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Environmental risk of diclofenac in European groundwaters and implications for environmental quality standards

Cláudia Duarte[1], Tiziana Di Lorenzo[1,2,3,4] & Ana Sofia P. S. Reboleira[1,5]✉

Groundwater harbours unique species adapted to perpetual darkness. Groundwater fauna plays a crucial role in global ecosystem services, but contamination poses a threat to this keystone ecosystem. Diclofenac is a common non-steroidal anti-inflammatory drug of particular concern, due to its presence in both surface and groundwater. We assess the environmental risk of diclofenac in European groundwaters using different scenarios, analyzing Measured Environmental Concentrations (MECs) of diclofenac and estimating the Predicted No Effect Concentration (PNECs) through two approaches: considering the sensitivity of the groundwater crustacean *Proasellus lusitanicus* (Isopoda: Asellidae), and using surface water species as proxies. Our results show that scenarios based on surrogate species predict that groundwater ecosystems are at risk due to diclofenac contamination. On the other hand, the MECs of diclofenac were consistently lower than the PNEC of *P. lusitanicus*, suggesting that the current MECs do not pose a significant threat to this groundwater-adapted species. However, risk scenarios differ considering the sensitivity of other groundwater species, emphasizing the importance of considering multiple species' sensitivities in risk assessment. Therefore, we recommend establishing an environmental quality standard for diclofenac in groundwater at 5 ng/L, a value that accounts the need for precautionary measures to safeguard groundwater ecosystems, essential for preserving their unique biota and services.

Keywords Pharmaceutical compounds, Stygofauna, Stygobitic, Subterranean environments, Groundwater ecosystems, Environmental risk assessment

Subterranean ecosystems, characterized by perpetual darkness, heavily rely on organic matter transported from the surface through percolating water[1]. These ecosystems exhibit narrower temperature variations and higher humidity compared to surface ecosystems[2]. Groundwater-obligate species possess distinct morphological, physiological, and behavioural traits that enable their survival in groundwater[3]. These species have longer life cycles and lower metabolic rates than their surface water relative species[4,5], and have likely lost circadian rhythm[6]. Morphological adaptations include depigmentation, reduction or absence of eye structures, elongation of appendages and body, increased sensory receptors with altered spatial distribution[3].

Groundwater fauna plays a crucial role in providing various ecosystem services, such as viruses and pathogens' removal, carbon recycling and environmental engineering and sediment remix through burrowing[7]. However, contamination from sources such as sewage wastewater, industrial activities, agricultural practices, stormwater runoff, salinization, and pesticide/fertilizer application pose a significant threat to this resource[1,8,9]. Although groundwater is often considered less vulnerable to contamination than surface water, traces of various pollutants, including veterinary and human medicinal products (VHMP), are frequently detected[10]. Of particular concern is diclofenac, a non-steroidal anti-inflammatory agent widely used in human and veterinary medicine since the 1970s[11]. In mammals, the majority of diclofenac is metabolized into various inactive metabolites before excretion,

[1]Centre for Ecology, Evolution and Environmental Changes & CHANGE – Global Change and Sustainability Institute, and Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Campo Grande, Lisbon, Portugal. [2]Research Institute on Terrestrial Ecosystems of the National Research Council of Italy (IRET-CNR), Via Madonna del Piano 10, Sesto Fiorentino, 50019 Florence, Italy. [3]NBFC (National Biodiversity Future Center), 90133 Palermo, Italy. [4]Department of Cluj-Napoca, "Emil Racoviță" Institute of Speleology, Str. Clinicilor 5, 400006 Cluj-Napoca, Romania. [5]National Museum of Natural History and Science, University of Lisbon, Rua da Escola Politécnica 56, 1250-102 Lisboa, Portugal. ✉email: asreboleira@fc.ul.pt

but a fraction of the drug is eliminated in its original form through the kidneys into the urine[11]. The primary source of diclofenac into the environment is through wastewater[12]. Although wastewater treatment plants partially remove diclofenac with removal efficiency ranging from 44.4% to around 90%[13], it is still detected in both surface water and groundwater[14].

