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In ovo transformation of two emerging flame retardants in Japanese quail (*Coturnix japonica*)

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ABSTRACT

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and Dechlorane Plus (DP) are two chlorinated, alternative flame retardants that have been found in wild birds and bird eggs. Little is known about the fate and effect of these compounds in birds, especially during the vulnerable stages of embryonic development. To investigate the ability of birds to biotransform these compounds, an *in ovo* exposure experiment with Japanese quail eggs was performed. Quail eggs were injected in the yolk sac with 1000 ng/g egg of TDCIPP (2.3 nmol/g ww), DP (1.5 nmol/g ww) or a mixture of both and were then incubated at 37.5 °C for 17 days. To get a time-integrated understanding of the *in ovo* transformation of the compounds, one egg per treatment was removed from the incubator every day and analyzed for TDCIPP and its metabolite bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) and/or for DP. By the end of the incubation period, TDCIPP was completely metabolized, while simultaneously BDCIPP was formed. The conversion of the parent compound into the metabolite did not occur proportionally and the concentration of BDCIPP showed a tendency to decrease when TDCIPP became depleted, both indicating that BDCIPP was further transformed into compounds not targeted for analysis. Further untargeted investigations did not show the presence of other metabolites, possibly due to the volatility of the metabolites. On the other hand, the DP concentration did not decrease during egg incubation. This study indicates that within the incubation period, avian embryos are able to biotransform TDCIPP, but not DP.

1. Introduction

Consumer products, such as textiles, building materials, electric and electronic equipment and furniture, consist largely of different types of polymers. Most of these polymers are petroleum-based, which renders them flammable (Alaee et al., 2003). To comply with the increasingly strict fire safety standards, flame retardants (FRs) are added to these products. Flame retardants are chemicals used to prevent ignition and slow down combustion in case of fire. However, these compounds are not always chemically bound to the material (additive FRs) and can therefore easily leach out into the environment during any point of the product's life cycle. In recent decades, it has been shown that some FRs are persistent and exert toxic effects in biota (Ezechiáš et al., 2014; Guigueno and Fernie, 2017; van der Veen and de Boer, 2012). This has created a paradox between fire safety regulations and environmental and health safety regulations. The strict regulations on the use of some brominated FRs by the Stockholm Convention (UNEP, 2017) and other directives have caused an increase in the production and usage of new and unrestricted alternatives, such as chlorinated and phosphorous FRs. Two alternative FRs that replace restricted compounds are tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and Dechlorane Plus (DP). TDCIPP (Fig. 1a) is a chlorinated organophosphate ester (OPE) that has been produced since the 1970s in order to replace tris(2,3-dibromopropyl) phosphate (or Tris) in textiles, after the latter was shown to have mutagenic properties (Blum and Ames, 1977). TDCIPP is mainly used in polyurethane foams and is one of the most commonly detected FRs in residential furniture (Stapleton et al., 2012). Multiple common and trade names (Fyrol FR-2, TDCP, TDCPP, *etc.)* have been used (van der Veen and de Boer, 2012) but for consistency, the acronym TDCIPP will be used throughout the present article. DP is a chlorinated FR that is produced since the mid-60s as a substitute for Dechlorane (or Mirex). It is used among others in electrical hard plastic connectors and cable coatings in televisions and computers (Feo et al., 2012). The commercial mixture of DP consists of two stereoisomers, *syn* and *anti* (Fig. 1b

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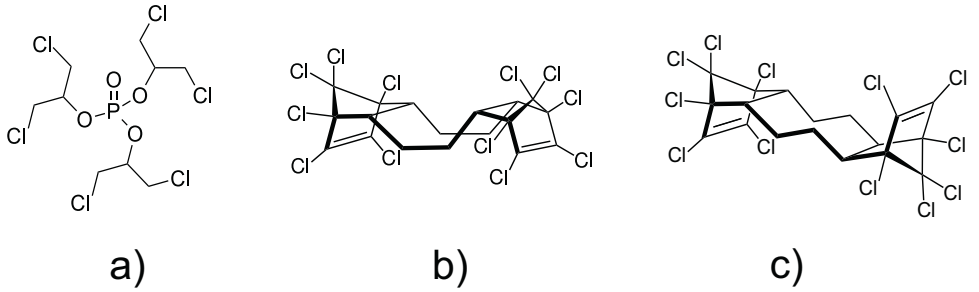

Fig. 1. Chemical structure of a) TDCIPP (CAS: 13674-87-8), b) *syn*-DP (CAS: 135821-03-3) and c) *anti*-DP (CAS: 135821-74-8).

and c) in a 35:65 ratio (Oxychem, 2013).

Both TDCIPP and DP have been detected in the environment and biota, including birds (Chen et al., 2013; Eulaers et al., 2014; Greaves and Letcher, 2014; Marteinson et al., 2016) and bird eggs (Barón et al., 2014; Champoux et al., 2017; Chen et al., 2012; Greaves and Letcher, 2014; Guerra et al., 2011; Muñoz-Arnanz et al., 2011, 2012; Su et al., 2015; Vorkamp et al., 2015). Their presence in bird eggs provides evidence for maternal transfer and may pose a risk to embryonic development and/or later developmental stages. This raises questions on the metabolizing capacity of avian embryos. OPEs have been shown to be rapidly metabolized in mammals by phase I and II metabolism (Hou et al., 2016). DP on the other hand, has shown not to metabolize easily in biota (Meeker et al., 2013; Tomy et al., 2008; Xian et al., 2011). Degradation products of DP have been detected in bird eggs (Guerra et al., 2011; Muñoz-Arnanz et al., 2011, 2012; Zheng et al., 2014a), but some studies suggest they are formed through biotic or abiotic processes prior to uptake or even through analytical impurities (Sverko et al., 2008, 2010; Tomy et al., 2008; Zheng et al., 2010, 2014b). Until now, information on exposure and metabolism of TDCIPP and DP during the embryonic development of birds is very scarce. Farhat et al. (2013) previously showed a decrease of TDCIPP *in ovo* during incubation and Zheng et al. (2014a) observed no change in DP concentrations in eggs from three different time points. However, neither the major metabolite of TDCIPP, bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), nor the general kinetics of the metabolism have been investigated in eggs prior to the present study. Other studies on the metabolism of these compounds had an *in vitro* approach (Chabot-Giguère et al., 2013; Greaves et al., 2016) or were focused on mammals (Lynn et al., 1981; Meeker et al., 2013; Van den Eede et al., 2013).

