

## Article

# Preliminary Analysis of the Diet of *Triturus carnifex* and Pollution in Mountain Karst Ponds in Central Apennines

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**Abstract:** Mountain karst ponds are sensitive environments, hosting complex trophic networks where amphibians play a major role, often as top predators. The diet of the Italian crested newt (*Triturus carnifex*) is still poorly known for populations occupying mountain karst ponds. These are traditionally used as livestock's watering points, leading to water pollution due to excreta and wading behavior. The aim of this paper is to understand the relationship between *T. carnifex* diet composition, assessed through the stomach flushing technique, and physical and chemical characteristics in mountain ponds, focusing on parameters altered by livestock pressure, such as ammonium concentration and dissolved oxygen. The high diversity of prey items found within the newts' gut contents confirms the generalist diet even in mountain ponds. The number of prey taxa, their relative abundance and Shannon–Wiener diversity index show variations among the sampled sites, related to livestock organic pollution. Moreover, we report the very first European records of microplastic items in amphibians' stomach content, which also represent the first evidence for Caudata worldwide. Our findings suggest that livestock pressure directly influences *T. carnifex* diet and highlight that the emerging issue of plastics is a threat even in remote high-altitude environments.

**Keywords:** diet; *Triturus carnifex*; mountain karst ponds; pollution; microplastics

## 1. Introduction

High-altitude ecosystems are nowadays more and more studied because of their susceptibility to ongoing climate change and habitat loss [1–3]. Many species have been proven to shift their ranges to higher altitudes [4–6] or, when the habitat does not permit these migrations, to possibly go extinct [7–9].

Karst mountain ponds are sensitive water bodies because of their exposure to several highly variable environmental factors. They are subject to seasonal snow coverage, noticeable daily and annual thermal excursion, UV-radiation and water-level changes [10–13]. As a result, the biotic component is often unique as well, showing adaptations to temperature fluctuation, long diapause or hibernation and short breeding phenology [14–16]. The species belonging to these animal communities are often of conservation concern, because of their specialization to mountain environments and to low connectivity among ponds [10,17].

In the Apennines, ponds are usually located within grasslands of karstic plateaus, and are traditionally used as watering points for livestock [18–20]. Therefore, the occurrence of cattle around

and inside the ponds determine the eutrophication of the water through the direct and indirect introduction of their excreta [21,22], as we also frequently observed during field campaigns. Even though most of these ponds fall within National and Regional Parks, they are still vulnerable and sensitive to many occurring environmental changes, because of their limited size [23,24]. Considering the alternation of turbid and clear water due to the alternative stable state of ponds [25], it is evident that the increase of non-managed livestock in ponds (especially horses and cows) determines nutrient enrichment, shifting towards turbid water conditions through sediment resuspension and alteration of chemicals, such as ammonium and oxygen concentrations [26,27]. Such physical and chemical modification affects both the macroinvertebrates and the vertebrates in ponds. In particular, the macroinvertebrate community structure changes and negatively influences the amphibians' community; this cascade effect depends on the environmental conditions of the ponds, because of a complex interaction between abiotic and biotic components [22,28,29]. Further, amphibians themselves show high sensitivity to climate [30,31], contaminants [32–34] and habitat changes [35,36].

Despite their highly threatened status [37,38] and their importance in food webs [39,40], amphibians' dietary ecology in high altitude lakes or ponds has been poorly investigated so far [41,42].

In this paper, we analyze how the ponds' alteration due to dense livestock presence could affect amphibians' diet, through the diet analysis of the Italian crested newt, *Triturus carnifex*, at mountain sampling sites. We study this aspect, which is still little known, focusing on both livestock-used ponds of different extents and artificial waters (rainwater collection cistern and troughs), characterized by different physical and chemical water conditions, in protected areas.

We explored, through a correspondence analysis, the association between physical and chemical variables and diversity in prey taxa in ponds with different degrees of livestock pollution. We also performed multiple statistical comparisons to test possible significant differences in the variability of newts' diet between natural and artificial sites and among the single sampling sites. We further assessed the presence of human-derived pollutants, such as plastics, which possibly affect freshwater ecosystems even in high altitudes.

## 2. Materials and Methods

### 2.1. Target Species

The Italian crested newt *Triturus carnifex* (Laurenti, 1768) was the target species of this study; it shows a disjunct distribution between the Italian and Balkan peninsulas [43]. In Italy, the species occurs from the sea level up to 1980 m [44]; in the Central Apennines, it reaches 1818 m a.s.l. in Lake Pantaniello [45,46].

