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Acute toxicity and histopathological effects of pyriproxyfen in adult male and female zebrafish (*Danio rerio*)†

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Pyriproxyfen (PPF) is a larvicide used to combat and control insects in agriculture, veterinary medicine and public health, particularly targeting vectors such as mosquitoes that transmit dengue fever. Despite being generally considered toxic to non-target organisms, the toxic effects of this substance have already been described. In this study, we investigated the effects of acute exposure (96 h) on the mortality and histopathology of different organs: the liver, gills, intestine, kidneys and gonads of adult individuals of the species *Danio rerio*. The results showed that the solubility of PPF prevents the accurate determination of $LC_{50-96\ h}$; however, despite causing slight toxicity to female gills and gonads, the substance presented deleterious effects on the other tissues analyzed. We conclude that PPF proved to be toxic even in acute exposures; however, more studies with this substance should be expanded to chronic experiments.

Environmental significance

Pyriproxyfen (PPF) is a broad-spectrum insect growth regulator (IGR) used in public health campaigns to control disease vectors responsible for the transmission of several arboviruses, as an antiparasitic in veterinary medicine, and as a pest control agent in domestic and agricultural environments. Due to its increasing use, PPF is now frequently detected in water bodies. Although its low water solubility limits acute toxicity to fish, our study confirmed that the median lethal concentration (LC_{50}) is above its solubility in water. While PPF is known to induce histopathological changes in the liver and gonads, we further investigated these effects and expanded our evaluations to include the gills, intestine, and kidneys. Changes in these organs and tissues can have direct ecological effects.

Introduction

Pyriproxyfen (PPF) (CAS RN: 95737-68-1; 2-[1-methyl-2-(4-phenoxyphenoxy)ethoxy]pyridine) is a broad spectrum insect growth regulator (IGR) that acts as a juvenile hormone agonist (JHA) and, due to its similarity to the natural insect hormone, PPF competes for juvenile hormone receptors.[1,2] Insect juvenile hormone plays an essential role in metamorphosis, sexual differentiation, courtship, locomotion, behaviour, and central nervous system physiology.[3] The mechanism of action of PPF affects the morphogenesis, reproduction, and embryogenesis of insects, leading to the inhibition of the development of adult characteristics, such as wings, maturation of reproductive organs and external genitalia.[2,4]

Even at low concentrations, PPF effectively controls a wide range of insect species.[5] This makes PPF a valuable tool for applications both as antiparasitic medicines in the veterinary field and pest control in domestic and agricultural environments, in addition to being used in public health campaigns to control insect vectors responsible for the transmission of various arboviral diseases, such as dengue, chikungunya, zika, and yellow fever.[2,5–7] However, even though PPF is considered safe for humans due to its low toxicity to mammals, PPF can negatively affect several non-target species of terrestrial and aquatic organisms.[2,5] And, due to the increasing use of PPF, mainly in public health campaigns and in agriculture, it raises concerns with regard to aquatic organisms due to its direct application in water to combat insect vectors or indirectly when it is applied to crops and, through runoff or leaching, reaches water bodies.[2,5]

Particularly in relation to different fish species, the acute toxicity of PPF is considered limited and the 50% lethal

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concentration (LC_{50}) values available in international scientific literature are often above the solubility limit of this larvicide in water.[5] For example, for the freshwater/estuarine fish species *Pseudomugil signifer* an $LC_{50-96\ h}$ of 0.854 mg L^{-1} was obtained[8] and for the freshwater fish species *Oreochromis niloticus*[9] and *Danio rerio* (zebrafish)[10] $LC_{50-96\ h}$ values of 2.77 mg L^{-1} and 2.80 mg L^{-1} were obtained respectively. However, these LC_{50} values should be used with caution due to the low water solubility of PPF. Nevertheless, physiological and behavioural effects can be observed at sub-lethal concentrations in different fish species.[5] Although histopathological damage was identified in the testes[11,12] and ovaries[11,13] of zebrafish; in the liver of *O. niloticus*,[9] zebrafish,[12] and *Labeo rohita*;[14] and also in the gills, kidneys, and brain of *L. rohita*,[14] a recent review of PPF fish toxicity demonstrated that the concentrations tested in several studies did not cover the range of environmentally relevant concentrations described in the literature.[15] The recommended concentration for use in mosquito larval control by the World Health Organization (WHO) is 0.01 mg L^{-1}. Nevertheless, the quantities of PPF used in agriculture are considerably higher than those used in mosquito population control.[5] While there are already data available in the international literature on acute toxicity and histopathological damage induced by PPF in fish, our objective was to evaluate the acute toxicity (mortality) and histopathological damage induced by PPF in the gills, liver, testes, ovaries, intestines, and kidneys of zebrafish at environmentally relevant concentrations to increase the database on the ecotoxicological effects of this pesticide.

Materials and methods

Animals

Wild type zebrafish of the AB strain (*D. rerio*), male and female, from the fish vivarium of the Federal University of São Paulo – São Paulo campus, were maintained in accordance with the CONCEA (National Council for the Control of Animal Experimentation) Normative Resolution No. 34/2017,[16] and the procedures were submitted and approved by the Ethics Committee on the Use of Animals of the Federal University of São Paulo (5220291122).

Chemicals

Pyriproxyfen with 96.0% purity was purchased from Toronto Research Chemicals (TRC-P 998850). Dimethyl sulfoxide (DMSO) with 99.9% purity was purchased from Synth (D1011.01.BJ). Benzocaine with >99% purity was purchased from Sigma-Aldrich (PHR1158).

Experimental design for PPF acute exposure

The experimental conditions established by the OECD TG 203 (ref. 17) were followed, and parameters such as temperature, photoperiod and food were maintained constant for all groups during the experiments. Five male and five female zebrafish, three months old, were used for each test concentration. These ten fish were housed together in a single 5 L aquarium for each concentration. Exposure duration was 96 hours. The concentrations used were calculated based on the maximum concentration allowed WHO for use as larvicide (0.01 mg L^{-1} PPF). The preparation of culture water for both control and treatments followed the reconstituted water protocol established by the UNIFESP Fish Animal Facility – campus São Paulo. This protocol involves adding 1.5 g of sea salt to every 5 L of reverse osmosis water. The pH was adjusted to 7.2 if necessary, using sodium bicarbonate.

