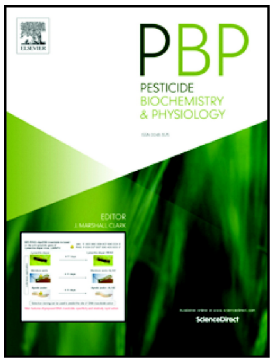


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PII: S0048-3575(16)30146-8
DOI: doi: 10.1016/j.pestbp.2017.04.002
Reference: YPEST 4049

To appear in: *Pesticide Biochemistry and Physiology*

Received date: 28 October 2016
Revised date: 27 February 2017
Accepted date: 13 April 2017

Please cite this article as: Sabrina Lavarías, Florencia Arrighetti, Augusto Siri , Histopathological effects of cypermethrin and Bacillus thuringiensis var. israelensis on midgut of Chironomus calligraphus larvae (Diptera: Chironomidae). The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Ypest(2017), doi: 10.1016/j.pestbp.2017.04.002

*Pesticide Biochemistry and Physiology*

# Histopathological effects of cypermethrin and *Bacillus thuringiensis var. israelensis* on midgut of *Chironomus calligraphus* larvae (Diptera: Chironomidae)

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**Abstract**

Pesticides are extensively used for the control of agricultural pests and disease vectors, but they also affect non-target **organisms**. Cypermethrin (CYP) is a synthetic pyrethroid used worldwide. Otherwise, bioinsecticides like *Bacillus thuringiensis* var. *israelensis* (*Bti*) have received great attention **as an environmentally benign and desirable alternative**. In order to evaluate the toxicity of those pesticides, *Chironomus calligraphus* was selected due to its high sensitivity to some toxicants. **Third and fourth instars larvae were** exposed to serial dilutions of CYP and *Bti* to determine $LC_{50}$ values. **In order to evaluate the potentially histopathological alterations as biomarkers**, after 96-h of exposure, live larvae were fixed for histological analysis of **the mid region of digestive tract**. The 96-h $LC_{50}$ values were 0.52 and 1.506 µg/L for CYP and *Bti*, respectively. **Midgut histological structure of the control** group showed a single layer of cubical cells with microvilli in their apical surface and a big central nucleus. The midgut epithelium of larvae exposed to **a** low concentration of CYP **(0.037 µg/L)** showed secretion activity and vacuolization while at high concentration **(0.3 µg/L)** cells showed a **greater** disorganization and a more developed fat body. On the other hand, *Bti* caused progressive histological damage in this tissue. *Chironomus calligraphus* is sensitive to *Bti* and CYP toxicity like other *Chironomus* species. The histopathological alterations could be a valuable tool to assess toxicity mechanism of different pesticides.

**Keywords**: Aquatic toxicology; *Chironomus*; Histopathological alterations; $LC_{50}$; Pesticides

## 1. Introduction

**Freshwater systems can be usually contaminated by direct overspray, runoff or accidental spillage of pesticides, thus aquatic organisms can be exposed to either sublethal or lethal concentrations of these toxicants** [1].

Cypermethrin (CYP), the α-cyano-3-phenoxybenzyl ester of 2,2-dimethyl-3-(2,2-dichlorovinyl) 2-2-dimethylcyclo- propane carboxylate, is a type-II pyrethroid insecticide. This pesticide is used worldwide in agriculture, home pest control, food stuff protection and disease vector control because of its relatively good photostability and broad spectrum insecticidal activity [2]. In Argentina, it was observed that CYP concentrations **can exceed aquatic ecological guidelines**, so non-target fauna could be affected [3]. Several studies have shown that aquatic invertebrates and fish are extremely sensitive to neurotoxic effects of CYP by altering foraging and prey-location behaviors, development, growth, and reproduction [4]. However, it is known that some pests, like mosquitoes, have developed resistance to pyrethroids [5]. Therefore, this fact also contributes to the challenge of searching alternatives to control harmful organisms that do not affect non-target ones.

The Gram-positive bacterium, *Bacillus thuringiensis* var. *israelensis* (*Bti*) is a popular tool for mosquito and black fly control in larviciding programs [6]. *Bti* has been approved by WHOPES for its use in drinking-water that receives little or no further treatment [7]. This biopesticide offers advantages over synthetic insecticides such as cost-effectiveness, target specificity, ease of production and amenability to formulation [8]. Moreover, *Bti* is generally considered safe to dipteran such as chironomids [9].

The cosmopolitan Chironomidae, also called non-biting midges, are commonly the most ubiquitous and the most abundant freshwater insect [10]. Larvae of these **dipterans** are benthic macroinvertebrates that live at the sediment-water boundary and they are important recyclers of organic matter, which is used by other detritivorous organisms of the freshwater

environments [11]. **Larval** stages also play an important role as link between primary producers and consumers [12]. Also, dipteran larvae are frequently used for sediment and water toxicity assessments due to their life-cycle and sensitivity so they are ideal test organisms for studying effects of pesticides on the life-history traits of non-target aquatic organisms [13].

The study of biomarkers at sub-cellular level could be used to assess the health status of single organisms, thereby anticipating changes at higher levels of biological organization in such ecosystems exposed to pesticides. In this context, several biomarkers have been studied in chironomids, mainly biochemical and molecular ones. **However, there is little information regarding cell and tissue biomarkers in this taxon [14].** Histological analyses could be very sensitive to detect sublethal effects of pollutants in organisms, in order to identify target organs and their toxic mechanisms. Thus the morphological expression of prior exposure to a toxicant is an integrator of biochemical and physiological changes. **In addition, histopathological alterations are considered useful biomarkers of effect because a small sample is required to identify specific lesions within the cells [15]. For this reason, these studies can be carried out on different types of organisms.**

*Chironomus calligraphus* Goeldi shows a Pan-American distribution, predominantly Neotropical [16,17]; it is commonly found in urban streams in the Pampean region. This species is highly sensitive to heavy metals and pesticides [18] so it was selected to evaluate the effect of both CYP and *Bti.* **Due to the fact that the digestive tract is one of the input pathways of toxicants into the organism, the aim of the present work was to study the differential histopathological effects of both insecticides in midgut larvae. Moreover, the lethal dose ($LC_{50}$) of CYP and *Bti* of *C. calligraphus* were assessed.**

## 2. Materials and methods

*2.1. Sample collection*

*Chironomus calligraphus* larvae were collected in an urban stream (Don Carlos stream; 34°52'25.88"S/ 58°01'29.18"W; 7 m. a.s.l.) in La Plata city, Buenos Aires, Argentina. Larvae were collected with a hand net, refrigerated, transported to the laboratory and kept in dechlorinated tap water ($CaCO_3$ hardness, 160 mg/L, pH between 6.9 and 7.05, and dissolved oxygen between 5 and 6 mg/L) at 22 ± 2 °C, and 12:12-h L:D photoperiod for at least two days before the experiments.

