Cell damage induced by polybrominated diphenyl ethers on *Chironomus sancticaroli* (Diptera: Chironomidae)

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Introduction

Chironomidae are the most common group of insects present in freshwater habitats. As sediment-dwelling organisms in the larval stages, they change the structure and properties of the substrate and act as significant organic matter recyclers (Armitage et al., 1995). In addition to their ecological ubiquity and species richness, chironomid larvae are capable of transferring contaminants present in sediment from larvae to adults (Reinhold et al., 1999) and likewise play an important role transporting contaminants through the food web that can modify ecosystem health (Clements et al., 1994; Pérez-Fuentetaja et al., 2015; Williams et al., 2018). These characteristics as well as their short lifecycle and ability to be easily cultured in the laboratory are advantages that make them good model organisms to indicate the quality of freshwater and sediment environments (Armitage et al., 1995; OECD, 2010; Serra et al., 2017). *Chironomus sancticaroli* Strixinho-Strixinho, 1981, including its synonym *Chironomus xanthus*, Rempel, 1939, is endemic to the Neotropical region (Trivinho-Strixino, 2011) and has been identified as an appropriate model to estimate local environmental conditions.

Among the chemicals in products (CiPs) which are emerging contaminants of public health concern, the polybrominated diphenyl ethers (PBDEs) stand out. These synthetic chemicals are incorporated either through reaction or addition into household and commercial polymer-based products like textiles, electronic devices and furniture to reduce flammability in case of fire (De Wit, 2002; WHO, 1997). It is estimated that plastic materials flameproofed by PBDEs can contain between 0.5 to 30% of PBDE of their total mass (Alaee et al., 2003). PBDEs can leach out into the environment during their production, use, waste disposal or during the recycling process (Rahman et al., 2001; Salvadoró et al., 2016; Tombesi et al., 2017). In addition, there are sources of PBDEs emission from combustion of waste electrical and electronic equipment (WEEE) and e-waste ignition (Redlern et al., 2017). Leaching rate of PBDEs that are covalently bonded to the polymer is lower than those added or mixed to the product (De Wit, 2002; Rahman et al., 2001). It has been estimated that the cumulative historical production
of mixtures containing PBDEs varied from 100 to 1600 kilotonnes (Abbasi et al., 2019; UNEP, 2018).

Depending on their average bromine content, 209 PBDEs can be produced. They used to be commercialised in the formulation of penta-BDE, octa-BDE and deca-BDE (Alae et al., 2003; Vonderheide et al., 2008; WHO, 1997). Currently, BDE-47 (2,2′,4,4′-tetrabromodiphenyl ether) and BDE-99 (2,2′,4,4′,5-pentabromodiphenyl ether) are most frequently encountered in environmental samples and animal tissues (Cunha et al., 2010; Dornbos et al., 2015; Kalantzis et al., 2009). Nevertheless, BDE-17 (2,2′,4-Tribromodiphenyl ether) has also been detected (Christianson et al., 2008; Mumford et al., 2015; Olukunle et al., 2015).

Even though some of the formulations of PBDEs were withdrawn from the market and banned by the Stockholm Convention, they are still present in environmental compartments (European Union, 2004; GLCC, 2003; Stockholm Convention, 2009). The PBDE levels in the environment depend on factors that include local anthropogenic activities, environmental photodegradation and presence of degrading microorganisms (Shi et al., 2015; Song et al., 2015). Cristale et al. (2019) measured PBDE levels in a Brazilian landfill describing concentrations in soil ranging from 3 to 715 ng g⁻¹ and in dust from 11 to 3500 ng g⁻¹. Similarly, levels of PBDEs in dust from houses ranged from 8 to 407 ng g⁻¹. A different study showed that the maximum level of PBDEs concentration in sediment samples from a groundwater water supply in Brazil was 5.4 ng g⁻¹ (Ferrari et al., 2019). Considering biological samples, PBDEs have been detected in species of fish, mussels and dolphins from Brazil (Silva et al., 2013; Magalhães et al., 2017; Quinet et al., 2011; Yogui et al., 2011).

