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Total Mercury Content in Amphibian Organs from the Ussuri Broadleaf and Mixed Forests Ecoregion

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Abstract The distribution of exposure to Hg in amphibians remains unknown on most continents. The Ussuri broadleaf and mixed forests ecoregion is a region of high biodiversity, the nine species of amphibia living on its territory are prey for over 25 threatened and rare vertebrate species. To understand the potential risks to wildlife, we measured the total mercury (THg) content in the Siberian wood frog (*Rana amurensis*) and the Sakhalin toad (*Bufo sachalinensis*) from the same locality. In total THg level was measured in 134 samples of liver, kidneys, and muscles via atomic absorption. Mean THg concentrations mg/kg dry weight (DW) were 0.055 ± 0.018 in muscles, 0.126 ± 0.040 in kidneys, and 0.140 ± 0.062 in liver in the immature Siberian wood frogs. In the adults of Siberian wood frogs, the mean THg concentrations were 0.035 ± 0.005 in muscles, 0.081 ± 0.006 in kidneys and 0.030 ± 0.005 in liver. Statistically significant differences in THg content between the two age groups were obtained only for the liver (p = 0.008). The mean THg of the Sakhalin toad was 0.058 ± 0.007 in muscles, 0.085 ± 0.009 in kidneys, and 0.137 ± 0.019 in liver. THg content in immature Siberian wood frogs and Sakhalin toads showed no statistically significant differences. THg correlations between tissues and organs were found. The THg content in amphibians was found to be comparable with the THg content of producers in terrestrial ecosystems and lower compared to species from other classes of terrestrial vertebrates. There is an assumption that other species of tailless amphibians of the genera *Rana* and *Bufo* may contain similar levels of total mercury in anthropogenically unpolluted or slightly polluted boreal landscapes of the Russia. For those boreal ecosystems that are already subject to pollution, our results may be used to justify specific actions when restoring ecosystem services, structures, and functions efficiently and effectively.

Keywords bioindicator, contaminant, Eastern Asian endemics, ecotoxicology, frogs, toads

1. Introduction

Amphibians are a specific group of animals that are threatened throughout their whole life cycle from living in water during the larval stage to adult stage mostly spent in terrestrial environment. Over the past half century, the status of many taxa has become of global conservation concern. The reduction of amphibians' abundance in different parts of the planet is known to be caused by multiple factors: diseases, invasive species, exotic pets trade, climate change and pollution. The decline in amphibian abundance is a cause for concern, but it may also indicate more serious

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environmental problems that may have systemic consequences (Hocking and Babbitt, 2014). Mercury (Hg) is a pollutant of great importance in the field of wildlife conservation (for example, Kalisińska, 2019). Almost 66% of all annual mercury emissions into the atmosphere come from anthropogenic human activity sources (Mason and Sheu, 2002). Several studies show that mercury exposure can negatively affect the behavior, physical fitness and survival of amphibians (Bergeron *et al.*, 2010; Burke *et al.*, 2010; Kalisińska, 2019; Yang *et al.*, 2020). In addition, many amphibians often occupy high trophic levels in their food webs, which may lead to elevated negative effects of toxic substances (Tornabene *et al.*, 2023). The concentration of mercury in biota has no direct connection with the concentrations of mercury in soil or water. Quite often soil Hg concentration and toxicity reference value displayed the broadest overall range (variability) (Sample *et al.*, 2014). Sediment total mercury (THg) values do not provide a clear measure of bioaccumulation or of the danger to amphibians and other aquatic animals (Bradford *et al.*, 2023; Tornabene *et al.*, 2023). For fish which are generally studied better than other vertebrates, the bioaccumulation coefficient, the ratio of THg (or MeHg) concentration in fish to concentration in their aquatic habitat, is dependent on several factors, such as the trophic level, size of the fish and selenium deficit (Smalling *et al.*, 2021). The shortage of selenium in vertebrates' diet is followed by an increase of mercury (Julshamn *et al.*, 1986). Still, reliable information on the dietary mercury exposure values that cause deleterious effects exists only for very few warm-blooded species. For birds the typical range of Hg levels in the diet (both in laboratory and field studies) that would affect reproduction fluctuated from 0.2 to more than 1.4 mg/kg wet weight (WW) (Fuchsman *et al.*, 2017). However, salamanders found to contain 4.519 ± 0.348 mg/kg WW THg, that exceeds the estimated threshold level for fish, have only shown sublethal effects such as hindered locomotor efficiency and feeding (Burke *et al.*, 2010). The study by Fuchsman *et al.* (2017) shows the existing toxicity reference values to be highly uncertain. Long-term research of the abiotic factors, toxicology of vertebrates and mercury ecotoxicology contain considerable gaps in our awareness on mercury behavior in nature, especially in amphibians. Overall, the distribution of exposure to Hg in amphibians remains unknown on most continents.

