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PRESENCE AND BIOTRANSFORMATION OF THREE HETEROAROMATIC COMPOUNDS COMPARED TO AN AROMATIC HYDROCARBON

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The presence of nitrogen, oxygen and sulfur containing aromatic compounds, namely carbazole, dibenzofuran, dibenzothiophene and their alkylated derivatives was investigated in potential environmental sources of these compounds. The persistence vs biodegradation of the parental heteroaromatic compounds was determined using bacterial consortia collected from three marine beaches from coastal Newfoundland. Experiments were performed at 25 and 4°C and bacterial populations derive from differently contaminated environments. In separate studies, rainbow trout, *Salmo gairdneiri*, were exposed to PACs through their diet and the bioelimination of glucuronide and sulfate conjugates followed in the gall bladder bile after a single and during continuous exposure. Comparison was done between the presence and fate of the three heterocyclic PACs and that of fluorene, a PAH counterpart with similar structure and physical-chemical properties. The tissue distribution of the PACs (not presented) provides information on the potential narcotic and/or reproductive effect of the unreacted compounds and the metabolites on the potential toxicity of the oxidation products.

Keywords: dibenzothiophene; dibenzofuran; carbazole; fluorene;

bioelimination; biodegradation

INTRODUCTION

Studies involving anthropogenic contaminants such as polycyclic aromatic compounds, PACs (including hydrocarbons, PAHs) are important due to the continuous input of these compounds in the environment, their bioaccumulation in different links of the food chain, and associated biological effects. PACs are continuously discharged in the environment, where their concentration predominates in marine sediments and is associated with toxicity^[1,2]. Research concerning the fate and effect of PACs has placed emphasis on the 16 parental PAHs which are recommended priority pollutants. Although these compounds predominate in combustion sources of PACs, a wide range of other aromatic structures such as alkylated PAHs, monocyclic derivatives, phenols, polycyclic aromatic sulfur, nitrogen and oxygen heterocycles (PASHs, PANHs and PAOHs, respectively) are also present in petroleum and combustion products^[3].

Bioaccumulation studies have demonstrated that non-polar, non-ionic, non-metabolised compounds will accumulate in tissues of biota according to their water solubility and lipid affinity^[4,5]. This statement covers, to a certain extent, the fate of PACs in invertebrates, which have less active mixed-function oxygenase enzymes than vertebrates and therefore require a higher level of contaminants to induce the enzymes, but does not cover the fate in vertebrates^[6,7,8]. PACs can undergo metabolism within tissues of an organism and become toxic through non-narcotic type effects and/or they can be converted to more polar derivatives in the environment and become more bioavailable for uptake^[9,10].

The present publication presents studies investigating the presence, biodegradation and bioelimination of three representative heterocyclic compounds, dibenzothiophene, dibenzofuran and carbazole in comparison to fluorene, with emphasis on their fate in a cold water environment. These compounds were chosen because of their relatively high water solubility compared to 2-6 membered ring PACs, making them less volatile and more readily bioavailable than smaller and larger molecular weight counterparts, respectively^[11]. Comparison of the physical-chemical properties that can be used to interpret reactivity are presented in Table 1. Of these properties, the octanol water partition

coefficient, $\log K_{ow}$ is used to interpret bioavailability and bioconcentration. The surface, volume and ovality describe the relative dimensions of these compounds and could be used in studies related to the binding of PACs with macromolecules or to the induction of enzymatic activity. The electronic energy, ionisation potential, HOMO and LUMO provide insight on the potential reactivity of the heterocycles. A combination of these properties with experimental results helps to understand differences in the fate of PAHs vs PACs and of lower vs higher molecular weight compounds.

TABLE I. Physical-chemical properties of the four PACs

	$\log K_{ow}$	Surf.	Vol.	Oval.	El. A ²	E. EV	I.P. EV	LUMO EV	HOMO EV
FL	4.18	193	160	1.35	-9872		8.8	-0.33	-8.84
CARB	3.51	189	156	1.35	-9938		8.5	-0.30	-8.49
DBT	4.49	189	163	1.32	-10107		9.0	-0.62	-8.60
DBF	4.12	183	152	1.32	-9738		8.6	-0.48	-9.02

-FL: fluorene, CARB: carbazole, DBT: dibenzothiophene, DBF: dibenzofuran, $\log K_{ow}$: octanol water partition coefficient^[11,12 and ref. within], Oval.: ovality, El. E.: electronic energy, I.P.: ionisation potential, LUMO: lowest unoccupied molecular orbital, HOMO: highest occupied molecular orbital, A: angstrom, EV: electron volt.

