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This report presents the results of marine and freshwater scientific work carried out by Marine Scotland Science.

Feasibility of Using a High-Performance Liquid Chromatography with Fluorescence Detection Based Method for Analysis of Polycyclic Aromatic Hydrocarbons in Bile Metabolites from Fish for the UK Clean Seas Environment Monitoring Programme.

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Executive summary

The aim of this report is to determine the feasibility of replacing the synchronous fluorescence scanning (SFS) method of analysis of biliary polycyclic aromatic hydrocarbons (PAHs) metabolites with a high-performance liquid chromatography with fluorescence detection (HPLC-F) method. This is being considered because it was highlighted during a recent international interlaboratory calibration (BEQUALM, 2020), organised by the ICES Working Group on Biological Effects of Contaminants (WGBEC), that overall the HPLC-F method performed better than SFS.

SFS is a rapid test and more cost effective than HPLC-F, however, HPLC-F is more specific and produces more reliable results than SFS. HPLC-F is also the recommended method for long term monitoring projects (OSPAR 2009, ICES 2005). However, Marine Scotland Science have a long time series of data using the SFS method which may be lost following a method change. Conversion factors can be applied to allow comparison between the two methods, however, these are not always reliable.

The Marine Environment Assessment Group currently have an HPLC instrument with a fluorescent detector, which could be used for PAH bile metabolites analysis. Although there will be some cost in implementing the HPLC-F method at MSS, this will be minimal as no capital expenditure is required. A method was developed at MSS in 2002 (Richardson 2002), which could be reviewed and once validated applied to routine monitoring.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants that pose significant health risk to fish. PAHs are natural components of coal and oil, and are also formed during the combustion of fossil fuels and organic material. PAHs occur as a result of natural processes such as forest fires. The main sources of PAHs in the environment include shipping traffic, combustion processes of fossil fuels, petroleum spills and “produced water” released from offshore oil drilling facilities (Maletić 2019). The lower molecular weight PAHs can taint the taste of fish and shellfish making them unsuitable for market (Davies et al. 2002). Whilst heavier PAHs such as benzo[*a*]pyrene are potentially carcinogenic to humans and animals (Harvey 1991).

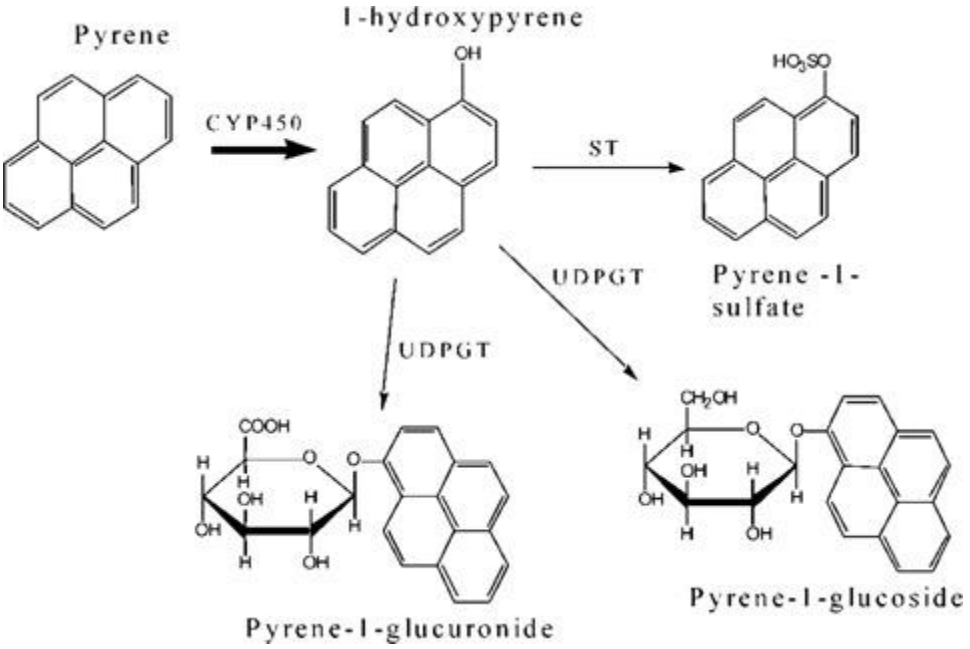
PAHs from different sources have different chemical properties and chemical analysis of PAHs allows source differentiation (Stogiannidis and Laane 2015). Due to their persistence and toxicity, PAHs are considered a hazardous substance in the marine environment and are included in international monitoring requirements. The Convention for the Protection of the Marine Environment of the North-East Atlantic or OSPAR Convention is the current legislative instrument regulating international cooperation on environmental protection in the North-East Atlantic.

