Effects of oil compounds and persistent organic pollutants (POPs) on phospholipid composition in liver and brain of Atlantic cod (*Gadus morhua*)

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Scientific environment

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Abstract

The work in this thesis is part of a project led by the Institute of Marine Research (IMR), with partners at the Department of Biomedicine (University of Bergen, UiB), the National Institute of Nutrition and Seafood Research (NIFES), and Department of Chemistry (UiB) financed by the Research Council of Norway.

Fish in the North Sea experience exposure to xenobiotic compounds from historic pollution and releases from industry and other human activities. A number of adverse biological effects of persistent organic pollutants (POPs) have been shown, including alterations in the cell membrane. This study is a follow-up of a previously published study from IMR that showed that Atlantic cod (*Gadus morhua*) that were exposed to short-chained alkylphenols had altered lipid composition and fatty acid distribution in the liver and brain compared to un-exposed fish. The study in this thesis consists of two exposure experiments where Atlantic cod were given pollutants in the diet through 4 weeks. In experiment 1 Atlantic cod were exposed to para-substituted nonylphenols (NPs), either the straight-chained isomer 4-*n*-NP, or a mixture of branched isomers, 4-T-NP. In experiment 2 Atlantic cod were given crude oil and/or a mixture of halogenated POPs. The POPs included polychlorinated biphenyls (PCB), chlorinated pesticides, polybrominated diphenyl ether (PBDE) and perfluorooctanesulfonic acid (PFOS). The fish were given doses corresponding to chronic pollution or higher doses analogous to acute spill accidents. The main focus of the thesis has been detailed studies of lipid composition, with emphasis on the phospholipids in the membranes, and fatty acid distribution in membrane lipids. Toxicogenomic studies have also been performed on the transcriptional levels, as well as biophysical studies of model lipid membranes as Langmuir monolayers and their interactions with selected POPs.

The treatments with NPs or oil/POPs did not induce large changes in membrane composition (lipid class composition and fatty acid distribution) in the liver and brain of male Atlantic cod. However, the transcriptional data suggest that the fish were

affected by the treatment at the molecular level. Differential expression in selected genes in phase I and II metabolism of xenobiotic compounds, PL biosynthesis and antioxidant responses were shown.

List of abbreviations

9-OH-P	9-OH-phenanthrene
AGPAT	Acylglycerophosphate acyltransferase
AHR	Aryl hydrocarbon receptor
AP	Alkylphenol
APE	Alkylphenol ethoxylates
CL	Cardiolipin
CT	CTP:phosphocholine cytidyltransferase
CTP	Cytidine triphosphate
CYP	Cytochrome P450
Cytc	Cytochrome c
DAG	Diacylglycerol
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DHA	Docosahexaenoic acid, 22:6(n-3)
DMPC	Dimyristoyl-*PC*
DMPE	Dimyristoyl-*PE*
DPPC	Dipalmitoylphosphatidylcholine
ELOVL	Elongation of very-long chain fatty acids
EPA	Eicosapentaenoic acid, 20:5(n-3)
ER	Estrogen receptor
EROD	Ethoxyresorufin-*O*-deethylase
ET	CTP:phosphoethanolamine cytidyltransferase
FA	Fatty acid
FADS	Fatty acid desaturases
FFA	Free fatty acid
G3P	Glycerol-3-phosphate
GC	Gas Chromatography
GC-FID	Gas Chromatography- Flame Ionisation Detector
GPAT	Glycerol-3-phosphate acyltransferase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
HA	Homeoviscous adaptation
HPTLC	High Performance Thin Layer Chromatography
HUFA	Highly unsaturated fatty acid
IMR	Institute of Marine Research
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPCAT	lysophosphatidylcholine acyltransferases

LPL	Lysophospholipid
LPLAT	Lysophospholipid acyltransferase
LPAAT	LPA acyltransferases
MMA	Mean molecular area
MT	Metallothionein
MUFA	Monounsaturated fatty acid
NP	Nonylphenol
OP	Octylphenol
PA	Phosphatidic acid
PAH	Polycyclic aromatic hydrocarbons
PBDE	Polybrominated diphenyl ether
PC	Phosphatidylcholine
PCB	Polychlorinated biphenyl
PE	Phosphatidylethanolamine
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PIS	Phospahtidylinositol synthase
PL	Phospholipid
PLA	Phospholipase A
POP	Persistent organic pollutant
POPC	1-Palmitoyl-2-oleoyl-PC
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acid
SPE	Solid phase extraction
TAG	Triacylglycerols
TLC	Thin Layer Chromatography
AA	Arachidonic acid, 20:4(n-6)
γ-HCH	Lindane (γ-hexachlorocyclohexane)

List of papers

Paper 1: Mari Bratberg, Li Liu and Sonnich Meier (2012): "Pitfalls in the use of polyethylene aminopropyl-coated columns for solid phase extraction separation of lipids."

Paper 2: Mari Bratberg, Pål A. Olsvik, Hans Kristian Brekken, Reidun Vadla and Sonnich Meier (2012):" Effects of branched and normal isomers of para-substituted nonylphenols on the glycerophospholipids in the liver and brain of male Atlantic cod (*Gadus morhua*)".

Paper 3: Mari Bratberg, Pål A. Olsvik, Rolf B. Edvardsen, Hans Kristian Brekken, Reidun Vadla and Sonnich Meier (2012): "Effects of oil pollution and persistent organic pollutants (POPs) on glycerophospholipids in liver and brain of male Atlantic cod (*Gadus morhua*)".

Contents

1. Introduction

1.1 Background

Fish and other organisms in the marine environment are exposed to a complex mixture of pollutants from human activity. Hazardous substances including heavy metals, organohalogens, pesticides and polycyclic aromatic hydrocarbons (PAHs) can be found in sediments, marine organisms and seawater. During the last decades many chemicals have been banned or phased out, however, historic pollution is still posing an environmental threat as e.g. polluted sediments may act as continued sources of release (OSPAR 2010). Pollution from hazardous substances might be local, regional or global (Vallack et al. 1998). Atmospheric long-range transport and ocean currents distribute chemicals from anthropogenic activities to remote areas like the Arctic. This has made especially persistent organic pollutants (POPs) like polychlorinated biphenyls (PCBs), perfluorooctanesulfonic acid (PFOS) and brominated flame retardants a global problem (Hung et al. 2010). PAHs are among the most widespread organic pollutants in the North-East Atlantic, and PAH pollution may be both a regional and global issue as they enter the sea from offshore activities, operational or accidental oil spills from shipping and as discharges from rivers and air (OSPAR 2010). Offshore oil production release oil and chemicals to the marine environment through routine operation in addition to occasional accidental oil spills. Most of the routine releases come from produced water discharges and some come from drill cuttings. Produced water is the term for the water that comes with the oil from the reservoir, and it contains hazardous substances that might be naturally occurring in the reservoir, or chemicals connected with the production process. Offshore oil and gas production is widespread in the North Sea and Norwegian Sea, and activities are expected to increase in the Barents Sea and in areas like Northern Norway, Greenland, the Faroe Islands and Iceland in the years to come (OSPAR 2010). In order to get a realistic picture of the pollution the fish is experiencing it is important to study the combined effects of all the different polluting compounds.

POPs are known to cause a number of adverse effects (see Section 1.5, and references therein), including disruption of biological membranes (Sections 1.6 and 1.7, and references therein). The effects of nonylphenols, oil pollution and/or mixtures of halogenated persistent organic pollutants, on the composition of membrane lipids in liver and brain of Atlantic cod (*Gadus morhua*) is the main focus of the thesis.

The work described in this thesis is a follow-up to a previously published study from IMR that showed that Atlantic cod that were exposed to short-chained alkylphenols had altered lipid composition and fatty acid distribution in the liver and brain compared to un-exposed fish. The observations included increases in the saturated fatty acids **(**SFA) and a decrease in (n-3)-polyunsaturated fatty acids (PUFA) in the phospholipid (PL) fraction of cod liver, and a significant reduction in brain cholesterol (Meier et al. 2007). Similar findings have also been observed in fish near oil installations in the North Sea (Grøsvik et al. 2009; Balk et al. 2011, see Section 1.7).

1.1.1 *In vivo* study

During November and December 2008 an *in vivo* exposure experiment with Atlantic cod was performed at IMR. The treatment consisted of one, or a mixture of, pollutant(s). The feed was administered with a tube directly to the stomach of anaesthetized fish. The different treatments included branched or straight-chained para-substituted NPs, chlorinated pesticides, PCBs, PBDEs, PFOS and weathered crude oil from Troll. The doses which were given to the fish corresponded to realistic levels of fish from Norwegian waters (*Paper 2*, *Paper 3*). Each fish was given one weekly dose for 4 weeks. Extensive chemical analyses were performed on the sampled tissues. Uptake and metabolism of the POPs were assessed by determination of concentrations of PCBs, chlorinated pesticides, PBDE, PFOS and NPs in the liver. Bile metabolites of NPs and PAH were quantified. Lipids were extracted from the liver and separated into lipid classes for which the fatty acid distribution and cholesterol content was determined by GC-FID of the corresponding fatty acid methyl esters (FAME). The fatty acid distribution, total lipid and cholesterol content in the brains

were determined by GC-FID of FAME prepared by direct methylation. Liver mRNA was extracted to be studied by the reverse transcription polymerase chain reaction (RT-PCR) (*Paper 2* and *Paper 3*) and a microarray (*Paper 3*). The focus in the genomic study was on genes in the phospholipid biosynthesis, in phase I and II metabolism of xenobiotics and in antioxidant responses.

1.2 A brief introduction to lipids

Lipids constitute a large class of compounds that may be defined, as Christie does; as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds" (Christie 2012). Other definitions also exist, often based on the (lack of) solubility in water for these compounds (Nelson & Cox 2008a). Lipids have important roles in biological functions, e.g. as energy stores and components in membranes. The main lipid class used as energy storage in eukaryotic cells is triacylglycerol (TAG) also called triglyceride. TAG has a glycerol backbone with three fatty acids in ester linkages (Figure 1) (Fahy et al. 2005).

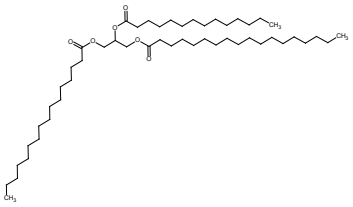

Figure 1: The molecular structure of a triacylglycerol with three saturated fatty acids attached to the glycerol backbone.

In biological membranes lipids form a semi-permeable bilayer. The lipids responsible for this structure are mainly phospholipids (notably the glycerophospholipids, Figure 2) characterized by a polar/hydrophilic "head group" (Figure 3) and fatty acyl chains as hydrophobic "tails" (Nelson & Cox 2008b).

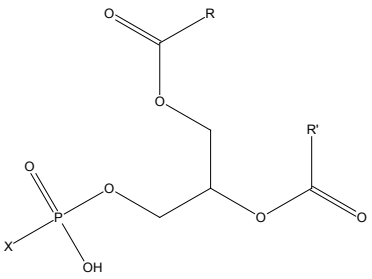

Figure 2: Schematic figure of a typical glycerophospholipid. R and R' denotes hydrocarbon chains. P-X bond denotes the bond to the oxygen in the hydroxyl group of choline (a), ethanolamine (b), serine (c), inositol (d) or glycerol (e) (shown in Figure 3).

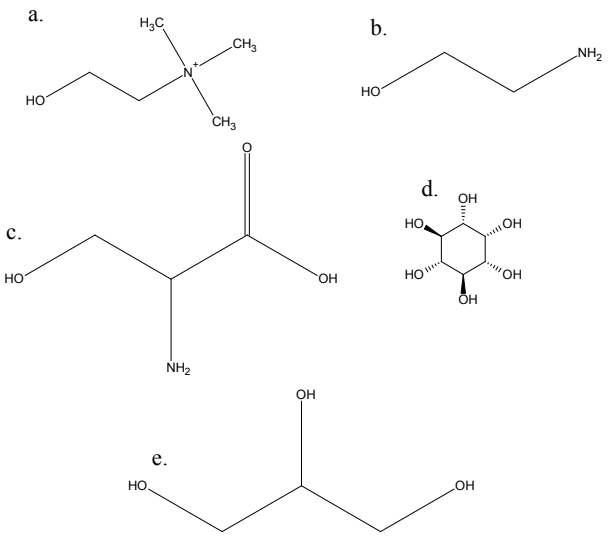

Figure 3: Molecular structures of a, choline; b, ethanolamine; c, serine; d, inositol; and glycerol.

Cholesterol (Figure 4) is another important lipid component of membranes (Bach & Wachtel 2003). It is recognized as a lipid that yields a more ordered structure in the

membrane and plays an important role in the formation of lipid rafts (Mouritsen & Zuckermann 2004; Nelson & Cox 2008b).

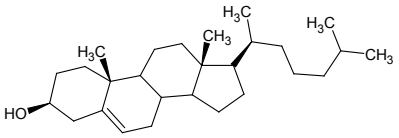

Figure 4: The molecular structure of cholesterol.

1.2.1 Nomenclature

The simple convention for naming fatty acids, which is used in this thesis, is to specify the chain length and the number of double bonds (if any), separated by a colon, and to give the position of the first double bond in parenthesis, counting from the carbon at the opposite end of the carboxyl carbon (Nelson & Cox 2008a); an example is given in Figure 5.

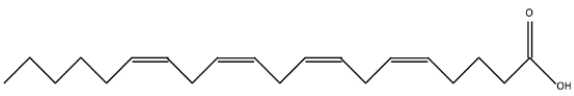

Figure 5: The molecular structure of arachidonic acid (AA) (trivial name); or 20:4(n-6) with the nomenclature convention used in this thesis.

Fatty acids are categorized by their degree of saturation. Saturated fatty acids (SFA) have no double bonds; mono-unsaturated fatty acids (MUFA) have one single double bond, whereas poly-unsaturated fatty acids (PUFA) have at least two double bonds. The double bonds in natural PUFA are rarely conjugated, rather they are most often separated by a methylene group, and the double bounds usually occur in the *cis*-configuration (Nelson & Cox 2008a).

1.3 Biosynthesis of phospholipids

Altering of the membrane lipids in an organism after exposure to toxicants might indicate alterations of the regulating mechanisms of the phospholipid biosynthesis pathways. Environmental impacts e.g. temperature changes have been shown to affect enzymatic activity of the PL biosynthesis. Thus an overview of the main pathways for biosynthesis is shown (Figure 6). Biological phospholipids can either be synthesized *de novo* through the Kennedy Pathway, or by remodeling in the Lands' cycle. In general, saturated and monounsaturated FAs are esterified at the *sn*1-position of a phospholipid, while PUFA are esterified at the *sn*2-position (Shindou et al. 2009b).

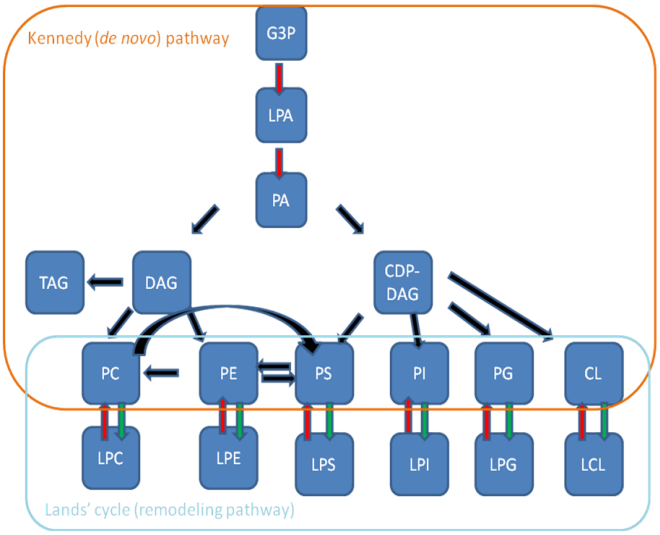

*Figure 6: Overview of the two main pathways in phospholipid biosynthesis, the Kennedy (*de novo*) pathway and the Lands' cycle (remodeling pathway). Red arrows indicate reactions catalyzed by acyltransferases, and green arrows indicate reactions catalyzed by phospholipases. The enzymes for the remaining reactions are described in the text. (This representation is based on figures in (Shindou & Shimizu 2009a) and (Tocher et al. 2008).)*

1.3.1 *De novo* pathway (Kennedy pathway)

The synthesis of phospholipids takes place near the membrane with either cytoplasmic or membrane-embedded enzymes (Moessinger et al. 2011; Vance & Vance 2004). Phosphatidic acid (PA) is the "starting point" for the *de novo* synthesis of phospholipids. PA itself is synthesized from glycerol-3-phosphate (G3P) catalyzed by acyltransferases; reaction is shown below (with enzymes in brackets):

- Glycerol-3-phosphate → [glycerol-3-phosphate acyltransferase, GPAT] → Lysophosphatidic acid (LPA) → [LPA acyltransferases, LPAATs] → phosphatidic acid (PA)

PA can either be dephosporylated to diacylglycerol (DAG) or it can be activated to CDP-diacylglycerol by CDP-diacylglycerol synthase (CDS). PC and PE are synthesized in the CDP-choline and CDP-ethanolamine pathways respectively, starting from DAG, and PI is made in a pathway from CDP-DAG and inositol catalyzed by a phospahtidylinositol synthase (PIS) (Tanaka et al. 1996; Yamashita et al. 1997; Zubay 1998). There is not much information known about the regulation of PI synthesis (Hermansson et al. 2011). CTP: phosphocholine cytidyltransferase (CT) converts phosphocholine into CDP-choline, and in the final step of this pathway phosphocholine is transferred from CDP-choline to diacylglycerol by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) (Vance & Vance 2004). The synthesis of PE has a similar pathway; CTP:phosphoethanolamine cytidyltransferase (ET) catalyzes the conversion of phosphoethanolamine to CDP-ethanolamine which is further transferred to DAG by CDP-ethanolamine:1,2-diacylglycerol ethanolamine phosphotransferase (EPT) to form PE. The CT and ET activity is regarded as the rate-limiting and thus regulatory step in the *de novo* synthesis of PC and PE respectively, provided adequate amounts of DAG (Hermansson et al. 2011). PS can be synthesized from PC or PE by exchange of the head group with L-serine (Lykidis 2007; Zubay 1998). The reaction is catalyzed by PS synthase-1 (PSS1) to convert PC or PS synthase-2 (PSS2) to convert PE, and it is

Ca^{2+}-dependent (Zwingelstein et al. 1998a). Studies on mice have shown that only animals lacking both of the PS synthases are not viable but it can be sufficient to have only PSS1 or PSS2 to function normally. It is indicated that in normal tissues there are PSS in excess and that they are regulated by feed-back inhibition by the product, PS (Hermansson et al. 2011).