Diclofenac was included in the European priority substances watchlist in 2015, which purposes to monitor the environmental concentrations of the most harmful chemical compounds in Europe[15]. Environmental quality standards (EQS), legally binding thresholds against which measured environmental concentrations (MECs) are compared, are set up for those chemicals that are considered to pose a Europe-wide risk[16,17]. Environmental Quality Standards refer to the concentration of a specific chemical substance in an environmental compartment that, if exceeded, may cause significant adverse effects. For diclofenac, an EQS of 0.050 μg/L has been determined for surface water bodies, although a higher value of 0.126 μg/L has been proposed based on a probabilistic approach[18]. The EQS for diclofenac in groundwater has not yet been determined. Environmental Quality Standards are typically derived based on scientific knowledge, considering the sensitivity of various organisms and ecosystems to the specific substance. Environmental Quality Standards are often derived based on PNEC (Predicted No Effect Concentration) values[19], where the PNEC is an estimate of the highest concentration of a substance in the environment below which no adverse effects are expected to occur in organisms or ecosystems. In turn, PNEC plays a crucial role in the process of Environmental Risk Assessment (ERA). ERA is a systematic evaluation of potential risks posed by chemical substances to the environment. It involves assessing the exposure of organisms or ecosystems to the measured environmental concentrations of a substance and determining the likelihood and magnitude of adverse effects[20,21]. By considering the PNEC values and additional factors, such as the potential for bioaccumulation and the specific environmental characteristics, regulatory bodies and environmental agencies determine appropriate EQS values to protect the environment.

Our objective was to enhance the understanding of the environmental risk posed by diclofenac to European groundwater ecosystems. First, we analysed the MECs of diclofenac in European groundwaters. Second, we conducted a PNEC estimation for diclofenac in groundwater through two approaches, where the first approach considered the sensitivity of a groundwater-adapted species *Proasellus lusitanicus* (Isopoda: Asellidae), while the second utilized the sensitivity of surface water species as substitutes, as recommended by the current European guidelines[20,21]. Further, we described four different scenarios of environmental risk of diclofenac in European groundwaters by combining the two approaches. Lastly, we explain our recommendations for establishing the EQS of diclofenac in groundwater in Europe.

Results

Time-independent assay

The assay conducted in our study satisfied the control validity criterion recommended by Di Lorenzo et al.[22] since we observed ≤ 20% mortality in the test control. The calculated LC_{50} values ranged between 194.61 (± 15.12) mg/L of diclofenac sodium at 14 days and 493.30 (± 310.41) mg/L at 4–5 days (Table 1). The increase of the LC_{50} values in the first days of observation (Table 1) is likely due to the poor fit of the probit models. To build the

Day	LC_{50} (mg/L)	SE (mg/L)	Parameter	Estimate	SE	p-value
1	293.93	78.65	*b*	3.96	1.12	< 0.001
3	403.69	184.70	*c*	198.87	13.60	< 0.001
5	493.30	310.41	*d*	1590.93	1843.62	0.388
6	354.48	100.84	*e*	3.57	1.77	0.044
7	279.55	46.67				
8	263.12	42.90				
11	225.19	24.70				
13	207.61	18.61				
14	194.61	15.12				
Day	**LC1$_0$ (mg/L)**	**SE (mg/L)**	**Parameter**	**Estimate**	**SE**	**p-value**
1	275.02	8.79	*b*	14.97	7.75	0.049
3	259.38	40.47	*c*	120.85	5.88	< 0.001
5	210.07	44.16	*d*	212.29	18.32	< 0.001
6	183.85	32.38	*e*	6.33	0.34	< 0.001
7	138.82	29.24				
8	120.78	29.13				
11	118.90	24.37				
13	121.24	21.49				
14	124.50	18.97				

Table 1. Lethal Concentration 50% (LC_{50}) and 10% (LC_{10}) of diclofenac sodium for *Proasellus lusitanicus* over a 14-day assay and parameters of the log-logistic models. LC_{50} and LC_{10} values are expressed in mg/L of diclofenac sodium and represent the concentration at which 50% and 10% of the tested organisms experience mortality; SE: standard error.

4-parameter log-logistic models, we excluded data of days 1 and 3. As expected, there was a noticeable decrease in LC_{50} values over time (Fig. 1). The parameters of the model for LC_{50} are reported in Table 1. All four parameters were significant ($p < 0.05$), except for the upper estimate (*d*), which was not ($p = 0.388$). This lack of significance may be due to the few data points near the upper limit (or LC_{50} values at time 0), making it challenging for the model to fit that part of the curve accurately. Importantly, the parameter of primary interest (*c*), representing the incipient LC_{50}, was highly significant and had a low standard error, indicating a reliable estimate. The incipient LC_{50} was 198.87 mg/L of diclofenac sodium (= 184.51 mg/L diclofenac). The calculated LC_{10} values per each observation day and the parameters of the log-logistic model are reported in Table 1. The parameters of the model were all significant ($p < 0.05$). The incipient LC_{10} was 120.85 mg/L diclofenac sodium (= 112.12 mg/L diclofenac).

