

Effect of Dispersed Oil on Fish Cardiac Tissue Respiration: A Comparison between a Temperate (*Dicentrarchus labrax*) and an Arctic (*Boreogadus saida*) Species

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Abstract

Chemical dispersants are considered as an appropriate technical response to oil spills in offshore environments. Nevertheless, since dispersant can increase the bioavailability of oil, its use is still a controversial issue. Furthermore, there is a lack of information related to the ecotoxicity of the oil-dispersant mixture especially for the Arctic area. Therefore, the aim of this study was to compare the impact on tissue respiration of a dispersed oil (weathered Crude Arabian Light) on two fish species, sea bass (*Dicentrarchus labrax*) and polar cod (*Boreogadus saida*) representative respectively of temperate and Arctic water ecosystems. Polar cod (n= 32; 94 ± 4 g) and sea bass (n= 32; 492 ± 20 g) were exposed for 48 hours to one of the following treatments: control, mechanically dispersed oil, chemically dispersed oil and dispersant alone. The impacts of these exposure conditions were assessed on heart energy metabolism using respirometry on permeabilized cardiac fibers. Following exposure, alteration in measurements of O₂ consumption by permeabilized cardiac fibers was found for the two species. The results show that for polar cod, oil alone decreased the activity of the respiratory chain (0.35 ± 0.05 O₂.min⁻¹.mg of dry tissues⁻¹ for control group; 0.20 ± 0.03 O₂.min⁻¹.mg of dry tissues⁻¹ for mechanically dispersed oil group) whereas the dispersant alone did not have any impact (0.30 ± 0.05 O₂.min⁻¹.mg of dry tissues⁻¹ for dispersant alone group). For sea bass, the results were different, dispersant alone decreased the activity of the respiratory chain (1.15 ± 0.11 O₂.min⁻¹.mg of dry tissues⁻¹ for control group; 0.47 ± 0.04 O₂.min⁻¹.mg of dry tissues⁻¹ for dispersant alone group) whereas the results for oil alone were not different from the control group (1.26 ± 0.32 O₂.min⁻¹.mg of dry tissues⁻¹ for mechanically dispersed oil group). These results show that oil and dispersants can alter mitochondrial activity and are a clear demonstration of the inter-specific variation in risk assessment.

1. Introduction

In Arctic regions a major reduction in ice areas has been observed (Comiso *et al.*, 2008, Perovich *et al.*, 2008), opening up new shipping routes and making possible the exploration and exploitation of new promising oil reserves (USGS, 2000; Pietri *et al.*, 2008). The increase in traffic and human activities in these highly sensitive regions enhances the risk of their contamination with petroleum compounds. In order to limit oil spill impact, dispersants are have mainly been used in offshore areas since the 1970s. However by increasing the bioavailability of oil compounds, they may enhance pollution damages (Chapman *et al.*, 2007). Thus, their use in Arctic regions is still debated due in part to the lack

of knowledge on their effects on organisms. Thus, the question of the effects of both fuel oil and dispersed oil on Arctic organisms should be raised. Most studies aimed at evaluating the impact of dispersant are performed on temperate species and only few have focused on polar areas (see for example Jonsson *et al.*, 2010; Nahrgang *et al.*, 2009; Gardiner *et al.*, 2013). In an emergency context, the temptation is high to extrapolate results of a study from temperate species to Arctic species. Olsen *et al.* (2011) investigated this question with determination of acute toxicity tests on eleven Arctic and six temperate species exposed to 2-methyl naphthalene. They concluded that values of survival metrics for temperate regions are transferrable to the Arctic for the chemical 2-methyl naphthalene with consideration of some uncertainties. Nevertheless, the question of the sublethal effects of petroleum compounds is still to be answered.

