In Vitro Digestion of Tire Particles in a Fish Model (Oncorhynchus mykiss): Solubilization Kinetics of Heavy Metals and Effects of Food Coingestion

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ABSTRACT: Tire and road wear particles (TRWP) have been shown to represent a large part of anthropogenic particles released into the environment. Nevertheless, the potential ecological risk of TRWP in the different environmental compartments and their potential toxic impacts on terrestrial and aquatic organisms remain largely underinvestigated. Several heavy metals compose TRWP, including Zn, which is used as a catalyst during the vulcanization process of rubber. This study investigated the solubilization potential of metals from cryogenically milled tire tread (CMTT) and TRWP in simulated gastric fluids (SF\textsubscript{GASTRIC}) and simulated intestinal fluids (SF\textsubscript{INTESTINAL}) designed to mimic rainbow trout (Oncorhynchus mykiss) gastrointestinal conditions. Our results indicate that the solubilization of heavy metals was greatly enhanced by gastrointestinal fluids compared to that by mineral water. After a 26 h in vitro digestion, 9.6 and 23.0% of total Zn content of CMTT and TRWP, respectively, were solubilized into the simulated gastrointestinal fluids. Coingestion of tire particles (performed with CMTT only) and surrogate prey items (Gammarus pulex) demonstrated that the animal organic matter reduced the amount of bioavailable Zn solubilized from CMTT. Contrastingly, in the coingestion scenario with vegetal organic matter (Lemna minor), high quantities of Zn were solubilized from L. minor and cumulated with Zn solubilized from CMTT.

KEYWORDS: tire, tyre, TRWP, heavy metals, microplastics

INTRODUCTION

The occurrence and fate in the environmental compartments and the potential threat for aquatic organisms of microplastic particles (<5 mm) have recently gained interest as plastic production has continued increasing throughout the past decades (359 million tons produced in 2018\textsuperscript{3}). Whether tire and road wear particles (TRWP) should be considered as microplastics is a point of discussion due to its composition being mainly elastomeric (polymers irreversibly hardened during the curing process), in contrast with thermoplastics (polymers undergoing deformation with temperature variations). However, TRWP are nowadays included in global and regional estimations of microplastics emissions into the environment. Similarly to microplastics, global TRWP emissions are constantly increasing and were recently estimated to reach 2.89 × 10\textsuperscript{9} tons/year in 2019\textsuperscript{2}. In Switzerland, TRWP were estimated to represent up to 61% of microplastics entering Lake Geneva\textsuperscript{3}.

TRWP are generated by the friction of tires onto the road pavement (RP) while driving. Thus, its composition consists of a rubbery polymeric backbone with inclusions of road minerals and bitumen\textsuperscript{4}. Several additives are used during the manufacture of tires in the rubber vulcanization process. Zinc oxide is commonly used as a sulfur vulcanization catalyst and represents up to 2.5 mass % of the final tire composition\textsuperscript{5}. Friction is an important factor that is responsible for the generation of TRWP and causes parts of the bituminous binder and minerals from the RP to be embedded in the tire matrix. Therefore, it was shown that metals originating from the pavement are incorporated into TRWP\textsuperscript{6}. Moreover, brake particles generated during braking have been shown to deposit on the road\textsuperscript{6,7}. These particles, which are composed of high amounts of Cu, Fe, Sb, Pb, and Ba\textsuperscript{6,8}, are also susceptible to be incorporated into TRWP.

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Most TRWP are emitted as nonairborne particles (90–99.9%) due to their size (4–120 μm) and density (1–2 g cm⁻¹). Indeed, high concentrations of TRWP (100–100,000 mg kg⁻¹) have been reported on roadside soil and in sediments (1000 mg kg⁻¹) after transport by runoff water. Therefore, involuntary ingestion of these particles by terrestrial and aquatic organisms may occur in the environment. The characterization of microplastics (polymer type, size, sources, and global distribution) ingested in situ by aquatic organisms is getting more attention, and quantitative data (number of particles/organisms or gut) are becoming available. However, although tire particles have also recently been detected in fish gut, the quantity of tire particles susceptible to be in contact with the gastrointestinal fluids of fish remains unknown.

