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Original Article

Results of the first analysis of tadpoles' diet and determination of microplastics presence of *Rana*, *Bufo* and *Bufotes* species from different localities in Serbia

Glorija Ćirković
Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, Kragujevac, Serbia
glorija.cirkovic@pmf.kg.ac.rs (corresponding author)

Aleksandra Rakonjac
Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, Kragujevac, Serbia

Rastko Ajtić
Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, Kragujevac, Serbia

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Abstract:

Nutrition is one of the main processes in living beings because it provides energy. Amphibians are an essential link in food chains, but they have not been sufficiently studied. It is believed that most anuran larvae and tadpoles are omnivorous, but the trophic status of many tadpoles has not been precisely determined. Microplastics (MPs) are one of the major threats to aquatic ecosystems and can be ingested by various groups of aquatic organisms, such as plankton, shellfish, fish, and amphibians, but current knowledge of MPs occurrence in these aquatic organisms is still scarce. In this research, the intestinal contents of tadpoles of four anuran species were analyzed to assess nutrition and the presence of microplastics. The results show that the largest percentage of tadpoles' diet consists of diatoms from the genera *Achnanthidium* and *Navicula,* and detritus. The remains of some invertebrates were also present in the material. Microplastic analysis proved the presence of MPs in all investigated samples. The data obtained in this research represent the first concrete results due to which it is necessary to continue extensive research at several levels.

Key words:
tadpoles, nutrition, microplastics, *Rana*, *Bufo*, *Bufotes*

Apstrakt:

Rezultati prve analize ishrane punoglavaca i utvrđivanje prisustva mikroplastike kod vrsta iz rodova *Rana*, *Bufo* i *Bufotes* sa različitih lokaliteta u Srbiji

Ishrana je jedan od ključnih procesa živih bića jer predstavlja glavni izvor energije. Vodozemci su važna karika u lancima ishrane, ali nisu dovoljno proučavani. Veruje se da je većina larvi bezrepih vodozemaca, odnosno punoglavaca, omnivorna, ali trofički status mnogih punoglavaca nije precizno utvrđen. Mikroplastika (MP) je jedan od glavnih zagađivača u vodenim ekosistemima pa je različite grupe vodenih organizama, kao što su plankton, školjke, ribe i vodozemci mogu uneti u organizam, ali trenutno znanje o prisustvu MP u ovim vodenim organizmima je još uvek nedovoljno. U ovom istraživanju analiziran je sadržaj creva punoglavaca četiri vrste bezrepih vodozemaca kako bi se procenila ishrana i prisustvo mikroplastike. Rezultati pokazuju da najveći procenat ishrane punoglavaca čine silikatne alge iz rodova *Achnanthidium* i *Navicula*, kao i detritus. Takođe, u sadržaju creva bili su prisutni i ostaci nekih beskičmenjaka. Analize mikroplastike dokazale su da su čestice MP prisutne u svim ispitivanim uzorcima. Podaci dobijeni u ovom istraživanju predstavljaju prve konkretne rezultate zbog kojih je neophodno nastaviti dodatna istraživanja na više nivoa

Ključne reči:
punoglavci, ishrana, mikroplastika, *Rana*, *Bufo*, *Bufotes*

Introduction

Knowing a species' trophic status and spectrum of nutrition is of particular importance, given that food is the primary link between the species and its habitat. Nutrition is an ongoing process and therefore can be crucial to the ecology of a particular species. Also, feeding is the most important activity of living beings, primarily because of obtaining the energy needed to perform life activities. Amphibians are vertebrates that can be at the top of food chains, although they are more often found in the middle of the food chains and form the link between lower taxa, such as insects, and higher ones, such as birds and mammals (Cogălniceanu et al., 2000). They are a very important link in food chains because, due to their life history, they connect aquatic and terrestrial ecosystems. Adults are, in most cases, carnivores. They feed on most invertebrates, and on some vertebrates. In contrast to adults, larvae of tailed and anuran amphibians show much greater variability in diet types. Some feed on plankton, some are microphagous, herbivorous, or detritivorous, and there are also predatory larvae (McDiarmid & Altig, 2010). Even though they are very important, they have been insufficiently investigated and the trophic status of many species of amphibians, both larvae and adults, has not been sufficiently studied.

In the literature related to the Balkan Peninsula, there is very little information about the diet of representatives of the order Anura (Popović et al., 1992; Simić et al., 1992; Bjelić et al., 1996; Paunović, 2000; Paunović et al., 2010; Paunović, 2011; Crnobrnja-Isailović et al., 2012; Čađenović, 2014), and the diet and morphology of the larval anuran, i.e. tadpoles, have been particularly poorly researched (Sidorovska et al., 2002; Ilić, 2020).

