



# Investigation of heavy metals in tissues and habitats of three edible frogs from Türkiye

Hale Tatlı<sup>1</sup> · Kenan Gedik<sup>2</sup> · Abdullah Altunışık<sup>1</sup>

Received: 11 August 2023 / Accepted: 20 November 2023 / Published online: 3 January 2024  
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2024

## Abstract

The soil, water, and organisms have been contaminated by heavy metals due to human activities and industrialization, which has produced a major environmental problem that has a deleterious effect on human health and food quality. Frogs, one of the good bioindicators for environmental pollution, are also among the alternative essential protein sources for humans. In Türkiye, three of these frogs are edible: *Pelophylax ridibundus*, *Pelophylax bedriagae*, and *Pelophylax caralitanus*, also known as Anatolian water frogs. Hence, to assess the possible health risks that might result from consuming frog legs in addition aquatic habitat of Anatolian water frogs, the water, sediment, and frog tissue samples (muscle and liver) were obtained from 11 different provinces covering all regions of Türkiye and analyzed to determine Cd, Cu, Cr, Zn, Pb, and As concentrations. The results revealed considerable variations in heavy metal concentrations among frog tissues, influenced by the sampling sites and species (ANOVA:  $p < 0.05$ ). The Estimated Daily Intake (EDI) values, calculated based on the average serving size, were also lower than the Provisional Tolerable Daily Intake (PTDI) levels for adult consumers. Furthermore, the study computed the Target Hazard Quotient (THQ) values for heavy metals, all of which were below the critical value of 1, indicating that consuming the hind leg muscles from these frog species would not pose an adverse health risk for humans.

**Keywords** Amphibian · Bioindicator · Metal · Pollution · Inland water

## Introduction

Heavy metals, the collective name for a group of metallic elements (e.g., mercury, lead, cadmium, chromium, etc.) known for their high density, atomic weight, and/or atomic number, are persistent pollutants released into aquatic and terrestrial environments through industrial discharges, agricultural runoff, and urban wastes. Due to the increase in anthropogenic activities and industrialization, soil, water,

and the atmosphere are exposed to heavy metal pollution, which creates a major environmental problem that affects human health and food quality (Wang et al. 2022). As these pollutants disperse in water, aquatic organisms absorb them and build up bioaccumulation in their tissues over time (Ali et al. 2019). Continuous exposure to heavy metals threatens not only the health and survival of individual organisms but also the integrity of entire aquatic ecosystems (Taiwo et al. 2014). Understanding how these heavy metals accumulate in organisms helps to assess their impact on the body (Rainbow 2018; Basaran 2022). Even at trace levels, heavy metals can be hazardous to most organisms because of the irreversible inhibition of certain enzymes (Henczová et al. 2008). Polluted water frequently contains combinations of multiple metals, significantly affecting aquatic organisms (Zeng et al. 2019; Fettweis et al. 2021). These heavy metals' interaction can influence their toxicity and accumulation (de Medeiros et al. 2020). Hence, studying how trace elements interact within tissues is essential, especially since their concentrations have risen in nature (Briffa et al. 2020).

The absorption of potentially toxic elements from the environment occurs in aquatic animals through their skin,

Responsible Editor: Philippe Garrigues

✉ Abdullah Altunışık  
abdullah.altunisik@erdogan.edu.tr

Hale Tatlı  
haticehale\_tatli22@erdogan.edu.tr

Kenan Gedik  
kenan.gedik@erdogan.edu.tr

<sup>1</sup> Faculty of Arts and Sciences, Department of Biology, Recep Tayyip Erdoğan University, Rize, Türkiye

<sup>2</sup> Vocational School of Technical Sciences, Recep Tayyip Erdoğan University, Rize, Türkiye

respiration, and nutrition. Because of their inability to break down naturally, metals tend to accumulate in the environment, leading to potential hazards for organisms as they enter the aquatic food chain (Al-Yousuf et al. 2000; Tao et al. 2012; Ali et al. 2019). Additionally, investigating the amount of metal in the tissues of consumed organisms is vital for evaluating their nutritional quality (Mani et al. 2022). Amphibians serve as vital bioindicators for monitoring water pollution (Simon et al. 2010; Şişman et al. 2015, 2021; Dönmez and Şişman 2021). Thanks to their permeable skin, which enables them to absorb substances from the environment rapidly (Hecnar 1995; Tatlı et al. 2022a), frogs can absorb heavy metals from their aquatic habitats, effectively accumulating these harmful substances in their bodies. Analyzing natural frog populations can offer valuable insights as reliable bioindicators (Lower and Kendall 1990; Vogiatzis and Loumbourdis 1998), shedding light on the persistent trend of metal buildup and pollution dynamics in aquatic ecosystems across an extended duration. This accumulation can lead to potential health risks for humans if the contaminated amphibians are ingested. Indeed, all amphibian species within the Ranidae family are suitable for human consumption, except for the frog species, which might be hazardous for humans (Neveu 2009). Frogs are recognized for their nutritional value, being low in fat and rich in protein and minerals (Özogul et al. 2008). Meat is one of the most basic foodstuffs for humans, and it is recommended to consume it from different sources to get the full benefit of its nutrients. However, due to the high consumption of red meat and its potential health implications when overindulged, researchers are now investigating alternative food sources (Şimşek et al. 2022). Frogs are emerging as a promising option, more affordable and safer for consumption than red meat (Stuart et al. 2004). The edible portions of frogs mainly consist of their legs, which offer a taste similar to chicken but with a slightly firmer muscle texture.

Research on the potential for heavy metal exposure from the consumption of frog legs has gained momentum in recent years (Borković-Mitić et al. 2016; Mani et al. 2022; Tatlı et al. 2022b; Zhelev et al. 2020). There are three edible frog species (*Pelophylax ridibundus*, *Pelophylax bedriagae*, and *Pelophylax caralitanus*) in Türkiye, and risk estimation for environmental pollution and human health by evaluating them together has not been explored in the literature up to now. Hence, this study aimed to analyze the concentration of heavy metals in the tissues (muscle and liver) and aquatic habitats (water and sediment) of three species of frogs belonging to the genus *Pelophylax*, with bioindicator properties, and also to evaluate if there are any health risks associated with the consumption of frog legs.

