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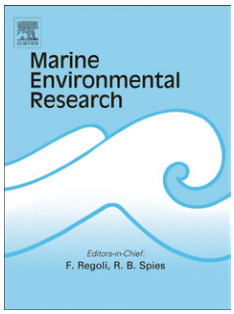

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High matrix interference affecting detection of PAH metabolites in bile of Atlantic hagfish (*Myxine glutinosa*) used for biomonitoring of deep-water oil production.

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Running head: bile fluorescence in hagfish

Key-words: Atlantic hagfish, *Myxine glutinosa*, bile fluorescence, PAH, biomonitoring, Barents Sea

Abstract

The characteristic biology and wide distribution of hagfish species makes them relevant for use in pollution biomonitoring at great water depths, particularly in regions where deep-water oil exploration may take place. The exposure of fish to petrogenic contaminants can normally be detected from the level of polycyclic aromatic hydrocarbon (PAH) metabolites in bile fluid. Some of these metabolites are strong fluorophores, allowing analytical detection by means of simple fluorometric techniques such as fixed wavelength fluorescence (FF) and synchronous fluorescence scanning (SFS). In the present study bile from Atlantic hagfish (*Myxine glutinosa*) collected in pristine areas (Barents Sea and southwestern Norway) displayed strong bile fluorescence levels, suggesting the presence of PAH contaminants. However, gas-chromatography-mass spectrometry (GC-MS) analyses ruled out PAHs as the origin for this fluorescence signal. Rather, the bile of *Myxine* contains components resulting in unusually strong background fluorescence interfering at the wavelength pairs used for detection of PAH metabolites. Possible background for the observed matrix interference and implications for detection of PAH metabolites in hagfish is discussed.

1. Introduction

The current increase in exploitation of deep-water hydrocarbon resources brings along the need for target study species for biomonitoring surveys. Due to the generally low species diversity and density of populations at great depths, selecting suitable sentinel species may be challenging. Several aspects of hagfish biology make the species potentially suitable for such purposes. The availability of Atlantic hagfish (*Myxine glutinosa*) is generally good as it can be caught with baited traps. The distribution range is wide and it can be found on both sides of the Atlantic Ocean at depths from 20-1100 m (Collette and Klein-McPhee, 2002). The potential for migratory movement is moderate, but the fact that hagfish are scavenging on carcasses of long lived species may expose hagfish to pollutants acquired by the prey in a wide area reflecting a regional pollution signal rather than that at a specific collection site. Furthermore, their burrowing behavior may increase the uptake of pollutants associated to the sediment, such as PAHs, through cutaneous absorption.

The presence of hagfish cytochrome P450 (CYP1A) genes and functional CYP1A enzymes (Andersson and Nilsson, 1989) or CYP1A homologues (Hahn et al., 1998) makes hagfish capable of metabolizing PAHs, although the CYP1A is not aryl hydrocarbon receptor (AHR) regulated (Hahn, 2002). Bile fluid of Atlantic hagfish should therefore contain detectable concentrations of PAH metabolites following exposure to these chemicals. The concentration of PAH metabolites in bile is a commonly used method to determine the level of exposure to petrogenic contaminants (Beyer et al., 2010). Bile fluorescence measured by fixed wavelength fluorescence (FF) has been useful for many fish species providing semi quantitative measures of PAH metabolites present in the bile of these species (Krahn et al., 1987; Aas and Klungsøyr, 1998; Beyer et al., 1998; van de oost et al. 2003; Insausti et al., 2009). A subset of samples from FF analysis is often subjected to GC-MS analysis in order to confirm the FF results and accurately determine the concentration of the various PAH metabolites in the bile. Experience from several research groups has led to the following recommended excitation/emission (ex/em) wavelength pairs: 290/335 nm for 2/3-ring PAH metabolites (e.g. naphthalene and phenanthrene), 341/383 nm for 4-ring PAH metabolites (e.g. pyrene), and 380/430 nm for 5/6-ring PAH metabolites (e.g. benzo[a]pyrene) (Krahn et al., 1984; Krahn et al., 1987; Lin et al., 1996; Aas et al., 1998; Aas et al., 2000; Ariese et al., 2005; Beyer et al., 2010).

As part of a baseline study prior to production start-up at the offshore oilfield *Goliat* in the Southern Barents Sea (Figure 1), the potential utility of Atlantic hagfish as a sentinel species in biomonitoring of petrogenic contamination was evaluated. Other fish species collected at the site displayed a low bile fluorescence signals typical for pristine areas, however, fluorescence signals from hagfish bile were unexpectedly high. In order to better understand the basis for this observation, additional Atlantic hagfish were collected in southwestern (SW) Norway. A group of this fish was exposed to 0.25 ppm dispersed crude oil for a week in order to investigate bile fluorescence responses to PAH exposure.