In this context, this work aimed at assessing the effects of a dispersed fuel oil on mitochondrial activity from an active aerobic organ *i.e.* the heart in two fish species chosen due to their representative place in their respective ecosystems, polar cod for Arctic areas and seabass *Dicentrarchus labrax* for temperate areas. The fish were exposed for 48 hours to mechanically dispersed oil, to one commercial formulation of dispersant and to the corresponding chemically dispersed oil. Cardiac energy metabolism was evaluated by respirometry on permeabilized cardiac fibers. The fish contamination status was evaluated through the presence of PAH (Polycyclic Aromatic Hydrocarbons) in the bile.

2. Materials and Methods

2.1 Animals

Sea bass ($n = 32$; weight: 492 ± 41 g; length: 33 ± 1 cm; mean \pm SD) were purchased from Gravelines hatchery (France) and polar cod ($n = 32$; weight: 94 ± 4 g; length: 24 ± 0 cm; mean \pm SD) were collected during summer 2011 in the waters of the Svalbard archipelago (Norway). Oxygen saturation was measured twice a day and was always higher than 90%. The temperature ($13.9 \pm 0.4^\circ\text{C}$ for seabass and $5.4 \pm 0.2^\circ\text{C}$ for polar cod) was measured daily.

2.2 Chemicals

The petroleum used in the study was a Crude Arabian Light (CAL). CAL is composed of 54% saturated hydrocarbons, 10% polar compounds and 36% aromatic hydrocarbons and has been used in previous studies (Claireaux *et al.*, 2013; Dussauze *et al.*, 2014; Theron *et al.*, 2014). In this study, CAL was evaporated (with air bubbling) until a weight loss of 7%. This process makes the lighter compounds evaporate, mimicking the weathering of an oil slick at sea. The weathered CAL contained 54% saturated hydrocarbons, 12% polar compounds and 34% aromatic hydrocarbons.

Finasol OSR 52, a commercial formulation from TOTAL Fluides, was used as dispersant in this study. It is a third generation oil-based dispersant combining surfactants (amphiphilic molecules) and solvents.

2.3 Experimental Design

The experiments were performed in a thermostated room at 14°C for seabass and at 5°C for polar cod. The fish were allocated to four experimental conditions: a control group (C), a group exposed to mechanically dispersed CAL (MD), a group exposed to chemically dispersed CAL (Finasol OSR 52: CD) and a group exposed to the dispersant alone (D). In the case of the MD and CD conditions, 25 g of oil was poured into 300L seawater tanks (concentration around 80 mg/L), in the CD condition 1.25 g of dispersant was also added (dispersant oil ratio: 1/20). For D condition, 1.25 g of dispersant was poured into the 300L seawater tank.

The fish feeding was stopped 24h before the experiment; the fish were then randomly assigned to their experimental conditions (8 fish per group) and placed in the 300 liter exposure tanks for 48 hours without water renewal. Each tank was equipped with a pumping system allowing continuous water homogenization (see Milinkovitch *et al.*, 2011 for details).

At the end of the exposure, the fish were killed. The heart was sampled and placed in ice cold medium iso-osmotic solution (in mM: NaCl 152, KCl 3.4, MgSO₄ 0.8, Na₂HPO₄ 0.44, KH₂PO₄ 0.44, NaHCO₃ 5, Hepes 10, Glucose 10, CaCl₂ 2.5, pH 7.8, 320 mosmol⁻¹). Gall-bladders were also sampled and stored at -80°C.

2.4 Fixed Wavelength Fluorescence Analysis of Bile

Bile contained in the gall-bladder was used to perform semi-quantitative analysis of PAH biliary metabolites (Vuorinen *et al.*, 2006). The bile was diluted in absolute ethanol (1/2000). Fluorescence measurements were performed with a Kontron Instruments SFM25. The measurements were made at wavelengths of excitation and emission, 343: 383 characteristic of 1-hydroxy-pyrene type metabolites (Lin *et al.*, 1996; Aas *et al.*, 2000). Values are expressed as Arbitrary Units (AU).

