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# BIOMARKERS RELEVANT TO OIL AND GAS INDUSTRIAL ACTIVITIES IN LOW TEMPERATURE MARINE ECOSYSTEMS

CAMUS, Lionel Andre Yves

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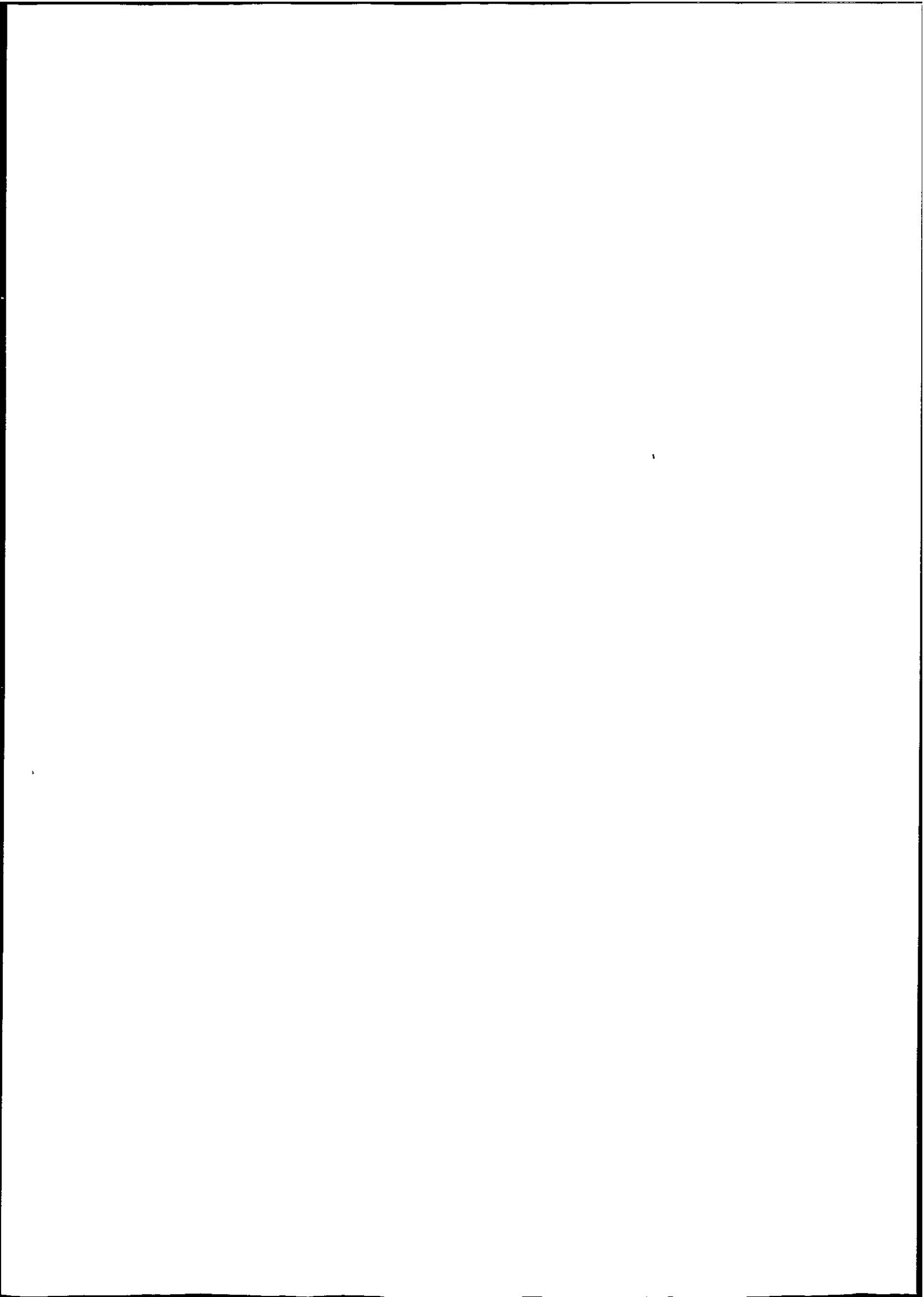
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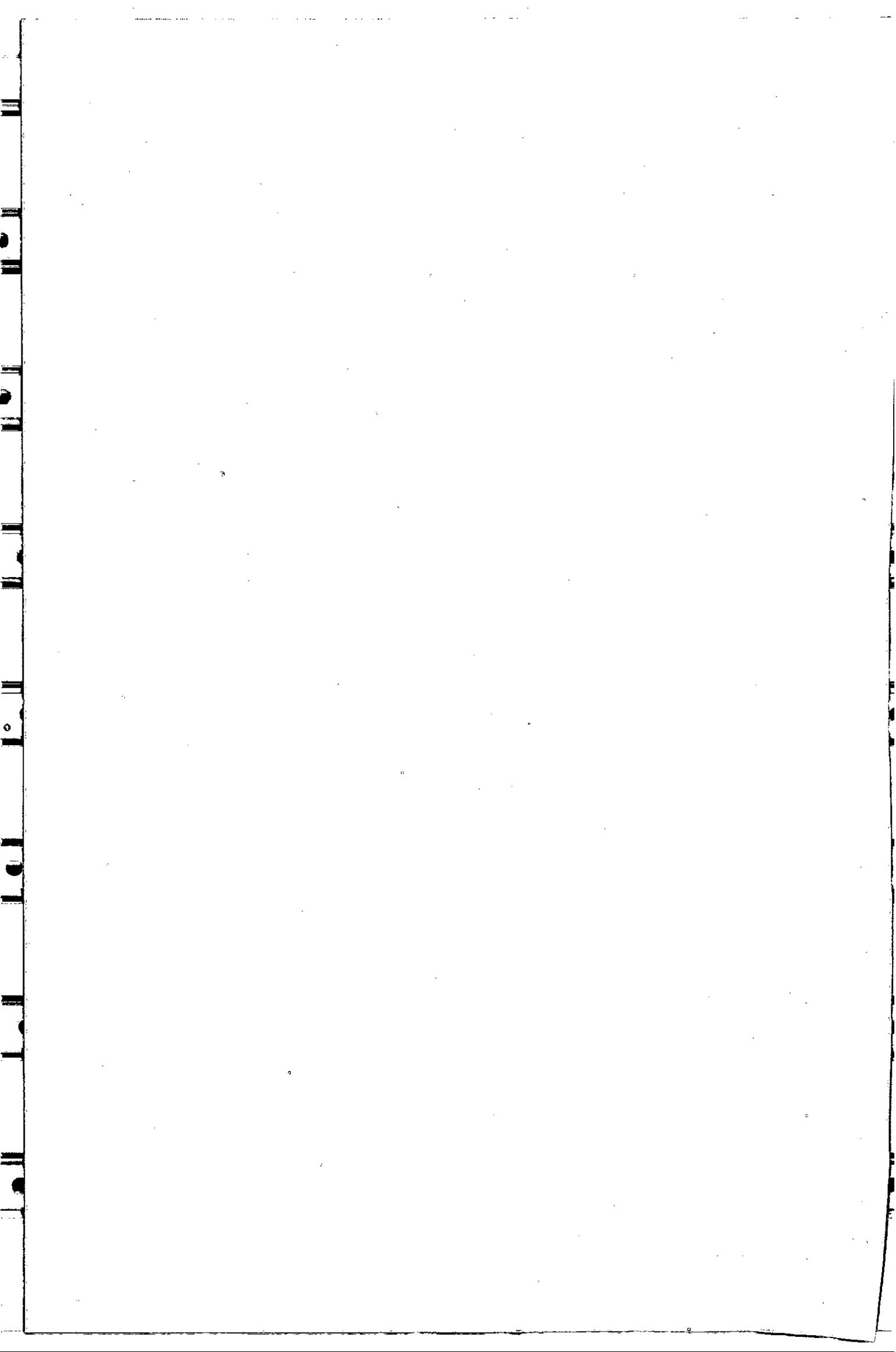
BIOMARKERS RELEVANT TO OIL  
AND GAS INDUSTRIAL ACTIVITIES  
IN LOW TEMPERATURE MARINE  
ECOSYSTEMS

L. A. Y. CAMUS

DOCTOR OF PHILOSOPHY

2002





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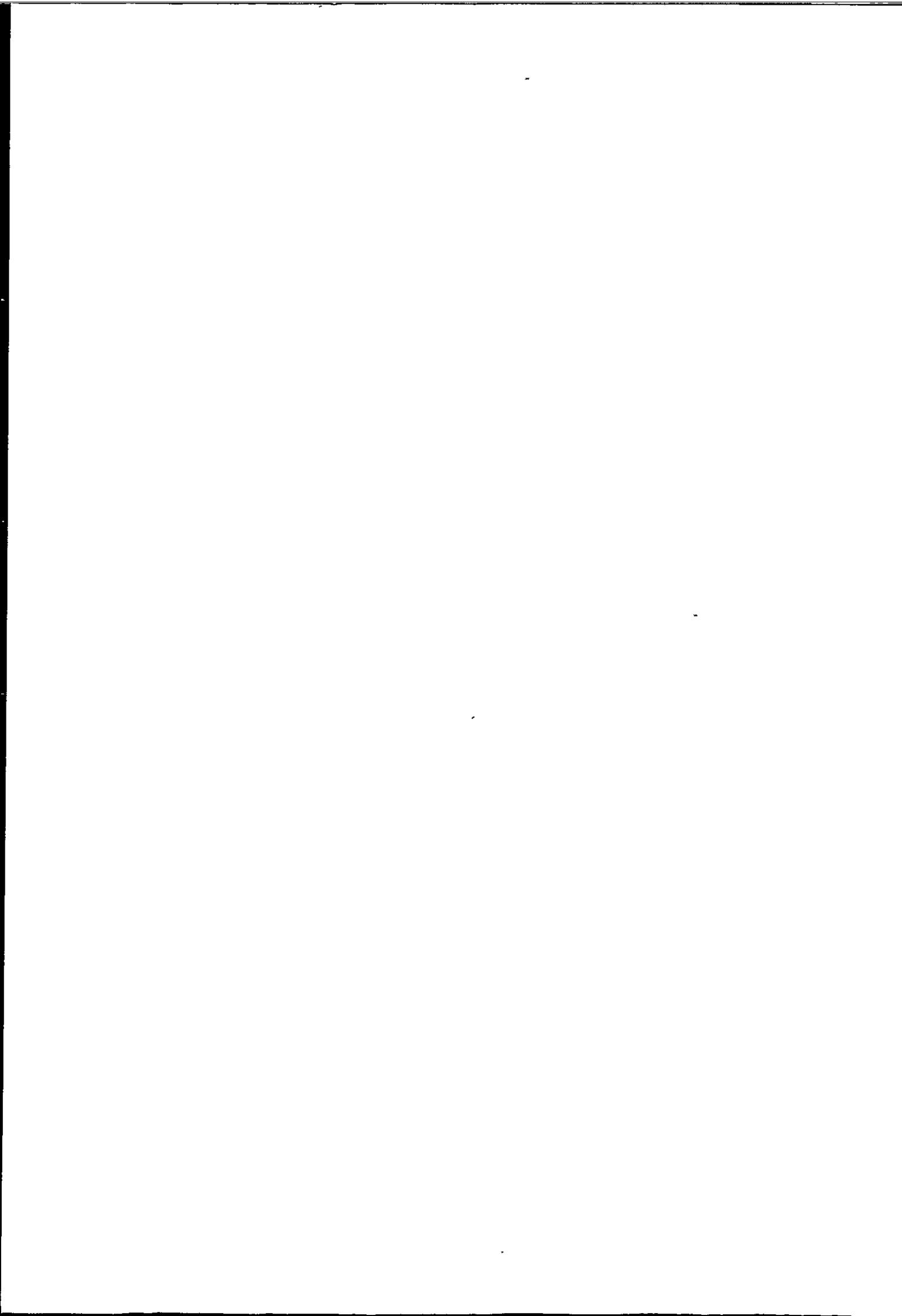
## Pharmacokinetics



Department of Biological Sciences  
University of Toronto  
1991

*"Le seul fait de rêver est déjà important.  
Je vous souhaite des rêves à n'en plus finir  
Et l'envie furieuse d'en réaliser quelques-uns.  
Je vous souhaite d'aimer ce qu'il faut aimer  
Et d'oublier ce qu'il faut oublier.  
Je vous souhaite des passions  
Je vous souhaite des silences  
Je vous souhaite des chants d'oiseaux au réveil  
Et des rires d'enfants.  
Je vous souhaite de résister à l'enlèvement, à l'indifférence,  
Aux vertus négatives de notre époque.  
Je vous souhaite surtout d'être vous."*

**Jacques Brel (1929-1978)**



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**BIOMARKERS RELEVANT TO OIL AND GAS INDUSTRIAL ACTIVITIES IN  
LOW TEMPERATURE MARINE ECOSYSTEMS**

By

**Lionel André Yves CAMUS**

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

**DOCTOR OF PHILOSOPHY**

Department of Biological Sciences

Plymouth Environmental Research Centre

Faculty of Science

And

Akvamiljø

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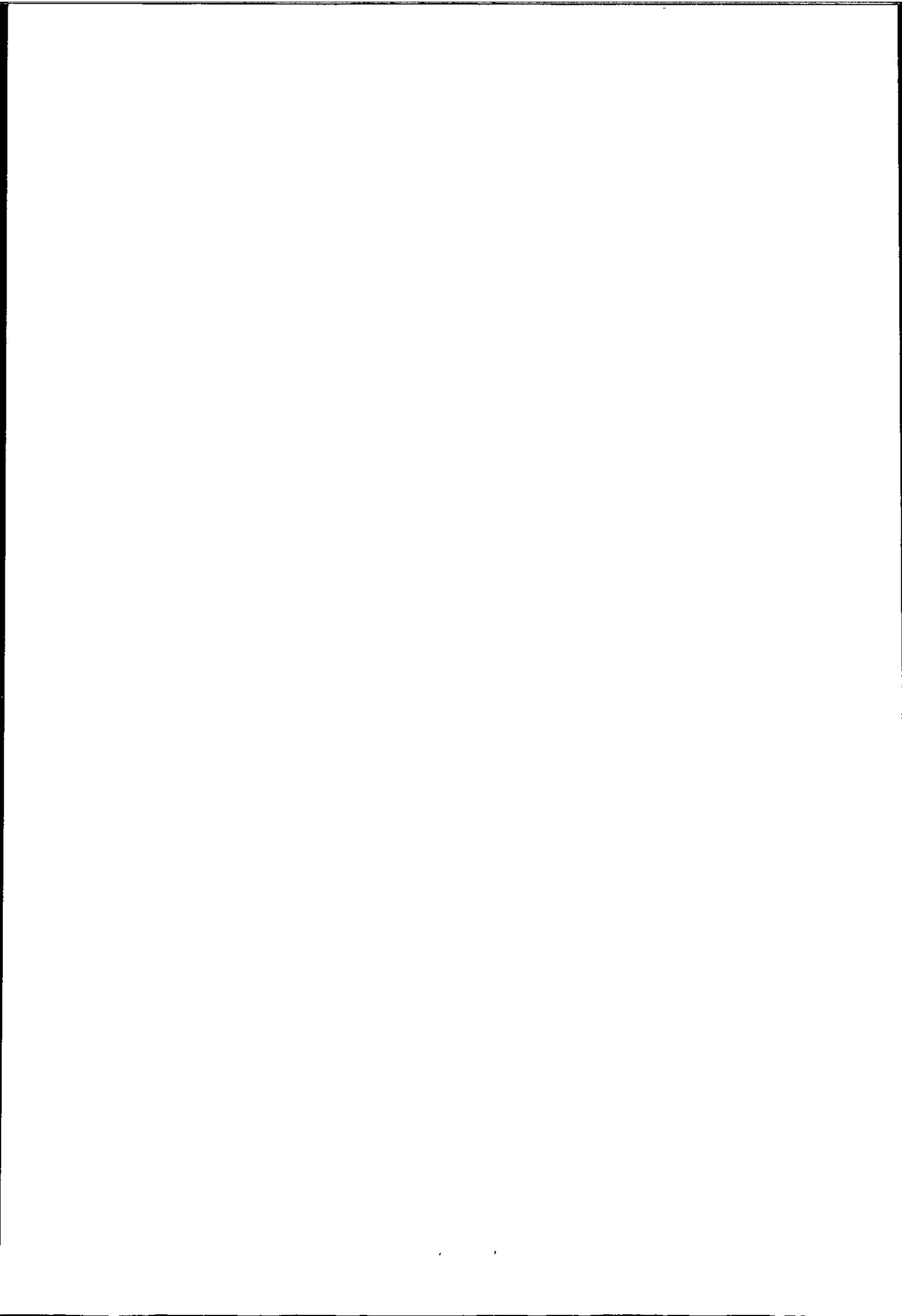
## BIOMARKERS RELEVANT TO OIL AND GAS INDUSTRIAL ACTIVITIES IN LOW TEMPERATURE MARINE ECOSYSTEMS

By

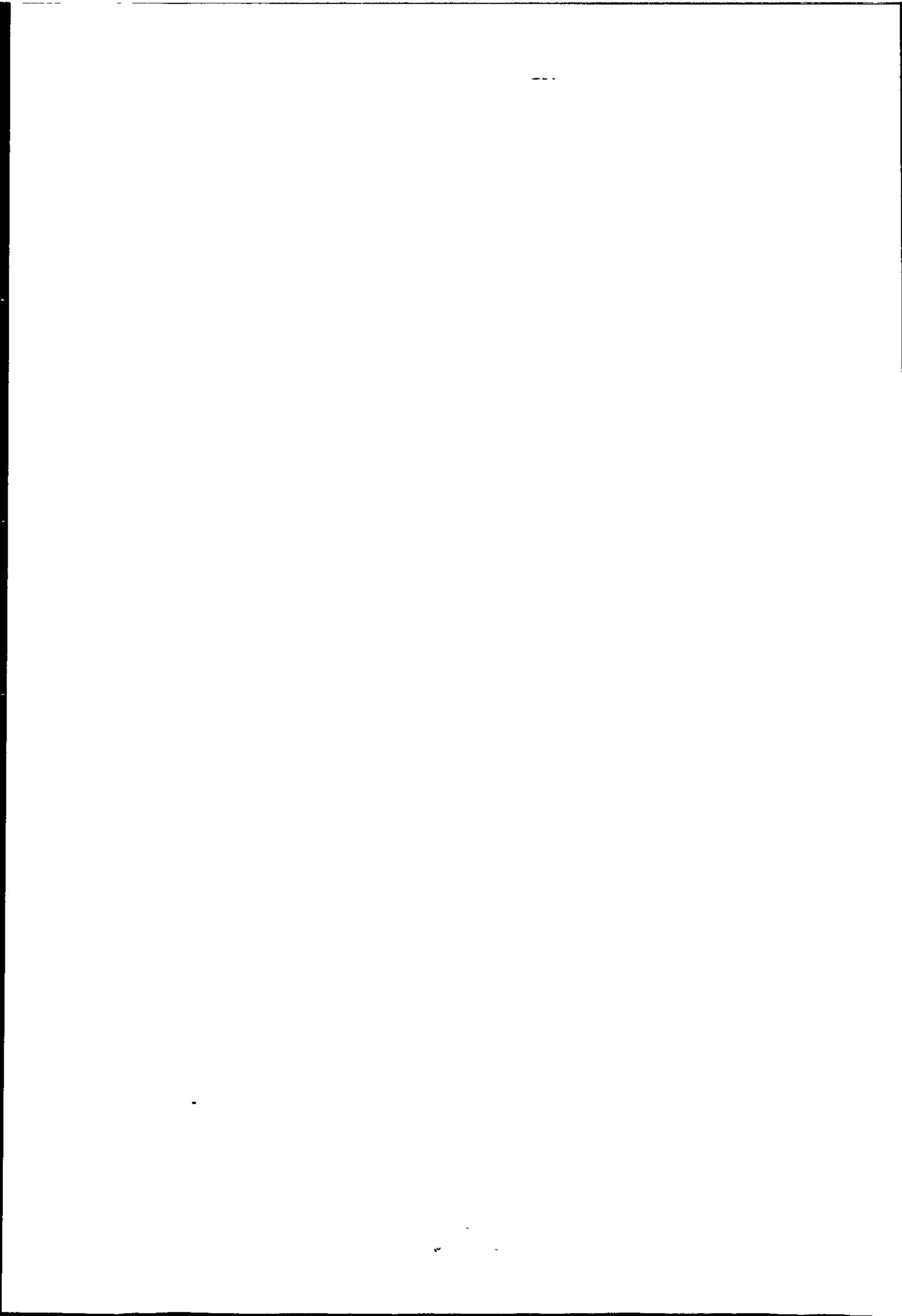
Lionel André Yves CAMUS

### ABSTRACT

Because of its geographical location, the Arctic environment is considered as pristine. However, expanding industrial activities in the Arctic require assessment of the toxicity of chemicals at low temperature. Biomarkers defined as "biological responses to a chemical or chemicals that give a measure of exposure or toxic effect" were shown to be relevant to measure *in situ* impact of oil discharges. Most biomarker studies have been performed with temperate organisms. The Arctic is characterised by low stable temperature, strong seasonality in light, resulting in a short primary production in Spring. Therefore, indigenous organisms have developed specific adaptations to live with a limited food supply in water near freezing point. Conversely, physical properties of petroleum hydrocarbons are affected by low temperature (i.e. reduced solubility). Consequently, the biological adaptation of cold-water organisms together with the altered oil behaviour, may affect typical biomarker responses. Because oil compounds are strongly prooxidant, the research strategy of this work was based on oxidative stress. The antioxidant defences were investigated by measuring the total oxyradical scavenging capacity (TOSC). The impact of reactive oxygen species was investigated by measuring the stability of the cell membranes. Finally, the physiology of the organisms was considered by looking at heart and respiration rates. Invertebrates were selected for study owing to their abundance in the polar ecosystem. They were sampled using dredges and Scuba diving from the research vessel Jan Mayen (University of Tromsø) in May and August 1999, and during May and September 2000 in the fjords of Svalbard and in Antarctica as well in January 2000. In the Arctic, two bivalves, *Mya truncata* and *Chlamys islandicus*, and two crustaceans, *Hyas araneus* and *Sclerocrangon boreas* were selected. In this work, the ecophysiology of Arctic and Antarctic marine invertebrates was investigated and compared to temperate organisms.



Polar marine invertebrates are characterised by low respiration and heart rates and a high TOSC. The elevated level of antioxidant defences is thought to reflect the oxidative pressure of the polar marine ecosystem; however, it suggested that a high TOSC may help to protect biomolecules from oxidative damage as repair mechanisms are limited due to the lack of food for 9 months. Organisms were exposed to poly aromatic hydrocarbons either dissolved, dispersed injected or *via* sediment. TOSC, cell membrane stability and heart rate were valid biomarkers to monitor the impact of poly aromatic hydrocarbons in Arctic marine organisms. The biomarker responses obtained in this study provide essential background information for monitoring the potential impact of oil and gas activities in the Arctic.



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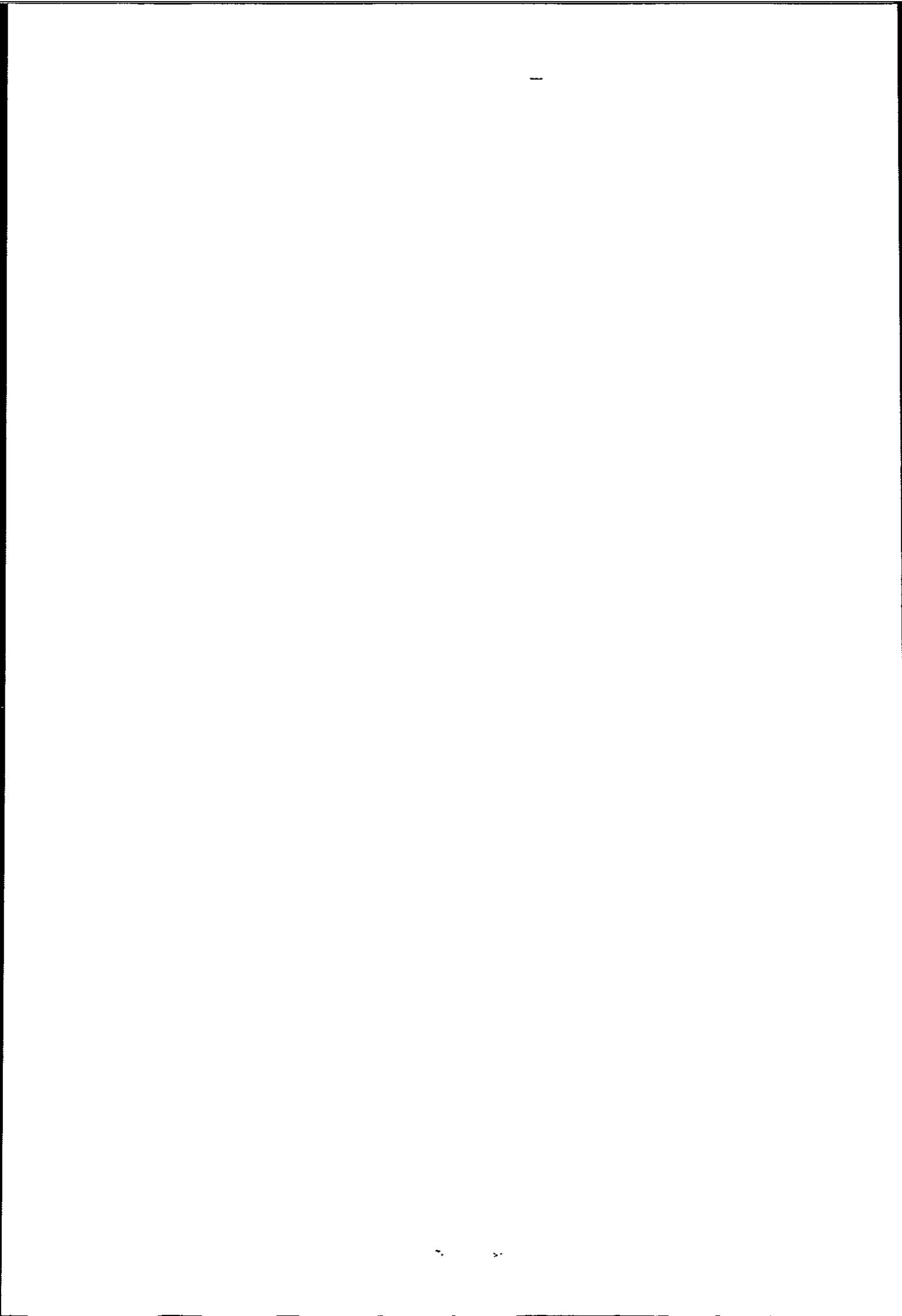
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## AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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A month course was undertaken in "biological adaptation of marine organisms to the Antarctic ecosystem", in the American Antarctic base at Mc Murdo.

Relevant scientific seminars and conferences were attended regularly and work presented as follows:

- Poster at Pollutant Responses In Marine Organisms 10 (Williamsburg, USA, 1999)
- Poster at Society of Environmental Toxicology and Chemistry (Madrid, Spain, 2001)
- Talk and poster at Pollutant Responses in Marine Organisms 11 (Plymouth, UK, 2001).

Collaboration with Prof. F. Regoli from the University of Ancona (Italy) was carried out to gain training in analytical work (TOSC-assay) and consultation purposes in the field of oxidative stress. Collaboration was also carried out with Prof. B. Gulliksen (University of Tromsø), Prof. O.J. Lønne (The University Courses on Svalbard) to participate to sampling cruises in the Arctic. Five papers have been prepared for publication; one is published, one is *in press*, three are submitted.

Signed



Date

13/08 - 2002

## Abbreviations

ABAP	2-2'-azo-bis-(2 methyl-propionamidine)-dihydrochloride
AMAP	Arctic Monitoring Assessment Programme
BFLV	body P-FL-verapamyl
CAPMON	computer aided physiological monitoring
CYP	cytochrome P
EthD-1	ethidium homodimer-1
GC	gas chromatography
HVA	homeoviscous adaptation
KMBA	$\alpha$ -keto- $\gamma$ -methiolbutiryc acid
MFO	mixed function oxygenase
MCA	metabolic cold adaptation
NRR	neutral red retention
PAC	poly aromatic compound
PAH	polyaromatic hydrocarbons
PCB	poly chloro byphenyl
POP	persistent organic pollutant
ROS	reactive oxygen species
TOSC	total oxyradical scavenging capacity



## Chapter 1

### The Arctic: environmental issues



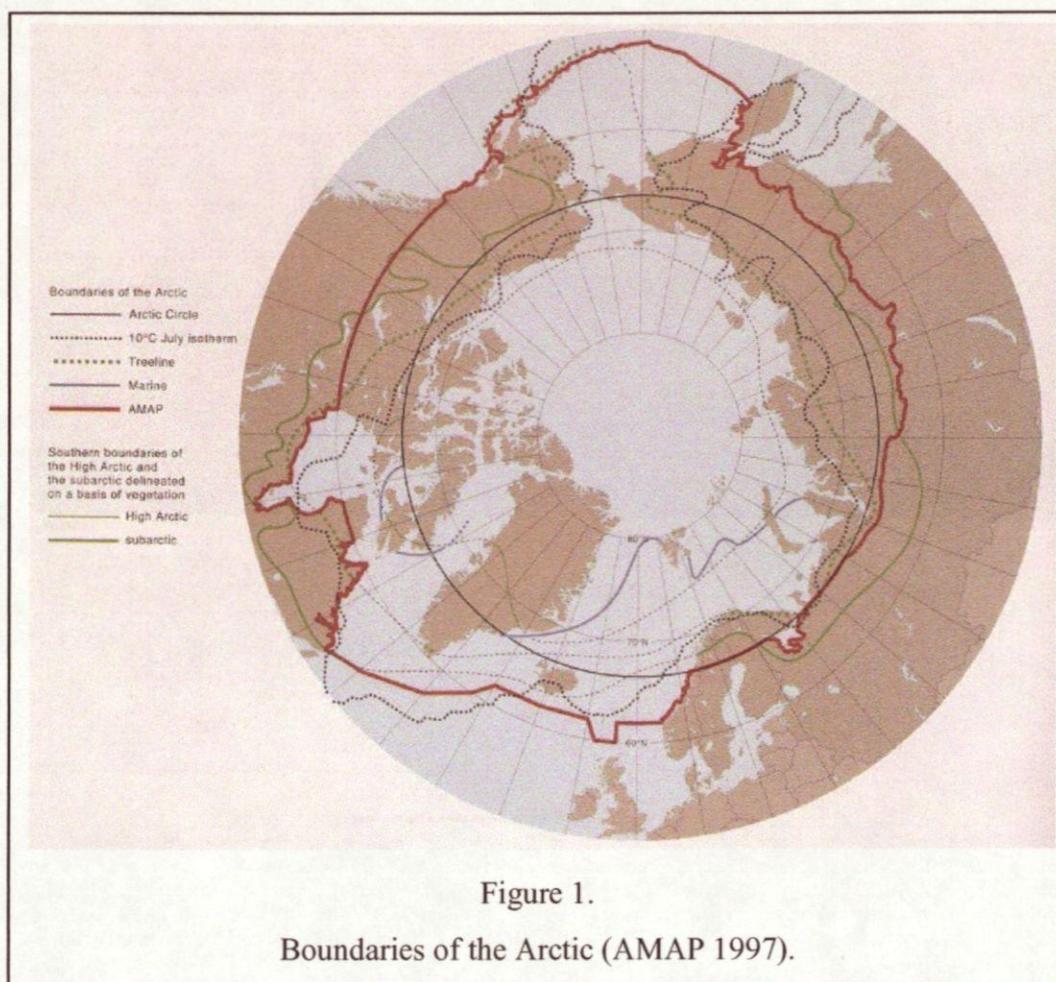
*“Men go out into the void of spaces of the world for various reasons. Some are actuated simply by a love of adventure, some have the keen thirst for scientific knowledge, and others again are drawn away from the trodden paths by the “lure of little voices,” the mysterious fascination of the unknown. I think that in my own case it was a combination of these factors that determined me to try my fortune once again in the frozen south.”*

**Sir Ernest H. Shackleton, English polar explorer (1874-1922).**



## 1.1. The Arctic

The word 'Arctic' has its origin in Greek, where *Arktos* means bear (Robert 1993). The Arctic region derives its name from the stellar constellation *Ursa major*, the Great Bear. In general, the Arctic is the area lying north of the Arctic Circle ( $66^{\circ} 32' N$ ) where the midnight sun occurs. Other definitions have been proposed. For example, the Arctic Monitoring Assessment Programme (AMAP) (1998) defined the Arctic as (Figure 1): i) climatically, the Arctic is the area north of the  $10^{\circ}C$  July isotherm; ii) on terrestrial aspects, the treeline boundary is also accepted as a definition; and iii) a marine boundary defined by the polar front, formed when the water of the Arctic Ocean, cool and dilute from melting ice, meets warmer, saltier water from the Southern Oceans. This boundary, however, moves according to the seasons, and does not take into consideration the fjords of the west coast of Svalbard though they are truly Arctic ecosystems. AMAP defined the Arctic on a more social, cultural and political basis (red line on Figure 1) to include people of the North.





## **1.2. The Arctic Ocean**

The Arctic Ocean system is an isolated sea surrounded by landmasses that leave only two outlets to other Oceans, the Bering Strait to the Pacific and Fram Strait to the Atlantic (Figure 1). The Arctic Basin is surrounded by extensive shallow shelves such that the deep water of the central basin is cut off from the other Oceans. The circulation pattern consists of two major anticyclonic currents, the Beaufort gyre over the Canadian Basin and a transport current across the Eurasian part of the basin that exists through the Fram Strait. The landmasses surrounding the Arctic Ocean have several large rivers whose discharges lead to the development of a low saline, stratified surface layer. The Arctic Ocean pack ice is a persistent multiyear system that is harder and thicker than that of the Antarctic. Antarctic pack ice is usually seasonal and only a year old or younger. The whole central Arctic Ocean is permanently covered by ice.

## **1.3. The Arctic environmental issues**

The Arctic is generally considered to be one of the last pristine regions on Earth. It is populated by indigenous people in relatively small numbers. The commercial fisheries are scarce due to ice presence and weather conditions. The industrial activity is limited compared to temperate regions although the Russian Arctic (Kola Peninsula, Pechora and Ob/Yenisey river basins, Murmansk) is industrialised. In spite of these relatively minor human activities in the Arctic region, growing evidence indicates the presence of anthropogenic contaminants that are not produced in the Arctic such as persistent organic pollutants (i.e poly chloro biphenyl) (AMAP 1998). This presence indicates that there has been long-distance atmospheric or oceanic transport of anthropogenic contaminants from mid and low-latitude sources where most of industrialised countries are located to the Arctic (Bard 1999).

### **1.3.1. Atmospheric transport of contaminants**

Volatile contaminants from mid-and low-latitudes reach the Arctic through a process known as "global distillation" (Goldberg 1975). Contaminants evaporate from soils in warm regions and become available for atmospheric transport to the pole where they condense out in the colder air. The adsorption of high molecular weight organic vapors to atmospheric particulate matter is enhanced by low temperatures. As the coldest Northern region area is a small proportion of the Earth's surface, these contaminants can



concentrate to surprisingly high levels in the Arctic. Pollution of the Arctic air is most pronounced during the coldest months of the year from December-April. Due to low temperature, low solar radiation and low precipitation, scavenging processes are inefficient and pollutants persist. Another process aiding atmospheric transport of contaminants in the Arctic is Haze (Shaw 1995). This phenomenon is explained by the accumulation of volatile chemicals, notably sulfur dioxide, in the Arctic atmosphere during winter when photochemical oxidation cannot occur. When the sun returns in the early spring, there is a high concentration of sulfur dioxide in the air ready to be converted into sulfate aerosols that will deposit in the Arctic in high amounts (Figure 2).

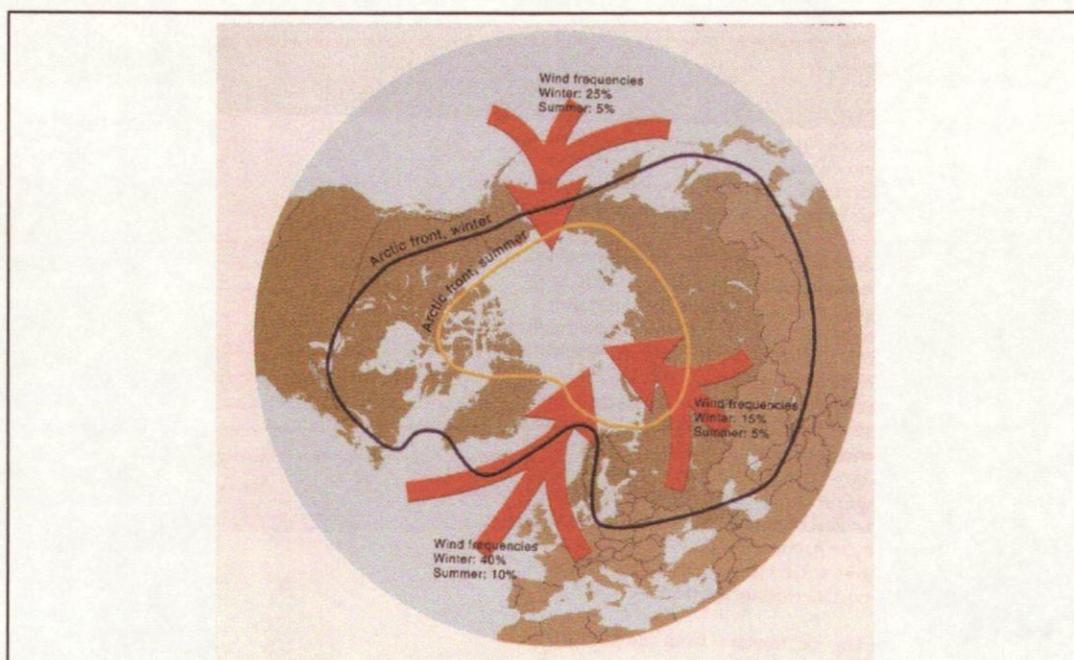


Figure 2.

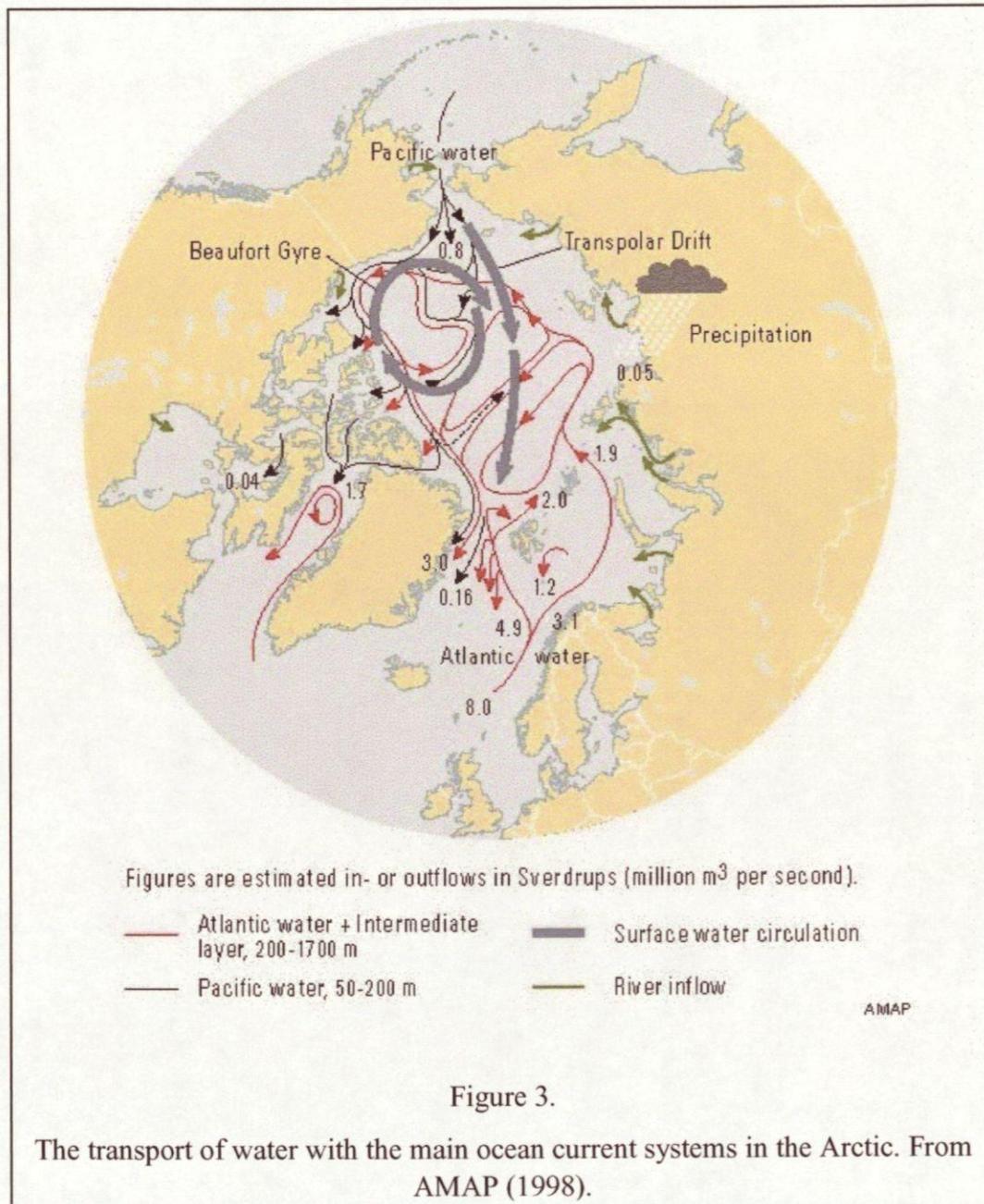
The position of the Arctic front influences contaminant transport in the atmosphere. The figure shows the mean position of Arctic air mass in January and July and the winter and summer frequencies of frequencies of winds driving the major south-to-north transport routes. From AMAP (1998).

### 1.3.2. Oceanic transport of contaminants

The Arctic Ocean is surrounded by land masses and receives a large input of freshwater from rivers that contribute to the input of pollutants (Yunker *et al.* 1996). Contaminants are also transported into the Arctic Ocean via narrow passageways: sea water enters the Arctic Ocean predominantly from the North Atlantic via the Barents Sea and the Fram



Strait with a smaller contribution from the North Pacific via the Bering Strait (Schlosser *et al.* 1995) (Figure 3).





### 1.3.3. Oil and gas industries

#### *Oil and gas development in the Arctic*

On a more local scale, there is a growing environmental concern over the possibility of oil exploitation in the Arctic marine ecosystem. Indeed, the increasing need for energy for the development of our modern industrialised society has already led to the exploration of new areas and to the exploitation of oil and gas resources located on the Arctic continental shelf (Figure 4). With the growing activities of oil and gas industries, there is an increasing risk of oil discharge in the marine environment via chronic production water discharge or through accidents such as oil spill. Activities related to this oil exploitation such as shipping for oil transport (tanker ship, pipeline) will also

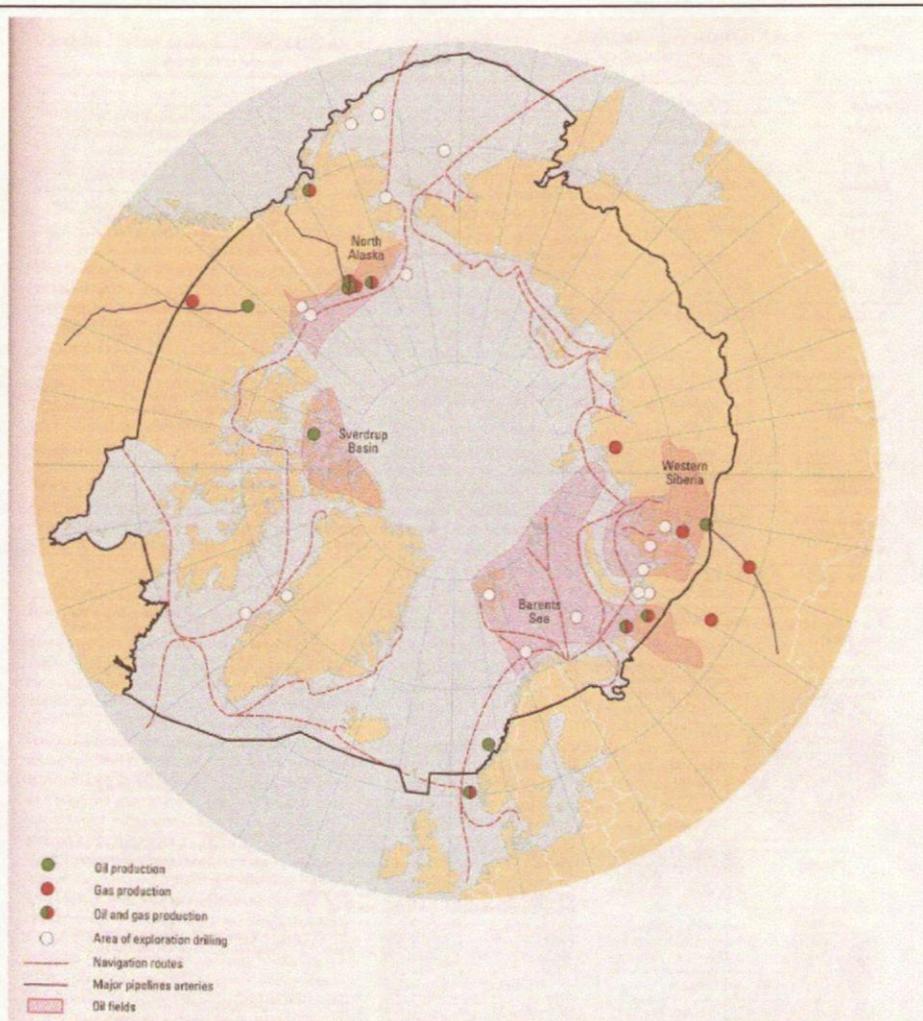


Figure 4

Major areas of oil and gas activities in the AMAP region (AMAP 1998).



increase.

#### ***Sources of poly aromatic hydrocarbons***

Crude oil contains poly aromatic hydrocarbons (PAH). PAHs are ubiquitous in the environment and, although they are naturally produced, a large number of human activities contribute to the release and increase of these substances in every biosphere compartment. Sources of PAH input to the ocean include: 1) incomplete combustion of fossil fuels; 2) forest and grass fires; 3) industrial effluents; 4) sewage effluents; 5) river-borne material from interior areas; 6) oil tanker operation routine and accidents; 7) waste barges; 8) offshore oil production routine and accidents; 9) natural oil seeps; and 10) diagenesis of organic matter in sediments (Farrington 1991). Once in the environment, the PAHs undergo a biogeochemical cycle which is not well characterized. Numerous parameters influence the fate of PAH in the environment, including temperature, salinity, photooxidation, and biological interaction with biota.

Because of the carcinogenic and mutagenic properties of PAH (Neff 1979) for marine biota and humans, the main objective of this thesis was to focus on petrogenic PAH. All of the 20-30 proven PAH carcinogens are in the high molecular weight PAH group. On the other hand, the low molecular weight PAHs have significant acute toxicity. Petrogenic PAHs are formed through a slow transformation of organic matter under pressure. Petrogenic mixtures consist mainly of smaller molecules with two and three rings molecules (Neff 1979). They are released chronically in the ocean via waste water during the oil offshore production processes. Current regulation set by the Paris-Oslo commission stated that the oil content of produced water should not exceed 40 mg l<sup>-1</sup> at the discharge point (Syvertsen 1996). This input contributes largely to the presence of oil in the water column, mainly in the dispersed form and, to a less extent, in the dissolved and adsorbed states. Other input is mediated through accidents such as oil spills and can lead to heavy sediment contamination when the spill arrives on the shore.

#### ***Oil in cold water***

Low temperature reduces the solubility of oil compounds. Therefore, they will tend to adsorb onto particulate matter and stay in the sediment. As a result, the bioavailability of oil at low temperature can be relatively reduced (Payne *et al.* 1991, Sydnes 1991). Nevertheless, the presence of ice in the Arctic Ocean results in unique interactions in the oil-water-ice system particularly when combined with the marked seasonal pattern in light and ice formation (Sydnes 1991) (Figure 5). When oil is trapped in the ice it can undergo a long transport in the Arctic Ocean. When ice melts, it will release the

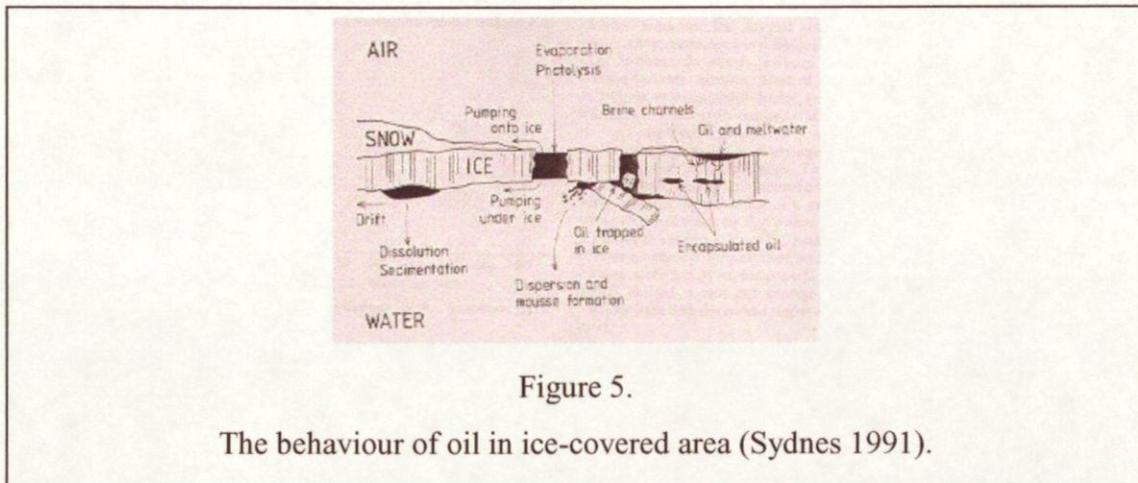


Figure 5.

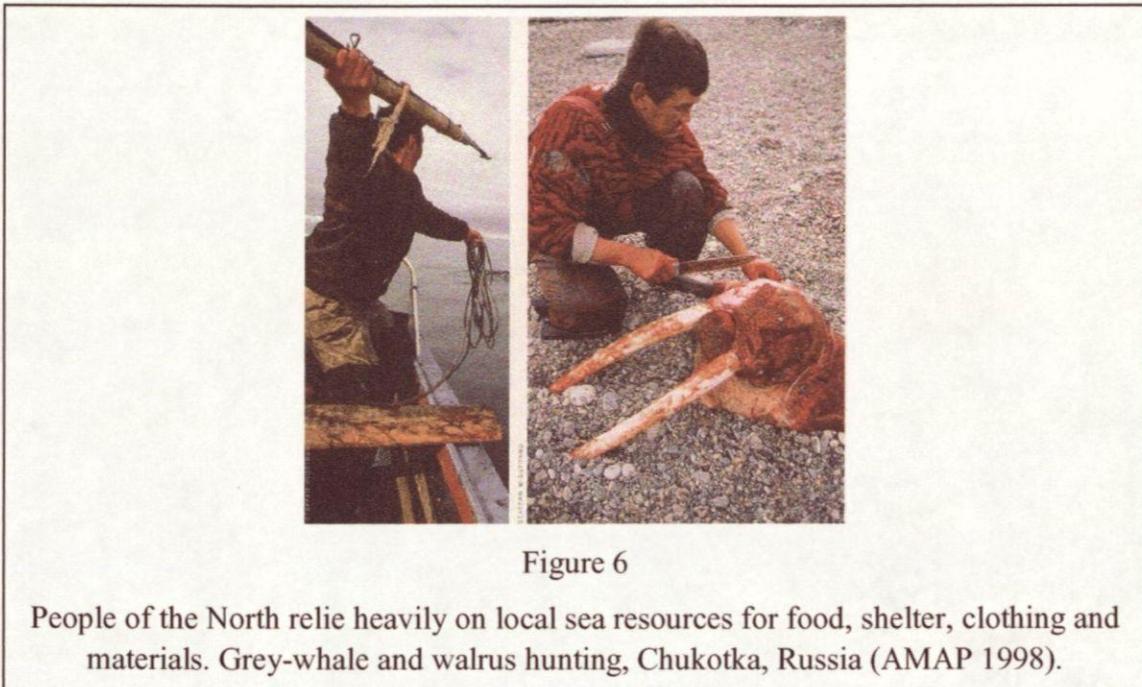
The behaviour of oil in ice-covered area (Sydnes 1991).

contaminants far way from their source of origin; most of the release will occur in the Beaufort Strait where the ice of the Arctic Ocean melts, thereby, concentrating the contaminants in one place. Fauna richness and production are important at the ice edge, and are unique features of the Arctic ecosystem. Intense blooms occur when the ice melts and retrieves and then attract fish, seals, whales and polar bear. As the contaminants trapped in the ice are released when the ice melts, the whole Arctic food chain is exposed to this pollution and will be threatened. The long illuminated summer coupled to an increased UV-B irradiance level may contribute to the photochemical degradation of PAH molecules, but it will also activate them rendering them more toxic (Sydnes 1991).



### 1.3.5. Environmental issues and human health

The Arctic region is populated with indigenous people that still rely on natural resources (whales, seals, walrus) for nutrition; therefore, human exposure to pollutants through the diet is of central concern in the Arctic (Figure 6). Many pollutants (POPs, heavy metals, PAHs) are biomagnified through Arctic food chains. Indigenous people of the Arctic still feed on natural resources which include animals located at the top of the food chain (seals, whales, walrus). Therefore, they are likely to be more exposed to relatively higher concentrations of pollutants than populations elsewhere in the world (Dewailly *et al.* 1996) and also because of their high fat body content. High levels of contaminants have been detected in local populations, notably PCBs in women breast milk (Dewailly



*et al.* 1992). While human exposures in the Arctic can be moderately reduced with some dietary modifications (provided these are culturally, socially and nutritionally suitable), some techniques are required to give early warning signals of food contamination.

### 1.4. Monitoring contaminant levels and effects in the Arctic

Facing the need for a global environmental approach to the management of the Arctic, eight countries (Canada, Denmark, Finland, Iceland, Norway, Sweden, The former USSR and the United States) signed the Declaration on the Protection of the Arctic Environment in Rovaniemi (Finland) on 14 June 1991. This document expressed particular concern for the effects of chemical pollution, including the effects of heavy



metals and for the assessment of potential impacts of development activities. At that time, the chairman of the International Arctic Science Committee (Dr. Fred Roots) indicated that much information necessary for the protection of Arctic regions was unknown, in particular their sensitivity to change. The Arctic Monitoring and Assessment programme (AMAP) was then launched to examine the levels and effects of anthropogenic pollutants.

The levels of the 5 major pollutant groups (persistent organic pollutants, heavy metals, radioactivity, acidifying gases and poly aromatic hydrocarbons) have been well documented through this Arctic Monitoring Assessment Programme (1998). All were detected and, for the first four groups, are of major concern as the observed concentration, in the biota were high and appeared to be a potential threat to the indigenous species. The measurements of polycyclic aromatic hydrocarbons in sediments of the Barents Sea and the Svalbard West Coast indicated that naphthalenes were detected in Isfjorden were due to the presence of coal mining in Svalbard (notably in Isfjorden) but levels were not of environmental concern (Stange and Klungsøyr 1997).

AMAP was unable to achieve a good understanding of the impact of the measured chemical levels on the Arctic biota because of a lack of knowledge about the biological effects of pollutants on the Arctic fauna. Warning signals of severely affected animals were measured in sea mammals (i.e. polar bears, seals or beluga) and birds (falcon) stressing the urgent need to focus on ecotoxicological studies. Few studies of the effects of pollution on Arctic marine ectotherms have been performed. Toxicity tests have been conducted to look at the effects of crude oil on some marine fauna (Percy 1976, Percy 1977, Aarset and Zachariassen 1983, Humphrey *et al.* 1987, Mageau *et al.* 1987, Riebel and Percy 1990, Aunaas *et al.* 1991). Studies of the toxicity of heavy metals to Arctic amphipods (Chapman and McPherson 1993) revealed that these organisms were surprisingly insensitive to metals (Chapman 1993). Some biomarker studies of Arctic fishes have been published (Christiansen and George 1995, George *et al.* 1995, Christiansen *et al.* 1996, Wolkers *et al.* 1996, Wolkers *et al.* 1998, Jorgensen and Wolkers 1999, Christiansen 2000, Ingebrigtsen *et al.* 2000).

Biomarker studies in Antarctica have been developed but mainly with an emphasis on the oxidative stress in mollusks (Viarengo *et al.* 1995, Regoli *et al.* 1997, Abele *et al.* 1998, Regoli *et al.* 2000), MFO/CYP1A induction in fishes (Focardi *et al.* 1989,



Focardi *et al.* 1992, Focardi *et al.* 1995, Jimenez *et al.* 1999), and metal toxicity to amphipods (Duquesne *et al.* 2000) and bivalves (Regoli *et al.* 1997).

The lack of data on the biological effects of pollutants on Arctic biota led AMAP, in 1998, to conduct a workshop on combined effects of chemicals in the marine environment to stimulate research in this domain. Recommendations arising from the workshop were to study not only the effects at the highest levels of the food web, but to investigate the effects at lower levels, notably in marine invertebrates.

### 1.5. The biomarker concept

For the last 30 years, there has been a growing concern with methods of evaluating the impact of human activities on the aquatic environment. The first approach, based on ranking the relative toxicities of chemical with laboratory acute tests performed on a few species, was generally not ecologically relevant. By measuring the level of chemicals in water, air, soils, sediments and biota, predictions were then made, based on extrapolations from toxicity tests (i.e. LC50), as to whether adverse effects were likely to occur in the considered ecosystem. The drawback of this approach was that it did not focus on the well being of individual organisms, populations and communities *in situ*. Another approach to measure the impact of pollution on the aquatic ecosystem has been the "bioindicator" method which does not aim to measure contaminant impact *in situ* at the community or ecosystem level (Warwick *et al.* 1988, Gray 1989, Gray *et al.* 1990). Bioindicators, relying on the presence or absence of a species, gives information on the impact in a late stage of disturbance once the ecosystem equilibrium has been disrupted. As a result, an ecotoxicological approach to pollutant studies was developed. The aim of ecotoxicology is to develop predictive tools which estimate the extent of contaminant exposure on the biota and monitor any biological effects *in situ* with the aim of improving environmental risk assessment. The main tool in the development of this approach was 'biomarkers'. The following biomarker definition was proposed: "an ecotoxicological biomarker is a biochemical, cellular, physiological or behavioral variation that can be measured in tissue or body fluid samples or at the level of whole organisms (either individuals or populations) that provides evidence of exposure to and/or effects of one or more chemical pollutants (and/or radiations)" (Depledge 1994). Basically, in environmental toxicology, the well being of a population is the main consideration and not the well being of the individual (as in medical sciences).

Biomarkers are classified in four classes: biomarkers of exposure, biomarkers of effects, biomarkers of exposure and effect and, finally, biomarkers of latent effects.

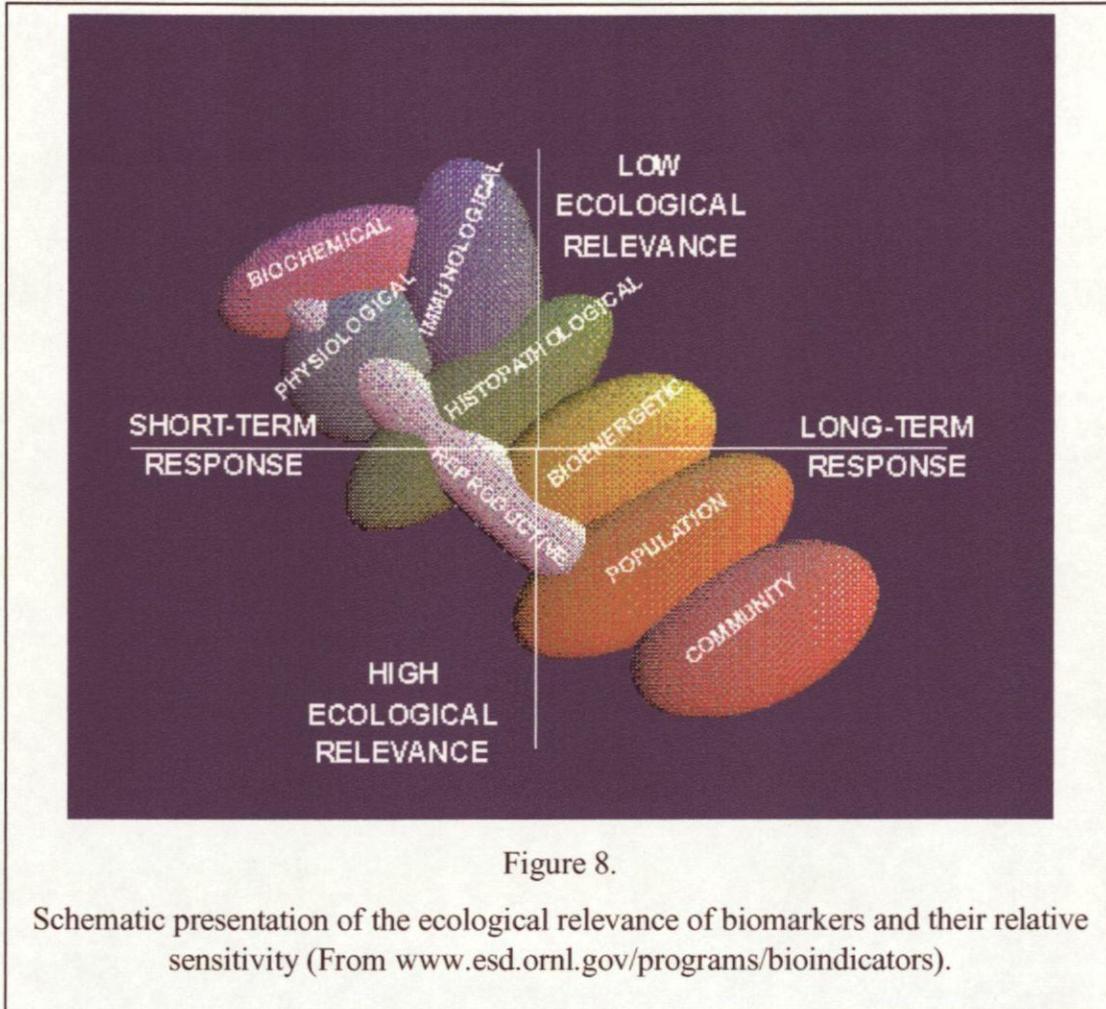
Exposure biomarkers signal exposure of an organism, a population or a community to chemical pollutants (Figures 7 and 8). They may range from generalized indicators of pollutant stress to specific indicators of exposure to a certain concentration of one particular pollutant. Effect biomarkers signal that an organism, a population or a community has been affected adversely by one or more pollutants. This type of biomarker does not necessarily provide information concerning the nature of the pollutant stress to which the organism was exposed. Exposure/effect biomarkers not only indicate that an organism, population or community has been exposed to one or more pollutants but specifically link the exposure to an effect. A range of possibilities exists with regards to the degree of pollutant specificity and exposure level that can be determined with such biomarkers. Finally, latent effect biomarkers indicate that apparently normal organisms have been exposed to a pollutant which, in other circumstances, may limit the ability of the organisms to adapt or survive.

In ecotoxicology, emphasis is placed on protecting some of the individuals in a very wide range of species, and also on the interrelations among the species and their environment. The selection of the test species should include numerous factors based on ecological considerations. Stegeman *et al.* (1992) and Lundebye Haldorsen (1996) proposed the following criteria for each candidate biomarker:





- 7) Wide applicability –should be applicable in a broad range of organisms.
- 8) Non-destructive –to facilitate work on species with low reproductive rates, endangered species and enable sequential sampling on the same individuals.
- 9) Easy, rapid and cost-effective to measure.



## 1.6. Biomarkers in the Arctic

### 1.6.1. Characterisation of biomarker responses in the Arctic

Little is known about the background levels of most typical biomarker responses and their seasonal variations in the Arctic. There is a need to gain fundamental knowledge on the selected biomarkers for each species before running any biomonitoring programme. The marked seasonality in food availability and the short food supply in the Arctic (Weslawski *et al.* 1988) may affect typical biomarker responses. The cold water adaptation of Arctic marine species may render the animal more vulnerable to pollution



(Christiansen *et al.* 1996). For instance, polar cod possess antifreeze compounds in their blood to prevent freezing. The kidney of this fish species is specifically adapted to retain these molecules in the blood during urine formation. Urine is one major excretion route for soluble compounds such as PAH metabolites, therefore, this kidney cold adaptation specificity prevents excretion of these xenobiotics. The alternative route of excretion of these contaminants is via the bile which is excreted into the digestive tract. Once in the gut, the contaminants can be activated by the gut microflora to become more toxic and reabsorbed into the organism (Christiansen *et al.* 1996) and this may result in adverse effects due to a prolonged depuration time of these compounds (Ingebrigtsen *et al.* 2000). Most marine organisms are able to metabolise poly aromatic hydrocarbons via enzymatic activities such as P-450 (Stegeman 1989). However, surprisingly Antarctic fish showed low baseline level of P-450 and the inducibility was limited (Focardi *et al.* 1989, Focardi *et al.* 1992). On the other hand, amphipods and mysids living under the ice possess a very efficient osmoregulation system to cope with large salinity fluctuation when ice freezes or melts that make them resistant to heavy metals (Chapman and McPherson 1993) normally known to disturb the osmoregulation processes (Bamber and Depledge 1997).

### **1.6.2. Selection of key biomarker responses**

Biomarkers have been developed for temperate marine animals for the last 20 years. Therefore, some assays are now well established and could be readily transferred and applied to Arctic marine species. Nevertheless, the selection of the biological responses is based on several aspects.

1. It should give signal of exposure/effects to poly aromatic hydrocarbons.
2. It should be easily measurable and sensitive in the considered species.
3. Arctic marine invertebrates possess specific biological adaptations to live in the polar environment, therefore, focus should be put on these biological adaptations.
4. From the biomarker definition, it is best to measure several biomarkers located at different levels of biological organisation on the same species, to be able to better understand the real effect of the compounds.

From this foregoing account, it is clear that biomarker studies of Arctic marine ectothermic invertebrates are lacking. Global pollution, due to the atmospheric transport



of contaminants released in industrialised countries, but also the increasing development of oil and gas industries in the Arctic Ocean, require monitoring of their potential impact on the marine Arctic fauna.

### **1.7. Aims of the thesis**

In this thesis, the chapter 2 will deal with the sources and implications of the oxidative stress in the Arctic. This will serve as a basis to set up a research strategy described in chapter 3. The materials and methods used in this research are covered in chapter 4. The research is divided in two main sections: the ecophysiology of the polar marine invertebrates, from chapter 5 to 7, and the biological effects of polycyclic aromatic hydrocarbons on the polar marine invertebrates from chapter 8 to 11. Finally, the chapters 12 to 13 are the general discussion and conclusion of this research.

## Chapter 2

### Oxidative stress in the Arctic: sources and implications



Smeerenburg glacier in Svalbard

*Jeg letted på hatten vi rakte hverandre hjertelig hånden: "How do you do?"- "How do you do?". ...Jackson: "I am dam'd glad to see you." - "Thank you, I also."- "Have you a ship here?"- "No, my ship is not here." Da stansed han med ett, ved en tilfældig ytring fra mig, så mig stivt ind i ansigtet og sa hurtig: "Arn't you Nansen?"- "Yes, I am."- Jackson: "By Jove, I am glad to see you."*

Fridtjof Nansen 1897 (Norwegian polar explorer).



## Introduction

Oxidative stress is a natural biological process that takes place during aerobic metabolism, and during general stressful conditions like hypoxia, ischaemia, and intoxication with contaminants (Viarengo *et al.* 1998). The well known consequences of the effects of oxidative stress are enhanced ageing, disruption of the cellular redox balance, oxidation of biomolecules (proteins, membrane lipids and nucleic acid) and promotion of carcinogenesis (Winston and Di Giulio 1991). Through evolution, most organisms have developed antioxidant defences to counteract the reactive oxygen species (ROS) to prevent damage. Earlier studies have revealed that the ability of an organism to respond to oxidative stress is related to its physiological conditions, food availability and environmental conditions (Viarengo *et al.* 1991, Regoli and Winston 1998, Sheehan and Power 1999). Although most contaminants, notably poly aromatic hydrocarbons, enhance the formation of ROS (Livingstone *et al.* 1990), the knowledge of oxidative stress in polar marine organisms is limited and requires investigation. For instance, the high oxygen content of cold water, and the intense illumination and UV irradiance over the summer, make the Arctic marine environment a strong prooxidant environment (Viarengo *et al.* 1998) which can affect ectothermic animals (Regoli *et al.* 2000). In this chapter, the sources and implications of oxidative stress in polar regions is reviewed as a basis for the biomarker research strategy.

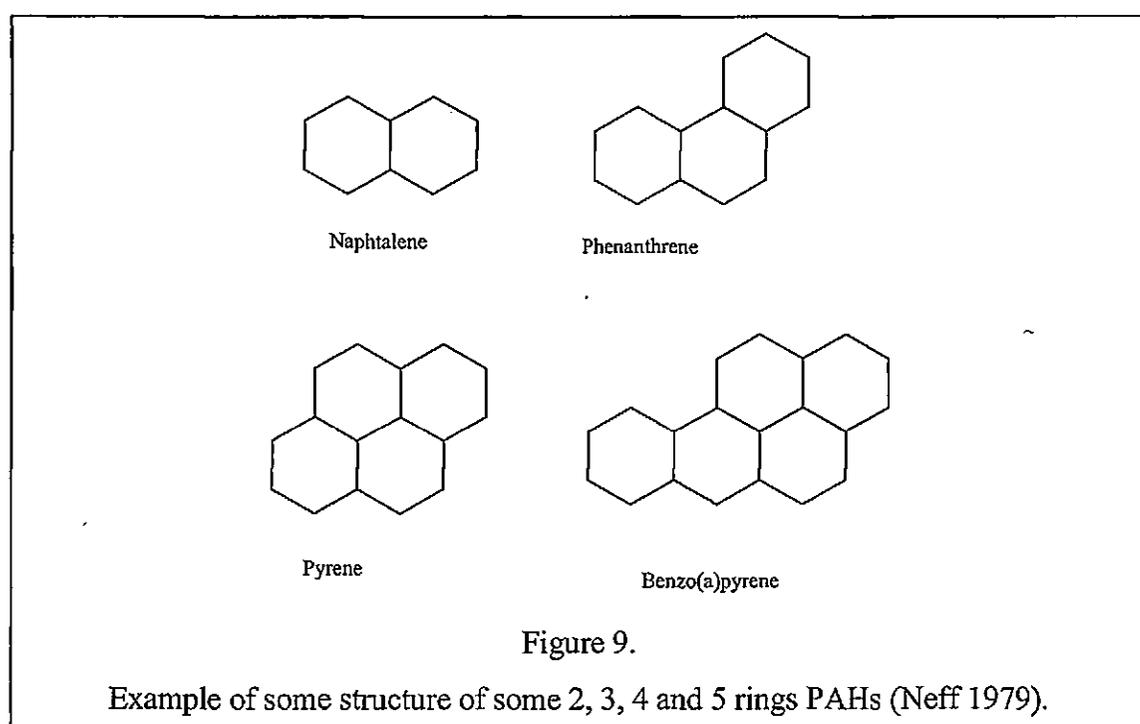
## 2.1. Oxidative stress sources

### 2.1.1. PAH mediated

#### *Nomenclature*

Poly aromatic hydrocarbons are composed of two or more fused aromatic (benzene) rings. Two aromatic rings are fused when a pair of carbon and hydrogen atoms is shared. The resulting molecule lies in a single plane. If nitrogen, oxygen or sulphur is associated with the molecule, they are often called poly aromatic compounds (PACs) (Lee *et al.* 1981). The physical and chemical properties of PAH depend on the molecular weight. For instance, solubility decreases with increasing molecular weight. Resistance to metabolism through biological processes (oxidation, reduction) decrease with increasing molecular weight. As a consequence of these differences, PAH

distribution and effects in the environment vary greatly. Nevertheless, because of their high hydrophobicity they have the tendency to be adsorbed onto particles and sediment out on the sea floor. Consequently, their hydrophobicity makes them highly lipophilic and, therefore, enhances their uptake by the biota resulting in accumulation at high levels in lipid components of animal tissues (i.e. cell membrane). These physical, chemical and biological properties led to the distinction of PAHs into two molecular weight classes (Figure 9): these are the lower molecular weight 2-3 ring aromatics (naphthalenes, fluorenes, phenanthrenes and anthracenes) and the higher molecular weight 4-7 ring aromatics (chrysene, benzo(a)pyrene, coronene). The  $K_{ow}$  octanol/water partition coefficient of a compound is a useful parameter to predict the environmental behavior of PAH and, notably interactions between these compounds and biota (Baussant *et al.* 2001).



#### *Invertebrates-PAH interactions*

Bivalves take up PAH mainly through respiration. Because bivalve filtering activity incorporates breathing and feeding, it is difficult to discriminate the PAH uptake route as for other organisms (i.e. fish) where dietary and respiratory uptake are separate routes (Baumard *et al.* 1999b). Filter feeding bivalves take up PAH from the water column present under several forms, dissolved (two and three ring compounds), and dispersed and particle adsorbed (i.e. adsorbed on unicellular algae) compounds (Baumard *et al.*

1998a, Baumard *et al.* 1998b, Baumard *et al.* 1998c, Baumard *et al.* 1999a, Baumard *et al.* 1999b, Baussant *et al.* 2001). Dissolved and low molecular weight molecules enter the organism through the gill epithelium, while adsorbed or dispersed compounds will follow the food ingestion path and, consequently, enter the organism through the digestive epithelium in the hepatopancreas (Connel 1988). The uptake varies with a diversity of factors like concentration of PAH, lipophilicity and solubility of the compounds, whether they are dissolved or adsorbed, seawater temperature, and filtering activity of the bivalve (Baumard *et al.* 1998a, b, c, Narbonne *et al.* 1999, Baumard *et al.* 1999a, b, Baussant *et al.* 2001). The accumulation and distribution of the PAH molecules will depend on fat content of the animal different tissues, and the metabolic capability to biotransform and excrete the compound (Baumard *et al.* 1999b, Baussant *et al.* 2001).

#### **PAH metabolism and ROS formation**

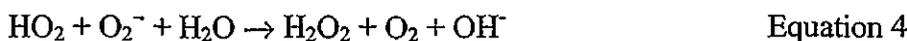
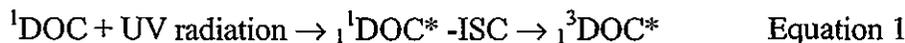
Once the PAH has entered the organism, it undergoes biotransformation processes that aim to render the molecule more water soluble to facilitate excretion. Bivalves, notably the mussel *Mytilus sp.*, possess multiple forms of the cytochrome P450 (CYP) enzyme system in the digestive gland (Wootton *et al.* 1995, 1996). An increased level of digestive gland CYP1A following exposure to PAH (indicating the capability of the bivalve to metabolise the PAH compounds) was reported by Sole *et al.* (1996). Cytochromes P450 were shown to be present in crustaceans (Lemaire *et al.* 1993, Sundt and Goksøyr 1998). The biotransformation reactions are enzymatic processes that add hydrophilic functional groups onto the parent PAH molecule to make it more water soluble. The enzymatic process is divided into two main phases. Phase I is cytochrome P450 dependent, the molecule is bioactivated following the addition of a hydroxyl group. In phase II (called conjugation), a larger molecule is added (i.e. glutathione). The metabolite is then soluble enough to be excreted through the gills. It is during the phase I that reactive oxygen species are produced. The production of oxyradical and its enhancement via redox cycling of xenobiotics, notably PAH, has been demonstrated in digestive gland microsomes of marine bivalves (Wenning *et al.* 1988, Livingstone *et al.* 1989). PAH, through the redox cycles are univalently reduced, often by the cytochrome P-450 reductase to a reactive intermediate which rapidly loses its electron to molecular oxygen thereby producing ROS and the parent compound that can undergo another redox cycle (Kappus and Sies 1981). Quinones are the major products of the

metabolism of PAH (Sjölin and Livingstone 1997). The electron transport chains of microsomes and mitochondria have long been recognized for their potential for ROS, however, it was shown recently that ROS production also occurs in lysosomes (Winston *et al.* 1996). It was identified that lysosomes of haemocytes produce ROS as a cytotoxic mechanism to kill and degrade foreign compounds like viruses and bacteria (Pipe 1992). Probably, ROS can also oxidise contaminants like PAH that have accumulated in the lysosomes (Fernley *et al.* 2000).

### 2.1.2. Natural occurrence of ROS in polar water

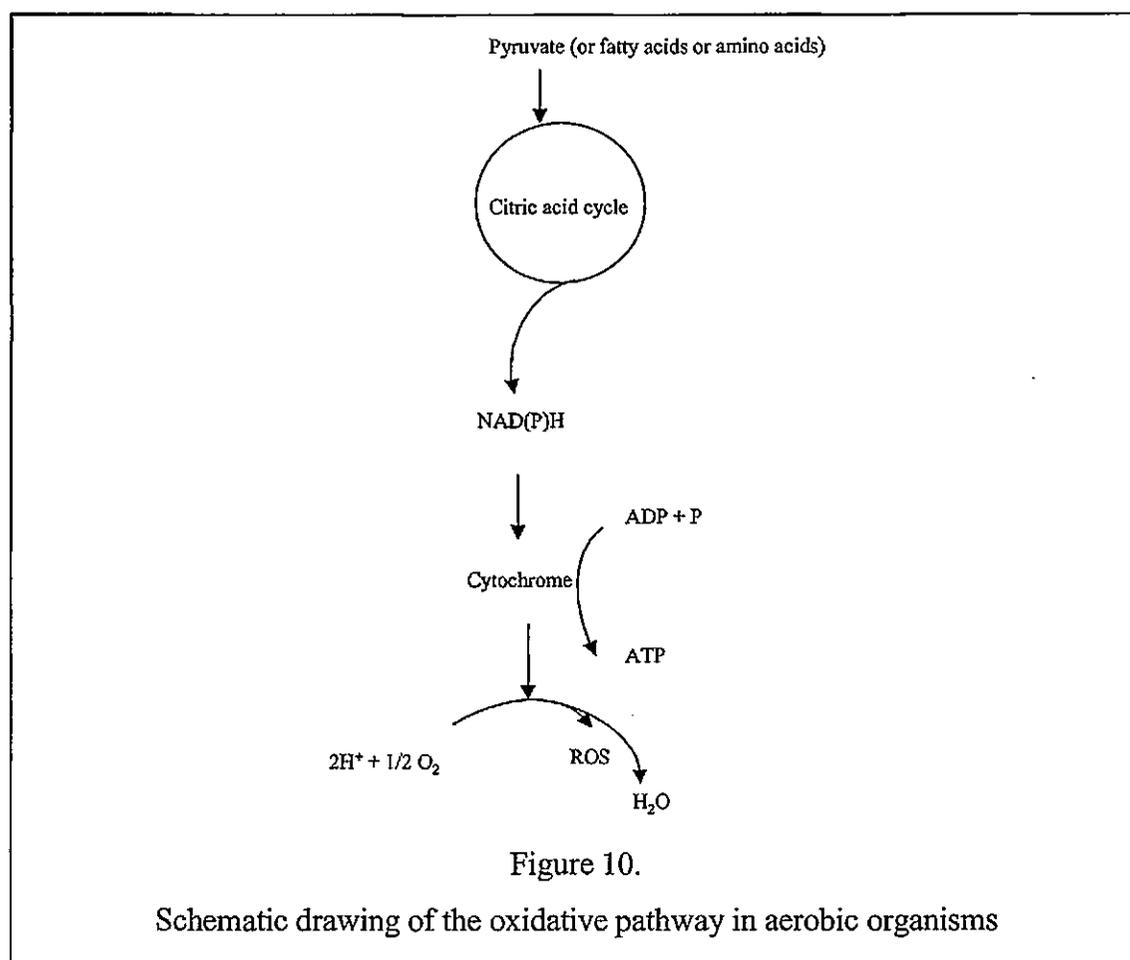
Reactive oxygen species are ubiquitous in sunlit natural waters and are important species formed from the photoreactions of dissolved organic carbon (DOC) in seawater. Recent stratospheric ozone depletion has caused an increase in ground-level ultraviolet-A and B radiation (Madronich *et al.* 1991, Gruzdev 1995). The depletion of ozone can be attributed to chlorofluorocarbons (CFCs) and is occurring to great extent in the North and South polar zones (Schoeberl and Hartmann 1991). Studies indicated that ROS production rates can be as high (or higher) than those at lower latitudes (Scully *et al.* 1996, Yocis *et al.* 2000, Qian *et al.* 2001). Cooper *et al.* (1994) suggested a simplified ROS formation following UV exposure:

the ground state of ( $^1\text{DOC}$ ) is excited by UV radiation to a singlet state ( $^1\text{DOC}^*$ ) and is transformed to the excited triplet state ( $^3\text{DOC}^*$ ) (Eq. 1). This triplet state may react with macromolecular oxygen to form superoxide ( $\text{O}_2^-$ ) or its conjugate acid  $\text{HO}_2$  (Eq. 2) which reacts with itself to give  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  (Eq. 3). Lastly, the interaction of  $\text{HO}_2$  and  $\text{O}_2^-$  Results in the formation of  $\text{OH}^-$  and additional  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  (Eq. 4).



### 2.1.3. Natural internal ROS generation

A consequence of aerobic life is the production of reactive oxygen species (ROS) as a result of normal oxygen metabolism (Figure 10). Essentially, the oxygen molecule is only partially reduced during the respiration processes. If  $O_2$  is only partially reduced by accepting two electrons, the product is hydrogen peroxide ( $H_2O_2$ ). If  $O_2$  accepts only one electron, the product is the superoxide radical ( $O_2^{\cdot -}$ ). As an estimated proportion, 2-3% of the oxygen consumed by aerobic cells is converted to ROS (Chance *et al.* 1979, Sohal and Weindruch 1996). ROS formation and subsequent oxidative stress is strongly linked with the metabolic rate of an organism, it is, therefore, of prime importance to



investigate the specific rate of the metabolism of polar marine organisms. Reactive oxygen species are also formed by lysosomes from haemocytes as a mechanism to kill pathogens (virus bacteria) (Winston *et al.* 1996).



**Low food supply and metabolic rate**

In Hornsund fjord, an Arctic fjord located in Svalbard, the sun stays above the horizon all day from 22 April to 15 August and stays beneath the horizon all day from 12 November to 13 February (Weslawski *et al.* 1988). As a result there is a marked seasonality in primary production. Primary production is characterised by a single, well timed maximum in May (Arctic) and January (Antarctic) (Figure 11), and a subsequent marked decrease in the number of cells of algae (Clarke 1988, Weslawski *et al.* 1988). Therefore, food is available for 8 to 10 weeks but is lacking for 9 months of the year for animals relying on phytoplankton. The consequences of a low food supply on the physiology of marine organisms has been a topic of dynamic debate. Many scientists consider low temperature to be the main factor influencing the physiology of polar organisms and not food availability. Krogh (1916) wrote that “one would expect that animals living at very low temperature should show a relatively high standard metabolism at that temperature compared with others living normally at high temperature”. This statement arose from the observation that temperate fish showed little swimming activity and lower respiration when cooled to low temperatures, whereas polar fish are active and, therefore, the hypothesis that polar fish show high metabolic rate at low temperature was put forward. This hypothesis was supported latter by numerous investigations and the concept of metabolic cold adaptation (MCA) was established by Scholander *et al.* (1953) and Wohlschlag (1964) who compared oxygen

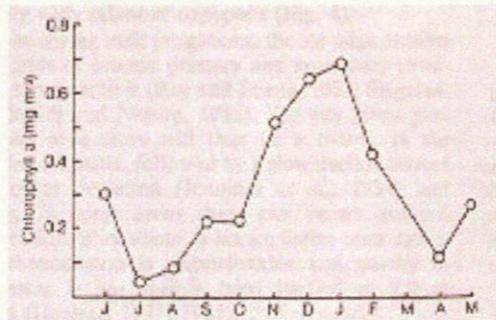


Figure 11.

Annual cycle of standing crop of chlorophyll a in the Southern Ocean (El-Sayed 1984).

consumption in polar and tropical fish. Studies performed in the 1970s on respiration in polar organisms, however, provided evidence for and against the MCA. Recently, Clarke (1980, 1983, 1987, 1991, 1993) summarised convincing arguments



demonstrating that, for polar ectotherms, metabolic rates were not elevated. This conclusion has received recent support from Antarctic bivalves (Davenport 1988, Peck *et al.* 1997, Ahn and Shim 1998, Pörtner *et al.* 1999) and fish (Clarke and Johnston 1999). The low metabolic rate measured in polar ectotherms appears to be an important adaptive strategy for energy conservation which might contribute to increasing the scope for growth and to maintaining high biomass in cold water where food is available in abundance for a very brief period of about 6 weeks and may be in short supply for up to 9 months of the year (Clarke 1991). The low food availability of polar water resulting in a low metabolic rate of the marine species leads to the hypothesis that the natural ROS formation, and the subsequent oxidative damages, can be expected to be low in polar marine invertebrates.

## 2.2. Implications of oxidative stress

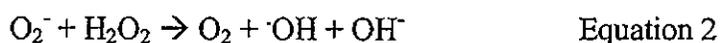
### 2.2.1. Biochemical adaptation to oxidative stress

ROS are potentially harmful products to cell components (Winston and Di Giulio 1991). Thus, in view of this harm, aerobic organisms have evolved a complex array of defences to protect them against ROS toxicity. Oxidative stress arises when antioxidant defences are overwhelmed.

