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Effects of oral exposure to brake wear particulate matter on the springtail Orthonychiurus folsomi[☆]

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ABSTRACT

Most of the heavy metals in urban environments derives from road traffic, particularly from tyres and brake wear (non-exhaust emission sources). These pollutants contaminate the soil, where several organisms have a primary ecosystem role (e.g., springtails, ants, earthworms). Springtails (Collembola) are soil-dwelling animals regulating soil fertility, flow of energy through above- and below-ground food webs, and they contribute to soil microbial community dispersion and biodiversity maintenance. In this study we investigated the ecotoxicological effects of oral exposure to particles emitted from brake pads and cast-iron brake discs in the euedaphic collembola species *Orthonychiurus folsomi* under laboratory conditions. Our results showed that chronic exposure to brake wear particles can have sub-lethal effects both at low and high concentrations and it can cause histological alterations. Here, SEM-EDX was applied to observe the particulate and we found its chemical markers in the gut and faeces of collembola, while histological analysis detected alterations of the digestive and reproductive systems and of the abdominal fat body at high concentrations.

1. Introduction

Air pollution is the main environmental risk to health in the world, causing respiratory and cardiovascular diseases (World Health Organization, 2020). Particulate matter (PM) may be also ingested through contaminated food, causing alterations of gut microbiota (Li et al., 2017; Papa et al., 2021c). In urban areas, vehicular traffic is one of the major sources of PM, which may derive from both exhaust (i.e., tailpipe emissions) and non-exhaust sources, the latter primarily including wearing down of brake pads, discs and tyres, and the resuspension of road dust. While stringent regulations have ensured that exhaust emissions are kept under control, lack of directives on non-exhaust emissions has led to an increase of the contribution of non-exhaust particulate to the overall PM in urban environments (OECD, 2020; Piscitello et al., 2021). Also, in cities, non-exhaust PM represents the main source of heavy metal contamination, including Cr, Cu, Fe, Mn, Sn, Ti, and Zn (Duong et al., 2006; Liati et al., 2018; Papa et al., 2021a). Many metals may also bioaccumulate in living organisms, such as plants (Bozdogan Sert et al., 2019; Cetin and Jawed, 2022; Sevik et al., 2020c) and it has been demonstrated that the outer bark of the road-facing part of plants displays higher metal concentrations (Cesur et al., 2021; Sevik et al., 2020a).

Roadside soils are an important reservoir for heavy metal pollution, which may affect pedestrians and residents through direct emissions and dust resuspensions (Chen et al., 2010; Papa et al., 2021a).

Road traffic may also compromise food safety because vegetables and fruit grown in heavily-trafficked areas show high concentrations of heavy metals (Sevik et al., 2020b).

Exposure to metal-based PM is of much concern as toxicological assessments indicate that it may promote oxidative stress caused by generation of free radicals and reactive oxygen species (ROS) (Jan et al., 2019; Soltani et al., 2018).

Moreover, exposure and bioaccumulation of heavy metals can cause cellular and tissue alterations, which often lead to impairment of

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biologically relevant systems in many species (Hernández-Plata et al., 2020; Le Saux et al., 2020; Nasr et al., 2020; Ranjeet et al., 2018; Shahjahan et al., 2022). Considering insects, histopathological examination of individuals exposed to lethal or sub-lethal doses of heavy metals have revealed abnormalities of different degrees in the digestive and reproductive organs, as well as in the fat body, the main detoxifying organ (Diener et al., 2015; El-Ashram et al., 2021; Fouda et al., 2011; Kheirallah and El-Samad, 2019; Polidori et al., 2018).

At present, only a few studies investigated the toxicological effects of non-exhaust PM emissions on microorganisms, plants, aquatic and terrestrial organisms exposed to media contaminated by wear debris from the braking system of vehicles (Dodd et al., 2014; Kukutschová et al., 2009; Maiorana et al., 2019; Shupert et al., 2013; Volta et al., 2020). To date, no information is available on the effects of chronic ingestion of low doses of non-exhaust PM on soil micro-arthropods which represent a key component of the soil edaphon. For example, Collembola are among the most abundant soil-dwelling animals regulating soil fertility, as well as flow of energy through above- and below-ground food webs (Filser, 2002). Given the high sensitivity of these insects to environmental pollution, many ecotoxicological studies have been carried out to assess the impact of soil contaminants on their reproduction and growth (Aspetti et al., 2010; Cutz-Pool et al., 2007; Frampton, 1997; Kopeszki, 1997). Some species are also used as bio-indicators for soil quality and health (Callejas-Chavero et al., 2022), and test guidelines are also developed for some species, including the well-known parthenogenetic species *Folsomia candida*, which is used for assessing the effects of chemicals on the reproduction of collembolans in soil (ISO, 2014, 2011; OECD, 2016).

Ecotoxicological studies assessing the consequences of pollutant ingestion by soil model organisms are mainly focused on plastics (Chang et al., 2022; Vaccari et al., 2022), whereas no data are available on the effects of non-exhaust emissions, especially brake and tire wear particles, acquired through the feeding route. Hence, in this work we evaluate the effects of the chronic ingestion of brake wear debris on the euedaphic collembola species *Orthonychiurus folsomi* Schäffer 1900 (Collembola: Onychiuridae). Euedaphic species are morphologically well adapted to soil having a significant reduction or loss of pigmentation and visual structures, reduced appendages, and reduced water-retention capacity. As a result of this adaptation, they are more sensitive to environmental variations, pollution and soil degradation than other living soil organisms and may quickly respond to low-level soil pollution (Callejas-Chavero et al., 2022; Menta, 2012). *O. folsomi* is a widespread species which may occur in great abundance in unpolluted soil and compost (Callejas-Chavero et al., 2022). The biology of the species is well studied and the whole mitochondrial genome is also available (Yao et al., 2020). Moreover, *O. folsomi* has been used in a number of toxicological tests and the potential of this species as a bio-indicator is widely acknowledged (Callejas-Chavero et al., 2022; Cermak et al., 2010; Dodd and Addison, 2010; Feisthauer et al., 2006; Huang et al., 2005; Sheppard et al., 2005).