To establish the range of concentrations testing, exploratory experiments were conducted with initial concentrations in a logarithmic wide range from 0.0001 mg L^{-1} to 10 mg L^{-1} of PPF, using DMSO as a cosolvent. Once the concentration range with observable effects was identified, four independent experiments were performed to determine the LC_{50}. The concentration range used in the experiments varied between 0.0001 and 10 mg L^{-1}; however, at the highest concentrations (5.0 and 10.0 mg L^{-1}), high turbidity was observed, indicating that the PPF water solubility limit had been exceeded, making it impossible to determine the PPF LC_{50}. Based on the results of the experiments conducted to establish the LC_{50}, five definitive test concentrations below the solubility limit of PPF in water (0.01; 0.1; 0.5; 1.0; and 1.5 mg L^{-1}) were selected to ensure that adequate dose–response curves for histopathological analysis were obtained. The larvicide solutions were prepared as a stock solution and dilutions were made for each aquarium from this solution. After the exposure periods, fish were anesthetized using a benzocaine solution (0.1 g of benzocaine in 1 mL of ethyl alcohol for every 100 mL of water). Finally, the fish were euthanized.

Histopathological analyses

Following euthanasia, the fish were collected whole and fixed with 10% buffered formalin. To facilitate sectioning, they were decalcified in 4% EDTA for 10 days and dehydrated in an alcohol series. Subsequently, tissues were processed for paraffin embedding using standard protocols. Subsequently, tissues were processed for paraffin for the paraffin technique with reactions in hematoxylin–eosin (H&E) according to Paulete and Beçak,[18] to evaluate potential morphological changes in the gills, liver, kidneys, gonads, and intestines.

Histopathological change index

To assess histopathological alterations, the reaction index of the organ ($I_{org\ rp} = alt\sum(a_{org\ rp\ alt} \times w_{org\ rp\ alt})$) was calculated for the liver and gills, following the methodology described by Bernet *et al.*,[19] with a scoring system adapted by Antunes *et al.*[20] For each organ evaluated, the index incorporated the reaction pattern (rp), the specific alterations observed (alt), the alteration score (a, ranging from 0 to 3, assigned according to severity and extent), and the alteration's importance factor (w, ranging from 1 to 3) as defined by Bernet *et al.*[19] was estimated.

Histopathological alterations were classified into five categories: (i) circulatory disturbances, (ii) regressive changes, (iii) progressive changes, (iv) inflammatory responses, and (v) neoplasms. When applicable, the reaction index was calculated separately for each reaction pattern. For the analysis, five

histological sections (5 μm thick) of each tissue were randomly selected by the evaluator per animal in each experimental group.

Quantitative analyses

For the liver the measurements of mean nuclear diameter, nuclear perimeter, nuclear area, nuclear volume, relationship between volume and area, shape coefficient, contour index and eccentricity of the liver cells were carried out according to Silva.[21] For this, 50 cells selected were quantified randomly from each section obtained from the fish specimens under analysis, totalling 250 cells per individual in each treatment. For the female gonads, the number and diameter of previtellogenic I, previtellogenic II and vitellogenic oocytes were counted, as well as the number of follicular cells surrounding the vitellogenic oocytes, according to Silva *et al.*[22] To the male gonads, quantification of the diameter of germinal cysts was carried out according to Nezzi.[23] For the secondary lamellae of the gills, the following parameters were measured according to Antunes *et al.*:[20] thickness of the secondary lamella, and distance between the lamellas. These measurements will be carried out with the aid of ImageJ software.

Statistical analysis

The data were subjected to statistical analysis, first passing through the Shapiro–Wilk normality test, and subsequently using the ANOVA with Tukey *post hoc* test or Kruskal–Wallis with Dunn *post hoc* tests, whether the data were parametric or non-parametric, respectively. Differences were considered significant for values of $p < 0.05$. The analyses were carried out using Stata version 11 and GraphPad Prism software.

Results

Acute toxicity (LC_{50})

For the first experiment carried out aiming to establish a range of PPF concentrations to determine the LC_{50} PPF value, we selected the following concentrations: 0.0001, 0.001, 0.01, 1.0, 5.0, and 10.0 mg L^{-1}. It is worth mentioning that in previous experiments carried out by other research group members using the PPF, there was a need to use DMSO as cosolvent (0.4% v/v) to promote larvicide dilution, so we added the co-solvent DMSO control, which was designated as the same DMSO concentration (0.4% v/v) used in the highest test concentrations of each independent test, going by the principle that, if the highest DMSO concentration had no effect on mortality, then the smaller concentrations shouldn't either. Concentrations of 1 mg L^{-1} and all below it showed no mortality in any of the replicates; however, concentrations of 5.0 mg L^{-1} and 10.0 mg L^{-1} showed mortality rates of 60% and 86.6% respectively (Table S1†). It is worth mentioning that as soon as we prepared the dilutions of 5.0 and 10.0 mg L^{-1}, the test medium became turbid (Fig. 1), which suggested that these higher concentrations exceeded the water solubility limit of PPF, even with the addition of the co-solvent DMSO. This effect was not observed in any of the other concentrations, not even in the DMSO control.

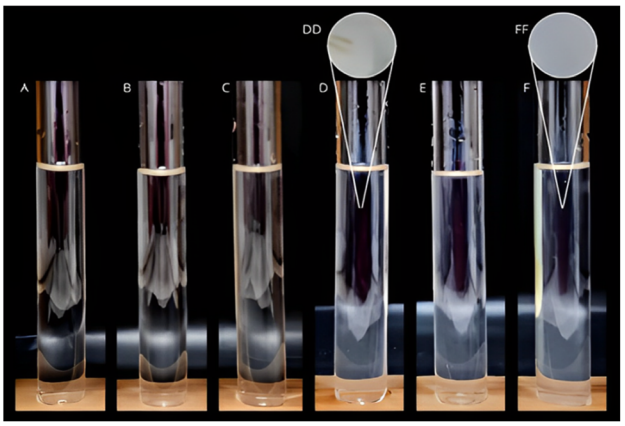

Fig. 1 Experimental aquariums for concentrations of 1.0; 2.0; 4.0; 5.0; 6.0; and 10.0 mg L^{-1} ((A–F), left to right). Turbidity as present in the 4 L volume exposure aquarium at concentrations of 5 (DD) and 10 (FF) mg L^{-1}. Notice that the test medium appears turbid after addition of PPF solution in DMSO.

