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PAH Metabolites in Bile of European Eel (*Anguilla anguilla*) from Morocco

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Abstract Environmental pollution of fish with organic contaminants is a topic of rising attention in Morocco. Polycyclic aromatic hydrocarbons (PAH) are prominent organic contaminants which are rapidly metabolized in fish. Their metabolites are accumulated in the bile fluid and can be used to assess PAH exposure. The two PAH metabolites 1-hydroxypyrene and 1-hydroxyphenanthrene were quantified in European eels (*Anguilla anguilla*) from two Moroccan river systems by high-performance liquid chromatography with fluorescence detection. Mean values ranged from 52 to 210 ng/mL 1-hydroxypyrene and from 61 to 73 ng/mL 1-hydroxyphenanthrene. The overall concentrations of PAH metabolites in eel from Morocco appeared moderate compared to eel from European rivers and coastal sites. The present study provides first information on concentrations of PAH metabolites in fish from Morocco.

Keywords PAH · Fish · Fresh water · Pollution · Morocco

Environmental pollution of fish is a topic of rising attention in Morocco (Bouachrine et al. 1998; El Morhit et al. 2009; Wariaghli et al. 2013). However, only few studies have been carried out so far, describing the potential threat of organic pollution to Moroccan aquatic ecosystem (Azdi et al. 2006; Chafik 2009; Er-Raioui et al. 2009; Hajjaj Hassani et al. 2006). The contamination with polycyclic aromatic hydrocarbons (PAH) has not yet been studied in fish from Moroccan waters. PAH are ubiquitous environmental contaminants found in marine sediments and waters associated with urbanized estuarine and coastal pollution as well as in rivers (Blahová et al. 2010; Meador et al. 1995). PAH are derived from both natural and anthropogenic sources. The latter can be related to pyrolysis and incomplete combustion of organic matter (Eisler 2007). Natural sources for PAH are e.g. forest fires and degradation of biological materials, which has led to the presence of these compounds in sediments and to the formation of fossil fuels (Eisler 2007). For the aquatic environment wastewater, atmospheric deposition and petroleum spillage are further prominent sources. PAHs and their intermediate degradation products have the potential to induce toxic or mutagenic effects in fish (Brinkmann et al. 2010, 2014; Monteiro et al. 2000) and in humans (Chen and Liao 2006).

PAH metabolites in the bile fluid are widely accepted as measures for PAH exposure in fish because of the rapid metabolization of PAH in vertebrates (Meador et al. 1995). As a consequence, PAH metabolites in fish are recommended as core monitoring parameters in European Seas (HELCOM 2012; OSPAR 2008). High performance liquid chromatography (HPLC) is widely used for the determination of PAH metabolites in different fish species (Harman et al. 2009; Kammann 2007; Pikkarainen 2006; Tairova et al. 2009; Vuorinen et al. 2006) and has been covered in a recent intercalibration exercise (Kammann et al. 2013).

The European eel (*Anguilla anguilla*) is probably one of the most vulnerable fish species to chemical pollution during its feeding and growth phase in freshwater as it is long-living, bottom dwelling, carnivore and has a high body fat content. Due to this, adverse effects of different

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contaminants have been speculated as a possible cause for the decline of the European eel stock, which is still considered as endangered and outside safe biological limits (ICES 2013). Since eels are quite heavily exploited in Moroccan inland waters (ICES 2011), a pollution assessment is needed, not only with regard to thresholds for human consumption but also to detect possible biological effects of contaminants on this species. Although some studies exist on PAH metabolites in eel (Kammann et al. 2014; Nagel et al. 2012a; Ruddock et al. 2003; Szlinder-Richert et al. 2014) up to now no data are available for PAH metabolites in fish from Moroccan waters. The aim of this study was to bridge this gap and to provide first information on concentrations of PAH metabolites of eels in Morocco; to evaluate spatial differences in PAH contamination and to discuss the possible threat for eel due to PAH contamination.

