UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Impacts of Veterinary and Human Medicinal Products in Groundwater Ecosystems

Cláudia Filipa dos Santos Duarte

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Dissertação orientada por:
Prof. Doctor Ana Sofia P. S. Reboleira
Doctor Tiziana Di Lorenzo

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This dissertation was only written thanks to the contribution, however indirect, of many (human) specimens. Similarly to the subterranean world, not all of the specimens will be named in the following list.

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Resumo

Os ecossistemas subterrâneos são uma importante fonte de água potável, da qual a maioria da população mundial depende. Os aquíferos são explorados para atividades como a agricultura e indústria, além dos usos domésticos, sofrendo enormes pressões pelas quantidades extraídas, e pela poluição que os atinge. Entre estes poluentes encontram-se os fármacos, administrados em humanos e animais, que os irão metabolizar e excretar através das fezes e urinas. Assim, as águas subterrâneas recebem compostos originais e metabolitos resultantes do metabolismo destes fármacos, sendo que ambas as formas poderão impactar o equilíbrio dos ecossistemas subterrâneos. Os efluentes domésticos e hospitalares são uma importante fonte de poluição, assim como a agricultura, que utiliza com frequência estrume contaminado com estes fármacos. Tudo isto atinge as águas subterrâneas por percolação e infiltração.

O ecossistema subterrâneo é caracterizado pela completa escuridão, elevada humidade e temperatura estável. Este ecossistema possui ainda cadeias tróficas truncadas, característica que leva a que exista pouca redundância nas funções desempenhadas por cada organismo. Devido à escuridão que caracteriza este ecossistema, existe ainda ausência de produção primária de origem fotossintética, sendo que quando existe produção primária, esta é de origem quimiolitotrófica. Estes ecossistemas possuem assim uma dependência dos compostos orgânicos externos que surgem das escorrências e da ação da gravidade. Adicionalmente, os organismos que habitam estes ecossistemas possuem eles próprios características adaptativas similares, tais como a despigmentação, o metabolismo lento, a ausência de visão, o alongamento dos apêndices, elevada longevidade, e a produção de menos ovos, ainda que maiores. Estas espécies possuem um elevado grau de endemismo, pelo que se encontram mais suscetíveis a eventos catastróficos.

Estes ecossistemas subterrâneos tão enigmáticos quanto desconhecidos, fornecem serviços de ecossistemas fundamentais. Para além de consistirem uma importante reserva de água potável, estes ecossistemas procedem à purificação de água e à manutenção do ciclo de nutrientes, possível devido aos animais que nestes ecossistemas habitam e que se alimentam de biofilmes e bactérias. Estes ecossistemas fornecem ainda refúgio a várias espécies, e são importantes locais espirituais e turísticos.

De momento, as indicações pela Agência Europeia do Medicamento sugerem a realização de testes ecotoxicológicos em organismos de superfície, como substitutos aos organismos subterrâneos, os troglóbios (terrestres) ou estigóbios (aquáticos). Na avaliação de risco ambiental são considerados os três níveis tróficos: produtores primários, consumidor primário e consumidor secundário. Ainda que nos ecossistemas aquáticos de superfície o nível trófico dos produtores primários (fotossintéticos) pode ser considerado bem representado, tal não acontece no ecossistema subterrâneo. Na determinação do quociente de risco ambiental de um certo fármaco para o ecossistema subterrâneo, é necessário a utilização de dois fatores de avaliação: para a obtenção do valor mínimo em que o organismo subterrâneo é afetado é necessária a divisão do valor mínimo em que o organismo de superfície é afetado por 10; para a obtenção da concentração existente nos aquíferos é necessária a multiplicação por 0,25 ao valor encontrado nas águas superficiais. A divisão pelo fator de avaliação de 10 tem o objetivo de proteger os organismos subterrâneos, considerados mais vulneráveis, enquanto a multiplicação pelo fator de 0,25 tem o intuito de considerar a percolação e infiltração nos solos, que poderão diminuir a concentração de fármacos ao longo do percurso até aos aquíferos. Ainda assim, as diferenças fisiológicas e metabólicas existentes entre os organismos de superfície e os organismos estigóbios levantam dúvidas sobre a adequação das indicações atuais da Agência Europeia do Medicamento para a proteção dos ecossistemas subterrâneos.

O objetivo principal desta dissertação é aumentar o conhecimento científico na área da avaliação de risco e gestão ambiental, através do estudo do impacto dos fármacos diclofenaco de sódio e acetaminofeno no estigóbio *Proasellus lusitanicus*, uma espécie aquática cavernícola endémica do centro de Portugal.

O estudo da mortalidade do *P. lusitanicus* após exposição a diclofenaco de sódio, um anti-inflamatório não-esteróide frequentemente encontrado nas águas subterrâneas, foi efetuado através de. um teste ecotoxicológico que. consistiu num teste sem limite temporal, sendo o término determinado pela estabilidade do fármaco ou pela observação da cessação da mortalidade. Esta abordagem foi complementada com a exploração de quatro cenários para o cálculo do quociente de risco para o fármaco diclofenaco de sódio. O cálculo do quociente de risco consiste na divisão da concentração do fármaco quantificada numa massa de água pela concentração em que se prevê não haver efeito no organismo. Assim, no cenário 1 e 3, a concentração medida nas massas de água foi estimada através da análise de literatura, enquanto no cenário 2 e 4 foi estimada com recurso a uma base de dados, disponibilizada pela Agência Europeia do Ambiente. A concentração em que não se prevê qualquer efeito no organismo foi estimada no cenário 1 e 2 através das indicações da Agência Europeia do Medicamento, e no cenário 3 e 4 através do teste ecotoxicológico realizado com estigóbios da espécie *P. lusitanicus.* Os resultados indicam que *P. lusitanicus* possui uma tolerância inicial elevada ao fármaco diclofenaco de sódio. No entanto, esta tolerância vai diminuindo à medida que o período de exposição aumenta. Entre os quatro cenários calculados, aquele que apresenta um quociente de risco maior foi o cenário calculado consoante as indicações da Agência Europeia dos Medicamento. Este resultado sugere que as indicações atuais são adequadas para a proteção dos ecossistemas subterrâneos.

Esta dissertação estudou também os efeitos subletais do fármaco acetaminofeno, um fármaco usado com frequência, e um contaminante recorrente das águas subterrâneas. Os efeitos subletais foram estudados através da quantificação de biomarcadores relacionados com a defesa, o stress oxidativo, a neurotoxicidade, e a produção de energia, após uma exposição de 14 dias a acetaminofeno. Após a exposição, foram analisados os níveis ou atividade dos biomarcadores glutationa total, glutationa *S*-transferase, peroxidase lipídica, sistema de transporte de eletrões, colinesterase e proteína total. O estudo dos níveis ou atividades de biomarcadores de dano e de defesa após exposição a acetaminofeno, quantificados nesta espécie pela primeira vez, indicam que este estigóbios possui um mecanismo de destoxificação adequado, visível pelas diferenças existentes na glutationa total e na glutationa *S*-transferase. Este mecanismo de destoxificação permitiu evitar dano celular em concentrações sub-letais, visível pela ausência de diferenças na peroxidase lipídica, no sistema de transporte de eletrões, e na colinesterase.

Até ao momento, as indicações existentes para a proteção do ecossistema cavernícola parecem ser adequadas para o efeito, sendo necessário ter em conta que os estigóbios utilizados nestes estudos são adultos, e que indivíduos mais juvenis poderão ser mais sensíveis a estes fármacos. Adicionalmente, é necessário ter em atenção que estes estudos foram realizados para concentrações de apenas um fármaco, e não para a mistura de vários em simultâneo, como tantas vezes ocorre nas águas subterrâneas. Estes estudos não permitem tirar conclusões sobre o efeito destes fármacos na fertilidade desta espécie, um fator que pode interferir nos números de uma população, nem concluir sobre o efeito conjunto do aumento da temperatura e da exposição a fármacos na sobrevivência e mortalidades destes indivíduos.

Esta dissertação representa o primeiro esforço no sentido de compreender o efeito pernicioso dos fármacos em organismos de águas subterrâneas em Portugal, testando os seus efeitos em espécies endémicas. Estudos futuros são fundamentais para tentar preencher estas e outras lacunas existentes,

que levarão à produção de indicações mais adequadas para a proteção dos enigmáticos ecossistemas subterrâneos tantas vezes esquecido, ainda que tão importante.

Palavras-chave: aquíferos, ecotoxicologia, fármacos, crustáceos, biomarcadores.

Abstract

Groundwater is an important source of fresh water, with the majority of world population depending on it. This resource sustains activities such as agriculture, industry, and domestic use, and suffers pressures from the high amount of water extracted, agriculture pollution, industry and domestic effluents, and hospital sewages, through percolation and leachate. This ecosystem is characterized by darkness, high humidity, and stable temperature, with truncated trophic chains and no primary photosynthetic production. This ecosystem is inhabited by organisms with unique traits, such as depigmentation, slow metabolism, and blindness, among others. Furthermore, groundwater ecosystems provide important ecosystem services, such as water purification, nutrient cycling, and cultural services. Currently, guidelines suggest using surface species as a proxy for groundwater organisms, the so-called stygobitic species, after using an assessing factor of 10 to account for the vulnerability of these species. This dissertation aims to increase the scientific knowledge in groundwater environmental risk assessing and management, by studying the mortality of diclofenac sodium and sublethal effects of acetaminophen in stygobitic species *Proasellus lusitanicus*. Besides studying mortality, by using a Time-Independent assay, this dissertation focused on sublethal effects as well, by quantifying levels and activity of defence and stress biomarkers. This species shows a remarkable tolerance to diclofenac sodium, which decreases over time of exposure. Furthermore, the current guidelines for groundwater ecosystem's protection are adequate. By studying defence and stress biomarkers after a 14-day exposure of acetaminophen, it was show that this species has a successful detoxification mechanism. This helps to explain this species' tolerance to diclofenac sodium. In the future, more studies are necessary to better understand the impacts of these groundwater pollutants.

Keywords: ecotoxicology, pharmaceuticals, crustaceans, groundwater, biomarkers.

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List of Abbreviations, Acronyms, and Symbols

VHMP – Veterinary and Human Medicinal Product

ERA – Environmental Risk Assessment

EMA – European Medicines Agency

MEC_{GW} – Measured Environmental Concentration in Groundwater

RQ_{GW} – Risk Quotient for Groundwater

AF – Assessing Factor

LC_{50} – Lethal Concentration

NSAIA – Non-Steroidal Anti-Inflammatory Agent

EC_{50} – Effective Concentration

EU – European Union

EC – European Commission

WFD – Water Framework Directive

$PNEC_{gw}$ - Predicted No-Effect Concentration of Groundwater

$PNEC_{sw}$ - Predicted No-Effect Concentration of Surface Water

SSD – Species Sensitivity Distribution

PRO – Propranolol

TI – Time-Independent

WWTP – Wastewater Treatment Plants

EEA – European Environmental Agency

EQS – Environmental Quality Standard

SSD – Species Sensitivity Distribution

DO – Dissolved Oxygen

NOEC – No-Observed Effect Concentration

TG – Total Glutathione

GST – Glutathione *S*-Transferase

ETS – Electron Transport System

LPO – Lipid Peroxidase

ChE – Cholinesterase

PROT – Total Protein

TBARS – Thiobarbituric Acid-Reactive Substances

ROS – Reactive Oxygen Species

1. Introduction

1.1. Groundwater ecosystems

Below the surface, there is a subterranean domain containing species with limited distribution and unique morphological and physiological traits (Castaño-Sánchez et al., 2020a; Hose et al., 2022). Subterranean-adapted species are considered good models for ecological studies (Reboleira et al., 2013; Mammola et al., 2019). The groundwater habitat is characterized by being perpetually dark, with a constant temperature, wet substrate, high humidity and low food-availability (Howarth, 1983; Ravn et al., 2020). Due to these characteristics, obligate groundwater species (stygobites) have several adaptions, such as loss of pigmentation and ocular structures, elongation of appendages, lack of circadian rhythm and ability to survive with low food resources (Hose et al., 2022).

Groundwater is used worldwide, serving as a solvent and cooling agent for industrial use and providing irrigation for agriculture (Griebler & Avramov, 2015). This resource constitutes 94-97% of the global freshwater resources available for direct human consumption (Griebler & Avramov, 2015; Castaño-Sánchez et al., 2020b). Furthermore, it harbours a unique and vulnerable ecosystem (Castaño-Sánchez et al., 2020b).

1.2. Veterinary and human medicinal products

As the human population grows, the amount of land allocated for agricultural, urban and industrial purposes enlarges, as well as the production of wastewater and exploitation of groundwater (Di Lorenzo et al., 2019). Pharmaceuticals are generally small organic polar compounds, critical both in human and veterinary modern medicine (Viana et al., 2021). The development of intensive farming and the use of veterinary and human medicinal products (VHMPs) has increased (Kim et al., 2018). In intensive farming, antibiotics are used to treat diseases, and also to enhance productivity by preventing them (Kim et al., 2018; Kivits et al., 2018).

1.3. Sources of groundwater contamination by veterinary and human medicinal products

The subterranean ecosystem is threatened by many anthropogenic activities such as domestic urban discharges, infiltration of industrial waste, agriculture and farming (Figure 1) (Castaño-Sánchez et al., 2020a). As animal manure and slurries contain nutrients essential for crop growth, it is commonly used as fertilizer (Gros et al., 2019). A common practice in animal production is the use of antibiotics and other pharmaceuticals in order to prevent disease and increase growth (Di Guardo & Finizio, 2017). The VHMPs are metabolized in the animal's organism and their original active substance and metabolites are excreted in the faeces and urine of the animal (Di Guardo & Finizio, 2017). Using animal manure and slurries as fertilizers, becomes an entryway of VHMPs in the soil, and later on into groundwater bodies due to its leachate from the surface (Gros et al., 2019).

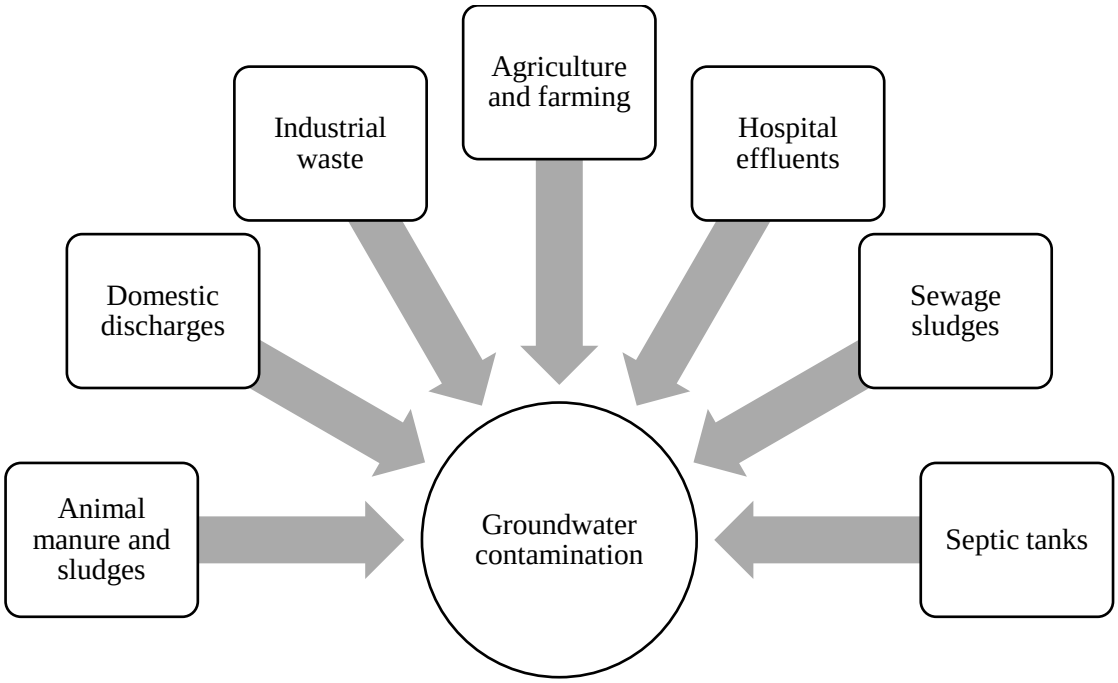

Figure 1. Schematic representation of sources of groundwater contamination.

1.4. Environmental Risk Assessment (ERA)

In Europe, the environmental risk assessment (ERA) of pharmaceuticals (human and veterinary) follows Directive 2001/83/EC (amended to Directive 2001/83/EC (2022)), where the evaluation of potential risks posed by pharmaceuticals and their environmental impact must be submitted and assessed. Additionally, specific arrangements to limit said impact should also be considered (European Medicines Agency, 2018; Directive 2001/83/EC, 2022). For groundwater ecosystems, ERA follows the guidelines of the European Medicines Agency (EMA) (Sebestyén et al., 2018; Di Lorenzo et al., 2019). The ERA is divided in two phases: I and II. In Phase I, the Measured Environmental Concentration in groundwater (MEC_{gw}) is compared to the value of 0.01µg/L; if the MEC_{gw} is lower, no risk is anticipated and there is no necessity to proceed to Phase II (Sebestyén et al., 2018). If the $MEC_{gw} \geq 0.1$ µg/L, Phase II is required, which corresponds to the calculation of the Risk Quotient for groundwater (RQ_{gw}) as in equation 1:

$$RQgw = \frac{MECgw}{PNECgw} \text{ (Equation 1)}$$

If $RQ_{gw} \geq 1$, an environmental risk due to that pharmaceutical is suspected (Sebestyén et al., 2018; Di Lorenzo et al., 2019); if $0.01<RQ_{gw}<0.1$, then the pharmaceutical represents medium risk; finally, if $0.01 \leq RQ_{gw}$, the pharmaceutical represents a low risk (Di Lorenzo et al., 2019).

Groundwater ecosystems are characterized by traits that lead species to evolve with low variability. This low variability is not problematic since these ecosystems are long-term stable environments, however, when facing sudden changes, in which anthropogenic changes and contamination with VHMPs are examples of, this low variability increases vulnerability and decreases the resilience of the community (Hose et al., 2022). Guidelines for ERA still recommend surface species as surrogate species, with differences and intrinsic vulnerability of stygobitic species being considered by assessing factor (AF) of 10 (European Medicines Agency, 2006). Furthermore, VHMPs have been detected in various

groundwater bodies around the world (Lopez et al., 2015; Bexfield et al., 2019), highlighting the urgency to understand their impact on groundwater ecosystems.