We generated an SSD curve for diclofenac sodium, as depicted in Fig. 2, to determine the assessment factor (AF_2) in Eq. (3). Our search in the U.S. EPA ECOTOX database yielded seven $L(E)C_{50}$ records, one of which was associated with the groundwater harpacticoid species *Nitocrella achaiae* Pesce, 1981[23]. Among the tested organisms, except for the algae *Chlamydomonas reinhard* P.A. Dangeard, 1888, *P. lusitanicus* exhibited the lowest sensitivity to diclofenac sodium. According to the guidelines[24], this indicates that an AF_2 of 1000 should be applied. Consequently, in scenarios 3 and 4 (Fig. 3), we considered a $PNEC_{gw}$ value of 112,120 ng/L of diclofenac, calculated by dividing the incipient LC_{10} (112.12 mg/L) used in lieu of NOEC value derived for *P. lusitanicus* by the AF_2 value of 1000.

Groundwater risk scenarios

Scenario 1 (Fig. 3) presented the highest risk, with an RQ_{gw} of 1060 in Germany (Table S1). Scenarios 2 and 3 (Tables S2 and S3) showed lower RQ_{gw} values compared to Scenario 1. However, the scenario with the lowest risk was Scenario 4 (Table S4). In Table 2, we summarized the minimum and maximum values for each scenario calculated in our study. Scenarios 1 and 2, relying on the sensitivity of surface water species as a proxy for groundwater organisms, revealed a range of moderate to very high environmental risks. In contrast, scenarios 3 and 4, based on the sensitivity of *P. lusitanicus* to diclofenac, indicated very low risks.

For scenarios 1 and 3, we exclusively considered studies that specifically quantified diclofenac concentrations in European groundwaters for our analyses. We excluded papers that focused on the physical and chemical properties of diclofenac, such as degradation, retention potential, adsorption and migration in the soil. Studies concentrating on the removal of diclofenac in wastewater treatment plants were also discarded. In total, we evaluated 11 papers that met our criteria for a total of 46 MEC values from seven European Member States encompassing the Mediterranean region and Europe (Tables S1 and S3). The majority of these MECs were related to Germany, while there was a shortage of MECs from southern Europe, except for Spain. The lowest MEC_{gw} reported in the literature was 1.4 ng/L, in France[25]. The highest MEC_{gw} was 5300 ng/L, in Germany[26]. For scenarios 2 and 4, we used the last version of WATERBASE database that provided 89 MEC values from four European countries: Czech Republic, France, Italy, and Slovakia, spanning the period from the year 2013 to 2019 (Tables S2 and S4). Among these countries, Slovakia recorded the highest MEC_{gw} of 200 ng/L in 2019, while France reported the lowest MEC_{gw} of 1 ng/L in 2014.

Discussion

We assessed the environmental risk of diclofenac in European groundwaters using different scenarios. Overall, we found that the MECs of diclofenac in European groundwaters in all the investigated scenarios of risk were consistently much lower than the NOEC of the groundwater-adapted asellid crustacean *P. lusitanicus*. Indeed, *P.*

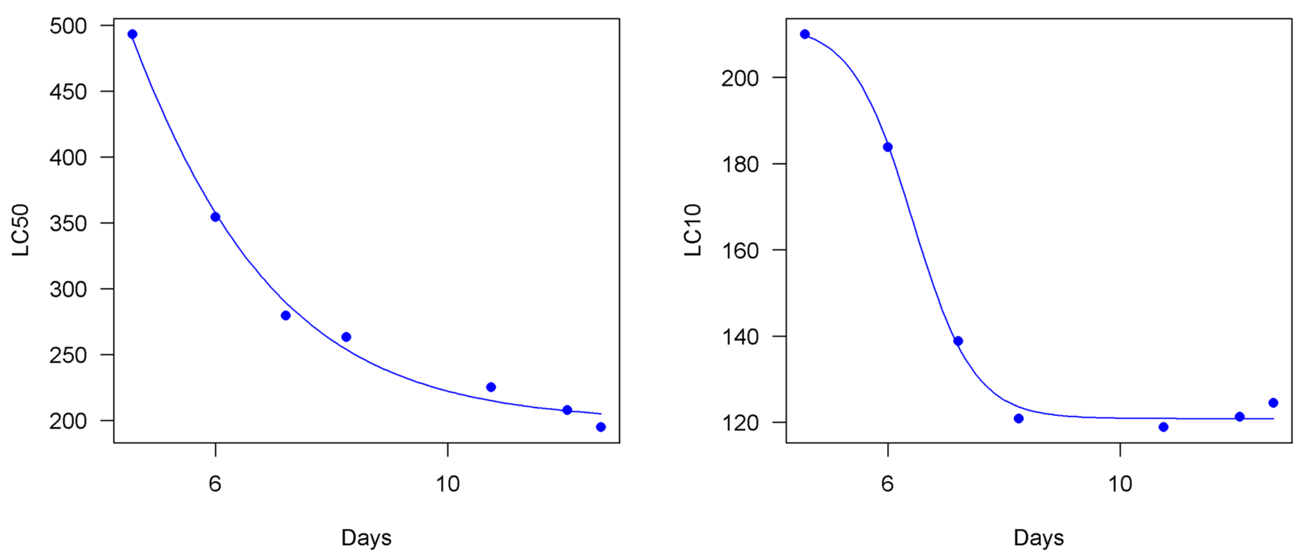

Fig. 1. LC_{50} and LC_{10} values (blue dots; in mg/L) for *Proasellus lusitanicus* exposed to diclofenac sodium (in mg/L) across various exposure days. Solid lines represent the fitted 4-parameter log-logistic models. Curve parameters provided in Table 1.

