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# Potential of A Trait-Based Approach in the Characterization of An N-Contaminated Alluvial Aquifer

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**Abstract:** Groundwater communities residing in contaminated aquifers have been investigated mainly through taxonomy-based approaches (i.e., analyzing taxonomic richness and abundances) while ecological traits have been rarely considered. The aim of this study was to assess whether a trait analysis adds value to the traditional taxonomy-based biomonitoring in N-contaminated aquifers. To this end, we monitored 40 bores in the Vomano alluvial aquifer (VO\_GWB, Italy) for two years. The aquifer is a nitrate vulnerable zone according to the Water Framework Directive. The traditional taxonomy-based approach revealed an unexpectedly high biodiversity (38 taxa and 5725 individuals), dominated by crustaceans, comparable to that of other unpolluted alluvial aquifers worldwide. This result is in contrast with previous studies and calls into question the sensitivity of stygobiotic species to N-compounds. The trait analysis provided an added value to the study, unveiling signs of impairments of the groundwater community such as low juveniles-to-adults and males-to-females ratios and a crossover of biomasses and abundances curves suggestive of an intermediate alteration of the copepod assemblages.

**Keywords:** crustaceans; copepods; stygobiotic; traits; groundwater; nitrate; ammonium; nitrite; nitrogen; contamination

## 1. Introduction

The taxonomy-based approach, which is traditionally focused on the analysis of taxonomic richness and abundances, has long been used to characterize and assess the impairment of freshwater ecosystems [1]. Following this approach, biological samples are collected from impaired sites and the taxa are identified, enumerated, and then compared to those of pristine reference sites (e.g., [2]). Trait-based approaches have been developed over the years alternatively to the taxonomy-based one or, more often, in combination with it. Traits are measurable physiological, morphological, and ecological characteristics of organisms, populations, or communities, such as the body size, the trophic role, the duration of life cycle, the reproductive rate, and so forth [3]. The trait-based approach allows understanding ecosystems in ways often unattainable by studies that are exclusively focused on taxonomic diversity. In the first place, traits i) reflect the species (population or community) adaptation level to their environment [4] and ii) can be used as measures of functional diversity [5]. Secondly, trait-based approaches work well across geographies since many traits are shared by widely

distributed taxa [3]. From this perspective, some traits can be even applied across ecoregions [6]. Other strengths are that they add diagnostic knowledge, do not require new sampling methodology, and can supplement taxonomic analyses, while weaknesses are redundancy, autocorrelation, and difficulty in the measurements [3]. One of the most useful and fascinating aspects of trait-based approaches is that they can reveal a substantive trait variation (e.g., metabolic changes) before the taxonomic composition of the community is impaired by the loss of some species [1]. Similarly, some traits may have an earlier diagnostic potential regarding ecosystem recovery [1]. However, the decision to prefer a trait-based approach over a taxonomy-based one must be taken with caution; rather, the best option is the combination of the two approaches, at least for freshwater ecosystems [1]. In fact, trait-based approaches may fail to diagnose impacts on imperiled species which are rare in terms of abundance, because of functional redundancy [1]. On the other hand, the diagnostic power of a taxonomy-based approach decreases if the community is not characterized at the species level, but rather described at the genus or even family resolution, as required in several biomonitoring programs [7,8].

Although trait-based approaches have an old tradition in freshwater ecology (reviewed in [1]), they have not been widely applied to groundwater ecosystems. Groundwater habitats, and alluvial aquifers in particular, are characterized by lack of light and by environmental conditions that are more stable compared to surface water environments [9]. The absence of light precludes primary production by photosynthesis, so groundwater ecosystems are generally reliant on allochthonous organic carbon from surface environments [10,11]. However, particulate organic matter is not always available to groundwater food webs because it is intercepted in the soil and the unsaturated zone [10]. Consequently, the basal food source for stygofauna (obligate groundwater-dweller fauna) consists mainly of bacteria and the organic matter that is assimilated by microorganisms [12–14]. Unlike surface waters, groundwater ecosystems support only a few vertebrate species and the invertebrate assemblages are generally dominated by crustaceans [15,16]. Groundwater invertebrate assemblages also may include mites, oligochaetes and, rarely, insects and mollusks [9,17]. Copepods, amphipods, isopods, and syncarids are crustacean taxa commonly collected in healthy groundwater ecosystems [18,19]. The response of stygobiotic communities to impairments due to anthropic pressures has often been examined through taxonomy-based approaches [20–23]. Recently, some stygofaunal traits have proven to be useful in assessing the response of groundwater communities to groundwater flooding [24], carbon input [25,26], habitat structure [27,28] and chemical contamination [20,29–31]. However, the selection of a set of traits that best characterize groundwater communities is still to come.

