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Comparative toxicity of imidacloprid and its transformation product 6-chloronicotinic acid to non-target aquatic organisms: Microalgae *Desmodesmus subspicatus* and amphipod *Gammarus fossarum*

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ABSTRACT

Neonicotinoids are widely applied pesticides due to their higher affinity for insect nicotinic acetylcholine receptors. These compounds are extensively applied to control pest insects in different agricultural crops; however they can also affect non-target invertebrates. Little is known about the toxicity effects of their transformation products on aquatic non-target organisms. Oxidative stress responses and behavioural changes in the crustacean amphipod *Gammarus fossarum* were investigated as well as the growth rate in freshwater algae *Desmodesmus subspicatus* after 96 h exposure to imidacloprid, its commercial formulation Confidor 200SL and its transformation product 6-chloronicotinic acid. Algal growth has shown significant sensitivity to Confidor 200SL and 6-chloronicotinic acid when compared to imidacloprid. In the case of amphipods, low doses of imidacloprid (102.2 $\mu g\,L^{-1}$) were sufficient to induce lipid peroxidation, while Confidor 200SL induced increased catalase activity (511.3 $\mu g\,L^{-1}$) and lipid peroxidation (255.6 $\mu g\,L^{-1}$). 6-Chloronicotinic acid altered significantly only antioxidant mechanisms (catalase activity) without changing lipid peroxidation levels. These different biochemical responses are helpful to understand the mechanism of imidacloprid and 6-chloronicotinic acid-induced oxidative stress. In addition, obtained data demonstrate potential harmful effects of neonicotinoid-based pesticides on non-target aquatic organisms.

1. Introduction

Pesticide producers are continuously replacing older generation pesticides with an array of newly developed pesticides. These products are characterised by selective action on target organisms in order to reduce their possible unwanted effects. One of these representatives is imidacloprid [IMI; 1-[(6-chloro-3 pyridynil) methyl]-*N*-nitro-2-imidazolidinimine] a nicotine-derived compound (neonicotinoid) with a large potential distribution due to its agonistic action on insect nicotinic acetylcholine receptors and its selective toxicity to insects over vertebrates [1].

Legislations related to the placement of pesticides on market focus only on parent compounds rather than formulations or transformation products. It is important to notice that the commercial formulations of IMI (such as Confidor 200SL, Admire, Merit and Gaucho) [2] are the ones applied in the environment with relevant soil (50–320 $g\,ha^{-1}$) and foliar concentrations (73–150 $mg\,L^{-1}$). These frequently used commercial mixtures of IMI contain co-formulants and other solvents that could modify its toxicity and bioavailability. Recent evaluation of the data relative to different formulations noted high levels of IMI in leaves and in blossoms of treated plants, and increases in residue levels over time [3]. Data indicated that the use of these IMI-formulations on annual basis may be at the end cumulative. Due to recent findings certain commercial products within the class of neonicotinoids (containing active ingredient IMI) were placed under re-evaluation and need further studies [3]. In addition, no particular control or monitoring over the presence of IMI transformation products is performed. The primary IMI breakdown products in soil are: imidacloprid urea, 6-hydroxynicotinic acid and 6-chloronicotinic acid (6CNA) [4]. 6CNA is one of the final transformation products of IMI and due to its high water solubility (2 $g\,L^{-1}$) it may leach from soil into the aquatic environment. Furthermore, IMI persistence in soil is affected by various factors such as temperature, organic matter, cropping and its solubility of 0.51 $g\,L^{-1}$. It can contaminate surface and ground water by runoff or leach from agricultural areas and lead to pulse-pesticide or localised contaminations [5,6].

Detected aquatic concentration indicate measured levels of IMI going from 14 $\mu g\,L^{-1}$ up to 0.3 $mg\,L^{-1}$ for surface waters [7,8], while the estimated concentrations for accidental spills reach high values going from 1.8 up to 7.3 $mg\,L^{-1}$ [9]. Different studies are

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referring to the persistence of IMI in the aquatic environment and its toxicity to different non-target aquatic invertebrates. These investigations span from single species toxicity tests in laboratory [10–13] to complete indoor/outdoor stream mesocosms studies under constant exposure [14] and short-pulse exposure conditions [15,16]. The authors observed generally modifications in survival, behaviour and population growth rate; while some of them evaluated biochemical alterations at molecular level and compared the toxic effects of pure compound IMI and its formulated version [11,12]. Although IMI is continuously studied, fewer investigations have been conducted on its transformation products. There is a single study relative to the toxicity of 6CNA on aquatic invertebrates performed on midge *Chironomus tentans* with LC_{50} (96 h) higher than 1 mg L^{-1} [17] which warrants expanding our knowledge in this field. In addition, major part of studies on IMI's transformation products are performed mainly on common honey bee *Apis mellifera* [18,19]. To this end, it is necessary to investigate their effects on freshwater biota, especially on non-target aquatic organisms.

Among the potential non-target organisms, unicellular green algae are commonly used for toxicity tests [20]. Any adverse impact on algae is likely to affect organisms at higher trophic levels and may have important consequences for the health status of the whole aquatic ecosystem [21]. In addition, aquatic non-target crustaceans of the genus *Gammarus* are frequently used in ecotoxicological studies [22–24]. They play a major role in leaf litter breakdown and are important for material transfer in the food web [25,26]. In this study *Gammarus fossarum* Koch, 1835 was used as a model organism considering its sensitiveness to several environmental pollutants. Furthermore, crustaceans as well as insects belong to arthropods and due to this crustacean-insect relationship they could present a potentially suitable non-target group for neonicotinoid testing [27].

Different classes of pesticides may be related to enhanced production of reactive oxygen species (ROS) which could contribute to the toxicity of these compounds [28]. Basic cellular metabolism in aerobic organisms involves the production of oxygen free radicals and non-radical ROS [29]. The imbalance between the generation and the neutralisation of ROS by antioxidant mechanisms within an organism generates the oxidative stress [30,31]. In order to have a better understanding of the toxic action of these compounds the involvement of induced ROS production was investigated by measurement of oxidative stress biomarkers such as antioxidant enzyme catalase (CAT), detoxifying enzyme glutathione-*S*-transferase (GST) and the levels of lipid peroxidation (LP) damage [28,32,33].

The aim of the study was to provide additional information on the possible toxic effects of IMI, its commercial formulation Confidor 200SL and its transformation product 6CNA on non-target aquatic organisms. For this reason physiological/biochemical biomarkers, mortality and behavioural alterations on the amphipods (acute toxicity) were evaluated as well as the growth rate of microalgae (chronic toxicity). Antioxidant defence system alterations and lipid peroxidative damage to cell membrane were studied because of their potential to serve as useful biochemical biomarkers that could be applied in environmental monitoring programmes.

