Arch Biol Sci. 2021;73(3):319-329 https://doi.org/10.2298/ABS210515025S

# Histopathology of *Chironomus riparius* (Diptera, Chironomidae) exposed to metal oxide nanoparticles

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**Received**: May 15, 2021; **Revised**: May 27, 2021; **Accepted**: May 28, 2021; **Published online**: June 10, 2021

**Abstract:** As the production of metal-based nanomaterials increases, it is inevitable that nano-scale products and byproducts will enter the aquatic environment. In terms of global production, the most abundant nano-oxides are $TiO_2$, $CeO_2$ and $Fe_3O_4$ nanoparticles. *Chironomus riparius* is commonly used for ecotoxicological assessment and defining its histopathological biomarkers that showcase the toxic effect of tested nanoparticles should lead to a better understanding of the consequences of nanomaterial accumulation in aquatic ecosystems. In this study, a histological description of the digestive and excretory systems as well as the fat body structure of *C. riparius* larvae is provided. In addition, potential histological biomarkers of nano-oxide toxicity were determined based on the obtained histopathological alterations in organs. Vacuolization was observed in epithelial cells of midgut region I that were treated with nano-$Fe_3O_4$ as well as in Malpighian tubules treated with nano-$Fe_3O_4$ and nano-$CeO_2$. Larvae exposed to nano-$TiO_2$ showed alterations in the fat body and midgut region II tissue architecture. Additionally, shortening of the intestinal brush border was determined in groups exposed to nano-$Fe_3O_4$. These results reveal the high sensitivity of these organs, which can be used as biomarkers in histopathological assessment and therefore lead to further improvement of existing methodology in ecotoxicological studies.

**Keywords:** Chironomidae; histopathology; ecotoxicology; nanomaterials; biomarkers

## INTRODUCTION

The development of nanomaterials was of tremendous importance for different fields of science. However, these substances enter aquatic ecosystems through different pathways that include direct disposal of industrial waste from factories, and indirect infiltration from the atmosphere, households and soil [1]. According to the International Organization for Standards (ISO), nanomaterials can be defined as materials with any external dimension on the nanoscale or with internal structure or surface structure on the nanoscale [2]. Nanosized materials are used in an array of industries, from cosmetics [3], electronics [4], medicine [4] chemical production [5], water treatment technologies [6] and others. Increased production of nanosized materials results in their expanded disposal as byproducts [7] and sublethal toxic effects on living organisms [8]. Materials in their reduced shape show different or enhanced mechanical, optical and electrical properties compared to their bulk counterparts. This occurrence is a consequence of the increased relative surface area, which involves increased reactivity [9].

Among the nanomaterials, the largest group includes metal-based products. Titanium dioxide ($TiO_2$) is the leading oxide in terms of global production in tons per year (about 10000 tons/year), followed by $SiO_2$ with production up to 10000 tons/year, and $CeO_2$, $Fe_xO_x$, $AlO_x$, ZnO, carbon nanotube nanoparticles (CNT) with production between 100 and 1000 tons/year [10]. $TiO_2$ is mostly used in cosmetics, especially sunscreens, the food industry (as an additive) and paints [11]. Its usage as a food coloring (E171) is of great concern since recent research has shown that this form of $TiO_2$ is toxic for living organisms [12,13]. For this reason, the French Government has instituted a regulation that forbids the use of E171 in the food industry [14]. The main mechanism of its cytotoxicity is oxidative damage that is induced

by reactive oxygen species (ROS) accumulation with simultaneous downregulation of antioxidative enzyme expression [12]. In this way, the generated ROS can damage cells at different levels, including organelles and DNA molecules [15].

The chemical and physical properties of cerium oxide ($CeO_2$) has led to its widespread use for different purposes – in the car industry (in the conversion process of carbon monoxide into carbon dioxide) [16], microchip production, as an abrasive [17], and in the cosmetic industry [18]. There is a great potential for $CeO_2$ use in medical treatments [19]. However, the high production of this oxide is worrying since it is capable of penetrating cells and inducing apoptosis through the generation of ROS [20].

Among the iron oxides, nanosized magnetite is widely applied in medicine due to its biocompatibility, chemical stability, high magnetic susceptibility and inexpensiveness [21]. Since production of these nanomaterials has increased only very recently, the information about its ecotoxicity is very scarce but the mechanism of its cytotoxicity is similar to the other two oxides mentioned above [22].