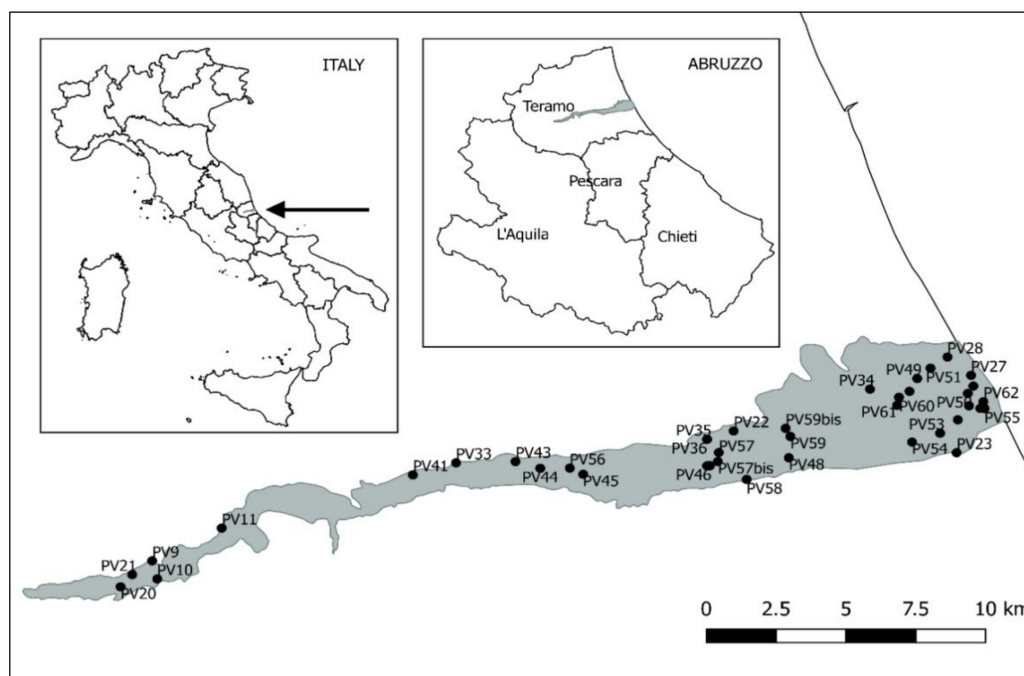
Alluvial aquifers are particularly prone to organic pollution as their outcrops are frequently cultivated or occupied by urban settlements [21,32]. As a result, an increase in the concentrations of nitrate, nitrite, and ammonium is often observed in groundwater, with long-lasting exceedances of the legal limits [22]. Organic contamination is known to cause a change in the structure and composition of stygobiotic assemblages. Elevated concentrations of N-compounds, due to contamination through sewage and septic tank leaks, or due to the overuse of natural and synthetic fertilizers, alter the structure of groundwater communities through favoring the proliferation of surface water organisms [22,33,34]. However, the ecological studies conducted on alluvial aquifers contaminated by N-compounds are still rather scarce and reliant on the taxonomy-based approach [21,35].

The aim of this study was to evaluate the performance of a trait-based approach in the ecological characterization of an alluvial aquifer with a ten-year history of N-compound contamination. To this end, we analyzed the biological assemblages of the Vomano alluvial aquifer (central Italy) that, in 2010, was designated as a Nitrate Vulnerable Zone (NVZ) under the Water Framework Directive (EC, 2000) due to a nitrate contamination affecting over 50% of the groundwater body volume [36]. We measured five ecological traits and compared the results with those deriving from the traditional approach based on taxonomic richness and abundance distribution.

## 2. Materials and Methods

### 2.1. Study Area

The study was performed on an alluvial aquifer (VO\_GWB; Figure 1) located in the hydrographic basin of the River Vomano (Italy). The outcrop of the aquifer is 30 km<sup>2</sup>. VO\_GWB is mainly fed by the River Vomano waters and rainfalls which reach the base of the aquifer in a few weeks [36,37]. The alluvial deposits of VO\_GWB consist mainly of gravel and sand interbedded by silty-clay lenses of limited extension [37]. The basement of the aquifer (at a depth of about 35 m) is made up of silty-clay deposits of very low permeability [37]. The groundwater flow is NW–SE directed, and the aquifer mean discharge is  $1.3 \times 10^{-2}$  m<sup>3</sup>/s [36]. VO\_GWB is used for drinking and industrial and agricultural purposes. The chemical and quantitative status of VO\_GWB is routinely monitored by the local environmental authority according to the requirements of the Water Framework Directive [38] and Groundwater Daughter Directive [39]. In 2010, VO\_GWB was designated as a Nitrate Vulnerable Zone (NVZ) under the Water Framework Directive [38] due to a persisting (>10 years) nitrate contamination [36].



**Figure 1.** Clockwise: the aquifer VO\_GWB in Italy and in the Abruzzo region; sampling sites (black dots) location.

Groundwater sampling occurred in 40 bores used for agricultural purposes (Figure 1). Groundwater organisms and water samples for chemical analyses were collected from shallow bores (up to 30 m deep) in autumn 2014 and spring and winter 2015 for a total of 97 samples. The altitude of the bores varied from 0 to 240 m above sea level and the depth from 2 to 30 m below the soil level. The piezometric level, measured using a freatimeter (PASI, Turin), was in the range 0.5–25.9 m below ground level. The bores' distance from the River Vomano varied from a minimum of 167 m to a maximum of 1733 m.

Groundwater flow of VO\_GWB is considered basal, meaning that the groundwater undergoes remixing at all aquifer depths [37]. Preliminary investigations in which the aquifer volume was arbitrarily subdivided into three (0–10 m, 11–20 m, and 21–30 m deep) or two (0–15 m, 16–30 m deep) sectors did not reveal any significant environmental or biological pattern (Supplementary Materials: PRELIMINARY). For this reason, the aquifer was investigated as a unique groundwater unit.

## 2.2. Biological Survey

Bores were sampled with either an immersion pump (IP), a manual pump (MP), or a modified Cvetkov net [40], depending on the bore type (drilled or hand-dug). Five hundred liters of groundwater were withdrawn using IP and 50 L using MP. The volumes were subsequently filtered by a 60 µm mesh net to collect groundwater fauna. The modified Cvetkov net, equipped with a weight anchored to the tail, was lowered into the hand-dug bores to the bottom and subsequently hauled to filter the entire column of bore water. Ten hauls were performed in each bore [41]. The collected samples were transported in 1 L plastic vials to the laboratory within 6 h and then fixed in 70% ethanol solution. In the laboratory, specimens were sorted under a stereomicroscope at 16× magnification and identified to the lowest taxonomic level possible. Copepods were identified to species level after dissection and subsequent observation with a Leica DM 2500 optical microscope, following dichotomous keys [42,43] and more recent taxonomic papers.

