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DNA adduct levels in fish from pristine areas are not detectable or low when analysed using the nuclease P1 version of the ^{32}P-postlabelling technique

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In order to understand and apply DNA adduct formation in fish liver as a biomarker for aquatic pollution, information concerning the natural background levels in non-contaminated organisms, caused by endogenous compounds, is of fundamental importance. In this study, DNA adducts were analysed in liver of 11 fish species from arctic and sub-arctic areas in the northern Atlantic using the nuclease P1 version of the ^{32}P-postlabelling technique. The collected fish were assumed not to have been influenced by anthropogenic pollution apart from possible long-range transported pollutants. As polycyclic aromatic hydrocarbons (PAHs) are thought to be fundamental in forming the type of DNA adducts detected by the method used, biliary PAH metabolite levels were measured in a selection of the investigated species. In all investigated individuals, the levels of PAH metabolites were undetectable. Controlled on-site exposure experiments with benzo[*a*]pyrene (polar cod) and laboratory experiments with crude oil (polar cod and Atlantic cod) were conducted. DNA adducts were formed in both these species. The field-sampled fish showed undetectable levels of DNA adducts or levels just above the detection limit. The present study supports the assumption that when DNA adducts are detected by the nuclease P1 version of the ^{32}P-postlabelling method in fish liver, it can be interpreted as DNA damage caused by pollutants.

Keywords: DNA adducts, polycyclic aromatic hydrocarbons, bile metabolites, fish, deep sea, biomarker background levels, Arctic.

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Introduction

DNA adducts are produced by the formation of covalent bonds between a variety of pollutants and the DNA molecule (Miller and Miller 1981). The negative biological significance of such adducts varies from direct cell death to the development of cancer or adverse effects in the next or subsequent generations (Kirkwood 1989, Bridges *et al.* 1990, Würgler and Kramers 1992, Wang *et al.* 1995). Pollutants can bind covalently to the DNA structure directly as the parent compound, or probably more often after cellular biotransformation to highly reactive metabolites (Varanasi *et al.* 1989a,b). Polycyclic aromatic hydrocarbons (PAHs), a ubiquitous and large group of environmental contaminants, are known to cause genetic toxicity through the formation of DNA adducts. Hence, DNA

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adduct formation has been used as a biomarker for PAH exposure and genotoxic effects.

PAHs are released into the environment due to incomplete combustion of fossil fuels in engines (Balk *et al.* 1994), discharges from electrochemical industry, creosote production and spillage of oil (Lorenz and Glovik 1972, Neff 1979). PAHs are taken up from the water phase directly over the gill membranes and to a lesser extent through the skin. The alimentary canal is another uptake route (Neff 1979). PAHs are found in several organs, but the major organ in which biotransformation occurs is the liver (Varanasi *et al*. 1989a); for this reason the liver is particularly suited for measurements of DNA adducts. The most sensitive method currently available for the detection of DNA adducts is the ^{32}P-postlabelling method (Reichert *et al*. 1998). Development of this method started in the early 1980s (Randerath *et al.* 1981, Gupta *et al.* 1982), and since that time a number of significant steps have been taken to improve it, including enrichment of aromatic and hydrophobic adducts by treatment with nuclease P1 (Reddy and Randerath 1986), use of storage phosphor imaging techniques for analysing and evaluating the DNA adducts (Reichert *et al.* 1992), and improved multidirectional thin-layer chromatography separation (Reichert and French 1994). The ^{32}P-postlabelling technique for DNA adduct detection has been used in fish in several field studies in recent years as a biomarker for genotoxic exposure, and has been found to be reliable in that exposed areas have shown elevated levels of adducts compared with control sites. This method is particularly suitable for the detection of adducts originating from PAH exposure (Reichert and French 1994).

Whether large hydrophobic DNA adducts can be formed naturally or if anthropogenic contamination is a prerequisite for DNA adduct formation is of prime importance when using DNA adducts as a biomarker for aquatic pollution. This is also true with regard to the use of DNA adduct formation in human medicine (Farmer and Shuker 1999). If DNA adducts, as detected by the nuclease P1 version of the ^{32}P-postlabelling method, can only be formed as a result of contact with contaminants, the finding of adducts would imply the presence of a contaminant source. Occasionally this source might of course be non-anthropogenic, e.g. PAH from forest fires, volcanoes or underwater hydrocarbon seeps.

PAH exposure can be monitored as the presence of PAH metabolites in gallbladder bile measured by direct fluorescence analysis such as fixed wavelength fluorescence (Ariese *et al*. 1993, Aas *et al*. 2000a), fluorescence preceded by high performance liquid chromatography separation, or by gas chromatography/mass spectrometry in selected ion mode (Jonsson *et al*. 2003a).

The aim of the present study was to investigate DNA adduct levels in fish from remote areas with no source of pollution other than possible long-range transported pollution and natural events. In the study, 11 fish species from the northern Atlantic and European Arctic were sampled during offshore cruises in 1999, 2000 and 2001. The fish were collected both from deep-sea sites, down to 1000 m, and shallower waters. PAH metabolite detection in bile samples was performed to obtain an indication of possible PAH exposure.

