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Effects of alkylphenols on glycerophospholipids and cholesterol in liver and brain from female Atlantic cod (*Gadus morhua*)

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Abstract

Offshore oil production releases large amounts of lipophilic compounds in produced water into the ocean. In 2004, 143 million m^3 produced water, containing approximately 13 tons of long-chain ($>C_4$) alkylphenols (AP), was discharged from installations in the Norwegian sector of the North Sea. Long-chain APs are known to cause endocrine disruption in a number of species. However, relatively little is known about their long-term effects in the marine environment. In the present study, Atlantic cod (*Gadus morhua*) were exposed (0.02 to 80 mg AP/kg) to a mixture (1:1:1:1) of APs (4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol) or 17 β-estradiol (5 mg E2/kg) for 5 weeks and the effect on the fatty acid profile and cholesterol content in the membrane lipids from the liver and the brain was studied. We also determined the interaction between different *para*-substituted APs and glycerophospholipids (native phospholipids extracted from cod liver and brain) and model phosphatidylcholine (PC 16:0/22:6 n-3) in monolayers with the Langmuir–Blodget technique. The study demonstrated that APs and E2 alter the fatty acid profile in the polar lipids (PL) from the liver to contain more saturated fatty acids (SFA) and less n-3 polyunsaturated fatty acids (n-3 PUFA) compared with control. In the brain of the exposed groups a similar effect was demonstrated, although with higher saturation of the fatty acids found in the neutral lipids (mainly cholesterol ester), but not in the polar lipids. The AP and E2 exposure also gave a decline in the cholesterol levels in the brain. The *in vitro* studies showed that APs increased the mean molecular areas of the PLs in the monolayers at concentrations down to 5 μM, most likely due to intercalation of the APs between PL molecules. The increase in molecular area increased with the length of the alkyl side chain.
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Keywords: Alkylphenol; Atlantic cod; Lipid; Fatty acid; Cholesterol; Membrane effects; Langmuir–Blodget technique

1. Introduction

Alkylphenols (AP) are some of the most intensively studied substances that have hormone-disrupting effects. There is good support from both *in vitro* and *in vivo* experiments that APs bind to and affect the estrogen receptors in a similar way as 17 β-estradiol (E2), although with much weaker responses, and thereby affect reproduction of fish (Jobling and Sumpter, 1993; Mueller and Kim, 1978; Nimrod and Benson, 1996).

There are several sources of APs in the environment. Nonylphenol (NP) and octylphenol (OP) ethoxylates are applied as surfactants and emulsifying agents in a large range of industrial products (Naylor, 1992). A large proportion of the degradation products of alkylphenol ethoxylates (APE) finally ends up in the aquatic environment, and NP and OP have therefore been found

Abbreviations: AP, Alkylphenols; APE, Alkylphenol ethoxylates; BHT, Butylated hydroxytoluene; BP, Butylphenol; CYP1A, Cytochrome P4501A; DAG, Diacylglycerol; DDT, Dichlorodiphenyltrichloroethane; E2, 17 β-estradiol; EROD, Ethoxyresorufin-*O*-deethylase activity; ELSD, Evaporative Light-Scattering Detection; FAME, Fatty acid methyl esters; FFA, Free fatty acids; HEPP, Heptylphenol; HEXP, Hexylphenol; MAG, Monoacylglycerol; MUFA, Monounsaturated fatty acids; NP, Nonylphenol; OP, Octylphenol; PAH, Polyaromatic hydrocarbons; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PL, Polar lipids; PP, Pentylphenol; PS, Phosphatidylserine; PUFA, Polyunsaturated fatty acids; SFA, Saturated fatty acids; TAG, Triacylglycerol.

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in a large number of freshwater systems and coastal marine areas all over the world, in concentrations of up to 369 μg/l in highly polluted areas, but with typical values in the low μg/l range (review in: Ying et al., 2002).

In addition to the degradation products of APE, APs are also natural components of crude oil (Ioppolo-Armanios et al., 1992). As a result of their solubility in water a high proportion will be found in the aqueous phase after water/oil separation and thus discharged into the sea with produced water. APs are typically found in concentrations of 0.6–10.0 mg/l in produced water. Some 80% of the total consists of the most water-soluble APs, phenol and cresol (C_1). Of the remaining components, the higher APs from butyl- to heptylphenols occur in low concentrations of 2–237 μg/l (Brendehaug et al., 1992; Røe and Johnsen, 1996).

We know very little about the fate and biological effects of APs in the marine offshore environment. Beste et al. (2001) measured NP in the North Sea (German Bight) and found concentrations in seawater of between 0.7 and 4.4 ng/l and up to 13 μg/kg in offshore sediment sampled more than 100 km offshore. The water concentration of NP from the Dutch coastal zone is found to be as high as 1700 ng/l (median concentration 77 ng/l) (Jonkers et al., 2005). Similarly, in the German part of the Baltic Sea the concentrations are up to 21 ng NP/l and 1 ng OP/l (Beck et al., 2005). Phenol and APs (sum C_1–C_4) are found in the sea around offshore installations in the North Sea at concentrations up to 486 ng/l and 140 ng/l, respectively (Riksheim and Johnsen, 1994).

The Institute of Marine Research, Norway started in 1997 a study aiming to find out whether APs in produced water from offshore oil industries are capable of interfering with hormonal processes in cod, and whether these substances affect central enzymatic systems involved in the metabolism of foreign compounds. The study demonstrated that AP induced estrogen-resembling effects in male fish affected steroid levels and disturbed gonadal development in both male and female fish, even at very low doses (Meier et al., 2007). The effects of AP exposure on enzymes that catalyze phase I and phase II reactions for detoxification and excretion of xenobiotics were also included in the analytical program and have been published elsewhere (Hasselberg et al., 2004a,b). In this paper, samples from the same investigation are examined for potential effects of APs on membrane lipids.

Fish have, like other ectothermic animals, high flexibility in remodelling the lipid composition of cell membranes. These homeoviscous adaptations (Sinensky, 1974) have been intensively studied in connection with thermal acclimation (Williams and Hazel, 1994). Many investigations also show that the fluidity of membrane lipids can be effected by xenobiotics like lindane (Suwalsky et al., 1998), α- and β-endosulfan (Videira et al., 1999), dichlorodiphenyltrichloroethane (DDT) (Nelson, 1987), polyaromatic hydrocarbons (PAH) (Engelke et al., 1996; Jimenez et al., 2002) and 2,4-dichlorophenol (Csiszar et al., 2002). In their capacity as amphiphatical molecules, APs would be expected to accumulate in the phospholipid bilayer of the membranes. Interactions of phenolic compounds with biological membranes have been well established from at least two intensively studied APs: the antioxidant BHT (butylated hydroxytoluene = 2,6-Di-*tert*-butyl-4-methylphenol; reviewed in Lanigan and Yamarik, 2002) and the intravenous anaesthetic propofol (2,6-diisopropylphenol) (James and Glen, 1980; Tsuchiya, 2001).

In the present study, we investigate how *in vivo* exposure of Atlantic cod to APs affects the fatty acid profile and cholesterol content in membrane lipids from the liver and the brain. In addition, we explore the interaction between different *para*-substituted APs and glycerophospholipids (native phospholipids extracted from cod liver and brain and model phosphatidylcholine (PC 16:0/22:6 n-3)) in monolayers with the Langmuir–Blodget technique.