The toxicity of TRWP on organisms remains uncertain after both acute and long-term exposure. Several studies demonstrated the toxic effects on aquatic and terrestrial organisms, whereas others showed no effects of the particles or its leachates. Determining the gastrointestinal bioaccessibility of particle-bound chemicals is the first critical step into the risk assessment process of TRWP-associated chemicals. The solubilization of Cd into natural and simulated gastrointestinal fluids of invertebrates from various types of microplastics has been reported, demonstrating the solubilization potential of polymer-bound elements during gut transit. In vitro digestion of tire particle/sediment mixtures has been performed to investigate the solubilization of Zn into the simulated gastrointestinal fluid of benthic invertebrates. Zn was shown to be highly solubilized into the gastrointestinal fluids compared to water. Taken together, the information regarding the bioaccessibility of TRWP-associated metals remains sparse. Therefore, this study aims to further elucidate the role of tire particle ingestion for metal bioaccessibility via (i) investigating the solubilization kinetics of metals from cryogenically milled tire tread (CMTT) and lab-generated TRWP into simulated gastric and intestinal fluids of a fish model species: the rainbow trout (Oncorhynchus mykiss) and (ii) assessing the impact of influencing factors, such as coinfection of natural food organic matter, on bioaccessible Zn (defined as soluble Zn available for uptake by epithelial cells in the gut).

**Materials and Methods**

**Materials.** Two different materials were used in this study. The tread of tires (cryogenically milled tire tread = CMTT), commonly used in exposure and toxicological studies related to tires, was produced along with lab-generated TRWP as a surrogate for environmental TRWP. As only a low amount of lab-generated TRWP could be obtained due to production limitations, most of the experiments were performed with CMTT, as explicitly stated hereafter. The generation of CMTT and TRWP has been described thoroughly in a previous study. Briefly, to generate CMTT, only the upper layer of the tire treads from Pirelli (Sottozero 3), Michelin (Primacy 3), and Bridestone (Saetta Touring 2) (ratio 1:1:2, respectively), excluding any other component of the tire (sub-tread and belt wedge), were removed using an applicable cutting tool and cut into small pieces of 1 cm³ using industrial scissors and a water jet machine. The tire pieces were then cryogenically milled using a hammer mill (model A, Pulva) to generate CMTT. The TRWP were produced on a road simulator at the Karlsruhe Institute of Technology (KIT). The road simulator consisted of a drum that was coated with RP, composed of a mixture of minerals and bituminous binder, representing the typical pavement from European roads. A tire was put in contact with the drum, and stone dust was added to the drum to increase the friction. Pressure was applied to the tire, and the rotation of the drum generated TRWP, which were collected using a vacuum system in accordance with ISO TS 22638. This process was applied to the three types of tires used to generate CMTT (see above), and a composite sample with the same tire ratio as that for CMTT was prepared. A bulk sample (1 g) of the RP used for the generation of the TRWP containing minerals and parts of the bituminous binder was also collected from the road simulator. All particles were collected and stored in amber glass vials in dark at room temperature. More details regarding the CMTT and TRWP physical/chemical characteristics (size distribution, polymer content, and electronic microscopy images) are presented in Tables S1 and S2 and Figures S1 and S2.

