HAVFORSKNINGSINSTITUTTET
INSTITUTE OF MARINE RESEARCH

Norges forskningsråd
Postboks 2700 St. Hanshaugen
0131 Oslo

Deres ref.: 152231/720   Vår ref.: 02/474-8/812-jk   Bergen, 28.10.2003
Your ref.:   Our ref.:

Sluttrapport for NFR-prosjekt nr. 152231/720: "Contamination of fish in the North Sea by the offshore oil and gas industry".

Vedlagt oversendes sluttrapport for overnevnte forskningsprosjekt. Vedlegg 1 gir et populærvitenskapelig sammendrag, og Vedlegg 2 er en foreløpig oppsummering av arbeidet som er gjennomført. Samlet databearbeiding og publisering pågår og en komplett teknisk rapport med alle data vil være klar i løpet av november 2003. Deretter vil det bli laget 2-3 manuskripter for vitenskapelig publisering av forskningsresultatene.

Prosjektgjennomføring og ressursbruk er gjennomført etter de opprinnelige planer for prosjektet. En rekke medarbeidere og institusjoner har hatt ansvar i gjennomføringen av de ulike delaktivitetene:
Havforskningsinstituttet: Jarle Klungsøyr (prosjektleder), Sonnich Meier, Guri Nesje
NIVA: Ketil Hylland, Knut Erik Tollefsen, Lasse Berglind, Alfhild Kringstad
NIFES: Marc Berntssen
RF-Akvamiljø: Jonny Beyer, Grete Jonsson
SINTEF: Alf. G. Melbye, Henrik Rye
Universitetet i Stockholm (ITM): Lennart Balk

Samarbeidet med Norges forskningsråd har vært godt og vi vil rette stor takk til NFR for den betydelige økonomiske støtten de har gitt til prosjektet.

Med hilsen

Ole Arve Misund
Forskningsdirektør

Jarle Klungsøyr
Prosjektleder

Prosjektnr.: 152231/720
Saksbehandler: Ian Gjertz, miljø og utvikling
Budsjettår: 2003
Prosjektperiode: 1.6.2002 - 1.9.2003
Program/aktivitet: Langtidsvirkn. av utslipp til
Prosjekttype: Prosjektstøtte
Fagkode: 475 - Zoofysiologi

Sluttrapport
Returneres ved prosjektslutt til Norges forskningsråd sammen med eventuelle vedlegg

Vennligst kontroller at alle forhåndsutfylte opplysninger stemmer. Ved feil og nye opplysninger krysser man av i korrekt boks og skriver inn den oppdaterte informasjonen enten på egne ark eller ved å fylle ut sluttrapportskjemaet som ligger på Forskningsrådets hjemmeside på Internett. Dette delvis forhåndsutfylte skjemaet skal altså returneres til Forskningsrådet sammen med eventuelle vedlegg.

A Grunnlagsopplysninger

Dersom navn på prosjektmedarbeidere (dvs. faglige medarbeidere på prosjektet som helt eller delvis er finansiert av Forskningsrådet) ikke framgår av grunnlagsopplysningene nedenfor, fylles opplysningene ut slik:

- Prosjektmedarbeidere: **Navn, stilling, arbeidssted, finansieringsandel fra Forskningsrådet.** Oppgi i tillegg om den ansatte er registrert som doktorgradsstudent.

Det er ingen endringer i grunnlagsopplysningene ❑ (kryss av)
Endringer og nye opplysninger er lagt ved på eget ark ❑ (kryss av)

Prosjektopplysninger:

Prosjektansvarlig
institusjon: Havforskningsinstituttet, Senter for marint miljø
Adm.ansvarlig: Forskningsdirektør Ole Arve Misund
Prosjektleder
(faglig ansvarlig): Forsker Jarle Klungsøyr
Prosjektmedarbeider(e):
Veileder:
Prosjekttittel: Contamination of fish in the North Sea by the offshore oil and gas industry

Finansieringsplan:

	2002	2003
Bevilgning/tilsagn fra Forskningsrådet:	1,500,000	1,000,000
Egne midler		
Andre offentlige midler		
Andre private midler		
EU-midler		

Følgende mål er avtalt for prosjektet:

Undersøke om kjemiske komponenter i operasjonelle utslipp fra oljeinstallasjoner bioakkumuleres og gir effekter på fisk, og om fiskens kvalitet som menneskeføde påvirkes negativt.

Delmål:
- Kartlegge nivåene av alkylfenoler og NPD/PAH i fisk fra Tampen- og Sleipnerregionen.
· Måle gallemetabolitter av NPD/PAH og bestemme sammenhengen med konsentrasjonene i lever/muskel.
· Måle ulike biomarkører for å bestemme biologiske effekter.
· Måle om kvaliteten på fiskemuskel forringes ved eksponering til oljekomponenter.
- Modellere konsentrasjonsfeltene av alkylfenoler og NPD/PAH i utslippsområdene, og koble dette til forurensningsgraden i fisk.

Vennligst adresser post til Norges forskningsråd, ikke til enkeltpersoner.

B Prosjektsammendrag

Prosjektsammendraget skal kunne brukes i prosjektkataloger/-registre og som grunnlag for annen informasjon fra Forskningsrådet. Nytt prosjektsammendrag utarbeides ved endringer i prosjektets mål/delmål, som medfører at tidligere prosjektsammendrag ikke kan benyttes.

Mindre endringer kan rettes direkte i teksten under. Dersom det utarbeides nytt prosjektsammendrag krysser man av i korrekt boks nedenfor og skriver inn det oppdaterte sammendraget som forklart øverst på side 1.

Sammendraget skal inneholde informasjon om følgende elementer: **Bakgrunn for prosjektet, problemstilling og betydning av forskningen.** Sammendraget skal være på maksimalt 200 ord.

Tittel og prosjektsammendrag på engelsk utarbeides ved første gangs framdriftsrapportering. Samme retningslinjer for innhold og evt endringer gjelder for engelsk som for norsk prosjektsammendrag.

Prosjektsammendrag, norsk

Nåværende prosjektsammendrag for prosjektet:

Prosjektet skal gi dokumentasjon på om nivåene av aromatiske hydrokarboner (NPD/PAH) og alkylfenoler er forhøyet i fisk fra havområder med store operasjonelle utslipp fra offshore olje- og gassindustri. Arbeidet vil gi informasjon om eksponering og belastningsgrad på fisk fra Tampen- og Sleipner-regionen, og vurdere dette opp mot ulike tilførselskilder og den generelle bakgrunnsbelastning av forurensning på fisk i Nordsjøen. Det skal gjennomføres målinger av biologiske effekter og faktorer som påvirker fiskens kvalitet som menneskeføde. Arbeidet skal kunne bidra til utforming av fremtidig overvåkning på forekomst og virkninger på fisk av oljeindustriens utslipp til sjø.

Det er ingen endringer i prosjektsammendraget   ☒ (kryss av)
Nytt prosjektsammendrag er lagt ved på eget ark   ☐ (kryss av)

Prosjektsammendrag, engelsk

Engelsk prosjekttittel: Contamination of fish in the North Sea by the offshore oil and gas industry

Det er ikke registrert engelsk prosjektsammendrag for dette prosjektet

Det er ingen endringer i engelsk tittel/prosjektsammendrag   ☒ (kryss av)
Nytt prosjektsammendrag (+ tittel) er lagt ved på eget ark   ☐ (kryss av)

C Faglig rapport

Den faglige rapporten skal skrives inn på egne ark merket "Faglig rapport" som returneres sammen med dette skjemaet, se veiledning øverst på side 1. Merk at det spørres etter periodiserte opplysninger i tiden fra siste framdriftsrapportering. Merk også at tabellene under punkt 2 og 3 SKAL fylles ut på skjemaet.

1 Oppnådde faglige resultater

Beskriv prosjektets oppnådde resultater i forhold til hovedmål, delmål og milepæler som er fastsatt i kontrakt og arbeidsplaner. Beskrivelsen skal også inneholde en samlet konklusjon/egen vurdering av prosjektgjennomføring og ressursbruk. Videre ønskes en vurdering av forholdet til Forskningsrådet i prosjektperioden. Se Vedlegg 2

Gi i tillegg en kort **populærvitenskapelig framstilling** av de viktigste FoU-resultatene (dvs nye funn, nye problemstillinger, ny kunnskap) som er oppnådd i prosjektperioden, og gi en vurdering av resultatenes nyhetsverdi. Framstillingen vil blant annet bli benyttet som underlag for Forskningsrådets årsrapportering til departementene, eksempelsamlinger på internett mv. Se Vedlegg 1

2 Vitenskapelige utgivelser og annen publisering

Gjør rede for vitenskapelige utgivelser og annen publisering (som er akseptert) fra prosjektet for hele prosjektperioden i en **publikasjonsliste** inndelt etter publikasjonstypene i tabellen nedenfor. De som har mottatt eget publikasjonsskjema fra NSD må fylle ut dette i tillegg. Følgende opplysninger bes oppgitt i listen:

For bøker/artikler i bøker/rapporter: *Forfatter(e), arbeidets tittel, tittel på bok/artikkelsamling, forlag/utgiver, redaktør, flerbindsverk/serie, sidenr., nr./bind/år, ISSN/ISBN, sted*
For artikler: *Forfatter(e), arbeidets tittel, tidsskrift/avis, sidenr., nr./vol./år, ISSN*
For foredrag, presentasjoner og lignende: *Forfatter(e), tittel, arrangement/dato/sted*

Oppgi antall utgivelser etter publiseringstype i tabellen nedenfor.

Publikasjonstyper:	Antall siden forrige rapp.	Antall hele prosjekt-perioden	Antall planlagt etter prosj.slutt
Artikler i vitenskapelige tidsskrifter med referee		(3)	3
Artikler i andre vitenskapelige tidsskrifter og antologier			
Bøker (monografier, lærebøker, antologier (red.))			
Publiserte foredrag fra internasjonale faglige møter/kongresser			
Andre rapporter, samt foredrag og presentasjoner fra vitenskapelige/faglige møter		(1)	1

3 Annen forskningsformidling

Gjør rede for andre formidlingstiltak enn publiseringsvirksomhet (dvs. deltakelse i vitenskapelige og allmennrettede/brukerrettede konferanser og møter, høringer, utstillinger og lignende) for hele prosjektperioden i en *liste* som skal inneholde følgende opplysninger:

For deltakelse i arrangementer: *Arrangement, arrangør og arrangementsdato*
Innslag om prosjektet i massemedia: *Mediets navn, type innslag og dato*

Oppgi antall formidlingstiltak etter tiltakstypene i tabellen nedenfor.

Andre forskningsformidlingstiltak:	Antall siden forrige rapport	Antall hele prosjekt-perioden	Antall planlagt etter prosj.slutt
Allmennrettede formidlingstiltak (populærvitenskapelige artikler/høringer/utstillinger)			
Brukerrettede formidlingstiltak (møter/seminarer i dep., næringsliv, organisasjoner)			2-3
Oppslag vedrørende prosjektet i massemedia			1-2

4 Andre resultater

Gi en kort beskrivelse av andre resultater i prosjektperioden som:
- Veiledning, kurs, undervisning
- Forskernettverk
- Brukernytte, patenter
- Annet

5 Prosjektmedarbeidere finansiert av Forskningsrådet

5.1 Doktorgrads- og postdoktorstipendiaters virksomhet
Gi opplysninger om *avbrudd, permisjoner og fratredelser.* Oppgi også tidspunkt for disputas for doktorgradsstipendiater.

5.2 Utenlandsopphold

Oppgi utenlandsopphold av mer enn tre måneders varighet siden forrige rapportering. Angi *navn på prosjektmedarbeideren, perioden, utenlandsk institusjon og land.* Det skal i tillegg redegjøres særskilt for utbyttet av oppholdet.

Prosjektleder (faglig ansvarlig):

For prosjektansvarlig institusjon (adm. ansvarlig):

Sted: .Bergen............. Dato: .28.10.03.........

Underskrift: ....

Forsker Jarle Klungsøyr

Sted: ....Bergen............Dato: 28. 10. 03........

Underskrift: ....

Havforskningsinstituttet
Senter for marint miljø
Forskningsdirektør Ole Arve Misund

NFR-prosjekt 152231/720: Contamination of fish in the North Sea by the offshore oil and gas industry.

Populærvitenskapelig fremstilling.

Offshore olje- og gassindustri medfører utslipp og belasting på miljøet. Det har vært reist spørsmål om utslippene kan forurense de viktige fiskebestandene som lever i områder med mange olje- og gassinstallasjoner. Det ble derfor høsten 2002 gjennomført en innsamling av ulike fiskearter fra Tampen- og Sleipner-regionen for å undersøke forurensningsgraden i fisk fra disse områdene sammenlignet med fisk fra et referanseområde ved Egersundbanken. Målet med undersøkelsen var å få dokumentasjon på om fisk fra Nordsjøen inneholdt forhøyete nivåer av forurensning som følge av utslipp fra petroleumsindustrien, og om denne forurensningen medførte biologiske effekter og forringet kvalitet på fisken. Arbeidet ble gjennomført som et samarbeide mellom en rekke institusjoner: Havforskningsinstituttet, NIFES, NIVA, RF-Akvamiljø, SINTEF og Universitetet i Stockholm (ITM).