Previous studies indicate that the current concentration of nanomaterials in the environment is low and may only produce sublethal effects [23]. Increased production followed by expanded discharge of such materials into water could have a detrimental effect on the health of aquatic biota [24]. Aquatic macroinvertebrates are widely used in ecotoxicology studies as model organisms for determining water quality [25]. Non-biting midges (Diptera, Chironomidae) are a widely distributed family of aquatic insects with a great ability to adapt to diverse water ecosystems. Their larvae are the most abundant benthic macroinvertebrates that link primary producers to consumers. Due to the relatively long life-cycle of non-biting midges, their low mobility, ubiquity and abundance in aquatic ecosystems, these organisms have a great potential in bioassessment [26].

To measure the toxicity of the studied agents, the parameters indicative of adverse effects were defined. These parameters, referred to as biomarkers, can be described at different levels of biological organization and presented as quantitative (number of dead individuals, number of laid eggs, changes in size and weight of individuals) and qualitative (behavioral changes and development rate) parameters [12]. Cell and tissue alterations can be important biomarkers of sublethal pollutant concentration effects [27]. Microscopic analyses of tissues show early warning signs for the severity of toxic effects on animals. This approach allows for the results describing the health condition of several studied individuals to be extrapolated to the whole community in the studied ecosystem [28]. Histopathology is frequently used in *in vivo* toxicity assessments of nanoparticles in animal models, including rats, mice and fish [29]. Studies have shown that chemical reactivity and ROS generation of the same particles increase with the decrease in their size [29,30]. The small size of these particles allows them to pass through body barriers and reach organs and tissues, thereby interacting with intracellular structures and altering their normal functioning and morphology in different ways [30]. These changes in the normal architecture of tissues can be visualized using histopathological assessment [31]. There are only a few studies about the histological description and histopathology of Chironomidae larvae [32-34]. *Chironomus riparius* Meigen, 1804 (Diptera, Chironomidae) is a species used as a standard model organism for ecotoxicity tests [35]. At present there are no references regarding the histological description of the main systems and organs in healthy individuals, or the histopathological changes that could be used as biomarkers of pollutant cytotoxicity for this species. Similar species have been described and these studies can be used as guidelines for further studies [28,36]. Since the histological patterns can vary even between close species [33], determination of the normal organ architecture of *C. riparius* is crucial as it will be used as the foundation for determining potential histopathological biomarkers.

The aim of this study was to establish reference histological structures of the main organs of *C. riparius* larvae and to determine the toxic effects of metal nano-oxides on the histopathological biomarkers of this species. Based on previous studies conducted on chironomid larvae, we determined that the target tissues and organs in our study should include the digestive system, Malpighian tubules and the fat body [34]. We predicted distinct histological changes in target tissues and organs caused by the toxic effects following nano-$TiO_2$, -$CeO_2$ and -$Fe_3O_4$ treatments, as it was already proven that these metal nano-oxides affect the standard biomarkers in *C. riparius* larvae

[12]. In order to realize the main aim of the study, we addressed the following problems: (i) to describe the main organs relevant of untreated *C. riparius* larvae for histopathological analysis; (ii) to describe the histological changes in digestive cells in the midgut region and parietal fat body exposed to metal nano-oxides; (iii) to determine vacuolization of Malpighian tubules in larvae exposed to metal nano-oxides, and (iv) to quantify differences in the digestive cell brush border as a response to toxic exposure.

## MATERIALS AND METHODS

### Nanoparticles

In this study, 3 metal oxides in nanosize form were used for treatment: $TiO_2$, $CeO_2$ and $Fe_3O_4$. $TiO_2$ nanoparticles were bought from Fiori Colori Spa, Italy, distributed by Pharmorgana GmbH, Germany. The material was in the form of commercial white food coloring (E171) with 99% purity. Detailed characteristics of the nanoparticles were stated in two previous studies that used the same nanomaterial batch [13,37]. Nano-$CeO_2$ with particles <25 nm in diameter were obtained from Sigma Aldrich, USA. Its characterization is described in detail in a previous study [38]. Nano-$Fe_3O_4$ was synthesized at the Department for Material Science and Nanotechnology, TOBB University of Economics and Technology, Ankara, Turkey. More information about the synthesis, chemical and physical properties of this nanoparticle can be found in [12].

