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Fixed wavelength fluorescence (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish: an evaluation of compound specificity, inner filter effect and signal interpretation

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Fixed wavelength fluorescence (FF) of bile has been evaluated as a monitoring tool for the screening of polyaromatic hydrocarbon (PAH) contamination in fish. The methodology was studied through laboratory and field experiments with Atlantic cod (*Gadus morhua* L.) and flounder (*Platichthys flesus* L.) exposed to various forms of PAH contamination. The present study demonstrates the ability of FF screening to discriminate between 2-, 4- and 5-ring PAH metabolites by using the wavelength pairs 290/335 nm, 341/383 nm and 380/430 nm, respectively. In general, the degree of fluorescence interference between these metabolite groups appears to be low. Dose– and time–response patterns of the FF signals were shown to give a good reflection of the PAH exposure. Further, the necessity of an appropriate dilution of bile samples prior to fluorescence measurements is demonstrated by a study of inner filter effect. Normally a dilution of 1000–2000-fold is necessary. Individual differences in the bile density, e.g. measured as the concentration of the bile pigment biliverdin, have to be allowed for when applying the FF method. However, it is shown that normalizing the FF signals to biliverdin concentrations on an individual basis added extra error to the data set. The simple, rapid and cost-effective FF method is found to be well suited for screening fish for PAH contamination.

Keywords: biomarker, PAH monitoring, fixed wavelength fluorescence, bile, Atlantic cod.

Introduction

Polyaromatic hydrocarbons (PAHs) constitute a group of ubiquitous environmental pollutants, some with mutagenic and carcinogenic properties. PAH contamination is a matter of concern also in the marine environment, and especially near industrialized areas (e.g. Krahn *et al.* 1987, Beyer *et al.* 1996). PAH exposure has been shown to cause induction of cytochrome P4501A, DNA adduct formation, as well as being carcinogenic, causing hepatic preneoplasms and neoplasms in several fish species (e.g. Reichert *et al.* 1998, Van der Oost *et al.* 1994, Beyer *et al.* 1996). The presence of PAH metabolites in bile has been shown to be a risk factor for such effects (Myers *et al.* 1998). Because of this toxicological relevance and the methodological simplicity involved in measuring PAH in fish bile, such measurements should represent a good biomarker for PAH contamination in fish that could be applied in larger monitoring programmes.

Fish metabolize PAHs, mainly in the liver. Subsequently, most of the PAH

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metabolites are excreted into the gallbladder bile, and stored there until the bile is emptied into the intestinal tract, usually during digestion of food (Varanasi *et al.* 1989). Because of their high capacity for PAH metabolism, PAH compounds do not accumulate to any extent in body tissues, and the exposure of fish to PAH is therefore difficult to detect by chemical analyses of liver or flesh. However, since the PAHs and their metabolites display strong and characteristic fluorescent properties, semi-quantitative measures of such compounds can be obtained by simple fluorescence analyses of bile samples. Lately, the monitoring of these substances in fish has to a considerable extent been performed by fluorescence detection of PAH metabolites in the bile (Krahn *et al.* 1986, Hellou and Payne 1987, Ariese *et al.* 1993, Lin *et al.* 1996). Detection of PAH metabolites in fish bile is now also included in the monitoring programme adopted by the OSPAR Commissions (Stagg 1998), and an EU programme on generation of fish bile reference material has recently been initiated (EU project: SMT 4-CT98-2250)

All PAH molecules absorb ultraviolet light followed by emission of light of a longer wavelength. This UV-fluorescence phenomena occurs because PAH molecules contain delocalized π-electrons. The fluorescence properties, i.e. optimal excitation and emission wavelengths and signal intensity, vary between PAH compounds and are dependent on size, structure and eventual substituents. Generally, the optimal excitation wavelength increases with increasing size of the PAH molecule (Vo Dihn 1978), i.e. smaller PAHs need more energy (shorter wavelength of the excitated light) than the larger compounds. This variability can be utilized in simple detection methods for PAHs.

Fluorescence analyses of PAH in bile can be performed by different techniques. By the fixed wavelength fluorescence method (FF) the fluorescence of the diluted bile sample is measured at a certain wavelength pair (Lin *et al.* 1996). Secondly, synchronous fluorescence spectrometry (SFS) of samples can be used to obtain a more detailed picture of the fluorescence pattern in the sample (Lloyd 1971, Ariese *et al.* 1993). In the SFS method a certain wavelength interval between emitted and excitated light, optimized for the type of PAH compounds being studied, is applied. For a further description of the sample metabolite pattern, an HPLC assay can be used (Krahn *et al.* 1986). By this method, the PAH metabolites are separated in a chromatographic column prior to being detected. The detection may be performed either as fluorescence (HPLC–F) or with a mass spectrometer (HPLC–MS). Generally, these three techniques for measurements of PAH metabolites in fish bile represent rapid and cost effective methods for the examination of PAH exposure of fish in laboratory experiments, as well as in field studies. Analysis time and cost is lowest for the FF method and highest for the HPLC method.

The objective of the present study has been to evaluate some methodological aspects of FF analysis of bile as a biomarker for screening PAH contamination in fish. In a concentrated bile fluid, absorption of the excitated and/or emitted light lead to a reduced fluorescence signal. Because of this inner filter effect (Bashford and Harris 1987), it is necessary to dilute the bile sufficiently prior to analysis. The inner filter effect is investigated and discussed. Bile samples from PAH exposed fish typically contain highly complex mixtures of PAH metabolites, some of which may have a significant degree of similarity in their fluorescence properties. The capability of the FF method to separate between 2-, 4- and 5-ring structures by the respective wavelength pairs 290/335, 341/383 and 380/430 nm was evaluated. The

selected PAH compounds which were investigated in this study were naphthalene, pyrene, chrysene and benzo[*a*]pyrene. The feeding status of the fish is also an important factor to consider as feeding activity will influence the concentration of the bile in the gall bladder, and thereby also the PAH concentration in the bile fluid (Collier and Varanasi 1991). The use of the bile pigment biliverdin for normalization of the FF signal is investigated. Further, dose– and time–response patterns of the FF signals are investigated in relation to PAH exposure.