The composition of simulated gastric fluid (SGASTRIC) and simulated intestinal fluid (SFINTESTINAL) is presented in Table S3. Briefly, both SGASTRIC and SFINTESTINAL consisted of a luminal buffer adapted from Leibovitz’s L15 cell culture medium to mimic the composition of the lumen of the fish intestine. The fluids were designed to be used in combination with a cell line isolated from the trout intestine, RTrungGC, which is cultured using L-15 medium. Purified pepsin (Sigma-Aldrich) was added to the luminal buffer at a concentration of 12.5 U mg⁻¹ of protein, and pH was adjusted to 2 with 32% HCl to obtain SFGASTRIC. A concentration of 4 mg mL⁻¹ of the porcine bile extract (Sigma-Aldrich) and 2 mg mL⁻¹ pancreatin (Sigma-Aldrich) were added to the luminal buffer to obtain SFINTESTINAL, with a pH of 7.4. Control experiments were performed in mineral water (MW) (Evian) to compare the solubilization of metals with simulated gastrointestinal fluids. The composition of Evian water is provided in Table S4. The surface tension of SFINTESTINAL was measured with a goniometer (EasyDrop, Kruss), and the presence, size, and stability of micelles were assessed by dynamic light scattering (Zetasizer ZS).

**Determination of Elemental Composition of Tire Particles.** To quantify the metal content of CMTT, TRWP, and RP, acid-digestion of the particles was performed. The following method ensured the complete mineralization of the samples as no sample remnants were collected during the postdigestion filtration step. Triplicates of 250 mg each material were placed into Teflon vessels, and 9 mL of 69% HNO₃ and 1 mL of Milli-Q water were added. The vessels were placed into a microwave reactor (multiwave Pro Anton Paar) and heated at 200 °C with the following conditions: 15 min heating up to 200 °C, 45 min steady conditions at 200 °C and 20 bar, and 10 min cooling to 70 °C. Then, the digesta were filtered through 0.45 μm filters (Chromafiltr), diluted 50-fold, and analyzed for metals as described below.

**Determination of the Morphology and Particle Size Analysis.** A qualitative analysis of the particle morphology was performed on CMTT and TRWP before and after the in vitro digestion using scanning electron microscopy (GeminiSEM 300, Zeiss). This was done in order to assess the general morphology of the particles and to investigate any morphological changes at the surface of the particles that might be
resulting from the exposure to the simulated gastrointestinal fluids. Particle size distributions of CMTT and TRWP were evaluated using laser diffraction, and the main composition of TRWP was evaluated by thermogravimetric analysis (Tables S1 and S2).

**Experimental Design.** The following experiments were designed to investigate the solubilization of metals originating from CMTT and TRWP into the simulated gastrointestinal fluids of rainbow trout. The solubilization kinetics of several metals, the impact of the CMTT concentration on Zn solubilization, and the impact of the coingestion of food on Zn bioaccessibility were explored.

**In Vitro Digestions. Solubilization Kinetics of Metals from CMTT and TRWP.** Overall, the in vitro digestion consisted of 3 h digestion in SF_GASTRIC to mimic the transit time in the fish stomach, followed by 23 h digestion in SF_INTESTINAL estimated as the average transit time in the fish small intestine. A total of 1.5 g of either CMTT or TRWP was added to 250 mL polypropylene flasks, and 150 mL of SF_GASTRIC was incorporated at t = 0 in order to obtain a solid/liquid ratio of 0.01. The flasks were immediately placed on an orbital shaker at 130 rpm in dark at room temperature (20 °C). Aliquots of 4 mL of the mixture were regularly collected by pipetting, immediately filtered at 0.45 μm (Chromafil), and stored in polypropylene tubes at 4 °C before analysis. After the 3 h in vitro digestion in SF_GASTRIC, the remaining SF_GASTRIC was removed by centrifugation and replaced by the appropriate amount of SF_INTESTINAL to maintain a solid/liquid ratio of 0.01. The same process (pipetting at regular intervals and filtration) was repeated until a digestion time of 23 h. The control experiment consisted of using MW instead of SF_GASTRIC and SF_INTESTINAL. All experiments were performed in triplicate.

**Impact of the CMTT Concentration on Zn Solubilization.** The relationship between the quantity of tire particles used in in vitro digestions and the quantity of metals solubilized was evaluated only with CMTT due to the limited quantity of TRWP available. The same protocol as that for the solubilization kinetics experiments was used with the exception that the digestions in SF_GASTRIC and SF_INTESTINAL were tested separately and not with a sequential approach in order to investigate the different solubilization patterns of Zn in both fluids. Three CMTT concentrations (0.1, 0.01, and 0.001 g mL⁻¹) were used for a total digestion time with SF_GASTRIC of 3 h. The same experiment was repeated with SF_INTESTINAL for 24 h. All experiments were performed in triplicate and conducted along with a MW control instead of SF_GASTRIC or SF_INTESTINAL.