2. Materials and methods

2.1. Animals

Experiments were carried out on two-year-old first-time spawning Atlantic cod (*Gadus morhua*, mean mass 691 g) from a strain of Atlantic cod produced at the Institute of Marine Research. The fish were divided into seven groups of 40 each, and transferred to 10 m^3 indoor tanks in which the lighting followed the natural light regime. From August until the experiments started, the fish were fed commercial fish feed (dry pellets from Felleskjøpet AS, Norway, 10% lipid). The tanks were supplied with seawater from 100 m depth and the water temperature was kept at 9–10 °C.

The exposed groups were given a mixture (1:1:1:1) of 4-*tert*-butylphenol (4-*tert*-BP) (Sigma-Aldrich, Norway), 4-*n*-pentylphenol (4-*n*-PP) (Sigma-Aldrich, Norway), 4-*n*-hexylphenol (4-*n*-HEXP) (Sigma-Aldrich, Norway) and 4-*n*-heptylphenol (4-*n*-HEPP) (TCI, Japan) in doses of 5, 500, 5000, 10 000 or 20 000 μg/kg body weight of each AP once a week. In addition to the control group we also had a positive control group that was given 17 β-estradiol (5 mg/kg body mass, once a week).

The fish were fed individually by oral intubation into the stomach under anaesthesia (benzocaine). The APs and E2 were dissolved in 1,2-propandiol and mixed into a paste consisting of ground dry pellets, water and fish oil (paste composition: 50.5% dry pellets, 40.5% water, 5% fish oil and 4% 1,2-propandiol/AP solution; the lipid content and fatty acid profile of the paste were close to that of the dry pellets). Both the fish and the paste were weighed immediately prior to feeding. The fish were fed once every seven days for four weeks. The fish were slaughtered a week after the final dose. The cods were anaesthetised with benzocaine and were killed after blood sampling by a blow on the head. Samples of liver and brain were rapidly removed by scalpel, frozen in liquid nitrogen and stored at −80 °C until analysis. The lipid studies were done only on the female cod.

2.2. Lipid extraction and analysis

Total lipid was extracted from liver and brain samples by a modified method (Folch et al., 1957) with chloroform/methanol (2:1 by vol.). An aliquot of the sample was separated into neutral lipids (NL: triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), cholesterol and cholesterol

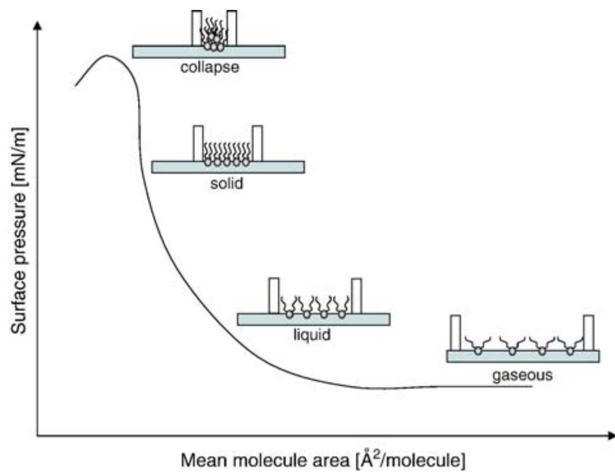

Fig. 1. Model of the Langmuir monolayer.

esters), free fatty acids (FFA) and polar lipids (PL) using Bond Elut columns (Supelclean LC-NH_2, Supelco) according to Kaluzny et al. (1985). Methyl esters of the fatty acids (FAME) from total lipids and the lipid classes were prepared and analysed on GC-FID as described by Meier et al. (2006).

Total cholesterol (sum of free and esters) was determined in the same GC analysis as the FAME and quantified by the same internal standard, nonadecanoic acid 19:0. Cholesterol decomposes to some degree under the methanolysis and the quantification is based on the sum of the areas of the cholesterol peak and its decomposition peaks (Meier et al., 2006). To analyse the amount of cholesterol in cod livers an aliquot of the NL fraction was saponified with KOH to remove the high excess of fatty acids before the analysis.

Alk-1-enyl ethers from plasmalogen in the brain samples are semi-quantitatively determined by GC-FID as dimethylacetals and their terminal decomposition derivatives, methyl-1-enyl ether (Maulik et al., 1995).

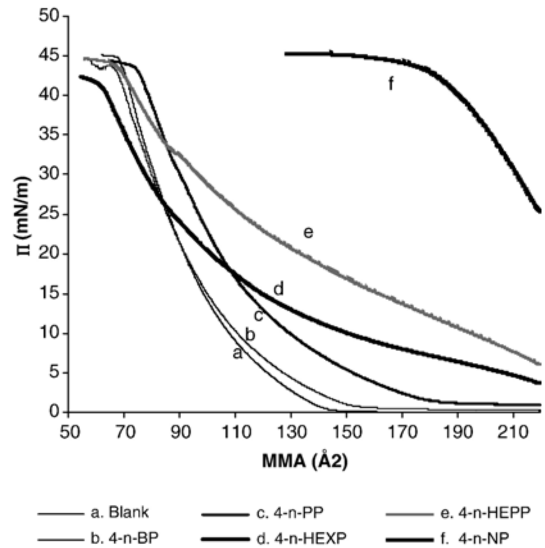

Fig. 3. Surface pressure π *versus* apparent surface area (MMA) at room temperature for the polar lipid fraction of cod brain with none (a) and 5 μM of 4-*n*-butylphenol (4-*n*-BP) (b), 4-*n*-pentylphenol (4-*n*-PP) (c), 4-*n*-hexylphenol (4-*n*-HEXP) (d), 4-*n*-heptylphenol (4-*n*-HEPP) (e), and 4-*n*-nonylphenol (4-*n*-NP) (f).

The PL fractions from the Bond Elut separation were evaporated to dryness under Ar (g) and dissolved in a small volume of chloroform. $CHCl_3/CH_3OH$ (2/1) and the phospholipids were separated by HPLC according to Arduini et al. (1996).

Twenty microliter portions were injected on a silica Nucleosil 100-7 column, 250×4 mm I.D. (Machery-Nagel, Germany) with a precolumn (Security Guard, Phenomenex KJO-4282). The mobile phase consisted of acetonitrile/hexane/methanol/phosphoric acid (920:30:30:12, by volume) delivered by an LKB 2151 HPLC pump (Bromma, Sweden) at a flow rate of 1.00 ml/min. The absorbance at 205 nm was measured in the eluate by a Pharmacia UV monitor (LKB

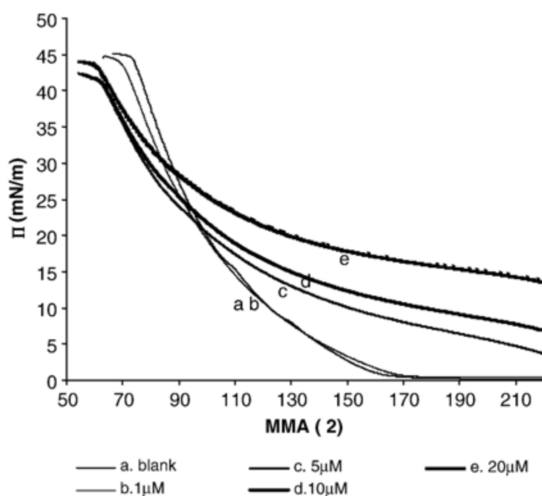

Fig. 2. Surface pressure π *versus* apparent surface area (MMA) at room temperature for the polar lipid fraction of cod brain with 0 (a), 1 (b), 5 (c), 10 (d), and 20 μM (e) of 4-*n*-heptylphenol (4-*n*-HEPP).

