

## Article

# Novel Protocol for Acute In Situ Ecotoxicity Test Using Native Crustaceans Applied to Groundwater Ecosystems

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**Abstract:** Current standardized laboratory test protocols use model species that have limitations to accurately assess native species responses to stressors. We developed and tested a novel acute *in situ* protocol for testing field-collected organisms. We used *Asellus aquaticus* and NaCl as a reference toxicant to test for the effects of location (laboratory vs. *in situ*), medium (synthetic vs. field water), substrate (presence vs. absence), and protocol replicability. We further tested the protocol using groundwater-adapted isopods: *Proasellus assaforensis* for the effect of location, *P. cavaticus* of medium and *P. lusitanicus* of substrate. Our results showed that *A. aquaticus*' lethality obtained with the novel acute *in situ* protocol did not significantly differ from those from laboratory testing. However, laboratory tested *P. assaforensis* showed a higher sensitivity, suggesting that its acclimation to laboratory conditions might have pernicious effects. *A. aquaticus* and *P. cavaticus* showed a higher mortality using synthetic medium *in situ* and under laboratory conditions, which overestimated the stressor's effect. Besides, substrate use had no significant effect. The novel acute *in situ* protocol allows the use of native species under realistic scenarios. It is particularly well adapted for assessing the risk of groundwater ecosystems but it can be applied to a wide range of ecosystems.

**Keywords:** groundwater ecology; groundwater ecotoxicology; stygofauna; in situ exposure; site-specific assessment

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## 1. Introduction

Ecotoxicological approaches are indispensable tools in environmental risk assessment (ERA). The inference of ecological impacts is challenged by the complex interactions within ecosystems [1]. The sensitivity to anthropogenic stressors varies markedly among species, and environmental variability, together with food web structure, plays an important role in determining the organisms' response [2,3].

Acute tests are well replicated and cost-effective tools, which are a reliable and essential support for ERA and decision making [4]. The use of acute tests has yielded a massive amount of ecotoxicological data, compiled and available in international databases (e.g., [5]). Despite the incremental complexity of ecotoxicological studies and the encouragement of ERA guidelines to use long-term studies [6], acute toxicity tests have remained an important source of data for species distributions models [5,7–10].

Nevertheless, ecotoxicological results provided in the databases usually correspond to the sensitivity of a reduced number of model species or of species reared under standard laboratory (lab) conditions, neglecting the use of native species [11]. Moreover, the

complex exposure dynamics of chemical mixtures observed in natural freshwater matrices leads to the application of site-specific assessment protocols, which experimentally prioritizes ecological realism [12].

In order to increase ecological realism, the use of relevant species for specific ecosystems is becoming widespread in ecotoxicity tests that focus on site-specific assessment [12–15]. Native organisms are presumably adapted to local environmental conditions and their use for *in situ* tests (e.g., *in situ* methodology using caged organisms) also prevents the introduction of exotic or invasive species [13]. However, the use of native species for ecotoxicology has some limitations since native organisms have often an unknown life history and some traits are difficult to assess prior to exposure to stressors, e.g., the organisms age [13].

Several studies are compelled to use field collected organisms in lab testing procedures because many species fail to adapt to lab holding conditions, and due to the lack of culturing methods for non-target species [16,17]. The use of field collected organisms under lab conditions potentially adds an extra stress associated to the manipulation, transportation and lab acclimation [13]. It may decrease the organisms' fitness, thereby overestimating the organisms' sensitivity to stressors [18]. Moreover, natural populations often show a limited number of specimens. Therefore, the use of field collected organisms often implies an increment in experimental costs compared to testing lab cultured organisms, due to repeated sampling [12,16].

Establishing lab cultures of organisms for ecotoxicological testing is particularly challenging for a number of ecosystems including subterranean, anchialine, and deep-sea ecosystems [16,17]. Environmental conditions in these difficult-to-access ecosystems cannot be reproduced in the lab and the ecological requirements of many species are not known. Still, a realistic ecotoxicological assessment of anthropogenic stressors is essential to define thresholds for establishing conservation measures in these ecosystems [16,19].

Generating ecotoxicological data for groundwater-adapted organisms is especially challenging [16]. Access to groundwater ecosystems is remotely small compared to their spatial extent, which makes their biodiversity largely unknown to science [20]. Furthermore, groundwater-adapted fauna is unique, composed of highly specialized organisms with characteristic morpho-physiological traits, small geographic ranges, and small populations [19]. The current scarcity of ecotoxicological data for ground-water organisms is strongly related to the difficulties in sampling, transporting, and rearing them in the lab [16,19,21]. Hence, groundwater ERA frequently relies on broad-based extrapolations such as using responses of surface organisms as surrogates and applying large assessment factors to accommodate data uncertainty [16,22]. Still, many studies have demonstrated that the use of surrogate organisms may under or overestimate the assessment of the ecological risk with considerable pernicious ecological and economic effects [7,8,23,24].

