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GC-MS determination of PAH- and alkylphenol-metabolites in fish bile for detection of produced water contamination

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Preface

This project, NFR 152449-720, has been funded by the Norwegian Research Council through the PROOF programme. The project was conducted at RF-Rogaland Research during 2002-2003 in the facility of Akvamiljø A/S in Stavanger (Norway). The project has been performed in collaboration with the NFR-152231 project, which is managed by the Institute of Marine Research. Some of the activities in the present project are continued in the PROOF funded NFR 153898-720 project (2003-2005).

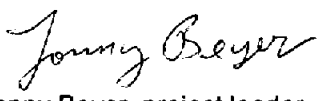
Résumé

Fish inhabiting waters downcurrent large operational discharges of produced water in the North Sea are potentially at risk for chronic low-dose contamination of PAHs and alkylphenols. This has been a matter of concern in recent years. There is a need for better analytical tools for monitoring this exposure. Gall bladder bile obtained from recipient fish may potentially serve as a target sample for assessments of both PAH and AP exposure in such situations. The present project report provides a GC-MS SIM protocol which is demonstrated as the best method for quantitative determination of petrogenic PAH metabolites in fish bile. Secondly, the present project includes an analytical pilot study on the feasibility of using GC-MS SIM detection of alkylphenols in fish bile as a monitoring tool of PW related AP exposure in fish. The initial method development results included in this report indicate a clear potential of the GC-MS with respect to this task, but further development of the assay is necessary before the method is operative. The testing and further development of the assay continues in the follow up project (153898-720) which also is funded by PROOF.

Acknowledges

I would like to thank all that have contributed to this study. NFR-PROOF is acknowledged for funding this project.

Stavanger / 30. September 2003



Jonny Beyer, project leader

Contents

1 Introduction and background

1.1 PAHs and alkylphenols in produced water

Formation water that comes with the hydrocarbon (HC) stream during oil and gas production at offshore installations must be removed before the HC product is further transported. Normally, the relative water content in the stream increases as a production field gets older. Subsequent to the separation from the HCs, the (produced) water is either re-injected or discharged to sea (Figure 1). The produced water (PW) effluent contains minor amounts of dispersed oil but also variable concentrations of water soluble organic and inorganic chemicals in various forms. The chemical composition of PW varies significantly between oil-fields, but certain organic chemical classes such as polyaromatic hydrocarbons (PAH) and alkylphenols (AP) is typically present at variable levels (Utvik 1999).

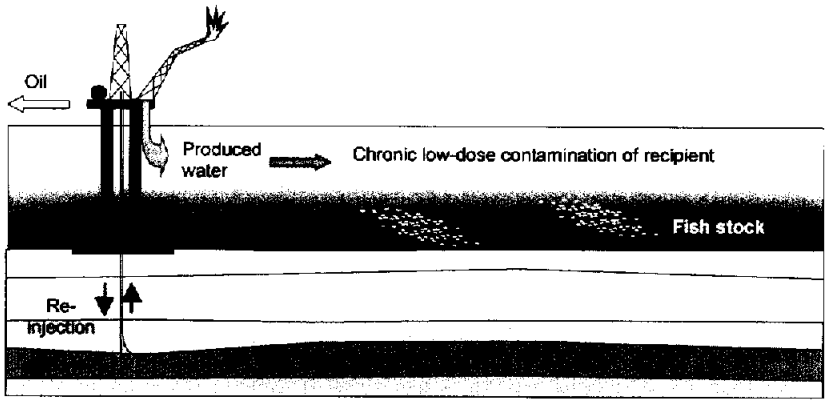


Figure 1: The produced water is separated from the hydrocarbons at the installation and re-injected or discharged to the recipient.

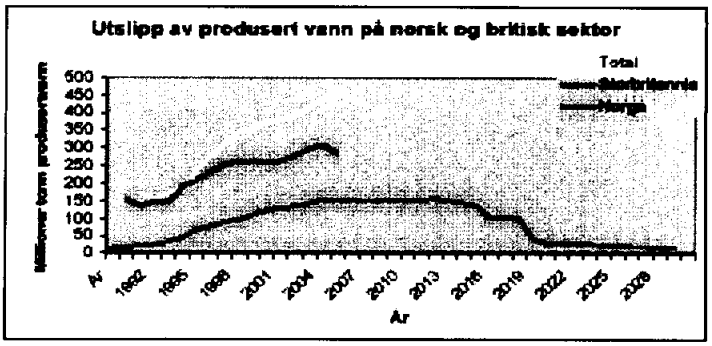


Figure 2: Historical discharges of produced water in the Norwegian and British sectors of the North Sea and total discharges. Data source: DTI and UKOOA. Graphical presentation obtained from (SFT et al. 2003).

The annual volume of PW discharged in Norwegian and British sector of the North Sea has increased sharply and is expected to stay high for a long period (Figure 2). The total amount of PAHs (Sum PAH_{16}), phenols (defined as C_0-C_3) and alkylphenols (C_4-C_7)

released in the Norwegian sector were in 1999 estimated to approximately 40, 470 and 15 tons, respectively (SFT 2000). Of the PAHs, naphthalene and alkylated naphthalenes contributed normally with approximately 90%, or more. With respect to the phenols/alkylphenols, the concentration in PW generally decreases with the increasing size of the alkyl chain, see Brendehaug et al. (1992) for details.

1.2 Environmental risk of PW - PAHs and APs

When PAHs and APs in a PW effluent enter the recipient environment their subsequent fate will depend on a range of factors; both related to the recipient as well as related to their chemical/physical characteristics. A full review of all relevant factors falls beyond the scope of this report, but among the most important recipient factor is the massive dilution that occurs in the recipient water mass ultimately after the discharge. Exposure markers in fish of PAH and AP contaminants from PW must therefore be highly sensitive when used in an offshore environment context. However, field studies with fish caged at various distances from PW discharge points (the BECPELAG study) have indicated that the recipient seawater even at a significant distance (10 km) downcurrent the discharge point may contain contaminants at concentration sufficient to induce increased bile metabolite levels in fish (Aas et al. 2003).

The question is, however, whether a chronic low-dose PAH and AP contamination of the offshore recipient represent a dose sufficient to cause long term impact. With respect to PAHs, it is known that long term exposure of fish can increase the amount of genetic lesions in vital organs, in particular in metabolic active tissues such as liver. These genetic lesions increase the frequency of mal-transcriptions of the genes and thus increase the frequency of cellular transformations and tumour developments (Figure 3). With respect to APs in PW, considerable attention has recently been directed towards their possible hormonal effects in fish. In particular, the possibility of PW-APs to act as oestrogen mimics and disturb vital hormonally regulated processes such as sexual development, maturation and spawning (Figure 3). In a study with Atlantic cod carried out by the Institute of Marine Research in Bergen, a mixture of four C_4-C_7 alkylphenols was found to reduce oestrogen levels and disturb spawning in females and to lower testosterone levels and induce vitellogenesis in males (Meier et al. 2002). The lowest AP concentration giving effect was estimated to be 0.032 ppb (calculated chronic exposure value).

