Environmental Pollution 303 (2022) 119137

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Differential effects of microplastic exposure on anuran tadpoles: A still underrated threat to amphibian conservation?☆

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ARTICLE INFO

Keywords:
Plastic polymers
Larval growth
Activity level
Rana latastei
Bufotes balearicus

ABSTRACT

Microplastics (MPs) have been reported to threaten a wide variety of terrestrial, marine, and freshwater organisms. However, knowledge about the effects of MPs on anuran amphibians, one of the most threatened taxa worldwide, is still limited. To assess the effects of MPs on the growth and survival of the Italian agile frog (*Rana latastei*) and green toad (*Bufotes balearicus)*, we exposed tadpoles to three different concentrations (1, 7, and 50 mg L^{-1}) of an environmental relevant mixture of microplastics (HPDE, PVC, PS and PES), recording data on their activity level, weight and mortality rates. While the effects of MPs on green toad tadpoles were negligible, Italian agile frog tadpoles were severely affected both in terms of growth and activity level, with high mortality rates even at the lowest MP density (1 mg L^{-1}). Our results suggest that MP contamination of freshwater habitats may contribute to the ongoing decline of anuran amphibians.

1. Introduction

Amphibian populations are globally declining (Hayes et al., 2010; Converse and Grant, 2019), with >30% of amphibian species categorized as at risk of extinction by the IUCN (Stuart et al., 2004). Several anthropogenic factors are contributing to this trend, including habitat loss (Houlahan and Findlay, 2003; Cushman, 2006), pollution (Bridges and Semlitsch, 2000; Slaby et al., 2019), disease epidemics and climate change (Kiesecker et al., 2001; Ficetola and Maiorano, 2016). Among the several pollutants that can contaminate freshwater habitats, microplastics (MPs), defined as plastic particles smaller than 5 mm in diameter (Thompson et al., 2004), have recently gained the attention of the scientific community (Eerkes-Medrano et al., 2015), raising concerns about their toxicity for aquatic species and potential bioaccumulation through food webs (Imhof et al., 2017; Windsor et al., 2019; Araújo et al., 2021).

Major input pathways for MPs into freshwaters are run offs from urban and agricultural areas, treatment plant effluents (Anderson et al., 2016) and atmospheric deposition (Dris et al., 2016). “Primary” MPs are manufactured for specific purposes, such as the microspheres contained in several cleaning products, while “secondary” MPs result from the biological and mechanical degradation of large plastic materials (Castro-Castellon et al., 2021). Out of the over 5300 synthetic polymers in commerce (Wagner and Lambert, 2018), polyethylene (PE), polyvinyl chloride (PVC), polypropylene (PP) and polystyrene (PS) are the most widespread in the environment. PE and PS can sorb high concentrations of priority pollutants, mainly polychlorinated biphenyls, while PVC and PS contain mutagenic vinyl chloride and vinyl benzene, respectively (Rochman et al., 2017). In addition, polyester fibers (PES) are dominant in waterbodies (Hu et al., 2018).

Microplastics can enter aquatic food webs at different trophic levels through direct ingestion, filter feeding, or predation on prey previously exposed to plastic debris (Wagner et al., 2014; Mattsson et al., 2015; Anderson et al., 2016). They adhere to periphyton, increasing the probability of ingestion by grazers (Boyero et al., 2019).

Most tadpoles have opportunistic feeding habits, filtering phytoplankton from the water column, scraping algae off the substrate, and ingesting a wide range of potentially edible particles, which they break into suitable sizes by their jaw sheaths and labial teeth (Altig et al., 2007; Wells, 2007). Their feeding habits make tadpoles prone to the ingestion of MPs, which has been demonstrated under field conditions. Although these pollutants have been reported to affect tadpole growth and development (Hu et al., 2018), available information is still scarce.

☆ This paper has been recommended for acceptance by Philip N. Smith.

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https://doi.org/10.1016/j.envpol.2022.119137
Received 15 December 2021; Received in revised form 19 February 2022; Accepted 10 March 2022

Microplastics may damage tadpoles through two major pathways: 1) exposing them to plasticizers or associated pollutants (e.g., persistent organic pollutants; Anderson et al., 2016); and 2) blocking their digestive tracts or inducing a false sense of satiation, thus impairing their feeding success (Watts et al., 2015; Welden and Cowie, 2016).