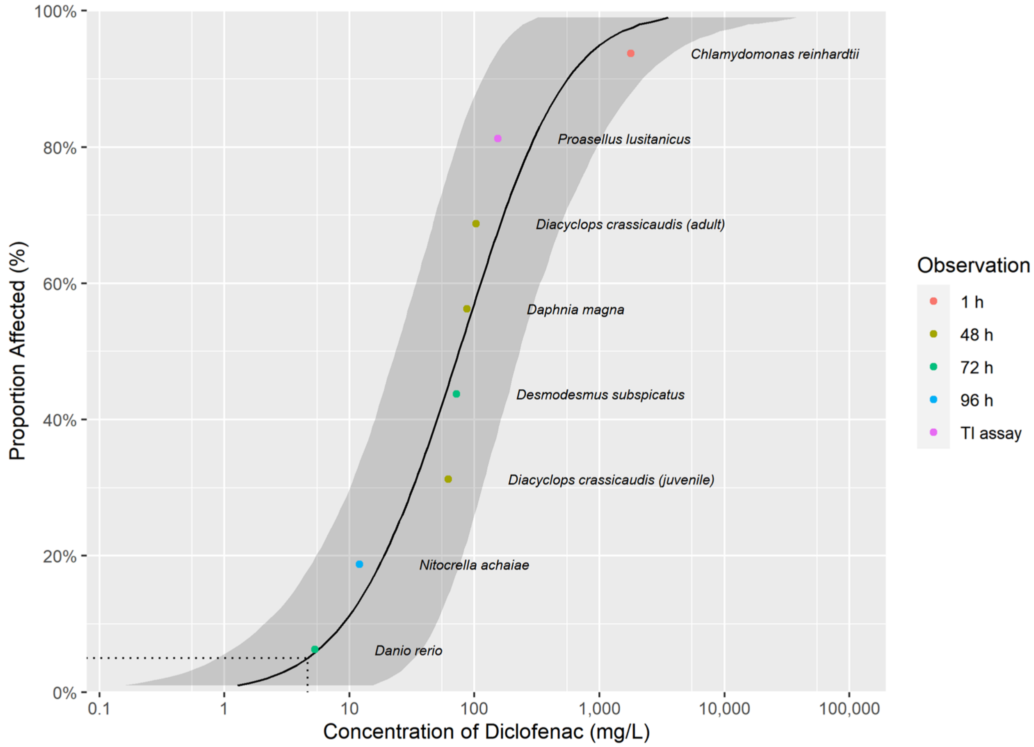

Fig. 2. Species Sensitivity Distribution (SSD) curve for aquatic species exposed to diclofenac. The curve illustrates the lethal response measured in hours (h) and using the incipient LC_{50} of this study for *Proasellus lusitanicus*.

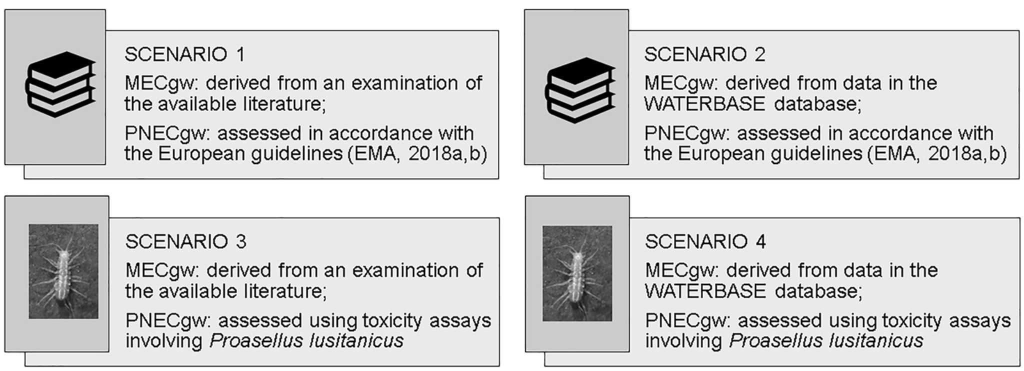

Fig. 3. Simplified description of the four scenarios for RQ_{gw} calculation (MEC_{gw}—measured environmental concentration in groundwater; $PNEC_{gw}$—predicted non-effect concentration of groundwater biota).

