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Hazard/Risk Assessment

Subchronic Effects of Tetrachloroethylene on Two Freshwater Copepod Species: Implications for Groundwater Risk Assessment

Mattia Di Cicco,[a,*] Agostina Tabilio Di Camillo,[a,b] Walter Di Marzio,[c] Maria Elena Sáenz,[c] Diana Maria Paola Galassi,[a] Giuseppe Pieraccini,[d] Angelo Galante,[a,e,f] Davide Di Censo,[g,h] and Tiziana Di Lorenzo[b,i,j,k]

[a]Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy
[b]National Research Council–Research Institute on Terrestrial Ecosystems (CNR-IRET), Florence, Italy
[c]Department of Basic Sciences, Program Research in Ecotoxicology, National University of Luján, National Scientific and Technical Research Council (PRIET-UNLU-CONICET), Luján, Argentina
[d]Mass Spectrometry Centre, University of Florence, Florence, Italy
[e]Gran Sasso National Laboratory, INFN, L'Aquila, Italy
[f]CNR-SPIN, c/o Department of Physical and Chemical Science, University of L'Aquila, L'Aquila, Italy
[g]Department of Neuroscience, Imaging, and Clinical Sciences, "G. D'Annunzio" University of Chieti-Pescara, Chieti, Italy
[h]Institute for Advanced Biomedical Technologies, "G. D'Annunzio" University of Chieti-Pescara, Chieti, Italy
[i]National Biodiversity Future Center, Palermo, Italy
[j]"Emil Racovita" Institute of Speleology, Cluj-Napoca, Romania
[k]Centre for Ecology, Evolution and Environmental Changes & CHANGE–Global Change and Sustainability Institute, and Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Lisbon, Portugal

Abstract: Aliphatic chlorinated hydrocarbons, notably tetrachloroethylene (also known as perchloroethylene [PCE]), are persistent, mobile, and toxic (PMT) and/or very persistent, mobile, and toxic (vPMT) groundwater pollutants, often exceeding safe drinking water thresholds. The present study delves into the groundwater risk assessment of PCE with a novel focus on the sensitivity of stygobitic species—organisms uniquely adapted to groundwater environments. Through a comparative analysis of the subchronic effects of PCE on the locomotion behavior of two copepod species, the stygobitic *Moraria* sp. and the nonstygobitic *Bryocamptus zschokkei*, we highlighted the inadequacy of the current European predicted-no-effect concentration of PCE for groundwater ecosystems. Our findings indicate significant behavioral impairments in both species at a concentration (32 ng/L PCE) well below the threshold deemed safe, suggesting that the current European guidelines for groundwater risk assessment may not adequately protect the unique biodiversity of groundwater habitats. Importantly, *B. zschokkei* demonstrated sensitivity to PCE comparable to or greater than that of the target stygobitic species, suggesting its utility as a substitute species in groundwater risk assessment. The present study adds to the limited research on the ecotoxicological sensitivity of groundwater species to PMT/vPMT chemicals and highlights the need for refined groundwater risk-assessment methodologies that consider the susceptibilities of stygobitic species. *Environ Toxicol Chem* 2024;00:1–13. © 2024 The Author(s). *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

Aliphatic chlorinated hydrocarbons, such as tetrachloroethylene (also known as perchloroethylene [PCE]), are persistent, mobile, and toxic (PMT) and/or very persistent, mobile, and toxic (vPMT) groundwater pollutants (Azzellino et al., 2019; Huang et al., 2023). These substances exhibit prolonged environmental persistence, a natural tendency to contaminate drinking water sources, and are often challenging to eliminate through standard water-treatment

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* Address correspondence to mattia.dicicco@univaq.it

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processes (Huang et al., 2023). Perchloroethylene is commonly used as an industrial solvent for chemical synthesis, metal degreasing, electronics cleaning, and textile dry cleaning and poses potential carcinogenic and mutagenic risks to humans (Cichocki et al., 2016). Due to its high density, PCE tends to migrate to and accumulate at the bottom of the aquifer by traveling through fractures (Walaszek et al., 2021). Natural attenuation of PCE in groundwater occurs under both reducing and oxidizing conditions and typically results in the formation of less chlorinated transformation products (Clement et al., 2000). However, previous studies have highlighted that, in groundwater, PCE concentrations may remain steady and consistent over time due to ample source availability at the surface and limited degradation in the aquifers (Walaszek et al., 2021). As a result, concentrations of aliphatic chlorinated hydrocarbons in groundwater, including trichloroethylene (TCE), frequently exceed the European water guideline for drinking water ([PCE] + [TCE] <10 μg/L; European Commission [EC], 2021), thereby restricting its suitability for drinking-water purposes (European Environment Agency [EEA], 2020).

Groundwater is not merely the largest reservoir of drinking water on our planet; it also constitutes a keystone ecosystem teeming with life (Saccò et al., 2024). Despite the absence of light and photosynthetic producers, as well as the limited quantity of organic matter—predominantly allochthonous, though an uncertain percentage appears to be generated onsite by chemolithotrophic bacteria (Overholt et al., 2022)—marine and freshwater groundwaters harbor >25,000 metazoan species (Martinez et al., 2018) along with countless microbes. Crustaceans dominate groundwater habitats, where stygobitic species (i.e., species that cannot complete their entire life cycle outside groundwater; Culver et al., 2023) exhibit morphological and physiological adaptations to these energy-limited yet environmentally stable ecosystems (Di Lorenzo, Avramov, et al., 2023). Stygobitic species are characterized by depigmentation, eyelessness (or nonfunctional eyes), and elongation of sensory organs (Fišer et al., 2023). Physiologically, they display a reduced range of trait modalities compared to their surface-water counterparts, also exhibiting lower fertility and metabolic rates and higher longevity (Hose et al., 2022). Stygobitic metazoan species represent a significant portion of global biodiversity at risk because of the rarity of many species and their restricted geographic distribution, which frequently extends no further than spot endemicity (Mammola et al., 2024). The role of groundwater microorganisms in degrading pollutants is well known (see Herzyk et al., 2017). Stygobitic metazoans also provide key ecosystem services, including stimulating microbial activities through grazing and the deposition of fecal pellets, sediment remixing through movement, preventing sediment clogging, and removing viruses and pathogens (Mermillod-Blondin et al., 2023). The efficiency of stygobitic metazoans in stimulating microbial activities is directly proportional to their abundance and functional fitness (Mermillod-Blondin et al., 2023). This implies that, to maintain their functional efficiency, metazoans should not perish nor should their activities (such as feeding and locomotion) be affected under chemical stress.

The risk posed by PCE to groundwater ecosystems can be assessed through environmental risk assessment (ERA), which involves computing the risk as the ratio (R) of the measured environmental concentrations (MECs) of PEC to the severity of adverse effects on groundwater species based on the predicted-no-effect concentration (PNEC). An R >1 indicates a potential risk to the groundwater ecosystem (EC, 2014). The PNEC is computed based on the no-observed-effect concentration (NOEC). In the absence of NOEC data for stygobitic species, the European guidelines (see European Medicines Agency [EMA], 2018) recommend using surface-water species as substitutes. Some guidelines also recommend applying a correction factor of 10 to account for the unique traits of groundwater communities (such as their low abundance, restricted distribution, etc.) that potentially make them more sensitive to toxicants compared to epigean communities (EMA, 2018).