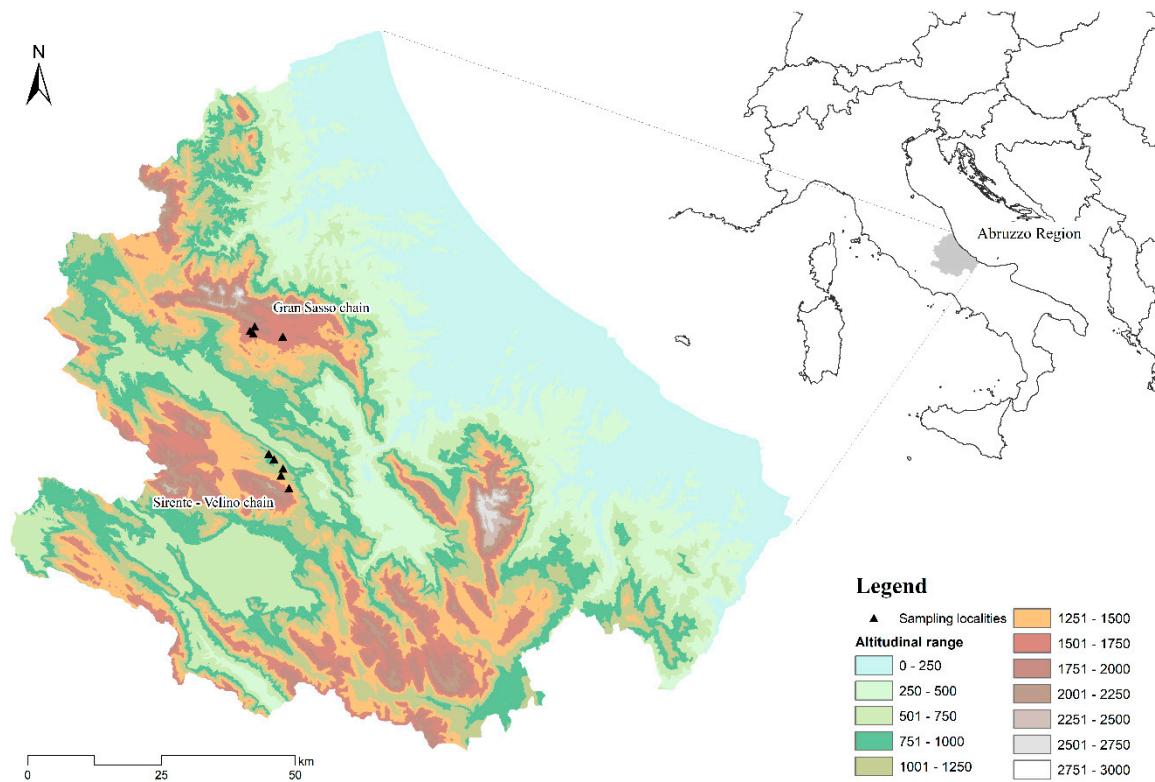
The terrestrial environment preferred by the Italian crested newt ranges from deciduous beech forests to typically Mediterranean xeric meadows; the shelters must be fresh and moist, often found under downed woods, in forest litter and in the burrows of small mammals. The aquatic phase occurs in lakes, ponds, springs and puddles, preferably rich in aquatic vegetation for egg deposition.

*Triturus carnifex* is classified as "Near threatened" by the Italian IUCN red list, with a declining population trend [47]. The main threats are the destruction or alteration of its reproductive sites at different levels, the shift to intensive agricultural practices, and climate change, which often leads to the desiccation of their preferred habitats [48–50].

### 2.2. Study Area

The study area included the Central Apennines' mountain karst plains (900–1700 m a.s.l.) (Figure 1), characterized by a scarcity of surface water. In this area, we selected 9 natural and artificial sampling sites, classified as ponds, troughs and a cistern; all of them fell within two protected areas, the Gran Sasso and Monti della Laga National Park and the Sirente Velino Regional Park. In the former, the ponds named Fossetta (two neighboring ponds, coded as GSA and GSB), Fossa di Paganica (GSC) and Racollo (GSD) were studied. In the latter, the Tempra pond (SVA), the Piane di Iano pond (SVB), the

Pagliare di Tione no-roof rainwater collection cistern, hereafter named only “cistern” (SVC), the Piano Canale (SVD) and Fonte dell’Acqua (SVE) troughs were sampled. Relevant pieces of information about the sampling sites are reported in Table 1.



**Figure 1.** Study area with altitude classes and sampling localities (black triangles).

**Table 1.** Sampling sites considered in the analyses and the corresponding geographic information.

Name	Code	Type	Area (m <sup>2</sup> )	Max. Depth (m)	Latitude	Longitude	Elevation (m a.s.l.)
Fossetta A	GSA	Pond	1870	1.2	42°24'17" N	13°35'14" E	1658
Fossetta B	GSB	Pond	358	1.2	42°24'19" N	13°35'13" E	1658
Fossa di Paganica	GSC	Pond	4500	1.5	42°24'55" N	13°39'27" E	1680
Racollo	GSD	Pond	7000	0.8	42°23'37" N	13°39'27" E	1570
Tempra	SVA	Pond	870	1.5	42°10'22" N	13°38'30" E	1183
Piane di Iano	SVB	Pond	780	1.2	42°11'21" N	13°37'22" E	960
Pagliare di Tione	SVC	Cistern	125	2.5	42°11'55" N	13°36'41" E	1080
Piano Canale	SVD	Trough	15	0.5	42°08'25" N	13°39'17" E	1342
Fonte dell’Acqua	SVE	Trough	26	0.5	42°08'21" N	13°39'10" E	1167

The GSA, GSB, GSD and SVB ponds show remarkable water level variations during summer; they are characterized by turbid waters (because of the silty bottom) and scarce vegetation (mainly composed by *Potamogeton*, *Ceratophyllum* and *Myriophyllum*) due to the constant presence of livestock (sheep, cows and horses), which enter the ponds for some meters from the banks (Figure 2). On the contrary, GSC and SVA show clearer waters, probably due to the wide extent (and the gravel bottom) of the former and due to the low livestock frequentation of the latter. In all these ponds, water is supplied only from snow melting and rainfall. SVC is not used by livestock as a watering point because of its limited accessibility due to perimeter walls; it has clear water and abundant aquatic vegetation. Finally, SVD and SVE show clear water and aquatic vegetation (including algae) as well; they are used as watering points, but livestock does not enter them.



