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Determination and safety evaluation of metal element contents of Oviductus Ranae produced from *Rana dybowskii* by pond culture–forest grazing relay model in Heilongjiang Province, China

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RESEARCH ARTICLE

Abstract

The aquatic products of grazing ecological culture exhibit a strong reliance on the production environment system, which may result in heavy metal contamination. In this study, 24 elements (Ag, Al, As, Ba, Cd, Co, Cr, Cs, Cu, Fe, Ga, K, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn) were determined for Oviductus Ranae (OR) from more than 1,000 individuals of *Rana dybowskii* produced by pond culture–forest grazing relay mode from six major production areas in Heilongjiang Province, China. Furthermore, we assessed the associated health risks. Our findings indicate that most of these 24 metal elements were detected in OR at a detection rate of 100%, with the exceptions being As (98.0%), Ga (98.0%), Co (98.0%), V (94.0%), Cd (89.0%), Pb (76.0%), Cs (74.0%), Tl (35.0%), and U (not detectable). Variations in element concentrations were significant across the six sampling sites, with characteristic elements identified to account for spatial differences. The total target hazard quotient was determined to be 0.152 in Heilongjiang Province, thereby suggesting a relatively low health risk associated with consuming OR from this region's production systems according to existing standards and guidelines. Nevertheless, special attention is needed for monitoring the habitat environments of *R. dybowskii* from two sampling locations showing heightened pollution levels.

Keywords: Oviductus Ranae; metal element; safety evaluation; recommended safe dosage

Introduction

The brown frog (*Rana dybowskii*) plays a significant role within the forest and water ecosystem of Heilongjiang Province, located in Northeast China, holding a long-standing cultural history and serving as a valuable economic resource due to the high economic value of the fallopian tube, known as Oviductus Ranae (OR), of its female species (Wei *et al.*, 2023). Studies have demonstrated that the OR is rich in proteins, unsaturated fatty acids, amino acids, phospholipids, steroids, nucleosides, vitamins, hormones, trace elements, and sugars, exhibiting a broad spectrum of physiological activities (Wang *et al.*, 2023), (Zhang *et al.*, 2024). Current research on OR is predominantly focused on the analysis of protein and lipid components, screening for bioactive substances, and the development of functional foods (Wang *et al.*, 2023). It is noteworthy that the brown frog, with its amphibious lifestyle, inhabits complex environments that may harbor potential pollutants (Hu *et al.*, 2022). However, there has been a paucity of attention given to the risks of bioaccumulation of contaminants within its habitat.

Recently, in China, a unique culture mode known as the pond culture–forest grazing relay model has emerged for producing OR. In this model, tadpoles naturally breed with their overwintering local parents in early spring, undergo metamorphosis into young frogs, and begin grazing in the forest (Hu *et al.*, 2022). During the forest grazing phase, the frogs primarily consume natural food sources such as insects and rely on natural water sources for hydration. They engage in a grazing process that revolves around the intricate dynamics of the environment-food-chain to sustain their feeding habits. *R. dybowskii* demonstrates sensitivity not only to a wide range of heavy metals but also, due to its amphibious nature, to these contaminants through various environmental pathways (Shang *et al.*, 2019). Previous studies have shown that these frogs can accumulate heavy metals through skin penetration and ingestion, resulting in significant genotoxic effects (Li, 2014, Wang *et al.*, 2017, Han *et al.*, 2018). Some investigations on the habitat environment and heavy metal content of various tissues of *R. dybowskii* in Jilin Province showed that the heavy metal content was correlated to the heavy metal content in its living environment (Han *et al.*, 2018). However, limited research has been conducted on the presence of metal elements in OR as byproducts of their reproductive tissues. Research on trace elements in OR has predominantly focused on documenting beneficial elements. Even when studies reveal that certain heavy metals are present at levels nearing or exceeding regulatory limits, there is a notable scarcity of scholarly work that advances to conduct an in-depth risk assessment of their intake (Zhang *et al.*, 2019). Excessive accumulation of heavy metals in the human body poses significant health risks (Witkowska *et al.*, 2021, Mitra *et al.*, 2022, Zaynab *et al.*, 2022). For instance, even low concentrations of lead (Pb), cadmium (Cd), arsenic (As), chromium (Cr), or mercury (Hg) can adversely affect human health by impairing the nervous system, disrupting both digestive and male reproductive systems, causing congenital disabilities, and increasing the likelihood of cardiovascular disease and cancer (Briffa *et al.*, 2020). Moreover, certain essential metals such as aluminum (Al) can induce toxic effects with excessive exposure (Igbokwe *et al.*, 2019). Overconsumption of Al may contribute to the development of Alzheimer's disease and other neurological disorders (Rahimzadeh *et al.*, 2022). Consequently, it is imperative to detect elements in OR, particularly heavy metal elements, and assess food safety risks.

This study involved the procurement of OR specimens from a sample size exceeding 1,000 individuals, collected from six prominent production regions of Heilongjiang Province, China, including Zhangguangcai, Lesser Khingan, and Wanda Mountains. A total of 24 elements (Ag, Al, As, Ba, Cd, Co, Cr, Cs, Cu, Fe, Ga, K, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn) were selected to investigate the presence of metal elements and assess the cumulative distribution of heavy metals in OR under a pond culture–forest grazing relay model. Subsequently, a health risk assessment was conducted based on edible safety standards for these metal contents. The findings have significant implications for developing quality safety and health culture standards related to OR within the pond culture–forest grazing relay model.

Materials and Methods

Study area and sample collection

Six main *R. dybowskii*-producing areas in Heilongjiang Province, China were selected, and a total of 1,080 adult female individuals from 54 locations were also selected. These individuals were gathered during their return from the forest to the hibernating habitat below the mountain (Fan *et al.*, 2022), specifically from September to October 2022. Each city had nine sampling points, and from each sampling point, a sample of 20 individuals with similar body size (average weight of 40 ± 4.5 g) was obtained to minimize individual variations. Prior to measuring the concentration of trace elements, the OR was extracted, dried, and stored in a dry oven at room temperature.

Sample pretreatment and analysis

The sample was precisely weighed to 0.5 g and underwent microwave digestion using Microwave Digestion Instrument MARS X (CEM, USA), as previously described in our study (Tang *et al.*, 2023). Briefly, after the sample is digested, the acid is removed to 0.5 mL, the volume of pure water is fixed to 10 mL, and the sample whose determination result exceeds the linear range of the standard curve is diluted and determined again. Subsequently, the solvent and reagent blank were analyzed using an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) 7500cx (Agilent, USA) equipped with an eight-stage rod collision/reaction cell system. Twenty-four elements (Ag, Al, As, Ba, Cd, Co, Cr, Cs, Cu, Fe, Ga, K, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn) were selected to reveal the presence of trace elements in OR in the present study. The detailed detection procedures and quality control were followed by our previous study (Table S1) (Tang *et al.*, 2023). Hg was determined by an overweight mercury analyzer (Hydra IIC, Leemanlabes, USA). A standard curve was generated prior to the determination and the test was conducted only when the fitting degree exceeded 0.998. For quality assurance, 5% to 10% of the samples were randomly selected and tested six times to ensure repeatability. A certified reference material (prawn, GBW 10050) provided by the Chinese Institute of Geophysical and Geochemical Survey was used to repeat the determination of this standard, and recoveries of 90.0 %–110 % were obtained (Table S2). The relative standard deviations (RSDs) for all elements were lower than 10%, except for Hg (10.1%), Cr (12.1%), and Ag (12.5%) that had low concentrations (Table S2). The final data were calculated based on dry weight.

