



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

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ABSTRACT

Histological effects of polybrominated diphenyl ethers (PBDEs) were observed in *Chironomus sancticarli* larvae which underwent acute exposure. 2,2',4'-triBDE (BDE-17), 2,2',4,4'-tetraBDE (BDE-47) and 2,2',4,4',5-pentaBDE (BDE-99) were evaluated at 0.5, 2.0 and 20 µg L⁻¹. Cytoplasm vacuolisation of oenocytes was observed in the larvae exposed to BDE-17 and BDE-47. Cuénot cells were disrupted at the brush border as an effect of the three evaluated congeners highlighting BDE-47 at 2.0 µg L⁻¹; 60% of larvae displayed this disruption. The midgut showed changes in the morphology of apex cells located next to the lumen of region I exposed to BDE-17 and BDE-47, while BDE-99 induced a narrowing of the lumen diameter. Significant cytoplasm vacuolisation of the larvae exposed to BDE-47 and BDE-99 was observed in region II of the midgut. Salivary glands showed acidophilic granules in the cytoplasm exposed to BDE-17 and BDE-47. The results showed that the tissues of *C. sancticarli* were sensitive to flame retardants; these histopathologies can compromise the health and physiology of this organism, highlighting the concern with the presence of PBDEs in freshwater sediments.

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 sancticarli* Strixino-Strixino, 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; Salvadó 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 (Redfern 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

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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 (Alaee 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; Kalantzi et al., 2009). Nevertheless, BDE-17 (2,2',4-Tribromodiphenyl ether) has also been detected (Christiansson 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 PBDEs 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; Quinete 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 & Fournie, 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 (Gutierrez 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; Richardi 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. sancticarloi* after short-term exposure.

Materials and methods

Larvae of *Chironomus sancticarloi* were obtained from the colony of the Laboratory of Morphology and Physiology of Culicidae and Chironomidae (LAMFIC²), maintained at the Federal University of Paraná (UFPR). The colony was kept in aerated aquaria 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 Sigma[®]. BDE-17, BDE-47 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±2 °C, 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 isoctane (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 Duboscq-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%