$O_2^-$  can dismutate via equation 1 to  $H_2O_2$  (Figure 10)



Hydrogen peroxide can serve as a precursor of the hydroxyl radical,  $\cdot OH$ , via the Haber-Weiss reaction, equation 2 (Haber and Weiss 1934) (Figure 10).



$O_2^-$ ,  $H_2O_2$  and  $\cdot OH$  are ROS capable of reacting with all kind of cellular components, possibly leading to protein degradation, enzyme inactivation, lipid peroxidation, DNA damage and ultimately cell death. Aerobic cells protect themselves against ROS by the action of adapted enzymes. Superoxide dismutase (SOD) catalyzes the reaction in equation 1. Catalase reduces  $H_2O_2$  according to equation 3 (Figure 10)





Glutathione peroxidases reduce peroxides according to equation 4 where GSH and GSSG are reduced and oxidized glutathione, respectively (Figure 10).



A wide array of non-enzymatic defences also contribute to scavenge ROS. These are fat soluble vitamins  $\alpha$ -tocopherol and  $\beta$ -carotene, as well as several low molecular weight compounds such as glutathione, ascorbic acid.

#### ***Antioxidant defences of polar marine invertebrates***

The oxidative stress of polar waters was shown to induce antioxidant responses in the Antarctic intertidal limpet *Nacella concinna* (Abele *et al.* 1998). Elevated antioxidant defences were measured in the polar scallops, *Adamussium colbecki* and *Chlamys islandicus* (Viarengo *et al.* 1995, Regoli *et al.* 1997, 2000) and in Antarctic fish (Colella *et al.* 2000). UV-B effects on Arctic marine life were investigated in the crustacean *Daphnia magna*. Individuals with melanic pigmented carapace were shown to be tolerant to UV-B exposure and antioxidant defences appeared to play little role in protection (Hessen 1996, Hessen *et al.* 1999, Borgeraas and Hessen 2000). More recently, the total oxyradical scavenging capacity assay provided supportive evidence of high prooxidative pressure of polar environments (Regoli *et al.* 2000).

#### ***Antioxidant defences and pollution monitoring***

Antioxidant defences of marine invertebrates were shown to respond to ROS formed during the metabolism of PAH (Livingstone *et al.* 1990). The sensitivity of these defenses made them ideal candidates for monitoring pollution impacts in the marine environment (Regoli and Principato 1995, Sole *et al.* 1998, Orbea *et al.* 1999, Sole 2000, Khessiba *et al.* 2001). The elevated level of antioxidant defences in polar marine bivalves, as shown by Regoli *et al.* (2000), makes antioxidant biochemical parameters ideal to monitor PAH impact in the polar water. Furthermore, the adaptation of polar organisms to high natural oxidative stress may render them resistant to PAH-originated ROS.

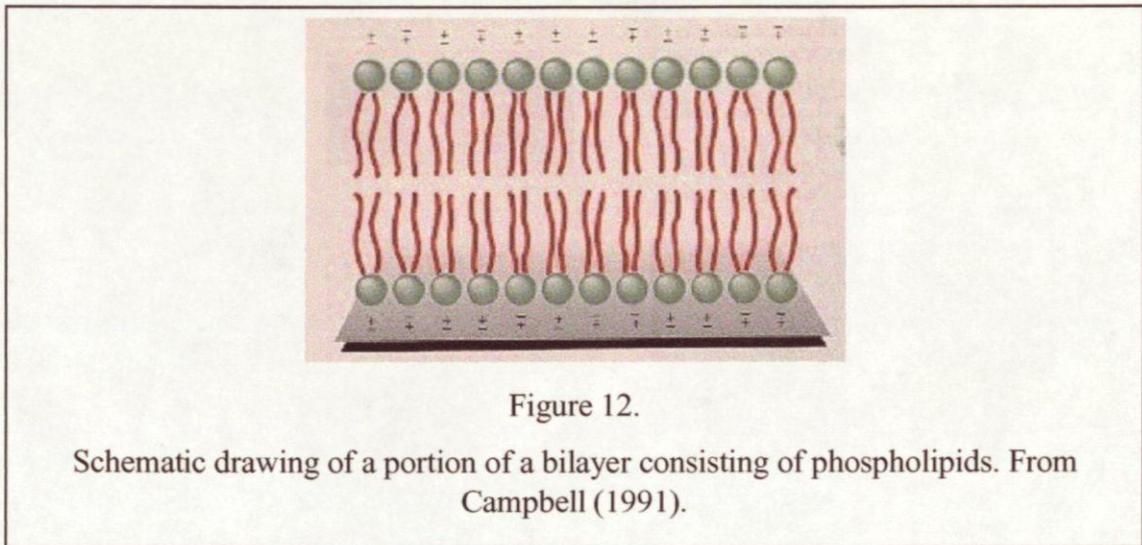
### **2.2.2. Impact of ROS on cold water adapted organisms**

Membrane lipid peroxidation is thought to be one of the main mechanisms by which toxicants exert damage when antioxidant defences are overwhelmed. ROS are known to

be powerful pro-oxidants in the peroxidation of unsaturated fatty acids (Slater 1984). The cell membranes of polar marine ectothermic animals are known to be specifically adapted to the low temperature of the polar water (Clarke 1983). In this section, the adaptation of membranes to low temperature is reviewed and the implications evaluated.

***The role of cellular membranes***

Membrane function is the basis of many vital cell activities. Four main activities were found to be regulated by cell membranes (Hazel 1995). 1) constraint of the free diffusion of solutes and catalyze specific exchange reactions, which together combine to determine the unique composition of both the cellular and subcellular compartment; 2) regulation of the utilization of energy stored in transmembrane ion gradients; 3) provision of an organizing matrix for the assembly of multicomponent metabolic pathways; and 4) governance of the transfer of information between the compartments they separate by possessing ligand-specific receptors. Cell membranes are made of



lipids (Figure 12). Membrane lipids play a role in mediating these functions by acting as: i) physical barriers to electrolyte diffusion, ii) solvents for a variety of membrane constituents, and iii) anchors, activators and conformational stabilizers of membrane proteins (Hazel 1995).

Membranes are composed mainly of phospholipids. The dynamic state of lipids in biological membranes is a central feature of membrane function and structure. Lipids are polymorphic and display phase behaviour and physical behaviors which are sensitive to alterations in the state physical state of the environment, notably, temperature (Campbell 1991).



**The concept of homeoviscous adaptation**

Ectothermic organisms, which either inhabit variable environments or which have adapted to extreme environmental conditions, exploit the chemical diversity amongst membrane lipid constituents so that lipids of appropriate physical properties are matched to prevailing environmental conditions. The active restructuring of membrane

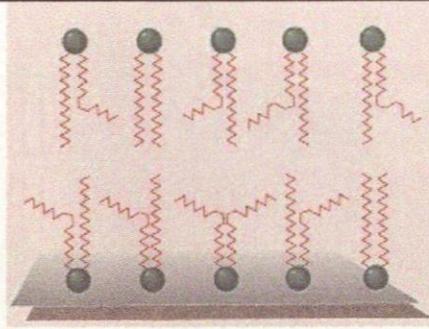


Figure 15.

Schematic drawing of a portion of a highly fluid phospholipid bilayer. The kinks in the unsaturated side prevents close packing of the hydrocarbon portions of the phospholipids. From Campbell (1991).

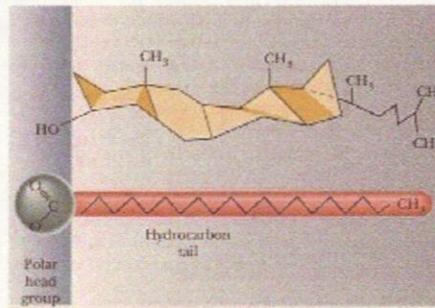


Figure 14.

Stiffening of the lipid bilayer by cholesterol. From Campbell (1991).

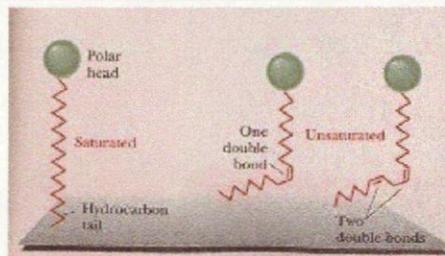


Figure 13.

The effects of double bonds on the conformation of the hydrocarbon tails of fatty acids. Unsaturated fatty acids have links in the tail region. From Campbell (1991)



lipid composition in response to environmental change preserves a suitable dynamic state of the bilayer and restores membrane function following environmental insult (Williams and Somero 1996). Figures 13, 14 and 15 illustrate some lipid adjustments to preserve the suitable membrane dynamic. The defense of a particular physical state of the membrane under variable environmental conditions has been termed homeoviscous adaptation (HVA) (Sinensky 1974). Numerous subsequent studies have indicated that the HVA hypothesis does not reflect adequately the specificity of lipid-protein interactions, the microdomain heterogeneity of biological membranes, or the diversity of membrane attributes that can influence function, and cannot, therefore explain several consistently observed patterns of temperature-dependent changes in membrane lipid composition (for review see Hazel 1995). For the scope of this thesis, however, HVA will be considered only to evaluate the consequences for cell membranes for cold-adapted ectotherms of living in water of very stable temperature below freezing point.

***Effect of low temperature on membrane composition of temperate organisms***

The temperate marine environment is characterized by a high daily and seasonal fluctuation in temperature. Therefore, ectothermic organisms inhabiting these waters are able to counteract temperature change by adjusting the cell membrane composition. *Mytilus californianus* adjust membrane composition on seasonal as well as hourly time scales and do so on the basis of their zonation in the intertidal region (Williams and Somero 1996). Membrane composition was found to be adjusted by temperate bivalve molluscs and crustaceans acclimatised to  $-1^{\circ}\text{C}$  (Pruitt 1990, Cuculescu *et al.* 1995, Gillis and Ballantyne 1999b).

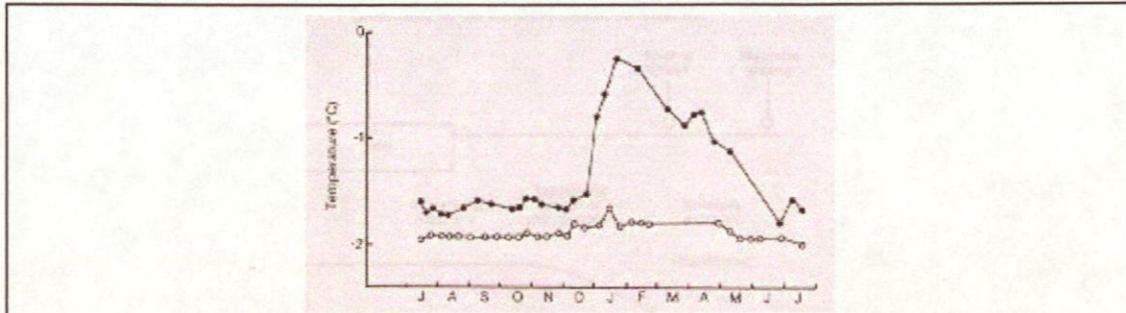
**Membrane composition of polar invertebrates and susceptibility to oxidative stress**

Figure 16.

Annual cycle of temperature at two sites in Antarctica. Open circle are from Mc Murdo Sound 1972/73 (Littlepage 1965). The other line is from Orwell Bight, Signy Island, 1973/1974 (in Clarke 1988).

In Hornsund fjord, Svalbard, seawater temperature is extremely stable from  $-1.88$  to  $3^{\circ}\text{C}$  (Weslawski *et al.* 1988). More stable temperature can be observed, for instance in Antarctica, in McMurdo Sound, the mean annual temperature is  $-1.8^{\circ}\text{C}$  with a standard deviation over the year of only  $0.2^{\circ}\text{C}$  (Littlepage 1965) (Figure 16). One physical consequence of this low temperature is the increased level of dissolved oxygen in the water. Saturated seawater at  $0^{\circ}\text{C}$  contains 1.6 times as much oxygen as it does at  $20^{\circ}\text{C}$ . This elevated oxygen concentration appears to affect the physiology of marine organisms as some ice fish in Antarctica can survive without respiratory pigments and rely instead on the greatly increased solution carrying capacity of the plasma. Polar gigantism of amphipods was shown to be related to oxygen availability (Chapelle and Peck 1999).

Several studies have investigated the membrane composition of cold adapted organisms living in polar water. Polar fish were shown to contain high levels of unsaturated fatty acids (Clarke 1983) but, surprisingly, results from a comparison study between Antarctic and Mediterranean scallops demonstrated that cold adaptation of the membranes from digestive gland cells implicate the regulation of the ratio of short/long and straight/branched fatty acid chains of the cholesterol content of membranes (Viarengo *et al.* 1994). In the Antarctic scallop *Adamussium colbecki* living at low temperature, the membrane fluidity was maintained in a physiological range by a higher cholesterol/phospholipid ratio, a higher amount of short-chain saturated fatty acids and, in particular, by a higher amount of unusual branched saturated fatty acids. Considering the vulnerability of unsaturated fatty acids to lipid peroxidation (Slater 1984), the



occurrence of branched-chain saturated fatty acids can be interpreted as a protective strategy against ROS (Viarengo *et al.* 1995). Similarly, studies performed on two Arctic marine bivalve molluscs, *Serripes groenlandicus* and *Mya truncata*, showed that polyunsaturated fatty acids, known to enhance membrane fluidity, were low (36%) compared with that of temperate bivalves; nevertheless, high levels of monounsaturated fatty acid were measured, monoenes were common and it was suggested they play a role in maintaining membrane function at sub zero temperatures (Gillis and Ballantyne 1999a). In crustaceans, comparison of the fluidity of neuronal and branchial membranes between an Antarctic amphipod and Baltic isopod indicated that there is homeoviscous adaptation of the Antarctic species to cold water (Lahdes *et al.* 1993) but no report to date on cell membrane composition is known.

### **2.3. Oxidative stress as a basis for a biomarker research strategy**

Oxidative stress appears to be a major challenge in polar waters compared to warmer water. The database characterising polar marine invertebrates in terms of adaptation to oxidative stress needs to be expanded to better understand the implications of ROS on the Arctic fauna and the impact of poly aromatic hydrocarbons. Therefore, in the next chapter, a strategy based on oxidative stress for a biomarker research is proposed.

## Chapter 3

### Research strategy



Preparing the Niskin bottle for water sampling under the Ross ice shelf (Antarctica).

*“Clark finds that with returning daylight the diatoms are again appearing. His nets and line are stained a pale yellow, and much of the newly formed ice has also a faint brown or yellow tinge. The diatoms cannot multiply without light, and the ice formed since February can be distinguished in the pressure ridges by its clear blue color. The older masses of ice are of a dark earthy brown, dull yellow, or reddish brown.”*

**Ernest Shackleton, in the Endurance expedition 1914-1916.**

## Chapter 3

### Research Strategy

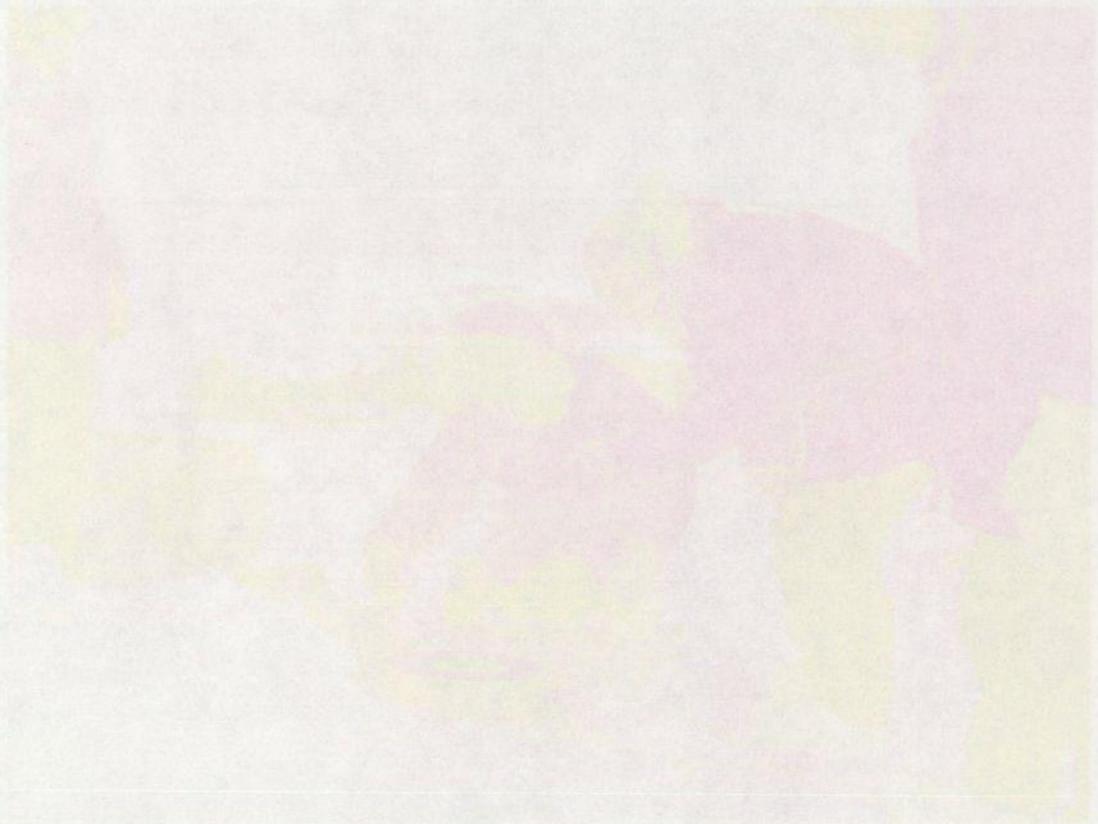


Figure 3.1: A researcher in a white lab coat and mask, possibly a scientist or researcher, working in a laboratory setting.

The first step in the research process is to identify the research problem. This involves a clear and concise statement of the problem to be investigated. The next step is to develop a research design, which includes the selection of the research method, the identification of the variables to be studied, and the determination of the sample and the data collection procedures. The final step is to analyze the data and draw conclusions from the results.

#### Research Objectives in the Laboratory Setting (Table 3.1)

Research Objective	Method	Sample	Data Collection
Identify the research problem	Literature review	Not applicable	Not applicable
Develop a research design	Experimental design	Random selection	Controlled environment
Analyze the data	Statistical analysis	Statistical software	Statistical software



### 2.1. Diagnostic evaluation

Child psychiatrists with the following research interests in the field of developmental psychopathology, including attention deficit hyperactivity disorder (ADHD), autism spectrum disorders (ASDs), and Tourette syndrome (TS), were invited to participate in the 2007 Workshop on the DSM-5 criteria for these conditions. The workshop was held at the University of California, San Diego, and was organized by the University of California, San Diego, Center for Autism and the University of California, San Diego, Center for Time Course Research in Psychopathology. The workshop was held on the campus of the University of California, San Diego, and was organized by the University of California, San Diego, Center for Autism and the University of California, San Diego, Center for Time Course Research in Psychopathology.

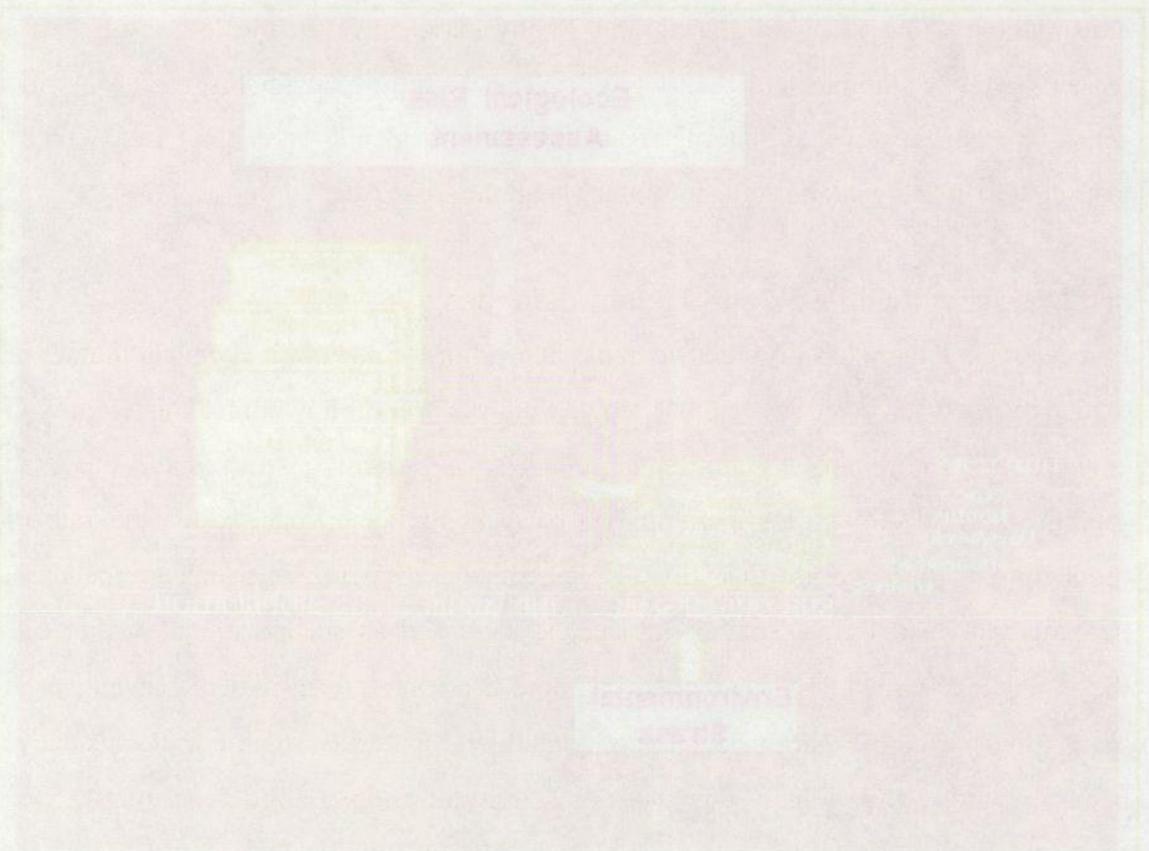


Figure 1

The diagram illustrates the relationship between DSM-5 criteria, developmental course, symptoms, and research.

The diagram illustrates the relationship between DSM-5 criteria, developmental course, symptoms, and research. The top box, 'DSM-5 criteria', is connected by arrows to two intermediate boxes: 'Developmental course' and 'Symptoms'. Both of these intermediate boxes are then connected by arrows to a final box at the bottom, 'Research'. This structure suggests that research is informed by both the developmental course and the symptoms of a condition, which are themselves defined by the DSM-5 criteria.

Therefore, the research strategy of this study was based on selecting biological responses related to oxidative stress and located at three different levels of biological organisation: 1) biochemical level: due to the strong oxidative pressure of the polar marine environment, polar marine invertebrates have developed efficient antioxidant defence systems which could confer them with an advantage to cope with ROS formed following metabolism of PAH; 2) cellular level: marine ectothermic invertebrates possess a specific lipid cellular membrane composition to maintain the fluidity of the membranes in relation to the low temperature, this adaptation may affect the lipid peroxidation of the cell membranes; and 3) physiological level: the low food supply appears to reduce the metabolic activity of polar ectothermic organisms, the respiration could indicate the level of internal ROS production. In addition, heart rate will be measured to investigate the concept of the Metabolic Cold Adaptation.

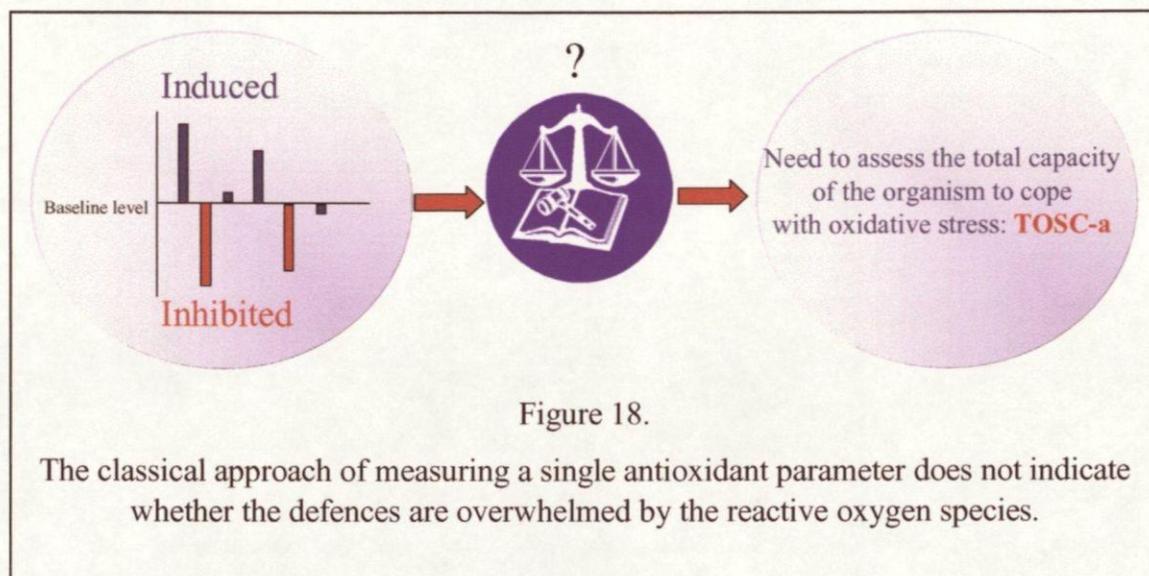
### **3.1.2. Biochemical level: antioxidant defences**

In recent years, there has been considerable interest in the use of biochemical indices of oxidative stress for invertebrates that provide information on how animals interact with environmental pollutants. Traditionally, studies of oxidative stress have analysed specific, single antioxidants, including primary antioxidants that reduce the rate of production of ROS and are specially adapted enzymes (superoxide dismutase, catalase, peroxidases). In addition, smaller molecules, classified as secondary antioxidants as they trap ROS directly, such as vitamin E and  $\beta$ -carotene (free radical scavengers in membranes), ascorbic acid, uric acid and glutathione for water soluble molecules have been measured. Variations in the levels or activity of antioxidant defences have been used largely to indicate reactive oxygen species (ROS) mediated toxicity. Moreover, depending on the availability of nutrients, reproductive status, growth, seasonality and other factors, levels of antioxidant parameters and oxidative stress may fluctuate significantly throughout the year (Sheehan and Power 1999). For instance in *Mytilus edulis*, the total glutathione level is low in winter and high in summer (Power and Sheehan 1996). Although this single antioxidant measurement is useful in understanding specific relationships between stressors and antioxidant parameters, their responses were shown to be very different and it is not unlikely to observe the contemporary depletion of some antioxidants together with the induction of others. For instance, a depletion of an antioxidant system may represent a first response to pollutants which can be followed by an induction of antioxidant systems (Livingstone *et*

*al.* 1992, 1993, Doyotte *et al.* 1997). Using antioxidant parameters as biomarkers to monitor pollution levels and exposure in the field appears possible (Porte *et al.* 1991, Regoli 1998). Numerous studies, however, have revealed the difficulty of interpreting the biological resistance to oxidative stress in quantifiable terms (Livingstone 1991, Porte *et al.* 1991, Regoli and Principato 1995, Sole *et al.* 1996, Doyotte *et al.* 1997, Regoli *et al.* 1997, Regoli 1998). Indeed, the relative contribution of each antioxidant to the total antioxidant capacity of a tissue cannot be elucidated by measuring a few antioxidant parameters and their implications, in terms of quantifiable resistance to oxidative stress, are difficult to evaluate (Power and Sheehan 1999). Toxicologists, working in the field of oxidative stress, have attempted to resolve this problem by developing an assay that could provide information on the total absorbance capacity of oxyradicals by a tissue (Figure 18). Wayner *et al.* (1985) proposed an experimental approach to measure total peroxy radical-trapping ability. The assay was based on the decomposition at 35°C of 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) to yield peroxy radicals at a constant rate in aqueous solution. Once ABAP was added to an aqueous solution of antioxidant (biological tissue) they measured the length of time that oxygen uptake by peroxidizable biological material was inhibited by the antioxidants in the biological material. This time was called the induction period and was measured using an oxygen electrode. By relating the results to the time of induction obtained with a known amount of the water soluble vitamin E analogue (Trolox) a quantitative measure of the total radical-trapping antioxidant parameter (TRAP), could be defined. The inconvenience of the method lies in the oxygen measurement devices. Glazer (1988) described a fluorescence-based method. The intensity of the fluorescence of phycoerythrin is proportional to the degree of damage caused by peroxy radicals. In presence of the antioxidant, the decrease in fluorescence is inhibited. Further development of the method was performed by Cao *et al.* (1993) and Ghiselli *et al.* (1995). Chevion *et al.* (1997) reported another approach based on cyclic voltammetry. Nevertheless, these methods were shown to have limits and the authors did not study the effects of biological tissues which could interfere with the measurements (Winston *et al.* 1998). Therefore, Winston *et al.* (1998) proposed a new, simple and reliable approach called the total oxyradical scavenging capacity assay (TOSC). The principle is based on the reaction between peroxy radicals (or hydroxyl or alkoxy radicals) and  $\alpha$ -keto- $\gamma$ -methiolbutyric acid (KMBA), which is oxidized to ethylene upon reaction with various ROS (eq. 1) ethylene is then measured by gas chromatography.



Winston *et al.* (1998) showed that their method is reliable for antioxidant solutions and biological tissues. It gives linear responses over a wide range of final dilutions of



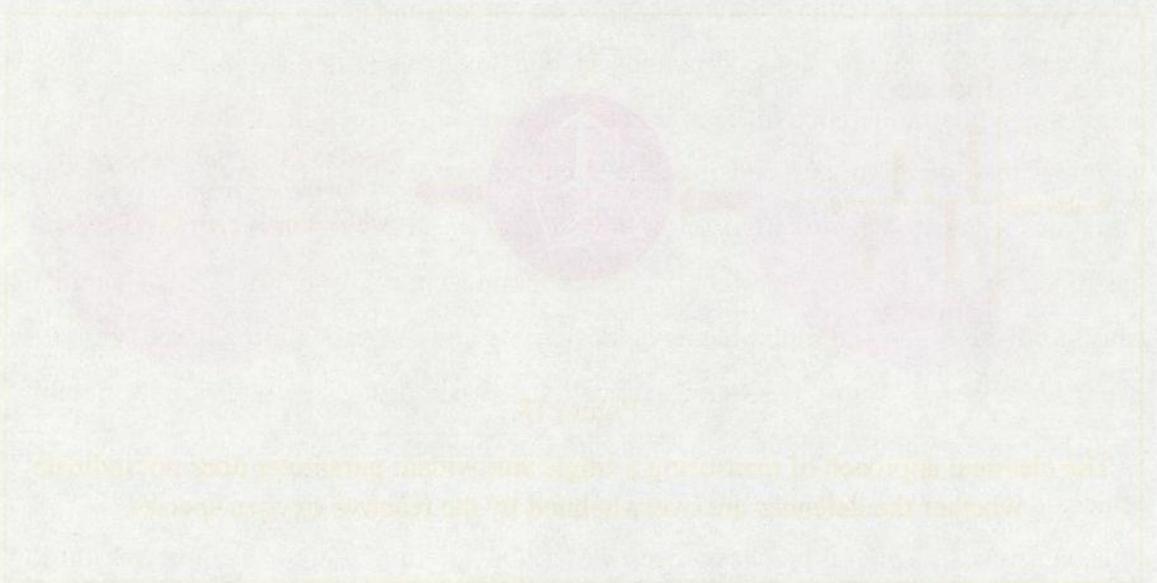
biological fluids. The ease of the analytical procedure, high reproducibility of the results and the ability to analyze 8-10 samples in less than 2 h make this a useful procedure for measurements of the total oxyradical scavenging capacity and its relationship with oxidative stress resistance. The TOSC assay was shown to be a useful biomarker with predictive validity at the organism level in *Mytilus galloprovincialis* exposed to a field heavy metal polluted site (Regoli 2000). TOSC induction was also noted in fish hepatocytes following exposure to oxidative stress (Winzer *et al.* 2001).

### Research questions

In this study, the following research questions are addressed:

1. What is the effect of the zero thermal acclimation of a temperate bivalve, *Mytilus edulis*, on the TOSC?
2. What is the TOSC characteristic of polar bivalves compared with temperate bivalves?
3. Can TOSC be used as biomarker of PAH exposure?

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### 3.1.3. Cellular level: stability of membranes

The potential of immune parameters as determinants of environmental contaminant impact has been an important focus in ecotoxicological studies. For the past 20 years, there has been a growing interest in immunocompetence in marine invertebrate populations. Invertebrates do not possess an inducible defence mechanism with the high degree of specificity and memory as found in vertebrate immune system (Marchalonis and Schluter 1990). Nevertheless, invertebrate blood cells (haemocytes) are particularly important as the immune system defends on organism against potential pathogens, parasites and various foreign compounds such as toxicants (Adema *et al.* 1991). Earlier studies of immune defence in invertebrates showed similarities in strategies to respond to injury and infection, and considerable attention has been placed on the structure and function of haemocytes of *Mytilus edulis*. Pipe *et al.* (1997) identified two classes of haemocytes in *M. edulis*: eosinophilic cells (agranular) and basophilic cells (granular). Basophilic cells showed high phagocytotic activity and oxyradical production. Granules were identified as lysosomes, small subcellular organelles. Haemocytes and, notably, lysosomes contain various enzymes (phosphatases, esterases, proteases and glucosidases) involved in cytotoxic mechanisms (Carballal *et al.* 1997). Two mechanisms for cell killing have been described for invertebrate haemocytes during phagocytosis: the release of lysosomal enzymes and other lysins, and the respiratory burst which involves the release of reactive oxygen species (Pipe 1992).

Laboratory studies indicate that basic immune functions are affected adversely by contaminant exposure (Coles *et al.* 1994) and similar observations were made in the field (Dyrynda *et al.* 1998). Organisms exposed to pollutants showed enhanced superoxide production and reduced activity of degradative enzymes, effects which may result in tissue damage or have implications for the efficient destruction of invading organisms. Concurrent with the studies performed on the immunocompetence of invertebrates, focus shifted to the lysosomes of haemocytes. In their role as components of the immune system, haemocyte lysosomes release acid hydrolases which degrade circulating pathogens and are also important organelles in the nutrition, tissue repair and turnover of cellular components (Cheung *et al.* 1998). Lysosomes are sites for ROS generation (Winston *et al.* 1996). Lysosomes have remarkable ability to sequester and accumulate a diverse range of toxic metals and organic chemicals (Moore 1990). However, concentrating such contaminants results in enhanced toxicity and cell injury



via damage to the lysosomes caused by lipid membrane peroxidation (Viarengo *et al.* 1989, Winston *et al.* 1996), impairment of the H<sup>+</sup> ion pump resulting in leakage of their acid hydrolases into the cytosol causing damages to the cell components (DNA, proteins, membranes) and eventually cell death. Moore and Willows (1998) developed further understanding of the role of bivalve lysosomes in marine pollution. They suggested that lysosomes were preadapted to stress and that animals with highly developed cellular lysosomal systems (bivalves, crustaceans) were more tolerant of pollutants than vertebrates. They highlighted the role of lysosomes as an intermediate, essential step in a detoxication process enhancing degradation and excretion of contaminants. They emphasised the ROS production of lysosomes that may contribute to lipid peroxidation and then production of lipofuscin that provides binding site for free micropollutants thereby, enhancing detoxication and protection (Moore and Willows 1998).

Cytochemical latency of lysosomal hydrolases was demonstrated in the digestive cells of the common mussel, *Mytilus edulis*, exposed to trace metals (Lowe and Moore 1979, Regoli 1992). The method published by Moore (1976) was based on measuring the activities of the lysosomal enzymes  $\beta$ -glucuronidase and glucosaminidase by a staining technique. Sections of the digestive gland were incubated in a medium containing naphthol AS-BI glucuronide and naphthol AS-BI glucosaminide as substrates, and fast red violet LB. The determination of lysosomal latency by cytochemical means provides a rapid and sensitive method to study the effects of stress at the cellular level.

Borenfreund and Puerner (1985) established the neutral red assay (NR) to measure the cytotoxicity of chemicals. The NR assay is based on the uptake of neutral red, a dye, and its accumulation in the lysosomes of viable uninjured cells. The cells were incubated with the dye for a certain amount of time. Then the cells were washed to extract the dye that was then measured with a microplate reader. The measured absorbance was linearly correlated with the number of surviving cells. The chemical mechanism is based on neutral red being a cationic dye that, as a weak base, accumulates within lysosomes where pH is low. The cellular uptake of neutral red is biphasic, with an initial rapid phase across the plasma membrane followed by a slower phase corresponding to accumulation in the lysosomes (Seglen 1983). This process involves both physical and facilitated diffusion, and results in the protonated form of the compound becoming trapped within the lysosomal compartment.

The method of Borenfreund and Puerner (1985) has the objective to investigate visually with a microscope the *in vivo* uptake and fate of neutral red in the cells (Lowe *et al.* 1992). Their assumptions were that if the lysosomally-accumulated dye could be visualised with a microscope, then the leakage back into the cytosol could be monitored and quantified using time as the determinant of effect (Lowe *et al.* 1992). The assay was performed with fish hepatocytes (Lowe *et al.* 1992) and mussel digestive gland (Lowe and Pipe 1994, Lowe *et al.* 1995). This assay, however, required sacrificing the animals, isolating the digestive glands cells by enzymic digestion of tissue, thereby, removing the opportunity of follow-up studies to investigate other parameters. Lowe *et al.* (1995) proposed investigating the viability of the neutral red method with haemocytes. These blood cells are easy to obtain, it is a non-destructive sampling method, and haemocytes are easy to maintain in physiological saline. Moreover, haemocytes are key cells in immunocompetence and one of their specific roles is to accumulate and degrade foreign compounds. Numerous subsequent studies have reported the effective application of the neutral red method to investigate the effects of contaminants on marine bivalves (Lowe *et al.* 1995, Grundy *et al.* 1996, Cheung *et al.* 1998, Wedderburn *et al.* 1998, Fernley *et al.* 2000, Lowe and Fossato 2000, Shepard and Bradley 2000).

The neutral red assay requires a well-trained scientist to interpret the cell morphology and color after it has been incubated with neutral red. One possible consequence is that the assay relies on subjective readings. Moreover, the neutral red assay is more difficult to perform on genera other than *Mytilus sp.* (Camus personal observation). Even though haemocyte function and structure between marine invertebrates species are not so diverse, differences do occur (Hine 1999). For instance, the morphology of haemocytes is different between genera. For instance, the small size of haemocytes of the Arctic scallop (*Chlamys islandicus*) renders the reading of the NRR difficult and too subjective. Shepard and Bradley (2000) used video image analysis with computer software to develop a quantitative lysosomal stability ratio based on the area of haemolymph cell lysosomes as index of lysosomal dysfunction. Although the method helped to measure lysosomal pathology, it required sophisticated equipment such as a microscope, camera, a computer and special software for image analysis. Grøsvik *et al.* (in prep) proposed an alternative assay where readings could be automated with a plate reader to avoid inconveniences caused by subjective reading. The latter assay is based on the hypothesis that under contaminant exposure, the lipid membranes (plasma and

nucleus membranes) of the cell (haemocytes or digestive gland) of bivalves may become destabilised due to lipid peroxidation, release of hydrolases from the lysosomes and lipid soluble xenobiotics that accumulate in the lipid membrane. A fluorescent probe, ethidium homodimer-1, crosses the membranes when they are unstable, and sticks to the DNA where it fluoresces red. The fluorescence can be monitored with a fluoroplate reader. The assay appeared to be correlated with NRR assay (Camus *et al.* 2000). In this study, the validation of the use of that method on several invertebrate species as a biomarker of pollution at the cellular level was performed.

### Research questions

In this study the following questions were addressed:

1. What are the consequences of sub-zero thermal acclimation on the stability of cellular membranes of the temperate bivalve, *Mytilus edulis*?
2. Can the plate reader and fluorescent probes method be used as biomarker of PAH exposure?
3. Can the stability of cellular membranes be used as biomarker of PAH exposure and effects in polar marine invertebrates?

#### 3.1.4. Physiological level: respiration and heart rates

##### *Respiration*

Respiration, measured as oxygen uptake, is considered a good indirect measure of the metabolic rate of animals which corresponds to their ATP demand. Nevertheless, numerous factors affect the measurement of oxygen uptake as pointed out by Clarke (1983). Reproduction, growth, feeding, and activity are all physiological states characterised by different rates of oxygen demand. If true basal metabolism is to be measured, other activities must be eliminated. Locomotory activity or feeding can be reduced by different techniques, however, it is impossible to reduce growth or gametogenesis. Therefore, a measure of true basal metabolism appears difficult. A reasonable approximation is then a measure of basal metabolism together with the instantaneous contribution from growth or gametogenesis taking place during the experiment (Clarke 1983). This is referred to as resting, standard or routine metabolic rate. A consequence of normal utilization of oxygen is the production of reactive

oxygen species. About 2 to 3% of the oxygen consumed by aerobic cells is converted to ROS (Chance *et al.* 1979) and low tissue oxygen consumption will decrease ROS production. Hence, polar invertebrates may be less threatened by oxidative stress due to their low respiration rate (Abele *et al.* 2001).

Respiration has been employed extensively in toxicity studies as health index and is a major parameter in scope for growth studies (Widdows *et al.* 1995). Copper decreased the respiration rate in *Mytilus edulis* (Scott and Major 1972) and decreased the scope for growth (Sanders *et al.* 1991). Zinc did not affect respiration in *Gammarus pulex* (Maltby and Naylor 1990) but the scope for growth was affected. No change in oxygen uptake was noted in *Mytilus edulis* and *Venus verrucosa* exposed to oil (Axiak and George 1987, Widdows *et al.* 1987), but the scope for growth was impaired due to lower feeding rate and food absorption efficiency. Exposure of *Mytilus edulis* to tributyltin increased the respiration rate (Widdows and Page 1993). *Neomysis integer* (mysid) showed elevated oxygen uptake following exposure to pesticides (Roast *et al.* 1999).

Few studies of the respiration rate of Arctic marine invertebrates have been performed and most of the work to evaluate the validity of the MCA hypothesis originated from Antarctic species. Hence, the first requirement of this study was to establish the metabolic rate of Arctic invertebrates to confirm or refute the MCA concept.

Numerous toxicity studies have reported that respiration, in water breathing animals that use gills for gas exchange, is a good indicator of contaminant uptake notably in bivalves (Baumard *et al.* 1998b, Baussant *et al.* 2001). Therefore, a low respiration rate can confer an advantage to the polar organism by reducing the uptake and subsequent bioaccumulation of pollutants.

Finally, a low metabolic rate could indicate that polar invertebrates may have a reduced capacity to metabolise PAHs, thereby, limiting the production of ROS and cellular damages such as lipid peroxidation.

#### **Heart rate**

In many areas of biological science, it is important to assess quickly and accurately the physiological state of animals. One of these measurements is often O<sub>2</sub> consumption. Although a good indicator of physiological state, oxygen uptake is not easy to assess

accurately and the diversity of methods makes data comparison difficult. Heart rate, on the other hand, is easy to measure, non-invasive, impacts little on the animals and offers the potential for measurement in the field. The cardiovascular system of an animal must provide: i) adequate perfusion of all tissues for the purposes of supplying O<sub>2</sub>, nutrients (amino acid, lipids, glucose), hormones and removal of wastes (urea, CO<sub>2</sub>); and ii) its performance must be changeable to meet the needs of its "host" as those needs change from time to time. In crustaceans, the cardiovascular system consists of three components, the haemolymph, a heart that generates the force to move the haemolymph, and the distribution circulatory system. Crustaceans possess an open circulatory system, with haemolymph returning to the heart via a series of sinuses. Therefore, measuring heart rate provides an estimate of numerous related physiological variables that are affected by intrinsic or extrinsic factors. Temperature is the main factor accounting for heart rate fluctuation *in situ* and in laboratory with a positive relationship (Ahsanullah and Newell 1971, DeFur and Mangum 1979, Wernick 1982, Eshky *et al.* 1996, Polhill and Dimock Jr 1996, Stillman and Somero 1996, Bojsen *et al.* 1998, Styrihave *et al.* 1999). Circatidal and circadian rhythmicity, and seasonal and daily variation in heart rate has been reported (Depledge 1992, Styrihave *et al.* 1999). Also, depth, light and salinity affect the cardiac activity in *Carcinus maenas* (Bojsen *et al.* 1998, Styrihave *et al.* 1999), as do aerial exposure (Ahsanullah and Newell 1971, Stillman and Somero 1996), and hypoxia (Taylor 1976, Paterson and Thorne 1995, Reiber and McMahon 1998, Harper and Reiber 1999). Increasing magnesium concentration of haemolymph leads to a decrease in heart rate by acting as an anaesthetic (Walters and Uglow 1981). Bradycardia was reported at reduced salinity in limpets (DePirro *et al.* 1999). Potential predators (fishes) are well able to detect minute electrical disturbance in their environment and would be able to detect a buried or hidden crab from the regular electrical pulses associated with the heart. Hence, brief simultaneous cessation of heart beat is associated with predator-like stimuli. It was also observed that heart rate was lower when measured *in situ* compared with measurements performed in the laboratory (Styrihave *et al.* 1999). It was shown that heart rate increases at commencement of locomotor activity (Aagaard *et al.* 1995, DeWachter and McMahon 1996, Styrihave *et al.* 1999). Cardiac frequency decreases with increasing size; female crustaceans show lower heart rate than males and intermoult phases also affect heart rate; decreasing cardiac activity was observed simultaneously with decreasing Darwinian fitness parameter and nutritional state (Ahsanullah and Newell

1971, Depledge 1985, Depledge and Lundebye 1996, Aagaard 1996). The temporary reverse of the direction of the scaphognathite beat (causing back flush of water over the gills to clean them) is often accompanied by a simultaneous inhibition of heart beat which prevents perfusion of the gill lamellae during the period in which deoxygenated water is backflushing the gills (McMahon 1999). Longer term rhythms involving heart and ventilatory pumping are also observed in lobster and crab. For instance, in the crab *Cancer productus*, periods of acardia lasting from seconds to 10-20 min occur commonly in quiescent but fettered animals in well aerated water (McMahon and Wilkens 1977). Heart rate may also cease during the tail flip or escape response in lobster (crayfish as well). This response could protect the heart from damage due to the large pulse of haemolymph returning from the abdomen (McMahon 1999).

#### **Heart rate as a biomarker of contaminants**

Contaminants affect heart rate and, therefore, heart rate offers the possibility of an early warning signal of detrimental effects of pollutants at the physiological level. Exposure to  $3\text{mg l}^{-1}$  copper ions induced continuous uninterrupted heart beat in quiescent *Carcinus maenas* (Depledge 1984). Copper ions of  $10\text{ mg l}^{-1}$  suppressed cardiac activity and oxygen consumption within 2 h, and alternating bradycardia and tachycardia were observed in *Carcinus maenas* (Depledge 1984). Heart rate of *Carcinus maenas* increased following exposure to copper and sodium arsenite (Bamber and Depledge 1997), while no effect was measured after oral exposure to benzo(a)pyrene (Bamber and Depledge 1997). A 15 to 20% decrease in median heart rate along a pollution gradient of an estuary and increased variability in response at the most contaminated sites was reported in *Hemigrapsus edwardsi* (Depledge and Lundebye 1996). Exposure to copper ions of *Carcinus maenas* decreased the mean interpulse duration reflecting an increase in heart rate, whereas maximum interpulse durations and coefficients of variation increased with increasing exposure concentration (Lundebye and Depledge 1998). Exposure to mercury ( $0.3\text{ mg l}^{-1}$ ) resulted in rapid and statistically significant increases in heart rate of *Gaetice depressus*, whereas exposure to a concentration of  $0.1\text{ mg l}^{-1}$  resulted in progressive reduction in heart rate similar to that of control crabs (Aagaard *et al.* 2000). Heart rate decreased significantly in a concentration-dependent manner following exposure to organophosphorous pesticide (Lundebye *et al.* 1997). Cardiac activity increased in *Carcinus maenas* exposed to crude oil and dispersant (Depledge 1984).

### ***Heart rate of polar species***

Numerous studies have been performed to investigate the heart rate of temperate marine invertebrates, notably bivalves molluscs and crustaceans, however, no data are available with polar crustaceans. Cardiac activity has been studied in Antarctic fish (Axelsson *et al.* 1992, Macdonald 1997), revealing that the cardiovascular adaptations that allow ectothermic animals to live in cold water are of particular interest to this study. For example, without any compensating mechanism, the hearts of cold-blooded animals might be expected to perform proportionately more work, since blood, in common with other fluids has an increased viscosity at low temperatures (Graham and Fletcher 1985). Studies on cardiac activity and morphology of the heart of 4 Antarctic fish species compared with temperate species revealed a temperature compensation (Axelsson *et al.* 1992, Macdonald 1997).

### ***A rapid, easy, non-invasive, non-destructive method***

Early attempts at cardiovascular monitoring in crustaceans were based on the use of electrodes that were implanted in the animal following drilling of a hole through the carapace. This invasive technique was rather destructive as the animal could be monitored only for a few hours (risk of infection and osmosis problems) and caused stress. It also lacked flexibility to monitor, for instance, the effect of locomotion on heart rate as the set up was hampered by the need to restrain or confine the animal to ensure the electrodes remained *in situ*. Also, numerous sources of interferences could interfere with the signal. Depledge and Andersen (1990) eliminated these problems by developing a non-invasive transducer which recorded heart rate using an optical, rather than an electrical, approach. In this new system, a transducer, glued on the carapace of the crab, emits infra red light which is reflected on the heart in proportion to its volume. The reflected light is detected by the same transducer, amplified and converted to a digital signal that is then displayed as a heart rate trace on a computer and recorded. The method is non-invasive, non destructive and does not stress the animal. Gluing the transducer requires only one or two minutes of handling. Other parameters can, therefore, be measured on the same individuals.

### **Research questions**

In this study, the following questions are addressed:

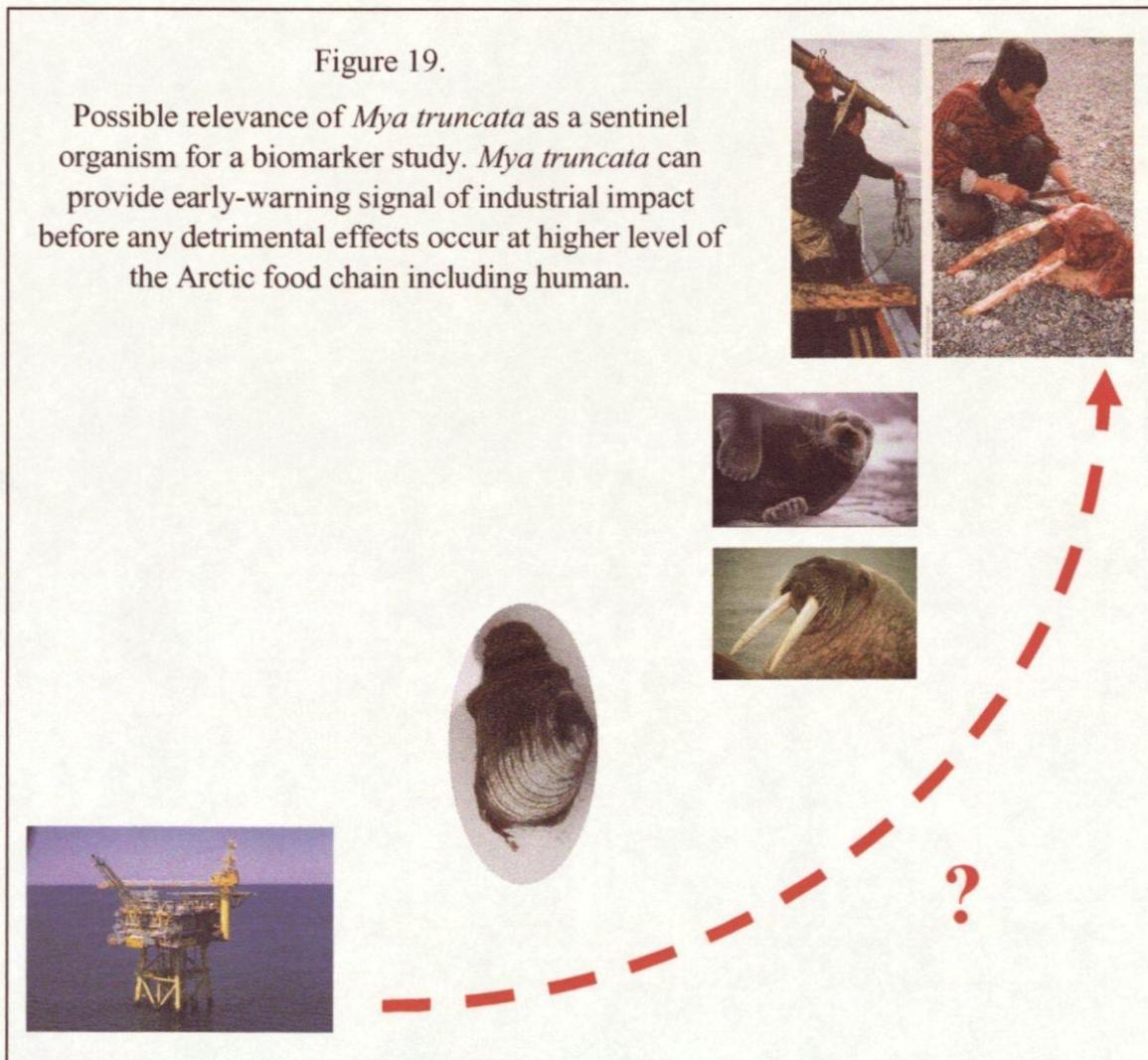
1. Is the heart rate of Arctic marine invertebrates low compared with temperate species?
2. Polar regions are characterised by low stable temperature, therefore, is heart rate of polar crustaceans sensitive to temperature change?
3. Does heart rate respond the same way to temperature change as oxygen uptake?
4. Can heart rate provide information to support the Metabolic Cold Adaptation hypothesis?
5. Can heart and respiration rates of polar crustaceans be used as a biomarker of organic pollution?

### **3.2. Species selection**

To date, most biomarker studies in the Arctic have concentrated on the pollution impact on the top predators (polar bears, seals, whales) (Bacon *et al.* 1992, Barron *et al.* 1995, Bandiera *et al.* 1997), including humans (Dewailly *et al.* 1992). To get early-warning signals before animals located at the higher levels of the food chain are impacted, requires research on lower levels of the food chain by selecting species that can be used as sentinel organisms. Ectothermal organisms, and essentially invertebrates, appear to be the best candidates for such a biomarker study for the following reasons:

1. They can be found virtually everywhere in the marine environment.
2. Many are sessile, therefore, biomarker responses will give an integrated response of pollutant exposure at a specific site.
3. They are relatively easy to handle both in the field and in the laboratory, and easy to sample and keep alive.
4. Many studies have confirmed that they possess compensatory responses to pollutants.
5. Many are low in the food chain and, therefore, provide early-warning signals of pollutant impacts before higher levels in the food web are impacted.
6. Some are economically important.

Four Arctic marine invertebrate species were selected in this study, two crustaceans and two bivalves. The bivalves were the Arctic soft shell clam, *Mya truncata*, and the Arctic scallop *Chlamys islandicus*. The crustaceans were the shrimp *Sclerocrangon boreas* and the spider crab *Hyas araneus*. The bivalves are filter feeders and are more likely to accumulate contaminants present in the water column, while the crustaceans are more in contact with the sediment and may ingest contaminated food. *Mya truncata* was selected as it is the main food source of seals and walrus which are the main nutritional basis of the indigenous people of the Arctic. Therefore, contaminants accumulated by *M. truncata* can be transferred rapidly to humans (Figure 19). The scallop and the shrimp were selected not only because of their commercial values but they are abundant, easy to sample, large enough to work with and resistant to the sampling treatment and transport.





### **3.3. Administration of pollutants in toxicological studies: advantages and disadvantages**

The main objective of laboratory toxicological studies is to investigate whether there is a causative link between the dose of the toxicant and the effects measured in an organism by reducing the number of variables (i.e. stable temperature, same feeding status etc.). Therefore, the method of exposure of the toxicant is critical. Any exposure regime needs to be ecologically relevant in terms of dose and administration route but also these needs to be clear biological responses that will help to answer the research hypothesis. Therefore, investigators often use high doses that may not be environmental realistic. Also, the route of exposure may not be ecologically relevant to ascertain that the animal has really been exposed to the pollutant (i.e. injection versus oral intake). Dealing with toxicants requires good laboratory practice and to strictly follow safety rules. For instance, benzo(a)pyrene is a highly potent carcinogenic compound. Thus, the administration of such chemical substance should be done in such a way that all kinds of contamination are strictly minimised. In such a case, the injection of the toxicant directly in the organism is probably the best practical option.

Toxicants have chemical and physical properties that affect their bioavailability. Oil compounds are poor water soluble molecules at low temperature. It is thus difficult to investigate their effect on marine fauna in the laboratory. To perform direct exposure procedures, the oil compound must be dissolved in a solvent. Ideally, the solvent should be inert and have no effects. For instance, benzo(a)pyrene is diluted in cod liver oil (Aas *et al.* 1998) and the mixture is injected directly into the organism. When studying the effect of dissolved oil compound acetone can be used as a carrier. An acetone control is then required to evaluate the biological effect of the solvent.

A more ecologically relevant procedure is to artificially design a polluted environment in the laboratory (i.e. contaminated sediment or contaminated food). Only the most water soluble oil component will be released from the sediment into the water column. Also, sediment on the field is inhabited with infauna such as worms, urchins etc. that may influence the bioavailability (i.e. bioturbation, metabolism, bio-activation). Ingestion of contaminated food is also more realistic but provides poor control on the real ingested dose (Bamber and Depledge 1997).

Since oil is a hydrophobic compound, it is mainly dispersed (oil droplets) when released at sea with discharge water originating from offshore platform during oil production.

Thus, it is probably ecologically relevant to investigate the effects of dispersed oil in the water column (Baussant *et al.* 2001). Even though, numerous parameters may affect bioavailability *in situ* (Skadsheim *et al.* 2000).

The ultimate objective of a biomarker is to validate laboratory observations with *in situ* studies on a potentially contaminated site. This requires access to a relatively polluted site. This appeared difficult in the European Arctic that is considered to be a pristine region. An alternative approach is to contaminate on a micro scale a part of the ecosystem where the organism lives but under good control.

### 3.4. Objectives

#### 3.4.1. Ecophysiology of polar marine invertebrates

One drawback of investigating biomarker responses in polar regions is the crucial lack of fundamental knowledge of the biochemistry, cellular biology, physiology and ecology of the selected organisms. Measuring a biomarker relies on measuring a biological response and it is essential to know the general variability of the response, for instance, the seasonal variation of the selected parameter. Polar ecosystems are highly seasonal in primary production, have low and stable seawater temperature, and are potentially highly prooxidant. These ecological features may influence the response of the selected biomarkers and these aspects need to be investigated. The ecophysiology of polar marine invertebrates will be investigated in this thesis. Comparison between polar and temperate species was carried out to characterise the biology of polar organisms. The following four chapters report aspects of the ecophysiology of polar organisms such as:

1. The influence of subzero acclimation of the temperate bivalve *Mytilus edulis* on the lysosomal and cell membranes stability, TOSC and PAH uptake.
2. The effect of acute temperature change on the heart and respiration rates of polar crustaceans.
3. Heart rate of polar crustaceans and the Metabolic Cold Adaptation.
4. Seasonal variation of the TOSC and metabolic rate of the Arctic clam, *Mya truncata* and comparison with Antarctic and temperate species.

### 3.4.2. Exposure studies

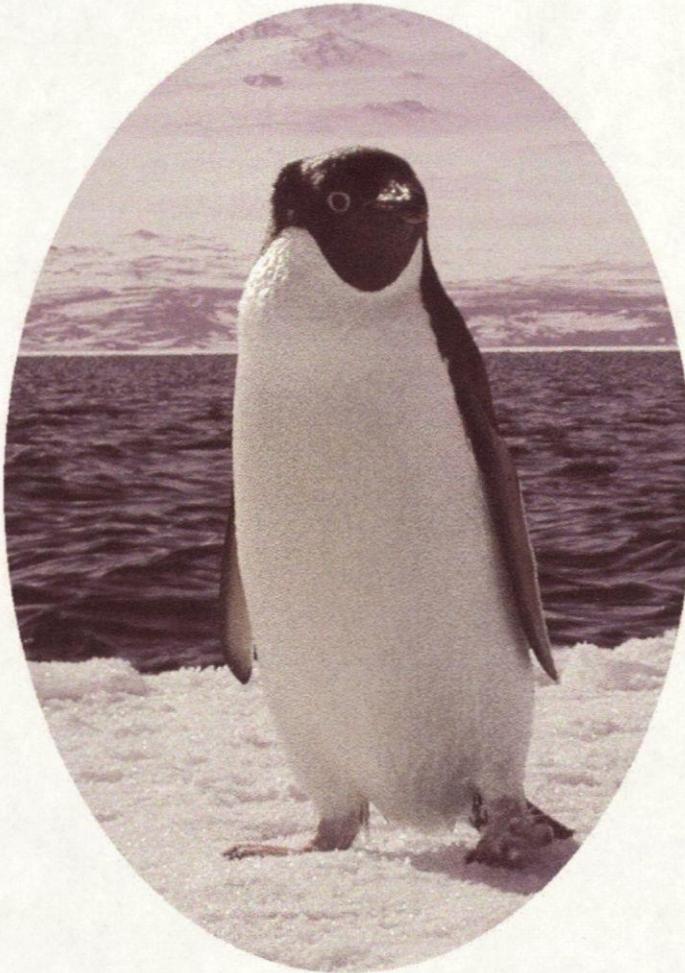
Four laboratory experiments were performed with different exposure systems and different species to evaluate the responses of the selected biomarkers in several exposure situations to PAH. The data are reported and discussed in the next 4 chapters.

1. The first experiment aimed at measuring the effect of a single carcinogenic PAH to establish evidence of ROS production following metabolism of the compound and two biomarker responses (TOSC and cell membranes stability).
2. The second one investigated the effect of PAH adsorbed onto sediment on a benthic species, the crab *Hyas araneus*; heart rate, respiration and TOSC were measured.
3. A field experiment was performed with the soft shell clam *Mya truncata*, by setting up a small-scale oil contaminated site.
4. The responses of TOSC, GST activity and cell membrane stability in *Sclerocrangon boreas* exposed to dispersed crude oil were measured using a continuous flow system. This experiment was undertaken under the responsibility of Dr. Grøsvik, the early draft manuscript is presented in the "published and submitted" paper section.



## Chapter 4

### Materials and Methods

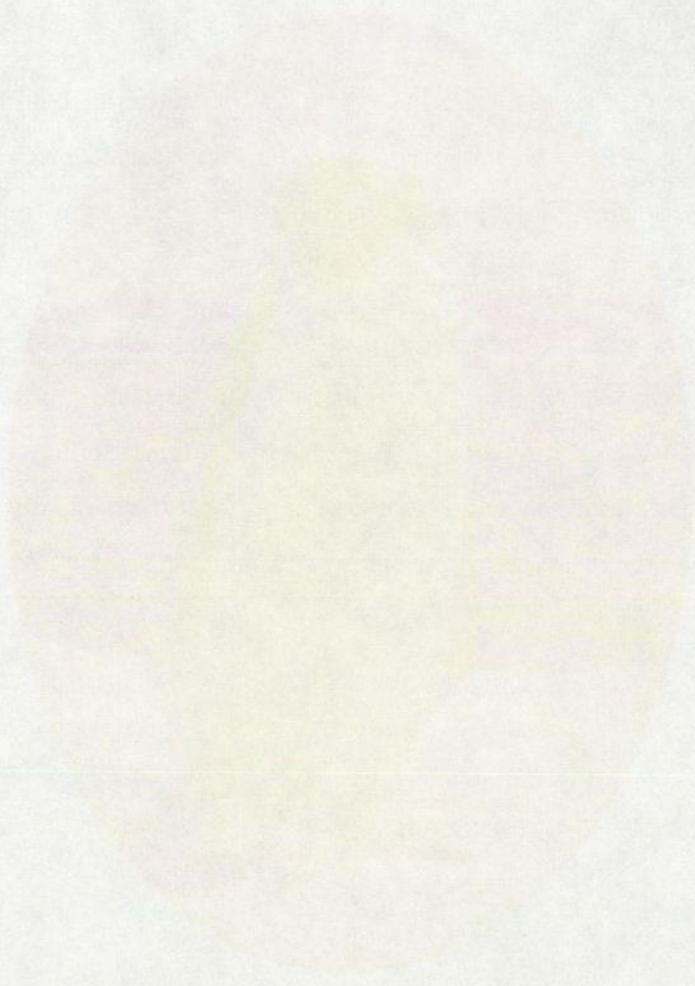


*“The Adelie is always comical. He pops out of the water with startling suddenness, like a jack in the box, alights on his feet, gives his tail a shake, and toddles off about his business. He always knows where he wants to go, and what he wants to do, and isn’t easily turned aside from his purpose.”*

**James Murray, Biologist of the Antarctic expedition of Ernest H. Shkelton in 1907-1909.**

Volume 40

Metabolism and Molecular Biology



The study of metabolism and molecular biology has been a central theme in the history of biology. This volume explores the evolution of these fields, from the early days of biochemistry to the modern era of molecular genetics and systems biology. It covers the discovery of the structure of DNA, the development of the genetic code, and the elucidation of the central dogma of molecular biology. The volume also discusses the role of metabolism in cellular processes and the evolution of metabolic pathways.

James Watson, Francis Crick, and Maurice Wilkins: The Discovery of the Structure of DNA

1953-1962

#### 4.1. Sampling

Four cruises were performed in the Arctic Ocean with the research vessel RV Jan Mayen (Plate 1) of the University of Tromsø for sampling Arctic marine organisms in the fjords of the West Coast of Svalbard. Four field expeditions took place in May and August 1999 and 2000. During these cruises, several techniques for sampling were used:

- Dredging with an Agassiz dredge (Plate 2) for sampling benthic fauna living on rocky bottom (*Hyas araneus*, *Sclerocrangon boreas*, *Chlamys islandicus*). The method is non-selective and rather destructive though it allows collection of a large amount of biological material in a very short time.
- Diving: this is a selective sampling method but not very efficient (Plate 3). It was used to sample *Mya truncata*, a member of a soft bottom fauna. *Mya truncata* was visually spotted and dug out by hand. Due to the difficulty to digging out large animals, only individuals of a size of less than 5 cm were usually sampled.



Plate 1.

Jan Mayen lying in the ice pack at 82° North

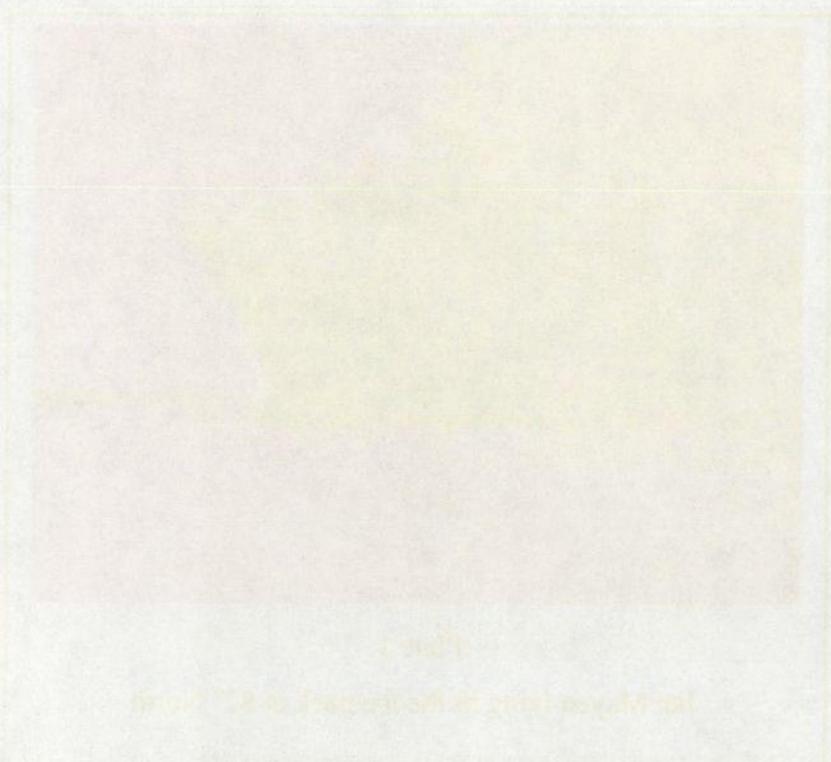
In Antarctica, due to the presence of fast ice, sampling could only be performed by divers. *Laternula elliptica*, *Glyptonotus antarcticus* were hand picked using this method of sampling.

### 4.1. Discussion

Four rivers were included in the study. Three of the rivers were in the same drainage basin (the Hudson River drainage basin) and the fourth was in a different drainage basin (the Delaware River drainage basin). The study was conducted in 1991 and 1992. The study was conducted in the same way as the study conducted in 1989 and 1990. The study was conducted in the same way as the study conducted in 1989 and 1990.

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Plate 2.

Agassiz dredge taken back into the ship after dredging the sea bottom at ca. 50 m depth at Hollenderbukta, Isfjorden (Svalbard). *Hyas araneus*, *Sclerocrangon boreas*, and *Chlamys islandicus* were sampled with this dredge.



Plate 3.

Divers getting ready to dive under the ice in Mc Murdo sound (Antarctica). *Laternula elliptica*, *Glyptonotus antarcticus* and *Mya truncata* (Arctic) were hand picked by scuba divers.



Figure 1

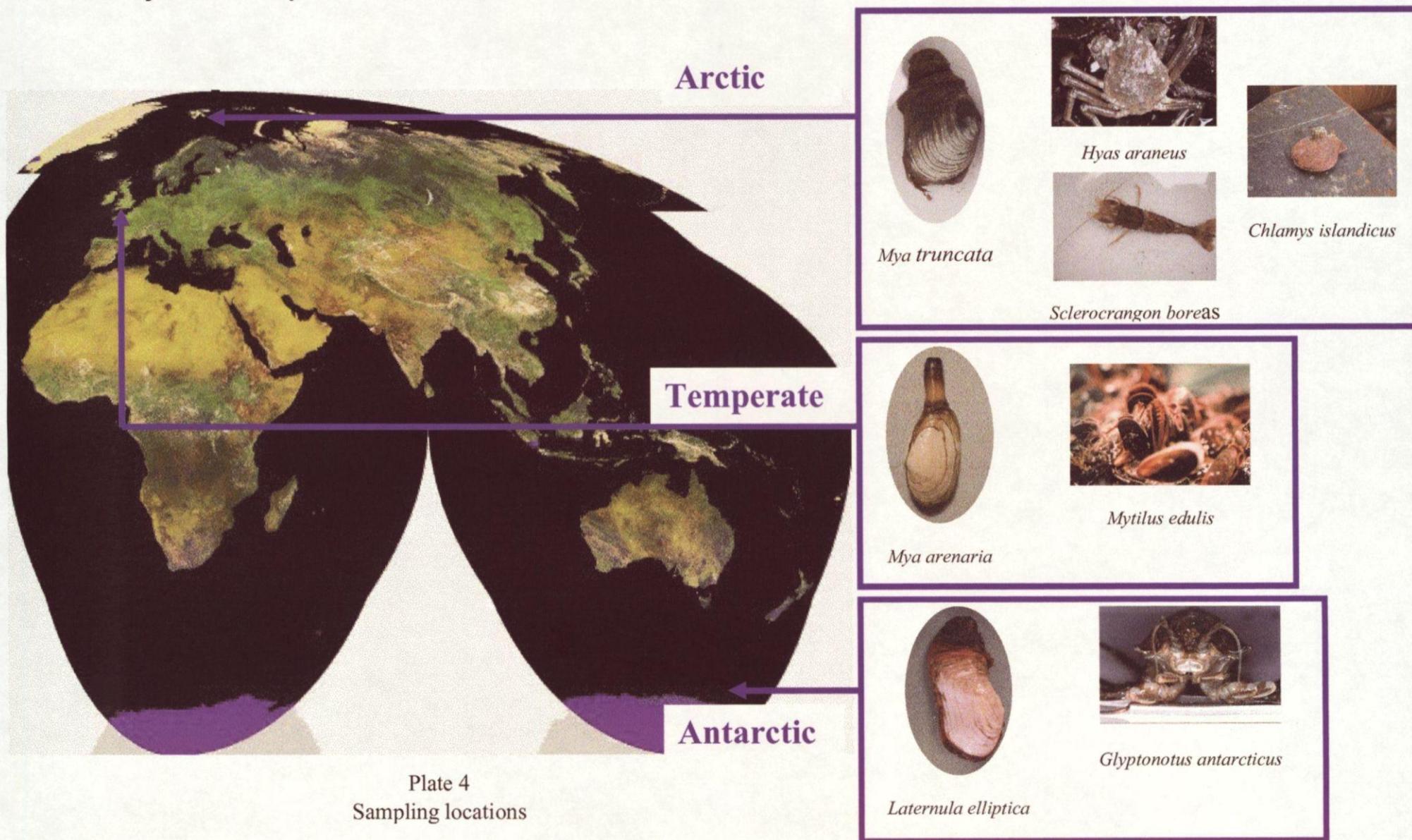
The figure shows a detailed view of the specimen's internal structure, with the highlighted areas representing the specific parts of interest. The drawing is a scientific illustration, likely from a historical biological text.

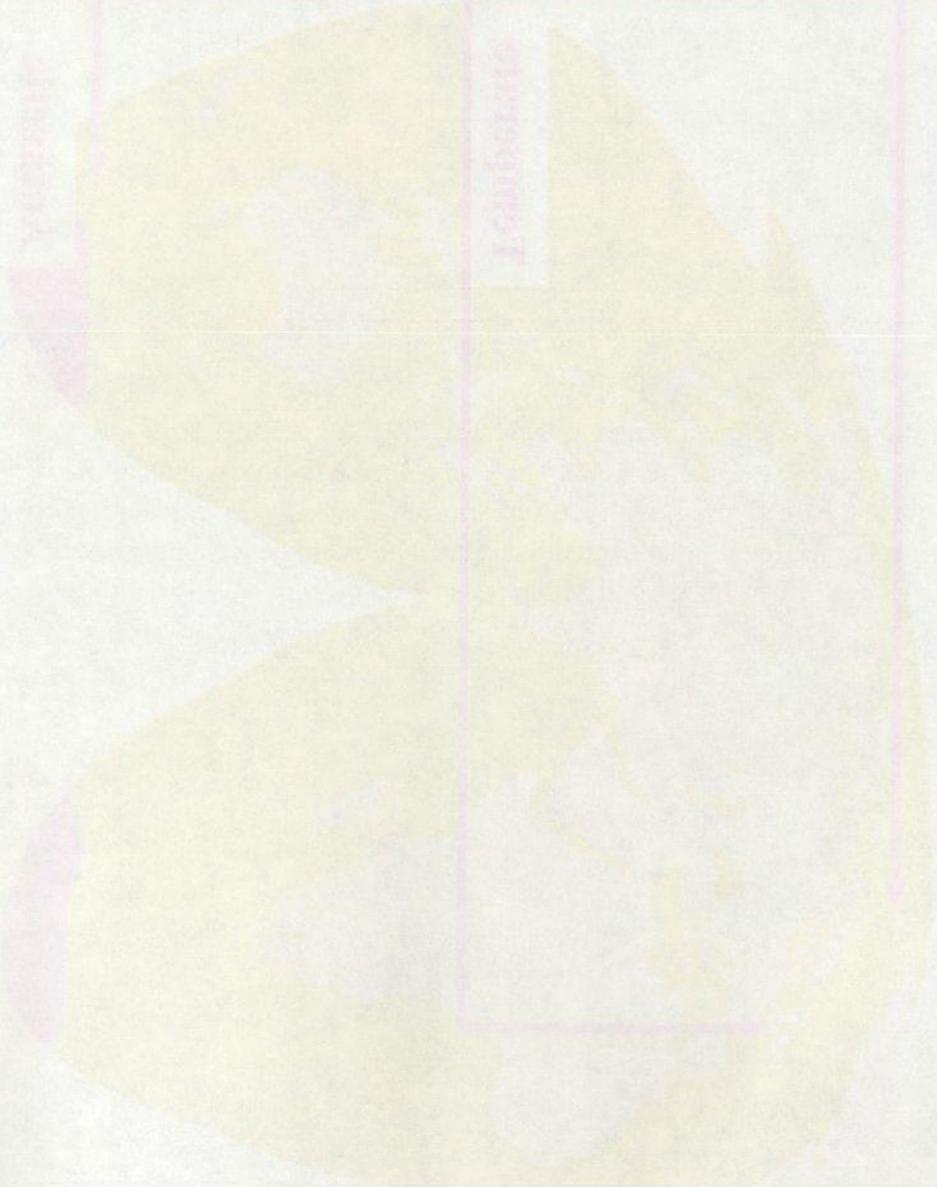


Figure 2

This figure provides a broader view of the specimen, showing its overall form and the distribution of the highlighted internal structures. The drawing is a scientific illustration, likely from a historical biological text.

## 4.2. Study site and species





Yucca

Yucca

Yucca

Yucca

#### 4.2.1. Arctic: Svalbard

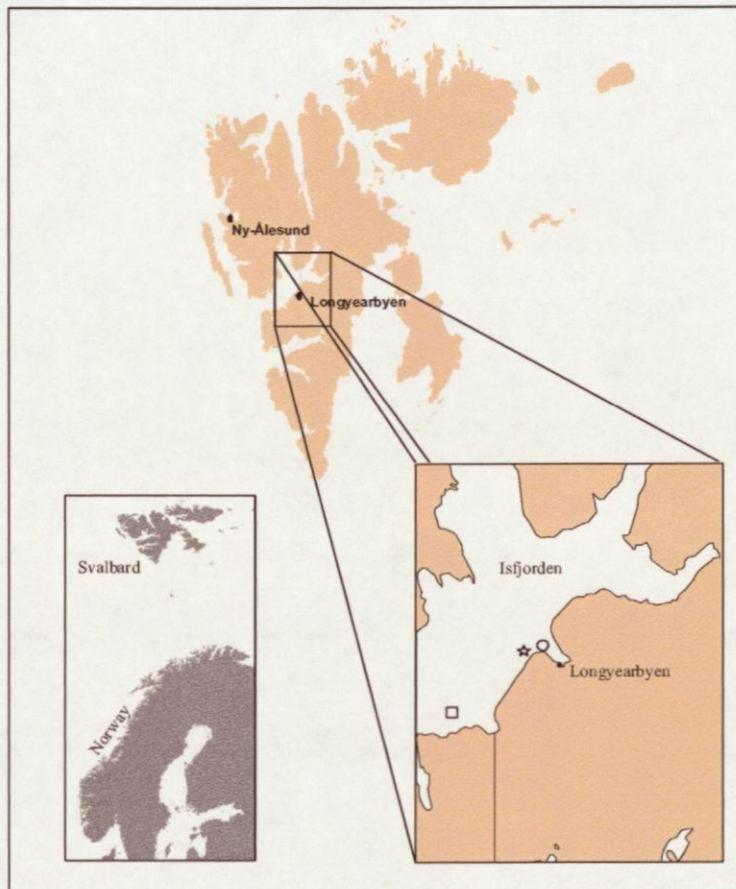


Figure 20.

Position of the sampling site in Isfjorden at Svalbard ( $78^{\circ}13'N$ ,  $15^{\circ}39' E$ ). *Hyas araneus*, *Sclerocrangon boreas*, *Chlamys islandicus* and *Hiatella arctica* were sampled in Hollenderbukta (square symbol) with an Agassiz dredge. *Mya truncata* were handpicked by divers at the location indicated by a star. The caging experiment was performed in Adventfjorden (open circle).

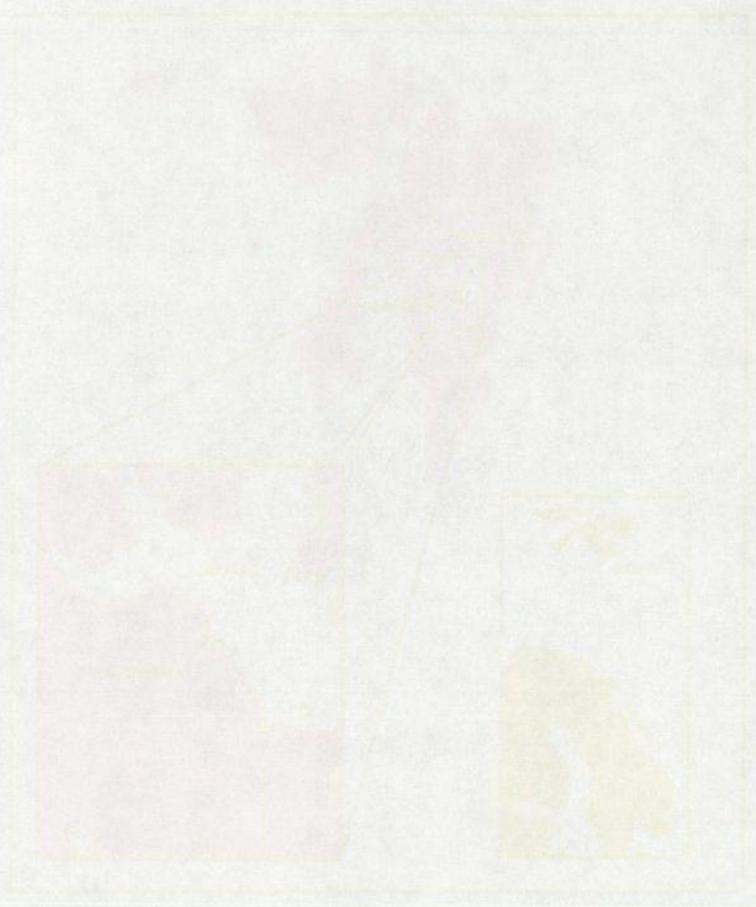
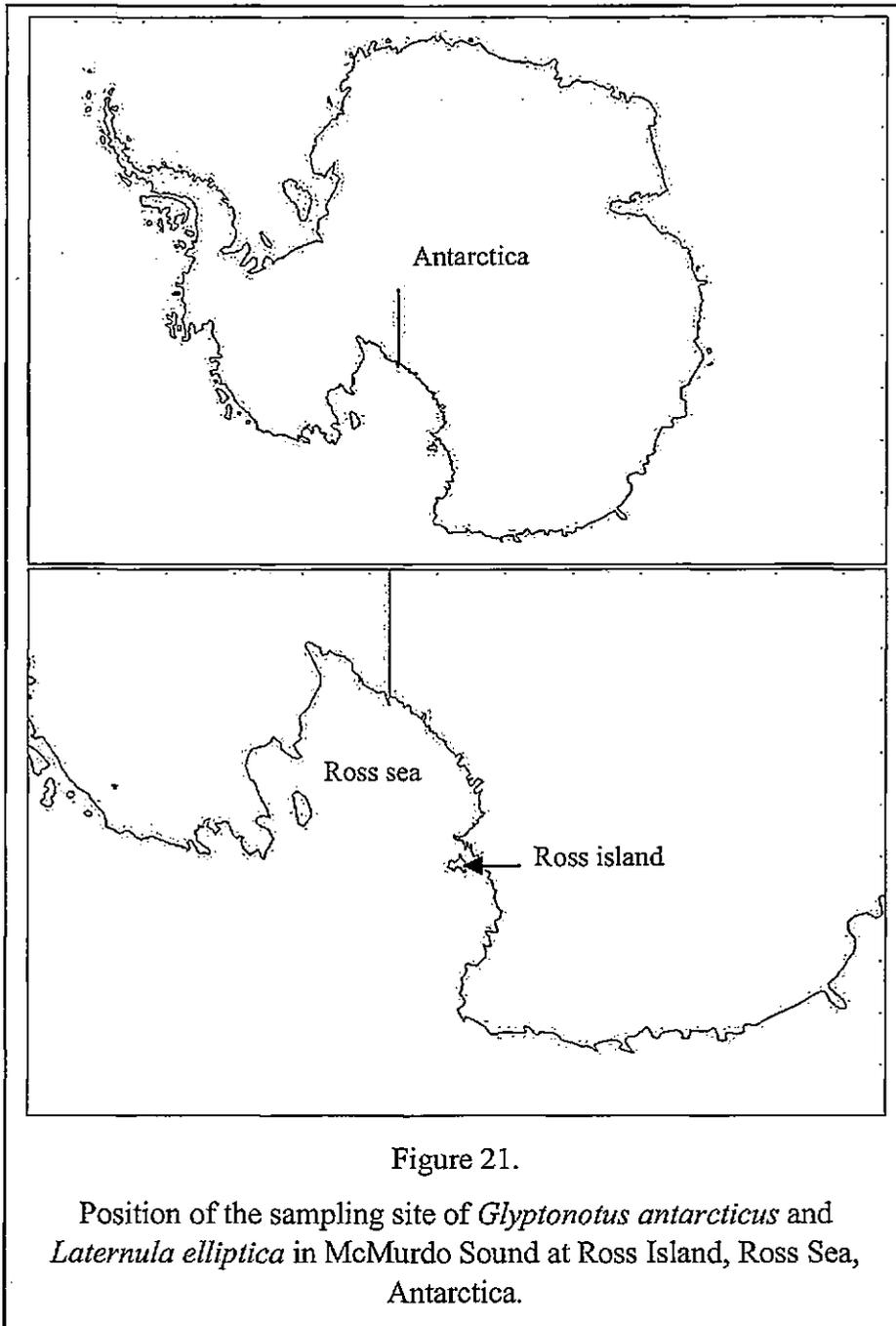


Figure 1

The chart displays the distribution of respondents across three categories: Total, Male, and Female. The 'Total' category represents the sum of all respondents, while 'Male' and 'Female' represent the gender-specific counts. The data indicates that the total number of respondents is significantly higher than the number of male respondents, which in turn is higher than the number of female respondents.