Resultatene viste at hyse, torsk, sei og sild fra Tampen ikke inneholdt forhøyete konsentrasjonen av aromatiske hydrokarboner sammenlignet med fisk fra Egersundbanken. Et tilsvarende bilde ble sett for hyse og torsk fra Sleipner. På Sleipner ble det ikke oppnådd prøver av torsk og sild. Konsentrasjonene av aromatiske hydrokarboner i fiskelever og galle var på vanlig forekommende bakgrunnsnivå for fisk fra Nordsjøen. Hysemuskel inneholdt også svært lite hydrokarboner som kunne relateres til utslipp fra oljeindustrien, nær målegrensene for analysemetodene som ble anvendt.

Det slippes ut store mengder produsert vann fra olje- og gassproduksjonen på norsk sokkel. Aromatiske hydrokarboner og alkylerte fenoler er naturlig forekommende komponenter i produsert vann, og disse stoffene har flere miljømessig betenkelige egenskaper. Den første stoffgruppen kan blant annet medføre skader på arvestoffet (DNA). Alkylerte fenoler kan medføre endokrine (hormonelle) effekter, med påfølgende virkninger på fiskens reproduksjon. Analyser av alkylerte fenoler i fiskelever ble gjennomført for å undersøke om stoffgruppen kunne spores i fiskelever. Resultatene viste at alkylerte fenoler fra produsert vann ikke var målbare i fisken, noen som betyr at konsentrasjonene av enkeltkomponenter var mindre enn 1-5 ng/g våtvekt. Dette kan bety at eksponeringsgraden til alkylerte fenoler er lav. En annen faktor som kan bidra til å forklare resultatet er at alkylerte fenoler i produsert vann forholdsvis hurtig skilles ut av fisk.

Det ble gjennomført en rekke målinger av biomarkører for ulike biologiske effekter i den samme fisken hvor det også var gjort kjemiske analyser. Målingene av enzymsystemer i fisk, som blant annet er viktige for metabolisme og utskillelse av fremmedstoffer (Cytochrome P4501A, GST, GR), viste små forskjeller mellom fisk fra Tampen og Sleipner sammenlignet med fisk fra referanseområdet. Et tilsvarende bilde ble funnet for plommemasseprotein (vitellogenin) som når det spores i hanfisk er en indikator på at fisken har vært utsatt for stoffer med østrogenlignende virkning.

Eksponering til polysykliske aromatiske hydrokarboner kan medføre dannelse av DNA-addukter. Slike genetiske skader anses som alvorlig siden de blant annet kan medføre kreftutvikling. Hyselever fra Egersundbanken viste et svakt forhøyet nivå av DNA-addukter, på Sleipner var antallet noe høyere, og på Tampen viste hyse et ganske høyt antall DNA-

addukter. Dette gir en klar antydning på at fisken har vært utsatt for forurensning, og det kan anses som sannsynlig at dette skyldes utslippene fra olje- og gassindustrien i området.

Målinger av parametre som indikerer oksydativt stress (lipid peroksydering) antydet også at fisk fra Tampen (torsk, hyse) var påvirket av forurensning. Lipidsammensetningen i muskel var vesentlig forskjellig fra fisk fra referanseområdet. Lipidene (fettet) i mager fiskemuskel består i vesentlig grad av strukturelle fosfolipider knyttet til cellemembranene. Det er i eksperimentelle studier vist at sammensetningen av disse kan forandre seg som følge av eksponering til blant annet alkylerte fenoler og aromatiske hydrokarboner, sannsynligvis som en kompensatorisk mekanisme for å opprettholde fluiditet og permeabilitet.

Konklusjonen fra undersøkelsene er derfor at selv om forurensning fra oljeindusrien ikke kan spores i fisken ved kjemiske analyser av oljekomponenter, så synes det som om fisk fra områdene med petroleumsindustri har vært eksponert for forurensning som medfører biologiske effekter.

NFR project No. 152231/720 – Contamination of fish in the North Sea by the offshore oil and gas industry.

Summary report to NFR.

Jarle Klungsøyr (Havforskningsinstituttet), Lennart Balk (ITM, University of Stockholm), Marc H.G. Berntssen (NIFES), Jonny Beyer (RF-Akvamiljø), Alf G. Melbye (SINTEF), Ketil Hylland (NIVA)

Introduction

This report to the Norwegian Research Council (NFR) gives a summary of the results from project No. 152231/720. The project started September 2002 and was finalized October 2003. The major aim of the project was to study to what extent contaminants from offshore petroleum industry bioaccumulate, cause effects in fish populations and affect food safety and quality. This main objective can be subdivided into:

- Determine the concentrations of aromatic hydrocarbons and alkylated phenols in fish from the Tampen and Sleipner region compared to fish from a reference area.
- Measure the levels of aromatic hydrocarbon metabolites in fish bile.
- Measure different biomarkers to determine biological effects.
- Investigate effects on fish quality.
- Model the concentration fields of aromatic hydrocarbons and alkylated phenols in the installation areas and compare this to levels found in fish.

The work was performed was performed as a joint project between Havforskningsinstituttet, NIVA, NIFES, RF-Akvamiljø, SINTEF and ITM (University of Stockholm). The contribution from each laboratory is indicated below.

Background

Since the late 1960s, the offshore oil and gas industry has become a major economic activity in the North Sea. Major oil developments have been in the northern parts of the North Sea in the United Kingdom and Norwegian sectors. Gas developments have taken place both in the southern and northern parts of the North Sea. In 2001 the total production of oil and gas were 244 million Sm^3 oil equivalents in at the Norwegian continental shelf, and a major part of this production was in the North Sea.

The offshore oil and gas industry result in operational and accidental discharges of contaminants. The most important operational discharges are oil, chemicals and metals. Particularly compounds within the first two groups cause environmental concern. During the 1970s and 1980s oil based drilling mud were extensively used. During that time oil contaminated drill cuttings was an important source of oil entering the sea. Since 1992 it has not been permitted to discharge oil based drilling mud in the Norwegian sector of the North Sea. Presently produced water is the most important source to oil while drilling operations are the most important activity causing discharges of chemicals. Accidental discharges are mostly smaller oil spills.

Produced water is water that follows with oil and gas from the reservoir and which is separated from the oil/gas flow on the platform or production vessel. Produced water consists of formation water, i.e. water that occurs naturally in the geological structure, and water that has been injected into the reservoir in order to maintain pressure within the formation. The

Produced water is cleansed of oil to a maximum content of 40 mg/l, and most of it is then discharged to the sea. In addition to the remaining oil, the produced water contains other substances that naturally occur in the reservoir, such as short chain organic acids, aromatic hydrocarbons, alkylated phenols, metals and traces of chemicals added in the production process.

Sampling (Havforskningsinstituttet)

R/V Michael Sars and R/V G.O. Sars were used for collection of fish and the cruises took place during the time periods 22 august - 4 September 2002 and 6-11 November 2002 respectively. Bottom trawl was used for collection of cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*) and saithe (*Pollachius virens*). Pelagic trawl was used for collection of herring (*Clupea harengus*). Complete samples of 25 (±10 %) individual fish were obtained for all four species at Tampen (close to Statfjord) and Egersundbanken. At Sleipner it was only possible to get complete samples of haddock and herring. The fish were kept alive in big tanks onboard the vessels until sampling took place. After killing the fish with a blow to the head, standard procedures were used for collection and storage of muscle, liver, kidney, blood and bile samples for the later chemical and biochemical analyses. Guidelines prepared by the International Council for Exploration of the Sea (ICES) were used to assure sampling quality. Table 1 gives the sampling stations for fish and the positions of the various platforms are shown in Figure 1.

Table 1. Sampling positions collection of fish. Site numbers and colour code (red – blue) refers to Figure 1.

M.Sars (red):

Site	Date	Position North	Position East
1	23-08-02	61°08'33"	002°10'04"
2	23-08-02	61°02'46"	002°28'29"
3	25-08-02	61°05'16"	002°15'48"
4	25-08-02	61°02'20"	002°20'32"
5	01-09-02	58°22'21"	002°01'13"
6	01-09-02	58°23'02"	001°49'30"
7	01-09-02	58°21'40"	001°42'55"
8	02-09-02	57°42'25"	005°09'36"
9	02-09-02	57°45'19"	005°50'27"
10	02-09-02	57°42'06"	005°09'49"
11	02-09-02	57°42'53"	005°09'46"
12	03-09-02	58°04'13"	004°35'16"

G.O.Sars (blue):

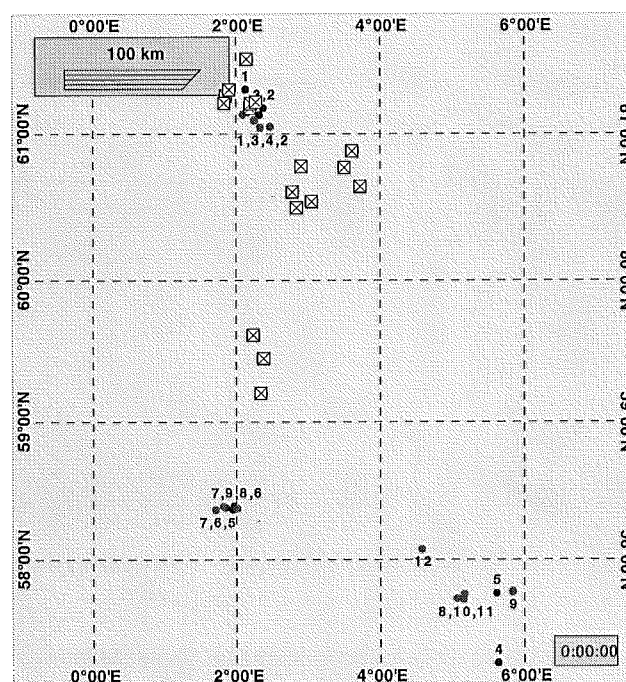| Site	Date	Position North	Position East
1	08-11-02	61°17'32"	002°08'28"
2	08-11-02	61°10'17"	002°22'55"
3	08-11-02	61°07'20"	002°19'39"
4	16-11-02	57°13'37"	005°38'52"
5	18-11-02	57°44'45"	005°37'23"
6	18-11-02	58°21'56"	001°58'34"
7	18-11-02	58°22'41"	001°51'39"
8	19-11-02	58°23'20"	001°58'14"
9	19-11-02	58°21'52"	001°57'05"

Figure 1. Sampling stations (Table 1) and offshore installations included in the simulations (Table 2).

Table 2. Location of release points with release depth and amounts of produced water

Release point	Latitude	Longitude	Depth of release (m)	Produced Water (tons/day)	Data from year*
Tampen area					
Statfjord A	61°15' 35"	1°51' 25"	40	70475	2002
Statfjord B	61°22' 42"	1°49' 87"	40	55029	2002
Statfjord C	61°17' 79"	1°54' 15"	40	79340	2002
Gullfaks A	61°10' 58"	2°11' 36"	18	129440	2002
Gullfaks B	61°12' 19"	2°12' 09"	20	33713	2002
Gullfaks C	61°12' 89"	2°16' 47"	28	111479	2002
Veslefrikk	60°46' 97"	2°53' 87"	6	12329	2002
Troll A	60°38' 75"	3°43' 59"	20	56	2002
Oseberg F	60°30' 00"	2°50' 17"	19	3603	2001
Oseberg C	60°36' 49"	2°46' 56"	14.5	2508	2001
Troll B	60°46' 47"	3°30' 18"	20	19834	2001
Troll C	60°53' 17"	3°36' 72"	12	29499	2001
Snorre TLP	61°26' 95"	2°08' 62"	15	18956	2001
Brage	60°32' 55"	3°02' 84"	1	4922	2001
Sleipner-area:					
Balder	59°12'21"	2°20'31"	5 m	2242	2001
Jotun	59°27'04"	2°22'53"	25 m	8524	2001
Sleipner A	58°22'04"	1°54'52"	15 m	350	2002
Sleipner T	58°22'04"	1°54'52"	5 m	230	2002
Varg	58°04'00"	1°54'00"	25 m	10400	2001
Heimdal	59°37'00"	2°14'00"	40 m	35	2001

* Applies to both volume and composition
Coordinates from NPD, controlled against Hydro and PPCoN data.

3

Modelling of concentration fields (SINTEF)

The DREAM model was used to calculate concentration fields resulting from produced water releases in the Norwegian sector of the Tampen- and Sleipner area. The simulations included organic compounds naturally occurring in the produced water (Table 3), and do not include production chemicals and metals. Data on the volume of produced water and site-specific composition of the produced water from the years 2001 and 2002 were used as input data. Three-dimensional current data for the area from 1994 and corresponding wind data from Gullfaks throughout the same year were used as physical input data. The depth of the release and the amount of produced water for the various platforms are shown in Table 2. We were not able to find data on the releases from the Frigg and Sigyn platforms in the Tampen area and these are thus not included in the simulations

Simulations.

