Effect of Sublethal Concentrations of the Antiparasitic Ivermectin on the Polychaete Species Hediste Diversicolor: Biochemical and Behavioral Responses

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Research Article

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Abstract

Pharmaceutical drugs have emerged as major micropollutants in aquatic ecosystems. Their presence has been systematically reported in monitoring survey, and their wide distribution and constant presence in the wild is a direct consequence of their massive use, in both human and veterinary therapeutics. Drugs used to treat parasitic infections in livestock are major contaminants, given the amounts in which they are administered, and reach the aquatic compartment in high amounts, where they may affect non target species. Some of these drugs are prone to find their final deposit in sediments of estuarine areas, exerting their toxic effects preferentially at these locations. Sediment dwelling organisms of coastal areas, such as polychaetes, are especially prone to have their major physiological functions compromised. Ivermectin is one of the most used antiparasitic drugs, and its effects are not limited to biochemical traits, but also behavioral features may be compromised considering their neurotoxic actions. Despite these putative effects, little is known about their toxicity on polychaetes. The present study aimed to characterizes the toxicity of realistic levels of ivermectin on the polychaete *Nereis diversicolor*, in biochemical and behavioral terms. The obtained results showed that low levels of ivermectin are capable of causing significant disturbances in mobility and burrowing activity of exposed worms, as well as alterations of metabolic and anti-oxidant defense efficacy of exposed animals, suggesting that its environment presence may mean a major environmental concern.

Introduction

Increasing agricultural production and population growth have led to increased environmental pollution due to the generation of large volumes of agricultural and household waste, with considerable potential for exerting deleterious effects on non-target biota (Durigan et al., 2012). Among these chemicals, one may find emergent pollutants, which are potentially toxic compounds released into natural waters due to low removal efficiency provided by conventional systems treatment sewage, with implications for the environment still poorly understood (Durigan et al., 2012). These contaminants include a broad group of compounds including personal care products, additives, nanomaterials, and particularly drugs for human and veterinarian use, which reach the environment as metabolites or in their unchanged form (Horvat et al., 2011; Fatoki et al., 2018), in concentrations in the order of the ng/L to µg/L (Boonstra et al., 2011; Horvat et al., 2011). Among the most abundant drugs that occur in the environment, specific classes stand out according to their use. This is the case of substances used primarily in veterinary, aquaculture, and livestock, where animals are medicated with high amounts of pharmacologically active substances, which contaminate the environment on a large scale. The importance of this issue is reinforced considering specific classes of drugs, namely antiparasitics that are regularly applied to the intensive livestock production, particularly cattle, pigs, sheep and horses, and cultured fish (Fent et al., 2006; Sherer, 2006).

Antiparasitics are chemicals used to control or kill endo- or ectoparasites (namely in cattle), being mainly used against a large number of helminths (e.g. nematodes) (Sherer, 2006; Horvat et al, 2011; Wolstenholme et al, 2016) or protozoa (Chen, 2016). These drugs either kill or immobilize/expel parasites

from the host's body, not causing any damage to the host (Abongwa et al., 2017). However, a potential adverse effect of these pollutants is the damage they may cause to non-target organisms environmentally exposed. This happens since these drugs have the ability to act in low concentrations (µg/L levels, or less) causing toxic chronic effects (Solomon et al., 2007; Horvat et al., 2011). Although antiparasitics are widely used, there is little data available regarding their environmental presence. However, Sherer (2006) conducted a study in the United States of America and determined their presence in concentrations of 0.12 mg/kg of doramectin, and 1.85 mg/kg of ivermectin in feces of medicated animals. At the same sites low concentrations of these compounds were found in soils, circa 0.046mg/kg Sherer, 2006). However, the information available regarding the concentration of these drugs in the environment is still limited and, since they have a wide applicability, their presence and potential impacts are expected to occur also in the aquatic environment (Horvat et al., 2011). In the particular case of the marine environment, where aquaculture activities are undertaken, estuaries are most subjected to contamination by such pollutants; animals at these locations, namely sediment-dependent organisms, are sometimes exposed throughout their life cycle (Fent et al., 2006).

Within the pharmacotherapeutic group of the antiparasitics, one may find macrocyclic lactones, constituted by avermectins (AVMs) and milbemycins, derived from natural fermentation of soil microorganisms of the genus Streptomyces (Abongwa et al., 2017). Damage caused by parasites in animals worldwide may be something significant, which makes AVMs extremely popular and necessary (Bai & Ogbourne, 2016). As a result of this massive use, large discharges of residues of these compounds have been reported, yet poorly documented. However, these pharmaceuticals have been already classified as potentially toxic to aquatic organisms, being necessary to undertake further studies on its environmental fate and effects (Maranho et al., 2014; Bai & Ogbourne, 2016), namely on aquatic organisms.

One of the most important antiparasitic drug is ivermectin (IVM), which is a legally approved drug, among those most commonly used in veterinary procedures against nematodes, namely aquaculture (e.g. sea lice, onchocerciasis; Bai & Ogbourne, 2016). The use of IVM has not always been legal, since previous evidences pointed also to massive illegal uses of IVM, prior to its approval, as described by Grant and Briggs (1998). IVM is a neurotoxin, and acts on glutamate gamma-aminobutyric acid mediated channels, present in invertebrates and vertebrates, thereby opening chloride channels (Bai & Ogbourne, 2016). This allows IVM to act as a neuromuscular inhibitor to promote hyperpolarization of the cell by anion input, hampering the transmission of nerve impulses, leading to paralysis of the muscles (Bai & Ogbourne, 2016; Chen, 2016; Crump, 2017). The inhibition of locomotion and muscle activity of parasites, is enough to interrupt the process of secretions, which is required to prevent the host immune system response (Degani-Katzav et al., 2016). The combination of such events leads to the death and expulsion of the parasite.

Because of its low metabolism, ivermectin is excreted in the feces almost unchanged (about 90% of the administered dose) and only about 2% of the dose is excreted in urine (González Canga et al., 2009). However, IVM has a high affinity for organic matter resulting in reduced bioavailability in water, with half-

