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Original article

Acute toxic effects of cadmium in larvae of the green toad, *Pseudepidalea variabilis* (Pallas, 1769) (Amphibia: Anura)

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The environmental impact of cadmium use and its accumulation in nature have increased to alarming levels. This study aimed to morphologically and histologically investigate the acute toxic effects of cadmium on green toad, *Pseudepidalea variabilis* (Pallas, 1769) larvae. Embryos were obtained from specimens collected in amplexus from nature and kept under laboratory conditions until stage 26, when they were exposed to cadmium (0, 1, 5, 10, 25, and 50 μg L^{-1}) for 96 h. The $LC_{10,}$ LC_{50}, and LC_{90} values of cadmium were calculated to be 26.98, 35.35, and 46.31 μg L^{-1}, respectively. Our results showed that cadmium had a negative effect on the body size of *P. variabilis* larvae (over 1 μg L^{-1}). Histological examination detected a fusion of gill lamellae, liver haemorrhage, oedema in the abdominal cavity, and deformations of pronephric tubules (over 10 μg L^{-1}). Our findings suggest that the green toad was sensitive to the cadmium treatment, with LC_{50} values lower than those reported by other studies. Thus, this species could be considered a reliable indicator species of environmental stress in aquatic ecosystem.

KEY WORDS: *embryotoxicity; heavy metal; histopathology; lethal concentrations*

The decline in amphibian populations has reached striking dimensions in recent years (1, 2). There are several hypotheses about the possible reasons behind this phenomenon (3, 4). Some of these hypotheses include impact of urbanization due to rapid increase in settlements and industrialization, chemical pollution, habitat degradation/destruction, and diseases (5-8). Among these, the destruction of habitats and increased pollution significantly threaten the survival of amphibians, which spend their embryonic and larval stages exclusively in water and play a significant role in biomonitoring programmes (9-11). That is why amphibian larvae are among the preferred bioindicators for aquatic toxicology studies (12-16).

Cadmium is present in the environment naturally; however, it is not an essential element for organisms (17). Increases in the amount of cadmium in nature might be either due to natural factors, like the ablation of rocks resulting from erosion and rain, or anthropogenic factors (18). In Turkey, for instance, one study (19) found the mean cadmium concentration in surface waters to be 110 μg L^{-1}.

The half-life of cadmium in organisms is 20 years (20). It cannot be excreted from the body and accumulates in the organism and throughout the food chain. Its biomagnification poses threats for human health as well as the ecosystem as a whole. Therefore, cadmium has recently become the subject of many investigations (16, 21-25). The toxic effects of cadmium investigated in freshwater fish include development abnormalities detected in many teleost species (26-28). Cadmium led to larval teratogenic and developmental abnormalities (29), to micronucleus induction at environmental levels of 2, 10, and 30 μg L^{-1}, and to increased concentration-dependent quantities of micronucleus after contamination with 10 and 30 μg L^{-1} in *Xenopus* larvae (30). In addition,

cadmium exposure caused a decline in the survival numbers of *Bufo americanus* (13), developmental delays in tadpoles of *Bufo raddei* (31), and toxic acute effects on early-life stages in *Duttaphrynus melanostictus* at levels of 0.2 mg L^{-1} (23). On the contrary, cadmium stimulated growth and development in amphibian larvae at concentrations from 0.25 to 5 μg L^{-1} (32).

Known as the green toad, *Pseudepidalea variabilis* is a common amphibian species in Turkey. This terrestrial and nocturnal species is water-dependent in the breeding season, when it is known to stay in water for long periods of time (33). This species generally inhabits slow-flowing and stagnant waters, seasonal ponds, and shallow pits filled with water for egg-laying (34). Its aquatic habitats are prone to increased heavy metal concentrations due to agricultural and industrial activities. To our knowledge, until now no related study on the acute toxic effects of cadmium on *P. variabilis* larvae has been conducted.

This study aimed to determine the lethal concentrations ($LC_{10,}$ LC_{50}, and LC_{90}) of cadmium and the acute toxic effects on the tissues and organs of *P. variabilis* larvae after a 96-h acute exposure.