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Materials and methods

Field-collected fish

Various marine fish species were caught in six different areas of the northern Atlantic Ocean/European Arctic by bottom trawling in the period from June 1999 to April 2001. The collection sites were the Faroe Islands, Jan Mayen, the west coast of Svalbard, a deep-sea site west of Svalbard, and two sites in the southern Barents Sea (figure 1). In total, 11 different species were investigated: polar cod (*Boreogadus saida*), daubed shanny (*Leptoclinus maculatus*), sea tadpole (*Careproctus reinhardti*), Atlantic spiny lumpsucker (*Eumicrotremus spinosus*), black seasnail (*Paraliparis bathybius*), Arctic rockling (*Gaidropsarus argentatus*), doubleline eelpout (*Lycodes eudipleurostictus*), polar sculpin (*Cottunculus microps*), Greenland halibut (*Reinhardtius hippoglossoides*), Atlantic cod (*Gadus morhua*) and capelin (*Mallotus villosus*). The approximate trawling depth and the number of individuals investigated for DNA adducts and PAH metabolites at the different sites are presented in table 1. The water temperatures at the depth of trawling were close to -1°C in the waters of Jan Mayen and at the deep-sea site off Svalbard, around $2-3^{\circ}$C in the Svalbard coastal waters, $1-4^{\circ}$C by the Faroe Islands and $3.5-4.5^{\circ}$C in the southern Barents Sea.

Positive control experiments

With the purpose of confirming the responsiveness to DNA adduct formation in these arctic areas, a limited exposure experiment was performed. A subsample of the polar cod originating from the Bay of Kongsfjorden were given an intraperitoneal injection of benzo[*a*]pyrene (B[*a*]P) dissolved in cod-liver oil (Møllers tran, Norway) at concentrations of 5 mg B[*a*]P/kg (August 2000, n = 5, 3–5 days' exposure) and 10 mg B[*a*]P/kg (August 1999, n = 6, 24 h' exposure). The injected fish were placed in tanks with circulating seawater on board the research vessel.

In two separate laboratory experiments, Atlantic cod and polar cod were exposed to 1 mg/l (1 p.p.m.) dispersed oil (North Sea crude oil, purchased from Statoil, Norway) in a continuous flow system for 2 weeks. The Atlantic cod were captured near Stavanger, Norway and kept in the laboratory for 2 weeks prior to the exposure, which was carried out in April 2002. The polar cod were trawled in the Barents Sea, near Bjørnøya, in December 2001, brought ashore in Tromsø, Norway, where they were placed in a fish tank until they were shipped by plane to Stavanger. The exposure experiment was carried out in February 2002 following a 2 week acclimation period. Further details on the continuous flow system for oil dispersion exposure can be found in Sanni *et al*. (1998).

Sampling procedure

The fish were killed with a blow to the head after being brought onboard or taken out of the exposure tank. The sex, size or age of the investigated fish were not taken into consideration for these studies, except for the Greenland halibut, for which 10 males and 10 females were analysed. From the central

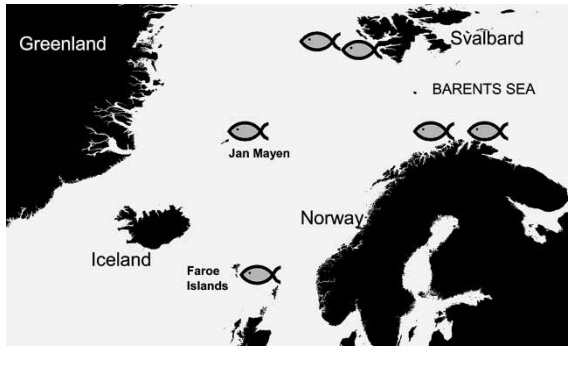

Figure 1. Sampling sites for fish in the northern Atlantic/European Arctic. Greenland halibut (*Reinhardtius hippoglossoides*) were caught near the Faroe Islands. Daubed shanny (*Leptoclinus maculatus*), sea tadpole (*Careproctus reinhardti*), Atlantic spiny lumpsucker (*Eumicrotremus spinosus*) and polar cod (*Boreogadus saida*) were caught in the Jan Mayen waters. Polar cod were caught on the west coast of Svalbard, while Arctic rockling (*Gaidropsarus argentatus*), doubleline eelpout (*Lycodes eudipleurostictus*), black seasnail (*Paraliparis bathybius*) and polar sculpin (*Cottunculus microps*) were caught further off the west coast of Svalbard. Atlantic cod (*Gadus morhua*) and capelin (*Mallotus villosus*) were collected in the western and eastern parts, respectively, of the southern Barents Sea.

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Table 1. Fish species captured in the northern Atlantic/European Arctic and the number of individuals analysed for DNA adducts in the liver using the ^{32}P-postlabelling technique and for PAH metabolites using fixed wavelength fluorescence (FF) and high performance liquid chromatography with fluorescence detection (HPLC/F).