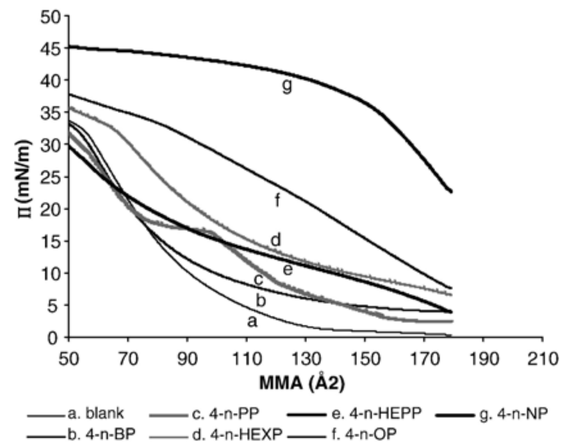

Fig. 4. Surface pressure π *versus* apparent surface area (MMA) at room temperature for the polar lipid fraction of cod liver with none (a) and 5 μM of 4-*n*-butylphenol (4-*n*-BP) (b), 4-*n*-pentylphenol (4-*n*-PP) (c), 4-*n*-hexylphenol (4-*n*-HEXP) (d), 4-*n*-heptylphenol (4-*n*-HEPP) (e), 4-*n*-octylphenol (4-*n*-OP) (f) and 4-*n*-nonylphenol (4-*n*-NP) (g).

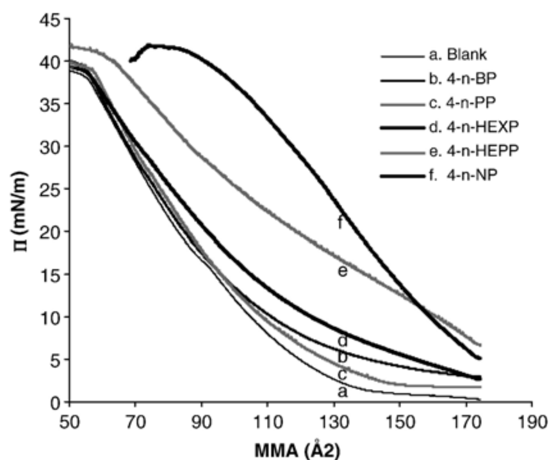

Fig. 5. Surface pressure π *versus* apparent surface area (MMA) at room temperature for commercial phosphatidylcholine (16:0/22:6 n-3) with none (a) and 5 μM of 4-*n*-butylphenol (4-*n*-BP) (b), 4-*n*-pentylphenol (4-*n*-PP) (c), 4-*n*-hexylphenol (4-*n*-HEXP) (d), 4-*n*-heptylphenol (4-*n*-HEPP) (e), and 4-*n*-nonylphenol (4-*n*-NP) (f).

VWM 2141, Bromma, Sweden). The column temperature was kept constant at 28 °C in a Croco Cil (Cluzeau Info-Lab, purchased from Teknolab, Oslo, Norway) thermostat. The phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) were manually collected to make sure that the separation of the phospholipids was optimal. The phospholipids in the fractions were separated from the phosphoric acid-containing elution fluid as follows: to one volume of eluate one volume of $CHCl_3$ was added followed by one volume of H_2O, and the phases were separated by centrifugation. The organic phase was collected and the aqueous phase extracted once more with $CHCl_3$. The organic phases were combined, evaporated to dryness by Ar (g) and the lipids stored at −20 °C.

The composition of the NL from the cod brain was analysed by HPLC with Evaporative Light-Scattering Detection (ELSD) according to Liu et al. (1993). An aliquot of the NL fractions from the Bond Elut separation was evaporated to dryness, dissolved in a small volume of isopropanol/hexane (1:10 v/v) and injected on a silica LiChrosorb Si 60, 150 mm × 4.6 mm (Varian-Chrompack). A Shimadzu LC-9A pump was connected to two mobile phases (M1, 100% hexane and M2, hexane: isopropanol:ethylacetate:formic acid, 80:10:9.9:0.1 v/v) that was eluted at a flow rate of 2 ml/min. The gradient was programmed as follows: a linear gradient of M1, 100–86% (M2, 0–14%) between 0 and 10 min, a linear gradient of M1, 86–0% (M2, 14–100%) between 10 and 15 min, and isocratic elution at M1, 0% (M2, 100%) between 15 and 20 min. Elution of TAG, cholesterol and cholesterol esters was detected with ELSD (Eurosep DDL 31). External standard curves were obtained for cholesterol (9–145 mg), cholesteryl palmitate (2–32 mg) and tristearin (0.2–4 mg).

2.3. Monolayer studies

Measurements were done with a KSV Langmuir instrument (KSV Instruments LTD, Helsinki, Finland) with a temperature-controlled Teflon trough (75 × 340 × 5 mm). A Pt Wilhelmy plate was used to measure the surface tension. The KSV software allows isobaric measurements by regulating the positions of the barriers (made of Delrin) that limit the film-covered area. Monolayers of native phospholipids extracted from cod liver and brain and commercial phosphatidylcholine (PC 16:0/22:6 n-3) (1 mg/ml of chloroform, 15–20 μl) were

Table 1
Lipid data from cod liver and cod brain (mean values ± standard deviation) expressed as: lipid % and cholesterol % relative to wet weight

		P.C.				
	Control (n = 10)	5 mg E2/kg (n = 8)	0.02 mg AP/kg (n = 5)	2 mg AP/kg (n = 9)	40 mg AP/kg (n = 9)	80 mg AP/kg (n = 8)
Cod liver						
Lipid %	66.7 ± 3.7	66.9 ± 7.4	69.1 ± 1.7	67.2 ± 4.5	70.3 ± 3.3	69.9 ± 2.3
Cholesterol %	0.58 ± 0.14	0.50 ± 0.11	0.43 ± 0.07	0.66 ± 0.15	0.51 ± 0.09	0.54 ± 0.12
% distribution of the fatty acids in different lipid classes						
NL	94.64 ± 0.90	94.99 ± 2.10	96.10 ± 1.53[a]	96.41 ± 1.20	95.69 ± 1.28	96.21 ± 0.67[a]
FFA	4.03 ± 0.84	3.19 ± 1.24	3.06 ± 1.47	3.31 ± 0.78	3.44 ± 1.32	3.07 ± 0.63[a]
PL	1.33 ± 0.20	1.07 ± 0.23[b]	0.84 ± 0.13[b]	0.96 ± 0.29[b]	0.87 ± 0.21[b]	0.72 ± 0.12[b]
Cod brain						
Lipid %	9.30 ± 0.69	9.31 ± 0.54	9.96 ± 0.66	9.36 ± 0.61	9.26 ± 0.32	9.43 ± 0.68
Cholesterol %	1.90 ± 0.42	1.52 ± 0.13[a]	1.55 ± 0.16[a]	1.48 ± 0.20[a]	1.38 ± 0.15[a]	1.40 ± 0.23[a]
% distribution of the fatty acids in different lipid classes						
NL	2.63 ± 0.21	2.75 ± 0.44	2.41 ± 0.20	2.23 ± 0.19[b]	1.96 ± 0.36[b]	1.89 ± 0.36[b]
FFA	0.81 ± 0.43	0.45 ± 0.52	0.49 ± 0.39	0.89 ± 0.44	0.48 ± 0.39	0.47 ± 0.36
PL*	96.56 ± 0.44	96.80 ± 0.67	97.10 ± 0.56	96.88 ± 0.42	97.56 ± 0.26[b]	97.63 ± 0.62[b]

Distribution of fatty acids in different lipid classes (NL = neutral lipids; FFA = free fatty acids; PL = polar lipids). AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. Statistically different (one-way ANOVA and Dunnet's test as a *post-hoc* test) from the control group is marked with letters ([a]: $P < 0.05$; [b]: $P < 0.01$).