1.3.2 Remodeling pathway (Lands' cycle)

The fatty acid composition of biological phospholipids does not fully reflect the composition in their precursor PA (Yamashita et al. 1997), indicating that the phospholipid molecular species is a result of further remodeling of the acyl chains after the *de novo* synthesis. These reactions are catalyzed by phospholipases degrading the PLs to lysophospholipids (LPLs), and by acyltransferases and transacylases reacylating the lysophospholipids. This is also called a deacylation-reacylation cycle (Yamashita et al. 1997). The phospholipases are characterized by where they cleave the phospholipid: Phospholipase A_1 (PLA_1) remove FAs at the *sn*1-position of the PL while PLA_2 remove FAs at the *sn*2-position. There also exists phospholipases C (PLC) and phospholipases D (PLD), respectively hydrolyzing the bond between the phosphate and the glycerol backbone, and the bond between the phosphate and the head group, though their roles in PL homeostasis are not as well-studied as the role of PLAs (Hermansson et al. 2011). An example of the deacylation/reacylation cycle is the removal of fatty acids at the *sn*-2 position of PC by phospholipase A_2 (PLA_2) to yield lysophosphatidylcholine (LPC) followed by re-acylation by lysophosphatidylcholine acyltransferases (LPCAT) (Moessinger et al. 2011).

Phospholipases A_2 degrades phospholipids and generates unsaturated free fatty acids (FFA) and lysophospholipids (LPL). At low concentrations FFA and LPL can be second messengers, but they are cytotoxic at higher concentrations. Lysophospholipids may alter membrane fluidity and permeability, or might be converted to bioactive

molecules; e.g. platelet activating factor, PAF. More than 30 different PLA_2 enzymes have been characterized in mammals to this date (Murakami et al. 2011) and they have been subdivided into different classes (Farooqui et al. 1997; Murakami et al. 2011). A usual way of distinction is "the big three" protein families; secretory PLA_2s ($sPLA_2$) that are low-molecular weight and Ca^{2+}-dependent, cytosolic PLA_2s (c PLA_2) that are unique to vertebrate species, and Ca^{2+}-independent PLA_2s (i PLA_2) (Murakami et al. 2011). Some types of PLA_2s do not fit into either of the aforementioned families e.g. because they are unique for lysosomes or adipose tissue, or substrate-specific to PAF (Murakami et al. 2011).

Acyltransferases and transacylases in Lands' Cycle

Transacylases catalyzes the reactions where fatty acids (acyl chains) are transferred from glycerophospholipids (phospholipids or lysophospholipids) to lysophospholipids to form new molecular species of phospholipids. There exists CoA-dependent and CoA-independent transacylase systems, and lysophospholipase/transacylases (Jackson et al. 2008; Yamashita et al. 1997). Acyltransferases transfers the acyl chain of an acyl-CoA to a lysophospholipid (Yamashita et al. 1997). Lysophospholipid acyltransferases (LPLATs) are divided into two different protein family groups, the acylglycerophosphate acyltransferases (AGPAT) and the membrane bound O-acyl transferases (MBOAT) (Hermansson et al. 2011). Some of the LPLATs are substrate-specific (e.g. LPIAT1 and LCLAT1) while others are able to acylate lysophospholipids with several different head groups; e.g. LPCAT3 and LPCAT4 may use LPE and LPS as well as LPC as substrates (Hermansson et al. 2011). Less is known about the other enzymes involved in the remodeling of LPLs, namely the proteins constituting CoA-independent acyltransferases and transacylases. The transacylases catalyzes reactions between PLs, "sending" an acyl group from one PL to another. The CoA-independent transacylase (CoA-IT) is often involved in transferring acyl chains from PCs to PE plasmalogens (Astudillo et al. 2011). Little is known about the regulation of LPLATs to this date (Hermansson et al. 2011).

Regulatory coordination in PL metabolism

The cross-regulation of the biosynthesis of the various PL classes is complex and not fully understood (Hermansson et al. 2011). Regulation of the pathways synthesizing PC, PE and PS or PI, phosphatidylglycerol (PG) and cardiolipin (CL) may happen at the step where PA is converted to DAG. Furthermore when the synthesis of PC or PE is inhibited, more TAG is produced from DAG. It is believed that the CPT/EPT reactions in the synthesis of PC/PE are reversible, thus making the bifunctional CEPT a regulatory mechanism, being able to convert PC back to DAG and further to PE when the PC levels are too high, or vice versa (Hermansson et al. 2011). There is evidence for cross-regulation of the pathways in which PE is synthesized (in mammals), the *de novo* (Kennedy) pathway and the decarboxylation of PS (see below). The total content of negatively charged PL species in the membrane seems to be regulated to maintain a constant charge of the membrane (Hermansson et al. 2011).

Phospholipid interconversions

The PE-to-PC-pathway (by methylation) is restricted to liver cells in mammals (Hermansson et al. 2011; Zubay 1998), and it has also been shown in hepatic cells in rainbow trout (Zwingelstein et al. 1998b). PE can be methylated to form PC in hepatic cells by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT) (Vance & Vance 2004) using *S*-adenosylmethionine as a methyl donor (Sundler & Akesson 1975; Zwingelstein et al. 1998b). Zwingelstein and co-workers showed that this conversion to PC was significantly slowed down in rainbow trout (*Oncorhynchus mykiss)* and European eel (*Anguilla anguilla*) when acclimated to high temperatures (Zwingelstein et al. 1998b).

PE can be synthesized by decarboxylation of PS catalyzed by PS-decarboxylase (PSD) (Vance & Vance 2004), an enzyme situated at the inner mitochondrial membrane (Hermansson et al. 2011). The rate-limiting step of this pathway is not the PSD activity but is rather considered to be the transport of PS from the endoplasmic reticulum (ER) and its subcompartment mitochondria-associated membranes (MAM) –

where PS is synthesized- to the mitochondria (Hermansson et al. 2011). However the decarboxylation pathway may be up-regulated when the CDP-PE pathway is compromised.

Desaturases and elongases

Fatty acid acyl chains may be modified by elongation with elongases, and by introduction of double bonds in the acyl chain with desaturases. In the elongation of a fatty acid, 2-carbon-units are added to a fatty acyl-CoA. Malonyl-CoA functions as the donor of 2-carbon-units, and NADPH is the reducing agent. The elongation mechanism involves four separate enzymatic reactions; condensation, reduction, dehydration and reduction (Guillou et al. 2010) where the rate-limiting step is the first condensation reaction, catalyzed by elongase enzymes (Elongation of very-long chain fatty acids (ELOVLs)). Seven ELOVLs are known to this date. ELOVL1, 3 and 6-7 prefer saturated and monounsaturated FAs as substrates while ELOVL2 and 4-5 prefer PUFAs. Some of the Elovl genes (Elovl1, 5 and 6) are expressed ubiquitously while others are tissue-specific (Guillou et al. 2010). Marine teleosts, including Atlantic cod, appear to lack the ELOVL2 enzyme that elongates C_{20} and C_{22} HUFAs and which is thus an essential enzyme for the synthesis of 22:6(n-3) (Monroig et al. 2011). ELOVL4 proteins have been characterized in zebrafish and are, in contrast to the human ELOVL4, able to participate in the synthesis of 22:6(n-3) as it can convert 22:5(n-3) to 24:5(n-3) (which can then be desaturated and shortened) (Monroig et al. 2010).

Desaturases are named after where the double bond they introduce is situated (Δ9, Δ6 and Δ5), and they can be divided into two different families, stearoyl-CoA desaturases (SCDs) and fatty acid desaturases (FADS). SCDs add a single double bond at position Δ9 (i.e. counting from the carboxyl carbon) to saturated fatty acids.

18:2(n-6) and 18:3(n-3) are essential fatty acids (EFA) for all vertebrates including fish, because they lack the Δ12- and Δ15-desaturases, and vertebrates must thus obtain the EFA from the diet (Tocher 2003).

The EFA are precursors for the physiologically important PUFAs such as 20:4(n-6), 20:5(n-3) and 22:6(n-3). Carnivores, that can eat other animals with high (enough) levels of the HUFAs, often have little or no ability to themselves synthesize the HUFA from 18:2(n-6) and 18:3(n-3). While freshwater fish species have evolved to be able to synthesize the HUFAs because of lack of these FAs in their diet, marine fish surrounded by HUFA-rich zooplankton have not "needed" this (Tocher 2003). So far, except of a bifunctional Δ5/ Δ6-FAD found in *Siganus canaliculatus* (Li et al. 2010), Δ5-FAD has not been isolated from a marine fish species (Monroig et al. 2011), a fact that has led to a hypothesis that some fish are not able to biosynthesize HUFA because of lack of certain genes in the biosynthesis pathway (Zheng et al. 2009). However Δ6-FAD has been isolated from all fish species studied, including Atlantic cod, but the activity and expression of this enzyme and gene is very low for cod compared to salmon (Tocher et al. 2006; Zheng et al. 2009). Also, while the salmon's expression of Δ6-FAD is regulated by the diet, with a low-HUFA-diet leading to up-regulation of the FAD, no such correlation is seen for cod (Tocher et al. 2006). Recently a third FAD, Δ4-FAD, have been isolated from a vertebrate for the first time, namely the herbivorous marine fish *Siganus canaliculatus*, indicating a more direct route for the biosynthesis of 22:6(n-3) from 22:5(n-3) (Li et al. 2010). Stimulation of desaturase activity when the membrane fluidity decreases, is proposed to be one of the mechanisms behind the regulation of membrane fluidity in homeoviscous adaptation (section 1.4) (Hulbert & Else 1999).

Arachidonic acid (AA) - a precursor to the eicosanoids

Arachidonic acid (Figure 5), a reaction product after PLA_2-catalyzation, metabolizes into eicosanoids. Eicosanoids are hormones, or hormone-like compounds, with local effects, targeting the cell where they are made or different neighboring cells, and

mediated through specific cell surface receptors (Zubay 1998). The eicosanoids are involved in inflammation, fever and pain (Funk 2001). The mechanisms of eicosanoid action are complex, e.g. depending on the context, prostaglandins may be both pro- and anti-inflammatory (Funk 2001; Stables & Gilroy 2011). Prostaglandins also evoke hyperalgesia (i.e. increased pain sensitivity). AA is found in the membrane, normally at the sn2-position of PLs. The amount of free AA depends on two competing regulatory reactions; the deacylation of PLs by PLA_2s and the reacylation by acyltransferases and transacylases. In a resting cell the acyltransferase mechanism is dominating, whilst the PLA_2-catalyzed degradation dominates in an active and stimulated cell (Astudillo et al. 2011; Balgoma et al. 2010). The CoA-independent transacylase system might also be active in a stimulated cell (Astudillo et al. 2011). In addition there exist lipid mediators that have EPA and DHA as precursors, resolvins (from both) and protectins (from DHA) (Stables & Gilroy 2011). These signal molecules reduce cardiovascular disease and the inflammations associated with it (Stables & Gilroy 2011).

Peroxisome proliferator-activated receptor (PPAR)

PPARs comprise a subfamily of nuclear receptors that are lipid-related transcription factors that might be activated also by xenobiotic molecules. The PPAR homologs α, β (or δ) and γ bind with the retinoid X receptor (RXR) to peroxisome proliferator response elements in genes. Fatty acids are the natural ligands for PPARs, but might also be responsive to POPs (Hahn et al. 2005). PPARs activate genes related to lipid metabolism, e.g. in fatty acid oxidation. PPARα might be anti-inflammatory (Ahmed et al. 2007; Arzuaga et al. 2007; Zambon et al. 2006; Zandbergen & Plutzky 2007).

1.4 Thermal acclimation in fish- effects on the membrane lipids

The membrane fluidity is an important property that may be defined as "*a measurement of the relative mobility of the phospholipid bilayer of the cell membrane. The fluidity of membranes allows movement within the plane of the membrane,*

providing the basis for lipid-lipid, lipid-protein and protein-protein interactions "(Hu et al. 2003). The membrane fluidity is a structural property that depends on the composition of the membrane, e.g. the ratio of cholesterol to phospholipids, and the ratio of saturated to un-saturated fatty acids. Fluidity is higher in a membrane with low ratios (cholesterol/phospholipids and saturated/unsaturated fatty acids) than in a membrane with the opposite properties. The transcriptional regulation of the biosynthesis of several lipids in the membrane is dependent of the physical state of the membrane (by feed-back signals) (Thewke et al. 2000).

The phospholipid cell membrane in poikilothermic organisms, including fish, is plastic to environmental impacts caused by thermal change. This plasticity has been explained by compensations of altering physical properties of the membrane such as fluidity. It has been hypothesized that the fluidity change in a PL membrane when exposed to POPs is analogous to the fluidity change when environmental temperature changes (from cold to warm) (Meier et al. 2007) thus an overview of the effects of temperature change in poikilotherms are given in the following.

Homeoviscous adaptation (HA) is a concept that has been used about biological membranes for decades (Sinensky 1974) in the context of ambient temperature changes. Poikilothermic organisms are able to regulate the composition of the membrane in order to maintain homeostasis and constant optimal viscosity of the membrane (Cossins & Prosser 1978).This means that the bulk property of the characteristic membrane fluidity remains the same. This is observed by e.g. change of the saturation degree or length of the acyl chains in the membrane lipids (Sinensky 1974) or by change in the head groups of the phospholipids (Pruitt 1988; Tocher 1995). There are both short-term "emergency" mechanisms and long term compensation to thermal acclimation. The mechanism(s) behind the HA seen in a large range of animals are not well known, but are believed to be common for the different species (Crockett 2008). Although there are also exceptions to the "rule" of homeoviscous adaptation, e.g. lack of HA in sarcoplasmic reticulum, and the

adaptation is not always perfect, HA is present in species from bacteria to animals (Hulbert & Else 1999). Aspects of membrane remodeling with temperature change that are not fully explained by HA include the accumulation of PUFA at cold temperatures (monoenes have approximately the same transition temperatures as PUFA, and are thus more effective per double bond) and increases of membrane-stabilising lipids when temperature decreases. This proves that HA cannot explain all the changes that happen in the membrane when temperature changes, but HA still is a paradigm that may explain much of the membrane alterations (Hazel 1995).

There are both short-term ("emergency") mechanisms of thermal acclimations (within hours) and the slower acclimatory thermal compensation that might be seen in seasonal fluctuations in temperature.

The *de novo* synthesis of phospholipids adapts its product to temperature, but is also slowed down when the temperature decreases. The remodeling pathway is faster (more energy-effective) and the simple reshuffling of already existing fatty acyl chains in the membrane to form new molecular species is able to alter the membrane fluidity. This has been shown for trout hepatocytes after only 6 hours cold acclimation (Williams & Hazel 1994).