Materials and Methods

PAH metabolites in bile were determined by the method described by Kammann et al. (2014) with modified HPLC conditions. In brief, 25 µL fish bile were mixed with 95 µL water and 5 µL β-glucuronidase/arylsulfatase solution (30–60 U/mL). The mixture was subsequently incubated for 2 h at 37°C on a heated shaker. The enzymatic reaction was stopped by the addition of 125 µL of ethanol. After centrifugation, the supernatants were used for HPLC analysis immediately. PAH metabolites 1-hydroxypyrene (1-OH-Pyr) and 1-hydroxphenantrene (1-OH-Phen) were separated by HPLC (Lachrom System; Merck Hitachi). Samples were chromatographed on a Nucleosil 100-3 C18 (3 × 125 mm) reversed phase column at a flow of 0.55 mL/min. The initial mobile phase was acetonitrile/0.1 % trifluoroacetic acid 50/50 (v/v) changing progressively after 10 min to 60 % actonitrile over 4 min and afterwards to 100 % acetonitrile within 2 min. A fluorimetric detector was attached to the HPLC system. Standard solutions were diluted in acetonitrile with 5 mg/mL ascorbic acid. The excitation/emission wavelength pairs for 1-OH-Pyr and 1-OH-Phen were 346/384 and 256/380 nm, respectively. For the quantification of bile pigments 25 µL of bile was added to 475 µL of water and the absorbance was recorded at 380 nm in microplates (Fluostar Optima, BMG Labtech, Offenburg, Germany).

Quantification was performed via external standards using a five point calibration. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to DIN 32645 (DIN 1994). The LOD and the LOQ for 1-OH-Pyr (1-OH-Phen) was 3.4 (0.5) and 22.5 (1.7) ng/mL bile, respectively. For internal quality assurance a fish bile sample as laboratory reference material was included in every sample batch to monitor the stability of the method (variation coefficient 15 % for 1-OH-Pyr). Participation in an intercalibration exercise leads to acceptable z-scores below ±2 for the laboratory performing the chemical analysis (Kammann et al. 2013). Every bile sample was analyzed twice. Blanks, five standard concentrations and the laboratory reference material were included in every sample batch. The recovery of 1-OH-Pyr was 98 %. Certified Standard solutions of 1-OH-Pyr and 1-OH-Phen were purchased by LGC (Dr. Ehrenstorfer Standards distributed by LGC, Middlesex, UK). All other chemicals were obtained from Merck (Darmstadt, Germany).

45 European eels (*A. anguilla*) in their yellow stage were collected from Sebou and Loukkos rivers/estuaries in Morocco during October/November 2009 by local fishermen using fyke nets. Yellow eels were chosen as this developmental stage is regarded as primarily sedentary (Belpaire and Goemans 2007). Sampling locations are shown in Fig. 1. Body length and weight were recorded for each fish (Table 1). After opening the body cavity, bile fluid was collected by a disposable syringe. Bile samples of approximately 0.1–0.5 mL were immediately frozen and stored at −20°C until analysis.

The Sebou estuary (34°16′N, 6°34′W) is located in the northwest of Morocco, at the Atlantic coast (Fig. 1), draining a surface area of 40,000 km^2. The Sebou is the main purveyor of water to the Gharb plain, a major agricultural region, where fertilizers and pesticides are widely used. Fish for the present study were sampled at two sites in Sebou river: "Sebou up" receive its pollution mainly by discharges from the cities Fes and Meknes whereas "Sebou down" represent an area with highly developed industrial activity (paper mills, sugar plants, tanneries, olive oil mills, oil refineries, alcohol industries etc.). The Loukkos river (35°09′N, 6°05′W) drains a catchment of 3730 km^2 (El Morhit et al. 2012). The landscape is composed of wetlands (coastal lagoons, swamps and rice fields), forests, and agricultural zones. The Loukkos watershed is less industrialized than "Sebou down".

One-way analysis of variance (ANOVA) was applied to test for differences between sampling stations using Scheffe's test ($p \leq 0.05$).

Results and Discussion

With the present study we addressed the question if a possible exposure of eel to PAH in Moroccan rivers is reflected in spatially different concentrations of PAH metabolites and if this contamination might be a potential threat for the Moroccan eels from Sebou and Loukkos, the most important eel catchments in Morocco (ICES 2011).