life DT_{50} of 39h in water and of 45d in sediment (Solomon et al., 2007). However, the bioavailability and half-life of IVM is seriously influenced by conditions such as low water solubility and instability when exposed to UV or visible light (Cui et al., 2018) and may be less than 2ng/L in surface waters (Solomon et al., 2007). Nevertheless, and in field conditions, namely in marine areas, IVM tends to stay for extremely long periods in the sediments, as shown by Roth et al. (1993). According to the estimates by Davies et al. (1998), the occurrence of IVM in marine sediment may exceed 100 days. A microcosm study conducted by Boonstra et al. (2011), showed that the values DT_{50} of IVM may vary from 1.1 to 8.3 days; predicted environmental concentrations (PEC) were of 25 to 60 ng/L, and a worst case scenario with concentrations of 1000 ng/L could also occur (Boonstra et al., 2011). However, the higher concentration of IVM reported in the literature was of 4.4 ng/L, and corresponded to runoff from farms (Nessel et al., 1989). Soil dependent bodies are exposed to IVM due to its persistence in soil, which can reach 7 days to a few months (Horvat et al., 2011). Despite its frequent and widespread use, ivermectin is not innocuous, being toxic to fish (Kennedy et al, 2014; Domingues et al, 2016; Massei et al, 2019), mammals (Trailovic & Nederljkovic 2010 ; Moreira et al., 2017; Cordeiro et al., 2018; Parisi et al., 2019) , birds (Sakin et al., 2012; Li et al., 2013; Liu et al., 2016), and insects (Strong & James, 1993; Solomon et al., 2007; Ishikawa & Iwasa, 2019). The data obtained by Black et al. (1997) showed that IVM caused a significant mortality in sediment polychaetes, suggesting that these organisms are highly susceptible to this drug, even after short exposures. In some of these studies IVM has been shown to cause oxidative stress with adverse effects mainly on mammalian behavior, reproduction and fecundity, deformation of fish embryos and larval death of some insects or even adult death, raising concerns about its fate and ecological effects. In addition, IVM has also been shown to be toxic to marine invertebrates, namely polychaetes that are responsible for bioturbation of sediments and mineralization of organic matter, as demonstrated by Black et al. (1997). Consequently, IVM presents a non-characterized risk to non-target organisms (Grant and Briggs, 1998; Lumaret et al., 2012). This scenario is even favored by a general lack of toxicity data of IVM, for aquatic species; in fact, the present day knowledge is scarce, and reliable data is still limited to a few studies, some of them with species of the genus *Daphnia*, which are not representative of the marine or estuarine environments; even more limited is the amount of information for marine sediment dwelling organisms (Roehr, 2011), which seem to be the major targets of this drug.

One of the already documented outcomes of IVM is oxidative stress (El-Far, 2013), a condition characterized by increased levels of reactive oxygen species (ROS), against which the antioxidant defense system is not effective to prevent damage. This imbalance causes damage to cells and macromolecules (Nunes et al., 2016). To quantify the level of oxidative stress in cells, biomarker studies that measure the levels of activation of the antioxidant defense system are used to measure biochemical sublethal changes resulting from individual exposure of organisms to xenobiotics (Hyne & Maher, 2003).

The organism *Nereis diversicolor*, described in 1776 by OF Muller, is a predator and filter feeder that inhabits intertidal zones in the temperate zones of the northern European and African coasts (Aberson et al., 2011; Ghribi et al., 2019), in sand or mud where it builds U-shaped or Y- channels, avoiding contact with other individuals (Patrick, 2002). Individuals of this species are key to support various predators

such as crabs, prawns, fish and birds (Carvalho et al., 2013), being important in the recycling of organic matter and nutrients, and in bioturbation (Bonnard et al, 2009). It is through burial that individuals promote sediment bioturbation that will move and irrigate the sediment affecting chemical flows (nutrients, pollutants) and microbiological activity (Aberson et al., 2011). Due to its high tolerance to temperature variations and hypoxia conditions (Patrick, 2002) it is considered a key species in bottom communities in almost all European estuaries (Patrick, 2002; Moreira et al., 2006). Moreover, this species has a great commercial interest since it is used as bait for anglers (Carvalho et al, 2013), and has a great potential to be produced as fish feed in aquaculture (Patrick, 2002; Bagarrão, 2013). Due to its high abundance and ecological relevance (in trophic chains and sedimentation processes), *N. diversicolor* was considered the one the appropriate species to serve as a test organism to study ecosystems exposed to various pollutants (Ghribi et al., 2019). Due to its responsiveness to pollutants, *N. diversicolor* is suitable for biomonitoring environmental ecosystems and management programs (Ghribi et al., 2019). It has been successfully used in biomonitoring programs, such as the assessment of environmental quality and to measure contaminant concentrations in the field, and to quantify biomarkers after exposure to pollution caused to a contaminant discharge in the Bay of Cadiz (Maranho et al., 2014). Given its importance in both the ecosystem, economy, and environmental sciences, *N. diversicolor* is an adequate species to assess the toxic effects of anthropogenic compounds, such as drugs such as IVM.

Considering that after excretion, IVM has the potential to contaminate aquatic sediments, it is important to study the potential effects of realistic levels of this drug on sediment dwelling organisms, such as polychaetes. For this purpose the present work used the quantification of biomarkers of oxidative stress and metabolism (namely, the activities of three key enzymes catalase (CAT), glutathione S-transferase (GSTs), and lactate desidrogenase, LDH) and behavioral changes as biomarkers in individuals of the polychaete *N. diversicolor*. The assessment of changes in the behavior of this organism is justified since IVM operates at the neuromuscular level, compromising the mobility of affected organisms. To attain this objective, behavioral tests focused both on locomotion and burial activity of exposed organisms.

Material And Methods

Chemicals

IVM [CAS number: 70288-86-7; degree of purity 98%], was purchased from Sigma Aldrich (Darmstadt, Germany). Since the solubility of IVM is slightly above 4 mg/l (Lumaret et al., 2012), two solutions were prepared with Milli-Q ultrapure water, at a concentration of 4,17mg/l for acute exposures, and 4,12mg/l for chronic exposures. All exposure media were contaminated using these two solutions, by dilution. Being photodegradable, IVM solutions were all wrapped in aluminum foil, and kept in the fridge at 4ºC. All other chemicals, namely those used for the preparation of buffers, media solutions for biomarkers, standards, were purchased from Sigma Aldrich, Panreac, and Applichem.

Test organisms

The organisms were manually captured with the help of a fork in the Douro Estuary Nature Reserve, in the Reserva Natural de São Paio, Afurada, Vila Nova de Gaia, Portugal, considered a reference site given the low levels of organic and inorganic contaminants, as demonstrated by Ghribi et al. (2019). Test organisms were collected during the low tide periods, in February and March 2018. This site is characterized by high hydrodynamism and is an area with environmental quality of coastal sediments, considered unpolluted (Ghribi et al., 2019). In addition to the capture of organisms, sediment was also collected at this site, which was subsequently transported to the laboratory. The organisms were transported in a plastic box and, upon arrival at the laboratory, were inspected and selected. Those that were unharmed and apparently healthy were sorted and selected to be sent to quarantine. These worms were then placed in a 50L box with sediment (previously and thoroughly washed with distilled water, and incinerated in the Ceramifor muffle, model MEC 85, at 450°C for 5 hours to remove all organic components), in reconstituted seawater (Tropic Marin ® salts, suitable for reverse osmosis water), temperature 20 ± 1ºC, with salinity 20 (similar to the value found in the field, suitable for the maintenance and growth of organisms; Bagarrão, 2013), continuous aeration, photoperiod 16 h D: 8 h N, and fed with commercial flake fish food (TetraMin ®) every 48 hours. Organisms were maintained under these conditions for 15 days for acclimatization/clearance/quarantine. Each box contained on average about 400 organisms/50cm^2.

