Bio accessibility of tire-associated organic chemicals in fish gut (*Oncorhynchus mykiss*): insights from an *in vitro* digestion model

Thibault Masset[a*], Benoit J.D Ferrari[b,c], William Dudefoi[d], Kristin Schirmer[d,e,f], Alan Bergmann[c], Etienne Vermeirssen[c] and Florian Breider[a]

[a] Ecole Polytechnique Fédérale de Lausanne – EPFL, Central Environmental Laboratory, IIE, ENAC, station 2, CH-1015 Lausanne, Switzerland. e-mail: thibault.masset@epfl.ch
[b] Ecotox Centre - EPFL ENAC IIE, GE, Station 2, CH-1015 Lausanne, Switzerland
[c] Ecotox Centre - Überlandstrasse 133, 8600 Dübendorf, Switzerland
[d] Eawag – Swiss Federal Institute of Aquatic Science and Technology, Department Environmental Toxicology, Überlandstrasse 133, 8600 Dübendorf, Switzerland
[e] EPFL, School of Architecture, Civil and Environmental Engineering, Lausanne, 1015, Switzerland
[f] ETHZ, Institute of Biogeochemistry and Pollutant Dynamics, Zurich, 8092, Switzerland

Abstract
Tire and Road Wear Particles (TRWP) account for an important part of the anthropogenic particles released into the environment. There are scientific knowledge gaps as to the potential bio accessibility of chemicals associated with TRWP to aquatic organisms. This study aimed to investigate the solubilization of five tire-associated chemicals into fish gut using an *in vitro* digestion model (*Oncorhynchus mykiss*). Our results show that the targeted compounds were partly and rapidly solubilized into simulated fluids (SF) present in the gastrointestinal tract within a typical gut transit time for fish (3h in $SF_{GASTRIC}$ and 24h in $SF_{INTESTINAL}$). The effects of food co-ingestion on the solubilization of tire-associated chemicals was compound-specific and either lowered or stimulated their solubilization into the gut fluids. Therefore, the uptake of the tire associated chemicals by the epithelial cells and related toxicity to fish need to be investigated.

Keywords
bio accessibility; tire; TRWP; tyre; 6PPD; 6PPD-Q.

INTRODUCTION

Tire and Road Wear Particles (TRWP) are produced during the wear of tire rubber on the road pavement. Thus, road pavement and road debris have been shown to be embedded in the rubber to form a heterogeneous material composed of rubber polymer, minerals, bitumen and various chemicals originating from the road environment or from the rubber itself. Among many other compounds, 2-mercaptobenzothiazole (MBT) and diphenylguanidine (DPG) are intensively used as vulcanization agents and can represent up to 0,5 %mass of the tire rubber [1]. Phenylenediamine compounds such as N-isopropyl-N′-phenyl-p-phenylenediamine (IPPD) and N-(1,3-dimethylbutyl)-N′-phenyl-1,4-phenylenediamine (6PPD) are commonly used as antioxidants or antiozonants and are added in large amounts (1 – 3%mass) to the final product in order to prevent cracking and degradation of the rubber during wear [2]. When generated, 95 – 99 % of the TRWP are expected to be deposited on the road side and be transferred into the nearby soil from which a fraction will eventually enter the aquatic environment[3]. To date, exposure to tire-related chemicals has been mainly studied *in vivo* with aquatic organisms exposed to leachates of tire particles[4,5]. A recent study concluded that a 6PPD oxidation product, namely 6PPD-quinone, is responsible for acute toxicity on *Coho salmon*[6]. Another study found microplastics in the stomach content of several wild fish species, including tire particles, suggesting that fish can also ingest TRWP [7]. Several studies showed that polymer-bound chemical solubilization was enhanced in fish gut fluids compared to water and could promote bio accessibility of the chemicals for uptake into the circulatory system[8,9]. Therefore, this study used a fish *in vitro* digestion model (*Oncorhynchus mykiss*) in order to (i) determine the solubilization kinetics of five antioxidants, vulcanization aids and transformation products from tire particles in the simulated gastrointestinal fluids of fish and (ii) assess the overall bio accessibility of organic compounds in fish gut with and without co-ingestion of food.

MATERIALS AND METHODS

Materials

Cryogenically Milled Tire Tread (CMTT) was produced as described by Kovochich et al.[10]. Briefly, the upper layer of the tire tread from Pirelli® (Sottozero 3), Michelin® (Primacy 3) and Bridgestone® (Saetta Touring 2) were cut into pieces of 1 cm^3 and cryogenically milled resulting in CMTT with a mean size of 188 µm. The composition of the fish simulated gastric fluid ($SF_{GASTRIC}$) and simulated intestinal fluid ($SF_{INTESTINAL}$) used in this study was similar to Siri et al.[11]. Briefly, both $SF_{GASTRIC}$ and $SF_{INTESTINAL}$ consisted of a luminal buffer adapted from Leibovitz's L15 cell culture medium to mimic the composition of the lumen of fish intestine.

