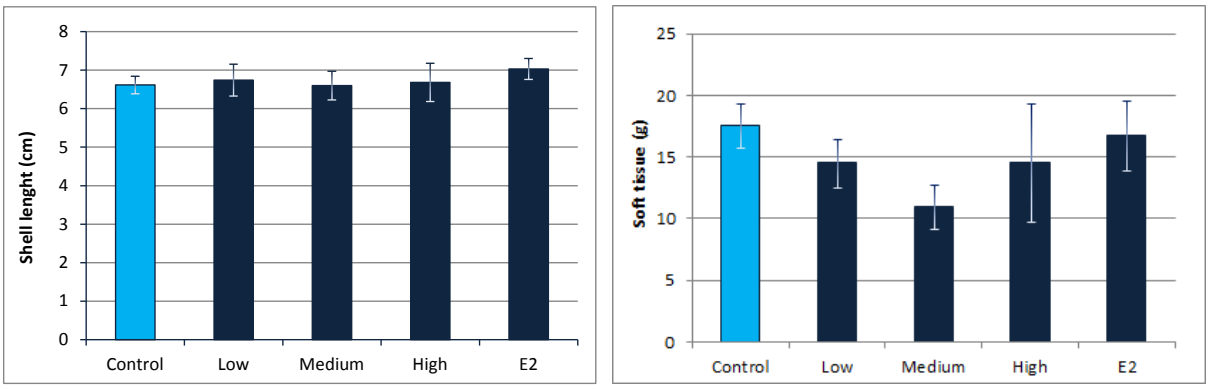

Figure 1A: Biological parameters of Chlamys islandica used in the experiment.