Simulations were performed to simulate the water concentration of produced water components in August and November. All simulations were run for a minimum of two months before the results were taken into account. The reason for this is that the system needs time to equilibrate in order to represent a continuous release. All release points were simulated simultaneously in order to include all interactions between dilution fields of different release points. The simulations were run without suspended particles in the water column. The resolution (grid element size) was approximately 1x 1.5 km.

The results from simulations of water concentrations in august for naphthalene, PAH 1, Phenol 1 and Phenol 2 are shown in Figure 2. The concentration of the groups PAH 2 and Phenol 3 did not exceed 0.001 ppb.

Table 3. Chemical components included in the simulations

Chemical group	Components included
Aliphates	Aliphates C10 – C36
BTEX	Benzene, toluene, ethylbenzene, xylene
Naphthalenes	Naphthalenes (C0 –C4)
PAH 1	Benzo(b)thiophene, C1-benzo(b)thiophenes, C2-benzo(b)thiophenes, C3-benzo(b)thiophenes, C4-benzo(b)thiophenes, C4-naphthalenes, Biphenyl, Acenaphthylene, Acenaphthene, Dibenzofuran, Fluorene, C1-Fluorenes, Phenanthrene, Anthracene, C1-phenanthrenes/anthracenes, Dibenzothiophene, C1-dibenzothiophenes
PAH 2	C2-Fluorenes, C3-Fluorenes, C2-phenanthrenes/anthracenes, C3-phenanthrenes/anthracenes, C4-phenanthrenes/anthracenes C2-dibenzothiophenes, C3-dibenzothiophenes,C4-dibenzothiophenes, Fluoranthene, Pyrene, C1-Fluoranthrenes/pyrenes, C2-Fluoranthenes/pyrenes, C3-Fluoranthenes/pyrenes, Benz(a)anthracene, Chrysene, C1-chrysenes, C2-chrysenes, C3-chrysenes, C4-chrysenes, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(e)pyrene, Benzo(a)pyrene, Perylene, Indeno(1,2,3-c,d)pyrene, Dibenz(a,h)anthracene, Benzo(g,h,i)perylene
Phenol 1	Phenols C0 – C4
Phenol 2	Phenols C5 – C6
Phenol 3	Phenols C7+

A

B

C

D

Concentration (ppb)
- 0.001 - 0.005
- 0.005 - 0.01
- 0.01 - 0.05
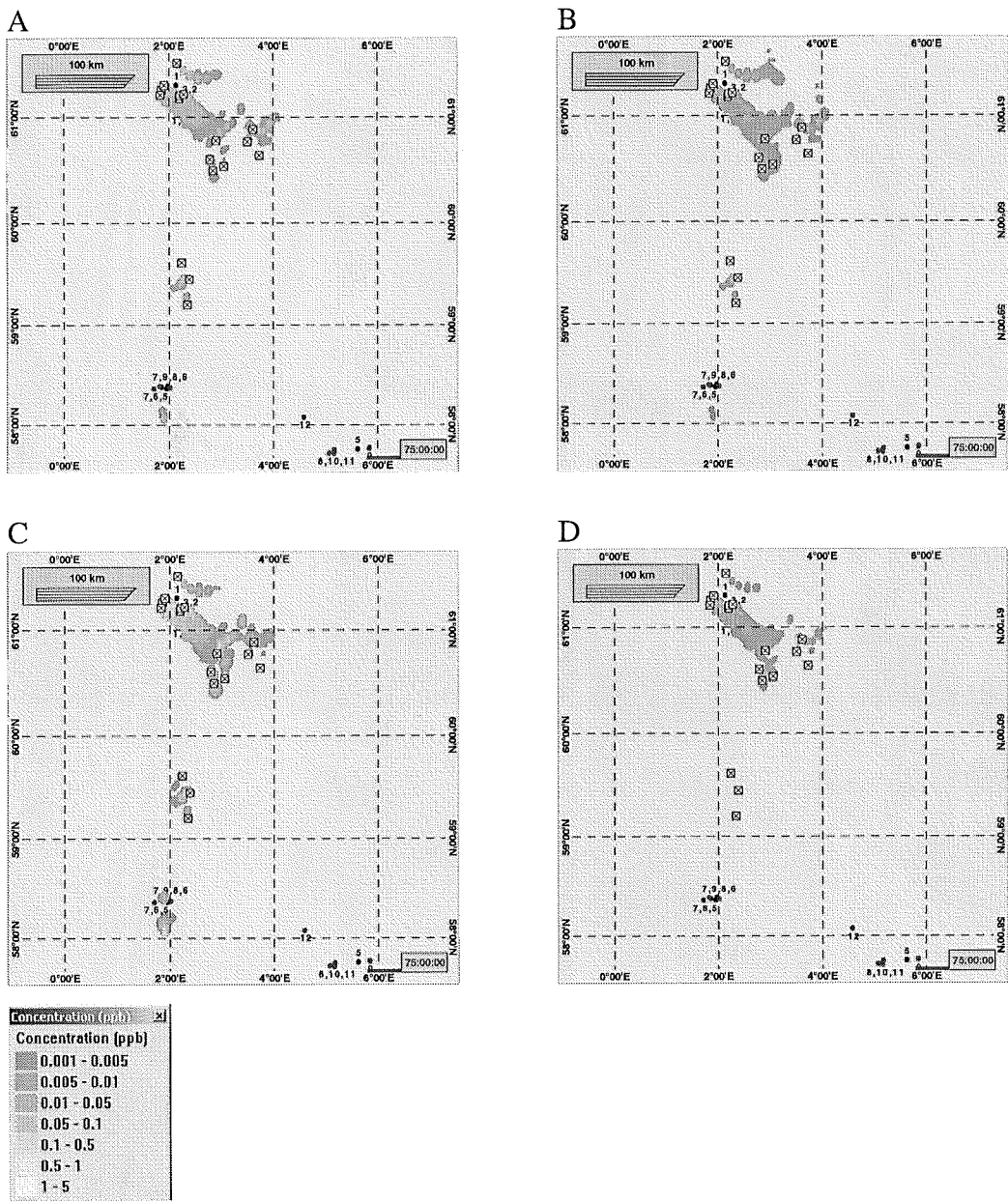- 0.05 - 0.1
- 0.1 - 0.5
- 0.5 - 1
- 1 - 5

Figure 2. Maximum concentration throughout the water column in the middle of august for Naphthalene (A), PAH 1 (B), Phenol 1 (C) and Phenol 2 (D). The concentration of PAH 2 and Phenol 3 was below 0.001 ppb. The colour code used is shown below the maps.

Aromatic hydrocarbons in fish (NIVA, SINTEF)

Introduction

In a study by Aabel *et al.* (1990) it was concluded that fish caught in the vicinity of the Oseberg field contained increased hydrocarbon levels compared to fish from reference sites. Increased hydrocarbon levels in fish from the Statfjord field were reported by Moe *et al.* (1994). In a study on fish collected 1993-1995 it was concluded that the levels of aromatic hydrocarbons in cod, haddock and saithe from oil installation areas was very low and not significant different from reference sites at Egersundbanken and in the Barents Sea (Klungsøyr and Johnsen, 1997). In the same study elevated levels of alkylated decalins were found in some fish from oil installation areas in the northern North Sea. In all the above studies mostly fish liver were analysed. In a resent study on haddock muscle collected from both oil installation areas and reference sites it was concluded that the fish not was contaminated by aromatic hydrocarbons (Klungsøyr *et al.*, 2001).

In the present study di- and polycyclic aromatic hydrocarbons were analysed in liver of cod, haddock, saithe and herring. Muscle samples of haddock were also analysed. The following compounds were included in the analysis:
NPD: Naphthalene, phenanthrene, dibenzothiophene and their C_1-C_3 alkylated homologs.
PAH (EPA list of 16 compounds): Acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, pyrene.

Only results for sums of NPD and PAH are presented in this summary report.

Materials and methods

In the NIVA laboratory the biological matter was homogenised, added internal standards and saponified. The PAHs were extracted with n-pentane and dried over sodium sulphate. The extraction volume was reduced, solvent exchanged to dichloromethane (DCM), and the extracts cleaned by GPC and solvent exchanged to cyclohexane. When necessary, the samples were further cleaned by dimethylsulfoxide (DMF) partitioning and backpartitioning to cyclohexane. The solvent was exchanged to isooctane and transferred to GC-vials for analysis by GC/MS. The MS detector was operated in selected ion monitoring mode (SIM), and the analyte concentrations in the standard solutions were in the range 0,01 to 10 ng/μl. The GC was equipped with a 30 m J&W DB-5MS column (0,25 mm i.d. and 0,25 μm film thickness), and an inlet operated in the splitless mode. The initial column temperature was 80°C, which after two minutes was raised to 160°C at a rate of 20°C/min and thereafter raised to 280°C at a rate of 3°C/min. The injector temperature was 300°C, the detector temperature was 290°C and the column flow rate was 1ml/min. Quantification of individual components was performed by using the internal standard method.

The SINTEF laboratory used an analytical method based on the same principles as the NIVA method. The solvent used (cyclohexane) was tested and approved by evaporation of an aliquot of 200 mL flowed by analysis by GC-FID. All glass equipment used was on beforehand baked at 400 °C (+/- 10°C) in at least 1 hour.

Fish tissue was partly thawed and weighed (approx. 15 grams). Dry weight of the tissue was determined by weighing approx 10 g on an aluminium foil beaker and dried in an oven at 100°C (+/-10°C) over night.

The fish tissue was finely cut and transferred to a round bottom flask and added a mixture of 10 mL 30% KOH in deionized water and 90 mL methanol. The mixture was added surrogate internal standards (SIS) (d_8-naphthalene, d_{10}-biphenyl, d_{10}-phenanthrene and d_{12}-chrysene). The mixture was then boiled on reflux in 4 hours. After cooling, the mixture was filtered through a baked Whatman GF/C- filter and washed using cyclohexane (3x30 mL). The filtrate was transferred to a separating funnel, and added deionized water (50 mL). The cyclohexane phase was collected, and the water phase was extracted twice with 30 mL cyclohexane. The combined extracts were dried over water free sodium sulphate (1 hour), filtered and evaporated using Turbovap and gentle Nitrogen evaporation to approx. 0,5 mL, prior to clean-up on a silica column (Bond Elut). The final extract was added recovery internal standards (RIS: fluorene-d_{10} and acenaphthene-d_{10}) prior to analysis.

Gas chromatography-mass spectrometry (GC/MS) was conducted using an Agilent 6890 Plus GC interfaced to an Agilent 5973 MSD. The GC was equipped with a 60 m x 0.25 mm ID, 0.25 μm film thickness, HP-5MS capillary column. The mass spectrometer was operated in the selected ion monitoring mode (SIM) to increase sensitivity and selectivity relative to full-scan operation. The detection limit of the individual component is < 0.005 μg/L.

Prior to sample analysis, an eight-point calibration curve (0.01 to 10 μg/mL) was established to demonstrate the linear range of the analysis and to determine the mean relative response factor (RRF) for individual compounds. The calibration solution contained the appropriate internal standards and all analysed compounds, except C_3-phenanthrene, C_1-, and C_3-dibenzothiophene. The response factors were generated for all targets and surrogates versus fluorene-d_{10}.

Quantification was performed following the method of internal standards using average RRFs from the initial calibration. Final data was determined (i.e. corrected) versus the appropriate surrogate compound to best represent the field sample compound concentration.

Results and discussion
Figure 3 shows the results for NPD and PAH in haddock muscle. The levels were generally very low and close to the analytical detection limits (LOD). No big differences were found between haddock from Tampen- and Sleipner region compared to the reference area, in fact the mean background levels at Egersundbanken were slightly higher than in fish from the petroleum installation areas.

A similar picture was found for NPD and PAH in haddock liver (Figure 4). Slightly higher concentrations compared to the reference site were detected in a few fish from Sleipner- and Tampen region but all samples contained very low concentrations of NPD and PAH.

For saithe the Concentrations of NPD and PAH in liver were very low and close to the detection limits of the analytical method (Figure 5). Slightly elevated concentrations were found in one fish from the Tampen region.

For cod the concentrations of NPD and PAH in liver were very low (Figure 6). One cod from the Tampen contained concentrations slightly higher than the other cod from the same region.
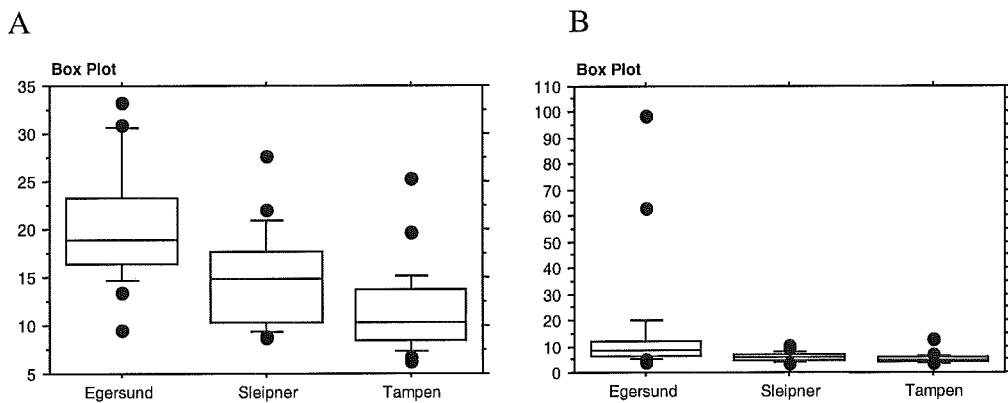
A   B

Figure 3. NPD (A) and PAH (B) in haddock muscle (ng/g ww). The figure shows median, quartiles, and 90/10 percentiles.