Heavy metal pollution evaluation

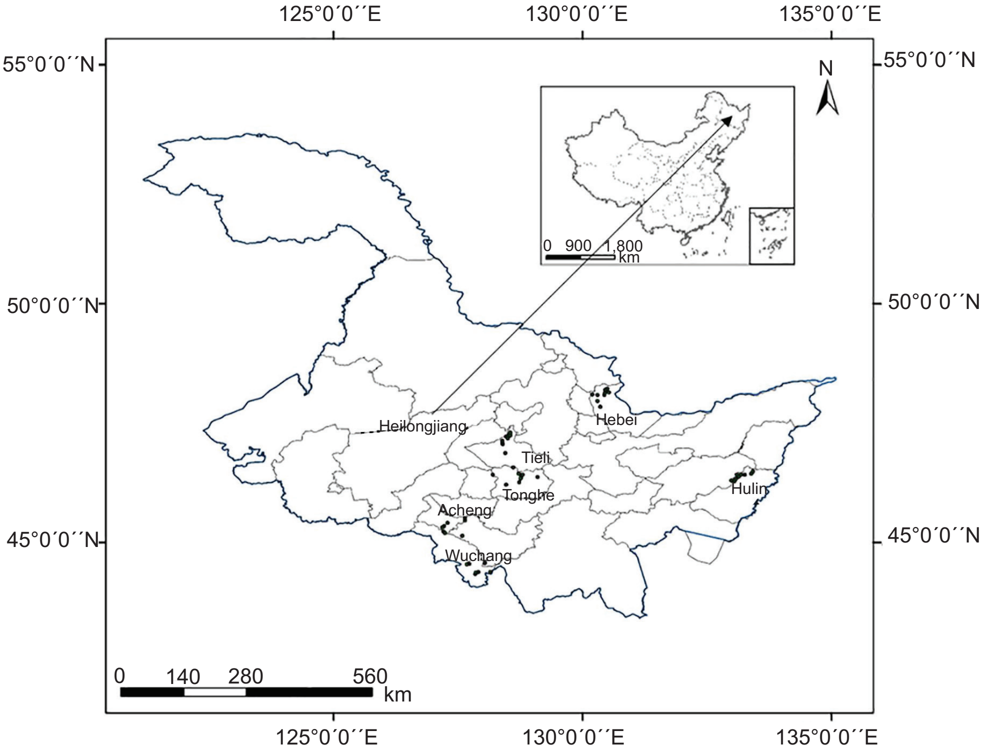
The heavy metals Cu, Pb, Cd, Cr, Hg, and As with relevant limit standards established in China were selected for the pollution index evaluation using the single-factor pollution index method:

$$P_i = \frac{C_i}{C_{si}} \tag{1}$$

In the formula (1), Pi is the pollution index of the ith heavy metal in OR; Ci is the content of the ith heavy metal or harmful element, mg kg^{-1}; Csi is the standard limit of the ith heavy metal or harmful element, mg kg^{-1}. The heavy metal limit standards in NY 5073-2006 (Limit of toxic and harmful substances in pollution-free food and aquatic products), NY/T 1516-2020 (Green food-frog and its processed products), GB/T 19507-2008 (Product of geographical indication-Jilin Changbai Mountain Oviductus Ranae), and GB 2762-2022 (National food safety standard limit of contaminants in food) were used as reference values. The pollution index Pi < 0.2 is the normal background level; Pi between 0.2 and 0.6 is a light pollution level; the medium pollution level was between 0.6 and 1.0 for Pi; Pi > 1.0 is the heavy contamination level (Figure 1).

Health risk assessment

The estimated daily intake (EDI, mg kg^{-1} Day^{-1}) of metal elements (Ag, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb,



Province	Region	Longitude(E)	Latitude(N)	Number of samples
Heilongjiang	Acheng	127.19–127.71	45.14–45.59	9
Heilongjiang	Wuchang	127.68–128.15	44.34–44.57	9
Heilongjiang	Tonghe	128.20–129.10	46.21–46.57	9
Heilongjiang	Tieli	128.39–128.63	46.87–47.30	9
Heilongjiang	Hebei	130.20–130.53	47.84–48.22	9
Heilongjiang	Hulin	132.99–132.60	46.28–46.60	9

Figure 1. Sampling regions of Oviductus Ranae in the Heilongjiang Province (n = 54).

Se, Sr, Tl, V, and Zn) depends on the concentrations of metal elements in each OR and the amount consumed daily. In the following formula, MC (mg kg^{-1}, dry weight) is the detected concentration of each metal element in the OR, and W (kg day^{-1}) refers to the daily consumption weight of OR, which is estimated by the daily consumption of aquatic products in China at 3 g day^{-1} (Li, 2014). The average weight (AW) of Chinese adults is 61.75 kg (Tang *et al.*, 2023).

$$\mathrm{EDI} = \frac{\mathrm{MC} \times \mathrm{W}}{\mathrm{AW}} \tag{2}$$

The target hazard quotient (THQi, i= Ag, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sr, Tl, V, and Zn) provides a quantitative standard to measure the exposure risk of metal elements. EFr is the exposure frequency (365 days year^{-1}. ED is the exposure duration which can last 70 years on average. RfD is the daily oral reference dose, and the average exposure time for noncarcinogens (ATn) equals ED × 365 days (Wang *et al.*, 2021). If the THQ points are <1, there would be a low risk of dietary exposure to metal elements. If not, the risk would be very high (Zhang *et al.*, 2023).

$$\mathrm{THQ_i} = \frac{\mathrm{EFr} \times \mathrm{ED} \times \mathrm{EDI}}{\mathrm{RfD} \times \mathrm{ATn} \times 1000} \tag{3}$$

Total target hazard quotient (TTHQ) is the sum of THQ of the 17 metal elements (i = Ag, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sr, Tl, V, and Zn) to assess the dietary risk of OR.

$$\mathrm{TTHQ} = \sum_{i=1}^{n} \mathrm{THQ_i} \tag{4}$$

In formula (4), TTHQ is the sum of THQ of multiple elements; TTHQ ≤ 1.0, indicates no significant negative impact; TTHQ > 1.0, indicates possible negative impact on human health; When TTHQ is greater than 10.0, it indicates that there is a chronic toxic effect.

Statistical analysis

Statistical analysis was conducted using SPSS Statistics 22.0 software. A one-way ANOVA (with Tukey post-hoc test) was employed to test the differences between different groups. For data that did not meet the normal distribution criteria, a nonparametric Kruskal–Wallis test and its post-hoc test were applied. In cases where the percentage of detection data below the limit of detection (LOD) was less than 60%, all results below the LOD were calculated as 1/2 LOD. To assess the correlations and group the metal element contents in OR from different sampling regions, Pearson correlation analysis and principal component analysis (PCA) were applied. A p-value less than 0.05 was considered statistically significant.

Results

Occurrence of metal elements in OR

Figures 2A,B illustrate the results of metallic analysis of OR samples collected from six sampling sites. Analysis of variance showed that there were significant differences in the elements of the six sampling sites ($p < 0.05$). Most of the 24 metal elements were detected in the OR at a rate of 100%, except for As, Ga, Co (98.0%), V (94.0%), Cd (89.0%), Pb (76.0%), Cs (74.0%), Tl (35.0%), and U (not detectable) (Table S3). The average concentrations of metal elements in OR were in the order of K > Na > Mg > Mn > Fe > Rb >Zn > Cu > Sr > Ba > Se > Ni > Cr > As > Hg > Cd > Ga > V > Co > Cs > Ag > Pb > Tl > U. (Table S3).

The macroelements (K, Na, Mg, Mn and Fe) and microelements (Rb, Zn, Cu, and Sr) had the highest concentrations (> 1 mg kg^{-1}) in OR samples from six cities (Table S3). Among these, the Rb content in Acheng, and the Cd, As, and Sr contents in Hulin were significantly lower than in the other cities ($p < 0.05$). Meanwhile, the Cd and Zn contents in Hebei; Cs, Rb, and Sr contents in Hulin; and As, Ba, and Tl contents in Tonghe were significantly higher than in the other cities ($p < 0.05$). The Ga content did not differ significantly across all sampling sites.