Another membrane effect that occurs after short time is a change in the PC/PE-ratio, which is decreased when the temperature decreases. The reason for this decrease can be that *de novo* synthesis of PE is less sensitive towards temperature than PC synthesis. Also decarboxylation of PS to PE is increased while the methylation of PE to PC in some cases is decreased as the temperature drops (Williams & Hazel 1994). However the PE-to-PC-methylation is not always positively correlated with temperature (Hazel & Williams 1990; Zwingelstein et al. 1998b) It is not straight-forward to explain a decrease in PC/PE ratio with cold-acclimation in light of the membrane fluidity. The phase transition temperatures of PC are generally lower than the fatty acids analogs of PE (Pringle & Chapman 1981; Silvius 1991) and studies of artificial lipid bilayers with homogenous SFA composition show more fluid bilayers at

low temperatures for PC-head-groups than for the PE analogs (Pringle & Chapman 1981; Pruitt 1988). It has also been shown that methylation of PE (to PC) increases the membrane fluidity in rat erythrocytes (Hirata & Axelrod 1978). A change in the PC/PE ratio may be an adaptation of membrane function to maintain an optimal balance of the membrane-stabilizing and –destabilizing lipids; PE tends to decrease the order of a lipid bilayer as it prefers a conical rather than a cylindrical geometry (Williams & Hazel 1994). It has also been hypothesized that the small head group size and anionic character of PE makes it able to interact with small molecules (e.g. small sugars) that increase membrane fluidity (Pruitt 1988).

The slower process of acclimating thermal compensation demands several days to weeks to function and is a helpful tool for poikilothermic organisms to cope with seasonal changes. The main characteristic of cold acclimated poikilotherms is an increased level of PUFA. The desaturase system does not work as an emergency HA as it needs days to desaturate SFA and MUFA to PUFA at low temperatures (Williams & Hazel 1994). An increase in the Δ9-desaturase transcription after cold-acclimation has been shown for several fish species (Logue et al. 1995; Williams & Hazel 1994; Wodtke & Cossins 1991; Zerai et al. 2010).

1.5 Persistent organic pollutants (POPs)

POPs may be defined as "*organic substances that possess toxic characteristics in a broad sense, are persistent, bioaccumulate, are prone to long-range transboundary atmospheric transport and deposition, and are likely to cause significant adverse human health or environmental effects near to and distant from their sources*" (Ballschmiter et al. 2002). The use of many "classic" POPs such as organochlorine pesticides and PCBs were banned or restricted in the 1970's in most Western countries and were globally banned by the Stockholm Convention on POPs in 2001 (Muir & Howard 2006). More POPs were added to the list in 2009, e.g. certain polybrominated diphenyl ethers (PBDE) congeners and perfluorooctanesulfonic acid (PFOS)

(United Nations Environment Programme 2009). However due to their persistent nature background levels of these compounds are still found in biological tissues in the marine environment (Cleemann et al. 2000; Julshamn et al. 2004; Voorspoels et al. 2004). Substances like PCBs may also still be released to the environment from pollutant-containing equipment that is still in use and from waste disposal and marine sediments contaminated from historic pollution (OSPAR 2010).

POPs comprise a large group of many thousand chemicals that may be further divided into several subgroups of compounds, such as the 209 PCB congeners. However, they have several important characteristics in common. POPs are characterized by relatively long half-lives in biota, sediments or air. These compounds are hydrophobic and lipophilic, and often resistant to metabolism rendering them prone to accumulate in the food chain. In the marine environment POPs prefer partitioning to solid organic matter rather than water, and in biota POPs stores in fatty tissues. POPs can be volatile and vaporize making them prone to atmospheric long-range transport. Reproductive impairment and carcinogenicity were among the first described effects of POPs (Jones & de Voogt 1999; Tyler et al. 1998) and later other effects such as neurotoxicity and endocrine disruption have been shown (Colborn 2004; Diamanti-Kandarakis et al. 2009; Fonnum & Mariussen 2009).

The following is an overview of representative POPs found in the environment with a description of their biological effects. Molecular structures are shown in Figure 7. This is merely a short overview; thorough reviews are given elsewhere (e.g. (Darnerud 2003; Lau et al. 2007; Reynaud & Deschaux 2006; Safe 1994; Servos 1999; Smith 1991). The effects of these compounds on biological membranes are treated separately in Section 1.6 (*in vitro* effects*)* and Section 1.7 (*in vivo* effects).

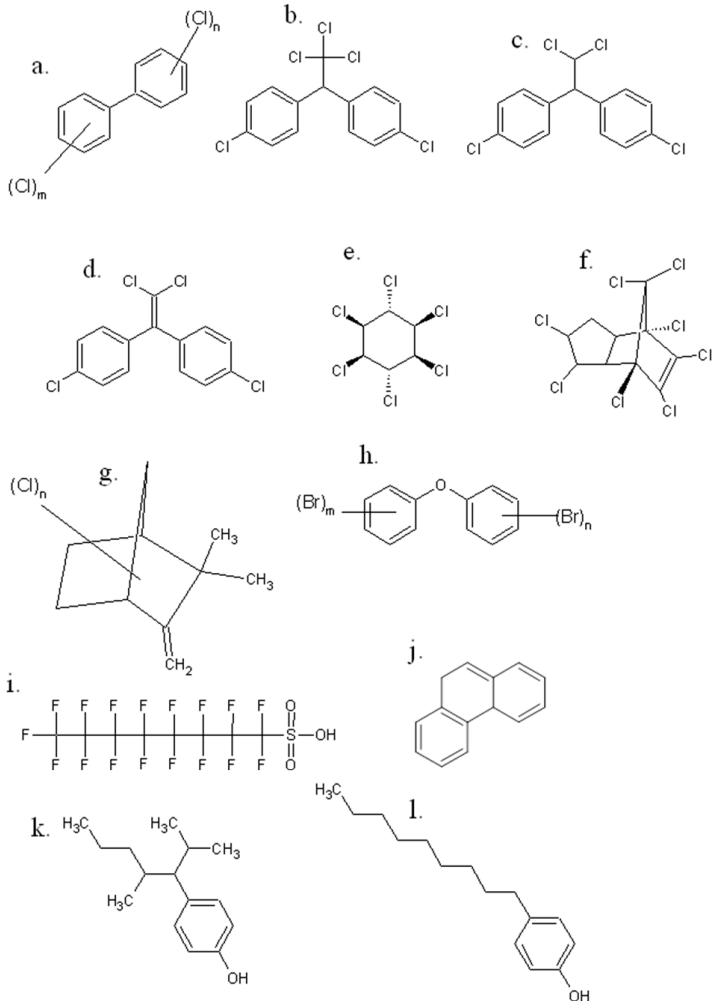

*Figure 7: Molecular structures of POPs included in this study; a, polychlorinated biphenyl (PCB); b, dichlorodiphenyltrichloroethane (DDT); c, dichlorodiphenyldichloroethane (DDD); d, dichlorodiphenyldichloroethylene (DDE); e, lindane; f, chlordane; g, toxaphene; h, polybrominated diphenyl ether (PBDE); i, perfluorooctanesulfonic acid (PFOS); j, phenanthrene, a polyaromatic hydrocarbon (PAH); k, 4-(2,4-dimethylheptan-3-yl)phenol which is an example of a 4-*tert-*nonylphenol (4-*tert-*NP); and l, 4-*n-*nonylphenol (4-*n-*NP).*

1.5.1 Polychlorinated biphenyls (PCBs)

There are 209 possible PCB congeners (Figure 7a), although a mere 36 of them are environmentally relevant. PCBs were introduced in the 1920's and gained popularity as e.g. capacitors, plasticizers in paint and transformer fluids (Kimbrough 1995). Even though the use of PCBs was banned in Western Europe in the 1980's it is still found in, and released to, the marine environment. Current sources of PCB contamination include waste disposals and releases from sediments (OSPAR 2010). PCBs are immuno- and neurotoxic, carcinogenic and affect the reproductive, developmental and endocrine systems (van den Berg et al. 1998). Toxicity differs for different PCB isomers, and especially ortho-substitution may determine toxicity. Non-ortho-substituted PCBs may have a co-planar configuration and are often referred to as dioxin-like, with biological effects similar to the toxicologically potent dioxins. The effects are mainly caused by interaction with the aryl hydrocarbon receptor (Safe 1994). The aryl hydrocarbon receptor is a transcription factor that induces phase I and II metabolism of xenobiotics (Ko et al. 1996). However, ortho-substituted PCBs are non-planar and their toxicities may be mediated by a different biological mechanism than the coplanar PCBs (Ganey et al. 1993; Tan et al. 2003; Voie et al. 2000a; Voie & Fonnum 2000b). It has been suggested that the effects of ortho-substituted PCBs are due to disruption of the lipid membrane (Campbell et al. 2008; Nishihara et al. 1985; Nishihara et al. 1992; Tan et al. 2003; Tan et al. 2004).

1.5.2 Organochlorine pesticides

Many effective pesticides, such as dichlorodiphenyltrichloroethane (DDT), lindane, chlordane and toxaphene are organohalogens. Their use is banned in most of the world, but there are restricted exceptions such as the use of DDT (Figure 7b) in malaria protection (Eskenazi et al. 2009).

Technical DDT formulations typically contained about 77 % of the para-para' substituted isomer (p-p'-DDT) with the remainder including para-ortho substituted isomers and the DDE (Figure 7d) and DDD (Figure 7c) analogs. Sublethal doses of DDT exposure cause effects on the nervous system, the reproductive system and is found to be mutagenic and carcinogenic (Smith 1991). DDT and metabolites may act on the estrogenic receptor (Klotz et al. 1996) or through other transcription factors e.g. activator protein1 (Frigo et al. 2002).

Lindane (γ-hexachlorocyclohexane, γ-HCH, Figure 7e) is an insecticide. By the year 2000 most Western European countries (the Oslo-Paris (OSPAR) Commissions) had phased out the use of lindane together with five other priority pesticides. The current marine and atmospheric levels of lindane are reduced, however some local "hotspots" remain (OSPAR 2010). Lindane may also affect nervous and reproductive systems in addition to carcinogenic effects (Smith 1991).

Chlordane (Figure 7f) is a chlorinated cyclodiene and has a *cis*- and a *trans*- isomer that metabolize with different efficiency (Murphy & Gooch 1995). Chlordane is structurally similar to other cyclodienic pesticides like dieldrin and endosulfan, and its biological effects are similar to those of other chlorinated hydrocarbon pesticides (Smith 1991). Chlordane-related metabolites (oxychlordane) may be more potent toxicants than chlordane itself (Gooch et al. 1990).

Toxaphene (Figure 7g) is a complex mixture of more than 200 different polychlorinated camphenes that historically has been used as an insecticide and piscicide (control of undesired fish stocks) (de Geus et al. 1999; Smith 1991). Toxaphene is not thermostable as it can dehydrochlorinate. As other organochloric pesticides toxaphene has been shown to have reproductive, behavioral and carcinogenic effects (Smith 1991).

1.5.3 Polybrominated diphenyl ethers (PBDEs)

Brominated flame retardants have been and are used in products such as polyurethane foams and adhesives and constitute a large group of chemicals. Commercially available mixtures of polybrominated diphenyl ether (PBDE) include PentaBDE (mostly tetra-, penta-, and hexaBDE congeners), OctaBDE (mostly heptaBDE plus hexa- and octa-BDEs) and DecaBDE (primarily the fully brominated BDE congener) (de Wit et al. 2010). The penta- and octa-BDE have been considered the most potentially hazardous substances in this group of chemicals and have been banned by the Stockholm Convention on POPs. However, the regulation of other flame retardants such as deca-BDE and hexabromocyclododecane (HBCD) has been less strict (OSPAR 2010). PBDEs are still found in the marine environment and are also subjects to long-range transport to the Arctic areas and tend to bioaccumulate in top predators (Boon et al. 2002; de Wit et al. 2010; Law et al. 2006; Voorspoels et al. 2003). PDBE (Figure 7h) can act through the aryl hydrocarbon receptor and the hydroxylated metabolites of PBDE may cause endocrine disruption through the thyroid system (Fowles et al. 1994; McDonald 2002; Meerts et al. 2000; Zhou et al. 2001). PBDE might also cause neurotoxic and reproductive effects (de Wit et al. 2010). PBDE have been shown to disrupt the Ca^{2+} homeostasis (Coburn et al. 2008) and to stimulate release of arachidonic acid by a mechanism dependent on phospholipase A_2 (Kodavanti & Derr-Yellin 2002) in rat brain.

1.5.4 Perfluorooctanesulfonic acid (PFOS)

Poly- and perfluorinated compounds (PFCs), like PFOS (Figure 7i), are stable molecules with water- and oil-repelling properties that are used in a large range of commercial products and in the industry. Their stability is due to the strong carbon-fluor-bonds. PFOS have been found in sewage sludge from wastewater treatment plants (Bossi et al. 2008; Kallenborn et al. 2004) but also in remote Arctic areas (Bossi et al. 2005; Butt et al. 2010; Young et al. 2007). PFOS have effects on the gene transcription in fish, notably genes related to energy metabolism in carp, (Hagenaars et al. 2008) and genes

related to stress responses, the Cytochrome P450 (CYP) family (phase I metabolism), phase II metabolism enzymes, lipid metabolism and ion regulation in salmon hepatocytes (Krovel et al. 2008).

1.5.5 Alkylphenols

Alkylphenols (APs, Figure 7k-l) are widespread xenobiotics found both in freshwater and coastal marine water all over the world (David et al. 2009; Servos 1999). The main environmental concern has been on the AP degradation products from non-ionic surfactants; alkylphenol ethoxylates (APE), nonylphenol (NP) and octylphenol (OP) (Ying et al. 2002). Nonylphenols have been shown to be among the molecular species in this group with the most toxic effects (Kvestak & Ahel 1994; Mcleese et al. 1981; Meier et al. 2007; Nimrod & Benson 1996; Staples et al. 2004). NP and OP have been reported in high concentration in marine sediment (up to 20 mg/kg), seawater (up to 4 µg/l) and marine biota (up to 1500 µg/kg) (David et al. 2009). APs are also found as natural compounds in crude oil and are discharged to the marine environment through produced water from of offshore oil production (Boitsov et al. 2007; Ioppolo-Armanios et al. 1992). The APs in produced water are by far dominated by short-chain APs (C_1-C_3), and they can be found in concentrations up to 50 ng/L around oil fields in the North Sea, while the long-chain APs (C_4-C_9) which constitute approximately 2 % of the total APs in produced water are not detected in seawater around the oil platforms (Harman et al. 2009). In 2010, approximately 0.3 tons of long-chain (C_6-C_9) APs were released in the produced water from the oil installations on the Norwegian shelf (Oljeindustriens Landsforening (OLF) 2011).

APs are identified to be xenoestrogens that can bind to the estrogen receptor (ER), and substantial amounts of evidence indicate that APs cause endocrine disruption in fish (Meier et al. 2011; Nimrod & Benson 1996; Servos 1999; Tollefsen & Nilsen 2008). Independent of the estrogenic pathways, APs can also induce biological effects by interfering with cell membranes. APs are amphipathic molecules with hydrogen bond

donor properties and this gives them high affinity to phospholipid membranes (Kwon et al. 2006; Nakane & Kubo 2009; Yamamoto & Liljestrand 2004).

1.5.6 Oil hydrocarbons: PAHs

Release of oil hydrocarbons to the environment might be categorized as either chronic or acute. Oil can be released to the sea from produced water discharges and an average concentration of approximately 15 mg/L dispersed oil is reported in produced water released from oil installations at the Norwegian Continental Shelf. The total release of oil from the Norwegian petroleum industry was 1563 tons in 2010 according to (Oljeindustriens Landsforening (OLF) 2011).

Accidental oil spills are also sources of crude oil releases to the environment. After a spill, the oil is subject to a weathering process, i.e. the combination of processes such as spreading of the spill, evaporation of volatile constituents in the oil, water/oil emulsifications, natural dispersion of oil in water, sedimentation, photo-oxidation and dissolution. Microbial degradation plays an important role in the degradation of spilled oil. Crude oils consist primarily of hydrocarbons but are complex mixtures that might also contain trace metals in addition to nitrogenous, sulphurous and oxygenic compounds (AMAP 2010). Biological effects such as oxidative stress, genotoxicity, lipid alteration and induction of biotransformation enzymes are observed in wild fish near oil installations in the North Sea (Balk, 2011) and in controlled laboratory experiments (Meier et al. 2007; Holth et al. 2009; Lie et al. 2009).

Polycyclic aromatic hydrocarbons (PAH, Figure 7j) found in crude oil are dominated by the small two- and three-ringed PAH and their alkylated derivatives, normally named NPD (=sum of naphthalene, phenanthrene, dibenzothiophene, and their C1-C3 alkylated homologs). The NPDs are considered to play a very important role in the toxicity to fish, even though it is also recognized that they do not explain the total toxicity, and there are many other toxic compounds in crude oil (Barron et al., 1999; Neff et al., 2000; Incardona et al., 2004; Melbye et al., 2009). The heavy 4- and 5-ringed PAH that have been identified to have carcinogenic and mutagenic properties

(Varanasi, 1980; Varanasi, 1982) are only present in very low levels in crude oil and originate mainly from combustion of organic material (Lima et al., 2005). Recognized biomarkers of PAH contamination in fish are the presence of PAH metabolites in bile, induction of CYP1A (measured as 7-ethoxy-resorufin-*O*-deethylation (EROD) activity) in liver and DNA adducts in liver (Aas et al. 2000; Stagg 1998). The elimination of PAH through metabolism is efficient in fish, and PAH do not tend to bioaccumulate to the same degree as e.g. organohalogenated POPs (Tuvikene 1995; van der Oost et al. 2003).