Materials and methods

Chemicals

Pyrene (> 97 %) and benzo[*a*]pyrene (> 98 %) were purchased from Sigma (St Louis, USA). Chrysene (>95%) was purchased from Fluka (Buchs, Switzerland) and naphthalene (> 99 %) from Merck (Schuchardt, Germany). Ethanol (96 %)was used as solvent for bile samples. All other chemicals used in preparation and analyses of samples were of analytical grade. Soybean oil (household type) was used as solvent for PAH compounds. Alkamuls EL-620 (emulphor oil) was obtained from Rhone-Poulenc Chimie, Paris, France.

Fish and sampling

Two marine fish species, Atlantic cod (*Gadus morhua* L.) and European flounder (*Platichthys flesus* L.), were used in the laboratory exposures and field investigations reported in the present paper. Juvenile cultured cod were obtained from the Institute of Marine Research's field station in Øygarden, Norway, whereas the flounder were caught at an unpolluted site, at Sotra, west of Bergen, Norway. Prior to experiments, the fish were acclimated for at least 7 days to the experimental conditions. For the laboratory experiments the fish were not fed for 48 h prior to sampling in order to avoid bile evacuation from the gall bladder.

When sampled, the fish were stunned by a blow to the head, and weight and length were measured. Subsequently, the fish were dissected and the gall bladder was removed with an artery clamp. The bladder was then pierced, and the bile drained into a sample tube. Bile samples were frozen at –20 °C or –80 °C prior to analysis.

Fixed wavelength fluorescence (FF) analysis

Prior to fluorescence measurements, the bile samples were diluted 1:1600 in 48 % ethanol (Ariese *et al*. 1993). Methanol may also be used, according to Lin *et al.* (1996). The FF analyses were performed on a Perkin Elmer LS50B luminescence spectrofluorometer. Slit widths were set to 2.5 nm for both excitation and emission wavelengths, and quartz cuvettes were used in all analyses. For detection of the naphthalene type of PAHs the excitation/emission wavelength pair 290/335 nm was used, and for detection of the benzo[*a*]pyrene type of compounds 380/430 nm was used, both according to the reports of Krahn *et al.* (1987) and Lin *et al.* (1996). In the time–response experiment the slightly different wavelength pair 379/425 nm was used for the detection of benzo[*a*]pyrene metabolites. This wavelength pair was found by optimizing the fluorescence detection of metabolites in bile from benzo[*a*]pyrene-exposed fish. For the pyrene type of metabolites, the wavelength pair 341/383 nm was selected, based on fluorescence analyses of pyrene-exposed fish. In the following account the FF measurements at a certain wavelength pair is denoted as, for example, $FF_{290/335}$. The FF values are expressed as arbitrary fluorescence units in all experiments and the signal levels of the solvent were subtracted in all cases. Synchronous fluorescence spectrometry (SFS) was used for studying compound specificity for the applied wavelength pairs. A difference between excitation and emission wavelengths ($\Delta\lambda$) of 42 nm was used. This $\Delta\lambda$ was found to be optimal for the detection of pyrene metabolites and also suitable for detection of naphthalene and benzo[*a*]pyrene metabolites. Eighteen bile samples from caged cod and flounder in Sørfjorden (Beyer *et al.* 1996) and benzo[*a*]pyrene-exposed cod (time–response experiment) were measured by means of HPLC–F at the detection wavelengths 380/430 nm in addition to $FF_{379/425}$ measurements. The HPLC–F analysis was performed according to the method described in Krahn *et al.* (1986).

Biliverdin concentration was estimated as absorbance at 660 nm according to Larson *et al.* (1947). Biliverdin absorbance can also be measured at 380 nm (Doumas *et al.* 1987, Freek Ariese pers. comm.), at which a more diluted sample is sufficient. The minimum amount of bile needed for performing both FF assays and biliverdin assays was approximately 25 µl when applying biliverdin absorbance at 660 nm and *ca* 10 µl when using 380 nm.

Table 1. Single PAH exposure studies performed with cod for studying compound specificity of $FF_{290/335}$, $FF_{341/383}$ and $FF_{380/430}$.

Compound	*n*, size	Route	Dose	Time
Naphthalene	3, 45±11 g	Intra-peritoneal	10 mg kg^{-1} in soybean oil	48 h
Chrysene	2, 116/64 g	Sub-cutaneous	2.5 mg kg^{-1} in acetone:emulphor (1:1)	48 h
Benzo[*a*]pyrene	3, 350±173 g	Intra-peritoneal	5 mg kg^{-1} in soybean oil	3 days
Control	3, 990±290 g	Intra-peritoneal	Soybean oil	3 days

Bile sample stability

Sixteen bile samples from a caging experiment near an aluminium works (Beyer *et al.* 1998) were reanalysed 1 year after first analysis to study sample stability at –80 °C. Eight samples from another experiment were reanalysed after 1 year's storage at –20 °C. In both cases the bile was stored as raw bile samples (not hydrolysed/deconjugated).

Exposure studies

Inner filter effect. Dilution series of several bile samples were measured by FF in order to study the relationship between concentration of the bile sample and fluorescence intensity. Eight samples for this study were taken from caged fish (Beyer *et al.* 1998) and three from the present dose–response experiment.

