Evaluation of metallic exposure in harbour seals (Phoca vitulina) from the North Sea using biomarkers of exposure and effects

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Faculté : Faculté des Sciences
Diplôme : Master en océanographie, à finalité approfondie
Année académique : 2017-2018
URI/URL : http://hdl.handle.net/2268.2/5542

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2017-2018

Cover illustration:

Harbour seal: https://www.ecopedia.be/dieren/gewone-zeehond

Acknowledgments

I would like to express my sincere gratitude to my promoter, **Dr Eric Gismondi,** for having welcomed me in his laboratory, for his support, encouragement , advice and assistance.

I am particularly thankful to my co-promoter, **Dr Krishna Das** for her participation at all levels of this master thesis, for having allowed me to work on this exciting subject, for having provided me with the samples needed for my work.

I extend my hearty thanks to **Dr France Damseaux** and to all members of the laboratories of oceanology and LEAE who have willingly helped me out with their abilities.

Furthermore, I want to express my gratitude to all **members of the jury** for their interest in my work and for their respective reading.

Last but not least, my thankfulness to **my parents Patricia and Eric** who has given me the chance to follow fascinating studies and has never stopped supporting me all along these past 2 years.

Abstract

Harbour seals bioaccumulate contaminants such as metal trace elements (MTEs), which are present in the North Sea and consequently they are often used as bioindicators for chemical exposure. For this reason, it is increasingly necessary to use biomarkers which are early indicators of exposure to toxic substances. The MTEs are known to cause oxidative stress, which is the result of an imbalance between the amount of reactive oxygen species (ROS) produced and the implementation of antioxidants defenses. This could cause lipid peroxidation and ultimately the death of the cell. Therefore, levels of antioxidants defenses could be good biomarkers to assess MTEs exposure. While oxidative damage could be a good biomarker of effects.

The aim of this study was to evaluate the relationship between the levels of MTEs contamination and biomarkers of exposure and effect. Selected biomarkers were: malondialdehyde (TBARS), total thiols, total glutathione (tGSH), Se dependent peroxydases (SeGPx), total glutathione peroxidases (GPx) and triglycerides. The first step of this study consisted to optimize assay protocols for those oxidative stress biomarkers in order to allow their assessments in the harbour seal serum. All biomarkers assays could be developed and analyzed by spectrophotometry.

In a second part, biomarkers analyses were conducted in a case study aiming to link their responses to contamination of MTEs in harbour seals of the Wadden Sea. The results demonstrated that MTEs concentrations in blood cells were significant different between males and females concerning Hg and Zn, with lower concentrations in females. Although, it has not been possible to identify how MTEs influenced oxidative stress in harbour seals, the lack of correlation between Se and SeGPx may indicate that Se is not limiting for harbour seals. However, a positive correlation was noted between Ni and GPx activities, probably caused by the formation of ROS by Ni. Moreover, the Hg contamination had potentially depleted tGSH in females (negative correlation) and finally, lipid peroxidation was increasingly important when it was exposed to Ni and Pb, however, this was only observed in March and not in October. An increase of lipid peroxidation could thus be due to environmental factors. Finally, even if the measurement of biomarkers is correct, the conservation of the serum may have permitted that few correlations were found between ETMs and biomarkers.

Résumé

Les phoques communs bioaccumulent des contaminants tels que les éléments trace métalliques (ETMs), présents en Mer du Nord et par conséquent, ils sont souvent utilisés comme bioindicateurs de l'exposition chimique. C'est pourquoi, il est de plus en plus nécessaire d'utiliser des biomarqueurs qui sont des indicateurs précoces d'exposition à des substances toxiques. Les ETMs causent un stress oxydatif, qui est le résultat d'un déséquilibre entre la quantité d'espèces réactives de l'oxygène (ROS) produites et les défenses antioxydantes mises en place. Cela pourrait entraîner la peroxydation des lipides et in fine à la mort de la cellule. Par conséquent, les niveaux des défenses antioxydantes pourraient être de bons biomarqueurs pour évaluer l'exposition à des ETMs. Alors que les dommages oxydatifs pourraient être un bon biomarqueur d'effet.

Le but de cette étude était d'évaluer la relation entre les niveaux de contamination des ETMs et les biomarqueurs d'exposition et d'effet. Les biomarqueurs choisis étaient: malondialdéhyde (TBARS), thiols totaux, glutathion total (tGSH), peroxydases dépendantes du sélénium (SeGPx), glutathion peroxydases totales (GPx) et les triglycérides. La première partie de l'étude consistait à optimiser les protocoles de dosage pour ces biomarqueurs de stress oxydatif afin de permettre leur évaluation dans le sérum du phoque commun. L'ensemble des dosages des biomarqueurs a pu être mise au point et analysés par spectrophotométrie.

En seconde partie, l'analyse des biomarqueurs fut réalisée dans une étude de cas visant à mettre en relation leur réponses et la contamination en ETMs des phoques communs de la mer des Wadden. Les résultats ont montré que les concentrations de ETMs dans les cellules sanguines étaient significativement différentes entre les mâles et les femelles pour le Hg et le Zn, avec des concentrations plus faibles chez les femelles. De plus, bien qu'il n'a pas été possible d'identifier comment les ETMs influençaient le stress oxydatif chez les phoques communs, l'absence de corrélation entre Se et SeGPx pourrait indiquer que le Se n'est pas limitant pour les phoques communs. Toutefois, une corrélation positive a été souligné entre le Ni et les activités GPx, probablement dû par la formation de ROS par le Ni. Par ailleurs, la contamination en Hg avait potentiellement appauvri le GSH total chez les femelles (corrélation négative) et finalement, la peroxydation lipidique était de plus en plus importante lorsqu'elle était exposée au Ni et au Pb; Cependant, ceci n'a été observé qu'en mars et non en octobre, une augmentation de la peroxydation lipidique pouvant être due à d'autres facteurs environnementaux. Enfin, même si la mesure des biomarqueurs est correcte, la conservation du sérum peut avoir permis de trouver peu de corrélations entre les ETMs et les biomarqueurs.

Glossary

AHR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CHP	cumene hydroperoxide
CRM	certified reference materials
DDT	Dichlorodiphenyltrichloroethane
DHA	Docosahexaenoic acid
DMA	Direct Mercury Analyzer
DNA	deoxyribonucleic acid
DTNB	dithiobis-(2-nitrobenzoic acid)
dw	dry weight
EDTA	ethylenediaminetetraacetic acid
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione tGSH total glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
GSTNB	Complex formed by reduced glutathione and 5-thio-2-nitrobenzoic acid
HCl	hydrogen chloride
Hg0	mercury vapour
$HgCl_2$	divalent mercury
HgSe	mercury selenide
HNO_3	nitric acid
HPLC	high performance liquid chromatography
HSP70	heat-shock-protein 70
H_2SO_4	sulfuric acid
IndSeGPx	selenium-independent glutathione peroxidase
ICP-MS	inductively coupled plasma mass spectroscopy
IL-2	cytokines interleukin-2
IL-10	interleukin-10
ITAW	Institute for Terrestrial and Aquatic Wildlife Research
KPi	potassium phosphate buffer
MDA	malondialdehyde
MES	2-(N-morpholino) ethanesulfonic acid
MeHg	methylmercury
MPA	meta-Phosphoric acid
MT	metallothionein
MTE	metallic trace element
µl	microliter
µM	micrometre
NADPH	nicotinamide adenine dinucleotide phosphate
NAG	N-acetylbeta-d-glucosaminidase

NaCl	sodium chloride
NaOH	sodium hydroxide
n-butanol	normal butanol
ng/g	nanogram/gram
nm	nanometre
nmol	nanomol
Na_2WO_4	sodium tungstate
OSPAR	Convention for the Protection of the Marine Environment of the NE Atlantic
PAHs	metals and polycyclic aromatic hydrocarbons
PCA	principal component analysis
PCB	polychlorinated biphenyl
PDV	phocine distemper virus
pH	potential hydrogen
POPs	persistent organic pollutants
PPARα	peroxisome proliferator-activated receptor
ROS	reactive oxygen species
rpm	revolutions per minute
SD	standard deviation
SeGPx	selenium-dependent glutathione peroxidase
SH	sulfhydryl group
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TBT	tributyltin
TCA	trichloroacetic acid
TEAM	triethanolamine
T-Hg	total mercury
TNB	5-thio-2-nitrobenzoic acid

Cd	cadmium
Cd	cadmium
Cr	chrome
Cu	copper
Fe	iron
Li	lithium
Ni	nickle
Pb	lead
Se	selenium
V	vanadium
Zn	zinc

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Context and objectives of this master thesis

Aquatic environments are more and more contaminated by pollutants from anthropogenic activities, and the North Sea is no exception. Harbour seals live and feed in polluted waters of the North Sea. They are considered as bioindicators for medium and long-term changes in ecosystems (Kakuschke & Prange, 2007). Therefore, biomonitoring occurs regularly in harbour seals, which is an essential step to identify trends of contaminants in the environment, but this does not provide information about the real impact of those contaminants on living organisms (Michèle & Giambérini, 2013). This is the reason for an increasing interest in the biological response of organisms to pollutants such as metallic trace elements (MTEs), and an effective way to obtain such information is by the use of biomarkers. MTEs can cause oxidative stress, which is often associated to the toxicity of MTEs (Ercal et al., 2001).

The purpose of this study was to evaluate the relationship between contamination levels of metallic trace elements and biomarkers of oxidative stress in harbour seals *Phoca vitulina* coming from the Wadden Sea.

As no oxidative stress biomarkers are available in *P. vitulina*, the first step of this study consisted to optimize assay protocols of oxidative stress biomarkers in order to allow their assessments in the harbour seal serum. The chosen biomarkers were: malondialdehyde (TBARS), total thiols, total glutathione, selenium-dependent and -independent glutathione peroxidases.

In a second step, these biomarkers of oxidative stress were measured in harbour seal serum of the Wadden Sea and results were combined to metallic trace element levels (determined by France Damseaux - FRS-FNRS PhD student – Laboratory of Oceanography – ULiège), in order to highlight possible correlations between the different parameters, and also evaluate the impact of MTEs on harbour seals. Finally, results of the present study will allow to determine if the stress oxidative biomarkers are relevant for the evaluation of metallic trace element exposure in harbour seals, and therefore if they could be included in future marine ecotoxicology researches.

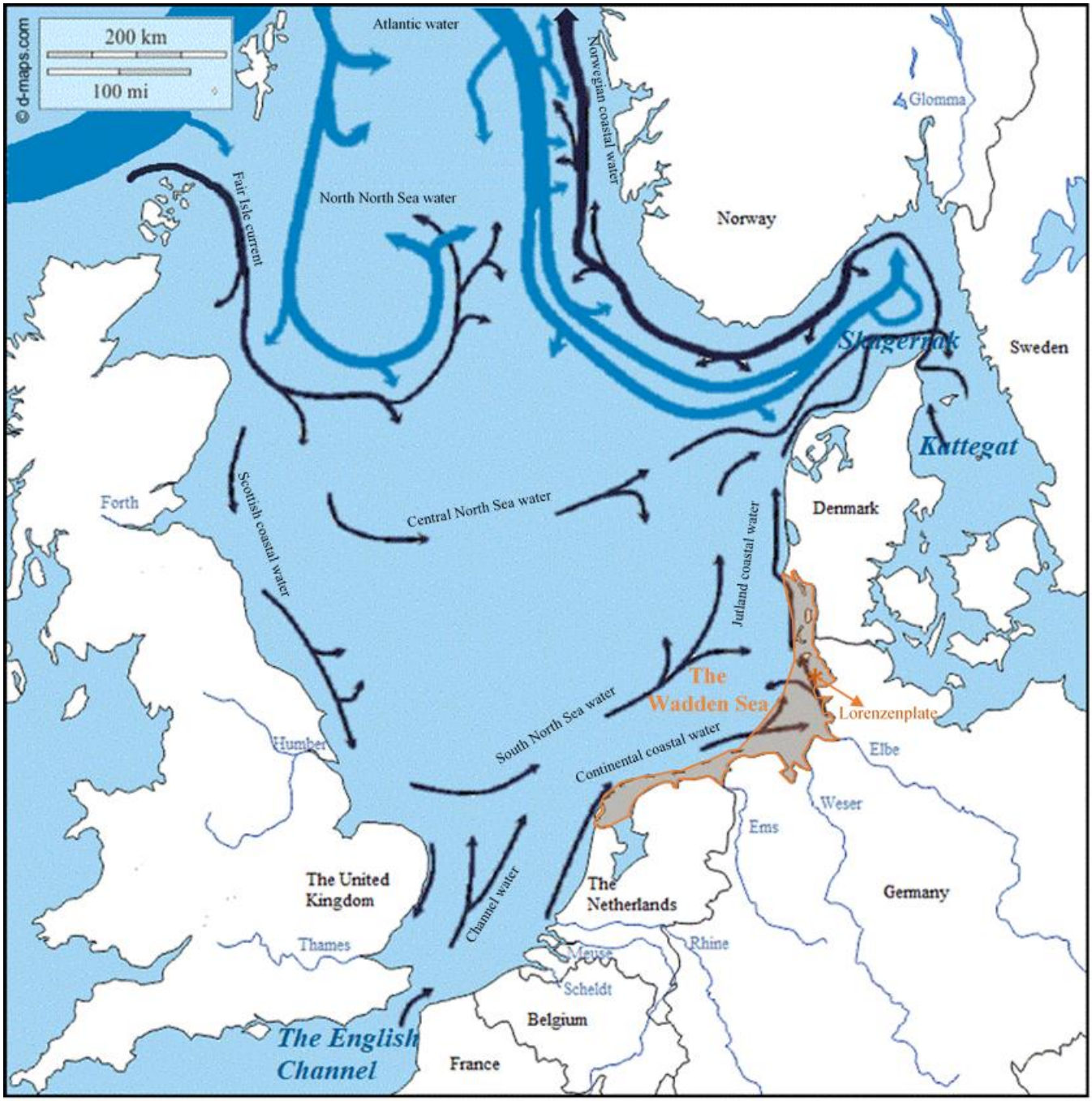

Figure 1: The North Sea with main rivers and currents. The study area (The Wadden Sea) is represented in orange. Arrow width represents the magnitude of the current. Blue arrows indicate the flow of Atlantic water and black arrows water of other types. Map adapted from webpage: d-maps.com, information about currents from Paramor et al. (2009)

Introduction

1. The North Sea

1.1 Generalities

The North Sea is a large semi-enclosed, but relatively shallow sea (average depth 90 meters) on the European continental shelf which is surrounded by eight countries (Figure 1) (Ducrotoy et al., 2000; Paramor et al., 2009; Quante et al., 2016; Walday & Kroglung, 2008). The English Channel in the west and Skagerrak and Kattegat in the east are considered by OSPAR (Convention for the Protection of the Marine Environment of the North-East Atlantic) to be part of the Greater North Sea (Quante et al., 2016). The Greater North Sea has a surface area of approximately 750 000 square kilometres and a total catchment area of 850 000 square kilometres (Ducrotoy et al., 2000; Paramor et al., 2009; Quante et al., 2016; Walday & Kroglung, 2008). The total annual input of freshwater is about 300 to 350 km^3, of which approximately one third comes from melt water of Scandinavia. The entire North Sea is an extremely productive region. More than 230 fish species are found in the North Sea, 20% of these species accounts for 95% of fish biomass in the North Sea (Ducrotoy et al., 2000; Paramor et al., 2009; Quante et al., 2016; Walday & Kroglung, 2008).

During this master thesis, the area of interest will be the Wadden Sea, which is a part of the North Sea. It has a trilateral form and extends from the Netherlands to Denmark for over 500km (Figure 1). The Wadden Sea is a very unique ecosystem, with a deposition system that is composed of tidal sand- and mudflats, barrier islands and salt marshes. 60% of all European intertidal areas can be found in the Wadden sea. The Wadden Sea sustains a rich and diverse flora and fauna, forms important nursery grounds for commercial fishes and is important for many species of migratory birds. For all these reasons, the Wadden Sea was added to UNESCO World Heritage in 2009 (De Jong et al., 2017; Quante & Colijn, 2016; Walday & Kroglung, 2008). Since 1978, the three surrounding countries (the Netherlands, Germany and Denmark) are working together to protect and conserve the Wadden Sea, from which the "Trilateral Monitoring and Assessment Program (TMAP)" flourished.

During the past few years, changes in the distribution and abundance of marine biota has occurred. This can be explained by climate change, but also by the increasing anthropogenic pressure on the North Sea environment (Ducrotoy et al., 2000; Paramor et al., 2009; Quante & Colijn, 2016; Salomons et al., 1988). Besides, the greatest pressure on the marine environment is exerted in the eastern and southern parts of the North Sea, partly corresponding to the Wadden

Sea. Even with this UNESCO status, the Wadden Sea is highly impacted by anthropogenic activities, as the rest of the North Sea.

1.2 Pollution of the North Sea

There are different pathways for contaminants to reach the marine environment, the main input is through rivers, direct discharge and deposition from the atmosphere. Many substances reach the marine environment, most are degraded effectively while others may be widespread in the marine environment due to their more persistent nature (OSPAR, 2000, 2010). Substances are hazardous because of their persistent nature, toxicity and ability to accumulate in living organisms (EEA, 2015). These substances can either be natural or anthropogenic. For instance, trace elements and nutrients are naturally occurring in the earth's crust, plants and animals. It is important to distinguish the natural concentrations from the part which is augmented by human activities (OSPAR, 2000).

Broadly, hazardous substances can be divided into trace elements, persistent organic contaminants (PCB, DDT, etc.) and degradable organic contaminants (organophosphates, alkylphenols, etc.) (Green et al., 2003). The impact of substances on biota depends on the bioavailability, bioaccumulation, biomagnification, toxicity and the capability of an organism to metabolise the substance. Organisms can take up contaminants directly, by absorption from sea water or by direct ingestion of particles, or indirectly when these same species are consumed by other organisms. For the oceanic parts, the atmospheric transport is the dominant source for several substances, such as mercury, lead, persistent organic pollutants (POPs) and some nitrogen compounds (OSPAR, 2000). Water and sediments with high contamination rates are generally found at inshore estuaries and coastal sites subjected to high industrialisation (Sheahan et al., 2001).

The North Sea is impacted by intense activities of 184 million people inhabiting the catchment area which drains into the sea. An additional 85 million people inhabit the catchment area of the Baltic sea which is connected to the North Sea. Among this, 3.7 million people live around the Wadden Sea area (De Jong et al., 2017). As a result, the North Sea has been subject to eutrophication, oil pollution, pollution by radioactive substances, hazardous substances and chemicals, posing threats to the marine environment. Associated industry and large towns have emptied their waste into the North Sea for over a long period. A wide variety of industries, are located in the basins of the main rivers discharging in the North Sea (Ducrotoy et al., 2000). In addition, the North Sea contains some of the busiest shipping lanes in the world, which cause mainly oil and air pollution (OSPAR, 1999, 2010; Quante et al., 2016; Walday & Kroglung, 2008). Furthermore, gas and oil platforms, a major economic activity in the North Sea, are

responsible for air pollution and the discharge of polluted water containing trace metals and a mix of chemicals (OSPAR, 2010). The North Sea also know an important historical pollution, after World wars I and II, large amounts of munitions and chemical weapons, including mustard gas and other chemical warfare agents, were dumped in the North Sea (EEA, 2015; Roose et al., 2011). In the Wadden Sea, many industries are found (e.g. oil refineries, container terminals, storage sheds, dockyards, chemical plants, incineration plants, power plants) resulting in emissions of different anthropogenic substances.