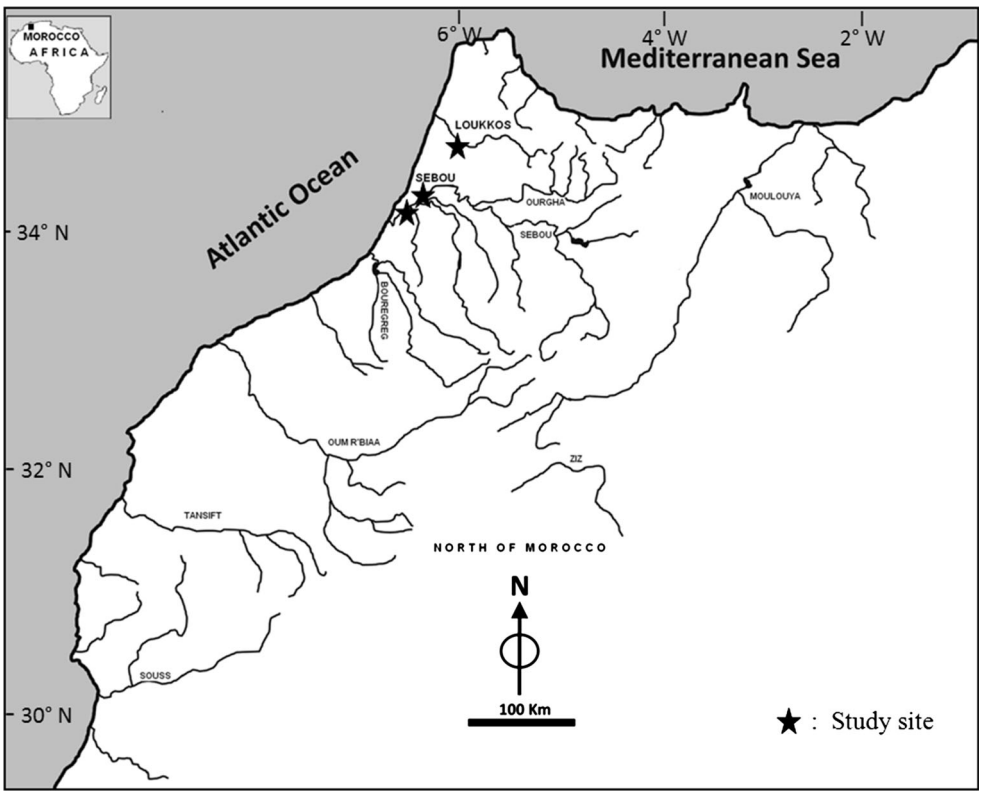

Fig. 1 Sampling locations of eel in Sebou and Loukkos (Morocco)

Table 1 Length, weight, PAH metabolites 1-hydroxypyrene (1-OH-Pyr) and 1-hydroxyphenantrene (1-OH-Phen), absorption of bile at 380 nm (AE380; arbitrary units/mL) of eel from Moroccan waters given as mean and range (in brackets)

Site	N	Length (mm)	Weight (g)	1-OH-Pyr (ng/mL)	1-OH-Phen (ng/mL)	AE380 (a.u./mL)
Sebou up	13	403 (335–550)	135 (66–296)	52 (12–221)	61 (18–152)	11 (2–36)
Sebou down	20	417 (337–610)	146 (69–490)	154 (59–545)	65 (29–183)	21 (8–125)
Loukkos	12	534 (350–900)	267 (73–556)	210 (56–453)	73 (10–305)	40 (7–144)
All fish	45	444 (335–900)	175 (66–556)	139 (12–545)	66 (10–305)	23 (2–144)

The PAH metabolites 1-OH-Pyr and 1-OH-Phen were detected in all samples (Table 1). The level of 1-OH-Pyr in eel from "Loukkos" were significantly higher when compared with "Sebou up" ($p < 0.05$; Fig. 2). The lower concentrations of 1-OH-Pyr observed at "Sebou up" reflect the less contaminated the river before entering the highly industrialized area around the Sebou basin at "Sebou down". However, highest concentrations of PAH metabolites with statistical significant difference to "Sebou up" were found in eel from Loukkos, which is a less industrialized area. Although the Loukkos estuary is known for its intense agricultural activity (El Bakouri et al. 2008) and the sampling site is in an area directly affected by discharged water especially from rice fields, this does not explain elevated PAH levels. Due to the size of the area, unknown sources of contamination cannot be fully excluded. At the same time, a movement of eels even in the yellow eel stage is another possible explanation for the detected results.