Despite the growing number of studies on mercury in Asian biota, they are mainly focused on mammals and birds, while amphibians remain poorly studied. There is no data on mercury in amphibians of the unique ecoregion Ussuri Broadleaf and Mixed forests (Dinerstein *et al.*, 2017), which includes the Primorsky Krai and the south of the Khabarovsk Krai in Russia. The Ussuri broadleaf and mixed forests ecoregion is a region of high biodiversity, of the nine amphibian species living on its territory, one is on regional and national lists for the protection of rare and endangered animals, the remaining species are common or numerous and are prey for over 25 threatened and rare vertebrate species (The Red Book of Primorsky Krai, 2005; Kuzmin and Maslova, 2005). Collection of tissues, organs and whole animals is needed to evaluate the possible risks of mercury exposure to wildlife. Non-lethal methods of hair and feather analysis which are chosen in most cases, especially for animals of conservation importance are often used in mammals, birds and reptiles (Hopkins and Babbitt, 2014). For amphibians, such methods are being developed and used for some species (Bergeron *et al.*, 2010; Tornabene *et al.*, 2023). However, while the search for non-lethal methods is underway, studies with the removal of small samples of non-endangered animal species are acceptable if there is a need to assess the risks associated with mercury.

The Siberian wood frog (*Rana amurensis*) and the Sakhalin toad (*Bufo sachalinensis*) are typical amphibians in the study area (Kuzmin and Maslova, 2005; Othman *et al.*, 2022; our data). These amphibians are medium-level predators that can make a significant contribution to the biomass of wetlands (Hocking and Babbitt, 2014). There is no information on bioaccumulation of pollutants in these species. Thus, the study aimed (1) to quantify the content of total mercury in two species: the Siberian wood frog and the Sakhalin toad from the same locality, and (2) to clarify the presence of THg correlations between tissues and organs.

2. Materials and Methods

The research area is located on an upland, non-wetland part of the West Primorye lowland plain. Therefore, natural factors that increase mercury methylation in the ecosystem: significant areas of swamps and acidic reservoirs are practically absent. After the almost complete destruction of the socio-economic infrastructure of the Russian Federation in the 1990s, small farms (crop and livestock) have been preserved here. There are no significant sources of anthropogenic mercury pollution. Therefore, the state of the environment is close to protected natural areas and the concentration of mercury in biota objects can probably be considered natural.

The material was collected in the Ussuri broadleaf and mixed forests ecoregion—in the south-west of the Primorsky Krai of Russia (44.44° N, 132.14° E) as part of a comprehensive study of the biological productivity of forest ecosystems by the Federal Scientific Centre of the East Asia Terrestrial

Biodiversity of Far Eastern Branch of Russian Academy of Sciences in 2022 and 2023. The captured animals were euthanized by cervical dislocation and kept frozen at −20°C until processed. Prior to dissection, the mass (± 0.1 g) of each sample was determined with electronic scales and body length was measured (from the tip of the muzzle to the anus: snout-vent length; hereafter, SVL) (± 1.0 mm).

Samples of kidneys, liver and muscles from captured amphibians were studied. 114 samples were taken from 42 individuals of the Siberian wood frog and 24 samples from eight individuals of the Sakhalin toad. Each sample was placed in an Eppendorf tube and stored frozen at temperatures from −4 °C to −16 °C. Then they were dried to a constant mass at a temperature of 37 °C for 1.5–2 d and the total mercury content in each sample was determined using a mercury analyzer RA-915M by the atomic absorption method of cold steam with a minimum mercury detection limit of 0.001 mg/kg. A sample of tissues weighing 10–30 mg was placed on a quartz dispenser, transferred to a thermolysis cell and burned at a temperature of 300 °C for 1–2 min. The accuracy of analytical measurement methods was controlled using DORM-4 certified biological material (Institute of Environmental Chemistry, Ottawa, Canada).