Depending on the degree of adaptation to groundwater, each taxon was classified either as stygobiont (SB: species strictly associated to groundwater habitats, where it completes the whole life cycle; [44]) or non-stygobiont (nSB: epigeal species accidentally or occasionally occurring in groundwater). Each specimen of the dominant taxon (Copepoda; see par. 4.) was individually photographed under a Leica M205C stereomicroscope. Length and width were measured with the software LAS (Leica Application Suite, Version 4.7.1 of Leica Microsystems, Wetzlar, Germany). Copepod lengths (in mm) were measured from the tip of the cephalic shield to the end of caudal rami, while width (in mm) was measured at the larger somite bearing legs. Sex was identified in adult individuals. Afterwards, copepod body dimensions (length and width) were converted to species-specific biovolume (in nL) using Equation (1) [45]:

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

where BV = biovolume, a = length (mm), b = width (mm), and CF is a correction factor that accounts for the body shape. CF varied from 230 to 750 according to the body shape outlines of each specimen [46]. The biovolumes were then converted into individual fresh weights assuming a specific gravity of 1.1 [24]. The dry mass (mg) was estimated assuming a dry/wet weight ratio of 0.25 and the dry weight carbon content (biomass) was assumed to be 40% of the dry mass [47].

## 2.3. Environmental Survey

Physicochemical parameters, such as electrical conductivity (µS/cm), pH, dissolved oxygen (mg/L), and temperature (°C), were measured in situ with a multiparametric probe (WTW 3430 SET G). Two liters of groundwater were collected and stored in high-density polyethylene bottles for further chemical analyses aimed at measuring the concentrations of the 110 chemical compounds indicated in the Supplementary Materials (ENV). Particulate organic matter (POM) was obtained as the difference between the sediment weight deprived of the faunal component and dehydrated at 105 °C and that obtained after combustion of the organic fraction at 540 °C.

## 2.4. Data Analysis: Environmental Variables

As subsequent samples from the same bore must be considered temporal pseudoreplicates producing bias in the statistics [48], the mean values of each abiotic variable were used in the statistical analyses in the place of the raw data. Only 22 variables out of 110 were analyzed, that is, only those showing a standard deviation (SD) ≠ 0 (Table 1) so as to exclude irrelevant variables in the statistical models. Potential environmental patterns were investigated through a principal component analysis (PCA) on the basis of the Euclidean distance matrix. Prior to PCA, data were normalized and a Draftsman's plot was used to examine the correlation among the environmental variables [49,50].

**Table 1.** Mean and standard deviations (SD) of the 22 environmental variables (Var.) retained in the statistical analyses. Alt: altitude (m above sea level); W.t.: water table (m below ground level.); T: temperature (°C); EC: electrical conductivity ( $\mu\text{S}/\text{cm}$ );  $\text{O}_2$ : dissolved oxygen (mg/L); POM: particulate organic matter (mg/L); TOC: total organic carbon (mg/L); DOC: dissolved organic carbon (mg/L);  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ;  $\text{NH}_4^+$ ,  $\text{SO}_4^{2-}$ ;  $\text{Cl}^-$ ,  $\text{PO}_4^{3-}$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$  (mg/L); DIC: cis-1,2-dichloroethylene ( $\mu\text{g}/\text{L}$ ); TCE: 1,1,2,2-tetrachloroethylene ( $\mu\text{g}/\text{L}$ ); CHL: trichloromethane ( $\mu\text{g}/\text{L}$ ); THC: total hydrocarbons (expressed as n-hexane in  $\mu\text{g}/\text{L}$ ). TV: legal threshold value. Mean values exceeding legal limits are indicated in bold.

Var.	Mean	SD	TV
Alt	72.7	72.3	
W.t.	8.3	5.3	
T	16.9	1.7	
EC	1166	231	
pH	7.3	0.2	
$\text{O}_2$	4.7	1.6	
POM	0.73	2.32	
TOC	3.02	2.39	
DOC	2.18	1.70	
$\text{NO}_2^-$	<b>10.25</b>	11.19	0.5
$\text{NO}_3^-$	<b>61.60</b>	30.07	50
$\text{SO}_4^{2-}$	103.75	51.97	250
$\text{Cl}^-$	55.76	37.49	
$\text{PO}_4^{3-}$	0.27	0.42	
$\text{NH}_4^+$	0.23	0.50	0.5
$\text{Ca}^{2+}$	144.99	30.13	
$\text{K}^+$	5.22	2.312	
$\text{Na}^+$	60.17	33.31	
DIC	0.03	0.043	0.05
TCE	0.07	0.132	1.1
CHL	0.04	0.046	1.5
THC	25.7	4.480	350

### 2.5. Taxonomy-Based Approach

The exhaustiveness of the biological sampling effort was assessed through taxonomic richness estimations, using nonparametric estimators, namely, Chao1, Chao2, Jackknife1 and Jackknife2, Bootstrap, Michaelis-Menton (MM), and Uglan-Gray-Ellingsen (UGE) [51]. Values were estimated by means of 999 randomizations without replacement.

Abundances were expressed as ind/L dividing the number of individuals of each sample by its volume (liters). Raw data are available in the Supplementary Materials (BIO\_VOGWB). Abundances of temporal replicates were summed per each sampling site prior to being used in the statistical analyses. Potential biological patterns were examined using a non-metric multidimensional scaling (nMDS) analysis, incorporating Bray-Curtis similarities after adding a dummy variable equal to 1 [49]. Prior to nMDS, data were  $\log(x + 1)$ -transformed to approximately conform the data to normality. BIO-ENV analyses [52], based on Spearman's rank correlations, were performed to find the 'best' match between the biological and the environmental data. The statistical significance of the results of the BIO-ENV routine was tested by the global BIO-ENV match permutation test whereby each set of samples was randomly permuted 99 times relative to the others [52]. In addition to multivariate analyses, univariate statistics was performed to assess correlation between individual environmental variables and cumulative abundances per sampling site. The significance was assessed through a permutation test performed by randomly shuffling 999 times the independent variable (i.e., the environmental variables). The permutation test was selected because it is more powerful than tests based on normal theory assumptions when data distribution departs substantially from normality, as it was the case for the data of this study. Regressions were performed with the *ape* library in R 3.0.2 [53].