## Materials and method

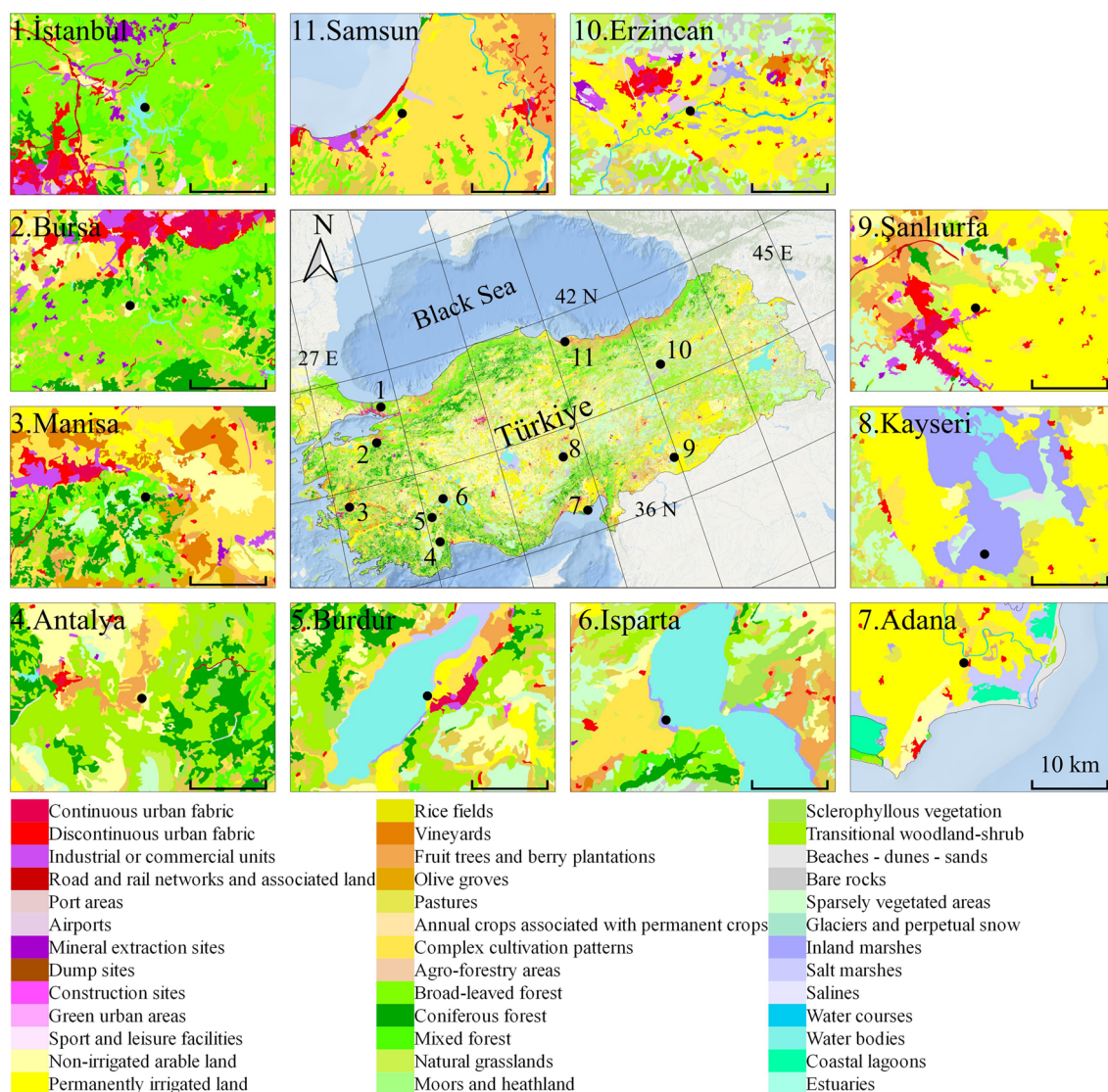
### Study area and sampling

The animal capture was approved with the permission of the local ethics committee of Recep Tayyip Erdogan University (Decision number: 2021/15). In this research, adult Anatolian water frogs (*Pelophylax ssp.*) were utilized, along with sediment and water samples from their natural habitats. Sampling was carried out between May and September 2022 at 11 different locations, namely Istanbul, Bursa, Manisa, Antalya, Burdur, Isparta, Adana, Kayseri, Şanlıurfa, Erzincan, and Samsun (Fig. 1), and the habitats' images were presented in Fig. 2. Also basic characteristics of the sampling sites were given on Table S1. Seven of these sampling stations were inhabited by *Pelophylax ridibundus* (locations 1, 2, 7, 8, 9, 10 and 11), two by *Pelophylax bedriagae* (locations 3 and 4) and two by *Pelophylax caralitanus* (locations 5 and 6).

A total of 110 frogs were sampled from 11 populations within the distribution range of *Pelophylax ssp.* Frogs living in lakes, ponds, puddles, or farmland/ditch (Fig. 2) were captured manually or with dip nets and put in plastic carrier bags. Samples of both water and sediment were taken from the same habitats. A polyethylene sampling bottle was used to collect 500 ml water samples. The sediment surface (0–3 cm depth) was excavated using a steel shovel to gather approximately 1 kg of sediments, which were then placed in plastic carrier bags. All samples (water, sediment, and frogs) were placed in insulated containers and conveyed to the lab, where muscle and liver tissue and sediment samples were kept in a freezer set to -20 °C for later experiments. Fifty milliliters of water were filtered through 0.45 mm PTFE filters of particle size, and five drops of HNO<sub>3</sub> (Suprapur, Merck) were added to reduce the pH to a level of less than two prior to cooling for further heavy metal analyses (US EPA 1994).

### Sample digestion

The Anatolian frog samples were thawed and washed with ultrapure water at room temperature (RT). The sediment samples were initially kept at RT and later dried in the oven. Each frog's snout-vent length (SVL) and weight were recorded (Table S2). Subsequently, with a stainless-steel dissection set, the frogs' livers and hind leg muscles were extracted with precision, and each frog was dissected individually. Two grams of fresh weight from the leg and liver tissues were allocated to separate digesting vessels. Digestion of the sediment was conducted using 0.5 g of the dry sample. 5 ml of HNO<sub>3</sub> (Suprapur, Merck) were added to each to tubes containing tissues and sediment samples.



**Fig. 1** Sampling locations in this study

The tubes were enclosed with polypropylene lids and were kept overnight. A block heater was used to incubate the tubes for 2.5 h at 95 °C. After being brought down to RT, the tubes underwent an additional 2 h of incubation at 95 °C with 2.5 mL of H<sub>2</sub>O<sub>2</sub> (Suprapur, Merck). The lids were taken off, and the tubes were left inside the block heater with the fluid content was reduced to approximately 2 mL. Afterward, the solutions were diluted with ultrapure H<sub>2</sub>O, strained with PTFE syringe filters (0.45 mm pore size), and placed in a +4 °C fridge until the measurements were completed (US EPA 1996).

### Heavy metal analysis and quality assurance

To assess and confirm the procedures' effectiveness at digesting the reference material (ERM-CE278k Mussel Tissue), the

same approach employed on the samples was likewise performed there (Table S3). ICP-MS (Agilent, 7800) was used to determine the concentration of TE. The internal standards and blank samples were also examined to look for potential interferences with the ICP-MS readings or sample preparation processes (Öztürk et al. 2021).

### Estimation and evaluation of health risk

By utilizing the heavy metal concentrations in frog legs, the Estimated Daily Intake (EDI) was assessed and contrasted with the provisional tolerable daily intake (PTDI) according to the FAO/WHO Joint Expert Committee on Food Additives (JECFA 1982, 2011a, b). Moreover, a Risk Coefficient (RK) method was employed to estimate the possible risk to human health from metals from consuming





**Fig. 2** Different habitat types in the sampling stations

of frog legs (USEPA 2015). The THQs, a quantification of heavy metal concentration compared to the dosage coefficient, illustrate the probability of persistent non-carcinogenic exposures (USEPA 2015). If the THQ is greater than 1, the studied heavy metal has deleterious impacts. EDI and THQ calculations were made as follows:

$$EDI = \frac{C \times FCR}{BW}$$

$$THQ_s = \frac{C \times EF \times ED \times FCR}{RfD \times BW \times EF \times ED} \times 10^{-3}$$

C: average metal concentration in the frog leg, FCR: Frog consumption (g/person/day), Average serving size for adults (227 g; US EPA 2000), ED: Duration of exposure (76 years/mean life expectancy in Türkiye) (WHO 2015), EF: frequency of exposure (365 days/year), BW: Mean body weight (72.5 kg Basara et al. 2016), RfD: Reference dose (mg/kg/day),  $10^{-3}$ : Conversion factor coefficient.