We tested the effects of long-term exposure to high and extremely low dose of wear debris generated from a commercially available brake pad, commonly sold in the EU market, on adults of *O. folsomi*. Wear debris were characterised in terms of mineral phases, size and morphology, and a high (typical of highly polluted soil) and a low dose (pollution level far below law limits) of exposure were considered. Effects on mortality, reproduction, and histological alterations in *O. folsomi* were investigated.

2. Materials and methods

2.1. Preparation of the yeast with and without brake pad powder

Brake wear particulate tested in this study was the same previously used in the study of Maiorana et al. (2019). In our experiments, springtails were fed with baker's yeast mixed with the brake wear particles, at two different concentrations, high (HiC) and an extremely low (LoC) (Table 1). Concentrations were established considering tin (Sn) as a reference, which has a stringent threshold for soil according to Italian legislation (i.e., 1 mg/kg for residential-recreative land use and at 350 mg/kg for the commercial-industrial land use; D. Lgs. n. 152/2006). The HiC dose was half the contamination threshold value for the commercial-industrial land use, which is set at 350 mg/kg (D.Lgs. n. 152/2006); the LoC dose was obtained by mixing 0.005 g of brake pad powder with 2.4 g of yeast and the resulting mixture stock (powder + yeast) was then diluted 1/100 (final tin concentration <1 mg/kg; Table 1). For the HiC dose, the resulting concentration of Cu and Zn was above safe regulatory limits, while none of the metals present in LoC exceeded such limits (Table 1).

2.2. Ecotoxicological test

The experiment was conducted in 2022, from June to July for the HiC dose, and from September to October for the LoC dose. *O. folsomi* was obtained from cultures reared at the Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore Piacenza, Italy.

To test the toxicity of the brake wear particulate, ten adults of *O. folsomi* per Petri dish per four replicates were used (four replicates per treatment and four replicates per control; 40 treatment and 40 control samples in total for each dose). Petri dishes were prepared with plaster of Paris (without carbon) and moistened with distilled water after including the adult springtails. Further, ~2.5 mg of rehydrated baker's yeast was added as food source. The baker's yeast was provided as it is and mixed with the brake wear particles in control and treated tests, respectively. All tests were incubated in a climate box at 20 ± 2 °C, with 97 ± 3% relative humidity for the duration of the experiment (four weeks for LoC dose, four days for HiC dose).

Every day, the number of alive adults, the number of eggs, and the number of juvenile stages were counted. At the end of the experiment, springtails were collected and prepared for histological analyses. Springtails and faeces were also analysed with scanning electron microscope (SEM) coupled with X-ray (EDX) analyses, as described below.

2.3. SEM-EDX analyses

SEM-EDX analyses were carried out on brake wear debris to assess the morphology, chemical composition, and size of the particles (Papa et al., 2021c). This technique was also used to identify the morphological and chemical markers of the brake wear debris in springtails and faeces samples. For each dose, four individuals (2 control + 2 treated) and four faeces samples (2 control + 2 treated) were mounted on an aluminium stub using double-adhesive carbon tape. Samples were covered with graphite for SEM-EDX analysis (Zeiss Gemini 500, Oberkochen, Germany - Bruker QuantaX-Flash 6|30, Berlin, Germany) (Capitani et al., 2021; Negri et al., 2015; Papa et al., 2021a, 2021b; Pellecchia and Negri, 2018). Secondary electrons (SE), backscattered electrons (BSE) images, EDX point analyses, and EDX maps were acquired (Papa et al., 2021c; Pellecchia and Negri, 2018). A sample of brake wear debris and treated yeast samples plus control were also

Table 1
Heavy metal concentrations in the high (HiC) and low (LoC) doses. *Traditional pad debris elemental composition (modified from table 2 by Maiorana et al., 2019). ** limits for residential-recreative land use and for commercial-industrial land use (D.Lgs. n. 152/2006).

Element	Presence in mg/kg		Law limit	Pad (% dry weight) *
	HiC	LoC		
Cu	297.22	6.271×10^{-1}	120 mg/kg	3.01
Cr	67.15	1.417×10^{-1}	150 mg/kg	0.68
Sn	179.71	3.792×10^{-1}	1–350 mg/kg **	1.82
Zn	276.48	5.833×10^{-1}	150 mg/kg	2.80

analysed.

2.4. Statistical analysis

A Wilcoxon signed rank test was performed to evaluate whether the difference in number of alive individuals, eggs and juveniles was statistically significant between treatment and control on LoC doses. Data analysis was performed using R v4.1.1 (R Core Team, 2020).

2.5. Histological analysis

Twenty alive adult control springtails, eighteen alive HiC-treated adult springtails and thirteen alive LoC-treated adult springtails were collected and shipped in sterile tubes to the laboratory of Veterinary General Pathology and Anatomical Pathology of the Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II" for histopathological analysis.

Upon reception of samples, evaluation of vitality was performed by observation at the stereomicroscope (Zeiss Stemi 305 trino, New York, NY, USA). Twelve HiC, five LoC and six control individuals (all females) which were identified as living and vital were further prepared as follows: all samples were 10% buffered formalin fixed for 24 h, paraffin-embedded for routine histological processing, and stained with hematoxylin and eosin (HE). Finally, tissue preparations were observed by light microscopy (Nikon Eclipse E−600, Nikon, Tokyo, Japan) to evaluate the presence of possible alterations.

