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Sensitivity of a widespread groundwater copepod to different contaminants

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Abstract

Groundwater is an indispensable resource for humankind and sustainable biomes functioning. Anthropogenic disturbance threatens groundwater ecosystems globally, but to which extent groundwater organisms respond to stressors remains poorly understood. Groundwater animals are rare, with small populations, difficult to find and to breed in the lab, which poses a main challenge to the assessment of their responses to pollutants. Despite the difficulties, assessing the toxicity of a large spectrum of stressors to groundwater organisms is a priority to inform towards appropriate environmental protection of these ecosystems. We tested the sensitivity to $CuSO_4$, diclofenac, and NaCl of a groundwater population of the copepod *Diacyclops crassicaudis crassicaudis* and compared its sensitivity with the model organism *Daphnia magna*. We ranked its sensitivity using a species sensitivity distribution (SSD) approach using the feasible data available for groundwater and surface crustaceans. Our results show that the most toxic compound was $CuSO_4$ for which higher amount of data was recorded and wider variability in response was observed. It was followed by diclofenac, largely lacking data for groundwater-adapted organisms, and the least toxic compound was NaCl. The differential sensitivity between *D. crassicaudis* and *D. magna* was contaminant-dependent. As a general trend *D. crassicaudis* was always distributed in the upper part of the SSD curves together with other groundwater-adapted organisms. Our results highlight that the widespread groundwater populations of the *D. crassicaudis* species complex, which can be successfully breed in the lab, may provide a reasonable approach to assess the ecological effects of anthropogenic stressors in groundwater ecosystems.

Keywords: Ecotoxicology, anthropogenic stressors, groundwater ecology, stygofauna, Copepoda, subterranean habitats.

Introduction

Groundwater is an indispensable resource for human survival and plays a fundamental role in sustaining surface ecosystems (Kundzewicz and Döll, 2009). Regretfully, its recharge is coupled with the incoming of anthropogenic disturbances, which threat the ecological sustainability of groundwater ecosystems worldwide (Castaño-Sánchez et al., 2020a, 2020b).

Groundwater ecosystems are characterized by permanent darkness, low food availability and relative thermal stability; these features, together with the pore size of the geological matrix, play a key role in determining the harbored biota (Korbel et al., 2019; Malard et al., 2009). The diverse fauna assemblages are typically originated by multiple independent colonizations from the surface and can be classified by the degree of adaptation to groundwater environmental conditions (Gibert and Deharveng, 2002). Obligate groundwater organisms, the so-called stygobionts have evolved convergent morpho-physiological traits (e.g., depigmentation, ocular regression, longer life span and lower metabolic rate); while stygophiles are able to establish stable populations in both surface and groundwater ecosystems, and stygoxenes are considered sporadic visitors (Galassi, 2001). Groundwater fauna plays important roles in ecological services, such as water purification, natural attenuation, potential bioindicators, and habitat refuge (Griebler and Avramov, 2015). Therefore, understanding the effect of anthropogenic disturbances in groundwater species is essential to implement appropriate thresholds that can ensure environmental protection of groundwater-dependent ecosystems (Castaño-Sánchez et al., 2020b; T. Di Lorenzo et al., 2019; Mammola et al., 2019).

Despite the growing awareness on the need for an appropriate management of groundwater ecosystems, these are of difficult access and such a constraint has been limiting the availability of ecological information needed to support the building and implementation of efficient protective policies (Griebler et al., 2010; Korbel and Hose, 2011; Mammola et al., 2020, 2019; Saccò et al., 2019). The limited knowledge of groundwater ecology at all levels, from individuals' biology to population and community structural dynamics, is a constraint to the development of suitable ecotoxicological methods with groundwater organisms to specifically address cause-effect relationships (Castaño-Sánchez et al., 2020b; Mammola et al., 2020, 2019). This is a critical line of evidence, along with ecological aspects and chemical quantification in groundwater to appropriately support regulatory guidelines and policy implementation (Stuart et al., 2012). The high endemism patterns of groundwater taxa, absence of cosmopolitan species, their long life cycles compared to their surface relatives, and the recognized difficulties sampling and in maintaining laboratory cultures of these organisms, are major constrains to establish an array of representative models for ecotoxicological approaches (Castaño-Sánchez et al., 2020b; Di Lorenzo et al., 2019). The lack of stable cultures inhibits the development and implementation of chronical approaches and standardized acute testing, translates into the current limitation of reliable comparison among results (Castaño-Sánchez et al., 2020b; Di Lorenzo et al., 2014).

