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Novel aspects of uptake patterns, metabolite formation and toxicological responses in Salmon exposed to the organophosphate esters—Tris(2-butoxyethyl)- and tris(2-chloroethyl) phosphate

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ABSTRACT

Given the compound differences between tris(2-butoxyethyl)- and tris(2-cloroethyl) phosphate (TBOEP and TCEP, respectively), we hypothesized that exposure of juvenile salmon to TBOEP and TCEP will produce compound-specific differences in uptake and bioaccumulation patterns, resulting in potential formation of OH-metabolites. Juvenile salmon were exposed to waterborne TCEP or TBOEP (0.04, 0.2 and 1 mg/L) for 7 days. The muscle accumulation was measured and bioconcentration factor (BCF) was calculated, showing that TCEP was less accumulative and resistant to metabolism in salmon than TBOEP. Metabolite formations were only detected in TBOEP-exposed fish, showing seven phase I biotransformation metabolites with hydroxylation, ether cleavage or combination of both reactions as important metabolic pathways. *In vitro* incubation of trout S9 liver fraction with TBOEP was performed showing that the generated metabolite patterns were similar to those found in muscle tissue exposed *in vivo*. However, another OH-TBOEP isomer and an unidentified metabolite not present in *in vivo* exposure were observed with the trout S9 incubation. Overall, some of the observed metabolic products were similar to those in a previous *in vitro* report using human liver microsomes and some metabolites were identified for the first time in the present study. Toxicological analysis indicated that TBOEP produced less effect, although it was taken up faster and accumulated more in fish muscle than TCEP. TCEP produced more severe toxicological responses in multiple fish organs. However, liver biotransformation responses did not parallel the metabolite formation observed in TBOEP-exposed fish.

1. Introduction

Organophosphate esters (OPEs) such as tris(2-butoxyethyl) and tris (2-cloroethyl) phosphate have been increasingly applied to replace halogenated flame retardants such as poly-brominated diphenyl ethers (PBDEs) that are almost phased out of production due to their proven toxicity, bioaccumulative and persistent properties (Hung et al., 2015). OPEs account for about 15% of global flame-retardants and are applied mostly in plastics, electronic parts, textiles and rubber products. OPEs are released from these materials and are spread into environment with partially unknown consequences for ecosystems and human health (Reemtsma et al., 2008a,2008b; Leonards et al., 2011a,2011b). In order to address the environmental and human health risks of OPEs, there is a need for scientific data on their occurrence and fate in the environment and biota. TCEP was shown to produce reproductive effects with potential deleterious consequences for fertility in animals and humans (Liu et al., 2012; Jin et al., 2013; Ta et al., 2014). Particularly, individual steps of steroidogenic pathways were influenced by TCEP in juvenile salmon (Arukwe et al., 2016). Other OPEs such as tris(1,3-dichloro-2-propyl) phosphate (TDCPP) produced developmental effects in zebrafish during embryogenesis (Dishaw et al., 2014), reduced circulating thyroxin (T4) levels in zebrafish larvae (Kim et al., 2015), interfered with the expression of thyroid hormone (TH)-responsive genes in cultured chicken embryos (Farhat et al., 2013) and modulated steroid hormone levels in H295R human adrenocortical carcinoma cells (Liu et al., 2012).

In mammals, bis(2-chloroethyl) carboxymethyl phosphate, bis(2-chloroethyl) hydrogen phosphate and bis(2-chloroethyl)-2-hydroxyethyl phosphate glucuronides were identified as urinary metabolites of TCEP (European Union Risk Assessment Report, 2009). These findings suggest metabolic pathways that involve oxidative and hydrolytic reactions, as well as glucuronidation through phase-II metabolism

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(ibid). Reported toxicological effects such as neurological dysfunctions, adverse reproductive effects, and endocrine disruptive and systemic responses (European Union Risk Assessment Report, 2009; Liu et al., 2012), prompted the classification of halogenated phosphate esters as risky substances in the Priority List of the Council Regulation (EEC, Council Regulation, 1993). On the other hand, non-halogenated OPEs, such as TBOEP are more hydrophobic than chlorinated OPEs (Fries and Puttmann, 2003; Qian et al., 2014). Thus, their spread in the environment is more associated with particle transport, precipitation and sorption to carbon-rich surfaces (Xu et al., 2017). The degradation of TBOEP in air and water is slow compared to biodegradation by microbes. Thus, based on available data, accumulation in aquatic organisms will not be expected (European Union Risk Assessment Report, 2009). In animal experiments, repeated exposure of TBOEP at high doses produced alterations in the liver and nervous system, with females showing higher susceptibility to toxic effects than males (Xu et al., 2017). TBOEP has been classified as a moderately toxic compound to aquatic organisms as shown for developing zebrafish (Ma et al., 2016). In salmon, low to no effects on steroid hormone synthesis in brain and kidney were observed (Arukwe et al., 2016). Data on *in vivo* uptake kinetics and metabolism of TBOEP in aquatic animals has not been previously reported. Further, despite the widespread occurrence of OPEs in the aquatic environment (van der Veen and de Boer, 2012), data on their bioaccumulation, metabolism and non-lethal effects on aquatic biota, especially fish, are limited to non-existent. Risk assessment has mainly focused on human health and the application of *in vitro* systems such as human liver microsomes or S9 liver fractions (van den Eede et al., 2013, 2015a, 2015b) or the excretion of metabolites in human urine samples (Reemtsma et al., 2011).

