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Microplastics, chlorpyrifos and their mixtures modulate immune processes in the terrestrial crustacean *Porcellio scaber*

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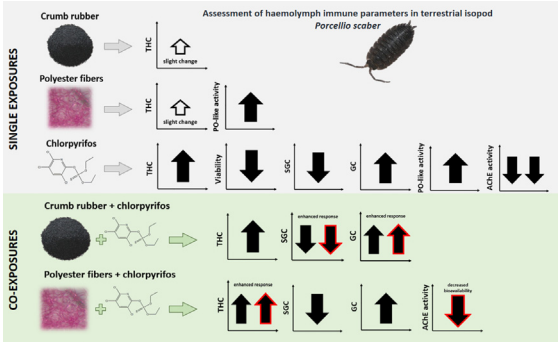
HIGHLIGHTS

- Immune parameters are slightly changed upon exposure to plastic fibres or crumb rubber.
- Chlorpyrifos caused significant changes in *Porcellio scaber* immune parameters.
- Microplastics decreased the bioavailability of chlorpyrifos for *P. scaber*.
- Mixtures of plastic fibres and chlorpyrifos resulted in greater response in haemocyte count.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics and agrochemicals are common pollutants in terrestrial ecosystems. Their interaction during coexistence in soils may influence their fate and adverse effects on terrestrial organisms. The aim of this study was to investigate how the exposure to two types of microplastics; polyester fibres, and crumb rubber; induce changes in immune parameters of *Porcellio scaber* and if the co-exposure of microplastics affects the response induced by the organophosphate pesticide chlorpyrifos. A number of immune parameters, such as total haemocyte count, differential haemocyte count, and phenoloxidase-like activity were assessed. In addition, the acetylcholinesterase (AChE) activity in the haemolymph was evaluated as a measure of the bioavailability of chlorpyrifos. After three weeks of exposure, the most noticeable changes in the measured immune parameters and also a significantly reduced AChE activity were seen in chlorpyrifos-exposed animals. Both types of microplastic at environmentally relevant concentrations caused only slight changes in immune parameters which were not dependent on the type of microplastic, although the two types differed significantly in terms of the chemical complexity of the additives. Mixtures of chlorpyrifos and microplastics induced changes that differed from individual exposures. For example, alterations in some measured parameters suggested a reduced bioavailability of chlorpyrifos (AChE activity, haemocyte viability) caused by both types of microplastics exposure, but the increase of haemocyte count was promoted by the presence of fibres implying their joint action. In conclusion, this study suggests that immune processes in *P. scaber* are slightly changed upon exposure to both types of microplastics and microplastics can significantly modulate the effects of other co-exposed chemicals. Further research is needed on the short-term and long-term joint effects of microplastics and agrochemicals on the immunity of soil invertebrates.

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1. Introduction

A number of studies over recent years have investigated the effects of microplastics on terrestrial invertebrates (Huerta Lwanga et al., 2017, 2016; Ju et al., 2019; Jemec Kokalj et al., 2018; Rodríguez-Seijo et al., 2017; Selonen et al., 2020; B.K. Zhu et al., 2018; Zhu et al., 2018). Most of these were applied at the level of the whole organism, in terms of survival, reproduction and feeding activity, so less is known about other sublethal effects after long-term exposure (Rodríguez-Seijo et al., 2017). One of the likely microplastic-induced changes in organisms are those related to immunity, as has been shown for aquatic organisms (Détrée and Gallardo-Escárate, 2017; Gomiero et al., 2018; Green et al., 2019; Liu et al., 2019; Mohsen et al., 2020; Revel et al., 2018; Von Moos et al., 2012). The responses of the immune system are the first line of protection against exogenous and endogenous threats, such as pathogenic infections, tissue damage or cancers, and thus the immune system offers a wide range of sensitive measures for the physiological state and environment of an organism (Boraschi et al., 2020; Canesi et al., 2015; Matozzo and Gagné, 2016). There are several ways in which microplastics might provoke an immune response leading to either immunosuppression or immunostimulation. For example, an immune response might arise through changes in the diversity and function of the intestinal microorganisms (Liu et al., 2019; Motta et al., 2018; Zhu et al., 2018), mechanical damage to the digestive tract (Davis and Engström, 2012; Lei et al., 2018; Qi et al., 2017), or changes in food quality (Pascual et al., 2004) and food intake (Matozzo et al., 2011). In addition to any effects of microplastic particles, the chemicals released from microplastics that are known as plastic additives (Hermabessiere et al., 2017) might themselves indirectly induce changes in the immune parameters (Sung et al., 2011).

This study was focused on the terrestrial isopod woodlouse *Porcellio scaber* (Crustacea: Isopoda), which is recognised as an important test species in soil ecotoxicology (Van Gestel et al., 2018) due to its indispensable ecosystem function in litter decomposition. Immune parameters have not been measured previously in *P. scaber* in response to a pollutant, but their modulation under infection has been investigated (Dolar et al., 2020; Kostanjšek and Pirc Marolt, 2015). The innate immunity of terrestrial isopods consists of humoral and cellular components, primarily the haemocytes, that first recognise and then respond to challenges through the activation of phagocytosis, nodulation, encapsulation, clotting and melanisation (Söderhäll, 2016). In *P. scaber*, three types of haemocytes have been described at the light microscopy level (Dolar et al., 2020). Semigranulocytes are responsible for encapsulation (Kostanjšek and Pirc Marolt, 2015) and phagocytosis (Chevalier et al., 2011), and they also synthesise and secrete the components of the prophenoloxidase (proPO)-activating system (Cerenius et al., 2003). Granulocytes are rich in cytoplasmic granules and are the main source of humoral molecules, such as antimicrobial peptides and enzymes involved in the proPO cascade (Havanapan et al., 2016; Qin et al., 2019). The third cell type is the hyalinocyte, which is mainly responsible for phagocytosis (Jia et al., 2017). The enzyme phenoloxidase (PO) is an important humoral component that is involved in many processes, such as cuticle sclerotisation, wound healing and defence against parasites, through induction of melanisation (Cerenius et al., 2008; Liu et al., 2007). The measured parameters in the present study are among the most commonly measured immune-related biomarkers in experimental studies with invertebrates: total haemocyte count (THC), differential haemocyte count (DHC), haemocyte viability, and PO-like activity (Le Moullac and Haffner, 2000; Matozzo et al., 2011; Revel et al., 2018).