3. Results

3.1. Ecotoxicological results

We recorded 50% mortality rates in HiC dose treated samples after four days from administering the treated yeast. The LoC treatment showed significant (p-value <0.05) sub-lethal effects when compared with the control, namely: a decrease in alive springtails and no reproduction (absence of eggs), and the consequent absence of juvenile stage springtails after four weeks of exposure. All survivor individuals for each dose were collected for SEM-EDX and histological analysis.

3.2. SEM-EDX results

SEM analyses on the brake wear particulate showed that it had different sizes from sub-micrometric to PM less 10 μm. EDX analyses and elemental mapping confirm the presence of heavy metals (including Fe, Cu, Zn, Cr, Sn), and other elements (Fig. 1). EDX spectra showed the presence of metal sulphides, including tin sulphides, and metal oxides (Fe- and Cu-oxides; Fig. 1).

SEM-EDX analyses demonstrated the presence of these brake markers both in the hindgut of HiC-treated springtails and their faeces (Fig. 2). In the hindgut, we found sub-micrometric PM (Fig. 2C) whose EDX spectra revealed the significant presence of Fe, Cu, Sn, Zn, Al, Mg, P, Cl, K, S (Fig. 2D). In the HiC-treated faeces, we found the same markers of brakes, including Sn, Fe, Cu, Cr, Zn, and crystals of calcium sulphate, with a typical elongated-euhedral habitus ($CaSO_4$) (Fig. 3A and D). In LoC dose springtails and faeces samples, we did not find the presence of brake wear PM.

3.3. Histological results

The histopathological analysis of LoC-treated samples did not show any significant alterations in any of the analysed tissues, compared to the control samples; on the contrary, HiC-treated samples revealed alterations of abdominal organs, namely midgut, ovaries, and abdominal fat body (Fig. 4A and B).

Considering the histological analysis of control samples, the midgut epithelium of *O. folsomi* appeared lined with an intact pseudostratified columnar epithelium strictly in contact with the basal lamina. Midgut epithelial cells showed brush border, and cytoplasm filled with many vesicles containing clear transparent material, while the midgut lumen presented food material and a well formed perithrophic membrane. Female gonads were composed of two sac-shaped ovaries, each constituted of a germarium, which contained clusters of germ cells, and a vitellarium, where mature oocytes were present and were nourished by nurse cells. Mature oocytes were stacked with yolk granules of different size and colour, and surrounded by nurse cells. Externally, a thin basal lamina and a double layer of flattened follicular cells was evident. The abdominal fat body of control samples was composed of groups of numerous trophocytes showing polyhedric nucleus and cytoplasm filled with small transparent vesicles (Fig. 4A).

The histological assessment of the midgut of HiC-treated samples revealed in some areas of the epithelium the presence of many intercellular gaps and detachment from the basal membrane. Moreover, the cytoplasm of the epithelial cells contained numerous vesicles filled with basophilic material while the lumen of the midgut was filled with food mingled with colourless, shiny, reflective material presenting different sizes and shapes, consistent with the brake pad powder administered. Sometimes, small highly basophilic cells, identified as haemocytes, were observed in the midgut lumen (Fig. 5A and B). Female gonads of HiC-treated samples showed different degrees of alteration ranging from inflammation to necrosis. Some oogonia of the germarium presented vacuoles of variable size in the cytoplasm and marginated chromatin, other presented nuclei undergoing shrinkage and becoming densely basophilic or fragmentated, suggesting pyknosis and karyorrhexis phenomena, respectively. Mature oocytes were filled with yolk granules homogenous in size and colour, while the external follicular epithelium appeared often hypertrophic and detached from the oocytes, in some cases disruption of the double layer leaded to fusion of oocytes' content. In the severest cases, necrosis and focal secondary dystrophic calcification was observed, as well as increase of empty spaces among the yolk granules (Fig. 5C–F). The fat body of HiC-treated samples presented signs of degeneration and necrosis, such as vacuolization, absence of nucleus, disruption of cell membranes and homogenisation of cytoplasm. Moreover, focal areas of melanisation were observed in the midgut lumen, ovaries and abdominal fat body (Fig. 5C–F).

4. Discussion

Brake wear particulate can affect air quality and its transport and deposition in the soil may have negative influence on the edaphic ecosystem, where some soil-dwelling species are particularly sensitive to stressors. Our work investigated the effects of chronic exposure to brake wear debris on the euedaphic species *O. folsomi* and its interactions with the springtail survival, reproduction, and the possible induction of histological alterations.

We found that long-term exposure to wear debris has a negative influence on the fitness of *O. folsomi*, both at the extremely low and high doses (Table 1). When the HiC dose was used, we recorded elevated mortality of the springtails after four days. Previous studies of ecotoxicological effects of particulate obtained from different brake systems (1000 mg/L) on the nematode *Caenorhabditis elegans* and the earthworm *Eisenia fetida* did not show any toxic effects after three days of exposure (Volta et al., 2020). *C. elegans* is a widespread free-living nematode that mostly lives in the liquid phase of soils and *E. fetida* is an epigeic earthworm, i.e., a species that inhabit the litter layer (Monroy et al., 2006; Queirós et al., 2019). Instead, *O. folsomi* is a euedaphic species, with morphological and behavioural adaptation to live in the deeper layers of the soil (Negri, 2004). Therefore, we cannot exclude that euedaphic species may display higher sensitivity to metal exposure, especially via the oral route.