Copepod crustaceans are ubiquitous in aquatic ecosystems. They are represented in planktonic, benthic and interstitial habitats, playing different roles in the food web (Galassi et al., 2009; Kulkarni et al., 2013). Despite their short life cycles, copepods have complex development including naupliar, juvenile and adult stages (Gutierrez et al., 2010). These different stages comprise behavioral and dietary changes useful for detecting the effect of the stressors along the transition periods, which can lead to a higher vulnerability (Kwok et al., 2015). Given these advantages and as per their ecological relevancy, copepods have been broadly used in environmental assessment, especially regarding marine ecosystems (Kwok et al., 2015). Copepods are present in all kinds of groundwater habitats and they are the most well represented taxa across groundwater invertebrates, assuming special relevance in the groundwater trophic web as dominant primary consumers and embracing also some predatory species (Galassi et al., 2009; Gibert and Deharveng, 2002). Among groundwater copepods, the species complex *Diacyclops crassicaudis* is broadly distributed in Europe, and it is also found in North America where several subspecies are known to have affinities with groundwater environments present in hyporheic zones and deeper in caves (e.g., the subspecies *D. crassicaudis crassicaudis* and *D. crassicaudis brachycercus*), frequently considered as stygophiles (Reid, 2004).

In the present study, we aimed to: i) assess the acute toxicity of different aquatic contaminants bearing worldwide distribution to a groundwater population of *Diacylops crassicaudis crassicaudis*; ii) directly compare its sensitivity with the freshwater cladoceran *Daphnia magna*, a standard tests species in ecotoxicological evaluations ; and iii) rank the sensitivity of the groundwater species using a species sensitivity distribution (SSD) approach that integrates data on the responses of surface and groundwater crustacean species to the tested contaminants. The selected stressors were copper sulfate, the nonsteroidal anti-inflammatory drug diclofenac and NaCl, covering for different chemical classes, as well as different inherent mechanisms of toxic action.

Methods

<u>*Diacyclops crassicaudis crassicaudis*</u>

Specimens of *D. crassicaudis crassicaudis* were sampled from a borehole in Mejorada del Campo, Madrid Community, Spain (40º23’32.52’’N 3º30’17.02W’’), which is part of the Guadalajara aquifer, located on a sedimentary basing (i.e., detrital aquifer) from the Tertiary period. The chemical characterization of the aquifer is available in Iepure et al. (2017), and the annual temperature ranges from 12.2 to 23.9 ± 0.2 (Supplementary material Table 1). The specimens were collected from the bottom of the water column using a 53-µm mesh plankton net. After collection, the specimens were placed in a sealable, 1-L plastic container filled with groundwater from the collection borehole and placed in a portable cooler for transportation to the laboratory. In the lab, specimens were successfully cultured in 250-mL vessels (max 100 individuals) with ASTM hard water medium (ASTM, 1980), and fed *ad libitum* with *Raphidocelis subcapitata* and TetraMin fish food. This maintenance setup was renewed three times per week and was kept at permanent darkness and constant temperature of 16 ± 0.2 ºC.

Test specimens were obtained from eight ovigerous females (each carrying 2 egg-sacs) separated from the original stock culture and maintained in a 200-mL glass container, under the same maintenance conditions as described above. After 12-15 days (the average development time to reach C1-C4 copepodite stages at 16 ºC) for juveniles, and 20 to 22 days for adults, the required number of specimens for the tests were randomly picked–up from the culture. Before testing, copepods were transferred to a 200-mL glass container for 3 days in the same medium conditions but deprived of food to clear the gut.

Daphnia magna

Laboratory cultures of *D. magna* (clone A *sensu* Baird et al., 1989a) were maintained in the laboratory for several generations in synthetic ASTM hard water medium (ASTM, 1980) supplemented with an organic additive (*Ascophyllum nodosum* seaweed extract) and vitamins (according to the receipt of Elendt and Bias, 1990). The cultures were maintained under a temperature of 20±2 °C and light:dark photoperiod of 16L:8D, and were renewed three times a week using neonates from the 3rd through 5th broods. The organisms were along with the renewal schedule, with a concentrated suspension of *R. subcapitata* (3x10^5 cells/mL), which was cultured in MBL medium under controlled conditions (Stein, 1973). All experiments were initiated with neonates (less than 24-h old), born between the 3rd and 5th broods, derived from a healthy bulk culture.