Finally, the structure of the biological assemblages of N-compound-contaminated alluvial aquifers other than VO\_GWB was obtained from literature by using i) ISI Web of Knowledge SM (© 2010 Thomson Reuters, Toronto, ON, CA) with the following strings applied to the topic field: “aquifer” and “stygo\*” and “nitrate” or “nitrite” or “ammon\*”; ii) Google with the same setting as for i).

### 2.6. Trait-Based Approach

Potential biological patterns were examined using the following traits and the statistical settings as for the taxonomy-based approach (par. 2.5): Trait#1 (crustaceans vs. non-crustaceans); Trait#2 (SB vs. nSB); Trait#3 (adults vs. juveniles); Trait#4 (males vs. females); Trait#5 (biomass). Since copepod crustaceans accounted for 98% of the aquifer biodiversity, Traits#2–5 were analyzed with respect to copepods only. We analyzed these traits since previous studies have demonstrated that they are reliable indicators of impairment of groundwater communities, with particular references to organic contamination. In detail: i) groundwater assemblages dominated by non-crustacean taxa and a high nSB/SB ratio should be considered impaired [34]; ii) the sex ratio of some populations of copepods may be skewed in favor of female individuals in impaired water bodies [54–56]; iii) the development of juvenile copepods is slowed down in populations exposed to ammonium with respect to control populations [57]; and the survival of copepodids under N-compound exposure is significantly lower than that of adult individuals of the same species [58]. Finally, we analyzed the biomass because its relationship with abundances provides key information about community impairment [49,50]. Distributions of copepod species abundances and biomasses were compared on the same terms with *k*-dominance curves, namely applying the Abundance/Biomass Comparison method (ABC) with the following assumptions [52]: i) in an undisturbed community, the *k*-dominance curve for biomass lies above the curve of abundances for its entire length; ii) as impairment occurs and becomes more severe, the abundance curve lies above the biomass curve, in part, or completely. The *W* statistic was used to algebraically interpret the position of the curves, the metric values being in the range  $-1,1$  (though neither limit is likely to be attained), with *W* close to 1 indicating undisturbed condition, *W* close to 0 indicating intermediate impairment, and *W* close to  $-1$  indicating severe impairment [52].

Eventual significant patterns of each trait were explored by nMDS plots performed on the Bray–Curtis dissimilarity matrices as in the taxonomy-based approach (par. 2.5). Similarly, BIO-ENV analyses [52] and permutational regressions were used to explore the correlations between the environmental variables and the data of the individual traits.

## 3. Results

### 3.1. Environmental Variables

Mean and SD values of the 22 variables with  $SD \neq 0$  are shown in Table 1.

Mean values of nitrites and nitrates were indicative of contamination according to the legal threshold values (respectively: 0.05 and 50 mg/L) set by Italian regulation [59].

Nitrite contamination was detected in 24 out of 40 bores, 20 of which also showed a nitrate contamination. Overall, bores contaminated by nitrates were 30 out of 40. Fifteen out of 40 bores were contaminated by ionized ammonium. Seven of these bores were also contaminated by nitrates while nine were contaminated by nitrites. Overall, contamination by N-compounds (nitrites, nitrates, and ionized ammonium) involved 36 bores out of 40. The PCA (matrix: 22 variables  $\times$  40 sampling sites; plot not shown) explained only 34% of the cumulative variance of the environmental data on the first two axes, failing to highlight a clear pattern of the environmental variables. This result implies that the chemical and physicochemical conditions of VO\_GWB were uniform through the aquifer and there were no areas with environmental peculiarities.

### 3.2. Taxonomy-Based Approach

The stygofauna collected in VO\_GWB accounted for 5724 individuals belonging to 38 taxa (35 crustaceans, 2 insect instars, and 1 water mite). Crustaceans were dominant in terms of abundances (99.70%), followed by insects (0.23%) and water mites (0.07%). Among crustaceans, the taxon Copepoda accounted for 97.93% of the total abundances, Amphipoda for 1.52%, Isopoda for 0.02%, Syncarida for 0.16%, and Ostracoda for 0.37%. Two out of seven non-parametric estimators reached the asymptotes, indicating the exhaustiveness of the sampling effort (Supplementary Materials: SRC). The remaining estimators indicated that the sampling effort was such to cover from 67% (Chao1) to 90% (Bootstrap) of the expected biodiversity of the aquifer.