**Figure 2.** Livestock near Fossa di Paganica pond, GSC (a); horses grazing aquatic plants (b) and cow cooling off during summer (c) in the Fossetta pond, GSA.

### 2.3. Sampling Methods

Newts (excluding larvae) were sampled starting from 2015 to 2019 (June–September, following the aquatic phase of the target species), employing both active and passive sampling methods. Dip netting sessions were performed, following a circular transect to cover the entire pond's perimeter. Funnel traps were also used as passive capture method, leaving them in the sampling sites for a 12-h period. The captured newts were stomach flushed using all the necessary equipment as described in [51]; the stomach contents were stored in Eppendorf safe-lock tubes with 70% ethanol.

A multi-parametric probe Eutech PD650 (EUTECH Instruments, Singapore, Singapore) was used to measure pH, dissolved oxygen (DO in mg/L), redox potential (mV) and the possible presence of ammonium ( $\text{NH}_4^+$  concentration, mg/L), resulting from livestock excreta directly released within the ponds (or nearby the corresponding banks). All of the parameters were measured once for each sampling session.

### 2.4. Diet Analysis

The prey items were identified at the lowest taxon allowed by the level of digestion, generally as orders.

The sampled newts for each sampling site, the number of different prey groups and the total number of prey items found within the stomach contents of each newt are reported in Supplementary Material Table S1.

Diversification of predated taxa was calculated for each sampled newt within each sampling site through the Shannon–Wiener diversity index ( $H'$ ), by means of the ‘diversity’ function of the ‘vegan’ [52] package in R [53]. The resulting  $H'$  values are reported in Supplementary Material Table S1.

Appropriate statistical tests were conducted in R to check for potential discrepancies in the diversification of prey groups among the sampling sites, as well as among the types of sampling sites

(i.e., ponds, cistern and troughs) and their “class” (i.e., natural, comprising the ponds, and artificial, including the cistern and the troughs).

A Shapiro–Wilk test and QQ plots were used to check the normality of the residuals from a linear model built considering the H' index as the response variable and the three above-mentioned grouping factors as covariates. Levene tests were performed to check homoscedasticity across the levels of the three grouping arrangements. Kruskall–Wallis tests were conducted to assess the significance of the considered grouping factors.

Subsequently, multiple pairwise Wilcoxon Rank Sum tests were conducted, using the ‘pairwise.wilcox.test’ function in R [53] with the Benjamini and Hochberg *p*-value adjustment method, to investigate differences among the single levels of the grouping factor(s) resulting as significant.

Moreover, we considered for each site the relative percentages of the different prey taxa per “average newt” (i.e., the ratio between the number of prey items, for each taxon, and the number of captured newts).

Correspondence analysis was performed through the ‘ca’ package [54] in RStudio [53], using both chemical parameters and relative prey abundance for each sampling sites as variables.

Together with prey items and other materials (e.g., parts of plants and sediments), we unexpectedly found microplastics in some stomach contents. All microplastics were observed and measured through a Leica M205C (Leica Microsystems, Wetzlar, Germany) binocular microscope. Photomicrographs were taken using a Leica DFC500 camera and the Zerene Stacker software (Zerene Systems LLC, Richland, WA, USA) version 1.04.

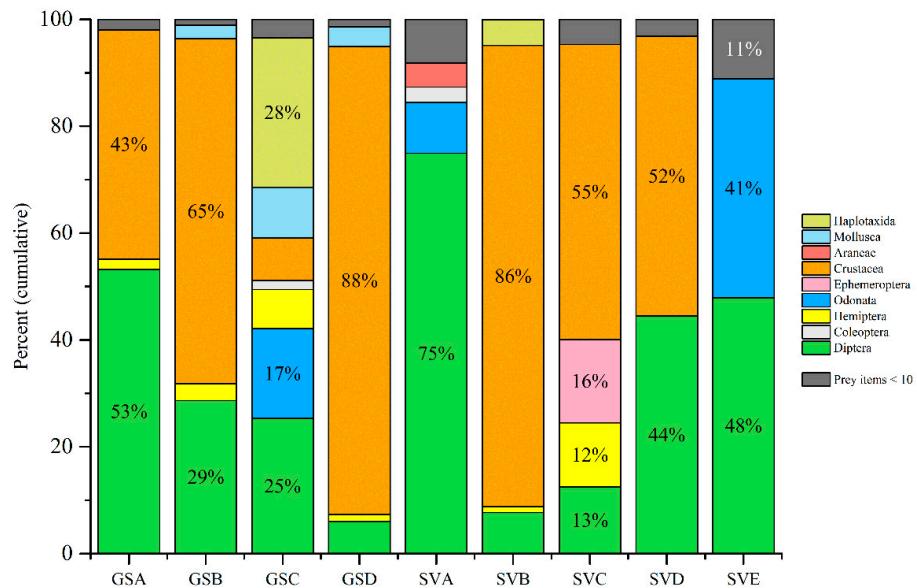
### 3. Results

The stomach contents of 239 Italian crested newts were collected from the nine sampling sites. The number of sampled newts and the total number of prey items for the different prey groups (for each site) are reported in Supplementary Material Tables S1 and S2.