### Test organisms

In this study, 4$^{th}$ instar larvae of *Chironomus riparius* Meigen, 1804, obtained from the stock culture housed at the laboratory of Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis, were used. The culture was formed and maintained according to OECD guidelines [39]. The larvae were housed in glass tanks with cellulose sediment and a mixture of tap and deionized water at 23˚C±2˚C, with a 16 h photoperiod and constant aeration. Tetra-Min® finely ground food was used for feeding.

### Bioassay design

This experiment was performed under laboratory conditions following the modified OECD 235 acute test [35]. Fourth instar larvae were used as they are the most resistant and large enough for further histopathological analyses. Given that this larval phase requires a substrate, a coarse quartz sand in a thin layer was provided. The use of the 4$^{th}$ larval stage is common in histopathological studies [28,32,33,36].

Glass jars of 700 mL volume (8 cm in diameter) with 105 $cm^3$ of coarse quartz sediment (with nanoparticles in treated groups and without them in a control group), and 4/5 mixture of tap and deionized water (1:1) were placed in a water bath at 23˚C±2˚C. Aeration was positioned at a minimum of 1 cm above the sediment to avoid turbulence. The experimental setup was formed 48 h before adding the larvae to stabilize the environment.

A total of 280 experimental individuals were arranged into 3 treatment groups with a defined concentration of metal oxide and one control group. Each treatment group contained 80 larvae divided into 8 replicates, while the control group contained 40 larvae divided into 4 replicates; each glass jar (replicate) contained 10 larvae. In the group treated with nano-$Fe_3O_4$, the concentration of nanoparticles was 0.0105 g/kg of the sediment, while in the groups treated with nano-$CeO_2$ and nano-$TiO_2$ the concentration was 0.2625 g/kg and 0.0525 g/kg of the sediment, respectively. The applied concentrations of nanoparticles were the maximal doses of oxides that were shown to produce no lethal effect on *C. riparius* larvae in previous studies [12,13,38]. Larvae were fed immediately after transfer to the glass jars to ensure sediment and nanoparticle intake. After 72 h of exposure, the larvae were fixed in 70% ethanol for further analyses.

### Histopathological analyses

Five larvae were selected from each replicate to be used for histopathological analyses. Larvae were dehydrated with a series of increasing concentrations of ethanol (70%, 80%, 90% and 96%), applied in succession for 45 min each. The samples were processed with toluene for 10 min and placed in tissue-embedding paraffin overnight. After mounting, longitudinal sections were cut to 5-µm thickness on a Leica® RM 2125RT microtome, stained with a combination of hematoxylin and eosin (H&E) and observed under a light photomicroscope (Leica® DM 2500). The brush border of the digestive

cells of midgut region II was measured using the imageJ program [40]. The length of microvilli and the cell area of the measured microvilli were determined, and the ratio was calculated. The brush border as a biomarker of cytotoxicity was already used in several studies [32-34]. The statistical difference of microvilli length between the control and treated larvae was examined with the Mann-Whitney test using the SPSS statistical software package Ver. 13.0 for Windows. The significance level was set at $P<0.05$. Sections of treated larvae were compared to the control and available data from studies on closely related species [28,32,33,36] and tissue alterations were recorded and described.

## RESULTS

### Internal morphology of untreated larvae of *Chironomus riparius*

The description of the internal morphology of untreated immature *C. riparius* includes the digestive system, the excretory system and the fat body. Histological analyses of the digestive system showed that 3 regions were unaltered: the foregut, midgut and hindgut. The foregut begins with the mouth and ends in the metathorax at the esophageal invagination. The esophagus is composed of flattened epithelial cells with large nuclei and surrounded by smooth muscle cells. On the border between the fore- and midgut, a stomodeal valve with an esophageal invagination was noted. It was accompanied by Cuénot cells that showed a strongly basophilic cytoplasm and a large nucleus with condensed chromatin. Following Cuénot cells there was gastric caeca, composed of diverticula arranged in rings (Fig. 1A).

The midgut was divided into 3 regions according to the shape of cells. Cells of region I had an irregular shape with a rounded base and a convex apical part of the cell with a short brush border. Region II contained cubic cells with a basophilic cytoplasm, a large nucleus with condensed chromatin and an extended brush border (Fig. 1B). The cells of region III were similar to those of region II but smaller and with a shorter brush border.