1.6 In vitro effects of POPs, oil and alkylphenols on membrane lipids

Several studies showing membrane disrupting effects of POPs in vitro are found in the literature, and an overview of the scientific literature on the subject is given in Supplementary data, Table A1.

1.6.1 PAHs

Monolayers of DOPC can be penetrated by PAHs, according to a study with anthracene, phenanthrene, pyrene, benzo[α]anthracene, fluoranthene and perylene (Nelson 1987). Korchowiek and co-workers studied monolayers of several disaturated model phospholipids exposed to five different PAHs (Korchowiec et al. 2008). They found that the monolayers were more expanded and in some cases were more liquid-like when in presence of PAHs. It was the largest molecule in their study, benzo(α)-pyrene that had the most severe effects. Several other studies have confirmed increased lipid membrane fluidity (Engelke et al. 1996) and decreased phase transition temperatures (Jimenez et al. 2002) after exposure to PAHs. (Weinstein et al. 1997) showed that ultrastructure (in gill cells of fathead minnow) can be altered by PAH (fluoranthene) e.g. by inducing the formation of lipid droplets.

1.6.2 APs

Membrane effects such as membrane swelling, increase in fluidity, lowering of the phase transition temperature, increased ion permeability and mitochondrial depolarization are found both for *ortho*-substituted APs (James & Glen 1980; Lanigan & Yamarik 2002; Singer 1977; Tsuchiya 2001) and *para*-substituted APs (Bragadin et al. 1999; Gong et al. 2008; Haavisto et al. 2003; Lamche & Burkhardt-Holm 2000; Xiao et al. 2011; Yao et al. 2006).

Increased membrane fluidity and disorder was shown in testicular Sertoli cells from rat (Gong et al. 2008). Morphological changes such as membrane swelling and an increased number of lipid particles have been shown in gill cells from the flounder (*Paralichthys olivaceus*) after exposure to NP (Xiao et al. 2011). Vesiculation of the Golgi apparatus has been shown in epidermis culture from rainbow trout after NP exposure (Lamche & Burkhardt-Holm 2000). In previous studies at IMR the Langmuir monolayer technique has been used to show that low concentration of different APs, from butylphenol to NP, increase the molecular areas of phospholipid monolayers, indicating that APs give a "looser packing" of the lipids and more fluid monolayer (Meier et al. 2007). The strongest effects were found for 4-*n*-NP. The branched isomers of NP, which are the most environmental relevant NPs, were not tested.

There are a number of reports from several groups showing that NPs have direct effects on membrane physical properties. Gong and co-workers (Gong et al. 2008) found that in rat testicular Sertoli cells the membrane fluidity increased and the microviscosity and molecular order decreased after exposure to 10, 20 and 30 μM NP. No significant effects were found at 0.1 and 1 μM NP. APs have earlier been found to have effects on the cell ultrastructure. NP is able to alter the cell morphology by swelling and increasing the number of lipid particles (Xiao et al. 2011) and induce vesiculation of the Golgi apparatus (Lamche & Burkhardt-Holm 2000). By enhancing the permeability of protons through the mitochondrial membrane, NPs are mitochondrial uncouplers that inhibit the ATP synthesis in mitochondria (Bragadin et

al. 1999). APs (C_4 and C_8) have also been shown to induce formation of lamellar bilayers in lipid droplets in rat Leydig cells and other morphological changes (Haavisto et al. 2003). NP ethoxylates are similarly found to change the ultrastructures of membrane compartment in amphibian heart induced by destabilization of the membrane lipids (Perrotta & Tripepi 2012). Watanabe et al. found that several genes in the lipid and fatty acid metabolism, e.g. acetyl-CoA-acyltransferase, were activated by nonylphenol but not estradiol, suggesting a mechanism independent of endocrine effects (Watanabe et al. 2004).

1.6.3 Halogenated POPs

Bonora and co-workers showed decreased melting temperature in DPPC(16:0/16:0-PC) liposomes with Aroclor 1254, a technical PCB-mixture (Bonora et al. 2003). Campbell and co-workers found that a di-saturated phospholipid bilayer that was added an ortho-substituted PCB (PCB-52) had two melting points whereas the non-ortho-substituted PCB (PCB-77) only had one, and proposed a model where the substitution pattern of the PCB is what determines the interaction with a lipid bilayer, suggesting that there is a stronger lipid-PCB interaction with an ortho-substituted PCB than for the co-planar "dioxin-like" PCBs (Campbell et al. 2008). Lindane, a hexachlorocyclohexane and pesticide, increased the membrane fluidity in model bilayers of DMPC (14:0/14:0-PC) and DMPE (14:0/14:0-PE) and seemed to prefer the inner leaflet of the human erythrocyte membrane (Suwalsky et al. 1998). Another chlorinated pesticide, DDT, decreased the melting points of model liposomes with DMPC and DMPE, and seemed to prefer the external layer of the liposome bilayer (Bonora et al. 2008). Endosulfan is an organochloric insectide and Differential Scanning Calorimetry studies prove that the α- and β-isomers both decrease the phase transition temperature on model phospholipid bilayers (DPPC) (Videira et al. 1999). Also fluorinated POPs can have effects on membranes. Hu and co-workers found increased membrane fluidity in fish leukocytes exposed to perfluorooctane sulfonic acid (PFOS) (Hu et al. 2003). Model phospholipid monolayers studied by means of the Langmuir technique also show increased fluidity when exposed to PFOS and

perfluorooctanoic acid (PFOA); the molecular area of the lipids are increased, and the phase transitions less pronounced (Matyszewska & Bilewicz 2008a; Matyszewska & Bilewicz 2009; Matyszewska et al. 2010). PFOS have greater effect than PFOA on the model monolayers (Matyszewska & Bilewicz 2008a). The poly-brominated diphenyl ethers (PBDEs) causes injuries related to cellular oxidative stress, mitochondrial damage and apoptosis (cell death) in rainbow trout gill cells (Shao et al. 2010).

In common for many of the in vitro studies mentioned here, is the relative high pollutant:lipid ratio in the systems studied, concentrations in the magnitude of 10 mol % PCB to lipid may not be environmentally relevant.

Relevance of alterations in membrane composition

There are different scenarios when a compound with potential membrane-altering effects is introduced to the membrane. Changes in the physical properties of the membrane and undesired variations in the permeability of the membrane can occur (Hu et al. 2003; Nelson 1996; Videira et al. 2002). Cellular functions such as carrier-mediated transport, membrane-bound enzymes and receptors might be altered when the membrane lipid composition is modified (reviewed in (Spector & Yorek 1985)). The consequences of membrane alterations are not always given, however, membrane lipid synthesis is under strict regulation and each type of membrane has its own characteristic composition (Stubbs & Smith 1984; Nelson & Cox 2008c) indicating that an optimal composition of the membrane is important for living organisms (Dowhan, 1997; Piomelli et al., 2007; Van Meer et al., 2008; Khalil et al., 2010).

1.7 In vivo effects of POPs, oil compounds and alkylphenols on membrane lipids

There are relatively few studies addressing the *in vivo* effects that xenobiotic pollutants have on biological membrane lipids. An overview of previously published reports on the subject is given in the Supplementary data, Table A2.

Dey and co-workers report significant differences in the lipid class composition in cod liver after the fish had been exposed to crude oil over 24 weeks with total hydrocarbon concentration 100-200 ppb (Dey et al. 1983). However, the lipid class composition that is reported for the control group deviates largely from other (and more recent) published reports. Dey et al. reports the mean total phospholipid content to be more than 25% of the total lipids whilst the triglycerides mean are reported to be a mere 35 % of the total lipids. In comparison Meier et al. found that neutral lipids/triglycerides and phospholipids constituted approximately 95 % and 1.3% respectively of the total lipids (Meier et al. 2007). Bell et al. also found similar lipid class distribution; with neutral lipids constituting more than 98 % of the total lipids in cod liver (Bell et al. 2006). Thus the results of Dey et al. may really tell us more about the technological development over the last 3 decades than about actual effects of oil pollution on lipid class composition in cod liver. Dey et al. also show the distribution of the FAME of the total lipids in the male cod liver, with large differences between control and exposed groups, notably an increase in the SFAs 14:0, 16:0 and 18:0, the MUFAs 16:1, 18:1 and 20:1 and the PUFA 20:5 and a decline in the MUFAs 14:1, and 17:1 and the PUFAs 16:4, 18:4, 20:4, 22:5 and 22:6. Similar, though more subtle, effects were seen in cod exposed to short-chained alkylphenols (Meier et al. 2007)**.** In a study from IMR, Atlantic cod were given weekly doses by oral intubation to the stomach of a mixture of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol through 5 weeks (Meier et al. 2007). Their main findings were a high increase in SFA and a decrease in (n-3)-PUFA, 22:6 in particular, in the PL fraction of cod liver. The brain cholesterol levels were reduced.

Membrane effects of POPs have also been studied in other animals. Examples of effects are accumulation of TAG and cholesterol in liver (Hinton et al. 1978; Kawashima et al. 1995; Kimbroug et al. 1972; Kudo & Kawashima 1997; Kudo et al. 1999), morphological alterations in the liver (Hacking et al. 1978; Hinton et al. 1978; Jonsson et al. 1981, Sylvie et al. 1996), alteration in the fatty acid profiles (Borlakoglu et al. 1990; Kakela & Hyvarinen 1999; Kudo et al. 1999; Kudo et al. 2011; Matsusue

et al. 1999) and changes in the activity of enzymes in lipid biosynthesis (Boll et al. 1998; Borlakoglu et al. 1991; Kawashima et al. 1995; Kudo et al. 1999; Kudo et al. 2011; Matsusue et al. 1999) (Supplementary data, Table A2).

Fatty acid alterations that are found in several studies are increases in SFA and/or MUFA (Dey et al. 1983; Kudo et al. 1999; Kudo et al. 2011) or decrease in PUFA (Kakela & Hyvarinen 1999; Meier et al. 2007). The studies are not always consistent with each other. Increased hepatic levels of AA in rats after administration of PCB are reported by (Borlakoglu et al. 1990) while reduced AA in total lipids are reported by (Matsusue et al. 1999).

Findings in wild fish

Lipid studies have been performed on wild fish from areas near oil installations with comparisons to fish from so-called clean areas. Balk and co-workers found a reduction in the ratio of (n-3)/(n-6)-PUFA in the muscle in both Atlantic cod and haddock near the oil installations at the Tampen field in the North Sea compared to a reference area at the Egersund bank (Balk et al. 2011). Also an elevation in the concentration of AA (20:4(n-6)) in the liver was found for both cod and haddock from near the Tampen field. Similar results were found by Grøsvik and co-workers where they found that the concentration of arachidonic acid (20:4(n-6)) was higher in haddock from the Tampen field than at reference areas. Also the (n-3)/(n-6)-PUFA levels were significantly lower in the neutral lipid, free fatty acids and PC/PE- fraction in haddock at the Tampen field (Grøsvik et al. 2009). Haddock from the Tampen field were in general lower condition than haddock from reference areas, with both relatively small livers and low hepatic lipid levels, and had approximately 50 % of the energy reserve compared with fish from the other areas (Grøsvik et al. 2009).

1.8 Membrane lipids and oxidative stress

Reactive oxygen species (ROS)

Common reactive oxygen species (ROS) include superoxide (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) (Huttemann et al. 2011). Most of the oxygen consumed by organisms is utilized in a 4 electron pathway producing energy, and water, in the eukaryotic mitochondrion (illustrated in Figure 8). Less than 10% of the consumed oxygen is reduced to ROS in a one electron pathway (Lushchak 2011). The "escaped" electron instead reacts with molecular oxygen to produce $O_2^{\cdot -}$ (Lushchak 2011), see Figure 8. Another place for ROS production is the endoplasmic reticulum (ER) where catabolism of both cellular and foreign chemicals by cytochrome P450 happens (Lushchak 2011). ROS may also be produced by oxidases in the cytosol and peroxisomes, and also by autooxidation of certain cell components or xenobiotics (Lushchak 2011).

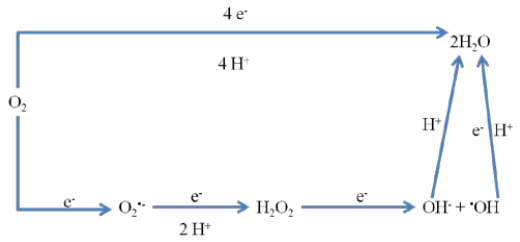

Figure 8: Schematic overview of oxygen metabolism. Two routes are possible: The upper part shows the 4-electron pathway that produce energy and water while the lower part shows the formation of reactive oxygen species (ROS), i.e. superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). (This representation is based on a figure in (Lushchak 2011)).

Important enzyme systems for the detoxification of ROS are superoxide dismutase (SOD), catalase, glutathione peroxidase (GPXs), and transferases, xanthine oxidase and glucose 6-phosphate dehydrogenase (G6PD) (Slaninova et al. 2009). No single parameter that alone functions as a biomarker of oxidative stress have been established to this date (Lushchak 2011; Slaninova et al. 2009). Several sensitive indicators have been suggested: Decreases in the ratio glutathione (GSH):glutathione disulfide

(GSSG) because healthy cells would contain about 100 times more of the reduced form GSH than the oxidized GSSG (Slaninova et al. 2009). Levels of metallothioneins (MT) or lipid peroxidation and also the activities of glutathione reductase (GR), glutathione S-transferase (GST) and GPX can indicate oxidative stress (Slaninova et al. 2009). Biomarkers of oxidative stress can be divided into two groups; those indicating damage by free radicals and those indicating the functions of the anti-oxidant defense systems (Slaninova et al. 2009). One of the most reactive ROS made *in vivo* is ·OH, a product of ionizing radiation (Halliwell 1994). Cytochrome c (Cytc) is a mitochondrial protein that is essential for aerobic energy production by taking part in the electron transfer in the electron transport chain (Huttemann et al. 2011). Cytc is also important in the progression of apoptosis, and it can function as a cardiolipin peroxidase and have phosphorylation sites (Huttemann et al. 2011). Cytc is both a ROS scavenger (superoxide and peroxide) and a ROS producer (Huttemann et al. 2011).

POPs can induce oxidative stress

Pesticides have several possible mechanisms to cause oxidative stress; entering the redox cycles by accepting or donating electrons, demand involvement of reductants such as glutathione, or inactivate antioxidants and associated enzymes and thus decrease the antioxidant capacity of the cell, decrease the metabolism and detoxification of the cell by disturbing energy-providing processes in the cell and finally pesticides may modify transcription and translation and indirectly increase the ROS level (Limon-Pacheco & Gonsebatt 2009; Lushchak 2011). Oxidative stress can be caused by pesticides and their biodegradation products, such as organochlorine and orgaonofluorine pesticides, organophosphates, carbamates, pyrethroids, bipyridyl herbicides, triazin and chloroacetanilide herbicides (Slaninova et al. 2009). Halogenated aromatic hydrocarbons and PAHs act on the aryl hydrocarbon receptor (AhR)/aryl hydrocarbon nuclear translocator (ARNT)-signaling pathway which involves genes such as cytochrome P4501A1, UDP-glucorunosyltransferase and NADPH quinine oxidoreductase (Limon-Pacheco & Gonsebatt 2009).

Lipid peroxidation

When a lipid is attacked by a free oxygen radical or another ROS a free radical chain reaction creating lipid peroxides is initiated. This chain reaction only stops when two (lipid) radicals react to create non-radical products (Crockett 2008).

The lipid peroxidation reaction chain may be described as

$-CH + R\cdot \rightarrow -C\cdot + RH$

$-C\cdot + O_2 \rightarrow -CO_2\cdot$

$-CO_2\cdot + -CH \rightarrow -CO_2H + C\cdot$

where –CH is a fatty acyl side chain, $R\cdot$ is a ROS capable of oxidizing PUFA. $R\cdot$ include ROS such as $\cdot OH$ and (lipid) peroxyl radicals ($-CO_2\cdot$) (Halliwell 1994).