*For the brain samples all the polar lipid fractions were used in the *in vitro* experiment. Therefore the amount of FA in PL was calculated from FA in total lipid subtracted by the amount of FA in NL and FFA.

Table 2
Fatty acid composition (wt.% of total fatty acids) of polar lipids from cod liver (mean values±standard deviation)

	Control	P.C.				
		5 mg E2/kg	0.02 mg AP/kg	2 mg AP/kg	40 mg AP/kg	80 mg AP/kg
14:0	2.23±0.42	2.50±0.67	2.83±0.38[a]	2.85±0.82	2.72±0.46[a]	3.02±0.87[a]
Iso 15:0	0.15±0.03	0.16±0.07	0.23±0.06[b]	0.23±0.11[a]	0.20±0.05[a]	0.21±0.04[b]
15:0	0.39±0.03	0.45±0.07[a]	0.47±0.08[a]	0.50±0.16	0.46±0.07[a]	0.53±0.11[b]
16:0	22.62±1.71	27.10±2.67[b]	26.14±2.38[b]	26.84±4.38[a]	28.10±2.85	28.97±3.64
Iso 17:0	0.27±0.02	0.26±0.05	0.24±0.05	0.29±0.03	0.25±0.05	0.30±0.03[a]
Antiso 17:0	0.19±0.07	0.30±0.06[b]	0.64±0.16[b]	0.29±0.14	0.22±0.14	0.36±0.09[b]
17:0	0.42±0.05	0.43±0.03	0.55±0.10[b]	0.54±0.11[a]	0.57±0.11[b]	0.58±0.10[b]
18:0	5.86±0.99	5.85±1.14	7.43±1.59[a]	8.31±1.85[b]	7.70±0.99[b]	8.51±1.03[b]
Σ SFA	32.14±2.37	37.05±2.64[b]	38.53±4.03[b]	39.86±6.98[b]	40.23±3.71[b]	42.47±4.52[b]
16:1 (n-9)	0.93±0.13	0.63±0.27[b]	0.79±0.12	1.00±0.75	0.66±0.10[b]	0.64±0.08[b]
16:1 (n-7)	1.52±0.47	1.41±0.46	1.54±0.36	1.44±0.26	1.53±0.29	1.77±0.87
16:1 (n-5)	0.13±0.03	0.16±0.02[a]	0.09±0.04[a]	0.10±0.02[a]	0.12±0.03	0.12±0.05
17:1 (n-9)	0.12±0.11	0.23±0.07[a]	0.36±0.14[b]	0.17±0.09	0.26±0.19[a]	0.29±0.05[b]
18:1 (n-9)	10.31±0.91	9.59±0.51	10.15±0.46	9.62±0.95	9.18±0.61[b]	9.73±0.91
18:1 (n-7)	3.40±0.22	3.20±0.20	3.84±0.40[a]	3.27±0.26	3.17±0.31	3.18±0.29
18:1 (n-5)	0.20±0.03	0.23±0.02[b]	0.20±0.04	0.22±0.04	0.20±0.02	0.26±0.07[a]
20:1 (n-11)	0.12±0.05	0.12±0.08	0.18±0.77	0.18±0.05[a]	0.20±0.07[b]	0.25±0.09[b]
20:1 (n-9)	0.75±0.25	0.68±0.07	0.84±0.07	0.86±0.21	0.81±0.21	1.13±0.38[a]
22:1 (n-11)	0.26±0.16	0.22±0.08	0.30±0.03	0.27±0.15	0.27±0.11	0.34±0.14
22:1 (n-9)	0.38±0.27	0.25±0.16	0.34±0.13	0.20±0.10	0.32±0.16	0.46±0.19
24:1 (n-9)	1.90±0.60	2.20±0.50	3.34±1.07[b]	2.67±0.30[b]	2.79±0.88[a]	2.63±0.23[b]
Σ MUFA	20.00±2.02	18.90±0.85	21.99±0.78	19.99±1.50	19.51±1.69	20.78±2.03
18:3 (n-3)	0.33±0.14	0.33±0.08	0.35±0.07	0.31±0.08	0.32±0.05	0.27±0.05
18:4 (n-3)	0.35±0.14	0.27±0.16	0.32±0.04	0.29±0.12	0.34±0.10	0.53±0.18[a]
20:4 (n-3)	0.36±0.05	0.38±0.07	0.28±0.08[a]	0.36±0.10	0.41±0.20	0.28±0.03[b]
20:5 (n-3)	13.64±1.51	14.59±1.11	11.59±0.91[a]	11.19±1.74[b]	11.43±1.19[b]	10.97±1.43[b]
22:5 (n-3)	1.43±0.24	0.91±0.14[b]	0.89±0.12[b]	0.91±0.33[b]	0.94±0.18[b]	0.83±0.62[a]
22:6 (n-3)	27.64±2.06	23.50±3.06[b]	21.97±3.69[b]	22.81±4.74[a]	22.67±3.75[b]	19.40±2.26[b]
Σ PUFA (n-3)	43.75±2.48	39.98±2.75[b]	35.41±4.71[b]	35.88±6.51[b]	36.11±4.48[b]	32.28±3.08[b]
18:2 (n-6)	2.27±0.32	2.05±0.30	2.03±0.24	2.04±0.33	2.01±0.19[a]	1.95±0.29[a]
20:2 (n-6)	0.16±0.02	0.16±0.04	0.26±0.09[b]	0.19±0.03[a]	0.22±0.06[a]	0.21±0.03[b]
20:4 (n-6)	1.12±0.14	1.25±0.19	1.06±0.14	1.00±0.10[a]	1.01±0.11	1.06±0.07
22:4 n-6	0.23±0.08	0.27±0.12	0.48±0.21[b]	0.71±0.57[a]	0.56±0.11[b]	0.87±0.30[b]
Σ PUFA (n-6)	3.78±0.43	3.72±0.52	3.84±0.26	3.94±0.38	3.80±0.21	4.09±0.37
16:4 (n?)	0.10±0.06	0.12±0.11	0.24±0.14[a]	0.10±0.06	0.13±0.10	0.06±0.05
18:2 (n?)	0.23±0.03	0.22±0.06	0.22±0.03	0.24±0.04	0.23±0.04	0.31±0.09[a]
Σ PUFA	47.86±2.43	44.05±2.90[b]	39.70±4.77[b]	40.15±6.30[b]	40.26±4.38[b]	36.74±3.02[b]
n-3/n-6	11.71±1.47	10.92±1.66	9.22±0.90[b]	9.26±2.27[a]	9.55±1.42[b]	7.97±1.23[b]

AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. Statistically different (unpaired Student's *t*-test) from the control group is marked with letters ([a]: $P<0.05$; [b]: $P<0.01$).

spread on a subphase of MilliQ water at 37 °C. The chloroform was allowed to evaporate before APs in various concentrations (dissolved in MilliQ water) were injected with a syringe under the subphase on the outer side of both barriers, and allowed to equilibrate for 10 min before the monolayers were compressed at 5 mm/min, while the surface tension as function of the mean molecular area was recorded (Agasosler et al., 2001). In addition to the four APs used in the *in vivo* experiment, a number of other APs were tested in the monolayer studies: 4-*n*-butylphenol (4-*n*-BP), 4-*sec*-butylphenol (4-*sec*-BP), 2-*tert*-butylphenol (2-*tert*-BP), 4-*n*-octylphenol (4-*n*-OP) and 4-*n*-nonylphenol (4-*n*-NP) (all from Sigma-Aldrich, Norway).