2.5 Tissue Respiration

Measurement of O₂ consumption of permeabilized cardiac fiber was performed using four thermostated respiration chambers (volume 1.4 mL) using an oxymeter Oxy-4 (Presens, Regensburg, Germany). Permeabilization of cardiac fibers was performed following a method adapted from Veksler *et al.*, (1987) and Kuznetsov *et al.*, (2008) at 4°C using saponin and collagenase. Briefly, cardiac fibers were cut in solution A (in mM: EDTA 5.5; MgCl₂ 2.5; Imidazol 10; HEPES 20; KCl 170; ATP 3.3; PCr 2; Dithiothreitol 0.5; pH 7.4 at 4°C) and were placed in incubation for 30 minutes in solution B (Solution A plus 0.05 mg/ml of Saponin and 2 mg/ml of Collagenase). The fibers were then washed for 10 min in solution A and 10 min in respiration medium (in mM: EDTA 0.08; MgCl₂ 7.5; KCl 150; Tris 20; NaH₂PO₄ 10; pH 7.4).

After permeabilization, the fibers were placed in respiration chambers filled with respiration medium. All measurements were performed in excess of ADP. Successive addition of substrates (Pyruvate/Malate, Succinate and Ascorbate/TMPD), cytochrome C and oligomycin were performed in the respiration chamber.

Cytochrome C was used to check the integrity of mitochondrial external membranes (Kuznetsov *et al.*, 2008). Pyruvate/Malate, Succinate and Ascorbate/TMPD allowed the analysis of different inputs in the mitochondrial respiratory chain. Oligomycine blocks the fifth complex of the respiratory chain and was used to measure the proton leak through the inner mitochondrial membrane. Proton leak was calculated as the O₂ consumption with Oligomycine divided by the O₂ consumption with Ascorbate/TMPD.

At the end of the experimentation, the fibers were dried and weighed to determine the O₂ consumption in $\mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of dry tissues.

2.6. Statistical Analysis

All analyses were performed using STATGRAPHICS software. Firstly all the data were tested with a Levene test to assess the variance homogeneity and a Kolmogorov-Smirnov and Shapiro-Wilk test were used to ensure that data followed a normal distribution. The samples with a normal distribution were tested with a one-way ANOVA followed by a Fisher test. Samples that did not follow a normal distribution were tested with a Kruskal-Wallis analysis. All data are expressed as the mean \pm standard error of the mean.

3. Results

3.1 Fixed Wavelength Fluorescence Analysis of Bile

The fluorescence intensities of bile samples at characteristic wavelengths of OH-pyrene metabolites (343:383 nm) are presented in Table 1 for seabass and polar cod.

Table 1: Biliary Fluorescence.

	343 : 383 nm	
	Seabass	Polar cod
C	13.6 ± 2.8 (a)	19.5 ± 0.8 (a)
D	13.3 ± 1.4 (a)	18.7 ± 0.6 (a)
CD	129.5 ± 11.9 (b)	179.7 ± 19.2 (b)
MD	160.0 ± 16.8 (b)	197.8 ± 23.2 (b)

Results expressed as arbitrary fluorescence units (mean ± standard error of the mean; n=8). C (Control), D (Dispersant alone), CD (Chemically Dispersed oil), MD (Mechanically Dispersed oil). Values not sharing common letters indicates a significant difference (P<0.05).

After the 48 hours of exposure, for both wavelengths and for the two species, the dispersant group (D) was not statistically different from the control group (C). In conditions CD and MD, bile fluorescence was significantly higher than the control group for both sea bass and polar cod. Moreover, for the two species, no statistical difference between the two CAL conditions (CD and MD) at both wavelengths was observed.

3.2 Tissue Respiration

3.2.1. Mitochondrial Integrity

Measurements of O₂ consumption after addition of Ascorbate/TMPD and Cytochrome C in the respiration chamber are reported in Table 2 for the two fish species.