Chemicals and test solutions

Acute toxicity tests were carried out with the metal copper dosed as $CuSO_4 \cdot 5H_2O$ (CAS 7758-99-8), the pharmaceutical compound diclofenac (CAS 15307-79-6), and the salt sodium chloride (CAS 7647-14-5), all from Sigma-Aldrich (Steinheim, Germany).

Stock solutions were freshly prepared in ASTM. The experimental concentrations were chosen based on preliminary range finding tests and taking into account LC_{50} values available for *Daphnia magna* in the U.S. EPA ECOTOX database (2020).

Experimental design

Methods for the 48-h acute toxicity tests to the different stressors were performed in general agreement with the standard protocol OECD (2004), under the same temperature and photoperiod regimes described for maintenance procedures. Briefly, five specimens were randomly assigned to the test vials filled with 10-mL of the appropriate test solution. Four replicates were set per treatment by diluting the stock solution with blank culture medium ASTM.

For *D. magna*, neonates younger than 24 h were used, 5 individuals being assigned to each replicate, and exposure was run for 48 h, at 20 ± 2 °C under and light:dark photoperiod of 16L:8D. For *D. crassicaudis crassicaudis*, experiments with the juveniles (copepodites) or adults were carried using also five organisms per replicate and the exposure was run for 48 h at 16 ± 1 ºC in the dark. No food was added before or during the assays and standard physico-chemical parameters according to requirements of standard procedures (OECD, 2004). Every 24 h, each vial test was observed for the presence of immobile animals (no movement after gentle stimulation by a sorting needle; surrogate for lethal effects). At the end of the trials, the records were used to determine the median effect (immobilization) concentrations (EC_{50}).

Data analysis

Dose response curves of the acute experiments were performed in R version 3.5.0 (R Team, 2013). For each tested compound, data from juveniles and adults of *D.*

crassicaudis crassicaudis and from *D. magna* neonates were pooled and fitted in a two parameter log-logistic model per species using a non-linear parametric functions from the drc package (Ritz et al., 2015). For each tested species and each life stage, effect concentrations (EC_x; x = 10, 50) were extrapolated from the fitted dose-response curves. One-Way ANOVA was also applied to compare among dose-response models (copepods adults vs. copepods juveniles vs. *D. magna*) within each contaminant, followed by Tukey's HSD multiple comparison tests using the function glht from the multcomp package (Ritz et al., 2019). The alpha level was set at 0.05 for all analyses.

The SSD approach was used to address comparison in the response to the studied contaminants between the available data for groundwater and surface freshwater crustaceans. Little data, limited to short term tests with field collected organisms are available for groundwater fauna, being neglected from wide databases. Therefore, we used median lethal or effective concentration data (LC/EC_{50}) for groundwater organisms available from literature and the EC_{50} values generated in this study for the groundwater population of *D. crassicaudis crassicaudis*. For surface freshwater crustaceans we used the generated data for *D. magna* and the obtained LC/EC_{50} from the U.S. EPA ECOTOX database (2020). Only well-supported LC/EC_{50} values following 48 h of exposure in laboratory test using static medium conditions were used. SSD curves were obtained by fitting a cumulative distribution to the ranked toxicity data using the spreadsheets provided by USEPA (2020).

Results

Differential sensitivity to the tested compounds was exhibited by *D. crassicaudis crassicaudis* and *D. magna* (Table 1, figure 1). *D. magna* was more sensitive to copper sulfate than both juveniles and adults of *D. crassicaudis crassicaudis.* Juveniles of *D.*

crassicaudis crassicaudis were more sensitive to diclofenac than adults and *D. magna*, but responses of the species did not differ significantly ($p = 0.604$). Finally, the response sensitivity to NaCl was very similar among the tested species. There was no statistical difference between the response to NaCl of *D. crassicaudis crassicaudis* juveniles or adults and *D. magna* ($p = 0.751$ and 0.266, respectively). In all cases, the copepodites were significantly more sensitive than adults (Table 2), with the EC_{50} at 48 h of the juveniles representing 59.9% of adults' values for diclofenac; 43.9% for copper sulfate; and 90% for NaCl.

