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**Physiological and metabolic alterations induced by commercial neonicotinoid formulations
in *Daphnia magna***

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Abstract

Neonicotinoid insecticides are widely used agents in agriculture to control a broad range of insect pests. Although use of neonicotinoid pesticides has resulted in the widespread contamination of surface waters, sublethal toxicity data of these products in relation to non-target aquatic biota are still poor. Therefore, the objective of this study was to assess the effects of two neonicotinoid pesticides with widespread use on the basic physiological functions: the thoracic limb activity and heart rate of *Daphnia magna*, and to screen for their potential to affect the cytochrome P450 monooxygenase system (ECOD activity) of daphnids. The considered pesticides were the acetamiprid- and thiacloprid based products Mospilan 20 SG and Calypso 480 SC, respectively. The dose-dependent variation in the three biological endpoints considered were assessed following 24h exposures. The two neonicotinoid formulations elicited significant depression on the thoracic limb activity and heart rate of daphnids at doses close to the immobility thresholds of formulations (48h-EC₅₀:

Mospilan 20 SG = 190 mg L⁻¹; Calypso 480 SC = 120 mg L⁻¹), an effect mainly attributable to the overall drop in the general health status of the organisms. The alterations in the physiological traits were significant at exposures to 190 mg L⁻¹ for Mospilan 20 SG and 48 mg L⁻¹ for Calypso 480 SC. The dose related variation in the ECOD activity of daphnids exposed to the selected neonicotinoid formulations followed a biphasic pattern, with starting effective doses for Mospilan 20 SG of 6.3 mg L⁻¹ (= 1/20 of 48h-EC₅₀ for *Daphnia* neonates), and for Calypso 480 SC of 0.034 mg L⁻¹ (= 1/4000 of 48h-EC₅₀). Maximal ECOD activity (2.2 fold increase vs. controls) was induced by Mospilan 20 SG in daphnids exposed to 114 mg L⁻¹ product (= 48h-EC₂₀), and by Calypso 480 SC (1.8 fold increase) at 5.2 mg L⁻¹ dose (= 1/20 of 48h-EC₅₀). Our results outlined significant alterations in the physiological traits and ECOD activity in exposed daphnids at concentrations below the immobility thresholds (48h-EC₅₀) of the products used as benchmarks to rate their toxicity risks to aquatic biota. Therefore, we think our findings might deserve consideration in the environmental risk evaluation of these products.

Keywords: neonicotinoids; *Daphnia magna*; thoracic limb activity; heart rate; ECOD activity

DECLARATIONS

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Animal Research (Ethics)

All applicable international, national and institutional guidelines for the care and use of animals were followed.

Consent to Participate (Ethics)

Not applicable.

Consent to Publish (Ethics)

Not applicable.

Plant Reproducibility

Not applicable.

Clinical Trials Registration

Not applicable.

Author Contribution

Anna Farkas: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. Dávid Somogyvári: Formal analysis, Investigation. Attila W. Kovács: Methodology, Formal analysis, Investigation, Writing - original draft. Mária Mörtl: Methodology, Formal analysis, Writing – original draft, Funding acquisition. András Székács: Resources, Writing – original draft. János Győri: Conceptualization, Funding acquisition, Writing – original draft. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

We have no competing interests to declare

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Competing interests statement

We have no competing interests to declare.

1. Introduction

Neonicotinoids are systemic insecticides widely used in crop production to control a broad range of insect pests (as reviewed by Morrisey et al., 2015). Owing to their high water solubility and proven persistence in soil and water, neonicotinoids became ubiquitous pollutants even in the aquatic environment, where they entered mainly through agricultural drainage (CCME, 2007; EFSA, 2008; Morrisey et al., 2015; Raby et al., 2018a). Neonicotinoids act on the nicotinic acetylcholine receptors in the central nervous system, through a mechanism highly conserved across many insect species, and similarly on other, non-target aquatic invertebrates (Zhang et al., 2000; Tomizawa and Casida, 2003, 2005; Vehovszky et al., 2015). Although most research has reported relatively lower toxicity of neonicotinoid insecticides to fish and crustaceans (Rico and Van den Brink, 2015), measurable toxicity to a range of other aquatic arthropods including insects (Diptera, Trichoptera, Ephemeroptera)

and some crustaceans (Amphipoda, Mysida, Podocopida) was demonstrated even at low environmentally relevant concentrations (Mineau and Palmer, 2013; Morrisey et al., 2015).