The lack of data concerning the effects of chlorinated aliphatic hydrocarbons on groundwater species aligns with the scarcity of ecotoxicological studies conducted with groundwater taxa. According to STYGOTOX, the database of toxicity data on groundwater organisms (Groote-Woortmann et al., 2024), only 43 chemicals were tested on groundwater organisms from 1976 to 2023. This paucity is remarkable compared with data from other existing ecotoxicological databases for epigean species (such as ECOTOX; Olker et al., 2022) that contain >10,000 chemicals and >6300 species. Numerous impediments contribute to the low number of chemicals tested on stygobitic species, including the extreme longevity of these species (often spanning years compared to weeks or months typical of standard test species such as *Daphnia*), low fertility (with many stygobitic species not reproducing in laboratory conditions), and the absence of standard food because most stygobitic species feed on microbes attached to sediment in their natural habitats (Di Lorenzo, Di Marzio, et al., 2019). Among standard test species, *Daphnia magna* exhibits the highest sensitivity to PCE in chronic toxicity assays, with a 28-day NOEC of 0.51 mg/L (Richter et al., 1983). Utilizing this finding and employing the correction factor of 10 (correction factor to the lowest endpoint of standard test species), the PNEC for aquatic ecosystems (PNECw) in Europe is determined to be 51 μg/L PCE (European Chemical Bureau, 2005). This PNECw may not be suitable for groundwater ecosystems due to the traits (high level of endemism, longevity, slow growth, and low reproduction rates; Hose et al., 2022) of stygobitic taxa that determine the limited tolerance of groundwater communities to chronic pressure from chemical stressors (Di Lorenzo, Avramov, et al., 2023). Groundwater communities may possess a reduced ability to recover from chemical stress, with any recovery potentially taking place over an extended time frame, spanning multiple seasons or even decades (Hose et al., 2022). Consequently, a higher correction factor should be recommended to extrapolate the PNEC for groundwater (PNECgw) from the PNECw. This approach parallels previous methodologies employed for deriving environmental quality standards in marine ecosystems (EC, 2011) and PNECgw for pharmaceutical compounds (EMA, 2018).

The present study aimed to fine-tune the groundwater risk assessment of PCE by assessing a PNECgw that better represents the sensitivity of groundwater species. To this end, we applied a correction factor of 1000 to the current PNECw and examined the subchronic effects of the resulting 50 ng/L PCE on the locomotion behavior of two copepod species with the intention of establishing it as the PNECgw. We aimed for a nominal concentration of 50 ng/L PCE. However, the measured concentration was 32 ng/L PCE. We designed an experiment to evaluate the subchronic effects of this concentration on the behavior of two freshwater copepod species: the stygobitic *Moraria* sp. and the nonstygobitic *Bryocamptus zschokkei*. *Moraria* sp. represents a species uniquely adapted to groundwater environments. *Bryocamptus zschokkei* belongs to the same family as *Moraria* sp. (Campthocamptidae) and may serve as a potential substitute in the risk assessment. To provide context for the computation of the environmental risk, we conducted a comprehensive screening of the MECs of PCE in European groundwaters using data sourced from the European database WATERBASE.

MATERIALS AND METHODS

Test organisms

Moraria sp. is endemic to the Apuan Alps (Italy), occurring in just two karst caves (Galmarini et al., 2023). This species is a detritus feeder (eating on microbial biofilm) and has low metabolic rates, scarce acclimation ability to temperature stress, low development (the development from Nauplius V to the adult takes >7 weeks at 8 °C), and low fertility (one or two large eggs per year at 8 °C; Di Lorenzo, Galassi, et al., 2023). In the laboratory, *Moraria* sp. lives for more than 2 years (Di Lorenzo, Galassi, et al., 2023). *Bryocamptus zschokkei* is a prevalent member of meiofaunal stream communities, showing a widespread distribution (Brown et al., 2003). The species is also commonly found in groundwater habitats, often transported from infiltrating surface waters. Its life cycle is relatively brief (6 weeks at 20 °C), whereas the life span is 5 to 11 months (Dole-Olivier et al., 2000). It easily breeds in the laboratory, with adult females typically producing 8 to 24 eggs and three broods within 6 weeks at 20 °C (O'Doherty, 1985). This nonstygobitic species primarily feeds on biofilm and has previously demonstrated sensitivity to contaminant exposure (Burton et al., 2002). Because of these characteristics, *B. zschokkei* has been recognized as an ecologically relevant test species for lotic freshwater environments (Burton et al., 2002).

In November 2022, we collected individuals of *Moraria* sp. from two drips in the Stalactites Gallery of the Antro del Corchia (44°01′31.98″N, 10°17′59.64″E), a karst cave in the Apuan Alps (Tuscany, Italy), utilizing the methods outlined in Pipan (2005) and Pipan and Culver (2005). In December 2022, we collected specimens of *B. zschokkei* from Sorgente del Tinello (44°01′42.5″N, 10°21′16.6″ E), a karst spring in the Apuan Alps. We disturbed the springbed sediments by foot and collected the dislodged fauna using a 60-μm mesh hand net, adhering to standard procedures for sampling spring fauna (Malard et al., 2002). Both samples were transferred into glass bottles, stored in a cooler box, and transported to the laboratory within 2 h.

Culturing conditions

In the laboratory, we promptly sorted the specimens of both species under a Leica M80 stereomicroscope at ×16 magnification. We then placed 58 individuals of *Moraria* sp. (54 adults and four nauplii) and five individuals of *B. zschokkei* (four ovigerous females and one nauplius) into two separate 100-mL glass vials, containing the dripping groundwater from the cave and the groundwater from the spring, respectively. The chemical characteristics of the cave water are reported in Supporting Information, Table S1. The animals were allowed to acclimate for 7 months at 7.8 ± 0.5 °C, which corresponds to the mean annual temperature of the cave (Di Lorenzo, Galassi, et al., 2023), in permanent darkness. This temperature regimen was set for both species based on the pronounced stenothermy observed for *Moraria* sp. (Di Lorenzo, Galassi, et al., 2023), contrasting with the clear eurythermy displayed by *B. zschokkei* (O'Doherty, 1985). Throughout the acclimation period, we changed 10% of the water volume in the rearing vessels every month. Under these conditions, the *B. zschokkei* cohort showed substantial growth, resulting in an increase in the number of individuals, which, by the end of the acclimation period, exceeded 150. In contrast, the cohort of *Moraria* sp. did not increase substantially in number as anticipated, likely because of the species' adaptations to groundwater habitats (Di Lorenzo, Galassi, et al., 2023). Although ovigerous females were frequently observed in the cohort of *B. zschokkei*, females of *Moraria* sp. carrying egg sacs were never observed during the culturing period.