Due to their physico-chemical properties, PBDEs are persistent and prone to long-range atmospheric transport. Therefore, they have been detected in aquatic and terrestrial organisms, including human blood and breast milk (De Wit et al., 2010; Dornbos et al., 2015; Hurley et al., 2017; Zheng et al., 2016). Several studies have shown that some congeners modify developmental and reproductive systems (Dufault et al., 2005; Usenko et al., 2011), are neurotoxic (Costa et al., 2014; Zheng et al., 2017) and carcinogenic (Man et al., 2011; Ward et al., 2014) and induce endocrine disruption (Fernie et al., 2005; Metcalfe et al., 2013). Studies related to the potential effects of PBDEs on population or community levels are rare, although it has been described that the presence of PBDEs in sediments can influence recruitment and fitness of marine benthic communities, specifically, polychaetes once PBDEs alter their settlement in a habitat (Lam et al., 2010).

The health status of a single organism as a result of sublethal effects caused by environmental contaminants can be measured at sub-cellular level allowing the recognition of target organs which in turn can be identified by histological analyses. The alterations can help to predict changes at higher levels of the species that make up the ecosystems affected by the contaminants (Hinton et al., 1992; Myers & Fournier, 2002). In particular, some cells or structures of chironomid larvae can be histologically analyzed to identify the effects of contaminants due to their importance in the physiology of the organism. For instance, oenocytes cells are associated with detoxification processes of larvae (Locke, 1969; Lycett et al., 2006) and are related to the formation of an impermeable surface in the epicuticle (Roma et al., 2010). Additionally, oenocytes contribute to lipid metabolism (Gutiérrez et al., 2007) and production of hormones such as ecdysteroids that induce the metamorphosis and activate the renovation of the larval tissues (Rachinsky et al., 1990; Romer & Bressel, 1994), and they influence the storage site of haemoglobins (Bergtrom et al., 1976).

Similarly, Cuénot cells, which are aggregates of oesophageal projection units, are responsible for producing the peritrophic matrix cells (Pierson, 1956; Richard et al., 2015). The salivary glands of chironomids are responsible for producing structural proteins that modulate the silk production involved in filter feeding and construction of housing tubes during the larval and prepupal stages (Wallace & Merritt, 1980; Wieslander, 1994). Malpighian tubules that lie freely in the body cavity, are related to the homeostasis regulation and removal of nitrogenous waste products and toxic substances, and some are also linked to the immune defence of insects (Beyenbach et al., 2010; Chapman, 2013; Wigglesworth, 1972). Because interaction with contaminants occurs after ingestion of food or water, we considered that the midgut should also be considered a target site for our analyses.

Taking all of the aforementioned aspects into account and the little information available on the influence of PBDEs in tissues of chironomids, the goal of this study was to assess the effects of BDE-17, BDE-47 and BDE-99 on different tissues of fourth instar larvae of C. sancticaroli after short-term exposure.

Materials and methods

Larvae of Chironomus sancticaroli were obtained from the colony of the Laboratory of Morphology and Physiology of Culicidae and Chironomidae (LAMFIC), maintained at the Federal University of Paraná (UFPR). The colony was kept in aerated aquarium following the protocol of Maier et al. (1990) under 25°C ± 2, 80% relative humidity and photophase:scotophase (12:12). Larvae were fed three times a week with DogChow® (Nestlé – Purina, Switzerland). Voucher specimens of this colony are in the Entomology Museum of the Zoology Department at the Federal University of Paraná (DZUP) with accession numbers 249269 to 249276.

To perform the bioassays, freshly laid egg masses from the colony were transferred to trays containing reconstituted water with 1.2 mg L⁻¹ hydrated CaSO₄, 0.08 mg L⁻¹ KCl, 2.44 mg L⁻¹ MgSO₄·7H₂O, 1.92 mg L⁻¹ Na₂CO₃, conductivity of 160 μS cm⁻¹, pH 7.2 and hardness 16 mg L⁻¹. Larvae were fed with TetraMin® fish three times per week and constant aeration was maintained until they reached the fourth-instar, when they were exposed to PBDEs.

