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Short Communication

Discovery of polycyclic aromatic acid metabolites in fish exposed to the petroleum compounds 1-methylphenanthrene and 1,4-dimethylphenanthrene

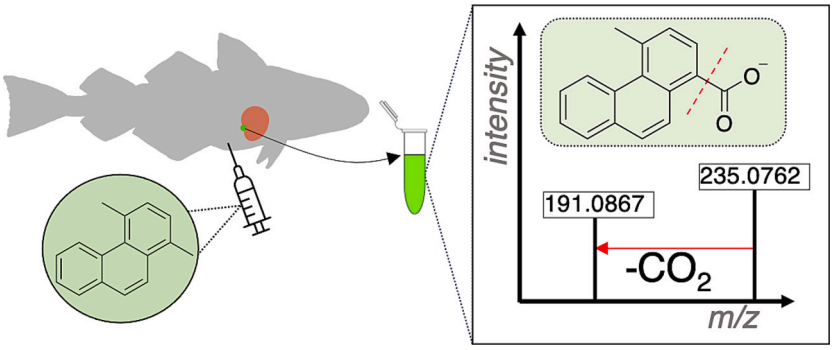
Charlotte L. Nakken [a,b], Sonnich Meier [b], Svein A. Mjøs [a], Lubertus Bijlsma [c], Steven J. Rowland [d], Carey E. Donald [b,*]

[a] Department of Chemistry, University of Bergen, Bergen, Norway
[b] Marine Toxicology, Institute of Marine Research, Bergen, Norway
[c] Environmental and Public Health Analytical Chemistry, Research Institute for Pesticides and Water, University Jaume I, Castellón, Spain
[d] Petroleum & Environmental Geochemistry Group, Biogeochemistry Research Centre, University of Plymouth, Plymouth, PL4 8AA, Devon, UK

HIGHLIGHTS

- Exposure of a marine fish to alkylated derivatives of phenanthrene produces novel carboxylic acid metabolites.
- Carboxylic acid groups occur singly, but also in hydroxylated and dihydrodiol metabolites.
- Incorporating *in-silico* tools into the data processing workflow led to successful suspect screening of potential new biomarkers.

GRAPHICAL ABSTRACT



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ABSTRACT

Most of the polycyclic aromatic hydrocarbons (PAHs) in petroleum are alkylated (alkyl PAHs), still the metabolism of these alkyl PAHs to the expected acid products (polycyclic aromatic acids; PAAs) has yet to be demonstrated in oil-exposed fish. Should these compounds be discovered in fish as they have in ragworm, rodents, and humans, they could present an indicative biomarker for assessing oil pollution. In this study, the ability to biotransform alkyl PAHs to PAAs was examined on Atlantic haddock (*Melanogrammus aeglefinus*). Exposure to phenanthrene, 1-methyphenanthrene or 1,4-dimethylphenanthrene was performed *via* intraperitoneal injection. An Ion Mobility Quadrupole Time-Of-Flight Mass Spectrometer (IMS-Q-TOF MS) was used in exploratory analysis of extracted bile samples. Acquisition of four-dimensional information by coupling liquid chromatography with the IMS-Q-TOF MS and *in-silico* prediction for feature prioritization in the data processing workflow allowed several tentative identifications with high degree of confidence. This work presents the first detection of PAAs in fish and suggests the importance of investigating alkyl PAHs in ecotoxicological studies of oil-polluted fish environments.

* Corresponding author.
E-mail address: carey.donald@hi.no (C.E. Donald).

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1. Introduction

Whilst unsubstituted polycyclic aromatic hydrocarbons (PAHs) are well-known environmental pollutants, most of the aromatic hydrocarbons in petroleum are in fact alkylated homologues (alkyl PAHs) (Boll et al., 2015; Hawthorne et al., 2006; Peng et al., 2023; Zakaria et al., 2002). The concentrations of total PAHs typically constitute between 0.2 % and 6.5 % by weight of oil, where alkyl PAHs accounts for 80–95 % of the contribution (Wang et al., 2003). Although the toxicity of alkyl PAHs may exceed that of the corresponding PAH (Donald et al., 2023a; Turcotte et al., 2011; Wassenaar and Verbruggen, 2021), comparatively little is known about the metabolism of alkyl PAHs, especially in marine fish.