In this context, the aim of our work was to develop a novel protocol for acute *in situ* testing that can be used as a reliable alternative to standard acute lab tests for the assessment of anthropogenic stressors in native groundwater crustaceans. Since groundwater organisms are difficult to sample, have small populations, and the number of organisms available for ecotoxicity testing are extremely limited [16,19], we used the freshwater isopod *Asellus aquaticus* to develop the protocol. NaCl was selected as a stressor and lethal responses of asellids to osmotic stress were estimated after 48 and 96 h of exposure. We tested for the most suitable acute exposure conditions, which included testing the effects of the following variables:

- (1) Location—*in situ* vs. standard lab conditions—since groundwater species are difficult to transport and maintain in the lab [16,19];
- (2) Test medium—synthetic vs. filtered field water—considering that organisms are presumably adapted to the physico-chemical conditions of the water in which they live [15,25];
- (3) Relevance of substrate—presence vs. absence—because asellids are thigmotactic benthic organisms with a preference for rough surfaces [26];

(4) Replicability: testing was repeated both in the lab and *in situ* in two consecutive years.

Then, we applied the protocol to three groundwater-adapted isopods living in caves, *Proasellus assaforensis*, *P. cavaticus* and *P. lusitanicus*, which we also used to test for the effect of location, medium, and substrate, respectively.

## 2. Materials and Methods

### 2.1. Development and Test Validation with *Asellus Aquaticus*

We selected as a test organism the freshwater species *A. aquaticus* (Linnaeus, 1758), and collected specimens from a population living in a small creek in the Botanical Garden in Copenhagen, Denmark (55°41'12.21" N, 12°34'23.83" E). Specimens were collected during the summer seasons of 2018 and 2019 (Figure 1). Specimens were individually picked from submerged stones or the streambed using brushes and placed randomly in sealable 1 L plastic containers filled with local freshwater. They were sorted under a portable stereomicroscope Leica EZ4. Specimens of similar size were selected to minimize age differences, and ovigerous females were excluded (i.e., those carrying eggs in the marsupium). Diseased and parasitized specimens were removed according to [27], and organisms were kept under observation for 24 h to discard the stock's use if 5% of the collected organisms were dead or showed signs of stress [28]. Specimens tested *in situ* were kept in a plastic container, which was deposited in the bottom of the stream (natural exposure temperatures available in Table S1). Specimens tested under lab conditions were transported in a portable cooler to the lab and acclimated in an environmental cabinet (Binder KB 240) at the mean summer temperature of the collection site, 17 °C [29], with a light : dark photoperiod of 16L : 8D during 24 h. All organisms tested were deposited in the collection of the Natural History Museum of Denmark.

Acute toxicity tests were carried out over 96 h using sodium chloride [CAS]: 7647-14-5 (Sigma-Aldrich, St Quentin-Fallavier, France, 99% purity) as a stressor. We tested for the effects of location, evaluating the differences between lab and *in situ* approaches; medium, using either synthetic or filtered field water; and substrate, performing the test either with or without a nylon net inserted into the testing vials. As recommended for ecotoxicity testing with groundwater-adapted isopods and to allow comparison with standard lab tests with crustaceans, we recorded mortality at two exposure times: 48 and 96 h [16,28,30]. To evaluate the replicability of the protocol, the acute *in situ* and lab tests were replicated one year later (summer of 2018 and 2019), using both synthetic water and filtered field water, while the use of substrate was discarded in the second replicate. In both replicates, tests were carried out at the same time *in situ* and under lab conditions (Figure 1).