Among metals typically present in brake wear debris, Fe, Cu, and Zn represent essential components for living organisms when present in trace amount, often functioning as protein cofactors in a wide variety of

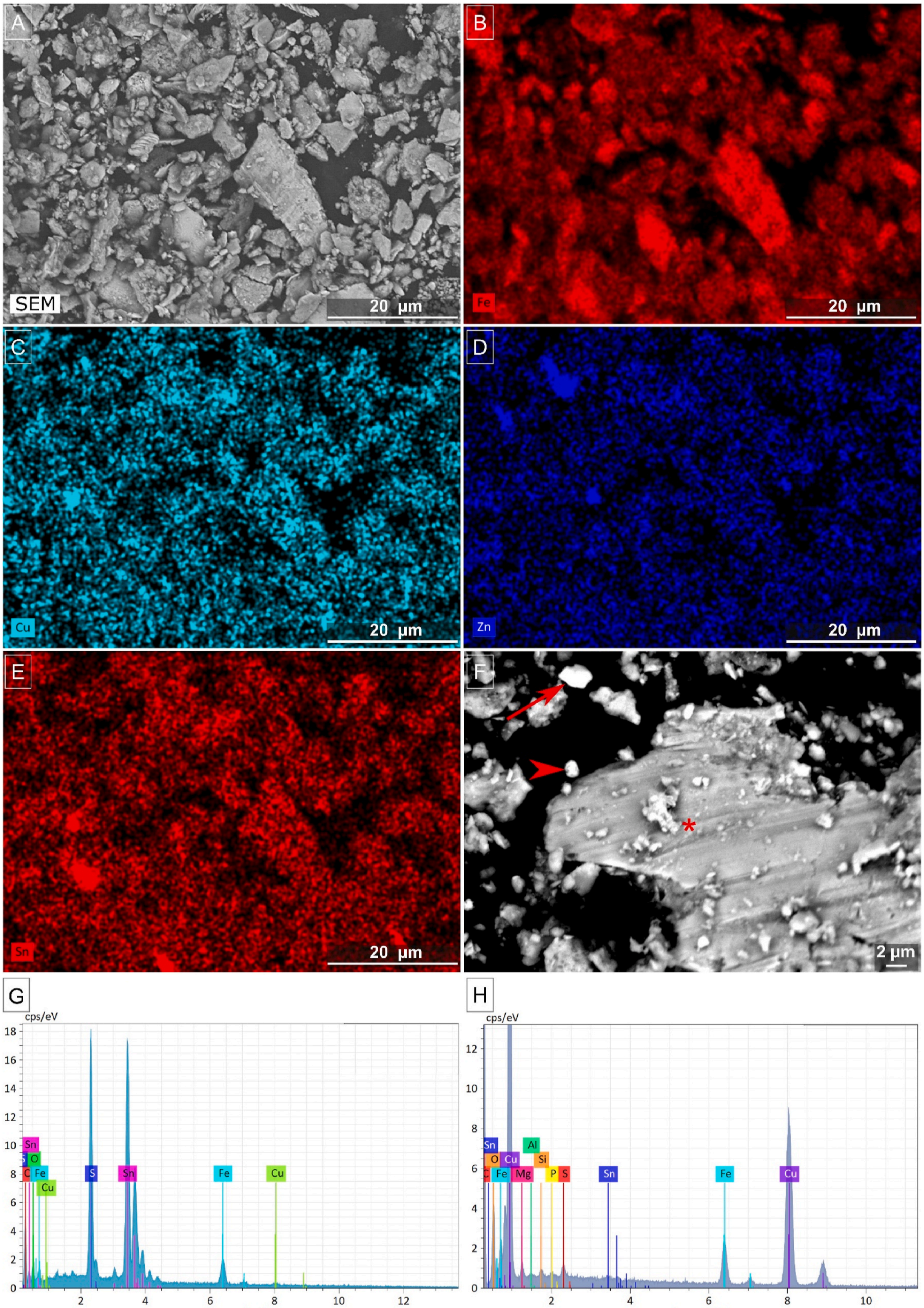

Fig. 1. Elemental mapping of brake wear debris and EDX spectra. A) SEM - SE image of brake wear debris showing particles of irregular shape with sharp edges; B-E) single element maps of Fe, Cu, Zn, and Sn present in the brake wear debris; F) SEM-BSE image of brake wear debris; G) EDX spectrum of a tin sulfide (arrow); H) EDX spectrum of copper oxide and iron oxide/hydroxide (arrowhead). Asterisk, a platelet with fairly smooth surface of iron oxide/hydroxide, possibly belonging to a brake disc.