Table 2. Measurements of O₂ consumption after addition of Ascorbate/TMPD and Cytochrome C for the 4 experimental conditions and the two fish species (seabass and polar cod).

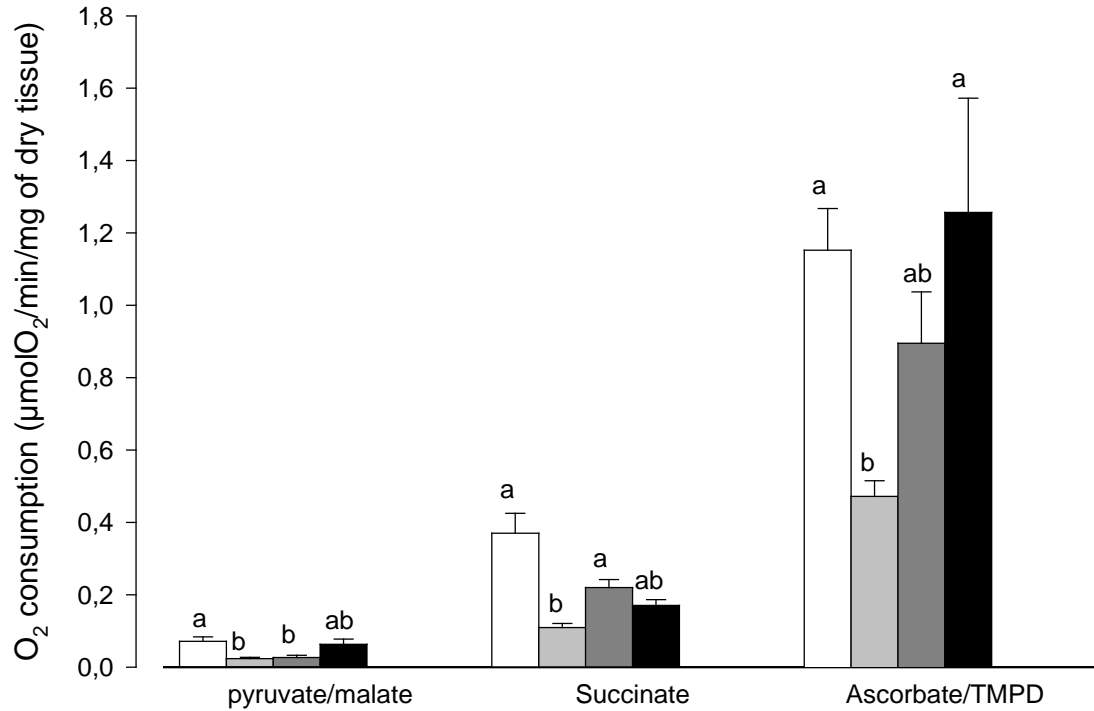
	O ₂ consumption (µmol O ₂ /min/mg of dry tissues)			
	Seabass		Polar cod	
	Ascorbate/TMPD	Cytochrome C	Ascorbate/TMPD	Cytochrome C
C	1.15 ± 0.11	1.19 ± 0.11	0.35 ± 0.05	0.31 ± 0.05
D	0.47 ± 0.043	0.51 ± 0.058	0.30 ± 0.05	0.24 ± 0.03
CD	0.89 ± 0.14	0.94 ± 0.13	0.23 ± 0.06	0.18 ± 0.03
MD	1.25 ± 0.32	1.35 ± 0.30	0.20 ± 0.03	0.18 ± 0.01

Results expressed as µmol O₂/Min/mg of dry tissues (mean ± standard error of the mean; n=8). C (Control), D (Dispersant alone), CD (Chemically Dispersed oil) and MD (Mechanically Dispersed oil)