#### 4.2.2. Antarctica: Ross Island



### 4.2.3. The temperate regions: The English Channel and the North Sea



Figure 23.

Position of the sampling site of *Mya arenaria* in the Yealm Estuary near Plymouth, Devon (United Kingdom).

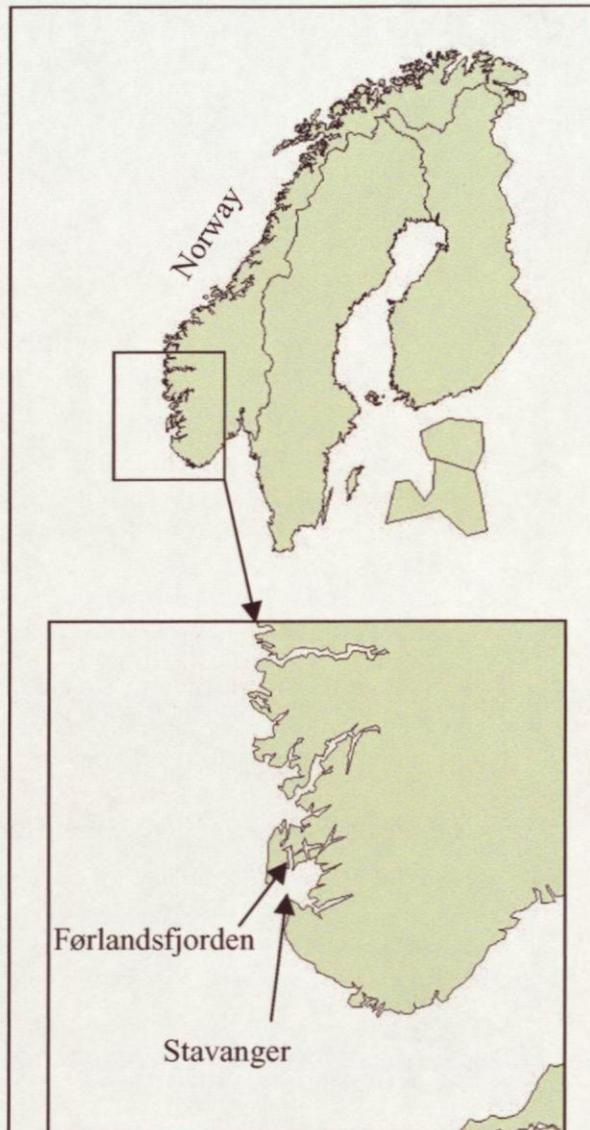


Figure 22.

Position of the sampling site of *Mytilus edulis*, in Førlandsfjorden, North of Stavanger, Rogaland, Norway.

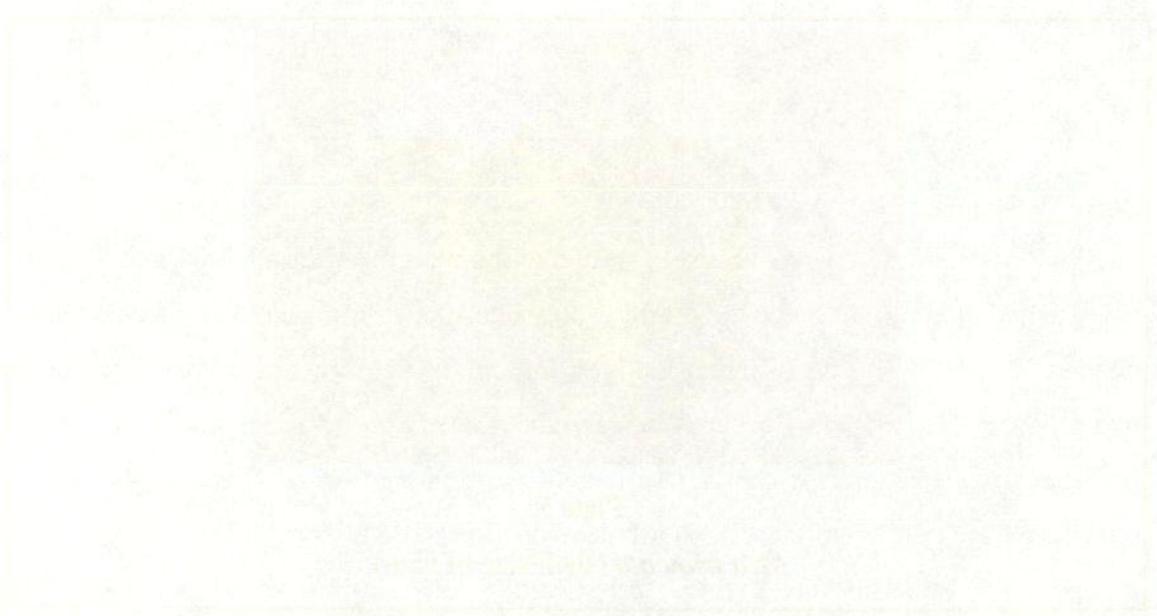




Introduction

Abstract

The purpose of this study was to investigate the relationship between job satisfaction and organizational commitment. The study was conducted in a large manufacturing company. The results showed that job satisfaction was a significant predictor of organizational commitment. The study also found that organizational commitment was a significant predictor of job satisfaction. The study was limited by its cross-sectional design and the use of self-reported data. The study has implications for organizations that want to improve job satisfaction and organizational commitment. Organizations should focus on improving job satisfaction to increase organizational commitment. Organizations should also focus on increasing organizational commitment to improve job satisfaction.



References

Allen, S. M., & Meyer, J. P. (1984). Organizational commitment and turnover intention: A comparison of self-reported and archival data. *Journal of Applied Psychology*, 69, 628-638.

Allen, S. M., & Meyer, J. P. (1985). Organizational commitment and organizational performance: A meta-analysis. *Journal of Applied Psychology*, 70, 155-165.

Allen, S. M., & Meyer, J. P. (1986). Organizational commitment and organizational performance: A meta-analysis. *Journal of Applied Psychology*, 71, 155-165.

Allen, S. M., & Meyer, J. P. (1987). Organizational commitment and organizational performance: A meta-analysis. *Journal of Applied Psychology*, 72, 155-165.

Allen, S. M., & Meyer, J. P. (1988). Organizational commitment and organizational performance: A meta-analysis. *Journal of Applied Psychology*, 73, 155-165.

brownish edge along the margin. The shell colour of *L. elliptica* is white with a pinkish or greenish shell covering (periostracum) when alive. This filter-feeder bivalve lives buried in gravelly or soft mud with a large siphon that cannot be fully retracted into its shell; there is a large gape where the two valves do not close. It has the ability to reburrow itself when exposed following ice scouring. This bivalve was sampled by scuba divers from the jetty of the American Antarctic research station of Mc Murdo (Ross sea, Ross Island) (Figure 21).



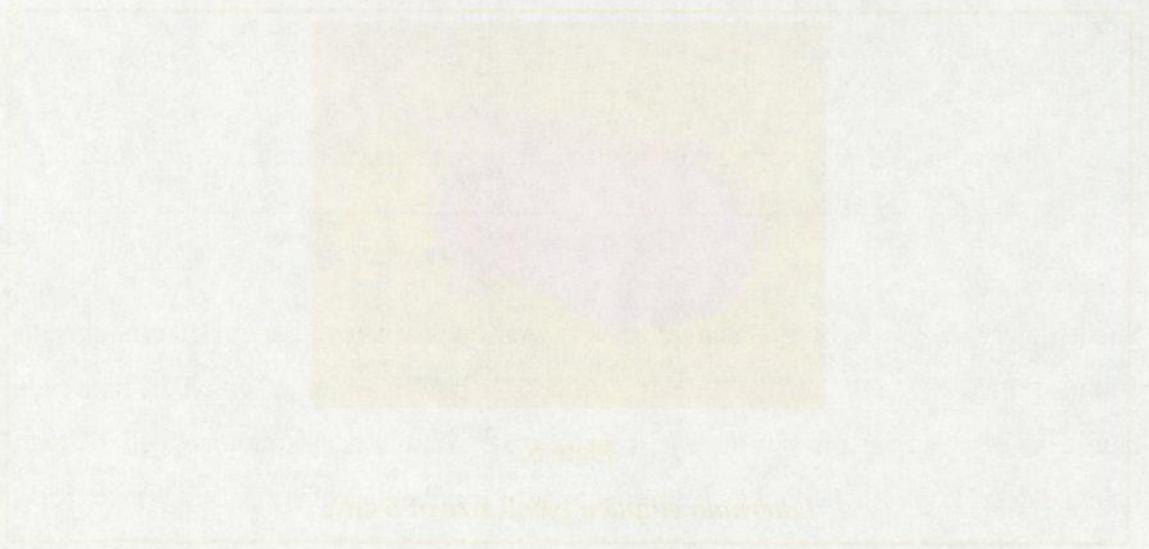
Plate 6.

*Laternula elliptica* (shell size of 5 cm).

***Chlamys islandicus***

Shell surface with 35 coarse ribs, split in two at the margin of the valve (Plate 7). Left valve usually pink, right one whitish-grey. Only one adductor muscle scar. Occurs at the depth of 2 to 1300 m, on different kinds of bottom, at water temperature below 10°C and salinity greater than 34. It is widely distributed Arctic species. Juveniles are predated by *Hyas araneus*; bearded seals and sea stars feed on adult scallops. It has a commercial value though farming is not developed yet. It was sampled with an Agassiz dredge from RV Jan Mayen from the university of Tromsø, at Hollenderbukta in Isfjorden at Svalbard (Figure 20).

The present study was designed to test the hypothesis that... (text is very faint and difficult to read)



Results were then... (text is very faint and difficult to read)



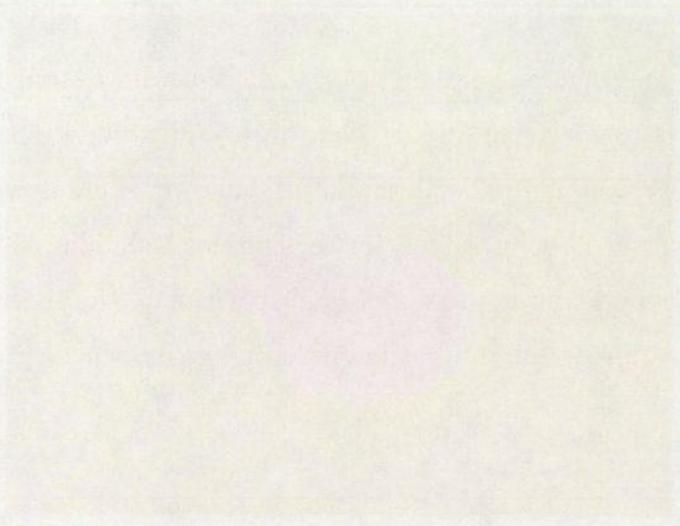


Figure 1. A line graph showing a trend over time. The x-axis is labeled 'Time' and the y-axis is labeled 'Performance'. The graph shows a curve that starts at a low point, rises to a peak, and then declines. The peak is labeled 'Peak Performance'.

The first section of the paper discusses the importance of understanding the relationship between performance and time. It highlights the fact that performance is not a static state but rather a dynamic process that changes over time. The second section focuses on the concept of 'peak performance' and how it is achieved. It discusses the various factors that contribute to peak performance, such as motivation, focus, and physical fitness. The third section explores the concept of 'burnout' and how it can affect performance. It discusses the signs and symptoms of burnout and offers strategies for preventing and recovering from it. The final section provides a summary of the key findings and offers some practical advice for improving performance over time.

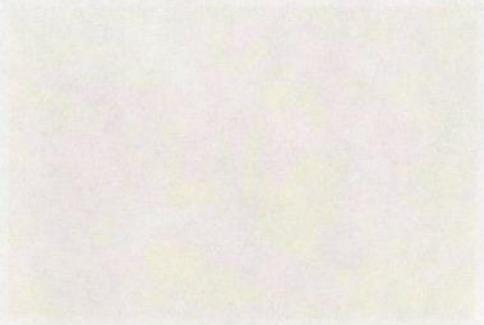


Figure 2. A line graph showing a trend over time. The x-axis is labeled 'Time' and the y-axis is labeled 'Performance'. The graph shows a curve that starts at a low point, rises to a peak, and then declines. The peak is labeled 'Peak Performance'.

***Mya arenaria***

Shell up to 15 cm long; more oval than *M. truncata* but with a similar arrangement of valves and hinge processes (Plate 9). It is white, dirty-white; interior brown. It is a filter feeder that lives burrowed in mud and sand from the lower shore down to 70 m and in estuaries. It can extend its long siphon for feeding and breathing. *Mya arenaria* is a temperate species common in the Atlantic, English Channel, North Sea and Baltic Sea. It was sampled by digging the mud at low tide in Yealm estuary (Plymouth, Devon, UK) (Figure 23).



Plate 9.

*Mya arenaria* (shell size of 5cm).

**4.3.2. Crustaceans**

***Sclerocrangon boreas***

This shrimp is grey to brown in colour (Plate 10). It occurs at depths of 2 to 450m, on different kinds of bottom, at temperatures below +4°C and salinities over 33. Eggs are incubated in winter. There is no pelagic larval stage and, juveniles appear in July. Rather common and numerous species, found in the food of fish and seals. Boreal,



Plate 10.

*Sclerocrangon boreas* (total length 9 cm).

It is well known that the rate of polymerization is affected by the concentration of the monomer and the initiator. In this study, the effect of the concentration of the monomer and the initiator on the rate of polymerization was investigated. The results are shown in Figure 1. It can be seen from Figure 1 that the rate of polymerization increases with the increase of the concentration of the monomer and the initiator. This is because the higher the concentration of the monomer and the initiator, the more active sites are available for the polymerization reaction.

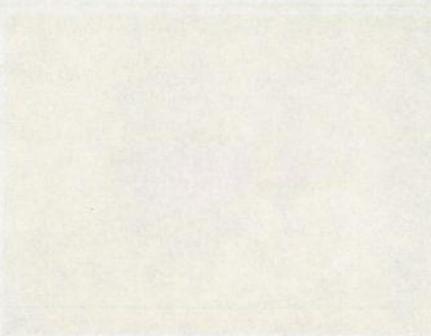


Figure 1. Effect of monomer and initiator concentration on the rate of polymerization.

4.2. Discussion

The results of the study are shown in Figure 1. It can be seen from Figure 1 that the rate of polymerization increases with the increase of the concentration of the monomer and the initiator. This is because the higher the concentration of the monomer and the initiator, the more active sites are available for the polymerization reaction. The results of the study are shown in Figure 1. It can be seen from Figure 1 that the rate of polymerization increases with the increase of the concentration of the monomer and the initiator. This is because the higher the concentration of the monomer and the initiator, the more active sites are available for the polymerization reaction.

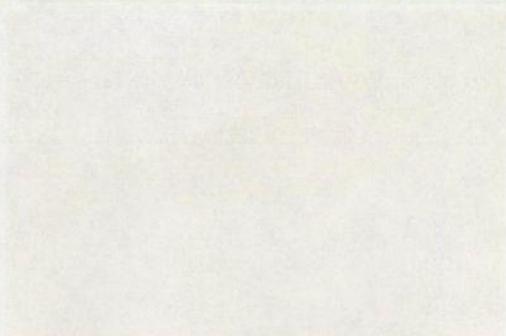
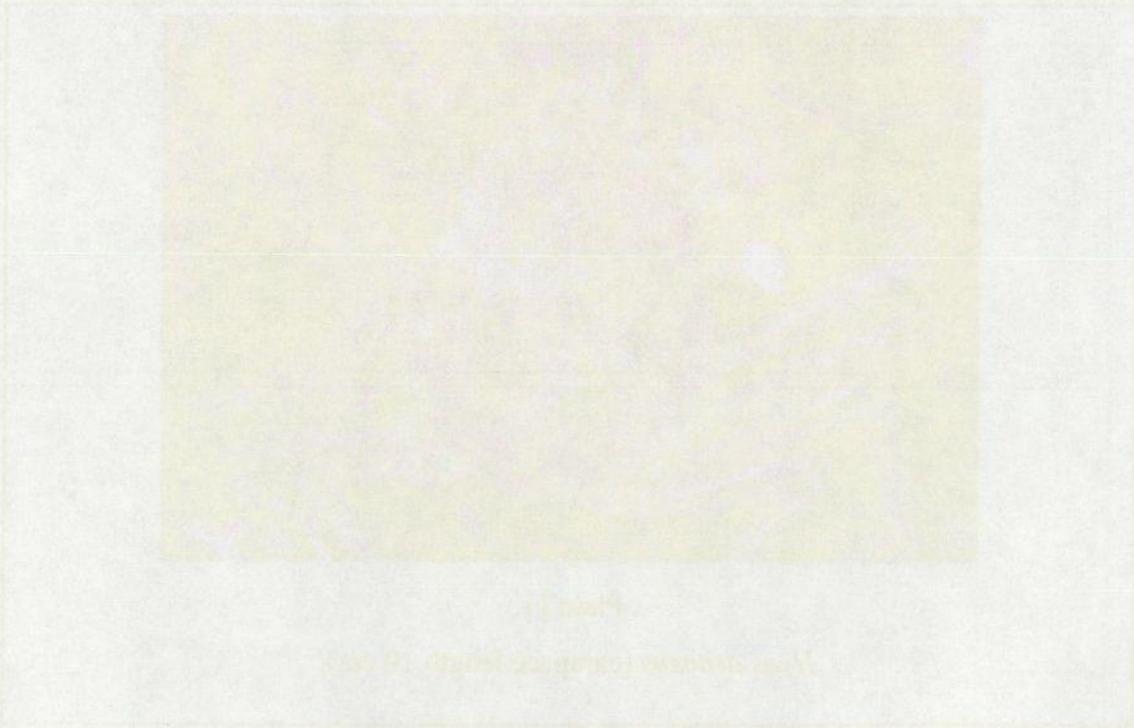


Figure 2. Effect of monomer and initiator concentration on the rate of polymerization.



...the extent to which the victim's perception of the perpetrator's behavior is related to the victim's perception of the perpetrator's behavior.

...the extent to which the victim's perception of the perpetrator's behavior is related to the victim's perception of the perpetrator's behavior. ...the extent to which the victim's perception of the perpetrator's behavior is related to the victim's perception of the perpetrator's behavior.





The first part of the paper discusses the importance of the study and the objectives of the research. It also outlines the methodology used in the study and the results obtained. The second part of the paper discusses the implications of the study and the conclusions drawn from the research. The third part of the paper discusses the limitations of the study and the areas for further research.

Figure 1

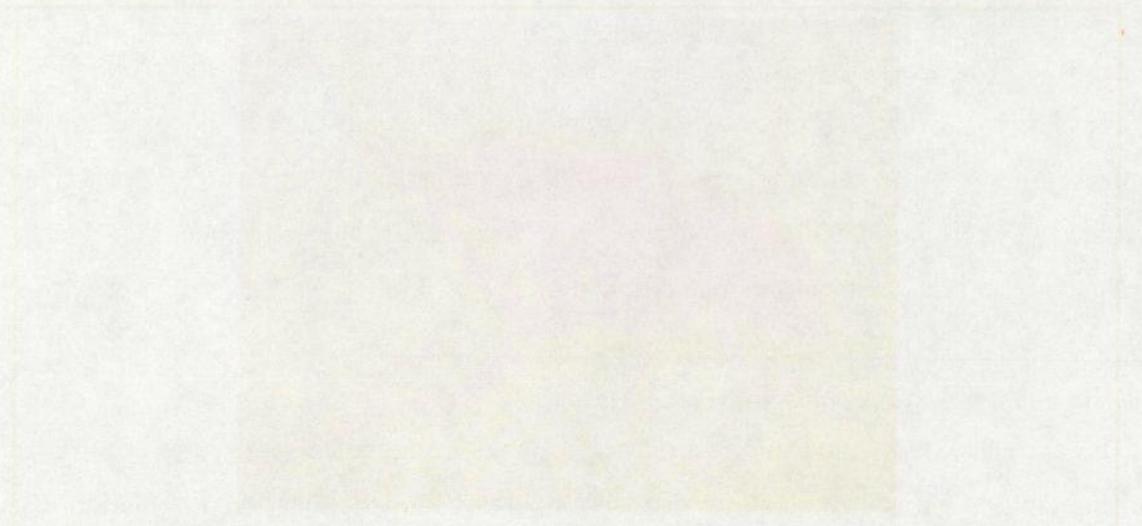


Figure 1

Figure 1 shows the relationship between the two variables. The curve indicates a positive correlation between the two variables.

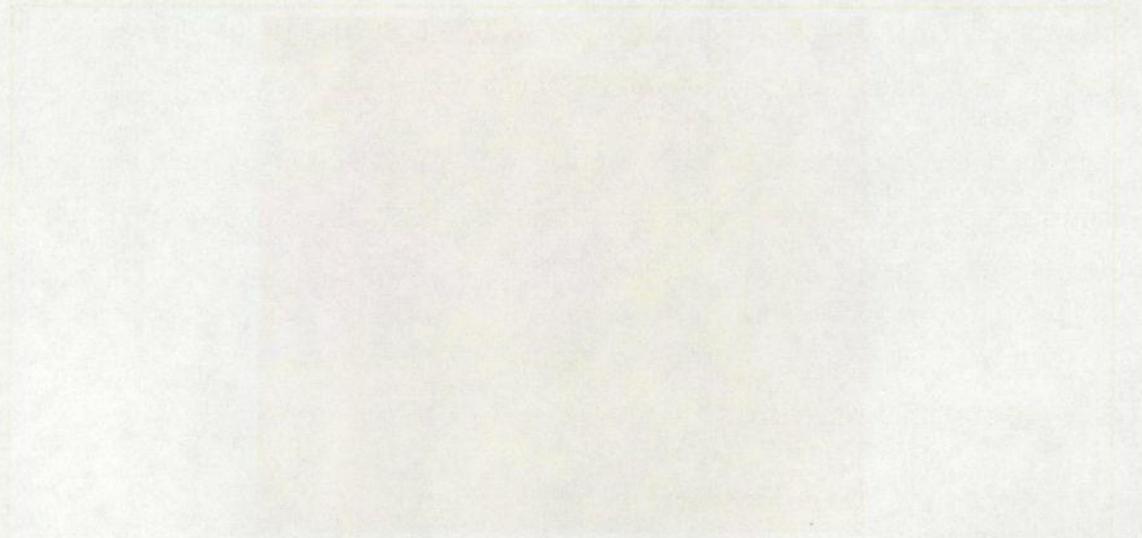


Figure 2

Figure 2 shows the relationship between the two variables.

## 4.4. Maintenance of species

### *In the field*

Once the animals were collected, they were stored in running seawater in a large aluminium tank on board RV Jan Mayen. The animals were transferred into large plastic buckets (200 l) and transported to the climate room at 2°C of the “University Courses on Svalbard”. The water was permanently aerated and renewed every two days with water pumped from 20 m deep in Adventfjorden.

### *Maintenance during transport*

Transport of organisms was carried out using 13 l plastic thermos (Walbro Automotive a/s, Norway). Crabs and bivalves were transported in habitat water while shrimps were packed between seawater moistened paper in several layers. Two plastic ice packs were added to keep the temperature close to zero. Temperature measurements indicated no change after plane transportation and transit of about 12 hr.

### *Maintenance of the species in the laboratory*

The animals were stored in large glassfibre tanks designed for aquaculture purposes. The climate room temperature was set at 2°C. Oxygenation and circulation was maintained with pumps. A protein skimmer helped remove biological wastes such as proteins and bacteria. The water was partially renewed twice a week with seawater pumped from the Stavanger fjord (-70m) and cooled down to 2°C. The bivalves were fed regularly (twice a week) with *Isochrysis sp.* The crabs and shrimps were fed with fresh crushed blue mussel (*Mytilus edulis*) or raw shrimp.

High mortality after transport was observed with *Chlamys islandicus*. Recent work showing the susceptibility of oysters to handling stress (Lacoste *et al.* 2001) could explain our observations.

## 4.5. Analytical Methods

### 4.5.1. On board measurement

Emphasis on this study was to optimise the methods so that they can be performed on board a research vessel, in the field and deployed in a basic research laboratory. Heart rate and respiration of most species were measured on board R/V Jan Mayen. The cell





management and the development of a sustainable fishery. The results of this study will be used to develop a management plan for the fishery.

### 4.1.3. Environmental and socio-economic factors

The results of the study show that the fishery is currently overfished and that the fish stock is declining. This is due to a combination of factors, including overfishing, habitat destruction, and climate change. The fishery is also facing socio-economic challenges, such as a decline in income and a loss of traditional knowledge. The study highlights the need for a sustainable management plan that takes into account both environmental and socio-economic factors. This plan should include measures to reduce fishing pressure, protect the fish habitat, and improve the livelihoods of the fishers. The study also emphasizes the importance of community-based management and the role of traditional knowledge in sustainable fisheries.

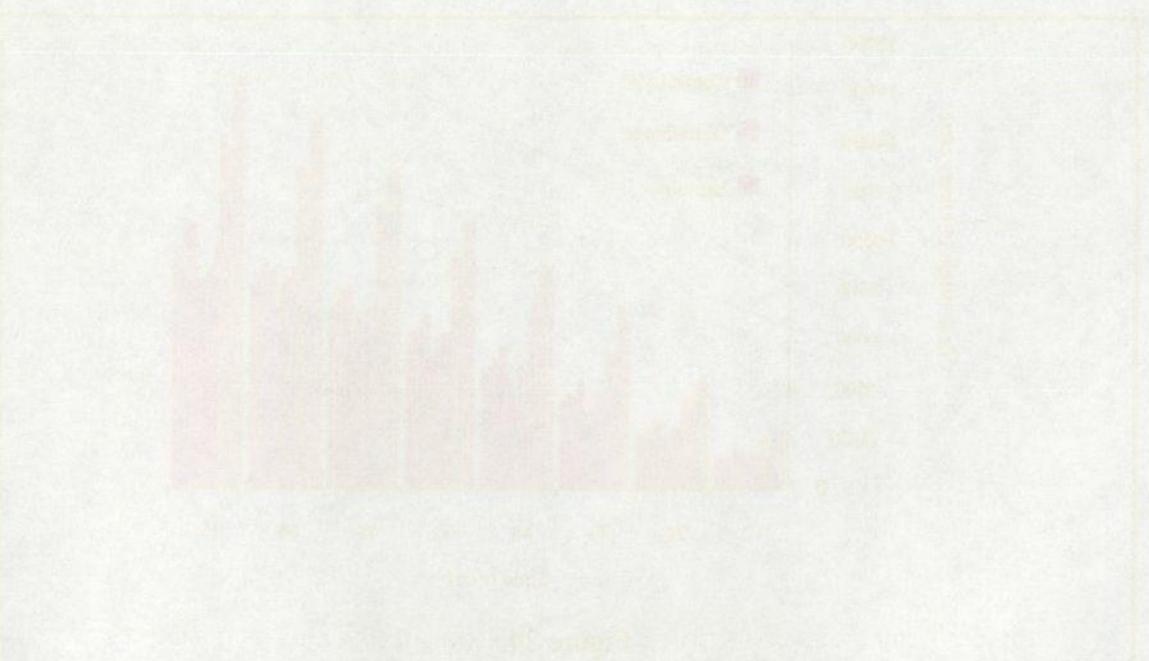


Fig. 1 Monthly catch of fish in the study area from 2008 to 2014. The Y-axis represents the number of fish (0 to 1000), and the X-axis represents the month (January to December). The chart shows a seasonal pattern in the catch, with higher catches in the winter months and lower catches in the summer months. The catch generally increased over the period from 2008 to 2014.

temperatures were 35, 160 and 220°C, respectively; helium was the carrier gas (1 ml/min flow rate) and a split ratio of 20:1 was used. The data acquisition system was run by the software Millennium32<sup>®</sup> (Waters). Each analysis required the measurement of control (no antioxidant in the reaction vial) and sample reactions (biological fluid in the vial). In the presence of antioxidant, ethylene production from KMBA was reduced quantitatively and higher antioxidant concentrations resulted in longer periods in which ethylene formation was inhibited relative to controls (Figure 24). By plotting the absolute value of the difference between the ethylene peak area obtained at each time point for the sample and control reactions it was possible to visualise whether the oxyradical scavenging capacity of the solution was changed (Figure 25). The area under the kinetic curve was mathematically calculated from the integral of the equation that best defines the experimental points for both the control and sample reactions (Figure 25). TOSC is then quantified according to Equation 1, where IntSA and IntCA are the integrated areas from the curve defining the sample and control reactions, respectively.

$$\text{TOSC} = 100 - (\text{IntSA}/\text{IntCA} * 100) \quad \text{Equation 1}$$

Thus, a sample that displays no oxyradical scavenging capacity would give an area

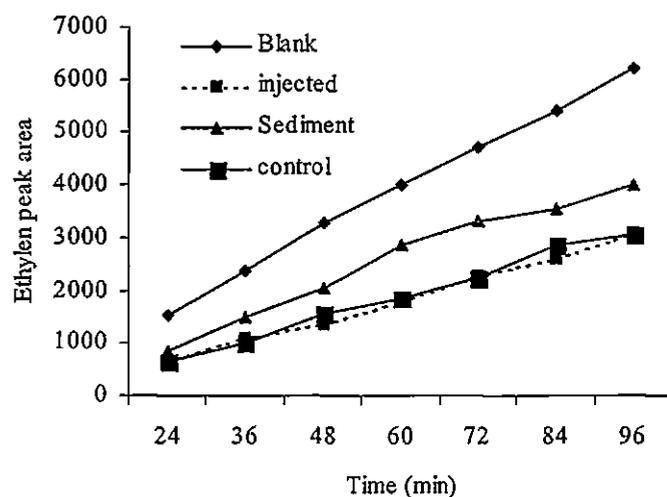


Figure 25.

Ethylene peak area versus time in blank vial (no antioxidant), two samples of hepatopancreas of *H. araneus* exposed to oil via injection or sediment or not exposed (control). Note the reduced scavenging capacity of the "sediment" curve compared to "control" and "injected" curves. The TOSC value as expressed in eq. 1 is directly calculated from this graph.









red working solution added to the area containing the attached cells. A 22 x 22 mm coverslip was then applied. After 15 min incubation in an insulated light proof chamber, the preparations were inspected under a microscope (x 500 magnification). Following a further 15 min incubation, the preparation was examined again and, thereafter, at 30 min intervals to determine the time at which the dye that had been taken up into individual lysosomes (turning them red; Plate 17) and leached out into the cytosol. The test was terminated when dye loss was evident in 50% (numerically assessed per field of view) of the small granular haemocytes and the time recorded (Plate 16); the study was concluded after 180 min. Following each inspection, the preparations were returned to the incubation chamber. The procedure, as described, does not produce a monoculture, but as the different cell types can be clearly distinguished through the microscope, this was not a problem.

#### 4.5.5. Respiration

A closed respirometer system was used in this study. Organisms were placed individually into glass vials (480 ml) (Plate 18), sealed with a screwed Teflon lid, and incubated at a regulated temperature. Empty vials, containing seawater but no



Plate 18.

Vial of 480 ml used for incubation  
and containing an individual of  
*Mya arenaria*.

organisms, were also incubated as controls and their oxygen concentration monitored. Incubation time was determined prior to each experiment to ensure oxygen tension was not reduced to less than 75% in the incubator (as such reduction would affect the respiration data). The oxygen concentration in each vial was measured by injecting a seawater sample taken from the vials into a respiration chamber (100  $\mu$ l) (Plate 21) connected to a polarographic Clark-type oxygen sensor (Plate 19) (Strathkelvin

...the study of aging and the elderly. The study of aging is a multidisciplinary field that draws on knowledge from psychology, sociology, anthropology, and other disciplines. The study of the elderly is a more specific area of research that focuses on the lives of older adults. This research can help us understand the challenges and opportunities that older adults face, and how we can best support them. The study of aging and the elderly is an important and growing field of research, and it has the potential to make a significant impact on the lives of older adults.

### Conclusion

The study of aging and the elderly is a complex and multifaceted field. It requires a deep understanding of the biological, psychological, and social factors that influence the lives of older adults. This research can help us better understand the needs and desires of older adults, and how we can best support them. The study of aging and the elderly is an important and growing field of research, and it has the potential to make a significant impact on the lives of older adults.

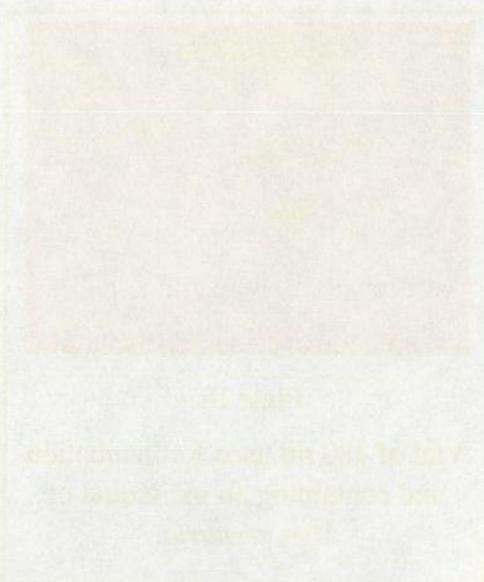


Figure 1  
The relationship between the study of aging and the study of the elderly.

The study of aging and the elderly is a complex and multifaceted field. It requires a deep understanding of the biological, psychological, and social factors that influence the lives of older adults. This research can help us better understand the needs and desires of older adults, and how we can best support them. The study of aging and the elderly is an important and growing field of research, and it has the potential to make a significant impact on the lives of older adults.

microcell MC100<sup>®</sup> and microcathode oxygen electrode SI130) maintained at a constant temperature by a recirculating refrigeration bath (Picture 20). The  $P_{O_2}$  was calibrated to oxygen-saturated sea water (i.e. 10.03 mg.l<sup>-1</sup> O<sub>2</sub>, 36‰ salinity, 5.5°C). Soft body tissue of bivalves and whole body of crustaceans were dried at 100°C for 24 h and respiration data were normalised to dry weight body mass. Data are expressed as mg O<sub>2</sub> per gram dry weight per hour.

Calculation of oxygen consumption rates was calculated from dissolved oxygen concentration, respirometer volume and time of incubation (Handy and Depledge 1999) as follows:

$$VO_2 = ([O_2]_a - [O_2]_b) \times V / T / dw$$

Where,  $VO_2$  = oxygen consumption rate (mg O<sub>2</sub> g d w<sup>-1</sup> h<sup>-1</sup>),  $[O_2]_a$  = oxygen concentration in water (mg l<sup>-1</sup>) at the start of the trial,  $[O_2]_b$  = oxygen concentration in water (mg l<sup>-1</sup>) at the end of the trial,  $V$  = volume of respirometer (l),  $T$  = duration of measurement in period (h), and  $dw$  = dry weight (g).

To convert oxygen concentration units between mg l<sup>-1</sup> and ml l<sup>-1</sup> the following relationship was used (Handy and Depledge 1999): mg O<sub>2</sub> = 1.428 x ml O<sub>2</sub>

Other conversions of oxygen concentration:

$$1 \text{ mg} = 0.700 \text{ ml (STP)} = 31.251 \text{ } \mu\text{mol} = 0.0625 \text{ mg atoms.}$$

The principle of operation of the Clark-type oxygen electrode consists of a probe at whose tip is an exposed gold or platinum cathode and a silver or silver/silver chloride anode. When the anode and cathode are polarised so that the cathode is held at a voltage of -0.6 to -0.8 volts relative to the anode, and connected via a solution of electrolyte such as KCl, the following reaction will occur at the anode:



Simultaneously, any oxygen which is present is reduced at the cathode:



Thus, for each oxygen molecule reduced, 4 electrons of current flow in the circuit. Oxygen is, therefore, continually consumed as it is reduced to OH<sup>-</sup> at the cathode.

In practice, the anode and cathode are covered by an oxygen permeable membrane to exclude other species with which it would interfere. The KCl electrolyte is buffered to remove the  $\text{OH}^-$  produced in the cathode reaction. As oxygen is removed at the cathode, a  $\text{PO}_2$  gradient is set up which extends outwards into the surrounding medium. In unstirred water, oxygen therefore diffuses inwards along the  $\text{PO}_2$  gradient, the outside of the electrode membrane is effectively sensing a very much lower  $\text{PO}_2$  than that in the surrounding water. For this reason, most Clark electrodes require the water to be stirred. The size of the signal generated by the electrode is proportional to the flux of oxygen molecules to the cathode. This oxygen flux is proportional to: 1)The  $\text{PO}_2$  of the water; 2)the permeability of the membrane; 3)the temperature of the water and 4)the surface area of the cathode.



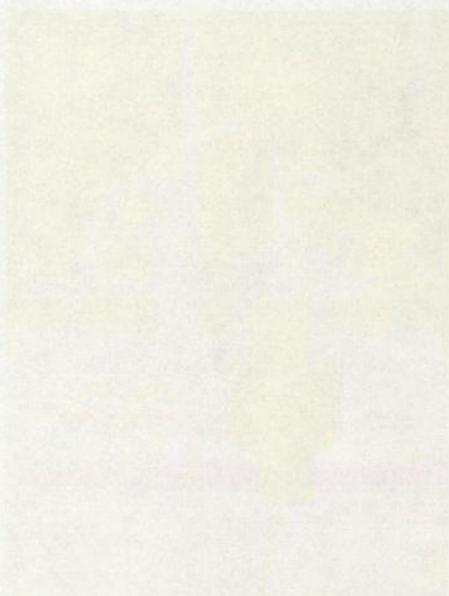


Figure 1

The figure illustrates four different scenarios of biomarker-phenotype relationships. The top-left plot shows a strong positive correlation, the top-right shows a strong negative correlation, the bottom-left shows no correlation, and the bottom-right shows a weak correlation.

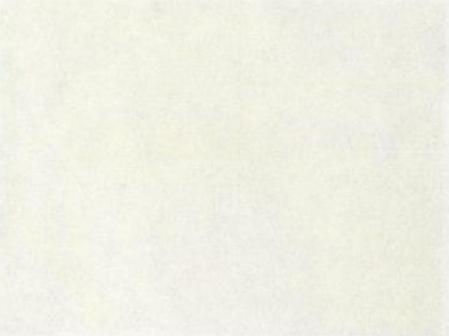


Figure 2

The figure illustrates four different scenarios of biomarker-phenotype relationships. The top-left plot shows a strong positive correlation, the top-right shows a strong negative correlation, the bottom-left shows no correlation, and the bottom-right shows a weak correlation.

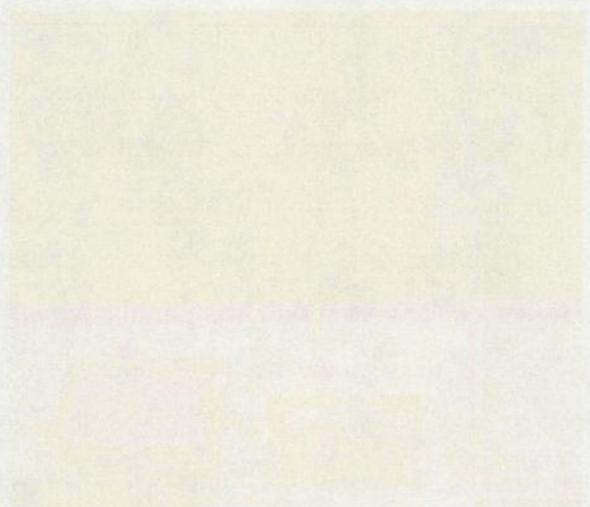


Figure 3

The figure illustrates four different scenarios of biomarker-phenotype relationships. The top-left plot shows a strong positive correlation, the top-right shows a strong negative correlation, the bottom-left shows no correlation, and the bottom-right shows a weak correlation.

#### 4.5.6. Heart rate

Heart rate was measured with the non-invasive CAPMON procedure (Computer Aided Physiological MONitoring system) developed by Depledge and Andersen (1990). The system, based on an optical rather than electrical approach, allows heart rate to be recorded with no physical or biological disturbance. An infra red light emitter/receptor was glued onto the carapace of the animal (Plate 24 and 27). Emitted light is reflected onto the heart in proportion to its volume. The signal is amplified and converted to a digital signal which is displayed on a computer (PC) (Plate 26). With the CAPMON unit, eight crabs were monitored simultaneously. The data were stored as ASCII files, and exported to a spreadsheet for statistical analysis and graphic plotting. Recording procedure was as follow: transducers were glued onto the carapace of eight crabs per group and connected to the interface. Each animal was transferred to a plastic non-

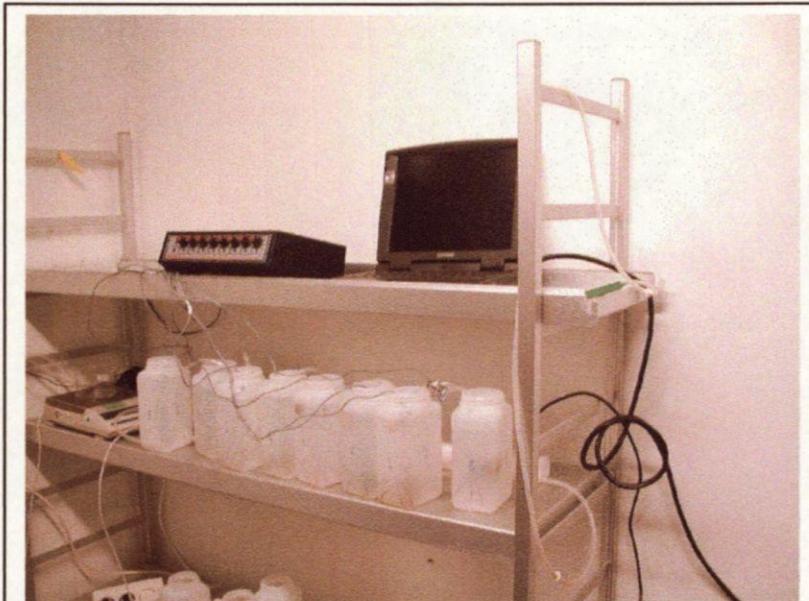


Plate 22.

Experimental set up deployed in the laboratory (climate room) to measure the heart rate of crustaceans. Eight non-transparent beakers contain one crab each glued to an infra red sensor that is connectd to the CAPMON-II interface (black box with red switches, top-left). A lab top connected to the CAPMON interface allow to visualise the heart signal. The room temperature is set at 2°C and the water of the beaker is permanently aerated.

transparent beaker filled up with one litre of seawater. Aeration of the water was performed during the experiment to provide oxygen (Plate 22, 23, 25 and 28). The size of the beaker prevented any locomotory or swimming activity that could affect

4.3.5. Results

There was no significant difference in the number of days spent in the hospital between the two groups. The mean number of days spent in the hospital was 12.5 (SD = 3.5) for the control group and 13.0 (SD = 4.0) for the intervention group. The mean number of days spent in the hospital was significantly higher for the control group than for the intervention group,  $F(1, 100) = 4.5, p < .05$ . The mean number of days spent in the hospital was significantly higher for the control group than for the intervention group,  $F(1, 100) = 4.5, p < .05$ . The mean number of days spent in the hospital was significantly higher for the control group than for the intervention group,  $F(1, 100) = 4.5, p < .05$ .

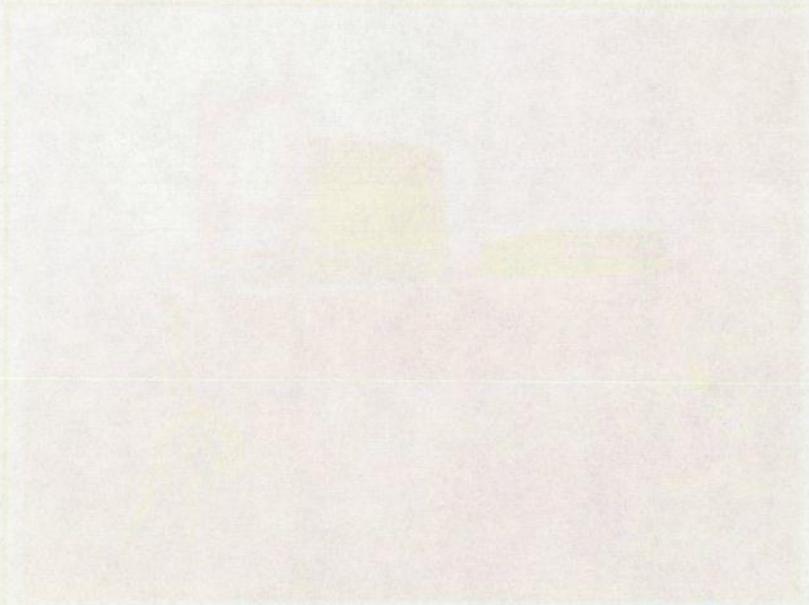


Figure 1

The number of days spent in the hospital was significantly higher for the control group than for the intervention group,  $F(1, 100) = 4.5, p < .05$ . The mean number of days spent in the hospital was significantly higher for the control group than for the intervention group,  $F(1, 100) = 4.5, p < .05$ . The mean number of days spent in the hospital was significantly higher for the control group than for the intervention group,  $F(1, 100) = 4.5, p < .05$ .

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recordings. The animals were given 2 h to acclimate to the new conditions before recording. Recordings of the heart rate lasted 4 h. The mean heart rate and the standard deviation were used in this study.

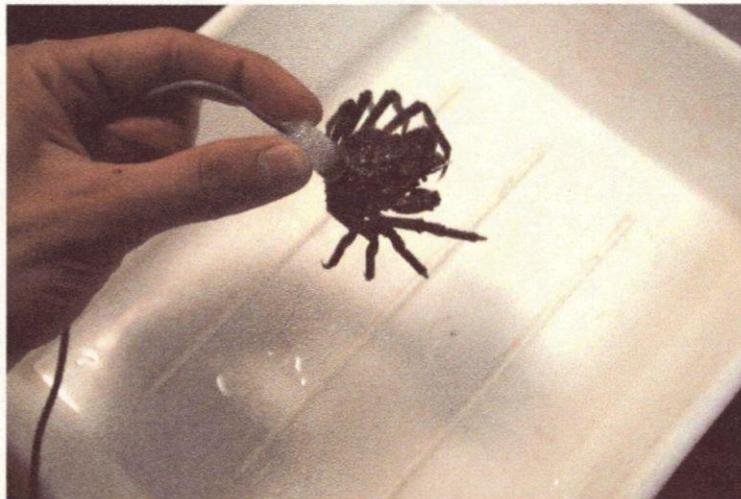


Plate 24.

Infra red sensor glued on the carapace of a juvenile *H. Araneus*.

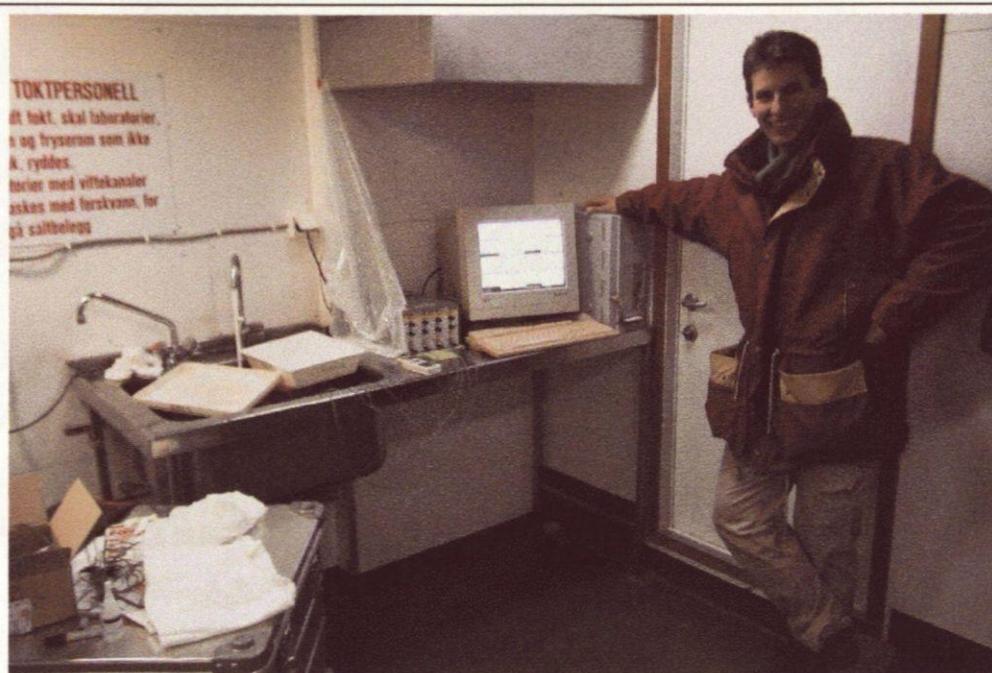


Plate 23.

Heart rate set-up deployed onboard the research vessel Jan Mayen. The sea water temperature was maintained constant with running water. The crabs were dispatched in the boxes placed in the sink.

The first of the series of photographs was taken on the 1st of August 1957. The second was taken on the 2nd of August 1957. The third was taken on the 3rd of August 1957. The fourth was taken on the 4th of August 1957. The fifth was taken on the 5th of August 1957.



Fig. 1

The first of the series of photographs was taken on the 1st of August 1957.

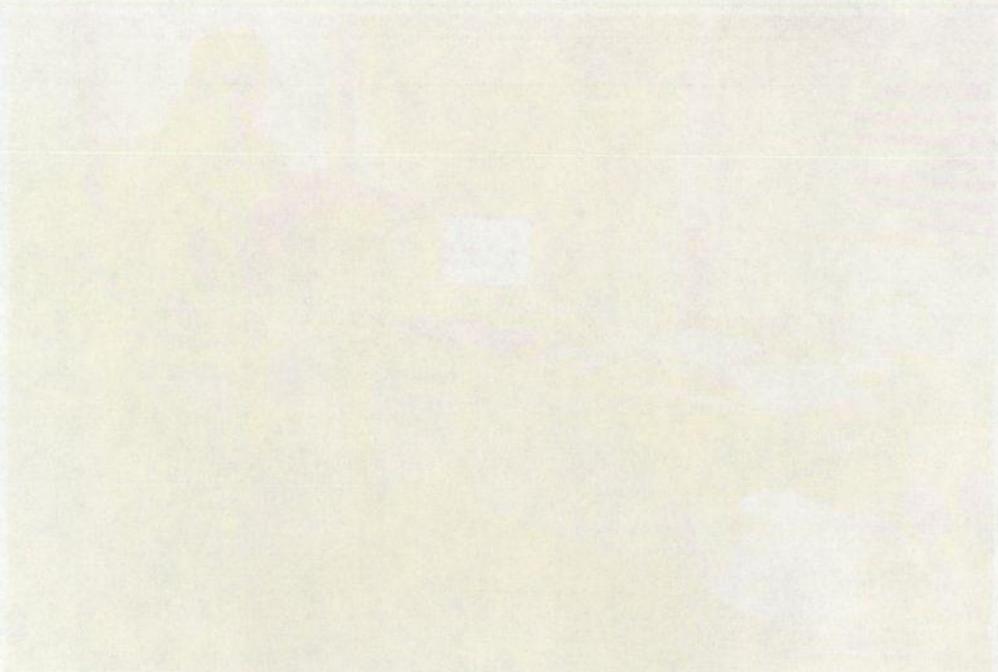


Fig. 2

The second of the series of photographs was taken on the 2nd of August 1957. The third was taken on the 3rd of August 1957. The fourth was taken on the 4th of August 1957. The fifth was taken on the 5th of August 1957.

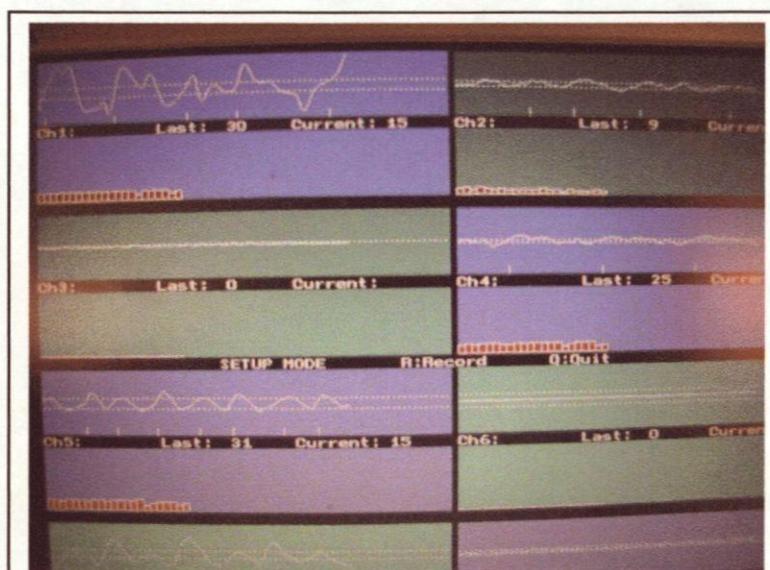


Plate 26.

Heart rate signal of *Glyptonotus antarcticus* displayed on the monitor of a laptop. The software shows the signal online (white plain line). The red bars correspond to the beat per minute recorded over one hour.

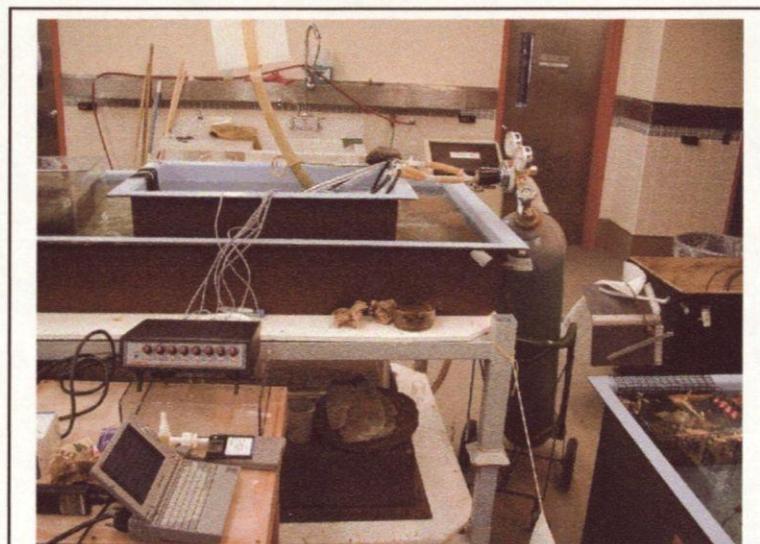


Plate 25.

Heart rate set-up deployed in McMurdo research station (Antarctica) to measure heart rate of *Glyptonotus antarcticus*. In the foreground lies the laptop connected to the CAPMON-II interface. The isopods are placed in beakers lying in the blue plastic aquarium filled up with running seawater (-1.5°C).

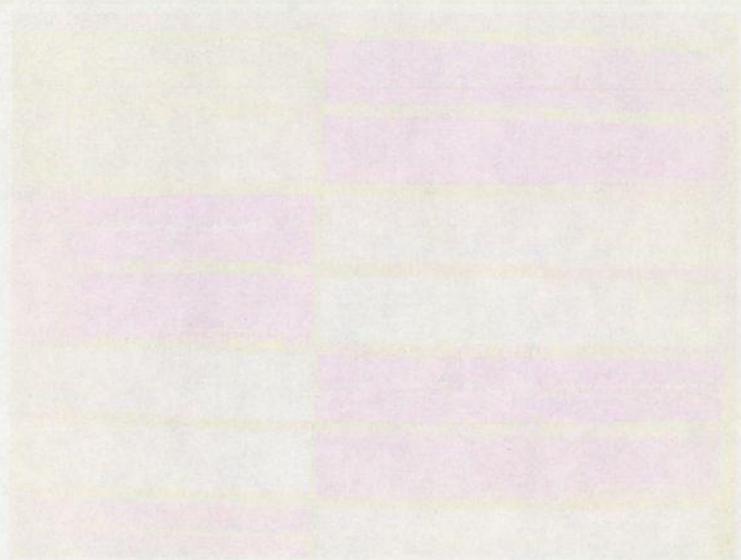


Figure 15

Figure 15 shows the percentage of correct responses over time for two subjects, A and B, under two conditions, C and D. The y-axis represents the percentage of correct responses (0-100), and the x-axis represents time in sessions (1-10). The data shows that both subjects performed at 0% correct responses across all sessions and conditions.

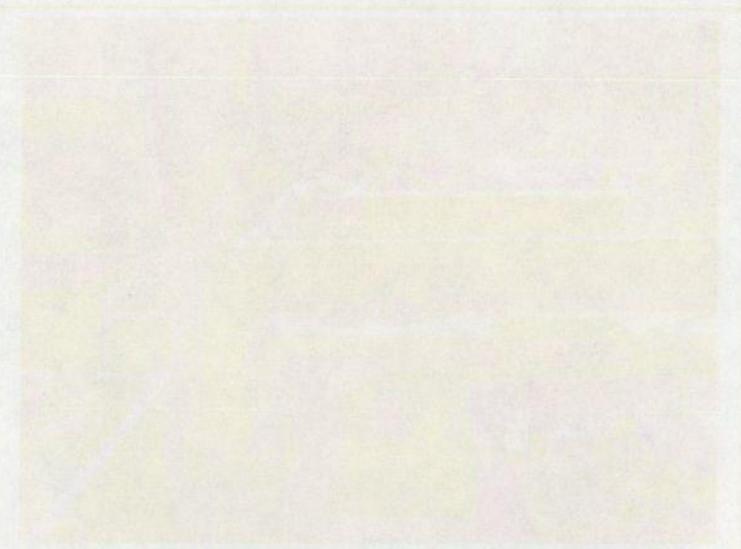


Figure 16

Figure 16 shows the percentage of correct responses over time for two subjects, A and B, under two conditions, C and D. The y-axis represents the percentage of correct responses (0-100), and the x-axis represents time in sessions (1-10). The data shows that both subjects performed at 0% correct responses across all sessions and conditions.

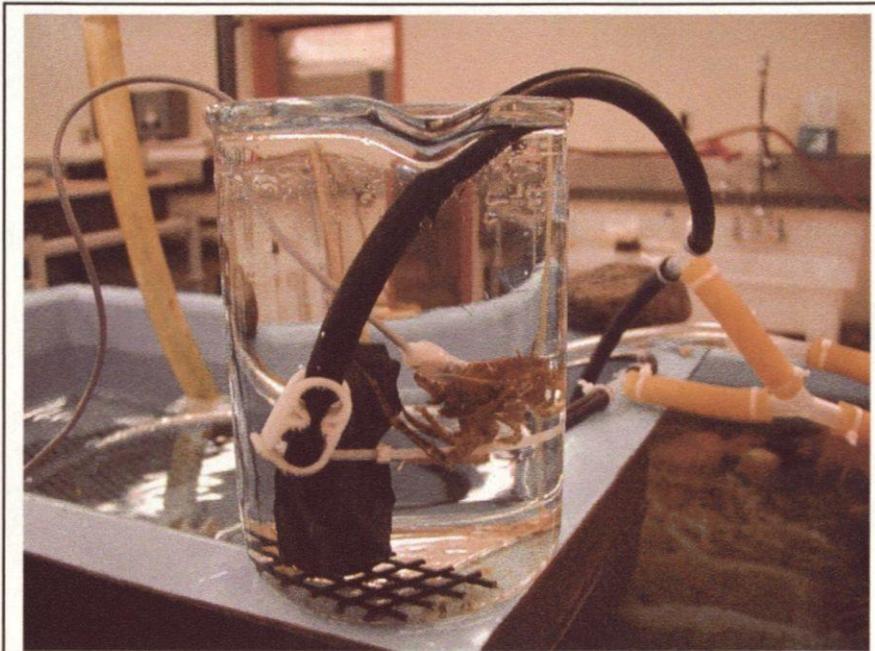


Plate 27.

Isopod connected to an infra red sensor. The water was aerated. A grid on the bottom helps the isopod to anchor to the bottom.



Plate 28.

Infra red sensor glued on the anterior part of an isopod for heart rate measurement.



Figure 1

Figure 1 shows a person in a white lab coat, likely a scientist or researcher, standing in a laboratory setting. The person is looking down at something on a table or piece of equipment. The background is slightly blurred, showing what appears to be laboratory equipment and a clean, professional environment.

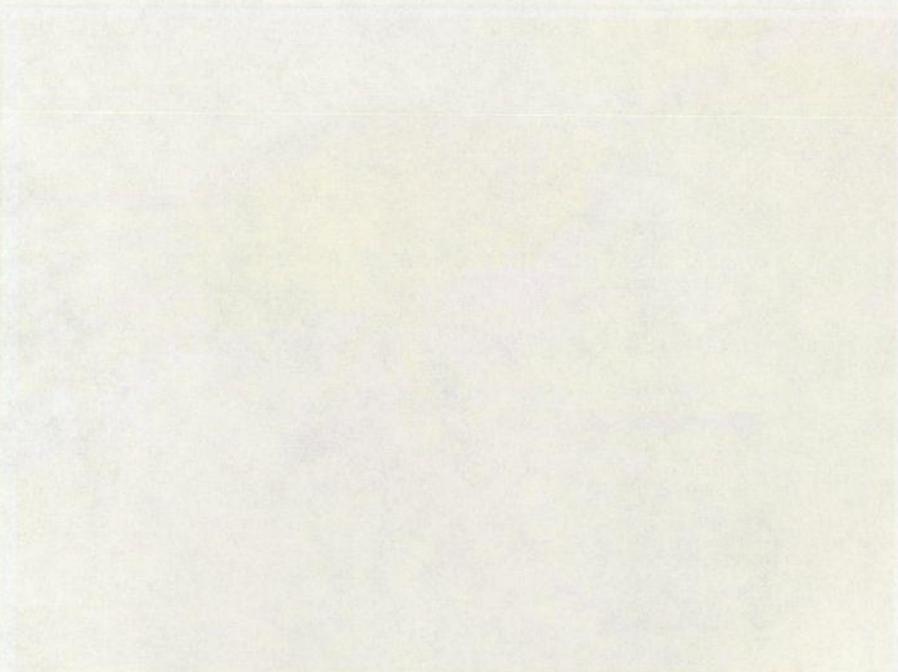


Figure 2

Figure 2 shows a person in a white lab coat, likely a scientist or researcher, standing in a laboratory setting. The person is looking down at something on a table or piece of equipment. The background is slightly blurred, showing what appears to be laboratory equipment and a clean, professional environment.

**Interpretation of experimental data:  $Q_{10}$  and the Arrhenius plot**

A commonly used mathematical model to describe the relationship between reaction rate and the temperature is due to Arrhenius (1889). It originates from chemistry where the higher the temperature, the faster a given chemical reaction will proceed. In other words, at higher temperatures, the probability that two molecules will collide is higher. This higher collision rate results in a higher kinetic energy, which has an effect on the activation energy of the reaction. The activation energy is the amount of energy required to ensure that a reaction happens. The Arrhenius equation has also been particularly used by physiologists studying the temperature behaviour of specific metabolic processes such as respiration (Clarke and Johnston 1999). The Arrhenius equation is as follows:

$$k = A e^{-E_a/RT}$$

Where  $k$  = the rate constant

$A$  = Frequency Factor ( $L \text{ mol}^{-1} \text{ s}^{-1}$ )

$E_a$  = Energy of Activation ( $\text{kJ mol}^{-1}$ )

$R$  = Gas Constant =  $8.315 \times 10^{-3} \text{ kJ mol}^{-1} \text{ K}^{-1}$

$T$  = Absolute Temperature (K)

The Arrhenius plot gives a straight line, the rearranged Arrhenius equation is as follows:

$$\ln(k) = \ln(A) - E_a/R (1/T)$$

$\ln(k)$  = Y axis

$\ln(A)$  = Intercept

$E_a$  = Slope

$1/T$  = X axis

Another mathematical relationship to describe data temperature dependant is the (Van't Hoff 1884). It describes the relationship between the rate of a process and temperature as the function  $Q_{10}$ , defined as the ratio of the rates of a process  $10^\circ\text{C}$  apart:

$$Q_{10} = \text{rate at } (T+10) / \text{rate at } T$$

Where, T = temperature in degrees Celsius.

$Q_{10}$  may be calculated for any temperature interval as:

$$Q_{10} = (k_2/k_1)^{10/(T_2-T_1)}$$

Where  $K_1$  and  $K_2$  are the rates at  $T_1$  and  $T_2$ , respectively.

The use of the  $Q_{10}$  relationship stems from the observation, formulated by Van't Hoff (1884), that the rate of a chemical process often approximately doubles for every 10°C rise in temperature ( $Q_{10} = 2$ ). A value of  $Q_{10}$  in the region of 2 to 3 reflects a system that behaves normally.

#### **4.5.7. Protein determination**

Total protein concentrations were determined spectrophotometrically following the method of Bradford (1976).

#### **4.5.8. PAH concentration analysis**

##### *Extraction of sediment and tissue samples for GC/MS-selective ion monitoring analysis*

Whole body tissue of the studied organism was cut into fine pieces by means of scissors until a homogenous sample was reached. Then, the following procedure was applied to bivalve tissue and sediment. Eight deuterated PAHs as quantitation internal standards (QIS) covering the molecular size of interest (naphthalene-d8, phenanthrene-d10, dibenzothiophene-d8, fluoranthene-d10, pyrene-d10, chrysene-d12, benzo(a)pyrene-d12 and dibenzo(a,h)anthracene-d14), were added to the homogenised tissue or sediment (5-10g) and boiled for 2 hr in a 10 % (w/v) solution of potassium hydroxide in methanol to achieve saponification. The digest was filtered and extracted repeatedly three times with cyclohexane. Cleanup of extracts was performed by solid phase extraction using 3 ml tubes containing 0.5 g of normal phase packing (Supelclean LC-Si, Supelco INC, Bellefonte, PA, USA). Further extraction was performed with a mixture of dichloromethane: cyclohexane (1:3). Recovery internal standard (RIS) mixture was added and samples were stored in capped vials until GC/MS-analysis.

**GC/MS-Selective ion monitoring analysis**

PAH analysis of all samples was performed by Gas Chromatography (HP5890, Hewlett Packard, USA) connected to a Mass Spectrometer (Finnigan SSQ7000, USA) and analysed in selected ion mode (GC/MS-SIM). The GC was equipped with a CP-SIL 8CB fused silica column (Chrompack, 50m x 0.25mm i.d., film thickness 0.25  $\mu$ m). Injector and detector temperatures were both 300°C. The column was held at 50°C for 1 min, ramped at 25°C min<sup>-1</sup> to 120°C, and at 3°C min<sup>-1</sup> to 320°C and held for 17 min at 320°C. Helium was used as carrier gas, with a flow rate of 0.6ml min<sup>-1</sup> at 50°C.

Tissue samples of *M. truncata* and sediment were analysed for 26 parent PAH compounds (including dibenzothiophenes), and expressed respectively as  $\mu$ g kg<sup>-1</sup> lipid and  $\mu$ g kg<sup>-1</sup> wet sediment. The sum of all analysed PAHs was designated as total PAH (TPAH).

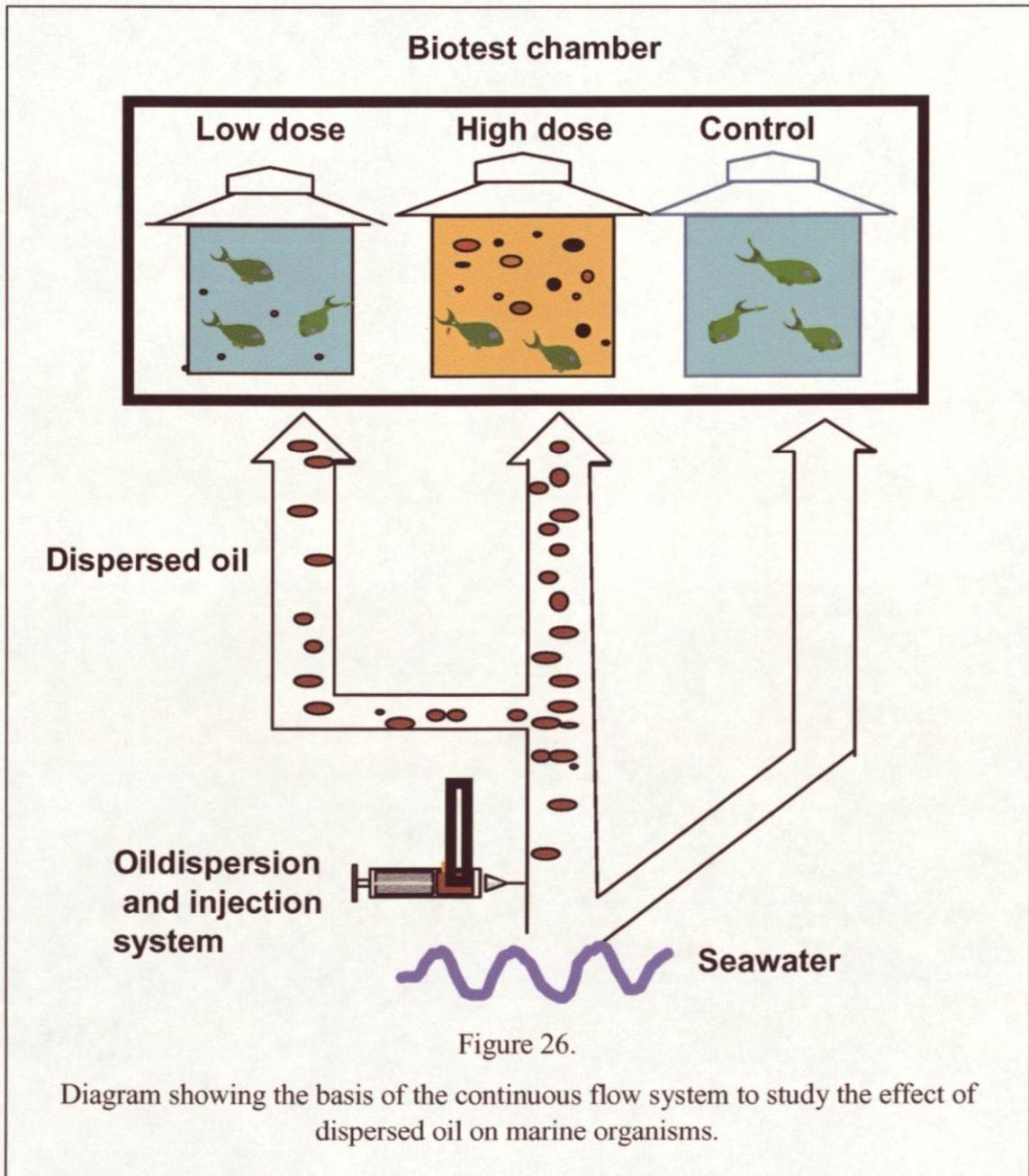
Lipid concentrations were measured in samples according to the method of Folch *et al.* (1957).

**Calculation and quality assurance of GC/MS analyses**

Calibration standards for all non-alkylated PAH compounds were prepared in seven different concentrations (10  $\mu$ g l<sup>-1</sup> – 2000  $\mu$ g l<sup>-1</sup>) and response factor curves were calculated. For calculation of alkylated PAH, the response factor curves for the respective non-alkylated homologues were used. The reproducibility of the response factors for three standards, covering the concentration range, was checked for each series of samples analysed, and the calculated concentrations were not allowed to exceed 20% deviation from the expected concentrations. RIS and QIS were added to the calibration standards in concentrations of approximately 500  $\mu$ g l<sup>-1</sup> of the individual deuterated compounds. One procedure blank and one control sample were extracted for each series of samples (n=12). The controls used were either a certified reference material (SRM2974, NIST, Gaithersburg, MD, USA) or an unexposed sample spiked with an appropriate amount of a certified mixture of PAH (Dr Eherenstorfer Reference Materials, Augsburg, Germany). The recovery of PAH in the samples was calculated by comparing the relative signal intensity of RIS and QIS in samples and calibration standards (defined to be 100%). Extraction recovery for 2-ring PAH compounds was at least 30 % in sediment samples and at least 40% in tissue samples. Extraction recovery for larger PAH compounds was at least 50% in both sediment and tissue samples.

#### 4.5.9. The Continuous Flow System (CFS) for dispersed oil exposure

The system described previously in Sanni *et al.* (1998) consisted of a series of three chambers closed with conical lids. Each exposure chamber contained a central cylinder with a propeller stirred at a speed of *ca.* 250 rpm for homogenization of the test solution and circulation of the water (Plate 29). The chambers (Figure 26) were connected to the continuous-flow system with test solution input at the bottom and output at the top. A flow rate of *ca.* 250 ml min<sup>-1</sup> was maintained through the chambers, enough to meet the oxygen requirement of the animals. Oil was kept in a dark, sealed storage bottle and was directly pumped in the stream of seawater at a constant flow rate according to the exposure concentration. No solvent was added. Droplets were created with an ultrasonic processor that produced dispersion (80 mg l<sup>-1</sup>) which was further diluted and delivered to the aquaria by peristaltic pumps (Taban *et al.* in prep). The total oil concentration, estimated from the concentration of sum PAH in the water, was approximately 8 times higher than the oil concentration estimated from volume of particles with size between 1.4 and 30 µm. The rest of the oil was either dissolved, had a smaller particle size than 1.4. The Multisizer measurements showed that 95 % of the oil droplets in the size range between 1.4 and 30 µm were less than 10 µm in size and the mean size, based on number of particles in size range 1.4 to 30 µm was 1.86 µm. The mean size of particles based on volume was 3.1 µm for particles in size range 1.4 to 10 µm and 4.1 for particles in size range 1.4 to 30 µm. The size distribution graphs indicated that few particles were larger than 10 µm and that many particles were smaller than 1.4 µm. Air and seawater temperatures were adjusted at 2°C. A sublethal concentration of oil was injected into the system during exposure periods. Water samples were collected in burned 2 l Duran glass bottles with glass stoppers, containing hydrochloric acid to ensure that the pH of the sample did not exceed 2. The samples were stored at 4°C in a dark room. Extraction of oil was performed the day after sampling. The total PAH concentration determined (sum PAH) in the treatment chamber was 10-26 µg l<sup>-1</sup>. The variations in oil exposure concentrations were due to instability with the oil pumps. PAH constitute 0.83% of the crude oil (Baussant *et al.* 2001) giving an oil concentration of 1-3 ppm. These data, together with the data from the Coulter Multisizer analyses, indicate an average oil exposure concentration of 2±1 ppm.



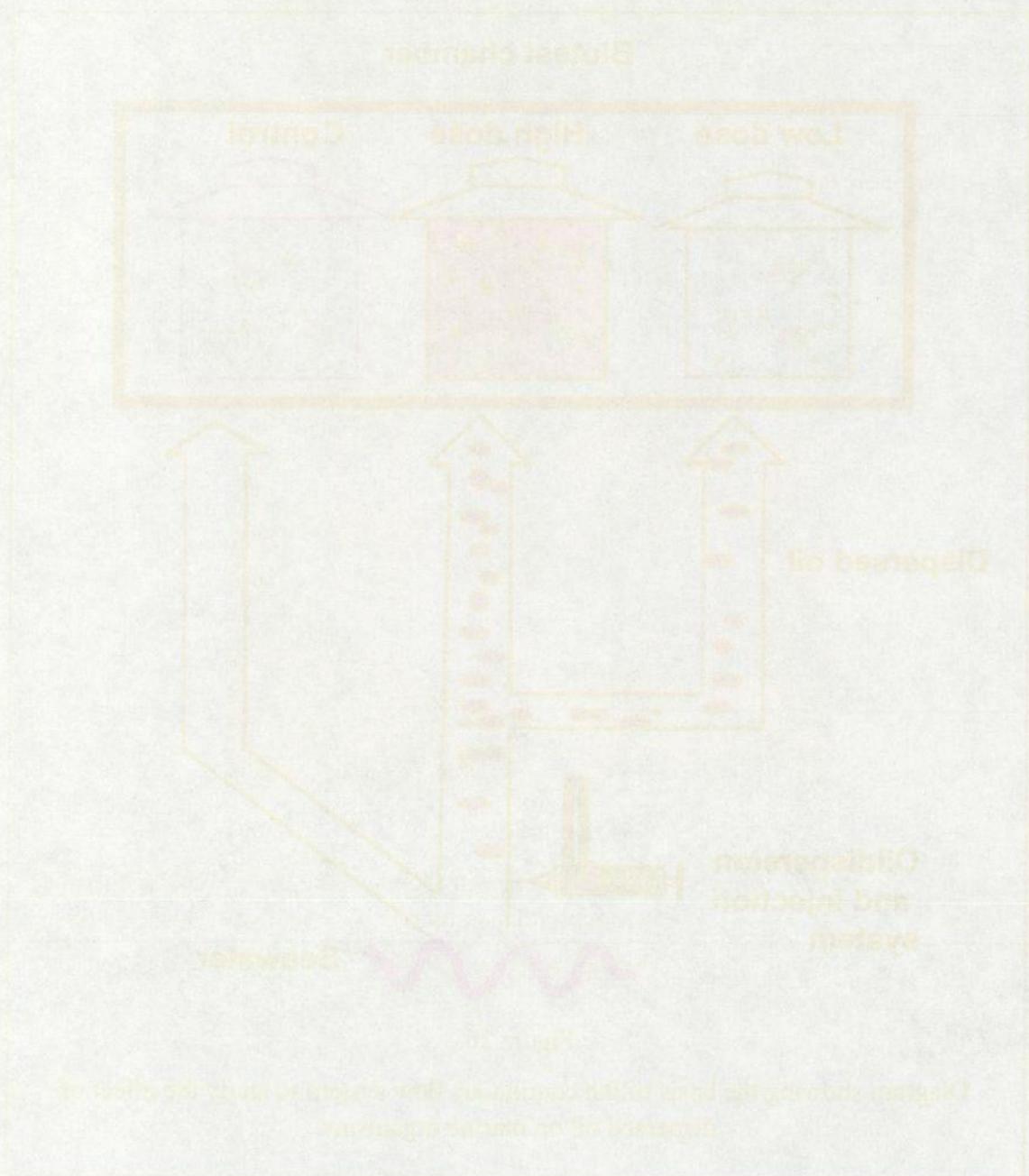


Diagram showing the flow of the control agent from the steamer to the biological chamber and the dispersal of the control agent.

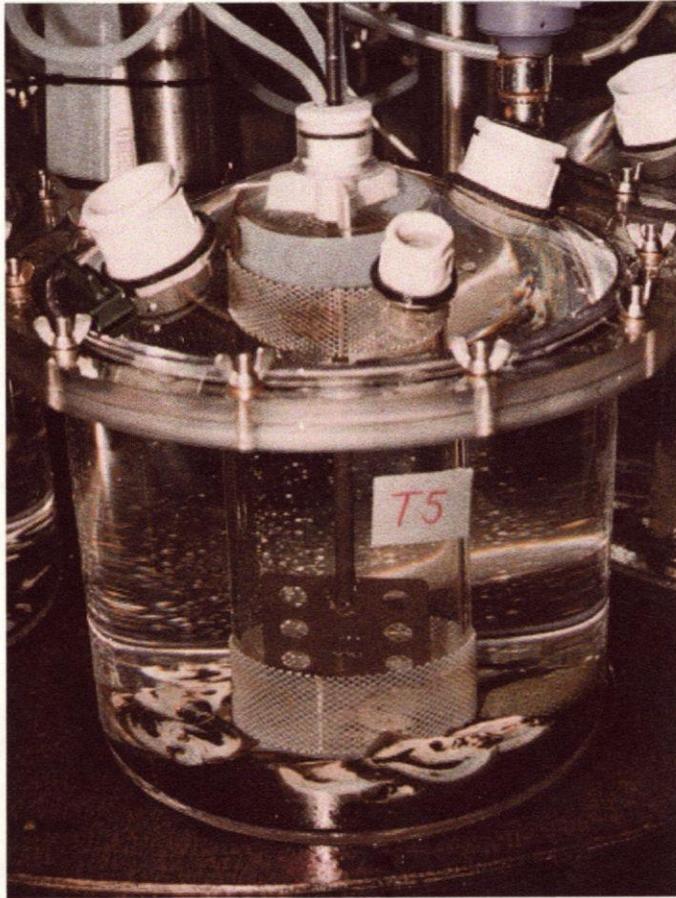


Plate 29.

A chamber of the CFS (with turbots). Note the propeller in the middle of the chamber for continuous agitation.

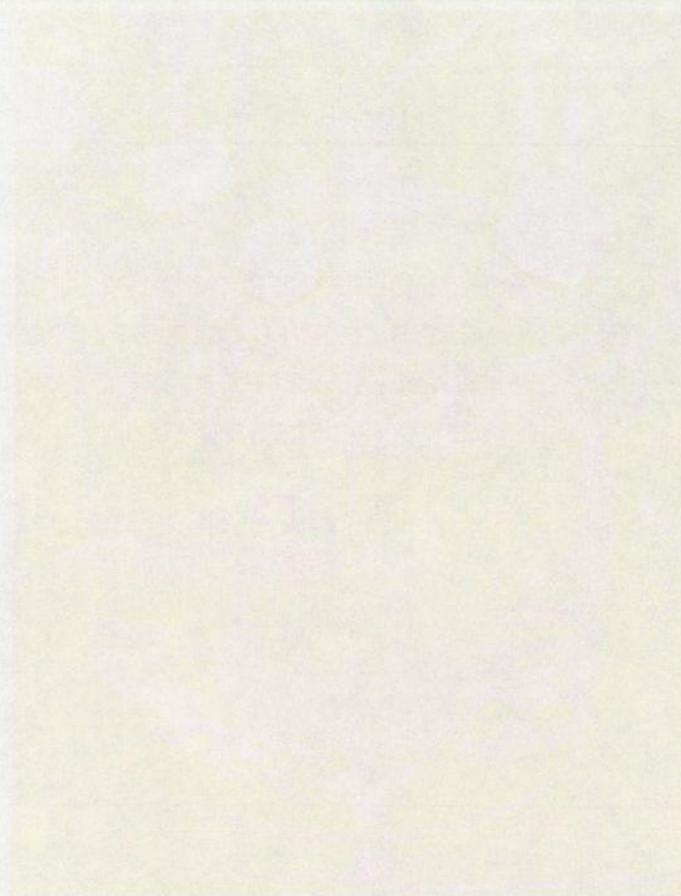


Table 1

A summary of the findings of the study, including the number of participants, the mean age, and the range of scores on the dependent variables.

## Chapter 5

### **Influences of low temperature acclimation of the temperate bivalve *Mytilus edulis* on the lysosomal and cell membranes stability and total oxyradical scavenging capacity**

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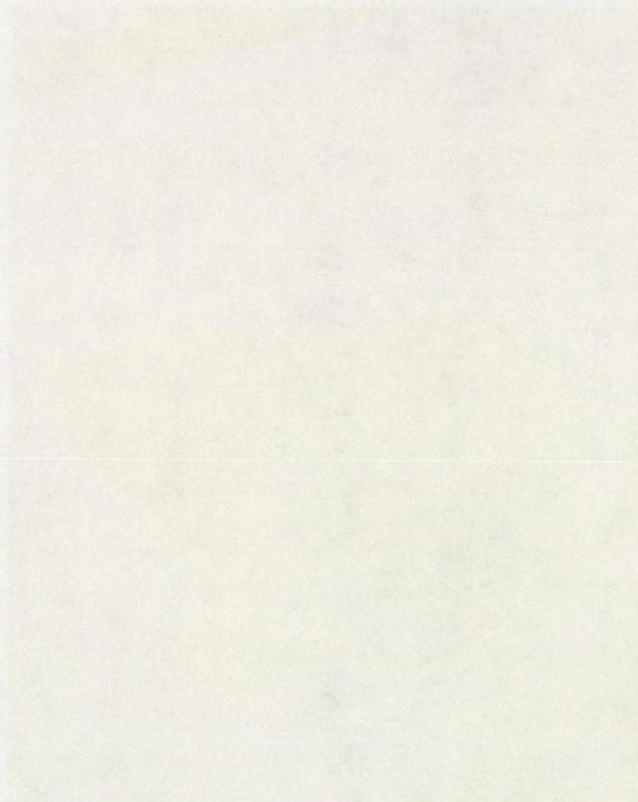
*“Quand à l’ours... le terrifiant ours polaire...Oublions-le! Il peut peser jusqu’à huit cents kilos! Et même quand la banquise est splendide de sérénité, j’ai toujours la pénible sensation qu’il se tient là, tapi quelque part, prêt a m’assaillir par-derrière...Dans le Grand Nord, c’est le danger par excellence, le symbolique gardien du pole.”*

**Jean Louis Etienne, polar French explorer.**

### Chapter 5

## Influence of low temperature acclimation of the temperate bivalve *Mytilus edulis* on the lysosomal and cell membrane stability and total oxidant scavenging capacity

Roberta M. Basso, Ludovico Basso, and Roberto M. Basso



Abstract: The present study was designed to evaluate the effects of low temperature acclimation on the lysosomal and cell membrane stability and total oxidant scavenging capacity of the temperate bivalve *Mytilus edulis*. The animals were acclimated to 10°C for 30 days. The results showed that low temperature acclimation led to a significant increase in the lysosomal membrane stability and total oxidant scavenging capacity, suggesting that the bivalve is able to adapt to low temperature by enhancing its antioxidant defense system.