2.4. Explanation of compression phases

Fig. 1 shows a typical surface pressure–molecular area curve for a glycerophospholipid obtained when the barriers are pressed towards each other, with the names and schematic appearance of the different phases. In the beginning, with the barriers most apart, the monolayer is in the gaseous phase with the acyl groups most apart in the air as indicated. Further compression (from right to left) forces the monolayer molecules into the liquid phase that causes a slight elevation of the surface pressure, starting with the so-called "lift-off" point. Further compression squeezes the lipid molecules into a solid lipid (they can not be forced further together), which gives a steep rise in the surface tension; by even more compression the layer collapses into a many-layered structure.

2.5. Statistical analyses

One-way ANOVA and Dunnet's test as a *post-hoc* test were used to analyse for statistical differences between the control group and the exposed groups for the lipid data and Student's *t*-test were used for the fatty acid profiles. Significance levels

are given in the figure and table legends. The statistical analyses were all performed using Statview software (SAS Institute, Cary, NC, USA) or XLSTAT software (Addinsoft, US). For the Langmuir isotherms at least three experiments were done for each condition, and typical results are shown in the figures.

3. Results

3.1. Monolayer studies

Fig. 2 shows the effects of increasing concentrations of 4-*n*-HEPP in the water subphase on surface tension/mean molecular area relationships in cod brain phospholipid monolayers. Increasing concentrations of the AP caused an increased surface tension at a given molecular area and the molecular area increased with increasing AP concentrations at a given surface tension. The mean molecular area at surface tensions at and below 20 mN/m also increased with increasing chain length of the 4-alkyl substituent (Fig. 3). The same concentration dependence was seen with the other APs studied (results not shown). As shown in Fig. 3, the changes in surface tension at a given molecular area as well as the change in molecular area of the cod brain polar lipids at constant surface pressure clearly increased with the chain length of the 4-substituted linear alkyl groups when the phenols were tested at the same concentration. It is noteworthy that this increase was rather modest when the chain length increased from 4 to 6 carbons, but very large when it increased from 7 to 9 (Fig. 3). 4-*n*-BP gave a small change in the isotherms (Fig. 3), while 4-*sec*- and 2-*tert*-BP decreased both the surface tension and the molecular area compared to the water control, and 4-*tert*-BP produced no change (data not shown). This shows that the bulkier the butyl-group is, the less it increases the mean molecular area.

The surface pressure/mean molecular area isotherms for polar lipids from cod liver increased with the concentration of 4-

Table 3
Fatty acid composition (wt.% of total fatty acids) of total lipids extract from cod brain (mean values ± standard deviation)

	Control	P.C. 5 mg E2/kg	0.02 mg AP/kg	2 mg AP/kg	40 mg AP/kg	80 mg AP/kg
14:0	0.46±0.02	0.53±0.03[b]	0.51±0.03[b]	0.49±0.02[b]	0.48±0.03	0.49±0.03[a]
15:0	0.14±0.01	0.16±0.02[b]	0.17±0.01[b]	0.15±0.02	0.13±0.01	0.14±0.02
16:0	15.16±0.48	15.42±0.55	15.49±0.50	15.54±0.69	15.40±0.31	15.55±0.76
Iso 17:0	0.24±0.02	0.25±0.02	0.25±0.02	0.25±0.03	0.25±0.02	0.24±0.02
17:0	0.15±0.01	0.15±0.02	0.14±0.01	0.15±0.01	0.15±0.01	0.14±0.01
18:0	8.52±0.23	8.50±0.29	8.40±0.27	8.65±0.25	8.42±0.29	8.72±0.20
20:0	0.13±0.01	0.13±0.01	0.13±0.02	0.13±0.02	0.13±0.01	0.13±0.02
22:0	0.15±0.02	0.14±0.02	0.14±0.02	0.14±0.02	0.1±40.01	0.15±0.02
Σ SFA	24.96±0.51	25.27±0.64	25.24±0.32	25.50±0.46	25.09±0.30	25.54±0.78
16:1 (n-9)	0.74±0.04	0.81±0.06	0.84±0.04	0.79±0.02	0.76±0.03	0.77±0.04
16:1 (n-7)	2.27±0.13	2.45±0.24	2.43±0.23	2.40±0.15	2.41±0.16	2.41±0.25
17:1 (n-9)	0.23±0.01	0.24±0.02	0.22±0.01	0.24±0.02	0.23±0.02	0.20±0.03
18:1 (n-9)	24.11±0.87	24.15±0.88	24.88±0.65	24.17±0.69	24.33±0.89	24.09±1.24
18:1 (n-7)	2.54±0.11	2.60±0.12	2.66±0.17	2.55±0.11	2.68±0.09[b]	2.63±0.09
18:1 (n-5)	0.09±0.00	0.09±0.00	0.09±0.00	0.09±0.01	0.09±0.00	0.09±0.00
20:1 (n-11)	0.09±0.01	0.08±0.01	0.04±0.04[b]	0.07±0.03	0.08±0.03	0.04±0.04[b]
20:1 (n-9)	2.61±0.28	2.59±0.21	2.74±0.46	2.56±0.25	2.82±0.20	2.46±0.29
20:1 (n-7)	0.20±0.03	0.20±0.02	0.21±0.02	0.19±0.02	0.20±0.02	0.18±0.02
22:1 (n-11)	0.12±0.02	0.11±0.04	0.09±0.05	0.11±0.02	0.11±0.06	0.08±0.06
22:1 (n-9)	0.88±0.11	0.85±0.08	0.90±0.15	0.82±0.08	0.91±0.08	0.87±0.11
22:1 (n-7)	0.17±0.02	0.17±0.02	0.16±0.02	0.16±0.02	0.17±0.02	0.17±0.02
24:1 (n-9)	9.38±0.46	8.82±0.56[a]	9.00±0.50	8.75±0.53[a]	8.86±0.36[a]	8.89±0.71
Σ MUFA	43.45±1.56	43.14±1.93	44.27±1.28	42.89±1.39	43.64±1.06	42.89±2.53
18:4 (n-3)	0.15±0.01	0.13±0.01[b]	0.12±0.01[b]	0.11±0.00[b]	0.10±0.01[b]	0.10±0.01[b]
20:4 (n-3)	0.15±0.06	0.13±0.01	0.15±0.02	0.13±0.05	0.13±0.02	0.14±0.02
20:5 (n-3)	6.22±0.24	6.58±0.20	6.27±0.14	6.26±0.33	6.36±0.31	6.35±0.25
22:5 (n-3)	0.97±0.06	1.05±0.07	0.94±0.05	0.98±0.06	1.01±0.06	0.97±0.10
22:6 (n-3)	23.02±1.24	22.51±1.41	21.81±0.96	22.97±1.20	22.46±0.74	22.83±1.81
Σ PUFA (n-3)	30.51±1.09	30.40±1.30	29.30±0.95	30.46±1.03	30.06±0.83	30.39±1.77
18:2 (n-6)	0.30±0.06	0.38±0.06[b]	0.39±0.02[b]	0.37±0.02[b]	0.41±0.02[b]	0.38±0.02[b]
20:2 (n-6)	0.17±0.01	0.17±0.01	0.17±0.01	0.17±0.02	0.18±0.02	0.17±0.02
20:4 (n-6)	0.62±0.01	0.62±0.03	0.63±0.04	0.61±0.04	0.61±0.03	0.62±0.02
Σ PUFA (n-6)	1.09±0.07	1.18±0.07[b]	1.19±0.05[b]	1.15±0.06[a]	1.20±0.06[b]	1.17±0.04[b]
Σ PUFA	31.59±1.07	31.58±1.30	30.49±0.98	31.61±1.02	31.27±0.83	31.57±1.77
n-3/n-6	28.22±2.20	25.89±2.02	24.67±0.86	26.50±1.68	25.04±1.31	25.95±1.83
Σ alk-1-enyl ethers	7.24±0.44	7.11±0.34	7.47±0.26	7.23±0.29	7.15±0.45	7.11±0.45

AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. The Σ alk-1-enyl-ethers originating from plasmalogens is the sum of dimethylacetal (DMA) and methylalk-1-enyl ethers (AME) with the chain length of 16:0. 18:0 and 18:1 and are given as percent of the total fatty acids. Statistically different (unpaired Student's *t*-test) from the control group is marked with letters ([a]: $P<0.05$; [b]: $P<0.01$).

n-HEPP in the same fashion (data not shown) as shown for cod brain in Fig 2. With cod liver polar lipids both the surface tension and the molecular areas increased with increasing alkyl length of linear 4-APs (Fig. 4). We also measured isotherms in monolayers of commercial 16:0/22:6 n-3 PC with 4-*n*-APs in the subphase, and also with a pure glycerophospholipid the surface tension and mean molecular area increased with increasing alkyl length (Fig. 5). The amounts of PC and PE from cod liver were too small for three monolayer experiments, but one experiment with each isolated phospholipid showed similar responses to 4-*n*-AP with increasing alkyl lengths as in Figs. 3 and 5.

3.2. In vivo effects

Table 1 gives an overview of the lipid composition in cod liver and brain, with total lipid amount, amount of cholesterol and the distribution of fatty acids between the different lipid classes: NLs, FFA and PLs. There was no significant difference in total lipid content of neither the liver nor the brain between the control and the exposed groups. There was no difference in cholesterol levels in the liver, but for the brain samples the levels of cholesterol were significantly lower in the E2 group (5 mg E2/kg) and the three highest exposed AP groups (2 mg AP/kg, 40 mg AP/kg and 80 mg AP/kg) compared with the control.

The AP exposure altered the distribution of fatty acids between the lipid classes in the liver. The exposed groups had a significant higher amount of NLs and lower amount of phospholipids than the control. There was a slight dose response in the AP groups. The E2 group also had lower amount of phospholipids than the control, but to a less degree compared to the AP groups. The situation was opposite in the brain samples. The three highest AP groups have significantly lower levels of NLs and more phospholipids compared with the control group. There was no effect on the oestrogen group and the lowest AP group (0.02 mg AP/kg).

Table 4
Fatty acid composition (wt.% of total fatty acids) of the neutral lipid fraction of the lipids extract from cod brain (mean values±standard deviation)

		P.C.				
	Control	5 mg E2/kg	0.02 mg AP/kg	2 mg AP/kg	40 mg AP/kg	80 mg AP/kg
14:0	1.27±0.13	1.48±0.20[a]	1.51±0.10[b]	1.58±0.20[b]	2.00±0.23[b]	1.85±0.19[b]
15:0	0.21±0.03	0.22±0.06	0.14±0.05[b]	0.24±0.05	0.27±0.03[b]	0.24±0.09[a]
16:0	22.63±2.19	23.07±3.16	25.76±0.89[b]	27.34±1.71[b]	31.29±3.58[b]	30.88±4.69[b]
Iso 17:0	0.06±0.04	0.10±0.01[b]	0.09±0.01	0.10±0.01[b]	0.12±0.02[b]	0.09±0.04
17:0	1.71±0.17	1.62±0.20	1.93±0.12[a]	1.94±0.15[b]	2.10±0.20[b]	2.17±0.22[b]
18:0	12.27±0.62	12.15±1.61	12.94±0.75	14.20±0.81[b]	14.42±1.88[b]	15.80±2.00[b]
20:0	0.70±0.05	0.64±0.08	0.76±0.04[a]	0.78±0.05[b]	0.83±0.08[b]	0.89±0.08[b]
22:0	0.54±0.05	0.47±0.08	0.55±0.10	0.57±0.07	0.73±0.13[b]	0.90±0.19[b]
24:0	3.29±1.20	2.43±0.16[a]	3.06±0.41	2.55±0.29	1.93±0.34[b]	1.97±0.36[b]
Σ SFA	42.68±2.20	42.18±5.41	46.73±1.50[b]	49.30±2.26[b]	53.68±5.32[b]	54.79±6.82[b]
16:1 (n-9)	0.42±0.03	0.37±0.03[b]	0.37±0.03[a]	0.33±0.02[b]	0.30±0.03[b]	0.29±0.02[b]
16:1 (n-7)	3.21±0.28	3.34±0.33	3.30±0.13	2.95±0.23[a]	2.75±0.31[b]	2.58±0.31[b]
17:1 (n-9)	0.09±0.02	0.09±0.01	0.09±0.01	0.09±0.01	0.10±0.03	0.04±0.05[a]
18:1 (n-9)	12.64±0.84	12.81±1.10	12.03±0.46	11.43±0.56[b]	10.36±1.15[b]	9.80±1.13[b]
18:1 (n-7)	2.17±0.15	2.34±0.19	2.14±0.09	1.92±0.11[b]	1.80±0.20[b]	1.66±0.20[b]
18:1 (n-5)	0.01±0.02	0.04±0.04[b]	0.01±0.03	0.02±0.03	0.00±0.00	0.00±0.00
20:1 (n-11)	0.13±0.09	0.18±0.07	0.12±0.12	0.15±0.08	0.21±0.11	0.04±0.10
20:1 (n-9)	3.54±0.40	3.50±0.41	3.50±0.45	3.12±0.18[b]	2.93±0.37[b]	2.59±0.40[b]
20:1 (n-7)	0.09±0.05	0.11±0.04	0.09±0.05	0.10±0.03	0.08±0.05	0.05±0.05
22:1 (n-11)	0.40±0.09	0.53±0.12[a]	0.44±0.12	0.45±0.09	0.64±0.14[b]	0.52±0.20
22:1 (n-9)	1.01±0.09	0.95±0.12	0.97±0.11	0.87±0.10[b]	0.81±0.12[b]	0.72±0.37
22:1 (n-7)	0.62±0.25	0.67±0.06	0.30±0.27[a]	0.34±0.19[b]	0.28±0.16[b]	0.37±0.16
24:1 (n-9)	12.49±1.20	10.74±1.14[b]	11.34±1.69	10.93±1.16[b]	10.09±1.84[b]	12.37±1.72
Σ MUFA	36.83±1.89	35.67±2.93	34.72±1.59	32.69±1.87[b]	30.34±3.51[b]	31.03±3.51[b]
18:4 (n-3)	0.13±0.01	0.11±0.01[b]	0.13±0.01	0.13±0.01	0.14±0.02	0.15±0.02[a]
20:4 (n-3)	0.02±0.05	0.11±0.08[a]	0.00±0.00	0.01±0.03	0.02±0.05	0.02±0.04
20:5 (n-3)	6.28±0.62	7.28±1.30[a]	5.71±0.53	5.14±0.43[b]	4.51±0.72[b]	4.07±1.15[b]
22:5 (n-3)	0.41±0.29	0.45±0.20	0.61±0.44	0.48±0.19	0.40±0.06	0.33±0.09
22:6 (n-3)	11.75±1.65	11.97±1.62	10.18±0.82	10.47±1.30	9.05±1.32[b]	8.12±2.73[b]
Σ PUFA (n-3)	18.59±1.99	19.92±2.84	16.63±0.81	16.24±1.36[b]	14.12±2.06[b]	12.68±3.81[b]
18:2 (n-6)	0.74±0.12	1.01±0.14[a]	0.81±0.18	0.76±0.09	0.96±0.13[b]	0.71±0.15
20:2 (n-6)	0.26±0.13	0.27±0.16	0.23±0.02	0.21±0.02	0.24±0.01	0.14±0.12
20:4 (n-6)	0.90±0.09	0.96±0.14	0.89±0.05	0.80±0.08[a]	0.66±0.10[b]	0.65±0.15[b]
Σ PUFA (n-6)	1.90±0.18	2.24±0.35[a]	1.92±0.19	1.77±0.10	1.86±0.20	1.50±0.33[b]
Σ PUFA	20.49±2.12	22.15±3.12	18.55±0.96	18.01±1.37[b]	15.98±2.24[b]	14.18±4.09[b]
n-3/n-6	9.82±0.77	8.95±0.89[a]	8.69±0.60	9.20±0.92	7.58±0.62[b]	8.38±1.65
Cholesterol	1478±187	1082±66[b]	1222±130[b]	1190±86[b]	1136±67[b]	1161±102