*2.2. Acute toxicity test*

Larvae **(3$^{rd}$ and 4$^{th}$ instars)** of *C. calligraphus* were exposed at different CYP and *Bti* concentrations for 96-h. Eight larvae were placed in small glass pots containing 60 ml of test solution which was replaced daily. Two glass pots were used for each treatment (16 animals per treatment). Larvae were exposed to at least six concentrations of each insecticide, whereas other groups were kept as control without any pesticide. **In order to calculate** $LC_{50}$ values, mortality was recorded and dead larvae were removed every 24-h. These bioassays for both pesticides were conducted in triplicate at 20-22 °C and a 12:12-h L:D photoperiod. Larvae were not fed for one day before **the assay nor during** the exposure period. **After the 96-h assays, live larvae exposed to different pesticides concentrations were fixed for histological analyses as described below in order to evaluate dose-response relationship.**

For CYP expositions (Glextrin 25 formulated, which was 25% of active principle purchased from GLEBA S.A. La Plata, Argentina), stock solution of 60 mg/L CYP was prepared in absolute ethanol (grade p.a.) at the beginning of each test and kept in darkness at 4 °C. The subsequent working stock solutions were obtained by diluting the main stock in absolute ethanol to the **effective** concentrations of exposure 0.037, 0.075, 0.15, 0.3, 0.6, 1.2

and 4.8 µg/L CYP. Additionally, a control group with ethanol and another water control group, were included. The final concentration of ethanol was always lower than 0.01% [19].

**CYP concentration in water samples from exposure experiments was analyzed as described by Hladik** et al., [20]. **Water samples (150 ml) were filtered using** a SPE C18 cartridge (UCT) and adsorbed compounds were eluted with ethyl acetate. The extract was dried and then solubilized in hexane. CYP concentrations were determined by gas chromatography (Hewlett Packard® 6890) equipped with electron capture detector (µECD), using a capillary column (HP-5MS) of 30m and 0.25 µm film thickness. Conditions and temperatures were: µECD 320 °C, injector 280 °C operated in splitless mode. The column temperature was programmed as follows: initial temperature 50 °C held for 1 min then increased at a rate of 15 °C $min^{-1}$; final temperature 300 °C held for 15 min. The carrier gas was helium at a flow of 2.5 ml/min. **Decachlorobiphenyl** was used as internal standard, and quantification was performed using the calibration curves of authentic standard run under the same conditions.

For *Bti* expositions (presentation 15 g/L of *Bti* 1200 UTI/mg purchased from BIOAGRO S.A. Gral Las Heras, Buenos Aires, Argentina), **a** stock solution of 3 mg/L *Bti* was prepared in water at the beginning of each test and kept in darkness at 4 °C. The subsequent working stock solutions were obtained by diluting the main stock in water to the concentrations for exposures 0.5, 1, 2, 4, 8 and 16 µg/L *Bti*.

In order to compare the sensitivity of *C. calligraphus* with other chironomids and target organisms such as mosquitoes and blackflies, the species sensitivity distributions (SSDs) for both insecticides were calculated using the USEPA SSD Generator software (http://www.epa.gov/caddis/da_software_ssdmacro.html) as described by Mugni et al. [21]. Comparisons were based on the acute 24-h toxicity data ($LC_{50}$) for freshwater **dipteran** larvae

in similar experimental conditions. Data for CYP were obtained from USEPA ECOTOX database (http://cfpub.epa.gov/ecotox/) while data for *Bti* were obtained from the literature.

### *2.3. Histological analyses*

Histological analyses of the digestive tract epithelium were studied, in particular the midgut region on control and exposed organisms to CYP and *Bti*. After 96-h of treatment, the entire live larvae were fixed in **Bouin's** solution and 4% formaldehyde for 4-h and subsequently stored in 70% alcohol. Larvae were dehydrated using an increasing series of ethanol concentrations and embedded in glycol-methacrilate resin (Leica Historesin®). Sections were cut at 5 µm with an electronic microtome (Leica® RM 2155), stained with hematoxyline-eosine and observed under light microscope (Leica AXIOPLAN 2 Zeiss®).

The degree of histopathology was scored according to the slides percentage with histological damage. The damage level was categorized as slight, moderate, and severe or no histopathology.

### *2.4. Statistical analyses*

Lethal concentration ($LC_{50}$) values and their 95% confidence limits for 24-h intervals were estimated with the standard method of PROBIT *analysis program version 1.5* (US, EPA) as described by Finney [22]. Results of biomarker analysis are shown as mean ± standard deviation (SD). Significant differences ($p < 0.05$) were compared using Tukey post hoc test. Data were analyzed using Instat v. 3.01.

## 3. Results

### *3.1. Determination of $LC_{50}$ values*

**No significant differences were observed between the replicates of each treatment within each trial.**

As expected, the sensitivity of *C. calligraphus* larvae to CYP and *Bti* increased with the exposure time (Table 1). Larval mortality started at 24-h to 0.037 µg/L CYP exposure and there was no survival at 96-h 4.8 µg/L CYP exposure. The variation between $LC_{50}$ values for CYP at 24-h and 96-h was 83.6% ($y = 5.123e^{-0.025x}$ $R^2 = 0.9507$). In *Bti* assay, **larval mortality occurred at** 24-h from the 1 µg/L *Bti* concentration and there was no survival at 96-h 8 µg/L *Bti* exposure. The variation between $LC_{50}$ values for *Bti* at 24-h and 96-h was 96.1% ($y = 105.46e^{-0.047x}$ $R^2 = 0.9743$).

