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Toxic effects of molluscicidal baits to the terrestrial isopod *Porcellionides pruinosus* (Brandt, 1833)

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Abstract
Purpose Methiocarb and metaldehyde are the most common molluscicides applied in agricultural and horticultural fields in Portugal and elsewhere in Europe. The application of molluscicidal baits to control slug and snail populations can pose a threat to non-target organisms like terrestrial isopods, because they are detritivorous and may feed on the toxic baits applied to the soil surface. The aim of this work was to evaluate the effects and understand the modes of action of these molluscicides to terrestrial isopods.
Materials and methods In this study, the terrestrial isopod *Porcellionides pruinosus* was exposed to these two molluscicides, and the time to lethality was evaluated. Biochemical indicators (biomarkers) are known to provide early warning signs of environmental pollution or stress conditions to the organisms, by measuring cellular or molecular responses of the target organism to xenobiotic agents. Therefore, to evaluate modes of action and effects and also to see if biomarkers can be used as early warning tools in molluscicidal exposures, three different enzymes, glutathione *S*-transferase (GST), acetylcholinesterase (AChE) and catalase (CAT), were analysed upon single exposures and binary mixtures tests.
Results and discussion These two molluscicides showed to be of extreme concern regarding terrestrial isopods, as all animals died after 24 h of exposure to methiocarb, and only 20% survived after 56 h of exposure to metaldehyde. Results indicate that the carbamate methiocarb inhibited significantly AChE activity, but no effects were observed in CAT and GST levels. The exposure to metaldehyde had no effects on AChE, but a decrease in GST activity as well as a general increase in CAT activity was observed at the higher exposure period tested (32 h). The combined exposure of the two molluscicides resulted in a general decrease in AChE and CAT activity, but no visible effects were observed in terms of GST activity.
Conclusions The LT50 values found in the single exposures to both molluscicides were very low, especially in the case of the carbamate methiocarb. The use of several biomarkers was a suitable tool to understand the mode of action of these two molluscicides in this isopod species.

Keywords Biomarkers · Metaldehyde · Methiocarb · Molluscicides · *Porcellionides pruinosus*

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1 Introduction

The chemical control of snails and slugs is mainly carried out with the use of molluscicidal pellets. The most commonly used molluscicides are metaldehyde and the carbamate methiocarb, which represent more than 90% of all European sales for molluscicides (Henderson and Triebskorn 2002). However, the use of these pellets can represent a hazard to non-target soil invertebrates such as woodlice that can feed on the pellets and thus be poisoned by these pesticides (Bailey 2002; Bieri 2003). In agricultural fields, molluscicide baits are randomly distributed on the soil surface (by manual or mechanical dispersion) in a way that in some surface areas, the number of baits available will be higher than the established recommended dose. The application of molluscicides in some cultures (vineyards and orchards) implies several applications within

a safety interval of 14 days. Moreover, in some vegetable crops (e.g. horticultural), baits are applied near the stem in agglomerates, to prevent snails and slugs from attacking the aerial part of the plants. Looking at this, it seems plausible to admit that isopods could encounter more than the recommended dose of baits (granules per area).

The modes of action of these two molluscicides in snail and slugs are well documented, with metaldehyde inducing severe alterations and ultrastructural destruction in mucocytes which leads to dehydration and subsequent death (Triebskorn et al. 1998), whereas methiocarb acts upon the central nervous system inhibiting acetylcholinesterase (AChE), which can cause overstimulation of the nervous system and ultimately the death of the animal (Engenheiro et al. 2005). Terrestrial isopods live on the surface layer of the soil and are detritivorous species responsible for nutrient recycling in soil ecosystems (Zimmer 2002). *Porcellionides pruinosus* has been used in ecotoxicological tests (Loureiro et al. 2002, 2005) and is considered a good test-species to evaluate pernicious effects of xenobiotics (Loureiro et al. 2009).

Biochemical indicators, known as biomarkers, can serve as early warning signs of environmental pollution or stress indication to soil organisms and can be divided in three classes: exposure biomarkers, effect biomarkers and susceptibility biomarkers (Schlenk 1999). Biomarkers of exposure are related with cellular or molecular responses indicating an interaction between an organism and a xenobiotic agent (Roberts and Oris 2004). Several studies have been made using biomarkers as tools to assess the effects of different pollutants to terrestrial isopods, as heavy metals (Köhler et al. 1996), PAHs (Stroomberg et al. 1999), organochlorine pesticides (Köhler et al. 1999), organophosphorous pesticides (Stanek et al. 2006) or titanium nanoparticles (Jemec et al. 2008).

Three enzymes were chosen to perform this study, based on their specific action, relevance and sensitivity to xenobiotic compounds. AChE is responsible for breaking down acetylcholine, an enzyme that is known to be inhibited by organophosphorous pesticides and carbamates. These pesticides can bind to the hydroxyl group of the functional part of this enzyme, resulting in a phosphorylated enzyme that has no activity and, hence, cannot hydrolyze the substrate acetylcholine (Fulton and Key 2001). Glutathione *S*-transferase (GST) is a phase II detoxification enzyme, which is involved in the cellular detoxification of several chemicals, catalysing the conjugation of reduced glutathione to several xenobiotic agents (Hayes and Pulford 1995), and is useful as an indicator of the effects of fungicides and insecticides (Schreck et al. 2008). Catalase (CAT) catalyses the decomposition of hydrogen peroxide derived from the formation of other reactive oxygen species (ROS), such as superoxide or hydroxyl radicals that were derived from phase I detoxification processes and led by the cytochrome P450 enzyme (Brown et al. 2004).

