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# Microplastics Occurrence in the European Common Frog (*Rana temporaria*) from Cottian Alps (Northwest Italy)

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**Abstract:** Microplastics (MPs) pollution is arousing growing attention, yet knowledge about its occurrence in amphibians is scant to date. With this study, we aimed to determine whether plastic (>5000 μm) and MPs (10–5000 μm) could be detected in adult *Rana temporaria* from a high-mountain ecosystem (the Cottian Alps, northwest Italy). To do this, aquatic compartments and the digestive tract of adult *R. temporaria* were analyzed. Water, sediment, periphyton, aquatic macroinvertebrates, and tadpoles tested negative for plastic and MPs. Microplastics were detected in all the adult frogs (n = 5); all the identified items (one per specimen) were fibers (size range: 550.91–2355.51 μm). A statistically significant positive correlation between the particle length and frog size was recorded. The predominant fiber color was blue. The chemical composition was polyamide (60%), polyethylene (20%), and polyethylene terephthalate (20%). Since both the biotic and the abiotic freshwater compartments (tadpoles included) revealed the absence of MPs, it can be assumed that adult frogs ingest MPs from the surrounding terrestrial environment.

**Keywords:** amphibians; fibers; micro-FTIR; polyamide; polyethylene; polyethylene terephthalate; remote ecosystems

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## 1. Introduction

Plastic is an extraordinarily versatile material, but the associated disadvantages are becoming increasingly evident, particularly for the consumption of non-renewable resources and fossil hydrocarbons [1,2]. Plastics generate an enormous quantity of waste: Researchers estimate that more than 8.3 billion tons of plastic have been produced since the early 1950s. About 60% of that plastic has ended up in either landfills or the natural environment [3]. These plastics that enter the environment contaminate the soil, rivers, lakes, and ultimately the oceans [3]. It has been estimated that approximately 8 million tons of plastic litter per year end up in different oceans, of which 80% of ocean plastics come from land-based sources [4]. The world annual production of plastics was 1.7 million tons in the 1950s [5] and totaled 368 million tons in 2019, 57.9 million tons (16%)

of which were from Europe [6]. In the first decade of the present century, plastic production was equated to the amount generated in the previous century, characterizing our current era as the "Age of Plastic" [7].

The widespread use of plastic in human activity has raised environmental concerns. After plastic enters (directly and/or indirectly) freshwater ecosystems through various sources (i.e., residential, domestic activities, tourism, etc.), it undergoes degradation (i.e., biological, mechanical, photooxidation by ultraviolet light) that alters the particle size and density [8]. Most plastics are highly resistant to (bio)chemical degradation, however, which is why they persist in the environment [9]. Plastics physically break up into smaller and smaller fractions, termed microplastics (MPs, 1 μm–5 mm) [10]. Microplastics are ubiquitous: From deep-sea sediments to high-mountain lakes in both temperate and tropical ecosystems [11–13]. Microplastics are classified into primary and secondary MPs [14]. Primary MPs are manufactured plastic particles that go into a variety of products (i.e., cosmetics), while secondary MPs are formed during the use and disposal of plastic products (i.e., degradation of plastic bottles) or in the decomposition of macroplastics into MPs [14].

The accumulation of MPs in both biotic and abiotic environmental compartments and the possible consequences are still barely known [15]. Terrestrial and freshwater and ecosystems are recognized as sources of plastics for the oceans. However, studies on MPs in such ecosystems are still scant to date [16]. This reflected a greater extent for mountain ecosystems, which provide a habitat for numerous species (i.e., insects and amphibians), many of which are endangered [17]. The monitoring of MPs in amphibians is of critical importance since they are in global decline [18] and the accumulation of MPs in amphibians may be a transfer path for these contaminants of emerging concern from freshwater to terrestrial ecosystems or vice versa [19].

The European common frog (*Rana temporaria*) has a widespread distribution in Europe from the Pyrenees to the Urals and West Siberia [20]. In southern Europe, this species is usually found in montane habitats [21]. Isolated populations may be vulnerable to regional or local impacts, for example, the introduction of salmonids [22], abandonment of pastoralism (with consequent disappearance of waterholes for reproduction), or diseases (i.e., ranavirus) [23]. Furthermore, *Rana temporaria* is a protected species listed in Annex V of the Habitats Directive-HD (92/43/EEC).