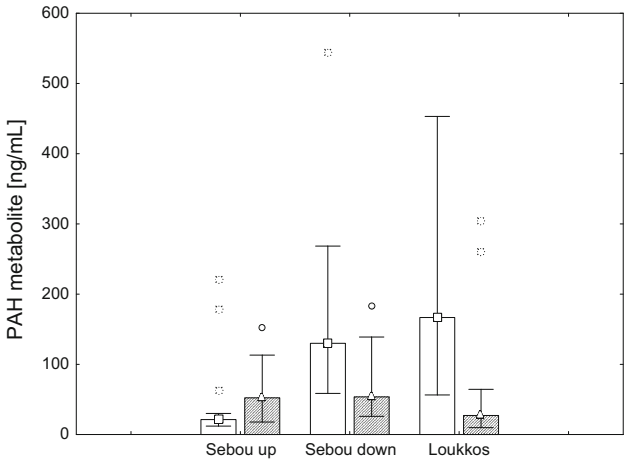

Fig. 2 Concentration of PAH metabolites (ng/mL) as median ± range (*whiskers*) extreme values (*rhomb*) and outliers (*asterisk*) in the bile of eel caught at three sites in Morocco; 1-hydroxypyrene (*white*) and 1-hydroxyphenanthrene (*grey*)

Table 2 PAH metabolite concentrations in eel (*Anguilla anguilla)* from different countries

Country	Sites	Stage	Length (cm)	1-OH-Pyr (ng/mL)	1-OH-Phen (ng/mL)	Reference
Morocco	River, lagoon	y	34–90	139 (12–545)	66 (10–305)	This study
Poland	Lagoon, lake	y	51–89	56 (3–378)	70 (13–328)	Szlinder-Richert et al. (2014)
Germany	River, lake	y	49–66	619 (111–2420)	227 (53–632)	Nagel et al. (2012a)
Germany	River, coast	y	39–77	1274 (466–3285)	302 (110–699)	Kammann et al. (2014)
United Kingdome	River, estuary	u	40–65	3200 (120–7610)	495 (23–2096)	Ruddock et al. (2003)

Means (ranges) of 1-hydroxypyrene (1-OH-Pyr) and 1-hydroxyphenantrene (1-OH-Phen). Stage: yellow eels (y) or unknown (u). Yellow eel data were selected according to maturation stage 1–3 (Durif et al. 2005)

In accordance with Nagel et al. (2012a) and Ruddock et al. (2003) 1-OH-Pyr was found to be the dominant compound in eel bile, except in samples from "Sebou up" where both metabolites are present in nearly equal concentrations (Table 1). Comparing the results of the present study to similar investigations from several European countries (Table 2), it is shown that the means of both PAH metabolite concentrations, especially in eel from the United Kingdome and Germany are higher compared to concentrations found in eel from Morocco. However, eel caught in Poland contained PAH metabolites in similar concentrations compared to the eels investigated in the present study. The studies cited in Table 2 cover eels caught in rivers, lakes or lagoons as well as in estuaries and can therefore be considered as directly comparable. Since PAH metabolite concentrations in eel bile are influenced by the developmental stage (Nagel et al. 2012b), such a comparison is only valid by looking at the same life history stage, in this case yellow eels.

To evaluate the measured PAH metabolite concentrations with respect to a possible adverse effect of PAH contamination for aquatic organisms, the results were compared to an internationally agreed threshold value for fish. Since no such thresholds exist for eel, the respective concentration for Atlantic cod (*Gadus morhua*) of 483 ng/mL bile was used (ICES 2012). Only few fish in the present study are close to this threshold value for 1-OH-Pyr. Even if this threshold has been calculated for another fish species, a comparison indicates that the PAH contamination in Moroccan eels might be of biological significance. However, the cited threshold value can only provide a rough guidance since it has been calculated for a marine teleost species with a different life history compared to the diadromous European eel. Therefore, it cannot be excluded, that PAH, especially together with other organic or inorganic contaminants or environmental impacts, may have some adverse effect for the eel.

With this study on PAH contamination in fish from Morocco information is provided on PAH metabolite amplitudes and spatial differences. Concentrations of PAH metabolites in eel appear moderate compared to fish caught in European waters and to internationally accepted threshold levels in other teleost species.

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