A follow up study has recently been conducted at RF-Akvamiljø to test the IMR findings; by studying the toxicokinetics of a radiolabelled mixture of the same four compounds exposed either via food (daily dose 5 µg/kg fish) or seawater (0.008 µg/l) (Sundt and Baussant 2003). The study revealed that substantial amounts of alkylated phenols were taken up and bioconcentrated in the body tissues of fish exposed via seawater, with modelled bioconcentration factors ranging from 100 to 500. On the other hand, the absorption efficiencies of the alkylated phenols via food were shown to be poor, with the lowest value observed for C_5-AP (8%) whilst for the other compounds absorption efficiency was between 12% to 14%. Based on this study, the exposure through water (and uptake over the gill membrane) seems to be the most relevant route to consider for APs contamination in fish, whereas uptake via food is less effective. The

study also showed that even the low AP concentration, 0.008 µg/l (ppb) in seawater, resulted in a markedly increase in AP concentrations in the bile fluid of the exposed fish, whereas the AP levels in other organs (such as liver) were generally low. The study utilised radiolabelled APs, and could therefore use highly sensitive radio-assays to trace the exposure compounds in the fish organs.

When fish are collected in the field, other analytical methods than radio-assays must of course be used, and this fact illustrates the rationale of the present project. Since increased levels of PAH and AP compounds in the bile practically is the first change that can be measured in fish exposed to these chemicals, a sensitive and selective detection of these biliary molecules offers an optimal early warning of eventual effects that might occur later in a hypothetical sequence of effects, as illustrated in Figure 3.

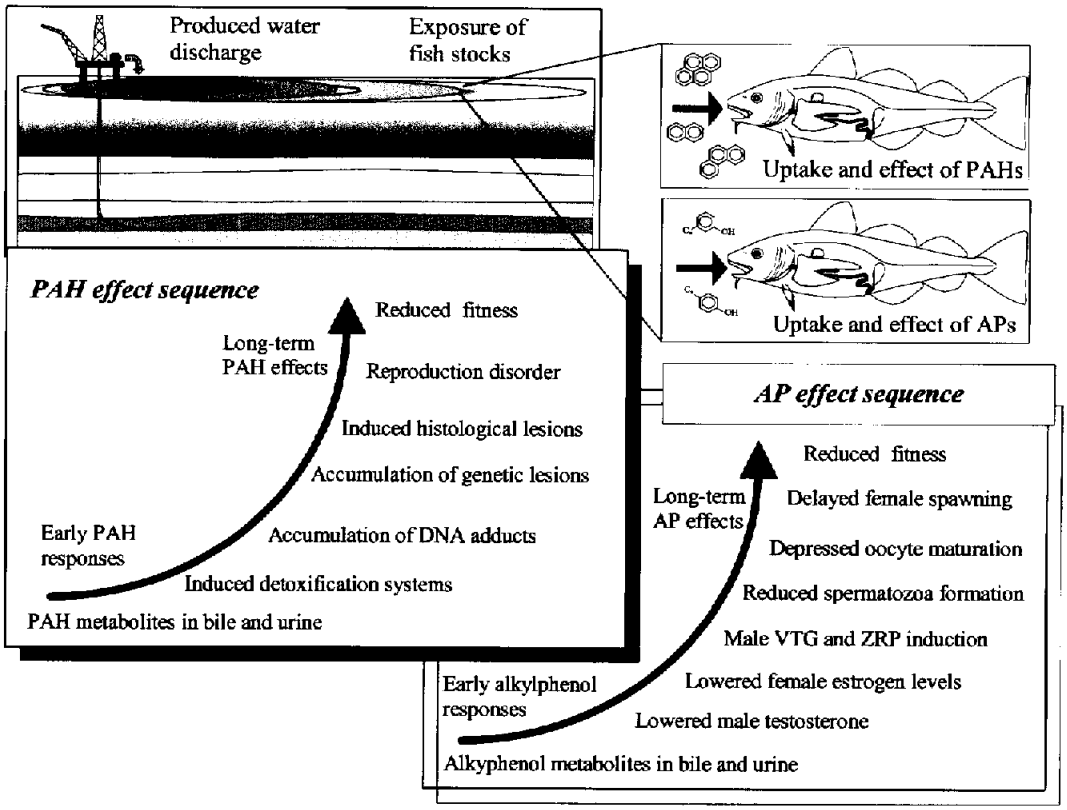


Figure 3: Principal scheme of effect sequence in fish exposed to PAH and to Alkylphenols.

1.3 Research need

There is a gap of knowledge concerning uptake efficiency, bioaccumulation and effects in marine fish of petrogenic PAHs (2-4 rings with large degree of alkylation) and the short-alkylchains APs; which are the PAH and AP species relevant to produced water (PW). Chemical analyses of PAH and AP parent compounds in fish tissue such as liver is a suboptimal approach since neither compound class are persistent in fish. The methods for detecting (and monitoring) PW related PAH and AP contamination in fish need improvements. There is also a need of field data on exposure levels (in the water

column), fate, bioavailability and eventual long term effects of produced water (PW) contaminants in fish in regions of the North Sea where large volumes PW are discharged. This need has been expressed by international bodies such as OSPARCOM and ICES as well as by the Norwegian pollution control authorities, the Ministry of Petroleum and Energy (OED) and recently also by the Norwegian Research Council working group on *Long term effects of discharges to sea from the offshore sector.*

2 Project objectives

- To compile an optimised protocol for GC-MS detection of bile metabolites of naphthalene and other small PAHs that typically characterise PW discharges.
- To perform laboratory exposures with Atlantic cod and alkylphenol (single) chemicals that typically occur in discharged produced water in order to produce bile samples for GC-MS determination of AP metabolites.
- To conduct pilot GC-MS analyses of AP standard compounds aimed at developing and optimising a GC-MS protocol for detection of selected AP compounds (parent compounds and metabolites) in the bile fluid.
- To use this GC-MS method in analyses of alkylphenol compounds in bile obtained from lab exposed fish and fish collected at relevant offshore locations (e.g. Tampen region).

Atlantic cod is selected as study species in this project but also other fish species, such as haddock, may later be included as suggested monitoring species, partly since this species is more frequently caught and since its sediment associated feeding habit render it relevant for pollution monitoring surveys in general.

3 Compilation of GC-MS & bile metabolite protocol for the monitoring of PW relevant PAHs in marine fish

In this work-package an improved protocol for bile PAH metabolite detection used as a monitoring tool of PW originated PAH pollution in fish is compiled. The compiled protocol drains the experience gained in several earlier projects where the use of biliary PAH metabolites in fish has been explored as a PAH exposure marker. Along with this process of method improvements two doctoral candidates have accomplished their studies (Aas 2000; Jonsson 2003a), and a number of papers from laboratory studies and field studies have been published in international journals (Beyer et al. 1998; Camus et al. 1998; Aas et al. 1998; Aas et al. 2000; Aas et al. 2001; Jonsson et al. 2003b; Jonsson et al. 2003c; Aas et al. 2003). Thus, the protocol given below is based on a detailed

insight in our group in how to best measure produced water related (i.e. crude oil related) PAH contamination in fish.

