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# Groundwater Amphipods of the Hyporheic Interstitial: A Case Study from Luxembourg and The Greater Region

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**Abstract:** Hyporheic interstitials are ecologically dynamic and often neglected aquatic environments. In this study, groundwater amphipods (Niphargidae, Pseudoniphargidae and Crangonyctidae) were sampled in hyporheic interstitials throughout Luxembourg and The Greater Region and specimens were analyzed by DNA barcoding. Sites characterized by gravel or coarse sand and high flow velocities of incoming water were the most species- and specimen-rich. A total of 11 species were detected, of which the *Niphargus aquilex* lineage EF of the *N. aquilex* cryptic species complex and juveniles of *N. schellenbergi* dominated the data set, but an unknown lineage of the *N. aquilex*-complex was also found. Some regions appeared to be devoid of groundwater amphipods. We hypothesize that underlying sandstone formations resulting in low sediment porosity may prevent physical colonization, but also that historical water pollution may have a long-lasting effect, either through the persistence of contaminants in the sediment or low recolonization rates of affected populations. In summary, our approach expanded regional species inventories, confirmed known occurrences, and validated previously questionable or historical morphology-based detections. In addition, the collection of absence data provided valuable insights into local extinctions. Finally, DNA-based distribution data are needed to gather information on the ecological affinities of groundwater amphipods to understudied hyporheic interstitial environments.

**Keywords:** subterranean biology; hyporheos; DNA barcoding; groundwater; Amphipoda

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## 1. Introduction

Amphipods are a very species-rich group of malacostracan crustaceans (Crustacea: Malacostraca: Amphipoda). They comprise at least 500 known obligate groundwater species, so called stygobionts, of which four families (Niphargidae, Pseudoniphargidae, Bogidiellidae and Crangonyctidae) are present in Central Europe [1,2]. As such, they can be frequently encountered in cave waters, springs and aquifers, where they predominantly but not exclusively act as predators [3]. Exact species numbers are hard to provide, and higher taxonomies are quickly outdated, as both species delimitations and taxonomic placements are the subject of frequent and ongoing scientific discussions [4–7]. However, the integration of molecular genetic methods (e.g., DNA barcoding) in recent years has led to the identification of many cryptic species complexes that are currently being unraveled, and, thus, to a significant increase in the number of known groundwater amphipod species [8–12]. Because of this unclear and dynamic taxonomic situation, many purely morphological records and their associated ecological information are questionable (e.g., [13–16]). This also means that there is a lack of reliable information on the habitat preferences and lifestyles of most groundwater amphipods. There are a few notable exceptions to the ecology of subterranean amphipods in the recent literature, where species identification has been based on molecular data (e.g., [2,17,18]).

The hyporheic interstitial is an often neglected environment, not only for groundwater amphipods, mainly due to sampling difficulties. It is found under and beside flowing water, where groundwater and surface water mix. Due to this mixing, it is usually considered an ecotone and is characterized by sharp gradients of biotic and abiotic factors, such as light, temperature, load of inorganic and organic substances or dissolved oxygen [19,20]. Dissolved oxygen and organic matter are transported into the hyporheic interstitial by downwelling water, whereas upwelling groundwater transports inorganic nutrients into the stream [19]. The type and size of the sediment particles forming the hyporheic interstitial are additional important parameters, as they determine the permeability and accessibility of this environment [19,21], ultimately affecting ecosystem functions. Healthy interstitials increase the resilience of a water body because they can act as a buffer for stressors in the water by filtering, storing and thus removing stressors from the water column [22–24]. Many species use the hyporheic interstitial as a nursery or spawning habitat or as a retreat during unfavorable environmental conditions in the above water column [19]. However, the hyporheic interstitial is also home to a variety of specialized meiofaunal species [19], but the biodiversity of hyporheic communities is poorly understood due to difficulties in the identification of minute individuals, habitat inaccessibility and declining taxonomic expertise [25].