Exposures

This work involved two durations of exposure: an acute exposure (with higher concentrations and a period of exposure of 96h), and a chronic exposure (with lower concentrations but with period of exposure of 28 days). The acute exposure involved a control group (animals not exposed to IVM), and five groups with ten replicates (with one individually exposed organism per replicate) exposed to five different concentrations of IVM (0.0625; 0.125; 0.250; 0.500; 1µg/L). This set of concentrations was defined considering the levels already described by Boonstra et al. (2011), including the worst case scenario that may be anticipated. Each replicate consisted of 1 animal exposed individually inside a 1.5L plastic container, previously used for drinking water. Animals were exposed in 750 ml of media (reconstituted artificial sea water), and each exposure vessel had circa 350ml of previously treated sediment. 60 organisms previously subjected to quarantine were selected randomly, and individually placed in each bottle. The containers were subjected to continuous aeration, photoperiod of 16 h D: 8 h N, and a temperature 20±1°C. During acute exposure, the organisms were not fed; the exposure media was changed and contaminated again at 48h after the onset of exposure. Since IVM is easily photodegradable, exposure vessels were covered with opaque plastic.

In chronic exposure, all experimental design and procedures were similar to those of the acute exposure, with the exception of the test concentrations (a control group not subjected to the test compound, and five groups exposed to different concentrations of IVM: 0.0156; 0.03120; 0.0625; 0.125; 0.250µg/L). The selected levels for the chronic exposure aimed at increasing the ecological relevance of data, considering the already reported values of 4,4ng/L in the literature, namely runoff water from livestock (Nessel et al.,

1989); in addition, according to Boonstra et al. (2011), concentrations up to 60ng/l can be reached in surface water near farms. In addition, animals were fed every two days with the same fish food, and the medium was renewed every 48 hours.

For both exposure modes, animals were removed from exposure apparatuses and were subjected to behavioral tests the day before the sacrifice, to avoid additional stress. After exposure, the organisms were sacrificed, placed in a petri dish on ice, and cut into small pieces with a scalpel. These tissue pieces were separated approximately in half and both halves were placed in Eppendorf microcubes and kept at -80°C until biochemical tests were performed.

Behavioral tests

Behavioral tests were performed the day before the animals were sacrificed to avoid an additional source of stress.

To check on the effects of IVM on the behavior of exposed organisms, a behavioral test was performed, to assess the distance that each animal travelled within a confined tube, for a period of one minute, to evaluate the interference of IVM on mobility. As already described, the mechanism of toxic action against parasites of IVM occurs through the interference on neuromuscular communication, reducing the mobility of exposed organisms. This distance was measured after 1 minute, from where the rear extremity (tail end) of the exposed animal was.

The second test aimed to determine how long took each individual to fully bury itself in sediment, according to the protocol described by Bonnard et al., (2009). This test was adopted since burial is a complex behavior based on instinctive reflexes and a change in the reflex response caused by exposure to a toxic agent can lead to a behavioral change (Bonnard, 2009).

Biochemical analysis

To quantify the activities of CAT and GST enzymes, the samples were thawed on ice, and homogenized with 50 mM phosphate buffer, pH=7.0, with 0.1% Triton X-100 (homogenization buffer), in a ratio of 1mL:0.15g of tissue, approximately. These were homogenized with an ultrasound Velp Scientifia, V5, and then samples were centrifuged in a centrifuge Thermo Scientific, at 15,000 G at 4°C for 10 minutes to obtain the supernatant fraction. Supernatants were then separated and stored at -80ºC until subsequent determinations.

To quantify the activity of LDH, the samples were thawed on ice and homogenized with TRIS buffer, pH=7.2 in a ratio of 1mL:0.15g of tissue. There were homogenized with an ultrasound Velp Scientifia V5, and then samples were centrifuged in a centrifuge Thermo Scientific, at 6000 rpm at 4ºC for 3 minutes to obtain the supernatant fraction. Supernatants were then separated and sored at -80ºC until subsequent determinations.

Every spectrophotometric reading for the determination of biochemical parameters were made on a microplate reader Thermo Scientific, model Multiskan Spectrum with the software SkanIt 2.4.4.

CAT activity determination

The activity of catalase (CAT) was determined by the method of Aebi (1984) that monitors the decomposition of H_2O_2 at 240 nm. CAT activity is expressed in terms of micromoles of hydrogen peroxide consumed per min per mg protein ($mmol^{-1}min^{-1}mg^{-1}$ protein) (Aebi, 1984).

Determination of GSTs Activity

Glutathione S-transferases (GSTs) are a group of related enzymes that catalyze the conjugation of glutathione (GSH) with various compounds with electrophilic centers. They catalyze the conjugate reaction of the substrate CDNB (2,4-dinitrochlorobenzene) with GSH (glutathione) to form a thioether which can be monitored by increasing absorbance at 340 nm. The activity of GSTs is expressed in terms of total soluble protein present in the samples ($mmol.min^{-1}mg^{-1}$ protein) (Habig et al., 1974).

Determination of LDH Activity

LDH (lactate dehydrogenase) activity was determined following the method of Vassault (1983). Determination of its activity was performed by measuring the reduction of absorbance caused by the oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) in the presence of pyruvate, at a wavelength of 340 nm. Activity was expressed in millimoles of β-NADH oxidized per minute, per milligram of protein.

Protein Quantification

Protein quantification was done in all samples. It is based on the method of Bradford (Bradford, 1976), which quantifies the formation of a complex stained resulting from the reaction of the Bradford reagent with total soluble protein. The measurement if performed by monitoring the absorbance of the samples are at 595 nm. Protein standards were prepared using γ globulin ($1mg.ml^{-1}$).

Statistical analysis

After the normality test (Shapiro-Wilk test) and homogeneity of variances (Levene test), biomarker and behavioral test data were compared using a one-way analysis of variance (ANOVA) followed by a Dunnett test, if any significant differences ($p < 0.05$) were found. Statistical analysis were performed using the SPSS 25 software.

Results

Behavioral tests

In terms of mobility of acutely exposed animals, none of the exposed organisms showed significant differences in relation to the control ($F_{5, 39}$=0.792; p=0.562; Figure 1). Similarly, no differences were noticed in terms of burial activity (F = 1.267; df = 5, 36; p = 0.299; figure 2).

In chronically exposed worms, there were no significant differences among treatments in the mobility test ($F_{5,32}$=1.272; p=0.3), despite the slight increase in the travelled distance along with the increase of IVM concentration (figure 3). In the burial test, the majority of exposed individuals were no longer capable of burying themselves as the concentration increased (Table 1). This event prevented the statistical analysis of this specific endpoint.