Analysis of PAH concentrations in sediment and biota are included as common indicators in the OSPAR’s Coordinated Environmental Monitoring Programme (CEMP). The CEMP includes OSPAR common indicators which are used to assess the sea by measuring certain parameters in the marine environment and comparing them to agreed assessment values. It is mandatory for OSPAR registered countries to monitor PAH concentrations in sediment and biota. PAHs can accumulate in shellfish, because they are taken in either directly from the marine environment or indirectly through food consumption, however, fish metabolise and excrete PAHs rapidly, therefore, the quantification of PAHs in fish tissues only provides limited information and does not provide a true representation of exposure. Alternatively PAH metabolites can be measured and used as biomarkers for recent environmental exposure to PAHs. This analysis is useful following an oil spill and also for long term monitoring.

In fish PAHs are taken up mainly via the gills then metabolised in the liver. During metabolism (Figure 1), enzymes efficiently convert PAHs to epoxides and hydroxylated derivatives and these products are further converted into highly water soluble conjugates to enable excretion (Lech and Vodicnik 1985). Different PAH parent

molecules are broken down into different metabolites. 1-Hydroxy pyrene (Figure 1), the major metabolite of the PAHs compound pyrene, is a commonly measured metabolite. It makes up a large percentage of the total PAH metabolites profile in bile of fish exposed to combustion related PAHs (Krahn et al. 1987). Metabolites are stored in the gall bladder in bile which can be extracted and analysed.

Figure 1: Pyrene biotransformation showing phase 1 and phase 2 metabolites.
Proposed biotransformation pathway of pyrene in sandworms (*Alitta virens*) (Jørgensen et al. 2005). In fish, phase 2 metabolites of pyrene are mainly pyrene-glucuronide and pyrene-sulfate however there are inter-species differences (Ikenaka et al. 2013).
CYP450 = cytochrome P450 enzymes; ST = sulfotransferaseenzymes; UDPGT = urinidinediphosphosphateglycuronosyl transferase enzymes.
Figure from Jørgensen et al. (2005).



Analysis of PAH bile metabolites in fish is one of the biological effects indicators included in the Joint Assessment and Monitoring Programme (JAMP) recommended by OSPAR (2009). This indicator is currently voluntary although it has been put forward as a potential common indicator which means it may become mandatory in the future. Nevertheless, the analysis of bile metabolites is recommended by the International Council for the Exploration of the Sea (ICES) and is included in the contaminants and effects monitoring which is undertaken in Scottish coastal and offshore areas as part of the UK Clean Seas Environment Monitoring Programme (CSEMP). The current analytical method at MSS uses synchronous fluorescence scanning (SFS) to determine PAH metabolites in fish bile. This method is not included in MSS's scope of international

standard ISO 17025 accreditation by the United Kingdom Accreditation Service (UKAS) however this would be a requirement if PAH bile metabolites did become an OSPAR common indicator.

Assessment criteria

There are internationally agreed OSPAR assessment criteria for PAH bile metabolites (Table 1). The assessment criteria were developed within the OSPAR framework with scientific advice from ICES. Background Assessment Criteria (BAC) were derived from reference sites (ICES 2010). Values below BAC indicate a background response whereas values above BAC indicate fish have been exposed to PAHs. Environmental Assessment Criteria (EAC) have been calculated from toxicological experiment data (Davies and Vethaak 2012). Values below the EAC indicate no chronic effect on the organism. If the EAC is exceeded, this indicates PAHs exposure at levels that cause significant harm, which may pose an unacceptable risk to the environment and its living resources. PAH bile metabolites assessment criteria are species specific however often a species specific BAC/EAC has been based on data from a different but similar species e.g. flounder and dab. The assessment criteria are also method specific.

Table 1: Method specific OSPAR Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC) for bile metabolites 1-hydroxy pyrene (1-OH pyrene) (equivalents) and 1-hydroxy phenanthrene (1-OH phenanthrene).
HPLC-F = High-performance liquid chromatography with fluorescence detection, SFS = synchronous fluorescence scanning and GC-MS = gas chromatography-mass spectrometry. Note different units used for each method.