PCE exposure and locomotion traits' measurements

Between September and October 2023, we randomly selected 20 adult females without any visible signs of impairment from both cohorts. Each female was then placed individually in a glass vial containing 10 mL of the cave water used for rearing the stygobitic species. The process of transferring *B. zschokkei* females into cave water mimics the natural process by which specimens of *B. zschokkei* are transported by infiltrating water and ultimately reach groundwater habitats. We allowed these females to acclimate in the new vials (and medium, for *B. zschokkei*) for an additional 30 days without changing the water (the vials were sealed), in permanent darkness and at 7.8 ± 0.5 °C. At the end of the acclimation period, we tracked the individual movements of each female as a control (see below, *Trajectory digitalization and analysis*). Following the initial tracking phase (Figure 1), each female was individually transferred into a 10-mL glass vial filled with a PCE solution. We prepared the solution by targeting a nominal concentration of 50 ng/L PCE by dissolving PCE (Chemical Abstracts Service no. 127-18-4; analytical standard purity 99%; Sigma-Aldrich) in ethyl alcohol (0.000678% ethanol v/v). Subsequently, we diluted this solution with cave water to achieve the desired nominal concentration of 50 ng/L PCE. After measuring (see below, *PCE*

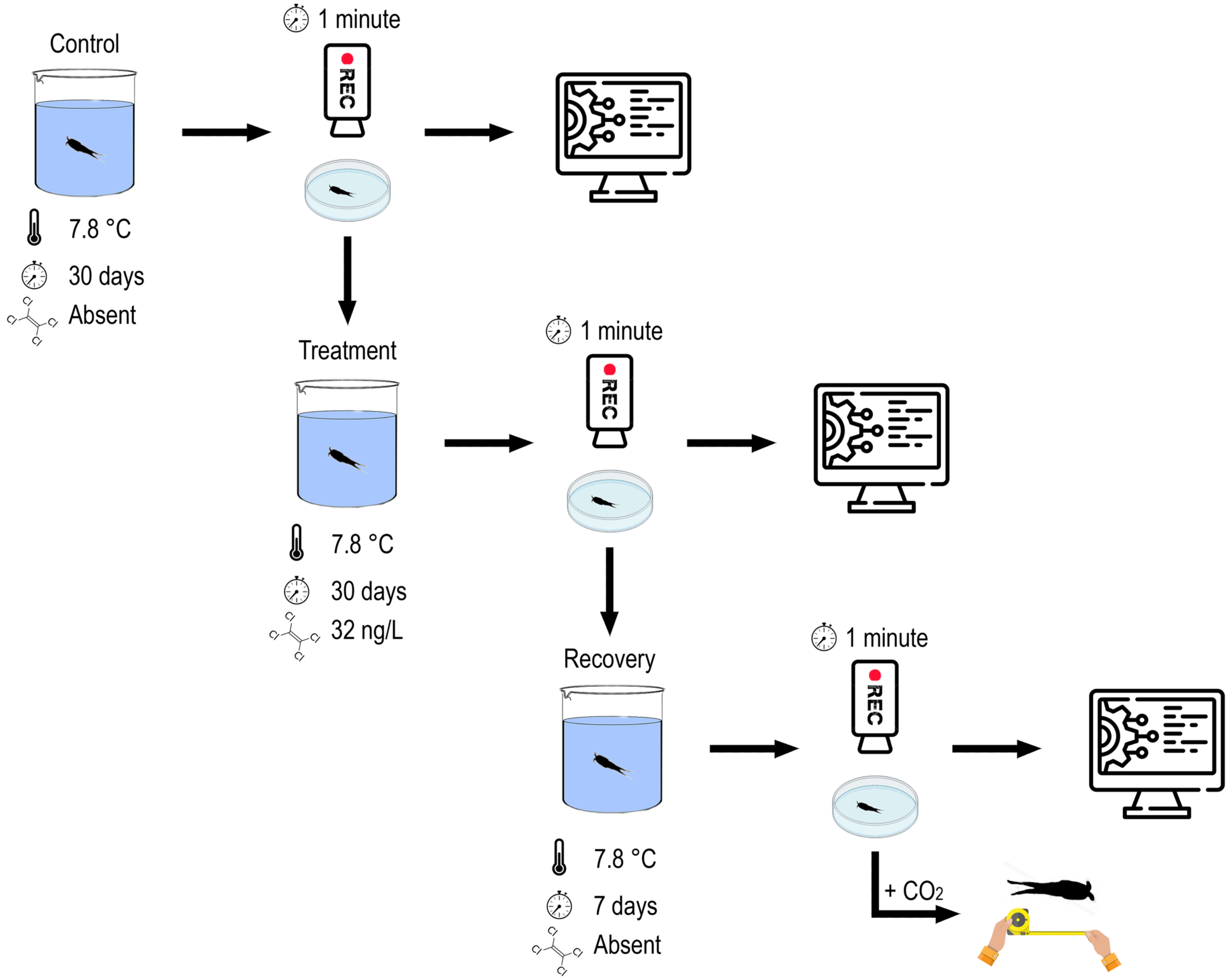

FIGURE 1: Experimental design for the assessment of *Bryocamptus zschokkei* and *Moraria* sp. behavior under control, treatment, and recovery conditions. Adult females were acclimated in cave water at 7.8 °C for 30 days (*Control*), followed by exposure to 32 ng/L perchloroethylene (PCE) solution under identical conditions for an additional 30 days (*Treatment*). Subsequent recovery was monitored for 7 days in cave water (*Recovery*), after which the specimens were anesthetized with CO_2 for morphometric analysis using ImageJ software (2024). Movement tracking was conducted at each phase to evaluate the behavioral response to PCE exposure and recovery.

solution and chemical analysis), we obtained a measured PCE concentration of 32 ng/L.

