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Toxicity of the insecticides Spinosad and Indoxacarb to the non-target aquatic midge *Chironomus riparius*

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Abstract

Spinosad and indoxacarb are two relatively new insecticides mainly used in agriculture to control insect pests. However, at their current application rates, non-target aquatic insect species may also be impacted. In this study, larvae of the non-biting midge *Chironomus riparius* were exposed in the laboratory to both insecticides and their effects evaluated at the organismal level, using standard ecotoxicological tests, and at the biochemical level, by monitoring specific oxidative stress, neuronal, and energy metabolism biomarkers. Chronic exposure to both insecticides compromised growth and emergence of *C. riparius*. Short-term exposures revealed alterations at a biochemical level that might be related to the toxicological targets of both insecticides. Growth and development time were the most sensitive endpoints at individual level for both pesticides, while at the biochemical level, the electron transport system activity was the most sensitive biomarker for spinosad exposure, suggesting an increase in energy demands associated with the activation of defense mechanisms, and Glutathione-S-transferase was the most sensitive biomarker for indoxacarb exposure, underlining the role of this enzyme in the detoxification of indoxacarb. Additionally, changes in lactate dehydrogenase and glutathione peroxidase activities were observed for both insecticides, and evidences of oxidative damage were found for spinosad. This study contributes to the growing knowledge on sublethal effects of novel insecticides on non-target aquatic invertebrates and strengthens the usefulness of biochemical biomarkers to support the interpretation of their potentially deleterious effects on aquatic insects near agricultural fields.

Keywords: aquatic invertebrates, biochemical biomarkers, insecticides, life-history effects, neurotoxicity

1. Introduction

One of the goals in integrated pest management is to find tailor-made and effective pesticides for specific pests while keeping adverse consequences on non-target species to a minimum (Chitgar and Ghadamyari, 2012; Stara et al., 2010; Wilkinson et al., 1979). Some non-target aquatic insects play vital roles in freshwater ecosystems, and are regularly subjected to significant concentrations of pesticides through runoff, drift, or leaching from adjacent agricultural fields (Cerejeira et al., 2003; Schulz, 2004). Ecotoxicological effects of pesticide exposure seen on higher levels of organization are often preceded by quantifiable alterations at biochemical levels and assessing earlier sub-organismal endpoints on key species may provide insights on the long-term consequences for natural populations (Lemos et al., 2010), hopefully providing regulators with early-warning tools for risk assessment.

Spinosad and indoxacarb are neurotoxic insecticides with distinct modes of action registered for agricultural use in Europe (European Commission, 2006a; 2008a). However, the toxicity data for these relatively novel insecticides on aquatic invertebrates is still very limited, considering that according to regulation (EC) No 1272/2008 (European Commission, 2008b) they are both classified as very toxic to aquatic life with long lasting effects.

Spinosad targets a unique site in nicotinic acetylcholine receptors (Copping and Menn, 2000; Orr et al., 2009), causing hyperexcitation of the nervous system

(Salgado, 1998; Salgado et al., 1998; Salgado and Sparks, 2005). It is very effective against several insect species (Hertlein et al., 2010), including chironomids (Bond et al., 2004; Lawler and Dritz, 2013; Pérez et al., 2007; Stevens et al., 2005). Recently, the European Food Safety Authority predicted an environmental concentration of 26.28 μg L^{-1} on surface waters resulting from the applications on leafy and fruity vegetables (worst case scenario) (EFSA et al., 2018a). Moreover, spinosad sorbs to the sediment where it seems to be more persistent (Cleveland et al., 2002), and where many sediment-dwelling organisms, including chironomid larvae, may be affected.

Indoxacarb acts by blocking voltage-dependent sodium channels, causing nervous system shutdown (Lapied et al., 2001; Wing et al., 1998; Wing et al., 2000). It is effective against several insect species (Anikwe et al., 2014; Dryden et al., 2013; Oxborough et al., 2015; Pridgeon et al., 2009), but particularly to lepidopterans (Dias, 2006; Wing et al., 1998; Wing et al., 2000). In 2003, indoxacarb estimated environmental long-term average concentrations in surface waters was of 3.7 μg L^{-1}, with peak values of 13.7 μg L^{-1} (EPA, 2003). More recently, levels up to 7.763 μg L^{-1} resulting from indoxacarb's application in lettuce crops were predicted for surface waters (EFSA et al., 2018b). Additionally, Indoxacarb also has a relatively high log Kow of 4.65 (Dias, 2006) suggesting it has a high tendency to sorb to sediments.

The freshwater midge *Chironomus riparius* Meigen (Diptera: Chironomidae) is a widely used model organism in ecotoxicology testing (Weltje et al., 2010) mainly due to its ecological relevance and easiness to handle in the laboratory. Additionally, *C. riparius* larvae have been previously used as a model to evaluate biochemical responses of insecticide exposure (Rodrigues et al., 2015a; Rodrigues et al., 2015b).

The main aim of this study was to investigate organismal and biochemical

effects of two neurotoxic insecticides on the aquatic invertebrate *C. riparius*. For that, environmentally relevant concentrations of spinosad and indoxacarb were used to investigate sub-lethal effects in terms of larval growth, development, and emergence using standard ecotoxicological tests. Moreover, the effects of these insecticides at the biochemical level were determined to evaluate their possible relation to the effects observed at the organismal level and their potential use in biomonitoring studies.

Biochemical biomarkers associated with key physiological functions were selected, such as: activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), essential in the protection against reactive oxygen species (ROS) induced by the exposure to xenobiotics; DNA damage and lipid peroxidation (LPO), as indicators of oxidative damage, resulting from excessive ROS; activity of glutathione-S-transferase (GST), a phase II biotransformation enzyme, involved in the detoxification of xenobiotics; acetylcholinesterase (AChE) activity, related to the cholinergic neurotransmission, as a biomarker of neuromuscular toxicity; the activities of lactate dehydrogenase (LDH) and electron transport system (ETS) were assessed as measures of energy metabolism.