				No. of analysed individuals		
					PAH metabolites	
Species	Latin name	Where captured	Depth (m)	DNA adducts	FF	HPLC/F
Daubed shanny	*Leptoclinus maculatus*	Jan Mayen (near shore)	325	7	—	—
Sea tadpole	*Careproctus reinhardti*	Jan Mayen	450	7	—	—
Atlantic spiny lumpsucker	*Eumicrotremus spinosus*	Jan Mayen	250	7	—	—
Polar cod	*Boreogadus saida*	Jan Mayen	250	7	—	—
Polar cod	*Boreogadus saida*	Svalbard west coast, 78°09′N, 014°28′E	240	7	—	—
Arctic rockling	*Gaidropsarus argentatus*	West of Svalbard, 79°45′N, 006°25′E	~1000	6	6	1
Doubleline eelpout	*Lycodes eudipleurostictus*	West of Svalbard, 79°45′N, 006°25′E	~1000	6	6	2
Black seasnail	*Paraliparis bathybius*	West of Svalbard, 79°45′N, 006°25′E	~1000	6	3	2
Polar sculpin	*Cottunculus microps*	West of Svalbard, 79°45′N, 006°25′E	~1000	6	1	1
Greenland halibut	*Reinhardtius hippoglossoides*	Faroe Islands, 60°50′N, 005°10′E	~1000	20	20	—
Atlantic cod	*Gadus morhua*	Barents Sea, 71°36′N, 021°10′E	320–340	10	10	—
Capelin	*Mallotus villosus*	Barents Sea, 70°52′N, 029°27′E	0–50	9	9	—

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part of the liver, approximately 1 g of tissue was dissected, placed in a cryo-tube and immediately transferred to liquid nitrogen. A relatively fast sampling procedure (approximately 100–150 s) is thought to reduce or prevent intracellular breakdown of DNA. Once at the laboratory the samples were stored at −80°C until DNA purification took place. The gallbladder was opened using a scalpel and a sample of bile was placed in a cryo-tube and transferred to liquid nitrogen. At the laboratory the bile samples were stored at −80°C prior to analysis.

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

DNA extraction and purification of the liver tissue was undertaken essentially as described previously (Dunn *et al.* 1987, Reichert and French 1994, Ericson *et al.* 1998, Ericson and Balk 2000). In brief, liver tissue was gently homogenized in 10 mM Tris chloride buffer and 100 mM ethylene diamine tetra-acetic acid (EDTA), followed by centrifugation to obtain a pellet of the nuclei. The crude nuclei pellet was resuspended and treated with sodium dodecyl sulphate, RNase T1, RNase A and α-amylase, followed by incubation with proteinase K. In order to avoid the formation of quinones that could cross-link nucleotides and result in elevated background levels (Harvey and Parry 1998), redistilled molecular biology grade phenol saturated with Tris chloride buffer, pH 8, plus 0.2% mercaptoethanol (v/v) and 0.1% 8-hydroxyquinoline (w/v) was used for subsequent extractions. DNA essentially free of cell debris was separated using a chronological extraction with phenol, a mixture of phenol, chloroform and isoamylalcohol (25:24:1, v/v/v), and lastly a mixture of chloroform and isoamylalcohol (24:1, v/v). Sodium chloride solution to a final concentration of 500 mM was then added, and the DNA was precipitated by gently mixing with cold (−20°C) absolute ethanol. The DNA was dissolved in 10 mM Tris chloride and 1.0 mM EDTA at pH 7.4, and quantified by its ultraviolet absorbance at 260 nm using a GeneQuant spectrophotometer.

Hydrolysation of the DNA to 3′-nucleoside monophosphates was then performed using micrococcal endonuclease (MN) and spleen phosphodiesterase (SPD). 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). 24 mU MN/μg DNA and 3.2 mU SPD/μg DNA, were incubated for 4 h at 37°C in 0.1 mM $CaCl_2$ and 10 mM succinate buffer at pH 6.0, was used. A $CaCl_2$ concentration of 0.1 mM was used to avoid later interference with the T_4 polynucleotide kinase activity (Hemminki *et al.* 1993). Nuclease P1 methodology (0.8 μg nuclease P1/μg DNA added and 45 min' additional incubation) was used to enrich the DNA adducts; this enzyme degrades the non-adducted 3′-nucleoside monophosphates to their nucleosides, while adducted 3′-nucleoside monophosphates originating from PAH exposure do not undergo such degradation (Reddy and Randerath 1986, Beach and Gupta 1992).

The adducts were then radiolabelled using 5′-[γ-^{32}P]triphosphate([γ-^{32}P]ATP) and T_4 polynucleotide kinase, resulting in a ^{32}P on the 5′ hydroxy group of the adducted 3′-nucleoside monophosphate. The labelling mixture (10 μl) contained 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, and was incubated for 30 min at a temperature of 37°C. As a control experiment, to ensure that the radiolabelled ATP was not limited to the amount of 3′-nucleoside monophosphates (adducted and non-adducted) present in the incubation, an aliquot of the labelled mixture was eluted with 250 mM ammonium sulphate, 20 mM Na_2HPO_4 and 20 mM NaH_2PO_4 (pH 6.4) on polyethyleneimine cellulose sheets, essentially as described by Reichert and French (1994). The presence of an ATP spot indicated that a significant amount was still remaining in the incubation in excess. Apyrase treatment was omitted; in our experience the activity of this enzyme seems to be too non-specific for degrading ATP.