A recent study that compared biota transfer of nine OPEs, reported TBOEP levels in both benthic and pelagic food web organisms in the range of 17 μg/kg ww (sculpin: *Myoxocephalus scorpius*) and 27 μg/kg ww (pouting: *Trisopterus luscus*) as the highest average concentrations (Brandsma et al., 2015). TCEP concentration was much lower, showing respective benthic and pelagic food web levels of 1 and 1.6 μg/kg ww in goby and herring (Brandsma et al., 2015). The toxicity and bioaccumulation of emerging contaminants (ECs) depend on their uptake, elimination and metabolism kinetics. Estimating bioaccumulation of ECs is crucial for food safety and ecosystem biomagnification perspectives. For risk assessment, a chemical bioaccumulation factor (BAF) is essential, either through experimentally based value or by estimation, based on the octanol-water partition coefficients (Kow) (TGD, 2003). Therefore, the aims of this study were to investigate the uptake, bioaccumulation, metabolism and metabolite formation in juvenile Atlantic salmon exposed to waterborne TBOEP and TCEP. Given the compound differences between these two OPEs, we hypothesized that exposure of juvenile salmon to TBOEP and TCEP will produce compound-specific differences in uptake and bioaccumulation patterns, resulting to potential formation of hydroxylated (OH) metabolites. TBOEP and TCEP were selected for the present study because they are commonly measured in a wide range of environmental and biota samples (both aquatic and terrestrial organisms) and with associated toxicity responses.

2. Materials and methods

2.1. Chemicals and reagents

Tris(2-butoxyethyl) phosphate (94%) (TBOEP; [CH3(CH2)3OCH2CH2O]3P(O)) and Tris(2-chloroethyl) phosphate (97%) (TCEP; (ClCH2CH2O)3P(O)), from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Trizol™ reagent was purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). Direct-zol™ RNA MiniPrep RNA isolation kit was purchased from Zymo Research Corporation (Irvine, CA, USA). iScript cDNA Synthesis Kit and iTaq SYBR Green Supermix with ROX were purchased from BioRad Laboratories (Hercules, CA, USA).

2.2. Animals

Atlantic salmon (*Salmo salar*) juveniles (parr; average length: 9.9 ± 0.1 cm; average weight: 7.76 ± 0.2 g) were obtained from Settefiskanlegget Lundamo AS (Lundamo, Norway) and brought at the animal holding facilities of NTNU's Department of Biology (Sealab, NTNU), where the acclimation and the exposure took place. The fish were acclimated for a period of 9 days, in a 100-l tank with continuously running freshwater at 10 °C using a 12 h:12 h photoperiod. The animals were given food once during the acclimation, and thereafter starved, even during exposure, to avoid possible interaction with the contaminant sorption process.

2.3. Exposure and sampling

The fish were divided into six exposure groups and one control group, of 16 individuals each; the treatment groups were exposed for a 7-day period to three different nominal concentrations of each of the two contaminants (0.04, 0.2 and 1 mg/L). The exposure was performed in replicates. Stock solutions (1 g/L) of both TBOEP and TCEP (Table 1) were prepared in Milli-Q water and stored at room temperature (18–20 °C) for the duration of the experiments. The concentrations tested were based on the levels of their environmental occurrence reported in previous literature (Regnery et al., 2011; Bollmann et al., 2012; Cristale et al., 2013), but also included a high concentration of 1 mg/L, that did not constitute acute toxicity risks to the experimental animals. Each group was kept in a 50-l glass tank containing continuously aerated tap water. The water (50%) was changed every three days, and new contaminant solution was added to restore the desired concentration within each tank. The tanks were kept at a constant temperature of 8 °C and a 12 h:12 h photoperiod was used. After the exposure period, lengths and weights were measured for each animal and organs were excised. Liver samples from six individuals (n = 6) of each experimental group were directly homogenized in Direct-zol reagent for RNA isolation. Livers from remaining animals were collected in eppendorf tubes, frozen in liquid nitrogen and stored at −80 °C for enzyme analysis.

2.4. Chemical analysis and metabolite formation

Fish muscle was used as a target organ for analysing contaminant accumulation to reflect food safety values of our study. In addition, the small size of the liver, which should have been the preferred organ for this kind of analysis, also made it difficult to use this organ for this analysis. Fish muscles were dried, crashed, homogenized in a mortar and extracted ultrasonically with methanol. The analysis of the target substances TBOEP, TCEP and their metabolites was performed by liquid chromatography-tandem mass spectrometry (HPLC–MS-MS) at positive electrospray ionisation and multiple reaction monitoring modes. Sample preparation and analysis are described in more detail in the supplementary information (SI). The resulting data were used to calculate bioconcentration factor (BCF). BCF_{fish} is usually expressed as concentration in fish (based on fresh weight: fw) over concentration in water phase. BCF can also be normalised to dry weight or lipid content (UK EPA 2011). Normalization to dry weight is common for risk assessment for food (UK EPA 2011) and normalization to lipid is often performed and relevant for lipophilic chemicals.

The HPLC–MS analysis did not indicate impurities that might have affected the analysis neither in quantification mode nor in MS modes used to identify the metabolites. One impurity was identified as butoxyethanol (measured with GC–MS) which had no importance for the LC–MS analysis and data interpretation. The stability of TCEP and TBOEP in water at 1 mg/L was examined to simulate fish culture conditions and time of exposure. The real concentrations of both OPEs in water were lower than the nominal concentrations by 22–46% caused probably by adsorption on glassware (Table S2), since after the initial