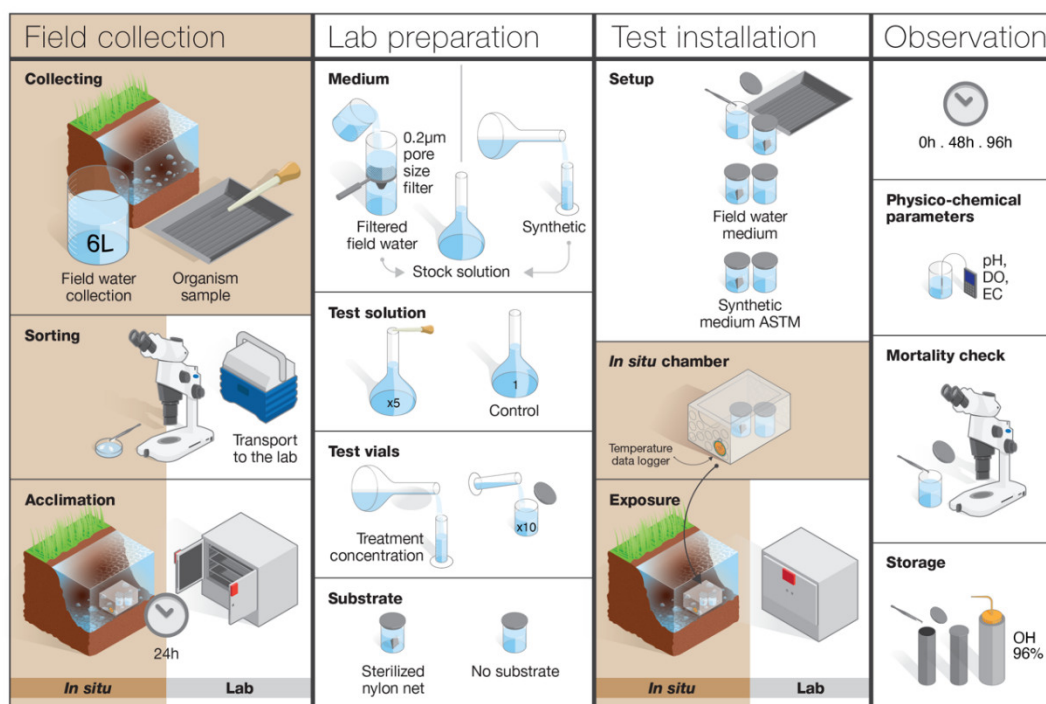


Figure 1. Overview of the experimental design.

The standard ASTM hard water medium (sterile distilled water adding 192 mg/L  $\text{NaHCO}_3$ , 120 mg/L  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 120 mg/L  $\text{MgSO}_4$ , and 8 mg/L KCl) was used as a synthetic medium. The field water was filtered immediately after collection, using a  $0.45 \mu\text{m}$  pore size, to ensure starvation conditions and remove potential predators. It was used as a control medium and diluent water. The salt concentration in field waters used as controls are provided in supplementary Table S2. The salt concentrations in the five treatments used during each test were checked using measurements of electrical conductivity (Table S3).

All tests were conducted in hermetic plastic vials (40 mL vol and 29 mm Ø) filled with 25 mL of testing medium, without aeration or food addition and a sterilized nylon net ( $2 \times 2 \text{ cm}$ ) was used as a substrate in the test where it was required. Organisms were tested individually to avoid cannibalism and interaction stress. A total of 10 specimens were tested per treatment concentration and control.

In the tests performed under lab conditions the test vials were placed in an environmental cabinet (KB 240, Binder, Hillerød, Denmark), with the same conditions as the acclimation period. For the test performed *in situ* during the experiment the vials were introduced in an *in situ* chamber. The *in situ* chamber was a transparent container with holes for the site water to flow around the vials. The chamber protected the vials and allowed them to be exposed to the natural environmental conditions (temperature and photoperiod). The temperature at which organisms were exposed was recorded hourly with a temperature datalogger placed in the chamber (TidbiT® v2 Temp UTBI-001, Onset, Bourne, MA, USA) (Figure 1, Table S1).

Test validation followed the OECD (2004) criteria, i.e., mortality rate in control below 10%, pH variation below 1.5 units, and DO variation smaller than 3 mg/L, throughout the test [16,30]. Mortality followed the OECD (2004) criteria, defined as a lack of movement or swimming after gentle stimulation by a sorting needle, and it was recorded at 48 h and 96 h in each test vial (Figure 1).

## 2.2. Testing Groundwater-Adapted Isopods

To evaluate the validity of the protocol for groundwater-adapted crustaceans, we selected three cave isopods of the genus *Proasellus* Dudich, 1925, which have been previously used in lab bioassays [31,32]. We used each of the three *Proasellus* species to test separately for the effect of medium, substrate, and location. Contrary to the surface water isopod *A. aquaticus*, groundwater isopods were present in too small numbers in their natural environment to test simultaneously for each species the effect of the three variables [15]. The study design and site for each species are provided in Table 1: *P. assaforensis* Afonso, 1988, *P. cavaticus* (Leydig, 1871) and *P. lusitanicus* (Frade, 1938) were used to test for the effect of location (lab versus *in situ* test), medium (synthetic vs. filtered field water), and substrate (with or without a nylon net), respectively.

**Table 1.** Collection sites and test conditions for the groundwater-adapted crustaceans *P. assaforensis*, *P. cavaticus*, and *P. lusitanicus*. T corresponds to the mean temperature during the *in situ* experiment or to the fixed temperature used in the lab. For each species, the larger “+” signs indicate the two modalities of the effect which was tested for.

| Species                | Site                 | Latitude       | Longitude      | T (°C)                  | Location |     | Medium    |             | Substrate |        |
|------------------------|----------------------|----------------|----------------|-------------------------|----------|-----|-----------|-------------|-----------|--------|
|                        |                      |                |                |                         | Cave     | Lab | Synthetic | Field Water | Net       | No Net |
| <i>P. assaforensis</i> | Assafora Cave        | 38°54′31.15″ N | −9°25′18.89″ E | 16.6 (cave)<br>16 (lab) | +        | +   |           | +           | +         |        |
| <i>P. cavaticus</i>    | Baume la Fraite Cave | 46°31′30″ N    | 5°44′02″ E     | 10.08                   | +        |     | +         | +           | +         |        |
| <i>P. lusitanicus</i>  | Olho de Mira Cave    | 39°32′29.01″ N | −8°43′19.93″ E | 13.05                   | +        |     |           | +           | +         | +      |