Most aquatic tadpoles are primary consumers that metamorphose into terrestrial adults, thereby transferring energy from aquatic habitats to food chains in terrestrial ecosystems (Trakimas et al., 2011). Because of their different habitats and the variety of life forms and feeding structures, different species of anuran amphibian larvae use several food sources including periphyton and detritus (Ranvestel et al., 2004).

Many tadpoles are benthic in their habitats, spending most of their time at the bottom of ponds, and relatively little in free water, so the composition of the benthos in the aquatic ecosystems they inhabit is important for their nutrition. Tadpoles from the Bufonidae and Ranidae families are, among others, with these characteristics. However, some species from these families sometimes, except for benthic organisms, feed on organisms from the surface of the water (bacterial film) (*Rana dalmatina*), scraping periphyton, corpses, and eggs of the same or other species or plankton (*Rana temporaria*, *Bufo bufo*) (Wells, 2007).

Species *R. dalmatina*, *R. temporaria* and *B. bufo* in Serbia can be syntopic. They enter aquatic habitats only during a short reproductive period in early spring, where tadpoles develop after hatching from eggs. The species *R. dalmatina*, *B. bufo* and *Bufotes viridis* inhabit the entire territory of Serbia, while the species *R. temporaria* is found on 10-50% of the entire territory of Serbia (Vukov et al., 2013).

The increased use of plastic products in human activities has caused concern for the environment. When plastic reaches (directly or indirectly) aquatic ecosystems, it undergoes degradation, after which there is a change in particle size and density. However, most plastic products are very resistant to biochemical degradation and therefore persist for a long time in the environment. Over time, they break down into smaller and smaller fractions called microplastics (from 1 to 5 μm in size) (Pastorino et al., 2022).

There are two types of microplastics: primary and secondary. The primary represents plastic particles that are produced and that enter the composition of various products (for example cosmetics), and the secondary is created by the decomposition of macroplastics or during the use and disposal of plastic products such as plastic packaging (Pastorino et al., 2022).

Microplastics (MPs) are one of the major threats to aquatic ecosystems and can be ingested by various groups of aquatic organisms, such as plankton, shellfish, fish, and amphibians, but current knowledge of MPs occurrence and impact on these aquatic organisms is still scarce (Hu et al., 2018; Kolenda et al., 2020). Also, despite the key role of amphibians in the food webs and the potential to transfer contaminants through trophic levels and from freshwater to terrestrial ecosystems, the data on their capacity to accumulate MPs are very scarce compared to other vertebrates (Pastorino et al., 2022). There are only a few published field studies about MPs accumulation by tadpoles (Hu et al., 2018; Karaoğlu & Gül, 2020; Kolenda et al., 2020).

The main goal of this research is to obtain results on the nutrition of tadpoles from different habitats in Serbia and thus supplement knowledge about the nutrition of anuran larvae and contribute to determining their trophic status. Another goal of this study was to determine whether tadpoles digest microplastics which can serve as a good initial step for further analyses of whether the MPs affect the development and metabolism of tadpoles.

Materials and Methods

Sampling

We used for the analysis 130 tadpoles of *Rana dalmatina, R. temporaria*, *Bufo bufo* and *Bufotes viridis* from 11 localities in Serbia (**Tab. 1**; **Fig. 1**). The tadpoles were collected using standard nets for amphibians and then preserved in alcohol. The collection was obtained during spring (April, May) 2021. and 2022. The 70 tadpoles were used to investigate tadpoles' diet, and 60 tadpoles

Table 1. Localities and a number of tadpoles used for intestinal content analysis, and developmental stages according to Gosner (1960)

Species	Localities	Number of specimens for nutrition analysis	Number of specimens for microplastic analysis	Developmental stages
Rana temporaria	Golijska River	10	20	34, 38
	Trešnjica River (Povlen)	5	10	25
	Srednji Povlen	5	/	29
Rana dalmatina	Medvednik	5	/	24,25
	Cer	5	5	25,38
	Golija	5	/	36
Bufo bufo	Tometino polje	5	/	37
	Cer	5	5	27
	Zlatibor	5	10	26
	Besna kobila	5	/	25
Bufotes viridis	Starac	10	10	28
	Zasavica	5	/	34