Scenario	Min RQ_{gw}	Max RQ_{gw}
1	0.3	1060
2	0.2	40
3	1.4×10^{-5}	0.0472
4	1.0×10^{-5}	0.00178

Table 2. Maximum and minimum RQ_{gw} calculated per scenario (RQ_{gw}—risk quotient for groundwater ecosystems).

lusitanicus exhibited a remarkably lower sensitivity to diclofenac compared to both surface and other groundwater species, as also suggested by the higher incipient LC_{50} compared to shorter exposure data in the SSD. This asellid species (adult mean body length: 5.2 mm)[4] was one order of magnitude more resistant to diclofenac than the groundwater copepod *N. achaiae* (adult mean body length: 0.5 mm)[23] and as resistant as the stygophile copepod *Diacyclops crassicaudis crassicaudis* (Sars G.O., 1863) (adult mean body length: 0.8 mm)[27]. Body size and metabolic rates likely play a role in determining such a sensitivity difference. The process of uptake involves the movement of diclofenac molecules from the surrounding water across the invertebrates' body surfaces, such as their gills, exoskeleton, or integument[28]. The drug can passively diffuse through cell membranes due to its small size and lipophilic (fat-soluble) nature and is transported to tissues and organs[29]. Smaller-bodied organisms, such as copepods, have a greater surface-to-volume ratio, which likely causes a higher passive diffusion of diclofenac and other substances in comparison to larger species[30]. Once inside the invertebrate's body, diclofenac reaches and affects tissues and organs. This process depends on the species' metabolic rates, which serve as a proxy for their physiological rates. Notably, *P. lusitanicus* exhibits metabolic rates (86 ng O_2/mg × h)[4] approximately one order of magnitude lower than those of groundwater copepod species (e.g., *Moraria* sp.: 913 ng O_2/mg × h)[5]. This may result in a reduced uptake rate of diclofenac and subsequent internal transport in *P. lusitanicus* compared to smaller invertebrate species, particularly under sub-chronic exposure conditions. Similar slower rates in the uptake of organic compounds have been reported in other groundwater invertebrate species[31]. We did not measure the size and weight of each tested specimen because all specimens were adults with similar sizes. However, we recognize that incorporating size and weight measurements could further elucidate the variability of sensitivity among individuals and recommend this as a direction for future research.