MATERIALS AND METHOD

The presence and fingerprint of heterocyclic compounds was determined by fractionating nearly 20 mg of oil or extract (containing p-terphenyl-d₄ as an internal standard) over 3g of activated silica gel in a 200 by 10 mm i.d. chromatography column prepared according to a variation of Wang et al^[13]. Five fractions were collected, evaporated gently, transferred to vials and concentrated or diluted as needed for GC-FID and GC-HRMS analyses. Quantification was performed using five point calibration curves and is based on ions representative of the parental heterocycles and these were used to quantify the C-1, C-2 and C-3 alkylated homologues (not presented). Abundances are also

expressed relative to the parental compounds.

Surficial sediments (5-10 top cm) were collected at low tide from three beaches around Newfoundland and the detailed procedure regarding bacterial degradation can be found in Meade's MSc thesis^[14]. One location, Come by Chance, is close to an oil refinery, the Port aux Basques location is near a railway terminal and petrochemical products, while the Bonne Bay location is near a ferry terminal. The experimental scale was 5 g of sediments in 500 ml screw capped vials containing 200 ml of mineral medium^[14,15], where collected sediments containing the local bacterial population were spiked with the PAC of interest. Metabolism of the compounds and disappearance of the starting material was observed using a Hewlett Packard 5890A GC coupled to a HP 5970 mass selective detector equipped with a 25 m, 0.25 µm i.d. CP-Sil 5CB column.

Five tanks with aerated, circulating fresh water (400 L) were used for single oral exposures. Each experiment contained twenty four rainbow trout, *Salmo gairdneri*, weighing 15-18g/per fish. Trout were fed to satiation and acclimated to 10°C for several weeks prior to the exposure of each group to olive oil containing fluorene, dibenzothiophene, dibenzofuran, carbazole or no additive. Fish were orally injected with 0.1ml of a 1% solution of the PAC and placed in the individual tanks, where they were not fed after exposure. Three fish were sacrificed from each tank on days 3, 4, 5, 6, 7, 10, 12 and 14, the gall bladder bile was removed and frozen at -40°C until analysis. Volumes of bile ranged between 20 and 30 µl/ fish.

Gall bladder bile metabolites were analysed after enzymatic hydrolysis^[16] using β-glucuronidase and sulfatase from limpets. The presence of PAC was quantified using ultra violet fluorescence spectroscopy at the wavelength pair of 280/305 nm and compared to 310/360 nm. for each PAC. Bile extracts were also analysed by GC-MS.

Two tanks containing thirty two rainbow trout weighing 300-330g each were acclimated at 5 and 10°C for several weeks prior to a long-term exposure. The amount of food required to reach satiation while feeding 5days/week was determined. Food spiked with a mixture containing the four PAC in a 1:1:1:1 ratio and representing 8µg PAC/ g fish/ day (each) was offered slowly at different corners of the tanks. Eight fish were sampled from each tank after feeding for 5, 10, 15 and 19 weeks. Fish were dissected and morphometrics obtained on whole body

weight, liver weight, fatty tissue and internal organs. Between 3 and 8ml of blood were also obtained from each fish.

RESULTS AND DISCUSSION

The relative abundance of DBT, DBF and carbazole and their alkylated homologues was determined in various sources of PACs such as coal, coke, crude oil, and in specific matrices of interest in previous exposure studies conducted in our laboratory (Hibernia crude oil, harbour sludge, weathered crankcase oil)⁽¹⁷⁻¹⁹⁾. Earlier interest concentrated on the presence and fate of PAHs. Column chromatography followed by GC-HRMS demonstrated an overlap in the fractionation of PAHs, PASHs and PAOHs in fraction 2 (1:10, hexane: CH_2Cl_2), but the GC-HRMS enabled the detection of the analytes of interest due to the high specificity of the isotopic masses and the efficient gas chromatographic separation.