Exposure to polyethylene MPs has been reported to cause histopathological changes (Araújo et al., 2020a), abnormalities in nuclear erythrocytes (Araújo et al., 2020b) and affect both growth and behavior in Cuvier's foam froglet (*Physalaemus cuvieri*) tadpoles (Araújo and Malafaia, 2020; Araújo et al., 2020b). In contrast, polystyrene MPs neither affected the growth nor the activity of African clawed frog (*Xenopus laevis*) larvae (De Felice et al., 2018).

Although some studies have shown that tadpoles are capable of egesting MPs fast (Hu et al., 2016; De Felice et al., 2018), recently it has been demonstrated that exposure to polystyrene microspheres can impair both feeding and growth of common midwife toad (*Alytes obstetricans*) tadpoles, with lethal effects at high MP concentration (1800 part. mL^{-1}; Boyero et al., 2019).

Furthermore, despite widespread concern for amphibian conservation, studies on the impact of MPs on threatened species are lacking. To assess the effects of MP exposure on both the growth and behavior of anuran tadpoles, we tested two species, differing in both habitat requirements and conservation status: the Italian agile frog (*Rana latastei*, Boulenger 1879) and Balearic green toad (*Bufotes balearicus*, Boettger 1880).

The Italian agile frog is an endemic species which occurs in residual lowland hygrophilous woods of northern Italy, Canton Ticino, Istria, Slovenia and Croatia (Barbieri and Mazzoti, 2006). Loss of suitable habitats caused by urbanization and intensive agriculture and non-native fish and crayfish are considered the main threats to this species and have been imputed as causes of some local extinction events (Edgar and Bird, 2005; Ficetola et al., 2012). Ongoing decline in both habitat quality and population size led the IUCN to list the Italian agile frog as "Vulnerable"; it is also included in Annexes II and IV of the Habitats Directive (EC 43/1992). The native range of the Balearic green toad includes the Italian peninsula and north-eastern Sicily, Sardinia, Corsica, and the Balearic Islands. This species is widespread along sandy coasts and lowland floodplains, also occurring in urbanized and agricultural areas. Breeding sites usually consist of small, temporary, and brackish water bodies (Sindaco et al., 2006; Gasparri et al., 2013). Despite being less vulnerable to anthropogenic environmental changes than other anurans, the Balearic green toad is included in Annex IV of the Habitats Directive.

Freshwater habitats where both species deposit their egg-clutches can be widely contaminated by MPs (Cera et al., 2020), especially in the proximity of urban areas (McCormick et al., 2016).

To mimic field conditions, both Italian agile frog and Balearic green toad tadpoles were exposed to three different environmental concentrations of a MP mix composed of four of the most common polymers (PVC, PS, High-Density PE and PES). We expected a reduction in activity, and therefore, foraging and growth of tadpoles exposed to increasing MP concentrations. We also expected *B. balearicus* to perform better than *R. latastei* under stressful conditions, due to its higher tolerance towards anthropic pressures.

2. Materials and methods

2.1. Animal collection

In February 2021, we collected 20 fragments of Italian agile frog egg clutches from three ponds located inside a natural protected area (Bosco del Vignolo, 45°13′N, 8°56′E; Lombardy, N Italy). Maximum water depth was 1 m, with moderate turbidity and low (<10%) aquatic vegetation cover. In April 2021, 20 fragments of green toad egg strings were collected from three artificial fountains located inside the Botanical Garden of Pavia (45°11′N, 9°10′E; Lombardy, N Italy); water depth was 20 cm with high turbidity and no vegetation cover. Animal collection, husbandry and experiments were authorized by ISPRA (Prot. 1790 of January 18, 2021).

Both egg clutches and string fragments were immediately brought to the laboratory and kept until hatching in ten 21 L rearing tanks (2 clutches or strings per tank), filled with dechlorinated tap water.

2.2. Production of MPs

We generated an environmentally relevant MP mixture using common plastic objects made of four different synthetic polymers: expanded polystyrene (PS) from black foam food tray, high-density polyethylene (HDPE) from red bottle caps, polyester fibers (PES) from blue-colored synthetic fabrics, and polyvinyl chloride (PVC) from common orange pipes for building. PS, HDPE, PES, and PVC had the following densities: 0.028–0.048, 0.94, 1.38, and 1.3–1.45 g cm^{-3}, respectively. HDPE, PVC and PS were fragmented into smaller pieces using a metal pincer, plunged into a bath of liquid nitrogen and subsequently cryomilled using a Fritsch Pulverisette 11 mill, at a speed of 10 000 rpm for 30 s. PES fibers from synthetic fabrics were generated by manually cutting a blue polyester t-shirt and collecting fibers from the exhaust filter of a dryer machine after the drying process of a mixture of synthetic fabrics.