Table 1

number of organisms at the end of both exposure regimes. exposed organisms – number of total organisms initially exposed; surviving organisms – number of organisms that survived to the exposure periods; displaced organisms – organisms that showed the ability, at the end of the exposure period, to move in the behavioral assay; buried organisms – organisms that at the end of the exposure periods, were able to bury themselves in the sediment.

	Treatment (µg/L)	Exposed organisms	Surviving organisms	Displaced organisms	Buried organisms
Acute Exposure	Control	10	10	10	10
	0.0625	10	9	9	9
	0.125	10	10	10	10
	0.250	10	9	9	9
	0.5	10	10	10	10
	1	10	7	7	7
Chronic Exposure	Control	10	10	10	9
	0.0156	10	7	7	4
	0.0312	10	8	8	3
	0.0625	10	4	3	1
	0.125	10	7	7	1
	0.25	10	8	7	0

Biochemical analysis

In terms of CAT activity of acutely exposed animals, there were only significant differences ($F_{5,44}$=5.148; p=0.001; figure 4) between the control group and the animals exposed to the concentration of 1 µg/L. The activity of GSTs and LDH in acutely exposed worms was not significantly altered when compared to control values ($F_{5,43}$=3.814: p=0.006; $F_{5,47}$=1.219; p=0.315; figures 5 and 6, respectively).

In chronic exposure, there were no significant differences in both activity of CAT ($F_{5,37}$=0.595; p=0.704; figure 7) and GSTs ($F_{5,33}$=0.908; p=0.488; figure 8). In terms of LDH activity ($F_{5,36}$= 2.384; p=0.05) only animals exposed to the highest tested IVM concentration had their enzymatic activity significantly impaired when compared to control values (figure 9).

Discussion

Behavioral testing is a type of response at the individual level influenced by a large number of factors, being thus sensitive to toxic effects; this endpoint is important in ecotoxicological assessment since it may indicate potential adverse effects at both the organism and the ecosystem, establishing causality effects between the alterations at the individual level that may have consequences at the population level (Bonnard et al., 2009). IVM is a well-known neurotoxic agent, and to address its putative behavioral impairments, we exposed individuals of *N. diversicolor* to different concentrations of this drug, and the locomotion and burrowing activities of exposed individuals were analyzed. Results of the locomotion and of the burrowing tests after acute exposure to IVM showed no significant behavioral changes. Somewhat similarly, no significant effects were reported following chronic exposure to IVM, but in this case, the most important observation was related to the loss of burrowing capacity shown by most exposed worms. The here reported absence of changes in locomotion behavior, is not in agreement with the described mechanism of therapeutic activity for this class of drugs. IVM acts specifically on ivermectin-sensitive channels, which are glutamate-activated chloride channels. By doing so, IVM causes an increased inflow of chloride ions, leading to the hyperpolarization of nerve and muscle cells of susceptible species (Ikeda, 2003). This effect result in paralysis of target species, namely of parasitic nematodes and insects (Turner and Schaeffer, 1989; Shoop et al., 1995). Results in line with the therapeutic activity of IVM were obtained by Ding et al (2001) after exposing individuals of the terrestrial worm species *Lumbriculus variegates* to 0.3nM IVM for 3h, which culminated in significant decreases of locomotor behavior, even after a short period of exposure. Animals exposed for longer period of 28 days showed a similar pattern, without significant changes in locomotion activity. However, and despite this absence of effects in the distance animals were able to travel following short and long term exposures, a large number of worms lost their natural ability to bury themselves after being chronically exposed to IVM. This result is not totally surprising, since the study conducted by Thain et al. (1997) already evidenced the deleterious influence of IVM on behavioral traits of the marine polychaete *Arenicola marina*. In this study, levels of exposure of 0.010 mg/kg were able to compromise the reburying activity of this organism. The methodological difference between the methods here used and those selected by the mentioned authors, namely the differences in exposure durations (animals were exposed for 28 days in our assays; and during 10 days in the study conducted by Thain et al) makes toxicity comparison particularly troublesome. However, it

seems clear that IVM seems to deleteriously interfere with this trait in worms, which is a particularly important alteration, with putative ecological consequences. The failure to achieve burying can constitute a dramatic challenge not only to affected individuals, but also to the population, since exposed individuals are much more vulnerable to predators (Bonnard et al., 2009). In addition, the burrowing capacity of estuarine/marine polychaetes is determinant for these environments, since it contributes decisively for the bioturbation of the sediment at these locations (Aberson et al., 2011). The bioturbation encompasses active movement of sediment and channel irrigation (Kristensen & Kostka, 2013) and is an essential process in estuarine ecosystems, acting in order to assure the cycling of nutrients (Fang et al., 2019). In a study conducted by Fang et al (2019), individuals from *N. diversicolor* were shown to be essential for the movement and irrigation of estuarine sediments, demonstrating its critical role to the functioning of the ecosystem in estuaries (Fang et al., 2019). The here reported absence of significant results concerning behavioral endpoints assessed after short term exposures to IVM suggests that the levels of contamination, or the exposure periods, were not sufficient to possibly change behavior of exposed worms. On the other hand, one must not forget the potential occurrence of interspecific differences between the organism we used and those that were described in the literature, which may justify this difference in sensitivity to IVM, and also in behavioral responses, which are extremely dependent upon the analyzed species. Behavioral traits, and most likely the way behavior is shaped by exposure to toxicants, is highly regulated by genetic and neural factors, as addressed by Yamamoto and Ishikawa (2013). Consequently, it is difficult to assume that distinct species may behave similarly when exposed to the same chemical.

Metabolism of xenobiotics (including pharmaceutical drugs) often results in excessive ROS production, due to the activation of specific metabolic pathways involving the cytochrome p450 complex (Hrycay and Bandiera, 2015). This leads to subsequent compensatory responses, that rely on the activation of antioxidant enzymes such as superoxide dismutase (SOD), a catalase (CAT) and glutathione peroxidase (GPx), which are natural scavengers of ROS (Regoli & Giuliani, 2014). If this compensatory response is not sufficient to eliminate ROS, a scenario of oxidative stress may be established (Regoli & Giuliani, 2014; Pires et al., 2016). Among the antioxidant enzymatic defensive system, CAT is extremely important since it converts hydrogen peroxide into water, providing effective protection against oxidative damage (Regoli & Principato, 1995; Nunes, 2019). An increase in CAT enzymatic activity is a natural response to the establishment of oxidative conditions. However, CAT inhibition, paradoxically it may seem, may also result inhibited after exposure to IVM. The inhibition of CAT activity was observed in the freshwater fish species *Danio rerio*, after being chronically exposed to 25 µg/L of IVM (Domingues et al., 2016). A study with rainbow trout exposed to 0.01 and 0.02 mg/kg also IVM of demonstrated a decrease in CAT activity (Sakin et al., 2012). This decrease may be related to the increase in the production and release of oxidative entities, namely ROS, and this decrease of enzymatic activity corresponds to the denaturation of such enzymes (Jemec et al., 2010; Regoli & Giuliani, 2014). However, our study clearly showed that *N. diversicolor* was not responsive, in terms of CAT, to IVM. In fact, some annelid species can tolerate exposures to relatively high concentrations of drugs from the avermectins family (namely, abamectin and ivermectin) without being significantly affected (Bai & Ogbourne, 2016). Sun et al. (2005) have shown

