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# Preliminary study of oxidative stress biomarkers and trace elements in North Sea Harbour Seals

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ABSTRACT

This preliminary study investigated the potential correlations between trace elements (mercury, zinc, cadmium, copper, selenium, lead, nickel, chromium, lithium and vanadium) concentrations, measured in red blood cells, and oxidative stress biomarkers (total thiols, total glutathione, total and selenium-dependent glutathione peroxidases, triglycerides, malondialdehyde) assessed in the respective serum, in males and females *P. vitulina*, sampled in the Wadden Sea in spring and autumn 2015.

Only concentrations of total mercury and zinc showed significant differences by sex, and only lipid peroxidation was different by season. Moreover, significant positive and negative correlations were observed between biomarkers (triglycerides, thiols, malondialdehyde, glutathione) and trace element concentrations (copper, lead, mercury, nickel, zinc). These findings suggest that the studied biomarkers could be useful for the assessment of oxidative stress in harbour seals exposed to trace elements, but further research with larger sample sizes is needed to better understand their specific associations.

## 1. Introduction

Trace elements (TEs) include essential elements for biochemical processes in organisms (e.g. Zn, Se, Cu), and non-essential elements (e.g. Cd, Pb, Hg) known to be toxic to exposed organisms (Richir and Gobert, 2016). Although TEs are naturally present in the environment (e.g. earth's crust), anthropogenic activities (e.g. industries, mining) have dramatically increased the rate of TE introduction into the environment. Their introduction leads to a global contamination of the environment, especially aquatic ecosystems (i.e. freshwater and seawater) (Green et al., 2003; Jaishankar et al., 2014), thus threatening aquatic biota. In organisms, TEs can generate reactive oxygen species (ROS), for example by directly reducing $O_2$ to $O_2^-$ (Kasprzak, 2002), and cause oxidative stress, which is an imbalance between ROS concentration and the organism's antioxidant defence capacity (Scandalios, 2005; Valko et al., 2005). In addition, it is now well established that oxidative stress occurring in organisms often leads to several adverse effects (e.g. DNA adducts, cell membrane destruction) that alter the health status of organisms (Valavanidis et al., 2006).

To mitigate oxidative stress (i.e. ROS products and their effects), organisms have enzymatic and non-enzymatic antioxidant systems, e.g. reduced glutathione (GSH), glutathione peroxidases (GPx), catalase or superoxide dismutase. However, when antioxidant defences are no longer sufficient to control ROS produced, the latter induce cellular damage including lipoperoxidation (Lushchak et al., 2011). Therefore, the use of the antioxidant defences measurement, as biomarkers of exposures or effects, is well established in the environmental risk assessment (Valavanidis et al., 2006; Monserrat et al., 2012), both in vertebrates (Martínez-Álvarez et al., 2005) and invertebrates (Franco et al., 2006; Sroda and Cossu-Leguille, 2011; Horion et al., 2015). Nevertheless, the analysis of these biomarkers is not developed in all aquatic organisms and their usefulness remains under question due to many abiotic and biotic confounding factors (e.g. temperature, sex, age) known to influence biomarker responses and to lead to inconclusive results if these factors are not considered (Martínez-Álvarez et al., 2005; Gismondi et al., 2012a, 2012b, 2012c, 2013, 2015).

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Marine mammals are often used as bioindicators for chemical exposure in the marine environment because of their widespread distribution, top position in the trophic food chain, potential for bioaccumulation of chemicals in their tissues, long-life span and relatively late maturity, including a low reproductive rate (Bossart, 2011). In the past, many studies have demonstrated that continuous exposure to TE affects the immune system (e.g. immunosuppression or immunoenhancement) of marine mammals such as the grey seal *Halichoerus grypus* and the harbour seal *Phoca vitulina* (Kakuschke and Prange, 2007; Kakuschke et al., 2005, 2006, 2008, 2009; Das et al., 2003, 2008; Weirup et al., 2013; Dupont et al., 2016; Lehnert et al., 2016). Consequently, several studies were conducted to develop the evaluation of biomarkers, such as blood cortisol values and transcription of cytokines, interleukin-10 (IL-10) or IL-2, to assess the immune system status of marine mammals exposed to TEs or persistent organic pollutants (Lehnert et al., 2014, 2016, 2018). To date, although studies have measured biomarkers of oxidative stress in marine mammals, they were mainly used to compare aquatic and terrestrial mammals or marine mammals with distinct diving capabilities or to assess the consequences of seal feeding (e.g. suckling, fasting) on oxidative stress (Wilhelm Filho et al., 2002; Cantú-Medellín et al., 2011; Vázquez-Medina et al., 2012). However, to our knowledge, no study has investigated the correlation between TE concentrations and levels of oxidative stress biomarkers measured in seals.

Blood sampling of marine mammals is a non-lethal sampling method, relevant for pollutant and biomarker analyses (Das et al., 2003). In general, TEs are measured in the whole blood of organisms, but this tissue does not always allow the use of commercial kits for the measurement of biomarkers necessary to evaluate sublethal effects. Indeed, although these kits are highly developed, quick to use and allow repeatability of analyses, most kits for antioxidant biomarkers can only be used in serum or plasma. Therefore, this preliminary study aimed at measuring TE concentrations in blood cells and antioxidant biomarkers levels in the respective serum of males and females harbour seal *Phoca vitulina* from the Wadden sea, in order to evaluate potential correlations between TE concentrations and antioxidant defence levels, and thus to study the usefulness and relevance of antioxidant biomarkers in the oxidative stress assessment related to metal contaminations.