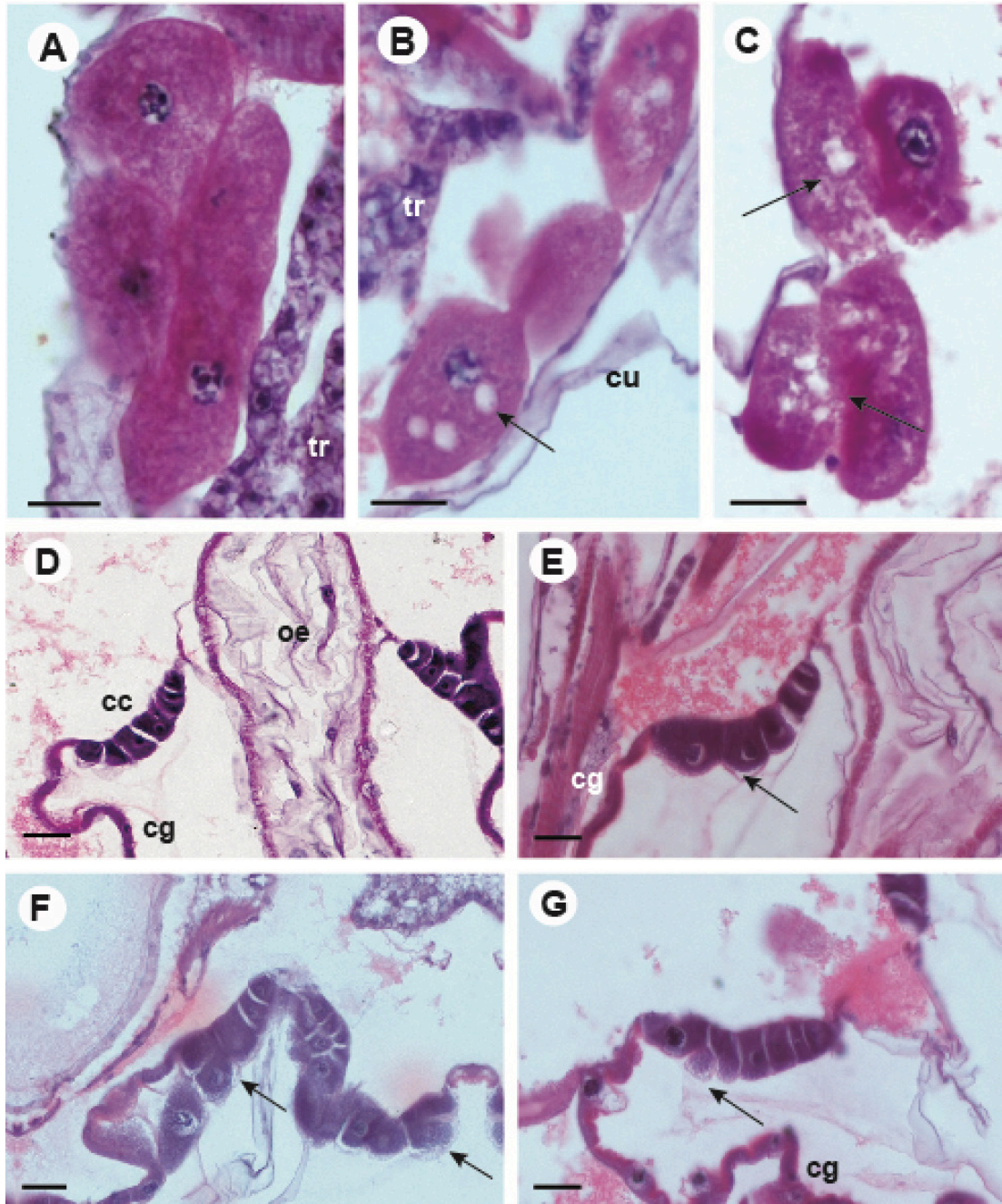


Figure 1. Micrographs of oenocytes and Cuénot cells of *Chironomus sancticaroli* larvae after PBDE exposure. **A.** Oenocytes of control (water). **B.** Oenocytes exposed to BDE-17 at 20 µg L⁻¹. **C.** Oenocytes exposed to BDE-47 at 2.0 µg L⁻¹. Arrows show cytoplasm vacuolization. **D.** Cuénot cells of control (water). **E.** Cuénot cells exposed to BDE 17 at 20 µg L⁻¹. **F.** Cuénot cells exposed to BDE 47 at 2.0 µg L⁻¹. **G.** Cuénot cells exposed to BDE 99 at 0.5 µg L⁻¹. Arrows show the brush border disruption and displacement in the apex cell. **tr:** trophocytes, **cu:** cuticle, **cc:** Cuénot cells, **oe:** esophagus, **cg:** gastric caeca. Scale bar: 20 µm.

of the larvae exposed at 0.5 and 20 $\mu\text{g L}^{-1}$, whereas the concentration 2.0 $\mu\text{g L}^{-1}$ caused alteration in 60% of the observed larvae ($\chi^2 = 5.06$, $df=1$, $p=0.167$). The BDE-47 caused alteration in 40% of the larvae exposed to the three concentrations ($\chi^2 = 3.46$, $p=0.325$), and the oenocytes of the larvae exposed to the different concentrations of BDE-99 did not present alterations ($\chi^2 = 6.66$, $df = 1$, $p=0.083$) (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 $\mu\text{g L}^{-1}$ ($\chi^2=5.6$, $df=1$, $p=0.132$). Equally, larvae treated with BDE-99 at 0.5 $\mu\text{g L}^{-1}$ and 2.0 $\mu\text{g L}^{-1}$ presented the same changes in the apex brush border ($\chi^2=4.53$, $df=1$, $p=0.209$), whereas BDE-47 at 2.0 $\mu\text{g L}^{-1}$ caused a significant alteration in 60% of the exposed larvae ($\chi^2=8.66$, $df=1$, $p=0.034$).

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 $\mu\text{g L}^{-1}$ and 2.0 $\mu\text{g L}^{-1}$ respectively ($\chi^2=4.0$ $df=1$, $p=0.261$). Similarly, BDE-47 at 0.5 $\mu\text{g L}^{-1}$ and 2.0 $\mu\text{g L}^{-1}$ caused changes in 40% of exposed larvae ($\chi^2=3.46$, $df=1$, $p=0.325$). 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 $\mu\text{g L}^{-1}$ ($\chi^2=5.06$, $df=1$, $p=0.167$) (Fig. 2). Furthermore, the midgut region II exhibited cytoplasmic vacuolisation in 60% of the larvae exposed at 2.0 $\mu\text{g L}^{-1}$ of BDE-47 while BDE-99 at 20 $\mu\text{g L}^{-1}$ induced vacuolisation of the cytoplasm in 40% of the exposed larvae ($\chi^2=5.6$, $df=1$, $p=0.133$) (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 $\mu\text{g L}^{-1}$ and 20 $\mu\text{g L}^{-1}$ ($\chi^2 = 9.86$, $df = 1$, $p = 0.019$). BDE-47 also induced formation of granules in 40% of the larvae exposed at 0.5 and 2.0 $\mu\text{g L}^{-1}$ respectively ($\chi^2 = 5.86$, $df = 1$, $p = 0.11$) (Fig. 3) (Table 1).