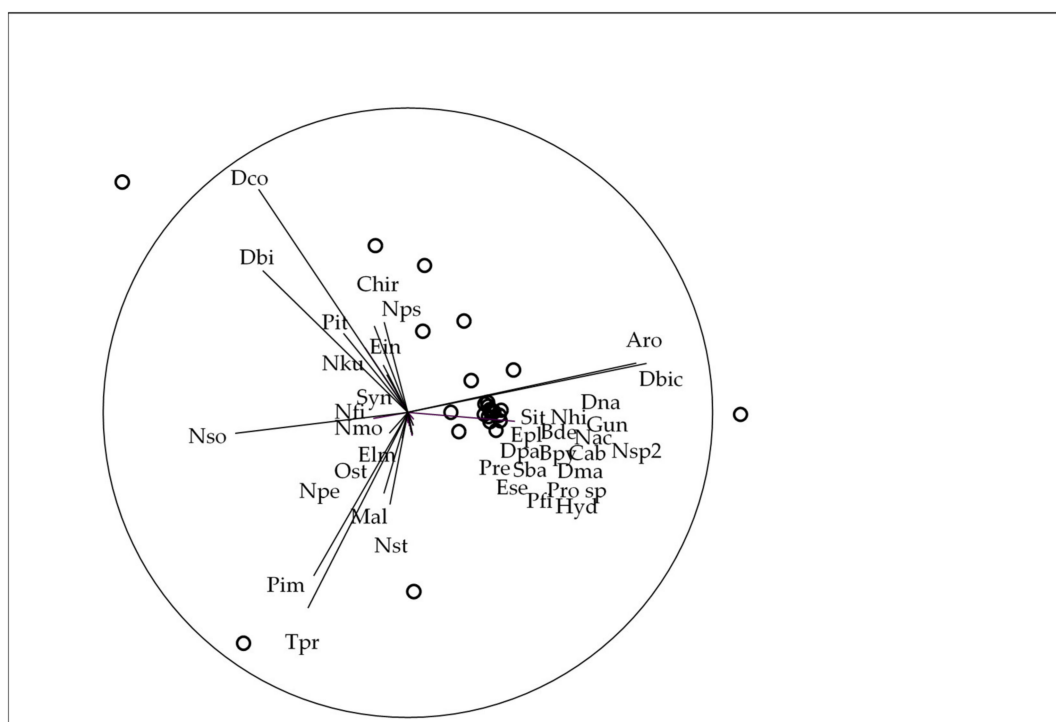
Literature data related to N-compound concentrations and groundwater taxonomic richness in 11 alluvial aquifers worldwide are reported in Table 2.

**Table 2.** Taxonomic richness (T: number of taxa; SB: number of stygobiotic taxa; nSB: number of non-stygobiotic taxa; C: number of crustacean taxa; Co: number of copepod taxa) and nitrate, nitrite, and ionized ammonium concentrations (mean values in mg/L) relating to 11 alluvial aquifers worldwide obtained from this study and from literature. Ref: references. N-compound exceedances are in bold. (ts): this study.

Country	Alluvial Aquifer	Area	T	SB	nSB	C	Co	NO <sub>3</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup>	NH <sub>4</sub> <sup>+</sup>	REF
Australia	Murray	97,000	11			8	2	2.4		0.04	[60]
Australia	Gwydir	26,600	21		>5	8	2	8.0			[61]
France	Ariège	250	10	10	0	10	0	<b>90.0</b>		<b>31</b>	[62]
France	Ariège and Hers	540	36	14	22		4	<b>&gt;50.0</b>			[35]
Germany	Rhine	5000	35	16	22	35	23	4.2		0.33	[63]
Italy	Vibrata	48	18	9	9	14	12	<b>72.5</b>			[21]
Italy	Adige	45	15	9	6	15	15	7.8	0.01	0.16	[22]
Italy	Lessinia	428		21		20	10	29.7			[64]
Italy	Vomano	30	38	21	17	35	28	<b>61.6</b>	<b>10.25</b>	0.23	(ts)
Slovenia	Brest	6	38	22	16	23	15	2.5			[65]
Spain	Tajo	201	11	3	8	11	6	11.4	0.02	0.19	[66]

The stygobiotic cyclopoid *Diacyclops cosanus* (Dco) occurred in 15 bores out 40 and was the species with the widest distribution in VO\_GWB. The remaining 35 taxa occurred in less than 12 bores out of 40 and 24 taxa (12 of which were stygobiotic) occurred in less than 4 bores. Bore PV28 (13 m deep) showed the highest biodiversity, with 11 taxa, 7 of which were stygobiotic, followed by PV48 (20 m deep) with 10 taxa, 9 of which were stygobiotic. More than 50% of the bores (namely, 23 out of 40) were characterized by a maximum of three taxa and no taxa were collected from three bores, namely, from P26 (18 m deep), PV53 (10 m deep), and PV62 (8 m deep).

The nMDS plot (Figure 2; 2D stress: 0.03; matrix: 38 variables × 40 sampling sites) showed a group of 27 bores of different depths (from 4 to 30 m deep), almost completely overlapping at the center of the plot and surrounded by 11 bores (from 2 to 25 m deep) located at different distances. Thirteen out of 38 taxa were exclusive of the bores overlapping in the center of the plot, namely, the copepods *Paracyclops fimbriatus* (Pfi), *Bryocamptus pygmaeus* (Bpy), *Eucyclops serrulatus* (Ese), *Diacyclops maggii* (Dma), *Graeteriella unisetigera* (Gun), *Nitokra hibernica* (Nhi), *Nitocrella fedelitaie* (Nfi), *Nitocrella achainae* (Nac), *Bryocamptus dentatus* (Bde), *Elaphoidella plutonis* (Epl), *Pseudectinosoma reductum* (Pre), the amphipod *Niphargus* sp. 2 (Nsp2), and a species belonging to the Coleoptera Elmidae (Elm). Three species, namely, the copepods *Diacyclops bicuspidatus* (Dbic) and *Nitocrella kunzi* (Nku), and the amphipod *Niphargus sodalis* (Nso), occurred exclusively in the not-overlapping bores (Figure 2 and Supplementary Materials: BIO\_VOGWB).



**Figure 2.** nMDS (2D stress: 0.03) ordination plot following the taxonomy-based approach in VO\_GWB. Acronyms are as in the Supplementary Materials: BIO\_VOGWB. Vector overlays are reported as exploratory tools to visualize potential linear monotonic relationships, based on Pearson correlation, between the taxa abundances and the nMDS ordination axes [52].