The age group of amphibians was determined based on the mass, SVL and morphology of the generative system (Kuzmin and Maslova, 2005). Since the sample of amphibians was not very large, we divided them into two groups: "adults" (adultus) (and "immature" (juvenile and subadultus).

Statistical analysis. The results were presented in the form of median and mean (or average) (arithmetic) and their errors (x ± mx). Distribution of empirical data on THg in the organs of the studied organisms did not follow the expected normal distribution, proven by the Kolmogorov-Smirnov test with Lilliefors correction. Thus, nonparametric Mann-Whitney *U* and Kruskal-Wallis tests were used. Statistical significance was determined at $p < 0.05$. Spearman correlation coefficient ($p \leq 0.05$) was used to determine correlations between the THg concentration in different pairs of organs and between the THg concentration and SVL and body weight (Sokal and Rolf, 1995).

All animal procedures were reviewed and approved by the Federal Scientific Centre of the East Asia Terrestrial Biodiversity's Institutional Animal Care and Use Committee (# 3_2023.02.21).

3. Results

The sample of the Siberian wood frog adults had the following parameters: SVL 52.7 ± 1.0 (45.0–65.0) mm and weight 14.2 ± 0.8 (9.0–26.1) g ($n = 29$); the group of immatures: SVL 41.6 ± 1.5 (31.0–49.0) mm and weight 5.7 ± 0.6 g (2.3–8.7) g ($n = 13$). In the sample of the Sakhalin toad, eight immature individuals were up to 10 g: SVL 37.6 ± 1.0 (31.0–46.0) mm and weight 5.9 ± 1.9 (2.2–9.2) g.

The individual content of THg in organs and tissues of the Siberian wood frog sample varies in a wide range —0.001–0.848 mg/kg dry weight (DW) in the liver, and the mean THg concentrations were 0.042 ± 0.007 mg/kg DW in muscles, 0.098 ± 0.014 mg/kg DW in kidneys, 0.069 ± 0.023 mg/kg DW in liver; the THg medians were 0.029 in muscles, 0.079 in kidneys, 0.028 in liver. In the group of immature Siberian wood frogs, the individual THg in organs varies in the same wide range of the entire sample 0.001–0.848 mg/kg DW in the liver, and the mean THg concentrations were 0.055 ± 0.018 mg/kg DW in muscles, 0.126 ± 0.040 mg/kg DW in kidneys, and 0.140 ± 0.062 mg/kg DW in liver; the THg medians were 0.033 in muscles, 0.087 in kidneys, 0.054 in liver (Figure 1). In the group of Siberian wood frog adults, the THg content in organs is lower than in the immature group, the individual THg content varies in a narrower range from 0.012 mg/kg DW in the liver to 0.165 mg/kg DW in the kidneys, and the mean THg concentrations were 0.035 ± 0.005 in muscles, 0.081 ± 0.006 in kidneys and 0.030 ± 0.005 in liver; the THg medians were 0.028 in muscles, 0.077 in kidneys, 0.026 in liver. Statistically significant differences in THg content between the two age groups were obtained only for the liver ($p = 0.008$). A direct positive correlation was found between THg in muscles and SVL and muscles and body weight in adult Siberian wood frogs (Figure 2). Significant correlations were found between the THg content in organ pairs of the Siberian wood frog sample (kidneys and muscles: $r = 0.47$, $p < 0.003$; liver and muscles: $r = 0.60$, $p < 0.0001$; kidneys and liver: $r = 0.63$, $p < 0.0001$). In pairs of organs in immature individuals, a strong positive correlation was also noted (r more than 0.96, $p < 0.05$); in adults, a moderate correlation was found only between liver and kidneys pair ($r = 0.589$, $p < 0.05$).

The individual THg content in the organs and tissues of the Sakhalin toad varies in the range from 0.041 mg/kg DW in muscles to 0.320 in the liver, and the mean THg concentration was 0.058 ± 0.007 in muscles, 0.085 ± 0.009 in kidneys, and 0.137 ± 0.019 in liver (Figure 1). A strong correlation of the THg content between all organs was revealed (muscles and kidneys: $r = 0.93$, $p < 0.002$; muscles and liver: $r = 0.82$, $p < 0.02$; kidneys and liver: $r = 0.90$, $p < 0.002$). The interspecies differences in Hg levels are of potential importance, but we have not identified them. Comparison of THg content in immature Siberian wood frogs and Sakhalin toads showed no statistically significant differences ($p = 0.057$ for muscles, 0.734 for kidneys, 0.104 for liver).