The mean concentrations of Pb (0.00898 ± 0.00709 mg kg^{-1}), Cd (0.0426 ± 0.0337 mg kg^{-1}), Cr (0.113 ± 0.0253 mg kg^{-1}), As (0.0670 ± 0.0376 mg kg^{-1}), Hg (0.0452 ± 0.00813 mg kg^{-1}), and Cu (5.92 ± 1.13 mg kg^{-1}) in all samples were much lower than the national standard limits of 0.5, 0.5, 2.0, 0.5, 0.5, and 50 mg kg^{-1}, respectively. However, 9.26% of the samples from Hebei exceeded the Chinese national safety standard for Cd (GB2762-2022).

Correlation and PCA of metal elements in OR

Pearson's correlation coefficients analysis was employed to investigate the bioaccumulation patterns of metal elements in OR under different cities. Among the 253 element pairs, 154 showed significant positive correlation and 63 showed significant negative correlation ($p <0.05$; Table 1). The degree of positive correlation between elements ranged from highest to lowest: Fe> Cu = Sr = Cr = V > Co > Rb = Ga = Cs > Hg = Na = Mn = Ni = Pb > Ba = Se = Cd = Tl > Zn = As > Mn > Ag. The degree of negative correlation ranged from highest to lowest: Cd > Ag > As > Na > Mn > Cr > Tl > Fe = Rb = Sr = Ba = Se = Ni = Cs > Zn = Hg = Pb > Mn = Cu > Ga = V = Co. All elements

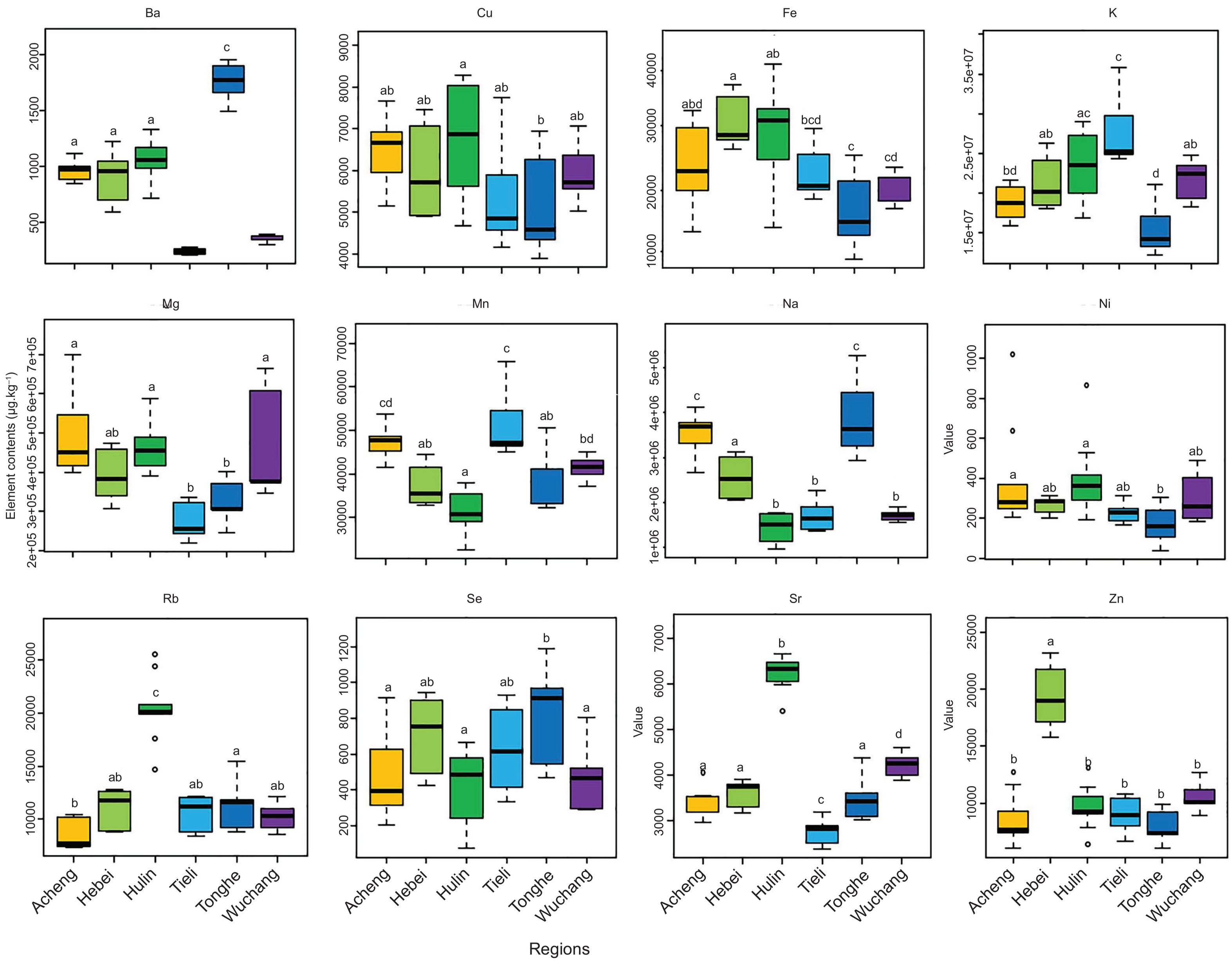


Figure 2. (A) Concentration levels of 12 macroelements and microelements in Oviductus Ranae from the Heilongjiang Province. (B) Concentration levels of 11 trace elements and microelements in Oviductus Ranae from the Heilongjiang Province.