Based on the results obtained in the first experiment, we designed a second experiment using PPF concentrations of 2.0, 3.0 and 4.0 mg L^{-1}. Before carrying out this experiment, we made several attempts to solubilize the PPF in ultrapure water by acidifying, increasing the temperature, and sonicating the solution; however, none of these methods proved effective. These concentrations showed close average mortalities with values around 60% (Table S1†).

The concentrations tested in the second experiment also did not allow us to establish a dose–response curve because there was no proportional variation in the mortality rate among the tested concentrations. Based on this result, we carried out a third experiment with the following concentrations: 2.0, 4.0, and 6.0 mg L^{-1}. In the third experiment, we obtained the average mortality rate of 62.5% for the concentration of 2.0 mg L^{-1}, 60% for the concentration of 4.0 mg L^{-1}, and 12.5% for the concentration of 6.0 mg L^{-1} (Table S1†).

The results of all of these three experiments indicated that the lowest concentrations showed higher mortality rates compared to higher tested concentrations, because concentrations of 2.0, 3.0, and 4.0 mg L^{-1} showed similar mortalities. These results suggest that at higher concentrations the non-soluble PPF may be precipitating in the form of particles, which causes, in addition to turbidity, lower bioavailability. Therefore, we designed one last (fourth) experiment to identify in which nominal concentration ranges we could find a dose–response curve. For this, we tested the following concentrations: 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; and 4.0 mg L^{-1}. We observed that at concentrations between 2.0 and 3.5 mg L^{-1} there was no variation in the mortality rate (~60%), and at the concentration of 4.0 mg L^{-1} there was an important decrease in the mortality rate (40%) (Table S1†). Thus, we can infer that the PPF $LC_{50-96\ h}$ for zebrafish is probably between 1.5 and 2.0 mg L^{-1}, and that the water solubility limit of the PPF should be around 2.0 mg L^{-1}.

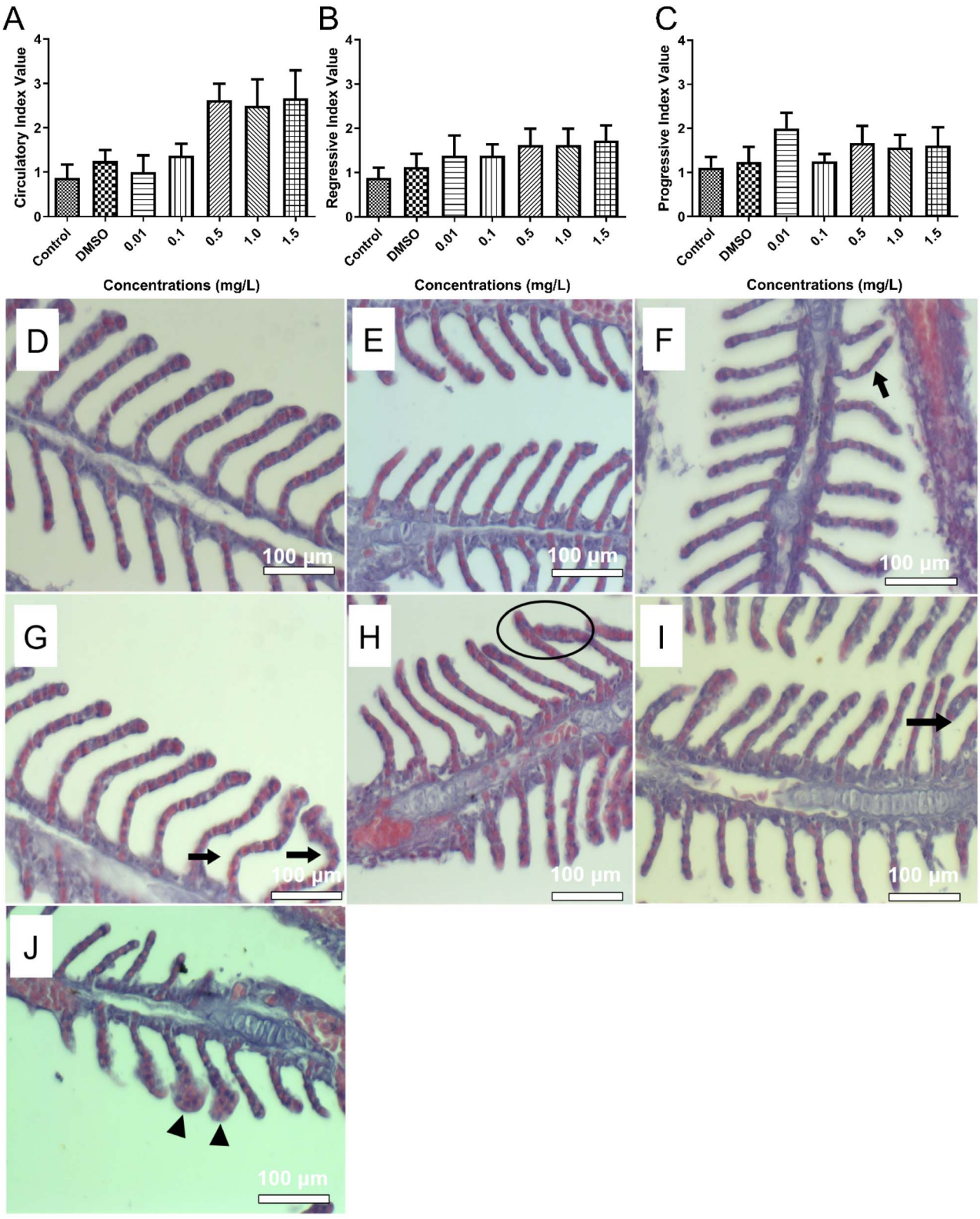

Fig. 2 Graphs of the histopathological alteration index in the gills (A–C). Note that for none of the parameters were there significant differences between the groups after the ANOVA/Tukey test with $p < 0.05$. ((A–C) circulatory, regressive and progressive indices respectively). (D–J) Normal appearance of the gills in the control group and DMSO control group ((D) and (E) respectively). Groups exposed to PPF: (F) group exposed to a concentration of 0.01 mg L^{-1}; (G) group exposed to a concentration of 0.1 mg L^{-1}; (H) group exposed to a concentration of 0.5 mg L^{-1}; (I) group exposed to a concentration of 1 mg L^{-1}; and (J) group exposed to a concentration of 1.5 mg L^{-1}. Note the alteration in curvature of the secondary lamellae (black arrows), partial fusion of the secondary lamellae (black circle), and dilation of the ends of the secondary lamellae (arrowheads). H&E technique. Scale bar = 100 µm.