2. Materials and methods

2.1. Chemicals

Imidacloprid (IMI) was purchased as the Pestanal® grade chemical (99.8% purity; Sigma–Aldrich, UK), and as a commercial formulation known as Confidor 200SL (200 g L^{-1} of active ingredient (a. i.) IMI, Bayer Crop Science Slovenia, Ljubljana, Slovenia) and 6CNA was obtained as pure compound (97%) from Fluka (Sigma–Aldrich, Switzerland). The following chemicals were all obtained from Sigma Aldrich: acetonitrile CHROMASOLV® for HPLC grade, dibasic and monobasic potassium and sodium phosphate, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide, 1-chloro-2,4-dinitrobenzene (CDNB), L-glutathione (reduced form), hydrogen peroxide (30%), bovine serum albumin (BSA), Bradford reagent, trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylhydroxytoluene (BTH), 96% ethanol, 1-butanol, hydrochloric acid (37%), dimethyl sulfoxide (DMSO). Potassium hydrogen phthalate was purchased from Alfa Aesar GmbH (Karlsruhe, Germany). Acetic acid glacial 100% p.a. was provided from Merck (Darmstadt, Germany). All chemicals were of the highest commercially available grade.

2.2. Stability study of tested chemicals during experimental trial

To ensure reliable toxicity data, the stability of IMI and 6CNA was checked. The exposure was confirmed measuring the concentrations of the specific chemicals at the beginning and end of the experimental trial, under the same condition as toxicity tests (described in Sections 2.4.1 and 2.4.2). IMI and 6CNA samples were taken in duplicates and all determinations were performed in four experiments.

IMI and 6CNA were extracted with the use of the miVac centrifugal concentrator Modular Series (Genevac). The water was evaporated (under maintained vacuum conditions at 70 °C for approximately 200 min). The dried leftover was re-dissolved in 500 μL of double deionised H_2O. Previous procedure was applied for samples with lower concentration of chemicals (used for tests with amphipods), while samples with higher concentration of chemicals (used for tests with algae) were analysed immediately without pre-concentration treatment. All prepared samples were stored in glass vials under dark at 4 °C until subjected to HPLC-DAD (UV–Vis). For quantification purposes, calibration curves were prepared. The r^2 value of the regression line for IMI was 0.9999 and for 6CNA was 0.9996.

2.2.1. HPLC-DAD analysis

Aqueous solutions of IMI and 6CNA were analysed by HPLC-DAD (UV–Vis) consisting of an Agilent 1100 Series chromatograph, coupled with a DAD detector operating in the UV–Vis range The separation was achieved using a Zorbax C8 column (250 mm × 4.6 mm) filled with a stationary phase Chromasil 100 (pore size 5 μm, end-capped) produced by BIA Separations d.o.o., Slovenia. The column thermostat was maintained at 25 °C and injection volume was 75 μL.

According to Žabar et al. methods for IMI [34] and 6CNA [35] detection were applied. For IMI detection the eluents consisted of 30% acetonitrile (A) and 70% acetic acid 0.75% v/v (B); isocratic elution; flow rate was 1 mL min^{-1}. The wavelength was 270 nm and the retention time was 8.9 min. While for the 6CNA detection the eluents consisted of acetonitrile (A) and acetic acid 1.5% v/v (B); flow rate was 1 mL min^{-1}. The gradient elution was as follows: 0–16 min 15% A; 16–20 min 70% A. The wavelength was 242 nm and the retention time for 6CNA was 13.2 min.

2.3. Test organisms

Desmodesmus subspicatus (Chodat) Hegewald *et* Schmidt (formerly *Scenesdesmus subspicatus*, CCAP 276/22) was kindly provided by the Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany. Microalgae were grown in a medium recommended by standard guidelines for freshwater algal growth inhibition test [36].

G. fossarum were collected in April–July 2011 using a net (by the kick sampling method) from the stream Vogršček (Slovenia). The

sampling site is in the lower Vipava Valley in Goriška region of Slovenia (45°90′ N; 13°70′ E). It is a small waterbed free of industrial, agricultural contamination or human activities and it can be considered unpolluted. This site has a good water quality according to ARSO data record (Agencija Republike Slovenije za okolje – Slovenian Agency for Environment; http://www.arso.gov.si/vode/poro%C4%8Dila%20in%20publikacije/povrsinske_letna.html) and high densities of gammarids are found.

All water samples from the sampling site and during the experimental trial were monitored for temperature, pH, conductivity, oxygen concentration and saturation with a multi-meter WTW 350i (with microelectrode replacements for small volumes). In addition, total organic carbon and total nitrogen (TOC and TN) were measured in water samples from the sampling site with a TOC Analitik Jena multi N/C 3100, calibrated with potassium hydrogen phthalate. Before being processed for the TOC and TN analyses, samples were acidified to pH 2–3 with hydrochloric acid.

Gammarids were kept during an acclimatisation period of at least 14 days in a 20-L glass aquarium supplied with thoroughly aerated original stream water. An 8/16 h light/dark natural photoperiod was maintained with the temperature at 12 ± 2 °C in a temperature and humidity controlled chamber and regular water renewal every two days. Animals were fed *ad libitum* twice a week using a pinch of dry food (e.g. TetraMina® flakes) or raw peas.

2.4. Experimental procedures

2.4.1. Algae toxicity test

Chronic toxicity of pesticides was conducted in 96 microwell plate. The algal inoculum was taken from an exponentially growing pre-culture and added into 25 mL of growth media in order to obtain an initial cell density of 10^4 cells mL^{-1}. Final volume of each well was 200 μL. Serial dilutions of tested pesticides were made in culture medium. Six replicates of controls (untreated) and three replicates of each test concentration were applied. All the plates with cover, control and treatments, were incubated for four days (96 h) at a temperature of 23 ± 1 °C and light intensity of 1100 lux. Algal growth was detected fluorometrically in intervals of 24 h over a period of 96 h in order to achieve a virtual kinetic data distribution. Analyses of chlorophyll fluorescence were performed by a Tecan Infinite® 200 PRO (Männedorf, Switzerland). Measurements were conducted using fluorescence excitation of 440 nm and by an emission of 680 nm. Before reading, tested microplates were shaken for 30 s at 100 rpm. Average of specific growth rates were calculated and subsequently used for calculation of percentage inhibition in comparison to control [37]. IC_{50} at 96 h (inhibition concentration that cause 50% inhibition of algal growth) was estimated for tested compounds using linear regression analysis [38].