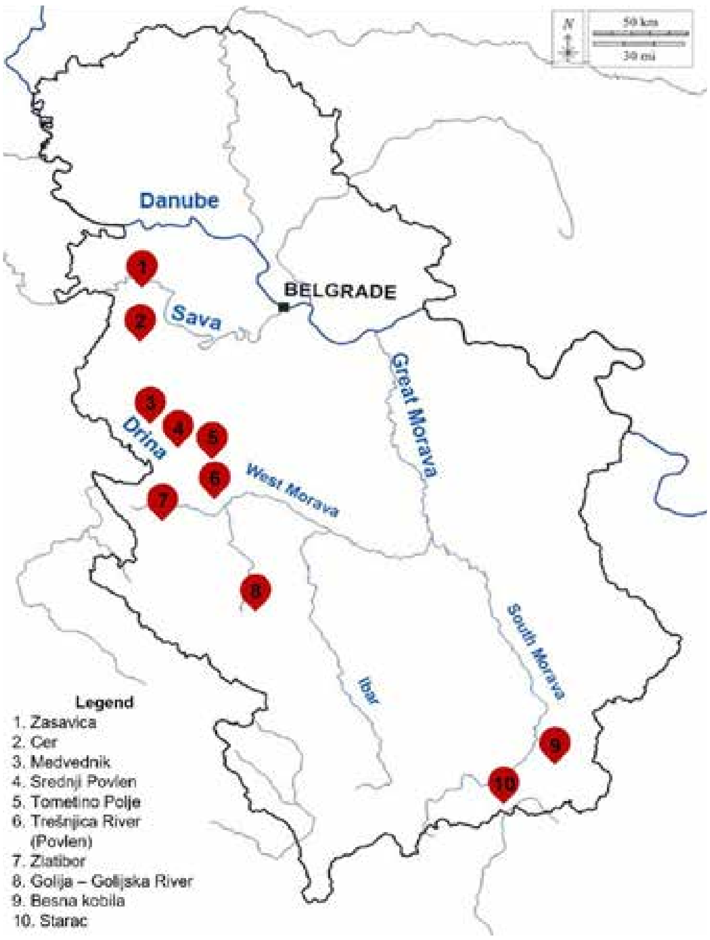

Fig. 1. Localities

were analyzed to estimate the presence of microplastics (**Tab. 1**). The selected four species for this study were the most adequate for analysis because they were the most abundant in investigated localities. For future research, we plan to include other species and more localities.

The tadpoles were from different developmental stages (most of them were 25 and 27), according to Gosner's simplified table for staging anuran embryos and larvae (Gosner, 1960).

Determination of species

Tadpoles can be identified using the details of the overall body proportions, but the position of the spiraculum and the arrangement of tooth rows and other mouthpart details allow a confident identification to be made. For the identification of the tadpole samples, we used an identification key (Ajtić & Ćirković, in prep.) based on the specific arrangement of tooth rows and other mouthpart details.

Nutrition analysis

To assess nutrition, intestinal contents were analyzed. A whole intestine was removed from each tadpole and contents were placed in ethanol (96%) and homogenized. Depending on the abundance of particles, either the entire sample or a subsample was observed under the microscope at

800x magnification. The observation was made by transects, photographed and, if possible, identified.

Algae were identified to the genera level using standard identification keys (Krammer & Lange-Bertalot, 1986, 1988, 1991; John et al., 2011; Jüttner et al., 2015; Wehr et al., 2015).

Microplastics analysis

The presence of microplastics was analyzed using the method described by Hu et al. (2018) with some modifications.

The specific number of tadpoles from each investigated species was separated in glass Petri dishes. Then, they were washed three times with distilled water and digested using H_2O_2 (30%) for no more than 72 h. The digested solutions were filtered through 47 mm diameter polycarbonate filters with a pore size of 5 µm. All filters were incubated at 60 °C for 4 h in a dryer and stored in dry glass Petri dishes until further observation. MPs were then searched for by means of a stereomicroscope.

To avoid contamination, a cotton laboratory coat was worn during procedures, and before analysis, all liquids (pure water and H_2O_2) were filtered using filter paper (5 µm porosity), and all the equipment used during analysis was rinsed in distilled water. The procedural blanks were performed to assess whether the materials, solutions, equipment, or workspace were contaminated with microplastics.

Results and discussion

The intestinal content analysis confirmed that the most significant percent of tadpoles' nutrition were diatoms, i.e., algae from class Bacillariophyceae, and the most present were specimens from the genera *Achnanthidium* and *Navicula*, and in a smaller amount, specimens from the genera *Surirella, Pinnularia,* and *Nitzschia* (**Tab. 2**). This may be a consequence of the period in which the tadpoles were feeding, as not all algae develop at the same time. Their development depends on the season, the physical properties of substrata, and different environmental factors (Murdock & Dodds, 2007). The presence of algae from Cl. Xanthophyceae, especially genus *Tribonema* was also noted. In some intestinal content, the representatives from the genus *Phacus* (Cl. Euglenophyceae, Phylum Euglenozoa; specimens from Srednji Povlen) (**Tab. 2**) were found as well. Detritus was also found in intestinal content, as well as the remains of invertebrates that could not be determined.