The females were kept in the test solution for 30 days under the same conditions as those during the control assessment. This duration cutoff was intended as subchronic exposure. The rationale was to observe potential toxic effects that may arise over a significant portion of the two species' life span without reaching the duration required for chronic exposure studies. Due to the low metabolic rates of stygobitic species, many researchers have concluded that acute exposure for these species is better represented by 21 days of exposure rather than the standard duration of 4 days (see Di Lorenzo, Di Marzio, et al., 2019). We did not aim for a longer exposure which could have resulted in significant PCE volatilization, potentially skewing the results. After the 30-day period elapsed, we tracked the individual movements of the females to evaluate their behavior under stress conditions (Treatment; Figure 1), as outlined below (see *Trajectory digitalization and analysis*). On completion of the second tracking phase, we assessed the effects of PCE clearance (i.e., PCE removal from the body of the organisms) and recovery (return to normal locomotory conditions after the chemical has been cleared). This involved returning the females to the 10-mL vials under normal control conditions (cave water, permanent darkness, 7.8 °C) for a 7-day period before conducting the final movement tracking assessment (Recovery; Figure 1). We considered a 7-day period enough for the clearance of PCE and to test the potential recovery of the individuals. We did not aim for longer recovery phases because *B. zschokkei* is known to not live as long as *Moraria* sp. (see above, *Test organisms*), and deaths occurring after the 7-day recovery phase could have been influenced by the natural senescence of the individuals of this species. At the conclusion of the third tracking phase, we anesthetized the animals using CO_2 and captured photographs of each specimen with a camera integrated into the stereomicroscope. Subsequently, we employed ImageJ software (Schneider et al., 2012) to assess the dimensions of each individual, namely the length (from the tip of the cephalic shield to the end of the caudal rami) and width (measured at the largest somite-bearing legs). The recorded body dimensions, in millimeters, were then converted to dry weight using an assumed dry-to-wet weight ratio of 0.25 (Reiss & Schmid-Araya, 2008). Wet weight was determined by dividing biovolume (BV) by 1.1 (Feller & Warwick, 1988). Biovolume was calculated using Equation (1) (Feller & Warwick, 1988):

$$BV = a \times b^2 \times CF \tag{1}$$

In Equation (1), a represents the length in millimeters, b is the width in millimeters, and CF is a correction factor set to 560 (Feller & Warwick, 1988; Reiss & Schmid-Araya, 2008).

Trajectory digitalization and analysis

Each female was recorded, at ×8 magnification, for 1 minute, at 30 fps, in a 0.8-cm diameter circular arena using a Leica M80 stereomicroscope equipped with a built-in camera (MC 170; Leica Microsystems). Video acquisition was done while maintaining a constant temperature of 7.8 ± 0.5 °C through a thermal bath. The length of the video was determined according to the protocol established by Di Cicco et al. (2022). Each video was analyzed in TrAQ (Di Censo et al., 2021), an open-source software developed in MATLAB (MathWorks) to obtain the two-dimensional, frame-by-frame coordinates of the animals' centroids. The positional data comprising 1800 coordinate pairs for each animal were saved in a .csv file. These .csv files were subsequently analyzed in RStudio (Ver 4.2.3; 2003) through the *trajr* package (McLean & Skowron Volponi, 2018). Initially, possible tracking errors due to incorrect animal identification were corrected manually by applying a moving average. Subsequently, trajectory smoothing was performed using the *TrajSmoothSG* function, which employs the Savitzky-Golay filter to remove noise from trajectories (Savitzky & Golay, 1964). This type of filter is particularly effective at preserving the trajectory's line shape and simultaneously removing high-frequency noise. This step is crucial when studying the trajectories of small animals such as copepods because the centroid identification, inferred through background subtraction algorithms, tends to produce noisy trajectories attributable to slight shifts in the animal cluster's shape caused by minor movements of their appendages (e.g., antennules). After smoothing, we proceeded to evaluate five parameters for each trajectory: Path length (PL, millimeters) calculated as follows:

$$\mathrm{PL} = \sum_{t=1}^{t=1800} \sqrt{(x_{t+1} - x_t)^2 + (y_{t+1} - y_t)^2} \tag{2}$$

In Equation (2), x_t and y_t are the spatial coordinates of the animal centroid at the frame t; x_{t+1} and y_{t+1} are the spatial coordinates of the animal centroid at the frame $t + 1$.

Average crawling speed (AS, millimeters per second) calculated as follows:

$$\mathrm{AS} = \frac{1}{1800} \sum_{t=1}^{t=1800} \frac{\mathrm{PL}_t}{\Delta t} \tag{3}$$

In Equation (3), PL_t is the path length of the t-th couple of timely adjacent centroid coordinates, and Δt is the time interval between the two abovementioned coordinates (frame time length). Basically, the AS is computed as the mean of the instantaneous speeds of the trajectory.

Percentage of activity (PA) calculated as follows:

$$\mathrm{PA} = \frac{af}{n} \tag{4}$$

In Equation (4), af is the sum of the frames of animal activity, and n is the total number of pairs of frames. The animal was considered active when its instantaneous speed in the i-th frame was ≥0.15 mm/second. This value was chosen after a preliminary assessment of the minimum speed below which the sole movement of the cephalic or swimming appendages of an inactive copepod individual is read by the software as active movement (Di Cicco et al., 2021; Di Lorenzo, Di Cicco, et al., 2019).

Trajectory convex hull (CH, mm^2) was calculated using the library *sp* in RStudio. A CH of a planar set was the minimum area of the convex polygon containing the planar set. The CH is a parameter that can be used as a proxy to establish the habitat exploration potential of the recorded individuals (Di Cicco et al., 2022). The *chull* command was used to find the vertex of the CH, and the command *Polygon* was applied to calculate the area.

Intensity use (IU, dimensionless) was used as a proxy to estimate path tortuosity and habitat exploitation (Almeida et al., 2010; Loretto & Vieira, 2005). It was calculated as follows:

$$\mathrm{IU} = \frac{\mathrm{PL}}{\sqrt{\mathrm{CH}}} \tag{5}$$

Statistical differences for each behavioral parameter were investigated through a two-way permutational analysis of variance (PERMANOVA; permutations = 9,999, α = 0.05, input distances = Euclidean, covariate = dry wt). The experimental design for the PERMANOVA was structured as follows: Factor 1—ecology (two levels), stygobite and nonstygobite; Factor 2—exposure (three levels), control, treatment, and recovery. We used Type III sum of squares to account for the variability of all factors and the interactions between them. When required, post hoc pairwise comparisons between all possible combinations of levels within Factor 2 and eventual interactions with the levels of Factor 1 were performed using permutational t tests at $\alpha = 0.05$ because permutational tests do not require α correction (Anderson, 2001).

PCE solution and chemical analysis

We measured the concentration at the end of the second tracking phase by headspace solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS) in duplicate. A 0.20-mL volume of the PCE solution was transferred into a 10-mL SPME glass vial containing 1.8 mL distilled water PCE-free and 0.5 g of NaCl. Headspace sampling was performed using a 75-µm Carboxen/polydimethylsiloxane fiber (Supelco, Sigma-Aldrich), which was conditioned at 300 °C for 30 minutes prior to use. For HS sampling, the fiber was exposed into the vial HS for 30 minutes at 50 °C under stirring. We used an Agilent Technologies GC-MS system, composed of an HP 6890 gas chromatograph coupled to a single-quadrupole HP 5973 Mass Selective Detector (Agilent Technologies, Cernusco sul Naviglio, Italy). The GC-MS was equipped with an RXI-5Sil MS with an integra-guard column (30 m × 0.25 µm inner diameter, film thickness 0.25 µm; Restek, Cernusco sul Naviglio, Italy). The oven temperature program was as follows: initial temperature 35 °C maintained for 4 minutes, then to 135 °C at 10 °C/minute, and to 300 °C at 20 °C/minute. The analyte was desorbed at 280 °C for 5 minutes into the GC injection port, equipped with a 0.75-mm inlet liner; injection was performed in splitless mode (3.5-minute valve off). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. Transfer line temperature was 280 °C. Data were recorded in full scan mode from 90 to 300 *m/z*, and the mass spectrometer was operating in positive ion mode; electron ionization at 70 eV was used. Data acquisition and processing were performed using HP ChemStation software (Ver. D.02.00; 2008). Perchloroethylene standard solutions in ethanol were used to build a calibration curve in PCE-free distilled water, used for external calibration. The four PCE standard solutions were freshly prepared at 4.0, 8.0, 16.0, and 32.0 ng/mL. Water samples for calibration were prepared adding 1 µL of each PCE standard solution to 1999 µL distilled water. The calibration points were analyzed as described for the solution in cave water: The area of the *m/z* 166 peak in the total ion chromatograms of PCE from the cave water sample and from the calibration curve points was measured. A linear calibration was obtained ($R^2 = 0.98$), and the concentration of PCE in the cave water sample was calculated, equal to 32 (±2.0) pg/mL (=32 ng/L). The instrumental limit of quantification was 0.5 ng/L.