The sensitivity comparisons between *C. calligraphus* with other dipterous species for CYP and *Bti* are shown in Figure 1 A and B, respectively. **Compared to other dipterans, *C. calligraphus* larvae were one of the most tolerant dipterans to CYP (Fig. 1A) and the most sensitive to *Bti* (Figure 1B).**

*3.2. General larval digestive tract histology of Chironomus calligraphus*

The digestive tract is divided in three areas: foregut, a large midgut and hindgut. In the anterior region of the digestive tract, the esophagus is formed by flattened cells with a large nucleus, and the Cuénot cells which present strongly basophilic cytoplasm and large round nucleus (Figure 2A). Three histological regions were distinguished in the midgut (Figures 2B-H). Anterior midgut epithelium showed a star-shaped outline, formed by columnar cells with large nucleus (Figures 2B,C). The basal region of such cells showed a more eosinophilic contour than the rest of the cells and their apical region is covered by short brush border (Figure 2C). The medial midgut was formed by cubical cells with conspicuous apical border, but they were smaller than those of the anterior region (Figures 2D,E). The posterior region of the midgut was formed by small-flattened cells containing a large central nucleus without

condensed chromatin (Figure 2F). In the transition between posterior midgut and hindgut, a valve region formed by cubical epithelium was observed (Figure 2G). The colon and rectum were observed in the final portion of the digestive tract (Figure 2H). This description corresponds to the digestive tract of control larvae; the effect of pesticides in exposed larvae midgut is described below.

*3.3. Histopathological effects of insecticides*

**Among the three areas of digestive tract (fore, mid and hindgut), the midgut is the most susceptible to toxic effects, so after 96-h exposure to CYP and *Bti,* histological analysis was carried out in such live larvae.** Several histological alterations in the midgut epithelium were observed (Table 2). The anterior midgut epithelium of the larvae treated with 0.037 µg/L and 0.075 µg/L of CYP showed vacuolization of the cytoplasm and a slight disorganization (Figures 3A-D). The medial region of the midgut presented cells with a high secretion activity at 0.037 µg/L of CYP (Figure 3B), and in some cases the cytoplasm seems vacuolated (Figure 3C). In larvae exposed at 0.075 µg/L of CYP the basal lamina began to detach from the epithelium (Figure 3E). At 0.3 µg/L of CYP treatment, a degenerative vacuolar hypertrophy of the anterior midgut cells was observed (Figure 3F) followed by a **complete** disorganization of the epithelia (Figure 3G). Additionally, a notable increment in the number of **fat bodies were** observed at 0.3 µg/L of CYP exposure (Figure 3H).

In the larvae exposed at **a** low concentration of *Bti* (0.5 µg/L) no major histological changes were observed in their midgut, except a slight vacuolization of the cytoplasm on the anterior midgut region (Figures 4A,B). Some minor histopathological changes in the epithelium were observed in larvae exposed at 1 µg/L of *Bti*, and these alterations became more severe **at an increasing dose. At 1 µg/L,** the cells of the anterior midgut became elongated and in the medial midgut region, the cells were closely attached to the basal lamina

following a sinuous labyrinth in the basal portion of the cell (Figures 4C,D). In the apical portion of the medial region the brush border was thinner and disrupted, in some cases was lost, and showed a high secretion activity (Figure 4D). At increasing concentrations of *Bti* (4 µg/L), the midgut epithelium started to become slightly disorganized (Figure 4E) and it was no longer connected to the basement membrane (Figure 4F).

## 4. Discussion

Histopathology could be an important tool in ecotoxicological assessment because it helps to detect and characterize biological endpoints of exposure to pollutants and lead us to understand the acute and chronic effects of a toxicant in different tissues and organs. For this type of **biomarker**, it is necessary to study the morphology and cell type composition of target organs in a model animal. As oral absorption of xenobiotics converts the midgut epithelium in a susceptible target organ, the digestive tract structure of *C. calligraphus* larvae was studied.

In control conditions, the midgut of *C. calligraphus* showed similar histological patterns to those described by Richardi et al. [14] in *C. sancticaroli*. Three morphological midgut regions were identified in this study. It is known that in other insect species such as *Rhynchosciara americana* [23] and *C. thummi* [24] these midgut regions are specialized in major functions. Exposure to xenobiotics could disrupt morphological structure as observed by da Silva Cruz et al. [25] in midgut of *Apis mellifera L.* larvae exposed to boric acid and fipronil. In honeybee larvae, vacuolization of the cytoplasm, dilation of the intercellular space, extrusion of cellular contents and chromatin compaction were observed [25]. On the other hand, boric acid caused the same changes mentioned above, in addition to narrowing the width of the epithelium and nuclear pyknosis in midgut of workers ants *Atta sexdens*

*rubropilosa* [26]. Such morphological alterations were similar to those observed in midgut of *C. calligraphus* exposed to CYP.

Pyrethroid insecticides are highly toxic to aquatic invertebrates, and their $LC_{50}$ values are usually lower than 1 µg/L including those ones for mosquito, blackfly, and tsetse fly larvae. For this reason, pyrethroids are often used in vector control [27]. Third-fourth instars larvae of *C. calligraphus* are also sensitive to CYP toxicity showing intermediate $LC_{50}$ values among some species of the genus.

CYP represents more than half of the total insecticide consumption in the Pampas region, and it is usually sprayed over the area to be treated. Under this practice, it was observed that **runoff water** is an important toxicity source in aquatic environments reaching concentrations up to 90 µg/L of CYP [28]. **Although** some studies have shown that CYP concentrations in water samples from the watercourses of affected area usually remained below detection levels, the concentrations in sediments could exceed 100 µg/Kg value [19]. This situation is due to the fact that pyrethroids are very hydrophobic and tend to be absorbed by the organic matter in sediments [29]. So this compartment could provide a dietary exposure route to benthic organisms such as chironomid larvae, and it could cause toxic effects on them [11]. Such proposal is in agreement with Maul et al. [30] who suggested that pyrethroid concentrations in sediments could negatively affect chironomid populations through reduced growth and reproduction.

The most important toxic effects of CYP in insects are mainly on both the peripheral and central nervous systems, because this compound affects voltage-dependent sodium channel and integral protein ATPase in the neuronal membrane [31]. Pyrethroids prolong the opening of these channels stimulating nerve cells, causing paralysis and possibly insect death [2]. Other mechanisms have been proposed to explain the effect of pyrethroids on the central nervous system of organisms. Such mechanisms include the antagonism of GABA mediated

inhibition, modulation of nicotinic cholinergic transmission, enhancement of noradrenalin release, action on calcium channels and inhibition of monoamine oxidase and acetylcholinesterase enzymes among others [32]. On the other hand, pyrethroid could affect the maintenance of homeostasis in freshwater organisms by altering ionic balance and osmoregulation [33].