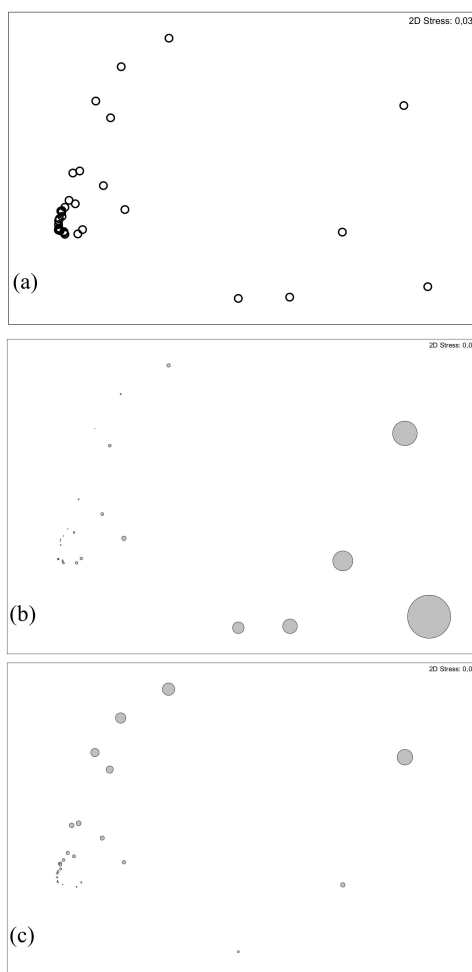
The BIO-ENV analysis showed that there was no significant explanatory relationship between the environmental variables and the matrix of the 38 taxa collected in VO\_GWB. In fact, the sole environmental variable which best correlated to the taxa abundances was POM ( $\rho = 0.436$ ) and the best  $n$ -dimensional combination consisted of five variables, namely, electrical conductivity, pH, nitrites, nitrates, and ionized ammonium. However, the real value of  $\rho$  fell within the 95% confidence limit of the normal distribution of randomly permuted values of  $\rho$  ( $p$ -value = 14%), indicating that the null hypothesis of no relationship between the best subset of environmental variables and the community structure should not be rejected. Univariate analyses gave significant results. In fact, cumulative densities were negatively correlated, although weakly, to electrical conductivity, dissolved oxygen, nitrates, sulphates, calcium, and sodium (Supplementary Materials: REGR). However,  $R^2$  values were  $<20\%$  for all parameters except for electrical conductivity ( $R^2 = 57\%$ ), meaning that the regression models had a low predictive potential.

### 3.3. Trait-Based Approach

Trait#1 (crustacean vs. non-crustacean taxa). VO\_GWB was dominated by crustaceans, occurring with 35 out of 38 taxa. The nMDS returned an arched, or horseshoe, pattern. This effect is very common in datasets in which there is a progressive turnover of a dominant variable [67]. In fact, the nMDS bubble plot showed that the arch effect was due to the turnover of crustacean taxa (Supplementary Materials: nMDS\_T1). The BIO-ENV analysis indicated no significant relationship between the best subsets of environmental variables (POM:  $\rho = 0.435$ ;  $p$ -value = 18%) and the abundances of crustacean and non-crustacean taxa. Univariate analyses did not highlight significant correlation between the cumulative abundances of crustaceans (or non-crustaceans) and the individual environmental variables.

Trait#2 (SB vs. nSB taxa). Seventeen out of 38 taxa were nSB and 21 were SB (Supplementary Materials: BIO\_VOGWB). The nMDS (2D Stress: 0.03) showed that SB and nSB taxa occurred almost in all bores (Figure 3a), however, with different abundances.





**Figure 3.** (a) nMDS plots (stress: 0.03) performed on copepod abundances data of both nSB and SB species; (b) nMDS bubble plot of the nSB species (ind/L); (c) nMDS bubble plot of the SB species (ind/L).

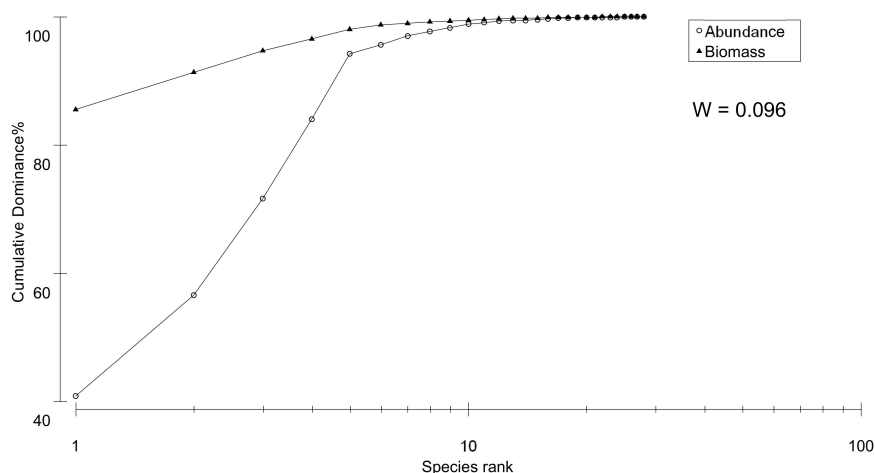
SB abundances (Figure 3b) were higher in the eight bores located in the upper sector of the plot while nSB abundances (Figure 3c) were higher in the lower sector of the plot, and in particular, in five bores. The group of eight bores with the highest abundances of SB taxa differed from the group of the five bores in the mean values of some environmental variables. Specifically, the average values of POM (0.44 mg/L), nitrite (9 mg/L), and ionized ammonium (0.031 mg/L) were lower in the bores with high densities of SB than in those with high densities of nSB (POM: 1.33 mg/L, nitrite: 13 mg/L, ionized ammonium: 0.644 mg/L). On the other hand, the mean values of electrical conductivity (1237  $\mu$ S/cm), nitrates (79 mg/L), sulphates (109 mg/L), chloride (51 mg/L), calcium (156 mg/L), and sodium (53 mg/L) were higher in the sites with high densities of SB than in those with high densities of nSB (electrical conductivity: 835  $\mu$ S/cm, nitrates: 43 mg/L, sulphate: 45 mg/L, chloride: 30 mg/L, calcium: 105 mg/L, and sodium: 31 mg/L). However, the BIO-ENV analysis indicated no significant relationship between the best subsets of environmental variables (POM:  $\rho = 0.443$ ;  $p$ -value = 16%) and the densities of the two ecological categories. The univariate analyses highlighted a significant negative correlation between SB copepod abundances and bore depth (Supplementary Materials: REGR), however, with an  $R^2$  equal to 11%. The abundances of nSB copepods were negatively correlated to electrical conductivity, dissolved oxygen, nitrate, sulphates, calcium, and sodium (Supplementary Materials: REGR).  $R^2$  values were <20% for all parameters except for electrical conductivity ( $R^2 = 57\%$ ).