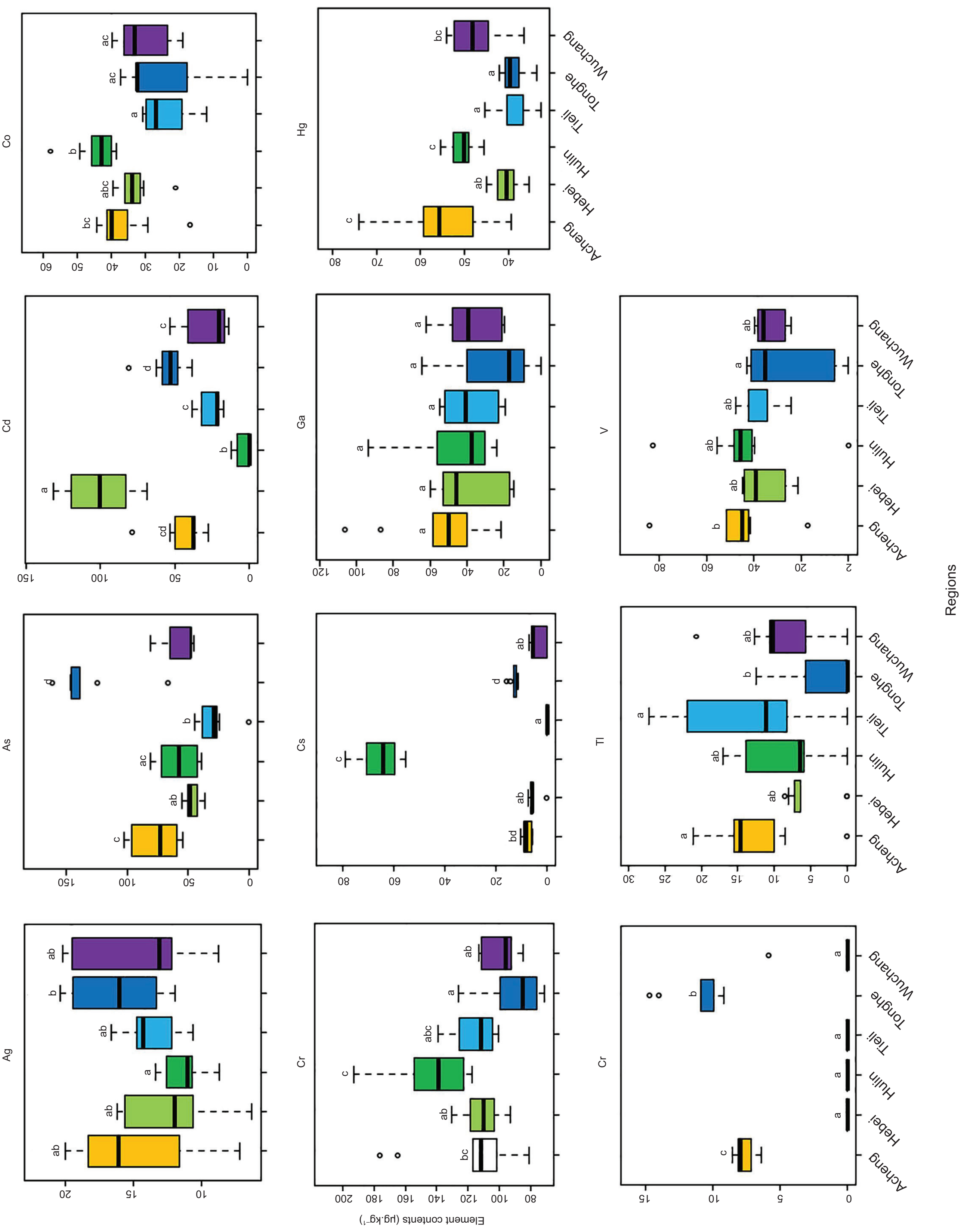


Figure 2. Continued.

Table 1. Pearson's correlation coefficients among metals in Oviductus Ranae (OR) from the Heilongjiang Province, China.

Element	K	Na	Mg	Mn	Fe	Rb	Zn	Cu	Sr	Ba	Se	Ni	Cr	As	Hg	Cd	Ga	V	Co	Cs	Ag	Pb
Na	–.432**	1																				
Mg	–0.086	–0.134	1																			
Mn	.508**	.284*	–0.183	1																		
Fe	.542**	–0.041	0.19	0.135	1																	
Rb	.305*	–.277*	–0.027	–.395**	.466**	1																
Zn	0.242	0.023	–0.014	–0.038	.670**	0.067	1															
Cu	.518**	0.18	0.161	.312*	.719**	.465**	.357**	1														
Sr	0.111	–.332*	.297*	–.554**	.381**	.881**	0.037	.447**	1													
Ba	–.650**	.604**	0.048	–.452**	–0.194	0.134	–0.145	–0.109	0.14	1												
Se	0.155	.545**	–.559**	.301*	0.246	0.125	.340*	0.263	–0.137	0.189	1											
Ni	0.159	–0.137	.739**	0.005	.476**	0.146	0.011	0.228	.308*	–0.081	–.283*	1										
Cr	.488**	–0.132	0.105	0.155	.710**	.559**	0.205	.665**	.473**	–0.195	0.024	0.243	1									
As	–.652**	.678**	–0.058	–0.184	–.394**	–0.074	–.323*	–0.124	–0.038	.774**	0.205	–0.202	–.325*	1								
Hg	–0.053	–0.064	.527**	–0.029	0.1	0.107	–0.171	.309*	.331*	–0.041	–.395**	.357**	.302*	–0.059	1							
Cd	–.365**	.409**	–0.188	–0.081	0.071	–.376**	.598**	–0.201	–.408**	.283*	.305*	–.271*	–.334*	0.138	–.323*	1						
Ga	0.135	0.111	–0.042	0.187	.472**	.367**	0.182	.438**	.354**	–0.118	0.259	0.189	.580**	–0.198	0.241	–0.02	1					
V	.335*	0.112	0.147	.307*	.687**	.325*	0.181	.571**	.298*	–0.202	0.205	.664**	.443**	–0.189	0.219	–0.158	.564**	1				
Co	0.06	0.029	0.1	–0.182	.466**	.499**	0.167	.540**	.608**	0.1	0	0.265	.460**	–0.078	.292*	–0.071	.542**	.508**	1			
Cs	0.042	–.303*	0.249	–.585**	.318*	.881**	–0.134	.305*	.894**	.287*	–0.238	.303*	.509**	0.036	.298*	–.457**	0.22	0.229	.506**	1		
Ag	–.455**	0.263	–.439**	0.004	–.480**	–0.207	–0.225	–.304*	–0.201	0.137	0.064	–.547**	–.312*	.354**	–0.096	.274*	0.154	–0.212	–0.076	–0.237	1	
Pb	.391**	–0.061	–0.022	.500**	.316*	0.064	–0.056	0.257	–0.007	–.429**	0.151	0.243	.381**	–.286*	0.045	–0.206	.447**	.451**	0.154	–0.049	–0.081	1
Tl	–.596**	.845**	–0.029	0.114	–.390**	–0.251	–.404**	–0.098	–0.259	.715**	.324*	–0.115	–.282*	.835**	0.039	0.135	–0.034	–0.088	–0.139	–0.158	.306*	–0.152

*Indicates correlation is significant at the 0.05 level (single–tailed), ** indicates correlation is significant at the 0.01 level (two–tailed).

showed significant positive correlations with each other, with Fe showing the strongest positive correlation and Cd showed the strongest negative correlation. There was no significant negative correlation between Ga, V, Co, and other elements. Tl showed strong positive correlation (rs ≥ 0.8) with As, Na, Sr, Cs, and Rb in all samples.

PCA analysis was used to reduce the dimensionality of data and identify the associated patterns between elements and sample classifications. The standardized data were subjected to PCA analysis (JECF, 2011) and the variance interpretation is shown in Figure 3. The first two principal components (PCs) were intercepted for cos2 visualization analysis (Figure 3A), which accounts for 45.5% of the cumulative variance. PC1 (explained variance of 28.9%) mainly characterized the information of Fe and Cr; PC2 (explained variance of 16.6%) mainly represented the information of Ba, Mn, Cs, K, Sr, and As; PC3 mainly represented Na and Se; PC4 mainly represented Ni and Hg; PC5 mainly represented Zn, Mg, Cd, and Ag. Accordingly, These 16 elements are characteristic elements of OR in the Heilongjiang Province (Figure 3B–D). The first two PC scores of all samples were plotted in a two-dimensional scatter plot for initial classification of the samples (Figure 4A). Although the samples could not be fully divided into six groups due to geographical differences, Hulin's samples were more dispersed than Wuchang's at the same sample size. Acheng, Wuchang, Tieli, and Hulin reflect the geographical distinction.

The clustering analysis was conducted on the top 10 contributing elements (Fe, Cr, Ba, Cs, Se, Na, Ni, Mg, Zn, and Cd) selected from the first five PCs (Figures 3D, 4B). The findings revealed three statistically significant clusters: Hulin was classified as a distinct cluster which aligns with the results obtained from Table S3 indicating relatively low pollution levels in this area. Hebei and Tonghe were assigned to cluster 2 signifying high pollution levels. Acheng, Wuchang, and Tieli were grouped under cluster 3 representing regions with moderate pollution. These observed spatial variations can be attributed to various factors including natural inputs, anthropogenic influences, geological enrichment features as well as factors regulating the bioavailability of these elements such as temperature fluctuations, water flux, and circulation dynamics along with organic matter content and differences in input sources.

