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Detection of oxidative stress in tissue homogenate from krill exposed to oil

Master's thesis in Biological Chemistry

By
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Universitetet i Stavanger

IRIS

HELSE STAVANGER
Stavanger universitetssjukehus

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Abstract

Krill are small crustacean animals, living all over the world. They are key organisms in the marine food web. The petroleum industry releases oil to the sea with the produced water and by accidental oil spill, and there is a concern about the effect the oil has on krill. Discharges of oil can lead to a situation of oxidative stress in marine organisms. Biomarkers such as malondialdehyde (MDA) and advanced oxidative protein products (AOPP) can be used to detect oxidative stress.

Existing methods for homogenization, total protein content, AOPP and MDA were modified and validated for use on krill homogenate. The methods were then utilized to analyze krill from a study performed at the International Research Institute of Stavanger (IRIS), where krill had been exposed to sublethal oil concentrations for eight days. The experiments were performed in spring, autumn and winter to investigate potential seasonal variations in biomarker response.

The following results were obtained:

- The homogenization process includes two successive centrifugation steps in order to get a clear and stable krill homogenate.
- The krill homogenate had to be pre-diluted for the spectrophotometric methods to get an absorbance within the linear range of the calibrators (1:10 or 1:15 for total protein and 1:8.3 or 1:13.3 for AOPP).
- Instrumental limits of detection and quantification for AOPP were 2.44 µM and 7.38 µM respectively, and the instrumental limit of quantification for MDA was 0.28 µM.
- The within-run variations were 7.8% and 4.0-13% for plasma control and krill homogenate respectively for AOPP, and 19% for krill homogenate control for MDA.
- Between-run variation was 8.8% for plasma control for AOPP, and 19% and 10% for krill homogenate control and plasma respectively for MDA.
- AOPP and MDA levels were significantly higher in krill that were frozen directly after capture (T0) in spring and autumn compared to krill kept in the laboratory (T1).
- Seasonal differences were detected with a significantly lower T1 MDA level in the spring krill, a significantly higher T0 MDA in autumn krill, and a significantly lower T0 AOPP in winter compared to T1 or T0 respectively of the other seasons.
- Any effect of the oil exposure however was not observed with MDA or AOPP.

Three methods, AOPP, MDA and total protein, were successfully adjusted and validated for analysis of krill homogenate. Due to the good sensitivity of the methods, individual krill could be analyzed for both AOPP and MDA, and the concentrations normalized with respect total protein content. AOPP was a simple and fast method, and with higher precision than the MDA. An improved AOPP could be the method of choice for the future monitoring of oxidative stress level in krill. However, changes in AOPP or MDA levels due to oil treatment for eight days were not detectable due to a high natural variation of these biomarkers in the krill homogenate.

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Abbreviations

AGE	Advanced Glycation End Product
ALE	Advanced Lipoxidation End Products
AOPP	Advanced Oxidative Protein Products
BCA	Bicinchoninic Acid
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
CA	Citric Acid
CV	Coefficient of Variation
DNPH	2,4-dinitrophenylhydrazine
DW	Dry Weight
HPLC	High Performance Liquid Chromatography
HPLC-F	High Performance Liquid Chromatography with Fluorescence detector
IRIS	International Research Institute of Stavanger
IUPAC	The International Union of Pure and Applied Chemistry
MDA	Malondialdehyde
NFR	Norges forskningsråd
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate Buffered Saline
PDA	Photodiode array
PROOF/PROOFNY	Long-term effects of discharges to sea from petroleum-related activities. Research programs under NFR (2002-2015).
PUFA	Polyunsaturated Fatty Acid
ROS	Reative Oxygen Species
SeaSens	Seasonal variation in sensitivity of krill to oil
SOP	Standard Operating Procedure
SUS	Stavanger University Hospital
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substance
TEP	Tetraethoxypropane
TProt	Total Protein
UiS	University of Stavanger
UV	Ultraviolet

1 Introduction

This thesis is part of the "SeaSens - Seasonal variation in sensitivity of krill to oil" project at International Research Institute of Stavanger (IRIS) funded by Norges forskningsråd (NFR). The SeaSens project will investigate krill response to oil in produced water, with a focus on seasonal variation.

1.1 Krill

Krill (euphausiids) are small shrimplike crustaceans (Figure 1-1), and in the same species class (*Malacostraca*) as shrimps, craps, lobsters and woodlice [1]. There are about 85 krill species spread all over the world [2], Northern krill (*Meganyctiphanes norvegica*) is the most common species in the north, where it habitats a large area in the North Atlantic from 30° N to 80° N, and the western part of the Mediterranean Sea [3]. The northern spawning limit for *M. norvegica* is around the Arctic Circle and it cannot truly be regarded as an Arctic species [4]. *Thysanoessa* is a genus that can be found in the north; where Arctic krill (*Thysanoessa raschii*) is found as far north as 80° N in the Arctic Ocean [2]. *T. inermis* and *T. longicaudata* are the most common species in the Barents Sea [5].

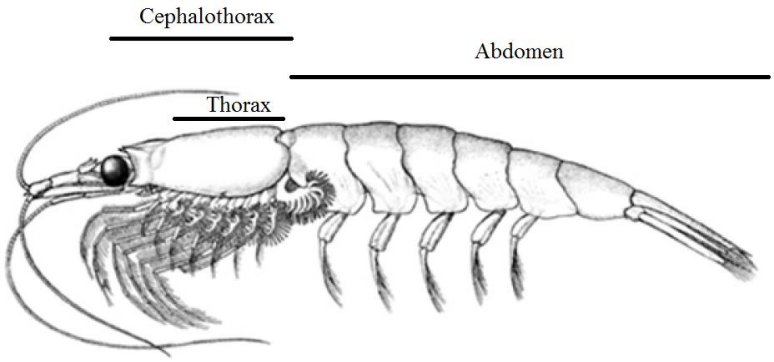

Figure 1-1 ***The Northern krill (Meganyctiphanes norvegica)****. Modified from [6]*

The majority of krill species live 0-400 m under the sea surface, with a diurnal vertical migration where they approach the surface at night [2]. Most krill are herbivorous with a diet of phytoplankton and algae, but some krill eat zooplankton as well [2]. The *M. norvegica* are carnivorous and can even eat their own species [2]. The krill are a part of the lower food chain, and they are prey for larger animals such as fish, squid, whale, penguin and seal [7].

Krill have a transparent body where the digestive system is visible (Figure 1-2). The food is mechanically processed in the stomach before the enzymes in the migut gland (hepatopancreas) further break it down [8]. Krill contain some red pigments (the carotenoid astaxanthin and its esters), that are assumed to provide UV protection [9]. Their astaxanthin source is digested algae [9]. The krill live in the dark, and the *Euphausiidae* family all possess light organs (photophores) to produce blue light, where the 10 separate light organs of *M. norvegica* allow it to bioluminescence spontaneously via a luciferin-luciferase-type of biochemical reaction [10].

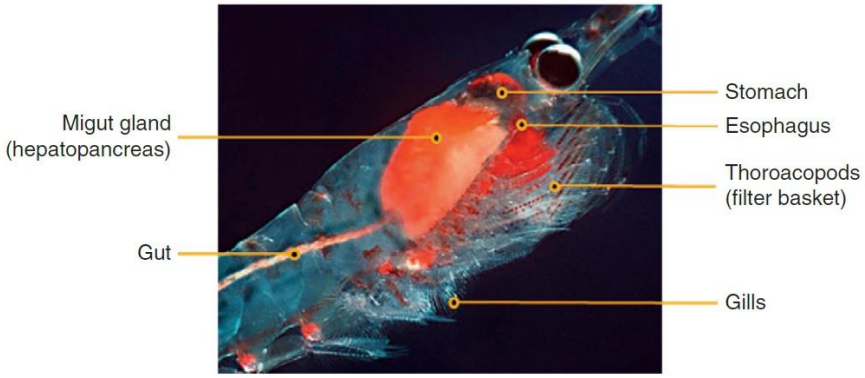

Figure 1-2 ***The digestive system of M. norvegica in the cephalothorax area*** *[8].*

The lipid composition in krill varies with season and food sources available. Falk-Petersen et al. [4] found a total lipid content (% of dry weight DW, Figure 1-3) in *M. norvegica* that was highest in the winter months, suggesting a more herbivorous diet during the summer. The cephalothorax contains the hepatopancreas that has the highest lipid content of all the krill organs (65% DW). The cephalothorax also contains the stomach (17% DW lipid content), the gonads (34% DW lipid content) and fat body of connective tissue (20% DW lipid content) [8]. The abdomen contains mostly muscle tissue with only 8% (DW) of lipids [8]. A high lipid store may leave the animals more disposed for oxidative stress, as lipids are preferred targets of reactive oxygen species (ROS) [11].

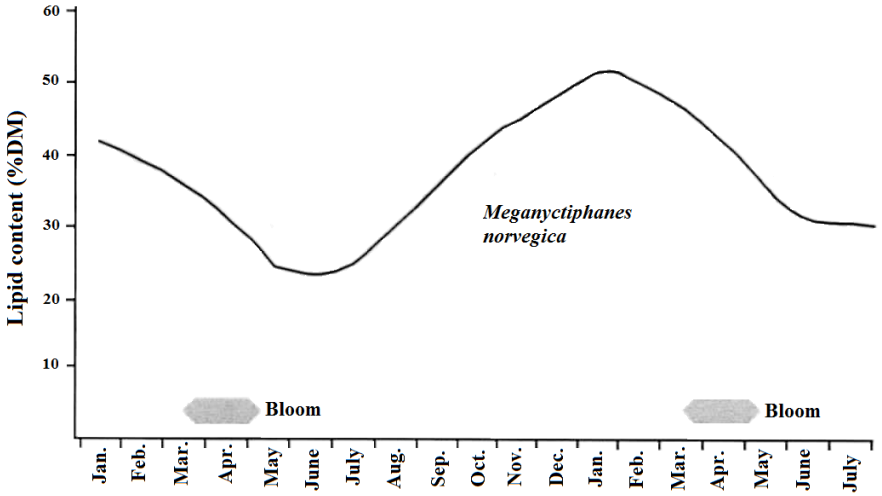

Figure 1-3 ***Lipid dynamics (total lipid) of M. norvegica in relation to season****. The spring bloom period of phytoplankton is indicated. Modified from [4].*

1.2 Produced Water

Krill from the SeaSens project were exposed to oil concentrations that represent discharges of produced water. Produced water is water brought to the surface with the crude oil or the natural gas. Either it was a natural part of the oil and gas in the reservoir, or water injected to the reservoir to increase the extraction of oil and gas. When the oil or gas is processed, this water is separated out, and the produced water must

be treated according to government regulations to remove oil and grease before the water is released to the environment [12].

The produced water contains naturally-occurring compounds such as inorganic salt, metals and metalloids, and a large variety of chemicals used in the water treatment offshore such as emulsifiers, surfactants, oil removing agents and scale inhibitors [12]. Typical organic compounds in the produced water include aliphatic hydrocarbons, carboxylic acids, phenols and low molecular weight aromatics, and polycyclic aromatic hydrocarbons (PAH). There is a maximum limit of 30 ppm (mg/L) oil in water in Norway [13].

The existing toxicity of PAH on different organisms can be drastically enhanced when the oil is exposed to sunlight, either by photosensitivity and production of singlet oxygen, or modification to a more toxic compound [14]. The photoinduced toxicity has been demonstrated in PAH like anthracene, fluoranthene and pyrene [15]. The toxicity of PAH increases from 2 to over 1000 times in the presence of UV light [16].

The long-term effects of discharges to sea from petroleum-related activities have been investigated in research programs PROOF and continued PROOFNY for over ten years [13]. The programs have found negative effects on different areas like bile metabolites in cod, sex hormone-mimicking effect on rainbow trout and egg development in mussels. Bechmann et al. [17] investigated Northern shrimp (*Pandalus borealis*) exposed to oil in water (0.015, 0.06 and 0.25 mg/L) and found that the amount (concentration and time) of the exposure correlated to PAH accumulation in tissue. The amount of the exposure also correlated to the biomarker responses; the lysosomal membrane stability (a general health indicator) and the alkaline unwinding (indication of DNA damage). When PAH is taken up by an organism, it can stimulate the production of reactive oxygen species (ROS) and lead to oxidative stress. This has been demonstrated in the Artic scallop (*Chlamys islandica*) with increasing lipid peroxidation over time when exposed to oil [18].

1.3 Krill Exposure Study

The SeaSens krill project is a part of the PROOFNY program, with a particular interest in studying Arctic and temperate species. To their knowledge at the time of application, no other studies had investigated the seasonal variation in response to oil for any species in the North Atlantic or Barents Sea. The krill are an important link in the marine food web [10] and are key organisms in temperate and Arctic ecosystems. The link between the phytoplankton, the krill and their predators such as the blue whale is potentially one of the shortest food chains involving a large marine mammal [19]. Krill are highly nutritious with their vitamin, mineral, essential amino acid and ω-3 polyunsaturated acid levels [20]. The effect of the oil exposure in different seasons could potentially vary as the physiological composition of the krill varies with the seasonal variation in type of food and the availability of the food.

The exposure study was conducted on the krill species *M. norvegica*, as a representative for all krill species in the Barent Sea. The oil concentrations the krill were exposed to reflect produced water discharges in low (0.015 ppm) and high (0.15 ppm) oil concentrations. In spring and summer seasons the oil might be photomodified due to UV radiation, potentially increasing the toxicity of the oil. The SeaSens project (Figure 1-4) will analyze several parameters in the krill that might be affected by the oil exposure. They are looking at parameters such as histology of gills and digestive glands, fatty acid composition, feeding rate, gene expression, oxidative stress and polycyclic aromatic hydrocarbons (PAH) in krill tissue. These parameters will be used in the investigation if krill has a seasonal variation in how it is affected, and

if the ultraviolet (UV) irradiation of oil in the water will increase the toxicity of oil. The relevant biomarkers will then be established as methods for monitoring the effects produced water discharges has in the field.

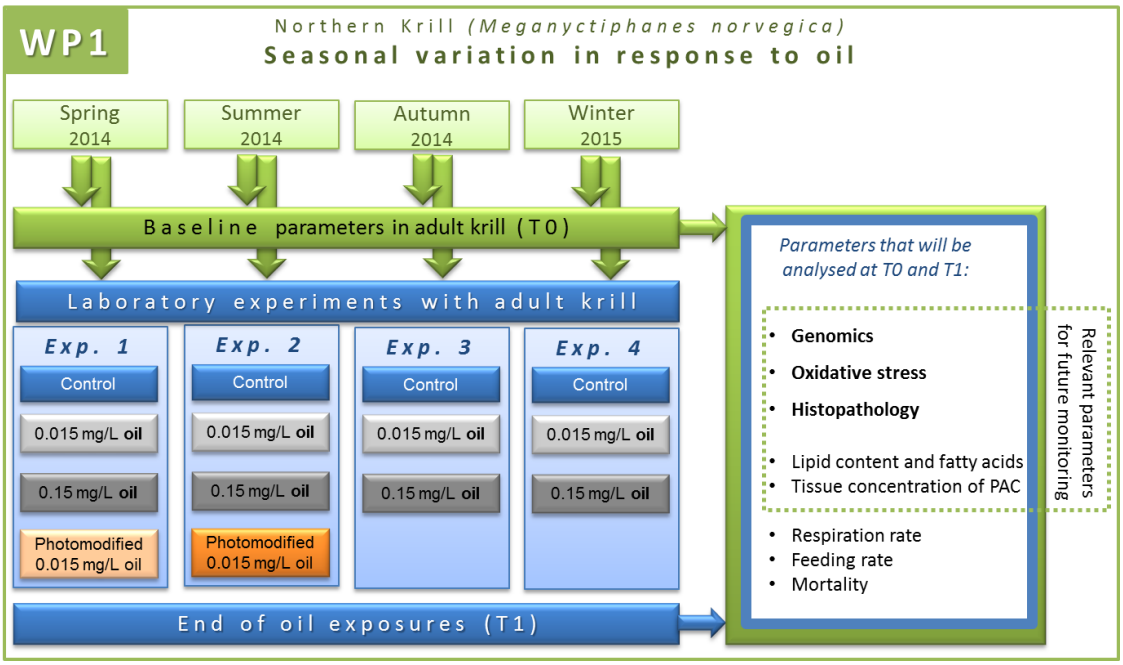

Figure 1-4 ***The SeaSens krill experiment.*** *The krill captured in each season are exposed to different oil treatments. After the exposure experiment the krill will be analyzed for several parameters, including oxidative stress. (Figure from the SeaSens project description, reused with permission.)*

1.4 Purpose of Thesis

Oxidative stress is an important component of the stress response in marine organisms exposed to pollution such as oil discharges from the petroleum industry [21]. By measuring the biomarkers of oxidative stress it can be possible to monitor the effects produced water has on krill in the field. The goal of this thesis is to establish a method for detecting oxidative stress in krill homogenate, by considering the biomarkers advanced oxidative protein products (AOPP) and malondialdehyde (MDA). MDA is a relatively stable end product of lipid peroxidation, and AOPP is a measurement of protein oxidation. The thesis used existing methods for measuring MDA and AOPP, and optimized them for use on krill homogenate. AOPP has never been measured in krill before. These methods should be easily implemented at the IRIS facilities by using the existing equipment. The aim is to find a method that can be used to monitor the effects of oil exposure on krill in the field.

The thesis involved multiple steps to achieve the end results of protein normalized AOPP and MDA values (Figure 1-5). The homogenate was prepared from the krill thorax, as the whole krill was used for both the genomics and oxidative stress analyses to be able to increase the number of samples. The aim was to prepare and store the homogenate under such conditions that total protein, AOPP and MDA could be analyzed with minor influence of pre-analytical factors. The homogenization process disrupt the cell wall, and is usually done at low temperatures (1 – 4 °C) to minimize the activity of krill endogenous proteases [22]. The homogenization could either be performed by a common, basic procedure using a grinder, or more extensive processes with liquid nitrogen. The centrifugation of the ground tissue separates the

different components of the tissue cells, leaving the insoluble parts such as shell, appendages and insoluble proteins in the pellet [22]. The existing methods for homogenization [23], total protein [24, 25], AOPP [26] and MDA (unpublished method from my supervisor) were modified and validated. It was important to find the optimal pre-dilutions of the homogenates to get absorbance within the calibration standards for the AOPP and the total protein methods. For the MDA method, the calibration standard concentrations were increased from the original method to reflect the MDA in the krill homogenates.

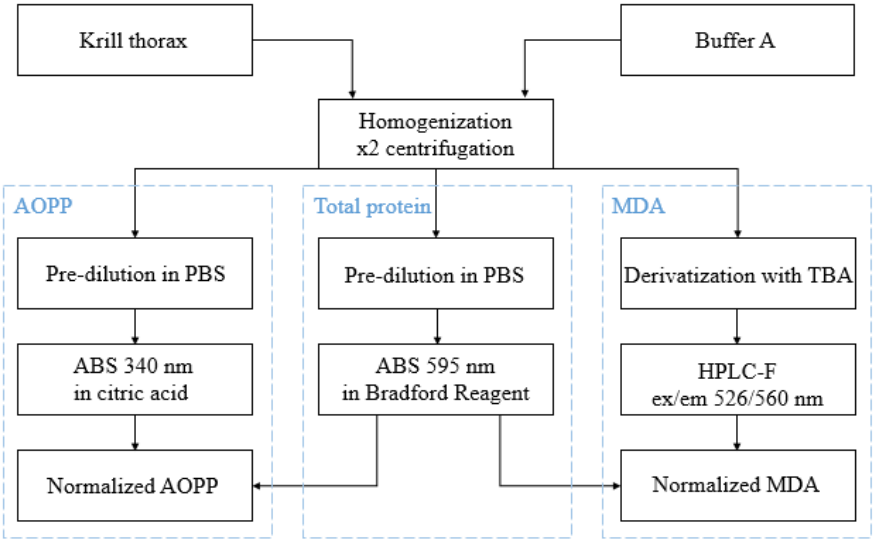

Figure 1-5 ***Overview of the processing of the krill****. Details of the methods in chapter 3.*

2 Theory

This chapter gives background information about oxidative stress and biomarkers for oxidative stress, and presents some measurement principles that will be utilized in this thesis.

2.1 Oxidative Stress

Oxygen is vital in biochemical processes of aerobic organisms, and reactive oxygen species (ROS) have a natural part in the cellular metabolism and functions as a defense against pathogens. The main source of ROS is leakage from the mitochondrial electron transport chain [27, 28]. ROS is oxygen in a more reactive state than molecular oxygen (Table 2-1). AOPP formation has been linked to chlorinated oxidants such as hypochlorous acid or chloramines [29] and not to $NO_2^{\cdot}$ [30]. Transition metals like iron (Fe^{2+}/Fe^{3+}) and copper (Cu^{+}/Cu^{2+}) are remarkably good promoters of free radical reactions, and they can convert $O_2^{\cdot-}$ and H_2O_2 into the highly reactive $^{\cdot}OH$ [31].

Through evolution the cells have developed several antioxidant defense mechanisms to protect it from ROS [32]. Antioxidants can significantly delay or prevent oxidation of a molecule [33] and can be divided into enzymatic and nonenzymatic antioxidants. The enzymatic antioxidants includes the superoxide dismutase which is a catalyst of $O_2^{\cdot-}$, the catalase which is a catalyst of H_2O_2 and the glutathione peroxidase working together with glutathione which is highly abundant in animal tissues and catalyze the reduction of H_2O_2 [21]. The nonenzymatic includes glutathione, ascorbic acid (vitamin C) which is a reductant source for H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$ and lipid hydroperoxides, and carotenoids, where the latter can either function as a light-harvesting pigment or quench ROS produced from an overexcitation by light [21]. Astaxanthin, a carotenoid found in krill, is a powerful antioxidant [34].

Table 2-1 ***List of some reactive oxygen species (ROS)****. Modified from [35].*

Free radicals	*Nonradicals*
Superoxide, $O_2^{\cdot-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\cdot}$	Hypobromous acid, $HOBr$
Hydroperoxyl, $OH_2^{\cdot}$	Hypochlorous acid, $HOCl$
Peroxyl, $RO_2^{\cdot}$	Ozone, O_3
Alkoxyl, $RO^{\cdot}$	Single oxygen, ($O_2^1\Delta g$)
Carbonate, $CO_3^{\cdot-}$	Organic peroxides, $ROOH$
Carbon dioxide, $CO_2^{\cdot-}$	Peroxynitrite, $ONOO^-$
Nitric oxide, $NO^{\cdot}$	Peroxynitrous acid, $ONOOH$
Nitrogen dioxide, $NO_2^{\cdot}$	Chloramines

Oxidative stress (Figure 2-1) is a situation that occurs when there is a serious imbalance between the antioxidants and the reactive oxygen species (ROS) that can damage the cell [35]. The imbalance means there are more of ROS than there normally should be. This could be due to an increase of ROS, or a decrease of antioxidants. The cell will be less capable of defending itself, and there could be extensive damage to nucleic acids, lipids and proteins [21]. Proteins are the major target for ROS (50 - 75% of all ROS), and all from primary to quaternary structure may be changed by mechanisms such as peptide backbone cleavage, cross-linking and/or modification of the side-chain of virtually every amino acid [36]. The pathogenesis or the progression of most human diseases such as cancer, cardiovascular and

neurodegenerating diseases, and even the aging process, have been linked with increased oxidative damage [37].

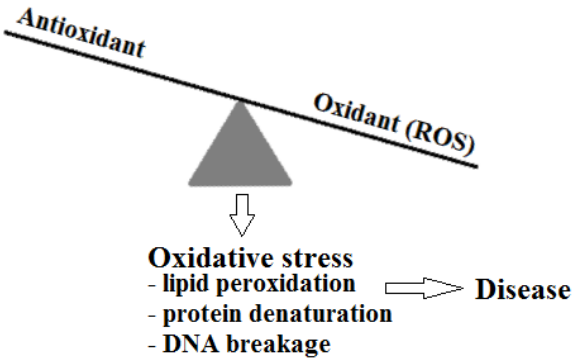

Figure 2-1 ***Imbalance between oxidants and antioxidants leads to oxidative stress.***

2.2 Biomarkers for Oxidative Stress

A commonly used definition of a biomarker is "a characteristics that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention" [38]. It could be biochemical, physiological, histological, morphological or behavioral measurements, and the biomarker could either be to indicate exposure of a chemical (without information on degree or effect), or to indicate effect of expose (the toxic effect on the organism) [39].

It is difficult to use the ROS as biomarkers for oxidative stress, as they are not stable long enough to be measurable. The fingerprint left by the ROS could be measured however. ROS leaves stable metabolites and oxidation target products like lipid peroxide end products and oxidized proteins [36]. Malondialdehyde (MDA) is the most commonly used biomarker for lipid peroxidation [32], and the most commonly utilized way of measuring protein oxidation is by detection of carbonyl groups [40] (Table 2-2).

Table 2-2 ***The most common biomarkers for the different types of oxidation.*** *Derived from [32].*

Type of oxidation	*Biomarker*	*Method*
Lipid	Malondialdehyde (MDA)	Derivatization with thiobarbituric acid (TBA), and detected by fluorometry, spectrophotometry, HPLC-VIS or HPLC-F*
DNA	Guanine hydroxylation (8-OHdG)	Detected by an electrochemical detector (ECD) connected with HPLC, or immunoassay with spectrophotometry
Protein	Protein carbonyl	Derivatization with 2,4-dinitrophenylhydrazine (DNPH), and detected by spectrophotometric assay, enzyme-linked immunosorbent assay (ELISA) electrophoresis followed by Western blot immunoassay

* high performance liquid chromatography (HPLC), visual light (VIS), fluorescence (F).