A   B
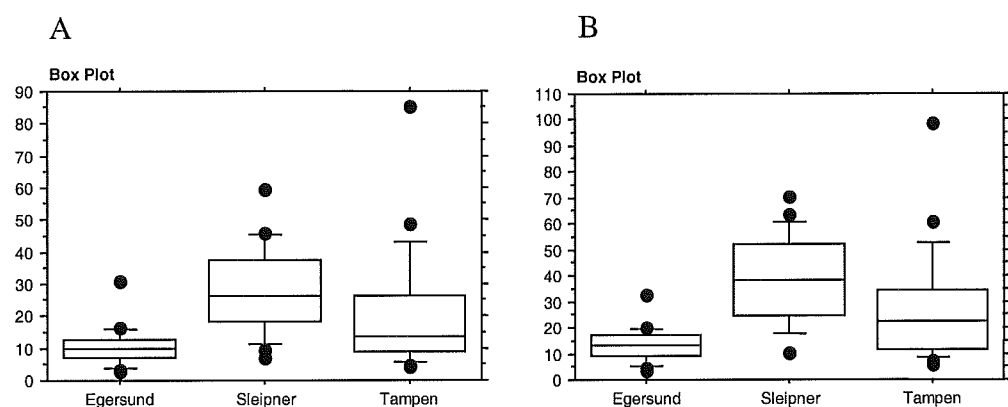

Figure 4. NPD (A) and PAH (B) in haddock liver (ng/g ww).
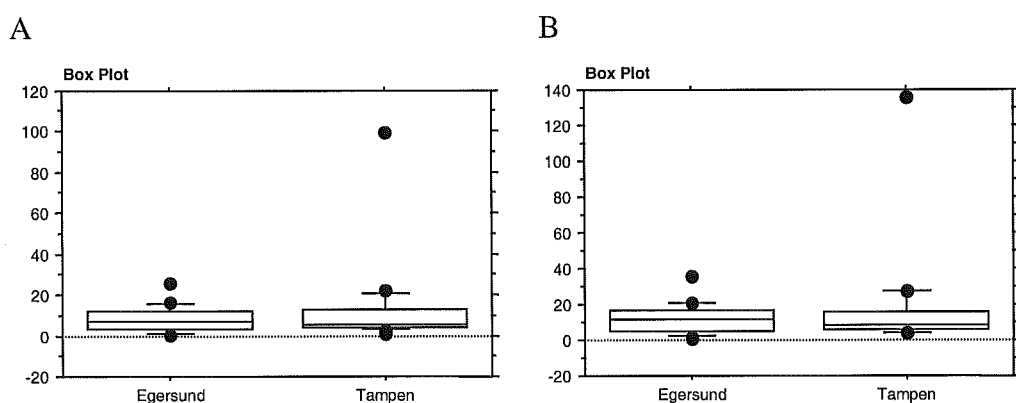
A   B

Figure 5. NPD (A) and PAH (B) in saithe liver (ng/g ww).

A similar result was seen for herring as for the other three species (results not presented), levels of NPD and PAH around detection limit. The preliminary conclusion is that fish from offshore oil installation areas generally contained very low background concentrations of oil hydrocarbons. This can be explained both by low exposure and/or a quite effective metabolic system in fish resulting in rapid excretion of aromatic hydrocarbons.
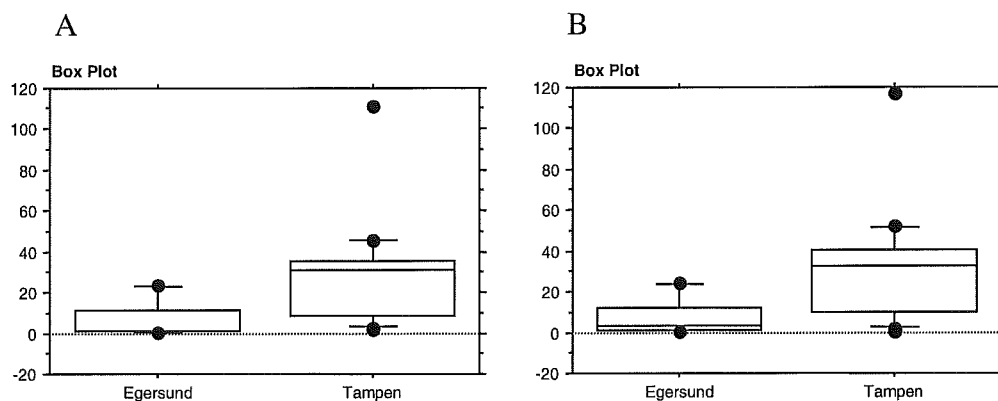
Figure 6. NPD (A) and PAH (B) in cod liver (ng/g ww)

Analysis of alkylated phenols (Havforskningsinstituttet)

Introduction

Alkylated phenols are found as natural components of crude oil. A high relative proportion will be found in the aqueous phase after separation of oil from produced water as a result of their water solubility. Produced water therefore contains low levels of alkylated phenols, typically found at concentrations of 0.6 - 10.0 mg/l. Some 80 % of the total consists of the most water-soluble phenols - phenol and cresols. Of the remaining components, the higher alkylated phenols from butyl- to heptylphenols occur in low concentrations of 2 - 237 µg/l. Estrogenic effects of alkylated phenols are known from a number of *in vitro* and *in vivo* studies. In this study the presence of alkylated phenols present in produced water was studied in fish liver. Degradation products of alkylphenol etoxylates (octyl- and nonylphenols) were also analysed.

Materials and methods

Pure standards (98-99%) of 48 alkylated phenols were from Sigma-Aldrich (Oslo, Norway). Deuterium labelled phenol (d5), p-cresol (d8), 2,4-dimethylphenol (d3), 4n-propylphenol (d12) and 4n-nonylphenol (d5) used as internal standards, were obtained from C/D/N ISOTOPES INC (USA). Pentafluorobenzophenone (recovery standard) and the derivatization reagent pentafluorobenzoyl chloride (PFBCL) were from Sigma-Aldrich. (Oslo, Norway). Pesticide grade solvents (hexane, toluene, dichloromethane, methanol) were from Merck (Oslo, Norway). Standards of pentafluorobenzoyl-derivatives of 4n-propylphenol, 4-isopropyl-3-metylphenol and 4n-nonylphenol were synthesized at the laboratory and purity were tested by GC-FID to be > 99 %. Sodium hydroxide (NaOH), sodium percarbonate (NaHCO₃) and sodium sulphate (Na₂SO₄) were from Merck (Oslo, Norway). The water used was cleaned using NANOPURE Ultrapure Water Systems (USA).

Samples of fish liver or muscle were taken with a scalpel immediately after killing the fish, frozen in liquid nitrogen and stored a t ÷ 80 °C until work up and analysis. Muscle samples were thawed and homogenized in a Waring blender before extraction. Samples of liver with high lipid content (up to 70 % lipid) were weighted directly into the test tubes in frozen condition, to avoid phase separation of lipids and proteins during thawing.

Approximately 1 g of tissue was weight into 25 ml test tubes and spiked with 100 μl of surrogate internal standard (SIS) in methanol, ca. 40 ng of each of the four deuterium labeled compounds. Dichloromethane (DCM, 10 ml) was added to the test tube and the samples homogenized and extracted for 1 min at 20000 rpm using a Sentry Microprocessor (Tempest LQ^2). The homogenization was repeated twice with 10ml of DCM in two new 25 ml test tubes. Water was removed from the extract by adding 1-2 g of Na_2SO_4 (activated over night at 400 °C) to the test tubes. The extracts were filtered through a filter funnel (Duran, porosities 4) into two new 25 ml test tubes. Before GPC the total volume (ca. 30 ml) was reduced by dry N_2 at 40 °C on a Turbovap LV evaporator (Zymark, USA) to 4 ml final volume.

GPC clean up were carried out on a HPLC system consisting of Gilson 232 autoinjector, Gilson 401 dilutor, Gilson 202 fraction collector, Pharmacia LKB 2150 HPLC pump, LKB 2252 LC controller and LKB 2144 fluorescence detector. GPC columns were obtained from Waters (Envirogel GPC cleanup 19 mm x 300 mm), and DCM at 5 ml/min was used as elution solvent. Two columns were coupled together using the Gilson 232 autoinjector as a switch vent. The majority of lipids from the first column are sent to waste before the alkylated phenols with some lipid residues enter the second column after valve switching. By this system a very good cleanup of the samples on the second column is obtained.

The fraction containing the alkylated phenols were collected from 19,4 – 28,4 min into two 25 ml glass tubes (22,5 ml in each glass). Following the GPC, the DCM-extracts were concentrated on a Turbovap LV evaporator to 1 ml and isooctane (1 ml) added as a keeper, and the evaporation continued ca. 0.5 ml to replace the DCM completely with isooctane.

Isooctane extracts of alkylated phenols were adjusted to 1 ml, and 2 ml of 1 M $NaHCO_3$ and 1 ml of 1 M NaOH added. After ½ min. of shaking (Retsch mixer), 2 ml of hexane and 50 μl of pentaflourobenzoyl chloride (10 % solution in toluene) were added and the test tube shaken vigorously for 1 min. The samples rested for ½ hour and were then added 8 ml of 1 M NaOH for hydrolysis of the surplus derivatization agent, and put in the refrigerator over night. The derivatives were extracted with 2 x 2 ml of hexane and prepared in GC vials.
The derivatization step is also functioning as secondary clean-up step by saponification of the lipid traces remaining after the GPC.

The pentafluorobenzoyl derivatives of the alkylated phenols were analysed by gas chromatography – mass spectrometry (GC/MS) in negative chemical ionization mode (NCI) and the instrumental conditions are given in Table 4.

Table 4. Instrumental condition in the GC-MS analysis.

Gas chromatograph	HP 6890 with 5973 MSD.
Column	50 m x 0,2 mm i.d. 0,33 μm film thickness DB5 MS from J & W Scientific (Folsom, CA. USA)
Injection	HP-7673A autosampler. 1 μl, pulsed splitless (50 psi. 2 min.), 250°C
Oven temperature program	40°C (2 min) – 10°C/min.- 110°C (0 min) – 3 °C/min – 250°C (0 min) – 10°C/min – 300°C (10 min)
Carrier gas	Helium, 1,0 ml/min
Interphase temperature	325°C (interphase), 150°C (ion-source), 150°C (quadropole)
Chemical ionization	Methane (40 %, HP default value)

Results and discussion

The results showed that all the samples of haddock, saithe, cod and herring analysed, contained levels of alkylated phenols originating from produced water below limit of detection. For most compounds listed in Table 5 this imply that the concentrations of individual compounds were below 1-2 ng/g ww. Some blank problems were experienced with cresols (C_1-phenols) and technical nonylphenols, resulting in higher detection limits for these compounds (20-50 ng/g ww). Technical nonylphenols are degradation products from alkylphenol etoxylates and are not originating from produced water. It was found necessary to reanalyze several samples of herring liver showing elevated concentrations of technical nonylphenols, and the results will be presented in the final technical report from the project.

Table 5. Alkylated phenols analysed in fish.

Name	Name	Name
Phenol	3-Isopropylphenol	2-tert-Butyl-6-methylphenol
o-Cresol	2,4,6-Trimethylphenol	4-tert-Butyl-2-methylphenol
m-Cresol	4-Isopropylphenol	4-(1,1-Dimethylpropyl)phenol
p-Cresol	3-Ethyl-4-methylphenol	4-n-Pentylphenol
2-Ethylphenol	2,3,6-Trimethylphenol	2,6-Diisopropylphenol
2,6-Dimethylphenol	2,3,5-Trimethylphenol	2,5-Diisopropylphenol
2,5-Dimethylphenol	4-n-Propylphenol	2-tert-Butyl-4-ethylphenol
2,4-Dimethylphenol	2-tert-Butylphenol	4-n-Hexylphenol
3-Ethylphenol	3-tert-Butylphenol	4-(1-Ethyl-1-methylpropyl)-2-methylphenol
3,5-Dimethylphenol	5-Isopropyl-3-methylphenol	4-n-Heptylphenol
4-Ethylphenol	4-tert-Butylphenol	4-tert-Octylphenol
2,3-Dimethylphenol	4-sec-Butylphenol	4-n-Octylphenol
3,4-Dimethylphenol	4-Isopropyl-3-methylphenol	2-Methyl-4-tert-octylphenol
2-Isopropylphenol	4-n-Butylphenol	4-n-Nonylphenol
2-n-Propylphenol	2-tert-Butyl-4-methylphenol	Technical nonylphenols (19 peaks)

Detection of PAH metabolites in bile (RF-Akvamiljø).