Prey items were counted and classified in 19 different taxa, namely Crustacea (4750 prey items), Diptera (1800), Haplotauxida (328), Odonata (220), Hemiptera (191), Mollusca (135), Ephemeroptera (75), Coleoptera (53), Araneae (35), Acarina (17), Hymenoptera (13), Dermaptera (five), Caudata (four), Trichoptera (three), Tricladida (three), Lepidoptera (two), Julida (two), Neuroptera (one) and Mermithida (one). A total of 641 items could not be determined, due to the high level of deterioration; 161 fragments of plants, 93 particles of inorganic sediments probably deriving from the bottom, 59 microplastic items in the form of fibres, splinters and other undeterminable fragments were also found.

Crustacea (among which 47% were Ostracoda, 33% Branchiopoda and 19% Maxillopoda) and Diptera (among which 81% were Chironomidae and 9% Culicidae) showed the highest frequencies per average newt in almost all the sampling sites, with Crustacea exceeding 85% of the total prey items in GSD and SVB; differently, the highest Diptera frequency is found in SVA (Figure 3). GSC showed the highest number of taxa (14) per average newt, followed by SVD (13) and an equal number for SVA and SVE (11); the remaining five sampling sites showed a total number of prey taxa  $\leq 10$  (GSA = 10, SVC = 10, GSD = 8, GSB = 7, SVB = 6).

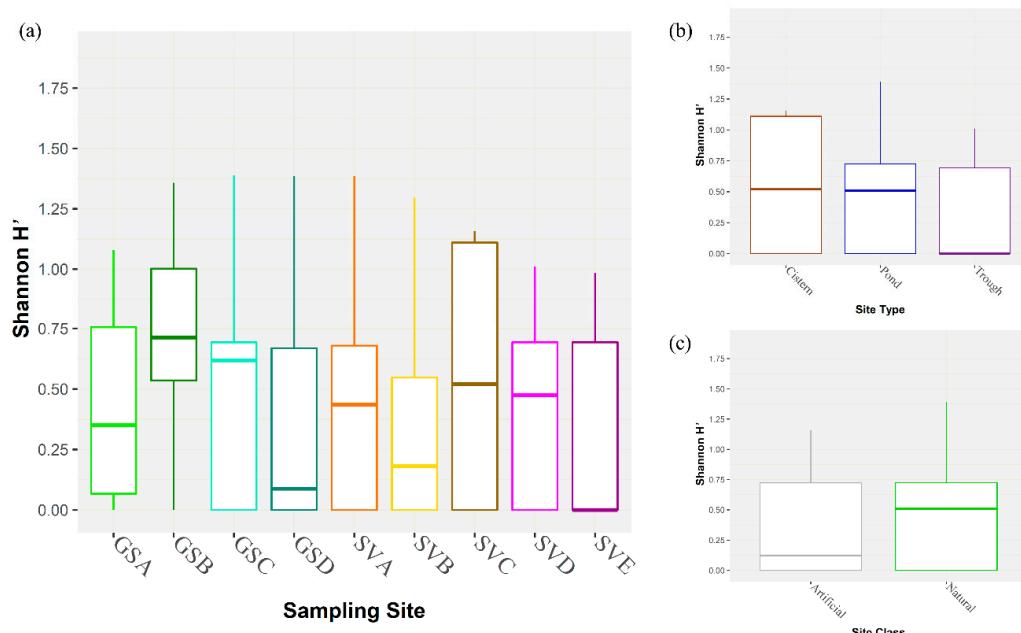
With respect to the analyses performed to assess the level of diversification within the diet of the single newts among the sampling sites, the performed Shapiro–Wilk test ( $W = 0.95$ ,  $p = 4.49 \times 10^{-7}$ ) and the inspected QQ plot led us to reject the hypothesis of the normality of the residuals from the linear model built upon the H' index, while the Levene tests did not reject homoscedasticity for the sampling site ( $F = 0.97$ ,  $p = 0.46$ ), site type ( $F = 1.35$ ,  $p = 0.26$ ) and site class ( $F = 0.06$ ,  $p = 0.80$ ) grouping factors.



**Figure 3.** Frequency of prey items (taxa) for each sampled site. Taxa < 10 prey items were aggregated into a single class (grey); percentages < 10% are not reported within the figure for graphical purposes.

Given the normality assumption was not met, a Kruskall–Wallis test was used to check the significance of the three grouping factors—significant differences in the means of the single levels resulted in only considering the sampling sites as a grouping factor ( $\chi^2 = 18.01$ ,  $df = 8$ ,  $p = 0.02$ ).

From the Wilcoxon Rank Sum tests successively performed on this grouping factor, GSB presented as significantly more diversified in terms of taxa predated by the single newts with respect to SVE ( $p = 0.04$ ); it is also worth noticing the low median  $H'$  value of GSA, GSD, SVB and SVE with respect to the remaining sampling sites (Figure 4a).



**Figure 4.** Boxplots of Shannon–Wiener  $H'$  index values across sampling sites (a), site types (b), and site classes (c).