The non-carcinogenic health risk of all elements contained in frog legs (Newman and Unger 2002) was evaluated using the Health Index (HI). This index is formulated by the sum of the Total Hazard Quotients (THQs), and is represented as:

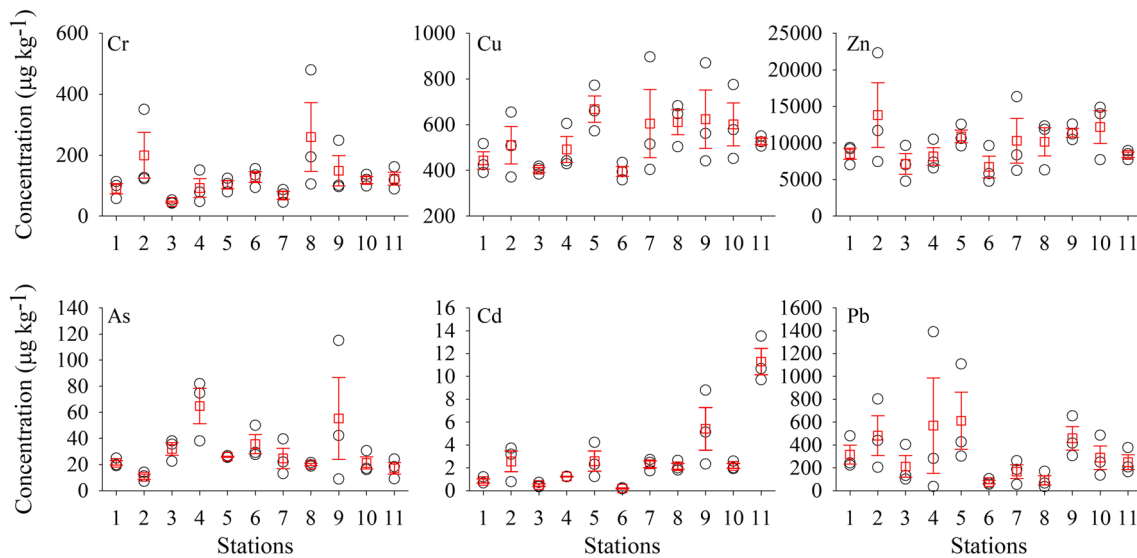
$$HI = THQ_{Cu} + THQ_{Pb} + \dots + THQ_x$$

## Data analysis

The Shapiro–Wilk and Levene tests were used to verify that the data conformed to a normal distribution and equal variance, respectively. When the data did not align with the normal distribution, it underwent the log10 transformation process. Detection and removal of outlier data were performed with the Boxplot technique. To investigate the variations in heavy metal contents among species and different stations, a one-way analysis of variance (ANOVA) was utilized, accompanied by a post hoc Tukey test. Additionally, to ascertain if any discrepancies in heavy metal amounts existed between the sexes, the independent sample t-test was used. The Pearson correlation method was applied to ascertain the links between elements. Furthermore, a linear regression analysis was implemented to analyze the potential connection between metal concentrations in Anatolian water frogs' liver and muscle tissues, considering their body lengths and weights. Using the SPSS 22 (IBM, USA) package program, statistical analyses were carried out and 5% ( $p < 0.05$ ) was chosen as the significance level.

## Results and discussion

Heavy metal concentrations in frog muscle and liver tissues were calculated in  $\mu\text{g kg}^{-1}$ , and the results are given in Figs. 3 and 4, respectively. The concentrations of heavy

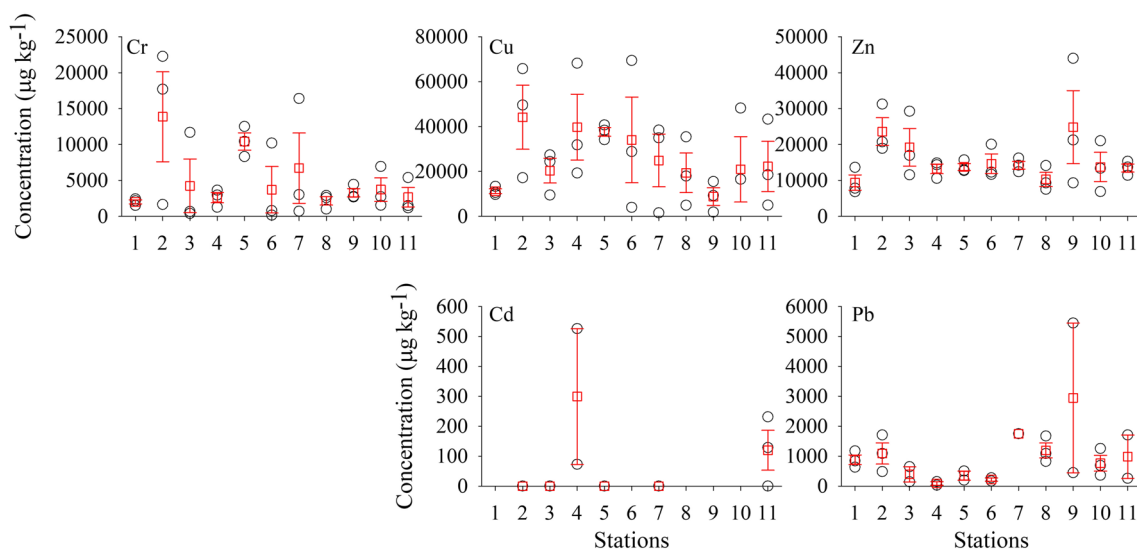


**Fig. 3** Heavy metal concentrations in the muscle tissue of Anatolian water frogs collected from 11 different stations across Türkiye. Circles represent the results for each frog. Red squares and error bars indicate mean and std error, respectively

metals in the muscle tissue of frogs obtained from multiple sites were as detailed: As, 7.08–114.90  $\mu\text{g kg}^{-1}$ ; Cd, under detection limits (UDL)–13.52  $\mu\text{g kg}^{-1}$ ; Cr, 41.75–479.35  $\mu\text{g kg}^{-1}$ ; Cu, 358.45–896.05  $\mu\text{g kg}^{-1}$ ; Pb, 34.74–1390.10  $\mu\text{g kg}^{-1}$ , and Zn 4744–22306.76  $\mu\text{g kg}^{-1}$  (Table S4; Fig. 3). The average concentrations ( $\mu\text{g kg}^{-1}$ ) of heavy metals in muscle tissue were determined in the following order: Zn (9785.11) > Pb (52.58) > Cu (25.01) > Cr (15.36) > As (3.92) > Cd (0.56). The heavy metal content in liver tissue was quantified as: Cd, UDL–526.31  $\mu\text{g kg}^{-1}$ ; Cr, 168.91–22268.04  $\mu\text{g kg}^{-1}$ ; Cu, UDL–69406.77  $\mu\text{g kg}^{-1}$ ; Pb,

29.23–5447.76  $\mu\text{g kg}^{-1}$ ; and Zn 6883.33–43955.22  $\mu\text{g kg}^{-1}$  (Table S4; Fig. 4). Following the mean concentration, the elements in the liver were sequenced from highest to lowest as Cu (25787.12) > Zn (15507.32) > Cr (5043.05) > Pb (938.02) > Cd (95.77).

Concentrations of metals in water samples varied (min–max) within following limits ( $\text{mg L}^{-1}$ ): As, 0.18–9.39  $\mu\text{g kg}^{-1}$ ; Cd, 0.01–0.05  $\mu\text{g kg}^{-1}$ ; Cr, 0.15–1.26  $\mu\text{g kg}^{-1}$ ; Cu, 0.99–3.13  $\mu\text{g kg}^{-1}$ ; Pb, 0.01–5.88  $\mu\text{g kg}^{-1}$ , and Zn 1.48–246.92  $\mu\text{g kg}^{-1}$  (Fig. 5). Heavy metal concentrations in sediments are listed as follows ( $\mu\text{g kg}^{-1}$ ): As, 478.74–8251.53; Cd,



**Fig. 4** Heavy metal concentrations in the liver tissue of Anatolian water frogs collected from 11 different stations across Türkiye. Circles represent the results for each frog. Red squares and error bars indicate mean and std error, respectively