This study aims to increase the knowledge on groundwater amphipods occurring in the hyporheic interstitials of Luxembourg and The Greater Region, the latter including Rhineland-Palatinate and Saarland (Germany), Lorraine (France) and Wallonia (Belgium). In principle, the groundwater amphipod fauna have been already extensively studied in Luxembourg in recent years. However, older taxonomic determinations were based on morphological characters alone [26], or investigations were mainly based on other habitat types, e.g., springs [27] or artificial caverns [28]. At the same time, it has been noted that the identification of groundwater amphipods without DNA-based methods is highly error-prone [28]. This fact was also emphasized by other authors for their regional groundwater amphipod fauna [29–32]. The current setting therefore provided a suitable framework for applying DNA barcoding to assess the diversity of groundwater amphipods in hyporheic interstitials of Luxembourg and The Greater Region. We hypothesized that the integration of molecular data would help identify frequently encountered juveniles and members of cryptic species complexes (e.g., *Niphargus aquilex*-complex and *N. kochianus*-complex), which are known to occur in the study region, and thus will increase the known species diversity of the study area (Hypothesis 1). Furthermore, we hypothesized that small-sized groundwater amphipod species (e.g., some members of *N. aquilex*-complex, *N. kochianus*-complex, *Niphargellus nolli* Schellenberg, 1938 and *Microniphargus leruthi* Schellenberg, 1934) would dominate the hyporheic interstitial due to size limitations of large-sized species (e.g., *Niphargus fontanus* Spence Bate, 1859, *N. schellenbergi* S. Karaman, 1932 and *N. puteanus* (C.L. Koch in Panzer, 1836)) (Hypothesis 2).

## 2. Materials and Methods

### *2.1. Sampling Sites and Sampling Method*

From June 2017 to July 2021, hyporheic interstitials [33] were investigated in our study area, which included the entire Grand Duchy of Luxembourg, the Province of Luxembourg and some sites in the immediate vicinity of the Province of Liège (both in Belgium), the states of Saarland and Rhineland-Palatinate and the neighboring areas in western Hesse and western Baden-Württemberg (all in Germany), and a single site in the Moselle department (France) (Figure 1, Table S1).

To benefit from low water levels, riverbanks were preferably investigated in the summer at a distance of a couple of m from the stream bank. Whenever possible, gravel banks located on river islands were included in the survey. The Karaman–Chappuis method was applied to collect groundwater amphipods [34,35]. A small pickaxe was used to dig pits in the gravel until they were about 10 cm below the water level. Wherever possible, pits were characterized according to their predominant substrate type ('silt' [<0.063 mm], 'fine and medium sand' [0.063–0.63 mm], 'coarse sand' [0.63–2 mm], 'gravel' [>2 mm]), the inflow direction of the water ('upwelling', 'from side', 'both') and the inflow rate ('<1 L/min', '1–10 L/min', '>10 L/min') (Table S2). The incoming water was investigated for groundwater amphipods for approx. 30 min, but less in the case of high inflow rates. Depending on the speed of the inflow, between 1 and about 50 L of water were examined. The water and a small part of the sediment collected at the bottom of the pit were sieved through a set of sieves (mesh sizes of 5, 1, 0.5 and 0.2 mm). The upper sieve retained coarse gravel and stones. In rare cases, larger amphipods were found in the 1 mm mesh sieve, while most individuals slipped through to the 0.5 mm mesh sieve and rarely to the 0.2 mm sieve. Gammarids (Amphipoda: Gammaridae) were sorted out on the spot. Eyeless white amphipods were transferred individually to 96% undenatured ethanol and later stored at −20 °C.