PAHs and alkyl PAHs can accumulate in the fatty tissues of fish, but the concentrations are generally low as fish can metabolize PAHs rapidly by excretion in the bile fluid. Determination of biliary PAH metabolites is therefore used as a biomarker for assessing both ongoing and recent PAH exposure in fish (Dearnley et al., 2022). No analytical method allows unambiguous identification of the biotransformation products of PAHs due to low availability of reference standards. Hence, routine environmental monitoring typically involves determination of a limited selection of hydroxylated derivatives of unsubstituted PAHs (Beyer et al., 2010). These conventional approaches, however, ignore assessment of any risks posed by the more abundant alkyl PAHs in petroleum and their metabolites (Achten and Andersson, 2015).

In general, PAHs are metabolized by cytochrome P450 (CYP) enzymes into oxygenated species. The phase I metabolites of phenanthrene in fish are dominated by dihydrodiols, accompanied by some monohydroxylated compounds (Donald et al., 2023b; Goksøyr et al., 1986). Alkyl PAHs can undergo metabolic transformations that differ from those of non-alkylated PAHs, by potentially featuring benzylic oxidations rather than on the condensed aromatic ring (Huang et al., 2017; Wang et al., 2022). This type of side chain oxidation enables additional action by CYP, resulting in a carboxylic acid group (Engst et al., 1999). Malmquist et al. (2013) showed how the marine ragworm *Nereis diversicolor* biotransformed 1-methylpyrene to the polycyclic aromatic acid (PAA) 1-pyrenecarboxylic acid. The same metabolic route, identified as double benzylic hydroxylation by CYP, was later demonstrated for alkylated phenanthrene and chrysene – and became generalized to other alkyl PAHs (Malmquist et al., 2015). PAAs have also been found in rodents and humans (Bendadani et al., 2016; Engst et al., 1999; Lin et al., 2021). To the best of our knowledge, they are yet to be reported in fish.

Recent advances in analytical techniques provide new strategies for the detection of environmental contaminants. In particular, some of these enable the shift from target analysis, using available reference standards, to non-target and suspect analyses. Unlike non-target analyses, the suspect screening approach in high-resolution mass spectrometry consists of processing the acquired data using lists of compounds with chemical formulas (*i.e.,* exact masses) expected to be present in a sample (Pourchet et al., 2020). Predictions of physical parameters and biotransformation routes from *in-silico* tools can refine data processing and further improve confidence in identifications (Bijlsma et al., 2019). Through utilization of ion mobility spectrometry (IMS), an additional 3D-characteristic of the ionized molecules can be achieved by ion collision cross section (CCS) measurements (Lanucara et al., 2014). Liquid chromatography (LC) and IMS hyphenated to quadrupole time-of-flight (LC-IMS-Q-TOF) ultimately provides four-dimensional peak detection: retention time, *m/z*, intensity, and CCS values. Moreover, it provides "cleaner" drift-resolved spectra which align the precursor and fragment ions based on the drift time separation (Gil-Solsona et al., 2021).

In this study, the ability of Atlantic haddock (*Melanogrammus aeglefinus*) to metabolize alkyl PAHs to PAA products was investigated. Controlled exposures were performed with individual treatments *via* intraperitoneal injection. The PAH phenanthrene (PHE) and two of its alkylated derivatives, 1-methylphenanthrene (1-MPHE) and 1,4-dimethylphenanthrene (1,4-DMPHE), were the model PAHs injected. Control fish had no PAHs administered. Bile extracts were screened using LC coupled with an IMS-Q-TOF mass spectrometer. A suspect library for peak picking was constructed from the *in-silico* predicted PAA metabolites of 1-MPHE and 1,4-DMPHE and their predicted CCS values. The results provide tentative identifications of PAA metabolites and introduce a new pathway of alkyl PAH metabolism in fish.