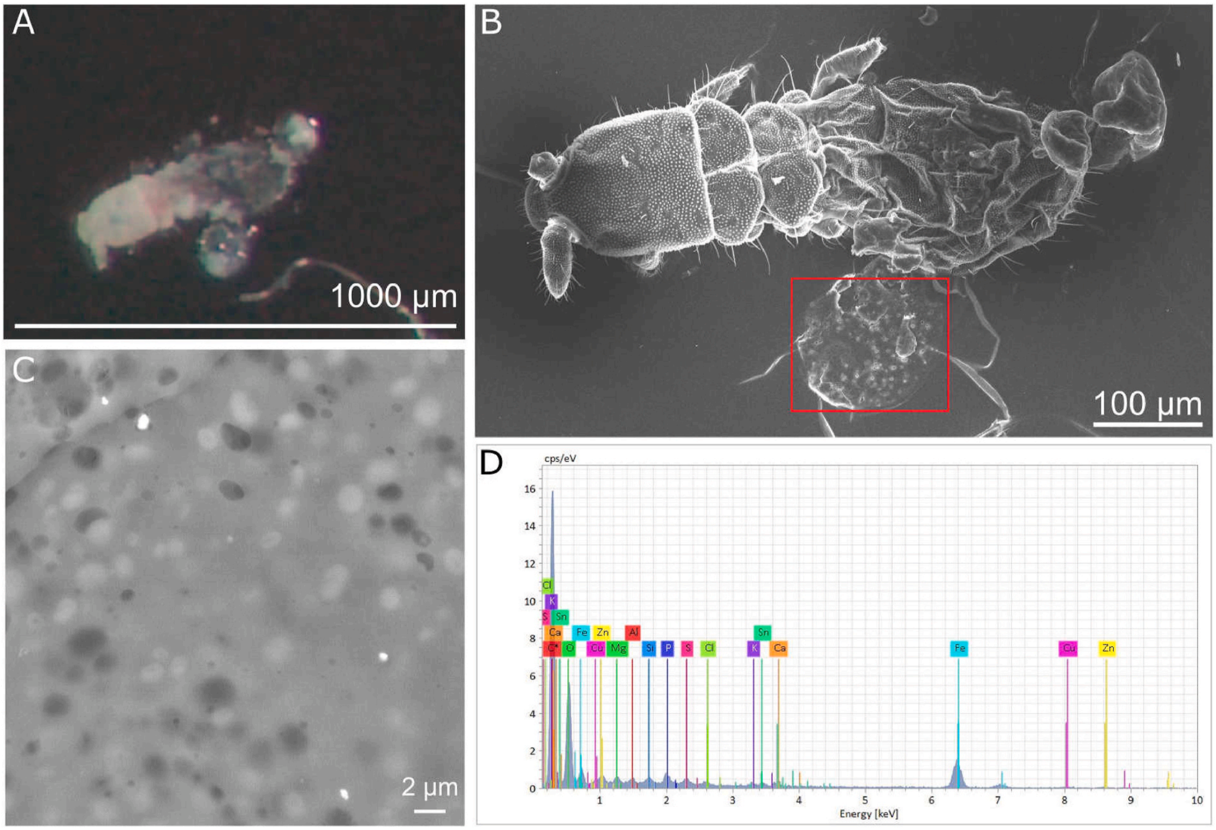

Fig. 2. Springtail treated with HiC dose. A) Optical microscope image of springtail adults. B) SEM with SE view of the same sample; the red area highlights the hindgut content. C) Magnification in BSE of the hindgut content; the bright spots were analysed through EDX. D) EDX spectrum of the bright spots identified in the hindgut. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

biological processes, but could become toxic when exceeding a threshold (Slobodian et al., 2021). For instance, Fe^{2+} and Fe^{3+} free ions can become toxic only under certain conditions and at very high concentrations (Volta et al., 2020) and iron-bearing PM is known to have an oxidant activity and subsequent toxicity by initiating the formation of ROS (Jan et al., 2019; Soltani et al., 2018). However, further studies are therefore needed to verify the oxidative potential of these particles also in the brake wear samples.

The other metals present in non-negligible amount in the brake wear debris used in our study were Cu and Zn, whose concentration in HiC dose was above the law limits. Due to their broad presence in the environment, often originated by vehicular traffic, several ecotoxicological studies explored the effect of these metals on collembola reproduction, survival and motility (Drapper et al., 2000; Hwang et al., 2016; Jośko et al., 2022).

Copper has been described to have an inhibitory effect on reproduction of *F. candida* at concentration 300 mg/kg (Jośko et al., 2022) and the LC50 of Cu reported for *F. candida* and *Lobella sokamensis* was 1.261 and 4472 mg/kg, respectively (An et al., 2013). Our data support inhibitory effect on reproduction and mortality of springtails at lower Cu concentrations (6.271E-01 and 297.22 mg/kg, respectively; Table 1), but these effects may be due to oral exposure of a complex mixture of metals characterizing the braking wear debris. Interestingly, a regulation of internal Cu concentration in collembola has been described by Ardestani and Van Gestel (2013). At 25 and 100 mg/kg of Cu in LUFA 2.2 natural soil, *F. candida* showed a slow accumulation with uptake rate constants of 0.02–0.17 g_{soil}/g_{animal}/day (Ardestani and Van Gestel, 2013). However, regulatory processes were not sufficient to prevent the accumulation of Cu above the normal environmental levels in soils (Ardestani and Van Gestel, 2013). Further, an antioxidant activity able to counteract the generated ROS due to Cu exposure has been described in *F. candida* (Maria et al., 2014), but no information is available for *O. folsomi* or other euedaphic springtails species.

Regarding Zn, ecotoxicological studies using ZnO nano and non-nano particles demonstrated that ions released from ZnO particles were responsible for the toxic effects rather than particle size (Ardestani et al., 2014). The survival of *F. candida* is not affected for concentrations up to 6400 mg Zn/kg dry weight, while the effect on reproduction is dose related: at concentrations below 1800 mg Zn/kg in the soil the numbers of juveniles produced were lower for ZnO nanoparticle than for non-nano ZnO, while reproductive effects of non-nano ZnO were higher above these concentrations (Kool et al., 2011). In our study, negative effect on reproduction and mortality of *O. folsomi* occurred at lower Zn concentrations (5.833E-01 and 276.48 mg/kg, respectively; Table 1). Zinc internal regulation in springtails can occur for concentrations up to 70–270 µg/g dry body weight (Ardestani et al., 2014).

In the present study, we showed that brake wear debris is ingested by springtails and eventually expelled with faeces, after interacting with the cells of the digestive epithelium causing sub-lethal effects. The elements found in the hindgut content such as Ca, S, P, K, Mg and Cl can be partly linked to the insect haemolymph that comprises water and inorganic salts (mostly Cl, K, and Mg) (Papa et al., 2021c), and partly due to ingested calcium sulphate ($CaSO_4$; gypsum and/or anhydrite), the typical ingredient for the plaster of Paris that we used as substratum.