Solution of 1 M IMI and 6CNA was prepared in DMSO. Afterwards, a 10 mM (2.55 g L^{-1} for IMI and 1.57 g L^{-1} for 6CNA) stock solution was prepared by the addition of IMI and 6CNA (1 M) or Confidor 200SL to standard algal medium, with constant mixing until complete dissolving. The test solutions were prepared by adding an appropriate volume of the stock solution in the algal medium to achieve final concentration. The following range of equal molar concentrations was prepared for all tested compounds: 7.6; 25.6; 51.1; 127.8 and 255.6 mg L^{-1} for IMI and 4.7; 15.7; 31.5; 78.7 and 157.5 mg L^{-1} for 6CNA. For Confidor 200SL the final concentrations were corresponding to 0.003–0.12% (v/v) which contained 7.6–255.6 mg L^{-1} of a. i. IMI. Lower concentrations of IMI than those monitored in this experimental trial were already tested on *D. subspicatus* and showed no effect on algal growth up to 10 mg L^{-1} and due to this fact were excluded [39]. The toxicity of co-formulants incorporated in Confidor SL 200 (as negative control – a solution consisting of 38.4% of dimethylsulfoxide, 37.5% of 1-methyl-2-pyrrolidone and 24.1% of double deionised water in place of IMI) was tested. In addition, as an internal quality control, the bioassays were also performed on the reference chemical potassium dichromate (positive control – $K_2Cr_2O_7$; 0.1–30 mg L^{-1}) [36].

2.4.2. Amphipods toxicity test

Gammarids were exposed for 24 h (acute toxicity) to equal molar concentrations of IMI, Confidor 200SL and 6CNA for better comparison. A short exposure period sufficient to promote early alterations (24 h) was used also to mimic runoff-related pulse exposures to pesticides [40,41]. The peak pesticide concentrations usually persist for about 24 h. Furthermore, *G. fossarum* from running water is greatly affected by short-term higher concentration of IMI [12]. Sub-lethal exposure concentrations were based on previously determined acute LC_{50} (48 h) and EC_{50} (24 h) values for IMI of 0.8 and 0.07 mg L^{-1} [12].

Solution of 1 M IMI and 6CNA was prepared in DMSO. Afterwards, a 10 mM (2.55 g L^{-1} for IMI and 1.57 g L^{-1} for 6CNA) stock solution was prepared by the addition of IMI and 6CNA (1 M) or Confidor 200SL to distilled water, with constant mixing until complete dissolving. The test solutions were prepared by adding an appropriate volume of the stock solution in the original stream water to achieve final concentration. The following range of concentrations was prepared for all tested compounds: 6.3; 12.7; 25.5; 51.1; 102.2; 153.3; 204.5; 255.6 and 511.3 μg L^{-1} for IMI and 3.9; 7.8; 15.7; 31.4; 62.8; 94.6; 126. 2; 157.7 and 315.5 μg L^{-1} for 6CNA. For Confidor 200SL the final concentrations were corresponding to 0.000003–0.0002% (v/v) which contained 6.3–511.3 μg L^{-1} of a. i. IMI. The tested concentrations of the negative control (co-formulants only) in case of amphipods were equivalent to concentrations of Confidor SL 200 used in the tests.

The experimental trial was performed using adult male specimens. After sex determination, total body length [42] and total wet weight was measured (animal were dried between two sheets of filter paper before being weighted). Fifty individuals per exposure concentration were used for every tested compound. Plastic Petri dishes (100 mm × 20 mm; 20 mL volume) covered in order to reduce water evaporation were used for exposure experiments. The bioassays were conducted in darkness, in a temperature and humidity-controlled chamber (12 ± 2 °C; 60% humidity). After a 24 h exposure period, immobility or moulting and mortality were observed. Live/dead organisms were determined by gently poking and observing movement of appendages. Organisms were counted as dead if none of the appendices were moving after poking for three times. Inactive/paralysed animals were identified when only respiration movements were left [43]. Moulted animals were counted based on the presence of the entire old *exuvia* in the exposure vessel (moulted amphipods were not used for biochemical parameters analyses). For each biochemical assay 10 randomly selected gammarids per concentration (from fifty individuals) were processed using whole-body homogenates due to their small body size.

2.5. Biochemical biomarker assays

Prior to individual homogenisation, excess chemicals present on the animals' surface were rinsed several times according to Jemec [11]. Whole-body specimens were homogenised in 500 μL of ice-cold phosphate buffer (pH 7.0) for 3 min using a glass–glass Elvehjem–Potter homogeniser. The homogenate was sonicated on ice (5–10 s) and centrifuged for 15 min at 3000 rpm and 4 °C. Freshly prepared clear supernatant was collected and kept on ice to be used for enzyme activities measurements.

Activity of acethylcholinesterase (AChE) was determined using DTNB and acetylthiocoline iodide as substrate according to Ellman et al. [44]. The reaction was followed on a Perkin Elmer Lambda 35

UV/VIS spectrophotometer at 412 nm for 8 min. AChE activity is expressed as μmol of substrate hydrolysed per minute per mg protein (ε = 13 600 L cm^{-1} mol^{-1} for DTNB).

CAT activity was determined according to the method of Jamnik and Raspor [45] by measuring the decrease in absorbance on spectrophotometer at 240 nm for 2 min due to the decomposition of H_2O_2 (ε = 40 L cm^{-1} mol^{-1}). The specific activity of CAT was expressed as μmol of H_2O_2 reduced per minute per mg protein.

GST activity was determined according to the protocol of Habig et al. [46]. The method is based on determination of the conjugated product dinitrophenyl-thioether at 340 nm produced from CDNB used as an artificial substrate and reduced glutathione. Values expressed as nmol of reduced glutathione and CDNB conjugate formed per min per mg protein (ε = 9600 L cm^{-1} mol^{-1} for CDNB).

All the data relative to the enzymatic activity are normalised to the total protein content based on the method of Bradford [47].

LP was estimated *in vitro* after the formation of malondialdehyde (MDA), a major by-product of lipid peroxidation that reacts with thiobarbituric acid [48], with slight modifications. Whole-body gammarids were rinsed, as described previously and homogenised individually in TCA–TBA–BTH reagent [15% (w/v) TCA, 0.37% (w/v) TBA, 1 M HCl, and 0.01% BTH]. Samples were incubated at 90 °C for 30 min, then chilled at room temperature, added 1.2 mL of 1-butanol and centrifuged at 12,000 rpm for 10 min. Absorbance of the supernatant was measured at 535 and 600 nm, the final one to correct the non-specific turbidity. Before the heating step, absorbance was measured at 280 nm for total protein concentration. These absorbance values of protein content were used to properly normalise absorbance values obtained for LP.