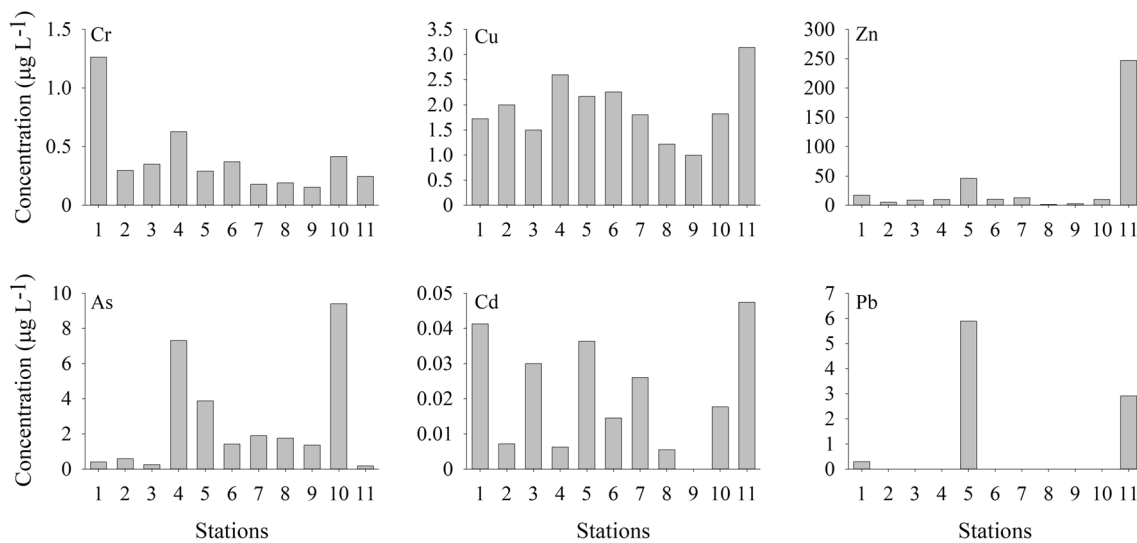


Fig. 5 Heavy metal concentrations in water samples collected from 11 different stations across Türkiye

78.01–549.90; Cr, 3420.81–189504.74; Cu, 1852.78–45617.42; Pb, 6182.68–27713.32; Zn, 7198.64–49863.42 (Fig. 6). Based on average concentration, heavy metals in water and sediment were ranked as follows ( $\mu\text{g L}^{-1}$  and  $\mu\text{g kg}^{-1}$ ): Zn (33.77) > As (2.58) > Cu (1.92) > Pb (1.82) > Cr (0.39) > Cd (0.02) and Cr (68867) > Zn (34544) > Cu (22821) > Pb (15545) > As (3700) > Cd (279), respectively.

Data analysis reveals that the aquatic environment has the lowest levels of heavy metals. Despite the low elemental concentration in water, it is common to find relatively significant quantities of several heavy metals in the tissues of frogs (Stolyar et al. 2008; Borković-Mitić et al. 2016; Prokić et al. 2016; Mani et al. 2022). Our findings align with previous research in this field. An analysis of water samples indicated heavy metal

concentrations that were up to 15,000 times smaller than the amount measured in edible tissues of frogs, with readings in liver tissue being up to 30,000 times lower. Accordingly, Stolyar et al. (2008) conducted a study that compared the concentrations of metals in two *P. ridibundus* populations in western Ukraine, with the distinction between rural and urban sites. Despite the low metal amounts in the water samples, they detected higher metal concentrations in the frog tissues they examined (Stolyar et al. 2008). The variation in metal levels between water and frog tissues could be ascribed to several factors. One possible reason is that frogs may accumulate heavy metals from anthropogenic or natural sources, or have experienced intermittent exposure to cumulative metal contamination in the past (Papadimitriou and Loumbourdis 2002;

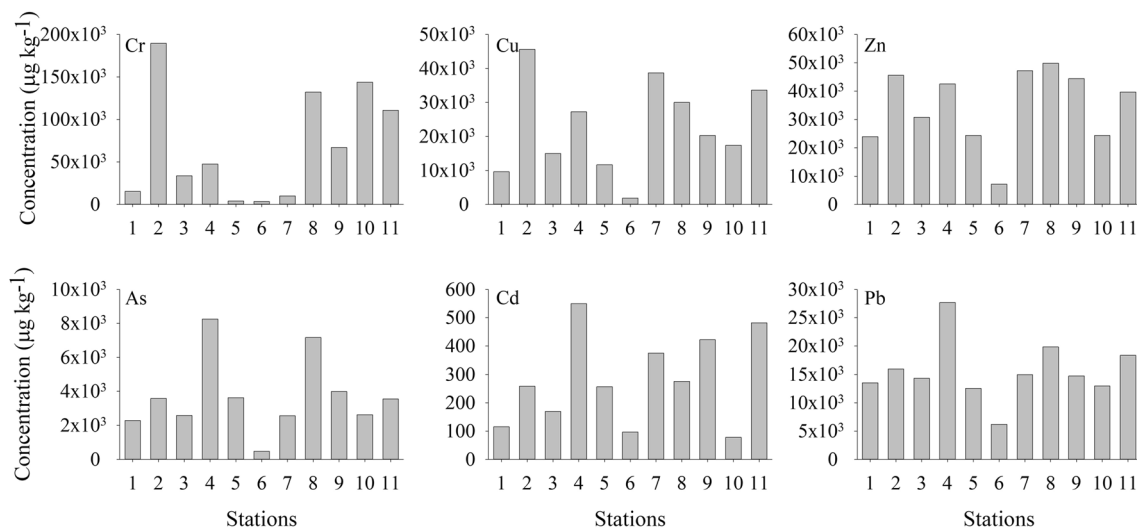


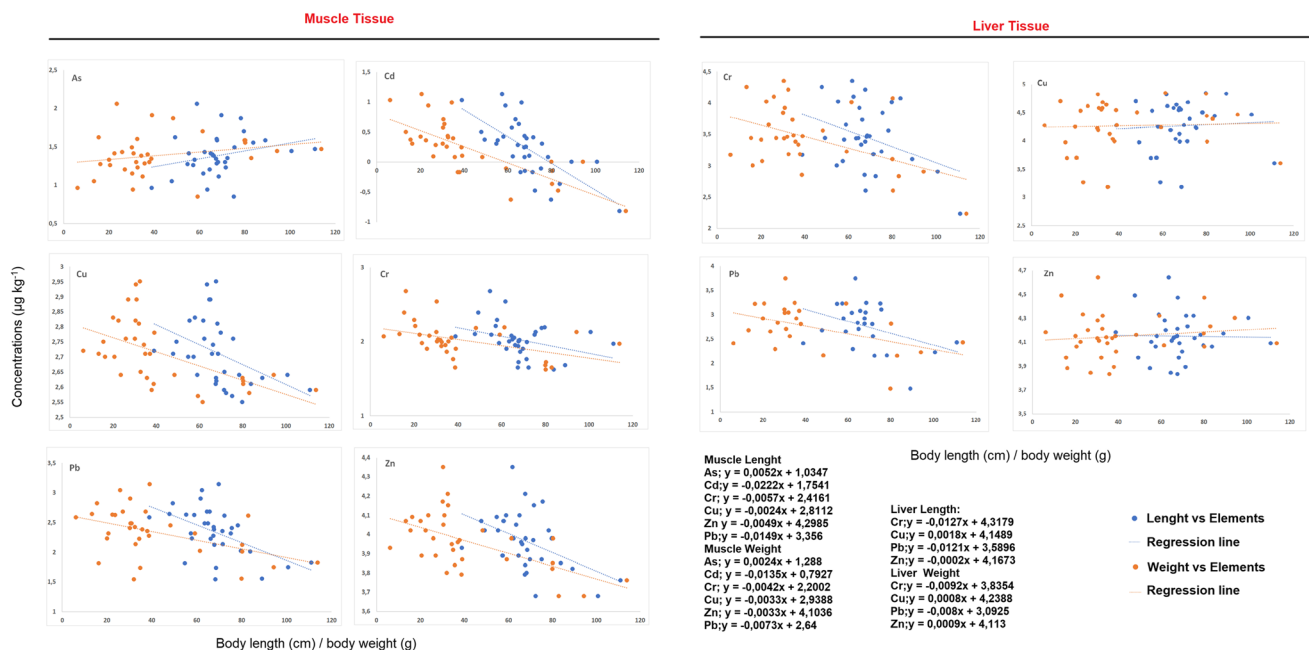
Fig. 6 Heavy metal concentrations in sediment samples collected from 11 different stations across Türkiye

Othman et al. 2009). Apart from directly absorbing heavy metals through their skin from water, frogs can also accumulate these metals in their bodies by consuming contaminated food or accidentally ingesting polluted sediment (Papadimitriou and Loumbourdis 2002). For this reason, metals accumulate much more in frog tissues over time than in the environment in which frogs live. Furthermore, sediment samples in this study were also found to have higher metal concentrations than water and frog samples. The findings of the current survey revealed increased metal concentrations in the sediment samples in comparison to both the water and frog samples. Because it is known that sediments act as a reservoir for pollutants, this is an expected result. Similar conclusions were reached in a recent study conducted in Nigeria, which discovered that sediment samples contained higher quantities of heavy metals than frog samples (Ediagbonya et al. 2022). However, Nummelin et al. (2007) suggested that frogs tend to accumulate metals in their tissues to much higher degrees than in the sediment, mainly through the consumption of aquatic plants or other creatures in the wetlands' food web.

