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Toxicokinetic of benzo[a]pyrene and fipronil in female green frogs (Pelophylax kl. esculentus)

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1. Introduction

Among freshwater media, wetlands are complex aquatic ecosystems often exposed to chemical stress caused by the accumulation of xenobiotics (e.g. PAHs, PCBs, personal care products, agrochemicals, natural compounds …) streamed by water (Zhang et al., 2010). To assess the potential toxicity of these pollutants, it is important to understand their accumulation and fate in aquatic organisms. It has been widely expected that the effects of xenobiotics depend on their uptake, tissue distribution and metabolism (biotransformation). A general consensus that an increased logKow led to an increased xenobiotic uptake and bioaccumulation is accepted. In this study we compared the toxicokinetics of two chemically different xenobiotics, i.e. benzo[a]pyrene and fipronil in female green frogs. Surprisingly, the uptake rates and the bioconcentration factors (BCF) of the two contaminants were not predicted by their logKow. The uptake rates obtained were of the same order of magnitude for the two contaminants and the BCFs measured for fipronil were about 3-fold higher than those obtained for benzo[a]pyrene. Fipronil appeared to be more recalcitrant than benzo[a]pyrene to detoxification processes leading to the accumulation of sulfone-fipronil especially in the ovaries. This phenomenon may explain reproductive influence of this contaminant described in other studies. Detoxification processes, including metabolism and the excretion of pollutants, are of importance when considering their persistence in aquatic organisms and trying to quantify their risks.

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Abstract

A general consensus that an increased logKow led to an increased xenobiotic uptake and bioaccumulation is accepted. In this study we compared the toxicokinetics of two chemically different xenobiotics, i.e. benzo[a]pyrene and fipronil in female green frogs. Surprisingly, the uptake rates and the bioconcentration factors (BCF) of the two contaminants were not predicted by their logKow. The uptake rates obtained were of the same order of magnitude for the two contaminants and the BCFs measured for fipronil were about 3-fold higher than those obtained for benzo[a]pyrene. Fipronil appeared to be more recalcitrant than benzo[a]pyrene to detoxification processes leading to the accumulation of sulfone-fipronil especially in the ovaries. This phenomenon may explain reproductive influence of this contaminant described in other studies. Detoxification processes, including metabolism and the excretion of pollutants, are of importance when considering their persistence in aquatic organisms and trying to quantify their risks.
Amphibians are key species in wetlands and constitute an important source of food for upper-level consumers. Therefore, it is important to quantify critical toxicokinetic parameters to determine how amphibians contribute to the transfer of persistent organic pollutants in the food web (Lenny et al., 2006). The sensitivity of amphibians to xenobiotics has been linked to the specific structure of their skin which is highly permeable (Hayes et al., 2006; Quaranta et al., 2009). It has been shown that a xenobiotic can diffuse through frog skin one or two orders of magnitude (depending of its hydrophobicity) faster than through mammal skin (Quaranta et al., 2009). This phenomenon is emphasized in amphibians by the slow rate of elimination of hydrophobic chemicals in anurans (Lenny et al., 2006). Indeed, understanding toxicokinetic processes is fundamental to better grasp xenobiotic fate and their potential effects on amphibians. Since very few toxicokinetic studies have been conducted in amphibians, the aim of the present work was to compare the toxicokinetics of two widespread contaminants present in contaminated wetlands, benzo[a]pyrene and fipronil, in adult green frogs. According to their physicochemical properties, we hypothesized both a higher uptake rate and BCF value for BaP (log Kow = 6), compared to fipronil (log Kow = 2.74). The uptake, distribution, metabolism, and activities of key enzymes involved in biotransformation were analyzed for these two chemically different xenobiotics. In light of these findings, potential reproductive dysfunction and embryo development impairment leading to decreased fitness in amphibian populations have been further discussed and linked to differences in the metabolism of these two model compounds.

2. Materials and methods

2.1. Chemicals

Benzo[a]pyrene (97% purity) and 7-14C-benzo[a]pyrene were purchased from Sigma Aldrich (France). 3-hydroxy-benzo[a]pyrene was purchased at NCI. Fipronil (85 mBq mL⁻¹) was purchased at Sigma Aldrich (France). Benzo[a]pyrene (97% purity) and 7-14C-benzo[a]pyrene were purchased from Sigma Aldrich (France). 3-hydroxy-benzo[a]pyrene was purchased at NCI. Fipronil (85 mBq mL⁻¹) was purchased at Sigma Aldrich (France).