Key words: *Mytilus edulis*; low temperature; lysosomal membrane stability; total oxidant scavenging capacity

## 5.1. Abstract

Expanding industrial activities in the Arctic require an urgent assessment of the toxicity of chemicals at low temperatures. Organisms acclimatized to low temperature exhibit specific adaptations. For example, the amount of unsaturated lipids is increased to maintain the fluidity of the cell membranes. It has been hypothesised that such temperature-induced alterations in membrane lipid composition may affect the stability of lysosomal and cell membranes in the common mussel, *Mytilus edulis*, an organism exposed to seasonal temperature extremes. Moreover, antioxidant defences are known to be low in winter, therefore, we tested whether low temperature reduced the total oxyradical scavenging capacity (TOSC) of *M. edulis*. As mussels may be exposed to petroleum compounds along industrialized coastlines, we tested the combined effects of exposure to low temperature and the petroleum compound, phenanthrene, on haemocyte membrane stability. Test animals, acclimated to either 0 °C or 10 °C, were exposed to phenanthrene (0=control or 500µg.l<sup>-1</sup>) and haemocytes were examined using the neutral red retention assay (lysosomal stability) and a fluorescence assay (cell membrane stability). The TOSC was performed according to the method of Winston *et al.* (1998). At 0 °C, lysosomal and cell membranes from uncontaminated mussels were destabilised compared with the same group at 10 °C ( $p=0.0005$ ); the TOSC was also significantly reduced ( $p<0.05$ ). No significant effects ( $p>0.05$ ) of phenanthrene were detected at either temperature. Possible mechanisms underlying membrane destabilisation include a weaker physical resistance of the membrane due to a higher amount of unsaturated lipids, an oxidative damage due to the reduced TOSC, a potentially higher level of reactive oxygen radicals at low temperature, and the higher susceptibility of unsaturated lipids to oxidative stress. More work is required to better understand the consequences of this membrane destabilisation at low temperature on the susceptibility of the organism to pollutants.

## 5.2. Introduction

At low temperatures, the cell membranes of ectothermic organisms undergo structural changes to maintain viscosity and enzyme kinetic properties (Hochachka and Somero 1984). Sinensky (1974) termed this response to temperature "homeoviscous adaptation" (HVA); this concept was reviewed recently by Hazel (1995). Early studies showed that the membrane composition of temperate bivalves, *Crassostrea virginica* and *Mercenaria mercenaria*, was affected by acclimation to subzero temperature (Gillis and Ballantyne 1999b). *Mytilus californianus* has the ability to adjust membrane composition on a seasonal basis (Williams and Somero 1996). The blue mussel (*Mytilus edulis*), is a temperate species that experiences large seasonal fluctuations in temperature; for instance, surface seawater temperature in Norwegian fjords can fluctuate from -1.88°C up to 20°C. Lysosomal membrane stability has been used thoroughly in biomarker studies to assess pollution impact in the field (Lowe *et al.* 1995, Fernley *et al.* 2000, Wedderburn *et al.* 2000). Lysosomes are subcellular organelles that accumulate and degrade contaminants (Lowe *et al.* 1995), and are sites of oxyradical production (Winston *et al.* 1996). The physical stress imposed on the lysosomal membrane by the accumulation of pollutants and lipid peroxidation due to oxidative damage leads to a breakdown of the membrane and severe impairment of the cell. Therefore, one objective of this study was to investigate whether cold-acclimated lysosomal and cell membranes are more vulnerable to stress than at warmer temperature.

Antioxidant defences prevent membrane lipid peroxidation, caused by reactive oxygen species (Winston and Di Giulio 1991). Low antioxidant defences and lipid peroxidation have been reported in *Mytilus sp.* in winter (Viarengo *et al.* 1991, Sheehan and Power 1999). The link existing between antioxidant defenses and membrane stability and the effects of season on these parameters raised the need to investigate the effect of low temperature on the total oxyradical scavenging capacity of *M. edulis*.

As blue mussels are exposed regularly to low temperatures during the winter in Norwegian Fjords, the present study set out to establish the effects of low temperature on: i) cell membrane stability of haemocytes, ii) lysosomal membrane stability in haemocytes, and iii) the total oxyradical scavenging capacity in the hepatopancreas of *M. edulis*. In addition, the effects of exposure to a polycyclic aromatic hydrocarbon (phenanthrene) were investigated at low temperature.

### 5.3. Materials and Methods

In October 1998, *Mytilus edulis* were collected from a clean reference site in F rlandsfjorden, Rogaland (Norway). The ambient temperature was around 10  C. Mussels were monitored in running seawater and fed with algae (*Isochrysis sp.*). For use in low temperature experiments, mussels were acclimated at 0  C for 1.5 months. Experiments were carried out simultaneously at 10  C and 0  C using 15 mussels per treatment and 500  g.l<sup>-1</sup> phenanthrene as the chemical exposure was used. Each group of mussels was placed in a tank containing 4.5 l of aerated seawater. The toxicant (diluted in acetone) was added first to 100 ml of algae solution and then transferred to the tank. An acetone control was also performed to measure the effect of acetone (no effect was measured, data not shown). A control with exposure to algae only was performed (data shown). The exposure medium was renewed every 24 h. After 4 days, the haemolymph from individual mussels was sampled and mixed (1:1) with physiological saline. Two assays were carried out on the haemocytes. Firstly, the retention of neutral red dye was measured in lysosomes following the procedure of Lowe *et al.* (1995) (see section (4.5.4.)). The second assay measured the membrane stability of haemocytes isolated from the various treatment groups (see section 4.5.3.). The hepatopancreas was excised, frozen and stored at -80 C prior analysis according to the method of Winston *et al.* (1998) and optimised for *Mytilus edulis* by Regoli *et al.* (1998) (see section 4.5.2.).

Non-parametric Wilcoxon tests were performed on these retention time measurements. Data of haemocyte membrane stability were distributed normally but were Boxcox transformed to meet equal variance conditions and analysed with one-way ANOVA test. Data of the TOSC were distributed normally and differences were identified with t-tests.

### 5.4. Results

Results showed that the neutral red retention time (Figure 27) and the ratio BFLV/EthD-1 (Figure 28) were both significantly reduced in the control groups at 0  C compared to 10  C ( $p < 0.05$ ). These results indicate that the membranes of lysosomes and haemocytes were destabilised at low temperature. TOSC was significantly reduced at 0 C ( $p < 0.05$ ) (Figure 29). No significant effects of phenanthrene on membrane stability could be detected by either method at 0  C or 10  C ( $p > 0.05$ ) (Figures 27 and 28).

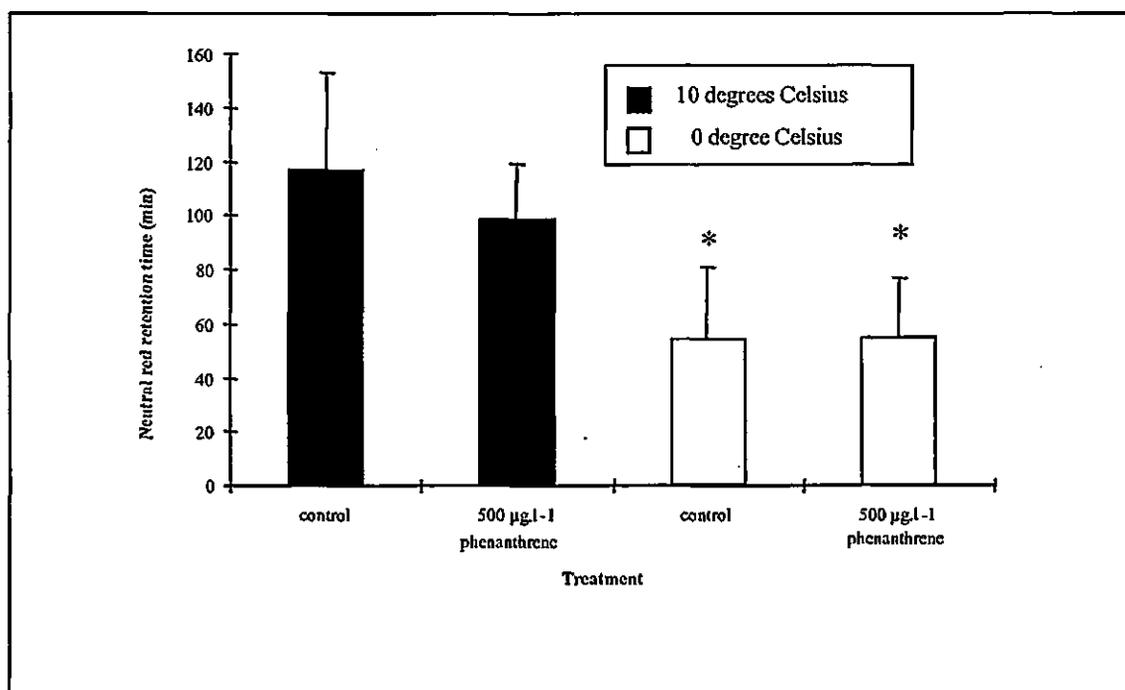


Figure 28.

Bar chart to show the lysosomal membrane stability (neutral red retention time) in haemocytes of *Mytilus edulis* exposed to 500 µg.l<sup>-1</sup> phenanthrene (mean and standard deviation,  $n=15$ ). Asterisks indicate significant effect of low temperature on the stability of the membrane compared with 10 °C ( $p=0.0005$ ).

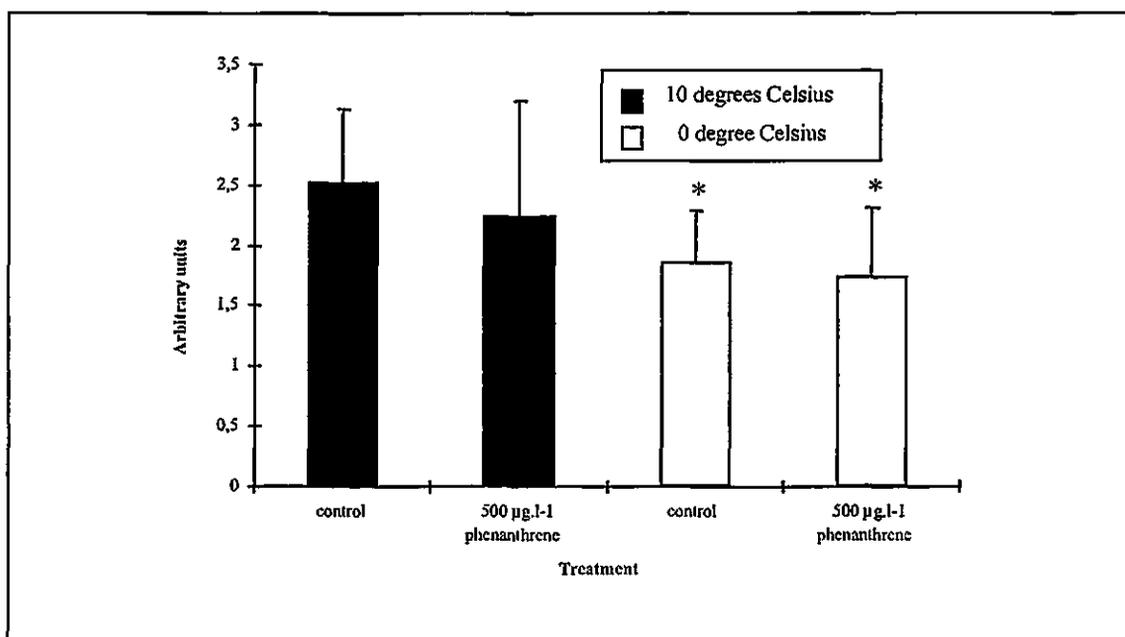
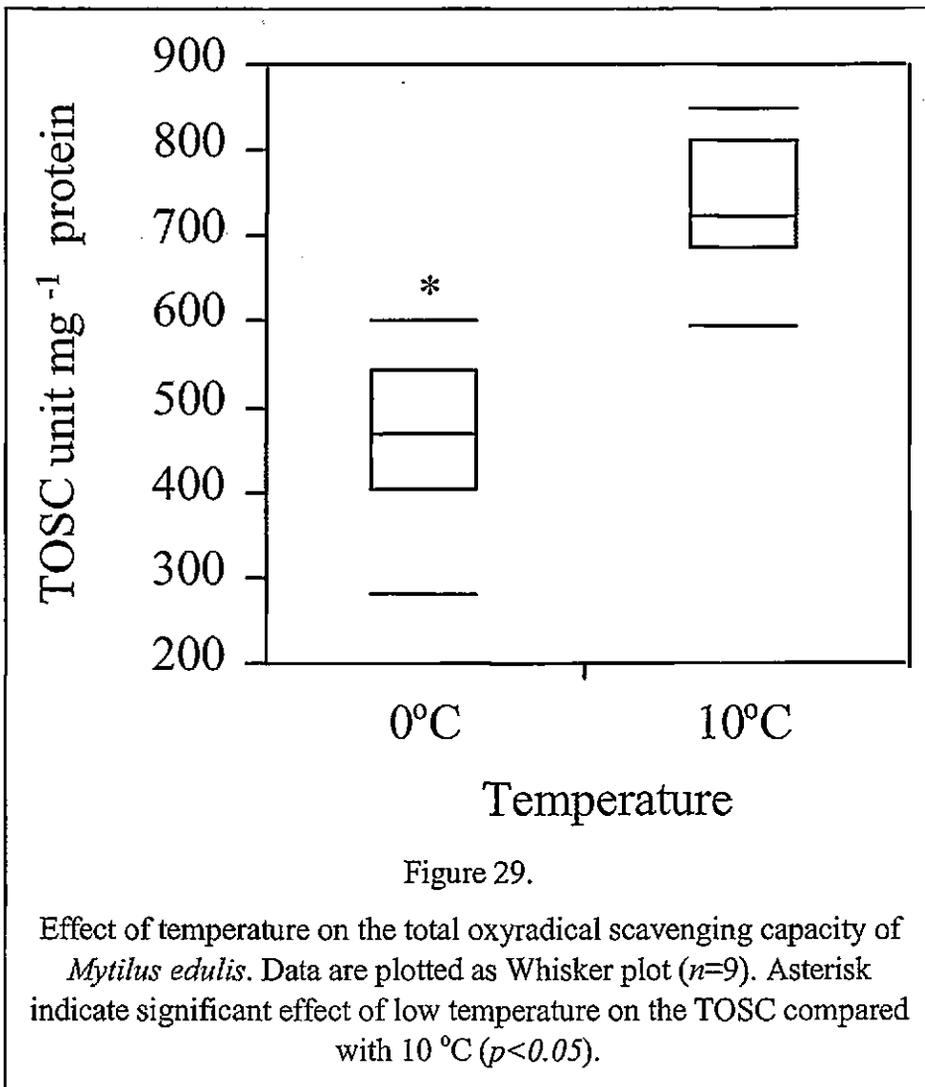


Figure 27.

Bar chart to show the cell membrane stability [ratio of fluorescence intensity of BFLV and EthD-1 (arbitrary unit)] of haemocytes of *Mytilus edulis* exposed to phenanthrene (mean and standard deviation  $n=15$ ). Asterisks indicate significant effect of low temperature on the stability of the membrane compared with 10 °C ( $p<0.05$ ).





### 5.5. Discussion

Present results indicate that lysosomal and cell membranes of the haemocytes of *Mytilus edulis* are more fragile at 0 than at 10°C. In addition, TOSC was significantly reduced at lower temperature. The membrane composition of bivalves adapted to low temperature is different compared with higher temperature to maintain fluidity of the membrane components (Gillis and Ballantyne 1999b). This metabolic compensation may help explain the more destabilised membrane at 0°C than at 10°C. In addition, at low temperatures, higher dissolved oxygen concentrations occur, providing a major source of reactive oxygen species (ROS) (Regoli *et al.* 2000). Organisms exposed to low temperatures, therefore, experience more oxidative stress than animals living at high



temperatures. ROS are known to be generated in lysosomes (Winston *et al.* 1996) and cause membrane lipid peroxidation. Since unsaturated lipids are more vulnerable to ROS than saturated lipids, it might be expected that membranes would be more susceptible to peroxidation at 0 °C than at 10 °C. Moreover, the decreased TOSC measured here indicate that *Mytilus edulis* is less capable of coping with ROS at low than at higher temperature. A decreased activity of antioxidant defences has been reported in winter conditions in *Mytilus sp* (Viarengo *et al.* 1991, Power and Sheehan 1996, Cancio *et al.* 1999, Sheehan and Power 1999). Viarengo *et al.* (1991) reported that a reduction of the antioxidant defence systems in winter was concomitant with an increase in lipid peroxidation. Therefore, the decreased membrane stability measured in the current study may be caused by lipid peroxidation due to the decreased TOSC. ROS are naturally produced due to aerobic metabolism. About 2 to 3% of the oxygen consumed are converted to ROS (Chance *et al.* 1979). A low respiration rate in *Mytilus edulis* at -1°C as observed by Loo (1992), could reduce the natural ROS production and, therefore, the TOSC as well.

In *Mytilus edulis*, the metabolism of PAH leads to the production of oxyradicals (Livingstone *et al.* 1990). Grundy *et al.* (1996) showed that the neutral red retention time was strongly reduced in the lysosomes of mussels exposed to phenanthrene for 14 days at 20 °C. No effects of phenanthrene on membrane destabilisation were recorded in the present experiment, nevertheless, bioaccumulated levels of phenanthrene were high and were not affected by the low temperature. The fact that bioaccumulation of phenanthrene was not affected by the temperature treatment supported the work of Loo (1992) who reported a filtration activity and high absorption efficiency for *M. edulis* experiencing seawater at -1°C. Thus, the exposure time to this compound in the present study was probably too short to induce any effects. A reduced endocytosis at low temperature (Rode *et al.* 1997) could have limited the uptake and accumulation of phenanthrene into the lysosomes.

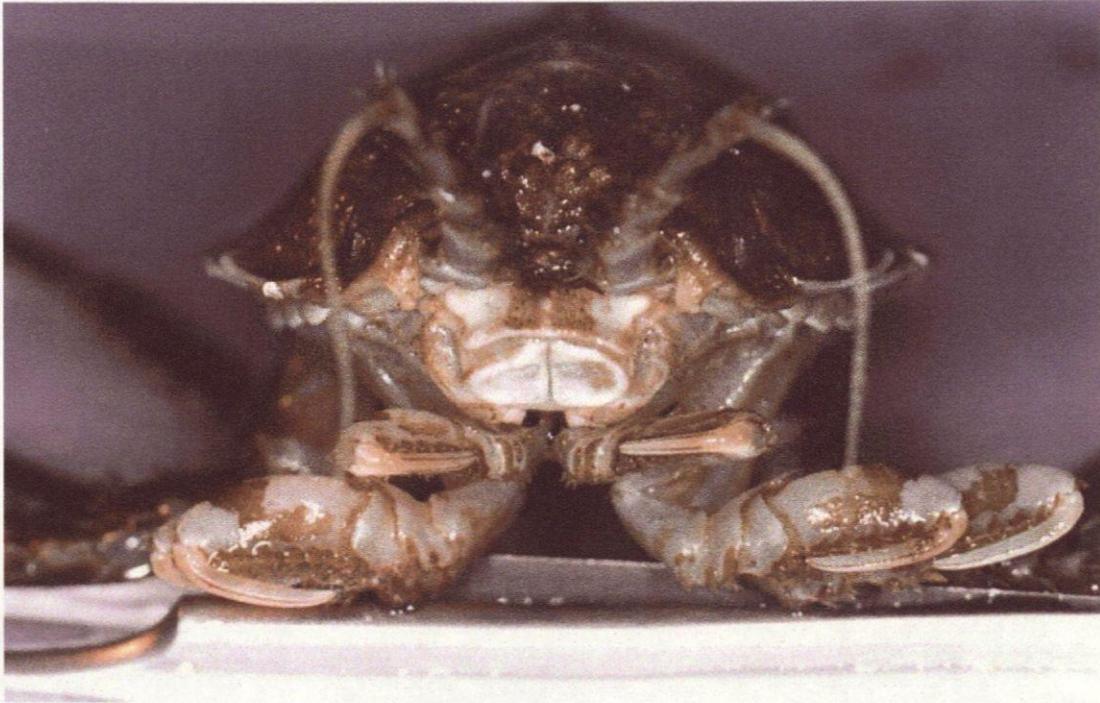
## 5.6. Conclusions

Assessing the cell membrane stability of haemocytes using BVFL and EthD-1 is a reliable method providing information of membrane stability with automated readings. Mussels acclimatized to 0 °C exhibited decreased stability of lysosomal and cell membranes in haemocytes compared with 10 °C. A decreased TOSC at low temperature

indicates that the decreased membrane stability may also be caused by lipid peroxidation. Phenanthrene exposure did not decrease further membrane stability at either temperature.

## Chapter 6

### Heart and respiration rates of polar crustaceans.



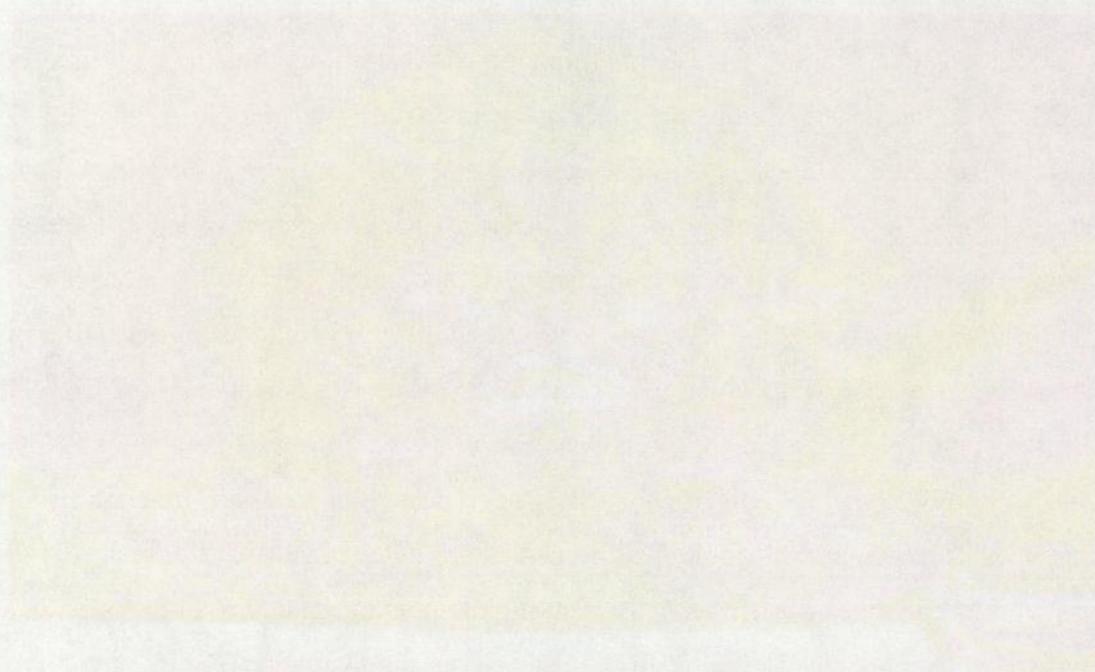
*Glyptonotus antarcticus*

*“As soon as the ice was strong enough to bear in the bay, Murray commenced his operations there. His object was the collection of the different marine creatures that rest on the bottom of the sea or creep about there.”*

**Sir Ernest H. Shackleton, Antarctic expedition 1907-1909.**

## Discussion

### Heart and respiration rates of polar charrs



**Fig. 1** Heart and respiration rates

of charrs in the Arctic and Antarctic regions. In the Arctic, charrs are found in a wide range of habitats, from high-altitude lakes to low-altitude streams. In the Antarctic, charrs are found in a more limited range of habitats, primarily in the sub-Antarctic islands.

The present study shows that heart and respiration rates of charrs increase with temperature.

## 6.1. Effect of acute temperature change on the heart and respiration rates of polar crustaceans

### 6.1.1. Introduction

Heart and respiration rates of temperate marine invertebrates have been thoroughly investigated. When measured simultaneously they can provide good understanding of the physiological condition of the animal, notably its respiratory functions (Widdows 1973, Eshky *et al.* 1996). Temperate marine organisms are physiologically adapted to temperature fluctuation and experience seasonal and daily variations in temperature (Bojsen *et al.* 1998, Styriehave *et al.* 1999). Cardiac activity and respiration rates of temperate invertebrates increase with increasing temperature (Ahsanullah and Newell 1971, DeFur and Mangum 1979, Wernick 1982). Similar information on heart rate of polar marine crustaceans is lacking. Polar marine ecosystems are characterized by very stable temperature (Clarke 1988, Weslawski *et al.* 1988). This temperature stability affects the physiology of indigenous species such that their rates of functioning of different biochemical and physiological processes are independent of temperature. For example, the heat-shock protein expression at high temperature, commonly viewed as a 'universal' characteristic of organisms, is absent in the Antarctic fish *Trematomus bernachii* (Hofmann *et al.* 2000). Evidence of an absence of thermoacclimatory responses to the changes in oxygen affinity was shown in the Antarctic isopod *Glyptonotus antarcticus* (Jokumsen *et al.* 1981). Numerous studies reported relatively low respiration rates for polar crustaceans, and these were interpreted as a selective advantage in an environment where temperature is low and resources limited (White 1975, Whiteley *et al.* 1996, 1997). Earlier studies reported the effect of temperature change on the respiration rate of polar crustaceans (White 1975, Aarset and Aunaas 1990), but no investigations have been performed on the heart rate of polar marine crustaceans. Therefore, in this present study, the sensitivity of the heart and respiration rates of polar crustaceans to acute temperature change was investigated to determine whether these physiological responses can provide information about polar adaptation. The selected species for the investigation were the Arctic benthic shrimp *Sclerocrangon boreas*, the Arctic crab *Hyas araneus* and the Antarctic isopod *Glyptonotus antarcticus*.

### 6.1.2. Materials and Methods

In summary, heart rate was measured with the CAPMON system from Depledge and Anderson (1990) (see section 4.5.6.) and respiration rate (see section 4.5.5.) was measured with a closed respirometer technique and a Clarke electrode coupled to a water cooled unit. Effect of temperature on the heart rate was measured before the measurements of the respiration rates. Any change (increase or decrease) in water temperature was performed slowly over 2h. Animals were acclimatised to the new temperature for one more hour before starting the heart rate measurement or incubation for respiration rate measurements.

As the upper lethal temperature limit for *G. antarcticus* is reported to be around 6°C (Arnaud 1985), the effect of temperature for this species was assessed within the range -1.5 to 4°C. Arctic crustaceans appear to be more tolerant to higher temperature (Aarset and Aunaas 1990) hence, the temperature was raised to 10°C in the current experiment. The same individuals were used to measure the heart and respiration rates except for *Hyas araneus*, though individuals with similar body size were selected. Animals of similar size were selected to compare the heart and respiration data (Table 1).

Table 1 : mean and standard deviation of dry and wet weight of *G. antarcticus*, *H. araneus* and *S. boreas*.

	Dry Weight (g)	Wet Weight (g)
<i>Glyptonotus antarcticus</i> (n=5)	4.61±0.73	22.92±4.88
<i>Hyas araneus</i> (n=5)	5.06±0.56	15.07±1.94
<i>Sclerocrangon boreas</i> (n=7)	3.32±0.56	15.07±1.94

### 6.1.3. Results

#### *Heart rate*

The heart rate of each species increased with temperature and the mean heart rate of each species was similar at low temperature. A  $Q_{10}$  of 2 indicated an increase of the heart frequencies of *S. boreas* and *H. araneus* in the temperature range 0 to 5°C (Figures

31 and 33). A  $Q_{10}$  of 1.54 between 5 and 10°C indicated a moderate effect of temperature on the heart rate. The overall  $Q_{10}$  in the temperature range 0 to 10°C is similar, 1.74 and 1.61 for *S. boreas* and *H. araneus*, respectively.

A different pattern was observed for *G. antarcticus* though the temperature range was narrower. An increase occurred between -1.5 to 2°C ( $Q_{10}=1.27$ ), followed by a marked rise from 2 to 4°C characterised by a  $Q_{10}$  of 4 (Figure 35). The  $Q_{10}$  between -1.5 to 4°C of 1.86 was comparable to the  $Q_{10}$  of the Arctic shrimp and crab within the same temperature range.

#### **Respiration rate**

The effect of temperature on the respiration rate of the Arctic species was different from the response measured in the heart rate. The oxygen uptake was stable from 0 to 5°C ( $Q_{10}=1.01$ ) in *S. boreas* but doubled from 5 to 10°C as indicated by a  $Q_{10}$  close to 4 (Figure 30). The  $Q_{10}$  from 0 to 10°C was 1.95, indicating a doubling of the metabolic rate over 10°C temperature increase (Figure 30).

The respiration rate of *H. araneus* was not affected by 5°C increase from 0 to 5°C as indicated by  $Q_{10}$  below zero (0.4) (Figure 32). This is confirmed by a  $Q_{10}$  of 1.05 over the temperature range 0 to 10°C, indicating that the increase of 10°C in temperature did not affect the respiration rate of *H. araneus* (Figure 32).

The  $Q_{10}$  of 2.41, calculated in the temperature range -1.5 to 4°C, indicates that the metabolic rate of *G. antarcticus* was temperature sensitive (Figure 34).



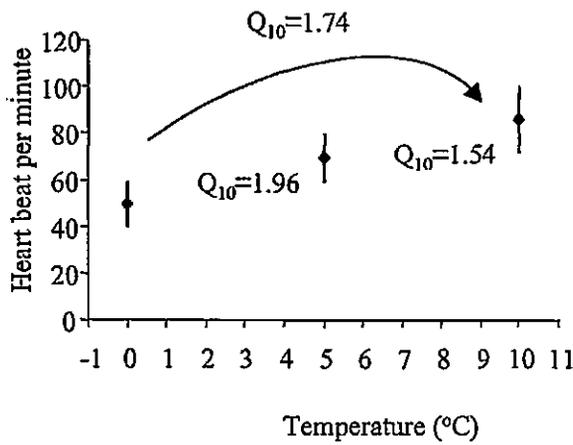


Figure 31. Effect of temperature on the heart rate of *Sclerocrangon boreas* (mean±standard deviation).

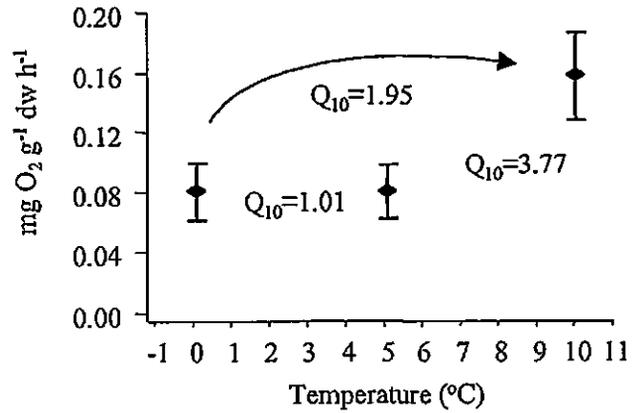


Figure 30. Effect of temperature on the respiration rate of *Sclerocrangon boreas* (mean±standard deviation).

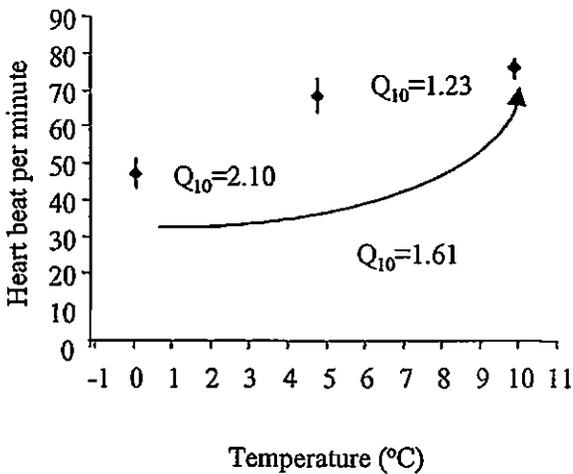


Figure 33. Effect of temperature on the heart rate of *Hyas araneus* (mean±standard deviation).

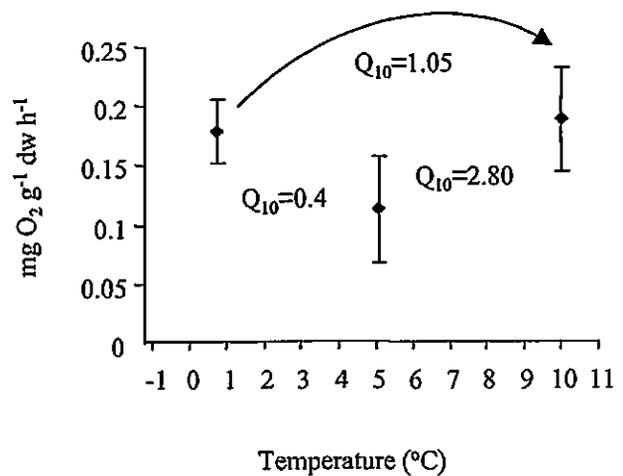


Figure 32. Effect of temperature on the respiration rate of *Hyas araneus* (mean±standard deviation).

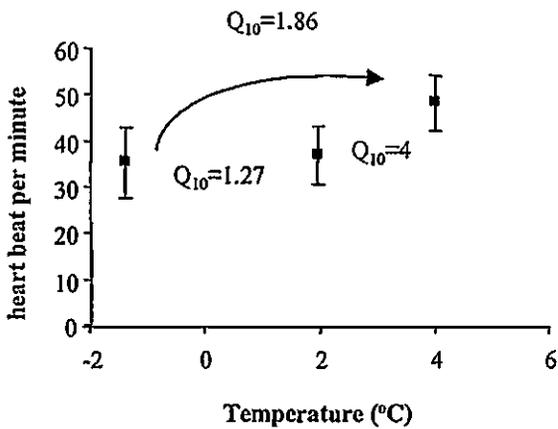


Figure 35. Effect of temperature on the heart rate of *Glyptonotus antarcticus* (mean±standard deviation).

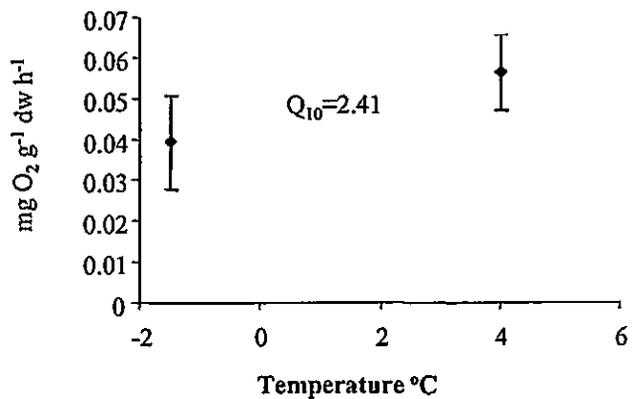


Figure 34. Effect of temperature on the respiration rate of *Glyptonotus antarcticus* (mean±standard deviation).

#### 6.1.4. Discussion

##### *Heart rate*

Acute temperature change affected the heart rate of all the Arctic crustaceans. However, the heart rate of *G. antarcticus* was shown to be insensitive to temperature change within the range  $-1.5$  to  $2^{\circ}\text{C}$ , reflecting a physiological adaptation to this temperature range that corresponds to the natural fluctuation of the Antarctic seawater temperature (Clarke 1988). Nevertheless, the overall  $Q_{10}$  for each species was close to 2. According to the Van't Hoff rule, the general response to increased temperature is an increased oxygen consumption rate, reflecting that enzymatic reaction rates approximately double with a temperature increase of  $10^{\circ}\text{C}$  indicated by a  $Q_{10}$  of 2. This response has been observed previously in the heart rate of *Carcinus maenas* (Ahsanullah and Newell 1971). The gradual reduction in  $Q_{10}$  with increasing temperature observed for *S. boreas* and *H. araneus*, is supported by the results of other studies (Ahsanullah and Newell 1971, Eshky *et al.* 1996), although the temperature ranges of the studies were different. The decrease in  $Q_{10}$  in the upper temperature range may indicate that the unusual elevated temperature imposes some physiological constraints to the organisms. For instance, the mechanical action of the heart is limited to a certain frequency that is optimized to run at low temperature in polar crustaceans. Earlier studies reported that the biochemical, metabolic and morpho-functional features of the heart of the Antarctic icefish was cold adapted (Tota *et al.* 1997). Examination of the effect of temperature change on the heart rate of porcelain crabs from temperate waters revealed some thermal tolerance limits in agreement with the natural environmental temperature that the crustaceans experience in the field. *Hyas araneus* is a temperate species that is migrating northward and colonising Arctic water. It is, therefore, well adapted to cope with temperature changes. *Sclerocrangon boreas* is a true Arctic species, although it is abundant in the Barents Sea where the temperature can fluctuate up to  $6^{\circ}\text{C}$  (Loeng 1991). The increased heart rate may be a physiological compensation to the decrease in dissolved oxygen levels in the water at high temperature.

##### *Respiration rate*

Temperature change affected the respiration rate of the Arctic species in a different manner compared with heart rate. *Sclerocrangon boreas* appeared insensitive to temperature change between 0 to  $5^{\circ}\text{C}$ . The respiration rate of *H. araneus* was not

affected in the temperature range 0 to 10°C and a decrease was noted from 0 to 5°C. The winter-acclimatized individuals of the benthic amphipod *Onissimus affinis* were relatively temperature insensitive between 0°C and 8°C with a  $Q_{10}$  of 1.36 (Percy 1975). Similar results have been demonstrated for the Arctic copepod *Calanus glacialis* (Tande 1988) and the Antarctic krill *Euphausia superba* (McWhinnies 1964). Relative insensitivity of the respiration rate to temperature change was also noted in the intertidal hermit crab *Clibanarius vittatus* (Wernick 1982). This relative insensitivity would help the organisms to keep the metabolism low in order to prevent energy expenditure where temperature fluctuations are narrow and food is limiting.

In contrast, a marked increase in the respiration rate of *G. antarcticus* indicated by a  $Q_{10}$  of 2.41 was observed. The  $Q_{10}$  value is in agreement with the work performed by White (1975). These observations indicate that *G. antarcticus* does not have the ability to maintain the metabolic rate constant when a temperature change occurs as for *S. boreas* and *H. araneus*. Temperature sensitivity of the respiration rate was demonstrated in 8 polar crustacean species (Hirche 1984, Aarset and Aunaas 1990). The increase in heart rate from -1.5 to 4°C helps explain the enhanced oxygen uptake measured in this study.

The heart rate is relatively comparable between species, the bigger size of the isopods compared with the two other Arctic species can explain a lower heart rate at low temperature. On the contrary, the respiration rate is different between species. *G. antarcticus* shows a very low respiration rate as reported by White (1975). The oxygen uptake of *S. boreas* is two times higher than *G. antarcticus* and two and half times lower than *H. araneus* at low temperature. In polar ectotherm organisms, a low respiration rate has been reported which appears to be an adaptive strategy to limit energy expenditure in a low food supply environment (Whiteley *et al.* 1996). The elevated metabolism of *H. araneus* and its temperature insensitivity is certainly attributable to its temperate origin. *Glyptonotus antarcticus* is a true Antarctic species (Whiteley *et al.* 1996), and *S. boreas* is in the Arctic and, therefore, expresses typical physiological adaptation to these specific polar regions. Nevertheless, the Antarctic isopod was shown to be stenothermal with an upper temperature limit of 6°C. *Sclerocangon boreas* experienced the rise in temperature up to 10°C without showing signs of lethal effects. Ice amphipods like *Gammarus wilkitzkii* experience extreme low temperatures of -1.88°C but sometimes lower as the brine water occurring in the ice crevasses during ice formation low. In spite of being adapted to low temperature, the ice



amphipods showed a very temperature sensitive respiration rate and were able to survive at 10°C (Aarset and Aunaas 1990). It is probable that the shorter evolutionary time of Arctic species compared with Antarctic species may explain this difference in stenothermality.



## 6.2. Heart rate of polar crustaceans and the metabolic cold adaptation

### 6.2.1. Introduction

Low temperature and limited seasonal temperature variation characterise the polar marine ecosystem. For McMurdo Sound in winter, reported temperatures were  $-2^{\circ}\text{C}$  (Littlepage 1965) and up to around  $0^{\circ}\text{C}$  in summer. The temperature in the Arctic fjord of Svalbard ranges from  $-1.86^{\circ}\text{C}$  to  $3^{\circ}\text{C}$  (Weslawski *et al.* 1988). As a consequence of this very stable temperature, most of endemic polar marine invertebrates are stenothermal and many benthic species have an upper lethal temperature limit of  $6^{\circ}\text{C}$  (Arnaud 1985).

The possibility of elevated metabolic rates in cold-water ectotherms has been a topic of debate over many years. The idea that metabolic rates of polar ectotherms should be elevated to compensate for the physiological constraints imposed by low temperature was first proposed by Krogh (1916). Scholander *et al.* (1953) and Wohlschlag (1964) advanced the concept of Metabolic Cold Adaptation (MCA) but this has not been accepted generally (Clarke 1980, 1983). Clarke and Johnston (1999) showed no metabolic cold adaptation for fish and the metabolic rate of cold-water bivalves was not elevated (Peck *et al.* 1997, Ahn and Shim 1998). In polar crustaceans, Whiteley *et al.* (1996) showed that the giant Antarctic isopod, *G. antarcticus*, does not show classic 'metabolic' cold adaptation but maintains relatively low oxygen uptake rates at  $0^{\circ}\text{C}$  as reported by White (1975). Because respiration rate is related to the heart rate in marine invertebrates (Widdows 1973, Eshky *et al.* 1996), the concept of MCA could be investigated by measuring the heart rate of crustaceans. Cardiac activity has been measured in many temperate marine decapod crustaceans and the conclusion is that the temperature is the main physical environmental factor that affects the heart rate of crustaceans (Ahsanullah and Newell 1971, DeFur and Mangum 1979, Morris and Taylor 1984, Aagaard 1996, Bojsen *et al.* 1998). While cardiac activity has been studied in Antarctic fish (Axelsson *et al.* 1992, Macdonald 1997), no data on cardiac activity of polar crustaceans have been reported. The cardiovascular adaptations that allowed Antarctic ectothermic animals to live in cold water are of particular interest. Without any compensating mechanism, the hearts of cold-blooded animals might be expected to perform proportionally more work, since blood, in common with other fluids, has an increased viscosity at low temperatures (Graham and Fletcher 1985). Moreover, it is

expected that *G. antarcticus* shows a low heart rate in relation to the low oxygen uptake demonstrated by White (1975). One role of the haemolymph circulation is to distribute biological material for growth, egg production and for the excretion of wastes. Because of the low protein production rate of *G. antarcticus* (Whiteley *et al.* 1996), the isopod may show a limited haemolymph circulation rate and consequently a low heart rate.

In this study, the *in vivo* cardiac activities of *G. antarcticus*, *H. araneus* and *S. boreas* were measured. Published studies on crustacean heart rate from temperate to tropical regions were compared and discussed with reference to the concept of metabolic cold adaptation.

### 6.2.2. Materials and Methods

Five individuals of *Glyptonotus antarcticus* were collected by divers from the jetty of McMurdo American research station (Ross island, Antarctica) in January 2000 (Figure 22). Isopods were kept in a large tank with running seawater, pumped from the Sound at a temperature of  $-1.5^{\circ}\text{C}$ , for at least 24 h before any experiments. The average length of the isopods was  $76.5 \pm 6.2$  mm and the average fresh weight was  $22.92 \pm 4.87$  g ( $n=5$ ). No food was provided during the experiment. Eight individuals of the spider crab *Hyas araneus* were collected from Hollenderbukta in Isfjorden (Svalbard, Arctic) in May 1999 (Figure 21). Crabs were taken with an Agassiz dredge from *ca.* 50m depth using the research vessel F/F Jan Mayen of the University of Tromsø. Seawater temperature was  $-1.5^{\circ}\text{C}$ . Small spider crabs (wet weight =  $13.00 \pm 3.91$ g, carapace length =  $37.79 \pm 3.20$  mm, carapace width =  $27.29 \pm 2.65$ mm) were selected for the experiments. Crabs were stored in cooled running seawater. No feeding was allowed and heart rate measurements were performed at  $0^{\circ}\text{C}$  on board 24 h after capture. In September 2000, eight individuals of the shrimp *Sclerocrangon boreas* were collected at the same location and handled the same way as *H. araneus* (wet weight =  $15.07 \pm 1.94$ ; dry weight =  $3.32 \pm 0.56$ ). Measurements of heart rate were performed in the laboratory at the "University Courses on Svalbard". Heart rate of the polar species was compared with the heart rate of 6 crustacean species living in temperate and tropical regions; data for temperate and tropical species were extracted from the literature (Table 2). Comparison was based on animal size ranging between 15 g and 25 g wet weight.

### ***Heart rate measurement***

Cardiovascular monitoring was performed with a non-invasive procedure (CAPMON) developed by Depledge and Andersen (1990) (see section 4.5.6.).

### **6.2.3. Results**

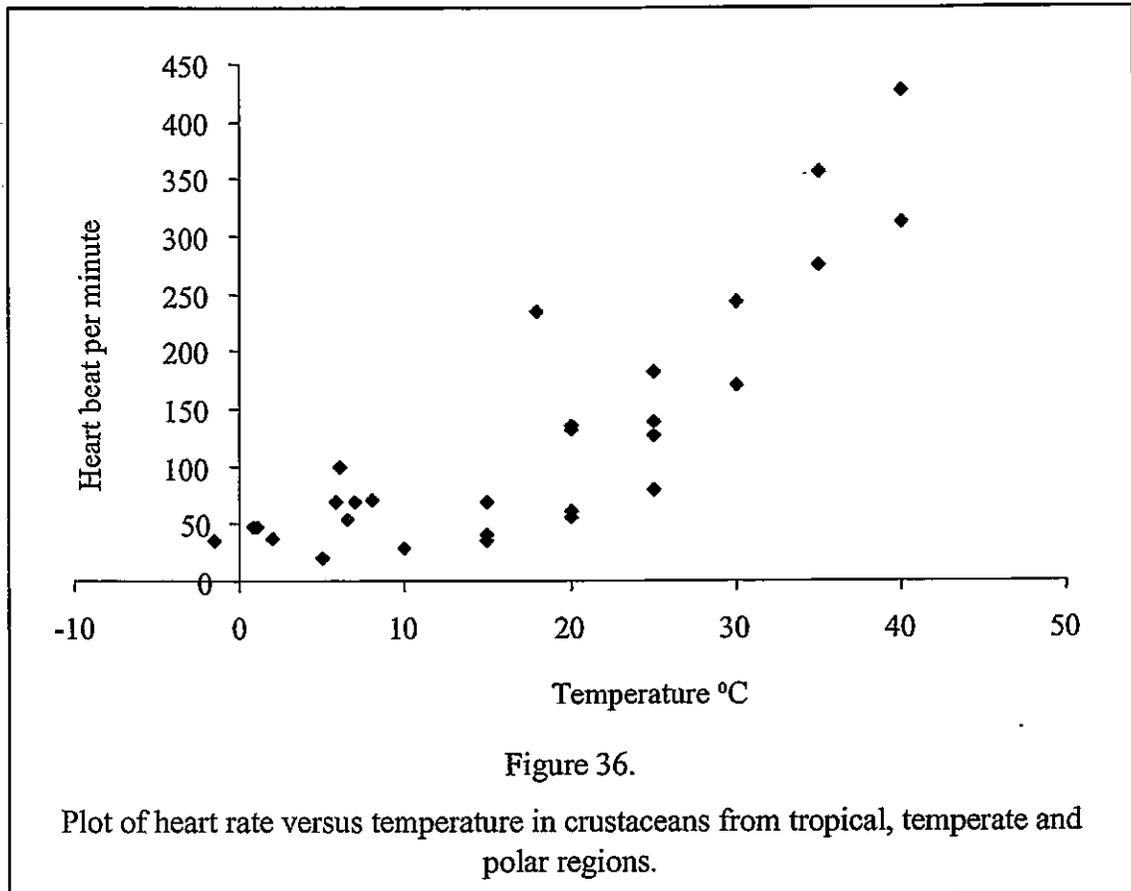
The plot of heart rate versus temperature (Figure 36) showed that heart beat frequencies of polar species are located in the lower left corner of the graph, indicating that heart rate at low temperatures are lower than heart rate at higher temperatures. An Arrhenius plot (Ln heart rate vs reciprocal of absolute temperature) produced a mathematical relationship between heart rate and temperature for polar species, and for tropical and temperate species (combined) which are significantly modelled by a linear relationship (Figure 37). An ANCOVA analysis showed evidence of no significant differences between slopes ( $p=0.978$ ) but a difference in y-intercept ( $p=0.021$ ). Hence, Polar crustaceans species show a low cardiac activity compared to other non-polar species but the higher intercept reveals a slight compensation in heart rate compared with expected values from extrapolation of the curve heart rate versus temperature of tropical and temperate species.

Regression line for temperate and tropical data:  $y = 24.89 - 5.96x$ ,  $r^2 = 0.68$ ,  $p < 0.0001$ ; Pearson coefficient = 0.83,  $p < 0.0001$ .

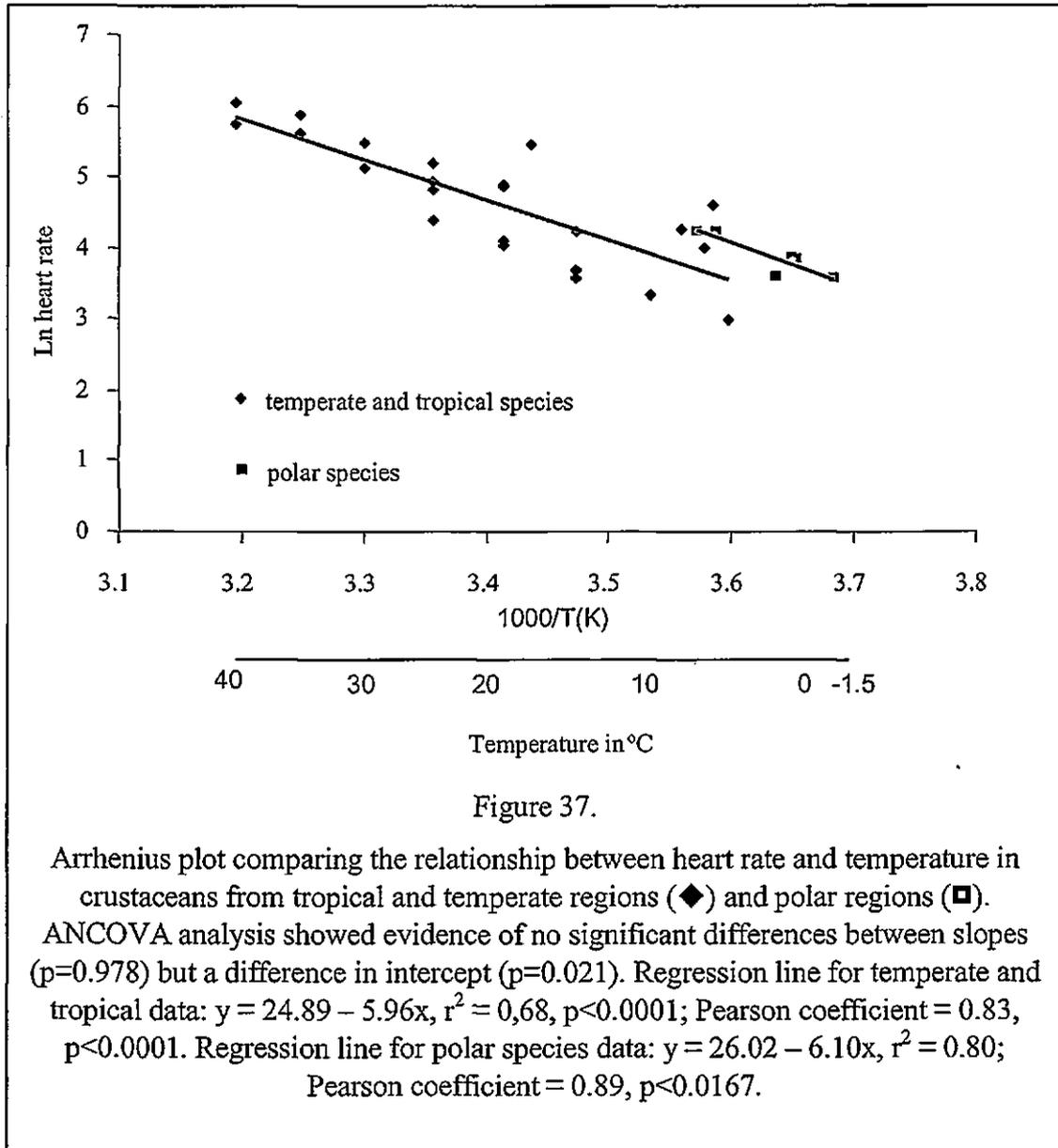
Regression line for polar species data:  $y = 26.02 - 6.10x$ ,  $r^2 = 0.80$ ; Pearson coefficient = 0.89,  $p < 0.0167$ .

Table 2: heart rate data were extracted from the literature. Species, temperature of measurement, mean heart rate and standard deviation and references are indicated.

Species	Reference	Temperature (°C)	Mean heart rate (bpm)
<i>Glyptonotus antarcticus</i>	Present study	-1.5	35.38±5.40
<i>Hyas araneus</i>	Present study	2	36.65±3.79
		1	47.26±2.94
		7	68.48±9.68
<i>Sclerocrangon boreas</i>	Present study	0.8	47.02±5.64
		5.8	68.99±4.35
<i>Carcinus maenas</i>	(Styrishave <i>et al.</i> 1999)	5	19.72
		10	27.99
		15	39.72
		20	56.36
		25	80
<i>Hemigrapsus edwardsi</i>	(Depledge 1978)	6.5	54
	(Depledge and Lundebye 1996)	8	70
<i>P. hebsti</i>	(DeFur and Mangum 1979)	6	100
		20	131.7
<i>M. nudus</i>	(DeFur and Mangum 1979)	18	235
<i>M messor</i>	(Eshky <i>et al.</i> 1996)	15	69.2
		20	134.71
		25	181.85
		30	242
		35	355
		40	427
<i>Uca inversa</i>	(Eshky <i>et al.</i> 1996)	15	36
		20	60
		25	139
		30	171
		35	274
		40	312
<i>Procambarus clarkii</i>	(Reiber and McMahon 1998)	25	125.6







#### 6.2.4. Discussion

##### *Resting heart rate at normal environmental temperature conditions*

In this study, data on *in vivo* heart rate of Antarctic and Arctic crustaceans are reported for the first time and compared with temperate and tropical species. Polar crustaceans show lower heart rate compared with temperate species. Little data exist on the heart rate of temperate species experiencing low temperature in winter although Depledge (1978) reported heart rate values for *Carcinus maenas* of 54 bpm at 6.5°C. Styriehave *et al.* (1999) measured a heart rate of *ca.* 20 bpm at 5°C and *P. hebsti* heart rate was 100 bpm at 6°C. In spite of the scarcity of heart frequency in crustaceans, temperate species seem to show similar heart rates at low temperature compared with summer heart frequency of Arctic species.

##### *Inter-species comparison of heart rate*

By plotting data of the 10 crustaceans species across the temperature range 0°C to 40°C (Figure 37), a positive significant temperature effect on heart rate was revealed. The Arrhenius mathematical relationship obtained in this study indicated that a rise in temperature from 0°C to 30°C causes an elevation in heart rate of 8 times for a standard crustacean of *ca.* 20 g wet weight. This would give a  $Q_{10}$  of 2 between the temperature range 0°C to 30°C. The Arrhenius plot of polar species shows a similar slope compared with the Arrhenius plot of the pooled data for tropical and temperate crustaceans. Nevertheless, the y-intercept of the polar species line is higher. This could reflect a higher heart rate in polar species than would be predicted from temperate and tropical species extrapolation. Calculated heart rate frequency at -1.5°C for temperate/tropical species would be 19 bpm. Heart frequency of *G. antarcticus* at the same temperature is 1.8 times higher. However, the compensation is weak compared to that reported for antarctic fishes that show a nearly complete temperature compensation, so that their rates at the ambient sub-zero temperatures are comparable with those of non-Antarctic fishes at temperatures 10-20°C higher (McDonald 1997). Acclimation of *Carcinus maenas* (a temperate crustacean species), to several temperatures between 5°C and 25°C for two weeks, showed that the heart rate was still markedly temperature dependent and a rather poor compensation had occurred (Ahsanullah and Newell 1971). Investigation of mussel heart rate acclimated to different temperatures revealed conflicting results



since no compensation was reported by Widdows (1973) while perfect compensation was shown by Pickens (1965).

The reasons for a low heart rate are unclear. The compensation may represent the need to keep up the heart rate at a certain level to sustain the physiological machinery which normally should be reduced at low temperature. But the limited compensation may reflect the importance of reducing the metabolic activities to save cost in an environment where food supply is limited. Indeed, the circulation system is part of the respiratory physiology and also carries biological material required by the animal for growth, locomotion and reproduction. Hemolymph carries molecules such as amino acids, glucose, lipids that originate from digested food. Therefore, the low heart rate of polar crustaceans reported in this study may reflect the low food availability and the necessity for the animal to restrict blood flow circulation to minimize energy expenditure.

Only 6 species were extracted from previous published work and combined with the polar crustacean data set to investigate the effect of temperature on heart frequency. This restricted number of species selected for comparison requires caution because: i) even though heart rate in crustaceans has been extensively studied, few species have been investigated; ii) direct comparison is limited by the size of the animal and a range size of 15 to 25 g was the optimal size to compare a maximum number of species. Due to the large diversity in body shape between crustaceans, it is difficult to establish a general mathematical relationship heart rate versus body mass; iii) very few relationships between heart rate and weight have been studied in the past that would allow calculation of the heart rate of a species for any realistic size (in our case, *ca.* 20g wet weight). For instance, in our data review, it has not been possible to include heart rate of species like *Cancer magister* and *Homarus americanus* (McMahon 1999) because only large individuals have been selected in earlier studies. An identical problem was faced with very small crustacean species like the grass shrimp *Palaemonetes pugio* (Harper and Reiber 1999). Therefore, some of the plots for the Arrhenius relationship are data calculated from mathematical models of heart rate versus temperature (Styrishave *et al.* 1999) or model heart rate versus weight (Eshky *et al.* 1996) (nevertheless calculations were carried out within realistic temperature or weight ranges).

**Heart rate vs oxygen uptake for metabolic rate**

Clarke and Johnston (1999) argued that an Arrhenius relationship is the best descriptor of whole animal metabolism. Therefore, it could be postulated that heart rate can provide similar information on whole animal metabolism. Ivleva (1977, 1980) calculated a  $Q_{10}$  of 2.26, with an Arrhenius plot of the oxygen consumption of a representative crustacean of 1 mg dry weight, which is similar to our calculated  $Q_{10}$  value of 2 between 0 to 30°C. Phylogenetic constraints and environmental history are important when investigating evolutionary adaptations. Comparisons should, as far as possible, be made within a phylogenetically consistent group (Clarke and Johnston 1999). The age and consistency of an environment are also important, because if current conditions have existed for only a short period (on evolutionary timescales) it is unlikely that either many species will have evolved adaptations, or that such adaptations will have reached a stable equilibrium. In this context, data for Antarctic crustaceans (*G. antarcticus*) can be interpreted differently to Arctic data. The Antarctic marine environment is one of extreme thermal stability and these conditions have been in existence for at least 5-10 million years. The Arctic marine ecosystem, however, is relatively new, about 3 million years (Clarke 1983), and largely influenced by Atlantic water. From the biological point of view, a large number of species living in the Arctic originates from Atlantic water while in Antarctica, most species are true endemic species. Further, decapod crustaceans do not occur in Antarctica, while isopods (and amphipods) are widely spread. These are therefore, limits to the present model since it is rather difficult to investigate heart rate in temperate and Arctic isopods due to their small size. Nevertheless, the similarity in heart rate of the Arctic species and the Antarctic species supports the model reported in this study. Frederich *et al.* (2000) reported a significant decrease in respiration in *Hyas araneus* collected from temperate water at 9°C and acclimatised to 2°C. The oxygen uptake of *H. araneus* at 2°C (0.025 mg O<sub>2</sub> g wet wt<sup>-1</sup> h<sup>-1</sup>) was comparable to the value reported by Frederich *et al.* (2000) (0.019 mg O<sub>2</sub> g wet wt<sup>-1</sup> h<sup>-1</sup>). Thus, the low temperature of the Arctic water reduces the metabolic activity of *H. araneus* as shown by the heart rate data. Respiration rate of *S. boreas* was shown to be low as well at 2°C (0.045 mg O<sub>2</sub> g wet wt<sup>-1</sup> h<sup>-1</sup>).

The concentration of the respiratory pigment (haemocyanin) of *G. antarcticus* was shown to be low but 50% of the oxygen was transported as dissolved oxygen reflecting the high oxygen concentration dissolved in the cold water of polar regions (Whiteley *et*

*al.* 1997). Antarctic icefishes were shown to lack hemoglobin and, therefore, their tissue oxygen demand relied on specific heart features (Tota *et al.* 1997). For instance, in the icefish, the low rate-reducing effect of low temperature has been compensated by large systolic volumes, and biochemical, and ultrastructural adaptations. It can be postulated that the low heart rate measured in polar crustaceans is a direct consequence of living in cold water but that cardiac compensations, at the structural level of the heart, comparable to icefishes occur; this remains unresolved in this study.

### **6.2.5. Conclusions**

The heart rate comparison between species across latitudes appears to be correlated to temperature. However, a compensation has occurred in the heart frequencies of polar species to maintain heart rate at a required minimum level to ensure a scope for activity. Nevertheless, the low compensation may reflect the need to minimise energy expenditure in an environment where food supply is limited. More data (number of species and on a wider temperature range) are required to confirm these findings.

## Chapter 7

### Variation of total oxyradical scavenging capacity and metabolic rate of the Arctic clam *Mya truncata* in relation to food availability: comparison with Antarctic and temperate species

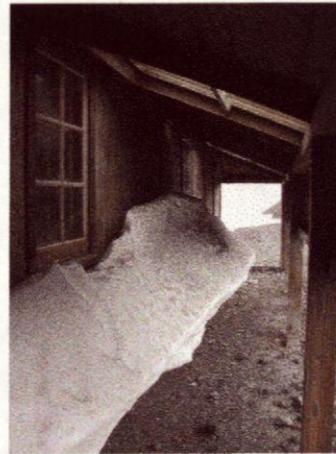
Submitted to Polar Biology



Discovery hut, Hut Point, Ross Island (Antarctica), built in 1901 by R.F.Scott.



Blubber stove of the Discovery hut.



Veranda of the Discovery hut.

*"Great God, this is an awful place".*

**Robert F. Scott, at the South Pole 16<sup>th</sup> of January 1912.**

### Chapter 1

Variation of total dry matter digestible energy (DMDE) and metabolizable energy (ME) of the diets in relation to feed availability, composition with Atlantic and temperate species

Table 1.1. Feed intake

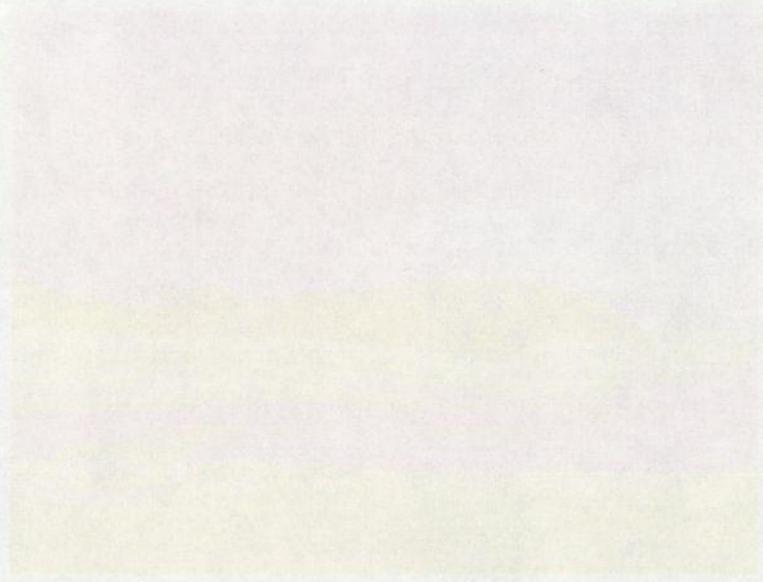


Table 1.2. Feed intake (continued)

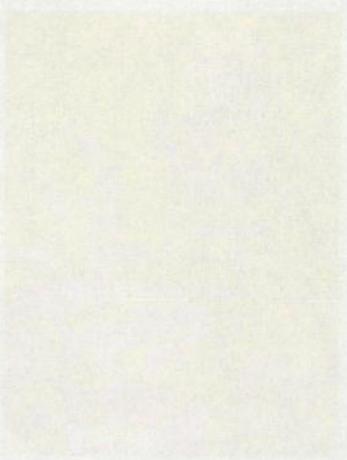
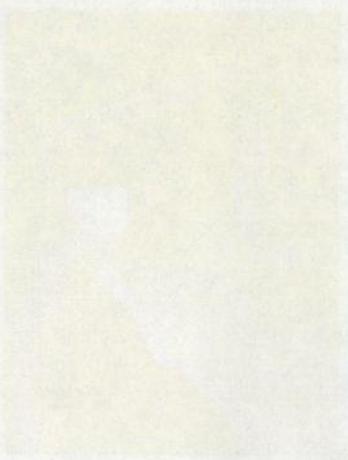


Table 1.3. Feed intake (continued)

Table 1.4. Feed intake (continued)

Table 1.5. Feed intake (continued)

## 7.1. Abstract

The Arctic marine ecosystem is characterised by the combination of a low but stable temperature and a markedly seasonal pattern of primary production (May). The 24 h illumination of summer days, and recent stratospheric ozone depletion, enhance the formation of elevated concentrations of reactive oxygen species (ROS;  $H_2O_2$ ,  $\cdot OH$ ,  $O_2\cdot^-$ ) in the water column. The seasonality of such ecological factors, and the stable low temperature, requires polar ectotherms, to develop metabolic and biochemical adaptations. While these adaptations have been addressed in Antarctic marine invertebrates, the Arctic ecosystem has received little similar attention. With increasing industrial exploitation of the European Arctic, there is a need to gain knowledge of all aspects of the biology of the indigenous fauna. In the present study, the effect of the food availability changes on the total oxyradical scavenging capacity (TOSC) and the metabolic rate of the Arctic clam *Mya truncata* was investigated and compared with similar bivalve species from temperate (*Mya arenaria*) and Antarctic (*Laternula elliptica*) ecosystems. Food availability did not affect significantly the respiration rate of *M. truncata* and values were relatively low ( $37.4 \mu g O_2 dw^{-1} h^{-1}$  at  $0^\circ C$ ). A significant difference in TOSC was identified between May (2000 unit  $mg^{-1}$  protein) and September (4000). The highest TOSC value was measured in the Antarctic species, *L. elliptica* ( $5854 \pm 449$  unit  $mg^{-1}$  protein, mean  $\pm$  S.D.), whereas the Arctic species, *M. truncata*, showed an intermediate TOSC value ( $4014 \pm 1334$  unit  $mg^{-1}$  protein), and the temperate species, *M. arenaria*, showed the lowest capability to scavenge peroxy radicals ( $1837 \pm 403$  unit  $mg^{-1}$  protein). The relative stability and low metabolic rate of *M. truncata* are typical of polar bivalves and are interpreted as an adaptation to cope with the limited food availability (low maintenance cost). The May to September difference in TOSC reflects the prolonged period of starvation but also the seasonality of the oxidative pressure of the environment due to high UV-B irradiance. The high TOSC levels in *M. truncata* and *L. elliptica* compared with *M. arenaria* confirm this hypothesis. The TOSC-assay provides clear understanding on the capability of *M. truncata* to cope with oxyradicals. This Arctic species appear vulnerable to anthropogenic compounds in early spring when antioxidant defences are depleted.

**Keywords:** Arctic, metabolic rate, *Mya truncata*, seasonality, TOSC

## 7.2. Introduction

In Hornsund fjord (Svalbard), surface seawater temperature ranges between  $-1.88$  and  $3^{\circ}\text{C}$  while bottom water temperatures remain constant at  $-1.88^{\circ}\text{C}$  (Weslawski *et al.* 1988). The sun largely controls this seasonality; at *ca.*  $78^{\circ}\text{C}$  North it stays above the horizon all day from mid April to mid August, and stays beneath the horizon all day from mid November to mid February. As a consequence of the illumination pattern, a single well-timed maximum of a dense but brief primary production is observed in May, followed by a rapid decline (Weslawski *et al.* 1988). The 24 h illumination of summer days enhances the formation of high concentrations of reactive oxygen species (ROS;  $\text{H}_2\text{O}_2$ ,  $\cdot\text{OH}$ ,  $\text{O}_2^{\cdot-}$ ) in the water column. Recent stratospheric ozone depletion has caused an increase in ultraviolet-B radiation, thereby, significantly augmenting the production of ROS in the water column (Yocis *et al.* 2000, Qian *et al.* 2001). One possible consequence of the concomitant high oxygen concentration and increase in ROS, is a high oxidative pressure of the marine environment.

The seasonality of food availability and ROS production, together with low stable temperature, has resulted in specific biological adaptations of polar marine organisms. The basal metabolism of Antarctic ectotherms (measured as oxygen uptake) is relatively low compared with temperate species (Peck *et al.* 1987, 1997, Davenport 1988, Ahn and Shim 1998, Clarke and Johnston 1999), and this is interpreted as an adaptive strategy to limit energy expenditure due to the brief period of food availability (Clarke 1991, 1993). The growth period is limited to the brief phytoplankton bloom (Peck *et al.* 1987, 1997). However, the effect of the short food availability on the metabolism of bivalves has received little attention compared with temperate bivalves. In this study, the metabolism of an Arctic bivalve was measured during (May) and after (September) the phytoplankton bloom to see whether food availability affects the metabolic rate.

The antioxidant defences of some polar fauna appears high compared with temperate species (Colella *et al.* 2000, Regoli *et al.* 2000) and seems to be related to elevated UV-B irradiance levels (Abele *et al.* 1998). While these adaptations have been studied in Antarctic organisms, Arctic marine invertebrates have received little attention. With increasing human activities in the European Arctic (AMAP 1998), there is an urgent need to gain knowledge of the physiological functions of Arctic marine invertebrates. Many contaminants are capable of modulating oxidative stress and, consequently, affecting the antioxidant defences of marine bivalves (Winston and Di Giulio 1991).

The marked seasonality of the Arctic environment is a factor that could seriously complicate the interpretation of biomonitoring data. The variability of the antioxidant defences is related to seasonality (temperature and food availability) and to the metabolic rate in temperate bivalves (Viarengo *et al.* 1991, Sheehan and Power 1999), however, the correlation between these parameters remains unexamined in polar bivalves. Until recently, oxidative stress has been investigated by measuring single antioxidant defences. While this approach provides a good understanding of the mechanistic link between antioxidant and prooxidant, it has limited ecological relevance for biomonitoring anthropogenic activities and for inter-species comparison. Therefore, in this investigation, the total oxyradical scavenging capacity (TOSC; Winston *et al.* 1998), which provides understanding and predictive capacity for adverse effects caused by oxidative stress at the individual level, measurements.

*Mya truncata* (Myidae), an Arctic suspension-feeder bivalve mollusc, relies on phytoplankton for its nutrition and, therefore, maybe expected to show seasonal biochemical and metabolic changes. It is widely distributed and present in high biomass in the Svalbard fjords ecosystem (Koszteyn *et al.* 1991) and is the main source of food for bearded seals (*Erignathus barbatus*) and walrus (*Odobenus rosmarus*) (Fisher and Stewart 1997); the latter species are an important source of food of the people of the North (AMAP 1998).

In the current investigation, the total oxyradical scavenging capacity (TOSC) and the metabolic rate of the Arctic clam, *Mya truncata*, was measured in May and in September to evaluate whether the brief food availability (May) affected the physiology of this bivalve. TOSC and the metabolic rate of *Mya truncata* were compared with similar bivalve species, *Mya arenaria* (temperate) and *Laternula elliptica* (Antarctica), to better understand the biological adaptation of *M. truncata* to the Arctic marine ecosystem.

### 7.3. Materials and Methods

#### Sampling site

*Mya truncata* was sampled at Isfjorden, Svalbard (78°13' N and 15°39' E) (Figure 20). Sampling was performed during the first week of May 1999 and in mid-September 2000. *Mya truncata* was hand sampled by SCUBA divers near the Norwegian

settlement of Longyearbyen at the outer part of Adventfjorden. Bivalves were stored in a large tank with running seawater and brought back to the laboratory for analysis. The respiration rate was measured in 7 (May) and 5 (September) individuals of similar size. The hepatopancreas of 7 (May) and 8 (September) individuals was dissected and frozen in liquid nitrogen for TOSC measurement. The field temperatures were 0°C in April and 5.5°C in September.

*Mya arenaria* was hand sampled at low tide in February 2001 from the Yealm Estuary, Devon, United Kingdom (50°05' N and 4°50' W, Figure 23). They were packed in humid and water-ice cooled boxes, airfreighted to Norway and stored at 10°C in running seawater for two days before any analysis. Respiration was measured in 6 individuals. Hepatopancreatic tissue from 8 individuals was frozen in liquid nitrogen for TOSC analysis.

*Laternula elliptica*, widely distributed in nearshore waters around the Antarctic continent in relatively high biomass, was hand sampled by scuba divers in January 2000 (Austral summer) at McMurdo, Ross island, Antarctica (166°30' E, 77°45' S, Figure 21). Animals were stored in running seawater at -1.5°C for two days before dissecting out the hepatopancreas. The latter was frozen in liquid nitrogen and airfreighted to Norway in liquid nitrogen. Respiration rate data were extracted from the literature.

### **Analyses**

Respiration was performed with a closed respirometer technique and a Clarke electrode (see section 4.5.5.). The TOSC assay was measured according to Winston *et al.* (1998) adapted for bivalves (Regoli *et al.* 1998) (see section 4.5.2.).

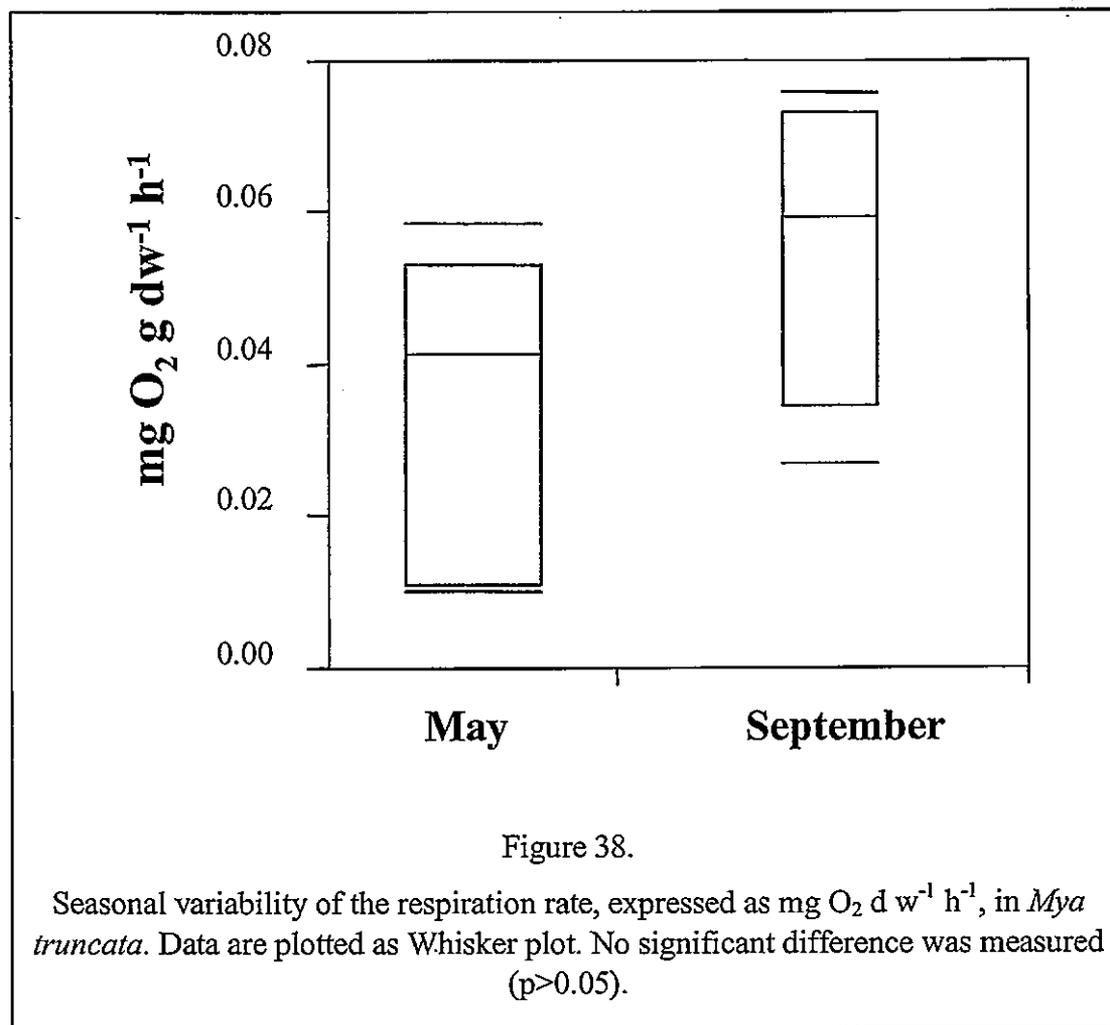
### **Statistical analysis**

Analyses were carried out with JMP, ver. (3.2.6), SAS Institute Inc., Cary, NC, USA. Statistical analyses were run after testing for normal distribution and homogenous variances. The parametric t-test was used (the significance level was  $p < 0.05$ ). Values of TOSC and respiration were plotted as Box and Whisker plots. The latter indicate the range of values and the median. The horizontal bars, that denote the upper and lower limits, include 95% of the data points. 50% of the data points are encompassed by the upper and lower limits of the boxes. The width of the boxes indicate the size of the samples.

## 7.4. Results

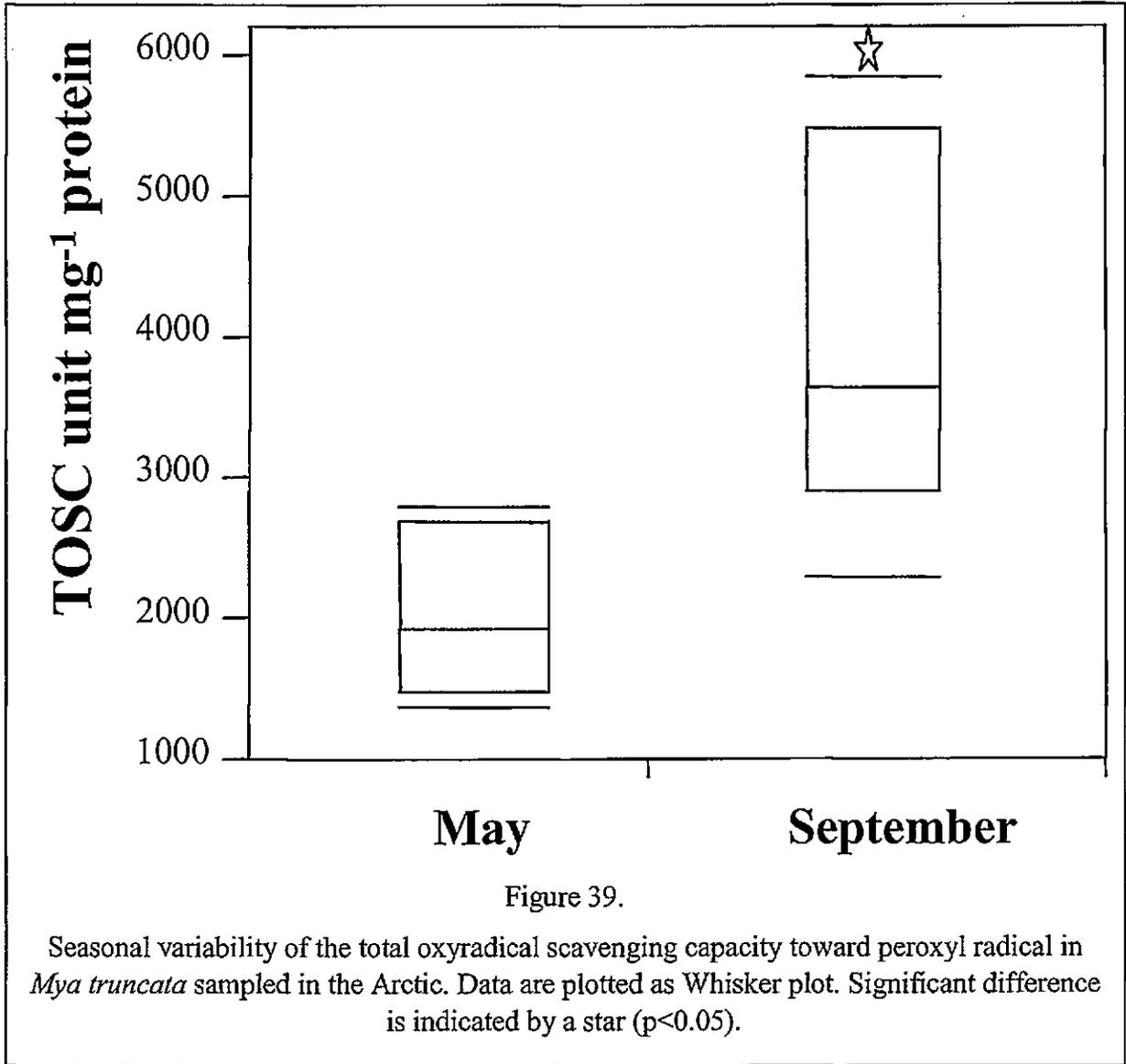
### Seasonal variability of metabolic rate

There was no significant difference in the respiration rates between the two months ( $p > 0.05$ ) (Figure 38). Nevertheless, respiration was higher in September than in May. These data indicate that the metabolic rate in *M. truncata* was not affected by a seasonal temperature rise of 5.5°C.



### Seasonal variability of TOSC

The September TOSC level was significantly ( $p < 0.05$ ) higher than that measured in May (Figure 39), suggesting that antioxidant defences of *M. truncata* are more efficient at scavenging peroxy radicals in September than in May.









### Interspecies variability of metabolic rate

The metabolic rate of *M. truncata*, *L. elliptica* and *M. arenaria* were compared with those of bivalve species living in polar or temperate regions (Table 3). Data are presented as  $\mu\text{g O}_2$  consumed per hour for standard organism of 1 gram (dry tissue), except for respiration data reported in this current study. The metabolic rates of polar bivalves are well below the values for temperate suspension feeding bivalve species living in warmer water. For example, the respiration rate of *M. truncata* at 0°C (4.10±1.32 g dry weight) is 4.8 times lower than *M. arenaria* at 10°C (1.50±0.25 g dry weight).

Table 3. Comparison of oxygen consumption rates ( $\text{Vo}_2$ ) of Arctic, Antarctic and temperate bivalves species

Species	T°C	Respiration $\mu\text{g O}_2 \text{ g d w}^{-1} \text{ h}^{-1}$	Ecosystem	Reference
<i>Mya truncata</i>	0	37.4	Arctic	This study
<i>Mya truncata</i>	5.5	55.3	Arctic	This study
<i>Chlamys islandicus</i>	0.5	63.7	Arctic	Personal data
<i>Hiatella arctica</i>	3.5	94	Arctic	Personal data
<i>Chlamys islandicus</i>	0	161.4	Arctic	(Schmid 1996)
<i>Chlamys islandicus</i>	2	271.3	Arctic	(Vahl 1978)
<i>Clinocardium ciliatum</i>	0	50-57	Arctic	(Schmid 1996)
<i>Laternula elliptica</i>	0.5	148	Antarctic	(Ahn and Shim 1998)
<i>Limopsis marionensis</i>	0	173	Antarctic	(Pörtner <i>et al.</i> 1999)
<i>Yoldia eightsi</i>	0	234.2	Antarctic	(Davenport 1988)
<i>Gaimardia trapesina</i>	0	417	Antarctic	(Ralph and Maxwell 1977)
<i>Crassostrea virginica</i>	10	244.2	Temperate	(Dame 1972)
<i>Crassostrea virginica</i>	20	531.2	Temperate	(Dame 1972)
<i>Crassostrea virginica</i>	30	604.0	Temperate	(Dame 1972)
<i>Mytilus edulis</i>	10	528.4	Temperate	(Vahl 1973)
<i>Mytilus edulis</i>	15	431.3	Temperate	(Bayne and Scullard 1977)
<i>Mytilus chilensis</i>	12	485.5	Temperate	(Navarro and Winter 1982)
<i>Mya arenaria</i>	10	181.3	Temperate	This study

## 7.5. Discussion

### Variability of respiration with food availability

The present study indicates that the metabolic rate of *M. truncata* in May was not significantly different from that in September. Therefore, the food availability in May did not induce a rise in metabolic rate in *M. truncata*. In temperate bivalves, summer to winter differences in metabolic rate have been related to food abundance, reproductive events and temperature (Bayne *et al.* 1973). Because of the stable polar seawater temperature, the increase in metabolism observed in some Antarctic species has been correlated with food abundance; however, many studies on the metabolic rate of polar bivalves have shown little seasonal variation. The metabolic rate of *Yoldia eightsi*, an Antarctic bivalve, was little affected by temperature change over the temperature range that it is likely to encounter in nature (Davenport 1988). Peck *et al.* (1997) were unable to correlate biomass growth increase in summer with metabolic rate in the Antarctic bivalve, *Liothyrella uva*. Ability to maintain low and stable metabolic rate even during periods of brief but important growth confers advantages (i.e. reduced maintenance cost allowing more growth) in low energy supply environments (Peck *et al.* 1997, Ahn and Shim 1998). Therefore, the metabolic rate of *M. truncata*, in this study, is typical of polar bivalves.