2.2.1 Malondialdehyde (MDA)

Malondialdehyde (MDA) is a small (72.07 g/mol), volatile, three carbon dialdehyde with pKa of 4.46 for the enolic OH group [41]. In a neutral or alkaline environment it will mainly be in its enol form (Figure 2-2) [42]. With pH at physiological conditions the MDA molecule is moderately reactive, and is considered highly toxic due to its potentially mutagenic and atherogenic interaction with DNA and proteins [42]. MDA is formed by lipid peroxidation (Figure 2-3), and is the most studied product of polyunsaturated fatty acid peroxidation [43].

Figure 2-2 ***The MDA molecule in its keto and enol form****. IUPAC name: propanedial.*

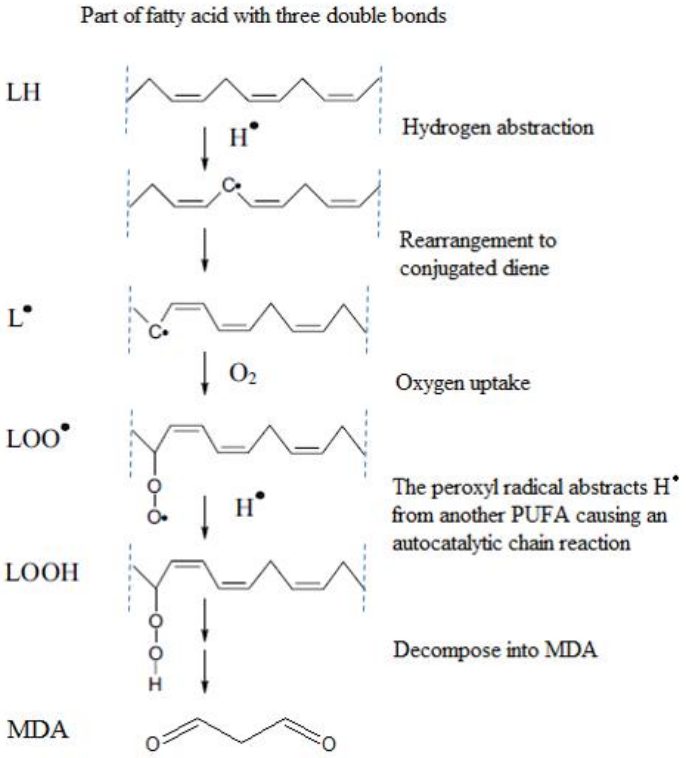

Figure 2-3 ***Lipid peroxidation.*** *LH is the polyunsaturated lipid, L˙ is the carbon-centered lipid radical, LOO˙ is the lipid peroxyl radical and LOOH is the lipid hydroperoxide. Termination of the lipid peroxidation happens when lipid radicals interacts and/or forms nonradical species [32]. The LOOH can easily decompose into lipid alkoxyl radicals (LO˙), aldehydes, alkanes, lipid epoxides and alcohols [32]. Modified from [44].*

The MDA molecule can exist in free form or bound to matrix molecules [42]. It is usually measured after derivatisation with thiobarbituric acid (TBA) which reacts with the free MDA under acidic conditions to create a $MDA(TBA)_2$ derivate (Figure 2-4), first described by Yagi (1976) [45]. The acid will hydrolyze some of the bound molecules to allow them to react with the TBA. A similar reaction is achieved with base hydrolysis. The $MDA(TBA)_2$ derivate has a red chromophore that can be detected spectrophotometrically at 532 nm [46], or fluorometrically with excitation/emission (ex/em) at 525/560 nm [47] or 515/553 nm [48]. The MDA molecule itself has no eletrophore, chromophore or fluorophore [43]. 1,1,3,3-Tetraethoxypropane or 1,1,3,3-tetramethoxypropane that quantitatively converts to MDA

when heated in acidic solution can be used as calibration standards [45, 48], and derivatized in the same manner as the samples. There are several different compounds that can react with TBA (TBA-reacting substances, TBARS), not just MDA. The other TBARS could be other aldehydes, sugars, amino acids, bilirubin and albumin [43], which will lower the specificity of the method, and is one of the reasons why the method has been criticized over the years.

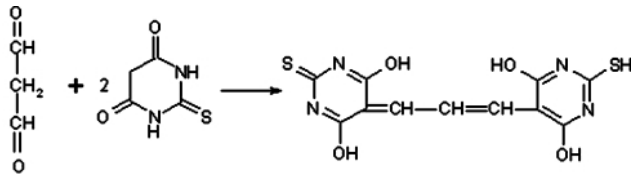

Figure 2-4 ***MDA reaction with TBA****[43]. Two TBA molecules reacts with the MDA molecule to form the MDA(TBA)$_2$ chromogen under acidic conditions.*

To improve the method specificity a chromatography step has been introduced. High pressure liquid chromatography (HPLC) or gas chromatography (GC) will separate some of the other chromogens from the MDA(TBA)$_2$ complex [41, 49]. The fluorescence detector (section 2.4) also improves the specificity compared to spectrophotometry by only detecting the chromophores with fluorescent abilities. The highest selectivity/specificity is achieved with mass spectrometry methods like GC-MS/MS or LC-MS/MS [37].

MDA in human plasma samples was reported stable for at least 6 months when stored at -80 °C [50]. The amount of MDA in a sample may vary due to how the sample is treated and stored. The derivatization step includes a hydrolysis step releasing the bound MDA. Acidic hydrolysis has been the most commonly used, but alkaline hydrolysis gives higher reported MDA [51]. This is either a more efficient way to free the bound MDA or it could be MDA formed from hydroperoxides in addition to the protein-bound MDA [52]. The lipid peroxidation could continue in vitro, triggered by the low pH and high temperature, which will give a higher MDA level; or the MDA molecule could be oxidized to a carboxylic acid or reduced to an alcohol, lowering the detectable MDA. Treating the samples with an antioxidant like butylated hydroxytoluene (BHT) could lower the artefactual lipid peroxidation [53]. However, the effect of BHT is under debate as some have not found any difference when not including the BHT in the derivatization process, and others found that the effect did not come from BHT but from the ethanol (or methanol) it was dissolved in [43].

The reported MDA level in human plasma varies depending on the method used. Del Rio et al. [42] presents a selection on MDA findings, where the level varies from 0.11 ± 0.03 μmol/L (TBA with HPLC-UV/Vis or with fluorimetry), 0.69 ± 0.13 μmol/L (TBA with HPLC-F) up to 13.8 ± 1.32 μmol/L (2,4-dinitrophenylhydrazine (DNPH) derivatization with HPLC-UV/Vis). TBARS levels in three different krill species have been reported as about 0.25 μmol/g protein in cold season (January), and 0.002 – 15 μmol/g protein in warm season (July and October) [54].

2.2.2 Advanced Oxidation Protein Products (AOPP)

The term ‘advanced oxidation protein products’ or AOPP was introduced by Witko-Sarsat et al. [55] in 1996 as novel oxidative stress marker of oxidized proteins. The plasma of uremic patients and healthy control was measured spectrophotometrically (section 2.3), and a peak at 340 nm was found for the uremic patients. This indication of a new chromogen correlated with plasma levels of the protein oxidation biomarkers dityrosine and pentosidine, but not with TBARS as the biomarker for lipid peroxidation. There is not necessarily correlation between biomarkers of protein oxidation and biomarkers of lipid

peroxidation, as there are different repair mechanisms and different degradation in vivo [35]. AOPP was further investigated in 2004 by Capeillère-Blandin et al. [30], and two overlapping chromophoric bands with maxima at 310 nm and 340 nm were found, corresponding to dityrosine, carbonyl proteins and pentosidine.

Dityrosine is a specific marker for free radical modification and oxidation of proteins, since it can only be formed by a reaction of two tyrosyl radicals [56]. The dityrosine is among other mechanisms synthesized by the myeloperoxidase-H_2O_2 system [57]. Dityrosine is metabolically stable, and increased concentrations have been found in various conditions as atherosclerosis, cystic fibrosis, end-stage renal disease and acute inflammation with or without sepsis [36]. Carbonyl group generation on proteins can be a result of:

1) The oxidation of amino acids, especially proline, arginine, lysine and threonine [40].
2) Advanced lipoxidation end-products (ALE) which are Michael adducts formed between lysine, histidine or cysteine and α,β-unsaturated aldehydes [36].
3) Advanced glycation end products (AGE) which are glycation/glycoxidation of lysine [36].

Pentosidine is a fluorescent AGE structure, which involves lysine and arginine residues combined with an imidazo-(4,5b)-pyridinium ring [58]. Pentosidine is considered a specific biomarker of protein glycation [29].

The AOPP levels in plasma were found stable for at least 6 months when stored at -80 °C [59], but protein carbonyls have been demonstrated as stable for 10 years when stored at -80 °C [36]. The original AOPP method was based on a dilution of plasma in phosphate buffered saline (PBS) and acetic acid, and calibration standards based on potassium iodide (KI) oxidized by chloramine-T (Figure 2-5) [55, 60]. The AOPP concentrations are expressed as μM chloramine-T equivalents, but commonly abbreviated to μM. Chloramine-T is a stable and strong oxidant and electrolyte in both acidic and alkaline media with E_{red}=1.138 at pH 0.65 and E_{red}=0.5 at pH 12, and with pKa estimated to 9.5 [61]. Chloramine-T can oxidize the iodide to iodine [61], which together with iodide forms the water-soluble triiodide (I_3^-) (eq 1 and eq 2). The triiodide can be measured spectrophotometrically, where it has absorption peaks at 290 nm and 351 nm [62].

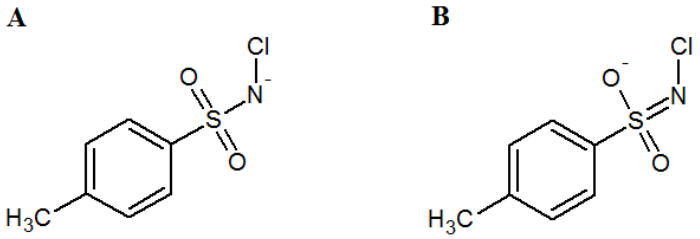

Figure 2-5 ***Two structures of chloramine-T****. Structure 'A' to the left is the most commonly depicted. Figure redrawn from Campbell and Johnson. [61].*

$$2\,I^-(aq) \xrightarrow{Chloramine-T} I_2(s) \qquad (1)$$

$$I_2(s) + I^-(aq) \rightleftharpoons I_3^-(aq) \qquad (2)$$

Hanasand et al. [26] modified the AOPP method by improving the solubility of lipoproteins. This was achieved by using citric acid (0.2 M) to dilute the plasma samples. Soluble lipoproteins prevents overestimation due to light scattering, and removing the lipoproteins with a protein precipitation step

would have caused an underestimation of the AOPP levels. The AOPP detected with this method was 82.6 ± 1.1 μM in medium AOPP plasma control.

There has been some confusion on how to use the method of Witko-Sarsat et al. [55]. In many cases the KI was added to the samples as well as to the chloramine-T [63-66], increasing the measured AOPP. This means that the reported AOPP results by others must be considered with this in mind, even for those who do not describe in detail how they prepared their samples (and thus reveal that KI was added to the sample). Altan et al. [67], Inkielewicz-Stępniak and Knap [68] and Benedetti et al. [69] all investigated AOPP levels in rat liver tissue homogenates, but reported 1.72 ± 0.32 nmol/g protein, about 110 μmol/g protein and about 15 μmol/g protein respectively in their healthy control samples, differing by a factor up to 100.

2.3 Spectrophotometry

Spectrophotometry is about measuring how light interacts with matter, it can be transmitted right through a solution, it can be reflected, scattered, absorbed and re-emitted [70]. Molecules that are capable of absorbing light are called chromophores, they excite to a higher energy state for a brief moment before returning to the ground state [71]. The energy is absorbed in double and triple bonds of unsaturated organic compounds [72]. The absorbance in a solution is defined as the logarithmic difference in radiant power of the incident beam and the beam that has passed through. This attenuation is dependent of how many chromophores it passed, as defined by the concentration of the solution and the path length, and is known as Beer-Lamberts law [72]:

$$A = \log (P_0/P) = \varepsilon bc \qquad (3)$$

where P_0 and P are the radiant power of incident beam and beam transmitted through respectively, ε is a proportionality constant for a given wavelength, b is the path length of the medium and c is the concentration of the absorbing species. The law holds as long as there is no interaction between solution molecules, and the concentration is not too high [73].

A microplate spectrophotometer (Figure 2-6) measures the intensity of light as a function of wavelength in a 96 well plate. This well plate includes calibration standard solutions with known concentrations of substance that absorbs at the given wavelength in addition to samples with unknown concentrations. The samples with unknown concentrations must have absorption within the range of the calibration standards. If not, the samples must be diluted, or the calibration standard concentrations adjusted. With the same path length (volume of the samples) and intensity of the incident light in all wells, the unknown concentrations of analyte in the samples can be determined using the line of regression. The spectrophotometry method has low specificity though, as multiple substances in the sample may absorb at the given wavelength [74]. In addition, particles or precipitation in the sample will increase the measured absorbance by scattering or reflection of the incident beam.

Figure 2-6 ***The Multiskan Ascent plate reader (Thermo LabSystems) at IRIS.*** *It is capable of measuring both in the ultraviolet (UV) area (340 nm) and in the visible (VIS) light range (595 nm).*

2.4 High Performance Liquid Chromatography – Fluorescence

Chromatography is a collective term for separation techniques based on distribution between two phases. The substances in the sample are transported by a mobile phase (gas or liquid) through the stationary phase where the substances adsorbs back and forward between the two phases. Separation occurs as the substances have different chemical qualities that affect the affinity to the different phases and thus the time spent in the stationary phase.

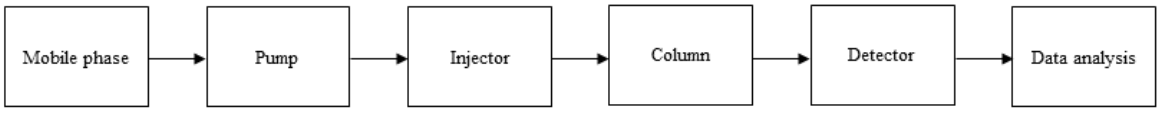

Figure 2-7 ***HPLC system - schematic setup.*** *The minimum of elements included in a HPLC system. Derived from [75].*

In a High Performance Liquid Chromatography (HPLC) system the solvent (liquid mobile phase) is pumped from a reservoir at a given flowrate, and the autosampler injects a small portion of the sample to the flow that is sent through the column with packed material (stationary phase) where the separation occurs under pressure (Figure 2-7). The separation could be done in several chromatographic modes based on the different properties of the molecule; by polarity, charge, size or ligand specificity. Separation by polarity with the reversed-phase chromatography (Figure 2-8) is the most commonly used [76], and is the method that was used for separation of the MDA derivate in this thesis. With reversed-phase chromatography, the polar mobile phase will be water mixed with a polar organic solvent like acetonitrile or methanol, and the non-polar column can have hydrophobic alkyl chains bound to very small porous particles [72]. The non-polar molecules in the mobile phase will tend to adsorb to the surface on the inside and outside of a particle if that surface is also non-polar. This means that the polar molecules will spend the least time in the stationary phase and will be eluted first. Elution of the non-polar molecules is accomplished by increasing the polar content of the mobile phase.

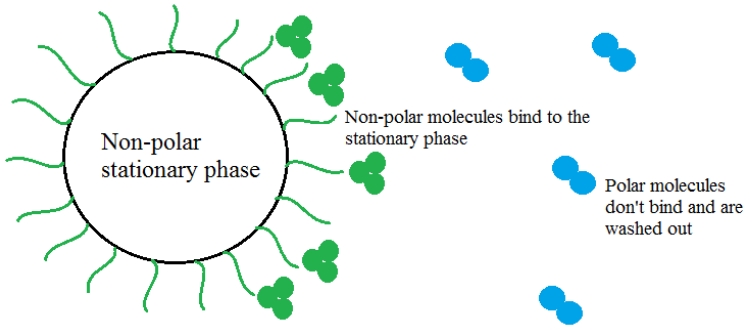

Figure 2-8 ***Reversed-phase chromatography principle****. The non-polar molecules (green) will bind to the non-polar hydrocarbon tails of the stationary phase (typically C8 or C18 tails on silica bead), while the polar molecules (blue) will not bind.*

After separation, the components of the sample elute from the column and are sensed by a detector. The detected signal forms a chromatogram with signal strength versus time. The components should ideally have been completely separated by this step, for each to have its own peak in the chromatogram. The signal should be higher than the baseline noise for a positive identification (Figure 2-9), with a signal-to-noise (S/N) above 3. The time to elute is called the retention time (t_{RT}), from once the sample was injected to the center of the peak, and should be at some distance from retention time t_0 of the non-retained compounds. The substances are normally identified based on the order they emerge from the column and by their retention time. The retention time can be adjusted by changing parameters such as type of mobile phase and flow, column, temperature and injection sample size. The reversed-phase column used in this thesis was 75 mm long with internal diameter of 2 mm, and had C18-coated particles of size 3 µm. The HPLC method had a gradient elution with phosphate buffer and methanol, and by varying the combination of the aquatic solvent (A) and organic solvent (B) over time the different substances were eluted.

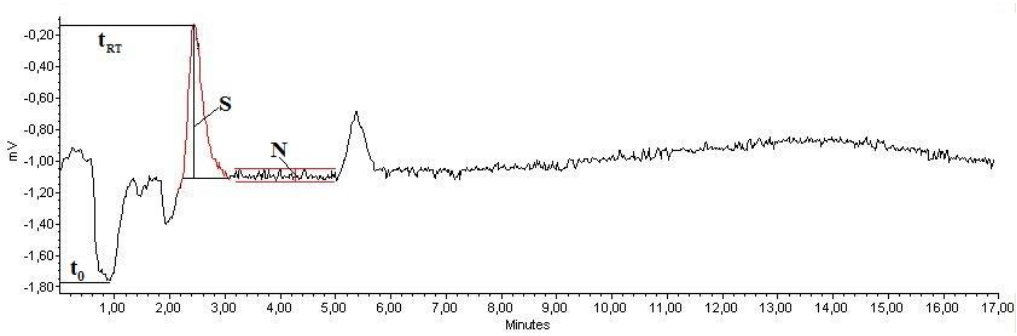

Figure 2-9 ***The signal (S), noise (N) and retention time (t_{RT}) of the MDA(TBA)$_2$ derivate.*** *The chromatogram (for the lowest concentration calibration standard) also show the retention time t_0 for the nonretained compounds.*

Fluorescence

In this thesis a fluorescence detector was used in combination with the HPLC (HPLC-F) to detect the chromophore of the MDA(TBA)$_2$ derivate.

A fluorescence detector is using the fluorescence abilities in the analyte, where energy in photons with a given wavelength can be absorbed, and then reemitted at a longer wavelength with some loss of energy (heat loss) (Figure 2-10). The exact wavelengths the fluorophore is excited at and emitted at is molecule specific. Unlike the UV/VIS detectors that measures in the direction of the incident light, a fluorescence sensor is usually at a 90° angle, to minimize the risk of detecting transmitted or reflected incident light.

The SpectraSYSTEM FL3000 fluorescence detector used in this thesis has monochromators for the excitation and emission wavelengths. The unique set of excitation and emission means that a fluorescence detector is more specific than a UV/VIS detector.

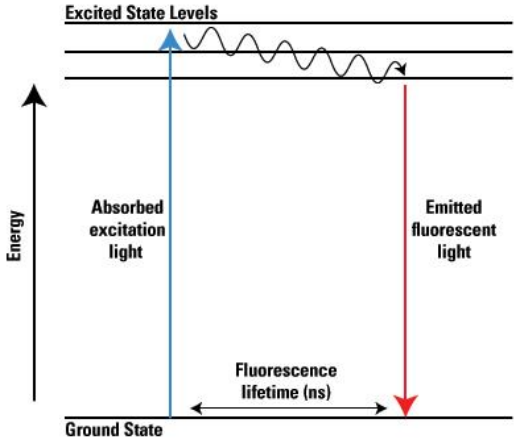

Figure 2-10 ***Jablonski energy diagram fluorescence*** *[77]. The incident light is absorbed, and then energy is emitted in all directions at a different wavelength.*

3 Materials and Methods

3.1 Chemicals and Reagents

Bradford Reagent, potassium dihydrogen phosphate (KH_2PO_4, anhydrous, ≥99.7%), chloramine-T ($CH_3C_6H_4SO_2NClNa \cdot 3H_2O$, ≥99%), potassium iodide (KI, ≥99.5%), 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT, ≥99.0%), 2-thiobarbituric acid (TBA, ≥98%) and 1,1,3,3-tetraethoxypropane (TEP, ≥96%) were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium hydrogen phosphate (K_2HPO_4, p.a.), sodium chloride (NaCl, ≥99.5%), citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$, ≥99.5%), glacial acetic acid (CH_3COOH, anhydrous, ≥99.8%), sulfuric acid (H_2SO_4, 95-97%, p.a.), 1-butanol ($CH_3(CH_2)_3OH$, p.a.), potassium hydroxide (KOH, ≥85.0%), methanol (CH_3OH, gradient grade for liquid chromatography, ≥99.9%) were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline (BupH™, 0.1 mol/L sodium phosphate, 0.15 mol/L sodium chloride, pH 7.2) was purchased from Thermo Scientific (Rockford, USA). Bovine serum albumin (BSA, Albumin fraction V fatty acid free) was purchased from Roche (Mannheim, Germany). Spinal Fluid Control (Liquichek, level 2) was purchased from Bio-Rad Laboratories (Irvine, USA). Absolute ethanol (prima) was purchased from Kemetyl (Vestby, Norway), and phosphotungstic acid ($H_3O_{40}PW_{12}.xH_2O$) was purchased from VWR (Leuven, Belgium).

3.2 Equipment

Table 3-1 ***List of equipment with details on model and manufacturer.***

Equipment	*Model*	*Manufacturer*
Overhead stirrer for homogenization. Used with VWR homogenization grinder (10 mL) and plain plunger/piston	Eurostar, 50-2000 rpm	IKA. Janke & Kunkel GMBH & Co
Centrifuge	5415 R	Eppendorf
Vacuum centrifuge	Concentrator 5301	Eppendorf
Microplate reader. Used with - PS Microplate 96 well flat bottom (Greiner Bio-One) for Total Protein - Costar UV Plate, 96 well, with UV transparent flat bottom (Corning Incorporated, VWR Norge) for AOPP	Multiskan Ascent	Thermo LabSystems
HPLC-F system processed with Empower software:		
-LC column	Gemini NX 3u C18 110A	Phenomenex
-PDA detector	WatersTM 996 Photodiode Array Detector	Waters
-Fluorescence detector	SpectraSYSTEM FL3000	Thermo Separation Products
-Autosampler	WatersTM 717plus Autosampler	Waters
-Controller	WatersTM 600 Controller	Waters
-Degasser	DEGASYS DG-2410	Uniflows

3.3 Solutions

Buffer A

Buffer A (0.1 M phosphate) for homogenization was prepared according to IRIS Standard Operating Procedure (SOP) 'Preparation of S12, microsomes and S100 by differential centrifugation' [23]. 3.4 g K_2HPO_4 (0.1 M) was dissolved to 0.5 L with distilled water, and 8.75 g KH_2PO_4 (0.1 M) was dissolved to 0.25 L with distilled water. Some of the KH_2PO_4 solution was added to the K_2HPO_4 solution until a pH of 7.4 was achieved in the mixture. The mixture was added NaCl (12.5 g pr. 0.5 L of mixture). The buffer was kept at +4 °C (stable for 6 months [23]).

PBS Buffer

The phosphate buffered saline (PBS) buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) was made by dissolving one pack of BupH™ phosphate buffered saline to a total volume of 500 mL with distilled water. The buffer was kept at +4 °C.

Total Protein Calibration Standards

Bovine serum albumin (BSA) was weight (1 g) and dissolved in 10 ml distilled water as a stock solution. Since BSA is hygroscopic, the accurate concentration was determined spectrophotometrically by diluting the stock solution 1:100 in PBS buffer (50 µL + 4950 µL = 1 g/L). Absorbance for 1 g/L at 280 nm should be 0.667 [78]. This was measured to 0.635, thus the BSA stock was 95.2 g/L. Calibration standard solutions (Table 3-2) were made from stock BSA diluted in PBS buffer. The calibration standards were made with five different concentrations between 0.143 mg/mL and 1.24 mg/mL. The stock BSA was stored in 500 µL aliquots at -20 °C, while the calibration standard solutions were kept at +4 °C (no longer than 3 months).

Table 3-2 ***The calibration standards for Total Protein****. The BSA stock solution had a protein content of 95.2 mg/mL.*

Standard	*Concentration (mg/mL)*	*BSA stock (µL)*	*PBS buffer (µL)*
2	0,143	7,5	4992,5
3	0,286	15	4985
4	0,571	30	4970
5	0,857	45	4955
6	1,24	65	4935

Citric Acid

Three different citric acid (CA) concentrations (0.1 M, 0.2 M and 0.5 M) were prepared in distilled water, and kept at +4 °C until use.

KI

Potassium iodide solution (KI, 1.16 M in PBS buffer) was prepared fresh each day, and protected from light exposure until use.

Chloramine-T Calibrator Standards

Chloramine-T stock solution (1 mM) was prepared in distilled water, and kept at +4 °C until use (no longer than 3 months). Seven calibration standards (Table 3-3) were prepared fresh each day from stock chloramine-T diluted in citric acid (0.2 M), with concentrations between 5 µM and 100 µM. When the

calibration standards were added to the microplates they were further diluted by the addition of KI, thus a different concentration were used to create the standard curve (Table 3-3).