The products of lipid peroxidation are widely used as biomarkers of damages from free radical reactions caused by e.g. pesticides (Slaninova et al. 2009). Such products include malondialdehyde, a secondary oxidation product of PUFAs, and other aldehydes and ketones (Slaninova et al. 2009). In wild fish it has been difficult to establish biomarkers for oxidative stress because of the variation in factors such as sex, reproductive condition, temperature, salinity, physiological or genetic adaptation to pollution, diet, (dissolved) oxygen and seasonal variation (Slaninova et al. 2009). Lipid oxidation products, such as resolvins, lipxins and isoprostanes, are also important as regulators in disease processes controlled by toxins (Berliner & Zimman 2007). PUFA are more prone to oxidation than MUFA and SFA, and if containing the same fatty acids PE is more likely than PC to undergo oxidative reactions (Crockett 2008). Vitamin E (or more precisely its major constituent; α-tocopherol) is an effective anti-oxidant when it comes to lipid peroxidation when there is balance between ROS and antioxidants in an organism. It works as a scavenger by removing the part of the fatty acid where the peroxyl is, and the product of this reaction is an α-tocopherol radical that is much less reactive than peroxyl radicals (Pamplona et al. 2002; Lushchak 2011).

1.9 Brief overview of membrane lipids in Atlantic cod (*Gadus Morhua*)

1.9.1 Cod liver

Cod liver membrane lipids are for the most part glycerophospholipids (phospholipids (PL) from here on) but the membranes also contain other lipids like sphingolipids and cholesterol. The molecular structure of a typical phospholipid with a glycerol backbone is shown in Figure 2, and the main head groups, choline, ethanolamine, serine, inositol and glycerol are shown in Figure 3. Phospholipids constitute 1-2 % of the total lipids in the liver of farmed cod (Meier et al. 2007). As the liver is the energy store of the cod, the liver lipids are dominated by triacylglycerols (TAG) constituting up to about 70 % of the liver wet weight. Clear correlations between the fatty acid composition of the TAG with the diet's FA profile have been shown (Lie et al. 1986), but there are no such clear correlations between diet and phospholipids (Lie & Lambertsen 1991). The main phospholipids found in cod liver are, in decreasing order of quantity in the membrane, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) (Lie & Lambertsen 1991). Each phospholipid also has a characteristic fatty acid profile (Bell & Dick 1990; Bell & Dick 1991; Lie & Lambertsen 1991); in common for all of them are a relatively high amount of PUFA such as DHA (22:6(n-3)) and EPA (20:5(n-3)). Briefly the PC is also characterized by a high proportion of the SFA 16:0, and the PI by high levels of arachidonic acid, 20:4(n-6) (Lie & Lambertsen 1991). The membrane lipid composition in cod as in other marine poikilothermic species has been shown to be flexible when exposed to thermal changes in the environment. Through homeostatic adaptation the membrane fluidity remains the same by altering the lipid composition in the membrane (see section 1.4).

1.9.2 Cod brain

Contrary to what is seen for the liver, the majority of the lipids found in brain tissue from cod are membrane lipids, and the relative PE amount is slightly higher than the

PC amount (47% and 41 % of the total polar lipids respectively) (Tocher & Harvie 1988). Total lipid content of cod brain is reported to range from 4.8% (Stoknes et al. 2004) to 7.7% (Tocher & Harvie 1988) to 9.30 % (Meier et al. 2007). The polar lipids constitute approx. 96-97 % of the total brain lipids (Meier et al. 2007) (an early report from 1988 found 75.7 % polar lipids (Tocher & Harvie 1988)). Characteristic for the brain is the presence of a relative large amount of cholesterol, and ether lipids (plasmalogens) in the PE-fraction (Bell & Dick 1993). Cholesterol and plasmalogens have important structural effects. Cholesterol has a rigidising effect on the lipid membrane. Plasmalogen-deficient cells have been shown to have more fluid membranes than those that contain plasmalogens (Hermetter et al. 1989), but plasmalogens also have a propensity to form inversed hexagonal phases (Lohner 1996). Plasmalogens may serve as a PUFA store, and some phospholipases (A2 family) can break the plasmalogen to yield e.g. arachidonic acid. Plasmalogens might also be protective against oxidative stress (Brites et al. 2004). The brain lipids also include sphingolipids, which include sphingomyelin, and glycosphingolipids (cerebrosides) (Olsen & Henderson 1989). Sphingolipids are often found in membrane microdomains (lipid rafts, caveolae) (Christie 2012; Sonnino & Prinetti 2009). It has been shown that lean fish (like Atlantic cod) have more accumulated POPs in brain than fattier fish, and POPs may also redistribute from liver to brain during periods of starvation (Elskus et al. 2005).

1.10 Analytical methods: In vitro study

This section covers the methodological background for an *in vitro* study that is part of the thesis. The results of the study are collected in Appendix 2 and discussed in the general discussion (Section 4.2).

1.10.1 The Langmuir technique

The Langmuir monolayer technique models one part of a biological membrane, the lipid monolayer (of which the cell membrane has two in its lipid bilayer structure). Phospholipids are spread on an aqueous surface, and form a monolayer with their polar

head-groups in the water and hydrophobic acyl chains in the air. The area of the monolayer is controlled by barriers that can compress or expand the monolayer while the surface pressure is measured. This technique can be used to study the effects of various compounds on the biophysical properties of the lipid monolayer.

Experimental procedure

The Langmuir trough used in the study was similar to that in Figure 9 (except that it did not have a dipper). In brief, the experimental procedure (similar to that described in (Broniec et al. 2007)) was to fill the trough with an aqueous solution (HEPES buffer) when the barriers were totally expanded (at each end of the trough). Temperature in the trough might be regulated by water that circulated underneath it. Dust particles were removed with a Pasteur pipette. Lipid solution (in chloroform) was slowly added with a syringe on top of the aqueous subphase and as the chloroform evaporated, a lipid monolayer would form. The barriers were coated with a hydrophobic material (Delran) so that the monolayer could not slide under the barriers. The barriers were driven towards each other by a motor controlled by the computer. Surface pressure was measured with a platinum plate (the Wilhelmy plate) connected to an electrobalance, and the computer calculated the Langmuir isotherms as functions of the mean molecular area (MMA) with applied mass and molar mass of the monolayer lipid as inputs.

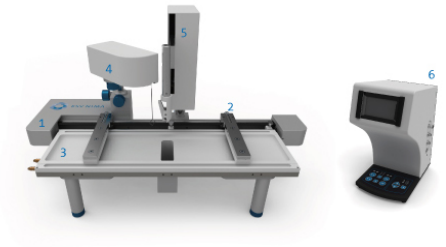

Figure 9: The Langmuir trough from KSV Nima (picture downloaded from company website (KSV Nima 2012)). The numbers in the figure show 1, Trough top; 2, Frame; 3, Surface pressure sensor; 4, Barriers and barrier drive; 5, Dipper (option); 6, Subphase cooling/heating mechanism.

Langmuir isotherms

The compression isotherms resulted from when the area of a lipid monolayer was being reduced as the barriers on the instrument were pressed toward each other. The lipids formed a monolayer with polar head-groups in the aqueous subphase and fatty acyl chains in the air. When the monolayer was expanded the lipids were in a gaseous phase (Broniec et al. 2007). With compression the lipids were pressed closer to conform a liquid phase. The transition between gas and liquid increased the surface pressure, a "lift-off". Further compression forced the lipid molecules into a solid phase and a steep rise in the surface pressure. The lipid molecules could not be pressed further toward each other and still remain a monolayer; which means that a further compression made the monolayer collapse as observed by decreased surface pressure (Blois et al. 2006; Broniec et al. 2007). An illustration of the compression isotherm is shown in Figure 10.

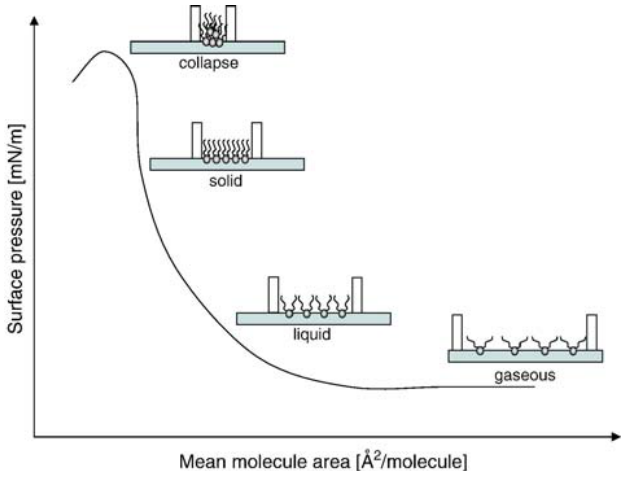

Figure 10: The Langmuir compression isotherm (from (Meier et al. 2007)). Before compression the lipid monolayer is in the gaseous phase, with the acyl chains far apart in the air. The compression barriers are pressed together (from right to left in the diagram) ordering the monolayer in a liquid phase. Further compression forces the monolayer to the solid phase, until the monolayer collapses when the barriers are so close to each other that the lipid molecules cannot be forced further together.

Effects of PCB congeners on lipid monolayers

To assess the impact of PCB on biological membranes, Langmuir studies of model lipids and native lipids from cod were performed. No attempts on dissolving the pollutants in the aqueous subphase were done as PCBs are very hydrophobic with high octanol-water and lipid-water partitioning constants (Jabusch & Swackhamer 2005). Instead the PCBs were dissolved in chloroform stock solutions. PCB stock solutions were mixed with lipid stock solutions to yield PCB-lipid solutions with controlled molecular ratios. The PCB-lipid solution was applied to the subphase in an identical manner to when only pure lipid solutions were applied. Three different PCB congeners were tested, PCB-52 (2,2',5,5'-substituted), PCB-77 (3,3',4,4'-substituted) and PCB 180 (2,2',3,4,4',5,5'-substituted). The most used model lipid was POPC (16:0/18:1-PC), and POPS and a POPE:POPC-mixture were also tested. Native PC extracted from cod brain with cholesterol, and PC from cod liver, were also tested. Molecular ratios PCB:lipid ranged from 1:2 to 1:1000 (Appendix 2). This concentration range covered (and went beyond) the theoretical concentrations of PCB:membrane lipid in the *in vivo* exposure study on Atlantic cod. Representative isotherms of the tests are shown in Appendix 2.

Effect of a synthetic PAH metabolite (9-OH-phenanthrene) on lipid monolayer

PAHs are metabolized more rapidly than PCB, and result in hydroxylated metabolites (Tuvikene 1995; van der Oost et al. 2003). To assess the potential effect of PAH metabolites on biological membranes, a pilot study was performed with 9-OH-phenanthrene (9-OH-P) and POPC. The molecular ratio 9-OH-P:POPC was 1:25 or 1:125. The Langmuir isotherms were similar with and without the 9-OH-P (Appendix 2). The purpose of the study was to look at environmentally relevant ratios of pollutants to lipid (similar to the expected ratios in our *in vivo* exposure experiment on Atlantic cod) so higher ratios were not tested. Higher ratios of 9-OH-P might have given important and interesting insights into the biophysical interference of 9-OH-P

with a lipid monolayer. However, this was regarded as to be beyond the environmental focus of this project.

2. Aims of the thesis

The work presented in this thesis is a part of a project financed by the Research Council of Norway. This is a follow-up study of an earlier IMR project that studied endocrine effects of alkylphenols in Atlantic cod and discovered that the membrane lipid composition in brain and liver was altered, by mechanisms apparently independent of the endocrine disruption. The biological material in this study comes from an exposure study performed in 2008 where Atlantic cod were orally exposed to

1. Branched or straight-chained nonylphenols

or

2. A mixture of POPs (PCBs, chlorinated pesticides, PBDE and PFOS) and/or weathered crude oil from the Troll installation. The exposure doses corresponded either to realistic background levels or 50 times higher concentrations (equivalent to large acute spills).

A detailed study of lipid classes in liver and fatty acid profiles of lipid classes from liver and from total lipids from brain from the exposed fish has been in focus.

The major aims of the thesis are

- Validate a method for separation of lipid classes from liver tissue from Atlantic cod (*Paper 1*).
- Study the effects of branched and straight-chained nonylphenols on the membrane lipid composition in liver and brain of male Atlantic cod. Transcription of genes involved in lipid biosynthesis, xenobiotic metabolism and the defense against oxidative stress is also studied in liver mRNA, as is the metabolism of the various NPs (*Paper 2*).
- Effects of persistent organic pollutants (POPs) and oil hydrocarbons on the membrane lipid composition in lipid classes in liver and total lipids in brain of male Atlantic cod. The bioaccumulation of POPs in liver and PAH metabolites

in bile have also been studied. Microarray analysis of mRNA from liver has been performed and data analysis has focused on important genes from the PL biosynthesis, phase 1 and II metabolism and oxidative stress (*Paper 3*).

- Biophysical studies of the effects of PCBs on monolayers made of model phospholipids and native membrane lipids from cod have been performed by the use of the Langmuir monolayer technique (*Appendix B*).

3. Summary of papers

Paper 1: Pitfalls in the use of polyethylene aminopropyl-coated columns for solid phase extraction separation of lipids

The background contaminations from three different solid phase extraction (SPE) columns were tested. Two of the columns were made of polyethylene and the third was made of glass. All columns released contaminations of fatty acids, notably 16:0 and 18:0, although the contamination released from the glass column was considerably lower than that from the polyethylene columns. The effectiveness of separation of the lipid classes neutral lipids, free fatty acids, PC, PE, PS and PI was tested. On the glass column two of the phospholipids, PC and PE, co-eluted.

Paper 2: Effects of branched and normal isomers of para-substituted nonylphenols on the glycerophospholipids in the liver and brain of male Atlantic cod (Gadus morhua)

Atlantic cod (*Gadus morhua*) were exposed *in vivo* to *para*-substituted nonylphenols (NP). The fish were fed with a weekly dose of either the normal isomer (4-*n*-NP) or a technical mixture of branched isomers (4-T-NP) of *para*-substituted NP, corresponding to a body burden of 1000 µg/kg for four weeks. Lipid class composition (TAG, FFA, PC, PE, PS and PI) and fatty acid distribution of the lipid classes in the cod livers were determined by High Performance Thin Layer Chromatography (HPTLC) followed by GC-FID. The fatty acid composition in the total lipids from brain was studied. NP-treatment did not induce significant changes in lipid composition in cod liver. Only a few minor changes were observed in the fatty acid profile of the brain and the lipid classes in the liver. Real-time PCR was used to assess the expression levels of selected genes from the CYP-family, the phospholipid biosynthesis pathway and the endocrine system in NP-exposed fish compared to unexposed control fish. Differential expression of CYP2N, AGPAT9, ELOVL1, PLA1A, PEMT and ZP3 after exposure to NP was reported. NP was detected in cod liver and bile. The NP concentration in the bile was 50 times higher than in the liver

for 4-T-NP and 80 times higher for 4-*n*-NP, indicating that the metabolism rate of 4-n-NP is likely to be higher for the straight chain NPs than for the branched isomers.

Paper 3: Effects of oil pollution and persistent organic pollutants (POPs) on glycerophospholipids in liver and brain of male Atlantic cod (Gadus morhua)

Eight groups of Atlantic cod were exposed *in vivo* to weathered crude oil and/or a mixture of halogenated POPs (PCB, chlorinated pesticides, PBDE and PFOS) for 4 weeks. The body burdens of exposure corresponded to environmentally realistic levels (low/high). One control group and one negative control (no force-feeding) group were also included in the study. The lipid class compositions in the livers (TAG, FFA and membrane lipids) were reported. Fatty acid distribution in the lipid classes from the liver, and from the total lipids in the brain, was determined by GC-FID of fatty acid methyl esters. Treatment with POPs and/or crude oil did not induce significant changes in lipid composition in cod liver, and only minor changes were observed in the fatty acid profile of the brain and the lipid classes in the liver. Isolated mRNA from cod liver was studied with microarray and RT-qPCR. When looking at the microarray dataset as a whole few differences were observed in fish between the groups exposed to POPs and/or Troll oil and there was large individual variation in gene expression, also within the same treatment groups. Central genes in the PL biosynthesis (phospholipases (A1, A2, C, D), elongases, desaturases, AGPATs, LPCATs, choline/ethanolamine phosphotransferases, PISD, PEMT and transacylases), related to defence against oxidative stress (superoxide dismutases, glutathione peroxidase and reductase, catalase, peroxiredoxin-1, peroxisome proliferator-activated receptors (PPARs) and heat shock proteins (HSP)) and phase I and II metabolism of xenobiotics (CYP-genes, glutathione s-transferases (GST), UDP-glucuronosyltransferases (UDP-GT) and sulfotransferases (SULT), were selected from the microarray dataset for closer examination. However, in line with the lipid composition data, the current exposure experiment mediated only modest transcriptional responses in liver of the fish. The contents of PCBs, PBDE, pesticides (DDTs, HCH and CHL) and PFOS in the cod livers, and hydroxylated PAH in bile, were reported. A combination of oil and

POPs induced the CYP1a detoxification system. There were differences in the accumulation factor between the different treatments suggesting that the combination of oil and POPs increased the metabolism of the different POPs.