Most antioxidant compounds, which play an important role in the detoxification of ROS in organisms, are composed of molecules containing a sulfhydryl (-SH) group, named "thiols". Among them, glutathione (GSH) is the most abundant and protects cells against oxidative stress caused by xenobiotics. GSH is a scavenger of toxic compounds (e.g. though glutathione oxidation) and also the cofactor for antioxidant enzymes such as glutathione peroxidases (GPx) (Meister and Anderson, 1983). GPx are enzymes involved in GSH-related antioxidant defence mechanisms (Margis et al., 2008). They are divided in two glutathione peroxidases: selenium-dependent (SeGPx) and selenium-independent (indSeGPx). SeGPx, which represent the main GPx total activity, can reduce organic peroxides (ROOH) and hydroperoxides ($H_2O_2$) while indSeGPx can only reduce $H_2O_2$ (Regoli et al., 2011). Since information on antioxidant defence levels is not always sufficient on its own to draw conclusions about whether or not the animal is suffering from oxidative stress (Espín et al., 2014), Costantini and Verhulst (2009) suggest associating at least one biomarker of oxidative damage (i.e. biomarker of effect) with biomarkers of antioxidant defences (i.e. biomarkers of exposure); therefore, in addition to analysing exposure biomarkers, we also assessed extent of lipoperoxidation by measuring the content of malondialdehyde (MDA), the end-product of the lipid peroxidation (Miyamoto et al., 2011). Furthermore, since organisms need energy for survival but can also use it to obtain the energy needed to mobilize defence mechanisms (Koop et al., 2008), the total triglyceride content was assessed, as the energy reserves available to the animal (De Coen et al., 2001). Finally, potential negative or positive correlations between concentrations of each TE and levels of each biomarker were assessed, considering sex and season, to discuss the relevance and usefulness of oxidative biomarkers in assessing oxidative stress caused by TE contamination in harbour seal.

## 2. Materials and methods

### 2.1. Blood sampling

Blood samples from 21 adult free-ranging harbour seals *P. vitulina* 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, using the methodology detailed in Hasselmeier et al. (2008). In spring, 8 males and 6 females were sampled, while in autumn, 2 males and 5 females were sampled. Six milliliters of whole blood samples were collected from the extradural vein in Vacutainer™ red top serum tubes including a silicone-coated interior and increasing silica act clot activator. Within 3 h of collection, samples were transported on ice to the laboratory at the University of Veterinary Medicine Hanover where serum and blood cells were prepared using a 5-min centrifugation at 3000*g* and 4 °C. The resulting supernatant and pellet, corresponding to the serum and the blood cells, respectively, were immediately transferred in new microtubes, frozen at −80 °C and transported on dry ice to the laboratory of the Liège University. Then, the serum was used to analyse biomarkers while blood cells were used for TE concentrations measurement, considering season (i.e. spring and autumn) and sex as confounding factors. This study was carried out under the relevant permits of the National Park Office Schleswig-Holstein and the animal experiment permit (AZ 312-72241.121-19) granted by the responsible agency the Ministry of Energy Transition, Agriculture, Environment, Nature and Digitization of the State of Schleswig-Holstein (MELUND).

### 2.2. Analysis of trace elements (TEs) in red blood cells

#### 2.2.1. Mercury

For total mercury (T-Hg) analysis, 10 mg of freeze-dried blood cells were accurately weighed (0.01 mg precision) and loaded into quartz boats (volume: 1500 μL) based on the method used by Damseaux et al. (2017). Total mercury levels were determined using atomic absorption spectrometry at 254 nm, in a Direct Mercury Analyser (DMA 80 Milestone, Minnesota, USA) according to the US EPA standard method 7473 (EPA, 1998). Quality assessment of analyses was assured by analysing samples in duplicates, and using standards (T-Hg 100 $mg{\cdot}kg^{-1}$), blanks (HCl 1%) and Certified Reference Materials (Seronorm L-3: Human, whole blood = 17.9 ± 1.8 $\mu g\ L^{-1}$ and DORM-2 Dogfish, muscle = 4.64 ± 0.14 $mg{\cdot}kg^{-1}$ dw of methylmercury) at the beginning and the end of each series. The percentage recovery for DORM-2 ranged between 101% and 105% showing optimal run of the analyses. Total-Hg concentrations in blood cells were expressed in $mg{\cdot}kg^{-1}$ dry weight (dw).

#### 2.2.2. Other trace elements

Concentrations of nine trace elements known to interact with antioxidant defences and cause toxic effects on exposed organisms were assessed in *P. vitulina* blood cells, based on Habran et al. (2012). This included: cadmium (Cd), chromium (Cr), copper (Cu), lithium (Li), nickel (Ni), lead (Pb), selenium (Se), vanadium (V) and zinc (Zn). Briefly, for each sample, 180–220 mg of blood cells were subjected to microwave-assisted digestion in Teflon™ vessels with 2 mL $HNO_3$ (65%), 1 mL $H_2O_2$ (30%) and 5 mL of 18.2 MΩ-cm deionized water. After cooling, samples were diluted to 50 ml with 18.2 MΩ-cm deionized water. The concentration of each TE was determined by inductively coupled plasma mass spectroscopy (ICP-MS, PerkinElmer, Sciex, DCR 2). Multiple-element ($^{74}Ge$, $^{103}Rh$, $^{209}Bi$, $^{69}Ga$) internal standards (CertiPUR®, Merck) were added to each sample and calibration standard solutions. Quality control and quality assurance for ICP-MS analysis were analysed twice (i.e. one at the beginning and one at the end of the analyses). This included method blanks and Certified Reference Materials (Seronorm L-3: Human whole blood and DORM-2: Dogfish muscle).

The percentages recovery for Seronorm L-3 and DORM-2 ranged between 98% and 110% and between 101% and 105%, respectively, showing optimal run of the analyses. TE concentrations in blood cells were expressed in $mg \cdot kg^{-1}$ or $\mu g \cdot kg^{-1}$ dry weight (dw).