The transition between the mid- and hindgut was marked by a proctodeal valve formed by cylindrical

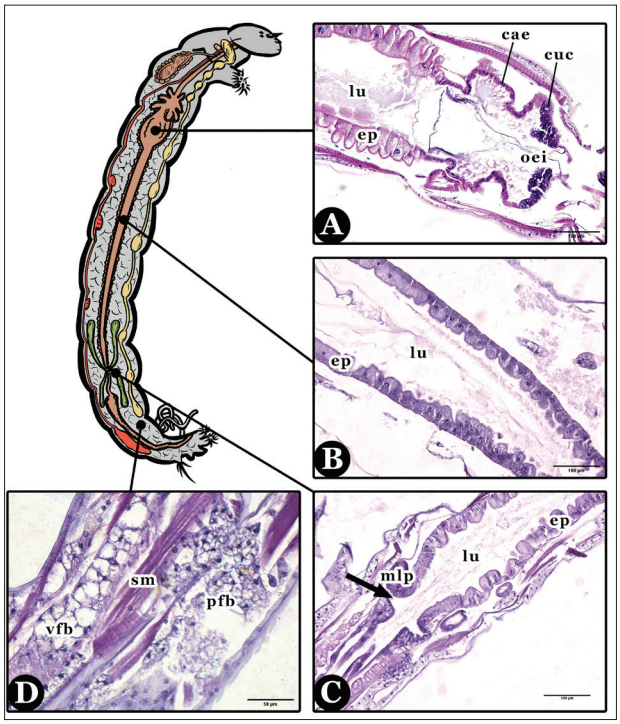


**Fig. 1.** Larval digestive and excretory system and fat body histology (untreated larvae). **A** – Longitudinal section of region I of the midgut showing tissue architecture of gastric caeca and the digestive tube; **B** – longitudinal section of region II of the midgut; **C** – longitudinal section of the proctodeal valve showing the place of insertion (arrow) of the Malpighian tubules and epithelial cells of region III of the midgut; **D** – longitudinal section showing the arrangement of fat body. cae: gastric caeca; cuc: Cuénot cells; e: epithelial cells of the midgut; lu: lumen; mlp: Malpighian tubules; oei: esophagus invagination; pfb: parietal fat body; sm: smooth muscle; vfb: visceral body fat.

basophilic cells. This was the place of insertion of the Malpighian tubules (Fig. 1C). The hindgut, formed by the ileum, colon and rectum, had a similar cell structure to the esophagus. The Malpighian tubules, composed of a single layer of pavement cells with a nucleus projecting to the lumen of the tubules, were located around the proctodeal valve (Fig. 1C).

The fat body of *C. riparius* was found along the whole-body length of larvae in 2 forms, the visceral fat body (VFB) that covered the visceral organs, and the parietal fat body (PFB) that was located between the epidermis and the intersegmental muscles. Both forms were composed of trophocytes that were larger in the visceral fat body. Trophocytes had a vacuolated cytoplasm while the perinuclear region was their only part with an affinity for hematoxylin stain. Parietal fat

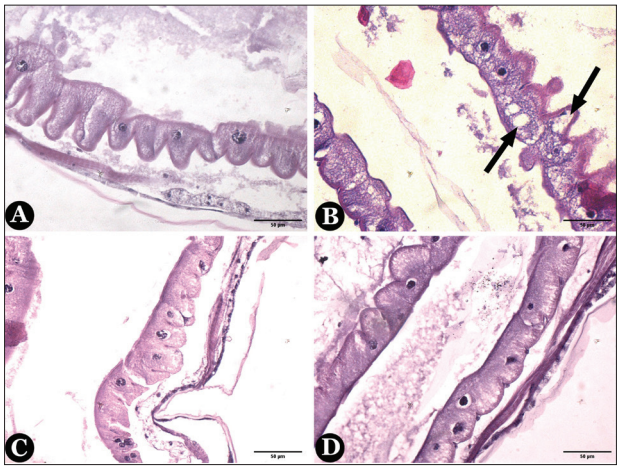


**Fig. 2.** Histopathological effect of nanoparticles on midgut region I. **A** – control group showing irregularly shaped digestive cells; **B** – treated group with nano-$Fe_3O_4$ showing vacuoles in all parts of digestive cells (black arrow); **C** – treated with nano-$CeO_2$; **D** – treated with nano-$TiO_2$ showing digestive cells similar to the control.