Nine bioassays were conducted in glass vessels containing ten larvae each, 50 mL of reconstituted water and 13 g of sand 50-70 mesh (CAS No 5436-43-1) and BDE-99 were tested at 0.5, 2.0 and 20 μg L⁻¹ after 48 hours of exposure. Two acetone and two water replicates were considered control groups. In total, 130 larvae were exposed. Bioassays were conducted in a BOD chamber under 25°C ± 2, 80% relative humidity and a photoperiod of 12 hours. Additionally, temperature, conductivity, pH and dissolved oxygen concentrations were determined at 0 hours and 48 hours after exposure.

Experimental solutions

Analytical grade standards of BDE-17 (CAS No. 147217-75), BDE-47 (CAS No 5436-43-1) and BDE-99 (CAS No 60348-60-9) in isooctane (50 μg mL⁻¹) were purchased from Accustandard® (New Heaven, CT). Stock solutions at 1000 μg L⁻¹ for dosing at 0.5 and 2.0 μg L⁻¹ were prepared in acetone and stored in amber glass vials at -20°C until the bioassays were initiated. Larvae exposed to 20 μg L⁻¹ were dosed directly from the pure standard.

Histopathology

Fresh larvae were fixed for 24 hours in Dubosq-Brazil solution, dehydrated with a graded series of ethanol, diaphanized in xylene, imbedded in Paraplast Plus® (Sigma) and cut into sections 7 μm thick. Sections obtained from whole larvae were stained with haematoxylin and eosin for light microscopic analyses. The histology preparation
was performed in the Laboratory of Reproduction and Community of Fish at UFPR.

Images were acquired under a light microscope (Scope.A1, Carl Zeiss, Germany) using a digital camera (AxioCam ERc5s, Carl Zeiss) and processed with Axion Vision SE64 software. Of the ten larvae exposed to each PBDE concentration, five were analysed. Of the control replicates, in water and solvent, also five of each were analysed, amounting to a total of 55 larvae analysed. Images of the exposed larvae were compared with those of the control treatments (water and solvent) as well as with those of the literature. A binomial test was used to assess the presence or absence of cell alterations. Significance level was set at 95%. Data were processed using BioEstat version 5.0 software (Ayres et al., 2005).

Results

The concentrations of PBDEs used in the experiment did not cause mortality to the fourth instar *C. sancticaroli* larvae. All concentrations of the test solutions were within 10% variation with respect to the nominal values.

The structures that evidenced some alteration after exposure to BDE-17, BDE-47 and BDE-99 were oenocytes, Cuénot cells, midgut regions and salivary glands. None of the larvae from water or solvent controls exhibited any cell alteration.

Larval oenocytes showed changes such as vacuolisation of the cytoplasm (Fig. 1). The BDE-17 caused this type of alteration in 20%
of the larvae exposed at 0.5 and 20 μg L\(^{-1}\), whereas the concentration 2.0 μg L\(^{-1}\) caused alteration in 60% of the observed larvae (\(X^2=5.06, df=1, p=0.167\)). The BDE-47 caused alteration in 40% of the larvae exposed to the three concentrations (\(X^2=3.46, p=0.325\)), and the oenocytes of the larvae exposed to the different concentrations of BDE-99 did not present alterations (\(X^2=6.66, df=1, p=0.034\)) (Table 1).

The Cuénot cells displayed disruption and displacement in the apex brush border (Fig. 1). These alterations were observed in 40% of the larvae exposed to BDE-17 at 20 μg L\(^{-1}\) (\(X^2=5.6, df=1, p=0.019\)). Equally, larvae treated with BDE-99 at 0.5 μg L\(^{-1}\) and 2.0 μg L\(^{-1}\) presented the same changes in the apex brush border (\(X^2=4.53, df=1, p=0.020\)), whereas BDE-47 at 20 μg L\(^{-1}\) caused a significant alteration in 60% of the exposed larvae (\(X^2=8.66, df=1, p=0.034\)) (Table 1).