With the present study, we wanted to determine whether plastic (>5000 μm) and/or MPs (10–5000 μm) are present in adults of *R. temporaria* inhabiting a high-mountain pond (Selleries Pond, northwest Italy) since MPs could interact with other stressors contributing to amphibian decline [24,25]. Generally, adult *R. temporaria* are usually found in ponds only during the reproductive period [23]. However, this species is present in the Selleries Pond (northwest Italy, Cottian Alps) throughout the ice-free season (April–October). Thus, we hypothesized that the aquatic compartment could be the main source of (micro)plastics if present. On this path, several aquatic compartments (water, sediment, periphyton, aquatic macroinvertebrates, and tadpoles) were assessed to understand the source of MPs.

## 2. Materials and Methods

### *2.1. Study Site*

Selleries Pond (45°02′53″ N 7°07′16″ E) is a high-mountain pond located at 1985 m a.s.l. in the municipality of Roure (Province of Turin, Piedmont) in the Cottian Alps (northwest Italy) (Figure 1). The site is classified as a Special Area of Conservation (SAC- IT1110006 "Orsiera Rocciavre") and can be reached only by foot. The pond measures 120 m in perimeter, has a maximum depth of 2.5 m, and is 815 $m^2$ in surface area. It is fishless, and provides the reproductive habitat for *Rana temporaria*, the only amphibian species recorded there. Trekking and pasturing are the two main anthropogenic activities, especially during the summer.

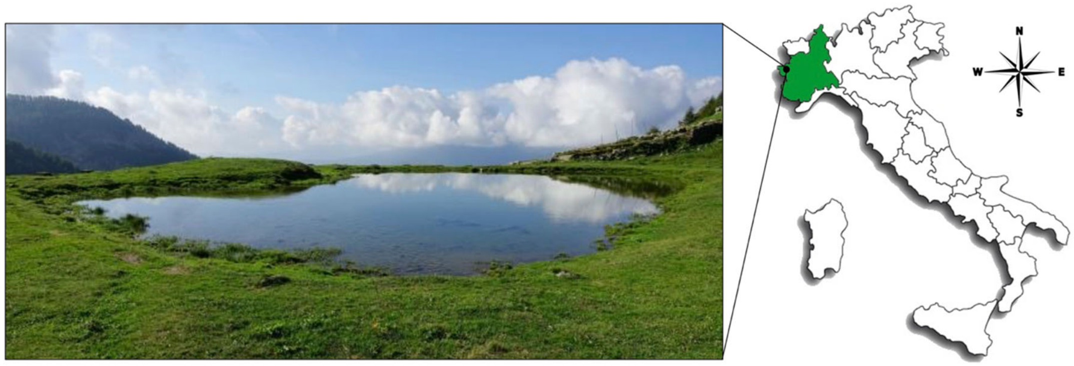



**Figure 1.** Location of the Selleries Pond (45°02′53″ N 7°07′16″ E), northwest Italy, Cottian Alps.

*2.2. Water and Sediment Sampling*

On 26 May 2020, sediment samples were collected with the Van Veen grab (SG-200; 250 $cm^2$ sampling surface) from five sampling sites (in triplicate) at differing depths (0–2.5 m) following the protocol proposed by Pastorino et al. [13]. The grab was cleaned with ultrapure water between each replicate to prevent contamination. The samples (n = 15) were placed in glass jars (1 L) covered with aluminum foil, transported to the laboratory, and frozen at −20 °C until MPs analysis.

Water samples were collected in triplicate (the same sites and time as the sediment samples) by means of an Apstein plankton net (mesh 45 μm) installed on a telescopic bar. The net was set directly below the water surface, taking in the entire water column, and spanning the entire lake perimeter (120 m; five random transects) [13]. The net was cleaned with ultrapure water between each transect to prevent contamination. The water samples (n = 15) were transferred into glass jars, covered with aluminum foil, transported to the laboratory, and refrigerated (+4 °C) until analysis.

*2.3. Sampling and Processing of Periphyton, Macroinvertebrates, Tadpoles, and Adult Frogs*

Biotic components were sampled the same day as the abiotic samples. Periphyton was sampled with a steel scalpel in 1 $m^2$ of the benthic substrate (cobbles and pebbles) at five sites (the same as the abiotic components) in triplicate (15 samples). Specimens of Diptera Chironomidae, the most abundant macroinvertebrates inhabiting high-mountain ponds [13], were sampled from the sediment using steel forceps at the same five sites (n = 20 per site; total samples: 100 individuals). Fifteen *Rana temporaria* tadpoles were sampled with a net (mesh size: 4 mm) at the same five sampling sites (total: 75 tadpoles) covering 1 $m^2$ of the pond bottom. All biological samples were placed in glass jars, covered with aluminum foil, and transported refrigerated to the laboratory and frozen (−20 °C) until analysis.