2.2. Animals

Green frogs (Pelophylax kl. esculentus (Linneé, 1758)) were obtained from the rearing center at Ardenay (France). The average weight of the frogs was 50 ± 1.05 g. The frogs were fed daily ad libitum with flies and worms. They were allowed to acclimatize for 3 weeks at 22 °C with a photoperiod of 12 h:12 h in constantly filtered water prior to starting the experiments.

2.3. Frog exposure to benzo[a]pyrene and fipronil

Frogs were exposed individually at room temperature (21 °C ± 2 °C) in 1 L glass jars protected from the light. The exposure solutions consisted of 500 mL tap water (pH 7.8, organic carbon – 0.6 mg L⁻¹, dissolved oxygen – 9.9 mg L⁻¹, nitrate 2.9 mg L⁻¹, nitrates < 0.02 mg L⁻¹) containing either BaP or fipronil at an initial concentration of 10 μg L⁻¹ enriched or not with either 14C-benzo[a]pyrene or 14C-fipronil (85 mBq mL⁻¹). These concentrations were chosen to match the BaP or fipronil concentrations usually found in highly polluted waters (Debenchke and Skrobialowski, 2003; Dorch et al., 2008). Control frogs were exposed to ethanol (vehicle) at 1/1000 (v/v) concentration. The frogs were not fed during the exposure period: 1–8 days.

2.4. Benzo[a]pyrene and fipronil uptake, bioconcentration and metabolism

2.4.1. Sample preparations

Water samples (N = 3) and biological samples (N = 3) were taken at the same time. Frogs were killed rapidly at 1, 2, 4, 6 or 8 day of exposure with a blow to the head. The organs of interest were quickly removed and weighed, and water homogenized and sampled. Blood was sampled directly in the heart. Three frogs were used for each time point and each treatment. BaP, fipronil and their 14C-derivatives were extracted from the blood by centrifugation using 1 mL acetonitrile and 1 mL ethanol. For the organs, extraction was done through homogenization in 3 mL acetonitrile, centrifugation (3000 g, 5 min, 4 °C), and homogenization of the resulting pellet with 3 mL ethanol. After centrifugation (3000 g, 5 min, 4 °C) the supernatant was pooled with the supernatant resulting from the first centrifugation. The pooled supernatants were then evaporated and the residue dissolved in 1 mL ethanol. The absence of residual radioactivity was systematically controlled in pellets resulting from the two successive extractions for each organ.

2.4.2. Uptake and bioconcentration factor (BCF) measurements

Appropriate volumes (Vwater) of water and ethanolic extracts were added to a liquid scintillation counting (LSC) cocktail (Ready Safe Scintillation liquid, Beckman) in scintillation vials. LSC measurements were done using a 1414 Wallac counter (Winspectral EG & G®Wallac). Total BaP or fipronil concentrations and their 14C-derivatives, noted as X thereafer, were measured in water (Cwater, fgL⁻¹) and in tissue (Ctissue, fg g⁻¹ fresh weight) using Eq. (1):

\[ C_X = \frac{LSC_X \times V_{\text{extract}}}{m_X} \frac{S_X}{S_W} \times \frac{m_X}{LSC_{\text{water}}} \]

where \( LSC_X \) is the measured signal in dpm; \( V_{\text{extract}} \) corresponds to the total volume of ethanolic extract and in the mass of the extracted organs in g. The specific activity of the compound X, \( S_X \) (fg dpm⁻¹) was measured at the beginning of each experiment (\( m_X \), total xenobiotic mass at T0). Xerobiotic uptake was also measured through the bioconcentration factor (BCF) according to Eq. (2):

\[ \text{BCF} = \frac{C_{\text{tissue}}}{C_{\text{water}}} \]

where \( C_{\text{tissue}} \) is the 14C-concentration measured from the total radioactivity count in the exposure water at the sample time and \( C_{\text{water}} \) is the 14C-concentration in frog tissue (dpm g⁻¹).

2.4.3. Benzo[a]pyrene and fipronil metabolism

BaP and fipronil metabolism was achieved using the thin layer chromatography (TLC) method. TLC was carried out using silica gel plates (G-25 UV254, Macherey-Nagel), at room temperature and in the dark. The volumes of ethanolic extract dropped on the plate were measured using corresponding LSC measurements, in order to obtain approximately 1000 dpm per spot. For BaP, a toluene/isopropanol (4:1, v:v) mixture was used as a mobile phase. Under these conditions, the BaP relative retention factors, \( R_f \)-value was 0.9. For fipronil, each plate was eluted successively with (1) petroleum ether (b.p. 40–60 °C) (diisocraethene (1:1:v:v) mixture and (2) petroleum ether/diisocraethene/ethyl acetate (24:24:1:v:v:v) mixture giving a final \( R_f \)-value of 0.3 for fipronil. Radioactive spots were revealed and quantitatively analyzed using a Phosphomager (Cyclone, PerkinElmer®). Using standard molecules, we found a \( R_f \)-value of 0.68 for 3-hydroxy-benzo[a]pyrene (3′-OH-BaP) and as previously described for the main fipronil metabolites, i.e. sulfone-fipronil, deshu-fipronil, sulfo-fipronil, the \( R_f \)-values obtained were 0.45, 0.65 and 0.90 respectively (Ravet et al., 2006).