There is substantial evidence that terrestrial ecosystems, especially agricultural soils, are contaminated with microplastics of different polymer compositions, shapes and sizes (Corradini et al., 2019; Nizzetto et al., 2016; Zhu et al., 2019). The reported concentrations of microplastics in agricultural soil range from 7100 to 42,940 particles kg^{-1} soil (Zhang and Liu, 2018), to 1600 to 56,000 particles kg^{-1} in sewage sludge (Li et al., 2018) and 0.03% to 6.7% soil weight in industrial areas (Fuller and Gautam, 2016). Among all of the microplastics found in the soils, synthetic fibres represent the major portion. For example, in agricultural soils amended with sewage sludge or biosolids, synthetic fibres have been reported to comprise 92% (Zhang and Liu, 2018), and even 97% (Corradini et al., 2019), of the microplastic particles present. The abrasion of tyre wear is also considered to be responsible for one of the greatest inputs of microplastics into the environment, in particular along the sides of roads (Kole et al., 2017; Sieber et al., 2020; Wagner et al., 2018). Tyre wear particles are not conventional thermoplastics, but are elastomers like rubber, which is not covered by the original definition of plastic. However, they have been considered as microplastics because synthetic polymers are an essential ingredient of tyres (Hartmann et al., 2019). Tyre wear particle concentrations of 0.2% to 12% (w/w) have been measured or estimated along roads, while 30 m from roads, the concentrations fall to 0.005% to 0.01% (w/w) (Unice et al., 2012; Wagner et al., 2018; Wik and Dave, 2009).

Apart from microplastics, agricultural soils are sinks for agrochemicals, many of which are very stable, with half-lives that range from a few months to several years, and in some cases even reach decades (Pose-Juan et al., 2015; Yadav et al., 2015). The interactions between microplastics and other pollutants during their co-existence influences their fate and toxicity (Ramos et al., 2015; Tourinho et al., 2019; Wang et al., 2018). Microplastics have been shown to act as carriers of hydrophobic substances due to their high hydrophobicity, small particle size and large specific surface area (Bakir et al., 2012; Hüffer et al., 2018; Teuten et al., 2009). Meso-particles and macro-particles made of low-density polyethylene plastic films used in agriculture have been shown to adsorb different types of pesticides (e.g., endosulfan, deltamethrin), and in addition to surface adsorption, diffusion also takes place within the plastic matrix (Ramos et al., 2015). The amounts of adsorbed pesticides correlate with the octanol-water coefficient (Ramos et al., 2015). Desorption of chlorpyrifos and trifluralin from plastic particles occurs upon contact with soil, although their migration largely depends on the type of pesticide formulation; this is higher for the chlorpyrifos registered product than for the analytical standard dissolved in an organic solvent (Ramos et al., 2015; Rodríguez-Seijo et al., 2019).

A number of studies have investigated the effects of microplastics pre-incubated with contaminants prior to exposure (also referred to as loaded microplastics) (Bellas and Gil, 2020; Besseling et al., 2019; Browne et al., 2013; Garrido et al., 2019; Kleinteich et al., 2018; Rivera-Hernández et al., 2019), while others have investigated the effects of simultaneous co-exposure of microplastics and chemicals on freshwater (Felten et al., 2020; Garrido et al., 2019; Horton et al., 2018; Kleinteich et al., 2018; Rehse et al., 2018; Zhang et al., 2019; Zocchi and Sommaruga, 2019) and marine organisms (Beiras and Tato, 2019; Bellas and Gil, 2020; Magara et al., 2019). At present, however, there are only a few studies that have investigated the effects of co-exposure of microplastics and other pollutants on terrestrial organisms (Wang et al., 2019a; Zhou et al., 2020).

In the present study, we exposed *P. scaber* to the pesticide chlorpyrifos, microplastics, and their mixtures. Chlorpyrifos is an organophosphorus insecticide that has been widely used since 1960, and is now frequently detected in water and food (Mahajan et al., 2019). The primary toxic mechanism of chlorpyrifos is based on its specific inhibition of acetylcholinesterase (AChE), an enzyme that is predominantly involved in neurotransmission, but that has many other physiological functions unrelated to the cholinergic system (Sepčić et al., 2019). Chlorpyrifos has already been investigated in mixtures with microplastics (Bellas and Gil, 2020; Garrido et al., 2019; Rodríguez-Seijo et al., 2019), which makes it a good model chemical for comparisons with other types of microplastics and organisms. Numerous effects of chlorpyrifos on selected terrestrial invertebrates have been reported (Gatti et al., 2002; Jager et al., 2007; Pelosi et al., 2014; Zhou et al., 2007), which include changes in immune parameters (Banaee et al., 2019; Galloway and Handy, 2003; Kankana Kalita and Devi, 2016). In earthworms, for example, reduced haemocyte viability (Booth and O'Halloran, 2001;

Eason et al., 1999) and phagocytic activity (Bunn et al., 1996) have been reported for chlorpyrifos exposure. However, although there have been some studies on the lethal effects of chlorpyrifos on terrestrial isopods (Morgado et al., 2016; Nair et al., 2002), the only study available on *P. scaber* provides little information on the sublethal effects of chlorpyrifos (Nair et al., 2002).

The aim of this study was to investigate the following questions: (i) Does a 3-week exposure of *P. scaber* to two types of microplastics with different shapes, sizes, polymer compositions and chemical additives (i.e., polyester fibres, crumb rubber) induce changes in their immune parameters; and (ii) Does co-exposure of microplastics affect the responses provoked by the organophosphate insecticide chlorpyrifos. We hypothesised that the effects of chlorpyrifos and microplastics mixtures will differ from their individual exposures, either with increased effects due to the interactions of these two pollutants, or with reduced effects due to decreased bioavailability of chlorpyrifos. We expect that the changes in the immune parameters will depend on the type of microplastic, due to the different physicochemical properties.

2. Materials and methods

2.1. Chemicals

The following chemicals were used: chlorpyrifos (98%; Cheminova Ltd, Denmark; CAS No. 2921-88-2; C9H11Cl3NO3PS), BCA protein assay reagents (Pierce, Rockford, ZDA), acetone, bovine serum albumin, Dulbecco's phosphate-buffered saline (DPBS; pH 7.1–7.5), sodium bicarbonate ($NaHCO_3$), potassium phosphate buffer (250 mM, pH 7.4; 100 mM, pH 7.0), Triton X-100, dopamine hydrochloride, sodium dodecyl sulphate, trypan blue, 5,5′-dithiobis-2-nitrobenzoic acid (for Ellman's reagent) and acetylthiocholine chloride (all Sigma-Aldrich).