2. Material and Methods

2.1 Test organism

Chironomus riparius larvae were collected from a laboratory culture long established at the University of Aveiro, Portugal. Larvae are kept in plastic aquaria filled with a fine layer of washed and burnt river sand (<1 mm) and American Society

for Testing Materials (ASTM) hard water. Cultures were maintained at 20 °C with a photoperiod of 16:8 h light-dark, with a constant inflow of air. Larvae are fed *ad libitum* with macerated fish food, Tetramin® (Melle, Germany).

2.2 Test chemicals

Spinosad (CAS number 168316-95-8), a mixture of spinosyns A and D (Crouse et al., 2001), two byproducts of the fermentation of *Saccharopolyspora spinosa* (Actinomycetales: Pseudonocardiaceae) (Mertz and Yao, 1990; Thompson et al., 2000), and Indoxacarb (CAS number 144171-61-9), an oxadiazine insecticide, were acquired from Sigma-Aldrich, UK. Stock solutions of the pesticides were prepared in ethanol (spinosad) and acetone (indoxacarb). Experimental solutions were prepared by diluting the stock solutions in ASTM hard water, and the final solvent concentration was kept at 0.01%.

2.3 Acute toxicity tests

Acute lethal toxicity was assessed following OECD guideline 235 (OECD, 2011) with water only exposures in crystalizing dishes, using 1st instar larvae. Larvae were exposed to concentrations of spinosad of 0 (solvent control), 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg L^{-1} and to 0, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μg L^{-1} of indoxacarb. After 48 h of exposure, mortality was checked by mechanical stimulation. To halt possible photodegradation of the chemicals, crystalizing dishes were protected from the light

during the test. The test was executed at 20 ± 1 °C, and larvae were not fed during the exposure.

2.4 Chronic toxicity tests

A 28-day chronic test was performed according to the OECD guideline 219 (OECD, 2004). First instar larvae of *C. riparius* (2 days old) were exposed to 0 (negative and solvent control), 0.5, 1.28, 3.2, 8, and 20 μg L^{-1} of spinosad in 150 mL of medium and layer of 1.5 cm of sediment (commercial river sand washed, sieved and burnt) in 200 mL glass vessels. A similar setup was made for indoxacarb, using 0, 1, 2, 4, and 8 μg L^{-1} treatments. Five larvae were used in each replicate, and five replicates were used for larval growth determination, while eight replicates were used for emergence endpoints. After ten days of exposure larvae growth was determined by measuring body length of the larvae with the aid of a stereomicroscope fitted with a calibrated micrometer and growth was calculated by subtracting the mean body length at the beginning (pool of 25 larvae of initial size). In the eight remaining replicates, adult *C. riparius* were collected daily, their gender determined and stored in 70% ethanol. Afterwards, adult midges were dried at 50 °C for 24 h and weighed with a microbalance (Mettler UMT2).

The tests were performed under the same conditions described for culturing: 20 ± 1 °C with 16:8h light:dark cycle with gentle aeration. Organisms were fed every two days at a ration of 0.5 mg Tetramin® $larvae^{-1}$ day^{-1}, and physicochemical parameters were checked throughout the experiment.

2.5 Biomarkers

For the determination of the biochemical biomarkers, the tests were initiated with 8 days old larvae (3rd instar). The concentrations used in these bioassays were 0, 0.5, 2, and 8 μg L^{-1} for spinosad and 0, 2, 4, and 8 μg L^{-1} for indoxacarb. Each crystalizing dish contained ten larvae and 80 mL of experimental solution and a fine layer of sediment. After 48 h, organisms from two replicates of the same treatment were pooled to give a total of twenty organisms per replicate. Six pooled replicates were used per treatment for spinosad, and seven pooled replicates for indoxacarb. Afterwards, excess water was gently removed with a filter paper, and organisms weighed, frozen with liquid nitrogen, and stored at -80 °C until further processing.

Samples were subsequently homogenized in 800 μL of 0.1 M of K-phosphate buffer (pH = 7.4) using a Ystral d-79282 homogenizer. This homogenate was divided into portions for ETS, LPO, and DNA damage determination. To LPO portion, 4% 2,6-Di-tert-butyl-4-methylphenol (BHT) in methanol was added to prevent subsequent lipid oxidation of the samples (Aloísio Torres et al., 2002). These three portions were immediately stored at -80 °C until used. The remaining homogenate was centrifuged at 10000 *g* for 20 min at 4 °C and the supernatant (post-mitochondrial supernatant) was collected and divided into portions for SOD, CAT, GST, GR, GPX, AChE, and LDH activities determination and for protein quantification. In every essay, reaction blanks were performed using K-phosphate buffer instead of the sample and all spectrophotometric measurements were made at 25°C using a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek® Instruments, Vermont, USA).

2.5.1 Protein quantification

Protein concentration was assessed following the Bradford protocol adapted to microplate, using γ-globuline as standard. Prior to AChE, CAT, GR, GPx, GST, and LDH activities determination, protein concentration was adjusted to approximately 0.8 mg L^{-1}. For these biomarkers, the exact protein concentration of the dilution was measured again at the end of the experiment.

2.5.2 Detoxification, oxidative stress and oxidative damage biomarkers

SOD activity was determined by following the method described by McCord and Fridovich (1969) adapted to microplate. Cytochrome c reduction was followed for 5 min at 550 nm, and results are expressed as SOD units (U) mg^{-1} protein. The determination of CAT activity was made according to Clairborne (1985). The consumption of H_2O_2 was assessed at 240 nm for 1 min, and results are expressed in μmol min^{-1} mg^{-1} of protein. For the assessment of GR activity, the method described by Cribb et al. (1989) was used. The oxidation of NADPH was monitored at 340 nm during 1 min, and results are expressed in nmol min^{-1} mg^{-1} of protein. Regarding GPx activity, it was determined by monitoring the oxidation of NADPH at 340 nm for 3 min, as a result of GR conversion of GSSG to GSH (Mohandas et al., 1984). Results are expressed in nmol min^{-1} mg^{-1} of protein. An adaption of Habig et al. (1974) protocol to microplate was used to determine GST activity. The formation of glutathione dinitrobenzene was measured at 340 nm during 3 min, and results are expressed in nmol min^{-1} mg^{-1} of protein. LPO levels were measured using thiobarbituric acid

reactive substances (TBARS) assay (Bird and Draper, 1984; Ohkawa et al., 1979). Absorbance was read at 535nm and results are expressed in nmol TBARS g^{-1} of wet weight. DNA damage was assessed following the protocols described by de Lafontaine et al. (2000) and Olive (1988). Fluorescence was measured using an excitation/emission wavelength of 360/460 nm, and results are expressed as ng of damaged DNA mg^{-1} of wet weight.