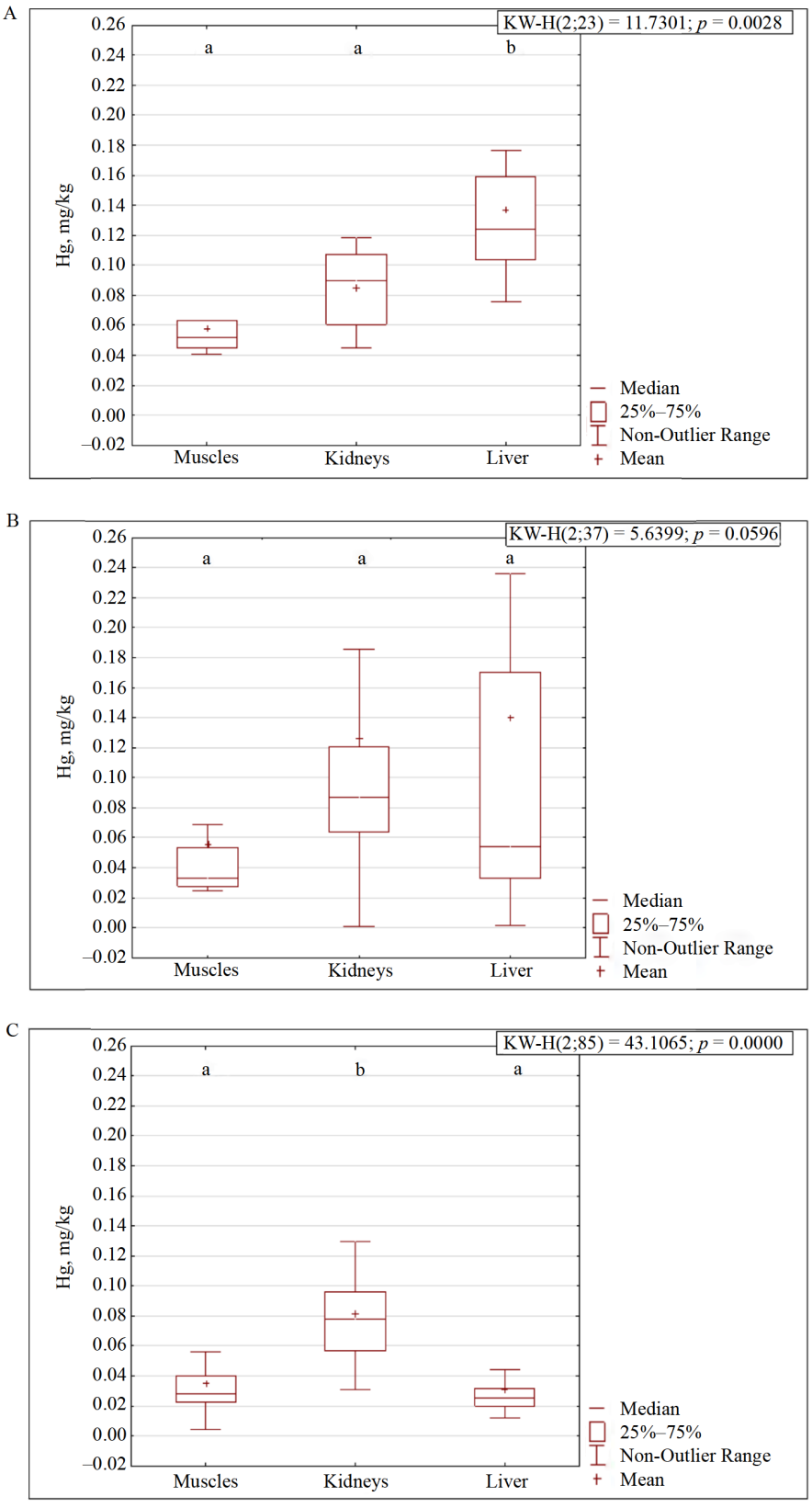


Figure 1 The total mercury content (mg/kg dry weight) in the organs. A: Immature Sakhalin toads (*Bufo sachalinensis*), B: Immature Siberian wood frogs (*Rana amurensis*), C: Adult Siberian wood frogs. Designations (a) and (b) are typical for statistically significant differences; (a) and (a) are not statistically significant differences.