This dissertation studies the effects of two VHMPs on a stygobitic species, in order to better understand lethal and sublethal responses to VHMPs on groundwater ecosystems.

2. Objectives and structure of the dissertation

The main objective is to increase the scientific knowledge in groundwater environmental risk assessment and management, by studying the impact of a VHMP on groundwater-adapted species. The specific objectives are to:

- review the current knowledge of the effects of VHMPs in groundwater ecosystems;
- test the acute ecotoxicological response of a groundwater-adapted crustacean to diclofenac sodium by inferring the lethal concentration to 50% of the population (LC_{50}) and comparing it with the LC_{50} values available for surface species;
- compare different scenarios of Risk Quotient (RQ) for diclofenac, using different available methods, in order to identify the most realistic and protective one for groundwater;
- evaluate sublethal responses of a groundwater-adapted crustacean to acetaminophen, using neurotoxicity, energy, and oxidative stress related biomarkers.

The dissertation is structured into introduction, three main chapters and final remarks:

- Introduction: provides a general state of the art about the problematic of contamination of VHMP in groundwater ecosystems.
- Chapter I: The environmental problematic of veterinary and human medicinal products in groundwater – A review.

This chapter revises the current knowledge on the effect of VHMPs in groundwater ecosystem, through a bibliographic revision and estimates of the relative sensitivity of groundwater-adapted species compared to surface species.

- Chapter II: New perspectives for environmental risk assessment of diclofenac in groundwater ecosystems.

This chapter estimates the acute toxicity (LC_{50}) of the groundwater crustacean *Proasellus lusitanicus* to the VHMP diclofenac sodium, a Non-Steroid Anti-Inflammatory Agent (NSAIA), selected due to previous reported high toxicity in crustaceans, and explores four different scenarios of RQ, to determine which scenario is more conservative for groundwater ecological conservation.

- Chapter III: Acetaminophen induced antioxidant and detoxification responses in a stygobitic crustacean.

This chapter determines for the first time if there are differences in a battery of biomarkers associated with important physiological functions after exposure to the VHMP acetaminophen, a popular and commonly used pharmaceutical and pollutant of groundwaters. In this study, total glutathione levels and glutathione *S*-transferase activity increased in the stygobitic species *P. lusitanicus* after a 14-day exposure to acetaminophen, preventing oxidative damage.

- Final remarks: provides a general overview of the results, significance of this work, and future perspectives in this research field.

Note: in Chapter II and in Chapter III, the lethal and sublethal assays were performed with the two VHMPs, but did not meet the assay validation criteria, reason why the unreliable results are not presented.

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Chapter I - The environmental problematic of veterinary and human medicinal products in groundwater – A review

Figure 1. 1. Entrance of Assafora Cave, located in Sintra-Cascais Natural Park. Credits: Rita Eusébio.

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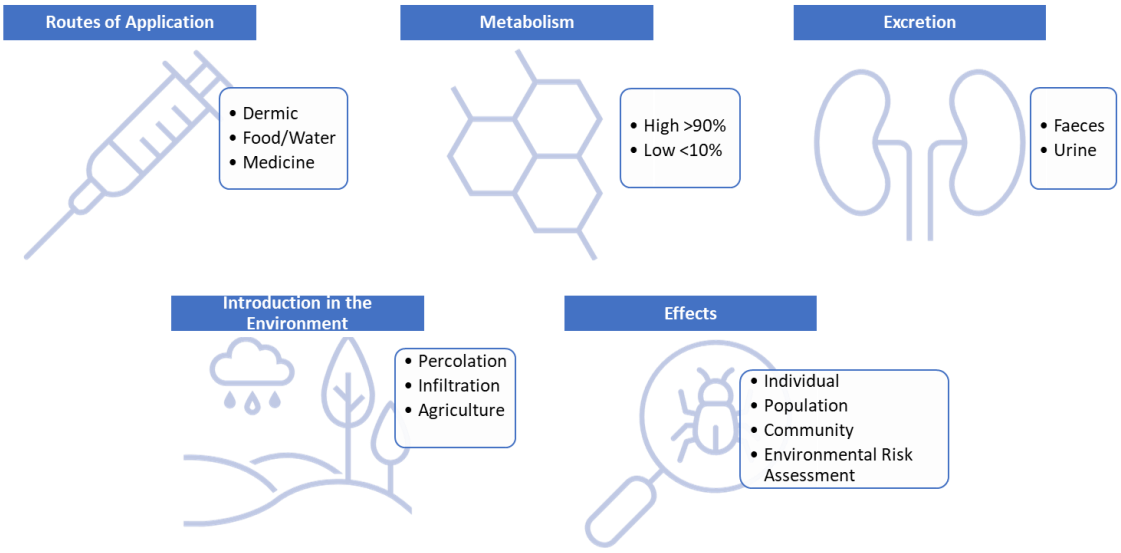
Graphical abstract



Abstract

Pharmaceuticals are used in veterinary and in human medicine to treat and prevent diseases. These products are often not fully metabolised by the organism, resulting in metabolites being excreted alongside the original active substance through urine and faeces. Once excreted, these may accumulate in soils, and reach groundwater through leachate. Groundwater is a habitat for highly adapted species with a set of peculiar traits. Studies show that veterinary and human medicinal products (VHMP) are recurrent in the environment, occurring also simultaneously in groundwater. The effects of VHMPs in groundwater ecosystems are still mostly unknown. Most studies focus on their effects on surface species, with effects on groundwater fauna being extrapolated from the usage of assessing factors on surface data. This review gathers knowledge on the occurrence and effects of VHMPs in groundwater and assesses its potential effects on groundwater biota.

1. Introduction

Veterinary and human medicinal products (VHMPs) are substances used to preserve animal health or to promote growth (Di Guardo & Finizio, 2017; Kim et al., 2018), which can be used in domestic animals or livestock production (Bexfield et al., 2019). With the development of intensive animal farming, there has been an increase in the usage of VHMPs (Kim et al., 2018). This increase leads to an increment of its bioavailability in the environment, whose values depend on the typology of the treated animal, on whether it is an intensive animal farming or pasture, on the route of application, on the animal metabolism, on the level of degradation during manure storage, and on the usage of contaminated manure for the fertilization of agricultural fields (Di Guardo & Finizio, 2017).

A vast number of these VHMP's are used in human medicine as well. The excretion of these pharmaceuticals occurs through urine and faeces, some of which in its original form, and since animal manure and slurries are commonly used as fertilizers in agriculture, this is an important route of entrance of VHMPs residues into the environment, allowing it to accumulate in soils, enter the human food chain, and leach to surface and groundwater bodies (Gaston et al., 2019; Gros et al., 2019). Other routes of entrance into the environment include domestic and hospital wastewater, wastewater treatment plant effluents, and landfill discharge. Possible destinies of these residues are surface waters and sediments (Rodríguez-Escales & Sanchez-Vila, 2016).

Pharmaceuticals' residues have a pernicious impact on the environment. In the case of antibiotics, their residues inhibit the growth of aquatic microorganisms, and promote the dissemination of antibiotic resistance genes (Rodríguez-Escales & Sanchez-Vila, 2016; Kim et al., 2018; Gros et al., 2019), a potential threat to human health. This kind of contamination has been shown to be positively correlated with other adverse conditions in human health, such as the generation of reactive oxidative species, hormone synthesis, neurotoxicity, and genotoxicity (Obimakinde et al., 2017). Besides the effects on human health, there are also nefarious effects on biodiversity, either by acute or chronic exposure (Obimakinde et al., 2017).

In groundwater, there is a relatively long residence time and persistence of VHMPs, due to the inexistence of photolysis (Carrara et al., 2008). A growing number of antibiotics have been detected, many of which in high concentrations, meaning that, like most toxic organic contaminants, they can bio-accumulate in exposed organisms, as well as bio-magnify along the food chain (Obimakinde et al., 2017).

We review the current knowledge on VHMPs and their potential effects on groundwater ecosystems. We analyse the maximum environmental values of VHMPs found in groundwater and their known ecotoxicological effects in subterranean species. We identify the major gaps and future perspectives in this field.

2. Material and Methods

Acute toxicity values were obtained via ECOTOX database, provided by Environmental Protection Agency (EPA (Environmental Protection Agency), 2022). Each pharmaceutical was searched via CAS number, and the information was filtered in order to obtain only LC_{50} and EC_{50} values for algae, crustaceans, and fish, for tests performed in fresh water, and endpoints within

96 hours. For species with more than one value for the same endpoint, the geometric mean was calculated.

Values of the detected concentration in water bodies were obtained via literature search in the Web of Science platform, by using the keywords “veterinary medicinal pharmaceuticals”, “groundwater”, “toxicity”, “crustaceans”, “aquatic environment”, “stygobitics” and “environmental risk assessment”.

All statistical analyses were performed using R Studio, version 4.0.3 (R Team, 2020). With the acute toxicity values obtained from the ECOTOX search, species sensitivity distribution (SSD) plots were created by using the packages “ssdtools” (Thorley & Schwarz, 2018) and “ggplot2” (Wickham, 2016).

3. Groundwater ecosystems

Below the surface lays a subterranean world, consisting of spaces and cavities, which vary largely in terms of size, with a common characteristic light absence (Culver & Pipan, 2019b).

Due to the absence of light, subterranean ecosystems lack photosynthesis and primary production (Culver & Pipan, 2019b; Castaño-Sánchez et al., 2020a). Most subterranean communities rely on nutrients that are transported into the habitat from the surface, with exceptions relating to chemoautotrophic bacteria (Culver & Pipan, 2019a; Castaño-Sánchez et al., 2020a). Percolating waters provide nutrients, as well as dissolved organic matter, and various microbes and invertebrates. Other sources of energy are flowing water, which provides energy to terrestrial and aquatic species; wind and gravity, when organic matter comes into the cave entrance due to their effect; and roots, which penetrate into some shallow caves and may be used as food sources (Culver & Pipan, 2019a; Ravn et al., 2020).

Groundwater is a vast habitat that extends below the land surface, harbouring very peculiar fauna. Stygobitic species are characterized by a group of morphological, physiological, and behavioural traits (Hose et al., 2022). The morphological adaptations are lack of pigmentation, with the possibility of complete transparency or opaque and white coloration; lack of vision, either by the complete lack of eyes or by their reduction; and the elongation of the appendages (Howarth & Moldovan, 2018; Di Lorenzo et al., 2019b). The physiological adaptations of stygobitic species relate to the metabolism, which is slower than the metabolism of surface species, a consequence of food stress, allowing these animals to save up energy (Howarth & Moldovan, 2018; Di Lorenzo et al., 2019b). The behavioural adaptations consist of changes in the circadian and seasonal rhythms (Howarth & Moldovan, 2018; Di Lorenzo et al., 2019b). Other adaptations relate to these animals’ longevity, which is longer than surface animals (Howarth & Moldovan, 2018; Di Lorenzo et al., 2019b), and the number and size of eggs, with stygobitic species producing fewer but larger eggs than their surface counterparts (Howarth & Moldovan, 2018).

Groundwater ecosystems are currently under pressure due to their excessive use, with the removal of water for irrigation in agriculture and industry occurring at a higher rate than the natural water recharge, and due to pollution (Griebler et al., 2014; Griebler & Avramov, 2015). Groundwater and its fauna are therefore at risk, as are the ecosystem services they provide (Griebler et al., 2014; Griebler & Avramov, 2015). These include supporting services, such as nutrient cycling; provisioning services, such as water, energy and genetic resources; regulating services, such as

drought attenuation, regulation of the water cycle, water purification and flood mitigation; and cultural services, such as spiritual and aesthetic value (Griebler et al., 2014; Griebler & Avramov, 2015).

4. Contamination pathways

VHMPs are designed to reach their specific sites of action in animals' organism, and are often optimized in terms of stability and mobility, not being easily biodegradable (Balzer et al., 2016).

The active compounds of VHMPs and their metabolites are excreted in faeces and urine, being able to reach the soil environment by leaching from containment during normal waste management operations or through its usage in fertilization of agricultural fields (Balzer et al., 2016; Tolls, 2001; Watanabe et al., 2010), effluents from municipalities, pharmaceutical industries, improper disposal of unused/expired pharmaceuticals, illegal untreated effluent discharge, treatment of crop diseases and leachates from solid waste landfills (Fernandes et al., 2021).

These substances are usually polar molecules with different structures and functions, often lipophilic or fairly soluble in water (Fernandes et al., 2021). Highly mobile VHMPs have the potential to reach groundwater or other water bodies, affecting non-target aquatic organisms (Tolls, 2001), as both metabolites and the original active compound remain active and with the ability to pass though cellular membrane after excretion to the environment (Fernandes et al., 2021). Furthermore, VHMPs may be present in drinking water, especially if they are highly mobile, stable in animal manure and soil, and resistant to water purification processes (Tolls, 2001). The percentage of removal rate in water purification processes depends on the VHMPs' polarity, water solubility and persistence. By the end of this process, these substances are introduced into surface water with the treated wastewater, being at risk of reaching groundwater (Balzer et al., 2016).

The presence of VHMPs in the environment occurs more often as mixtures of various compounds and metabolites than as a single compound or metabolite, and their synergetic/antagonistic effects relate to the geographical area, climatologic conditions and occurrence of wastewater discharges (Fernandes et al., 2021).

VHMPs' persistence depends on environmental factors, physiological properties of the molecule, the presence of other pharmaceuticals in the same matrix, and the presence and activity of microorganisms with the ability to degrade these substances. The presence of biodegradable organic carbon sources may improve these substances' degradation, due to microorganisms' growth or co-metabolic processes (Fernandes et al., 2021).

Furthermore, VHMPs suffer natural attenuation and detoxification through sorption, hydrolysis, photolysis, dispersion, and biodegradation. Less commonly, radioactive decomposition may occur (Fernandes et al., 2021). However, continuous introduction into the aquatic environment results in these substances' persistence and wide distribution in all water bodies (Pereira et al., 2020).

5. Environmental regulation of VHMPs at European Union level

In order to be consumable, drinking water is treated through the process of disinfection, which does not remove pharmaceuticals (Gaston et al., 2019). Additionally, emerging contaminants, the category in which pharmaceuticals are included, have been found in aquifers since 1990s (Gaston et al., 2019).

The European Commission (EC) has created regulations in order to tackle this problem, with the aim of avoiding the deterioration of water bodies' status. Water Framework Directive (WFD) (2000/60/EC) requires Member States to manage water with an integrated ecosystem-based approach, and to consider all water and their dependent ecosystems as interlinked and interdependent. This Directive has as a main objective to establish a good ecological status of all surface waters, as well as a good chemical and quantitative status of all groundwater (Directive 2000/60/EC, 2000).

Even though there has been a rising concern about the effect of pharmaceuticals on the subterranean environment, the current guidelines for the Environment Risk Assessment (ERA) still recommend using surface organisms as surrogates for the groundwater environment (EMA, 2018). By using an assessing factor (AF) of 10, the predicted no-effect concentration of groundwater ($PNEC_{gw}$) species can be obtained through the predicted no-effect concentration of surface water ($PNEC_{sw}$) species, as shown in equation 1.1.

$$PNECgw = \frac{PNECsw}{10} \text{ (Equation 1.1)}$$

Values of measured environmental concentration for groundwater (MEC_{gw}) may also be extrapolated from available values of measured environmental concentration for surface water (MEC_{sw}), by multiplying by an AF of 0.25, as shown in equation 1.2.

$$MECgw = MECsw\ X\ 0.25 \text{ (Equation 1.2)}$$

$PNEC_{gw}$ and MEC_{gw} are fundamental for the calculation of the risk quotient for groundwater (RQ_{gw}) ecosystems, whose calculation is shown below, in equation 1.3.

$$RQgw = \frac{MECgw}{PNECgw} \text{ (Equation 1.3)}$$

6. Pharmaceutical classes

6.1. Non-Steroidal Anti-Inflammatory Agents (NSAIA)

NSAIAs are a class of pharmaceuticals with anti-inflammatory, antipyretic and analgesic properties (Rastogi et al., 2021). Even though these substances are generally considered to have medium to high mobility, the local environmental conditions, such as redox conditions, aquifer pollution sources and groundwater residence time are relevant to predict their fate in groundwater (Jurado et al., 2021). NSAIAs are regarded as a major concern, due to their high and increasing consumption (Jurado et al., 2021). Furthermore, this class of pharmaceuticals has been found to be persistent, being detected in various water sources in the range of ng/L and µg/L (Jurado et al., 2021; Rastogi et al., 2021).

Microbial degradation is a relevant pathway for NSAIA removal. By using *Trametes versicolor* (Lloyd, 1920) and *Phanerochaete sordida* (Karst, 1889) YK-624, it was possible to successfully degrade and mineralize diclofenac, ibuprofen, and naproxen. *T. versicolor* also showed an effect on ketoprofen degradation (Rastogi et al., 2021). The degradation is reached through processes such as decarboxylation, oxidation and deoxidation, hydrolysis, elimination of HCL group, dichlorination and dehydrogenation (Rastogi et al., 2021).

Acetaminophen, commonly known as paracetamol, is a popular analgesic used in the treatment of fever, headaches, and other light pains (Wu et al., 2012). Since it is one of the most prescribed pharmaceuticals, acetaminophen has been detected in various water bodies, from rivers to groundwater, in concentrations as high as 1036 µg/L, raising concerns about its effects on the environment and in public health (Lin et al., 2015). Although acetaminophen has been detected in groundwater, there is no data about its effects on groundwater ecosystems. The available data, modelled in Species Sensitivity Distribution (SSD) curves in Figure 1. 2.A), show that the most sensitive organism to acetaminophen is *Daphnia magna* (Straus, 1820), after 48 hours exposure to 18.89 mg/L. The least sensitive organism is the rotifer *Branchionus calycifloris* (Pallas, 1766), with lethal consequences only at the concentration of 5305,82 mg/L. To the best of our knowledge, there is no current data on acetaminophen exposure to stygobitic species. All RQ determined correspond to surface species, and groundwater ecosystems risk assessments are evaluated through surface species' values with the corresponding AF.