### 2.3. Biomarker assessments in the serum

#### 2.3.1. TBARS assay

The thiobarbituric acid (TBA) assay was used to assess lipid peroxidation using a modified protocol of Lepage et al. (1991). First, 240 µL of serum were mixed with 240 µL 0.5% butylated hydroxytoluene (BHT) and 960 µL distilled water. Then, 165 µL of 1.4 M trichloroacetic acid (TCA) were added, the samples were mixed, and centrifuged for 10 min at 1000*g*. A volume of 1200 µL of supernatant was transferred to a new test tube and 1 mL of 1% thiobarbituric acid (TBA) was added, as well as 200 µL of 1 N NaOH used to adjust the pH to 2. Reaction was performed by heating the test tubes to 100 °C for 30 min and stopped by cooling on ice for 4 min. After having added 150 µL of 5 N HCl to reach a pH below 0.75, TBARs complexes were extracted with 750 µL *n*-butanol and a 10-min centrifugation at 1000*g*. The optical density of the resulting supernatant was measured at 535 nm in duplicates for each sample, and MDA concentrations were expressed in pmol/mg protein, by using a MDA standard curve ranging from 0.31 µM to 10 µM.

#### 2.3.2. Total thiols

The total thiols assay was adapted from the protocol describe in Costa et al. (2006). This assay is based on the interaction between thiol and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) forming a highly coloured complex with maximum absorbance at 412 nm. Briefly, 50 µL of serum was mixed with 770 µL of 100 nM phosphate buffer-EDTA pH 7.5 and 20 µL of 10 mM DTNB. Mixture reaction was incubated for 5 min at room temperature before reading the optical density at 412 nm. Total thiols contents were measured in duplicates for each sample and expressed as µmol eq. GSH/mg protein using a standard curve ranging from 3.9 µM to 500 µM of reduced glutathione as thiol reference.

#### 2.3.3. Total glutathione

The total glutathione concentration was measured using the Total Glutathione kit (Cayman, Sanbio, Netherlands) performed in 96-well plate and following the manufacturer's instructions. This "indirect" assessment is based on the reaction between the sulfhydryl group of reduced glutathione (GSH) and DTNB, producing a yellow coloured 5-thio-2-nitrobenzoic acid (TNB) having an absorption peak between 405 and 414 nm. 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. Furthermore, oxidized glutathione (GSSG) is also reduced to GSH by glutathione reductase; hence, the obtained result reflects both GSH and GSSH, called hereafter "total glutathione" (tGSH).

First, samples were deproteinized by adding 100 µL of 10% metaphosphoric acid to 100 µL of serum, kept at room temperature for 5 min, and centrifuged at 10,000 ×*g* for 10 min. Then, 6 µL of 4 M triethanolamine were added per 100 µL of supernatant. Total GSH (tGSH) measurement was performed by placing 50 µL of each deproteinized sample in two wells (duplicates) mixed with 150 µL reactional mixture containing MES buffer (0.4 M 2-(N-morpholino)ethanesulphonic acid, 0.1 M phosphate buffer and 2 mM EDTA, pH 6), $NADP^+$ and glucose-6-phosphate as co-factors, glutathione reductase, glucose-6-phosphatase and DTNB. Reaction was incubated for 25 min while agitating, and absorbance was measured at 405 nm. Results were estimated by using a GSSG standard curve ranging from 0.25 µM to 8 µM and expressed as pmol tGSH/mg protein.

#### 2.3.4. Total and selenium-dependent glutathione peroxidases

Total glutathione peroxidase (GPx tot) and selenium-dependent glutathione peroxidase (Se-GPx) activities were assayed according to Regoli et al. (2011): cumene hydroperoxide (200 mM) and hydrogen peroxide (100 mM) were used as substrates for the determination of GPx-tot and Se-GPx activities, respectively. Reactive mixtures were mixed with reduced glutathione (100 mM), NADPH (27 mM) and glutathione reductase (100 U/mL). For the Se-GPx assay, sodium azide (100 mM) was also added. The activity was monitored by following the decrease in NADPH concentration (absorbance at 340 nm), which is consumed during the generation of GSH from oxidized glutathione (extinction coefficient 6.2 $mM^{-1} \cdot cm^{-1}$). Each sample was measured in duplicate and the average was calculated. Both GPx-tot and Se-GPx activities were expressed in nmol/min/mg protein.

#### 2.3.5. Triglyceride content

Triglyceride concentrations were determined in serum by using the DiaSys kit (Diagnostic System, Grabels, France) in 96-well plate. Briefly, 10 µL of serum diluted 4 times in 0.9% NaCl were placed in well and mixed with 240 µL of reaction mixture (DiaSys kit solution) as recommended by the manufacturer. Next, 96-well plate was incubated for 5 min at 37 °C while agitating. Absorbance was measured at 492 nm, and results were estimated by using a standard curve ranging from 62.5 to 71.5 mg/L of triglyceride, and expressed as mg/L.

#### 2.3.6. Protein content

The total protein content of each sample was quantified according to Bradford (1976) using bovine serum albumin (BSA) for the standard curve. Results were expressed in mg/L.

### 2.4. Statistical analysis

Due to the low sample sizes, statistical analyses were carried out considering on the one hand sex (pooled season data) and on the other hand season (pooled sex data). First, data were tested for normality and homogeneity of variance using Shapiro-Wilk and Bartlett tests, respectively. Normality and homoscedasticity were respected for both TE concentrations and biomarkers results; therefore, Student's *t*-tests were performed. In addition, correlations between each TEs and biomarkers were estimated using Pearson coefficient correlation test. When no significant differences were observed according to sex and/or season, correlations were analysed using all data set. Otherwise, they were performed considering season and/or sex. All analysis was performed using XLStat 2019.2.1 software.