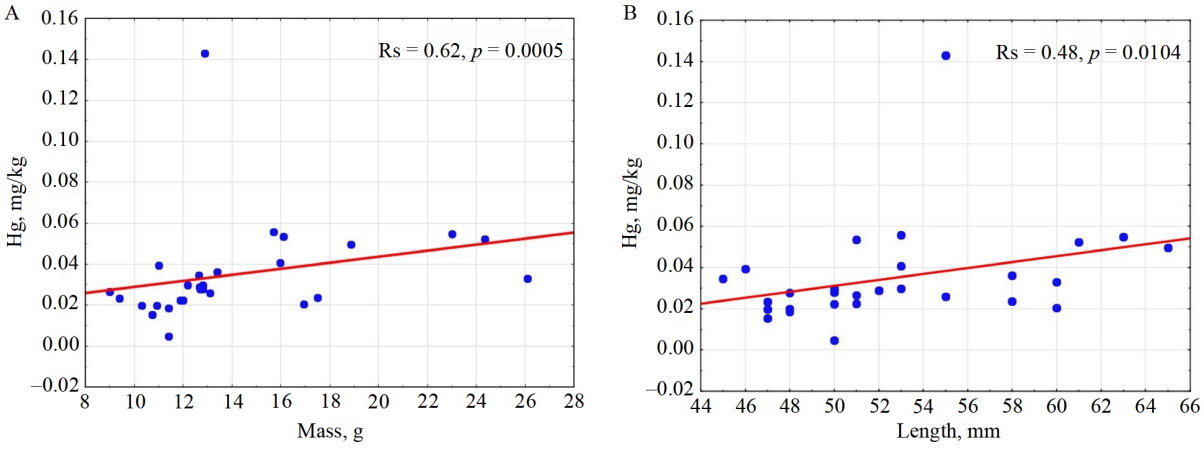


Figure 2 Correlation between the total mercury content in muscles. A: Body weight, B: Snout-vent length in adult Siberian wood frogs (*Rana amurensis*).

4. Discussion

Amphibians are one of the key links between terrestrial and aquatic ecosystems, performing habitat and diet shifts during migration and ontogenesis (Trakimas *et al.*, 2011). The transition to feeding on terrestrial invertebrates is accompanied by a decrease in THg concentrations in the organs of terrestrial tailless amphibians compared with their larvae (Bergeron *et al.*, 2010; Faccio *et al.*, 2019). Our research has shown, that such a decrease occurs in a single-step manner, the first observed in the immature individuals of the Siberian wood frog and the consecutive one in adult amphibians. In other species, it may also increase throughout the whole life, as was observed by Tornabene *et al.* (2023) in a long-term study. The correlations between THg levels and body weight or SVL in adult Siberian wood frogs (Figure 2) are potentially interesting, it maintains a position to increase Hg levels throughout the whole life in a terrestrial environment. Similar findings were reported by Hothem *et al.* (2010) where the content of mercury compounds was found to increase with age in Bullfrogs (*Lithobates catesbeianus*). The mean THg concentration in adults was 142% greater than that in larvae, and the mean THg concentration for immatures was 76% greater than that in larvae. The mean THg concentration in adults was 38% greater than the mean THg concentration in immatures. However, neither SVL nor mass was significantly correlated with THg concentration (Hothem *et al.*, 2010).

Despite the specific features of the young Siberian wood frogs' and Sakhalin toads' diets, their organs and tissues contain comparable concentrations of total mercury, which are apparently determined by the overall mercury level of one locality, including the waterbodies in which larvae developed, and land—the habitat of the terrestrial form. If we study a larger number of species of tailless and tailed amphibians from the same locality, the mercury level may or may not be comparable (Tornabene *et al.*, 2023).

The highest concentrations of mercury in most of the studied animals were observed in the liver, like that in other tailless amphibians from different regions (Byrne *et al.*, 1975; Terhivuo *et al.*, 1984; Hothem *et al.*, 2010; Komov *et al.*, 2017; Tornabene *et al.*, 2023), the adult Siberian wood frogs were an exception, as they had higher levels of mercury in their kidneys. At the same time, the level of THg in the organs of immature Siberian wood frogs was approximately the same as the level of MeHg in adult wood frogs (*R. sylvatica*) from Vermont and *L. catesbeianus* from northern California and Arizona (Tornabene *et al.*, 2023). The role of the geological component in the formation of the level of mercury compounds in amphibian tissues is not determining, as shown by Tornabene *et al.* (2023).