In Figure 1. 2.B), the most sensitive species reported to diclofenac sodium is the fish *Danio rerio* (Hamilton, 1822), with a lethal concentration (LC_{50}) of 5.3 mg/L, and the least sensitive species is the algae *Chlamydomonas reinhardtii* (P.A. Dangeard, 1888), with an LC_{50} of 1776 mg/L.

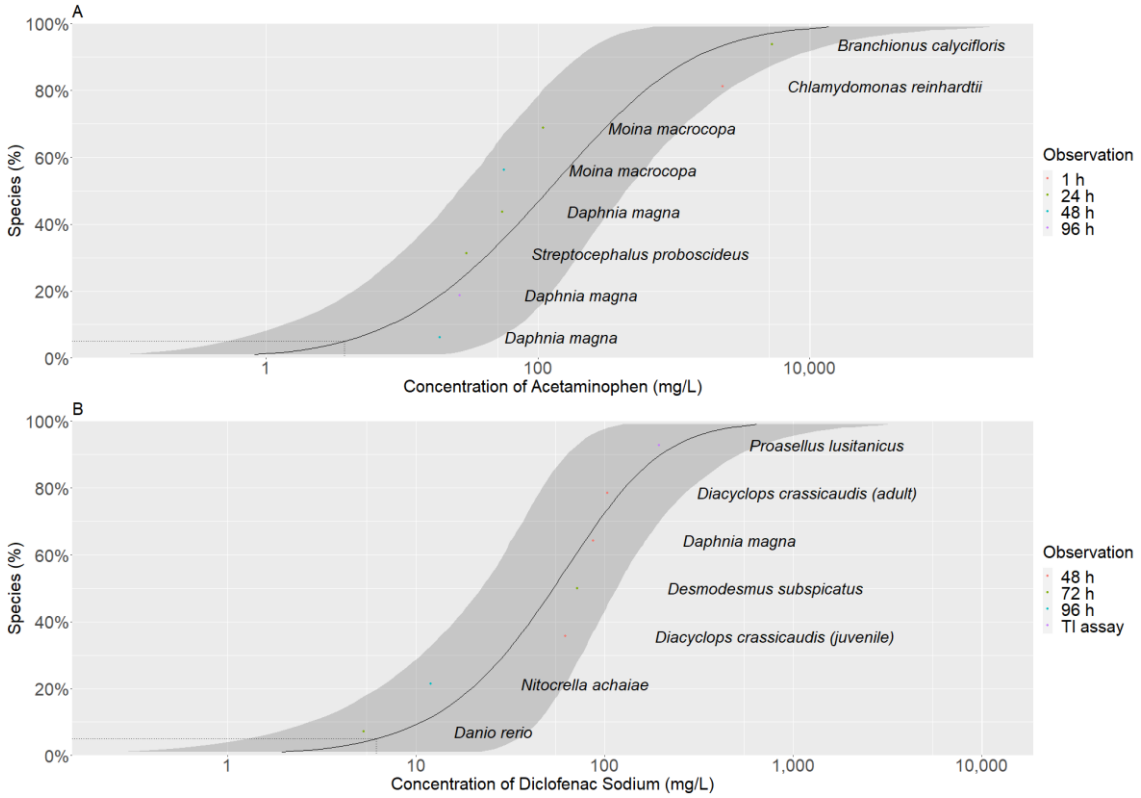


Figure 1. 2 A) Species Sensitivity Distribution (SSD) curve for acetaminophen based on acute toxicity data. Red represents the values obtained after one hour of exposure; darker green represents 24 hours of exposure; lighter green represents 48 hours of exposure; purple represents 96 hours of exposure. B) SSD curve for diclofenac based on acute toxicity data. Red represents 48 hours of exposure; darker green represents 72 hours of exposure; lighter green represents 96 hours of exposure; purple represents time-independent (TI) assay (compiled through ECOTOX database).

In Table 1. 1, it is possible to see the available information on the sensitivity of groundwater crustaceans to diclofenac sodium. The epigean cyclopoid *Diacyclops crassicaudis crassicaudis* (Sars GO, 1863) (juvenile) and the groundwater harpacticoid *Nitocrella achaiae* (Pesce, 1981) are more sensible than *D. magna*. In addition, the epigean *D. crassicaudis crassicaudis* (adult) has a higher resistance to this NSAIA than *N. achaiae*.

Table 1. 1 Available LC_{50}/EC_{50} for groundwater crustaceans to diclofenac sodium.

Species	LC_{50}/EC_{50} (mg/L)	Reference
Diacyclops crassicaudis crassicaudis (juvenile)	61.9	(Castaño-Sánchez, Pereira, et al., 2021a)
Diacyclops crassicaudis crassicaudis (adult)	103.3	(Castaño-Sánchez, Pereira, et al., 2021a)
Nitocrella achaiae	12.01	(Di Lorenzo et al., 2021a)

6.2. Antibiotics

Antibiotics are applied to treat infectious diseases and to promote growth in animals (Balzer et al., 2016; Liu et al., 2018; Viana et al., 2021). These are frequently used in human and veterinary medicine.

These compounds suffer low metabolism and are weakly absorbed in the gut, being excreted mainly through urine and faeces (Liu et al., 2018; Viana et al., 2021). Waste water treatments do not completely remove these compounds, resulting in their continuous release into the environment (Liu et al., 2018; Viana et al., 2021).

As a consequence, the increase in the availability of antibiotics may produce pressure on water bacteria communities, leading to the formation of antibiotic resistance bacteria, which in turn would affect the therapeutic effect against human and veterinary pathogens (Liu et al., 2018; Viana et al., 2021).

Chlortetracycline is a broad-spectrum antibiotic from the tetracycline class, used in the treatment against various gram-positive and gram-negative bacteria (Dzomba & Zaranyika, 2021). Another use for this antibiotic is in veterinary medicine, as a growth promotor. In Figure 1. 3.A), it is possible to observe that the most sensitive organism is the fish *Oryzias latipes* (Temminck & Schlegel, 1846), with an LC_{50} of 78.9 mg/L. On the opposite end, the most resistant organism is *Moina macrocopa* (Strauss, 1820), with a LC_{50} of 515 mg/L.

Another antibiotic of great concern is erythromycin, a macrolide antibiotic used in both human and veterinary medicine (Minski et al., 2021). In Figure 1. 3.B), it is possible to see that the available data indicates that concentrations lower than 100 mg/L cause lethal effects in various organisms, being the most resistant *B. calycifloris*, with an LC_{50} of 27.53 mg/L, and the most sensitive *Anabaena* sp. (Nostoc) with an LC_{50} of 0.022 mg/L.

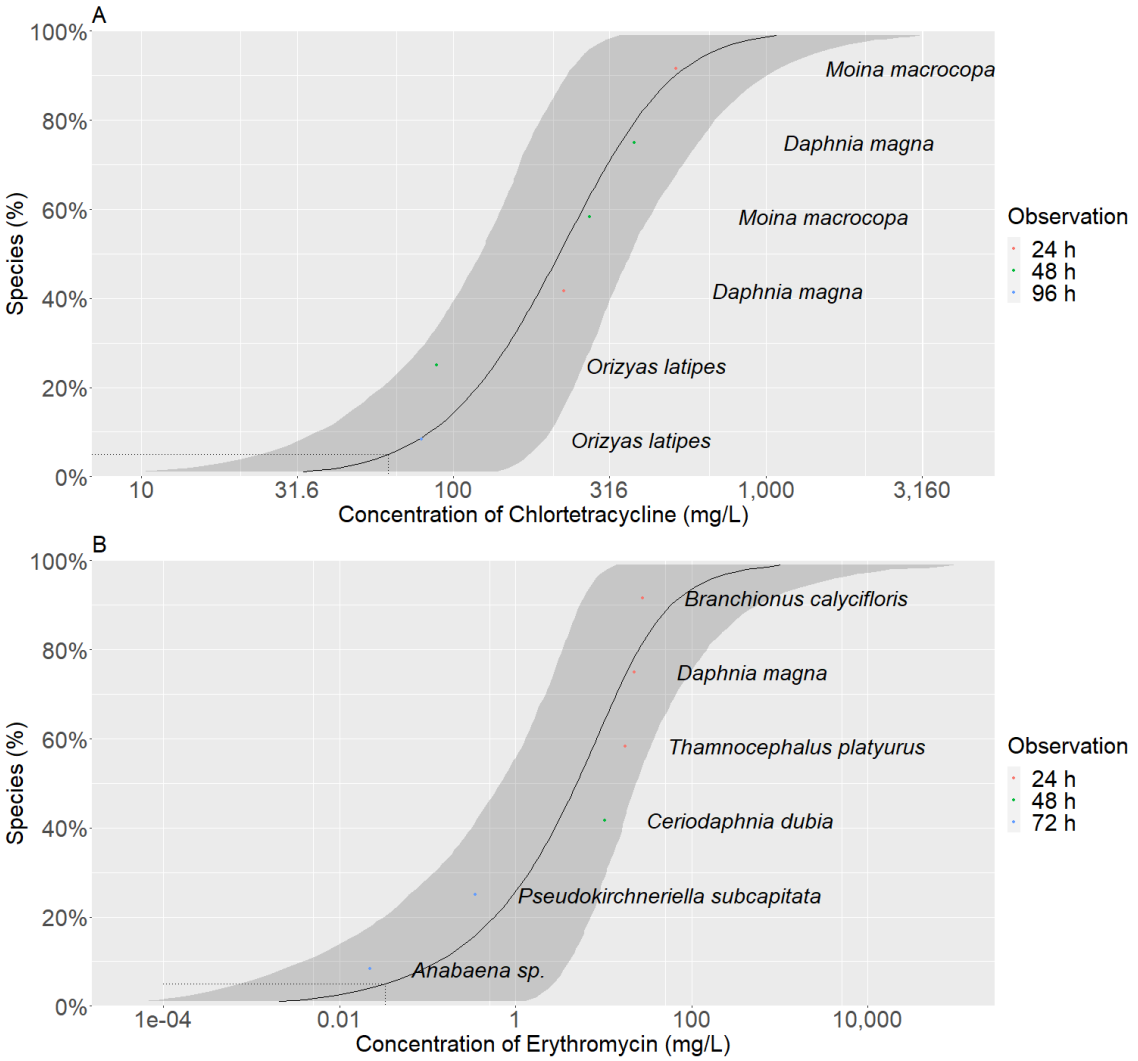


Figure 1. 3 A) Species Sensitivity Distribution (SSD) curve for chlortetracycline based on acute toxicity data. B) SSD curve for erythromycin based on acute toxicity data. Red represents the values obtained after 24 hours of exposure; darker green represents 48 hours of exposure; blue represents 72 hours of exposure (compiled through ECOTOX database).

6.3. B-blockers

β -adrenergic receptor blockers, also known as β-blockers, are widely used in the treatment of cardiovascular disorders, such as abnormal heart rhythms, high blood pressure and angina pectoris (Piram et al., 2008; Ramil et al., 2010; Yi et al., 2020).

These pharmaceutical compounds are excreted mainly through urine, of which 1-75% is released in their original form, without metabolization (Yi et al., 2020). Furthermore, wastewater treatments do not completely remove these compounds. Coupled with the fact that this pharmaceutical class has a high rate of consumption and a relatively high persistence, its detection in water bodies is frequent (Godoy et al., 2015).

As fish have β-adrenergic receptors similar to mammals, their exposure to this class of pharmaceuticals is hypothesized to lead to cardiovascular disfunction. When exposed to propranolol (PRO), fecundity of *O. latipes* and the heart rate of *D. magna* suffered a decrease

(Ramil et al., 2010). Other effects on aquatic organisms include a decrease in reproduction rates, abnormal behaviours, and disruption of testosterone levels (Yi et al., 2020).

Over 80% of PRO is excreted via urinary metabolites in humans, and only about 20% is removed in wastewater treatments. Di Lorenzo et al. (2019a) studied the toxicity of the groundwater crustacean *Diacyclops belgicus* (Kiefer, 1936) to PRO. The LC_{50} at 96 h for *D. belgicus* was observed to be 5 mg/L, at 15ºC while the EC_{50} at 48 h was 5 mg/L for *D. magna*.

6.4. Psychoactive Agents

Psychoactive agents, also known as psychiatric pharmaceuticals, have been detected in the aquatic environment, alongside their metabolites (Tanoue et al., 2019). These detections are expected since there are many different pharmaceuticals available on the market, used to treat mental disorders such as anxiety and insomnia, induce anesthesia, and treat/manage pain (Cunha et al., 2017; Tanoue et al., 2019). These compounds are used in the veterinary industry to induce anesthesia and stimulate animal appetite (Cunha et al., 2017). Psychoactive agents may be used recreationally, with stimulant and hallucinogenic effects (Tanoue et al., 2019).

These compounds are considered difficult to degrade (Cunha et al., 2017). One of the by-products of diazepam's degradation is oxazepam, which is itself a commercialized compound (Cunha et al., 2017). This emphasizes that metabolites should not be ignored, as they are active ingredients and could have pernicious effects themselves. Furthermore, psychoactive agents are difficult to remove during the waste water treatment, resulting in their release into the surface water, and later infiltration into groundwater (Grabicová et al., 2020; Wang et al., 2020).

Exposure to these compounds led to the observation of changes in organism behaviour, e.g. in predator-prey relationships, social traits, feeding rate, circadian rhythms, reproduction and migration strategies, which could lead to important ecological changes (Grabicová et al., 2020; Tanoue et al., 2019). However, there is some controversy about the effects of these substances (Tanoue et al., 2019). Most of these compounds are hydrophobic, which means that aquatic organisms will readily absorb them (Tanoue et al., 2019).

To the best of our knowledge, there is no information about the effect of this pharmaceutical class in groundwater species.

7. Discussion

Using surface water species as surrogates for the estimation of stygobitic taxa's sensitivity is recommended in the EC guidelines, being necessary the division of a AF of 10 from the $PNEC_{sw}$ of the most sensitive algae, *Daphnia* or fish, when calculating the $PNEC_{gw}$ (European Medicines Agency, 2006). This AF aims to consider the higher vulnerability of groundwater species and inability to recover from interfering events, when comparing to surface species (European Medicines Agency, 2006). Additionally, the multiplication of an AF of 0.25 is used in order to estimate the MEC_{gw}, by using MEC_{sw} (European Medicines Agency, 2006). However, karst areas are characterized by a low or no soil cover, leading to poor filtration and high infiltration (Kogovšek & Petrič, 2013). Direct infiltration via stream sinks, shafts and caves make karst aquifers particularly vulnerable to pollution, with deep groundwater being more vulnerable than initially thought (Reberski et al., 2022). Usually, more pollutants are detected and in higher

concentrations in surface water in comparison to groundwater, except in the specific cases of fenofibrate (Reberski et al., 2022). More studies are needed in order to clarify which are the real concentrations of VHMPs in groundwater. Table 1.A. 1, with MEC_{gw} and Measured Environmental Concentration in water resources (MEC_w) values, is available in 10.1 Appendix 1.

Conducting ecotoxicological tests on groundwater species has several obstacles. These species tend to be difficult to find and collect, since their spatial distribution is usually restricted (Di Lorenzo et al., 2019a,b). This results in the collection of specimens in fewer numbers than necessary to perform ecotoxicological tests. In addition, these species are extremely difficult to rear and reproduce in the laboratory, becoming near impossible to perform ecotoxicological tests with juveniles individuals of stygobitic species (Di Lorenzo et al., 2019a, b). Furthermore, it is complicated to protect species unknown to scientific community. Subterranean environments are difficult to access, with most subterranean habitats remaining poorly explored (Griebler et al., 2014; Ficetola et al., 2019). This affects our knowledge on biodiversity: a phenomenon known as the Racovitzan impediment. These habitats are assumed to hold a large and underestimated portion of Earth' species (Ficetola et al., 2019). These obstacles help to understand the paucity of studies in the subterranean ecotoxicology assays.

Differences in the metabolic rate and oxygen consumption between hypogean and epigean crustacean species also raise concerns (Avramov et al., 2013). Hypogean crustacean species were observed to have a rate of oxygen consumption up to five/seven times slower than epigean species (Di Lorenzo et al., 2015) as well as 1.2 to 8.6 times lower levels of the enzymatic activity of the main key regulatory enzymes (Avramov et al., 2013). It can be debated that these differences result in a delayed manifestation of toxicity effect (Avramov et al., 2013), and that a time-independent (TI) test should be considered when evaluating the effect of a pharmaceutical product on subterranean-adapted species. On the other hand, the low metabolism of hypogean species could delay the onset of defence mechanisms, such as detoxification and metabolite excretion (Di Lorenzo et al., 2015).

8. Future perspectives

Unique traits demand unique protocols (Di Lorenzo et al., 2019b; Castaño-Sánchez et al., 2020b). Conservative AFs are important to protect an ecosystem that is still so vastly unknown, however, further research is necessary.

Obstacles in subterranean ecology include the lack of knowledge related to behaviour and life cycles, which causes difficulties in the performance of acute toxicity assays in juveniles rather than in adults lifeforms, as well as in the performance of chronic toxicity assays (Castaño-Sánchez et al., 2020a).

Additionally, monitoring and assessing groundwater quality is a practical action to identify the current threats and evaluate potential new ones. It is also useful to define and quantify the fate and transportation of VHMPs in the aquifer systems (Castaño-Sánchez et al., 2020a).

Lastly, identifying the effects that these VHMPs produce in subterranean species is only possible by setting ecotoxicology protocols adequate for the peculiar traits that these species have. Guidelines have been previously suggested (Di Lorenzo et al., 2019b).