2.5.3 Neurotransmission and energy related biomarkers

Effects of spinosad and indoxacarb on cholinergic neurotransmission were evaluated monitoring AChE activity, following Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). The absorbance was read at 414 nm for 5 minutes, and results are expressed in nmol min^{-1} mg^{-1} of protein. To determine the activity of anaerobic metabolism-related enzyme LDH, oxidation of NADH was monitored at 340 nm as proposed by Vassault (1983) and Diamantino et al. (2001). Results are expressed in nmol min^{-1} mg^{-1} of protein. ETS activity was determined following De Coen and Janssen (1997) with some adaptations (Rodrigues et al., 2015b). Absorbance was read at 490 nm for 5 minutes, and results are expressed mJ h^{-1} mg of $protein^{-1}$.

2.6 Statistical analysis

Effects of insecticide exposure on life history and biochemical endpoints were evaluated by one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc

test to determine statistically significant differences between solvent controls and treatments, and/or by a test for linear trend to discriminate if there is a linear increase or decrease in response as the concentration increases. Data were checked for residual normality using D'Agostino-Pearson and Shapiro-Wilk normality tests and for homoscedasticity with Brown-Forsythe test. Unpaired *t*-tests did not find differences between negative and solvent controls for any of the endpoints analysed, therefore solvent control was used as the control for all analysis. Spinonad's DNA damage data were log-transformed to correct for normality. For spinosad LPO data and for indoxacarb percentage of emergence data, transformations did not correct for normality, but since homogeneity of variances was verified, one-way ANOVA was executed. Since all larvae in the spinosad chronic test exposed to 20 $\mu g\ L^{-1}$ died, this treatment was excluded from analysis. Statistical analysis was made in GraphPad Prism® 7 for Mac and significance level was set at 0.05.

3. Results

3.1 Spinosad

For spinosad, in the highest concentration tested, there was 40% mortality after 48 h of exposure (Supplementary data, table I). Because of the gradients of concentrations used for spinosad, the 48 h LC50 could not be estimated and thus is higher than 256 $\mu g\ L^{-1}$. Concerning the chronic bioassay, at day 10 no larvae were alive at the highest concentration tested (20 $\mu g\ L^{-1}$) while 92% of the larvae were recovered from the control. Additionally, at day 10 of exposure, statically significant differences

were found for growth between control and the 8 μg L^{-1} treatment ($F_{(4,20)} = 7.640$, $p < 0.001$) (Table I). Regarding emergence parameters, there was a significant increase in time to emergence at 8 μg L^{-1} for both males ($F_{(4,27)} = 3.831$, $p < 0.05$) and females ($F_{(4,26)} = 3.606$, $p < 0.05$) (Table I). No adults have emerged in the 20 μg L^{-1} treatment, and although overall ANOVA was not significant for the remaining treatments ($F_{(4,34)} = 2.295$, $p = 0.079$), Dunnett's test discriminated differences between control and 8 μg L^{-1} treatments in terms of percentage of emerged adults. No effects were found on adult weight (NOEC = 8 μg L^{-1}) (Table II).

To what concerns biochemical biomarkers, exposure to spinosad significantly increased GPx activity in *C. riparius* larvae in the highest concentration ($F_{(3,20)} = 7.601$, $p < 0.01$; Fig. 2b), and LDH activity in the 2 μg L^{-1} treatment ($F_{(3,19)} = 8.357$, $p = 0.001$; Fig. 1d). There was also a significant increase in LPO at the two highest concentrations tested ($F_{(3,20)} = 4.87$, $p < 0.05$; Fig. 1a) and, although not significant, DNA damage also increased in the same treatments ($F_{(3,20)} = 2.651$, $p = 0.077$, Fig 1b). ETS activity was the most sensitive biomarker, with a significant increase observed for all tested concentration ($F_{(3,20)} = 31.76$, $p < 0.001$; LOEC = 0.5 μg L^{-1}, Fig 1e). Moreover, this increase was concentration-dependent ($r^2 = 0.83$, $p < 0.001$). No significant alterations were detected for AChE, CAT, GR, GST, and SOD activities (Figures 1-2).

2.2 Indoxacarb

In acute tests and for the highest concentration of indoxacarb tested, there was 47% mortality after 48 h of exposure (Supplementary data, table I). The 48 h LC50 of indoxacarb was estimated to be higher than 128 μg L^{-1}. Chronic exposure to

indoxacarb led to a decrease in larval growth in the highest concentration tested (8 μg L^{-1}; $F_{(4,19)} = 4.746$, $p < 0.01$) (Table III). Moreover, indoxacarb exposure led to a delay in emergence of males ($F_{(4,32)} = 11.96$, $p < 0.001$) and females ($F_{(4,33)} = 6.031$, $p < 0.001$). No effects were observed for the percentage of emerged adults (Table III) nor for adult weight (NOEC = 8 μg L^{-1}) (Table IV).

Regarding biochemical biomarkers, GPx activity increased in the highest concentration tested ($F_{(3,24)} = 5.055$, $p < 0.01$; Fig. 4b). Exposure to indoxacarb significantly increased LDH activity in the highest concentration tested ($F_{(3,23)} = 3.331$, $p < 0.05$; Fig. 3d), and this increase was dose-dependant ($r^2 = 0.30$, $p < 0.01$; Fig. 3d). GST activity increased from concentration 4 μg L^{-1} onwards ($F_{(3,24)} = 4.81$, $p < 0.01$; Fig. 3c). For SOD activity, although ANOVA was significant, the post hoc test did not find any significant differences between the control and the experimental treatments ($F_{(3,24)} = 4.21$, $p < 0.05$; Fig. 4d). No significant alterations were detected for the remaining biomarkers studied.