MATERIALS AND METHODS

Animals and treatment

The green toad, *Pseudepidalea variabilis* (Pallas, 1769), is included in the "data deficient" (DD) category of the IUCN Red List (35). Tadpoles were obtained in the laboratory from 4 adult (2 ♂♂, 2 ♀♀) *P. variabilis* caught in amplexus in March 2010, in a seasonal pond in Çanakkale, north-western Turkey. *P. variabilis* specimens were transferred to the laboratory and kept in polypropylene containers until ovulation. After ovulation, embryos were transferred to 30×30×25 cm glass aquaria. After hatching, the larvae were fed with fish feed of plant origin (100 g/ carbohydrate: 30.16 g; sugars: 7.33 g; dietary fibre: 9.3 g; fat: 19.94 g; protein: 36.49 g; energy: 450 kcal) and boiled lettuce leaves until stage 26 (36).

Cadmium chloride ($CdCl_2$) was supplied from Sigma-Aldrich Chemical Company Inc. (St. Louis, MO, USA). A stock solution of 100 μg L^{-1} was prepared by dissolving $CdCl_2$ powder in distilled water. Larvae at stage 26 were exposed to concentrations of 1, 5, 10, 25, and 50 μg L^{-1} of cadmium. The dosing solutions were prepared by appropriate dilutions of the stock solution.

Twenty randomly selected *P. variabilis* larvae were exposed to control (water) and each cadmium concentration, in acute (96 h) exposure experiments, in a static bioassay test system. Aquaria were filled with 2 L of water or cadmium solutions and during exposure, temperature, pH, and dissolved oxygen levels were measured daily with an Elmetron CO-401 meter (analysio GmbH, Greifswald, Germany). The treatment was carried out in a 14:10-h light-dark cycle and the number of dead tadpoles was recorded and faeces removed every 24 hours.

Morphology and histology

Ten randomly selected larvae from the control and each treatment group were measured for total length, width, and tail length. Furthermore, wet weights of the larvae were determined (0 and 96 h). Wet weight (precision 0.001 g) and total length (precision 0.01 mm) were measured after euthanasia with a digital scale and digital calipers. Ten larvae survived after the 96 h of exposure were fixed in Bouin's solution for histological examination after macroscopic evaluation. Afterwards, these larvae were processed through alcohol, xylene, and paraffin series and then paraffin blocks were prepared. Serial cross sections 6-8 μm in thickness were obtained from these blocks, stained with hematoxylin and eosin (H&E), and histopathologically examined under a light microscope. Finally, the histological imaging of the preparations was carried out using a camera mounted on an Olympus BX51 light microscope (Japan) and analysed using the DP2-BSW software.

Statistical analysis

Finney's Probit analysis method was used to calculate 96-h LC_{10}, LC_{50}, and LC_{90} values of cadmium for *P. variabilis* larvae. Lethal concentrations were calculated at 95 % confidence intervals. Comparisons of total length, tail length, width and weight between control and treatment groups were done using one-way analysis of variance (ANOVA). Discriminant analysis was done in order to show the possible total differences of the concentrations tested. Statistical analyses were performed using SPSS software (ver. 16.0) and alpha was set at 0.05.

RESULTS

Acute toxicity

Mean water temperature was 21.2±2 °C, while pH and dissolved oxygen in aquaria were 6.8±0.3 and 8.2±0.4 mg L^{-1}, respectively. No mortality was recorded in the control group and in the cadmium groups exposed to 1 and 5 µg L^{-1}. The highest mortality was observed at 50 µg L^{-1}, where 10 larvae died within the first 24 h and only one larva survived at the end of the exposure period. After 72 h, a dead larva was found in the 10 µg L^{-1} group, whereas 8 larvae died in the 25 µg L^{-1} group. Values of 96-h LC_{10}, LC_{50}, and LC_{90} are presented in Table 1.

Morphology and histology

Total length was the only parameter found to be significantly different between treatments ($p<0.05$). Comparisons of total length, width, tail length, and wet weight between control and treatment groups are presented in Table 2. Since only one larva survived the 50 µg L^{-1} concentration to the end, this group was not included in the statistical analysis.

The canonic discriminant analysis was performed to assess differences in total body length among groups. It showed two functions, which explained 100 % of the total variance (Figure 1) and the *p*-value of function 1 was significant (Table 3).

No abnormalities were found in the histological examinations of larvae from the control group. Likewise, no significant histological findings were detected in the groups exposed to 1 and 5 µg L^{-1} of cadmium. However, deformations and gill lamellar fusions were recorded in some larvae at 10 and 25 µg L^{-1} (Figure 2).

Furthermore, effects such as deformations of pronephric tubules structure were prominent and clearly observed at increasing concentrations (Figure 3).