Risk assessment

We sourced MECs of PCE in European groundwaters from WATERBASE (last updated in July 2023). WATERBASE is the comprehensive repository of the EEA, which stores information about the chemical conditions of European rivers, lakes, groundwater bodies, transitional zones, coastal areas, and marine waters. The database also includes data on the quantity of water resources in Europe and records emissions into waters from both point and diffuse sources of contamination. Specifically, we extracted our data set from WATERBASE–Water Quality ICM (EEA, 2023), which archives information on chemical compounds found in European water bodies, spanning the temporal range from 2000 to 2022. From 2000 to date, PCE has been monitored by member states on a facultative basis, in accordance with the provisions outlined in the Water Framework Directive (EC, 2000). We computed the risk of PCE in groundwater by using the ratio of MECs extracted from the WATERBASE database and a PNECgw of 32 ng/L. For comparative purposes, we also calculated the risk using the ratio of MECs to 51 µg/L, which is the current PNECw for European aquatic ecosystems according to the European guidelines for risk assessment (EC, 2014).

RESULTS

Locomotion traits

The females of the species *B. zschokkei* were 22% larger (average dry wt = 0.94 ± 0.58 µg) than the females of *Moraria* sp. (average dry wt = 0.73 ± 0.19 µg). All females from both species survived during the control phase. A substantial portion of the mortality occurred during the treatment with PCE and, for *B. zschokkei*, in the recovery phase (Table 1). Throughout the duration of the experiment (67 days), no female of *B. zschokkei* became ovigerous. In contrast, four females of *Moraria* sp. became ovigerous, each carrying two large eggs. Of these, two females became ovigerous during the treatment phase, while the other two did so during the recovery phase.

In total, we analyzed 97 trajectory recordings (174,600 frames). Both *ecology* and *exposure* factors had a significant effect on PL (Factor 1 × Factor 2: pseudo-$F_{(2,80)} = 5.84$; $p = 0.005$). In detail, the PL of *B. zschokkei* in the control group was significantly longer (33%) than that of *Moraria* sp. (Tables 1 and 2 and Figure 2). However, during the treatment and recovery phases, no significant difference was observed between the two species or between the two phases (Tables 1 and 2 and Figure 2). The PL of *B. zschokkei* was significantly shorter in the treatment (92%) and recovery (87%) phases compared with the

TABLE 1: Average values and standard deviations of path length, average speed, percentage of activity, convex hull, and intensity of use (dimensionless)

Ecology	Exposure (M%)	PL (mm)	AS (mm/s)	PA (%)	CH (mm^2)	IU
BRY	C (0)	11.8 ± 5.32A	0.2 ± 0.09A	44.93 ± 22.04A	7.59 ± 8.56A	5.51 ± 2.04A
BRY	T (50)	0.94 ± 1.9B	0.02 ± 0.03B*	3.83 ± 8.33B	0.26 ± 0.61B	1.23 ± 2.02B
BRY	R (30)	1.57 ± 3.72B	0.03 ± 0.06B	5.26 ± 13.87B	0.76 ± 1.97B	1.05 ± 1.84B
MOR	C (0)	7.86 ± 3.5C	0.13 ± 0.06C	34.19 ± 22.85A	6.73 ± 8.81A	4.16 ± 1.32C
MOR	T (25)	2.43 ± 3.48B	0.04 ± 0.06B	10.31 ± 17.48B	1.72 ± 4.24B	2.02 ± 2.99B
MOR	R (0)	4.11 ± 4.28B	0.07 ± 0.07B*	14.45 ± 22.14B	1.89 ± 4.03B	4.45 ± 4.14ACB

Different letters and the asterisk indicate significant differences in the pairwise *t* tests.

M% = percentage of mortality (in parentheses); PL = path length; AS = average speed; PA = percentage of activity; CH = convex hull; IU = intensity of use; BRY = *Bryocamptus zschokkei* (nonstygobitic); C = control; T = treatment; R = recovery; MOR = *Moraria* sp. (stygobitic).

TABLE 2: The percentage decrease (rounded to the nearest integer) relative to the control within the ecology levels of each behavioral parameter

Ecology	Exposure (M%)	PL	AS	PA	CH	IU
BRY	C (0)	–	–	–	–	–
BRY	T (50)	92%	90%	91%	97%	78%
BRY	R (30)	87%	85%	88%	90%	81%
MOR	C (0)	–	–	–	–	–
MOR	T (25)	69%	69%	70%	74%	51%
MOR	R (0)	48%	46%	58%	72%	7%

M% = percentage of mortality (in parentheses); PL = path length; AS = average speed; PA = percentage of activity; CH = convex hull; IU = intensity of use; BRY = *Bryocamptus zschokkei* (nonstygobitic); C = control; T = treatment; R = recovery; MOR = *Moraria* sp. (stygobitic).