2.2. Microplastics

The polyester fibres used in this study were prepared by cutting a fleece blanket followed by cryo-milling using a homogeniser (MillMix 20; Domel, Slovenia), as described by Selonen et al. (2020). The fibres had the shapes of narrow strips with a mean sample length of 220 ± 200 μm, a length range of 12 μm to 2870 μm, and a thickness of 6 μm.

The crumb rubber (particle size, <180 μm) was produced by Genan (Denmark) from mixed end-of-life car tyres by cryo-milling. The powder contained several different synthetic rubbers, which included styrene-butadiene rubber, butadiene rubber and butyl rubber, with 10% to 35% natural rubbers, and 25% to 35% carbon black. Scanning electron microscopy showed that the crumb rubber particles were irregular fragments (Supplementary information, Fig. S1). According to the particle size analysis, the dominant fraction of particles was from 80 μm to 110 μm (mean, 102.9 μm; volumetric distribution). A number of metal trace elements were also measured in the crumb rubber. The largest proportions were for Zn (22,700 μg g^{-1}), Al (1300 μg g^{-1}), Co (139 μg g^{-1}) and Cu (130 μg g^{-1}). Eight different polycyclic aromatic hydrocarbons were detected in the crumb rubber: benzo[*ghi*]perylene (0.492 μg g^{-1}), fluorene (0.189 μg g^{-1}), benzo[*a*]pyrene (0.152 μg g^{-1}), phenanthrene (0.127 μg g^{-1}), benzo[b+j]fluoranthenes (0.119 μg g^{-1}), benzo[*k*]fluoranthene (0.083 μg g^{-1}), pyrene (0.048 μg g^{-1}) and fluoranthene (0.0135 μg g^{-1}).

2.3. Test organisms

Porcellio scaber (woodlice) were collected from a compost heap in a non-contaminated, pollution-free garden in Kamnik, Slovenia (46° 13′ 32.988″ N; 14° 36′ 42.12″ E). Before the experiments, they were synchronised for several months under constant temperature (20 ± 2 °C) and illumination (16:8 h, light:dark) in a climate-controlled chamber at the University of Ljubljana. They were caged in glass containers with a mixture of loamy sand and peat at the bottom (at 40% water holding capacity), and fed on dry leaves from common hazel (*Corylus avellana*) and common alder (*Alnus glutinosa*), and on carrots, as described by Jemec Kokalj et al. (2018). The soil and dry leaves were dry sterilised at 105 °C for 3 h before the woodlice were introduced into the glass containers. Only healthy, adult woodlice (30–60 mg fresh body mass) of both sexes were used. Moulting woodlice, females with marsupia, and those showing symptoms of bacterial or viral infection were excluded.

2.4. Experimental design

Two experiments were carried out, one with chlorpyrifos and fibres, and the other with chlorpyrifos and crumb rubber. Each experiment had three different treatments: chlorpyrifos alone, microplastics alone, and chlorpyrifos + microplastics. The concentrations used for both of the microplastics were 0.05%, 0.5% and 1.5% (w/w) in the microplastic alone treatments, and 0.5% (w/w) in the chlorpyrifos + microplastics treatments. The microplastics concentrations were chosen based on a previous study (Selonen et al., 2020). The nominal concentrations of chlorpyrifos were 0.2, 0.4, 0.6, 0.8 and 2.0 mg kg^{-1} dry soil in both the chlorpyrifos alone and the chlorpyrifos + microplastics treatments. These concentrations were based on a previous study that reported a 120-h median lethal concentration (LC_{50}) for woodlice of 2.0 mg kg^{-1} (Nair et al., 2002). Chlorpyrifos concentrations in the test soil were measured at the beginning of the experiments for the two highest concentrations of chlorpyrifos; 0.8 and 2.0 mg kg^{-1}. These samples were analysed by a certified commercial analytical laboratory (Groen Agro Control, The Netherlands), using liquid chromatography–tandem mass spectrometry and chlorpyrifos-ethyl as the standard. The detection limit was 0.01 mg kg^{-1} dry soil. As the measured concentrations did not differ by more than 10% from the nominal values, the nominal concentrations were used in all calculations. The following controls were run: negative control without chlorpyrifos and without solvent, solvent control #1 (acetone), and solvent control #2 (acetone, 0.5% microplastics).

Standard agricultural soil (Lufa 2.2; Lufa Speyer, Germany) was used in all of the experiments. For the individual microplastics exposure, the fibres and crumb rubber were first mixed with the dry soil, and prior to exposure, the moisture content was adjusted to 40% of the water holding capacity, by addition of deionised water and mixing. Chlorpyrifos was dissolved in 30 mL acetone (measured stock concentration, 65 ± 5 g L^{-1}) and added to the soil. The acetone was left to evaporate off overnight in a fume hood. The solvent control was prepared by adding the same amount of acetone as in the chlorpyrifos treatments, to reach the final concentration of 7.5% acetone (v/w). The solvent control was also left for the acetone to evaporate off overnight. For the chlorpyrifos + microplastics co-exposures, the microplastics were added the next day, and the moisture content was adjusted to 40% water holding capacity. After a day of stabilisation of the soil with these additions, the soil was placed into the test jars, as 20 g to 30 g moist soil into each 100-mL jar, and the woodlice were then introduced into the prepared jars. Five replicate jars were prepared for each treatment, as each chlorpyrifos concentration (without or with microplastics), and the negative and solvent controls. Five woodlice were placed into each replicate jar and some dry leaves of common hazel were added for food. Altogether, 625 woodlice were used for this study, including the controls and the individual and mixture conditions (Supplementary Information, Fig. S2).

The soil moisture content was checked every 3 days, and the common hazel leaves were replaced each week. The pH of the soil was from 5.2 to 5.6 for chlorpyrifos + polyester fibres, and 6.0 to 6.3 for chlorpyrifos + crumb rubber, across all of the chlorpyrifos concentrations tested. Thus, the pH did not differ according to the chlorpyrifos concentration, which is line with our previous study on individual chlorpyrifos exposure (Broerse and Van Gestel, 2010).

2.5. Sampling of haemolymph

After 3 weeks of exposure of the woodlice in the jars, their haemolymph was collected using a glass micropipette. The intersegmental membrane between the 5th and 6th dorsal segment was punctured with a sterile syringe, and the woodlouse was gently squeezed. Haemolymph from a single woodlouse or pooled from several woodlice (depending on the volume collected from each woodlouse) was diluted with DPBS (pH 7.1–7.5; see details below). The cellular immune parameters (i.e., THC, DHC, haemocyte viability) were assessed immediately after collection of the haemolymph. For PO-like and AChE activities, the haemolymph was stored for up to 2 weeks at −20 °C.