Bile Metabolites	Species	Common Name	Method	Units	BAC	EAC
1-OH pyrene	*Gadus morhua*	Cod	HPLC-F	ng ml^{-1}	21.00	-
1-OH pyrene	*Gadus morhua*	Cod	GC-MS	ng g^{-1}	-	483
1-OH pyrene	*Scophthalmus maximus*	Turbot	GC-MS	ng g^{-1}	-	909
1-OH pyrene	*Hippoglossus hippoglossus*	Halibut	GC-MS	ng g^{-1}	-	745
1-OH pyrene	*Limanda limanda*	Dab	HPLC-F	ng ml^{-1}	16.00	-

1-OH pyrene	*Platichthys flesus*	Flounder	HPLC-F	ng ml^{-1}	16.00	-
1-OH pyrene	*Melanogrammus aeglefinus*	haddock	HPLC-F	ng ml^{-1}	13.00	-
1-OH pyrene equivalents	*Gadus morhua*	cod	SFS	µg ml^{-1}	1.10	35
1-OH pyrene equivalents	*Scophthalmus maximus*	Turbot	SFS	µg ml^{-1}	-	29
1-OH pyrene equivalents	*Hippoglossus hippoglossus*	Halibut	SFS	µg ml^{-1}	-	22
1-OH pyrene equivalents	*Limanda limanda*	dab	SFS	µg ml^{-1}	0.15	22
1-OH pyrene equivalents	*Platichthys flesus*	flounder	SFS	µg ml^{-1}	1.30	29
1-OH pyrene equivalents	*Melanogrammus aeglefinus*	haddock	SFS	µg ml^{-1}	1.90	35
1-OH phenanthrene	*Gadus morhua*	cod	HPLC-F	ng ml^{-1}	2.70	-
1-OH phenanthrene	*Gadus morhua*	cod	GC-MS	ng g^{-1}	-	528
1-OH phenanthrene	*Scophthalmus maximus*	Turbot	GC-MS	ng g^{-1}	-	1832
1-OH phenanthrene	*Hippoglossus hippoglossus*	Halibut	GC-MS	ng g^{-1}	-	262
1-OH phenanthrene	*Limanda limanda*	dab	HPLC-F	ng ml^{-1}	3.70	-
1-OH phenanthrene	*Platichthys flesus*	flounder	HPLC-F	ng ml^{-1}	3.70	-
1-OH phenanthrene	*Melanogrammus aeglefinus*	haddock	HPLC-F	ng ml^{-1}	0.80	-

- denotes that assessment criteria have not been developed

Analytical methods to determine biliary PAH metabolites

There are a number of methods suitable for determination of PAH metabolites in fish bile. Simple fluorescent based techniques can be suitable, depending on the testing requirement. These semi-quantitative methods do not look at individual compounds, they simply measure anything that fluoresces so will include all PAH metabolites. If analysis of specific metabolites is required, quantitative methods such as high-performance liquid chromatography with fluorescence detection (HPLC-F) or gas chromatography-mass spectrometry (GC-MS) are required.

Different methods require different sample preparation of the bile. The PAH metabolites present in bile exist as hydrophilic conjugates such as pyrene glucuronides (Figure 1). For quantitative techniques, the bile must be pre-treated with a hydrolysis step, which can be time consuming. Simple rapid fluorescence based methods do not require a pre-treatment step as conjugated metabolites can be measured directly. For these methods, non-hyrdolysed bile can be simply diluted and analysed.

The ICES TIMES review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile (ICES 2005) discusses the advantages and disadvantages of the various existing methods, rather than providing standard operating procedures for a specific technique. MSS currently use the SFS method, which is also used by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) so there is a consistent approach across the UK.

Synchronous fluorescence scanning (SFS) method

The SFS method is primarily a screening method and measures the total presence of several fluorescent compounds as the 1-hydroxy pyrene equivalent concentration. An external calibration is performed with 1-hydroxy pyrene standard solutions and the results reflect the sum of all PAH metabolites calibrated against 1-hydroxy pyrene. Therefore the results are expressed in equivalents of 1-hydroxy pyrene.

The benefits of SFS is that is it sensitive, cost effective, rapid and allows to distinguish between exposed and un-exposed fish. OSPAR assessment criteria including BACs and EACs (Table 1) have been developed for dab (*Limanda limanda*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*), three out of four species sampled in the current Scottish CSEMP. MSS have a long history of using this method and some time

series have almost 20 years of data. However, this method cannot identify specific metabolites if this is required.

High-performance liquid chromatography with fluorescence detection (HPLC-F

HPLC-F is another method suitable for screening large numbers of fish for exposure to PAHs by analysis of total PAH metabolites in non-hydrolysed bile. However, with the addition of a pre-treatment hydrolysis step, specific metabolites can be identified. HPLC-F uses a combination of excitation/emission wavelengths to identify specific PAH metabolites including 1-hydroxy pyrene. 1-Hydroxy phenanthrene, a metabolite of phenanthrene, is also another common metabolite identified with HPLC-F. BAC have been developed for both these metabolites in a range of species including dab, flounder and cod (Table 1). Although the HPLC-F method is more labour intensive and requires more time for instrument operation than the SFS method.