The separation and clean-up of the adducts was performed using multidirectional thin-layer chromatography (TLC) on polyethyleneimine cellulose sheets as described previously suitable for adducts formed by PAHs (Reichert and French, 1994, Ericson *et al.* 1999a). In order to improve the resolution and reproducibility of DNA adducts on TLC separations, laboratory-produced polyethyleneimine cellulose sheets prepared as described by Reichert and French (1994) were used.

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 homogenized liver tissue from perch exposed for 14 days to 50 mg B[*a*]P/kg by intraperitoneal injection. Additionally, the chromatography adduct standard 7R,8S,9S-trihydroxy-10R-(N^2-deoxyguanosyl-3′-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (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. The detection limit was calculated for each individual sample from the actual background signal of the chromatogram.

Chemicals for ^{32}P-postlabelling analysis

Standard DNA (salmon sperm, D-1626), spermidin (S-2626), Rnase A (R-4642), MN (N-3755) and SPD (P-9041) were obtained from the Sigma Chemical Company, St Louis, Missouri, USA. RNase

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T1 (109193), nuclease P1 (236225), proteinase K (1000144), α-amylase (102814), T_4-polynucleotidekinase (3′-phosphatase free, 838292) 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 B[*a*]PDE-dG-3′p was obtained from Midwest Research Institute, Kansas City, Missouri, USA. Cellulose (MN-301) was bought from Machery-Nagel, Düren, Germany. Vinyl strips (polyvinyl chloride 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. Crude oil was obtained from Statoil, Stavanger, Norway. 2-Hydroxy-naphthalene, 1-hydroxy-phenanthrene, 3-hydroxy-B[*a*]P and 1.5% butylated hydroxytoluene were purchased from Fluka Chemie AG (Buchs, Switzerland). Perylene and 1-hydroxy-pyrene were purchased from Acros, Geel, Belgium. Chrysene 1,2-diol was obtained from NCI (MRI), Kansas, Michigan, USA. β-Glucuronidase with 5% sulphatase activity (type HP-2) was obtained from Sigma-Aldrich (Steinheim, Germany). All other solvents and chemicals for DNA purification and adduct analysis as well as bile metabolite analysis were purchased from common commercial sources and were of analytical purity.

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Equipment and apparatus for ^{32}P-postlabelling analysis

Purified DNA was quantified on the basis of its absorption at 260 nm using 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 the Packard Instrument Company (Meriden, Connecticut, US). DNA adduct levels and 'fingerprint' patterns were evaluated by a storage phosphor imaging technique using a PhosphorImager SI instrument and ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, California, USA).

Analysis of PAH metabolites

Bile samples were screened for the presence of PAH metabolites by direct fluorescence analysis as fixed wavelength fluorescence (FF) (Aas *et al.* 2000a). A subsample was analysed by high performance liquid chromatography with fluorescence detection (HPLC/F) (Krahn *et al*. 1987, Jonsson *et al*. 2003a).

FF. Bile samples were diluted 1:1600 in methanol:water (1:1). Slit widths were set at 2.5 nm for both excitation and emission wavelengths, and samples were analysed in a quartz cuvette. All bile samples were analysed by FF on a Perkin Elmer LS50B luminescence spectrometer (Llamtrisant, UK). The wavelength pair 341 nm/383 nm, optimised for the detection of pyrene metabolites, was used. Synchronous fluorescence spectrography (SFS) was performed on a representative selection of the bile samples. The same bile dilution and slit widths were applied. A difference between excitation and emission wavelength ($\Delta\lambda$) of 42 nm was used.

HPLC/F. A smaller subset of bile samples were validated by use of quantitative HPLC/F methodology. The method used was as described in Jonsson *et al*. (2003a, b). The target PAH metabolite compounds were 2-hydroxy-naphthalene, 1-hydroxy-phenanthrene, 1-hydroxy-pyrene, 3-hydroxy-B[*a*]P and 1,2-dihydrodiol chrysene.

Calculations and statistical analysis

DNA adducts were quantified 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 the respective autoradiogram. A spot-specific (area/zone) electronic signal, corresponding to 1.5 times the representative background (spot/area/zone) electronic level, on the same autoradiogram was considered to be the limit of detection and limit of quantification of DNA adducts. In practice, the different detection limits obtained amongst the different fish species investigated depended mainly on the radioactive purity of the polyethyleneimine cellulose sheets. The data are presented as the mean ± SD derived from *n* animals.

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Results

DNA adducts

The livers sampled from all fish species were visually inspected and found to be without any obvious disorders, such as bleeding, abnormal shape or occurrence of tumours.

The field-collected teleost fish species investigated showed levels of DNA adducts below or just above the detection limit as analysed by the nuclease P1 version of the ^{32}P-postlabelling method (table 2). In contrast to the other species, doubleline eelpout and polar sculpin captured west of Svalbard showed detectable levels of DNA adducts in nearly all the individuals analysed. In Atlantic cod caught in the southern Barents Sea, adducts levels just above the detection limit were found in six of the 10 fish analysed.