## 2.3. Field Water Characterization

The physico-chemical content of field water used as a medium was analyzed for each location (Table S2). Concentrations of TOC, N-NH<sub>4</sub><sup>+</sup>, N-NO<sub>2</sub><sup>−</sup>, N-NO<sub>3</sub><sup>−</sup>, and P-PO<sub>4</sub><sup>3−</sup> were determined spectrophotometrically (Varian Cary 50 UV-Vis, Agilent Technologies, Santa Clara, CA, USA). Chlorides were analyzed by the argentometric method and concentration of total organic carbon (TOC) by TOC analyzer (Multi N/C 3100, Analytik Jena, Jena, Germany). The concentrations of Na<sup>+</sup> K<sup>+</sup> Mg<sup>2+</sup> Ca<sup>2+</sup> and metals—Ag, Al, As, Cd, Co, Cu, Fe, Mn, Pb and Zn—were analyzed by ICP-MS (Agilent 7900ce, Agilent Technologies, USA). All methods were performed according to the American Water Works Association standards [33]. Temperature, pH, electrical conductivity, and dissolved oxygen were measured using a multi-parameter probe (Aquaprobe AP-2000, Aquaread, Broadstairs, England).

## 2.4. Statistical Analyses

Dose-response data for the first replicate test with *A. aquaticus* were fitted using a generalized linear model (glm) and probit link, with time (48 and 96 h), location (lab vs. *in situ*), medium (synthetic vs. filtered field water), and substrate (net vs. no net) as factors, and log (salinity treatment concentrations) as covariate, and interactions between all of them. A second glm model was performed including time, location, medium, and replicate (first—2018 and second—2019) as factors with their interactions, to analyze replicability. Dose response data for *P. assaforensis*, *P. cavaticus*, and *P. lusitanicus* were fitted with glm models including the controlled variables (either medium, substrate, or location) and time (48 and 96 h) as factors and their interactions were considered. All statistical analyses were performed in R 3.5.0 using the “stats”, “emmeans”, and “ggplot2” packages [34]. Lethal concentrations which affect 50% of the population (LC<sub>50</sub>) were obtained using the LabApplStat package [35]. Statistical differences (alpha level set at 0.05) in response to salt dose

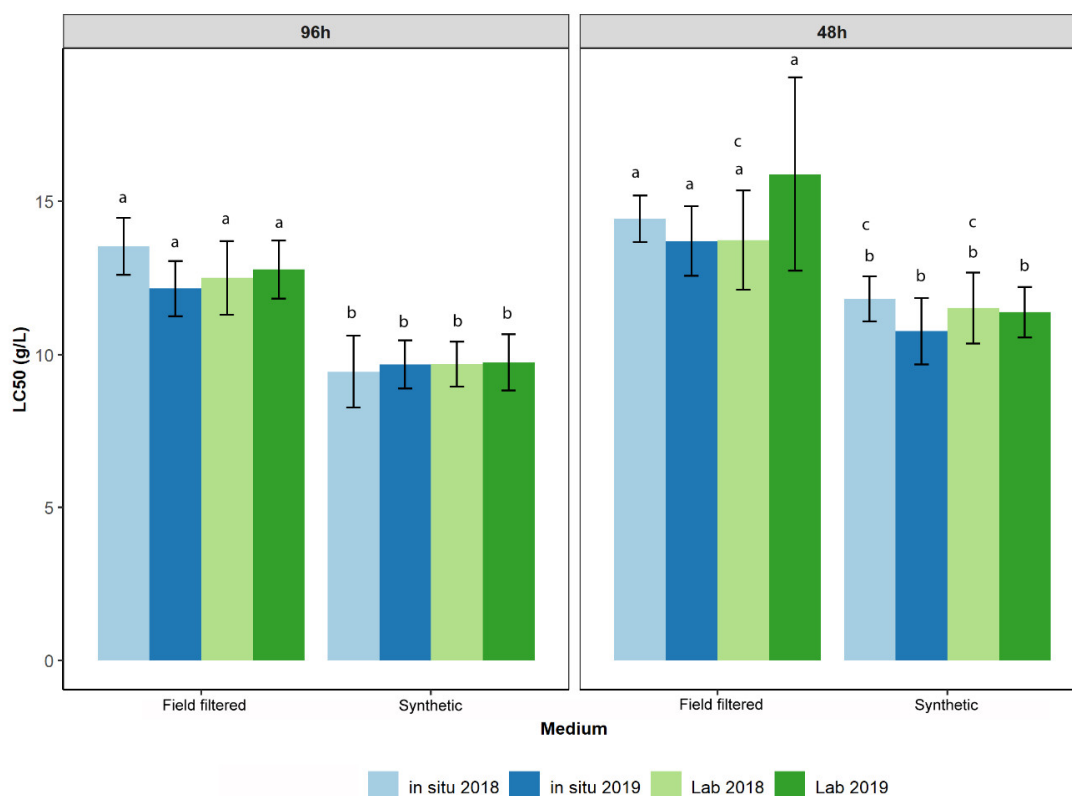
between different variables were tested using a paired one-way ANOVA with the emmeans package [36]. Data were visualized using the ggplot2 package [37].