Table 3-3 ***The AOPP chloramine-T calibration standards****. The calibration standards were prepared from stock (1 mM) and citric acid (0.2 M). 190 µL of calibration standard was added to the microplate, and when the KI (1.16 M, 10 µL) was added the actual chloramine-T concentration on plate changed accordingly. The 'Chloramine-T on plate' concentration was used to create the standard curve.*

Standard	*Chloramine-T on plate (µM)*	*Chloramine-T stock (µL)*	*CA (0.2 M) (µL)*	*Chloramine-T (µM)*
Cal 1	4,75	5	995	5
Cal 2	9,5	10	990	10
Cal 3	19	20	980	20
Cal 4	38	40	960	40
Cal 5	57	60	940	60
Cal 6	76	80	920	80
Cal 7	95	100	900	100

Butylated hydroxytoluene

The butylated hydroxytoluene solution (BHT, 0.7 mM in 40% ethanol) was prepared from a stock solution of 2,6-bis(1,1-dimethylethyl)-4-methylphenol (7.13 mM in absolute ethanol). The solution was kept at room temperature.

Thiobarbituric acid

2-Thiobarbituric acid (TBA) (46 mM in 50/50 glacial acetic acid/distilled water) was dissolved with magnetic stirring and gentle heating. The solution was kept at room temperature protected from light exposure.

Phosphotungtic acid

Phosphotungstic acid (2 g) was dissolved in 20 mL distilled water (10% w/v). The solution was kept at +4 °C.

Tetraethoxypropane

All solutions were prepared daily. The 1,1,3,3-tetraethoxypropane (TEP) was used to prepare several stock solutions in 40% ethanol (Table 3-4). With a TEP weight of 10.21 mg for approximately 10 µL, stock 1 has a concentration of 29.7 mM. Eight calibration standards were prepared from stock 3 diluted in 40% ethanol (Table 3-5), with a concentrations from about 0.29 µM to 22.8 µM, in addition to blank (40% ethanol).

Table 3-4 ***Stock solutions for TEP****. Stock 1, 2 and 3 were prepared from TEP and the previously made stock solutions.*

Stock	*40% ethanol (µL)*	*TEP (µL)*	*Stock 1 (µL)*	*Stock 2 (µL)*
1	1490	10		
2	990		10	
3	900			100

Table 3-5 ***The MDA calibration standards****. Stock 3 has a TEP concentration of approximately 29 µM in 40% ethanol.*

ID	*Stock 3 (µL)*	*40% ethanol (µL)*	*Concentration (µM)*[a]
Cal 2[b]	10	990	0,29
Cal 3	20	980	0,57
Cal 4	40	960	1,14
Cal 5	80	920	2,28
Cal 6	160	840	4,56
Cal 7	320	680	9,13
Cal 8	500	500	14,3
Cal 9	800	200	22,8

[a] The concentration changes with the exact weight of the stock solution, example concentration with 9.80 mg TEP to stock.
[b] The standard was not used when analyzing the first four MDA analysis of krill exposed to oil (18 analyses in total).

Phosphate buffer for MDA
KH_2PO_4 (50 mM, distilled water) was adjusted to pH 7.0 with KOH (6 M, distilled water). The buffer was filtered through a membrane filter (pore size 0.45 µm) and kept at +4 °C.

Mobile phase 'A' for MDA HPLC
Mobile phase 'A' was 10% methanol and 90% phosphate buffer for MDA. The solution was kept at room temperature.

Mobile phase 'B' for MDA HPLC
Mobile phase 'B' was 90% methanol in distilled water. The solution was kept at room temperature.

3.4 Krill

The *M. norvegica* krill caught at night in the fjords outside of IRIS' locations in Mekjarvik was used in the experiment. The krill were part of the SeaSens experiment (Figure 1-4, Table 3-6), but the summer setup was excluded from the SeaSens experiment due to poor krill harvest. An additional test was included in the autumn where krill were exposed to a higher oil concentration (1 mg/L of produced oil) for two days. The T0 krill were frozen in liquid nitrogen right after capture and kept in a -80 °C freezer in cryovials. The T1 krill were acclimatized for about a week in the laboratory, prior to the eight days of exposure study. T1 krill were kept in separate containers, fed with EZ larvae and Artemia, and frozen in liquid nitrogen once the exposure was over.

Table 3-6 ***The different krill groups.*** *In total 75 krill were dedicated to each T1 treatment (Control, Low, High and Low UV) per season, where 15 krill of each treatment were allocated to oxidative stress analysis. The additional High High treatment consisted of 16 krill to oxidative stress analysis.*

Season	*Group*	*Use*
Spring	T0 method	Frozen after capture. Used to develop the methods.
(captured 11.03.14)	T0 control	Frozen after capture.
	T1 experiment	Frozen after eight days of exposure. Four treatments; Control (no oil exposure), Low (0.015 mg/L oil in seawater), High (0.15 mg/L oil in seawater) and Low UV (0.015 mg/L oil in seawater where the oil is treated with UV light).
Autumn	T0 method	Frozen after capture. Used to develop the methods.
(captured 23.09.14)	T0 control	Frozen after capture.
	T1 experiment	Frozen after eight days of exposure. Three treatments; Control (no oil exposure), Low (0.015 mg/L oil in seawater) and High (0.15 mg/L oil in seawater). Additional High High experiment of two days of exposure (1 mg/L oil in seawater).
Winter	T0 control	Frozen after capture.
(captured 14.01.15)	T1 experiment	Frozen after eight days of exposure. Three treatments; Control (no oil exposure), Low (0.015 mg/L oil in seawater) and High (0.15 mg/L oil in seawater).

3.5 Homogenization

Homogenization was performed according to IRIS Standard Operating Procedure (SOP) [23] with some small modifications, using buffer A. Once removed from the -80 °C freezer, the krill were kept on ice (maximum four krill at once to avoid thawing quickly). The head and abdomen were cut off to get the thorax containing the hepatopancreas (Figure 1-2, Figure 3-1). The krill used during method development and the krill from the exposure study were treated slightly different. During method development the krill were kept on ice, then the thorax was separated and further homogenized. The krill from the exposure study had their head and abdomen separated from the thorax in a separate job where the krill were kept frozen on dry ice the whole time, as the head and abdomen were included in the separate genomics test. The homogenization was done on thorax segments from the -80 C freezer (maximum four out at once, kept on ice).

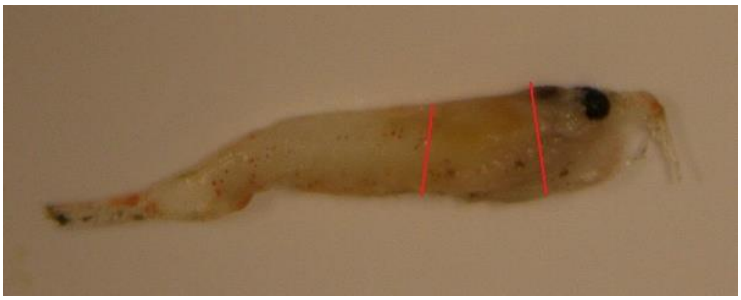

Figure 3-1 ***Cut sections on krill****. The thorax was separated from head and abdomen while still frozen.*

The thorax was weighed and put into the homogenization grinder. Ice-cold homogenization buffer was added 4:1 (4 mL to 1 g krill), with the minimum amount of 0.44 mL buffer. The krill was homogenized

with 10 slow strokes (900 rpm) where the grinder was surrounded by ice to avoid temperature increase. The homogenate was poured into pre-cooled LoBind eppendorf vials, kept on ice until all four krill were homogenized, and then centrifuged for 20 minutes at 12 000 g (4 °C). The supernatant, carefully avoiding the fatty layer, was transferred to a new vial and the centrifugation repeated. The second supernatant was aliquoted in three cryovials (Table 3-7) for storage at -80 °C. Note that every time the krill homogenate was analyzed for total protein, AOPP or MDA, the nature of the homogenate made it necessary to mix (using the pipette) before use to regain homogeneity.

Table 3-7 ***Distribution of krill homogenate.*** *There was a small volume of krill homogenate available. The minimum volume necessary to perform each analysis was aliquoted to TProt (total protein), AOPP and MDA. The drops were transferred with a glass Pasteur pipette.*

Krill thorax (g)	TProt (drops)	AOPP (drops)	MDA (drops)
0.02-0.07	2	5	remaining
0.08-0.17	2	4	remaining

3.5.1 Total Protein Content

The total protein content in the homogenate was measured based on the principle of Bradford [24] and the Sigma protocol "96 Well Plate Assay Protocol" [25], using BSA as calibration standards (Table 3-2). The krill homogenate was thawed on ice, and pre-diluted in PBS buffer (Table 3-8) to fall within the range of the standard curve (0,1428 -1,2376 mg/mL). The pre-diluted krill homogenate was vortexed and centrifuged (5000 g, 5 minutes). Blank (PBS buffer), calibration standards, control (Liquichek with expected protein content 0.873 mg/mL) and pre-diluted krill homogenate were all added in triplicates (5 µL), then Bradford Reagent (250 µL) was added to all wells using inverted pipetting with a multichannel pipette. The plate was covered with aluminium foil and shaken on the plate reader (300 rpm, 15 minutes) prior to the measurement at 595 nm.

Table 3-8 ***Total Protein krill homogenate pre-dilution****. The krill homogenate was diluted in PBS buffer based on the weight of the thorax, to ensure the absorbance would fall within the range of the standard curve.*

Krill thorax (g)	*Krill homogenate (µL)*	*PBS buffer (µL)*	*Dilution*
0.02 - 0.07	20	180	1:10
0.08-0.17	20	280	1:15

3.5.2 Pre-Dilution of Krill Homogenate for Total Protein

The optimal pre-dilution of the krill homogenate in PBS for total protein content measurement was tested with dilutions 1:10 (15 µL homogenate and 135 µL PBS), 1:15, 1:20, 1:25 and 1:30 on each of 10 krill homogenates in PBS.

3.5.3 Sedimentation of Krill Homogenate

The robustness of the krill homogenate with respect to total protein content was investigated by how the samples were prepared and stored.

The Stability of Undiluted Homogenate stored in the Refrigerator
The homogenization process was done with either one centrifugation or two centrifugations. With a single centrifugation the supernatant was frozen directly as the krill homogenate. With two centrifugations the first supernatant transferred to a clean vial and centrifuged a second time, and the second supernatant was frozen as the krill homogenate. The krill homogenates were taken from the -80 C freezer, thawed and total protein content measured with a 1:15 dilution in PBS. The undiluted homogenates were kept in refrigerator, and reanalyzed two days later diluted 1:15 in PBS.

The Stability of Pre-Diluted Homogenate stored in the Refrigerator
The krill homogenates were taken from the -80 °C freezer, thawed and the total protein content measured in a small portion after 1:20 dilution in PBS. The undiluted homogenates were kept in refrigerator, and another small portion reanalyzed at dilution 1:20 in PBS on four executive days.

Pre-Diluted Homogenate Stability in Refrigerator
The krill homogenates were taken from the -80 °C freezer, thawed and pre-diluted to 1:20 in PBS. The pre-diluted homogenates were kept in refrigerator, vortexed and centrifuged (5000 g, 5 minutes) prior to analysis. The total protein content were analyzed in small aliquots of the diluted homogenate at day 1 (the same day as thawed), day 2, day 3, day 8, day 9, day 10 and day 11.

3.6 Measurement of AOPP

The AOPP content was analyzed as described by Hanasand et al. [26], with some modifications for the krill samples. The samples were added in triplicates to the wells of the UV transparent 96-microtiter plate in two steps (Table 3-9). The first step was to add 190 µL of blank (0.2 M citric acid) and calibration standards (Table 3-3) to the first three columns, and then 40 µL of control and pre-diluted krill homogenate (Table 3-10) were added to the remaining columns of the microplate. The second step was to add KI (1.16 M, 10 µL) to the blank and the calibration standards, and then protect the plate from further light exposure by gradually covering the plate with aluminium foil while adding citric acid (0.2 M, 160 µL) to the pre-diluted homogenate and controls. After 2 minutes (300 rpm) on the shaker in the plate reader, the wells were briefly inspected for bubbles. The absorbance (340 nm) was measured 5, 10, 15 and 20 minutes after adding KI. The resulting AOPP concentration is reported in µM chloramine-T equivalents, abbreviated to µM.

Table 3-9 ***Outline of 96-microtiter plate for AOPP****. Columns 1 to 3 were used for 190 µL of calibration standards and blank in triplicates, and 10 µL KI was added in the second step. Columns 4 to 12 were used for 40 µL of control samples and krill homogenates in triplicates, and 160 µL of citric acid (0.2 M) was added in the second step.*

	1 - 3	4 – 6	7 - 9	10 -12
A	Blank x 3	Control x 3	Krill homogenate x 3	Krill homogenate x 3
B	Cal 1 x 3	Krill homogenate x 3	Krill homogenate x 3	Krill homogenate x 3
C	Cal 2 x 3	Krill homogenate x 3	Krill homogenate x 3	Krill homogenate x 3
D	Cal 3 x 3	Krill homogenate x 3	Krill homogenate x 3	Krill homogenate x 3
E	Cal 4 x 3	Krill homogenate x 3	Krill homogenate x 3	Krill homogenate x 3
F	Cal 5 x 3	Krill homogenate x 3	Krill homogenate x 3	Krill homogenate x 3
G	Cal 6 x 3	Krill homogenate x 3	Krill homogenate x 3	Krill homogenate x 3
H	Cal 7 x 3	Krill homogenate x 3	Krill homogenate x 3	Krill homogenate x 3

Table 3-10 ***AOPP krill homogenate pre-dilution****. The krill homogenate was diluted in PBS buffer based on the weight of the thorax, to ensure the absorbance would fall within the range of the standard curve.*

Krill thorax (g)	*Krill homogenate (µL)*	*PBS buffer (µL)*	*Dilution*[a]
0.02 - 0.07	24	16	1:8.3
0.08 -0.17	15	25	1:13.3

[a] Dilution refers to the final dilution of krill homogenate in the well when 160 µL of citric acid is added.

A large human plasma pool was frozen in small aliquots and used as control. During method development, the krill homogenate was pre-diluted in different solvents to find out if the solvent had any effect on the sample stability and absorbance reading; citric acid (0.2M and 0.5 M), buffer A and PBS buffer. The combination of 40 µL pre-diluted homogenate and 160 µL citric acid was fixed.

3.6.1 Stability of KI and Calibration Standards

Chloramine-T with KI as calibration standard was investigated by looking at how light sensitivity and storage of KI influenced the absorbance over time. The AOPP absorbance readings (340 nm) were performed at short time intervals. Freshly made KI (1.16 M, 10 µL) was compared to one and three weeks old KI (1.16 M, 10 µL) protected from light exposure on sets of calibration standards. The light sensitivity of KI was tested by comparing absorbance of calibrator solutions on plates covered with aluminium foil to the absorbance of the calibrator solution on plates not covered with aluminum foil.

3.6.2 Pre-Dilution of Krill Homogenate for AOPP

The optimal pre-dilution and solvent was investigated for plasma and for krill homogenate. The pre-dilution with PBS and buffer A was done prior to samples added to microplates. The citric acid 'pre-dilution' was done by first adding only plasma sample to the well (< 40 µL). The additional volume of citric acid for pre-dilution was added at the same time as the 160 µL of citric acid was added.

- The plasma control was pre-diluted in citric acid (0.2 M), PBS buffer and buffer A respectively, to achieve the final dilution on plate as 1:5, 1:10, 1:13.3 or 1:20, after 40 µL of the pre-diluted plasma had been added 160 µL citric acid (0.2 M). The AOPP content was measured.
- The krill homogenate was pre-diluted in citric acid (0.2 M) and PBS respectively, to achieve the final dilution on plate as to 1:10, 1:13.3 or 1:20 after 40 µL of the pre-diluted krill homogenate had been added 160 µL citric acid (0.2 M). The AOPP content was measured.

3.6.3 Citric Acid Concentration

The pre-diluted krill homogenates (40 µL) were dissolved in citric acid (160 µL) in the AOPP procedure. The optimal citric acid concentration was investigated by considering the stability of measured AOPP and by visual inspection of the clarity the diluted samples in cuvettes.

- 40 µL of pre-diluted krill homogenate in PBS (1:13.3) was added 160 µL of citric acid (0.1, 0.2, 0.5 or 1 M) to achieve a total dilution of 1:15. The AOPP content was measured.
- The pre-diluted krill homogenate in PBS (1:13.3) was diluted with citric acid concentrations (0.1 or 0.2 M). Solutions with different concentrations were visually compared in cuvettes.

3.7 Measurement of MDA

The krill homogenate was prepared for MDA analysis according to a procedure provided by my supervisor, which was a reversed-phased HPLC-F based on Yagi [48] and Lykkesfeldt [46]. The calibration standard concentrations were modified for use with krill homogenate (Table 3-5). The krill homogenate was carefully thawed on ice, and 30 µL of sample were transferred to a 1.5 mL eppendorf vials with screw-caps. H_2SO_4 (350 µL, 42 mM), BHT (50 µL, 0.7 mM) and TBA (100 µL, 46 mM) were added the vial. The mix was vortexed, incubated (95 °C, 1 hour), and then cooled on ice (6 minutes). 1-butanol (500 µL) and phosphotungstic acid (10 µL, 10%) was added, and the mix vortexed and centrifuged (16.000 g, 3 minutes). The butanol layer was carefully transferred (400 µL) to clean vials, and evaporated to dryness in vacuum centrifuge (1 hour and 20 minutes, 60 °C). The samples was dissolved in 200 µL of 20% methanol in phosphate buffer for MDA (50 mM), and centrifuged (16.000 g, 3 minutes). 150 µL was transferred to HPLC vial while carefully avoiding the pellet.

A set of 20 derivatized standards and samples were injected (10 µL) on the HPLC-column (Table A-10, appendix A.3), and run with a gradient program (Table 3-11). The detection was done with fluorescence at ex/em 526/560 nm, and with a photodiode array (PDA) detector in range 490 nm to 550 nm. Once the set were finished, the system changed to the shutdown method with flowrate at 0.1 mL/minutes and 20% A and 80% B. A large krill homogenate pool were frozen in aliquots and used as control of method reproducibility.

Table 3-11 ***The HPLC gradient****. Total time for a run was 17 minutes. There was no wash between the injections. Mobile phase A was 10% methanol in phosphate buffer for MDA (50 mM), and mobile phase B was 90% methanol. Flow rate was set to 0.25 mL/min.*

Time (min)	*A (%)*	*B (%)*
0	95	5
4	50	50
7	50	50
8	25	75
12	25	75
13	95	5
17	95	5

3.7.1 Derivate Stability

Each sample run was 17 minutes, and with two injections each for the 20 samples of derivatized standards and krill homogenates, the total runtime for the set was over 15 hours. The sample derivate stability over time was investigated in two different ways. First, a single test was conducted where HPLC injections of a set of six calibration standards and five krill homogenates (pool) were repeated three times, at time zero, after 30 hours and after 43 hours. Second, any potential changes in control sample and calibration standards 5 - 7 were monitored as part of the standard MDA method; they were injected both in the beginning and in the end of every set.

Color Intensity of Krill Homogenate

The color intensity of krill homogenates prior to derivatization was registered. The intensity/color of the krill homogenate was registered in five groups, with '1' as the weakest color, almost transparent, and up to

'5' as the darkest red color. The color intensity was used to assess if the color of the krill homogenate would have impact on the MDA results.

3.8 Validation of the Methods

Validation was done for the measurements of total protein, AOPP and MDA. Each method's performance was validated to provide its characteristics such as precision, recovery, linearity of the calibration curve, limit of detection and limit of quantification.

Precision
A method should produce consistent results every time. The within-day variation (repeatability) and the between-day variation (intermediate precision) were used to define this. The within-day and between-day variations are expressed as the coefficient of variation (CV, %) of the analyzed control samples (n=9 and 25 respectively for total protein, n=18 and 21 respectively for AOPP and n=5 and 20 respectively for MDA). The krill homogenates were split to multiple cryovials during the homogenization phase, to avoid thawing multiple times for between-day testing.

Recovery
Samples of krill homogenate was spiked [79] with known concentrations of analyte. The analyte was added in low and high concentration, and the spiked sample was compared to krill homogenate with added blank in the same volume to define the recovery (%) (eq 4). The recovery gives an indication of the accuracy of the method, of how close to the true value of the analyte the method can detect.

$$\text{Recovery (\%)} = \frac{(conc.\ spiked\ sample - conc.unfortified\ sample)}{conc.fortification} \cdot 100\% \quad (4)$$

Calibration Curve
The line of regression y = a + bx was defined for the calibration standards, and the coefficient of determination (R2) and the residual between theoretical and experimental concentration for each calibrator evaluated.

Limit of Detection (LOD) and Limit of Quantification (LOQ)
The limit of detection (LOD, 3.3 σ/S) and quantification (LOQ, 10 σ/S) was calculated directly from the regression line, where σ was the standard deviation of the intercept ('a') and S was the slope of the line ('b') [80]. For the MDA method a signal-to-noise (S/N) at 3 indicate LOD, and S/N at 10 indicate LOQ.

3.9 Statistical Methods

The total protein and the AOPP were measured in triplicates, while the MDA was a single measurement. The results are reported as mean ± standard deviation (SD) unless otherwise noted. MDA results are based on mean of the two injections. All data were processed on Excel®. Student's t-test was used to look for significant differences ($p < 0.05$) between groups as it was the available statistical tool with Excel. Paired t-tests were used to compare different treatments such dilution factors, solvents or change over time. Unpaired t-tests (using the 'heteroscedastic' attribute in Excel) were used to compare groups of krill with different treatments such as control versus high, or spring low versus autumn low.

4 Results

The krill was homogenized to make it suitable for biomarker analysis. The homogeneity and stability of the krill homogenate were investigated with total protein measurements, and the total protein content in the homogenate was later used to normalize the AOPP and MDA contents of the sample when the methods were utilized on krill from the exposure study.

4.1 Homogenization

4.1.1 Pre-Dilution of Krill Homogenate for Total Protein

Ten different krill homogenates were diluted to various concentrations to find a dilution or a dilution range where the measured total protein concentration in krill homogenate was stable (Figure 4-1). To be able to compare the individual krill homogenates, all total protein concentrations were converted to percentage numbers, where the 100% value for each krill homogenate was the mean value from its five dilutions. Measured concentration increased as the dilution factor increased. The 1:15, 1:20 and 1:25 dilutions had similar concentration levels with small SD. The measured total protein concentration in 1:10 dilution was 83 ± 7% versus the 1:15 concentration of 95 ± 3%. There was no significant difference between 1:20 and 1:25 dilutions, but all other dilutions were significant different from each other. Krill homogenates that was not pre-diluted or diluted 1:4 gave absorption measurements above the highest calibration standard, and pre-dilution 1:35 and 1:40 gave absorption measurements below the lowest concentration calibration standard. These pre-dilutions were not investigated further.

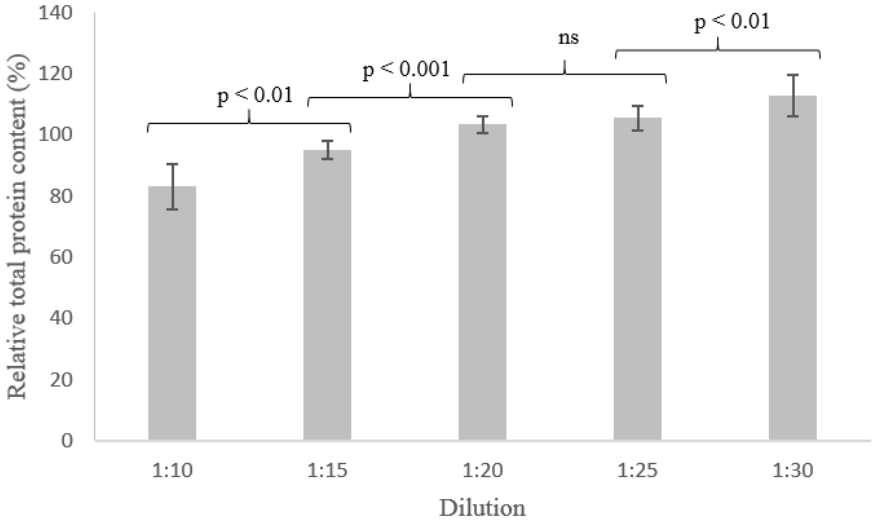

Figure 4-1 ***Dilution series total protein****. The percentage value of each dilution based on the average of all five dilutions of homogenate in PBS. (Error bars indicate ±SD. n=10). Significant differences are marked with p < 0.05. ns= not significant..*

4.1.2 Sedimentation of Krill Homogenate

Homogenate Centrifugation Effect on Storage

The krill homogenate is a thick, pink liquid of suspended particulate matter. One centrifugation result in a large pellet and a liquid supernatant with a thin fatty layer on top. Centrifuging the supernatant a second time produced a clearer supernatant with no or barely small traces of pellet or fatty layer. The supernatants

were analyzed for total protein the day of preparation and after two days storage at 4 °C. A single centrifugation resulted in a total protein concentration of 52 ± 12% on day three compared to the concentration on day one, while two centrifugations resulted in a concentration of 73 ± 10% (Figure 4-2, Table A-1, appendix A.1). Two centrifugations were implemented as part of the homogenization method.