In the territory of Primorsky Krai, the total mercury content in biota was studied on small samples of plant litter and plants (Baranovskaya *et al.*, 2022b; Hansei *et al.*, 2021). In the plant litter in the three studied protected areas of Sikhote-Alin, the mean concentration of total mercury was 0.045 ± 0.004 (0.041–0.053) mg/kg DW, 0.076 ± 0.033 (0.035–0.136) mg/kg DW, 0.092 ± 0.046 (0.027–0.161) mg/kg DW; and the mean mercury concentration in plants decreased in a row (mg/kg DW): lichens (0.135) > ferns (0.0383) > laburnum (0.023) > sedges (0.016) (Baranovskaya *et al.*, 2022b). On the islands, the average mercury content was 26.3 ng/g for hornbeam, 24.5 for Mongolian oak, 9.1 for Gmelin wormwood (Hansey *et al.*, 2021). That is, the average total mercury content in producers such as laburnum, ferns, hornbeam, Mongolian oak turned out to be less than in immature Siberian wood frogs and

Sakhalin toads and comparable to the mean THg in the muscles and liver of adult Siberian wood frogs.

As for vertebrates, the mercury content was studied mainly in the fur of various groups of mammals (Poddubnaya *et al.*, 2021; Poddubnaya and Timoshkin, 2023 and references therein), which is usually higher than in the internal organs, or in some species close to the content in the liver (Poddubnaya, Timoshkin, 2023 and references therein). Comparison of available data on THg the fur of mammals (Table 1), and in the liver of amphibians showed that the average THg content in the liver of immature amphibians is almost 20 times higher than in the fur of a water deer (*Hydropotes inermis argyropus* Heude, 1884), slightly higher than the mercury content in the fur of the Korean mouse (*Apodemus penninsulae* Thomas, 1907), 2–3 times lower than in predatory mammals and almost 5 times lower than in fur of shrews of genus *Sorex*. The average content of THg in the liver of adult amphibians was only 4 times higher than in the fur of a water deer, and 4 to 30 times lower than in other mammals (Table 1). It could have been expected that insectivorous mammals and "insectivorous" amphibians may contain total mercury in approximately the same concentrations, but this has not been confirmed and is probably since shrews of the genus *Sorex* contain more mercury from preying on earthworms, known to contain high concentrations of mercury compounds (Roodbergen *et al.*, 2008). The results close to our data were obtained by Komov *et al.* (2017) in the Vologda Oblast: as a rule, amphibians contained lower concentrations of mercury compounds, compared to species from other classes of terrestrial vertebrates (Ivanova *et al.*, 2014).

Many studies on mercury content in amphibians contain information on wet weight, so we used a conversion factor of 1.3 calculated from the ratio of total mercury in dry and wet weight, taken from the work of Baranovskaya *et al.* (2022a) for comparison with our data. The THg in the organs of the Sakhalin toad (Table 1) was higher than that of the Common toad (*Bufo bufo*) from the Vologda region: 0.009–0.087 mg/kg DW, in the liver 0.007–0.087 mg/kg DW (Komov *et al.*, 2017) (hereafter calculated using a coefficient of 1.3). Liver of Common toads from a relatively clean area of Finland contained 0.12 mg/kg WW (0.156 mg/kg DW) (Terhivuo *et al.*, 1984), which is very close to our data. In another ecologically clean area in the former Yugoslavia, the liver of Common toads contained 0.74 mg/kg WW (0.962 mg/kg DW) (Byrne *et al.*, 1975), this is much higher than that of Sakhalin toads in the south of Primorsky Krai; in the area of the mercury deposit in the former Yugoslavia, the mercury content in the liver of Common toads was almost a hundred times more–21.75 mg/kg WW (28.28 mg/kg DW) than in Sakhalin toads in the Primorsky Krai (Table 1).