**Food Organic Matter Coingestion Scenarios.** To investigate the effect of the coingestion of food organic matter on the bioaccessible fraction of Zn, in vitro digestions with CMTT only and with the addition of either Gammarus pulex (JBL) or Lemna minor obtained from a culture in a fish store were performed. These species were chosen as surrogates for animal and vegetal food items. Briefly, whole freeze-dried G. pulex and L. minor were ground into powder with a mortar and pestle and were incorporated into an SF_GASTRIC solution with CMTT (75 mg) (CMTT/liquid ratio = 0.01 and 0.0033 for G. pulex and L. minor, respectively). The flasks were immediately placed on an orbital shaker at 130 rpm in dark at room temperature (20 °C) for 3 h. The mixtures were then passed through 0.45 μm filters (Chromafil) and stored in polypropylene tubes at 4 °C until analysis. The same protocol was repeated with SF_INTESTINAL instead of SF_GASTRIC for digestion for 23 h. Three food/CMTT ratios of 2:1, 5:1, and 10:1 with the CMTT concentration of 0.01 g mL⁻¹ held constant were tested. Although higher ratios are likely to occur in the environment, the introduction of high amounts of food in our in vitro digestion vessels led to a very viscous solution, preventing the processing of the samples. Therefore, scenarios considering a food/CMTT ratio >10 could not be tested. A negative control containing no addition of food organic matter and only CMTT was performed. Control experiments with food organic matter only (without CMTT) whose quantities corresponded to the different ratios of 2:1, 5:1, and 10:1 were also performed. All experiments were performed in triplicate.

**Metal Analyses.** Analyses of Zn were performed on an inductively coupled plasma (ICP) optical emission spectrometer (Shimadzu ICPE-9000) for microwave digested extracts since Zn was present in high amounts in CMTT and TRWP. Analyses of Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo, Ba, and Pb, present in low quantities in particles and in simulated fluids, were performed on a triple quadrupole ICP mass spectrometer (Agilent 8900). For both instruments, calibration curves were established with 10 points of appropriate concentration from a multi-element standard solution (Inorganic Ventures). Quality control solutions were processed between a series of 10 samples. More information regarding the instrument settings and QA/QC are available in Text S1.

## RESULTS AND DISCUSSION

**Metal Content of the Particles.** Elemental analysis of CMTT revealed high quantities of Zn (7780 ± 69 μg g⁻¹), as expected from its use in the vulcanization process of rubber (Table 1). All other metals were detected in quantities of <20 μg g⁻¹ with the exception of Fe (83 ± 4 μg g⁻¹). Concentrations of metals measured in CMTT were consistent with the concentrations reported for tire granulates and for tire tread.

In contrast to CMTT, TRWP contained higher quantities of Fe (4863 ± 432 μg g⁻¹) and Ti, Cr, Mn, Co, Ni, Cu, Ba, and Pb (20–300 μg g⁻¹). Lower concentrations of Zn (1448 ± 76 μg g⁻¹) were measured in TRWP than that in CMTT. This was related to the very low concentration of Zn detected in the RP (<250 μg g⁻¹) (Table 1). When incorporated into the polymer matrix of the tire, the RP likely acted as a dilution material and lowered the overall Zn concentration in TRWP. Kreider et al. (2010) also observed a dilution effect of the road...
Figure 1. Solubilization kinetics of Zn in SF \textsubscript{GASTRIC} and SF \textsubscript{INTESTINAL}, represented by the concentration of Zn solubilized in the fluid (left axis) and the total percentage solubilized (right axis) from (a) CMTT and (b) TRWP. Blue dots represent Zn solubilized into SF \textsubscript{GASTRIC} for the first 3 h, and red dots represent Zn dissolved into SF \textsubscript{INTESTINAL} for the following 23 h of the \textit{in vitro} digestion. Black dots represent Zn solubilized into MW (control) and are not displayed in (b) because values were less than the limit of quantitation. Error bars represent the standard deviation of the replicates ($n = 3$).