that when organisms of the species *Eisenia fetida* were exposed to sediments contaminated with concentrations between 0 and 5mg/kg of avermectin B1a, a percentage of 80% to 95% of this drug was absorbed, but no significant effects were reported in the organisms. The here presented study did not demonstrate any response involving the activation of CAT as a defensive mechanisms towards the putative metabolism of IVM. In fact, both durations of exposure, acute and chronic, did not yield significant alterations in terms of catalase activity, suggesting the absence of pro-oxidative alterations. The here reported absence of effects may be also due to alternative mechanisms, that exist in polychaetes, but were not measured in this study. These mechanisms involve enzymatic or non-enzymatic systems (Sun and Zhou, 2008). Several studies have showed that not only enzymatic antioxidants act against ROS species but that some polychaetes have other defense mechanisms (Abeleoeschger et al., 1994; Moraes et al., 2006). Moraes (2006) suggested that the species *Laeonereis acuta* (nereididae) secretes mucus with antioxidant properties. This effect is due the fact that it is a favorable substrate for the growth of bacteria with the ability of degrading H_2O_2, by expressing specific enzymes such as catalase. Polychaetes, including *H. diversicolor*, go through sexual maturation processes during their life cycle, which are evidenced by a change in brownish to dark green color caused by increased biliverdin pigment (Abeleoeschger et al., 1994). The pigment biliverdin is known for its already established antioxidant properties (Jansen and Daiber, 2012), and its effects may have contributed for the absence of clear oxidative effects following exposure to IVM. The same physiological role of ROS scavenging can be attributed to vitamin A, the precursor of β caroten (Abeleoeschger et al., 1994). Although these non-enzymatic mechanisms were not quantified in this study, we cannot rule out the hypothesis that they may exist and play an important role in IVM-induced oxidative stress scenarios.

Glutathione-S-transferases (GSTs) are a set of biotransformation enzymes that perform a dual protective action because they catalyze the process of cell detoxification by conjugating toxicants with reduced glutathione (GSH) (Townsend and Tew, 2003), making them more hydrophilic, easier to excrete and therefore less toxic (Ghribi et al., 2019). In addition, these isoenzymes are capable of inactivating lipid peroxidation products by the use of GSH as a reducing agent (Sturve et al., 2008), contributing to the antioxidant defense efficacy. GSH itself is the most abundant scavenger that participates in the antioxidant defense system, directly neutralizing ROS through its oxidation, resulting in the formation of a glutathione dimer of oxidized glutathione (GSSG) (Regoli & Giuliani, 2014). The role of GSTs mediated metabolism of IVM was already made clear; in fact, these isoenzymes seem to play a crucial part in the resistance of helminth parasites against IVM, as evidenced by Perbandt et al. (2005). However, and similarly to what occurred for CAT, no significant effects were reported after both acute and chronic exposures to IVM in terms of GSTs activities. Previous data from the literature evidenced that IVM exposure could result in significant metabolic alterations. According to the data published by Mounsey et al. (2010), GSTs have a prominent role in the detoxification of IVM in the human parasitic species *Sarcoptes scabiei*. IVM-resistant animals were exposed to a GSTs inhibitor, and their susceptibility to this insecticide strongly increased; in addition, IVM-exposed individuals evidenced significant increase in GSTs activity, demonstrating that this pathway is responsible for the metabolism and excretion of IVM in this insect species. Similarly, GSTs mediated conjugation was also shown to be important in the

resistance of the parasitic insect *Rhipicephalus microplus* to IVM, as demonstrated by Le Gall et al. (2018). This same mechanisms was responsible for the resistance of the nematode *Caenorhabditis elegans* to not only IVM, but also to another macrocyclic lactone with antihelmintic action, namely moxidectin (Ménez et al., 2016). On the contrary, GSTs activity of the fish *D. rerio* exposed to 25 µg/L and 2.5 µ g/L IVM, was significantly inhibited, according to the study conducted by Domingues et al. (2016). The reduction of the activity of GSTs can be related to the depletion of the cofactor GSH (Domingues et al., 2016). According to the suggested mechanism underlying this inhibition of GSTs, the authors suggested that GSTs activity might decrease with the depletion of GSH, which is no longer available during the time course of the intoxication to be conjugated with IVM or its metabolites (Domingues et al., 2016). A possible interpretation for the here obtained results, in comparison with literature data, may be due to the metabolism of IVM by *H. diversicolor*. Despite the absence of data concerning this specific pathway of GSH conjugation, these organisms may not privilege this specific GSH conjugation pathway for detoxification of IVM. One alternative metabolic route for detoxification of IVM may involve CYP450, which was demonstrated to play a key role in the biotransformation of IVM in human body lice (Yoon et al., 2011). In addition to GSTs and cytochrome P450, other alternative metabolic routes also seem to be of critical significance, namely esterases, and ATP Binding Cassette Transporters, as shown by Ménez et al. (2016), and by Le Gall et al. (2018).

Absence of toxicity was also reported after measuring the activity of lactate dehydrogenase in worms, following both acute and chronic exposures. Again, any comparison of the here obtained data with patterns found in previous study is made difficult given the absence of literature on this theme. However, previous data focused on the toxic effects of IVM on mammalian cell lines, using LDH activity as effect criterion. The study by Mattei and Rodrigues (1994) showed that IVM could exert a significant inhibitory effect on LDH activity of the cell line IB-RS-2. However no changes were reported in glucose-6-phosphate dehydrogenase and glucose-6-phosphatase activities, suggesting that IVM did not cause any significant impairment of intracellular respiratory activity, namely by inducing anaerobiosis. Exposure of rodents to abamectin, another antiparasitic drug of the avermectin group, yielded significant alteration of mitochondrial processes, namely by compromising the aerobic cellular respiration, with decreased mitochondrial membrane potential, impairment of ATP biosynthesis and disruption of intracellular Ca(2+), which culminated in necrosis (Maioli et al., 2013). Considering that the aerobic pathway seems to be an indirect target for avermectins, is important to hypothesize that the activity of lactate dehydrogenase may be enhanced in exposed organisms, but this assumption was not validated by our data. Though mechanistically possible, the importance of this inhibitory effects remains to be further elucidated.