Experimental design

Solubilization kinetics. Solubilization kinetics of organic chemicals were investigated both in $SF_{GASTRIC}$ and $SF_{INTESTINAL}$ separately to investigate the effects of pH and the various bile salts on the kinetics. *In vitro* digestion was performed by introducing 150 mg of CMTT in amber vessels containing 15 mL of $SF_{GASTRIC}$ or $SF_{INTESTINAL}$. The digestion was performed at 20°C under gentle agitation for 3 h ($SF_{GASTRIC}$) and 24 h ($SF_{INTESTINAL}$). Each vessel content corresponding to a kinetic timepoint was centrifuged and passed through 0.45 µm GFF filters. All experiments were performed in triplicates and control experiments were performed in mineral water (Evian®) for comparison with digestive fluids.

Co-ingestion experiments. The environmentally realistic scenario of co-ingestion of food and CMTT was explored. 4 g of ground *Gammarus pulex* (*G.pulex*) and 0.4 g of CMTT were introduced in digestion vessels. 20 mL of $SF_{GASTRIC}$ was added in the vessels and the digestion was performed at 20°C under gentle agitation. After 3 h, 20 mL of $SF_{INTESTINAL}$ was added to the vessel and the pH was adjusted to 7.4 with addition of NaOH. The digestion was stopped after 27 h in total and all samples were centrifuged and passed through 0.45 µm GFF filters. Control experiments were performed with ground *G. pulex* alone and with CMTT only for comparison. All experiments were performed in triplicates.

Chemical analysis. For both experiments, the samples were analysed without further treatment for the following compounds 2-mercaptobenzothiazole (MBT), Benzothiazole (BTH), 1,3 diphenylguanidine (DPG), N-(1,3-dimethylbutyl)-N′-phenyl-1,4-phenylenediamine (6PPD) and 6PPD-quinone (6PPD-Q) with UPLC-MS/MS. Appropriate deuterated internal standards were added before analyses to account for potential losses during sample treatment and for matrix effects.

RESULTS

Solubilization kinetics

All compounds were readily solubilized into $SF_{GASTRIC}$ and $SF_{INTESTINAL}$. Tentative fitting of the data with a diffusion-controlled model, a logarithmic model and 0^{th}, 1^{st} and 2^{nd} order kinetic models was performed. Best fit models are provided in Table 1. In $SF_{GASTRIC}$, BTH and 6PPD-Q solubilization was best fitted by a logarithmic model indicating that these compounds were rapidly solubilized and that a plateau was quickly reached. MBT and 6PPD were best fitted by a diffusion-controlled model suggesting that solubilization was limited by intra-particle diffusion leading to a concentration gradient at the surface of the particles. In contrast, DPG was continuously solubilized during the 3h digestion and followed a pseudo 0^{th} order kinetic. In $SF_{INTESTINAL}$, all compounds were solubilized following a logarithmic kinetic except DPG that followed a diffusion-controlled model. Overall, the

solubilization of all compounds was enhanced in the simulated fluids compared to water except for the polar BTH (log K_{ow} = 2.0) where solubilization in water was similar. The presence of bile salts and surfactants forming micelles in $SF_{INTESTINAL}$ likely stimulated the solubilization of the more apolar compounds (log K_{ow} = 2.5 to 5.4). This experiment demonstrated that tire-associated chemicals were partly but rapidly solubilized into simulated gastrointestinal fluids within a typical gut transit time for fish (3h in $SF_{GASTRIC}$ and 24h in $SF_{INTESTINAL}$).

	$SF_{GASTRIC}$					$SF_{INTESTINAL}$				
	model	**k**	**95% CI**	**r_{adj}^2**	**p-value**	**model**	**k**	**95% CI**	**r_{adj}^2**	**p-value**
BTH	Log (μg^{L-1} log (min^{-1})	110.6	103.8-117.4	0.95	$< 10^{-10}$	Log (μg^{L-1} log (min^{-1})	90.0	76.1-104.0	0.92	$< 10^{-9}$
MBT	Diff. (μg^{L-1} $min^{-1/2}$)	28.0	24.8-31.2	0.96	$< 10^{-10}$	Log (μg^{L-1} log (min^{-1})	358.4	267.5-449.4	0.80	$< 10^{-6}$
DPG	0th order (μg^{L-1} min^{-1})	3.3	2.7-3.9	0.91	$< 10^{-8}$	Diff. (μg^{L-1} $min^{-1/2}$)	82.6	74.5-90.7	0.96	$< 10^{-12}$
6PPD	Diff. (μg^{L-1} $min^{-1/2}$)	1018	863-1173	0.93	$< 10^{-8}$	Log (μg^{L-1} log (min^{-1})	26.1	20.9-31.3	0.88	$< 10^{-7}$
6PPD-Q	Log (μg^{L-1} log (min^{-1})	0.9	0.8-0.9	0.97	$< 10^{-10}$	Log (μg^{L-1} log (min^{-1})	2.9	2.4-3.4	0.90	$< 10^{-8}$