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10. Appendix

10.1. Appendix 1

Table 1.A. 1. Measured Environmental Concentration in groundwater ([MEC)gw) and Measured Environmental Concentration in water resources ([MEC]w)

Active substance	Therapeutic class	CAS nr.	Route of application	[MEC] gw (ug/L)	[MEC] w (ug/L)	Location	Reference
Acetaminophen	NSAIA	103-90-2	Oral	1036	NA	Taiwan	doi:10.1007/s10661-015-4497-3
				0.0103	0.0147	France	doi:10.1016/j.envpol.2011.04.033
				0.38	NA	United States	doi:10.1016/j.scitotenv.2008.04.028
				NA	0.527	Portugal	doi:10.1016/j.scitotenv.2016.08.089
				1.89	NA	United States	doi:10.1016/j.scitotenv.2011.05.053
				0.188	12.43	Nigeria	doi:10.1016/j.emcon.2020.02.004
				NA	0.048	Baltic sea (Germany)	doi:10.1016/j.marpolbul.2014.06.024
				NA	2.983	Aegean Sea & Dardanelles (Greece and Turkey)	doi:10.1016/j.marpolbul.2014.06.024
				NA	0.375	Venice (Italy)	doi:10.1016/j.marpolbul.2014.06.024
				NA	0.085	San Francisco Bay (USA)	doi:10.1016/j.marpolbul.2014.06.024

				NA	0.012	Mediterranean Sea (Israel)	doi:10.1016/j.marpolbul.2014.06.024
				NA	0.11	Spain	doi:10.1016/j.scitotenv.2012.08.036
				NA	3.59	Italy	doi:10.1016/j.scitotenv.2014.02.053
Atenolol	B-blocker	29122-68-7	Oral	0.0036	NA	Taiwan	doi:10.1007/s10661-015-4497-3
				0.0055	0.0047	France	doi:10.1016/j.envpol.2011.04.033
				0.106	NA	Spain	doi:10.1016/j.scitotenv.2010.04.041
				NA	0.013	Baltic sea (Germany)	doi:10.1016/j.marpolbul.2014.06.024
				NA	0.194	Aegean Sea & Dardanelles (Greece and Turkey)	doi:10.1016/j.marpolbul.2014.06.024
				NA	0.022	Venice (Italy)	doi:10.1016/j.marpolbul.2014.06.024
				NA	0.057	San Francisco Bay (USA)	doi:10.1016/j.marpolbul.2014.06.024
				NA	NA	Italy	doi:10.1016/j.scitotenv.2016.09.168
				NA	0.036	United States	doi:10.1021/es801845a
				NA	0.006	Netherlands	doi:10.1016/j.scitotenv.2012.04.010
				ND	0.2419	Italy	doi:10.1016/j.scitotenv.2014.02.053

Amoxicillin	antibiotic	26787-78-0	Oral	0.1	NA	Germany	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/amoxicillin/26787-78-0/none/Water%20(Ground%20Water)
				NA	NA	Italy	doi:10.1016/j.scitotenv.2016.09.168
Chlortetracycline	antibiotic	57-62-5	Oral	0.0866	NA	China	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/chlortetracycline/57-62-5/none/Water%20(Ground%20Water)
Diclofenac	NSAIA	15307-86-5	Oral and Dermic Use	0.0097	0.0054	France	doi:10.1016/j.envpol.2011.04.033
				NA	0.038	Portugal	doi:10.1016/j.scitotenv.2016.08.089
				0.042	0.2	Nigeria	doi:10.1016/j.emcon.2020.02.004
				0.024	NA	Europe	doi:10.1016/j.watres.2010.05.032
				0.477	NA	Spain	doi:10.1016/j.scitotenv.2010.04.041
				0.00314	NA	Spain	doi:10.1016/j.envpol.2012.11.022
				NA	0.0092	Baltic sea (Germany)	doi:10.1016/j.marpolbul.2014.06.024
				NA	0.0097	Aegean Sea & Dardanelles (Greece and Turkey)	doi:10.1016/j.marpolbul.2014.06.024
				NA	0.0061	Mediterranean Sea (Israel)	doi:10.1016/j.marpolbul.2014.06.024

				NA	0.0012	United States	doi:10.1021/es801845a
				NA	0.0169	Spain	doi:10.1016/j.scitotenv.2012.08.036
				ND	0.158	Italy	doi:10.1016/j.scitotenv.2014.02.053
Eprinomectin	antiparasitic	123997-26-2	Dermic Use	NA	0.015-0.019	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.045	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.02	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.01-0.016	France	doi:10.1016/j.scitotenv.2019.01.303
Florfenicol	antibiotic	73231-34-2	Injectable/ Oral	NA	0.021	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.022-0.434	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.007	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.287	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.434-0.930	France	doi:10.1016/j.scitotenv.2019.01.303
Flumequine	antibiotic	42835-25-6	Injectable	NA	0.01-0.013	France	doi:10.1016/j.scitotenv.2019.01.303

				0.066	NA	Taiwan	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/flumequine/42835-25-6/none/Water%20(Ground%20Water)
Flunixin	NSAIA, analgesic, antipyretic	42461-84-7	Injectable	NA	0.005	France	doi:10.1016/j.scitotenv.2016.09.168
				NA	0.014	France	doi:10.1016/j.scitotenv.2016.09.168
Ivermectin	antiparasitic	70288-86-7	Injectable	NA	0.021	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.014	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.013	France	doi:10.1016/j.scitotenv.2019.01.303
Ketoprofen	NSAIA, analgesic, antipyretic	22071-15-4	Oral	0.061	NA	Germany	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/ketoprofen/22071-15-4/none/Water%20(Ground%20Water)
Lincomycin	antibiotic	154-21-2	Injectable	0.207	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.045	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.096	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				NA	0.006	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.005	France	doi:10.1016/j.scitotenv.2019.01.303
				1.9	NA	United States	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/lincomycin/154-21-2/none/Water%20(Ground%20Water)

Spiramicyn	antibiotic	8025-81-8	Injectable	<LOQ	NA	Germany	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/spiramycin/8025-81-8/none/Water%20(Ground%20Water)
Oxytetracycline	antibiotic	79-57-2	Oral	NA	0.036	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.325	France	doi:10.1016/j.scitotenv.2019.01.303
				0.13	NA	United States	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/oxytetracycline/79-57-2/none/Water%20(Ground%20Water)
Sulfadiazine	antibiotic	68-35-9	Oral	NA	0.508	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	2.946	France	doi:10.1016/j.scitotenv.2019.01.303
				0.0144	NA	Taiwan	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/sulfadiazine/68-35-9/none/Water%20(Ground%20Water)
Sulfamethazine	antibiotic	57-68-1	Injectable	NA	0.006-0.066	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.035	France	doi:10.1016/j.scitotenv.2019.01.303
				0.063	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.031	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.033	NA	United States	doi:10.1016/j.jconhyd.2010.12.010

				0.025	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.616	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.131	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.073	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.11	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.076	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				0.055	NA	United States	doi:10.1016/j.jconhyd.2010.12.010
				3.6	NA	United States	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/sulfadimidine/57-68-1/none/Water%20(Ground%20Water)
Sulphadimethoxine	antibiotic	122-11-2	Oral	0.13	NA	United States	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/sulfadimethoxine/122-11-2/none/Water%20(Ground%20Water)
Tilmicosin	antibiotic	108050-54-0	Oral	NA	0.007	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.007	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.005	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.007	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.009	France	doi:10.1016/j.scitotenv.2019.01.303

				NA	0.006	France	doi:10.1016/j.scitotenv.2019.01.303
Tiamulin	antibiotic	55297-95-5	Oral	0.113	NA	France	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/tiamulin/55297-95-5/none/Water%20(Ground%20Water)
Triclabendazole	antiparasitic	68786-66-3	Dermic Use	0.005	NA	France	doi:10.1016/j.scitotenv.2019.01.303
Trimethoprim	antibiotic	738-70-5	Oral	NA	0.009	France	doi:10.1016/j.scitotenv.2019.01.303
				NA	0.121	France	doi:10.1016/j.envpol.2011.04.033
				NA	0.468	France	doi:10.1016/j.envpol.2011.04.033
				NA	0.018	France	doi:10.1016/j.envpol.2011.04.033
				0.1948	NA	United States	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/trimethoprim/738-70-5/none/Water%20(Ground%20Water)
				NA	0.003	Spain	doi:10.1016/j.scitotenv.2012.08.036
Tylosin	antibiotic	1401-69-0	Oral	0.05	NA	United States	https://ipchem.jrc.ec.europa.eu/#databaseConsole/PHARMSUBA/tylosin/1401-69-0/none/Water%20(Ground%20Water)

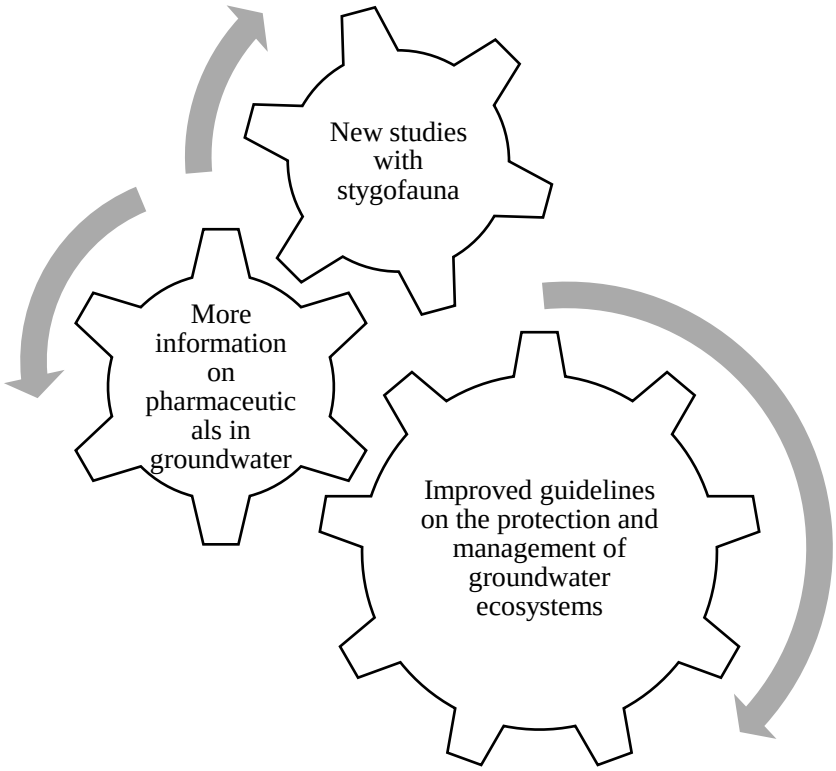
Chapter II – New perspectives for environmental risk assessment of diclofenac in groundwater ecosystems

Figure 2. 1 Prospection and collection of stygobionts in Assafora Cave, located in Sintra-Cascais Natural Park. Credits: Ana Sofia P.S. Reboleira.

Paper in preparation for submission:

Duarte, C., Di Lorenzo, T. & Reboleira, A.S.P.S., New perspectives for environmental risk assessment of diclofenac in groundwater ecosystems.

Graphical abstract



Abstract

Groundwater is the habitat of a number a stygobitic species that possess a variety of unique traits. Aquifers are susceptible to anthropogenic contamination, such as those originating from veterinary and human medicinal product (VHMP). The environmental risk assessment (ERA) refers to the analysis of the potential effects that VHMPs pose on a given environment by comparing their measured environmental concentrations to their predicted no-effect concentration (PNEC). We tested the acute toxicity in a time-independent (TI) assay of the widely used non-steroidal anti-inflammatory diclofenac sodium on a stygobitic asellid, *Proasellus lusitanicus*, and ranked its sensitivity with available ecotoxicity data of aquatic biota. Our results show that the lethal concentration (LC_{50} = 191.39 mg/L) decreases over exposure time. Furthermore, we explore four scenarios of ERA, to evaluate which scenario is the most conservative and if current guidelines are appropriate for groundwater ecosystems. Of the scenarios explored, the most conservative one is based on the current ERA guidelines (Risk Quotient (RQ) = 1060), while the least conservative one was computed by using measured environmental concentration (MEC) values from databases and the PNEC obtained from the performed TI assays (RQ = 8.03×10^{-6}). Our results raise questions about the optimal way to test groundwater species' sensitivity to anthropogenic contaminants, since the toxicology assays guidelines for surface taxa are likely not adequate for stygobitic species due to the intrinsic differences of both.

1. Introduction

Subterranean ecosystems are characterized by complete darkness which prevents energy production from photosynthesis. They rely mostly on organic matter transported via percolating water from the surface, and occasionally via chemolithotrophic processes (Ravn et al., 2020; Brad et al., 2021). Additionally, subterranean ecosystems have a narrower temperature amplitude throughout the day and year when compared with surface ecosystems, and higher humidity (Badino, 2004; Mammola et al., 2019).

Stygobitic species are obligate groundwater organisms which have morphological, physiological and behavioural traits to life in groundwater (Hose et al., 2022). Stygobitic species have a longer life cycle than their surface relatives, lower metabolic rates, and loss of circadian rhythm (Wilhelm et al., 2006; Di Lorenzo et al., 2015). They have the absence of pigmentation, loss or reduction of eye structures, elongation of appendages and body, elongation and increase in the number of sensory receptors, as well as a different spatial distribution of these receptors, and cuticle thinning and scale reduction (Hose et al., 2022).

Groundwater ecosystems provide essential ecosystem services, such as water purification, through biodegradation and elimination of pathogens, water balance of groundwater dependent ecosystems, with a role in buffering floods and droughts and sustaining wetlands, biodiversity, by providing habitat to rare and endemic species, as well as recreational services, with the provision of hot springs, tourism, and spa (Griebler & Avramov, 2015). This resource is under pressure due to contamination by sewage wastewater, through leakage from sewer pipes, industry and/or animal farms, the application of sewage sludge and animal manure in agriculture fields, urban and rural storm water run-off, infiltration by contaminated river water, and application to pesticides and/or fertilizers onto soils (Burri et al., 2019).

Although groundwater is often considered less vulnerable to contamination than surface water, traces of various pollutants are frequently detected (Lukač Reberski et al., 2022). Among these pollutants, veterinary and human medicinal products (VHMP) are included, being often detected in ng/L or µg/L (Bexfield et al., 2019).

Diclofenac sodium is a non-steroidal anti-inflammatory agent (NSAIA), in use since the 1970s, both in human and veterinary medicine (Sathishkumar et al., 2020; Castaño-Sánchez et al., 2021). This pharmaceutical may be applied dermally or taken orally. However, due to its hydrophilic nature, diclofenac sodium is not fully absorbed by the skin (Hui et al., 1998). Nowadays, veterinary uses of diclofenac sodium in Europe are greatly restricted, so wastewater is the key exposure route for wildlife. Removal techniques performed in wastewater treatment plants (WWTP) are not entirely efficient in diclofenac sodium removal, causing it to be detected in both surface water and groundwater. Depending on the performed process, diclofenac sodium's removal varies from 44.4% to around 90% (Alessandretti et al., 2021).

In 2015, diclofenac sodium was included in the European priority substances watchlist (Official Journal of the European Union, 2015). The list aims to gather measured environmental concentrations (MECs) of the most harmful chemical compounds in Europe and is regularly updated (Official Journal of the European Union, 2020). The European Environmental Agency (EEA) gathers the MECs in a database called WATERBASE, which is available online (*Waterbase - Water Quality ICM*, 2022). The chemicals that are deemed to present a Europewide risk are withdrawn from the watchlist and selected for Environmental Quality Standard (EQS) derivation. The EQS is the concentration below which the

ecological functions and the community structure of a water body are not changed (Directive 2000/60/EC, 2000; Lepper, 2005). EQS represents the legally binding threshold against which to compare the MECs. Either "good" or "poor" quality status is assigned to a water body based on the EQS, according to the European Water Framework Directive (Directive 2000/60/EC, 2000). Diclofenac sodium has been monitored for a while and finally withdrawn from the watchlist and selected for EQS derivation. An EQS equal to 0.050 µg L^{-1} has been assessed for this compound in surface water bodies. This value has been much criticized and a higher value equal to 0.126 µg L^{-1} has been recently proposed based on a probabilistic (species sensitivity distribution (SSD)) approach that accounts for all of the reliable and relevant data in accordance with the European guidelines (Leverett et al., 2021). The EQS for diclofenac sodium in groundwater has not been assessed yet.

Environmental risk assessment (ERA) is part of the requirements for all new marketing authorization for medicinal products (European Medicines Agency, 2018). In ERA, the MEC of a pharmaceutical compound is divided by its PNEC (the concentration below which adverse effects are not expected to occur). From this ratio, it is possible to obtain values of risk; if the ratio is ≤ 1, no risk is expected. Otherwise, if the ratio > 1, there is a risk associated with the exposure (Pereira et al., 2020). For new pharmaceuticals, PNEC and EQS concentrations should be the same. Diclofenac sodium is not a new pharmaceutical compound; however, since the EQS of diclofenac for groundwater bodies has not been assessed so far, the procedure to derive it must be the same as for deriving the PNEC (De Bruijn et al., 2002). To derive the PNEC of a pharmaceutical compound in groundwater, the EMA guidelines (European Medicines Agency, 2018) recommend using: i) long-term toxicity data; ii) surface water species as surrogates of stygobitic taxa; iii) an assessment factor to account for uncertainties related to the extrapolation from the laboratory to the field and iv) a further assessment factor to extrapolate from surface to groundwater species. This latter has been assessed as equal to 10 by (Kolar & Finizio, 2017) and reported in the guidelines. The use of surface water species as proxies of groundwater taxa is necessary because of the few available data. Performing ecotoxicological assays with stygobitic species poses several obstacles, such as the difficulty in collecting specimens in enough number and maintaining and rearing them in the laboratory (Di Lorenzo et al., 2019a; Castaño-Sánchez et al., 2020).

In this study, we aimed to advance the knowledge about the environmental risk posed by diclofenac sodium to groundwater ecosystems. To this end, we: 1) reviewed the scientific literature on the pernicious effects of diclofenac on freshwater species, including stygobitic species; 2) analysed the MECs of diclofenac sodium in European groundwaters to detect higher risk zones and provide evidence that diclofenac sodium represents a risk for groundwater bodies; 3) estimated the PNEC of diclofenac sodium based on the acute toxicity of the groundwater crustacean *Proasellus lusitanicus* (Frade, 1938) in a time-independent (TI) assay (long-term toxicity assay), and compared this values with that indicated in the guidelines. Finally, we provided four different scenarios of environmental risk of diclofenac sodium in groundwater by computing the Risk Quotient (RQ) where: a) the MECs were obtained from either literature or WATERBASE and b) the PNECs were obtained from either the EU guidance or from the test assays performed in this study.