2. Materials and methods

2.1. Chemicals

A lock mass standard, leucine-enkephalin, and the instrument calibration solution (CCS Major Mix) were purchased from Waters (Manchester, U.K.). HPLC- and UHPLC-grade solvents (Chromasolv) were obtained from Honeywell (Seelze, Germany). Formic acid (>98 %) was purchased from Merck (Darmstadt, Germany). Phenanthrene (CAS 85–01-8), dimethyl sulfoxide (DMSO) and tricaine methanesulfonate (MS-222) were purchased from Sigma-Aldrich (Oslo, Norway). 1-methylphenanthrene (CAS 832–69-9) and 1,4-dimethylphenanthrene (CAS 22349–59-3) were purchased from Chiron AS (Trondheim, Norway). As described previously (Aitken et al., 2018), 3- and 4-phenanthrenecarboxylic acids were purchased from Sigma-Aldrich (Poole, UK). 2- and 9-phenanthrenecarboxylic acid were synthesised by refluxing a mixture of either 2-acetylphenanthrene or 9-acetylphenanthrene (Sigma-Aldrich, Poole, U.K.) with a 5 % solution of sodium hypochlorite for 24 h according to the procedures of Dixon and Neiswender Jr. (1960). 1-Phenanthrenecarboxylic acid was not available.

2.2. Experiment, sample collection and preparation

Exposures were performed as described by Meier et al. (2020). Adult Atlantic haddock were supplied from a brood stock at the Austevoll Research Station. All animal experiments within the study were approved by NARA, the governmental Norwegian Animal Research Authority (reference number FOTS ID 5924), in accordance with approved guidelines. The Austevoll Research station has the following permission for catch and maintenance of Atlantic haddock: H-AV 77, H-AV 78, and H-AV 79, given by the Norwegian Directorate of Fisheries. Furthermore, the Austevoll Research station has a permit to run as a Research Animal facility using fish (all developmental stages), with code 93 from the National IACUC; NARA.

Each fish (number of fish exposed indicated in parenthesis) was injected with individual PAH or alkyl PAH compounds: PHE (3), 1-MPHE (2), or 1,4-DMPHE (2). The fourth treatment group was the Control (1), administered without PAHs. PAHs were dissolved in DMSO and fish oil and were injected into the abdominal cavity of each fish corresponding to a dose of 5 mg/kg fish weight. Fish were anesthetized before injection (60 mg/L tricaine methanesulfonate). The fish were killed three days after injection by using a high dose of anaesthetic. Bile was sampled and immediately frozen in liquid nitrogen and stored at −80 °C until metabolite extraction. All fish underwent a one-day fasting period prior to the sampling.

2.3. Bile extraction

The PAH metabolite extraction followed the protocol of da Silva et al. (2023). Briefly, 50 μL bile was diluted, cleaned for proteins and phospholipids in a phospholipid removal cartridge, hydrolyzed with glucuronidase/sulfatase enzyme (1 h, 40 °C), and extracted and cleaned up using solid phase extraction. Extracts were stored in the dark at −20 °C until analysis.

2.4. Instrumentation

Analyses were performed on a Waters Acquity UPLC I-Class system

coupled to a Vion IMS-Q-TOF mass spectrometer. Chromatographic separation was performed using a reverse-phase C18 BEH column (Waters, 100 × 2.1 mm, 1.7 μm) maintained at 45 °C. Binary mobile phases consisting of water (mobile phase A) and methanol (mobile phase B) were used at a flow rate of 0.45 mL/min. The organic fraction was kept at 30 % for 0.1 min and linearly increased to 80 % by 20.1 min. At 20.2 min, it was increased to 98 % until 23.1 min. The column was then allowed to equilibrate, resulting in a total run time of 28.0 min. The sample temperature was 10 °C and the injection volume was 5 μL.