4. Discussion

Spinosad and Indoxacarb are neurotoxic insecticides highly effective in controlling insect pests which were initially deemed as relatively safe for non-target species (Bacci et al., 2016; Boucher and Ashley, 1999; Jones et al., 2005; Lahm et al., 2000; Liu and Zhang, 2012; Sarfraz et al., 2005). Although there is no recent literature available on measured levels of both chemicals in natural freshwater environments, the concentrations used in this study are within the estimated environmental levels,

and clearly impaired *C. riparius* life-history traits with alterations at the biochemical level also observed.

Chronic exposures to indoxacarb and spinosad produced comparable outcomes in terms of *C. riparius* life history traits: growth reduction observed after 10 days of exposure at 8 μg L^{-1} of spinosad and at 8 μg L^{-1} of indoxacarb translated into a delay in development of both males and females, but interestingly, did not result in a reduction of imagoes weight. This suggests that *C. riparius* larvae were capable of recovering and reaching the desired weight, at the expense of longer development time. This trade-off is not unusual as body weight is associated with the reproductive output of chironomids (Sibley et al., 2001) - nonetheless, a delay in development time is still an important ecological driver as it can have direct consequences on synchrony of emergence and mating success, thus affecting population dynamics (Sibley et al., 1997). This is particularly relevant on protandrous species such as *C. riparius*. Still, a full life-cycle test contemplating reproductive endpoints such as fecundity and fertility could provide a better estimate of population-level effects and should be considered in future studies. The main dissimilarity observed between the effects of the two compounds at the organismal level, was that spinosad exposure also affected *C. riparius* survival: there was a reduction in the number of emerged adults at 8 μg L^{-1}, and at 20 μg L^{-1} no imagoes have emerged. Previous data indicated a NOEC (no observed effect concentration) of 0.62 μg L^{-1} for *C. riparius* (EFSA et al., 2018a), however, in the present study a NOEC of 3.2 μg L^{-1} and a LOEC (lowest observed effect concentration) of 8 μg L^{-1} for larval growth and emergence were observed under exposure to spinosad. Information on the chronic risk to aquatic organisms, including sediment dwellers is lacking (EFSA et al., 2018a). Regarding other dipterans, a spinosad

concentration of 17 μg L^{-1} has been demonstrated to decrease the emergence of *Polypedilum nubifer* (Duchet et al., 2015). Concentrations ranging from 3.7 to 45 μg L^{-1} seem to affect *Culex pipiens* emergence (Hertlein et al., 2010), while at 60 μg L^{-1} Cetin et al. (2005) reported a complete inhibition of *Culex pipiens* adult emergence. Tomé et al. (2014) determined that exposure to spinosad compromises swimming behavior of *Aedes aegypti*. Behavioral changes have been demonstrated for many neurotoxic compounds and can lead to a reduction in food intake (Pestana et al., 2009; 2010; Tomé et al., 2014; Werner and Moran, 2009), which, although not addressed, might have also occurred here with *C. riparius* and contributed to the reduced growth and developmental rates. Considering other aquatic invertebrates, impairment of population growth rate by spinosad was described for *Daphnia pulex* and *Daphnia magna* at 8 μg L^{-1} (Duchet et al., 2010) and for *Ceriodaphnia dubia* at 1 μg L^{-1} (Deardorff and Stark, 2011).

Regarding the long-term effects of indoxacarb on chironomids, the information available is very limited. Still, a 28-day EC10 of 1.68 μg L^{-1} (endpoint not specified) and a 28-day NOEC (development rate) of 1.8 μg L^{-1} (active substance) were previously determined for *C. riparius* (EFSA et al., 2018b). In the present study, a NOEC of 4 μg L^{-1} was observed for development and emergence endpoints. Ding et al. (2011) investigated the effects of pesticide-contaminated sediments on *C. dilutus*, and the authors concluded that indoxacarb was amongst the most toxic sediment-associated pesticides to *C. dilutus* they tested (10-day LC50 of 11.3 μg g_{oc}^{-1}; growth NOEC of 3.2 μg g_{oc}^{-1}). Available information shows that commercial formulations of indoxacarb affect life history traits of some insect pest species (Gamil et al., 2011; Martin et al., 2006; Saryazdi et al., 2012), however present results clearly show that life history of non-

target aquatic insects may also be altered.

Although the biomarkers exposure experiment does not reflect the entire exposure duration of the chronic toxicity test, short exposures to low concentrations of both insecticides tested induced several biochemical changes in *C. riparius* larvae. As expected, due to their distinct modes of action, different responses were observed at the biochemical level.

GPx, CAT, and SOD are first-line defense antioxidant enzymes against reactive oxygen species. SOD catalyzes the conversion of superoxide anions to hydrogen peroxide (H_2O_2), which is subsequently detoxified by CAT and GPx (Ighodaro and Akinloye, 2007). The increase in GPx activity induced by spinosad exposure may have occurred to prevent the accumulation of H_2O_2 due to increased oxygen metabolism. GPx has a higher affinity for H_2O_2 than CAT (Lushchak, 2012), which may explain why GPx activity increased while catalase activity remained unchanged. The increase in GPx activity was, however, insufficient to prevent oxidative damage, as indicated by the increase of LPO levels and the perceptible increase of DNA damage. The concomitant increase in LPO and GPx has been previously observed in the kidney of *Oreochromis niloticus* (Piner and Uner, 2014) and in mammalian cell lines (Pérez-Pertejo et al., 2008) exposed to the same insecticide. Increased LPO levels are indicative of cellular damage, which may have contributed to the effects observed at the organism level.