After recommendations imposed by OSPAR during the 1980s and 1990s, environmental concentrations of monitored substances have fallen, but are still above acceptable levels (OSPAR, 2010). OSPAR's objective is to reduce discharges, emissions and losses of hazardous substances, thereby reaching near background values for natural occurring substances and close to zero for anthropogenic substances (OSPAR, 2010). Moreover, not all substances are monitored and there is a growing scientific concern for emerging substances (Roose et al., 2011).

2. Metallic trace elements

Metallic trace elements (MTEs) are the metallic portion of trace elements. In the past they were often referred to 'heavy metals', however this appellation is now discussed, as metallic trace elements also contains some metals which are not 'heavy' (e.g., Ni, Al) and elements which are not metals (e.g., As, Se) (Richir & Gobert, 2016). Many MTEs are essential (e.g., Fe, Zn, Se and Cu) for biochemical processes in organisms and are required in very small quantities, however they become noxious when a certain threshold concentration is exceeded and are deficient when concentrations are too low. Others are non-essential, such as Hg, Cd and Pb, and tissue levels are therefore generally not well regulated by the bodies of organisms (Green et al., 2003; Richir & Gobert, 2016). Other MTEs may or could be essential (e.g., V, As, Ni), however the knowledge about those is still evolving (Richir & Gobert, 2016). MTEs, essential and non-essential, are naturally present in the environment (e.g. earth's crust, natural chemical processes). However, human activities have effectively increased the rate at which MTEs are introduced into the environment by human-induced chemical processes and synthetization for use in industrial processes, agriculture and customer goods. Although MTEs are hazardous and may cause many adverse biological effects on an organism's activity (e.g. growth, survival, reproduction), and are persistent, tend to bioaccumulate and some even biomagnif(throughout the food chain, MTEs are still widely used and exposure has increased in many parts of the world (Green et al., 2003; Jaishankar et al., 2014).

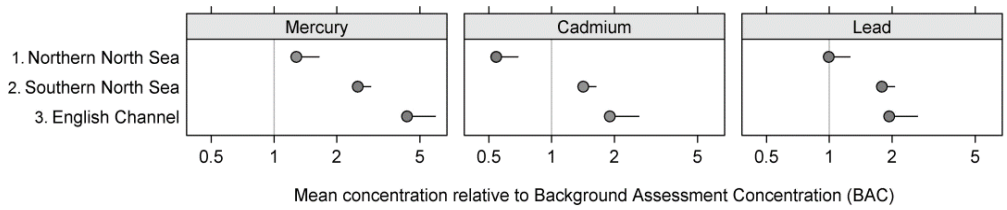

Figure 2: Mean concentrations in sediment relative to the Background Assessment Concentration (BAC) OSPAR (2017b).

In general, higher concentrations of MTEs are measured in estuaries compared to intermediate and offshore sites. Higher concentrations are found in the southern North Sea compared to the northern North Sea. This can be attributed to a more important fresh water input from major rivers in the south and to the anti-clockwise circulation of the North Sea which confined eastern British river discharges to the southern North Sea (Figure 1) (Sheahan et al., 2001). At offshore areas, atmospheric inputs are more likely to dominate, while near shore and coastal areas the situation is likely to be reversed (OSPAR, 2000; Sheahan et al., 2001). The main anthropogenic sources of MTEs into the atmosphere are fossil fuel combustion, non-ferrous metal production and waste incineration. There is a general reduction in direct, riverine and atmospheric inputs due to general efforts, such as OSPAR's work on hazardous substances and the EU covering this actions (OSPAR, 2010).

As for the most hazardous substances, some MTEs are still above acceptable levels in the North Sea. This is the case for the three most toxic metallic trace elements (mercury, cadmium and lead) which are on OSPAR's List of Chemicals for Priority Action, even if atmospheric and water input of mercury, cadmium and lead have halved since the start of the 1990s (OSPAR, 2017a). Recent measurements indicated that mercury and lead concentrations in sediments are at or above the Background Assessment Concentrations (BACs) in all contaminants assessment areas of the North (Figure 2). Mean concentrations of cadmium in sediment are below the BAC in the Northern North Sea but still above in the Southern North Sea (OSPAR, 2017b).

Although concentrations of mercury, cadmium and lead have decreased, their concentrations are still 2 to 5 times the background concentration and in some areas of the Southern North Sea cadmium and mercury concentrations in fish and shellfish have risen these last few years (OSPAR, 2017c).

2.1 Mercury

The main anthropogenic sources of mercury (Hg) are metallurgic industry, fossil fuels and in particular coal, incineration, chlor-alkali industry, dental amalgams, etc. (OSPAR, 2010). Consequently, atmospheric deposition is the dominant source of mercury contamination in the North Sea and is estimated to be 10.5 tonnes per year compared to 6.8 to 8.5 tonnes a year for the riverine and direct inputs (Green et al., 2003). In the environment, Hg can be found in organic form as methylmercury (MeHg) or in inorganic forms as di- and monovalent ionic Hg and metallic Hg. In the marine environment, MeHg is the main Hg form found in the food web, due to a continuous microbial transformation from inorganic to organic Hg, particularly under anoxic conditions (Green et al., 2003).

Hg is a non-essential element to all organisms. Due to its potential for bioaccumulation and biomagnification (as methylmercury), and its high toxicity, Hg is considered as one of the most dangerous metals in the aquatic environment. The organic form is known to be more toxic for animals than inorganic forms and it's why total mercury (T-Hg) is a poor indicator for the toxic effect (Das et al., 2003). The toxicity of Hg will depend on the form in which it's taken up, the route of entry and on the extent to which mercury is absorbed (Das et al., 2003). Additionally, excretion rates of Hg are relatively slow, which explains high levels in long living animals, such as marine mammals. MeHg is a well-known neurotoxin in mammals, causing anorexia and lethargy (Das et al., 2003). It is also known that MeHg has a high affinity for sulfhydryl groups in addition to being fat-soluble. MeHg is transferred through the placenta and is known to cross the blood-brain barrier, this may eventually end in foetal death. In marine mammals, Hg is most concentrated in the liver, followed by the kidneys and muscles (Das et al., 2003; Green et al., 2003; Mahurpawar, 2015).

Marine mammals have a mechanism for detoxifying the Hg as mercury selenide (HgSe). Namely, the presence of high Hg and Se concentrations in marine mammals does not result in poisoning, these two elements are mutual antagonists. Almost all Hg taken up by marine mammals is in the organic form, but in the liver, only less than 10% of Hg is found as MeHg. This implies a demethylation process, which is achieved by the formation of HgSe granules accumulated in the liver. High levels of Hg will be found in the liver but they are non-toxic because of this form which is less bioavailable (Das et al., 2003).

2.2 Cadmium

Different sources of cadmium (Cd) are: mining, metal industry, coating industry, production of batteries, burning of fossil fuels, phosphate fertilisers, waste incineration and leaching from waste deposits (Green et al., 2003).

Non-essential and highly toxic, Cd is known to effect vital biological processes such as ion exchange, energy production and protein synthesis, because it often interacts with the metabolism of essential trace metals. Long-term exposure in humans can lead to the disease called 'Itai-Itai', kidney damage and painful bones and joints (Green et al., 2003). Cd is more soluble in water than other metals and it's more bioavailable, it also tends to bioaccumulate in the proximal tubular cells from the kidney (Jaishankar et al., 2014). The potential for biomagnification is uncertain since intestinal absorption of Cd is low in vertebrates (Faroon et al., 2012).

Despite high Cd concentrations in kidneys of marine mammals (e.g. 2000 µg/g dry weight measured in Artic ringed seals by Dietz et al. (1998)), no obvious Cd toxic effects have been registered in marine mammals, suggesting highly efficient detoxification mechanisms. For

instance, high concentrations of Cd in marine mammals do not lead in renal damage (Das et al., 2003). This could be achieved by metallothionein, a low-molecular-weight, cysteine-rich, metal-binding protein present in the whole animal kingdom. Metallothioneins play an important role in Cd retention in tissues, thanks to the cysteine residues which have a high binding affinity for divalent ions such as Zn^{2+} and Cd^{2+}. Metallothionein (MT) will bound to Cd and by doing so, is responsible for Cd accumulation in tissues (Das et al., 2003).

2.3 Lead

Once used for water pipes, sweetener in wine and colouring for skin-cream (OSPAR, 2017a), key sources of lead are now mining and petrol (OSPAR, 2010), but also: metal plating, wastes from battery industry, fertilizers and pesticides, exhaust from automobiles and smelting of ores (Jaishankar et al., 2014). In the North Sea, riverine and direct discharges have respectively decreased by 50% and 80% between 1990 and 2006 (OSPAR, 2010). However, lead (Pb) does not degrade which can explain high concentration in the environment by former use. In the environment, inorganic form of Pb is predominantly found, the organic form (alkyl lead) is less common (Abadin et al., 2007a). Pb also tends to be trapped nearshore because it quickly binds to particulate matter. Pb will not be transported in dissolved phase and hereby will not be transported by sea currents, and consequently atmospheric inputs of Pb are increasingly important away from the coast (Sheahan et al., 2001).

Pb is non-essential and highly toxic, furthermore, it bioaccumulates in biota but is not biomagnified in terrestrial or aquatic food chains (Abadin et al., 2007b). Pb predominantly accumulates in bones and blood from vertebrates. However, mobilisation of Pb in bones can occur later and bones can thus be a pool for Pb even long after Pb exposure (Abadin et al., 2007a). Decreased synthesis of haemoglobin and eventually anaemia will occur when exposed to high concentrations. Encephalopathy and mental retardation could also occur due to severe exposure to inorganic lead (Green et al., 2003). Pb which is not stored in bones leaves the body through urine or faeces. The toxicity of Pb is engendered by oxidative stress, which is caused by the imbalance between the production of antioxidants and free radicals. in addition, Pb metal ions can replace other bivalent and monovalent cations like Ca^{2+}, Mg^{2+}, Fe^{2+} and Na^{+} which leads to distribution of the biological metabolism of the cell (Jaishankar et al., 2014).

2.4 Other metallic trace elements

Although Cd, Pb and Hg are considered to be the most toxic MTEs, aquatic environments are also contaminated by other MTEs, which could be as toxic and accumulated in biota. This study

contains another 8 MTEs including: Zn, Fe, Cu, Se, Ni, Cr, Li and V. An overview of their biological function, major sources and toxicity can be found in Table 1.

Table 1: Overview of biological function, major sources and toxicity of Zn, Fe, Cu, Se, Ni, Cr, Li and V.

MTEs	Biological function	Major sources	Toxicity	Sources
Zn	Indispensable for normal growth and reproduction of all living organisms. Vital for more than 300 enzymes and the role in stabilization of DNA.	Natural sources are erosion, forest fires and volcanic eruptions. The main anthropogenic source is galvanization.	Zn is considered to be relatively nontoxic, bur high Zn intake may cause nausea, vomiting, lethargy, etc. It could induce Cu deficiency and an increase in high density lipoproteins (cholesterol).	(Fosmire, 1990; Frassinetti et al., 2006)
Fe	Vital for enzymes such as cytochromes and catalase, but also for haemoglobin and myoglobin.	The main anthropogenic source is mining.	When Fe cannot bind to proteins, free Fe will cause the formation of harmful free radicals which can attack DNA, furthermore free Fe can cause lipid peroxidation and can disrupt oxidative phosphorylation. Conversion of ferrous iron in to ferric iron releases hydrogen ions and thus increase metabolic acidity.	(Jaishankar et al., 2014)
Cu	Essential for many biological processes such as: cross-linking collagen and elastin, formation and maintenance of myelin and is part of the copper, zinc-superoxide dismutase which scavenges the free radical superoxide.	Natural sources are windblown dust, volcanoes and forest fires. The anthropogenic sources are Cu water pipes, Cu cookware, drinking water, birth control pills, vitamin and minerals supplements and fungicides.	Homeostasis is highly regulated through Cu transporters and chaperone proteins, however when disrupted, Cu can be toxic by initiate oxidative damage.	(Gaetke et al., 2014)
Se	Se is important as selenium proteins. For example, it is essential for GPx (protecting from reactive oxygen species) and more than 30 selenoproteins. Se acts as an agent that helps to neutralize toxic effects and remove metals such as Pt and Hg.	Naturally present in the earth crust. Anthropogenic sources are pigmentation of paint, metallurgy, agriculture and electronics.	Se can be highly toxic at higher concentrations. Not fully studied, however Se could block the sulfhydryl groups of tissue proteins and induce the formations of superoxide radicals in reaction with glutathione. Furthermore, the formation of methyl selenium would lead to the formation of free oxygen radicals such as superoxide anion and induces oxidative stress. Se is able to give electrons and act as a metal and when it accepts them, it acts as a non-metal.	(Minor metals trace association, 2018; Sobolev et al., 2018)

Ni	Non	Anthropogenic sources are production of nickel (used in electroplating, electroforming, batteries and stainless steel) and Ni contaminated waste disposal.	Ni induces oxidative DNA damage, further it induces oxidative stress which depletes glutathione and activates oxidative sensitive transcription factors. It has also been suggested that Ni substitutes for Fe in a hypothetical oxygen sensor, imitating permanent hypoxia.	(Denkhaus & Salnikow, 2002)
Cr	Non	Anthropogenic sources are sewage, burning of oil and coal, petroleum, fertilizers, oil well drilling and metal plating.	The most commonly occurring forms of Cr are Cr(III) and Cr(VI), both being toxic to animals, humans and plants. However, Cr(III) has a weaker membrane permeability than Cr(VI). The reactions between Cr(VI) and biological reductants like thiols and ascorbate, result in the production of reactive oxygen species such as superoxide ions, hydrogen peroxide, and hydroxyl radical, ultimately leading to oxidative stress.	(Jaishankar et al., 2014)
Li	Non	Natural sources are natural brines and lakes. The anthropogenic source is waste of Li batteries.	Li is considered to have a medium toxicity and is still used as a drug for bipolar disorders. However, in humans it is associated with increased risk of reduced urinary concentrating ability, hypothyroidism, hyperparathyroidism and weight gain.	(Aral & Vecchio-Sadus, 2008; McKnight et al., 2012)
V	The essentiality of this element in cellular functions is yet to be established.	The anthropogenic sources are power producing plants using fossil fuels, burning of coal wastes and dumps of coal dust in mining areas.	Vanadium is medium toxic, at high concentrations, V may be a reproductive and developmental toxicant in mammals.	(Domingo, 1996; Venkataraman & Sudha, 2005)

2.5 Metallic trace elements in marine mammals

Marine mammals are often used as bioindicators for chemical exposure (such as metallic exposure) in the marine environment. They are adequate due to their widespread distribution, their top position in the trophic food web, their potential to bioaccumulate and eventually biomagnify chemicals in their tissues, their long life span and relatively late maturity including a low reproduction rate (Bouquegneau et al., 1996; Kakuschke & Prange, 2007). Also, there has been an increased interest in studying trace metals in marine mammals because of large scale die-offs and reproductive failure and the finding of relatively high concentrations of metals in those animals (Agusa et al., 2011; Bouquegneau et al., 1996). For instance, the mass mortalities of

harbour seals in 1988 and 2002, where respectively 18 000 and 22 000 harbour seals from the North Sea died of phocine distemper virus (a morbillivirus), have risen the concern of immunodeficiency engendered by pollutants (Agusa et al., 2011). Different studies demonstrated that continuous metal exposure can lead to immunoenhancement or immunosuppression effects in marine mammals, depending on the metal, its speciation, concentrations, bioavailability and some other factors, such as the duration of exposure, route of exposure, species, sex, etc. (Das et al., 2008; Dupont et al., 2016; Kakuschke et al., 2005, 2006; Kakuschke, Valentine-Thon, et al., 2009; Kakuschke, Valentine-Thon, Fonfara, et al., 2008; Kakuschke, Valentine-Thon, Griesel, et al., 2008; Kakuschke & Prange, 2007). Killer cell activities, phagocytosis and transformation of lymphocytes in marine mammals were shown to be adversely affected. Kakuschke et al. (2008) determined lymphocyte inhibition of new-born harbour seal by Be, Cd, Hg, and Sn and stimulation was induced by Mo and Ni.

MTEs concentrations vary greatly within marine mammals as they are affected by numerous factors such as geographic location, diet, age, sex, the tissues considered and metabolic rates (Das et al., 2003). In addition, the toxicity of the metal will depend on the route of entry and its form. Marine mammals can take up trace metals through the lungs from the atmosphere, through the skin, across the placenta before birth, via milk through lactating, through ingestion of sea water and ingestion of food. With the last one being the major route of entry for metals in marine mammals (Das et al., 2003). Furthermore, some diets can influence the metal uptake, for instance cadmium concentrations are elevated in the viscera of molluscs and cephalopods. Leading to high Cd concentrations in animals feeding on molluscs and cephalopods. Besides, seals could be contaminated before birth by transfer of metals through female marine mammals placenta and lactating off to their pups. For this reason, metal concentrations will decrease in mature gestating females (Das et al., 2003).

3. Harbour seal

The harbour seal, and more precise the subspecies *Phoca vitulina vitulina*, will be the studied species of this master thesis as it lives and feeds in the polluted waters of the Wadden Sea. Within the “Trilateral Monitoring and Assessment Program” from the Wadden Sea, the harbour seal is used as bioindicator for chemical exposure and is considered as an indicator for medium and long-term changes in the ecosystem. This is due to its widespread distribution over the coastal areas, its high trophic level, resulting in bioaccumulation and biomagnification of chemicals in its tissues, its long-life span, its relatively late maturity and low reproduction rate (Kakuschke & Prange, 2007).

Figure 3: Harbour seals in Nieuwpoort. Picture taken by Lucienne Daneels on 8 January 2014.

Figure 4: Geographic distribution of the 5 sub-species of harbour seals with P. v. vitulina represented in pink, P.v. concolor represented in purple, P. v. mellonae represented in yellow, P. v. richardsi represented in green and P. v. stejnegeri represented in red. Source: http://online.sfsu.edu/bholzman/courses/Fall01%20projects/HSeal.htm.

3.1 Anatomy

Just like all phocids, the harbour seal do not have an external ear flap, therefore phocids are often referred to the earless seals (Berta et al., 2015). The harbour seal average length varies among populations, ranging from 1.4 meter to 2 meters with females about 156 cm (± 90 kg) and males about 170 cm (± 120 kg) (Bonner, 1989b; Burns, 2009; Fey et al., 2016). The harbour seal contains a coat with thick short hairs and no colour differences between male and females are observed (Bonner, 1989a; Burns, 2009). The harbour seal has a round head with a short snout, has a slightly upturned nose and narrow nostrils which form a V shape from above (Figure 3) (Fey et al., 2016).

3.2 Geographic distribution

The harbour seal inhabits cold-temperate and temperate waters on both sides of the North Atlantic and North Pacific Ocean (Bonner, 1989a). However, the five recognised subspecies (*P. v. concolor, P. v. mellonae, P. v. vitulina, P. v. stejnegeri and P. v. richardii*) have distinct geographic distributions (Figure 4). In the eastern North Atlantic, *P. v. vitulina* occurs from the English Channel, throughout the North Sea and northward up to the Barents Sea. The most northern population can be found in Svalbard. They also occur in the southern Baltic Sea and around the coast of Ireland and Great Britain. From all pinnipeds, the harbour seal has the broadest distribution and range of different habitats (Burns, 2009), but remains mostly in the coastal waters of the continental shelf and slope. They are commonly found in bays, rivers, estuaries and intertidal zones. The total worldwide population of harbour seals is estimated up to 500 000 individuals (Lowry, 2016), of which 38 126 animals would live in the Wadden Sea (Jensen et al., 2017).