Within aquatic biota, cladocerans were proven to be quite tolerant to a range of neonicotinoids and their technical formulations (Jemec et al., 2007; Pavlaki et al., 2011; Raby et al., 2018a, 2018b), with most data reporting on more general endpoints as survival and reproduction success determined in both acute and chronic toxicity studies. However, to better understand the real risks of neonicotinoids to cladocerans, knowledge on their effects on the basic physiological functions of these organisms and on the efficiency to metabolize this class of xenobiotics are also essential. As earlier outlined by Ankley et al. (2010) mapping of linkages between impairments of physiological functions and alterations at cellular and molecular levels is essential to better understand the mechanisms of biological features of organisms in adverse outcome pathways (AOP). For example, in terrestrial insects it was already evidenced that differences in the sensitivity to neonicotinoids is primarily dependent on the differential affinity of neonicotinoids for the nicotinic acetylcholine receptor (nAChR) as the target site (Taillebois et al., 2014; Crossthwaite et al., 2017; Manjon et al., 2018). In addition, differential susceptibility of various insects to neonicotinoids was also found to strongly depend on the speed and efficiency to metabolize neonicotinoids by particular types of microsomal cytochrome P450 monooxygenases (CYP P450) (Sparks et al., 2013; Bass et al., 2015). We have hypothesized therefore, that even in the case of daphnids their low sensitivity to neonicotinoids is attributed not only to a low density- or lack in specific receptor configurations, but also the xenobiotic metabolizing system of the organism will affect the toxicity potential of neonicotinoid pesticides.

To date, information on the effects of neonicotinoid insecticides on the basic physiological functions of daphnids are scarce. A single study by Bownik et al. (2017) reports alterations in the heart rate and thoracic limb activity of daphnids under acute exposure to an acetamiprid based formulation (Mospilan 20 SP). In the study by Bownik et al. (2017) impairments in the heart rate and thoracic limb activity of daphnids were assessed within the 25 – 100 mg L⁻¹ concentration range, at concentrations approaching the 48h-EC₅₀ toxicity threshold of the product (*Daphnia magna* 48h-EC₅₀ > 100 mg L⁻¹, as per the OECD Guideline 202).

In ecotoxicity studies with *Daphnia magna* as indicator organisms, the thoracic limb activity and heart rate proved to be sensitive endpoints to rate the deleterious effects of toxicants (Paul et al., 1997; Lovern et al., 2007; Lari et al., 2017a; 2017b; Bownik, A., 2020). Lowered heart contraction frequency of *D. magna* (considered as a physiologic indicator of the metabolic rate of the organism) imply significant disturbances in the haemolymph and nutrient circulation throughout the body of daphnids (Gruithuisen, 1828 as cited by Steinkey et al., 2019). Thoracic limb activity determines both the feeding and respiration rate of daphnids (Smirnov 2013; Lari et al., 2017a), as the movement of thoracic limbs directs food particles to the food groove of the organism and, at the same time ensures a propulsion for gas exchange over the body surface of daphnids (Pirow et al., 1999a; 1999b, Lari et al., 2017a).

Based on knowledges mentioned above, the purposes of this study were first to investigate the potential of two commercial neonicotinoid formulations: Mospilan 20 SG (active ingredient (a.i.) acetamiprid), and Calypso 480 SC (a.i. thiacloprid) to alter the heart rate and thoracic limb activity of *Daphnia magna* following acute (24 hours) exposure. In addition to physiologic traits evaluation, we have assessed also the CYP P450 enzyme activity variation

(as ECOD) in exposed daphnids as a cellular marker of xenobiotic metabolism. In this study we have intentionally tested the formulated products in order to address also the potential harm of additives present in the product. Previously, in acute bioassays with *Daphnia magna* (conform OECD Guideline 202) we have already demonstrated for Mospilan 20 SG a toxicity potential 1.3 and 19.6 times higher than that explained by its acetamiprid and linear alkylbenzene sulfonate content (Mörtl et al., 2019), while for Calypso 480 SC antagonism was evidenced, with the toxicity of the formulation 2 – 3 times lower than that expected according its thiacloprid content (Takács et al., 2017). In addition, testing of pesticides not only as pure active compounds but also as commercial formulations is increasingly supported in order to avoid the misinterpretation of their toxicological profile (as reviewed by Nagy et al., 2020).

The two neonicotinoid formulations were selected owing their widespread use in both intensive crop production as well as in households as pest control agents resulting in increased incidences of active compound detections in surface waters. Globally, environmental concentrations of acetamiprid were reported in approximately 50% of water monitoring studies ranging from 0.008 to 44.1 $\mu\text{g L}^{-1}$, with maximal concentrations as high as 225 $\mu\text{g L}^{-1}$ (Anderson et al., 2013; Morrissey et al., 2015, Mörtl et al., 2020). In Australian rivers draining horticulture and vegetable cultivating regions, thiacloprid detections were evidenced in 27 – 93% of water samples, reaching concentrations up to 1.4 $\mu\text{g L}^{-1}$ (Sanchez-Bayo and Hyne, 2014).