As a lipophilic compound, CYP could penetrate into the lipid bilayer of tissues and affect fluidity membrane [34]. In fact, it has been observed that CYP induced fragility and destabilization of hemocyte lysosomal membrane in freshwater mollusks [35]. It has also been reported that CYP metabolism in invertebrates causes oxidative stress by production of reactive oxygen **species (ROS) [36], that are represented** by superoxide anion radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) and highly reactive hydroxyl radical (OHs) which are able to induce DNA damage, protein degradation and lipid peroxidation [37]. Therefore, such toxic mechanisms could explain morphological alterations observed in the midgut of *C. calligraphus* larvae. In addition to the effects described above, the development of the fat body in larvae of *C. calligraphus* exposed to high concentration of CYP was observed. This alteration may be related to the storage of secondary metabolite derivatives from pyrethroid detoxification that were absorbed and metabolized through the midgut epithelium, as suggested in the honeybee [25]. In fact, the fat body is responsible for producing the enzymes that participate in the biotransformation of chemicals [14]. Additionally, fat body adipocytes are cells with great plasticity, able to store large amounts of triglycerides, but some physiological situations can cause fat body hypertrophy by extreme lipid deposition [38]. Such metabolic pattern could explain the alterations caused by CYP in midgut of *C. calligraphus* larvae. **Alves et al. [39] also observed that in mosquitoes *Culex quinquefasciatus* exposed to CYP for very short time, the morphological features of midgut and fat body were affected.**

On the other hand, **the** intestinal tract is a common target for pest control strategies due to the eating habits of insects [40]. One of such strategies is the use of bacterial biopesticides like *Bti* which was found to be toxic to mosquitoes [41]. Some studies have shown that it is possible to control mosquitoes by *Bti* without affecting beneficial insects as chironomids [9]. However, the response of chironomid communities to *Bti* is highly variable due to both, environmental factors and variations in the susceptibility of individual chironomid taxa [42]. Among the genus *Chironomus,* there are many different $LC_{50}$ values reported which depend on the *Bti* formulations tested. For instance, Hughes et al. [9] reported values for 48-h $LC_{50}$ ranging from 0.040 to >200 mg/L, for *C. tepperi*. Ali et al. [43] also observed a wide 48-h $LC_{50}$ range (4.46-14.63 mg/L) for *C. salinarius*. Another study in *C. thummi* showed that 24-h $LC_{50}$ values were 0.77 mg/L and 0.33 mg/L for two *Bti* preparations [44]. *Chironomus kiiensis* showed a wide range of $LC_{50}$ values related with the commercial *Bti* formulation evaluated, ranging from 0.92 to 3.58 mg/L at 24-h, and 0.13 to 1.57 mg/L at 48-h [45]. The results of this study demonstrated the high sensitivity of larvae of *C. calligraphus* to *Bti* compared to other species of the genus. Anyway, as mentioned above, it is difficult to compare the sensitivity of larvae to *Bti* because reported values were obtained from different bioassay conditions. Some of these factors could be attributed to different toxin content and type formulations, larvae states associated with the maturity of the defense mechanisms, and the biodisponibility of the toxicant [41]. Such biodisponibility in chironomids could be affected by their larval tubes. Tubes play an important role in feeding, respiration and they evolved as, not only anti-predator adaptations but also protection against toxicants, as suggested by Halpern et al. [46]. It is important to note that in these test conditions, larvae of *C. calligraphus* were not given substrate to build their tubes, so this fact could be the reason why they showed high sensitivity to CYP and *Bti* compared to other chironomids.

The larvicidal activity of *Bti* is due to a large group of proteinaceous toxins which are synthesized during sporulation and accumulated in parasporal inclusions. After the ingestion by a susceptible host, the parasporal body is solubilized and protoxins are released and activated by proteases [41]. The active delta-endotoxin goes through the peritrophic matrix, binds with the cadherin protein located on the brush border membrane of midgut cells and as a consequence, it induces the formation of a pore. Therefore, the ion gradient and osmotic balance of the apical membrane are disrupted by pore formation, increasing water absorption, causing rupture and disintegration of midgut cells [47]. The disruption of the midgut epithelium barrier allows bacterial invasion of the haemocoel, leading to septicemia and death of the insect.

Most studies related to pathogenesis and midgut histopathology caused by *Bti* concluded that the action mode of delta-endotoxins is similar irrespective of the taxonomy of the target insect [41, 48–51]. As observed in other organisms, *C. calligraphus* larvae exposed to *Bti* showed dose-dependent histopathological damage [51]. In larvae exposed at higher concentration than 1 µg/L of *Bti*, the brush border of the medial region of the midgut epithelium became thinner or directly was lost, but the most drastic histopathological symptom was the swelling of columnar cells. Similar alterations were observed in dipteran larvae *Simulium pertinax* exposed to *Bti* [52]. In that organism, *Bti* caused the loss of defined internal fibrillar arrangement in cellular microvilli, possibly due to dissolution of their cytoskeleton, leaving only remnants of distorted membranes [53]. The presence of a well-developed brush border in that digestive tract region would indicate that this would be the main absorption site of the midgut. Therefore, this could be the reason why the medial midgut region was the most affected tissue in *C. calligraphus* larvae by *Bti* infection.

Different environmental pollutants act through similar toxic mechanisms causing a limited number of histological alterations **(biomarkers of effect)** [15]. So, the challenge is to

differentiate the specific etiologic agents responsible for such lesions. The results of this study could be useful to compare the toxic mechanism between two compounds, one anthropogenic and the other one with biological origin. Therefore, it might be interesting to analyze differential responses of both types of insecticides at sublethal concentrations in other animal models.

As mentioned above, the biocide *Bti* has many advantages that make it useful for the control of aquatic pests. However, it could affect non-target organisms at concentrations for routine field applications in control programs [54]. In addition, the resistance developed by some pest organisms to this biocide, requires the application of higher concentrations increasing the ecological risk [55]. Consequently, biological pest control using *Bti* and synthetic insecticides should be carefully studied prior to inoculation and subsequent follow-ups in order to assess their effects on the whole biota of such ecosystems.

## Conclusion

These results indicate that histological midgut structure in *C. calligraphus* larvae is sensitive to both pyrethroid CYP and biopesticide *Bti*. It could be useful to study larvae collected from environments exposed to those insecticides. Thus the relationship between mechanisms of toxicity and histopathological changes could be confirmed in midgut of *C. calligraphus*.



