Acetaminophen induced antioxidant and detoxification responses in a stygobitic crustacean

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Abstract

A variety of veterinary and human medicinal products (VHMPs) are found in groundwater, an often-neglected habitat inhabited by species with unique traits, stygobitic species. It is crucial to understand the effect of VHMPs on stygobitic species because they may respond differently to stressors than surface species. Our hypothesis is that groundwater species may be more susceptible to environmental contaminants due to less plasticity in their detoxification response and acquisition of energy because subterranean habitats are more stable and isolated from anthropogenic activities. We performed a battery of biomarkers associated with important physiological functions on the stygobitic

asellid crustacean *Proasellus lusitanicus,* after a 14-day exposure to acetaminophen, a commonly used pharmaceutical and pollutant of groundwaters. Our results show an decrease in total glutathione levels and an increase in glutathione *S*-transferase activity, suggesting a successful detoxification response. This helps explaining why acetaminophen did not cause oxidative damage, as well as had no effect cholinesterase activity nor in aerobic production of energy. This study shows the remarkable capacity of *P. lusitanicus* to tolerate sublethal concentrations of VHMP acetaminophen. Most ecotoxicological studies on stygobitic species focused on the lethal effects of these compound. The present study focus on consequences at sublethal concentrations. Future studies should assess the stress levels induced to better predict and estimate the impacts of contaminants on groundwater ecosystems.

Keywords: Groundwater, Oxidative stress, Biomarkers, Sublethal effects, Subterranean ecosystems

1. Introduction

Groundwater consists of 94-97% of all the liquid freshwater globally available (Castaño-Sánchez et al., 2020b; Griebler & Avramov, 2015). This is a major source of drinking water for many European Union (EU) citizens (European Environment Agency, 2018). Half of European drinking water is obtained from groundwater, and many large cities depend on it. The majority of public water and agriculture supplies come from groundwater abstraction (European Environment Agency, 2018). Although it is tempting to see groundwater ecosystems only as an important human resource, it is a habitat for peculiar and unique species. It is an isolated ecosystem and the species inhabiting groundwater tend to have short-range distributions and a high degree of endemism, as result of multiple colonization events (Hose et al., 2022). The so-called stygofauna have adaptations to life in groundwater, which includes dramatic changes in morpho-

physiological traits when compared to surface species (Hose et al., 2022). These species are characterized by low metabolic rates, low fertility and slow population growth. If submitted to habitat degradation, catastrophic and/or stochastic events, they are at higher risk of extinction or significant population reduction (Mammola et al., 2019). Stygofauna has a key role on ecosystem services related to water purification and nutrient turn-over (Smith et al., 2016). Therefore, it is vital to ensure the good ecological quality status of groundwater (Hose et al., 2023).

Anthropogenic disturbances can threaten groundwater ecosystems' equilibrium, and threaten their ecosystem services, such as water purification and provision, important since many citizens depend on groundwater, and its biodiversity, with the potential discovery of new processes and future knowledge (Griebler & Avramov, 2015, Hose et al., 2023).

Groundwaters are contaminated with VHMPs from a variety of classes, such as antibiotics (amoxicylin, chlortetracycline), non-steroidal anti-inflammatory (acetaminophen, diclofenac), β-blockers (atenolol, propranolol) (IPCheM Portal, 2023). Concentrations may vary from below level of detection to values as high as 3.6 µg/L (Sulphamethazine, in the United States (Watanabe et al., 2010).

Agriculture is an important consumer of groundwater, putting pressure on groundwater provisions, but also consists of a relevant pollutant, pressing the quality of the same groundwater it consumes (European Environment Agency, 2018; Marmonier et al., 2018). A potential source of veterinary and human medicinal products (VHMP) is manure, frequently used in agriculture as fertilisers. VHMPs are widely used in cattle to prevent and treat diseases, as well as increasing growth (Gros et al., 2019). These VHMPs often accumulate in the soils where manure is used as a fertiliser, later leaching to both surface and groundwater bodies (Gros et al., 2019). Other sources of contamination that