Spinosad also led to the increase in ETS activity, an indicator of cellular oxygen metabolism, and LDH activity, involved in the anaerobic pathway of energy production, indicating high levels of energy consumption and high metabolic demand (Rodrigues et al., 2015a; Silva et al., 2016). This increase in energy demand may be associated with the activation of antioxidant mechanisms, as implicit by the increase of GPx and/or

other defense mechanisms that were not addressed here. Moreover, an increase in metabolic costs due to the induction of defense mechanisms may also, in part, explain observed reductions in growth and development, since less energy will be available for other physiological processes.

Spinosad's inhibitory effects on AChE activity have been reported for other insect species (El-Mageed and Elgohary, 2006; Maiza et al., 2013; Rabea et al., 2010; Tine et al., 2015), and as a nicotinic acetylcholine receptor modulator, some alterations in AChE activity were anticipated. However, the 48h exposure to the tested concentrations did not induce changes in AChE activity of *C. riparius* larvae. Azevedo-Pereira et al. (2011) work with *C. riparius* larvae has also revealed that a 48h exposure to imidacloprid, an insecticide that also targets nicotinic acetylcholine receptors, did not induce alterations in AChE activity. The authors indicated that inhibitory effects of imidacloprid on AChE were only detected after 96h of exposure and in the post-exposure period, and yet behavioral changes were linked to AChE activity (Azevedo-Pereira et al., 2011). Given the information available in the literature, it is possible that 48h exposure to spinosad was not enough to impair AChE activity. Follow-up tests should be performed with prolonged exposure periods, to evaluate the possible extent of spinosad toxic effect on *C. riparius* AChE. Nonetheless, this short-exposure triggered alterations on other biochemical biomarkers, indicating that secondary mechanisms might also be accountable for spinosad's toxicity to *C. riparius*, such as the interference with gamma-aminobutyric acid receptors or others (Salgado and Sparks, 2005).

Regarding the effects of indoxacarb at the biochemical level, GST was the most sensitive endpoint. GST, an enzyme involved in biotransformation and detoxification (Clark, 1989), has been categorized as an ineffective biomarker of pesticide exposure in

C. riparius (Hirthe et al., 2001), and some works endorse this assumption due to its disparate responses to different pesticides (Planelló et al., 2013). Regardless, in this study, an increase in GST activity as a result of indoxacarb exposure was observed in *C. riparius* larvae. An identical response to indoxacarb was observed in *Blattella germanica* and *Spodoptera littoralis* larvae (Gamil et al., 2011; Maiza et al., 2013). Additionally, Nehare et al. (2010) and Pang et al. (2012) postulated that the detoxification by GST might play a relevant role in indoxacarb resistance. GPx activity also increased in larvae exposed to indoxacarb. Since there were no changes in oxidative damage indicators (LPO and DNA damage), it is suggested that GPx activity and detoxification by GST contributed to preventing oxidative damage in a short-term exposure. As opposed to spinosad, only the anaerobic metabolism (LDH) was induced by indoxacarb in *C. riparius* larvae, since no changes were detected in ETS activity. This induction of LDH may occur due to higher and more readily available energy demands for the activation of GPx and GST, and again this might have contributed to the effects observed at the individual level (reduction in larval growth and increase in time to emergence).

This study elucidates some biochemical responses to spinosad and indoxacarb exposure that may be associated with the effects observed at the organismal level. The induction of defense mechanisms and higher energy expenditures are most likely direct responses *of C. riparius* larvae to cope with the exposure, while oxidative damage may be a direct consequence of spinosad's mechanism of action and may have contributed to the slightly more severe effects observed. Although not specific for insecticide exposure, biochemical biomarkers addressed in the present study may

be valuable early-warning tools in biomonitoring studies, since changes were observed in short exposures and at lower concentrations than the ones causing effects at the organismal level. ETS was the most sensitive biochemical biomarker for spinosad, as it was responsive to 0.5 $\mu g\ L^{-1}$, while for indoxacarb GST was the most sensitive biomarker (LOEC of 4 $\mu g\ L^{-1}$), underlining the role of GST in the detoxification of indoxacarb.

Our findings revealed that under controlled laboratory conditions, spinosad is slightly more toxic to *C. riparius* than indoxacarb since, besides the reduction of larval growth and the increase in time to emergence, a reduction in emergence rate was also observed. Chironomids larvae play a vital role in freshwater ecosystems due to their abundance and food chain position (Péry et al., 2002), and therefore the application of spinosad and indoxacarb near freshwater systems at current rates should be monitored and reviewed, since the concentrations used in this work and that elicited clear deleterious effects are within the estimated environmental levels. Moreover, through their emergence, chironomids represent an important food source for riparian predators, and therefore changes in the emergence of aquatic insects can also have implications for the terrestrial food webs (Schulz et al., 2015).

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Figure captions

Table I – Growth (n = 5) and emergence endpoints (n = 8) of *C. riparius* larvae exposed to Spinosad. All values are presented as mean ± SEM. An asterisk denotes statistically significant differences to the control treatment (0 μg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test). A number sign denotes statistically significant differences to the control treatment (0 μg L^{-1}; Dunnett's test) when overall ANOVA is not significant ($p = 0.079$).

Spinosad Concentrations (μg L^{-1})	Growth (mm)	Total emergents (%)	Development time (days)	
			Males	Females
0	12.3 ± 0.2	80.0 ± 7.6	15.3 ± 0.4	16.8 ± 0.4
0.5	11.6 ± 0.3	65.0 ± 9.1	15.2 ± 0.3	17.1 ± 0.4
1.28	11.7 ± 0.3	65.0 ± 7.3	15.3 ± 0.3	17.2 ± 0.3
3.2	10.9 ± 0.5	72.5 ± 8.4	15.0 ± 0.3	17.9 ± 0.6
8	8.8 ± 0.9*	45.0 ± 9.8#	17.1 ± 0.7*	19.9 ± 1.1*
20	N.C.	N.C.	N.C.	N.C.