2. Materials and methods

2.1 Fish collection

Atlantic hagfish were collected in the Southern Barents Sea (71° 16` N, 22° 06`E) at approximately 290 meters depth (22 individuals, average length 36 cm, SD = 3.4) in October 2008, and in SW Norway (Åmøyfjord, 59° 01` N, 05° 44`E) at approximately 100 meters depth (26 individuals, average length 26 cm, SD = 3.0) in March 2009 (Figure 1). The hagfish were caught in traps made up from 150 mm perforated PE pipe with a 26 mm one-way funnel entrance. The entrance was fitted with splintered 1 mm plankton mesh to avoid animals escaping once inside the trap. Tusk (*Brosme brosme*) was collected at the same Barents Sea location using two-chamber traps (Furevik 1997). For both types of traps, frozen mackerel was used as bait.

<<Insert Figure 1>>

2.2 Crude oil exposure

Hagfish collected in SW Norway were used for this study. 11 Fish , average length 30 cm (SD = 2.6) were exposed for one week (26th March to 2nd April 2009) to 0.25 ppm mechanically dispersed crude oil from the Goliat oil field in the Barents Sea. Parallel with the exposure of hagfish, halibut (*Hippoglossus hippoglossus*) originating from a local fish farm were identically exposed. The exposure setup is described in detail by Sanni et al. (1998). The control group for the exposure study consisted of 15 hagfish (average length 26 cm, SD = 3.0). In order to document the exposure conditions, the size distribution of oil droplets in the control, exposure and header tank were monitored by Multisizer™ (Beckman Coulter) on day 7. In order to provide PAH profile and concentration information, a single water sample taken on day 5 of the exposure experiment was analysed by GC-MS for PAHs.

2.3 Analyses

2.3.1 Fixed wavelength fluorescence (FF)

Fixed wavelength fluorescence (FF) was conducted as described by Aas et al. (2001). Briefly, bile samples were diluted in 50% methanol (1:1600) followed by measuring FF in a quartz cuvette on a Perkin Elmer LS50B luminescence spectrometer at

excitation/emission (ex/em) wavelength pairs 290/335 nm, 341/383 nm, and 380/430 nm. A slit width of 2.5 nm was used for both ex and em wavelengths.

2.3.2 Mass spectrometric analyses

Samples for mass spectrometric analysis of PAH metabolites in bile were prepared according to the method of Jonsson et al. (2003) and the analyses were run according to the method described by Jonsson et al. (2004). The extraction and analysis of PAHs in exposure water was performed as described by Jonsson et al. (2004). For this analysis, 2 L samples of seawater were extracted with cyclohexane. The analysis included quantitative determination of 16 PAH species/species groups (Table 2).

3. Results and discussion

3.1 Analysis of bile from Atlantic hagfish collected in the Barents Sea

Samples of tusk collected at the Barents Sea site displayed low background bile fluorescence signals typical for pristine areas, however, fluorescence signals from hagfish bile were unexpectedly high (Figure 2). The high fluorescence levels observed in hagfish could incorrectly be interpreted and lead towards the conclusion that the analyzed specimens had high concentrations of PAH metabolites in bile. The data also show considerable variation of fluorescence profile among individual hagfish samples. This variability becomes clear when data from synchronous fluorescence spectrometry from selected samples is compared (Figure 3). Compared with the fluorescence levels obtained for tusk samples, which indicate normal background signals for fish collected in pristine waters, the levels measured in hagfish are approximately one order of magnitude higher (Figure 4).

<<Insert Figure 2>>

<<Insert Figure 3>>

<<Insert Figure 4>>

The unusually high fluorescence levels and the individual variation in fluorescence profile prompted a more thorough investigation of the sample material. Bile from ten individual samples of Barents Sea hagfish were therefore subjected to GC-MS analysis. This analysis failed to confirm the presence of PAH metabolites included in the analysis, except C_1-OH-phenanthrene and C_2-OH-phenanthrene (Table 1). No typical petrogenic PAH metabolite profile was observed in the chromatogram window where peaks of C_1-OH-phenanthrene and C_2-OH-phenanthrene are normally observed. Instead, interference from other unknown compounds with slightly different retention times from the mentioned phenanthrene derivatives was observed. Signals from these compounds were dominating that region of the chromatogram preventing accurate quantification of the C_1-OH-phenanthrene and C_2-OH-phenanthrene levels in the samples.

<<<Insert Table 1>>

<<Insert Table 2>>

3.2 Exposure experiments

To better understand the matrix interference phenomenon, hagfish were collected in SW Norway. One group was exposed to 0.25 ppm dispersed crude oil from the Goliat oil field and one group was kept in sea water only. The mean size of oil particles in the exposure water were 9.3 µm. Presence of PAHs in the exposure water was confirmed both by GC-MS analysis of water (Table 2) and by presence of metabolites in the halibut receiving the same water (Table 1).