## Acknowledgements

This work received partial financial assistance from Agencia Nacional de Promoción Científica y Técnica, Argentina: PICT 2015-0669 through grant to Dr. Florencia Arrighetti. We are grateful to Norma Tedesco for language revision and Sergio Mijailovsky for chemical analysis. We also thank G. Tarelli from GLEBA for supplying CYP and V. Geloso from

Biagro for supplying *Bti*. This paper is Scientific Contribution N° 977 of the Institute of Limnology "Dr. Raúl A. Ringuelet" (ILPLA, CCT-La Plata CONICET, UNLP).

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## FIGURE CAPTIONS

**Figure 1.** Sensitivity of *C. calligraphus* compared to other dipteran, calculated by species sensitivity distributions (SSDs) for CYP (A) and *Bti* (B) using acute 24-h toxicity data.

**Figure 2.** Digestive tract of *C. calligraphus* in control group. **A.** Longitudinal section of the foregut and gastric caeca region, showing the esophagus and the caecum, note Cuénot cells. **B**. Longitudinal section of the anterior midgut epithelium. **C**. Detail of the columnar cells of the anterior midgut with a short brush border in the apical region of the cell. **D**. Longitudinal section of the medial midgut epithelium. **E**. Detail of the cells of the medial midgut, note the dense brush border projecting into the lumen of the midgut. **F**. Posterior region of the midgut epithelium showing small-flattened cells. **G.** Longitudinal section of the transition region between posterior midgut and hindgut showing the proctodeal valve (arrow). **H**. Longitudinal section of the colon and rectum. cae: gastric caeca, cc: Cuenót cells, fd: food, lu: lumen, mf: muscle fibers, mg: midgut, mt: Malpighian tubule, oe: oesophagus

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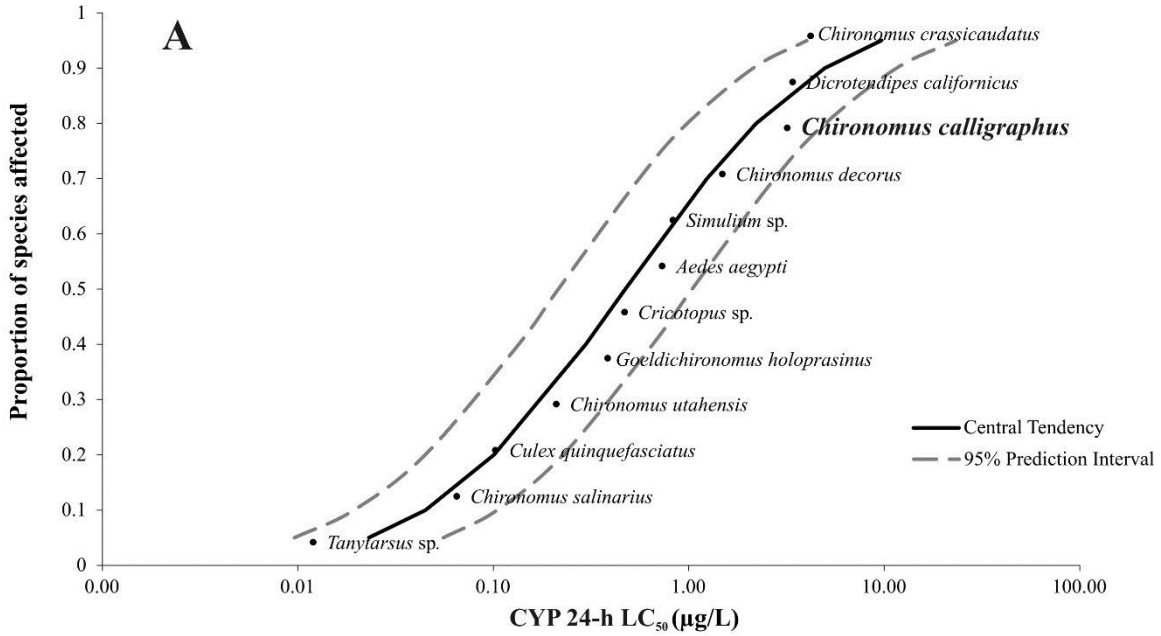
**Figure 3.** Midgut epithelium of *C. calligraphus* after cypermethrin exposure. **A-C.** Midgut epithelium at 0.037 µg/L of CYP treatment. **A**. Anterior midgut epithelium showing vacuolization of the cytoplasm. **B.** Medial region of the midgut epithelium, showing intense active synthesis of some acidophilic secretion (arrow). **C.** Medial region of the midgut showing vacuolization of the cytoplasm. **D.** Anterior midgut at 0.075 µg/L of CYP treatment showing cells disorganized. **E.** Medial region of the midgut epithelium that lost contact with their basal membrane at 0.075 µg/L of CYP treatment. **F.** Anterior midgut showing hypertrophy of cells at 0.3 µg/L of CYP treatment. **G**. Disorganized midgut epithelium at 0.3

µg/L of CYP treatment. **H**. Detail of the fat body surrounding a ganglion in the nerve cord. fb: fat body, fd: food, g: ganglion lu: lumen, mf: muscle fibers.

**Figure 4.** Midgut epithelium of *C. calligraphus* after *Bti* exposure. **A.** Columnar cells of the anterior midgut epithelium after treatment with 0.5 µg/L of *Bti*. **Inset**: several cytoplasmic vacuoles (arrow). **B**. Medial midgut epithelium showing extended microvilli and several vacuoles in the cytoplasm after treatment with 0.5 µg/L of *Bti*. **C** and **D**. Anterior (C) and medial (D) midgut epithelium at 1 µg/L showing infolds in the plasma membrane forming a basal labyrinth. **E.** Anterior midgut epithelium at 4 µg/L of *Bti* treatment starting to become disorganized. **F.** Medial midgut epithelium showing dilated intercellular spaces in the basal at 4 µg/L of *Bti* treatment. fd: food, lu: lumen, mf: muscle fibers.