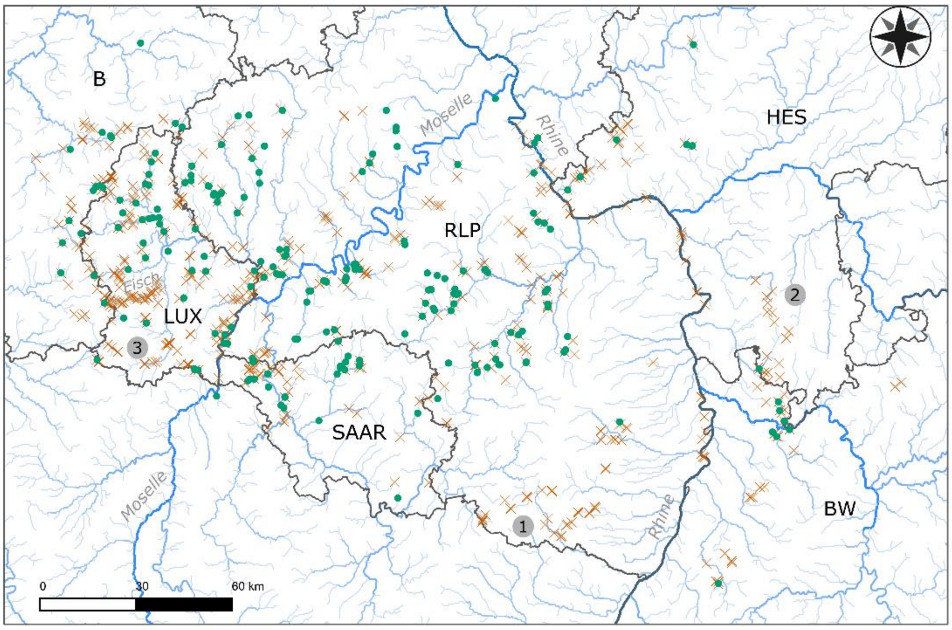


**Figure 1.** Overview of sampling sites in hyporheic interstitials. Green circles: groundwater amphipods present; orange crosses: no groundwater amphipods found. B = Belgium, BW = Baden-Württemberg (Germany), HES = Hesse (Germany), LUX = Luxembourg, RLP = Rhineland-Palatinate (Germany), SAAR = Saarland (Germany). 1 = Palatinate Forest mountain range (Germany), 2 = western Odenwald mountain range (Germany), 3 = Minett and southern Gutland regions (Luxembourg). The hydrological network and administrative boundaries are indicated, and the map is cropped to show the sampling sites of our core study area.

Pearson's Chi$^2$ test [36] was used to compare the observed patterns of groundwater amphipod detection with the expectation that the assessed parameters of the collection pits (i.e., sediment type, inflow direction and inflow rate) had no effect on the presence (or the probability of detection) of groundwater amphipods.

### 2.2. Molecular Identification of Specimens

Specimens were morphologically investigated using a Stereo Zoom Microscope 4002 (Wang BioMedical, Wageningen, The Netherlands). In general, one specimen per site was barcoded, but in the case of different morphotypes, several specimens from the same site were barcoded, even though they may represent different developmental stages of the same species. 273 specimens from 267 sites were DNA barcoded in this study, and enriched by 19 barcoded specimens from 9 interstitial sites in Luxembourg from another source [28]. Specimens were shipped to the laboratory of the Aquatic Ecosystem Research Group (University of Duisburg-Essen, Germany) for DNA barcoding of the 658 bp-long cytochrome *c* oxidase subunit 1 (COI) marker typically used for animals. The obtained COI sequences were trimmed and edited using Geneious R6 version 6.1.8 [37]. The Neighbor Joining algorithm in Mega X [38] was run with p-distance, 1000 bootstrap replicates and the pairwise deletion option to cluster sequences and identify species according to the latest regional taxonomic revisions and annotations [11,28,30,39,40]. Members of the *N. aquilex*-complex were annotated as the respective (sub)lineage.

## 3. Results

### 3.1. Characterization of Hyporheic Interstitials and Regional Patterns

A total of 1006 pits were created, of which 267 contained a total of 957 groundwater amphipods (Figure 1). It was not possible to characterize all the investigated hyporheic interstitials according to their predominant sediment type, since in some places, sediment types were highly intermixed, with no predominate sediment type present (Figure 2, Table S2). However, given the respective pool of characterized sampling sites, groundwater amphipods were underrepresented in silty sediments and medium and fine sands, and more frequent than expected in coarse sands [$X^2$ (3, $N$ = 225) = 22.1698, $p < 0.0001$]. There was also a higher chance of collecting amphipods under upwelling conditions [$X^2$ (2, $N$ = 172) = 14.3335, $p < 0.001$] and at high inflow rates [$X^2$ (2, $N$ = 225) = 22.2656, $p < 0.0001$] (Figure 2, Table S2), taking the variable sampling times and investigated inflow volumes into consideration.