Knowledge on the sublethal effects of these neonicotinoids in *D. magna* may contribute to a better judgement of the real environmental risks posed by their intensive use and help to

develop safer water quality guidelines to protect aquatic biota from this unintended pollution pressure.

2. Materials and methods

2.1. Agrochemical formulations and chemical analysis

Experiments were performed with the neonicotinoid formulations Mospilan 20 SG (acetamiprid 20.2%, Nippon SODA Co. Ltd., Japan), and Calypso 480 SC (thiacloprid 40.40%, Bayer AG, Germany) purchased from local distributors. Stock solutions were freshly prepared for each exposure series by dissolving the commercial formulations in the media used for *D. magna* culturing. Exposure media were prepared from fresh stock solutions with nominal concentrations of 2000 mg L⁻¹ for Mospilan 20 SG and 1000 mg L⁻¹ for Calypso 480 SC. The concentration of stock solutions was verified in each test series by means of the active ingredients (acetamiprid, thiacloprid) quantified in 1 ml subsamples stored at -22 °C until chemical analysis. In addition, each test dilution was verified both at test initiation and end to verify the real concentrations of neonicotinoid exposure.

Acetamiprid and thiacloprid quantification was performed using a Younglin YL9100[®] HPLC system equipped with a YL9150 autosampler as previously described in Mörtl et al. (2016). Active ingredients were separated on a C₁₈ column (150 mm x 4.6 mm i.d., 5 µm) at 40 °C. UV signals were recorded at λ = 252 and 269 nm. Eluent flow rate was 1.0 mL min⁻¹ with isocratic elution for 10 min (65:35 = A:B eluents, A = 90% water:10% MeOH, B = MeOH). Exposure media of control treatments did not contain any interfering matrix component therefore, the quantitation of active compounds was performed based on external calibrations with standard dilutions within the 0.050 – 120 mg L⁻¹ concentration range. The limits of detection (LODs) for standards were 0.020 and 0.030 mg L⁻¹ and the corresponding

limits of quantification (LOQs) were 0.060 and 0.090 mg L⁻¹ for acetamiprid and thiacloprid, respectively. For Calypso 480 SC exposures in the lowest dose, where the expected thiacloprid concentrations fell below LOQ, the active compound was extracted three times from 5 ml of sample by using 100 µl methanol and 2 ml dichloromethane. The lower layers were combined, the organic phase was evaporated to dryness and the residue was resolved in 500 µl of HPLC eluent used as mobile phase. Recoveries were determined in triplicates with spiked solutions containing 0.010 mg L⁻¹ of thiacloprid. Average recovery was 93 ± 4%.

2.2. Test organisms and exposure conditions

Daphnia magna Straus were cultured at a density of 15 individuals L⁻¹ in a mixture (1:1 v/v) of reverse-osmosis water and a commercial spring water (Mizse, Hungary – conductivity 520 µS cm⁻¹ at 20°C; pH 7.6; 342 mg L⁻¹ HCO₃⁻; 62 mg L⁻¹ Ca²⁺; 23.9 mg L⁻¹ Mg²⁺). During culturing the main quality parameters of the mixed media varied within the following ranges: conductivity 280 – 330 µS at 20 °C, hardness 80 – 84 mg L⁻¹ as CaCO₃, alkalinity 150 – 170 mg L⁻¹ as CaCO₃, pH 7.4 – 8.2, dissolved oxygen 7.4 – 7.8 mg L⁻¹. *D. magna* were raised in a temperature-controlled room at 22 ± 1 °C and 16:8 hours light-dark photoperiod with white fluorescent lamps as a light source. The freshwater media was changed twice weekly and neonates were removed every 24 h. Before renewal, the fresh media was aerated for two hours to ensure the appropriate oxygen saturation. *D. magna* cultures were fed daily with *Raphidocelis subcapitata* algae at a minimum concentration of 10⁵ cells mL⁻¹ and additional supplementation of powdered commercially available fish food (0.9 – 1.2 mg L⁻¹) formulated for juvenile tropical fish (Fix 1., distributor Aqua-Life Kft, Hungary). Semi-static *R. subcapitata* cultures were grown at 22 °C in BG11 medium (Ripka et al., 1979) without nitrate.