The dataset available for each tested stressor differed in amount of species tested and concentration ranges. For copper sulfate, we obtained a total of 32 records for 11 freshwater crustaceans, in which only data for two groundwater species was available (Supplementary material Table 2). For diclofenac we obtained eight records from three species without representatives of groundwater ecosystems (Supplementary material Table 2). Finally, for NaCl, a total of 12 records was obtained from eight species, including three from groundwater species (Supplementary material Table 2, Figure 2).

The lowest lethal values were observed for copper, but the range of sensitivity of the freshwater biota to copper spreads within three orders of magnitude (0.01 to 26 mg/L) (Supplementary material Table 2). Intermediate sensitivity was found for diclofenac (EC_{50} range: 22 - 142 mg /L) and the least toxic compound was NaCl, with an EC_{50} range between 1 to 6 g /L (Supplementary material Table 2).

The groundwater population of *D. crassicaudis crassicaudis* was always located in the upper section of the sensitivity distribution curves (i.e., it has a lower relative sensitivity), which is consistent with other field collected groundwater crustaceans (Figure 2).

Discussion

Historically, the effects of anthropogenic stressors in groundwater ecosystems have been neglected. Toxicity data for groundwater species is extremely scarce and there is a lack of standardization in test methods, hindering the comparison of sensitivity among species (Castaño-Sánchez et al., 2020b; Di Lorenzo et al., 2019). The present study represents a two-fold contribution in this context as (i) it provides data on the sensitivity of a groundwater copepod to add to the scarce existent database; (ii) it encloses a comparison with surface water ecotoxicological models, allowing an appraisal on the suitability of stygophile organisms to represent the groundwater fauna.

Copper sulfate was the most toxic stressor for all tested organisms. Soluble free ion Cu^{2+} is the most toxic form of copper, affecting directly gas exchange surfaces and playing a major role as osmoregulatory toxicant (Brooks and Lloyd Mills, 2003). Additionally, Accumulation and physiological impairment occur as long as internal exposure occurs, regardless of how copper reach cells. It can be through burdened food ingested, but filtration can also favour the entry of copper apart from immediate effects in exchange mechanisms in the gill epithelium (Canli, 2006). Adults and copepodites of *D. crassicaudis crassicaudis* were around 100 times more tolerant to copper sulfate than *D. magna*. The highest tolerance to copper sulfate of *D. crassicaudis crassicaudis* compared to *D. magna* is consistent with the broad sensitivity responses observed for copper sulfate in SSD curves. Copper was the tested compound with more records in the SSD curves. The copper sensitivity found for *Daphnia magna* corresponds to the EC_{50} values from the ECOTOX database, which reflects a good quality and relevance of the data provided. Due to their historic use in industrial activities, anthropogenic pollution by metals has been largely studied; copper sulfate gained particular attention as per its broad use as a fungicide in agricultural management (De Oliveira-Filho et al., 2004).

Moreover, concentrations of copper that produce lethal toxicity for freshwater crustaceans has been observed areas in intensely pressured by human activities (e.g., Krčmar et al., 2018; Mansouri et al., 2012). This translates into a wider availability of ecotoxicological data for copper sulfate, which is also amongst the most studied compounds when the focus are groundwater ecosystems (Castaño-Sánchez et al., 2020b). Furthermore, copper sulfate is the contaminant producing wider differences in species sensitivity amongst those tested, with at least three orders of magnitude found across species' responses compared to one order of magnitude for NaCl and diclofenac. Among copepods, adults of *D. crassicaudis crassicaudis* were about 10 times more tolerant to copper sulfate than adults of the freshwater calanoid *Notodiaptomus conifer* (Gutierrez et al., 2010).

Organisms collected in the field can be naturally exposed to copper in contrast with laboratory organisms cultured in synthetic medium, bearing no Cu in its composition (Bossuyt and Janssen, 2005). It has been observed that organisms become adapted to copper exposure at low levels, which translates into a higher tolerance in further assessments compared to naïve organisms; this enhanced tolerance is lost after few generations in the absence of copper (Bossuyt and Janssen, 2005; LeBlanc, 1982; Sun et al., 2014). Field exposure to copper may have occurred and therefore potentially explaining the observed high relative tolerance of *D. crassicaudis crassicaudis*, which was collected in an area with intense agricultural practices where copper concentrations in the hyporheic zone ranged from 0.0 to 2 mg/L (Iepure et al., 2017, 2013).