Notwithstanding, the site type ( $\chi^2 = 4.29$ ,  $df = 2$ ,  $p = 0.12$ ) and site class ( $\chi^2 = 2.32$ ,  $df = 8$ ,  $p = 0.13$ ) were shown to be non-significant grouping factors by the Kruskall–Wallis tests, considering

the differences among the levels of these two factors emerging from boxplots in Figure 4b,c, single Mann–Whitney U tests were performed between natural and artificial sites, and among the three site types.

With respect to site class, the diets of the single newts within natural sites could be considered to be more diversified than in artificial ones ( $U = 5798.5, p = 0.06$ , alternative = ‘greater’) under a less constraining 0.1 threshold for the  $p$ -value; differently, considering site types, diets of the single newts in ponds were seen to be significantly richer than in (averages across the replicates) troughs ( $U = 4781, p = 0.02$ , alternative = ‘greater’), while no significant pattern emerged for the other two pairs (i.e., ponds–cistern and cistern–troughs).

The chemical parameters measured for each sampling site (averages across the replicates) are reported in Table 2. GSA, GSB, GSD and SVB show very low values of dissolved oxygen, along with relatively high values of ammonium (up to 0.26 mg/L in SVB), leading to classify them as “low water quality sites”.

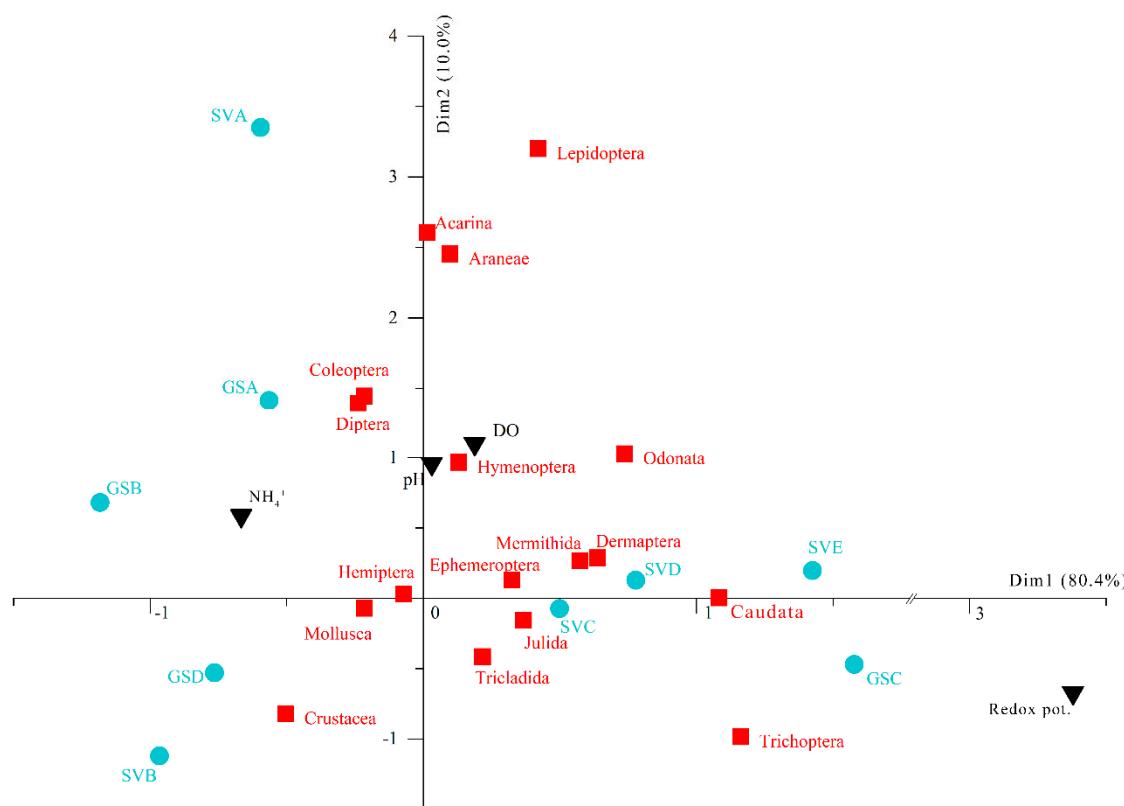
**Table 2.** Chemical parameters measured for each sampling site.

Name	Code	pH	Redox Potential (mV)	DO (mg/L) (DO (%sat), Temp. (°C))	NH <sub>4</sub> <sup>+</sup> (mg/L)
Fossetta A	GSA	7.3	−6.9	3.0 (38%, 20.8 °C)	0.12
Fossetta B	GSB	7.6	−17.7	3.6 (47%, 21.1 °C)	0.17
Fossa di Paganica	GSC	8.3	38.1	4.5 (62%, 24.0 °C)	0.00
Racollo	GSD	7.6	−7.9	2.3 (32%, 23.2 °C)	0.03
Tempra	SVA	9.5	−5.1	7.0 (90%, 24.2 °C)	0.06
Piane di Iano	SVB	7.5	−28.7	3.7 (45%, 17.9 °C)	0.26
Pagliare di Tione	SVC	8.5	16.2	7.2 (87%, 21.4 °C)	0.00
Piano Canale	SVD	8.1	27.2	7.8 (96%, 20.3 °C)	0.00
Fonte dell’Acqua	SVE	7.9	24.7	7.4 (93%, 23.4 °C)	0.00

SVA and GSC can be classified as “halfway sites”, while SVC, SVD and SVE, which represent the artificial sites, could be grouped as “high water quality sites” (Table 2).