Bile fluorescence in laboratory hagfish (hagfish collected in SW Norway) (both exposed and control) confirmed the high signals observed in the Barents Sea samples, although levels were slightly lower than what was observed for the hagfish kept in the laboratory. The individual differences were also less pronounced for the hagfish collected in SW Norway compared with the Barents Sea hagfish (Figure 4). The fluorescence levels in the hagfish were considerably higher than levels typically found in specimens of bony fish collected in pristine areas e.g. tusk collected at the Barents Sea site (Figure 4). Bile fluorescence in hagfish exposed to crude oil also showed individual variation in both level and profile. Another interesting finding was the low levels of PAH metabolites in bile from oil exposed hagfish compared to results from three individual bile samples

from identically exposed halibut (Table 1). This indicates that the bioavailability of petrogenic PAHs in water is low for hagfish under the given laboratory conditions, or that it is not metabolized as efficiently.

3.3 Uptake and excretion of PAHs in hagfish

Currently little information is available regarding the biochemistry and metabolite formation in hagfish, although some studies have described the isolation of bile salts (Haslewood, 1966; Cross, 1966; Anderson et al., 1967; Haslewood, 1967; Anderson and Haslewood, 1969; Hofmann and Hagey, 2008) and antimicrobial peptides (Shinnar et al., 2003; Uzzel et al., 2003; Subramanian et al., 2009). Catalytically active CYP1A has been detected in Atlantic hagfish and is believed to contribute to the metabolism of some compounds in oil (Andersson and Nilsson, 1989; Hahn et al. 1998). The present laboratory exposure experiment indicates that the uptake of dispersed oil over the gill epithelium is significantly lower for hagfish than for halibut, or that aromatic compounds originating from the oil are not metabolized similarly to what occurs in other fish species (Table 1).

The low levels of PAH metabolites in bile of oil exposed hagfish from the present study may have several explanations. Compared to bony fishes Hagfish may have unusually efficient PAH excreting capability through alternative pathways , or PAH may be accumulated in the liver or other lipid rich organs. Under the given exposure conditions, this species may not have an efficient uptake of these compounds from the surrounding water. The former may be caused by the species ability to close the flow over the gills and for periods of time relying only on cutaneous respiration, an adaptation to its sediment burrowing behavior. Further investigations are necessary prior to formulating conclusions regarding the physiological explanation for these findings.

3.4 Investigation towards identification of interfering compounds

Differences in individual fluorescence profiles indicate that several unknown compounds interfering with the fluorescence readings are present in the bile samples at different concentrations (Figure 3). The fluorescence profiles with up to three major peaks also indicate that the compounds are highly conjugated.

In an attempt to elucidate the structure of the unknown compounds resulting in the high fluorescence signal, one sample was subjected to GC-MS analysis utilizing full ion scan mode. By such means we detected two compounds not normally found in bile samples from fish, in the C_1-OH-phenanthrene and C_2-OH-phenanthrene region of the GC chromatogram containing one bromine atom each (*m/z* 397/399 and *m/z* 280/282, respectively). The presence of bromine was confirmed by the typical isotope pattern (^{79}Br and ^{81}Br) and loss of 79/81 Da in both spectra. It was also determined that both compounds contained one hydroxyl group due to the fact that both compounds showed loss of 73 Da in the MS spectra, which was caused by the fragment $C_3H_9Si^{\cdot}$(TMS·). The TMS group is derived from sample preparation where the bile sample post enzymatic digestion is treated with bis(trimethylsilyl)-trifluoroacetamide in order to convert all free hydroxyl groups to the corresponding TMS-ether (Beyer et al., 2010). These analyses did not provide sufficient information in order to elucidate the structure of these two compounds.

The present work could not determine if these compounds are naturally found in Atlantic hagfish or are metabolites of pollutants acquired from the environment. However, due to the fact that brominated flame retardants usually contain 4-10 bromine atoms, it is unlikely that these compounds originate from such a source. It is possible that the brominated compounds are formed *in situ* since it is documented that Atlantic hagfish possess the enzyme haloperoxidase (Shinnar et al., 2000; Shinnar et al., 2003; Bittner et al., 2007) that catalyses the post-translational bromination of tryptophan in cathelicidins (antimicrobial peptides).

The large variability among individuals in concentration of these compounds indicates that their presence may be related to biological status such as recent food availability, exposure to pathogens or reproduction status, rather than exposure to pollutants. Further work is required in order to reveal the structure of these compounds, their origin, and possibly function.