2.5. Measurement of biotransformation activity

2.5.1. Preparation of subcellular fractions

Frogs were killed at 1, 2, 4 or 6 day of exposure with a blow to the head. The livers and kidneys were quickly removed and washed in ice-cold 0.15 mM KCl. Five frogs were used for each time point and treatment. The liver and kidneys were homogenized in buffer A (250 mM sucrose; 10 mM HEPES: 1 mM EDTA; PMSF; 1 mM DTT, pH 7.4) using a motor-driven Ultra Turrax glass-Teflon homogenizer (Ika Labortechnik). The homogenate was centrifuged at 100,000 g for 20 min at 4 °C and the resulting supernatant was ultracentrifuged at 100,000 g for 1 h at 4 °C. The resulting supernatant containing the cytosolic fraction was stored at −80 °C for further analysis of the glutathione S-transferase (GST) activity. The microsomal pellet was suspended in buffer A containing 20% glycerol (w/v), and the fresh microsomal suspension obtained was examined immediately for ethoxyucoumarin O-deethylation (ECOD) activity.

2.5.2. Protein assay

The microsomal and cytosolic protein content was determined using Bradford’s colorimetric method (1976) using bovine serum albumin as standard.

2.5.3. ECOD activity

ECOD activity was determined in duplicate and modified from Desousa et al. (1995). Sixty μg microsomal proteins were added to 0.05 M phosphate buffer (pH – 7.2) with 0.4 mM 7-ethoxycoumarin (7-Ec, Fluka) giving a total reaction volume of 200 μL and incubated at 30 °C. The production of 7-hydroxycoumarin (7-OH) was stopped after 4 min and the concentration of 7-OH obtained by measuring the fluorescence intensities of both samples and calibration standards of.
7–OH (sigma) at \( \lambda_{\text{ex/em}} = 380/460 \) nm with a Varioskan flash (Thermoscientific). Activity was expressed in pmole of 7–OH formed per min, per \( \mu \)g of protein.

2.5.4. GST activity

Glutathione S-transferase (GSTs) activity was measured on the cytosolic fraction using 1-chloro-2,4-dinitrobenzene (CDNB, Sigma) as substrate. Reduced glutathione (Sigma) was added to 0.1 M phosphate buffer (pH 7.2) containing 20 \( \mu \)g cytosolic proteins and 3 \( \mu \)M CDNB, for a total reaction volume of 200 \( \mu \)L. After 1 min incubation at 30 °C, the absorbance of glutathione conjugated with 1-chloro-2,4-dinitrobenzene (GS-CDNB) was recorded at 340 nm with a Varioskan flash (Thermoscientific). An extinction coefficient of \( \varepsilon_{\text{GS-CDNB}} = 9.6 \text{ mM cm}^{-1} \) was used to obtain the concentrations. The activity was expressed in pmole of GS-DNB per min, per \( \mu \)g of protein.

2.6. Statistical analysis

Data is expressed as the mean ± SEM. For biotransformation activity, each value was derived from five individual experiments. Since the nature of the distribution of the results was unknown (Gaussian or not), the statistical significance between the means obtained for the different treatments was assessed using the non-parametric Mann–Whitney’s \( U \) test of significance (\( p < 0.05 \) was considered statistically significant).

For the kinetics of BCF, each value was derived from three individual experiments. The effect of time was examined using the non-parametric Kruskal Wallis test followed by the non-parametric Mann–Whitney’s \( U \) test for multiple comparisons (\( P < 0.05 \) was considered statistically significant).

3. Results

3.1. BaP and fipronil uptake

Water clearance of BaP and fipronil was used as a surrogate for uptake kinetics in frogs (Fig. 1a). Concentrations of parent molecules were derived from a TLC analysis of the water of exposure (Tables 1 and 2). The observed decreases in concentrations were best fitted using two component exponential decay, and shows that the initial rate constants were statistically equivalent with values of \( 2.6 ± 0.3 \text{ d}^{-1} \) for BaP (Eq. (3); Fig. 1a) and \( 3.0 ± 1.4 \text{ d}^{-1} \) for fipronil (Eq. (4); Fig. 1a), indicating similar rapid initial uptake for the two xenobiotics. The remaining fipronil concentrations after 1 day of exposure were always statistically higher than those measured for BaP, but both were completely taken up by the frogs within 6 days of exposure.