4. General discussion

4.1 Analytical methods

4.1.1 Lipid class separation

Solid phase extraction (SPE) vs. High Performance Thin Layer Chromatography (HPTLC)

Solid Phase Extraction (SPE) with aminopropyl-linked silica as a stationary phase and various solutions that elute compounds according to degree of polarity is a well established method for separation of lipid classes (Christie 1992). This method has also been the preferred method for lipid analysis in our laboratory at the IMR for more than a decade. An elution regime as described by (Perez-Palacios et al. 2007) has been modified to be optimal for the separation of marine lipids (by increasing the solvent volume that elutes the PC fraction, description is given in *Paper 1*). This SPE regime was also intended to be used as the main method for lipid class separation in this project. However, the detection of severe contamination by short-chained saturated fatty acids in the SPE-columns that we documented in *Paper 1* and that to some extent have been documented by others (Russell & Werne 2007; Vonk et al. 2008; Vonk et al. 2010; Karlsson et al. 2011) made it necessary to apply other analytical methods. HPLC-MS is a good method for separation of lipid molecular species (Hermansson et al. 2005), however we had no validated methods for this in our laboratory, and the initiation of such a method validation was regarded as too complex for this project. High performance thin layer chromatography (HPTLC, further abbreviated to TLC) was considered a proper alternative, with an established method for separation of lipids from marine tissues (Olsen & Henderson 1989).

Advantages with this technique are that it is relatively time- and cost-effective and quite simple to perform. Only small amounts of solvents are necessary, which makes the TLC method a good “green” candidate. Disadvantages with the TLC method are that TLC is sensitive to the ambient temperature and humidity. This can be controlled

to a certain degree by keeping the plate in the oven (120°C) before analysis, in a desiccator between first and second elution and by reducing the time the plate is exposed to the ambient air in the lab to a minimum, but variations will still be present. The problems these variations cause with identifying different compounds can be minimized by adding (external) lipid standards on each plate. More severe are the consequences these variations have on the quality of the elution. Humidity can lead to poorer resolution. In the samples of lipids from cod liver this made the separation of especially phosphatidylethanolamine (PE) from the dominating triglycerides fraction difficult. The triglyceride-rich liver lipid is a challenging tissue to analyze for the much lower levels of phospholipids present in the cell membrane. Triglycerides make up about 95-99 % (Meier et al. 2007) of the total lipids, and the phospholipids only about 1 %. When adding enough lipid extract in order to obtain detectable amounts of phospholipids, one may overload the chromatographic system resulting in an improper separation of the lipid classes. In order to get good chromatographic bands of all lipid classes, so that GC-FID analysis of the fatty acid methyl esters could be performed, each liver lipid extract was analyzed on three different plates; one each to collect for neutral lipid (triglycerides), phospholipids (PC, PE and PS/PI) and free fatty acids. (Cholesterol content was detected and calculated as a pooled average from the two total lipid fractions.) When demanding plural TLC analyses for each sample this method was not efficient compared to the previously described SPE method, and the analytical variation is also larger for the HPTLC procedure than the SPE method. However, as one of the main aims of the thesis was to separate between the different membrane lipids, the HPTLC worked well for the purpose. As described in *Paper 1*, experiments with SPE columns made of glass did not yield satisfactory separation between the major membrane lipids PC and PE (even if the contamination of fatty acids were minor in the glass columns compared to the polyethylene columns).

High Performance Thin Layer Chromatography

TLC is a simple method for rapid separation of analytes, with many applications in the separation of lipid classes (Touchstone 1995; Fried 2003; Fuchs et al. 2011). The

method was first described and published in 1938 and has been a “mainstream” method since the 1960’s (Shostenkot et al. 2000). The development of High Performance Thin Layer Chromatography (HPTLC) with commercial and standardized adsorbent plates has renewed the interest for the technique and HPTLC is in extensive use in e.g. drug research (Fuchs et al. 2011). In brief, the TLC method is based on the movement of a mobile phase by capillary forces on a thin layer of a stationary phase (Kowalska et al. 2003). The laboratory environment, including parameters such as ambient temperature and humidity, might affect the (HP)TLC separation. E.g. did (de Zeeuw et al. 1992) show that the retention factor (Rf) values and the reproducibility was drastically changed in the tropics relative to a moderate climate. High relative humidities increased most Rf values and changes in the humidity had more consequences for the Rf values than temperature change (de Zeeuw et al. 1992).

4.1.2 Lipid extraction

The details of the lipid extraction method are given in *Paper 2* and *Paper 3*. In order to remove some of the dominating triglycerides before the lipid class separation, the cod livers were centrifuged before the lipid extraction. The centrifugation made the triglycerides separate as oil on top of the liver tissue. This top layer consisted of triglycerides, and some cholesterol, and the fatty acid profile and quantity of cholesterol was determined by GC-FID. The remaining tissue was further extracted using a modified Folch method (Bligh & Dyer 1959; Folch et al. 1957). The total lipid percent (as weight of total sample) of the liver was determined gravimetrically, as the sum of the oil that separated on top after the centrifugation, and the lipid extract from the remaining tissue after the centrifugation . By this method approximately 50 % of the TAG were removed. However there were still much TAG left to impede the separation of the minor membrane lipids. For future work, more effort should be done in order to separate off more of the TAG before further analysis. Recently a method where triglycerides are extracted with hexane in this step have been developed in our lab at the Institute of Marine Research (*Meier et al. 2012, unpublished data*).

4.1.3 Statistical methods

One-factor Analysis of Variance (ANOVA) is a common method used to compare the means of populations that e.g. have received different treatments. Assumptions of ANOVA are that the populations are independent and normally distributed with common variance (Walpole et al. 2002). To test the normality assumption one can perform numerical hypothesis tests or plots of the empirical data (Jarque & Bera 1987; Lilliefors 1967). The hypothesis tested in an ANOVA is that the populations come from the same distribution. However, ANOVA does not point to which population(s) that are different, if any. After ANOVA have declined the null hypothesis of equally distributed populations, several so-called post-hoc tests may be used to point out which groups are different. Examples are Dunnett's test, that compares each population to a control population (Dunnett 1980a) and Tukey's test that makes paired comparisons between populations (Dunnett 1980b; Walpole et al. 2002). Pre-processing of data before performing ANOVA might be necessary in order to meet the assumptions of the ANOVA (Bland & Altman 1996). Examples of pre-processing are data transformations such as logarithmic (log) transformation (van den Berg et al. 2006; Zahurak et al. 2007) and arcsine root transformation of proportion data (Osborne 2002; Pires & Amado 2008). In this thesis the first (log) transformation was used on microarray data, and the latter (arcsine) transformation on the fatty acid distribution profiles.

4.2 Langmuir monolayer isotherms

Effects of PCB congeners

The effects of various PCB congeners were tested on Langmuir monolayers of synthetic phospholipids and native membrane lipids from cod liver and brain. Representative Langmuir compression isotherms are shown in Appendix B (Figures B1-B5). In common for all the Langmuir monolayer experiments was that the compression isotherms were similar with and without PCBs. No large effects of the

PCB could be observed even with molecular ratios that were beyond what is environmentally relevant. Most of all, this observation is a proof that the Langmuir monolayer is not an optimal means to study effects of PCB on biological membranes, and to our knowledge there are no published peer-reviewed studies of use of Langmuir monolayer technique for this purpose. A usual procedure when the effect of a given chemical on a lipid monolayer is tested with the Langmuir technique, is that the chemical is dissolved in the aqueous subphase before the lipid film is applied (Steinkopf et al. 2008) or it might be injected in the subphase when the monolayer has reached a desired surface pressure (Glomm et al. 2009). However given the low aqueous solubility of PCB (Huang & Hong 2002), the methods to introduce PCB to the lipid monolayer were restricted. One possible explanation to the lack of observable effect of PCB on Langmuir monolayers might be that the PCB molecules are too hydrophobic to interact with the hydrophilic head groups of the phospholipids and prefer the hydrophobic fatty acyl chains where they might be too small to have an impact on the MMA of the lipid monolayer. It should also be noted that Langmuir monolayers are only a very simplified model of only half of a biological membrane, so there cannot be drawn clear conclusions on grounds of these observations regarding whether PCB might disrupt a lipid membrane or not.

Results were reproduced on another instrument

Part of the study was repeated at the Department of Chemical Engineering at NTNU (Trondheim, Norway) on a similar instrument (except this instrument had a dipper (Figure 9) and the Wilhelmy plate was made of paper). The results were reproduced on this instrument, thus eliminating the probability of a systematic instrumental error.

Effects of 9-OH-PAH

A pilot study was performed with the PAH metabolite 9-OH-phenanthrene and POPC as a model membrane lipid. Representative Langmuir compression isotherms are presented in Appendix 2 (Figure B6). However as no significant effects were observed at the molecular ratios tested, we did not proceed to test the PAH-metabolites further.

As noted earlier, studies with higher molecular (PCB:lipid) ratios might yield interesting biophysical insights, however was considered to be outside the focus of the thesis.

4.3 Effects of NPs in liver and brain of male Atlantic cod

The study concerning the exposure to branched or straight-chained NPs is reported and discussed in *Paper 2*. The article emphasized three aspects of the effects on Atlantic cod after the exposure; the uptake and metabolism of NPs, the effect on lipid composition in brain and liver, and gene transcription.

4.3.1 Uptake and metabolism of NPs in Atlantic cod

The uptake of NP in the fish was confirmed by GC-MS (NCI) analysis and NPs were found in clearly detectable levels in the liver. The concentrations of NPs in bile were 50 (4-T-NP) and 80 (4-n-NP) times higher than what was found in the liver. This result agrees well with earlier reports showing that APs are readily taken up by fish and also very rapidly metabolised. The results also suggest that the metabolism depend on the structure of the NPs as the isomer distribution in the bile is not identical to the isomer distribution the liver of the fish administered the technical mixture of NPs.

4.3.2 Effects on membrane lipids

The effects of branched and straight-chained 4-NP exposure on the membrane lipid composition in Atlantic cod are presented in *Paper 2*. One important aim of the study was to determine quantitatively the distribution of the lipid classes, notably the PL constituting the membrane lipids, to see if a change analogous to homeoviscous adaptation (HA) could be found. However, no alterations in the lipid composition in the cod liver were observed after the NP exposure in this study. This was in contrast to the previous study from our group where female cod exposed to short-chained APs had significantly decreased levels of PLs. Some minor alterations in the fatty acid profiles in the different lipid classes were shown in this study, such as relative proportions of 20:1(n-11) was decreased and 20:5(n-3) was increased in the NL, and

22:5(n-3) and 22:6(n-3) was increased in the PE fraction of the fish exposed to 4-n-NP. No significant differences were shown in the PC fractions of the animals exposed to NPs, nor were any fatty acid profile alterations observed for any of the lipid classes in the liver of cod exposed to the technical mixture of NPs (4-T-NP). The few alterations seen in the PE fatty acid profile of 4-n-NP exposed fish (the increase in two PUFAs) may not be explained by the HA theory if the expected increased membrane fluidity is assumed. Rather would the opposite be expected; a decrease in PUFAs is observed in fish when going from a cold to a warmer environment (Farkas & Csengeri 1976).

4.3.3 Effects of NPs on gene transcription in liver of male Atlantic cod

Transcriptional effects of NP on liver mRNA were assayed with RT-qPCR (presented in *Paper 2*). Several genes from the CYP family and genes involved in PL biosynthesis and xenoestrogenic biomarkers were studied. CYP enzymes are important in phase 1 metabolism catalyzing the oxidation of xenobiotic compounds (Ghanayem et al. 2000; Guengerich 2008). As NP already has a hydroxyl group it might be metabolized directly by phase II enzymes, by conjugation of the phenol group to glucuronic acid (Cravedi & Zalko 2005). In *Paper 2* no up-regulation of the transcription of the studied CYP genes were shown, indicating that phase 1 metabolism was not the main biotransformation pathway of neither the straight-chained or branched NPs. A further indication of the phase II metabolism pathway was the preferred accumulation of NP metabolites in bile over liver; there were 50 times more NPs in bile than in liver in fish exposed to 4-T-NP, and likewise 80 times more in bile than liver for fish exposed to 4-n-NP.

Estrogenic effects of NPs and other APs are well-studied, but are not the main focus of this thesis. However 3 genes considered being biomarkers of xenoestrogenic exposure were assayed and are presented in *Paper 2*. The transcription of the zona radiata gene,

ZP3, was significantly down-regulated in the fish exposed to 4-n-NP but there was not observed differential gene expression of VtgA nor ESR1.

Genes involved in PL biosynthesis were assayed in *Paper 2*, and four of the genes we selected (AGPAT9, ELOVL1, PEMT and PLA1A) showed differential expression after the *in vivo* NP exposure. This indicates that the biosynthesis of PLs was affected by the NP treatment at the transcriptional level although we could not find correlating results from our lipid studies. This might suggest that there were effects on the PL composition in the liver of cod exposed to NPs that we were not able to detect with the methods used.

4.4 Effects of oil and halogenated POPs in liver and brain of male Atlantic cod

The effects of weathered crude oil and/or halogenated POPs on the membrane lipids in liver and brain of cod are presented in *Paper 3*.

4.4.1 Uptake of POPs in liver and metabolism of PAH from Troll oil

Chemical analyses confirmed uptake of POPs in liver. It was also shown apparent differences in metabolic rates for the different components. The bioaccumulation data suggest that metabolism of PBDEs and HCHs are dose-dependent, with increasing metabolic rate with high doses. The metabolism of DDT appeared to be faster when a high dose of Troll oil was given in addition.

4.4.2 Effects on membrane lipids

As for APs, many POPs have been shown to have membrane altering effects, both *in vivo* and *in vitro* (reviewed in Appendix A). In the study reported in *Paper 3*, the total lipid and cholesterol content in liver of cod were not affected by exposure to POPs and/or Troll oil. The relative lipid class composition was significantly different only between the control and the negative control. The relative PL distribution was similar

in all groups exposed to POPs/crude oil and the controls (however the relative PE levels were higher in the fish exposed to a high level of crude oil and low levels of POPs). Supposing an increase in membrane fluidity as a result of POPs exposure one would expect a rise in the PC/PE ratio, and similar membrane ordering alterations, due to the homeoviscous adaptation (HA) theory (section 1.4), however such effects are not observed. The FA profile of the NL fraction from the cod livers were similar for all the fish, both exposed and un-exposed. Some small differences in minor FAs were seen in the FA profile of the PC fraction for some of the exposed groups relative to the control. In the PE fraction from the livers of the cod exposed to high levels of the POPs mixture, the FA profile was significantly different from the control, notably was there an increase in the total SFA levels and a decrease in the PUFA 20:5(n-3), a discovery that might be consistent with the HA theory. However there were no observed differences in the other exposed groups compared to the control.

The FA profiles of the total lipids from the brain were similar for all groups, exposed and un-exposed. However some small differences in minor FAs were significant. The fish exposed to high levels of both oil and POPs had significantly increased levels of total SFA compared to the control.

There were no large differences in the membrane lipid composition in the fish that had been exposed to POPs and/or oil, compared to the fish in the control group.

4.4.3 Effects of oil and halogenated persistent organic pollutants on gene transcription in liver of male Atlantic cod

Differential expression of genes in the fish exposed to high doses of POPs and/or oil were studied with a microarray assay, in addition to the same real-time RT-qPCR assay that was used on the fish exposed to NPs. When looking at a subset of selected data with genes in PL biosynthesis, phase I and II metabolism of xenobiotic compounds, and in the antioxidant response system, some differences from control were observed.

The transcription of CYP1A was increased in the fish in all groups that had been treated with the Troll oil. No significant increase was seen in the group only given the POPs-mixture. However, treatment with POPs in addition to Troll oil increased the effect in CYP1A expression. This is partly in agreement with the relative accumulation pattern of the POPs (reported in *Paper 3*). These data indicate lower relative accumulation (and possibly higher metabolism rate) of lindane and PBDE when given high doses of the POPs mixture (as opposed to low doses of the mixture). The relative accumulation of DDT is lower in fish given high doses of the Troll oil in addition to the POPs mixture. It is also possible that a part of the dose-dependent differences in relative accumulation can be explained by not all of the POPs being taken up in the fish. To find this out analyses of feces from the fish could have been analysed, however this could cause practical challenges and have not been performed in this study.

All in all, not many treatment-dependent differences were seen in the microarray study. The data rather show large individual differences independent of treatments. There are only small differences in the transcription of genes in the PL biosynthesis which is consistent with the results in the composition of the membrane lipid classes and fatty acid distribution.

4.5 Conclusions

The chemical analyses confirmed uptake of POPs by the Atlantic cod, thus making the study a successful exposure experiment. However, the prior reported effects on membrane composition (Meier et al. 2007) were not confirmed in this study. The main results of the thesis are summed up below:

- Methodological:
    - SPE with columns of a HDPE material cannot be used for the separation of lipid classes from cod liver due to a significant contamination of fatty acids.

- Aminopropyl-coated SPE columns made of glass cannot be used for the separation of glycerophospholipids, because PC and PE are not separated properly.
- Cod liver lipids may be separated with HPTLC, and the fatty acid distribution may be determined by scraping each lipid class sample off the plate.