Adult specimens of *Rana temporaria* were identified by scanning the banks and exposed rocks with binoculars while walking around the pond. Five frogs morphologically identified as *Rana temporaria* [26] were caught with a hand net (mesh size 15 mm) and were immediately stored in a precleaned glass 5L-container covered with a steel lid (rinsed 3 times with filtered water) placed in a cold box (+10 °C) and transported to the laboratory. Each specimen was euthanatized using buffered tricaine methanesulfonate (MS-222) at a concentration of 6 g/L [27]. The specimens were weighted to determine the body mass (g) and measured for their snout–vent length (cm), and then necropsied under air-controlled conditions to avoid airborne microplastic pollution. The entire digestive tract was gently removed, placed in a precleaned glass container, and stored at −20 °C until analysis. The adult frogs sampled for this study are unique samples and part of a monitoring survey of the health status of amphibians in high-mountain ponds/lakes.

### *2.4. Microplastic Determination*

Sediment samples were extracted in triplicate by a saturated NaCl pre-filtered solution; the whole extract was then recovered and filtered on 6-µm pore paper disks (Whatman®, Sigma-Aldrich, St. Louis, MO, USA). The water samples were filtered without digestion using a 6-µm pore paper disk filter as described in Pastorino et al. [13].

Biological samples (periphyton, macroinvertebrates, tadpoles, digestive tracts from adult frogs) were pretreated and sonicated as described in detail in Bertoli et al. [16]. Briefly, the Creon enzyme (Creon 40’000 at 20 mg per g of tissue; TRIS–buffered pH at 8.00; 37 °C; Mylan, Italia S.r.l, Milano, Italy) was selected as a digestion technique since it allows the removal of the tissue without affecting the identification of plastic polymers. The digested tissues were filtered using a paper fiber filter disk (6 µm) (Whatman®, Sigma-Aldrich, St. Louis, MO, USA), stored in glass Petri dishes, dyed with Rose–Bengal, and dried overnight at 40 °C [28]. Presorting was performed using stereomicroscopy (10–80×; SMZ-800 N, software NIS-elements D, Nikon, Tokyo, Japan). Potential target items were measured by a digital webcam and analyzed via microscopy coupled with Fourier transform infrared spectroscopy (µFT-IR; Nicolet iN 10MX®, Thermo Fischer Scientific, Waltham, MA, USA). The µFT-IR system was equipped with a liquid nitrogen-cooled MCT-A detector (spectral range 7800–650 $cm^{-1}$) operated via an OMNICTM Picta (Thermo Fisher Scientific, Waltham, MA, USA) user interface. Identifications were performed by determining and comparing the spectral differences of targeted items to a library of known materials (only matches >90% were considered). The limit of quantification (LOQ) for the chemical analysis of particles was 10 µm. Recovered items were classified according to size, shape, color, and chemical type as reported by Galgani et al. [29].

### *2.5. Quality Assurance/Quality Control (QA/QC)*

The general quality of the method adopted to determine the microplastic used in this study was optimized and/or checked according to guidelines reported in the recent literature [30,31]. Following the quality criteria reported by the literature concerning sample treatments and analyses, lab preparation, clean air conditions, negative/positive controls, and polymer identification, the total accumulated score obtained by this study was 10/10 [31].

Three positive and three negative controls were performed for each batch of analyses.

Negative controls had to be microplastic free within the target dimensional range to be considered acceptable; on the contrary, positive controls were considered acceptable if the mean particle recovery of spiking microplastics particles (orange PP and PE fragments mixture spiked in a digested aliquot of tissues, n = 3) were recovered over 80% of the initial spiked number.

The sample treatments were performed under air-controlled conditions to avoid airborne microplastic pollution using a dedicated clean chamber equipped with double HEPA-II filtration to minimize microplastic pollution. Thoroughly rinsed glassware was used at each stage of the process, and both blanks and spiked samples were analyzed to evaluate the performance of the whole process. Blanks were performed using extraction solutions as samples (n = 5), and because of the results obtained on blanks (mean 0.004 ± 0.009 items/L, recovered items were a white filament of polyethylene terephthalate-PET), data reported in this study were not corrected by microplastics recorded in blanks because it was negligible. Spiked samples were extracted to evaluate recovery with pink polypropylene (PP) and PET blue microplastic fragments; the recoveries of spiked samples were >90%.