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In Vitro Digestion of CMTT and TRWP. Zn was solubilized in SF\textsubscript{GASTRIC} and SF\textsubscript{INTESTINAL} both from the CMTT and the TRWP, which resulted in high concentrations in the gastrointestinal fluid after the \textit{in vitro} digestion (Figure 1). Four kinetic models (zeroth-, first-, and second-order kinetics and the diffusion-controlled model\textsuperscript{[19,28]}) were applied to fit our data in order to investigate the mechanisms driving Zn solubilization (Table S5). The solubilization kinetics of Zn from CMTT into SF\textsubscript{GASTRIC} and SF\textsubscript{INTESTINAL} were best represented with the diffusion-controlled model (Table 2). As the mixing was ensured during the time course of the experiment, this suggests that the solubilization was limited by intraparticle diffusion, leading to a concentration gradient at the surface of the particles.

For CMTT, after the 3 h digestion in SF\textsubscript{GASTRIC}, 2.1% of the total Zn was solubilized, and at the end of the digestion (3 h in SF\textsubscript{GASTRIC} only followed by 23 h in SF\textsubscript{INTESTINAL} only), 9.6% of the total Zn was solubilized, which represented a final concentration of 6.7 mg L\textsuperscript{-1} in the gastrointestinal fluids.

Fe, Pb, Mn, and Co were also solubilized into the SF\textsubscript{GASTRIC} and SF\textsubscript{INTESTINAL} but due to their much lower concentrations in CMTT, concentrations of only 38.4, 5.1, 2.5, and 0.7 $\mu$g L\textsuperscript{-1} (corresponding to 4.6, 9.0, 15.8, and 0.9% of the total metal content of CMTT, respectively) were measured after 3 h in SF\textsubscript{GASTRIC} and no significant desorption occurred in SF\textsubscript{INTESTINAL}.

The solubilization kinetics of Zn from TRWP exhibited a different pattern from that of CMTT (Figure 1). The equilibrium was rapidly reached after 30 min of digestion with SF\textsubscript{GASTRIC} leading to 14.5% of total Zn solubilized. Once again, the replacement of SF\textsubscript{GASTRIC} by SF\textsubscript{INTESTINAL} reinforced the solubilization of Zn to reach 23% of the total Zn solubilized into the gastrointestinal fluids at the end of the experiment.

The different solubilization kinetics between CMTT and TRWP were likely related to the size distribution of the types of particles. Indeed, as the size of TRWP was much smaller than that of CMTT (mean sizes of 45 and 188 $\mu$m, respectively), the specific surface area of TRWP was larger and hence probably explained the higher Zn solubilization rate. Additionally, it is likely that the rougher aspect and higher porosity of the TRWP than those of the smoother CMTT (Image S1) also contributes to its larger specific surface area. The impact of the particle size on the solubilization kinetics of the pollutants from other types of microplastics has recently been observed and corroborates the results obtained in our study since an increase in the solubilization rate with smaller particles was reported.\textsuperscript{[29,30]}

As a consequence, Zn solubilization...
from TRWP was best fitted by a pseudo-zero-order model (Table 2) before the equilibrium was reached after 30 min in SF\textsubscript{GASTRIC} and by a diffusion-controlled model before the equilibrium was reached after 6 h in SF\textsubscript{INTESTINAL} (Figure 1b). This result indicated that Zn was constantly solubilized without any solubility constraint and that a pseudo-equilibrium state was rapidly reached. Despite the fact that the solubilization of Zn from TRWP (23\%) was greater than that from CMTT (9.6\%), the concentration of Zn in the gastrointestinal fluids at the end of the digestion was twice as high with CMTT (6709 μg L\textsuperscript{-1}) as that with TRWP (3308 μg L\textsuperscript{-1}). This is due to the larger initial concentration of Zn in CMTT (7780 μg g\textsuperscript{-1}) than that in TRWP (1448 μg g\textsuperscript{-1}).