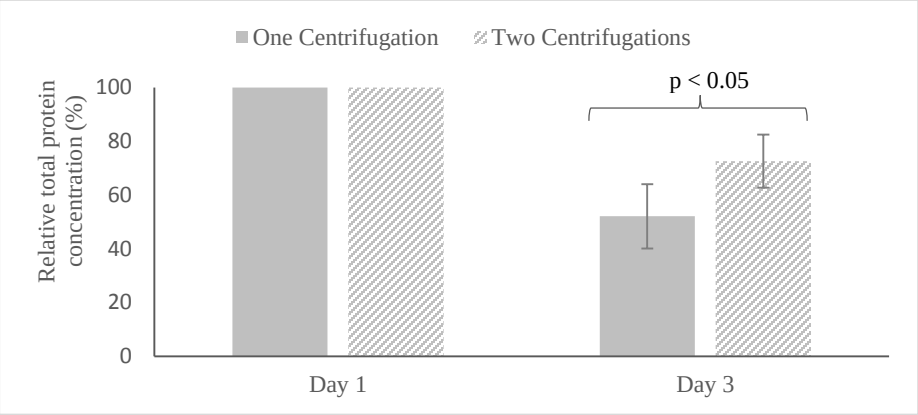

Figure 4-2 ***One or two centrifugations of the homogenate****. The total protein concentration each krill homogenate had on day one was defined as 100%, and the value on day three was relative to this value. (Error bars indicate ±SD, n=4). Homogenates diluted 1:15.*

The Stability of Undiluted Homogenate stored in the Refrigerator

Five krill homogenates were kept in cryovials in the refrigerator once they were out of the freezer. To be able to compare the individual krill homogenates, all total protein concentrations were converted to percentage numbers. The initial protein concentration measured on day one was defined as 100%. The protein content decreased with time, down to 57 ± 10% on the fourth day (Figure 4-3 and Table A-2, appendix A.1). Some of the homogenates had a visible change in color (Figure 4-4).

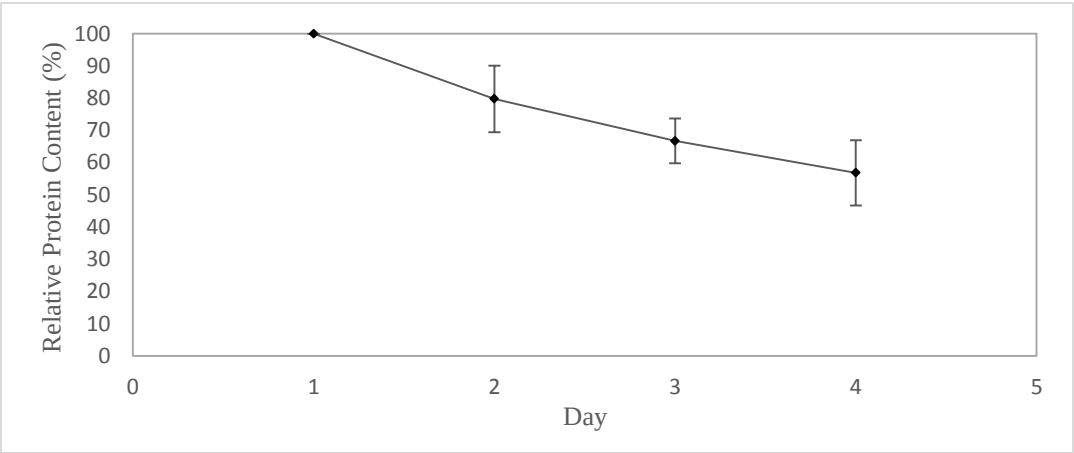

Figure 4-3 ***Homogenate kept in refrigerator.*** *Homogenate pre-diluted 1:20 when analyzed for total protein content. (Error bars indicate ±SD, n=5).*

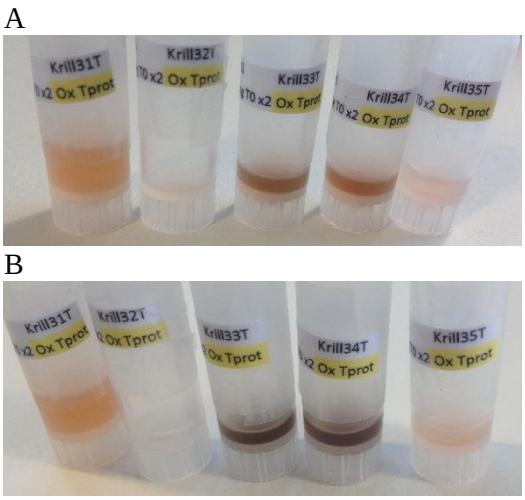

Figure 4-4 ***The color of the krill homogenate after being kept in refrigerator****. 'A' shows the cryovials with the homogenates on the third day, and 'B' on the fourth day.*

The Stability of Pre-Diluted Homogenate stored in the Refrigerator

Four different pre-diluted (1:20) krill homogenates were stored in the refrigerator and analyzed for total protein concentration on seven occasions during a ten days period. The initial value for a krill homogenate on day one was set to be 100%, and the percentage values on the other days were found for each of krill homogenates. In general, the protein content decreased from the initial value, and on the eleventh day it was 56 ± 1% of the initial concentration (Figure 4-5 and Table A-3, appendix A.1).

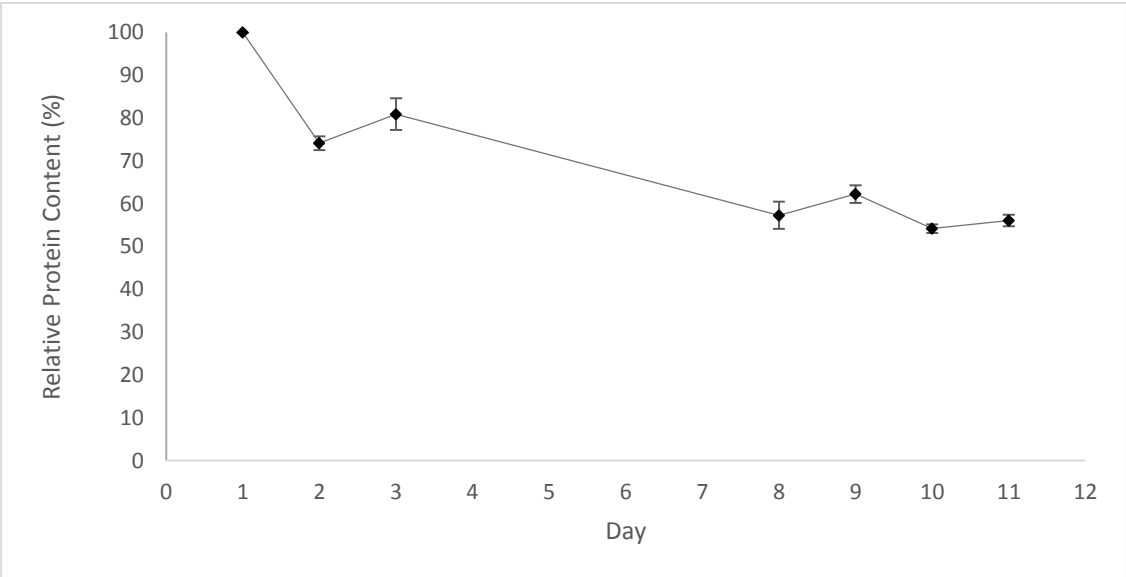

Figure 4-5 ***Degradation in pre-diluted homogenate kept in refrigerator****. Homogenate pre-diluted 1:20. (Error bars indicate ±SD, n=4).*

4.1.3 Validation of Total Protein

Precision

The within-day and between-day variation of the total protein content was 6.5% and 6.1% respectively for the control (Table 4-1) during the method development. In addition the within-day and between-day variation of krill homogenates were 2.8 – 7.4% (Table A-4, appendix A.1) and 7.0 - 11% (Table A-5, appendix A.1) respectively. The total protein content was measured in a 1:20 pre-dilution of the homogenates.

Table 4-1 ***The within-day and between-day variation of the total protein content****.*

	Within-day variation[a]				*Between-day variation*[b]			
Sample type	*Mean (mg/mL)*	*SD (mg/mL)*	*CV (%)*	*R2*	*Mean (mg/mL)*	*SD (mg/mL)*	*CV (%)*	*R2*
Control	0.711	0.046	6.5	0.992	0.733	0.044	6.1	0.985-1.000
Krill homogenate			2.8-7.4	0.987			7.0-11	0.992-1.000

[a]: n=9 for control, and n=4 for three different krill homogenates.
[b]: n=25 for control, and n=4 for four different krill homogenates.

A control card was established for the 96-well plates for the total protein in krill from the exposure study (Figure 4-6). The control card was based on the mean and SD of the between-day variation of the control from the method development (0.733 ± 0.044 mg/mL, Table 4-1), where 2SD was the warning limit and 3SD the action limit. The concentration of the control measurements on the microplates from the exposure experiment were all in the area between the mean and mean - 2SD. This was within acceptable distance but indicated a systematic shift. The concentration of the 14 controls from the exposure experiment was 0.681 ± 0.028 mg/mL, with a CV of 4.1%.

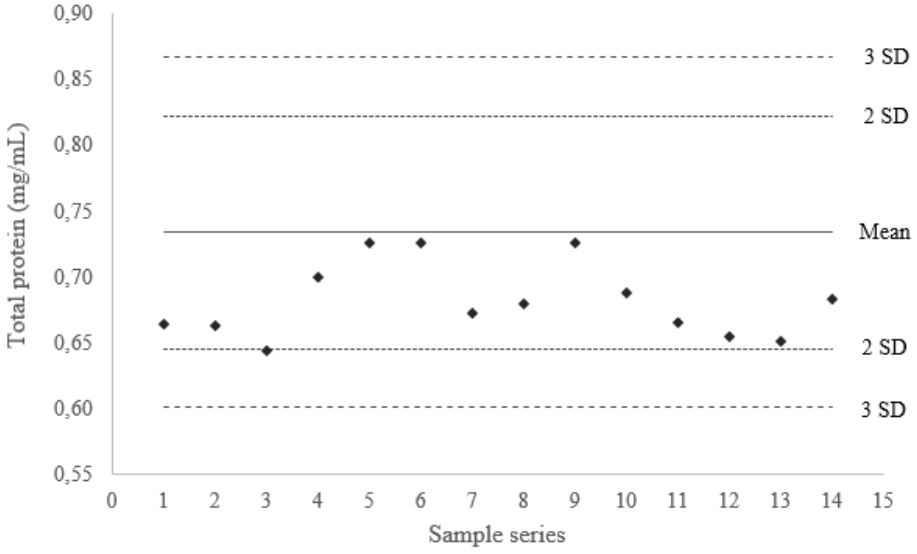

Figure 4-6 ***Control card for the total protein measurements for krill exposed to oil****. Mean and SD established from the method development (n= 25) are shown with the straight lines. (n=3).*

Calibration Curve

The coefficient of determination (R2) for the calibration curve of total protein was between 0.985 and 1.000 for all 96 well plates analyzed within the project (Table A-6, appendix A.1). The calibration curve

had a slope 'b' of 0.398 ± 0.024, and intercept 'a' of 0.361 ± 0.014. The absorbance range for the calibration standards was 0.419 – 0.848, corresponding to 0.143 – 1.24 mg/mL. Within the project, all residuals for the three highest concentration calibrators were less than 15%, while the two lowest concentration calibration standards were less than 20% (Table A-6, appendix A.1).

LOD/LOQ

The instrumental limits of detection (LOD) and quantification (LOQ) of the method were 0.113 mg/mL and 0.343 mg/mL respectively, based on 'a' and 'b' values (Table A-6, appendix A.1). The LOD is below the lowest concentration calibration standard (Cal 2 at 0.143 mg/mL), while the LOQ is between the second and third calibration standard (Cal 3 at 0.286 mg/mL and Cal 4 at 0.571 mg/mL). The overall method LOD and LOQ increased according to the dilution factor (Table 4-2).

Table 4-2 ***The overall method LOD and LOQ of total protein in krill homogenate****.*

Pre-dilution	*LOD (mg/mL)*	*LOQ (mg/mL)*
1:10	1,13	3,43
1:15	1,70	5,15

4.2 Measurement of AOPP

4.2.1 Stability of KI and Calibration Standards

The calibration standards are based on chloramine-T oxidizing KI, and the absorbance of the resulting triiodide measured at 340 nm. KI was in excess while chloramine-T concentrations varied to create the regression line. It was discovered that the measured absorbance was not stable over time. By using freshly made KI solutions and by protecting the entire microplate from light-exposure this issue was reduced to a minimum. The method was implemented to measure absorbance ten minutes after KI had been added to chloramine-T.

Solutions of KI are sensitive to light and were stored protected from light-exposure until mixed with the chloramine-T standards. The effect of storage on KI was determined by comparing results from three different microplates each containing sets of blank and chloramine-T calibration solutions mixed with three different KI (all 1.16 M, but either freshly made, stored one week, or stored three weeks). The effect was prominent in the blank (Figure 4-7), where the initial absorbance (measured three minutes after addition of KI) in the freshly made KI (ABS 0.037 ± 0.001) was lower than in the two stored KI (ABS 0.040 ± 0.002 in both) (Table A-8, appendix A.2). It was concluded that KI solution had to be made fresh daily.

Protecting the entire microplate from light-exposure as soon as KI was added had an effect on the absorbance of the calibration standards and the blank (Figure 4-7 and Figure A-1, appendix A.2). With no protection, the absorbance of the blank increased with a slope of 0.00126 (ABS/min) or 3.4 %/min. The light-protected blank had a slope of 0.000234 (ABS/min) or 0.6 %/min. The absorbance continued to rise for the lower concentration standards (Cal 1 to Cal 5) in the unprotected plates. For the higher concentrations (Cal 6 and Cal 7) the effect was opposite. The absorbance of the higher calibration standards protected from light-exposure was slightly decreasing, while the absorbance of calibration standards not protected rose to a level where they stayed more constant. The changes in absorbance of calibration standards and blank in the light-protected plates were small, and only of importance the first ten minutes after KI addition (Figure A-8, appendix A.4).

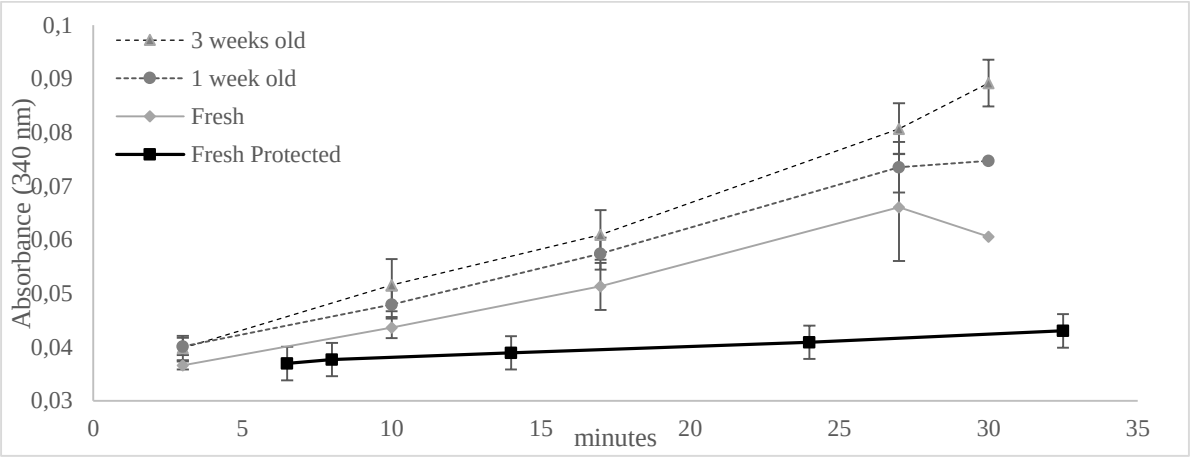

Figure 4-7 ***Absorbance of blank with different KI-solutions over time****. All KI-solution of same concentration (1.16 M) stored protected from light-exposure. '3 weeks old', '1 week old'and 'Fresh' were used on microplates not protected from light-exposure. 'Fresh Protected' were measured on light-protected plates. (Error bars indicate ±SD, n=3 different plates).*

4.2.2 Pre-Dilution of Krill Homogenate for AOPP

Initially a plasma control was used to test the effect of pre-diluting a sample for AOPP analysis using three different solvents; PBS, buffer A and citric acid (0.2 M). The results indicated that PBS was a good choice of solvent, and that a 1:10 dilution provided the same concentration results as undiluted plasma.

The AOPP absorbance measurement was done ten minutes after KI had been added to the calibration standards. There was no significant difference in the AOPP concentration between the 1:5 sample (not pre-diluted, 40 µL added to well) and the 1:10 dilution for any of the solvents (Figure 4-8), nor was there any difference between the different solvents at 1:10 dilution. For each of the solvents though, the detected AOPP concentration decreased when the plasma sample was diluted 1:13.3 compared to 1:10. The lower AOPP concentration at 1:13.3 was only significant for the citric acid when compared to 1:5. Further dilution of the plasma sample in PBS (1:20) gave an AOPP concentration as 79% of the 1:5 value, but then the absorbance was just below the lowest calibration standard.

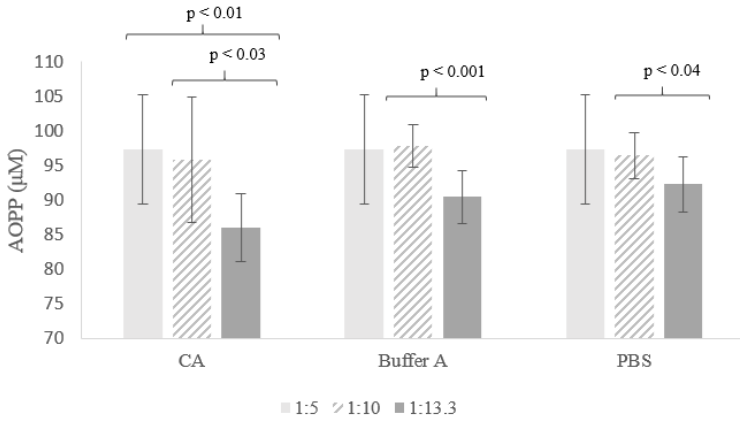

Figure 4-8 ***AOPP in diluted plasma samples in three different solvents****. The 1:5 sample (not pre-diluted) is included for comparison for all three solvent types. (Error bars indicate ±SD, n=8-9). Significant differences are marked with p < 0.05.*

Krill homogenates pre-diluted 1:10 in PBS resulted in stable absorption (Figure 4-9) within the standard calibration curve range and provided the highest AOPP concentration (Figure 4-10). An adjusted version of these conditions with the pre-dilution split to 1:8.3 and 1:13.3 based on thorax weight was later used when analyzing krill from the exposure study. Alternative dilutions and solvents were tested, and the absorbance was recorded for a period after the KI had been added to the calibration standards.

Three different krill homogenates were pre-diluted in citric acid (0.2 M) and PBS to 1:10 and 1:13.3 (Figure 4-9). The absorbance at 340 nm was measured between 0.265 and 0.596. To be able to compare the individual krill homogenates, all absorbance measurements (340 nm) were converted to percentage numbers, where the 100% value for each krill homogenate was the absorbance for 1:10 dilution in citric acid (2.5 minutes after KI was added). The absorbance measurements were constant over the entire period with less than 3% change. Krill homogenates that was not pre-diluted (thus measured as 1:5) gave absorption measurements above the highest calibration standard in six out of seven krill homogenates tested, and was not investigated further.

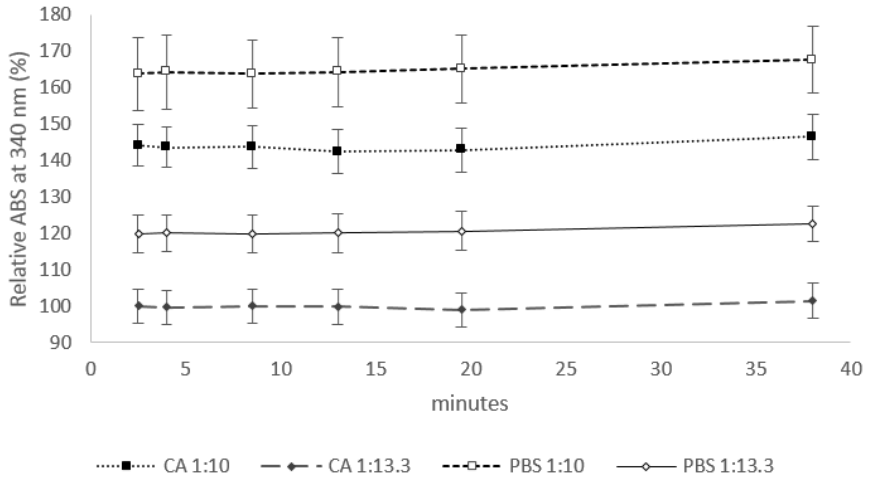

Figure 4-9 ***Relative absorbance at 340 nm over time for diluted krill homogenates in two different solvents****. (Error bars indicate ±SD, n=9 for 1:10 and n=6 for 1:13.3)*

The absorbance values were converted to AOPP concentrations, and the highest AOPP concentration was found in the 1:10 dilution in PBS (Figure 4-10). The numbers were compared as percentage values of the AOPP concentration in 1:10 dilution in citric acid for the individual krill. The AOPP concentrations in PBS were significantly higher than in citric acid ($p < 0.05$), and there was a significant decrease in AOPP concentration from dilution 1:10 to 1:13.3 ($p < 0.04$).

A second dilution series in PBS at 1:10, 1:13.3 and 1:20 (Figure A-2, appendix A.2) also showed a decrease in AOPP concentration as the krill homogenates became more diluted ($p < 0.0001$). At 1:20 one of the krill homogenates was around the absorbance level at the lowest chloramine-T calibration solution. A single krill homogenate had absorbance around the absorption level of the highest chloramine-T standard at 1:10 dilution during method development.

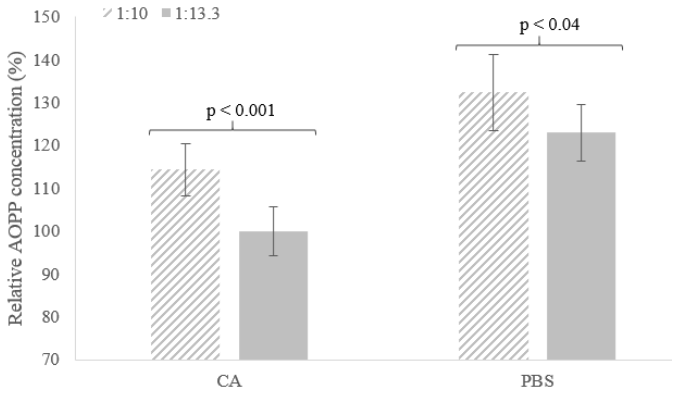

Figure 4-10 ***Relative AOPP concentration in diluted krill homogenate in two different solvents****. Measured 8.5 minutes after KI was added to calibration standards. (Error bars indicate ±SD, n=9 for 1:10 and n=6 for 1:13.3). Significant differences are marked with p < 0.05.*

4.2.3 Citric Acid Concentration

A citric acid concentration of 0.2 M was found as the optimal concentration for dissolving the pre-diluted krill homogenates in the AOPP method. This concentration gave a clear solution with absorbance within the calibration curve range. Several citric acid concentrations (0.1 M, 0.2 M, 0.5 M and 1 M, all 160 µL) were tested to dissolve the pre-diluted krill homogenate (1:13 in PBS, 40 µL). Nine different krill homogenates were used, and to compare the numbers all absorbance values were converted to percentage values of the absorbance measured with the 0.2 M citric acid. The AOPP measurements were the highest in the weakest citric acid concentration, and decreased as the concentration increased (Figure 4-11). A t-test confirmed significant difference ($p < 0.0001$) between absorbance of krill homogenates dissolved in different citric acid concentrations.

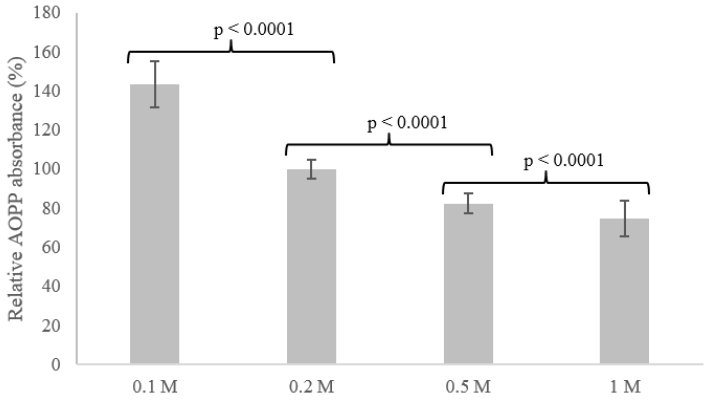

Figure 4-11 ***Relative AOPP absorbance at 340 nm in krill homogenate, in different citric acid concentrations****. Measured 7 minutes after KI was added to calibration standards. (Error bars indicate ±SD, n=30 for 0.2 M, n=36 for 0.5 M and n=18 for 0.1 M and 1 M). Significant differences are marked with p < 0.05.*

The dissolved krill homogenates (n=5, 40 µL pre-diluted (1:13.3 in PBS) krill homogenate + 160 µL citric acid) was transferred to 4 mL cuvettes and visually inspected. The homogenates had a pale pink color. For one krill homogenate three different citric acid concentrations were compared, where the 0.1 M gave a solution with a somewhat milky and cloudy appearance, the 0.2 M solution had a much clearer appearance and the 0.5 M gave a clear and less colored solution (Figure 4-12). The other four krill homogenates were

compared using only 0.1 M and 0.2 M citric acid. For one krill there was no visible difference between the two, but for the other three there was a small but visible difference where homogenate dissolved in 0.2 M citric acid solution was clearer than in 0.1 M citric acid.