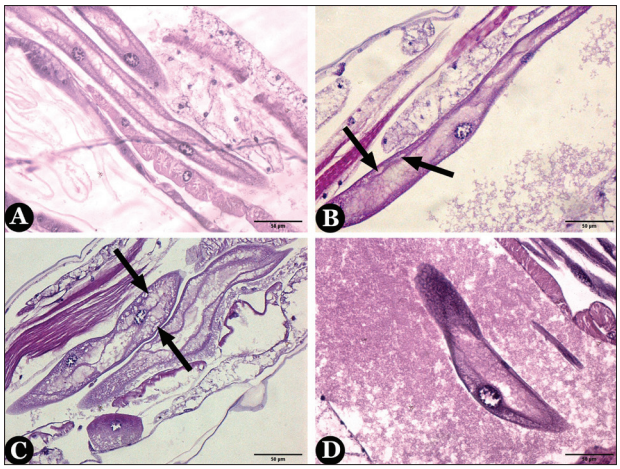


**Fig. 3.** Histopathological effect of nanoparticles on Malpighian tubules. **A** – Malpighian tubules of the control group; **B** – treated group with nano-$Fe_3O_4$ showing vacuoles in Malpighian tubule cells (black arrow); **C** – treated with nano-$CeO_2$ showing intensive vacuolization of epithelial cells (black arrow); **D** – treated with nano-$TiO_2$ without vacuolization of epithelial cells of Malpighian tubules.

**Table 1.** Histological changes in *Chironomus riparius* larvae exposed to sublethal concentrations of $TiO_2$, $CeO_2$ and $Fe_3O_4$ nanoparticles. Results are presented as the number of larvae with tissue alterations relative to the total number of treated larvae.

| Tissue alteration | $TiO_2$ | $CeO_2$ | $Fe_3O_4$ |
|---|---|---|---|
| Midgut region I vacuolization | 0/40 | 0/40 | 17/40 |
| Malpighian tubules vacuolization | 0/40 | 19/40 | 21/40 |
| Parietal fat body alteration | 20/40 | 0/40 | 0/40 |
| Change of midgut architecture | 11/40 | 0/40 | 0/40 |

body cells were small, clustered and associated with the larval integument (Fig. 1D).

## Histopathological effect of nanoparticles

Morphological analyses of the tissues of the digestive and excretory systems and the fat body revealed histopathological changes in the treated groups.

Alterations in the digestive system were recorded in regions I and II of the midgut. Larvae treated with a sublethal concentration of $Fe_3O_4$ nanoparticles exhibited changes in the digestive cells of the midgut region I, while nano-$TiO_2$ and nano-$CeO_2$ did not produce changes in this region. In the control group, the epithelial cells of the midgut region I had an irregular shape, with an eosinophilic basal and apical border, a granular cytoplasm and a large, rounded nucleus with condensed chromatin. A short brush border facing the lumen was noted (Fig. 2A).

Larvae treated with nano-$Fe_3O_4$ showed vacuolization of region I midgut cells in different percentages. Large vacuoles were observed in the basal and apical parts of the cell in 17/40 of treated larvae (Fig. 2B, Table 1). Digestive cells of larvae exposed to nano-$CeO_2$ and nano-$TiO_2$ were similar to those in the control group (Fig. 2C, D).

Histological analyses of Malpighian tubules exhibited alterations in epithelial cells of this excretory organ in larvae exposed to nano-$Fe_3O_4$ and nano-$CeO_2$. In these groups, vacuolization of Malpighian tubules was evident (Fig. 3B, C). Vacuoles were observed in 21/40 individuals in the group treated with nano-$Fe_3O_4$, and in 19/40 individuals treated with nano-$CeO_2$ (Table 1). Larvae exposed to the nano-$TiO_2$ treatment exhibited no vacuolization of Malpighian tubules (Fig. 3D), like the larvae in the control group. The Malpighian tubules in the control group consisted of a single layer of plate-like cells filled with a granular cytoplasm. The large nucleus of epithelial cells extended to the lumen of the tubule (Fig. 3A).