Trait#3 (adults vs. juveniles). All copepod species were collected with adults and juveniles with a few exceptions, such as the nSB species *Nitokra hibernica* and the SB species *Nitocrella fedelitae*, *Nitocrella morettii*, *Nitocrella kunzi*, *Bryocamptus dentatus*, and *Pseudectinosoma reductum*, which were

collected with adults only. The nMDS plot (Supplementary Materials: nMDS\_T3) showed no clear separation of the bores, however, the bubble plot highlighted that adult copepods were slightly more numerous than juveniles almost in every bore. The univariate analyses highlighted a significant negative correlation between adult copepod abundances and electrical conductivity, dissolved oxygen, nitrate, sulphates, calcium, and sodium (Supplementary Materials: REGR). A significant negative correlation was assessed between juvenile abundances and electrical conductivity, sulphates, calcium, and sodium as well (Supplementary Materials: REGR). However,  $R^2$  values were all  $<20\%$ , except for electrical conductivity ( $R^2 > 50\%$ ).

Trait#4 (males vs. females). Twelve copepod species occurred with more females than males, nine species with more males than females, and seven species with the same number of males and females. Overall, the average abundance  $\pm$  SD of males was  $0.26 \pm 0.70$  ind/L and that of females was  $0.67 \pm 2.13$  ind/L, with a sex ratio equal to 0.38. The nMDS did not show a clear pattern (Supplementary Materials: nMDS\_T4). The BIO-ENV analysis indicated no relationship between the best subsets of environmental variables (POM:  $\rho = 0.437$ ;  $p$ -value = 18%) and the abundances of males and females. The univariate analyses highlighted a significant negative correlation between male abundances and electrical conductivity, dissolved oxygen, nitrate, sulphates, and calcium (Supplementary Materials: REGR). A significant correlation was assessed between female abundances and electrical conductivity, dissolved oxygen, nitrates, sulphates, calcium, and sodium as well (Supplementary Materials: REGR). However,  $R^2$  values were  $<20\%$  for all parameters and for both males and females, except for electrical conductivity ( $R^2 > 43\%$ ).

Trait#5 (biomass). The total dry weight carbon (mean  $\pm$  SD) was  $0.012 \pm 0.061$  mg/L C. Multivariate analyses did not reveal significant patterns. The univariate analyses highlighted a significant negative correlation between biomass and electrical conductivity, dissolved oxygen, and calcium (Supplementary Materials: REGR). However,  $R^2$  values were all  $<20\%$ , except for electrical conductivity ( $R^2 = 42\%$ ). The biomass and abundance curves partially crossed at about the 20th species but since both curves were close to 100% at this point, the crossover was unclear (Figure 4). However, the  $W$  metric equal to 0.096 was indicative of a moderate impairment of the copepod assemblages.



**Figure 4.** Abundances-biomasses curves of copepod species in GWB\_VO aquifer. The two curves' crossover occurring at the 20th species and the metric  $W = 0.096$  were indicative of moderate impairment of the copepod assemblages [52].

#### 4. Discussion

The taxonomic richness of VO\_GWB was high and consistent with previous studies in other European and non-European alluvial aquifers [60,64,66,68,69], both at the local and the regional scales [15,70]. However, the number of species collected in VO\_GWB is surprisingly high if considering the persisting N-compound contamination which occurred in 90% of the aquifer. This study highlighted

the high taxonomic richness and abundances of the assemblages of VO\_GWB with respect to those of other alluvial aquifers worldwide contaminated by N-compounds. The taxonomic richness (38 taxa) and the abundances (5725 individuals) of VO\_GWB were comparable to those of pristine alluvial aquifers. Such a result is in contrast with previous data showing that freshwater invertebrates are highly sensitive to N-compounds [71]. From this point of view, the results of this study represent a novelty and require further ecotoxicological investigations. In fact, to date, ecotoxicological studies with N-compounds have been focused mainly on non-stygobiotic invertebrates, with the exception of the study carried out by [58], who analyzed the sensitivity of the stygobiotic cyclopoid *Diacyclops belgicus* (not found in VO\_GWB) to ionized ammonium. The low number of ecotoxicological data concerning stygobiotic species, and the reasons behind the difficulty in carrying out such studies, have recently been reviewed by [72], who have also claimed as unrealistic the probability of gathering new data in the near future. Thus, the high taxonomic richness and abundances observed in VO\_GWB will likely remain unsupported by ecotoxicological considerations for some more time.