Our results showing that the diet of tadpoles is not limited to algae is consistent with other studies (Altig et al., 2007), where it was reported that tadpoles consume a range of different particles in their diet, including benthic or planktonic algae, vegetation, bacteria, fungi, zooplankton, and animal flesh, as well as inorganic particles, such as sand. It is assumed that the diverse diet of most tadpoles actually reflects an indiscriminate diet. The type and dynamics of tadpole nutrition are related to the structure and morphology of the oral apparatus, but also to the availability and diversity of nutritional resources in the habitats where metamorphosis takes place. A smaller number of studies (Peterson & Boulton, 1999) have focused on tadpoles that feed on algae by scraping from the substrate, but herbivorous tadpoles have been shown to alter the structure and/or biomass of algae in permanent lentic aquatic ecosystems. Some researchers claim that the contents of tadpoles' guts represent an inexhaustible sample of algae (Huang et al., 2003; Skelly & Golon, 2003), which is confirmed by our results.

It is believed that tadpoles, many of which are primary consumers, greatly influence on the structure and functioning of ecosystems by changing the composition of algae, i.e. the composition of primary producers, as well as on the dynamics of organic matter in many aquatic habitats. Previous research has shown that primary production, nutrient cycling, plant residue decomposition, and invertebrate populations change when tadpoles are removed from habitats or reduced in numbers (Whiles et al., 2006).

The substrate in aquatic ecosystems is a significant source of food for tadpoles. The tadpoles use organic matter from detritus in their nutrition, benthic algae from phyla Bacillariophyta and Chlorophyta, and remains of all other algae, invertebrates, and vertebrates. This refers to tadpoles of many species in the families Bufonidae, Discoglossidae, Hylidae, Leptodactylidae, Megophryidae, Myobatrachidae, Pelobatidae, Ranidae, and Rhacophoridae (Wells, 2007). The results of the Skelly & Golon (2003) study confirm that tadpoles are affected by variations in the benthic particles as a food source. The same authors showed that tadpoles are nevertheless selective when it comes to nutrition and that the variety and availability of nutrient sources in and between aquatic habitats significantly impact their development.

In Serbia, there are no studies of microplastics' influence on anurans, especially not on their larvae. There is some information about the presence of MPs in some fish species (Nikolić et al., 2022) which suggests that there are particles of MPs in our aquatic ecosystems and that's why we could assume that it can also be found in tadpoles because they are extremely sensitive to changes in the environment. After examination of the remains of tadpoles used for microplastic analyses, it was confirmed that

Table 2. Algae composition in tadpoles' intestine

	Bufo bufo				*Bufotes viridis*		*Rana dalmatina*			*Rana temporaria*		
	Cer	Tometino polje	Besna kobila	Zlatibor	Starac	Zasavica	Medvednik	Cer	Golija	Golijska River	Trešnjica River (Povlen)	Srednji Povlen
Cl. Bacillariophyceae												
Achnanthidium	+++	+++	+++	++	++	+++	+++	+++	+++	+++	+++	-
Navicula	+++	+++	-	-	+	+++	+++	+++	+++	+++	+++	+++
Melosira	+	-	-	-	-	+	-	-	-	-	++	-
Denticula	-	+++	-	-	-	-	-	-	-	++	-	-
Surirella	-	+	+	-	+	+	-	-	+	+	-	++
Nitzschia	++	-	+	-	+++	+	+	+	+	+	-	-
Pinnularia	++	+	+	-	+	+	+	+	+	+++	+	+++
Gomphonema	-	++	-	++	-	+	+	+	+	+	-	-
Ulnaria	-	-	+	-	-	+	+	+	+	+	-	-
Fragilaria	-	-	-	-	++	+	-	-	+	+	-	-
Hannaea	-	+	++	+	-	-	-	-	-	+	-	-
Cl. Chlorophyceae												
Microspora	-	-	-	-	+++	-	-	-	-	-	-	-
Cl. Xanthophyceae												
Tribonema	+	-	+++	+	+++	+++	+	+	+	-	+	++
Cl. Euglenophyceae												
Phacus	-	-	-	-	-	-	-	+	-	+	-	+++

$^{+++}$ present in each sample; $^{++}$ present in every sample, but in a smaller percentage; $^{+}$ present in less than 50% of samples; $^{-}$ not present the elements of microplastics were present in all investigated species. The MPs in most cases were fibrils in different colors (**Tab. 3, Fig. 2**). This finding provides novel insights into the accumulation of MPs in tadpoles from different localities in Serbia.

