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MASTEROPPGAVE

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1.1 Summary

The North Sea is becoming heavily contaminated by organic pollutants. The dominating sources of contamination are land-based releases, transport and offshore oil and gas industry. This creates a complex spectrum of different persistent contaminants accumulating in the fish in the North Sea. The dominating pollutants are Persistent Organic Pollutants (POPs) such as organic chlorinated (PCB, DDT), brominated (PBDE) and fluorinated (PFOS) compounds from industrial waste products. There are also significant discharges of hydrocarbons like poly aromated hydrocarbons (PAH) and alkylphenols (AP) from the offshore oil production and oil spills. Because organic chemicals are lipophilic they have the ability to bioaccumulate in the fatty tissue and the cell membranes in organisms making them persistent and a severe threat to the marine environment.

In this study, the effect of chlorinated, brominated and fluorinated pollutant chemicals as well as crude oil and alkylphenols, upon the mitochondrial membrane in Atlantic cod have been investigated. Fish are the most at threat from aquatic pollution and together with their long-term exposure in natural habitat they are suitable biomonitores of environmental pollution and the toxic effects. Liver is known to be a metabolic active organ and performs a number of important and complex biological functions like energy metabolism that is essential for survival. It also provides the liver cells with large amount mitochondria. In cod, this organ consists of about 70% fat, making in a natural habitat for organic pollutants entering the organism. It also plays a major role against the toxic compounds induced free radical damage by virtue of having a variety of antioxidants.

Liver samples were collected from both exposed cod and control cod. From these samples mitochondria were isolated using centrifuge techniques. The presence of mitochondria in the pellet fraction was confirmed by the assay of cardiolipin (CL) by thin layer chromatography (TLC) and citrate synthase. From the isolated mitochondria, membrane lipids were extracted and the different classes of phospholipids were separated by TLC. The fatty acid analyzes of

the selected phospholipid classes CL, phosphatidyethanolamine (PE) and phosphatidylcholin (PC) were performed using gas chromatography (GC).

Biomarker analyses related to oxidative stress and antioxidants; Superoxide dismutase (SOD), catalase and cytochrome c oxidase (CytC oxidase), was to be examined using Enzyme-linked immunosorbent assay (ELISA). Western Blot analyses were made to verify the antibodies, but because of unreliable results alternative methods for identifying oxidative stress were used. Lipid peroxidation was therefore examined with malondialdehyde (MDA) assay. As this method also involved some uncertainties, an additional method, carbonyl assays, measuring protein oxidation was included.

The lipid peroxidation and carbonyl assays indicated that there was no oxidative damage, but additional examination is needed to verify the results. The fatty acid composition did not seem to alter significantly in any of the treatments and correlates with the findings in the oxidative damage study. Due to a limited amount of parallels, and great variations within the group, further study is needed before a conclusion is made.

Keywords: Mitochondria, Cardiolipin, phosphatidyethanolamine, phosphatidylcholine,

Oxidative stress, POP

Contents

Abbreviations

ADP Adenosine diphosphate

AP Alkylphenols

ATP Adenosine triphosphate

DDT Dichloro-Diphenyl-Trichloroethane

CL Cardiolipin

CytC Cytochrome C

ER Endoplasmatic reticulum

FA Fatty Acid

G3P Glycerol-3-phosphate

GC Gas Chromatograph

HRP Horse radish peroxides

OECD Organization for economic co-operation and development

MDA Malondialdehyde

MS Mass Spectrometry

MUFA Monounsaturated FA

MeOH Methanol

PA Phosphatidic acid

PAH Polyaromatic hydrocarbon

PAGE Polyacrylamide gel electrophoresis

PBDE Polybrominated Diphenyl Ethers

PC Phosphatidylcholin

PCB Polychlorinated biphenyls

PE Phosphatidylethanolamine

PFOS Polyfluooctane sulphonic acid

PG Phosphatidylglycerolphosphate

PGPS PGP synthase

PL Phospholipid

POP Persistent organic pollutants

PUFA Polyunsaturated FA

PVDF Polyvinylidene fluoride

ROS Reactive oxygen species

RSD Relative standard derivation

SDS Sodium dodecyl sulfate

SD Standard derivation

SFA Saturated FA

SOD Superoxid Dismutase

TLC Thin Layer Chromatorgaphy

1.2 Background and aim of the study

The Marine research institute have accomplished many successful studies on the effects of persistent organic pollutants such as AP's and PAH's from offshore oil production on Atlantic cod (Gadus morhua) (Lie et al 2009; Meier et al. 2007; Boitsov et al.2007; and Bohne-Kjersem et al. 2009). With focus on fatty acid composition, protein expression and biomarkers, important knowledge about the hazardous effects has been discovered. Because pollutants are capable of movement over considerable distances, changing between different elements including water, air, soil and biota, oil and gas industry are not the only contributors to pollutant chemicals. Transport and land-based releases from industry and discharged waste play an important part in the man-made emission of hazardous chemicals threatening the organisms and the ecosystem.

A pilot study involving mixtures of different chemicals from transport, land-based releases and crude oil, was designed. The chemicals are all expected to be found in our waters and a more realistic picture of the complex nature of pollutants is created. As a part of this study, the overarching objective of this thesis is to generate new knowledge about the long term effects on lipid composition in Atlantic cod mitochondria caused by these complex mixtures of POP's, with focus on CL as well as PE and PC. A 50 fold of the occurring concentrations of the pollutant chemicals were given to the fish to reflect the long term effect as these chemicals have the ability to accumulate in the organism. Because many of the toxicants that are involved in this study are known to induce oxidative stress, in addition to lipid analysis, a biomarker study was included to create a better understanding of the mechanisms in action, this including the antioxidant proteins catalase, SOD and CytC oxidase. A detection method for lipid and protein oxidative damage (MDA and Carbonyl) was included as an as an alternative to the antioxidants.

CL also has a tight connection with Cyt C in the mitochondria. The release of Cyt C from the mitochondria is a necessary requirement to initiate cell apoptosis and may be induced by reactive oxygen species (ROS) (Petrosillo et al 2003).

2.1 Lipid composition and Metabolism

Mammalian cell membranes contain around 1,000 different phospholipids. This large mixture of phospholipids species is primarily the result of the distinct fatty acyl chains esterified to the sn-1 and sn-2 positions of the glycerol backbone as well as the different polar head groups attached to the sn-3 position of the glycerol backbone. Phospholipids were, for many years, thought to play a primarily structural role in biological membranes. The amounts of the various phospholipids in a membrane define the fluidity of the membrane and, consequently, the functions of the embedded proteins. Studies preformed in the last 20 years have revealed, however, that these lipids mediate important regulatory functions in cells, partly because of their important roles in biological processes such as apoptosis and cell signaling (Vance et al 2010) which is regulated by their conversion into key lipid second messengers (Berridge et al. 1984; Nishizuka et al.1986). Phospholipids also have a functional impact on the membrane bound enzymes. Cardiolipin located in the inner membrane of the mitochondrion plays an important role in the regulation of Cytochrome C Oxidase activity, ATP-ase and adenine nucleotide translocase levels (Schlame et al. 2000).

2.1.1 Mitochondrion

The Mitochondrion is an organelle percent in almost all the cells found in eukaryotic organisms (Alberts 2002). Descendent from bacteria, the mitochondria has its own DNA and divides by binary fission (division into two parts which each have the potential to grow to the size of the original cell) similar to bacterial cell division, but unlike bacteria, the mitochondria can also fuse with other mitochondria (Chen et al. 2009; Hermann et al 1998). The organelles consist of two highly specialized membranes creating two separate mitochondrial compartments; the internal matrix and the intermembrane space. Because of its unique structure, the mitochondrion has the ability to metabolize acetyl groups via the citric acid cycle found in the intermembrane space, producing CO_2, NADH and$FADH_2$, and release energy to power the ATP synthase (Alberts 2002).

Mitochondrial membrane biogenesis requires the import and synthesis of proteins as well as phospholipids. How the mitochondrion regulates phospholipid levels and maintains a tight protein-to-phospholipid ratio is not fully understood. The lipid composition contains the major classes of phospholipids found in all cell membranes, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA). Mitochondria also contain Phosphatidylglycerol (PG) and cardiolipin (CL), which are predominantly if not exclusively found here (Zinser et al. 1991). The presence of CL and the absence of cholesterol in the fish mitochondria are likely to contribute to the membrane flexibility (Ballantyn 2004). PE, PG and CL are synthesized in the mitochondria whereas the others are synthesized in the endoplasmatic reticulum (ER) and must be imported (Shlame et al 1997).

2.1.2 Metabolism

The citric acid cycle or Krebs cycle, is found in the mitochondria and includes a series of oxidation and reduction reactions. The end product is a result of the oxidation of an acetyl group creating two molecules of carbon dioxide as a waste product and three molecules of NADH, which is used in the electron-transport chain. Krebs cycle is always followed by oxidative phosphorylation in aerobic conditions (Alberts 2002; Berg et al. 2000).

As a high-energy electron is passed along the electron-transport chain, some of the energy released is used to drive the three respiratory enzyme complexes that pump H^+ out of the matrix. It is the complexes I, III and IV coupled to the electron transport chain reactions that generate the electrochemical proton gradient across the mitochondrial inner membrane. (Eble et al. 1990). The enzyme Cytochrome C oxidase, also known as the Complex IV, is a large transmembrane protein found in the mitochondrion. It is the last enzyme in the respiratory electron transport chain of mitochondria, converting the molecular oxygen to two molecules of water. Complex IV has been shown to require two associated CL molecules in order to maintain its full enzymatic function

(Shlame et al. 2000) The production of energy in the mitochondrial innermembrane is a result of the Krebs cycle, but also of oxidative phosphorylation. This metabolic pathway utilizes energy released by redox reactions and the proton gradient in the earlier stages to synthesize ATP by complex V, the mitochondrial F_1F_0-ATP synthase from adenosine diphosphate (ADP) to ATP (Alberts 2002)

2.1.3 Cardiolipin

Cardiolipin (CL) is found almost exclusively in the mitochondrial inner membrane where it contributes to about 20% of the total lipid composition. It is a unique phospholipid synthesized from phosphatidylglycerol and cytidinediphosphat-diacylglycerol, the entire CL synthesis is shown in figure 2.1.3b (Houtkooper et al 2008). As there are four distinct alkyl chains in CL (figure 2.1.3a) the potential for complexity of this molecule species is enormous. However, in most animal tissues, CL contains 18-carbon fatty alkyl chains with 2 unsaturated bonds on each of them (Alberts 2002). Because of its structure; CL is involved in many important stages of maintaining the mitochondrial membrane dynamics and providing a stabile environment for individual enzymes and enzyme complexes in the metabolic processes. CL is also thought be involved in the apoptotic processes in connection with cytochrome c (Cytc) release (Shlame et al 2000).

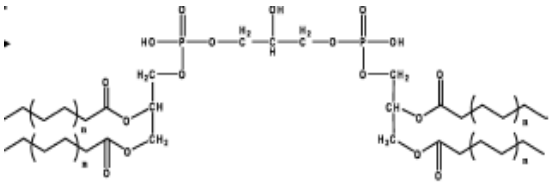


Figure 2.1.3a molecular structure of CL. (Houtkooper et al 2008).

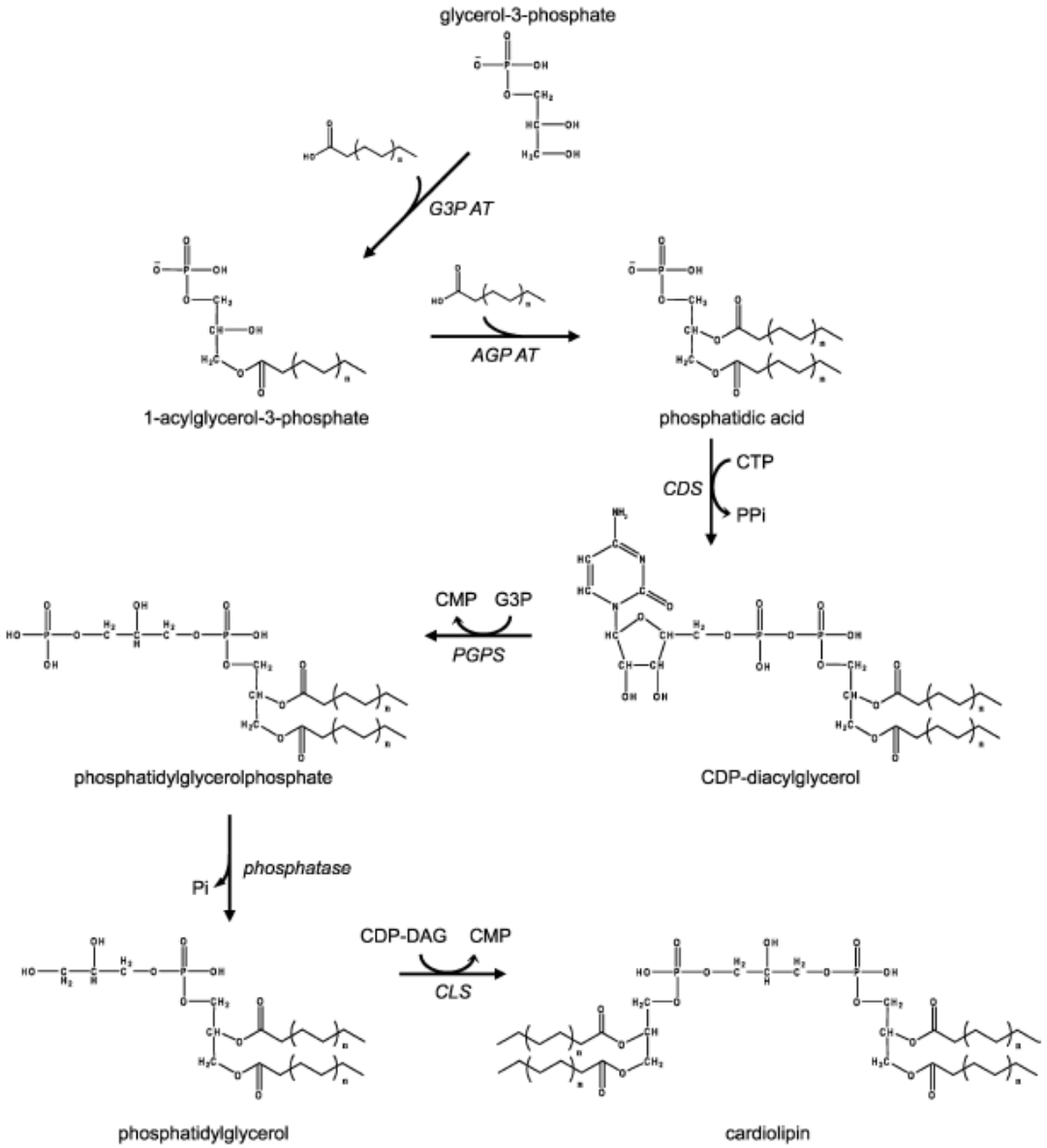


Figure 2.1.3b Cardiolipin synthesis in eukaryotes undergoes 6 steps from the common precursor of CL in biosynthesis; glycerol-3-phosphate (G3P) before it results in the formation of CL. In contrast to most phospholipid biosynthesis, which takes place in the endoplasmatic reticulum (ER), the CL biosynthesis from the end of step two (phosphatidic acid) takes place in mitochondria (Houtkooper et al 2008).

2.1.4 Cardiolipid and the metabolic pathway

By stabilizing the physical properties of membranes and its specific interactions with proteins and modulation of their functions, CL appears to have important roles in both mitochondrial structure and function (Shlame et al 2000). Newly synthesized CL undergoes a remodeling process in which saturated acyl chains are replaced with more unsaturated chains, thereby establishing a high degree of acyl chain symmetry (Shlame et al 1993). In this way CL may accommodate its structure accordingly to the cell or organelles demand.

The tight connection between CytC and CL forming a CytC - CL complex indicate an important role of CL in the metabolic processes. It also leaves the phospholipid exposed to reactive oxygen spesies (ROS). When H_2O_2 interacts with the complex, the cardiolipin peroxidase oxidizes H_2O_2 and forms H_2O, resulting in a peroxidation of CL (Kagan et al. 2005). CL peroxidation will in turn lead to a decrease in electron transport chain activity of the complexe (Petrosillo et al., 2003; Paradies et al. 2004; Lesnefsky et al. 2004). This leads to a weakening of mitochondrial complex activity which may increase electron leak from the electron transport chain, generating more superoxide anion radical and feeding the cycle of oxygen-radical-induced damage.

A novel mechanism of regulation was demonstrated by the last step in CL biosynthesis, catalyzed by CL-synthase which is stimulated by the increase in matrix pH that accompanies respiration (Gohil et al., 2004). This mechanism demonstrates the interdependence of CL biosynthesis and electron transport chain function; CL promotes supercomplex formation and cytochrome oxidase activity, which, in turn, upregulate CL synthesis. The phospholipid also seems to play an important part in stabilizing the ADP/ATP carrier. In a study preformed on yeast it was found that under optimal conditions, CL increases the efficiency of oxidative phosphorylation by at least 35% (Claypool et al 2008).

2.1.5 Phosphatidylethanolamine

The phospholipid phosphatidylethanolamine (PE) comprises > 25% of total mitochondrial phospholipids and is the second most abundant mammalian membrane phospholipid. Mitochondrial PE (figure 2.6.1) is synthesized by the Psd1p-catalyzed decarboxylation of PS (Vance et al 2010) and consists of 4 major parts; a head amino group attached to a phosphate group, a glycerol group and two hydrocarbon tails (Alberts 2002). In general, PE tends to contain higher proportions of polyunsaturated fatty acids (PUFA) than PC. In mitochondria of rainbow trout PUFA contributes to over 60% of the fatty acids with 22:6 (n-3) being the dominating PUFA. The saturated fatty acids (SFA) contribute to approximately 20% (Kraffe et al. 2007). Although CL plays a key role in many mitochondrial functions, recent evidence suggests that at least some functions overlap with those of PE. Both PE and CL have the ability to form nonbilayer structures in membranes (Gohil et al 2009). PE is also thought to play important roles in biological processes such as apoptosis and cell signaling (Vance et al 2010).

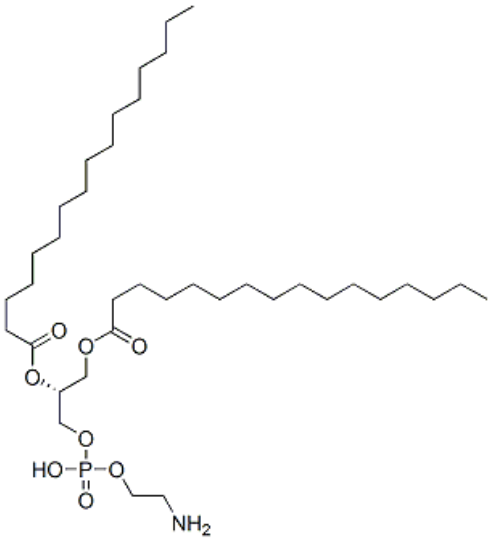


Figure 2.1.5 The chemical structure of PE.

2.1.6 Phosphatidylcholine

Phosphatidylcholine is the most common and abundant phospholipid in mammalian cell membranes, constituting 40–50% of total phospholipids. In mitochondria of rainbow trout PUFA contributes to over 50% of the fatty acids with 22:6 (n-3) being the dominating PUFA. PC also contains 30% SFA dominated by 16:0 which contributes to over 25%. (Kraffe et al. 2007). The structure is shown in figure 2.1.7 and is constructed from four major parts; A head of choline linked to a phosphate group and a glycerol group which is connected to two hydrocarbon tails of fatty acids (Alberts 2002). PC is synthesized via the choline pathway. Choline may be produced through the methylation of PE to PC catalyzed by phosphatidylethanolamine N-methyltransferase or from PC, choline is generated via the action of phospholipases (Bremer et al. 1961; Li et al. 2008).

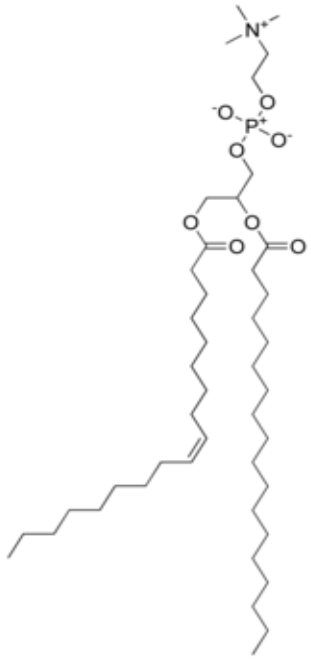


Figure 2.1.6 The chemical structure of PC.

2.1.7 The fluidity of the lipid membrane

The lipids in the membrane have the ability to move within the plane of the bilayer. This function contributes to the fluidity of the membrane and is highly dependent on the composition of the bilayer; the type of phospholipids and the number of double bonds (unsaturated) between carbon atoms in the hydrocarbon tails. The more saturated (no double bonds) and the longer the tails are the closer the packing of the hydrocarbon tails, and the less fluid and more viscous the bilayer becomes. A shorter chain reduces the tendency to interact with the other tails and increases the fluidity. The tails will wary in length between 14 and 24 carbons, with 18-20 being the most common. Most phospholipids also have one or more double bonds on one of the hydrocarbon chains and no double bonds on the other. The double bonds contribute to the membrane fluidity by creating a kink in the tail which in turn makes it harder to pack the lipids tight (Alberts 2002). Flexibility and fluidity is important for the cells ability to live, grow and reproduce. It enables membrane proteins to fuse in the bilayer and interact with other proteins. Proteins and lipids synthesized at other locations are able to fuse into the membrane in other regions of the cell and are distributed evenly in the membrane and between the mother and daughter cell at cell division (Alberts 2002).

2.1.8 External effects on membrane fluidity and environmental adaptation

Many environmental factors induce the production of reactive oxygen species (ROS). As internal temperature varies along with the ambient environmental temperature in poikiloterms, most fish must routinely cope and adapt to a great variety in temperature. This also effects the metabolic rate and causes fluctations in ROS levels (Filho et al 1993). Therefore, ROS generation, oxidation rates and antioxidant status seem to be directly related to ambient temperature or metabolic activity (Filho et al 2000).