- Biological effects of *in vivo* exposure:
  - Chemical analyses of the NP-exposed fish showed NPs in liver and bile, and indicated faster metabolism of the straight-chained isomer (4-*n*-NP) than of the branched isomers.
  - Chemical analyses confirmed uptake of PCBs, PBDE, PFOS, lindane, DDTs and chlordane in the fish that had been exposed to a mixture of these POPs.
  - Analysis of hydroxylated PAH metabolites in bile documented the exposure to Troll oil.
  - Induction of CYP1A in the liver of exposed fish showed a dose-dependent effect on the metabolism of oil and the POPs.
  - No effects on lipid class composition in cod liver after *in vivo* exposure to NPs or POPs and/or Troll oil.
  - No effects on membrane lipid class composition in cod liver after *in vivo* exposure to NPs or POPs and/or Troll oil.
  - No large effects on fatty acid distribution in neutral lipids or membrane lipids from cod liver after the *in vivo* exposure
  - No effects on fatty acid distribution in the brain after the *in vivo* exposure
- *In vitro* technique
  - The Langmuir monolayer technique was not an ideal method to study the effects of PCB congeners upon a monolayer of membrane lipids.

- Apparently PCB had no significant interactions with monolayers of commercial phospholipids or native membrane lipids from cod brain and liver.

Appendix A: *In vivo* and *in vitro* studies on the membrane effects of POPs

Table A1: In vitro studies on the effects of POPs on membrane lipids

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
Human erythrocyte; DMPC; DMPE	Lindane	1:10; 1:5; 1:1	Lindane binds to inner part of membrane bilayer in blood cells, more impact on DMPE than DMPC. Decrease in reflection intensities of DMPE and DMPC which indicate increased membrane fluidity		(Suwalsky et al. 1998)
DPPC +/- cholesterol	Endosulfan (α and β)	1:40 to 1:1	Increased membrane fluidity, but the isomers show different effects in different parts of the bilayer. Lowered phase-transition temperature.		(Videira et al. 1999)
DOPC	PAH: Anthracene, phenanthrene, pyrene, benzo[α]anthracene, fluoranthene, perylene	0,2 µM aqueous solution of PAH	PAHs penetrate lipid monolayers		(Nelson 1987)
Monolayers of several disaturated model phospholipids	PAH: Anthracene, phenanthrene, pyrene, chrysene, benzo[α]pyrene	1:9	Monolayers were more expanded and in some cases were more liquid-like when in presence of PAHs. It was the largest molecule in their study, benzo(α)-pyrene that had the most severe effects		(Korchowiec et al. 2008)
PC from egg	Pyrene and pyrene derivatives	3 mol%	Increased membrane fluidity		(Engelke et al. 1996)
DMPC, DPPC, DSPC	benzo[α]pyrene	2-50 mol%	Decreased phase transition temperature		(Jimenez et al. 2002)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
DPPC and DMPE	PCB (Arochlor 1254)	0,25-30% (w/w) or ca 6 × 10−3 to	Decreased melting point and Δ enthalpy of transition for DPPC	Complex phase transitions for DMPE, also DMPE shows lower melting point with PCB	(Bonora et al. 2003)
DMPC and DMPE liposomes	DDT	0,25-30 %(w/w)	Decreased melting point	Interaction especially in the external layer of the liposome bilayer	(Bonora et al. 2008)
DPPC, DMPC and DMPE	PFOA and PFOS	10^-4 M PFOS/PFOA in aqueous subphase of Langmuir monolayer.	More fluidic layer (air-water interface). Greater molecular area of lipid with PFOS or PFOA in subphase.	PFOS more effective than PFOA)	(Matyszewska & Bilewicz 2008a)
DMPC	PFOA and PFOS	10^-4 M PFOS/PFOA in aqueous subphase of Langmuir monolayer.	Increased membrane fluidity and -thickness. More pronounced for PFOS than PFOA		(Matyszewska et al. 2008b)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
DMPC	PFOS	10^-4 M PFOS in aqueous subphase of Langmuir monolayer.	Topographically higher PFOS-enriched domains were clearly distinguishable among lower pure DMPC regions		(Matyszewska et al. 2010)
Isolated lipid rafts from rat liver F258 epithelial cells	Benzo[a]pyrene		Altered the composition of plasma membrane microstructures		(Tekpli et al. 2010)
Isolated lipid rafts from rat liver F258 epithelial cells	Benzo[a]pyrene		Na^+/H^+ exchanger 1 (NHE-1), a regulator of apoptosis is activated by benzo[a]pyrene; this happens by relocation of the NHE-1 from cholesterol-rich micro-domains to more fluid zones in the plasma membrane		(Tekpli et al. 2012)
Liposomes prepared with the polar lipid fraction of membranes from bacterial cells (*Bacillus stearothermophilus*)	DDT		Increased disorder in membrane lipids		(Donato et al. 1997a)
DPPC	Chlorobenzenes	20-60 mmol/kg membrane	Lowered, and broadened, transition temperatures.		(van Wezel et al. 1996)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
Liposomes of DMPC, DPPC and DSPC; native membranes (erythrocytes, brain microsomes, myelin, sarcoplasmic reticulum, and mitochondria)	Lindane		Broadened and shifted main phase transition. Lowered transition temperature. No effect of lindane in liposomes with much cholesterol (>30 mol %), but some effects with low cholesterol (<30 mol %). No perturbation of lindane on fluid phase in native membranes.		(Antunes-Madeira & Madeira 1989)
Liposomes of DMPC, DPPC and DSPC	DDT		Decrease phase transition midpoint temperature		(Antunes-Madeira & Madeira 1990)
DOPC	DDT and benzo[a]pyrene		Altered permeability of phospholipid monolayer which was modified by gramicidin, an antibiotic known to increase membrane permeability. DDT decrease membrane permeability.		(Nelson 1996)
DPPC, DMPC, DSPC, DAPC	PCB	PCB in excess (solubility study)	Membrane-water partitioning process (of PCBs) dependent on membrane fluidity (more than hydrophobicity of membrane bilayer)		(Dulfer & Govers 1995)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
DMPC, DLPC, DMPE	PCB-52 and PCB-77		Reduction in phase transition temperature of bilayer when PCB-52 was added. The authors propose that the substitution pattern (ortho/non-ortho) determines how the PCB interact with the bilayer; ortho-substituted interacts preferably with the bilayer lipid interior, while coplanar PCB interact with the polar head groups.		(Campbell et al. 2008)
DMPC, DPPC and DSPC with or without cholesterol. Native membranes from pig erythrocyte, porcine peripheral blood lymphocytes, brain microsome from sheep, fragmented sarcoplasmic reticulum from white muscle of rabbit,	Bromfenvinfos and methyl bromfenvinfos		Lower phase transition temperature. Cholesterol inhibited the effects of the insecticide.		(Blasiak 1995)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
Polar lipids from *Bacillus stearothermophilus*	α- and β-endosulfan		Disordering effects on membrane below, and at, transition temperature.	Inhibition of bacterial growth.	(Martins et al. 2003)
Polar lipids from *Bacillus stearothermophilus*	DDT, DDE		Alterations in lipid class and fatty acid composition, and dependent on Ca^{2+}. DDT more effective than DDE. Bacteria grown on DDT showed increased membrane order.		(Donato et al. 2000)
Phospholipids (PE, lyso-PE and cardiolipin) liposomes from *Escherichia coli*	Cyclic hydrocarbons (benzene, naphthalene, biphenyl, tetralin, anthracene, decalin, cyclohexane, ethylbenzene, o-Xylene, o-di-ethylbenzene, α-pinene, β-pinene, γ-terpinene, limonene)	0.1-150 µmol/mg phospholipids	Swelling of membrane bilayer. Increased membrane fluidity		(Sikkema et al. 1994)
Polar lipids from *Bacillus stearothermophilus*	DDT		Increased levels of straight-chained fatty acids, decreased levels of branched fatty acids. Increased membrane fluidity.		(Donato et al. 1997b)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
Liposomes made of DLPC/POPS, DMPC/POPS and POPC/POPC, all mixtures PC:PS(80:2, mol:mol)	Pentachlorobenzene, 2,6-dichlorobenzoic acid, 2,3-dichlorophenol, iprodione, endosulfan, vinclozolin, procymidone, tetradifon, p-chlorobenzoic acid, i-chloro-4-nitrobenzene, 4-chlorobenzyl chloride, 4-chloroacetanilide, 0-bromobenzoic acid, p-bromoaniline, chloramine T,		Liposome electrokinetic capillary chromatography with phospholipid liposomes as carrier and halogenated pesticides as analytes showed little interaction between liposome and analytes with OH- and/or acidic groups, and stronger interactions between liposomes and larger compounds with several Cl-substituents		(Wiedmer et al. 2008)
Cell cultures					
Alcaligenes xylosoxidans	PAHs (naphthalene, acenaphtylene, pyrene, fluoranthene)		Decrease in fatty acid unsaturation		(Certik et al. 2003)
Pseudomonas sp. LE2	Lindane		Increase in the ratio total saturated to total unsaturated fatty acids. Less fluidizing effect of lindane on the membranes of cells that had adapted to lindane.		(Kim et al. 2002a)
Ralstonia Eutropha H850	PCB (2,2',5,5'-tetrachlorobiphenyl)		Increase in the ratio total saturated to total unsaturated fatty acids Increase in saturated fatty acids, and decreased membrane fluidity		(Kim et al. 2001; Kim et al. 2002b)
Corynebacterium sp.	Crude oil and n-alkanes		Increase of odd-numbered fatty acids, modifications of phospholipid classes		(Mazzella et al. 2005a)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
Rainbow trout gill cells	2,2',4,4'-tetrabromodiphenyl ether BDE-47		Lipid peroxidation in mitochondrial membrane. Induction of apoptosis		(Shao et al. 2010)
Cultured hepatocytes from fetal quail *(Coturnix coturnix japonica)*	PCB (Aroclor 1254) and pesticides (Phenobarbital, lindane, dieldrin, α-endosulfan, pentachlorophenol, Trichlorfon, Ethyl-azinphos, 2,4,5-trichlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid, Methyl-parathion, Atrazine, Diflubenzuron, Diuron, Amitraz and Carbofuran)		Ultrastructural modifications		(Hugla et al., 1996)
Rat renal tubular cell cultures	Aroclor 1248, PCB-153, PCB-77		Increased Arachidonic acid release (only by di-ortho-congener)		(Sanchez et al. 1997)
Fish leukocytes (carp) used as model membrane system	PFOS		Increased membrane fluidity. Increased permeability.		(Hu et al. 2003)
Arthrobacter sp. (strain Sphe3)	PAH (phenanthrene)		Increase in the proportion of diphosphatidylglycerol, at the expense of phosphatidylglycerol. The ratio of iso-fatty acids to anteiso-fatty acids was decreased.		(Kallimanis et al. 2007)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
Corynebacterium sp. (Strain 8)	Crude oil		Altered fatty acid composition. (e.g. decrease in MUFA)		(Mazzella et al. 2005b)
Microcosm culture	Crude oil		Modifications in fatty acid profile		(Syakti et al. 2006)
The bacterium *K. varians*	Chlorophenols		Decreased levels of unsaturated fatty acids in the PC- and PE-fraction	Reduced growth. Effects were different and depending on when the xenobiotic was added; at zero time or 3 days of culture.	(Dercova et al. 2004).
Testicular Sertoli cells from rat (Sprague Dawley)	Nonylphenol		Increased membrane fluidity and disorder		(Gong et al. 2008)
Gill cells from flounder (*Paralichthys olivaceus*)	Nonylphenol		Morphological changes such as membrane swelling and an increased number of lipid particles	Cytotoxicity was shown.	(Xiao et al. 2011)
Epidermis culture from rainbow trout	Nonylphenol		Vesiculation of the Golgi apparatus		(Lamche & Burkhardt-Holm 2000)

Lipid	POP	Ratio POP:lipid	Effects	Comment	Reference
Submitochondrial particles from bovine heart	Linear alkylbenzene sulfonates, nonylphenol polyethoxylates and their biodegradation derivatives (sulphophenyl carboxylates, nonylphenol, nonylphenoxy acetic acid)		Inhibition of reverse electron transfer (step in the respiratory chain) by NP	Authors suggest that membrane permeability is altered.	(Argese et al. 1994)
Rat liver F258 epithelial cells	Benzo[a]pyrene		Increased membrane fluidity. Co-treatment with cholesterol significantly reduced (benzopyrene-induced) apoptosis, and inhibited membrane fluidization.		(Gorria et al. 2006)
Thymocytes from mice	Toxaphene and 2,5-dichloro-3-biphenylol	In concentrations causing cell death.	Toxaphene caused decreased membrane fluidity while 2,5-dichloro-3-biphenylol had the opposite effect		(Sandal et al. 2004)

Table A2: In vivo studies on the effects of POPs on membrane lipids

Species and tissue	POP	Dose and exposure time	Membrane effects	Other effects (Comment)	Reference
Rat					
Rat (Sprague-Dawley) liver	PCB: Aroclor 1254	0.6 -1.5 mmol/kg body mass; killed after 48-120 hours	Increase in the proportion of arachidonic acid. Linoleate desaturase increase.	Increase in cytochrome P-450	(Borlakoglu et al. 1990; Borlakoglu et al. 1991)
Rat (Wistar) liver	PFOA	0.0025- 0.04% PFOA in diet for week	Increase in PC, PE, PS, PI and TAG in liver. Increase in activities of glycerol-3-phosphate acyltransferase, diacylglycerol kinase, PS decarboxylase. Decrease in CTP:phosphoethanolamine cytidyltransferase and PE N-methyltransferase. NO CHANGE for activity of CTP:phosphocholine cytidyltransferase. Increase in 16:0/18:1-PC. Decrease in 18:2-PC.	Body burden not given	(Kudo et al. 1999)
Rat liver (Sprague-Dawley)	PCB (Aroclor 1254)	Six daily i.p. injections of 25 - 50 mg PCB/kg body weight	Increased PL, cholesterol and TAG. Morphological alterations.	Increased HSI	(Hinton et al. 1978)

Species and tissue	POP	Dose and exposure time	Membrane effects	Other effects (Comment)	Reference
Rat (Wistar/Sprague Dawley) liver	PCB (Clophen-A30, A50 og A60)	Up to 2000 ppm PCB in diet for 4 days.	Activities of lipogenic enzymes were induced by PCBs. Effect greater with ME, G6PDH and PGDH (10-fold up) than for FAS and ACL (2-fold)	Body burden determined after exposure, differs between individuals. Activities of gluconeogenic enzymes (PEPCK and FdPase) were dose-dependently decreased by PCBs.	(Boll et al. 1998)
Rat (Wistar) liver	PCB: 3,3',3,3',5-pentachlorobiphenyl (PenCB)	A single i.p. of 0.5-25 mg/kg (dissolved in corn oil)	20:4(n-6) reduced in total lipids. Linoleic acid (18:2) and bishomo-γ-linolenic acid (18:3) increased. Activity of Δ5- and Δ6-desaturase decreased		(Matsusue et al. 1999)
Rat (Wistar) liver and blood	PFOA, PFDA	Diets containing 0.0025-0.04% (w/w) PFOA and 0.00125-0.01% (w/w) PFDA for 1 week	Activities of glutathione (GSH) S-transferases towards l-chloro-2,4_dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobnzene (DCNB) were depressed. Increased hepatic concentration of TAG. Accumulation of cholesterol in liver.		(Kawashima et al. 1995)

Species and tissue	POP	Dose and exposure time	Membrane effects	Other effects (Comment)	Reference
Liver from rat (Sherman strain)	PCB (Aroclor 1254 or 1260)	Exposure through diet, up to 72 mg/kg/day for 30 weeks	Large, and soft, livers. Lipid vacuoles. Accumulation of lipid.		(Kimbroug et al. 1972)
Rat (Sprague Dawley) liver	PCB (Aroclor 1242) and/or DDT	75-150 ppm PCB/DDT for 36 weeks	Ultrastructural modifications, necrosis, "numerous lipid deposits"		(Jonsson et al. 1981)
Liver of channel catfsh and rat (Sprague Dawley)	PCB (Aroclor 1254)	Daily i.p. injections for 7 days, 50 mg/kg body weight	Rat: Lipid accumulation (lipid droplets), increase inn smooth endoplasmic reticulum. Fish: Lipid accumulation, increased profiles of rough endoplasmic reticulum	Strongest effects in rats.	(Lipsky et al. 1978)
Rat (Sherman strain) liver	DDT	At first mixed in food, and offered "*ad libitum*". When the animals reached a body weight of 150-250 g: 100-2500 ppm in diet. Duration:1-6 months.	Increased fat storage. Ultrastructural modifications		(Ortega 1966)