The aim of this study was to evaluate the effects of two molluscicides on the terrestrial isopod *P. pruinosus* (Brandt, 1883). This assessment was made in three different steps: first, to evaluate lethal effects of both molluscicides in time; second, to detect changes on the activity of three enzymatic biomarkers AChE, GST and CAT upon molluscicidal exposure, understand their modes of action in isopods and question the use of biomarkers as early warning tools in this kind of exposures; and finally, to identify the combined effect of the two chemicals to this terrestrial isopod.

2 Materials and methods

2.1 Test organisms

The isopods used in this experiment were obtained from a laboratorial culture, maintained in a climatic chamber at 25° C, 60% moisture content and with a 16:8 h light:dark photoperiod. Only adult animals (15–25 mg wet weight) with antenna were used. In the beginning of the test, no sex differentiation was done, although pregnant females were not used in the experimental procedure.

2.2 Test chemicals

Two molluscicides were used in the experiment: metaldehyde and methiocarb, as the commercial formulations of CARAKOL® (Impex™) containing 5% of metaldehyde and MESUROL® (Bayer™) with 4% of methiocarb, respectively. Tests were performed in LUFA 2.2 soil, commercialised by the German Institution LUFA Speyer. The soil used was characterised by the following properties: pH of 5.5, organic matter content of 3.9% and 6% of clay, 17% of silt and 77% of sand.

2.3 Experimental procedure

2.3.1 Acute bioassay

An acute bioassay was performed to establish the sensitivity of *P. pruinosus* to the molluscicide baits through time. Twenty isopods were exposed to five metaldehyde baits for a period of 56 h, and 20 isopods were exposed to three baits of methiocarb for 24 h. Isopods' fitness was routinely checked and registered, and the lethal time (LT_{50}) after the beginning of exposure to the molluscicide baits was calculated. Considering the LT_{50} values calculated, several single exposure periods were chosen to evaluate the sublethal effects of each of the pesticides.

2.3.2 Single chemical exposure

Each animal was placed individually in a plastic box, with a surface area of 0.0064 m^2 containing 30 g wet soil. In this experiment, we used five baits of metaldehyde per test-box, which gives a concentration 20 times higher than the recommended dose (39 pellets per square metre). The recommended dose for the carbamate methiocarb is 20 baits per square metre, and in the experimental procedure, we tested three pellets per test-box, giving a concentration 30 times higher than the field recommended dose. Even though, this scenario can be widely found in agriculture fields or gardens, because molluscicide application is done randomly, and baits are usually found at high densities in some areas, forming agglomerates.

In this experimental setup, isopods were collected 8, 16, 24 and 32 h after metaldehyde exposure, and 1, 2, 3 and 4 h after methiocarb exposure. Ten isopods were sampled per time of exposure. After each of these sampling times, five isopods were collected from a control test-box, maintained under the same conditions although without any molluscicide baits. Thus, a total of 60 isopods was analysed per molluscicide. Isopods were then frozen at −80°C until the biomarker analyses were performed. In addition, several test-boxes with different exposure periods to control situations were also included in this experiment to determine if there were any shifts in the enzymatic activities throughout time.

2.3.3 Experimental procedure—mixture toxicity exposure

For the mixture toxicity tests, isopods were exposed to the two molluscicides in six different combinations for time of exposure. Each combination comprised ten isopods exposed individually to three baits of methiocarb (Mb) and five baits of metaldehyde (Md), alternatively and never simultaneously (Table 1). In addition, several isopods were also kept in a test box without any contamination (control conditions) as the mixture experiment went on. Five isopods were collected after 17 h, five isopods were collected after 25 h and finally, ten isopods were collected after 34 h. Again, after each sampling time, animals were stored at −80°C until the biomarkers analyses were performed.

2.4 Preparation of the animals

For the biomarker analysis, isopods were divided in two sections: head and body. The head was used for the AChE assay, and the remaining body was used for GST and CAT assays. Homogenisation of the animals was made using a sonicator (KIKA Labortechnik U2005 Control™).

Table 1 Experimental design used for the six combined exposures performed with three baits of methiocarb and five baits of metaldehyde, to the terrestrial isopod *Porcellionides pruinosus*, using two consecutive exposures

Combination	First exposure period	Second exposure period
Mb1hMd16h	1 h to methiocarb	16 h to metaldehyde
Mb1hMd32h	1 h to methiocarb	32 h to metaldehyde
Md32hMb1h	32 h to metaldehyde	1 h to methiocarb
Md32hMb2h	32 h to metaldehyde	2 h to methiocarb
Mb1hMd24h	1 h to methiocarb	24 h to metaldehyde
Mb2hMd24h	2 h to methiocarb	24 h to metaldehyde

2.5 Determination of AChE activity

After sonication, samples using isopods' heads were centrifuged at 1,700 g for 3 min at 4°C. The obtained supernatant was immediately assayed for AChE activity according to the Ellman technique (1961) adapted to the microplate (Guilhermino et al. 1996). In a 96-well microplate, 250 μl of the reaction solution was added to 50 μl of the sample, and absorbance was recorded at 414 nm, after 10, 15 and 20 min. The reaction solution had 1 ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10 mM solution, 1.280 ml of 0.075 M acetylthiocholine iodide solution and 28.920 ml of 0.1 M phosphate buffer. The enzyme activity is expressed as unit (U) per milligramme of protein. A unit corresponds to a nanomole of substrate hydrolyzed per minute, using a molar extinction coefficient of 1.36×10^{-3} M^{-1} cm^{-1}.