### 3. Results

We found large variation in  $LC_{50}$  for each species depending on the test conditions. The acute sensitivity to NaCl, expressed as  $LC_{50}$  ranged from 9.4 g NaCl/L at 96 h to 15.8 g NaCl/L at 48 h for *A. aquaticus*; from 10.1 g NaCl/L at 96 h to 13.9 g NaCl/L at 48 h for *P. cavaticus*; from 12.7 g NaCl/L at 96 h to 16.9 g NaCl/L at 48 h for *P. lusitanicus*; and from 12.3 g NaCl/L at 96 h to 15.7 g NaCl/L at 48 h for *P. assaforensis* (Table S4).

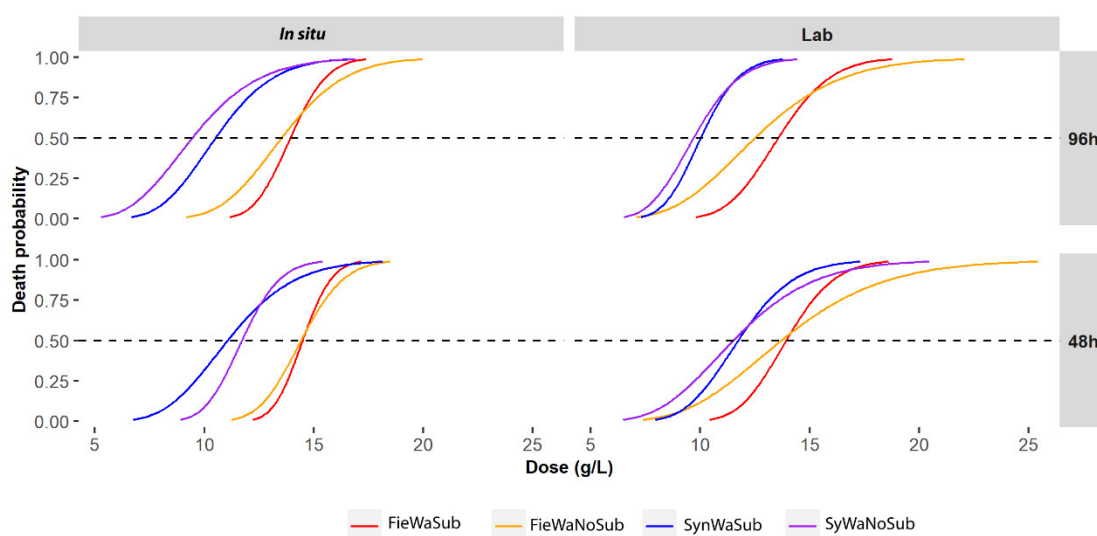
#### 3.1. Development and Test Validation with *Asellus Aquaticus*

For *A. aquaticus*, we found no significant differences in  $LC_{50}$  between *in situ* and lab experimental setups under the same conditions including comparison across the two replicates (Figures 2 and 3, Tables S5a and S6a). The  $LC_{50}$  were significantly lower with synthetic medium, except for the two comparisons between the 48 h filtered field-water lab test of the first replicate and the 48 h synthetic-water *in situ* and lab tests of the first replicate ( $p = 0.626$  and  $0.695$ , respectively) (Figures 2 and 3, and Tables S5b, S6b and S6c).



**Figure 2.** Differences in the lethal NaCl concentration values affecting 50% of test populations ( $LC_{50}$ ) at 48 and 96 h for *A. aquaticus* considering the different test medium conditions (field filtered or synthetic medium) and replicate tests performed in 2018 and 2019. Different letters near error bars indicate significant differences between lethal concentrations at 96 h and 48 h ( $p < 0.05$ ).