The environmental risk assessment conducted using the sensitivity of *P. lusitanicus* to diclofenac has yielded scenarios indicating no significant risk. These findings suggest that the current measured environmental concentrations of diclofenac in European groundwaters do not pose a substantial threat to the survival of this groundwater species. However, our study shows that the sensitivity of other groundwater species to diclofenac varies. The differing sensitivities of species within a given ecosystem can greatly influence the assessment of risks associated with specific contaminants. In the case of diclofenac, if the focus had been on the groundwater copepod species *N. achaiae*[23], the risk scenarios would have likely portrayed a higher level of concern and highlighted a greater potential risk to groundwater ecosystems. These observations emphasize the need for comprehensive assessments that consider the sensitivities of multiple species within an ecosystem. A critical aspect regarding the ERA procedures is the recommendation to use three model taxa representing three trophic levels when determining the PNEC of pharmaceutical compounds[20,21]. While surface water ecosystems typically feature algae, crustaceans and fish (primary photosynthetic producers, primary consumers and predators), in groundwater environments, primary production is limited to chemolithoautotrophic processes, if present[1]. As a result, these ecosystems rely on the transport of organic matter from the surface[32]. Additionally, microorganisms in groundwater ecosystems are believed to play a significant role in transforming organic matter, which can support entire food webs[33]. Because Crustacea is the dominant taxon in groundwater, and copepods are highly abundant in all aquatic ecosystems, the environmental risk assessment in groundwater could be effectively based solely on crustaceans. It implies that using freshwater copepods for risk assessment would eventually be more appropriate than using *Daphnia* species. However, conducting ecotoxicological studies with groundwater species presents numerous challenges, as discussed in detail in Di Lorenzo et al.[22]. These challenges arise from the unique physiological characteristics of groundwater species, such as their low reproduction rates, long life spans, and low metabolism[34–36], which make them ill-suited for tests designed for surface water invertebrates[22]. Accessing groundwater habitats and collecting groundwater species require expertise and specific equipment, making the process more complex and time-consuming compared to sampling surface water organisms[7]. Employing surface water species as substitutes to estimate the sensitivity of groundwater species to chemical contaminants, while not without limitations, is a practical approach given the current challenges in conducting ecotoxicological studies with groundwater organisms. Previous studies have demonstrated the relevance of this method, suggesting that despite inherent differences, it can provide valuable insights into potential risks[23,37]. Nonetheless, it remains crucial to consider the unique traits and sensitivities of stygobitic species when interpreting these surrogate-based assessments[38,39]. The findings of this study highlight that the European guidelines[20,21] for the environmental risk assessment of pharmaceutical compounds in groundwater present the most concerning environmental risk scenario of diclofenac. Based on Scenarios 1 and 2, it becomes evident that a significant number of European groundwaters are at risk from diclofenac contamination. These scenarios indicate that the presence of diclofenac in these groundwater systems poses a high risk at concentrations exceeding 5 ng/L. The implication arising from this is that the EQS for diclofenac in groundwater should be < 5 ng/L. This value may initially appear overly restrictive, especially considering the higher resistance observed in groundwater species like *P. lusitanicus* and *N. achaiae*. However, we believe that an EQS of 5 ng/L covers the need for precautionary measures to safeguard groundwater ecosystems, which are delicate and vulnerable[3,7]. Pharmaceuticals frequently co-occur in groundwater[40,41]. However, the potential effects of pharmaceutical mixtures on *P. lusitanicus* or groundwater fauna as a whole remain poorly understood (e.g.[42,43]). Additionally, several studies have highlighted the potential for synergistic or additive effects of pharmaceutical mixtures, including diclofenac, which justifies the establishment of an EQS for diclofenac that is significantly lower than the PNEC for individual species[44,45]. In addition, the results of ecotoxicological trials may not fully represent the potential effects of diclofenac on the real populations of groundwater species. Our toxicity test specifically focused on the adult stages of the groundwater crustacean *P. lusitanicus*, while it has been observed that the sensitivity of juveniles to diclofenac can differ from that of adults in other crustaceans[27]. Studies on the epigean cyclopoid species *D. crassicaudis crassicaudis* have shown that juvenile stages are approximately twice as sensitive to diclofenac compared to adults[27]. This difference can be attributed to various factors, including the role of calcium in crustaceans. Calcium is essential for the mineralization of the new cuticle, and the pathway of calcium accumulation may inadvertently lead to the uptake of contaminants. The higher rate of moulting and growth during the earlier stages of life makes juvenile crustaceans more susceptible to the toxic effects of substances like diclofenac[30]. Finally, the effects of diclofenac in more natural or semi-natural conditions (such as those reproduced in a mesocosm) may be more severe compared to what is observed in ecotoxicological trials where the diclofenac ingestion is not considered because the animals are not fed during the experiments.

Following this reasoning, the EQS for diclofenac in groundwater (5 ng/L) would be 10 times more restrictive than the current EQS in surface water (50 ng/L). This 10 × difference should be the rule of thumb to be incorporated into the new law revising the Water Framework Directive, the Groundwater Directive, and the Environmental Quality Standards Directive (Surface Water Directive) in Europe[46]. Notably, to enhance the protection of the EU's groundwater resources, members of the European Parliament have called for threshold values applicable to groundwater to be set at levels ten times lower than those established for surface water. This step means a significant commitment to safeguarding groundwater ecosystems and species[47].

One important aspect to consider in our study is the geographical bias of the depicted scenarios. The extent of monitoring for diclofenac in European groundwaters varies significantly among different countries. The WATERBASE database, for instance, initially had limited information, with only one collection site in France in 2013. However, in 2019, more countries contributed to the database, with Italy, Slovakia, France, and the Czech Republic providing a substantial number of sampling sites. It is also important to acknowledge that regulatory monitoring programs often focus on potentially problematic sites, which may not fully depict a realistic scenario. This selective monitoring approach might result in missing higher concentrations that could occur in specific locations, such as those near emission sources like hospitals.

Conclusion

In this study, we assessed the environmental risk of diclofenac in European groundwaters through various scenarios. Our findings suggest that setting an environmental quality standard of 5 ng/L for diclofenac in European groundwaters is reasonable and necessary to protect this vulnerable ecosystem. The potential for synergistic or additive effects of pharmaceutical mixtures and the varying sensitivities of groundwater invertebrates and life stages underscores the need for precautionary measures. However, we acknowledge that our study has limitations, including the geographical bias in monitoring data and the challenges in conducting ecotoxicological studies with groundwater species. Using surface water species as surrogates, while applying appropriate corrections for specific groundwater traits, seems a practical approach to address these challenges. Moving forward, comprehensive assessments considering multiple species' sensitivities and the role of microorganisms in groundwater ecosystems will be crucial to effectively protect these valuable habitats. To better understand potential risks and protect groundwater ecosystems, it is essential to expand monitoring efforts, especially in alluvial aquifers recharged by stream waters with high diclofenac concentrations from wastewater treatment plants.