Assessment of heavy metal pollution level

To determine the levels of heavy metal pollution in OR under the pond culture–forest grazing relay culture model in the Heilongjiang Province, we used Equation (1) to calculate the pollution index (Pi) for Cu, Pb, Cd, Cr, Hg, and inorganic As. As shown in Table 2, the main pollutant is Cd, while all other elements are within the normal background levels (Pi < 0.2). Except for the absence of heavy metal pollution in the Hulin sample, Cd is lightly polluted (0.2 < Pi < 0.6) in the Acheng, Wuchang,

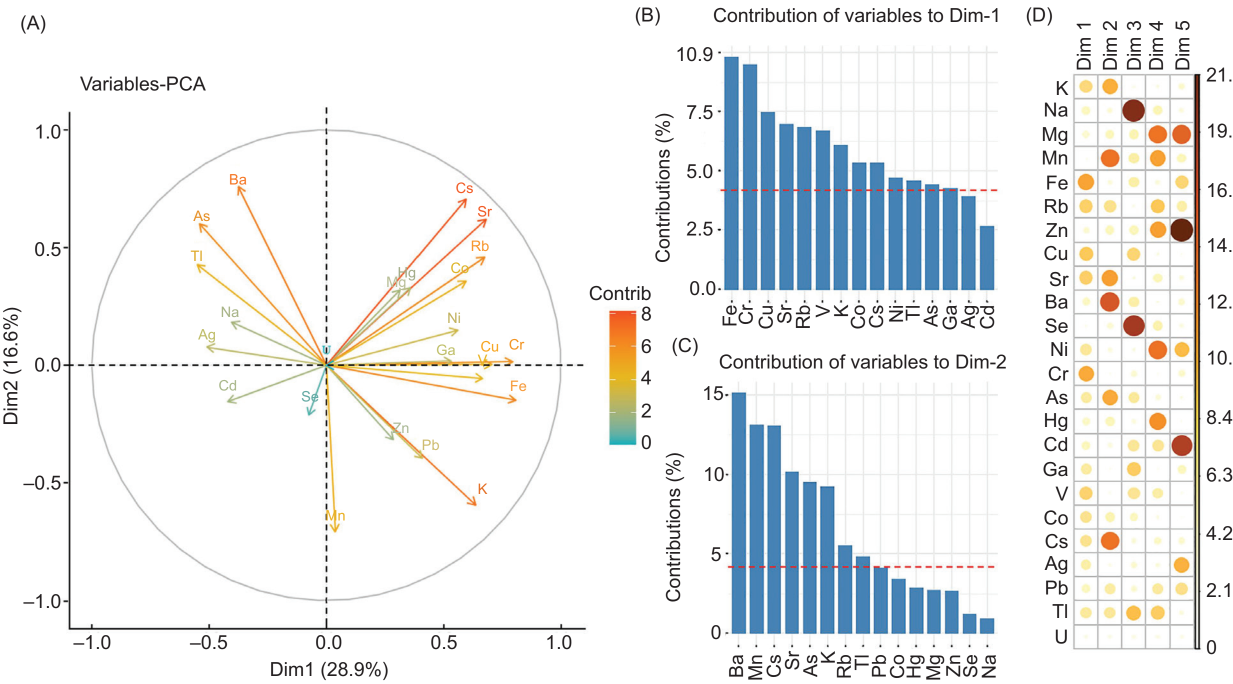


Figure 3. Principal component analysis (PCA). (A) Scatter plot scores of the first two principal components (PCs), (B) variance interpretation rate of the first axis PCA, (C) variance interpretation rate of the second axis PCA, and (D) the representation of the first five major components as elements.

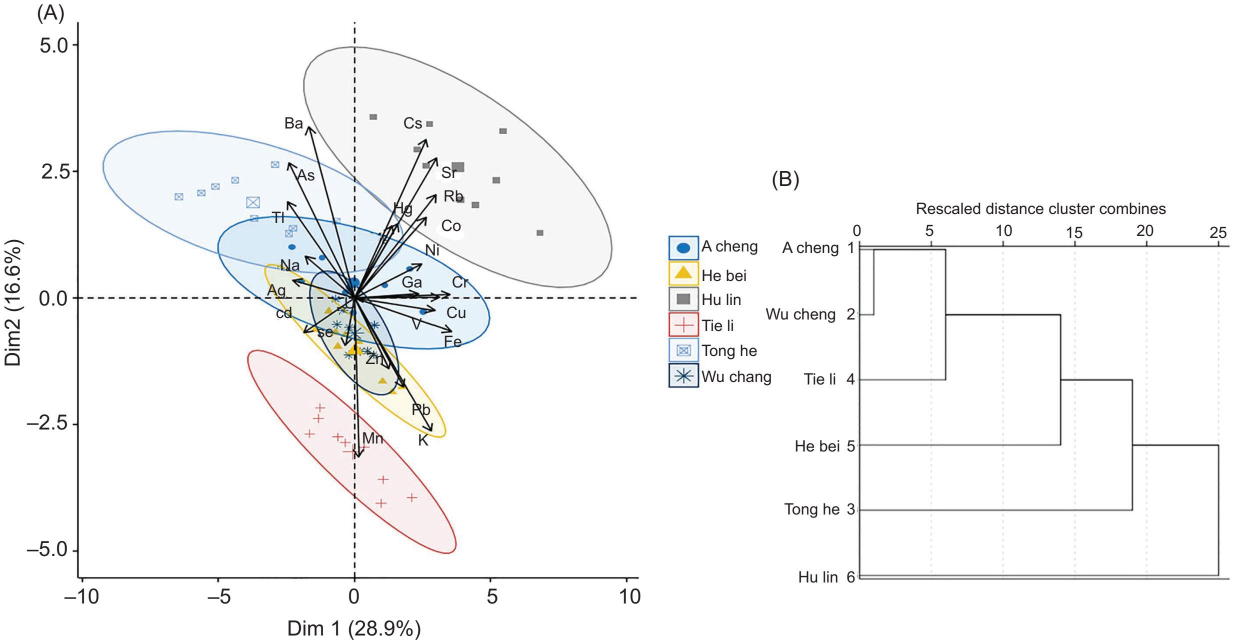


Figure 4. Scores plot of the principal component analysis (PCA) and hierarchical cluster analysis of Oviductus Ranae from in Heilongjiang Province. (A) Variance explained by the first (PC1) and second (PC2) principal components is indicated on the axis of scores plot. (B) Dendrogram of the sampling sites from the Heilongjiang Province.

Tonghe, and Tieli samples, and heavily polluted (Pi > 1.0) in the Hebei sample.

Risk to human health through consumption

Table 3 presents the EDI values of metal elements obtained from OR consumption. All metal elements demonstrate EDI values that are significantly below the provisional tolerable daily intake (PTDI) limits set by the Joint FAO/WHO Expert Committee on Food Additives and the recommended oral reference dose of elements (RfDo) established by the US Environmental Protection Agency (U.S. Environmental Protection Agency, 2016; Joint FAO/WHO Expert Committee on Food Additives, 2011). To further assess the potential adverse impacts of these elements on human health, the quantitative target risk coefficient (TRC) method proposed by the USEPA was utilized in this study. Both total hazard quotient (THQ) and TTHQ were calculated. All elements showed a THQ less than 1 and the TTHQ values of metal elements in the OR from the Heilongjiang Province were 0.152, indicating that the samples analyzed in this study did not pose any toxicological risks to human health.