Introduction

Detection of biliary PAH metabolites in fish serve as an early warning parameter that indicates a recent or ongoing PAH exposure which subsequently may lead too more adverse effects such as DNA lesions (e.g. 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 OSPARCOM (1998).

In this project, PAH metabolites in bile were analysed in fish (cod, saithe and haddock) collected at three North Sea field locations in 2002 (Tampen, Sleipner and Egersundbanken). The overall aim was to assess whether a PAH exposure signal in the collected fish could be detected; special emphasis was put on metabolites of typically crude oil related PAHs (naphthalenes and phenanthrenes) in order to decide the contribution from produced water discharges at the Tampen and Sleipner fields. In addition, representative metabolites of four ring PAH (1-OH-pyrene) and five ring PAHs (3-OH-benzo(a)pyrene) were analysed.

Materials and methods

Chemicals

1-hydroxy-naphthalene (1-OH-NPH) and 2-Hydroxy-naphthalene (2-OH-NPH) were 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 b-glucuronidase with 5 % sulphatase activity (Type HP-2) were bought from Sigma-Aldrich (Steinheim, Germany). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 2,6-dibromophenol (2,6-DBP) were obtained from Varian (Morton Grove, IL, USA) and Avocado (Heysham, England), respectively. Sodiumacetate, anhydrous sodiumsulphate (pesticide grade) and ethylacetate (pesticide grade) were provided by Merck (Darmstadt, Germany).

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

Sampling of fish and bile

Bile samples were obtained from the collected fish specimens directly after the trawl haul. The fish were prior to sampling kept alive on board the ship by means of a seawater tank. After sampling the bile samples were immediately frozen and stored at liquid nitrogen. At return to laboratory the bile samples were transferred to a -80°C freezer and stored until analysis.

Sample preparation

Gall bladder bile from individual fish was prepared for analysis as described by (Jonsson et al. 2003). Briefly, 20–30 µl of bile was 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) were added and the solution left at 40°C for 2 hours. The OH-PAHs were extracted with ethylacetate (4 times 0.5 ml), the combined extract dried with anhydrous sodium sulphate and concentrated to 0.5 ml. Trimethylsilyl (TMS) ethers of OH-PAHs were prepared by addition of 0.2 ml BSTFA and heating for two hours at 60°C. TPA was added as a GC-MS performance standard before transferring the prepared samples to capped vials. 2,6-DBP was used as internal standard for quantification, but could not fully adjust for recovery loss. Adjustment was done by established recoveries from former work (Jonsson et al. 2003). The validity of the established recoveries was regularly controlled (every 20 sample) by analysis of one out of two certified reference material (CRM 720 and CRM 721). Alkylated OH-PAHs were not adjusted for recovery loss.

GC-MS analysis

Trimethylsilyl ethers of non-alkylated OH-PAHs (TMS-OH-PAHs) in fish bile samples were analysed by a GC-MS system consisting of a HP5890 series II Gas chromatograph, Finnigan A200S autosampler and a Finnigan MAT SSQ7000 mass spectrometer (Thermo Finnigan, Huddinge, Sweden). The system was controlled by a DEC station 5000. Helium was 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) were injected on a split/splitless injector with splitless mode on for one minute. The temperatures for the injector, transfer-line and ion source were held at 250°C, 300°C and 240°C, respectively, and the GC oven temperature programme was 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 were

obtained at 70 eV in selected ion mode (SIM), and the ions selected for the determination of TMS-OH-PAHs were 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 were 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 were unknown. Based on the fragmentation pattern of non-alkylated TMS-O-PAHs (Jonsson et al. 2003) and studies performed by Krahn et al. (1992) and Yu et al. (1995), the molecular ions were selected for determination of the alkylated TMS-O-PAHs. Delineation of the RT windows for integration of metabolites of alkylated PAHs was obtained by analysis of bile extract from fish exposed to a North Sea crude oil.

Results and discussion

The concentrations of PAH metabolites detected in the bile samples from cod, haddock and saithe collected at Tampen, Sleipner and Egersund fields in 2002 are shown below (Table 6). The result overview shows metabolites of naphthalenes, phenanthrenes and pyrene, whereas the levels of benzo[a]pyrene metabolites were generally below the detection limit and are not shown in the table. Generally, the results are presented by the calculated mean (with standard deviation) of each species at each site and show generally that the fish collected at the two study sites Statfjord and Sleipner did not have any significant increase of petrogenic PAH metabolite signals as compared to the reference fish that were collected at the Egersundbanken.

As judged from earlier experience with PAH metabolite detection in fish bile the various classes of aromatic metabolites displays some variability, i.e. some compounds exists in higher concentrations than others in field collected fish also in non-polluted fish. An overview of typical background levels of the various metabolite classes is provided below (Table 7). Most importantly, the alkylated forms of smaller PAHs are normally found in higher concentrations in the bile as compared to the concentration of the non-alkylated isomers. Overall, the results provided in Table 6 illustrates most of all a situation where the detected PAH metabolites at all three sites and in all three fish species analysed fluctuate at and sometimes just above the typical background level. Recent experience from the BECPELAG survey at Tampen showed that cod that were caged in the plume downcurrent an offshore installation with produced water discharges displayed a moderate increase of the biliary PAH metabolite level after five weeks of caging (Aas et al. 2003). In the same BECPELAG study, however, it was also found that feral saithe that were collected at the same locations as were used for caging did not show the same signals. In the feral saithe, the levels were more or less the same at all sites, as also is the overall case in the present study. In other words, fish that moves freely around oil installations will be exposed if and whenever they enter the produced water (PW) plume. Apparently, this happens rather rarely and a significantly increase of the contamination level in the regional population of fish is therefore not seen.

From the present results and supported by the data from the BECPELAG study, it therefore seems clear that the regional PAH contamination of feral fish around PW discharging offshore installations is rather limited, except for the water mass within the plume. This is most valid for pelagic and semi-pelagic fish species such as saithe and cod. With respect to haddock, we know that this species has a feeding habit that brings it much closer in contact to the sediment than the cod and the saithe. Indeed, the intestine of haddock is most often filled up with

Table 6. PAH metabolites in fish bile from cod, haddock and saithe. All concentrations are given as ng/g bile.

Atlantic cod	Statfjord (n=10)		Sleipner (n=3)		Egersund (n=22)	
Compound (ng/g bile)	Mean	STDEV	Mean	STDEV	Mean	STDEV
1-OH-naphthalene	6.5	2.7	7.6	1.8	5.7	2.3
2-OH-naphthalene	18	11	12	5	24	11
C1-OH-naphthalene	366	54	412	39	1318	551
C2-OH-naphthalene	128	26	146	18	310	115
C3-OH-naphthalene	764	88	719	17	620	143
1-OH-phenanthrene	0	0	0	0	0	0
C1-OH-phenanthrene	83	11	96	18	123	31
C2-OH-phenanthrene	100	19	102	33	139	46
1-OH-pyrene	2.3	3.8	0.0	0.0	3.9	5.4

Haddock	Statfjord (n=21)		Sleipner (n=22)		Egersund (n=23)	
Compound (ng/g bile)	Mean	STDEV	Mean	STDEV	Mean	STDEV
1-OH-naphthalene	6.8	2.4	8.6	3.6	6.6	3.1
2-OH-naphthalene	21	12	14	6	12	6
C1-OH-naphthalene	545	258	371	99	498	205
C2-OH-naphthalene	177	75	147	48	157	44
C3-OH-naphthalene	713	123	721	155	790	105
1-OH-phenanthrene	1.74	4.39	0	0	0	0
C1-OH-phenanthrene	204	147	235	201	290	169
C2-OH-phenanthrene	174	104	142	85	283	158
1-OH-pyrene	3.06	6.97	10.4	12.0	5.71	5.53

Saithe	Statfjord (n=24)		Sleipner		Egersund (n=15)	
Compound (ng/g bile)	Mean	STDEV			Mean	STDEV
1-OH-naphthalene	12.4	3.1			7.8	2.1
2-OH-naphthalene	47	13			31	14
C1-OH-naphthalene	342	97			394	297
C2-OH-naphthalene	242	88			215	103
C3-OH-naphthalene	634	162			726	186
1-OH-phenanthrene	0	0			0	0
C1-OH-phenanthrene	88	24			92	16
C2-OH-phenanthrene	128	67			119	89
1-OH-pyrene	0	0			1.5	3.2

Table 7. Interpretation basis for petrogenic PAH metabolite signals in bile from feral marine fish.

	Noice signal	Trace signal	Quantified
Compound	ng/g bile	ng/g bile	ng/g bile
1-OH-naphthalene	5	<10	>= 10
2-OH-naphthalene	5	<10	>= 10
C1-OH-naphthalene	250 - 300	< 1000	>= 1000
C2-OH-naphthalene	200 - 250	< 1000	>= 1000
C3-OH-naphthalene	400 - 600	< 2000	>= 2000
1-OH-phenanthrene	0	< 10	>= 10
C1-OH-phenanthrene	100-150	< 500	>= 500
C2-OH-phenanthrene	70 - 150	< 500	>= 500
1-OH-pyrene	0	<10	>= 10

sediment material, which has been ingested during the search for small infaunal invertebrates. Larger and more lipophilic PAHs have a higher tendency of adhering to particles that may settle and sediment at the bottom in the area downcurrent the produced water discharge. It could thus be expected that metabolites of four and five rings PAHs such as pyrene and benzo[a]pyrene could come out higher in the haddock than in cod and saithe. As judged from the results (Table 6) this appears to be the case, at least with respect to pyrene. As mentioned earlier, the BaP metabolite levels in the bile samples were too low for secure concentrations to be measured (not shown). It is nevertheless important to stress that the haddock for this reason seem to be a more appropriate species than cod and saithe for assessing the general PAH contamination around oil production installations offshore where produced water is discharged.

The overall conclusion is that the concentrations of typical petrogenic PAH metabolites in bile from feral fish collected at Tampen and Sleipner in 2002 were not significantly increased as compared to the reference fish collected at the Egersundbanken.

Biomarker responses (NIVA)

Introduction
Exposure of environmental contaminants to biological systems will first affect molecular and cellular processes. If adaptation and/or repair mechanisms are overloaded, detrimental effects may accumulate in biological systems. Effects at higher hierarchical levels (e.g. population, community) are always preceded by sublethal changes in organisms, allowing the development of markers for such effects, i.e. biomarkers.

There exist several definitions for a "biomarker". The most commonly used is similar to that suggested by Mayer (1992), in which biomarkers were *"quantifiable biochemical, physiological, or histological measures that relate in a dose- or time-dependent manner to the degree of dysfunction that contaminants have produced"*. These measures are quickly evident, but not readily interpreted at the population level. Measures at higher hierarchical levels will, on the other hand, become evident too late to have a diagnostic or preventive value, and thus not function as the wanted "early warning signal".

In the current project, a range of different biomarkers was used to identify possible effects from estrogenic substances (vitellogenin), PAHs or other planar substances (EROD, GST) and any radical-generating substance (glutathione reductase, GST). Cytochrome P4501A activity (EROD) links exposure to e.g. PAHs with subsequent generation of DNA adducts. Glutathione reductase and glutathione *S*-transferase (GST) links with the generation of lipid peroxides and antioxidant stress.

Materials and methods

Sampling
Fish were sampled on board ship. Following anaesthetisation, length and weight was determined. Blood was drawn from the caudal vein using a heparinised and aprotinised syringe. The body cavity was opened and liver, kidney and bile removed. Slices from the central part of the liver were transferred to vials and frozen in liquid nitrogen. The remainder of the liver was transferred to clean glasses and frozen at -20°C. Liver samples were kept below 80°C during all subsequent transport, handling or storage prior to analyses.

Blood was transferred to a vial and kept on ice until centrifugation. Following centrifugation, plasma was withdrawn and frozen in liquid nitrogen.

The biomarkers analysed were EROD, glutathione S-transferase, glutathione reductase and vitellogenin. Haddock, cod and saithe were selected for biomarker analyses.

EROD

Cytochrome P450 1A catalyses the deethylation of 7-ethoxyresorufin to resorufin. Cytochrome P4501A activity in microsome fractions can be quantified from the amount of resorufin produced (Andersson & Förlin, 1985). Ethoxyresorufin-O-deetylase (EROD) activity was assayed fluorimetrically as described by Burke and Mayer (1974) using an internal NIVA-method adapted for plate-reader.

Glutathione S-transferase

Total glutathione S-transferase (GST) activity was determined in the cytosolic fraction using CDNB as substrate. The method used was that described by Habig et al. (1974) adapted to plate-reader (using a quartz microtiter-plate). Activity was determined kinetically and calculated as mmol/min/mg cytosolic protein.

Glutathione reductase

Glutathione reductase was determined in livers of haddock, cod and saithe using a kinetic assay adapted for plate-reader, a modification of the method described by Cribb et al. (1989) using a quartz microtiter-plate. Activity was determined kinetically as decrease of NADPH in each well compared to blanks and calculated as nmol/min/mg cytosolic protein.

Vitellogenin

Vitellogenin was determined in plasma from haddock, cod and saithe using a competitive ELISA with cod vitellogenin as standard and competing antigen. The protocol used is an adaption of that described by Scott & Hylland (2002).