2.6. Statistical analyses

All statistical tests were performed using STATISTICA 7 StatSoft software. Results from each exposure trial are presented in graphs as mean ± standard error (SE). Statistical comparisons were conducted between control and exposure data using the Student's *t*-test or the Mann–Whitney rank sum test after the software direct choice of parametric or nonparametric data, respectively. In addition, multiple comparisons were analysed with the One-way ANOVA and Tukey post-test. $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*) were accepted as levels of statistical significance and shown in graphical representations.

3. Results

3.1. Water quality parameters and stability study

Water quality parameters were measured for all water samples from the sampling site and during toxicity tests. No significant changes were observed across the whole experimental trial (n = 10). Mean values were as follows: pH 7.9 ± 0.1, temperature 14.7 ± 0.3 °C and water conductivity of 378.3 ± 21.7 μScm^{-1}. The water had average oxygen concentration of 9.8 ± 0.2 mg L^{-1} and saturation of 95.8 ± 2.3%. Mean values of TOC and TN at the water source location were 8.7 ± 0.1 and 0 mg L^{-1}, respectively. Moreover, dissolved oxygen concentration during whole experimental trial was between 70% and 80% of saturation. These were all acceptable conditions for toxicity test [49].

Our experiments showed no significant changes in concentration of IMI and 6CNA in test solutions during 24 h (amphipods) and 96 h (algae) exposure (Table 1). The actual exposure concentrations of both chemicals did not differ by more than 3.4 ± 0.3% (for concentrations in tests with amphipods) and by 15.8 ± 0.4% (for concentrations in tests with algae) from the initial concentrations. IMI and 6CNA concentrations were consistent over time in all tests. Therefore the results are given in nominal concentrations, as suggested by ISO 10706 [50].

3.2. Algae toxicity test

Algal chronic toxicity revealed a high toxic potential of 6CNA at the highest concentration (Fig. 1C). 6CNA induced some perceivable alterations in algae growth, causing slight and temporary inhibition effects at lower doses (4.7 and 15.7 mg L^{-1}) already after 24 h compared to control ($p < 0.05$) (Fig. 1C). The highest dose of 6CNA extensively suppressed the algal growth. 6CNA induced acidification of the algal medium (pH up to 5.5 ± 0.1; n = 3). In all other groups, pH did not deviate significantly from the initial values as in the case of 6CNA at the highest dose. Overall 6CNA effects were stimulatory on algae growth. Major stimulatory effect of 6CNA was observed at 31.5 mg L^{-1} (48 h) reaching 176.4 ± 3.4% and stayed significantly increased also after 72 h compared to control ($p < 0.001$) (Fig. 1C). It was not possible to calculate the IC_{50} value for IMI due to its low inhibitory effects within the entire range of tested concentrations (Fig. 1A). Furthermore, the toxicity of Confidor 200SL ranged from 27.9% up to 49.72% (Fig. 1B). Inhibition of algal growth was significant at 127.8 and 255.6 mg L^{-1} compared to control ($p < 0.01$). Higher toxicity of Confidor 200SL was possibly induced by the co-formulants present in the commercial formulation which contributed as a major part to toxicity for algae. The co-formulants alone induced a significant inhibition of 82.3% and 89.7% (at 0.06 and 0.12%; v/v) compared to control ($p < 0.001$) (Fig. 1B).

3.3. Amphipods toxicity test

3.3.1. Survival rate and behavioural alterations

After 24 h of acute toxicity test, monitored in all groups were: (1) the number of dead amphipods (mortality) and (2) the number

Table 1
Mean ± standard error detected concentrations expressed as μg L^{-1} and mg L^{-1} of IMI and 6CNA in aqueous samples for the 24 h *G. fossarum* and 96 h *D. subspicatus* static toxicity tests (n = 3).

Nominal concentration μ L^{-1}	Dark T = 22 °C [μ L^{-1}]		Nominal concentration (mg L^{-1})	Light T = 22 °C [mg L^{-1}]	
IMI	0 h	24 h	IMI	0 h	96 h
102.2	105.5 ± 2.5	99.7 ± 0.7	7.6	7.5 ± 0.1	6.4 ± 0.1
153.3	154.7 ± 0.7	148.5 ± 1.4	25.6	26.3 ± 0.5	21.9 ±0.5
204.5	203.9 ± 1.8	198.1 ± 0.5	51.1	51.4 ± 1.2	44 ± 1.4
255.6	254.2± 1. 6	250.8 ± 0.4	127.8	127.4 ± 0.7	103.6 ± 2.2
511.3	511.7 ± 0.18	481.2 ± 0.6	255.6	255.1 ± 0.8	240.4 ± 2.9
6CNA	0 h	24 h	6CNA	0 h	96 h
62.8	62.4 ± 0.5	63.3 ± 0.9	4.7	4.5 ± 0.1	4.1 ± 0.1
94.6	93.5 ± 0.8	92 ± 0.8	15.7	14.8 ± 0.5	14 ± 0.2
126.2	127.3 ± 0.4	120 ± 0.9	31.5	29.9 ± 0.9	28.7 ± 0.6
157.7	157.4 ± 0.9	152.6 ± 1.1	78.7	77.1 ± 1.3	71.1 ± 0.8
315.5	315.7 ± 0.3	310.1 ± 1.2	157.5	156.1 ± 0.8	122.3 ± 2.6