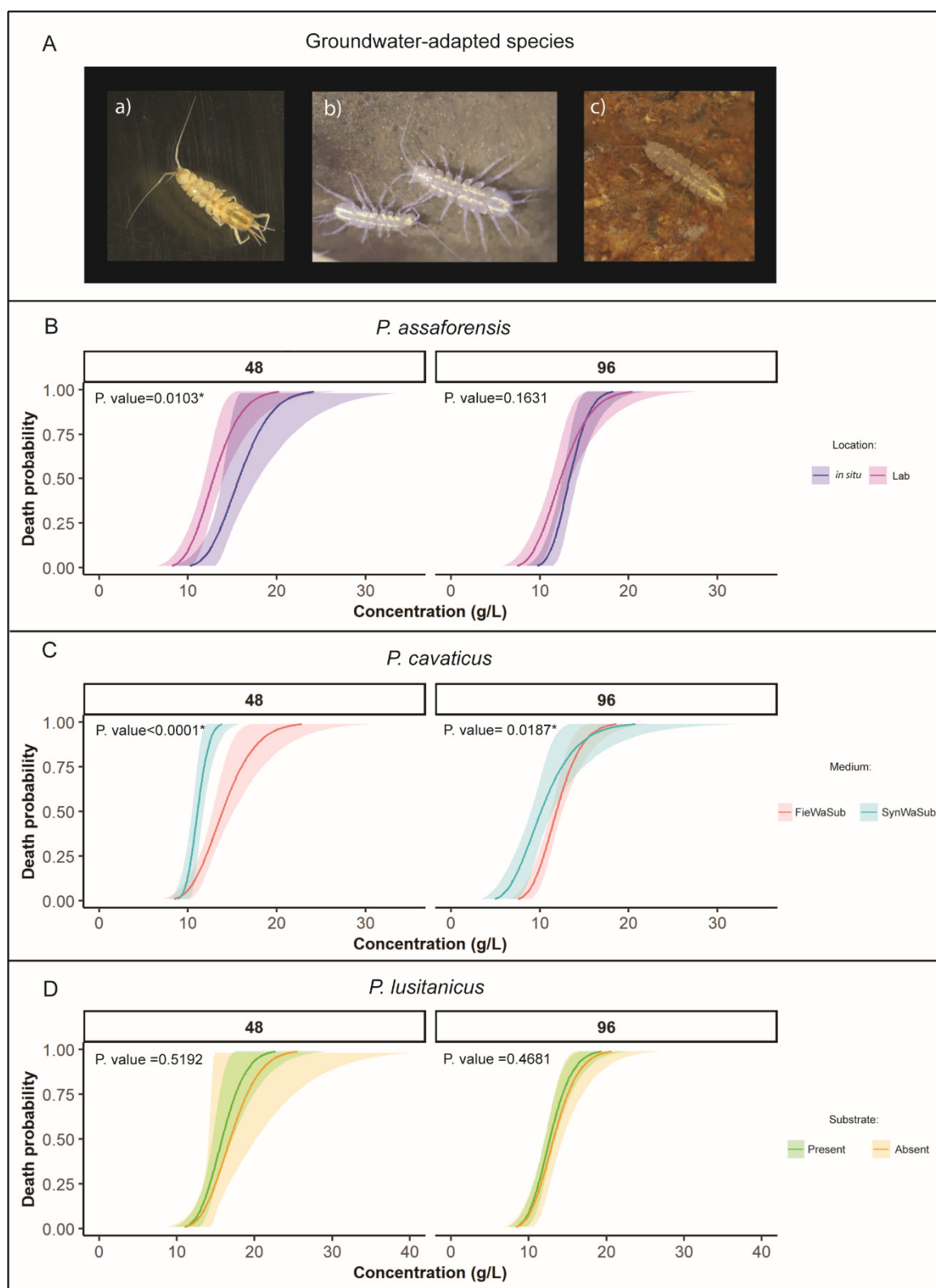
The effect of substrate (presence vs. absence of the net) tested in the first replicate experiment with *A. aquaticus* did not provide any significant differences on salinity sensitivity under the same experimental setups (Figure 3, Table S5c). In the study of the replicability, no significant differences in sensitivity were observed between the replicate lab experiments performed in 2018 and 2019, neither between the replicates for the *in situ* experiments, even though the temperature was slightly different between them (Figure 2, and Tables S1 and S6a).



**Figure 3.** Acute NaCl (g/L) dose-response curves for *A. aquaticus* *in situ* and under lab conditions at 48 h and 96 h for the first replicate experiment. Different test conditions: SynWaSub, synthetic ASTM water with substrate; SynWaNoSub, synthetic ASTM water without substrate; FieWaSub field collected water with substrate; FieWaNoSub, field collected water without substrate.

### 3.2. Testing Groundwater-Adapted Isopods

Contrarily to the results observed for *A. aquaticus*, *P. assaforensis* showed a higher sensitivity to NaCl under lab conditions than *in situ* at 48 h (Figure 4B). For the two other factors tested using groundwater-adapted species, the results were consistent with those observed for *A. aquaticus*. *P. cavaticus* was more sensitive to increasing concentrations of salt when using synthetic water as a medium (Figure 4C). Moreover, we found no statistical differences in the sensitivity of *P. lusitanicus* between the tests with and without substrate (Figure 4D).