As early life stages of amphibians, tadpoles show extensive feeding activity that exposes them to MPs elements present in the aquatic environment (Hu et al., 2018). Our results match with the results of Da Costa Araújo et al. (2019) which have shown that MPs can be ingested by *Physalaemus cuvieri* (fam. Leptodactylidae) tadpoles. MPs can accumulate in their organs and cause morphological, cytotoxic, and mutagenic changes that may affect their health and development. Kolenda et al. (2020) study showed that tadpoles of pond-breeding anurans from Central Europe are exposed to MPs pollution. The same authors also suggest that tadpoles can be a significant vector of MPs transfer from aquatic to terrestrial ecosystems. Investigation of MPs in tadpoles of species *Pelophylax ridibundus* and *Rana macrocnemis* (fam. Ranidae) from Turkey confirmed that MPs particles are present in tadpoles (Karaoğlu & Gül, 2020). Pastorino et al. (2022) showed that MPs can be found in the digestive tract of adult specimens of *Rana temporaria*.

The presence of microplastics in tadpole samples from different localities in Serbia represents the first concrete results, so it is necessary to continue extensive research at several levels. For future analysis, we plan to include other species and more localities.

Tadpoles are extremely sensitive to changes in the environment and can give scientists valuable insight into how an ecosystem is functioning, and because they have an important role in food chains, many other animals are affected by them.

The investigation of MPs in amphibians is of critical importance due to their endangerment and decline, and the accumulation of MPs in amphibians can be a pathway for transfering these important pollutants between freshwater and terrestrial ecosystems (Pastorino et al., 2022).

Table 3. Results of microplastic analysis

Species	Localities	Number of specimens	Presence of microplastics	Characteristics of microplastics (shape, color)
Bufo bufo	Cer	5	+	black particles
Bufotes viridis	Starac	10	+	red filaments
Rana dalmatina	Cer	5	+	black filament
Rana temporaria	Golijska River	20	+	blue and red filaments
	Trešnjica (Povlen)	10	+	black filaments

⁺ present

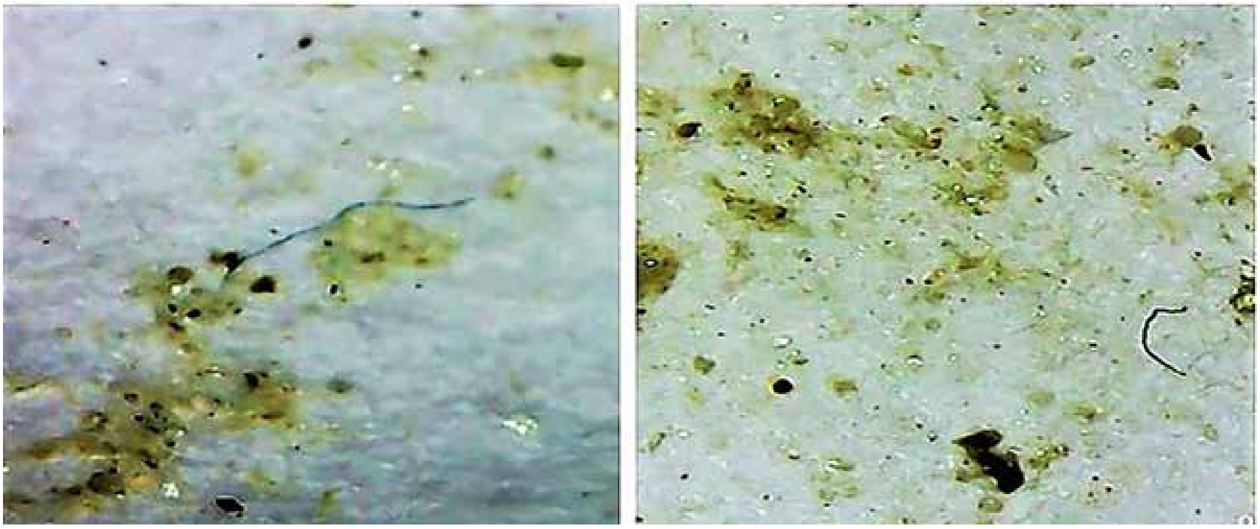

Fig. 2. Microplastic filaments in tadpole samples

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