2.6 GST analysis

GST activity was determined based on the method described by Habig et al. (1974) and adapted to microplate (Diamantino et al. 2001). We mixed 100 μL of PMS in 200 μL of a reaction solution. The reaction solution was a mixture of 4.950 ml K-phosphate 0.1 M (pH 6.5) with 900 μL GSH 10 mM, and 150 μL 1-chloro-2,4-dinitrobenzene (CDNB) 10 mM and was measured at 340 nm. The enzyme activity is expressed as unit (U) per milligramme of protein. A unit corresponds to a nanomole of substrate hydrolyzed per minute, using a molar extinction coefficient of 9.6×10^{-3} M^{-1} cm^{-1}.

2.7 Catalase analysis

CAT activity was determined based on the method described by Clairborne (1985), consisting in mixing 50 μL of PMS with 500 μL H_2O_2 0.030 M, and 950 μL K-phosphate 0.05 M (pH 7.0) and measuring the decomposition of the substrate (H_2O_2) at 240 nm. The enzyme activity is

expressed as unit (U) per milligramme of protein. A unit corresponds to a micromole of substrate hydrolyzed per minute, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

2.8 Statistical analyses

The time after which 50% of the animals were found dead in the test-boxes following molluscicide application (LT_{50}) was calculated using a logistic equation (STATISTICA 7).

Enzymatic activities obtained in the different controls (different time periods) were compared using a one-way analysis of variance (ANOVA; SIGMA STAT 3.5.) to detect possible differences between animals collected in different periods from the control test-boxes. If enzymatic activities in control animals were not different in any of the experiment periods, these values were pooled and used as a total mean control to compare changes in enzymatic activities of animals exposed to the molluscicides also using a one-way ANOVA (SIGMA STAT 3.5.). Whenever data were not normally distributed and data transformation did not correct for normality, a Kruskal–Wallis ANOVA on Ranks was performed, followed by the Dunnett's method when significant differences were found.

3 Results

3.1 Acute bioassay

After a period of 24 h of continuous exposure to methiocarb baits, all the animals in the test-boxes were found dead, and a LT_{50}=7.27 (5.38–9.18 h) was obtained (Fig. 1). This result clearly indicates the severe toxicity of this molluscicide to the terrestrial isopod *P. pruinosus*. The application of metaldehyde baits also resulted in a high mortality rate (Fig. 1) after the 56-h period of exposure with a calculated LT_{50} of 50.49 h (50.48–50.50 h).

It was observed that upon exposure, isopods detected (by smell or vision) the blue baits, and almost immediately direct themselves to the baits, walking around them and bite a small fraction of one bait. After this episode, isopods start to show effects from baits toxicity like changes in pattern mobility.

3.2 AChE activity

No statistical differences were found between activities observed in controls periods of metaldehyde (P=0.473), methiocarb (P=0.824) and combined experiments (P=0.463).

Metaldehyde did not have any effects in the activity of AChE of the isopod *P. pruinosus*. After comparing the four exposure periods with the correspondent control values, no differences were found in this enzyme activity. The results in AChE activity were homogenous during the 32-h of exposure, and no variations were found between the animals exposed (Fig. 2).

Methiocarb significantly inhibited AChE activity in *P. pruinosus* already after the first hour of exposure to the molluscicide baits and throughout the experimental period. In all sampling times, the enzymatic activity of exposed animals was smaller than the control, with its lowest activity being observed after 4 h of exposure (53,997 U/mg protein), showing an inhibition of 42% compared with control activity (Fig. 2).

In the joint toxicity experiments, a significant inhibition of AChE activity was found in almost all the combinations performed (H=60.19, $P\leq$0.001), with the exception of the combination of Md32hMb1h, where the activity obtained in the exposed animal (132.81 U/mg protein) was similar to control animals. Although not expected, a significant increase in AChE activity in comparison to the control was observed in the combined effect of 32 h of metaldehyde plus 2 h of exposure to methiocarb (Fig. 2). In this combination, the value calculated (281.47 U/mg protein) was more than the double observed in the control (130.50/mg protein). This experiment was repeated twice, and the pattern of response of increased AChE activity in the referred combination was consistent in all experiments made.

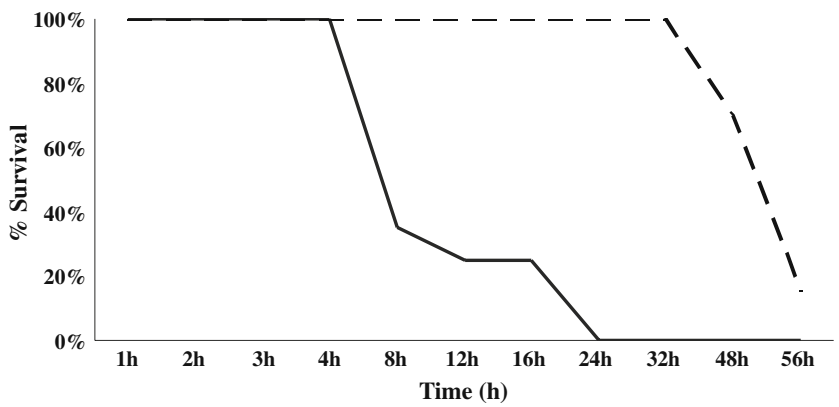

Fig. 1 Percentage of survival of the isopod *Porcellionides pruinosus* exposed to three methiocarb baits (*straight line*) and five metaldehyde baits (*dashed line*) in time (hours)