control. Similarly, the PLs of *Moraria* sp. in the treatment (70%) and recovery (48%) phases were significantly shorter than the controls (Tables 1 and 2 and Figure 2). The two factors also had a significant effect on AS (Factor 1 × Factor 2: pseudo-$F_{(2,80)} = 6.56$; $p = 0.006$). The AS of *B. zschokkei* was significantly faster (35%) than that of *Moraria* sp. in the control group. However, no substantial differences were observed during the treatment and recovery phases within each species or between species, except for the AS of *B. zschokkei* during the treatment phase, which was significantly slower (72%) than that of *Moraria* sp. in the recovery phase (Tables 1 and 2 and Figure 2). The AS of *B. zschokkei* was significantly lower in the treatment (90%) and recovery (85%) phases compared with the control. Similarly, the AS of *Moraria* sp. was significantly lower in the treatment (70%) and recovery (47%) phases compared with the control. The PA was significantly affected by the *exposure* factor (Factor 2: pseudo-$F_{(2,80)} = 23.6142$; $p = 0.001$), while no substantial differences were observed between the two species (Factor 1: pseudo-$F_{(1,80)} = 1.16$; $p = 0.270$). The interaction of the two factors was also not significant (Factor 1 × Factor 2: pseudo-$F_{(2,80)} = 2.2710$; $p = 0.109$). Post hoc tests revealed that the PA of both species was lower during the treatment (92% for *B. zschokkei* and 70% for *Moraria* sp.) and recovery (88% for *B. zschokkei* and 58% for *Moraria* sp.) phases compared with their respective control group (Tables 1 and 2 and Figure 2). However, no significant differences were observed between the treatment and recovery phases (Tables 1 and 2 and Figure 2). Similarly, the *ecology* factor did not have a significant effect on the trajectory CH (Factor 1: pseudo-$F_{(1,80)} = 0.10$; $p = 0.753$), whereas the *exposure* factor significantly influenced this variable (Factor 2: pseudo-$F_{(2,80)} = 8.67$; $p = 0.001$). The interaction of the two factors was not significant (Factor 1 × Factor 2: pseudo-$F_{(2,80)} = 0.30$; $p = 0.728$). The trajectory CH values were significantly smaller in the treatment (97% for *B. zschokkei* and 75% for *Moraria* sp.) and recovery (90% for *B. zschokkei* and 72% for *Moraria* sp.) phases compared with the respective control, consistent with the observations for PA. However, no significant differences in the trajectory CH were detected between the treatment and recovery phases for both species (Tables 1 and 2 and Figure 2). Finally, both the *ecology* and *exposure* factors had a significant effect on IU (Factor 1 × Factor 2: pseudo-$F_{(2,80)} = 5.38$; $p = 0.005$). In detail, the IU of *B. zschokkei* in the control group was significantly higher (25%) than that of *Moraria* sp. (Tables 1 and 2 and Figure 2). In the treatment group, no significant difference was observed between the two species (Tables 1 and 2 and Figure 2). However, the IU of *Moraria* sp. in the recovery group was significantly higher than that of *B. zschokkei* in the recovery

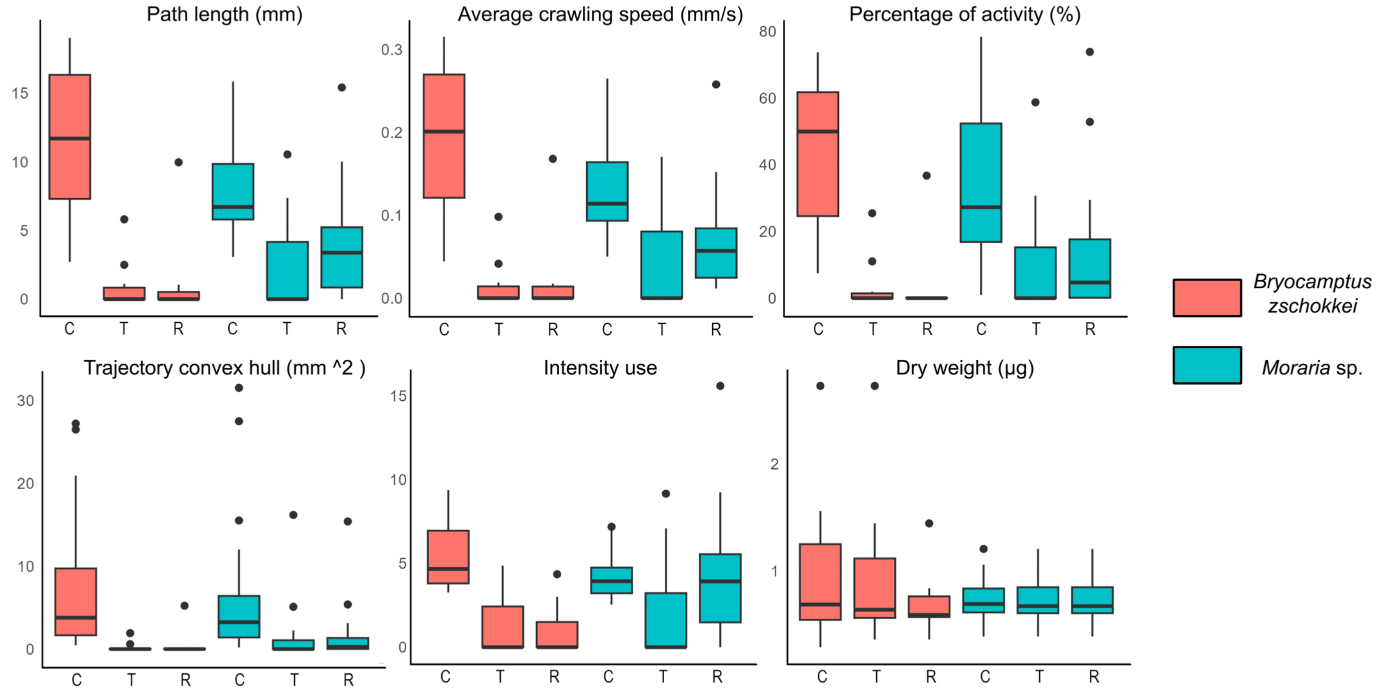

FIGURE 2: Comparative boxplots for five behavioral parameters and dry weight of *Moraria* sp. (stygobite, in blue) and *Bryocamptus zschokkei* (nonstygobite, in red). Each boxplot displays the median (central line), interquartile range (box boundaries), and outliers (individual points) for the levels of the exposure factor. C = control; T = treatment; R = recovery.

phase (77%; Tables 1 and 2 and Figure 2). The IU of *B. zschokkei* was significantly lower in the treatment (78%) and recovery (81%) phases compared to the control. The IU of *Moraria* sp. was lower in the treatment (52%) compared with the control; however, we highlighted no significant difference in the IU in the control phase with respect to the recovery phase for this species (Tables 1 and 2 and Figure 2).

Risk ratios

We extracted 116,832 MEC values of PCE from WATERBASE. The data were from 2007 to 2020 for five countries (Austria, Germany, Spain, Italy, Romania), involving 19,471 samples (Supporting Information, Table S2). Austria and Spain showed substantial and consistent PCE monitoring activity across the whole period, while Germany showed a late uptick. In contrast, Italy and Romania showed minimal monitoring activity, while the remaining member states seem not to have yet commenced the monitoring of this PMT/vPMT substance in their groundwaters. Based on the data extracted from the database, PCE was identified at concentrations exceeding the instrumental limit (0.3 ng/L) in all five countries (Supporting Information, Table S2). Perchloroethylene concentrations varied widely, ranging from 0.0003 (Austria in 2009) to 414 µg/L (Austria in 2015). The majority of the samples (>90%) showed a risk ratio <1 when using the PNECw (51 µg/L PCE), indicating no risk (Supporting Information, Table S2; Figure 3). However, when the PNECgw (32 ng/L PCE) was applied, >80% of the samples exhibited a risk ratio >1, highlighting a widespread risk to groundwater ecosystems (Supporting Information, Table S2; Figure 3). In addition, a small percentage of samples showed risk ratios exceeding 100 and 1,000 when the PNECw was used, while the risk ratios exceeded 10,000 and even 100,000 when the PNECgw was applied (Figure 3).