2. Material and Methods

2.1. Animal collection and acclimation

Individuals of the stygobitic species *Proasellus lusitanicus* (Figure 2. 2) were collected in Olhos d'Água Cave (39°32'28.4"N 8°43'20.0"W; Central Portugal) in October 2021. The species is endemic to Portugal, inhabiting caves from the Estremenho karst massif (Magniez 1967), where the annual average

temperature is 17º C (Reboleira et al., 2011). This species has been previously used to test the effects of copper sulphate and potassium dichromate (Reboleira et al., 2013). At the collection site, temperature, pH, dissolved oxygen (DO), and electrical conductivity were measured by using a portable multiparameter probe (AQUAREAD - WTW MULTI 3430). Water properties are presented in Table 2.A. 1, in Appendix 7.1.

Figure 2. 2. Specimens of *Proasellus lusitanicus* (Frade, 1938).

About 300 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 the collection. Afterwards, the specimens were acclimated to the laboratory conditions by keeping them in permanent darkness and at the temperature of the collection site. A few spoons of the sediment from the cave were provided since *P. lusitanicus* is a deposit-feeder. No artificial food was supplied. The individuals remained in these stable conditions for a month (duration of preliminary tests), after which TI assays started.

2.2. Time Independent assay

Acute toxicity tests were carried out with the pharmaceutical compound diclofenac sodium (CAS number: 15307-79-6; 2-[(2,6-dichlorophenyl) amino] benzeneaceticacid sodium salt (1:1); $C_{14}H_{10}Cl_2NaNO_2$)) purchased from Sigma-Aldrich (Steinheim, Germany). The solution was prepared fresh.

We tested the acute toxicity of diclofenac sodium on *Proasellus lusitanicus*. Previous to exposure, the specimens were acclimatized in filtered groundwater in order to clear the contents of the digestive tract and to avoid decrease in bioavailability of the pharmaceutical product during the experiment. Assays were performed in glass vials in order to avoid adsorption of the pharmaceutical product. The vials were not aerated during the assays to avoid stress and no food was provided. Throughout the assay, glass vials were maintained at a temperature of 17ºC and kept away from the light.

Three runs of range-finding tests were performed prior to the final test with the following nominal concentrations: 1, 10 and 100 mg/L, plus blank control (range-finding test #1); 125, 175, 225 and 275 mg/L, plus control (range-finding test #2) and 325, 375, 425 and 475 mg/L, plus control (range-finding test #3). Each concentration was tested with four specimens, which were loaded individually with a soft brush in the vials with 6 mL of the appropriate solution.

The range-finding tests did not result in 100% mortality within 96 hours. This led us to the decision to replace the acute toxicity tests for TI assays, an assay previously performed by Avramov et al. (2013) and Hose et al. (2019), which allows accounting for the possible delayed toxic effects in groundwater fauna. A TI test assesses acute toxicity without a predetermined temporal end point, i.e., the test continues until the toxic response has ceased or other (practical) considerations dictate its terminus (Rand, 1995). The test was terminated at day 14 when the mortality nearly ceased and was not prolonged to avoid impairing the stability of diclofenac concentrations.

The final TI assay was carried out with the following nominal concentrations 75, 125, 175, 225 and 275 mg/L. A stock solution was prepared fresh with a concentration of 275 mg/L was prepared by dissolving 0.0715 g in 260 mL of commercial water. Ten specimens were distributed per concentration, plus 10 in the control with commercial water, following recommendations by Di Lorenzo et al. (2019b). In total, 60 specimens were used, each in an individual vial containing 6 mL of the appropriate solution, following the protocol recently available (Castaño-Sánchez et al., 2021). Dissolved oxygen and pH were measured prior and after the tests with AQUAREAD - WTW MULTI 3430. Mortality in each test vial was recorded during the first 14 days with a maximum gap of 3 days. Death was defined as complete immobility of the animal without any agitation of the uropods over 1-2 min of observation. The assay was considered valid if the mortality in the control group was ≤20%, and if the variation of the concentration of the DO was within 20% (Di Lorenzo et al., 2019b).

2.3. Statistical analysis and Risk Quotient (RQ) calculation

All statistical analyses were performed using R Studio, version 4.0.3.

Mortality data from the TI assay was used to estimate the ultimate lethal concentration for 50% of the population (LC_{50}) (Sprague, 1969). For each day of observation, an LC_{50} value was calculated using the packages "drc" (Ritz et al., 2015), and the LC_{50} function was plotted with the package "ape" (Paradis & Schliep, 2019), version 5.6.2. To estimate the ultimate LC_{50}, the daily values were plotted versus time and a linear regression was fitted by ordinary least squares.

For the ERA, four different risk scenarios of diclofenac sodium in European groundwater bodies were explored where the risk was computed as quotient RQ, through equation 1:

$$RQgw = \frac{MECgw}{PNECgw} \text{ (Equation 2.1),}$$

where MEC_{gw} stands for measured environmental concentration of diclofenac sodium in groundwater, and $PNEC_{gw}$ stands for predicted no-effect concentration for groundwater biota. Both values must be in the same unit. In Table 2. 1, it is specified how the four scenarios differ in detail.

a. In Scenario 1, the MEC_{gw} corresponds to the values obtained through literature research in Web of Science platform by using the keywords "diclofenac" and "groundwater". Only papers written in English were selected, containing data from European Union (EU) Members States, and published in peer-reviewed scientific journals. The $PNEC_{gw}$ is obtained from Equation 2:

$$PNECgw = \frac{PNECsw}{AF1} \text{ (Equation 2.2),}$$

where PNECsw is equal to 50 ng/L according to (Carvalho et al., 2016), and AF1 is an assessment factor equal to 10 that accounts for the uncertainties related to using freshwater species laboratory assays to estimate the sensitivity of groundwater communities (EMA, 2018).

b. In Scenario 2, the MEC_{gw} is obtained through the WATERBASE (*Waterbase - Water Quality ICM*, 2022) database by the European Environmental Agency (EEA). The $PNEC_{gw}$ value is the same as in Scenario 1.
c. In Scenario 3, the MEC_{gw} value is the same as in Scenario 1, whereas the $PNEC_{gw}$ value is obtained via TI assay performed with *P. lusitanicus* in this study (see paragraph 2.1.3). According to the EU guidelines (EMA, 2018), the $PNEC_{gw}$ must be calculated following equation 3:

$$PNECgw = \frac{NOEC}{AF2} \text{ (Equation 2.3)}$$

where NOEC stands for no-observed effect concentration and AF2 is an assessment factor that accounts for the uncertainties related to using acute sensitivity to estimate chronic sensitivity. In this study, the NOEC was estimated from the LC_{10} obtained via the TI assay with *P. lusitanicus*. The LC_{10} was computed on day 14 of the TI assay (see Section 2.1.3) using the packages "drc" (Ritz et al., 2015) of the R software. According to the EU guidelines (European Medicines Agency, 2018), the AF2 was set as follows "*An assessment factor of 100 applies to a single long-term NOEC/EC10 if this NOEC was generated for the trophic level showing the lowest L(E)C50 in the short-term tests[…]. If the only available long-term NOEC is from a species which does not have the lowest L(E)C50 from the short-term tests, […] the assessment of the effects is based on the short-term data with an assessment factor of 1000*" (ECHA (European Chemicals Agency), 2017). To decide which AF2 to use, we compared taxa sensitivity (from both surface and groundwater) from different trophic levels. We considered only values obtained from acute tests with a maximum duration of 96 h, testing the active substance in a freshwater medium without renovation. Data was obtained through ECOTOX database (EPA (Environmental Protection Agency), n.d.). For the species that had more than one value for the same endpoint, the geometric mean was calculated. The comparison of the sensitivity of *P. lusitanicus* with the sensitivity of other species was performed by analysis of Species-Sensitivity Distribution (SSD) model. The SSD curve was produced by using the packages "ssdtools" (Thorley & Schwarz, 2018) and "ggplot2" (Wickham, 2016).

d. In Scenario 4, the MEC_{gw} is the same as in Scenario 2, and the $PNEC_{gw}$ is as in Scenario 3.

In Appendix 2, Table 2.A. 2. Ecotoxicity values for diclofenac sodium (data compiled through ECOTOX database) (EC50 – Effective Concentration; LC50- Lethal Concentration). contains the available ecotoxicology acute data, and in Appendix 3, Table 2.A. 3 contains the available MEC_{gw} available in the consulted literature.

Table 2. 1 Simplified description of the four scenarios for RQ calculation (MECgw – Measured Environmental Concentration in groundwater; PNECgw – Predicted Non-Effect Concentration of groundwater biota).

Scenario 1	**Scenario 2**
MECgw is estimated through literature analysis **PNECgw** is obtained according to the EU guidance (EMA, 2018)	**MECgw** is estimated through the WATERBASE surface water data **PNECgw** is obtained according to the EU guidance (EMA, 2018)
Scenario 3	**Scenario 4**
MECgw is estimated through literature analysis **PNECgw** is obtained through the time independent assays with *P. lusitanicus*	**MECgw** is estimated through the WATERBASE surface water data **PNECgw** is obtained through the time independent assays with *P. lusitanicus*

3. Results

3.1. TI assay

The test met the control validity criterion recommended in Di Lorenzo et al. (2019b) since we observed ≤ 20% mortality in the test control. The LC_{50} values ranged between 194.61 (±15.12) after 14 days and 493.30 (±310.41) mg/L, obtained at 4-5 days (Table 2. 2). As expected, there was a clear LC_{50} decreasing over time (Figure 2. 3). The linear model parameters were as follow: intercept = 451.507 mg/L, with a p-value = 8.48×10^{-7}; slope = -18.58 mg/L, p-value = 0.0043; $R^2 = 0.53$. The ultimate LC_{50} was computed using the model equation, and was calculated as 191.37 mg/L.

Table 2. 2. Calculated LC_{50} (in mg/L) of *Proasellus lusitanicus* in 14 days for diclofenac sodium (LC_{50} – Lethal Concentration).

Days	**LC_{50} (mg/L)**	**Error**
1	293.93	78.65
2-3	403.69	184.70
4-5	493.30	310.41
6	354.48	100.84
7	279.55	46.67
8	263.12	42.90
11	225.19	24.70
12-13	207.61	18.61
14	194.61	15.12

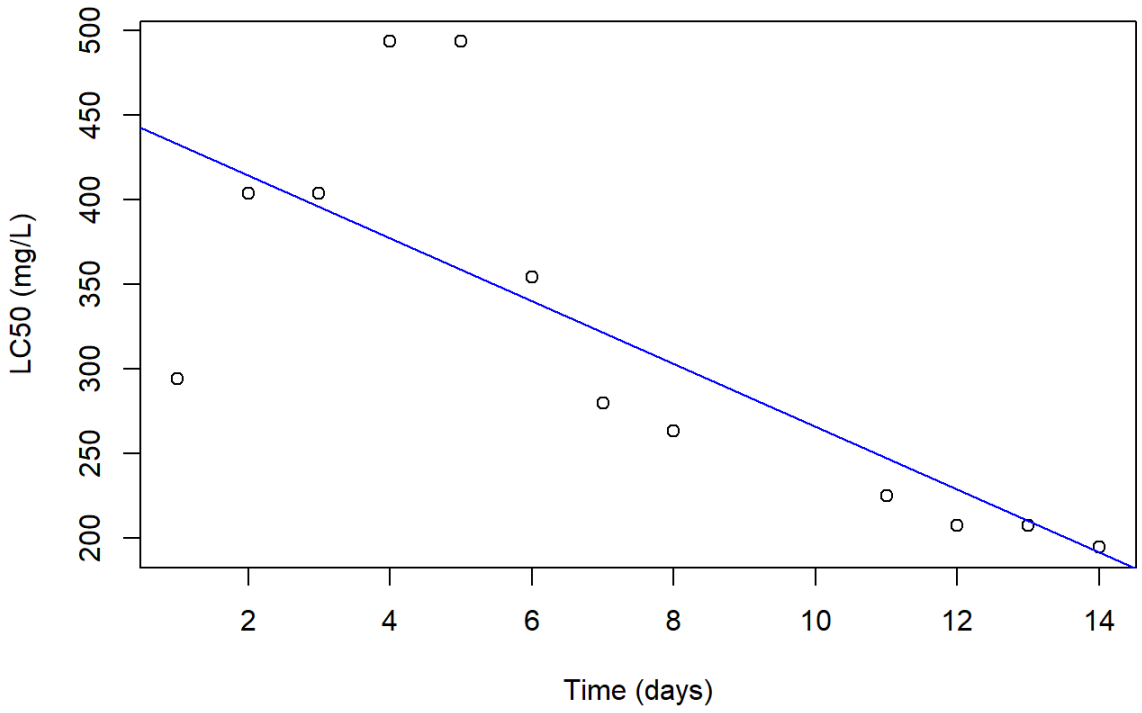


Figure 2. 3. LC50 throughout 14 days for *Proasellus lusitanicus* to diclofenac sodium.

To compute the AF2 of Equation 3, we produced an SSD curve for diclofenac sodium, shown in Figure 2. 4. The search in the U.S. EPA ECOTOX database resulted in seven records of L(E)C_{50}, one of which related to groundwater harpacticoid *Nitocrella achaiae* (Di Lorenzo et al., 2021a) (view Appendix 7.2). Except for *Chlamydomonas reinhard*, P.A. Dangeard, 1888, an algae, *P. lusitanicus* is the most resilient

to diclofenac sodium. According to the guidelines (ECHA (European Chemicals Agency), 2017), this means that the AF_2 to be used must be equal to 1000.

Thus, in scenarios 3 and 4 the considered $PNEC_{gw}$ is 124500 ng/L, obtained by dividing the NOEC (= 124.50 mg/L) of *P. lusitanicus* by the AF_2 of 1000.

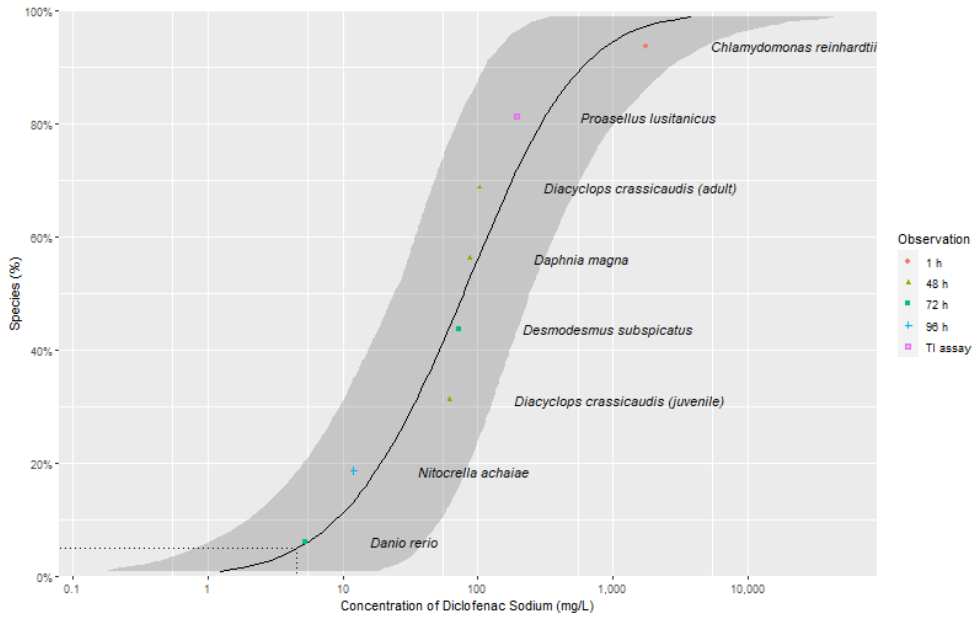


Figure 2. 4. Species Sensitivity Distribution (SSD) curve for aquatic crustaceous in response to diclofenac sodium (h – hours; TI assay – time independent assay).

3.2. Environmental Risk Assessment (ERA) Scenarios

3.2.1. Scenarios 1 and 2 ($PNEC_{gw}$ = 5 ng/L)

Only studies that quantified diclofenac sodium in European groundwaters were considered. From the available literature, papers aiming to study the physico-chemical properties of diclofenac (such as degradation, retention potential, adsorption and migration in the soil) were discarded, as were studies that focused on diclofenac's removal in wastewater treatment plants. A total of 11 papers were evaluated, in which the lowest MEC_{gw} available in the literature was 1.4 ng/L, in France (Rabiet et al., 2006a). The highest MEC_{gw} was 5300 ng/L, in Germany (Müller et al., 2012). More detailed data on the occurrence of diclofenac sodium is available in Appendix 7.3.

3.2.2. Scenarios 3 and 4 ($PNEC_{gw}$ = 124500 ng/L)

The last version of WATERBASE database contains information on contamination of groundwater from diclofenac sodium from four countries: Czech Republic, France, Italy, and Slovakia, from the year 2013 up to 2019. The highest MEC_{gw} was measured in Slovakia, in 2019, with a value of 0.2 µg/L. On the other hand, the lowest MEC_{gw} was measured in France, in 2014 with a value of 0.001 µg/L. In surface waters, the lowest MEC available in WATERBASE was detected in Croatia, in 2016, with a value of 0.00085 µg/L. The highest value was measured in Czech Republic, in 2018, with a value of 2240 µg/L. A total of 24 countries provided information about the presence of diclofenac sodium in surface waters, which include lake and river waters. Of the 3828 monitoring sites, 2256 were groundwater sites. In this study, only groundwater values were used.

Among the calculated scenarios, the highest risk is depicted in Scenario 1, with an RQ of 1060. This value was calculated with a MEC_{gw} of 5300 ng/L, in Germany, (Müller et al., 2012), and the PNEC of 5 ng/L. The least risk is depicted in Scenario 4, with a RQ of $8.03x10^{-6}$, where the MEC_{gw} was of 0.0001 µg/L, measured in France in 2014, and reported in WATERBASE, and the $PNEC_{gw}$ was the $PNEC_{gw}$ computed through the toxicity from *P. lusitanicus*. In Table 2. 3 is available the minimum and maximum values for each scenario calculated.

Table 2. 3. Maximum and minimum RQ calculated per scenario (RQ – Risk Quotient).