The benefits of using HPLC-F is that it provides more information about the specific metabolites present. Metabolite patterns are readily discernible and these patterns can assist in establishing the source of pollution. HPLC-F is most suitable when combustion is the main source of PAHs and it gives poor results when PAHs source is mainly petrochemical (ICES, 2005). The HPLC-F method has been trialled at MSS in the past (pre 2010) for specific studies but has not been used in long term monitoring. A detailed method has been developed by a PhD student at the Marine Laboratory (Richardson 2002).

Gas chromatography–mass spectrometry (GC-MS)

GC-MS is another method, which can identify specific metabolites. EAC have been developed for 1-hydroxy pyrene and 1-hydroxy phenanthrene determined by GC-MS in cod (Table 1). Similarly to HPLC-F it is more labour intensive and requires more instrument time than SFS, it also requires more pre-treatment of samples including derivatisation. It is most suitable when identifying bile metabolites in complex chromatograms and the method of choice for identifying PAH metabolites following an oil spill.

Conversion factor

In order to compare the results of specific metabolites, for example 1-hydroxy pyrene identified by HPLC-F or GC-MS, to results from SFS methods, where 1-hydroxy pyrene equivalents are reported, a conversion factor can be applied. Studies have used both methods to determine 1-OH pyrene (equivalents) and by comparison of results have concluded suitable conversion factors. Ariese et al. (1993) suggest that the SFS results need to be corrected by dividing by 2.2 in order to compare the HPLC-F method. This conversion factor is widely accepted in fish and has successfully demonstrated converting SFS results to allow comparison to HPLC-F and GC-MS during intercalibration exercises (Kammann et al. 2013). Tairova et al. (2012) used a study specific conversion factor of 1.7 to convert SFS to compare to HPLC-F. They analysed samples by both method and concluded that dividing SFS results by 1.7 was sufficient to compare to HPLC-F. However neither conversion factor can be used to convert between assessment criteria (Table 1). Often, conversation factors do not account for all variation and can be affected by seasonal or local issues.

International interlaboratory calibration exercise

During a recent Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) laboratory intercalibration comparison exercise (BEQUALM 2020) organised by the ICES Working Group on Biological Effects of Contaminants (WGBEC) it was highlighted that the SFS method of analysis of 1-hydroxy pyrene equivalents in bile metabolites did not perform as well as the HPLC-F method. To assess performance in the intercalibration exercise, individual Z scores were calculated for statistical comparison between participating laboratories. The Z scores were calculated using the formula

$$z\ score = \frac{(measured\ value - mean\ value\ from\ all\ laboratories)}{standard\ deviation\ from\ all\ laboratories}$$

using 1-hydroxy pyrene data from HPLC-F analysis, and corrected (divided by 2.2) 1-hydroxy pyrene equivalent data from SFS analysis. MSS Z score results from the comparison exercise were satisfactory (<2), however, Z scores were tighter for labs using the HPLC-F method (between -1 and 1) compared to the SFS method (between one and two) and MSS reported two samples which had Z scores closer to 2. Z scores greater than two are unacceptable.

In the exercise, SFS was only used by two of the participating laboratories, MSS and CEFAS. Whereas the majority (four out of eight) of laboratories that participated in the exercise used HPLC-F. None of the participating laboratories used GC-MS.

International use and recommendations

HPLC-F is increasingly becoming a popular method for analysis of biliary PAH metabolites. Data submitted to ICES from Norway, Germany and the Netherlands shows that these countries are using HPLC-F for PAH bile metabolites monitoring. Whilst in the UK both CEFAS and MSS use SFS. Although it is ideal that there is consistency in the UK, it would be more beneficial to all countries if the same method was used internationally so that results can easily be compared without the need for conversation factors. HPLC-F has also been used in a number of recent publications including work in the Integrated Assessment of Contaminant Impacts on the North Sea (ICON) project (Kammann et al. 2017), in Brazil (de Albergaria-Barbosa et al. 2018) and in Finland (Vuorinen et al. 2017). Overall HPLC-F has become the standard international method for analysis of PAH bile metabolites.