2.6. Analysis of immune markers

The THC, DHC and haemocytes viability were determined in duplicate for each haemolymph sample obtained from a single woodlouse or pooled from a maximum of three woodlice (immediately after haemolymph collection) according to Dolar et al. (2020). The total number of samples analysed per controls and treatments was 8–12. Five μL haemolymph was diluted 1:5 with DPBS (pH 7.1–7.5) with 0.4% trypan blue, which stained dead haemocytes, while viable haemocytes remained unstained. Ten μL of this 30-μL haemocyte suspension was pipetted into a haemocytometer (Neubauer) to determine the THC, DHC and haemocyte viability under light microscope (Axio Imager Z1; Zeiss). DHC was obtained using differential interference contrast microscopy, by counting the numbers of the different types of haemocytes: (1) small hyalinocytes without cytoplasmic granules; (2) larger semigranulocytes with low density of cytoplasmic granules; and (3) granulocytes with high density of cytoplasmic granules (Dolar et al., 2020).

The PO-like activity included both the plasma PO and haemocyanine-derived PO activities, as measured in triplicate in the haemolymph samples collected from individual woodlice. The total number of samples analysed per controls and treatments was 10–13. Due to the large volume of haemolymph needed and the lack of woodlice left for this analysis, PO-like activity was only measured under certain treatment conditions: chlorpyrifos alone (all concentrations except the highest, of 2.0 mg kg^{-1}), and 0.5% (w/w) fibre alone, and 0.05% and 1.5% (w/w) crumb rubber alone; this assay was not carried out for the mixture treatments. The PO-like activity was determined photometrically (Dolar et al., 2020). Freshly collected haemolymph (3 μL) was diluted 1:39 with a solution containing DPBS (pH 7.1–7.5), 8 mM dopamine hydrochloride, and 2 mM sodium dodecyl sulphate, for in vitro PO activation. Forty μL of this 120-μL reaction mixture was transferred to a 384-well plate. The formation of reddish-brown pigment was measured using an imaging reader (Cytation 3; Biotek, USA), at 475 nm and 25 °C, over at least 3 h. The PO-like activity in the haemolymph was calculated as the change in absorbance from the linear part of the absorbance slope per min per μL haemolymph, as described by Charles and Killian (2015), then normalised to the control, and expressed as percentages.

2.7. Analysis of acetylcholinesterase activity in haemolymph

Acetylcholinesterase (AChE) activity was measured in duplicate in the haemolymph samples collected from an individual woodlouse. The total number of samples analysed per controls and treatments was 8–14. Due to the limited amount of haemolymph sample (as it was also used for the other immune parameters), AChE activity could not be measured for all of the chlorpyrifos concentrations and for the chlorpyrifos + crumb rubber co-exposure. AChE activity was determined according to Ellman et al. (1961), using microtitre plates, and as described by Madžarić et al. (2018). Ellman's reagent was prepared by dissolving 91 mg 5,5′-dithiobis-2-nitrobenzoic acid in 100 mL 250 mM potassium phosphate buffer (pH 7.4), with addition of 37.5 mg $NaHCO_3$. The solution was diluted to 1 L with deionised H_2O and stored in a dark glass bottle at 4 °C. Prior to measurement, the collected haemolymph (3 μL) was diluted 1:20 with 100 mM potassium phosphate buffer (pH 7.0), vortexed, and centrifuged at 16,000 ×*g* for 15 min at 4 °C. The reaction mixture in each well of the 384-well microtitre plates included: 20 μL sample, 15 μL Ellman's reagent and 5 μL acetylthiocholine chloride with a final concentration of 1 mM. The reactions were followed photometrically using an imaging reader (Cytation 3; Biotek, USA) at 405 nm for 15 min at 25 °C. The specific AChE activity was calculated as nmol hydrolysed acetylthiocholine chloride min^{-1} mg^{-1} protein (ε_{405} = 13,600 M^{-1} cm^{-1}).

Protein concentrations in the haemolymph were determined using BCA protein assay kits (Pierce, Rockford, IL, USA). After 30 min of incubation at 37 °C, the absorbance was measured at 562 nm. The protein concentrations in the samples were calculated from the standard curves with bovine serum albumin (25–2000 μM).

2.8. Data analysis and reporting

Total haemocyte counts and PO-like activity are expressed as the percentage changes from the control group, while DHC is expressed as the proportions (%) of each of the three haemocyte types in the THC. Haemocyte viability is presented as the proportions (%) of viable haemocytes within the THC. The data were analysed using the OriginPro v2020 software (OriginLab, Northampton, Massachusetts, USA). For normal distributions and homoscedasticity of the data, one-way ANOVA was performed followed by Tukey tests; otherwise, non-parametric Kruskal-Wallis tests were used, followed by Mann-Whitney *U* tests (Supplementary information, Tables S1–S5). $p < 0.05$ was considered as significantly different. Within each treatment (chlorpyrifos alone, chlorpyrifos mixtures with fibres/crumb rubber), the data were compared to the respective controls. Chlorpyrifos treatments without and with fibres or crumb rubber were compared at the effects of the same chlorpyrifos concentration. Outliers were defined as those with 1.5-fold difference between the first and third quartiles, and were excluded from the analysis.

3. Results

3.1. Immune parameters

3.1.1. Individual microplastics exposure

Exposure of the woodlice to polyester fibres or crumb rubber for 3 weeks altered the profiles of the immune parameters. At 0.05%, 0.5% and 1.5% polyester fibres and crumb rubber, THC increased by 38%, 60% and 40%, and by 61%, 36% and 21%, respectively. With the low sample numbers, when compared to the relevant control, these differences were statistically significant only for 0.05% crumb rubber (Fig. 1A). No clear dose-related responses were seen between THC and the microplastic concentrations. The DHC pattern, which includes information on granulocytes, semigranulocytes and hyalinocytes, was very similar to the respective controls for the microplastic exposure. No statistically significant changes in the viabilities of the haemocytes were seen (Fig. 2). The PO-like activity was significantly increased only at 0.5% polyester fibres in the soil, and was not affected by any of the crumb rubber concentrations tested (Fig. 3A).