The levels of THg in the organs of the Siberian wood frog (Table 1) were 4–8 times lower than in the liver in three species of frogs from the European part of Russia (*Rana temporaria*, *Pelophylax lessonae* and *P. ridibundus*) from the Vologda oblast, the average values of THg in the liver were 0.715 ± 0.023 – 0.131 ± 0.004 mg/kg DW (Komov *et al.*, 2017). Liver of frogs *R. temporaria* from a relatively clean area of Finland contained 0.06 mg/kg WW (0.08 mg/kg DW) from other areas—up to 0.19 mg/kg WW (0.25 mg/kg DW) (Terhivuo *et al.*, 1984). This is slightly higher than that of the Siberian wood frog in the Primorsky Krai (Table 1). The liver of frogs *R. temporaria* from an ecologically clean area in the former Yugoslavia contained 0.48 mg/kg WW (0.62 mg/kg DW) (Byrne *et al.*, 1975), which is much more than in the south-west of Primorsky Krai (Table 1). The liver of Siberian wood frogs contained slightly less total mercury than the liver

Table 1 Total mercury content (mg/kg of dry weight) in the liver of amphibians and fur of mammals from the south of Primorsky Krai (* species of conservation importance (The Red Book of Primorsky Krai, 2005)).

Species	*N* samples	Limits, mg/kg	Mean ± SE, mg/kg dry weight	Reference
Siberian wood frog *Rana amurensis*, entire sample	37	0.001–0.848	0.069 ± 0.023	This study
Siberian wood frog *R. amurensis*, adult	25	0.012–0.127	0.030 ± 0.005	This study
Siberian wood frog *R. amurensis*, immature	12	0.001–0.848	0.141 ± 0.062	This study
Sakhalin toad *Bufo sachalinensis*, immature	9	0.041–0.354	0.164 ± 0.026	This study
Korean field mouse *Apodemus peninsulae*	75	0.004–0.502	0.118 ± 0.020	Poddubnaya, Timoshkin, 2023
Shrews *Sorex* sp.	58	0.14–3.79	0.90 ± 0.16	Poddubnaya, Timoshkin, 2023 and references therein
* Water deer *Hydropotes inermis argyropus*	7	0.001–0.026	0.008 ± 0.003	Poddubnaya, Timoshkin, 2023 and references therein
* Tiger *Panthera tigris altaica* from coastal area	12	0.115–0.918	0.434 ± 0.067	Poddubnaya *et al.*, 2021
* Tiger *P. t. altaica* from inland area	12	0.057–0.950	0.239 ± 0.075	Poddubnaya *et al.*, 2021
* The Asian black bear *Ursus thibetanus*, 1823	22	0.055–1.233	0.336 ± 0.056	Poddubnaya, Timoshkin, 2023 and references therein

of *R. temporaria* frogs from two types of landscapes within the Vologda region: 0.082–0.100 mg/kg WW (0.107–0.13 mg/kg DW) in lake-rich wetlands and 0.017–0.310 mg/kg WW (0.022–0.403 mg/kg DW) in in areas with few lakes (Komov *et al*., 2017). But as the levels are in a close range, this may also support the assumption that in anthropogenically unpolluted or slightly polluted boreal landscapes, tailless amphibians of the genera *Rana* and *Bufo* may contain similar levels of total mercury. It is also necessary to consider the period of data collection as the Hg levels became lower after the 2000s, in many regions compared to the 1970s and 1990s (Tornabene *et al*., 2023).

The fact that the concentration of mercury in amphibians from different areas of the boreal zone of the Holarctic is roughly at the same level allows assuming that such a level can be used as a background and considered safe for toads and frogs. It is very important for environmental research in the absence of information on mercury levels that cause disorders of amphibian metabolism and development. For those boreal ecosystems that are already affected by pollution, our results may be used to justify specific actions when restoring ecosystem services, structures, and functions efficiently and effectively.

5. Conclusion

Thus, the THg in the organs of immature individuals of the Siberian wood frog and Sakhalin toad from the same locality within the Southern Primorsky Krai was approximately the same. Adult Siberian wood frogs contain statistically significantly lower concentrations of THg in liver than immature ones. The THg content of adult Siberian wood frogs is comparable to the THg content of producers in the Primorsky Krai terrestrial ecosystems. Correlations between THg concentrations in organ pairs revealed in the present study provide support for the utilization of the non-lethal methods of THg assessment in rare and protected species, like those used by Pfleeger *et al*. (2016), Tornabene *et al*. (2023). However, more research on this issue is needed. The degree of mercury compounds' accumulation in the tissues of amphibian-eating animals, especially those in the group of rare and threatened species, needs to be studied.

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