Five-days-old daphnids were exposed for 24 hours to five serial dilutions of neonicotinoid formulations within the following concentration ranges: Mospilan (0.63 – 190 mg L⁻¹), Calypso (0,025 – 120 mg L⁻¹). Exposures were performed in 6 well plates (NUNC BioLite 6 well Multidish plate, Thermo Fisher Scientific, Rochester, NY) by incubating two daphnids in 10 ml of exposure media. Each treatment was applied in duplicate in a test series, and the tests were repeated four times (resulting 16 replicates per treatment and per endpoint). Exposures were run similarly in a temperature-controlled room at 22 ± 1 °C and 16:8 hours light-dark photoperiod with white fluorescent lamps as a light source. Each test series contained two control wells with untreated daphnids, finally resulting in 16 control datasets for heartbeat and thoracic limb activity.

In general, equitoxic concentrations of the two pesticides were preselected for assays at dilutions of 1/4000-, 1/400-, 1/20- and 1/4 of the 48h-EC₅₀-s, as well as the 48h-EC₂₀ and 48h-EC₅₀ thresholds of the formulations, which were previously determined for neonates in acute immobilization tests as per the OECD 202 Guideline (OECD202, 2004). According preliminary tests, some differences in the final exposure concentrations of pesticides was introduced to better outline the dose-dependent variations of targeted endpoints. Based on immobilization tests the 48h-EC₂₀ and EC₅₀ thresholds for Mospilan 20 SG were 114 (103 – 131) and 186 (CI: 171 – 214) mg L⁻¹, while for Calypso 480 SC were 52 (41 – 71) and 119 (108 – 130) mg L⁻¹ (as mean and 95% confidence limits (CI); personal unpublished data). Finally, the nominal exposure concentrations varied for Mospilan 20 SG between 0.63 – 190 mg L⁻¹ and for Calypso 480 SC between 0.034 – 120 mg L⁻¹ ranges.

Within independent exposures, the concentration of formulations remained relatively constant, as revealed by active ingredient quantifications in old exposure media. The

concentrations of neonicotinoid active compounds in control media were always lower than the limits of quantification (<LOQ). As mean measured concentrations of acetamiprid and thiacloprid in exposure media varied within $108 \pm 27\%$ and $103 \pm 11\%$ of the preselected nominal doses, the mean measured concentrations in old exposure media were used in data presentation and statistical analysis.

2.3. Video microscopy of the heartbeat and thoracic limb activity of *D. magna*

Following exposures, the thoracic limb activity and heartbeat of daphnids was recorded using a Leica M205C stereo microscope equipped with a Leica DFC450 camera (Leica Microsystems, Germany) at a 40 x total magnification. Thirty-second video records with a frame rate of 100 frames per second were taken simultaneously for two daphnids transferred in 100 μl media on a glass slide. Stabilization of daphnids during recording was assured by the surface tension of the media as previously established by Villegas-Navarro et al. (2003). The heartbeat and thoracic limb activity of control- and treated daphnids was evaluated by reviewing the video records in slow motion at 25% of normal speed, and visually counting the number of thoracic limb beats and heart contractions within 10 and 5 seconds time intervals arbitrarily selected in each record.

2.4. *In vivo* cytochrome P450 (as ECOD) activity estimation

The ECOD activity of daphnids exposed to neonicotinoid formulations was indirectly evaluated as per the methods of Gottardi et al. (2016) and adapted from Gagnaire et al., (2010). The assay principle is based on assessing *in vivo* the conversion of 7-ethoxycoumarin-

O-deethylase (as the substrate) to 7-hydroxycoumarin by the cytochrome P450 enzymes of exposed daphnids. For this purpose, five-days-old daphnids (5 individuals per 20 ml exposure media) were exposed for 24 hours at identical test concentrations as those used in the physiological traits assessment tests. Each treatment was applied in duplicate in a test series and the tests were repeated three times (resulting in 6 replicates per treatment). Following pre-exposure, daphnids were incubated in groups of five individuals in 2 ml volumes of exposure media spiked with 7-ethoxycoumarin (0.01 mM final concentration) in 24 well plates (NUNC BioLite 6 well Multidish plate, Thermo Fisher Scientific, Rochester, NY). Incubation was performed in complete darkness for three hours at 22 ± 1 °C. Parallel subsamples of incubation media devoid of organisms served as blanks for determining the background fluorescence of the substrate. During incubation, 100 μ L aliquots of incubation media were transferred every 30 minutes into wells of a black microplate (OptiPlate 96F, PerkinElmer Inc., USA) for fluorescence recording using a microplate reader (VICTOR³_{TM} 1420 Multilabel Counter, PerkinElmer Inc., USA). Fluorescence measurements were performed at room temperature, using the following fluorescence filter pairs: excitation = 355 ± 40 nm, emission = 460 ± 25 nm. Blank corrected fluorescence values recorded over time were then converted to total amount of 7-hydroxycoumarin by means of a calibration curve of 7-hydroxycoumarin standard (0 – 50 pmol range, in distilled water). Here, the progressive decrease in incubation volume was taken into consideration. ECOD activity was finally determined as the slope of a linear regression (Origin Pro 95E, OriginLab Co., USA) fitted in the linear range of the 7-hydroxycoumarin concentrations determined during the three hours incubation time. ECOD activity was expressed in pmol 7-hydroxycoumarin h⁻¹ organism⁻¹).