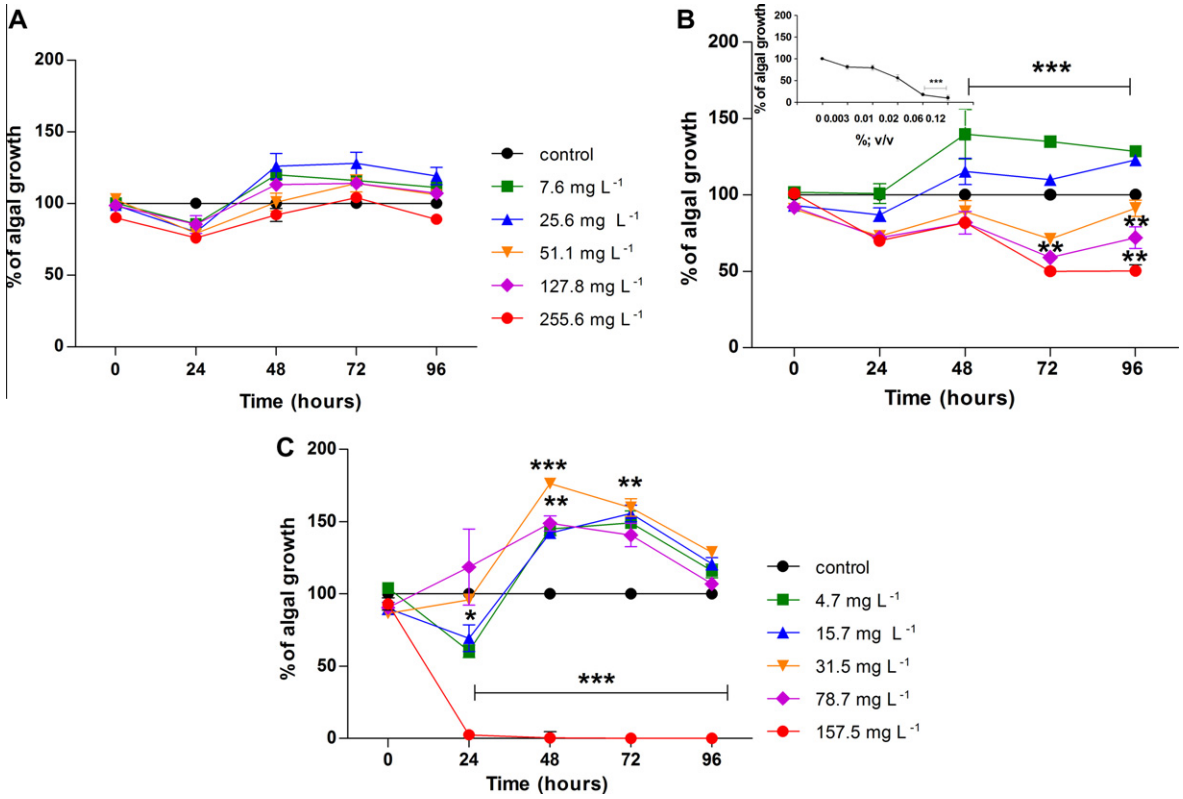

Fig. 1. *D. subspicatus* % of algal growth compared to control after exposure to IMI (A) Confidor 200SL (B) and 6CNA (C) at 24, 48, 72 and 96 h. The inside graph represents exposure to negative control-co-formulants only. Data are reported as mean ± standard error ($n = 3$). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

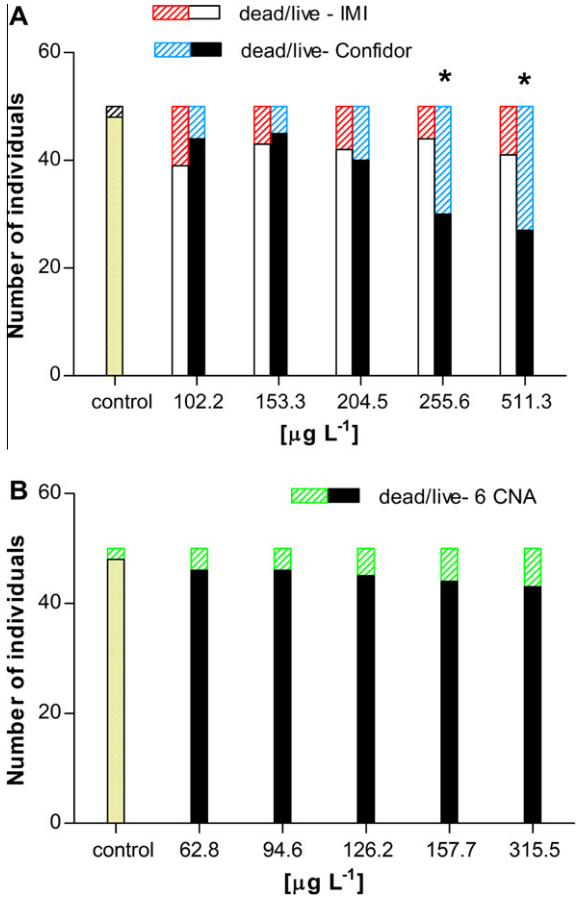

Fig. 2. Mortality rate of *G. fossarum* after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). ($n = 50$). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

of immobile/paralysed or recently moulted amphipods. Only male adult specimens were used for laboratory tests. All specimens presented a mean total body length of 12.35 ± 0.25 mm and mean weight of 0.029 ± 0.002 g. Individuals which sex was not possible to determine were classified as juveniles and not used for this research.

The negative control (co-formulants mixture) did not have any adverse effects on *G. fossarum* at all tested concentrations (data not presented). Due to this fact, all values were compared to control (only stream water). Furthermore, concentrations of all tested compounds lower than 102.2 µg L^{-1} for IMI and 62.8 µg L^{-1} for 6CNA did not induce significant effects compared to control (data not shown).

Average mortality in control groups was between 2.2 ± 1.1 and 4.3 ± 1.9% in all bioassays. Our data demonstrated slight toxicity of IMI with minor changes in mortality rate (Fig. 2A). IMI induced only 22.3% ± 5.09 of dead organisms at 102.2 µg L^{-1}. Commercial formulation Confidor 200SL demonstrated an increased effect on mortality, especially at higher concentrations. Percentages of dead organisms at 255.6 and 511.3 µg L^{-1} of a. i. reached 40 ± 5.7% and 45.5 ± 7.3%, respectively (Fig. 2A). This increased mortality was significant for the both concentrations ($p < 0.05$). On the contrary 6CNA showed an overall low toxicity, ranging from 8.6 ± 1.9% up to 14.1 ± 1.1% (at 62.8 and 315.5 µg L^{-1}, respectively; Fig. 2B).

At 511.3 µg L^{-1} of IMI and Confidor 200SL was present a high number of inactive animals with only respiration movements. These values were of 76.6 ± 6.6% for IMI and of 90 ± 5.7% for Confidor 200SL ($p < 0.001$; compared to control) (Table 2). It is also interesting to report the number of animals that underwent moult (leaving the entire old *exuvia*) after treatment with tested compounds, apparently stimulatory effect on moult processes was due to the action of transformation product 6CNA. Number of moulted amphipods after 24 h exposure to 6CNA at 315.5 µg L^{-1} was of 56.6 ± 3.3% ($p < 0.001$) (Table 2). Number of moulted

Table 2
Number of immobile/paralysed, hyperactive and moulted individuals of *G. fossarum* (% of total treated animals) exposed to IMI, Confidor 200SL and 6CNA for 24 h. Data are expressed as mean ± standard error ($n = 30$).