Figure 1



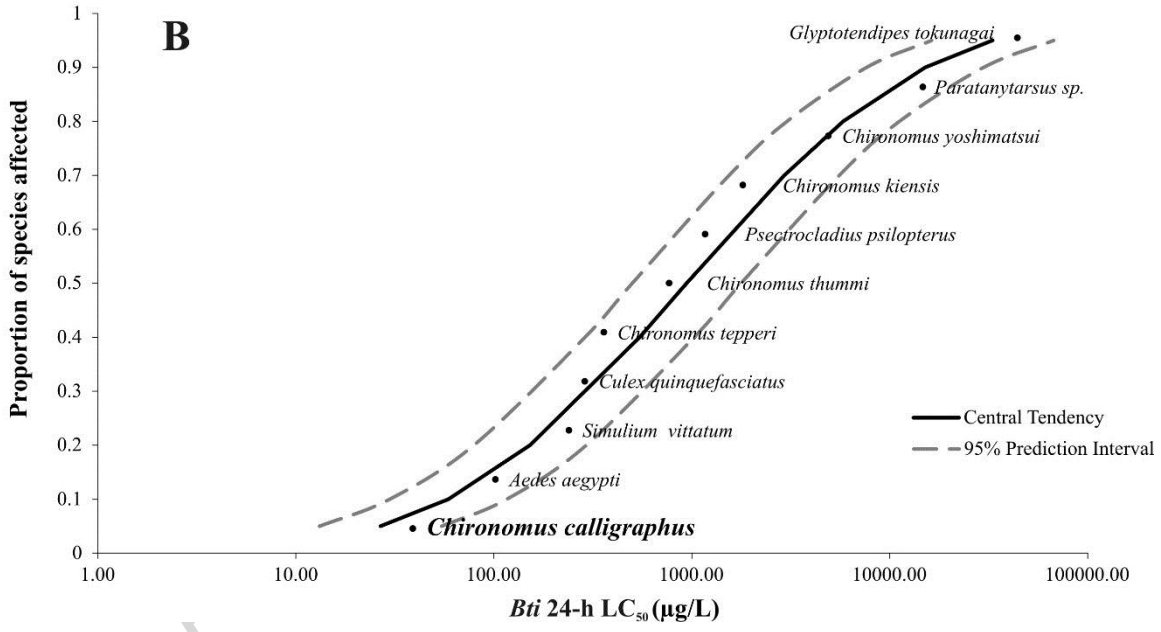



Figure 2

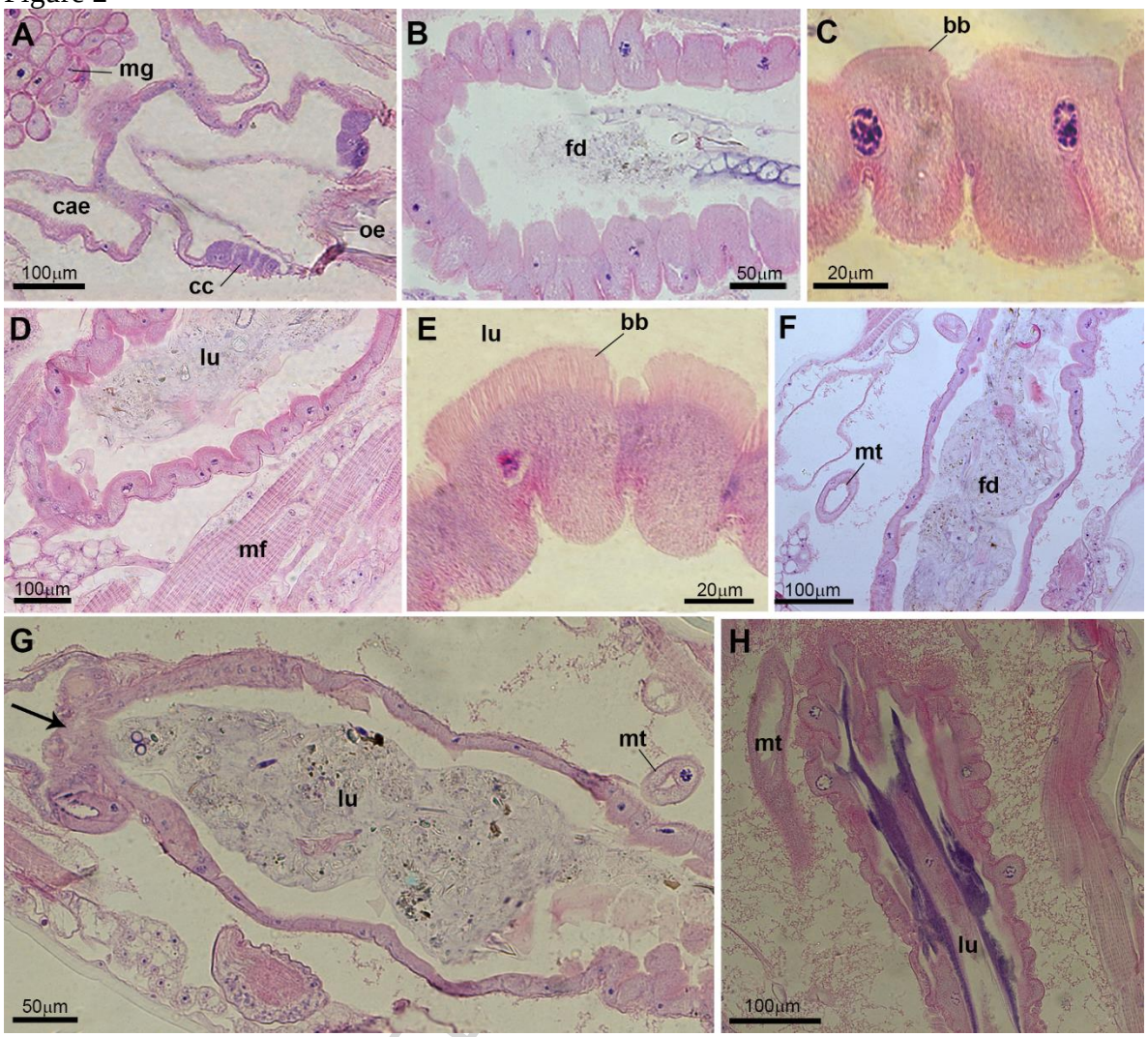


Figure 3

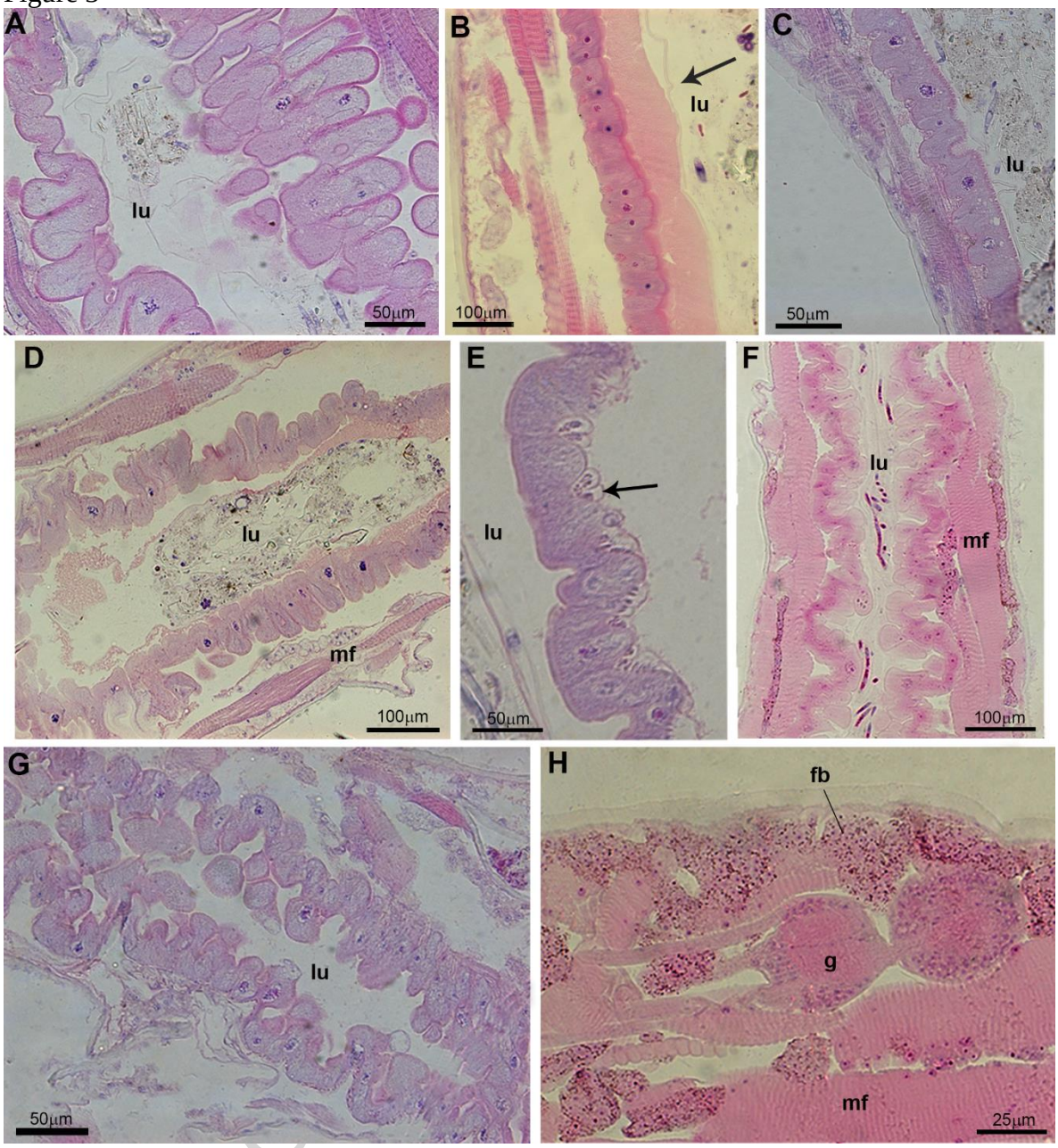


Figure 4

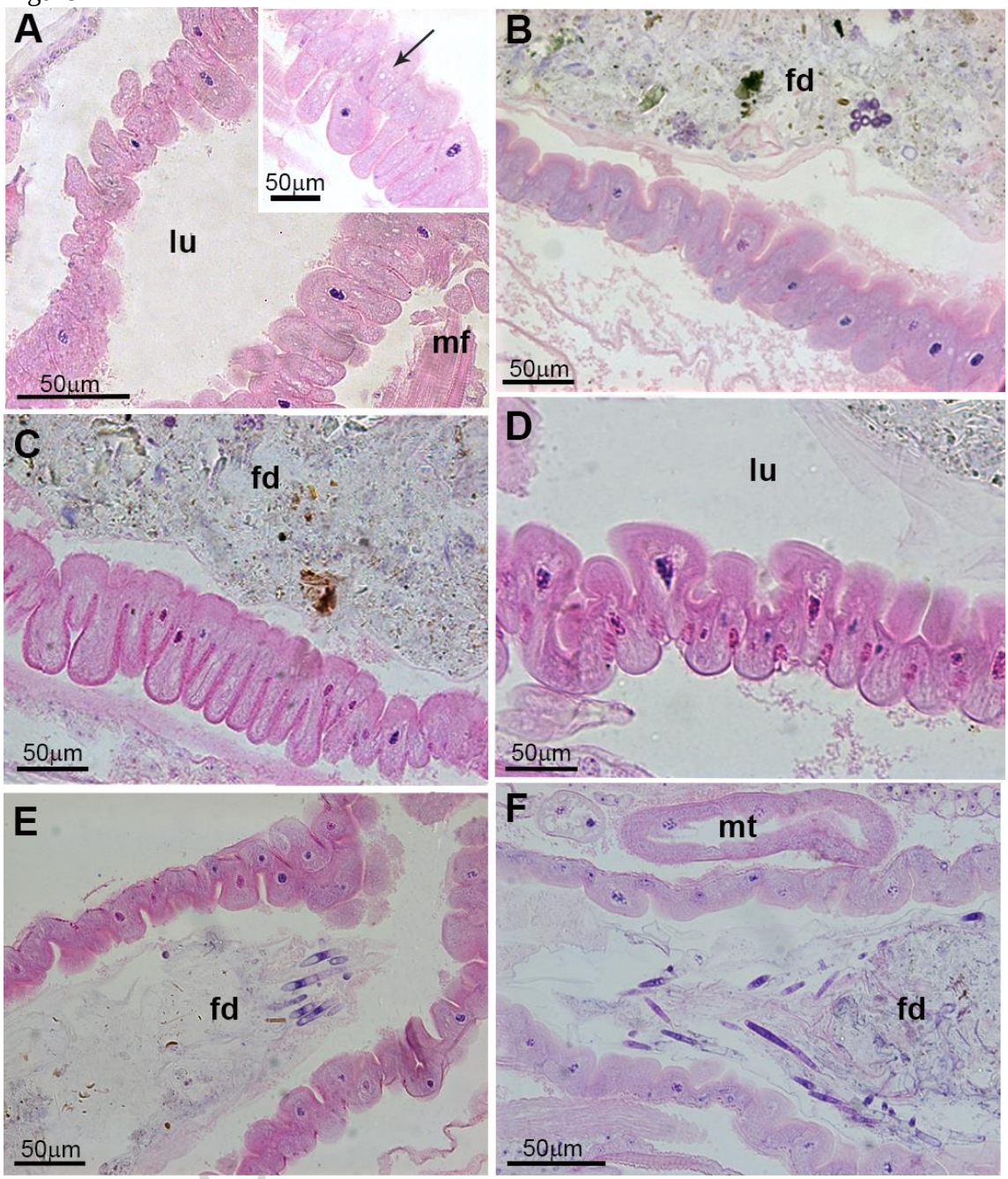


**Table 1** $LC_{50}$ values for 3rd and 4th instars larvae of *C. calligraphus* exposed to CYP and *Bti* for 96 h.

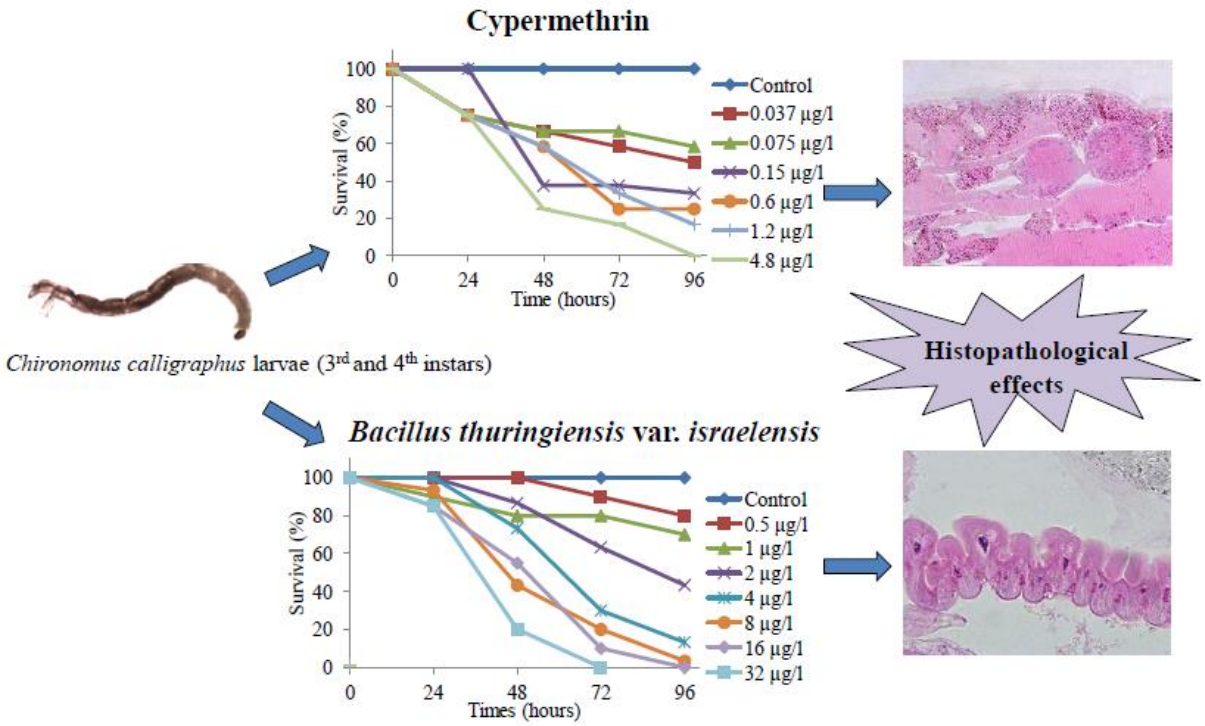
| $LC_{50}$ | CYP µg/L | *Bti* µg/L |
|---|---|---|
| 24-h | 3.19 (Nd) | 38.63 (19.41 - >10000) |
| 48-h | 1.35 (0.69 - 8.04) | 11.18 (6.92 - 31.78) |
| 72-h | 0.67 (0.36 - 2.74) | 2.67 (1.82 - 3.62) |
| 96-h | 0.52 (0.21 - 0.85) | 1.5 (1.01 - 1.98) |

(Confidence intervals) Equation of the Probit analysis for CYP $y = 5.123e^{-0.025x}$ $R^2 = 0.9507$; for *Bti* $y = 105.46e^{-0.047x}$ $R^2 = 0.9743$