3.2. BaP and fipronil bioconcentration in frogs

For BaP and fipronil, the evolution of BCF was time dependent (\( p < 0.05 \)). After one day of exposure, the frog’s bioconcentration factor [BCF, Eq. (2)] for BaP was around 16.1, it then increased over time until it reached a maximum value after 6 days of exposure (BCF\(_{\text{MAX}} = 5.6 ± 0.9 \) ), and significantly decreased from day 6 to day 8 (\( p < 0.05 \) ) (Fig. 1b). The BCF values obtained for fipronil were significantly 2–3 times higher than those found for BaP (\( p < 0.05 \)) but varied in a similar way over time, with a regular increase over the first 6 days of exposure (BCF\(_{\text{MAX}} = 11.7 ± 1 \) ) and a decrease from day 6 to day 8 (\( p < 0.05 \) ) (Fig. 1b). The general BCF trend for both BaP and fipronil concurred with the rapid depletion occurring in the surrounding water (>80% of initial native pollutant) which most likely limits pollutant uptake after one day of exposure.

Biocconversion distribution in frog organs was heterogeneous and characterized by a large disparity in BCF values (e.g. 0.2–1300) (Fig. 2a and Supplementary Table 1). For most organs, BCF variations over time followed the general trend observed for whole frogs, with the exception of the gall bladder, fat bodies and intestine for which the values remained constant after 6 days, or increased until the end of the experiment. BCF were always found to be maximal in the gall bladder (e.g. BCF\(_{\text{MAX}} = 1353.7 ± 556.5 \) at day 8), surpassing by a factor of ten the BCF of the organs involved in xenobiotic excretion such as the intestine, which had a BCF\(_{\text{MAX}} = 92.6 ± 3.3 \) at day 8 and the kidneys for which a BCF\(_{\text{MAX}} = 77.1 ± 10.9 \) was reached after 6 days (Fig. 2a and Supplementary Table 1). For all other organs including the ovaries, oviducts and fat bodies, the BCF measured were around or below 10. Fipronil repartition within the frog was more homogeneous than that observed for BaP even though the biocconversion factors in the organs varied widely from 4 to 500 (Fig. 2b and Supplementary Table 2). The BCFs varied over time according to the same general trend as for the BCFs of whole frogs for this pesticide, and reached their highest value after 6 days of exposure except for fat bodies for which BCF\(_{\text{MAX}} \) equal 161.0 ± 77.0 after 4 days exposure. Along with the fat bodies, the highest BCFs were found in the gall bladder (517.0 ± 218.0), ovaries (71.7 ± 21.5), liver (46.8 ± 4.2), intestine (38.9 ± 12.9) and kidneys (30.2 ± 3.8). Overall, BCFs measured for fipronil were higher than those found for BaP for the organs not involved in xenobiotic excretion (gall bladder, intestine and kidneys).

3.3. BaP and fipronil metabolism by frogs

BaP was metabolized into a large variety of products, as revealed by their different \( Rf \) on thin layer chromatography (from 0 to 0.75, Table 1). The majority of the \( ^{14}\text{C} \)-products found in water and in all organs after 1 day of exposure had low \( Rf \)-values of 0 and/or 0.1
(Table 1). Hydrolyses of these polar metabolites were experimentally performed on several samples (notably water) using β-glucuronidase (EC 3.2.1.31) and arylsulfatase (EC 3.1.6.1) enzymes (Roche). The results showed that the level of 3-OH-BaP increased after enzyme hydrolysis, demonstrating that these polar metabolites could correspond to glucuronono- and sulfate-conjugates (data not shown). Low quantities of metabolites displaying intermediate RF-values (from 0.5 to 0.75) were found in the intestine and liver. Among them, 3-OH-BaP (RF = 0.68) corresponding to 2–8% of total metabolites in the intestine were identified. Finally, BaP in its native form (RF = 0.9) was detected in large proportions in fat bodies (~55%) and in the skin where its proportion increased in time.