2.5. Statistical analysis

Datasets were first subjected to an outlier analysis according to Chauvenet's criterion and extreme values excluded, resulting in final n data from 14 to 16 for physiological endpoints and 5 to 6 data for ECOD activity. Datasets were further tested for normality and homogeneity of variances by a Shapiro-Wilk and Levene's tests. Thoracic limb beat frequency- and heart rate datasets met the assumptions for parametric analysis thus, differences in physiological endpoint values observed within different treatment conditions were analysed for significance by one-way ANOVA ($p = 0.05$), followed by Tukey's means comparisons test.

For ECOD activity data the population variances were significantly different at $p \leq 0.05$ therefore, the significance of differences between the datasets of different treatments was assessed using a Kruskal-Wallis test and Mann-Whitney U test. Statistical analyses and graphical plotting were performed in OriginPro (OriginPro 95E, OriginLab Co., USA).

3. Results

3.1. Physiological responses in *D. magna* exposed to neonicotinoid formulations

Control daphnids were characterised by a mean thoracic limb beating frequency of 233 ± 28 beats min^{-1} and a baseline heart rate of 403 ± 29 beats min^{-1} . Compared to control treatments, both neonicotinoids elicited significant decrease in the thoracic limb activity of daphnids at concentrations equal-, or close to the 48h-EC₅₀ thresholds of the formulations (Fig. 1).

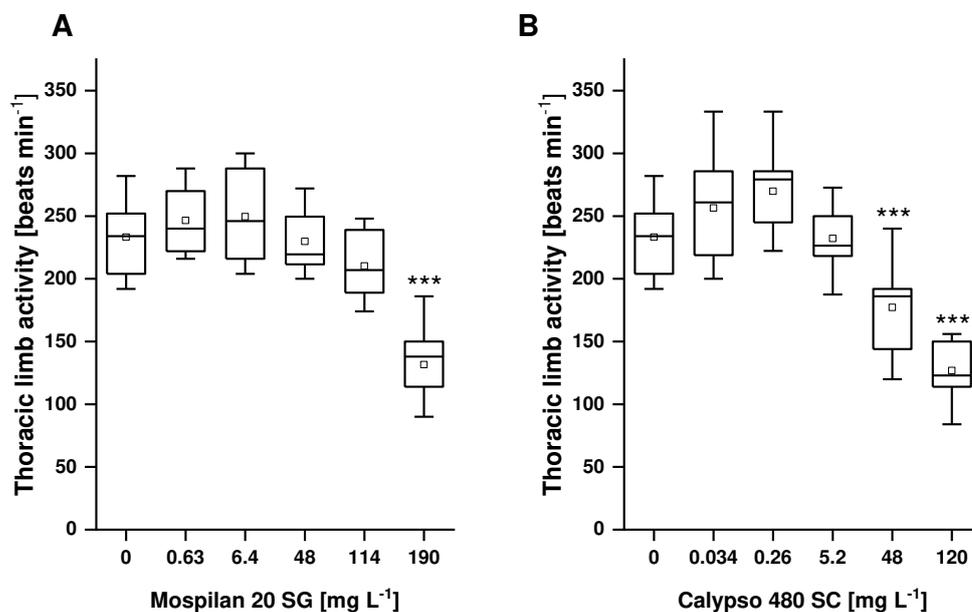


Fig.1. Thoracic limb activity in *Daphnia magna* following 24 h exposure to A: Mospilan 20 SG and B: Calypso 480 SC. Plots represent the median (square), 25-75% percentiles (box) and 10th-90th percentiles range (whiskers) values (n = 14 – 16 individual recordings per treatment). Asterisks indicate significant differences (***) $p < 0.001$ between data recorded in unexposed individuals and those exposed to formulations (ANOVA, Tukey test). For each datasets normality and homogeneity of variances were met (Shapiro-Wilk, Levene's test, $p < 0.05$).

Mospilan 20 SG caused significant drop in the thoracic limb activity of daphnids at the 190 mg L⁻¹ dose (by 44%) as compared to the control (Fig. 1 A: $F(5,93) = 39.7$; $p < 0.001$). In daphnids exposed to Calypso 480 SC statistically significant decrease in thoracic limb activity (by 24%) was observed at the 48 mg L⁻¹ exposure dose, which corresponds to the 48h-EC₂₀ of the formulation, while in the highest dose applied (120mg L⁻¹) the thoracic limb activity decreased by 46% as compared to the control (Fig. 1B: $F(5,92) = 43.6$; $p < 0.001$). For both

formulations, low dose exposures elicited increases in thoracic limb activity of daphnids (by 7 – 16%) as compared to control; however, the differences were not statistically significant.