Summary and conclusions

This study showed a lack of correspondence between bile fluorescence levels, an indicator of oil exposure, and actual levels of PAH metabolites in bile from environmentally exposed hagfish (*Myxine glutinosa*). Due to possible interference from unknown compounds in the bile, care must be taken when interpreting bile fluorescence

data in hagfish. Furthermore, caution should be exercised when interpreting the data from GC-MS analysis in the C_1-OH-phenanthrene and C_2-OH-phenanthrene region of GC chromatograms. Further work is required in order to determine the structure of the unknown compounds. Results demonstrate the importance of biomarker baseline investigations prior to use of new indicator species.

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Figure legends:

Figure 1.

Sampling locations for Atlantic hagfish (*Myxine glutinosa*) and tusk (*Brosme brosme*, Barents Sea only).

Figure 2.

Fixed wavelength fluorescence (FF, given as pyrene equiv. μg/mL bile) results for Atlantic hagfish and tusk from the Barents Sea, Atlantic hagfish from SW Norway (control fish from the exposure experiment), and Atlantic hagfish from SW Norway exposed to 0.25 ppm crude oil.

Figure 3.

Synchronous fluorescence spectrometry (SFS) of bile samples of hagfish (*Myxine glutinosa*) and tusk (*Brosme brosme*), collected in the Southern Barents Sea, indicating the extent of the inter-individual differences in signal profiles. A difference of 42 nm between excitation and emission ($\Delta\lambda$) was applied. Excitation wavelength typical for metabolites of 2, 4, and 5 ring PAHs are indicated (Aas et al., 2000).

Figure 4.

Average levels of bile fixed wavelength fluorescence (FF, given as pyrene equiv. μg/mL bile) in tusk (*Brosme brosme*), Atlantic hagfish (*Myxine glutinosa*) from Southern Barents Sea, Atlantic hagfish from SW Norway (negative laboratory control), and Atlantic hagfish from SW Norway exposed to 0.25 ppm crude oil.

Table legends:

Table 1.

Bile PAH metabolites (ng/g) analyzed by GC-MS single ion mode (SIM): Results from Atlantic hagfish (*Myxine glutinosa*) collected in the Barents Sea, SW Norway and from exposure to 0.25 ppm Goliat field crude oil). PAH metabolite levels in samples of halibut (*Hippoglossus hippoglossus*) from the same oil exposure is included for comparison. na = not analyzed.

Table 2.

PAH concentrations in laboratory sea water supplemented with 0.25 ppm Goliat field crude oil and concentration of the various compounds in crude oil from the Goliat field. Data express mean values (µg/L seawater) of two independent GC-MS analyses conducted on water samples taken on day 5 of the experiment. Quantification limit (LOQ) for single compounds 0,005 g/L. nd = not detected; * = compound below the quantification limit (values in brackets are approximate concentrations). Levels of all 5 and 6 ringed PAHs included in the analysis were found to be below LOQ.