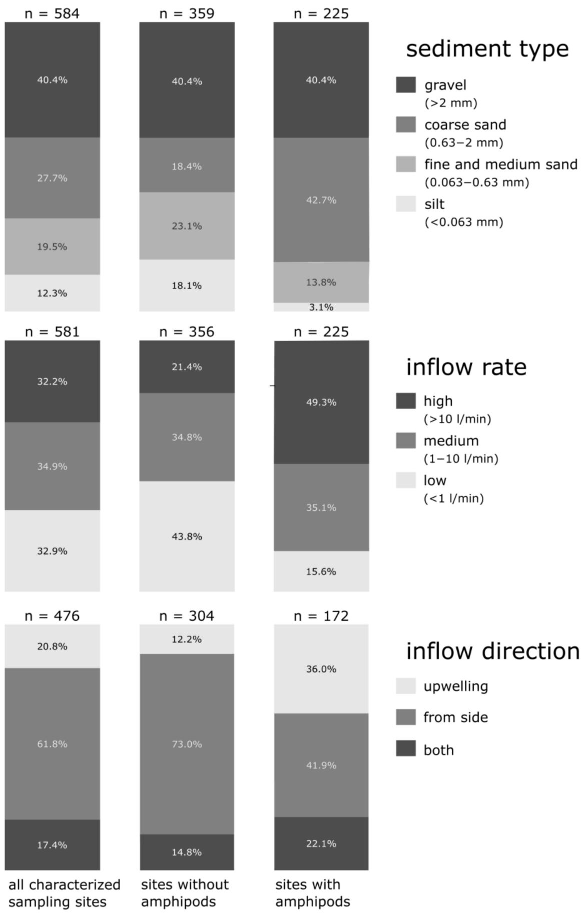


**Figure 2.** Characterization of collection pits. Indicated are the relative proportions of sediment types (gravel, coarse sand, fine and medium sand, silt), speed of inflow (high, medium, large) and direction of inflow (upwelling, from side, both), respectively, for all characterized collection pits, for sites without groundwater amphipods, as well as for sites with groundwater amphipods. The number of sites characterized (n) is given for each parameter individually, as not all sites could be evaluated for all parameters. More information can be retrieved from Tables S1 and S2.

Groundwater amphipods were collected in the hyporheic interstitials across the whole study area. However, in some areas, there was no accessible hyporheic interstitial due to geological and anthropogenic features, e.g., in deep incisions of straightened streams (Upper Rhine lowlands and West Palatinate moor lowlands, both in Germany) (Figure 1). In other regions, no groundwater amphipods were found in the hyporheic interstitial despite the high sampling effort, e.g., in the Eisch River (Belgium, Luxembourg), in the Rhine River, in the Palatinate Forest and in the western Odenwald mountain range (all three in Germany), as well as in the Minett and southern Gutland regions (both in Luxembourg) (Figure 1).

### *3.2. Groundwater Amphipod Species Diversity*

The genetically investigated specimens formed 11 lineages (species richness S = 11), which we were able to taxonomically annotate as *M. leruthi*, five members of the *N. aquilex*-complex *sensu* Schiødte, 1855, *N. puteanus*, *N. fontanus*, *N. schellenbergi*, *N. nolli* and *Crangonyx subterraneus* Spence Bate, 1859 (Table 1, Figure S1). The five entities of the *N. aquilex*-complex were *N. aquilex* A, *N. aquilex* B, *N. aquilex* EF (*sensu* [28,30]), *N. aquilex* G (*sensu* [39]) and a single specimen of a so-far-undescribed lineage, collected in the hyporheic interstitial of the Udelfanger Bach River (the state of Rhineland-Palatinate, Germany). *Niphargus aquilex* EF and *N. aquilex* G contain two separate sublineages (F-1 and F-2, as well as G-1 and G-2, respectively) each, but available data are not sufficient to unambiguously infer the species status of either sublineage. We therefore decided to treat *N. aquilex* EF and *N. aquilex* G as one species each, but to separate our findings of the respective sublineages (Tables 1 and 2, Figure S1). *Niphargus aquilex* EF (sublineage F-1) and *N. schellenbergi* were the most frequently observed taxa in the investigated hyporheic interstitial, occurring at 39.7 (106/267; 114 specimens) and 22.8% (77/267; 101 specimens), respectively, of all sites where groundwater amphipods were detected. The species diversity of groundwater amphipods detected in the interstitials in this study was compared with the known regional groundwater amphipod fauna of the two best-studied areas in our target region, i.e., Luxembourg and Saarland (Table 2). To ensure a fair comparison, only the regionally observed taxa from our study were used for comparison. The findings of *N. aquilex* A and *N. nolli* in Luxembourg are the first records for the country. *Microniphargus leruthi sensu stricto* [11] has been found again in Luxembourg, 60 years after its first finding [41]. Six groundwater amphipod species have been recorded for Saarland despite a decade-long and intensive research in the most diverse biotopes and using DNA-based tools for species identification. While we were not able to detect *N. aquilex* A and I, *N. kochianus* Spence Bate, 1859 (questionable record) and *N. virei* Chevreux, 1896 in the investigated hyporheic interstitials, we can confirm the presence of the species *N. schellenbergi*, *N. aquilex* EF (sublineage F-1) and G (sublineage G-1), as well as *N. nolli* (so-far-questionable record). Furthermore, *N. aquilex* B, which refers to the type species of the cryptic species complex [28], must be added to the regional checklist.

**Table 1.** Overview of groundwater amphipod species found in the hyporheic interstitial of Luxembourg and The Greater Region. Taxonomic annotations: *Niphargus aquilex* A, B, EF *sensu* [28,30], *aquilex* G *sensu* [39]. The single finding of *Microniphargus leruthi* originates from a cave interstitial. B-Wal = Wallonia (Belgium), D-BW = Baden-Württemberg (Germany), D-Hes = Hesse (Germany), D-RLP = Rhineland-Palatinate (Germany), D-Saar = Saarland (Germany), LUX = Luxembourg, n = number of barcoded specimens, x = species detected in the target region. Included are barcoded specimens from our study and [28].

| Species | n | LUX | B-Wal | D-RLP | D-Saar | D-Hes | D-BW |
|---|---|---|---|---|---|---|---|
| *Crangonyx subterraneus* Spence Bate, 1859 | 4 | | x | x | | x | |
| *Microniphargus leruthi* Schellenberg, 1934 | 1 | x | | | | | |
| *Niphargellus nolli* Schellenberg, 1938 | 6 | x | | x | x | x | |
| *Niphargus aquilex* A | 7 | x | | x | | | |

**Table 1.** *Cont.*

| Species | n | LUX | B-Wal | D-RLP | D-Saar | D-Hes | D-BW |
|---|---|---|---|---|---|---|---|
| *Niphargus aquilex* B (*sensu stricto*) | 28 | x | x | x | x | x | |
| *Niphargus aquilex* EF (sublineage F-1) | 114 | x | x | x | x | x | |
| *Niphargus aquilex* EF (sublineage F-2) | 11 | x | x | x | | | |
| *Niphargus aquilex* G (sublineage G-1) | 12 | | | x | x | x | x |
| *Niphargus aquilex* G (sublineage G-2) | 1 | | | | | | x |
| Undescribed species of the *N. aquilex*-complex | 1 | | | x | | | |
| *Niphargus fontanus* Spence Bate, 1859 | 1 | x | | | | | |
| *Niphargus puteanus* (C.L. Koch in Panzer, 1836) | 5 | | | x | | | x |
| *Niphargus schellenbergi* S. Karaman, 1932 | 101 | x | x | x | x | x | x |

**Table 2.** Comparison of groundwater amphipod species diversity from this study and the literature. Indicated are the species found in the hyporheic interstitials in Luxembourg and Saarland from this study, as well as from published data on groundwater amphipod diversity from Luxembourg [27,28] and Saarland [12]. Taxonomic annotations: *Niphargus aquilex* A, B, EF *sensu* [28,30], *N. aquilex* G, I *sensu* [39]. x = present; (x) questionable morphodetermination.

| Species | Luxembourg Interstitial (This Study) | Luxembourg Interstitial [27] | Luxembourg All [28] | Saarland Interstitial (This Study) | Saarland All [12] |
|---|---|---|---|---|---|
| *Microniphargus leruthi* Schellenberg, 1934 | x | | | | |
| *Niphargellus nolli* Schellenberg, 1938 | x | | | x | (x) |
| *Niphargus aquilex*-complex *sensu* Schiødte, 1855 | | x | | | |
| *Niphargus aquilex* A | x | | | | x |
| *Niphargus aquilex* B (*sensu stricto*) | x | | x | x | |
| *Niphargus aquilex* EF (sublineage F-1) | x | | x | x | x |
| *Niphargus aquilex* EF (sublineage F-2) | x | | x | | |
| *Niphargus aquilex* G (sublineage G-1) | | | x | x | x |
| *Niphargus aquilex* I | | | | | x |
| *Niphargus fontanus* Spence Bate, 1859 | x | | x | | |
| *Niphargus kochianus*-complex *sensu* Spence Bate, 1859 | | | x | | (x) |
| *Niphargus puteanus* (C.L. Koch in Panzer, 1836) | | | x | | |
| *Niphargus schellenbergi* S. Karaman, 1932 | x | x | x | x | x |
| *Niphargus virei* Chevreux, 1896 | | | | | x |

## 4. Discussion

### 4.1. Overall High Species Diversity in the Hyporheic Interstitial

We hypothesized that the application of DNA barcoding for the groundwater amphipod fauna of the hyporheic interstitial will lead to an increase in species diversity compared to the older literature, as members of cryptic species complexes and juveniles can be readily identified down to the species level (Hypothesis 1) [28,30]. With 11 putative groundwater amphipod species, species richness (*S*) was remarkably high in the interstitials of the study area. In the specific case of Luxembourg (*S* = 7), our study led to the first finding of *N. aquilex* A and *N. nolli* in the country, as well as to the redetection of *M. leruthi sensu stricto* [11] 60 years after its first finding in the country [41]. A recent integrative taxonomic study using DNA barcoding and traditional morphology has already discovered much more species than were previously known for the country [28], although mainly artificial cavities (e.g., mines) and springs were studied. On the contrary, another study systematically investigated the amphipod fauna of 41 springs and 30 hyporheic interstitial sites in Luxembourg, but did not apply DNA barcoding [27]. The detection of two additional new species in the hyporheic interstitials in Luxembourg by our study must thus be related to the combination of a systematic sampling in this specific environment and a DNA-based identification of specimens. We expect that a more systematic investigation of the hyporheic interstitials in Belgium, France and other parts of Germany will likely reveal further unrecognized groundwater amphipod species. However, the finding of *M. leruthi* is not linked to a hyporheic interstitial site, but the species was revealed from a cave interstitial. This may indicate that this environment has not been sufficiently studied in the study area and also warrants further investigation. Most often, caves and artificial cavities are opportunistically studied [42], and samplings are made when 'something' is encountered. This strategy thereby can easily overlook small species/specimens such as the 1.2–2-mm long *M. leruthi* [41,43]. Alternatively, cave lakes or subterranean streams could be sampled by the aid of either bait boxes or plankton nets. In both cases, mainly the fauna of cave lakes and subterranean streams are surveyed, but not those of cave interstitials. Since the transportation of a Bou–Rouch pump [35,44] into caves is not practical, the Karaman–Chappuis method [34] seems to be a suitable and effective alternative. In practice, however, this sampling method may encounter other difficulties, as cave soles are often cemented or, in the case of the presence of calcium$^{2+}$ or iron$^{2+}$, gravel banks may be heavily sintered.

In addition to the specific case of Luxembourg, we also compared our findings with the recently compiled amphipod checklist of the state of Saarland (Germany) [12]. Our data thus led to a slight increase in the total groundwater amphipod species richness in Saarland ($S$ = 6 to $S$ = 7), in addition to confirming previously questionable records. Furthermore, the finding of *N. aquilex* B highlights the importance of the hyporheic interstitial acting as a habitat of so far poorly known groundwater amphipod species. *Niphargus aquilex* B is considered a rare species in the study area, and was known in the study area only in Luxembourg, where a single specimen was collected by [28] in the Sauer River. However, in the context of our study, we collected 28 specimens of this species throughout the hyporheic interstitials of the whole study area (Luxembourg and The Greater Region). A preference for the hyporheic interstitial can be also revealed for *N. aquilex* EF (sublineage F-1), which was the predominant taxon in our study. However, our still-limited knowledge of the biodiversity of hyporheic interstitials is best illustrated by our discovery of a species new to science within the *N. aquilex*-complex.

### *4.2. Small Individuals but Not Small Species Dominate in the Hyporheic Interstitial*

We speculated that small-sized groundwater amphipod species would dominate in the hyporheic interstitial due to the size benefits when compared to adults of larger species (Hypothesis 2). In the study area, several small species were expected, e.g., *M. leruthi* (adult length 1.2–2 mm) [43,45,46], *C. subterraneus* (1.2–6 mm) [47], *N. nolli* (2.8 mm) [48,49], *N. dimorphopus* Stock & Gledhill, 1977 (3.5–4.5 mm) [50], *N. kieferi* Schellenberg, 1936 (7 mm) [48], *N. kochianus* (5–8 mm) [48–50], *N. laisi* Schellenberg, 1936 (5–7 mm) [48,49] and *N. pachypus* Schellenberg, 1933 (7 mm) [51]. While both *C. subterraneus* and *N. nolli* were indeed found in the hyporheic interstitial in Luxembourg and The Greater Region, albeit in low numbers, the other species were absent. In addition, *M. leruthi* was found at a single site from a cave interstitial. While large(r) adult individuals of groundwater amphipods are physically excluded by the small spaces offered by most hyporheic interstitials, their juveniles are principally able to colonize them. However, those taxonomic findings are usually not recorded, because the morphodetermination of juvenile groundwater amphipods is known to be a very challenging or even impossible task. For example, almost 25 years ago, [27] investigated the hyporheic interstitial of Luxembourg and recorded a total of 154 niphargids, but with several juvenile specimens of *Niphargus* sp. present in the collection (pers. obs. AW). *Niphargus aquilex sensu lato* dominated their dataset (146 specimens, 95%), whereas just eight specimens (5%) were morphologically identified as *N. schellenbergi*. In our study, we genetically identified *N. schellenbergi* as one of the two predominant species in the hyporheic interstitial of Luxembourg. It is considered a large-sized species whose adults reach a length of about 15 mm (according to own investigation, they can be up to 18 mm long) [12,29,52], but apparently juveniles seem to strive well in the hyporheic interstitial. Since a faunal change between the time of the study of [27] and our sampling and in all interstitial habitats in Luxembourg seems unlikely, the more likely explanation is that juveniles of *N. schellenbergi* cannot be identified due to their size or life-period morphological characteristics.

Two other large(r)-sized species, *N. fontanus* and *N. puteanus*, were also revealed, but in low numbers. *Niphargus fontanus* is generally rare, but seems to be slightly more common in Belgium [26]. While the species could not be detected by morphological identification of the material from Luxembourg [27], it was sequenced from one hyporheic interstitial site in the Minett region (Luxembourg). Additionally, *N. puteanus* is generally rare in the study area, with more frequent records in the eastern part [18,28,41]. We detected this species only in hyporheic interstitials located in Rhineland-Palatinate and Baden-Württemberg (both in Germany), which is in accordance with the results of [18].

In summary, the present data indicate that there is no clear pattern of small-sized groundwater amphipod species dominating at least the shallow hyporheic interstitials (<0.5 m depth) that we sampled, but the medium-sized *N. aquilex* EF (sublineage F-1) and juveniles of the large-sized *N. schellenbergi* were predominant. *Niphargus schellenbergi* is by far the most common species in the study area and has so far been found mainly in caves, artificial cavities and springs [12,28,29,52]. Due to the predominance of juveniles in the hyporheic interstitial, it is likely that it has often been overlooked in the past. *Niphargus schellenbergi* must be considered an ecological generalist, colonizing and surviving in different and distinct groundwater-dependent ecosystems and environmental conditions (see [28] and this study). The strong dominance of *N. aquilex* EF among the members of the *N. aquilex*-complex in our data set is striking. Based on our data, *N. aquilex* EF seems to be an interstitial specialist. However, its specialization is not reflected at the morphological level, since other members of the *N. aquilex*-complex are cryptic or at least pseudocryptic [40]. The environmental specializations for hyporheic interstitials might originate from eco-physiological adaptations to better cope with the often fluctuating environmental conditions and steep abiotic and biotic gradients [20,22–24], or the increased numbers of host–parasite interactions when being confronted with the parasites of epigean taxa [39,52]. In this context, we can assume that the hyporheic interstitial represents the main habitat of *N. aquilex* EF (sublineage F-1), which completes its entire lifecycle in this environment. On the contrary, only juveniles of *N. schellenbergi* seem to be physically able to inhabit interstitial sediments, whereas its adults have to migrate into groundwater-dependent environments with larger porosity (e.g., aquifers), connected to hyporheic interstitial sites. This difference in the ecological connection to the hyporheic interstitial also affects the likeliness of recolonization events after environmentally unsuitable conditions, which should be higher for *N. schellenbergi* when compared to interstitial specialists such as *N. aquilex* EF.

### *4.3. Regional Absence of Groundwater Amphipods in Hyporheic Interstitials*

The Karaman-Chappuis method used proved to be effective for collecting groundwater amphipods from different hyporheic interstitial sites. However, only groundwater close to the sediment surface and near the riverbanks can be investigated, whereas a Bou-Rouch pump is closed and reaches down to a depth of 2 m, enabling the investigation of hyporheic interstitials at a certain distance from the river or even of submerged sediments. Acknowledging these sampling limitations and the fact that our results must be interpreted in this context as well, we were able to identify some geographical regions and rivers without any groundwater amphipods in the hyporheic interstitials, although groundwater amphipods are common in other biotopes in these areas (e.g., springs, mines, caves) [12,28,29,41,52]. Their absence in the Palatinate Forest mountain range (Rhineland-Palatinate, Germany) and the western Odenwald mountain range (Baden-Württemberg and Hesse, both in Germany) can be best explained by the geologies of the mountain ranges: Buntsandstein weathers into small sand grains, which results in overall low porosity and thus accessibility of sediments. This situation is enhanced by the weathering clay of the overlying Muschelkalk, which can close the remaining small pores in the hyporheic interstitial and ultimately lead to sediment clogging. As such, a high fraction of silt and fine sands and a low inflow speed characterized the collection pits in those regions, not allowing even small-sized groundwater amphipods to be present. The occurrence of sandstone environments might also be responsible for the apparent absence of groundwater amphipods in interstitial sites in the Minett and southern Gutland regions (both in Luxembourg). In addition, the Minett region is characterized by an intense historical iron industry [53,54], and today is highly populated and urbanized. Both might have led to an accumulation of stressors affecting the regional aquatic fauna in general [55,56].

In addition to the absence of groundwater amphipods in the hyporheic interstitials of some mountain ranges or geographical regions, groundwater amphipods were also absent from a few but well-sampled rivers. In the Eisch River (Belgium and Luxembourg), 24 sites in the main channel and another 9 sites in its tributaries in the close vicinity to the main channel were investigated, but no specimen was found. The Eisch River was contaminated multiple times between 1948 and 1961 by the phenol industry located in Steinfort (upstream from our sampling sites), either directly by the slightly water-soluble phenol with its safety classification H411 (i.e., toxic to aquatic organisms with long-lasting effects [57]), or indirectly by its degradation products, leading to the extinction of the local aquatic fauna [58]. It can be expected that traces of these contaminations are still present in the sediments and aquifers, potentially also influencing the presence of groundwater amphipods or affecting the probability of their recolonization. A similar anthropogenically induced situation could be found in the Rhine River (Germany). Although the river has also been heavily straightened, there are many larger gravel banks along the riverbanks. We were able to examine 31 pits on different gravel banks, all of which remained free of living amphipods. It is unlikely that the interstitials of the Rhine River were never colonized by groundwater amphipods [18]. The most plausible hypothesis is therefore similar to the situation of the Eisch River. Namely, municipal wastewater and frequent pollution by the chemical industry in the 1950s–1970s [58] led to an accumulation of toxic substances in the aquifers and sediments. Although the epigean aquatic fauna have largely recovered [59,60], groundwater amphipods apparently have not returned there yet. Even in the case of a high ecological suitability of the investigated hyporheic interstitials in the Rhine River, it is most likely that a generally slow migration rate prevents groundwater amphipod populations from recolonizing them faster after serious pollution events. Future research should focus on identifying the ecophysiological or physical factors that prevent groundwater amphipod species from being more abundant in hyporheic interstitials. Similarly, one has to generate more knowledge on the actual species diversity in hyporheic interstitials and the ecological preferences of individual groundwater amphipods for this specific environment. Of particular interest could be species from the hyporheic interstitial that are more widely distributed, which could possibly represent complexes of cryptic species, or which can provide new insights into the dispersal capabilities, resiliencies and migration corridors of groundwater amphipods. Likewise, their areas of distribution or regional absence should be investigated in more detail. How deep are the potential chemical pollutants and do they affect the quality of groundwater? If previous pollution still has a significant effect, this could indicate that groundwater (and drinking water) pollution is a long-term process with a slow recovery rate. If the water quality is fine, it might indicate that the recolonization of groundwater and the recovery of ecosystem functions are long-lasting processes. Both aspects are important for groundwater monitoring, especially in relation to drinking water.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15030411/s1, Table S1: Overview of sampling sites and specimen metadata; Table S2: Additional data on the characterization of the collection pits underlying Figure 2; and Figure S1: Neighbor Joining-tree reconstruction based on COI of groundwater amphipod species detected in the hyporheic interstitial of Luxembourg and The Greater Region.

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**Data Availability Statement:** Metadata of the specimens analyzed in this study can be found in the Supplementary Materials Table S2. DNA barcodes and the associated metadata (i.e., collector, date of collection, locality, coordinates) are additionally deposited in the Barcode of Life Data System project 'MNHNL'.

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