PAH compound specificity. SFS was performed on bile samples from cod exposed to various PAHs in order to study the possible interference of fluorescence signals between the different selected wavelength pairs. In addition to the pyrene exposed fish, described in the dose–response experiment, a few individuals were exposed to naphthalene, chrysene and benzo[a]pyrene. Bile samples were pooled for each exposure compound. Table 1 presents an overview of the exposures. Synchronous fluorescence spectrometry (SFS) ($\Delta\lambda = 42$ nm) was applied to investigate possible interference between the wavelength pairs.

In addition, SFS of bile samples from cod chronically exposed to dispersed crude oil in water (1 ppm for 3 days, Aas *et al.* in prep.) were compared to SFS from cod exposed to PAH originating from an aluminium works (Beyer *et al.* 1998).

Time–response experiment—benzo[a]pyrene. Atlantic cod (140 ± 60 g) were exposed *per os* (in gelatine capsules which were force fed) to 0.2 mg benzo[*a*]pyrene kg^{-1} fish dissolved in soybean oil. The control group received soybean oil only. Six fish from each group were sampled 1, 3, 7, 14 and 28 days after exposure.

Dose–response experiment—pyrene. In a dose–response experiment Atlantic cod (95 ± 25 g) were intraperitoneally dosed with pyrene at three concentrations; 0.01 mg kg^{-1} fish (group 1), 0.1 mg kg^{-1} (group 2) and 1 mg kg^{-1} (group 3) for 24 h. Pyrene was emulsified in soybean oil. The control group (4) received soybean oil only ($n = 7$–9).

Normalization of FF levels towards biliverdin concentration. In order to investigate the effect of normalizing the FF data towards bile density, the coefficient of variance (relative size of standard deviation compared to the mean) was calculated before and after normalizing the data for some selected exposure groups. Data sets from the time– and dose–response experiments and a caging experiment (Beyer *et al.* 1996) were applied, and the data were normalized to the concentration of the bile pigment biliverdin. An increase in the coefficient of variance within exposure groups, as a result of normalization of the data, will indicate an addition of error to the data set.

Statistical analyses

Statistical tests for the time– and dose–response experiments were performed with the use of JMP® software, version 3.1.6.2, SAS Institute, Inc., Cary, NC, USA. Parametrical tests were preceded by tests for normal distribution within each sample of values (per sampling day and exposure group), and for homogeneity of variance (across treatments and days). All data were subjected to log transformations. The data were tested day-wise by one-way analysis of variance (ANOVA) with treatment as independent variable. Student's *t*-test was applied day-wise for comparisons of means in the time–response experiment, while Dunnet's test was used for comparison of exposed groups to control in the dose–response experiment. The following significance levels were used (**): $p < 0.0001$, (*): $p < 0.01$.

Results

Inner filter effect

Constituents other than PAHs may absorb the incoming light in the cuvette when measuring fluorescence in bile samples. This is called inner filter effect and is illustrated in figure 1 by $FF_{341/383}$ levels from dilution series of selected bile samples from a field experiment. Four samples are presented. As can be seen from the figure, the inner filter effect is generally most pronounced for the most dense samples, i.e. the ones with the highest measured biliverdin concentrations. Similar patterns were also observed for $FF_{290/335}$ and $FF_{380/430}$ (results not presented). The linear part of the curve starts at around 1000–2000-fold dilution for these samples. Bile samples from the dose–response experiment, where the biliverdin concentrations were approximately 10-fold lower, showed linearity already at 400-fold dilution (results not shown).

PAH compound specificity

Synchronous fluorescence spectra (SFS) ($\Delta\lambda = 42$ nm) of bile from cod exposed to naphthalene, pyrene, chrysene and benzo[*a*]pyrene are shown in figure 2. The three peaks originating from naphthalene-, pyrene- and benzo[*a*]pyrene-exposed fish are relatively well separated from each other, implying a low degree of interference between the metabolites of these compounds. Chrysene, however, interferes significantly with the peak of naphthalene metabolites and also to some extent with the peak of pyrene metabolites. The pyrene metabolite peak is the most clearly defined peak of the four. The peaks of naphthalene, pyrene and benzo[*a*]pyrene metabolites correspond to optimized detection by $FF_{290/335,}$ $FF_{341/383}$, and $FF_{380/430}$, respectively. Figure 3 compares the SFS of cod exposed to crude oil in the laboratory and caged cod exposed to a pyrolytic PAH source. As can be seen from the figure the two SFS have totally different profiles. The main peak of the oil exposed fish corresponds to the 290/335 nm wavelength pair, i.e. naphthalene-type signals, and a minor peak is seen at 341/383 nm. The caged fish have a major peak corresponding to the 341/383 nm wavelength pair, indicating the predominance of pyrene metabolites. The ratio between $FF_{341/383}$ and $FF_{290/335}$ can be used to indicate the origin of PAH contamination. For the two bile samples scanned in figure 3 these ratios were 0.5 and 20 for the oil-exposed and the aluminium works-exposed fish, respectively.

Time–response experiment—benzo[a]pyrene

Benzo[*a*]pyrene metabolites were detected by fluorescence at the excitation/emission wavelength pair 379/425 nm ($FF_{379/425}$) as shown in figure 4. A peak level in fluorescence signal was observed at day 3 after oral administration, followed by a gradual decrease until day 28. The FF level was still significantly different from the control at day 28. Two individuals from the exposed group at day 3 were removed from the data, since background bile fluorescence signals were measured in these and the oral administration clearly had failed. Biliverdin concentrations in the bile samples did not differ significantly between the sampling days (not shown).