3.1.2. Individual chlorpyrifos exposure

Total haemocyte counts, DHC, haemocyte viability and PO-like activity were all significantly affected after 3 weeks exposure to chlorpyrifos. The increase in THC reached 89% at the highest chlorpyrifos concentration of 2.0 mg kg^{-1} dry soil (Fig. 1B). At this concentration, the haemocyte viability was decreased by 17% (Fig. 4D), while the PO-like activity was significantly increased only at 0.6 mg kg^{-1} chlorpyrifos (Fig. 3B). The most evident changes were for DHC, with an increasing trend in granulocyte counts and decreasing semigranulocyte and hyalinocyte numbers (Fig. 4A–C). None of the measured immune

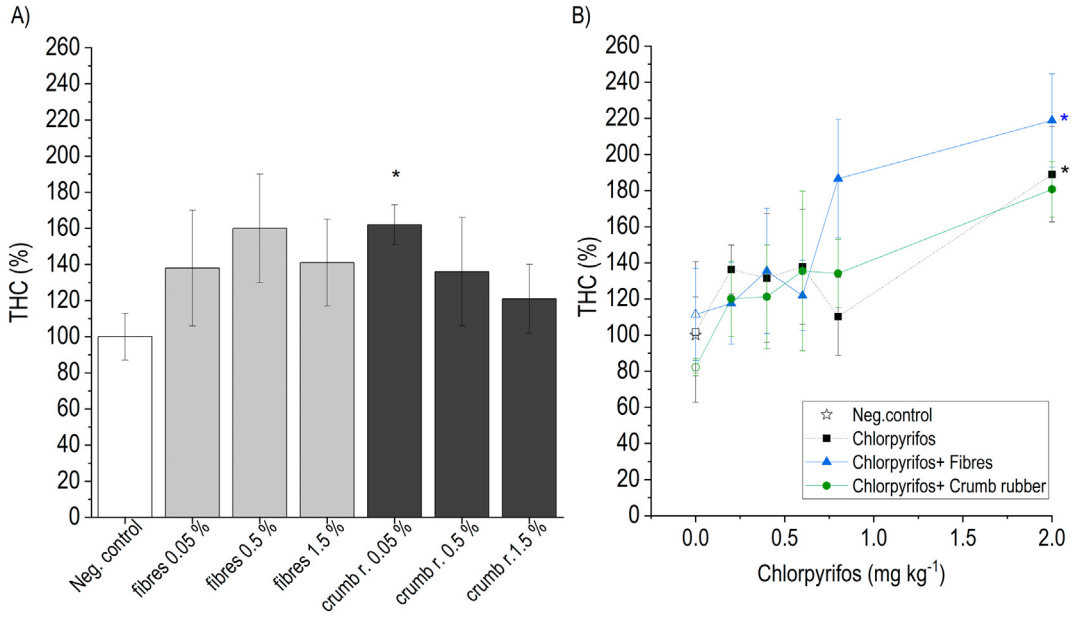

Fig. 1. Total haemocyte count (THC) in the woodlice (*Porcellio scaber*) following 3 weeks exposure to the microplastics alone (A), and to chlorpyrifos alone and plus microplastics, as polyester fibres and crumb rubber (B). Controls (shown at 0.0 chlorpyrifos): negative control (no solvent, no microplastics; empty black star); solvent control (no microplastics; empty black square), plus solvent and fibres (no chlorpyrifos; empty blue triangle); and plus solvent and crumb rubber (no chlorpyrifos; empty green circle). Data are means ±SE (n = 8–12). *, $p < 0.05$, versus relevant control.

parameters were affected in the solvent control (acetone) compared to the negative controls.

3.1.3. Chlorpyrifos and microplastics co-exposure

The woodlice exposed to chlorpyrifos + crumb rubber showed similar concentration-related trends of THC increase as for the woodlice exposed to chlorpyrifos alone (Fig. 1B), but different DHC profiles. This was seen as more pronounced increases in granulocytes and decreases in semigranulocytes at some chlorpyrifos concentrations in the chlorpyrifos + crumb rubber mixtures. Statistically significant differences between chlorpyrifos alone and chlorpyrifos + crumb rubber were seen at 0.4 mg chlorpyrifos kg^{-1} dry soil for granulocytes, and 0.2 mg and 0.6 mg chlorpyrifos kg^{-1} dry soil for semigranulocytes (Fig. 4A–B). Joint exposure to chlorpyrifos + polyester fibres resulted in higher THC (at 0.8 and 2.0 mg kg^{-1} chlorpyrifos) than for chlorpyrifos alone, but these differences did not reach statistical significance due to the high variability of the data (Fig. 1B). The DHC profiles were similar for chlorpyrifos alone and chlorpyrifos + fibres, with statistically significant differences between these only at 0.8 mg chlorpyrifos kg^{-1} dry soil for hyalinocytes (Fig. 4C). The viability of the haemocytes was affected only at the highest concentration of chlorpyrifos, but not when polyester fibres or crumb rubber were included (Fig. 4D).

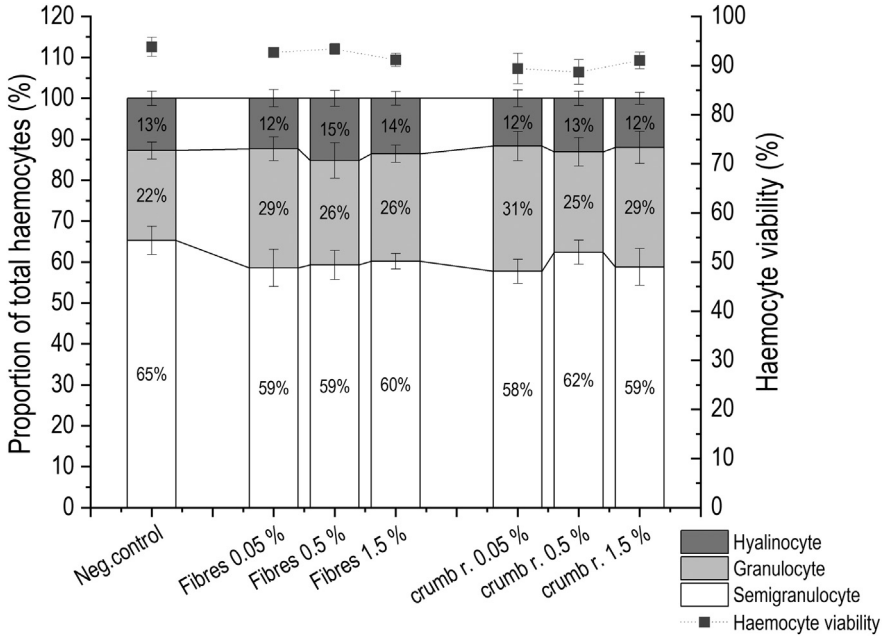

Fig. 2. Proportions of different cell types within the total haemocytes populations, as hyalinocytes, granulocytes and semigranulocytes, and viability of haemocytes in the woodlice (*Porcellio scaber*) following 3 weeks exposure to the microplastics alone, as polyester fibres and crumb rubber. Data are means ±SE (n = 8–12).