RQ Scenario	Minimum	Maximum
1	0.28	1060
2	0.2	40
3	$1.12 x10^{-5}$	0.043
4	$8.03x10^{-6}$	0.002

4. Discussion

Overall, the MECs reviewed in this study were significantly lower than the estimated NOEC (i.e. LC_{10}) and the measured LC_{50} of *Proasellus lusitanicus*. This asellid species was more resistant to diclofenac sodium than the stygobitic copepod *Nitocrella achaiae* (Di Lorenzo et al., 2021). Although the reason is unknown, it may be suspected that size is the main reason for this. Smaller-bodied organisms have a greater surface-to-volume ratio, which causes a higher uptake of the substances, and makes these smaller organisms to be more sensitive to toxicants (Taddei et al., 2021). The probabilistic species sensitivity distribution highlighted that *P. lusitanicus* is insensitive to diclofenac sodium. However, it is important to take into consideration that in the environment various pharmaceuticals may occur in the same aquifer simultaneously (Bartelt-Hunt et al., 2011; Bexfield et al., 2019), and that the effect of mixtures of pharmaceuticals on *P. lusitanicus*, or in any subterranean fauna, is poorly known (e.g., (Di Lorenzo et al., 2014)). Furthermore, this test was performed using adults and the effect of diclofenac sodium in juveniles may vary from this predicted one. In a study with the epigean cyclopoid *Diacyclops crassicaudis crassicaudis* (Castaño-Sánchez et al., 2021), juvenile copepods had a lower LC_{50} of 61.9 mg/L, when adult copepods LC_{50} value was determined as 103.3 mg/L. Calcium is used by crustaceans to mineralise the new cuticle, and the calcium accumulation pathway may lead to the accidental uptake of contaminants. Due to the higher rate of moulting and growth in the earlier stages of life, juveniles tend to be more sensible to toxicants (Taddei et al., 2021).

The calculated scenarios suggest that the EU guidance may be considered the most conservative for the protection of groundwater ecosystems, with Scenario 1 obtaining the highest values of RQ (view appendix 7.4, Table 2.A. 4. Risk Quotient for Scenario 1 (MECgw – Measured Environmental Concentration in groundwater bodies; PNEC – Predicted No-Effect Concentration; RQ – Risk Quotient).). In this scenario both PNEC and MECgw used to calculate the RQ were obtained via literature search. RQ values for Scenarios 2 and 3 are available in appendix 7.4, Table 2.A. 5 and Table 2.A. 6, respectively. The least conservative scenario is Scenario 4, which uses MECgw obtained via the WATERBASE database, and the PNEC obtained through the exposure of *P. lusitanicus* to diclofenac sodium (view appendix 7.4, Table 2.A. *7*). We can suspect that the groundwater asellid *P. lusitanicus* does not seem to be the appropriate bio-indicator for groundwater quality.

In 2017, the EMA tasked an expert group to derive guidelines for assessing the ERA of veterinary pharmaceutical compounds in groundwater (European Medicines Agency, 2018) and a guideline, still in draft, on the ERA of medicinal products for human use (*Environmental Risk Assessment of Medicinal Products for Human Use*, 2018). Both guidelines have been under debate (Di Lorenzo et al., 2019b; Di Lorenzo et al., 2021), namely due to its recommendation of estimating the environmental risk of pharmaceutical compounds in groundwaters using the sensitivity, either LC_{10} or NOEC, of surface water species as a proxy of stygofauna sensitivity, by using an AF equal to 10, applied to account for uncertainties in extrapolating from species with morphological, physiological and behavioural traits different from their groundwater counterparts. For example, the lifecycle of stygobitic species is typically longer, and the metabolic rates are significantly lower than those of surface water relatives (Di Lorenzo et al., 2015; Simčič et al., 2005). This leads to stygobitic organisms having longer exposures to substances than their surface relatives. However, lower metabolic rate does not necessarily make groundwater organisms more vulnerable than surface water ones (Avramov et al., 2013). Low metabolism might both delay the onset of the detoxification mechanism and delay the uptake of toxicants (Avramov et al., 2013). A further critical aspect concerning the ERA guidelines is the recommendation of using three model taxa covering three trophic levels when estimating the PNECgw: algae, crustaceans, and fish. In surface water ecosystems, these three trophic levels are well represented; however, they are not good representatives of the groundwater ones, with food chains being typically truncated. The absence of light prevents photosynthesis, and with the exception of chemolithoautotrophic production, primary production does not occur (Ravn et al., 2020). This means that these ecosystems depend on the transportation of organic matter from the surface (Culver & Pipan, 2019; Ravn et al., 2020). Bacteria and fungi have a poorly understood role in these ecosystems, although they seem to be responsible for transforming organic matter into biofilm, being able to support entire food webs (Venarsky & Huntsman, 2018). In these ecosystems, crustaceans have the highest species richness and population abundance (Malard et al., 2009; Stoch & Galassi, 2010). While acknowledging that the chronic lack of ecotoxicological data concerning stygobitic species prevents the use of methodologies alternative to the ones indicated in the EMA guidelines, the new data about the sensitivity of *P. lusitanicus* to diclofenac sodium presented here provides a degree of context for further considerations about the ERA of pharmaceutical compounds in groundwater. The EMA guidelines for the risk assessment in groundwater could further improve by considering bacteria instead of algae as primary producers and non-parthenogenetic crustaceans instead of *Daphnia* as primary consumers.

The NSAIA diclofenac sodium has been detected in groundwaters around Europe, with values often around the ng/L and the µg/L. According to the WATERBASE database, values vary from 0.001 µg/L, in France, to 0.2 µg/L, in Slovakia (*Waterbase - Water Quality ICM*, 2022), and according to available literature, from 1.4 ng/L, in France (Rabiet et al., 2006), to 5300 ng/L, in Germany (Müller et al., 2012). The extent of monitoring is highly variable among different countries. In the WATERBASE database, the first year with available information on the amounts of diclofenac sodium present in groundwater was in 2013, with a single collection site in France. In 2019, more countries added information to this database, with Italy adding 6 collection sites, Slovakia adding 25 sample sites, France having 329 sample sites, and Czech Republic adding 635 collection sites (*Waterbase - Water Quality ICM*, 2022). The dataset of MECs of diclofenac reviewed in this study provided a reasonable scenario of diclofenac contamination in European groundwater bodies. However, regulatory monitoring programmes are routinely targeted toward the most potentially problematic sites, and this may prevent depicting an entirely realistic scenario. In addition, higher concentrations which could be encountered locally, where there are specific emission sources, such as hospitals, could have been missed. Data on concentrations of diclofenac in European groundwaters suggest that there are potential risks to aquatic receptors from

surface water. It would therefore be prudent to monitor diclofenac sodium concentrations in those groundwater bodies (mainly alluvial aquifers) known to be recharged by stream waters with high concentrations of diclofenac sodium from WWTPs.

5. Final Remarks

Diclofenac sodium has been detected in groundwater, which is inhabited by stygobitic species different to surface species and that are suspected to be affected differently to pharmaceuticals. In this study, we set out to determine the LC_{50} of stygobitic species *Proasellus lusitanicus* to diclofenac sodium, as well as determine if the current guidelines for ERA are adequate. TI assays were used to account for the slower metabolism of subterranean organisms, and an LC_{50} of 191.37 mg/L was determined. As per our results, current ERA guidelines are adequate.

This study does not take into consideration sub-lethal effects of pharmaceuticals, which can limit organisms' fitness and population's survival. Lack of knowledge of stygobites' life cycle is a barrier to the studying of pharmaceuticals' effect on fertility. However, analyses of stress and defence biomarkers of this species after exposure to pharmaceuticals is an option. Furthermore, studying the effect of pharmaceuticals mixtures should also be considered, as in the environment, they are rarely present alone.

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7. Appendix

7.1. Appendix 1

Table 2.A. 1 Water properties from groundwater of specimen and groundwater collection site, and commercial water.

Properties	Olho d'Água (specimen collection site)	Alviela spring (groundwater collection site)	Commercial water
Temperature (ºC)	15.40	18	NA
pH	5.15	5.4	6.40
Dissolved oxygen (%)	94.5	79	101
Dissolved oxygen (mg/L)	9.36	7.49	9.14
Electric conductivity (ms/cm)	400	609	92

7.2. Appendix 2

Table 2.A. 2. Ecotoxicity values for diclofenac sodium (data compiled through ECOTOX database) (EC_{50} – Effective Concentration; LC_{50}- Lethal Concentration).

Species scientific name	Species group	End-point	Concentration (mg/L)	Timepoint (hours)	DOI
Daphnia magna	Crustaceans	EC_{50}	68	48	https://doi.org/10.1016/S0378-4274(03)00068-7
Daphnia magna	Crustaceans	EC_{50}	22.43	48	10.1016/s0147-6513(02)00082-9
Daphnia magna	Crustaceans	EC_{50}	68	48	10.1016/S0147-6513(03)00141-6
Danio rerio	Fish	EC_{50}	5.3	72	10.1016/j.ecoenv.2010.08.031
Danio rerio	Fish	LC_{50}	7.8	72	10.1016/j.ecoenv.2010.08.031
Dugesia japonica	Planarian	LC_{50}	6.8	24	https://doi.org/10.1080/02772248.2013.857671

Dugesia japonica	Planarian	LC_{50}	4.7	72	https://doi.org/10.1080/02772248.2013.857671
Dugesia japonica	Planarian	LC_{50}	5.3	48	https://doi.org/10.1080/02772248.2013.857671
Dugesia japonica	Planarian	LC_{50}	4.2	96	https://doi.org/10.1080/02772248.2013.857671

7.3. Appendix 3

Table 2.A. 3. Measured environmental concentrations (MEC) in groundwater (EU- European Union).

Reference	EU Member State	MEC_{GW} (ng/L)
Sathishkumar *et al*., 2020	Europe	24
Sathishkumar *et al*., 2020	France	9.7
Sathishkumar *et al*., 2020	Germany	590
Sathishkumar *et al*., 2020	Germany	15.4
Sathishkumar *et al*., 2020	Germany	45
Sathishkumar *et al*., 2020	Luxembourg	11
Sathishkumar *et al*., 2020	Poland	2.77
Sathishkumar *et al*., 2020	Serbia	18
Sathishkumar *et al*., 2020	Spain	477
Sathishkumar *et al*., 2020	Spain	380
Candela *et al*., 2016	Spain	74
Jurado *et al*., 2019	Spain	225.2
Jurado *et al*., 2019	Spain	49
Jurado *et al*., 2019	Germany	3050
Jurado *et al*., 2019	Germany	15.5
Jurado *et al*., 2019	France	24
Kapelewska *et al*., 2018	Poland	2100
Kapelewska *et al*., 2018	Poland	108.34
Rozman *et al*., 2015	Czech Republic	26.3
Rozman *et al*., 2015	Czech Republic	13.1
Rozman *et al*., 2015	Czech Republic	30
Banzhaf *et al*., 2013	Luxembourg	3
Muller *et al*., 2012	Germany	3050
Muller *et al*., 2012	Germany	499
Muller *et al*., 2012	Germany	216
Muller *et al*., 2012	Germany	1700
Muller *et al*., 2012	Germany	214

Muller *et al*., 2012	Germany	325
Muller *et al*., 2012	Germany	88
Muller *et al*., 2012	Germany	89
Rabiet *et al*. 2006	France	2.5
Einsiedl *et al*. 2010	Germany	15
Einsiedl *et al*. 2010	Germany	10
Einsiedl *et al*. 2010	Germany	6
Einsiedl *et al*. 2010	Germany	4
Lonappan *et al*. 2016	Mediterranean region	2
Lonappan *et al*. 2016	France	0.9
Lonappan *et al*. 2016	Spain	1.7
Lonappan *et al*. 2016	Spain	3.1
Heberer *et al*., 1998	Germany	380

7.4. Appendix 4

Table 2.A. 4. Risk Quotient for Scenario 1 (MECgw – Measured Environmental Concentration in groundwater bodies; PNEC – Predicted No-Effect Concentration; RQ – Risk Quotient).

Scenario 1			
Country	MECgw (ng/L)	PNEC (litera-ture;ng/L)	RQ
Europe	24	5	4.8
France	2.5	5	0.5
France	9.7	5	1.94
Germany	590	5	118
Germany	15.4	5	3.08
Germany	45	5	9
Luxembourg	11	5	2.2
Poland	2.77	5	0.554
Serbia	18	5	3.6
Spain	477	5	95.4
Spain	380	5	76
Spain	74	5	14.8
Spain	225.2	5	45.04
Spain	49	5	9.8
Spain	256	5	51.2
Germany	3050	5	610
Germany	15.5	5	3.1
France	24	5	4.8
Poland	2100	5	420
Poland	108.34	5	21.668
Czech Republic	26.3	5	5.26
Czech Republic	13.1	5	2.62
Czech Republic	30	5	6

Luxembourg	3	5	0.6
Germany	5300	5	1060
Germany	499	5	99.8
Germany	216	5	43.2
Germany	1700	5	340
Germany	214	5	42.8
Germany	325	5	65
Germany	88	5	17.6
Germany	89	5	17.8
Germany	15	5	3
Germany	10	5	2
Germany	6	5	1.2
Germany	4	5	0.8
Germany	380	5	76
Mediterranean region	2	5	0.4
Spain	1.7	5	0.34
Spain	3.1	5	0.62
France	2.1	5	0.42
France	2.4	5	0.48
France	2.3	5	0.46
France	1.4	5	0.28
France	2.5	5	0.5
France	2.1	5	0.42

Table 2.A. 5. Risk Quotient for Scenario 2 (MECgw – Measured Environmental Concentration in groundwater bodies; PNEC – Predicted No-Effect Concentration; RQ – Risk Quotient).

Scenario 2				
Country	Year	MECgw (ug/L)	PNEC (literature; in ug/L)	RQ
France	2013	0.02	0.005	4
France	2014	0.02	0.005	4
France	2014	0.001	0.005	0.2
France	2017	0.018	0.005	3.6
France	2017	0.02	0.005	4
France	2017	0.01	0.005	2
France	2017	0.011	0.005	2.2
France	2017	0.03	0.005	6
France	2017	0.033	0.005	6.6
France	2017	0.013	0.005	2.6
France	2017	0.012	0.005	2.4
France	2017	0.014	0.005	2.8
France	2017	0.016	0.005	3.2
France	2017	0.04	0.005	8
France	2017	0.063	0.005	12.6
France	2017	0.019	0.005	3.8

France	2016	0.02	0.005	4
France	2017	0.023	0.005	4.6
France	2017	0.05	0.005	10
France	2017	0.113	0.005	22.6
France	2017	0.038	0.005	7.6
France	2017	0.039	0.005	7.8
France	2017	0.024	0.005	4.8
Czech Republic	2018	0.04	0.005	8
Czech Republic	2018	0.01	0.005	2
Czech Republic	2018	0.03	0.005	6
Czech Republic	2018	0.027	0.005	5.4
Czech Republic	2018	0.018	0.005	3.6
Czech Republic	2018	0.035	0.005	7
Czech Republic	2018	0.02	0.005	4
Czech Republic	2018	0.014	0.005	2.8
Czech Republic	2018	0.05	0.005	10
Czech Republic	2018	0.029	0.005	5.8
Czech Republic	2018	0.073	0.005	14.6
Czech Republic	2018	0.026	0.005	5.2
Czech Republic	2018	0.028	0.005	5.6
Czech Republic	2018	0.015	0.005	3
Czech Republic	2018	0.08	0.005	16
Czech Republic	2018	0.086	0.005	17.2
Czech Republic	2018	0.011	0.005	2.2
Czech Republic	2018	0.023	0.005	4.6
Czech Republic	2018	0.174	0.005	34.8
Czech Republic	2018	0.06	0.005	12
Czech Republic	2018	0.017	0.005	3.4
Czech Republic	2018	0.055	0.005	11
Czech Republic	2018	0.034	0.005	6.8
France	2019	0.02	0.005	4
France	2019	0.01	0.005	2
France	2018	0.02	0.005	4
France	2018	0.161	0.005	32.2
France	2018	0.01	0.005	2
France	2018	0.011	0.005	2.2
France	2018	0.044	0.005	8.8
France	2018	0.014	0.005	2.8
France	2018	0.017	0.005	3.4
France	2018	0.026	0.005	5.2
France	2018	0.032	0.005	6.4
France	2018	0.025	0.005	5
France	2018	0.105	0.005	21
France	2018	0.048	0.005	9.6
France	2018	0.013	0.005	2.6

France	2018	0.012	0.005	2.4
France	2018	0.033	0.005	6.6
France	2018	0.022	0.005	4.4
France	2018	0.021	0.005	4.2
France	2018	0.015	0.005	3
Slovakia	2019	0.04	0.005	8
Slovakia	2019	0.2	0.005	40
France	2019	0.039	0.005	7.8
France	219	0.015	0.005	3
France	2019	0.007	0.005	1.4
France	2019	0.018	0.005	3.6
Czech Republic	2019	0.04	0.005	8
Czech Republic	2019	0.01	0.005	2
Czech Republic	2019	0.037	0.005	7.4
Czech Republic	2019	0.015	0.005	3
Czech Republic	2019	0.013	0.005	2.6
Czech Republic	2019	0.143	0.005	28.6
Czech Republic	2018	0.026	0.005	5.2
Czech Republic	2019	0.041	0.005	8.2
Czech Republic	2019	0.014	0.005	2.8
Czech Republic	2019	0.03	0.005	6
Czech Republic	2019	0.112	0.005	22.4
Czech Republic	2019	0.11	0.005	22
Czech Republic	2019	0.012	0.005	2.4
Czech Republic	2019	0.021	0.005	4.2
Czech Republic	2019	0.019	0.005	3.8
Czech Republic	2019	0.024	0.005	4.8
Italy	2019	0.005	0.005	1

Table 2.A. 6. Risk Quotient for Scenario 3 (MECgw – Measured Environmental Concentration in groundwater bodies; PNEC – Predicted No-Effect Concentration; RQ – Risk Quotient).