3.3 Hauling-out

The harbour seal displays a high fidelity towards its local haul-out region, but varies between individuals. They tend to travel in average 30 km away from their haul-out site, however some seals may travel over longer distances up to 500 km (Burns, 2009; Lowry, 2016; Sharples et al., 2012). Throughout the year, harbour seals haul out regularly to rest, breed and moult. Nevertheless, the abundance of seals will be higher during the breeding and moulting seasons (Brasseur & Kirkwood, 2015; Burns, 2009; Thompson et al., 1991). Harbour seals haul out on rocks, beaches, sand bars, mud flats, vegetation, sea ice, glacial ice and man-made structures (Burns, 2009; Lowry, 2016). In the Wadden Sea, harbour seals mainly haul out on undisturbed sand banks, from where they conduct foraging trips into the North Sea (Jensen et al., 2017).

3.4 Foraging behaviour and diet

The harbour seal is considered to be a top predator. As opportunists and generalists, harbour seals prey mainly on abundant and easily available food. Prey consists generally of small to medium size fishes, such as members of the codfish family, hake, mackerel, herring, sardines, melt, shad, capelin, sand lance, culpins, flatfishes, salmonids, etc. Cephalopods are the second most important source after fishes, crustaceans such as shrimps are believed to be important for recent weaned pups (Burns, 2009). Their diets will vary strongly by season and region, since it seems to be mainly linked to availability. Foraging trips mainly occurs quite near their haul-out, most seals stay within a 30 km range. Also, the use of a wide range of different foraging habitats by individuals was observed, this may indicate an individual specialization in foraging techniques. Furthermore, the foraging site fidelity may be related to previous foraging experiences (Hall et al., 1998; Sharples et al., 2012; Tollit et al., 1998).

Spatial changes of harbour seals in the German part of the Wadden Sea were observed by de la Vega et al. (2016). The harbour seals foraging during spring and summer is closer to their haul-out site, since the biomass of prey is at highest in the Wadden Sea at that period of time. In fall and winter, the harbour seals tend to forage more into the North Sea, when the biomass of prey items is relatively low in the Wadden Sea. On the other hand, they observed a shift from a pelagic based diet in spring to a benthic based diet in summer, fall and winter (de la Vega et al., 2016).

Each day, the harbour seal consumes about 5% of its weight, corresponding to approximately 4-5 kg of fish (Aarts et al., 2018; Fey et al., 2016). Finally, it has been demonstrated that whiskers play an important role in food research and prey capture (Zimmer, 2001).

3.5 Reproduction and reproductive behaviour

In general, female harbour seals reach sexual maturity at 3 to 4 years, while males reach sexual maturity at 4 to 5 years. Mating occurs in the water at about the time when pups are weaned (Marine Mammal Science Education Committee, 2018). Pregnancy rates exceed 85%, which means most female harbour seals bear a pup every year. Once fertilization has occurred, it is followed by a prolonged period of delayed implantation (embryonic diapause) that lasts up to about 2.5 months. The total gestation period, starting from the fertilization, is about 10.5 months (Burns, 2009). New-borns measure 75 cm on average and weight about 10 kg (Fey et al., 2016). A nursing period of about 4 weeks (sometimes up to 6 weeks) takes place, where mothers feed their pup with rich, fatty milk. (Burns, 2009; Marine Mammal Science Education Committee, 2018). For harbour seals of the European coast, most pupping occurs during late June and early July (Burns, 2009).

3.6 Threats

The maximum life span of harbour seals is around 35 years, although few animals live that long in the wild. They are threatened by anthropogenic activities and natural phenomena. The most important natural threat is diseases, the European population experienced two epidemics of phocine distemper virus (PDV) in 1988 and 2002, which respectively resulted in death of 23000 and 30000 harbour seals (Härkönen et al., 2006) and reduced the population in the Wadden Sea by 57% and 50% respectively. After the epizootics, the Wadden Sea population showed a strong recovery and has kept growing. An epizootic of influenza A virus subtype H10N7 reached the Wadden Sea in 2014, which caused increased mortality within the harbour seal population. Fortunately, the harbour seal population recovered quickly, the population grew at an annual rate of 12% (Aarts et al., 2018; Jensen et al., 2017).

Moreover, fisheries and aquaculture exert a big pressure on harbour seals. The tendency to prey on commercial important species has resulted in longstanding conflicts with fishermen (Burns, 2009). These fishing activities may also cause shifts and changes in community structures which are potentially irreversible and are seriously impacting harbour seals prey populations. In addition, fisheries cause bycatch of many species, including the harbour seal (Ducrotoy et al., 2000; Lowry, 2016; OSPAR, 2000).

Like other marine mammals, harbour seals are threatened by anthropogenic contaminants such as organochlorines (PCBs, DDT, etc.), MTEs and polycyclic aromatic hydrocarbons (PAHs) which can have influences on immune functions and reproductive ability. Marine mammals are usually at the top of the food chain, and often accumulate large amounts of those contaminants. Some suggests a possible immunosuppressive role of contaminants to explain the severity of pinniped epidemics (De Guise et al., 2003; Ross, 2002), while other linked the reduced pup production between 1950 and 1975 in the Wadden Sea to high PCB concentrations (Reijnders, 1986). An experiment where harbour seals were fed with PCB-contaminated fish for 2 years, reported reproductive failure in those animals. Another study where harbour seals were fed with highly contaminated Baltic Sea fish versus much less contaminated Atlantic Ocean fish, demonstrated that animals fed with Baltic fish resulted in impaired NK-cell activity, T-lymphocyte function and delayed type hypersensitivity (De Guise et al., 2003; Gregory & G. Cyr, 2003).

4. Biomarkers

Until the late of the 1980s, environmental monitoring of chemical substances was limited to the measurement of concentrations of those substances in water, sediments and living organisms.

However, this is an essential step to identify trends of contaminants in the environment, but this does not provide information of the real impact of those contaminants on living organisms. Physicochemical assessment is thus insufficient to evaluate the health in an organism with a mixture of contaminants potentially leading to disease (Michèle & Giambérini, 2013). For instance, the measurement of metal concentrations in tissues from organisms do not give a correct view of the metal toxicity. Some metals are known to interact, such as Se and Hg where elevated concentrations of both metals do not lead to toxicity. Additionally, measuring the total metal concentrations is not always relevant since it is a poor indicator for the toxic effect, for instance, MeHg is known to be much more toxic for organisms than inorganic Hg (Das et al., 2003).

This is why studies started to be interested in the biological response of organisms to pollutants, and this is how biomarkers were born. Biomarkers are early warning systems and can be noticed before the further deterioration of the structure and function of an organism. Further, they integrate the interactive effects of complex mixtures of chemicals (Bartell, 2006). As the definition of biomarkers has changed over the last 30 years, the definition proposed by Lagadic et al. (1997) remains the most complete until today, and indicates that: "*a biomarker is an observable and/or measurable change at the molecular, biochemical, cellular, or physiological level, which reveals a present or past exposure of an individual to at least one pollutant*". Further, biomarkers can be divided in different classes depending on what they indicate, and the biomarkers of exposure and effects are the most commonly used in ecotoxicology (Table 2).

Table 2: The two most common types of biomarkers, their definitions and the biomarkers used in this study (Schmidt, 2006).

Type of biomarker	**Definition**	**Used in this study**
Biomarkers of exposure	Indicative of exposure to one or more substances.	Total thiols, total glutathione, Selenium-dependent and -independent glutathione peroxidases.
Biomarkers of effects	Reflect health impairment and thus the consequences of exposure.	Malondialdehyde (TBARS)

In the case of harbour seals, different molecular biomarkers were used to evaluate pollutant exposure, health effects and immune status. For instance, mRNA transcription of aryl hydrocarbon receptor (AHR), aryl hydrocarbon receptor nuclear translocator (ARNT), peroxisome proliferator-activated receptor (PPARα), cytokines interleukin-2 (IL-2), interleukin-10 (IL-10) and heat-shock-protein 70 (HSP70) were investigated several times in blood from seals to assess xenobiotic exposure such as persistent organic pollutants (POPs) (Lehnert et al., 2014, 2016, 2017; Weirup et al., 2013). In Nyman et al. (2003), cytochrome P4501A activity and

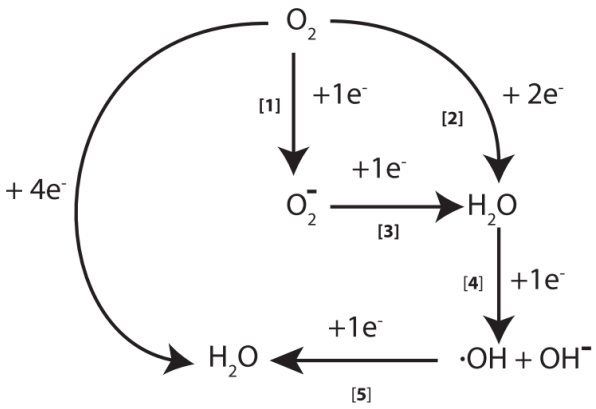

Figure 5: Reaction from the oxygen reduction: [1] production of superoxide anion radical, [2] formation of hydrogen peroxidase by reduction of oxygen, [3] formation of hydrogen by reduction for superoxide anion, [4] formation of hydroxyl radical, [5] reduction of hydroxyl radical to water (Blasco et al., 2016).

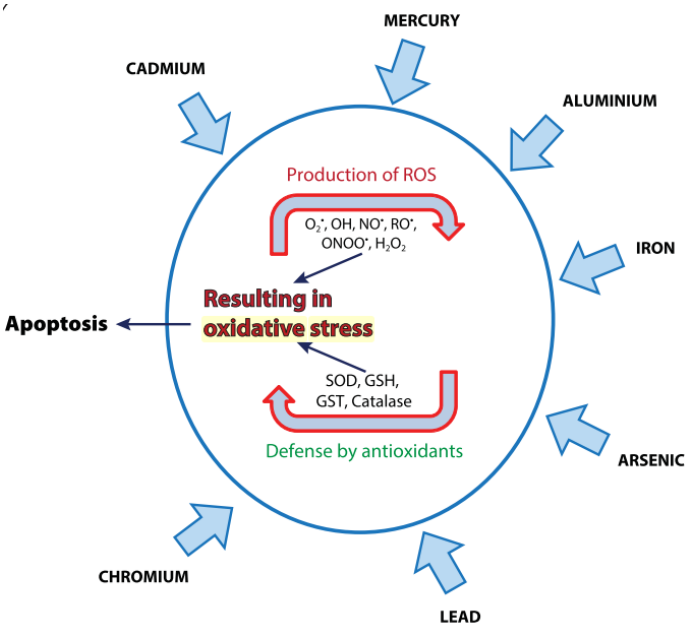

Figure 6: The attack from metallic trace elements on a cell and the balance between ROS production and antioxidant defence (Jaishankar et al., 2014).

vitamin E levels, in blubber or plasma, were proposed as exposure biomarkers for polychlorinated biphenyls (PCBs) and dichlorodiphenyl-trichloroethane (DDT) in Baltic ringed and grey seals.

However, the only specific biomarker used to evaluate metallic exposure in seals was metallothionein's (MTs) (Sonne et al., 2009; Teigen et al., 1999; Tohyama et al., 1986), a low-molecular-weight (6–7 kDa), cysteine-rich, non-enzymatic metal-binding proteins represented in the whole animal kingdom (Klaassen et al., 1999).

4.1 Oxidative stress

Aerobic organisms depend upon oxygen (O_2) for the provision of energy, where the energy transfer occurs via the phosphorylation of ADP. O_2 is than reduced to water by the mitochondrial electron transport chain. The partial reduction, caused by endogenous processes, results in a constant production of reactive oxygen species (ROS) such as hydrogen peroxidase, hydroxyl- and superoxide radicals (Figure 5), which could result in lipid peroxidation, DNA damage, depletion of sulfhydryl, and altered calcium homeostasis if antioxidant defenses are no longer effective (Blasco et al., 2016; Espín et al., 2014). Oxidative stress is thus a situation where there is an imbalance between the amount of ROS produced and the part which is eliminated (Lushchak, 2011).

It's known that production of reactive oxygen species (ROS) can be increased by MTEs exposure (Figure 6 and Figure 8). Indeed, redox-active metals such as iron, copper and chromium can catalyse Fenton-like reactions generating directly reactive oxygen species (Figure 7), whereas redox-inactive metals such as Pb, Cd, Hg and others induce oxidative stress indirectly by depleting the major antioxidants of cells, such as glutathione and other thiol-containing antioxidants and protein-bound thiol groups (Espín et al., 2014).

$$M^{(n)} + O_2^{\cdot -} \rightarrow M^{(n-1)} + O_2$$
$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$
$$M^{(n-1)} + H_2O_2 \rightarrow M^{(n)} + HO^{\cdot} + OH^-$$

Figure 7: Fenton-like reactions

4.2 Antioxidants

Indeed, to protect themselves against negative effects of ROS, organisms have developed an antioxidant defence system, to prevent, neutralize and remove harmful toxicants (Espín et al., 2014). The antioxidant defence system consists of low molecular mass and high molecular mass antioxidants. Low molecular mass antioxidants include both endogenous and dietary antioxidants such as reduced glutathione (GSH), ascorbic acid (vitamin C), carotenoids (including β-carotene), retinol (vitamin A) and α-tocopherol (vitamin E). These usually operates as free radical

scavengers, but other mechanisms can be implicated. In addition, the high molecular mass antioxidants consists of two groups. A specific group includes antioxidant enzymes superoxide dismutase, catalases, Se-dependent glutathione peroxidases, DT- diaphorase and needed cofactors such as glutathione reductase and glucose-6-phosphate dehydrogenase. Non-specific high molecular mass antioxidants are represented by proteins that prevent ROS-induced damage by binding to transition metal ions (mainly iron and copper) such as metallothionein's and ferritin (Lushchak, 2011).

Glutathione peroxidases

Glutathione peroxidases (GPx) are the major enzymes in the antioxidative defence mechanism depending on glutathione (Simona et al., 2008), the large family contains selenium-dependent (SeGPx) and selenium-independent (indSeGPx) enzymes. They protect cells from oxidative damage by reducing both free hydrogen peroxide (H_2O_2) to water and organic hydroperoxides to their corresponding alcohols.

$$2GSH + H_2O_2 \rightarrow GSSG + H_2O$$

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O$$

However, SeGPx can reduce organic peroxides (ROOH) and H_2O_2, while indSeGPx can only reduce organic hydroperoxides. GPx can be found in different cellular locations, including the cytosol, mitochondrial matrix and membranes (Regoli et al., 2011). Different types of GPx can be found: cytosolic, gastrointestinal, plasmatic and phospholipid (Simona et al., 2008).

Thiols

Thiols are a group of organic compounds containing a sulfhydryl group (-SH) attached to a carbon atom. In addition to glutathione, thiols group includes other sulphur-containing antioxidant compounds such as cysteine, methionine, taurine, lipoic acid, mercaptopropionylglycine, N-acetylcysteine, and the three major organosulfur compounds of Gallic oil (diallyilsulfide, diallyldisulfide and diallyltrisulfide) (Costa et al., 2006). Among all the antioxidants, thiols constitute the major portion of the body antioxidant, they play a significant role in de defence against ROS. They are both intracellular and extracellular, free or bound to proteins (Prakash et al., 2009).

Total glutathione

Glutathione, L-y-glutamyl-L-cysteinylglycine, is the most abundant cellular thiol and protects cells against toxic effects of a variety of endogenous and exogenous compounds, including trace metals and ROS. Glutathione can be found in two forms: reduced as GSH and oxidised as GSSG. GSH will protect cells against oxidative stress by acting as a direct scavenger of ROS. GSH will

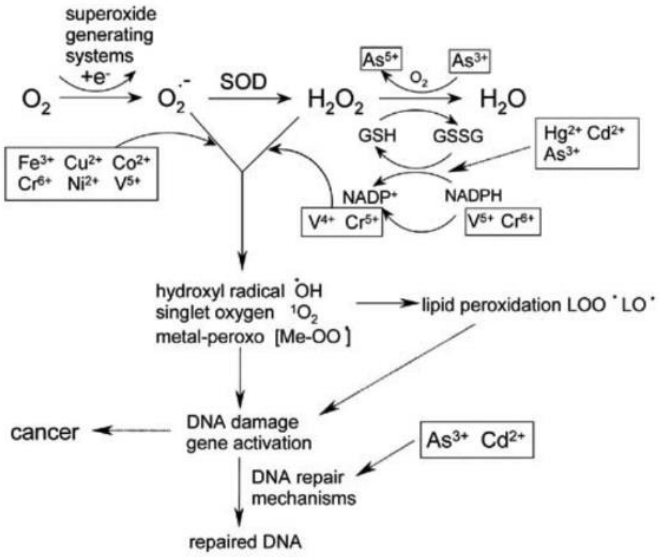

Figure 8: Pathway for metal induced oxidative stress (Valko et al., 2005).

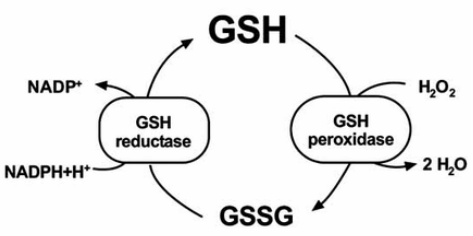

Figure 9: GSH:GSSG recycling is catalyzed by GSH reductase & peroxidase, and requires NADPH + H + (Anjaneyulu et al., 2008).