Our results suggest that the LC_{50} is likely close to the water solubility of PPF. Considering the low PPF water solubility and the impossibility of establishing an accurate LC_{50} value, we selected the concentrations of 0.01; 0.1; 0.5; 1.0; and 1.5 mg L^{-1} for carrying out the histopathological experiments.

Gill histopathological evaluation

The gill histopathological qualitative analysis demonstrated that PPF was slightly toxic (Fig. 2). Dilations of the ends of the secondary lamella, partial lamellar fusion, and alterations in the curvature of the secondary lamellae were observed at the two

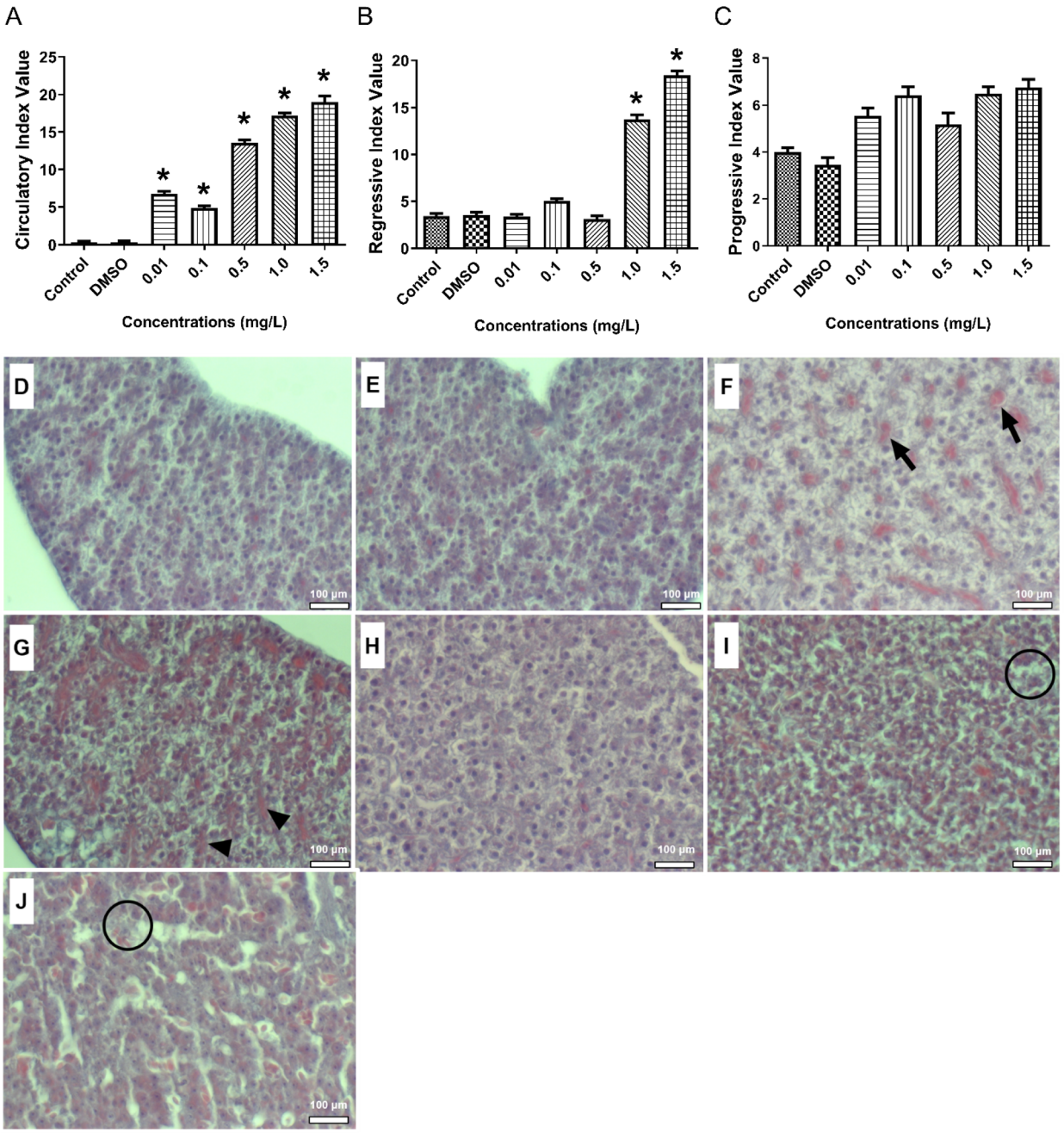

Fig. 3 Graphs of the histopathological alteration index in the liver (A–C). (A–C) circulatory, regressive and progressive indices respectively. Note that there were significant differences between all groups exposed to PPF compared to controls in circulatory alterations (*) and only in groups 1 and 1.5 mg L^{-1} for regressive alterations (*) after the ANOVA/Tukey test with $p < 0.05$. (D–J) Normal appearance of the liver in the control group and DMSO control group ((D) and (E) respectively). Groups exposed to PPF: (F) group exposed to a concentration of 0.01 mg L^{-1}; (G) group exposed to a concentration of 0.1 mg L^{-1}; (H) group exposed to a concentration of 0.5 mg L^{-1}; (I) group exposed to a concentration of 1 mg L^{-1}; and (J) group exposed to a concentration of 1.5 mg L^{-1}. Note the blood congestion (arrows) and dilation of blood vessels (arrowheads), as well as degeneration of hepatic tissue (black circle). In (H) and (I), we can see a visual decrease in hepatocyte nuclei compared to the other groups, and in (H–J), we can see disorganization of hepatic tissue. Scale bar = 100 µm.

highest concentrations (1.0 and 1.5 mg L^{-1}), but at low frequencies (Fig. 2). No inflammatory changes or neoplasia were found. The histopathological index supports these analyses, as no significant differences were identified in any of the evaluated parameters (circulatory, regressive, progressive alterations, and total histopathological alteration index) (Fig. 2). The parameters of thickness of the secondary lamella, and distance between the lamellas also did not show significant changes between the groups (Fig. S1†).