**Figure 4.** NaCl dose-response curves for groundwater-adapted species at 48 and 96 h. **(A)** Habitus: (a) *Proasellus assaforensis*; (b) *P. cavaticus*; and (c) *P. lusitanicus*; **(B)** Comparison between *in situ* and lab conditions using *P. assaforensis*; **(C)** Medium comparison using *P. cavaticus in situ*; **(D)** Substrate use comparison using *P. lusitanicus*. See Table 1 for the experimental design used for each groundwater species. FieWaSub corresponds to field collected water and SynWaSub to synthetic ASTM medium. \* Significant value.

#### 4. Discussion

Acute *in situ* testing approaches have the wide advantage of using realistic environmental conditions. Additionally, it also allows the use of local specimens avoiding transportation and lab acclimation. This is particularly critical for groundwater-adapted fauna, which often have a very narrow thermal niche breadth [10,38,39].

*Asellus aquaticus* was selected as a model organism to develop and validate the protocol because it has recently been suggested as a model for biomonitoring environmental pollution [40]. *A. aquaticus* has been successfully used in lab and *in situ* ecotoxicological studies using field collected organisms [41–43], and stable lab cultures have been successfully raised [27,44]. *A. aquaticus* responded similarly to increasing NaCl concentrations *in situ* and under lab conditions using the same medium conditions. This indicates that transportation and acclimation to the lab did not affect the organisms' fitness, dismissing a possible overestimation of sensitivity for the tests performed under lab conditions.

Contrary to the results observed with *A. aquaticus*, the groundwater-adapted species *P. assaforensis* showed higher mortalities at 48 h in lab conditions than in cave conditions. Because thermal and light conditions were the same between the cave and the lab, we suggest that either the transportation or the acclimation process affected the fitness of *P. assaforensis*. This finding corroborates the difficulties encountered in a number of studies when running ecotoxicological testing under lab conditions with groundwater-adapted fauna [15,16,19]. It also suggests that *in situ* tests developed in this study are likely to generate more reliable acute data than lab tests for native crustaceans that are highly sensitive to transportation and rearing conditions.

The use of synthetic medium consistently resulted in higher sensitivity to NaCl than field water in both surface and groundwater isopods. Synthetic mediums are aimed to isolate the stressor effect from the interaction with the water matrix, therefore they are composed only of few salts [41,45,46]. However, the use of field collected water as a medium is already integrated in some standardized acute ecotoxicity tests, e.g., "clean surface water, groundwater or reconstituted water are acceptable as dilution water" [28]. Nevertheless, [25,47] suggested that the lack of trace and essential elements such as Cu in synthetic mediums may limit the organisms' survival. Moreover, synthetic medium has different physico-chemical properties than the natural medium in which the organisms are collected. Hence, the physico-chemistry of synthetic water may influence the organisms' physiology and interfere with the effect of the toxicants [48]. The synthetic water used in the present protocol was consistently less concentrated in salts than the site water of the four tested species. Hence, the acclimation to the synthetic water may have induced a first osmotic stress to which we added a second inverse osmotic stress when submitting the organisms to increasing concentrations of NaCl.

We found no differences in mortality between tests performed with and without substrate both for *A. aquaticus* and *P. lusitanicus*. The use of a substrate is recommended when testing epibenthic organisms [26] because they can hardly attach to the vials surface. In natural conditions, many epibenthic organisms also tend to hide below diverse substrates (e.g., stones, leaves). Hence, the lack of a substrate may increase energetic expenditures due to increased locomotory activity or increased levels of stress, thereby increasing mortality. In the present study, we observed that intense pleopod movements at high NaCl concentrations raised the organisms in the water column and that they could no longer stabilize themselves. However, substrate may also bias mortality if the substance to be tested can adsorb onto it. As long as the potential for adsorption is low, as is the case with NaCl, we recommend using substrates when testing epibenthic organisms.

By repeating the test after a year with *A. aquaticus* we were able to confirm the response patterns observed in the first replicate study. Although the mean environmental temperatures recorded during the two *in situ* tests of *A. aquaticus* were slightly lower (−1.3 °C, in 2018) or higher (+4 °C, in 2019) than the medium summer temperature used as a fixed temperature in lab test, we found no significant difference in the response to NaCl

between replicates at the lab and *in situ* testing. This suggests that the difference in temperature between *in situ* and lab tests was small enough to avoid causing a thermal shock that would have influenced the response of *A. aquaticus* to salt (see [39]).

Acute lab tests are performed at stable temperatures within the range of the organism's optimal conditions. Yet, temperature stability in ecotoxicity testing lacks environmental realism [48,49]. Temperature affects the physiological processes of ectotherm organisms such as metabolic rate and locomotory and feeding activities, which influence the stressors' effects on organisms in terms of uptake, elimination, and detoxication rates of chemicals [50–52]. Therefore, acute *in situ* testing can be used to study the response of stressors considering a realistic thermal exposure. However, the uncertainty of the temperature variability of *in situ* test disappears when working with groundwater organisms. The stable annual temperatures usually observed in groundwater habitats [10] and the lack of fast temperature fluctuation facilitate the comparison between *in situ* and lab tests.