Fig. 1

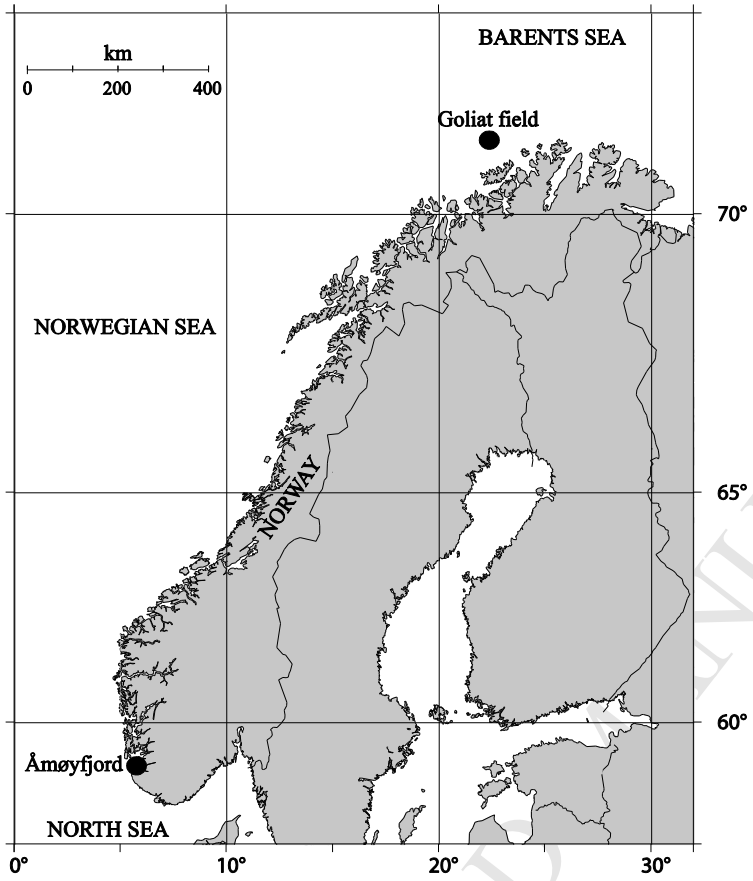


Fig. 2

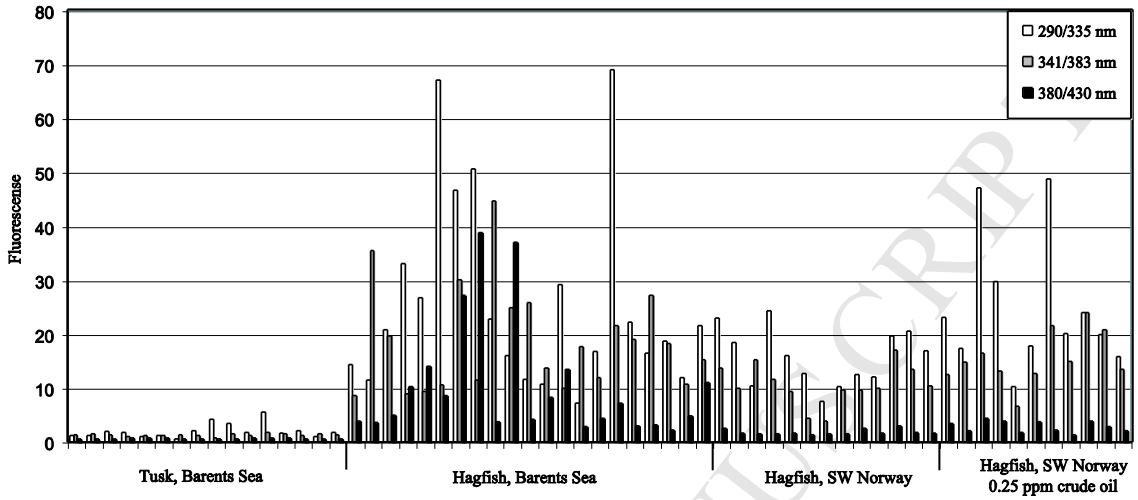


Fig. 3

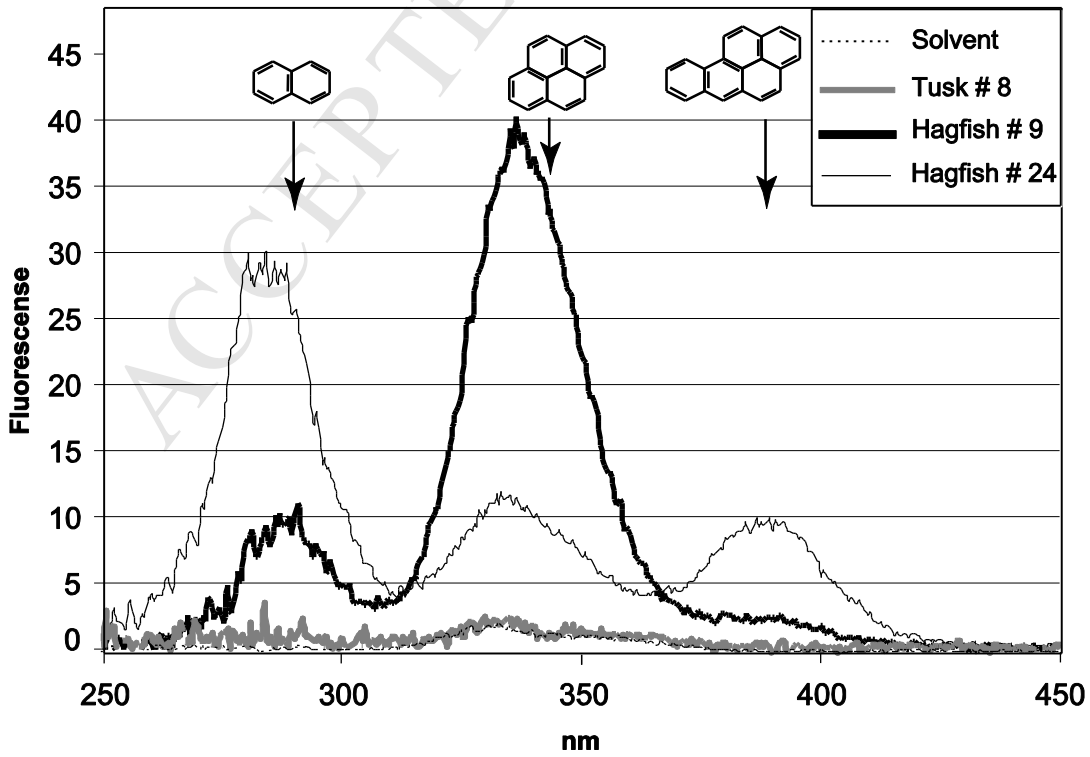


Fig.4

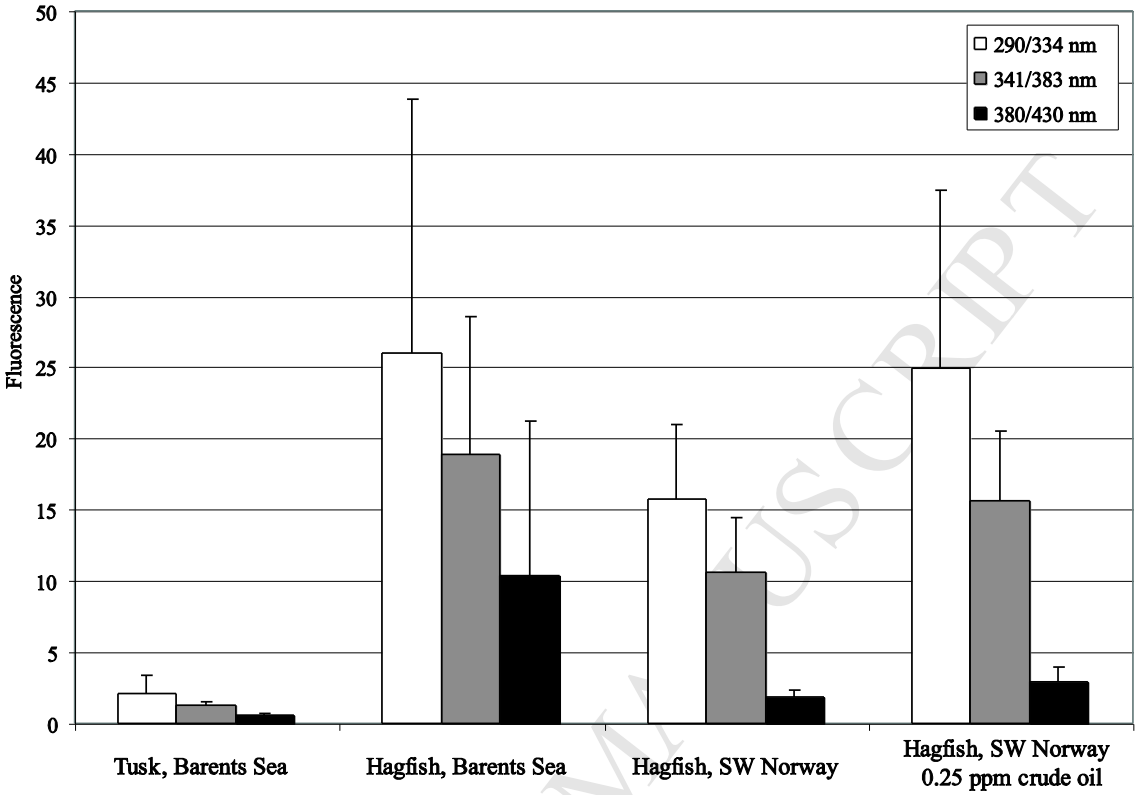


Table 1

		Hagfish, Barents Sea field samples										Hagfish, SW Norway laboratory control							Hagfish, SW Norway 0.25 ppm crude oil					Halibut (farmed) 0.25 ppm crude oil		
Sample #		9	11	14	16	18	19	20	22	27	30	1	2	5	6	10	11	12	1	3	6	7	8	1	2	3
PAH-matabolite (ng/g)	LOQ																									
1-OH-naphthalene	30	< *	88	< *	< *	< *	< *	< *	59	63	42	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *
2-OH-naphthalene	30	217	155	261	333	< *	718	< *	< *	247	704	322	51	139	64	155	601	69	188	79	194	212	33	< *	< *	< *
C_1-OH-naphthalene	300	536	< *	< *	442	655	558	557	612	442	1085	551	417	< *	425	< *	865	321	732	370	453	< *	< *	2441	757	1654
C_2-OH-naphthalene	500	< *	< *	< *	< *	< *	609	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	17603	4796	10381
C_3-OH-naphthalene	1000	< *	< *	1062	< *	< *	1930	< *	< *	< *	2071	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	36067	10282	26675
1-OH-phenanthrene	30	< *	< *	< *	< *	< *	534	34	32	na	74	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	316	132	193
1-OH-pyrene	30	< *	< *	< *	< *	< *	na	< *	< *	na	< *	133	100	116	169	113	332	97	126	241	318	160	88	1118	370	898