76 risk one of the most enigmatic ecosystems are domestic and hospital waste (Castaño-
77 Sánchez et al., 2020a; Mammola et al., 2019). Both of these may reach groundwater
78 through percolation and leaching, transporting within it VHMPs (Paíga et al., 2016;
79 Verlicchi et al., 2012).
80 VHMPs are responsible for negative side effects on non-target aquatic organisms, with
81 lethality being one of them. Acetaminophen, also known as paracetamol, is one of the
82 most popular prescribed and self-medicating drugs (Wu et al., 2012). Acetaminophen is
83 lethal for *Daphnia magna* at value of 33.8 mg/L. Furthermore, chronic exposure was
84 shown to affect moulting frequency, days until the first brood and first egg production
85 (Ding et al., 2020). This was connected to the expression of reproduction-related genes
86 CYP_{314} and EcR, which increased, and Vtg, an endocrine biomarker that was up-
87 regulated. Additionally, detoxification-genes CUP360A8, CYP314, MRP4 and P-gp
88 were also shown to be related to acetaminophen's metabolism in *D. magna* (Ding et al.,
89 2020). No previous study focused on the effect of paracetamol on stygobitic species has
90 been made. Acetaminophen and its metabolites remain to be continuously released into
91 the aquatic environment via domestic wastewater and hospital effluents, where, due to its
92 hydrophilicity and high solubility, it may accumulate (Wu et al., 2012). Therefore,
93 acetaminophen has been found in many water bodies, from surface waters to
94 groundwaters, in the order of ng/L up to µg/L (Nödler et al., 2014; Paíga & Delerue-
95 Matos, 2016; Rabiet et al., 2006). In French groundwaters, a study found a maximum
96 concentration of 481 ng/L, being in the top five of the most detected pharmaceuticals in
97 groundwaters across all seasons (Lopez et al., 2015).
98 We determine the sublethal effects of acetaminophen in the stygobitic species *Proasellus*
99 *lusitanicus* (Frade, 1938). The biomarkers we use measure the basic functioning of cells,
100 therefore we can analyse the cells' response to any type of stress, including chemicals

with very different modes of action (Silva et al., 2021, Calado et al., 2022). The advantage is that independently on the analysed parameter, we know if the animal suffers from the induced stress (Abe et al., 2018, Almeida et al., 2015). This stygobitic crustacean was exposed to a range of concentrations, some of which environmentally relevant concentrations (at the same level found in groundwaters around Europe), for 14 days and the following common biomarkers associated with important physiological functions were determined for the first time in this species: total glutathione level (TG; a non-enzymatic antioxidant), glutathione *S*-transferase activity (GST; a phase II conjugation enzyme), electron transport system activity (ETS; aerobic production of energy), lipid peroxidation level (LPO; oxidative damage), cholinesterase activity (ChE; neurotransmission), and total protein (PROT). This battery of biomarkers allows us to gather novel information and better understand how this stygobitic species is affected by VHMPs and may bring new light to groundwater management and the inclusion of early-warning biomarkers in Environmental Risk Assessment (ERA) guidelines.

2. Material and Methods

2.1. Animal collection and acclimation

Individuals of the stygobitic asellid *Proasellus lusitanicus* were collected in May 2022, in Olhos d'Água Cave (Fig. 1) in the Estremenho karst massif, Portugal. It is endemic and found in several caves from that massif (Magniez, 1967; Reboleira et al., 2011). It is a benthic species that feeds on biofilms, improving water quality and being a prey for other stygobitic species (Reboleira et el., 2011, Reboleira et al., 2013). Among stygobitic crustaceans, *P. lusitanicus* is metabolically well characterized under incremental temperature scenarios (Di Lorenzo et al., 2019, Di Lorenzo & Reboleira, 202), and has been previously used in lethal ecotoxicity testing (Reboleira et al., 2013; Castaño-Sánchez et al., 2021). Moreover, it is the only stygobitic species liable to be collected in

the geographic area in sufficient number to perform ecotoxicity tests according to the current recommendations by Di Lorenzo et al. (2019).