3.1 Introduction about biliary PW - PAH metabolites in fish

Detection of biliary PAH metabolites in fish may serve as an early warning parameter that indicates a recent or ongoing PAH exposure. As illustrated previously (Figure 3), a long term PAH stress may lead to more adverse effects such as DNA lesions (e.g. covalent bound DNA adducts) and later irreversible histopathological effects (e.g. PAH induced tumors). Because of this, the determination of PAH metabolite levels in fish bile has been proposed as a biomarker of PAH exposure by international bodies such as ICES and the Oslo Paris Commissions, e.g. (OSPARCOM 1998).

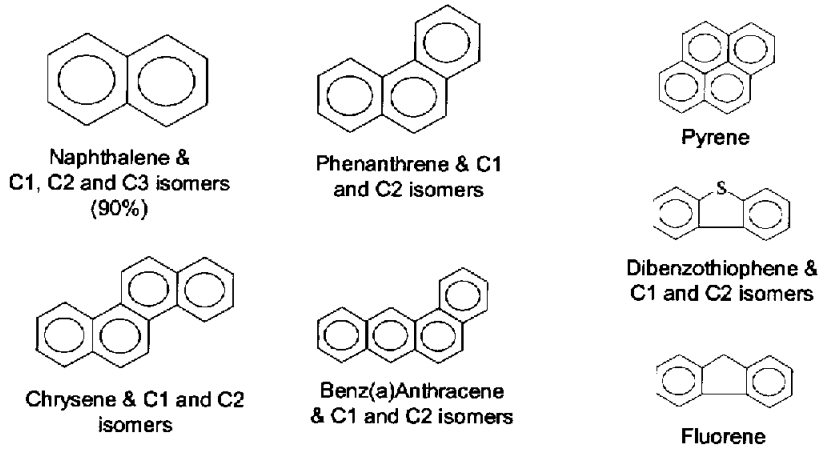


Figure 4: Molecular structures of some of the most common PAHs in produced water.

With respect to produced water, a characteristic PAH profile is always dominated by smaller, typical petrogenic, PAH compounds with a high degree of alkylation. In particular, high relative concentrations are found for the naphthalenes and phenanthrenes (Figure 4). The domination of the smaller PAH forms is also representative for the PAH profile seen in the bile from oil exposed fish. In this protocol, special emphasis is therefore put on the detection of these typically crude oil related PAHs; i.e. naphthalene, phenanthrene and alkylated isomers of these.

3.2 GC-MS protocol for PW – PAH metabolites

3.2.1 Chemicals

1-hydroxy-naphthalene (1-OH-NPH) and 2-hydroxy-naphthalene (2-OH-NPH) are obtained from Fluka Chemie AG (Buchs, Switzerland), and 1-hydroxy-phenanthrene (1-OH-PHE) from Promochem (Wesel, Germany). 1-hydroxy-pyrene (1-OH-PYR), triphenylamine (TPA), and β-glucuronidase with 5 % sulphatase activity (Type HP-2)

are bought from Sigma-Aldrich (Steinheim, Germany). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 2,6-dibromophenol (2,6-DBP) are obtained from Varian (Morton Grove, IL, USA) and Avocado (Heysham, England), respectively. Sodiumacetate, anhydrous sodiumsulphate (pesticide grade) and ethylacetate (pesticide grade) are obtained from Merck (Darmstadt, Germany).

Multilevel (seven) calibration curves are prepared for trimethylsilyl ethers of OH-PAHs, TMS-O-PAHs, (5 – 550 ng/g) in ethylacetate. Response factor curves are calculated for the non-alkylated TMS-O-PAHs, and used for calculation of sample concentration. Semi-quantitative concentrations of alkylated TMS-O-PAHs are calculated by use of response factor curves for the corresponding non-alkylated compounds.

3.2.2 Sampling of fish and bile

Gall bladder bile samples are obtained from fish specimens directly after the trawl haul or from fish kept alive prior to necropsy (e.g. in seawater tanks). The bile samples should always be taken from fresh material; i.e. avoid using bile from fish that have been dead for a long time, i.e. several hours. Sometimes, the bile volume in collected fish is empty or very limited. In this case it is recommended to keep the fish alive overnight, if this is possible with the vessel used for the survey. After the sampling, the bile samples should immediately be frozen and stored in a freezer (-20°C is fine) or at liquid nitrogen. When kept frozen, the bile samples stay stable for a long time and can thus be stored several years before analysis (if required).

3.2.3 Sample preparation and TMS derivatisation for GC-MS

The gall bladder bile from individual fish is prepared for analysis as described in detail by Jonsson et al. (2003b). Briefly, 20–30 µl of bile is weighed accurately into a microcentrifuge vial. Internal standard, 2,6-dibromophenol (2,6-DBP), and β-glucuronidase (3000 units) in sodium acetate buffer (0.4 M, pH = 5) are added and the solution is left at 40°C for 2 hours. The OH-PAHs are then extracted with ethylacetate (4 times 0.5 ml), the combined extract dried with anhydrous sodium sulphate and subsequently concentrated to 0.5 ml. Trimethylsilyl (TMS) ethers of OH-PAHs are prepared by addition of 0.2 ml BSTFA and heating for two hours at 60°C. TPA is added as a GC-MS performance standard before transferring the prepared samples to capped vials. 2,6-DBP is used as quantization internal standard, but may not fully adjust for recovery loss. Consider the details in Jonsson et al. (2003b) for recovery adjustments. The validity of the established recoveries should be regularly controlled (every 20 sample) by analysis of one out of two certified reference materials (e.g. CRM 720 and CRM 721, see Jonsson et al. (2003b) for details). In the present protocol, alkylated OH-PAHs are not adjusted for recovery loss.

3.2.4 GC-MS analysis

Trimethylsilyl ethers of non-alkylated OH-PAHs (TMS-OH-PAHs) in fish bile samples are analysed by an appropriate GC-MS system. In the present protocol, the system consists of a HP5890 series II Gas chromatograph, Finnigan A200S autosampler and a Finnigan MAT SSQ7000 mass spectrometer (Thermo Finnigan, Huddinge, Sweden). The system is controlled by a DEC station 5000. Helium is used as carrier gas and the applied column was CP-Sil 8 CB-MS, 50 m x 0.25 mm and 0.25 µm film-thickness (Instrument Teknikk A.S., Oslo, Norway). Samples and calibration standards (1 µl) are injected on a split/splitless injector with splitless mode on for one minute. The

temperatures for the injector, transfer-line and ion source are held at 250°C, 300°C and 240°C, respectively, and the GC oven temperature programme is as follows: 80°C to 120°C at 15°C min-1, 120°C to 300°C at 6°C min-1 and held at 300°C for 30 min. Mass spectra are obtained at 70 eV in selected ion mode (SIM), and the ions selected for the determination of TMS-OH-PAHs are M+, [M-15]+ and [M-29]+, *m/z* 245.1 for TPA and *m/z* 308.9 and 323.9 for TMS-2,6-dibromophenol. These masses are selected on the basis of preliminary analysis in full scan mode in order to identify the most abundant ions.