Liver histopathological evaluation

In the liver, we observed relevant alterations at concentrations of 1.0 and 1.5 mg L^{-1}, where there was a decrease in hepatocyte nucleus size, dilation of blood capillaries, and disorganization and degeneration of hepatic tissue (this last one was also observed at a concentration of 0.5 mg L^{-1}), along with blood congestion (Fig. 3). These data were corroborated by quantitative analyses of nuclear area and volume. The parameters of nuclear diameter, nuclear perimeter, relationship between volume and area, shape coefficient, contour index and eccentricity did not show significant alterations (Fig. 4). The histopathological alteration index showed significant differences in the circulatory alteration index for all exposed groups and a regressive alteration index only for the groups exposed to concentrations of 1.0 and 1.5 mg L^{-1} (Fig. 3). No inflammatory changes or neoplasia were found.

Gonad histopathological evaluation

No morphological changes were observed in either the ovarian or testicular tissue across any of the parameters analysed. In addition, no changes were detected in the diameter of the ovarian follicles or germinal cysts (Fig. S2 and S3†). Quantitative analyses of ovarian follicles revealed that the average number of pre-vitellogenic follicles 1 and 2 remained consistent between all the groups. In contrast, a decrease in the vitellogenic follicles was observed in the females exposed to concentrations of 0.5, 1.0 and 1.5 mg L^{-1} (Fig. 5). No changes were identified in male gonads in any of the parameters analysed (Fig. S4†).

Intestine and kidney histopathological evaluation

For the intestine and kidneys, only qualitative analyses were conducted, focusing on the identification of histopathological changes. For the intestine, fracture of the villus and lysis of the epithelium were identified at concentrations $\geq$ 0.5 mg L^{-1} (Fig. 6). Regarding the kidneys, at concentrations $\geq$ 0.5 mg L^{-1} PPF, several collecting ducts with epithelial degeneration were identified (Fig. 6).

Discussion

PPF larvicide is a yellow, waxy solid with a melting point of 47.4 °C, and with a density greater than that of water (1.242 g mL^{-1} at 25 °C).[5] At room temperature (25 °C) and pH 6, PPF becomes slightly soluble in water (0.367 $\pm$ 0.004 mg L^{-1});[5] however, it is highly soluble in organic solvents such as hexane (400 g kg^{-1} at 20 °C) and xylene (500 g kg^{-1} at 20 °C).[24] PPF is a potent juvenile hormone agonist,[6] inhibiting the development of maturing larvae.[25] Due to its mode of action, it is considered safe for numerous non-target species, and is not genotoxic or carcinogenic.[26] For fish species specifically the PPF acute toxicity is considered to be quite limited, with the effects described in the international literature often occurring at concentration ranges exceeding its water solubility.[5] According to a literature review by Moura and Souza-Santos,[2] zebrafish is particularly resistant to PPF-induced damage. Our results suggest that the PPF LC_{50} is probably between 1.0 and 2.0 mg L^{-1}, a value similar to the LC_{50} obtained by Wei *et al.*[10] (LC_{50} = 2.80 mg L^{-1}) for zebrafish. The high mortality rates observed at a concentration of 10 mg L^{-1} are likely related to particle formation and subsequent intake through the gills and digestive tract.

Gills and intestines are widely studied mucosal organs in fish, since they provide an interface between the internal and external environment. They act as a protective barrier, playing a crucial role in the intake and absorption of pollutants.[27] Our results demonstrated low toxicity when we analysed the gill histology, considering that although we found some morphological changes, their frequency was low in the tested groups. In the literature, there is a lack of studies related to histopathological changes in acute toxicity studies. Nevertheless, some studies have shown sub-chronic or chronic exposure to PPF.[15] Naseem *et al.*[14] found several changes in the gills of *L. rohita*, after exposure to PPF, such as necrosis, folding in the secondary lamellae, and lesions in the cartilage of the primary lamella. Their study, despite using some lower concentrations than ours, carried out exposure for 30 days, and that extended exposure duration may explain the more severe damage observed compared to our findings.

Regarding changes in intestinal morphology, our results are unprecedented for PPF and suggest potential impacts on organ function at concentrations above 0.5 mg L^{-1}. The intestine is both a digestive organ and one of the largest immune organs of fish, which is responsible for nutrient absorption, immune regulation, and catabolism.[28] Our results indicate that the histopathological alterations represent defence mechanisms, including villi rupture, structural loss, and tissue degeneration. Previous studies have also reported histopathological alterations in the intestines of *Heteropneustes fossilis* adults exposed to triazophos, another insecticide.[29] Several changes, such as epithelial degeneration, necrosis, atrophy, and leukocyte infiltration, were observed after 10 days of exposure to 5 mg L^{-1}. At a higher concentration (10 mg L^{-1}), additional alterations were observed, including vacuolization, leukocyte infiltration, and mucosal autolysis. At 15 mg L^{-1}, further damage was observed, such as degenerated and necrotic villi, epithelial cell degeneration, and pronounced atrophy.[29]

The liver plays a central role in several physiological processes, including macronutrient metabolism, hormone regulation (endocrine control) and immunity.[30] It is also essential for detoxification and xenobiotic metabolism, making it a primary target for toxic effects caused by environmental contaminants.[30,31] In the liver, we find several relevant changes, mainly circulatory and in the reduction of the hepatocyte