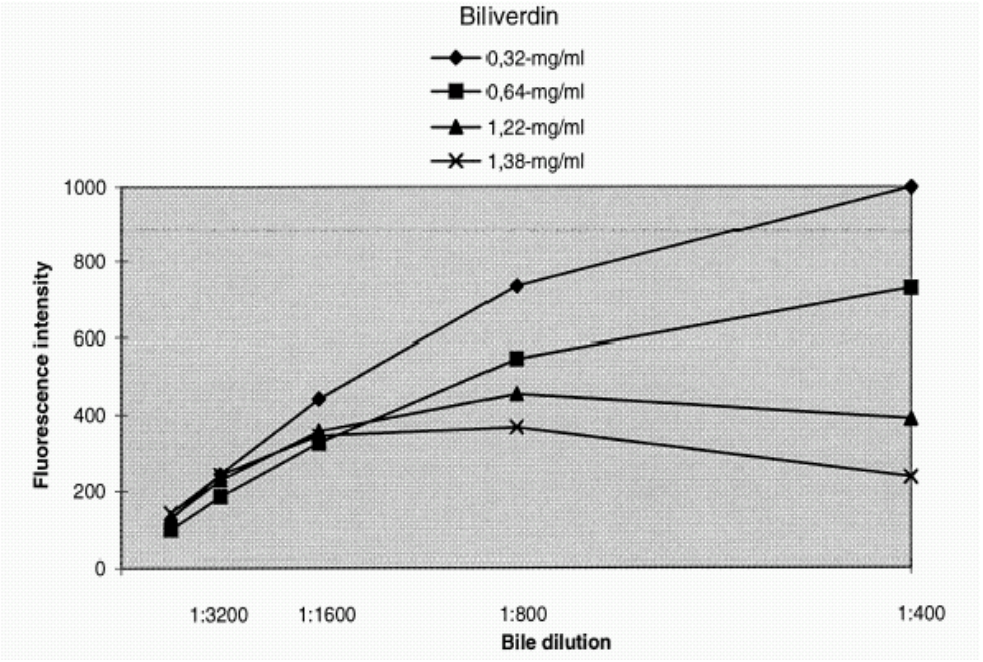

Figure 1. The inner filter effect dependence upon bile density (biliverdin concentration) is illustrated by $FF_{341/383}$ levels of dilution series made from four selected bile samples. The samples are taken from a caging experiment with Atlantic cod near an aluminium works (Beyer *et al.* 1998).

Dose–response experiment—pyrene

As can be seen from figure 5, the dose–response experiment with pyrene shows an increasing response in $FF_{341/383}$ levels by increased exposure dose. The dose–response correspondence was best between the two highest exposure groups. The group receiving 0.01 mg pyrene per kg fish showed a 2.3-fold increase compared with the control. The 10-fold increase in dose between the exposed groups, was reflected by a 3.6- and 8.8-fold increase in the response levels, between 0.01 mg kg^{-1} and 0.1 mg kg^{-1} and between 0.1 mg kg^{-1} and 1 mg kg^{-1} respectively. The biliverdin concentrations in the bile samples showed no significant differences between the exposure groups (not shown).

Normalization of FF levels towards biliverdin concentration

As a general rule the coefficient of variance within exposure groups was higher for FF data that were normalized to biliverdin concentration compared with non-normalized data. The coefficient of variance decreased as a result of normalization in only a few exposure groups. This occurred only in highly exposed groups, such as day 1 in the time–response experiment and the most contaminated station in the caging experiment (results not shown). The coefficient of variance within groups generally increased by decreasing exposure dose. This phenomenon is illustrated in figure 6 with data from the dose–response experiment. This is due to the greater relative importance of the biliverdin concentration when FF levels are low. Most important to notice, however, is the fact that the coefficient of variance generally increased when FF data were normalized.

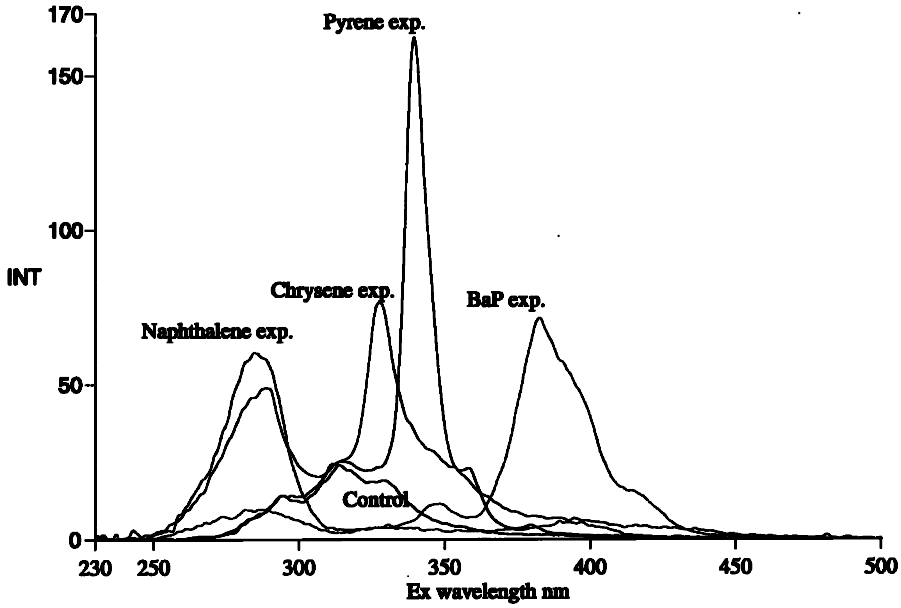

Figure 2. Synchronous fluorescence spectrometry (SFS) of bile samples from cod exposed to naphthalene, chrysene, pyrene and benzo[*a*]pyrene. A difference of 42 nm between excitation and emission ($\Delta\lambda$) was applied. This $\Delta\lambda$ was selected for an optimal detection of pyrene metabolites, but metabolites from naphthalene, chrysene and benzo[*a*]pyrene was also identified using this $\Delta\lambda$. As can be seen from the figure, the different compounds fluoresced at increasing wavelengths according to molecular size, from the 2-ring naphthalene to the 5-ring benzo[*a*]pyrene metabolites. The inter-compound interference was generally low, but chrysene interfered with the signal of both naphthalene and pyrene metabolites, most strongly with naphthalene metabolites.