The retention times (RT) of alkylated TMS-O-PAHs are generally unknown. But based on the fragmentation pattern of non-alkylated TMS-O-PAHs (Jonsson et al. 2003b) and studies performed by Krahn et al. (1992) and Yu et al. (1995), it is still feasible to select a set of molecular ions for determination of the alkylated TMS-O-PAHs. Delineation of the RT windows for integration of metabolites of alkylated PAHs can be obtained by analysis of appropriate reference samples, such as bile extracts from fish exposed to a North Sea crude oil as illustrated below (Figure 5).

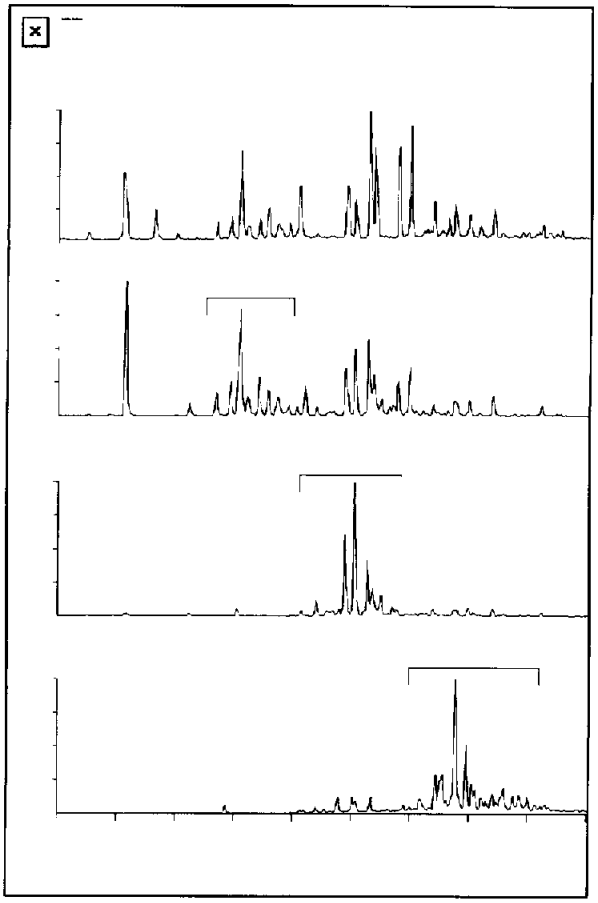


Figure 5: GC-MS determination of deconjugated metabolites from naphthalene and its C_1-C_3 alkylated homologues in bile from fish exposed to crude oil. Delineation of the RT windows for integration of metabolites of alkylated are indicated by brackets on the ion chromatograms.

4 Feasibility study: GC-MS detection of alkylphenols and AP metabolites in bile of marine fish

4.1 Exposure of cod to alkylphenols in laboratory

4.1.1 Exposure agents and fish exposure

For fish exposure, a set of alkylphenol single compounds were selected based on their reported high concentrations in PW. The APs were: para-cresol, ortho-cresol, 3,5-dimethylphenol, 2,4,6-trimethylphenol, para-tert-butylphenol and 4-tert-butyl-2-methylphenol (Figure 6). In addition a mixture of alkylphenols which was obtained (Chiron AS) with a composition designed to be representative for a "typical" AP profile in PW (mix composition shown in Table 1). Small amounts of each single APs and the AP mixture were weighed in glassware and subsequently added a minimum (a few droplets) of acetone until fully solved. A certain volume of codliver oil was added to reach a wanted concentration of the exposure solution (Table 2). The glass was capped, mixed well and then left open overnight at roomtemperature to evaporate the acetone.

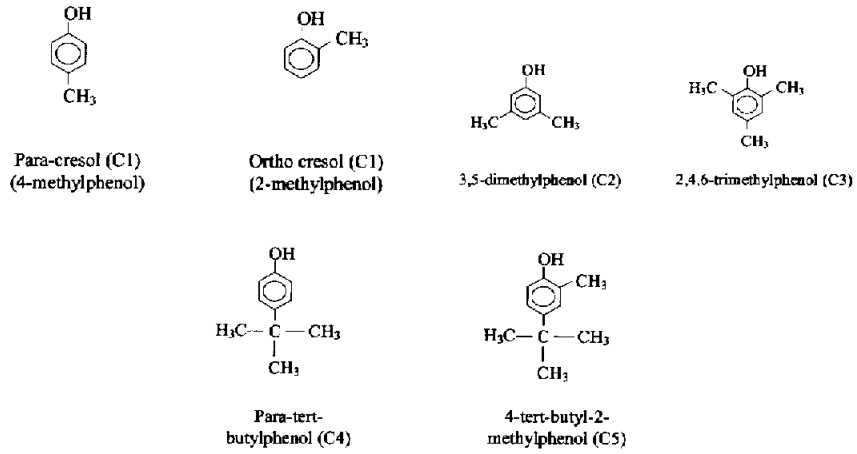


Figure 6: Molecular structure of alkylphenols used for fish exposure.

Table 1: Composition of the alkylphenol mix delivered by Chiron AS. Concentration of alkylphenols in acetone.

	CAS no	mg alkylphenol pr liter acetone	gram alkylphenol pr 6.3 liter acetone
p-cresol	106-44-5	33800	212.94
m-Ethylphenol	620-17-7	6540	41.20
3,5 dimethylphenol	108-68-9	6540	41.20
2,4,6-Trimethylphenol	527-60-6	3800	23.94
2-(1,1-dimethyl)ethylphenol	88-18-6	373	2.35
3-(1,1-dimethyl)ethylphenol	585-34-2	373	2.35
4-butylphenol	1638-22-8	373	2.35
4-penthylphenol	14938-35-3	1620	10.21

Table 2: Concentration of the various AP exposure solutions and the injected doses used for the subcutaneous/inter-muscular injection exposure of cod.