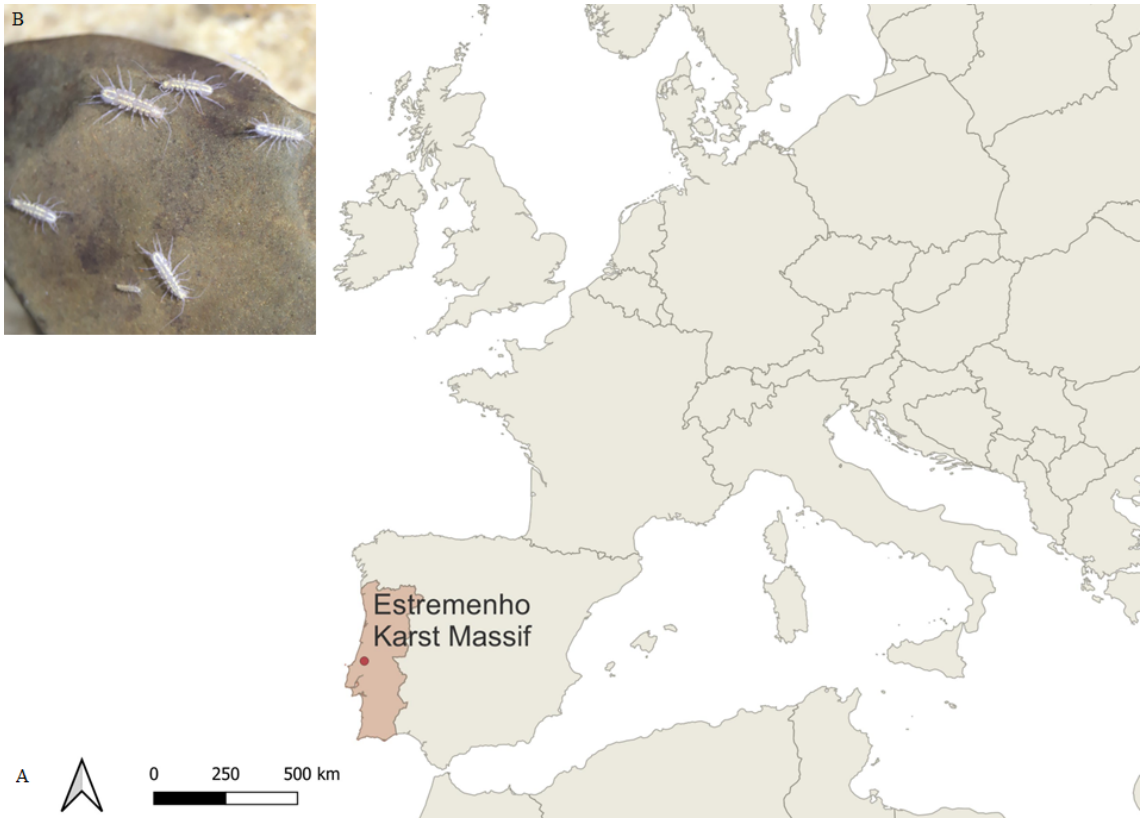

Figure 1. A) Estremenho Karst Massif, collection site of *Proasellus lusitanicus* (map rendered with QGIS 3.24.3); B) Stygobitic species *Proasellus lusitanicus* live habitus.

Temperature, pH, dissolved oxygen (DO), and electrical conductivity were measured by using a portable multiparameter probe AQUAREAD – WTW MULTI 3430, at the collection site. About 200 individuals were collected in the field with a macro-pipette (capacity of 30 mL) and transported to the laboratory in plastic containers with groundwater from the collection site in a cooler within five hours from collection. Afterwards, the specimens were acclimated to the laboratory conditions for two weeks by keeping them in permanent darkness and at the average temperature of the collection site, 17 ºC, to undergo acclimation. Further information on water parameters of acclimation medium is available in Supplemetary Material 1, Table S.1. Sediment from the cave was

provided in the vials of *P. lusitanicus*, which presumably feeds them and allows them to be at their best fitness (Castaño-Sánchez et al., 2021). No artificial food was supplied.

2.2.Test solution, sample preparation and analyses

The pharmaceutical compound acetaminophen (CAS 103-90-2; $C_8H_9NO_2$) was purchased from Sigma-Aldrich (Steinheim, Germany). The solutions were prepared fresh with commercial water, at concentrations of 100 mg/L, 10 mg/L, 1 mg/L, 0.1 mg/L and 0.01 mg/L. Ten specimens were exposed individually in glass vials to 12 mL of each concentration. Because the experimental design was extended until 14 days, we renewed the medium to ensure that there was no reduction of the concentration of acetaminophen in the water.

Exposure to acetaminophen ended after 14 days. This time period is based on preliminary assays for this species, following the recommendations for ecotoxicological testing with stygobitic crustaceans (Di Lorenzo et al., 2019b). Each surviving specimen was individually placed in 2 mL microtubes, frozen in liquid nitrogen and weighed in a scale A&D, model ER-120A. Samples were homogenised (1200 µL) in ultrapure water using the tissue lyser Retsch MM400 for 1 minute at 30 Hz (8 microspheres per microtube). The homogenized sample was divided into different microtubes to allow the following quantifications: total glutathione (TG; 250 µL), glutathione *S*-transferase (GST; 250 µL), lipid peroxidation (LPO; 100 µL), electron transport system (ETS; 150 µL), cholinesterase (ChE; 250 µL), and total protein (PROT; 100 µL). Samples were kept at -80ºC until further analyses.