Validation of FF signal towards HPLC–F

Eighteen bile samples from PAH-exposed fish were analysed by HPLC–F in addition to the FF measurements (figure 7). A relatively good correlation was found between the two methodologies (R^2: 0.86, $p < 0.0001$).

Bile sample stability

After 1 year at –80 °C, the average change in fluorescence signal in the 16 bile samples reanalysed by $FF_{341/383}$ was –0.3 %. The eight samples which were stored at –20 °C for 1 year showed an average change by $FF_{341/383}$ of + 2.5 %. This indicates a very good stability of PAH compounds when stored as raw bile samples (not hydrolysed/deconjugated). The storage should be equally safe at both –80 °C and –20 °C.

Discussion

In several studies the presence of PAH metabolites in fish bile has been shown to correlate with both CYP1A induction (Upshall *et al.* 1993, Beyer *et al.* 1996) and DNA adduct formation (Van der Oost *et al.* 1994, French *et al.* 1996) as well as the prevalence of hepatic lesions, e.g. neoplasms (Krahn *et al.* 1986, Myers *et al.* 1998).

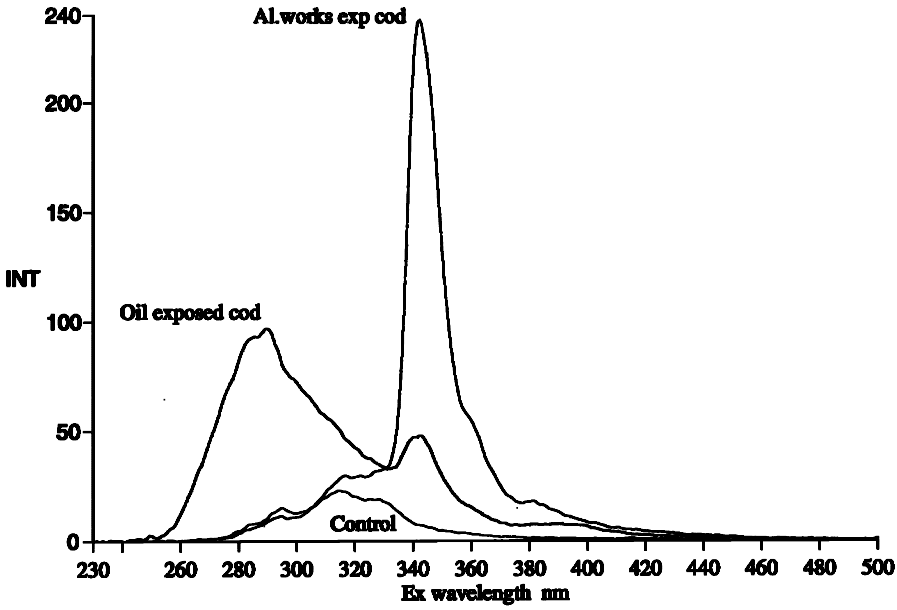

Figure 3. Synchronous fluorescence spectrometry (SFS) of bile from Atlantic cod exposed to oil in the laboratory (1 ppm dispersed crude oil in water for 3 days, Aas *et al.* in prep) and cod caged near an aluminium works (Beyer *et al.* 1998). The different exposure regimes were reflected by different SFS profiles. Bile from oil-exposed fish had a major fluorescence peak in the area where naphthalene metabolites are known to fluoresce, corresponding to $FF_{290/335}$. A minor peak was also seen at the pyrene wavelengths 341/383 nm in these fish. Bile from the fish caged near the aluminium works showed its major fluorescence peak at 341/383 nm, reflecting the presence of pyrene metabolites.

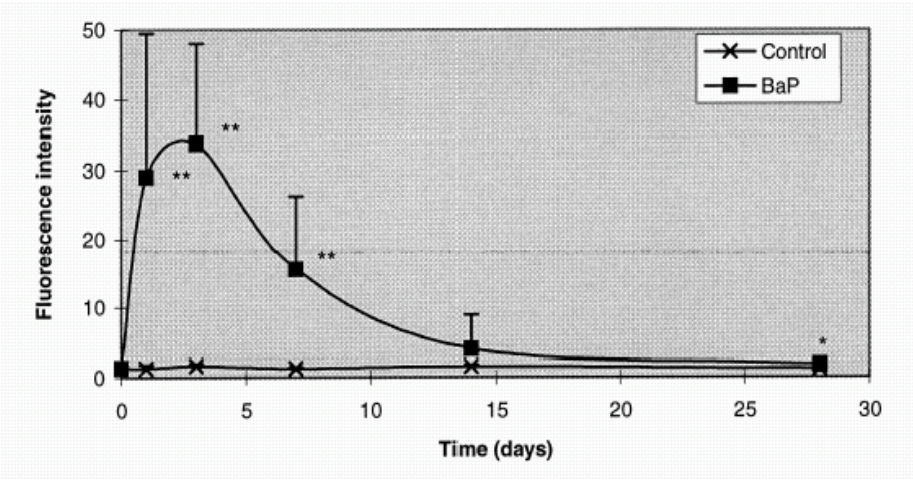

Figure 4. Time–response $FF_{379/425}$ levels (mean + SD) from Atlantic cod orally exposed to benzo[*a*]pyrene. The control group received the solvent soybean oil only. Levels are expressed as fluorescence intensity. Two individuals were removed from the benzo[*a*]pyrene group at day 3 since these showed background levels and obviously had not taken up any benzo[*a*]pyrene. Except for day 3 all sampling groups contained six fish. Significance levels are indicated by (**) $p < 0.0001$, (*) $p < 0.01$.