	Carrier concentration	Injected dose in fish
4-methylphenol (para-cresol)	10 mg/ml	10 mg/kg
2-methylphenol (ortho-cresol)	10 mg/ml	10 mg/kg
3,5 dimethylphenol	10 mg/ml	10 mg/kg
2,4,6 trimethylphenol	10 mg/ml	10 mg/kg
para-tert-butylphenol	1 mg/ml	1 mg/kg
4-tert-butyl-2-methylphenol	10 mg/ml	10 mg/kg
AP mixture	10 mg/ml	10 mg/kg

Juvenile Atlantic cod (25-30 g) was obtained from Sagafjord Sea Farm (Leirvik, Stord) and transported to the facility at Akvamiljø (Stavanger). The water temperature in fish tanks during acclimation were 13°C (12-14°C). The fish were fed with cod pellets during the acclimation period. In the first exposure experiment, randomly split groups of minimum 8 individuals were exposed by means of inter-muscular injection to para-cresol and to para-tert-butylphenol as single compound exposures and to a mixture of alkylphenols. Each fish was exposed by means of a single intermuscular injection (Figure 7). A reference group was exposed to vehicle only. The fish were sampled after four days. The fish were not fed during the last five days of acclimation and the exposure period in order to ensure a maximum volume of bile for the sampling.

The cod were exposed in two separate exposure experiments. The first in December 2002 with para-cresol, para-tert-butylphenol and the AP mixture; and the second in May 2003 with ortho-cresol, 3,5-dimethylphenol, 2,4,6-trimethylphenol, and 4-tert-butyl-2-methylphenol (Table 2). In each of the two rounds control fish were injected with codliver oil only. Each fish received 1 ml cod-liver oil / kg fish (also control fish) by means of one single subcutaneous/inter-axial-muscular injection (Figure 7).

During sampling the fish was first stunned by a head blow and the external biological data were recorded. Subsequently, blood was obtained from the caudal vein by means of a heparinised syringe. The blood samples were held cold on ice and plasma fractions were subsequently prepared with a centrifugation at 855*g* x 4 min (at 4°C). The plasma samples were then frozen and stored at -80°C until analysis. The liver was excised and weighed for measurement of organ-somatic index and then frozen and stored at -80°C until analysis. The gall bladder was carefully excised and the bile was evacuated into a cryovial and subsequently frozen and stored at -80°C until analysis.

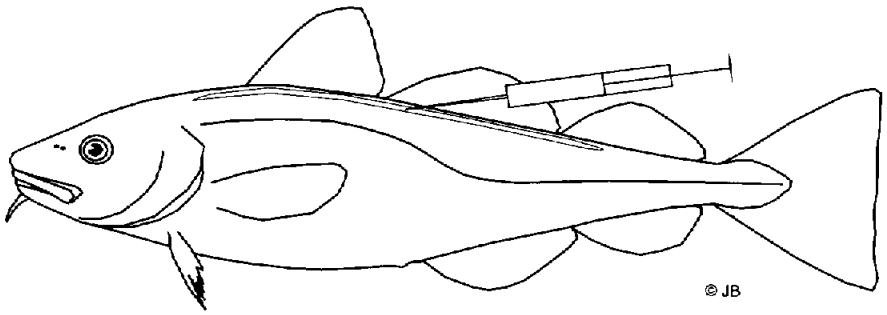


Figure 7: The fish were exposed by means of injecting AP compounds into the area between the two major axial muscles. The exposure agents were first solved in acetone and subsequently transferred to the cod-liver oil vehicle. The vehicle injection volume was always 1 ml/kg fish.

4.2 GC-MS method for AP analysis

From the experience in studies reported by Pedersen and Hill (2000) and Ferreira Leach and Hill (2001), it is expected that the majority of alkyl phenol residues in bile samples will be in the form of various glucuronide conjugated metabolites. These authors described bile metabolites of 4-tert-octylphenol, whereas the present study focus on six specific produced water related alkylphenols (2-methylphenol, 4-methylphenol, 3,5-dimethylphenol, 2,4,6-trimethylphenol, 4-tertbutylphenol, 4-tertbutyl-2-methylphenol). Preparation of bile samples will include enzymatic hydrolysis (deglucuronation) of conjugates and TMS (trimethylsilyl) derivatisation. The extraction and derivatisation procedure will based on the methods described for analyses of PAH metablites in bile by Krahn et al. (1987), with modifications as described by Jonsson et al. (2003b). Prior to establishment of an analytical method there is a need for thorough investigation of the different steps within the sample preparation procedure as well as the GC-MS protocol. The present study report the results obtained during the initial steps toward the development of a GC-MS method for determination of metabolites of alkylated phenols in fish bile.

4.2.1 Mass spectrometry detection of alkylated phenols

Six alkylated phenols (APs) were prepared in ethylacetate as single compound solutions. The concentrations were ranging from 0.6 - 1.7 µg/g. Trimethylsilyl derivatives (TMS-APs) were thereafter prepared by mixing 200 µl bis(trimethylsilyl)trifluoroacetamide, BSTFA, (to 500 µl of the AP solutions (0.4 – 1.2 µg/g). The most abundant ions for APs and TMS-APs were determined by GC-MS analysis in full scan mode (Table 3).

Table 3: The most abundant ions of derivatised and non-derivatised alkylated phenols

	g/mol	m/z (% relative intensities)		
2-methylphenol	108.1	108.1 (100)	107.1 (84)	79.1 (55)
4-methylphenol	108.1	107.1 (100)	108.1 (94)	77.1 (34)
3,5 dimethylphenol	122.1	107.1 (100)	122.1 (90)	77.1 (26)
2,4,6 trimethylphenol	136.1	121.1 (100)	136.1 (83)	135.1 (24)
4-tert-butylphenol	150.1	135.1 (100)	107.1 (35)	150.1 (10)
4-tert-butyl-2-methylphenol	164.2	149.1 (100)	121.1 (38)	162.4 (26)
TMS-derivatives				
2-methylphenol	180.1	165.1 (100)	180.1 (80)	135.1 (47)
4-methylphenol	180.1	165.1 (100)	180.1 (50)	73.1 (22)
3,5 dimethylphenol	194.1	179.1 (100)	194.1 (58)	105.1 (26)
2,4,6 trimethylphenol	208.2	208.2 (100)	193.1 (84)	119.1 (67)
4-tert-butylphenol	222.2	207.2 (100)	73.1 (74)	222.2 (16)
4-tert-butyl-2-methylphenol	236.2	221.2 (100)	236.2 (16)	73.1 (14)

4.2.2 Chromatographic separation of alkylated phenols

GC-MS separation was achieved for a mixture of six alkylated phenols dissolved in ethyl acetate (Figure 8). The chromatographic peaks were right-side tailed and we therefore decided to derivatise the alkylated phenols in order to improve the peak shapes. Derivatives were prepared and the resulting chromatogram revealed sharper and more symmetrical peaks (Figure 9).

4.2.3 Instrumental limits of detection (LODs)

Instrumental limits of detection (LODs) were determined for both derivatised and non-derivatised alkylated phenols (Table 4). Chromatograms for derivatised (Figure 9) and non-derivatised (Figure 8) alkylated phenols are shown for concentrations close to the LODs. The right-side tailing of non-derivatised alkylated phenols was pronounced when analysing low concentrations and is resulting in poor detection limits for free alkylphenols as compared to their TMS-derivatives (10 to 100 times decreased).