Species and tissue	POP	Dose and exposure time	Membrane effects	Other effects (Comment)	Reference
Male Wistar rats	Lindane	Daily injection with 1 mg/100 g body weight for 12 days	Ventral prostate membranes: PL content increased, cholesterol:PL ratio decreased (cholesterol unchanged). Increased membrane fluidity.		(Gutierrezocana et al. 1992)
Mouse					
Mouse (ddY strand) liver	Perfluorinated fatty acids (PFCAs) (C_6-C_9)	I. p.: Once a day for 5 d with PFCAs at the doses of 150 mg/kg of body weight for PFHeA, of 20 and 50 mg/kg of body weight for PFHA, and 0.5- 10 mg/kg of body weight for PFOA and PFNA.	Increased contents and proportions of 18:1(n-9), 16:1(n-7) and 20:3(n-6). 16:0 were also increased. Perfluorooctanoic acid elevated the expressions of mRNA encoding acetyl-CoA carboxylase, fatty acid synthase, malic enzyme, stearoyl-CoA desaturase (SCD) (SCD1 and 2), chain elongase (ELOVL5), D6 desaturase (Fads2), 1-acylglycerophosphocholine acyltransferase (LPCAT) (LPCAT3).	The authors suggest that the alterations in fatty acid composition are caused by up-regulation of SCD, de-novo fatty acid synthesis, chain elongase and D6-desaturase, and that SCD induction is partly mediated by PPARα.	(Kudo et al. 2011)

Species and tissue	POP	Dose and exposure time	Membrane effects	Other effects (Comment)	Reference
Mouse (ddY strand) liver and blood serum	PFOA	I.p.: 1.25- 10 mg/kg body weight once a day for 7 days	Marked accumulation of TAG in liver of mice fed vegetable oils, but the level of TAG remained low in the mice fed a fish oil diet.		(Kudo & Kawashima 1997)
Guinea pig					
Guinea pig (*Cavia procellus)*	Toxaphene	Acute: Single dose of300 mg/kg body weight. Subacute: 2-5 mg/kg daily for 60 days	Brain: Increased levels of neutral lipids, and decrease of PL Liver and kidney: Decrease in PL. Alterations in PL composition in brain, kidney and liver		(Chandra & Durairaj 1995)
Mink					
Adipose tissue around lymph nodes from mink (*Mustela vison*)	PCB (Aroclor 1242) and copper	1 mg PCB in food each day for 28 days	Decrease in PUFA in PL in females	Copper apparently opposite effect than PCB. No significant effects in males.	(Kakela & Hyvarinen 1999)

Species and tissue	POP	Dose and exposure time	Membrane effects	Other effects (Comment)	Reference
Birds					
Pigeon (*Columba livia*)	PCB: Aroclor 1254	Single i. p.: 0.6-3.0 mmol/kg body mass; killed after 24-120 hours.	Increase in the proportion of arachidonic acid.Linoleate desaturase increase. Correlation between concentrations of cytochrome b5 and cytochrome P450 and activity of pigeon linoleate desaturase	Increase in cytochrome P-450	(Borlakoglu et al. 1990; Borlakoglu et al. 1991)
Chick embryos (*Gallus gallus domesticus*)	PCB-126	Single injection: 1.6 µg PCB/kg egg; incubated for 19 days.	Increased hepatic lipid peroxidation, increased membrane fluidity		(Katynski et al. 2004)
Fish					
Yellowtail flounder *(Pleuronectes ferrugineus)*	Toxaphene	0.02-0.2 µg/g body weight each day for 2 weeks	Altered lipid composition, with differences between males and females.		(Scott et al. 2002)
Cod (*Gadus morhua*) liver and brain	Alkylphenols (C_4-C_7)	0.02-80 mg/kg for 5 weeks	Liver: More SFA, less PUFA(n-3) in PL. Brain: Declined cholesterol levels		(Meier et al. 2007)

Species and tissue	POP	Dose and exposure time	Membrane effects	Other effects (Comment)	Reference
Liver from rainbow trout (*Oncorhynchus mykiss)*(yolk-sac fry)	Lindane	1 mg/L (in water) for 3 days	Ultrastructural changes		(Sylvie et al. 1996)
Liver from rainbow trout (*Oncorhynchus mykiss)*	PCB (Aroclor 1254)	1-100 ppm in diet for 229-330 days	Altered ultractructure		(Hacking et al. 1978)
Liver from cod (*Gadus morhua*)and winter flounder *(Pseudopleuronectes americanus)*	Crude oil	100-200 ppb total hydrocarbons in water (cod) or up to 3375 µg/g in sediments (flounder) for 24 weeks	Alterations in lipid composition, e.g. in total lipids from male cod: an increase in the SFAs 14:0, 16:0 and 18:0, the MUFAs 16:1, 18:1 and 20:1 and the PUFA 20:5 and a decline in the MUFAs 14:1, and 17:1 and the PUFAs 16:4, 18:4, 20:4, 22:5 and 22:6	Lipid class composition that is reported for the control group deviates largely from other (and more recent) published reports (e.g. more than 25 % PL)	(Dey et al. 1983)
(i.p. = intraperitoneal injections)					

Appendix B: Representative Langmuir isotherms from the *in vitro* study.

The figures show monolayers of synthetic phospholipids, or native membrane lipids from cod, with or without PCB congeners.

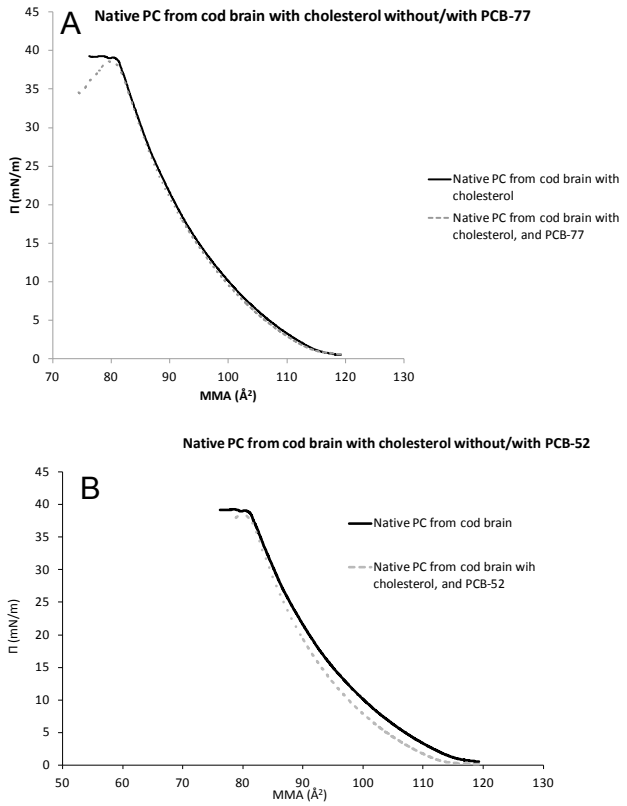

Figure B1: Langmuir isotherms of native PC from cod brain with cholesterol and PCB-77 (A) or PCB-52 (B). Surface pressure versus apparent surface area (MMA) at room temperature (T=20 °C). Molecular ratio PCB:cholesterol:PC (1:2:3).

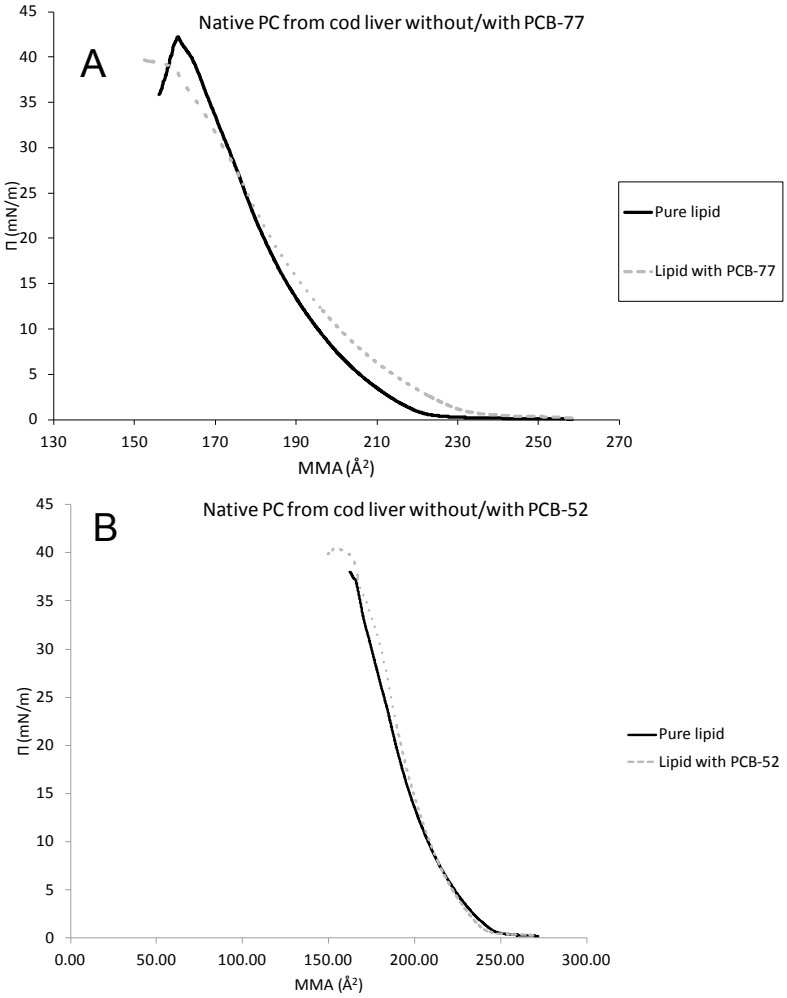

Figure B2: Langmuir isotherms of native PC from cod liver with PCB-52 (A) or PCB-77 (B). Surface pressure versus apparent surface area (MMA) for pure PC (solid line) and PC with PCB (dashed line) at room temperature (T=20 °C). Molecular ratio PCB:PC (1:2).

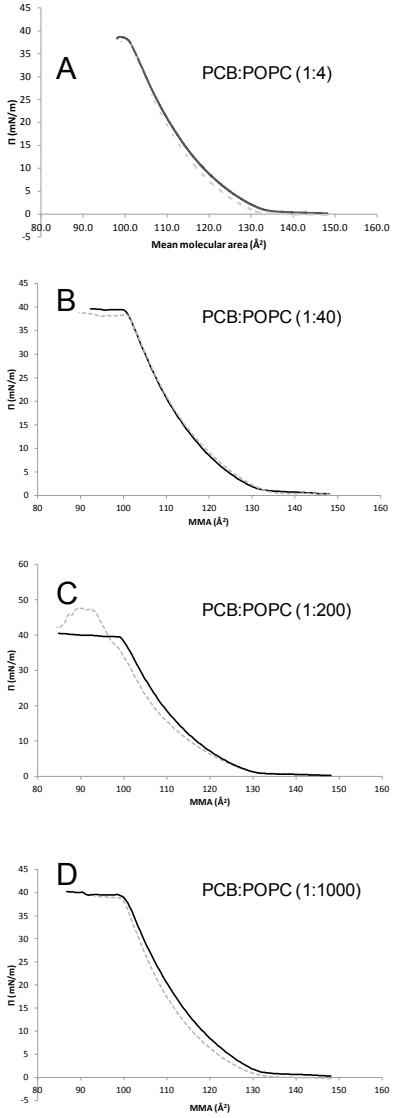

Figure B3: Langmuir compression isotherms for a commercial phosphatidylcholine (POPC) without (solid line) or with PCB-52 (dashed line) at 4 different molecular ratios. Molecular ratios PCB:POPC are A, (1:4) at room temperature (T=20 °C); B, (1:40) at T=11°C; C, (1:200) at T=11 °C; and D, (1:1000) at T=11 °C.

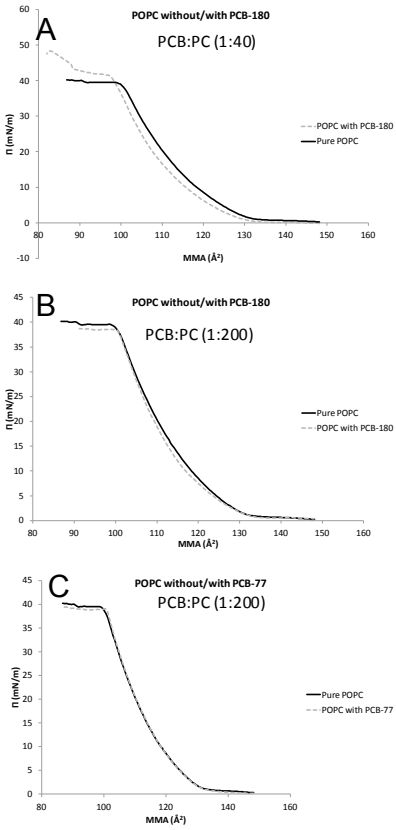

Figure B4: POPC without or with PCB-180 or PCB-77. Surface pressure (Π) versus apparent surface area (MMA) of POPC without (solid line) or with (dashed line) PCB at T=11 °C. Molecular ratios are A, PCB-180:POPC (1:40); B, PCB-180:POPC (1:200); and, C, PCB-77:POPC (1:200).

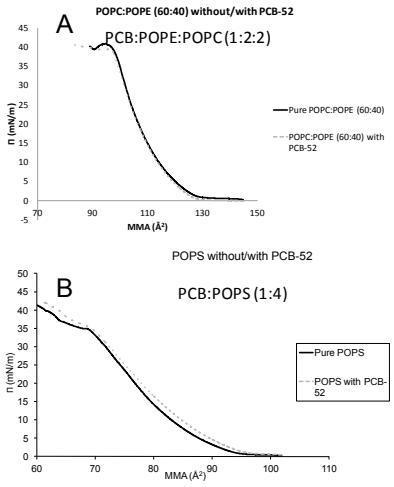

Figure B5: Surface pressure (Π) versus surface area (MMA) at room temperature of A, mixture of two commercial phospholipids (POPE:POPC, (40:60) without (solid line) or with PCB-52 (dashed line); and, B, POPS without (solid line) or with (dashed line) PCB-52, at room temperature.

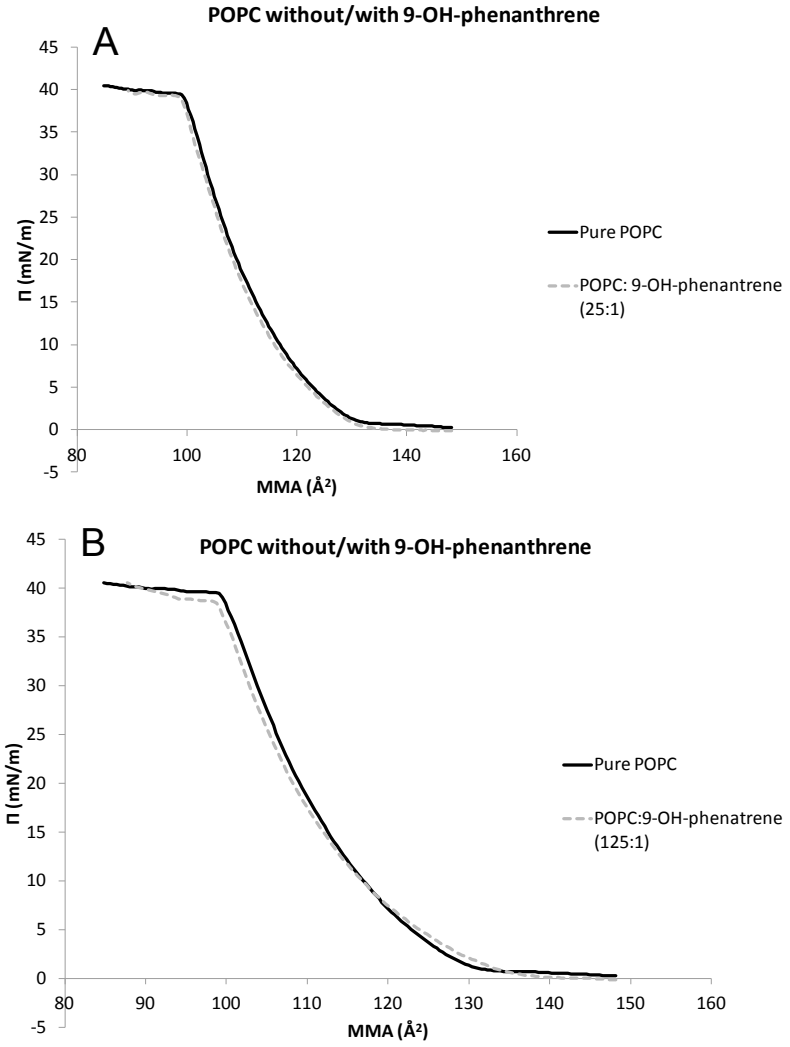

Figure B6: Langmuir compressions isotherms of POPC monolayer without (solid line) or with (dashed line) 9-OH-phenanthrene (9-OH-P) at T=11°C. Molecular ratios POPC:9-OH-P are A, 25:1; and, B, 125:1.

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