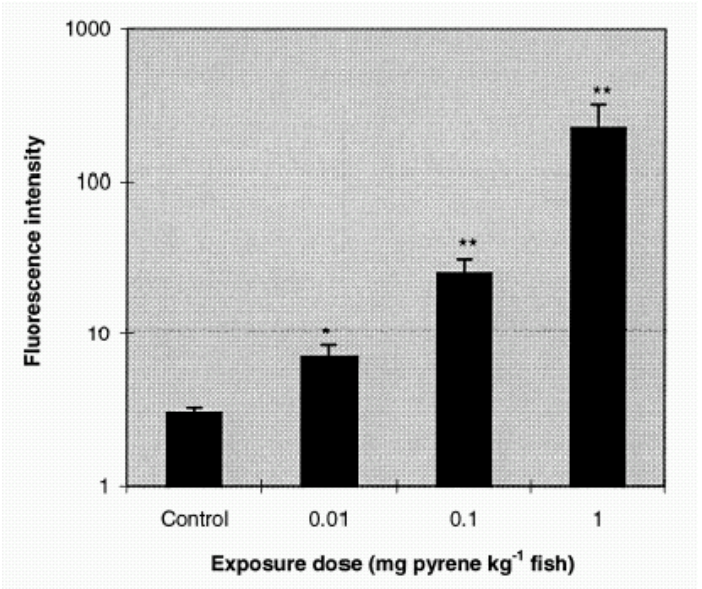

Figure 5. Dose-response $FF_{341/383}$ levels (mean + SD) from Atlantic cod intraperitoneally exposed to pyrene. Levels are expressed as fluorescence intensity, $n = 7–9$ per group. A clear dose dependent response was observed. Significance levels were (**) $p < 0.0001$.

There are also examples showing significantly increased PAH metabolite levels in samples where no induction of CYP1A were found (McDonald *et al.* 1996, Boleas *et al.* 1998, own unpublished results). This demonstrates the relatively high sensitivity of fluorescence analysis of PAH metabolites, but also that adverse biological effects not necessarily will follow their presence. Thus, increased levels of PAH metabolites should be regarded as an indication of potential negative effects, which may trigger follow-up investigations. The discussion will further focus on methodological aspects of the FF method.

Inner filter effect

The presence of the inner filter effect (Bashford and Harris 1987) depends on the density of the bile sample and the applied dilution factor, as described earlier. The density of the bile is in turn dependent on the feeding status of the fish, which again may vary between individual fish and according to daily and seasonal fluctuations. Thus, the dilution necessary for avoiding the inner filter effect will vary according to these factors. The inner filter effect has not previously been mentioned in connection with fluorescence analyses of PAHs in fish bile. Authors have, though, generally applied a dilution factor which would normally prevent an inner filter effect. Ariese *et al.* (1993) diluted all bile samples to > 97 % transparency corresponding to 500–2000-fold dilution. Lin *et al.* (1996) diluted the samples 1:1000 prior to FF analyses. From the present study we can conclude that a sample dilution of 1:1000 to 1:2000 generally should be sufficient to avoid inner filter effect. By using a too high dilution, the sensitivity of the measurements will decrease. To ensure that the inner filter effect is avoided, it is recommended that a concentration-response curve is plotted before analysing the bile samples.

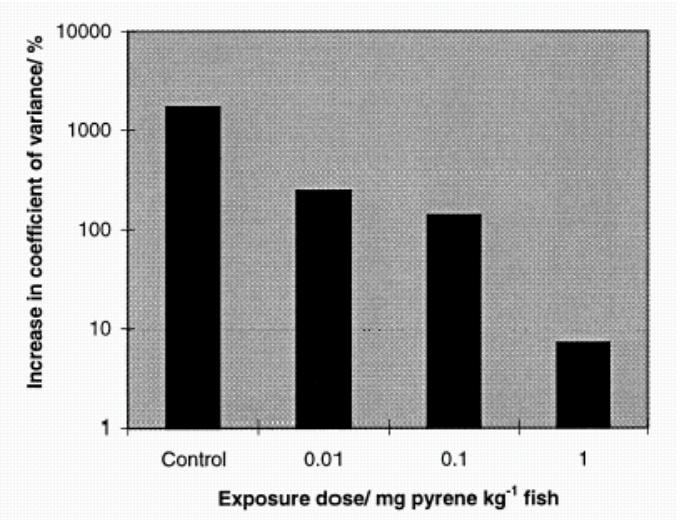

Figure 6. Increase in the coefficient of variance (standard deviation/mean) per exposure group from not normalized to normalized individual $FF_{379/425}$ levels. Data are taken from the dose–response experiment and are presented group-wise. As can be seen from the figure the coefficient of variance increased for all groups and mostly for the lowest exposed groups. An increase in the coefficient of variance within exposure groups indicate that noise/ error is added to the data when normalizing.