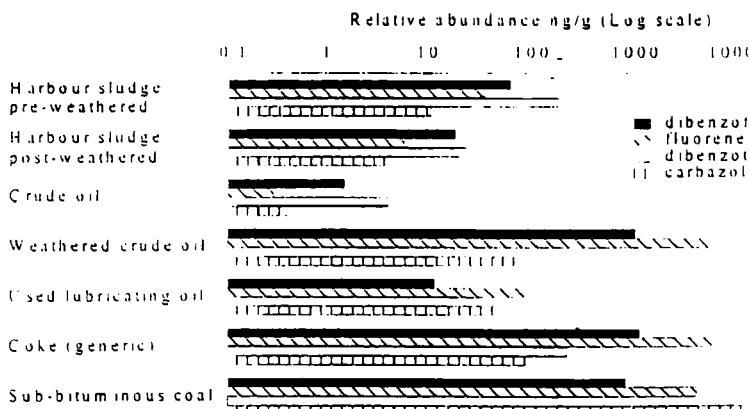


FIGURE 1 Relative abundance of PACs

The presence of PANHs was predominantly observed in fraction 3 (1:1, hexane: CH_2Cl_2), but some tailing was apparent in the next two fractions. This result reflects the range of polarities present within the nitrogen series and indicates that a study focusing on PAHs could determine the presence of PASHs and PAOHs, but not easily that of PNAHs. Of the matrices analysed, coal contained the largest proportion

of carbazole, followed by coke, weathered crude oil and used lubricating oil (Figure 1). DBT was present in a relatively similar abundance in harbour sludge, coke and coal. DBF was relatively as abundant in coal, coke and weathered Hibernia crude oil, present at a lower level than fluorene, but higher than DBT. The proportion of the 3 heterocycles varied between sources and was substantial when compared to fluorene, emphasizing the importance of determining the environmental fate of heterocycles. The fingerprint obtained for the alkylated homologues after four months weathering of some sources confirms the higher bioavailability (solubility) and/or reactivity (degradation) of the lower molecular weight parental compounds, since the sediments become enriched in the alkylated derivatives (not presented).

In the biodegradation study, microbial degradation of the heterocycles was investigated using local marine bacterial consortia at a conventional temperature of 25°C and at one more typical of locations in the Northwest Atlantic slated for petroleum exploration, 4°C^[14,20]. This study represents the first attempt to investigate the ability of local bacteria to biotransform PACs spiked in collected sediments. In general, bacterial degradation was 3 or 4 times slower at 4°C than at 25°C, and each target compound was affected differently according to location. At 25°C, carbazole degraded the least at all locations over the 12 day experiment, DBF degraded the most near the ferry terminal and DBT near the oil refinery. At 4°C, DBT degraded the least at all locations. DBF degraded the most near the ferry terminal and carbazole near the refinery. Bacterial degradation using the local consortia tended to be complete, with no isolation of intermediates, except for a small percentage of sulfoxide collected from the biodegradation of another PASH, benzothiopene^[14]. The variation in degradation demonstrates the importance of studying these heterocyclic components along with the PAHs^[21].

A short-term experiment concentrated on the bioelimination of bile metabolites after a single oral exposure of rainbow trout to a PAC. Metabolism was followed as with PAHs, using β -glucuronidase and some sulfatase to hydrolyse conjugated derivatives. Phase 1 oxidation products known to form glucuronides include alcohols, carboxylic acids, secondary amines and primary sulfides, while alcohols and secondary amines can also form sulfate conjugates. The fate of xenobiotics in terms of biotransformation products can include

methylation, acetylation, glutathione and amino acid derivatives. The analytical approach used here would detect the presence of methylation and acetylation products.