The conjugation of all these data, after acute and chronic exposures, reinforces the assumption previously made, that *H. diversicolor* is not particularly responsive to IVM. Thus, it is possible to suggest that the lack of sensitivity of this species to exposure to this class of compounds results precisely from not being able to metabolize and bioactivate IVM (given the short period of exposure, or the low levels of drug) in a great extent, thereby preventing the overproduction of ROS and thus avoiding the deleterious effects arising from their physiological action. However, other defensive mechanisms of unspecific nature

may also have prevented the absorption of IVM in a large extent. In fact, animals from this species eat by directly ingesting food particles from the external media, or by creating a mucus film around their bodies to collect food particles (Patrick, 2002). This mucus, released by the epidermal cells, is also used to build and stabilize the canals where they live (Kristensen & Kostka, 2013), but also protects the animal against absorption of compounds (e.g. metals) and pathogens (Coutinho et al., 2018). In addition, IVM absorption may be strongly influenced by P-glycoprotein (P-gp), present mainly in the intestinal epithelium, causing a slower absorption (González Canga et al., 2009) already documented in experimental animals. The P-glycoprotein is an active carrier and is implicated in the emergence of multi-drug resistance phenomena in several species, acting as a physiological barrier, sending a wide range of molecules of different therapeutic drugs to the outside of cells playing a major role in absorption, distribution, metabolism and excretion of drugs in the body (Edwards, 2003). According to Edwards (2003), the severe CNS side effects that have been reported in several vertebrates following IVM treatment (e.g. anorexia in dogs) are due to an absence or functional deficiency of P- gp (Edwards, 2003), that favors its absorption. However, the existence of this P-gp prevents the accumulation of this drug, thereby limiting its toxic effects. Despite not being characterized in polychaetes, it is possible to suggest that a similar functional structure may also exist, and contribute for the here observed lack of adverse effects.

Conclusions

The major conclusion from this study is that ivermectin major effect was the behavioral alteration, observed in exposed individuals. In comparison with all biochemical parameters, behavior seemed always to be more impacted, and no biochemical changes were even noticeable. The importance of behavior as effect criterion is thus high, since chronically exposed animals took considerably longer periods of time to bury themselves, a finding of indisputable ecological significance. In the case of acutely exposed animals, the absence of behavioral and biochemical changes was reported after a short period of only 4 days, which may not be sufficient to significantly alter the physiology of the animals. In fact, IVM caused only significat effects flowing prolonged periods of exposure, which is exactly the most likely scenario to happen in the environment. Considering the general absence of effects on the antioxidant and metabolic defensive systems in exposed worms, other antioxidant and conjugation mechanisms were most probably activated to combat the adverse effects (namely, oxidative stress) in *N. diversicolor*. Thus, it is suggested that more studies should be carried out in which non-enzymatic antioxidant mechanisms (such as the deployment of mucus, or the biosynthesis of pigments present in this species) are quantified in order to understand their effect on oxidative stress. Despite the relevance of the data obtained, the present study did not establish any causal link between behavioral and biochemical effects. Our results support the hypothesis that IVM may cause chronic toxicity, becoming an additional challenge for animals that live in the sediment. The greater propensity of IVM to act on sediment organisms is justified by the fact that this compound may remain for months at this location due to the affinity of IVM for soil/sediment particles. As a result of these changes, it is expected that

changes in the ecosystem may occur, including changes in the food chain, bioturbation of estuarine waters and consequently in sediment and nutrient recirculation.

Declarations

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Ethics approval and consent to participate

Not applicable. Animals used in this assay are from an invertebrate species, which do not require previous ethics approval.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Ana Gomes was involved in formal analysis; investigation; methodology; and writing of the original draft.

Diana Pinheiro was involved in formal analysis; investigation; methodology; and writing of the original draft.

Bruno Nunes was involved in conceptualization; data curation; formal analysis; funding acquisition; project administration; resources; supervision; validation; and in writing, namely, reviewing and editing the manuscript.

All authors read and approved the final manuscript.

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Figures

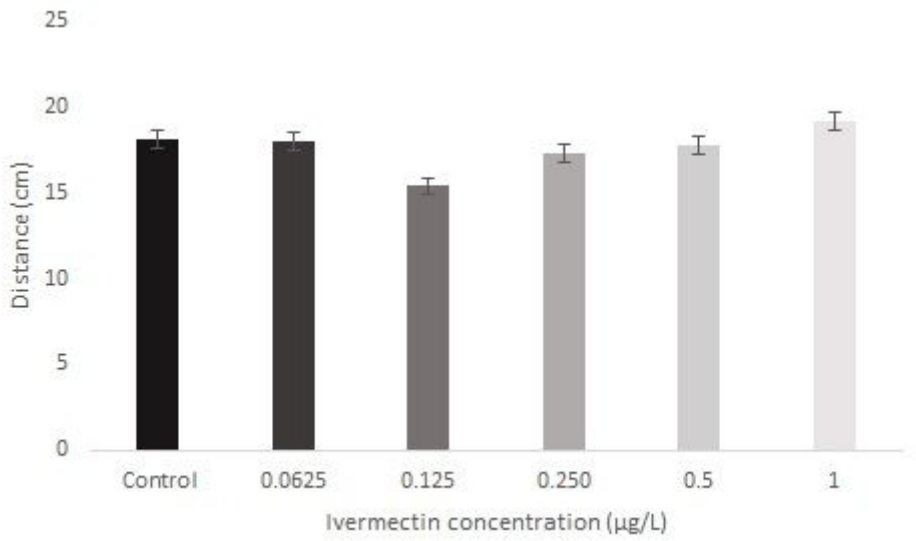

Figure 1

Displacement of individuals of H. diversicolor acutely exposed to IVM. Average distances traveled by different groups as a function of ivermectin concentration, expressed in centimeters. Values are the mean distance, 10 replicates, and corresponding standard error bars.

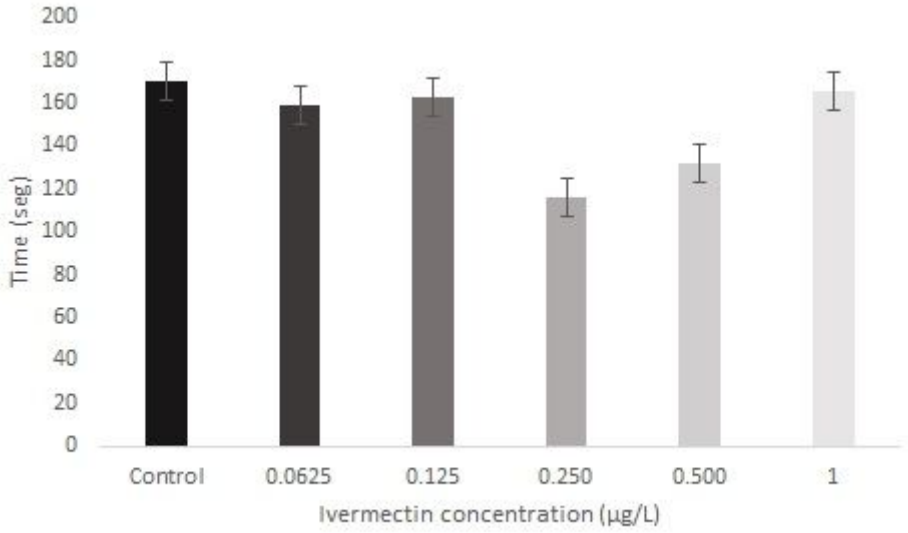

Figure 2

Burrowing time of individuals of H. diversicolor acutely exposed to IVM.. Mean time each group took to bury as a function of ivermectin concentration, expressed in seconds. Values are the mean distance, 10 replicates, and corresponding standard error bars.