No histopathological alterations were encountered in the cross sections of the livers of larvae in the control group. Nevertheless, deformation and haemorrhage in liver were observed in most of the larvae exposed to 10 and 25 µg L^{-1} of cadmium. In addition, vacuolization and increments in the distance of the intercellular area were observed in the hepatocytes of the same sections (Figure 4).

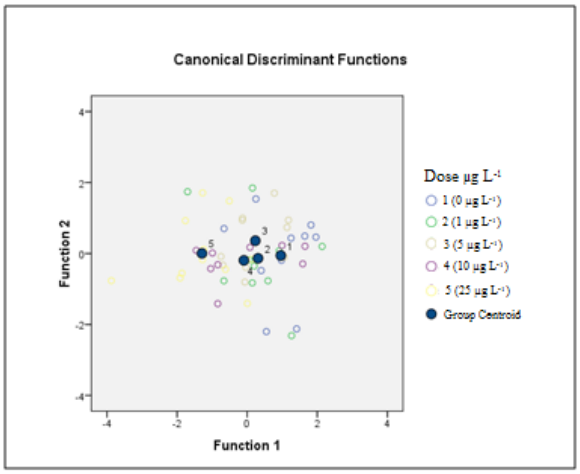

Figure 1 *According to total length measurements, the total differences of exposure concentrations between groups*

A severe visceral oedema was detected in larvae at 25 µg L^{-1} in the cross-sections of the internal organs (Figure 5). As only one larva survived at 50 µg L^{-1}, the histopathological findings are not presented here.

The incidence of observed histopathological findings of the ten larvae is presented in Table 4.

DISCUSSION

There is a strong relationship between increasing concentrations of cadmium and the decline in the hatching success of frog embryos (37). Cadmium LC_{50} values determined in previous acute toxicity studies on amphibian larvae vary considerably. The 96-h LC_{50} of cadmium for *Bufo arenarum* larvae was reported in the range from 2.19 to 6.77 mg L^{-1} (38). The LC_{50} values for cadmium in *R. ridibunda* larvae calculated in two different studies were 0.45 mg L^{-1} (39) and 71.8 mg L^{-1} (40). This last value is very close to the 90-h LC_{50} reported in *Xenopus laevis* larvae - 80 to

Table 1 *Lethal concentrations after cadmium exposure in* P. viridis *for 96 hours*

Lethal concentrations	Concentration (µg L^{-1})	95 % Confidence interval	
		Lower bound	Upper bound
LC_{10}	26.98	21.05	31.24
LC_{50}	35.35	30.41	41.09
LC_{90}	46.31	40.05	59.36

Table 2 *Comparison of morphological measurements of treatment groups*

Measurements	Cd (µg L^{-1})	n	mean	SD	SE	95 % Confidence interval for mean		Min.	Max.	*p*
						Lower bound	Upper bound			
Total length (mm)	0	10	15.76	0.89	0.28	15.12	16.40	13.95	16.77	
	1	10	15.08	1.18	0.37	14.23	15.92	12.72	16.99	
	5	10	14.88	0.75	0.26	14.25	15.51	13.95	15.88	0.00
	10	10	14.78	1.30	0.45	13.69	15.86	13.18	16.47	
	25	10	13.45	1.38	0.48	12.29	14.61	10.70	14.91	
Width (mm)	0	10	3.08	0.31	0.09	2.85	3.30	2.51	3.61	
	1	10	3.20	0.85	0.27	2.59	3.82	0.92	4.04	
	5	10	3.03	0.37	0.13	2.72	3.35	2.14	3.27	0.69
	10	10	3.29	0.25	0.09	3.07	3.50	3.04	3.77	
	25	10	2.97	0.36	0.12	2.66	3.27	2.15	3.37	
Tail length (mm)	0	10	9.62	0.78	0.24	9.07	10.18	7.90	10.38	
	1	10	9.19	0.78	0.24	8.64	9.75	7.72	10.11	
	5	10	9.39	0.87	0.35	7.75	9.53	7.54	10.78	0.34
	10	10	9.43	0.87	0.30	8.70	10.15	7.90	10.70	
	25	10	8.78	1.19	0.42	7.79	9.78	6.25	10.20	
Wet weight (g)	0	10	0.03	0.01	0.01	0.02	0.03	0.03	0.04	
	1	10	0.03	0.01	0.01	0.02	0.03	0.03	0.04	
	5	10	0.03	0.01	0.01	0.02	0.03	0.03	0.04	0.33
	10	10	0.03	0.01	0.01	0.02	0.03	0.02	0.04	
	25	10	0.02	0.01	0.01	0.01	0.03	0.01	0.04	