The electrospray ion source was operated in negative mode with capillary voltage 2.6 kV; cone voltage of 40 V; desolvation temperature 475 °C; source temperature 110 °C; desolvation gas flow (N_2) 950 L/h; cone gas flow (N_2) 50 L/h. Traveling-wave ion mobility spectrometry (TWIMS) separated the analytes at default parameters. Automatic lock correction to ensure mass measurement accuracy was acquired by the reference mass of the lock mass standard, leucine-enkephalin (*m/z* 554.2615) at half-minute intervals. The TOF analyzer was operated in the sensitivity mode using the high-definition MS^E mode, with scan settings from *m/z* 50–800 at 0.3 s per scan for two independent scans with different collision energies: the low energy scan was set at 6 eV CE to monitor the parent adduct ions, and the high energy ramp (8–45 eV) to monitor fragmentation of the precursor ions. Nitrogen was used as drift gas and argon as collision-induced dissociation (CID) gas.

2.5. Suspect library and CCS predictions

A library of phase I PAA metabolite predictions for 1-MPHE and 1,4-DMPHE was created (Table S1 Supporting information). The metabolites were each predicted to occur with a carboxylic acid in one of three forms: i) singly, ii) with a hydroxyl group, or iii) with a dihydrodiol moiety. The structures of the predicted PAAs are given in Figs. S1 and S2. Predictions were performed by Meteor Nexus version 3.1.0 (KB 2018 1.0.0, Lhasa Limited) using the prediction method "Absolute/Relative Reasoning". Structure files (.mol) were uploaded to the UNIFI™ software (Waters, UK), which provided expected mass and fragmentation of the molecules. Potential adducts included deprotonated ions. Predictions for CCS values in Table S3 were performed by the CCS_{H-} model presented by Celma et al. (2022).

2.6. Data processing

Data acquisition and analyses were performed using UNIFI™ software (version 1.9.4.053). The minimum intensity threshold was set to 5 counts in the mass range *m/z* 50–650. Suspect screening by mass and CCS was performed at 5 mDa and 6 % tolerance, respectively. First, the applied library generated a list of "Identified Components" matching detected accurate masses within the CCS tolerance in the suspect list. Second, a common neutral loss filter was applied to eliminate candidates without relevant fragment ions. The criterion included the characteristic neutral loss 43.9898 Da, corresponding to loss of CO_2, within 2 mDa mass tolerance. Finally, manual investigations of the low- and high energy spectra were conducted to filter out the final candidates (*e.g.,* evaluating deprotonated molecules, relative retention times, adducts, and in-source fragmentation).

The fit of the experimental data to the theoretical isotope distribution was inspected by Waters' commercial algorithm (i-FIT) for elemental composition analysis using common elements (C, H, N, O, P, S, Cl, Br, and F). By selecting the suspect deprotonated molecules and the most prominent fragments, output data presented chemical formulas based on exact mass, isotopic patterns, and chemical rules (carbon/hetero-atom and carbon/hydrogen ratio filter). Confidence in the chemical formula assignment is reported as an i-FIT confidence (%).

Identification criteria required: i) mass accuracy $\leq$1 mDa for the suspect ion, ii) mass loss corresponding to a CO_2 neutral loss within 2 mDa, iii) 6 % ΔCCS tolerance from predicted values, iv) top ranked i-FIT confidence, v) not detected in the Control.

3. Results and discussion

3.1. Identifications of polycyclic aromatic acid (PAA) metabolites

The hypothesis that fish can form PAAs following exposure to alkyl PAHs is confirmed by the present laboratory experiments with Atlantic haddock. Detection information (*m/z*, retention time, CCS) for all candidate PAAs is reported in Table S2. The exact position of the substituents cannot be deduced from the MS data. All predicted isomers are presented in Table S1 as both SMILES (Simplified Molecular Input Line Entry System) and InChI Key (International Chemical Identifier).

Five PAA metabolites were detected under the defined identification criteria (Table 1). The unsubstituted parent molecule, PHE, is not expected to produce PAAs as it is not alkylated. None of the listed PAAs were detected in the PHE or the Control treatment. The UNIFI software predicted plausible fragments and collected the matching fragment ions of the high-energy spectra, resulting in the refined spectra presented in Figs. S3–S7.