We filtered the obtained materials on cellulose membranes and assessed the size of MPs (maximum diameter or length for powders and fibers, respectively), using a Leica EZ 4D stereomicroscope equipped with a digital camera and ImageJ (Fig. S1). Mean size was 0.75 mm (SD 0.36) for HDPE fragments ($n = 59$), 0.28 mm (SD 0.24) for PVC ($n = 105$) and 0.59 mm (SD 0.40 for PS ($n = 85$). Mean length of PES fibres ($n = 60$) was 1.01 mm (SD 0.73) (Fig. S1).

We then pooled powders and fibers to obtain a mixture of MPs in three concentrations: 1, 7, and 50 mg L^{-1}; the lowest was consistent with mean concentrations at the outlet of wastewater treatment plants (6400 MP m^{-3}, Schmidt et al., 2020; assuming spherical MPs with a mean diameter of 700 μm and a density of 1 g cm^{-3}, 6400 MP m^{-3} correspond to 1.15 mg L^{-1}), while the other two concentrations (7 and 50 mg L^{-1}) followed a geometrical increase and were tested as worst-case scenarios based on the highest concentration (450 000 MP m^{-3}) reported by Schmidt et al. (2020). The highest concentration was also consistent with previous toxicity studies (Besseling et al., 2014; Araújo et al., 2020a).

To prevent low-density polymers from being overrepresented in terms of particle numbers, for each concentration we weighed the MPs using a high-precision scale and metal cutlery as to obtain the following constant weight-ratio: 3 PVC: 3 HDPE: 3 PES: 1 PS.

2.3. Experimental procedure

To test for the effects of MP exposure on tadpole growth, behaviour and survival, we performed two experimental trials, one using Italian agile frog tadpoles and one using Balearic green toad tadpoles. Each trial consisted of 24 tanks (38 × 28 × 20 cm), filled with aged well water, positioned in groups of four on six tables (blocks) in the same room, with natural light and air temperature conditions. Following a randomized and balanced design, we assigned MP treatments to tanks so that each block included one replicate for each of the three densities to test (1, 7, and 50 mg L^{-1}) and one control (0 mg L^{-1}), with 6 replicates per treatment. During trials, we recorded water temperature twice every day, at 10am and 4pm, using a digital thermometer, and provided a fixed quantity of rabbit chow (60 mg, i.e. ca.15% of the wet mass of tadpoles at the start of the trials) to each tank every day.

We recorded mortality rates by carefully inspecting all tanks twice a day. To assess the number and type of ingested MPs, dead tadpoles were collected separately for each tank and frozen until analysis. We assessed the lethal concentration expected to cause 50% mortality in the tested agile frog population (LC50) at 240 h by plotting mortality vs. MP density and Log-probit regression. MP concentrations causing 10% and

90% mortality were also reported.

To assess the activity levels of tadpoles belonging to different treatments, tadpoles were recorded for ten consecutive minutes using a digital camera (Olympus Tough TG-5) hung up 1 m above each block. We assessed the proportion of active (swimming or foraging) tadpoles every second within a 10-s interval each minute (10 s × 10 min = 100 observations per tank). Activity was recorded five times per trial.

2.3.1. *Trial I*

One week after hatching, 480 *R. latastei* tadpoles were moved into the 24 experimental tanks (20 tadpoles per replicate). A further sample of 30 tadpoles was used to assess mean ± SD wet-weight (20 mg, SD 1) and stage (27.1, SD 0.65; min-max: 26–28, following Gosner, 1960) at the start of the trial. Mean water temperature was 16.8 °C (SD 0.7, min-max: 16.0–18.0) throughout the trial period (from 17 to March 24, 2021).

At the end of the trial, we wet-weighed (precision of 0.01 mg) all survived tadpoles from both the medium and high-density treatments and 60 randomly chosen tadpoles from low density and control treatments (10 tadpoles per tank). To assess their capacity to recover, we moved all survived specimens from the highest MP exposure and as many randomly selected individuals from the other treatments into four 21 L tanks filled with 8 L of aged well water (one tank per treatment). During the two successive weeks we fed tadpoles with rabbit chow and recorded mortality rates.