In the genus *Pelophylax*, different frog species exhibit significant variations in mean Cr, As, and Cd concentrations in their muscle tissues, as determined by a One-Way ANOVA (Cr:  $F_{2,30} = 4.317$ ,  $p < 0.05$ ; As:  $F_{2,30} = 5.529$ ,  $p < 0.01$ ; Cd:  $F_{2,30} = 6.771$ ,  $p < 0.01$ ). Conversely, the mean Cu, Zn, and Pb concentrations among the frog species were found to be similar (One-Way ANOVA; Cu:  $F_{2,30} = 1.654$ ,  $p = 0.208$ ; Zn:  $F_{2,30} = 2.401$ ,  $p = 0.108$ ; Pb:  $F_{2,30} = 0.076$ ,  $p = 0.927$ ). The results of the statistical analysis to determine

whether there was any difference in the mean heavy metal concentrations of male and female muscle tissue showed that Cu (t-test;  $t = 2.690$ ,  $df = 31$ ,  $p < 0.05$ ) and Cd (t-test;  $t = 2.462$ ,  $df = 31$ ,  $p < 0.05$ ) were higher in males and As (t-test;  $t = -2.443$ ,  $df = 31$ ,  $p < 0.05$ ) was higher in females. On the other hand, in the liver tissue, no difference was found between the heavy metal concentrations of male and female individuals, except for Pb (t-test;  $t = 3.131$ ,  $df = 23$ ,  $p < 0.01$ ). In addition, it was found that the mean Cr, As, and Cd (One Way Anova; Cr:  $F_{2,30} = 4.317$ ,  $p < 0.05$ ; As:  $F_{2,30} = 5.529$ ,  $p < 0.01$ ; Cd:  $F_{2,30} = 6.771$ ,  $p < 0.01$ ) concentrations in the muscle tissue of the species in the same genus were different from each other, and the mean Cu, Zn, and Pb (One Way Anova; Cu:  $F_{2,30} = 1.654$ ,  $p = 0.208$ ; Zn:  $F_{2,30} = 2.401$ ,  $p = 0.108$ ; Pb:  $F_{2,30} = 0.076$ ,  $p = 0.927$ ) values were similar. Metal concentrations of the three species were found to be similar in liver tissue, except for Pb (One Way Anova;  $F_{2,22} = 11.654$ ,  $p < 0.001$ ). The findings of the study conducted in Nigeria indicated that Zn and Cd contents were higher in male frogs, while Cr, Cu, and Pb values were higher in female frogs (Ediagbonya et al. 2022). The precise reason for this contrast remains unknown; however, researchers have proposed that it might be linked to genetic differences between the sexes.

The inability of organisms to effectively eliminate toxic elements from their bodies leads to their buildup in tissues, leading to bioaccumulation. This process continues up the food chain, resulting in biomagnification (Burger and Snodgrass 2001; Stolyar et al. 2008; Mani et al. 2022). The



**Fig. 7** The relationship between body size and mass and the heavy metal concentrations in the tissues of Anatolian water frogs collected from 11 different stations across Türkiye. Results were obtained using

linear regression analysis. Each dot shows the result of different samples. X-axis shows length or weight of frog; y-axis shows concentration ( $\mu\text{g kg}^{-1}$ )



concentrations of Cu and Zn in the sediment, water, and frog tissues were notably higher than other metals, likely due to their abundance in the earth's crust and extensive industrial use (Hoffman et al. 2003).

Amphibians occupy both aquatic and terrestrial ecosystems, with water playing a vital role in supporting their lives for the majority of their lifespan (Altunışık 2018; Altunışık and Kara 2021; Tatlı et al. 2022a, b). Their dependence on water during a significant part of their life makes them susceptible to potential toxic element concentrations in aquatic environments (Wells 2007; Mani et al. 2022). The statistical analysis indicates noteworthy discrepancies in muscles' heavy metal levels among sampling locations (ANOVA, post hoc Tukey; Fig. 3). It is possible that the deviations in metal concentrations at the sampling sites are due to the extent of contamination in the development habitats. Typically, the metal concentrations in frog tissues collected from different sites mirror the pollution levels in the area (Hodkinson and Jackson 2005; Vershinin 2007). In light of this information, the variations in heavy metal levels in water and sediment samples, as shown in Fig. 5 and 6, appear to be of significant concern. Considering frogs' close interaction with their surroundings and their role as reliable bioindicators of pollution (Othman et al. 2009; Şişman et al. 2021), variations in metal concentrations in their tissues can serve as important indicators of overall environmental quality.

Another influencing factor is the variation in frog sizes across different sampling locations, which depends on certain variables. To investigate this, a linear regression analysis was performed to explore the relationship between frog length/weight and metal levels in the liver and hind limb muscles (Fig. 7). The data indicated negative correlations between frog length/weight and Cd, Cr, Cu, Pb, and Zn concentrations in hind limb muscles, with a positive correlation observed between length–weight and As concentrations (Cd:  $r = -0.725, p < 0.001$ ; Cr:  $r = -0.359, p < 0.05$ ; Cu:  $r = -0.490,$

**Table 2** The relationship among heavy metal concentrations detected in frog liver tissue

	Cr	Cu	Pb	Zn
Cr	<b>1</b>	<b>0.430</b> 0.014*	<b>0.172</b> 0.410	<b>0.052</b> 0.775
Cu		<b>1</b>	<b>-0.408</b> 0.048*	<b>0.173</b> 0.344
Pb			<b>1</b>	<b>0.005</b> 0.983
Zn				<b>1</b>

The numbers are given in bold show the correlation coefficient. Light colored numbers show the  $p$  value; \*  $p < 0.05$

$p < 0.01$ ; Pb:  $r = -0.520, p < 0.01$ ; Zn:  $r = -0.526, p < 0.01$ ; As:  $r = 0.269, p = 0.13$ ). Furthermore, negative correlations were found between length/weight and Cr and Pb levels in liver tissue. However, positive correlations were observed between length/weight and Cu and Zn concentrations (Fig. 7). Nevertheless, these correlations lacked statistical significance ( $p > 0.05$ ), except for Cr ( $r = -0.527, p < 0.01$ ). Similar to our findings, a previous study reported a negative correlation between frog length–weight and As, Cd and Pb concentrations in muscle tissues, and a positive correlation between length–weight and Cr, Cu and Zn concentrations (Mani et al. 2022). In addition, the study found that there was a negative correlation between As, Cd, Pb, Cr, and Zn concentrations and the length–weight of frog's liver tissue (Mani et al. 2022).