Discussion

The guarantee of food safety is contingent upon the meticulous scrutiny of agricultural commodities via quality inspection and health risk assessment (Sheng *et al.*, 2019). It is imperative to undertake a comprehensive evaluation of the inorganic and heavy metal components found in OR to ascertain the absence of toxic and deleterious substances. This analysis serves to provide early warnings and risk assessments for potential quality and safety issues of the product that may arise from improper grazing behaviors of the brown frog as well as heavy metal contamination in their habitat. Concurrently, when examining through the lens of sustainable development, the preservation of the ecological environment and the sustainable advancement of agricultural production are intrinsically linked to the utmost significance of quality and safety. For instance, the adoption of unsound agricultural production methods can give rise to soil contamination, misallocation of water resources, and disturbance of ecological equilibrium (Piñeiro *et al.*, 2020). These repercussions not only hinder the sustainable development of the *R. dybowskii* pond culture–forest grazing relay model but also hold the capacity to cause irreparable damage upon the water and forest ecosystems.

The concentration of heavy metals in OR can be influenced by the heavy metal background value of the habitat and the feeding habits of *R. dybowskii*, which may have potential implications for human health given their widespread occurrence in nature (Wang *et al.*, 2017, Han *et al.*, 2018). In order to evaluate the quality and safety of OR, this study utilized an ICP-MS method to analyze the elemental composition of OR obtained from major

Table 2. Pollution index (Pi) for Oviductus Ranae (OR) from the Heilongjiang Province, China.

Elements	Limit standards (mg kg^{-1})	A cheng		Wu chang		Tong he		Tie li		He bei		Hu lin	
		Pi	Pollution level	Pi	Pollution level	Pi	Pollution level	Pi	Pollution level	Pi	Pollution level	Pi	Pollution level
Cu	50^{a}	0.129	Clean	0.118	Clean	0.103	Clean	0.106	Clean	0.119	Clean	0.135	Clean
Cr	2^{abd}	0.0595	Clean	0.0497	Clean	0.0446	Clean	0.0581	Clean	0.0555	Clean	0.0714	Clean
As	0.5^{abcd}	0.0156	Clean	0.0112	Clean	0.027	Clean	0.00592	Clean	0.00938	Clean	0.0114	Clean
Hg	0.5^{bcd}	0.109	Clean	0.0952	Clean	0.0778	Clean	0.0784	Clean	0.0813	Clean	0.101	Clean
Cd	0.5^{bcd}	0.435	Slight	0.28	Slight	0.544	Slight	0.254	Slight	1.012	Heavy	0.032	Clean
Pb	0.5^{abcd}	0.0255	Clean	0.019	Clean	0.00627	Clean	0.0287	Clean	0.0114	Clean	0.0168	Clean

[a] NY 5073 - 2006;
[b] NY/T 1516 - 2020;
[c] GB/T 19507 - 2008;
[d] GB 2762 - 2022.

production areas in the Heilongjiang Province, China. The results indicated that OR exhibited significant levels of K, Na, Mg, Mn, Fe, Rb, Zn, Cu, and Se. The aforementioned elements are of utmost importance in upholding the physiological function and biochemical metabolism of the body, with their tissue content remaining relatively stable (Zoroddu *et al.*, 2019). OR is rich in these vital elements, and prolonged consumption of it presents a considerable opportunity for augmenting essential and advantageous nutrients. Furthermore, the presence of residual heavy metals in OR is predominantly composed of Ni, Cr, As, Hg, Cd, and Pb, with a 100% detection rate for Ni, Cr, and Hg (refer to Table S3). The concentration of Cd in OR exceeded the national pollutant limit standard by 9.26%, while the levels of other heavy metals remained within the standard limits. Similar findings have been documented by other researchers in terms of elemental analysis of *R. dybowskii* or OR products (Li *et al.*, 2014 & Wang *et al.*, 2017, Han *et al.*, 2018, Shang *et al.*, 2019). However, variations in habitat environment and frog diet are likely influential factors contributing to the disparities in heavy metal content observed across different locations.

The assessment findings of the single-factor pollution index revealed that Cd was the predominant heavy metal contaminant in OR. In adherence to national guidelines, the concentrations of heavy metal constituents in OR should adhere to the pertinent stipulations outlined in the "Regulations on the Limit of Heavy Metals in Aquatic Products." Although the levels of Cu, Cr, As, Hg, Cd, and Pb in the samples collected from Hulin were within acceptable background values, indicating the absence of pollution concerns, other samples exhibited contamination specifically attributed to Cd. Notably, Hebei demonstrated a significantly elevated degree of Cd pollution (refer to Table 2). In this study, we employed the Pearson correlation coefficient to examine the correlation among 23 identified elements in OR sourced from the primary producing regions of the Heilongjiang Province. These correlations are influenced by the interactions of organisms, both synergistic and antagonistic, as well as the impact of environmental microorganisms and enzyme activity on physiological processes such as heavy metal enrichment and metabolism in organisms. Out of the total, 154 pairs of elements displayed a statistically significant positive correlation, whereas 63 pairs of elements exhibited a statistically significant negative correlation. Among these pairs, Cd demonstrated the most pronounced negative correlation, while Fe showcased the most robust positive correlation.

Cd, a heavy metal known for its toxicity and carcinogenic properties, presents a substantial threat to human health. Its main sources are industrial processes, such as landfill leachate, fossil fuel combustion, mining waste, and

Table 3. Estimated daily intakes (EDI) and target hazard quotient (THQ) of trace elements due to Oviductus Ranae (OR) consumption.

Element	RfDo/ μg kg^{-1}·d^{-1}	A cheng		Wu chang		Tong he		Tie li		He bei		Hu lin		Heilongjiang Province	
		EDI	THQ	EDI	THQ	EDI	THQ	EDI	THQ	EDI	THQ	EDI	THQ	EDI	THQ
Mn	140[a]	3.48	0.0249	3.03	0.0216	2.77	0.0198	3.74	0.0267	2.71	0.0194	2.28	0.0163	3	0.0214
Fe	800[b]	1.76	0.0022	1.45	0.00182	1.18	0.00148	1.65	0.00206	2.27	0.00284	2.14	0.00268	1.74	0.00218
Zn	300[a]	0.633	0.00211	0.766	0.00255	0.577	0.00192	0.652	0.00217	1.4	0.00468	0.708	0.00236	0.79	0.00263
Cu	500[b]	0.47	0.00094	0.43	0.00086	0.375	0.00075	0.388	0.00078	0.435	0.00087	0.49	0.00098	0.431	0.00086
Sr	600[a]	0.245	0.00041	0.308	0.00051	0.257	0.00043	0.201	0.00034	0.263	0.00044	0.454	0.00076	0.288	0.00048
Ba	200[a]	0.0702	0.00035	0.0262	0.00013	0.127	0.00064	0.0179	9E-05	0.066	0.00033	0.076	0.00038	0.0639	0.00032
Se	5:00 AM	0.0357	0.00715	0.0335	0.00671	0.0602	0.012	0.0466	0.00931	0.0524	0.0105	0.0305	0.00609	0.0431	0.00863
Ni	20[a]	0.0289	0.00144	0.0219	0.00109	0.0122	0.00061	0.0167	0.00084	0.0194	0.00097	0.0295	0.00148	0.0214	0.00107
Cr	3:00 AM	0.00868	0.00289	0.00724	0.00241	0.0065	0.00217	0.00846	0.00282	0.00809	0.0027	0.0104	0.00347	0.00823	0.00274
As	0.3[a]	0.00568	0.0189	0.00407	0.0136	0.00983	0.0328	0.00216	0.00719	0.00342	0.0114	0.00416	0.0139	0.00488	0.0163
Hg	0.571[b]	0.00396	0.00694	0.00347	0.00607	0.00283	0.00496	0.00286	0.005	0.00296	0.00519	0.00368	0.00645	0.00329	0.00577
Cd	1:00 AM	0.00317	0.00317	0.00204	0.00204	0.00397	0.00397	0.00185	0.00185	0.00737	0.00737	0.00023	0.00023	0.00311	0.00311
V	9:00 AM	0.00347	0.00039	0.00246	0.00027	0.00192	0.00021	0.00273	0.0003	0.0026	0.00029	0.00325	0.00036	0.00274	0.0003
Co	0.3[a]	0.00264	0.0088	0.00222	0.00742	0.00188	0.00626	0.00177	0.00591	0.0024	0.00801	0.00323	0.0108	0.00236	0.00786
Ag	5:00 AM	0.00107	0.00021	0.00107	0.00021	0.00119	0.00024	0.001	0.0002	0.00091	0.00018	0.00082	0.00017	0.00101	0.0002
Pb	4[c]	0.00093	0.000233	0.000694	0.00017	0.00023	0.0000571	0.00105	0.00026	0.00041	0.0001	0.00061	0.00015	0.00065	0.00016
Tl	0.003[a]	0.000559	0.186	0.0000472	0.0157	0.00081	0.269	0	0	0	0	0	0	0.00024	0.0785
TTHQ			0.267		0.0832		0.357		0.0658		0.0752		0.0664		0.152