Desaturation of fatty acids is an important adaptation mechanism for fish to maintain membrane fluidity under thermal stress. Most poikilotherms respond to thermal changes by adapting the physical properties of their membranes to the new situation to preserve the functional and structural integrity of these structures, a phenomenon that Sinensky et al termed "homeoviscous adaptation." The homeoviscous efficacy, the extent to which the cells compensate for temperature

changes, varies among the tissues and membranes (Cossins et al 1982; Lee et al 1990). Adjustment of the physicochemical properties of the membranes to the temperature is expected to be rapid and reversible to ensure proper functioning under fluctuating thermal conditions in fish (Wodtke et al 1991). Kraffe et al. 2007 have shown that the fluidity of the mitochondria in rainbow trout red muscle follows the changes in the environmental temperature (table 2.1.10). After short term and long term (8 weeks) acclimation (5°C to 15°C) it is clear that the levels of 16:0 and 18:1n-7 are significantly decreased in the warm acclimation, at the same time there is a increase to over 20% of 18:2n-6 of the total FA (Kraffe E. et al 2007).

	CL		
Fatty acids	Cold acclimated	Short-term warm exposed	Warm acclimated
16:0	8.9±0.7[a]	7.4±0.5[a]	3.2±0.2[b]
18:0	2.0±0.4	1.3±0.1	2.2±0.4
16:1n-7	1.2±0.1[a]	1.8±0.1[a]	3.2±0.4[b]
18:1n-9	7.4±0.2[a]	7.0±0.3[a]	6.1±0.2[b]
18:1n-7	6.4±0.5[a]	6.3±0.3[a]	2.4±0.2[b]
20:1n-9	2.4±0.2[a]	2.1±0.2[ab]	1.7±0.2[b]
22:1n-11	3.1±0.5	3.3±0.4	2.2±0.2
18:2n-6	13.4±0.5[a]	14.4±0.5[a]	21.4±0.6[b]
18:3n-3	1.2±0.4[a]	1.7±0.1[a]	3.1±0.2[b]
20:4n-6	0.5±0.1	0.3±0.1	0.5±0.2
20:5n-3	1.2±0.1	1.0±0.1	1.2±0.1
22:4n-6	Trace	Trace	Trace
22:5n-6	0.6±0.1	0.5±0.1	0.6±0.1
22:5n-3	2.1±0.1[a]	1.4±0.1[b]	1.7±0.1[c]
22:6n-3	44.8±0.9	44.5±1.2	43.3±0.8
Others***	5.5±0.4	5.9±0.2	5.7±0.4
Total SFA	12.3±1.3[a]	9.7±0.7[b]	6.8±0.4[c]
Total MUFA	20.4±0.7[a]	21.3±0.6[a]	15.9±0.2[b]
Total PUFA	67.2±0.7[a]	69.0±1.0[a]	77.3±0.6[b]

Figure 2.1.8 Changes in the properties of mitochondrial CL from oxidative muscle of rainbow trout *Oncorhynchus mykiss* (Kraffe et al 2007).

2.2 Oxidative Stress

Oxidative stress is a result of free oxyradicals or reactive oxygen species (ROS). ROS occurs when electrons are passed on to molecular oxygen, causing a generation of the highly reactive oxyradical such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Walker et al third edition).

All cells are dependent on the complex balance between the pro-oxidant factors and the antioxidant defense mechanisms. Oxidative stress occurs when there is a disturbance in this normal red-ox state and the production of ROS exceeds the capacity of the defense system. The cells then encounter a stress condition with subsequent damage on all components of the cell, including proteins, lipids and DNA (Walker et al. third edition; Dhalla et al. 2000). The defense system consists of a group of enzymes that are able to detoxify oxyradicals, such as superoxide dismutase, catalase and peroxidase. They are however, not necessarily able to cope with an increase in oxyradical formation caused by the action of persistent organic pollutants. The effects of ROS on a cell depend on the size of the damage. A small amount of stress is something most cells are able to overcome, and can is still regain its original state. Chronic oxidative stress however, may lead to severe inflammation conditions which may ultimately lead to cellular apoptosis, tissue degeneration and necrosis.

2.3 Oxidative biomarkers

Fish as species are on top of the aquatic food chain; as vertebrates and poikilotherms, they strongly respond environmental changes and stress conditions (Weber et al 1992). Therefore, they are often used as indicator species of pollutant exposure in the aquatic environment. There are still gaps in the knowledge of cellular damage response mechanisms, repair processes and diseases related to oxidative stress. The use of biomarkers will hopefully provide a more complete picture of the various effects in action (Almroth et al 2008). Biomarkers can be defined

as any detectable biological response to an environmental chemical, including biochemical, physiological, histological, morphological and behavioral effects (Walker et al. third edition).

2.3.1 MDA

One of the many biological targets of oxidative stress and the prime target of peroxidation is the polyunsaturated fatty acids (PUFA) in the membrane. Lipid oxidation and generation of peroxides gives rise to a number of secondary products which can decompose in to lipid hydroperoxides and malondialdehyde (MDA). MDA reflects the degree of lipids peroxidized, and is the principal and most studied product of PUFA peroxidation. This aldehyde is a highly toxic biomolecule and should be considered as more than just a marker of lipid peroxidation. Its interaction with DNA and proteins has often been referred to as potentially mutagenic and atherogenic (Gupta et al 2009). Although the method is widely utilized, the use a MDA as a marker for lipid peroxidation is controversial. MDA can be formed during eicosanoid metabolism (cellsignaling molecules) and the analytical methods for measuring MDA are exposed to artifactual errors (Janero et al 1990).

2.3.2 Carbonyl

ROS can also lead to oxidation of amino acid residue side chains and formation of protein-protein cross-linkages, as well as oxidation of the protein backbone resulting in protein fragmentation. Direct oxidation of some amino acids such as lysine, arginine, proline, and threonine residues may result in carbonyl derivatives. When carbonyl groups already exist, they also have the ability to be induced into proteins by reactions with aldehydes (f.ex, malondialdehyde) produced during lipid peroxidation. The presence of carbonyl groups in proteins has therefore been used as a marker of ROS-mediated protein oxidation, and several sensitive methods for the detection and quantification of protein carbonyl groups have been developed (Levine et al 1994).

2.3.3 Cytochrome C Oxidase

Cytochromes are membrane-bound hemoproteins that consists of heme groups and are able to carry out electron transport. They may exist in monomeric proteins or as subunits of bigger enzymatic complexes that catalyze redox reactions. They are found in the mitochondrial inner membrane and ER of eukaryotes. Cytochrome C oxidase ia able to convert two highly reactive oxygen atoms to harmless molecules of water. By holding the superoxide radical at a special bimetallic center, between a heme-linked iron atom and a copper atom, the oxygen picks up a total of four electrons which may be converted into to two molecules of water (Alberts 2002)

2.3.4 Manganese Superoxide Dismutase – Mn-SOD

Two types of Superoxide dismutases (SOD) have been isolated from mammalian liver; one enzyme contains manganese and is found primarily in the mitochondrial matrix, while the other contains copper and zinc and is localized primarily in the cytosol. Superoxide dismutases catalyze the reaction of the Superoxide anion to hydrogen peroxide (Fridovich et al 1975). Because of its catalytic function, SOD is thought to be involved in the protection of cells against damage from lipid peroxidation. When SOD activity is absent or depressed, Superoxide anions may react with hydrogen peroxide to produce hydroxyl radicals that could subsequently initiate lipid peroxidation and potentially producing deleterious effects on membranes (DeRosa et al. 1980).

2.3.5 Catalase

Catalase is an enzyme promoting the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen and can be used as a biomarker of oxidative stress. A number of studies reported an increase of superoxide dismutase and catalase activities when an excess of ROS was observed in bivalves (Cheung et al. 2004; Pellerin-Massicotte et al. 1997). The presence of pollutants in the environment contributed to an increases of catalase activity and lipid peroxidation (malondealdehyde) levels in bivalves (Cossu et al 1997).

2.4 Pollutants– potential promoters of oxidative stress

The term ecotoxicology appeared in scientific society about 40 years ago and was introduced by Renè Truhaut. The need for a new common term combining ecology and toxicology reflect the growing concern of environmental chemicals and their effects upon species other than man. Ecotoxicoloy is the study of the presence, behaviour and harmful effects of man-released pollutant chemicals in organisms and the natural environment. Many different chemicals are regarded as pollutants, ranging from simple inorganic ions to complex organic molecules. Their effects upon ecosystem and individuals also differ causing acute, sub-acute or chronic toxicity. (If nothing else is stated the reference is C.H Walker, third edition).

2.4.1 The fate of pollutants

As pollutants move and distribute in the environment they are transported in and out of different abiotic and biotic phases. There are five major types of environmental phases: water, air, soil, sediment and biota. The environmental fate of pollutants depend on their chemical properties and the properties of the environmental phase in which they occur;

Physicochemical properties of the pollutant

– Volatility, fugacity, hydrophobicity, polarity, hydrophilicity
– Bioavailability (Availability for uptake into biota)
– Ability to bioaccumulate in biota
– Ability to biomagnifiy within biota (to increase by the trophic levels)
– Potential for reaction with biological molecules

Properties related to persistence and degradation

– Persistence towards physical degradation and biodegradation
– Ability to produce adverse degradation products

Ability for inducing toxicity/adverse conditions in biota

– By direct or indirect interaction with biological molecules
– By direct or indirect interaction with subcellular or cellular processes
– By direct or indirect interaction with biological tissues and/or physiological processes

(C.H Walker, third edition, Jonny Beyer, UiS)

2.4.2 Persistent Organic Pollutants

POP's (Persistent organic pollutants) is the generic term for a number of pollutant compounds that resist biodegradation and remaining in the environment, taking centuries to fully degrade. The toxin accumulates in the lower hierarchy and migrates up the food chain as it builds up in body fat. POP's include many pesticides, industrial chemicals, organochlorines, and by-products like dioxins. POPs in the Arctic environment may persist for decades due to their low reactivity and slow reaction rates at low temperatures. Many of these chemicals have the potential to increase the oxidative stress in the effected organism. Several classes of pollutants, including trace metals and organic compounds, are known to enhance the formation of ROS resulting from xenobiotic redox cycling (Almroth et al 2008).

2.4.2.1 Organic pollutants

Organic molecules are characterized as molecules containing carbon atoms. The same goes for pollutants, as a great majority of pollutants containing carbon are described as organic pollutants. Because carbon atoms may easily form stable bonds with other carbons as well as hydrogen, oxygen, phosphorus and nitrogen atoms, organic pollutants have the ability to enter and react with a great diversity of complex organic compounds found in living organisms. Carbon can form single, double or triple covalent bonds, as well as complex linear, circular or aromatic chain structures. These organic structures may consist of carbon alone or together with other atoms such as fluor, nitrogen, hydrogen, oxygen or phosphorus. This creates an enormous amount of possible structures and compounds.

From a small abridgement of the many industrial chemicals, we find Hydrocarbons, Polychlorinated biphenyls (PCBs), Polychlorinated naphthalenes (PCNs), Chloroparaffins, Brominated flame retardants, Polybrominated biphenyls (PBBs) and Detergents (anionic, cationic and non-ionic), but there are many more. The substances mentioned also have several different congeners, PCB alone have altogether 209 possible congeners. About 120 of these are percent in commercial products such as Aroclor 1254 and Aroclor 1260. When carbon is being the principal element, they often have very little polarity and low water solubility.

2.4.3 Polychlorinated Biphenyls – PCB's

PCBs, shown in figure 2.4.3, are mixtures of commercially related compounds developed in the 1920s**.** Today traces of the substance are found in soil, seabed, and in plants, animals and humans all over the world. Because of its unique properties; this stable, unreactive viscous liquid of low volatility, were applied in a number of applications such as hydraulic fluids, coolant-insulation fluids in transformers and plasticizers in paint**.** When the products later were discarded, they became a permanent source of pollution. PCB's are slow-working toxins, with a low solubility in water in contrast to oils and organic matter. This property causes the PCB to be stored in the body's fatty tissue after being absorbed in the organism. Because of its many congeners it varies in both chemistry and structure depending on the number and position of chlorine atoms. This also affects the ability to cause harm to the exposed organism. In animals, exposure to these environmental toxins may interfere with the function of various organs and systems such as the endocrine and immune system, nervous systems (Miller-Perez et al. 2009), reproductive system (Hansen et al. 1998) as well as hormone disturbance (Sridhar et al. 2004). The metabolites are also thought to be indirectly carcinogenic as they enhance the effects of other substances through the generation of ROS that can induce DNA oxidative damage (Miller-Perez et al 2009). It has also been shown that mono- and dichlorinated biphenyls can be metabolized to dihydroxy compounds and further oxidized to reactive metabolites which form adducts with nitrogen and sulfur nucleophiles including DNA .These results demonstrate that both free radicals and oxidative DNA damage are produced during the oxidation of lower chlorinated biphenyls (Oakley el al. 1997). As PCB's are broken down at a slow rate, they are likely to be retained in

the organism its entire lifespan. The toxin is also passed on to the next generation as PCB's passes on to the offspring through the fat in the placenta as well as in breast milk (Walker et al third edition).

PCB in Norway

Norway has been affected by PCB pollution in several ways:

1. The cold and damp climate has led to widespread use of PCB in construction materials such as isolation glass and grouting. These products were later discharged of as normal waste, creating an active source of pollution.
2. Several of the Norwegian fjords are polluted because of emissions from shipyards, industrial activities and waste grounds.
3 The country's northern location makes it susceptible to long-distance pollution via air and sea currents.

(PCB.no, PCB Sanering AS)

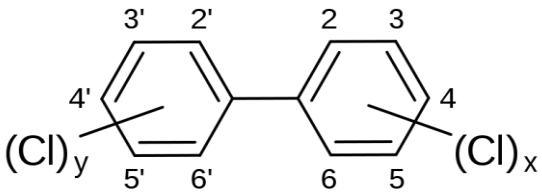


Figure 2.4.3 The structure of PCB consists of two phely-rings covalently bound together and a number of Cl atoms (x,y) on each ring.

2.4.4 Organochlorine Insecticides - DDT, DDD and DDE

Organochlorine Insecticides is a common description of a large group insecticides including DDT (Dichloro-Diphenyl-Trichloroethane) and its biproducts; DDE (1,1-dichloro-2,2-bis (pdichlorodiphenyl) ethylene) and DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane). DDT,

shown in the figure 2.4.4, was one of the first modern pesticides and is considered to be moderately toxic to vertebrates. The pesticide effect the exposed organism by opening sodium ion channels in the neurons, causing them to react spontaneously which leads to spasms and eventual death. The half life is 2-15 years, and when broken down it produces DDE and DDD which are also highly persistent and have similar chemical and physical properties. The DDT has a low solubility in water (Walker et al third edition).

In a study on redox environment and survival of hepatocytes from Hoplias malabaricus (wolf fish) using 50 nM of DDT, the antioxidant enzymes catalase, glucose-6-phosphate dehydrogenase (G6PDH), glutathione reductase and SOD were all affected. The data showed that despite of some protective responses, the increased disturbance on membrane lipids and proteins increased hydrogen peroxide levels, and decreased glutathione concentration. The cell viability strongly indicated that the presence of oxidative stress induced by DDT was the reason of hepatotoxicity (Filipak et al. 2008).

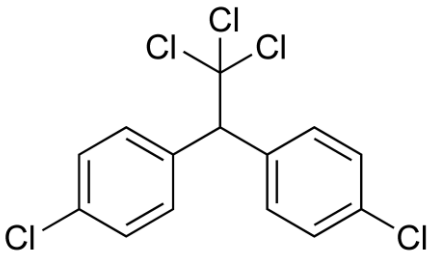


Figure 2.4.4 The structure of DDT also contain two phenyl rings, but has a set number of Cl atoms (DDT;5 Cl atoms, DDE and DDD have 4 Cl atoms).

2.4.5 Polybrominated Diphenyl Ethers - PBDEs

The polybrominated diphenyl ethers (PBDEs), represent an important group of industrial chemicals that have been massively produced and extensively used in plastics, textiles, furniture, and electronic devices, including computers and TV's a as way of reducing the risk of fire (McDonald et al 2002). Mixtures of polybrominated biphenyls have therefore been marketed as Brominated flame retardants (BFRs). PBDEs, shown in figure 2.4.5, share structural similarity to

the persistent PCBs and have high heat stability, high lipid solubility, and low vapor pressure, which contribute to their environmental persistence and bioaccumulation abilities (Darnerud et al. 2003). PBDE congeners also have the ability to cause oxidative damage. In experiments with *in ovo* and post-hatch captive American kestrels, the organisms developed hepatic oxidative stress reflected by elevated lipid peroxidation after they were exposed to PBDE congeners (predominantly BDE-47, -99, -100, -153) (Fernie et al. 2005).

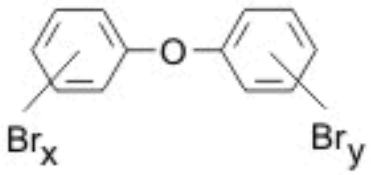


Figure 2.4.5 The chemical structure of PBDE. Like PCB, a different number of Br atoms (x,y) contribute to a great variety of different structures.

2.4.6 Perfluorooctane sulphonic acid – PFOS

Perfluorooctanesulfonate (PFOS) shown in figure 2.4.6 and related perfluorinated compounds are an emerging group of persistent environmental contaminants that have been detected in various environmental matrices, including wildlife and humans (Lehmler et al. 2005; OECD rapport 2002). They have been widely used in consumer products such as carpets, furniture, household cleaner, fabrics, and paper products as well as manufacturing processes (industrial surfactants and emulsifiers) (Andersen et al 2006). This has contributed to a widespread distribution and persistence of these compounds in the environment. The mammalian toxicity of PFOS has been investigated *in vitro* and *in vivo*. In the rat and Cynomolgus monkeys, reduction of body weight, liver hypertrophy, and decreased serum cholesterol and triglycerides have been reported after exposure to PFOS (Seacat et al 2002,2003). *In vitro* studies have shown that PFOS interferes with mitochondrial bioenergetics (Starkov et al 2002) and fatty acid protein binding in the liver (Luebker et al 2002). A study by Giesy and Kannan in 2001 which included several different wildlife species (fish, birds and marine mammals), showed higher levels of PFOS in the

predatory animals then what was found in their diet. These findings indicate a bioaccumulative property in the higher trophic levels in the food chain.

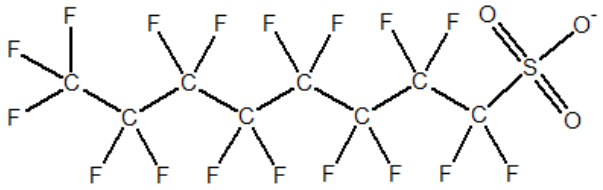


Figure 2.4.6 Chemical structure of PFOS.

2.4.7 Nonylphenol

Nonylphenol is a compound used in cleaning products and belong to a large group of Alkylphenol ethoxylates (APEO) which are one of the major groups of nonionic surfactants. Worldwide, approximately 360,000 tons APEO are used annually (Richtler et al 1988) in the manufacturing of plastics, textiles, agricultural chemicals, and paper. Nonylphenol is a persistent biodegradation product of the nonylphenol ethoxylates. The nonylphenol ethoxylates are released directly into the aquatic environment after use via normal waste water discharges (Talmage et al 1994). Alkylphenols are toxic to animals and plants (Lewis et al 1992), probably due to the hydrophobic alkyl residue that induces alteration of the cell membranes (Argese et al 1994). In trout species, nonylphenol was found to accumulate in the liver, gill, skin, gut, fat, and kidney tissue (Ahel et al. 1993; Coldham et al. 1998; Lewis et al. 1996). Nonylphenol have also been found to have estrogenic properties (Burkhardt-Holm et al. 2000).

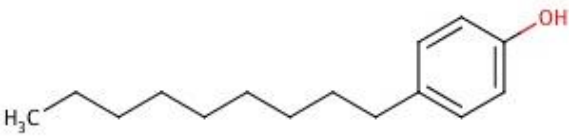


Figure 2.4.7 The chemical structure of 4-nonylphenol.

2.4.8 Crude Oil

Crude oil is a naturally occurring liquid consisting of a complex mixture of various hydrocarbons and other organic compounds, that are found in geologic formations beneath the earth's surface. The hydrocarbons in crude oil are mostly alkanes, cycloalkanes and various aromatic hydrocarbons while the other molecular compounds may be nitrogen, oxygen and sulfur, as well as trace amounts of metals such as iron, nickel, copper and vanadium. The exact molecular composition varies widely from formation to formation but the proportion of each chemical element varies over much more narrow limits. (Speight et al.1999). Polycyclic aromatic hydrocarbons (PAHs), derived largely from fossil fuels and their combustion, are pervasive contaminants in rivers, lakes, and near shore marine habitats. Many studies have followed the *Exxon Valdez* oil spill (EVOS) on March 24, 1989 were about 40 million liters of crude oil was spilled into the sea, and covered 400 km^2 of ocean. Studies initiated after EVOS demonstrated that fish embryos exposed to low levels of PAHs in weathered crude oil develop a syndrome of edema and craniofacial and body axis defects. Studies also highlight the relative toxicity of low-molecular-weight tricyclic PAHs which has earlier been considered to have a low toxicity (Incardona et al. 2005)

A study preformed on juvenile Atlantic cod induced by crude North Sea oil and North Sea oil spiked with alkyl phenols and PAH's showed a change in protein expression after low levels of exposure. The protein expression profiles after exposure indicated that there were effects on fibrinolysis and the complement cascade as well as the immune system, fertility-linked proteins and bone resorption. It also showed some effect on fatty acid metabolism, increased oxidative stress, impaired cell mobility and increased levels of proteins associated with apoptosis (Bohne-Kjersem et al 2010). Resent events in the Mexico Gulf have given the world yet another oil spill, which might become one of the greatest disasters in the oil industry. April 20, BP's Deepwater Horizon oil rig exploded. The explosion resulted in an oil spillage estimated by the government to reach 210,000 gallons (5,000 barrels, a much discussed number) a day sending more than 6 million gallons of crude oil into the Gulf of Mexico. (Natural Resources Defense Council).

2.5 Analytical separation

2.5.1 The centrifuge

Centrifugation is based on a basic principle; the centrifugal force (g-force) and separation is achieved by the means of the accelerated gravitational force created by a rapid rotation. The action of centrifugal force is used to promote the accelerated settling of particles in a solid-liquid mixture. Two distinct major phases are formed in the centrifugation tube during centrifugation, a pellet (sediment) and the supernatant (the liquid). This technique is widely used to isolate suspended particles from their surrounding medium on either a batch or a continuous-flow basis. Applications for centrifugation are many and may include sedimentation of cells and viruses, separation of subcellular organelles, and isolation of macromolecules such as DNA, RNA, proteins, or lipids.