## 3. Results

### 3.1. Trace element concentrations

The concentrations of TE in harbour seal blood cells are presented in Table 1. No differences in TE concentrations according to sex or season were observed, except for T-Hg and Zn. Indeed, when combining season data, males on average had higher levels of T-Hg and Zn than females (*p*-values = 0.009 and 0.036, respectively).

### 3.2. Antioxidant biomarkers

All biomarker results considering season and sex are presented in Fig. 1. Only TBARs content showed significant differences by season (pooled by sex), being higher in autumn than spring (Fig. 1B, *p*-value = 0.002).

### 3.3. Correlations between TE and biomarkers

Correlations were examined on the overall results (males and females combined or spring and autumn combined) when no statistical differences were observed by sex and/or season. In opposite, correlations for T-Hg and Zn were analysed considering sex, while correlations for TBARs were analysed considering season.

**Table 1**
Mean trace elements (TE) concentrations in red blood cells of *Phoca vitulina* males and females sampled in spring and autumn 2015 in the Wadden sea. Italic values indicate standard deviation.

| | | T-Hg (mg/kg) | Zn (mg/kg) | Cd (μg/kg) | Cu (mg/kg) | Se (mg/kg) | Pb (μg/kg) | Ni (μg/kg) | Cr (μg/kg) | Li (μg/kg) | V (μg/kg) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Spring | Males | 1.3 | 12.4 | 3.0 | 2.2 | 3.3 | 17.3 | 16.6 | 63.9 | 48.3 | 2.2 |
| | (n = 8) | 0.3 | 0.4 | 3.8 | 0.1 | 0.6 | 3.4 | 9.7 | 50.3 | 10.2 | 0.6 |
| | Females | 1.1 | 11.5 | 1.9 | 2.3 | 3.2 | 25.1 | 29.0 | 42.7 | 53.9 | 1.8 |
| | (n = 6) | 0.2 | 0.7 | 0.8 | 0.2 | 0.3 | 9.7 | 40.2 | 12.1 | 9.4 | 0.8 |
| Autumn | Males | 1.3 | 13.1 | 1.3 | 2.4 | 3.1 | 16.6 | 25.1 | 32.6 | 41.9 | 1.1 |
| | (n = 2) | 0.4 | 0.3 | 0.01 | 0.01 | 0.1 | 0.01 | 1.01 | 2.01 | 3.01 | 4.01 |
| | Females | 0.8 | 12.5 | 6.2 | 2.3 | 2.8 | 18.8 | 40.8 | 69.9 | 44.5 | 3.4 |
| | (n = 5) | 0.3 | 0.4 | 11.0 | 0.2 | 0.7 | 5.3 | 29.3 | 21.8 | 12.4 | 4.9 |
| | MeanSpring | 1.2A | 12.1A | 2.5A | 2.3A | 3.3A | 20.6A | 21.9A | 54.8A | 50.7A | 2.0A |
| | (n = 14) | 0.3 | 0.7 | 2.9 | 0.2 | 0.5 | 7.6 | 26.7 | 39.2 | 9.9 | 0.7 |
| | MeanAutumn | 1.0A | 12.6A | 4.8A | 2.3A | 2.9A | 18.2A | 36.3A | 59.3A | 43.7A | 2.8A |
| | (n = 7) | 0.4 | 0.5 | 9.3 | 0.1 | 0.5 | 4.5 | 28.5 | 25.5 | 12.2 | 4.2 |
| | MeanMales | 1.3a | 12.6a | 2.7a | 2.2a | 3.3a | 17.2a | 18.3a | 57.6a | 47.0a | 2.0a |
| | (n = 10) | 0.3 | 0.5 | 3.4 | 0.1 | 0.5 | 3.0 | 14.5 | 46.3 | 10.9 | 0.7 |
| | MeanFemales | 0.9b | 11.9b | 3.9a | 2.3a | 3.0a | 22.2a | 34.4a | 55.1a | 49.6a | 2.5a |
| | (n = 11) | 0.3 | 0.7 | 7.3 | 0.2 | 0.5 | 8.3 | 34.5 | 21.6 | 11.4 | 3.3 |

Lowercase: Significant differences between seasons in males.
Uppercase: Significant differences between seasons in females.
*: Significant differences between males and females sampled the same season.