### Variability of TOSC with food availability

TOSC values were relatively low in May and elevated in September. Previous studies on temperate bivalves have demonstrated a decrease of antioxidant defences during the winter months, and this has been correlated with increased biological damage (i.e. lipid peroxidation) followed by a subsequent increase corresponding to the phytoplankton bloom (Viarengo *et al.* 1991, Power and Sheehan 1996, Cancio *et al.* 1999). This decrease in antioxidant defence for temperate bivalves was observed in the depletion of scavenger compounds (glutathione, vitamin E and carotenoid) and reduced enzyme activities (superoxide dismutase, catalase and peroxidase) (Viarengo *et al.* 1991, Power and Sheehan 1996, Cancio *et al.* 1999). Spring increases in antioxidant capacities in temperate bivalves have been linked to a need to cope with the excess of oxyradicals arising from an increase in the metabolic rates caused by a temperature rise and a phytoplankton bloom (Orbea *et al.* 1999). In the present experiment, the relatively low TOSC level may represent the typical decrease of antioxidant defences in temperate

bivalves after a long winter. Given that food was absent for the previous 9 months for *M. truncata*, the cost of maintaining a stock of antioxidant defences during winter can be too high and the energy may be preferentially used in gamete production and individual survival.

Seasonal fluctuation of protein content, with a low protein level in late winter, indicated that proteins can be the major substrate used to fuel metabolic requirements in polar bivalves (Peck *et al.* 1987). This conclusion supports the low level of antioxidant defences measured here in May indicating that enzymatic proteins may also be a source of energy for maintaining the basal metabolism of *M. truncata* during winter. The seasonal pattern of growth of polar invertebrates, characterised by a steady state in winter and a relatively fast increase during the phytoplankton bloom (Peck *et al.* 1997), further supports the hypothesis that sufficient energy was available during the phytoplankton bloom to allow *M. truncata* to build up antioxidant defences as measured in September.

Regoli *et al.* (2000) put forward the hypothesis that polar bivalves require high TOSC to cope with the high level of ROS that are photochemically produced in the water during summer. ROS are ubiquitous in sunlit natural waters and are important species formed from the photoreactions of dissolved organic matter (DOM) in seawater. Previous studies indicated that ozone hole events, occurring in polar regions, increase UV-B irradiance contributing, therefore, to enhance ROS production (Yocis *et al.* 2000, Qian *et al.* 2001). High levels of H<sub>2</sub>O<sub>2</sub> were measured in the Antarctic intertidal zone during summer (Abele *et al.* 1998). The high TOSC measured in September in *M. truncata* may be an adaptative strategy to avoid biological damage caused by the intensive production rate of ROS during the long illumination period from mid April to mid August. Abele *et al.* (1998) reported an increase in antioxidant defences and lipid peroxidation in the Antarctic limpet exposed to hydrogen peroxide. High antioxidant defence systems have been reported in polar bivalves (Regoli *et al.* 2000) and fish (Colella *et al.* 2000). Furthermore, the relatively low DOM level in polar water, once the phytoplankton bloom is finished, allows a deeper penetration of UV-B, causing ROS to be formed closer to the benthic fauna, and yielding higher volume of ROS (Scully *et al.* 1996). The low TOSC value in May compared with September can also be explained by the limited ROS formation due to the dark winter and the fast ice preventing UV-B to penetrate the water column.

### Interspecies variability of TOSC

A clear interspecies difference in TOSC values indicated a greater capability of polar species to counteract ROS toxicity, with a high, intermediate and low TOSC value for the Antarctic, Arctic and temperate species, respectively. These data corroborate earlier comparative studies of TOSC in scallop species (Regoli *et al.* 2000). The comparison of the metabolic rate in the current study indicates that *M. arenaria* is more likely to face a natural high oxyradical production due to its higher respiration rate. However, the TOSC value of *M. arenaria* is the lowest one of the range studied. Therefore, the difference in the capacity of *M. truncata*, *M. arenaria* and *L. elliptica* to deal with oxidative stress is related mainly to external prooxidant factors. High UV-B irradiance level is the likely main cause of the large amount of ROS produced as hypothesised above (Viarengo *et al.* 1995, Regoli *et al.* 1997, 2000, Colella *et al.* 2000). The difference in TOSC between *M. truncata* and *L. elliptica* may reflect differences in seawater temperature stability. Littlepage (1965) reported a temperature variation of *ca.* 0.3°C in McMurdo Sound while in Isfjorden temperature ranges from -1.8 to 5°C. Seawater temperature can affect the dissolved oxygen concentration and, therefore, modulate ROS generation.

### Interspecies variability of metabolic rate

Previous studies have reported low metabolic rates in polar bivalves compared with temperate and tropical species (Davenport 1988, Schmid 1996, Peck *et al.* 1997, Ahn and Shim 1998, Pörtner *et al.* 1999). Reduction of metabolism in polar poikilotherms has been considered an important adaptative strategy for energy conservation in an environment where food availability is very limited (Clarke 1991, 1993). It is in polar water that a great energetic benefit is obtained by living in cold water, as maintenance costs are so low compared with temperate or tropical species with elevated metabolic rate. It is interesting to note that *M. truncata* and *L. elliptica* showed the lowest respiration rate. These bivalves are present in high biomass, confirming that low metabolic rate may contribute to a high scope for growth when food is available to develop a high biomass (Brey and Clarke 1993). Consequently, seal and walrus populations of the Arctic can use this bivalve stock as an important source of nutrition (Fisher and Stewart 1997).



## 7.6. Conclusions

Oxidative stress is a general chemical threat to all biological systems (Winston and Di Giulio 1991). Previous studies have demonstrated the presence of antioxidant parameters such as enzymes (catalase, superoxide dismutase) or scavengers (glutathione), especially in the digestive gland of bivalves. Hence, oxidative damage reflects an imbalance between the production of oxidants and removal or scavenging of those oxidants by molecular defences. The investigation of oxidative stress by the measurement of a single antioxidant parameter does not allow full assessment of the total capacity of an organism to cope with oxyradical production. While some parameters are elevated, others may be depressed, consequently, the use of a panel of single antioxidant is ambiguous and will not provide sufficient information on the prooxidant factors (Power and Sheehan 1996). Measurement of the total oxyradical scavenging capacity provides a better understanding of the basal capability to neutralize ROS (Winston *et al.* 1998, Regoli 2000). Numerous chemical contaminants enhance ROS production when metabolised by bivalves (Livingstone *et al.* 1990). Hence, it is of prime importance that antioxidant defences are effective to avoid biological damage caused by ROS. Further studies are required to investigate the direct link between UV-B irradiance increase and elevated antioxidant defences. UV-B affects marine Antarctic organisms (Smith *et al.* 1992), but it is not known whether the enhanced ROS formation causes biological damage to the polar marine fauna. In the present study, it appears that polar bivalves have a high capability to deal with ROS but the lower TOSC value of *M. truncata* in May renders this species vulnerable to potential release of contaminants caused by anthropogenic activities during that period.

## Chapter 8

### **Total oxyradical scavenging capacity and plasma membrane stability of haemocytes of the Arctic scallop, *Chlamys islandicus*, following benzo(a)pyrene exposure**

In press in Marine Environmental Research (2002)



Minke whale

*“The vigorous slaughter of whales both in the sub-Antarctic and in the sub-tropics, for the one area reacts on the other, call for universal legislation to protect the whales from early commercial extinction, and the industry which is of worldwide economic importance, from having to be abandoned.”*

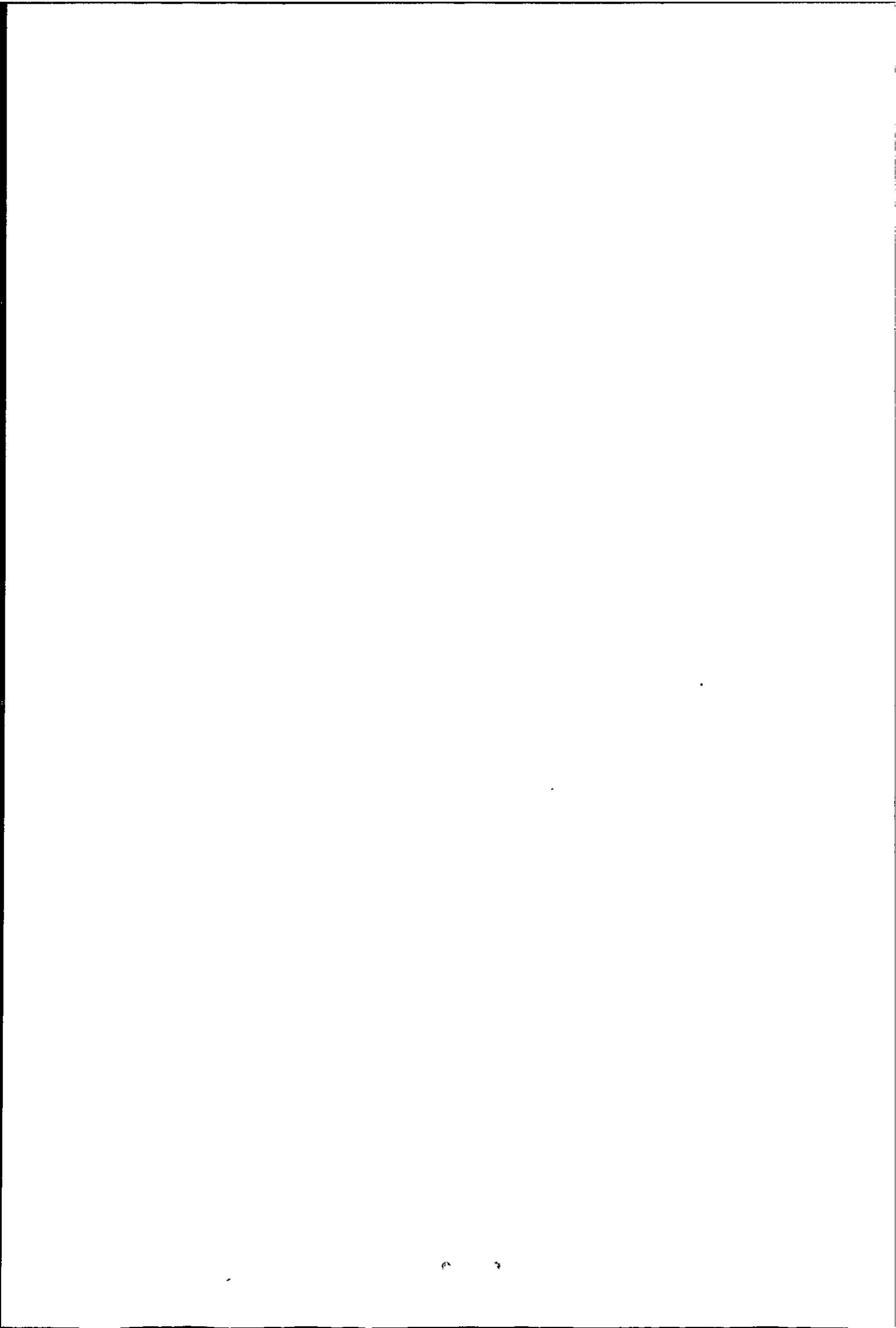
**Robert S. Clark, Lieut. R.N.V.R., Endurance expedition of Shackleton, 1914-1917.**



### 8.1. Abstract

Industrial activities, notably oil and gas industries, are expanding in the Arctic. Most of biomarkers were developed using temperate organisms living at temperatures above 10°C. Little is known about the biomarker responses of organisms living between -1.88 to 5°C. Therefore, assessment of the toxicity of chemicals to cold-water adapted species is required. In this study, the Arctic scallop, *Chlamys islandicus*, was selected as a key species for biomonitoring because of wide distribution in Arctic waters and its commercial value. Test animals, stored in seawater at 2°C, were injected with benzo(a)pyrene (diluted in cod liver oil 5 mg ml<sup>-1</sup>) in the adductor muscle every 24 h for four days giving a final dose of 0, 74 and 90.6 mg kg<sup>-1</sup> wet weight for control, low and high dose, respectively. The biomarkers used were total oxyradical scavenging capacity (TOSC) in the digestive gland and cell membrane stability of haemocytes. TOSC values were significantly reduced (ca. 30%) in exposed groups (p<0.05), indicating a depletion of oxyradical molecular scavengers. The antioxidant defences appeared to be overwhelmed by the reactive oxygen species as the plasma membranes of haemocytes were destabilised (p<0.05). These data indicate that reactive oxygen species (ROS) were produced by Arctic scallops via the metabolism of benzo(a)pyrene at 2°C; the antioxidant defences had the capacity to scavenge the ROS as they get depleted. Nevertheless, haemocytes plasma membranes were destabilised probably due to lipid peroxidation.

**Key words:** TOSC, cell membrane, benzo(a)pyrene, *Chlamys islandicus*, Arctic.



## 8.2. Introduction

The growing interest of oil and gas industries in the European Arctic has raised concerns of the potential impact of chemicals at low temperature. For the past 20 years, biomarkers [defined as “biological responses to a chemical or chemicals that give a measure of exposure or toxic effect” (Peakall 1994)] have been developed in temperate species to provide early-warning signals of detrimental impacts on the marine ecosystem. Few toxicity studies in the Arctic have been performed, raising the need to investigate chronic biological effects of chemicals with resident organisms (Chapman 1993). Laboratory studies are needed to investigate the link between cause and effects of PAH on the Arctic fauna. For instance, polycyclic aromatic hydrocarbons (PAH), notably benzo(a)pyrene are known to enhance reactive oxygen species (ROS) production in bivalves (Livingstone *et al.* 1990) and to cause lysosomal membrane disruption (Grundy *et al.* 1996). ROS may cause the total oxyradical scavenging capacity (TOSC) to decrease (Regoli 2000) or to increase (Winzer *et al.* 2001). Polar bivalves possess an elevated TOSC (Regoli *et al.* 2000) which may render them less susceptible to ROS. At the cellular level, adaptation to low temperatures is associated with modified composition of the membrane lipid (Viarengo *et al.* 1994). When antioxidant defences are overwhelmed, ROS can cause cell membrane lipid-peroxidation. Thus, the vulnerability of Arctic bivalves to PAH-mediated ROS production has to be questioned. In this study, responses of the total oxyradical scavenging capacity and plasma membrane stability of haemocytes have been measured in the Arctic scallop, *Chlamys islandicus*, exposed to a carcinogenic PAH, benzo(a)pyrene, known to enhance ROS production.

## 8.3. Materials and methods

In September 1999, *Chlamys islandicus* ( $7.42 \pm 0.57$  cm shell height,  $n=30$ ) was collected with an Agassiz dredge from Isfjorden at Svalbard ( $78^{\circ}13'N$ ,  $15^{\circ}39'E$ ) (Figure 20). After one day of acclimatization in  $2^{\circ}C$  aerated seawater with no sediment, 3 groups, each of 10 individuals, were established as control, low and high dose of B(a)P (Figure 41). The chemical [5 mg of B(a)P diluted in 1 ml of cod liver oil] was injected into the adductor muscle once a day for four days giving a final dose of 0, 74 and  $90.6 \text{ mg kg}^{-1}$  wet weight; no feeding was allowed and water was renewed every day.

After 4 days, haemolymph was sampled from the adductor muscle of the 10 individuals from each group, and mixed (1:1) with physiological saline. 200  $\mu$ l of cell suspension were added per well of a dark microplate and incubated with 50  $\mu$ l of Ethidium homodimer-1 (EthD-1) for 5 min at room temperature. Fluorescence was read at ex544/em612. EthD-1 is taken up by haemocytes with destabilized membranes and the fluorescence increases upon binding to nucleic acids (Camus *et al.* 2000) (Figure 41) (see section 4.5.3.). At the same time as the blood was sampled, the hepatopancreas of seven individuals per group was excised, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  and, following Winston *et al.* (1998), the TOSC assay was performed (Figure 41) (see section 4.5.2.).

Statistical analyses were made using JMP v3.2.6., SAS Institute, Inc., Cary, NC, USA. Normal distribution and homogeneity of variances was established before statistical treatment. The Dunnett's test was performed for both sets of data. Data are plotted as Box and Whisker plots. The significance level was  $p < 0.05$ .



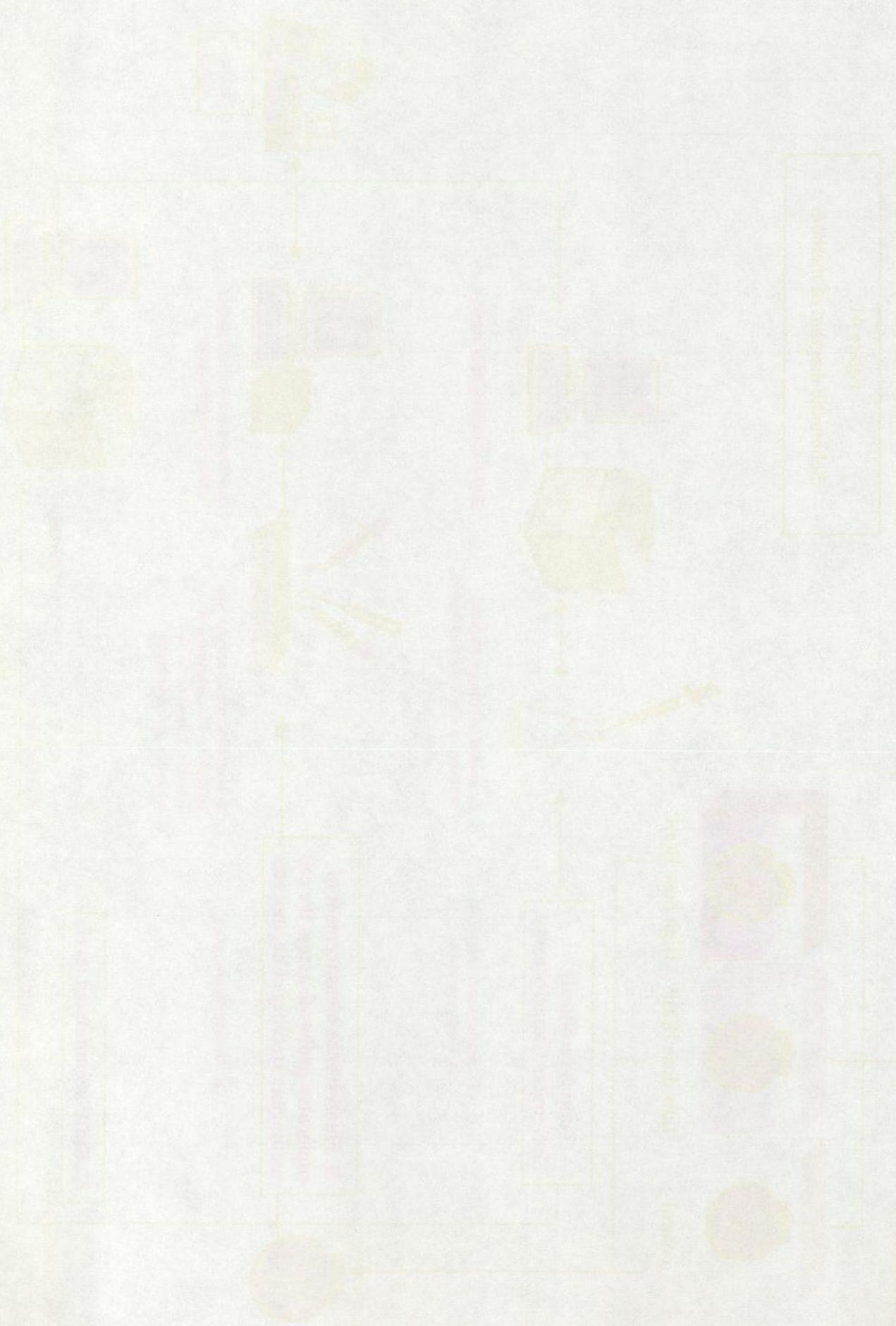


Figure 1. A flowchart illustrating the relationship between the variables of the study.

### 8.4. Results

#### TOSC

A significant decrease of 33% and 26% in TOSC value was measured in benzo(a)pyrene in the low and high dose exposed groups, respectively ( $p < 0.05$ ) (Figure 42). No significant difference was noted between treated groups. A TOSC depression can be indicative of depletion of oxyradical scavengers. The mean TOSC value of the control group was  $2683 \pm 703$  unit  $\text{mg}^{-1}$  protein.

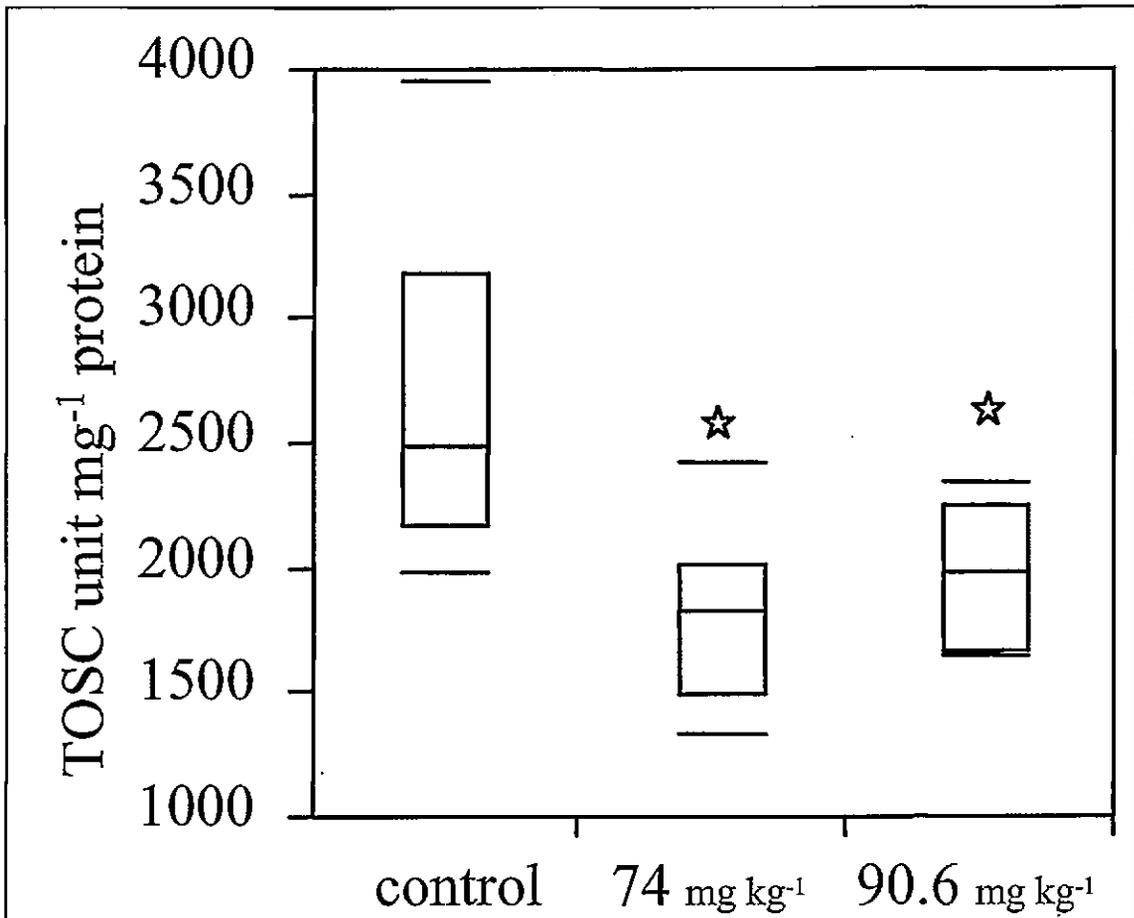


Figure 42.

Total oxyradical scavenging capacity of *Chlamys islandicus* following exposure for 4 days to two concentrations of benzo(a)pyrene. Data are plotted as Whisker plot. Significant effect is indicated by a star ( $p < 0.05$ ).

### Membrane stability

A significant increase in uptake of EthD-1 into the haemocytes of *Chlamys islandicus* was measured in the low dose treated group ( $p < 0.05$ ) (Figure 43). An increase of fluorescence occurred in the high dose but was not significant ( $p > 0.05$ ). Thus, the plasma membranes of the haemocytes of *C. islandicus* were strongly and slightly destabilised by the low and high dose benzo(a)pyrene exposure, respectively.

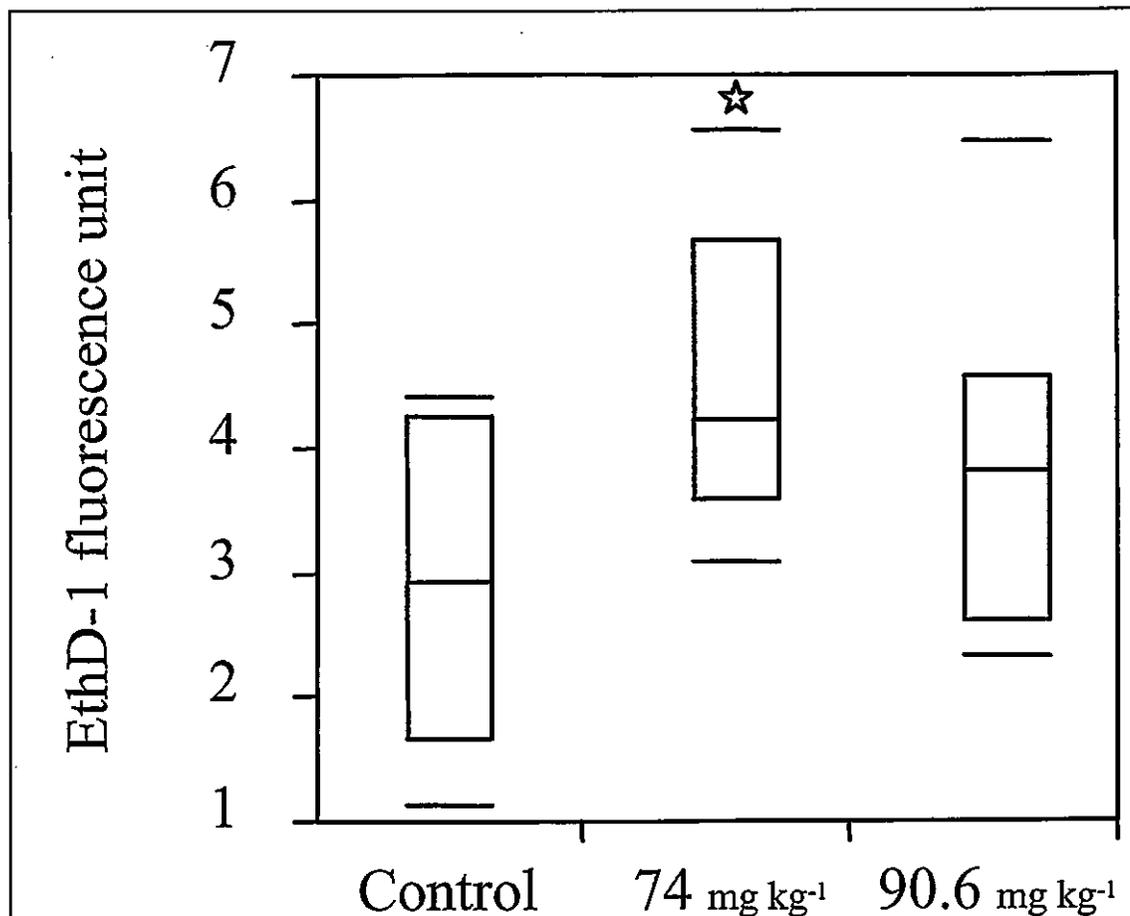


Figure 43.

Stability of the plasma membrane of the haemocytes of *Chlamys islandicus* measured as fluorescence units of Ethidium homodimer-1 (EthdD-1) following 4 days exposure to two concentrations of benzo(a)pyrene. Data are plotted as Whisker plot. Significant effect is indicated by a star ( $p < 0.05$ ).

## 8.5. Discussion

The rational basis of the TOSC assay is to measure the balance between antioxidant parameters and prooxidant factors (Winston *et al.* 1998). The significant decrease in TOSC value reported in the present study indicates that antioxidant defences are depleted (i.e. glutathione) due to ROS production. This depletion confirms the enhanced ROS production caused by the metabolism of B(a)P as reported by Livingstone *et al.* (1990). Therefore, one possible consequence of this ROS production, and depressed antioxidant defences, is that the protection of the cells against the damaging effect of ROS is no longer covered. This is confirmed by the decreased plasma membrane stability of haemocytes measured in this study and demonstrated by others (Viarengo *et al.* 1989, Winston *et al.* 1996, Regoli 2000). Nevertheless, the direct effect of B(a)P could be related to the binding of this highly lipophilic chemical to the membrane (lipid or proteins) thus compromising the basic functions (fluidity, ionic pumps). The indirect effects could be mediated by the alteration of lysosomal membranes due to accumulation of B(a)P in these organelles and subsequent loss of hydrolytic enzymes into the cytosol (Grundy *et al.* 1996, Camus *et al.* 2000). Impaired function of the plasma haemocyte membrane can have consequences on the cellular immunity of *C. islandica* (Dyrynda *et al.* 1998). The reduction of the antioxidant cellular defences could also lead to protein and enzymes inactivation, damage to DNA and ultimately carcinogenesis (Winston and Di Giulio 1991). The lack of dose response may be due to the relatively high doses of B(a)P used in this experiment; these may have a strong toxic effect such as enzyme inhibition or reduction in whole metabolism (reduction in the oxygen uptake).

The present study demonstrated the link between the effects of benzo(a)pyrene on the redox status of the organisms and susceptibility to oxidative stress of cell membranes. Thus, TOSC appears to be an ecological relevant biomarker as it provides understanding and predictive capacity for adverse effects experienced by an Arctic bivalve. The TOSC assay and the plasma membrane stability of haemocytes in *C. islandicus* could represent useful tools for biomonitoring the impact of industrial activities, notably oil offshore production operations, in the Arctic marine ecosystem.

## Chapter 9

### Heart rate, respiration and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to crude oil via sediment and injection

In press in Aquatic Toxicology (2002)



Killer whale in the McMurdo Sound

*"...all around we could hear the killers [whale] blowing, their short, sharp hisses sounding like sudden escapes of steam. The killers were a source of anxiety, for a boat could easily have been capsized by one of them coming up to blow. (...) Shipwreck mariners drifting in the Antarctic seas would be things not dreamed of in the killers' philosophy, and might appear on closer examination to be tasty substitutes for seal and penguin."*

**Sir Ernest H. Shackleton, The Endurance expedition, 1914-1917.**

### Chapter 10

## Heart rate regulation and the autonomic nervous system: an overview of the autonomic nervous system, including the sympathetic and parasympathetic divisions, and the role of the heart rate variability (HRV) in health and disease.

(1996) (1996) (1996)

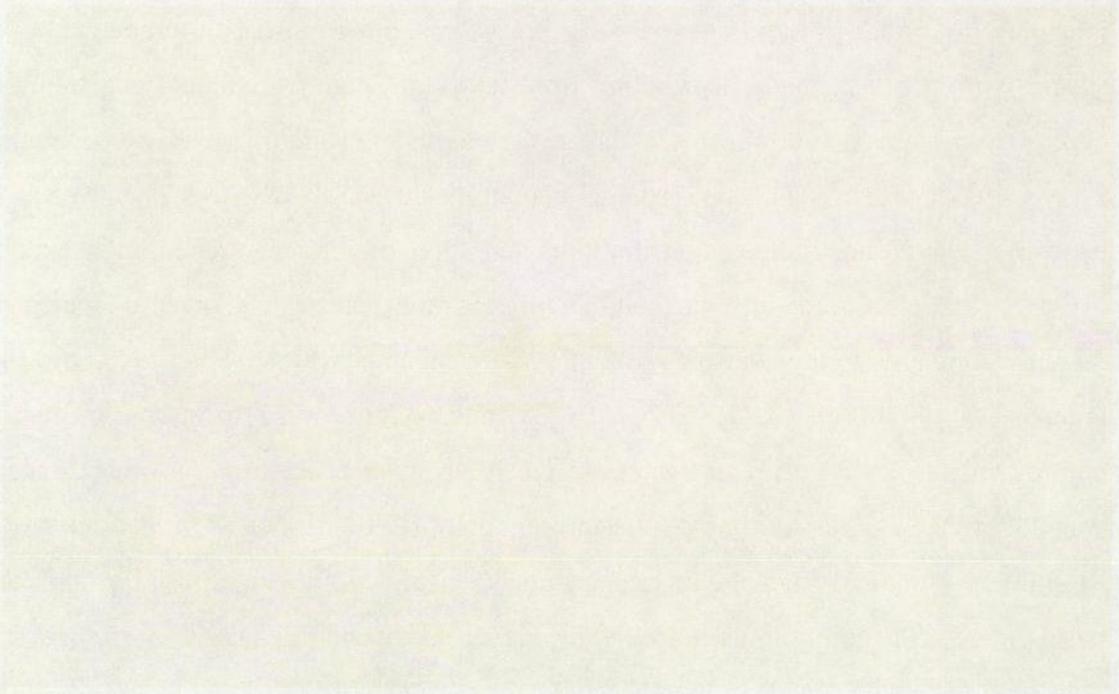


Figure 10.1: A diagram illustrating the autonomic nervous system and its control of heart rate.

The autonomic nervous system (ANS) is a complex network of nerves that controls the body's internal organs and functions. It is divided into two main branches: the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). The SNS is responsible for the 'fight or flight' response, while the PNS is responsible for the 'rest and digest' response. The heart rate is regulated by the ANS, with the SNS increasing heart rate and the PNS decreasing it. Heart rate variability (HRV) is a measure of the variation in time between successive heartbeats. It is an important indicator of overall health and stress levels. High HRV is associated with good health and low stress, while low HRV is associated with poor health and high stress.

Figure 10.1

The diagram illustrates the autonomic nervous system and its control of heart rate.

## Abstract

Increasing industrial activity in the European Arctic has raised concerns of the potential anthropogenic impact of chemicals on this polar marine ecosystem. For the past 20 years or so, biomarkers have been developed to provide early-warning signals of detrimental impacts on the marine ecosystem, however, most biomarker methods have been developed for organisms living in temperate rather than polar waters. Little is known about biomarker responses in organisms living within the temperature range of  $-1.88$  to  $+5^{\circ}\text{C}$ . In this study, biomarkers from temperate studies were tested on the Arctic spider crab *Hyas araneus* to validate their use in polar ecosystems. *Hyas araneus* is commonly found in the Svalbard fjord ecosystems, although it is a temperate water species with a geographical distribution from northern Spain to Svalbard and found at depths from 10 m to 1200 m. In this paper, the effects of crude oil were investigated at  $2^{\circ}\text{C}$  via two routes: i) injection and ii) contaminated sediment. After two weeks of exposure, heart rate, oxygen consumption and total oxyradical scavenging capacity (TOSC) were measured. In both methods of exposure, heart rate showed a significant increase compared to the control ( $P < 0.0001$ ,  $n = 7$ ); mean heart rate values ( $\pm$  S.D.) of *H. araneus* were  $49.06 (\pm 13.72)$ ,  $57.56 (\pm 7.28)$  and  $63.30 (\pm 6.57)$  beats per minute in control, injected and sediment-treated groups, respectively. Respiration of *H. araneus* was not affected significantly by either oil treatment ( $P > 0.05$ ), but two individuals ( $n = 8$ ) showed a marked increase in oxygen uptake in the sediment-exposed group. The basal oxygen consumption of control *H. araneus* was lower ( $0.025 \text{ mg O}_2 \text{ g wet wt}^{-1} \text{ h}^{-1}$ ) than reported for *H. araneus* living in temperate water. TOSC of *H. araneus* was not affected by either exposure treatment ( $P > 0.05$ ) although large variability in the injected group may indicate a concomitant induction and depression of TOSC. The mean TOSC value in the sediment-exposed group was lower than the control, indicating a certain saturation of the oxyradical scavenging system in the former group. The higher ecological relevance of TOSC, compared with heart rate, leads to the conclusion that the arctic crab was experiencing a moderate pollution effect. Low temperature may be the main factor reducing: i) the bioavailability of poly-aromatic hydrocarbons and ii) the metabolic rate of *H. araneus* and, consequently, the uptake and metabolism of oil compounds into reactive oxygen species.

**Keywords:** Arctic, Biomarkers, *Hyas araneus*, Heart Rate, Respiration, TOSC, Crude oil.

## 9.1. Introduction

Increasing industrial activity, notably oil and gas exploration, in the European Arctic requires better assessment methods for the potential anthropogenic impact of chemicals on this polar marine ecosystem (AMAP 1998). For the past 20 years or so, biomarkers [defined as "biological responses to a chemical or chemicals that give a measure of exposure or toxic effect" (Peakall 1994)] have been developed in temperate species to provide early-warning signals of detrimental impacts on the marine ecosystem. The application and value of biomarkers at low temperatures have been little studied. For example, the seawater temperature in Hornsund, an Arctic fjord at Svalbard, ranges from  $-1.88$  to  $3^{\circ}\text{C}$  (Weslawski *et al.* 1988). Chemicals, notably oil, behave differently at low compared to higher temperature; for example, the viscosity of oil is a temperature-dependant property (Payne *et al.* 1991). Thus, at low temperature, oil does not flow as freely, spread or dissolve in water to the same extent as at higher temperatures and such physical differences have important consequences for the bioavailability of oil components. Marine organisms living at low temperatures have developed specific adaptations, such as hysteresis antifreeze agents (Denstad *et al.* 1987), to prevent freezing. Such adaptations to cold water may render organisms more vulnerable to xenobiotic exposure as reported for polar cod, *Boreogadus saida* (Christiansen *et al.* 1996). On the other hand, studies have shown that Arctic invertebrates may be more resistant to heavy metals due to their eopontic behaviour (Chapman and McPherson 1993). In the case of oil, the cold-water adaptations of marine organisms, together with the altered behaviour of oil at low temperature, may affect the typical biomarker responses developed for temperate species. This hypothesis needs to be tested before biomarkers are deployed in the Arctic. In this study, established biomarkers have been applied to the Arctic spider crab, *Hyas araneus* (Decapoda, Brachyura, Majidae), a species found commonly in Svalbard fjord (Dyer 1985). This benthic crab, a temperate species with a geographical distribution from northern Spain to Svalbard, is found at depths from 10 m to 1200 m. It feeds on juvenile scallop (Nadeau and Cliche 1998) and may scavenge dead animals (Nickell and Moore 1992), and is predated by seabirds and bearded seals (Hjelset *et al.* 1999). The high abundance and wide distribution of *H. araneus* make it a potential sentinel species for monitoring the Arctic marine ecosystem.

In crustaceans, biomarkers have been developed based upon a single response, however, it is the integrated repertoire of the full compensatory responses which determines the

survival potential of individual, as emphasised by Hebel *et al.* (1997). Therefore, in the present study, two physiological biomarkers (heart rate and respiration) and one biochemical biomarker [Total Oxyradical Scavenging Capacity (TOSC)], were measured in the same individual to gain understanding of the responses of *H. araneus* to oil at different levels of biological functioning. Crustacean heart rate is a useful biomarker. Cardiac activity increased in *Carcinus maenas* following exposure to the water soluble fraction of crude oil (Depledge 1984b), while bradychardia and arrhythmia were measured in *Pugettia producta* when the gills were exposed to the water soluble fraction of crude oil (Zimmer *et al.* 1979). Numerous studies have reported heart rate disruption following exposure to metal contamination (Depledge 1984a, Depledge and Lundebye 1996, Bamber and Depledge 1997). Respiration is another sensitive biomarker for oil exposure of marine invertebrates; the respiratory rate of *Carcinus maenas* increased when exposed to the water soluble fraction of crude oil (Depledge 1984b). Molecular oxygen is vital for most living organisms, providing energy through the coupling of oxidation to the phosphorylation of ADP into ATP (main source of stored energy directly available for the cell). Partial reduction results in the formation of various potentially toxic reactive oxygen species (ROS). The cytotoxic consequences of oxyradical production include alterations in enzyme functions, lipid peroxidation (membrane destabilisation), DNA damage and cell death. Nevertheless, the extent of such damages depends on the effectiveness of antioxidant defences. Decapod crustaceans are able to activate xenobiotics, notably benzo(a)pyrene, to reactive mutagenic products (i.e. Marsh *et al.* 1992, Fossi *et al.* 1997, Sundt and Goksøyr, 1998), and measurements of oxidative stress are based upon single antioxidant parameters (i.e. glutathione, catalase, superoxide dismutase) (i.e. Arun *et al.* 1999, Orbea *et al.* 2000). The sensitivity of single antioxidative parameters enables their use as rapid and easy-to-use biomarkers. However, their ecological relevance is limited, as it is impossible to obtain a complete assessment of the oxidative stress based on a few biochemical responses. As a result, Winston *et al.* (1998) proposed a new assay, the total oxyradical scavenging capacity assay (TOSC), to measure the balance between antioxidant parameters and prooxidant factors. The TOSC assay measures the capability of a tissue to neutralize ROS in quantifiable term; this provides better understanding, and predictive capacity, of the effects of environmental conditions on the redox status of the organisms and their susceptibility to oxidative stress (Regoli 2000). The method has been validated successfully as a biomarker (Regoli 2000). In addition, Regoli *et al.*

(2000) showed that the basal total oxyradical scavenging capacity was higher in a polar than a temperate marine bivalve, indicating that the natural prooxidant pressure of cold water-ecosystems was elevated compared with temperate water.

In this study, biomarker responses of *Hyas araneus* to oil were investigated via two routes: contaminated sediment and injection.

## 9.2. Materials and Methods

### Sampling and maintenance of crabs

In May 2000, *Hyas araneus* were collected from Hollenderbukta in Isfjorden at Svalbard (Figure 20). Crabs were taken with an Agassiz dredge from *ca.* 50m depth using the research vessel F/F Jan Mayen of the University of Tromsø. Seawater temperature was  $-1.5^{\circ}\text{C}$  at the time of collection. Small spider crabs [wet weight =  $13.00 \pm 3.91\text{g}$ , carapace length =  $37.79 \pm 3.20\text{mm}$ , carapace width =  $27.29 \pm 2.65\text{mm}$  ( $n=24$ )] were selected for the experiments. Crabs were stored in cooled ( $1^{\circ}\text{C}$ ) seawater and air freighted to the laboratory (no mortality occurred during transport). In the laboratory, crabs were stored for one month prior to their use in experiments in a glass fibre tank (200 l); the seawater and air temperatures were maintained at  $2^{\circ}\text{C}$ . Illumination reproduced the summer Arctic light regime of 24 h daylight. Crabs were fed every two days with a mixed diet of crushed mussels (*Mytilus edulis*) or pieces of dead fish and shrimp. Seawater was filtered and a protein skimmer was coupled to improve water quality. Seawater was changed weekly.

### Exposure system

24 crabs were divided into three groups, each of 8 individuals, and each group was placed into one of the following 40 l aquaria: 'control', 'sediment exposure' and 'injection' (Figure 45). To obtain the contaminated sediment, 500 ml of crude oil, originating from the oil field Frøy (North Sea), were mixed thoroughly with 6 kg of wet sediment (particulate size diameter  $> 0.5\text{mm}$ ) sampled on a beach located at Stavanger (Norway). The contaminated sediment was left standing at  $2^{\circ}\text{C}$  with no animals for 24 h to allow the oil to adsorb onto the sediment particles. The seawater was then poured and any excess oil on the surface was removed. Crabs were placed in the tank and left for 15 days. For the injection exposure, 8 crabs were injected with  $5\ \mu\text{l}$  of the same crude oil at

day 0, day 1 day 3 and day 6. In total, 20 µl of oil were injected for a mean wet weight of 12.73 ( $\pm 2.84$ ) (1.6 µl per gram wet weight). Injected crabs provided a positive control of oil uptake and sediment-exposed crabs simulated an acute oil spill. Irrespective of treatment, crabs were fed twice a week with freshly crushed mussels (*Mytilus edulis*); seawater was renewed the day after feeding and the excess food was removed. After 15 days, the respiration and heart rate of 8 crabs from each group was measured within 24 h (using the same individuals). After completion of the cardiac activity recordings, the fresh weight, length and width of each crab were measured and the midgut gland was removed, frozen and stored at  $-80^{\circ}\text{C}$  for the TOSC assay.

### **Biomarker analyses**

Heart rate was measured with the non-invasive CAPMON procedure (Computer Aided Physiological MONitoring system) developed by Depledge and Andersen (1990) (Figure 44) (see section 4.5.6.).

TOSC was measured according to Winston *et al.* (1998) (Figure 44) (see section 4.5.2.). A closed respirometer technique was used to measure the respiration rate (Figure 44) (see section 4.5.5.).

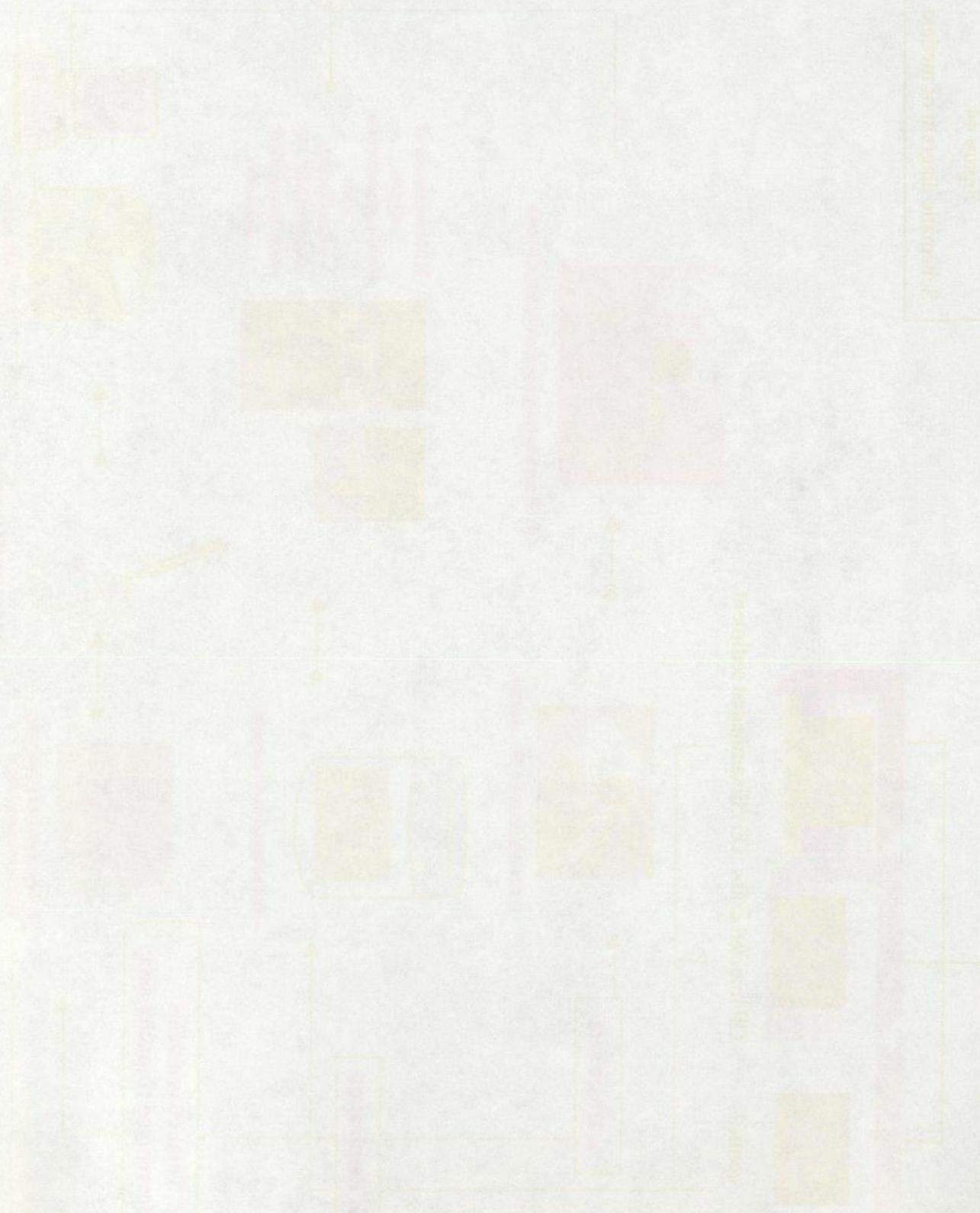
### **PAH in sediment analysis**

Sediment was sampled and stored in aluminium paper at  $-80^{\circ}\text{C}$  prior to analysis. The PAH analysis was performed by gas chromatography and mass spectrometry (GC-MS) according to the method of Douglas *et al.* (1994) (see section 4.5.7.).

### **Statistical analysis**

All statistical analyses were made using JMP v3.2.6., SAS Institute, Inc., Cary, NC, USA. Heart rate data were not normally distributed and variances were not equal, as a result, the non-parametric Wilcoxon test was used. Respiration and TOSC data were normally distributed and variances were equal therefore, the Dunnett's test was used to test for statistical differences. Values of heart rate, respiration and TOSC were plotted as Box and Whisker plots. These indicate the range of values and the median. The horizontal bars, that denote the upper and lower limits, include 95% of the data points. 50% of the data points are encompassed by the upper and lower limits of the boxes. The significance level was  $p < 0.05$ .





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## 9.4. Results

### Heart rate

Heart rate increased significantly in injected and sediment-treated crabs ( $p < 0.0001$ ) compared with controls (Figure 45). The mean heart rates of *H. araneus* were  $49.06 \pm 13.72$ ,  $57.56 \pm 7.28$  and  $63.30 \pm 6.57$  beats per minute (mean  $\pm$  S.D.) in control, injected and sediment-treated group respectively (see values reported in Table 4). Also, the heart rate was significantly higher in sediment-exposed crabs compared with injected crabs ( $p < 0.0001$ ). In each exposure group, the variability decreased by 50% compared with the control, indicating reduced inter- and intra-individual variability.

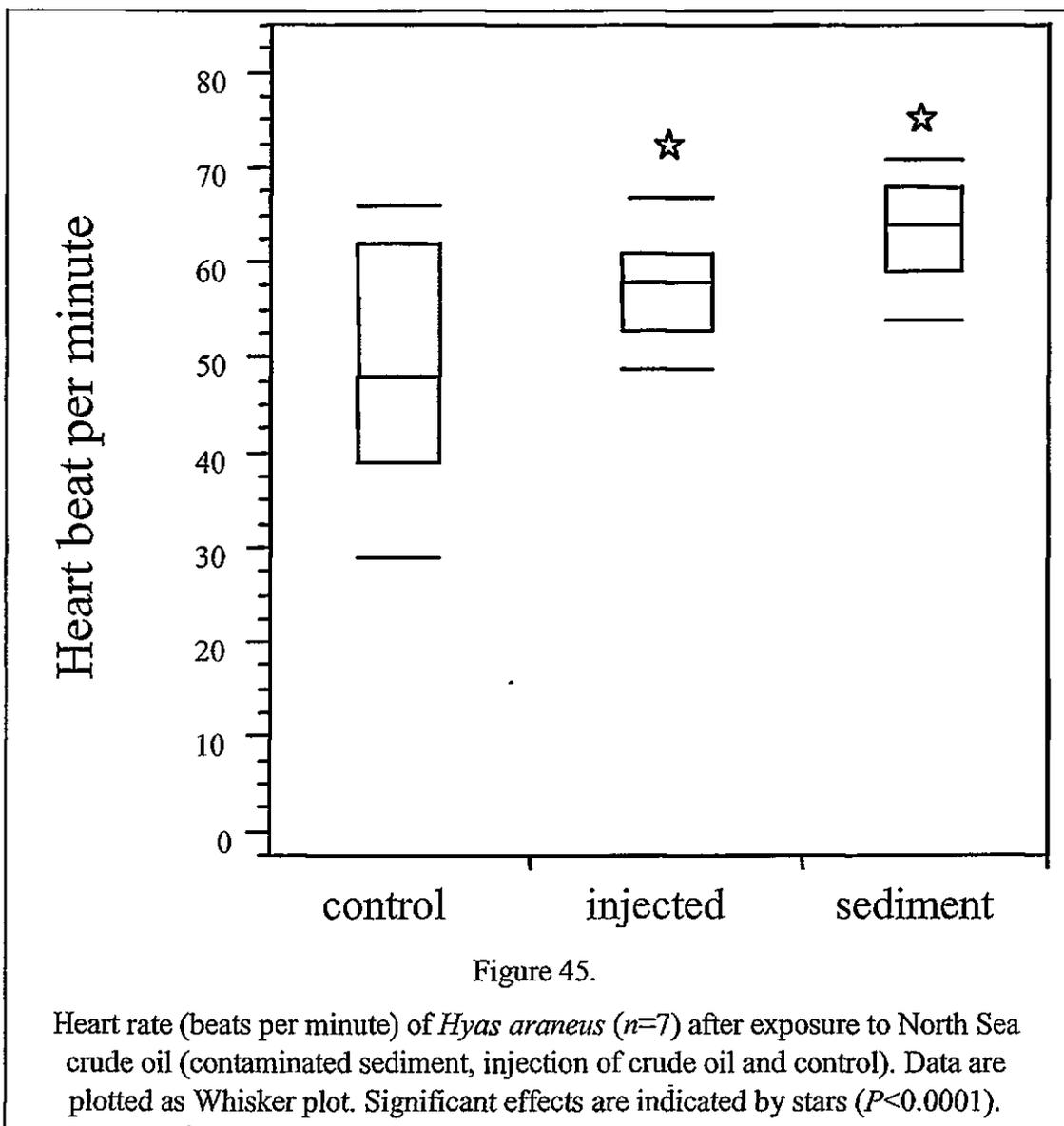


Table 4. Heart rates of individual *Hyas araneus* [fH = heart frequency (beats per minute); C% = Coefficient of variability].

Control		Injected		Sediment	
fH ± SD	C%	fH ± SD	C%	fH ± SD	C%
46.8 ± 14.51	31.00	52.89 ± 1.45	2.75	62.35 ± 2.92	4.69
42.90 ± 8.50	19.82	66.21 ± 4.45	6.72	68.00 ± 3.25	4.77
66.43 ± 4.05	6.09	47.23 ± 7.96	16.85	63.43 ± 2.32	3.65
33.83 ± 11.72	34.63	56.95 ± 6.29	11.04	69.15 ± 1.42	2.05
43.48 ± 6.48	14.91	59.38 ± 1.89	3.18	54.27 ± 5.93	10.93
45.47 ± 9.50	20.90	60.69 ± 4.74	7.81	71.47 ± 2.48	3.47
61.48 ± 1.57	2.55	58.98 ± 1.62	2.75	58.88 ± 3.71	6.30

**Respiration**

Oxygen consumption by *H. araneus* was not significantly affected by either oil treatment ( $p>0.05$ ) (Figure 46). Nevertheless, the variability in the sediment-exposed group was twice as high as the control, with high values indicating an increase in

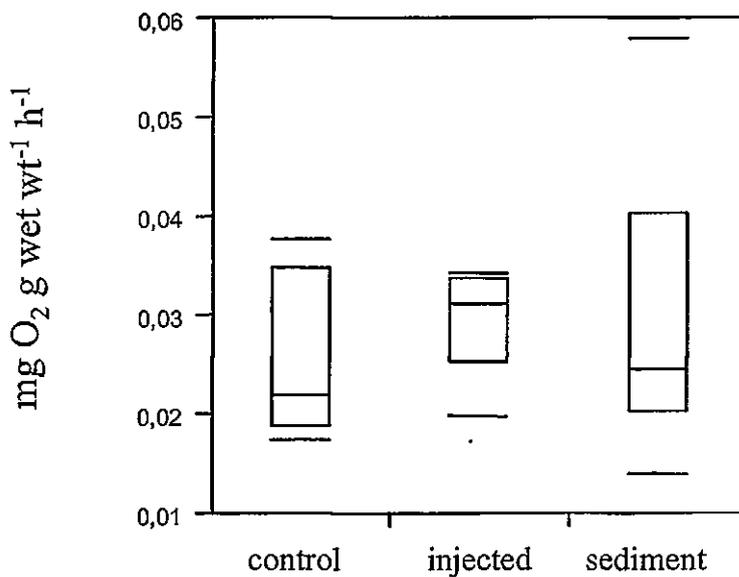


Figure 46.

Respiration of *Hyas araneus* measured as uptake of mg of oxygen per g wet weight per hour ( $n=8$ ) after exposure to Northe Sea crude oil (contaminated sediment, injection of crude oil and control). Data are plotted as Whisker plot.

oxygen uptake (0.045 and 0.058 mg O<sub>2</sub> g wet wt<sup>-1</sup> h<sup>-1</sup>) for two individuals. The basal oxygen consumption in control *H. araneus* at 2°C is 0.025 mg O<sub>2</sub> g wet wt<sup>-1</sup> h<sup>-1</sup> (Table 5).

**TOSC**

TOSC was not affected by either exposure ( $p > 0.05$ ) (Figure 47 and Table 5). Nevertheless, variability of TOSC increased in the injected group with a high TOSC value of 7000 unit mg<sup>-1</sup> protein and a low value of 3200 unit mg<sup>-1</sup> protein. This variability indicates different responses of the antioxidative defences in various individuals which could be interpreted as a transient phase. Nevertheless, the mean TOSC value of the injected group (4942 unit mg<sup>-1</sup> protein) was similar to that of controls (4837 unit mg<sup>-1</sup> protein). The sediment-treated group showed a decrease in the median (4133 unit mg<sup>-1</sup> protein) and in the mean (4377 unit mg<sup>-1</sup> protein), indicating a certain depression in TOSC, although this was not statistically significant.

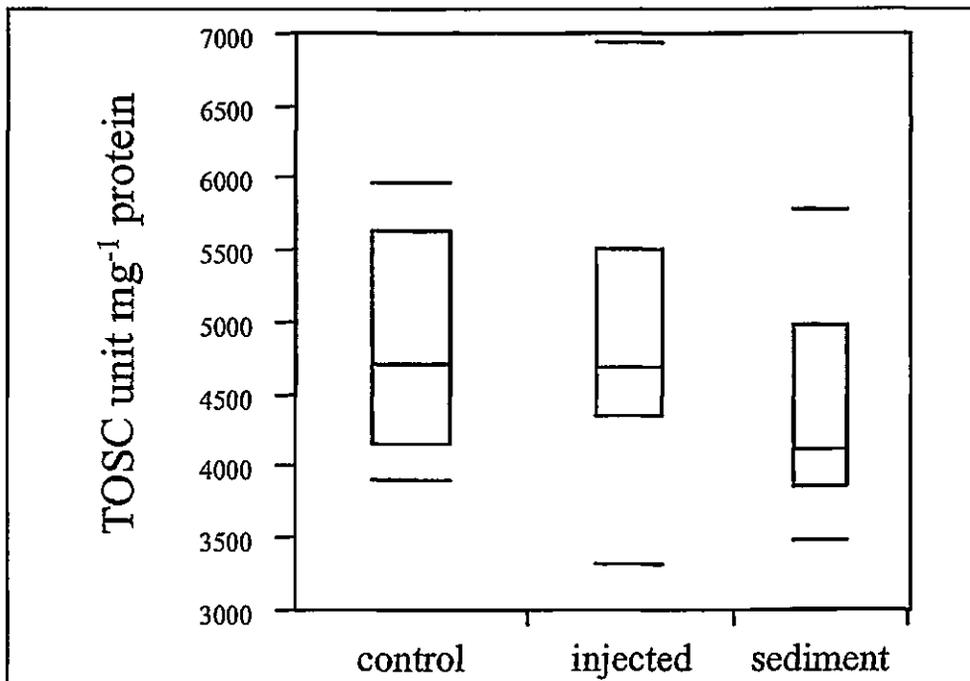


Figure 47.

Variations of the total oxyradical scavenging capacity (TOSC) towards the peroxy radicals in the digestive gland of *Hyas araneus* after exposure to North Sea crude oil [contaminated sediment ( $n = 6$ ), injection of crude oil ( $n = 7$ ) and control ( $n = 8$ )].

Table 5. Mean and standard deviation of respiration of *Hyas araneus* in different treatments ( $\mu\text{g O}_2$  wet weight $^{-1}$  hr $^{-1}$ ,  $n=8$ ) and TOSC measured in hepatopancreas (TOSC unit per mg protein,  $n=8$ ).

	control	injected	sediment
Respiration	$26 \pm 8.2$	$30 \pm 5.1$	$29 \pm 14.6$
TOSC	$4837 \pm 758$	$4942 \pm 1121$	$4376 \pm 801$

### Polycyclic aromatic hydrocarbon in sediment

The polycyclic aromatic hydrocarbon composition (PAH) of the crude oil used in the experiment and concentration in sediment are reported in Table 6. The total PAH is 234 195  $\mu\text{g kg}^{-1}$  of wet sediment. Two rings molecules (i.e. naphthalenes) are the main compounds of this crude oil as they represent 95% of the total amount of oil compounds. The three and four ring compounds represented 5.5% only of the total PAH. Five rings compounds (i.e. benzo(a)pyrene) were under the detection limit. The two ring molecules are known to be the most soluble PAHs (Neff 1979). Siron *et al.* (1993, 1996) have reported that naphthalenes were the major dissolved compounds in seawater at low temperature ( $<3^\circ\text{C}$ ). In our study, it is likely that *H. araneus* was mainly exposed to naphthalenes. The low molecular weight of naphthalenes make them more bioavailable and they could be taken up readily through the gills of the crabs.

Table 6. PAH composition, measured by GC/MS, of the applied North Sea crude oil, in the sediment after 2 weeks exposure. Values are in  $\mu\text{g}$  of PAH per kg of sediment. Total PAH (TPAH) is the sum of all 26 measured PAHs. Values in brackets correspond to signal below detection limit.

Compound	Concentration ( $\mu\text{g kg}^{-1}$ )
Naphthalene	15465
C1-naphthalene	53842
C2-naphthalene	85514
C3-naphthalene	65760
Acenaphthylene	289
Acenaphthene	437
Fluorene	3338
Phenanthrene	3528
Anthracene	0
C1-phen/anthr	1347
C2-phen/anthr	971
Dibenzothiophene	552
C1-dibenzothiophene	276
C2-dibenzothiophene	273
Fluoranthene	204
Pyrene	384
Benzo(a)anthracene	(48)
Chrysene+Triphenylene	346
C1-chrysene	770
C2-chrysene	897
Benzo(b)fluoranthene	(58.8)
Benzo(k)fluoranthene	0
Benzo(a)pyrene	0
Indeno(1,2,3-cd)pyrene	0
Benzo(g,h,i)perylene	0
Dibenzo(a,h)anthracene	0
<b>TPAH</b>	<b>234195</b>

## 9.5. Discussion

Heart rate appeared to be the most sensitive biomarker to oil exposure in *Hyas araneus*. Oil-injected and sediment-exposed crabs showed a significant increase in heart rate compared with controls. Very little data on the effect of oil components on cardiac activity of crustaceans have been published. The increased heart rate measured in the current study supports Depledge (1984b) who reported an increased heart rate in *Carcinus maenas* exposed to the water-soluble fraction (WSF) of crude oil. However, bradycardia and arrhythmia were induced in *Pugettia producta* when low levels of the WSF of oil was passed over the gills (Zimmer *et al.* 1979). Bamber and Depledge (1997) investigated the dietary exposure of *Carcinus maenas* to benzo(a)pyrene and failed to record any obvious effects on heart rate over 7 days of exposure. The high variability in heart rate of the control group in the present experiment supports Depledge (1984a) who reported a similar response for *Carcinus maenas*. The decreased variability in the injected and sediment-exposed groups has been observed by Depledge (1984a). Lundebye and Depledge (1998) showed that increased heart beat in *Carcinus maenas* was due to a decline in mean interpulse duration. The elevated heart frequencies, reported in the current experiment, may indicate respiratory stress that was demonstrated by an increased respiration rate in *Carcinus maenas* exposed to the WSF (Depledge 1984b). However, no change in respiration rate was measured in the present experiment. Exposure of *Carcinus maenas* to copper induced increased heart frequency and tissue hypoxia (Nonnotte *et al.* 1993, Bamber and Depledge 1997). A major concern of oil pollution is that it might affect oxygenation of seawater (F.A.O. 1977). The lack of oxygen could influence respiratory and cardiac physiology resulting in a greater volume of blood flow demanded per unit of oxygen required for tissue respiration. Cho *et al.* (1994) reported that naphthalene metabolites bind to large proteins such as haemoglobin, probably altering functions of the respiratory pigment leading to hypoxia. Nevertheless, several authors have reported a decrease in heart rate due to hypoxia and a concomitant bradycardia, with the heart stroke volume increasing to compensate for the lack of oxygen (Reiber and McMahon 1998).

The increased heart frequency of sediment-contaminated and injected *H. araneus* may have resulted from voluntary reduction of the ventilatory stream to the gills to prevent chemical uptake. This compensatory behaviour would minimise the exchange with the external media while maintaining basal oxygen supply to the tissues. Increased heart

rate is associated with locomotor activity in crustaceans. Aagaard *et al.* (1995) recorded an elevated heart rate prior to locomotor activity in *Carcinus maenas* and on no occasion did locomotor activity increase prior to elevation of heart rate. In our study, similar observations were made. Aagaard *et al.* (1995) postulated that the circulatory and respiratory systems of the shore crab are prepared in advance to deal with the metabolic demands of locomotor activity. Such responses would be regulated by the neuro-endocrine system. The cardiac response of *H. araneus* could be regarded as an avoidance response. The animal has initiated its physiological machinery for escaping the oil-induced stress. As crabs remain quiescent, the energetic demand is not increased as shown in the steady state of oxygen uptake measured in the present study.

Changes in the variability of physiological parameters may provide an additional useful insight into the impact of chemical contamination (Depledge and Lundebye 1996). Fredrich *et al.* (2000) reported a decrease in variability of heart rate and oxygen consumption in *Hyas araneus* under physiological stress. Depledge and Lundebye (1996) explained the increasing inter-individual variability of heart rate with increasing contaminant concentration as an increasing proportion of affected crabs in the sampled group. In the current study, the reduced inter and intra-individual variability indicated that oil exposure has induced a homogeneous physiological response in crabs.

Fredrich *et al.* (2000) reported a significant decrease in respiration in *Hyas araneus* collected from temperate water at 9°C and acclimatised to 2°C. In the current study, the oxygen uptake of *H. araneus* at 2°C (0.025 mg O<sub>2</sub> g wet wt<sup>-1</sup> h<sup>-1</sup>) was comparable to the value reported by Fredrich *et al.* (2000) (0.019 mg O<sub>2</sub> g wet wt<sup>-1</sup> h<sup>-1</sup>). Thus, the low temperature of the Arctic water has reduced the metabolic activity of *H. araneus*. This observation was reported also by Whiteley *et al.* (1996) for the giant Antarctic isopod (*Glyptonotus antarcticus*) compared with a temperate isopod (*Idotea rescata*). The low oxygen uptake rate in *H. araneus* certainly reduced the uptake of poly-aromatic hydrocarbons through the gills compared with temperate individuals. Moreover, the low metabolic rate of the spider crab may result in reduced metabolism of oil compounds into reactive oxygen species (ROS). The low uptake and reduced metabolism of PAH could explain the lack of TOSC response measured in the present study. ROS is naturally produced during respiration when O<sub>2</sub> is reduced (Winston and DiGiulio 1991) but ROS generation is also known to be enhanced via the metabolism of oil compounds (Livingstone *et al.* 1990). Babu and Brown (1995) reported an oxidant-stress toxicity of

naphthalenes. There is a large literature reporting the varied activity of antioxidant defences and activation of contaminants to reactive metabolites in decapod crustaceans following pollutant exposure (e.g. Marsh *et al.* 1992, Gamble *et al.* 1995, Sundt and Goksøyr 1998, Arun *et al.* 1999, Orbea *et al.* 2000). Orbea *et al.* (2000) localised four main antioxidant enzymes (catalase, Cu/Zn-superoxide dismutase, Mn-superoxide dismutase and glutathione peroxidase) in the digestive gland of the shore crab *Carcinus maenas*, indicating the capacity of decapod crustaceans to cope with ROS. In the present study, no significant TOSC response was measured indicating that the possible enhancement of ROS production (notably peroxy radicals) was limited and did not saturate the scavenging system capacity of *H. araneus*. The basal oxyradical scavenging capacity of *H. araneus* is relatively high compared with other marine invertebrates. For example, Regoli (1998) reported TOSC values of *ca.* 600 unit  $\text{mg}^{-1}$  protein for the blue mussel (*Mytilus edulis*) and *ca.* 700 unit  $\text{mg}^{-1}$  protein for a starfish (*Leptasterias epichlora*). Elevated TOSC values in a cold-water species may be indicative of an enhanced resistance of toxicity in ROS generation. This would confirm a natural high prooxidant pressure for cold-water species (Regoli *et al.* 2000) and might also provide *H. araneus* with the capability of dealing with moderate variations in ROS generation. The lower mean TOSC value in the sediment-treated group is indicative of a certain degree of saturation of the antioxidative system. It is likely that such saturation corresponds to the partial consumption of the fast-acting scavengers of peroxy radical, for instance glutathione, uric acid and ascorbic acid (Winston *et al.* 1998). To date, reported data on the use of TOSC as a biomarker of ROS indicate a significant reduction in antioxidant capacity (Regoli 2000, Camus *et al.* *in press*, Grosvik *et al.* *in prep*) or induction (Winzer *et al.* 2001). Grosvik *et al.* (*in prep*) observed a depression in TOSC in the hepatopancreas of the Arctic shrimp (*Sclerocrangon boreas*) exposed to 2 ppm dispersed oil for 5 days at 2°C. Similar observation were reported in Arctic scallop exposed to benzo(a)pyrene (Camus *et al.* *in press*). With a depression in TOSC, Regoli (2000) reported a greater depletion of low molecular weight molecules during the first phase of exposure of Mediterranean mussels to high levels of pollutants, indicating ROS production and activation of antioxidant defences. In the present experiment, the absolute TOSC mean value may not be the appropriate parameter to use as a biomarker and the inter-individual variability may be more indicative of exposure (Depledge and Lundebye 1996). In the injected group, the variability in TOSC measurements was twice that of the control. These data suggest a concomitant induction

and depression in TOSC, and support the transient response of antioxidant defences as reported by other authors (Livingstone *et al.* 1992, Doyotte *et al.* 1997). A depletion or induction of antioxidant systems may represent a first response to pollutants that can be followed by a more integrated response. It would have been interesting to extend the exposure period beyond 14 days to follow the change in TOSC. TOSC was measured in the hepatopancreas whereas injection was performed into the haemolymph where haemocytes represent the main defence system against foreign substances (Dyrynda *et al.* 1998). Thus, the hepatopancreas may have not been the correct target tissue to measure TOSC and the haemocytes may have been more appropriate.

In this study, heart rate (a physiological biomarker) was very sensitive in reflecting the first effects of oil in each exposure context. It is interesting to note that the TOSC assay did not provide any distinct signal, however, this latter biomarker has more ecological relevance than heart rate. McMahon (1999) highlighted the difficulty of interpreting cardiac performance in crustaceans by measuring one single parameter, such as heart rate. McMahon (1999) stated that haemolymph flow through any particular decapod arterial system does not necessarily vary with heart rate or even cardiac output but is, in each artery, modified from moment to moment by a variety of hormonal and neural drives. Heart rate on its own cannot provide a meaningful diagnostic of pollution exposure. TOSC is a tool for quantitatively assessing the biological resistance to toxicity of different forms of ROS. Up to now, the efficiency of the antioxidant system has been based upon analysis of single components, including enzymes (catalase, superoxide dismutase, etc), and smaller molecules such as vitamin E, C, uric acid and reduced glutathione. Numerous studies have reported responses to oxidative stressors that can be very different and, while depletion is measured in one parameter, a concomitant induction could be measured in another one. The overall budget of ROS versus anti-oxidant defences can, therefore, barely be estimated and the complexity of the interpretation prevents any prognosis at the individual or population level. Single antioxidant analysis indicates exposure to oxidative stress but cannot provide a diagnostic measure of the health of the organism. The benefit of the TOSC assay is that it provides a prediction of the effects of environmental conditions on the redox status of the whole organism and its susceptibility to oxidative stress disease (Regoli 2000). The increase in heart rate may reflect the initial effect of oil notably the naphthalene compounds; however, the lack of change in TOSC associated with a low oxygen uptake

reveals a limited toxic effect probably due to a low uptake and metabolism of PAH into ROS.

## 9.6. Conclusions

This investigation of the impact of oil on Arctic *Hyas araneus* at low temperature revealed an increase in heart rate, a steady state in respiration and a transient response in TOSC in the injected group, and a moderate decrease in the sediment-exposed group compared with the controls. The higher ecological relevance of TOSC, compared with heart rate, leads to the conclusion that the Arctic crab was experiencing a moderate pollution effect. Low temperature may be the main factor reducing the bioavailability of poly-aromatic hydrocarbons in the water however, the relatively low metabolic rate of *H. araneus* may have reduced the uptake, metabolism of PAH and the production of ROS.

## Chapter 10

### **Biomarker responses and PAH uptake in *Mya truncata* following exposure to oil-contaminated sediment in an Arctic fjord (Svalbard)**

Article submitted to Science of the Total Environment



Erebus volcano (smoking) and castle rock

*“We stood on the verge of a vast abyss, and at first could see neither to the bottom nor across it on account of the huge mass of steam filling the crater and soaring aloft in a column 500 to 1000 ft. high. As the result of averaging aneroid levels, together with the hypsometer determination at the top of the old crater, Erebus may be calculated to rise to a height of 13,370 ft. above sea level.”*

**Professor David, first man on top of Erebus volcano. Antarctic expedition of Ernest Shackleton, 1907-1909.**

### Discussion

Findings for caregivers and PBL relate to five areas: following exposure to the intervention, caregiver and PBL (Swanson)

Findings related to caregiver and PBL (Swanson)

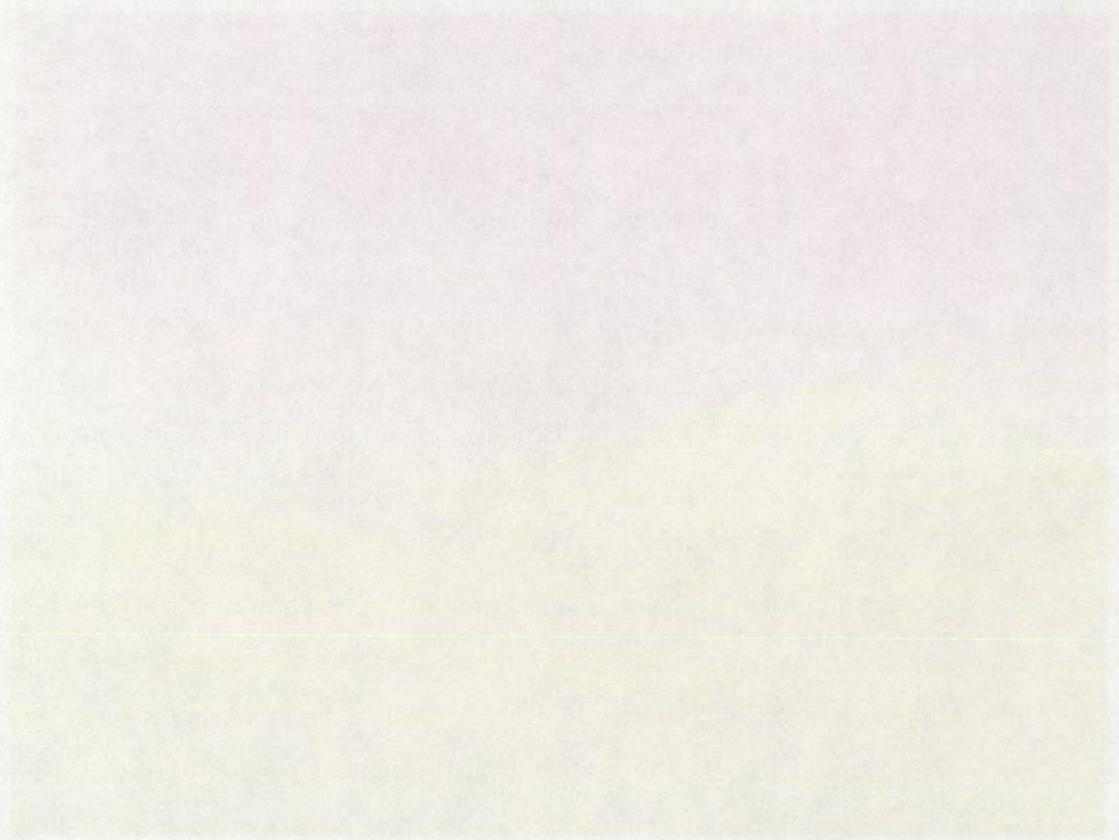


Table 1. Summary of findings for caregivers and PBL

The findings for caregivers and PBL are presented in Table 1. The results indicate that caregivers and PBL showed significant improvements in various areas following the intervention. These improvements were observed in both the short-term and long-term follow-up periods. The findings suggest that the intervention had a positive impact on the well-being and functioning of caregivers and PBL.

These findings have important implications for practice and research. They suggest that interventions targeting caregivers and PBL can be effective in improving their well-being and functioning. Further research is needed to explore the mechanisms of change and to evaluate the long-term sustainability of these improvements.

### 10.1. Abstract

Expanding industrial activities in the Arctic require assessment of the toxicity of chemicals at low temperature. Little is known about the potential impact of chemicals on marine organisms living in seawater at temperatures of ca.  $-1.88$  (and seldom rising above  $3-4^{\circ}\text{C}$ ). The arctic bivalve *Mya truncata* is common in the Svalbard fjord ecosystem where it experiences low temperature throughout the year, and is the main food resource for walrus and seals. To measure the impact of polycyclic aromatic hydrocarbons (PAH) on *M. truncata*, the response of three biomarkers was investigated from bivalves exposed to sediment contaminated with a PAH mixture (crude oil): i) total oxyradical scavenging capacity-assay (TOSC), ii) plasma membrane stability of haemocytes and iii) respiration rates. TOSC showed no change after two weeks of exposure to the contaminated sediment. The high TOSC value ( $4010 \pm 1339$  unit  $\text{mg}^{-1}$  protein) of the control group is indicative of the presence of high levels of ROS in the water formed naturally by increased levels of UV-B irradiance in polar regions. Cellular membranes of the haemocytes of exposed bivalves were significantly destabilised compared with controls ( $p < 0.05$ ), indicating that internal cellular antioxidant defences were overwhelmed. Respiration rate was low ( $0.055 \pm 0.020$   $\text{mg O}_2 \text{ dw}^{-1} \text{ h}^{-1}$ ) for control and PAH-exposed individuals, and typical of polar bivalves, reflecting a strategy to minimise energy expenditure and to cope with 9 months of starvation. Bioaccumulation of PAH by *M. truncata* was low, probably due to low metabolic rate and poor solubility of the oil compounds at low temperature. Data indicated an uptake of mainly low molecular weight compounds (two and three ring molecules). A good correlation of  $\log\text{BAF}_{\text{lipid}}$  (bioaccumulation factor) and  $\log K_{\text{ow}}$  (octanol/water partition coefficient) was shown ( $r^2 = 0.87$ ). Tissue sensitivity and/or functional differences (hepatopancreas versus haemocytes), PAH uptake route (dietary versus gills), the low temperature reducing metabolic rate of *M. truncata* and the bioavailability of PAH, are factors that help explain these findings.

*Keywords:* Biomarkers, bioaccumulation, PAH, sediment, Arctic, *Mya truncata*.

## 10.2. Introduction

Human activities are increasing in the European Arctic and, as a result, reliable environmental monitoring tools are required to identify the potential impact of industrial developments, notably oil and gas activities (AMAP 1998). Biomarkers [defined as "biological responses to a chemical or chemicals that give a measure of exposure or toxic effect" (Peakall 1994)] are relevant measures of the *in situ* impact of oil discharges (Aas *et al.* 2001). Although biomarkers have been employed in environmental toxicology for the last 20 years, most of the research with marine ecosystems has been performed in temperate regions characterised by high variations of the seawater temperature (i.e. from 10 to 20°C). Little is known about the potential impact of contaminants in marine organisms living in polar waters. The Arctic marine ecosystem is characterised by a combination of a low, but stable, temperature and a single, well-timed maximum of dense, but very brief, primary production in May, followed by a rapid decline (Weslawski *et al.* 1988). Recent stratospheric ozone depletion in polar areas has caused an increase in UV-B radiation, thereby, significantly augmenting the production of toxic reactive oxygen species (ROS) in the water column (Yocis *et al.* 2000, Qian *et al.* 2001) threatening marine life (Abele *et al.* 1998). Hence, polar marine invertebrates possess specific biological adaptations to live in this marine ecosystem. Respiration is low presumably to cope with the low food supply and 9 months of starvation (Peck *et al.* 1997, Ahn and Shim 1998, Clarke and Johnston 1999). Antioxidant defences are high compared with temperate species to cope with oxidative stress (Regoli *et al.* 2000). Cell membrane composition differs from temperate ectotherms to maintain fluidity at low temperature (Gillis and Ballantyne 1999a). Conversely, physical properties of petroleum hydrocarbons behave differently at low temperature; for instance, crude oil does not dissolve in cold water to the same extent as at higher temperatures and such differences have important consequences to the bioavailability of oil components (Neff 1979). Consequently, the biological adaptation of cold water organisms, together with the altered oil behaviour, may affect the typical biomarker responses. Earlier studies have attempted to test this hypothesis by looking at effect of poly aromatic hydrocarbons (PAH) on fish (Christiansen *et al.* 1996, Wolkers *et al.* 1996) and amphipods (Aunaas *et al.* 1991), but little similar work has been carried out with Arctic bivalves.

Numerous studies with temperate bivalves show that uptake, metabolism and excretion of PAH depend upon the metabolic rate and antioxidant defences properties. Therefore, in this study, to understand what the impact of PAH on cold water adapted bivalve is, the research strategy was to focus on oxidative stress by investigating the metabolic rate, antioxidant defences and impact of ROS. All these three biological features are closely linked (Sohal and Weindruch 1996, Abele *et al.* 1998, Regoli 2000, Winzer *et al.* 2001). The metabolism of PAH enhances ROS production (Livingstone *et al.* 1990) but the relatively low metabolism of Arctic bivalves may reduce the metabolism of PAH, consequently minimising the oxidative stress. Conversely, The relatively high level of antioxidant defences of polar bivalves (Regoli *et al.* 2000, Camus *et al.* submitted) may confer on them a potential resistance towards oxidative stress. Further, it is established that lipid membranes are targets of ROS (Viarengo *et al.* 1989) and because of the lower unsaturated lipid content (Gillis and Ballantyne 1999a, Viarengo *et al.* 1995), cell membrane of polar bivalves appear to be more resistant to oxidative stress (Viarengo *et al.* 1995). These three hypotheses need to be resolved to gain a better understanding of what the potential impact of PAH can be on Arctic bivalves.

Because of their capacity to accumulate organic compounds, filter-feeder bivalves have been used for monitoring contaminant levels and effects in temperate water (Baumard *et al.* 1999b, Lowe and Fossato 2000), therefore, the Arctic bivalve *Mya truncata* (Myidae) a suspension filter-feeder living buried in the sediment was selected. It is widely distributed in Svalbard's fjords ecosystem (Koszteyn *et al.* 1991) and represents the main source of food of bearded seals (*Erignathus barbatus*) and walrus (*Odoboenus rosmarus*) (Fisher and Stewart 1997) which are still the major nutritional basis of the indigenous people of the North (AMAP 1998). Previously, *M. truncata* has been used as a monitoring species in the Arctic to measure petroleum hydrocarbon levels (Humphrey *et al.* 1987) following an experimental oil spill in the Canadian Arctic but scientists failed to measure the effects because of an inadequate biomarker selection strategy (Mageau *et al.* 1987). Its capacity to accumulate chemicals that could be readily transferred to high levels of the food web, including human, make *M. truncata* a key species for monitoring the potential impact of industrial activities in the Arctic.

In this study, a small-scale field study of sediment contamination was performed in an arctic fjord (Svalbard) by exposing the Arctic clam, *Mya truncata*, to PAH-contaminated sediment. The antioxidant defence responses were measured as total

oxyradical scavenging capacity according to Winston *et al.* (1998) as it will provide information with a predictive validity at the organism level. The effect of PAH on the metabolic rate was investigated by measuring the oxygen uptake. Finally, ROS toxicity was investigated by looking at the cell membranes stability of haemocytes with a method reported in Camus *et al.* (2000). This biological holistic approach consisting in measuring biological responses located at different levels of biological hierarchy (biochemical, cellular and physiological) will help to gain a broad understanding of the biological responses of *M. truncata* and to determine the survival potential in a PAH contaminated environment (Depledge 1994).

### 10.3. Materials and Methods

#### Field programme

In September 2000, the field experiment was established at Isfjorden at Svalbard (78°13' N and 15°39' E) (Figure 20). *Mya truncata* were hand sampled by scuba divers near the Norwegian settlement of Longyearbyen at the outer part of Adventfjorden. The bivalves were stored in large plastic tanks filled with sea water with permanent aeration for 24 h at 2°C. Grab samples of sediment were taken from the research vessel RV Jan Mayen of the University of Tromsø at the same location as the bivalves were sampled. The field site was at the mouth of Adventfjorden at 5 m depth near the shore to provide easy access to the divers. Seawater temperature was 6.5°C during establishment and 5.5°C when sampling was performed, 14 days later.