Unlike CMTT, several other metals were detected in the gastrointestinal fluids of TRWP digestion due to the contribution of the road constituent to the overall metal burden of these particles (Table 1). The solubilization kinetics of Mn, Co, Ni, and Ba displayed a similar pattern to that for Zn with a rapid increase in the concentration over time to reach a steady state or a pseudo-steady state after a contact time of 26 h (Figure S2). The amount of metals solubilized was not proportional to their concentration in TRWP as some metals (Mn and Co) were more prone to solubilization than others (Ni and Ba) (Figure S3). The solubilization of other metals (Cr, Fe, and Cu) present in TRWP could not be investigated because their concentration in the digesta matrix was high. Therefore, for these metals, it was not possible to distinguish the relative contribution of TRWP from the matrix background.

Overall, the solubilization of all metals was greatly enhanced (from a factor of 3.75 for Co to a factor of 12.4 for Zn) both in SF\textsubscript{GASTRIC} and SF\textsubscript{INTESTINAL} compared to that in the MW control (Figures 1 and S3). The main differences between MW and SF\textsubscript{GASTRIC} were pH (7.4 and 2.0, respectively) and the presence of Pepsin in SF\textsubscript{GASTRIC}. As pH is a strong determinant of metal solubilization,\textsuperscript{28,31} the low pH of SF\textsubscript{GASTRIC} likely favored the solubilization of the metals. The negative correlation between the pH of aqueous solutions (including gut fluids) and solubilization of metals from polymeric matrices has been demonstrated for thermoplastics\textsuperscript{28,33} as well as tire particles.\textsuperscript{3,24,35} The SF\textsubscript{INTESTINAL} solution was a neutral (pH = 7.4) complex matrix composed of high amounts of proteins, phospholipids, and bile acids. The critical micelle concentration in SF\textsubscript{INTESTINAL} calculated from contact angle measurements was approximately equal to 2000 mg\textsubscript{ bile} L\textsuperscript{-1} (Figure S4). The SF\textsubscript{INTESTINAL} used in this study contained 5000 mg \textsubscript{ bile} L\textsuperscript{-1} concentration, thus well above the critical micelle concentration, indicating that SF\textsubscript{INTESTINAL} was a micellar solution. The presence of stable micelles was confirmed by dynamic light scattering, which revealed a high concentration of micelles. Although the micelle-mediated solubilization of hydrophobic organic compounds has been demonstrated,\textsuperscript{36,37} it is possible that micelles also positively impact the solubilization of metals due to their capacity to act as a ligand.\textsuperscript{38} Furthermore, the solubilization of metals could be enhanced by complexation with natural constituents of SF\textsubscript{INTESTINAL} such as proteins. More specifically, amino acids, such as histidine, have been identified as complexing molecules for metals.\textsuperscript{39–41}

Overall, Zn was solubilized in much higher amounts than every other metal in the digestive fluids due to its high concentration in CMTT and TRWP (7780 and 1448 μg g\textsuperscript{-1}, respectively). Although Zn is an essential metal and is tightly regulated, excess Zn can be deleterious for fish. Diet-borne Zn toxicity to fish has been investigated and showed contrasting results among fish species, with the effects on growth observed from 30 μg Zn g\textsubscript{ digesta}\textsuperscript{-1} for guppies (Poecilia reticulata) to no observable effects on growth with up to 1500 μg Zn g\textsubscript{ digesta}\textsuperscript{-1} for rainbow trout (O. mykiss).\textsuperscript{42,43} Therefore, due to (i) the high concentration of Zn measured in CMTT and TRWP and (ii) the high solubilization potential of Zn into fish simulated gastrointestinal fluids, this study aimed to further elucidate the role of tire particle ingestion for Zn bioaccessibility.