Diclofenac is a an anti-inflammatory pharmaceutical compound specifically designed to be applied in vertebrate systems, including in humans (Sathishkumar et al., 2020). Crustaceans are affected by diclofenac following absorption in the intestinal tract and consequent induction of oxidative stress and/or negative effects in neurotransmission

(Oliveira et al., 2015). Our data indicate that juveniles of *D. crassicaudis crassicaudis* were almost twice as sensitive as neonates of *D. magna*, whose sensitivity was similar to the adults of *D. crassicaudis crassicaudis.* However, in the SSD curve, the available acute toxicity for *D. magna* was positioned all along the SSD curve. A very limited number of experimental records were available for the fitting of the diclofenac SSD curve, and these are restricted to cladocerans and the tested copepod *D. crassicaudis crassicaudis*. Our study provides the first toxicity record for a pharmaceutical compound in groundwater organisms. Even though diclofenac is used worldwide and included in the watch list of substances of emerging concern for EU-wide monitoring (EU, 2015; Loos et al., 2010), the immense variability of emerging compounds requiring attention and their frequent presence at low concentrations in complex mixtures that are challenging to assess may contribute to explain the scarcity of data (Stuart et al., 2012; Sui et al., 2015). Nevertheless, diclofenac concentrations in freshwater and groundwater ranged from 0.0005 to 0.5 mg/L, which are known to produce sub-lethal effects on crustaceans (Oliveira et al., 2015; Sathishkumar et al., 2020).

NaCl was the least toxic of the tested stressors. Na^+ and Cl^- are the main ions in charge of controlling osmolarity regulation though the active ion-transport in the organisms cell membranes (Lignot et al., 2000). Still, high concentrations of NaCl concentrations, yet much lower than sea level, can disrupt the osmotic regulation process producing lethal toxicity in freshwater organisms (Griffith, 2017). The response to NaCl was very similar between *D. magna* and *D. crassicaudis crassicaudis*, a trend that was confirmed by the SSD approach. The SSD for NaCl, although not as complete as that for copper, collected records for 10 species, including three stygobionts (Castaño-Sánchez et al., 2020a; Loureiro et al., 2015). The toxicity of NaCl has usually been addressed to understand how different marine and freshwater crustaceans cope with changing concentrations to maintain the osmotic balance (Griffith, 2017). Moreover, it has recently gained further attention as salinization has been appraised as one of the major emerging threats for freshwater and groundwater ecosystems worldwide (Castaño-Sánchez et al., 2020a; Li et al., 2020; Loureiro et al., 2015; Reid et al., 2019). Contrarily to the pattern observed for the other tested contaminants, *D. magna* was the most tolerant to NaCl across cladocerans, being allocated to the upper part of the curve in between all groundwater organisms. This may be explained because *D. magna* is an euryhaline species which lives in fresh, brackish and thalassohaline waters being also found in coastal rock-pools, and that this species may therefore be better adapted to salinity fluctuations than other *Daphnia* species (Gonçalves et al., 2007). Both adults and juveniles of *D. crassicaudis crassicaudis* were slightly more tolerant to NaCl than the stygobiont cyclopoid copepod (*Diacyclops* n. sp.) and twice more tolerant than the stygobiont harpacticoid copepod (*Ameiridae* n. sp). The similarity in the response between both groundwater species from the same genus (*Diacyclops*) may suggest a similarity in the response to NaCl between stygobiont and stygophile copepods.

Considering the ecotoxicity data used to perform the SSD curves, 75% of the data correspond to cladocerans and approximately 11% to copepods, where only two of them are surface species and four are groundwater inhabitants. The absence of standardized acute or chronic toxicity testing protocols for freshwater copepods has been leading to an overlooking of copepods sensitivity in freshwater ecotoxicological assessments. This suggests an impediment to realistically compare sensitivity with organisms from other aquatic compartments where cladocerans are frequently scarce or missing, andcopepods are more broadly studied (Castaño-Sánchez et al., 2020a, 2020a; Di Lorenzo et al., 2019; Kulkarni et al., 2013).