[a] RfDo: oral reference dose of elements as established by the USEPA. (Retrieved May 1, 2023 from: https://iris.epa.gov/AtoZ/?list_type=alpha)

[b] RfDo for Fe, Cu and Hg were used the provisional tolerable daily intake (PTDI) limits set by JECFA (Retrieved May 1, 2023 from: https://apps.who.int/food-additives-contaminants-jecfa-database/.).

[c] RfDo for Pb were used (Wang et al., 2021).

human activities like pesticide application. Each year, an estimated 25,000 tons of Cd are discharged into the environment, where it can infiltrate organisms through respiration, ingestion of contaminated food, and consumption of contaminated drinking water, leading to bioaccumulation. Cd exposure through food consumption has been linked to the occurrence of neurotoxicity, the development of osteoporosis, gastrointestinal damage, renal failure, and systemic dysfunction. In addition, cadmium exposure has been found to cause organ-specific injuries in different animal species, such as head kidney injury in carp, cardiac toxicity in mice, increased peripheral blood lymphocyte necrosis in chickens, and effects on the human reproductive system (Noverita *et al.*, 2021 & Muhammad *et al.*, 2023). In frogs, toxicological effects similar to those observed in other vertebrates have been identified. Heilongjiang Province, being a significant heavy industry and commodity grain hub in China, has maintained a longstanding concern regarding heavy metal contamination in water and soil. Numerous studies and monitoring findings suggest that the overall extent of heavy metal pollution in water, sediment, and soil within the Heilongjiang Province, as well as its associated ecological risks, are relatively minimal. However, it is worth noting that the concentration of Cd is comparatively elevated. Hence, the findings of this study align with the broader outcomes of environmental monitoring (Zhang *et al.*, 2018 & Zhao *et al.*, 2021). Given its significant impact on human health in the soil and water ecosystems of China, the exposure to Cd should be duly acknowledged during the forest grazing phase of *R. dybowskii* in Heilongjiang Province.

The PCA conducted on the OR identified the presence of numerous distinctive elements, namely, Fe, Cr, Ba, Mn, Cs, K, Sr, As, Na, Se, Ni, Hg, Zn, Mg, Cd, and Ag. The comprehensive examination of a wide range of elements can be attributed to the relatively low variance interpretation rate. It is noteworthy that each element made a substantial contribution to the outcomes derived from the PCA analysis. PC1 (Fe and Cr) and PC2 (Ba, Mn, Cs, K, Sr, and As) are likely influenced by exogenous pollution originating from geographical factors, industrial activities, and agricultural practices such as mining, fertilization, and pesticide use. PC3 (Na and Se) may be associated with the utilization of commercial feed. Lastly, PC4 (Ni and Hg) along with PC5 (Zn, Mg, Cd, and Ag) appear to be primarily linked to activities in the electronics industry, printing, dyeing, and metallurgy. Therefore, it can be concluded that the PCs are closely intertwined with both the characteristics of the natural habitat and human activities.

Excessive consumption of essential elements, despite their significance in the human diet, can detrimentally impact human health (Abbaspour *et al.*, 2014). Prolonged exposure to toxic elements, including arsenic, cadmium, lead, and mercury, even at minimal concentrations, can be deleterious (Briffa *et al.*, 2020). The THQ serves as a benchmark for assessing the potential harm associated with exposure to both essential and toxic elements. A TTHQ value below 1 signifies a negligible or minimal risk for human consumption. It is crucial to acknowledge that the evaluation of risk is contingent upon the dietary patterns observed within distinct populations in respective regions. In the present study, it was found that Mn and As exhibited the highest contribution to the TTHQ. Mn, being an advantageous element for the human body, primarily functions as an activator for numerous enzymes within the human system. In addition, it is intricately associated with the synthesis of sugar, amino acids, proteins, cholesterol, and fat metabolism. Conversely, As and its compounds possess toxic properties, whereby excessive levels of arsenic can disrupt normal cellular metabolism, impede respiration and oxidation processes, and induce cytopathic alterations. Furthermore, it should be noted that excessive intake of As can lead to detrimental effects on small arteries and capillary walls, as well as its impact on vasomotor centers, ultimately causing an elevation in vascular permeability. Consequently, this can lead to a decrease in blood volume and exacerbate organ damage. It is worth considering that adhering to a daily consumption of less than 210 g of OR for adults, with an average body weight of 61.75 kg, would align with the recommended daily oral reference dose for manganese consumption set by the United States Environmental Protection Agency (USEPA, Retrieved May 1, 2023 from: https://www.epa), thereby minimizing potential risks associated with excessive manganese intake. We prudently decreased this quantity by a factor of 20 (Wang *et al.*, 2021), resulting in a maximum daily intake of 10.5 g, which is considered a safe dosage. This amount aligns with the daily dose of 5–15 g recommended by the Chinese Pharmacopeia (ChP, 2020).

Conclusions

The present study conducted a comprehensive analysis of the content and health risk assessment of 24 metal elements in OR obtained from six major producing areas within the Heilongjiang Province, China. The findings demonstrated notable disparities in the metal element concentrations among OR samples originating from distinct production regions. Despite the detection of Cd contamination in the majority of samples, the TTHQ risk assessment results indicate a relatively low health risk associated with the consumption of OR produced through the pond culture–forest grazing relay model in the Heilongjiang Province, China. To improve the precision of monitoring and evaluation, future studies will focus on expanding the sampling range and enhancing accuracy.

Author Contribution

G.H. was in charge of conceptualization; S.T, P.L., D.Q., and Z.X. were responsible for methodology; S.T. was in charge of software; S.T, D.Q., and Z.X. did the validation; G.H., Z.X., and S.T. were concerned with formal analysis; F.C., P.L., S.T, D.Q., and Z.X. did the investigation; G.H. and P.W. were responsible for resources; G.H., Z.X., and S.T. did data curation; G.H. and S.T. wrote the original draft; G.H. and S.T. did the writing—review and editing,; G.H., Z.X., and S.T. did the visualization; G.H., Z.X., and P.W. supervised the study; project administration and funding acquisition were done by G.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The methodology employed in this work was approved by the Ethics Committee on the Use of Animals of Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences (Protocol numbers 2022-12-01-03). All methods were carried out in accordance with relevant guidelines and regulations. The study is reported in accordance with ARRIVE guidelines.

Data Availability Statement

All the data have been fully presented in the article.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary

Table S1. Operation parameters of ICP-MS (Agilent 7500 cx).