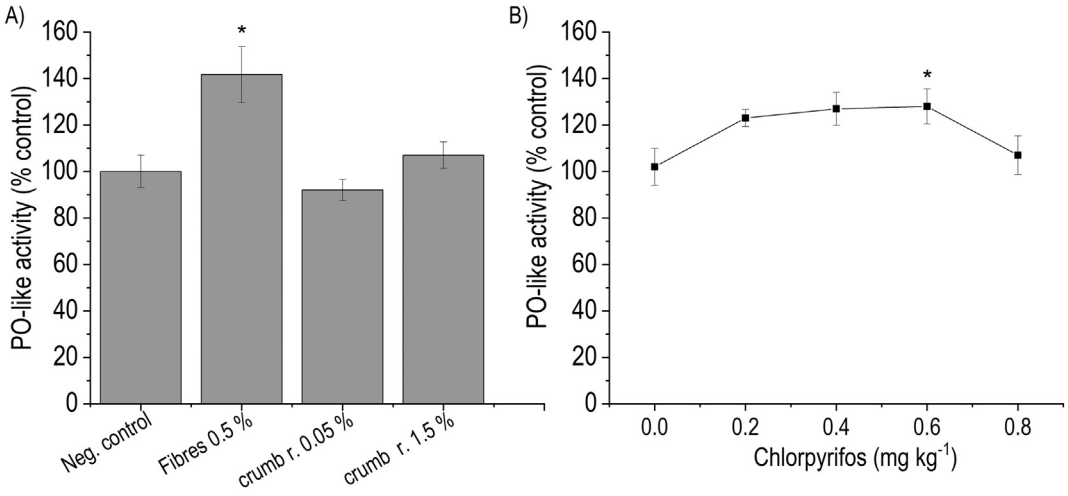

Fig. 3. Phenoloxidase (PO)-like activity in the woodlice (*Porcellio scaber*) following 3 weeks exposure to the microplastics alone (A), and to chlorpyrifos alone (B). Data are means ±SE (n = 10–13). *, $p < 0.05$, versus relevant control.

3.2. Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity in the haemolymph was significantly decreased by about 60% (±4.8% SE) upon exposure to 0.8 mg chlorpyrifos kg^{-1}. Following the chlorpyrifos + polyester fibres co-exposure, inhibition of the AChE activity showed a similar trend, but was less pronounced, at 43% (±8.5% SE) at the same concentration of chlorpyrifos. However, this difference between the two treatments at 0.8 mg chlorpyrifos kg^{-1} was not statistically significant (Fig. 5).

4. Discussion

This study investigated the immunomodulatory changes in *P. scaber* following 3-week exposure to chlorpyrifos alone and in mixtures with microplastics, as polyester fibres and crumb rubber. This resulted in significant changes following chlorpyrifos alone, but only small changes in the immune parameters for microplastics alone. Mixtures of chlorpyrifos and microplastics induced different changes for some of the immune parameters compared to the individual exposures. While a lower reduction in haemocyte viability and AChE activity at the highest chlorpyrifos

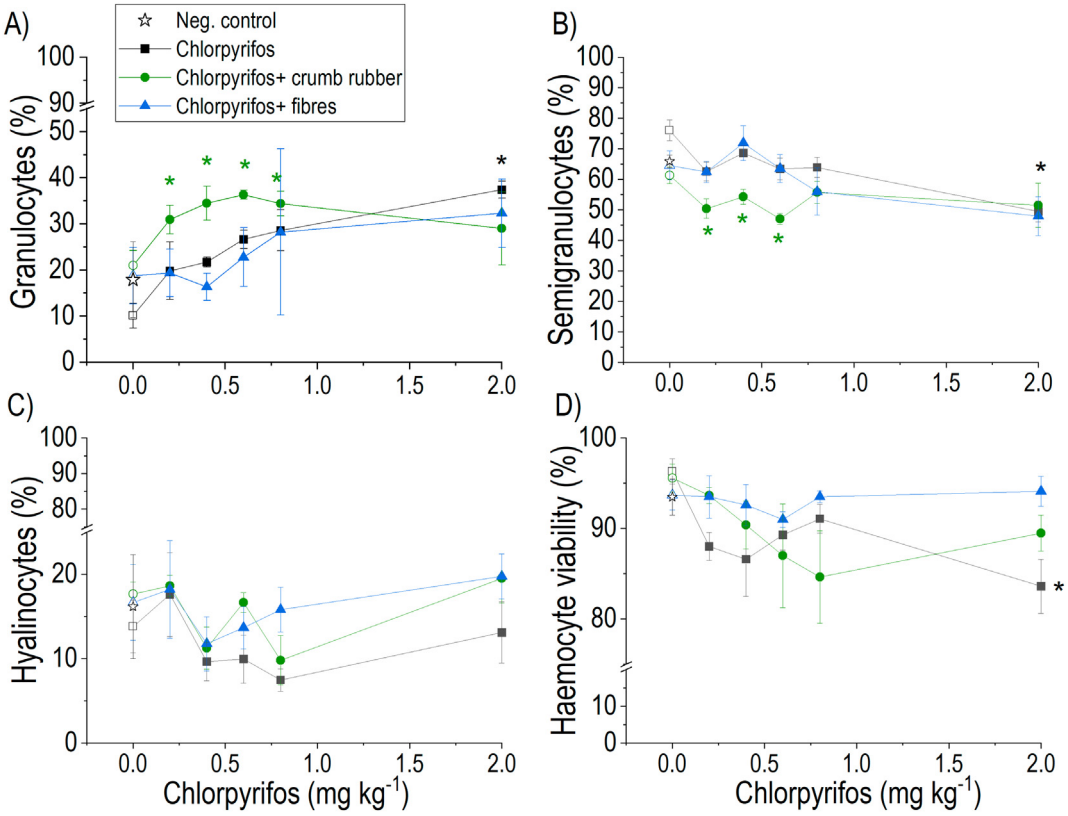

Fig. 4. Differential haemocyte counts for the granulocytes (A), semigranulocytes (B) and hyalinocytes (C), and viability of haemocytes (D) in the woodlice (*Porcellio scaber*) following 3 weeks exposure to chlorpyrifos alone and plus microplastics, as polyester fibres and crumb rubber. For controls, see legend to Fig. 1. Data are means ±SE (n = 8–12). *, $p < 0.05$, versus relevant control. Statistically significant differences between treatments at individual chlorpyrifos concentrations (not shown) are described in the main text.