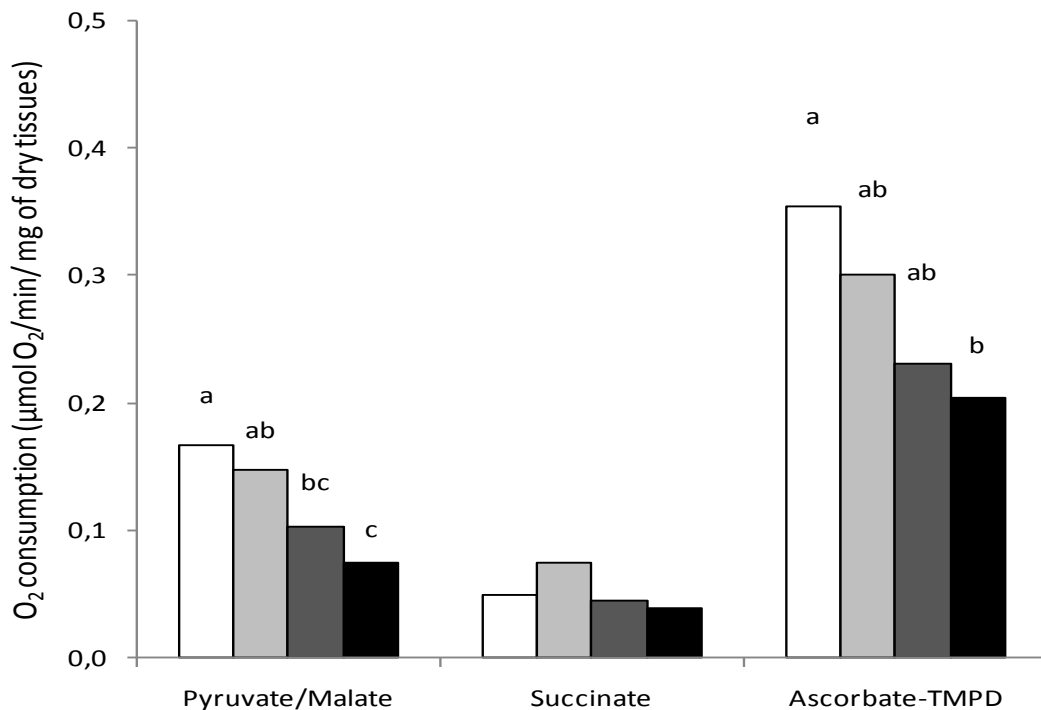
For each condition and for the two species, no significant difference was observed between O₂ consumption with Ascorbate/TMPD and after addition of Cytochrome C.

3.2.2 O₂ Consumption of Permeabilized Cardiac Muscle Fibers





The results of O₂ consumption after addition of Pyruvate/Malate, Succinate and Ascorbate/TMPD are reported in Figure 1A for seabass and 1B for polar cod.



1A



1B

Figure 1A and 1B. O₂ consumption of permeabilized cardiac muscle fibers of seabass (1A) and polar cod (1B) for the four experimental conditions after addition of substrates. Results expressed as µmol O₂/min/mg of dry tissues (mean ± standard error of the mean; n=8). C (Control, ) , D (Dispersant alone, ) , CD (Chemically Dispersed oil, ) , and MD (Mechanically Dispersed oil, ) . Values not sharing common letters in the column indicate a significant difference (P<0.05).

After Pyruvate/Malate addition, a significant decrease in O₂ consumption was observed in conditions D and CD compared to the control. The MD condition is not statistically different from the others for seabass measurements. For polar cod, after Pyruvate/Malate addition, no difference was observed between the D and C conditions. For these two conditions, O₂ consumptions were significantly higher compared to the MD condition. The results for the chemically dispersed oil were not statistically different from conditions D and MD.

After addition of succinate, a significant decrease in O₂ consumption in condition D compared to C was observed without significant effect in conditions CD and MD for sea bass measurements. For polar cod, other the other hand, no difference was observed between the four conditions after addition of Succinate.