2.3.2. *Trial II*

One week after hatching, 480 *B. balearicus* tadpoles were moved into experimental tanks (20 tadpoles per replicate). We assessed mean Gosner's stage (27, SD 0.75; min-max: 26–28) and weight (21 mg, SD 2.5) were assessed following the same protocol used for trial I. Tadpoles were exposed to MP treatments for one week, from 13 to May 20, 2021. During the trial, mean water temperature was 20.7 °C (SD 0.3, min-max: 20–21.4). At the end of the trial each tadpole was moved from its tank to a numbered (from 1 to 20), white plastic cup. Then, we used a Random Integer Set Generator to select and weight half of the tadpoles (for a total of 240 tadpoles).

2.4. *MP extraction from dead tadpoles*

To remove the organic content and extract the MPs, dead *R. latastei* tadpoles from trial I were chemically digested using hydrogen peroxide (30% H_2O_2). In total, we collected 53 daily samples, out of which 30 (from different days, blocks and treatments; Table S1) were processed for MP analysis. Each sample included between 1 and 10 tadpoles, depending on daily mortality rates. Samples were placed in beakers, filled with 20 mL of H_2O_2, for 1 h at 50 °C and overnight at room temperature. Using a glass filtration apparatus, we then filtered the solutions on a cellulose membrane (pore size: 0.45 μm; filtering area: 1193.985 mm^2). Both the beaker and reservoir flask of the filter were rinsed with filtered Milli-Q. Filter membranes were placed in a glass Petri dish with a closed lid and left to dry in a desiccator for 48 h. To assess the number and size range of ingested MPs, for each sample we took ten images at 20× magnification (19 228 mm^2, 1,6% of filtering area) and one at 8× magnification (119 983 mm^2, 10% of filtering area). We used the latter for PVC and HDPE MPs, while PS and PES items were analyzed using 20x images. The total number of MPs was then extrapolated for the whole filtering area, except for 1 mg L^{-1} samples, for which, expected numbers of PVC and HDPE particles being low, whole membrane areas were inspected. Finally, for each polymer, we calculated the number of ingested MPs per tadpole by dividing the total number of MPs per the number of dead tadpoles in each daily sample.

2.5. *Statistical analysis*

We used linear mixed models (LMMs) to explore variation in both weight and activity levels. In each model, we included 'Treatment', 'species' and their interaction as fixed factors, and 'tank within block' as a random factor. We used Akaike's Information Criterion (AIC) to select the best model. For each experimental trial, comparisons among MP treatments were extracted from the models using "emmeans" package in R (Lenth et al., 2018). Degrees-of-freedom were calculated using Kenward and Roger's method (1997), while Tukey's test was used for post-hoc comparisons. Mortality rates were compared by Kruskal-Wallis' test, using Mann-Whitney tests for post-hoc comparisons. Residual plots were inspected to check model assumptions. To assess treatment-related variation in the number of ingested MPs per dead tadpole of *Rana latastei*, the normality of variables (size distribution of each polymer) was tested using Shapiro-Wilk's normality test. We applied one-way ANOVA (followed by Tukey's post hoc test for multiple comparisons) to test for normally distributed MPs (PVC and HDPE), otherwise (PES and PS) data were tested by Kruskal-Wallis' test (followed by Dunn's pairwise test). We calculated Bonferroni's confidence intervals for the proportion of use (White and Garrot, 1990) to compare the frequency of use of each size class with its availability in the experimental tanks. Statistical analyses were conducted using R (version 3.2.1; R Core Development R Development Core Team, 2013) and lme4 package (Bates, 2010).

3. Results

Italian agile frog tadpoles were highly affected by exposure to MPs, showing significant negative effects in terms of growth, activity, and survival even at low concentrations, while Balearic green toad tadpoles did not.

The models (LMMs) showed that final weights were significantly affected by both treatment and species (treatment x species: $F_{3,\ 34} = 10.44$, $p < 0.001$; Table 1). The weight of frog tadpoles decreased with MP density (Fig. 1), while none of the treatments differed significantly from controls for toad tadpoles. However, we recorded a slight increase in final weight for both low and medium MP concentrations (Table 1, Fig. 1).

Activity levels varied among treatments and species (treatment x species: $F_{3,\ 30} = 3.9$, $p = 0.018$; Table 2). Activity levels of frog tadpoles exposed to any MP density (treatment) were significantly lower than controls (Fig. 2), while in toad tadpoles a reduction in activity level was recorded only for those exposed to the highest MP density (Table 2, Fig. 2).