Histopathological examination of *O. folsomi* samples revealed the presence of alterations of different severity in the midgut, ovaries and fat body in HiC-treated samples and the absence of significant tissue modifications in LoC-treated samples.

In our study, the midgut of HiC samples showed areas with many intercellular gaps, probably due to cell shrinkage, and detachment of epithelial cells from the basal lamina. These same modifications have been associated to the process of degeneration that precedes

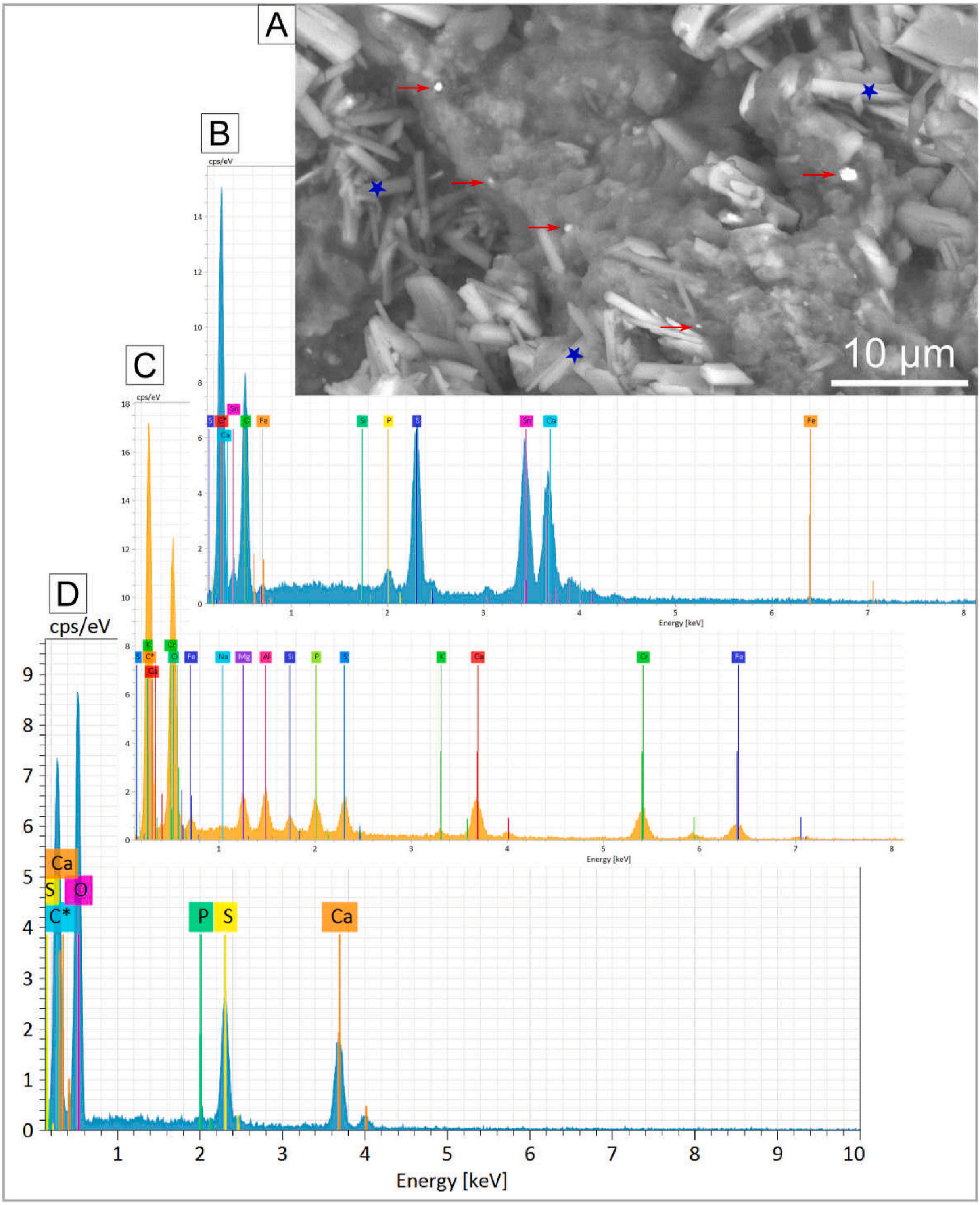

Fig. 3. Springtail faeces treated with HiC dose. A) SEM with BSE view of the faeces sample; the red arrows indicate the brake wear particles found in faeces, and the blue star indicates the calcium sulphate crystals. B–C) EDX spectrum of the bright spots (brake markers) identified in the faeces. D) EDX spectrum of the gypsum ($CaSO_4$. $2H_2O$)or anhydrite ($CaSO_4$) crystal. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regeneration of the midgut epithelium which occurs as a defence mechanism against toxic substances. Comparing HiC-treated samples with LoC and control samples, it appears that the process of degeneration is increased in HiC-treated samples, suggesting activation of the afore-mentioned defensive mechanism. Moreover, the activation of a "midgut immune system" is also supported by the presence of haemocytes and melanisation in the lumen of the midgut, where the supposed brake pad power material was observed. Furthermore, in HiC samples changes in the cytoplasmic content were also observed and they could be connected to functional impairment of the organelles and subsequent alterations of the metabolism of the cell, as well as to accumulation of surplus of essential and non-essential metals (Hopkin,