N.C. – not calculated due to 100% mortality

Table II – Adult weight of *C. riparius* exposed as larvae to Spinosad. All values are presented as mean ± SEM, n = 8.

Spinosad Concentrations (μg L^{-1})	Males dry weight (mg)	Females dry weight (mg)
0	0.542 ± 0.012	1.084 ± 0.037
0.5	0.540 ± 0.013	1.084 ± 0.019
1.28	0.568 ± 0.017	1.063 ± 0.036
3.2	0.524 ± 0.014	1.035 ± 0.033
8	0.521 ± 0.027	1.118 ± 0.091
20	N.C.	N.C.

N.C. – not calculated due to 100% mortality

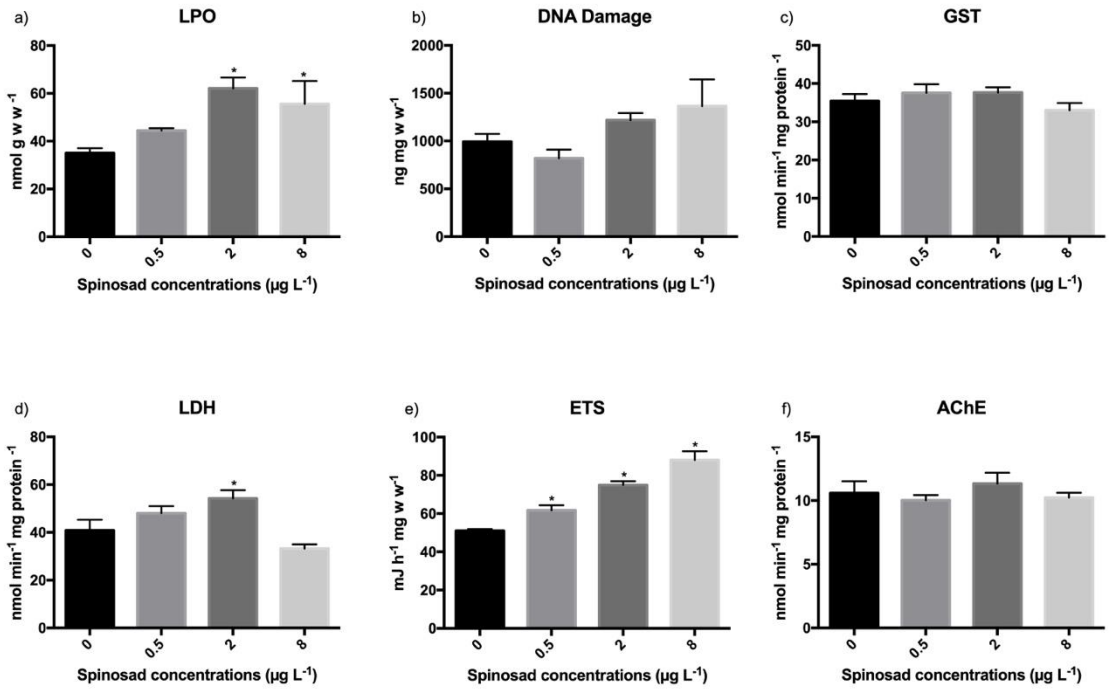

Figure 1 – Oxidative damage, biotransformation, energetic metabolism and neuronal biomarkers in *C. riparius* larvae after 48h exposure to spinosad: a) Lipid Peroxidation; b) DNA Damage; c) Glutathione-S-Transferase; d) Lactate Dehydrogenase; e) Electron Transport System; f) Acetylcholinesterase. All values are presented as mean + SEM, n = 7. An asterisk denotes statistically significant differences to the control treatment (0 µg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