The positive control experiments, in which polar cod were intraperitoneally injected with B[*a*]P, demonstrated that it was possible to induce the formation of DNA adducts in this species (table 3). An internal standard containing B[*a*]P was analysed (data not shown) and confirmed that the spot seen for the B[*a*]P-exposed polar cod was indeed the most commonly formed B[*a*]P adduct in fish liver. Representative autoradiograms from field-collected fish and B[*a*]P-exposed polar cod are shown in figure 2.

Both Atlantic cod and polar cod exposed to 1 mg/l dispersed crude oil for 14 days in the laboratory showed increased levels of DNA adducts compared with the non-exposed control groups (table 3 and figure 3). Polar cod exposed to oil for 2 weeks demonstrated a 10-fold increase in adduct levels compared with experimental control fish, while the polar cod exposed to B[*a*]P onboard the research vessel showed a 2- to 3.5-fold increase versus the non-exposed individuals from the same group.

Table 2. DNA adducts in the liver tissue of a range of fish species captured at different locations in the northern Atlantic/European Arctic, measured using the nuclease P1 version of the ^{32}P-postlabelling technique. Adduct levels and detection limits are expressed as the mean ± SD. Detection limits are calculated per individual sample (i.e. per autoradiogram) and are dependent on the background signal for each sample.

Species	No. of analysed individuals	No. of individuals with DNA adducts	DNA adduct level in individuals with DNA adducts (nmol/mol normal nucleotides)	Detection limit for individuals without DNA adducts (nmol/mol normal nucleotides)
Daubed shanny	7	1	3.1	0.61 ± 0.15
Sea tadpole	7	0	—	1.34 ± 0.03
Atlantic spiny lumpsucker	7	1	0.24	0.54 ± 0.12
Polar cod, Jan Mayen	7	0	—	0.55 ± 0.07
Polar cod, Svalbard	7	1	1.26	0.72 ± 0.10
Arctic rockling	6	1	0.75	1.02 ± 0.47
Doubleline eelpout	6	5	0.34 ± 0.13	0.64
Black seasnail	6	2	0.69 ± 0.35	0.66 ± 0.24
Polar sculpin	6	6	1.72 ± 1.04	—
Greenland halibut, females	10	0	—	0.41 ± 0.08
Greenland halibut, males	10	1	0.54	0.40 ± 0.08
Atlantic cod	10	6	0.75 ± 0.58	0.43 ± 0.19
Capelin	9	0	—	0.55 ± 0.27

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Table 3. Positive control exposure experiments. DNA adduct levels measured using the nuclease P1 version of the ^{32}P-postlabelling technique in liver samples of polar cod (*Boreogadus saida*) exposed intraperitoneally to B[*a*]P, and polar cod and Atlantic cod (*Gadus morhua*) exposed to dispersed crude oil. Levels are expressed as the mean ± SD.

Species	Exposure	No. analysed individuals	No of individuals with DNA adducts	DNA adduct level (nmol/mol normal nucleotides)
Polar cod	10 mg/kg B[*a*]P (1999)	6	6	1.2 ± 0.7
Polar cod	5 mg/kg B[*a*]P (2000)	5	4	2.0 ± 1.7
Polar cod	1 mg/l dispersed crude oil	6	6	12.2 ± 3.5
Polar cod	Control	6	3	1.2 ± 0.5
Atlantic cod	1 mg/l dispersed crude oil	6	6	17.5 ± 11.0
Atlantic cod	Control	6	6	1.6 ± 1.1

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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. Labelling and TLC migration of the standard DNA adduct B[*a*]PDE-dG-3′p on the polyethyleneimine cellulose sheets showed the normal labelling frequency as well as the expected migration (R_f values) and spot shape on the sheets. In addition, results from positive control samples (adducted liver tissue from perch) processed in parallel to the field and laboratory exposure samples served as quality assurance for all the analytical steps in the ^{32}P-postlabelling method, as expected responses were observed (results not shown). Based on this, it is unlikely that the low adduct levels observed in the field-collected individuals in these studies were due to any kind of artefact.

PAH metabolites in bile

FF screening. All bile samples screened for PAH metabolites in bile using the FF method showed background fluorescence signals typical for unexposed fish. Some representative synchronous fluorescence scans of bile from field and experimentally exposed fish are shown in figure 4. Naphthalene and phenanthrene-type metabolites have a fluorescence optimum around 290/335 nm (ex/em) nm. The oil-exposed Atlantic cod showed a peak in this area, indicating that PAH from the oil is taken up and metabolized. The peak observed for the B[a]P-exposed polar cod indicates uptake and biotransformation of B[a]P. Black seasnail, representing the field-collected fish, had a SFS spectrum typical for fish not exposed to PAH.