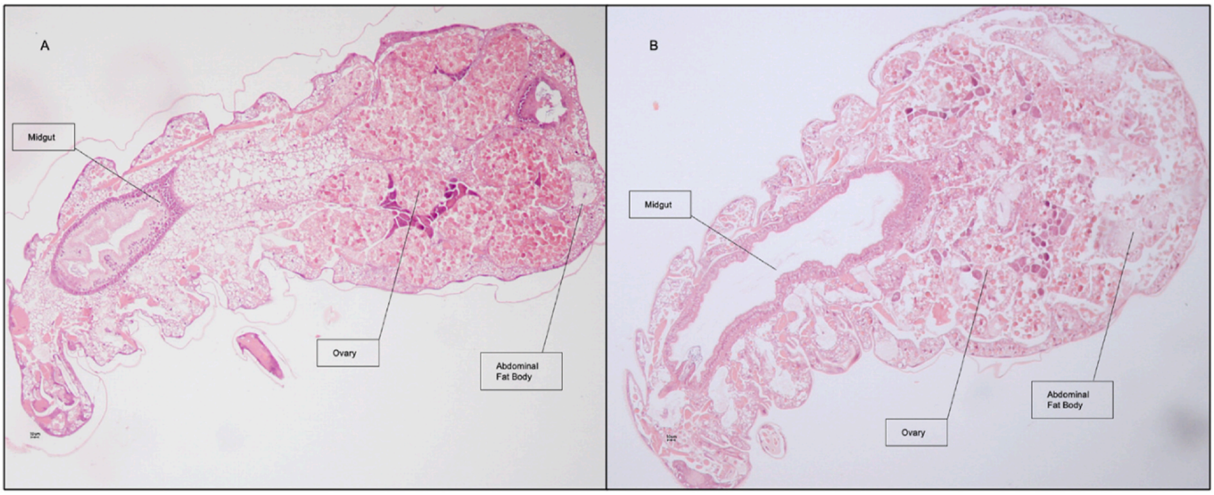

Fig. 4. *O. folsomi* longitudinal section. A) *O. folsomi* control sample showing localisation of analysed organs. 100*x*, HE; B) *O. folsomi* HiC treated sample showing localisation of analysed organs. 100*x*, HE. Scale bar 10 μm.

1997; Pawert et al., 1996). Similar alterations connected to heavy metal exposure, have been described in other insects confirming the toxic action of these elements on the midgut and the unbalance in the absorption and excretion of substances (El-Ashram et al., 2021; Kheirallah and El-Samad, 2019; Nasr et al., 2020; Wu et al., 2009). Altered nutrient metabolism, negatively influences reproduction through impairment of vitellogenesis, yolk production and consequently oocyte maturation (Badisco et al., 2013; Dong et al., 2008; Fei et al., 2005). Our samples treated with HiC doses showed yolk granules homogenous in size and colour, suggesting decreased variability in content of the yolks, and increase of empty spaces among yolk granules, probably due to lack of primary nutrients. Moreover, as previously described by Osman et al. (2015), the observed detachment of the external follicular epithelium from the oocytes and disruption of the double layer may also negatively influence the passage of nutrients, and consequently impair nourishment of oocytes and delay in oocyte maturation. As previously demonstrated in other species (Kheirallah and El-Samad, 2019; Kheirallah et al., 2019; Massa et al., 2019; Osman et al., 2015; Siekierska and Urbańska-Jasik, 2002) ovaries presenting severe abnormalities, such as the ones found in our study, are unable to correctly function and guarantee effective reproduction. Moreover, it appears that turnover of oocytes is also impaired as oogonia of the germinarium presented vacuolization, pyknosis and karyorrhexis, as already described by Poprawa et al. (2022) in *Lithobius forficatus*.

As for the ovaries, the abdominal fat body of HiC samples presented signs of degeneration and necrosis. The insect fat body, together with the midgut and haemolymph, plays an essential role in detoxification (Dubovskiy et al., 2011; Enayati et al., 2005; Keeley, 1985) and in the synthesis of yolk protein precursors (e.g., vitellogenin), which determine correct development of the oocyte and of the egg (Pan et al., 1969; Sun et al., 1991; Valle, 1993). Therefore, alterations of the throphocytes imply not only modification of energy metabolism but could also impair correct functioning of detoxification and reproductive activity. We can then suggest that alterations found in the ovaries could be connected, at least in part, to fat body malfunctioning, and reduced production of vitellogenin.

Conversely to HiC-treated samples, LoC-treated samples did not show alterations of the analysed organs nor activation of immune response, suggesting a dose-dependent effect of heavy metals on tissues (Balali-Mood et al., 2021; Esposito et al., 2012) and the existence of a threshold of tolerance below which individuals are able to balance the toxic effects of heavy metals. However, more studies are needed to better define this hypothesis.

5. Conclusion

The use of Collembola and especially deep-living species as bioindicators of soil pollutants is increasing as they tend to be more sensitive than other living organisms and may quickly respond to low-level soil pollution. We investigated the toxicological effects of brake wear debris, i.e., a mixture of metals frequently found as pollutants in urban and road-side soils, on *O. folsomi*, a euedaphic species with a well-known biology, ecology and behaviour.

By applying a multidisciplinary approach, we were able to characterise: (i) the size and mineralogical composition of brake wear debris administered to the collembola, (ii) to verify its ingestion and presence in the collembola gut, and (iii) to determine lethal and sub-lethal effects.

Brake wear debris is a heterogeneous mixture of metal-based particles below 10 μm in length, in form of metal-oxides and -sulphides, which includes Fe-, Cu-, Zn-oxides, and Sn-sulphides. Oral exposure to environmentally-relevant concentrations of brake wear debris can cause dose-dependent effects on the midgut, ovaries, and fat body of *O. folsomi*, which can impair the correct functionality of these organs and lead to reduction of reproductivity, humoral immunity and survival rates.