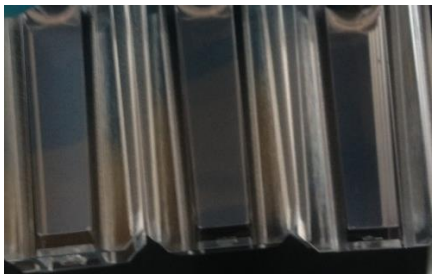

Figure 4-12 ***Cuvettes with krill homogenate solved in different citric acid concentrations****. Left cuvette: 0.1 M citric acid, middle cuvette: 0.2 M citric acid and right cuvette: 0.5 M citric acid.*

4.2.4 Validation of AOPP

Precision

The within-day and between-day variation of the plasma control were 7.8% and 8.8% respectively (Table 4-3), measured in plates protected from light-exposure. For krill homogenates the within-day variation of the AOPP concentration was 4.0-13% (Table 4-3). When considering the absorbance at 340 nm, the CV was 5.9% and 6.5% for within-day and between-day variation of plasma respectively.

Table 4-3 ***The within-day and between-day variation of the AOPP content****. The plasma control and three krill homogenate pools (1:13.3 in 0.2 M citric acid, measured at 8 minutes after KI added) were analyzed. The plasma controls were measured 5-11 minutes after KI was added.*

Sample	*Within-day variation*[a]				*Between-day variation*[b]			
type	*Mean*	*SD*	*CV (%)*	*R2*	*Mean*	*SD*	*CV (%)*	*R2*
Krill pool K1 (µM)	294	12	4,0	0,999				
Krill pool K2 (µM)	248	11	4,4	0,999				
Krill pool K3 (µM)	179	24	13	0,999				
Plasma (µM)	92,0	7,2	7,8	0,997	91,9	8,0	8,8	0.997 - 1.000
Plasma (ABS 340 nm)	0,236	0,014	5,9	0,997	0,233	0,015	6,5	0.997 - 1.000

[a] n=6 for the krill homogenate pools, and n=18 for plasma

[b] n=13 for plasma

A control card was established for the 96-well plates for AOPP analysis of krill from the exposure study (Figure 4-13). The control card was based on the mean and SD of the between-day variation of the plasma control from the method development (91.9 ± 8.0 µM) (Figure 4-13) where 2SD was the warning limit and 3SD the action limit. The AOPP control measurements (plasma) on the microplates from the exposure study were all close to the method development mean.

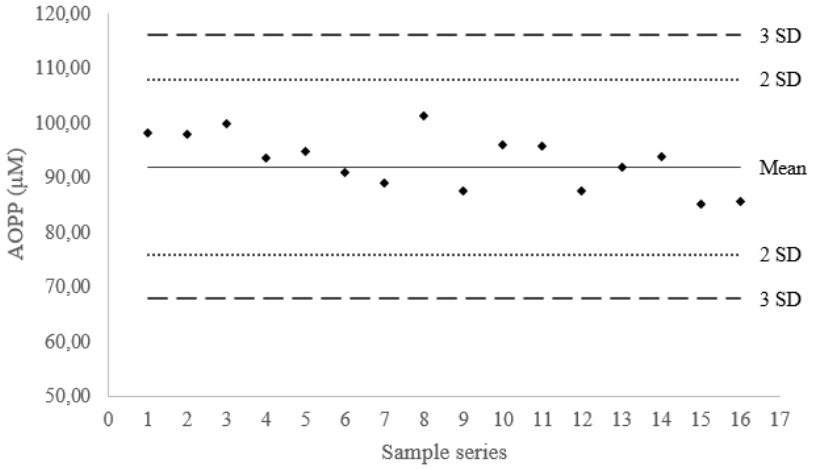

Figure 4-13 ***Control card for the AOPP measurements for krill exposed to oil****. Mean and SD established from the method development (n= 13) are shown with the straight lines. (n=3 for each microplate).*

Calibration Curve

The coefficient of determination (R2) for the calibration curve of the AOPP concentration was between 0.994 and 1.000 for the 96 well plates within this study (Table A-9, appendix A.2). The calibration curve had a slope 'b' of 0.0096 ± 0.0005, and intercept 'a' of 0.053 ± 0.007. The absorbance value range was 0.092 – 0.951, corresponding to 4.75 – 95 µM chloramine-T. Residuals for the lowest concentration calibration standard (Cal 1) varied between -2 and 39%, and were below 15% for the remaining six calibrations standards (Table A-9, appendix A.2).

LOD / LOQ

The instrumental limits of detection (LOD) and quantification (LOQ) were 2.44 µM and 7.38 µM respectively, based on 'a' and 'b' values (Table A-9, appendix A.2). The LOD is below the lowest concentration calibration standard (Cal 1 at 4.75 µM), while the LOQ is below the second lowest calibration standard (Cal 2 at 9.5 µM). The overall method LOD and LOQ increased according to the dilution factor (Table 4-4).

Table 4-4 ***LOD and LOQ of AOPP in krill homogenate****.*

Pre-dilution	*LOD (µM)*	*LOQ (µM)*
1:8.3	20,2	61,3
1:13.3	32,4	98,2

4.3 Measurement of MDA

4.3.1 Chromatograms

The krill homogenates and MDA calibration standards were derivatized with TBA to add a fluorescent chromophore to the MDA molecules. The separation and detection was done on a reversed-phase HPLC with a fluorescence detector. The chromatogram acquired with the fluorescence detector (ex/em 526/560 nm) had a distinct peak for the $MDA(TBA)_2$ derivate both in krill homogenate (Figure 4-14) and in the MDA calibration standards (Figure 4-15 and Figure A-3, appendix A.3). The chromatograms of the MDA calibration standards (Figure 4-15) differed from chromatogram of the krill homogenate (Figure 4-14) with a negative peak just before the $MDA(TBA)_2$ derivate peak. There were traces of other (unknown)

fluorescent compounds in the chromatogram, but the peak of the $MDA(TBA)_2$ derivate was always the highest. The $MDA(TBA)_2$ derivate was eluted at 2.57 ± 0.20 minutes. The t_0 retention time for the nonretained compounds was between 0.80 and 1.0 minutes.

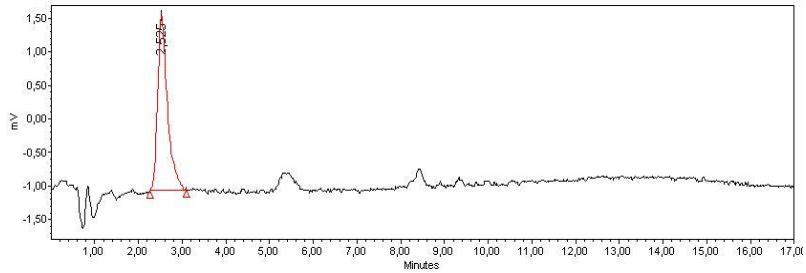

Figure 4-14 ***Chromatogram for krill pool control.*** *Fluorescence detector with ex/em 526/560 nm. MDA t_{RT} = 2.525 minutes.*

The peak intensities were 775 – 34 721 µV for the calibration standards, 2 498 – 8 842 µV for the control krill homogenate and 1 013 - 13 132 µV for the various krill homogenates from the exposure study. The baseline noise level was less than 80 µV.

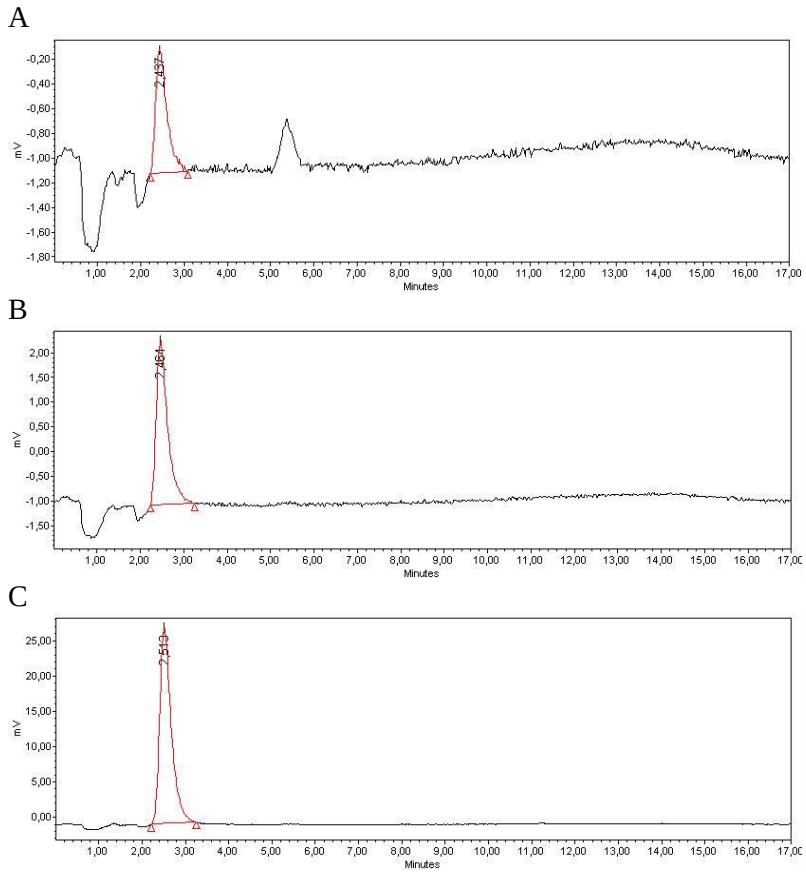

Figure 4-15 ***Chromatograms for MDA calibration standards with fluorescence detector (ex/em 526 nm/560 nm)****. 'A' is the chromatogram for Std2 (0.275 µM), 'B' is the chromatogram for Std5 (2.203 µM) and 'C' is the chromatogram for Std9 (22.032 µM).*

4.3.2 Derivate Stability

The derivatized samples were stored in room temperature in the autosampler prior to injection. The $MDA(TBA)_2$ derivate stability over time in this environment was investigated for krill homogenate and MDA calibration standard solutions. There was less than 9.2% change in measured $MDA(TBA)_2$ peak area in krill homogenate when analyzed within 11 hours (Figure 4-16). Increases of 16% and 43% in $MDA(TBA)_2$ peak area in krill homogenate and calibration standards respectively was detected after 30 hours storage (Figure 4-17).

The sample stability within a run was investigated by measuring the krill homogenate pool control, MDA calibration standard 5, 6 and 7 both in the beginning of the run, and 11 hours later at the end of the run. The relative change in MDA area (%) after 11 hours storage was 0.6 ± 3.6% for krill homogenate and 2.9 ± 3.6% for the highest concentration MDA calibration standard (Figure 4-16). A paired t-test between the first and second measurement confirmed significant changes ($p<0.05$) for all the MDA calibration standards over time, but not for the krill homogenate.

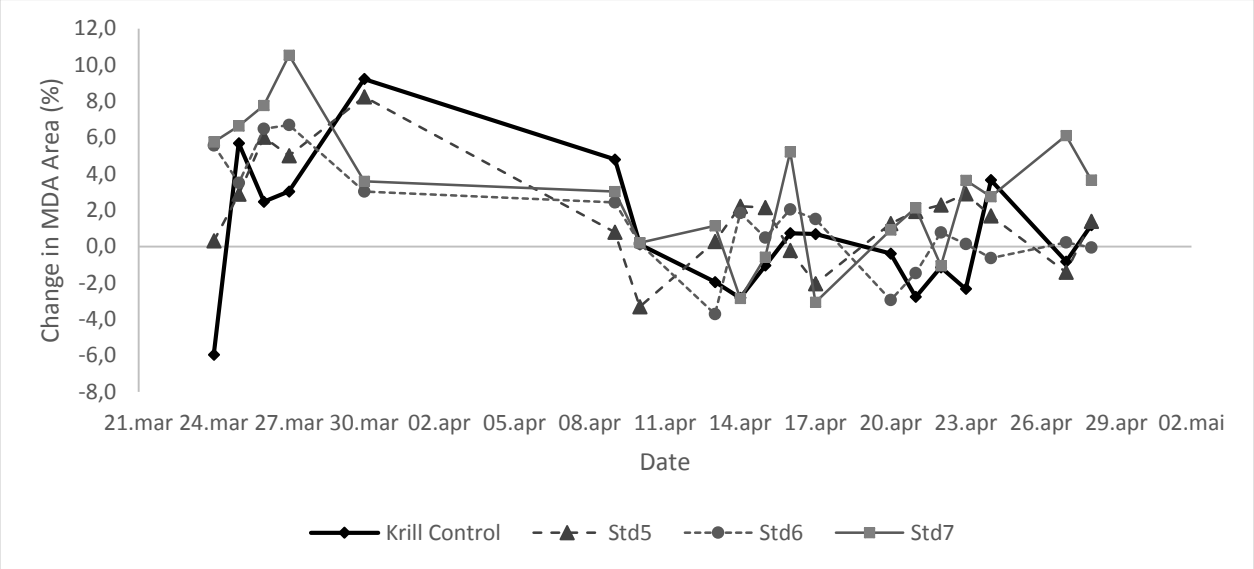

Figure 4-16 ***Short-term $MDA(TBA)_2$ derivate stability****. Change in MDA area after 11 hours storage (n=19 sample sets, mean value of two injections.)*

The long term sample stability (>24 hours in room temperature) was investigated by measuring $MDA(TBA)_2$ in five krill homogenates and six MDA calibration standards three times; first time defined as 0 hour, second time after 30 hours and third time after 43 hours. All MDA peak areas were converted to percentage numbers, where the 100% value for each sample was the first measurement. There was an increase in relative area for both types of sample over time ($p < 0.03$) (Figure 4-17 and Table A-11, appendix A.3). The krill homogenate increased to 116 ± 6% after 30 hours, and 118 ± 8% after 43 hours compared to the initial area, while the MDA calibration standards increased to 143 ± 14% and 151 ± 37% after 30 hours and 43 hours respectively.

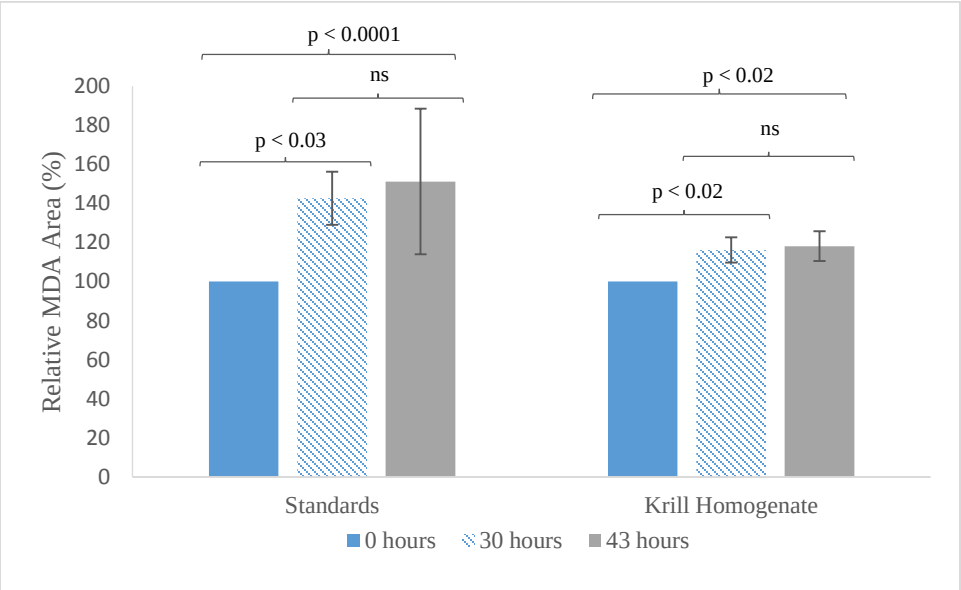

Figure 4-17 ***Long-term MDA(TBA)$_2$ derivate stability****. Increase in MDA peak area (%) from time 0 to 43 hours after initial measurement. (Error bars indicate ±SD, n=5 krill homogenates, n=6 MDA calibration standards). Significant differences are indicated with p < 0.05. ns = not significant.*

Color Intensity of Krill Homogenate

The color intensity of different krill homogenates was registered prior to each derivatization, with '1' as the weakest color, almost transparent, and up '5' as the darkest red color. The red color is the astaxanthin in the krill. There was no apparent correlation between the intensity of the color and the measured MDA concentration (µM) in each of these homogenates (Figure 4-18).

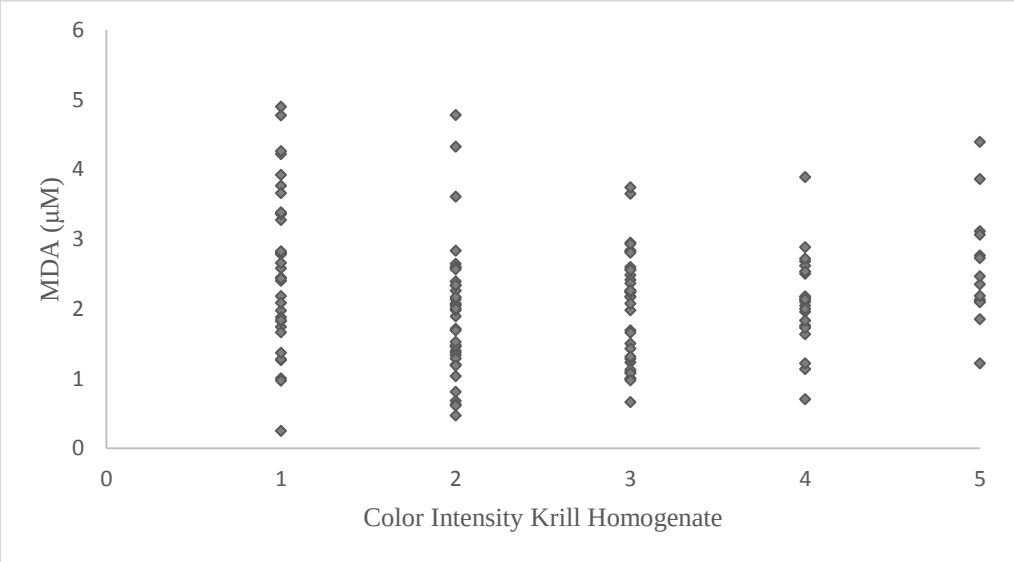

Figure 4-18 ***Color intensity krill homogenate versus measured MDA concentration.*** *(n=143).*

4.3.3 Validation of MDA

Precision

A pool of krill homogenates was used as a control in each sample set (Figure 4-19). The four initial measurements showed a high MDA content compared to the remaining period, and were discarded from the between-day calculation and from the results for the exposure study. The within-day and between-day variation of the MDA was 19% (n=5) and 19% (n=16) respectively for the krill homogenate pool control (Figure 4-19). Human plasma samples were measured in four sample sets (Figure A-4, appendix A.3), with a between-day variation of 10% (n=3) after the first sample set was discarded.

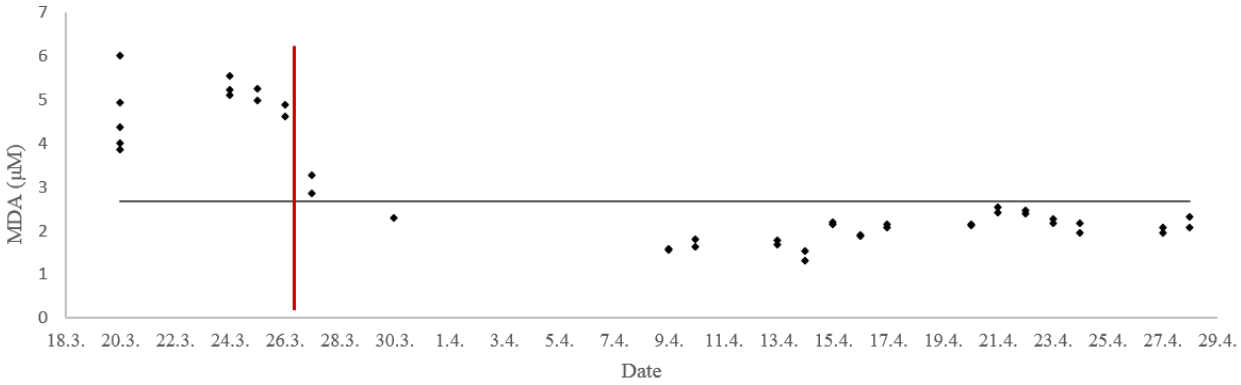

Figure 4-19 ***MDA in krill homogenate pool control****. The overall mean MDA (2.67 µM) is shown as a straight line. (n=20 sample sets). The red line between March 26, 2015 and March 27, 2015 marks the sample sets that were discarded from the exposure study results and the between-day calculation.*

Recovery

Samples from a krill homogenate pool (25 µL) were spiked with low concentration of MDA standard (1,15 µM, 5 µL) and high concentration MDA standard (28,64 µM, 5 µL). Blank (40% ethanol, 5 µL) was added to the unfortified samples. The recovery was 189% and 102% for the low and high spike concentration respectively (Table 4-5).

Table 4-5 ***MDA recovery in krill homogenate****. Spiked with low and high concentration of MDA. n=3.*

MDA unfortified sample (µM MDA ±SD)	*MDA added (µM MDA)*	*MDA detected (µM MDA ±SD)*	*Recovery (%)*
3.96 ± 0.19	0.19	4.33 ± 0.16	189
3.96 ± 0.19	4.77	8.83 ± 0.51	102

Calibration Curve

The coefficient of determination (R2) for the calibration curve of MDA was between 0.989 and 1.000 for all sample sets analyzed during the method development and the utilization of the method on krill exposed to oil (Table A-12, appendix A.3). The measured area range was 21 917 – 505 591 (µV·sec), corresponding to 0.28 – 22.7 µM. The calibration curve had a slope 'b' of 21 832 ± 1 543, and intercept 'a' of 12 699 ± 5 814. There was an increase of 1417% from the initial value to the maximum value of 'a', and a 17% decrease from the initial value to the minimum value of 'b' (Figure A-6, appendix A.3).

Residuals for the four lowest concentration calibration standard (Cal 2, Cal 3, Cal 4 and Cal 5) had a large offset from the regression line and varied between -130 – 43%, -6.7 – 59%, -5.0 – 33% and -23 – 12%

respectively (Table A-12, appendix A.3). The highest concentration calibration standard (Cal 9) was closer to the regression line and had residuals that varied between -4.6 and 1.7%.

LOQ
The instrumental limit of quantification was 0.28 µM. This was based on the lowest calibration standard with S/N at 10.

4.4 Oxidative Stress in Krill Exposed to Oil

The krill from the exposure study was homogenized, and analyzed for total protein, AOPP and MDA content. The exposed krill had different weight profiles each season (Table 4-6). Results that were below LOD for each method were not included, and based on the MDA control only MDA results from March 27, 2015 and onwards were included. Each of the seasons had T0 krill (frozen directly after capture), and T1 krill from the exposure study as 'C' control (0 ppm oil), 'L' low (0.015 ppm oil), 'LUV' low UV (0.015 ppm UV treated oil), 'H' high (0.15 ppm oil) and 'HH' high high (1 ppm oil).

Table 4-6 ***Overview of krills exposed to oil analysis.*** *'A': how many krill homogenates analyzed in total. 'L': how many analyzed were between LOD and LOQ. Values below LOD were excluded. The dashed line marks the boundary between the two different kinds of pre-dilutions for the total protein and the AOPP methods.*

Treatment	*Total amount*	*Thorax weight (g) distribution*				*Min weight (g)*	*TProt*		*AOPP*		*MDA*	
		0.02-0.04	*0.05-0.07*	*0.08-0.10*	*0.11-0.17*		*A*	*L*	*A*	*L*	*A*	*L*
Spring T0	75	0	4	38	33	0,06	75	7	70	1	6	0
Spring T1	58	1	11	30	16	0,04	58	10	57	16	36	0
Autumn T0	70	14	51	5	0	0,02	70	30	70	2	11	0
Autumn T1	43	15	25	3	0	0,03	43	32	39	4	36	0
Autumn T1 HH	16	14	2	0	0	0,03	16	13	15	8	12	0
Winter T0	30	1	19	8	2	0,04	30	8	30	4	11	0
Winter T1	45	7	25	9	4	0,02	45	19	43	7	36	0
SUM	337	52	137	93	55		337	119	324	42	148	0

4.4.1 Total Protein in Krill Homogenate

The total protein content in the krill homogenates was measured and used to normalize the AOPP and MDA concentrations (Figure A-7, appendix A.4). The total protein concentration in krill homogenates from the three seasons were significantly different from each other, where krill homogenates from the spring had the highest total protein concentration and from the autumn the lowest (Figure 4-20).

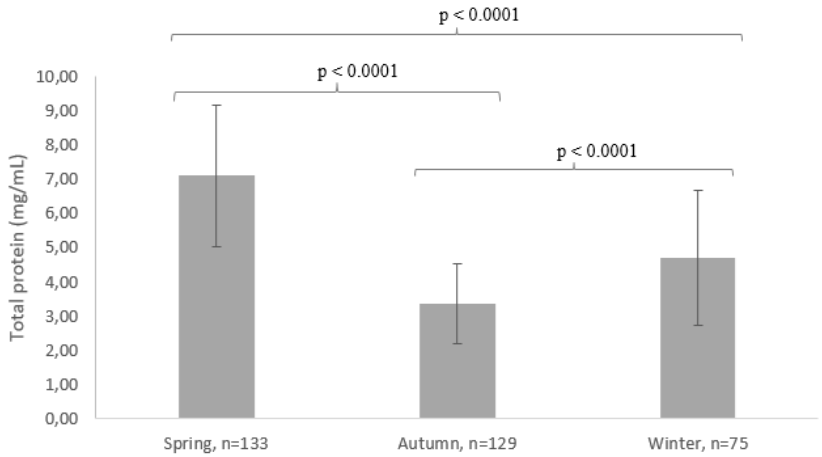

Figure 4-20 ***Total protein (mg/mL) per season.*** *(Error bars indicate ±SD). Significant differences are indicated with $p < 0.05$.*

4.4.2 AOPP

The AOPP concentration (µM) in krill homogenates from the exposure study was measured, and it was found that the concentration was the highest in the krill caught in the spring (Figure 4-21). The T1 treatments within each season were significantly different from each other. There were no significant differences between the autumn and winter AOPP levels in the T0 krill (frozen directly after capture).