2.5.1.1 Centrifugation forces

Rpm stands for Rotations per minute. The g-force of the centrifuge stands for the standard acceleration due to gravity. This force must be calculated individually for each centrifuge as two rotors with different diameters running at the same rotational speed will subject samples to different accelerations. The g-force can be calculated by

Eq.(2.3) $$g = \frac{r\,(2\pi N)^2}{RCF}$$

r = radius N = rotational speed measured in revolutions per unit of time.

Eq.(2.4) $$RCF = \frac{(2\pi v)^2 .r}{981}$$

RCF = Relative centrifugation field, v = velocity, 981 = gravitational field (cm/s^2)

2.5.2 Differential Centrifugation

This is one of the most common methods for fractionating cells, separating the different organelles within the cell and making them available for individual analyses. The separation is based on the application of a gravitational field on the particles and the medium, separating the organelles or fraction according to size, shape and density. The larger and denser the particles are, the greater the centrifugation force is experienced and the faster the particles move. Demonstrated in the figure 2.3.2, cellular membranes are spun down with only 7000g and for a short period of time, were as small ribosomes need 300 000g for 2h.

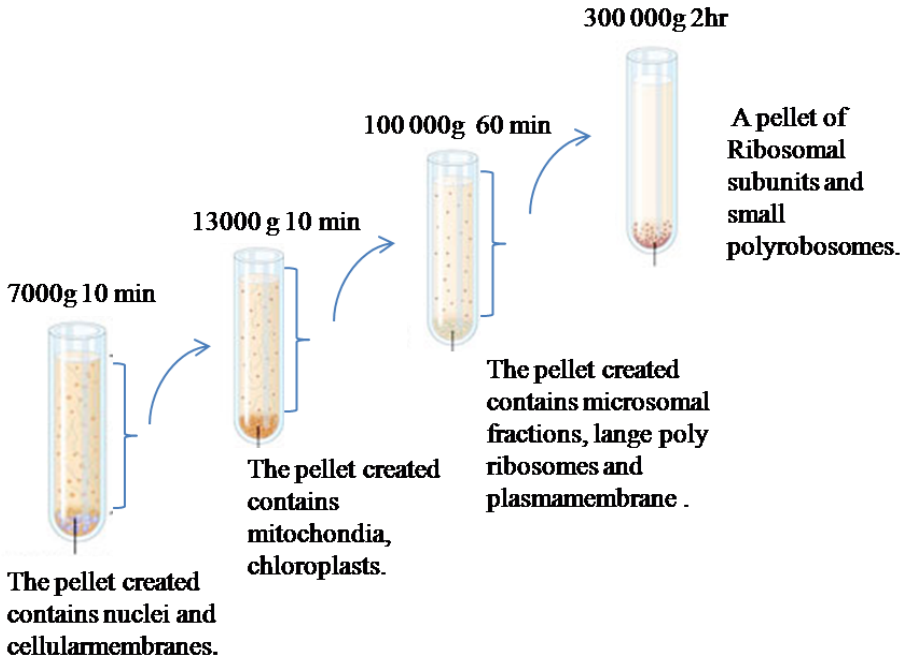


Figure2.5.2. Different fractions and organelles are separated at different speeds and time intervals. The supernatant or pellet may be used for further analysis.

2.6 Chromatography

Chromatography is a method also used to separate different substances in a homogenous solution. The principle is the same as in extraction methods; using a stationary phase and a mobile phase, separating the compound according to their interaction with the two phases. They all have a stationary phase which may be a solid or a liquid supported on a solid, and a liquid or a gas mobile phase. The mobile phase flows through the stationary phase and carries the components of the mixture with it. In Absorption chromatography a solid stationary phase is used together with a liquid or a gaseous mobile phase. The solute is absorbed on the surfaces of the solid particles and the more strongly the solute is absorbed the slower it travels through the stationary phase/column. When different components travel at different rates they are separated and made possible to identify (if nothing else is stated the reference for this section is D.C Harris).

2.6.1 Thin layer chromatography - TLC

Thin Layer Chromatography (TLC) is one of many separation methods used to separate mixtures of substances into their components. The name of this method is quite describing as the basic principle of the method is using a thin, uniform layer of silica gel or alumina coated onto a piece of glass, metal or rigid plastic as a stationary phase. Like all types of chromatography, a mobile phase is also used to transport the analytes, in this case, upwards the plate.

2.6.1.1 Stationary phase

In this study it was used HPTLC (high performance TLC) plates. HPTLC and TLC plates use the same type of silica gel 60. But in HPTLC particle sizes range between 4-8 mm, and the mean particle size measures 5-6 mm, giving a smoother surface and a higher separation than conventional TLC plates (Merck). The silica gel is a form of silicon dioxide. The silicon atoms are joined via oxygen atoms in a covalent structure. On the surface, silicon atoms are attached to -OH groups, creating Si-O-H bonds instead of Si-O-Si bonds. Because of these -OH groups, the

surface of the silica gel very polar, allowing hydrogen bindings with suitable compounds as well as van der Waals dispersion forces and dipole-dipole attractions.

2.6.1.2 Mobile phase

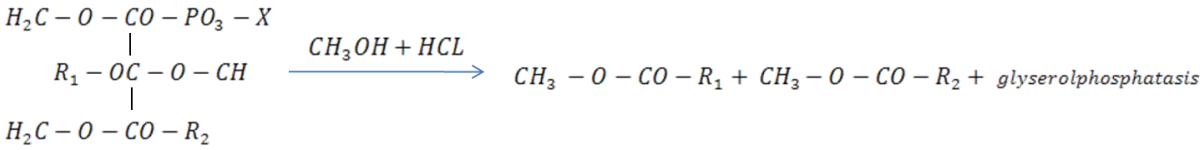
The method often includes both a polar and a nonpolar mobile phase, running the polar phase just over the middle of the plate, and the nonpolar up to 1 cm from the edge of the plate. This ensures that both polar and nonpolar components are separated. The entire TLC process is preformed in an enclosed glass beaker, insuring that the atmosphere in the beaker is saturated with solvent vapor from the mobile phase.

2.6.1.3 The separation

As the solvent begins to soak up the plate, it first dissolves the compounds in the sample spot on the base line. The compounds present will then tend to get carried up the chromatography plate as the solvent mobile phase continues to move upwards. How fast the compounds travel up the plate depends on two things: the stationary phase and the mobile phase. How hydrogen bonds forming between the compound and the surface of the silica gel have a big impact on the retention time. More frequent hydrogen bindings to the gel in a substance create a stronger binding to the stationary phase. The substance is then *adsorbed* more strongly than the others. That means that the more strongly a compound is adsorbed, the less distance it can travel up the plate. The mobile phase is also important as it affects how easily the compound is pulled back into solution away from the surface of the silica.

2.7 Methanolysis

Methanolysis, transesterification or methylation refers to the process in which free esterified fatty acids are converted into methyl esters. The fatty acids to be analyzed by GC are often analyzed as their corresponding fatty acid methyl esters (FAME). In this study an optimized methanolysis, acid catalyzed by HCl is used (equation 3) based on the study by Meier et al (2006).



Equation 3. The reaction in methanolysis. X- is a phospholipid group and R_1 and R_2 demonstrate two different fatty acids.

2.8 Laemmli SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a frequently utilized method in molecular biology. The technique is used to separate proteins accordingly by size and charge. The proteins which are to be analyzed are mixed with Laemmli, which is a mixture of SDS, DDT, glycerol, 2-mercaptoethanol and bromphenol blue. The SDS detergent binds to all the proteins positive charges giving each protein an overall negative charge so that proteins will separate based on size and not by charge. SDS, DTT and beta 2-mercaptoethanol denaturizes the proteins and subunits enabling the gel to separate them based on size, not on shape. Bromphenol blue serves as an indicator dye, and glycerol increases the density of the sample so that it will fall to the bottom of the well (Laemmli et al 1970).

2.8.1 PAGE

PAGE functions in the same way as normal electrophoresis, with one positive pole and one negative pole. All proteins will now have a negative charge and move towards the positive pole. The gel is constructed to serve two functions; stacking and separation. The stacking gel has a large pore size so that the proteins are concentrated and all start move at the same time giving sharper bands. The separating gel separates the different proteins by size, making them detectable according to the relative molecular mass (Gallagher et al 2001).

2.9 Western Blotting

Western blotting, involves the separation of a protein mixture by gel electrophoresis, and then transferring the proteins to a suitable membrane. In this case it is used a nitrocellulose membrane. The transfer is carried out using electricity and a 90 degrees current making the proteins migrate from the gel and onto the membrane (figure 2.4).

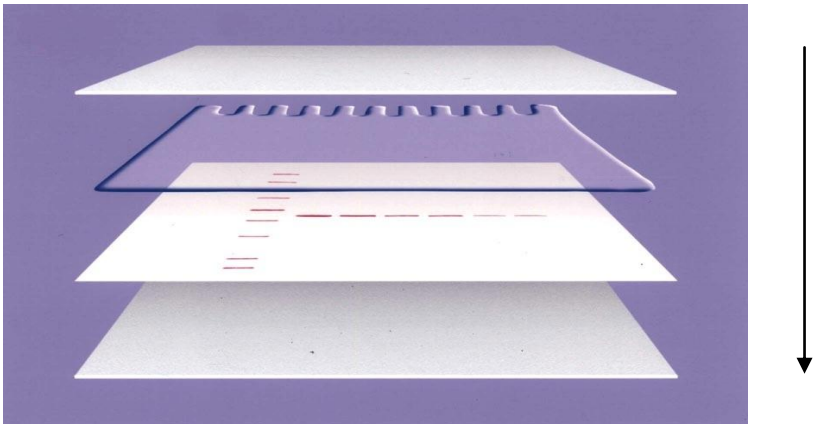


Figure 2.9 The different layers in a western blotting reaction (sandwich). The top and bottom layer is filter paper. Number two from the top demonstrates the gel and number three the membrane. The arrow gives the direction of electricity transfer (news.thomasnet.com).

Western blot analysis can detect one protein in a mixture of any number of proteins and also give information about the size and molecular mass. The protein is identified through its reaction with a specifically labeled antibody, polyclonal or monoclonal. This method is dependent on the use of a high-quality antibody specifically directed against the target protein. Non-specific

binding of antibodies can be reduced by blocking the unoccupied membrane sites with a protein or non-ionic detergent. So before adding primary antibody the nitrocellulose membrane is incubated it in a blocking solution. One of the best blotting agents around is Nonfat Dry Milk.

Chemiluminescent detection uses an enzyme to catalyze a reaction that results in the production of visible light. Some chemiluminescent systems are based on the formation of peroxides by HRP (horseradish peroxidase). When the enzyme substrate is incubated with the membrane, the secondary antibodies containing HRP, catalyses the oxidation of luminol giving an emission of light making the corresponding bonds visible on the developed film. ELC (enhanced chemiluminesensce) is a way of detecting proteins using chemical enhancers like phenols together with the oxidation of luminal. This reaction gives a maximum light emission at a wavelength of 428 nm making it possible to measure and detect with Hyperfilm ELC (Gallagher et al 2004).

2.10 Citrate Synthase enzyme activity

Citrate Synthase (CS) enzyme and enzyme activity was essayed in the mitochondrial pellets as described in Kuznetsov, Lassnig and Gnaiger (2006). CS is an enzyme found in the citric acid cycle and localized in the mitochondrial matrix. Because the enzyme is synthesized on cytoplasmatic ribosomes and transported into the mitochondrial matrix, it is commonly used as a quantitative marker for intact mitochondria (Holloszy et al. 1970; Williams et al 1986; Hood et al. 1989) In this study it was only used as a verification of the isolation method.

CS catalyses a specific reaction of 2 carbon acetyl CoA with 4 carbon oxaloacetate forming 6 carbon citrate which regenerates CoA-SH. The absorbing compound is the reaction product thionitrobenzoic acid (TNB) from the irreversible reaction;

CoA-SH + DTNB → TNB + CoA-S-S-TNB.

The working wavelength is 412nm as TNB is strongly absorbent at this wavelength. An linearly increase of absorbance, up to 0,6-0,8 units, is seen in the sample with time.

2.11 BCA Protein Assay

The bicinchoninic acid Protein Assay was introduced by Smith, et al. in 1985. The assay uses a two step reduction reaction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium and BCA. The reaction results in an intense purple-colored product which exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The intensity of the color produced is proportional to the number of peptide bonds in the reaction. Only tripeptides and larger polypeptides or proteins will react where as single amino acids and dipeptides do not give the biuret reaction (manufactures protocol BCA Protein Assay, Thermo scientific, Rockford, USA).

2.12 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a widely used immunology based detection method for antibody or antigen detection. As this part of the experiment was not executed, the principles of the method is only shortly described.

In ELISA special microtiter plate wells are added antigens or antibodys diluted in buffer. As the microplate is covered with a special solid phase, the antigens attache to the well after incubation. When the antibodys are added they bind to the specific antigen in the well. Both enzyme linked antibodies or an enzyme linked secondary antibody may be used for detection. They are also specific and will bind to the target antibodys. Washing procedures between each step are done to prevent unspecific binding. Quantification and detection is now possible when using the enzyme labeled reactants and the enzymatic reactions to yield color which can be measured spectrophotometricaly (Crowther, 1995).

2.13 Analytical calculation

2.13.1 Quantitative analysis

Although there has been remarkable advancement in instrument technology, automation and computer science, there will always be some uncertainties involved in quantitative analysis, especially when dealing with biological material. Ideally, the experiment would be repeated such a number of times, that if all errors were random, an accumulation around the average value will occur. Such a Gaussian distribution requires a large number of samples, and in lab experiments we are often forced to work with a much smaller amount of material. When working with living organisms there are also many ethical aspects to be taken in to consideration. Economics and time are also often the restricting factor. By making statistical analysis we may estimate the results from a much smaller amount of samples.

2.13.2 Precision and Accuracy

Precision is a measurement of the reproducibility of the results. If the method is reducible, one would be able to repeat the study and achieve the same results in another lab. Accuracy and precision go hand in hand, if the reproducibility is good, but the results produced are inaccurate, it does no help that the standard derivation is low. Accuracy is defined as the closest to the true value, given that the true value is already known. By performing a number of different measurements on the same analyte and using well known procedures, a good agreement between the methods may give a good indication of the true value. In the ideal method the procedure is both, reproducible and accurate.

2.13.3 Uncertainties

Every method has its weaknesses; it may be interference by material other then the compound of interest, such as triglycerides interfering with spectrometric measurements, the detection level of the GC instrument or unspecific binding in western blot analyses. This contributes to a number of uncertainties involved with the method and the results produced.

2.13.4 Preanalytical errors

The lack of standardized procedures for sample collection, including specimen acquisition, handling and storage, may account for over 90% of the errors currently encountered within the entire diagnostic process in humans (Lippi et al 2006). Preanalytic refers to everything that is preformed prior to the actual analysis.

2.13.5 Analytical Errors

Analytical errors during the analyses and are referred to as systematical or random errors. Systematical errors occur in one or several steps in the experiment and is caused by errors in the method it self, making it reducible and a potential problem in all the samples. The use of a contaminated internal standards or standards with the wrong concentration may cause such an error. Random errors, as the name suggests, is a result of random and uncontrolled variables that occur during the measurements. This may be caused by a pipetting error when adding a substrate in an enzymatic reaction, or a sudden change in temperature in the laboratory leading to a speed up in the reaction.

2.13.6 Questionable data

When performing statistical analysis on biological material, there will always be some natural variations making gaps in the data points. An organism is an individual, shaped by its heritage and by its surrounding environment. These natural variations are important to take in consideration when studying the results. Inconsistency between data points is unavoidable, and to discard data one should be certain that there has occurred an error in the procedure that may have led to the particular result. Statistical analyses are also available to rule out outsiders.

3. Materials and methods

3.1 The fish

Atlantic cod were purchased from a local breeding source, and kept in a saltwater tank at the Marine Research Institute. The water temperature was kept at approximately 9°C. Over a period of four weeks the fish were exposed to complex mixtures of POP's (persistent organic pollutants) and oil components through feeding, given one treatment a week. The food pasta consisted of commercial fish pellets (18% lipid), distillated water and fish oil (table 3.1 a). The amounts corresponded to approximately 0,5% of the fish total body mass. To get wide spectra of effects from the different pollutants, a number of fish were exposed to 5 different mixtures of pollutants based on findings in our waters. It was also included a control group and two mixtures (table 3.1 b). Chlorinated compounds consisted of PCB's, DDT, DDE and DDD, Chlordane, Lindane and Toxaphene. The brominated compounds consisted of; PBDE and DE-71, the fluorinated compounds of PFOS and Alkylphenols were represented by 4-nonylphenol and 4-*tert*-nonylphenol. One group was also exposed to crude oil from Trold Olje. The concentrations are shown in table 3.1c and are based on background studies performed at the Norwegian cost and North Sea. The chlorinated and brominated compounds are based on a study by Green et al (2003) chlordane is based on findings by Kallenborn et al (2007) and the fluorinated compounds are based on a study by Falandysz et al (2006). The oil components are reference values from a study performed on cod in the Statfjord area in the North Sea (Grøsvik et al 2007). The values are used as references, and a 50 fold of the concentrations found have been used in this study to simulate sudden or larger discharges of pollutants and bioaccumulation. Because PFOS was not able to dissolve in the fish oil, propadiol was used instead. Equal amounts of propadiol were added to the other treatments to prevent a possible interference. Only male cod fed the high concentrations was used in the experiment which was randomly chosen from each group.

Table 3.1 a) Contents of the food pasta.

Food pasta	Wight (g)
fishpellet (18 % lipid)	120
Destillatet water	94
Fish oil	24
Propadiol	2
Total	240

Table 3.1 b) Mixtures of POP and oil components and the number of cod from each group.

Treatment	Number of fish
Control	8
Chlorinated compounds	5
Brominated compounds	5
Fluorinated compounds	5
Oil	5
Oil + mixture* High	5
Mixture* high	5
Alkylphenols	10
Total	48

*The mixture consisted of the high concentrations of the chlorinated, brominated and fluorinated compound

Table 3.1c Dose exposed to the fish.

Treatment	Total body burden (µg/kg)
Chlorinated compounds	High
PCB (mix of Aroclors 1242, 1254, 1260)	11385
4,4`DDT	264
4,4`DDE	842
4,4`DDD	182
Chlordane (technical)	990
Lindane (gamma-HCH)	99
Toxaphene (DE-TOX 483 (technical))	1320
Brominated compounds	
Polybrominated diphenyl ethers (PBDE, DE-71)	990
Fluorinated compounds	
PFOS (Perfluorooctane sulphonic acid)	660
Alkylphenols	
4-nonylphenol	500
4-*tert*-nonylphenol	500
Oil	
Trold olje (200 +)	25000

3.2 Isolation of mitochondria

Isolation of mitochondria from cod liver was performed by differential centrifugation according to the method of Pon and Schon (2001) with some modifications. Directly after sacrifice, the liver was removed and stored in eppendorf tubes at -80°C, approximately 2,0 grams in each tube. Immediately after the samples were thawed on ice, the liver was washed in 10 ml washing solution (0,25 M sucrose, 0,01 M Tris-HCl, 1 mM EDTA, pH 7,4). After removing the washing solution, 18 ml of homogenizing buffer (0,25 M sucrose, 24 mM Tris-

HCl, 1mMEDTA pH 7,4 and 0,5 mg/ml BSA) was added and the sample homogenized using a Ultra Turrax homogenizer. The homogenizing was preformed in a cool room at 4 °C.

The centrifugation was preformed with the use of Sorvall RC 5C plus/ Sorvall RC 5B plus with a HB-4 1300 Rotor. The homogenate was transferred to a 50 ml centrifuge tube and centrifuged at 750g for 10 min at 4°C to remove membranes, nucleus and fat. The supernatant was filtered using a gas bin (9 x 10 cm) and re-centrifuged for 10 min at 750g at 4°C to remove additional fat. The supernatant was transferred into a new centrifuge tube using a syringe and centrifuged at 1300g. Before the last centrifugation the supernatant was removed and the pellet resuspended in 5ml homogenizing solution. The pellet was transferred to a new tube using a pipette and centrifuged at 1300g for 10 min. The mitochondria pellet was then resuspended in 300µl homogenizing solution and stored at -80°C for further analysis.

3.3 Citrate Synthase – Mitochondrial marker enzyme

The enzyme activity was measured spectrophotometrically using Varian Cary 50 Bio UV-spectrometer. All samples were measured at two concentrations; 50µg/ml and 100µg/ml, from the mitochondrial fraction and the second supernatant from the centrifugal isolation of the mitochondria pellet. To start the enzymatic reaction we added; 100µl 0,1 mM DTNB, 25µl 10% Triton-X-100, 50µl Oxalacetate, 25µl acetyl CoA, 800 µl distillated water and 100µl sample making a total volume of 1000µl. The samples were mixed carefully before transferred to a 1 ml glass cuvette and measured. Distillated water was used as a blank, and the samples were measured for 200 sec. at 412nm. A commercial citrate synthase was used as standard diluted 1:500 in 0,1 M Tris-HCl buffer, pH 7,0.

3.4 Protein Concentration

Themo Sientific BCA™ Protein Assay Kit, microplate procedure was used to measure protein concentration (sample to washing reagents ratio 1:8) 25 µl of sample and 200µl working reagent containing 50 parts reagent A (sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0,1 M sodium hydroxine) and one part reagent B (4% cupric sulfate) was added to each well. The microplate was placed on a gentle shaker for 30 sek and incubated for 30min at 37°C covered in aluminum foil. Two dilutions of each mitochondrial pellet were measured (1:1, 1:2). A commercially available BSA was used as standard to determine the concentration (table 2.4) and the samples were measured spectrophotometrically at 562 nm within 10 min after incubation on Asys UVM340 microplate reader. The 1:2 dilution was used for further analysis.

Table 2.4 Standard dilutions.

Standard	µg/ml
1	0
2	25
3	125
4	250
5	500
6	750
7	1000
8	1500
9	2000

3.4 Lipid Analysis

3.4.1 Lipid extraction

200µl of the mitochondria pellet was solved in 2ml Chloroform/Methanol (2:1) in a glass tube and N_2 was added before stored at -20°C for further analysis. The lipids were extracted by a modified Folch method (J.Folch et al 1957). A glass filter connected to a vacuum-pump was used to filter the samples into a 15ml glass tube using Chloroform:Methanol (2:1) (1x2ml, 1x1ml). 2 ml 0,88% KCl (aq) was added to the sample before vortexed, and centrifuged 2000rpm for 5 min creating a two phase system. It was used Beckman CS15R centrifuge with rotor F0850. The upper phase was discharged, removing non-lipid material, and the lower phase used for further analyses. The volume was decreased and the sample dried using N_2 gas then solved in 50µl chloroform and stored at -20°C.