PAH compound specificity

For fluorescence detection of the naphthalene and benzo[*a*]pyrene types of metabolites in bile the wavelength pairs 290/335 nm and 380/430 nm have been applied, respectively (Krahn *et al.* 1987, Lin *et al.* 1996). Ariese *et al.* (1993) showed that conjugated 1-OH pyrene, which is a dominant metabolite in fish bile after biotransformation of pyrene, can be detected and measured by synchronous fluorescence spectroscopy (SFS). In the exposure experiments described in this study the conjugated 1-OH pyrene is detected by $FF_{341/383}$, representing the height of the pyrene peak seen in the SFS shown in figure 3. Metabolites of the naphthalene and benzo[*a*]pyrene types could also be identified when using this excitation–emission difference ($\Delta\lambda$) of 42 nm, since their optimal wavelength differences are relatively similar, i.e. 45 nm for naphthalene and 50 nm for benzo[*a*]pyrene, respectively.

As can be seen from the SFS of bile from fish exposed to naphthalene, pyrene and benzo[*a*]pyrene separately (figure 3), there is minor fluorescence interference between their assigned wavelength pairs, 290/335 nm, 341/383 nm and 380/430 nm, respectively. In nature, however, fish may be exposed to a whole range of different PAH compounds (e.g. Lee 1972, Stegeman 1977, Varanasi 1989), and compounds with similar structure and size may interfere if they fluoresce in the same wavelength region. Fluorene, phenanthrene and anthracene metabolites will for instance give an additional fluorescence signal at 290/335 nm (Lin *et al.* 1996, own unpublished results). As was demonstrated in figure 2, chrysene metabolites have a major fluorescence interference at the naphthalene wavelength pair 290/335 nm, and will also to some extent interfere at the pyrene wavelength pair 341/383 nm. At 380/430 nm, 3-OH benzo[*a*]pyrene is shown to be the most dominant fluorescence contributor, but also, for instance, perylene will fluoresce at this wavelength (Lin *et*

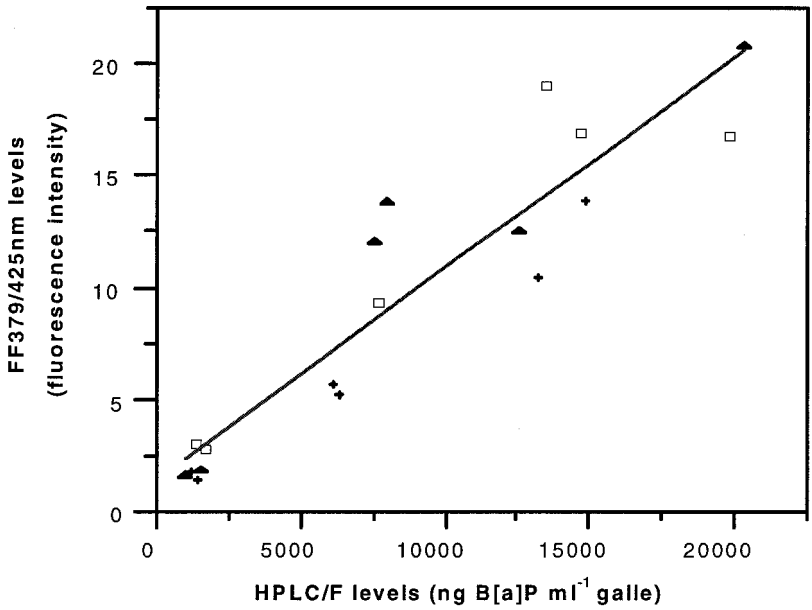

Figure 7. Correlation between HPLC–F (380/430 nm) and $FF_{379/425}$ analyses of benzo[*a*]pyrene type of metabolites in bile measured in the same samples. (+) Atlantic cod, Sørfjorden (Beyer *et al.* 1996), (□) Flounder, Sørfjorden (Beyer *et al.* 1996), (▲) Benzo[*a*]pyrene exposed Atlantic cod (time–response experiment). All groups were included. $R^2 = 0.86$, $p < 0.0001$.

al. 1996). Also, biogenic compounds like cholesterol, progesterone and testosterone are shown to cause fluorescence that can interfere with the PAH signal (Hellou and Upshall 1995). Since these were found to have a fluorescence peak at 280/305 nm, there is a risk for interference at 290/335 nm. However, these compounds are thought to contribute to the background fluorescence signal equally for all exposure groups (Hellou and Upshall 1995).

Cod and flounder that were caged near a metal smelter (Beyer *et al.* 1996) showed the same major peak at 341/383 nm that was observed for fish exposed to single pyrene (figures 2 and 3). This indicates that conjugated 1-OH pyrene is a major contributor to biliary fluorescence in fish exposed to PAH of pyrolytic origin. This is in accordance with the findings of Ariese *et al.* (1993). PAH contamination originating from a petrogenic source show a dominance of 2- and 3-ring compounds over 4-and 5-ring compounds, while pyrogenic PAH is dominated by 4- and 5- ring compounds (Neff 1990 and figure 3). The ratio between $FF_{341/383}$ or $FF_{380/430}$ and $FF_{290/335}$ levels can therefore be used as an indicator of the source of PAH contamination, as shown in the results. Because of the possibility of overlapping signals from different PAH compounds, FF analyses must be regarded as measures of groups of PAH metabolites. It seems, however, reasonable to postulate that the $FF_{290/335}$ will mainly be comprised of 2- and 3-ring structures, $FF_{341/383}$ mainly 4-ring structures and $FF_{380/430}$ mainly 5-ring structures. By applying these three fluorescence wavelength pairs in environmental monitoring, one will include the most frequent and prioritized PAH compounds associated with environmental contamination.