#### Experimental design

The sediment was divided in two equal amounts, each of 40 kg, and placed into two identical boxes (30 cm deep, 60 cm long and 30 cm large); one labelled 'control' and the other 'treatment' (Figure 20). Crude oil (0.4 l from a North Sea oil field) was mixed with 40 kg of sediment (10 ml of oil per kg of sediment). Individuals of *M. truncata* were hand buried into the sediment (20 individuals per box). Each box was lowered to the sea bottom and anchored at 5 m depth by divers. The treatment box was placed downstream of the control box and 10 m apart to prevent any risks of contamination. To reduce predation (i.e. seals), a grid was placed on top of each box. A red buoy, connected to the boxes by a rope, allowed easy relocation of the site for latter sampling (Figure 48). Animals were left in the boxes for two weeks. Sediment samples were taken to determine levels of PAH at the start and at the end to monitor oil sediment concentration in both boxes. To achieve this, surface sediment was sampled and placed in aluminium paper and stored in the freezer at -80°C. After 14 days, the bivalves were removed from the boxes, stored in sea water and brought back to the laboratory for biomarker and PAH level analyses. 5, 8 and 6 individuals from each group were used for respiration measurement, membrane stability and the TOSC assay, respectively. Hepatopancreas was dissected out for the TOSC-assay, frozen in liquid nitrogen and stored at -80°C prior to analysis. Following the two weeks exposure, two individuals per group were frozen at -80°C for PAH analysis (Figure 48).

## Analyses

### **TOSC**

The method is based on Winston *et al.* (1998) (Figure 48), except that buffers were adjusted for marine bivalves (Regoli *et al.* 1998) (see section 4.5.2.).

### **Haemocyte cellular membrane stability**

The method measures the stability of the cell membranes of the haemocytes and was reported by Camus *et al.* (2000). The fluorescent probe Ethidium homodimer-1 (EthD-1) enters cells with destabilised membranes (plasma and nucleus membrane) and sticks to the DNA in the nucleus. The intensity of the fluorescence indicates the extent of impaired membranes (Figure 48) (see section 4.5.3.).

### **Respiration**

A closed respirometer was used in this study. Five bivalves were placed individually into glass vials (480 ml), sealed with a screwed Teflon lid and incubated for 4 h at 5.5°C (Figure 48) (see section 4.5.5.). Five empty vials, containing seawater but no bivalves, were also incubated as controls and the oxygen concentration monitored.

### **Statistical analysis**

The analyses were carried out with JMP, ver. (3.2.6), SAS Institute Inc., Cary, NC, USA. Statistical analyses were run after testing normal distribution and homogenous variances. Parametric Dunnett's test was used; the significance level was  $p < 0.05$ . Values of TOSC, EthD-1 fluorescence and respiration were plotted as Box and Whisker plots. These indicate the range of values and the median. The horizontal bars that denote the upper and lower limits of the boxes include 95% of the data points. 50% of the data points are encompassed by the upper and lower limits of the boxes.

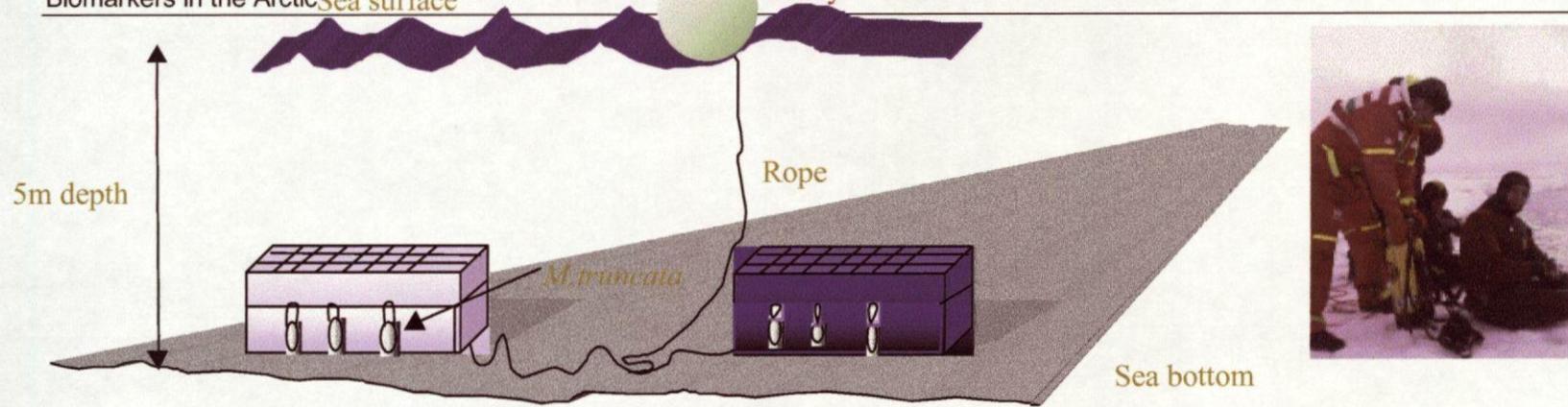
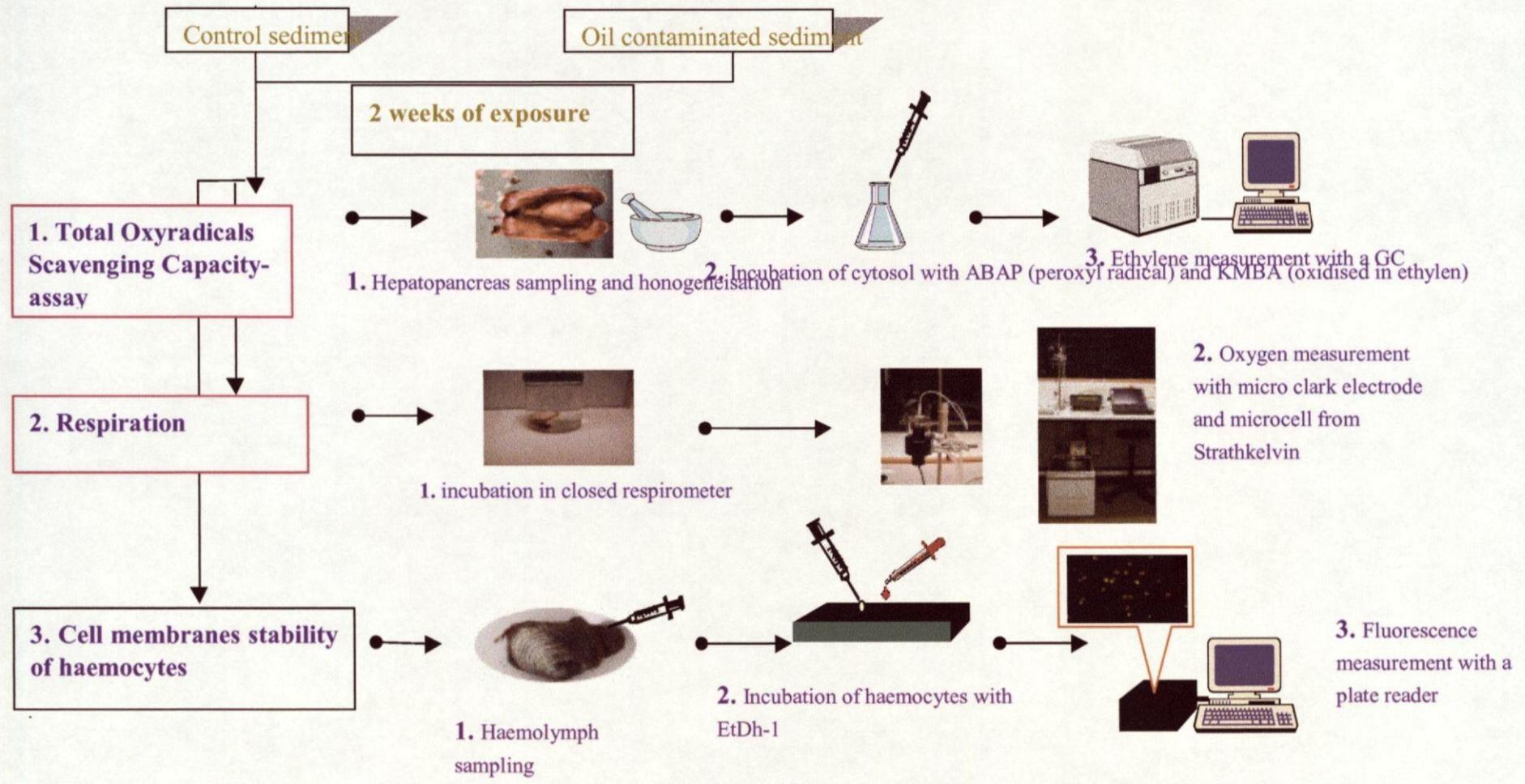
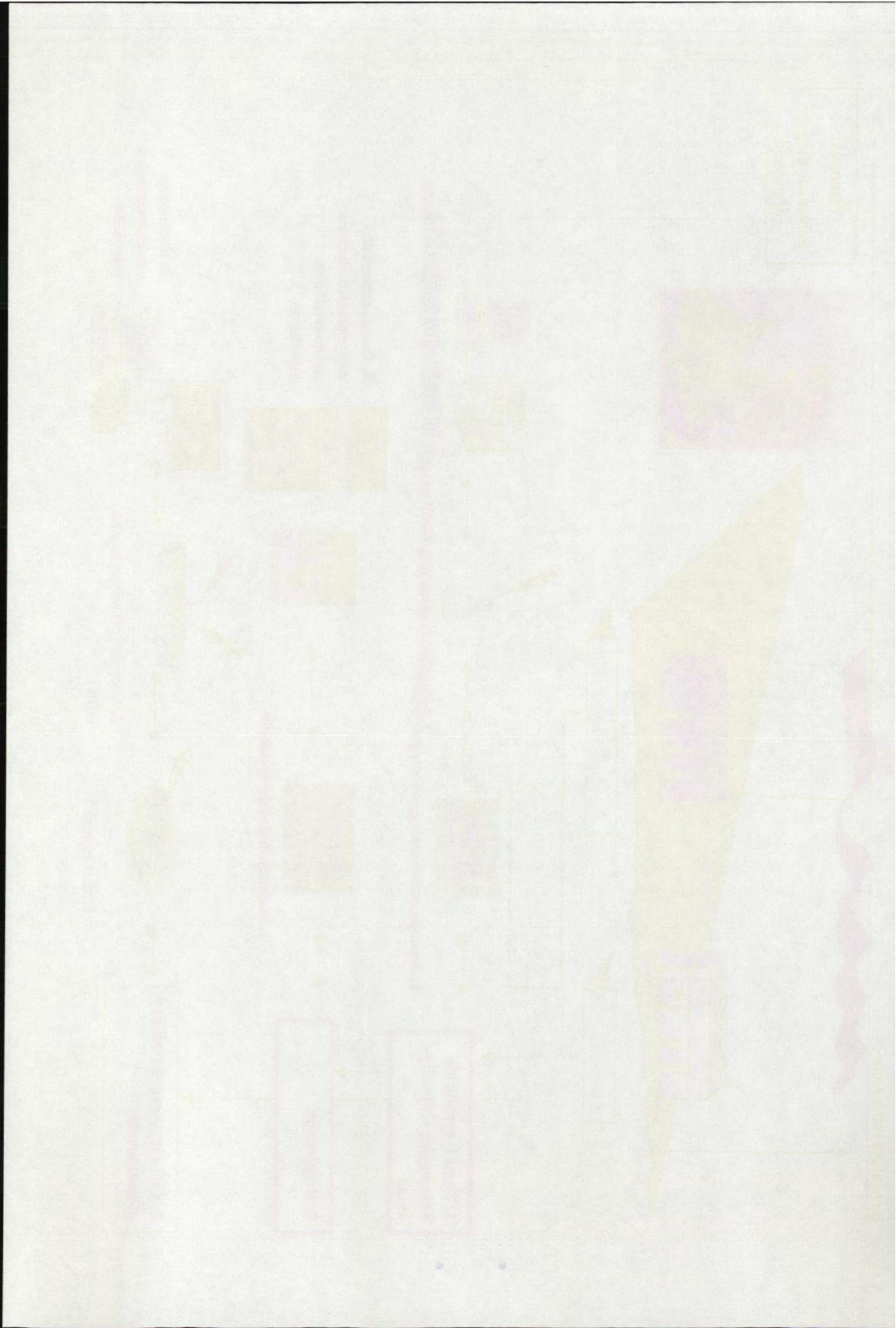


Figure 48.  
Schematic diagram  
of method.





## 10.4. Results

### TOSC

No significant difference in TOSC values was noted ( $p>0.05$ ) in the exposed group compared with the control (Figure 49 and Table 7). Interestingly, variability of the control group was twice that of the exposed group. The specific mean TOSC value of *M. truncata* in the control group was  $4\ 014 \pm 1339$  unit  $\text{mg}^{-1}$  protein (mean  $\pm$  S.D.).

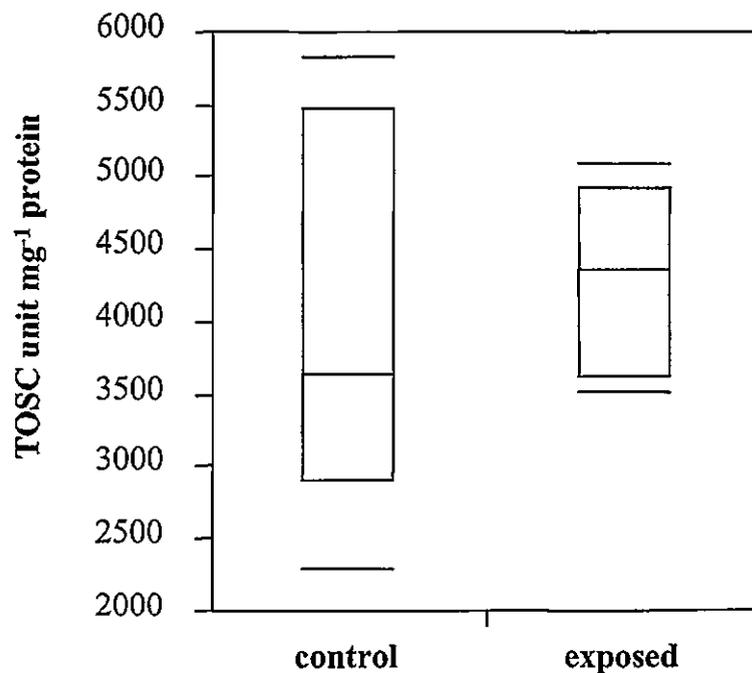
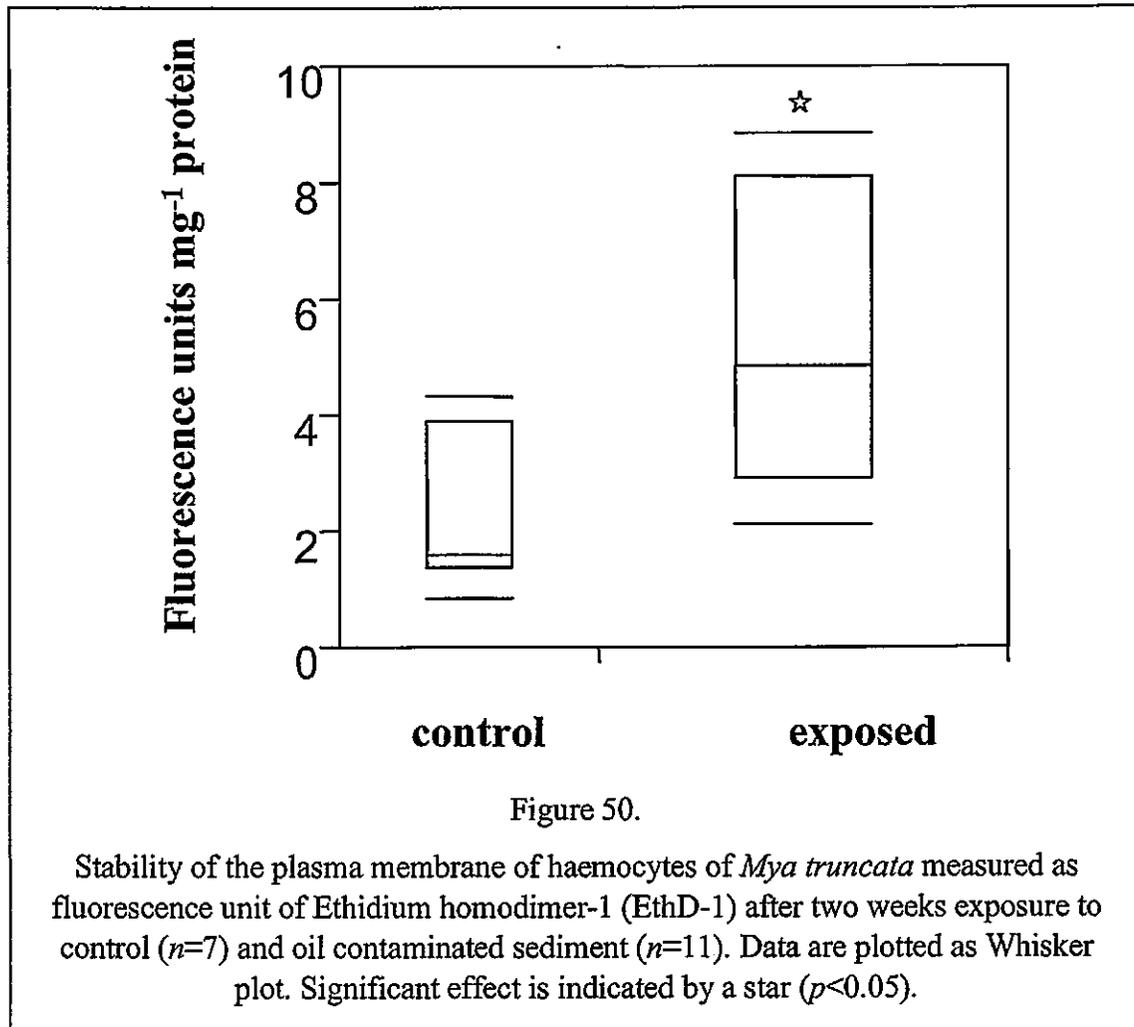


Figure 49.

Total Oxyradical Scavenging Capacity (TOSC) values towards peroxy radicals in the digestive gland of *Mya truncata* following two weeks of field deployment [control ( $n=6$ ); exposed=oil-contaminated sediment ( $n=6$ )]. Data are plotted as Whisker plot.

### Membrane stability

A significant increase in uptake of EthD-1 into the haemocytes of *M. truncata* was measured in the exposed group ( $p < 0.05$ ) (Figure 50 and Table 7). In the exposed group, the measured fluorescence was twice that of the control. Thus, the cellular membranes of haemocytes of *M. truncata* exposed to oil are permeable to EthD-1, indicating destabilisation of the membranes.



### Respiration

No significant differences were observed in the oxygen uptake of *M. truncata* in either group (Figure 51 and Table 7). Nevertheless, there was a decrease in the median of the exposed group, indicating a depressed respiration in some individuals. The basal summer oxygen uptake of *M. truncata* at 5.5°C was  $0.055 \pm 0.020$  mg O<sub>2</sub> g dw<sup>-1</sup> h<sup>-1</sup> (mean±S.D.).

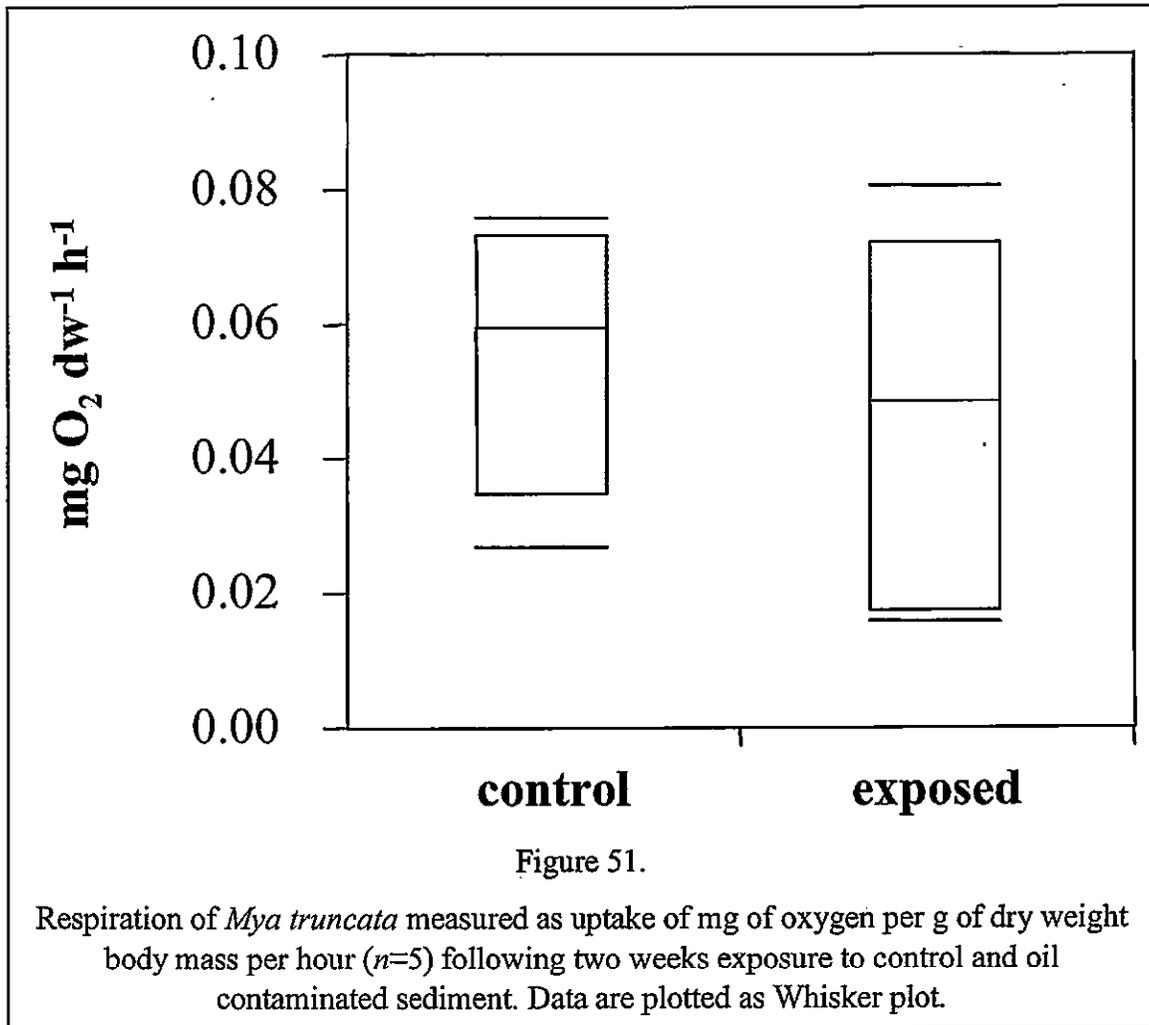
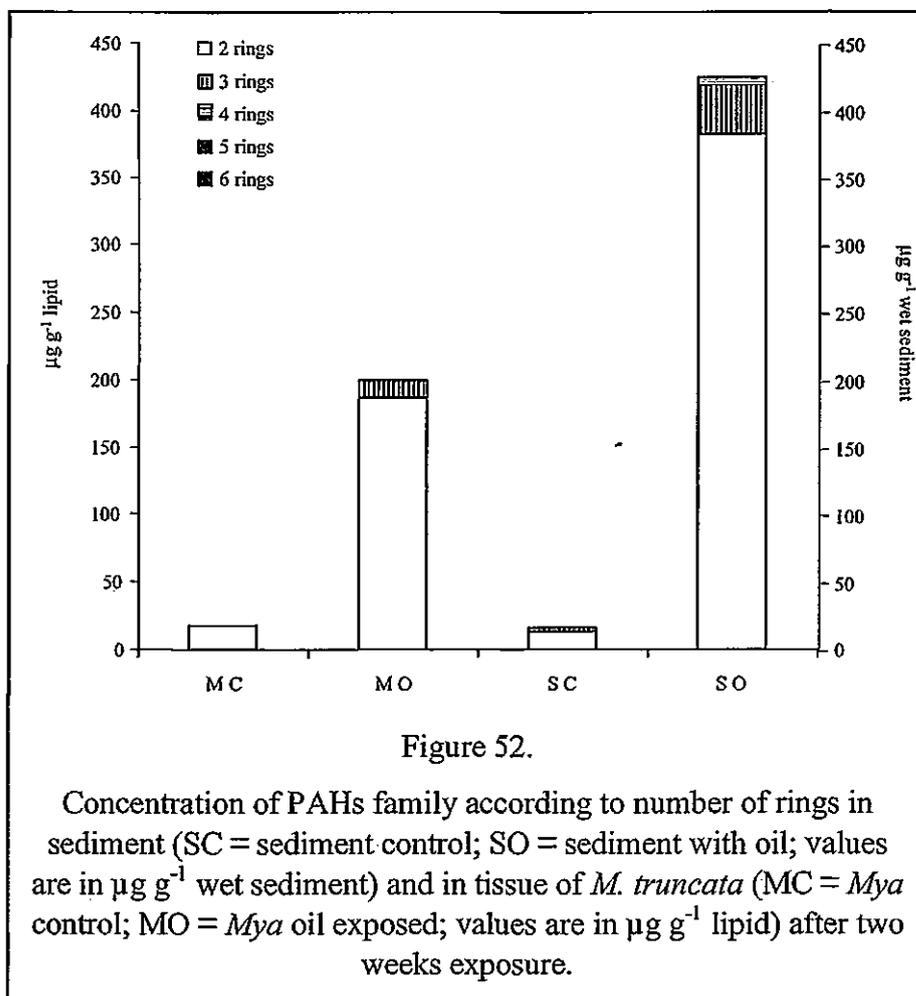


Table 7. Mean and standard deviation of TOSC, membrane stability and respiration in *Mya truncata* following a two-week exposure to control and oil-contaminated sediment.

	TOSC (TOSC unit mg <sup>-1</sup> protein)	Membrane stability (Fluorescence unit mg <sup>-1</sup> protein)	Respiration (mg O <sub>2</sub> dw <sup>-1</sup> h <sup>-1</sup> )
Control	4014 ± 1339	2.37 ± 1.35	0.055 ± 0.020
Exposed	4376 ± 642	5.40 ± 2.54	0.046 ± 0.028

### PAH in sediment

The total concentration of PAH in the oil-contaminated sediment at day 0 was 940 435 µg kg<sup>-1</sup> wet sediment; two ring and three ring molecules represent 83 and 16% of TPAH respectively. After two weeks at sea, the remaining total PAH amount in the surface sediment of the exposed group was 431 677 µg kg<sup>-1</sup> wet sediment. The four and five ring molecules remained adsorbed in the sediment after two weeks while the concentrations of the two and three ring compounds decreased by 50% (Figure 52).



### PAH uptake in *M. truncata*

The total amount of PAH in the clam tissue was  $200\,089\ \mu\text{g kg}^{-1}$  lipid, ten times higher than control clams after two weeks of exposure to contaminated sediment. Nevertheless, the PAH level in the clam tissue is half the concentration of oil in the sediment after two weeks. Only two and three ring molecules were detected (Figure 52) in the exposed *M. truncata*. 93 % of TPAH were two ring compounds.

### Relationships between bioaccumulation factor and log $K_{ow}$

The bioaccumulation factors (BAFs; ratio oil compounds in bivalve tissue and compound levels in sediment) were calculated on lipid weight of the different samples. The BAFs were lower than 0.6 indicating that *M. truncata* poorly accumulated PAH from the sediment (Table 2). Large molecules (four to six rings) were not detected in the present experiment, therefore, were not included in the calculations. The logBAF was plotted against log  $K_{ow}$  (hydrophobicity of PAH expressed by the octanol/water partition coefficients). A significant polynomial of degree two relationship was found ( $r^2=0.87$ ,  $p<0.05$ ), indicating that lipid-based bioaccumulation factors increase with hydrophobicity and reach a plateau for log  $K_{ow}$  above 4.5 (Figure 53).

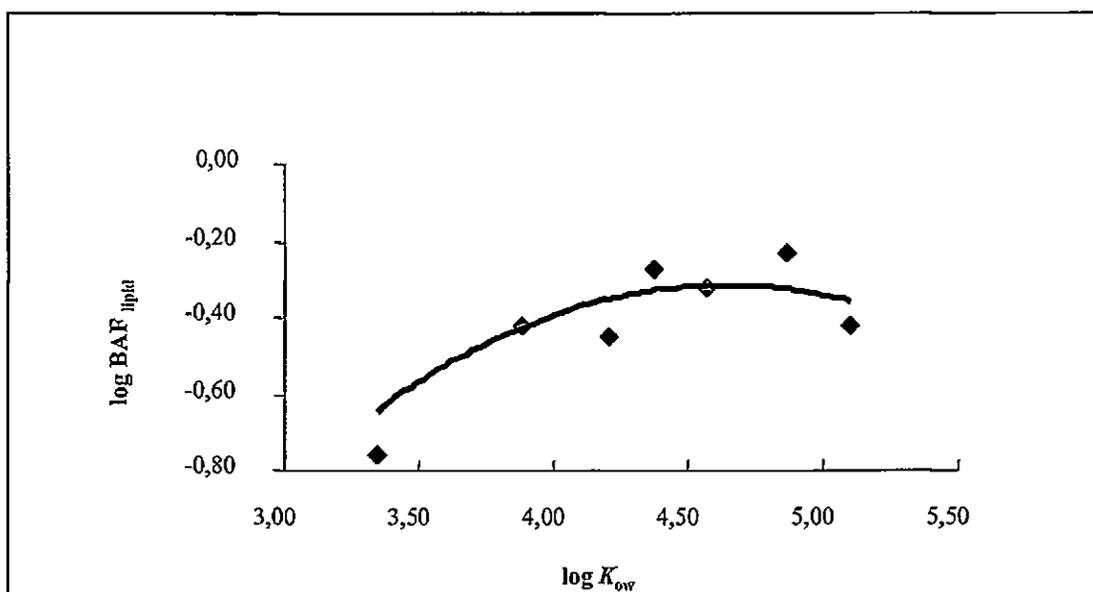


Figure 53.

Relationship between the bioaccumulation factor ( $\text{BAF}_{\text{lipid}}$ ) and the octanol/water partition coefficient ( $K_{ow}$ ) plotted on a log-log scale, in *Mya truncata* exposed to two weeks crude oil contaminated sediment. The adjustment (polynomial of degree 2) was significant at the threshold level of  $p < 0.05$ .

## 10.5. Discussion

The metabolic rate of *M. truncata* falls within the range measured for other Arctic bivalves. For example, 50 to 57  $\mu\text{g O}_2 \text{ g dw}^{-1} \text{ h}^{-1}$  was the range reported for *Clinocardium ciliatum* at 0°C (Schmid 1996), and between 63.7 and 94  $\mu\text{g O}_2 \text{ g dw}^{-1} \text{ h}^{-1}$  for *Chlamys islandicus* (0.5°C) and *Hiatella arctica* (3.5°C) (Camus *et al.* submitted). The metabolic rate of Arctic bivalves is low compared with temperate equivalents (Dame 1972, Vahl 1973, Bayne and Scullard 1977, Navarro and Winter 1982). The oxygen consumption rate of *M. truncata* (Arctic) at 5°C and *M. arenaria* (temperate) at 10°C was four times lower (Camus *et al.* submitted). Reduced metabolism of polar bivalves is considered an important mechanism for energy conservation in cold water where food is in short supply (Clarke 1991, Peck *et al.* 1997, Ahn and Shim 1998, Peck *et al.* 2000). Such low metabolic rates enable Arctic organisms to survive long periods of no food supply and facilitate the transfer of more energy into growth when food is available during the brief phytoplankton bloom.

In suspension feeding bivalves, the main route of PAH uptake is via filtering activity and, therefore, in a PAH sediment contaminated context, the uptake of PAH occurs only after the chemical has desorbed into interstitial or overlying water (Foster *et al.* 1987, Narbonne *et al.* 1999, Baumard *et al.* 1999b). As observed by Narbonne *et al.* (1999), at the end of the present experiment, 50% of the initial sediment burden of two and three ring hydrocarbon compounds were desorbed and transferred to the water compartment. Chemical tissue analysis revealed that some of these compounds were bioaccumulated by *M. truncata*. The logarithmic shape of the  $\log\text{BAF}_{\text{lipid}}$  versus  $\log K_{\text{ow}}$  curve confirms that uptake of the PAH molecules is based upon their degree of lipophilicity and size. The shape of the curve, similar to the one reported for *Mytilus edulis* and for semipermeable membranes in general, is typical of organisms that have a poor capability to metabolise PAH (Baussant *et al.* 2001).

*Mya truncata* is a sediment burrower that uses a long siphon to reach the surface of the sediment to access oxygenated water. Oil compounds, dissolved in the water, may have induced an avoidance behaviour consisting of retracting the siphon and cessation of breathing as observed in *Mya truncata* exposed to oil (Mageau *et al.* 1987).

No difference in oxygen uptake was shown between exposed and non-exposed *M. truncata*, indicating that respiration rate was not affected by oil exposure. Exposure to

PAH increases the rates of oxygen consumption of other bivalves species such as *Venus verrucosa* (Axiak and George 1987), *Mytilus edulis* (Widdows *et al.* 1982), *Mya arenaria* (Fong 1976, Stainken 1978) and *Macoma balthica* (Steckoll *et al.* 1980). It has been suggested that this increased basal metabolic rate is due to inhibition of membrane-bound respiratory enzymes (Steckoll *et al.* 1980). Hence, the PAH uptake did not affect the respiratory processes in *M. truncata*.

The basal TOSC value measured in *M. truncata* (4000 unit mg<sup>-1</sup> protein) is in the same range measured for other Arctic bivalve species (i.e. 2800 unit mg<sup>-1</sup> protein for *Chlamys islandica* and 2000 for *Hiatella arctica*; Camus *et al.* submitted), however, it is higher than values reported for temperate bivalves. Regoli and Winston (1998) reported 600 unit mg<sup>-1</sup> protein in the mussel *Mytilus edulis* and 700 for the starfish *Leptasterias epichlora*. This higher capacity to scavenge ROS in Arctic species supports the view that marine ecosystem is highly prooxidant (Abele *et al.* 1998, Colella *et al.* 2000, Regoli *et al.* 2000). In Antarctica, numerous studies have reported that recent increase in ozone hole has enhanced the formation of reactive oxygen species in the water column due to the increased UV-B radiations (Yocis *et al.* 2000, Qian *et al.* 2001). Therefore, organisms in environments with elevated levels of ROS may require elevated antioxidant defences to prevent biological damage. However, recently, Camus *et al.* (submitted) suggested that polar bivalves require a high TOSC to protect efficiently biomolecules, characterised by a low turnover rate (Whiteley *et al.* 1996), from natural oxidation in an environment where food availability is low.

Considering the uptake of PAH and that metabolism of PAH enhances the production of ROS (Livingstone *et al.* 1990, Winston and Di Giulio 1991) a change in TOSC value was expected in the exposed bivalve as measured by others (Regoli 2000, Winzer *et al.* 2001, Camus *et al.* *in press*) but this was not observed in this study. The low metabolic rate of *M. truncata*, measured in this study, may result in a low ROS production caused by the metabolism of PAH. Further, the logarithmic shape of the logBAF<sub>lipid</sub> versus logK<sub>ow</sub> indicates a low PAH metabolic capability of *M. truncata*. The high level of antioxidant defence measured in this experiment provides *M. truncata* with the capability to deal with moderate variations in ROS generation resulting in no difference of TOSC between treatments. Depledge and Lundebye (1996) emphasised the need to consider the variability of a biomarker response to provide additional useful insight into the chemical impact of pollutant. TOSC values variability was reduced in the exposed

group. As oxidative stress can induce (Winzer *et al.* 2001) or depress TOSC (Regoli 2000), it is possible that concomitant TOSC induction and inhibition was occurring in the control group. The decrease in variability of the exposed group may reflect a homogeneous induction of the antioxidative system in each individual.

Oxidative damage occurs when ROS production exceeds the total ROS scavenging capacity of an organism (Winston and Di Giulio 1991). Deleterious consequences can arise and, one of these, is lipid membrane peroxidation (Viarengo *et al.* 1989, Regoli 2000, Winzer *et al.* 2001). The present study observation of the destabilised haemocyte membranes is supported by studies that demonstrated that the white blood cells are the main site of defence against foreign substances in marine bivalves (Lowe *et al.* 1995, Carballal *et al.* 1997, Dyrzynda *et al.* 1998). Using the neutral red retention method, Lowe *et al.* (1995) provided clear evidence of contaminant-induced lysosomal membrane damage in the haemocytes of *Mytilus galloprovincialis*. Lysosomes, subcellular organelles that contain various enzymes (i.e. acid hydrolases), are sites of intense ROS production (Winston *et al.* 1996); the latter are claimed to be a cytotoxic mechanism against invaders and foreign compounds (Pipe 1992, Van Der Knaap *et al.* 1993); Another role of lysosomes is to accumulate contaminants that damage cells. The production of ROS can compromise the functional integrity of the lysosomal membrane following lipid peroxidation (Viarengo *et al.* 1989, Winston *et al.* 1996) resulting in release of acid hydrolases in the cytosol and further damage and disruption to the cell. Furthermore, under conditions of persistent xenobiotic challenge, oxidant stress might far exceed the normal antioxidant defences in bivalve haemocytes thereby, not preventing lipid peroxidation (Winston *et al.* 1996). The fluorescent assay used in the present study, showed good correlation with the neutral red retention method in haemocytes of *Mytilus edulis* (Camus *et al.* 2000), revealing that the destabilised plasma membrane of the haemocytes, measured in exposed *M. truncata*, is a direct consequence of decreased lysosomal membrane stability.

Previous studies have investigated the relationship between oxyradical toxicity and membrane alterations: reduced capability to scavenge oxyradicals in mussels (*Mytilus galloprovincialis*) was associated with destabilisation of lysosomal membranes (Regoli 2000), similar findings were reported in the Arctic scallop *Chlamys islandicus* (Camus *et al.* in press). Increased lipid peroxidation was measured in flounder hepatocytes following oxidative stress while TOSC increased (Winzer *et al.* 2001). In *M. truncata*,

antioxidant defences of hepatopancreatic tissue were not overwhelmed by ROS production (as indicated by the stable TOSC value), nevertheless, destabilised haemocytes cellular membranes were measured. This lack of correlation may reflect functional differences and/or different PAH route of uptake. Dissolved PAHs are the main compounds that were taken up by *M. truncata*, therefore, these PAH molecules entered the animal via breathing into the gills and haemolymph system resulting in impacting directly the haemocytes. Hepatopancreatic biochemical properties are affected mainly by the dietary uptake of PAH associated with ingested food-particles; the haemocytes are predominantly affected by water-soluble molecules taken up through the gills. The link between oxyradical toxicity and membrane destabilisation and our data suggests that the tissue sensitivity and/or functional differences have to be taken into consideration if we want to show evidence of a relationship between TOSC and membrane destabilisation.

Viarengo *et al.* (1995) suggested that the low level of unsaturated lipids observed in polar bivalves confer a better protection to the membranes against oxidative stress. Nevertheless, in this experiment, haemocytes cell membranes were the most sensitive tissue of *Mya truncata* to PAH exposure.

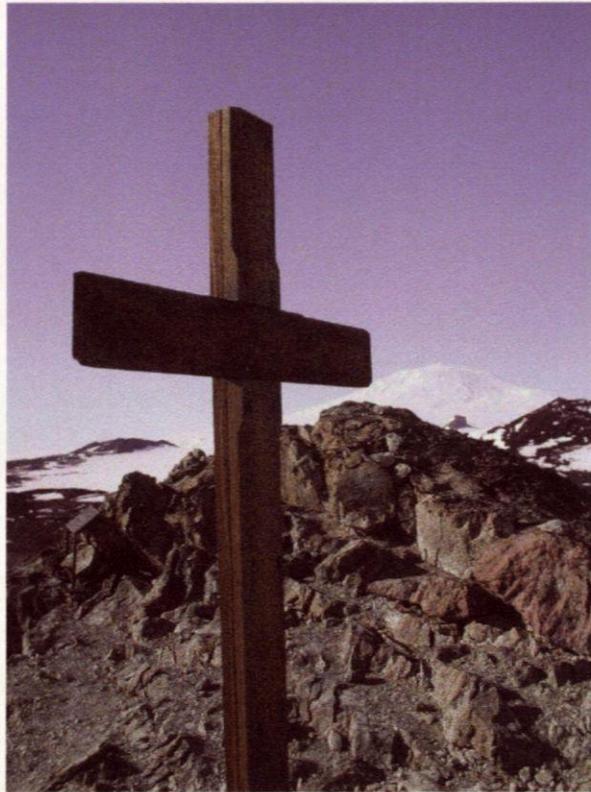
## 10.6. Conclusions

The study showed that PAHs were taken up by *M. truncata* and resulted in destabilisation of the haemocytes membranes but did not affect the total oxyradical scavenging capacity of the hepatopancreas. Tissue sensitivity and functional differences (hepatopancreas versus haemocytes), PAH uptake route (dietary versus gills), the typical low metabolic rate of *M. truncata* and low temperature reducing the bioavailability of PAH, are factors that explain these findings. Impaired function of the cellular haemocyte membranes can have consequences on the phagocytosis of micro-organisms (Grundy *et al.* 1996). Given the important role of haemocytes in cellular immunity of bivalves (Dyrynda *et al.* 1998), PAH-induced cell injury will contribute to a loss of immune protection and finally to carcinogenesis in *M. truncata* as observed in *M. arenaria* (Van Beneden 1997).

## Chapter 11

### **Antioxidant defence responses and cell membranes stability in Arctic shrimp *Sclerocrangon boreas* following exposure to dispersed crude oil**

This chapter is presented under the publication format as Dr. B.E. Grøsvik was leader of this experiment.



Cross on Observation Hill, Ross Island (Antarctica), erected in January 1913 by the Terra Nova expedition in memory of Scott, Bowers, Wilson, Oates, Evans who perished on the return journey from the South Pole 29<sup>th</sup> March 1912.

”To strive, to seek, to find, and not to yield” (inscription on the cross).

Chapter 11

Antioxidant defense responses and cell membrane stability in Arctic shrimp *Gammarus borealis* following exposure to diesel oil

This chapter is included in the book *Antioxidant Responses in Aquatic Organisms*, edited by Dr. H. G. O. Schuster, Wiley-Interscience, 1998.

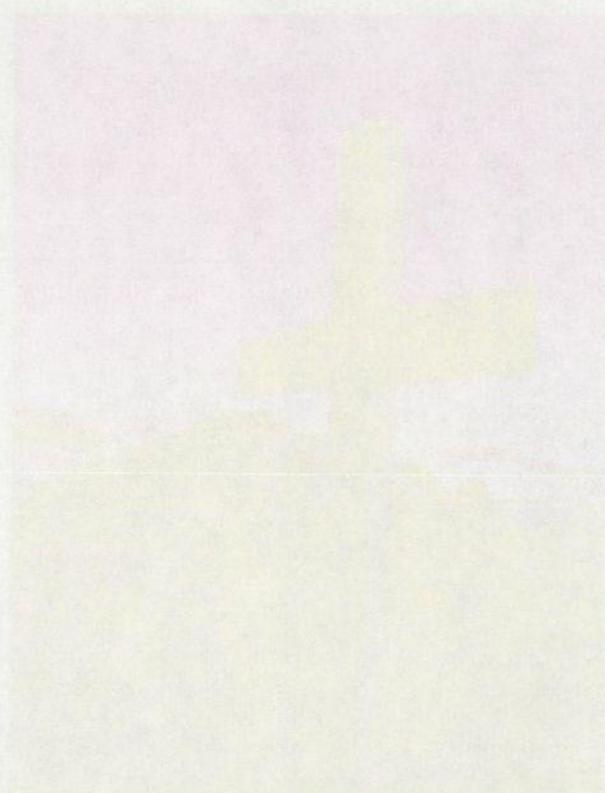


Figure 11.1. Antioxidant defense responses and cell membrane stability in Arctic shrimp *Gammarus borealis* following exposure to diesel oil. The figure shows a 3D bar chart with three bars of increasing height, representing different levels of exposure or time points. The bars are colored in shades of purple and blue. The y-axis represents the measured response, and the x-axis represents the different experimental groups or time points.

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## Antioxidant defence responses and cell membrane stability in Arctic shrimp *Sclerocrangon boreas* following exposure to dispersed crude oil

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### Abstract

Knowledge of how resident invertebrate species like Arctic shrimp *Sclerocrangon boreas* respond to oil compounds is important for future risk assessment studies in the Arctic. The objective of this work was to measure antioxidant defence responses and cell membranes stability in the Arctic shrimp *S. boreas* exposed to dispersed crude oil in water by means of a continuous flow system. Antioxidant defences were investigated by measuring glutathione *S*-transferase (GST) and total oxyradical scavenging capacity (TOSC). As a biomarker for cellular damage, membrane destabilisation measured as increased uptake of the fluorescent probe ethidium homodimer-1 (EthD-1) to a cell suspension of hepatopancreatic cells was used. Shrimp were exposed for five days, and the oil concentration in the exposed chamber was  $2 \pm 1$  ppm. It should be noted that this high oil concentration was selected in order to achieve a positive induction of biomarker signals, and that the concentration is considerably higher than what can be expected in the sea as a result of produced water discharges. A significant induction of GST activity ( $p < 0.05$ ) was observed in exposed shrimps. Thus, *S. boreas* is able to metabolise, and conjugate the activated molecule with glutathione to facilitate excretion. The mean GST activities were 20.3 and 26.4  $\text{nmol min}^{-1} \text{mg}^{-1}$  in control and exposed group, respectively. The TOSC values were significantly reduced ( $p < 0.05$ ) following oil exposure. The mean TOSC values were 8795 and 6284  $\text{unit mg}^{-1}$  protein in control and exposed, respectively. Uptake of EthD-1 was two times higher in cells of exposed shrimps compared with control, indicative of cellular damage to exposed shrimp.

**Keywords:** Arctic, *Sclerocrangon boreas*, dispersed oil, GST, TOSC, cell membrane stability

## Introduction

Due to possible future oil and gas exploration in the Arctic, it is important to perform controlled laboratory studies on endemic and representative species, in order to establish knowledge on susceptibility and response pattern to oil contamination. Such data is needed to select which effect parameters to use for field measurements. Knowledge of how resident invertebrate species like Arctic shrimp *Sclerocrangon* sp. respond to oil compounds is important for future risk assessment studies.

One approach being applied is the use of biomarkers. Most studies of biological responses have been produced with temperate organisms. Little is known about toxicity of contaminants on marine organisms living in an environment characterised by a low and stable temperature (from  $-1.88$  to  $5^{\circ}\text{C}$ ) and a strong seasonality in light and primary production (Weslavski et al. 1988). A few studies have revealed that the ectothermic marine organisms inhabiting the polar ocean possess specific biological adaptations (Pörtner and Playle 1998). Polycyclic aromatic hydrocarbons (PAH) are affected by temperature and light exposure (Neff 1979). For instance, the solubility decreases at low temperature and photo oxidation make molecules more biologically reactive. Consequently, the biological adaptation of polar marine species and the altered oil behaviour may affect the typical biomarker responses and require investigation.

Current oil and gas activities in the North Sea discharge produced water, which should not exceed 40 ppm of oil according to the regulation defined by the Oslo-Paris Commission. Low levels of oil in water dispersion have been shown to be bioaccumulated in controlled laboratory experiments with fish and mussels (Baussant et al. 2001a,b). Formation of DNA adducts have been observed in Atlantic cod *Gadus morhua* after exposure to oil water dispersion at 0.06 ppm for 30 days. Adduct formation have been correlated to more adverse effects, as occurrence of liver neoplasia (Myers et al. 1998).

In this study, *Sclerocrangon boreas* (Crangonidae) was selected. It was first described by C.J. Phipps (1774) as *Cancer boreas*. The genus *Sclerocrangon* was described by G.O. Sars (1885). They are benthic and frequent at depths from 30-100 meters in the Barents Sea and Svalbard fjords (Dons 1915; Grieg 1932).

PAH are known to enhance the formation of the deleterious reactive oxygen species (ROS) following metabolism by marine invertebrates (Livingstone et al. 1990,

Winston and DiGiulio 1991). One consequence of the ROS production is the induction or inhibition of the antioxidant defences to scavenge these deleterious compounds (Livingstone et al. 1990, Winston and DiGiulio 1991). Glutathione is one of the most important antioxidant agents involved in the neutralisation of ROS (Meister 1989). Moreover, glutathione is the cofactor of many enzymes catalysing the detoxification and excretion of several toxic compounds.

Glutathione *S*-transferases (GST), acts as the catalyst of a very wide variety of conjugation reactions of glutathione with xenobiotic compounds containing electrophilic centres (Habig et al. 1974). Changes in the activities of GST can reflect exposure of organisms to xenobiotics and these responses are used as a tool to measure effects of pollution on various organisms. The level of activity may be affected after exposure to several types of environmental stress including oil contamination. Such effects have been reported with mussels (*Mytilus edulis*) deployed in cages in the North Sea (Børseth et al. 2000). GST activity was measured in this study to know whether PAH have been metabolised and conjugated to glutathione by *S. boreas*.

Antioxidant defence systems have been extensively used to monitor the *in situ* impact of chemicals in the marine environment (Regoli and Principato 1995, Sole et al. 1998, Orbea et al. 1999, Sole 2000, Khessiba et al. 2001). However, the difficulty to interpret the biological resistance to oxidative stress in quantifiable terms led to the development of the total oxyradical scavenging capacity (TOSC) assay by Winston et al. (1998). In this study, the TOSC assay was selected to measure the total capacity of the shrimp hepatopancreatic tissue to neutralize ROS formed following metabolism of PAH.

One deleterious consequence of ROS is the lipid peroxidation (Slatter 1984). Earlier biomarker studies have investigated the effects of contaminants on membranes and in particular the lysosomal membrane with the neutral red retention assay in haemocytes of blue mussels (Viarengo 1989, Lowe et al 1995, Grundy et al. 1996, Regoli 1992) and fish hepatocytes (Lowe 1992). In this study, we have tested whether membrane destabilisation also may be measured in a suspension of hepatopancreatic cells by a fluorescent plate reader after addition of the fluorescent probe ethidium homodimer (EthD-1). EthD-1 intercalates between the bases of the DNA to yield red fluorescence, and is generally excluded from viable cells. It binds with little or no sequence preference at a stoichiometry of one dye per 4–5 base pairs of DNA. Once the dye is bound to nucleic acids, its fluorescence is enhanced many fold. EthD-1 is taken up

generally, and the fluorescence will increase upon binding to nucleic acids, however, damaged cells take up EthD-1 to a larger extent.

The objective of this work was to study effects in Arctic shrimp *S. boreas* after exposure to dispersed crude oil on the selected biomarker responses; GST activity, TOSC and cell membrane stability in hepatopancreatic tissue.

## Material and methods

### Sampling and maintenance of *S. boreas*

*S. boreas* were collected in Isfjorden (Svalbard, 78°13'N, 15°39'E), in September 1999 with an Agassiz dredge deployed from the research vessel F/F Jan Mayen from the University of Tromsø at ca. 40 m depth and sea water temperature of 0°C. The shrimp were transported by plane to the laboratory (Stavanger, Norway) packed in humid paper in a cooled thermos. They were maintained for three months in the laboratory at 2°C and fed raw shrimp prior to the experiment.

### Production of oil in water dispersion

An ultrasonic processor (Misonix Sonicator XL2015 Generator) produced oil in water dispersion (80 mg/l), which was further diluted and delivered to the aquaria by peristaltic pumps according to (Taban I. C., R.K. Bechmann, K.B. Øysæd, G. Jonsson, A. Skadsheim, S. Sanni, and B. Gaudebert. *in prep*) Reproduction effects, growth, PAH metabolites in the bile, and body burden of PAH for sheepshead minnow *Cyprinodon variegatus* chronically exposed to a dispersion of oil). A Coulter Multisizer (model TA II) was used to count and size all particles with diameter between 1.4 and 30 µm. Filtered seawater was used as the electrolyte solution. The blank was a water sample from the inlet to the control aquarium. The multisizer measurements showed that 95% of the oil droplets in the size range between 1.4 and 30 µm were less than 10 µm in size, and the mean size based on number of particles of size range 1.4 to 30 µm was 1.86 µm. The mean size of particles based on volume was 3.1 µm for particles in size range 1.4 to 10 µm, and 4.1 µm for particles in size range 1.4 to 30 µm. The size of the droplets was in the size range observed for produced water (Kirsten Mönig, RF-Rogaland Research, pers. comm.).

### **PAH Measurements and oil content in exposed chambers**

Water samples were collected in burned 2 l Duran glass bottles, containing hydrochloric acid to ensure that the sample did not exceed pH 2. The samples were stored protected from light at 4°C and extracted the day after sampling. An appropriate amount of eight internal standards were added to the water bottles and stirred for 15 min. Cyclohexane (50 ml) were added to the water and stirred for another 30 min., and then poured into a separating funnel. The water phase was drained back into the sampling flask and extracted two additional times. Anhydrous sodium sulphate was added in excess and allowed to rest for 30 min. before filtering through glass sinter filter (pore size 2) and concentration to 0.5 ml by use of TurboVap 500 (Zymark Corporation, Hopkinton, MA, USA). A mixture of three additional deuterated internal standards (RIS, recovery internal standards), were added to the extract prior to GC/MS – analysis. PAH analysis of all samples was performed by Gas Chromatography (HP5890, Hewlett Packard, USA) connected to a Mass Spectrometer (Finnigan SSQ7000, USA) and analysed in selected ion mode (GC/MS-SIM). Response factor curves were calculated for the individual PAH and used for calculation of sample concentrations. The reproducibility of the response factors for at least three standards covering the concentration range is regularly checked for each series of samples analysed. The control was an unexposed sample spiked with an appropriate amount of a certified mixture of PAH (Dr Eherenstorfer Reference Materials, Augsburg, Germany).

The concentration of PAH compounds and sum PAH in the water samples of both control and oil exposed chambers are shown in Table 1. The total PAH concentration determined (sum PAH) in the chamber was 10-26 µg/l. The variations in oil exposure concentrations were due to instability with the oil pumps. PAH constitute 0.83% of the crude oil used (North Sea crude oil) (Baussant et al. 2001). These data together with the data from the Coulter Multisizer analyses gave an oil exposure concentration of low and high dose to be 2±1 ppm.

### **Exposure and sampling**

The shrimps were exposed for five days in the CFS to 2±1 ppm dispersed crude oil. Due to the energy input to produced dispersed oil, the water temperature in the exposure chamber was slightly elevated; control: 2.0±0.1°C, 2 ppm: 2.2±0.3°C. No mortality was observed. The length (74±17 mm), weight (6.7±5.3 g) and gonadal status were

measured, and hepatopancreas sampled. The hepatopancreas was divided in two parts. One was used immediately for isolation of single cells for studies of membrane stability, while the remaining part was frozen in liquid nitrogen for GST and TOSC analyses. During all procedures, care was taken to work on ice to avoid any temperature increase.

### **Analytical part**

#### **Measurements of glutathione-S-transferase (GST) activity**

Glutathione-S-transferase (GST) activities were measured using 1-chloro-2,4-dinitrobenzene (CDNB,  $C_6H_3ClN_2O_4$ ) as a substrate and glutathione-S-hydroxylase (GSH,  $C_{10}H_{17}N_3O_6S$ ) as a co-factor. Before analysis, the cytosol was diluted 1:5 in 100 mM potassium phosphate buffer (pH 7.4), in order to obtain linear UV signal with time during the spectrometric measurements. Enzyme activity measurements were performed according to (Habig et al. 1974; Habig and Jakoby 1981). After the addition of all the components, the cuvette (plastic) was capped, the content rapidly mixed and the increase in absorbance due to the formation of the conjugate product (GST-CDNB) was recorded by continuous registration for 1 minute at 340 nm, on a Perkin-Elmer Model Lambda 2S UV/VIS spectrometer. For every series of samples ( $n=10$ ) analysed, 3 blank samples (without cytosol) were analysed. GST activities were expressed as moles of substrate converted per minute per mg of protein in the cytosol. All samples were analysed three times.

#### **Measurements of total oxyradical scavenging capacity (TOSC)**

Hepatopancreas were sampled and stored at  $-80^{\circ}C$  for 3.5 months. Samples were homogenised in four volumes of 100 mM potassium phosphate buffer, pH 7.5, 2.5% NaCl and protease inhibitors. The homogenate was centrifuged at  $100\ 000 \times g$  for 1 hr, cytosolic fractions were aliquoted and stored at  $-80^{\circ}C$ .

Peroxy radicals generated by thermal homolysis of 2-2'-azo-bis-(2 methyl-propionamide)-dihydrochloride (ABAP) cause the oxidation of  $\alpha$ -keto- $\gamma$ -methylbutyric acid (KMBA) to ethylene. Ethylene formation is monitored by gas chromatographic analysis from the reaction vial. The partial inhibition of ethylene formation in the presence of antioxidants that compete with KMBA for oxyradicals is the basis of the assay (Winston et al. 1998). The appropriate assay conditions were 0.2 mM KMBA and 20 mM ABAP in 100 mM potassium phosphate buffer, pH 7.4.

Reactions were carried out in 10 ml rubber septa sealed vials in a final volume of 1 ml. The reactions were initiated by injection of 100  $\mu$ l of 200 mM ABAP in water and incubation in water bathed thermostated at 35°C for a constant peroxy radical generation from ABAP. Ethylene production was measured by gas-chromatographic analysis of 200  $\mu$ l aliquots taken directly from the top space of the reaction vials. Ethylene formation was monitored for 96 min. Each analysis requires measurement of control reactions (no antioxidant in the reaction vessel) and sample reactions (antioxidant in the reaction vessel). In the presence of antioxidants, ethylene production from KMBA was quantitatively reduced and higher antioxidant concentrations resulted in longer periods in which ethylene formation was inhibited relative to controls.

### Quantification of TOSC

The area under the kinetic curve was mathematically calculated from the integral of the equation that best defines the experimental points for both the control and sample reactions. TOSC is then quantified according to equation 1, where IntSA and IntCA are the integrated areas from the curve defining the sample and control reactions, respectively.

$$\text{TOSC} = 100 - (\text{IntSA}/\text{IntCA} \times 100) \quad (1)$$

Thus, a sample that displays no oxyradical scavenging capacity would give an area equal to the control ( $\text{IntSA}/\text{IntCA} = 1$ ) and a resulting TOSC = 0. On the other hand, as  $\text{IntSA}/\text{IntCA}$  goes to 0 the hypothetical TOSC value approaches 100. Because the area obtained with the sample is related to that of the control, the obtained TOSC values are not affected by small variations in instrument sensitivity, reagents, or other assay conditions. The specific TOSC was calculated by dividing the experimental TOSC by the molar concentration of the antioxidant or by the amount of protein in mg used in the assay (Regoli 2000).

### Membrane stability

#### Preparation of single cell suspension of hepatopancreas

Hepatopancreas (one third) was taken out, rinsed in physiological saline solution (PSS) pH 7.4 (4.77 g/l Hepes, 25.48 g/l NaCl, 13.06 g/l MgSO<sub>4</sub>, 0.75 g/l KCl and 1.47 g/l CaCl<sub>2</sub>) containing 1% BSA and 1 mM EDTA. Tissue was added approx. 1 ml PSS

(volume depending of weight), cut with scissors till homogenous solution, put in Eppendorf tube, let stand for 30 sec., supernatant withdrawn and stored on ice until analysis.

### **Plate reader assay**

50 µl cell suspension, 150 µl PSS and 50 µl of Ethidium homodimer-1 (EthD-1) (Molecular Probes, Eugene, Or, USA) to final concentration of 0.5 µM, was added per microplate well. The plate was gently shaken by hand. Fluorescence of EthD-1 was read at ex544/em612 nm, measured successively after incubation in dark at room temperature at different time intervals. The background fluorescence was corrected for, and membrane destabilisation expressed as delta uptake per mg protein in samples, i.e. uptake after 75 min. of incubation subtracted uptake after 5 min. of incubation.

### **Protein determination**

Total protein concentrations of the samples were determined according to Bradford (1976), using BSA as standard.

### **Statistical analysis**

Statistical analyses were run with the Student's t test after testing for normal distribution and homogenous variances (TOSC values log transformed). The analyses were performed with JMP, ver. 3.1.6.2, SAS Institute Inc., Cary, NC, USA.

## **Results**

### **Crude oil concentration**

Chemical measurements of PAH content in oil contaminated water together with data from Coulter Multisizer analyses, demonstrated oil concentration in the exposed chamber to be 2±1 ppm.

### **GST**

A significant induction of GST activity ( $p < 0.05$ ) was observed in the shrimps exposed to 2 ppm crude oil (Figure 1A). Mean GST activities were 20.3 and 26.4 nmol/min/mg protein for control and exposed shrimps, respectively.

## TOSC

The TOSC was significantly reduced at 2 ppm compared with control ( $p < 0.05$ ) (Figure 1B). Mean TOSC values were 8795 and 6284 u/mg protein in control and exposed group respectively.

## Cell membrane stability

The results demonstrated 2 times higher (but not statistically significant) uptake in hepatopancreas cells from shrimps exposed to 2 ppm oil, indicating cellular damage to exposed shrimp (Figure 1C).

## Discussion

GST catalyses reactions to conjugate activated PAH following metabolism by cytochrome P-450 enzymes, to render them less toxic and more soluble for excretion. The increased activity of GST for the exposed group, reported in this study, indicated that *S. boreas* was able to metabolise and excrete PAH by conjugating the activated metabolite to glutathione. Earlier studies have reported induced GST activity in crustaceans exposed to heavy metals and organochlorine (Ishizuka et al. 1998; Reddy 1999). However, inconsistent results on GST activity of molluscs from different field and mesocosm exposures have been reported (Lee 1988, Suteau et al. 1988, Regoli and Principato 1995). GST comprise a multigene family encoding for many isozymes with different, but often overlapping specificities for the commonly used substrates (CDNB in our study). Thus, GST activity is related to the expression pattern of isozymes.

A depression in TOSC values in digestive gland of molluscs following exposure to PAH (Camus et al. submitted) and heavy metals (Regoli 2000) has been demonstrated. This decrease in TOSC values suggests a depletion of low molecular weight molecules and notably glutathione that is an important antioxidant parameter to scavenge ROS (Meister 1989). The increased activity of the GST, reported in this study, supports that glutathione was used for conjugation reactions. The consequence of a depressed TOSC is that antioxidant defences can be overwhelmed by ROS production and can no longer protect the various cell components against deleterious effects of ROS such as lipid peroxidation (Winston and DiGiulio 1991, Regoli 2000). High correlation has been demonstrated between reduced TOSC values and reduction of lysosomal stability in mussels sampled from a metal polluted site (Regoli 2000). Concomitant to a decrease in

TOSC, a decrease in cell membrane stability was observed in *Chlamys islandicus* exposed to benzo(a)pyrene (Camus in press). The increased uptake of EthD-1 in hepatopancreatic cells of *S. boreas*, indicative to a destabilisation of cell membranes, support the TOSC assay to be a predictive biomarker for oxidative stress in these organisms. Membrane destabilisation may be due to lipid peroxidation or damage to membrane proteins.

Levels of EthD-1 measured are a function of uptake and efflux. Efflux of different chemicals is often performed by membrane pumps called multixenobiotic resistance (MXR) (Kurelec 1992). These proteins belong to a class of high molecular weight membrane glycoproteins associated with the multidrug resistance (MDR). They have been conserved through evolution from bacteria to man and is a part of the cellular defence system (Endicott and Ling 1989). Both induction and inhibition of MDR proteins could possibly effect measurements of EthD-1 uptake, and measurements of MDR activities may have supplemented the interpretation.

Temperature in the exposed chamber was 0.2 °C higher compared with control chamber due to energy input to produce dispersed oil in water. It is unlikely that these changes had an effect on the results obtained. However, other studies showed that enzymatic properties are highly susceptible to temperature increase in stenothermal organisms (Abele et al. 2001).

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## References

- Aas, E., T. Baussant, L. Balk, B. Liewenborg, and O.K. Andersen (2000) PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. *Aquatic Toxicol.* 51(2): 241-258.
- Abele, D., C. Tesch, P. Wencke and H. O. Pörtner (2001) How does oxidative stress relate to thermal tolerance in the Antarctic bivalve *Yoldia eightsi*? *Antarctic Science* 13(2): 111-118.
- Baussant, T., S. Sanni, G. Jonsson, A. Skadsheim, J.F. Børseth, (2001a) Bioaccumulation of polycyclic aromatic compounds: 1. Bioconcentration in two marine species and in semipermeable membrane devices during chronic exposure to dispersed crude oil. *Environmental Toxicology & Chemistry* 20(6): 1175-1184.
- Baussant, T., S. Sanni, A. Skadsheim, G. Jonsson, J. F. Børseth and B. Gaudebert (2001b). Bioaccumulation of polycyclic aromatic compounds: 2. Modeling bioaccumulation in marine organisms chronically exposed to dispersed oil. *Environmental Toxicology & Chemistry* 20(6): 1185-1195.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.
- Børseth, J. F., B. E. Grøsvik, L. Camus, S. Le Floch and B. Gaudebert (2000). Biomarkers: A new Approach to assess Environmental Impact of Petroleum E&P Activities. *Society of Petroleum Engineers* 61202: 1-7.
- Camus, L., M. Jones, J. F. Børseth, B. E. Grøsvik, F. Regoli and M. H. Depledge (*in press*) Total oxyradical scavenging capacity and plasma membrane stability of the haemocytes of the Arctic scallop, *Chlamys islandicus*, following benzo(a)pyrene exposure. *Marine Environmental Research*.
- Dons, C. (1915). Nord-Norges Decapoder. Tromsø, J. Kjeldseths Boktrykkeri: 56-57.
- Endicott, J. E. and V. Ling (1989). The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annual Review of Biochemistry* 58: 137-71.

Grieg, J. A. (1932). Decapoda Crustacea fra bankene ved Svalbard. Bergens museums årbok 1932. Naturvitenskapelig rekke nr. 2: 12-14.

Grundy, M. M., M. N. Moore, S. M. Howell and N. A. Ratcliffe (1996). Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. *Aquatic Toxicology* 34(4): 273-290.

Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974). Glutathione-S-transferase. The first enzymic step in mercaptic acid formation. *Journal of Biology and Chemistry*, 249, 7130-7139.

Habig, W. H. and Jakoby, W. B. (1981). Assays for differentiation of glutathione S-transferases. *Methods in Enzymology*, 77, 398-405.

Habig, W.H. and Jakoby, W.B. (1981). Assays for differentiation of glutathione S-transferases. *Methods in Enzymology* (77): 398-405.

Ishizuka, M., T. Sakiyama, H. Iwata, M. Fukushima, A. Kazusaka and S. Fujita (1998). Accumulation of halogenated aromatic hydrocarbons and activities of cytochrome P450 and glutathione S-transferase in crabs (*Eriocheir japonicus*) from Japanese rivers. *Environmental Toxicology & Chemistry* 17(8): 1490-1498.

Khessiba, A., P. Hoarau, M. Gnassia-Barelli, P. Aissa, and M. Romeo (2001) Biochemical response of the mussel *Mytilus galloprovincialis* from Bizerta (Tunisia) to chemical pollutant exposure. *Archive of Environmental Contamination & Toxicology* 40(2): 222-229.

Kurelec, B. (1992). The multixenobiotic resistance mechanism in aquatic organisms. *Critical Review of Toxicology* 22(1): 23-43.

Livingstone, D. R., P. G. Martinez, X. Michel, J. F. Narbonne, S. Ohara, D. Ribera and G. W. Winston (1990) Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* (L.), and other mollusks. *Functional Ecology* 4(3): 415-424.

Lowe, D. M., V. U. Fossato and M. H. Depledge (1995). Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. *Marine Ecology Progress Series* 129: 189-196.

Lowe, D. M., M. N. Moore and B. M. Evans (1992). Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. *Marine Ecology Progress Series* 91: 135-140.

Lowe, D. M., C. Soverchia and M. N. Moore (1995). Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. *Aquatic Toxicology* 33: 105-112.

Meister, A. (1989) On the biochemistry of glutathione. In: *Glutathione Centennial. Molecular Perspectives and Clinical Implications*, edited by N. Taniguchi, T. Higashi, S. Sakamoto and A. Meister. Academic Press. San Diego, CA. pp 3-22.

Moore, M. N. (1988). Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in molluscan digestive cells to environmental and experimental exposure to xenobiotics. *Marine Ecology Progress Series* 46: 81-89.

Moore, M. N., R. J. Wedderburn, D. M. Lowe and M. H. Depledge (1996). Lysosomal reaction to xenobiotics in mussel hemocytes using BODIPY-FL-verapamil. *Marine Environmental Research* 42(1-4): 99-105.

Mourente, G. and E. Diaz-Salvago (1999). Characterization of antioxidant systems, oxidation status and lipids in brain of wild-caught size-class distributed *Aristeus antennatus* (Risso, 1816) Crustacea, Decapoda. *Comparative Biochemistry and Physiology. B-Biochem. & Mol. Biol.* 124(4): 405-416.

Myers, M.S., L.L. Johnson, T. Hom, T.K. Collier, J.E. Stein and U. Varanasi (1998) Toxicopathic hepatic lesions in subadult anglic sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: Relationships with other biomarkers of contaminant exposure. *Marine Environmental Research.* 45(1): 47-67.

Orbea, A., I. Marigomez, C. Fernandez, J. V. Tarazona, I. Cancio and M. P. Cajaraville (1999) Structure of peroxisomes and activity of the marker enzyme catalase in digestive epithelial cells in relation to PAH content of mussels from two Basque estuaries (Bay of Biscay): Seasonal and site-specific variations. *Archives of Environmental Contamination and Toxicology* 36(2): 158-166.

Phipps, C. J. (1774). *A Voyage Towards the North Pole Undertaken by His Majesty's Command 1773*. J. Nourse: 189-191, Plate 12.

Pörtner, H.O. (2001) Climate change and temperature-dependant biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften*. 88(4): 137-146.

Pörtner, H. O., L. Peck, S. Zielinski and L. Z. Conway (1999) Intracellular pH and energy metabolism in the highly stenothermal Antarctic bivalve *Limopsis marionensis* as a function of ambient temperature. *Polar Biology* 22(1): 17-30.

Reddy, P. S. (1999). Modulations in antioxidant enzymes in the hepatopancreas and gill of the fresh water crab *Oziotelphusa senex senex* during exposure to cadmium and copper. *Freshwater Environmental Bulletin* 8(7-8): 445-452.

Regoli, F. (1992). Lysosomal responses as a sensitive stress index in biomonitoring heavy-metal pollution. *Marine Ecology Progress Series* 84(1): 63-69.

Regoli, F. (2000). Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. *Aquatic Toxicology* 50(4): 351-361.

Regoli, F. and G. Principato (1995) Glutathione, Glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions – Implications for the use of biochemical markers. *Aquatic Toxicology* 31(2): 143-164.

Regoli, F., M. Nigro, S. Bompadre and G. W. Winston (2000) Total oxidant scavenging capacity (TOSC) of microsomal and cytosolic fractions from Antarctic, Arctic and Mediterranean scallops: differentiation between three potent oxidants. *Aquatic Toxicology* 49(1-2): 13-25.

Sars, G. O. (1885). Description of new and imperfectly known species. - The Norwegian North -Atlantic Expedition 1876-1878. *Crustacea I. Christiania*. 6: 5-280 + 21 plates.

Slatter, T.F. (1984) Free-radical mechanisms in tissue injury. *Biochem. J.* 222:1-15.

Sole, M. (2000) Assessment of the results of chemical analyses combined with the biological effects of organic pollution on mussels. *Trends in Analytical Chemistry* 19:1-9

Sole, M., L. D. Peters, K. Magnusson, A. Sjölin, Å. Granmo and D. R. Livingstone (1998) Responses of the cytochrome P450-dependent monooxygenase and other protective enzyme systems in digestive gland of transplanted common mussel (*Mytilus*

*edulis* L.) to organic contaminants in the Skagerrak and Kattegat (North Sea). *Biomarkers* 3(1): 49-62.

Viarengo, A., M. Pertica, L. Canesi, A. Mazzucotelli, M. Orunesu and J. M. Bouquegneau (1989) Purification and biochemical characterisation of a lysosomal copper rich thionein like protein involved in metal detoxification in the digestive gland of mussels. *Comparative Biochemistry and Physiology* 93C(2): 389-395.

Weslawski, J. M., M. Zajackowski, S. Kwasniewski, J. Jezierski and W. Moskal (1988) Seasonality in an Arctic fjord ecosystem: Hornsund, Spitsbergen. *Polar Research* 6:185-189.

Winston, G. W. and R. T. Di Giulio (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology* 19:137-191.

Winston, G. W., F. Regoli, A. J. Dugas, J. H. Fong and K. A. Blanchard (1998). "A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids." *Free Radical Biology and Medicine* 24(3): 480-493.

## Legend to figure

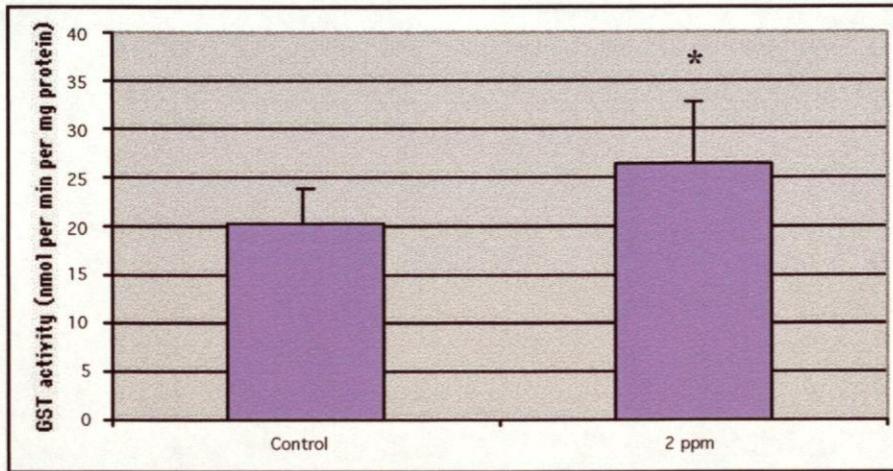
**Figure 1. Biomarker responses in hepatopancreas of Arctic shrimp after five days of exposure to 2 ppm dispersed crude oil.** Presented as means + standard deviation. (A) GST activity (nmol/min/mg protein). (B) TOSC values towards peroxy radicals per mg protein (units per mg protein). (C) Membrane destabilisation in single cells measured as uptake of the EthD-1 per mg protein relative to control. The number of individuals (n) was 9 for the control group and 10 for the 2 ppm exposure group, except for TOSC measurements for the control group, where n=5. (\*) indicates significant difference,  $p < 0.05$ .

**Table 1. PAH levels in oil exposed chambers**

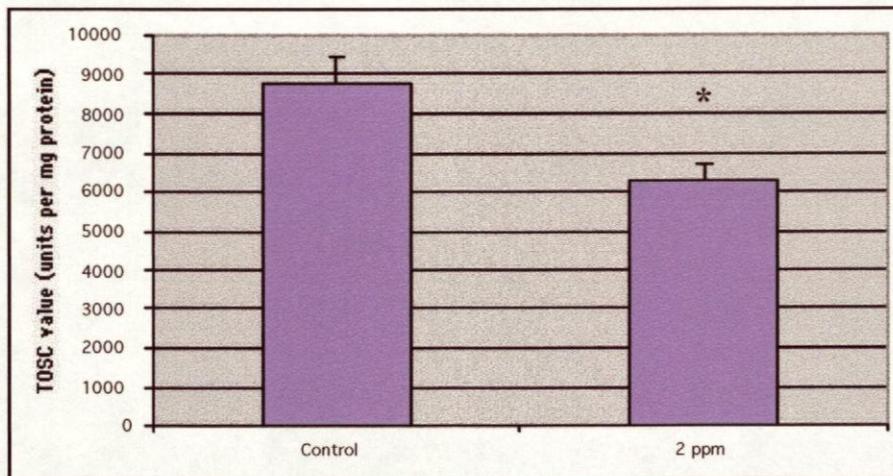
Treatment	Control	Crude oil	Crude oil	Crude oil
Sampling date:	17.12.99	17.12.99	19.12.99	20.12.99
Compound	µg/L	µg/L	µg/L	µg/L
Naphthalene	*<	0.71	1.81	2.24
C1-naphthalene	*<	2.24	6.37	7.19
C2-naphthalene	*<	3.81	8.38	8.25
C3-naphthalene	0	2.41	6.29	5.82
Acenaphthylene	0	0	*<	*<
Acenaphthene	0	*<	*<	*<
Fluorene	0	*<	0.49	0.49
Phenanthrene	0	0.12	0.27	0.29
Anthracene	0	0.15	0.34	0.36
C1-phen/anthr	0	0.15	0.48	0.48
C2-phen/anthr	0	0.09	0.44	0.42
Dibenzothiophene	0	*<	0.05	0.05
C1-dibenzothiophene	0	*<	*<	*<
C2-dibenzothiophene	0	*<	*<	*<
Fluoranthene	0	*<	*<	*<
Pyrene	0	*<	*<	*<
Benzo(a)anthracene	0	*<	*<	*<
Chrysene	0	*<	*<	*<
C1-chrysene	0	0	*<	*<
C2-chrysene	0	0	0	0
Benzo(b)fluoranthene	0	0	0	0
Benzo(k)fluoranthene	0	0	0	0
Benzo(a)pyrene	0	0	0	0
Indeno(1,2,3,cd)pyrene	0	0	0	0
Benzo(g,h,i)perylene	0	0	0	0
Dibenzo(a,h)anthracene	0	0	0	0
<b>Sum PAH</b>	<b>0</b>	<b>9.68</b>	<b>24.91</b>	<b>25.58</b>

\*< = Below levels of quantification

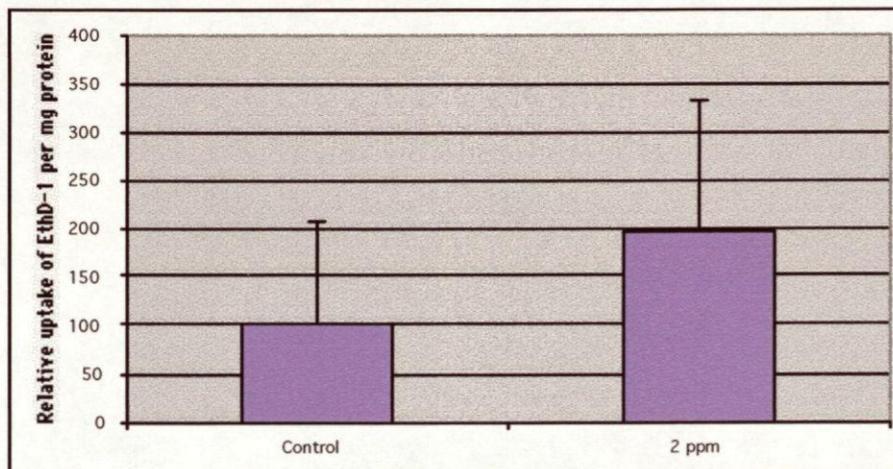
Figure 1.



A



B



C

Figure 1

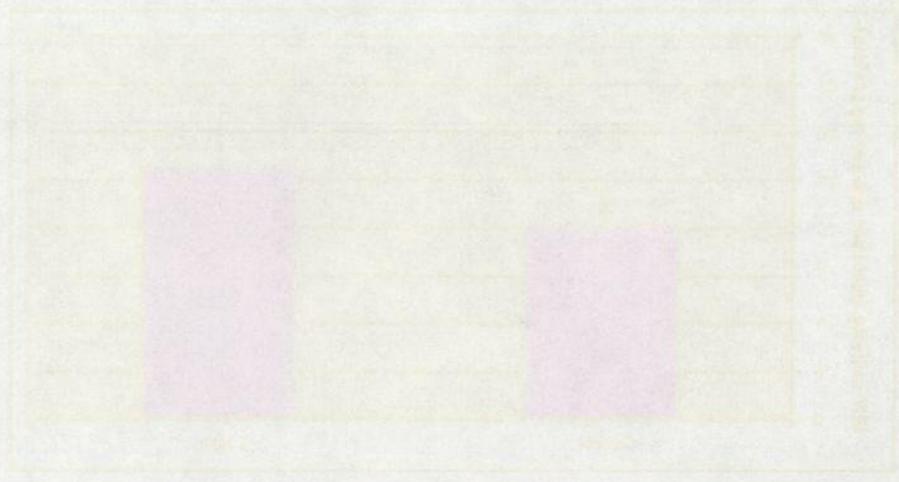


Figure 2

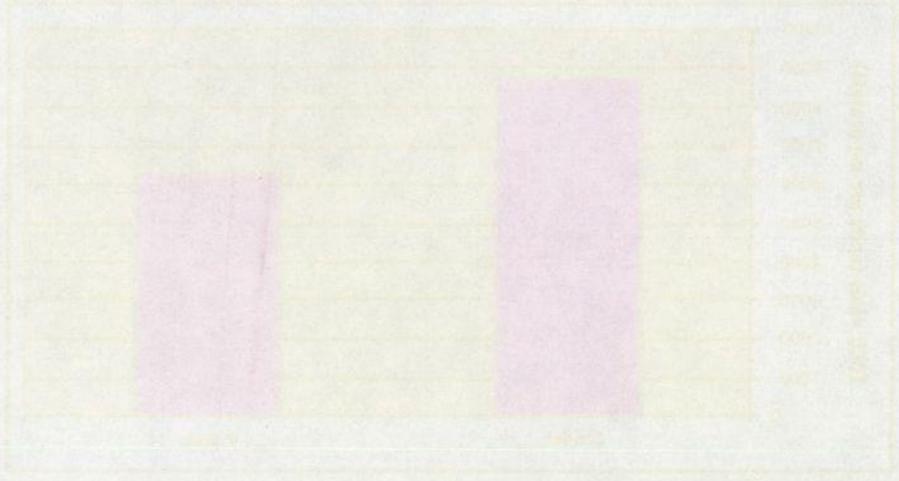
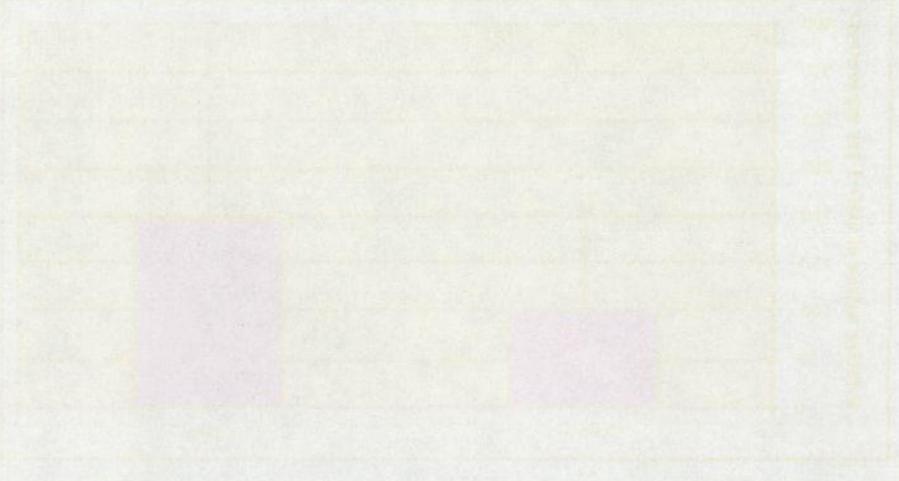


Figure 3



## Chapter 12

### Biomarkers and monitoring



Weddell seal breathing through an ice hole (Ross sea)

*“That day, by means of galvanized iron wires, we slung the inner pot from our aluminium cooker over the lighted wicks of our blubber cooker, thawed down snow in it, added chips of seal meat and made a delicious bouillon. This had a rich red color and seemed very nutritious, but to me was indigestible.”*

**Professor David**, on the way to the magnetic South Pole, Antarctic expedition of E. H. Shackleton (1907-1909).

### Chapter 13

## Diagrams and Monitoring

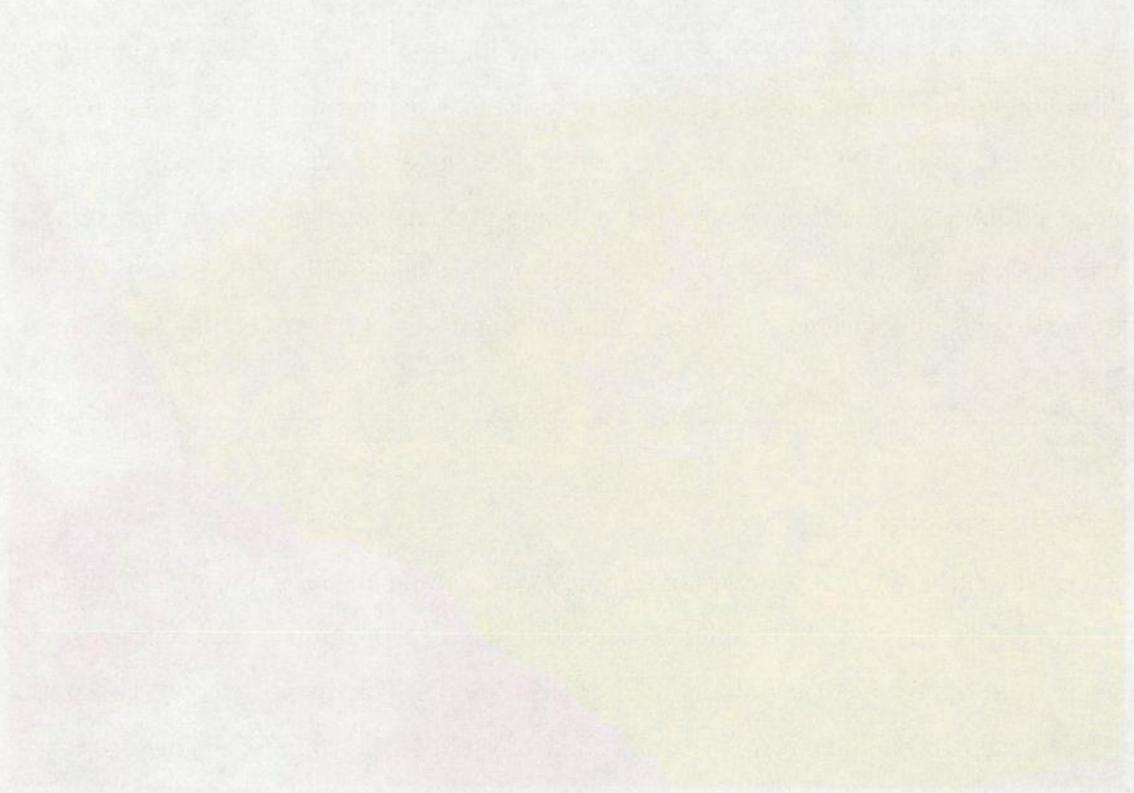


Figure 13.1: A diagram showing the layout of a field or area.