3.4.2 Lipid class separation by Thin Layer Chromatography

Several methods were tested to establish an optimized method for the separation of the lipids of interest. Two 1D methods and one 2D method were included in the tests. As the 2D method requires one silica plate for each sample, a more efficient and economic use of the plates was achieved in the 1D method. Some of the steps were included in all the methods;

A saturated atmosphere in the glass-chambers was created using filter paper soaked in solvent. The vapor stops the solvent from evaporating as it rises up the plate. The plates were dried between each applications of mobile phase, as they may make the one or the other more or less polar which in turn may affect the results. The plates were developed in the same matter with 3% cupric acetate in 8% phosphoric acid (Fewester et al. 1969) and placed in a heater for 20min at 160°C. Because this developing technique is based on a charring response with the lipids, further analysis of the lipids is not possible. Another developing technique using fluorescent lipid marker and UV light, was applied when further analysis were needed.

2.4.2.1 2D TLC

Lipids were separated by 2D-TLC method on silica gel plates according to Guschina et al. (2009) with some modifications. Glass-chambers and E.Merck (10x10) silica gel 60 plates were used. Plates were then activated and dried for 1 h. in 100°C before applying 50µl lipid extract.

Chloroform/methanol/water (65:25:4, by volume) solution was used in the separation of the polar components. The TLC-plate was placed with the baseline down in the same way as in the 1D-method. The first phase was run up to 1 cm from the top of the silica plate. To remove the remains of solution-I, plates were placed in an exicator under vacuum for approximately 30 min. When separating the nonpolar components the plate was placed with it's left side (seen from the front) down in the beaker. The second dimension was run up to 1 cm from the top using a chloroform/acetone/methanol/acetic acid/water (50:20:10:10.2, by volume) solution. The plates were dried shortly before sprayed with cupric acid or a fluorescent lipid marker when used for further analysis.

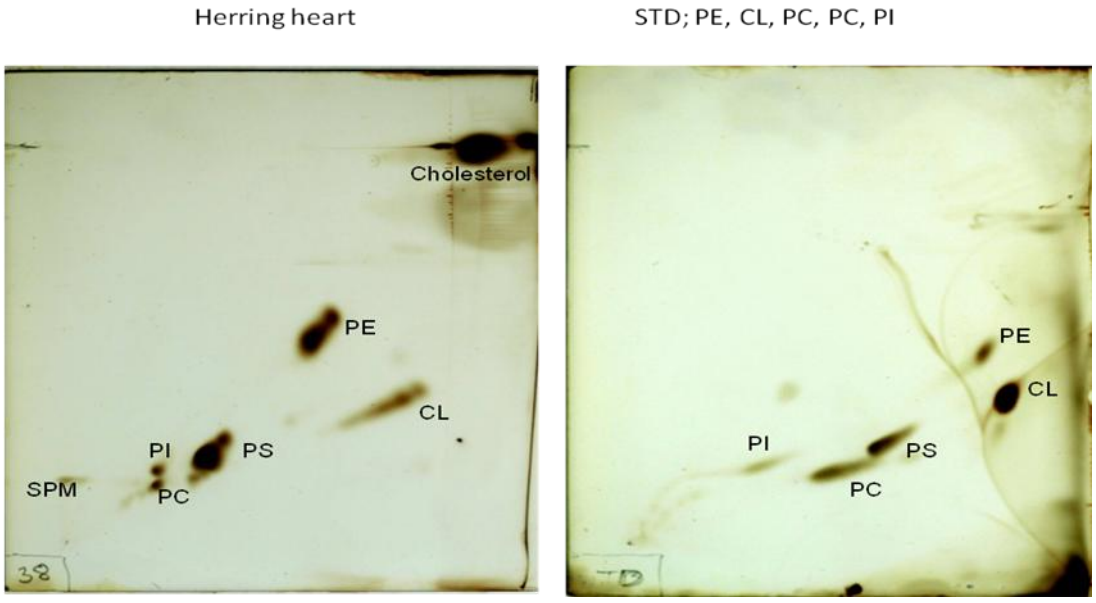


Figure 2.4.2.1 2D TLC from lipids extracted from Herring heart (left) and lipid standers (right) stained with cupric acid.

3.4.2.2 1D TLC

The lipids were separated by a two different 1D methods according to Barcelòn-Coblijn et al. (2008) and Olsen et al. (1989). The two methods are shown in figure 2.4.2.2. Because the lipid fractions also were used for further analysis using gas chromatography, proper separation was important to prevent contamination from the other lipid classes. As a better separation was achieved with a combination of the two methods this was the method used for further analysis.

Polar Lipids were separated on E.Merck silica gel 60 plates (10x10). Plates were activated and dried for 1 h. in 100°C before loading the silica gel with 50 µl lipid extract. The phospholipids were separated using a polar chloroform:methanol:acetic acid:water (55:37,5:3:2 by volume) solution (Barcelòn-Coblijn et al. 2008). The polar phase was run up to 7 cm on the silica plate. To remove the remains of the polar solution the plate was placed for 30min in an exicator under vacuum. The plate was then developed in an non-polar solution; hexane : diethyl ether : acetic acid (80:20:2 by volume) (Olsen et al. 1989) up to 9cm on the silica plate separating the neutral lipids (NL), free fatty acids (FFA), cholesterol esters and triglycerides. The plates were dried shortly before sprayed with cupric acid or a fluorescent lipid marker when used for further analysis.

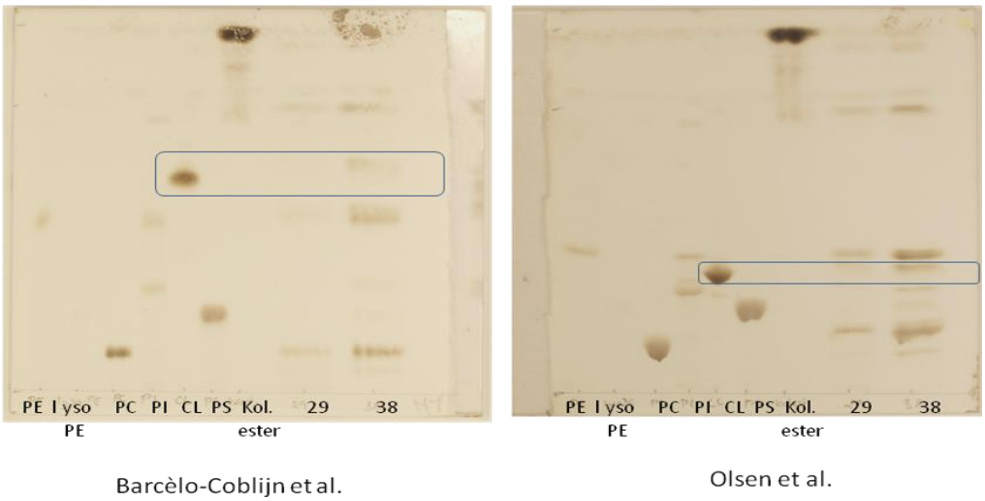


Figure 2.4.2.2 1D TLC plates with lipid standers and two heart samples named 38 and 29, separated in the two different methods and detected with cupric acid. The lipid class outlined in the picture show CL.

3.4.3 Fatty acid analysis by GC

Gas Chromatograph GC7890 (Agilent) with split less injector and flame ionization detector (GC-FID) was utilized for detection and quantification of the fatty acids. The columns were 25mx0,25 mm polar silica carbonwax columns with 0,20 µm poly ethyl glycol as stationary phase and helium 5.0 was used as mobile phase. Commercial available Nuchek standards (Nu463 Prep, Elysian, MN, USA), which are known metylesters, were used as controls and for identification as well as calculation of the response factor (equation 3.4). GC-MS was also used to verify the results. With each run there was included a blank only containing the internal standard.

Eq. (3.4) $$RF\,(x) = \frac{A(X)\ x\ M(18:0)}{A(18:0) x\ M(x)}$$

A= area of the FA top

M (18:0) = relative mass of the FA in the standard

M(x) = relative mass of the FA in the standard

By using an acid-catalyzed trans-esterification of the lipids with HCl in methanol, the phospholipids were analyzed as methyl esters. Directly after detection by TLC, the lipid extracts were scraped of the silica plates and transferred into a 15 ml reagent tube containing 50 µl, 0,1µg/µl 19:0 internal standard. The standard was later used for quantification of the FA. The samples were solved in 1 ml 2M HCl/MeOH and placed in an incubator preheated to 100°C for 2 hours. The tap was tightened after 10 min. After cooling, the samples were reduced to half its volume with N_2- gas and replaced with dH_2O. FA-methyl esters were extracted with 2 x 2 ml hexane. The solution was mixed using a whirl-mixer before centrifuged at 2000 rpm for 5 min creating a two phase system. It was used Beckman CS15R centrifuge with rotor F0850. The upper phase (hexane-phase) was transferred to a new tube using a glass pipette before reduced to 100µl with N_2-gas and transferred to a GC sample vial.

The mobile phase held a constant flow at 1ml/min. The injector temperature was set at 270°C and the detector had a temperature at 300°C. The GC program started at 90°C for 2min before rising the temperature 30°C/min to 165°C, then 2,5°C/min to 225°C and held for 3min. The temperature was then raised 10 °C/min to 240° and held there for 50min. Total run time approximately 90 min. EZ Chrom chromatography software (Agilent Technology) was used for creating methods, designing custom reports, view the calibration curves, acquire and process data and create and run batch sequences. The metanolysis reagent was made by converting liquid HCL to HCL gas by a reaction with H_2SO_4 and dissolving the HCL gas in dry methanol kept on ice (Kishimoto et al 1959).

3.5 Biomarker study

All antibodies were tested on western blotting prior to the ELISA analysis verifying the binding to the correct protein. Western Blotting combines gel electrophoresis to separate the proteins in the mitochondrial fraction with immunological detection of the separated proteins. Gel electrophoresis was run on SDS-polyacrylamide gels using 4-10% polyacrylamide and the buffer system of Laemmli. The samples were denaturated by boiling in the presence of SDS. Polyclonal anti-cod CytC oxidase anti-cod catalase and anti-cod Cu/Zu SOD was developed by Bjørn Einar Grøsvik and Prof. Anders Goksøyr, University of Bergen. (All solutions are describes in the appendix)

3.5.1 SDS-PAGE

Each sample was mixed with Laemmeli (1:3), mixed and vortexed for 20 sek. The samples were then placed in a sonicator for 3 min before they were heated to 60 °C for 5 min. After cooling samples were centrifuged at 40rpm for 5 min in a table centrifuge.

1,5 mm gels with 10 wells were made, enabling each well to carry 54µl sample with approximately 100µg protein. It was used a 4% stacking gel and a 10% separating gel. The gel was placed in a tank (BioRad) with running buffer and run for approximately 30 min on 200V. See appendix for a detailed description of reagents, stacking and separating gel.

3.5.2 Western Blotting

The SDS-PAGE gel containing separated proteins was equilibrated in blotting buffer (see appendix) for at least 10 min together with the filter paper and fiber pads. The PVDF (Polyvinylidene fluoride, GE Osmonics) membrane was cut to the size of the gel, wet in methanol for 10 seconds and soaked in dH_2O for 2 minutes before equilibrated in the blotting buffer for at least 10 min. A sandwich was made laying the different components in a specific order; Cathode side- filter pad- filter paper- pre-equilibrated gel - filter paper- filter pad (figure 3.5.2). To prevent air bubbles the air was removed rolling a glass tube over the membrane and filter paper. The cassette was placed in a tank (Biorad) filled with cold blotting buffer and an ice container to prevent the buffer running warm. The transfer tank was connected to a power supply and transferd for approximately 1,5 h at a constant voltage of 100V.

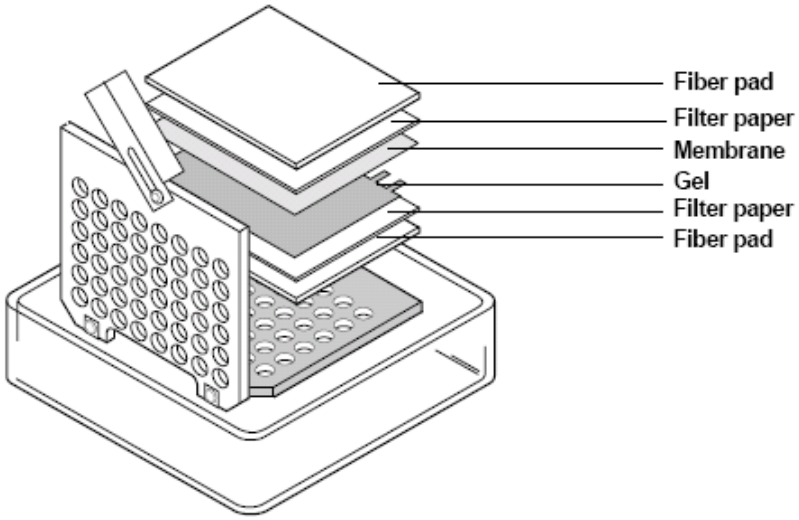


Figure 3.5.2 The order in which the membrane and gel is placed is crucial for a correct transfer from the gel as the electric current will move the proteins in one direction independent on its position.

The membrane was removed from the sandwich, and equilibrated for 5 min in TBS-T. To block sights on the membrane not occupied bye sample protein to prevent unspecific binding the membrane was soaked in the blocking (3% dry milk in TBS) solution for 2 h in room

temperature on a gentle shaker. Prior to adding the primary antibody the membrane was washed 2x5 min in TTBS. Primary antibody was diluted in 3% dry milk in TBS, according to the optimum concentration for the specific antibody (table 3.2). Antibody solution was added to the membrane, just enough to cover the membrane and incubated over night at room temperature on a gentle shaker.

Table 3.2 Cu/Zn SOD and Catalase were diluted accordingly to previous studies and CytC Oxidase was diluted after testing different consentrations 1:2500, 1:1000 and 1:200.

Antibody	Cu/Zn SOD	Catalase	CytC Oxidase
Dilution	1:400	1:1000	1:500
Size	~ 16kDa.	~50kDa.	~57 kDa

Before adding the secondary antibody the membrane was washed 2x5 min in TBS-T. Two types of secondary antibody was used; HRP-goat-anti rabbit diluted 1:2000 and alkaline phosphatases- goat-anti rabbit 1:20 000 secondary antibodies. HRP (horse radish peroxides) was detected using an ECL-method (Enhanced chemiluminescence). ECL is based on the emission of light during the HRP- and hydrogen peroxide-catalyzed oxidation of luminol. The emitted light was captured on a CCD (charge coupled device) camera and Fluor-S Max (Biorad), for qualitative analysis. Alkaline phosphatases was developed using a CDP-Star/Nitroblock substrate solution (Applied Biosysthem), and visualized using Chemiluminescence (Fujinon LAS3000). Membranes were also stained with Ponceau S, Acid Red for a rapid reversible detection of the protein bands on the PVDF membrane.

3.5.6 MDA – Malondealdehyde

A commercially available assay kit; NWLSS™ NWK-MDA, was used to measure MDA in the samples. The method is based on the reaction between MDA and thiobarbituric acid (TBA); forming a MDA-TBA_2 adduct that absorbs strongly at 532 nm (figure 3.5.6). Interference can be a significant problem in some biological samples, and some of the reagents may cause some concern for the credibility of the results. The samples were kept at lower temperatures to prevent lipid peroxidation artifacts, and samples were also added antioxidant BHT and ion chelator EDTA to prevent any additional oxidation. A data reduction method was applied to reduce non-specific TBARS related background interference by using the 3rd derivative in a $SCAN_{400\text{-}700}$ analysis (figure 3.5.6). The analysis was performed according to manufactures protocol and scanned on Varian Cary 50 Bio UV- spectrometer.

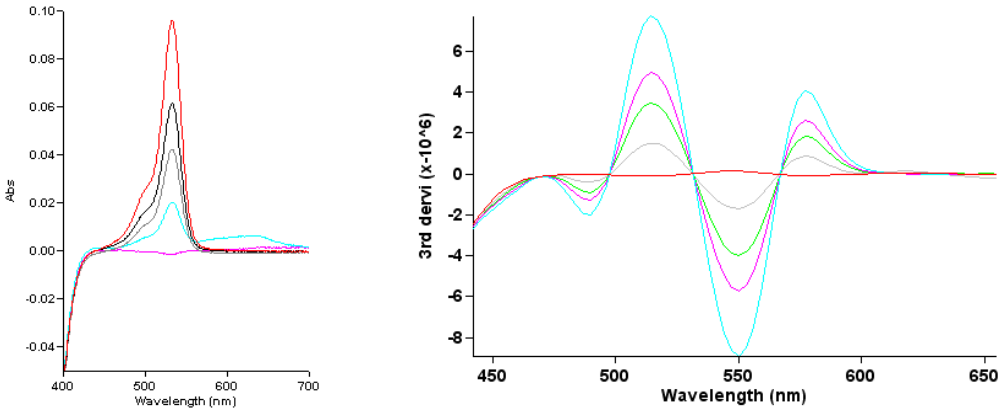


Figure 3.5.6 Absorption spectra from 400 to 700 nm of the standards used in the measurement of MDA. The standards range in concentrations 1-4µg/µl, lowest concentration shown in turquoise and the highest shown in red (right). The spectra to the left show the 3rd derivate of the absorption, the lowest concentration is here shown in gray and the highest in turquoise.

3.5.7 Carbonyl

Protein Carbonyl Assay Kit (Cayman Chemical Company, USA) was used to measure protein oxidation product carbonyl according to the manufactures protocol. The method is based on the reaction between 2,4-dinitrophenylhydrazine (DNPH) and the protein carbonyls resulting in a Shiff base, which produces a corresponding hydrazoe. The hydrazoe was measured spectrophotometrically with Asys UVM340 microplate reader at an absorbance between 365-380 nm and the amount of carbonyl is quantified using a standardized equation;

Eq (3.5) Protein Carbonyl (nmol/ml) = $[(CA)/(0.011\ \mu M^{-1})]\ [500\mu l/200\mu l]$

CA is the corrected absorbance, and the control value has been subtracted from the sample value to prevent interference from the background.

3.6 Statistical analyses

27 of the most dominating FA were included in the statistical analysis and FA making less that 1% were ignored. Samples consisting of less that 0,3µg total FA were also ignored as there appared to be oxidative damage in samples of low concentrations. When interpreting quantitative differences between two sets of data, statistical analyses are needed to determine the significant of the difference and to verify that there has been a change.

There was not a normal distribution in the ∑FA as the p-value; 0,001, was lower than the significance level alpha=0,05 (Shapiro-Wilk test, Jarque-Bera test, Lilliefors test) and the variables did not meet the assumptions of anova. A Kruskal-Wallis test was therefore used to determine the significance of the alterations in FA composition. The method is non-parametric and does not assume normal distribution. The measurement of ∑SFA, ∑MUFA and ∑PUFA data from all groups as well as n6/n3 was tested. In a factorial design the number

of experiments in 2^k, k being the number of variables investigated. The variables are studied at two levels designated by +1 and -1. The effect of each variable is shown by K_x which is the difference between the mean values of all experiments at +1 and -1. K_x also indicate the importance of the variables investigated. The response is shown by y, and n is the number of samples.

Eq (3.6)

$$K_x = \frac{\Sigma y\,(+)}{n(+)} - \frac{\Sigma y\,(-)}{n(+)}$$

To detect the dominating variables in the data set Principal component analysis was performed in Sirius 8.0. By a using a multidimensional mathematical procedure the amounts data that can be analyzed exceeds and multiple variables may be compared in the same plot. This is achieved by making principal components. The first principal component is constructed so it accounts for as much of the variability in the data as possible, the next component explains for as much of the remaining variability as possible, and so on. The sample values were transformed to level out the quantitative differences among the fatty acids to prevent the smaller samples from beeing over ruled by the more dominating FA. The FA values were standardized by dividing the value of the FA on the mean value of the group. The data was imported into the program, the fatty acids as variables and the sample as objects. The results are presented in plots of object scores and variable loadings. A short distance and small angle (minimum 0°) from the center of the plot indicated a positive correlation between objects and variables. A longer distance and a larger angle (maximum 180 °) would indicate a negative correlation. If the sample was encountered at an angle of 90°, no correlation was observed.

4. Result

4.1 Protein Concentration

Table 4.1 a-i) The protein concentration was measured using BCA™ Protein Assay Kit (Thermo Sientific) and the microplate procedure.

a) Control			
Samples	**Average mg/ml**	**Samples**	**Average mg/ml**
9.46 p	2,16	9.23 p	2,53
9.4 p	1,44	9-27 p	0,47
9.7 p	3,71	9-28 p	1,39
9.11 p	1,98	9-45 p	1,59

b) Chlorine		c) PBDE		d) PFOS	
Samples	**Average mg/ml**	**Samples**	**Average mg/ml**	**Samples**	**Average mg/ml**
H1-19 p	0,50	H2:7 p	1,24	H3-1 p	1,79
H1:10 p	0,35	H2:11 p	2,08	H3-14 p	1,03
H1:13 p	2,68	H2:13 p	0,86	H3-16 p	1,43
H1:18 p	1,06	H2:20 p	0,48	H3-21 p	1,05
H1:2 p	0,66	H2-2 p	0,47	H3-23 p	1,11

e) 4-tert-nonylphenol		f) 4-nonylphenol	
Samples	**Average mg/ml**	**Samples**	**Average mg/ml**
T4-3 p	1,99	N4-7 p	1,38
T4:9 p	1,58	N4-19 p	0,73
T4:13 p	1,31	N4-17 p	1,05
T4:21 p	1,19	N4-20 p	0,79
T4:22 p	1,24	N4-25 p	0,84

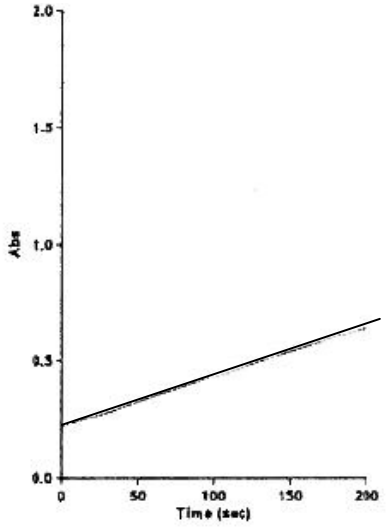
g) Crude oil		h) Mix + oil		i) Mix	
Samples	**Average mg/ml**	**Samples**	**Average mg/ml**	**Samples**	**Average mg/ml**
H5-3 p	0,91	H7.9 p	1,87	H8:4 p	1,58
H5-7 p	1,48	H7.12 p	2,97	H8:10 p	0,65
H5-18 p	1,26	H7.14 p	2,26	H8:11 p	1,17
H5-21 p	1,33	H7.16 p	4,48	H8:14 p	0,74
9-22 p	2,36	H7.22 p	1,29	H8:23 p	0,96

4.2 Citrat synthase activity

The rate of enzymatic activity and change in concentration dc_B/dt was calculated from the absorption rate. The mitochondrial pellets clearly show a higher citrate synthase activity than the supernatants (super 2 in the table 4.2). Figure 4.2 a give a graphic representation of the enzyme activity. The citrate synthase was only measured in control samples.