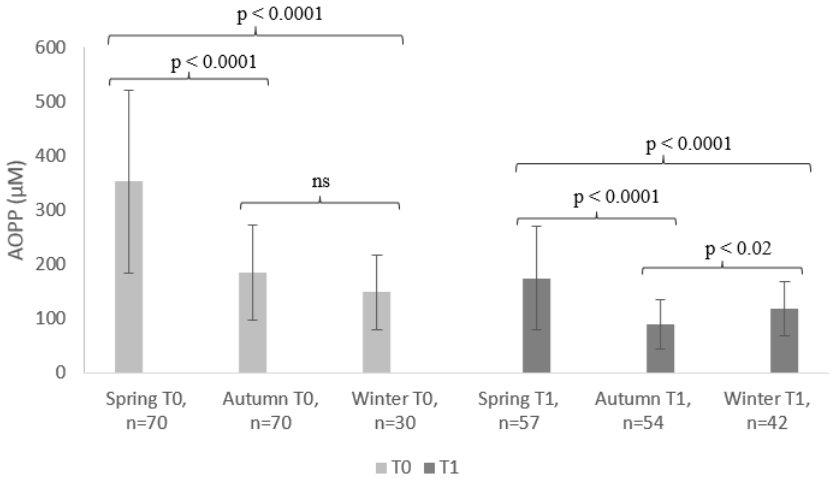

Figure 4-21 ***AOPP concentration (µM) in krill homogenates per season (T0 and T1)****. (Error bars indicate ±SD). Significant differences are indicated with $p < 0.05$. ns = not significant.*

When the AOPP concentrations were normalized to total protein content, there were less significant difference between the different seasons for each treatment (Figure 4-22). The AOPP in krill homogenates from the Low treatment in spring and autumn were significantly different, and the AOPP in T0 krill homogenates caught in winter were significantly different from spring and autumn T0 krill.

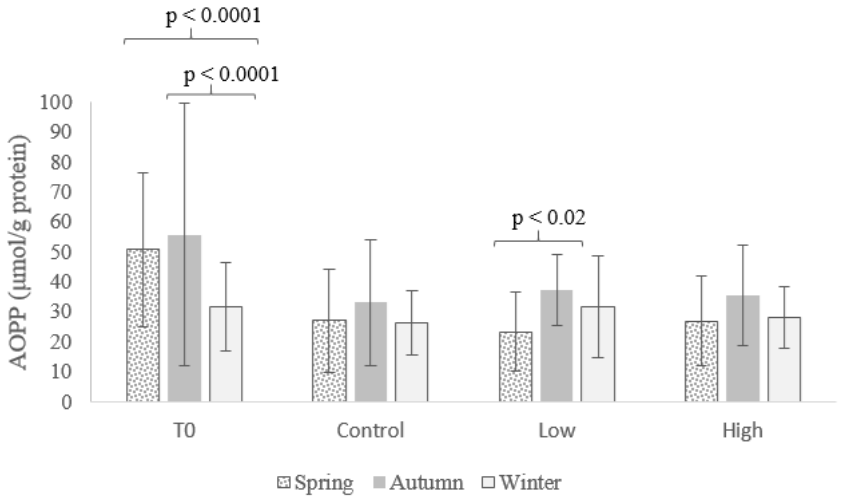

Figure 4-22 ***Normalized AOPP (µmol/g protein) in krill homogenates per treatment.*** *(Error bars indicate ±SD, n=11-70). Significant differences are indicated with p < 0.05.*

Normalized AOPP concentrations in krill homogenate samples grouped according to season show a significantly higher level in the T0 krill (frozen directly after capture) than the T1 krill kept in the laboratory for the samples from spring and autumn (Figure 4-23). In addition, normalized AOPP concentrations in krill homogenate from the autumn High High treatment were different from all other autumn treatments except the control group. For the winter season, none of the normalized AOPP concentrations in krill homogenates from different treatments were significantly different.

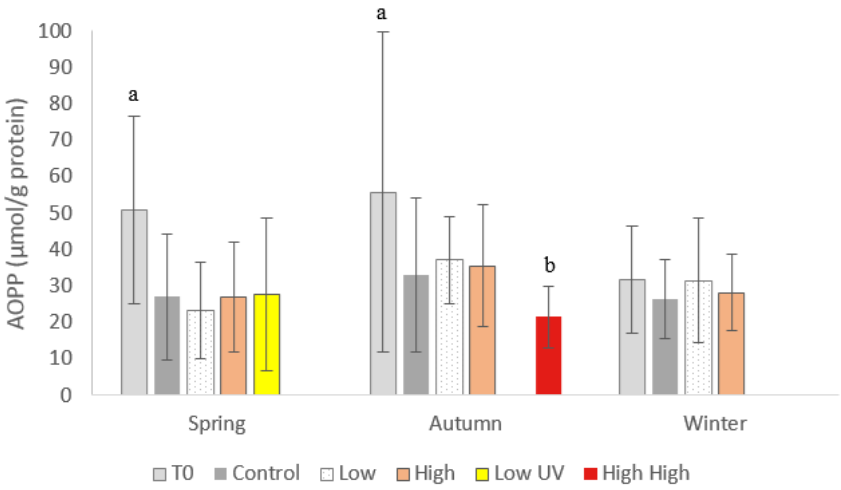

Figure 4-23 ***Normalized AOPP (µmol/g protein) in krill homogenates from the different treatments per season.*** *'a' indicates significant difference from the other treatments within the season, and 'b' indicate significant difference from the other treatments within the autumn season except from autumn control. (Error bars indicate ±SD, n=11-70.)*

4.4.3 MDA

A subset of the samples from each exposure treatment from the three seasons were analyzed for MDA. Each sample set run included nine calibration standards, two krill homogenate pool controls and nine krill samples mixing different treatments in random order. The MDA values were grouped by season and by T0

or T1 (frozen directly or kept in the laboratory for the exposure study) (Figure 4-24). The MDA concentration in theT1 krill homogenates kept in the laboratory were significantly different for each seasons. For the krill that was frozen directly after capture (T0) it was only the autumn and winter MDA concentrations that were different.

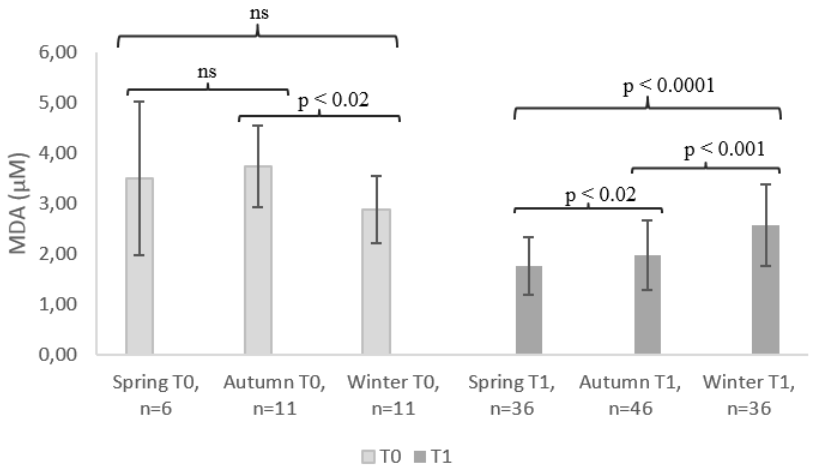

Figure 4-24 ***MDA concentration (µM) in krill homogenates per season (T0 and T1)****. (Error bars indicate ±SD) Significant differences are indicated with p < 0.05. ns: not significant.*

The MDA concentrations in krill homogenate samples were normalized to the total protein content and grouped according to exposure treatment (Figure 4-25). MDA concentrations in T1 spring samples for control, low and high are significantly lower than in the corresponding autumn T1 and winter T1 samples. The spring T0 MDA however only differed from the autumn T0 MDA. The autumn and winter samples had significant differences in MDA levels for T0 and for T1 low treatments.

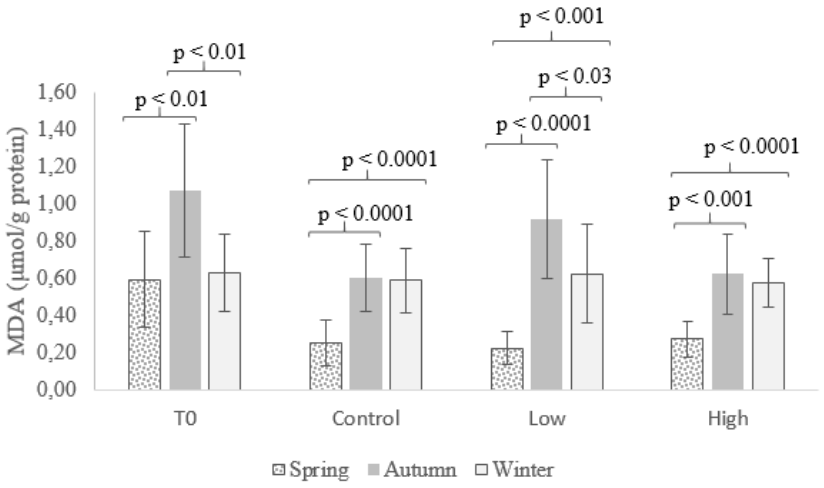

Figure 4-25 ***Normalized MDA (µmol/g protein) in krill homogenates per treatment.*** *(Error bars indicate ±SD, n=6-12). Significant differences are indicated with p < 0.05.*

Normalized MDA concentrations in krill homogenate samples were grouped according to season (Figure 4-26). For the spring samples there was a significantly higher level (p < 0.001) in the T0 krill frozen directly after capture than in the T1 krill kept in the laboratory. In addition, the autumn T0 and Low MDA

were significantly different from the other autumn MDA values ($p < 0.03$) but not from each other. None of the MDA values for the winter season treatments were significantly different from each other.

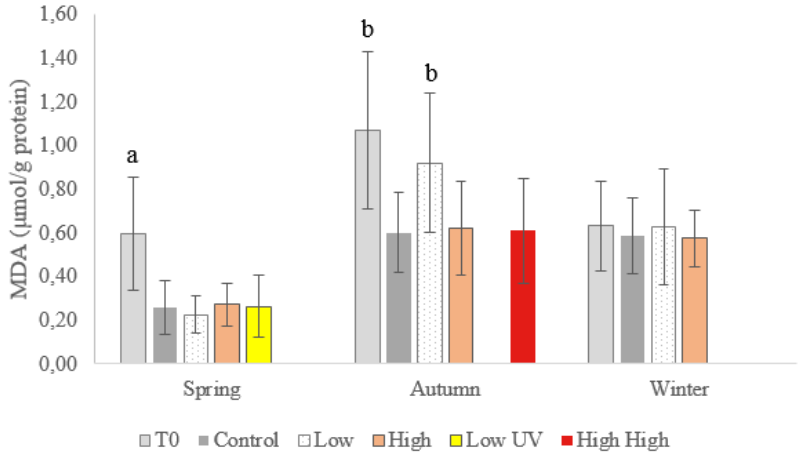

Figure 4-26 ***Normalized MDA (µmol/g protein) in krill homogenates from the different treatments per season.*** *'a' indicates significant difference from the other treatments within the season, and 'b' indicate significantly different from the other treatments within the season but not the ones marked with 'b'. (Error bars indicate ±SD, n=6-12.)*

4.4.4 Comparison of MDA and AOPP Levels

The measured AOPP and the measured MDA levels for the krill homogenates were compared by plotting the MDA versus the AOPP concentrations. The correlation was higher for protein normalized AOPP and MDA value than for the non-normalized (Figure 4-27, Figure 4-28). The spring krill showed some correlation between the MDA and the AOPP levels with R2 at 0.72 for the low UV and the high treatments (normalized values), while the R2 was 0.59 and 0.51 for the low and the T0 respectively. There was no correlation for the spring control treatment, nor in any of the autumn and winter treatments for the protein normalized MDA and AOPP values (Figure 4-28 - Figure 4-30).

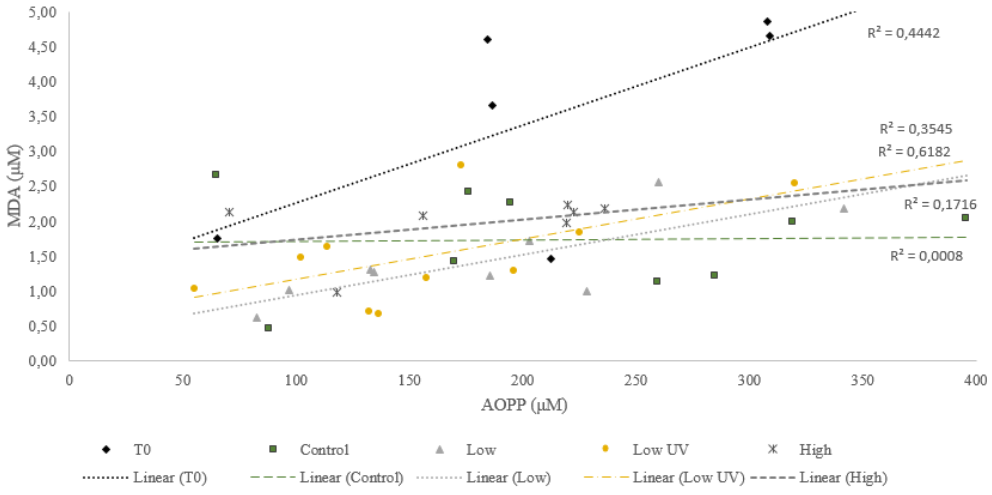

Figure 4-27 ***Non-normalized MDA versus AOPP concentrations in krill homogenates from spring experiment.***

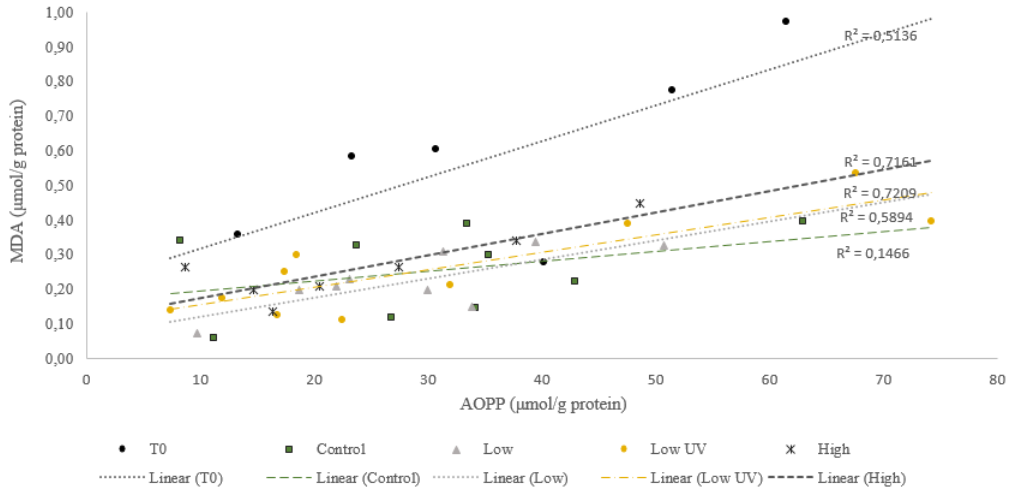

Figure 4-28 ***Protein normalized MDA versus AOPP concentrations in krill homogenates from spring experiment.***

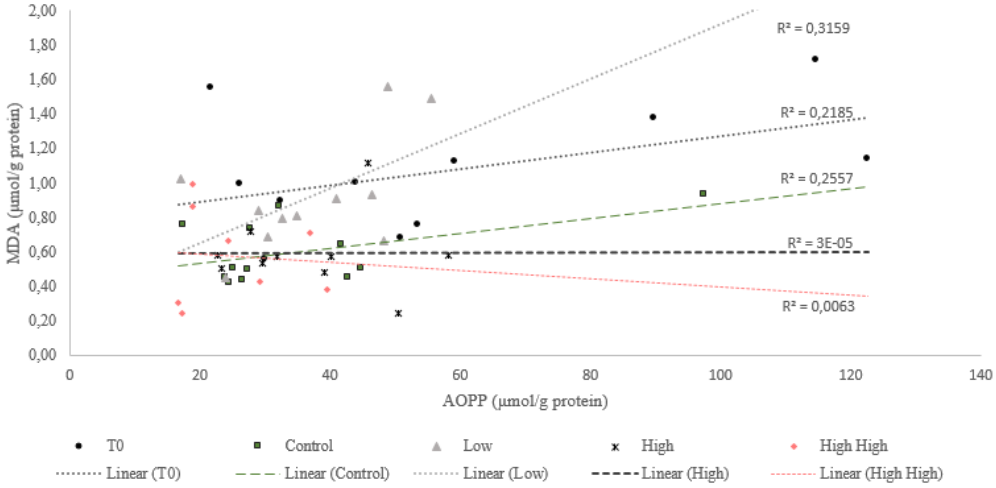

Figure 4-29 ***Protein normalized MDA versus AOPP concentrations in krill homogenates from autumn experiment.***

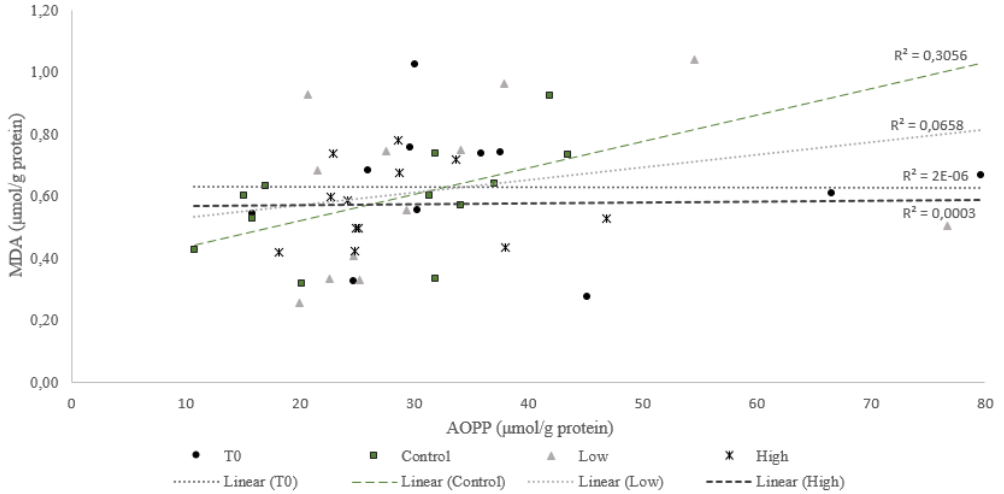

Figure 4-30 ***Protein normalized MDA versus AOPP concentrations in krill homogenates from winter experiment.***

5 Discussion

The goal of this thesis has been to investigate the level of oxidative stress in krill homogenate by use of two biomarkers, AOPP as a measure of protein oxidation and MDA as a measure of lipid peroxidation. The work has been based on existing methods, but special adjustments for krill homogenate have been made during this project.

5.1 Homogenization

The homogenization method chosen for krill was based on a well-established IRIS method [23], with homogenization in ice-cold phosphate buffer, centrifugation and removal of supernatant two successive times (section 3.5). Many different basic homogenization methods are mentioned in the literature (Table A-7, appendix A.1). Some homogenize the krill in large bulks combining several whole animals, like Bargu et al. [19]. This provides larger volumes of homogenate, but disables individual krill results. Most methods use a tris or phosphate buffer with a pH at physiological conditions. Some methods also includes sucrose and substances like ethylenediaminetetraacetic acid (EDTA) for enzyme activity inhibition, dithiothreitol (DTT) to break disulfide bond between proteins, trichloroacetic acid (TCA) for precipitation of macromolecules and/or phenylmethanesulfonyl fluoride (PMSF) as protease inhibitor. Evaluation of homogenization processes are seldom presented in articles. Draper et al. [81] however has a small note where the MDA levels found when homogenizing pig liver was significantly higher than when the liver was minced with scissor (7.80 ± 0.71 μg/g versus 4.22 ± 1.08 μg/g respectively, n=5).

Although the homogenization method was well established for various types of sample material at IRIS, it needed further investigation of several important parameters before being used for krill;

1) One or two centrifugation steps
2) Storage temperature
3) Storage time

The aim was to prepare and store the homogenate under such conditions that total protein, AOPP and MDA could be analyzed with minor influence of pre-analytical factors.

Total protein was used as a parameter to evaluate the homogeneity and stability of the krill homogenate. Two successive centrifugation steps yielded a clearer and more stable supernatant compared to one centrifugation (section 4.1.2). The krill homogenate was not stable over time when stored in the refrigerator. Both undiluted and diluted homogenate kept in refrigerator decreased in measured total protein (section 4.1.2). This shows that the krill homogenate must be analyzed very quickly after it has been thawed.

Although the total protein was measured by a standard Bradford 96 well assay, it was optimized and validated with respect to precision, detection limits and linear response. Then, the optimal pre-dilution of the krill homogenate prior to analysis was investigated to ensure that the absorbance would fall within the range of the calibrators. If the concentrations in the pre-diluted homogenate were too low, they would not be distinguishable from the background noise. The dilutions analyzed were from 1:10 to 1:30, higher or lower dilution factors gave absorption out of range of the calibration standards. Pre-dilution from 1:15 to 1:25 resulted in very stable total protein concentrations (section 4.1.1). The 1:10 pre-dilution gave a slightly lower result with 83 ± 7% of the mean concentration. It was initially expected that insufficient

dilution (such as 1:10) could absorb falsely high due to incomplete dissolution and light scattering. That was not experienced.

Pre-dilution 1:20 of krill homogenate resulted in reproducible protein concentrations and was therefore initially chosen. However, at a late stage in the method development, adjustments had to be made when it was discovered that the autumn krill were significantly smaller than the spring krill. The 1:20 pre-dilution could not be used as its absorbance was below LOD for many krill. The 1:15 pre-dilution also had a few cases of absorbance below LOD for the autumn krill, but 1:10 had a case of absorbance above the highest calibration standard for spring krill. The solution was to split how the krill were diluted based on their thorax weight.

A commercial protein control (Liquichek Spinal Fluid Control, Bio-Rad) was used to determine within-day and between-day variations. The control had an expected protein content within a range of 0.698 - 1.05 mg/mL (Bio-Rad). The between-day variation was 6.1% while the within-day variation was 6.5%. Both within-day and between-day concentrations were within the range from the supplier. In this method the between-day variation were slightly lower than the within-day variation, which is opposite of what was expected. They both were low variations however, indicating reliable results from the method. The control card for the commercial protein control for the microplates from the exposure study (Figure 4-6, section 4.1.3) shows that the values were within two standard deviations from the mean, but shifted in the negative direction thus indicating a systematic error. This could be caused by the control material not having 100% homogeny in the different aliquots, as a new vial from the -20 C freezer was used for these plates. All krill homogenates from krill exposed to oil experiment were measured in the same period and would all be effected similarly should there by another cause for the systematic deviation. The results can be compared with each other without further adjustments.

The within-day and between-day variation of the krill homogenate were 2.8 – 7.4% and 7.0 - 11% respectively. The precision is close to the commercial protein control precision, but the subtle differences could indicate that the homogeny in the homogenate may be different between vials.

The range of the calibration standard concentrations is within an area where the Coomassie brilliant blue dye has a close to linear response. This has been demonstrated with the R2 values all above 0.985, where the mean R2 was 0.996. There were however some residual for the two lower calibration standards; but all below 20% (Table A-6, appendix A.1). The total protein content in these two calibration standards were both below LOQ, but above LOD. The residuals for Cal 3 were positive in 34 of 36 plates with a mean value of 9.6%, indicating that there might be a systematic error with the calibration standard solution. The same but opposite effect was seen in Cal 4, but to a lesser extent. The two cancelled each other out.

The use of the Bradford assay for protein content in some marine animals has been up for debate; Compère et al. [82] compared Biuret's method, the bicinchoninic acid (BCA) protein assay and the Bradford assay for protein content in crab cuticle extracts. Biuret and BCA method gave similar results, while the Bradford assay gave 5-10 times lower result. This lower detection with Bradford was also noted for marine diatoms ([83]) and krill ([84, 85]). Compère et al. [82] mention that the difference might be due to "the presence of many acidic proteins in the extracts, since the Coomassie blue is known to stain such proteins weakly". As long as the Bradford assay was used in the same way for all samples in this thesis, the numbers are comparable.

5.2 AOPP

The AOPP method used in this thesis was the method from Hanasand et al. [26], which was a modification of the original AOPP method of Witko-Sarsat et al. [55] by using citric acid to better solve the plasma lipids. In this thesis plasma was replaced by krill homogenate, which has not been tested for AOPP content previously according to best of our knowledge. The AOPP method was therefore tested and validated for analysis of krill homogenate. Initially, the results did not seem reliable. It was discovered that the calibrators were not stable over time, and this had to be further investigated before the method could be used for analyses of krill homogenate. Several factors were discovered as important (section 4.2.1):

1) Protecting the microplate from light-exposure as soon as possible after KI was added to the calibration standards
2) The KI solutions should be made fresh every day
3) The measurements must be done at the exact same time after addition of KI for the results to be comparable

The plate should be protected from light-exposure as soon as possible after the addition of KI to the calibration standards, to avoid the additional effect of light-induced iodide oxidation to iodine by oxygen. There was no notion on this in the articles presenting the method. Oxidation should ideally only occur with chloramine-T, to achieve a linear response to the increasing chloramine-T concentrations in excess KI. The stability of the KI solution was also investigated. It was declared stable for one month by Witko-Sarsat (in private email to my supervisor). The results however indicated that some oxidation of iodide had already occurred in the elder KI solutions, as the absorbance was higher than for the freshly made KI (section 4.2.1). From that point on freshly made KI solution was used for the calibration standards.