The differential sensitivity between the surface water model *D. magna* and the groundwater representative *D. crassicaudis crassicaudis* was found to be contaminant-dependent, highlighting that no feasible extrapolations about sensitivity to contamination can be assumed straightforwardly between species. Adults and copepodites of *D. crassicaudis crassicaudis* were more tolerant to copper sulfate, but less tolerant to diclofenac, than *D. magna*. For NaCl, similar sensitivity was observed between species, regardless of the copepod stage considered. Therefore, the groundwater species was not consistently more sensitive than the model species for freshwater *D. magna*, although in previous studies such consistency was argued for different chemicals (Di Lorenzo et al., 2019). Di Lorenzo et al. (2019) found that *D. crassicaudis crassicaudis* was more tolerant to caffeine and propranolol than *D. magna* by one order of magnitude. Essentially, appropriate assessment of groundwater is needed to provide realistic criteria for ensuring groundwater ecosystems quality and protection. The view that wide ecotoxicological databases available for a widely considered freshwater model such as *D. magna* cannot support accurate regulatory benchmarks for the sustainable protection of the groundwater biota is corroborated by our results. Still, taking up the integrative perspective provided by the SSD curves, juveniles and adults of *D. crassicaudis crassicaudis* were always at the upper half of the curves together with other groundwater species, meaning that they are more tolerant than most of their surface freshwater counterparts.

The current knowledge on responses to stressors for groundwater organisms correspond to data obtained from field collected specimens or from their first generations cultured in laboratory. Therefore, the lowest sensitivity can reflect an adaptive response to environmental contamination (Bossuyt and Janssen, 2005). On the other hand, the acute data for stygobiont organisms is largely based in the responses of adults, in comparison with most of the data available for surface freshwater organisms, which correspond to juvenile stages. This bias direct comparisons because adults are known to be less sensitive than juveniles (Arzate-Cárdenas et al., 2011; Haque et al., 2018; Hoang and Klaine, 2007) as it was also observed with *D. crassicaudis crassicaudis* among the tested compound. Despite this, our results suggest that groundwater organisms may have a high tolerance to environmental stressors, which has been seen as an advantage for a successful colonization of groundwater ecosystems (Reboleira et al., 2013). In fact, such enhanced tolerance fits with the traits needed for a species to colonize a harsh environment.

A higher ratio between non-stygobiont/stygobiont abundance has been proposed as a trait for biomonitoring groundwater ecosystems, regarding the presence of organic carbon and nutrients (e.g., nitrate) as signals of anthropogenic impact (Stein et al., 2010). However, complex assemblages of stygobiont and stygophile organisms are frequently found in groundwater, which has been specially studied when assessing species richness and biodiversity pattern of copepod species (Galassi et al., 2009; Iepure et al., 2017; Pipan and Culver, 2013). Given the limitation of implementing ecotoxicity studies with stygobiont organisms, the use of stygophile copepods may provide a realistic approach to assess the ecological effect of anthropogenic stressors in groundwater. The wide distribution of *D. crassicaudis crassicaudis* dominantly found in detrital aquifers (Iepure et al., 2017) and the successful cultivability in the lab provides new opportunities to assess sub-lethal endpoints and to increase our understanding of the biological response of groundwater ecosystems to stressors.

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Figures caption

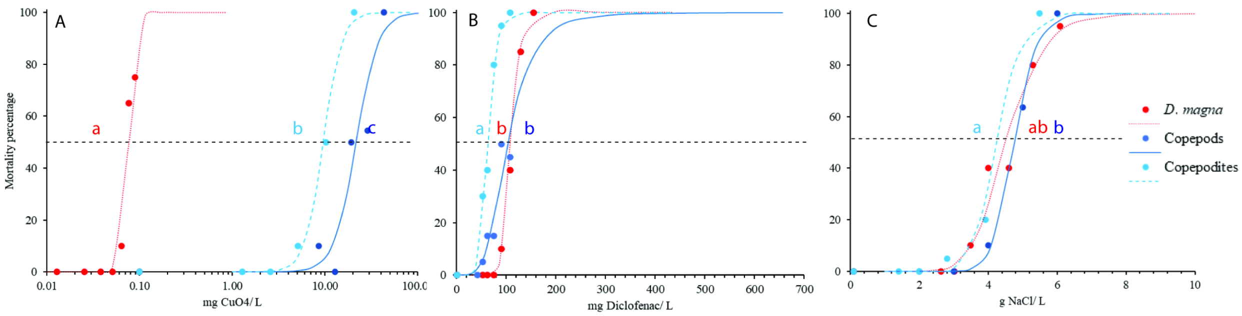
Figure 1. Experimental acute response (filled circles) and model fits (lines) following 48 h of exposure to copper sulfate (A), diclofenac (B) and NaCl (C) with the new born *Daphnia magna* (red), juveniles copepodites (light blue) and adults copepods (dark