Table 4.2 Citrate Synthase activity measured in two different fractions from the centrifugation, the mitochondrial fraction (pellet) and supernatant.

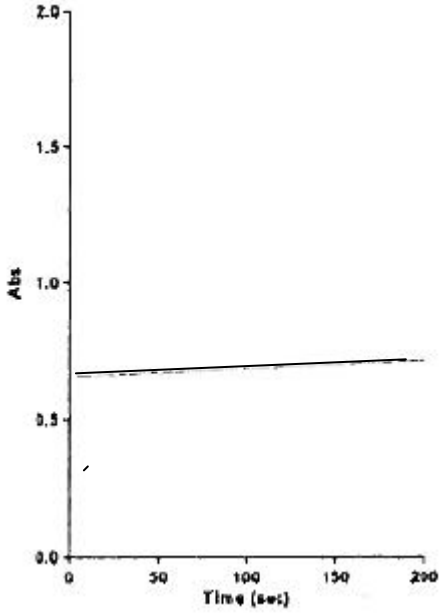
Sample	Concentration protein µg/µl	Abs/min	Average Abs/min	µmol/min/mg	Average µmol/min/mg
Standard 1	172	0,0022		1,88098	
Standard 2	172	0,0021	0,00215	1,79549	1,83823
9.2 pellet	50	0,0012		0,00173	
9.2 pellet	50	0,0012	0,0012	0,00173	0,00173
9.2 pellet	100	0,0023		0,00169	
9.2 pellet	100	0,0025	0,0024	0,00184	0,00177
9.2 Super. 2	50	0,0002		0,00029	
9.2 Super. 2	100	0,0004	0,0003	0,00029	0,00029
9.6 pellet	50	0,0012		0,00175	
9.6 pellet	50	0,0012	0,0012	0,00175	0,00175
9.6 pellet	100	0,0023		0,00168	
9.6 pellet	100	0,0022	0,00225	0,00161	0,00164
9.6 Super. 2	50	0,0002		0,00030	
9.6 Super. 2	100	0,0003	0,00025	0,00023	0,00026



a)

b)

c)



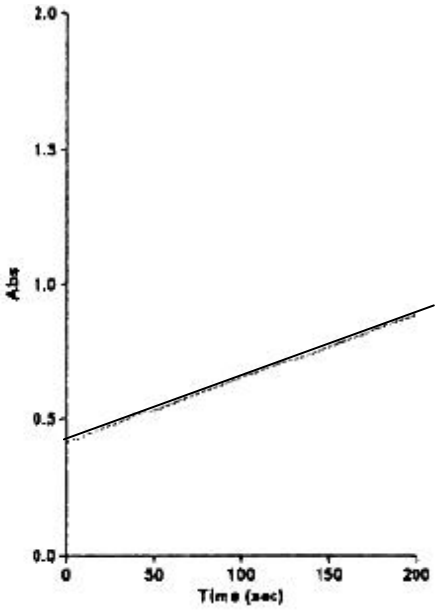


Figure 4.2 a) The absorbance of commercial available citrate synthase standard 1,795 µmol/min/mg. b) measurements from the mitochondrial fraction at a concentration of 100 µg/µl and the supernatant c) from the same sample as the mitochondrial fraction 100 µg/µl.

4.3 Biomarker analyses

The results of the biomarker study including CytC oxidase, Zn/Cu SOD and catalase are reported in the figures below. CytC oxidase is presented in figure 4.3.1 a-b and figure 4.3.2. Despite the variety of tests, there were no bonds on any of the membranes. It is uncertain if the antibody would not attach to the target protein or if the lack of interaction with the secondary antibody caused the problem. The Zn/Cu SOD antibody was tested on both total homogenate (figure 4.3.2) and isolated mitochondria (figure 4.3.3). Catalase was only tested on isolated mitochondria (figure 4.3.4). All membranes were incubated with alkaline phosphate secondary antibody and developed using a CDP/Nitroblock substrate solution and visualized using Chemiluminescence.

a) b)

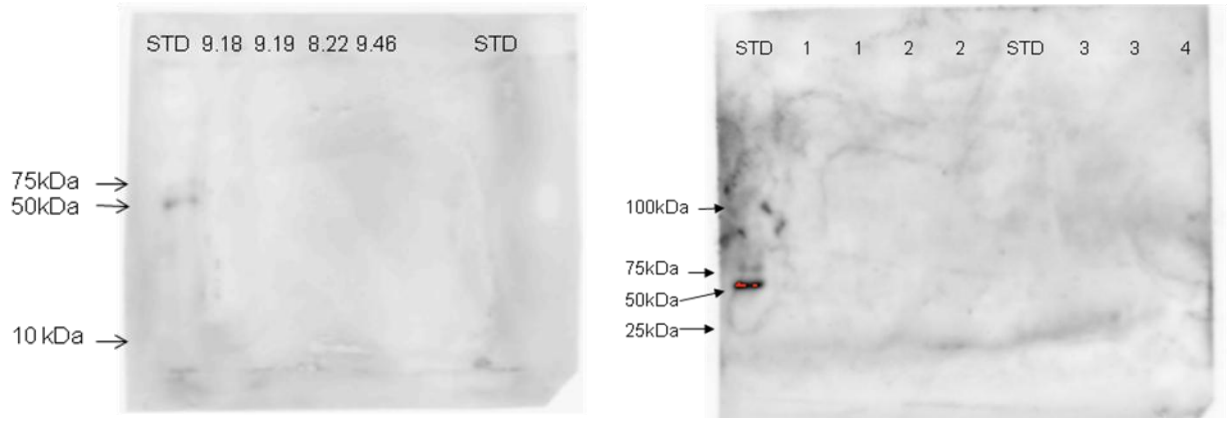


Figure 4.3.1 Western Blots incubated with anti-cod CytC oxidase. On the first membrane (a), mitochondria pellets from control (9.18, 9.19, 9.46) and exposed sample (8.22) are presented. On the second membrane (b) shown the fractions from the centrifugation process ; Nr. 1 and 2 are total homogenate from the first and second centrifugation (2300rpm), 3; supernatant from the third centrifugation (9200rpm) and 4; the mitochondrial pellet.

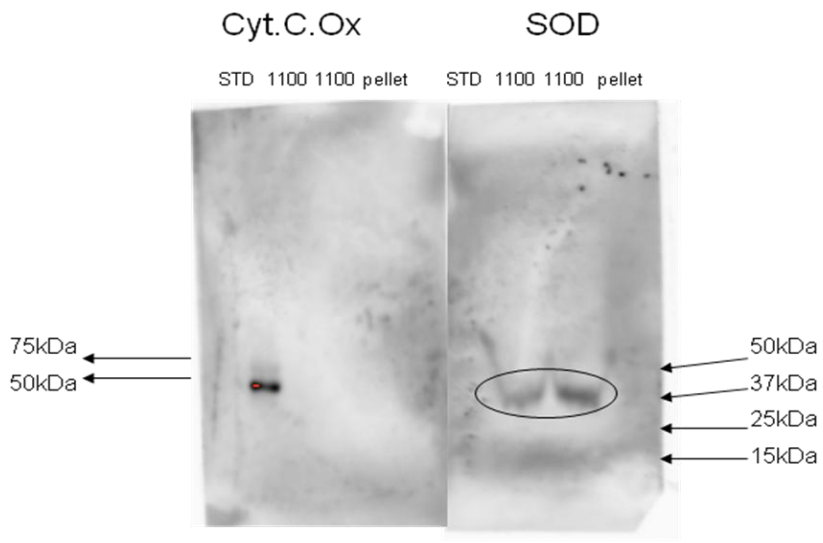


Figure 4.3.2 Western Blot incubated with anti-cod CytC oxidase (left) and anti-cod Cu/Zn SOD (right). A different centrifugation technique has been applied to the total homogenate, which was centrifuged at 1100 g for 20 min (samples marked 1100). The pellet followed normal procedures.

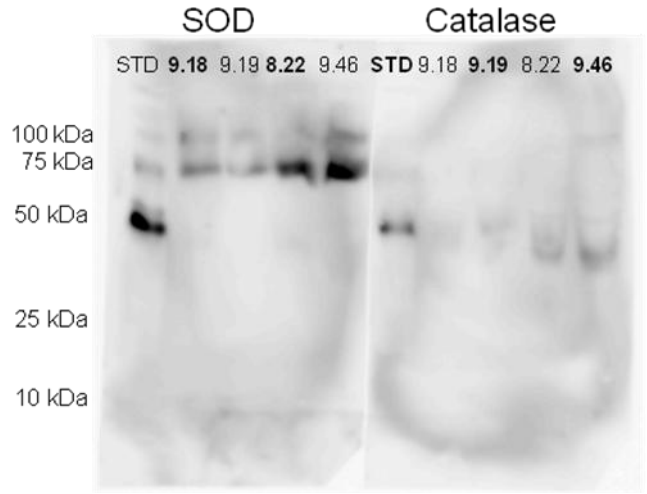


Figure 4.3.3 Western Blot incubated with anti-cod Cu/Zn SOD and anti-cod Catalase. Both control (9.18, 9.19, 9.46) and exposed sample (8.22) are presented on the membrane. An increase in the concentration of the primary antibodies was made, SOD 1:200 and Catalase 1:500.

4.4 MDA

MDA was measured in both in isolated mitochondria (figure 5.4 a-d) and in total homogenate (figure 5.4 e). Control samples (figure 5.4 a-c) and exposed samples (figure 5.4 d) were included. The absorption spectra was set to 400-700nm and the MDA-TBA_2 adduct should give a significant increase in the absorption around 532nm.

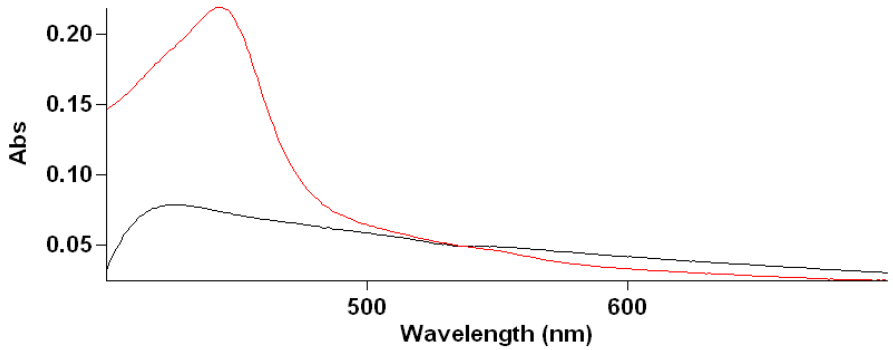


Figure 4.4 a) The spectra show Abs/wavelength in a control sample. The red line shows the sample and the black line show the blank.

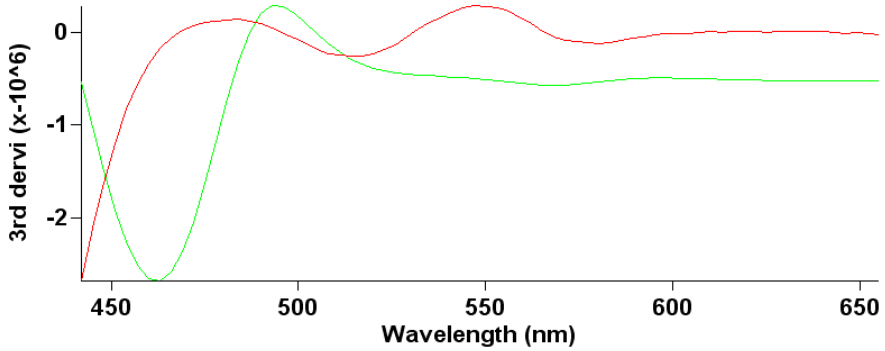


Figure 4.4 b) Measurements of MDA in a control sample. The spectra show 3rd derivate of Abs/wavelength, the red line is the blank. There is a small top around 490nm.

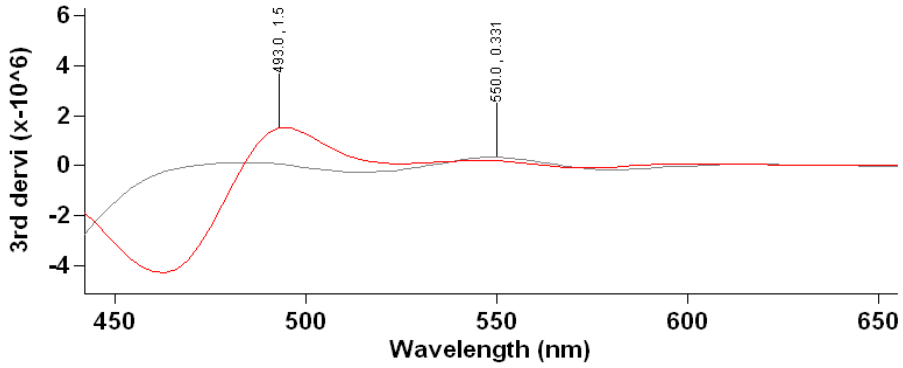


Figure 4.4 c) 3rd derivate/wavelength of the same control sample as shown in the previous spectra. The sample has been extracted with butanol to remove interfering components such as hemoglobin. There is still an absorption top around 490nm and no significant increase in the absorption around 530nm. The spectra show both Abs/wavelength (top) and 3rd derivate of Abs/wavelength.

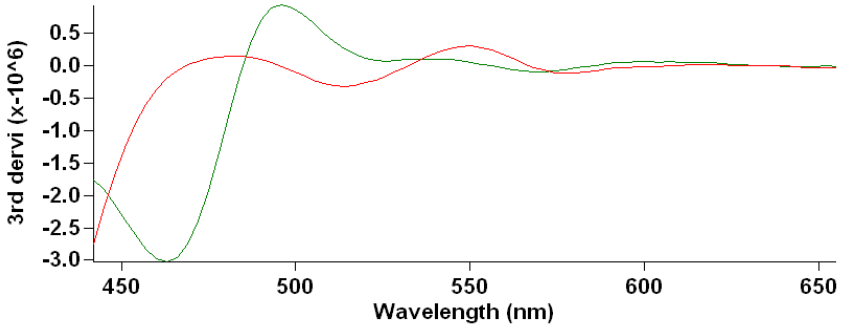


Figure 4.4 d) MDA measurement of a sample exposed to the mixture (chlorinated brominated and fluorinated compounds). The spectra show the 3rd derivate/wavelength and the sample show a peak similar to the one in the control sample around 490nm. The sample has been extracted to remove potential interference

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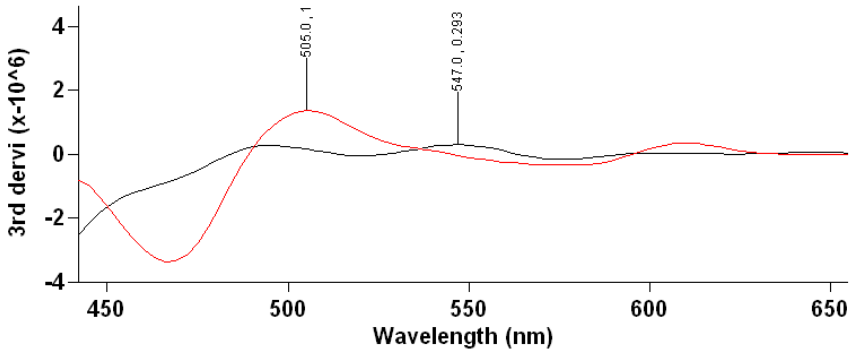


Figure 4.4 e) Measurements of a more lipid rich fraction from the isolation were made to see if the shift in the absorption top would increase with the amount of triglycerides. The top has shifted but in the opposite direction of what we anticipated. The top is now around 505nm. This sample is also extracted to remove other potential interference.

4.5 Carbonyl

Table 4.5 a) Carbonyl measured in control samples and exposed samples at 380nm.

380 nm	Carbonyl	Protein mg/ml	carbonyl/protein nmol/mg
Control	3,864	4,513	0,856
	3,636	5,126	0,709
	3,182	5,350	0,595
Mix	3,182	4,169	0,763
	2,727	4,938	0,552
	5,000	6,092	0,821

Table 4.5 b) Carbonyl measured in control sample and exposed sample at 365nm.

365nm	Carbonyl	Protein mg/ml	carbonyl/protein nmol/mg
Control	2,273	2,229	1,020
Mix + Crude oil	3,864	4,246	0,910

4.6 FA analysis

The FA analysis from CL, PE and PC is reported in this section. The concentrations have only been measured by protein concentration (Table 4.1) and by the total µg FA from the TLC. The samples varied greatly in the protein concentrations, which in turn affected the amounts of FA in the samples. When plotting the protein concentration and FA µg there was a correlation between the protein and FA (figure 4.6). As some of the samples were quite low in protein concentration, this affected in particular CL, which is present in much lower concentrations then PE and PC. In the PCA score plots the amount of FA (µg) and amount of protein (µg) in the pellet was plotted as the dependent variables and the samples as the objects. In CL (a) this contributed to ~ 25% of the variance in the sample. In PE and PC the same plot explained respectivly ~22% and ~30% of the total variance. In all phospholipid classes there seemed to be no correlation or a positive correlation between the protein concentration and the amounts of FA. There were however also samples that were negatively correlated, this might be due to loss of the sample during filtration, extraction or TLC

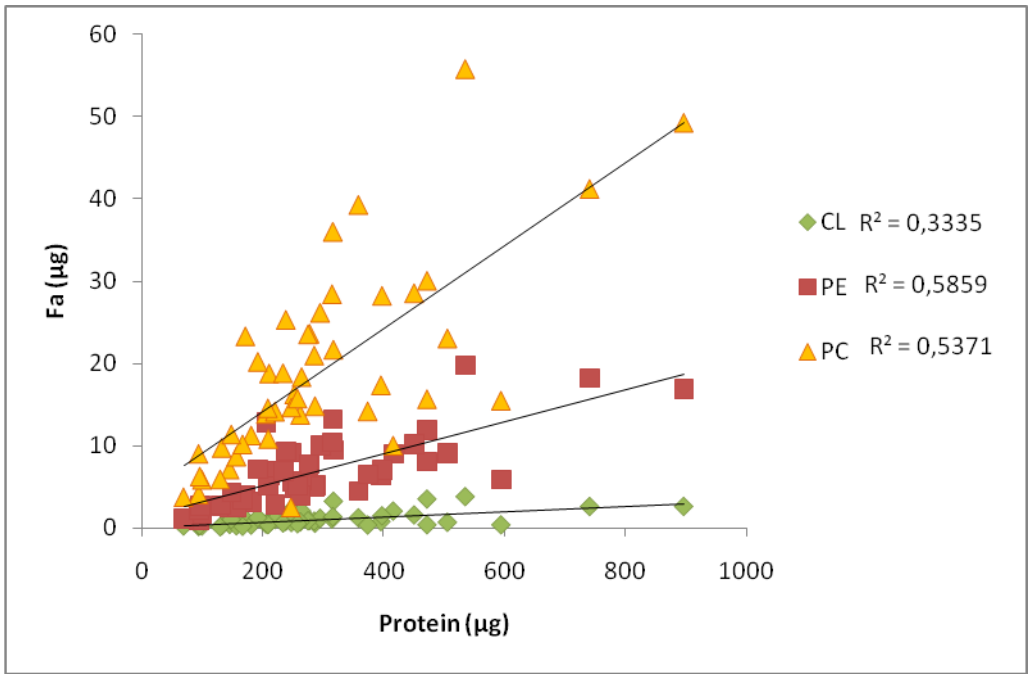
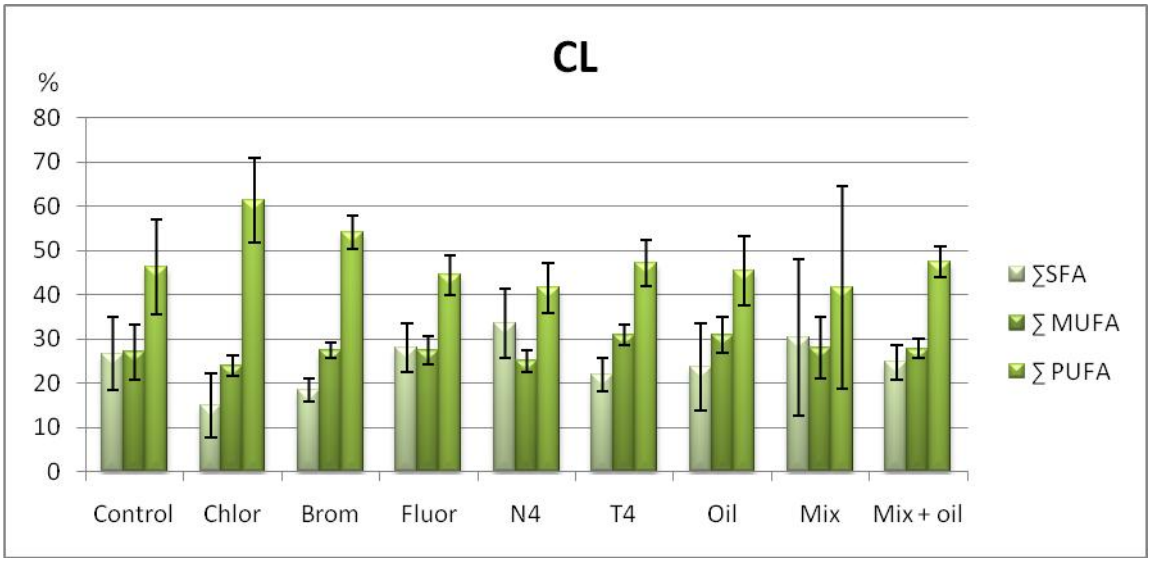


Figure 4.6 a Protein concentration µg and FA µg in CL, PE and PC.