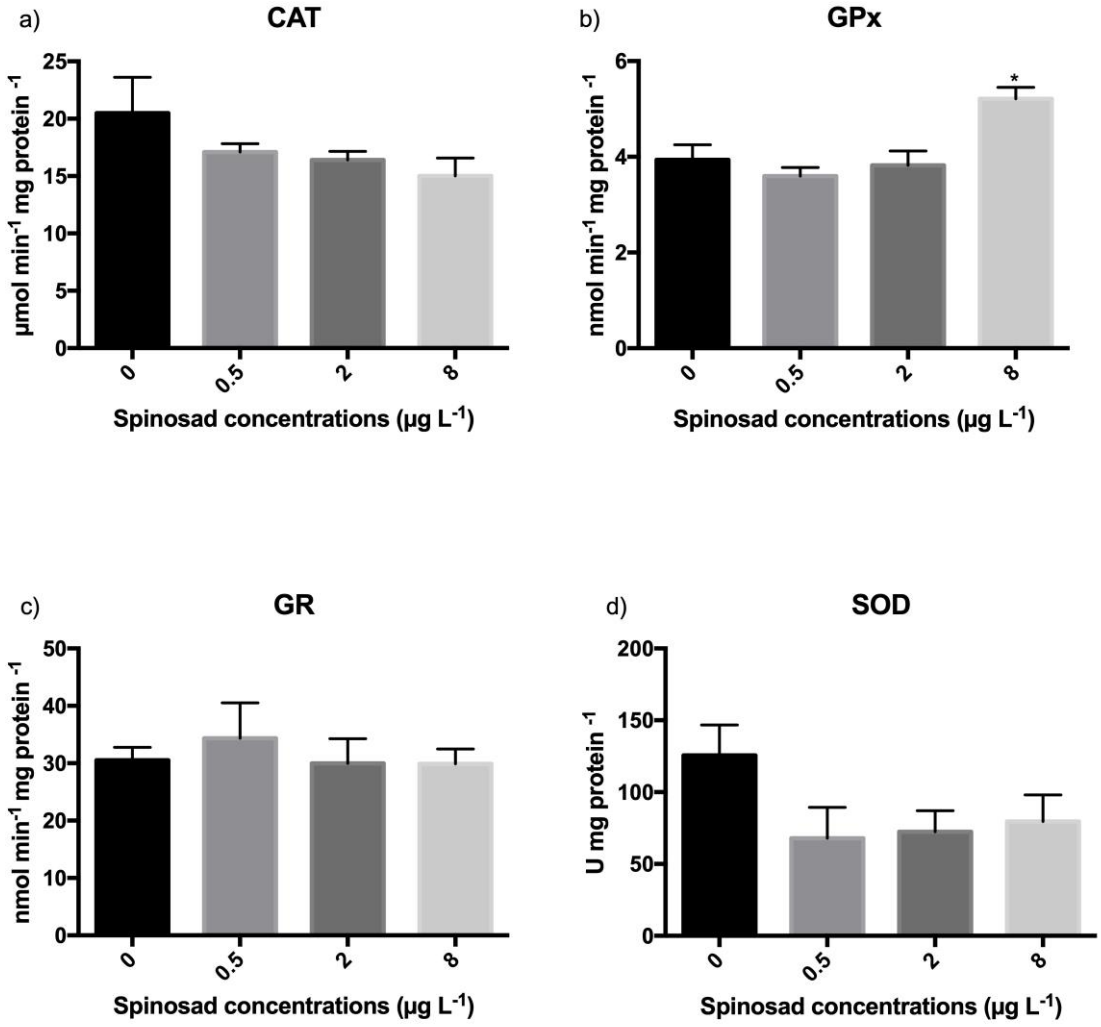

Figure 2 – Oxidative stress biomarkers in *C. riparius* larvae after 48h exposure to spinosad: a) Catalase; b) Glutathione Peroxidase; c) Glutathione Reductase; d) Superoxide Dismutase. All values are presented as mean + SEM, n = 7. An asterisk denotes statistically significant differences to the control treatment (0 µg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

Table III – Growth (n = 5) and emergence endpoints (n = 8) of *C. riparius* larvae exposed to Indoxacarb. All values are presented as mean ± SEM. An asterisk denotes statistically significant differences to the control treatment (0 µg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

Indoxacarb Concentrations (µg L^{-1})	Growth (mm)	Total emergents (%)	Development time (days)	
			Males	Females
0	11.7 ± 0.3	90.0 ± 5.4	17.0 ± 0.3	18.4 ± 0.6
1	11.3 ± 0.1	90.0 ± 3.8	16.0 ± 0.3	19.5 ± 0.5
2	11.6 ± 0.6	85.0 ± 6.3	16.2 ± 0.3	18.5 ± 0.4
4	10.9 ± 0.3	82.5 ± 7.0	17.4 ± 0.4	19.9 ± 0.6
8	9.9 ± 0.4*	95.0 ± 3.2	19.0 ± 0.4*	22.1 ± 0.9*

Table IV – Adult weight of *C. riparius* exposed as larvae to Indoxacarb. All values are presented as mean ± SEM, n = 8.

Indoxacarb Concentrations (μg L^{-1})	Males dry weight (mg)	Females dry weight (mg)
0	0.478 ± 0.012	0.943 ± 0.049
1	0.502 ± 0.014	0.998 ± 0.030
2	0.461 ± 0.018	0.981 ± 0.051
4	0.503 ± 0.022	0. 999 ± 0.039
8	0.469 ± 0.012	0.954 ± 0.049

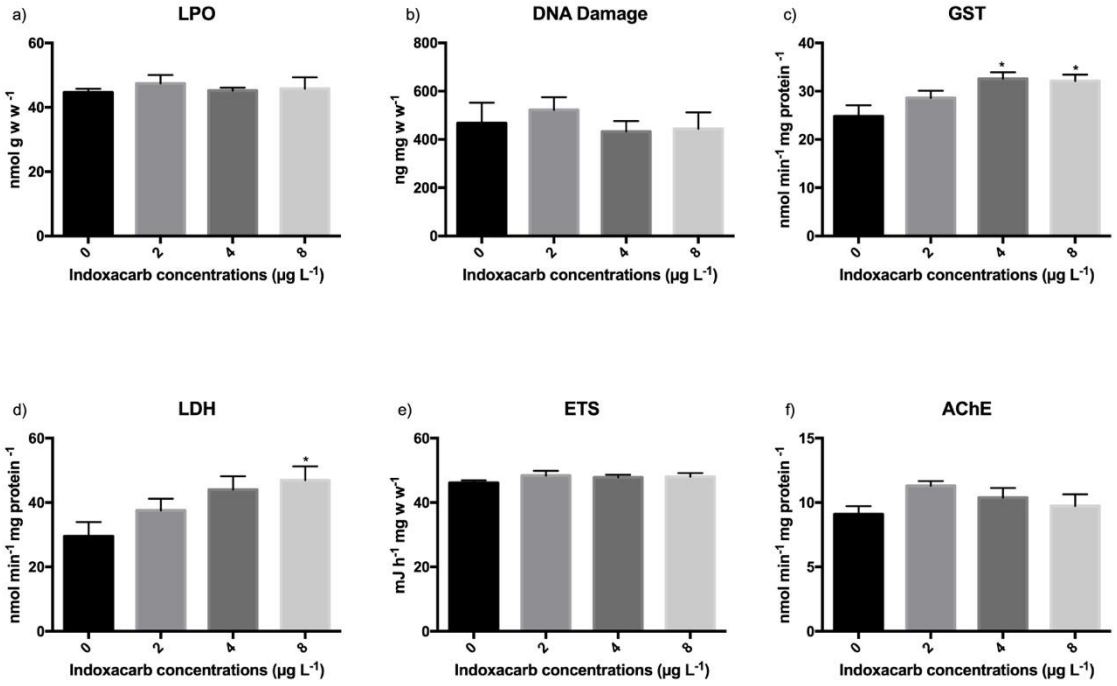

Figure 3 – Oxidative damage, biotransformation, energetic metabolism and neuronal biomarkers in *C. riparius* larvae after 48h exposure to indoxacarb: a) Lipid Peroxidation; b) DNA Damage; c) Glutathione-S-Transferase; d) Lactate Dehydrogenase; e) Electron Transport System; f) Acetylcholinesterase. All values are presented as mean + SEM, n = 7. An asterisk denotes statistically significant differences to the control treatment (0 μg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