Changes in trophocyte morphology were noted in larvae exposed to nano-$TiO_2$, with 20/40 individuals showing histological alterations in the PFB (Fig. 4D, Table 1). These fat body cells were rounder than in the control group and disconnected from each other with clearly visible intercellular spaces. The PFB of control

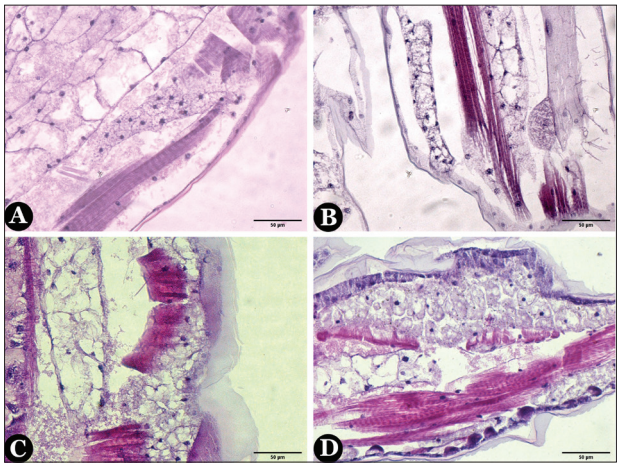


**Fig. 4.** Histopathological effect of nanoparticles on trophocytes of parietal fat body. **A** – control group showing clustered small, polygonal trophocytes; **B** – treated group with nano-$Fe_3O_4$ and **C** –treated with nano-$CeO_2$ with a parietal fat body similar to the control group; **D** – group treated with nano-$TiO_2$ showing rounder and detached trophocytes with considerable intercellular space.

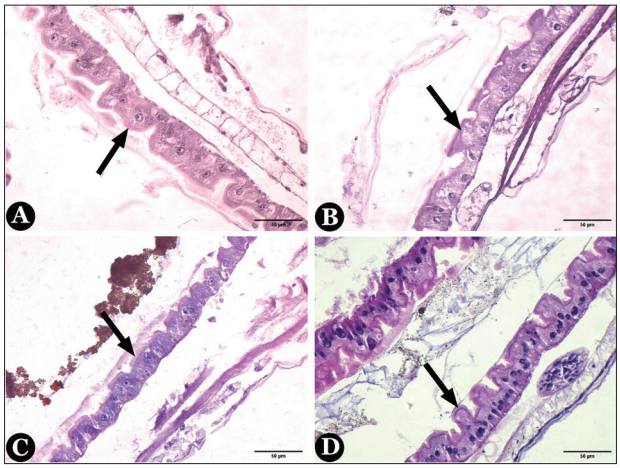


**Fig. 5.** Histopathological effect of nanoparticles on midgut region II. **A** – control group showing cubic cells with brush border (black arrow) and secretion to the lumen; **B** – treated group with nano-$Fe_3O_4$ showing cubic digestive cells covered with a brush border; **C** – treated with nano-$CeO_2$ with digestive cells apparently similar to the control group; **D** – treated with nano-$TiO_2$ showing numerous cells elongated towards the lumen and secretion of brush border.

group larvae and larvae treated with nano-$Fe_3O_4$ and nano-$CeO_2$ consisted of polygonal cells with a vacuolated cytoplasm and a small nucleus (Fig. 4A, B, C).

Among the 20 larvae with PFB alterations, morphological changes in the midgut region II were observed simultaneously in 11 larvae. These changes were presented as a greater number of cells that were compressed closer together in comparison to the control group (Fig. 5A, D, Table 1).

The brush border of the epithelial cells in region II showed a significant difference in the length of brushes in comparison to the group treated with nano-$Fe_3O_4$ and the control group. The treated group had significantly shorter brushes than the control group (Mann-Whitney test, $P<0.05$). Larvae from the groups treated with nano-$CeO_2$ and nano-$TiO_2$ showed no significant differences in the length of the brush border (Fig. 5).