Fipronil was also metabolized into other products with a higher polarity with an RF of 0, but was mainly found in the form of its major metabolites in organs and tissue: sulfone-fipronil, desthiophipronil, sulfide-fipronil. Metabolites of low RF-values represented 63 ± 4% of 14C-compounds present in water after one day of exposure and were mostly found in the gall bladder and intestine. After 2 days of exposure and for the majority of the organs tested, fipronil (native form) was detected in large proportions (from 2% in the gall bladder to 28% in the ovaries), but it progressively decreased from day 2 to day 6 (Table 2). In most of the extracted organs, the main metabolite was sulfone-fipronil, for which the proportions increased regularly from day 1 to day 6, and tended to accumulate in fat bodies, the skin and the ovaries (Table 2). Desthio-fipronil was also present in relatively large proportions in all the organs studied and was detected in exposure water after only 1 day of exposure (3 ± 2%).

3.4. Effects of BaP and fipronil on enzymatic activities

Cytochrome P450 (Fig. 3a and b) and GST (Fig. 3c and d) activities were measured in the liver and kidneys 1, 2, 4 and 6 days after BaP or fipronil exposure. ECOD activity was twice significantly induced in the liver (Fig. 3a) and kidneys (Fig. 3b) by BaP at two day-exposure. It had to be noted that ECOD basal activities are always 3 times higher in the liver than in kidneys. Surprisingly, exposure to fipronil led to a marked depression in liver basal ECOD activity, equivalent to 61–81% of the controls, over the full exposure time (Fig. 3a) with no change in activity in the kidneys (Fig. 3b).

Basal activity of cysteolic GST in the liver (Fig. 3c) and kidneys (Fig. 3d) were equivalent with values of 250 and 300 pmol min⁻¹ µg proteins⁻¹ respectively. BaP exposure induced an increase in GST activity, but the values obtained significantly

### Table 1

<table>
<thead>
<tr>
<th>Organs</th>
<th>Days of exposure</th>
<th>BaP</th>
<th>Polar metabolites</th>
<th>RF 0.1</th>
<th>RF 0.4</th>
<th>RF 0.5</th>
<th>RF 0.6</th>
<th>3-OH BaP</th>
<th>RF 0.75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1</td>
<td>–</td>
<td>65.7 ± 4.8</td>
<td>34.2 ± 4.8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>60.7 ± 19.2</td>
<td>39.2 ± 19.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>–</td>
<td>82.6 ± 3.8</td>
<td>17.3 ± 3.8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>73.2 ± 22.3</td>
<td>26.8 ± 22.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1</td>
<td>–</td>
<td>55.6 ± 3.0</td>
<td>44.3 ± 3.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>9.5 ± 0.5</td>
<td>90.4 ± 0.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>–</td>
<td>15.4 ± 0.8</td>
<td>84.5 ± 0.8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>40.9 ± 13.5</td>
<td>59.1 ± 13.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Intestine</td>
<td>1</td>
<td>1.4 ± 1.0</td>
<td>83.1 ± 8.3</td>
<td>7.6 ± 3.5</td>
<td>–</td>
<td>2.5 ± 0.7</td>
<td>3.3 ± 1.2</td>
<td>1.9 ± 2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>66.8 ± 2.4</td>
<td>17.3 ± 4.2</td>
<td>–</td>
<td>2.2 ± 2.2</td>
<td>5.9 ± 1.5</td>
<td>1.6 ± 1.6</td>
<td>5.9 ± 0.5</td>
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<tr>
<td></td>
<td>4</td>
<td>–</td>
<td>78 ± 10.5</td>
<td>10.7 ± 0.4</td>
<td>–</td>
<td>2.2 ± 2.2</td>
<td>4.7 ± 1.6</td>
<td>2.1 ± 2.1</td>
<td>4.2 ± 4.2</td>
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<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>61.5 ± 5.9</td>
<td>18.6 ± 6.5</td>
<td>–</td>
<td>2.2 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>8.3 ± 2.4</td>
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<td>61.6 ± 5.2</td>
<td>33.9 ± 1.8</td>
<td>1 ± 1.4</td>
<td>1.9 ± 2.6</td>
<td>–</td>
<td>1.6 ± 2.2</td>
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<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>66.9 ± 2.3</td>
<td>30.6 ± 4.7</td>
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<td>–</td>
<td>–</td>
<td>2.5 ± 2.5</td>
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<tr>
<td></td>
<td>4</td>
<td>–</td>
<td>74.8 ± 7.5</td>
<td>25.1 ± 5.0</td>
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<td>–</td>
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<td>6</td>
<td>–</td>
<td>79.2 ± 7.8</td>
<td>20.7 ± 7.8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>1</td>
<td>–</td>
<td>79.9 ± 1.5</td>
<td>20.1 ± 1.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>81.4 ± 3.3</td>
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<td></td>
<td>4</td>
<td>–</td>
<td>79.1 ± 1.4</td>
<td>20.9 ± 1.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>77 ± 0.9</td>
<td>22.9 ± 0.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>1</td>
<td>54.2 ± 32.5</td>
<td>32.9 ± 23.3</td>
<td>12.8 ± 9.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>47.9 ± 14.5</td>
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<td>86.4 ± 13.5</td>
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<td>91.5 ± 8.4</td>
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Each value represents the percentage mean of the total 14C-quantity ± SEM of 3 frogs.
Table 2
Fipronil metabolism in water and in frog in function of time exposure (10 µg L⁻¹).