HPLC/F. To achieve a more compound-specific and quantitative detection, a subset of the bile samples was analysed using HPLC/F (table 1). The limit of detection (LOD) for the method was 1 μg/kg solvent, while the limit of quantification (LOQ) for the actual samples was 10 μg/kg bile. The LOD used was based on the standards, while the LOQ was based on spiked bile samples. Both the LOD and LOQ were set at a level corresponding to three times the background signal in the chromatogram. None of the five selected PAH metabolites could be detected with HPLC/F in any of the bile samples analysed (details not shown). Bile

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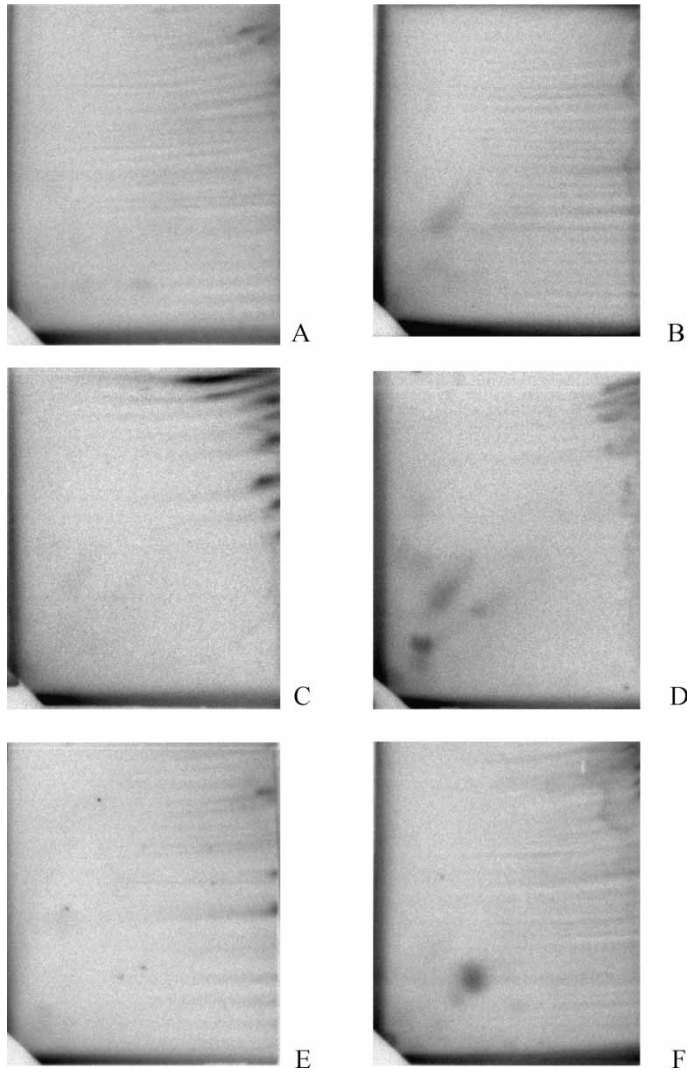

Figure 2. Representative autoradiograms showing the presence of absence of DNA adducts in liver of selected arctic fish species. (A) Arctic rockling (*Gaidropsarus argentatus*); (B) doubleline eelpout (*Lycodes eudipleurostictus*); (C) black seasnail (*Paraliparis bathybius*); (D) polar sculpin (*Cottunculus microps*); (E) polar cod (*Boreogadus saida*), non-exposed; (F) polar cod, exposed intraperitoneally to 5 mg/kg B[*a*]P.

samples from the exposure experiments described in the present study were not analysed by HPLC/F.

Discussion

The ability to form DNA adducts in liver in response to PAH exposure has been demonstrated in a range of fish species, both in field and laboratory studies. English sole (*Parophrys vetulus*) from Puget Sound, near the city of Seattle, showed highly

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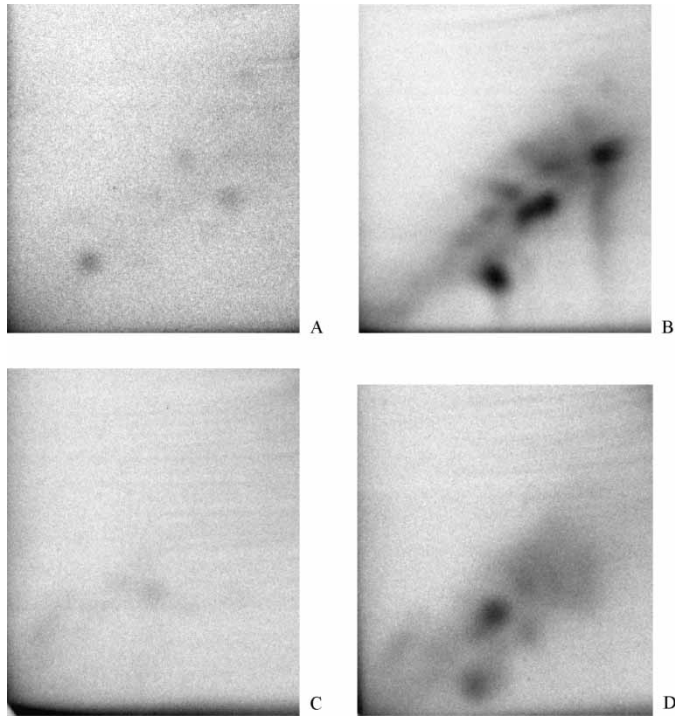

Figure 3. Representative autoradiograms showing DNA adducts in the liver of Atlantic cod (*Gadus morhua*) and polar cod (*Boreogadus saida*) exposed to dispersed crude oil for 14 days in a continuous flow exposure system. (A) Control cod; (B) exposed Atlantic cod; (C) control polar cod; (D) exposed polar cod.