**Table 2** Histological alterations in midgut epithelium of *Chironomus calligraphus* larvae after 96-h of exposure to different concentrations of CYP and *Bti*. (−) no histopathology, (+) slight histopathology present in <25% of the slides, (++) moderate histopathology present in 25%-75% of the slides and +++ = severe histopathology present in >75% of the slides.

| | CYP (µg/L) | | | | | | | *Bti* (µg/L) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Histopathology | Control | 0.037 | 0.075 | 0.15 | 0.3 | 0.6 | 1.2 | Control | 0.5 | 1 | 2 | 4 |
| *High secretion* | - | +++ | +++ | + | +++ | +++ | +++ | - | - | ++ | ++ | + |
| *Disintegration of the brush border* | - | - | - | - | - | - | - | - | - | ++ | +++ | +++ |
| *Cytoplasmic vacuolization* | - | ++ | ++ | ++ | +++ | +++ | +++ | - | + | - | + | - |
| *Detachment of basal lamina* | - | - | ++ | +++ | ++ | +++ | +++ | - | - | ++ | + | ++ |
| *Epithelial disorganization* | - | + | + | + | +++ | +++ | +++ | - | - | - | + | ++ |
| *Numerous fat body* | - | - | - | - | ++ | +++ | +++ | - | - | - | - | - |

**Graphical abstract**



Highlights

- Toxicity effects of different pesticides were studied in *Chironomus calligraphus.*
- CYP and *Bt*i caused histological damage in midgut epithelium of larvae.
- Toxicity mechanisms and histological changes could be related with the nature of insecticides.