Results and discussion

EROD, GST, GR and vitellogenin were determined in all sampled individuals from all three areas. In addition, general condition indices were calculated. The fish sampled appeared generally to be comparable. Sizes were similar within each of the species and condition similar for the three species in the three (two) areas. Liver-somatic index did however differ substantially for haddock in the three areas (Figure 7). It is generally thought that a high LSI in an otherwise unstressed environment indicates good nutritional status. In the current study, that would indicate that haddock from the Tampen region, in the vicinity of Statfjord, had fed less well than haddock from the other two areas. It should however be noted that gonad and stomach weight should be detracted for optimal use of this index. These data were not available.
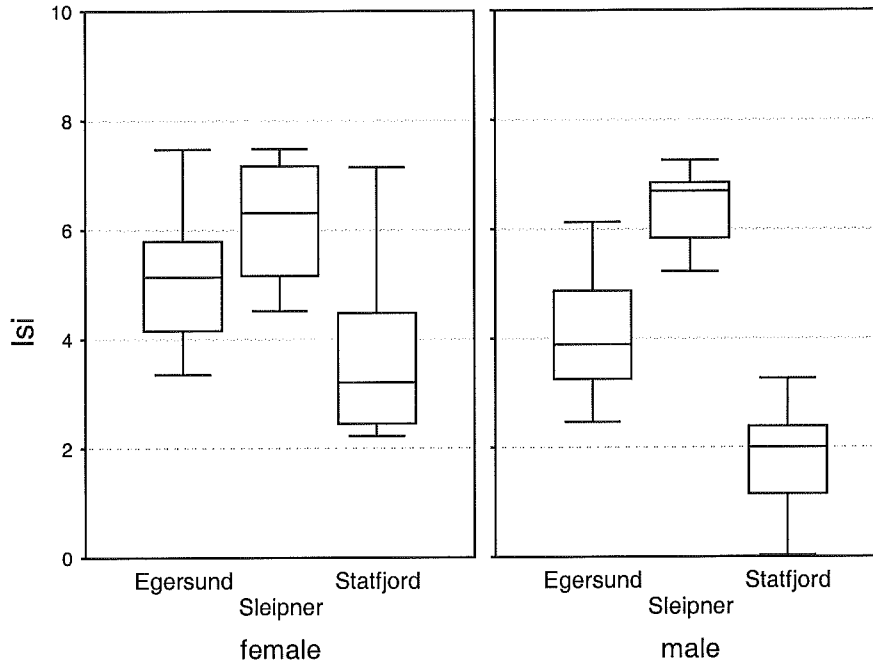
Figure 7. Liver somatic index (lsi) in haddock collected in the indicated areas. The figure indicates median, quartiles and 90/10 percentiles.

There were no large differences between the areas in the levels of hepatic EROD. The only difference was observed for haddock, in which males from Sleipner had somewhat lower activity (Figure 8, Kruskal-Wallis, p=0.02). EROD was similar at the reference site and at Statfjord in this species.
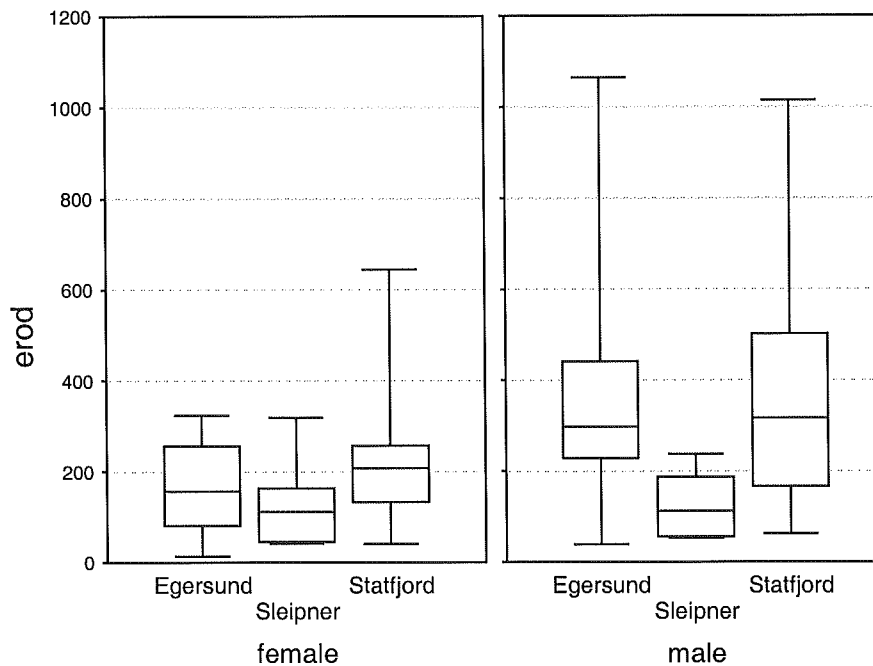

Figure 8. Hepatic EROD in haddock collected in the indicated areas, determined as pmol/min/mg protein. Further information about the figure from the legend to Figure 7.

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For hepatic glutathione S-transferase (GST) there were also minor, although significant, differences between fish collected in the three the areas. Haddock of both sexes collected in the Statfjord area had higher activity than haddock collected in the other two areas (Figure 9, Kruskal-Wallis, p=0,01). The effect was more pronounced in female than male haddock. An increase in GST was seen in caged cod analysed during the BECPELAG workshop (Danischewski et al., submitted). A similar pattern was seen for female cod (less clear for males of that species), but not for saithe.
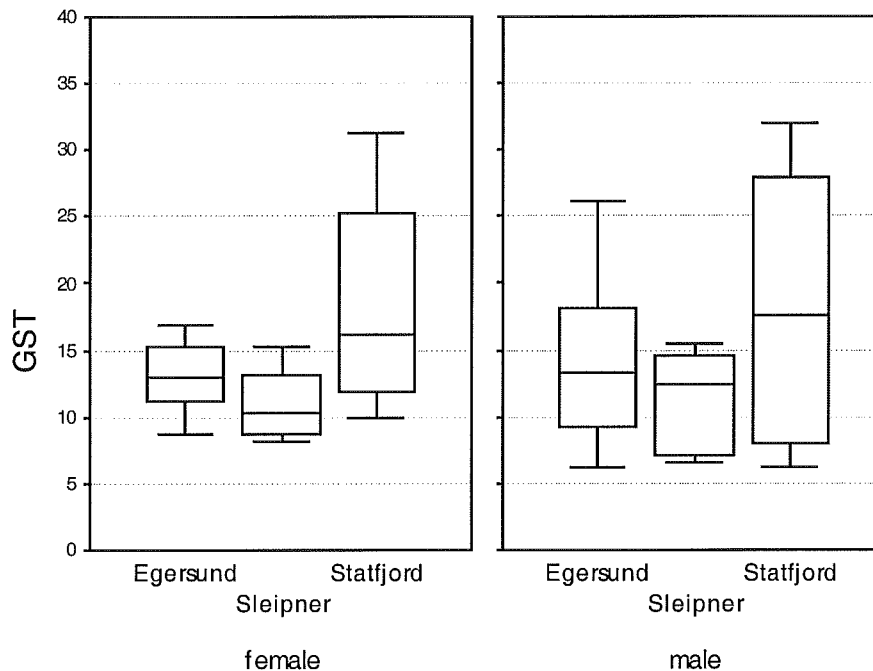
Figure 9. Hepatic GST in haddock collected in the indicated areas (arbitrary units). Further information about the figure from the legend to Figure 7.

Hepatic glutathione reductase (GR) was significantly lower in saithe collected in the Statfjord area compared to saithe from the Egersund bank (Kruskal-Wallis, p=0.03), whereas GR activity was significantly elevated in cod from Statfjord compared to cod from Egersund (Kruskal-Wallis, p=0.01). There were no significant differences between haddock from different areas for this biomarker. There is no clear reason for the observed increase in GR found in cod and decrease found in saithe. Glutathione reductase is an enzyme involved in the regeneration of reduced glutathione and is thus intimately involved in regulating the availability of this chelator and antioxidant.

The differences in vitellogenin-levels found in the three species could mostly be explained from natural processes (maturation status, etc). Vitellogenin appeared to be somewhat increased, although not significantly, in male haddock from Statfjord compared to the other two areas.

In vitro screening of bile metabolites (NIVA).

The objective of this study is to screen fish bile for the presence of chemicals with acute toxic, estrogenic and PAH-like properties using a fish in vitro bioassay. The fish in vitro bioassay is based on liver cells that are isolated from rainbow trout and kept in a primary mono-layer culture (see Tollefsen et al., In press, for details). The monolayer culture of hepatocytes are

exposed to ethylacetate extracts of bile containing potentially toxic chemicals accumulated in the bile as metabolites or mother compounds (For extraction methodology, see Beyer et al., this volume). The endpoints of study include the measurement of acute toxicity (metabolic inhibition and membrane integrity), and induction of the estrogenic biomarker vitellogenin and EROD activitiy as a measure for CYP1A inducing chemicals (PAH, dioxins and other Arylhydrocarbon receptor agonists) in the extracts. The in vitro bioassay is thus measuring both acute and sub-letal endpoints.

The results from the analysis show that the in vitro screening methodology may detect several classes of environmental toxicants. Initial in vitro screening of bile from fish exposed intraperitoneally to beta-naphtoflavone (BNF), a well known inducer of EROD activity and the potent estrogen 17β-estradiol (E2), show that the bile accumulate high levels of both CYP1A inducers (BNF) and estrogenic chemicals (E2). Using the same methodology, screening of bile extracts from Haddock sampled at the Statfjord, Sleipner and Egersund area show that bile from single fish contain chemicals that are estrogenic (measured as vitellogenin induction) and acute toxic (metabolic inhibition, but not membrane disruption) to liver cells in culture. The presence of both estrogenic and acute toxic chemicals in these samples were only found in a few fish, and in samples from all sampling stations, thus suggesting that toxic effects seen in these samples are due to presence of endogenous chemicals rather than exposure to exogenous toxins. In fact, presence of estrogenic chemicals in bile was only found in samples from mature female fish, which suggest that endogenous produced estrogens are responsible for the observed activity. None of the tested samples from the study area was able to induce EROD activity in the cells, thus suggesting that bile from these fish contain low levels of CYP1A inducers like PAH, dioxins and other CYP1A inducers. In summary, in vitro screening of bile from fish sampled at the Statfjord, Sleipner and Egersund area show low toxic activity in general, and there seems to be no clear relationship between potential exposure to acute toxic, estrogenic and CYP1A inducers and the measured toxic activity in the samples.

Fish quality, fatty acid profile and lipid peroxidation (NIFES)
Marine cold-water fish are characterised by high concentrations of polyunsaturated fatty acids, and fish may, therefore be particularly susceptible to toxicant enhanced lipid peroxidative stress. Induction of the endogenous anti-oxidant has been shown in fish caught near pollution sites that seem to be mostly related to organic chemicals such as PAH (Regoli *et al.* 2002). PAH itself can act as antioxidant or pro-oxidant, causing possible lipid peroxidative stress and/or alteration in the fatty acid profile. Research at the Institute of Marine Research has shown that under controlled conditions alkylphenol exposures cause a reduction in polyunsaturated fatty acids as well as increased membrane fluidity. This was explained as a counter mechanism to compensate for accumulation of PAH in membranes causing increased membrane fluidity.

The present study aimed to assess lipid peroxidative stress and fatty acid composition in 3 lean fish species in relation to areas with and without offshore activity.

Materials and methods
TBARS, α–tocopherol, CAT, SeGSH-Px, fatty acid analysis
Lipid peroxidation was measured colorimetrically as thiobarbituric acid reactive substances using a modification of the method of Schmedes and Hölmer (1989). After lipid extraction of 0.3 g frozen (-80°C) tissue sample in 4 ml of a solution containing 66% (v/v) chloroform:

methanol (2:1), 0.005% butylated hydroxytoluene, and 1mM EDTA (in an N_2 atmosphere), 2 ml aliquots of the water extract were added to 2 ml TBA reagent (5% TCA, 1% thiobabituric acid in 0.25 N HCl). The absorbance of the sample was compared to that of a malondialdehyde standard curve at 532 nm with a spectophotometer (Shimadzu, Graplicord, UV 240, Japan). The standard curve was prepared by dissolving 50 μl 1,1,-3,3,-tetraethoxyporpon in 50 ml 0.1 M HCl (0.1 mM malondialdehyde stock solution) which was diluted in TBA reagent to give a 0, 2.5, 5 7.5, 10, and 12.5 mM malondialdehyde standard solution. .

α–Tocopherol was determined using the method from Lie et al. (1994). Frozen (-80 °C) tissue or feed samples (0.1 g) were saponified at 100 °C for 20 min in a pyrogallol, ascorbic acid, and EDTA saturated solution containing 80% (v/v) ethanol and 20 mM KOH. After lipid extraction in hexane, α–tocopherol was separated by normal-phase HPLC and subsequently detected by fluorescence (excitation: 289 nm, emission: 331 nm).