Quality Assurance/Quality Control activities, performed to ensure the general quality of the used method, were optimized to ensure the high performance of recovery within the range of size of specific interest; particles below 6 µm, if present, were lost during the filtration step. Nevertheless, a 6 µm particle size was lower than the LOQ of the µFT-IR technique and this loss was considered unsignificant for the specific purposes of this study.

### 2.6. Statistical Analysis

The Shapiro–Wilk test was used to verify whether the data followed a normal distribution. Based on the results ($p = 0.83$ and $p = 0.79$ for frog snout–vent length and MPs size, respectively), it was assumed that the assumption was met. Based on such assumptions, the Pearson correlation coefficient was used to check the correlation between the snout–vent length of adult frogs and MPs size [32]. Statistical significance was set at $p < 0.05$. R software (RStudio, Inc., Boston, MA, USA, version 3.5.2.) was used to perform the statistical analyses.

## 3. Results and Discussion

Despite the key role of amphibians in the food web and the potential to transfer contaminants through trophic levels and from freshwater to terrestrial ecosystems and vice versa [33,34], there are scarce data on their capacity to accumulate MPs compared to other vertebrates.

With this study, we measured the occurrence of plastic and MPs in adult specimens of *Rana temporaria* and analyzed biotic and abiotic aquatic compartments in a high-mountain pond. The water and sediment samples tested negative for plastic and MPs, indicating their absence in the abiotic freshwater compartments. Plastic and MPs also tested negative in the samples of periphyton, macroinvertebrates, and tadpoles. Since tadpoles feed mainly on periphytic algae and litter on sediment [35], the absence of MPs in these primary food sources was consistent with the failure to find plastic and MPs in the tadpoles. Diptera Chironomidae, which are classified as collector–gatherers in the functional feeding groups (FFG) [36], forage on particles in sediments. Based on this assumption, we assume that if MPs were present in the sediment, we would have found them in the specimens of Diptera Chironomidae as well.

Differently, MPs were found in the digestive tract of all the adult specimens of *Rana temporaria*: Five adult males (range of snout–vent length: 8.2–9.2 cm; range of body mass: 88–94 g) were analyzed (Table 1). The diet of adult *R. temporaria* includes terrestrial insects (i.e., spiders, snails, worms) and aquatic macroinvertebrates [26]. Adult frogs may ingest MPs mistaking them as food (accidentally) or via prey containing MPs. Since both the biotic and the abiotic freshwater compartments (tadpoles included) revealed the absence of MPs, it can be assumed that adult frogs ingest MPs from the surrounding terrestrial environment (i.e., terrestrial prey).

Table 1 presents the total items and the item per frog; it also reports the particle shape, color, size, and chemical type. All the items were fibers (Figure 2).

The predominant colors were blue (60%), light blue (20%), and black (20%). While it is difficult to identify the source of blue particles, their higher prevalence compared to the other colors has already been shared in other studies on freshwater ecosystems [37,38]. The particle size ranged from 550.91 to 2355.51 μm, and a significant positive correlation between the particle length and frog size was found (Pearson's correlation; $r = 0.991$, $p = 0.001$). Presumably, larger frogs catch larger prey, which, in turn, can ingest larger particles [39]. Similarly, Jâms et al. [32] found a positive relationship between the animal size and length of ingested plastic, and that body length describes over 40% of the variance in the size of the largest plastic item an animal can ingest.

**Table 1.** Biometric features and microplastics in adult *Rana temporaria* from the Selleries Pond. Microplastics are reported as total number of items, shape, color, size, and chemical composition. PE denotes polyethylene, PET is polyethylene terephthalate, and PA is polyamide.

| Specimen | Snout–Vent Length (cm) | Body Mass (g) | Total Items | Shape | Color | Size (μm) | Chemical Composition |
|---|---|---|---|---|---|---|---|
| 1 | 9.0 | 94 | 1 | fibre | blue | 2056.70 | PA |
| 2 | 8.4 | 89 | 1 | fibre | light-blue | 1049.74 | PA |
| 3 | 9.2 | 98 | 1 | fibre | black | 2355.51 | PET |
| 4 | 8.2 | 88 | 1 | fibre | blue | 550.91 | PE |
| 5 | 8.7 | 92 | 1 | fibre | blue | 1689.12 | PA |