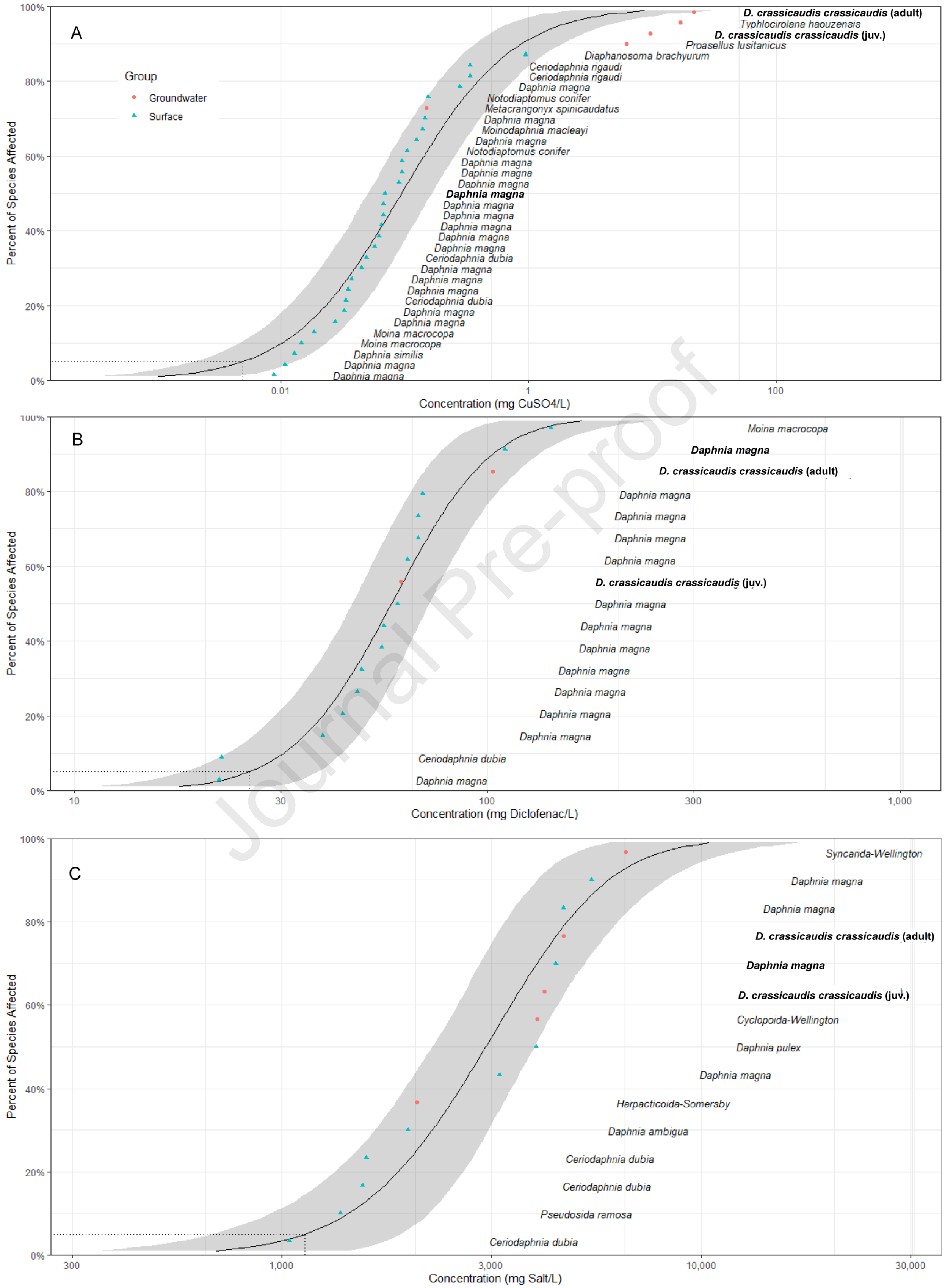
blue) of *D. crassicaudis crassicaudis.* a,b,c notation is used to distinguish statistically significant differences among organisms' response based on model comparison.