Table 2

PAH group	Compound	*Goliat* crude oil	Control	0.25 ppm oil
2-Ring PAHs	Naphthalene	1030 mg/kg	* (0.002 µg/L)	0.190 µg/L
	C_1-naphthalene	2700 mg/kg	* (0.002 µg/L)	0.541 µg/L
	C_2-naphthalene	4200 mg/kg	* (0.002 µg/L)	0.808 µg/L
	C_3-naphthalene	2800 mg/kg	*	0.479 µg/L
3-Ring PAHs	Acenaphthylene	*	nd	*
	Acenaphthene	* (12 mg/kg)	nd	*
	Fluorene	75 mg/kg	nd	0.011 µg/L
	Phenanthrene	175 mg/kg	nd	0.028 µg/L
	Anthracene	*	nd	nd
	C_1-phen./anthr.	280 mg/kg	nd	0.037 µg/L
	C_2-phen./anthr.	310 mg/kg	nd	0.039 µg/L
DBTs	Dibenzothiophene	27 mg/kg	nd	* (0.004 µg/L)
	C_1-dibenzothiophene	73 mg/kg	nd	0.010 µg/L
	C_2-dibenzothiophene	74 mg/kg	nd	0.006 µg/L
4-Ring PAHs	Fluoranthene	* (6 mg/kg)	nd	*
	Pyrene	*	nd	*

Table

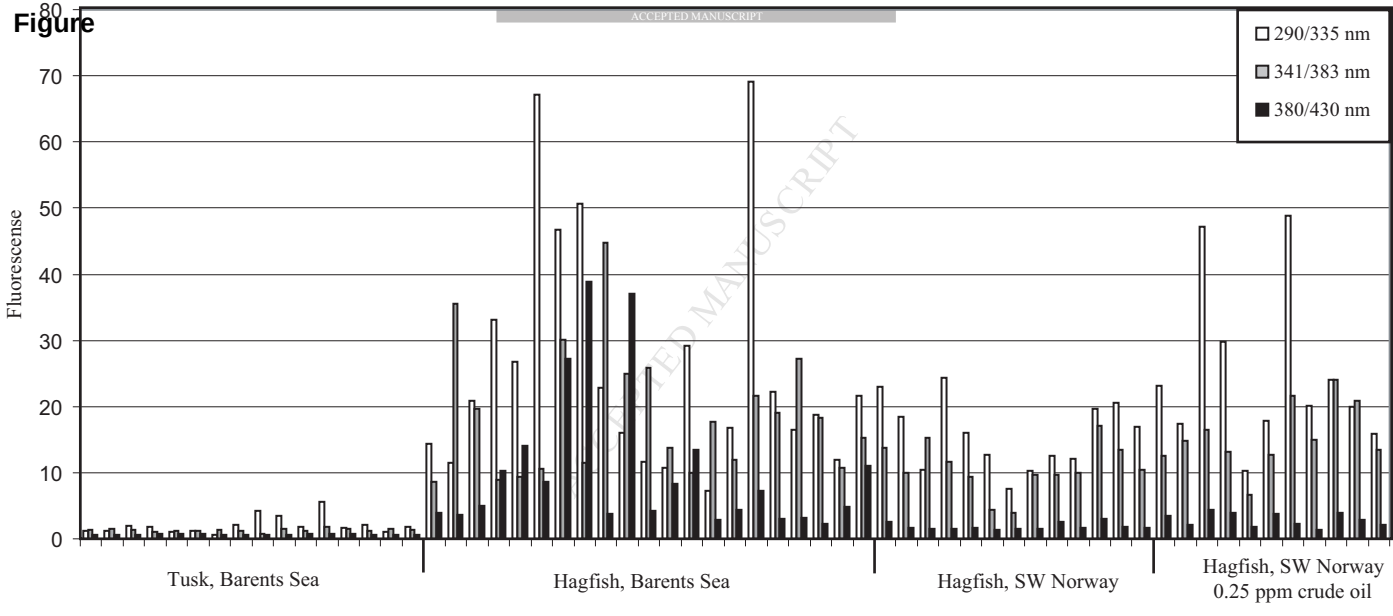
		Hagfish, Barents Sea field samples										Hagfish, SW Norway laboratory control							Hagfish, SW Norway 0.25 ppm crude oil					Halibut (farmed) 0.25 ppm crude oil		
Sample #		9	11	14	16	18	19	20	22	27	30	1	2	5	6	10	11	12	1	3	6	7	8	1	2	3
PAH-matabolite (ng/g)	LOQ																									
1-OH-naphthalene	30	< *	88	< *	< *	< *	< *	< *	59	63	42	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *
2-OH-naphthalene	30	217	155	261	333	< *	718	< *	< *	247	704	322	51	139	64	155	601	69	188	79	194	212	33	< *	< *	< *
C_1-OH-naphthalene	300	536	< *	< *	442	655	558	557	612	442	1085	551	417	< *	425	< *	865	321	732	370	453	< *	< *	2441	757	1654
C_2-OH-naphthalene	500	< *	< *	< *	< *	< *	609	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	17603	4796	10381
C_3-OH-naphthalene	1000	< *	< *	1062	< *	< *	1930	< *	< *	< *	2071	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	36067	10282	26675
1-OH-phenanthrene	30	< *	< *	< *	< *	< *	534	34	32	na	74	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	< *	316	132	193
1-OH-pyrene	30	< *	< *	< *	< *	< *	na	< *	< *	na	< *	133	100	116	169	113	332	97	126	241	318	160	88	1118	370	898