2.2.1. Neurotoxicity and oxidative stress related biomarkers

Quantification of TG levels was performed by using an adapted protocol from Rodrigues et al., (2022): the reaction of reduced glutathione with DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) in the presence of an excess of glutathione reductase was read at 412 nm for 3 minutes every 30 s, with agitation between readings. A standard curve using reduced glutathione (concentrations of 10000 μM, 100 μM, 10 μM, 1 μM and 0.1 μM) was used to determine samples' concentration (Rodrigues et al., 2017).

Determination of GST activity followed an adapted protocol (Rodrigues et al., 2022), which relies on the conjugation of reduced glutathione with CDNB (1-chloro-2,4-dinitrobenzene). This reaction was read at an absorbance of 340 nm, for 5 minutes, at intervals of 30 seconds, with agitation between readings. The molar extinction coefficient used for calculation was $9.6x10^3$ $M^{-1}cm^{-1}$.

LPO levels were measured by reading at an absorbance of 535 nm, which measures thiobarbituric acid-reactive substances (TBARS), similarly to Rodrigues et al. (2022). In order to avoid further lipid peroxidation during storage, BHT (2,6-Di-tert-butyl-4-methylphenol 4% in methanol) was previously added to the samples. The molar extinction used was $1.56x10^5$ $M^{-1}cm^{-1}$.

ChE activity was assessed by adapting a protocol from Silva et al. (2021). Microplates were read for a period of 3 minutes, with 30-second intervals with agitation, at an absorbance of 414 nm. The molar extinction applied was $13.6x10^3$ $M^{-1}cm^{-1}$.

PROT was determined with an adapted protocol from Rodrigues et al. (2022). A standard curve with γ-globulin was used (0 mg/mL, 0.2 mg/mL, 0.5 mg/mL and 1 mg/mL), and the reaction with BioRad was measured at an absorbance of 600 nm.

All absorbances were read with Thermo Scientific Multiskan Sky Microplate Spectrophotometer at 25ºC.

2.2.2. Energy-related biomarkers

Quantification of ETS activity was performed by adapting the protocol of Rodrigues et al. (2022). Microplates were read for 6 minutes at an absorbance of 490 nm, with intervals of 30 seconds and agitation. Quantification is based on the reduction of iodonitrotetrazolium chloride in the presence of Triton X-100, a non-ionic detergent. Furthermore, for the quantification of ETS, the molar extinction coefficient is 1.59×10^4 $M^{-1}cm^{-1}$. Oxygen consumption rate was calculated following the following proportion: for each µmol of oxygen consumed, 2 µmol of INT-formazan was formed. Absorbance was read with Thermo Scientific Multiskan Sky Microplate Spectrophotometer at 25 ºC.

2.3.Statistical analyses

All analysis and plots were performed with R Studio, version 4.0.3 (RStudio Team, 2020). Data's normality was analysed with Shapiro-Wilk's test, and homoscedasticity was analysed with Bartlett's test. Whenever the criteria were met (normal distribution and equal variances), one-way ANOVA was performed, followed by a Dunnett's and a Tukey's test to determine if there were differences between the control group (0 mg/L) and the remaining groups, subjected to rising concentrations of acetaminophen (0.01, 0.1, 1, 10, 100 mg/L of acetaminophen); when criteria were not met, data was analysed with Kruskal-Wallis' test, followed by a Dunn's test, to determine if there were differences between the control group and the remaining groups. Significance level was set at $p < 0.05$; Bonferroni correction was applied.

3. Results

Total glutathione (TG) levels were significantly affected (p-value = 0.00127). Organisms exposed to 0.01 mg/L exhibited a significantly higher level of TG than the ones exposed to 0.1 mg/L (p-value = 0.0029), and 1 mg/L (p-value = 0.0219). No significant difference was detected between the control group and any other group (Figure 2A; suppl. table S2, S3).