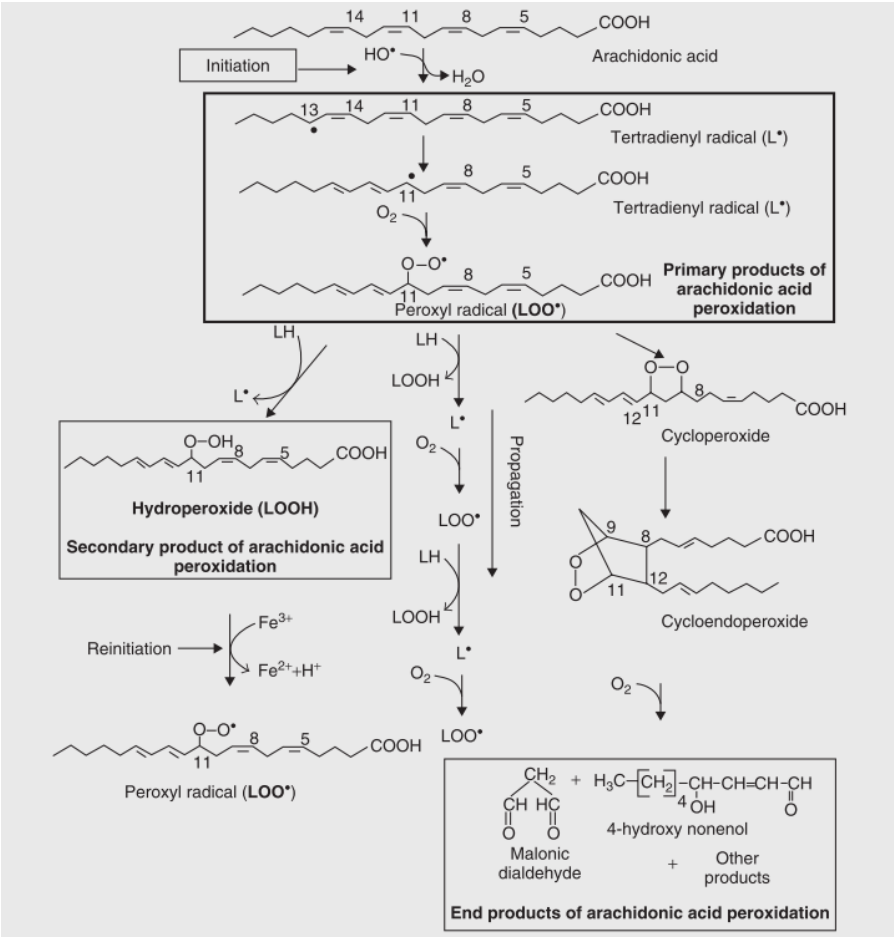

Figure 10: Suggested pathways of lipid peroxidation (Lushchak et al., 2011).

also participate in enzymatic detoxification reaction for ROS as it will act as a cofactor or coenzyme for enzymes such as glutathione S-transferase (GST) and glutathione peroxidases (GPx). Glutathione reductase (GR) allows the recycle of GSH once uses. Indeed, it will reduce GSSG to GSH by using NADPH (Figure 9) but when oxidative processes exceed the reducing capacity of GR, the excess of GSSG is excreted resulting in a net loss of total GSH from the tissue (Glutathione Assay Kit by Cayman chemical (No. 703002); Meister & Anderson, 1983; Regoli et al., 2011).

Levels of antioxidants and activities of antioxidant enzymes could be a useful biomarker of metal related oxidative stress. Information on antioxidant capacity by itself is not sufficient to make conclusions about oxidative stress (Espín et al., 2014). Costantini & Verhulst (2009), suggests that a marker of antioxidant capacity should always be associated with at least a marker of oxidative damage when the aim is to make conclusions about oxidative stress.

4.3 Oxidative damage

The most frequently used method to evaluate oxidative damage is to determine oxidation of lipids or lipid peroxidation (Figure 10), which is usually done by measuring the end products, such as malondialdehyde (MDA) and 4-hydroxynonenal (Lushchak, 2011).

Malondialdehyde (MDA)

MDA is an aldehyde that has been formed at the end of the lipid peroxidation. Polyunsaturated fatty acids with more than two methylene interrupted double bods will produce MDA if lipid peroxidation occurrs. In general, arachidonic acid (20:4) and docosahexaenoic acid (22:6) are the major precursors of MDA, where fatty acid with less than two double bounds will be poor precursors. MDA is the most commonly used product to determine lipid peroxidation and is considered to be an excellent pollutant biomarker of effect to indicate the presence of contaminants, including metals (Miyamoto et al., 2011).

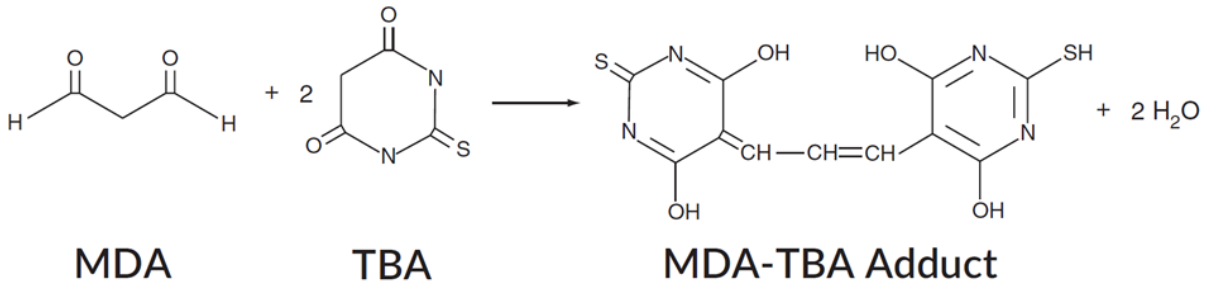

Figure 11: Formation of the (TBA)2-MDA adduct under high temperature and acidic conditions ((figure obtained from TBARS Assay Kit (No.10009055) by Cayman Chemical).

Materials and methods

1. Setting up of biomarkers assays

All biomarkers were analysed by spectrophotometry. Furthermore, this was the first time MDA, thiols, GPx and glutathione were measured in serum from harbour seals. For this reason, protocols had to be tested and adapted to harbour seal serum. Optimization of biomarker assessments were based on known protocols, preferable for serum. To perform the protocol optimizations, harbour seal serums dating from 2013 stored in -80°C were used. The sampling and sample preparation were performed as described in "B. Case study on harbour seals form the Wadden Sea" (see below). Sex, age, length, weight and date of sampling have not been taken in account for the optimisation. Moreover, if not mentioned, the reagents were prepared in distilled water.

1.1 TBARS

The TBARS assay is one of the most popular methods to measure end products of lipid peroxidation. This method is based on the formation of a reddish compound by the reaction of thiobarbituric acid (TBA) with MDA and other aldehyde products derived from secondary products of lipid peroxidation (Figure 11). Because this assay is not specific, MDA and the other aldehydes are regrouped as thiobarbituric acid reactive substances (TBARS). During the heating step of the assay, TBARS may form, this is why antioxidants such as butylated hydroxytoluene (BHT) are often added. The MDA assay used in this study was based on Lepage et al. (1991), which was developed for high-performance liquid chromatography (HPLC) with human plasma.

Initial protocol:

1) Add 240 µl of serum/blank (distilled water)/standard, 240µl of 0,5%BHT (prepared in methanol) and 960ml distilled water into a microtube and vortex.
2) Add 165 µl of TCA (trichloroacetic acid, 1.4M) to reach a final concentration of 150mM TCA and immediately vortex.
3) Centrifuge for 10 minutes at 1000 g.
4) Transfer 1200 µl of supernatant to a reaction tube.
5) Add 1 ml of 1% TBA (thiobarbituric acid) to the supernatant.
6) Adjust pH between 2.5 and 4.5 by adding approximately 220 µl of 1N NaOH.
7) Close tubes and heat for 60 minutes at 100°C.
8) Cool reaction tubes on ice for 4 minutes.

9) Add 150 µl of HCl (5N) to reach a pH lower than 0,75 (since n-butanol is only active at low pH).

10) Extract TBARS complexes from mixture by adding 750 µl of n-butanol, vortex for 1 minute and centrifuge for 10 minutes at 1000 g at 4°C.

11) Transfer the supernatant to a spectrophotometer cuvette and read absorption measured at 535 nm.

TCA, TBA and BHT solution were made freshly before performing the assay. Each sample and standard was analysed in duplicate and means were used for statistical analyses.

Calculations:

A MDA standard curve ranging from 0.3125 µM to 10 µM MDA was performed using a commercial 500 µM MDA solution, and the same assay as described above, in order to calculate the MDA concentrations which were expressed in µM TBARS/ mg proteins.

Protocol optimisation for harbour seal serum:

In order to obtain an optimized protocol to assay lipid peroxidation in harbour seals, several parameters have been tested. The parameters which were optimized are presented in Table 3.

Table 3: Optimization of different parameters of the TBARS assay aiming to obtain an adequate assay for serum of harbour seals.

Optimized parameter	Tested conditions	Why optimize this parameter?
1. Addition of 1N NaOH to standard	+0 µl (pH≈1.2) / +100 µl (pH≈1.5)/ +150 µl (pH≈1.7) / +200 µl (pH≈2)/ +250 µl (pH≈2.9) / +265 µl (pH≈3.6)	- The formation of the MDA-TBA complex is sensitive to pH. - Same pH conditions are needed for the sample as for the standard.
2. Addition of 1N NaOH to sample	+0 µl (pH≈1.1) / +150 µl (pH≈2)/ +200 µl (pH≈2.5) / +220 µl (pH≈3.5) / +230 µl (pH≈5.5)	- The formation of the MDA-TBA complex is sensitive to pH. - Same pH conditions are needed for the sample as for the standard.
3. Heating time at 100°C	30 min/40 min/50 min/60 min	- Optimal reaction time.
4. Extraction by n-butanol	Performed / not performed	- To check if this step is necessary.

Finally, to validate the assay, the standard addition technique was applied (see below for detailed method).

1.2 Total thiols

Total thiol group assay was adapted from the protocol described in Costa et al. (2006), established for human serum. This assay is based on the fact that thiol interacts with 5,5'-dithiobis-

(2-nitrobenzoic acid) or DTNB, forming a highly coloured anion with maximum absorbance at 412 nm.

Initial protocol:

1) Add 25 µl of serum/standard and 1 ml in KPi-EDTA buffer in a spectrophotometry cuvette and mix vigorously.
2) Make a blank by adding 1025 µl KPi-EDTA buffer to a spectrophotometry cuvette.
3) Set instrument autozero for 412 nm with the blank.
4) Read absorbance of the sample at 412 nm (=A1).
5) Add 25 µl of DTNB (10 mM in absolute methanol) to the solution and to the blank.
6) Incubate for 15 minutes at room temperature.
7) Read the absorbance of the sample (A2) and blank (B) at 412 nm.

For each sample, as for the standard curve, a duplicate was made and means were used for statistical analyses.

Calculations:

Results were calculated as follows: Absorption = A2-A1-B

where B is the absorbance of the background reaction; A1 is the background absorption of the serum used and A2 is the absorbance of the formed coloured anion with background absorption of the serum and the background reaction of the blank.

A standard curve, ranging from 3.9 µM to 500 µM, was performed using GSH as thiol reference. Each standard concentration was prepared in KPi-EDTA buffer (100 mM potassium phosphate buffer (KPi), 1mM ethylenediaminetetraacetic acid (EDTA), pH 7,5). The standard curve allowed to calculate the total thiol concentration expressed in µM eq. GSH/mg proteins.

Protocol optimisation for harbour seal serum:

In order to obtain an optimized protocol for total thiols in harbour seals, several parameters have been tested (Table 4).

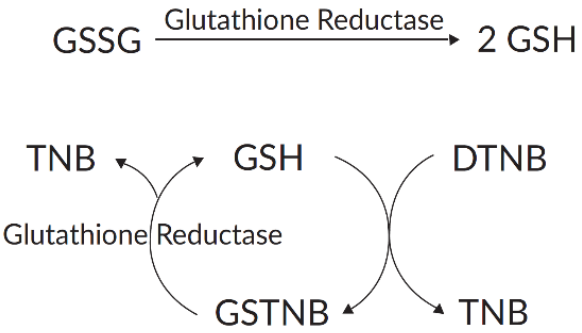

Figure 12: GSH recycling (figure obtained from Glutahione Assay Kit (No.703002) by Cayman Chemical).

Table 4: Optimization of different parameters of the total thiol assay aiming to obtain an adequate assay for serum of harbour seals

Optimized parameter	Tested conditions	Why optimize this parameter?
1. Serum/ standard dilutions and final reaction volume	- 25 µl of serum/standard + 1 ml KPi-EDTA +25 µl of DTNB (1/41, total reaction volume 1025 µl) - 125 µl of serum/standard + 900 µl KPi-EDTA +25 µl of DTNB (1/8.2, total reaction volume 1025 µl) - 100 µl of serum/standard +720 µl KPi-EDTA +20 µl of DTNB (1/8.2, total reaction volume 820 µl)	- To obtain measurable values but use as less serum as possible.
2. DTNB concentration	10 mM / 15 mM	- To test if reaction was not limited by a to low DTNB concentration.
3. Incubation time	5 min/ 10 min/ 15 min/ 20 min	- Optimal reaction time.
4. Absorption wave length	405 nm / 412 nm	- Optimal absorption wave length.

To validate the assay, the standard addition technique was applied (see below for detailed method).

1.3 Total glutathione

The total glutathione concentration was measured using the Total Glutathione kit by Cayman chemical (No. 703002). Briefly, this "indirect" assessment is based on the fact that the sulfhydryl group of GSH reacts with DTNB and produces a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) having an absorption peak between 405 and 414 nm (Figure 12). In order to measure the total glutathione concentrations (i.e. reduced and oxidized forms), the produced disulfide GSTNB is reduced by glutathione reductase to recycle the GSH and produce also TNB (Figure 12). Furthermore, GSSG is also reduced to GSH by glutathione reductase (Figure 12); hence, the assay reflects both GSH and GSSH, called thereafter "total glutathione" (tGSH).

Initial protocol:

The assay starts with a deproteinization step, recommended for serum and other tissues rich in proteins, in order to avoid potential interactions:

1) Add 100 µl 10 % metaphosphoric acid to 100 µl of serum sample.
2) Allow the mixture to stand at room temperature for 5 minutes.
3) Centrifuge at 10 000 g for at least 10 minutes.
4) For each 1ml of supernatant collected, add 50 µl of triethanolamine (TEAM 4M), this increased the pH of the sample. The sample is now ready to use.

The assay is performed as followed:

5) Add 50 µl of each standard concentration in each designated well, on the 96-well plate.

6) Add 50 µl of deproteinised sample to each of sample wells.

7) Add 150 µl of assay cocktail providing in the kit and containing MES buffer, Cofactor mixture, enzyme mixture, water and DTNB, using a multichannel pipette.

8) Cover the plate and incubate for 25 minutes while agitating.

9) Read absorbance at 405 nm in Multiskan EX (Thermo Fischer).

Each sample and standard was analysed in duplicate and means were used for statistical analyses.

Calculations:

A standard curve ranging from 0.25 µM to 8 µM GSSG was made with a 25 µM GSSG standard which was further diluted with MES buffer (0.2M 2-(N-morpholino) ethanesulphonic acid, 50 mM phosphate and 1 mM EDTA, pH 6) and expressed in equivalent total GSH. With the obtained equation of the standard curve, total glutathione concentration could be calculated and expressed in µM GSH/mg proteins.

Protocol optimisation for harbour seal serum:

In order to obtain an optimized protocol for total glutathione in harbour seals, several parameters have been adjusted (Table 5).

Table 5: Optimization of different parameters of the total glutathione assay aiming to obtain an adequate assay for serum of harbour seals

Optimized parameter	Tested conditions	Why optimize this parameter?
1. Standard curve	Replace GSSG with GSH	- The standard curve with GSSG was not so accurate and stable, therefore GSH was tested.
2. Addition of 4M TEAM to standard and sample	+5 µl TEAM / +5.5 µl TEAM / +6 µl TEAM / +6.5 µl TEAM per 100µl of supernatant	- TNB formation is sensitive to pH - Same pH conditions are needed for the sample as for the standard.

To validate the assay the standard addition technique was applied (see standard addition technique later).

1.4 Glutathione peroxidase (GPx)

To determine the selenium-dependent (Se-GPx) and selenium-independent (indSe-GPx) activities, protocols were obtained from Regoli et al. (2011). This assay is considered as a general assay and can be used for different tissues and organisms. However, Se-GPx reacts with a wide variety of hydroperoxides, including both H_2O_2 and organic peroxides, whereas the Se-independent forms reduces only organic hydroperoxides.

$$2GSH + H_2O_2 \rightarrow GSSG + H_2O$$
(Se-GPx)

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O$$
(both Se-GPx and indSe-GPx)

This characteristic was exploited in this assay to distinguish both forms of GPx. The change in absorbance measured will be due to the consumption of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is used by glutathione reductase (GR) to reconvert glutathione disulphide (GSSG) to GSH. This decrease in NADPH concentration, followed at 340 nm, is proportional to the GPx activity.

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

Before measuring GPx activities, a blank reaction had to be determined. This was done for the two GPx forms by following the protocol and replacing the sample by KPi buffer ($= \Delta abs_{blank}$), and performed 5 times for each biomarker before starting. Reactives were always freshly prepared before the assay, except for KPi buffer (100 mM) and EDTA (100 mM), which both could be stored up to 3 months at 4°C.

Initial protocol:

Se-dependent GPx	**Se-dependent and Se-independent GPx**
1) Add to a spectrophotometry cuvette (final volume 1 ml)	
- 885 µl of KPi buffer (100 mM) - 10 µl of EDTA (100 mM) - 20 µl of GSH (100 mM) - 10 µl of GR (100 $U.ml^{-1}$) - 10 µl of NaN_3 (100 mM) - 50 µl of blank (KPi buffer) or sample	- 896 µl of KPi buffer (100 mM) - 10 µl of EDTA (100 mM) - 20 µl of GSH (100 mM) - 10 µl of GR (100 $U.ml^{-1}$) - 50 µl of blank (KPi buffer) or sample
2) Vigorously mix cuvette and set instrument autozero for measurements at 340 nm.	
3) Add 10 µl of NADPH (20 mg/ml) and read absorption at 340 nm, this should give a value between 0,9 and 1,2.	

4) Add 5µl of H_2O_2 (100 mM) and vigorously mix.	4) Add 4 µl of cumene hydroperoxide (CHP, 200 mM, diluted in methanol) and vigorously mix.

5) Measure the decrease in absorbance for at least 1 minute at 340 nm, the reactions should be linear, if not the sample should be diluted.

Each sample was measured in triplicate, and means were used for statistical analyses.

Calculations:

The real change in absorption from the sample ($= \Delta abs_{final\ sample}$) is obtained by subtracting the rate of blank reaction ($= \Delta abs_{blank}$) from the rate in absorbance per minute from the sample ($= \Delta abs_{sample}$). With the extinction coefficient of NADPH at 340 nm (6220 M^{-1} cm^{-1}), the activity could then be expressed in nmol min^{-1} mg^{-1} protein, using the Beer-lambert law:

$$C = \frac{\varepsilon * l}{A}$$

With:

- C = concentration of NADPH in mol/l
- ε = extinction coefficient of NADPH at 340 nm (= 6220 M^{-1} cm^{-1})
- l = Length of the optical path in centimetre
- A = absorbance

Protocol optimisation for harbour seal serum:

Sins no verification could be done with the standard addition technique, because GPx is an enzyme, and no standard curve was used. No parameters were changed.

1.5 Triglyceride

Triglycerides are the main source of stored energy in organisms and are thus needed to maintain the oxidative defence mechanisms of the organisms, to see if those are affected by oxidative stress, they were decided to be added to this study. Triglyceride concentrations were determined in serum samples by using a commercial kit from DiaSys. This kit is known to work well on serum and this is why no optimisation was performed.

Initial protocol:

Before starting, the serum samples were diluted 4 times in 0.9% NaCl because of the high lipid content in serum.

1) Add 10 µl of diluted sample or standard in a dedicated well of a 96 plate.
2) Add 240 ml of the FS solution (DiaSys) to each well.

3) Put the plate in the Fluoroskan programmed for 5 cycles of 1 minute incubation at 37°C followed by mixing at 600 rpm for 1 minute (10 minutes in total).
4) Put plate in the Multiskan and read absorbance at 492 nm.

Each sample and standard was analysed in duplicate.

Calculations:

A standard curve ranging from 6.25 to 71.43 mg/dl triglyceride was made using a standard solution of 200 mg/dl triglyceride. All dilutions were made in 0.9% NaCl. The obtained equation of the standard curve was used to calculate triglyceride concentrations which were expressed in mg/dl serum.