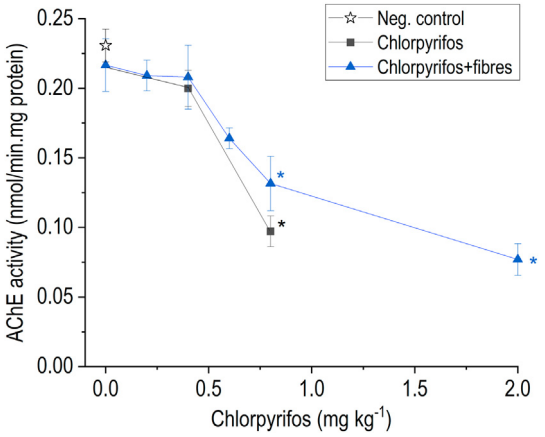

Fig. 5. Acetylcholinesterase (AChE) activity in the haemolymph in the woodlice (*Porcellio scaber*) following 3 weeks exposure to chlorpyrifos alone and plus microplastics, as polyester fibres. Data are means ±SE (n = 8–14). *, $p < 0.05$, versus relevant control.

concentration implied decreased chlorpyrifos bioavailability in the presence of the microplastics, enhanced changes in haemocyte counts were observed for the chlorpyrifos and fibres mixtures (Table 1).

Exposure to chlorpyrifos led to obvious changes in the immune parameters of *P. scaber*. A significant increase in THC was observed at the highest concentration of chlorpyrifos (2.0 mg kg^{-1}). Some studies have suggested that THC increases represent a feedback loop to replace non-viable haemocytes (Fatima et al., 2014). However, the haemocyte count is a frequently influenced parameter due to the altered physiological state of an organism, for example during moulting or due to environmental challenges, such as starvation (Matozzo et al., 2011), temperature stress (Hernroth et al., 2012) and even the composition of the diet (Pascual et al., 2004). A similar THC increase has been documented upon chlorpyrifos injection in the moth *Spodoptera litura* (Irfan et al., 2019), as well as for other invertebrates following exposure to pesticides (George and Ambrose, 2004). The significant decrease in haemocyte viability in *P. scaber* at the highest concentration of chlorpyrifos, however, can be considered as a direct adverse effect of the insecticide on haemocytes, which is a commonly observed phenomenon in crustaceans (Jose et al., 2011). One of the possible modes of action of chlorpyrifos is the induction of oxidative stress and subsequent cell apoptosis, which has been demonstrated both *in vitro* with cell lines (Ki et al., 2013) and *in vivo* for mussel haemocytes (Patetsini et al., 2013).

Table 1
Summary of woodlice (*Porcellio scaber*) responses upon exposure to chlorpyrifos alone and in combination with the microplastics, as polyester fibres and crumb rubber. Overall trends are shown, although some did not reach statistical significance under all exposure concentrations (see main text).

Parameter	Chlorpyrifos		
	Alone	Plus fibres	Plus crumb rubber
Total haemocyte count	↑	↑↑	↑
Granulocytes	↑	↑	↑↑
Semigranulocytes	↓	↓	↓↓
Hyalinocytes	=	=	=
Viability of haemocytes	↓	=	=
Acetylcholinesterase activity	↓↓	↓	n.d.

The most significant changes in *P. scaber* exposed to chlorpyrifos were seen for the differential haemocyte counts, with an apparent dose-dependent increase in granulocytes and decrease in semigranulocytes. Such opposite trends in granulocyte and semigranulocyte counts are frequently observed, as granulocytes and semigranulocytes appear to represent two consecutive phases of haemocyte maturation (Rebelo et al., 2013). For this chlorpyrifos exposure, a shift in metabolic processes might lead to the formation of granules, and thus to an increase in the granulocytes (George and Ambrose, 2004). A similar increase in granulocytes was reported for silkworm larvae (*Philosamia ricini*) when they were fed on chlorpyrifos-contaminated food for 96 h (i.e., sprayed with 1.5 and 2.0 mg L^{-1} chlorpyrifos) (Kankana Kalita and Devi, 2016). Along with other cell types, granulocytes secrete components of the proPO-activating system (Qin et al., 2019). Here, we observed an increase in PO-like activity up to 0.8 mg chlorpyrifos kg^{-1}, which follows the increased THC and granulocytes. A positive correlation between THC (and granulocytes) and PO-like activity was previously reported for bacterial and viral infections in crustaceans (Dash et al., 2015; Dolar et al., 2020). Similarly, Arambourou and Stoks (2015) reported increased body PO-like activity in *Ischnura elegans* damselfly larvae after acute chlorpyrifos exposure. In contrast, some other studies have reported decreases in PO-like haemolymph activity after short-term exposure to chlorpyrifos and glyphosate, with an additional reduction in THC (Banaee et al., 2019; Kankana Kalita and Devi, 2016). PO is involved in many processes, which include cuticle sclerotisation, wound healing, defence against parasites via melanisation, and production of components with antimicrobial, cytotoxic, opsonic, and encapsulation-promoting activities (Cerenius and Söderhäll, 2004). Due to the complex role of this enzyme, it remains difficult to explain the biological consequences of increased PO activity in an organism, although it might be an indication of immunostimulation.

The bioavailability of chlorpyrifos for *P. scaber* was indirectly assessed by measuring the AChE activity in the haemolymph, which is a known specific target for organophosphates like chlorpyrifos (Banaee et al., 2019; Muangphra et al., 2016; Rodríguez-Seijo et al., 2019). Here, this AChE activity was significantly and dose-relatedly decreased by chlorpyrifos. Inhibition of haemolymph AChE activity is an indirect indication that the haemocytes were exposed to chlorpyrifos and therefore their viability might be directly compromised, as discussed above. Currently, the physiological role of the haemolymph AChE activity is still unclear (Glavan et al., 2018; Moreira et al., 2001). However, a link to immune processes is possible, as it has been shown that acetylcholine, which is the substrate of AChE, is involved in immune response (Rajendran et al., 2015; Shi et al., 2014). Wu et al. (2019) measured cholinesterase activity in granulocytes of two Asian horseshoe crab species, and they proposed that this activity is a haemocyte immune parameter. In addition, Wang et al. (2019b) showed up-regulation of genes that encode three AChE subunits in haemocytes in the shrimp *Litopenaeus vannamei* when infected with white spot syndrome virus. However, the association between AChE inhibition and other immune-related changes in *P. scaber* remains to be investigated.