The activity of glutathione S-transferase (GST) was also significantly affected (p-value = 0.0093) after exposure to acetaminophen. Organisms that were exposed to 0.1 mg/L (p-value = 0.042), 1 mg/L (p-value = 0.026) and 10 mg/L (p-value = 0.026) showed a significant increased activity of GST when compared to the control (Figure 2B, suppl. table S4).

Levels of lipid peroxidation (LPO) levels were not significantly affected by acetaminophen treatments if compared to the control group. However, significant differences occurred (p-value = 0.0459). Specifically, organisms exposed to 1 mg/L exhibited a significantly increased (p-value = 0.0386) LPO level when compared to 0.1 mg/L (Figure 2C, suppl. table S5).

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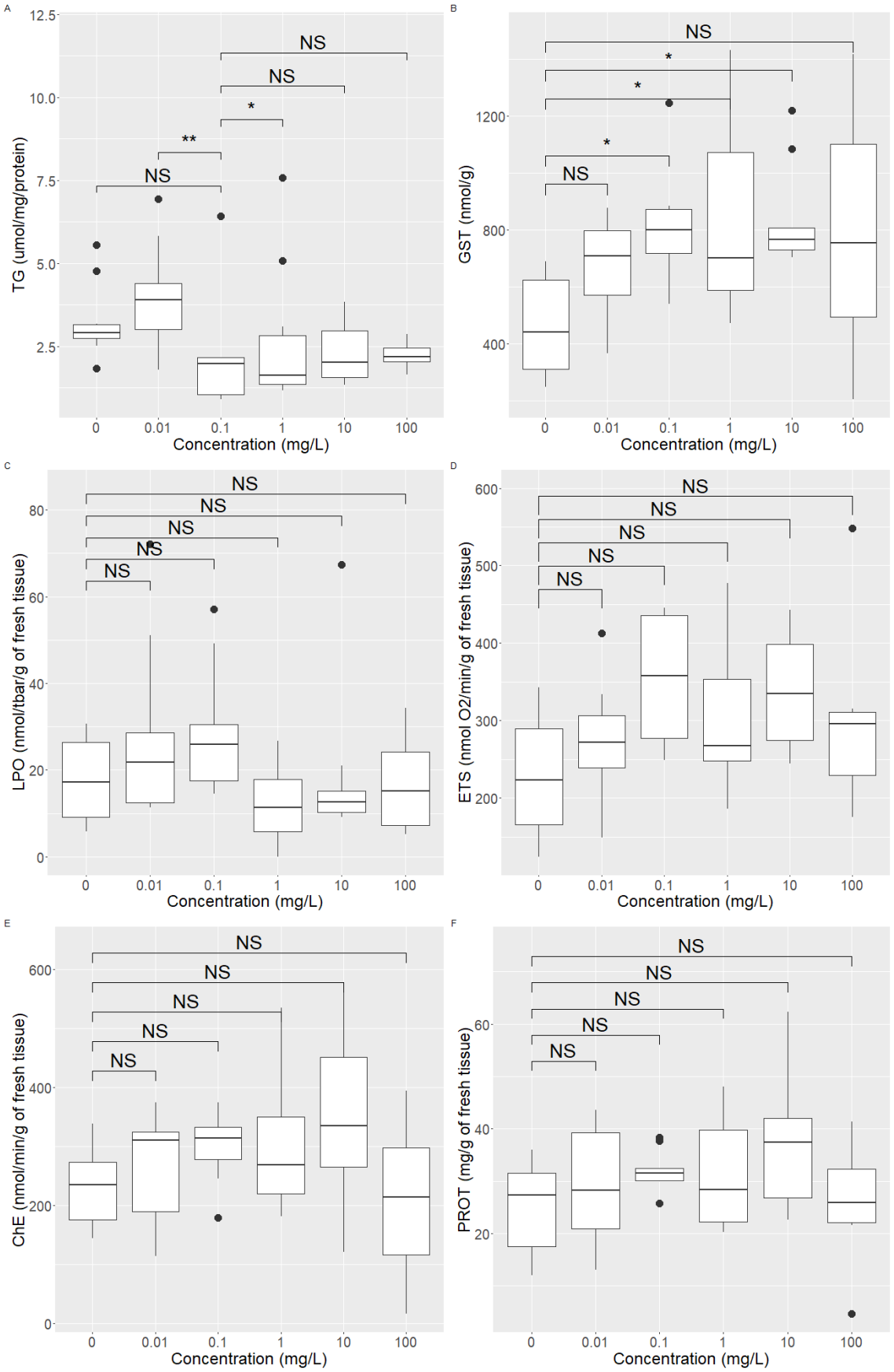
A
TG (umol/mg/protein)
12.5
10.0
7.5
5.0
2.5
NS
NS
*
**
NS
0
0.01
0.1
1
10
100
Concentration (mg/L)
B
GST (nmol/g)
1200
800
400
NS
*
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*
NS
Concentration (mg/L)
C
LPO (nmol/tbar/g of fresh tissue)
80
60
40
20
0
NS
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Concentration (mg/L)
D
ETS (nmol O2/min/g of fresh tissue)
600
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200
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Concentration (mg/L)
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ChE (nmol/min/g of fresh tissue)
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Figure 2. Results of biomarkers analysis of *Proasellus lusitanicus* after exposure to acetaminophen for 14 days. A) Level of total glutathione (TG); B) Activity of glutathione S-transferase (GST); C) Level of lipid peroxidation (LPO); D) Activity of the electron transport system (ETS); E) Activity of cholinesterase (ChE); F) Levels of protein (PROT) content. (Circles – Outliers; NS – Not significant; ** $p < 0.01$; * $p < 0.05$).