1.6 Analysis of proteins

In order to normalize all the measured biomarkers, protein concentrations were determined in serum samples by the Bradford protein assay (1976), which is a spectroscopic analytical procedure. For this purpose, a ”Bradford solution” was prepared as followed::

- 100 mg Coomassie blue
- 50 ml ethanol (95%)
- 100 ml phosphoric acid 85%
- 850 ml Milli-Q water

The solution was than filtered with a 0.45 µm filter, and let for 1 night at 4°C to stabilize it before the use. The Bradford solution is stored at 4°C and is stable for 1 year.

Initial protocol:

1) Add 100 µl of sample or standard to a reaction tube.
2) Add 5 ml of Bradford solution and let solution rest at room temperature for 10 minutes.
3) Read absorbance at 595 nm.

For each sample as for the standard curve a triplicate was made.

Calculations:

A standard curve ranging from 32.25 to 1000 µg/ml BSA (bovine serum albumin) was prepared in KPi buffer (pH 7.5, 100 mM). The obtained equation of the standard curve, allowed to calculate the protein concentrations which were expressed in µg/ml serum.

Protocol optimisation for harbour seal serum:

Different dilutions of serum samples were tested, because of the high protein content in serum. Serum dilutions of 4, 20, 50 and 100 times were tested.

1.7 Standard addition technique

Except for GPx, triglyceride and protein assessments, all optimized protocol were verified using a standard addition technique, consisting to add a known concentration standard to the sample (serum in this case). The absorbance measured of this mix (absorption C) minus the absorbance of the serum (absorbance A), should than be equal to the absorbance of standard at its final concentration (absorption B) (Figure 13). The obtained value may maximum differ for 10% from the expected value of the standard at this final concentration (measured during standard curve).

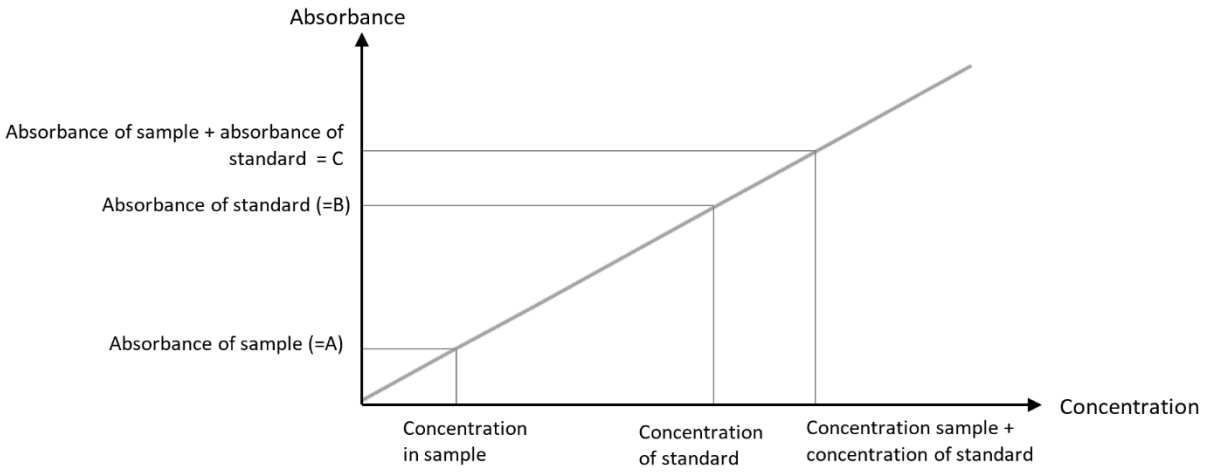

Figure 13: Graphical representation of the standard addition technique.

2. Case study on harbour seals form the Wadden Sea

Sampling, serum preparation and analysis of metallic trace elements was performed by France Damseaux (FRS-FNRS PhD student – Laboratory of Oceanography – ULiège).

2.1 Sampling

Blood samples from 21 free-ranging harbour seals were collected on the sandbank Lorenzenplate (54°25’ N and 8°38’ E) in the German part of the Wadden Sea, during spring (March) and autumn (October) 2015. This was done in collaboration with professor Ursula Siebert from the Institute for Terrestrial and Aquatic Wildlife Research (ITAW) of the University of Veterinary Medicine Hanover. Sex, length and weight were determined before blood samplings. In total, 10 males and 11 females were sampled, from which 7 seals sampled during October (males=2, females=5) and 14 in March (males=8, females=6). The manipulation was performed

as fast as possible and the seals were under continuous veterinary observation. Blood samples designated for serum and blood cells were transferred to Vacutainer BD red top tubes and were put on ice until arrival at the laboratory. The field studies were carried out under the relevant permits of the National Park Office Schleswig-Holstein and the animal experiment permit (AZ 312-72241.121-19).

2.2 Sample preparation and storage

Serum and blood cells were prepared in the laboratory at the University of Veterinary Medicine Hanover by allowing the blood to clot at room temperature. The clotted blood was than centrifugated during 5 minutes at 3000 g in a refrigerated centrifuge. The resulting supernatant and pellet correspond to the serum and the blood cells, respectively. The serum of each individual was apportioned in 3 microtubes of 0.5 ml, 0.5 ml and 1 ml. Both tissues were transported on dry ice to the laboratory of the Liège University, where the serum was stored at -80°C and the blood cells at -20°C until analysis.

2.3 Analysis of metallic trace elements

Mercury

For the total mercury, about 10 mg of freeze-dried blood cells were accurately weighed and loaded into quartz boats. These were preheated to 400°C for 5 minutes to remove any impurities of mercury. Total Hg (T-Hg) levels were determined by atomic absorption spectroscopy, AAS, with the DMA-80 (Direct Mercury Analyzer, Milestone). The method has been validated for solid samples using US EPA Method 7473. Quality assurance methods included evaluating by measuring blanks (HCl 1%), duplicates and CRMs – Seronorm L-3 with every 10 samples. T-Hg concentrations are expressed in $ng.g^{-1}$ dw.

Other metallic trace elements

Concentrations of metallic trace elements Cd, Cr, Cu, Fe, Ni, Pb, Se, V and Zn were measured in blood cells. The method used is based on Habran et al. (2012), where about 0.15 - 0.18 g of freeze-dried blood cells were weighed to the nearest 0,1 mg. All these samples were subjected to microwave-assisted digestion in Teflon™ vessels with 3 ml HNO_3 (65%), 1 ml H_2O_2 (30%) and 4 ml of 18,2 MΩ-cm deionized water. After cooling, samples were diluted to 50 ml with 18,2 MΩ-cm deionized water in a volumetric flask. The levels of Cd, Cr, Cu, Fe, Ni, Pb, Se, V and Zn were determined by inductively coupled plasma mass spectroscopy (ICP-MS, PerkinElmer, Sciex, DCR 2). Multiple elements (74Ge, 103Rh, 209Bi, 69Ga) of internal standards (CertiPUR®, Merck) were added to each sample and calibration of standard solutions. Quality control and quality assurance for ICP-MS included field blanks, method blanks, certified reference materials

(CRMs) – Seronorm L-3 andDOLT-3. Reported concentrations for all elements in blood were expressed on a dry weight (dw) basis in ng. g^{-1}.

2.4 Analysis of biomarkers

TBARS, total thiols, total glutathione, triglyceride and proteins concentrations, as well as Se-GPx and indSe-GPx activities were performed by using the optimized protocols developed in the first part of this Master thesis. The obtained protocols can be found in the second part of the results, and completed protocols are presented in appendix 1.

2.5 Statistical analysis

Analyses were carried out using XLSTAT 2018. Descriptive statistics such as mean ± standard deviation, median and range were reported for MTE concentrations and oxidative stress biomarkers. The data were tested for normality using the Shapiro–Wilk test. When the data did not meet the normality (p-value > 0.05), differences were analysed by the Mann-Whiney U test, while a T-test was used when normality was respected. A principal component analysis (PCA) was also carried out in order to observe potential correlation between all the measured parameters (biomarkers and MTE concentrations). To go further in the evaluation of the effects of each metal on the oxidative stress biomarkers, a Pearson correlation matrix was made. This also allowed to know which biomarkers were relevant for this purpose and how they were impacted. Pearson correlation matrix was also made between the oxidative stress biomarkers.

Results

1. Setting up of biomarkers assays

1.1 TBARS

In the TBARS assay various parameters were investigated, such as the addition of different amounts of NaOH to serum and standard, incubation time and extraction by n-butanol. Conditions which were considered to be most optimal can be found in Table 7, these parameters were therefore used for the case study on harbour seals.

1. Addition of 1N NaOH to standard

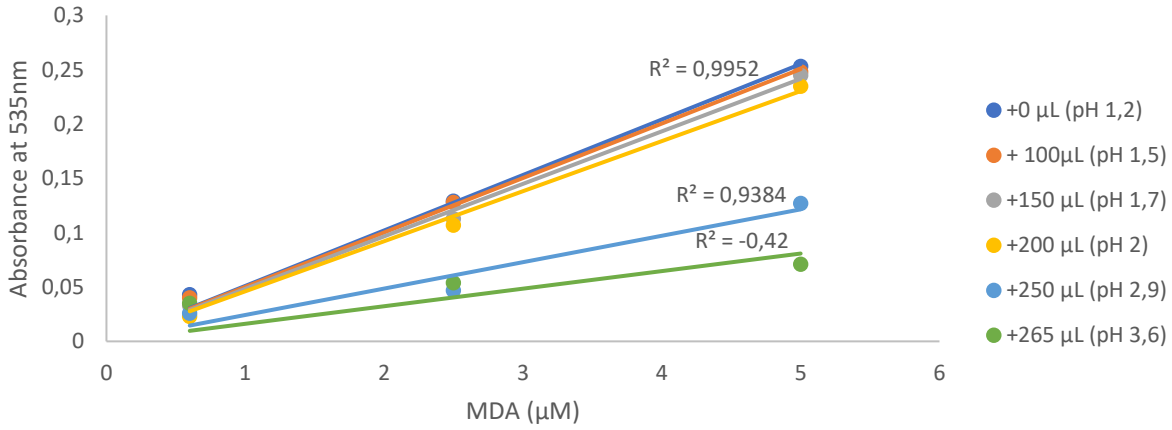

Figure 14: Optimization of volumes of 1N NaOH to the standards (with corresponding pH values) and their regression.

Considerable lower absorbances and less good linear fit of standard curves were obtained when pH was adjusted before the heating step to pH's higher than 2.9. Similar standard curves were observed at pH 1.2, 1.5, 1.7 and 2 by respectively addition of 0 µl, 100 µl, 150µL and 200 µl 1N NaOH to the mixture of supernatant and TBA before the heating step, those gave higher absorbances and a correct linear fit was obtained (Figure 14).

2. Addition of 1N NaOH to sample

Addition of 1N NaOH to the sample before the heating step gave similar results at pH 1.1 and 2. With increased pH, a decrease, in absorbance was observed (Table 6). Further, that same pH of standard and sample were not reached by addition of the same volumes of NaOH. A pH of 2 in sample was reached by addition of 150 µl of 1N NaOH, while a pH of 2 in standard was obtained by addition of 200 µl 1 N NaOH. To be comparable with literature, the pH was further adjusted to 2, for both standard and sample and a standard addition was tested with this.

Table 6: Optimization of volumes of 1N NaOH to serum (with corresponding pH values) and its influence on the absorbances.

Addition of 1N NaOH to sample	Test on sample 1	Test on sample 2
+0 µl (pH≈1.1)	0.017/0.018	0.043/0.048
+150 µl (pH≈2)	0.018/0.019	0.049/0.042
+200 µl (pH≈2.5)	0.016/0.015	0.049/0.037
+220 µl (pH≈3.5)	0.007/0.006	
+232 µl (pH≈5.5)	0.005/0.005	0.031/0.029

Values on both sides of the '/' represents duplicates.

3. Heating time at 100°C

Slightly higher absorbances were measured in sample 1 at 30 minutes compared to 60 minutes. For the gain of time and the slightly higher observed values, 30 minutes were chosen as heating time and tested for the standard addition technique.

4. Extraction by n-butanol

Higher absorbance values were found when n-butanol extraction was performed. For this reason and because it compensates the dilution caused by adding different quantities of NaOH to sample and serum, it was chosen to test a standard addition with the extraction with n-butanol.

Table 7: Summary of selected condition to be potentially optimal

Optimized parameter	Conditions considered as potentially optimal
1. Addition of 1N NaOH to standard	+0 µl (pH≈1.2) / +10 0µl (pH≈1.5) +150 µl (pH≈1.7) / **+200 µl (pH≈2)**/ +250 µl (pH≈2.9) / +265 µl (pH≈3.6)
2. Addition of 1N NaOH to sample	+0 µl (pH≈1.1) / **+150 µl (pH≈2)** /+200 µl (pH≈2.5) / +220 µl (pH≈3.5) / +230 µl (pH≈5.5)
3. Heating time at 100°C	**30 min**. / 40 min. / 50 min. / 60 min.
4. Extraction by n-butanol	**Performed** / not performed

Standard addition performed with all conditions considered to be potentially optimal:

As can be seen in Table 8, the difference between estimated and expected absorbance, in this case for a 5 µM MDA solution, was relatively small, with 6% and <1% differences for the two tested samples. In order to confirm the performance of the optimized protocol, a similar standard addition was performed by adding 230 µl of serum and 10 µl of a 60 µM MDA solution. Difference between obtained and expected absorbance of the 2.5 µM MDA solution were 10% and 4% respectively for Pv 20080 and 20081. The standard addition was thus considered to be correct and therefore those conditions were considered as optimal.

Table 8:Standard addition with all conditions considered as optimal (Table 7) for the TBARS assay.

	Test 1: Pv 20080	**Test 2: Pv 20081**
Mean Absorbance of standard addition (230 µl serum + 10 µl 120 µM MDA)	0.238	0.273
Mean absorbance sample (240 µl serum)	0.023	0.035
Estimated absorbance for MDA concentration at 5 µM	0.238 – (0.023*0.958[a]) = 0.216	0.273 – (0.035*0.958[a]) = 0.239
Mean absorbance of standard at 5 µM MDA	0.229	0.240
Difference between estimated and expected absorbance of a 5 µM MDA solution	$\|0.216 - 0.229\| =$ 0.013 (6%)	$\|0.239 - 0.240\| =$ 0.001 (<1%)

Mean absorbance represents the mean of duplicates.

[a] corresponds to the dilution of the serum by performing the standard addition: 230 µl/240 µl serum.

1.2 Total thiols

Serum dilutions, reaction volume, DTNB concentrations, incubation time and abortion wave length are the parameters which were investigated for the total thiols assessment.

1. Serum/ standard dilutions and reaction volume

Dilutions at 1/41 gave absorbances at the limit of detection, dilution at 1/8.2 gave absorbances in detection range of our spectrophotometer (Figure). As 1/8.2 dilutions in reaction volume of 820 µl gave the same results as the reaction in 1025 µl final volume (Figure). The standard addition technique was tested with a 1/8.2 dilution in a final volume of 820 µl.

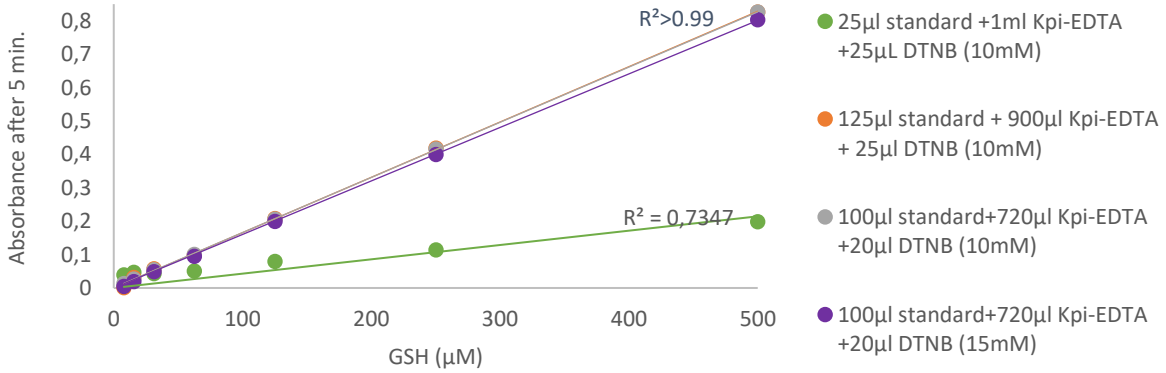

Figure 15: Standard curves with different serum dilutions and different DTNB concentrations

2. DTNB concentration

No significant difference in standard curve between the two concentrations were observed (Figure). As 10 mM was described in the original protocol, the standard addition technique was tested with this condition.

3. Incubation time

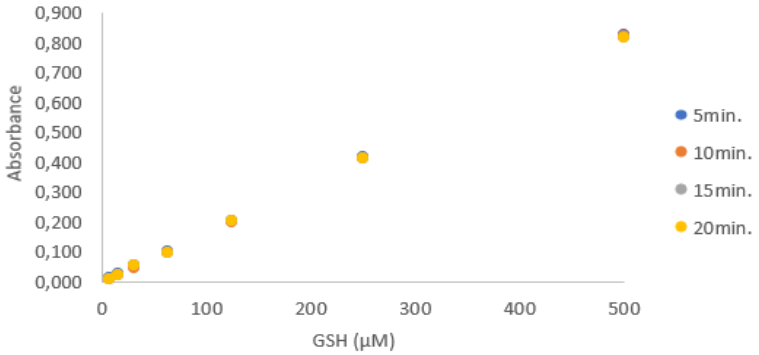

Figure 16:Standard curves after 5, 10, 15 and 20 minutes of incubation.

No difference in standard curves between 5, 10,15 and 20 minutes of incubation (Figure), the same results were obtained for tests on serum. The standard addition was performed with an incubation time of 5 minutes.

Table 9: Summary of selected condition to be potentially optimal

Optimized parameter	**Conditions considered as most optimal**
1. Serum/ standard dilutions and reaction volume	- 25 µl of serum/standard + 1 ml KPi-EDTA +25 µl of DTNB (1/41, total reaction volume 1025 µl) - 125 µl of serum/standard + 900 µl KPi-EDTA +25 µl of DTNB (1/8.2, total reaction volume 1025 µl) - **100 µl of serum/standard +720 µl KPi-EDTA +20 µl of DTNB (1/8.2, total reaction volume 820 µl)**
2. DTNB concentration	**10 mM** / 15 mM
3. Incubation time	**5 min.** / 10 min. / 15 min. / 20 min.

Standard addition performed with all conditions considered to be potentially optimal:

Table 10: Standard addition with all conditions considered as optimal (Table 9) for the total thiol assay.

	Test: Pv 20098
Absorbance of standard addition (50 µl serum + 50 µl 500µM GSH, final concentration 250 µM GSH)	0.539
Mean absorbance sample (100 µl serum)	0.254
Estimated absorbance for GSH concentration at 250 µM	0.539 – (0.254*0.5[a]) = 0.412
Mean absorbance of standard at 250 µM GSH	0.423
Difference between obtained and expected absorbance of a 250 µM GSH solution	$\|0.412 - 0.423\| = 0.011$ (2.6%)

Mean absorbance represents the mean of duplicates.

[a] corresponds to the dilution of the serum by performing the standard addition: 50 µl/100 µl serum.

From this standard addition (Table 10) we can consider the condition used as optimal as a correct standard addition was obtained with only a 2.6% difference between estimated and expected absorbance, in this case for a 250 µM GSH solution.

1.3 Total glutathione

In Table 11 the most optimal conditions for the different investigated parameters of the total glutathione assessment on harbour seal serum can be found.