Influence of the CMTT Concentration on Zn Solubilization. In order to investigate the influence of the concentration of CMTT on the solubilization of Zn, in vitro digestions with three different concentrations (0.1, 0.01, and 0.001 g mL\textsuperscript{-1}) were performed.

In SF\textsubscript{GASTRIC} the solubilization kinetics were different for the three scenarios, and a positive trend between the percentage of Zn (% Zn) solubilized and CMTT concentration was observed except for the last data point after 3 h (Figure 2a). It is possible that the equilibrium was reached for the highest concentration tested (0.1 g mL\textsuperscript{-1}). After 3 h, % Zn solubilized was 0.37–2.32% for all three scenarios. In SF\textsubscript{INTESTINAL} the solubilization kinetics were similar, and the equilibrium was not reached after 24 h digestion for the two lower concentrations (0.01 and 0.001 g mL\textsuperscript{-1}) (Figure 2b). A slower solubilization rate was observed for the highest concentration (0.1 g mL\textsuperscript{-1}), likely due to the near-equilibrium between CMTT and SF\textsubscript{INTESTINAL} with a high Zn concentration measured in the solution (33.6 mg L\textsuperscript{-1} at 24 h), preventing faster solubilization. A decrease in the percentage of metal solubilized was also observed with the increasing CMTT concentration for various metals and was
related to the saturation of the liquid phase. By adding up % Zn solubilized in SFGASTRIC and SFINTESTINAL for the three scenarios, on average, 8.7 ± 2.8% of Zn was solubilized in gastrointestinal fluid after a gut transit time of 27 h (3 h in stomach +24 h in intestine). These results show that % Zn solubilized in gastrointestinal fluids is mostly independent of the amount of ingested CMTT, unless saturation of the liquid phase occurs—a scenario unlikely to happen in natural environments where concentrations of TRWP in water are estimated to range from 0.5 to 5 mg L⁻¹. Under the assumption that these results can be extrapolated to TRWP, one can expect that 20−25% of Zn would be solubilized from these particles independently of the particle concentration measured in the gut (Figure 1).

**Effects of Food Organic Matter Coingestion on Zn Solubilization.** Two coingestion experiments of CMTT with either animal (G. pulex) or vegetal (L. minor) food organic matter were conducted. Alongside, experiments without CMTT but with food organic matter only were performed for comparison. The coingestion of L. minor resulted in a significant increase in the Zn concentration in both SFGASTRIC and SFINTESTINAL (Figure 3a,c). As can be deduced from Figure 3a,c, Zn contribution from CMTT was cumulative and added up to the Zn contribution from L. minor in SFINTESTINAL. The amount of Zn solubilized was dependent on the quantities of L. minor added as the concentration of soluble Zn increased with increasing food/CMTT ratios. Soluble Zn was released from L. minor; 31−72 and 72−93% of its total Zn content were solubilized in SFGASTRIC and SFINTESTINAL, respectively (Figure S5). These results indicate that no complexation or adsorption of Zn solubilized from CMTT occurred on the vegetal organic matter.

In contrast, coingestion with G. pulex led to a reduction of the soluble Zn concentrations compared to that with the digestion of CMTT only, both in SFGASTRIC and SFINTESTINAL at food/CMTT ratios of 2 and 5 (Figure 3b,d). For the coingestion scenario with a food/CMTT ratio of 10, the soluble Zn concentration was similar to that with CMTT only. These results show that Zn solubilized from CMTT did not cumulate with Zn solubilized from G. pulex but was partly reabsorbed or complexed by the organic matter added via G. pulex.