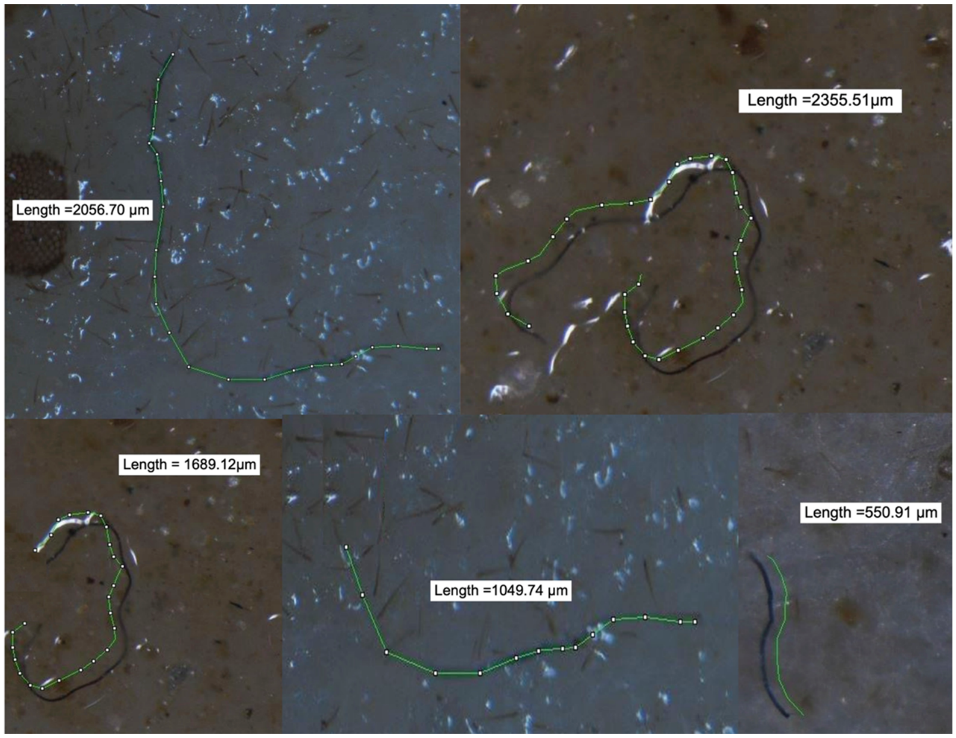
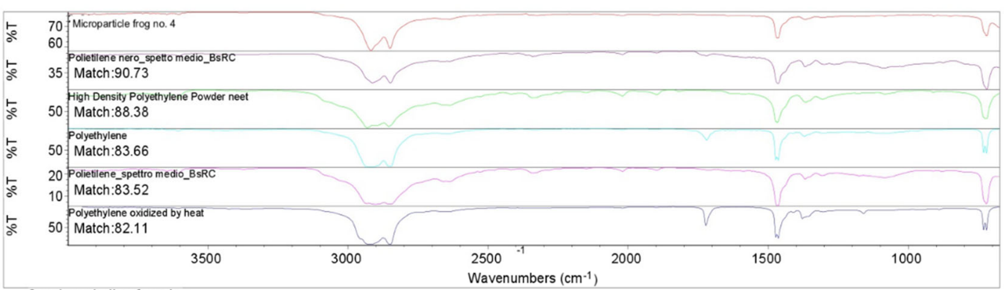



**Figure 2.** Fibers detected in the five adult specimens of *Rana temporaria* from the Selleries Pond (northwest Italy).

The chemical composition of MPs, in decreasing order, was 60% polyamide (PA), 20% polyethylene (PE), and 20% polyethylene terephthalate (PET). An example of the µFT-IR analysis is given in Figure 3.




**Search results list of matches**

| | Index | Match | Compound Name | Library Name |
|---|---|---|---|---|
| 1 | 4 | 90.73 | Polietilene nero_spetto medio_BsRC | Plastics_BsRC |
| 2 | 59 | 88.38 | High Density Polyethylene Powder neet | STI-Diffuse |
| 3 | 482 | 83.66 | Polyethylene | HR Hummel Polymer and Additives |
| 4 | 6 | 83.52 | Polietilene_spettro medio_BsRC | Plastics_BsRC |
| 5 | 764 | 82.11 | Polyethylene oxidized by heat | HR Nicolet Sampler Library |
| 6 | 3 | 81.96 | Polietilene 6000000_spettro medio_BsRC | Plastics_BsRC |
| 7 | 132 | 81.25 | Poly(ethylene:acrylic acid) (6% acrylic acid) | HR Sprouse Polymers by Transmission |
| 8 | 29 | 80.72 | OCTACOSANE, 99% | HR Aldrich Hydrocarbons |
| 9 | 2 | 80.71 | Polietilene 1000_spettro medio_BsRC | Plastics_BsRC |
| 10 | 1 | 80.64 | TRIACONTANE, 99% | Aldrich Condensed Phase Sample Library |

**Figure 3.** Spectra of a microparticle found in a frog (no. 4; top red line) and the spectral match with the reference library (other colors).