No histopathological changes in the Malpighian tubules of the *C. sancticaroli* larvae were observed as an influence of the acute PBDEs exposure.

Discussion

Histological analysis revealed injuries in the diverse tissues of *C. sancticaroli* larvae after exposure to PBDE in acute conditions. Some of the most sensitive structures observed were the oenocytes, the BDE-17 and BDE-47 standing out in particular at each tested concentration that prompted cytoplasmic vacuolisation and alterations of the oenocytes format. These alterations might modify larval growth due to physiological functions in which oenocytes are involved. Furthermore, oenocytes that are injured might trigger the larval dispersal syndrome and reduce feeding as they affect lipids, increasing haemolymph as described in larvae of *Drosophila melanogaster* Meigen, 1830 (Gutierrez et al., 2007). The injuries caused to chironomid oenocytes can be correlated with lipophilicity of PBDEs towards lipid-rich structures (ATSDR, 2004) and their role of defence of the organism as the first structure affected in the presence of xenobiotics as described in *Bombus morio* (Swederus, 1787) (Hymenoptera: Apidae) (Abdalla & Domingues, 2015).

BDE-99 did not induce alterations in the larvae's oenocytes despite its known toxic effects, this fact can be explained in part due to the specific debromination (bromine loss) throughout the metabolic pathway of PBDEs which influences their toxicity and bioaccumulation rate (Roberts et al., 2011; Stapleton et al., 2004; Usenko et al., 2013). It is probable that *C. sancticaroli* larvae transform BDE-99 to less brominated congeners such as BDE-47 or BDE-17 via metabolic pathways, as demonstrated in fish species (Roberts et al., 2011) or chemical models (Gandhi et al., 2006).

Table 1
Percentage of cell damage of *Chironomus sancticaroli* after 48h of exposure to BDE-17, BDE-47 and BDE-99 at three different concentrations.

Structure	BDE-17			p value	χ^2
	Concentration ($\mu\text{g.L}^{-1}$)				
	0.5	2.0	20		
Percentage of damage					
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	BDE-47			p value	χ^2
	Concentration ($\mu\text{g.L}^{-1}$)				
	0.5	2.0	20		
Percentage of damage					
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	BDE-99			p value	χ^2
	Concentration ($\mu\text{g.L}^{-1}$)				
	0.5	2.0	20		
Percentage of damage					
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 ($p<0.05$)