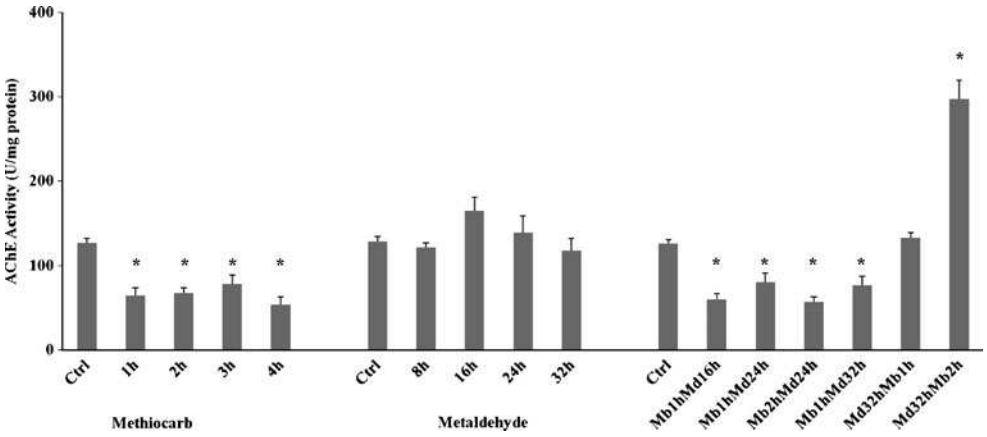

Fig. 2 Acetylcholinesterase (AChE) activity in *Porcellionides pruinosus* exposed to metaldehyde, methiocarb and six different combinations of the two baits. Results are expressed as the mean value of AChE activity (units per milligramme protein) with associated standard error. *Asterisk* indicates significant differences between control and treatments ($P \leq 0.05$)

3.3 GST activity

No statistical differences were found between activities observed in controls periods of metaldehyde ($P=0.853$) methiocarb ($P=0.581$) and combined experiments ($P=0.682$).

An increase in GST activity after giving metaldehyde baits to the isopods was observed after 16 h of exposure exhibiting 125% of the activity calculated for the control animals (Fig. 3). Extending the exposure periods to 24 and 32 h, GST activity decreased. In these two experimental periods, the enzymatic values calculated for 24 and 32 h was 37.98 and 39.92 U/mg protein, resulting in an inhibition of 73% and 78%, respectively.

Methiocarb did not have any effects in GST activity (see Fig. 3); values calculated for 24 and 32 h and respective control periods were consistent during the experimental period without any oscillation of the calculated values of activity. In addition, the combination of the two molluscicides did not provoke any effects in GST activity; in all combinations, the values were similar to those observed in the respective control (see Fig. 3).

3.4 CAT activity

No statistical differences were found between activities observed in control periods of metaldehyde ($P=0.506$) methiocarb ($P=0.122$) and combined experiments ($P=0.433$).

The application of metaldehyde baits caused an increase in CAT activity in all exposure periods but the 16 h sampling time (Fig. 4). This increase in CAT activity was more evident after the periods of 24 h, where the mean values in exposed animals (409.05 U/mg protein) were six times higher than the values in the control (86.63 U/mg protein). Also, in the last sampling time (32 h), the mean values of the antioxidant enzyme in the animals exposed to the baits were twice the values in control situation (228.47 U/mg protein).

CAT activity values were consistent along the 4 h of exposure to methiocarb baits, since no variation in this enzyme activity was observed (see Fig. 4). Although an inhibition of 71% and 63% of CAT activity was found after 2 and 3 h of exposure to this molluscicide, no significant differences were found after the statistical procedure.

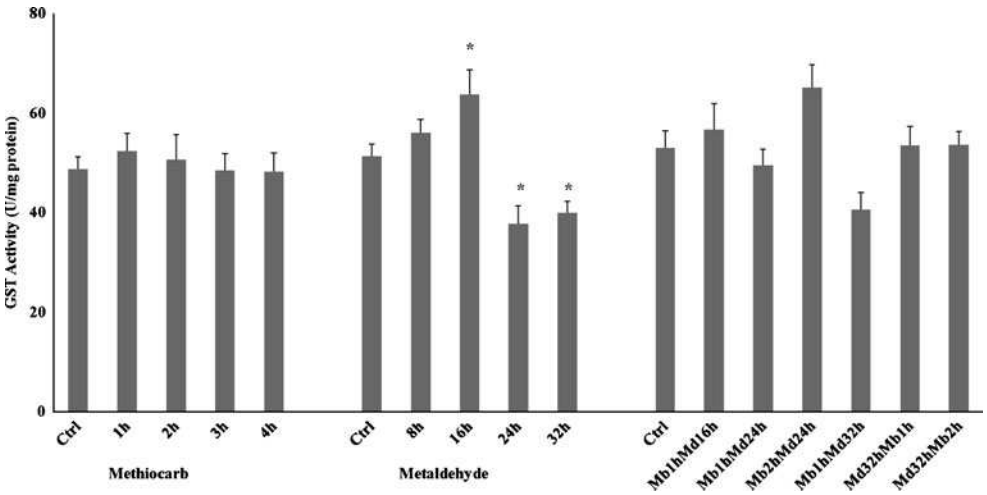

Fig. 3 Glutathione *S*-transferase (GST) activity in *Porcellionides pruinosus* exposed to metaldehyde, methiocarb and six different combinations of the two baits. Results are expressed as the mean value of GST activity (units per milligramme protein) with associated standard error. *Asterisk* indicates significant differences between control and treatments ($P \leq 0.05$)