Polyamide is a polymer held together with amide bonds and is highly resistant to abrasion. Widely employed in textile manufacturing, it is also used in the automotive and the transportation industry. Because polyamide fibers were originally developed as an alternative to silk, they are soft and flexible, which enhances wearing comfort. We can assume that the polyamide fibers probably came from the technical apparel tourists to the area wear. The pond is, in fact, a popular destination during the summer season. It cannot be excluded, however, that the presence of MPs in such remote areas as Selleries can derive from the atmospheric transport of fibers from urban/lowland point-source contamination, as previously reported [40].

Polyethylene (PE) and polyethylene terephthalate (PET) were also detected in the frogs' digestive tracts, indicating just how widespread these polymers are. Indeed, PE and PET are the main MPs contaminants in European freshwater ecosystems [41]. Polyethylene is the most common plastic in use today for packaging (i.e., plastic bags), while PET is used in applications ranging from packaging to electronics.

Unfortunately, we are unable to compare our findings with other field studies since the present study is the first to report MPs in adult frogs. However, three published field studies about MPs accumulation by tadpoles were performed: Hu et al. [42] reported an average of 0–2.73 particles in tadpoles of four species (*Bufo gargarizans*, *Microhyla ornata*, *Pelophylax nigromaculatus*, and *Rana limnochari*) from 25 waterbodies from the Yangtze River (China); Karaoğlu and Gül [43] recorded an average of 302.62–306.69 items/g in tadpoles of *Pelophylax ridibundus* and *Rana macrocnemis* from Turkey; and Kolenda et al. [38] highlighted how 53 out of 201 tadpoles belonging to five species (mainly *Bufo bufo*) from Poland waterbodies ingested at least one particle. The other available studies in the literature are experimental trials. For example, Hu et al. [44] investigated the uptake, accumulation, and elimination of MPs (polystyrene) by *Xenopus tropicalis*. Moreover, Boyero et al. [45] studied the effects of polystyrene microspheres on the survival, body condition, and function of the tadpole *Alytes obstetricans*.

Microplastics are often composed of a complex mixture of chemicals: Additives and monomers included in the ingredients of the plastic material and by-products of manufacturing and chemical contaminants in water accumulate on plastic when it becomes litter (i.e., persistent organic pollutants and metals) [46].

## 4. Conclusions

Despite the small sample size, being *Rana temporaria* listed in Annex V of the HD, our findings provide novel insights into the accumulation of MPs in adult frogs from a high-mountain ecosystem. We noted the absence of MPs in the freshwater compartment; accordingly, the fibers detected in the adult frogs may have derived from the terrestrial environment. Further studies are needed to better understand the distribution of the chemical type, size, and color of MPs in terrestrial compartments, the trophic transfer of MPs from freshwater and terrestrial ecosystems (and vice versa), their harmful effects on both tadpoles and adult frogs (also analyzing MPs in different tissues), their interactions with other threats [47–49], and their consequences for ecosystem functioning. Moreover, fibers up to 2 mm in size were detected. Thus, further research is needed to assess the potential effect of such large particles on the digestive tract of frogs (i.e., intestinal damage).

**Author Contributions:** Conceptualization, P.P. and M.R.; data curation, P.P. and M.R.; formal analysis, P.P.; investigation, P.P., M.P., A.D.B., D.B., S.A. (Serena Anselmi), S.C., S.A. (Silvia Alberti), A.D., M.O., E.P., and M.R.; methodology, P.P., A.D.B., D.B., S.A. (Serena Anselmi), S.C., G.T., and M.R.; project administration, P.P. and M.P.; writing—original draft, P.P.; writing—review and editing, M.P. and M.R. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** ARRIVE and European Directive 2010/63/EU guidelines [27,50] were the reference documents for all experimental procedures. Permission for sampling the tadpoles and adult frogs and conducting necropsy was obtained from the "Ente di Gestione delle Aree Protette delle Alpi Cozie" (Executive Determination no. 106-24/05/2021).

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**Data Availability Statement:** Not applicable.

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