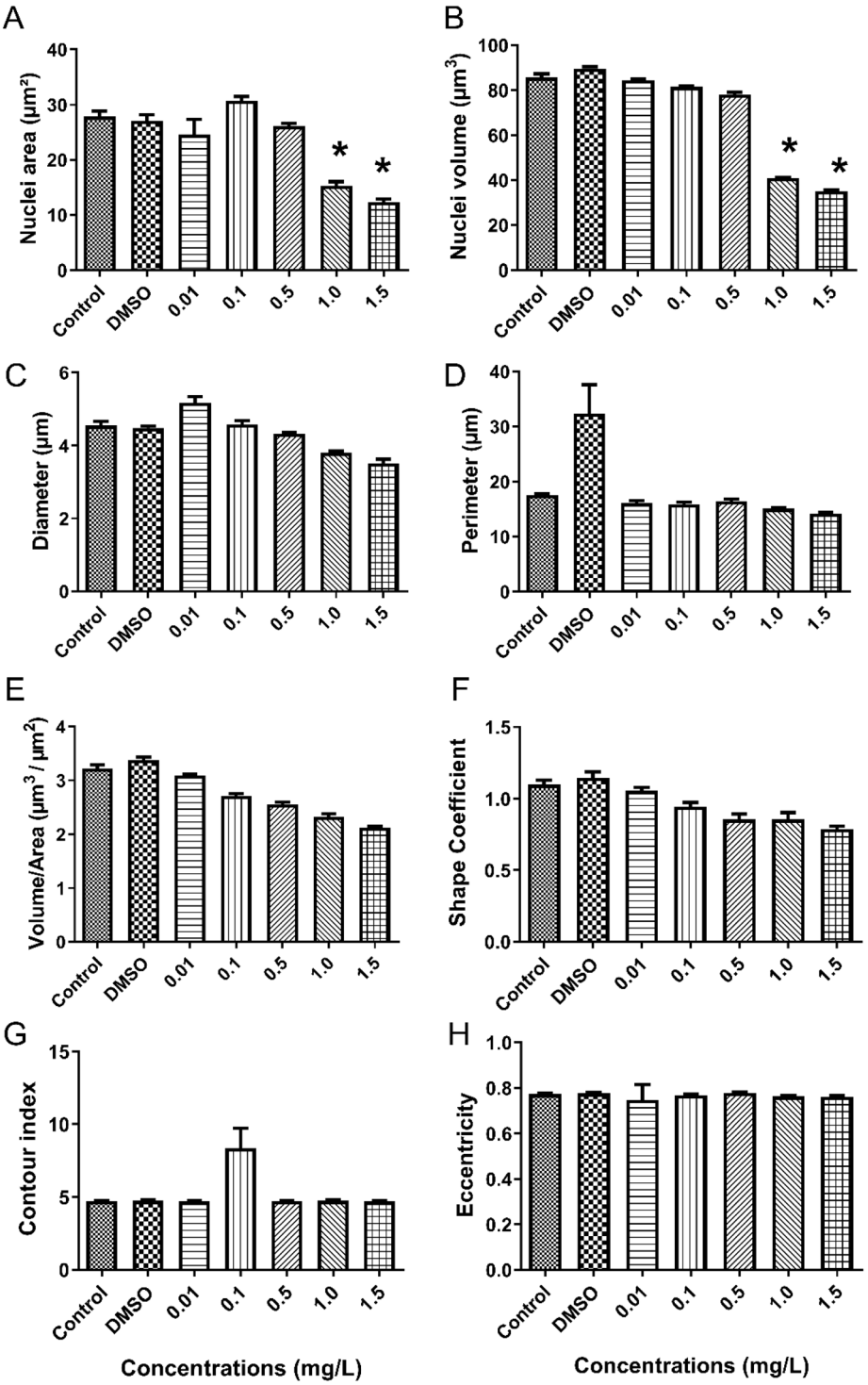

Fig. 4 Graphs of the quantitative analyses performed on hepatocyte nuclei. (A) nuclei area; (B) nuclei volume; (C) diameter; (D) perimeter; (E) volume/area relationship; (F) shape coefficient; (G) contour index; (H) eccentricity. Note that only groups 1 and 1.5 mg L^{-1} showed a significant difference compared to the other groups (*) in terms of nuclear area and volume, which shows a decrease in the nucleus size in these groups. ANOVA/Tukey test with $p < 0.05$.

nucleus. To our knowledge, no acute studies related to the histopathology of PPF have been reported. Most acute toxicity studies focus on lethality and biomarkers. Histopathological analysis has been described primarily in sub-chronic or chronic exposure studies. For instance, Silva *et al.*[9] in studies with *O. niloticus* found, after 28 days of PPF exposure, several histopathological changes, such as blood congestion, areas of inflammatory infiltrate, loss of tissue organization and fibrosis. Some of these findings are consistent with those found in our results; the most serious findings described by the authors are