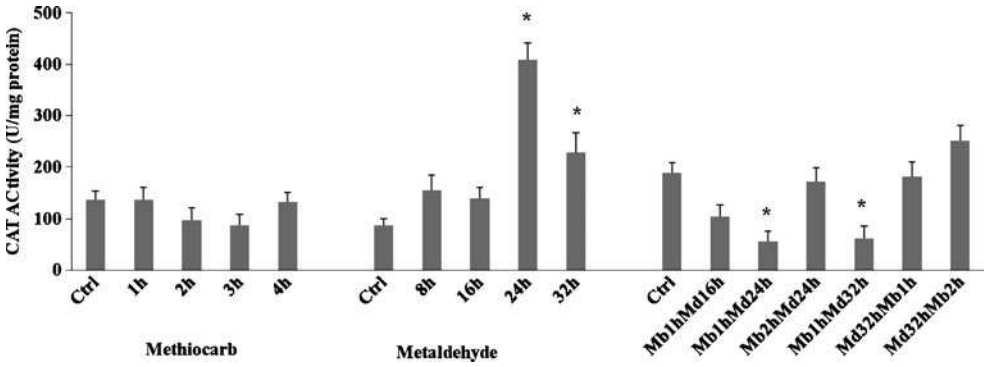

Fig. 4 Catalase (CAT) activity in *Porcellionides pruinosus* exposed to metaldehyde, methiocarb and six different combinations of the two baits. Results are expressed as the mean value of CAT activity (units per milligramme protein) with associated standard error. *Asterisk* indicates significant differences between control and treatments ($P \leq 0.05$)

In the mixture experiments, only two combinations (Mb1hMd24h and Mb1hMd32h) were statistically different from the control (see Fig. 4), showing and inhibition of 30% and 33% when compared to control mean values.

4 Discussion

Methiocarb and metaldehyde caused severe lethal effects to the terrestrial isopod *P. pruinosus* in short-term exposures, although methiocarb and metaldehyde are considered non-harmful to terrestrial invertebrates, mainly due to its rapid dissipation in soil (Meredith 1996; Bieri 2003). The scarce amount of time needed to observe lethal effects after bait consumption in the acute bioassay performed clearly indicates that molluscicide application represents a risk for these terrestrial invertebrates. Regarding the rapid effects that these compounds induce, a regular risk assessment procedure might not be possible as dose–effects assessments are not clear. Instead, time to death can be considered a useful approach in situations similar to this one, where short time periods (e.g. few hours) are enough to induce lethality. In addition, chronic evaluation, using sub-lethal concentrations, will be improbable, as dosages are not controlled. As explained before, it was observed that after some minutes of exposure, isopods were attracted by the baits and bitted a small portion of one of the bait, resulting is almost immediate effects (changing in mobility patterns). Thus, the dose applied in this study cannot be related to the effects and will be difficult to quantify.

Metaldehyde did not have any effect in the AChE activity during the exposure period of 32 h, since no variations in AChE levels were observed in the exposure periods sampled. This seems to indicate that metaldehyde and its metabolic secondary product aldehyde, tested in the conditions described, do not affect this enzyme activity.

Following application of the molluscicide metaldehyde, some authors indicate a diminution in AChE activity in the nervous tissue of the snail *Lymnaea acuminata* (Tiwari et al. 2008). Others (Putchakayala and Ram 2000) found that although having been responsible for an excitatory effect in muscle contraction in the zebra mussel, metaldehyde did not enhance or inhibit the effects of acetylcholine, probably because the two stimulants activate different cells within the organism. The application of methiocarb strongly inhibited AChE activity being reduced after 1 h of exposure to almost 50% compared to the control. This drastic inhibition of AChE activity surely can be linked with the mode of action of the carbamate methiocarb, which is designed specifically to inhibit this enzyme, as it was attested by several research papers concerning the effects of this pesticide in AChE activity of various non-target species (Taylor 1996; Wellman and Heimbach 1996; Jensen 1998; Hyne and Maher 2003; Boran et al. 2007; Radwan et al. 2008). The strong decrease in AChE activity within a small exposure period is also in agreement with previous studies that illustrated the decrease in the activity of this enzyme when terrestrial isopods were exposed to organophosphate insecticides, that have a similar inhibitory influence in AChE as carbamates, even at sub-lethal concentrations (Fischer et al. 1997; Ribeiro et al. 1999; Stanek et al. 2003; Engenheiro et al. 2005). The depression in AChE activity as a direct result of *P. pruinosus* ingestion of methiocarb pellets and the sensitivity of this enzyme to exposure represents a specific response and is of critical importance in the determination of the mode of action of this molluscicide to terrestrial isopods and, therefore, can be considered a precise biomarker of exposure (Peakall 1999).

In the combinations where the methiocarb pellets were first given to the animals, AChE activity decreased in comparison with the control animals. In these cases, apparently, the subsequent period of exposure to metaldehyde baits had no effect in the enzymatic activity, since enzyme activity was comparable with the single experiments with methiocarb tested with the same exposure period. It is noteworthy, however, to observe that in one mixture, AChE activity increased in comparison to control values. The combination comprised 32 h of exposure to metaldehyde plus 2 h of exposure to methiocarb. In this treatment, the values obtained for AChE activity were the

highest of all the experiments performed and almost twice the activity registered in the single toxicity experiment with metaldehyde for 32 h. Previous works using the fresh water snail *Lymnaea stagnalis* (Mills et al. 1990, 1992) described an increase in firing activity and development of paroxysmal depolarising shifts in buccal motoneuron, indicating severe alterations in the central nervous system (CNS) after application of metaldehyde. This suggests that these alterations could provide a useful model for screening the effects of molluscicides on a functional neuronal network. An induction in the AChE activity has been recorded in cytochemical experiments, in which sequence analysis revealed that AChE expressed in apoptotic cells could be considered a biomarker and a regulator of apoptosis (Zhang et al. 2002). The results seem to indicate that the application of this molluscicide has an impact on the CNS of *P. pruinosus*, and that increasing time of contact with the pellets induces an augment of AChE activity. From the above, one can hypothesise that the increase in AChE activity might be a result of intra-specific responses of the CNS of this isopod species to the application of metaldehyde baits in combination with methiocarb baits.