The traditional taxonomy-based approach showed that most taxa were collected only in a few bores and showed, therefore, restricted distributions as already observed in other studies (e.g., [64,66]). The most widespread species in VO\_GWB, *D. cosanus*, is known to be highly tolerant to elevated values of salinity and electrical conductivity, being collected in other N-contaminated alluvial aquifers, in brackish coastal groundwater in southern Italy, and in sulfidic saturated karst [21]. The occurrence of epigeal species in the deeper sectors of VO\_GWB is recurrent in alluvial aquifers, especially in those polluted by contaminants of organic origin (e.g., [22,64]). The weak correlations of the environmental and biological variables are a common result in groundwater studies, in particular, in aquifers that are highly homogeneous from a physicochemical point of view [22,66,73]. In this study, the variance explained by the univariate models was low and this indicated that further factors, in addition to those already investigated, must be taken into account in the future to better explain the observed biological patterns. For instance, copepod species interactions could play a role in determining the biological assemblages in VO\_GWB as already observed in other aquifers [74]. Similarly, previous studies showed that the grain size composition of sediments [75–77], and the lowering of the groundwater table [21,75], could influence the biological assemblage's patterns in alluvial aquifers.

Trait#1 (crustaceans vs. non-crustaceans) highlighted the predominance of crustaceans in VO\_GWB. This result is in contrast with previous studies [21,33,62] which showed that crustacean abundances are lower than those of non-crustaceans in aquifers polluted by N-enriched infiltration waters.

Trait#2 provided additional information to this study, highlighting that stygobiotic species seemed to thrive in the bores where non-stygobiotic species were less abundant. The environmental variables did not entirely explain such a pattern. Hence, additional factors should be investigated such as the isolation of the bores from the surface. In fact, groundwater habitats characterized by intense water exchanges with the surface environment often exhibit a predominance of generalist non-stygobiotic fauna, while isolated groundwater sites show a predominance, or even an exclusive occurrence, of stygobiotic species [19,61,78,79].

Trait#3 showed that juveniles were less abundant than adult copepods and that some species of stygobiotic copepods were collected exclusively with adults. This is a peculiar result because stygobiotic copepod species are known to reproduce in groundwater, albeit showing a low fertility [80]. In unaltered ecosystems, juveniles are usually more abundant than adults, while the opposite was observed in this study, at least for a few species. This result could be related to the N-compound contamination of the aquifer. In fact, juvenile stages are generally more sensitive to pollution than adults [81,82] as it was observed for the stygobiotic cyclopoid *Diacyclops belgicus* when exposed to ammonium nitrate [58].

Trait#4 highlighted that the sex ratio departed from 1 and was equal to 0.38 in favor of females. In diploid populations, sex ratio is usually equal to 1 [83,84], however, surface water populations' sex ratio tends to be skewed as result of seasonality, environmental conditions, and contamination level [54–56].

In general, seasonality does not affect stygobiotic populations [44]. However, N-compounds could have a role in determining a sex ratio <1 in VO\_GWB. For example, the sex ratios of the populations of *Cyclops vicinus* and *Thermocyclops crassus* were skewed in favor of female individuals in some altered surface water bodies [54]. Although no correlations were assessed between nitrites, nitrates, or ionized ammonium and abundances of adult male or female copepods in this study, these pollutants may nevertheless have had an effect on the populations in the past, reducing the number of male copepods during the ten-year-long contamination (still persisting in 2015) and inducing the skewed sex ratio <1 observed in 2014–2015.

Trait#5 highlighted that VO\_GWB had an average carbon content equal to  $0.012 \pm 0.061$  mg/L C, accounting for 0.049 g C. With respect to the carbon content (0.004 g) measured in an unpolluted chalk aquifer in southern England [24], the amount measured in VO\_GWB is high. However, a fair part of this value, namely 0.047 g C, was contributed by the individuals of *Acanthocyclops robustus*, a large non-stygobiotic copepod species collected in bore PV36. Excluding the carbon contributed by this species, the average dry carbon found in VO\_GWB was equal to 0.006 g C which is comparable to the amount measured in southern England [24]. However, the partial crossover of the biomasses and abundances curves and the W metric indicated impairment of copepod assemblages although not as severe as expected in a long-term contaminated alluvial aquifer as VO\_GWB is.

Based on these results, we can conclude that the trait-based approach provided an added value to this study, allowing a better characterization of the biological assemblages of VO\_GWB. The main difficulties that we encountered were i) the high taxonomic expertise required and ii) the long time needed for measuring individuals. In fact, the attribution of the two categories “stygobiotic” and “non-stygobiotic”, at least for copepods, necessitated identifications at species level and a solid knowledge of the autecology of each species.

## 5. Conclusions

In this study, we pursued two objectives, namely, i) to provide new data concerning the biodiversity of an alluvial aquifer affected by a persistent N-compound contamination, and ii) to assess whether an approach based on some selected ecological traits may provide an added value to the traditional taxonomy-based methodology. The alluvial aquifer investigated in this study showed a surprisingly high biodiversity, dominated by crustaceans, comparable to that of alluvial aquifers in good chemical state. This result is in contrast with the observations of previous studies. The results call into question the real sensitivity of stygobiotic species (copepods, in particular) to N-compounds, which turned out to be lower than expected. The trait-based approach provided new details, unveiling signs of impairments of the groundwater community such as low juveniles-to-adults and males-to-females ratios and a crossover of biomasses and abundances curves suggestive of an intermediate alteration of the copepod assemblages. The signs of community alteration were not clearly imputable to the N-compound contamination affecting the aquifer for 10 years so that further investigations are needed, including additional factors such as sediment size and species interactions, in the analyses. The application of a trait-based approach to copepod-dominated groundwater assemblages requires a long time for data acquisition and high taxonomic skills. A trait database could ease the application of such approaches in studies concerning groundwater ecosystems and its setup should be strongly encouraged among researchers.

**Supplementary Materials:** The Supplementary Materials are available online at <http://www.mdpi.com/2073-4441/11/12/2553/s1>.

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