Selenium dependent glutathione peroxidase (SeGSH-Px) activity was measured after Bell et al. (1985), modified for microplate assay, using H_2O_2 as the substrate and sodium azide as a catalase inhibitor. Frozen tissues were homogenised with 9 volumes of ice-cold 50mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.5 mM dithiothreitol and 1% (v/v) Triton-X. The tissue homogenates were centrifuged at 10 000 x g for 30 min at 4 °C. The supernatant was removed and diluted with a 100 mM phosphate buffer, pH 7.4, containing 2mM EDTA before being used for the assay. Twenty μl of diluted homogenate was added to 160 μl of assay mixture (4 replicates per sample). The assay mixture consisted of 1 mM GSH, 0.1 mM NADPH, 0.5 units GSH-reductase, 1 mM EDTA, 2 mM sodium azide and 50 mM phosphate buffer, and pH 7.4. This assay mixture was acclimated for 6 min to 20 °C; the reaction was initiated with 20 μl 2.5 mM H_2O_2 to produce a final concentration of 0.25mM. The rate of NADPH oxidation was measured every 20 sec for 3 minutes at 340 nm. Protein was measured by the method of Lowry et al. (1951).

The fatty acid composition in fillet of the samples was determined according to Lie & Lambertsen (1991). Briefly, lipids were extracted form approximately 0.4 g fillet from 10 randomly chosen fish from each of the three locations by adding chloroform/methanol (2:1) and kept at -20°C over night. The samples were filtered, saponified and methylated using 12% BF_3 in methanol. TAnalyses of the methyl esters fatty acid derivatives were separated performedon a Carlo Erba gas chromatograph using "cold on column" injection, heated at 69°C for 20 seconds, increased by 25°C/min to 160°C for 28 min, increased by 25°C/min to 190°C, held at 190°C for 17 min, finally increased with a 25°C/min to 220°C and then held at 220°C for 9 min. The chromatograph was equipped with a 50-m-CP-Sil88 fused silica capillary column (i.d. 0.32 nm; Chromopack, the Netherlands). The fatty acids were identified against the retention time of a standard mixture of methyl esters (Nu-Chek-Prep. Elysian, MN, US).

Results and discussion
In general, there is indication that lipid peroxidative stress, as seen from increased liver GSH-Px and muscle a-tocopherol, occurs in fish (especially cod and haddock) caught near Statfjord (Tampen), compared to Sleipner (only for haddock) or Egersund area. A clear difference in fatty acid profile was seen in cod and haddock caught near Statfjord compared to Sleipner (only haddock) or Egersund area. PCA analyses showed significant differences in fatty acid

profile in cod, saithe and haddock from Egersund compared to Statfjord. No differences were found in fatty acid profile in saithe from Egersund or Sleipner. No material of cod and haddock from Sleipner was available. The differences in fatty acid composition was explained by differences in n3/n6 ratio, sum long chained polyunsaturated fatty acids, saturated fatty acids, and archidonic fatty acid.

The ratio n3/n6 fatty acid decreased substancially in all fish species caught near Statfjord compared to Egersund. Moreover, the total amount and relative proportion of saturated fatty acids compared to polyunsaturated fatty acids was higher in cod and haddock caught in the Statfjord area compared to the Egersund bank area. Although an overall lower level in polyunsaturated fatty acids was observed, archidonic acid was higher in cod and haddock form Statfjord compared to Egersund. The meaning of the latter is unclear, however it ought to be noted that archidonic acid is a well-known signalling role in regulation of several metabolic processes including lipid metabolism itself.

Muscle lipids of lean cod, saithe and haddock contain mostly only phospholipids, so differences observed in locations might be mostly related to membrane (phosphor) lipids. Several general environmental factors are known to affect the phospholipids profile. Temperature and typical cold water adaptation is known to increase both position as well as level of polyunsaturated fatty acids. Furthermore, diets are the main factor deciding the final composition of lipids in diets. However, feeding experiments with large variations in lipid composition have not be shown to cause the such distinct difference in fatty acid profile e.g. n3/n6 ratio (Lie et al. 1987) as was observed among the different locations. In addition, wild ranging saithe caught around fish farms that only predated on commercial fish feed showed only marginal differences compared to saithe from an area without farming activity (Skog et al. 2002). Temperature differences were little with a tendency to decreased temp at locations with relative higher level of saturated fatty acids.

It is striking that the change in fatty acid profile coincides with the indicators of lipid peroxidative stress. The reduction in vitamin E and increased SeGSH-PX indicate a using of anti-oxidants and onset of lipid radical scavenger enzymes. However, no differences in MDA were observed indicating that no severe propagation of lipid oxidation occurred.

Table 8. Results for cod. Data in the same rows with different superscripts are significantly (p<0.05) from each other (one-way ANOVA, Tukey's HSD, unequal, mean±SD)

	Statfjord	Sleipner	Egersund
liver			
GSH-Px (nmolNADPH min^{-1} mg prot.$^{-1}$	407.42±128.48[a]		217.42±98.84[b]
CAT (μmol H_2O_2 min^{-1} mg prot.$^{-1}$)	114.48±35.44[a]		87.44±34.81[a]
TBARS (nmol MDA g^{-1} ww)	12.02±8.30[a]		16.52±7.92[a]
Muscle			
a-tocopherol (μg g^{-1} ww)	4.15±0.95[a]		5.13±1.69[b]
n3/n6 fatty acids (ratio %)	11.17±2.63[a]		17.88±3.29[b]

Table 9. Results for haddock. Data in the same rows with different superscripts are significantly (p<0.05) from each other (one-way ANOVA, Tukey's HSD, unequal, mean±SD)

	Statfjord	Sleipner	Egersund
liver			
GSH-Px (nmolNADPH min^{-1} mg prot.$^{-1}$	326.7±117.43[a]	270.37±75.19	541.44±109.25[b]
CAT (μmol H_2O_2 min^{-1} mg prot.$^{-1}$)	102.65±20.56[a]	73.78±24.50[a]	172.23±31.94[a]
TBARS (nmol MDA g^{-1} ww)	21.02±19.40[a]	11.26±6.573[a]	12.40±5.33[a]
Muscle			
a-tocopherol (μg g^{-1} ww)	2.84±0.70[a]		6.86±1.56[b]
n3/n6 fatty acids (ratio %)	9.13±1.49[a]	11.78±1.13[b]	18.09±0.98[b]

Table 10. Results for saithe. Data in the same rows with different superscripts are significantly (p<0.05) from each other (one-way ANOVA, Tukey's HSD, unequal, mean±SD)

	Statfjord	Sleipner	Egersund
liver			
GSH-Px (nmolNADPH min^{-1} mg prot.$^{-1}$	707.68±79.37[a]		541.44±109.26[b]
CAT (μmol H_2O_2 min^{-1} mg prot.$^{-1}$)	212.21±42.15[a]		192.22±31.95[a]
TBARS (nmol MDA g^{-1} ww)	11.96±4.63[a]		12.40±5.33[a]
Muscle			
a-tocopherol (μg g^{-1} ww)	8.02±0.54[a]		8.86±1.56[a]
n3/n6 fatty acids (ratio %)	16.40±2.78[a]		18.10±0.99[a]

DNA adduct formation (ITM, Stockholm University)

Introduction

DNA adducts are formed through covalent bonds between a variety of pollutants and the DNA molecule (Miller and Miller 1981). The negative biological significance of such adducts could vary, from direct cell death to the development of cancer or adverse effects in the next generation(s) (Kirkwood 1989, Bridges *et al.* 1990, Würgler and Kramers 1992, Wang *et al.* 1995). Pollutants can bind covalently to the DNA structure directly, as a parent compound, or probably more often after cellular biotransformation as highly reactive metabolites (Varanasi *et al.* 1989a, b). Polycyclic aromatic hydrocarbons (PAH), an ubiquitous and large group of environmental contaminants, are known to cause genetic toxicity through the formation of DNA adducts. Hence, DNA adduct formation has been applied as a biomarker for PAH exposure and genotoxic effects.

The most sensitive method for the detection of DNA adducts is presently the ^{32}P-postlabelling method (Reichert *et al.* 1998). The development of this method started in the early 1980s (Randerath *et al.* 1981, Gupta *et al.* 1982). Since that time a number of significant steps to improve the ^{32}P-postlabelling methodology have been taken. For instance, enrichment of aromatic and hydrophobic adducts by treatment with nuclease P1 (Reddy and Randerath 1986), use of storage phosphorous imaging technique for analysing and evaluation of DNA adducts (Reichert *et al.* 1992) and improved multidirectional thin-layer chromatography separations (Reichert and French 1994).

The present study aimed at investigating DNA adduct levels in a feral fish species, haddock (*Melanogrammus aeglefinus*) in 3 areas in the Northern Atlantic. Two areas were selected on the basis that they constitute possible sedimentation areas of contaminated seston particles released from offshore oil activities, i.e., Statfjord and Sleipner, while one area was selected as a reference area, the Egersund bank.

Materials

Standard DNA (salmon sperm, D-1626), spermidin (S-2626), Rnase A (R-4642), micrococcal endonuclease (N-3755) and spleen phosphodiesterase (P-9041) were obtained from Sigma Chemical Company, St. Louis, MO, USA. RNase T1 (109 193), nuclease P1 (236 225), proteinase K (1000144), α-amylase (102814), T$_4$-polynucleotidekinase (3´-phosphatase free, 838 292) and phenol (1814303) were bought from Roche Diagnostics, Scandinavia AB, Bromma, Sweden. Radiolabelled ATP ([γ-^{32}P]ATP) with specific activity 3000 Ci/mmol (110 TBq/mmol) was obtained from Amersham Biosciences, Uppsala, Sweden. The B[a]P standard adduct, 7R, 8S, 9S-trihydroxy, 10R-(N^2-deoxyguanosyl-3´-phosphate)-7,8,9,10-tetrahydro-B[a]P (B[a]PDE-dG-3´p), was obtained from Midwest Research Institute, Kansas City, MO, USA. Cellulose (MN-301) was bought from Machery-Nagel, Düren, Germany. Vinyl strips (PVC foil, 0.2 mm thickness), used for the groundwork of the polyethyleneimine cellulose sheets were obtained from Andren & Söner, Stockholm, Sweden. Scintillation fluid (Ultima gold) was bought from CIAB, Lidingö, Sweden. B[a]P and pyrene were obtained from Sigma, St Lois, USA

Equipment and apparatus

Purified DNA was quantified on the basis of its absorption at 260 nm by use of microcuvettes in a GeneQuant spectrophotometer supplied by Pharmacia Biotech, Uppsala, Sweden. Liquid scintillation spectroscopy was performed in a Packard Tri-Carb 2100TR liquid scintillation counter from Packard Instrument Company. DNA adduct levels and "fingerprint" patterns were evaluated by the storage phosphor imaging technique using a PhosphorImager™ SI instrument and ImageQuant, 5.0 software, Molecular Dynamics software, Sunnyvale, CA, USA.

DNA purification and ^{32}P-postlabelling analysis of adducts

The liver tissue underwent DNA extraction and purification essentially as described in previously published information (Dunn *et al.*, 1987, Reichert and French 1994, Ericson *et al.* 1998, Ericson and Balk 2000). In brief, liver tissue in 10 mM Tris-Cl, 100 mM etylenediaminetetraacetic acid (EDTA) was gently homogenised, followed by centrifugation to obtain a pellet of the nuclei. The crude nuclei pellet was resuspended and treated with SDS, RNase T1, RNase A and α-amylase, followed by incubation with proteinase K. In order to avoid formation of quinones that could cross link nucleotides and result in elevated background levels (Harvey and Parry 1998), redistilled molecular biology grade of phenol, saturated with Tris-Cl buffer, pH 8, with 0.2% mercaptoethanol (v:v) and 0.1% 8-hydroxyquinoline (w:v) was used for subsequent extraction. DNA was extracted essentially free of cell debris with the chronological extraction with phenol, a mixture of phenol, chloroform and isoamylalcohol (25:24:1, v:v:v), and at last, a mixture of chloroform and isoamylalcohol (24:1, v:v). Thereafter, sodium chloride solution to a final concentration of 500 mM was added, and by gently mixing with cold (-20° C) absolute ethanol, DNA was precipitated. DNA was dissolved in 10 mM Tris-Cl, 1.0 mM EDTA at pH 7.4 and the amount was identified by its UV absorbance at 260 nm, using a GeneQuant spectrophotometer. Hydrolysation of the DNA to 3´-nucleoside monophosphates with micrococcal endonuclease (MN) and spleen phosphodiesterase (SPD) was then accomplished. This is a key step in the

procedure since unbalanced hydrolysation could give rise to a loss of adducts (Perin-Roussel *et al.* 1990), as well as to false positive adducts (Pfau *et al.* 1994). With this background we used 24 mU MN/µg DNA and 3.2 mU SPD/µg DNA, incubated during four hour at 37° C in a 0.1 mM CaCl$_2$, 10 mM succinate buffer at pH 6.0. To avoid later interference with the T$_4$ polynucleotide kinase activity the CaCl$_2$ concentration was 0.1 mM (Hemminki *et al.* 1993). To enrich DNA adducts we used the Nuclease P1 (0.8 µg Nuclease P1/µg DNA added and 45 min additional incubation) methodology since this enzyme degrades the non adducted 3´-nucleoside monophosphates to their nucleosides, while adducted 3´-nucleoside monophosphates originating from polycyclic aromatic hydrocarbon exposure do not undergo such degradation (Reddy and Randerath 1986, Beach and Gupta 1992). Adducts were then radiolabelled by use of 5´-[γ-^{32}P]triphosphate([γ-^{32}P]ATP) and T$_4$ polynucleotide kinase to a ^{32}P in the 5´hydroxy group of the adducted 3´-nucleoside monophosphate. The labelling mixture (10 µL) contains, DNA corresponding to 12.5 µg purified DNA, 8 units of T$_4$ polynucleotide kinase (3´-phosphatase free, 3.1 Mbq [γ-^{32}P]), 14 mM MgCl$_2$, 14 mM dithiothreitol and 3.5 mM spermidine in a 70 mM Tris base with a final pH of 7.6, which was incubated for 30 minutes at a temperature of 37° C. Separation and clean-up of adducts was performed by multidirectional thin-layer chromatography (TLC) on polyethyleneimine cellulose sheets as described previously suitable for adducts formed by polycyclic aromatic hydrocarbons (Reichert and French, 1994). In order to improve resolution and reproducibility of DNA adducts on TLC separations we used laboratory produced polyethyleneimine cellulose sheets prepared according to Reichert and French (1994).