	Scenario 3		
Country	MECgw (ng/L)	PNEC (*Proasellus lusitanicus*)	RQ
Europe	24	124500	0.000193
France	2.5	124500	2.01E-05
France	9.7	124500	7.79E-05
Germany	590	124500	0.004739
Germany	15.4	124500	0.000124
Germany	45	124500	0.000361
Luxembourg	11	124500	8.84E-05
Poland	2.77	124500	2.22E-05
Serbia	18	124500	0.000145
Spain	477	124500	0.003831
Spain	380	124500	0.003052

Spain	74	124500	0.000594
Spain	225.2	124500	0.001809
Spain	49	124500	0.000394
Spain	256	124500	0.002056
Germany	3050	124500	0.024498
Germany	15.5	124500	0.000124
France	24	124500	0.000193
Poland	2100	124500	0.016867
Poland	108.34	124500	0.00087
Czech Republic	26.3	124500	0.000211
Czech Republic	13.1	124500	0.000105
Czech Republic	30	124500	0.000241
Luxembourg	3	124500	2.41E-05
Germany	5300	124500	0.04257
Germany	499	124500	0.004008
Germany	216	124500	0.001735
Germany	1700	124500	0.013655
Germany	214	124500	0.001719
Germany	325	124500	0.00261
Germany	88	124500	0.000707
Germany	89	124500	0.000715
Germany	15	124500	0.00012
Germany	10	124500	8.03E-05
Germany	6	124500	4.82E-05
Germany	4	124500	3.21E-05
Germany	380	124500	0.003052
Mediterranean region	2	124500	1.61E-05
Spain	1.7	124500	1.37E-05
Spain	3.1	124500	2.49E-05
France	2.1	124500	1.69E-05
France	2.4	124500	1.93E-05
France	2.3	124500	1.85E-05
France	1.4	124500	1.12E-05
France	2.5	124500	2.01E-05
France	2.1	124500	1.69E-05

Table 2.A. 7. Risk Quotient for Scenario 4 (MECgw – Measured Environmental Concentration in groundwater bodies; PNEC – Predicted No-Effect Concentration; RQ – Risk Quotient).

Scenario 4				
Country	Year	MECgw (ug/L)	PNEC (*Proasellus lusitanicus;* ug/L)	RQ
France	2013	0.02	124.5	0.000160643
France	2014	0.02	124.5	0.000160643
France	2014	0.001	124.5	8.03213E-06

France	2017	0.018	124.5	0.000144578
France	2017	0.02	124.5	0.000160643
France	2017	0.01	124.5	8.03213E-05
France	2017	0.011	124.5	8.83534E-05
France	2017	0.03	124.5	0.000240964
France	2017	0.033	124.5	0.00026506
France	2017	0.013	124.5	0.000104418
France	2017	0.012	124.5	9.63855E-05
France	2017	0.014	124.5	0.00011245
France	2017	0.016	124.5	0.000128514
France	2017	0.04	124.5	0.000321285
France	2017	0.063	124.5	0.000506024
France	2017	0.019	124.5	0.00015261
France	2016	0.02	124.5	0.000160643
France	2017	0.023	124.5	0.000184739
France	2017	0.05	124.5	0.000401606
France	2017	0.113	124.5	0.000907631
France	2017	0.038	124.5	0.000305221
France	2017	0.039	124.5	0.000313253
France	2017	0.024	124.5	0.000192771
Czech Republic	2018	0.04	124.5	0.000321285
Czech Republic	2018	0.01	124.5	8.03213E-05
Czech Republic	2018	0.03	124.5	0.000240964
Czech Republic	2018	0.027	124.5	0.000216867
Czech Republic	2018	0.018	124.5	0.000144578
Czech Republic	2018	0.035	124.5	0.000281124
Czech Republic	2018	0.02	124.5	0.000160643
Czech Republic	2018	0.014	124.5	0.00011245
Czech Republic	2018	0.05	124.5	0.000401606
Czech Republic	2018	0.029	124.5	0.000232932
Czech Republic	2018	0.073	124.5	0.000586345
Czech Republic	2018	0.026	124.5	0.000208835
Czech Republic	2018	0.028	124.5	0.0002249
Czech Republic	2018	0.015	124.5	0.000120482
Czech Republic	2018	0.08	124.5	0.00064257
Czech Republic	2018	0.086	124.5	0.000690763
Czech Republic	2018	0.011	124.5	8.83534E-05
Czech Republic	2018	0.023	124.5	0.000184739
Czech Republic	2018	0.174	124.5	0.00139759
Czech Republic	2018	0.06	124.5	0.000481928
Czech Republic	2018	0.017	124.5	0.000136546
Czech Republic	2018	0.055	124.5	0.000441767

Czech Republic	2018	0.034	124.5	0.000273092
France	2019	0.02	124.5	0.000160643
France	2019	0.01	124.5	8.03213E-05
France	2018	0.02	124.5	0.000160643
France	2018	0.161	124.5	0.001293173
France	2018	0.01	124.5	8.03213E-05
France	2018	0.011	124.5	8.83534E-05
France	2018	0.044	124.5	0.000353414
France	2018	0.014	124.5	0.00011245
France	2018	0.017	124.5	0.000136546
France	2018	0.026	124.5	0.000208835
France	2018	0.032	124.5	0.000257028
France	2018	0.025	124.5	0.000200803
France	2018	0.105	124.5	0.000843373
France	2018	0.048	124.5	0.000385542
France	2018	0.013	124.5	0.000104418
France	2018	0.012	124.5	9.63855E-05
France	2018	0.033	124.5	0.00026506
France	2018	0.022	124.5	0.000176707
France	2018	0.021	124.5	0.000168675
France	2018	0.015	124.5	0.000120482
Slovakia	2019	0.04	124.5	0.000321285
Slovakia	2019	0.2	124.5	0.001606426
France	2019	0.039	124.5	0.000313253
France	219	0.015	124.5	0.000120482
France	2019	0.007	124.5	5.62249E-05
France	2019	0.018	124.5	0.000144578
Czech Republic	2019	0.04	124.5	0.000321285
Czech Republic	2019	0.01	124.5	8.03213E-05
Czech Republic	2019	0.037	124.5	0.000297189
Czech Republic	2019	0.015	124.5	0.000120482
Czech Republic	2019	0.013	124.5	0.000104418
Czech Republic	2019	0.143	124.5	0.001148594
Czech Republic	2018	0.026	124.5	0.000208835
Czech Republic	2019	0.041	124.5	0.000329317
Czech Republic	2019	0.014	124.5	0.00011245
Czech Republic	2019	0.03	124.5	0.000240964
Czech Republic	2019	0.112	124.5	0.000899598
Czech Republic	2019	0.11	124.5	0.000883534
Czech Republic	2019	0.012	124.5	9.63855E-05
Czech Republic	2019	0.021	124.5	0.000168675
Czech Republic	2019	0.019	124.5	0.00015261

Czech Republic	2019	0.024	124.5	0.000192771
Italy	2019	0.005	124.5	4.01606E-05

Chapter III – Acetaminophen induced antioxidant and detoxification responses in a stygobitic crustacean

Figure 3. 1 Specimens' collection in Olho de Mira cave, located at Serra de Aire e Candeeiros Natural Park. Credits: Ana Sofia P.S. Reboleira.

Paper submitted:

Duarte, C., Gravato, C., Di Lorenzo, T. & Reboleira, A.S.P.S. Acetaminophen induced antioxidant and detoxification responses in a stygobitic crustacean.

Graphical abstract

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 response of stygobitic species to VHMPs, as it is likely to be different from surface species' response. 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 increase in total glutathione levels and glutathione *S*-transferase activity, indicating a successful detoxification response. This helps explaining why acetaminophen did not cause oxidative damage, as well as did not affect cholinesterase activity nor the aerobic production of energy. This study shows the remarkable capacity of *P. lusitanicus* to tolerate sublethal concentrations of VHMP acetaminophen. A few ecotoxicological studies focused on lethal effects of these compounds, however damage occurs at sublethal concentrations and future studies should assess the stress levels induced to better predict and estimate impacts of contaminants on groundwater ecosystems.

1. Introduction

Groundwater consists of 94-97% of all the liquid freshwater globally available (Griebler & Avramov, 2015; Castaño-Sánchez et al., 2020b), and is the major source of drinking water for many European Union (EU) citizens (European Environment Agency, 2018). In fact, 50% of European drinking water is obtained from groundwater, and many large cities depend on it (European Environment Agency., 2018), and 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. Due to the isolation and fragmentation that are part of these ecosystem traits, species that inhabit these ecosystems tend to have short-range distributions and a high degree of endemism (Hose et al., 2022). The so-called stygofauna have adaptations to life in groundwater, which include dramatic changes in morpho-physiological traits (Hose et al., 2022). These species are characterized by low metabolic rate, low fertility and slow population growth (Mammola et al., 2019), and as a result, they are at higher risk of extinction or significant population reduction, if submitted to habitat degradation, and catastrophic and/or stochastic events (Mammola et al., 2019). Therefore, it is vital to ensure groundwater is in a good quality status.

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

From these anthropogenic disturbances, agriculture is not only 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 to fertilise the soils. VHMPs are 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 risk one of the most enigmatic ecosystems are domestic and hospital waste (Mammola et al., 2019; Castaño-Sánchez et al., 2020a). Both of these may reach groundwater through percolation and leaching, transporting within it VHMPs (Verlicchi et al., 2012; Paíga et al., 2016).

VHMPs are responsible for negative side effects on non-target aquatic organisms, with lethality being one of them. Many studies have focused on the determination of LC_{50} and EC_{50} values for VHMPs in aquatic organisms (Di Lorenzo et al., 2019, 2021; Castaño-Sánchez, Pereira, et al., 2021). There have been other studies focusing on more subtle effects, such as morphological alterations (Nogueira et al., 2019), DNA methylation (Nogueira et al., 2019), as well as swimming behaviour alteration (Nogueira et al., 2019; Di Cicco et al., 2021). This, it is suspected that stygobites are affected by these VHMPs as well, with mortality being the biggest concern. However, sublethal effects mustn't be forgotten, as they can limit individual's fitness and population numbers.

Acetaminophen, also known as paracetamol, is one of the most popular prescribed and self-medicating drugs (Wu et al., 2012). Acetaminophen's removal has been studied, by means of ozonation, H_2O/UV oxidation (Andreozzi et al., 2003), electrochemical (Brillas et al., 2005) and photocatalytic methods (Yang et al., 2008). However, acetaminophen and its metabolites remain to be continuously released into the aquatic environment via domestic wastewater and hospital effluents, where, due to its hydrophilicity and high solubility, it may accumulate (Wu et al., 2012). It comes as no surprise that this VHMP has been found in many water bodies, from surface waters to groundwaters, in the order of ng/L or µg/L (Rabiet et al., 2006; Nödler et al., 2014; Paíga & Delerue-Matos, 2016). In surface water, acetaminophen was found in concentrations as high as 527 ng/L in Lis River, Portugal (Paíga et al., 2016). Both

wastewater treatment plants (WWTP) and livestock production were pointed out as being the most probable culprit of the contamination due to the proximity of both facilities. In French groundwaters, a study found a maximum concentration of 481 ng/L, being in the top five of the most detected pharmaceuticals in groundwaters across all seasons (Lopez et al., 2015).

This study aims to determine the sublethal effects of acetaminophen in the stygobitic species *Proasellus lusitanicus* (Frade, 1938). This asellid stygobitic crustacean was exposed to environmentally relevant concentrations of acetaminophen for 14 days and the following 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 species *Proasellus lusitanicus* were collected in Olhos d'Água Cave (39°32'28.4"N 8°43'20.0"W, Figure 3. 1) in the Estremenho karst massif, Portugal, in May 2022. The species is endemic to Portugal, inhabiting caves from that massif (Magniez 1967) where the average temperature is 17ºC (Reboleira et al., 2011). This species has been previously used in lethal ecotoxicity testing (Reboleira et al., 2013; Castaño-Sánchez et al., 2021). Temperature, pH, dissolved oxygen (DO), and electrical conductivity were measured by using a portable multiparameter probe AQUAREAD – WTW MULTI 3430, at the collection site (Table 3.A. 1, Appendix 7.1)). 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, 17ºC, to undergo acclimation. 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, Malard, et al., 2021). No artificial food was supplied.

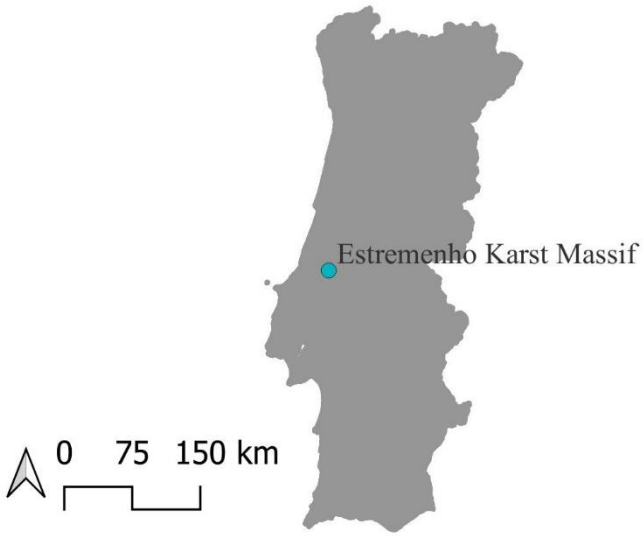

Figure 3. 2. Sampling site (map rendered with QGIS 3.24.3).

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 solution was 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. The solution was renovated every seven days, in order to avoid degradation of the test solution. at this point.

At the end of the 14-day exposure, each specimen was individually placed in 2 mL microtubes, frozen in liquid nitrogen and weighted 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 excess of glutathione reductase was read at 412 nm for a period of 3 minutes, with light agitation between readings. A standard curve (concentrations of 10000 µM, 100 µM, 10 µM, 1 µM and 0.1 µM) was used as was the molar extinction coefficient of $14{,}1x10^3\ M^{-1}cm^{-1}$ in order to determine samples' concentration (Rodrigues et al., 2017).

Determination of GST activity followed an adapted protocol by (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 a period of 5 minutes, at intervals of 20 seconds, with light agitation between readings. The molar extinction applied was of $9.6x10^3\ M^{-1}cm^{-1}$.

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 added to the samples. The molar extinction applied was of $1.56x10^5$ $M^{-1}cm^{-1}$.

ChE activity was assessed by adapting a protocol from Silva et al. (2021). Microplates were read for a period of 3 minutes, with 20 second intervals with light agitation, at an absorbance of 414 nm. The molar extinction applied was of $13.6x10^3$ $M^{-1}cm^{-1}$.

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

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

2.2.2. Energy related biomarkers

Quantification of ETS levels was performed by adapting the protocol of Rodrigues et al. (2022). Microplates were read for 6 minutes, with intervals of 30 seconds of light agitation, at an absorbance of 490 nm. 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 $1.59x10^4$ $M^{-1}cm^{-1}$. Oxygen consumption rate was calculated following the following proportion: for each µmol of oxygen consumed, 2 µmol of INT-formazan was formed.

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

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 $p < 0.05$.

3. Results

TG levels were significantly affected (p-value = 0.00127). Organisms exposed to 0.01 mg/L exhibited a significant higher level of TG than the ones exposed to 0.1 mg/L (p-value = 0.0029), 1 mg/L (p-value = 0.0205), and 10 mg/L (p-value = 0.0490; Figure 3. 3). No significant difference was detected between control group and any other group.

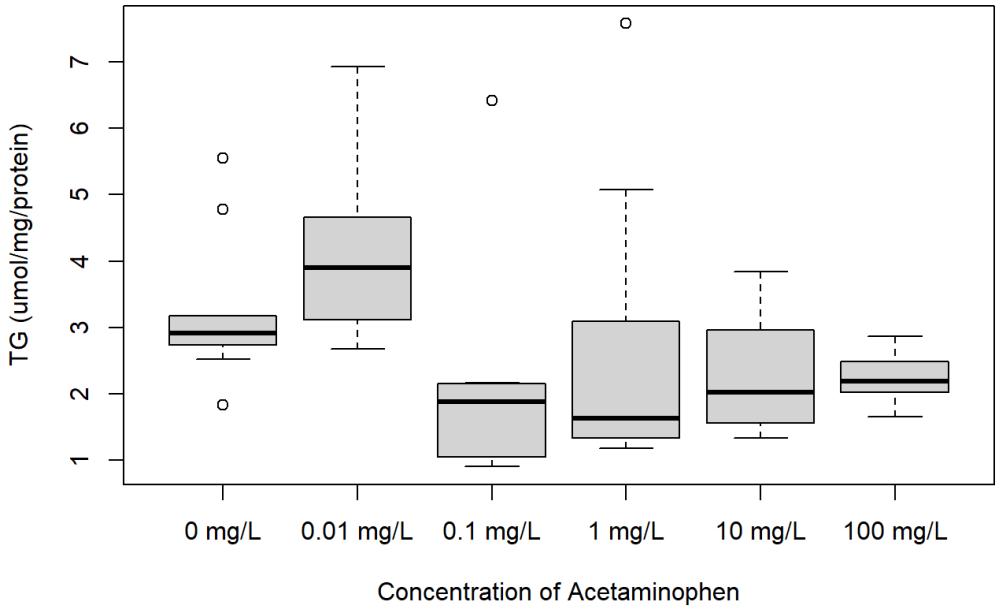

Figure 3. 3. Levels of total glutathione (TG) in *Proasellus lusitanicus* exposed to 0, 0.01, 0.1, 1, 10, and 100 mg/L of acetaminophen. Circles represent the outliers. Data did not show a normal distribution, as Shapiro-Wilk's test's p-value = 5.376x10-5. Presence of significant differences between 0.01 mg/L and all treatment groups except the control after exposure.

The activity of GST was also significantly affected (p-value = 0.0093) after exposure to acetaminophen (Figure 3. 4). Organisms that were exposed to 0.1 mg/L (p-value = 0.0126), 1 mg/L (p-value = 0.0079) and 10 mg/L (p-value = 0.0080) showed a significant increased activity of GST when compared to the control (Figure 3. 4).