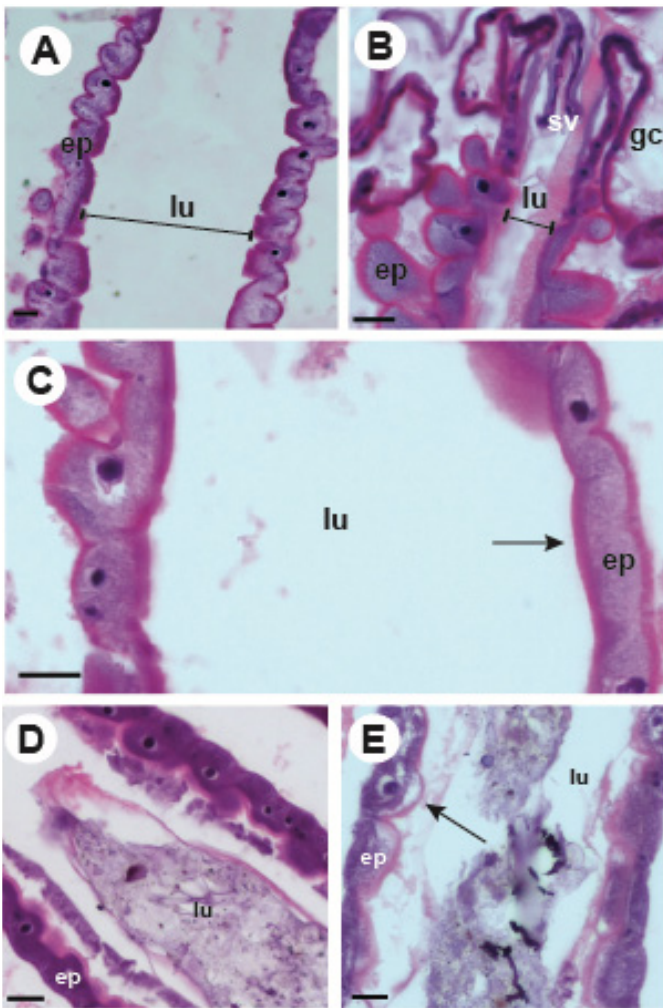


Figure 2. Micrographs of larval *Chironomus sancticaroli* midgut after acute exposition to PBDEs. **A.** Region I midgut lumen belonging to control treatment (water). **B.** Region I midgut lumen exposed to BDE-99 at $0.5 \mu\text{g L}^{-1}$ exhibiting its narrowing. **C.** Epithelial cells of midgut, region I, exposed to BDE 47 at $0.5 \mu\text{g L}^{-1}$ showing changes in cell morphology. **D.** Midgut cells, region II, of control treatment (water). **E.** Midgut cells, region II, exposed to BDE-47 at $2.0 \mu\text{g L}^{-1}$ showing the cytoplasm vacuolization. Bars shows the width of the lumen. **gc:** gastric cecum. **ep:** gut epithelia. **lu:** lumen. **sv:** stomodaeal valve. Scale bar: 20 μm .

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 $2.0 \mu\text{g L}^{-1}$. 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 larvae lumen in region I and BDE-47 and BDE-99 caused cytoplasmic vacuolization of region II of the larvae midgut. This vacuolization was more evident in the larvae exposed to BDE-47. Cell alteration in region II of the midgut was not observed. The cytoplasmic vacuolization observed can be associated with an imbalance of the osmotic process as recognised in larvae of *Aedes aegypti* (Linnaeus, 1762) after exposure to *Annona*

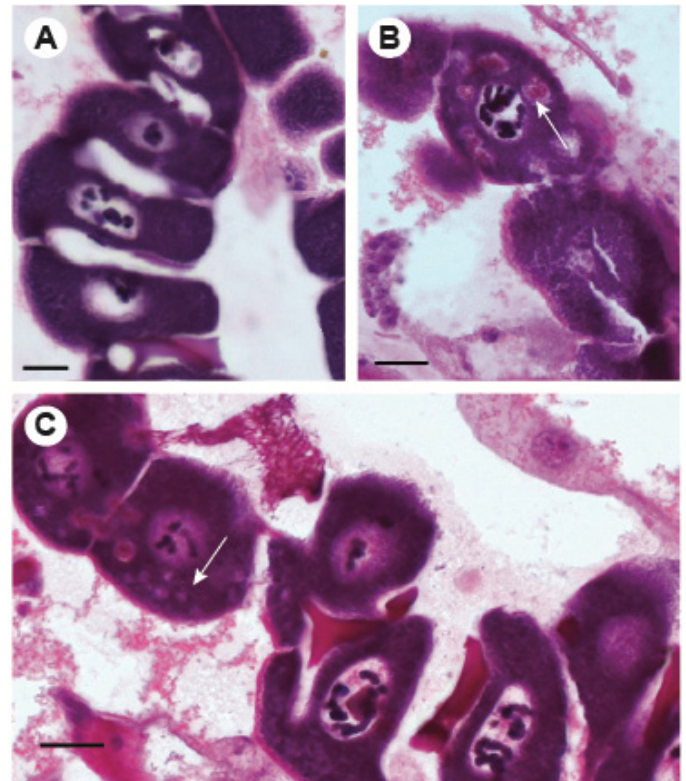


Figure 3. Micrographs of the salivary gland of larval *Chironomus sancticaroli* after acute exposition to PBDEs. **A.** Salivary gland of control treatment (water). **B.** Salivary gland exposed to BDE 47 at $0.5 \mu\text{g L}^{-1}$ showing formation of the cytoplasm acidophilic granules. **C.** Salivary gland exposed to BDE 17 at $20 \mu\text{g L}^{-1}$ shows formation of the cytoplasm acidophilic granules. Arrow. Scale bar: 20 μm .

coriacea Mart. (Annonaceae) and *Schinus terebinthifolius* Raddi (Anacardiaceae) extracts (Costa et al., 2012; Procópio et al., 2015). Similarly, larvae of *Ceraeochrysa 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 (Armbruster et al., 1986; Meirelles 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 Ameer et al., 2015; Liney et al., 2006; Schwaiger 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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