Mortality occurred from day 3. Mean mortality rates of frog tadpoles at the end of the experimental period increased with MP concentration (control: 1.67%, SD 1.0; 1 mg L^{-1}: 20.8%, SD 5.5; 7 mg L^{-1}: 63.3%, SD 7.8; 50 mg L^{-1}: 69.2%, SD 4.2), differing significantly between any treatment and controls (Kruskal-Wallis' $\chi^2 = 18.74$, 3 df, $p < 0.001$; $p < 0.01$ for all paired tests). After the recovery period, mortality rate was null in the control tank, while reached 1.94%, 61.11%, and 97.22% in tadpoles from the 1 mg L^{-1}, 7 mg L^{-1}, and 50 mg L^{-1} treatments, respectively. LC_{50} at 10 days was assessed as 2.5 mg L^{-1} ($p < 0.001$; LC_{10} - $LC_{90} = 0.5$–12.6 mg L^{-1}).

In toad tadpoles, mortality rate was null in control and low-density tanks, while it reached 2.5% and 6.7% in the 7 and 50 mg L^{-1} treatment groups, respectively.

The number of ingested MPs per dead Italian agile frog tadpole was recorded for different treatments, replicates and dates (Table S1). The smallest, lethal mean intake was 253 MPs (1 mg L^{-1}) while the highest was 760 MPs (50 mg L^{-1}), MPs mostly consisting of PES fibers (76% and 88%, respectively). The treatment did not affect the number of ingested HDPE and PS, while the number of ingested PVC particles increased with MP concentration ($F = 19.7$, 2 df, $p < 0.001$; Tukey's post hoc tests: 1–7 mg L^{-1}: $p = 0.047$; 1–50 mg L^{-1}: $p < 0.001$; 7–50 mg L^{-1}: $p = 0.006$), and the number of ingested PES particles differed significantly between the lowest and highest concentrations ($\chi^2 = 12.6$, 2 df, $p = 0.002$; Dunn's multiple comparison test: $Z = 3.54$, $p = 0.001$; Fig. 3).

Table 1
Estimated means from LMMs for tadpole weight after exposure to four MP treatments and comparisons for all treatment pairs (N = number of weighted tadpoles.

	Rana latastei				Bufotes balearicus			
Treatment	mean (N)	df	Lower CL	Upper CL	mean (N)	df	Lower CL	Upper CL
0	69.3 (60)	35	60.2	78.4	84.3 (120)	35	75.2	93.5
1	58.4 (60)	35	49.3	67.5	91.0 (120)	35	81.9	100.1
7	32.5(49)	43.4	22.8	22.8	90.4 (120)	35	81.3	99.5
50	20.9 (47)	45.9	11.2	30.7	74.2 (120)	35	65.1	83.3
Contrast	**estimate**	**df**	**t ratio**	***p***	**estimate**	**df**	**t ratio**	***p***
0-1	10.95	32.6	1.82	0.28	−6.65	32.6	−1.11	0.68
0-7	36.82	36.9	5.89	<0.001	−6.05	32.6	−1.01	0.74
0-50	48.37	38.1	7.69	<0.001	10.12	32.6	1.69	0.35
1-7	25.87	36.9	4.14	0.001	0.60	32.6	0.10	0.99
1-50	37.42	38.1	5.95	<0.001	16.77	32.6	2.79	0.04
7-50	11.55	42.2	1.77	0.3	16.17	32.6	2.69	0.05

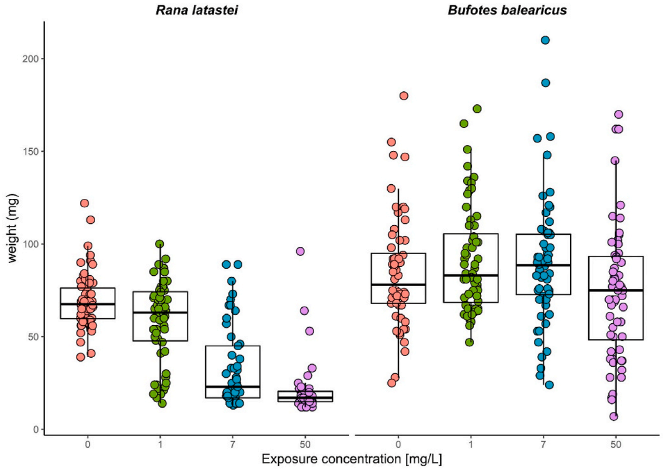

Fig. 1. Final tadpole weights for each of the four MP concentrations (0, 1, 7, 50 mg L^{-1}).