Following the application of metaldehyde, GST activity increased significantly after 16 h of exposure, and this induction might be related to the presence of the xenobiotic and subsequent activation of the natural detoxifying defence system (Van der Oost et al. 2003). After 24 and 32 h of exposure, the values of GST activity decreased in comparison with the values in the control animals. It seems that after triggering GST levels, the continuous exposure to metaldehyde led to an inhibition of GST activity, which is in conformity with other studies where early induction of GST was not sustained during longer exposure periods (Ferrari et al. 2007). In adults of the isopod species *Porcellio scaber*, a decreased GST activity was also found after application of the neonicotinoid imidacloprid (Drobne et al. 2008), which is in conformity with our results. It is important to state that this induction of GST activity and subsequent decrease with time of exposure not only reveals the role of this detoxifying enzyme in the biotransformation of metaldehyde but also is a valuable contribution to understand the mode of action of this molluscicide to *P. pruinosus*.

The relatively homogenous response throughout the test period seems to point out that methiocarb does not have an effect on GST activity, since no induction or reduction in the basal levels of this enzyme was detected. This indicates that at least in the tested exposure period, no activation of this xenobiotic metabolising enzyme occurs, which also has been observed in previous works where this enzyme conjugate was proven to be insensitive to the application of carbamates (Ribera et al. 2001) and organophosphorous pesticides (Massa et al. 2008) in earthworms and lepidopters. The lack of sensitivity of GST to some classes of insecticides may be related to intrinsic and molecular properties of this enzymatic complex (Crane et al. 2002), and in some cases, the time of exposure or concentration of the xenobiotic is not enough to activate this enzymatic complex.

In the binary experiments with the two molluscicides, no changes in GST activity were observed, even in the combinations where metaldehyde baits were given at first, at concentrations that caused a decrease in the GST levels in single toxicity experiments. The subsequent period of exposure to methiocarb baits could be responsible for a 'compensation' in the enzymatic levels, and thus the inhibitory effects are not evident in the combination experiments. This response in all combinations tested, with no variations found along the exposure periods, seems to confirm that this enzymatic conjugate cannot always be used to detect an impairment caused by pesticide application, and thus, results should be interpreted carefully (Hyne and Maher 2003).

Among biomarkers used in toxicological evaluation, those based on antioxidant defences, like CAT, reveal potentially impairment in an organism's fitness mediated by the formation of ROS (Bocchetti and Regoli 2006). The increase in ROS production is known to result in an increased oxidative damage to macromolecules and alterations in critical cellular processes (Howcroft et al. 2009). Following application of metaldehyde, an increase in CAT activity was observed in the first two periods of exposure, with a significant increase in the 24- and 32-h exposure periods. This result is in conformity with previous studies that also observed a significant increase in CAT activity in terrestrial snails after the administration of metaldehyde (el-Wakil and Radwan 1991) and might be indicative of a defence mechanism towards cellular damage occurring in the organism as a consequence of ROS formation.

CAT activity did not have statistical differences in the four sampling times of methiocarb exposure when compared with control values, although a decrease was observed after 2 and 3 h of exposure to the carbamate. A decrease in CAT activity upon pesticide exposure has been described for other isopod species, like *P. scaber* (Jemec et al. 2008), mussels and daphnids (Khessiba et al. 2005; Jemec et al. 2007). This non-activation of CAT activity in this isopod species might also be related to the results obtained in the enzyme GST, which was also not induced by the exposure to methiocarb and the fact that the short period of exposure to the carbamate was not sufficient to induce changes, since it is known that carbamate pesticides do cause oxidative stress and are responsible for the activation of CAT (Maran et al. 2009).

An inhibitory pattern of CAT activity was observed in the combinations experiment where the animals first

received methiocarb baits. In only two of the sampling times, the decrease on CAT activity was statically significant (Mb1hMd24h and Mb1hMd32h), and the following period of exposure to metaldehyde baits was not enough to reverse the effects obtained in these combinations. The decreasing activity of CAT observed in the single exposure to methiocarb seems to be confirmed in this combined experiment, since statistical differences from the control were observed. This could be a consequence of a longer period of exposure of the animals to the baits in the combined experiment in comparison with the single administration of methiocarb. In the combinations where metaldehyde baits were given, at first, no differences in CAT activity were found in comparison with the enzymatic levels observed in the control. These results were not in accordance with the extreme increase in CAT activity found in single toxicity experiments with metaldehyde, but probably, the subsequent exposure to methiocarb baits could be responsible for the mitigation of an increased CAT activity during the exposure to metaldehyde baits.

5 Conclusions

The LT50 values found in the single exposures to both molluscicides were very low, especially in the case of the carbamate methiocarb. This clearly indicates the severity of the exposure to these baits by terrestrial isopods, since even a short exposure to the baits redounded to an extremely high mortality rate. So, it seems pertinent to take this into account in risk assessment schemes for molluscicide application in agricultural fields or gardens and its impact on macrofauna like terrestrial isopods. As expected, due to the specificity of this enzyme, AChE activity seems to be an accurate biomarker of exposure to the carbamate methiocarb. The two enzymes, GST and CAT, involved in protecting cellular damages from oxidative stress confirmed also to be useful in assessing metaldehyde exposure. The use of several biomarkers was a suitable tool to understand the mode of action of these two molluscicides in this isopod species.