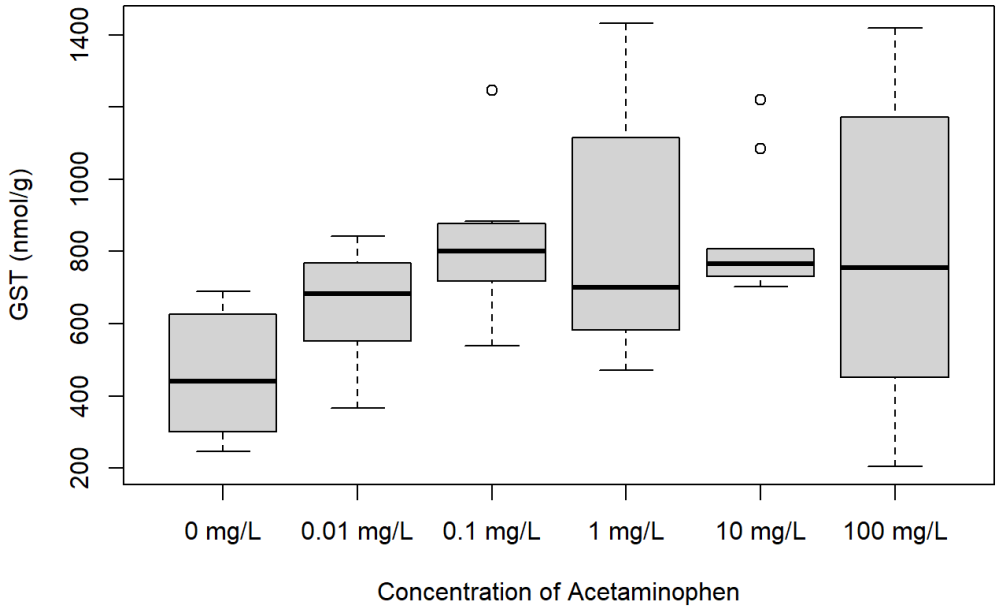

Figure 3. 4. Activity of glutathione S-transferase (GST) in *Proasellus lusitanicus* exposed to 0, 0.01, 0.1, 1, 10, and 100 mg/L of acetaminophen. Data showed a normal distribution, as Shapiro-Wilk's p-value = 0.05, and equal variances, as Bartlett's test p-value = 0.013. Presence of significant differences between control and groups exposed to 0.01, 0.1, 1, and 10 mg/L of acetaminophen.

Levels of LPO levels were not significantly affected on acetaminophen treatments if compared to control group (Figure 3. 5). 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.

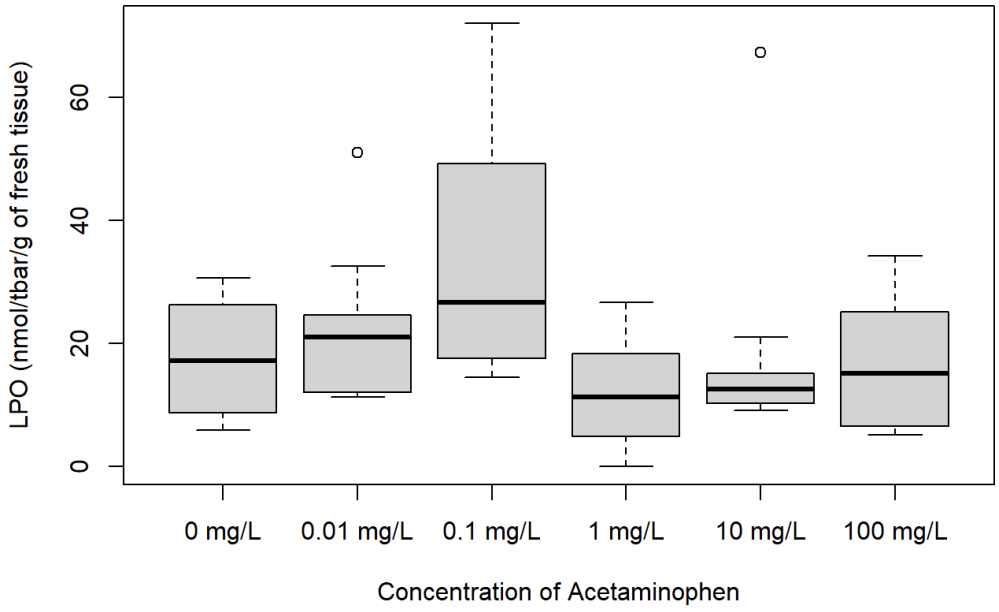

Figure 3. 5. Levels of Lipid Peroxidase (LPO) in *Proasellus lusitanicus* exposed to 0, 0.01, 0.1, 1, 10, and 100 mg/L of acetaminophen. Data did not show a normal distribution, as Shapiro-Wilk's test's p-value = 5.282x10-6. Significant differences present between 1 mg/L group and 0.1 mg/L group after exposure.

The ETS activity (p-value = 0.1114; Figure 3. 6), ChE activity (p-value = p-value = 0.1114; Figure 3. 7), and protein content (p-value = 0.3526; Figure 3. 8) were not significantly different in organisms exposed to all concentrations of acetaminophen and control group.

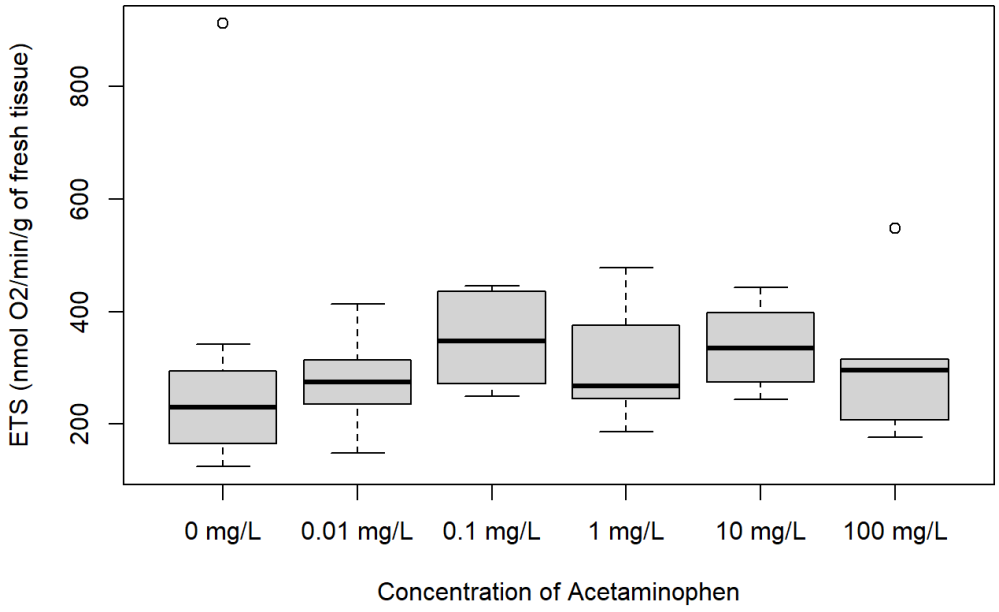

Figure 3. 6. Levels of electron transport system (ETS) in *Proasellus lusitanicus* exposed to 0, 0.01, 0.1, 1, 10, and 100 mg/L of acetaminophen. Data did not show normal distribution, as Shapiro-Wilk's test's p-value = 2.078x10-6. No presence of significant differences.

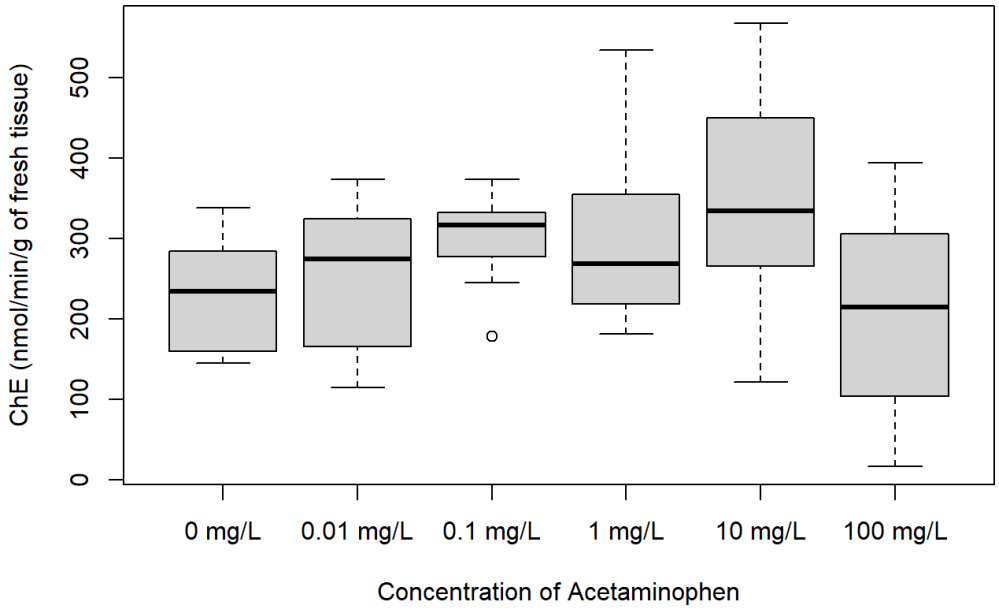

Figure 3. 7. Levels of cholinesterase (ChE) in *Proasellus lusitanicus* exposed to 0, 0.01, 0.1, 1, 10, and 100 mg/L of acetaminophen. Data did not show equal variances, as Bartlett's test's p-value = 0.071. No presence of significant differences.

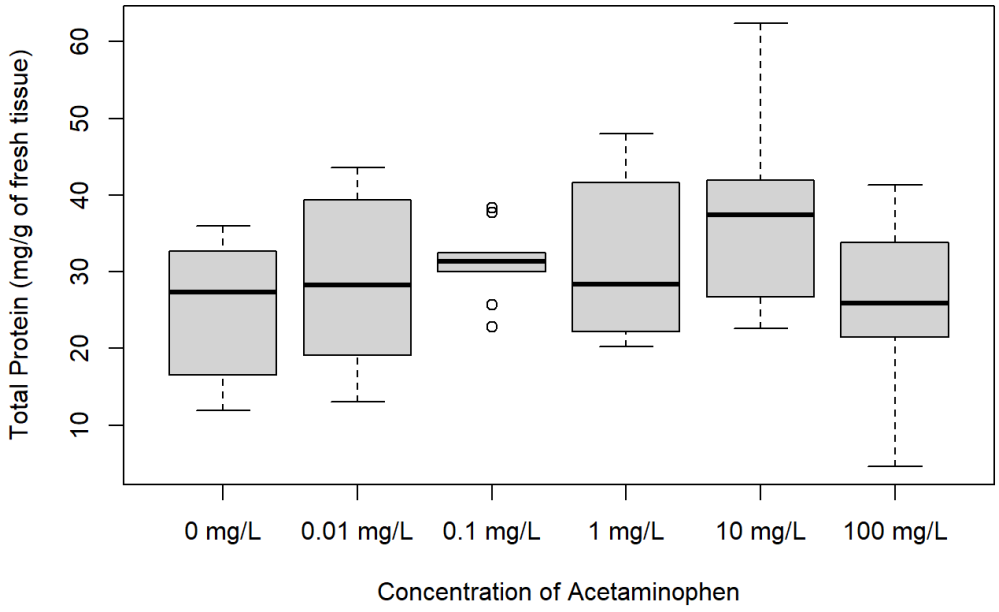

Figure 3. 8. Levels of total protein (PROT) in *Proasellus lusitanicus* exposed to 0, 0.01, 0.1, 1, 10, and 100 mg/L of acetaminophen. Data did not show equal variances, as Bartlett's test's p-value = 0.1241. No presence of significant differences.

4. Discussion

Biomarkers assessed in *Proasellus lusitanicus* showed increased levels of total glutathione (TG) on organisms exposed to 0.01 mg/L, and glutathione *S*-transferase (GST) activities on organisms exposed to low and intermediate concentrations of acetaminophen, ranging between 0.01 mg/L and 100 mg/L. Lipid peroxidase (LPO) levels were not significantly higher in organisms exposed to acetaminophen compared to control. These alterations suggest the activation of detoxification processes and antioxidants to cope with exposure to acetaminophen, which were partially successful in preventing further oxidative damage. In addition, all biomarkers analysed showed a dumb-bell shaped curve which suggests that higher concentration of acetaminophen seem to compromise cell integrity that would result in values in biomarkers somehow similar to control or even lower than control. In fact, at the concentration of 10 mg/L, mortality reached 10%, while at the concentration of 100 mg/L, mortality was as high as 40%.

GSTs are associated with metabolic detoxification processes, being one of the most relevant phase II enzymes (Gravato & Santos, 2002; Frova, 2006). In our study, GST activity was significantly increased for all the tested concentrations of acetaminophen in good agreement with previous results observed using the freshwater crustacean *Daphnia longispina*, when exposed to 20 µg/L (Sousa & Nunes, 2021) for 48 hours, and *Daphnia magna*, when exposed to concentrations ranging from 0.8 mg/L 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 µg/L and 40 µg/L (Sousa & Nunes, 2021), and 21-days acetaminophen exposure for concentrations ranging from 5 mg/L 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 control. This reduction of GST activity has been previously explained via glutathione reductase reduction (Daniel et al., 2019) and might also show the pattern of a dumb-bell shape curve that seems to be observed for all the biomarkers determined in the current study Furthermore, the levels of TG could 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 cells, being representative of 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-benzoquinoemine) accumulation. 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), but will also justify enzymatic inhibition that seems to be our case when all biomarkers show a dumb-bell response. 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 (Claude Amiard-Triquet, Jean-Claude Amiard et al., 2013).

Recently, there has been a growing number of studies regarding the sensitivity of stygobitic species to a number of contaminants, including VHPs (Reboleira et al., 2013; Di Lorenzo et al., 2019; Castaño-Sánchez et al., 2020b; Di Lorenzo et al., 2021). These studies usually focus on lethal endpoints, LC_{50}, or on effective concentrations, EC_{50}. Sensitivity of stygobitic species is not always superior or inferior to the one of surface species.

In this study, no significant changes were observed in ETS activity, findings similar to Lukančič et al. (2009), where there was not difference in ETS activity when *Asellus aquaticus* and *Gammarus fossarum* were exposed to atrazine. The same study found differences in ETS activity when both species were exposed to imidacloprid. Therefore, 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 and energy was sufficient to cope with 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 (Claude Amiard-Triquet, Jean-Claude Amiard et al., 2013) fuelled with energy that was not compromised at least for the concentrations tested. Moreover, the PROT was kept constant within organisms exposed to acetaminophen when compared to control, which might mean that cellular allocation of energy was not compromising synthesis despite the tendency to decrease in organisms exposed to the highest concentration.

Acetaminophen has no evident direct mechanism of neurotoxicity. In this present study, we did not observe significant changes in ChE activity, which is consistent with two previous work 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, the latter being 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. In Daniel et al. (2019), organisms were exposed both acutely and chronically, and the results of both period exposures yielded different results.

In our study the range varied between 10 ng/L and 100 mg/L which might mean that a broader range would facilitate non-monotonic responses of biomarkers as we obtained.

For this current study, it was only possible to perform one period of exposure, having been decided on a 14 day-exposure. This was due to only after this period of time was possible to determine a preliminary LC_{50} for this species. Unfortunately, a previous acetaminophen TI assay may not be considered valid as the amount of dissolved oxygen varied >20% from the value in the beginning of the TI assay. In the present study, such variation was not observed but led us to test a broader range of concentrations due to the uncertainty of the LC_{50} for acetaminophen. Despite all the constraints of using a stygobitic crustacean species, this research work allows us to determine a battery of biomarkers that could be useful to understand the ecotoxicity of several other classes of pollutants to this species.

5. Final remarks

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. It seems acetaminophen induced defence biomarkers related to detoxification that prevented oxidative damage. More studies are still needed and desirable in this area, in order to quantify biomarkers levels at different times of exposure (both shorter and longer), allowing to determine intraspecific fluctuations and comparison with other species.

Furthermore, it would be relevant to use this methodology with others VHMPs, as well as other pollutants. Sublethal effects should be analysed to truly assess VHMPs' effects, as death is an extreme endpoint, and other less extreme effects may also be compromising to an individual's survival. Thus, biochemical endpoints such as biomarker can be used as early warning tools of health effects at the organism level and higher levels of biological organization.

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7. Appendix

7.1. Appendix 1

Table 3.A. 1 Water properties from groundwater and specimen collection site.

Properties	Alviela spring
Temperature (ºC)	16.50
pH	5.27
Dissolved oxygen (%)	81.6
Dissolved oxygen (mg/L)	8.01
Electric conductivity (ms/cm)	489

8. Final Considerations

Groundwater ecosystems are habitats to peculiar species with unique traits. Although often forgotten, these ecosystems are exposed to veterinary and human medicinal products (VHMP), used to treat and prevent diseases in humans and in animals. After excretion, both the original active substance and metabolites are excreted, after which they reach groundwater through leachate. Once in groundwater, their effects on groundwater fauna are still vastly unknown, since most studies focus on their effects on surface species, extrapolating the effects on stygobitic species by using assessing factors.

Studying groundwater organisms has several obstacles, such as difficulty in their collection, laboratorial maintenance, and rearing. This means that getting organisms in enough numbers to be able to perform ecotoxicological assays that yield strong and reliable results is a major difficulty in subterranean ecosystems. One of the reasons why studies in this area are scarce.

In this dissertation, a time-independent (TI) assay was used to estimate the acute toxicity of the non-steroidal anti-inflammatory agent (NSAIA) on a stygobitic asellid crustacean, *Proasellus lusitanicus*. Results show that the lethal concentration (LC_{50}) of diclofenac sodium is estimated at 191.39 mg/L, with this value decreasing over time. From the four explored scenarios of Environmental Risk Assessment, the most appropriate for protecting groundwater ecosystems was Scenario 1, which is in accordance with the current guidelines indicated by the European Medicine Agency.

It was also performed a set of defence and stress biomarkers on *P. lusitanicus*, after a 14-day exposure to acetaminophen, a commonly used and groundwater pollutant VHMP. The results show a significant increase of total glutathione and glutathione *S*-transferase, indicating a successful detoxification mechanism. This explains the absence of oxidative stress and the absence of differences in aerobic production energy. This study shows that *P. lusitanicus* is remarkably tolerant to sublethal concentrations of acetaminophen.

More information on the effects of VHMPs on groundwater ecosystems will lead to an improvement of current guidelines and management. The lethal and sublethal effect of mixtures of VHMPs in stygobitic species are still vastly unknown, yet very relevant, as various VHMPs are found in the same aquifer simultaneously. Furthermore, it would be interesting to study stygobitic species' response to VHMPs in combination with increasing temperature, as it is predicted that this will occur in the future.

As damage may occur at sublethal concentrations, future studies should assess stress levels induced by these factors to better predict and estimate the impact of VHMPs on groundwater ecosystems.