QA procedures

For quality assurance a positive control, consisting of DNA adducted liver tissue, was run in parallel to the investigated samples. This material consisted of liquid nitrogen homogenised liver tissue from perch (*Perca fluviatilis*) exposed for 14 days to 50 mg B[a]P/kg by intraperitoneal injection. Additionally, an chromatography adduct standard, B[a]PDE-dG-3´p, was processed in parallel to the field and laboratory exposure samples. As a negative control, salmon sperm DNA was used to confirm that no false adducts were produced in parallel to the investigated samples. Finally, to ensure that radiolabelled ATP was not limited to the amount of 3´-nucleoside monophosphates present (adducted and remaining non-adducted) in the incubation, an aliquot of the labelled mixture was eluted with 250 mM ammonium sulphate: 20 mM Na$_2$HPO$_4$: 20 mM NaH$_2$PO$_4$ (pH 6.4), on polyethylenimine cellulose sheets, essentially according to Reichert and French, 1994. The presence of an ATP spot indicated that significant amount was still remaining in the incubation in excess.

DNA adducts were calculated using the ratio of adducts to the total number of analysed nucleotides, nmol adducts/mol normal nucleotides. The detection limit of DNA adducts was calculated for each individual sample, from the actual background signal which was selected from a representative area of their respective autoradiogramm. A spot-specific (area/zone) electronic signal, corresponding to 1.5 times the representative background (spot/area/zone) electronic level, on the same autoradiogramm was considered as the limit of detection and limit of quantification of DNA adducts. The data are presented as individual levels for the 7 individuals analysed at each area.

DNA adduct formation

DNA adducts were determined in female haddock from all three areas. The positive (QA) control experiments where liver tissue from perch (processed in parallel to the liver samples from haddock), exposed via intraperitoneally injected B[a]P, demonstrated that all steps in the ^{32}P-postlabelling methodology was well functioning during the analytical protocol, as

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expected results were obtained. Labelling and TLC migration of the standard DNA adduct, B[a]PDE-dG-3′p, on the PEI-cellulose sheets showed the normal labelling frequency as well as the expected migration (R_f values) and spot shape on the sheets. TLC separations confirmed that radiolabelled ATP was not a limiting factor in our incubations. Pure salmon sperm DNA did not show any spots, indicative of false negative adducts (results not shown). Based on this, it is highly unlikely that the adduct levels observed in the liver of the field collected haddock in these studies were due to any kind of artefact.

The ^{32}P-postlabelling methodology has been applied for fish in several field studies as a biomarker for genotoxic exposure in recent years, and found to be reliable in the way that exposed areas have shown elevated levels of adducts as compared to control sites. Examples are English sole (*Parophrys vetulus*) in Puget Sound, near the city of Seattle, which showed highly significant increased DNA adducts in liver tissue from contaminated areas (Varanasi *et al.* 1989b, Stein *et al.* 1992). Tilapia (*Tilapia mossambica*), brown bullhead (*Ictalarus nebulosus*), oyster toadfish (*Opsanis tau*), European eel (*Anguilla anguilla*), white sucker (*Castostomus commersoni*), perch (*Perca fluviatilis*), Northern pike (*Esox lucius*) and Atlantic cod (*Gadus morhua*) living in contaminated areas have also demonstrated significantly elevated levels of DNA adducts in the liver tissue compared to fish from assumed less exposed sites (Dunn *et al.* 1987, Liu *et al.* 1991, Collier *et al.* 1993, Van der Oost *et al.* 1994a, El Adlouni *et al.* 1995, Ericson *et al.* 1996, 1998, 1999, Aas *et al.* 2001).

In the present study DNA adduct formation, as analysed by the ^{32}P-postlabelling nuclease P1 version, was investigated in liver tissue from feral haddock. Although the biological material was limited in number, DNA adducts could be observed on the autoradiogramm from all three investigated areas with quite large differences between the areas. In Figure 10 representative DNA adduct "fingerprints" are presented from the three areas. The average level of DNA adducts were above "true" background areas, also in the Egersund area (2.1 nmol adducts/mol normal nucleotides) where it was comparative low levels, although with one striking exception. Since that exception was a individual with a specific DNA adduct (*see white ring*) resembling a typical adduct often observed at the other sites there is reason to suspect that this individual has a history of living more analogous to individuals from Sleipner and/or Statfjord areas, as illustrated in Figure 11. Since DNA adduct measurements with the ^{32}P-postlabelling methodology gives information of the genotoxic substances source(s), it is possible to exclude this individual from the control group when it is compared with the two potentially more exposed groups. In Figure 12 all individuals are presented. Preliminary statistical calculations indicate difference between all stations, although the difference between Egersund and Statfjord are of the highest magnitude ($p < 0.0048$).

In order to understand and apply DNA adduct formation in fish liver as a biomarker for aquatic pollution, it is of fundamental importance to hold knowledge of eventual natural background levels in non-contaminated organisms, i.e. caused by endogenous compounds. Absolute detection level of DNA adducts may vary according to the actual method and method modifications applied. With the purpose of obtaining such knowledge, with the methodology we regularly use, we have recently analysed liver samples from several different fish species from assumed non-exposed areas in the arctic and sub-arctic areas in Northern Atlantic, and find undetectable levels of DNA adducts or average levels just above the detection limit, *i.e.*, ~1 nmol adducts/mol normal nucleotides (Aas et al. 2003, *In press*).
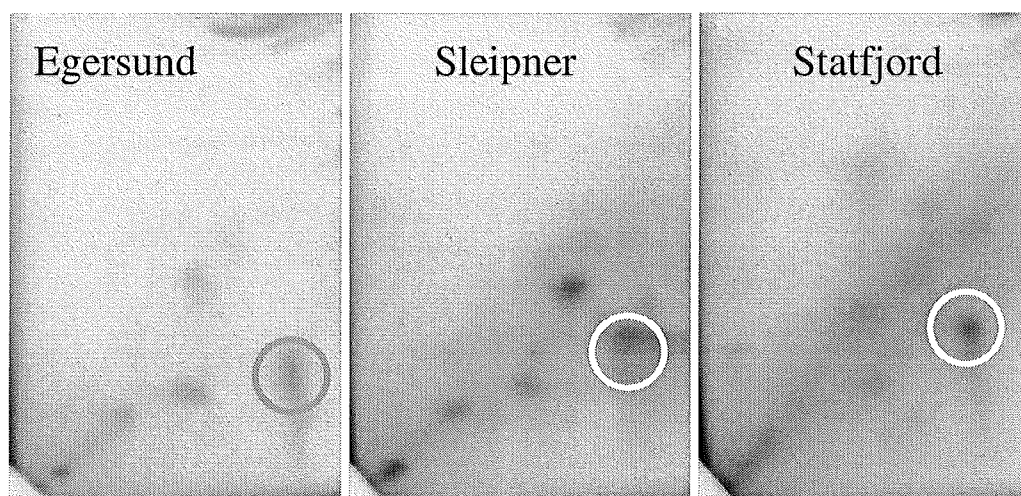
Figure 10. Typical DNA adduct pattern from the three areas presented as autoradiogramm. One commonly observed and specific analysed DNA adduct at Sleipner and Statfjord are encircled with a white line (see also Figure 11).

In comparison with these levels, the obtained DNA adducts levels at Sleipner and Statfjord, strongly suggests a situation of increased genotoxicity since all 14 individuals showed higher levels. In fact the levels found among haddock in the Statfjord area (~20 nmol adducts/mol normal nucleotides) are on the same levels as outside specific point sources of PAHs/DNA binding xenobiotics such aluminum smelter (Ericson et al. 1998), pulp and paper mills (Ericson et al. 1995), and creosote contaminated bottom sediment (Ericson et al. 1999).
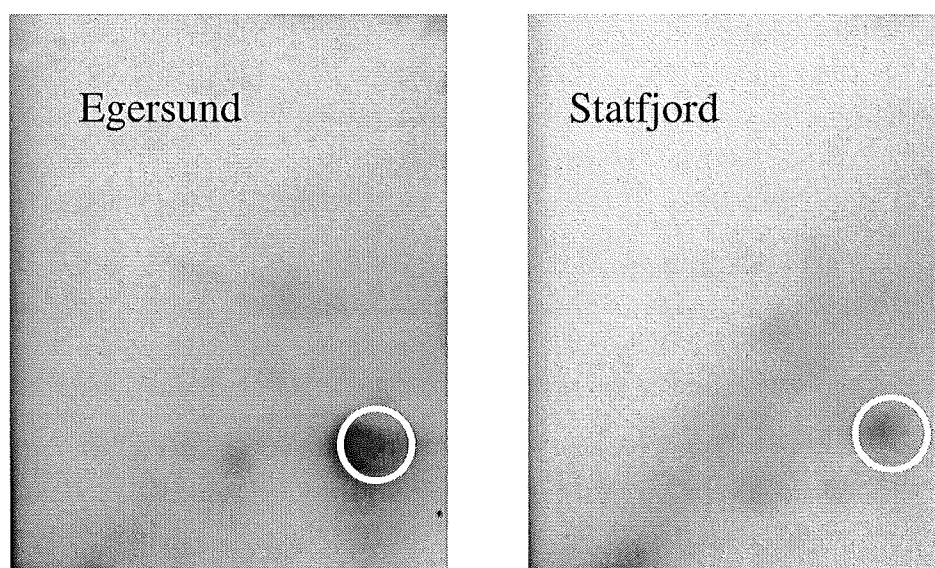

Figure 11. Comparison of the high level of a specific DNA adduct in a individual haddock caught in Egersund compared with a Statfjord individual.
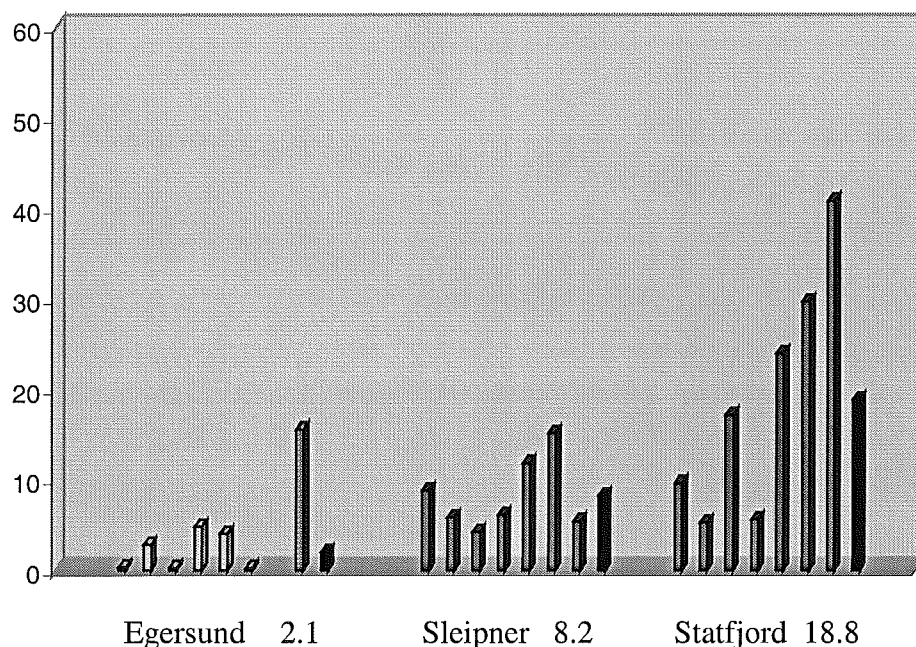
Figure 12. Hepatic DNA adducts in haddock (*Melanogrammus aeglefinus*). Average values are presented by the black bar and indicated by the number adjacent to the area, although excluding the extreme individual (red/green bar) at Egersund.

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