Although the ICES TIMES publication (ICES 2005) is not for a specific method of analysis, it does make some recommendations. Exploratory studies can use a rapid screening method (including SFS), especially if large numbers of samples are to be analysed in the same laboratory. However these screening methods are not recommended if interlaboratory comparability is important. For long-term, international monitoring programmes specific metabolites should be determined, requiring more elaborate chemical analysis (including HPLC-F and GC-MS).

The main source of PAHs should also be considered when deciding which method to use. If the main source is pyrolytic and there is a higher proportion of heavier parent PAHs then HPLC-F techniques are preferred. However, if there is a higher proportion of smaller PAHs, which would be the case following an oil spill, the HPLC-F method gives poor resolution and therefore the GC-MS method is preferred. For routine monitoring most sites will show a predominately pyrolytic source of PAHs, and, therefore, HPLC-F is a more suitable technique.

Furthermore, HPLC-F is also the recommended methodology in the JAMP guidance (OSPAR 2009) for long term monitoring projects.

Feasibility of implementing HPLC-F at MSS

A high-performance liquid chromatography (HPLC) system with fluorescence detector (Shimadzu) is available at MSS which could be used for PAH bile metabolites analysis (https://www.shimadzu.com/an/hplc/prominence/uflc.html). This instrument was purchased 5-6 years ago to analyse algal toxins but is no longer in use. The method developed as part of the PhD project (Richardson, 2002) used a Vydac C18 column for the separation of the PAH metabolites. Shimadzu recommend a C18 column for the analysis of PAH metabolites. This costs approximately £500 and would last for around 500-1000 injections working out at about £0.50-£1 per sample. The service cost would be about £2,000-£3,000 annually depending on chosen package. This is twice the current SFS instrument service cost, however still relatively small in comparison to other instruments. Another cost to consider is the solvent used to prepare the sample, and the standard used to calibrate the results. There is little difference in these costs between the two methods, although the HPLC-F method does use a greater volume of solvent. The total cost per sample would be dependent on the number of samples processed annually.

Drawbacks of implementing HPLC-F

The main issue in implementing a HPLC-F method at MSS is there will be a significant change in our time series. The new time series would not be directly comparable with previous data and time series of almost 20 years could be lost. It may be possible to use a conversation factor (Ariese et al. 2013; Tairova et al. 2012) or alternatively, both method could be run simultaneously and a specific conversation factor for the Scottish data determined.

Another obstacle with the HPLC-F method is that there is limited assessment criteria. The assessment criteria for bile metabolites are method specific (Table 1). The current MSS fish monitoring programme focusses on dab, however, plaice, flounder and cod are also sampled. Currently there is only a BAC for dab, flounder and cod from HPLC-F data whereas there is BAC and EAC developed for SFS in these species. There are no assessment criteria available for plaice for either method. This is a major drawback to implementing HPLC-F at MSS as EACs are very useful in assessments to determine if significant harm is being caused.

However, the assessment criteria for some species were actually based on data from another species (Davies and Veethak 2012) as follows:

- SFS EAC for dab was based on data from halibut.
- SFS EAC for flounder was based on data from turbot.
- HPLC BAC for flounder was based on data from dab.

Therefore, going forward during assessments the BAC derived from dab data which has already also been used for flounder could also be used for plaice. This could be further developed as data from plaice is gathered provided background sites are included in the monitoring programme. A proposed BAC for plaice would need to be agreed at international level.

Determining EAC would be more difficult, the previously established EACs were determined from toxicology experiment data (Davies and Vethaak 2012). MSS has only had a single result (2012-2018) above the SFS EAC. It may be feasible to convert the SFS EAC if a suitable conversation factor is determined from running the two methods simultaneously. EAC have been developed for turbot and halibut using GC-MS analysis. Since GC-MS and HPLC-F are comparable (Kammann et al. 2013) then these assessment criteria may be suitable considering SFS EAC for flounder and dab that has been used was originally determined from turbot and halibut data (Davies and Vethaak 2012). This would require discussion at WGBEC.

If MSS do change to HPLC-F and CEFAS continue to use SFS this will make UK assessments very difficult however CEFAS are currently undertaking a similar review and discussions will continue through the UK Biological Effect of Contaminants on the Marine Environment group (BECME).

Conclusions

The HPLC-F method is the recommended method for analysis of PAH bile metabolites for long term monitoring studies (ICES 2005, OSPAR 2009). Although there will be some cost in implementing the HPLC-F method at MSS this will be minimal as capital expenditure is not required. Implementation of the HPLC-F method is necessary to bring our CSEMP in line with international recommendations. However, the major drawback to this will be the loss of the current time series therefore ways of minimising the impact

of this must also be considered. Running the two methods in parallel during validation may help compensate this.

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