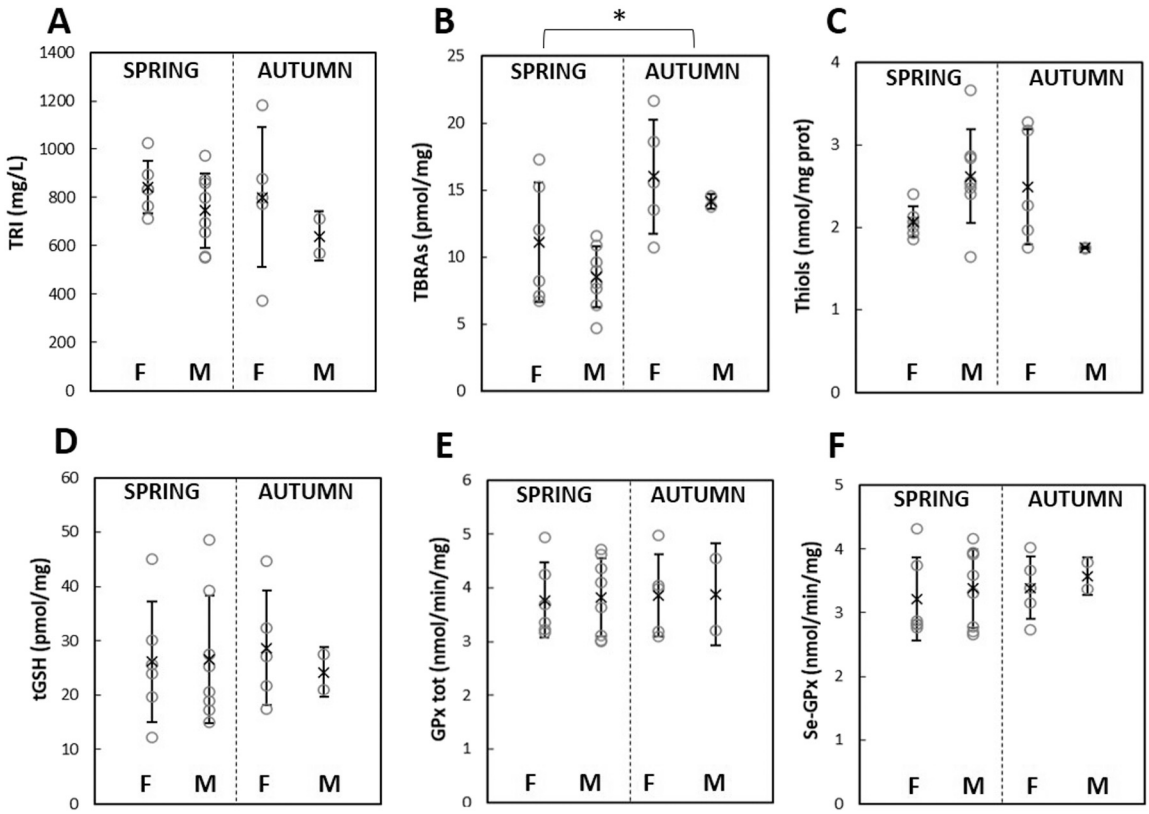


**Fig. 1.** Biomarkers assessments in serum of *Phoca vitulina* males and females sampled in spring and autumn 2015 in the Wadden sea. Replicates values (open circles) and means ± standard deviations were indicated. Asterisk indicates season differences. TRI: triglyceride, TBARS: lipid peroxidation, tGSH: total glutathione, GPx tot: total glutathione peroxidase, Se-GPx: selenium-dependent glutathione peroxidase.

Among all the biomarkers measured in this study, only GPx-tot and Se-GPx showed no correlation with any of the TE concentrations (Table S1). Significant positive correlations were observed between triglyceride content and concentrations of Cu and Pb ($\rho = 0.489$, $p$-value $= 0.024$; $\rho = 0.543$, $p$-value $= 0.01$, respectively) (Fig. 2). TBARS concentrations were positively correlated with Pb and Ni concentrations ($\rho = 0.63$, $p$-value $= 0.016$; $\rho = 0.55$, $p$-value $= 0.04$, respectively) in the spring (Fig. 3A), but no correlations were observed in the autumn (Fig. 3B). Moreover, a negative correlation was measured between total thiols contents and Pb concentrations ($\rho = -0{,}448$, $p$-value $= 0.04$) (Fig. 4). Finally, considering sex differences in T-Hg and Zn concentrations (Table 1), only correlations with tGSH and TBARs contents, respectively, were observed (Fig. 5). Levels of tGSH were negatively correlated with T-Hg concentration in females ($\rho = 0.66$, $p$-value $= 0.02$), while no correlation was observed in males ($\rho = 0.43$, $p$-value $= 0.20$) (Fig. 5A). Similarly, TBAR levels and Zn concentrations were positively correlated in females ($\rho = 0.65$, $p$-value $= 0.03$) but not in males ($\rho = 0.362$; $p$-value $= 0.30$) (Fig. 5B).

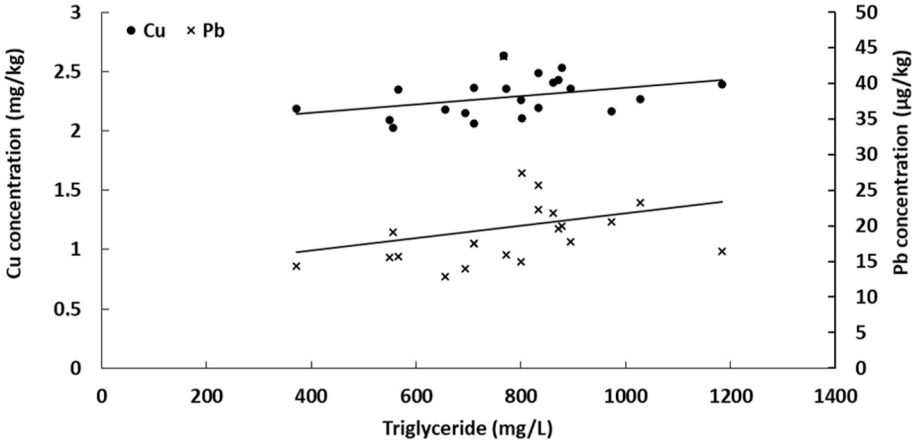


**Fig. 2.** Correlations between triglyceride content and Cu and Pb concentrations in *Phoca vitulina* considering all dataset. The coefficients of correlation for Cu and Pb were ρ = 0.489 (*p*-value = 0.024) and ρ = 0.543 (*p*-value = 0.011), respectively. Lines indicate significant linear regressions.