DISCUSSION

When drafting the present study, we found a lack of previous studies focusing on the behavioral effects of subchronic exposure to PCE on freshwater invertebrates. This research gap was also highlighted by Sárkány-Kiss et al. (2012), who noted the limited availability of literature on the impact of chlorinated hydrocarbons on aquatic ecosystems. While the environmental and public health consequences of widespread PCE contamination have been extensively studied because of its association with human health risks and the impairment of groundwater suitability for drinking purposes (Chiu et al., 2013), the effects on stygobitic species have been predicted (Di Lorenzo, Borgoni, et al., 2015) but had remained unexplored until now.

We observed significant mortality in both *Moraria* sp. and *B. zschokkei* under treatment with 32 ng/L PCE, with the non-stygobitic species *B. zschokkei* experiencing particularly high losses (50% mortality). Previous research has shown that PCE can cause 50% mortality in *Ceriodaphnia dubia* and *D. magna* at concentrations >0.8 mg/L within a maximum of 7 days (Niederlehner et al., 1998; Richter et al., 1983). The difference in PCE sensitivity between the daphnids and the copepod species of the present study is substantial. The reason for this remarkable difference is not fully understood. However, several key factors may contribute. Water flea species, such as those from the genus *Daphnia* or *Ceriodaphnia*, exhibit a higher ecological tolerance than the copepod species we investigated. They have a nearly worldwide distribution, inhabiting diverse water bodies ranging from fresh to brackish environments, including small temporary pools and large lakes (Ebert, 2022). In addition, studies have revealed the rapid adaptive evolution of different *Daphnia* species to various environmental factors, including the density of predatory fish, heavy metal pollution, increased density of toxic cyanobacteria, and temporal dynamics in eutrophication (Brede et al., 2009).

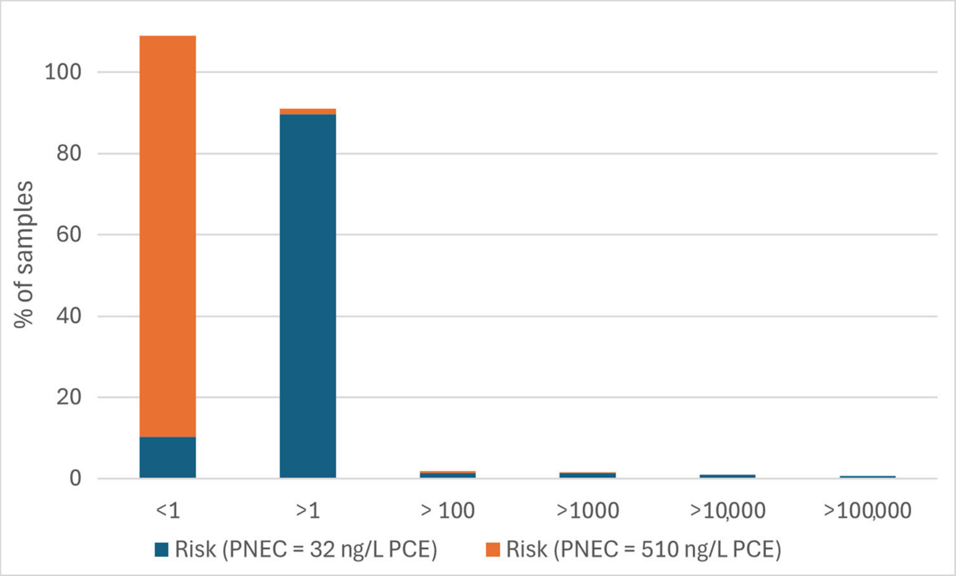

FIGURE 3: Percentage of samples exhibiting varying levels of risk based on ratios of measured environmental concentration to a predicted-no-effect concentration (PNEC) for groundwater of 32 ng/L (present study; blue bars) and a PNEC for aquatic ecosystems of 51 µg/L (orange bars). PEC = perchloroethylene.

This rapid adaptability might confer a broader range of tolerances to contaminants.

The substantial mortality observed in our subchronic trial underscores the high PCE toxicity for freshwater copepod species. We found that subchronic exposure to 32 ng/L PCE also affected the crawling behavior of the two species. Although the impact of PCE was comparable for both species in terms of tortuosity (IU) and CH, differences in the species' ecophysiology appeared to influence PL, AS, and PA. In detail, PCE substantially reduced the PL, AS, and PA of both species ≥70%. However, although *Moraria* sp. partially improved during recovery, *B. zschokkei* showed a persistent reduction in the three variables after the clearance phase. Reduced PL, AS, and PA in many invertebrate species have been linked to decreased energy reserves due to impaired foraging, which can lead to reduced growth and reproductive success (Bertram et al., 2022). Both species exhibited ≥70% reduction in CH and a consistent (≥50%) reduction in the tortuosity (IU) during exposure to PCE, with only partial recovery observed for the stygobitic species after the clearance. A reduction in tortuosity and CH typically means that the path has become more linear and that the organism explores a smaller portion of its environment (Almeida et al., 2010; Heuschele et al., 2020; Loretto & Vieira, 2005). This means that (i) the organism is not utilizing its habitat to its full potential, (ii) it may be less efficient at avoiding predators (the organism is more predictable and easier for predators to catch), (iii) it may miss potential food resources and (iv) sexual mates (Almeida et al., 2010; Heuschele et al., 2020; Loretto & Vieira, 2005). The reduction in encounters between mating partners leads to a decrease in population growth while hindering the ability of both species to relocate in response to disturbances (Gerritsen & Strickler, 1977; Mermillod-Blondin et al., 2023). Impairments in locomotion traits may indicate lethargy (narcotic effect) or impaired mobility (neurotoxicity). Our results suggest that PCE might have a neurotoxic effect on both species at 32 ng/L, likely associated with the disruption of neurotransmitters such as acetylcholine, dopamine, and serotonin (Altmann et al., 1990; Honma et al., 1980; Perrin et al., 2007). This specific mode of action is more plausible than nonpolar narcosis at low effect concentrations, as documented in rats (Honma et al., 1980). Because of the limited understanding of PCE toxicokinetics in aquatic invertebrates, we can only cautiously hypothesize that a similar mechanism may be at play for *B. zschokkei* and *Moraria* sp. On the other hand, a range of toxicants is recognized for its potential to disrupt neurotransmitter systems in both stygobitic and nonstygobitic copepods, leading to changes in swimming behaviors (Di Cicco et al., 2021, 2022). The existing literature focusing on *D. magna* demonstrates a clear link between impaired swimming capabilities and altered neurotransmission attributable to sublethal exposure to various toxic substances (see Bownik & Pawlik-Skowrońska, 2019; Parolini et al., 2018; Ren et al., 2017).