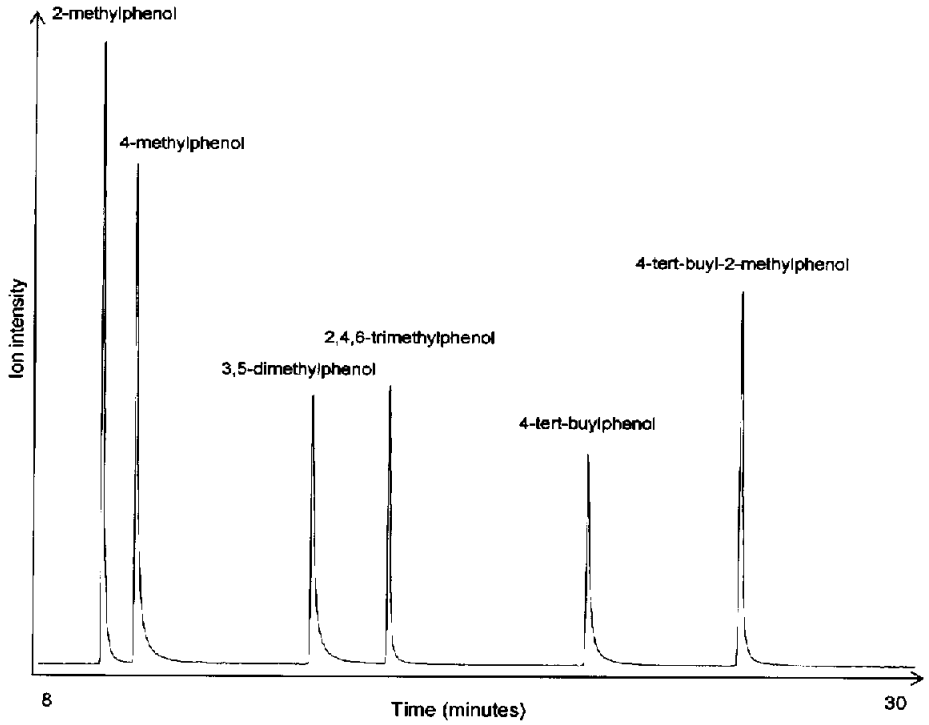


Figure 8: GC-MS SIM of six alkylated phenols. Ions determined: m/z 107.1, 108.1, 121.1, 122.1, 135.1 and 136.1

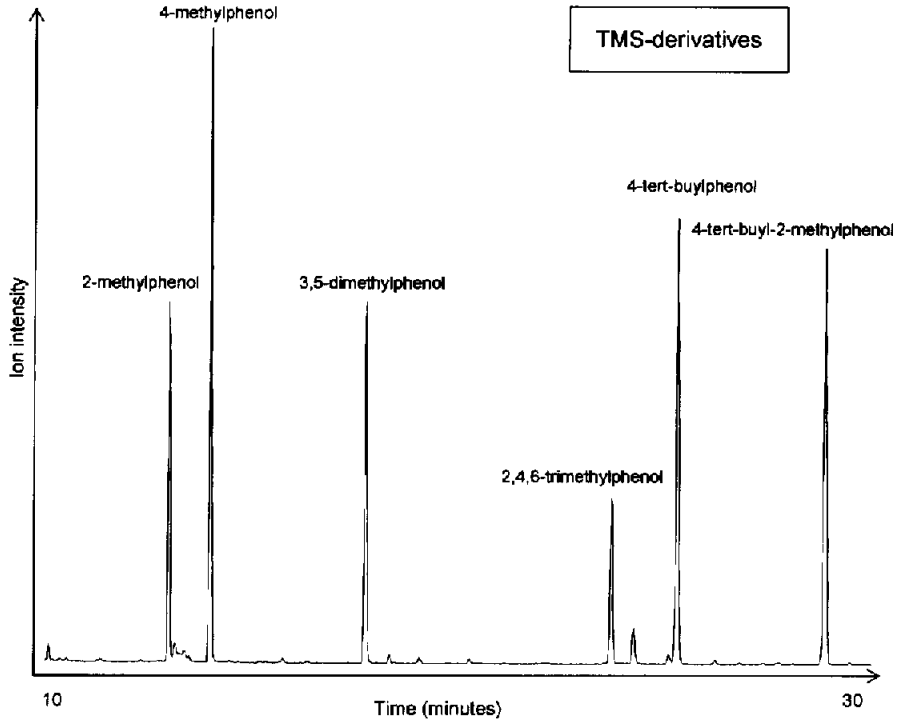


Figure 9: GC-MS SIM of TMS-derivatives of six alkylated phenols. Ions determined: m/z 165.1, 179.1, 207.1, 208.1 and 221.1

Table 4: Limits of detection for derivatised and non-derivatised alkylated phenols

	pg injected
2-methylphenol	15
4-methylphenol	40
3,5 dimethylphenol	10
2,4,6 trimethylphenol	18
4-tert-butylphenol	30
4-tert-butyl-2-methylphenol	15
TMS-derivatives	
2-methylphenol	0.4
4-methylphenol	0.4
3,5 dimethylphenol	0.3
2,4,6 trimethylphenol	1.3
4-tert-butylphenol	1.2
4-tert-butyl-2-methylphenol	0.3

4.2.4 Derivatisation method

Two derivatisation reagents, BSTFA (bis(trimethylsilyl)trifluoroacetamide) and TMSI (N-trimethylsilylimidazole in pyridine, 21 % W/V), were compared in order to achieve an optimal derivatisation. The amount of reagent and time needed to complete deravatisation were determined as well.

The efficiency of the derivatisation was measured as the peak areas of individual compounds when analysed by GC-MS in SIM mode. BSTFA resulted in a more successful derivatisation of the six alkylated as compared to TMSI. The yield increased slightly with amount of derivatisation reagents (25 – 400 µl BSTFA) and the amount of TMS derivative was stable after two hours reaction time at 60°C (Figure 10).