1. Standard curve

The standard curve with GSSG presented more variations in duplicates, which was not the case for GSH (Figure 17:).

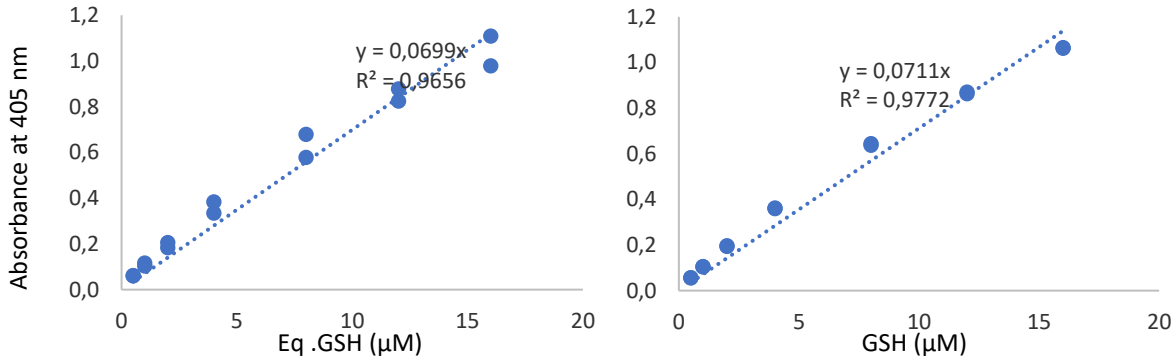

Figure 17:Standard addition curve for the tGSH assay performed with GSSG (left) and GSH (right).

2. Addition of 4 M TEAM to standard/sample

A pH difference was obtained between standard and serum when adding 5µl of TEAM to 100 µl of supernatant of standard and serum. A pH of 4 was obtained in the standard while a pH of 6 was obtained in the sample. Addition of 6µl TEAM to both standard and serum gave a pH of 7 to both of the solutions. The pH difference was important as a standard addition with addition of 5µl TEAM to both standard and sample gave considerable bad results. The standard addition (Table 11) when both standard and serum had same pH of 7, gave correct results.

Table 11: Summary of selected condition to be potentially optimal for the total glutathione assay in serum of harbour seals.

Optimized parameter	Conditions considered as most optimal
1. Standard curve	1. **With** / without **deproteinization** 2. Standard curve made with GSSG / **GSH**
2. Addition of 4 M TEAM to standard/sample	1. Standard: +5 µl TEAM (pH≈ 4) / +5.5 µl TEAM (pH≈ 6)-/ **+6 µl TEAM (pH≈ 7)** / +6.5 µl TEAM (pH≈ 7) 2. Sample: +5 µl TEAM (pH≈ 6) / +5.5 µl TEAM (pH≈ 7) / **+6 µl TEAM (pH≈ 7)** / +6.5 µl TEAM (pH≈ 7)

Standard addition performed with all conditions considered as optimal:

Table 12: Standard addition with all conditions considered as optimal (Table 11) for the total glutathione assay.

	Test 1: PV 21503	**Test 2: PV 21504**
Mean Absorbance of standard addition (90µl serum +10 µl 80µM GSH, final concentration of 8µM added GSH)	0.292	0.302
Mean absorbance sample (100µl)	0.055	0.070
Estimated absorbance for GSH concentration at 8µM	0.292 – (0.055*0.9) = 0.243	0.302 – (0.070*0.9) = 0.239
Mean absorbance of standard at 8µM	0.232	0.232
Difference between estimated and expected absorbance of a 8 µM GSH solution	$\|0.243 - 0.232\|$ = 0.010 (4%)	$\|0.239 - 0.232\|$ = 0.007 (3%)

Mean absorbance represents the mean of duplicates.

[a] corresponds to the dilution of the serum by performing the standard addition: 90µl/100µl serum.

Conditions were considered as optimal, a correct standard addition was obtained with only a 4% and 3% difference between estimated and expected absorbance, in this case for a 8 µM GSH solution.

1.4 Glutathione peroxidase

No tests could be effectuated on those protocols because no standard addition could be performed and thus correctness of tests could not be verified, however the protocol gave measurable values.

2. Case study on harbour seals

2.1 Metallic trace metals

MTE concentrations in blood cells of the harbour seal considering sex and season are presented in Table 13. Significant differences among sexes were observed in T-Hg (p= 0.015), and Zn (p= 0.049) concentrations, with the highest median levels of both MTEs in males. Significant difference in weight between M/F are observed (p=0.004), however the possibility that difference between male/female of T-Hg and Zn are due to weight differences is excluded since no significant correlations are found between T-Hg, Zn and weight. From the analysed metallic trace elements, only Zn showed significant differences between seasons (p=0.032), with the highest median in October. Further when we divided Zn following the gender, only seasonal variation were observed for the females (p=0.019) with the highest concentrations in October, while no significant variation was found for males between March and October (p=0.064).

Table 13: Metal concentrations in blood cell samples (ng/g, dry weight) of harbour seals for all individuals and according to sex and season.

Metal concentrations (ng/g) in blood cells of harbour seal

			T-Hg	Zn	Cd	Fe	Cu	Se	Pb	Ni	Cr	Li	V
All individuals (n=21)		Mean ± SD	1123 ± 332	12249 ± 690	10.73 ± 34.14	2990983 ± 65653	2287 ± 165	3146 ± 523	19.81 ± 6.72	26.73 ± 27.49	56.28 ± 34.62	48.36 ± 10.96	2.25 ± 2.37
		Median	1060	12319	1.64	2984320	2270	3183	17.82	15.79	48.29	49.36	1.76
		Range	365 - 1891	10389 - 13341	0.23 - 157.66	2819105 - 3103939	2028 - 2637	1831 - 4185	12.83 - 43.79	1.56 - 110.93	20.31 - 145.95	28.59 - 67.98	0.57- 12.15
Sex	Female (n=11)	Mean ± SD	950 ± 250*	11954 ± 738*	3.85 ± 7.34	2992050 ± 74341	2327 ± 181	3022 ± 494	22.22 ± 8.29	34.37 ± 34.47	55.06 ± 21.58	49.60 ± 11.40	2.52 ± 3.26
		Median	937	12189	1.64	2984320	2354	3102	19.93	18.33	51.96	50.25	1.68
	Male (n=10)	Mean ± SD	1312 ± 316*	12574 ± 478*	18.29 ± 49.09	2989809 ± 58607	2242 ± 140	3281 ± 545	17.16 ± 3.00	18.33 ± 14.47	57.61 ± 46.28	47.00 ± 10.89	1.96 ± 0.68
		Median	1253	12488	1.41	2998618	2222	3201	16.63	13.38	32.62	46	1.95
Season	March (n=14)	Mean ± SD	11993 ± 297	12054 ± 716*	2.53 ± 2.89	2977226 ± 73918	2266 ± 177	3261 ± 486	20.62 ± 7.63	21.94 ± 26.70	54.79 ± 39.21	50.68 ± 9.93	2.00 ± 0.69
		Median	1190	12158	1.64	2976442	2230	3266	19.34	13.38	43.03	49.81	2.20
	October (n=7)	Mean ± SD	971± 370	12640 ± 458*	27.12 ± 58.29	3018497 ± 34703	2327 ± 140	2916 ± 554	18.18 ± 4.45	36.30 ± 28.53	59.26 ± 25.46	43.73 ± 12.21	2.76 ± 4.15
		Median	942	12654	1.45	3014643	2354	3025	16.43	31.40	59.00	47.27	1.35

n= number of samples

* Significant differences in metal concentrations according to sex and season: $p<0.05$.

2.2 Oxidative stress biomarkers

Proteins, triglyceride, TBARS, total thiols, total glutathione concentrations and GPx activities are presented in Table 14, considering sex and season. Only TBARS showed significant differences ($p<0.001$) between season, with higher concentrations in fall. All other measured biomarkers highlighted, no difference between males and females, and considering the sampling season.

Table 14: Proteins, triglyceride concentrations, enzyme activities, glutathione levels and lipid peroxidation in serum of harbour seals, and values according to sex and season.

Proteins, triglyceride, lipid peroxidation, thiol levels and enzyme activities and in serum									
			Proteins[a]	Triglyceride[b]	TBARS[c]	tThiols[d]	tGSH[e]	GPx[f]	SeGPx[g]
All individuals (N=21°)		Mean ± SD	81035 ± 4745	77.56 ± 18.53	0.0116 ± 0.0046	2.35 ± 0.56	0.0267 ± 0.0102	3.82 ± 0.68	3.36 ± 0.55
		Median	81413	78.61	0.0107	2.27	0.0253	3.69	3.37
		Range	69132 - 89414	37.22 - 118.33	0.0054 - 0.0216	1.64 - 3.66	0.0122 - 0.0486	3.00 - 4.97	2.66 - 4.32
Sex	Female (N=11°)	Mean ± SD	82185 ± 4865	82.72 ± 21.12	0.01 ± 0.01	2.26 ± 0.51	0.03 ± 0.01	3.81 ± 0.69	3.30 ± 0.56
		Median	81688	83.33	0.0100	2.07	0.03	3.69	3.16
	Male (N=10)	Mean ± SD	79771 ± 4510	72.39 ± 14.81	0.0097 ± 0.0030	2.45 ± 0.62	0.0261 ± 0.0105	3.83 ± 0.72	3.42 ± 0.55
		Median	80801	70.28	0.0093	2.5	0.0231	3.87	3.48
Season	March (N=14)	Mean ± SD	81781 ± 3757	78.77 ± 14.22	0.0095 ± 0.0033*	2.38 ± 0.52	0.0264 ± 0.0110	3.80 ± 0.69	3.31 ± 0.61
		Median	81590	81.67	0.0086	2.41	0.0246	3.67	3.09
	October (N=7°)	Mean ± SD	79544 ± 6370	74.72 ± 27.65	0.0158 ± 0.0040*	2.28 ± 0.67	0.0274 ± 0.0091	3.87 ± 0.73	3.45 ± 0.43
		Median	79097	74.17	0.0145	1.97	0.0271	3.99	3.38

N= number of samples.
° Number of samples for triglyceride= N-1
* Significant differences in biomarker concentrations according to season : $p \leq 0.001$.
[a] Proteins (µg /ml).
[b] Triglyceride (mg/dl).
[c] Lipid peroxidation, estimated as thiobarbituric acid-reactive substances (nmol/mg proteins).
[d] Total thiols (nmol/mg proteins).
[e] Total glutathione (nmol/mg proteins).
[f] Glutathione peroxidase (nmol/min/g protein).
[g] Se-dependent glutathione peroxidase (nmol/min/g protein).

2.3 Oxidative stress biomarkers response to MTE exposure

Despite some low levels of metals, several oxidative stress biomarkers correlated with metallic trace element concentrations. The principal component analysis (Figure 18) for all individuals, all sex and seasonal groups combined, shows that component 1 explains 21.78% of the variability of the samples and component 2 explains 15.23% of this variability. Together, these two factors explain 37.01% of the total variability of the samples. All metals, except for T-Hg and Se, are positively correlated with component 1. We can observe a strong positive correlation between GPx and SeGPx, positive correlations between those enzymes and tGSH is also found, further Cd,

Ni and Zn are following the same tendency. Negatively correlated to those, we found T-Hg. Se shows negative correlations with V, Li, Fe, CR, Pb, Cu and triglycerides. Further an opposite correlation is found between TBARS and total thiols.

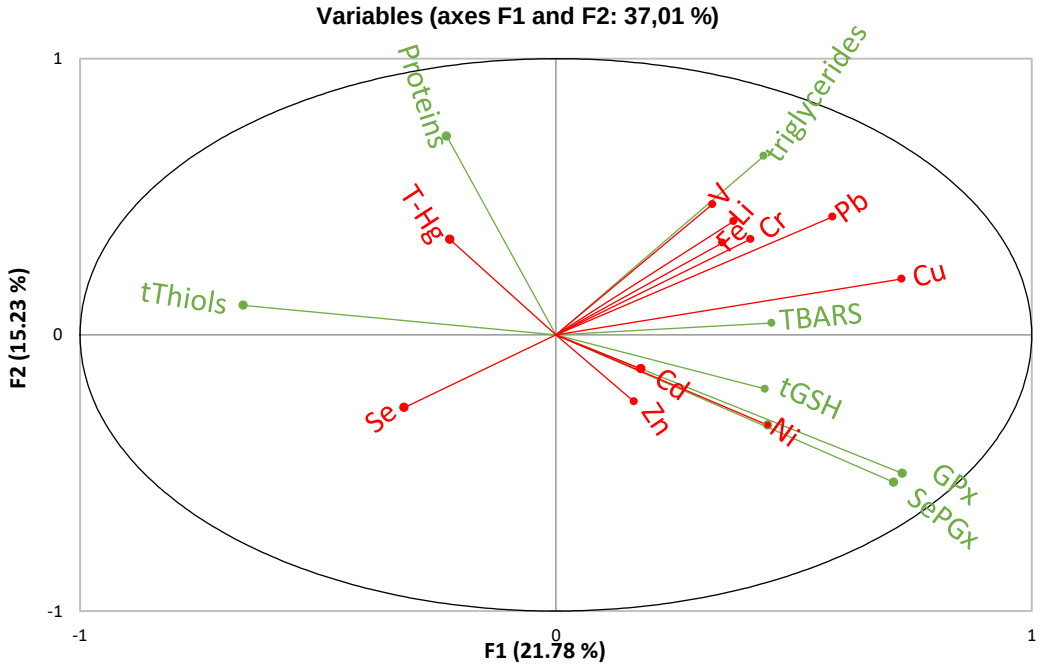

Figure 18: Principal component analysis of 11 metallic trace elements, five oxidative stress biomarkers, proteins and triglyceride from 21 harbour seal samples represented on a factorial plane defined by factors F1 and F2.

To confirm these correlations found in the PCA, a Pearson correlations matrix between biomarkers and metals from the harbour seal was calculated (Table 15). Except for triglyceride and tThiols, all biomarkers showed some significant correlation with MTE contamination. Indeed, significant negative correlation between proteins and Cd are observed. Further, significant positive relationships between TBARS from March and Pb and Ni can be found. Furthermore, significant negative relationships were found between tGSH and Se and female Hg. Also, a correlation matrix between biomarkers was performed, however no significant correlations were obtained (Appendix 2).

Table 15: Pearson correlation matrix between metallic trace element and oxidative stress biomarkers from 21 harbour seal samples (to be continued on next page).

	triglyceride	**March TBARS**	**October TBARS**	**tThiols**	**tGSH**	**GPx**	**SePGx**
Female T-Hg	0.192 (0.595)	0.002 (0.997)	0.516 (0.374)	0.012 (0.973)	**-0.652 (0.030)**	-0.019 (0.955)	-0.053 (0.876)
Male T-Hg	-0.115 (0.752)	0.448 (0.265)	/	0.365 (0.299)	0.542 (0.106)	-0.422 (0.225)	-0.372 (0.290)
Female Zn	0.109 (0.765)	0.559 (0.249)	0.487 (0.406)	0.224 (0.507)	-0.210 (0.535)	0.273 (0.417)	0.293 (0.382)
Male Zn	-0.134 (0.713)	0.337 (0.414)	/	-0.339 (0.339)	0.107 (0.769)	0.207 (0.567)	0.276 (0.441)
March Zn	-0.014 (0.962)	0.150 (0.608)	/	0.264 (0.361)	0.002 (0.995)	0.146 (0.619)	0.182 (0.533)

October Zn	-0.219 (0.677)	/	0.083 (0.859)	-0.228 (0.623)	-0.731 (0.062)	0.491 (0.264)	0.752 (0.051)
Cd	0.013 (0.958)	-0.039 (0.895)	-0.173 (0.710)	-0.207 (0.367)	-0.019 (0.936)	-0.169 (0.465)	0.047 (0.841)
Fe	0.050 (0.834)	0.320 (0.264)	0.588 (0.165)	-0.274 (0.230)	0.002 (0.994)	0.049 (0.834)	0.006 (0.978)
Cu	0.412 (0.071)	0.447 (0.109)	0.419 (0.350)	-0.286 (0.208)	0.078 0.736)	0.339 (0.133)	0.386 (0.084)
Se	0.010 (0.968)	-0.323 (0.259)	0.281 (0.541)	0.063 (0.786)	**-0.566** **(0.008)**	0.122 (0.597)	0.201 (0.382)
Pb	0.236 (0.317)	**0.672** **(0.008)**	-0.432 (0.332)	-0.406 (0.068)	0.258 (0.259)	0.308 (0.174)	0.265 (0.246)
Ni	-0.289 (0.216)	**0.606** **(0.022)**	0.588 (0.165)	-0.235 (0.305)	0.008 (0.971)	**0.586** **(0.005)**	**0.530** **(0.013)**
Cr	0.289 (0.216)	0.186 (0.525)	-0.168 (0.719)	-0.189 (0.413)	0.257 (0.261)	0.299 (0.189)	0.284 (0.212)
Li	0.434 (0.056)	0.207 (0.477)	-0.178 (0.702)	-0.156 (0500)	0.124 (0.591)	0.068 (0.771)	0.054 (0.815)
V	0.212 (0.370)	0.314 (0.274)	0.599 (0.155)	-0.260 (0.256)	0.155 (0.502)	0.094 (0.687)	0.048 (0.836)

Values in bold are significant at p <0.05.
P-values are indicated between brackets.

TBARS

When considering the difference of TBARS concentrations between March and October, a significant correlation with TBARS and Ni in March (r=0.606, p=0.022) is observed. However, when eliminating a value considered to be an outlier (indicated as a triangle in (Figure 19), no correlation is found between TBARS and nickel in March. Further, when considering differences between March and October, positive correlations are found between Pb and TBARS in March (r= 0.672, p=0.008) (Figure 19), again when removing the outlier in Figure 19, no correlation is found between TBARS in march and lead. This outlier corresponds to one individual (Pv 21001), a heavy adult female of 89.5 kg (mean female weight is 58 kg).

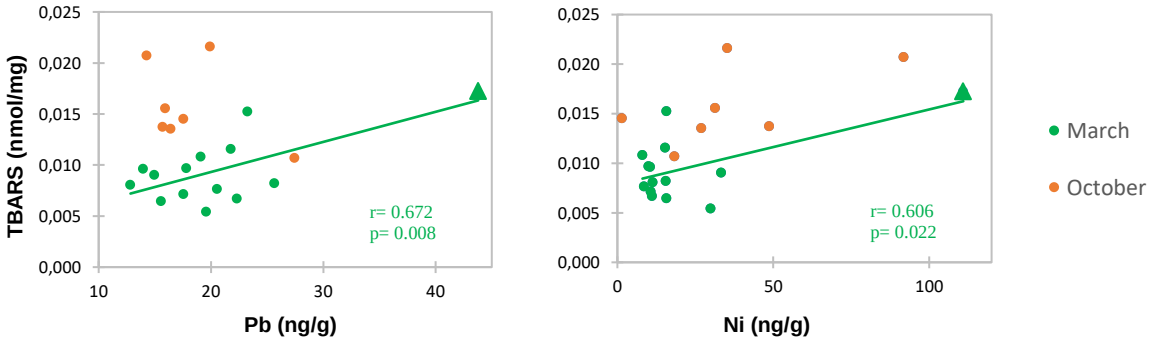

Figure 19: Relationship between TBARS (nmol/mg) in serum and Pb and Ni concentrations (ng/g) in blood cells of harbour seals. Data is set is divided in march and October which is respectively represented in green and orange.