Excluding PES fibers, 50% of ingested MPs were in the size range of 80–245 μm. Mean fragment size was 0.097 mm (SD 0.06) for PS (n = 120), 0.27 mm (SD 0.12) for HDPE (n = 36), 0.24 mm (SD 0.11) for PVC (n = 121); mean length of ingested PES fibres (n = 121) was 0.51 mm (SD 0.36). The smallest ingested MP particles were less than 0.2 mm, while the largest fragment was 0.853 mm; the longest ingested fiber was 1.65 mm. Intake frequency tended to decrease with increasing MP size (Fig. 4); in general, tadpoles selected particles in the range 0.2–0.5 mm, while fibers shorter than 0.2 mm and between 0.5 and 0.7 mm were ingested more than expected based on their availability (Fig. 4).

4. Discussion

Anuran tadpoles usually eat almost continuously, needing a constant food intake, which is pumped with water through the oral cavity and then sorted by size to be channelled into the esophagus either directly or through the pharynx (Wells, 2007). Food intake is based on random searching, which exposes tadpoles to the ingestion of a wide variety of non-food particles (Kinne et al., 2004; Savage, 2009). Moreover, MPs are in the same size range as plankton, increasing the risk for unintentional ingestion (Browne et al., 2007).

Despite being the same size and stage and raised using the same protocol, Italian agile frog and Balearic green toad tadpoles behaved and

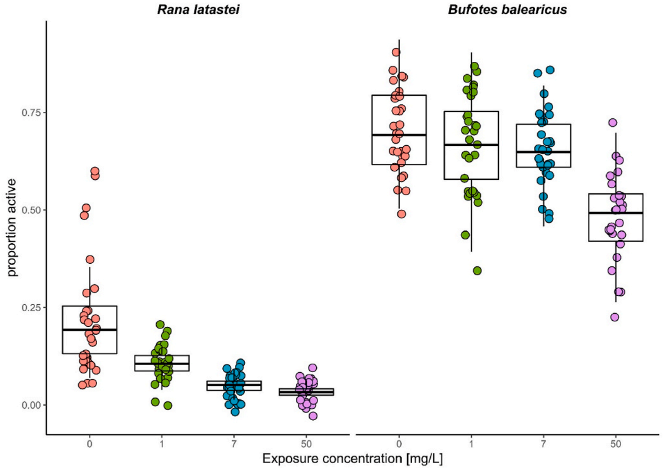

Fig. 2. Tadpole activity levels for each of the four MP concentrations (0, 1, 7, 50 mg L^{-1}).

Table 2
Estimated means from LMMs for tadpole activity level after exposure to four MP treatments and comparisons for all treatment pairs.

Treatment	*Rana latastei*				*Bufotes balearicus*			
	mean	df	Lower CL	Upper CL	mean	df	Lower CL	Upper CL
0	0.22	39.2	0.17	0.28	0.70	43.3	0.65	0.76
1	0.11	39.2	0.05	0.17	0.66	39.2	0.61	0.72
7	0.05	39.2	−0.004	0.1	0.66	39.2	0.60	0.71
50	0.03	39.2	−0.02	0.09	0.48	39.2	0.42	0.53
Contrast	**estimate**	**df**	**t ratio**	***p***	**estimate**	**df**	**t ratio**	***p***
0–1	0.11	30.2	3.07	0.02	0.036	31.9	0.98	0.76
0–7	0.17	30.2	4.65	<0.001	0.045	31.9	1.23	0.61
0–50	0.19	30.2	5.08	<0.001	0.222	31.9	6.07	<0.001
1–7	0.06	30.2	1.58	0.4	0.009	30.2	0.24	0.99
1–50	0.07	30.2	2.02	0.2	0.186	30.2	5.02	<0.001
7–50	0.02	30.2	0.43	0.97	0.177	30.2	4.78	<0.001

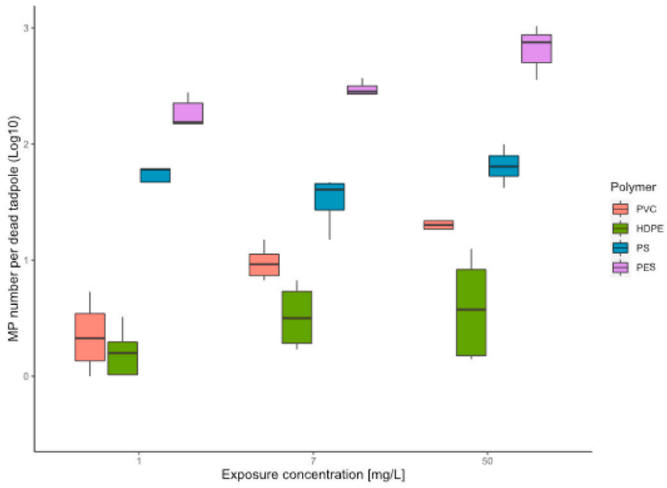

Fig. 3. Ingested MPs per dead Italian agile frog tadpole for the three exposure concentrations (1, 7, 50 mg L^{-1}). MP numbers are Log10 transformed for visual purposes.