The electron transport system (ETS) activity (p-value = 0.1114) (suppl. table S6), cholinesterase (ChE) activity (p-value = 0.1114) (suppl. table S7), and protein (PROT) content (p-value = 0.3526, suppl. table S8) were not significantly different in organisms exposed to all concentrations of acetaminophen and control group (Figure 2D, E, F). Overall mortality is available in suppl. table S9.

4. Discussion

Biomarkers assessed in *Proasellus lusitanicus* showed decreased levels of total glutathione (TG) on organisms exposed to 0.01 mg/L of acetaminophen, and increased glutathione S-transferase (GST) activities on organisms exposed to concentrations between 0.01 and 100 mg/L. Lipid peroxidation (LPO) levels were not significantly higher in organisms exposed to acetaminophen compared to the control. These alterations suggest the activation of detoxification processes and antioxidants to cope with exposure to acetaminophen, which was partially successful in preventing further oxidative damage. At the concentration of 10 mg/L, mortality reached 10%, while at the concentration of 100 mg/L, mortality was as high as 40%. This increasing mortality might explain the decline of levels and activities of the biomarkers.

GSTs are associated with metabolic detoxification processes, being one of the most relevant phase II enzymes (Frova, 2006; Gravato & Santos, 2002). In our study, GST

activity was significantly increased for all the tested concentrations of acetaminophen meaning that conjugation and elimination of acetaminophen or its metabolites was being efficient because no oxidative damage was observed. Moreover, GST seems to be playing an important role also conjugating the potential lipid peroxides resulting from the direct (electrophilic metabolites of acetaminophen) and/or indirect (Reactive oxygen species) attack of the membranes. Previous results observed for the freshwater crustacean *Daphnia longispina*, when exposed to 20 µg/L for 48 hours (Sousa & Nunes, 2021), and *Daphnia magna*, when exposed to concentrations ranging from 0.8 and 2.56 mg/L for 48 hours (Daniel et al., 2019). In contrast, the activity of GST decreased in *D. magna* in 48 hours acetaminophen exposure at concentrations of 20 and 40 µg/L (Sousa & Nunes, 2021), and 21-days acetaminophen exposure for concentrations ranging from 5 to 20 mg/L (Daniel et al., 2019). GSTs' activity has been previously associated with acetaminophen and its metabolites' excretion, via conjugation with reduced glutathione (Daniel et al., 2019), although its response is not always straightforward to predict, as both patterns (increasing and decreasing of activity) were observed in different studies (Sousa & Nunes, 2021). In our study, 100 mg/L of acetaminophen did not show a significant increase nor decrease of GST activity when compared to the control. This reduction of GST activity has been previously explained via glutathione reductase reduction (Daniel et al., 2019). Furthermore, the levels of TG may limit in part the conjugation process of GST since reduced glutathione is a substrate of GST. Despite all of these, there were no differences in LPO levels between the control group and the remaining groups. LPO is a biomarker that shows damage to cell membrane, being a consequence of the increased levels of reactive oxygen species (ROS) modifying lipids due to an inefficient antioxidant capacity (Brandão et al., 2014). It is known that acetaminophen's overdose causes hepatic injury in mammals (Nunes et al., 2014), potentially by intracellular glutathione's exhaustion,

leading to NAPQI (N-acetyl-p-benzoquinone imine) accumulation. Acetaminophen presumably causes endocrine disruption in crustaceans and interfer with ecdysis (Nunes et al. 2014). Our results also showed the total glutathione depletion at particular concentrations of acetaminophen. This will lead to multiple toxic effects, such as DNA and RNA damage, oxidation of membrane lipids, necrosis and cell death (Nunes et al., 2014). A rise in GST for almost all the concentrations tested explains why there was no significant difference in LPO levels, as damage only occur when all detoxification defences are overwhelmed according to previous research works (Amiard-Triquet et al., 2013).