Nominal concentration (Vg L^{-1})	Immobile/paralysed individuals	Hyperactive individuals	Moulted individuals
IMI			
Control	None		None
102.2	16.6 ± 3.3**	None for all groups	10 ± 5.7
153.3	16.6 ± 8.8		13.3 ± 3.3
204.5	13.3 ± 3.3		23.3 ± 8.8
255.6	43.3 ± 3.3***		26.6 ± 3.3**
511.3	76.6 ± 6.6***		23.3 ± 3.3**
Confidor 200SL			
Control	None		None
102.2	23.3 ± 3.3**	None for all groups	6.6 ± 3.3
153.3	33.3 ± 3.3**		13.3 ± 3.3
204.5	46.6 ± 14.5**		13.3 ± 8.8
255.6	56.6 ± 3.3***		10 ± 0
511.3	90 ± 5.7***		13.3 ± 3.33
6CNA			
Control		None	None
62.8	None for all groups	16.6 ± 3.3**	20 ± 5.7**
94.6		23.3 ± 3.3**	33.3 ± 3.3**
126.2		43.3 ± 3.3***	43.3 ± 12**
157.7		43.3 ± 3.3***	46.6 ± 3.3***
315.5		80 ± 5.7***	56.6 ± 3.3***

** $p < 0.01$.
*** $p < 0.001$.

animals was minor after 24 h of exposure to IMI and Confidor 200SL at 511.3 μg L^{-1} (23.3 ± 3.3% and 13.3 ± 3.3%, respectively; $p > 0.05$). 6CNA seemed to induce overall hyperactivity and rapid swimming (with numerous sideways and back-and-forth movements) which affected 80 ± 5.7% of total treated gammarids at 315.5 μg L^{-1} 6CNA (compared to control; $p < 0.001$). Numbers of counted individuals which presented the described behavioural characteristic are summarized in Table 2. It is important to emphasise that this data need further quantification with technologies that allow a more detailed analyses and recording of behavioural patterns.

3.3.2. Effects on enzyme activities and lipid peroxidation

Results of enzyme activities were expressed per protein content, since changes in the protein were not significant as a result of 24 h exposure to all tested compounds.

In this study a possible indirect effect of IMI on AChE activity in neonicotinoid exposed gammarids was tested as a biomarker of the cholinergic system. *G. fossarum* exposed to IMI displayed no significant changes of AChE activity at all concentrations (data not presented in graph). The AChE values at all exposure concentrations of IMI ranged between 70.6 ± 7.8 and 78.2 ± 11.6 μmol/min/mg proteins ($p > 0.05$; compared to control). CAT activity was not modified after IMI exposure (Fig. 3A). The values ranged between 22.04 ± 1.5 μmol/min/mg protein for control and 28.4 ± 8.6 μmol/min/mg protein at 255.6 μg L^{-1}. Commercial formulation induced a moderate change in CAT at 511.3 μg L^{-1} a. i. going up to 48.06 ± 9.7 μmol/min/mg protein compared to control ($p < 0.05$). Values of CAT activity in the case of exposure to 6CNA reached 48.9 ± 6.7 μmol/min/mg protein already at 157.7 μg L^{-1} ($p < 0.001$) (Fig. 3B). After exposure to Confidor 200SL two different outcomes for GST activity at 255.6 and 511.3 μg L^{-1} were evident (Fig. 4A). At 255.6 μg L^{-1} was present an observable, but statistically not significant decrease in GST activity ($p = 0.053$). The values of GST went from control values of 419.1 ± 101.8 nmol/min/mg protein

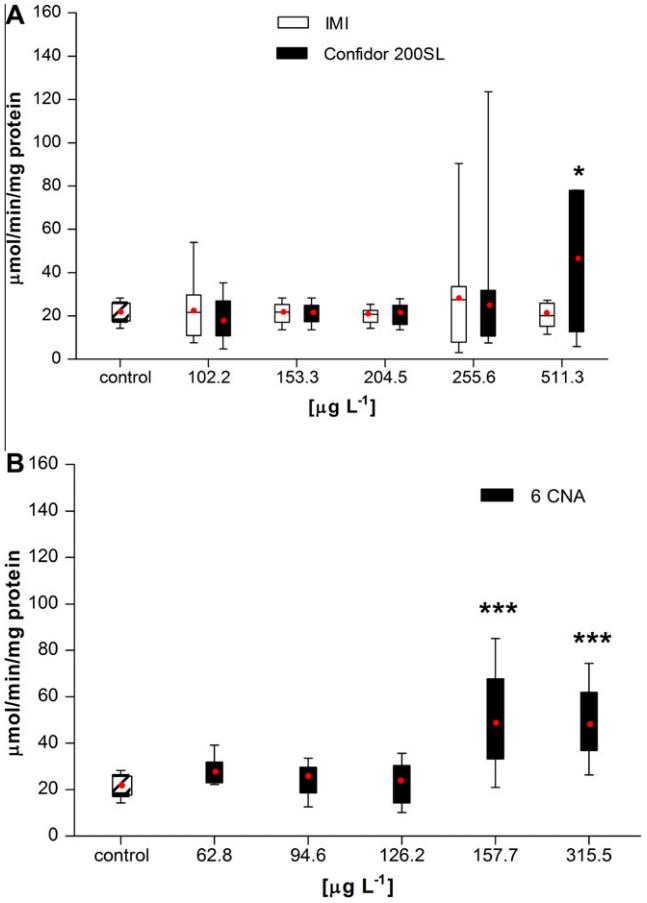

Fig. 3. Whole-body CAT activity (μmol/min/mg protein) of *G. fossarum* measured after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). ($n = 10$). $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

to 286.8 ± 92.71 nmol/min/mg protein at 255.6 μg L^{-1}. Higher concentration of Confidor 200SL (511.3 μg L^{-1} of a. i.) induced an increase of GST activity up to 831.4 ± 117.2 nmol/min/mg protein ($p < 0.05$). IMI and 6CNA exposure provoked no significant changes in GST activity compared to control ($p > 0.05$) (Fig. 4A and B, respectively). IMI induced at 102.2 μg L^{-1} an increase in lipid peroxidation (LP) levels (Fig. 5A). This increase was 2.7-fold higher in contrast to the control group ($p < 0.01$). On contrary, Confidor 200SL induced significant rise of thiobarbituric acid reactive substances (TBARS) only at higher dose (255.6 μg L^{-1} of a. i.; $p < 0.05$). This increase was lower than the significant peak induced by IMI at 102.2 μg L^{-1} (Fig. 5A). No significant effect of 6CNA on LP increase was noted after 24 h at all concentrations (Fig. 5B). However, it was detected a significant decrease of LP values at 315.5 μg L^{-1} ($p < 0.001$).