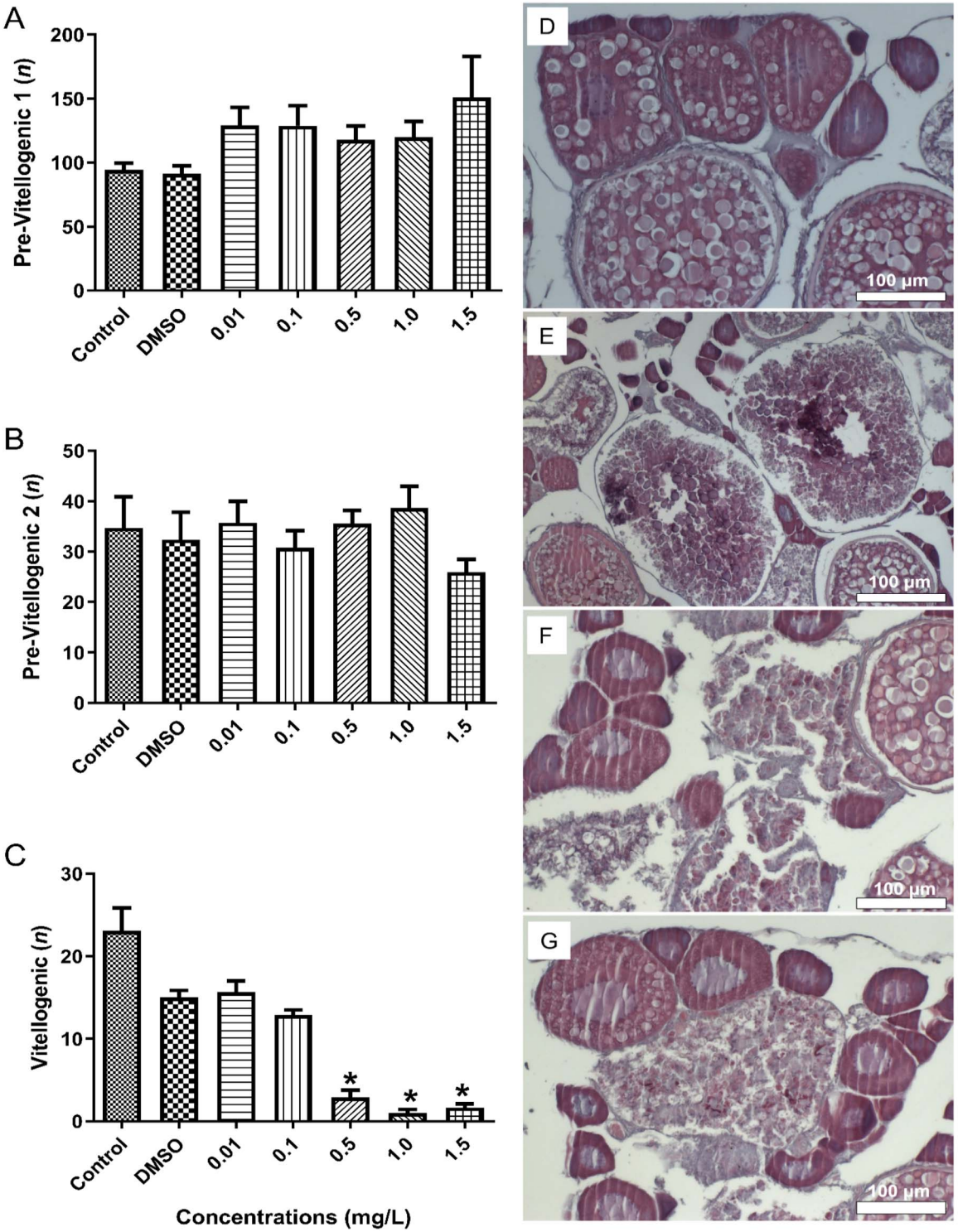

Fig. 5 Number of pre-vitellogenic 1 (A), pre-vitellogenic 2 (B) and vitellogenic (C) follicles. Note the decrease in the number of vitellogenic follicles at concentrations above 0.5 mg L^{-1}. ANOVA/Tukey test with $p < 0.05$. Female gonads stained with H–E (D–G). In (D) the control group, (E) DMSO group and in (F) and (G) groups exposed to concentrations of 1 and 1.5 mg L^{-1} respectively. Note that despite variations in follicle types, no significant morphological changes are observed. Scale bar = 100 µm.

directly correlated to longer exposure. Arman,[32] Guo *et al.*,[33] Hamid *et al.*,[34] and Yang *et al.*[35] described blood congestion and dilation of capillaries in the liver, as common changes in zebrafish, after exposure to various contaminating agents, as described in our results. According to Arman,[32] both capillary dilation and congestion can be associated with impaired venous flow and heart failure and extrahepatic inflammatory conditions. Mishra and Mohanty,[36] Ostaszewska *et al.*,[37] and Rašković