After addition of Ascorbate/TMPD, a significant decrease in O₂ consumption in condition D compared to C was observed without significant effect in conditions CD and MD for sea bass measurements. For polar cod, after addition of Ascorbate/TMPD, O₂ consumption was similar for C, D and CD. The MD condition was not statistically different from conditions D and CD. Exposure to MD induced a significant decrease in O₂ consumption of permeabilized cardiac muscle fibers compared to C conditions.

3.2.3 Proton Leak

The proton leak results for the 4 experimental conditions are reported in Figure 2A for sea bass and Figure 2B for polar cod.

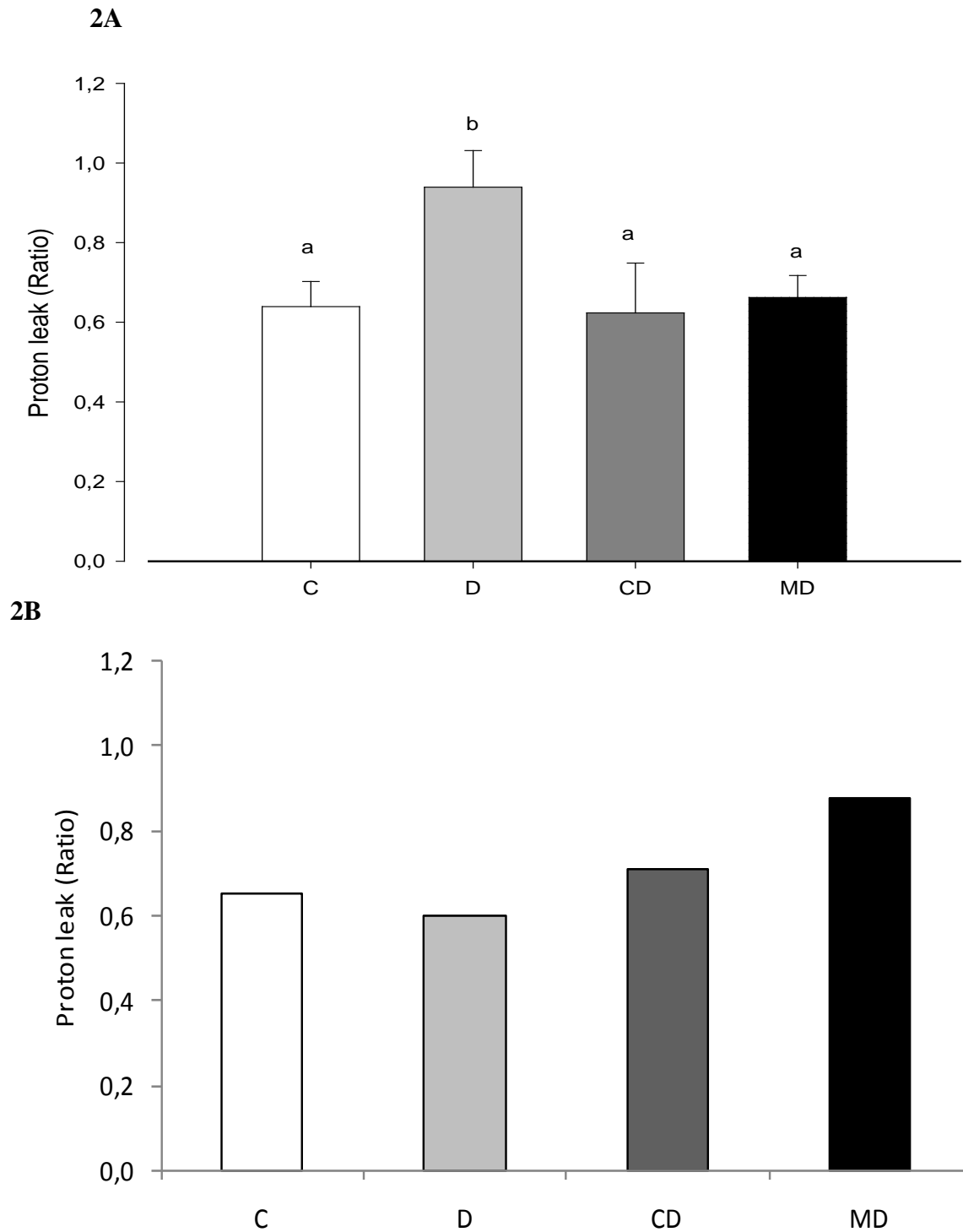






Figure 2A and 2B: Measurement of proton leak of permeabilized cardiac muscle fibers for the four experimental conditions for sea bass (2A) and polar cod (2B). Results expressed as (mean \pm standard error of the mean; n=8). C (Control, ) , D (Dispersant alone, ) , CD (Chemically Dispersed oil, ) and MD (Mechanically Dispersed oil, ) . Values not sharing common letters in the column indicate a significant difference ($P < 0.05$).

For seabass, a significant increase in proton leak was observed in condition D compared to the three others, whereas no change in proton leak was observed whatever the condition was for polar cod.

4. Discussion

For several decades, the use of chemical dispersants has been a technique commonly used in case of oil spills in offshore areas (Chapman *et al.*, 2007). However, their use is still debated and could lead to important damages. It is therefore of primary importance to understand the effects of dispersants on organisms, especially on Arctic fish species. The aim of this study was to compare the impact of dispersed oil on a major physiological function, the cardiac function, in the seabass *Dicentrarchus labrax* and the polar cod *Boreogadus saida*. For this, fish were exposed for 48 hours to mechanically dispersed weathered CAL, to one dispersant and to the corresponding chemically dispersed oil. Furthermore, one group was performed without CAL or dispersant and used as a control.

In this work, individual contamination levels were estimated by measuring biliary fluorescence, an efficient biomarker of exposure to petroleum compounds in fish (Aas *et al.*, 2000; van der Oost *et al.*, 2003). The results of OH-pyrene metabolites are in the same pattern for the two species of fish. Looking at measurements of bile fluorescence, only auto-fluorescence was observed for groups C and D whereas a significant increase in OH-pyrene metabolites was observed in conditions CD and MD.