Methods

Animal collection and acclimation

We collected 300 individuals of the groundwater asellid *Proasellus lusitanicus* (Frade, 1938) (Fig. 4) 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, where the annual average temperature is about 17 °C[48]. *Proasellus lusitanicus* has been previously used to test the ecophysiological effects of copper sulphate, potassium dichromate, acetaminophen, NaCl and temperature on groundwater fauna[4,43,48,49]. We measured chemical and physical parameters, such as temperature, pH, dissolved oxygen, and electrical conductivity, using a portable multiparameter probe (AQUAREAD—WTW MULTI 3430) at the collection site. Water properties are presented in Table S5.

We collected the individuals with a macro-pipette (capacity of 30 mL) and transported them to the laboratory in plastic containers filled with groundwater from the collection site. We placed the containers in a cooler to maintain temperature during the transportation process within five hours. Upon arrival at the laboratory,

Fig. 4. Specimens of the groundwater asellid *Proasellus lusitanicus* (Isopoda: Asellidae) from Olhos d'Água Cave, Estremenho karst massif (Portugal).

we acclimated the specimens to the laboratory conditions by keeping them in permanent darkness and at the same temperature as the collection site. To meet the dietary requirements of *P. lusitanicus*, we provided a small amount of the sediment from the cave as *P. lusitanicus* is a deposit-feeder. No artificial food was supplied. We acclimated the individuals in these stable conditions for one month (duration of preliminary tests), before the commencement of the toxicity testing (time-independent assays).

Time-independent assay

We conducted the acute toxicity tests using 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). We prepared fresh solutions for the tests.

Before exposure, we acclimated the specimens in 2-μm filtered groundwater to clear their digestive tract and ensure consistent bioavailability of the pharmaceutical compound during the experiment. We used glass vials to prevent adsorption of the pharmaceutical compound. To minimize stress due to handling, we did not aerate the vials during the assays, and provided no food. We maintained the vials in darkness and at 17 °C, which corresponds to the mean annual temperature of the collection site.

We conducted three runs of range-finding tests as a preliminary step before the final test with the following nominal concentrations: 1, 10 and 100 mg/L (range-finding test #1); 125, 175, 225 and 275 mg/L (range-finding test #2) and 325, 375, 425 and 475 mg/L (range-finding test #3). We included blank controls in all tests. We tested each concentration with four specimens individually, using a soft brush to load them into the vials containing 6 mL of the appropriate solution.

As the range-finding tests did not result in 100% mortality within 96 h, we took the decision to replace the acute toxicity tests with a time-independent assay[31,50], which allows for accounting for the possible delayed toxic effects in groundwater fauna. We considered a time-independent assay to be an acute toxicity test that lacks a predetermined time limit and continues until either the toxic response has stopped, or practical reasons necessitate ending the test[51]. In our study, the toxic response had not totally ceased after 14 days; however, the test solution started changing colour and this was assumed to be a practical reason for ending the test. Hence, the assay was terminated at day 14 and was not prolonged to avoid impairing the stability of diclofenac sodium concentrations.

We carried out the final assay using nominal concentrations of 75, 125, 175, 225 and 275 mg/L. We prepared a stock solution of 275 mg/L diclofenac sodium by dissolving 0.0715 g of the salt in 260 mL of commercial water, which had been previously used in long-term trials with *P. lusitanicus*[4]. We tested each concentration and the blank control with ten individuals as recommended by Di Lorenzo et al.[22]. In total, we used 60 individuals, with each specimen placed in an individual vial containing 6 mL of the appropriate solution, following the protocol described by Castaño-Sánchez et al.[27]. We measured dissolved oxygen and pH before and after the tests using the AQUAREAD—WTW MULTI 3430. We recorded mortality in each test vial every two/three days over 14 days. We considered death as the complete immobility of the animal without any uropod movement over 1–2 min of observation. We determined the validity of the assay by assessing the control group mortality, which was required to be $\leq 20\%$, and by ensuring that the variation in dissolved oxygen concentration was within 20% as per the criteria outlined by Di Lorenzo et al.[22]. We calculated LC_{50} and LC_{10} values (the concentrations that cause death in 50% and 10% of the test organisms, respectively) in every observation day using mortality data and a probit analysis. Probit analysis is a type of regression used to analyse binary (dead/alive) response variables. It is commonly used in dose–response studies to determine the concentration or dose of a substance that produces a specific effect in a given percentage of the population (in our case, LC_{50} and LC_{10})[52]. Then, we plotted these LC_{50} and LC_{10} values over time and estimated the respective curves by fitting the following 4-parameter log-logistic model with a Poisson error structure to account for the non-negative nature of the LC_{50} and LC_{10} values:

$$f(x) = c + \frac{d - c}{1 + \exp(b \times (\log(x) - \log(e)))}$$

where: *f(x)* is the predicted LC_{50} (or LC_{10}) at time *x*; *c* represents the lower asymptote, which we used to estimate the incipient LC_{50} (or LC_{10}) value (the stabilized long-term response); *d* is the upper asymptote, representing the initial LC_{50} (or LC_{10}) value at the earliest time points; *b* is the slope parameter, controlling the steepness of the curve; *e* represents the time point (in days) at which the LC_{50} (or LC_{10}) is at its midpoint between the lower and upper asymptotes. The incipient LC_{50} (or the incipient LC_{10}) refers to the LC_{50} (or LC_{10}) value that is observed or estimated when the response has stabilized after a prolonged exposure period. It represents the "ultimate" or long-term toxicity level[31,50].

All analyses and visualizations were conducted in R, utilizing the 'drc' package, within the RStudio environment (version 5.6.3)[53].

Groundwater environmental risk

We explored four different risk scenarios of diclofenac in European groundwaters (Fig. 3), by computing the groundwater risk (RQgw) as in Eq. (1):

$$RQgw = \frac{MECgw}{PNECgw}, \tag{1}$$

where MEC_{gw} stands for measured environmental concentration of diclofenac in groundwater, and $PNEC_{gw}$ stands for predicted no-effect concentration for groundwater biota. Both values must be in the same unit. We have employed standard criteria in Hernando et al.[54] for interpreting the risk. These criteria establish different risk levels as follows: "low risk" when RQ_{gw} falls between 0.01 and 0.1, "medium risk" when RQ_{gw} ranges from

0.1 to 1, and "high risk" when RQ_{gw} exceeds 1. In our study, we introduced two additional risk categories: "very low" for RQ values below 0.01 and "very high" for RQ values above 10. These new categories serve the purpose of better characterizing and interpreting the environmental risks identified in our analysis.

In Scenario 1 (Fig. 3), we determined the MEC_{gw} based on a literature search in Web of Science platform. We used the keywords "diclofenac" and "groundwater" to select papers written in English, specifically focusing on data from European Union Member States, and published in peer-reviewed scientific journals. To determine the $PNEC_{gw}$, we employed Eq. (2):

$$PNECgw = \frac{PNECsw}{AF1}, \tag{2}$$

where $PNEC_{sw}$ was set at 50 ng/L, according to Carvalho et al.[55]. To account for uncertainties associated with using freshwater species to estimate the sensitivity of groundwater communities, we applied an assessment factor (AF_1) of 10, as recommended by the European Medicines Agency[20,21].

In Scenario 2, we acquired the MEC_{gw} from the WATERBASE, a comprehensive water quality database maintained by the European Environmental Agency[56]. The $PNEC_{gw}$ remains the same as in Scenario 1.

In Scenario 3, the MEC_{gw} remains consistent with Scenario 1. However, for the $PNEC_{gw}$, we conducted a time-independent assay specifically for this study using *P. lusitanicus*, as previously described. Following the guidelines provided by the European Medicines Agency[20,21], the $PNEC_{gw}$ is determined using Eq. (3):

$$PNECgw = \frac{NOEC}{} \tag{3}$$

where NOEC stands for no observed effect concentration and AF_2 is an assessment factor that considers the uncertainties associated with using acute sensitivity to estimate chronic sensitivity. In our study, we estimated the NOEC using the incipient LC_{10} derived from the time-independent assay with *P. lusitanicus*. According to the EMA guidelines[20,21], the AF_2 is determined 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 $L(E)C_{50}$ from the short-term tests, […] the assessment of the effects is based on the short-term data with an assessment factor of 1000*"[24]. To determine AF_2 to use, we compared the sensitivity of taxa from different trophic levels, considering both surface and groundwater species. We used data (Table S6) reported as mg/L of diclofenac from acute tests with a maximum duration of 96 h conducted in a freshwater medium without renovation from the ECOTOX database[57]. For species with multiple values for the same endpoint, we calculated the geometric mean. We compared the sensitivity of *P. lusitanicus* with that of other species using the Species-Sensitivity Distribution (SSD) model. The SSD curve was generated using the packages "ssdtools"[58] and "ggplot2"[59].

Data availability

All data is available in supplementary material.

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Author contributions

All authors contributed to the writing and reviewed the manuscript. C.D. Methodology, Visualization, Data curation, Investigation, Formal analysis, Writing—Original Draft. T.D.L. Methodology, Conceptualization, Data curation, Co-supervision, Formal analysis, Writing—Original Draft, Writing—Review & Editing. A.S.P.S.R. Conceptualization, Methodology, Fieldwork, Resources, Investigation, Funding acquisition, Project administration, Supervision, Writing—Review & Editing.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.S.P.S.R.