The pattern of alterations in the heart beating frequency of daphnids exposed to the formulations revealed a similar variation pattern to that observed for thoracic limb activity (Fig. 2 A,B).

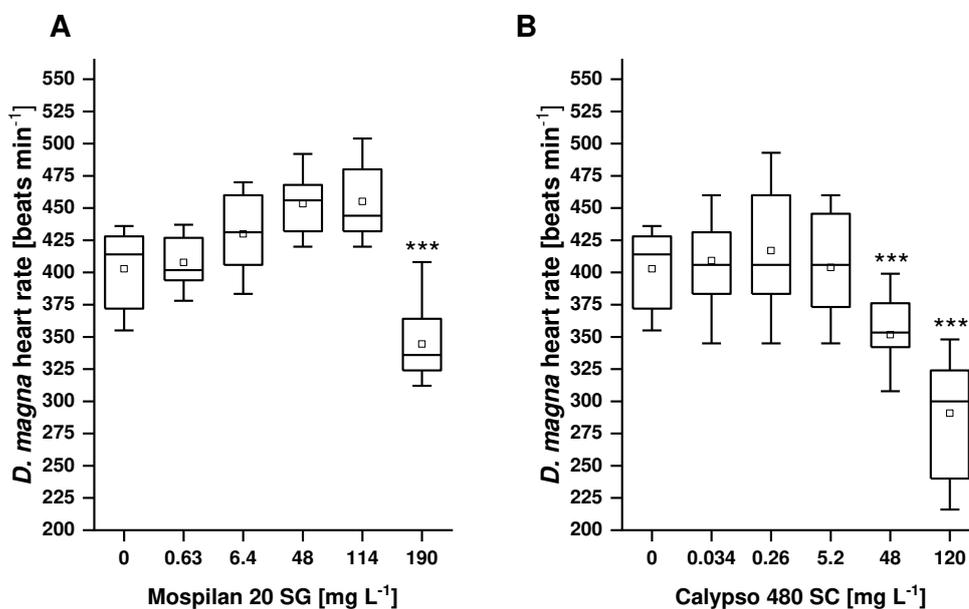


Fig.2. Heart rate in *Daphnia magna* following 24 h exposure to A: Mospilan 20 SG and B: Calypso 480 SC. Plots represent the median (square), 25-75% percentiles (box) and 10th-90th percentiles range (whiskers) values (n = 14 – 16 individual recordings per treatment). Asterisks indicate significant differences (***) $p < 0.001$ between data recorded in unexposed individuals and those exposed to neonicotinoids (ANOVA, Tukey test). For each

datasets normality and homogeneity of variances were met (Shapiro-Wilk, Levene's test, $p < 0.05$).

In daphnids exposed to Mospilan 20 SG a progressive but statistically insignificant increase in heart rate (by maximum 13%) was observable up to the 48h-EC₂₀ threshold of the product (114 mg L⁻¹) compared to control then, a significant drop (by 14%) in heart beating frequency was recorded at the highest 190 mg L⁻¹ exposure concentration (Fig. 2A: $F(5,87) = 37.6$; $p < 0.001$). For daphnids exposed to Calypso 480 SC statistically significant decrease in the heart beating frequency (by 13%) relative to the control was observed at the 48 mg L⁻¹ exposure dose (corresponding to the 48h-EC₂₀ of the product) followed by further decline (by 28%) upon exposure to 120 mg L⁻¹ (Fig. 2B: $F(5,95) = 29.08$; $p < 0.001$). In the low dose range (0.034 – 5.20 mg L⁻¹) Calypso 480 SC had no apparent effect on the heart rate of daphnids.

3.2. Modulation of ECOD activity in *D. magna* exposed to neonicotinoid formulations

The mean ECOD activity of control daphnids was 1.26 ± 0.10 pmol h⁻¹ organism⁻¹, a baseline level which is comparable to data previously reported by Gottardi et al. (2016; 2019) for organisms of similar developmental stage. Exposure to both neonicotinoid pesticides elicited significant induction in the ECOD activity of daphnids compared to controls within the whole dose-range tested, with the enzyme activity displaying a biphasic pattern (Fig. 3).