Midgut cells exhibited changes in the morphology of the apex cells located near the lumen of region I (Fig. 2). This change was observed in 20% and 40% of larvae exposed to BDE-17 at 0.5 μg L\(^{-1}\) and 2.0 μg L\(^{-1}\) respectively (\(X^2=4.0, df=1, p=0.261\)). Similarly, BDE-47 at 0.5 μg L\(^{-1}\) and 2.0 μg L\(^{-1}\) caused changes in 40% of exposed larvae (\(X^2=3.46, df=1, p=0.325\)). On the other hand, BDE-99 did not cause changes in the morphology cells of region I of the midgut but induced narrowing of the lumen diameter in 20% of the larvae exposed exclusively at 0.5 μg L\(^{-1}\) (\(X^2=5.06, df=1, p=0.167\)) (Fig. 2). Furthermore, the midgut region II exhibited cytoplasmic vacuolisation in 60% of the larvae exposed at 2.0 μg L\(^{-1}\) of BDE-47 while BDE-99 at 20 μg L\(^{-1}\) induced vacuolisation of the cytoplasm in 40% of the exposed larvae (\(X^2=5.6, df=1, p=0.033\)) (Fig. 2) (Table 1).

The salivary glands were altered showing acidophilic granules in their cytoplasm. These granules were observed in 60% of the larvae exposed to BDE-17 at 0.5 μg L\(^{-1}\) and 20 μg L\(^{-1}\) (\(X^2=9.86, df=1, p=0.019\)). BDE-47 also induced formation of granules in 40% of the larvae exposed at 0.5 and 2.0 μg L\(^{-1}\) respectively (\(X^2=5.86, df=1, p=0.11\)) (Fig. 3) (Table 1).

Table 1

| Structure   | Concentration (μg.L\(^{-1}\)) | BDE-17 | 0.5 | 2.0 | 20 | p value | \(X^2\) |
|-------------|------------------------------|--------|-----|-----|----|---------|--------|
| Oenocytes   |                              |        | 20  | 60  | 20 | 0.167   | 5.06   |
| Cuénot cells |                             |        | 0   | 0   | 40 | 0.132   | 5.60   |
| Midgut region I |                         |        | 20  | 40  | 0  | 0.261   | 4.00   |
| Midgut region II |                        |        | 0   | 0   | 0  | 0.083   | 6.66   |
| Salivary glands |                         |        | 60  | 0   | 60 | 0.019*  | 9.86   |

| Structure   | Concentration (μg.L\(^{-1}\)) | BDE-47 | 0.5 | 2.0 | 20 | p value | \(X^2\) |
|-------------|------------------------------|--------|-----|-----|----|---------|--------|
| Oenocytes   |                              |        | 40  | 40  | 40 | 0.325   | 3.46   |
| Cuénot cells |                             |        | 0   | 60  | 0  | 0.034*  | 8.66   |
| Midgut region I |                         |        | 20  | 20  | 0  | 0.325   | 3.46   |
| Midgut region II |                        |        | 0   | 60  | 0  | 0.040*  | 8.26   |
| Salivary glands |                         |        | 40  | 40  | 0  | 0.118   | 5.86   |

| Structure   | Concentration (μg.L\(^{-1}\)) | BDE-99 | 0.5 | 2.0 | 20 | p value | \(X^2\) |
|-------------|------------------------------|--------|-----|-----|----|---------|--------|
| Oenocytes   |                              |        | 0   | 0   | 0  | 0.083   | 6.66   |
| Cuénot cells |                             |        | 40  | 40  | 0  | 0.209   | 4.53   |
| Midgut region I |                         |        | 20  | 0   | 0  | 0.167   | 5.06   |
| Midgut region II |                        |        | 0   | 0   | 40 | 0.133   | 5.6    |
| Salivary glands |                         |        | 0   | 0   | 0  | 0.083   | 6.66   |

*indicates statistical difference (\(p<0.05\))
Disruption of the brush border of Cuénot cells was observed in larvae exposed to the three PBDE congeners, being the most noteworthy alteration in larvae exposed to BDE-47 at 2.0 μg L⁻¹. The injury to the Cuénot cells structure by PBDEs can compromise the insect gut physiology as a consequence of failure in the production of the peritrophic matrix that protects the epithelium from mechanical abrasion (Agrawal et al., 2014; Tellam, 1996; Terra, 2001). Moreover, the transport of ions, nutrients, water and digestive enzymes (Zhuzhikov, 1964), as well as inactivation of ingested toxins (Hegedus et al., 2009), can also be altered in the larvae.