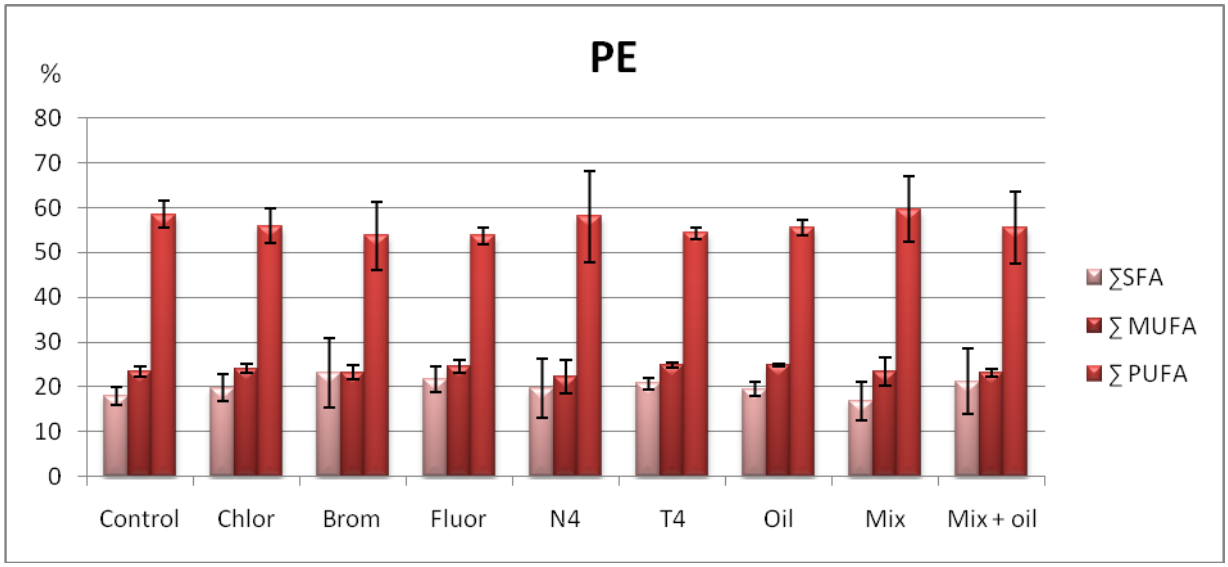
The saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in each of the treatments are represented in figure 4.6.1 a-c for respectively CL, PE and PC. N4 and T4 represent the treatments with 4-Nonylphenol and 4-tert-nonylphenol. The values show the average of the total sum from FA in the samples within the group, the error bars rapport +/- standard derivation. The PUFA was the dominating FA in all the phospholipid groups. PC had a slightly higher level of SFA than CL and PE as expected from the high levels of 16:0 found in PC (Kraffe et al. 2007). There was a slight increase of SFA in the treatments with 4-Nonylphenol (N4) and the Mixture group in CL, but these changes were not significant (p=0,05).

The relationship between PUFA (n-6) and PUFA (n-3) in the three PL classes CL, PE and PC is reported in figure 4.6.2 a-c. In the PUFA's we would expect a decrease in n6 and an increase in n3 which leads to a decrease in the n6/n3 ratio. Althougt it appared to be a a slight decrease in the Chlorinated and Brominated samples in CL, these changes were not significant (p=0,05).

a)



b)



c)

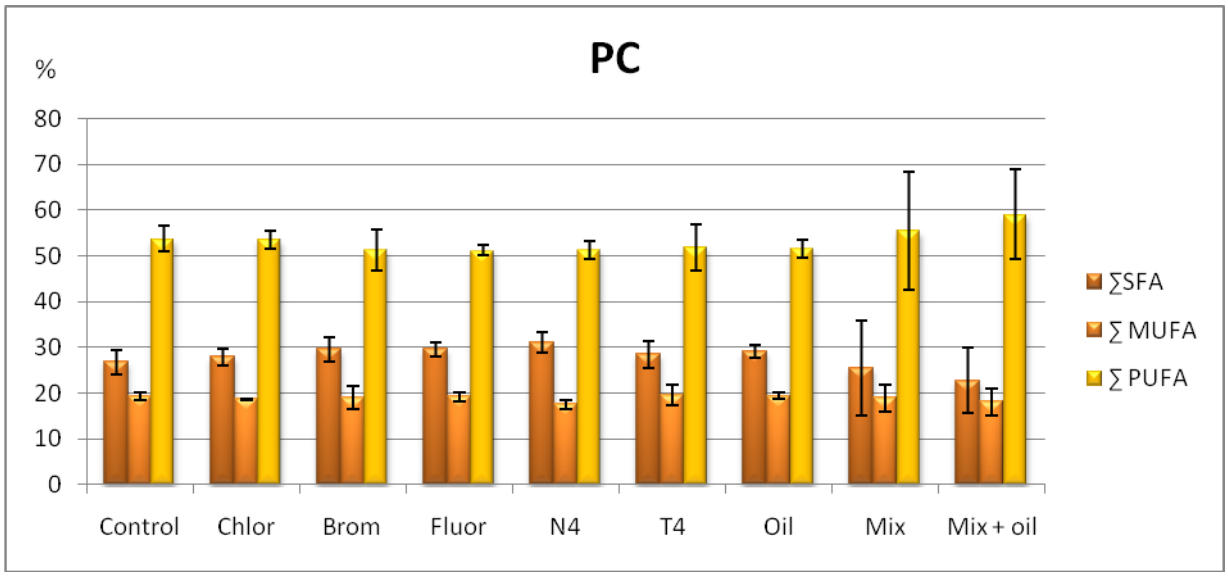


Figure 4.6.1 The relationship between satureted (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in CL(a), PE (b) and PC (c) in %. The error bars show +- SD.

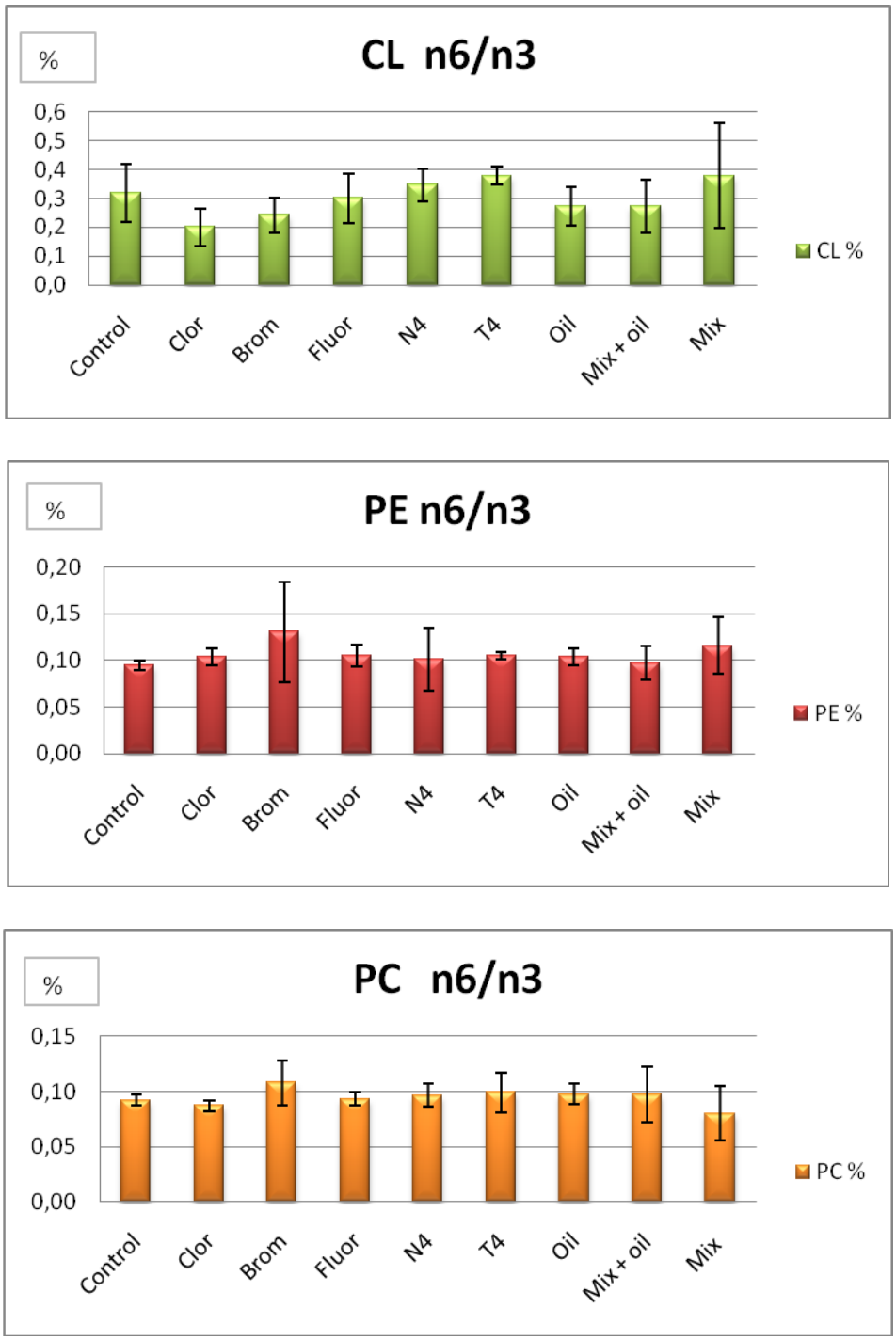


Figure 4.6.2 The relationship between PUFA (n-6) and (n-3) found in the CL fraction (top), PE fraction (middle) and PC fraction (bottom). The errorbars show the +- SD of the sample.

Table 4.6 Explanation of the labels used in the PCA-plots. In PE, the *C* is swhiched out with an *E*, in PC a *P*.

Treatment	Phospholipid class	PCA-name
Controls	**CL**	**9 ** C**
Chlorinated compounds	**CL**	**1 ** C**
Brominated compounds	**CL**	**2 ** C**
Fluorinated compounds	**CL**	**3 ** C**
4-Nonylphenol	**CL**	**N ** C**
4-tert-Nonylphenol	**CL**	**T ** C**
Crude Oil	**CL**	**5 ** C**
Mixture + crude oil	**CL**	**7 ** C**
Mixture	**CL**	**8 ** C**

** the spesific number of the sample in the group.

The FA composition of the individual FA is rapported in the Table A.2 in the appendix. In the CL phospholipid class the SFA in all treatment groups were dominatinated by 16:0 and 18:0 which contributed with ~20% with exeption to the chlorinated and brominated samples which were slighly lower ~10%. The dominating MUFA was 18:1 (n-9) contributing with ~ 7% and 18:2 (n-3), 20:5 (n-3) and 22:6 (n-3) were the dominating PUFA making up to ~40% of the total FA. In the PCA plots with FA's as the dependent variabel, the principal component 1 (PC1) contributed to 30% and PC2 to 22% of the total variation. There did not appare to be any systematic correlations in any directions in the different treatmens nor controls (blue) and several of the treatmets overlaped. In the loading plot 24:1(n-9) was negativly correlated to the total FA, whear as 22:1 (n-9), 20:1 (n-11), 18:1(n-11) and 16:1(n-9) were positively correlated. There also seemed to be a connenction between the saturated FA which were clusterd togeather and slightly posivily correlated.

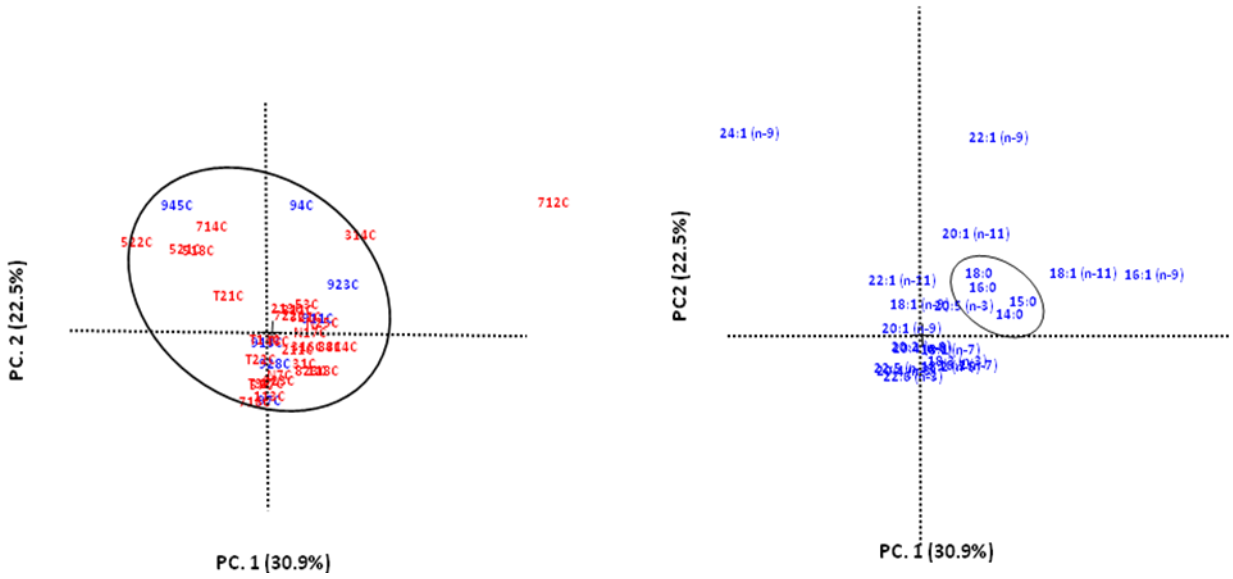


Figure 4.6.3 PCA score plot and loading plot (right) for CL from all the reatments with the FA as dependent variable and the samples as objects. The controls are shown in blue and are marked by the circel. The rest of the groups are marked with red. The labels are explained in table 4.6

In PE (table A.3) 16:0 and 18:0 also dominated the SFA contributing with ~20%, 18:1 (n-9) and 18:1 (n-11) made respectivly ~8 and ~6 % of the total FA and were dominating in the MUFA group. The dominating PUFA were 20:5 (n-3) and 22:6 (n-3) making almost 50% of the total FA. This correlated with all the treatments. In the PCA score plot with FA as the dependent variabels PC1 contributed to 53% of the variation. The controls were less scatered in PE than in the two other phospholipid classes. The reason for his is uncertain. There was also a strong positive correaltion in the samples from group 1 and 2, which were the chlorinated (1) and brominated (2) treated samples. The loading plot showed a positivly correlation by 22:1(n-9), 16:1(n-9), 16:1(n-7) and the saturated FA 14:0, 15:0 and 16:0.

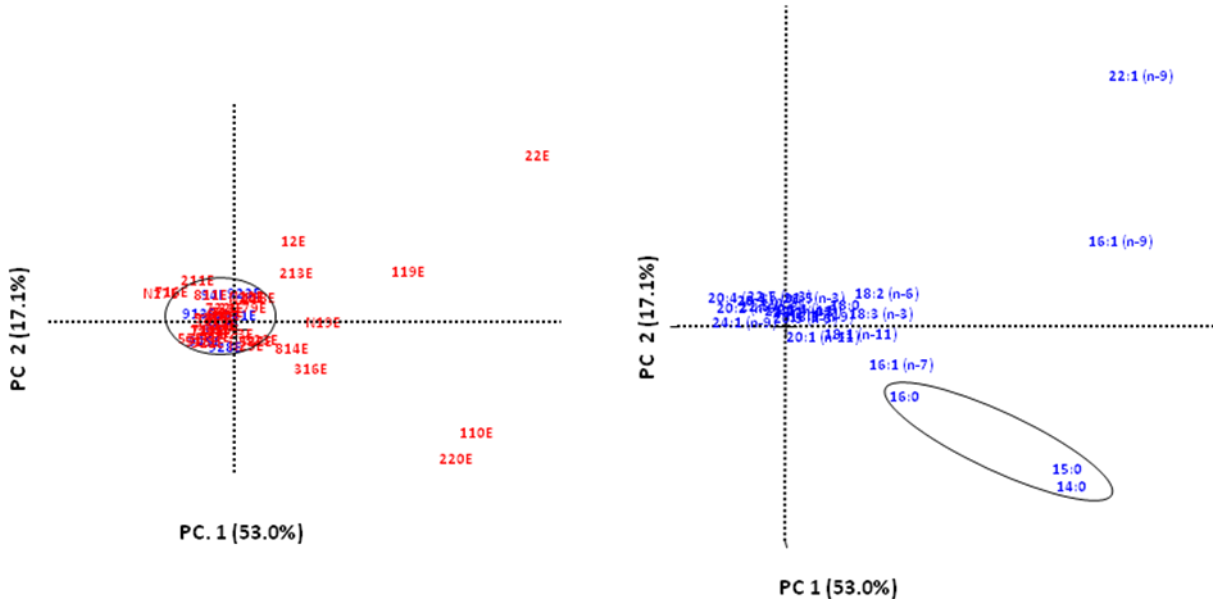


Figure 4.6.4 PCA score blot of all the treatments from PE with the relative amounts of FA as dependent variable (left) and PCA loading plot (right). The controls are shown in blue and are marked by the circel, and the rest of the groups in red. The labels are explained in table 4.6

In the PC class, 16:0 alone contributed to over 20% of the total FA. 18:1 (n-9) dominated amoung the MUFA's with ~9% and 20:5 (n-3) togeather with 22:6 (n-3) dominated in the PUFA with over 45% of total FA. These findings correlated in all the treatments. In the PCA score plot with FA as the dependent variabel PC1 contributed to ~40% of the variation. There appared to be a positive correlation in some of the samples from the mix+oil group and mix (group 7 and 8), but only including some of the samples in the groups. PC2 explained ~20% of the variation. In the chlorinated and brominated samples (group 1 and 2) there was a posetivly correlation to the total FA. In the loading plot it was found a positive correlation of the 22:1 (n-9) and 24:1(n-9). A cluster of the saturated FA 14:0, 15:0 and 16:0 was found to be slightly negaivly correlated in contrast to the other phospholipis classes.

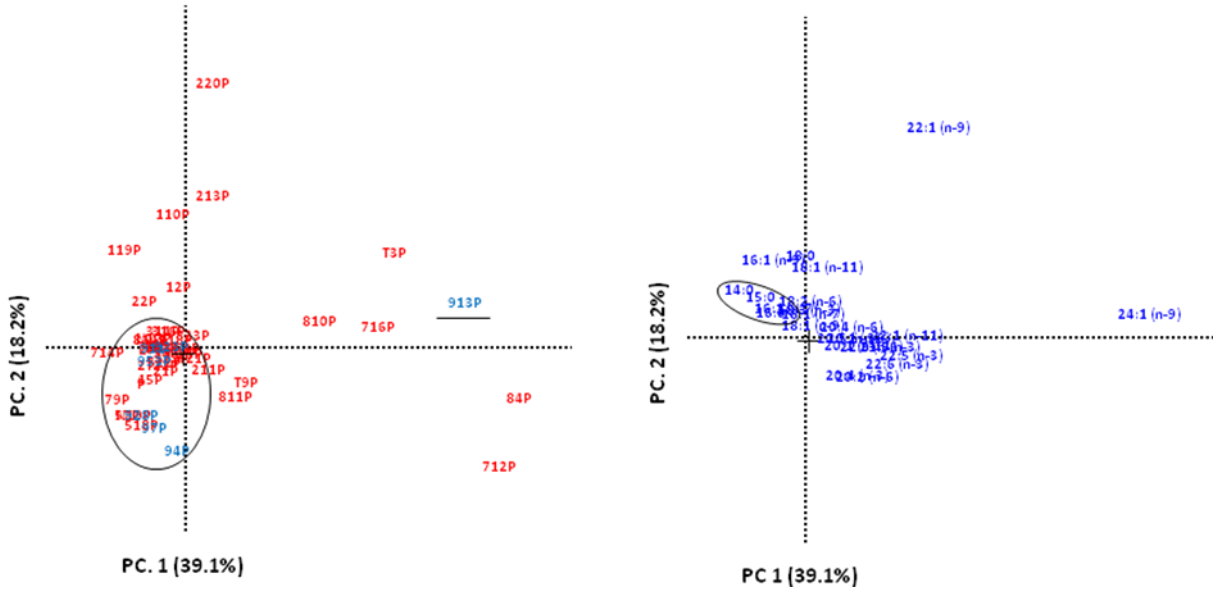


Figure 4.6.5 PCA score blot of all the treatments from PC with the relative amounts of FA as dependent variable (left) and PCA loading plot (right). The controls are shown in blue and are marked by the circel, with exeption of one (marked in blue with underline) and the rest of the groups in red. The labels are explained in table 4.6

4.7 Contaminants

There was found a number of unknown compounds in all the phospholipid classes. PE and PC did not seem to be affected by the contamination but as there were particulary low concentrations of CL, the unknown contributed to a consideralble amount (up to 50%) in these samples. Some were identified as hydrocarbons and phtalate by GC-MS. The chromatogram is shown in the appendix, table A.1 and figure A.1-A.3. The phtalate is tought to decend from plastic in some of the equipment, possably the centrifuge tubes as it was precent in all samples.

5 Discussion

In the biomarker study there were several issues that did not correlate with the expected results. The Western blot analyses that were preformed to verify the antibodies before starting with ELISA analysis showed that the primary antibodies did not serve their purpose. As stated previously in the results, there was both unspecific binding, and no binding at all. The membranes were all stained with Ponceau S, Acid Red for a rapid reversible detection of the protein to verify the transfer from the gel to the membrane. It was found protein on all gels. There were also used two different types of secondary antibody to reassure that the problem was not lying here. The anti-cod catalase and anti ZnCu-SOD was produced at the university of Bergen (UiB) in connection with another study on liver homogenate from cod. Although there was some unspecific binding with both catalase and SOD, the target protein band was much stronger then the other unspecific bands and reliable results were produced (Grøsvik BE. et al 2007). The same procedure was repeated in isolated mitochondria with some modifications, using the same concentrations of antibodies. The unspecific binding in the isolated mitochondria was much stronger that the actual target protein (figure 4.3.3). As catalase is also found in the Peroxisomes in the cells (Alberts 2002) there might have been a higher concentration in the total homogenate that in the isolated mitochondria. This would explain the stronger bands and the more insignificant meaning of the unspecific binding in total homogenate in the previous study. The antibodies used are also polyclonal and have not been affinity purified this may be the reason for the unspecific binding. As stated earlier in the chapter on biomarkers (2.3.4), there are two types of SOD; Mn-SOD and ZnCu-SOD. Mn-SOD is found in the mitochondria, where as ZnCu-SOD is found in the cytosol. In this study we tested if the anti-cod ZnCu-SOD was able to detect Mn-SOD. Because polyclonal antibodies are often less specific than monoclonal, a cross binding to the Mn-SOD is what we hoped to achieve. This unfortunately did not work for our benefit. On the other hand it shows that the antibody is specific for ZnCu-SOD which might be useful in another study. When

applying the same centrifugation method as in the previous study (Figure 4.3.2), binding to the protein was achieved but at a slightly higher weight than expected, around 37kDa. Mn-SOD is expected to appear at 25kDa.As the band most likely was not Mn-SOD, no further attempts were made to detect the protein. The anti-cod CytC Oxidase was the antibody which there has been the least research on, and it had previously not been used in cod. The lack of cross-reactivity with the antibody was probably due to the inability to bind to the expected sequence in the protein. Because of economic reasons, the antibody had been produced with binding affinity towards only one epitope, which increases the possibilities that the binding site is missed (Grøsvik et al 2007).