After a single oral exposure of rainbow trout, bile metabolites were analyzed over a two weeks period (Table II). The level of fluorene metabolites (4 detected) increased starting on day 5 and reached a maximum on day 10. The production of DBF metabolites (4 detected) increased starting on day 7 and peaked on day 10. In the case of carbazole, less metabolites were eliminated than with DBF or fluorene. Finfish exposed to DBT produced no detectable uv/f response in hydrolyzed bile extracts during the 2 weeks (DL of DBT: 1 ng/ μ l compared to 0.01 ng/ μ l for the others). Bile extracts analysed by GC-MS indicated that the phase I oxidation products were due to the addition of one oxygen to the original PACs. Under the present analytical conditions, after a short-term single oral exposure, heterocyclic aromatic compounds appear to display a lower rate of elimination of glucuronide and sulfate conjugates than the corresponding PAH. This observation needs follow up, since this is a semi-quantitative method using the starting material as standard.

Table 2. Metabolites ng/ μ l of bile

Compounds	Days post exposure							
	3	4	5	6	7	10	12	14
Carbazole	NA	NA	2	2.5	2.2	3.8	4.5	3.8
DBF	2.4	2.4	2.5	3.1	3.3	5.8	4.8	2.2
Fluorene	7	7	8	11	14	15	10	8
DBT	ND	ND	ND	ND	ND	ND	ND	ND

NA: not available. ND: not detected.

The short-term experiment represented an acute type exposure and was followed by a longer exposure of trout to food pellets containing a mixture of the four PACs. In this case, we examined the bioelimination and bioaccumulation of contaminants in muscle, liver, fatty tissue, internal organs and blood, after 5, 10, 15 and 19 weeks exposure and amount of material eliminated in faeces, due to lack of absorption through the digestive system. Mainly the results relating to the bioelimination of metabolites are presented here. Morphometrics obtained on fish collected from the chronic exposure displayed

significant positive Pearson correlations between fish weight and the above organ weights. However, a significant negative correlation was observed between the gall bladder weight and the level of metabolites observed in each fish ($p<0.01$), as well as a negative correlation between liver index and level of metabolites in whole bile, although less significant ($p<0.1$). The ensuing result was that a constant bioelimination of metabolites was observed over time, when expressing concentrations in terms of whole gall bladder bile content. Figure 2 presents the intensity of the fluorescing bile metabolites expressed in terms of microliter of bile, in comparison to the intensity expressed in terms of total bile volume.

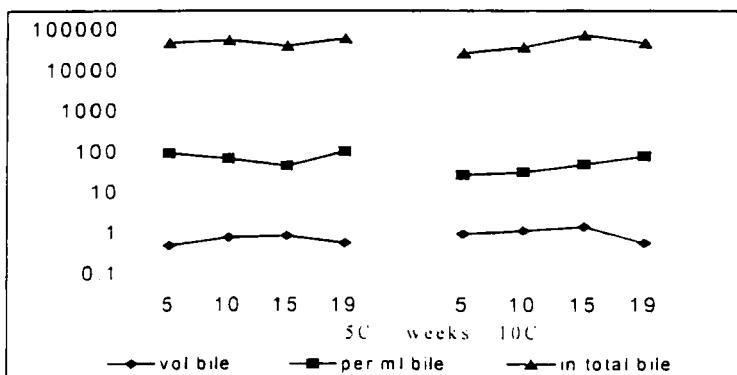


Figure 2: Mean fluorescence intensities and volumes (ml) observed in gall bladder bile

Levels of detected bile metabolites tended to be 0.5 to 1.8 times higher at the 5 compared to 10°C. GC-MS analysis of these extracts indicated the predominance of fluorene and DBF metabolites, much lower levels of carbazole and traces of DBT metabolites. This relative order is in agreement with observations from the short-term experiment. However, direct HPLC analysis of conjugates present in the gall bladder bile indicates the presence of uncharacterized derivatives for each of the used heterocycles.

The toxicity of phase I metabolites, that is of oxidation products, is related to the time they persist in the body and to the reactivity of products formed. A comparison of the production of metabolites in time could indicate the relative potential toxicity of the compounds, although

a higher reactivity and toxicity is associated with the larger molecular weight PAHs (>3 rings)^[23]. Interpretation would also necessitate investigations of biological effects due to exposure and comparison to the fate of larger molecular weight heterocycles. As well, since the fate of xenobiotics in finfish involves bioaccumulation, biotransformation and reactivity with macromolecules or biotransformation and bioelimination, as well as excretion, investigation of the other fates will enhance our ability to model fate and assess the possible toxicity of these anthropogenic contaminants^[23,24].

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