100 mg L^{-1} (41), but both were substantially above the LC_{50} calculated for *X. laevis* embryos - 850 µg L^{-1}. The cadmium 96-h LC_{50} for stage 26 *P. variabilis* larvae in our study was 35.35 µg L^{-1}. The dissimilar lethal concentration values calculated in all of these studies might have been due to differences in environmental conditions in habitats, such as temperature and pH, or perhaps due to general differences in species. The developmental stage at the moment of exposure also influences lethal concentration estimations. In a study on juvenile *R. ridibunda*, the LC_{50} calculated after a 96-h exposure to cadmium was 51.2 mg L^{-1} (42), a value greater than or similar to the LC_{50} found for the larvae of this species. It is therefore important to analyse the water at several stages; not only once. According to Turkish regulations, the highest acceptable value is 100 µg L^{-1} (43), while this value was set at 200 µg L^{-1} in the 2008 TSE limits (the Turkish Standards Institution) (43).

This study detected serious declines in survival percentages of the larvae resulting from cadmium exposure at between 25 and 50 µg L^{-1}. We could say that cadmium levels within 25-50 µg L^{-1} were critical for *P. variabilis* larvae. This is most strongly confirmed by the survival of one larva in the group exposed to 50 µg L^{-1} of cadmium. James and Little (13) reported that 540 µg L^{-1} of chronically applied cadmium reduced survival and extended the metamorphosis period in *Bufo americanus* larvae, but prematurely induced metamorphosis at 5 and 54 µg L^{-1}. Cadmium exposure at 0.25-5 µg L^{-1} stimulated growth and metamorphosis in *Rana pipiens* larvae (16, 32). In another study (15), chronic exposure to 0-855 µg L^{-1} of cadmium did not affect the survival rate of *X. laevis* larvae. When discussing the complexity of amphibian larval development (under extensive hormonal regulation), it may be concluded that small concentrations of cadmium exposure may even have a stimulating effect on this process.

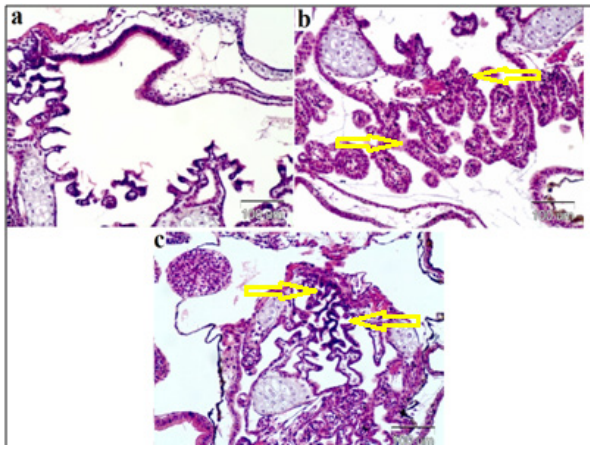

Figure 2 *Cross sections of the gill lamellae of (a) control group, (b) 10 µg L^{-1} cadmium, and (c) 25 µg L^{-1} cadmium (lamellar fusion shown with arrows), H&E staining*

Table 3 *Statistically significant values of the discriminant analysis made so as to reveal the difference among the groups following cadmium exposure in* P. viridis *larvae for 96 hours*

Function	Eigenvalue	Variance (%)	Total (%)	Canonical Correlation	Wilks Lambda	Chi-square	df	*p*
1	0.648	94	94	0.627	0.583	24.556	8	0.00
2	0.041	6	100.0	0.199	0.960	1.835	3	0.60