The diagram illustrates the layout of a field or area, showing various regions and boundaries. The regions are color-coded, with a large yellow area at the top, a purple area on the left, and a green area at the bottom. The diagram is used to illustrate the concept of monitoring in the field.

The diagram is used to illustrate the concept of monitoring in the field. It shows the layout of a field or area, with various regions and boundaries. The regions are color-coded, with a large yellow area at the top, a purple area on the left, and a green area at the bottom.

### **12.1. Biomarker sensitivity**

In this thesis, four biological responses to PAH were investigated in different exposure situations (dissolved PAH, dispersed, adsorbed, injected) in 4 different Arctic marine invertebrates. Because of the differences between each experiment, comparison of the biomarker responses appears difficult. Nevertheless, the cell membrane stability technique responded in every exposure situation to PAH, independent of the selected species. This may be explained either by the high sensitivity of the technique, or by the fact that cell membranes of marine invertebrates are highly susceptible to lipid peroxidation. In the field experiment performed with *Mya truncata*, the cell membrane stability appeared more sensitive than the biomolecular marker the (TOSC assay). This confirms that the detoxication process, of marine invertebrates, to metabolise and excrete pollutants, relies heavily on the immune system, haemocytes and lysosomes, where contaminants are sequestered and degraded. This sensitivity may also reflect that cell membranes of polar marine invertebrates are highly susceptible to contaminant stress and ROS.

### **12.2. Biomarker hierarchy**

The typical chronological biomarker responses, according to the cause-effect concept and flow of events in the hierarchy of the biological organisation in ecotoxicology, is such that on exposure to a pollutant, biomolecules (i.e. enzymes, receptors) respond first, followed by cellular responses and then physiological responses. This sequence did not occur in the crab experiment. Indeed, heart rate (a physiological biomarker) was more sensitive than TOSC (a biomolecular response). It is of course highly probable that the molecular interaction occurred on other system, not measured in this experiment but the effect was measured directly at the level of physiology. For example, the crab may have detected the presence of oil by means of a neuroreceptor (located in its antennules) that induced a "stress signal" corresponding to an elevated heart frequency. This finding highlights the importance of selecting physiological assays (such as heart rate) which are easy, fast and cheap to perform. Even though it is not a biomarker specific to a certain class of pollutants, heart rate can be used as a screening biomarker. Impaired heart rate indicates that the health status of the organisms is deteriorated and that further investigation is required to elucidate the cause.

### 12.3. Recovery

Biomarkers should be selected optimally to provide the best information on the health of the individual. This means that scientists should know where all biomarker responses lie within the zone of compensatory reactions (Figure 7). The threshold of irreversibility must be identified to be able to answer the questions: is the measured effect irreversible or not? How important is the impact? Therefore, recovery experiments are needed to locate the threshold of "reversible damage". Considering the low food availability in the polar regions and the slow growth and longevity of most ectothermic organisms, recovery may take longer than for temperate ecosystem. This needs to be investigated.

### 12.4. Species complex

Although it is difficult to compare directly the responses of each species used in this study, different species showed different responses due to their different feeding strategy, life strategy (epibenthic, burried in the sediment etc.) and also due to the difference in exposure context (dissolved, dispersed, adsorbed oil). Species occurrence in the field is highly patchy and, when biomonitoring, scientists have to work with the species that are available. For instance, *S. boreas*, *H. araneus* and *C. islandicus* are more abundant on hard rocky bottoms while *M. truncata* is abundant in a muddy habitat. In this work, we provide evidence for the possibility of using different polar marine species for monitoring potential industrial activities occurring in different polar marine habitat: *H. araneus*, *S. boreas*, *C. islandica* and *M. truncata*.

### 12.5. Exposure context

PAHs are ubiquitous compounds with different physical forms (dissolved, dispersed, adsorbed). Hence, laboratory experiments have to be performed to investigate the effect of PAHs at stages. The effects of three main physical forms of oil were investigated in this study but not on all species nor with all biomarkers; this remains to be performed.

### 12.6. Background level of biomarker responses

The variability of the TOSC in relation to food availability was shown in *Mya truncata*. This raises the importance of investigating further the background level of most biomarkers in the polar marine ecosystem (that is characterised by a strong food



seasonality). For instance, it is probable that the cell membrane stability varies with food availability and/or reproduction cycle. Fundamental knowledge is lacking and more work in parallel with biomarker investigations is required to better understand the specificity of the biology of polar marine invertebrates. In addition, the strong seasonality of the ecological parameters of the polar ecosystem may affect the oil distribution (i.e. ice transport) and fate (i.e. photooxidation casue by intense UV radiations) in the environment. Albeit, the physical and chemical properties of oil at different season in the Arctic are known, the effects on the fauna at different time of the year are not.

### **12.7. Conclusions**

This account highlights the need for use of a biomarker suite and a holistic approach (molecular, cellular and physiological biomarker) when biomonitoring with biomarkers in the field. Further, the study has shown the need to gain better understanding of the fundamental aspects of the biology of polar marine invertebrates. This approach will help understand the impact of contaminants on the integrated response of an animal. Such a monitoring protocol will help create a better diagnostic of the situation and, eventually, a full prognosis to decide what is the best action to be taken to prevent any impact at higher levels of biological organisation (populations, communities and human) in the Arctic.

## Chapter 13

### Oxidative stress and food availability



*“Il faut se tromper, être imprudent. Les hommes prudents sont des infirmes”*

**Jacques Brel**

### Chapter 13

## Oxidative stress and food availability

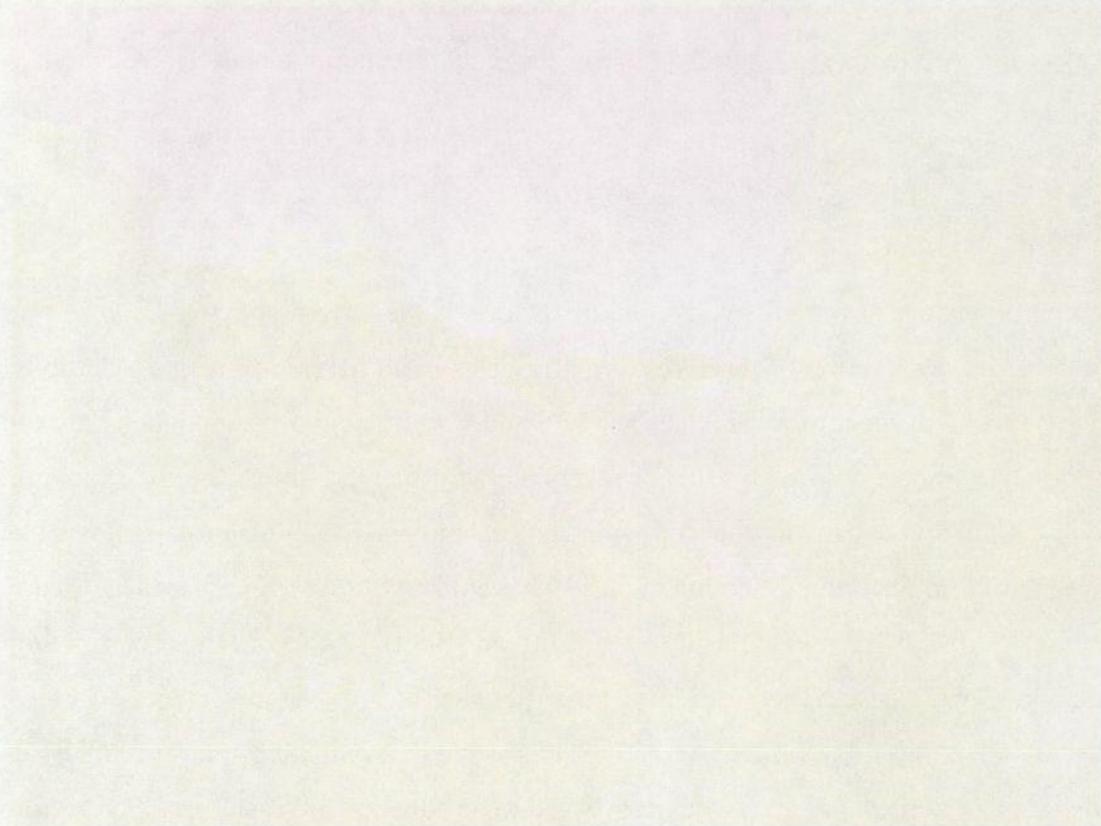


Figure 13.1. Oxidative stress and food availability.

### 13.1. Introduction

In this study, the elevated TOSC level measured in polar bivalves was thought to reflect the high levels of ROS in the water column formed by the high level of UV-B in polar regions. This hypothesis is based upon the assumption that elevated levels of antioxidant defences reflect elevated prooxidant pressures on the environment as reported by most scientists working in the field of oxidative stress. However, is this always true? Are there any other hypotheses that could explain the high TOSC levels of polar marine bivalves?

### 13.2. The problem

A common feature of the life cycle of all molecules, is the progressive oxidation caused by the natural production of reactive oxygen species in all aerobic processes. Carbonyl groups, arising mostly from the attack of ROS and low molecular weight aldehydes and ketones, accumulate on proteins in an irreversible way, thereby, impairing their function and structure, and enhancing their turnover. For example, *ca.* 10% of protein molecules may exhibit carbonyl modifications, caused by oxidative stress, every day in steady state conditions in a typical cell rat (Stadtman 1992). However, this observation (Stadtman 1992) raises the question of the efficiency of antioxidant defences. Indeed, scientists working in the field of oxidative stress state that oxidative stress occurs when antioxidant defences are overwhelmed by ROS production (Winston and Di Giulio 1991). But, if 10% of the proteins are oxidised every 24 h in normal rat cell (Stadtman, 1992) this means that antioxidant defences are not efficient ROS scavengers, and that enough biological material is available to produce new biomolecules to replace the oxidised ones. Therefore, if the food availability is high enough to provide biological material to produce biomolecules, it may not be necessary to spend energy in protecting these biomolecules. Protection against oxidative stress would be the result of a trade off between biomolecules turnover rate and antioxidant defence efficiency. Oxidative stress would be defined as a balance between ROS production level, food availability, biomolecule turnover rate and antioxidant defences levels.

### 13.3. Polar ectotherms show a low protein turnover

Evidence from comparative work between a temperate and an Antarctic isopod revealed that the polar crustacean showed extremely low protein synthesis and low metabolism (Whiteley *et al.* 1996). The rate of protein synthesis of *G. antarcticus*, the Antarctic isopod, was 75 times lower than the values obtained for *Idotea rescata*, the temperate isopod. Animals that have low metabolic rates, and low rates of whole body protein synthesis, may show low rates of protein degradation and by inference low rates of protein turnover with reduced energy requirements for protein turnover (Hawkins 1991). This low protein turnover appears to be an adaptation to the low food supply available in polar regions. A low protein turnover implies that the proteins will be used for a longer time to assure the function and structure of the cells. Furthermore, the protein synthesis cost for *G. antarcticus* at 0°C was 4 times higher compared with *I. rescata* at 4°C. The proportion of oxygen uptake, attributable to total whole body protein synthesis, was 66% in *G. antarcticus* and 22% in *I. rescata*. Previous studies have shown a correlation between oxygen consumption rates and rates of protein synthesis in whole animals with the maintenance of a constant ratio between the two variables within a species (Houlihan 1991). Therefore, the low metabolic rate of *Mya truncata* and *Laternula elliptica* is indicative of a low protein turnover rate.

### 13.4. High TOSC and low, expensive turnover of protein

From the foregoing account, it can be hypothesised that by having an extremely low turnover rate, polar marine invertebrates increase drastically the risk of exposing proteins to oxidative stress. Consequently, they may experience problems to produce sufficient proteins to replace the damaged biomolecules. Furthermore, as the cost of protein synthesis in polar species is high, it is therefore essential that the newly-formed proteins be protected from oxidative damage in an efficient way. The low respiration rate of polar species may help reduce ROS as 2 to 3% of inhaled O<sub>2</sub> is transformed into ROS (Chance *et al.* 1979). The elevated TOSC levels measured in this study in polar marine invertebrates support the hypothesis that polar marine invertebrates require a very efficient protection against oxidative stress to protect their biomolecules. An elevated total oxyradical scavenging capacity would allow polar marine invertebrates to have a low protein turnover rate and live with low food supply. Therefore, this high TOSC would not reflect the high prooxidant pressure of the environment but, rather,

the essential need to protect the biomolecule pool in a very efficient way from oxidative damage. Evidence of reduced oxidative damage was reported by (Bluhm and Brey 2001) who investigated the age of the Antarctic shrimp *Notocrangon antarcticus*, a long-lived crustacean, by measuring the concentration of lipofuscin accumulated in the tissues. Lipofuscin is a pigment produced by the oxidation of macromolecules (lipids, proteins) by ROS (Sitte *et al.* 2001). The average yearly lipofuscin accumulation in the tissue of *N. antarcticus* was nearly linear and estimated as 0.02% area fraction year, which is considerably lower than rates published for species from lower latitudes (Bluhm and Brey 2001).

### **13.5. Why do polar marine ectotherms show longevity?**

Polar marine invertebrates show a tendency for longevity. The bivalves, *Laternula elliptica* can live for more than 10 years, *Yoldia eightsi* up to 60 years (Nolan and Clarke 1993) and some may reach 120 years (Peck and Bullough 1993). Age measurements for *Mya truncata* suggest a lifespan of 50 years (Sundet personal communication). To date, polar researchers have explained this longevity by a low metabolism or low protein turnover rate, however, we need to ask what is meant by ageing and how is ageing related to low metabolism. Ageing which is a progressive and irreversible physiological decline in the latter part of life, was shown to be mainly caused by the damaging effect of ROS, produced naturally during the respiration (Sohal and Weindruch 1996). For instance, lipofuscin, a pigment produced by the oxidation of macromolecules (lipids, proteins) by ROS (Sitte *et al.* 2001), shows increasing concentration in the tissue with age (Terman and Brunk 1998). Therefore, all kind of mechanisms that help reduce oxidative damage may enhance longevity. Low metabolism, low food availability and elevated TOSC appears to explain the longevity of polar marine ectotherms.

#### **13.5.1 Low metabolism and longevity**

Pearl (1928) put forward the theory the “rate of living” that states that “the rate of energy expenditure would determine the length of life”. It is interesting to note, however, that the rates of ROS production is directly correlated to the metabolic rate and inversely related to the maximum life span of the species of study (Ku *et al.* 1993). The low metabolic rates of polar marine ectotherms may reduce ROS production,

resulting in minimised oxidative damage and increased life expectancy. In addition, the low stable temperature, with little or no rise in the polar oceans, prevents any rise in metabolic activity. Furthermore, a decreased activity, as noticed in most polar ectotherms, helps decrease ROS production. Moreover, feeding activity, during the brief phytoplankton bloom, did not affect the level of activity measured in winter.

### **13.5.2. Caloric restriction in polar waters enhances longevity**

Caloric restrictions, defined as a low food supply, metabolic activity and therefore ROS production in mammals and extend life span (Koizumi *et al.* 1992). In polar regions, feeding occurs for a very brief time of about 2 to 3 months, leaving the animals to starve for the following 9 months. In polar ectotherms, metabolic activity was shown to slightly increase during the phytoplankton bloom though this occurs only for 3 months, while for the rest of the year, metabolism was kept very low, diminishing ROS formation.

### **13.5.3. High level of antioxidant defences and longevity**

If ageing is linked to oxidative damage, then longevity will be enhanced by the elevated capability of an organism to scavenge oxyradical to prevent biomolecules oxidation. Retardation of ageing process by elevated antioxidant defences was investigated in *Drosophila melanogaster* (Orr and Sohal 1994). Flies, over-expressing superoxide dismutase (removal of  $O_2^-$ ) and catalase (removal of  $H_2O_2$ ) by carrying three copies of each of these genes, exhibited as much as a one-third extension of life span, a longer mortality rate doubling time, a lower amount of protein oxidative damage, and a delayed loss in physical performance. Our present data indicating that polar organisms possess an elevated TOSC, compared with temperate species, are in agreement with other studies (Colella *et al.* 2000, Regoli *et al.* 2000). Thus, high TOSC level, can contribute largely to the longevity of polar ectotherms by scavenging a great deal of ROS.

### **13.6. Adaptation to polar water: a trade off between low food availability and oxidative stress?**

From the above question, new hypotheses to explain the adaptations of marine ectotherms to the polar environment and the sequence of biological adaptation required to achieve this polar adaptation can be proposed:

- i) Either, a high TOSC value insures good protection from oxidative damage, allowing marine invertebrates to reduce the rate of turnover of protein, thereby, reducing their metabolism, hence, they were best fitted to colonise environments characterised by a low food availability.
- ii) Or, living in an environment with poor food availability required a low protein turnover and a low respiration rate to minimise energy expenditure and, therefore, an elevated TOSC to protect proteins from oxidative damages was developed.

Antioxidative defences are probably one of the first detoxication systems developed when the Earth's atmosphere and water started to be strongly oxidant about 2.2 billions years ago. Oxygen was a poison to life and the phenomenon of oxygen toxicity is referred to the "oxygen paradox". For instance, haemoglobin was produced as an oxygen scavenger to prevent the molecules of oxygen to enter cells and create damage. The Circum-Antarctic current has probably isolated the marine fauna for about 2 billion years, that is 0.2 billion years latter than the period when the oxygen production started. These figures would indicate that antioxidative defenses may have been developed by marine organisms, probably at a high level since oxygen levels were high, and latter the marine fauna located on the Antarctic shelf experienced a decrease in temperature resulting in a speciation toward cold water. Therefore, the polar adaptation may have been achieved because of an elevated TOSC that helped organisms to live with low food availability and have a low biomolecule turnover rate.

### **13.7. Conclusions**

The limited food availability in polar regions appears to be an important environmental factor that affects the physiology of polar marine invertebrates as highlighted by Clarke (1983, 1991) in his reviews of the metabolic cold adaptation. Indeed, because of the lack of food allowing high biomolecule turnover rate, polar ectothermic organisms have

developed a life strategy aimed at minimizing the oxidative damage on biomolecules by reducing internal production and impact of ROS. For example, the low metabolic rate reduces natural internal ROS production, and low and stable temperature contributes to the caloric restriction which helps keeping a low metabolic rate. To minimize energy expenditure, marine ectothermic organisms show a reduced activity and oxidative damages are prevented by a high level of antioxidant defences

The primary goal of this reduced oxidative stress is to protect, in a very efficient way, the protein pool. This is characterised by a low protein turnover rate and expensive production costs and offers the possibility to colonise an ecosystem characterised by low food availability. One outcome of this adaptation is the longevity of polar organisms compared with temperate organisms. The hypothesis, in terms of evolution and adaptation, that organisms with a high TOSC were conferred with advantages to colonize polar environments but requires more evidence.

### **13.8. Furtherwork**

Owing to the cost of performing research in polar region and to sample marine organisms, the ecophysiology and the biological effects of contaminants on the polar marine invertebrates still require investigation. Nevertheless, in the chapter 13 of this thesis, a new hypothesis to explain the role of a high TOSC level in polar ectotherms was highlighted. This hypothesis is mainly based on literature survey, therefore, it requires experimental evidences. The main research question would be the following one: is there a link between TOSC level and the rate of turnover of biomolecules in marine ectotherms originating from different latitudes?



## References

### A

Aagaard, A. (1996) In situ variation in heart rate of the shore crab *Carcinus maenas* in relation to environmental factors and physiological condition. *Marine Biology* 125:765-772.

Aagaard, A., B. Styrihave, C. G. Warman and M. H. Depledge (2000) The use of cardiac monitoring in the assessment of mercury toxicity in the subtropical pebble crab *Gaetice depressus* (Brachyura: Grapsidae: Varuninae). *Scienta Marina* 64(4): 381-386.

Aagaard, A., C. G. Warman, M. H. Depledge and E. Naylor (1995) Dissociation of heart rate and locomotor activity during the expression of rhythmic behaviour in the shore crab *Carcinus maenas*. *Marine and Freshwater Behaviour and Physiology* 26(1): 1-10.

Aarset, A. V. and T. Aunaas (1990) Metabolic responses of the sympagic amphipods *Gammarus wilkitzkii* and *Onisimus glacialis* to acute temperature variations. *Marine Biology* 107:433-438.

Aarset, A. V. and K. E. Zachariassen (1983) Synergistic effects of an oil dispersant and low temperature on the freezing tolerance and solute concentrations of the blue mussel (*Mytilus edulis* L.). *Polar Research* 1 n.s.:223-229.

Aas, E., J. Beyer and A. Goksoyr (1998) PAH in fish bile detected by fixed wavelength fluorescence. *Marine Environmental Research* 46(1-5): 225-228.

Aas, E., J. Beyer, G. Jonsson, W. L. Reichert and O. K. Andersen (2001) Evidence of uptake, biotransformation and DNA binding of polyaromatic hydrocarbons in Atlantic cod and corkwing wrasse caught in the vicinity of an aluminium works. *Marine Environmental Research* 52: 213-229.

Abele, D., B. Burlando, A. Viarengo and H. O. Pörtner (1998) Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal limpet *Nacella concinna*. *Comparative Biochemistry and Physiology* 120B(2): 425-435.

Abele, D., C. Tesch, P. Wencke and H. O. Pörtner (2001) How does oxidative stress relate to thermal tolerance in the Antarctic bivalve *Yoldia eightsi*? *Antarctic Science* 13(2): 111-118.

Adema, C. M., W. P. W. Van Der Knaap and T. Sminia (1991) Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. *Reviews in Aquatic Science* 4(2-3): 201-223.

Ahn, I. Y. and J. H. Shim (1998) Summer metabolism of the Antarctic clam, *Laternula elliptica* (King and Broderip) in Maxwell Bay, King George Island and its implications. *Journal of Experimental Marine Biology and Ecology* 224(2): 253-264.

Ahsanullah, M. and R. C. Newell (1971) Factors affecting the heart rate of the shore crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology* 39A:277-287.

AMAP (1998) AMAP Assessment Report: Arctic Pollution Issues. Arctic Monitoring and Assessment Programme (AMAP), Oslo, Norway. xii+ 859 pp.

Arnaud, P. M. (1985) Essai de synthese des particularites eco-biologiques (adaptations) des invertebres benthiques antarctiques. *Science* 11:117-124.

Arrhenius, S. A. (1889) *Zoology Physics and Chemistry* 4:226-248.

Arun, S., P. Krishnamoorthy and P. Subramanian (1999) Properties of glutathione peroxidase from the hepatopancreas of freshwater prawn *Macrobrachium malcolmsonii*. *International Journal of Biochemistry and Cell Biology* 31(6): 725-732.

Aunaas, T., A. Olsen and K. E. Zachariassen (1991) The effects of oil and oil dispersants on the amphipod *Gammarus oceanicus* from Arctic waters. *Polar Research* 10(2): 619-630.

Axelsson, M., W. Davison, M. E. Forster and A. P. Farrell (1992) Cardiovascular responses of the red-blooded Antarctic fishes *Pagothenia bernacchii* and *P. borchgrevinki*. *Journal of Experimental Biology* 167:179-201.

Axiak, V. and J. George (1987) Bioenergetic responses of the marine bivalve *Venus verrucosa* on long-term exposure to petroleum hydrocarbons. *Marine Environmental Research* 23:33-47.

## B

Babu, B. N. and O. R. Brown (1995) Quantitative effects of redox-cycling chemicals on the oxidant-sensitive enzyme dihydroxy-acid dehydratase. *Microbios* 82(332): 157-170.

Bacon, C. E., W. M. Jarman and D. P. Costa (1992) Organochlorine and polychlorinated biphenyl levels in pinniped milk from the Arctic, the Antarctic, California and Australia. *Chemosphere* 24(6): 779-791.

Bamber, S. D. and M. H. Depledge (1997) Responses of shore crabs to physiological challenges following exposure to selected environmental contaminants. *Aquatic Toxicology* 40:79-92.

Bandiera, S. M., S. M. Torok, R. J. Letcher and R. J. Norstrom (1997) Immunoquantitation of cytochromes P450 1A and P450 2B and comparison with chlorinated hydrocarbon levels in archived polar bear liver samples. *Chemosphere* 34(5-7): 1469-1479.

Bard, S. M. (1999) Global transport of anthropogenic contaminants and the consequences for the Arctic marine ecosystem. *Marine Pollution Bulletin* 38(5): 356-379.

Barron, M. G., H. Galbraith and D. Beltman (1995) Comparative reproductive and developmental toxicology of PCBs in birds. *Comparative Biochemistry and Physiology* 112C:1-14.

Baumard, P., H. Budzinski and P. Garrigues (1998a) Polycyclic aromatic hydrocarbons in sediments and mussels of the western Mediterranean sea. *Environmental Toxicology and Chemistry* 17(5): 765-776.

Baumard, P., H. Budzinski, P. Garrigues, J. C. Sorbe, T. Burgeot and J. Bellocq (1998b) Concentrations of PAHs (Polycyclic Aromatic Hydrocarbons) in various marine organisms in relation to those in sediments and to trophic level. *Marine Pollution Bulletin* 36(12): 951-960.

Baumard, P., H. Budzinski and P. Garrigues (1998c) PAHs in Arcachon Bay, France: origin and biomonitoring with caged organisms. *Marine Pollution Bulletin* 36(8): 577-586.

Baumard, P., H. Budzinski, P. Garrigues, H. Dizer and P. D. Hansen (1999a) Polycyclic aromatic hydrocarbons in recent sediments and mussels (*Mytilus edulis*) from the Western Baltic Sea: occurrence, bioavailability and seasonal variations. *Marine Environmental Research* 47:17-47.

Baumard, P., H. Budzinski, P. Garrigues, J. F. Narbonne, T. Burgeot, X. Michel and J. Bellocq (1999b) Polycyclic hydrocarbons (PAH) burden of mussels (*Mytilus* sp.) in different marine environment in relation with sediment PAH contamination, and bioavailability. *Marine Environmental Research* 47:415-439.

Baussant, T., S. Sanni, G. Jonsson, A. Skadsheim and J. F. Børseth (2001) Bioaccumulation of polycyclic aromatic compounds: 1. Bioconcentration in two marine species and in semipermeable membrane devices during chronic exposure to dispersed crude oil. *Environmental Toxicology and Chemistry* 20(6): 1175-1184.

Bayne, B. L. and C. Scullard (1977) Rates of nitrogen excretion by species of *Mytilus* (Bivalvia: Mollusca). *Journal of the Marine Biological Association of the United Kingdom* 57:355-369.

Bayne, B. L., R. J. Thompson and W. J. (1973) Some effects of temperature and food on the rate of oxygen consumption by *Mytilus edulis* L. In *effects of temperature on Ectothermic organisms* (Edited by Wieser, W.), pp. 181-193. Springer-Verlag, Berlin.

Bluhm, B. A. and T. Brey (2001) Age determination in the Antarctic shrimp *Notocrangon antarcticus* (Crustacea : Decapoda), using the autofluorescent pigment lipofuscin. *Marine Biology* 138(2): 247-257.

Bojsen, B. H., H. Witthøfft, B. Styrisshave and O. Andersen (1998) *In situ* studies on heart rate and locomotor activity in the freshwater crayfish, *Astacus astacus* (L.) in

relation to natural fluctuations in temperature and light intensity. *Freshwater Biology* 39:455-465.

Borenfreund, E. and J. A. Puerner (1985) Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicology Letter* 24:119-124.

Borgeraas, J. and D. O. Hessen (2000) UV-B induced mortality and antioxidant enzyme activities in *Daphnia magna* at different oxygen concentrations and temperatures. *Journal of Plankton Research* 22(6): 1167-1183.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248-254.

Brey, T. and A. Clarke (1993) Population-dynamics of marine benthic invertebrates in Antarctic and sub-Antarctic environments-are there unique adaptations? *Antarctic Science* 5(3): 253-266.

## C

Campbell, A. (1994) *Seashores and shallow seas of Britain and Europe*. The Hamlyn Publishing Group Limited, 320pp.

Campbell, M.K. (1991) *Biochemistry*. Saunders College Publishing, 619pp.

Camus, L., B. E. Grøsvik, J. F. Børseth, M. Jones and M. H. Depledge (2000) Stability of lysosomal and cell membranes in haemocytes of the common mussel (*Mytilus edulis*): effect of low temperatures. *Marine Environmental Research* 50:325-329.

Camus, L., M. Jones, J. F. Børseth, B. E. Grøsvik, F. Regoli and M. H. Depledge (*in press*) Total oxyradical scavenging capacity and plasma membrane stability of the haemocytes of the Arctic scallop, *Chlamys islandicus*, following benzo(a)pyrene exposure. *Marine Environmental Research*.

Cancio, I., A. Ibabe and M. P. Cajaraville (1999) Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels *Mytilus galloprovincialis* and its relationship with the lipid content. *Comparative Biochemistry and Physiology* 123C(2): 135-144.

Cao, G., A. H.M. and R. G. Cutler (1993) Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology & Medicine* 14:303-311.

Carballal, M. J., C. Lopez, C. Azevedo and A. Villalba (1997) Enzymes involved in defense functions of hemocytes of mussel *Mytilus galloprovincialis*. *Journal of Invertebrate Pathology* 70(2): 96-105.

Chance, B., H. Sies and A. Boveris (1979) Hydroperoxide metabolism in mammalian organs. *Physiological Review* 59(3): 527-605.

Chapelle, G. and L. S. Peck (1999) Polar gigantism dictated by oxygen availability. *Nature* 399(6732): 114-115.

Chapman, P. M. (1993) Are Arctic marine invertebrates relatively insensitive to metals? *Environmental Toxicology and Chemistry* 12(4): 611-613.

Chapman, P. M. and C. McPherson (1993) Comparative zinc and lead toxicity tests with Arctic marine invertebrates and implications for toxicant discharges. *Polar Record* 29(168): 45-54.

Cheung, V. V., R. J. Wedderburn and M. H. Depledge (1998) Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo harbour, Hong Kong. *Marine Environmental Research* 46(1-5): 237-241.

Chevion, S., E. M. Berry, N. Kitrossky and R. Kohen (1997) Evaluation of plasma low molecular weight antioxidant capacity by cyclic voltammetry. *Free Radical Biology & Medicine* 22(3): 411-421.

Cho, M., R. Jedrychowsky, B. Hammock and A. Buckpitt (1994) Reactive naphthalene metabolite binding to hemoglobin and albumin. *Fundamental and Applied Toxicology* 22(1): 26-33.

Christiansen, J. and S. George (1995) Contamination of food by crude oil affects food selection and growth performance, but not appetite, in an Arctic fish, the polar cod (*Boreogadus saida*). *Polar Biology* 15:277-281.

Christiansen, J. S. (2000) Sex differences in ionoregulatory responses to dietary oil exposure in polar cod. *Journal of Fish Biology* 57A(167-170):

Christiansen, J. S., R. A. Dalmo and K. Ingebrigtsen (1996) Xenobiotic excretion in fish with glomerular kidneys. *Marine Ecology Progress Series* 136(1-3): 303-304.

Clarke, A. (1980) A reappraisal of the concept of metabolic cold adaptation in polar marine invertebrates. *Biological Journal of Linne Society* 14:77-92.

Clarke, A. (1983) Life in cold water: the physiological ecology of polar marine ectotherms. *Oceanography and Marine Biology Annual Review* 21:341-453.

Clarke, A. (1987) temperature, latitude and reproductive effort. 38 08.12.98):

Clarke, A. (1988) Seasonality in the Antarctic Marine-Environment. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 90(3): 461-473.

Clarke, A. (1991) What is cold adaptation and how should we measure it? *American Zoologist* 31(1): 81-92.

Clarke, A. (1991) Cold adaptation. *Journal of Zoology* 225:691-699.

Clarke, A. (1993) Seasonal acclimatization and latitudinal compensation in metabolism: do they exist? *Functional Ecology* 7:139-149.

Clarke, A. and N. M. Johnston (1999) Scaling of metabolic rate with body mass and temperature in teleost fish. *Journal of Animal Ecology* 68(5): 893-905.

Colella, A., M. Patamia, A. Galtieri and B. Giardina (2000) Cold adaptation and oxidative metabolism of Antarctic fish. *Italian Journal of Zoology* 67:33-36.

Coles, J. A., S. R. Farley and R. K. Pipe (1994) Effects of fluoranthene on the immunocompetence of the common marine mussel, *Mytilus edulis*. *Aquatic Toxicology* 30:367-379.

Connel, D. V. (1988) Bioaccumulation behavior of persistent organic chemicals in aquatic organisms. *Review of Environmental Contamination and Toxicology* 101:117-160.

Cooper, W. J., C. W. Shao, D. R. S. Lean, A. S. Gordon and F. E. Scully (1994) Factors affecting the distribution of H<sub>2</sub>O<sub>2</sub> in surface waters. *Advances in Chemistry Series* 237:391-422.

Cuculescu, M., D. Hyde and K. Bowler (1995) Temperature-acclimation of marine crabs changes in plasma membrane fluidity and lipid composition. *Journal of Thermal Biology* 20(1-2): 207-222.

## D

Dame, R. F. (1972) The ecological energetics of growth, respiration and assimilation in the intertidal American oyster *Crassostrea virginica*. *Marine Biology* 17:243-250.

Davenport, J. (1988) Oxygen-consumption and ventilation rate at low-temperatures in the Antarctic protobranch bivalve mollusk *Yoldia* (= *Aequiyoldia*) *eightsi* (Courthouy). *Comparative Biochemistry and Physiology* 90(3A): 511-513.

DeFur, P. and C. Mangum (1979) The effects of environmental variables on the heart rates of invertebrates. *Comparative Biochemistry and Physiology* 62A:283-294.

Denstad, J. P., T. Aunaas, J. F. Børseth, A. V. Aarset and K. E. Zachariassen (1987) Thermal hysteresis antifreeze agents in fishes from Spitsbergen waters. *Polar Research* 5 n.s.:171-174.

DePirro, M., G. Santini and G. Chelazzi (1999) Cardiac responses to salinity variations in two differently zoned Mediterranean limpets. *Journal of Comparative Physiology* 169B(7): 501-506.

Depledge, M. H. (1978) Cardiac activity of the shore crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology* 60A:65-67.

Depledge, M. H. (1984) Disruption of circulatory and respiratory activity in shore crabs (*Carcinus maenas* (L.)) exposed to heavy metal pollution. *Comparative Biochemistry and Physiology* 78C(2): 445-459.

Depledge, M.H. (1984) Changes in cardiac activity, oxygen uptake and perfusion indices in *Carcinus maenas* (L.) exposed to crude oil and dispersant. *Comparative Biochemistry and Physiology* 78C(2): 461-466.

Depledge, M. H. (1985) The influence of nutritional state on the circulatory and respiration physiology of the shore crab, *Carcinus maenas*. *Journal of the Marine Biological Association of the United Kingdom* 65:69-78.

Depledge, M. H. (1992) On the tidal, diurnal and seasonal modulation of endogenous cardiac activity rhythms in the shore crab (*Carcinus maenas* L.). *Oecologia* XVIII(N.S.): 53-67.

Depledge, M. H. (1994) The rational basis for the use of biomarkers as ecotoxicological tools. In: *Non-destructive biomarkers in vertebrates*. Edited by M.C. Fossi and C. Leonzio, Lewis Publishers, Boca Raton, Florida, pp. 271-295.

Depledge, M. H. and B. B. Andersen (1990) A computer-aided physiological monitoring-system for continuous, long-term recording of cardiac activity in selected invertebrates. *Comparative Biochemistry and Physiology* 96A(4): 473-477.

Depledge, M. H. and A. K. Lundebye (1996) Physiological monitoring of contaminant effects in the individual rock crabs, *Hemigrapsus Edwardsi*: the ecotoxicological significance of variability in response. *Comparative Biochemistry and Physiology* 113C(2): 277-282.

DeWachter, B. and B. McMahon (1996) Haemolymph flow distribution, cardiac performance and ventilation during moderate walking, activity in *Cancer magister* (Dana) (Decapoda, Crustacea). *The Journal of Experimental Biology* 199:627-633.

Dewailly, E., P. Ayotte, C. Blanchet, J. Grondin, S. Bruneau, B. Holut and G. Carrer (1996) Weighing contaminant risks and nutrient benefits of country food in Nunavik. *Arctic Medical Research* 55(Suppl. 1): 13-19.

Dewailly, E., A. Nantel, S. Bruneau, C. Laliberte, L. Ferron and S. Gingras (1992) Breast milk contamination by PCDDs, PCDFs and PCBs in Arctic Quebec: a preliminary assessment. *Chemosphere* 25:7-10.

Douglas, G. S., R. C. Prince, E. L. Butler and W. G. Steinhauer (1994) The use of internal chemical indicators in petroleum and refined products to evaluate the extent of biodegradation. In *Hydrocarbon Bioremediation*. Boca Raton, FL, US. CRC Press Inc.

Doyotte, A., C. Cossu, M. C. Jacquin, M. Babut and P. Vasseur (1997) Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquatic Toxicology* 39(2): 93-110.

Duquesne, S., M. Riddle, R. Schulz and M. Liess (2000) Effects of contaminants in the Antarctic environment-potential of the gammarid amphipod crustacean *Paramorea walkeri* as a biological indicator for Antarctic ecosystems based on

toxicity and bioaccumulation of copper and cadmium. *Aquatic Toxicology* 49(1-2): 131-143.

Dyer, M. F. (1985) The distribution of *Hyas araneus* (L.) and *Hyas coarctatus* Leach (Crustacea: Decapoda: Brachyura) in the North Sea and the Svalbard region. *Journal of the Marine Biological Association of the United Kingdom* 65:195-201.

Dyrynda, E. A., R. K. Pipe, G. R. Burt and N. A. Ratcliffe (1998) Modulations in the immune defences of mussels (*Mytilus edulis*) from contaminated sites in the UK. *Aquatic Toxicology* 42(3): 169-185.

## E

El-Sayed, S.Z. (1984) Productivity of Antarctic waters-a reappraisal. In *Marine Phytoplankton and Productivity Lecture Notes on Coastal and Estuarine Studies*, 8 (Edited by Holm-Hansen O., Bolis L. and Gilles R.) pp. 19-34. Springer, Berlin.

Eshky, A. A., A. C. Taylor and J. A. Atkinson (1996) The effects of temperature on aspects of respiratory physiology of the semi terrestrial Crabs *Uca inversa* (Hoffmann) and *Metopograpsus messor* (Forskål) from the Red Sea. *Comparative Biochemistry and Physiology* 114A(4): 297-304.

## F

Farrington, J. W. (1991) Biogeochemical processes governing exposure and uptake of organic pollutant compounds in aquatic organisms. *Environmental Health and Perspectives* 90:75-84.

Fernley, P. W., M. N. Moore, D. M. Lowe, P. Donkin and S. Evans (2000) Impact of the *Sea Empress* oil spill on lysosomal stability in mussel blood cells. *Marine Environmental Research* 50:451-455.

Fisher, K. I. and R. E. A. Stewart (1997) Summer foods of Atlantic walrus, *Odobenus rosmarus rosmarus*, in northern Foxe basin, northwest territories. *Canadian Journal of Zoology* 75(7): 1166-1175.

Focardi, S., C. Fossi, L. Lari, L. Marsili, C. Leonzio and S. Casini (1992) Induction of mixed-function oxidase (MFO) system in 2 species of Antarctic fish from Terra-Nova bay (Ross Sea)-induction of MFO activity in Antarctic fish. *Polar Biology* 12(8): 721-725.

Focardi, S., C. Fossi, C. Leonzio and P. Disimplicio (1989) Mixed function oxidase activity and conjugating enzymes in 2 species of Antarctic fish. *Marine Environmental Research* 28(1-4): 31-33.

Focardi, S., M. C. Fossi, L. Lari, S. Casini, C. Leonzio, S. K. Meidel and M. Nigro (1995) Induction of MFO activity in the Antarctic fish *Pagothenia Bernachii*-Preliminary results. *Marine Environmental Research* 39(1-4): 97-100.

Folch, J., M. Lees and G. H. S. Stanley (1957) A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry* 226:497-509.

Fong, W. C. (1976) Uptake and retention of Kuwait crude oil and its effects on oxygen uptake by the soft-shell clam, *Mya arenaria*. *Journal of Fisheries and Research Board of Canada* 33:2774-2780.

Fossi, M. C., C. Savelli, S. Casini, E. Franchi, N. Mattei and I. Corsi (1997) Multi-response biomarker approach in the crab *Carcinus aestuarii* experimentally exposed to benzo(a)pyrene, polychlorobiphenyls and methyl-mercury. *Biomarkers* 2(5): 311-319.

Foster, G. D., S. M. Baksi and J. C. Means (1987) Bioaccumulation of trace organic contaminants from sediment by baltic clams (*Macoma balthica*) and soft-shell clams (*Mya arenaria*). *Environmental Toxicology and Chemistry* 6(12): 969-976.

Frederich, M., F. J. Sartoris, W. E. Arntz and H. O. Pörtner (2000) Haemolymph Mg<sup>2+</sup> regulations in decapod crustaceans: physiological correlates and ecological consequences in polar areas. *The Journal of Experimental Biology* 203:1383-1393.

## G

Gamble, S. C., P. S. Goldfarb, C. Porte and D. R. Livingstone (1995) Glutathione peroxidase and other antioxidant enzyme function in marine invertebrates (*Mytilus edulis*, *Pecten maximus*, *Carcinus maenas* and *Asterias rubens*). *Marine Environmental Research* 39(1-4): 191-195.

George, S. G., J. S. Christiansen, B. Killie and J. Wright (1995) Dietary Crude-oil Exposure During Sexual-Maturation Induces Hepatic Mixed-Function Oxygenase (cyp1a) activity at very-low Environmental Temperatures in Polar Cod *boreogadus-saida*. *Marine Ecology-Progress Series* 122(1-3): 307-312.

Ghiselli, A., M. Serafini, G. Maiani, E. Azzini and F.-L. A. (1995) A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radical Biology & Medicine* 18(1): 29-36.

Gillis, T. E. and J. S. Ballantyne (1999a) Mitochondrial membrane composition of two arctic marine bivalve mollusks, *Serripes groenlandicus* and *Mya truncata*. *Lipids* 34(1): 53-57.

Gillis, T. E. and J. S. Ballantyne (1999b) Influences of subzero thermal acclimation on mitochondrial membrane composition of temperate zone marine bivalve mollusks. *Lipids* 34:59-66.

Glazer, A. N. (1988) Fluorescence-based assay for reactive oxygen species: A protective role for carnitine. *FASEB Journal* 2:2487-2491.

Goldberg, E. D. (1975) Synthetic organohalides in the sea. *Proceedings of the Royal Society of London, Series B* 189:277-289.

Graham, M. S. and G. L. Fletcher (1985) On the low blood viscosity of two cold water marine sculpins. A comparison with the winter flounder. *Journal of Comparative Physiology-B* 155:455-459.

Grant, J. and B. Thorpe (1991) Effects of suspended sediment on growth, respiration, and excretion of the soft-shell clam (*Mya arenaria*). *Canadian Journal of Fisheries and Aquatic Sciences* 48(7): 1285-1292.

Gray, J. S. (1989) Effects of environmental-stress on species rich assemblages. *Biological Journal of the Linnean Society* 37(1-2): 19-32.

Gray, J. S., K. R. Clarke, R. M. Warwick and G. Hobbs (1990) Detection of initial effects of pollution on marine benthos-an example from the Ekofisk and Eldfisk oilfields, North Sea. *Marine Ecology Progress Series* 66(3): 285-299.

Grundy, M. M., M. N. Moore, S. M. Howell and N. A. Ratcliffe (1996) Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. *Aquatic Toxicology* 34(4): 273-290.

Gruzdev, A. N. (1995) Possible changes in the dose of biologically active ultraviolet radiation received by the biosphere in the summer time Arctic due to total ozone interannual variability. *Science of the Total Environment* 161:669-675.

Grøsvik, B.E., L. Camus, R.K. Bechmann, I.C. Taban, F. Regoli, J.F. Børseth and B. Gaudebert (*in prep*) Antioxidant defence responses and cell membrane stability in Arctic shrimp *Sclerocrangon boreas* following exposure to dispersed crude oil.

## H

Haber, F. and J. Weiss (1934) The catalytic decomposition of hydrogen peroxide by iron salts. *Proceedings of the Royal Society of London A*(147): 332-351.

Harper, S. L. and C. Reiber (1999) Influence of hypoxia on cardiac functions in the grass shrimp (*Palaemonetes pugio* Holthuis). *Comparative Biochemistry and Physiology Part A* 124:569-573.

Hawkins, A. J. S. (1991) Protein-turnover-a functional appraisal. *Functional Ecology* 5(2): 222-233.

Hazel, J. R. (1995) Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annual Review of Physiology* 57:19-42.

Hebel, D. K., M. B. Jones and M. H. Depledge (1997) Responses of crustaceans to contaminant exposure: A holistic approach. *Estuarine Coastal and Shelf Science* 44(2): 177-184.

Hessen, D. O. (1996) Competitive trade-off strategies in Arctic *Daphnia* linked to melanism and UV-B stress. *Polar Biology* 16(8): 573-579.

Hessen, D. O., J. Borgeraas, K. Kessler and U. H. Refseth (1999) UV-B susceptibility and photoprotection of Arctic *Daphnia* morphotypes. *Polar Research* 18(2): 345-352.

Hine, P. M. (1999) The inter-relationships of bivalve haemocytes. *Fish and Shellfish Immunology* 9(5): 367-385.

Hirche, H. J. (1984) Temperature and metabolism of plankton-I. Respiration of Antarctic zooplankton at different temperatures with comparison of Antarctic and Nordic krill. *Comparative Biochemistry and Physiology* 77A(2): 361-368.

Hjelset, A. M., M. Andersen, I. Gjertz, C. Lydersen and B. Gulliksen (1999) Feeding habits of bearded seals (*Erignathus barbatus*) from the Svalbard area, Norway. *Polar Biology* 21(3): 186-193.

Hochachka, P. W. and N. Somero (1984) *Biochemical Adaptation*. Princeton University Press, New Jersey.

Hofmann, G. E., B. A. Buckley, S. Airaksinen, J. E. Keen and G. N. Somero (2000) Heat-shock protein expression is absent in the Antarctic fish *Trematomus bernachii* (family Nototheniidae). *Journal of Experimental Biology* 203(15): 2331-2339.

Houlihan, D. F. (1991) Protein turnover in ectotherms and its relationships to energetics. In *Advances in Comparative and Environmental Physiology*, Vol. 7 (Edited by R. Gilles), pp. 1-43. Springer, Berlin.

Humphrey, B., P. D. Boehm and M. C. Hamilton (1987) The fate of chemically dispersed and untreated crude oil in Arctic benthic biota. *Arctic* 40 suppl. 1:149-161.

## I

Ingebrigtsen, K., J. S. Christiansen, Ö. Lindhe and I. Brandt (2000) Disposition and cellular binding of 3H-benzo(a)pyrene at subzero temperatures: studies in an agglomerular arctic teleost fish-the polar cod (*Boreogadus saida*). *Polar Biology* 23:503-509.

Ivleva, I. V. (1977) Quantitative correlation of temperature and respiratory rate in poikilothermic animals. *Polskie Archiwum Hydrobiologii* 20:283-300.

Ivleva, I. V. (1980) The dependance of crustacean respiration on body mass and habitat temperature. *International Revue der Gesamten Hydrobiologie und Hydrographie* 65:1-47.

## J

Jimenez, B., M. C. Fossi, M. Nigro and S. Focardi (1999) Biomarker approach to evaluating the impact of scientific stations on the Antarctic environment using *Trematomus bernachii* as a bioindicator organism. *Chemosphere* 39(12): 2073-2078.

Jokumsen, A., R. M. G. Wells, H. D. Ellerton and R. E. Weber (1981) Hemocyanin of the giant Antarctic isopod, *Glyptonotus antarcticus*: structure and effects of temperature and PH on its oxygen affinity. *Comparative Biochemistry and Physiology* 70A:91-95.

Jorgensen, E. H. and J. Wolkers (1999) Effect of temperature on the P4501A response in winter- and summer-acclimated Arctic char (*Salvelinus alpinus*) after oral benzo[a]pyrene exposure. *Canadian Journal of Fisheries and Aquatic Sciences* 56(8): 1370-1375.

## K

Kappus, H. and H. Sies (1981) Toxic drug effects associated with oxygen metabolism, Redox cycling and lipid peroxidation. *Experientia* 37:1233-1241.

Khessiba, A., P. Hoarau, M. Gnassia-Barelli, P. Aissa and M. Romeo (2001) Biochemical response of the mussel *Mytilus galloprovincialis* from Bizerta (Tunisia) to chemical pollutant exposure. *Archives of Environmental Contamination and Toxicology* 40:222-229.

Koizumi, A., M. Tsukada, Y. Wada, H. Masuda and R. Weindruch (1992) Mitotic activity in mice is suppressed by energetic restriction induced torpor. *Journal of Nutrition* 122(7): 1446-1453.

Koszteyn, J., S. Kwasniewski, O. Rozycki and J. M. Weslawski (1991) Atlas of the marine fauna of Southern Spitsbergen. Vol. 2 Invertebrates Part 1. Edited by R. Z. Klekowski and J. M. Weslawski. Polish Academy of Sciences Institute of Ecology, Institute of Oceanology.

Krogh, A. (1916) *Respiratory exchange of animals and Man*. London, Longmans, Green.

Ku, H. H., U. T. Brunk and R. S. Sohal (1993) Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radical Biology and Medicine* 15(6): 621-627.

## L

Lacoste, A., F. Jalabert, S. K. Malham, A. Cueff and S. A. Poulet (2001) Stress and stress-induced neuroendocrine changes increase the susceptibility of juvenile oysters (*Crassostrea gigas*) to *Vibrio splendidus*. *Applied and Environmental Microbiology* 67:2304-2309.

Lahdes, E. O., L. A. Kivivuori and M. Lehti-Koivunen (1993) Thermal tolerance and fluidity of neuronal and branchial membranes of an Antarctic amphipod (*Orchomene plebs*); a comparison with a baltic isopod (*Saduria entomon*). *Comparative Biochemistry and Biology* 105A(3): 463-470.

Lee, M. L., M. V. Novotny and K. D. Bartle (1981) Analytical chemistry of polycyclic aromatic compounds. Academic Press Inc. New York.

Lemaire, P., P. J. Den Besten, S. G. M. O'Hara and D. R. Livingstone (1993) Comparative metabolism of benzo(a)pyrene by microsome of hepatopancreas of teh shore crab *Carcinus maenas* L. and digestive gland of the common mussel *Mytilus edulis*. *Polycyclic Aromatic Compounds* 3:1133-1140.

Littlepage, J. L. (1965) Oceanographic investigations in McMurdo Sound, Antarctic. In *Biology of the Antarctic Seas II. Antarctic Research Series 5* (Edited by Llano G.A.), pp. 1-37. American Geophysical Union, Washington, DC.

Livingstone, D. R. (1991) Towards a specific index of impact by organic pollution for marine-invertebrates. *Comparative Biochemistry and Physiology* 100C(1-2): 151-155.

Livingstone, D. R., P. Lemaire, A. Matthews, L. Peters, D. Bucke and R. J. Law (1993) Prooxidant, antioxidant and 7-ethoxyresorufin o-deethylase (EROD) activity responses in liver of dab (*Limanda limanda*) exposed to sediment contaminated with hydrocarbons and other chemicals. *Marine Pollution Bulletin* 26(11): 602-606.

Livingstone, D. R., F. Lips, P. G. Martinez and R. K. Pipe (1992) Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. *Marine Biology* 112(2): 265-276.

Livingstone, D. R., P. G. Martinez, X. Michel, J. F. Narbonne, S. Ohara, D. Ribera and G. W. Winston (1990) Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* (L.), and other mollusks. *Functional Ecology* 4(3): 415-424.

Livingstone, D. R., P. G. Martinez and G. W. Winston (1989) Menadione-stimulated oxyradical formation in digestive gland microsomes of the common mussel *Mytilus edulis* L. *Aquatic Toxicology* 15:213-36.

Loeng, H. (1991) Features of the physical oceanographic conditions of the Barents Sea. *Polar Research* 10(1): 5-18.

Loo, L. O. (1992) Filtration, assimilation, respiration and growth of *Mytilus edulis* L. at low temperatures. *Ophelia* 35(2): 123-131.

Lowe, D. M. and V. U. Fossato (2000) The influence of environmental contaminants on lysosomal activity in the digestive cells of mussels (*Mytilus galloprovincialis*) from the Venice Lagoon. *Aquatic Toxicology* 48:75-85.

Lowe, D. M., V. U. Fossato and M. H. Depledge (1995) Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis*

from the Venice Lagoon: an *in vitro* study. *Marine Ecology Progress Series* 129:189-196.

Lowe, D. M. and M. N. Moore (1979) The cytochemical distribution of zinc (Zn<sup>2+</sup>) and iron (Fe<sup>3+</sup>) in the common mussel, *Mytilus edulis*, and their relationship with lysosomes. *Journal of Biological Association of the United Kingdom* 59:851-858.

Lowe, D. M., M. N. Moore and B. M. Evans (1992) Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. *Marine Ecology Progress Series* 91:135-140.

Lowe, D. M. and R. K. Pipe (1994) Contaminant-induced lysosomal membrane damage in marine mussel digestive cells: an *in vitro* study. *Aquatic Toxicology* 30:357-365.

Lundebye, A. K., T. M. Curtis, J. Braven and M. H. Depledge (1997) Effects of the organophosphorous pesticide, dimethoate, on cardiac and acetylcholinesterase (AChE) activity in the shore crab *Carcinus maenas*. *Aquatic Toxicology* 40(1): 23-36.

Lundebye, A. K. and M. H. Depledge (1998) Molecular and physiological responses in shore crabs *Carcinus maenas* following exposure to copper. *Marine Environmental Research* 46(1-5): 567-572.

Lundebye, A. K. and M. H. Depledge (1998) Automated interpulse duration assessment (AIDA) in the shore crab *Carcinus maenas* in response to copper exposure. *Marine Biology* 130(4): 613-620.

Lundebye Haldorsen, A. K. (1996) Relationships between molecular and physiological responses to chemical stress. Ph.D. thesis, Odense University, Denmark.

## M

Macdonald, J. (1997) Intrinsic rates of heartbeat in Antarctic fishes: is an icefish different? *Comparative Biochemistry and Physiology* 118A(4): 1051-1066.

Madronich, L. O., M. Bjorn, M. Ilyas and M. M. Caldwell (1991) Changes in biologically active ultraviolet radiation reaching the earth's surface, p. 1-14. In *Environmentla effects of ozone depletion: 1991 update*. UNEP.

Mageau, C., F. R. Engelhardt, E. S. Gilfillan and P. D. Boehm (1987) Effects of short-term exposure to dispersed oil in Arctic invertebrates. *Arctic* 40 suppl. 1:162-171.

Maltby, L. and C. Naylor (1990) Preliminary observations on the ecological relevance of the *Gammarus* scope for growth assay: effect of zinc on reproduction. *Functional Ecology* 4:393-397.

Marchalonis, J. J. and S. F. Schluter (1990) Origins of immunoglobulins and immune recognition molecules. *Bioscience* 40:758-768.

Marsh, J. W., J. K. Chipman and D. R. Livingstone (1992) Activation of xenobiotics to reactive and mutagenic products by the marine invertebrates *Mytilus edulis*, *Carcinus maenas* and *Asterias rubens*. *Aquatic Toxicology* 22(2): 115-128.

McMahon, B. R. (1999) Intrinsic and extrinsic influences on cardiac rhythms in crustaceans. *Comparative Biochemistry and Physiology Part A* 124(4): 539-547.

McMahon, B. R. (1999) Heart-rate: is it a useful measure of cardiac activity performance in crustaceans? In: Schram, Federic R, von Vaupelklein JC, editors. *Crustaceans and the Biodiversity Crisis*. Leiden: Brill 807-822.

McMahon, B. R. and J. L. Wilkens (1977) Periodic respiratory and circulatory performance in the red rock crab *Cancer productus*. *Journal of Experimental Biology* 202:363-374.

McWhinnies, M. A. (1964) Temperature responses and tissue respiration in Antarctic crustaceans with particular reference to the krill *Eupausia superba*. *Antarctic Research Serie* 1:63-72.

Moore, M. N. (1976) Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the comon mussel, *Mytilus edulis*, and changes induced by thermal stress. *Cell and Tissue Research* 175:279-287.

Moore, M. N. (1990) Lysosomal cytochemistry in marine environmental monitoring. *Histochemical Journal* 22:189-191.

Moore, M. N. and R. I. Willows (1998) A model for cellular uptake and intracellular behaviour of particulate-bound micropollutants. *Marine Environmental Research* 46(1-5): 509-514.

Morris, S. and A. C. Taylor (1984) Heart rate response of the intertidal prawn *Palaemon elegans* to simulated and *in situ* environmental changes. *Marine Ecology Progress Series* 20:127-136.

## N

Nadeau, M. and G. Cliche (1998) Predation of juvenile sea scallops (*Placopecten magellanicus*) by crabs (*Cancer irroratus* and *Hyas sp.*) and starfish (*Asterias vulgaris*, *Leptasterias polaris*, and *Crossaster papposus*). *Journal of Shellfish Research* 17(4): 905-910.

Narbonne, J. F., J. E. Djomo, D. Ribera, V. Ferrier and P. Garrigues (1999) Accumulation kinetics of polycyclic aromatic hydrocarbons adsorbed to sediments by the mollusk *Corbicula fluminea*. *Ecotoxicology and Environmental Safety* 42:1-8.

Navarro, J. M. and J. E. Winter (1982) Ingestion rate, assimilation efficiency and energy balance in *Mytilus chilensis* in relation to body size and different algal concentrations. *Marine Biology* 67:255-266.

Neff, J. M. (1979) Biological effects of PAH in the aquatic environment. Applied Science Publishers Ltd, London

Nickell, T. D. and P. G. Moore (1992) The behavioural ecology of epibenthic scavenging invertebrates in the Clyde Sea area-laboratory experiments on attractions to bait in moving water, underwater TV observations in situ and general conclusions. *Journal of Experimental Marine biology and Ecology* 159(1): 15-35.

Nolan, C. P. and A. Clarke (1993) Growth in the bivalve *Yoldia-eightysi* at Signy island, Antarctica determined from internal shell increments and Ca 45 incorporation. *Marine Biology* 117(2): 243-250.

Nonnotte, L., F. Boitel and J. P. Truchot (1993) Waterborne copper causes gill damage and hemolymph hypoxia in the shore crab *Carcinus maenas*. *Canadian Journal of Zoology-Revue Canadienne de Zoologie* 71(8): 1569-1576.

## O

Orbea, A., H. D. Fahimi and M. P. Cajaraville (2000) Immunolocalization of four antioxidant enzymes in digestive glands of mollusks and crustaceans and fish liver. *Histochemistry and Cell Biology* 114(5): 393-404.

Orbea, A., I. Marigomez, C. Fernandez, J. V. Tarazona, I. Cancio and M. P. Cajaraville (1999) Structure of peroxisomes and activity of the marker enzyme catalase in digestive epithelial cells in relation to PAH content of mussels from two Basque estuaries (Bay of Biscay): Seasonal and site-specific variations. *Archives of Environmental Contamination and Toxicology* 36(2): 158-166.

Orr, W. C. and R. S. Sohal (1994) Extension of life span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science* 263(5150): 1128-1130.

## P

Paterson, B. D. and M. J. Thorne (1995) Measurements of oxygen uptake, heart and gill bailer rates of the callinassod burrowing shrimp *Trypaea australiensis* Dana and its responses to low oxygen tensions. *Journal of Experimental Marine and Ecology* 194:39-52.

Payne, J. R., G. D. McNabb and J. R. Clayton (1991) Oil-weathering behavior in Arctic environments. *Polar Research* 10(2): 631-662.

- Peakall, D. B. (1994) the role of biomarkers in environmental assessment.1. Introduction. *Ecotoxicology* 3(3): 157-160.
- Pearl, R. (1928) Experiments on longevity. *Q Review of Biology* 3:391-407.
- Peck, L. S., S. Brockington and T. A. Brey (1997) Growth and metabolism in the Antarctic brachiopod *Liothyrella uva*. *Philosophical Transactions of the Royal Society of London Series B* 352(1355): 851-858.
- Peck, L. S. and L. W. Bullough (1993) Growth and population structure in the infaunal bivalve *Yoldia eightsi* in relation to iceberg activity at Signy island, Antarctica. *Marine Biology* 117(2): 235-241.
- Peck, L. S., A. Clarke and L. J. Holmes (1987) Summer metabolism and seasonal changes in biochemical composition of the Antarctic brachiopod *Liothyrella uva* (Broderip, 1833). *Journal of Experimental Biology and Ecology* 114:85-97.
- Peck, L. S., J. G. Colman and A. W. A. Murray (2000) Growth and tissue mass cycles in the infaunal bivalve *Yoldia eightsi* at Signy Island, Antarctica. *Polar Biology* 23(6): 420-428.
- Percy, J. A. (1975) Ecological physiology of Arctic marine invertebrates. Temperature and salinity relationships of the amphipod *Onisimus affinis* H.J. Hansen. *Journal of Experimental Marine Biology and Ecology* 20:99-117.
- Percy, J. A. (1976) Responses of Arctic marine crustaceans to crude oil and oil-tainted food. *Environmental Pollution* 10:155-162.
- Percy, J. A. (1977) Responses of Arctic marine benthic crustaceans to sediments contaminated with crude oil. *Environmental Pollution* 13:2-10.
- Pickens, P. E. (1965) Heart rate of mussels as a functions of latitude, intertidal height, and acclimation temperature. *Physiological Zoölogy* 38:390-405.
- Pipe, R. K. (1992) Generation of reactive oxygen metabolites by the haemocytes of the mussel *Mytilus edulis*. *Developmental Comparative Immunology* 16:111-122.
- Pipe, R. K., S. R. Farley and J. A. Coles (1997) The separation and characterisation of haemocytes from the mussel *Mytilus edulis*. *Cell Tissue Research* 289:537-545.
- Polhill, J. B. and R. V. Dimock Jr (1996) Effects of temperature and pO<sub>2</sub> on the heart rate of juvenile and adult freshwater mussels (Bivalvia: Unionidae). *Comparative Biochemistry and Physiology* 114A(2): 135-141.
- Porte, C., M. Sole, J. Albaiges and D. R. Livingstone (1991) Responses of mixed function oxygenase and antioxidase enzyme system of *Mytilus sp* to organic pollution. *Comparative Biochemistry and Physiology* C 100(1-2): 183-186.
- Pörtner, H. O., L. Peck, S. Zielinski and L. Z. Conway (1999) Intracellular pH and energy metabolism in the highly stenothermal Antarctic bivalve *Limopsis marionensis* as a function of ambient temperature. *Polar Biology* 22(1): 17-30.

Power, A. and D. Sheehan (1996) Seasonal variation in the antioxidant defence systems of gill and digestive gland of the blue mussel, *Mytilus edulis*. *Comparative Biochemistry and Physiology* 114C(2): 99-103.

Pruitt, N. (1990) Adaptations to temperature in the cellular membranes of crustacea: membrane structure and metabolism. *Journal of Thermal Biology* 15(1): 1-8.

## Q

Qian, J. G., K. Mopper and D. J. Kieber (2001) Photochemical production of the hydroxyl radical in Antarctic waters. *Deep-Sea Research Part I-Oceanographic Research Papers* 48(3): 741-759.

## R

Ralph, R. and J. G. H. Maxwell (1977) The oxygen consumption of the Antarctic lamellibranch *Gaimardia trapesina* in relation to cold adaptation in polar invertebrates. *British Antarctic Survey Bulletin* 45:41-46.

Regoli, F. (1992) Lysosomal responses as a sensitive stress index in biomonitoring heavy metal pollution. *Marine Ecology Progress Series* 84:63-69.

Regoli, F. (1998) Trace metals and antioxidant enzymes in gills and digestive gland of the mediterranean mussel *Mytilus galloprovincialis*. *Archives of Environmental Contamination* 34:48-63.

Regoli, F. (2000) Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. *Aquatic Toxicology* 50(4): 351-361.

Regoli, F., M. Nigro, S. Bompadre and G. W. Winston (2000) Total oxidant scavenging capacity (TOSC) of microsomal and cytosolic fractions from Antarctic, Arctic and Mediterranean scallops: differentiation between three potent oxidants. *Aquatic Toxicology* 49(1-2): 13-25.

Regoli, F. and G. Principato (1995) Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology* 31:143-164.

Regoli, F., G. B. Principato, E. Bertoli, M. Nigro and E. Orlando (1997) Biochemical characterization of the antioxidant system in the scallop *Adamussium*

*colbecki*, a sentinel organism for monitoring the Antarctic environment. vol. 17 received):

Regoli, F. and G. W. Winston (1998) Applications of a new method for measuring the total oxyradical scavenging capacity in marine invertebrates. *Marine Environmental Research* 46(1-5): 439-442.

Reiber, C. L. and B. R. McMahon (1998) The effects of progressive hypoxia on the crustacean cardiovascular system: A comparison of the freshwater crayfish, (*Procambarus clarkii*), and the lobster (*Homarus americanus*). *Journal of Comparative Physiology* 168B(3): 168-176.

Riebel, P. N. and J. A. Percy (1990) Acute toxicity of petroleum-hydrocarbons to the Arctic shallow water mysid, *Mysis oculata* (Fabricius). *Sarsia* 75(3): 223-232.

Roast, S. D., J. Widdows and M. B. Jones (1999) Scope for growth of the estuarine mysid *Neomysis integer* (Peracarida: Mysidacea): effects of the organophosphate pesticide chlorpyrifos. *Marine Ecology Progress Series* 191:233-241.

Robert, L. N. P. (1993) Dictionnaire alphabetique et analogique de la langue francaise. Dictionnaires Le Robert, 1993, Paris, pour le Nouveau Petit Robert, edition entierement revue et amplifiee du petit Robert.

Rode, M., T. Berg and T. Gjoen (1997) Effect of temperature on endocytosis and intracellular transport in the cell line SHK-1 derived from salmon head kidney. *Comparative Biochemistry and Physiology* 117A(4): 531-537.

## S

Sanders, M. B., L. S. Martin, W. G. Nelson, D. K. Phelps and W. Welch (1991) Relationships between accumulation of a 60 kDa stress protein and scope for growth in *Mytilus edulis* exposed to a range of copper concentration. *Marine Environmental Research* 31:81-97.

Sanni, S., K. B. Oysaed, V. Hoivangli and B. Gaudebert (1998) A continuous flow system (CFS) for chronic exposure of aquatic organisms. *Marine Environmental Research* 46(1-5): 97-101.

Schlosser, P., J. Swift, D. Lweis and S. L. Pfirman (1995) The role of the large-scale Arctic Ocean circulation in the transport of contaminants. *Deep-Sea Research II* 42:1337-1367.

Schmid, M. K. (1996) On the distribution and oxygen consumption of ecologically important benthic animals in the waters around Svalbard (Arctic). *Berichte Zur Polarforschung*, Edited by Dr. Franz Rieman, Alfred-Wegner-Institut, Bremerhaven

Schoeberl, M. R. and D. L. Hartmann (1991) The dynamics of the stratospheric polar vortex and its relation to springtime ozone depletions. *Science* 251:46-52.

Scholander, P. F., W. Flagg, V. Walters and L. Irving (1953) Climatic adaptation in Arctic and tropical poikilotherms. *Physiology and Zoology* 26:67-92.

Scott, D. M. and C. W. Major (1972) The effects of copper (II) on survival, respiration, and heart rate in the common blue mussel, *Mytilus edulis*. *Biological Bulletin* 143:679-688.

Scully, N. M., D. J. McQueen, D. R. S. Lean and W. J. Cooper (1996) Hydrogen peroxide formation: The interaction of ultraviolet radiation and dissolved organic carbon in lake waters along a 43-75 degrees N gradient. *Limnology and Oceanography* 41(3): 540-548.

Seglen, P. O. (1983) Inhibitors of lysosomal function. In: Fleischer S, Fleischer B (eds) *Methods in enzymology. Biomembranes, Part I. Membrane biogenesis: assembly and targeting.* 96:737-763.

Shaw, G. E. (1995) The Arctic haze phenomenon. *Bulletin of the American Meteorological Society* 76(12): 2403-2413.

Sheehan, D. and A. Power (1999) Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. *Comparative Biochemistry and Physiology* 123C(3): 193-199.

Shepard, J. L. and B. P. Bradley (2000) Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. *Marine Environmental Research* 50:457-463.

Sinensky, M. (1974) Homeoviscous adaptation. A homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proceeding of the National Academy of Science U.S.A.* 71:522-525.

Siron, R., E. Pelletier, D. Delille and R. S. (1993) Fate and effects of dispersed crude oil under icy conditions simulated in mesocosms. *Marine Environmental Research* 35:273-302.

Siron, R., E. Pelletier and S. Roy (1996) Effects of dispersed and adsorbed crude oil on microalgal and bacterial communities of cold seawater. *Ecotoxicology* 5(4): 229-251.

Sitte, N., K. Merker, T. Grune and T. vonZglinicki (2001) Lipofuscin accumulation in proliferating fibroblasts in vitro: an indicator of oxidative stress. *Experimental Gerontology* 36(3): 475-486.

Sjölin, A. M. and D. R. Livingstone (1997) Redox cycling of aromatic hydrocarbon quinones catalysed by digestive gland microsomes of the common mussel (*Mytilus edulis* L.). *Aquatic Toxicology* 38:83-99.

Skadsheim, A., T. Baussant, R. K. Bechmann, A. Bjørnstad, B. Gaudebert, C. Labes-Carrier, I. C. Jensen and G. Jonsson (2000) Density and size dependent uptake of various PAC in organisms of a model food chain. *Polycyclic Aromatic Compounds* 18:161-175.

Slater, T. F. (1984) Free-radical mechanisms in tissue injury. *Biochemical Journal* 222:1-15.

Smith, R. C., B. B. Prezelin, K. S. Baker, R. R. Bidigare, N. P. Boucher, T. Coley, D. Karentz, S. Macintyre, H. A. Matlick, D. Menzies, M. Ondrusek, Z. Wan and K. J. Waters (1992) Ozone depletion-ultraviolet-radiation and phytoplankton biology in Antarctic waters. *Science* 255(5047): 952-959.

Sohal, S. R. and R. Weindruch (1996) Oxidative stress, caloric restriction and aging. *Science* 273:59-63.

Sole, M. (2000) Assessment of the results of chemical analyses combined with the biological effects of organic pollution on mussels. *Trends in Analytical Chemistry* 19:1-9

Sole, M., L. D. Peters, K. Magnusson, A. Sjölin, Å. Granmo and D. R. Livingstone (1998) Responses of the cytochrome P450-dependent monooxygenase and other protective enzyme systems in digestive gland of transplanted common mussel (*Mytilus edulis* L.) to organic contaminants in the Skagerrak and Kattegat (North Sea). *Biomarkers* 3(1): 49-62.

Sole, M., C. Porte, X. Biosca, C. L. Mitchelmore, J. K. Chipman, D. R. Livingstone and J. Albaiges (1996) Effects of the "Aegean Sea" oil spill on biotransformation enzymes, oxidative stress and DNA-Adducts in digestive gland of the mussel (*Mytilus edulis* L.). *Comparative Biochemistry and Physiology* 113C(2): 257-265.

Stadtman, E. R. (1992) Protein oxidation and aging. *Science* 257(5074): 1220-1224.

Stainken, D. M. (1978) Effects of uptake and discharge of petroleum hydrocarbons on the respiration of the soft-shell clam *Mya arenaria*. *Journal of Fisheries and Research Board of Canada* 35:637-642.

Stange, K. and J. Klungsøyr (1997) Organochlorine contaminants in fish and polycyclic aromatic hydrocarbons in sediments from the Barents Sea. *ICES Journal of Marine Science* 54:318-332.

Steckoll, M. S., L. E. Clement and D. G. Shaw (1980) Sublethal effects of chronic oil exposure on the intertidal clam *Macoma balthica*. *Marine Biology* 57:51-60.

Stegeman, J. J. (1989) Cytochrome P-450 forma in fish: catalytic, immunological and sequence similarities. *Xenobiotica* 19:1093-1110.

Stegeman, J. J., M. Brouwer, T. D. G. Richard, L. Förlin, B. A. Fowler, S. B.M. and P. A. Van Veld (1992) Molecular responses to environmental contamination: Enzyme and protein systems as indicators of chemical exposure and effect. In *Biomarkers: Biochemical, physiological and histological markers of anthropogenic stress* (ed. R.J. Hugget, R.A. Kimerle, P. M. Mehrle Jr and H.L. Bergman), Lewis publishers Inc., Chelsea, MI, USA. pp.235-335.

Stillman, J. H. and G. N. Somero (1996) Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (Genus

*Petrolisthes*): correlation of physiology, biochemistry and morphology with vertical distribution. *The Journal of Experimental Biology* 199:1845-1855.

Styrishave, B., A. Aagaard and O. Andersen (1999) *In situ* studies on physiology and behaviour in two colour forms of the shore crab *Carcinus maenas* in relation to season. *Marine Ecology Progress Series* 189:221-231.

Sundt, H. and A. Goksøyr (1998) *In vivo* and *in vitro* biotransformation of polycyclic aromatic hydrocarbons in the edible crab, *Cancer pagurus*. *Marine Environmental Research* 46(1-5): 515-519.

Sydnes, L. K. (1991) Oil, water, ice and light. *Polar Research* 10(2): 609-618.

Syvertsen, E. E. (1996) Regulation of produced water on the Norwegian continental shelf. In Reed M, Johnsen S, eds, *Produced water 2: Environmental Issues and Mitigation Technologies*. Plenum, New York, NY, USA, pp 7-11.

## T

Taban, I. C., R. K. Bechmann, K. B. Øysæd, G. Jonsson, A. Skadsheim, S. Sanni and B. Gaudebert (*in prep*) Egg production, growth, PAH metabolites in the bile and body burden of PAH for *Cyprinodon variegatus* (sheepshead minnow) chronically exposed to a dispersion of oil.

Tande, K. (1988) The effect of temperature on metabolic rates of different life stages of *Calanus glacialis* in the Barents Sea. *Polar Biology* 8:457-461.

Taylor, A. C. (1976) The respiratory responses of *Carcinus maenas* to declining oxygen tension. *Journal of Experimental Biology* 65:309-322.

Terman, A. and U. T. Brunk (1998) Lipofuscin: Mechanisms of formation and increase with age. *Apmis* 106(2): 265-276.

Tota, B., M. C. Cerra, R. Mazza, D. Pellegrino and J. Icardo (1997) The heart of the Antarctic icefish as paradigm of cold adaptation. *Journal of Thermal Biology* 22(6): 409-417.

## V

Vahl, O. (1973) Pumping and oxygen consumption rates of *Mytilus edulis* L. of different sizes. *Ophelia* 12:45-52.

Vahl, O. (1978) Seasonal changes in oxygen consumption of the Iceland scallop (*Chlamys islandica* (O.F. Muller)) from 70°N. *Ophelia* 17:1-24.

Van Beneden, R. (1997) Environmental effects and aquatic organisms: investigations of molecular mechanisms of carcinogenesis. *Environmental Health Perspectives* 105(3): 669-674.

Van Der Knaap, W. P. W., C. M. Adema and T. Sminia (1993) Invertebrate blood-cells-morphological and functional-aspects of the hemocytes in the pond snail *Lymnaea stagnalis*. *Comparative Haematology International* 3(1): 20-26.

Van't Hoff, T. H. (1884) *Etudes de dynamic chimique*. Muller, Amsterdam, 214pp.

Viarengo, A., D. Abele-Oeschger and B. Burlando (1998) Effects of low temperature on prooxidant processes and antioxidant defence systems in marine organisms. In *Cold Ocean Physiology*. Edited by H.O. Pörtner and R.C. Playle. Cambridge University Press. 213-235.

Viarengo, A., R. Accomando, G. Roma, U. Benatti, G. Damonte and M. Orunesu (1994) Differences in lipid-composition of cell membranes from Antarctic and mediterranean scallops. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular biology* 109(4): 579-584.

Viarengo, A., L. Canesi, M. P.G., L. D. Peters and D. R. Livingstone (1995) Prooxidant processes and antioxidant defence systems in the tissues of the Antarctic scallop (*Adamussium colbecki*) compared with the Mediterranean scallop (*Pecten jacobaeus*). *Comparative Biochemistry and Physiology* 111B(1): 119-126.

Viarengo, A., L. Canesi, M. Pertica and D. R. Livingstone (1991) Seasonal variations in the antioxidant defences systems and lipid-peroxidation of the digestive gland of mussels. *Comparative Biochemistry and Physiology* 100C(1-2): 187-190.

Viarengo, A., M. Pertica, L. Canesi, A. Mazzucotelli, M. Orunesu and J. M. Bouqueneau (1989) Purification and biochemical characterisation of a lysosomal copper rich thionein like protein involved in metal detoxification in the digestive gland of mussels. *Comparative Biochemistry and Physiology* 93C(2): 389-395.

## W

Walters, N. J. and R. F. Uglow (1981) Haemolymph magnesium and relative heart activity of some species of marine decapod crustaceans. *Journal of Experimental Biology and Ecology* 55:255-265.

Warwick, R. M., M. R. Carr, K. R. Clarke, J. M. Gee and R. H. Green (1988) A mesocosm experiment on the effects of hydrocarbon and copper pollution on a sublittoral soft-sediment meiobenthic community. *Marine Ecology Progress Series* 46(1-3): 181-191.

Wayner, D. D. M., G. W. Burton, K. U. Ingold and S. Locke (1985) Quantitative measurement of the total, peroxy radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. *FEBS Letter* 187:33-37.

Wedderburn, J., V. Cheung, S. Bamber, M. Bloxham and M. H. Depledge (1998) Biomarkers of biochemical and cellular stress in *Carcinus maenas*: an in situ field study. *Marine Environmental Research* 46(1-5): 321-324.

Wedderburn, J., I. McFadzen, R. C. Sanger, A. Beesley, C. Heath, M. Hornsby and D. Lowe (2000) The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stage bioassays and adult histopathology. *Marine Pollution Bulletin* 40(3): 257-267.

Wenning, R. J., R. T. DiGiulio and E. P. Gallagher (1988) Oxidant-mediated biochemical effects of paraquat in the ribbed mussel, *Geukensia demissa*. *Aquatic Toxicology* 12:157-170.

Wernick, A. M. (1982) The oxygen consumption of the hermit crab *Clibanarius vittatus* (BOSEC) (Decapoda, Diogenidae) in relation to temperature and size. *Revista Brasileira de Biologia* 42(2): 267-273.

Weslawski, J. M., M. Zajaczkowski, S. Kwasniewski, J. Jezierski and W. Moskal (1988) Seasonality in an Arctic fjord ecosystem: Hornsund, Spitsbergen. *Polar Research* 6:185-189.

White, M. G. (1975) Oxygen consumption and nitrogen excretion by the giant Antarctic Isopod *Glyptonotus antarcticus* Eights in relation to cold-adapted metabolism in marine polar poikilotherms. Proceeding of the 9th European Marine Biology Symposium. Harold Barnes, Editor. Aberdeen University Press 707-724.

Whiteley, N. M., E. W. Taylor and A. J. ElHaj (1996) A comparison of the metabolic cost of protein synthesis in stenothermal and eurythermal isopod crustaceans. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* 40(5): R1295-R1303.

Whiteley, N. M., E. W. Taylor and A. J. ElHaj (1997) Seasonal and latitudinal adaptation to temperature in crustaceans. *Journal of thermal biology* 22(6): 419-427.

Widdows, J. (1973) Effect of temperature and food on the heart beat, ventilation rate and oxygen uptake of *Mytilus edulis*. *Marine Biology* 20:269-276.

Widdows, J., T. Bakke, B. L. Bayne, P. Donkin, D. R. Livingstone, D. M. Lowe, M. N. Moore, S. V. Evans and S. L. Moore (1982) Responses of *Mytilus edulis* to exposure to WAF of North Sea oil. *Marine Biology* 67:15-31.

Widdows, J., P. Donkin, M. D. Brinsley, S. V. Evans, P. N. Salkeld, A. Franklin, R. J. Law and M. J. Waldock (1995) Scope for growth and contaminated levels in North Sea mussels *Mytilus edulis*. *Marine Ecology Progress Series* 127:131-148.

Widdows, J., P. Donkin and S. V. Evans (1987) Physiological responses of *Mytilus edulis* during chronic oil exposure and recovery. *Marine Environmental Research* 23:15-33.

Widdows, J. and D. S. Page (1993) Effects of tributyltin and dibutyltin on the physiological energetics of the mussel, *Mytilus edulis*. *Marine Environmental Research* 35:233-249.

Williams, E. E. and G. N. Somero (1996) Seasonal-, tidal-cycle- and microhabitat-related variation in membrane order of phospholipid vesicles from gills of the intertidal mussel *Mytilus californianus*. *Journal of Experimental Biology* 199(7): 1587-1596.

Winston, G. W. and R. T. Di Giulio (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology* 19:137-191.

Winston, G. W., M. N. Moore, M. A. Kirchin and C. Soverchia (1996) Production of reactive oxygen species by hemocytes from the marine mussel, *Mytilus edulis*: Lysosomal localization and effect of xenobiotics. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology and Endocrinology* 113(2): 221-229.

Winston, G. W., F. Regoli, A. J. Dugas, J. H. Fong and K. A. Blanchard (1998) A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. *Free Radical Biology and Medicine* 24(3): 480-493.

Winzer, K., G. W. Winston, W. Becker, C. J. F. Van Noorden and A. Köhler (2001) Sex-related responses to oxidative stress in primary cultured hepatocytes of European flounder (*Platichthys flesus* L.). *Aquatic Toxicology* 52:143-155.

Wohlschlag, D. E. (1964) Respiratory metabolism and ecological characteristic of some fishes in McMurdo Sound, Antarctica. *Antarctic Research Serie American Geophysical Union* 1:33-62.

Wolkers, J., E. H. Jorgensen, S. M. Nijmeijer and R. F. Witkamp (1996) Time-dependent induction of two distinct hepatic cytochrome P4501A catalytic activities at low temperatures in Arctic charr (*Salvelinus alpinus*) after oral exposure to benzo(a)pyrene. *Aquatic Toxicology* 35(2): 127-138.

Wolkers, J., E. H. Jorgensen, S. M. Nijmeijer and R. F. Witkamp (1998) Dose and time dependency of cytochrome P4501A induction in liver and kidney of B(a)P exposed Arctic charr (*Salvelinus alpinus*). *Marine Environmental Research* 46(1-5): 117-120.

Wootton, A. N., P. S. Goldfarb, P. Lemaire, S. C. M. OHara and D. R. Livingstone (1996) Characterization of the presence and seasonal variation of a CYP1A-like enzyme in digestive gland of the common mussel, *Mytilus edulis*. *Marine Environmental Research* 42(1-4): 297-301.

Wootton, A. N., C. Herring, J. A. Spry, A. Wiseman, D. R. Livingstone and P. S. Goldfarb (1995) Evidence for the existence of cytochrome-P450 gene families (CYP1A, 3A, 4A, 11A) and modulation of gene-expression (CYP1A) in the mussel *Mytilus spp.* *Marine Environmental Research* 39(1-4): 21-26.