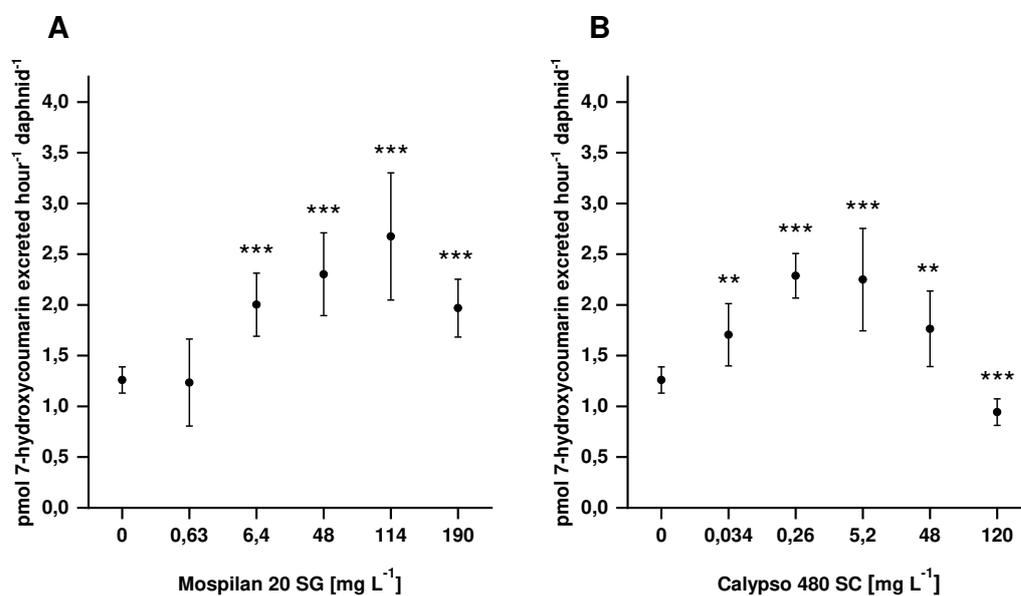


Fig. 3. (A) Effect of Mospilan 20 SG exposure (24h) on ECOD activity in five days old *Daphnia magna*. (B) Effect of Calypso 480 SC exposure (24h) on ECOD activity in five days old *Daphnia magna*. Data are presented as mean \pm SD for 5 – 6 replicates per treatment. Exposure groups labelled by ** and *** significantly differed from control datasets at $p < 0.01$ and $p < 0.001$ respectively (Mann-Whitney U test).

Lowest effective concentrations causing significant induction in ECOD activity were of 6.4 mg L⁻¹ for Mospilan 20 SG (1/20 of 48h-EC₅₀) and of 0.034 mg L⁻¹ for Calypso 480 SC (1/4000 of 48h-EC₅₀). For Mospilan 20 SG, maximal 2.2 fold increase in ECOD activity compared to controls was detected at the 114 mg L⁻¹ exposure dose (= 48h-EC₂₀ of the product), which was followed by a fall-off in enzyme activity (by 26% vs. the maximal ECOD activity level) upon exposure to the highest exposure dose of 190 mg L⁻¹ product (= 48h-EC₅₀) (Fig. 3A;

Mann-Whitney U test, $p < 0.001$). In daphnids exposed to Calypso 480 SC maximal 1.8 fold increase in enzyme activity was measured at the 5.2 mg L^{-1} dose ($=1/20$ of 48h-EC_{50}). Further increase in treatment dose resulted in a progressive decrease in ECOD activity even significantly below the baseline level (by 25%; Mann-Whitney U test, $p < 0.001$) characteristic for untreated daphnids (Fig. 3B).

4. Discussion

The tested neonicotinoid formulations exerted significant alterations in the thoracic limb activity and the heart beating rate of five days old daphnids only in concentrations approaching the *D. magna* 48h-EC_{50} thresholds (Mospilan 20 SG: $186 \text{ (CI: } 171 - 214) \text{ mg L}^{-1}$; Calypso 480 SC: $119 \text{ (CI: } 108 - 130) \text{ mg L}^{-1}$). At the highest doses applied, both neonicotinoids elicited closely similar reduction (by 44- and 46% respectively) in the beating frequency of the thoracic limbs. Such a drop in thoracic limb activity reduces both the overall food uptake and gas exchange by organisms, leading to reduced energy allocation, growth performance and reproduction, which finally may severely compromise the chances of survival of affected organisms (Friberg-Jensen et al., 2010; Lari et al., 2017b). Reduction in thoracic limb activity of daphnids was also reported in relation to high concentrations of particulate matter including food (Kirk, 1991; Peñalva-Arana et al., 2007; Lovern et al., 2007; Lari et al., 2017a) but also in response to dissolved toxicants as well (Friberg-Jensen et al., 2010; Lari et al., 2017a; Bownik and Stępniewska, 2015; Bownik et al., 2018; Bownik et al., 2019a; 2019b). A decrease in the thoracic limb activity of daphnids in the presence of high particulate matter densities is considered a natural response (Lari et al., 2017a), while in the case of dissolved substances the inhibition of this physiological feature is supposed to be

linked to the depressive action of bioactive compounds on the Na⁺/K⁺ ATP-ase activity resulting in a decreased transmission between neurons and muscles of the thoracic limbs (Bownik et al., 2019a).