4. Discussion

Chronic testing was performed on freshwater microalgae *D. subspicatus*. Generally, it appears that algae are some orders of magnitude less sensitive to IMI than arthropod species and exhibiting no effects of IMI on their growth rate [36,51]. Tišler et al. [38] determined for *D. subspicatus* an IC_{50} (72 h) for IMI a. i. at 389 mg L^{-1} (in comparison highest applied concentration in this study was 255 mg L^{-1}). Data presented in this research confirmed the same action of IMI as pure compound causing no significant adverse effects on algal growth. On the contrary, Confidor 200SL was highly toxic to algae due to the presence of co-formulants which

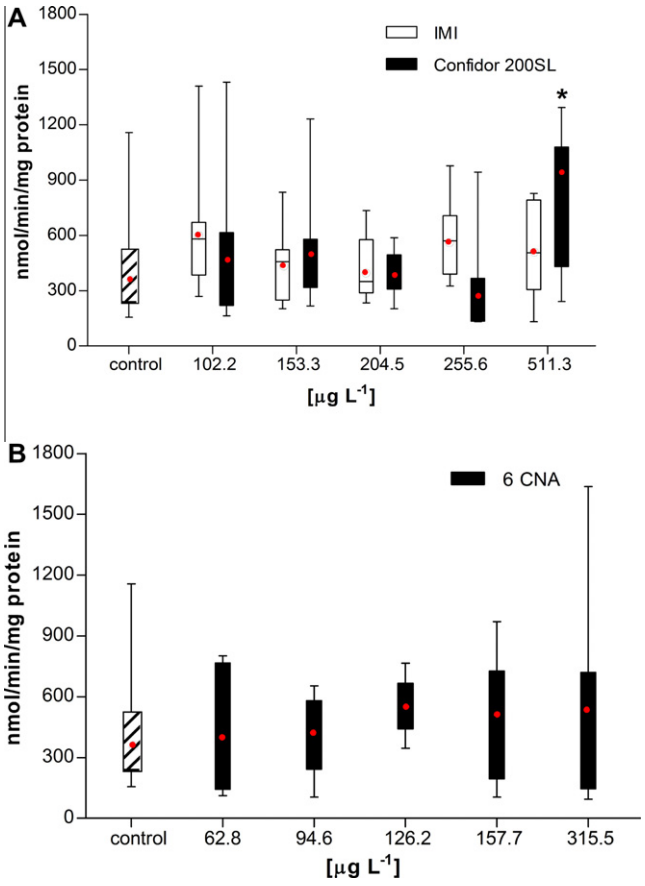

Fig. 4. Whole-body GST activity (nmol/min/mg protein) of *G. fossarum* measured after 24 h of exposure to IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). ($n = 10$). $p < 0.001$ (∗∗∗), $p < 0.01$ (∗∗), and $p < 0.05$ (∗).

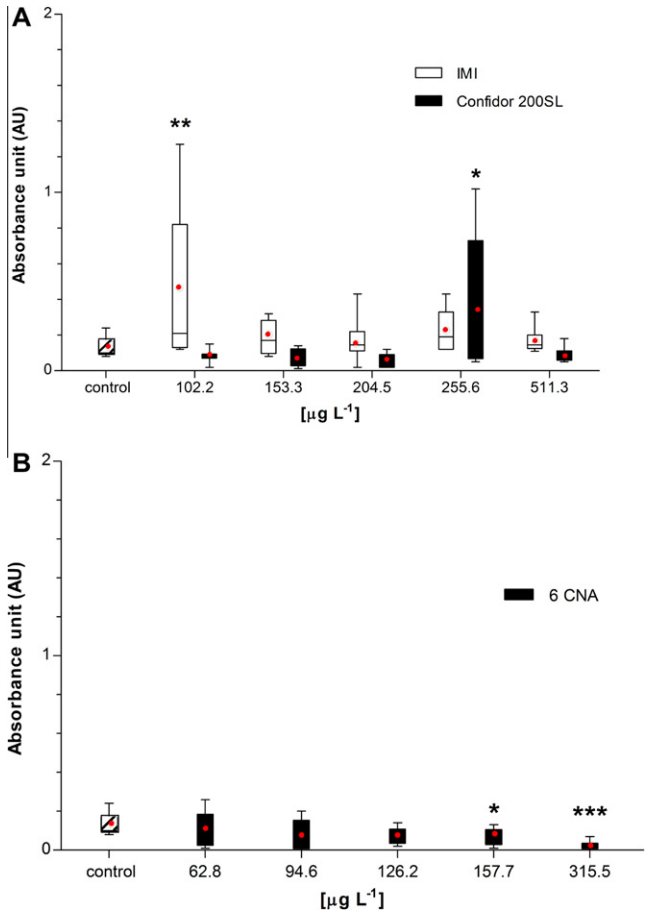

Fig. 5. Whole-body LP of *G. fossarum* (expressed in absorbance units of TBARS products) measured after 24 h of exposure IMI or Confidor 200SL (A) and 6CNA (B). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (●). ($n = 10$). $p < 0.001$ (∗∗∗), $p < 0.01$ (∗∗), and $p < 0.05$ (∗).

started to inhibit their growth already at 0.003 v/v%. On the other hand, 6CNA induced algal growth and proliferation after a 96 h period of exposure at all concentrations, while at the highest dose (157.5 mg L^{-1}) already after 24 h induced a significant inhibition and algae death. Presumably the algal growth was inhibited because of the dissociation of the carboxylic group present in 6CNA [52]. This issue induced acidic changes in pH of the algal media and adversely influenced the sensitive microalgae. 6CNA is a final transformation product formed in environment that does not act as nicotinic agonist but may also contribute to the toxicity effects [18]. 6CNA contains the 6-chloropyridinyl moiety and based on its structural/chemical consideration may be of toxicological significance. This transformation product is included in the tolerances established for the IMI residues, although should be considered on its own in order to recognise additional IMI-toxicity effects. Algae as primary producers contribute substantially to aquatic habitats and their sensitivity to Confidor 200SL and 6CNA found in this study could cause environmental problems.