Thiols and GSH

No significative correlations were found between total thiols and MTEs. However, on the PCA (Figure 20) we can observe a general opposite tendency with all MTEs, except for Se and Hg. Negative correlations were observed between the total GSH concentration and Se (r= -0.556;

p=0.008). In the same way, GSH concentration and T-Hg were negatively correlated in females (r=-0.652; p=0.030) while no correlation was highlighted in males (Figure 20).

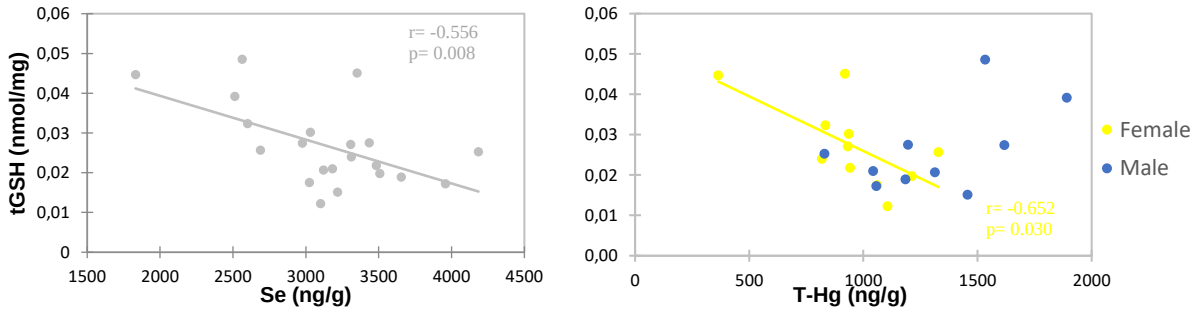

Figure 20: Relationships between tGSH concentrations (nmol/mg) in serum and Se and total Hg in blood cells from harbour seals. Data is set and divided in females and males which is respectively represented in yellow and blue,. A grey colour represents the whole population.

GPx

Both, GPx and SeGPx, are positively correlated with Ni (Figure 21). No other correlations were found.

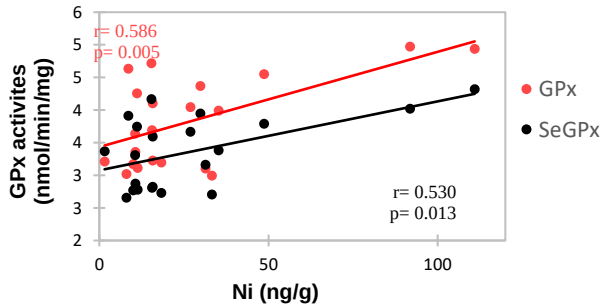

Figure 21: Correlation between GPx activities (nmol/min/mg) in serum and Ni in blood cells (ng/g) from harbour seals, with GPx in red and SeGPx in black.

Triglyceride

No significant correlations are found with triglyceride. However when removing the highest Pb value (corresponding to Pv 21001), significant correlation was observed with Pb (Figure 22). Further almost significant correlations were found with Cu and Li (Figure 22).

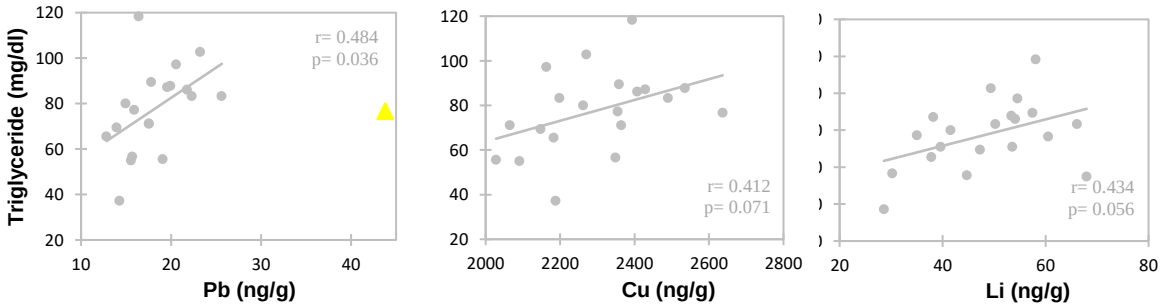

Figure 22: Relationship between triglyceride concentrations (mg/dl) in serum and Pb, Cu and Li concentrations (ng/g) in blood cells from harbour seals.

Discussion

To evaluate the relationship between contamination levels of metallic trace elements and biomarkers of oxidative stress in harbour seal *Phoca vitulina*, an optimization of assay protocols of oxidative stress biomarkers was first performed in order to allow their assessments in the harbour seal serum. Then, the second part consisted to evaluate the correlations between these oxidative stress biomarkers and the metallic trace element levels, in harbour seals.

1. Setting up of biomarkers assays

1.1 TBARS

Thiobarbituric acid reactive substances (TBARS) is a biomarker of effect as it quantify lipid peroxidation, through malondialdehyde assessment (Miyamoto et al., 2011). Many variations on the TBARS assay are possible: different sample pre-treatments, choices of acid type and concentrations, heating temperature and time, and the addition of antioxidants or not.

An important component of this assay seemed to be the pH, which is known to influence the formation of the pink chromogen $(TBA)_2$-MDA adduct (Miyamoto et al., 2011). Literature refers to pH ranging from 2 to 4.5 during the heating step (Cheeseman & Hermann, 1990; Lepage et al., 1991; Miyamoto et al., 2011), however in this study the optimal pH was not higher than 2 (Figure 14). Lepage et al. (1991) had an optimal pH around 3.5 for retrieving the standard, but the only difference with this study was the choice of acid used for deproteinization, as the used assay was based on Lepage et al. (1991). In the latest study, a mixture of H_2SO_4 and Na_2WO_4 (30 and 15 mM) was used instead of TCA (150 mM) in this present study, which is commonly used in many other studies evaluating TBARS (Cheeseman & Hermann, 1990; Costa et al., 2006; Mendes et al., 2009). The acid chosen may therefore influence the optimal pH for the $(TBA)_2$-MDA adduct to form during the heating step. Another important factor was to have the standard and serum at the same pH during the heating step, otherwise no correct standard addition was obtained, however this is not mentioned in literature. In Lepage et al. (1991), the optimum pH for the complete formation of the adduct with MDA standard was limited to the narrow pH range of 3.3 to 3.7, while with plasma samples, it was found to be complete from pH 2.5 to pH 4.5. It is surprising to note that few authors have paid attention to the pH of the reaction mixture which seems to be of major importance. The heating time of 30 minutes was considered sufficient in this study as it was also in Arbor (2017) and Miyamoto et al. (2011). The reaction time is considered to be influenced by pH and TBA concentrations (Grotto et al., 2009; Mendes et al., 2009), with lower pH and higher TBA concentrations accelerating the reaction. As a pH of 2 was used in this

study instead of a pH of 3.5 in Lepage et al. (1991), our reaction could be faster than the 60 minutes proposed by Lepage et al. (1991). Change in temperature was not considered to be helpful, since Lepage et al. (1991) reported a decrease of 25% of the adduct formation at 70°C. Further, other studies all used temperatures between the 95 and 100°C (Cheeseman & Hermann, 1990; Grotto et al., 2009; Mendes et al., 2009). The extraction of the $(TBA)_2$-MDA adduct formed was considered to be necessary. Therefore, this was done by n-butanol, which is used for this purpose as described in many different studies (Cheeseman & Hermann, 1990; Lepage et al., 1991; Miyamoto et al., 2011; Yagi, 1998) and it is known to increase the recovery of the adduct. In our study, this was not only performed for this reason, but different volumes of NaOH were added to serum and standard and this led to different dilutions in both. However, when adding n-butanol, all the adducts formed, no matter in which volume, were retained in the upper layer of n-butanol and thus negating the effect of the different dilutions. Furthermore, this TBARS assay lacks specificity, as TBA Reactive Substances are measured and not only MDA (Arbor, 2017; Lepage et al., 1991; Miyamoto et al., 2011). Also, artefactual formation of MDA from lipid hydroperoxides during the acid heating step can give an incorrect perception of the MDA amount in a tissue. Therefore, antioxidants (butylated hydroxytoluene, BHT) can be added (Miyamoto et al., 2011). Lepage et al. (1991) indicated an overestimating of MDA of 47% when no BHT was used. Moreover, not all commonly used protein precipitants are optimal for the TBARS assay. The pH of the supernatant after addition of TCA was 1.1, while in Lepage et al. (1991), where H_2SO_4 and Na_2WO_4 (30 and15 mM) were used, the supernatant was at a pH of 2.2. In the same study, it was demonstrated that TCA at our final concentration ensures a retention of about 60% of the adduct, while with H_2SO_4 and Na_2WO_4 they obtained a full retention. However, this effect is neglected as the standard is submitted to the same conditions and thus considered to have the same retention of MDA. Lepage et al. (1991) shown the importance of the deproteinization preparative step, which was not always used in past studies. Indeed, this could be explained by protein interactions that could occurred during the assay; therefore, the deproteinization allowed to decrease this biased without losing MDA molecules, which is too small to precipitate. Yet, even with this problem, the assay is still widely used because of its simplicity and cheapness, but contains numerous pitfalls. In addition, since few years, the use of HPLC is known to increase specificity and sensitivity of the assay (Grotto et al., 2009; Lepage et al., 1991; Miyamoto et al., 2011; Sochor et al., 2012), Sochor et al. (2012) even demonstrated significantly different results obtained by HPLC versus the spectrophotometric method. Therefore, for future studies it may be recommended to develop this technique in serum harbour seals.

1.2 Total thiols

Thiols are a group of organic compounds containing a sulfhydryl group (-SH) attached to a carbon atom. Constitute the major portion of the total body antioxidants and they play a significant role in defence against reactive oxygen species (Costa et al., 2006; Prakash et al., 2009). By binding with DTNB, thiols form a highly coloured anion with maximum absorbance at 412 nm (Costa et al., 2006). Assays using DTNB of Ellman's reagent are commonly used for spectrophotometric assay of thiols (Hu, 1994). After having tested the original protocol proposed by Costa et al. (2006) in harbour seals, it appeared that serum dilution proposed was not appropriate for serum of harbour seals, and thus more serum was needed to obtain measurable absorbances. This adaptation was necessary since the assay described in Costa et al. (2006) is an automated procedure adapted for a chemical analyser, which needs much less reagent than for a manual procedure. The higher DTNB concentration tested (*i.e.* 15 mM) did not improve the total thiols detection as compared to the 10 mM DTNB concentration described in Costa et al. (2006). Therefore, 10 mM DTNB solution is not test limiting and was thus used in this work. Absorbance of the formed complex was read after 15 minutes in Costa et al. (2006), but optimization results demonstrated that the reaction was stable after 5 minutes and remained stable at least for 15 minutes after this. This is useful information, if measurement could not be performed after 5 minutes the absorbance can still be read up to 15 minutes after and still give correct values (Figure 16). In a similar protocol developed by Hu (1994) in human plasma, absorbance was read at 15 minutes and remained stable for at least 30 minutes thereafter. The stability of the colour formed may be influenced by the proteins present in the sample, so Hu (1994) suggested to dilute plasma more than 20 times. However, the dilution of 8.2 used in this work did not cause stability problems as was demonstrated over time, and confirmation was obtained by testing a dilution of 16.4 which gave proportional values to the 8.2 dilution.

1.3 Total glutathione

Glutathione, is the most abundant cellular thiol and protects cells against toxic effects of a variety of endogenous and exogenous compounds, including trace metals and ROS. Can be found in two forms: reduced as GSH and oxidised as GSSG, here we determined the total glutathione which contains thus both forms (Meister & Anderson, 1983; Regoli et al., 2011). For the determination of total glutathione, the Glutathione Assay Kit by Cayman chemical (No. 703002), based on a spectrophotometry method, was used. This kit contains explanations about performing the assay with serum, however some technical constraints were encountered. Nevertheless, the results were not satisfying as no correct standard addition was obtained. Consequently, pH was checked and this showed different pH values between serum and standard after the addition of

TEAM reagent, used to increase the pH after the acid deproteinization, as prescribed by the kit. After the prescribed addition of TEAM, a pH of 4 was obtained in the standard and a pH of 6 was obtained in the serum. The reaction between GSH and DTNB is sensitive to pH and only occur at a neutral pH (Hu, 1994). The lower pH in standards gave too low values to obtain a correct standard addition, which can be explained by a reduced interaction of DTNB at low pH. It was decided to increase the added volume of TEAM to increase the pH of the standard and when adding 6 µl of TEAM to 100 µl supernatant, a pH of 7 was obtained in both serum and standard. Ultimately, this optimization gave a correct standard addition. Further, HPLC assays for tGSH assessment can be found and hey provide robust and specific analytical methodology for the quantification of intracellular thiols (De Almeida et al., 2011).

1.4 Glutathione peroxidase

Glutathione peroxidases (GPx) are the major enzymes in the antioxidative defence mechanism depending on glutathione, the large family contains selenium-dependent (SeGPx) and selenium-independent (indSeGPx) enzymes (Simona et al., 2008). Glutathione peroxidases were tested as described in Regoli et al. (2011) on harbour seal serum. Parameters optimization was not necessary as test assessments gave measurable values. Besides, as no standard curve could be used in an enzymatic assay, no standard addition could be performed to check the measurement.

2. Case study on harbour seals

After having optimized several biomarker protocols in serum of harbour seals, a case study was conducted on harbour seals from the Wadden Sea. The obtained biomarker levels were measured and correlated with metallic trace elements in order to evaluate relationships and to form an idea whether those biomarkers are useful tools to assess metallic exposure of harbour seals.

2.1 Metallic trace elements

MTEs concentrations in marine mammals are influenced by numerous factors such as geographic location, diet, age, sex, tissues considered and metabolic rates (Das et al., 2003). Furthermore, MTEs are not routinely measured in blood cells from marine mammals, but the results obtained here followed the same tendency as MTEs concentrations measured in whole blood from harbours seals of the Wadden sea (Griesel et al., 2008) (Table 16). However, all MTEs concentrations in blood cells were higher than previously found concentrations in whole blood of harbour seals, except for Cd, Cu and V, but they remained in the same order of magnitude (Table

16). Concentration were about 2.5 times higher for Hg and Ni, up to 10 times higher for Pb, and twice higher for Fe and Cr concentrations, than concentrations in whole blood.

Table 16: Comparison between whole blood MTEs concentrations in harbours seals studied by Griesle et al.(2008) with the blood cell MTEs concentrations in harbour seals from the present study.

MTEs	Blood concentrations in harbour seals from the Wadden Sea by Griesel et al. (2008)	Blood cell concentrations in harbour seals from the Wadden Sea in present study
Hg	172 ± 143 µg/l (n=22)	468 ±138 µg/l
Zn	3.4 ± 0.5 mg/l (n=85)	5.1 ± 0.3 mg/l
Cd	<0.12 -3.10 µg/l (n=28)	0.1 - 65.73 µg/l (median = 0.68 µg/l)
Fe	733 ± 86 mg/l (n=14)	1247 ±27 mg/l
Cu	527 - 1371 µg/l (n=28)	845 - 1099 µg/l
Se	885 µg/l (n=85)	1327 µg/l
Pb	0.73 µg/l (n=28)	7.43 µg/l
Ni	2.41 µg/l (n=28)	6.58 µg/l
Cr	8.74 µg/l (n=28)	20.13 µg/l
Li	/	20.58 µg/l
V	<0.0 5- 3.03 µg/l (n=28)	0.24 - 5.03 µg/l

Sources: All data about harbour seal blood concentrations are from Griesel et al. 2008, except for Hg concentrations which were obtained from Das et al. 2008.

Higher concentration of Hg in blood cells compared to whole blood concentration could be explained by the fact that 70-95% of the Hg is bound to haemoglobin in red blood cells. Furthermore, 95% of Pb is bound to red blood cells which can explain concentrations up to 10 times higher in blood cells. In addition, Fe is part of haemoglobin, Ni can bind with blood cells as well as Cr (Devoy et al., 2016; Pyle & Couture, 2011; Zava, 2016), that explains the concentrations measured in blood cells of *P. vitulina*.

Mercury concentration in blood cells was significantly higher in males compared to females, which is consistent with results obtained by McHuron et al. (2014) in harbour seals of central California (*P. v. richardsi*) . However, Das et al. (2008) observed the opposite results in harbour seals from the Wadden Sea. Less commonly reported, results highlighted also the difference in Zn concentrations between different genders, with lower concentrations in females than males for both MTEs. These Hg and Zn differences may result from differences in foraging behaviour, the offload of Hg and Zn from females to their pups via gestation and lactation, and physiological variations at the molecular and biochemical levels (Kakuschke, Griesel, et al., 2009; McHuron et al., 2014). Still, the offload of Zn to pups is not well documented. Kakuschke et al. (2009) reported higher concentrations in blood from pups compared to adults and presumed that anyhow higher concentrations of Zn in pups may be due to the importance of Zn during development for high metabolism and blood formation of pups, and thereby not transferred to pups (Kakuschke, Griesel, et al., 2009). Further, only females showed a significant difference in Zn concentrations between March and October, with lower concentrations in March compared to October; However, this does

not correspond to the pupping season of harbour seals from the North Sea, were pupping occurs during summer months (Jensen et al., 2017). Moreover, Vega et al. (2016) observed a clear change in harbour seals diet and foraging behaviour from pelagic during spring to benthic during summer, as seals tend to stay in Wadden Sea during summer but during winter they tend to increase the use of North Sea resources. These dietary variations and changes in foraging location between season could lead to a different MTEs exposure between seasons. After all, it is complicated to make conclusions as individuals caught during March and October are not the same, the observed variation between seasons could thus be due to interindividual differences. For instance, age is also an important factor influencing T-Hg concentrations, as it accumulates strongly with age in most marine mammals (Das et al., 2003). However, in this study, ages of the individuals were not known. Additionally the study suffered from small and unequal sample sizes between seasons (n=14 for March and n=7 for October).

2.2 Oxidative stress biomarkers

In the present study, four potential oxidative stress biomarkers of exposure (total glutathione, total thiols, glutathione peroxidases and se-dependent glutathione peroxidase) and one potential biomarker of effect (lipid peroxidation as TBARS) were tested on harbour seal serum. MTEs are known to induce oxidative stress, but response varies depending on the concentration of exposed metals, duration of exposure and species (Espín et al., 2014). Oxidative stress, with the aim to evaluate MTEs exposure, has been poorly studied in marine mammals. Some studies reported oxidative stress biomarkers such as GPx activities, TBARS and total glutathione concentration on northern elephant seal, ringed seal, clymene dolphin and some other marine mammals to assess the impact of fasting and apnea-induced hypoxemia (Elsner, 2002; Filho et al., 2002; Vázquez-medina et al., 2011; Vázquez-Medina et al., 2012). However, as far as known, no oxidative stress biomarkers were assessed on harbours seals.

Total glutathione, total thiols and glutathione peroxidases are part of the antioxidant defence system. In general, induction of these antioxidant defence systems is interpreted as an adapatpion of the organisms to environmental disturbances, while inhibition reflects the toxic effect of pollutants and may cause cell damage such as lipid peroxidation (Amiard-Triquet & Amiard, 2013).