The time-varying absorbance of the calibration standards was investigated by measuring the same plate multiple times for up to one hour. The initial graphs when not protected from light-exposure showed a rise of about 0.1 units of absorbance at 340 nm between initial and maximum measurements for all blank and calibration standards (Figure A-1, appendix A.2). This effect was most likely the light-induced iodide oxidation by oxygen as it was of the same order for the blank. When the calibration standards were protected from light-exposure the change was minimal, but should be considered. The inflection point at ten minutes in the graph of absorption versus time was chosen as the method measurement time. The absorption of the krill homogenate was constant at this time. This change in absorbance of the calibration standards incite a strict measurement protocol; where care must be taken to measure at the exact same time after addition of KI, to achieve the same absorbance measurements and comparable results. Others report that they measure the absorbance immediately [55] or after two minutes on a microplate shaker [26].

Dilutions of krill homogenates did not result in the same concentration (Figure A-2, appendix A.2). One potential reason for this is that the calibrators used (chloramine-T oxidizing iodide to iodine/triiodide) is not the same type of molecules as the AOPP, and has a different response to change in concentration than the krill homogenate. Halving the concentration in the well with the krill homogenate may not necessary reduce the absorbance the same way half the concentration chloramine-T does. Due to this there should ideally only be one dilution factor used for all samples for the results to be comparable. This was not possible though due to the different sizes of the krill thorax. Two different pre-dilutions were chosen to avoid absorbance values below or above those of the calibration standards, and to get a more similar concentration in the samples. Note that the dilution factor does not refer to the pre-dilution with PBS, but the total dilution with both PBS and citric acid. Witko-Sarsat et al. [55] reported that there was a linear

response when diluting the plasma from 1:2 up to 1:20 in PBS, this was confirmed with the plasma control in this thesis, but it was not true for the krill homogenate.

The precision of the method determined by analysis of a plasma control and expressed by the within-day and between-day variations were 7.8% and 8.8% respectively. The within-day variation of the krill homogenate ranged between 4.0 and 13%. These numbers are higher than the reported within-day and between-day variations (0.9-1.4% and 1.3-2.2% respectively) for plasma [26]. The original method of Witko-Sarsat et al. using PBS and acetic acid as solvent had within-day and between-day variations less than 5% and 10% respectively [55]. The Hanasand et al. [26] method was basically the same method as the one used in this thesis, with the exceptions of measurement after ten minutes in this thesis and not two minutes, and that the plate was protected from light-exposure. These two exceptions may be some of the reason why the precision was different. When the plate was protected using the aluminium foil, it was only briefly inspected for bubbles to avoid much light-exposure, and small bubbles could influence the results. The controls from the exposure study measurements (92.7 ± 5.0 μM) stayed within two standard deviations from the mean on the control card (Figure 4-13). The plasma control AOPP concentration is of the same order as the medium plasma control AOPP in Hanasand et al. [26] of 82.6 ± 1.1 μM.

The AOPP method is a relative new method. The range of the calibration standards is the area where chloramine-T oxidation was found to give a linear response [55], and were not changed when utilizing the method. This linearity has been demonstrated with the R2 values all above 0.994, where the mean R2 was 0.998. Hanasand et al. [26] reported a correlation coefficient r^2>0.999 for all analysis. The variation in the intercept of the regression line gave LOQ above the lowest calibration standard. This caused uncertainty in the lower concentration area, reflected by the high residuals of the lowest calibration standard.

5.3 MDA

The chromatograms showed a clear, defined peak for the $MDA(TBA)_2$ derivate in all samples and MDA calibration standards. MDA-TBA spectrophotometry methods have a reputation as not very specific due to the other TBARS that can react with the TBA. With a HPLC-F separation and detection the specificity will increase, and some of the small unidentified peaks on the chromatograms (section 4.3.1) might be derivates of other TBARS separated from the $MDA(TBA)_2$ derivate.

One sample set analyzed daily on the HPLC-F included nine krill homogenates from the exposure study, nine MDA calibration standards with blank and two control samples. The set size of 20 was chosen based on the long sample derivation time, and some concern about the stability of the samples during the derivatization process. The derivate stability was investigated for samples kept in room temperature, and showed no significant changes in measured MDA within 11 hours for the krill samples. There was a significant increase when the samples were stored more than 24 hours though. Possibly, more bound MDA was slowly released and reacted with TBA even after the sample preparation was completed. This indicates that although the samples were stable for the current sample set runtime, adding more samples to the set could lead to increase in MDA and less reliable measurements. The MDA in the calibration standards showed a minor increase after 11 hours, to minimize any impacts this would have on the regression line the calibration standards were the first to be analyzed.

The calibration standards had a range of 0.28 – 22.7 μM MDA. There was no detected signal in blank. The linearity was shown with a coefficient of determination (R2) above 0.989, and with a mean value of 0.997. Seljeskog et al. [47] reported a R2 of 0.9991 for a HPLC-F method with a different isocratic elution. The

limit of quantification in this thesis was 0.28 µM, the same as the lowest calibration standard. Mendes et al. [49] reported lower LOD and LOQ of 0.10 µM and 0.17 µM respectively for a HPLC-F method (with a different gradient). The residuals for the lower MDA calibration standards were high, as was the variation of the intercept value. The calibration standards were single samples, and any deviation in one of them could have large impact on the regression line.

The krill control samples were analyzed for every sample set and the concentration varied between 1.42 and 5.29 µM (Figure 4-19, section 4.3.3). Due to high MDA concentrations in the first sample sets, the results were divided in two. The first collection was discarded from the exposure study results and from the between-day calculations. The within-day and between-day variations were both 19%. Others have reported within-day and between-day variations of less than 5% and 10% respectively for MDA analyzed on a HPLC system [47, 86]. The normalized krill MDA levels are in agreement with reported MDA concentrations. Tremblay et al. [54] reported values from 0.002 µmol/g protein to 15 µmol/g protein depending on krill species and season, while the MDA values in this thesis have varied from 0.06 µmol/g protein to 1.71 µmol/g protein in the krill from the exposure study.

Plasma samples were analyzed four times over a period of 39 days, but the first sample set was discarded from the between-day variation calculation. The MDA concentration in plasma in the last three measurements (1.01 ± 0.10 µM) are in agreement with the plasma values found with the same method by my supervisor (1.16 ± 0.35 µM, n=103, unpublished data). The MDA level found in human plasma in a healthy control have been reported between 0.11 and 4.45 µM depending on the method used [43].

The issues with the high variation in the control and the high increase of the intercept could be caused by the HPLC system not fully equilibrated. The intercept values were initially low and then gradually rose. There was no guard column ahead of the analytical column to protect it from contaminants and molecules that bind irreversibly to the column. However, the pressure in the system prior to the injection did not rise to the warning level of 2500 psi (Figure A-5, appendix A.3). The baseline level was relatively stable, with a small tendency to baseline drift after seven minutes (Figure 4-15 'A', section 4.3.1). Another cause of error could be loss of MDA during the sample preparation. The large variation in the control could also indicate that the homogeny in the krill homogenate pool may be different between the aliquots, but the aliquots were randomized before use.

The HPLC system had a minor software bug that was discovered during this thesis; prior to injection there should be 95% mobile phase A and 5% mobile phase B, but the display showed 95% A and 0% B. It was verified that no mobile phase B was injected (no sound to indicate pump activity). This situation lasted only about 30 seconds, and was corrected to 5% B as the samples were injected. As this was the same for all samples, it should not have a large impact on the results. The emission wavelength should be 525 nm according to the procedure, but 526 nm had to be used due to limitations in the settings of the fluorescence detector. This should not be an issue.

The krill samples were spiked with low and high concentration of MDA, with recovery at 189% and 102% respectively. The recovery was good at high concentration, and similar to other reported MDA recovery factors. Seljeskog et al. [47] reported analytical recovery of 90 – 94% in plasma, while Mendes et al. [49] reported 100 – 108% recovery in fish sample extract. The MDA recovery in this thesis was too high at low concentration though. The too high recovery factor could reflect the more complex media of krill homogenate, where more substances give signal than in the pure MDA standard. The low concentration MDA was the same as the standard deviation of the unfortified sample. Finally, the high recovery could be

due to mistakes in the sample preparation. The dissolving step in the last stage of sample preparation is a potential candidate.

5.4 Oxidative Stress in Krill

The total protein content, the AOPP concentrations and the MDA concentration have been analyzed in krill homogenates from the exposure study. The most interesting findings were that T0 krill from the spring and the autumn had high AOPP and MDA contents within their season, and that there was little difference between the T1 control and the different treatments within a season.

A potential reason for the higher levels in T0 could be the feeding of the animals; in the laboratory the animals were fed twice daily and were not starving. In fish liver samples, starvation has been shown to increase the MDA level from about 2.5 μM in control to about 15 μM in fish starved for five weeks [87]. The MDA level in refed fish returned to 2.5 μM. The lipid level in the *M. norvegica* krill is at its highest in January (40% of dry mass) [4], and this high lipid reservoir could explain why there were no significant differences for the T0 winter krill. The total protein content was higher in the spring krill homogenates, which was natural due to the higher thorax weight. All of the autumn krill thorax weights were below the threshold value of 0.11 g for the homogenization method, which made the krill homogenate more diluted. The single high normalized MDA value for a T1 treatment (autumn 'low') could be elevated due to its low total protein content (77% below LOQ). It was only in the spring krill that there was some correlation between the normalized MDA and AOPP concentrations. For autumn and winter krill there was no correlation.

The small differences between T1 treatments and control were not what was initially expected; it was assumed that krill exposed to increasing oil toxicity would have an increase in the oxidative stress biomarkers. This kind of increase has been found in the MDA levels in fish [88] and in the AOPP levels in rat liver [68] when exposed to toxins. Others have reported no significant differences in MDA levels between control and exposed animal. Dorts et al. [89] exposed black tiger shrimps to two different pesticides, and found no significant difference in the MDA levels in the shrimp hepatopancreas. Almroth et al. [90] exposed fish (eelpout) to various concentrations of oil (0, 0.010, 0.10 and 1.0 ppm) for four days, but found no significant differences in the MDA/g liver levels. The protein oxidation measured as protein carbonyl levels in the fish liver however did rise with increasing oil concentrations. Tremblay and Abele [91] treated Antarctic krill *Euphausia superba*, *Euphausia mucronata* and *Euphausia pacifica* with hypoxia, reoxiganation and temperature increase, and found only some limited increases in MDA levels.

The total protein concentrations in krill from the exposure study have been under LOQ for 17%, 76% and 42% of the T1 spring, autumn and winter samples respectively. This introduce an uncertainty when comparing normalized numbers between seasons. The normalized AOPP level has been similar between seasons for the different treatments, but the normalized MDA level was lower in spring treatments than the other two seasons. MDA has been reported to vary with season, in Icelandic scallop the MDA levels were significantly lower in March than in September or December [92]. There has been no distinguishing for age, sex or reproductive state in the krill, but the smallest krill were not selected for the exposure study. Food availability and season will also make a difference on lipid content [4, 93, 94]. A possibly low lipid level in spring could explain the low MDA level. Tremblay and Abele [91] found higher MDA level in cold season than in warm season for the krill species *Euphausia mucronata*.

5.5 Future Work

One of the challenges during this thesis has been the small volume of the krill homogenate to work with, in some cases less than 200 µL. This had to be split for analysis of total protein, AOPP and MDA. The small volume made it impossible to repeat an analysis that failed, as there were no spare material. In retrospect, the entire process could have been done differently with a more diluted homogenate. The pre-dilutions for total protein and AOPP could have been adjusted, and a different set of concentrations for the calibration standards could have been applied for the MDA method. Other ways to increase the volume of homogenate could be to combine several krill thorax to one sample, or by using the whole krill.

The AOPP method could be changed to have a pre-dilution based on total protein content, to ensure equal density in the homogenates. This would make the results more comparable, as the response from dilution was not linear for krill homogenate.

The MDA method could be further investigated for use on krill by changing several parameters:

- Different solvents/mobile phase buffers and different gradients for separation, to increase the retention time and try to further separate any potential substances eluted at the same time as the $MDA(TBA)_2$ derivate.
- Guard column to protect the analytical column.
- Try with a shorter runtime, as the MDA(TBA)2 derivate eluted in less than three minutes, and the whole runtime was 17 minutes. With a faster method more samples could be analyzed each day.
- Triplicates of each sample.

6 Conclusion

Existing methods for detecting oxidative stress with the biomarkers AOPP and MDA have been evaluated and modified for the use on krill homogenate. The krill thorax was homogenized and could be split to three different analysis with 20, 15-24 and 30 µL homogenate for the total protein, AOPP and MDA methods respectively.

The AOPP method was a simple, fast and reproducible method, with within-day and between-day variations of 7.8% and 8.8% respectively. The instrumental limits of detection and quantification were 2.44 µM and 7.38 µM respectively, which correspond to 20.2 and 61.3 µM respectively for AOPP in 1:8.3 pre-diluted krill homogenate. The AOPP levels have been measured in krill homogenate for the first time, and the samples had a constant absorbance for at least 20 minutes. The initial issue with a varying absorbance of the calibration standards were minimized by protecting the samples from light-exposure, and by measuring the absorbance at the same time after KI had been mixed with the calibration standards. There were some indication that the krill homogenates did not result in the same concentrations when diluted, but this could be compensated for by adjusting the dilution factor according to density in the homogenate.

The $MDA(TBA)_2$ derivate was detected as a clear, defined peak in the chromatogram in all samples and calibration standards. The derivatized krill samples were found as stable for at least 11 hours. The within-day and between-day variation were both 19%. The instrumental limit of quantification was 0.28 µM

Normalized AOPP and MDA levels were higher in spring and autumn krill frozen directly after capture, compared to the krill participating in the exposure study within the seasons. These differences could be due to the feeding of the animals when kept in the laboratory. The difference between T0 and T1 control krill demonstrates how important it is to keep control krill under the same conditions as the exposed krill. Seasonal variations were detected as the spring T1 MDA levels significantly lower than autumn and winter T1 levels. In addition, the MDA levels for T0 autumn krill were higher than T0 spring and winter krill, and the AOPP levels for T0 winter krill were lower than T0 spring and autumn krill. However, the differences in biomarker levels between each of the exposure treatments and the control were small. It could be that the potential increases in biomarkers were too subtle to be detected within the natural variation of a season, or simply that the krill had high tolerance for produced water exposure of the oil concentrations in the experiment.

Of the two methods, AOPP seems like the most promising method to implement for future monitoring of oxidative stress due to its precision, simplicity and efficiency in large numbers of samples analyzed each day. The existing AOPP method could be used, with only a small modification using spare material to ensure that the levels would stay above LOQ for both total protein and AOPP. Neither of the methods seem to be able to detect oxidative stress effects in krill from exposure to produced water for eight days though.

7 References

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Appendices

A.1 Homogenization

Table A-1 ***Total protein content of krill homogenate centrifuged one or two times****. The plates were analyzed 13th of October 2014 and 15th of October 2014, both with coefficient of determination R2 of 0.996. n=3*

Number of centrifugations	*Krill ID*	*Day 1 (mg/mL)*	*Day 3 (mg/mL)*	*Relative Protein Content Day 3 (%)*
One	Krill13T	9,09	5,48	60,3
One	Krill14T	6,88	3,30	48,1
One	Krill15T	11,97	4,43	37,0
One	Krill16T	7,04	4,44	63,1
Two	Krill17T	8,32	4,91	59,0
Two	Krill18T	8,38	6,35	75,8
Two	Krill19T	8,29	6,06	73,1
Two	Krill20T	5,93	4,89	82,5

Table A-2 ***Total protein content of krill homogenate kept in refrigerator.*** *The plates were analysed October 27, 2014 to October 30, 2014, and day 1 is the same day they were taken out of the -80 °C freezer. n=3*

Day	*Krill31 (mg/mL)*	*Krill32 (mg/mL)*	*Krill33 (mg/mL)*	*Krill34 (mg/mL)*	*Krill35 (mg/mL)*	*Corr. coeff. R2:*
1	7,49	8,69	6,39	5,38	12,07	0,998
2	5,03	8,23	4,78	4,22	10,11	0,992
3	4,37	5,84	4,22	3,47	9,36	0,994
4	3,92	4,37	3,11	3,19	8,87	1,000

Table A-3 ***Total protein of diluted krill homogenate kept in refrigerator****. The plates were analysed October 20,2014 to October 30, 2014, and day 1 is the same day they were taken out of the -80 °C freezer. n=3 unless specified otherwise. R2 ≥ 0.992.*

Day	*Total protein content (mg/mL)*				*Relative protein content (%)*				*Mean*	*SD*
	Krill22	*Krill23*	*Krill24*	*Krill25*	*Krill22*	*Krill23*	*Krill24*	*Krill25*	*(%)*	*(%)*
1	6,44	7,38	6,96	12,32	100	100	100	100	100	0
2	4,79	5,43	5,00*	9,31*	74,5	73,6	71,7*	75,6*	73,8	1,6
3	4,84	6,13	5,73	10,08	75,1	83,1	82,2	81,8	80,6	3,7
8	3,45	4,20	3,81	7,50	53,6	57,0	54,7	60,9	56,5	3,2
9	3,89	4,60	4,17	7,94	60,4	62,3	59,9	64,5	61,8	2,1
10	3,53	3,97	3,67	6,77	54,8	53,8	52,7	54,9	54,0	1,0
11	3,62	4,09	4,05	6,81	56,3	55,3	58,1	55,3	56,2	1,3

* n=2 due to bubbles in well.

Table A-4 ***The within-day variation of the total protein content in krill homogenate****. The plate was analyzed January 26,, 2015. n=1 for each measurement no.*

Measurement No.	*Krill50T (mg/mL)*	*Krill73T (mg/mL)*	*Krill74T (mg/mL)*	*Corr. coeff. R2*
1	4,62	4,20	2,45	0,987
2	5,10	4,35	2,88	0,987
3	4,58	4,46	2,75	0,987
4	4,69	4,45	2,86	0,987
Mean (mg/mL)	4,75	4,36	2,73	
SD (mg/mL)	0,24	0,12	0,20	
CV (%)	5,0	2,8	7,2	

Table A-5 ***The between-day variation of the total protein content in krill homogenate****. The plates were analyzed October 27, 2014 to October 30, 2014. n=3 for each parallel.*

Day	*Krill36 (mg/mL)*	*Krill37 (mg/mL)*	*Krill38 (mg/mL)*	*Krill39 (mg/mL)*	*Corr. coeff. R2:*
1	7,27	6,46	5,90	5,77	0,998
2	8,55	7,25	6,26	6,59	0,992
3	9,52	7,55	6,82	6,70	0,994
4	8,57	7,48	6,81	6,93	1,000
Mean (mg/mL)	8,48	7,19	6,45	6,50	
SD (mg/mL)	0,93	0,50	0,45	0,50	
CV (%)	11	7,0	7,0	7,8	

Table A-6 ***The calibration curve parameters for total protein content****. Absorbance at 595 nm. The residual mean, SD and CV are calculated from absolute values of the residuals. n=36 microplates. y = bx + a.*

Variable	*Mean*	*SD*	*CV (%)*	*Min*	*Max*
R2	0,996	0,003	0,3	0,985	1,000
a	0,361	0,014	3,8	0,342	0,400
b	0,398	0,024	6,1	0,335	0,433
Blank ABS	0,377	0,015	3,9	0,358	0,410
Cal 2 ABS	0,419	0,013	3,2	0,400	0,448
Cal 3 ABS	0,464	0,013	2,7	0,442	0,495
Cal 4 ABS	0,599	0,014	2,3	0,571	0,642
Cal 5 ABS	0,706	0,020	2,9	0,642	0,753
Cal 6 ABS	0,848	0,027	3,2	0,782	0,903
Cal 2 Residual (%)	6,5	5,5	84	-16	18
Cal 3 Residual (%)	9,4	3,8	41	-4,1	20
Cal 4 Residual (%)	5,1	3,4	66	-14	3,8
Cal 5 Residual (%)	2,4	2,0	84	-6,8	6,4
Cal 6 Residual (%)	1,4	1,1	77	-2,7	3,8

Table A-7 ***Overview of some homogenization methods from literature.***

Method	*Sample type*	*Weight to volume*	*Buffer*	*pH*	*Centrifugation*	*Analysis*
IRIS method (this thesis) [23]	Krill thorax	1:4 (1 g krill to 4 mL buffer)	Phosphate buffer (0.1 M)	7,2	12 000 g for 20 min, two times	MDA and AOPP
Tremblay et al. [54]	Krill	100 mg krill in 2 volumes buffer	Phosphate buffer (50 mM) with 50 mM EDTA, 1 mM PMSF	7,5	1 500 g for 20 min	TBARS
Bargu et al. [19]	Bulk of whole krill	1:1 (50 g krill and 50 g distilled water)	Distilled water.	-	No centrifugation	Domoic acid toxin analysis
Dorts et al. [89]	Bulk of shrimp hepatopancreas and gills	1:5 (w:v)	Phosphate buffer (50 mM) with protease inhibitor cocktail	7,5	No centrifugation	MDA
Mendes et al. [49]	Minced fish (hake, sea bream or sardine)	1:2 (15 g fish and 30 mL solution)	TCA solution (7.5% (p/v) TCA, 0.1% (p/v) EDTA, 0.1% (p/v) propyl gallate)	-	Filter paper prior to 6 000 rpm for 10 min	MDA
Paital and Chainy [95]	Mud crab hepatopancreas and abdominal muscle tissue (separately)	10% (w/v) or 20% (w/v)	50 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, 150 mM KCl, 1 mM PMSF and 0.5 mM sucrose	7,8	1 000 rpm for 10 min	MDA
Hussein et al. [96]	Rat brain	1:5 (1 g tissue in 5 mL buffer)	Phosphate buffer (0.02 M)	7,4	4000 rpm for 15 min	MDA and AOPP
Yanar et al. [66]	Rat brain (hippocampus, frontal lobe and parietal lobe separately)	10% (w/v)	Potassium phosphate buffer (0.1 M) with 0.1% (w/v) digitonin	7,4	5 000 g for 10 min	AOPP
Mateos et al. [97]	Rat liver	1:5 (0.5 g liver and 2.5 mL buffer)	Trizma base buffer (0.25 M) with 0.2 M sucrose and 5 mM DDT	7,4	10 000 g for 30 min	MDA
Altan et al. [67]	Rat liver	20% (w/v)	20 mM Tris-HCl	7,4	5 000 g for 10 min	AOPP
Benedetti et al. [69]	Rat liver	1:10 (100 mg tissue in 1 mL buffer)	0.25 M Tris, 0.2 M sucrose, 5 mM DTT	7,4	yes (no details)	MDA
Benedetti et al. [69]	Rat liver	1:10 (100 mg tissue in 1 mL buffer)	20 mM PBS	7,4	yes (no details)	AOPP

A.2 AOPP

Table A-8 ***Effect of storage of KI on AOPP measurements****. The ABS were measured on three sets of calibrators (all in triplicate samples) on plate using three different KI solutions; one KI that had been stored (protected from light exposure) for about three weeks, one KI that had been stored for about one week, and a freshly made KI. The slope of the measurements over time were found between the initial measurement (at 3 minutes) and at 27 minutes. The measurements (n=3 different plates, with the three sets of KI on each) were all done without aluminium foil. R2 ≥ 0.996.*

	Three weeks old						*One week old*						*Fresh*					
	Initial (3 minute) ABS			*ΔABS/min*			*Initial (3 minute) ABS*			*ΔABS/min*			*Initial (3 minute) ABS*			*ΔABS/min*		
	Mean	*SD*	*CV (%)*	*Mean*	*SD*	*CV (%)*	*Mean*	*SD*	*CV (%)*	*Mean*	*SD*	*CV (%)*	*Mean*	*SD*	*CV (%)*	*Mean*	*SD*	*CV (%)*
Blank	0,040	0,002	5,6	0,00178	0,00020	11	0,040	0,002	4,1	0,00139	0,00018	13	0,037	0,001	2,0	0,00126	0,00039	31
Cal 1	0,088	0,006	6,5	0,00245	0,00037	15	0,090	0,005	5,1	0,00196	0,00014	7,2	0,088	0,006	6,6	0,00197	0,00016	7,9
Cal 2	0,135	0,013	9,5	0,00304	0,00045	15	0,141	0,010	7,0	0,00232	0,00012	5,3	0,136	0,016	12	0,00237	0,00012	5,0
Cal 3	0,227	0,034	15	0,0034	0,0011	33	0,242	0,023	9,4	0,00219	0,00025	12	0,235	0,030	13	0,00242	0,00025	10
Cal 4	0,417	0,067	16	0,0031	0,0021	67	0,415	0,041	9,9	0,00172	0,00070	41	0,423	0,091	21	0,0020	0,0013	64
Cal 5	0,62	0,11	18	0,0019	0,0032	163	0,585	0,050	8,5	0,0012	0,0012	104	0,61	0,15	24	0,0013	0,0028	214
Cal 6	0,79	0,12	15	0,0019	0,0039	202	0,767	0,083	11	0,0012	0,0022	185	0,77	0,18	23	0,0014	0,0038	280
Cal 7	0,98	0,18	19	0,0013	0,0055	417	0,984	0,082	8,3	-0,0011	0,0018	-172	0,94	0,21	22	0,0009	0,0049	536

