

Article

Unraveling Functional Diversity Patterns in Hyporheic Zones: A Trait-Based Approach Applied to Copepods from the Rio Gamberale Creek

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Abstract: Despite the recognized ecological significance of hyporheic zones, biological investigations into their ecology, especially concerning functional diversity, remain limited. This is particularly true for copepod assemblages, abundant in the transitional zone between groundwater and surface waters, requiring more thorough exploration. To bridge this knowledge gap, our study extensively monitored a hyporheic zone within a mountain creek and examined nine functional traits across twelve copepod species found in this environment. Through the application of RLQ and fourth-corner analysis, as well as functional diversity indices including functional richness, evenness, divergence, and dispersion, we aimed to establish links between functional traits and environmental factors. Our findings revealed relationships between environmental features—specifically electrical conductivity, pH, and ammonium concentration—and the trait composition of copepod assemblages, which were influenced by species abundances. Considering the intimate connection between functional traits and ecosystem services, assessing functional diversity within hyporheic zones offers valuable insights into its functionality in terms of services rendered. This study emphasizes the importance of understanding and managing functional diversity in hyporheic zone dynamics to ensure the health and stability of ecotones and, by extension, riverine and groundwater ecosystems.

Keywords: ecotone; meiofauna; body flexibility; functional traits; functional diversity



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1. Introduction

In 1959, Orghidan introduced the term “hyporheic zone” to denote a habitat defined by the interaction between surface water and the underlying shallow groundwater, occurring below the stream’s active channel and spreading into the surrounding riparian zones [1]. This ecotone—an interface between ecosystems that represents dynamic gradients and hosts unique assemblages of fauna (e.g., [2])—has gradually garnered attention from the scientific community (e.g., [3–6]). Surface water infiltrates the hyporheic zone through downwelling spots, where low hydrostatic pressure allows for the transport of essential nutrients and oxygen, while water within this zone flows parallel to the surface, extending residence times [7]. Hyporheic water undergoes oxygen depletion, triggering biogeochemical reactions, and resurfacing in nutrient-rich but oxygen-deficient upwelling zones, interacting with groundwater from the aquifer below [4]. The fauna of the hyporheic

zone display distinctive characteristics, with certain traits typical to surface water ecosystems and others representative of groundwater ones, collectively setting it apart from both [8]. Groundwater-dwelling fauna, adapted to darkness, low oxygen levels, and low energy inputs, are common in upwelling areas of hyporheic zones [9]. The hyporheic zone often hosts meiobenthic crustaceans, such as copepods and ostracods, with some species displaying varying degrees of specialization [10,11]. Copepods, a diverse group of small crustaceans abundant in freshwater habitats [12], are usually prevalent in the hyporheic zone. Copepods exhibit remarkable adaptability to diverse environmental conditions and represent pivotal primary consumers in aquatic ecosystems, crucial for transferring energy from primary producers to higher trophic levels [12,13]. Further, these crustaceans provide essential ecosystem services, such as organic matter processing and nutrient cycling across all surface water–groundwater interfaces. They contribute to water purification and bioremediation by stimulating microbial activities and consuming pathogens. Additionally, interstitial copepods influence the hydraulic conductivity of sediments by bioturbation, supporting the functioning of hyporheic zones [14]. Lastly, copepods are widely distributed across different regions and ecosystems [13]. For all these reasons, copepods serve as valuable models for studying the functionality of hyporheic zones.

Ecological studies on the hyporheic zone have examined its ecotonal nature, as well as its role as a refuge for taxa in intermittent streams [15,16] and as an environmental filter [17,18]. Further studies have delved into the taxonomic composition of hyporheic assemblages, also linking the ability of organisms to colonize the hyporheic zone to their biological traits [19]. The use of functional traits to characterize biological assemblages has revolutionized ecologists' perception of freshwater systems, offering predictive insights into ecosystem functioning, stability, and ecological change, particularly when observing alterations in functional rather than taxonomic structures (e.g., [20]). Trait-based functional analyses have been applied across diverse organisms (microorganisms [21], plants [22], invertebrates [23], vertebrates [24]), freshwater ecosystems (rivers [25], lakes [26], groundwaters [27]), and ecotones, including the hyporheic zones [19,28].

Despite the growing number of studies on the meiofaunal community of the hyporheic zone [29–31], investigations into its functional ecology at the species level remain limited, primarily due to the need for elevated taxonomic and functional expertise to correctly identify the collected taxa and assign them the corresponding trait scores [31]. However, this type of research is crucial as taxonomic approaches may fall short in elucidating the response of faunal assemblages to environmental changes [32]. Previous research has indicated that the functional and taxonomic diversity of hyporheic assemblages may be influenced by environmental factors such as grain size and nitrogen levels [19,28]. Di Lorenzo et al. [28] specifically highlighted ammonium impact on these assemblages in a study of a small Apennine stream, the Rio Gamberale Creek. This mountain stream, transitioning from a pristine upstream section to a downstream area impacted by agriculture and urban runoff, showcases ammonium concentrations exceeding regulatory thresholds. The present study builds upon Di Lorenzo et al. [28], examining the taxonomic and functional variation within the hyporheic copepod assemblage of the Rio Gamberale Creek. By focusing on a single taxon, we were able to analyze a variety of functional traits not assessable at broader taxonomic scales. In this manner, we were able to test the hypothesis that both the taxonomic and trait compositions of hyporheic copepod assemblages vary along an environmental gradient, from upstream to downstream, due to ammonium, suggesting potential biological mechanisms driving the observed taxonomic changes.

2. Materials and Methods

2.1. Study Area

This study focused on Rio Gamberale (42°14'00" N, 13°32'10" E), an Apennine creek (Figure 1) with a length of 10.04 km, a discharge of 360 L/s and a hydrographic basin spanning 51.08 km² [33]. Originating in Vado di Pezza (Abruzzo, Italy) at an elevation of 1500 m a.s.l., the creek flows through a protected area (Sirente-Velino Regional Park,

Abruzzo, Italy) and a karst plain. Along its course, Rio Gamberale Creek encounters various human activities, including farms and crop fields, and two downstream urban wastewater treatment plants. The stream completely sinks underground through the Pozzo Caldaio sinkhole (1253 m a.s.l.) and after 2600 m of underground flow, it resurfaces in the Grotta di Stiffe cave (661 m a.s.l.). The area encompassing the Rio Gamberale watershed experiences an average annual rainfall of around 800 mm, with rainfall in winter potentially three times higher than in summer [34].

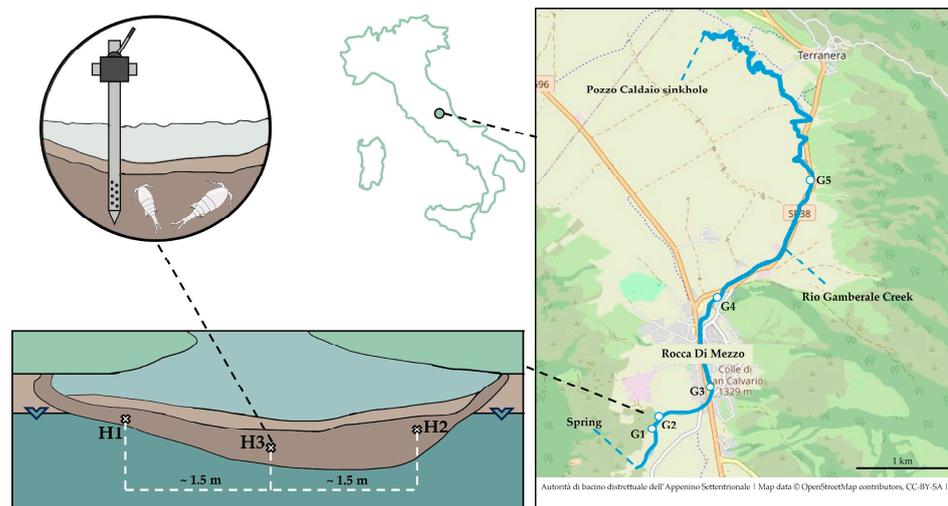


Figure 1. Clockwise from the right: Rio Gamberale Creek (Italy) and location of the five sampling stations (G1, G2, G3, G4, and G5) in the hyporheic zone of the creek course; location of the three samples (H1, H2, H3) collected in each site along an oblique transect (the arrows indicate the level of the water table); detailed illustration of the faunistic sampling method using a membrane pump connected to a steel piezometer.

2.2. Sampling Methods

We monitored the hyporheic zone (HZ) of Rio Gamberale at five sampling stations (Figure 1), each located approximately 1.5 km apart, except for G1 and G2, which were about 300 m apart. At each station, we randomly collected three samples (H1, H2, and H3) along an oblique transect, each sampling site separated from the others by 1.5 m at least. We conducted winter and summer sampling surveys in December 2014 and June 2015, respectively. Previous studies revealed similar streambed sediment composition at all sampling stations [28], dominated by sand ($63 \mu\text{m} < \varphi \leq 2 \text{ mm}$; range: 60–81%), clay-silt ($\varphi \leq 63 \mu\text{m}$; range: 18–40%), and gravel ($2 \text{ mm} < \varphi \leq 64 \text{ mm}$; range: 0–3%). European Sampling Manual guidelines for subterranean ecosystems were followed to collect environmental and biological data at each sampling station [35].

To collect hyporheic samples, we employed a modified Bou-Rouch pump [36] connected to removable steel piezometers (equipped with 5 cm-screened tips), which were hammered down to 40 cm below the streambed [35]. Shortly after setting up the piezometer, we pumped 10 L of interstitial water maintaining a rate of up to 0.66 L/s to remove hyporheic organisms effectively [35]. The pump collected samples of both swimming organisms and specimens closely associated with sand particles. Typically, a Bou-Rouch pump captures 76–100% of taxa in 10 L of pumped water [35]. Subsequently, we filtered the pumped interstitial water using a 60 μm -mesh net; the filtered water sample was then bottled and preserved in a 70% alcohol solution. In the laboratory, we sorted the samples using a stereomicroscope (Leica M80, Wetzlar, Germany) set at 16 \times . Using a glass pipette, copepod individuals were collected and placed onto a microscope slide, then dissected for taxonomic identification. Subsequently, they were observed under an optical microscope, referring to taxonomic keys [37–41] and recent literature, to identify them at the species level.

We measured electrical conductivity, pH, dissolved oxygen, saturation, and temperature using a WTW Multi 3430 SET G multiparametric probe. Additionally, we collected a 10 L sample of hyporheic water for chemical analysis immediately after biological sampling. These samples were sealed in glass vials and transported in a refrigerated box to a private accredited laboratory within two hours from collection. The laboratory analyzed the samples for 108 potential pollutants, reflecting the anthropogenic pressures in the catchment area, and measured concentrations of dissolved organic carbon (DOC) and total organic carbon (TOC). The analysis followed methods specifically adhering to the European analytical standard [42]. In our laboratory, we measured particulate organic matter (POM) after removing all animals from the biological samples. We dried the sediments, weighed them, burned them at 540 °C, and reweighed them to calculate POM content [43].

2.3. Functional Traits of Hyporheic Copepods

We measured all copepod specimens using LAS software (Leica Application Suite, version 4.7.1), capturing images with a LEICA M205C stereomicroscope equipped with a built-in camera. Body dimensions (length and width) were converted to species-specific biovolume (in nL) using the regression (Equation (1)) indicated in Reiss and Schmid-Araya [44]:

$$BV = a \times b^2 \times CF, \quad (1)$$

where a = length (mm), b = width (mm), and CF is a correction factor that accounts for the body shape. Since CF values may vary from 230 to 750 according to Warwick and Gee [45], we selected a CF of 490 for cyclopoids and a CF of 560 for harpacticoids based on the best approximation of body shape. Biovolume was then converted into individual fresh weight assuming a specific gravity of 1.1 [46]. The dry mass (mg) was estimated assuming a dry/wet weight ratio of 0.25 and a dry weight carbon content was assumed to be 40% of the dry mass [47]. Subsequently, we determined the life stage (juvenile or adult) of each specimen and the sex (male or female) of each adult.

We defined nine functional traits, arranged into four main groups:

1. Life history: i. juveniles-to-adults ratio (J/A), ii. males-to-females ratio (M/F);
2. Morphological: i. average biomass (B), ii. sexual dimorphism (SD);
3. Behavioral: i. body flexibility (BF);
4. Physiological: i. diet (D), ii. feeding habits (FH), iii. ecology (E), iv. thermal preference (TP).

We attributed the traits to the identified species as follows: we calculated J/A as the ratio of the total number of copepodites to the total number of adults; M/F was calculated as the ratio of the total number of adult males to the total number of adult females. The average biomass represented the mean of the biomasses of all individuals. In contrast, SD was defined as the ratio between the mean length of males and the mean length of females. We defined body flexibility as the maximum body angle measured in degrees, based on photographed specimens [23]. The diet trait included four modalities: fine sediment + microorganisms, living microinvertebrates + protists, microinvertebrates + protists + benthic epiphytic + algae, and algae. The feeding habit included five modalities: deposit-feeder, deposit-feeder + scraper, herbivorous, predator, and omnivorous. Ecology encompassed three modalities based on species that complete the life cycle exclusively in groundwater (stygo-bites; [48]), species that might complete their life cycle in groundwater but are not obligate-dwellers (stygophiles), and species that do not complete their life cycle in groundwater (stygoxenes). Lastly, we identified four modalities of thermal preference: stenothermal, moderately stenothermal, eurythermal, and highly eurythermal. The extrapolation of trait modalities relied on both the literature review and empirical observations. Detailed references are provided in Table S3.

2.4. Statistical Analysis

For each sampling site, we calculated the average values of the measured environmental parameters, observed abundance for each species, and measured quantitative traits (e.g., J/A) from the two surveys. Then, we examined the relationships among species abundance (Table L: 12 species \times 15 samples; Supplementary Table S1), environmental (Table R: 17 variables \times 15 samples; Supplementary Table S2), and trait variables (Table Q: 9 traits \times 15 samples; Supplementary Table S3) using multivariate ordination techniques combined into an RLQ analysis [49]. The RLQ analysis is a co-inertia analysis that provides multivariate associations of traits and environmental variables weighted by the abundance of the species [49]. Within these constraints, the RLQ analysis identified axes that maximized the covariance between the R and Q tables, establishing a connection between environmental variables and functional traits [50]. To feed the RLQ algorithm, we performed dimensionality reduction of the environmental, abundance, and trait data using a correspondence analysis (CA) for the L table and a principal component analysis (PCA) for the R and Q tables [51]. To avoid multiple linearities, before performing the analyses, we explored the correlation between seventeen z-score-transformed environmental variables exhibiting a non-zero standard deviation using Draftsman's plot [52]. To further assure the integrity of our results, we examined the variance inflation factors for each variable.

Afterward, we performed a fourth-corner analysis to examine the degree of correlation between the environmental variables and the traits. While RLQ analysis offers a condensed overview of multivariate associations, the fourth-corner method enables testing the statistical significance of bivariate correlations [53]. Following Dray et al. [51], we used the sequential approach (Model 6) proposed by ter Braak et al. [54], where Model 2 was utilized to permute the samples and test the null hypothesis, with fixed traits, that species distribution is not correlated to the environmental variables, while Model 4 was employed to permute the species and test, with fixed environmental variables, the null hypothesis of uncorrelated species distribution and traits. If the largest of the two p -values from Models 2 and 4 was lower than the fixed threshold for Type I error ($\alpha = 0.05$), the null hypothesis that species distribution is not correlated to either traits or environmental characteristics should be rejected. We also applied Bonferroni and FDR p -value corrections to avoid inflation of the Type I error [51]. All analyses were executed using the *ade4* package [55], available in R software v. 4.3 [56]. Additionally, we computed functional diversity indices, namely Functional Richness (FRic), Functional Evenness (FEve), Functional Divergence (FDiv), and Functional Dispersion (FDis), to provide insights into the functional aspects of the assemblages [57,58]. The functional diversity indices were computed using the *FD* package [59] in R. To evaluate potential statistically significant differences in the functional diversity indices between groups of sites being segregated along the main axes resulting from the RLQ analysis, we applied a one-way PERMANOVA ($\alpha = 0.05$, permutations = 999; factor: Group; levels: R and L), followed by permutational pairwise post hoc t -tests. We used unrestricted permutations of raw data and Type I Sums of Squares on a Euclidean distance matrix [60]. To assess the potential heterogeneity of the variances among groups before PERMANOVA, we performed the permutational Levene's test with a PERMDISP routine ($\alpha = 0.05$, permutations = 999) [60].

3. Results

We collected a total of 261 individuals spanning twelve copepod species (Table S1), among which two were stygobitic, one stygophile, and nine stygoxene (Table S3). Of these individuals, 58 were copepodites and 201 were adults, the latter comprising 126 females and 75 males (Table S3). The individual biomass values ranged from 0.11 μg to 22.54 μg (mean $B = 2.12 \mu\text{g}$), while sexual dimorphism was observed with a ratio ranging from 0.70 to 1.10 (mean $SD = 0.89$). The M/F ratio varied from 0.20 to 6.00 (mean $M/F = 1.18$), while the J/A ratio extended from 0 to 1.25 (mean $J/A = 0.35$). Regarding body flexibility, the angles ranged from a minimum of 31° to a maximum of 103° (mean $BF = 65^\circ$). Furthermore, all the environmental parameters we evaluated exhibited a non-zero standard deviation (Table 1).

The concentrations of the 108 potential pollutants were found to be below the detection limits and thus were excluded from further analyses. The Draftsman's plot unveiled a negative correlation exceeding 99% between the percentages of clay and silt and that of sand, and a positive correlation between DOC and TOC (94%). Consequently, we decided to eliminate the sand percentage and TOC from further consideration. Furthermore, we excluded POM, DOC, Cl, Na, Ca, and K variables, which showed a variance inflation factor > 10, from our analysis. This adjustment allowed us to proceed with 15 samples and 11 predictor variables, effectively reducing variance inflation.

Table 1. Minimum (min), maximum (max), mean (μ), and standard deviation (SD) of the 17 environmental variables showing non-zero standard deviation in the hyporheic zone of the Rio Gamberale Creek.

Environmental Variable ¹	Min	μ	SD	Max
T (°C)	4.00	18.40	9.79	4.70
EC ($\mu\text{S cm}^{-1}$)	420.00	942.00	511.80	107.19
pH	7.37	8.25	7.92	0.23
DO (mg L^{-1})	0.15	10.80	6.09	2.70
GRA (%)	0.00	3.20	0.53	0.86
CLA (%)	18.07	39.83	29.18	7.25
POM (mg L^{-1})	20.00	1202.00	347.40	275.65
DOC (mg L^{-1})	1.00	2.80	1.67	0.44
NO ₂ (mg L^{-1})	0.03	0.78	0.15	0.19
NO ₃ (mg L^{-1})	0.25	8.80	3.67	3.02
NH ₄ (mg L^{-1})	0.03	2.25	0.42	0.67
SO ₄ (mg L^{-1})	1.80	43.00	7.98	7.73
Cl (mg L^{-1})	0.25	17.00	4.38	4.47
PO ₄ (mg L^{-1})	0.03	0.25	0.16	0.10
Ca (mg L^{-1})	70.00	145.00	101.07	14.75
K (mg L^{-1})	0.50	5.00	1.31	1.00
Na (mg L^{-1})	3.80	3.80	3.80	3.80

¹ T: Temperature, EC: Electrical Conductivity, DO: Dissolved Oxygen, GRA: Gravel, CLA: Clay and Silt, POM: Particulate Organic Matter, DOC: Dissolved Organic Carbon, NO₂: Nitrites, NO₃: Nitrates, NH₄: Ammonium, SO₄: Sulfates, Cl: Chlorides, PO₄: Phosphates, Ca: Calcium, K: Potassium, Na: Sodium.

The total inertia of the RLQ was 5.499, most of which was explained by the first axis (90%), while that of the second axis accounted for just 10% of the variance. Hence, we decided only to interpret the scores of the first axis. The RLQ analysis unveiled a sample partitioning along this axis, where five samples from the G4 and G5 sampling stations clustered on the left half of axis 1 (L group), while the remaining samples aligned on the right half of axis 1 (R group; Figure 2a). Sites on the right of the axis 1 were primarily characterized by high dissolved oxygen, pH, and nitrites (Figure 2b). Body flexibility was the trait with the highest association with these environmental parameters (Figure 2c). The corresponding species were the stygobitic species *Eucyclops intermedius* Damian, 1955 and *Diacyclops clandestinus* (Yeatman, 1964), along with the stygoxene *Attheyella crassa* (Sars G.O., 1863), *Bryocamptus pygmaeus* (Sars G.O., 1863), and *Canthocamptus staphylinus* (Jurine, 1820) (Figure 2d), collectively exhibiting higher values of body flexibility (Table S3). The left part of the first axis appeared to be mainly associated with high values of ammonium, percentage of gravel, electrical conductivity, phosphates, and sulfates (Figure 2b). Traits of diet and feeding habits, along with high values of biomass, seemed to be associated with these environmental variables (Figure 2c). Species on the left side of the first axis were all non-stygobitic cyclopoids, while those on the right were all non-stygobitic harpacticoids, except for the two stygobitic cyclopoid species (Figure 2d). Nitrates, clays, and temperature mainly described the axis 2 (Figure 2b).

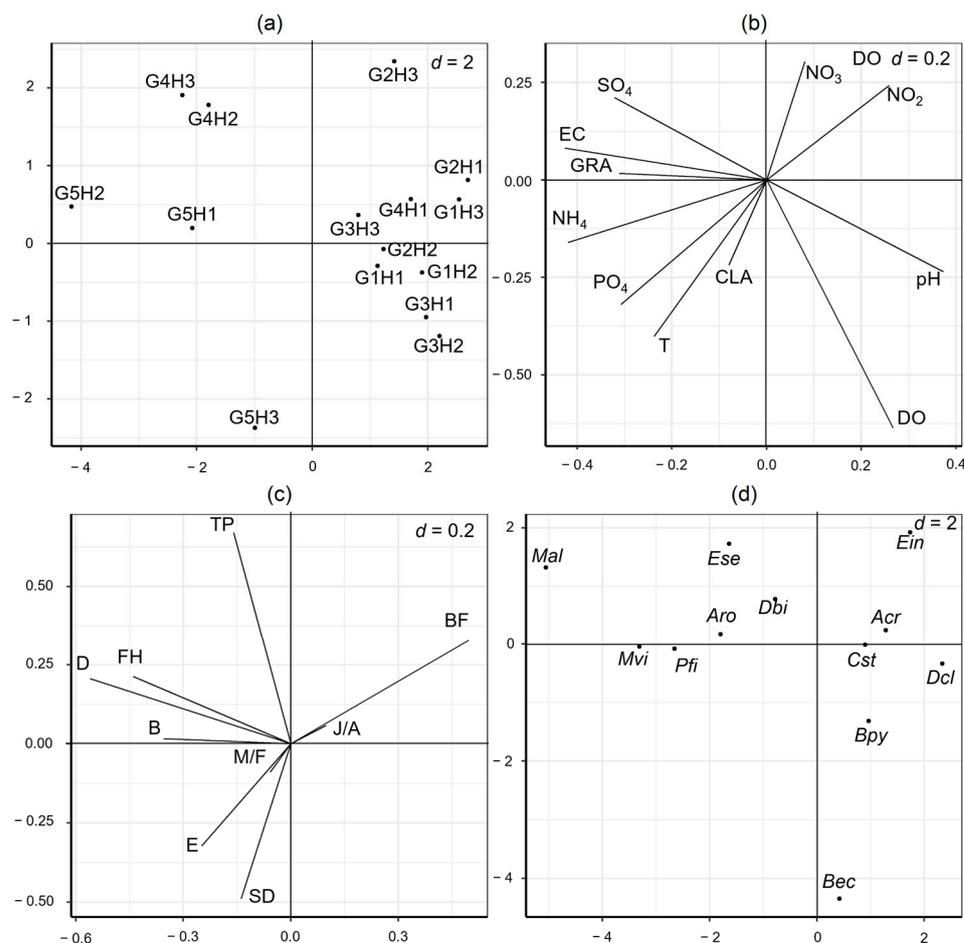


Figure 2. Results of the first two axes of RLQ analysis: (a) scores of samples, (b) coefficients for environmental variables, (c) coefficients for traits, and (d) scores of the copepod species. The values of d denote the grid size. Aro: *Acanthocyclops robustus* (Sars G.O., 1863), Acr: *Attheyella crassa* (Sars G.O., 1863), Bec: *Bryocamptus echinatus* (Mrázek, 1893), Bpy: *Bryocamptus pygmaeus* (Sars G.O., 1863), Cst: *Canthocamptus staphylinus* (Jurine, 1820), Dbi: *Diacyclops bisetosus* (Rehberg, 1880), Dcl: *Diacyclops clandestinus* (Yeatman, 1964), Ein: *Eucyclops intermedius* (Damian, 1955), Ese: *Eucyclops serrulatus* (Fischer, 1851), Mal: *Macrocyclus albidus* (Jurine, 1820), Mvi: *Megacyclops viridis* (Jurine, 1820), Pfi: *Paracyclops fimbriatus* (Fischer, 1853). T: Temperature, EC: Electrical Conductivity, DO: Dissolved Oxygen, GRA: Gravel, CLA: Clay and Silt, NO₂: Nitrites, NO₃: Nitrates, NH₄: Ammonium, SO₄: Sulfates, PO₄: Phosphates. J/A: juveniles-to-adults ratio, M/F: males-to-females ratio; B (μ g): average biomass; SD: sexual dimorphism; BF ($^{\circ}$): body flexibility; D: diet; FH: feeding habit; E: ecology; TP: thermal preference.

The fourth-corner analysis revealed no significant ($\alpha = 0.05$) correlations between the eleven environmental variables and the nine functional traits after applying p -value corrections.

Variations in functional diversity indices calculated on the averaged samples were observed between the L and R groups (Table S4). Specifically, samples within the R group seemed to display a higher median value of functional richness, compared to those within the L group. Contrarily, samples from the L group seemed to exhibit a higher median value of functional evenness. Meanwhile, the median values of both groups seemed not to differ in functional divergence and functional dispersion (Figure S1). However, the PERMANOVA analysis did not reveal any significant differences in functional diversity indices between the two groups of sites.

4. Discussion

In our study, we observed variations in the taxonomic composition of the hyporheic copepod assemblage occurring in an Apennine mountain stream, the Rio Gamberale Creek, across a gradient of environmental parameters. Specifically, harpacticoid species appeared to be segregated from most of the cyclopoid ones within a two-dimensional RLQ-derived environmental space primarily characterized by ammonium, gravel, electrical conductivity, phosphate, sulfate, dissolved oxygen, pH, and nitrites. Our analyses revealed that these taxonomic differences were associated with changes in certain functional traits, notably body flexibility (behavioral trait), diet, feeding habits, and ecology (physiological traits), and biomass (morphological trait) along this gradient. Interestingly, other traits, such as life history, sexual dimorphism, and thermal tolerance, did not seem to substantially contribute to the observed patterns.

The trait ecology appeared to be the primary determinant in the positioning of two stygobitic species, *Eucyclops intermedius* Damian, 1955 and *Diacyclops clandestinus* (Yeatman, 1964), at one extreme of the environmental gradient defined by the first axis derived from the RLQ analysis, distinctly separated from other non-stygobitic cyclopoid species on the other extreme. We attribute this ecological pattern primarily to the ammonium gradient, which, increasing towards the left half of axis 1, seems to be the main driver shaping the distribution. Our observation aligns with previous findings in the literature, indicating the potential intolerance of these two stygobitic species to ammonium [61]. Stygobitic copepods have been shown to be significantly more sensitive to ammonium—at least of one order of magnitude—compared to non-stygobitic ones [61,62]. While the exact reasons for these differences remain unclear, it is known that stygobitic copepods exhibit metabolic rates five to seven times lower than their surface water counterparts [61]. This suggests that detoxification processes may occur at a slower rate in stygobitic copepods, rendering them more susceptible to ammonium intoxication than non-stygobitic ones even at similar exposure levels. The toxic effects of ammonium on crustaceans primarily manifest through the impairment of respiratory metabolism [63], cell membrane osmoregulation [64], and, in freshwater copepods, endocrine regulation of growth factors [65]. In this study, stygobitic species were absent where ammonium concentrations exceeded 0.03 mg/L. Consistently, Di Lorenzo et al. [61] noted that ammonium concentrations above 0.032 mg/L appeared to have a detrimental impact on the copepod assemblage in general, particularly affecting stygobites.

Biomass and body flexibility appear to be the primary traits influencing the separation of harpacticoid and cyclopoid species along a gradient primarily determined by gravel content. In our investigation, we observed that harpacticoid species tend to be smaller in size and exhibit higher body flexibility compared to cyclopoid species, except for the two stygobitic cyclopoid species *E. intermedius* and *D. clandestinus*, which are smaller and more flexible than the others. The presence of less gravel typically results in smaller pore spaces, potentially limiting the occurrence of species with large biomass and low flexibility, which may encounter difficulty in moving within the narrow interstices. Body flexibility is a trait frequently studied in macroinvertebrates and aquatic insects. For example, Lemes da Silva et al. [66] associated higher body flexibility with a behavioral adaptation useful under extreme or unstable environmental conditions, such as high flow rates or degraded habitats. Increased flexibility allows organisms to seek refuge in small interstitial voids and pores within the substrate, adopting a more stable position relative to the substrate [67]. Thus, this trait is likely to confer an adaptive advantage in facing environmental changes, even under degraded conditions [19]. Studies have also indicated that organisms with smaller body sizes and higher body flexibility thrive in heterogeneous environments rich in fine sediment, as flexibility enables them to navigate small interstices within the substrate [19,68]. In the literature, body flexibility is often categorized into discrete classes. For instance, Magliozzi et al. [23] defined three flexibility classes: “none” for values below 10°, “low” for values between 10° and 45°, and “high” for values above 45°. However, this categorization may not adequately capture the variability of this trait in copepod assemblages, as most

species in our study exhibited a body flexibility greater than 45° . Our decision to consider body flexibility as a non-categorical trait expressed by continuous values provides a more accurate representation of the variability of this trait in copepods.

The traits of diet and feeding habits notably influence the separation of harpacticoid and cyclopoid species along a gradient, which we primarily attribute to electrical conductivity, ammonium, sulfate, phosphate, and gravel. In particular, all harpacticoid species and two stygobitic cyclopoids were characterized as deposit feeders or scrapers, primarily feeding on fine sediments and microorganisms. Conversely, the epigeic cyclopoid assemblage exhibited a broader range of functional categories related to diet, including living macroinvertebrates, protists, benthic epiphytic algae, and algae. Similarly, they displayed a wider array of feeding habits, encompassing herbivory, predation, and omnivory. Species with diverse feeding traits may be adapted to varying levels of mineralization. For instance, herbivorous species and those that feed on a combination of macroinvertebrates, protists, benthic epiphytes, and algae may thrive in environments with higher electrical conductivity, where mineral dissolution makes more nutrients available. Accordingly, previous studies have underscored the relationship between electrical conductivity and macroinvertebrate assemblages with diverse feeding traits [69]. Phosphate often dictates the growth of algae and other autotrophic organisms. Variations in feeding traits might be directly linked to how copepods exploit these resources, with herbivorous copepods particularly favored in environments where phosphate-induced algal blooms occur [70]. Conversely, species that primarily feed on microorganisms—located on the right side of the first axis—may be influenced by sulfate concentrations. High sulfate levels can alter the types of bacteria and microorganisms present in the water, thus affecting the food sources available for different copepod species [71]. In this study, copepod species that feed on algae, macroinvertebrates, protists, and benthic epiphytic algae showed larger biomass compared to those feeding primarily on fine sediments and microorganisms. Consequently, the presence of gravel, which typically results in wider pore spaces, tended to favor the occurrence of larger herbivorous, predators, and omnivorous copepods, as observed in previous studies [30]. Finally, the cyclopoid assemblage on the left sector of the first RLQ axis demonstrated less specialization in terms of diet and feeding habits compared to the harpacticoid and stygobitic cyclopoid species. In our study, the enrichment in ammonium favored species exhibiting a more generalist approach to diet and feeding habits, as also observed for meiobenthic assemblages [28].

Life history traits, such as the ratios of juveniles to adults and males to females, showed weak correlations with the environmental variables investigated. This could be attributed to the lack of consideration for seasonal variations in our analysis. Indeed, in surface-dwelling meiofauna, the ratio of juveniles to adults exhibits periodic changes over the course of the year, which are closely linked to seasonal trends [46]. Similarly, sex ratios in epigeic populations tend to be influenced by factors such as seasonality and the presence of pollutants [72,73]. For example, Di Lorenzo et al. [74] reported a sex ratio below 1 in an alluvial aquifer where nitrogen compounds were present. Since our study did not incorporate seasonal variations, we could not assess whether these life history characteristics have a significant role in shaping the dynamics of the hyporheic copepod community throughout the year.

None of the indices of functional diversity revealed statistically significant changes between the two groups of sites located at the opposite extremes of the environmental gradient identified by the first axis of the RLQ analysis. This suggests that, overall, the copepod assemblage in the ammonium-rich hyporheic waters of Rio Gamberale Creek exhibited a functional diversity comparable to that found in ammonium-poor waters, indicating that the functional diversity of the assemblages within those groups remains relatively consistent despite variations in environmental conditions [75]. This finding aligns with existing research indicating that functional diversity in stream invertebrate assemblages might not significantly fluctuate in response to environmental stress gradients [76]. For instance, a recent review [77] highlighted that functional diversity was either unresponsive or only

weakly sensitive to anthropogenic stress in a majority of the studies examined. The subdued reaction of functional diversity indices could be attributed to several factors. Firstly, studies limited to only one taxon might constrain the scope for observing pronounced functional reactions. Secondly, the high functional redundancy observed among the copepod community members might obscure any potential impacts arising from environmental pressures acting on few species. Thirdly, functional diversity indices may not effectively capture functional shifts depending on a small subset of trait modalities due to the information loss inherent in summarizing diverse trait modalities into a singular diversity measure. We might have observed a pronounced response in functional diversity indices if the analysis had focused solely on traits predominantly affected by the stress gradient, such as ecology, body flexibility, biomass, diet, and feeding habits. Additionally, certain functional traits, such as body shape and locomotion, were not considered in this study, as we opted to use body flexibility as a proxy. However, exploring these aspects would be valuable, given their significance for ecosystem services, particularly bioturbation [14]. Moreover, life history traits related to fertility, such as egg biomass and the percentage of ovigerous females, have emerged in previous research as significant descriptors of groundwater and ecotonal ecosystem functionality [78]. Unfortunately, we were unable to obtain this information for the purposes of this study. Therefore, expanding the understanding of the hyporheic zone by incorporating a greater number of traits, especially those related to fertility and locomotion, and planning the experimental design to include seasonal variables would be beneficial. Nevertheless, we are confident that the results presented here can serve as a starting point for assessing trait responses to environmental variations in ecosystems that are still not sufficiently understood and explored, such as the hyporheic zone. Understanding which functional traits respond the most to changing environmental conditions enables us to focus attention on the overall functioning of ecotonal ecosystems and understand the early indicators of an unhealthy assemblage in response to anthropogenic stress.

5. Conclusions

In this study, we explored the complex relationships between taxonomic, functional, and environmental factors within a copepod assemblage residing in the hyporheic zone of an Apennine creek. Our analyses revealed that both the taxonomic and functional features of the copepod assemblage mirrored changes along an environmental gradient, which reflected an enrichment in ammonium. This was mainly due to the sensitivity of stygobitic copepods to ammonium, further reinforcing available evidence about their value as ecological indicators. Additionally, our investigation emphasized the role of functional traits, such as biomass and body flexibility, in driving the differentiation between harpacticoid and cyclopoid species, particularly in areas where gravel content significantly influences the environment. Our findings stressed the necessity of considering multifaceted trait responses to environmental gradients for a comprehensive understanding of meiofaunal dynamics in ecotonal ecosystems. Despite some limitations, such as not accounting for seasonal variations and unexplored traits like locomotion and fertility, our study provides significant insights into the responses of copepod traits to environmental variability within the hyporheic zone. Additionally, it sheds light on the potential ecosystem services rendered by copepods in ecotonal regions. These findings lay the groundwork for further research aimed at unraveling ecosystem functioning and identifying early indicators of stress in response to human impacts.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d16050289/s1>. Table S1: Table L of the RLQ analysis with the abundances of the hyporheic copepod species within the averaged samples; Table S2: Table R of the RLQ analysis with the values of environmental variables in the averaged samples; Table S3: Table Q of the RLQ analysis with the values of the nine functional traits for the twelve copepod species of Rio Gamberale Creek. Abund: abundances, J/A: juveniles-to-adults ratio, M/F: males-to-females ratio; B (μg): average biomass; SD: sexual dimorphism; BF ($^{\circ}$): body flexibility; D: diet (FS+M: fine sediment + microorganisms; LM+P: living microinvertebrates + protists; Mi+P+A: microinvertebrates + protists + algae;

A: algae); FH: feeding habit (Df: deposit-feeder; Df+S: deposit-feeder + scraper; He: herbivorous; Pr: predator; Om: Omnivorous); E: ecology (SB: stygobite; SP: stygophile; SX: stygoxene); TP: thermal preference (St: stenothermal; MSt: moderately stenothermal; Eu: eurythermal; Heu: highly eurythermal); Table S4: Functional diversity indices related to the averaged samples. Group R: samples to the right of the first axis of the RLQ analysis, Group L: samples to the left of the first axis of the RLQ analysis. FRic: Functional Richness, FEve: Functional Evenness, FDiv: Functional Divergence, FDis: Functional Dispersion. Figure S1. Boxplots depicting functional diversity indices in the R (right of the first axis of the RLQ analysis) and L (left of the first axis of the RLQ analysis) groups. Each boxplot comprises median lines, 25th–75th percentile boundaries, and whiskers extending to the 10th–90th percentiles [79–84].

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