Tables 1 and 2 show the interrelationship between metals in liver and muscle tissues, respectively. In muscle tissue, there was a positive significant correlation between Cr–Zn and Cu–Zn (Table 1). As has a non-significant positive relationship with Cd, Pb and Zn, and a non-significant negative relationship with Cr and Cu. Cd has a non-significant

**Table 1** The relationship among heavy metal concentrations detected in frog muscle tissue

	As	Cd	Cr	Cu	Pb	Zn
As	<b>1</b>	<b>0.008</b> 0.963	<b>-0.114</b> 0.526	<b>-0.148</b> 0.410	<b>0.281</b> 0.113	<b>0.041</b> 0.819
Cd		<b>1</b>	<b>0.094</b> 0.603	<b>0.215</b> 0.229	<b>0.016</b> 0.930	<b>0.175</b> 0.329
Cr			<b>1</b>	<b>0.144</b> 0.424	<b>0.058</b> 0.747	<b>0.499</b> 0.003*
Cu				<b>1</b>	<b>0.274</b> 0.123	<b>0.460</b> 0.007*
Pb					<b>1</b>	<b>0.208</b> 0.246
Zn						<b>1</b>

The numbers are given in bold show the correlation coefficient. Light colored numbers show the  $p$  value; \*  $p < 0.05$



positive correlation with Cr, Cu, Pb and Zn. Cr has an insignificant positive relationship with Cu and Pb. Cu has an insignificant positive correlation with Pb. Pb Cu has a non-significant positive correlation with Zn (Table 1). On the other hand in liver tissue, Cu has a significant positive relationship with Cr and a significant negative relationship with Pb (Table 2). Relationship between Cr-Pb, Cr-Zn, Cu-Zn and Pb-Zn was insignificant (Table 2). Similarly, a significant positive correlation was reported between Cu-Cr and Cu-Pb in the frog *Pyxicephalus edulis* (Ediagbonya et al. 2022). Metals with strong correlations point to comparable sources of contamination.

Concentrations of heavy metals in the frog's muscle and liver tissues have been documented in various research studies, as presented in Table S4. An investigation in Northern Greece (Loumbourdis and Wray 1998) reported higher levels of Cd, Cr, Cu, Pb, and Zn in both liver and muscle tissues compared to our study. A further study conducted in the vicinity of an e- waste dump has found that the content of As, Cu, Pb and Zn in the leg muscle tissues of three diverse species (*Hoplobatrachus rugulosus*, *Fejervarya limnocharis* and *Occidozyga lima*) was higher than in this study (Thanomsangad et al. 2020). In addition, *X. laevis* and *R. esculentus* in Nigeria (Tyokumbur and Okorie 2011) and *R. tigrina* and *E. cyanophlyctis* species in Pakistan (Qureshi et al. 2015) had relatively high content of Cd, Cu, Cr, Zn, and Pb in liver and muscle tissues. On the other hand, the results of our study were similar in terms of many metals such as As, Cd, Cu, Pb, and Zn when compared with studies conducted in Bulgaria (Zhelev et al. 2020) and northeastern Türkiye (Mani et al. 2022).

Turkey plays an important role as a supplier and trader of frogs (Çiçek et al. 2020). For over four decades, Anatolian water frogs (*Pelophylax spp.*) have been gathered

for consumption either from nature or through aquaculture (Akin and Bilgin 2010, Kürüm 2015). According to the data of the Turkish Statistical Institute, in 2022, 210 tons of frog legs were obtained by hunting and 25 tons by aquaculture (TUIK 2023). Various agencies have established guidelines to ensure that food organisms do not contain excessive levels of certain trace elements to ensure consumer safety. Some trace elements, including Cu, Co, Cr, Zn, V, Mn, and Ni play crucial roles in the organisms' metabolism, but they can become toxic at high doses. Additionally, Pb, Cd, and As can have damaging impacts on humans even when present in minuscule concentrations, earning them a spot in the top 10 of the "Substance Priority List" released by ASTDR (2019). Several global entities have also established the uppermost tolerable concentrations for Pb, Cd, and As. For instance, the European Commission (EC) has issued guidelines stating that the concentrations of Cd and Pb in fishery products should not exceed  $1 \text{ mg kg}^{-1}$  and  $1.5 \text{ mg kg}^{-1}$ , respectively (EC 2006). Our data, compared to these limits (Table 3), showed that the Cd and Pb values remained within the specified thresholds. To determine the Estimated Daily Intake (EDI) levels for average serving size (227 g), we used the measured values of Pb, Cd, Cu, Zn, and As. Additionally, we assessed the EDI levels based on the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Provisional Tolerable Daily Intake (PTDI) norms (Table 3). Remarkably, the calculated EDI rates for adults were below the JECFA thresholds (Table 3).

To determine the potential non-carcinogenic health hazard associated with the consumption of the hind leg muscles of frogs gathered from 11 different sites, THQ values were calculated and presented in Table 3. These THQ values represent the risk associated with each potentially

**Table 3** Estimated Daily Intake (EDIs), Target Hazard Quotients (THQs), and Hazard Index (HI) through frog consumption from Türkiye

		Cu	Pb	As	Zn	Cd	Cr	HI
Concentrations in muscle tissue	Average	0.534	0.320	0.003	9.785	0.003	0.125	
	Minimum	0.358	0.035	0.001	4.745	0.0002	0.042	
	Maximum	0.896	1.390	0.011	22.307	0.0135	0.479	
EDI	Average	$2.39 \times 10^{-4}$	$1.43 \times 10^{-4}$	$1.34 \times 10^{-6}$	$4.38 \times 10^{-3}$	$1.33 \times 10^{-6}$	$5.59 \times 10^{-5}$	
	Minimum	$1.60 \times 10^{-4}$	$1.55 \times 10^{-5}$	$3.17 \times 10^{-7}$	$2.12 \times 10^{-3}$	$6.83 \times 10^{-8}$	$1.87 \times 10^{-5}$	
	Maximum	$4.01 \times 10^{-4}$	$6.22 \times 10^{-4}$	$5.14 \times 10^{-6}$	$9.98 \times 10^{-3}$	$6.05 \times 10^{-6}$	$2.14 \times 10^{-4}$	
PTDI		0.5	0.004	0.002	1	0.001		
RfD		0.040	0.020	*0.0003	0.300	0.001	*0.003	
THQs	Average	0.006	0.007	0.004	0.015	0.001	0.019	0.052
	Minimum	0.004	0.001	0.001	0.007	0.000	0.006	0.019
	Maximum	0.010	0.031	0.017	0.033	0.006	0.071	0.169

THQs < 1 metal is unlikely any adverse health effects; THQs > 1 metal is likely adverse health effects. Rfd (mg/kg/d), \*Inorganic As and Cr (VI) Rfd values (mg/kg/day), US EPA (2015); Average concentration of inorganic As estimated as 10% of total As (US FDA 1993). \*EDI (mg/kg/day); PTDI (mg/kg/day): provisional tolerance daily intake, Cu and Zn (JECFA 1982), As (JECFA 2011a), Cd and Pb (JECFA 2011b)

toxic element in the frogs. The results indicate that the regular portion size of frog consumption is not expected to be detrimental to adult consumers since all THQ values for the elements were less than 1. Additionally, we examined the HI levels, which represent the cumulative non-carcinogenic risk from multiple elements. All HI values lower than 1 (the limit value) were observed to range from 0.019 to 0.169, depending upon the mean portion size. This suggests that the consumption of frogs is not expected to impact human health negatively.

## Conclusion

In this study, heavy metal bioaccumulation in the tissues and habitats of three commercially collected and exported edible frog species (*Pelophylax ridibundus*, *Pelophylax bedriagae*, *Pelophylax caralitanus*) in Türkiye was meticulously investigated. Additionally, the research aimed to assess the potential non-carcinogenic health risk associated with consuming the hind leg muscles of these frog species. The results revealed considerable variations in heavy metal concentrations among frog tissues, influenced by the sampling sites and species. Among the three edible species, *P. ridibundus* and its habitats have relatively higher heavy metal concentrations than other species. However, a reassuring outcome was the discovery that the concentrations of Cd and Pb in the edible tissues were well below the permissible limits set by the European Commission (EC). The Estimated Daily Intake (EDI) values, calculated based on the average serving size, were also lower than the Provisional Tolerable Daily Intake (PTDI) levels for adult consumers. Furthermore, the study computed the Target Hazard Quotient (THQ) values for heavy metals, all of which were below the critical value of 1, indicating that consuming the hind leg muscles from these frog species would not pose an adverse health risk for humans.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11356-023-31226-9>.

**Authors contributions** Hale Tatlı: conceptualization, methodology, validation, formal analysis, investigation.

Kenan Gedik: validation, resources, writing—review and editing, supervision, visualization Abdullah Altunışık: conceptualization, funding acquisition, methodology, validation, writing—review and editing, supervision, project administration.