However, to date, the exact pathological mechanism underlying histological lesions is unknown and more studies focusing on pathogenesis and the mechanisms of action of metal mixtures are needed. We foresee that using multiple collembola species and exploring the contaminants effects on collembola through epigenetics, transcriptomics and microbiota analysis could provide additional information on the long-term interaction and toxicological effects.

Finally, our data may prompt new approaches to define soil quality, in order to establish more acceptable threshold concentrations of soil pollutants.

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Author Contribution statement

Giulia Papa and Ilaria Negri designed research; Bartolo Forestieri, Giulia Papa, Karen Power and Ilaria Negri performed research; Giancarlo Capitani, Paola Maiolino and Ilaria Negri contributed analytic tools; Bartolo Forestieri, Giulia Papa, Karen Power, Giancarlo Capitani, Paola Maiolino and Ilaria Negri analysed data; Giulia Papa, Karen Power

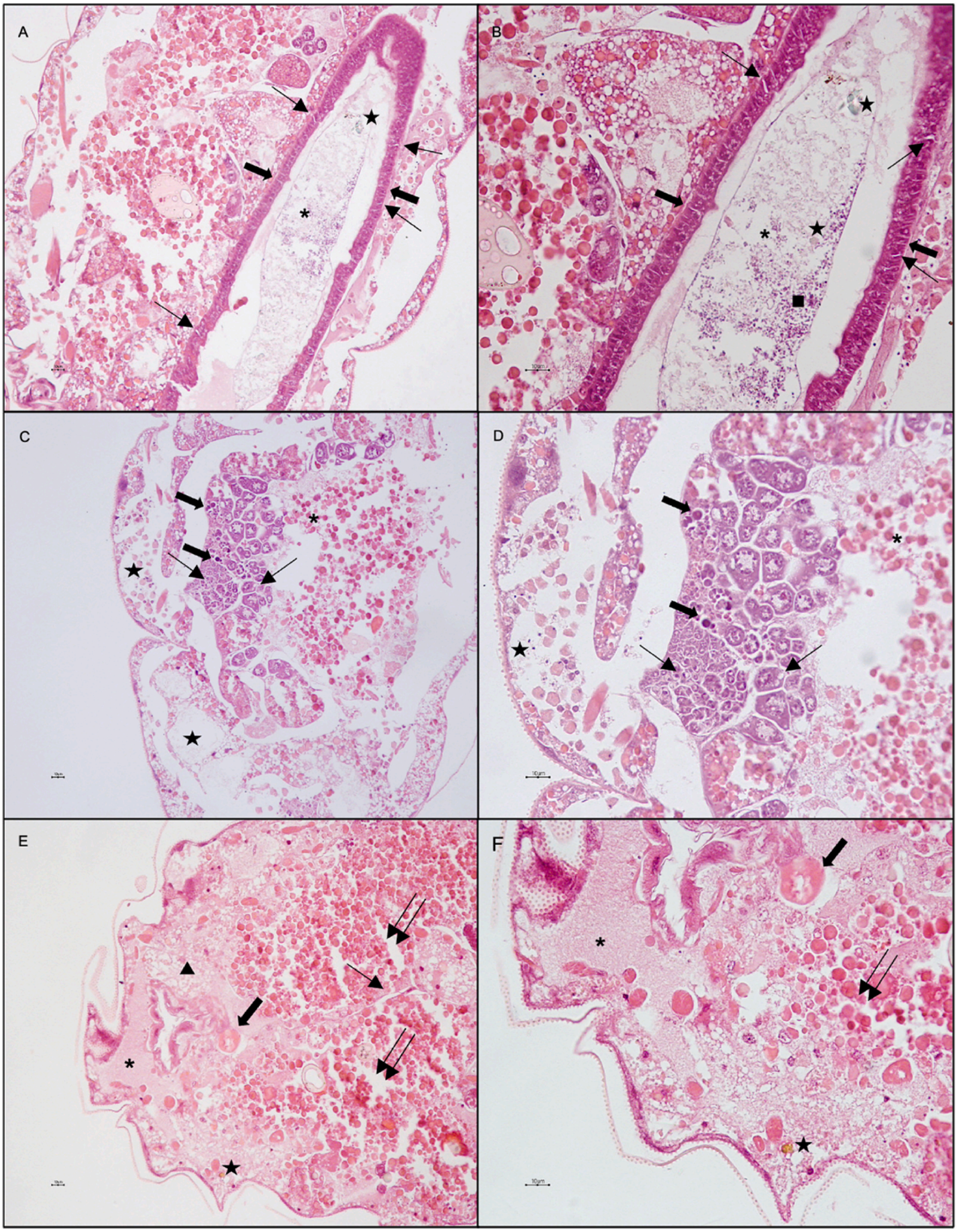

Fig. 5. *O. folsomi* HiC treated sample. A-B) Midgut: presence of intercellular gaps (thin arrow) and detachment from the basal membrane (fat arrow), lumen filled with food (asterisk) mingled with reflective material (star) and small basophilic cells (square). 200*x* and 400*x*, HE. C-D) Ovary and fat body: oogonia presenting vacuoles (thin arrow) and pyknotic nuclei (fat arrow); mature oocytes presenting fusion of oocytes' content (asterisk). Trophocytes showing homogenisation of cytoplasm (star). 200*x* and 400*x*, HE. E-F) Ovary and fat body: mature oocytes presenting external follicular epithelium detached from the oocytes (thin arrow), necrosis (asterisk), secondary dystrophic calcification (fat arrow) and increase of empty spaces among the yolk granules (double arrow). Trophocytes showing homogenisation of cytoplasm (triangle) and melanisation (star). 200*x* and 400*x*, HE. Scale bar 10 μm.

and Ilaria Negri wrote the manuscript. All authors reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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