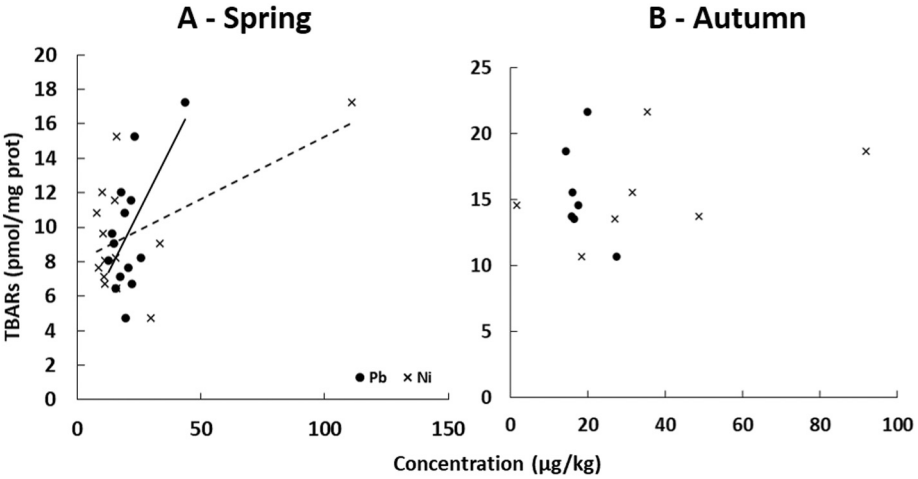


**Fig. 3.** Correlations between lipid peroxidation (TBARs) and Pb and Ni concentrations in *Phoca vitulina*, considering season. In spring, correlations with Pb and Ni were ρ = 0.628 (*p*-value = 0.016) and ρ = 0.554 (*p*-value = 0.04) respectively, while in autumn, correlations were ρ = −0.393 (*p*-value = 0.384) and ρ = 0.461 (*p*-value = 0.298), respectively. Line and dote-line indicate significant linear regressions for Pb and Ni, respectively.

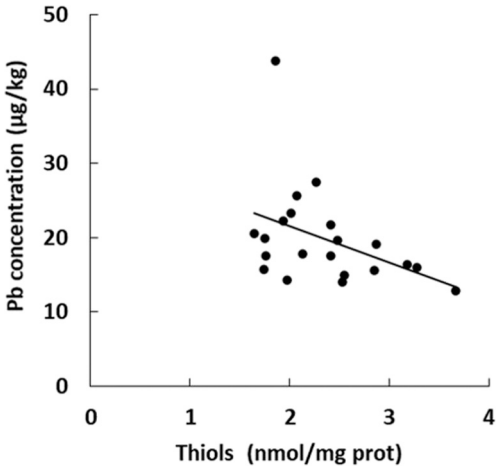


**Fig. 4.** Correlations between total thiols content and Pb concentrations in *Phoca vitulina*, considering all dataset (ρ = −0.448; *p*-value = 0.04). Line indicates significant linear regression.

## 4. Discussion

### 4.1. Trace element (TE) concentrations

Although TE concentrations are not routinely measured in blood cells from marine mammals, results of the present study are in the same pattern than those measured in whole blood from harbour seals of the Wadden sea (Griesel et al., 2008). However, considering that many TEs, such as Hg, Pb, Ni or Cr, bind to red blood cells (Pyle and Couture, 2011; Devoy et al., 2016), TE concentrations measured in blood cells are expected to be slightly higher than those measured in whole blood.

The results highlighted differences in T-Hg and Zn concentrations according to sex (pooled season), i.e. higher T-Hg and Zn concentration in *P. vitulina* males than females (Table 1). Although these results are not consistent with those of Das et al. (2008), who did not observe differences between male and female harbour seals, they are consistent with results obtained by McHuron et al. (2014) in harbour seals *P. vitulina* of central California and Kopec and Harvey (1995) in harbour seals in San Francisco Bay. Several factors might influence TE concentrations in blood of seals including sex, body size, season, moulting, diet, behaviour and sampling location (e.g. Thompson et al., 1989; Das et al., 2008; Habran et al., 2012). The difference in T-Hg concentrations between males and females could resulted of the reproduction process, especially

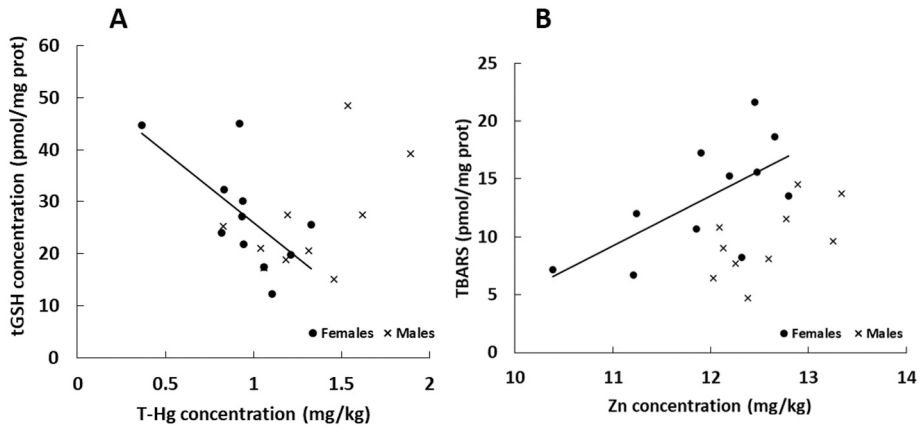


**Fig. 5.** Correlations between (A) total glutathione content and T-Hg concentrations in *Phoca vitulina* males and females ($\rho = 0.43$; *p*-value = 0.20; $\rho = -0.66$; *p*-value = 0.02, respectively) and (B) lipoperoxidation (TBARs) and Zn concentrations in *Phoca vitulina* males and females ($\rho = 0.36$; *p*-value = 0.30; $\rho = 0.65$; *p*-value = 0.03). Lines indicate significant linear regression.

the maternal transfer of T-Hg from females to their pups during gestation and lactation (Wagemann et al., 1988; Kakuschke et al., 2009; Habran et al., 2012; McHuron et al., 2014). Although no significant differences were observed between seasons, probably due to the small sample size, diet and foraging behaviour of harbour seals could to different contamination levels according to the season. In their study, de La Vega et al. (2016) showed a net change in the diet and foraging behaviour of harbour seals from a pelagic diet in spring to a benthic diet in summer, as seals tend to remain in the Wadden Sea in summer but tend to increase the use of North Sea resources in winter. These dietary variations and changes in foraging locations between seasons could therefore result to different exposure to TE.