AP doses are given as the sum of 4-*tert*-butylphenol, 4-*n*-pentylphenol, 4-*n*-hexylphenol and 4-*n*-heptylphenol concentrations. The cholesterol is normalised relative to the amount of total fatty acids. Statistically different (unpaired Student's *t*-test) from the control group is marked with letters ([a]: $P<0.05$; [b]: $P<0.01$).

The fatty acid profiles of the PLs in the liver were clearly affected by the AP exposure (Table 2). All the exposed groups had lower relative levels of n-3 family polyunsaturated fatty acids (n-3 PUFA) and corresponding higher levels of saturated fatty acids (SFA) compared with the control group. There were only minor differences between the groups in the relative amount of monounsaturated fatty acids (MUFA) and the n-6 family PUFA. The different influences on n-3 and n-6 PUFA implied that the n-3/n-6 PUFA ratio decreased from 12 in the control group to 8 in the high AP group (80 mg AP/kg). All the SFA increased in the exposed groups, the highest AP group had 32% higher levels of total SFA than the control. All long-chain n-3 PUFA (=C_{20}) decreased, while 18:3 (n-3) and 18:4 (n-3) were not affected or increased. The highest AP group had 26% lower levels of n-3 PUFA than the control group. The E2 group showed lower but otherwise similar changes in the fatty acid profile of the PLs as that for AP exposed groups.

The liver fatty acid profiles of the NLs and the FFA did not differ significantly from the control (data not shown).

The fatty acid profiles of the total brain lipids showed only minor differences between the treatments (Table 3). No group differences in the amount of alk-1-enyl ethers from plasmalogens were found. All of the phospholipid fractions from the brain samples were used in the *in vitro* experiments. However, because PLs amount to more than 96% of the total content of the fatty acid, the fatty acid profiles of the total extract represent well the composition of membrane lipids. No group differences in the composition of the FFA isolated from the brain could be shown (data not shown). The fatty acid profiles of the NLs, on the other hand, showed highly significant differences between the groups and with a clear dose dependent response (Table 4). The AP exposed groups had higher relative levels of SFA than the control group and lower levels of MUFA and PUFA. The fatty acid profiles from the E2 group differed only marginally from the control groups demonstrating minor effects of E2 exposure on the saturation levels of fatty acids. The NLs are dominated by cholesterol. HPLC analysis of the NL showed that approximately 98% of the total NL was free cholesterol and 2% was cholesterol esters. The amount of TAG was under the detection limit of this method. The fatty acid profiles of the NL do therefore mainly reflect the fatty acid composition of cholesterol esters.

4. Discussion

The high affinity for phospholipid monolayer reflects the amphipathic nature of APs. As could be expected, the capability for membrane interference increased with the chain length of the alkyl group. APs clearly have a specially high effect on both the surface tension and the mean molecular area of the monolayers.

We found the same pattern in all the monolayers tested, but the increase in surface area after adding AP was higher in the two native mixtures of PLs from brain and liver than from commercial PC (16:0/22:6 n-3). Large effects of increased unsaturation were found in the partitioning of AP between liposome membrane vesicles and water. The uptake of NP in PC (16:0/18:1) vesicles was 5 fold higher compared with PC (16:0/16:0) vesicles (Yamamoto and Liljestrand, 2004). This shows that the composition of the lipid phases is very important in studying membrane effects and that the use of native extracts can be more comparable to the physiological situation.

An explanation of the observed (and consistent) effects of the APs on the mean molecular area of monolayers is that the amphiphilic APs penetrate the lipid–water boundary of the layers by inserting the lipophilic alkyl-benzene domain of AP among the fatty acids in glycerophospholipids in the monolayer and with the hydrophilic OH groups being positioned among the hydrophilic phospholipid headgroups. This explains the increase in the mean molecular area since the computer program calculates the area per number of *phospholipid molecules*, which is the only information given to the program, and not the number of AP molecules inserted. Thus, the area (perpendicular of the monolayer) for each single phospholipid molecule is not altered; the program registers the combined area of the lipid *plus* the area of the inserted molecules. Clearly, this insertion is very small for alkyl groups equal to and lower than hexyl, but above this alkyl chain length the increment becomes large. The observed increase in surface tension at a given molecular area with increasing AP concentration is not straightforward to explain, but indicates that the monolayer gets “harder” more AP that penetrates. APs are quickly taken up by the intestine after oral exposure and transported by the blood to the liver (Sundt and Baussant, 2003). The liver is the main target of lipophilic xenobiotics in cod, but lean fish like cod are also accumulating high amounts of xenobiotic in the brain compared with fat fish (Ingebrigtsen et al., 1990).

The extremely high lipid content (67–70%) found in the liver of the cod is normal for cod held in captivity, where the fish are fed high-energy lipid-rich diets (Lie et al., 1996). The liver is the lipid energy store of the cod and does mainly contain TAG. The fatty acid profile of TAG is strongly influenced by the profile of the diet (Lie et al., 1996). The composition of PLs is more independent of the diet and the fatty acid profiles found in this paper show a typical picture of phospholipids from cod with its very high levels of n-3 PUFA (Lie and Lambertsen, 1991). The PLs from cod liver and brain have very different compositions of phospholipids. The major phospholipids in the liver are dominated by PC (54%), PE (28%), phosphatidylinositol (PI) (11%) and PS (7%) (Lie and Lambertsen, 1991), while the compositions in the brain are PC (41%), PE (47%), PI (9%) and PS (3%) (Tocher and Harvie, 1988). Large amounts of the PE in the brain are ether lipids (plasmalogens) (Bell and Dick, 1993). Typical for fish phospholipids is the very high amount of PUFA, and especially the brain contains much phospholipids with long-chain PUFA both in *sn*-1 and *sn*-2 positions of the glyceride (Bell and Dick, 1991).