Operation parameters (optimized daily)	
RF power	1,500 W
Reflected power	<15 W
Plasma gas flow rate	15 L min^{-1}
Nebulizer gas flow rate	0.98 L min^{-1}
Auxiliary gas flow rate	0.24 L min^{-1}
Collision gas H_e	4.0 mL min^{-1}
Makeup gas	0.15 L min^{-1}
Nebulizer pump	0.10 rps
Uptake speed	0.40 rps
Uptake time	45 s
Stabilization Time	30 s
Acquisition	Spectrum(Multi Tune)
Peak Pattern	Full Quant(3)

Table S2. Summary of analyte masses, elements for internal standard method (ISTD), analytical conditions for octopole reaction system (ORS), correlation coefficient of standard curve(R), limits of detection (LOD), and results of quality control for study elements.

Analyte	Isotope	ISTD	ORS	R	LOD (mg·kg^{-1})	RSD (%)	GBW10050 (mg·kg^{-1})	Found value (mg·kg^{-1})	Recovery (%)
Na*	23	^{45}Sc	He	0.9998	2.04	8.42	0.31 ± 0.02	0.30 ± 0.03	96.8
Mg*	24	^{45}Sc	He	1.0000	0.079	2.22	0.169 ± 0.006	0.158 ± 0.004	93.5
K*	39	^{45}Sc	He	0.9998	0.306	8.11	0.49 ± 0.01	0.47 ± 0.04	95.9
V	51	^{45}Sc	He	0.9999	0.003	8.56	0.24 ± 0.07	0.24 ± 0.02	100
Cr	53	^{45}Sc	He	0.9999	0.012	**12.1**	0.35 ± 0.11	0.33 ± 0.04	94.3
Mn	55	^{45}Sc	He	1.0000	0.105	1.96	8.9 ± 0.3	9.06 ± 0.18	102
Fe	56	^{45}Sc	He	**0.9980**	1.26	7.56	112 ± 12	113 ± 8.54	101
Co	59	^{45}Sc	He	0.9999	0.001	8.59	0.044 ± 0.005	0.042 ± 0.004	95.4
Ni	60	^{72}Ge	He	0.9999	0.025	2.71	0.23	0.21 ± 0.026	91.3
Cu	63,65	^{72}Ge	He	0.9998	0.128	8.15	10.3 ± 0.7	10.1 ± 0.82	98.1
Zn	64,66	^{72}Ge	He	0.9998	0.004	5.92	76 ± 4	76.6 ± 4.54	101
Ga	71	^{72}Ge	He	1.0000	0.001	5.13	0.01^a	0.009 ± 0.001	**90.0**
As	75	^{72}Ge	He	0.9999	0.003	8.16	2.5	2.54 ± 0.21	102
Se	82	^{72}Ge	He	0.9997	0.020	5.29	5.1	4.94 ± 0.26	96.9
Rb	85	^{72}Ge	He	0.9998	0.002	4.69	1.4 ± 0.1	1.39 ± 0.06	99.3
Sr	88	^{72}Ge	He	1.0000	0.003	6.68	20 ± 2	19.5 ± 1.31	97.5
Ag	107	^{103}Rh	He	0.9998	0.001	**12.5**	0.017	0.017 ± 0.002	100
Cd	111,114	^{103}Rh	He	0.9998	0.001	4.44	0.039 ± 0.002	0.039 ± 0.002	100
Cs	133	^{103}Rh	He	0.9998	0.001	6.66	0.027 ± 0.002	0.026±0.002	96.3
Ba	137,138	^{103}Rh	He	0.9999	0.004	5.50	2.3 ± 0.3	2.37±0.13	103
Tl	205	^{209}Bi	He	0.9998	0.001	5.41	0.01^a	0.011±0.001	**110**
Pb	206,207,208	^{209}Bi	He	0.9998	0.003	9.76	0.20 ± 0.05	0.21±0.02	105
U**	238	^{209}Bi	He	0.9996	0.001	8.20	9.7 ± 0.8	9.9 ± 0.8	102
Hg**	202	/	/	0.9991	0.001	**10.1**	49 ± 8	50 ± 5	102

The data are represented as means ± standard deviation.
^{a}Spiked test sample in 0.010 mg·kg^{-1}.
*The unit of concentration of element is 10^{-2}.
**The unit of concentration of element is 10^{-9}.

Table S3. Mean concentrations of heavy metals in Oviductus Ranae (μg kg^{-1} d.w).n = 54.

Group	Elements	A cheng	Wu chang	Tong he	Tie li	He bei	Hu lin	MIN	MAX	Average	SD	Detection rate/%
Macro-elements	K	1.87E+07	2.17E+07	1.53E+07	2.75E+07	2.13E+07	2.34E+07	1.23E+07	3.58E+07	2.13E+07	4.89E+06	100
	Na	3.55E+06	1.71E+06	3.88E+06	1.72E+06	2.57E+06	1.45E+06	9.71E+05	5.27E+06	2.48E+06	1.04E+06	100
	Mg	4.91E+05	4.65E+05	3.27E+05	2.74E+05	3.91E+05	4.64E+05	2.19E+05	6.98E+05	4.02E+05	1.13E+05	100
	Mn	4.78E+04	4.15E+04	3.80E+04	5.13E+04	3.72E+04	3.12E+04	2.25E+04	6.58E+04	4.12E+04	8.24E+03	100
	Fe	2.41E+04	2.00E+04	1.62E+04	2.27E+04	3.12E+04	2.94E+04	8.21E+03	4.13E+04	2.39E+04	7.41E+03	100
Micro-elements	Rb	8.60E+03	1.03E+04	1.15E+04	1.07E+04	1.12E+04	2.04E+04	7.37E+03	2.55E+04	1.21E+04	4.33E+03	100
	Zn	8.69E+03	1.05E+04	7.92E+03	8.94E+03	1.93E+04	9.72E+03	6.04E+03	2.32E+04	1.08E+04	4.29E+03	100
	Cu	6.45E+03	5.90E+03	5.15E+03	5.32E+03	5.97E+03	6.73E+03	3.90E+03	8.29E+03	5.92E+03	1.13E+03	100
	Sr	3.36E+03	4.22E+03	3.52E+03	2.76E+03	3.60E+03	6.22E+03	2.38E+03	6.67E+03	3.95E+03	1.16E+03	100
	Ba	9.64E+02	3.59E+02	1.75E+03	2.46E+02	9.05E+02	1.04E+03	2.15E+02	1.96E+03	8.77E+02	5.16E+02	100
	Se	4.90E+02	4.60E+02	8.25E+02	6.39E+02	7.19E+02	4.18E+02	7.39E+01	1.19E+03	5.92E+02	2.60E+02	100
	Ni	3.96E+02	3.00E+02	1.68E+02	2.29E+02	2.66E+02	4.05E+02	3.81E+01	1.02E+03	2.94E+02	1.67E+02	100
Trace-elements	Cr	119	99.4	89.2	116	111	143	71	193	113	25.3	100
	As	77.9	55.8	135	29.6	46.9	57.0	ND	161	67.0	37.6	98.0
	Hg	54.3	47.6	38.9	39.2	40.6	50.5	32.6	74.0	45.2	8.13	100
	Cd	43.5	28.0	54.4	25.4	101	3.20	ND	132	42.6	33.7	89.0
	Ga	53.7	37.9	26.6	36.5	38.2	48.9	ND	106	40.3	22.1	98.0
	V	47.7	33.8	26.4	37.4	35.6	44.6	ND	84.3	37.6	15.6	94.0
	Co	36.2	30.5	25.8	24.3	33.0	44.3	ND	57.8	32.4	10.4	98.0
	Cs	7.84	3.43	12.9	ND	5.45	65.9	ND	79.1	15.9	23.2	74.0
	Ag	14.7	14.7	16.3	13.7	12.5	11.3	6.28	20.4	13.9	3.65	100
	Pb	12.8	9.52	3.13	14.4	5.68	8.41	ND	27.2	8.98	7.09	76.0
	Tl	7.68	0.648	11.1	ND	ND	ND	ND	14.7	3.23	4.63	35.0
	U	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0

ND: not detectable.