Table

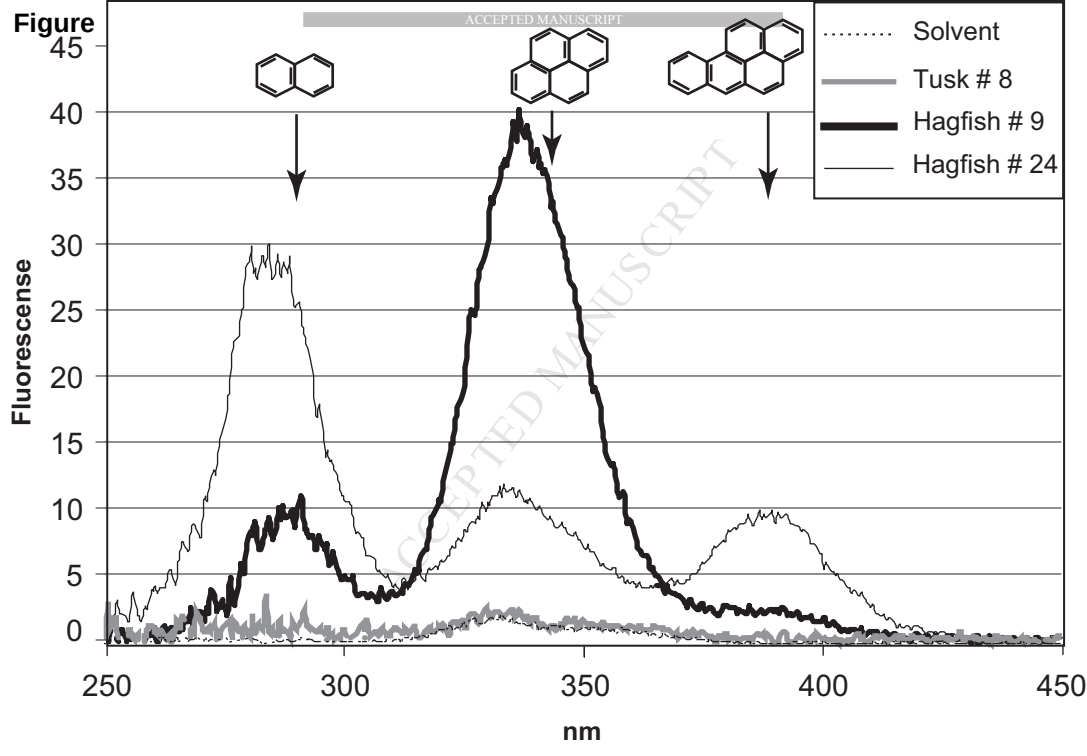
PAH group	Compound	*Goliat* crude oil	Control	0.25 ppm oil
2-Ring PAHs	Naphthalene	1030 mg/kg	* (0.002 µg/L)	0.190 µg/L
	C_1-naphthalene	2700 mg/kg	* (0.002 µg/L)	0.541 µg/L
	C_2-naphthalene	4200 mg/kg	* (0.002 µg/L)	0.808 µg/L
	C_3-naphthalene	2800 mg/kg	*	0.479 µg/L
3-Ring PAHs	Acenaphthylene	*	nd	*
	Acenaphthene	* (12 mg/kg)	nd	*
	Fluorene	75 mg/kg	nd	0.011 µg/L
	Phenanthrene	175 mg/kg	nd	0.028 µg/L
	Anthracene	*	nd	nd
	C_1-phen./anthr.	280 mg/kg	nd	0.037 µg/L
	C_2-phen./anthr.	310 mg/kg	nd	0.039 µg/L
DBTs	Dibenzothiophene	27 mg/kg	nd	* (0.004 µg/L)
	C_1-dibenzothiophene	73 mg/kg	nd	0.010 µg/L
	C_2-dibenzothiophene	74 mg/kg	nd	0.006 µg/L
4-Ring PAHs	Fluoranthene	* (6 mg/kg)	nd	*
	Pyrene	*	nd	*

Figure

Figure



Figure



Figure