## DISCUSSION

Previous studies on nano-$TiO_2$, -$CeO_2$ and -$Fe_3O_4$ treatments on non-biting midge larvae revealed their toxicity at different levels. High concentrations of nano-$TiO_2$ were lethal to chironomid larvae, while environmentally-relevant concentrations of the same nano-oxide caused morphological changes in mouthpart structures [13]. A study with nano-$CeO_2$ showed its genotoxicity on *Chironomus riparius* larvae [38], while morphometric analyses revealed deformities on the mouthpart structures in treated larvae [12]. Nano-$Fe_3O_4$ showed its toxic effect at the morphological level, altering the larvae oral apparatus, as well as at the biochemical and molecular levels, inducing oxidative stress and DNA damage [12]. Histopathological analyses are important for identifying the toxic effect of the nano-oxides at a cellular level and for comprehending the consequences of nanoparticle accumulation in the aquatic environment on living organisms that directly or indirectly come into contact with them. While histological analyses revealed tissue alterations in all treated groups of *C. riparius* larvae, different nano-oxides caused different extents of histological changes on target tissues and organs.

In the present study, histological analyses of non-treated *C. riparius* larvae did not show any differences in internal morphology in comparison to existing data [28,33,36]. These findings are of great potential for defining the general histopathological biomarkers that could be used in the histopathological assessment of related species.

Most food digestion and absorption takes place in the midgut [41], and the control group had a similar

morphology of this region compared to the previously described *Chironomus sancticaroli* and *Chironomus columbiensis* [28,36]. Intense vacuolization was noted in the digestive cells of the midgut region I in a significant percentage of larvae treated with $Fe_3O_4$ nanoparticles. Vacuoles found in the nano-$Fe_3O_4$ group did not appear in the other treatments or in the control group. Earlier studies have shown that nano-$Fe_3O_4$ can induce oxidative stress and DNA damage in *C. riparius* larvae [12], so this vacuolization of the midgut cells points to a toxic cellular effect of $Fe_3O_4$ nanoparticles. Vacuoles in the anterior digestive cells were also observed in other studies with water pollutants. The larvae of *Polypedilum* sp., sampled from tributaries affected by mining, showed a high degree of cytoplasm vacuolization in the midgut epithelia [33]. Studies with insecticides and *Chironomus calligraphus* provided similar results [32]. Vacuoles may also be found in normal cells of the midgut [42], and their presence is probably the result of transcytosis [41]. However, the occurrence of intense vacuolization may indicate that these cells are undergoing cell death [43].

The peritrophic matrix (PM) is a semipermeable membrane that protects midgut cells from mechanical and chemical damage. The pore size of the PM slowly decreases towards the hindgut and allows small molecules to pass to midgut cells. Therefore it does not represent a barrier for nanoparticles [44]. It was shown that nanosized particles are capable of entering midgut digestive cells [45]. These properties of the midgut makes it the most susceptible to the harmful effect of ingested toxins, as was shown in previous studies [32,33]. The foregut and hindgut did not show any tissue alterations, probably because of the cuticle layer that covers the epithelium [41].

Morphological examination of the Malpighian tubules revealed no alterations in the control group and the group treated with nano-$TiO_2$. However, vacuolization of epithelial cells was recorded in larvae exposed to $CeO_2$ and $Fe_3O_4$ nanoparticles. Malpighian tubules possess a mechanism of active transport of toxins and, along with the midgut, respond rapidly to their presence [46,47]. This property makes them great biomarkers for ecotoxicological studies [46] as was shown in other studies on insects [33,34]. Vacuolization of the epithelial cells in Malpighian tubules was noted in bees exposed to insecticides, as an indicator of the initiation of the cell death process [48]. Similar results were obtained with mosquitoes [49]. In general, it appears that strong toxic agents induce cell death in Malpighian tubules by generating numerous vacuoles that can be observed in the cytoplasm. Nano-$CeO_2$ cellular uptake and cytotoxic activity were already shown [20], and the main mechanism behind its toxicity is the generation of ROS that causes oxidative stress and cell damage [50]. A similar effect was noted in the case of $Fe_3O_4$ nanoparticles as well [22].