Figure 2. Species sensitivity distribution (SSD) for copper sulfate (A), diclofenac (B) and NaCl(C) based on acute toxicity data. In red stygobiont crustacean species tested are indicated. Blue are surface water organisms tested in the juvenile stage. Bold represent the tested species in this study. The solid line indicates the central tendency of SSD, and shaded areas indicate the 95% confidence interval.

Table 1: EC_{50} and EC_{10} values for 48 h of the tested organisms and 95% confidence intervals within brackets.

Species	**$CuSO_4$ EC_{10} (mg/L)**	**$CuSO_4$ EC_{50} (mg/L)**	**Diclofenac EC_{10} (mg/L)**	**Diclofenac EC_{50} (mg/L)**	**NaCl EC_{10} (g/L)**	**NaCl EC_{50} (g/L)**
Diacyclops crassicaudis juveniles	5.8 (4.1-7.4)	9.6 (8.0-11.2)	45.7 (39.6-51.8)	61.9 (57.8-66)	3.5 (3.1-3.9)	4.23 (3.95-4.5)
Diacyclops crassicaudis adults	18.6 (11.9-25.4)	21.7 (17.3-26.3)	60.7 (54-67.4)	103.3 (87.6-119.1)	4 (3.9-4.2)	4.7 (4.6-4.8)
Daphnia magna	0.06 (0.05-0.7)	0.07 (0.07-0.08)	92.7 (89.4-95.9)	110.8 (109.1-112.5)	3.5 (3.2-3.8)	4.5 (4.2-4.7)


A
B
C
Mortality percentage
100
80
60
40
20
0
0.01
0.10
1.00
10.00
100.0
mg CuO4/ L
0
100
200
300
400
500
600
700
mg Diclofenac/ L
0
2
4
6
8
10
g NaCl/ L
a
b
c
a
b
b
a
ab
b
D. magna
Copepods
Copepodites


A
Group
Groundwater
Surface
Percent of Species Affected
100%
80%
60%
40%
20%
0%
D. crassicaudis crassicaudis (adult)
Typhlocirolana haouzensis
D. crassicaudis crassicaudis (juv.)
Proasellus lusitanicus
Diaphanosoma brachyurum
Ceriodaphnia rigaudi
Ceriodaphnia rigaudi
Daphnia magna
Notodiaptomus conifer
Metacrangonyx spinicaudatus
Daphnia magna
Moinodaphnia macleayi
Daphnia magna
Notodiaptomus conifer
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Ceriodaphnia dubia
Daphnia magna
Daphnia magna
Daphnia magna
Ceriodaphnia dubia
Daphnia magna
Daphnia magna
Moina macrocopa
Moina macrocopa
Daphnia similis
Daphnia magna
Daphnia magna
0.01
1
100
Concentration (mg CuSO4/L)
B
Percent of Species Affected
100%
80%
60%
40%
20%
0%
Moina macrocopa
Daphnia magna
D. crassicaudis crassicaudis (adult)
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
D. crassicaudis crassicaudis (juv.)
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Daphnia magna
Ceriodaphnia dubia
Daphnia magna
10
30
100
300
1,000
Concentration (mg Diclofenac/L)
C
Percent of Species Affected
100%
80%
60%
40%
20%
0%
Syncarida-Wellington
Daphnia magna
Daphnia magna
D. crassicaudis crassicaudis (adult)
Daphnia magna
D. crassicaudis crassicaudis (juv.)
Cyclopoida-Wellington
Daphnia pulex
Daphnia magna
Harpacticoida-Somersby
Daphnia ambigua
Ceriodaphnia dubia
Ceriodaphnia dubia
Pseudosida ramosa
Ceriodaphnia dubia
300
1,000
3,000
10,000
30,000
Concentration (mg Salt/L)

Highlights

- Widespread groundwater copepod had differential sensitivity to the tested compounds
- Cu was the most toxic compound tested, followed by diclofenac and NaCl
- The sensitivity of *Diacyclops crassicaudis* and *Daphnia magna* was compound-specific
- Groundwater species are located at the top of the SSD curves, over surface species
- *D. crassicaudis* may be useful for assessing stressors in groundwater

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: