Taxonomic and functional trait variation along a gradient of ammonium contamination in the hyporheic zone of a Mediterranean stream

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ABSTRACT

Ammonium has detrimental effects on the survival and physiology of freshwater invertebrates, however, the effects of ammonium on the functionality of hyporheic invertebrate assemblages have never been investigated. In this study, we applied a mixed (taxonomy- and trait-based) approach to explore the taxonomic and functional trait variation along a gradient of ammonium contamination in the hyporheic zone of a Mediterranean stream. We analysed fifty-one trait modalities of thirty-eight invertebrate taxa. We tested a priori hypotheses on the functional traits that should be affected. We observed a severe erosion of taxonomic and functional diversity with the loss of 12 taxa and 11 trait modalities related to size and body form, fecundity and reproduction, resistance forms and respiration, diet, locomotion and feeding habits. The effect on taxonomic and trait modality abundances was also relevant; in particular, the decrease in detritivores seemed to have led to a low content in dissolved organic matter. Synergic detrimental effects of ammonium and the summer season were also observed. Despite some uncertainties, we found out that the mixed approach responded consistently along a gradient of ammonium contamination. Since functional traits link community organization to ecosystem goods and services, the results of our study could be prodromal to the inclusion of ammonium as indicator of functional stress in a health index of hyporheic zones in Mediterranean rivers.

1. Introduction

Ecological studies exploring the functionality of the hyporheic zone (HZ; i.e., the region of sediments and porous space beneath and alongside a stream bed, where there is mixing of shallow groundwater and surface water) have covered main aspects such as: i) the ecotonal nature of the HZ, which is modulator for most metabolic stream processes (e.g., Williams et al., 2010; Marzadri et al., 2014; Ward et al., 2019; Thomle et al., 2020; Wu et al., 2021); ii) the role of HZ as a refuge for taxa, especially in intermittent streams (e.g., Wood et al., 2010; Stubbington et al., 2011; Peralta-Maraver et al., 2018; Nelson et al., 2019; Bruno et al., 2020), and iii) the relations between hyporheic invertebrate assemblages and environmental conditions such as geomorphological processes (Magliozzi et al., 2019), pore water volume, organic carbon content and microbial assemblages (e.g., Boeker et al., 2016; Mathers et al., 2019; Schmid-Araya et al., 2020). Assessing the effect of contamination on the functionality of the HZ is an important avenue of

research that, however, in the last years, has mainly focused on metals (e.g., Piló et al., 2016; Jin et al., 2020).

Ammonium contamination in the HZ has recently come to the fore for the implications connected with the riverbank filtration of water for human consumption (Covatti and Grischek, 2021). Ammonium occurring in the HZ, as well as in stream water and groundwater, has a few natural (e.g., nitrate reduction, peat) and several anthropogenic (fertilizers, livestock manure, leachate from landfills and septic tanks, industrial and domestic wastewater) sources (e.g., Zhu et al., 2013). In the HZ, ammonium is undesirable because it causes the formation of anoxic microzones since 1 mg/L of ammonium leads to the consumption of about 4 mg/L of dissolved oxygen (Roy Chowdhury et al., 2020). Oxygen consumption by ammonium causes, in turn, changes in reducing conditions with potential negative consequences on the release of manganese and iron from the hyporheic sediments (Liu et al., 2020). Ammonium is toxic to aquatic invertebrates and, depending on the concentration and time of exposure, induces variation in gene

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expression (Di Lorenzo et al., 2017), rapid death (Di Marzio et al., 2009, 2018; Di Lorenzo et al., 2014, 2015a), as well as alteration of functional traits such as respiration (Di Lorenzo et al., 2016), development (Di Marzio et al., 2014), biomass, reproduction and dispersion (Arenas-Sánchez et al., 2021).

To the best of our knowledge, no studies have investigated the effects of ammonium on hyporheic assemblage functionality if not marginally, focusing on broad contamination cases in which ammonium was involved along with other pollutants (Williams and Fulthorpe, 2003), mainly nitrogen (Caschetto et al., 2017) and metals (Iepure et al., 2013). These studies have relied on a taxonomy-based approach, that is on examining eventual variations in taxonomic richness and abundance distribution. Trait-based and mixed approaches (i.e., coupled taxonomy- and trait-based approaches) have been only recently used in studies concerning the HZ (e.g., Descloux et al., 2014; Magliozzi et al., 2019; Bruno et al., 2020) but never applied to an ammonium contamination context. On the other hand, mixed approaches have been successfully used to target anthropogenic stress in freshwater benthic communities (e.g., Usseglio-Polatera et al., 2000; Dolédec and Statzner, 2008; Archaimbault et al., 2010; Van den Berg et al., 2020).

In this study, we investigated the effects of ammonium on hyporheic invertebrate assemblages through a mixed approach. We took advantage of an ideal study area, a small Apennine stream with an upstream pristine stretch located in a protected area and a downstream stretch surrounded by anthropogenic pressures that release ammonium by runoff (agricultural areas) and wastewater discharge (from an urban wastewater treatment plant). The Italian regulation (Repubblica Italiana, 2006), which transposes the European Water Framework Directive 2000/60/EC (EC, 2000), establishes that the water quality of a water body is poor when ammonium concentration $\geq$ 0.5 mg/L. In the downstream stretch of the Rio Gamberale, the stream waters are rich in ammonium, up to concentrations that exceed the quality threshold set by the Italian regulation (0.5 mg/L). First, we predicted that the taxonomic and trait composition of the hyporheic assemblages of the upstream pristine stretch of the stream could be significantly different from that of the downstream stretch (hypothesis H1: upstream vs downstream). Second, we predicted that the differences assessed in the previous analysis could be due to a linear shift in both taxonomic and trait composition along the increasing gradient of ammonium contamination (hypothesis H2: gradient analysis). We based our hypotheses on the results of previous studies that highlighted the effect of ammonium on the survival, development, respiration, biomass, reproduction and dispersal of hyporheic invertebrates (e.g., Di Marzio et al., 2009, 2014, 2018; Di Lorenzo et al., 2014, 2015a, 2015b, 2016; Arenas-Sánchez et al., 2021). Hypothesis H1 was based on a previous field study in which we observed that the Italian freshwater quality threshold for ammonium ($=$ 0.5 mg/L) was not protective for crustacean assemblages in alluvial aquifers underlying densely cultivated areas (Di Lorenzo et al., 2015a). In a further laboratory study, we also observed that, for some crustacean species, the negative effects of ammonium occurred at concentrations $<$ 0.5 mg/L (Di Lorenzo et al., 2014). For this reason, we also considered it appropriate to perform a gradient analysis (hypothesis H2).

2. Materials and methods

2.1. The study area

Rio Gamberale (42°14′00′′ N, 13°32′10′′ E; length: 10.04 km; discharge: 360 L/s; Fig. 1) is an Apennine stream that originates at 1500 m a.s.L in Vado di Pezza (Abruzzo, Italy).

According to the EU CIS Guidance on pressures and impacts (EC, 2003), the Rio Gamberale stream can be divided into an upstream and a
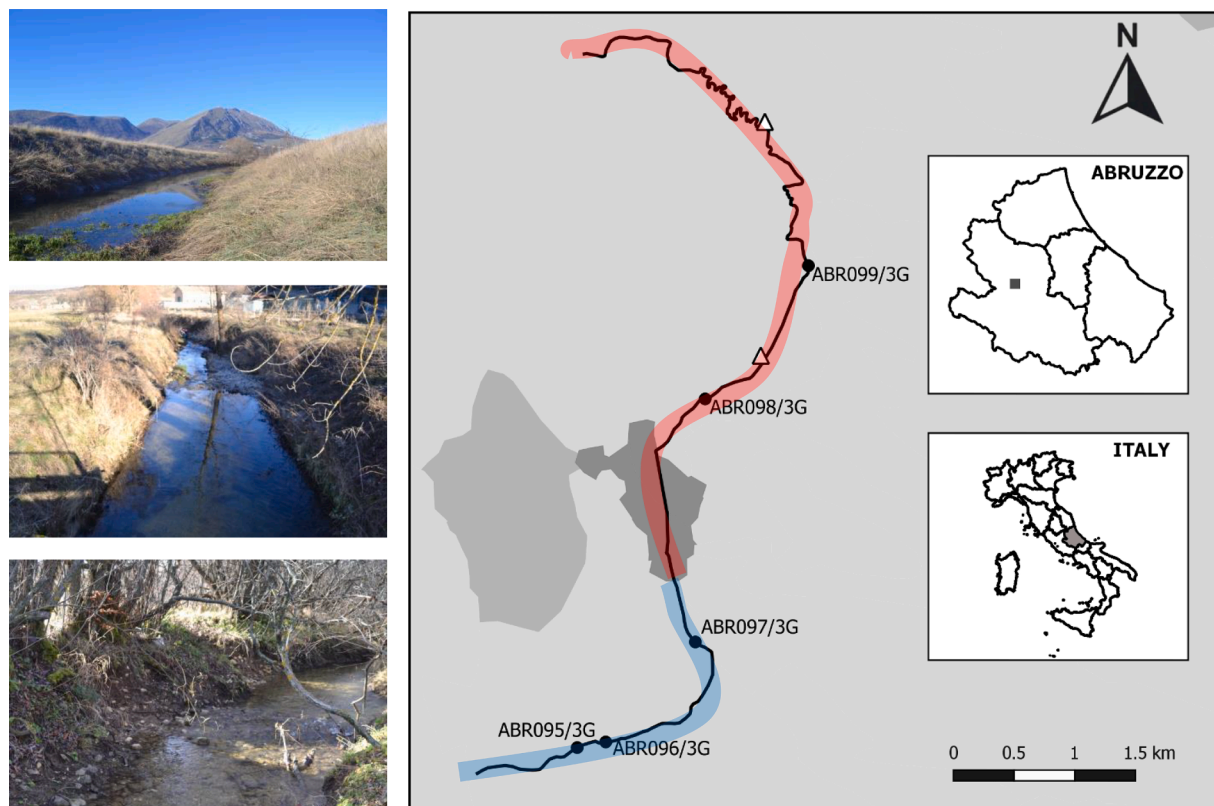
Fig. 1. Rio Gamberale stream (Abruzzo - Italy) and sampling stations. The land use is represented in shades of grey as follows: stable meadows, wooded areas and areas characterized by shrub and/or herbaceous vegetation in the full background of silver grey, heterogeneous agricultural areas in light grey and urbanized areas in dark grey. The sampling stations are represented by black dots and the wastewater treatment plants by white triangles. The upstream stretch is highlighted in blue; the downstream stretch is highlighted in red.

downstream stretch. In the upstream stretch, the stream runs in a protected area established in 1989 and denominated Parco Naturale Regionale Sirente-Velino (Abruzzo, Italy). In the downstream stretch, at an altitude of 1350 m a.s.L., the stream crosses the Altopiano delle Rocche, a vast plain of karst origin and once site of an ancient lake basin (Late Pleistocene and Holocene sediments). The Meso-Cenozoic carbonate mountains that encircle the hydrographic basin of the stream reach altitudes up to 2271 m a.s.l.. Along its path in the downstream stretch, the stream encounters several anthropogenic activities (agriculture and two urban wastewater treatment plants) which release ammonium to its waters. Near the mountain village of Terranera (1270 m a.s.l.), the stream sinks underground through the Pozzo Caldaio sinkhole (1253 m a.s.L.) and, finally, emerges in Grotta di Stiffe cave (661 m a.s.L.) after 2600 m (Scorzini and Leopardi, 2017). The hydrographic basin of the Rio Gamberale stream is 51.08 km^2 and the mean flow is 360 L/s. The average rainfalls are about 800 mm/year in the catchment area, with more intense rainfalls occurring during the winter period, up to 3-fold higher than those in the summer period (Regione Abruzzo, 2010).

2.2. Sampling survey

We monitored the HZ of the Rio Gamberale stream in five sampling stations, of which: i) three (ABR095/3G, ABR096/3G, ABR097/3G; Fig. 1) located in the upstream pristine stretch not subject to any anthropogenic activities except for a disused quarry which, however, does not pose a risk to the water body (EC, 2003) and ii) two (ABR098/3G, ABR099/3G; Fig. 1) located in the downstream stretch surrounded by agricultural areas and situated downstream of the discharge of an urban wastewater treatment plant (ABR099/3G). The sampling stations were about 1.5 km apart from each other, except for ABR095/3G and ABR096/3G which were about 300 m apart.

At each sampling station, we took three samples (H1, H2, H3) from three sampling sites located on an oblique transect (at the left, right and centre of the transect), at a distance of at least 1.5 m from each other. We carried out two sampling surveys, in December 2014 (winter) and June 2015 (summer). The month of December 2014 had a mild climate with 190 mm rainfalls. The month of June 2015 was hot and dry with thermal values above the seasonal average and rainfalls < 20 mm (Regione Abruzzo, 2010). The stream never went dry during our survey. Since the hyporheic sampling sites were not fixed, the hyporheic samples were considered true replicates and not pseudoreplicates (Anderson et al., 2008). Preliminary surveys showed that the streambed sediment composition was not significantly different among the five stations, all being dominated by sand (63 μm < φ ≤ 2 mm; range: 60–81%), followed by clay-silt (φ ≤ 63 μm; range: 18–40%) and gravel (2 mm < φ ≤ 64 mm; range: 0–3%). The detail of the granulometric composition of the streambed for each hyporheic replicate in each station is reported in the Supplementary File (GRAN). The results of a multivariate permutational ANOVA performed during the preliminary survey indicated that there was no significant difference in the granulometric composition of the five sampling stations. The results of the analysis are reported in the Supplementary File (GRAN). We collected both environmental and biological data as described in the next paragraphs following the procedures outlined in the European sampling manual concerning subterranean ecosystems (Malard et al., 2002).

2.3. Biological data

Hyporheic samples were collected by pumping 10 L of interstitial water with a Bou-Rouch pump (Bou and Rouch, 1967) connected to steel piezometers hammered in the streambed at a depth of 40 cm (Malard et al., 2002). The piezometers were mobile, provided with a closed tip and a screened section of 5 cm with holes of 5 mm (Mugnai et al., 2015). The Bou-Rouch pump created a disturbance and maintained an interstitial flow around the piezometer (up to 0.66 L/s; Malard et al., 2002)

that was sufficient to dislodge hyporheic organisms. The pump sampled both swimming organisms and species intimately linked to sand particles; in 10 L of pumped water, 76–100 % of the taxa are usually collected by a Bou-Rouch pump (Malard et al., 2002). The pumped interstitial water was filtered with a 60 μm-mesh net. Samples were preserved in a 70% alcohol solution. In the laboratory, the samples were sorted with the aid of a stereomicroscope at 16×. Organisms were picked up by a glass pipette and identified to the lowest possible taxonomic resolution based on updated literature (e.g., Kiefer, 1960; Amoros, 1984; Schmid, 1993; Di Sabatino et al., 2004; Dussart and Defaye, 2006). Afterward, 12 functional traits, described by the modalities reported in Appendix 1, following Descloux et al. (2014 and references therein) and Di Lorenzo et al. (2020), were measured for each specimen. Traits reflected life history, locomotion, reproduction, diet, physiology and habitat affinity of the collected taxa. The trait profiles of the 38 invertebrate taxa collected in the HZ of the Rio Gamberale stream were described mainly at species and genus levels and reported in the Supplementary File (TRAITS). The traits of Nematoda, Oligochaeta, Crustacea Ostracoda, Acari Hydrachnidia and Diptera were described using the mean trait profiles of their families in the corresponding biogeographic region (Descloux et al., 2014). Each collected specimen was photographed using a LEICA M205C stereomicroscope provided with an integrated camera and subsequently measured using the LAS software (Leica Application Suite, version 4.7.1). The body length (in mm) was converted into taxon-specific biovolume (in nL) using the formulas indicated in Reiss and Schmid-Araya (2008), assuming a specific gravity of 1.1; the dry carbon content (biomass) was set equal to 40% of the dry mass with a dry/wet mass ratio equal to 0.25.

The trait profile of each sample was obtained by weighing the individual trait profiles of taxa by their abundance in the sample. The weighted scores were summed per each trait in each sample and then rescaled to sum up to 100% for each sample, following Descloux et al. (2014 and references therein).

For each sample, we also measured a set of univariate diversity measures, namely Margalef's (d), Pielou's (J'), Shannon's (H' where logs are to base e) and Simpson's (1-λ) indices.

2.4. Environmental data

We withdrew 10 L of hyporheic water for the chemical analyses after the biological sampling, using the same procedure. We used a multiparametric probe (WTW Multi 3430 SET G) to measure electrical conductivity at 25 °C (μS/cm), pH, dissolved oxygen (mg/L), oxygen saturation (%) and temperature (°C) for each hyporheic sample. We measured the concentrations of 108 potential contaminants (Supplementary File: CHEM) by retaining a volume of water equal to 2 L for the chemical analyses. The compounds were selected based on the anthropogenic pressures (agriculture, urban settlements and wastewater treatment plants) in the catchment area. We also measured the concentrations of particulate organic matter (POM), total organic carbon (TOC) and dissolved organic carbon (DOC) to account for the carbon content in the HZ of the Rio Gamberale stream. The particulate organic matter was measured after the removal of all the fauna from the samples; the remaining sediments were dried in an oven at 105 °C for 24 h, cooled down for further 24 h and finally weighed. The dry weighted samples were incinerated at 540 °C and weighed again to determine the amount of POM as the difference between dry mass and ash mass (Fischer et al., 2002).

2.5. Data analysis

2.5.1. Sampling effort

Exhaustiveness of the biological sampling effort was assessed through examining the increasing of total number of different taxa observed (S_{obs}) as samples were successively pooled. To assess how much larger S could get with repeated sampling, five non-parametric

(Chao1, Chao2, Jackknife1, Jackknife2 and Bootstrap) and one parametric (Michaelis-Menten) estimators were used, each of which was calculated as every new sample was added, so the result was again a curve of the evolution of the S predictor as sample size increases (Magurran and McGill, 2011 and references therein). Values were estimated by means of 999 randomizations without replacement.

2.5.2. Testing H1: upstream vs downstream

Our first hypothesis (H1) was that the taxonomic and trait composition of the hyporheic assemblages in the upstream pristine stretch of the stream could be different from that of the downstream stretch. To test this hypothesis, we used three-way PERMANOVAs (Anderson, 2001). PERMANOVA is a routine for testing the simultaneous response of one or more variables to one or more factors in an ANOVA experimental design based on resemblance measures, using permutation methods (Anderson, 2001). Biological and environmental data were analysed separately, using the design described later in the text. Resemblance matrices were based on: i) Euclidean distances of normalized environmental data; ii) Euclidean distances of non-transformed percentages of trait modalities and iii) Bray-Curtis distances prior $\log(x + 1)$ transformation for the taxonomic data (species abundances and diversity indices) and biomasses. A dummy variable equal to 1 was added, when necessary, to include otherwise empty cells in the Bray-Curtis distance computation (Anderson et al., 2008). Multicollinearity among environmental variables were inspected through Draftsman's plots. Since highly collinear variables (i.e., correlation $|r| \geq 0.95$) contain effectively the same information and are redundant for the purposes of the analysis, we dropped redundant variables before proceeding, keeping in mind that the variables which were retained in the analyses might be acting as proxies for the ones that were dropped (Anderson et al., 2008).

We applied a mixed hierarchical design consisting of two fixed and one random factor: "season" (SE) with two levels (*summer*, *winter*), "stretch" (ST) with two levels (*upstream*, *downstream*), and "sampling stations" (SS), nested in ST, with five levels (*ABR095/3G*, *ABR096/3G*, *ABR097/3G*, *ABR098/3G*, *ABR099/3G*). The model equation was as follows:

$$SS_{tot} = SS_{SE} + SS_{ST} + SS_{SE(ST)} + SS_{SE \times ST} + SS_{SE \times SS(ST)} + SS_{res} \quad (1)$$

where SS is the sum of squares and SS_{res} is the residual sum of squares (the sum of squared deviations of observations from their own group mean). We examined the model under the Type I partitioning of sum of squares that is the most sensitive for hierarchical unbalanced mixed design (Anderson, 2001). We also used permutation of residuals under a reduced model as it yields the best power and the most accurate type I error for multi-factorial designs (Anderson, 2001). Although PERMANOVA does not rely on assumptions relating to the distribution of data, a PERMDISP routine was applied prior to PERMANOVAs to account for the potential heterogeneity of the variances within each factor (Anderson, 2001) and strong skewness in the distribution were avoided through $\log(x + 1)$-transforming the biological data, when necessary (Anderson, 2001). The significance level (α) was set at 0.05. Correct p-values were obtained through Monte Carlo random draws from the asymptotic permutation distribution (Anderson and Robinson, 2001) when too few permutations were available for a given test. We inspected both multivariate and univariate models. However, we refrained from analysing the variation in the abundances of every single taxon because when the abundances of the individual taxa are < 1% of total abundances the chance of correctly interpreting the outcomes of the analysis are reduced (Clarke and Gorley, 2015). We analysed the abundances of all taxa accounting for at least 4% of the total abundances in any sample (Clarke and Gorley, 2015). When deemed useful to improve understanding, the most significant results of the PERMANOVAs were visualized through PCO (Principal Coordinates Analysis) plots. The concentrations of ammonium over the sampling stations were visualized

by a bubble plot, in which circles were drawn at each sample, of size related to the ammonium concentrations at that sample (Clarke and Gorley, 2015).

2.5.3. Testing H2: gradient analysis

To test the hypothesis H2 (i.e., we expected linear correlation between taxonomic or trait composition and the increasing gradient of ammonium contamination), we performed a gradient analysis using the DistLM (Distance-based Linear Models) routine (McArdle and Anderson, 2001). The DistLM routine analyses and models the linear relationships between the dissimilarity matrices of biological data and the predictor environmental variable(s). The routine allows to fit one or more environmental predictors to one or more biological variables. We first inspected multivariate (all predictors together) and then univariate (ammonium vs every trait modality) models. For multivariate models (conditional tests; Anderson et al., 2008), we applied the BEST selection procedure which examines, for each model, the value of the selection criterion for all possible combinations of predictor variables. We applied the AIC criterion (Akaike, 1973) and used R^2 (variance explained by the models) to assess the fit of the best-solution models. When deemed useful to enhance comprehension, the most significant models were showed through dbRDA (distance-based Redundancy Analysis; Legendre and Anderson, 1999) plots. Afterwards, univariate linear models (marginal tests; Anderson et al., 2008) were examined to assess the Pearson correlation between ammonium and the abundances of each taxon or trait modalities. We used p-values obtained through 999 permutations to assess the fit of the univariate models (the significance was set at $\alpha = 0.05$).

All analyses and plots were performed with E-PRIMER and PERMANOVA + software v. 6 (Anderson et al., 2008) and the software R v. 3.5.0.0 (R Development Core Team, 2013).

3. Results

3.1. Sampling effort

Overall, we collected a total of 815 invertebrate specimens belonging to 38 taxa (Supplementary File: TAXA). Crustaceans were dominant with 371 individuals, followed by insects (296 ind.), annelids (131 ind.), molluscs (13 ind.) and mites (4 ind.). Rotifera and Tardigrada did not occur in the HZ of the Rio Gamberale stream, at least during this study. The Michaelis-Menten estimator indicated that we captured the totality of the expected biodiversity in the HZ of the Rio Gamberale stream. The other estimators indicated a non-exhaustive sampling effort with a missed percentage of biodiversity in the range of 12% to 32% (expected number of taxa: Chao1: 48; Chao2: 50; Jacknife1: 51; Jacknife2: 56 and Bootstrap: 44; Fig. 2).

3.2. Testing H1: upstream vs downstream

3.2.1. Environmental variables

The values of the 116 environmental variables are reported in the Supplementary File (CHEM). The concentrations of 99 chemical compounds, including metals, pesticides, volatile organic compounds and hydrocarbons, did not exceed the instrumental limit of detection, indicating that the compounds did not occur in the HZ of the Rio Gamberale stream, at least during our survey. For this reason, the 99 contaminants were not included in the statistical analyses. The reaming compounds occurred with detectable concentrations which were, however, lower than the legal limits (Table 1); they were all included in the analyses. Ammonium was the only pollutant found in the HZ of the Rio Gamberale stream; it showed concentrations > 0.5 mg/L (=quality threshold for surface waters set by the Italian regulation; Repubblica Italiana, 2006) in the samples of the two sampling stations located in the downstream stretch of the stream (Table 1). TOC and DOC were highly correlated (r = 0.954) and TOC was dropped from the PERMANOVA analyses. The
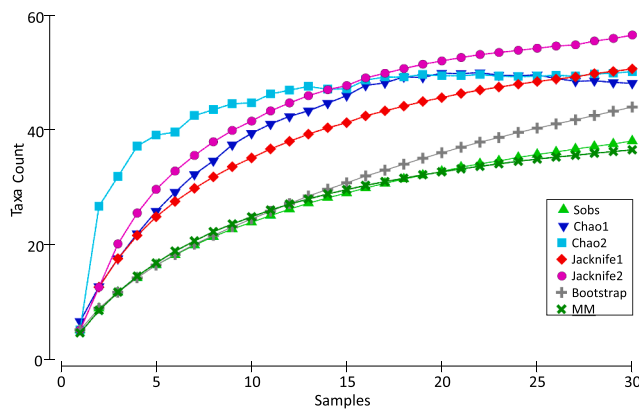
Fig. 2. Trends of the accumulation curves of the observed (S_{obs}) and expected taxonomic richness. The results of the taxonomic richness estimators in the hyporheic zone of the Rio Gamberale stream were as follows (expressed in taxa counts): Chao1 = 60; Chao2 = 57; Jacknife1 = 57; Jacknife2 = 64; Bootstrap = 50; MM (Michaelis-Menten) = 41. The reader should refer to the online version for colours.

Table 1

Mean (μ) and standard deviation (SD) of the environmental variables (EV) and diversity indices (DIVERSE) in the hyporheic zone of the Rio Gamberale stream. The mean and SD values are also reported for the two levels (w: winter; s: summer) of the factor "season" and the two levels (u: upstream; d: downstream) of the factor "stretch". EC: electrical conductivity; POM: particulate organic substance; TOC: total organic carbon; DOC: dissolved organic carbon; d: Margalef's index; J': Pielou's index; H': Shannon's index; 1-λ: Simpson's index. Ammonium concentrations $\geq$ 0.5 mg/L (Italian legal limit) are indicated in bold. The asterisks indicate significant differences based on the results of the PERMANOVAs.

EV	μ	SD	μ_s	μ_w	μ_u	μ_d
T (°C)	9.62	4.72	14.22*	5.30*	10.28	9.45
EC (μS/cm)	513.00	105.60	474.07	549.50	582.41*	464.72*
pH	7.93	0.23	8.01	7.86	7.82	7.98
O_2 (mg/L)	6.22	2.74	5.22	7.15	4.84	6.92
POM (mg/L)	338.45	275.55				
O_2 (%)	64.39	22.33	58.70	69.72	48.21*	73.83*
POM (mg/L)	338.45	275.55	348.60	328.94	306.58	374.61
DOC (mg/L)	1.67	0.44	1.78	1.93	1.81*	1.56*
TOC (mg/L)	1.85	0.50	1.62	1.72	1.92	1.80
NO_2^- (mg/L)	0.15	0.19	0.27*	0.03*	0.09	0.19
NO_3^- (mg/L)	3.81	3.09	6.14*	1.64*	3.90	3.51
SO_4^{2-} (mg/L)	8.08	7.62	8.76	7.44	13.02*	4.62*
Cl$^-$ (mg/L)	4.49	4.44	2.09*	6.74*	7.04	2.60
PO_4^{3-}(mg/L)	0.17	0.10	0.09*	0.24*	0.19	0.14
NH_4^+ (mg/L)	**0.45**	0.70	0.34	**0.56**	0.03*	**1.02***
Ca^{2+} (mg/L)	101.35	14.59	97.60	104.88	107.12	97.00
K^+ (mg/L)	1.31	1.00	1.31	1.31	1.81	0.98
Na^+ (mg/L)	8.26	7.36	10.11	6.53	10.57	6.75
		36				
DIVERSE	μ	SD	μ_s	μ_w	μ_I	μ_{II}
d	1.43	0.61	1.67	1.19	1.40	1.44
J'	0.79	1.15	0.82	0.76	0.78	0.80
H'	1.22	0.41	1.43	0.99	1.29	1.16
1-λ	0.67	0.18	0.73	0.60	0.66	0.67

multivariate PERMANOVA highlighted significant differences in the values of the environmental variables between winter and summer (SE: Pseudo-$F_{1,29}$ = 10.4, p = 0.0002). The variability between the two levels of the factor "stretch" was also significant (SS$_{ST}$: Pseudo-$F_{1,29}$ = 5.31, p = 0.0050) while no differences resulted for the factor "sampling stations" nor for the factors' interactions (p-values > 0.05). The result was visualized through PCO plots (Fig. 3). The percentage of the total variation explained by the first two PCO axes was 53.4%. The plots showed a clear separation of 'winter' and 'summer" samples (Fig. 3a) and of "upstream" and "downstream" samples (Fig. 3b). This pattern was

mainly due to the variation of ten environmental variables. In detail, PERMANOVA highlighted significant differences in the mean values of temperature (T), NO_2^-, NO_3^-, Cl$^-$ and PO_4^{3-} between summer and winter (Appendix 2): T, NO_2^- and NO_3^- mean concentrations were higher in summer than in winter while Cl$^-$ and PO_4^{3-} mean concentrations were higher in winter than in summer (Appendix 2).

PERMANOVA also highlighted significant differences in the mean values of electrical conductivity (EC), oxygen (O_2 %), DOC, SO_4^{2-} and NH_4^+ between the upstream and downstream stretches (Appendix 2): EC, DOC and SO_4^2 showed higher values in the upstream than in the downstream stretch (Table 1) while O_2 % and NH_4^+ showed higher values in the samples of the downstream stretch (Table 1). In particular, NH_4^+ showed increasing concentrations from the first (ABR095/G3) to the last (ABR098/G3) station (see Supplementary File; PCO_NH4).

3.2.2. Taxonomic composition

Overall, 32 taxa were collected in the upstream stretch and only 20 in the downstream stretch; 26 taxa were collected in winter and 33 in summer (Supplementary File: TAXA). However, PERMANOVA did not highlight significant differences in the values of the diversity indices, for any of the investigated factors (Table 1; Appendix 2). On the contrary, the multivariate PERMANOVA performed on the overall taxonomic composition showed significant differences between winter and summer and between the two levels of the factor "stretch" (Appendix 2). The result was shown through PCO plots (Fig. 3). The percentage of the total variation explained by the first two PCO axes was 39%. The plots showed some separation of 'winter' and 'summer" samples (Fig. 3c) and "upstream" and "downstream" samples (Fig. 3d). Differences were attributable to changes in both taxonomic richness and abundance distribution. Molluscs occurred only in the upstream stretch of the stream (Appendix 2; Supplementary File: TAXA). Nematodes (represented by the sole family Mermithidae) were significantly more abundant in summer and in the downstream stretch (Supplementary File: TAXA). Crustacean abundances were significantly higher in summer than in winter (Supplementary File: TAXA), while annelid abundances were significantly higher in the downstream stretch of the stream than in the upstream one (Appendix 2; Supplementary File: TAXA). The results of PERMANOVA conducted on the abundances of insects and acari did not show significant differences (Appendix 2). The univariate analyses carried out on individual taxa gave significant results only for the abundances of naidids, which were significantly higher in the downstream stretch, and those of the harpacticoid copepod *Bryocamptus pygmaeus*, which were significantly higher in summer (Appendix 2; Supplementary File: TAXA). Non-significant statistical results of the remaining 36 taxa were not shown.

3.2.3. Functional trait composition

Four trait modalities out of 51 were not represented in the HZ of the Rio Gamberale stream, namely: parthenogenesis + asexual reproduction (trait "reproduction"), dead animal > 1 mm and living macrophytes (trait "diet") and piercers (trait "feeding habits").

PERMANOVA highlighted significant differences in the overall abundances of the traits "maximal potential size", "fecundity" and "diet" due to the factor "stretch" (Appendix 2). In detail: i) taxa with body sizes ranging from 0.5 to 1.0 cm (class 3) were not present in the downstream stretch while occurring in the upstream one (Appendix 2; Fig. 4); ii) taxa producing < 100 eggs were significantly more abundant in the downstream stretch as opposed to those producing 100–1000 eggs, which were significantly less abundant, and those producing > 1000 eggs, which were completely absent in the downstream stretch though occurring in the upstream one (Appendix 2; Fig. 4); iii) taxa feeding on dead plants (>1 mm) were completely absent in the downstream stretch though sporadically occurring in the upstream one and those feeding on fine detritus (<1 mm) were less abundant as opposed to those feeding on living macroinvertebrates which were significantly more abundant in the downstream stretch (Appendix 2; Fig. 4).
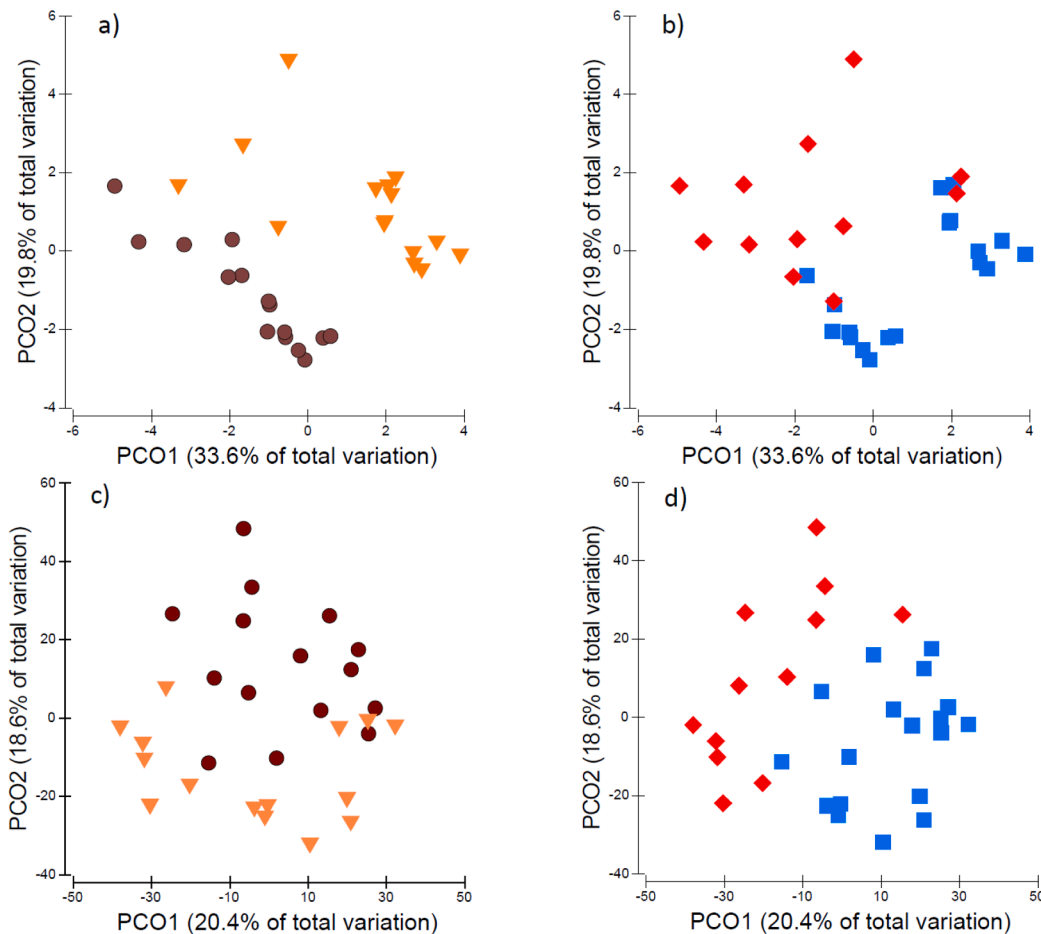
Fig. 3. PCO plots showing the separation of "summer" (orange triangles) and "winter" (brown dots) samples and "upstream" (blues squares) and "downstream" samples (red diamonds) based on: a, b) Euclidean distances of the environmental variables; c, d) Bray-Curtis similarities of the taxonomic abundances. The cumulative variance explained by the first two axes is 53% for the environmental variables (a, b) and 39% for the taxonomic abundances (c, d). The reader should refer to the online version for colours.

The trait "locomotion and substrate relation" showed significant differences in abundances between winter and summer and between the two levels of the factor "stretch" (Appendix 2). The pattern was mainly described by the burrowers, which were significantly less abundant in the downstream stretch as opposed to taxa that live attached to the sediments, which were significantly more abundant in the downstream stretch and in winter (Appendix 2; Fig. 4). At the multivariate level (that is considering all the modalities of a given trait altogether), no significant differences related to the remaining traits were highlighted (Appendix 2). However, when individually analysed, some modalities of the traits "reproduction", "feeding habits" and "resistance form" revealed significant differences (Appendix 2): ovoviviparous taxa did not occur in the downstream stretch while taxa producing free clutches were significantly more abundant in summer than in winter (Appendix 2; Fig. 4); shredders were significantly less abundant in the downstream stretch and in winter (Appendix 2; Fig. 4).

PERMANOVA did not detect significant differences in the abundance of the rarest traits, i.e., those represented by few individuals or represented in a single sample. However, it is worth mentioning that further seven modalities represented in the upstream stretch were not found in the downstream one, namely: 1) spherical organisms (trait "body form"); 2) clutches - terrestrial (+in vegetation); 3) dormancy and 4) housing against desiccation (trait "resistance form"); 5) plastron + spiracle (trait "respiration form"); 6) scrapers and 7) filter feeders (trait "feeding habits"). On the contrary, all the modalities represented in the downstream stretch also occurred in the upstream one. Concerning the season, a single modality (filter-feeders of the trait "feeding habits") was present in winter but was not found in summer. On the contrary, all the modalities that were represented in summer were also represented in winter.

3.3. Testing H2: gradient analysis

The outputs of the linear models are reported in Appendix 3. We examined 71 multivariate (conditional tests) and as many univariate (marginal tests) models. Ammonium was one of the predictors in 26 out of the 71 multivariate models (Appendix 3). However, in none of these models, with the sole exception of the one describing the linear relationships with the abundances of the taxon Nematoda (Appendix 3; Supplementary File: NEMATODA), was ammonium the main descriptor. In the remaining 25 models, ammonium explained <10% of variance (data not shown).

As for the univariate models, 19 were significant (Appendix 3). In detail: i) the abundances of nematodes, taxa performing 1 cycle/year (trait "number of cycles per year"), streamlined organisms (trait "body form"), organisms breathing through tegument (trait "respiration"), organisms attached to sediments (trait "locomotion and substrate relation"), organisms producing isolated free eggs (trait "reproduction") and parasites (trait "feeding habits") were positively correlated to ammonium (i.e., their abundances increased with increasing ammonium concentrations) while ii) the abundances of taxa with body sizes ranging from 0.5 to 1.0 cm (class 3), organisms breathing through gills (trait "respiration"), burrowers (trait "locomotion and substrate relation") and organisms feeding on fine detritus (trait "diet") were negatively correlated (Fig. 5).

4. Discussions

In this study, we aimed at investigating whether ammonium induces variations in the taxonomic and functional compositions of hyporheic assemblages and whether the extent of such variations is proportional to
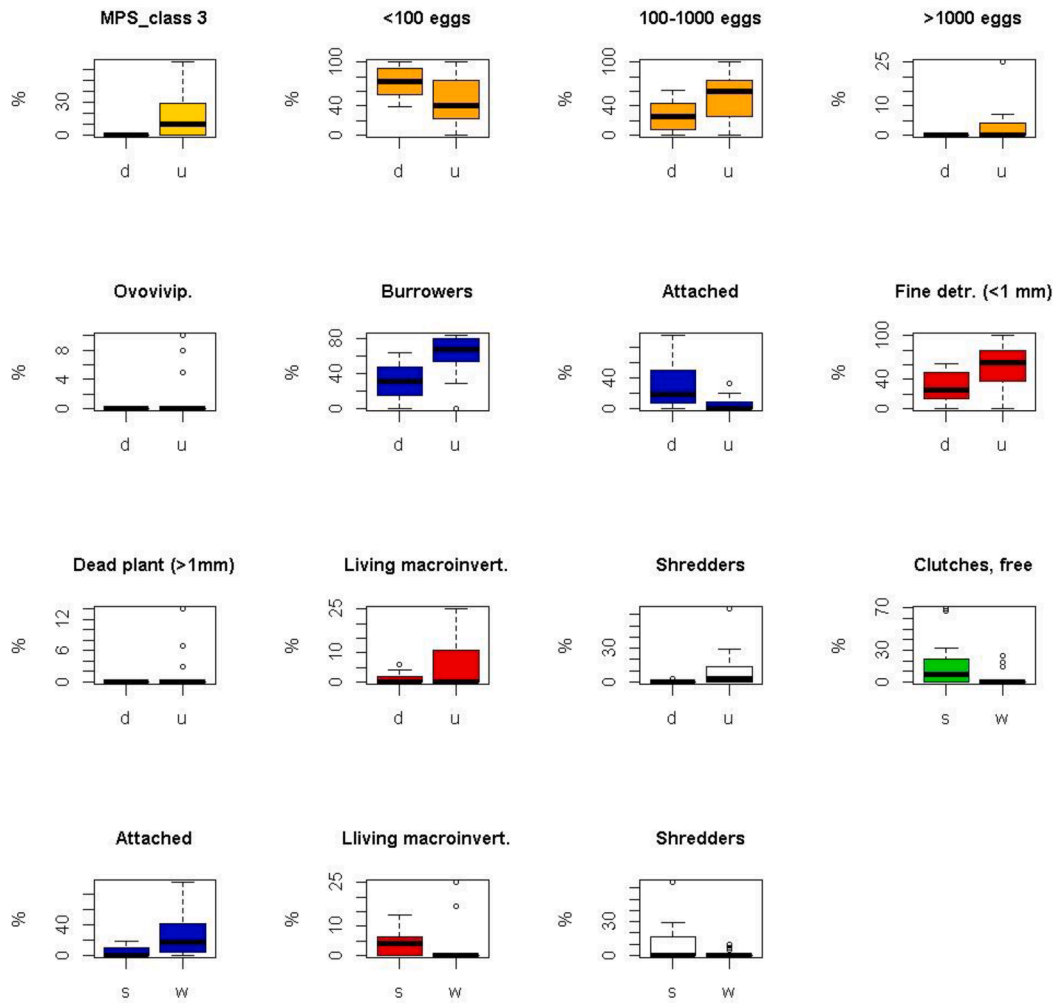
Fig. 4. Boxplots representing the abundances (in %) of the trait modalities that resulted significantly different between the upstream (u) and downstream (d) stretches and/or winter (w) and summer (s). Modality names as in Appendix 1. Colours represent traits: maximal potential size (MPS: yellow), fecundity (orange), reproduction (green), locomotion and substrate relation (blue), diet (red), feeding habits (white). The reader should refer to the online version for colours.

ammonium concentrations. To this end, we compared the composition and functionality of the hyporheic assemblages of two stretches (an uncontaminated upstream stretch vs a contaminated downstream stretch) of the Rio Gamberale stream, and analysed the linear correlations along an increasing ammonium concentration gradient. We observed a severe erosion of taxonomic composition and functional diversity in the downstream stretch compared to the upstream one; variations in some taxa and trait modalities were also proportional to ammonium concentrations. We concluded that our hypotheses, H1 and H2, were supported. In Table 2, we provided a summary of the most relevant results.

4.1. Taxonomic composition

We observed lower taxonomic richness and abundances in the downstream ammonium-contaminated stretch (Table 2) as also observed by Di Lorenzo et al. (2013) in the hyporheic zone of an Italian Alpine stream, as opposed to an increase in the abundance of nematodes and annelids, mainly leeches, as also detected in other studies (Gaudes et al., 2013; Jacks et al., 2021). Hirudinida Erpobdellidae are usually among the most tolerant taxa to organic pollution with unspecific modes of action (Van den Berg et al., 2019). Nematode abundances, which in this study significantly increased with increasing ammonium concentrations, are known to depend on inorganic nutrient (ammonium, nitrates and phosphates) enrichment (Gaudes et al., 2013). We expected