were consequently affected by MP exposure in sharply different ways. As recorded for common goby (*Pomatoschistus microps*) juveniles (de Sà et al., 2015), frog tadpoles actively fed on MPs, whilst toad tadpoles gathered around edible food, apparently ignoring MPs. Hence, interspecific variation in the effects of MP exposure seemed to depend on differential attractiveness and uptake rather than efficacy in the egestion of MPs.

The size of mouth opening may be excluded as a cause of variation in feeding behavior, as toad tadpoles are macrophagous, while *Rana* species usually ingest smaller particles (Savage, 1950). Knowledge is still insufficient to understand whether variation in other mouth features, such as the pattern of marginal papillae, which should have tactile and chemosensory functions and thus may control the flow of food particles towards the mouth (Altig and McDiarmid, 1999), may involve more efficient discrimination of edible particles by bufonid larvae (Bonacci et al., 2008). The two patterns may also depend on interspecific differences in behavioral plasticity (Luniak, 2004), with such a typical forest-dwelling species as the Italian agile frog being less able than habitat-generalist green toads to cope with polluted habitats and avoid anthropogenic materials, a hypothesis which is worthy of further testing.

Intake by Italian agile frog tadpoles increased with MP concentration. Mortality rates were by far higher than expected, increasing exponentially since the third day of exposure and with the lowest number of survivors for the highest treatment. Although available data for several taxa show that in freshwater habitats the number of MPs per individual is usually lower (ranging between 0.2 and 24; Hu et al., 2018) than that suspected to be lethal for anuran larvae (>200), our data demonstrate that short-term exposure to low, environmentally relevant MP concentrations can affect tadpole growth and survival.

Impaired growth indicated that MPs altered the feeding efficiency of tadpoles, either causing direct damage (Araújo et al., 2020a; 2020b), or inducing satiety, as suggested by the lowering of activity levels with MP density. The post-treatment phase allowed us to observe that the effects of MP entanglement are little reversible, suggesting that even short-term exposure to high MP concentrations may severely affect the reproductive success of agile frogs.

The highest MP numbers in dead tadpoles were recorded for the smallest PS (<0.2 mm) fragments and PES fibers up to 1.7 mm long, suggesting that particle size, morphology or color may affect MP intake. Fibers are the most spread MPs in freshwaters (Hu et al., 2018), where more than 100 fibers per liter of effluent were recorded (Browne et al., 2011). Elongated PES fibers may be mistaken with aquatic plants and their morphology may enhance the ingestion of particles longer than the average recorded for the other tested polymers.