Overall, Zn solubilized from CMTT was adsorbed on, or complexed by, animal organic matter (G. pulex) but not vegetal organic matter (L. minor). One explanation for this observation could be that G. pulex is composed of 40% proteins, whereas L. minor has a lower protein content (25%). Proteins and particularly metallothionein, found in animals, are strong ligands for Zn. Thus, it is possible that the complexation on metal-binding proteins was more important with the coingestion of G. pulex than that with L. minor. More importantly, G. pulex exoskeletons are composed of chitin, a fibrous substance consisting of polysaccharides, which has been proven to bind metal cations by the process of chelation.
Therefore, it is plausible that the Zn cations solubilized from CMTT were subsequently bound by proteins or chitin from G. pulex. In a general manner, animal organic matter reduced the amount of bioavailable Zn solubilized from CMTT. Contrastingly, in the coingestion scenario with vegetal organic matter, high quantities of Zn were solubilized from L. minor for all food/CMTT ratios and cumulated with the Zn solubilized from CMTT.

To date, experiments investigating the coingestion of food organic matter in desorption studies of micropollutants from microplastics or tire particles remain scarce and mainly refer to organic compounds such as polychlorinated biphenyls37 or explore the particular case of deposit feeder organisms being exposed to sediment organic matter.39−41 Accounting for coingestion scenarios when performing in vitro digestion experiments of contaminated polymeric matrices is critical as it is a way to better represent the situations likely to occur in the aquatic environment. Our results demonstrated the complexity of the interactions between the different phases in the fluids, which are highly dependent on the nature of the food organic matter (vegetal vs animal). The concentrations of Zn in the food organic matter used for the coingestion scenarios in this study were consistent with the Zn contents reported in the environment for L. minor49 and G. pulex50,51 as well as with other prey types susceptible to be ingested by fish, such as various zooplankton species.52,53 However, the three food/CMTT ratios investigated were, for technical reasons (see the Material and Methods section), low and likely below the ratios encountered in the environment. Nevertheless, our results show that the addition of food organic matter could act as a strong complexant for Zn and might contribute to the lower bioaccessibility of this metal once released from CMTT. It was found that G. pulex and L. minor remnants could eventually be digested and act as a source of bioaccessible Zn. For coingestion scenarios with L. minor, the Zn contribution from the food could significantly outweigh the Zn contribution from CMTT.

Environmental Implication. Our coingestion studies used CMTT as a surrogate for the more environmentally relevant TRWP. As TRWP contained lower amounts of Zn (Table 1) than CMTT, it is likely that the Zn contribution from TRWP in coingestion scenarios will be lower than what was demonstrated in this work with CMTT. Nevertheless, our study shows that tire rubber contains high amounts of Zn and that its solubilization within the fish digestive fluids is enhanced compared to its leaching potential in water. This pattern was also observed for other metals such as Mn, Co, Ni, and Ba. If future studies were to confirm that environmental TRWP contain these metals, the contribution of TRWP to the exposure of Mn, Co, Ni, and Ba would need to be investigated. It is important to note that low concentrations of heavy metals could induce toxicity, potentially related to mixture effects.

In an environmentally relevant coingestion scenario of tire rubber with food organic matter, the Zn contribution from CMTT will represent only a fraction of the total Zn solubilized into the gastrointestinal fluids. Indeed, in an effort to reproduce environmentally relevant coingestion scenarios, it was shown that most of the Zn solubilized in the gastrointestinal fluids would be released from diet and that 20−30% of the total Zn solubilized would originate from CMTT. However, various organic compounds are also added as additives during the manufacture of the tire tread and are suspected to cause toxic effects to aquatic organisms. Recently, 6-PPD quinone, an oxidation product of the widely used antiozonant N-(1,3-dimethylbutyl)-N’-phenyl-1,4-benzenediamine (6-PPD) was shown to be highly poisonous to Coho Salmon,54 highlighting the need for further studies on the bioavailability of organic additives used in the production of rubber. The methods used in this study could be applied to investigate the solubilization potential and bioaccessibility of such organic compounds in the fish gastrointestinal fluids.
Sustainable Developments (WBCSD) Tire Industry Project (TIP), TIP is a global CEO-led initiative undertaken by leading tire manufacturing companies. TIP drives research on potential human health and environmental impacts of tires throughout their lifecycle. The study design, execution, interpretation, and manuscript preparation were conducted solely by the authors.

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