The immune parameters in *P. scaber* exposed to both types of microplastics only showed slight changes compared to the controls. PO-like activity was increased in response to polyester fibre exposure, but not to crumb rubber. Similarly, Liu et al. (2019) reported increases in PO-like activity in the crab *Eriocheir sinensis* when exposed to polystyrene microspheres for 7 days, while a decrease was shown after the longer exposures for 14 days and 21 days. PO-like activity and haemocyte viability were also reduced in the marine worm *Hediste diversicolor* when exposed to a mixture of polyethylene and polypropylene microplastics for 10 days (Revel et al., 2018). We observed a significant increase in THC in *P. scaber* only at 0.05% (w/w)

crumb rubber, but not for fibres or the other exposures. THC did not follow a dose-related trend here, which means that even low concentrations of microplastics (e.g., 0.05% w/w) are sufficient to induce immune-related changes. Microplastics might provoke changes in immune parameters through a release of additives (Capolupo et al., 2020) that can cross the gut barrier and directly act on immune cells, like chlorpyrifos, and/or they might have effects through physical interactions of the microplastics with the gut. As shown by the chemical analysis, crumb rubber is a very complex material that is also rich in organic and inorganic additives that might be released into the soil and result in changes to immune processes. However, if this was the case, a dose-related change in these immune parameters should have been observed, as the concentrations of plastic-associated chemicals released into the soil would differ significantly between the lowest (0.05%) and highest (1.5%) microplastic concentrations used here. It is therefore more likely that the observed changes in immune parameters upon exposure to the microplastics were predominately due to alterations to the microenvironment of the gut; e.g., due to changes in gut chemistry and microbiome (Van der Zande et al., 2020), responses to the changed diet (Pascual et al., 2004), or an immune response to damage of the gut cuticle (Davis and Engström, 2012; Lei et al., 2018).

When woodlice were exposed to a mixture of chlorpyrifos and microplastics, the changes in immune parameters differed from the individual exposures (Table 1). The THC increase was greater for combined chlorpyrifos + polyester fibres than for chlorpyrifos or the fibres alone. On the other hand, the reduced inhibition of haemolymph AChE activity and the lower effects on haemocyte viability following chlorpyrifos + fibres compared to chlorpyrifos alone indicate that the fibres reduced the bioavailability of chlorpyrifos. The same conclusion can be drawn for the chlorpyrifos + crumb rubber co-exposure, where there were no effects on the viability of haemocytes, which was evident for chlorpyrifos alone. Unfortunately, AChE activity could not be measured in the crumb rubber co-exposure, which might have provided further evidence of reduced bioavailability. Interestingly, the chlorpyrifos + crumb rubber co-exposure resulted in significantly different DHC profiles compared to chlorpyrifos alone, with significantly greater increases in granulocytes and decreases in semigranulocytes at some of the chlorpyrifos concentrations. Both types of microplastics thus decreased the bioavailability of chlorpyrifos, while some of the parameters examined showed joint additive actions. Although polyester fibres and crumb rubber differ in their physicochemical properties, which might affect their pollutant adsorption potential (Bakir et al., 2014; Tourinho et al., 2019), we did not detect any significant differences for the effects of these two microplastics on the chlorpyrifos toxicity.

Studies on microplastic–chemical mixtures have reported a variety of outcomes in comparison to individual pollutant exposure. Compared to individual pollutant exposures, the effects of mixtures can be enhanced (Bellas and Gil, 2020; Felten et al., 2020; Zhang et al., 2019; Zhou et al., 2020; Zocchi and Sommaruga, 2019), reduced (Rehse et al., 2018; Wang et al., 2019a) or the same (Beiras et al., 2019; Beiras and Tato, 2019; Horton et al., 2018; Magara et al., 2019). As can be seen from these studies, the results of co-exposure are not dependent on the polymer type, pollutant (e.g., metal vs. organic pollutant) or exposure route (aquatic vs. terrestrial). Also, contrasting results have been reported for chlorpyrifos mixtures with microplastics. Simultaneous exposure for 48 h to a mixture of chlorpyrifos + polyethylene microplastics and chlorpyrifos-loaded microplastics (i.e., chlorpyrifos pre-incubated with the microplastics) had greater effects on survival, egg production and feeding activity of the copepod *Acartia tonsa* than chlorpyrifos alone (Bellas and Gil, 2020). However, the opposite was reported for marine algae, which were significantly less affected when exposed to chlorpyrifos-loaded microplastics than to chlorpyrifos alone; in this latter case, reduced bioavailability was suggested to be the main reason (Garrido et al., 2019). In the present study, microplastics co-exposure appears to have reduced chlorpyrifos bioavailability, but also increased the responses provoked by chlorpyrifos.

The choice of test parameters might also partly explain the variety of responses observed upon individual pollutant exposure in comparison to their mixtures with microplastics. For example, inhibition of AChE activity is a very specific marker of organophosphate exposure, and a reduction in its inhibition is direct proof of reduced chlorpyrifos bioavailability. Also, haemocyte viability is a very sensitive marker here, as chlorpyrifos acts directly upon haemocytes. On the other hand, haemocyte counts (as THC or DHC) represent more general, and less specific, markers as these can reflect many different physiological alterations (Hernroth et al., 2012; Pascual et al., 2004; Sequeira et al., 1995), including for the joint actions of chlorpyrifos and microplastics, as shown by the present study. It appears that the final conclusions on how microplastics might modulate the changes in these immune parameters provoked by chlorpyrifos depends on the specificity and sensitivity of the parameter tested, which was also previously suggested by Cedergreen and Streibig (2005).

In conclusion, we have provided evidence here that the immune processes of *P. scaber* are only slightly altered after 3 weeks of exposure to environmentally relevant concentrations of polyester fibres and crumb rubber. These microplastics modulate the effects of chlorpyrifos exposure on the immune processes examined in *P. scaber*, although the results here appear not to be internally consistent at times: while some of the parameters indicated that microplastics reduce the bioavailability of chlorpyrifos, the responses of some other parameters were enhanced in the presence of these microplastics. This study thus indicates the need for further research into the short-term and long-term effects of microplastics and chemical mixtures on the immune processes of soil invertebrates.

CRediT authorship contribution statement

Andraž Dolar: Methodology, Formal analysis, Writing – original draft, Visualization. **Salla Selonen:** Conceptualization, Writing – review & editing. **Cornelis A.M. van Gestel:** Conceptualization, Writing – review & editing. **Valentina Perc:** Methodology, Validation. **Damjana Drobne:** Writing – review & editing, Funding acquisition. **Anita Jemec Kokalj:** Conceptualization, Methodology, Writing – review & editing, Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.144900.

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