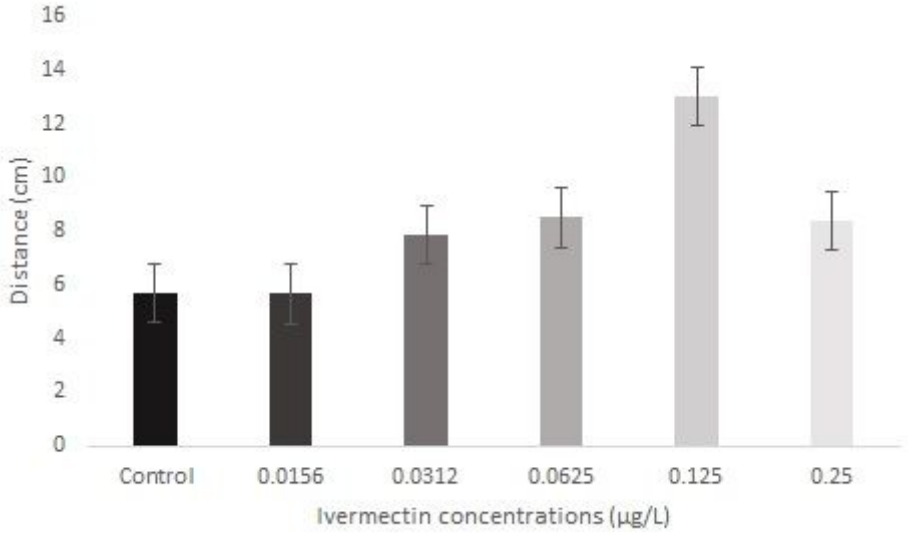

Figure 3

Displacement of individuals of H. diversicolor chronically exposed to IVM. Average distances traveled by different groups as a function of ivermectin concentration, expressed in centimeters. Values are the mean distance, 10 replicates, and corresponding standard error bars.

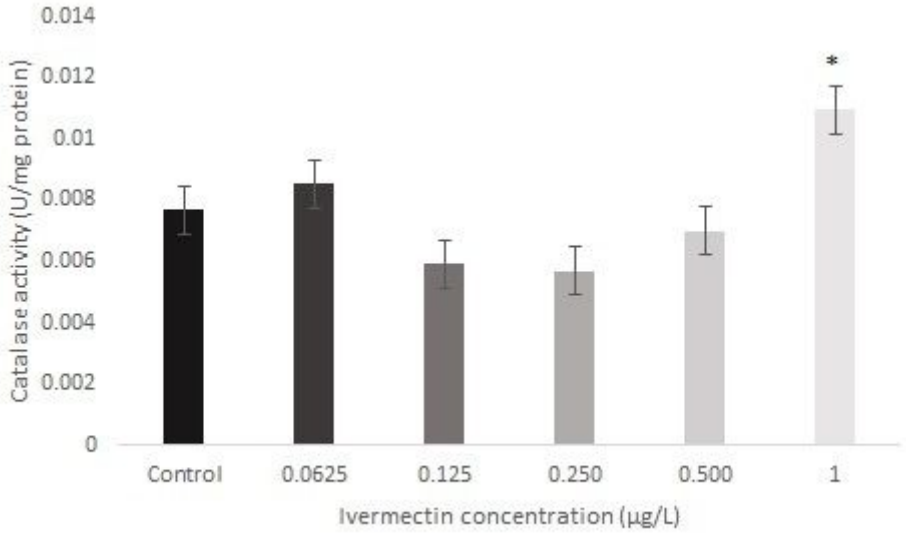

Figure 4

Catalase activity of individuals of H. diversicolor acutely exposed to IVM. Values are the mean activity, 10 replicates, and corresponding standard error bars. * - statistical differences in relation to the control treatment, $p<0.05$.

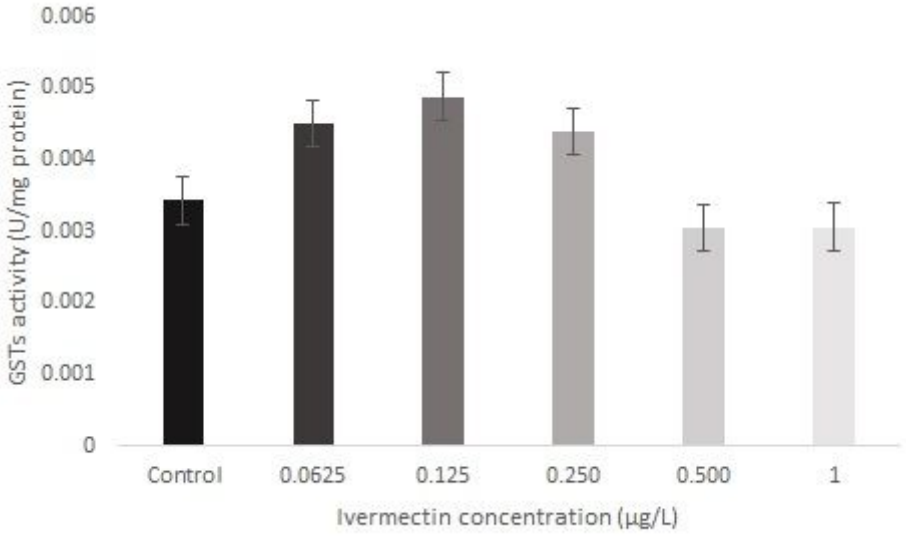

Figure 5

GSTs activity of individuals of H. diversicolor acutely exposed to IVM. Values are the mean activity, 10 replicates, and corresponding standard error bars.