<table>
<thead>
<tr>
<th>Organs</th>
<th>Days of exposure</th>
<th>Fipronil</th>
<th>Polar métabolites</th>
<th>Sulfone-fipronil</th>
<th>Destho-fipronil</th>
<th>Sulfide-fipronil</th>
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<tr>
<td>Blood</td>
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<td>36.4 ± 2.9</td>
<td>8.4 ± 0.5</td>
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<tr>
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<td>2</td>
<td>16.7 ± 0.5</td>
<td>22.7 ± 2.9</td>
<td>43.1 ± 1.5</td>
<td>6.5 ± 4.6</td>
<td>11.1 ± 2.7</td>
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<td>67.7 ± 1.2</td>
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<td>38.8 ± 2.6</td>
<td>58.1 ± 3.4</td>
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<tr>
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<td>51.6 ± 2.1</td>
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</tr>
<tr>
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<td>4</td>
<td>4.2 ± 2.9</td>
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<td>64.9 ± 4.1</td>
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<td>5.2 ± 1.1</td>
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<tr>
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<td>77.6 ± 2.4</td>
<td>22.3 ± 2.6</td>
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<td>85.7 ± 4.4</td>
<td>142 ± 4.48</td>
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<td>28.3 ± 1.5</td>
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<td>88.1 ± 2.7</td>
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<td>86.5 ± 0.6</td>
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<tr>
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<td>2.8 ± 2</td>
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<td>53.1 ± 12.3</td>
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<td>—</td>
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</table>

Each value represents the percentage mean of the total 14C-quantity ± SEM of 3 frogs.

Fig. 2. Evolution of bioconcentration factor in organs of frogs exposed to benzo(a)pyrene (a) or fipronil (b). BCF was calculated from Eq. (2). Each value represents the mean ± SEM of 3 replicates. GB, gall bladder.
differed from the control in the liver and kidneys just 1 and 2-day-exposure. Fipronil seemed to be a weak inducer of GST activity in the liver 4-day-exposure and probably in the kidneys but this last value was statistically non-significant.