Due to the unavailability of reference standards for the suspects, we established rigorous criteria for the tentative identifications. The combination of these criteria ensured a comprehensive assessment for proper annotation of the PAAs. First, the mass tolerance was expressed in mDa to ensure high mass accuracy and consistency in mass error, opposed to using the relative unit ppm which depends on molecular weight. Secondly, testing of the diagnostic common neutral loss of -CO_2 from the carboxylic acid moiety was validated by mass spectra obtained for the standards of the phenanthrene carboxylic acids. Increased confidence was further improved by including predicted CCS_{H-} values (Celma et al., 2020). All observed CCS values had deviations below Δ6% (Table S3), which is in line with the published prediction accuracy *i.e.,* 6 % relative error within the 95th confidence interval (Celma et al., 2022). Further, the use of i-FIT calculations (Table S2) assisted in eliminating false positives. The i-FIT algorithm takes the mass accuracy and the isotope model into consideration and provides the most confident chemical formula. According to requirement iv) of the annotation requirements, only candidates with top i-FIT ranking matching the chemical formula in the suspect list were included. The fact that all the detections presented in this study provided a number 1 ranking increases the credibility of the results.

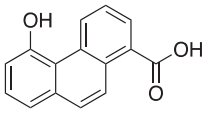
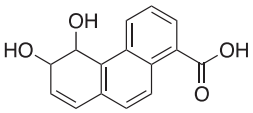
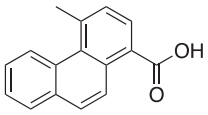
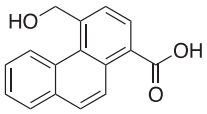
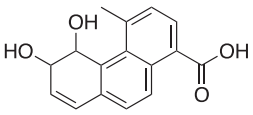
No mobile phase additives were used in this study for two reasons: i) to avoid ion suppression, and ii) ensure deprotonated electrospray adducts. When 0.1 % formic acid in water and acetonitrile was used, it led to ion suppression in addition to a predominance of sodium formate adducts ($[M\text{-}H + HCOONa]^-$) of the phenanthrene carboxylic acid standards. Due to a lower signal strength and an absence of CCS prediction models for such adducts, the original mobile phase system without additives was preferred. As a result, the isotopes had better intensity and could be utilized to elucidate the chemical formula.

The extraction method used herein was designed for environmentally relevant samples containing lower concentrations of metabolites, whereas in this study fish were exposed to high concentrations of alkyl PAH by injection. The amount of glucuronidase and sulfatase enzymes to convert phase II products into their corresponding phase I products was therefore probably insufficient. The results here can thus not be used quantitatively to evaluate the significance of the metabolic capacity to carboxylic acids. The aim of the study was to examine whether PAAs were formed or not, and not to assess the relative amounts of the different predicted metabolites. In such cases, a wider range of both intrinsic (*e.g.* age and sex) and extrinsic (*e.g.* dose, duration, and exposure route) factors requires consideration (Oost et al., 2003).

3.2. Relevance to environmental monitoring

Environmental monitoring of biliary PAH metabolites has historically focused on a suit of monohydroxylated compounds (Beyer et al., 2010). The restricted selection of metabolites may therefore lead to an

Table 1
Tentatively identified polycyclic aromatic acid (PAA) metabolites produced by Atlantic haddock after exposure to 1-methylphenanthrene (1-MPHE) and 1,4-dimethylphenanthrene (1,4-DMPHE), and example molecular structures. Mass error is calculated from monoisotopic mass. The exact substitution position of the hydroxy and carboxylic acid groups is indeterminable.