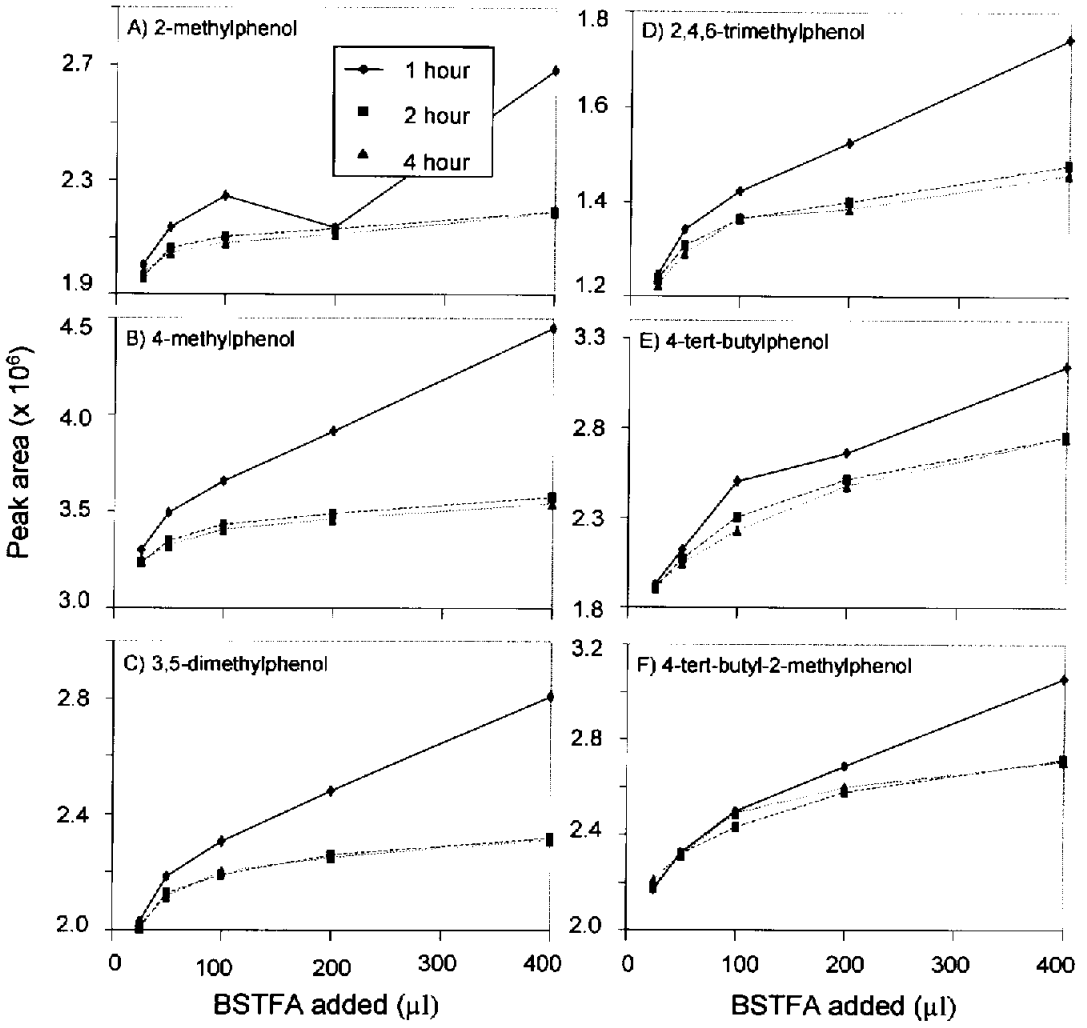


Figure 10: Optimization of TMS derivatisation. Five different amounts of BSTFA (25, 50, 100, 200 and 400 µl) were added to a mixture of six alkylated phenols (A-F). The reaction took place at 60°C for 1, 2 and 4 hours. Amount of derivatised alkylphenol was measured as peak area after GC-MS analysis in SIM mode.

The peak shapes were symmetrically and the noise level low (Figure 11A) by addition of 200 or 100 µl TMSI to 100 and 200 µl sample solution, respectively (final concentration of alkylated phenols were equal). However, peak areas for TMS derivatives of alkylated phenols decreased significantly by increasing the volume of TMSI stepwise from 10 to 200 µl. Figure 11B illustrate the skewed peak shape obtained when the amount of TMSI was decreased to 50 µl. Furthermore, a decrease of TMSI volume to 20 or 10 µl resulted in double or multiple shaped peaks (Figure 11C).

The yield, measured as peak area, was higher for all alkylated phenols by use of BSTFA, independent of reagent volume. In addition, no disturbance of the peak shape was observed; although an increased volume of BSTFA resulted in a more pronounced noise level. Conclusively, BSTFA was considered to be a better derivatising reagent than TMSI for alkylated phenols dissolved in ethyl acetate.

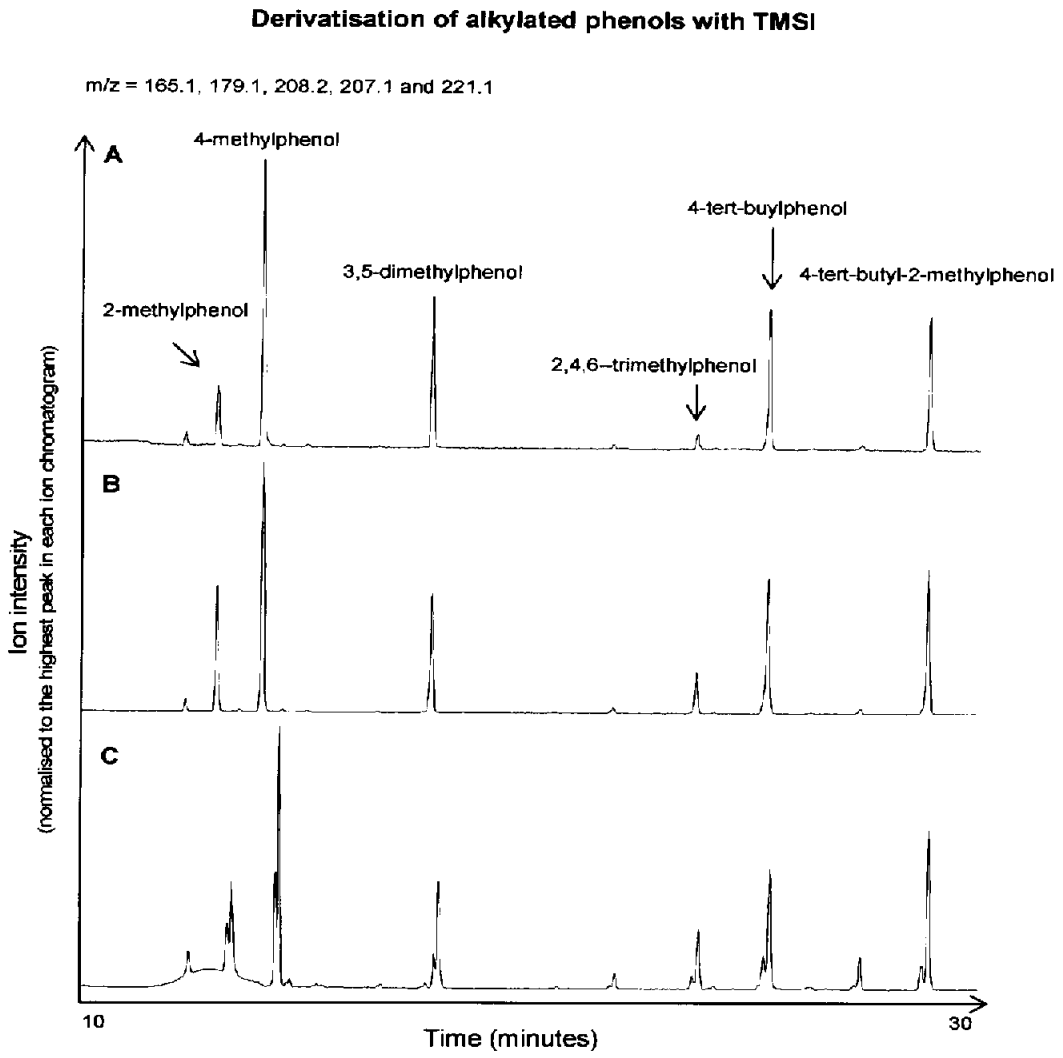


Figure 11: GC-MS analysis following derivatisation of six alkylated phenols with A) 100 µl, B) 50 µl and C) 20 µl of TMSI reagent. The concentration of individual compounds range between 297 – 430 ng/g in A, B and C.