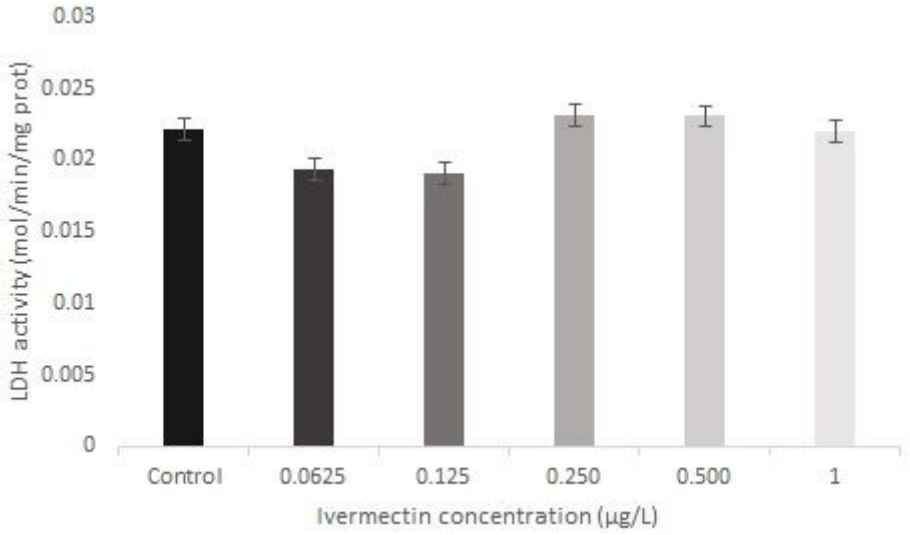

Figure 6

LDH activity of individuals of H. diversicolor acutely exposed to IVM. Values are the mean activity, 10 replicates, and corresponding standard error bars.

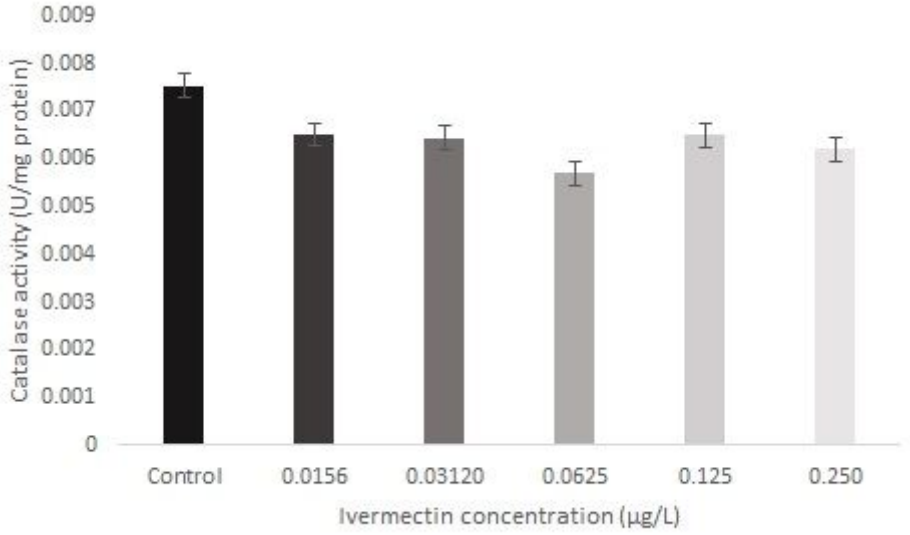

Figure 7

Catalase activity of individuals of H. diversicolor chronically exposed to IVM. Values are the mean activity, 10 replicates, and corresponding standard error bars.

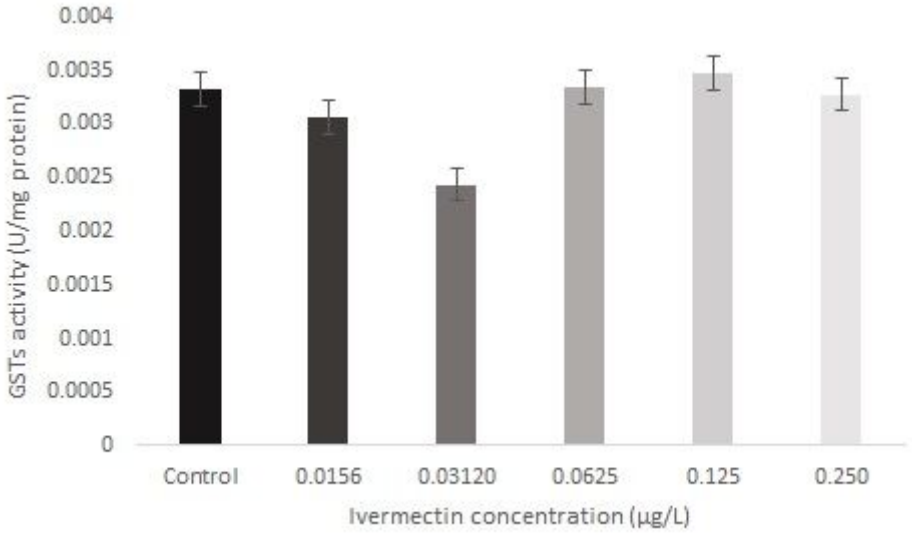

Figure 8

GSTs activity of individuals of H. diversicolor chronically exposed to IVM. Values are the mean activity, 10 replicates, and corresponding standard error bars.

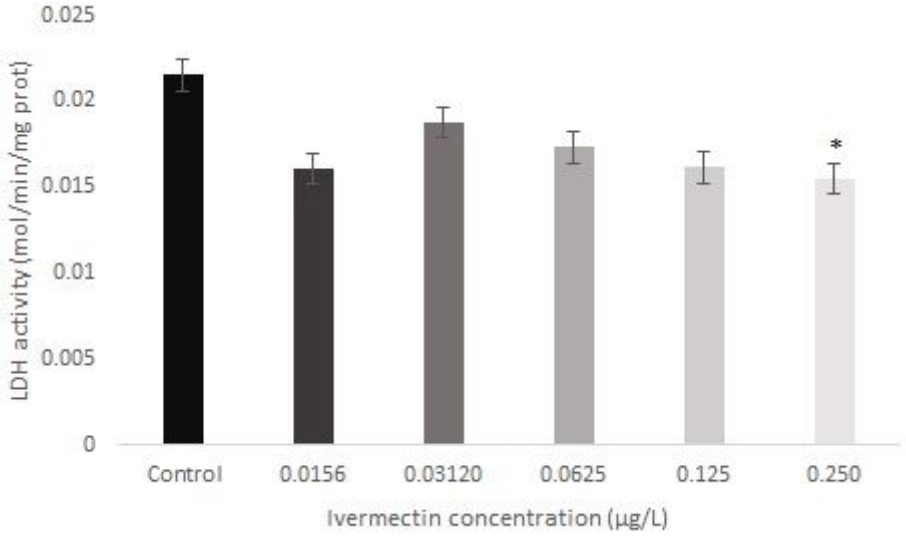

Figure 9

LDH activity of individuals of H. diversicolor chronically exposed to IVM. Values are the mean activity, 10 replicates, and corresponding standard error bars.