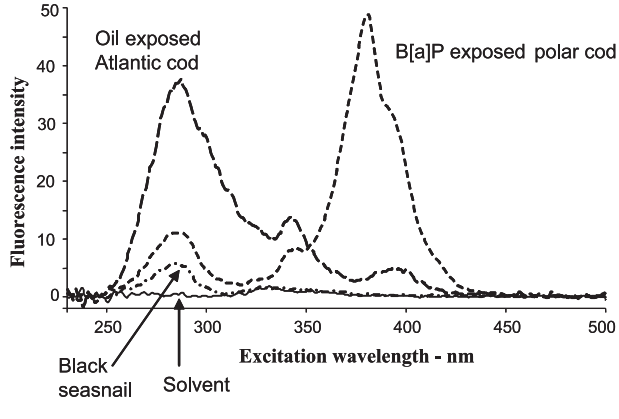

Figure 4. Synchronous fluorescence spectrography (Δλ 42 nm) of bile samples from field-collected and experimentally exposed fish. Fluorescence intensity is expressed as arbitrary units. Two- and three-ring PAHs fluoresce just below the 300 nm excitation wavelength, as can be seen for the oil-exposed Atlantic cod (*Gadus morhua*). The small peak in the same area seen for black seasnail (*Paraliparis bathybius*) can be regarded as a background fluorescence signal, and does not represent the presence of PAHs. B[*a*]P-exposed polar cod demonstrated a clear peak just below 400 nm, where B[*a*]P fluoresces, demonstrating that B[*a*]P has been taken up and metabolized.

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significant increased DNA adducts in liver tissue from contaminated areas (Varanasi *et al.* 1989b, Stein *et al.* 1992, French *et al*. 1996). Tilapia (*Tilapia mossambica*), brown bullhead (*Ictalurus nebulosus*), oyster toadfish (*Opsanis tau*), European eel (*Anguilla anguilla*), white sucker (*Catostomus commersoni*), perch (*Perca fluviatilis*), northern pike (*Esox lucius*) and Atlantic cod (*Gadus morhua*) living in contaminated areas have also been shown to have significantly elevated levels of DNA adducts in liver tissue compared with 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.* 1998, 1999, Aas *et al*. 2001). In some studies, relatively high levels of DNA adducts have been reported in control/reference fish (>30 nmol adducts/mol normal nucleotides). In some of these studies the average control values are even comparable to levels in polluted sites (Kurelec *et al.* 1989, Stein *et al.* 1992, Van der Oost *et al.* 1994b, El Adlouni *et al.* 1995, Eufemia *et al.* 1997). This may be explained either by contaminated control sites, migration of fish from other contaminated areas, variation in biotransformation capacity between fish species (Van der Oost *et al*. 2003), or by the fact that DNA adducts may be formed at non-contaminated sites by natural endogenous compounds.

Documentation of background levels in non-contaminated fish is, however, more sparse. Knowledge about the presence or absence of background level of DNA adducts is necessary for their interpretation as a biomarker and their subsequent use in environmental risk assessment. According to Farmer and Shuker (1999), eventual DNA adducts of endogenous origin are dominated by low molecular weight molecules, molecules that are not detected as DNA adducts by the nuclease P1 version of the ^{32}P-postlabelling method. In that article, Farmer and Shuker (1999) postulate that very low levels of large hydrophobic DNA adducts may have toxicological implications, such as an increased risk of liver tumour formation. However, there are also indications that the presence of DNA adducts is not necessarily associated with toxic effects for the individual (Van der Oost *et al.* 2003). The finding of absence of DNA adducts is still valuable from a risk assessment point of view, in that toxicological effects acting through this pathway can be ruled out (at least with regard to PAH DNA adducts).

The absolute detection level of DNA adducts may vary according to the actual method and modifications applied. In the present studies the nuclease P1 version of the ^{32}P-postlabelling technique was used, and the number of normal nucleotides was calculated based on ultraviolet determination of the DNA. This methodology is optimized for the detection of larger PAHs. The total adduct number may be underestimated, as the attached TLC separation excludes adducts with a low hydrophobicity, such as hydroxyradical-based adducts (e.g. 8-hydroxy-2′-deoxyguanosine adducts) as well as adducts based on few (i.e. two or three) aromatic rings. These kinds of adducts will be washed away from the TLC sheets and therefore are not detected. Similarly, adducts with a very high hydrophobic character (approximately seven or more aromatic rings) will be retained at the origin on the TLC sheet and thereby avoid detection. Aromatic amines, a group of DNA adduct-forming xenobiotics, are not resistant to nuclease P1 treatment (Gupta 1985) and hence are not detected by this method. For these kinds of

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adducts, an alternative method of enrichment of adducts should be used, such as the butanol extraction of adducts as described in Gupta (1985). The ^{32}P-postlabelling technique can also be used for determining the amount of normal nucleotides. Different techniques used to determine total DNA could affect the absolute level of calculated DNA adducts. However, it should not affect the relative adduct levels between polluted and non- or low-polluted sites (Ericson *et al.* 1998, 1999, Stein *et al.* 1993, Varanasi *et al.* 1989c).