The substantial impairment of the crawling activity observed in both *Moraria* sp. and *B. zschokkei* as a result of PCE exposure has significant implications for the ecosystem services these species provide. Immobility and decreased habitat exploration diminish the efficiency of sediment remixing, crucial for preventing sediment clogging (Mermillod-Blondin et al., 2023) and porewater displacement (Giere, 2009). Crawling limitation also likely impairs feeding rates with consequences on carbon recycling (Mermillod-Blondin et al., 2023). The reduced PL and CH impede the dispersal of feces, which serve to boost microbial activity (Mermillod-Blondin et al., 2023). Although a reduction in ecosystem services provided by *B. zschokkei* may be buffered by the high abundance of this nonstygobitic species, that of *Moraria* sp. is likely beyond recovery. Stygobitic populations inherently show low abundances, due to low fertility rates, which makes recovery after disturbance nearly unattainable (Hose et al., 2022). Consequently, this results in a permanent depletion of the ecosystem services this species may offer. Sublethal laboratory-based toxicity assays, though effective at isolating and assessing specific pollutant risks, are not suitable for predicting cascading effects at the population, community, and ecosystem levels (see Ellison et al., 2016). Hence, the exact ecological consequences of sublethal PCE exposure on groundwater communities might be more severe than expected. Given that many PMT chemicals are ubiquitous in groundwaters (Huang et al., 2023), there is a substantial risk that other stygobitic species, which are currently understudied, may also be threatened. The presence of multiple PMT chemicals in groundwater could lead to compounded effects, further endangering these delicate ecosystems. Finally, persistent and widespread groundwater contamination might pose a chronic risk to groundwater communities (Fleeger et al., 2003).

The absence of recovery in the movement variables following PCE clearance in *B. zschokkei* is likely attributable to its higher susceptibility to intoxication compared to the stygobitic *Moraria* sp. Previous research has demonstrated that nonstygobitic species generally exhibit higher metabolic rates than their epigean counterparts (Hose et al., 2022). For copepods specifically, the metabolic rates of nonstygobitic cyclopoids can be up to seven times higher than those of related stygobitic species within the same family (Di Lorenzo, Di Marzio, et al., 2015). We lack direct metabolic rate comparisons between *Moraria* sp. and *B. zschokkei*. However, our results suggest significantly higher metabolic rates in *B. zschokkei* and, likely, more elevated PCE uptake during exposure. Despite potentially higher detoxification rates, they were evidently insufficient for *B. zschokkei* to ensure complete PCE clearance within the 7-day period. The greater reduction in all movement variables observed in *B. zschokkei* compared to *Moraria* sp. under PCE exposure further supports the hypothesis of higher uptake rates.

Our findings did not show significant differences in the path tortuosity and CH of the two species. Our finding suggests that the nonstygobitic *B. zschokkei* shows some behavioral traits akin to those of *Moraria* sp., albeit showing higher speed, PL, and PA. This similarity supports the use of this species as a surrogate for *Moraria* sp. In addition, organisms that typically exhibit more dynamic and varied movements are sensitive indicators of environmental changes because deviations from their normal behavior are more evident. This further supports the use of *B. zschokkei* as a substitute for *Moraria* sp. in groundwater ERA.

The observation of egg production during the treatment and recovery phases of *Moraria* sp. represents a novel finding. The time frame between insemination and egg extrusion in *Moraria* sp. in natural conditions remains unknown. In the present study, eggs were extruded from two females during the treatment with PCE, between 31 and 60 days after the initial isolation, and during recovery, between 61 and 67 days, for an additional two females. It is unclear whether this time frame is typical for this species or if egg extrusion was stimulated by a hormetic effect of the toxic compound (Calabrese & Mattson, 2011), which may have persisted during the recovery period in cave water. Brown et al. (2003) found that at very low lindane concentrations (3.2 and 10 μg/L), there was a significant increase in the number of offspring produced per female of *B. zschokkei* compared with the controls. This outcome was interpreted as a hormetic effect. *Bryocamptus zschokkei* typically releases eggs shortly after insemination and can produce multiple broods from a single insemination. Our results showed that no females produced egg sacs during either the control or treatment/recovery phases, indicating that egg extrusion in *B. zschokkei* is possibly hindered by 32 ng/L PCE.

The analysis of risk revealed significant insights into the potential ecological impact of PCE contamination in groundwater. The comparison between the two PNEC values (32 ng/L and 51 μg/L) highlighted a stark difference in risk scenarios. with the majority of groundwater samples deemed unsafe for stygobitic species. This discrepancy underscores the need for revising regulatory standards to better protect groundwater environments. Furthermore, the presence of extreme risk ratios exceeding 10,000 and even 100,000 when using the PNECgw value of 32 ng/L highlighted the urgency for targeted remediation efforts in highly contaminated areas. Existing literature reveals that PCE is a PMT/vPMT groundwater pollutant globally, impacting thousands of sites across North America and other industrialized regions, with concentrations reaching up to milligrams per liter (Carter et al., 2012; Pecoraino et al., 2008; Svetina et al., 2024). These findings call for policymakers and environmental regulators to reconsider current PNECw values and implement more protective measures to safeguard groundwater ecosystems from the adverse effects of PCE contamination.

We acknowledge the presence of persisting knowledge gaps on PMT/vPMT chemicals that require attention. Specifically, the physiological and molecular mechanisms underlying the observed effects remain unknown and warrant further exploration. In addition, quantifying the loss of ecosystem services resulting from the reduction in crawling activities is crucial for a comprehensive understanding of the effective impacts. Conducting additional comparative dose–response analyses involving a broader range of groundwater species could offer more insights into species-specific vulnerabilities, thereby enhancing the efficacy of risk assessments. We emphasize that future research should explore a broader PCE concentration range and expand on other PMT/vPMT chemicals to establish unharmful levels and further refine the risk assessment of these substances in groundwater.

CONCLUSIONS

The present study has elucidated the subchronic effects of PCE exposure on two freshwater copepod species, *Moraria* sp. (stygobitic) and *Bryocamptus zschokkei* (nonstygobitic), offering insights into the ecological risks posed by PCE in groundwater ecosystems. Our findings reveal that PCE, even at concentrations as low as 32 ng/L, significantly impairs the crawling behavior and survival of both species, with some notable differences in their recovery postexposure. The implications of the present study are twofold. Firstly, the observed sensitivity of both copepod species to PCE underscores the inadequacy of current PNECs for protecting groundwater ecosystems. Our results advocate for a fine-tuning of the European groundwater risk-assessment guidelines to encompass the ecological significance and vulnerability of stygobitic species, which exhibit unique physiological adaptations to groundwater and contribute to maintaining its quality and functionality. Secondly, the sensitivity of *B. zschokkei* to PCE, which is comparable to or greater than that of *Moraria* sp., indicates its potential as a substitute species in groundwater risk assessments.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5977.

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Data Availability Statement—The raw data can be obtained from the corresponding author (mattia.dicicco@univaq.it) on reasonable request.

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