The MDA and Carbonyl study also had some difficulties. The MDA assay is based on a spectrophotometrical method measuring the reaction between MDA and TBA forming a MDA-TBA_2 adducts which absorbs strongly at 532 nm (3.5.6). MDA is a result of peroxidised lipids. The measurements were made in both control samples and exposed samples. The mitochondria fraction in all samples gave an absorption peak around 490 nm (figure 5.3.1 a). An extraction method for removing potential interference from hemoglobin was included, but the absorption peak remained around 490nm (figure 5.3.1 b-d). In the controls the results were positive as no obvious oxidation had occurred during the isolation. In the exposed samples the observed peak (490nm) differed from what expected (532nm) was based on the manufactures protocol (Northwest Life Science Specialties, LCC, and Vancouver). As the measurements were made with a spectrophotometrical method, triglycerides may interfere with the measurements giving false low values. Fat may interact the sample causing the absorbance at to increase and give a high level of background noise covering the absorption top. A lipid rich fraction from the mitochondria isolation was measured to compare and investigate if an increase in triglycerides would cause an increase in background noise or a shift in the absorption peak. The protocol explains that there might be a shift in the peak absorbance depending on the sample content. The spectra however seemed similar to the control and the exposed sample with exception of the absorption peak, now 505nm, which seemed to have shifted slightly towards the expected peak 532nm (figure 5.3.1e). As there were no difference between the control and the exposed sample, this may imply that there is no peroxidation of the lipids in neither of the samples, or both. Antioxidants

such as EDTA and BHT are also added to prevent additional oxidation, this can also have resulted in a down regulation of the already oxidized lipids and MDA. Since the background was too high to make reliable results, we did not make any conclusions based on the results of the measurement. A different method was instead tested for similar effects.

The carbonyl assay is also a measure on oxidative damage, but on proteins and aminoacids. Similar to the MDA assay, this is also a spectorphotometrical method, based on the reaction between carbonyl and DNPH (2,4-dinitrophenylhydrazine). Also here both control samples and exposed samples were tested and the results in both groups were similar (table 5.4.1). The control samples showed slightly higher values than the exposed samples, but there was no significant difference. The samples were kept on ice at all times and the centrifugation was performed at 4°C to prevent additional oxidation. Streptomycin treatment was also performed to prevent false high estimation of carbonyl caused by nucleic acid. Although this procedure is a commercial kit, it includes many steps to prepare the samples and errors and damage to the samples may occur. Too low protein concentrations may also cause false negative results. The manual, does, however recommend concentrations between 1-10µg/µl, leaving the samples in the lower range, but still within the recommended range. BSA (bovine serum albumin) has also shown to interfere with the assay giving slightly higher levels of carbonyl. 0,05% BSA was added in the homogenization solution during the isolation of mitochondria and was present in equal amounts in both controls and exposed samples. In a study performed on rat liver mitochondria the baseline levels of carbonyl were approximately 1,40 nmol/mg protein, slightly higher than measured in cod 0,72nmol/mg. After liver ischemia-reperfusion, which is known to cause ROS, the carbonyl levels were 3,34 nmol/mg protein (Domenicali et al 2000), a significant increase. Although rat mitochondria differs from cod mitochondria, the high values found it the rats indicate that there was not a significant oxidation of the proteins in the cod samples, in controls nor exposed liver mitochondria.

Fatty acid composition

Desaturation of fatty acids is an important adaptation mechanism for fish to maintain membrane fluidity under thermal stress (Sinensky et al. 1874; Kraffe et al 2007; Wodtke et al 1991). As we believe that the same adaptation is seen with oxidative stress, a decrease in PUFA and a slight increase in the MUFA and SFA were expected. In CL, the 4-Nonylphenol and the mixture +oil group it was observed an upregulation of SFA and a down regulation of the PUFA. It has previously been reported effects of alkylphenols in Atlantic cod (Meier et al 2007; Bhone-Kjersem et al 2009) and the mixture contain a high dose of the chlorinated, brominated and fluorinated compounds in addition to the crude oil and would appeaed to be one of the more toxic treatments. From the n6/n3 ratio, the treatments with the chlorinated and brominated compounds together with the oil and mixture+oil treatments there appeared to be an increase in n3 FA and a decrease in the n6/n3 ratio. Although the results correlates with the hypothesis, there were no significant differences with the treatments in any of the exposure groups (p=0,05).

In the two other phospholipid classes; PE and PC, no significant effects were observed with any of the treatments. In the PCA-plots it was found considerable less variation in the PE fraction that in the two other fractions. The reason for this is uncertain. All phospholipid classes seemed to have a cluster of the SFA 14:0, 15:0 and 16:0 (18:0 was also included in CL). This might be explained by their connection in the FA synthesis. The SFA were positively correlated in CL and PE, but not in PC. In CL 24:1(n-9) was negatively correlated where as the FA was positively correlated in PC. 24:1 (n-9) is connected to the FA 22:1(n-9) through the FA synthesis. 22:1(n-9) was positively correlated in all phospholipid classes.

As with all biological material, there are natural variations between the individuals, also in fish species. As there were a substantial amount of samples and a relatively comprehensive analytical method, only 5 samples from each of the treatments were chosen with exception of the control group where there were 8 samples. This contributed to the rather high standard derivation in the samples, making the results less reliable and statistical analysis became difficult to perform. The many steps involved in the preparations and analyses also increase the possibilities of oxidation and contamination. If there already exist oxidative damage or oxidation products such as MDA and carbonyl, it may enhance additional oxidation in the sample (Gupta et al. 2009; Levine et al 1994). Extra precaution was taken to prevent

additional oxidation by keeping the samples on ice, using centrifuges with cooling system and storing the samples in -20°C and -80°C with N_2. The assay of carbonyl also indicated that there was no oxidative damage in exposed or control samples although these results should be interpreted with caution.

Due to the extremely low concentrations found in CL, the GC analyses were detecting FA in the lower area of the detection limit. Interference from contaminating hydrocarbons (HC) and plastic subcomponents such as phthalate became a particularly problem in these samples as the contamination peaks dominated in some of the samples. Many extra hours were put into manually detect the low peaks as the chromathography software missed and overlooked the low values or the contaminant overlapped the sample peak.

Compared to rats and microorganisms, the amount of research performed on fish and fish biology, is very narrow and limited. As fare as I'm concerned, studies on the FA composition in mitochondrial cod (Gadus morhua) liver has not been published. Although there has been published articles concerning the mitochondrial FA composition in trout (Kraffe et al. 2007), the mitochondria are isolated from red muscle, and differ slightly from the findings in this study. The lack of reference values makes it more difficult to interpret the results when there is great variation between the parallels. When excluding samples from the data set, a criteria should be determined for discharging samples that does not meet the standard of the rest of the samples. One of the control samples in the study appeared to be oxidized, as it was found very high levels of SFA ~60% and low levels of PUFA ~15% in CL (data not represented in the paper). The same effect was seen in PE and PC. The sample was also found to have low protein concentrations and µg FA (0,15µg). Similar effects were seen in other samples with low protein and FA concentrations, and a criteria of 0,3 µg FA was therefore determined. When including oxidized or broken samples in PCA plots, the extreme variations seen in these samples overshadow the actual differences in the other samples.

In a future study greater amounts of liver sample should be used when isolating mitochondria, especially if the phospholipid of interest is CL.

6. Conclution

In an aquatic environment, despite the presence of constitutive or enhanced antioxidant defense systems, increased levels of oxidative damage will occur in the organisms exposed to contaminants that stimulate the production of ROS (Livingstone et al. 2001). The responses of fish are dependent on the species, enzymes and single or mixed contaminants. As cardiolipin is located in a highly ROS trafficated pathway, oxidation of this particular lipid seems probable. After 8 different treatments involving ROS inducing pollutants there were not found significant effects in any of the treatments in any of the pholpholipid groups CL, PE or PC. Small effects may be difficult to observe when the number of parallels is limited and standard derivation is high. As this was a part of a pilot study, important knowledge concerning analytical methods and sample material however, has been gained. A potential FA composition model for the three phospholipids from cod mitochondria has also been described. The results should however be interpreted with caution.

7. Future aspects

In collaboration between Institute of Marine Research and The Department of Biomedicine a study concerning the toxic effects of pollutants upon mitochondria in cell cultures. The study has a similar combination of the contaminants used in this study, both individually and in combination with others. After exposure the viability and potential ROS is to be measured. The study will also focus on fatty acid composition and the cell signaling pathways in action. As he study is performed *invitro*, there is a more controlled environment and possibilities for a greater number of samples.

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Appendix

Chemicals used at Department of Biomedicine

The reagents have been purchased from Sigma Aldrich if nothing else is stated

Western Blotting

Blotting buffer

3,03 g Tris

14,4g glycine

20% (v/v) methanol

make up for final volume of 1000mL

with dH_2O.

Store at 4°C.

Tris-buffered saline (TBS) pH 7,5

4,8 g Tris base

58,4 g NaCl

1800mL dH_2O

Adjust pH with 5M HCl, adjust volume to 2000mL with dH_2O.

Store at 4°C.

TTBS:

TBS containing 0,05% (v/v) Tween-20.

Dissolve 1mL Tween-20 in 2000mL TBS.

Store at 4°C.

DEA solution

400ml H_2O

44 ml DEA (10,6gr

Adjust pH to 9,4 with HCl

800mg $MgCL_2$

Blocking solution

3% (w/v) dry milk in TTBS.

May be stored at 4°C for 5 days.

Developing solution HRP

Dissolve 30mg 4-chloro-1-naphtol in 10ml methanol

Add 30µl cold 30% H_2O_2 to 50mL TBS.

Mix before use and pour immediately over the membrane.

Developing Solution Alkalinephosphateses

49,25 ml DEA

250µl CDP Star (Tropix)

500µl Nitroblock II (Tropix)

Laemmli Gel

10% 1,5mm separating gel

3,33 ml Acrylamide/bis solution

2,5 ml 1,5 mM TRIS pH 8,8

4,0 ml dH_2O

100µl 10% SDS

50µl 10% ammonium persulfate (APS)

5µl TEMED (Biorad)

4% 1,5mm stacking gel

0,67 ml Acrylamide/bis solution

1,25 ml 0,5 mM TRIS pH 6,8

3,0 ml dH_2O

50µl 10% SDS

50µl 10% ammonium persulfate (APS)

2µl TEMED (Biorad)

10xRunning buffer

30,28g TRIS

144,13g Glycine

10g 0,1% SDS (Biorad)

1 l dH_2O

Chemicals used at IMR

(Extraction, TLC, GC)

Merck;

Chloroform

Acetic Acid

Diethylether

Hexane

Methanol (Sigma)

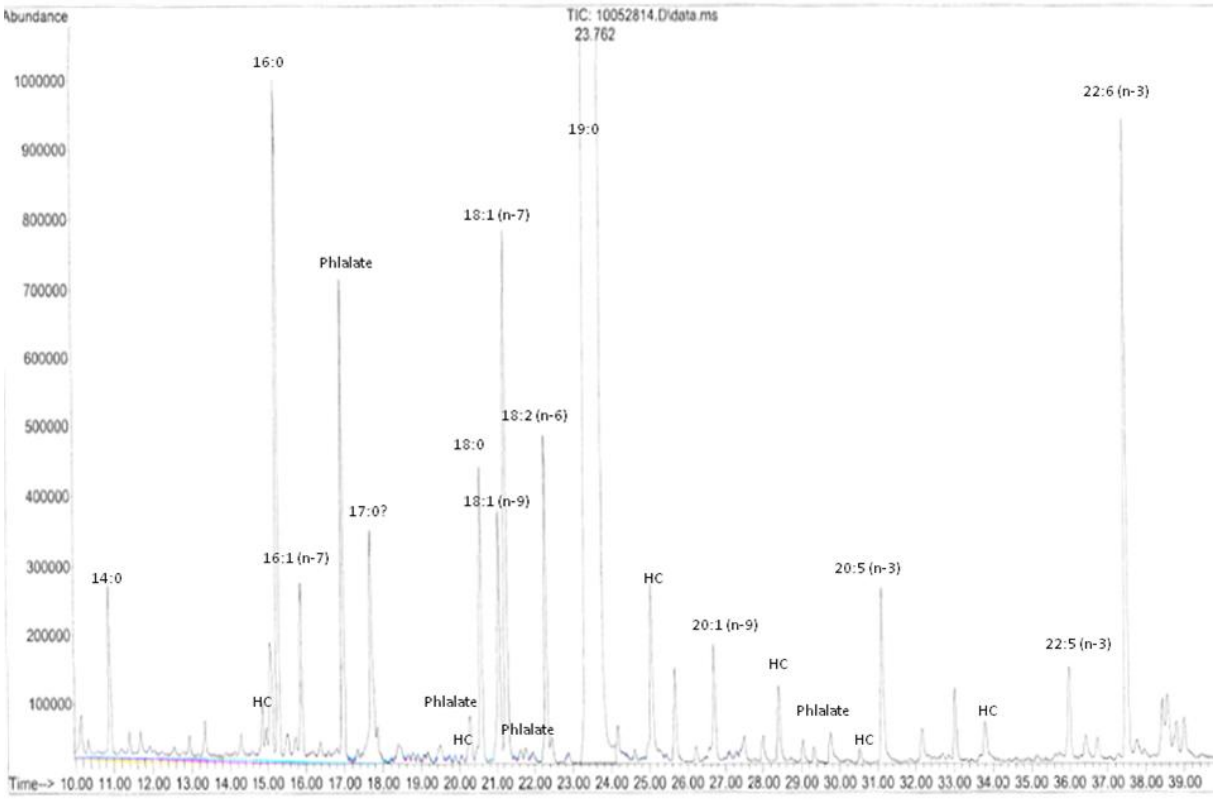

Figure A.1 GC-MS spectra of a pooled CL sample. Hydrocarbons =HC

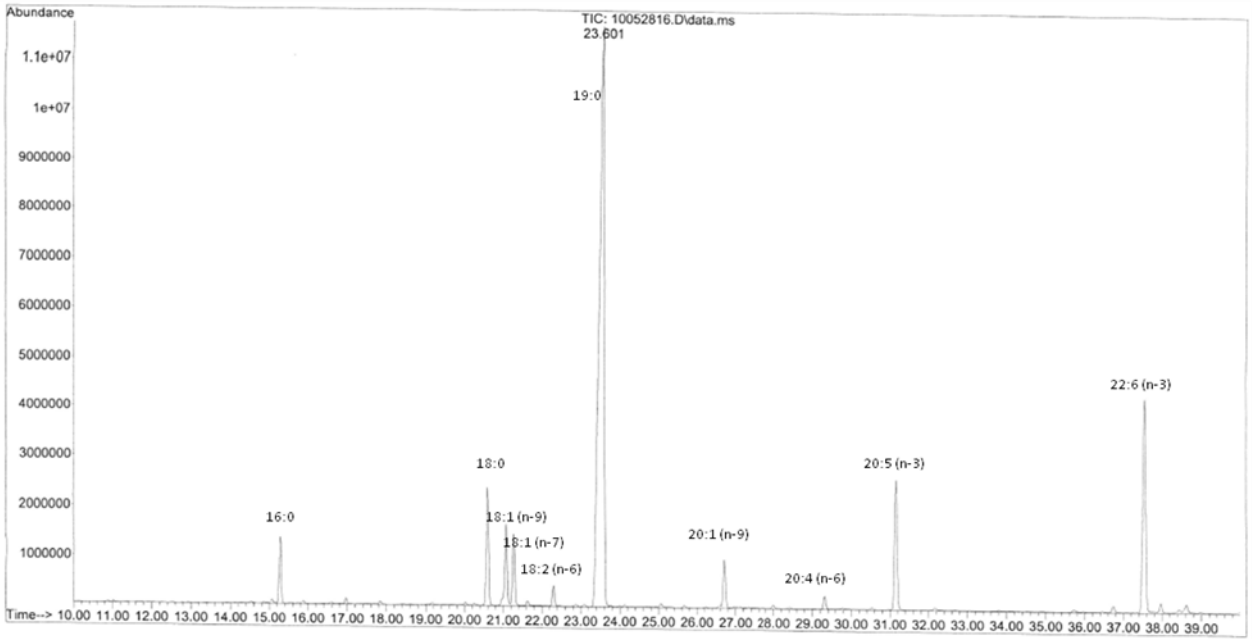

Figure A.2 GC-MS spectra of a pooled PE sample.

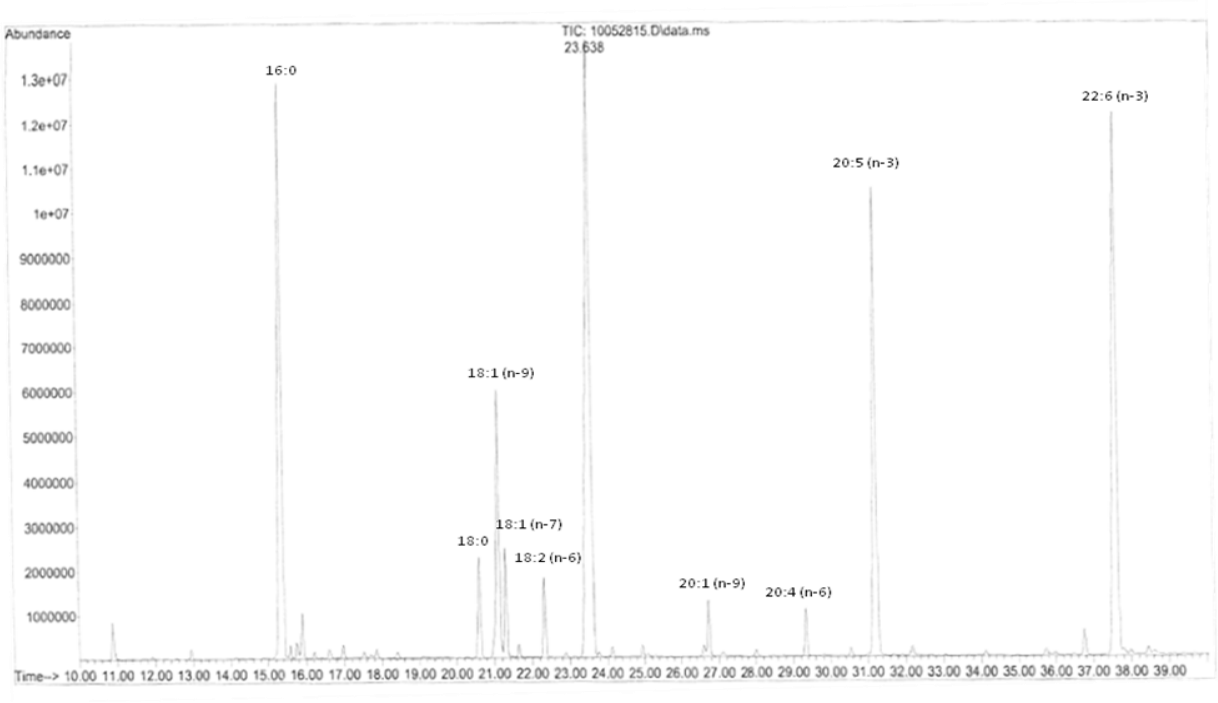

Figure A.3 GC-MS spectra of a pooled PC sample.

Table A.1 Mass spectra diagnostics (m/z) and retentiontime in CL, PE and PC.

		Retention time (min)			Mass spectra , diagnostic ion (m/z)				
Peak No.	Identity	CL	PE	PC	M+	Base top	omega	alpha	
1	14:0	10,88	10,88	10,88	242	74			
2	15:0	12,93	12,91	12,93	256	74			
3	16:0	15,29	15,28	15,34	270	74			
4	16:1 (n-11)		15,58	15,58	270	74			
5	16:1 (n-9)	15,74	15,75	15,75	268	55			
6	16:1 (n-7)	15,89	15,89	15,89	268	55			
7	16:1 (n-5)	16,38		16,22	268	55			
8	iso 17:0		16,586	16,602	284	74			
9	Futan	17,69	17,67	17,69	326	101			
10	17:0	17,85	17,84	17,85	284	74			
11	17:1 (n-9)		18,40	18,40	282	55			
12	18:0	20,58	20,60	20,60	298	74			
13	18:1 (n-11)	20,98	20,96	21,01	296	55			
14	18:1 (n-9)	21,06	21,09	21,16	296	55			
15	18:1 (n-7)	21,27	21,28	21,31	296	55			
16	18:1 (n-5)		21,64	21,64	296	55			
17	18:2 (n-6)	22,30	22,29	22,32	294	67			
18	19:0	23,76	25,54	23,50	312	74			
19	18:3 n3	24,16	24,11	24,13	292	79	108	236	
20	18:4 (n-3)	24,95			290	79	108	194	
21	20:0	26,27	26,24	26,22	326	74			
22	20:1 (n-11)		26,58	26,59	324	55			
23	20:1 (n-9)	26,70	26,71	26,72	324	55			
24	20:1 (n-7)			26,95	324	55			
25	20:2 (n-6)	28,02	27,98	28,01	322	67			
26	21:0	29,04	29,05	29,07	340	74			
27	20:4 (n-6)	29,33	29,32	29,34	318	79	150	180	
28	20:4 (n-3)		30,51	30,54	318	79			
29	20:5 n3	31,20	31,18	31,21	316	79	108	180	
30	22:0			31,87	354	74			
31	22:1 (n-11)		32,15	32,18	352	55			
32	22:1 (n-9)	32,20		32,34	352	55			
33	22:1 (n-7)			32,86	352	55			
34	21:5 (n-3)		34,10	34,12		79	108	194	
35	22:5 (n-6)		35,72	35,73	342	79	150		
36	22:5 (n-3)	36,75	36,74	36,76	342	79	108	208	
37	24:0			37,31	382	74			
38	22:6 (n-3)	37,51	37,53	37,59	342	79	108	166	
39	24:1 (n-9)	37,78	37,76	37,80	348	55			
	Hydrocarbons				M+	Base top (M1)	M2	M3	M4
	H1	15,10	15,07	15,19		57	71	85	99
	H2	20,26	20,25	20,28		57	71	85	99
	H3	25,68	25,67	25,69		57	71	85	99
	H4	28,42	28,42	28,45		57	71	85	99
	H5	31,14				57	71	85	99
	H6	33,79				57	71	85	99
	Phtalate				M+	Base top (M1)	M2	M3	M4
	P1	16,97	17,00	16,98	194	163	77	92	
	P2	20,46			194	163	149	181	77
	P3	22,44	22,45	22,46	194	163	149	181	77
	P4	29,74	29,78	29,75		163	149	181	70

Table A.2 Average of FA composition as percent ± SD of total FA in CL. n=the number of samples in the group.