some impairment in the composition of crustacean assemblages as the survival of freshwater crustaceans, especially copepods, is seriously impaired by chronic exposure to low (in the order of a few µg/L) ammonium concentrations (Di Lorenzo et al., 2014). In fact, we observed an erosion of the taxonomic richness with the disappearance of *Gammarus elvirae*, *Niphargus* sp.1 (gr. *longicaudatus*), *Bryocamptus echinatus* and *Moraria poppei meridionalis* in the downstream stretch of the stream. The pattern observed for the molluscs, which were absent in the downstream stretch while occurring in the upstream one, is also in accordance to the results of previous studies on benthic taxa belonging to the same group (Alonso and Camargo, 2009). The taxa *Radix labiata*, *Bythinella opaca* complex, *Ancylus fluviatilis* and *Pisidium* sp. 1, which occurred in the upstream stretch, were not found in the downstream one, likely because molluscs are known to be the most sensitive to ammonium (Yan et al., 2019). The taxonomic richness of the instars of Ephemeroptera, Trichoptera, Plecoptera (EPT) and Coleoptera was significantly lower in the downstream stretch than in the upstream one. The disappearance of several species belonging to the EPT may be related to an increase in ammonium concentration, as observed in the Ephemeroptera (Beketov, 2004), and in several other insect taxa in Malaysian headwaters (Ab Hamid and Md Rawi, 2017), despite EPT have been frequently found in polluted freshwater bodies in the Mediterranean area (e.g., Kalogianni et al., 2017; Karaouzas et al., 2018). Moreover, their disappearance in the downstream stretch of the Rio Gamberale stream could also be due to an increase in predatory pressure
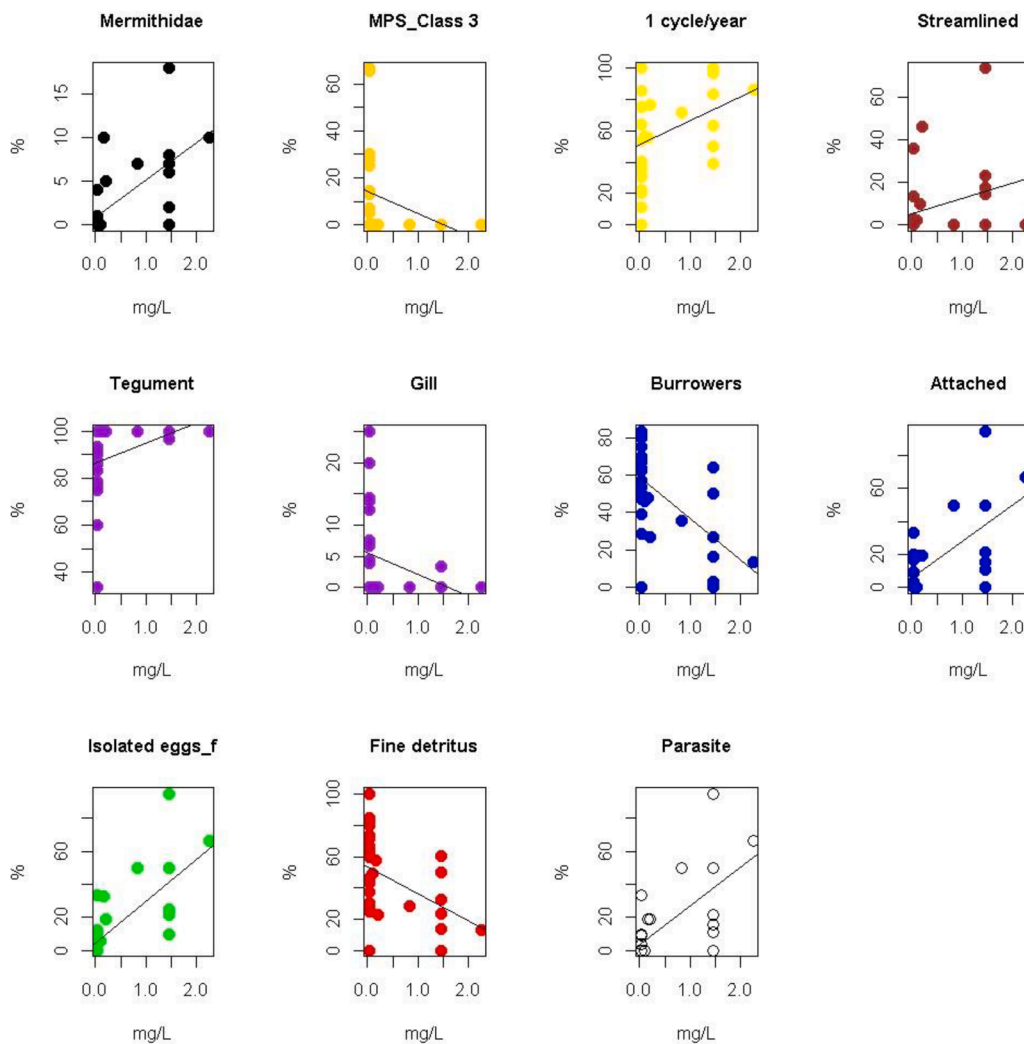
Fig. 5. Plots showing the significant linear correlations (Pearson) between ammonium and the abundances of Mermithidae (Nematoda) and ten trait modalities. Refer to Appendix 3 for tests' significance and correlation values and Appendix 1 for modalities explanation. Colours represent traits: maximal potential size (MPS: orange), fecundity (yellow), reproduction (green), locomotion and substrate relation (blue), diet (red), feeding habits (white), respiration (purple), body form (brown). The reader should refer to the online version for colours.

because organisms that feed on living macroinvertebrates (e.g., mainly leaches) were significantly more abundant in this stretch than in the upstream one.

In our study, indices of community structure seemed to be insensitive to ammonium. It is difficult to say why. Some authors claimed that classical diversity indices, which typically condense all relevant information about an ecosystem's diversity into a single real number, could be affected by both the failure to assess the taxa disparity (Daly et al., 2018) and small sample sizes (Hosokawa et al., 2021). The results of our studies indicated that the sampling effort should have been increased a little while we did not identify all taxa to the species level; these two factors could be the main reasons why the diversity indices masked the assemblage variability in the HZ of the Rio Gamberale stream.

4.2. Functional trait composition

Sixteen modalities belonging to nine traits ("body size", "body form", "fecundity", "reproduction", "resistance forms", "respiration", "diet", "locomotion" and "feeding habits") were either completely eroded or significantly decreased in the abundances in the downstream stretch of the stream. In particular, the disappearance of taxa producing >1000 eggs, along with the decreasing in the abundances of those producing 100–1000 eggs, was suggestive of a low internal recovery capacity through the lack of highly-reproductive species of the hyporheic community (Lange et al., 2014). The decrease in fecundity was somehow expected because previous studies observed that the negative effect of

ammonium on invertebrate egg production significantly exceeds that of other contaminants, for example, chlorobenzene (Williams and Fulthorpe, 2003). The disappearance of small (0.5–1.0 cm) and spherical organisms in the downstream stretch was likely due to their high sensitivity to ammonium because of the larger surface/volume ratio (Dolédec and Statzner, 2008). The loss of taxa producing clutches (both terrestrial + in vegetation and free), in contrast to an increase of those producing free isolated eggs, seemed counter intuitive considering the role of the surface/volume ratio on contaminant exposure rates (Bonada et al., 2007). The loss of ovoviviparous molluscs, which are also able to enter in dormancy or housing against desiccation, was the main factor affecting the disappearance of these traits in the downstream stretch due to the high sensitivity of molluscs to ammonium in freshwaters (Beggel et al., 2017 and references herein).

The loss of organisms that breathe through aerial organs such as plastron was related to the disappearance of molluscs and coleopteran elmids. In accordance, this trait modality is less frequent in stream sites surrounded by agricultural areas (Ladrera et al., 2019). The disappearance of scrapers and taxa feeding on dead plants > 1 mm was related to the loss of many insects and microcrustacean species in the downstream stretch of the stream. Furthermore, we assume that higher concentrations of ammonium in the downstream stretch of the stream also contributed to lowering the abundances of burrowers and shredders, as also observed by Dehedin et al. (2013). Ammonium seemed not to affect taxa feeding on living macroinvertebrates, that is large predators such as leeches, likely because of the larger biomass which makes these animals

Table 2

Summary of the significant results of the analyses with a focus on the effect of ammonium. H1 (upstream vs downstream analysis) and H2 (gradient analyses: marginal tests). The significant differences detected by PERMANOVA are reported as either U > D or U < D where, for instance, U > D indicates that the taxon/trait was more abundant in the upstream stretch (U) than in the downstream one (D). In column H2, significant Pearson coefficients are reported.

H1		H2	
Taxonomic composition	U vs. D	Taxonomic composition	Pearson
Overall taxa	U > D		
Annelida	U < D		
Mollusca	U > D		
Nematoda	U < D	Nematoda	0.64
Naididae	U < D		
Functional traits	**U vs. D**	**Functional traits**	
Max potential size 0.5–1.0 cm	U > D	Max potential size 0.5–1.0 cm	−0.36
Fecundity <100	U < D		
100–1000	U > D		
>1000	U < D		
Diet Fine detritus (<1 mm)	U > D	Diet Fine detritus (<1 mm)	−0.45
Living macroinvertebrates	U < D		
Dead plants (>1 mm)	U > D		
Locomotion and substrate relation Burrower	U > D	Locomotion and substrate relation Burrowers	−0.59
Attached (temporarily or permanently)	U < D	Attached (temporarily or permanently)	0.65
Reproduction Ovoviviparity	U > D	Reproduction Isolated eggs, free	0.73
Feeding habits Shredders	U > D	Feeding habits Parasite	0.68
		Number of cycles per year 1	0.47
		Body form Streamlined	0.32
		Respiration Tegument	0.40
		Gill	−0.37

less sensitive to the ammonium concentrations found in this study (Jacks et al., 2021). The lower abundances of shredders and organisms feeding on fine detritus, along with those feeding on dead plants, highlighted a decrease in the overall abundances of detritivores in the downstream stretch which could lead to a less efficient recycling of organic matter; the significant decrease in DOC revealed by PERMANOVA in the downstream stretch could be attributable to this pattern.