Cardiac function is essential for organisms; any alteration or modification of this function may affect the homeostasis of the animal due to the role of heart in all physiological and metabolic functions (Milinkovitch *et al.*, 2013). In addition, various scientific studies suggest that the analysis of cardiac function is a sensitive and relevant indicator for observation of the impact of hydrocarbon compounds both in the field and the laboratory (Carls *et al.*, 2008; Incardona *et al.*, 2012). For these reasons, the heart is considered as a target organ in environmental toxicology and particularly for toxicology related to oil (Hicken *et al.*, 2011; Milinkovitch *et al.*, 2012). Furthermore, the analysis of mitochondrial function is central to the study of the intracellular energetic metabolism (Kuznetsov *et al.*, 2008). Studies on mammalian cells have shown that after contamination, PAHs are preferentially located in the mitochondria (Zhu *et al.*, 1995). Once in these organelles they can be metabolized by mitochondrial cytochrome P450 (Jung and Di Giulio, 2010) leading to more reactive and more toxic compounds. Consequently, the mitochondrion appears to be a particularly interesting target for studies of PAH effects and particularly at a cardiac level.

The technique of permeabilized fibers, using saponin and collagenase, can be used to study *in situ* mitochondrial function (Toleikis *et al.*, 1996). This approach makes it possible to preserve the interactions between different cellular compartments. A preliminary step for this type of approach is the verification of the mitochondrial integrity after the permeabilization process. This can be done by the addition of cytochrome C under maximal oxygen consumption conditions with Ascorbate and TMPD as substrates (Kuznetsov *et al.*, 2008). For both species, the addition of cytochrome C showed that mitochondrial outer membranes were not degraded during the process of cell membrane permeabilization. Indeed, if these membranes are intact, the endogenous cytochrome C is still in the inter-membrane space and the addition of exogenous cytochrome C had no effect on respiration (Kuznetsov *et al.*, 2008), which is the case for polar cod and sea bass.