In the present study, DNA adduct formation as analysed by the nuclease P1 version of the ^{32}P-postlabelling technique, was investigated in 11 species from presumably pristine areas of the northern Atlantic. A total of 98 individual liver samples of fish were analysed. On the whole, the results demonstrated DNA adduct levels below (approximately three-quarters of the fish samples) or just above (approximately one-quarter of the fish samples) the detection limit for the method. Polar sculpin and doubleline eelpout captured west of Svalbard, and Atlantic cod sampled in the southern Barents Sea, had the highest percentage of the individuals demonstrating detectable adducts, though at low levels. Analyses of PAH metabolites in bile, measured both by the FF method and the HPLC/F method, demonstrated levels below detection limits for all analysed individuals, supporting the presumption of low ongoing PAH contamination in the study areas. The capability of the HPLC/F method for detecting PAH metabolites both from oil and combustion sources has been documented by Jonsson *et al.* (2003a).

Where adducts were detected, the adduct levels in individuals can be regarded as low compared with levels found in fish from contaminated areas and fish exposed to oil (e.g. Varanasi *et al.* 1989a, Aas *et al.* 2000b, 2001). Possible sources for these low DNA adduct levels could be natural compounds, such as from hydrocarbon cold seeps, discharges from mining activity at Svalbard, or eventually long-range transported organic pollution. Organic pollutants, including PAHs, have been shown to occur in the sediment and biota of the European Arctic (Stange and Klungsøyr 1997). The variation in adduct levels among different species can be correlated with differences in factors such as age, habitat and feeding strategies, as well as the biotransformation/metabolism of xenobiotics such as enzymatic activation and deactivation processes. Differences in DNA repair systems and efficiency is also a factor. The somewhat different DNA adduct pattern of Atlantic cod and polar cod when both species were exposed to the same crude oil for 14 days suggests different biotransformation/metabolism in these two species (figure 3B and 3D). Most of the species included in the present study are benthic, and uptake of sediment-bound PAH might be a source of contamination. Recent studies on fish species inhabiting water depths of 300–1500 m in remote areas of the western North Atlantic have indicated that these species are exposed to xenobiotics at levels that might be active at the molecular level (Stegeman *et al.* 2001). *CYP1A* induction and inhibition with α-naphthoflavone *in vitro* were observed, demonstrating the ability for the molecular activation of pollutants and hence the possibility of DNA-adduct formation in these deep-sea species.

In brook trout (*Salvelinus fontinalis*) caught in a remote fresh water system, DNA adducts of possible PAH origin were found both in brain and liver tissue (Ray *et al.* 1995). In a similar study on northern pike (*Esox lucius*) caught in the

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national park of Sauna, a wildlife reserve located in the northern part of Sweden far from any pollution source, liver DNA adducts were detected (L. Balk, unpublished data). Atmospheric fallout of PAH was suspected to be the source of these increased adduct levels (L. Balk, unpublished data).

There are, however, also earlier studies in which DNA adducts were found to be undetectable or very low in fish. Juvenile northern pike from several humus-rich lakes and small ponds in the southern part of Sweden, without any known exposure of xenobiotics, did not show detectable DNA adducts in either the liver or extrahepatic tissues such as gill, brain, intestine and kidney (Ericson *et al.* 1999b, Ericson and Balk 2000, E. Aas *et al*., unpublished data). Feral cod embryos and larvae of Atlantic cod living along the coast of northern Norway have been shown to have very low levels of DNA adducts when compared with cod from the generally more polluted Baltic Sea (Ericson *et al.* 1996).

Controlled exposure experiments in the present study in which polar cod were intraperitoneally exposed to B[*a*]P, and Atlantic cod and polar cod were exposed to dispersed crude oil, demonstrated the ability of these species to form DNA adducts. The higher level observed in the crude oil-exposed fish compared with the B[*a*]P-exposed fish could be explained by a number of factors. Crude oil contains a range of different PAHs that could bind to DNA, and the exposure time was also longer for the oil exposure. Uptake from water could also be more efficient than uptake after an intraperitoneal injection of B[*a*]P dissolved in cod-liver oil (Randall *et al.* 1998), especially in cold water. In fact, for one individual polar cod exposed to B[*a*]P, no increased adduct level was observed compared with the control. The results showed that the main adduct formed after B[*a*]P exposure was B[*a*]PDE-dG-3′p, a common adduct with has been frequently detected in the liver of other fish species (Ericson *et al.* 1999a, b).

In conclusion, the findings of the present study showed that fish from areas without local pollutant sources did not possess significant levels of DNA adducts in liver as analysed by the nuclease P1 version of the ^{32}P-postlabelling method, except for slightly increased levels in some species. The obtained results substantiate the assumption that when DNA adducts are detected by the nuclease P1 version of the ^{32}P-postlabelling method in fish liver, it can be interpreted as a DNA damage caused by pollutants in the area.

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