Acute lab testing typically evaluates the lethal effect of a chemical under barren conditions, using controlled environmental variables. This allows comparing the obtained results with those available in databases [49]. A major caveat of acute lab testing is the lack of environmental realism [13], which is provided by *in situ* methodologies. However, standardization is more difficult because of the environmental heterogeneity, especially regarding the physico-chemical water conditions (and temperature in surface environments). *In situ* testing approaches have been performed by exposing model or non-native organisms to site-specific conditions (e.g., *in situ* caging experiments [14]). Compared to lab and *in situ* testing using caged organisms exposed to a continuum water flux (i.e., open water circulation), our novel *in situ* protocol has the advantage of using site-specific organisms and field water as medium (i.e., with known physico-chemical characteristics). The novel acute *in situ* testing provides an efficient method for evaluating site-specific scenarios for potential biodiversity changes in response to variation in the concentration of selected stressors. In the present study, NaCl was selected for developing the *in situ* test, but the use of the test can be expanded and applied to other stressors. Moreover, the use of native organisms under *in situ* conditions reduces transportation, acclimation and the risk of introducing species into the ecosystems.

There are three prerequisites for deploying acute *in situ* testing:

- (i) Site and stressors' selection: broad knowledge of the selected site including the environmental characterization of the anthropogenic hazard. It implies the characterization of sources and pathways of contaminants which affect the ecosystem and the identification of potential new pollutants [53–57];
- (ii) Water matrix characterization: description of the physico-chemical characteristics of water used as a medium and ensuring the stability of the stressor under study [28,30]. Indeed, the behavior of the stressor can be affected by interactions with several characteristics of the water matrix, including pH and temperature, thereby modifying the concentrations to which the organisms are exposed. Chemical analyses also provide baseline data of the chemicals to which organisms are exposed;
- (iii) Selection of the test organisms: an ecosystem characterization is recommended for selecting organisms that are representative of the study ecosystem and have an important role in the food web [23,58]. In the present study, we focused on crustaceans because of their abundance and ecological relevance in groundwater ecosystems [59]. Specifically, isopods are primary consumers, an important food source for other trophic levels, and are included in regulatory assessment schemes for chemicals [41]. However, the novel acute *in situ* protocol can be adapted for other organisms, including other crustacean groups.

## 5. Conclusions

The proposed protocol for acute *in situ* testing has the potential to increase the availability of ecotoxicological data for ERA of groundwater ecosystems. This is fundamental because the use of surface organisms as surrogates for groundwater ecosystem risk assessment is increasingly debated. The proposed protocol is actually being used to test for differences in the sensitivity of multiple surface and groundwater species of asellid crustaceans to different stressors. When analyzed within the context of a comparative phylogenetic approach, such sensitivity data may prove to be essential to test for different sensitivity among surface and groundwater organisms, thereby bringing robust arguments into the debate on the use of surrogate organisms in ERA.

Our protocol represents a major step towards generating acute ecotoxicity data for groundwater species (and other native crustaceans in a wide range of ecosystems) that can be used for ERA. However, future research on groundwater ecotoxicity should also focus on developing new methods for assessing chronic impacts and sublethal endpoints [19].

**Supplementary Materials:** The following are available online at [www.mdpi.com/2073-4441/13/8/1132/s1](http://www.mdpi.com/2073-4441/13/8/1132/s1), Table S1: Temperatures measured in the *in situ* climatic chamber during the experiments. (a) *A. aquaticus* first tests replicate, (b) *A. aquaticus* second tests replicate, (c) *P. assaforensis*, (d) *P. cavaticus*, and (e) *P. lusitanicus*; Table S2: Chemical analysis and parameters of synthetic and field water of each locality used as medium. EC, electrical conductivity; DO, dissolved oxygen; TOC, total organic carbon; n.d., not detected; Table S3: NaCl nominal concentration (g NaCl/L) and electrical conductivity (EC mS/cm) for the different experiments; Table S4: Variability in the lethal NaCl concentration values affecting 50% of test populations (LC<sub>50</sub>) at 48–96 h exposure for the tested species. Location: *in situ* or laboratory (lab) conditions. Medium: synthetic water (SynWa) or field water (FilWa); Substrate: with a substrate (net) or without (absence); Table S5: pairwise comparisons for the first replicate test with *A. aquaticus*. Variables analyzed: (a) the effect of location within time and medium; (b) the effect of medium within time; and (c) the effect of the substrate within time, medium and location. Pink pattern: Statistically significant results; Table S6: pairwise comparisons considering the two replicates test with *A. aquaticus*. Variables analyzed: (a) the effect of location considering replicates independently within medium and time; (b) effect of medium within time; and (c) the effect of all variables across each other. Pink pattern: Statistically significant results.

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