The variation in abundances of ten trait modalities belonging to eight different traits were linearly correlated to ammonium concentrations (Table 2). Four of these trait modalities (organisms with a size in the range of 0.5–1.0 cm, burrowers, attached organisms and those feeding on fine detritus) were also in accordance with the predictions of H1 hypothesis (Table 2) and have been already discussed. Taxa producing free isolated eggs (trait "reproduction") progressively increased their abundance with increasing ammonium concentrations in contrast to our expectations related to the higher surface/volume ratios of the eggs (Arenas-Sánchez et al., 2021). Increasing in the abundances of parasites along the increasing ammonium gradient was mainly related to the presence of the Nematoda Mermithidae (Gaudes et al., 2013) while the increasing of taxa performing one cycle per year was due to higher abundances of oligochaetes and leaches, many species being tolerant to ammonia and other pollutants (Glasby et al., 2021). Streamlined organisms were more abundant than spherical organisms likely because of the lower surface/volume ratio which reduces the exposure to ammonium (Dolédec and Statzner, 2008). Finally, while the positive correlation with the abundances of tegument-breathers was expected because they are likely more affected by gaseous pollutants rather than soluble contaminants such as ammonium, the negative correlation with gill-breathers seemed to be counterintuitive for the same reason (Rico and Van den Brink, 2015).

The total absence of four trait modalities in the HZ of the Gamberale stream (parthenogenesis + asexual reproduction of the trait "reproduction"; dead animal > 1 mm and living macrophytes of the trait "diet" and piercers of the trait "feeding habits") is not attributable to ammonium or to any of the other determinants in this study. At the moment, we cannot know if this is a peculiarity of the HZ of the Rio Gamberale stream or a characteristic shared by other HZs of other Mediterranean mountain streams. Further studies are needed to shed some light on this point.

In the multivariate models, ammonium did not emerge as the main descriptor except for nematodes. Nevertheless, ammonium was one of the predictors in 26 models and the sole contaminant detected in our survey. In our opinion, its role in the functioning of complex systems, such as the HZ, deserves to be better investigated by virtue of these results. Statistical models including ammonium, even those with a low explained variance, suggest that NH_4^+ should always be included in the monitoring of the functionality of the hyporheic zone of surface water bodies, at least in the Mediterranean zones. In particular, more studies are needed on ammonium toxicity. Ammonium is acutely toxic to freshwater invertebrate species at concentrations (uncorrected for pH and temperature) ranging from 0.5 to 23 mg/L (US EPA, 1985), therefore chronic effects were expected at the concentrations measured in this study. Chronic effects including a reduction in growth rates and morphological development have been already observed for some freshwater copepods (Di Marzio et al., 2014); studies are needed to investigate further effects, such as reduction in reproduction rates and respiration.

4.3. Other environmental predictors and season effect

Ammonium appeared not to affect the traits "biomass" and "affinity to groundwater" and this contrasts with our expectations based on previous studies (Di Lorenzo et al., 2015a) and on laboratory experiments in which we observed a delayed development of some species of hyporheic copepods when long-exposed to sub-lethal ammonium concentrations (Di Marzio et al., 2014). However, biomass may be more related to other factors, such as the concentration of organic matter (Di Lorenzo et al., 2020); in fact, our analyses showed that TOC and DOC were the main predictors of biomass along with nitrites and chloride (Appendix 3). The low abundance of stygobitic taxa (three species, only) was likely due to the low extent of the exchanges of groundwater-surface water through the HZ (e.g., Dole-Olivier, 2011; Di Lorenzo et al., 2014); however, this factor has not been investigated in this study. On the other

hand, low stygobitic species richness and abundances were observed in an alluvial aquifer contaminated by ammonium (Di Lorenzo et al., 2015a); therefore, we cannot exclude that ammonium had a role in shaping the low stygobitic abundances in the HZ of the Rio Gamberale stream as well.

The higher concentration of nitrates and nitrites detected in summer, which may be the result of lower dilution capacity of the HZ during the hottest season, suggests that the observed combined effects of season and ammonium should be expected (Holmstrup et al., 2010; Arenas-Sánchez et al., 2019). In fact, the strongest detrimental effect of ammonium on taxonomic richness and total abundances in the HZ of the Rio Gamberale stream occurred in summer (higher temperatures, higher nitrate and nitrite concentrations) and in the downstream stretch of the stream (higher ammonium concentrations). This result is in line with previous studies where we observed that temperature may boost the sensitivity of hyporheic copepods to ammonium (Di Lorenzo et al., 2015b).

It is difficult to disentangle the effect of the other environmental parameters from that of ammonium in this study. Complex multivariate models with large numbers of descriptors exhibiting negative and positive interactions with one another do not allow an easy explanation of the observed patterns. Therefore, complex multivariate models should be always accompanied by univariate analyses of simpler, causative factors underpinning mechanisms that drive multiple higher-order network properties (Clarke and Gorley, 2015), as we did in this study. Although the explained variances, or the correlations, were not always high, the models indicated ammonium as a descriptor of the observed biological patterns. Further evaluations to better evaluate the individual and combined effects of the other predictors should be done under semi-controlled conditions (e.g., mesocosms). Interactions of ammonium with temperature should be primarily investigated in mesocosm in Mediterranean areas. Further traits that were not measured in this study, such as the female to male and adult to juvenile ratios, which proved to be affected by nitrogen compounds in groundwater copepod assemblages (Di Lorenzo et al., 2020), could be usefully involved in the next studies. Finally, our study was based on taxonomic identifications done to the lowest practical resolution but Ostracoda and Acari Hydracnidia, for instance, remained described at the class/order level. It is evident that we have possibly missed some diversity (as also highlighted by the non-parametric estimators); deepening in the taxonomic determinations of these taxa could help in enlightening the observed patterns since crustacean sensitivity to ammonium is known to differ markedly between species belonging to the same family, as demonstrated for copepods by Di Marzio et al. (2009); Di Marzio et al. (2018) and Di Lorenzo et al. (2014).

5. Conclusion

Our study highlighted the significant effect of ammonium on the taxonomic and functional composition of hyporheic assemblages of Mediterranean streams, although further mesocosm studies could be fundamental to shed light on the counter-intuitive results of our research. The outcomes of our study indicated that summer seems to be the period during which ammonium had the strongest detrimental effect on the functionality of the hyporheic zone of the Rio Gamberale, with severe repercussion on the detritivorous components. Despite some uncertainties, functional traits can help point to potential mechanisms at the base of cause–effect relationships between ammonium and hyporheic communities. Since functional traits link community organization to ecosystem goods and services, the results of our study could be prodromal to an ecosystem quality index for the hyporheic zone. Under this perspective, this study supports the use of mixed (taxonomy-based and trait-based) approaches for the ecological status assessment of the hyporheic zones in Mediterranean streams.

CRediT authorship contribution statement

Tiziana Di Lorenzo: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing - review & editing. **Barbara Fiasca:** Methodology, Validation, Investigation, Data curation, Writing - review & editing, Project administration. **Mattia Di Cicco:** Methodology, Investigation, Writing - review & editing. **Marco Cifoni:** Methodology, Investigation, Writing - review & editing. **Diana M.P. Galassi:** Methodology, Validation, Investigation, Resources, Data curation, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2021.108268.

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