The fat body of treated larvae was examined and alterations of the PFB were visible only in larvae exposed to $TiO_2$ nanoparticles. In comparison to the control group with polygonal trophocytes tightly attached to each other, these cells in the treated group showed a rounder shape with some intercellular space. A similar manifestation was described in *Drosophila melanogaster* during the prepupal stage in the disaggregation phase, but as a part of the normal life cycle [51]. However, previous research did not reveal any statistically significant changes in the length of the period from egg-hatching to metamorphosis into the adult form in *C. riparius* larvae treated with the same nano-oxides at sublethal concentrations [12]. Previous studies have elucidated that the holometabolous insect prepupal stage implies remodeling of the midgut region by the development of the pupal midgut and degeneration of larval digestive cells [52]. Histological observations of larvae treated with nano-$TiO_2$ have shown a markedly higher number of compacted midgut cells in comparison to the control group. This occurrence was observed in 11/20 larvae that also showed changes in the PFB. However, it was seen that transition to the pupal stage in *Apis mellifera* induces degeneration of the Malpighian tubules [53], which was not recorded in larvae exposed to $TiO_2$ nanoparticles.

It was revealed that one of the general forms of the stress response in honeybees is the release of fat body content in an attempt to increase energy immobilization [54]. The types and concentrations of lipids in the insect fat body are variable under stress situations such as exposure to insecticides [55]. Such variations in fat body content may cause morphological changes in the tissue [56]. Nano-$TiO_2$ particles can penetrate cells of the midgut and accumulate there, as noted in a study of *Ceriodaphnia dubia* [57]. Additionally, it

was shown that larvae have the ability to regenerate midgut epithelial cells when infected or exposed to xenobiotics [58], which could explain the changes in the midgut region. The results of the present study indicate that $TiO_2$ displayed visible tissue alterations in the fat body and midgut region, but it is unclear whether this oxide has an indirect impact on tissues by provoking metamorphosis or as the result of a direct impact caused by its toxic potential.

Microvilli cover the midgut cells and have various functions such as the production and secretion of digestive enzymes, assimilation of nutrients, ion homeostasis and signaling [40]. In the control group, the midgut region II had a continuous, long brush border on the apical side of the digestive cells that was turned toward the intestinal lumen. Measuring the length of the region II brush border of the midgut revealed a statistically significant shortening of microvilli in larvae treated with $Fe_3O_4$. It was shown that toxins can cause brush border disruption in Chironomidae [34]. Microvilli length reduction was observed in a *D. melanogaster* larvae starvation study [59]. Transmission electron microscope analysis revealed the formation of abnormal droplets or small vesicles at the end of microvilli, which caused their breakage and probably shortening. Similar results were obtained in the same experiment with *D. melanogaster* and *Callosobruchus maculatus* treated with plant defense inhibitors [59]. Since microvilli are not static structures, actin filaments that modulate their length respond in certain situations of physiological stress, such as fasting or pathological infections [60]. The insect gut epithelium plays an important role in immunity, therefore shortening of the brush border may be a kind of defense against toxins that are present in the intestinal lumen [41]. Thus, the absorption process is minimized and the possibility of toxin entry is reduced. Several studies have described the cytotoxicity of $Fe_3O_4$, which lies in its potential to induce oxidative stress through the generation of ROS [22].

The organs that were the most sensitive to acute exposure in a previous study of *C. sancticaroli* were the organs of the digestive system, the Malpighian tubules and the fat body [34]; our results correlate with these findings. Further analyses (including chronic tests) are necessary for a better understanding of the histopathological changes in *C. riparius* larvae. Comparison of the assessment of the chronic response with acute response can lead to a better understanding of the effects of metal nano-oxide on the studied species. This study supports the use of *C. riparius* larvae, as one of the most sensitive organisms, in the examination of sediment toxicity [35], and points the way to the development of histopathological biomarkers that can improve the existing methodology in ecotoxicological studies.

**Funding:** This study was supported by the Serbian Ministry of Education, Science and Technological Development, Grant 451-03-9/2021-14/200124.

**Acknowledgements:** We wish to thank students Jelena Đorđević and Nađa Milenković (Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš, Serbia) for their assistance in realization of the experiment.

**Author contributions:** JSS: investigation, visualization, formal analysis, writing of original draft. ĐM: supervision, methodology, visualization, formal analysis, writing the original draft, review and editing. JSV: methodology, data curation visualization, writing of the original draft**,** conceptualization. DNSZ: visualization, writing of the original draft. NRS: methodology, visualization. JBS: methodology**,** visualization. PJV: supervision, methodology, visualization, formal analysis, writing of the original draft, review, editing.

**Conflict of interest disclosure:** The authors declare no conflict of interest.

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