2.3 Oxidative stress biomarkers response to MTE exposure

Glutathione peroxidase enzymes (GPx) are major components for cell protection against oxidative damage, as they reduce hydroperoxide and a variety of organic hydroperoxides using glutathione (GSH) as cofactor (Regoli et al., 2011). In the harbour seal serum, it could be observed

that the majority of the GPx consisted of the Se-dependent GPx. The Se-dependent, GPx-3 is believed to be the most important extracellular antioxidant enzyme in mammals (Tabet & Touyz, 2007). On the other hand, Se is thus necessary for Se-GPx forms, several authors have demonstrated the decreased GPx activity when selenium is poor or not bioavailable and others have demonstrated that Se supplementation could increase GPx activity (Zachara et al., 2004; Krofic et al., 2014; Lloyd et al., 1989; McMurray & Blanchflower, 1976; Thomson et al., 1977). McMurray & Blanchflower (1976) observed significant positive correlations between blood Se concentration and GPx activities in sheep and cow, as well as Thomson et al. (1977) reported this same observation in humans. However, this was not the case in this study, no correlation was found between Se in blood cells and Se-dependent GPx in serum. This could be because Se is not a limiting factor in harbour seals, but Lloyd (1989) found correlations between human blood selenium and GPx activities when the Se concentrations were less than 1.26 µmol/l, above that value no correlation was found. As harbour seals had higher Se concentrations in their blood compared to humans, it could be that selenium is not limited to the harbour seal. Further, measurements of Se in blood did not indicated that Se was bioavailable for GPx, Levander et al. (1983) used GPx activity to evaluate bioavailability of selenium as peroxidase activity appeared to be strongly related to dietary selenium.

Furthermore, GPx activities are known to be induced by ROS production, created by MTEs, while inhibited by some other MTEs depending on the species, the tissue considered, the doses and duration of the exposure (Carolina et al., 2015). In this study, a positive correlation was observed between Ni and total GPx. Tsao et al. (2017) found that under oxidative stress caused by nickel exposure in humans, GPx stored in cytoplasm of tissues was released into the blood circulation to reduce the formed hydrogen peroxides. However, as the nickel exposure increased, the GPx release slowed down. On the contrary, Misra et al. (1990) found that rats injected with nickel(II)acetate showed a decrease of GPx activities in kidney and no change was observed in blood. GPx activities can be influenced by other MTEs inducing oxidative stress, which may trigger the GPx activities to cope with the produced hydro peroxides. In addition, Pb is known to influence GPx, In a review from Carolina et al. (2015) 8 studies on human blood indicated an increase in GPx, while 5 studies reported a decrease and 2 studies did not observed differences in blood activities of humans with control groups. A general tendency was to have an increase of GPx at lower Pb exposure and a decrease at higher exposure levels. Moreover, Pb, Hg and Cd can form insoluble complexes with Se, reducing the Se bioavailability and consequently Se-GPx activities could be negatively impacted (Carolina et al., 2015; Espín et al., 2014).

GPx can also be negatively impacted by an insufficient increase of glutathione reductase activity, enzyme which restores GSSG to GSH, with GSH being a cofactor for GPx. A decrease in activity of GPx in presence of Pb, Hg and Cd could also be due to the competition for GSH (Reddy et al., 1981). As divalent ions are well known to have high affinity for -SH groups and thus also for GSH which is a thiol involved in cell protection against reactive oxygen species (ROS) and metallic trace metals (Regoli et al., 2011). However, in this study no correlations was found between GPx and GSH.

Total GSH is only a part of the total thiols found in harbour seal serum from this study. Other thiols are for example metallothionein's, which are widely used as biomarkers of exposure to MTEs, as their thiol groups enable them to bind particular metallic trace metals (Amiard-Triquet & Amiard, 2013). Total thiols are not significantly correlated to a specific MTE in this present study. However, total thiols have shown a (non-significative) negative correlation with all measured MTEs except for Hg and Se, suggesting a possible depletion of thiols by MTEs. The absence of total thiols depletion caused by Hg and Se could be explained by the fact that mercury has namely a lower affinity for thiol groups and higher affinity for selenium containing groups by several orders of magnitude (Amiard-Triquet & Amiard, 2013). Furthermore, tGSH is known to be depleted by inactive MTEs such as Cd, Hg and Pb (Valko et al., 2005). However, only a negative correlation between T-Hg and tGSH was observed in female individuals. In males, a trend of positive correlation between T-Hg and tGSH was observed that could be due to an increased production of GSH when exposed to mercury or a more efficient recycling of GSSG to GSH by glutathione reductase using NADPH. was. But, more often, the ratio between GSH and GSSH is utilized as a biomarker, under normal conditions the molar GSH:GSSG ratio exceeds 100:1, while under oxidative stress, this ratio has been demonstrated to decrease to values of 10:1 and even 1:1 (Zitka et al., 2012). A decrease in total glutathione will only occur as the glutathione reductase cannot follow the rate at which GSSG is produced and excess of GSSG will then be excreted.

When the antioxidant defence is overwhelmed and not capable of eliminating excess of ROS, this can lead to different types of cellular damage, such as lipid peroxidation which is the most widely studied and therefore also determined in this study. One of the degradation compounds of lipid peroxidation is malondialdehyde (MDA), which was estimated by the TBARS assay, which reacts with MDA but also with MDA-like peroxides (Amiard-Triquet & Amiard, 2013).

In the present study, a correlation between Pb and Ni concentrations and TBARS levels in harbour seals was observed (Figure 19). This is consistent with other investigations which showed

that both Pb (Asperczyk & Asperczyk, 2004; Carolina et al., 2015) an Ni (Chen et al., 2003; Das & Das, 2001; Misra et al., 1990; Tsao et al., 2017) induce oxidative stress as lipid peroxidation (MDA) in humans and rats, probably through a ROS production (Das & Das, 2001). However, Pb and Ni were correlated to MDA only in March, and no correlation was measured in October when lipid peroxidation were higher. The lipid peroxidation may then be caused by other environmental factors than MTEs. It has been demonstrated in bovines and rats that high temperatures in summer were accompanied with higher oxidative stress and lower antioxidant enzymes activity compared to winter season (Bihari et al., 2016). This could thereby explain the significant higher lipid peroxidation in October as compared to March. In addition, TBARS can also be induced by pollutants others than MTEs, organic compounds and pesticides are known to induce lipid peroxidation (Miyamoto et al., 2011). Other studies shown increased lipid peroxidation with increased Hg concentration (Espín et al., 2014; Stohs & Bagchi, 1995; Valavanidis et al., 2006), however this was not observed in this study. Increased lipid peroxidation is engendered by Hg due to its capacity to promote free radicals, interact with antioxidant enzymes and by the reduced bioavailability of selenium (Carolina et al., 2015; Espín et al., 2014). Indeed, it was demonstrated in marine mammals that the formation of mercury selenide granules are accumulated in the liver (Nigro & Leonzio, 1996).

Triglycerides are the main source of stored energy in organisms, and as energy is needed to antitoxic defences, therefore triglyceride assessment was measured to represent the energy reserves of harbour seals. In the present study, triglyceride was positively correlated to Pb, and tend to be positively correlated to Cu and Li. Kristal-Boneh et al. (1999) and Sharma et al. (2012), both reported increased trigyceride concentrations in human exposed to Pb, however this correlation was not significant in both sutdies, but the correlation with cholesterol was, wich is a trigycerlide. They both suggests that the lead exposed persons mau have altered lipid profile. Further, is tis also commonly found that Cu and tryglycerides are correlated, however the causality between the variables, remains to be studied (Burkhead & Lutsenko, 2013). On humans an increase in plasma triglyceride in observed with age (Greenfield et al., 1980), this could also be possible within marine mammals. And as Pb is known to bioaccumulate and thus increas with age, the underlying cause of the corealation between Pb and triglyceride could be age.

3. Conclusions

The purpose of this study was to optimize assay protocols of oxidative stress biomarkers in order to allow their assessments in the harbour seal serum, and also to evaluate the relationship

between contamination levels by metallic trace elements and biomarkers of oxidative stress in harbour seal *Phoca vitulina* coming from the Wadden Sea.

This master thesis has demonstrated that oxidative stress biomarkers of effects and exposure (malondialdehyde (TBARS), total thiols, total glutathione, selenium-dependent and -independent glutathione peroxidases) could be correclty mesured in harbour seal serum using the obtained optimized protocols. This is the first study developping oxidative stress biomarkers in harbour seal serum and the firts results obtained here showed that these biomarkers could be useful tools in future investigations.

Results also highlighted that MTEs concentrations in blood cells were in the same order of magnitude as concentrations observed in blood from free ranging harbours seals, however reflecting the biological difference between whole blood and blood cells. Besides, MTE contaminations seemed to be the same between males and females, except for Hg and Zn concentrations which were higher in males than females.

Oxidative stress biomarkers are known to be useful tools to access MTEs exposure in many organisms, but oxidative stress may be influenced by numerous environmental factors. From this study, it could not be identified how MTEs influence oxidative stress in harbour seals. However, remarkable was the absence of correlation between selenium and selenium-dependent glutathione peroxidase, which could indicate that selenium is not limiting in harbour seals. Further, only Ni was positively correlated to GPx activities. However, a general tendency may be the depletion of thiols by MTEs, but no significant observations were found for particulate MTEs. Hg had potentially depleted total GSH in female individuals. Finally, it was observed that lipid peroxidation in harbour seals may be increasingly important when exposed to Ni and Pb; however, this was only observed in March and not in October, where increased lipid peroxidation may be a result of other environment factors.

To interpreter biomarker results, it is important to known by which environmental factors they are impacted, if those environment factors are known, biomarkers of oxidative stress could represent an efficient way to represent the pollution by MTEs.

Finally, even if the measurement of the biomarkers is correct, the conservation of the serum may have ensured that few correlations were found between MTEs and the biomarkers. Between the collection of serum samples and the oxidative stress biomarkers analysis, samples were sotred for about 3 years in -80°C. Ansarin et al. (2015) for instance observed fluctation of TBARS in serum samples after only 3 days at -20°. In general, it is recomanded to store serum samples ony

for one month at -80°C for the tested biomarkers, for total gluathione assay it is even recomanded to deprotenise the serum before storage (Cayman Chamical Kits No . 10009055, 700340, 703102 and 703002).

4. Perspectives

Although this study allowed to develop new biomarker assays in harbour seal serum, and the first case study results highlighted their potential useful in monitoring the health status of organisms, many works are need to improve their uses. For example, it would be useful to adapt these protocols to multi plate assays to reduce needed sample volumes and to gain time. In addition, in the present work, the sample storage was probably the most important parameter impacted the biomarker assessment, therefore in future, it could be interesting to perform a similar study being careful to the sample storage. Besides, in order to increase the relevance of the biomarker assessment in a context of metallic element traces, it will be wise to perform all the measurement (i.e. MTEs and biomarkers) on the same tissue (e.g. whole blood).

To have a better understanding on the impact of MTE pollution on oxidative stress biomarkers is would be necessary to compare MTEs and biomarker effects on two or more populations of harbour seals with districted difference in MTE contaminations. However, this must be chosen to limit the difference between the environmental conditions of both sites. To add a species (for instance the grey seal) could made it possible to visualise how both species react differently or not on the presence of MTEs.

Finally, in the present study, each biomarker shown a specific response face to MTEs, suggesting that each oxidative stress biomarker tested here could be useful. However, other oxidative stress biomarkers, such as glutathione-S-transferase (GST), superoxide dismutase (SOD) or catalase (CAT), would be develop in harbour seal. In addition, although total thiols have been evaluated here, it could be interesting to develop metallothionein assessment, which is the main commonly biomarker specifically used for MTEs pollution. Consequently, all these biomarkers could be used in a multi-biomarkers approach (in addition to immune-relevant molecular biomarkers already develop) in order to increase the relevance of conclusions regarding the health status of organisms exposed environmental contaminations.

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Appendices

1. Appendix 1: Obtained protocols

1.1 TBARS

Assay:

1) Add 240 µl of serum/blank (distilled water)/standard, 240µl of 0,5%BHT (prepared in methanol) and 960ml distilled water into a microtube and vortex.
2) Add 165 µl of TCA (trichloroacetic acid, 1.4M) to reach a final concentration of 150mM TCA and immediately vortex.
3) Centrifuge for 10 minutes at 1000 g.
4) Transfer 1200 µl of supernatant to a reaction tube.
5) Add 1 ml of 1% TBA (thiobarbituric acid) to the supernatant.
6) Adjust pH to 2 by adding respectively 150 and 200 µl of 1N NaOH, to serum and standard.
7) Close tubes and heat for 30 minutes at 100°C.
8) Cool reaction tubes on ice for 4 minutes.
9) Before extraction, add 150 µl of HCl (5N) to reach a pH lower than 0,75 (since n-butanol is only active at low pH).
10) Extract TBARS complexes from mixture by adding 750 µl of n-butanol, vortex for 1 minute and centrifuge for 10 minutes at 1000 g at 4°C.
11) Transfer the supernatant to a spectrophotometer cuvette and read absorption measured at 535 nm.

TCA, TBA and BHT solution were made freshly before performing the assay. For each sample as for the standard curve a duplicate was made and means were used for statistical analyses.

Calculations:

A MDA standard curve ranging from 0.3125 µM to 10 µM MDA was performed using a commercial 500 µM MDA solution, and the same assay as described above, in order to calculate the MDA concentrations which were expressed in µM TBARS/ mg proteins.

1.2 Total thiols

Assay:

1) Add 50 µl of serum/standard and 770 µl in KPi-EDTA buffer in a spectrophotometry cuvette and mix vigorously.
2) Make a blank by adding 820 µl KPi-EDTA buffer to a spectrophotometry cuvette.
3) Set instrument autozero for 412 nm with the blank.
4) Read absorbance of the sample at 412 nm (=A1).
5) Add 20 µl of DTNB (10 mM in absolute methanol) to the solution and to the blank.
6) Incubate for 5 minutes at room temperature.
7) Read the absorbance of the sample (A2) and blank (B) at 412 nm.

For each sample, as for the standard curve, a duplicate was made and means were used for statistical analyses.

Calculations:

Results were calculated as:

Absorption = A2-A1-B

where B is the absorbance of the background reaction; A1 is the background absorption of the serum used and A2 is the absorbance of the formed coloured anion with background absorption of the serum and the background reaction of the blank.

A standard curve, ranged from 3.9 µM to 500 µM, was performed using GSH as thiol reference. Each standard concentration was prepared in KPi-EDTA buffer (100 mM potassium phosphate buffer (KPi), 1mM ethylenediaminetetraacetic acid (EDTA), pH 7,5). The standard curve allowed to calculate the total thiol concentration expressed in µM eq. GSH/mg proteins.

1.3 Total glutathione

Assay:

Deproteinization:

1) Add 100 µl 10 % metaphosphoric acid to 100 µl of serum sample /standard.
2) Allow the mixture to stand at room temperature for 5 minutes.
3) Centrifuged at 10 000 g for at least 10 minutes.
4) For each 1ml of supernatant collected, add 60 µl of triethanolamine (TEAM 4M), this increased the pH of the sample and standard to approximately 7. The sample was now ready to use.

The assay is performed as followed:

5) Add 50 µl of deproteinised sample / standard to each of sample wells, on the 96-well plate.
6) Add 150 µl of assay cocktail containing MES buffer, Cofactor mixture, enzyme mixture, water and DTNB, using a multichannel pipette.
7) Cover the plate and incubate for 25 minutes while agitating.
8) Read absorbance at 405 nm in Multiskan EX (Thermo Fischer).

Each sample and standard was analysed in duplicate and means were used for statistical analyses.

Calculations:

A standard curve ranging from 0.25 µM to 8 µM GSSG was made with a 25 µM GSSG standard which was further diluted with MES buffer (0.2M 2-(N-morpholino) ethanesulphonic acid, 50 mM phosphate and 1 mM EDTA, pH 6) and expressed in equivalent total GSH.

1.4 Glutathione peroxidase (GPx)

Assay:

Se-dependent GPx	**Se-dependent and Se-independent GPx**
1) Add to a spectrophotometry cuvette (final volume 1 ml)	
- 875 µl of KPi buffer (100mM) - 10 µl of EDTA (100mM) - 20 µl of GSH (100mM) - 10 µl of GR (100U.ml^{-1}) - 10 µl of NaN_3 (100mM) - 50 µl of blank (KPi buffer) or sample	- 896 µl of KPi buffer (100mM) - 10 µl of EDTA (100mM) - 20 µl of GSH (100mM) - 10 µl of GR (100U.ml^{-1}) - 50 µl of blank (KPi) or sample
2) Vigorously mix cuvette and set instrument autozero for measurements at 340 nm.	
3) Add 10 µl of NADPH (20 mg/ml) and read absorption at 340 nm, this should give a value between 0,9 and 1,2.	
4) Add 5µl of H_2O_2 (100 mM) and vigorously mix.	4) Add 4 µl of CHP (200 mM, diluted in methanol) and vigorously mix.
5) Measure the decrease in absorbance for at least 1 minute at 340 nm, the reactions should be linear, if not the sample should be diluted.	

Each sample was measured in triplicate, and means were used for statistical analyses.

Calculations:

The real change in absorption from the sample ($= \Delta abs_{final\ sample}$) is obtained by subtracting the rate of blank reaction ($= \Delta abs_{blank}$) from the rate in absorbance per minute from the sample ($= \Delta abs_{sample}$). With the extinction coefficient of NADPH at 340 nm (6220 M^{-1} cm^{-1}), the activity could then be expressed in nmol min^{-1} mg^{-1} protein, using the Beer-lambert law:

$$C = \frac{\varepsilon * l}{A}$$

With:

- C = concentration of NADPH in mol/l
- ε = extinction coefficient of NADPH at 340 nm (= 6220 M^{-1} cm^{-1})
- l = Length of the optical path in centimetre
- A = absorbance

1.5 Analysis of proteins

Assay:

1) Dilute serum 100 times in KPi buffer (pH 7.5, 100mM).
2) Add 100 µl of diluted sample or standard to a reaction tube.
3) Add 5 ml of Bradford solution and let solution rest at room temperature for 10 minutes.
4) Read absorbance at 595 nm.

For each sample as for the standard curve a triplicate was made.

Calculations:

A standard curve was made with concentrations ranging from 32.25 to 1000 µg/ml BSA (bovine serum albumin) prepared in KPi buffer (pH 7.5, 100mM). With the obtained equation of the standard curve, protein concentration could be calculated and expressed in µg/ml serum.

2. Appendix 2: Pearson correlation matrix of biomarkers

	Proteins	**triglyceride**	**March TBARS**	**October TBARS**	**tThiols**	**tGSH**	**GPx**	**SePGx**
Proteins	**1**	0,176	0,913	0,614	0,141	0,459	0,111	0,060
triglyceride	0,315	**1**	0,477	0,518	0,876	0,825	0,761	0,956
March TBARS	0,032	0,207	**1**		0,339	0,604	0,758	0,726
October TBARS	0,234	-0,334		**1**	0,494	0,744	0,250	0,257
tThiols	0,332	-0,037	-0,276	-0,313	**1**	0,867	0,052	0,146
tGSH	-0,171	-0,053	-0,152	-0,153	-0,039	**1**	0,205	0,220
GPx	-0,358	-0,072	0,090	0,503	-0,429	0,289	**1**	**<0.0001**
SePGx	-0,418	-0,013	0,103	0,496	-0,328	0,280	**0,932**	**1**

Values above diagonal correspond to the p-values, values under the diagonal correspond to Pearson's r.