Acute toxicity (24 h) of IMI and 6CNA was evaluated on the freshwater amphipod *G. fossarum*. After exposure to the highest dose of IMI (511.3 μg L^{-1}) and 6CNA (315.5 μg L^{-1}), an overall low mortality was noticed. Most significant effect, as in algae, was observed in case of Confidor 200SL. Increased mortality induced by Confidor 200SL supports the idea that major side effects could be caused by additives such as dimethyl sulfoxide (DMSO) and *N*-methylpyrrolidone (NMP). These co-formulants mixture alone induced no toxicity in amphipods, while the combined action of IMI and co-formulants increased the toxicity of the commercial formulation. In the case of another amphipod crustacean *Hyalella azteca*, Stoughton et al. [15] confirmed its higher sensitivity to formulated product than to technical IMI. Also, other tests have shown formulated pesticides to be more toxic to aquatic organisms [11,37,53]. These supplementary substances in commercial formulations often represent the highest proportion in pesticide mixtures, so even a minor concern regarding their toxicity and possible synergistic effects with other ingredients should be considered [54,55]. Additional studies will be needed to address the potential effect of additives, but such studies are not easily feasible since identity and quantity of other ingredients is most often regarded as confidential information and therefore rarely revealed in easily accessed literature or product labels.

Neonicotinoids are agonist of nAChRs [1] and do not exert a direct inhibition of the AChE activity as for example organophosphates. In our study we tested possible indirect inhibitory effects on freshwater amphipods exposed to neonicotinoids. This measurement was also performed on gills of neonicotinoid exposed mussels and showed an interesting outcome with 'U-shape dynamics' of AChE activity [56]. In this study Dondero et al. observed significant inhibition at the lowest and at the intermediate tested concentration. On contrary, in our case the outcome of IMI effect on AChE activity presented no indirect effect or changes at all exposure concentrations compared to control group.

It is well-known that pesticides can induce oxidative stress by the generation of ROS, which can induce oxidant-mediated effects (such as increased activities of antioxidant enzymes) and oxidant-mediated toxicities (such as oxidation of lipids) [57]. Only a few

previously published data are available regarding the IMI-induced oxidative stress and these merely relate to mammalian model organisms. These studies showed a slight increase in intracellular ROS and nitric oxide production after IMI exposure [58,59]. A study of Lukančič et al. on *G. fossarum* demonstrated that IMI influenced not only the respiration but also the electron transport system (ETS) activity [12]. This effect was a consequence of different processes, including oxidative stress. Partial damage to the inner mitochondrial membrane by lipid peroxidation possibly impaired the function of ETS. For better understanding of ROS involvement in the toxicity mechanisms of neonicotinoids, antioxidant enzyme activity, detoxifying GST mechanism and lipid peroxidative damage were monitored in amphipods. In this study, CAT activity after Confidor 200SL and 6CNA exposure at highest doses was significantly increased and indicated action of the protection mechanisms involved in cellular repair processes. El-Gendy et al. [60] reported a similar increase of CAT after neonicotinoid exposure, but again only in IMI-treated mice. Enhanced GST activity after Confidor 200SL exposure reflects the detoxification processes in treated gammarids and this induction may be due to the glutathione dependent enzyme system that provides major protection against xenobiotic agents. A recent study on the mosquito, *Aedes aegypti*, demonstrated that exposure to IMI increased glutathione transferase mRNA levels as well as other genes coding for antioxidant proteins [61]. In addition was also noticed a slight decrease in antioxidant enzyme GST after exposure to Confidor 200SL (at 255.6 $\mu g\ L^{-1}$ of a. i.). This decrease of the GST activity, although not significant was evident with 1.5-fold lowered GST activity at 255.6 $\mu g\ L^{-1}$ of a.i. in Confidor 200SL. This decrease could be interpreted as being overwhelmed by conspicuous ROS production. An additional explanation of enzyme's indirect inhibition is related to their binding with ROS produced also during pesticide metabolism. Metabolism of IMI involves many processes of hydroxylation, i.e. the hydroxylation of the imidazolidine ring at position 4 or 5 leading to the formation of hydroxylated compounds and subsequent loss of important amounts of hydroxyl radicals [62]. Concurrently, with slightly diminished GST activity increased lipid peroxidation levels occurred (at the same exposure concentration of Confidor 200SL). IMI and Confidor 200SL exposure provoked an increase of LP in amphipods. During IMI exposure LP increase occurred at 102.2 $\mu g\ L^{-1}$ and was represented by a similar-to-hormetic effect. This increase was induced at lower concentrations of IMI and not at higher doses as expected. On the other hand, Confidor 200SL induced an increase of TBARS products, which was highest at 255.6 $\mu g\ L^{-1}$ of a. i. Higher TBARS levels at 255.6 $\mu g\ L^{-1}$ suggested that exposure to Confidor 200SL resulted in a different time-course of cellular ROS generation or in a possible direct lipid oxidation due to the interactive action of co-formulants and IMI. It is important to notice potentially different toxicity pathways or time-course effects of the parent compound and its transformation product that were observed during this study. After a 24 h exposure 6CNA provoked strong induction of antioxidant enzyme CAT, while its effect was completely absent on the LP, probably due to highly active CAT. On the contrary, Confidor 200SL altered all parameters confirming its higher toxicity compared to active ingredient.

Behaviour is considered as a useful tool in ecotoxicology since is one of the early warning indicators of toxicant stress [14]. During experimental pesticide exposure analysed individual biochemical biomarkers should be linked to behavioural responses whenever this is possible [63]. In this study individuals with modified behaviour were counted. During exposure, animals treated with 511.3 $\mu g\ L^{-1}$ (IMI) exhibited an increase in immobility and inactivity that can be a direct IMI effect on neuro-muscular acetylcholine receptors provoking impairment of locomotion and food filtration, with consequent animal starvation and difficulties in ventilation [64]. Alternatively, 6CNA at the highest dose induced rapid movements and animal hyperactivity, as well as disorientation. This disoriented behaviour was also shown in non-target organisms, such as *Apis mellifera*. Honey bees treated with IMI were confused and failed to return to their homing site [65]. Hyperactivity in swimming may also be linked to an avoidance response towards present chemicals [66]. Interestingly, short-term 6CNA exposure stimulated amphipods moulting processes. Moulting is an essential physiological process for crustaceans controlled by the neuroendocrine system, on which different toxicants, such as pesticides, can act [67]. Moreover, moulted or recently moulted animals could be more susceptible to pesticide action.

This research confirmed the importance of testing commercial formulations of IMI and IMI's transformation products as they interfere with pure compound safety characteristics. Our present results show that commercial formulation of IMI and its by-product 6CNA exert oxidative stress in freshwater amphipods as well as negative effects on algae growth. The induction of CAT, GST and LP levels demonstrates that exposure of *G. fossarum* to Confidor 200SL leads to peroxidation of membrane lipids and triggers antioxidant and detoxifying cellular mechanisms. Amphipods exposed to 6CNA experienced mainly the activation of catalase scavenging protection mechanism. In general, the major toxic effects were due to the commercial formulation Confidor 200SL both in case of algae and amphipods. This issue is relevant as these marketed mixtures are the one applied directly in the environment and should be further monitored.

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