Optimal membrane fluidity is essential in maintaining normal physiological functions. Fish have high flexibility in remodelling the lipid composition of cell membranes (Cossins, 1994). The mechanisms responsible for the perception of changes in membrane fluidity have not been fully characterized, but include changes in fatty acid unsaturation, changes in the proportions of phospholipid classes or cholesterol and changes in the lipid–protein ratio (Williams and Hazel, 1994). Warm adaptation is increasing the membrane fluidity, and typically

results in effects like increasing the saturation of the membrane fatty acids, increasing the relative amount of PC relative to PE, and decreasing the amount of cholesterol in the membrane (Williams and Hazel, 1994).

The high increase of SFA and decrease in n-3 PUFA (particularly 22:6 (n-3)) that are found in PLs from the liver of the AP and E2 exposed fish are similar to the response of heat acclimatization in fish. Lie and Lambertsen (1989) found very similar effects on the FA composition of erythrocyte phospholipids in cod held at different water temperatures. At 16 °C PC contained 29% SFA and 38% PUFA while at 8 °C the corresponding figures were 27% and 46%. Similarly, PE at 16 °C contained 25% SFA and 47% PUFA while at 8 °C the corresponding figures were 17% and 56% (Lie and Lambertsen, 1989). From our data it is not possible to find out if the changes are general changes in the levels of saturation of all membrane lipids or simply an exchange of PE with PC. The fatty acid composition of the total lipids (>96% PLs, Table 1) in the brain samples was not significantly affected. However, the extent of saturation of the fatty acids in the NLs (mainly cholesterol esters) increased a lot. A decline in cholesterol content of the brain lipids was demonstrated. The down-regulation of cholesterol in the brain may be a response to increased fluidity of the membrane caused by the AP exposure. Nevertheless, the possibility of being the consequence of different maturation rates in the female control and AP exposed fish can not be ruled out (see below). We do not have any information on seasonal fluctuations of cholesterol in the brain of fish.

In microorganisms there is increasing evidence that physical state membranes control the expression of genes that in turn have direct effect on the membrane fluidity. It has been shown that alcohols that induce membrane perturbation induce similar changes in gene expression as heat shock (Vigh et al., 1998).

The effects found on the lipid composition are most likely not driven by an estrogenic pathway. Even though similar effects in the E2 group are observed, these effects are much weaker.

Noteworthy, the estrogen levels of the E2 group were extremely high (after a dose of 5 mg/kg) compared to physiological plasma concentrations of E2 in sexually mature female cod (25 µg/l plasma). Therefore the estrogenic effect in this group was much higher than even in the AP group given the highest dose. The plasma concentration of vitellogenin was 5 times higher in the E2 treated fish than in the control group and the AP groups (Meier et al., 2007). E2 may also affect membrane fluidity (Dicko et al., 1999; Liang et al., 2001) but have lower affinity for biological membranes than APs (Yamamoto and Liljestrand, 2004).

The mechanisms behind the AP effects are several. Other studies done at this lab have shown that exposure to APs has major effects on the levels of sex hormones in female fish. Even very low doses of APs lowered the levels of E2 in female fish. Oocytes of exposed fish developed more slowly than normal and this effect was manifested in their low gonadosomatic index and delayed estimated time of spawning (Meier et al., 2007). APs may also affect the redox status in the female cod. For example, the total hepatic glutathione concentration was increased after one week of exposure, but no effect was found after 5 weeks of exposure (Hasselberg et al., 2004a). *In vivo* and *in vitro* effects of AP exposure in cod have been studied in Hasselberg et al. (2004b). Fish from this experiment showed a dose-related increase in hepatic CYP1A and CYP3A protein in male cod, but no effect was observed in the females. However, this increase of CYP1A protein levels in the male fish was not linked to an increase in CYP1A-mediated ethoxyresorufin-*O*-deethylase (EROD) activity, implying that APs inhibit the CYP1A activity *in vivo*.

The estrogenic effects of long-chain *para*-substituted APs are well known (Nimrod and Benson, 1996). There are also good support for this that APs can be related to membrane effects that are independent of the estrogenic pathways: NP provokes a vesiculation of the Golgi apparatus of epidermis cells from fish at a concentration of 20 µM (Lamche and BurkhardtHolm, 2000). Similar doses of 4-*tert*-BP and 4-*tert*-OP cause the formation of lipid droplets and other changes in Leydig cell membrane structures of rats (Haavisto et al., 2003). Schwaiger et al. (2000) suggest that anemia found in NP exposed fish is a consequence of an interaction between NP and the erythrocyte membrane. NP is increasing the membrane permeability of mitochondria membranes to protons and acts therefore as an uncoupler of the oxidative phosphorylation (Bragadin et al., 1999). Mitochondrial depolarization by NP is also suggested as one of the mechanisms behind NP induced thymocyte apoptosis (Yao et al., 2006). Several investigations have shown that APs are disrupting Ca^{2+} homeostasis by affecting Ca^{2+} channels (Beeler and Gable, 1993; Michelangeli et al., 1990; Kirk et al., 2003). Gap junctional intercellular communication is reduced in a murine Sertoli cell line by NP (between 1 and 50 µM). The effect is partly explained by the reduction in phosphorylation of connexin 43 (Aravindakshan and Cyr, 2005), but the gap junction may also be affected by the changes in the membrane lipid bilayer (Cascio, 2005). Microarray analysis of gene expression profiles in mouse exposed to NP or E2 reveals that the gene expression in the liver was more affected by NP than E2, and activation of many genes involved in lipid and fatty acids metabolism was only found in the NP groups. These include genes for apolipoprotein A-IV, peroxiredoxin, granulin and lecthin cholesterol acyltransferase and acetyl-CoA acyltransferase (Watanabe et al., 2004).

There is also support for those similar changes in the lipid composition as reported in the present article which can be found in fish exposed to oil pollution. Dey et al. (1983) reported decreasing levels of PUFA and higher levels of SFA in the PLs of the liver of cod and winter flounder (*Pseudopleuronectes americanus*) after a long term (24 weeks) exposure to low levels of crude oil (100–200 µg/l). Wild fish (cod and haddock (*Melanogrammus aeglefinus*)) caught in the North Sea around the Tampen field (high oil activity and discharge of produced water) had relatively higher proportions of SFA to PUFA in the muscle (unpublished data) and the n-3/n-6 PUFA ratio in the muscle was also lower compared with fish caught at Egersund bank (reference area) (Hylland et al., 2006).

5. Conclusion

The *in vitro* studies showed that APs are easily incorporated into monolayers of PL at concentrations down to 5 µM. The

study demonstrated that APs and E2 alter the fatty acid profile of the PLs from the liver containing more SFA and less n-3 PUFA compared with control. In the brain of the exposed groups similar effects were demonstrated with higher saturation of the fatty acids of the NLs (mainly cholesterol esters), but not of the PLs. AP and E2 exposure also caused a decline in the cholesterol levels in the brain.

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