Moreover, it can be concluded that despite the effects observed in our species, the aerobic production of energy was not compromised in organisms exposed to acetaminophen at any tested concentration, and energy was sufficient to cope with the demands required for detoxification processes and survival. This will also be important for the organism to avoid oxidative damage. Lack of oxidative damage may be due to a successful detoxification mechanism, which can be partly corroborated by the changes observed in GST activity (Amiard-Triquet et al., 2013) fuelled with energy that was not compromised, at least for the concentrations tested. Nevertheless, our results do not exclude the hypothesis that growth, behavioural and/or reproduction of the organisms exposed to acetaminophen might not be compromised. Compromising physiological functions due to acetaminophen exposure is not unlikely because *P. lusitanicus*, like many other groundwater species, has a considerably lower metabolism than surface water species as a consequence of food and oxygen limitation in groundwater (Di Lorenzo & Reboleira, 2022; Hose et al., 2022). Therefore, further studies should be performed in order to understand how much energy *P. lusitanicus* allocated for acetaminophen detoxification by deviating it from other important physiological functions. Acetaminophen has no

evident direct mechanism of neurotoxicity. We did not observe significant changes in ChE activity, which is consistent with two previous studies using daphnids (Daniel et al., 2019; Sousa & Nunes, 2021).

It is important to note that these previous studies used for our discussion were performed on surface water crustaceans, while this study was performed on a stygobitic species, characterized by their slower metabolism (Hose et al., 2022). Other differences between ours and previous studies are the exposure period and the range of concentrations tested. Daniel et al. (2019) exposed daphnids both acutely and chronically to acetaminophen, and the results of both period exposures yielded different results. Our concentrations ranged between 0.01 and 100 mg/L, which might mean that a broader range would facilitate non-monotonic responses of biomarkers as we obtained.

Despite all the constraints of using a stygobitic crustacean species, such as low number of organisms available for testing, difficulty in collection, and in rearing them in the laboratory setting (Di Lorenzo et al., 2019b; Castaño-Sánchez et al., 2020a), this research work allows us to determine a battery of biomarkers that are useful to understand the ecotoxicity of several other classes of pollutants to this species and by extension on their impacts in groundwater ecosystems.

Finally, the results of our study suggest that the environmental risk related to acetaminophen is low in groundwater ecosystem. The Predicted No Effect Concentration (PNEC) of acetaminophen in groundwater is equal to the PNEC in surface water reduced by a factor of 10 (EMA, 2018), i.e. 0.0134 mg/L (ECHA, 2022). *Proasellus lusitanicus* seems to well tolerate this concentration, as evidenced by our study. The low environmental risk associated with acetaminophen has also been observed for other pharmaceutical compounds, such as propranolol and diclofenac (Di Cicco et al., 2021; Di Lorenzo et al., 2019a). However, the contamination of groundwater environments is

rarely caused by a single compound (Loos et al., 2010). More frequently, mixtures of VHMPs occur.

5. Conclusions

The current results shed light on a fairly unexplored area: the sublethal effects of VHMPs in stygobites by assessing a battery of biomarkers of important physiological functions. Acetaminophen induced defence biomarkers related to detoxification that prevented oxidative damage, as aerobic energy production was not compromised. Further studies in quantifying biomarkers levels at different times of exposure (both shorter and longer) will allow to determine intraspecific fluctuations and comparison with other species. Moreover, future research should also target the effect of mixtures of pollutants on stygobitic species to provide more environmentally realistic insights about the fate of groundwater ecosystems in a changing world. Sublethal effects should be analysed to truly assess VHMPs' effects, as death is an extreme endpoint, and other less extreme effects may also compromise for individual's survival. Thus, assessing biochemical endpoints, such as biomarkers, are extremely useful as early warning tools of health effects at the organism level and higher levels of biological organization.

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