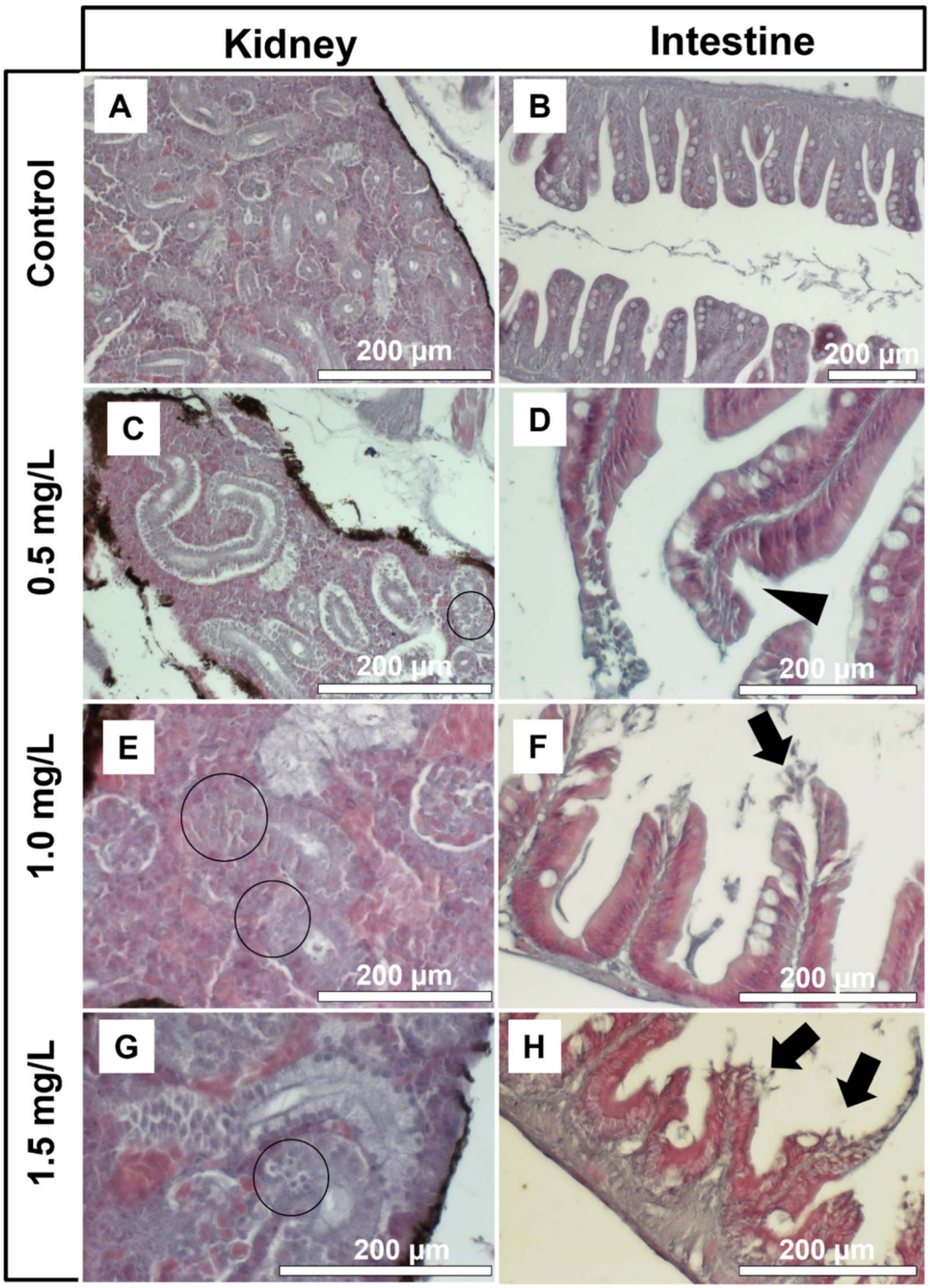

Fig. 6 Kidney and intestine stained with H&E (A–H). In (A) and (B) control group. In (C), (E) and (G) Group exposed to concentrations of 0.5, 1.0 and 1.5 respectively, showing degeneration of the tubular epithelium (circle). In (D) group exposed to a concentration of 0.5 mg L^{-1} showing fracture of the villus 9 circle). In (F) and (H) lysis of the villus epithelium (arrow) at concentrations of 1 and 1.5 mg L^{-1} respectively. Scale bar = 200 µm.

et al.[38] pointed out the appearance of pyknotic nuclei and the loss of tissue organization and liver parenchyma. In light of the critical damage to liver function, our results appear to show that the highest concentrations are extremely toxic to the liver.

The kidneys perform a range of physiological functions essential for maintaining homeostasis, including the removal of metabolic waste from the bloodstream, regulation of fluid balance, and maintenance of electrolyte levels.[39] As a result, exposure to xenobiotics can severely damage these organs. Our

results indicated epithelial degeneration on collecting ducts. Acute kidney injury may occur due to acute loss of kidney epithelial cells.[40] Loganathan *et al.*[29] reported histopathological alterations in the kidneys of *H. fossilis* adults exposed to the insecticide triazophos. At a concentration of 5 ppm, several changes were observed, including vacuolization, necrotic tubules, degeneration of renal epithelial cells, distortion and dilation of renal tubules, and cloudy degeneration. At 10 ppm, necrosis of hematopoietic tissue and dilation of glomeruli were noted. Exposure to 15 ppm resulted in narrowing of the tubular lumen, pyknotic nuclei, hypertrophy, degeneration of renal epithelial and glomerular cells, intercytoplasmic vacuolization, and necrosis. Concerning PPF toxicity on fish, Naseem *et al.*[14] observed moderate to severe histopathological lesions in the kidney after 20 and 30 days of exposure. The authors reported lesions such as degeneration and necrosis of renal tubules, distortion of the glomeruli, pyknosis, sloughing of the renal tubular epithelium, and edema.[14]

Gonadal differentiation refers to the developmental process through which undifferentiated gonads become either testes or ovaries, under the influence of sex-determining signals.[41,42] Gamete production, regarding spermatogenesis and oogenesis, plays a central role in reproduction.[43] PPF may act as an endocrine-disrupting compound, with the potential to induce morphological alterations in zebrafish gonads, including increased formation of spermatogonial cysts in the testes and oocyte atresia in the ovaries,[15] potentially causing deleterious effects on reproduction. Our findings suggest a sex-specific gonadal response to acute PPF exposure, particularly in females. To our knowledge, no other studies have reported the effects of acute PPF exposure on gonadal tissue.

In females, a significant reduction in vitellogenic follicles was observed in the exposed groups. In contrast, male gonads did not exhibit significant morphometric alterations, with all stages of spermatogenesis being preserved. The selective reduction of vitellogenic follicles, with preservation of pre-vitellogenic stages, suggests a targeted disruption of vitellogenesis—a hormone-dependent process crucial for oocyte maturation,[44] as also reported by Oliveira *et al.*,[12] after a 7 day exposure experiment. Similarly, Maharajan *et al.*[11] reported a decrease in the proportion of vitellogenic stage oocytes at concentrations starting from 10 $\mu g\ L^{-1}$ after a 21 day exposure. Conversely, both Oliveira *et al.*[12] and Maharajan *et al.*[11] reported morphometric and histological alterations in the testes, indicating that sub-chronic and chronic exposures can adversely affect male reproductive physiology. These findings, in agreement with our results, suggest that although male gonads appear more resilient to PPF acute exposure—possibly due to lower endocrine sensitivity or differences in gametogenic dynamics—prolonged or higher-dose exposures to PPF are still capable of eliciting detectable testicular changes.

Conclusions

Our study highlights the low water solubility of PPF, which affects the understanding of its general effects on parameters such as mortality, considering that in addition to the real concentration, it must consider the effects of particle formation and their interactions with organisms. This larvicide has harmful effects on several tissues, when we evaluate their morphology, particularly the gonads and intestines, which can have direct ecological effects. Therefore, further research on PPF, especially chronic exposure studies, is needed to gain a more comprehensive understanding of its ecological impacts.

Ethical statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of the National Council for the Control of Animal Experimentation (CONCEA no. 61) and approved by the Animal Ethics Committee of the Federal University of São Paulo, protocol no. 5220291122.

Data availability

The data supporting this article have been included as part of the ESI.† Link for the raw data: **https://repositorio.unifesp.br/communities/417e7122-f137-40de-9978-8eb7da600044** (acute toxicity and histopathological effects of pyriproxyfen in adult male and female zebrafish (*Danio rerio*)).

Author contributions

Conceptualization: FK, BFP; methodology: BGG, AAV, FKT, FK, DLP, NAD; formal analysis: BGG, AAV, FKT, FK, DLP, NAD, RSB, GG; writing – original draft: BGG, RSB; writing – review & editing: all authors; project administration: FK, BFP; funding acquisition: FK, BFP, GG.

Conflicts of interest

There are no conflicts to declare.

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