The midgut of C. sancticaroli larvae showed variation in the morphology of the apex cells located next to the lumen after BDE-17 and BDE-47 exposure. BDE-99 produced the narrowing of the larval lumen in region I and BDE-47 and BDE-99 caused cytoplasmic vacuolisation of region II of the larval midgut. This vacuolisation was more evident in the larvae exposed to BDE-47. Cell alteration in region II of the midgut was not observed. The cytoplasmic vacuolisation observed can be associated with an imbalance of the osmotic process as recognised in larvae of Aedes aegypti (Linnaeus, 1762) after exposure to Annona coriacea Mart. (Annonaceae) and Schinus terebinthifolius Raddi (Anacardiaceae) extracts (Costa et al., 2012; Procópio et al., 2015). Similarly, larvae of Ceraceochrysa claveri (Navás, 1911) also displayed vacuoles in the cytoplasm after neem oil intake (Scudeler & Santos, 2013). At the same time, cytoplasm vacuolisation can be caused by synthetic insecticides targeting ion channel proteins as reported in Culex quinquefasciatus Say, 1823 (Alves et al., 2010) and compounds that interact with the nicotinic acetylcholine receptors in the nervous system as described in Apis mellifera Linnaeus, 1758 (Catae et al., 2014; Kakamand et al., 2008).

Salivary glands exhibited granule formation in the cytoplasm prompted by BDE-17 and BDE-47 characterised by a high eosin affinity indicating its acid nature. Salivary glands of larvae exposed to BDE-17 exhibited more granules than those exposed to BDE-47. The present study describes for the first time the acid grains growth in chironomid salivary glands. Some alterations reported in the salivary glands of insects encompass the disruption of the membrane cells, cytoplasm vacuolisation and nuclear deformation after exposure to neonicotinoids in Musca domestica Linnaeus, 1758 (Mohammed Xider, 2018). In addition to cellular modifications caused by the direct ingestion of xenobiotics, the capacity of salivary glands to remove substances from the haemolymph that were not eliminated by the Malpighian tubules can also compromise the cellular integrity of the glands (Armbuster et al., 1986; Meirles et al., 2001). Moreover, failures in silk production can compromise the anti-predator function of tubes and compromise the survival of individuals (Dillon, 1985; Hershey, 1987).

Our study did not detect any alterations in the Malpighian tubule cells as an effect of the PBDE exposure. The maintenance of the integrity may be correlated with the presence of a large quantity of mitochondria, which means that cells are very active (Chapman, 2013).
and rich in genes, expressed in this organ, that are responsible for regulating enzymes like glutathione S-transferase and Cytochrome P450 as described in Drosophila (Wang et al., 2004). These enzymes are involved in the metabolism and detoxification of toxic substances present in organisms (Boelsterli, 2007). It was demonstrated that BDE-17, -47 and -99 at concentrations ranging from 0.5 to 3 µg L⁻¹ caused an increase of the glutathione S-transferase activity of the C. sancticaroli larvae (Palacio-Cortés et al., 2017).

Despite histological alterations which commonly provide powerful tools for distinguishing and describing the long-term effects of environmental contaminants (Ben Ameur, 2015; Liney et al., 2006; Schweiger et al., 2000), the current study detected histopathologic lesions of PBDEs in C. sancticaroli larvae in short-term exposure. In conclusion, this study demonstrates that BDE-17, BDE-47 and BDE-99 in an in vivo model induced histological alterations along the evaluated concentrations. This paper contributes to the knowledge of the effects of PBDEs in cell structures of the Chironomus sancticaroli larvae as biomonitoring species of sediment systems.

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Conflict of interest

The authors declare no conflict of interest.

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