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References

Bailey SER (2002) Molluscicidal baits for control of terrestrial gastropods. In: Barker GM (ed) Molluscs as crop pests. CABI Publishing, Wallingford, pp 33–54

Bieri M (2003) The environmental profile of metaldehyde. In: Henderson IF (ed) Slugs and snails. In World Agriculture, British Crop Protection Council, Symposium Proceedings 80, pp 255–260

Bocchetti R, Regoli F (2006) Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. Chemosphere 65:913–921

Boran M, Altinok U, Çapkin E, Karaçam H, Biçer V (2007) Acute toxicity of carbaryl, methiocarb, and carbosulfan to the rainbow trout (*Oncorhynchus mykiss*) and Guppy (*Poecilia reticulata*). Turk J Vet Anim Sci 31:39–45

Brown PJ, Long SM, Spurgeon DJ, Svendsen C, Hankard PK (2004) Toxicological and biochemical responses of the earthworm *Lumbricus rubellus* to pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon. Chemosphere 57:1675–1681

Clairborne A (1985) Catalase activity. In: Greenwald R (ed) Handbook of methods for oxygen radical research. CRC Press, Boca Raton, pp 283–284

Crane M, Sildanchandra W, Kheir R, Callaghan C (2002) Relationship between biomarker activity and developmental endpoints in *Chironomus riparius* Meigen exposed to an organophosphate insecticide. Ecotox Environ Saf 53(3):361–369

Diamantino TC, Almeida E, Soares AVMV, Guilhermino L (2001) Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* straus. Chemosphere 45:553–560

Drobne D, Blažič M, Van Gestel CAM, Lešer V, Zidar P, Jemec A, Trebše P (2008) Toxicity of imidacloprid to the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea). Chemosphere 71:1326–1334

Ellman GL, Courtney KD, Andres VJ, Featherstone R (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmac 7:88–95

El-Wakil HB, Radwan MA (1991) Biochemical studies on the terrestrial snails *Eobania vermiculata* (Müller) treated with some pesticides. J Environ Sci Health Part B 26(5–6):479–489

Engenheiro EL, Hankard PK, Sousa JP, Lemos MF, Weeks JM, Soares AMVM (2005) Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environ Toxicol Chem 24(3):603–609

Ferrari A, Venturino A, D'Angelo AMP (2007) Effects of carbaryl and azinphos methyl on juvenile rainbow trout (*Oncorhynchus myskiss*) detoxifying enzymes. Pest Biochem Physiol 88:134–142

Fischer E, Farkas S, Hornung E, Past T (1997) Sublethal effects of an organophosphorus insecticide dimethoate on the isopod *Porcellio scaber*. Comp Biochem Physiol 116C(2):161–166

Fulton MH, Key PB (2001) Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ Toxicol Chem 20(1):37–45

Guilhermino L, Lopes MC, Carvalho AP, Soares AMVM (1996) Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32:727–738

Habig H, Pabst MJ, Jakoby WB (1974) Glutathione *S*-transferase, the first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139

Hayes JD, Pulford DJ (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol 30:445–600

Henderson I, Triebskorn R (2002) Chemical control of terrestrial gastropods. In: Barker GM (ed) Molluscs as crop pests. CABI Publishing, Wallingford, pp 1–31

Howcroft CF, Amorim MJB, Gravato C, Guilhermino L, Soares AMVM (2009) Effects of natural and chemical stressors on

Enchytraeus albidus: can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils? Environ Int 35:318–324

Hyne RV, Maher WA (2003) Invertebrate biomarkers: links to toxicosis that predict population decline. Ecotoxicol Environ Saf 54:366–374

Jemec A, Drobne D, Tišler T, Trebše P, Ros M, Sepčić K (2007) The applicability of acetylcholinesterase and glutathione S-transferase in *Daphnia magna* toxicity test. Comp Biochem Physiol Part C: Toxicol Pharmacol 144:303–309

Jemec A, Drobne D, Remskar M, Sepčić K, Tisler T (2008) Effects of ingested nano-sized titanium dioxide on terrestrial isopods (*Porcellio scaber*). Environ Toxicol Chem 27(9):1904–1914

Jensen S (1998) Acetylcholinesterase activity associated with methiocarb resistance in a strain of Western Flower Thrips, *Frankliniella occidentalis* (Pergande). Pestic Biochem Physiol 61:191–200

Khessiba A, Roméo M, Aïssa P (2005) Effects of some environmental parameters on catalase activity measured in the mussel (*Mytilus galloprovincialis*) exposed to lindane. Environ Poll 133:275–281

Köhler HR, Hiittenrauch K, Berkus M, Gräff S, Alberti G (1996) Cellular hepatopancreatic reactions in *Porcellio scaber* (Isopoda) as biomarkers for the evaluation of heavy metal toxicity in soils. Appl Soil Ecol 3(1):1–15

Köhler HR, Knödler C, Zanger M (1999) Divergent kinetics of hsp70 induction in *Oniscus asellus* (Isopoda) in response to four environmentally relevant organic chemicals (B[a]P, PCB52, g-HCH, PCP): suitability and limits of a biomarker. Arch Environ Contam Toxicol 36:179–185