Table 1: Best fit models and solubilization rates of tire-associated chemicals in $SF_{GASTRIC}$ and $SF_{INTESTINAL}$ from CMTT. Log = Logarithmic kinetic model, Diff = Diffusion-controlled kinetic model.

Co-ingestion experiments

In vitro co-ingestion of CMTT along with surrogate fish preys (*G. pulex*) showed contrasting results. The solubilization of BTH was not impacted by the addition of food (figure 1) whereas the solubilization of MBT, DPG and 6PPD-Q was 1.9, 2.8 and 5.6-times lower, respectively, in the co-ingestion scenario. This could be explained by the adsorption of the compounds on the food particles and organic matter that are removed by filtration before analysis. Contrastingly, the solubilization of 6PPD was enhanced by the addition of food. Dissolved organic matter originating from the food could have promoted the solubilization of this apolar compound (K_{ow} of 6PPD = 5.4) as it was shown to be the case for chemicals with similar hydrophobicity such as PAHs[12].

Overall, the effects of the environmentally realistic co-ingestion scenario on the solubilization of tire-associated chemicals was compound-specific Only a small to moderate percentage of tire associated chemicals was found to be solubilized in the simulated fluids during *in vitro* digestion (between 0.4% and 22.8% of their total concentration in the CMTT). However, the *in vitro* digestion of 10 g of CMTT/L of digestive fluids resulted in significant concentrations of 6PPD, DPG, 6PPD-Q, BTH and MBT in the digestive fluids. Therefore, the uptake of the tire-associated chemicals by the epithelial cells and related toxicity to fish need to be investigated.

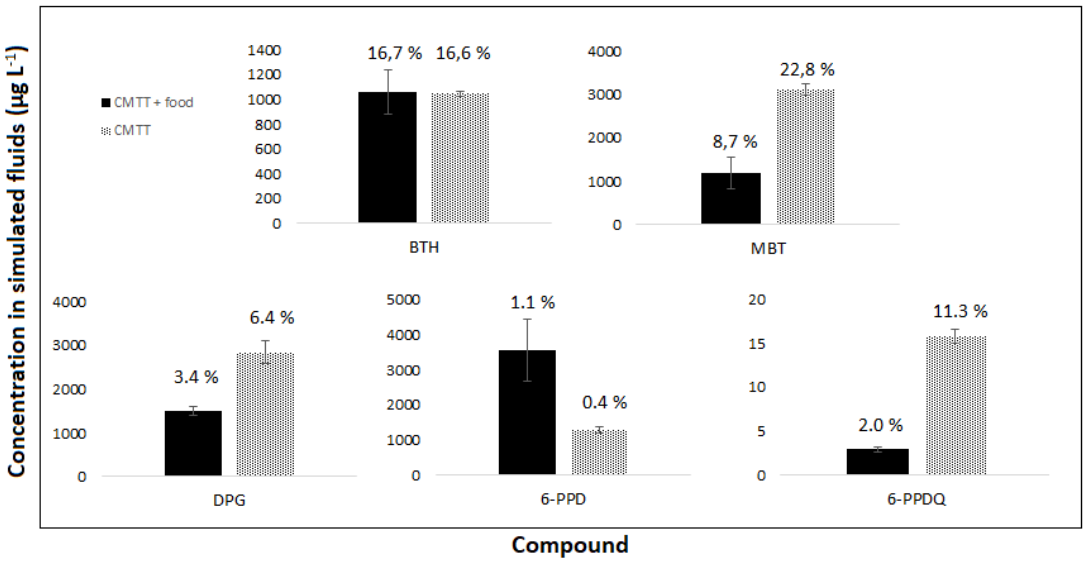

Figure 1: Concentration of tire-associated chemicals (µg L^{-1}) solubilized from CMTT into simulated digestive fluids after an *in vitro* digestion of 27 h with (black bars) and without (grey bars) co-ingestion of food. Percentages represent the amount of the total compound concentration in CMTT solubilized in the simulated fluids during the *in vitro* digestion.

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