The correspondence analysis (Figure 5) resulted in a neat prevalence of Dim 1 (80.4%) along which natural sites (GSA, GSB, GSD, SVA and SVB) and artificial ones (SVC, SVD and SVE) are clearly separated, except for the GSC pond, which groups with the latter. Ammonium is distant from DO and redox potential and placed near to the ponds group; differently, pH does not seem to form any relevant group with the other variables. Mollusca, Crustacea, Hemiptera, Coleoptera and Diptera are placed in the negative portion of Dim 1, near the ponds group; on the contrary, Odonata, Trichoptera and Caudata are the main taxa placed close to the artificial sites. Tricladida, Mermithida, Haplotauxida, Julida, Dermaptera, Ephemeroptera and Hymenoptera are located between the two groups of sampling sites, in proximity to the artificial sites. Acarina, Araneae and Lepidoptera are distant from both the ponds and artificial sites groups, while Neuroptera is completely separated along the positive portion of the Dim 2 (not shown), probably due to their little contribution in diet diversity.

Microplastics were found in the stomach contents of newts inhabiting four sampling sites, namely GSC, GSD, SVD and SVE. A sample for each type of microplastic found is represented in Figure 6. Two net-like plastic fragments were found in the stomach flush of a newt sampled in GSD, measuring 3.7 mm and 3.1 mm, respectively (Figure 6a). Two textile-like fibres (out of many others found in all the sampling sites) are shown in Figure 6b, measuring 1.8 mm (blue) and 1.2 mm (red). Finally, a blue microplastic fragment (1.44 mm in size), was found in the stomach contents of a newt sampled in the SVE trough along with a little (0.1 mm) yellow fragment (Figure 6c).



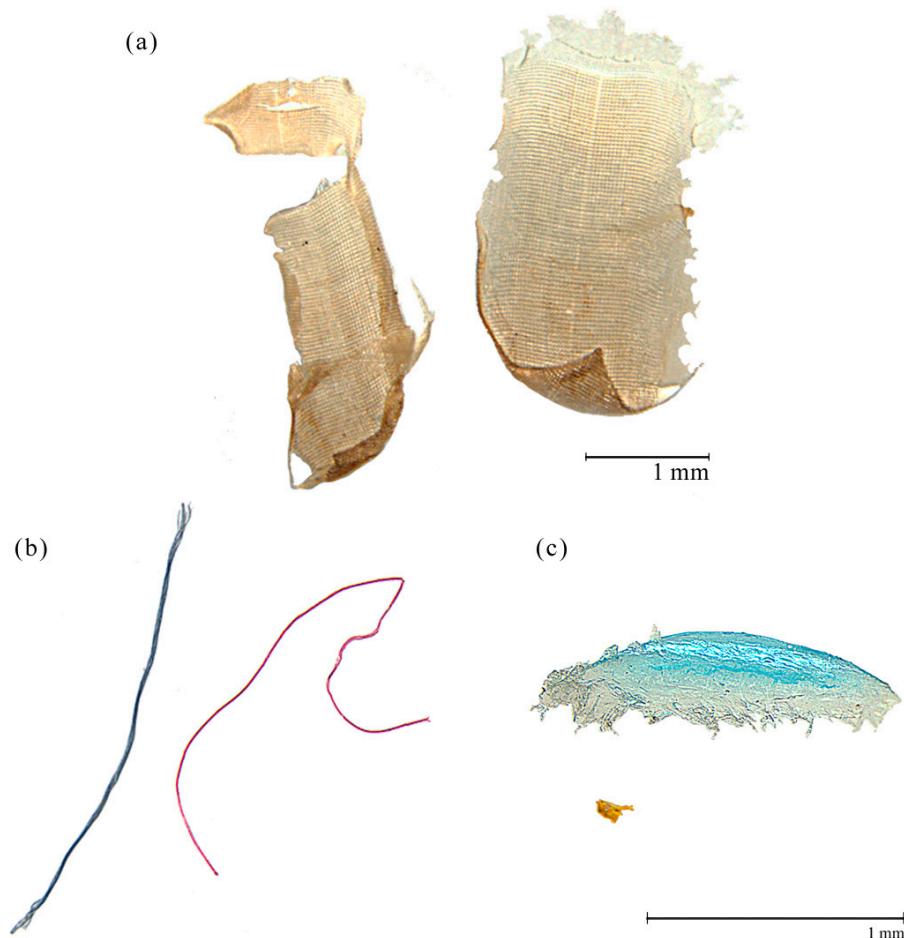
**Figure 5.** Correspondence analysis for both prey items and chemical parameters measured in all sampling sites. Blue circles: sampling sites, red squares: prey taxa and black triangles: physical and chemical parameters.

To characterize these items, ATR-FT-IR analyses were carried out using a Perkin Elmer (USA) Spectrum Two™ FTIR (PerkinElmer, Waltham, MA, USA) instrument equipped with the Attenuated Total Reflectance (ATR) accessory with a single bounce diamond crystal. The microplastics were analyzed at a  $1\text{ cm}^{-1}$  resolution within the  $4000\text{--}400\text{ cm}^{-1}$  range, with air used for the background spectrum. A consistent force was applied using the pressure monitoring system integrated with the instrument to maximize the spectrum intensity; the optimal number of scans was determined by collecting the spectra of the plastic fragments. An increase in the quality of the signal-to-noise ratio between 10 and 50 scans at a spectral resolution of  $1\text{ cm}^{-1}$  was observed. After 50 scans, the improvement in spectral quality was less evident, thus, all samples were collected at 50 scans.

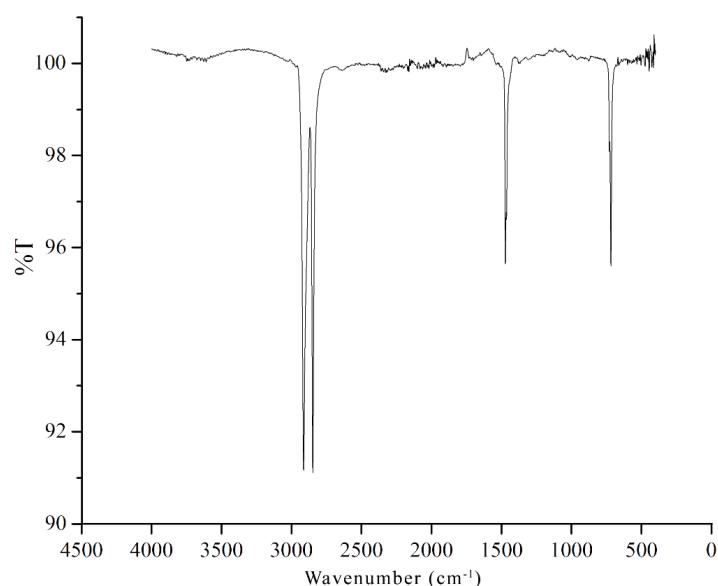
Analyses by ATR-FT-IR spectroscopy enabled the identification of microplastics by detecting absorbance bands. In Figure 7, the IR spectra of a fragment of polyethylene resulting from the analysis of the blue item pictured in Figure 6c are reported, corresponding to the bands of absorbance intrinsic of the stretching of C–H ( $3000\text{--}2770\text{ cm}^{-1}$ ) and the bending of C–H ( $1500\text{--}1450\text{ cm}^{-1}$ ) bonds. Additionally, ATR-FT-IR measurements enabled the detection of bands attributed to the scissoring of C–H bonds ( $750\text{--}700\text{ cm}^{-1}$ ).

The IR analysis of the fragments pictured in Figure 6a shows the characteristic bands of a medical pressure-sensitive adhesive (spectrum in Supplementary Material Figure S1a). The fibres reported in Figure 6b are probably part of mountain technical clothes. The analysis of the corresponding IR spectrum (Supplementary Material Figure S1b) shows the presence of the typical bands of polyacrylic polymers, although the signal-to-noise ratio is moderately low.

Finally, the chop of Figure 6c is likely to be a part of a plastic bottle cap, considering its polyethylene composition revealed by the IR spectra.



**Figure 6.** Microplastics found within *Triturus carnifex* stomach contents. (a) the biggest fragments found, probably deriving from a medical patch; (b) blue and red fibres, probably deriving from mountain clothes; (c) chop, probably deriving from a plastic bottle and very small yellow fragment (bottom) from an undeterminable origin.



**Figure 7.** IR spectrum of the polyethylene chop (displayed in Figure 6c, above) found within an Italian crested newt's stomach contents.

#### 4. Discussion

The Italian crested newt exhibits a generalist diet [44,55,56], consuming a considerable variety of preys [57]. In our samples, aquatic invertebrates presented the great majority of the collected preys; the most predicated taxa were Crustacea, followed by Diptera, with a neat prevalence of small-sized prey items for both, while the others 17 taxa found in the stomach contents were less represented.

The study area has been used for centuries for grazing, and its management has been subject to national regulation for at least 100 years [58]; while in the past the studied territory was mainly pastured by sheep, nowadays cattle and horses represent the main grazers. This determines a change in grazing management, since usually only sheep are looked after by the shepherd during the whole day (personal field observations). The differences in the chemical and physical conditions observed in the sampled sites may be the direct consequence of such kind of pasture management: in fact, while sheep do not usually wade the ponds, cows and horses are frequently observed in them. Thus, ponds are subject to livestock wading and defecation, determining an alteration of water quality, while drinking troughs or tanks does not usually undergo these pressures [29,59]. This could explain the notable differences between the natural sampled sites and the artificial ones, in terms of abiotic conditions.

The correspondence analysis permitted us to segregate two main groups. Sites showing good physical and chemical parameters (i.e., the three artificial sites and the GSC pond) are associated along the positive half of Dim 1 with taxa with narrow ecological tolerances to disturbance and organic pollution (e.g., Odonata, Ephemeroptera and Trichoptera). Differently, GSD and SVB are placed on the negative portion of Dim 1 and Dim 2 and are mainly associated with Crustacea, which showed predominance in the diet of newts from these sites, leading to low prey diversity (Figures 3 and 4a). These two sites are part of a larger group comprising also GSA and GSB (as shown by the correspondence plot), in which all sites are associated with ammonium, probably reflecting the ease of livestock wading in these ponds. SVA, which shows low ammonium and high DO concentrations when compared to the aforementioned sites, is distant from this group along Dim 2; this can be explained by the low livestock density around this site (personal observation).