Loureiro S, Sousa JP, Nogueira AJA, Soares AMVM (2002) Assimilation efficiency and toxicokinetics of 14C-lindane in the terrestrial isopod *Porcellionides pruinosus*: the role of isopods in degradation of persistent soil pollutants. Ecotoxicol 11:481–490

Loureiro S, Soares AMV, Nogueira AJA (2005) Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ Poll 138:121–131

Loureiro S, Amorim MJB, Campos B, Rodrigues SMG, Soares AMVM (2009) Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ Pollut 157(2):625–632

Maran E, Fernández M, Barbieri P, Font G, Ruiz MJ (2009) Effects of four carbamate compounds on antioxidant parameters. Ecotox Environ Saf 72:922–930

Massa R, Blevins S, Chao SL (2008) Role of acetylcholinesterase and glutathione S-transferase following exposure to nicosulfuron and diazinon in *Helicoverpa zea*. Ecotoxicol Environ Saf 71:230–235

Meredith RH (1996) Testing bait treatments for slug control. In: Henderson IF (ed) Slug and snail pests in agriculture. British Crop Protection Council, Farnham, pp 157–164

Mills JD, McCrohan CA, Bailey SER, Wedgwood MA (1990) Effects of metaldehyde and acetaldehyde on feeding responses and neuronal activity in the snail *Lymnaea stagnalis*. Pestic Sci 28:189–199

Mills JD, McCrohan CR, Bailey SER (1992) Electrophysiological responses to metaldehyde in neurones of the feeding circuitry of the snail *Lymnaea stagnalis*. Pestic Biochem Physiol 42(1–2):35–42

Peakall DB (1999) The use of biomarkers in hazard assessment. In: Peakall DB, Colin HW, Migula P (eds) Biomarkers: a pragmatic basis for remediation of severe pollution in Eastern Europe. Kluwer Academic Publishers, Dordrecht, Netherlands, pp 123–133

Putachakayala SM, Ram JL (2000) Toxic and excitatory effects of the molluscicide metaldehyde on the biofouling bivalve *Dreissena polymorpha* Pallas. Pest Manag Sci 56:39–42

Radwan MA, Essawy AE, Abdelmeguied NE, Hamed SS, Ahmed AE (2008) Biochemical and histochemical studies on the digestive gland of *Eobania vermiculata* snails treated with carbamate pesticides. Pestic Biochem Physiol 90:154–167

Ribeiro S, Guilhermino L, Sousa JP, Soares AMVM (1999) Novel bioassay based on acetylcholinesterase and lactate dehydrogenase activities to evaluate the toxicity of chemicals to soil isopods. Ecotoxicol Environ Saf 44:287–293

Ribera D, Narbonne JF, Arnaud C, Saint-Denis M (2001) Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil, effects of carbaryl. Soil Biol Biochem 33:1123–1130

Roberts AP, Oris JT (2004) Multiple biomarker response in rainbow trout during exposure to hexavalent chromium. Comp Biochem Physiol Part C: Toxicol Pharmacol 138:221–228

Schlenk D (1999) Necessity of defining biomarkers for use in ecological risk assessments. Mar Pollut Bull 39(1–12):48–53

Schreck E, Geret F, Gontier L, Treilhou M (2008) Neurotoxic effect and metabolic responses induced by a mixture of six pesticides on the earthworm *Aporrectodea caliginosa nocturna*. Chemosphere 71:1832–1839

Stanek K, Gabrijelcic E, Drobne D, Trebše P (2003) Inhibition of acetylcholinesterase activity in the terrestrial isopod *Porcellio scaber* as a biomarker of organophosphorous compounds in food. Arh Hig Rada Toksikol 54:183–188

Stanek K, Drobne D, Trebše P (2006) Linkage of biomarkers along levels of biological complexity in juvenile and adult diazinon fed terrestrial isopod (*Porcellio scaber*, Isopoda, crustacea). Chemosphere 64:1745–1752

Stroomberg GJ, de Knecht JA, Ariese F, van Gestel CAM, Velthorst NH (1999) Pyrene metabolites in the hepatopancreas and gut of the isopod *Porcellio scaber*, a new biomarker for polycyclic aromatic hydrocarbon exposure in terrestrial ecosystems. Environ Toxicol Chem 18:2217–2224

Taylor P (1996) Agents acting at the neuromuscular junction and autonomic ganglia. In: Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Goodman GA (eds) The pharmacological basis of therapeutics. McGraw-Hill, New York, pp 177–198

Tiwari F, Singh K, Singh DK (2008) Enzyme inhibition by different bait formulations in the nervous system of the snail *Lymnaea acuminate*. In: Joshi BD, Joshi PC, Joshi N (eds) Environmental pollution and toxicology. A.P.H. Publishing Corporation, New Dehli, pp 115–128

Triebskorn R, Christensen K, Heim I (1998) Effects of orally and dermally applied metaldehyde on mucus cells of slugs (*Deroceras reticulatum*) depending on temperature and duration of exposure. J Molluscan Stud 64:467–487

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Wellmann P, Heimbach F (1996) The effects of methiocarb slug pellets on the earthworm *Lumbricus terrestris* in a laboratory test. In: Henderson IF (ed) Slug and snail pests in agriculture. British Crop Protection Council, Farnham, pp 181–188

Zhang XJ, Yang L, Zhao Q, Caen JP, He HY, Jin QH, Guo LH, Alemany M, Zhang LY, Shi YF (2002) Induction of acetylcholinesterase expression during apoptosis in various cell types. Cell Death Differ 9:790–800

Zimmer M (2002) Nutrition in terrestrial isopods (Isopoda:Oniscidea): an evolutionary-ecological approach. Biol Rev Camb Philos Soc 77:455–493