## Y

**Yocis, B. H., D. J. Kieber and K. Mopper (2000) Photochemical production of hydrogen peroxide in Antarctic Waters. Deep-Sea Research Part I- Oceanographic Research Papers 47(6): 1077-1099.**

**Yunker, M. B., L. R. Snowdon, R. W. Macdonald, J. N. Smith, M. G. Fowler, D. N. Skibo, F. A. Mclaughlin, A. I. Danyushevskaya, V. I. Petrova and G. I. Ivanov (1996) Polycyclic aromatic hydrocarbon composition and potential sources for sediment samples from the Beaufort and Barents Seas. Environmental Science and Technology 30:1310-1320.**

## **Published Article**

### **Paper I**

**Published in Marine Environmental Research**

## Stability of lysosomal and cell membranes in haemocytes of the common mussel (*Mytilus edulis*): effect of low temperatures

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### Abstract

Expanding industrial activities in the Arctic require an urgent assessment of the toxicity of chemicals at low temperatures. Organisms acclimatized to low temperature exhibit specific adaptations. For example, the amount of unsaturated lipids is increased to maintain the fluidity of the cell membranes. It has been hypothesized that such temperature-induced alterations in membrane lipid composition may affect the stability of lysosomal and cell membranes in the common mussel, *Mytilus edulis*, an organism exposed to seasonal temperature extremes. As mussels may be exposed to petroleum compounds along industrialized coastlines, we tested the combined effects of exposure to low temperature and the petroleum compound, phenanthrene, on haemocyte membrane stability. Test animals, acclimated to either 0 or 10°C, were exposed to phenanthrene (0 = control or 500 µg l<sup>-1</sup>) and haemocytes were examined using the neutral red retention assay (lysosomal stability) and a fluorescence assay (cell membrane stability). At 0°C, lysosomal and cell membranes from uncontaminated mussels were destabilized compared with 10°C ( $P = 0.0005$ ). No significant effects ( $P > 0.05$ ) of phenanthrene were detected at either temperature. Possible mechanisms underlying membrane destabilization include a weaker physical resistance of the membrane due to a higher amount of unsaturated lipids, a potentially higher level of reactive oxygen radicals at low temperature and the higher susceptibility of unsaturated lipids to oxidative stress. More work is required to better understand the consequences of this membrane destabilization at low temperature on the susceptibility of the organism to pollutants. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** Membrane stability; Low temperature; Lysosomes; Haemocytes; *Mytilus edulis*

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## 1. Introduction

As oil and gas development in the European Arctic regions gains momentum, reliable environmental monitoring tools are required to identify the potential impact of such disturbance on organisms inhabiting these regions (AMAP, 1997). One approach currently being applied is the use of biomarkers [defined as “biological responses to a chemical or chemicals that give a measure of exposure or toxic effect” (Peakall, 1994)]. Biomarkers may also provide an early warning of detrimental health effects in biota and humans. Although biomarkers have been employed in environmental toxicology for the last 20 years, most of the research with marine ecosystems has been performed in temperate regions at temperature ranging from 10 to 15°C. Little is known about the potential impact of chemicals on Arctic marine organisms living in seawater at temperatures of ca. –2°C (and seldom rising above 3–4°C). Some studies have indicated that low temperature appears to enhance the effect of environmental pollutants (e.g. Aarset & Zachariassen, 1983) while some others showed that Arctic invertebrates could be more resistant due to their epontic behaviour (e.g. Chapman & McPherson, 1993). At low temperatures, cell membranes undergo structural changes to maintain viscosity to keep enzyme kinetic properties (Hochachka & Somero, 1984). Lowe, Fossato and Depledge (1995) showed that the lysosomal membrane stability in the haemocytes of blue mussels (*Mytilus galloprovincialis*) was compromised following pollutant exposure. Therefore, it has been hypothesized that low temperature may affect the stability of lysosomal and cell membrane in haemocytes of the common mussel (*Mytilus edulis*) as well. Blue mussels are exposed regularly to low temperatures during the winter in Norwegian fjords. In the present study, the effects of low temperature on cell membrane stability and lysosomal membrane stability were investigated in haemocytes of *M. edulis*. In addition, the effects of exposure to a poly-aromatic hydrocarbon (PAH; phenanthrene) were investigated at low temperature.

## 2. Materials and methods

In October 1998, mussels (*M. edulis*) were collected at a clean reference site in Førlandsfjorden, Rogaland (Norway). The ambient temperature was around 10°C. They were kept in running seawater and fed with a regular diet of algae (*Isochrysis* sp.). For use in low temperature experiments, mussels were acclimated at 0°C for 1.5 months. Experiments were carried out simultaneously at 10 and 0°C using 15 mussels per treatment and 500 µg l<sup>-1</sup> phenanthrene as the chemical exposure was used. Each group of mussels was placed in a tank containing 4.5 l of aerated seawater. The toxicant (diluted in acetone) was added first to 100 ml of algae solution and then transferred to the tank. The exposure medium was renewed every 24 h. After 4 days, the haemolymph from individuals mussels was sampled and mixed (1:1) with physiological saline.

Two assays were carried out on the haemocytes. Firstly, the retention of neutral red dye was measured in lysosomes following the procedure of Lowe et al. (1995).

When 50% of the haemocytes showed signs of lysosomal leaking (the cytosol becoming red and the cells rounded), the time was noted and examination of the cells was concluded. The second assay measured the membrane stability of haemocytes isolated from the various treatment groups. The method consisted of incubating haemocytes with the probes BODIPY® FL verapamil (BFLV) and ethidium homodimer-1 (EthD-1), and measuring the fluorescence ratio of the two probes. Healthy granulated haemocytes take up BFLV efficiently into lysosomes while uptake is decreased in haemocytes of exposed mussels. EthD-1 is taken up gradually over time and the fluorescence increases upon binding to nucleic acids. Cells with destabilized membranes take up EthD-1 to a greater extent while cells with a compromised membrane show a decreased ratio compared with cells from untreated mussel.

Non-parametric Wilcoxon test was performed on retention time measurements. Data of haemocyte membrane stability were normally distributed but were Boxcox-transformed to meet equal variance conditions and analysed with one-way analysis of variance (ANOVA) test.

### 3. Results

Results showed that the neutral red retention time (Fig. 1) and the ratio BFLV/EthD-1 (Fig. 2) were both significantly reduced in the control groups at 0°C compared to 10°C ( $P < 0.05$ ). These results indicate that the membranes of lysosomes and haemocytes were destabilized at low temperature. No significant effects of phenanthrene could be detected by either method at 0 or 10°C ( $P > 0.05$ ) (Figs. 1 and 2).

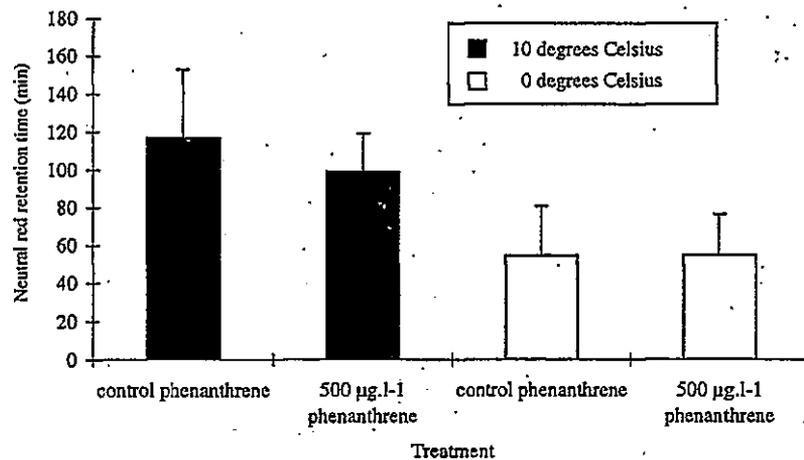


Fig. 1. Bar chart to show the lysosomal membrane stability (neutral red retention time) in haemocytes of *Mytilus edulis* exposed to 500 µg l<sup>-1</sup> phenanthrene (mean and standard error,  $n = 15$ ). Asterisks indicate significant effect of low temperature on the stability of the membrane compared with 10°C ( $P = 0.0005$ ).

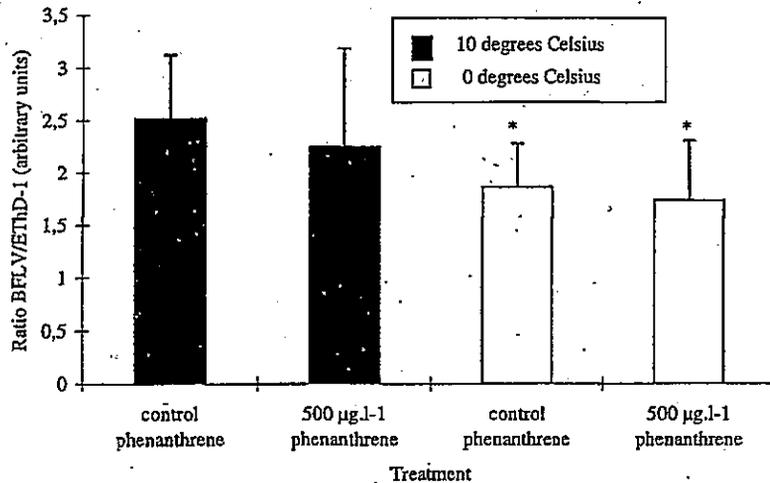


Fig. 2. Bar chart to show the cell membrane stability [ratio of fluorescence intensity of BODIPY® FL verapamil (BFLV) and ethidium homodimer-1 (EthD-1) (arbitrary unit)] of haemocytes of *Mytilus edulis* exposed to phenanthrene (mean and standard error,  $n=15$ ). Asterisks indicate significant effect of low temperature on the stability of the membrane compared with 10°C ( $P < 0.05$ ).

#### 4. Discussion

Present results indicate that lysosomal and cell membranes of haemocytes of *M. edulis* are more fragile at 0 than at 10°C. Organisms adapted to low temperature contain a higher level of unsaturated lipids to maintain fluidity of the membrane components (Hochachka & Somero, 1984). This metabolic compensation may help explain the more destabilized membrane at low temperature. In addition, at low temperatures higher dissolved oxygen concentrations occur, providing a major source of reactive oxygen species (ROS) (Regoli, Nigro, Bompadre & Winston, 1999). Organisms exposed to low temperatures, therefore, experience more oxidative stress than animals living at high temperatures. ROS are known to be generated in lysosomes (Winston, Moore, Kirchin & Soverchia, 1996) and cause membrane lipid peroxidation. Since unsaturated lipids are more vulnerable to ROS than saturated lipids, it might be expected that membranes would be more susceptible to peroxidation at 0 than at 10°C. In *M. edulis*, the metabolisation of PAH leads to the production of oxyradicals (Livingstone et al., 1990). Grundy, Moore, Howell and Ratcliffe (1996) showed that the neutral red retention time was strongly reduced in lysosomes of mussels exposed to phenanthrene for 14 days at 20°C. No effects of phenanthrene on membrane destabilization were recorded in our experiment. Thus, either our exposure time to this compound was too short or the bioavailability of phenanthrene was reduced at 10 and 0°C. Additionally, the metabolic activity of the mussels could be reduced at the low temperature. To isolate the mechanism, it is important to compare the levels of bioaccumulated chemical within the tissue at low and higher temperatures (study in progress, data not shown). Antioxidant defences may contribute to scavenge ROS efficiently as a more elevated capacity to cope with

oxyradicals in organisms acclimatized at low temperature was reported by Regoli et al. (1999).

The similar results produced by each method demonstrated that: (1) the technical procedure of neutral red retention time was valid; and (2) assessing the cell membrane stability of haemocytes using BVFL and EthD-1 is a reliable method providing information with automated readings.

In conclusion, mussels acclimatized to 0°C exhibited decreased stability of lysosomal and cell membranes in haemocytes compared with 10°C. Phenanthrene exposure did not decrease further membrane stability at either temperature.

### Acknowledgements

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### References

- Aarset, A. V., & Zachariassen, K. E. (1983). Synergistic effects of an oil dispersant and low temperature on the freezing tolerance and solute concentrations of the blue mussels (*Mytilus edulis* L.). *Polar Research*, 1 n.s., 223–229.
- AMAP, (1997). Arctic Pollution Issues: A State of the Arctic Environment Report. Arctic Monitoring and Assessment Programme, Oslo.
- Chapman, P., & McPherson, C. (1993). Comparative zinc and lead toxicity tests with Arctic marine invertebrates and implications for toxicant discharges. *Polar Record*, 29(168), 45–54.
- Grundy, M. M., Moore, M. N., Howell, S. M., & Ratcliffe, N. A. (1996). Phagocytic reduction and effects of lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. *Aquatic Toxicology*, 34, 273–290.
- Hochachka, P. W., & Somero, N. (1984). Temperature adaptation from biochemical adaptation. New Jersey: Princetown University Press.
- Livingstone, D., Garcia, R., Martinez, P., Michel, X., Narbonne, J. F., O'hara, S., Ribera, D., & Winston, G. W. (1990). Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other molluscs. *Functional Ecology*, 4, 415–424.
- Lowe, D. M., Fossato, V. U., & Depledge, M. H. (1995). Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. *Marine Ecology Progress Series*, 129, 189–196.
- Peakall, D. B. (1994). The role of biomarkers in environmental assessment (1). Introduction. *Ecotoxicology*, 3, 157–160.
- Regoli, F., Nigro, M., Bompadre, S., & Winston, G. (2000). Total oxidant scavenging capacity (TOSC) of microsomal and cytosolic fractions from Antarctic, Arctic and Mediterranean scallops: differentiation between three potent oxidants. *Aquatic Toxicology*, 49(1–2), 13–25.
- Winston, G. W., Moore, M. N., Kirchin, M. A., & Soverchia, C. (1996). Production of reactive oxygen species by haemocytes from the marine mussel, *Mytilus edulis*: Lysosomal localization and effect of xenobiotics. *Comparative Biochemistry and Physiology*, 113C(2), 221–229.

## **Paper II**

*In press in Marine Environmental Research*



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## Total oxyradical scavenging capacity and cell membrane stability of haemocytes of the Arctic scallop, *Chlamys islandicus*, following benzo(a)pyrene exposure

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### Abstract

Industrial activities, notably oil and gas industries, are expanding in the Arctic. Most of the biomarkers were developed using temperate organisms living at temperatures above 10 °C. Little is known about the biomarker responses of organisms living between -1.88 and 5 °C. Therefore, assessment of the toxicity of chemicals to cold-water adapted species is required. In this study, the Arctic scallop, *Chlamys islandicus*, was selected as a key species for bio-monitoring because of wide distribution in Arctic waters and its commercial value. Test animals, stored in seawater at 2 °C, were injected with benzo(a)pyrene (diluted in cod liver oil 5 mg ml<sup>-1</sup>) in the adductor muscle every 24 h for four days giving a final dose of 0.74 and 90.6 mg kg<sup>-1</sup> wet weight for control, low and high dose, respectively. The biomarkers used were total oxyradical scavenging capacity (TOSC) in the digestive gland and cell membrane stability of haemocytes. TOSC values were significantly reduced (ca. 30%) in exposed groups ( $P < 0.05$ ), indicating a depletion in oxyradical molecular scavengers. The antioxidant defences appeared to be overwhelmed by the reactive oxygen species as the plasma membranes of haemocytes were destabilised ( $P < 0.05$ ) probably due to lipid peroxidation. These data indicate that reactive oxygen species (ROS) were produced by Arctic scallops via the metabolism of benzo(a)pyrene at 2 °C. © 2002 Published by Elsevier Science Ltd.

Keywords: TOSC; Cell membrane; Benzo(a)pyrene; *Chlamys islandicus*; Arctic

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## 1. Introduction

The growing interest of oil and gas industries in the European Arctic has raised concerns of the potential impact of chemicals at low temperature. For the past 20 years, biomarkers [defined as “biological responses to a chemical or chemicals that give a measure of exposure or toxic effect” (Peakall, 1994)] have been developed in temperate species to provide early-warning signals of detrimental impacts on the marine ecosystem. Few toxicity studies in the Arctic have been performed, raising the need to investigate chronic biological effects of chemicals on endemic organisms (Chapman, 1993). Laboratory studies are needed to investigate the link between cause and effects of poly aromatic hydrocarbons (PAH) on the Arctic fauna. For instance, PAH, notably benzo(a)pyrene, is known to enhance the production of reactive oxygen species (ROS) (Livingstone et al., 1990) and to cause lysosomal membrane disruption in bivalves (Grundy, Moore, Howell, & Ratcliff, 1996). Polar bivalves possess an elevated total oxyradical scavenging capacity (TOSC) (Regoli, Nigro, Bompadre, & Winston, 2000) which may make them less susceptible to ROS. At the cellular level, adaptation to low temperature is associated with modified composition of the membrane lipid (Viarengo et al., 1994) which may render the membrane more susceptible to oxidative stress due to the higher level of unsaturated fatty acids. Thus, the capacity of Arctic bivalves to metabolise PAH, and their vulnerability to ROS, has to be established. In this study, responses of the total oxyradical scavenging capacity (Winston, Regoli, Dugas, Fong, & Blanchard, 1998) and cell membrane stability of haemocytes were measured in the Arctic scallop, *Chlamys islandicus*, exposed to benzo(a)pyrene.

## 2. Materials and methods

In September 1999, *Chlamys islandicus* ( $7.42 \pm 0.57$  cm shell height,  $n=30$ ) was collected with an Agassiz dredge from Isfjorden at Svalbard ( $78^{\circ}13'N$ ,  $15^{\circ}39'E$ ). After one day of acclimatization in  $2^{\circ}C$  aerated seawater with no sediment, three groups, each of 10 individuals, were established as control, low and high dose of B(a)P. The chemical [ $5 \mu g$  of B(a)P diluted in 1 ml of cod liver oil] was injected into the adductor muscle once a day for four days giving a final dose of 0, 74 and 90.6  $ng\ kg^{-1}$  wet weight; no feeding was allowed and water was renewed every day.

After 4 days, haemolymph was sampled from the adductor muscle of the 10 individuals from each group and mixed (1:1) with physiological saline. Cell suspension (200  $\mu l$ ) were added per well of a dark microplate and incubated with 50  $\mu l$  of Ethidium homodimer-1 (EthD-1) for 5 min at room temperature. Fluorescence was read at ex544/em612. EthD-1 is taken up by haemocytes with destabilized membranes and the fluorescence increases upon binding to nucleic acids (Camus, Grøsvik, Børseth, & Depledge, 2000). At the same time as the blood was sampled, the hepatopancreas of seven individuals per group was excised, frozen in liquid nitrogen and stored at  $-80^{\circ}C$ . The cytosolic fraction was incubated with 20 mM 2,2'-azo-bis-amidinopropane (ABAP, that generates peroxy radicals at  $35^{\circ}C$ ) and 0.2 mM

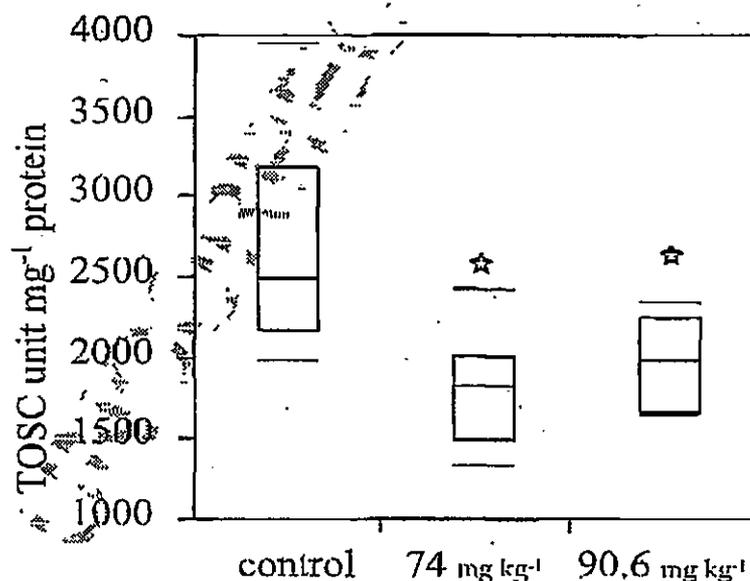
1  $\alpha$ -keto- $\gamma$ -methiolbutyric acid (KMBA, that produces ethylene when oxidised) (Win-  
 2 ston et al., 1998). In the presence of antioxidants, ethylene production is reduced  
 3 quantitatively and may be monitored by gas chromatography.

4 Statistical analyses were made using JMP v3.2.6., SAS Institute, Inc., Cary, NC,  
 5 USA. Normal distribution and homogeneity of variances was established before  
 6 statistical treatment. Dunnett's test was performed for both sets of data. Data are  
 7 plotted as Box and Whisker plots. The significance level was  $P < 0.05$ .

### 8 9 10 3. Results

11  
12 A significant decrease in TOSC value was measured in the low (33%) and high-  
 13 dose (26%) benzo(a)pyrene exposed group ( $P < 0.05$ , Fig. 1). No significant differ-  
 14 ence was noted between treated groups. A TOSC depression is taken to indicate  
 15 depletion of oxyradical scavengers. The mean TOSC value of the control group was  
 16  $2683 \pm 703$  unit  $\text{mg}^{-1}$  protein.

17 A significant increase in uptake of EthD-1 into the haemocytes of *Chlamys islandicus*  
 18 was measured in the low-dose treated group ( $P < 0.05$ , Fig. 2). No significant  
 19 increase was measured in the high dose ( $P > 0.05$ ). Thus, the plasma membranes of  
 20 haemocytes of *C. islandicus* were destabilised following exposure to B(a)P.



44 Fig. 1. Total oxyradical scavenging capacity of *Chlamys islandicus* following exposure for 4 days to two  
 45 concentrations of B(a)P. Significant effects are indicated by a star ( $P < 0.05$ ).

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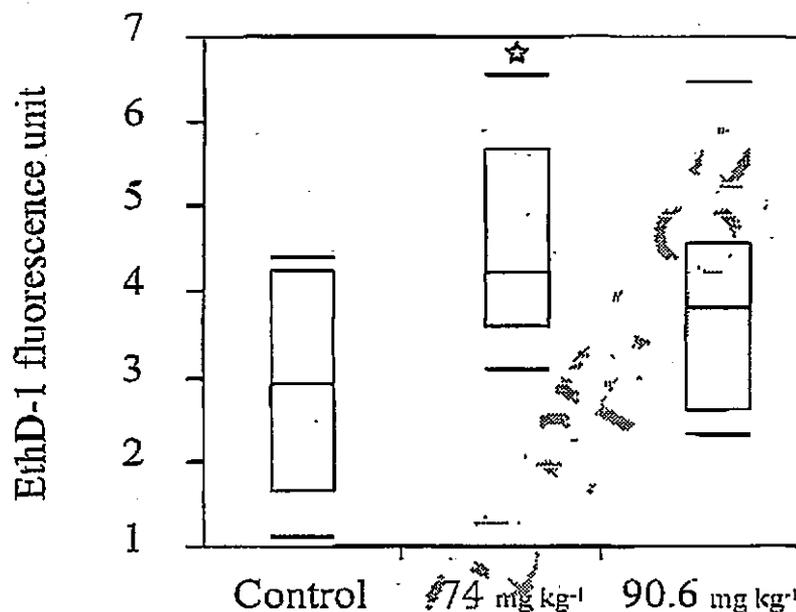


Fig. 2. Stability of plasma membrane of haemocytes of *Chloris islandicus* measured as fluorescence unit of Ethidium homodimer-1 (EthD-1) following 4 days' exposure to two concentrations of B(a)P. Significant effect is indicated by a star ( $P < 0.05$ ).

#### 4. Discussion

The TOSC assay measures the balance between antioxidant parameters and prooxidant factors (Winston et al., 1998). The significant decrease in TOSC value reported in the present study indicates, therefore, that antioxidant defences were depleted (i.e. glutathione) due to ROS production. This depletion could indicate enhanced ROS production caused by the metabolism of B(a)P in bivalves (Livingstone et al., 1990). The capability of *C. islandicus* to metabolise PAH and to produce ROS in an environment characterised by low temperature and low food supply is expected to be limited, as indicated by a low respiration rate of  $63.7 \mu\text{g O}_2 \text{ g dw}^{-1} \text{ h}^{-1}$  ( $0.5^\circ\text{C}$ ; Camus et al., in preparation). One possible consequence of this ROS production and depressed antioxidant defences is reduced protection of the cells against the damaging effect of ROS. This is confirmed by the decreased plasma membrane stability of haemocytes measured in this study and demonstrated by others (Regoli et al., 2000; Viarengo et al., 1989; Winston, Moore, Kirchin, & Soverchia, 1996). B(a)P could have had a different effect on the stability of the membranes by binding on lipophilic molecules thus compromising the basic functions (fluidity, ionic pumps). An indirect effect could have occurred by altering the lysosomal membrane due to accumulation of B(a)P in these organelles, thereby, causing the loss of hydrolytic enzymes into the cytosol (Camus et al., 2000; Grundy et al. 1996), further impairing cell membrane stability. Impaired function of the

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1 plasma haemocyte membrane has consequences for the cellular immunity of bivalves  
2 by reducing the phagocytosis ability of the haemocytes (Dyrynda, Pipe, Burt, &  
3 Ratcliff, 1998); this remains to be investigated in *C. islandicus*. The reduction of the  
4 antioxidant cellular defences could also lead to protein and enzyme inactivation,  
5 damage to DNA and, ultimately, carcinogenesis (Winston & Di Giulio, 1991). The  
6 lack of dose response may be due to the toxic effect of the high dose causing enzyme  
7 inhibition or reduction in the metabolism.

8 The present study demonstrated a link between the effects of benzo(a)pyrene on  
9 the redox status of an organism and the susceptibility to oxidative stress of cell  
10 membranes. The TOSC assay, and the plasma membrane stability of haemocytes in  
11 *C. islandicus*, appear to provide potential for biomonitoring the impact of industrial  
12 activities in the Arctic marine ecosystem.

#### 15 Uncited reference

17 Regoli (2000)

#### 20 Acknowledgements

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26 ERBFMBICT983185 and by TotalFinaElf's "Cold Water" project.

#### 29 References

- 31 Camus, L., Grøsvik, B. B., Børseth, J. F., & Depledge, M. H. (2000). Stability of lysosomal and cell  
32 membranes in haemocytes of the common mussel (*Mytilus edulis*): effect of low temperatures. *Marine  
33 Environmental Research*, 50, 325-329.
- 34 Camus, L., Regoli, F., Børseth, J. F., Jones, M., & Depledge, M. H. (in preparation) Comparison of the  
35 variation of the total oxidant scavenging capacity and metabolic rate of the Arctic clam *Mya truncata*  
36 with Antarctic and temperate species in relation with food availability.
- 37 Chapman, P. M. (1993). Are Arctic marine invertebrates relatively insensitive to metals? *Environmental  
38 Toxicology and Chemistry*, 12(4), 611-613.
- 39 Dyrynda, E. A., Pipe, R. E., Burt, G. R., & Ratcliff, N. A. (1998). Modulations in the immune defences  
40 of mussels (*Mytilus edulis*) from contaminated sites in the UK. *Aquatic Toxicology*, 42(3), 169-185.
- 41 Grundy, M. M., Moore, M. N., Howell, S. M., & Ratcliff, N. A. (1996). Phagocytic reduction and effects  
42 on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*.  
43 *Aquatic Toxicology*, 34(4), 273-290.
- 44 Livingstone, D. R., Martinez, P. G., Michel, X., Narbonne, J. F., Ohara, S., Ribera, D., & Winston, G. W.  
45 (1990). Oxylradical production as a pollution-mediated mechanism of toxicity in the common mussel,  
*Mytilus edulis* (L.), and other mollusks. *Functional Ecology*, 4(3), 415-424.
- 46 Peakall, D. B. (1994). The role of biomarkers in environmental assessment. 1. Introduction. *Ecotoxicol-  
47 ogy*, 3(3), 157-160.

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L. Camus et al. / Marine Environmental Research □ (□□□□) □-□

- 1 Regoli, F. (2000). Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a  
2 predictive biomarker of oxidative stress. *Aquatic Toxicology*, 50(4), 351-361.
- 3 Regoli, F., Nigro, M., Bompadre, S., & Winston, G. W. (2000). Total oxidant scavenging capacity  
4 (TOSC) of microsomal and cytosolic fractions from Antarctic, Arctic and Mediterranean scallops: dif-  
5 ferentiation between three potent oxidants. *Aquatic Toxicology*, 49(1-2), 13-25.
- 6 Viarengo, A., Accomando, R., Roma, G., Benatti, U., Damone, G., & Orunesu, M. (1994). Differences  
7 in lipid-composition of cell membranes from Antarctic and mediterranean scallops. *Comparative Bio-  
8 chemistry and Physiology B-Biochemistry and Molecular biology*, 109(4), 579-584.
- 9 Viarengo, A., Perlica, M., Canesi, L., Mazzacotelli, A., Orunesu, M., & Bouqueneau, J. M. (1989). Pur-  
10 ification and biochemical characterisation of a lysosomal copper rich thionein like protein involved in metal  
11 detoxification in the digestive gland of mussels. *Comparative Biochemistry and Physiology C-Pharmacology  
12 Toxicology and Endocrinol.*, 93(2), 389-395.
- 13 Winston, G. W., & Di Giulio, R. T. (1991). Prooxidant and antioxidant mechanisms in aquatic organ-  
14 isms. *Aquatic Toxicology*, 19, 137-191.
- 15 Winston, G. W., Moore, M. N., Kirchin, M. A., & Soverchia, C. (1996). Production of reactive oxygen  
16 species by hemocytes from the marine mussel, *Mytilus edulis*: Lysosomal localization and effect of  
17 xenobiotics. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology and Endocrinology*,  
18 113(2), 221-229.
- 19 Winston, G. W., Regoli, F., Dugas, A. J., Hong, J. H., & Blanchard, A. (1998). A rapid gas chroma-  
20 tographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids.  
21 *Free Radical Biology and Medicine*, 24(3), 480-493.
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**Paper III**

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## Heart rate, respiration and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to polycyclic aromatic compounds via sediment and injection

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### Abstract

Increasing industrial activity in the European Arctic has raised concerns of the potential anthropogenic impact of chemicals on this polar marine ecosystem. For the past 20 years or so, biomarkers have been developed to provide early-warning signals of detrimental impacts of chemicals on the marine ecosystem, however, most biomarker methods have been established for organisms living in temperate rather than polar waters. Little is known about biomarker responses in organisms living within the temperature range of  $-1.88$  to  $+5$  °C. In this study, established biomarkers from temperate studies were tested on the Arctic spider crab *Hyas araneus* to validate their use in polar ecosystems. *H. araneus* is common in Svalbard fjord (Norway), although it is a temperate water species occurring from northern Spain to Svalbard at depths from 10 to 1200 m. In this paper, the effects of oil were investigated at 2 °C via two routes: (i) injection and (ii) contaminated sediment. After 2 weeks of exposure, heart rate, oxygen consumption and total oxyradical scavenging capacity (TOSC) were measured in the same individuals. In both methods of contaminant exposure, heart rate showed a significant increase compared with the control ( $P < 0.0001$ ,  $n = 7$ ); mean heart rate values ( $\pm$  S.D.) of *H. araneus* were 49.06 ( $\pm 13.72$ ), 57.56 ( $\pm 7.28$ ) and 63.30 ( $\pm 6.57$ ) beats per minute in control, injected and sediment-treated groups, respectively. Respiration of *H. araneus* was not affected significantly by either oil treatment ( $P > 0.05$ ), but two individuals ( $n = 8$ ) showed a marked increase in oxygen uptake in the sediment-exposed group. The basal oxygen consumption of control *H. araneus* was lower ( $0.025$  mg O<sub>2</sub> g wet wt.<sup>-1</sup> h<sup>-1</sup>) than reported for *H. araneus* living in temperate water. Although TOSC of *H. araneus* was not affected significantly by either exposure treatment ( $P > 0.05$ ) the mean TOSC value in the sediment-exposed group was lower than the control, indicating some saturation of the oxyradical scavenging system. Results indicate that although low

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temperature appears to be the main factor reducing the bioavailability of polycyclic-aromatic hydrocarbons, the relatively low metabolic rate of Arctic *H. araneus* is also implicated in decreased uptake and metabolism of oil compounds into reactive oxygen species (ROS). © 2002 Published by Elsevier Science B.V.

**Keywords:** Arctic; Biomarkers; *Hyas araneus*; Heart rate; Respiration; TOSC; Polycyclic aromatic hydrocarbons

## 1. Introduction

Increasing industrial activity, notably oil and gas exploration, in the European Arctic requires better assessment methods for the potential anthropogenic impact of chemicals on this polar marine ecosystem (AMAP, 1997). For the past 20 years or so, biomarkers (defined as 'biological responses to a chemical or chemicals that give a measure of exposure or toxic effect', Peakall, 1994) have been developed in temperate species to provide early-warning signals of detrimental impacts on the marine ecosystem. The application and value of biomarkers at low temperatures have been little studied. For example, the seawater temperature in Hornsund, an Arctic fjord at Svalbard, ranges from  $-1.88$  to  $3$  °C (Weslawski et al., 1988). Chemicals, notably oil, behave differently at low compared with higher temperature; for example, the viscosity of oil is a temperature-dependant property (Payne et al., 1991). Thus, at low temperature, oil does not flow as freely, spread or dissolve in water to the same extent as at higher temperatures and such physical differences have important consequences for the bioavailability of oil components. Marine organisms living at low temperatures have developed specific adaptations, such as hysteresis antifreeze agents (Denstad et al., 1987), to prevent freezing. Such adaptations to cold water may render organisms more vulnerable to xenobiotic exposure as reported for polar cod, *Boreogadus saida* (Christiansen et al., 1996). On the other hand, studies have shown that Arctic invertebrates may be more resistant to heavy metals due to their epontic behaviour (Chapman and McPherson, 1993). In the case of oil, the cold-water adaptations of marine organisms, together with the altered behaviour of oil at low temperature, may affect the

typical biomarker responses developed for temperate species. This hypothesis needs to be tested before biomarkers are deployed in the Arctic. In this study, established biomarkers have been applied to the Arctic spider crab, *H. araneus* (Decapoda, Brachyura, Majidae), a species found commonly in Svalbard fjord (Dyer, 1985). This benthic crab, a temperate species with a geographical distribution from northern Spain to Svalbard, is found at depths from 10 to 1200 m. It feeds on juvenile scallop (Nadeau and Cliché, 1998), and may scavenge dead animals (Nickell and Moore, 1992), and is predated by seabirds and bearded seals (Hjelset et al., 1999). The high abundance and wide distribution of *H. araneus* make it a potential sentinel species for monitoring the Arctic marine ecosystem.

In crustaceans, biomarkers have been developed based upon a single response, however, it is the integrated repertoire of the full compensatory responses which determines the survival potential of individual, as emphasised by Hebel et al. (1997). Therefore, in the present study, two physiological biomarkers (heart rate and respiration) and one biochemical biomarker [(total oxyradical scavenging capacity (TOSC)), were measured in the same individual to gain understanding of the responses of *H. araneus* to oil at different levels of biological functioning. Crustacean heart rate is a useful biomarker. Cardiac activity increased in *Carcinus maenas* following exposure to the water soluble fraction of crude oil (Depledge, 1984b), while bradychardia and arrhythmia were measured in *Pugettia producta* when the gills were exposed to the water soluble fraction of crude oil (Zimmer et al., 1979). Numerous studies have reported heart rate disruption following exposure to metal contamination (Depledge, 1984a; Depledge and Lundebye, 1996; Bamber and De-

pledge, 1997). Respiration is another sensitive biomarker for oil exposure of marine invertebrates; the respiratory rate of *C. maenas* increased when exposed to the water soluble fraction of crude oil (Depledge, 1984b). Molecular oxygen is vital for most living organisms, providing energy through the coupling of oxidation to the phosphorylation of ADP into ATP (main source of stored energy directly available for the cell). Partial reduction results in the formation of various potentially toxic reactive oxygen species (ROS). The cytotoxic consequences of oxyradical production include alterations in enzyme functions, lipid peroxidation (membrane destabilisation), DNA damage and cell death. Nevertheless, the extent of such damages depends on the effectiveness of antioxidant defences. Decapod crustaceans are able to activate xenobiotics, notably benzo(a)pyrene, to reactive mutagenic products (i.e. Marsh et al., 1992; Fossi et al., 1997; Sundt and Goksøyr, 1998), and measurements of oxidative stress are based upon single antioxidant parameters (i.e. glutathione, catalase, superoxide dismutase; i.e. Arun et al., 1999; Orbea et al., 2000). The sensitivity of single antioxidative parameters enables their use as rapid and easy-to-use biomarkers. However, their ecological relevance is limited, as it is impossible to obtain a complete assessment of the oxidative stress based on a few biochemical responses. As a result, Winston et al. (1998) proposed a new assay, the TOSC assay, to measure the balance between antioxidant parameters and prooxidant factors. The TOSC assay measures the capability of a tissue to neutralise ROS in quantifiable term; this provides better understanding, and predictive capacity, of the effects of environmental conditions on the redox status of the organisms and their susceptibility to oxidative stress (Regoli, 2000). The method has been validated successfully as a biomarker (Regoli, 2000). In addition, Regoli et al. (2000) showed that the basal TOSC was higher in a polar than a temperate marine bivalve, indicating that the natural prooxidant pressure of cold water ecosystems was elevated compared with temperate water.

In this study, biomarker responses of *H. araneus* to oil were investigated via two routes: contaminated sediment and injection.

## 2. Material and methods

### 2.1. Sampling and maintenance of crabs

In May 2000, *H. araneus* were collected from Hollenderbukta (Isfjorden) at Svalbard (Fig. 1). Crabs were taken with an Agassiz dredge from ca. 50 m depth using the research vessel F/F Jan Mayen (University of Tromsø). Seawater temperature was  $-1.5\text{ }^{\circ}\text{C}$ . Small spider crabs [wet weight =  $13.00 \pm 3.91\text{ g}$ , carapace length =  $37.79 \pm 3.20\text{ mm}$ , carapace width =  $27.29 \pm 2.65\text{ mm}$  ( $n = 24$ )] selected for the experiments were stored in cooled ( $1\text{ }^{\circ}\text{C}$ ) seawater and air freighted to the laboratory (no mortality occurred during transport). In the laboratory, crabs were stored in a glass fibre tank (200 l) for 1 month prior to their use in experiments; the seawater and air temperatures were maintained at  $2\text{ }^{\circ}\text{C}$ . Illumination reproduced the summer Arctic light regime of 24 h daylight. Crabs were fed every 2 days with a mixed diet of crushed mussels (*Mytilus edulis*) or pieces of dead fish and shrimp. Seawater was filtered and a protein skimmer was coupled to improve water quality. Seawater was changed weekly.

### 2.2. Exposure system

Twenty-four crabs were divided into three groups, each of eight individuals, and each group was placed into one of the following 40 l aquaria: 'control', 'sediment exposure' and 'injection'. To obtain the contaminated sediment, 500 ml of oil, originating from a North Sea oil field, were mixed thoroughly with 6 kg of wet sediment (particle size diameter  $> 0.5\text{ mm}$ ) sampled on a beach located at Stavanger (Norway). The contaminated sediment was left standing at  $2\text{ }^{\circ}\text{C}$  with no animals for 24 h to allow the oil to adsorb onto the sediment particles. The seawater was then poured and any excess oil on the surface was removed. Crabs were placed in the tank and left for 15 days. For the injection exposure, eight crabs were injected with  $5\text{ }\mu\text{l}$  of the same oil at day 0, day 1, day 3 and day 6. In total,  $20\text{ }\mu\text{l}$  of oil were injected for a mean wet weight of  $12.73 (\pm 2.84)$  ( $1.6\text{ }\mu\text{l g}^{-1}$  wet weight). Injected crabs provided a

positive control of oil uptake and sediment-exposed crabs simulated an acute oil spill. Irrespective of treatment, crabs were fed twice a week with freshly crushed mussels (*M. edulis*); seawater was renewed the day after feeding and the excess food was removed. After 15 days, the respiration and heart

rate of eight crabs from each group was measured within 24 h (using the same individuals). After completion of the cardiac activity recordings, the fresh weight, length and width of each crab were measured and the midgut gland was removed, frozen and stored at  $-80^{\circ}\text{C}$  for the TOSC assay.

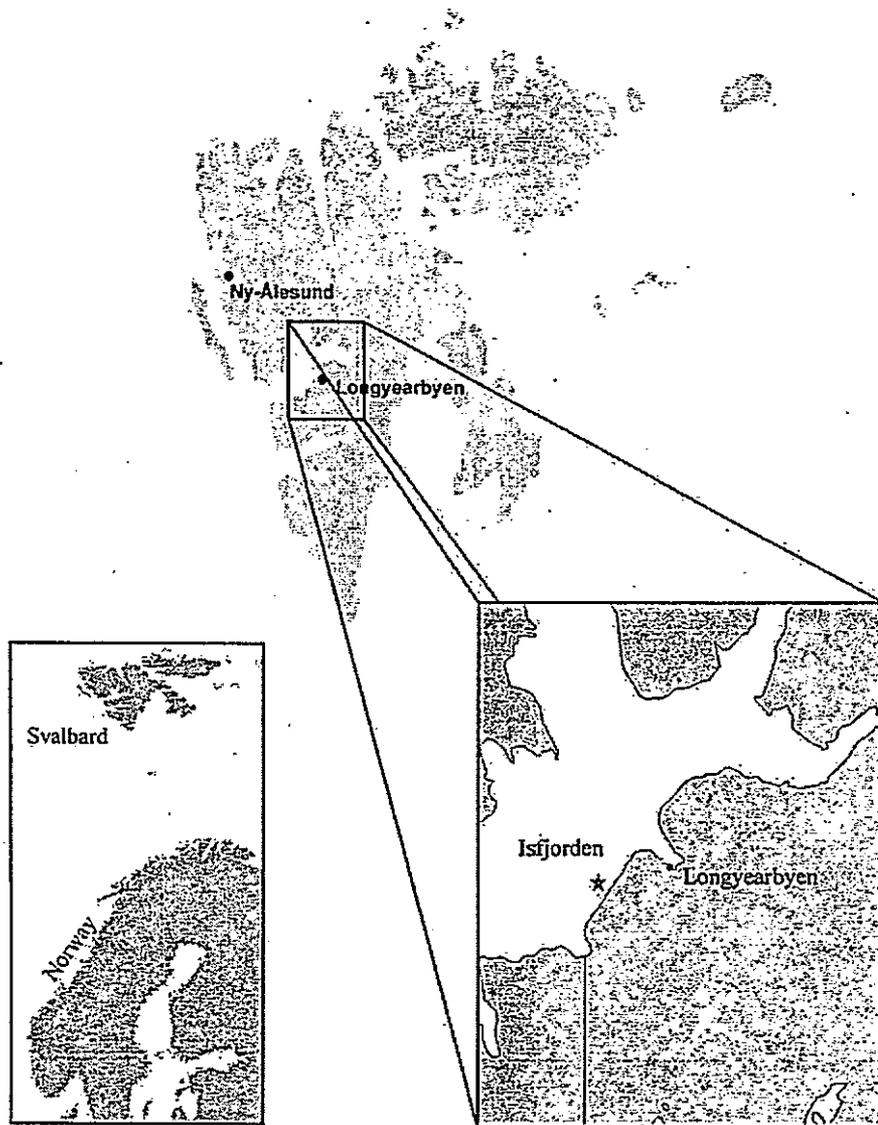


Fig. 1. Position of the sampling site, Hollenderbukta (see star), in Isfjorden at Svalbard ( $78^{\circ}13'N$ ,  $15^{\circ}39'E$ ).

### 2.3. Heart rate

Heart rate was measured with the non-invasive Computer Aided Physiological MONitoring procedure (CAPMON system) developed by Depledge and Andersen (1990). The system, based on an optical rather than electrical approach, allows heart rate to be recorded with no physical or biological disturbance. An infra red light emitter/receptor was glued onto the carapace of the animal. Emitted light is reflected onto the heart in proportion to its volume. The signal is amplified and converted to a digital signal which is displayed on a computer (PC). With the CAPMON unit, eight crabs were monitored simultaneously. The data were stored as ASCII files and exported to a spreadsheet for statistical analysis and graphic plotting. Recording procedure was as follows: transducers were glued onto the carapace of eight crabs per group and connected to the interface. Each animal was transferred to a plastic non-transparent beaker filled up with 1 l of seawater. During the experiment, the water was aerated constantly to provide oxygen. The size of the beaker prevented any locomotory or swimming activity that could affect recordings. The animals were given 2 h to acclimate to the new conditions before recording. Recordings of the heart rate lasted 4 h. Due to technical problems (inefficiency of some sensors), heart rate data of seven crabs per group were analysed and are reported.

### 2.4. Respiration

Eight crabs were placed individually into glass vials (240 ml), sealed with a screwed Teflon lid and incubated for 2 h at 2 °C. Eight empty vials, containing seawater but no crabs, were also incubated as controls and the oxygen concentration measured. In each vial, the oxygen concentration was measured by injecting a seawater sample taken from the vials into a respiration chamber (75 µl) connected to a polarographic Clark-type oxygen sensor (Strathkelvin® microcell MC100 and microcathode oxygen electrode SI130) maintained at 2 °C by a recirculating refrigeration bath. The  $P_{O_2}$  (1 mmHg = 0.1333 kPa) was calibrated to oxygen-saturated seawater (337 µmol

$l^{-1}$  at 2 °C and a salinity of 36 ‰). Data are expressed as mg  $O_2$  per gram of wet weight per hour.

### 2.5. TOSC-assay

The midgut glands of *H. araneus* were homogenised with a potter in four volumes of potassium phosphate buffer (100 mM  $KH_2PO_4$ , pH 7.5, 2.5% NaCl). The homogenate was centrifuged at  $100\,000 \times g$  for 1 h, and the cytosolic fractions were aliquoted and stored at -80 °C. Peroxyl radicals were produced at a constant rate at 35 °C by the thermal homolysis of 2,2'-azo-bis-(2-methylpropionamide)-dihydrochloride (ABAP). Peroxyl radical can oxidise the substrate  $\alpha$ -keto- $\gamma$ -methylolbutyric acid (KMBA) to ethylene gas which is measured by gas chromatography. The optimal assay conditions were 0.2 mM KMBA, 20 mM ABAP in 100 mM  $KH_2PO_4$  buffer, pH 7.4. Reactions were carried out in 10 ml vials sealed with gas-tight Minimert® valves (Supelco, Bellefonte, PA) in a volume of 1 ml. Ethylene production was measured by gas-chromatographic analysis of 200 µl taken from the head space of the reaction vials. Ethylene formation was monitored for 96 min with a Hewlett-Packard (HP 5890 series II) gas chromatograph equipped with a Supelco SPB-1 capillary column (30 m  $\times$  0.32 mm  $\times$  0.25 µm) and a flame ionisation detector (FID). The oven, injection and FID temperatures were 35, 160 and 220 °C, respectively; helium was the carrier gas (1 ml  $min^{-1}$  flow rate) and a split ratio 20:1 was used. The data acquisition system was run by the software Millennium32® (Waters). Each analysis required the measurement of control (no antioxidant in the reaction vial) and sample reactions (biological fluid in the vial). In the presence of antioxidants, ethylene production from KMBA was reduced quantitatively and higher antioxidant concentrations resulted in longer periods in which ethylene formation was inhibited relative to controls. By plotting the absolute value of the difference between the ethylene peak area obtained at each time point for the sample and control reactions, it is possible to visualise whether the oxyradical scavenging capacity of the solution is changed. The area under the

kinetic curve (ethylene surface area of the peak vs. time) was calculated mathematically from the integral of the equation that best defines the experimental points for both the control and sample reactions. TOSC is quantified according to Eq. (1), where IntSA and IntCA are the integrated areas from the curve defining the sample and control reactions, respectively.

$$\text{TOSC} = 100 - \left( \frac{\text{IntSA}}{\text{IntCA}} 100 \right) \quad (1)$$

Thus, a sample that displays no oxyradical scavenging capacity would give an area equal to the control ( $\text{IntSA}/\text{IntCA} = 1$ ) and a resulting TOSC of 0. On the other hand, as  $\text{IntSA}/\text{IntCA}$  goes to 0, the hypothetical TOSC value approaches 100. Because the area obtained with the sample is related to that of the control, the obtained TOSC values are not affected by small variations in instrument sensitivity, reagents or other assay conditions. The specific TOSC was calculated by dividing the experimental TOSC by the concentration of protein used for the assay. Protein concentration was measured according to the method of Bradford (1976).

## 2.6. PAH in sediment analysis

Sediment was sampled and stored in aluminium paper at  $-80^\circ\text{C}$  prior to analysis. The polycyclic aromatic hydrocarbon (PAH) analysis was performed by gas chromatography and mass spectrometry (GC-MS) according to the method of Douglas et al. (1994).

## 2.7. Statistical analysis

All statistical analyses were made using JMP v3.2.6., SAS Institute, Inc., Cary, NC, USA. Heart rate data were not normally distributed and variances were not equal, as a result, the non-parametric Wilcoxon test was used. Respiration and TOSC data were normally distributed and variances were equal, therefore, the Dunnett's test was used to test for statistical differences. Values of heart rate, respiration and

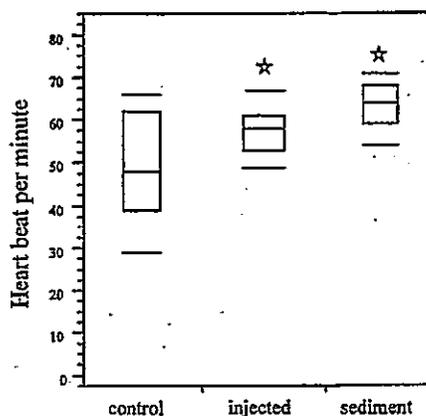


Fig. 2. Heart rate (beats per minute) of *H. araneus* ( $n = 7$ ) after exposure to North Sea oil (contaminated sediment, injection of oil and control). Significant effects are indicated by stars ( $P < 0.0001$ ).

TOSC were plotted as Box and Whisker plots. These indicate the range of values and the median. The horizontal bars that denote the upper and lower limits of the boxes include 95% of the data points; 50% of the data points are encompassed by the upper and lower limits of the indentations in the vertical sides of the boxes. The significance level was  $P < 0.05$ .

## 3. Results

### 3.1. Heart rate

Heart rate increased significantly in injected and sediment-treated crabs ( $P < 0.0001$ ) compared with controls (Fig. 2). The mean heart rates of *H. araneus* were  $49.06 \pm 13.72$ ,  $57.56 \pm 7.28$  and  $63.30 \pm 6.57$  beats per min (mean  $\pm$  S.D.) in control, injected and sediment treated group, respectively (see values reported in Table 1). The heart rate of sediment exposed-crabs was significantly higher than injected crabs ( $P < 0.0001$ ). In each exposure group, the variability in heart rate decreased by 50% compared with the control, indicating reduced inter- and intra-individual variability in contaminated-exposed individuals.

Table 1  
Heart rates of individual *H. araneus*

Control		Injected		Sediment	
fH ± S.D.	C%	fH ± S.D.	C%	fH ± S.D.	C%
46.8 ± 14.51	31.00	52.89 ± 1.45	2.75	62.35 ± 2.92	4.69
42.90 ± 8.50	19.82	66.21 ± 4.45	6.72	68.00 ± 3.25	4.77
66.43 ± 4.05	6.09	47.23 ± 7.96	16.85	63.43 ± 2.32	3.65
33.83 ± 11.72	34.63	56.95 ± 6.29	11.04	69.15 ± 1.42	2.05
43.48 ± 6.48	14.91	59.38 ± 1.89	3.18	54.27 ± 5.93	10.93
45.47 ± 9.50	20.90	60.69 ± 4.74	7.81	71.47 ± 2.48	3.47
61.48 ± 1.57	2.55	58.98 ± 1.62	2.75	58.88 ± 3.71	6.30

fH, heart frequency (beats per minute); C%, coefficient of variability.

### 3.2. Respiration

Oxygen consumption by *H. araneus* was not significantly affected by either oil treatment ( $P > 0.05$ ; Fig. 3). Nevertheless, the variability in the sediment-exposed group was twice as high as the control with high values indicating an increase in oxygen uptake (0.045 and 0.058 mg O<sub>2</sub> g wet wt.<sup>-1</sup> h<sup>-1</sup>) for two individuals. The basal oxygen consumption in control *H. araneus* at 2 °C was 0.025 mg O<sub>2</sub> g wet wt.<sup>-1</sup> h<sup>-1</sup> (Table 2).

### 3.3. TOSC assay

TOSC was not affected by either exposure ( $P > 0.05$ ; Fig. 4 and Table 2). Nevertheless, TOSC variability was increased in the injected group with a relatively high TOSC value of 7000 unit mg<sup>-1</sup> protein and a low value of 3200 unit mg<sup>-1</sup> protein. This variability may indicate a transient phase of the antioxidative defences in some individuals. Nevertheless, the mean TOSC value of the injected group (4942 unit mg<sup>-1</sup> protein) was similar to that of control (4837 unit mg<sup>-1</sup> protein). Although not statistically significant, the sediment-treated group showed a decrease in the median (4133 unit mg<sup>-1</sup> protein) and in the mean (4377 unit mg<sup>-1</sup> protein), compared with control.

### 3.4. Polycyclic aromatic hydrocarbon in sediment

The PAH composition and concentration in sediment are reported in Table 3. The total PAH

is 234 195 µg kg<sup>-1</sup> of wet sediment. Two ring molecules (i.e. naphthalenes) are the main compounds of this oil as they represent 95% of the total amount of oil compounds. The three and four ring compounds represented 5.5% only of the total PAH. Five ring compounds (i.e. benzo(a)pyrene) were under the detection limit of the apparatus. The two rings molecules are known to be the most soluble PAHs (Neff, 1979). Siron et al. (1993, 1996) reported that naphthalenes were the major dissolved compounds in seawater at low temperature (< 3 °C). Thus, in our study, it is likely that *H. araneus* was exposed mainly to naphthalenes. Their low molecular weight make them more bioavailable through the gills of the crabs.

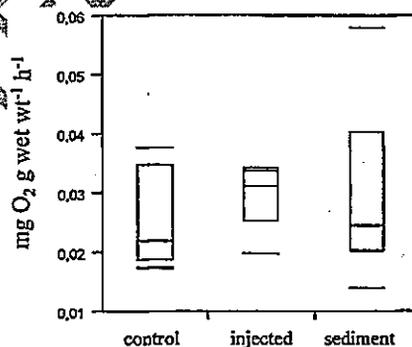


Fig. 3. Respiration of *H. araneus* measured as uptake of milligram of oxygen per gram wet weight per hour ( $n=8$ ) after exposure to North Sea oil (contaminated sediment, injection of oil and control).

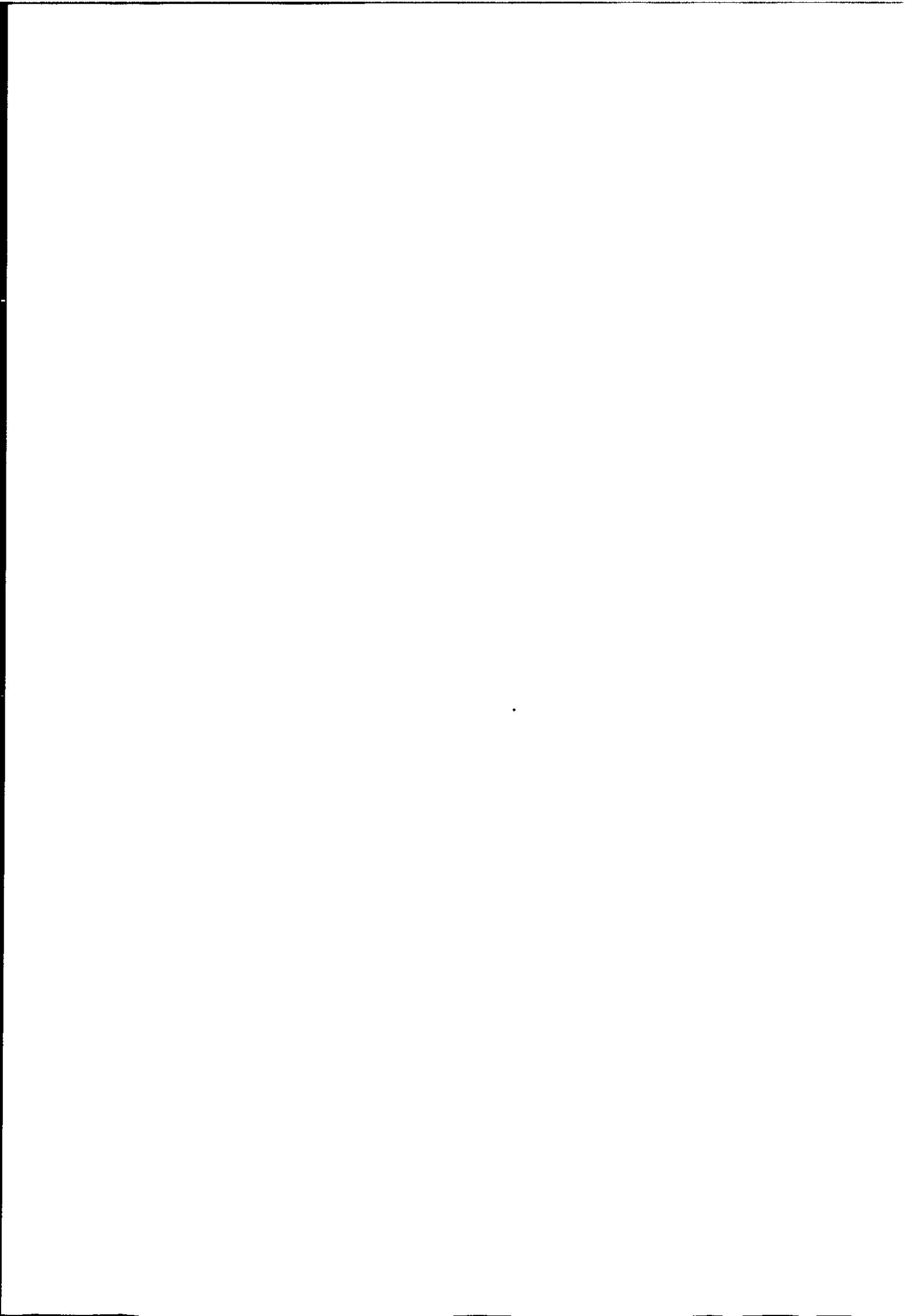


Table 2

Mean and S.D. of respiration of *H. araneus* in different treatments ( $\mu\text{g O}_2$  wet wt.<sup>-1</sup> h<sup>-1</sup>,  $n=8$ ) and TOSC measured in hepatopancreas (TOSC unit per mg protein,  $n=8$ )

	Control	Injected	Sediment
Respiration	26 ± 8.2	30 ± 5.1	29 ± 14.6
TOSC	4837 ± 758	4942 ± 1121	4376 ± 801

#### 4. Discussion

Heart rate appeared to be the most sensitive biomarker to oil exposure in *H. araneus*. Oil-injected and sediment-exposed crabs showed a significant increase in heart rate compared with controls. Very little data on the effect of oil components on cardiac activity of crustaceans have been published. The increased heart rate measured in the current study supports Depledge (1984b) who reported an increased heart rate in *C. maenas* exposed to the water-soluble fraction (WSF) of crude oil. However, bradycardia and arrhythmia were induced in *P. producta* when low levels of the WSF of oil was passed over the gills (Zimmer et al., 1979). Bamber and Depledge (1997) investigated the dietary exposure of *C. maenas* to benzo(a)pyrene and failed to record any obvious effects on heart rate over 7 days of exposure. The high variability in heart rate of the

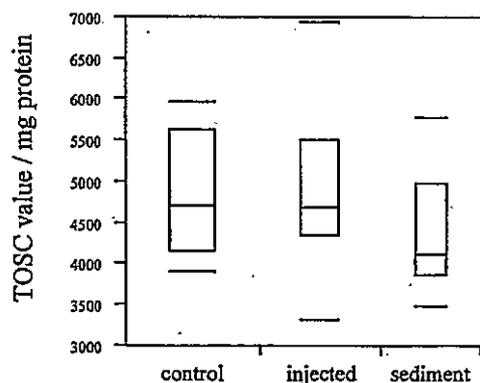


Fig. 4. Variations of the TOSC towards the peroxy radicals in the digestive gland of *H. araneus* after exposure to North Sea oil [contaminated sediment ( $n=6$ ), injection of oil ( $n=7$ ) and control ( $n=8$ )].

Table 3

PAH composition, measured by GC-MS, of the applied North Sea oil, in the sediment after 2 weeks exposure

Compound	Concentration ( $\mu\text{g kg}^{-1}$ )
Naphthalene	15 465
C1-naphthalene	53 842
C2-naphthalene	85 514
C3-naphthalene	65 760
Acenaphthylene	289
Acenaphthene	437
Fluorine	3338
Phenanthrene	3528
Anthracene	0
C1-phen/anthr	1347
C2-phen/anthr	971
Dibenzothiophene	552
C1-dibenzothiophene	276
C2-dibenzothiophene	273
Fluoranthene	204
Pyrene	384
Benzo(a)anthracene	(48)
Chrysene + triphenylene	346
C1-chrysene	770
C2-chrysene	897
Benzo(b)fluoranthene	(58.8)
Benzo(k)fluoranthene	0
Benzo(a)pyrene	0
Indeno(1,2,3-cd)pyrene	0
Benzo(g,h,i)perylene	0
Dibenzo(a,h)anthracene	0
TPAH	234 195

Values are in  $\mu\text{g}$  of PAH per kg of sediment. Total PAH (TPAH) is the sum of all 26 measured PAHs. Values in brackets correspond to signal below detection limit.

control group in the present experiment supports Depledge (1984a) who reported a similar response for *C. maenas*. The decreased variability in the injected and sediment-exposed groups has been observed by Depledge (1984a). Lundebye and Depledge (1998) showed that increased heart beat in *C. maenas* was due to a decline in mean interpulse duration. The elevated heart frequencies, reported in the current experiment, may indicate respiratory stress that was demonstrated by an increased respiration rate in *C. maenas* exposed to the WSF (Depledge, 1984b). However, no change in respiration rate was measured in the present experiment. Exposure of *C. maenas* to copper induced increased heart frequency and tissue hypoxia (Nonnotte et al., 1993; Bamber and Depledge,

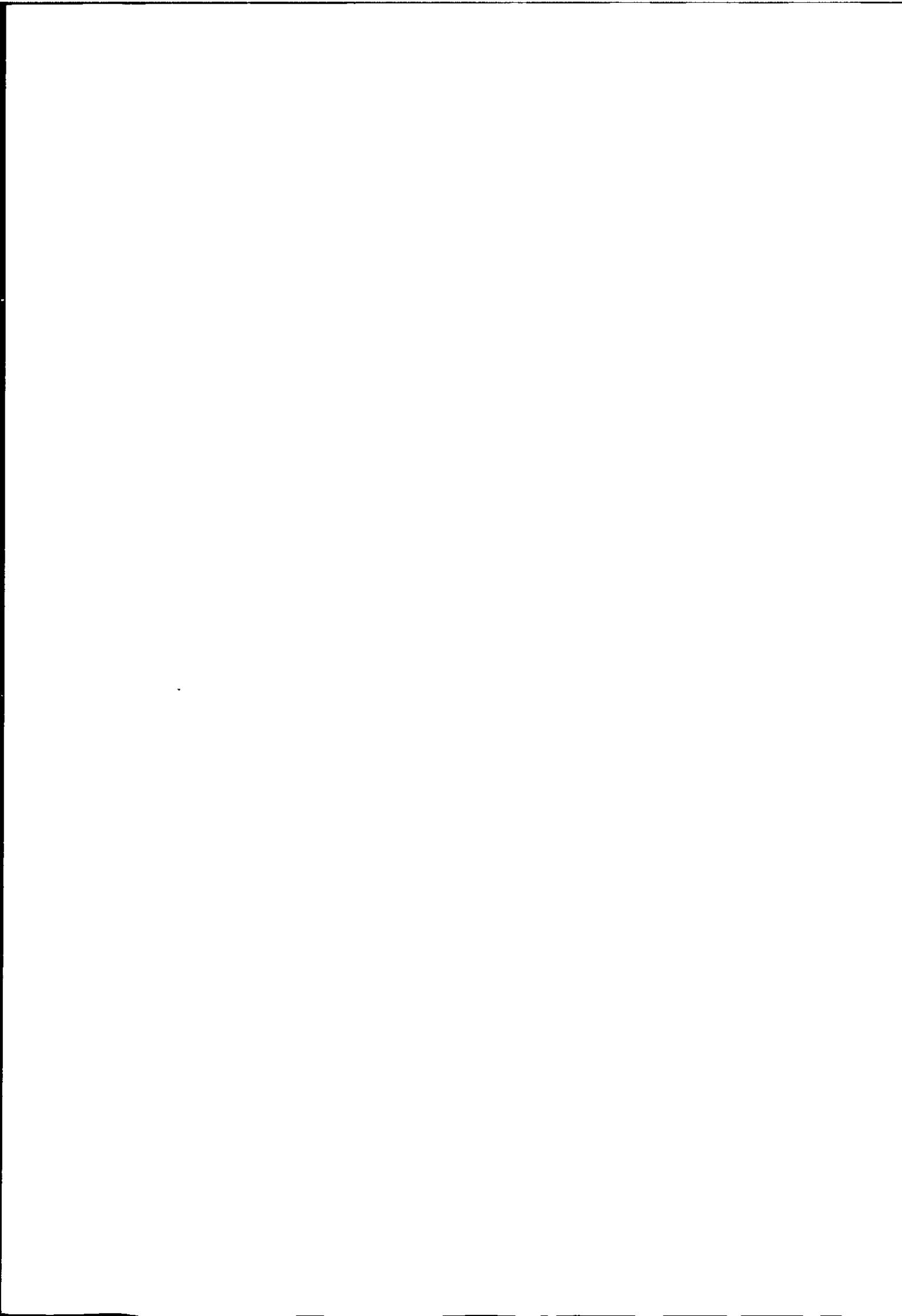
1997). A major concern of oil pollution is that it might affect oxygenation of seawater (FAO, 1977). The lack of oxygen could influence respiratory and cardiac physiology resulting in a greater volume of blood flow demanded per unit of oxygen required for tissue respiration. Cho et al. (1994) reported that naphthalene metabolites bind to large proteins such as haemoglobin, probably altering functions of the respiratory pigment leading to hypoxia. Nevertheless, several authors have reported a decrease in heart rate due to hypoxia and a concomitant bradycardia, with the heart stroke volume increasing to compensate for the lack of oxygen (Reiber and McMahon, 1998).

The increased heart frequency of sediment-contaminated and injected *H. araneus* may have resulted from voluntary reduction of the ventilatory stream to the gills to prevent chemical uptake. This compensatory behaviour would minimise the exchange with the external media while maintaining basal oxygen supply to the tissues. Increased heart rate is associated with locomotor activity in crustaceans. Aagaard et al. (1995) recorded an elevated heart rate prior to locomotor activity in *C. maenas* and, on no occasion, did locomotor activity increase prior to elevation of heart rate. In our study, similar observations were made. Aagaard et al. (1995) postulated that the circulatory and respiratory systems of the shore crab are prepared in advance to deal with the metabolic demands of locomotor activity. Such responses would be regulated by the neuro-endocrine system. The cardiac response of *H. araneus* could be regarded as an avoidance response. The animal has initiated its physiological machinery for escaping the oil-induced stress. As crabs remain quiescent, the energetic demand is not increased as shown in the steady state of oxygen uptake measured in the present study.

Changes in the variability of physiological parameters may provide an additional useful insight into the impact of chemical contamination (Depledge and Lundebye, 1996). Frederich et al. (2000) reported a decrease in variability of heart rate and oxygen consumption in *H. araneus* under physiological stress. Depledge and Lundebye (1996) explained the increasing inter-individual variability of heart rate with increasing contami-

nant concentration as an increasing proportion of affected crabs in the sampled group. In the current study, the reduced inter- and intra-individual variability indicated that oil exposure has induced a homogeneous physiological response in crabs.

Frederich et al. (2000) reported a significant decrease in respiration in *H. araneus* collected from temperate water at 9 °C and acclimatised to 2 °C. In the current study, the oxygen uptake of *H. araneus* at 2 °C (0.025 mg O<sub>2</sub> g wet wt.<sup>-1</sup> h<sup>-1</sup>) was comparable to the value reported by Frederich et al. (2000) (0.019 mg O<sub>2</sub> g wet wt.<sup>-1</sup> h<sup>-1</sup>). Thus, the low temperature of the Arctic water has reduced the metabolic activity of *H. araneus*. This observation was reported also by Whiteley et al. (1996) for the giant Antarctic isopod (*Glyptonotus antarcticus*) compared with a temperate isopod (*Idotea rescata*). The low oxygen uptake rate in *H. araneus* certainly reduced the uptake of PAH through the gills compared with temperate individuals. Moreover, the low metabolic rate of the spider crab may result in reduced metabolism of oil compounds into ROS. The low uptake and reduced metabolism of PAH could explain the lack of TOSC response measured in the present study. ROS is naturally produced during respiration when O<sub>2</sub> is reduced (Winston and Di Giulio, 1991) but ROS generation is also known to be enhanced via the metabolism of oil compounds (Livingstone et al., 1990). Babu and Brown (1995) reported an oxidant-stress toxicity of naphthalenes. There is a large literature reporting the varied activity of antioxidant defences and activation of contaminants to reactive metabolites in decapod crustaceans following pollutant exposure (e.g. Marsh et al., 1992; Gamble et al., 1995; Sundt and Goksøyr, 1998; Arun et al., 1999; Orbea et al., 2000). Orbea et al. (2000) localised four main antioxidant enzymes (catalase, Cu/Zn-superoxide dismutase, Mn-superoxide dismutase and glutathione peroxidase) in the digestive gland of the shore crab *C. maenas*, indicating the capacity of decapod crustaceans to cope with ROS. In the present study, no significant TOSC response was measured indicating that the possible enhancement of ROS production (notably peroxy radicals) was limited and did not saturate the



scavenging system capacity of *H. araneus*. The basal oxyradical scavenging capacity of *H. araneus* is relatively high compared with other marine invertebrates. For example, Regoli and Winston (1998) reported TOSC values of ca. 600 unit  $\text{mg}^{-1}$  protein for the blue mussel (*M. edulis*) and ca. 700 unit  $\text{mg}^{-1}$  protein for a starfish (*Leptasterias epichlora*). Elevated TOSC values in a cold-water species may be indicative of an enhanced resistance of toxicity in ROS generation. This would confirm a natural high prooxidant pressure for cold-water species (Regoli et al., 2000) and might also provide *H. araneus* with the capability of dealing with moderate variations in ROS generation. The lower mean TOSC value in the sediment-treated group is indicative of a certain degree of saturation of the antioxidative system. It is likely that such saturation corresponds to the partial consumption of the fast-acting scavengers of peroxy radical, for instance glutathione, uric acid and ascorbic acid (Winston et al., 1998). To date, reported data on the use of TOSC as a biomarker of ROS indicate a significant reduction in antioxidant capacity (Regoli 2000; Camus et al., 2002; Grosvik et al., 2002) or induction (Winzer et al., 2001). Grosvik et al. (2002) observed a depression in TOSC in the hepatopancreas of the Arctic shrimp (*Sclerocrangon boreas*) exposed to 2 ppm dispersed oil for 5 days at 2 °C. Similar observation were reported in Arctic scallop exposed to benzo(a)pyrene (Camus et al., 2002). With a depression in TOSC, Regoli (2000) reported a greater depletion of low molecular weight molecules during the first phase of exposure of Mediterranean mussels to high levels of pollutants, indicating ROS production and activation of antioxidant defences. In the present experiment, the absolute TOSC mean value may not be the appropriate parameter to use as a biomarker and the inter-individual variability may be more indicative of exposure (Depledge and Lundebye, 1996). In the injected group, the variability in TOSC measurements was twice that of the control. These data suggest a concomitant induction and depression in TOSC, and support the transient response of antioxidant defences as reported by other authors (Livingstone et al., 1992; Doyotte et al., 1997). A depletion or induction of

antioxidant systems may represent a first response to pollutants that can be followed by a more integrated response. It would have been interesting to extend the exposure period beyond 14 days to follow the change in TOSC. TOSC was measured in the hepatopancreas whereas injection was performed into the haemolymph where haemocytes represent the main defence system against foreign substances (Dyrynda et al., 1998). Thus, the hepatopancreas may have not been the correct target tissue to measure TOSC and the haemocytes may have been more appropriate.

In this study, heart rate (a physiological biomarker) was very sensitive in reflecting the first effects of oil in each exposure context. It is interesting to note that the TOSC assay did not provide any distinct signal, however, this latter biomarker has more ecological relevance than heart rate. McMahon (1999) highlighted the difficulty of interpreting cardiac performance in crustaceans by measuring one single parameter, such as heart rate. McMahon (1999) stated that haemolymph flow through any particular decapod arterial system does not necessarily vary with heart rate or even cardiac output but is, in each artery, modified from moment to moment by a variety of hormonal and neural drives. Heart rate on its own cannot provide a meaningful diagnostic of pollution exposure. TOSC is a tool for quantitatively assessing the biological resistance to toxicity of different forms of ROS. Up to now, the efficiency of the antioxidant system has been based upon analysis of single components, including enzymes (catalase, superoxide dismutase, etc.), and smaller molecules such as vitamin E, C, uric acid and reduced glutathione. Numerous studies have reported responses to oxidative stressors that can be very different and, while depletion is measured in one parameter, a concomitant induction could be measured in another one. The overall budget of ROS versus anti-oxidant defences can, therefore, barely be estimated and the complexity of the interpretation prevents any prognosis at the individual or population level. Single antioxidant analysis indicates exposure to oxidative stress but cannot provide a diagnostic measure of the health of the organism. The benefit of the TOSC assay is that it provides a prediction of the effects of

environmental conditions on the redox status of the whole organism and its susceptibility to oxidative stress disease (Regoli, 2000). The increase in heart rate may reflect the initial effect of oil notably the naphthalene compounds; however, the lack of change in TOSC associated with a low oxygen uptake reveals a limited toxic effect probably due to a low uptake and metabolism of PAH into ROS.

## 5. Conclusions

This investigation of the impact of oil on Arctic *H. araneus* at low temperature revealed an increase in heart rate, a steady state in respiration and a transient response in TOSC in the injected group, and a moderate decrease in the sediment-exposed group compared with the controls. The higher ecological relevance of TOSC, compared with heart rate, leads to the conclusion that the Arctic crab was experiencing a moderate pollution effect. Low temperature may be the main factor reducing the bioavailability of PAH in the water however, the relatively low metabolic rate of *H. araneus* may have reduced the uptake, metabolism of PAH and the production of ROS.

## 6. Uncited references

Depledge (1985), Frederick et al. (2000), Guerin and Stickle (1995), Morris and Bridges (1989), Roast et al. (1999).

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## References

- Aagaard, A., Warman, C.G., Depledge, M.H., Naylor, E., 1995. Dissociation of heart rate and locomotor activity during the expression of rhythmic behaviour in the shore crab *Carcinus maenas*. *Marine and Freshwater Behaviour and Physiology* 26 (1), 1–10.
- AMAP, 1997. Arctic pollution issues: a state of the Arctic environment report. Arctic Monitoring Assessment Programme. AMAP Publisher, Oslo, Norway.
- Arun, S., Krishnamoorthy, P., Subramanian, P., 1999. Properties of glutathione peroxidase from the hepatopancreas of freshwater prawn *Macrobrachium malcolmsonii*. *International Journal of Biochemistry and Cell Biology* 31 (6), 725–732.
- Babu, B.N., Brown, O.R., 1995. Quantitative effects of redox-cycling chemicals on the oxidant-sensitive enzyme dihydroxy-acid dehydratase. *Microbios* 82 (332), 157–170.
- Bamber, S.D., Depledge, M.H., 1997. Responses of shore crabs to physiological challenges following exposure to selected environmental contaminants. *Aquatic Toxicology* 40, 79–92.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254.
- Camus, L., Børseth, J.F., Grosvik, B.E., Regoli, F., Jones, M.B., Depledge M.H., 2002. Total oxyradical scavenging capacity and cell membrane stability of haemocytes of the Arctic scallop, *Chlamys islandicus*, following benzo(a)pyrene exposure. *Marine Environmental Research*, in press.
- Chapman, P.M., McPherson, C., 1993. Comparative zinc and lead toxicity tests with Arctic marine invertebrates and implications for toxicant discharges. *Polar Record* 29 (168), 45–54.
- Cho, M., Jedrychowsky, R., Hammock, B., Buckpitt, A., 1994. Reactive naphthalene metabolite binding to hemoglobin and albumin. *Fundamental and Applied Toxicology* 22 (1), 26–33.
- Christiansen, J.S., Dalmo, R.A., Ingebrigtsen, K., 1996. Xenobiotic excretion in fish with glomerular kidneys. *Marine Ecology Progress Series* 136 (1–3), 303–304.
- Denstad, J.P., Aunaas, T., Børseth, J.F., Aarset, A.V., E.Z.K., 1987. Thermal hysteresis antifreeze agents in fishes from Spitsbergen waters. *Polar Research* 5, 171–174.
- Depledge, M.H., 1984a. Disruption of circulatory and respiratory activity in shore crabs (*Carcinus maenas* (L.)) exposed to heavy metal pollution. *Comparative Biochemistry and Physiology* 78C (2), 445–459.
- Depledge, M.H., 1984b. Changes in cardiac activity, oxygen uptake and perfusion indices in *Carcinus maenas* (L.) exposed to crude oil and dispersant. *Comparative Biochemistry and Physiology* 78C (2), 461–466.
- Depledge, M.H., 1985. The influence of nutritional state on the circulatory and respiration physiology of the shore crab, *Carcinus maenas*. *Journal of the Marine Biological Association of the United Kingdom* 65, 69–78.

- Depledge, M.H., Andersen, B.B., 1990. A computer-aided physiological monitoring-system for continuous, long-term recording of cardiac activity in selected invertebrates. *Comparative Biochemistry and Physiology A Physiology* 96 (4), 473–477.
- Depledge, M.H., Lundebye, A.K., 1996. Physiological monitoring of contaminant effects in the individual rock crabs, *Hemigrapsus Edwardsi*: the ecotoxicological significance of variability in response. *Comparative Biochemistry and Physiology* 113C (2), 277–282.
- Douglas, G.C., Prince, R.C., Butler, E.L., Steinhauer, W.G., 1994. The use of internal chemical indicators in petroleum and refined products to evaluate the extent of biodegradation. In: Hinchee, R.E., Alleman, B.C., Hoeppe, R.E., Miller, R.N. (Eds.), *Hydrocarbon Bioremediation*. CRC Press, Boca Raton, FL, USA, pp. 219–326.
- Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M., Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquatic Toxicology* 39 (2), 93–110.
- Dyer, M.F., 1985. The distribution of *Hyas araneus* (L.) and *Hyas coarctatus* Leach (Crustacea: Decapoda: Brachyura) in the North Sea and the Svalbard region. *Journal of the Marine Biological Association of the United Kingdom* 65, 195–201.
- Dyrynda, E.A., Pipe, R.K., Burt, G.R., Ratcliffe, N.A., 1998. Modulations in the immune defences of mussels (*Mytilus edulis*) from contaminated sites in the UK. *Aquatic Toxicology* 42 (3), 169–185.
- FAO (Food and Agricultural Organisation of the United Nations), 1977. Impact of oil on the marine environment (No. 6). FAO, Rome, pp. 1–250.
- Fossi, M.C., Savelli, C., Casini, S., Franchi, E., Mattei, N., Corsi, I., 1997. Multi-response biomarker approach in the crab *Carcinus aestuarii* experimentally exposed to benzo(a)pyrene, polychlorobiphenyls and methyl-mercury. *Biomarkers* 2 (5), 311–319.
- Frederich, M., Sartoris, F.J., Arntz, W.E., Pörtner, H.O., 2000. Haemolymph  $Mg^{2+}$  regulations in decapod crustaceans: physiological correlates and ecological consequences in polar areas. *The Journal of Experimental Biology* 203, 1383–1393.
- Gamble, S.C., Goldfarb, P.S., Porte, C., Livingstone, D.R., 1995. Glutathione peroxidase and other antioxidant enzyme function in marine invertebrates (*Mytilus edulis*, *Pecten maximus*, *Carcinus maenas* and *Asterias rubens*). *Marine Environmental Research* 39 (1–4), 191–195.
- Grosvik, B.E., Camus, L., Bechman, R.K., Taban, I.C., Regoli, F., Borseth, J.F., Gaudebert, B., 2002. Antioxidant defence and cell response and cell membrane stability in Arctic shrimp *Sclerocrangon boreas* following exposure to dispersed crude oil. In preparation.
- Guerin, J.L., Stickle, W.B., 1995. Effects of cadmium on survival, osmoregulatory ability and bioenergetic of juvenile blue crabs *Callinectes sapidus* at different salinities. *Marine Environmental Research* 40 (3), 227–246.
- Hebel, D.K., Jones, M.B., Depledge, M.H., 1997. Responses of crustaceans to contaminant exposure: a holistic approach. *Estuarine Coastal and Shelf Science* 44 (2), 177–184.
- Hjelset, A.M., Andersen, M., Gjertz, I., Lydersen, C., Gulliksen, B., 1999. Feeding habits of bearded seals (*Erignathus barbatus*) from the Svalbard area, Norway. *Polar Biology* 21 (3), 186–193.
- Livingstone, D.R., Martinez, P.G., Michel, X., Narbonne, J.F., Ribera, S.D., Winston, G.W., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* (L.), and other mollusks. *Functional Ecology* 4 (3), 415–424.
- Livingstone, D.R., Lips, F., Martinez, P.G., Pipe, R.K., 1992. Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. *Marine Biology* 112 (2), 265–276.
- Lundebye, A.K., Depledge, M.H., 1998. Molecular and physiological responses in shore crabs *Carcinus maenas* following exposure to copper. *Marine Environmental Research* 46 (1–5), 567–572.
- Marsh, J.W., Chipman, J.K., Livingstone, D.R., 1992. Activation of xenobiotics to reactive and mutagenic products by the marine invertebrates *Mytilus edulis*, *Carcinus maenas* and *Asterias rubens*. *Aquatic Toxicology* 22 (2), 115–128.
- McMahon, B.R., 1999. Heart-rate: is it a useful measure of cardiac activity performance in crustaceans. In: Schram, Federic, R., von Vaupelklein, J.C. (Eds.), *Crustaceans and the Biodiversity Crisis*. Brill, Leiden, pp. 807–822.
- Morris, S., Bridges, C.R., 1989. Interactive effects of temperature and L-lactate on the binding of oxygen by the hemocyanin of 2 Arctic boreal crabs *Hyas araneus* and *Hyas coarctatus*. *Physiology Zoology* 62 (1), 62–82.
- Nadeau, M., Cliché, G., 1998. Predation of juvenile sea scallops (*Placopecten magellanicus*) by crabs (*Cancer irroratus* and *Hyas* sp.) and starfish (*Asterias vulgaris*, *Leptasterias polaris*, and *Crossaster papposus*). *Journal of Shellfish Research* 17 (4), 905–910.
- Neff, J.M. (Ed.), 1979. Polycyclic aromatic hydrocarbons in the aquatic environment. Sources, fates and biological effects. Applied Science Publishers, Essex, UK.
- Nickell, T.D., Moore, P.G., 1992. The behavioural ecology of epibenthic scavenging invertebrates in the Clyde Sea area: laboratory experiments on attractions to bait in moving water, underwater TV observations in situ and general conclusions. *Journal of Experimental Marine Biology and Ecology* 159 (1), 15–35.
- Nonnotte, L., Boitel, F., Truchot, J.P., 1993. Waterborne copper causes gill damage and hemolymph hypoxia in the shore crab *Carcinus maenas*. *Canadian Journal of Zoology-Revue Canadienne de Zoologie* 71 (8), 1569–1576.
- Orbea, A., Fahimi, H.D., Cajaraville, M.P., 2000. Immunocalcification of four antioxidant enzymes in digestive glands of mollusks and crustaceans and fish liver. *Histochemistry and Cell Biology* 114 (5), 393–404.
- Payne, J.R., McNabb, G.D., Clayton, J.R., 1991. Oil-weathering behaviour in Arctic environments. *Polar Research* 10 (2), 631–662.

- Peakall, D.B., 1994. The role of biomarkers in environmental assessment. 1. Introduction. *Ecotoxicology* 3 (3), 157–160.
- Regoli, F., 2000. Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. *Aquatic Toxicology* 50 (4), 351–361.
- Regoli, F., Winston, G.W., 1998. Applications of a new method for measuring the total oxyradical scavenging capacity in marine invertebrates. *Marine Environmental Research* 46 (1–5), 439–442.
- Regoli, F., Nigro, M., Bompadre, S., Winston, G.W., 2000. Total oxidant scavenging capacity (TOSC) of microsomal and cytosolic fractions from Antarctic, Arctic and Mediterranean scallops: differentiation between three potent oxidants. *Aquatic Toxicology* 49 (1–2), 13–25.
- Reiber, C.L., McMahon, B.R., 1998. The effects of progressive hypoxia on the crustacean cardiovascular system: a comparison of the freshwater crayfish, (*Procambarus clarkii*), and the lobster (*Homarus americanus*). *Journal of Comparative Physiology B Biochemical Systemic and Environmental* 168 (3), 168–176.
- Roast, S.D., Widdows, J., Jones, M.B., 1999. Scope for growth of the estuarine mysid *Neomysis integer* (Percarida: Mysidacea): effects of the organophosphate pesticide chlorpyrifos. *Marine Ecology Progress Series* 191, 233–241.
- Siron, R., Pelletier, E., Delille, D., Roy, S., 1993. Fate and effects of dispersed crude oil under icy conditions simulated in mesocosms. *Marine Environmental Research* 35, 273–302.
- Siron, R., Pelletier, E., Roy, S., 1996. Effects of dispersed and adsorbed crude oil on microalgal and bacterial communities of cold seawater. *Ecotoxicology* 5 (4), 229–251.
- Sundt, H., Goksøyr, A., 1998. In vivo and in vitro biotransformation of polycyclic aromatic hydrocarbons in the edible crab, *Cancer pagurus*. *Marine Environmental Research* 46 (1–5), 515–519.
- Weslawski, J.M., Zajaczkowski, M., Kwasniewski, S., Jezierski, J., Moskal, W., 1988. Seasonality in an Arctic fjord ecosystem: Hornsund, Spitsbergen. *Polar Research* 6, 185–189.
- Whiteley, N.M., Taylor, E.W., ElHaj, A.J., 1996. A comparison of the metabolic cost of protein synthesis in stenothermal and eurythermal isopod crustaceans. *American Journal of Physiology Regulatory Integrative and Comparative Physiology* 40 (5), R1295–R1303.
- Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology* 19, 137–191.
- Winston, G.W., Regoli, F., Dugas, A.J., Fong, J.H., Blanchard, K.A., 1998. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. *Free Radical Biology and Medicine* 24 (3), 480–493.
- Winzer, K.G., Winston, G.W., Becker, W., Van Noorden, C.J.F., Köhler, A., 2001. Sea-related responses to oxidative stress in primary cultured hepatocytes of European flounder (*Platichthys flesus* L.). *Aquatic Toxicology* 52, 143–155.
- Zimmer, R.K., Cook, D.K., Case, J.F., 1979. Chemosensory induced bradycardia in the kelp crab, *Pugettia producta* (Randall). *Journal of Experimental Marine Biology and Ecology* 38, 135–150.

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