**Funding** We thank the following for funding: Scientific and Technical Research Council of Turkey (TÜBİTAK, Grant number: 122Y406) and Grant BAP (Grant number: FBA-2022–1315) from Recep Tayyip Erdogan University.

**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethical approval** Sampling was performed with the permission of the local ethics committee of Recep Tayyip Erdogan University (decision number: 2021/15) for animal experiments.

**Consent to participate** Not applicable.

**Consent to publish** Not applicable.

**Competing interests** There are no conflicts of interest to declare.

## References

- Akin Ç, Bilgin CC (2010) Preliminary report on the collection, processing and export of water frogs in Turkey. (Presented to KKGGM). ODTÜ, Ankara, Turkey. [in Turkish]
- Ali H, Khan E, İlahi I (2019) Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation. *J Chem* 2019. <https://doi.org/10.1155/2019/6730305>
- Altunışık A (2018) The first demographic data and body size of the southern banded newt, *Ommatotriton vittatus* (Caudata: Salamandridae). *Acta Herpetol* 13:13–19. <https://doi.org/10.13128/Acta>
- Altunışık A, Kara Y (2021) Unusual winter activity of *Bufo bufo* (Anura: Bufonidae). *Turk J Biodivers* 4:105–107
- Al-Yousuf MH, El-Shahawi MS, Al-Ghais SM (2000) Trace metals in liver, skin and muscle of *Lethrinus lentjan* fish species in relation to body length and sex. *Sci Total Environ* 256:87–94. [https://doi.org/10.1016/S0048-9697\(99\)00363-0](https://doi.org/10.1016/S0048-9697(99)00363-0)
- ATSDR (2019) The ATSDR 2019 substance priority list. Atlanta. <https://www.atsdr.cdc.gov/spl/index.html>
- Basaran B (2022) Comparison of heavy metal levels and health risk assessment of different bread types marketed in Turkey. *J Food Compos Anal* 108:104443. <https://doi.org/10.1016/j.jfca.2022.104443>
- Basara BB, Guler C, Soyutun I, Aygün A, Özdemir TA (2016) Republic of Turkey ministry of health statistics yearbook 2015. Ankara (Turkey): Ministry of Health Publication, p 248
- Borković-Mitić SS, Prokić MD, Krizmanić II et al (2016) Biomarkers of oxidative stress and metal accumulation in marsh frog (*Pelophylax ridibundus*). *Environ Sci Pollut Res* 23:9649–9659. <https://doi.org/10.1007/s11356-016-6194-3>
- Briffa J, Sinagra E, Blundell R (2020) Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon* 6:e04691. <https://doi.org/10.1016/j.heliyon.2020.e04691>
- Burger J, Snodgrass J (2001) Metal levels in southern leopard frogs from the Savannah River site: Location and body compartment effects. *Environ Res* 86:157–166. <https://doi.org/10.1006/enrs.2001.4245>
- Çiçek K, Ayaz D, Afsar M et al (2020) Unsustainable harvest of water frogs in southern Turkey for the European market. *Oryx*. <https://doi.org/10.1017/S0030605319000176>
- de Medeiros AMZ, Coa F, Alves OL et al (2020) Metabolic effects in the freshwater fish *Geophagus iporangensis* in response to single and combined exposure to graphene oxide and trace elements. *Chemosphere* 243:1–8. <https://doi.org/10.1016/j.chemosphere.2019.125316>
- Dönmez M, Şişman T (2021) The morphometric and erythrometric analyses of *Pelophylax ridibundus* living in anthropogenic pollution resources. *Turk J Zool* 45:314–328. <https://doi.org/10.3906/zoo-2104-16>