The use of selected substrates and inhibitors allowed the analysis of different elements of the respiratory chain. Pyruvate/malate, succinate and ascorbate/TMPD are respectively substrates of the first, second and fourth complexes of the respiratory chain. The measurement of maximal ADP-Stimulated (state 3) respiration with these substrates made possible the analysis of different parts of the respiratory chain. The results show that for polar cod, oil

alone decreased the activity of the respiratory chain whereas the dispersant alone did not have any impact. Oxygen consumption results show that the dispersant groups (D and CD) exhibited intermediate results between the control and the MD condition. It appears that, in most cases, the dispersant did not induce a decrease in energy production by mitochondria and that the dispersants did not increase the oil toxicity. For sea bass, the results were different. Dispersant alone decreased the activity of the respiratory chain whereas oil alone was not different from the C group. Taken together, these results seem to indicate that, for seabass, dispersant could alter the activity of the complexes I, II and IV of cardiac mitochondria.

Concerning proton leak (when the fifth complex of the respiratory chain is inhibited by oligomycin, Kuznetsov *et al.*, 2008), for polar cod, no modification could be observed in any groups. For seabass, an increase in proton leak was observed in the D group compared to the control whereas no change was observed for CD and MD groups.

Petroleum compounds are known to induce mitochondrial alteration. For example, Stabenau *et al.* (2008) observed an alteration of muscle mitochondria oxygen consumption and membrane potential in *Rana pipiens* exposed to pyrene; and a study of Knecht *et al.* (2013) has demonstrated a reduction of mitochondrial oxygen consumption rate in zebra fish embryos following exposure to a cocktail of PAHs. A possible mechanism of alteration of the respiratory chain by petroleum compounds is the production of ROS. In fact these highly reactive products are known to have a negative impact on complexes I, III and IV of the respiratory chain (Musatov and Robinson, 2012). Dispersants could also have the same mechanism of action: they appear to stimulate ROS production since a study of Milinkovitch *et al.* (2013) has shown increased antioxidant defenses in the hearts of *Liza aurata* exposed to chemical.

In this study, the heart mitochondrial respiratory chain appeared to be altered both by the dispersant and the oil but surprisingly in the sea bass the dispersant had an effect but not the oil while in the case of the polar cod the results were the exact opposite. The higher sensitivity of polar cod mitochondria to oil could be explained based on the hypothesis that polar cod mitochondria are more sensitive to ROS. In fact, acclimation to cold environments leads to modifications of mitochondrial properties and in particular to an increased unsaturation index of mitochondrial membranes, and phospholipid unsaturations are known to be preferential targets for ROS (Guderley, 2004). This hypothesis would have to be verified with the measurement of membrane peroxidation and does not explain why sea bass appears to be more sensitive to dispersant alone. Another possible explanation of the observed differences could also be the consequence of the temperature of experimentation (14 °C for seabass and 5 °C for polar cod). The lower temperature of exposure during polar cod experimentation certainly had effects on the physico-chemical properties of the oil. Low temperatures, by increasing the viscosity of the oil, may make it less dispersible (Chandrasekar *et al.*, 2006) but, the most volatile petroleum compounds (known to be the most toxic) may have been in contact with polar cod for longer due to lower evaporation conditions. Further experiments are clearly needed to explain the physiological bases of this interesting interspecies difference.

In conclusion, our results show that the oil and dispersants may alter mitochondrial activity in two fish representative of distinct ecosystems. This interspecific variation found in this study clearly shows that it is not judicious to make an assumption about the environmental impact of dispersed oil in polar areas based only on the results obtained using temperate species and shows the need for ecotoxicological investigations in polar regions.

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