4. Discussion

The purpose of this study was to compare the interactions of two model contaminants, with differing physiochemical properties, in adult green frogs. During our experiments, the frogs were not fed and their thin, permeable skin interacted directly with the xenobiotics present in solution (Fenoglio et al., 2009). Since anuran amphibians do not drink water (Ogushi et al., 2010) the major uptake route for BaP and fipronil should be the skin. A general consensus that an increased octanol/water partitioning coefficient (log Ow) led to an increase in BCF, resulting from higher miscibility with grease and an ability to cross biological membranes, has been established (Lu et al., 2000; Leney et al., 2006). Evidence that there is a linear increase in the pollutant permeability of isolated frog skin in correlation to the increase in their log Ow has recently been reported (Quaranta et al., 2009). Indeed, both a higher uptake rate and BCF value were expected for BaP (log Ow = 6), compared to fipronil (log Ow = 2.74, Raveton et al., 2006). Surprisingly, the uptake rates obtained were of the same order of magnitude for the two contaminants (Fig. 1a), and the BCFs measured for fipronil were about 2 or 3 times higher than those obtained for BaP (Fig. 1b). Despite its relatively close log Ow value, a BCF = 1.6 has been reported for atrazine (log Ow = 2.7) in Xenopus laevis stage 66 larvae (Edginton and Rouleau, 2005), that is 10 times lower than that found for fipronil herein. This further example, as well as the rapid limitation of uptake due to depletion of the surrounding media confirmed that important processes other than uptake velocity and partitioning, related to log Ow values, were involved in BaP and fipronil bioconcentration in frogs. Detoxification processes, including metabolism and the excretion of pollutants, are of importance when considering their persistence in aquatic organisms (Landrum et al., 1992; Baussant et al., 2001; Sundt et al., 2009). In vertebrates, the entero-hepatic cycle, involving the liver and kidneys, plays a central role in catabolism and the evacuation of contaminants. For both BaP and fipronil, the highest BCF values were measured in the gall bladder during the first day of exposure (Fig. 2). In fact the high values obtained for gall bladder BCFs were due to the retention of bile resulting from the starvation of the frogs during the exposure period (Jonsson et al., 2003). However, it indicated that for the two contaminants in the present study, fecal elimination by the frog take place rapidly, as it was described for atrazine with Xenopus laevis (Edginton and Rouleau, 2005) and with Bufo americanus (Mendez et al., 2009). The lower BCF values obtained for BaP compared to fipronil in the liver, concomitantly with the highest BCFs for gall bladder content and the intestine, indicated that this general evacuation process was more efficient for BaP than for fipronil in frogs, and that these differences may result from the involvement of detoxification enzymes in the liver (Reynaud and Deschaux, 2006). These results were consistent with previous studies which suggested that frogs eliminate BaP much more rapidly than any other PAH whatever their log Ow (Leney et al., 2006). In the liver we found a low proportion of BaP intermediary products (Rf from 0.4 to 0.75), certainly resulting from oxidative metabolism (Phase I). The majority of polar compounds (Rf 0.1 or Rf 0) retained in the gall bladder could be considered of a conjugated nature, considering that conjugation (Phase II) is the main way of producing biliary hydrophilic evaluable derivatives (Zamek-Gliszczynski et al., 2006). Overall, BCFs in the liver were lower for BaP due to the massive excretion of these more water soluble metabolites in the gall bladder (Phase III), and in the intestine for evacuation (if normal digestion had occurred). This was consistent with the induction observed for both Phase I and Phase II enzymes in the liver after BaP exposure (Fig. 3) and with previous studies in amphibians (Camatini et al., 1998). The presence
of BaP intermediary products in the intestine certainly arose after the partial hydrolysis of polar metabolites originating from bile. This allowed their re-absorption by intestine mucosa, as it has already been described for mammal and fish models (Massaad et al., 1992; James et al., 2001). The intestine had also been shown to absorb some conjugates into the blood flow which are finally expelled in the urine after filtration in the kidneys (James et al., 2001; Marie et al., 2010). The $Rf = 0.1$ metabolites were mainly found in the blood and tended to accumulate in the kidneys, with a characteristic saturation shape of enzyme or transporter kinetics, as described for some BaP metabolites in mammal models (Gosselin et al., 2005; Marie et al., 2010). Since only the most polar metabolite(s) ($Rf = 0$) was present in the exposure solution, this result sheds light on the role of the kidneys in the transformation of $Rf = 0.1$ metabolite(s) into ready-to-expulse compounds. Those excrated metabolites could correspond to glucuronopo-BaP, sulfate- and glutathione-conjugates as described in many other organisms (Hornung et al., 2004; Kennedy et al., 2004; Bichon et al., 2008; Kennedy and Tierney, 2008; Zhu et al., 2008) and supported by the results we obtained when enzymatic digestion with β-glucuronidase and arylsulfatase was performed. In addition, the increase in both P450 and GST activity in this organ indicated that the benzo[a]pyrene was rapidly deurilated by Pelophylax esculentus entero-hepatic systems, and consistent with a low further distribution of the contaminant in other organs.

Fipronil was rapidly transformed into its sulfone derivative in the liver (Table 2). This metabolite has been described as more recalcitrant and therefore with a higher BCF than its parent compound in rainbow trout (Konwick et al., 2006), probably due to a relative increase in log$K_{ow}$ (2.81; Aajoud et al., 2003; Raveton et al., 2006). Numerous studies both in mammals and aquatic animals have described the oxidation of fipronil into sulfone-fipronil by cytochromes P450 (Tang et al., 2004; Das et al., 2006; Konwick et al., 2006), but we observed a high depression in ECD0 activity for fipronil exposed frog livers (Fig. 3a). In the study of Das et al. (2006), the induction of P450 observed at the mRNA level was reflected by inhibition at the protein activity level. It was possible that the depression in ECD0 observed in frog livers after fipronil treatment was the result of competition between fipronil or sulfone-fipronil and the substrate used for the test and could reflect the very low metabolism of fipronil by frogs. Further results are needed to confirm this hypothesis. In water, only $Rf = 0$ derivatives were found, suggesting that only these metabolites are excreted by frogs, indeed it seemed that sulfone-fipronil also needs to be conjugated by phase 2 enzymes before excretion. GST conjugation of sulfone-fipronil has been described in rootworms (Scharf et al., 2000) and we found a slight increase in GST activity in the liver after 4 days of exposure to fipronil. Consequently, fipronil distribution was controlled by the slow conjugation of sulfone-fipronil by the liver. Desthio-fipronil was also detected but in lower proportions in all the organs studied. This photo-product of fipronil (Aajoud et al., 2003; Raveton et al., 2006) was present in the exposure water after only 1 day of exposure. Bearing in mind its high lipophilicity, this product is rapidly absorbed by frogs after its formation in water, but similarly to sulfone-fipronil, could not be further metabolized due to its sequestration in non-efficient detoxification organs, or is slowly metabolized in polar metabolites such as its amide-form (Aajoud et al., 2003; Bichon et al., 2008).