- EC (2006) European Commission regulation no. 1881/2006 of 19 December 2006 setting 450 maximum levels for certain contaminants in foodstuffs. Off J Eur Union L364-5-L364-24. Available at <https://eur-lex.europa.eu/legalcontent/EN/ALL/?uri=Dcelex%3A32006R1881>
- Ediagbonya TF, Ogunjobi JA, Odinaka CV, Adenikinju CA (2022) Bioaccumulation of elemental concentrations in sediment and frog (*Pyxicephalus edulis*) in Igbebo River, Ondo state, Nigeria. *Chem Africa* 5:1153–1165. <https://doi.org/10.1007/s42250-022-00406-4>
- Fettweis A, Bergen B, Hansul S et al (2021) Correlated Ni, Cu, and Zn sensitivities of 8 freshwater algal species and consequences for low - level metal mixture effects. *Environ Toxicol Chem* 40:2013–2023. <https://doi.org/10.1002/etc.5034>
- Hecnar SJ (1995) Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. *Environ Toxicol Chem* 14:2131–2137. <https://doi.org/10.1002/etc.5620141217>
- Henczová M, Kiss A, Filla A et al (2008) Comparative biochemistry and physiology, part C effects of Cu 2+ and Pb2+ on different fish species: Liver cytochrome P450 -dependent monooxygenase activities and FTIR spectra. 148:53–60. <https://doi.org/10.1016/j.cbpc.2008.03.010>
- Hodkinson ID, Jackson JK (2005) Terrestrial and aquatic invertebrates as bioindicators for environmental monitoring, with particular reference to mountain ecosystems. *Environ Manage* 35:649–666. <https://doi.org/10.1007/s00267-004-0211-x>
- Hoffman D, Rattner B, Burton G, Cairns J (2003) Hoffman et al. *Handb. Ecotoxicology*. Lewis Publ. Boca Raton
- JECFA (1982) Evaluation of certain food additives and contaminants 26th Report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Report Series No. 683. World Health Organization, Geneva, Switzerland. Available at [http://apps.who.int/iris/bitstream/10665/41546/1/WHO\\_TRS\\_683.pdf](http://apps.who.int/iris/bitstream/10665/41546/1/WHO_TRS_683.pdf)
- JECFA (2011a) Evaluation of certain food additives and contaminants 72nd Report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series No. 959. World Health Organization, Rome, Italy. Available at [http://apps.who.int/iris/bitstream/10665/44514/1/WHO\\_TRS\\_959\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/44514/1/WHO_TRS_959_eng.pdf). Accessed 2 Sept 2023
- JECFA (2011b) Evaluation of certain food additives and contaminants 73rd Report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series No. 960. World Health Organization, Geneva, Switzerland. Available at [http://apps.who.int/iris/bitstream/10665/44515/1/44515/1/WHO\\_TRS\\_960\\_eng.pdf](http://apps.who.int/iris/bitstream/10665/44515/1/44515/1/WHO_TRS_960_eng.pdf). Accessed 2 Sept 2023
- Kürüm V (2015) Statistics of frog trade in Turkey. Republic of Turkey Ministry of Agriculture and Forestry General Directorate of Fisheries and Aquaculture, Ankara, Turkey. [in Turkish]
- Loumbourdis NS, Wray D (1998) Heavy-metal concentration in the frog *Rana ridibunda* from a small river of Macedonia, Northern Greece. *Environ Int* 24:427–431. [https://doi.org/10.1016/S0160-4120\(98\)00021-X](https://doi.org/10.1016/S0160-4120(98)00021-X)
- Lower WR, Kendall RJ (1990) Sentinel species and sentinel bioassay. In: McCarthy JF, Shugart LR (eds) *Biomarkers of environmental contamination*, CRC Press Inc., Boca Raton, FL, pp 309–331
- Mani M, Altunışık A, Gedik K (2022) Bioaccumulation of trace elements and health risk predictions in edible tissues of the marsh frog. *Biol Trace Elem Res* 200:4493–4504. <https://doi.org/10.1007/s12011-021-03017-1>
- Neveu A (2009) Suitability of European green frogs for intensive culture: Comparison between different phenotypes of the esculenta hybridogenetic complex. *Aquaculture* 295:30–37. <https://doi.org/10.1016/j.aquaculture.2009.06.027>
- Newman MC, Unger MA (2002) *Fundamentals of ecotoxicology*. Lewis Publishers, Boca Raton, p 480
- Nummelin M, Lodenius M, Tulisalo E et al (2007) Predatory insects as bioindicators of heavy metal pollution. *Environ Pollut* 145:339–347. <https://doi.org/10.1016/j.envpol.2006.03.002>
- Othman MS, Khonsue W, Kitana J et al (2009) Cadmium accumulation in two populations of rice frogs (*Fejervarya limnocharis*) naturally exposed to different environmental cadmium levels. *Bull Environ Contam Toxicol* 83:703–707. <https://doi.org/10.1007/s00128-009-9845-y>
- Özogul F, Prof A, Özogul Y, Olgunoglu AI (2008) Comparison of fatty acid, mineral and proximate composition of body and legs of edible frog (*Rana esculenta*). *Int J Food Sci Nutr* ISSN 59:558–565. <https://doi.org/10.1080/09637480701403277>
- Öztürk RÇ, Gedik K, Şahin A, Özvarol Y, Mutlu E (2021) Comparative trace element trends in *Posidonia oceanica* and its sediments along the Turkish-Mediterranean Sea coast. *Environ Sci Pollut Res* 28(43):61397–61407
- Papadimitriou E, Loumbourdis NS (2002) Exposure of the frog *Rana ridibunda* to copper: Impact on two biomarkers, lipid peroxidation, and glutathione. *Bull Environ Contam Toxicol* 69:885–891. <https://doi.org/10.1007/s00128-002-0142-2>
- Prokić MD, Borković-Mitić SS, Krizmanić II et al (2016) Antioxidative responses of the tissues of two wild populations of *Pelophylax kl. esculentus* frogs to heavy metal pollution. *Ecotoxicol Environ Saf* 128:21–29. <https://doi.org/10.1016/j.ecoenv.2016.02.005>
- Qureshi IZ, Kashif Z, Hashmi MZ et al (2015) Assessment of heavy metals and metalloids in tissues of two frog species: *Rana tigrina* and *Euphyllactis cyanophlyctis* from industrial city Sialkot, Pakistan. 14157–14168. <https://doi.org/10.1007/s11356-015-4454-2>
- Rainbow PS (2018) *Trace metals in the environment and living organisms: the British Isles as a case study*. Cambridge University Press. <https://doi.org/10.1017/9781108658423>
- Simon E, Braun M, Tóthmérész B (2010) Non-destructive method of frog (*Rana esculenta* L.) skeleton elemental analysis used during environmental assessment. *Water Air Soil Pollut* 209:467–471. <https://doi.org/10.1007/s11270-009-0214-6>
- Şimşek E, Alkaya A, Şereflişan H, Özyılmaz A (2022) Comparisons of biochemical compositions in marsh frog (*Pelophylax ridibundus*) (Anura; Ranidae) grown in different conditions; wild, semicultured and cultured ones. *Turk J Zool* 46:261–269. <https://doi.org/10.55730/1300-0179.3054>
- Şişman T, Keskin MÇ, Dane H et al (2021) Marsh frog (*Pelophylax ridibundus*) as a bioindicator to assess pollution in an agricultural area. *Pak J Zool* 53:337–349
- Şişman T, Aşkın H, Türkez H et al (2015) Determination of nuclear abnormalities in peripheral erythrocytes of the frog *Pelophylax ridibundus* (Anura: Ranidae) sampled from Karasu River Basin (Turkey) for pollution impacts. *J Limnol Freshw Fish Res* 1:75–75. <https://doi.org/10.17216/limnofish-5000115825>
- Stolyar OB, Loumbourdis NS, Falfushinska HI, Romanchuk LD (2008) Comparison of metal bioavailability in frogs from urban and rural sites of Western Ukraine. *Arch Environ Contam Toxicol* 54:107–113. <https://doi.org/10.1007/s00244-007-9012-6>
- Stuart SN, Chanson JS, Cox NA et al (2004) Status and trends of amphibian declines and extinctions worldwide. *Science* (80-) 306:1783–1786
- Taiwo IE, Henry AN, Imbufe AP (2014) Heavy metal bioaccumulation and biomarkers of oxidative stress in the wild African tiger frog, *Hoplobatrachus occipitalis*. 8:6–15. <https://doi.org/10.5897/AJEST2013.1603>
- Tao Y, Yuan Z, Xiaona H, Wei M (2012) Distribution and bioaccumulation of heavy metals in aquatic organisms of different trophic levels and potential health risk assessment from Taihu lake, China. *Ecotoxicol Environ Saf* 81:55–64. <https://doi.org/10.1016/j.ecoenv.2012.04.014>
- Tatlı H, Altunışık A, Gedik K (2022a) Trace element bioaccumulation and health risk assessment derived from leg consumption of the

- marsh frog, *Pelophylax ridibundus* (Pallas, 1771 ). Ege J Fish Aquat Sci 39:182–190. <https://doi.org/10.12714/egejfas.39.3.02>
- Tatlı HH, Altunışık A, Gedik K (2022b) Microplastic prevalence in anatolian water frogs (*Pelophylax spp.*). J Environ Manage 321. <https://doi.org/10.1016/j.jenvman.2022.116029>
- Thanomsangad P, Tengjaroenkul B, Sriuttha M, Neeratanaphan L (2020) Heavy metal accumulation in frogs surrounding an e-waste dump site and human health risk assessment. Hum Ecol Risk Assess 26:1313–1328. <https://doi.org/10.1080/10807039.2019.1575181>
- TURKSTAT (2023) Turkish Statistical institute. Ankara, Turkey
- Tyokumbur ET, Okorie T (2011) Bioconcentration of trace metals in the tissues of two leafy vegetables widely consumed in south west Nigeria. Biol Trace Elem Res 140:215–224. <https://doi.org/10.1007/s12011-010-8683-4>
- U.S. EPA (1994) Method 200.8: Determination of trace elements in waters and wastes by inductively coupled plasma-mass spectrometry, Revision 5.4. Cincinnati, OH
- US EPA (2000) Guidance for assessing chemical contaminant data for use in fish advisories volume 2 risk assessment and fish consumption limits, 3rd edn. US Environmental Protection Agency
- US EPA (1996) Acid digestion of sediments, sludges, and soils. US Environmental Protection Agency. EPA Method 3050B. Washington
- US FDA (US Food and Drug Administration) (1993) Guidance Document for Chromium in shellfish. Center for Food Safety and Applied Nutrition, Washington
- USEPA (2015) Regional screening level (RSL) summary table (TR D 1E-6, HQ D 1) June 2015(revised). Available at <https://semsub.epa.gov/work/03/2218422.pdf>
- Vershinin V (2007) Biota of urban areas UroRAN, Ekaterinburg, Russia, 85
- Vogiatzis AK, Loumbourdis NS (1998) Cadmium accumulation in liver and kidneys and hepatic metallothionein and glutathione levels in *Rana ridibunda*, after exposure to CdCl<sub>2</sub>. Arch Environ Contam Toxicol 34:64–68. <https://doi.org/10.1007/s002449900286>
- Wang Z, Luo P, Zha X et al (2022) Overview assessment of risk evaluation and treatment technologies for heavy metal pollution of water and soil. J Clean Prod 379:134043. <https://doi.org/10.1016/j.jclepro.2022.134043>
- Wells KD (2007) The ecology and behaviour of amphibians. Chicago University Press, Chicago
- WHO (2015) Countries. Available at <http://www.who.int/countries/en/>
- Zeng J, Chen F, Li M et al (2019) The mixture toxicity of heavy metals on *Photobacterium phosphoreum* and its modeling by ion characteristics-based QSAR. PLoS One 14:1–13
- Zhelev ZM, Arnaudova DN, Popgeorgiev GS, Tsonev SV (2020) In situ assessment of health status and heavy metal bioaccumulation of adult *Pelophylax ridibundus* (Anura: Ranidae) individuals inhabiting polluted area in southern Bulgaria. Ecol Indic 115:106413. <https://doi.org/10.1016/j.ecolind.2020.106413>

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.