The controversial impact of grazing on amphibians' communities is still debated, as reviewed by Howell, Mothes, Clements, Catania, Rothermel and Searcy [29]; some studies highlighted the negative effects of grazing on amphibians, e.g., [60,61], while others documented a positive or intermediate response, due to the species-specific requirements of the target amphibians [62–64].

Based on our research, ponds, which represent typical habitats for the newts in grasslands of karst plains and show high biodiversity in uncontaminated conditions [13], are negatively influenced by livestock pressure, especially when they can be easily waded. Indeed, we found livestock contamination altering the chemical and physical water parameters in ponds (e.g., low redox potential, high ammonium and low DO); this can be related to the absence of highly sensitive taxa and the consequent low number of different predicated taxa per site.

Another important aspect is that the only water supply for these ponds derives from snow melting and rain, resulting in a low rate of water replacement, especially during late summer, when the pasture pressure increases.

On the other hand, the artificial sampling sites, notwithstanding their small extent, present a high number of prey taxa, probably due to the constant water flow, which positively contributes to the high quality of water in these sites. This is further confirmed by the presence of some taxa which exhibit a reduced tolerance to the disturbances found in ponds, as also shown by the correspondence analysis.

Fossa di Paganica (GSC) is the only pond which does not follow this trend, showing conditions similar to the artificial sampling sites; in fact, it presents the highest number of prey taxa among all sites, and good chemical parameters. This could be explained by the large dimensions of the pond, which allow for the buffering of the negative effects of cattle and horses wading and defecating. Furthermore, the diffused presence of macrophytes found in GSC could favour macroinvertebrate diversity, as already observed in other karst ponds [65]. This is reflected by the high number of prey taxa found in this site, which could anyway be an underestimation of the total macroinvertebrate diversity.

Notwithstanding the evidence of livestock contamination of ponds and the corresponding influence on the number of prey taxa, the analysis of the Shannon H' variations among the sites showed that prey diversification for each newt is occasionally high if compared to the number of prey taxa per sampling site (e.g., GSB and SVA, see Figure 4a).

Previous studies showed that the low diversity of available prey may hamper larval development, due to low intake of dietary antioxidants [66,67] leading to reduced immune response capability and mass growth [68]. Thus, we speculate that newts, also considering their generalist diet, may be led to diversify their preys as much as possible, to integrate a possible lack of nutrients and avoid the negative effects mentioned before.

Furthermore, amphibian feeding is also conditioned by interspecific competition [69], which can be changed by the noticeable disequilibria found in most of the sampled sites, considering the absence of invertebrate predators (sometimes also acting as preys), such as Coleoptera, Hemiptera and Odonata.

In this work, we present the first European record of microplastics in amphibians' stomach contents and the first one ever recorded for Caudata.

While several studies about microplastics in marine ecosystems have been published in recent decades [70], field studies examining their presence in freshwater ecosystems started only recently [71]. Despite both Caudata and Anuran being among the most threatened animal groups worldwide, the only papers assessing the presence of microplastics in gut content referred to Anuran species [71,72]. Moreover, the previous records of microplastics in Anuran stomach contents were collected in human-altered freshwater habitats, such as farmlands surrounding river deltas [72] and plantations [71]; on the contrary, we found evidences of microplastic ingestion in scarcely populated, high-elevation territories, even covered by protected areas.

The chemical composition of the collected microplastics, emerging from the performed ATR-FT-IR spectroscopy analyses, shows that they are associated to human activities carried out in mountain environments, such as pastoralism and tourism; indeed, the majority of microplastic items are textile fibres (Figure 6b). The presence of these fibres in sampling sites may be due to waste displacement by the wind from the neighbouring roads and mountain retreats, or to livestock-related items, such as horse bridles or cow tags.

While the effects of microplastic ingestion by amphibians have been already assessed in laboratory studies with contrasting results [73,74], there is no information about the potential negative impacts on amphibians' communities in freshwater habitats.

## 5. Conclusions

High-altitude environments are nowadays proven to be very sensitive to ongoing environmental changes; the alteration of their freshwater habitats leads to rapid and dramatic loss of biodiversity. While, on one hand, pasture has shaped some peculiar environments for centuries, on the other hand it may represent a serious threat if not correctly managed. Human presence has, in this case, a dual role; it may cause a loss of diversity (e.g., unmanaged pasture) but it may also lead to the persistence of amphibian populations, because of the construction of artificial watering points, which currently host highly diverse communities. This is the very first assessment of the livestock-related detrimental effects on newts' diets in mountain karstic ponds; ongoing targeted sampling campaigns will enrich the knowledge about these threats, which widely affect mountain ecosystems.

The unexpected discovery of microplastics in many stomach contents reinforces the alarm about the ubiquitous plastic pollution of natural habitats, even in high-altitude protected territories; it is thus worth conducting future targeted research about this emerging issue to properly adapt conservation strategies.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4441/12/1/44/s1>, Figure S1: IR spectra of (a) medical pressure-sensitive adhesive and (b) fibres pictured in Figure 6a,b, respectively, found within Italian crested newts' stomach contents. Table S1: Sampled newts for each sampling site, number of

different prey groups, Shannon H', and total number of prey items found within the stomach content of each newt. Table S2: Macroinvertebrates found within stomach contents for all the sampled newts within each sampling site.

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