Time– and dose–response experiments

A peak in PAH levels 1–3 days after administration, as found in our study, corresponds well to other studies where fish were exposed to benzo[*a*]pyrene (Collier and Varanasi 1991, Beyer *et al.* 1997). In the experiment of Beyer *et al.* (1997) the FF signals remained at an elevated level until day 16. This finding contrasts the results in the present study where a rapid decrease was observed after day 3. Lack of feeding is the probable reason for a persistent high level of biliary fluorescence. In our study the fish were fed regularly except for 48 h prior to sampling. The result of starvation is an increased density of the bile and thereby also an increased concentration of benzo[*a*]pyrene metabolites (see section on normalization of FF levels).

A prerequisite for application of the FF method for monitoring purposes is the establishment of a reasonable dose–response relationship within the monitoring range. Parameters that influence this relationship are rate and capacity of uptake, metabolism and depuration of the actual compounds as well as the detection limit of the method. Several authors have shown good dose–response relations for fluorescence detection of PAH in fish bile. Collier and Varanasi (1991) found a good correlation for English sole between fluorescence response in bile, measured by HPLC–F at the wavelength pair 380/430 nm and exposure doses ranging from 0.1 to 25 mg benzo[*a*]pyrene per kg fish (intermuscular injection). Beyer *et al.* (1997) also demonstrated a well correlated response in $FF_{379/425}$ levels in bile of flounder for doses of 0.02 to 2.5 mg benzo[*a*]pyrene per kg fish (intramuscular injection). The FF signals in the present dose–response study were well correlated with the dose, and the lower range of concentration response linearity was identified around the lowest exposure group, 0.01 mg pyrene kg^{-1}. The FF method has also been applied in time– and dose–response experiments where cod and turbot (*Scophthalmus maximus* L.) were exposed to different concentrations of dispersed crude oil in water (Camus *et al.* 1998, Aas *et al.* in prep). In these studies the FF responses were found to reflect the nominal oil concentrations in water very well.

Normalization of FF levels towards biliverdin concentration

Bile is a digestive secretion, which is released episodically to the gut. If the fish is starving, the bile will not be evacuated, and the total volume and also density of bile will increase with time (e.g. Grossbard *et al.* 1987, Avery *et al.* 1992). As a result of the increase in bile density, the biliary concentration of PAH metabolites will also increase as demonstrated by Collier and Varanasi (1991). It has been suggested that biliary fluorescence data should be normalized to biliverdin or protein concentration in the bile (Collier and Varanasi 1991, Lin *et al.* 1996). The recognition of biliverdin as a good correlation factor for biliary PAH metabolites assumes a proportional relationship between production of biliverdin, i.e. catabolism of haemoglobin (Grossbard *et al.* 1987), and the level of biliary PAH metabolites. As reported in this study, the coefficient of variance within exposure groups generally increased by normalizing the FF levels on an individual basis to biliverdin concentrations (figure 6). This may indicate that additional errors are introduced to the data set when normalizing. The increase in the coefficient of variance was generally more pronounced in groups with low PAH exposure since the variation in biliverdin concentration in these groups will have relatively more influence. On these

grounds it was chosen not to normalize the data to bile density in this study. However, measurements of bile density, as biliverdin or protein concentration, should always be made to allow for differences in feeding status between exposure groups since this will still influence the metabolite levels in the bile.

Quantification

Because several PAH parent compounds and their metabolites may contribute to the fluorescence at the same wavelength pair, as discussed earlier, an absolute quantification of FF levels to a single PAH compound is impossible. However, the main objective of a screening method is not to give absolute quantification of selected PAH compounds, but rather being able to discriminate between exposed and non-exposed individuals. For this purpose relative measures of the PAH levels, as provided by the FF method, are sufficient. The relative accuracy of the FF method was investigated by Lin *et al.* (1996), who found a good correlation between PAH metabolites in bile measured by FF and the more compound-specific method HPLC–F. Also the present study showed that reliable relative measures of PAH contamination in fish can be provided by the FF method.

The fluorescence values obtained by the FF method can be expressed as equivalents of a chosen standard compound. This can be done either directly by the FF method or indirectly by analyses of a selection of the samples with HPLC–F. Ariese *et al.* (1993) and Lin *et al.* (1994) have used 1-OH pyrene as a standard for quantification of pyrene metabolites by SFS analyses. Lin *et al.* (1996) used naphthalene and benzo[*a*]pyrene as standards for FF analyses at 290/335 and 380/430 nm, respectively. It is important to be aware of the differences in both fluorescence intensity and wavelength optimums between the standard and the conjugated metabolites that fluoresce in the bile sample. Therefore, the values must be interpreted as equivalents of the chosen standard, and comparisons to levels of parent compounds or comparisons between different metabolites cannot be done directly. Hydrolysing the bile sample before analysis can be performed in order to deconjugate the metabolite into a compound which is possible to obtain commercially as a standard, e.g. free 1-OH pyrene (Ariese *et al.* 1993). However, hydrolysing the bile sample prior to analysis has been shown to cause a reduced fluorescence signal as well as decreased storing stability (Freek Ariese personal comm., own unpublished results). The analytical procedure will also become more complicated when including a deconjugation step, and hydrolysation of samples prior to FF analysis is for these reasons not recommended. An alternative way of quantifying the FF signals is simply to express the FF levels as compared to a background level, either from the control samples from the actual study or some standard bile samples.

In order to develop a methodology applicable for standardized monitoring, it is of prime importance to carry out inter-laboratory standardization. A 3 year project supported by the EU-commission was initiated in 1998 aiming at achieving this goal (EU project: SMT 4-CT98-2250). HPLC–F methodology is the prioritized analytical method in this work. The FF method, which may be better suited for screening purposes than the more time- and cost-consuming HPLC–F method, should also be subjected to inter-calibration.

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