4.3 GC-MS detection of AP compounds in bile

The results obtained in the initial analytical studies have been a necessary step toward the development of a method for analysis of alkylphenol metabolites in fish bile. We have so far established a GC-MS method and we have determined the instrumental detection limits for derivatised and non-derivatised alkylated phenols. Separation performance and detection limits were improved for derivatives and BSTFA was a more efficient reagent as compared to TMSI.

Bile samples from fish exposed to individual alkylphenols and field exposed fish from Tampen area will be analysed within the PROOF funded NFR 153898-720 project (2003-2005).

5 Discussion

Neither PAHs nor alkylphenols are persistent in fish. The metabolic system (preferentially in the liver) rapidly transforms these compounds mostly into more water soluble glucuronide conjugates that are easier to excrete. The main excretion route is through the gallbladder bile which is emptied into the intestine during feeding. Thus, before being emptied the bile contains a metabolite pattern of the nonpersistent lipophilic pollutants recently metabolised in the fish body. This phenomenon has been utilised in the development of biliary PAH metabolites as early-warning markers of PAH exposure in fish, and this exposure marker is presently being recommended for environmental monitoring by international bodies such as ICES and OSPARCOM (OSPARCOM 1998). A development of an assay for AP compounds and AP metabolites in the bile fluid will further strengthen the potential of using fish bile screening for evaluating environmental contamination situations in aquatic environments.

Three methodological approaches can be utilised for measuring PAH metabolites in fish bile. The easiest and by far the most straightforward approach is simple fluorescence screening of diluted bile (non-hydrolysed or hydrolysed) by using fixed fluorescence (FF) detection or synchronous fluorescence scanning (SFS) (Ariese et al. 1993; Aas 2000). These assays offer semi-quantitative measures of certain fluorescent PAH metabolites in bile from PAH exposed fish and should be used as first step screening assays to discriminate exposed and non-exposed specimens and situations. These screening markers are not described in details in the present report. The second approach for biliary PAH metabolite measurement is HPLC-FD, which normally is the recommended analytical procedure for quantitative determination of fluorescent PAH metabolites. However, several studies in our lab clearly indicate the limitations of HPLC for the analysis of metabolites of naphthalene and other small PAH species that typically occur in PW (Jonsson et al. 2003b). The main problem is the low separation efficiency normally seen for these compounds on HPLC columns. The third methodological approach, the GC-MS SIM, have in our studies been found to be the optimal quantitative assay of these petrogenic PAH species (Jonsson et al. 2003b). Experience and analytical results also from other projects in our group, including a recently finished EU project on PAH metabolites in fish bile (Ariese et al. 2001) and the ongoing field project BECPELAG in the North Sea which is organised by ICES (Aas et al. 2003), support this observation. The results collected in the various studies indicate the GC-MS method to be both more sensitive and have significantly better separation and detection power for the smaller PAH species than the HPLC-FD method which presently is recommended by OSPARCOM. The GC-MS SIM method described in the present report should therefore be considered as the method of choice for quantitative determination of PAH metabolites in fish bile in connection to environmental monitoring of oil and produced water contamination of marine waters.

Exposure studies with radiolabelled alkylphenols both in our group (Sundt and Baussant 2003) as well as elsewhere, e.g. (Lewis and Lech 1996; Coldham et al. 1998; Ferreira Leach and Hill 2001), shows that AP contaminants after being taken up by fish preferentially are excreted through the bile pathway. Consequently, the bile fluid may

be used also for the determination of the AP exposure of the fish, or more exactly, to estimate the flux of APs passing through the fish body. Our above described experience with GC-MS detection of low molecular weight PAH compounds, such as naphthalene metabolites has indicated a potency of using this analytical approach also for the biliary AP metabolites. Support to this assumption is given by the initial method development results presented in this report. Due to the present lack of well-functional and sensitive methods for AP quatitation in biotic tissues, the development of a GC-MS based analysis of biliary AP metabolites would be a significant step forward in the making of an early warning marker for AP exposure in fish. This development is an ambiguous task. Our ultimate aim is a fully operative method optimised for typical produced water APs and the results in this initial study have so far been encouraging. We have established an operative GC-MS method for a PW-relevant set of AP standards. We have determined the instrumental detection limits for non-derivatised as well as for derivatised forms of these selected APs. Our results so far also demonstrate that both separation performance and detection limits were significantly improved for AP derivatives. Interestingly, bis(trimethylsilyl)trifluoroacetamide (BSTFA) was found to be a more efficient derivatising reagent as compared to N-trimethylsilylimidazole (TMSI), indicating BSTFA to be the reagent of choice for the further method development process.

The last objective in the present pilot project was to utilise a new GC-MS protocol for determination of AP metabolites in bile samples from the fish exposed to the selected PW-relevant alkylphenols in the laboratory and bile samples from fish collected at PW contaminated offshore locations. However, we consider the method not yet to be sufficiently developed for this analytical task. The method development process will now continue within the frame of the PROOF funded NFR 153898-720 project (2003-2005), in which a one-year post-doctoral study is fully dedicated for this task. The analyses of the bile materials produced in the present project will be conducted in this follow up project.

6 Summary and conclusions

Fish inhabiting waters downcurrent large operational discharges of produced water in the North Sea are potentially at risk for chronic low-dose contamination of PAHs and alkylphenols. There is a need for better analytical tools for monitoring this exposure. Gall bladder bile obtained from recipient fish may potentially serve as a target sample for assessments of both PAH and AP exposure in such situations. The present project report provides a GC-MS SIM protocol which is demonstrated as the best method for quantitative determination of petrogenic PAH metabolites in fish bile. Secondly, the present project includes a pilot study on the feasibility of using GC-MS SIM detection of alkylphenols in fish bile as a monitoring tool of PW related AP exposure in fish. The method development results included in this report indicate a clear potential of the GC-MS with respect to this task, but further development of the assay is necessary before the method is operative. The testing and further development of the assay continues in the follow up project which also is funded by PROOF.

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