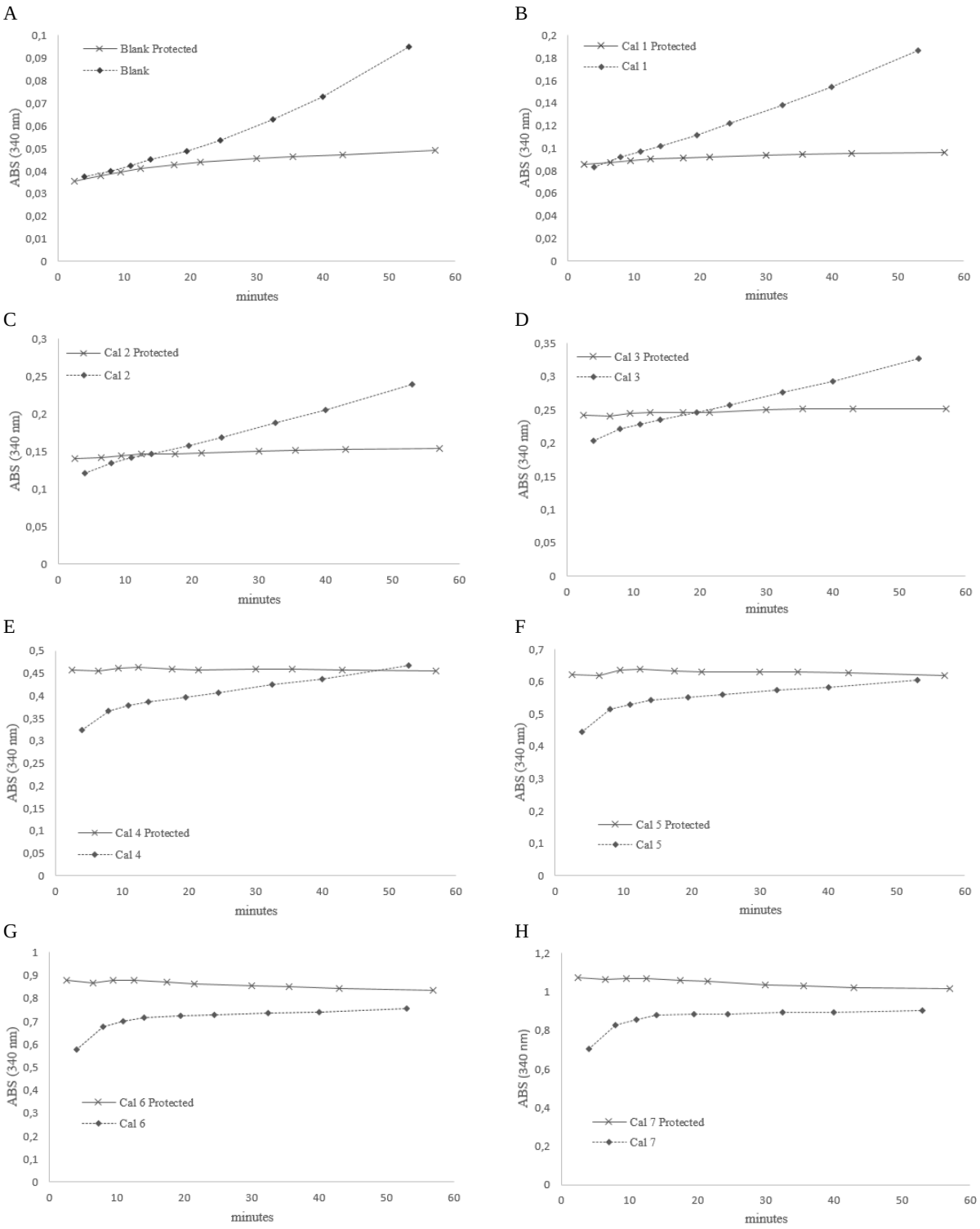

Figure A-1 ***Absorbance at 340 nm for AOPP calibration standards and blank with and without protection from light-exposure****. The 'Protected' curves were covered with aluminium foil once KI (freshly made) had been added, the others not. 'A' to 'H' show blank and chloramine-T calibration standard 1 (Cal 1) to chloramine-T calibration standard 7 (Cal 7) respectively, with combined graphs for light-protected and not protected plates. The light-protected plate was measured December 19, 2014, and the plate not protected was measured December 17, 2014. n=3 for each data point.*

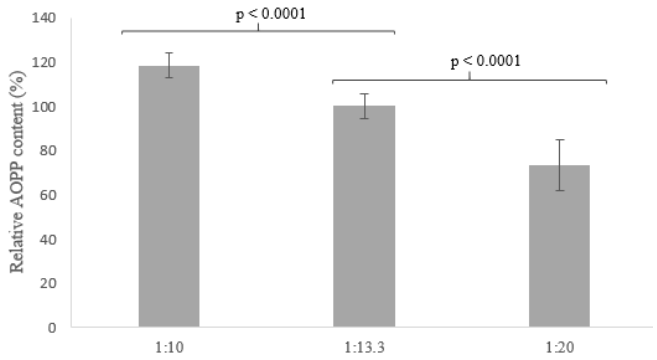

Figure A-2 ***Relative AOPP concentration in diluted krill homogenate in PBS****. The measurements were done 8 minutes after KI was added.. Relative AOPP content is calculated from the 1:13.3 dilution. (Error bars indicate ±SD, n=8 for 1:10 and n=18 for 1:13.3 and 1:20). Significant differences are marked with p < 0.05*

Table A-9 ***The calibration curve parameters for AOPP concentration****. Absorbance was read at 340 nm, 7 to 11 minutes after KI was added. The residual mean, SD and CV are calculated from absolute values of the residuals. n=21 microplates. y = bx + a.*

Variable	*Mean*	*SD*	*CV (%)*	*Min*	*Max*
R2	0,998	0,001	0,1	0,994	1,000
a	0,053	0,007	13	0,038	0,067
b	0,0096	0,0005	5,3	0,0087	0,0109
Blank ABS	0,039	0,002	5,5	0,034	0,042
Cal 1 ABS	0,092	0,003	3,1	0,087	0,098
Cal 2 ABS	0,141	0,005	3,2	0,135	0,153
Cal 3 ABS	0,236	0,011	4,6	0,219	0,262
Cal 4 ABS	0,421	0,019	4,5	0,386	0,461
Cal 5 ABS	0,605	0,025	4,1	0,557	0,653
Cal 6 ABS	0,783	0,044	5,6	0,714	0,880
Cal 7 ABS	0,951	0,046	4,8	0,879	1,069
Cal 1 Residual (%)	14	11	75	-2,4	39
Cal 2 Residual (%)	3,9	3,7	96	-4,5	14
Cal 3 Residual (%)	2,4	2,4	100	-8,4	6,9
Cal 4 Residual (%)	2,1	1,3	61	-4,3	3,3
Cal 5 Residual (%)	2,3	1,6	67	-6,3	3,8
Cal 6 Residual (%)	1,8	1,2	71	-4,7	2,7
Cal 7 Residual (%)	1,6	1,2	75	-1,4	4,1

A.3 MDA

Table A-10 ***The order of injection of MDA derivatized samples****. 20 unique samples. The 'Control 1' sample was injected at three different occasions; in the beginning, middle and at the end. The calibration standards 5 to 7 were injected at two different occasions; in the beginning and at the end. Calibration standard 2 were added to the set later, replacing a krill homogenate sample, to not change the difference in time between the samples injected on multiple occasions. n=1, but all samples injected two times.*

Sample	*Injections*
Blank	2
Cal 3	2
Cal 4	2
Control 1	2
Cal 5	2
Cal 6	2
Cal 7	2
Cal 8	2
Cal 9	2
Control 1	2
Control 2	2
Cal 2	2
Sample 1	2
Sample 2	2
Sample 3	2
Sample 4	2
Sample 5	2
Sample 6	2
Sample 7	2
Sample 8	2
Sample 9	2
Control 1	2
Cal 5	2
Cal 6	2
Cal 7	2

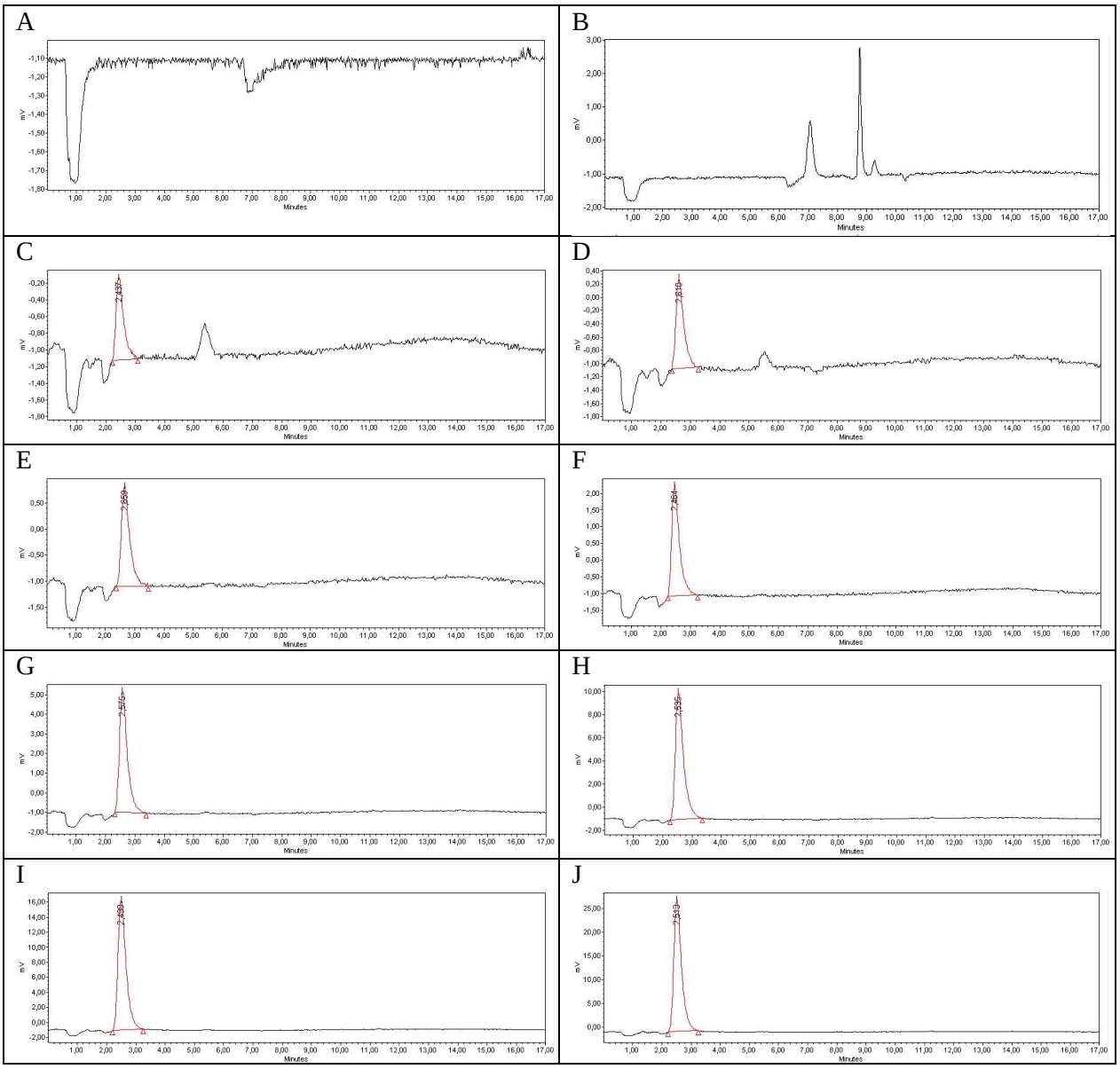

Figure A-3 ***Chromatograms for MDA calibration standards with fluorescence detector (ex/em 526 nm/560 nm)****. 'A' is the chromatogram for Std1 (0 µM) injection 1, 'B' is the chromatogram for injection 2 of Std1, and 'C' to 'J' are the chromatograms for injection 2 for Std 2 (0.275 µM), Std 3 (0.551 µM), Std4 (1.102 µM), Std 5 (2.203 µM), Std 6 (4.406 µM), Std 7 (8.813 µM), Std 8 (13.770 µM) and Std 9 (22.032 µM) respectively.*

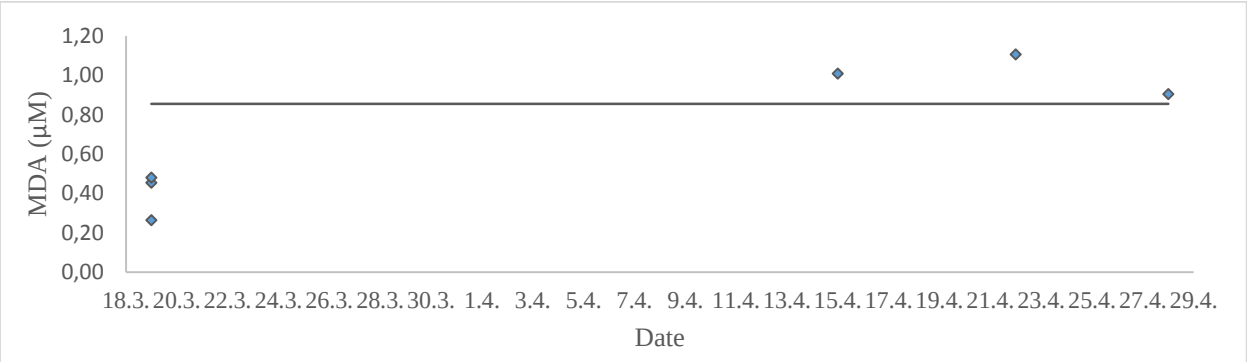

Figure A-4 ***MDA in human plasma control****. The overall mean MDA (0.856 µM) based on the four daily means is shown as a straight line. The first sample set contained three plasma samples, the other three sample sets contained one plasma sample each. n=4 sample sets.*

Table A-11 ***MDA stability over time.*** *Area under curve registered for six MDA calibration standards, and five krill homogenates. The area at time 0 was defined as 100%, and the areas at time 30 hours and 43 hours were calculated relative to time 0. The values are a mean of the two injections of each sample (n=1). The standards used were not the final method standards.*

	*MDA Area (µV * sec)*			*MDA relative area (%)*		
Hours (h)	*0*	*30*	*43*	*0*	*30*	*43*
Std2	11 983	16 723	21 577	100	139,6	180,1
Std4	32 916	47 989	50 927	100	145,8	154,7
Std5	37 242	61 299	76 416	100	164,6	205,2
Std5_2	60 662	90 290	87 176	100	148,8	143,7
Std6	103 723	133 685	119 371	100	128,9	115,1
Std8	260 365	334 689	282 410	100	128,5	108,5
Sample1	113 569	139 058	144 155	100	122,4	126,9
Sample2	84 273	92 445	97 535	100	109,7	115,7
Sample3	74 827	91 834	91 811	100	122,7	122,7
Sample4	77 682	90 493	92 008	100	116,5	118,4
Sample5	94 221	103 288	100 805	100	109,6	107,0

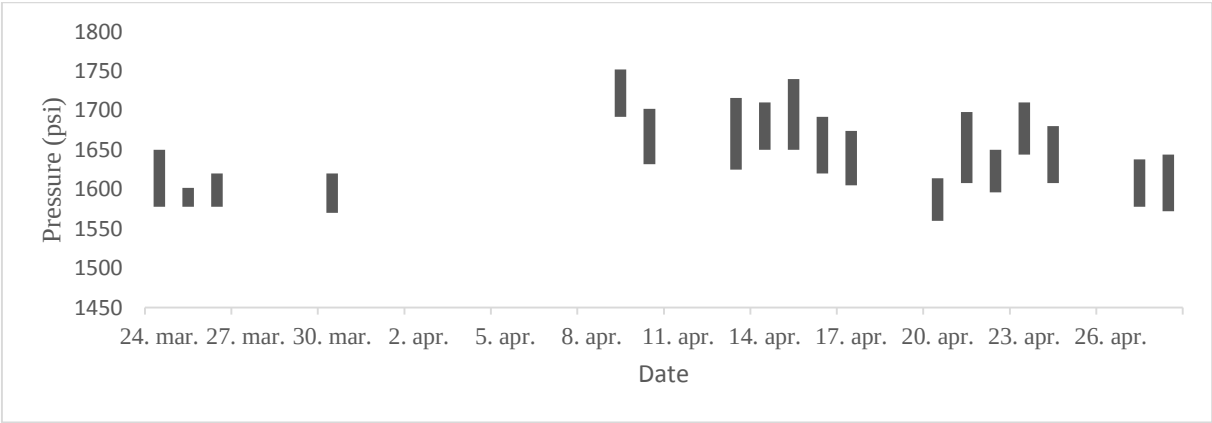

Figure A-5 ***Pressure profile HPLC****. Variation in pressure registered prior to injection.*

Table A-12 ***The calibration curve parameters for MDA****. The residual mean, SD and CV are calculated from absolute values of the residuals. n=21 sample sets.*

Variable	*Mean*	*SD*	*CV (%)*	*Min*	*Max*
R2	0,997	0,003	0,3	0,989	1,000
a	12 699	5 814	46	1 329	20 164
b	21 832	1 543	7,1	17 976	24 500
Blank Area (µV * sec)	0	N/A	N/A	0	0
Cal 2 Area (µV * sec) [a]	21 917	3 114	14	15 034	26 068
Cal 3 Area (µV * sec)	28 147	4 796	17	18 166	35 442
Cal 4 Area (µV * sec)	39 685	5 101	13	26 673	46 973
Cal 5 Area (µV * sec)	64 477	5 250	8,1	53 250	72 028
Cal 6 Area (µV * sec)	115 962	9 976	8,6	94 772	146 740
Cal 7 Area (µV * sec)	219 462	12 655	5,8	192 209	246 664
Cal 8 Area (µV * sec)	328 895	22 629	6,9	289 207	393 281
Cal 9 Area (µV * sec)	505 591	37 122	7,3	443 879	581 187
Cal 2 Conc (µM) [a]	0,28	0,01	4,6	0,27	0,32
Cal 3 Conc (µM)	0,57	0,02	4,1	0,54	0,64
Cal 4 Conc (µM)	1,13	0,05	4,1	1,07	1,29
Cal 5 Conc (µM)	2,27	0,09	4,1	2,15	2,58
Cal 6 Conc (µM)	4,54	0,19	4,1	4,29	5,16
Cal 7 Conc (µM)	9,07	0,37	4,1	8,59	10,3
Cal 8 Conc (µM)	14,2	0,6	4,1	13,4	16,1
Cal 9 Conc (µM)	22,7	0,9	4,2	21,5	25,8
Cal 2 Residual (%)[a]	28	33	118	-130	43
Cal 3 Residual (%)	23	16	70	-6,7	59
Cal 4 Residual (%)	9,1	7,6	83	-5,0	33
Cal 5 Residual (%)	5,0	5,4	108	-23	12
Cal 6 Residual (%)	4,8	3,6	76	-11	14
Cal 7 Residual (%)	4,4	5,4	124	-8,5	19
Cal 8 Residual (%)	2,7	3,2	118	-3,7	16
Cal 9 Residual (%)	1,5	1,2	79	-4,6	1,7

[a] Sample set n=16.

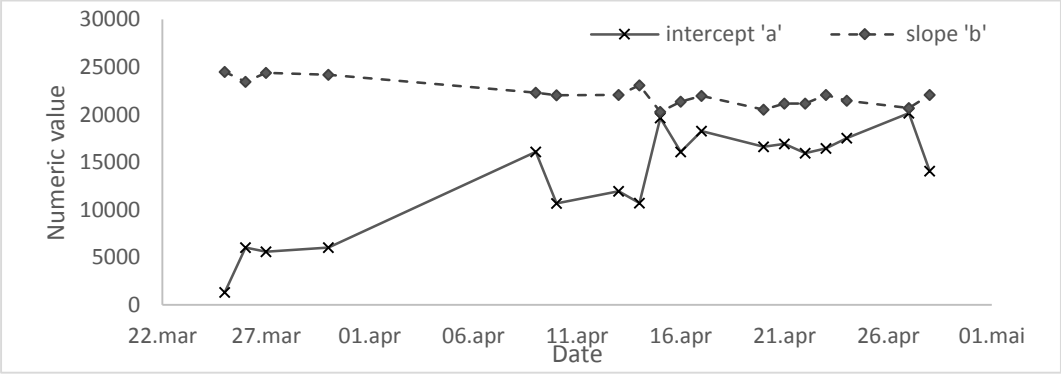

Figure A-6 ***MDA regression line 'a' and 'b' over time.***

A.4 Oxidative Stress in Krill Exposed to Oil

Total Protein

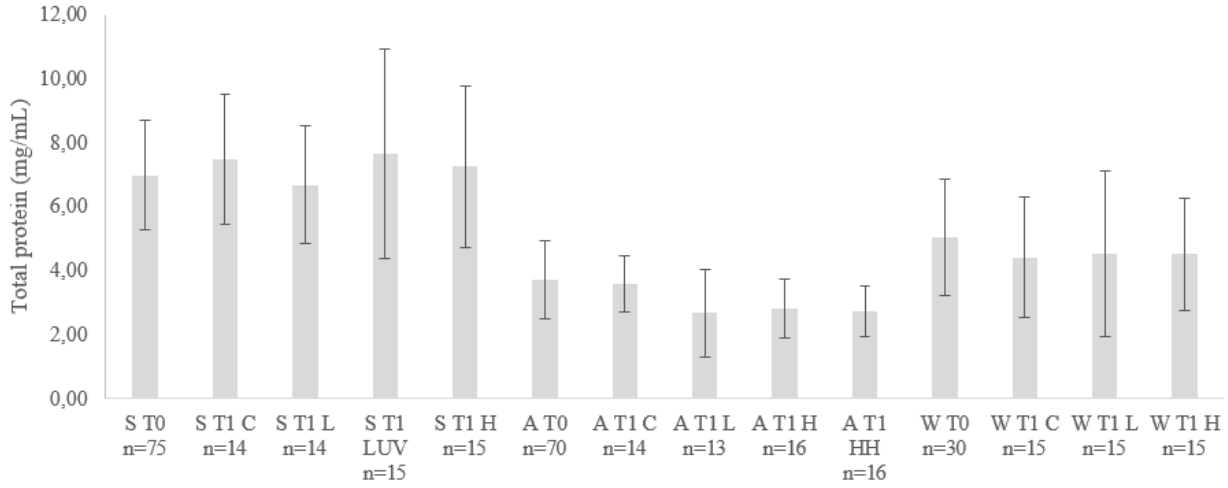

Figure A-7 ***Total protein (mg/mL) in every treatment and for each season****. Seasons 'S' spring, 'A' autumn and 'W' winter, and treatments 'T0' frozen directly after capture, 'T1' kept in laboratory, 'C' control (0 ppm oil), 'L' low (0.015 ppm oil), 'LUV' low UV treated (0.015 ppm oil), 'H' high (0.15 ppm oil) and 'HH' high high (1 ppm oil for 2 days). (Error bars indicate ±SD).*

AOPP

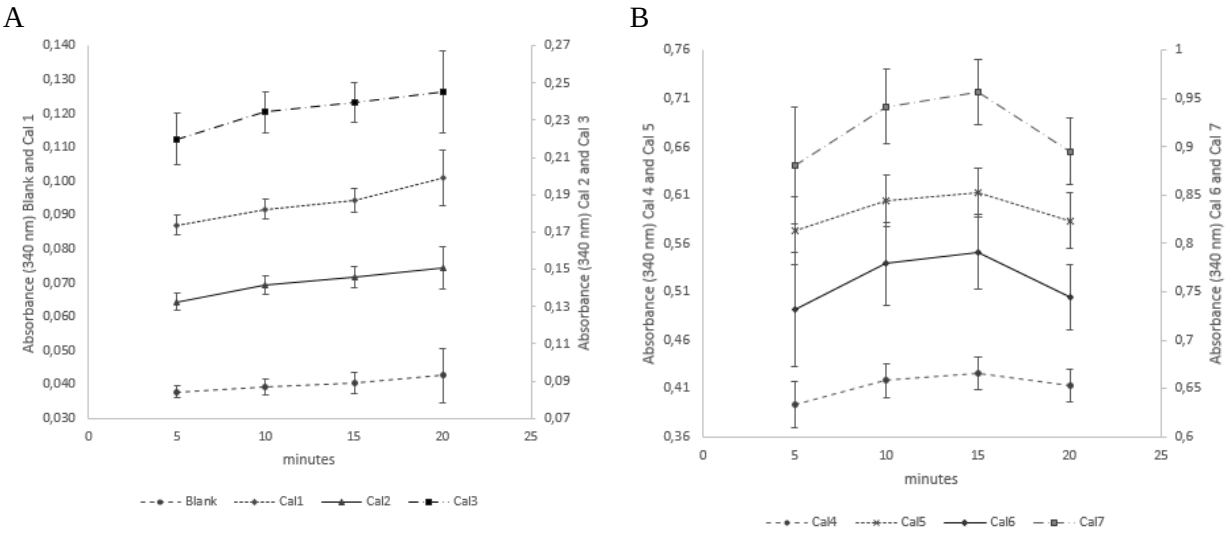

Figure A-8 ***AOPP absorbance (340 nm) for blank and chloramine-T calibration standards over time****. 'A' shows the blank and the lower three calibration standards Cal 1 to Cal 3, and 'B' shows the higher concentration calibration standards Cal 4 to Cal 7. Both 'A' and 'B' show four curves, where the primary y-axis (left) is for the lower two, and the secondary y-axis (right) is for the higher two. n=15 for at 5 minutes, n=16 at 10 and 15 minutes, and n=3 at 20 minutes.*