Both BaP and fipronil were measured in fat bodies, the ovaries, and the oviducts (Fig. 2 and Supplementary Tables). For female frogs, the maternal transfer of lipophilic contaminants has been reported in the field, and it is correlated to their ability to be integrated into lipoproteins (including high density lipoproteins, HDL) synthesized by the liver (Kadokami et al., 2004; Hopkins et al., 2006; Wu et al., 2009). Most of the lipids and lipoproteins contained in the yolk come from fat bodies, reservoirs for egg build-up. The BCFs of fipronil and its sulfone derivative in the ovaries corresponded to 3.4 the value obtained for whole frog bodies, with an impressive value of 9 for fat bodies. For BaP this value was of 1.2 in the ovaries and slightly higher in the oviducts (2.5) and fat bodies (2.3). These results were in line with those previously obtained, which showed a lower partition in the eggs related either to a difficult transfer from fat bodies to eggs (Kadokami et al., 2004) or to their higher metabolism and evacuation rate (Wu et al., 2009). More surprising was the accumulation of BaP low $Rf$-value metabolites in frog skin, ovaries and in fat bodies. This result was different from previous studies in xenopus injected with BaP in which it accumulated in ovaries and fat bodies in its native form (Camatini et al., 1998). These results suggest that the route of exposure significantly influences xenobiotic distribution and metabolism. Similarly a high level of BaP metabolite contamination was found in mammal gonads and testes, probably due to their higher affinity for HDL (Busbee et al., 1990; Ramesh et al., 2001). The effects of fipronil described in mammals and aquatic animals concern endocrine disruption and developmental toxicity (Leghait et al., 2010; Ohi et al., 2004; Stehr et al., 2006). In mammals, fipronil has been shown to directly affect female reproduction at high concentrations (Ohi et al., 2004), due to the modification of estradiol and progesterone plasma levels that finally result in a decrease in the pregnancy index. In addition, fipronil has also been described as interacting directly with cytochrome P450 involved in steroid metabolism (Tang et al., 2004). Finally, fipronil has been described to disrupt thyroid function in mammals, and this hormonal system is also involved in amphibians’ early development (Duarte-Guterman et al., 2010). As a final result, it could be expected that fipronil and sulfone-fipronil’s high accumulation capacity in frog organs and tissue involved in reproduction might lead to reproductive dysfunction, the developmental impairment of embryos and thereby to decreased fitness in the amphibian populations exposed to this xenobiotic.

The persistence of organic pollutants is a recognized concern, yet the consequences of their metabolism processes and products with regards to the biological effects or toxicity are not straightforward. BaP is known to require activation by means of oxidation in order to become reactive and if not immediately conjugated, may have deleterious effects on biomolecules (Willett et al., 2000; Nebert and Dalton, 2006). For example, the presence of huge quantities of benzo[a]pyrene diol epoxide, known to be the most reactive BaP metabolite is a pre-requisite for degrading more precisely the risk of the formation of protein or DNA adducts that may in turn lead to cancerogenesis. The presence of different intermediates, such as 3′–OH–BaP, in the intestine and the liver of the frogs (Table 2), was demonstrated in our study but further experiments are still needed in order to clearly identify which kind of metabolites are present in these organs. In addition, the toxicity of fipronil as compared to its photo-product desthio-fipronil is interspecies dependent (Konwick et al., 2005) and, in case of amphibians, needs further investigation.

5. Conclusions

Our results showed that metabolism of benzo[a]pyrene by adult green frogs follows the general process of the entero-hepatic systems and relates to the induction of several detoxification enzymes (e.g. P450 and GSTs). This general process is limited by the conjugation step for sulfone-fipronil. In all the organs and tissue (despite their involvement in the depuration process) increased bioconcentration factors for fipronil were at least 10 times higher than for BaP. Considering the high level of sulfone-fipronil and the
presence of desthio-fipronil in the organs and tissue involved in female frog reproduction, further studies are needed to assess whether these metabolites, together with their parent molecule, have long-term effects on the overall population, through a decrease in the success of fecundation, and on the development of embryos. Finally, further studies of these two contaminant types are needed in order to better understand the relationship between metabolite internal doses and toxic events under chronic exposure.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envpol.2011.10.029.

References