The objective of this study was to investigate the ability of avian embryos to biotransform two alternative flame retardants, TDCIPP and DP, by means of an *in ovo* experiment. An *in ovo* experiment offers the advantage of mimicking exposure through maternal transfer and allows us to study the effect of a compound or a mixture of compounds on the development of the bird. Here, the Japanese quail (*Coturnix japonica*) was used as an avian model species. Because of its short developmental period and small egg size, this precocial species is very suitable for toxicokinetic studies in the early development (Huss et al., 2008; Jaspers, 2015).

2. Materials and methods

2.1. Chemicals and standards

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP, CAS no. 13674-87-8; 100 µg/mL in toluene) and Dechlorane Plus *syn*- (*syn*-DP, CAS no. 135821-03-3; 50 µg/mL in toluene) and *anti*- (*anti*-DP, CAS no. 135821-74-8; 50 µg/mL in toluene) isomers used for egg injections were purchased from AccuStandard (New Haven, CT, USA). Lecithin and peanut oil were purchased from Merck (Darmstadt, Germany). Ethanol was purchased from VWR International LLC (Radnor, PA, USA).

Individual standards of TDCIPP, BDCIPP, *syn*- and *anti*-DP and the corresponding labelled internal (TDCIPP-d_{15}, BDCIPP-d_{10}, ^{13}C-*syn*-DP and ^{13}C-*anti*-DP) and recovery (triphenyl phosphate-d_{15} / TPhP-d_{15}, 2,2′,3,3′,4,4′,5,6,6′-nonachlorobiphenyl / CB207, β-hexabromocyclododecane-d_{18} / β-HBCDD-d_{18} and tris(2-chloroethyl) phosphate-d_{12} / TCEP-d_{12}) standards for targeted and untargeted chemical analysis were obtained from Wellington Laboratories (Guelph, Ontario, Canada). The recovery standard 1,3-dichloro-2-propanol-d_5 (1,3-DCP-d_5) for untargeted chemical analysis was purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents used (*n*-hexane, acetone acetic acid, ammonia, methanol, dichloromethane / DCM, sulfuric acid, *iso*-octane, cyclohexane and ethyl acetate) were of SupraSolv (Merck, Darmstadt, Germany) or Picograde (LGC Promochem, Wesel, Germany) grade. Sodium sulfate (Na_2SO_4, Merck) and silica gel (0.063 − 0.200 mm, Merck) were pre-washed with *n*-hexane and heated overnight at 150 °C before use.

2.2. Vehicle preparation

An emulsion of peanut oil and water, using lecithin as an emulsifier, was used as a vehicle to dissolve the compounds in the egg yolk. The emulsion was prepared in accordance to Brunström and Örberg (1982) by dissolving lecithin (L-α-phosphatidylcholine from egg yolk) in DCM and peanut oil. Then DCM was evaporated under a stream of clean air, using a heating plate at 35 °C and a stirring magnet. The compounds in solution were then added to the lecithin/peanut oil mixture and the toluene was evaporated using a rotary evaporator (RV 10 digital, IKA) at 150 mbar and approximately 50 °C. In the framework of a larger experiment where the effect of mixture toxicity was assessed, we also investigated the biotransformation of the two compounds when occurring in a mixture. Therefore, the final emulsions contained TDCIPP or DP (*syn*- and *anti*-isomer, nominal proportion 30:70) singly or in an equal mixture of the two, in a concentration of 500 µg/mL. Subsequent chemical analysis indicated that the actual proportions of *syn*- and *anti*-DP in the emulsion ranged between 31:69 and 34:66. After autoclave sterilization (25 min, 120 °C), two parts (1.6 mL) of emulsion were mixed with three parts (2.4 mL) of sterile distilled water to mimic the lipid: water proportion of egg yolk (Brunström and Örberg, 1982). The emulsions were sonicated (Ultrasonic Cleaner, VWR) during 30 s after which they were injected. Chemical analysis of the control emulsions did not show any peaks.

2.3. Egg injection and incubation

The egg experiment was performed in the animal laboratory facilities at the Department of Biology at NTNU, Norway. Fertilized Japanese quail eggs were obtained from a breeder (Birkeland, Norway) and were stored for a maximum of two days in a dark refrigerated room at a temperature of 13 °C. Fifty eggs were divided into three injection treatments: 1000 ng/g egg of TDCIPP (n = 16), 1000 ng/g egg of DP (n = 17) and an equal mixture of 1000 ng/g egg TDCIPP and DP (n = 17). The maximum injected volume was 2 µL/g egg, therefore this volume was adjusted according to the individual egg mass (mean ± SD: 14.3 ± 1.5 g). Eggs were injected in the yolk sac at embryonic day (ED) zero, so before the start of incubation. Prior to injection, the blunt