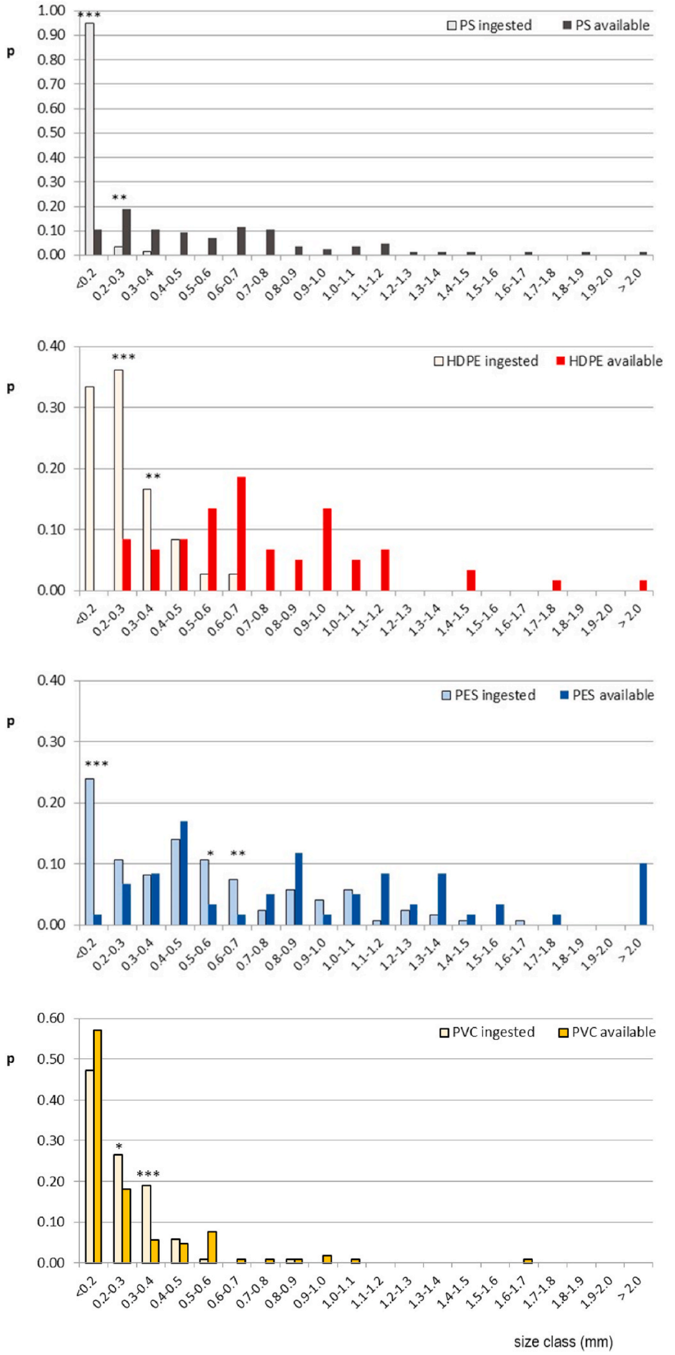

Fig. 4. Percentage size distribution of MPs ingested by Rana latastei (use) vs. MP availability for all tested polymers (Bonferroni's confidence intervals for the proportion of use; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

The long intestine of anuran tadpoles (>10 times their body length) forms a tight double spiral (Pretty et al., 1995), the *ansae* of which may favor the entanglement of fibers. PES fibers have been demonstrated to adhere to the intestinal epithelium of *X. laevis* tadpoles (Bacchetta et al., 2021), forming "balls" which are likely to affect gut functions. As a benchmark, the highest number of fibers recorded in a dead Italian agile frog tadpole corresponded to a total length (ca. 340 mm) which was assessed to be more than twice the length of its intestine.

The pattern of mortality in Balearic green toad tadpoles was by far less dramatic and consistent with the previous study by Boyero et al.

(2019), who reported high (7 out of 8 tested individuals) mortality in common midwife toad tadpoles only when exposed to high MP density (1800 MP mL^{-1}). Dead Balearic toad tadpoles were not analyzed, but visual inspection using a stereomicroscope confirmed the ingestion of blue fibers, suggesting that PES played a major role as a cause of mortality also for this species.

5. Conclusions

With the aim of addressing the potential adverse effects of waterborne MP on freshwater species, we tested two amphibian species under realistic contamination conditions. We showed that adverse effects of MP depended on the species and that MP intake varied depending on MP characteristics such as density, size, shape and color. Our results suggest that MP pollution may contribute to the decline of vulnerable anuran species, highlighting the need for further studies on the effects of these pollutants on the reproductive success of amphibian populations.

To point out the actual threat posed by these pollutants, we stress the need to test a variety of polymers, as to mimic environmental conditions and assess shapes and sizes that are more prone to be mistaken with food by anuran larvae. Concentrations being equal, MP availability or attractiveness to tadpoles may differ between laboratory and natural conditions. Mesocosms may be used to bridge this gap between the laboratory and real ecosystems.

Amphibian populations face many threats, which, acting synergistically, have been imputed of their worldwide decline. We note that the role that most factors play in affecting amphibian abundance and diversity in relatively undisturbed areas such as those usually exploited by Italian agile frogs is still poorly understood. Therefore, we suggest that future studies should not focus only on highly polluted habitats.

Author contribution

A. Balestrieri: Conceptualization, Investigation, Writing - original draft; A. Winkler: Investigation, Methodology; G. Scribano: Investigation, Methodology, Data curation; A. Gazzola: Conceptualization, Writing - original draft; G. Lastrico: Investigation; A. Grioni: Investigation; D. Pellitteri-Rosa: Project administration, Supervision, Writing - review & editing; P. Tremolada: Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

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

Acknowledgements

We thank F. Pistoja (Bosco del Vignolo), F. Bracco, N. Ardenghi and P. Cauzzi (Botanical Garden of Pavia) for giving us access to breeding sites for the collection of egg clutches. We also thank S. Camazzola (Department of Chemistry of the University Milan) for providing us with cryo-milled polymer particles. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.119137.

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