### 4.2. Oxidative stress biomarkers and TE exposure

To our best knowledge, no biomarkers of oxidative stress have been evaluated on harbour seals to date. Therefore, six common biomarkers of exposure and effect, used in ecotoxicological risk assessment (Amiard-Triquet et al., 2012), were evaluated in *P. vitulina* serum considering sex and season (Fig. 1). Despite the fact that studies have already focused on antioxidant defence levels in marine mammals (Wilhelm Filho et al., 2002; Vázquez-Medina et al., 2006; Cantú-Medellín et al., 2011; Righetti et al., 2014), very few analysed the levels of oxidative stress biomarkers by separating males and females. Our results revealed no significant differences between males and females, suggesting that sex does not influence the assessment of these six biomarkers in *P. vitulina* serum. The results seem in contradiction with previous works showing sex differences in the use of biomarkers in aquatic vertebrates (Figueiredo-Fernandes et al., 2006) or invertebrates (Gismondi et al., 2012a, 2012b, 2012c, 2013, 2015). However, even if our results might be caused by the small sampling sizes (i.e. 11 females and 10 males), results are in agreement with those obtained by Sharick et al. (2015) and Kanerva et al. (2012) who observed no significant difference in oxidative stress biomarkers (e.g. glutathione peroxidase, catalase and superoxide dismutase activities, glutathione concentration) measured in males and females of the elephant seal *Mirounga angustirostris* and the ringed seal *Pusa hispida*, respectively. These results assume that males and females could face oxidative stress in a similar way, which is not necessarily the case as shown by Costantini (2018), who combined results from many studies, conducted on vertebrates, and often observed differences between males and females in oxidative damage or antioxidant regulation. However, the reasons for these differences remain poorly understood to this day.

The effect of season, as a confounding factor, was also evaluated on biomarkers (Fig. 1). No significant season difference was observed in biomarker, except for TBARS which was higher in autumn than spring. The result is surprising if you consider that seasonal changes in antioxidant defences are commonly highlighted in many invertebrates and vertebrates (see review Chainy et al., 2016). For example, it was observed that rats exposed to high temperature in summer had low levels of antioxidant enzymes and higher oxidative stress as compared to winter season (Bhat et al., 2008). Similarly, Belló-Klein et al. (2000) observed higher lipoperoxidation in heart and liver of rats during summer as compared to winter. Nevertheless, the results indicated seasonal differences in TBARS levels, underlying higher lipid peroxidation in the autumn than in the spring (Fig. 1B). In fact, several seasonal factors can influence the energy and physiology of harbour seals including water temperature, reproduction and prey availability (Thompson et al., 1997; Kershaw and Hall, 2016; Jensen et al., 2017). Water temperatures in the North Sea show pronounced seasonal changes. Heat loss by seals must be compensated by increased food intake or catabolism of protein and fat to maintain a constant internal temperature (Harding et al., 2005). The absence of increased antioxidant enzyme activities in autumn as compared to spring supports the assumption of oxidative stress occurring in autumn (i.e. higher TBARs observed in autumn as compared to spring), probably due to increase in ROS production. In fact, when the antioxidant defences are overwhelmed and unable to eliminate excess ROS (i.e. lack of activity increase), cellular damage occur, particularly lipid peroxidation (Scandalios, 2005).

In the present study, several biomarkers were positively or negatively correlated with TE concentrations. Indeed, triglyceride content was positively correlated to Cu and Pb concentrations in red blood cells of *P. vitulina* (Fig. 2). The positive correlation between triglyceride and Cu is consistent with results from Lei et al. (2017) who observed that dietary copper addition significantly increased blood triglyceride levels in rabbits, probably through upregulation of carnitine palmitoyltransferase gene expression (key enzymes for lipolysis). In the same way, the correlation between triglyceride content and Pb contamination is similar to that observed by Skoczyńska et al. (1993) who showed an increase in serum triglycerides in rats exposed to lead. This correlation could be the consequence of an inhibition of the lipoprotein lipase enzyme (Kihara et al., 1989), responsible for the degradation of triglycerides and which is linked to the β-adrenergic receptor, known to be inhibited by lead in rats (Skoczyńska et al., 1986).

The results also revealed that the higher the concentrations of Pb and Ni, the higher the levels of TBARs in *P. vitulina* (Fig. 3). This is consistent with other studies that have shown that both Pb an Ni induce lipid peroxidation (MDA) in humans and rats (Das et al., 2001; Chen et al., 2003; Kasperczyk et al., 2004; Lopes et al., 2016; Tsao et al., 2017). The toxicities of Pb and Ni are well established and may result in carcinogenicity, neurotoxicity, embryotoxicity or reproductive toxicity