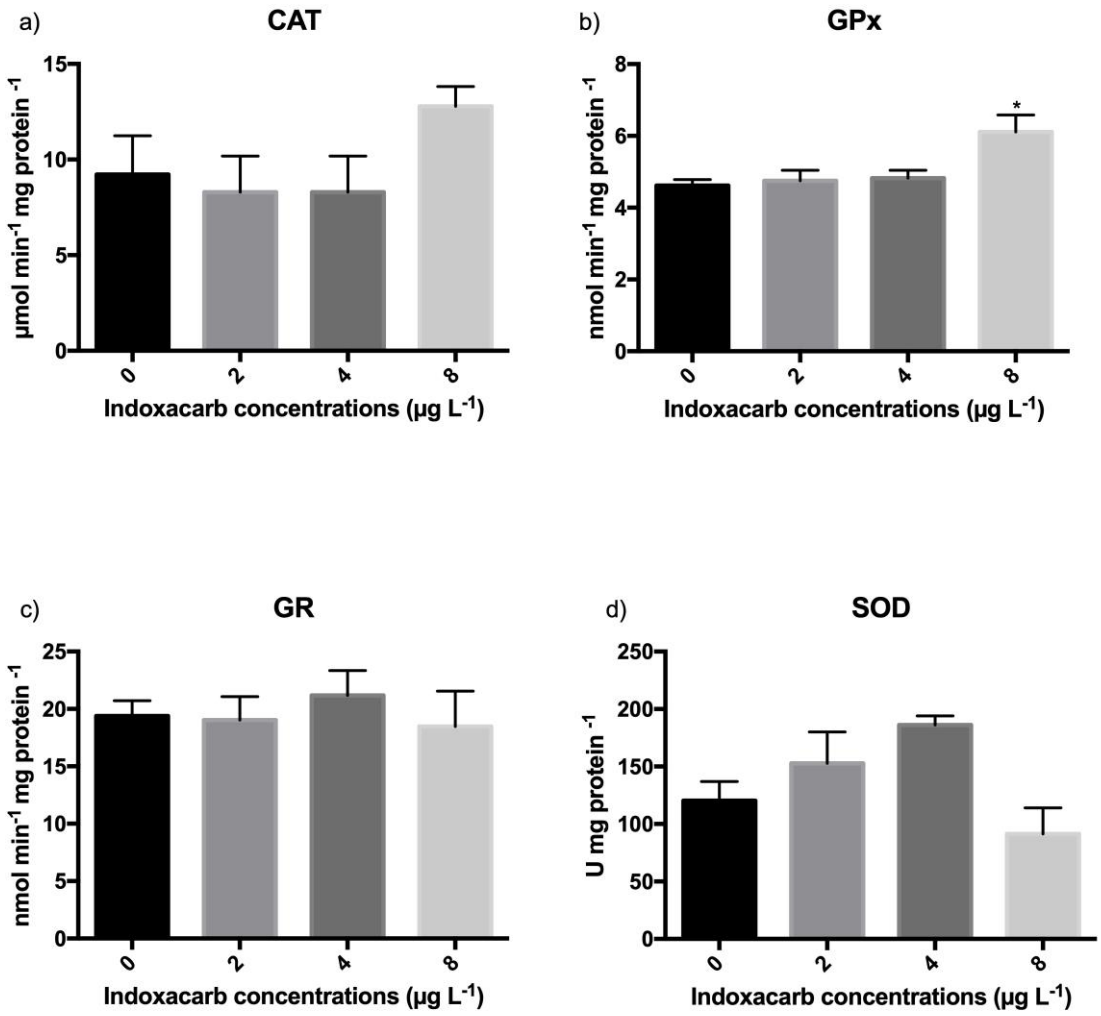

Figure 4 – Oxidative stress biomarkers in *C. riparius* larvae after 48h exposure to indoxacarb: a) Catalase; b) Glutathione Peroxidase; c) Glutathione Reductase; d) Superoxide Dismutase. All values are presented as mean ± SEM, n = 7. An asterisk denotes statistically significant differences to the control treatment (0 µg L^{-1}; $p < 0.05$, ANOVA, Dunnett's test).

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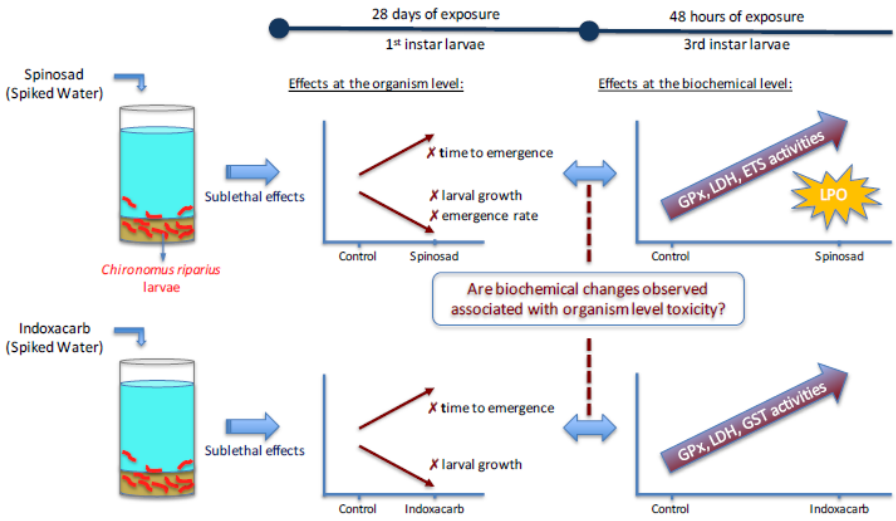

Graphical abstract

Highlights

Responses of *C. riparius* to spinosad and indoxacarb exposure were investigated.

Exposure to both insecticides compromised *C. riparius* life-history traits.

Both insecticides induced defense mechanisms and cellular oxygen consumption.

Non-target aquatic insect species may be impacted by spinosad and indoxacarb use.