Treatment	Metabolite	Molecular structure	Observed mass (*m/z*)	Mass error (mDa)	Diagnostic fragment (*m/z*)
1-MPHE	*Hydroxyphenanthrene-carboxylic acid*		237.0557	0.0	193.0669
	Dihydroxy-dihydrophenanthrene-carboxylic acid		255.0658	−0.5	211.0761
1,4-DMPHE	*Methyl-phenanthrene-carboxylic acid*		235.0762	−0.2	191.0867
	Hydroxy-methyl-phenanthrene-carboxylic acid		251.0712	−0.2	207.0813
	Dihydroxy-dihydromethyl-phenanthrene-carboxylic acid		269.0823	0.4	225.0919

incomplete PAH exposure assessment. Particularly since PAAs are primary metabolites in marine organisms like the ragworm *Nereis diversicolor* (Malmquist et al., 2015). Not considering PAAs in such cases would result in an underestimated exposure as alkyl PAHs typically represent >80 % of total PAHs in petroleum sources (Wang et al., 2003). Recently, 2-phenanthrenecarboxylic acid has been proposed as a biomarker of methylated PAH exposure in humans (Lin et al., 2022). Similarly, data from the present study suggests PAAs in bile may represent biomarkers for alkyl PAH exposure in fish.

Alkyl PAHs are often considered more toxic than unsubstituted PAHs (Peng et al., 2023). Moreover, metabolites of alkyl PAHs have gained attention as they exceed the toxicity of their unsubstituted parents as reactive intermediates (Bendadani et al., 2014). PAAs present a metabolite group not previously considered in the discussion about causative toxicants in crude oil. Knowledge about PAA toxicity is consequently very limited. The petroleum acid group, known as naphthenic acids, is a complex mixture of carboxylic acids that forms a highly polar fraction in petroleum. Naphthenic acids are found in pollution sources such as production water discharges, as a result of oil weathering or spills of biodegraded oils (Aeppli et al., 2012; Rowland et al., 2011; Scarlett et al., 2013). The class includes PAAs, like phenanthrene carboxylic acids, which have been detected in degraded crude oil (Aitken et al., 2018). However, the importance of PAAs in such mixtures and degradation products is uncertain as many of the compounds are still unidentified at a molecular level (Melbye et al., 2009). Dogra et al. (2018) investigated the toxicity and developmental abnormalities in *Danio rerio* (zebrafish) embryos after exposure to six individual naphthenic acids, including the two PAAs 1-pyrenecarboxylic acid and 2-naphthalenecarboxylic acid. For 1-pyrenecarboxylic acid, deformities were observed at 3 mM, with a half maximal effective concentration (EC_{50}) determined to be 2.3 mM. The exposure of 2-naphthalenecarboxylic acid at 25.5 mM gave an EC_{50} of 6.4 mM, representing approximately three times less potency. This highlights the diverse toxic properties of PAAs.

4. Conclusion

We have carried out an exploratory investigation of potential PAAs from exposure of a marine fish species to two alkylated derivatives of the PAH phenanthrene. Further studies are needed to investigate whether the metabolites formed in these laboratory experiments occur naturally in wild-caught petroleum-exposed fish. Improved insight into PAAs can contribute to a better design of pollution monitoring. It also paves the way for new considerations in understanding the toxic mechanisms of oil spills so that more refined assessments of the ecological risks and effects can be accomplished. The albeit tentative identifications presented here were carried out with strict identification criteria. To our knowledge, this is the first report of PAAs in fish. By this work, we emphasize the importance of including alkyl PAHs in ecotoxicological assessments as they may produce a wide array of metabolites with biomarker potential.

CRediT authorship contribution statement

Charlotte L. Nakken: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Sonnich Meier:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing. **Svein A. Mjøs:** Funding acquisition, Supervision, Writing – review & editing. **Lubertus Bijlsma:** Methodology, Resources, Writing – review & editing. **Steven J. Rowland:** Resources, Writing – review & editing. **Carey E. Donald:** Conceptualization, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Overview of predicted metabolites (Figs. S1 and S2), suspect library from predicted metabolites with SMILES and InChI Key (Table S1), supplemental spectra (Fig. S3–S7), detection results of identified metabolites (Table S2), and overview of collision cross section predictions and measurements (Table S3).

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