CL	Control n=7		Chlor n=2		Brom n=3		Fluor n=5		4-Nonyl n=3		4-tert-nonyl n=5		Crude Oil n=5		Mix n=5		Mix + oil n=4	
Fatty acid	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
14:0	3,37	± 1,73	3,31	± 1,33	3,07	± 0,31	5,35	± 0,99	5,45	± 1,10	3,50	± 0,66	3,00	± 1,24	4,02	± 2,34	3,39	± 2,02
15:0	0,70	± 0,23	0,43	± 0,24	0,45	± 0,05	0,93	± 0,30	1,07	± 0,25	0,44	± 0,25	0,60	± 0,39	0,89	± 0,49	0,65	± 0,20
16:0	12,37	± 3,63	6,15	± 2,87	8,27	± 0,75	14,14	± 2,86	16,23	± 3,36	10,79	± 2,29	11,65	± 5,28	13,33	± 6,83	11,04	± 1,70
18:0	9,00	± 4,49	4,38	± 2,73	5,94	± 1,61	6,40	± 1,75	9,15	± 2,77	6,52	± 1,30	7,32	± 2,62	9,05	± 5,40	8,31	± 3,52
20:0	0,61	± 0,40	0,21	± 0,12	0,30	± 0,09	0,56	± 0,25	0,74	± 0,27	0,46	± 0,16	0,44	± 0,35	1,05	± 1,21	0,45	± 0,64
24:0	0,64	± 0,56	0,37	± 0,05	0,38	± 0,25	0,71	± 0,25	0,89	± 0,22	0,19	± 0,27	0,67	± 0,25	2,02	± 3,56	0,88	± 0,58
16:1 (n-11)	0,77	± 0,21	0,94	± 0,38	0,74	± 0,31	0,97	± 0,41	0,91	± 0,18	0,73	± 0,06	0,51	± 0,37	0,52	± 0,25	0,69	± 0,25
16:1 (n-9)	1,07	± 0,59	2,62	± 1,10	1,64	± 0,31	1,64	± 0,75	1,16	± 0,39	0,82	± 0,47	0,71	± 0,51	3,32	± 3,25	2,50	± 0,63
16:1 (n-7)	4,13	± 1,17	4,50	± 0,64	4,29	± 1,46	5,05	± 1,25	4,60	± 0,88	4,86	± 0,45	3,55	± 1,17	2,25	± 2,01	5,09	± 2,24
18:1 (n-11)	1,20	± 0,20	0,87	± 0,00	1,50	± 0,91	1,33	± 0,23	1,15	± 0,26	1,46	± 0,44	1,11	± 0,11	2,64	± 2,58	1,50	± 0,25
18:1 (n-9)	6,74	± 2,22	5,90	± 1,19	8,20	± 1,25	6,10	± 1,84	5,45	± 0,72	7,79	± 0,90	8,60	± 2,14	5,62	± 1,70	6,18	± 0,97
18:1 (n-7)	4,38	± 0,98	4,36	± 0,09	4,41	± 0,93	4,83	± 1,37	4,45	± 0,95	6,15	± 0,80	4,27	± 0,97	3,77	± 0,68	5,26	± 0,79
20:1 (n-11)	0,81	± 0,68	0,50	± 0,32	0,94	± 0,40	0,73	± 0,20	0,82	± 0,18	0,77	± 0,26	0,86	± 0,20	1,00	± 0,68	0,68	± 0,37
20:1 (n-9)	2,82	± 1,01	2,59	± 0,07	2,68	± 0,09	3,27	± 0,84	3,21	± 0,70	4,28	± 0,39	3,73	± 0,19	1,96	± 1,28	2,89	± 0,44
22:1 (n-11)	1,50	± 0,85	0,89	± 0,13	1,10	± 0,19	1,50	± 0,33	1,71	± 0,27	1,59	± 0,45	2,19	± 0,41	3,01	± 4,11	1,42	± 0,51
22:1 (n-9)	1,65	± 1,58	0,36	± 0,11	0,92	± 0,56	0,93	± 1,08	0,54	± 0,28	0,62	± 0,35	0,74	± 0,27	1,54	± 1,33	0,69	± 0,41
24:1 (n-9)	2,02	± 2,39	0,70	± 0,30	1,05	± 0,89	1,09	± 0,77	0,96	± 0,05	1,92	± 1,35	4,68	± 3,29	2,39	± 2,47	0,92	± 0,06
18:2 (n-6)	8,49	± 2,03	8,20	± 0,86	8,73	± 1,75	8,46	± 2,23	8,51	± 0,91	10,85	± 1,90	7,84	± 1,95	7,18	± 3,09	11,83	± 3,10
20:2 (n-6)	1,12	± 0,42	0,95	± 0,01	0,85	± 0,11	1,19	± 0,28	1,40	± 0,31	1,41	± 0,24	1,01	± 0,24	0,71	± 0,39	0,02	± 0,02
20:4 (n-6)	0,58	± 0,11	0,71	± 0,20	0,84	± 0,22	0,44	± 0,27	0,65	± 0,14	0,70	± 0,12	0,68	± 0,14	0,43	± 0,33	0,58	± 0,35
20:4 (n-3)	0,71	± 0,21	0,79	± 0,06	0,88	± 0,03	0,56	± 0,29	0,58	± 0,17	0,66	± 0,41	0,74	± 0,21	0,68	± 0,63	0,71	± 0,09
18:3 (n-3)	1,20	± 0,26	1,18	± 0,18	1,30	± 0,05	0,97	± 0,27	1,07	± 0,23	1,20	± 0,13	0,99	± 0,28	0,93	± 0,59	1,46	± 0,37
20:5 (n-3)	4,64	± 1,07	5,81	± 0,15	6,16	± 1,27	11,28	± 8,29	8,40	± 3,88	6,01	± 1,41	7,12	± 1,45	6,69	± 6,36	7,71	± 4,01
21:5 (n-3)	0,00	± 0,00	0,00	± 0,00	0,00	± 0,00	0,00	± 0,00	0,00	± 0,00	0,00	± 0,00	0,00	± 0,00	0,00	± 0,00	0,00	± 0,00
22:5 (n-3)	1,61	± 0,33	2,44	± 0,63	2,16	± 0,32	1,20	± 0,30	1,16	± 0,30	1,54	± 0,15	1,54	± 0,38	1,19	± 1,16	1,51	± 0,38
22:6 (n-3)	27,39	± 10,40	40,85	± 10,72	33,19	± 4,77	20,36	± 5,20	19,74	± 7,10	24,73	± 3,69	25,44	± 7,21	23,80	± 15,15	23,64	± 5,01
∑SFA	26,68	± 11,04	14,85	± 7,35	18,41	± 3,05	28,10	± 6,40	33,52	± 7,97	21,91	± 4,93	23,68	± 10,13	30,36	± 19,82	24,73	± 8,65
∑MUFA	26,30	± 11,67	23,29	± 3,96	26,74	± 6,99	26,46	± 8,66	24,06	± 4,68	30,26	± 5,85	30,44	± 9,26	27,50	± 20,09	27,13	± 6,67
∑PUFA	45,75	± 14,83	60,93	± 12,81	54,11	± 8,54	44,47	± 17,14	41,50	± 13,04	47,10	± 8,05	45,37	± 11,85	41,62	± 27,69	47,45	± 13,34
∑PUFA (n-6)	10,19	± 2,55	9,86	± 1,07	10,42	± 2,09	10,10	± 2,79	10,56	± 1,37	12,95	± 2,27	9,54	± 2,33	8,32	± 3,81	12,43	± 3,47
∑PUFA (n-3)	35,56	± 12,27	51,06	± 11,74	43,68	± 6,45	34,37	± 14,36	30,94	± 11,68	34,15	± 5,78	35,83	± 9,52	33,30	± 23,88	35,02	± 9,87
n6/n3	0,29	± 0,21	0,19	± 0,09	0,24	± 0,32	0,29	± 0,19	0,34	± 0,12	0,38	± 0,39	0,27	± 0,24	0,25	± 0,16	0,35	± 0,35

Table A.3 Average of FA composition as percent ± SD of total FA in PE. n=the number of samples in the group.

PE	Control n=7		Chlor n=5		Brom n=5		Fluor n=5		4-Nonyl n=5		4-tert-nonyl n=5		Crude Oil n=5		Mix n=5		Mix + oil n=5	
Fatty acid	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
14:0	0,25	0,09	0,27	0,03	0,73	0,76	0,40	0,15	0,45	0,27	0,33	0,13	0,31	0,14	0,23	0,11	0,35	0,22
15:0	0,11	0,04	0,12	0,04	0,21	0,18	0,18	0,10	0,13	0,12	0,12	0,05	0,12	0,04	0,10	0,06	0,12	0,04
16:0	7,18	1,09	7,76	0,23	10,73	7,33	9,05	2,18	7,91	3,90	7,84	0,72	7,70	1,39	6,58	2,46	7,83	1,73
18:0	9,94	2,29	11,03	2,53	10,81	3,39	11,37	1,57	10,52	2,65	11,89	1,21	10,93	0,64	9,43	1,86	12,28	5,87
20:0	0,25	0,05	0,26	0,10	0,37	0,25	0,29	0,09	0,31	0,06	0,25	0,03	0,22	0,06	0,22	0,02	0,29	0,27
24:0	0,24	0,07	0,39	0,32	0,23	0,04	0,43	0,30	0,34	0,12	0,31	0,07	0,24	0,05	0,25	0,06	0,34	0,10
16:1 (n-11)	0,21	0,06	0,12	0,10	0,30	0,22	0,16	0,05	0,13	0,08	0,23	0,32	0,16	0,05	0,20	0,09	0,21	0,10
16:1 (n-9)	0,27	0,16	0,45	0,26	0,88	0,66	0,28	0,14	0,22	0,10	0,31	0,12	0,21	0,04	0,26	0,20	0,28	0,21
16:1 (n-7)	0,46	0,11	0,51	0,05	0,70	0,49	0,63	0,17	0,68	0,57	0,53	0,06	0,46	0,09	0,46	0,18	0,56	0,22
18:1 (n-11)	0,88	0,18	0,70	0,09	1,02	0,19	0,96	0,35	0,84	0,39	1,09	0,21	0,90	0,14	0,88	0,16	0,79	0,39
18:1 (n-9)	8,72	0,52	9,07	0,72	8,44	0,96	9,33	0,54	7,35	2,30	9,09	0,81	9,30	0,38	8,61	1,94	9,14	1,50
18:1 (n-7)	6,07	0,41	6,40	0,29	5,32	0,93	6,61	0,86	5,48	1,78	6,65	0,29	7,17	0,28	6,11	1,20	5,75	0,34
20:1 (n-11)	0,42	0,06	0,47	0,07	0,48	0,09	0,49	0,04	0,53	0,08	0,50	0,06	0,50	0,08	0,47	0,06	0,53	0,18
20:1 (n-9)	5,68	0,52	5,27	0,25	4,78	1,89	5,26	1,00	5,77	0,94	5,53	0,13	5,44	0,52	5,59	0,76	5,16	0,91
22:1 (n-11)	0,54	0,08	0,62	0,14	0,60	0,12	0,55	0,04	0,80	0,22	0,57	0,14	0,49	0,13	0,59	0,16	0,55	0,08
22:1 (n-9)	0,13	0,12	0,30	0,33	0,47	0,51	0,16	0,07	0,23	0,19	0,12	0,06	0,06	0,06	0,19	0,10	0,12	0,04
24:1 (n-9)	0,16	0,06	0,19	0,02	0,18	0,10	0,14	0,09	0,25	0,07	0,27	0,25	0,23	0,02	0,16	0,12	0,12	0,07
18:2 (n-6)	2,49	0,14	2,87	0,09	3,48	1,76	2,76	0,35	2,43	0,92	2,37	0,23	2,73	0,23	2,41	0,64	3,14	1,44
20:2 (n-6)	0,62	0,05	0,62	0,04	0,40	0,36	0,58	0,11	0,68	0,04	0,61	0,05	0,60	0,02	0,62	0,10	0,53	0,09
20:4 (n-6)	1,84	0,29	1,69	0,16	1,97	0,52	1,70	0,20	1,74	0,21	2,08	0,05	1,82	0,20	2,08	0,27	1,84	0,35
22:5 (n-6)	0,10	0,05	0,06	0,01	0,08	0,05	0,07	0,07	0,21	0,10	0,12	0,17	0,05	0,06	0,08	0,03	0,19	0,27
20:4 (n-3)	0,46	0,12	0,39	0,00	0,32	0,29	0,44	0,07	0,50	0,12	0,39	0,16	0,43	0,11	0,50	0,04	0,45	0,06
18:3 (n-3)	0,32	0,03	0,40	0,02	0,42	0,17	0,35	0,05	0,32	0,10	0,30	0,03	0,35	0,05	0,34	0,09	0,43	0,16
20:5 (n-3)	15,98	2,18	15,84	0,64	16,63	2,48	16,42	1,38	16,81	0,90	17,26	0,95	15,05	0,82	17,01	1,02	16,67	3,26
21:5 (n-3)	0,21	0,06	0,28	0,10	0,20	0,07	0,16	0,04	0,19	0,05	0,17	0,02	0,09	0,08	0,16	0,02	0,18	0,01
22:5 (n-3)	1,48	0,20	1,51	0,27	1,49	0,36	1,34	0,14	1,57	0,52	1,59	0,33	1,45	0,15	1,71	0,80	1,40	0,21
22:6 (n-3)	34,99	2,50	32,23	4,32	28,75	6,96	29,90	2,62	33,60	10,60	29,50	0,59	32,99	1,77	34,79	6,25	30,73	4,88
∑SFA	17,97	3,47	19,17	2,89	22,13	11,15	20,89	3,94	18,88	6,73	20,11	2,00	18,97	2,13	16,33	4,39	20,53	7,91
∑MUFA	23,33	2,20	26,40	2,04	25,48	7,03	26,89	3,50	24,37	7,46	26,71	2,25	27,28	1,94	25,45	5,32	25,86	5,17
∑PUFA	58,50	5,49	53,02	5,56	50,25	11,26	50,95	4,67	55,62	12,64	52,00	2,35	52,84	3,26	57,28	8,61	52,42	9,28
∑PUFA (n-6)	2,56	0,40	2,37	0,21	2,45	0,93	2,34	0,37	2,62	0,35	2,80	0,27	2,48	0,29	2,77	0,39	2,56	0,71
∑PUFA (n-3)	52,99	4,97	50,26	5,36	47,49	10,04	48,17	4,23	52,49	12,18	48,81	1,92	49,93	2,87	54,01	8,18	49,40	8,51
n6/n3	0,05	0,08	0,05	0,04	0,05	0,09	0,05	0,09	0,05	0,03	0,06	0,14	0,05	0,10	0,05	0,05	0,05	0,08

Table A.4 Average of FA composition as percent ± SD of total FA in PC. n=the number of samples in the group.

PC	Control n=7		Chlor n=5		Brom n=5		Fluor n=5		4-Nonyl n=5		4-tert-nonyl n=5		Crude Oil n=5		Mix n=5		Mix + oil n=5	
Fatty acid	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
14:0	1,49	0,63	2,24	0,71	1,86	0,31	2,08	0,46	2,27	0,26	1,48	0,26	1,72	0,20	1,56	0,79	1,30	0,92
15:0	0,35	0,09	0,43	0,06	0,41	0,00	0,45	0,06	0,46	0,03	0,37	0,06	0,39	0,03	0,36	0,15	0,29	0,13
16:0	21,85	2,41	22,39	1,26	22,78	2,51	23,81	1,23	25,11	1,71	23,16	2,45	23,70	1,41	20,52	9,07	18,10	6,11
18:0	2,95	0,68	2,69	0,51	4,32	6,02	2,97	0,46	3,06	0,41	3,27	0,46	3,05	0,26	2,86	0,65	2,81	0,58
20:0	0,04	0,01	0,02	0,02	0,08	0,01	0,05	0,00	0,05	0,01	0,04	0,01	0,04	0,01	0,04	0,01	0,05	0,03
24:0	0,18	0,07	0,15	0,02	0,22	0,02	0,22	0,04	0,17	0,02	0,18	0,04	0,15	0,03	0,17	0,08	0,27	0,13
16:1 (n-11)	0,36	0,07	0,34	0,31	0,34	0,04	0,34	0,22	0,46	0,10	0,31	0,17	0,37	0,15	0,20	0,15	0,26	0,09
16:1 (n-9)	0,56	0,12	0,82	0,14	0,68	0,09	0,66	0,09	0,64	0,08	0,54	0,05	0,54	0,14	0,53	0,23	0,47	0,22
16:1 (n-7)	1,54	0,40	1,88	0,15	1,52	0,08	1,76	0,17	1,54	0,19	1,50	0,19	1,55	0,25	1,66	0,74	1,33	0,61
18:1 (n-11)	0,93	0,29	0,89	0,10	1,17	0,08	1,01	0,33	0,82	0,14	1,05	0,31	0,77	0,14	0,82	0,18	0,88	0,33
18:1 (n-9)	9,50	0,75	8,99	0,40	9,37	0,16	9,33	0,74	8,39	0,66	9,78	1,70	9,88	0,79	9,17	2,05	8,45	2,19
18:1 (n-7)	3,06	0,32	2,83	0,24	2,54	2,05	3,01	0,21	2,79	0,20	2,95	0,29	3,13	0,11	2,80	0,50	2,67	0,66
20:1 (n-11)	0,48	0,06	0,43	0,04	0,50	0,00	0,46	0,04	0,47	0,08	0,53	0,10	0,52	0,11	0,60	0,11	0,57	0,15
20:1 (n-9)	2,06	0,23	1,67	0,12	1,96	0,10	1,83	0,17	1,73	0,17	1,87	0,17	1,93	0,27	2,02	0,50	2,21	0,47
22:1 (n-11)	0,45	0,05	0,48	0,10	0,51	0,00	0,48	0,04	0,45	0,04	0,49	0,11	0,44	0,07	0,51	0,23	0,65	0,26
22:1 (n-9)	0,07	0,05	0,10	0,05	0,17	0,01	0,07	0,02	0,06	0,02	0,09	0,04	0,06	0,02	0,08	0,03	0,11	0,05
24:1 (n-9)	0,36	0,36	0,25	0,01	0,27	0,01	0,25	0,05	0,23	0,06	0,45	0,39	0,21	0,04	0,53	0,52	0,52	0,38
18:2 (n-6)	2,50	0,13	2,42	0,16	2,92	0,35	2,47	0,15	2,46	0,18	2,54	0,30	2,64	0,23	2,56	0,58	2,40	0,74
20:2 (n-6)	0,31	0,05	0,26	0,02	0,18	0,03	0,27	0,03	0,29	0,03	0,30	0,04	0,29	0,03	0,31	0,07	0,34	0,07
20:4 (n-6)	1,62	0,13	1,53	0,08	1,74	0,04	1,51	0,07	1,59	0,09	1,68	0,03	1,57	0,07	1,71	0,14	1,39	0,78
22:5 (n-6)	0,09	0,02	0,07	0,01	0,08	0,00	0,10	0,04	0,17	0,07	0,08	0,10	0,07	0,04	0,12	0,04	0,12	0,08
20:4 (n-3)	0,41	0,02	0,44	0,00	0,25	0,05	0,40	0,05	0,37	0,04	0,41	0,05	0,41	0,04	0,49	0,10	0,43	0,03
18:3 (n-3)	0,37	0,02	0,47	0,04	0,35	0,02	0,39	0,03	0,38	0,03	0,36	0,04	0,38	0,03	0,41	0,08	0,37	0,11
20:5 (n-3)	19,12	1,58	18,48	1,72	18,76	2,26	18,09	0,54	19,19	0,58	19,74	1,46	18,48	0,85	20,61	1,89	20,02	1,14
21:5 (n-3)	0,23	0,02	0,23	0,02	0,23	0,00	0,22	0,01	0,21	0,01	0,25	0,02	0,18	0,10	0,26	0,09	0,28	0,08
22:5 (n-3)	1,16	0,08	1,30	0,24	1,26	0,04	1,16	0,10	1,15	0,10	1,24	0,17	1,18	0,05	1,32	0,60	1,68	1,01
22:6 (n-3)	27,93	1,96	28,37	1,17	25,53	11,00	26,62	1,53	25,51	2,30	25,32	5,28	26,35	2,35	27,78	10,80	32,05	9,80
∑SFA	26,87	3,89	27,93	2,58	29,66	8,88	29,57	2,26	31,12	2,44	28,51	3,29	29,06	1,94	25,51	10,75	22,81	7,91
∑MUFA	19,38	2,71	18,69	1,67	19,03	2,62	19,20	2,05	17,58	1,74	19,57	3,52	19,40	2,08	18,92	5,24	18,11	5,41
∑PUFA	53,75	3,99	53,57	3,47	51,31	13,80	51,23	2,57	51,30	3,42	51,91	7,48	51,55	3,79	55,56	14,39	59,08	13,83
∑PUFA (n-6)	4,52	0,32	4,28	0,27	4,92	0,42	4,36	0,30	4,50	0,37	4,61	0,47	4,57	0,38	4,70	0,83	4,24	1,66
∑PUFA (n-3)	48,81	3,65	48,84	3,19	46,14	13,32	46,47	2,22	46,43	3,01	46,90	6,96	46,56	3,38	50,38	13,46	54,40	12,14
n6/n3	0,09	0,09	0,09	0,09	0,11	0,03	0,09	0,13	0,10	0,12	0,10	0,07	0,10	0,11	0,09	0,06	0,08	0,14