The characteristic effects of the two neonicotinoid formulations on the heart beating frequency of daphnids was slight (but statistically insignificant) increase in the low dose exposures, followed by significant depression at doses close to the 48h-EC₅₀ of the products.

Early studies by Ermakov (1937 as cited by Smirnov, 2013) outlined that Ca²⁺ concentration plays a role in adjusting the heart rate of *D. magna*. Ingle et al. (1937 as cited by Steinkey et al., 2019) stated that an increase in the heart rate of *Daphnia* is most probably due to a downregulation in Ca²⁺ concentration, as an increase in Ca²⁺ concentration was proven to reduce the heart rate of daphnids through induction of diastolic arrest. Bekker and Krijgsmann (1951), in their investigation focused on mapping the mechanism of the heart in *Daphnia*, reported heart rate depression in daphnids exposed to nanomolar concentrations of acetylcholine or micromolar concentrations of tetraethylpyrosphosphate, a phenomenon presumed to be a specific toxic response of the myogenic pacemaker of the *Daphnia* heart.

As significant alterations in thoracic limb activity and heart rate in exposed daphnids were detected at doses close to the immobility thresholds of the products, it is assumed that these responses are attributable to a significant drop in the general health status of organisms, rather than a specific toxic action of the products.

In contrast to the physiological responses, a significant dose-dependent alteration in the ECOD activity of daphnids exposed to the neonicotinoid formulations was detected at lower (much below EC₅₀ thresholds) doses of pesticide formulations. Exposure of daphnids to the neonicotinoid formulations elicited significant dose-dependent induction in the ECOD

activity of organisms, suggesting that the cytochrome P450 mediated metabolism in daphnids was activated. Effective concentrations that significantly induced the ECOD activity of daphnids were well below the acute toxicity thresholds of the products, which in terms of active ingredient contents corresponded to 1.3 mg L⁻¹ acetamiprid and 0.014 mg L⁻¹ thiacloprid. These data seem to have environmental relevance particularly for Calypso 480 SC because this effective dose already approaches thiacloprid detections of 0.02 – 4.50 µg L⁻¹ reported in surface waters worldwide (Sanchez-Bayo and Hyne, 2014; Velisek and Stara, 2018). This presumption is further supported by Morrissey et al. (2015) who reported for individual neonicotinoid concentrations in surface waters a geometric mean of 0.13 µg L⁻¹ and a geometric mean for peak concentrations of 0.63 µg L⁻¹. Moreover, it was also outlined that these mean concentrations, computed from grab or spot samples, may underestimate by 50% the mean values (Xing et al., 2013) and mean peak loads by 1-3 orders of magnitude. Taking into consideration this last presumption, effective concentration thresholds that altered the ECOD activity in daphnids established in our study do not entirely exclude the possibility of toxic incidences caused by these commercial pesticides even under field conditions therefore, these data may have relevance even in relation to environmental risk assessment.

In daphnids, upregulation of certain cytochrome P450 genes were reported upon chronic exposure to acetaminophen (Kim et al., 2018) or to polystyrene nanoplastics (Wu et al., 2019). In addition, net inhibition toward cytochrome P450 activity (ECOD) by azole fungicides was demonstrated by Dalhoff et al., (2016). The results of our study demonstrate that the cytochrome P450 monooxygenase system of daphnids was significantly induced by the tested neonicotinoid formulations, and this metabolic pathway may contribute to the

detoxification of the bioactive compounds present in the formulations, thus serving some protection against the toxicity of these pesticides.

5. Conclusions

The above data revealed that although sublethal concentrations of the acetamiprid- and thiacloprid based pesticides (Mospilan 20 SG and Calypso 480 SC, respectively) elicited only minor physiological alterations (decreased the thoracic limb beating frequency and heart rate), the biochemical compensatory mechanisms (activation of the cytochrome P450 monooxygenase system) started to respond from concentrations comparable to neonicotinoid concentrations already reported for surface waters. Our results therefore, suggest a relevant role for this metabolic pathway in the detoxification of the bioactive compounds present in formulations, moreover, support the concept to consider this biochemical marker (ECOD activity) to be used as a bioindication trait for environmental risk evaluation when neonicotinoid insecticides are concerned.

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