(Outridge and Scheuhammer, 1993; Brix et al., 2017; Singh et al., 2018; Rizvi et al., 2020). Among the mechanisms related to their toxicity is the production of ROS (Xi et al., 1993; Lopes et al., 2016), which are well known to induce lipid peroxidation. However, Pb and Ni concentrations were only correlated with TBARs in spring, and no correlation was measured in autumn. This season-related correlation is surprising if we consider that the season didn't influenced TE concentrations. However, even if no significant correlation was observed in the autumn between any TE concentrations and TBARs and that no higher TE concentrations were measured in autumn, it cannot be ignored that other factors could be correlated with this parameter and explain the higher levels in autumn than in spring, such as contamination by organic pollutants (e.g. polychlorinated biphenyls), also known to induce oxidative stress (Miyamoto et al., 2011). In addition, TBARs levels were also positively correlated to Zn concentrations in females (Fig. 5B), which is in contradiction with many experiments showing a protective role of Zn (Jemai et al., 2007; Mansour and Mossa, 2010). Zn is known as an essential trace element having several basic functions in metalloenzymes, transcription factors, immunoregulation, growth, and cytoprotection, with antioxidant, anti-apoptotic, and anti-inflammatory roles. For example, administration of Zn to rats exposed to chlorpyrifos has been shown to reduce the lipid peroxidation caused by the pesticide (Goel et al., 2005). However, conversely, too high concentration of Zn could cause inhibition of mitochondrial respiration and induce oxidative stress (Franco et al., 2006), which could probably explain the positive correlation observed in the present study. However, it is difficult to assess the basal concentration (and therefore tolerated concentrations) of elemental TE such as Zn, in wild populations living in continuously contaminated areas.

Total thiols represent proteins such as metallothionein or glutathione (GSH) that are able to bind to TEs (Amiard-Triquet et al., 2012). Total thiols showed a significant negative correlation with Pb concentration (Fig. 4), suggesting a mobilization of thiols to cope with metal contamination. However, the detoxification through glutathione pathways seemed not involved in this depletion, as no significant correlation was highlighted between tGSH levels or GPx activities and Pb concentration (Table S1). Among thiols proteins, tGSH showed a significant negative correlation with T-Hg concentration in females, but not in males (Fig. 5), indicating a possible sex-related difference in the antioxidant defences mobilization, as already underlined in aquatic vertebrates (Figueiredo-Fernandes et al., 2006) and invertebrates (Gismondi et al., 2012a, 2012b, 2012c, 2013, 2015), but probably not observed here due to the small sample size.

Finally, no correlation was measured between GPx activity and TE concentration, whereas GPx are one of the major components of cell protection against oxidative damage (Regoli et al., 2011), especially the Se-GPx activity. Indeed, the results revealed that the majority of GPx activities came from the Se-dependent form (Se-GPx) (Fig. 1). Therefore, it could be expected that Se concentrations influence Se-GPx activities as observed in mammals and described by several authors (i.e. a decrease in Se-GPx activity when Se concentration is low or not bioavailable, or an increase of Se-GPx activity when Se concentrations increase) (Thompson et al., 1976, 1977; Lloyd et al., 1989; Krofič Žel et al., 2014). However, the concentration of Se was not correlated with the activities of GPx or Se-GPx in *P. vitulina*. This could be explained by an excessively high Se concentration in the blood of *P. vitulina*, as observed in humans by Lloyd et al. (1989). These authors did not show a significant correlation between Se and GPx activity when Se concentrations were higher than 1.26 μmol/L, whereas a very strong association was measured at a Se concentration below this threshold. The lack of correlation could also be the consequence of a complexation of Se with other TEs (e.g. Pb, Cd, Hg) leading to an unavailability of Se for GPx activity (Espín et al., 2014; Gajdosechova et al., 2016; Lopes et al., 2016). Nonetheless, Se concentrations tended to be negatively correlated to tGSH levels ($\rho = -0.478$, $p$-value $= 0.053$, Table S1), suggesting a mobilization of glutathione pathways due to Se toxicity (e.g. ROS production). Finally, it cannot be ignored that the absence of correlation between Se concentration and GPx activity could result from measurements carried out in different tissues (i.e. Se in red blood cells and GPx in serum), suggesting in the same way that serum does not appear to be the appropriate tissue for using GPx activity as biomarkers.

## 5. Conclusion

This preliminary study aimed to evaluate potential correlations between TE concentrations, measured in blood cells, and biomarkers of oxidative stress measured in the respective serum, in the harbour seal *P. vitulina*, considering sex and season as confounding factors. Indeed, the use of biomarkers in environmental risk assessment is often controversial due to the influence of these confounding factors (Amiard-Triquet and Berthet, 2015). However, the biomarkers of oxidative stress measured in serum of *P. vitulina* did not seem to be influenced by sex and season, and showed significant positive or negative correlations with TEs concentrations. Nevertheless, although these results are promising for the use of these biomarkers in environmental risk assessment, this finding needs to be further investigated especially due to the small sample sizes. Similar investigations should be conducted with increased sample size to improve understanding of the relationships between blood TE concentrations, oxidative stress, and antioxidant biomarker levels observed in this work. Similarly, other biomarkers of antioxidant defences (e.g. superoxide dismutase, catalase) should be developed and evaluated in serum of *P. vitulina* in the future, to obtain an overview of antioxidant enzyme activities, and thus significantly increase the confidence of results assessing oxidative stress. Finally, all the biomarkers of oxidative stress studied here could be useful in a multi-biomarker approach (e.g. complementing the molecular biomarkers relevant to the immune system already developed in Lehnert et al., 2018) to increase the significance of conclusions regarding the global health status of *P. vitulina* exposed to environmental contamination.

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## CRediT authorship contribution statement

**Eric Gismondi:** Conceptualization, Methodology, Supervision, Formal analysis, Writing – original draft, Writing – review & editing. **Lucienne Daneels:** Data curation, Formal analysis, Writing – original draft. **France Damseaux:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Kristina Lehnert:** Methodology, Resources, Writing – original draft. **Ursula Siebert:** Methodology, Resources, Writing – original draft, Writing – review & editing. **Krishna Das:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Resources, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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