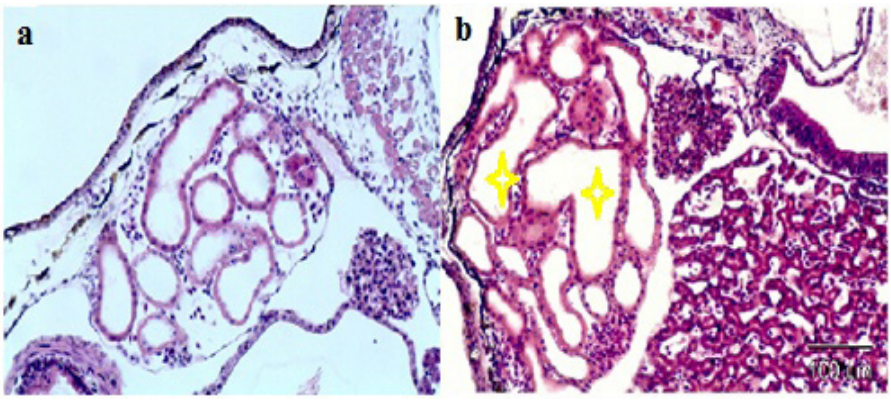

Figure 3 *Cross sections of the pronephric tubules of (a) control group, and (b) 25 µg L^{-1} cadmium (deformations of pronephric tubules shown with asterisk), H&E staining*

Table 4 *The incidence of observed histopathological findings in the gill, kidney, and liver of larvae administered cadmium*

Lesions	Cadmium (µg L^{-1})				
	0	1	5	10	25
Gills lamellar fusions	0	0	0	5	7
Kidney deformation of pronephric tubules	0	0	1	3	6
Liver deformation and haemorrhage	0	0	1	3	6
Vacuolisation	0	0	0	6	8
Visceral oedema	0	0	3	5	8

n=10. Values indicate the numbers of larvae with observed lesions in their sections

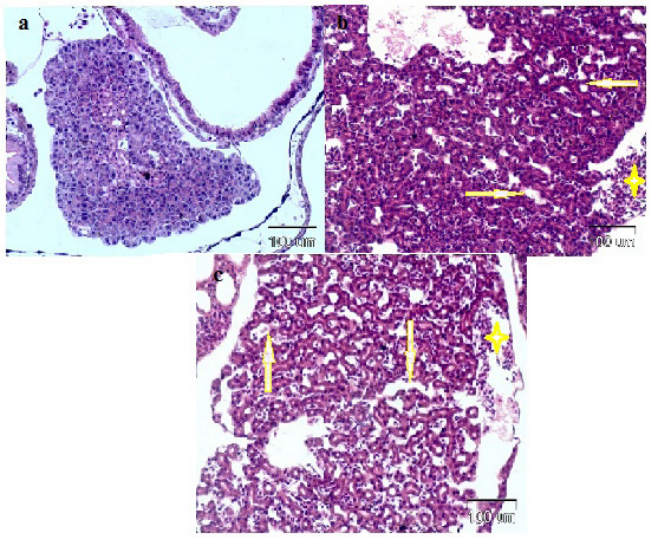

Figure 4 *Cross sections of the liver of (a) control group, (b) 10 µg L^{-1} cadmium and (c) 25 µg L^{-1} cadmium (haemorrhage shown with asteriks, vacuolization shown with arrows), H&E staining*

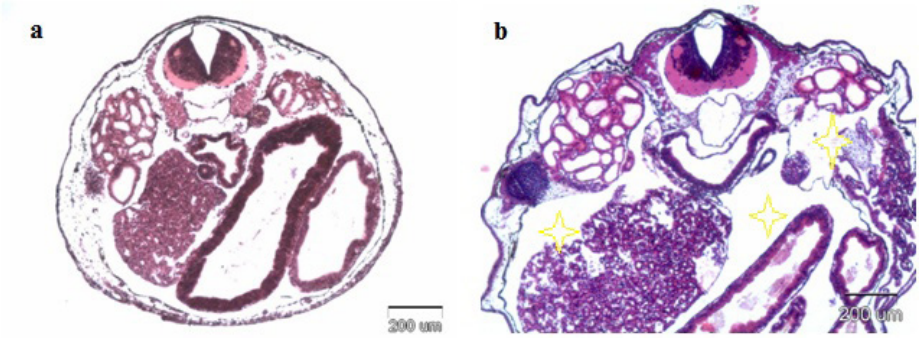

Figure 5 *Cross sections of the internal organs of (a) control group, and (b) 25 μg L^{-1} cadmium (visceral oedema shown with asterisk), H&E staining*

Heavy metal exposure at sub-lethal concentrations initially affects the gills, with the potential risk of accumulation (44). Gill damage, in particular fusion of secondary lamellae, has been reported in *Channa punctatus* exposed to 172 mg L^{-1} of cadmium during 10 days (45). The most important cause of gill lamellar fusion due to pollutant exposure is their function as a barrier for preventing pollutant entry into the body (46). Likewise, fusions in gill lamellae in our study were found in larvae exposed to 10 and 25 μg L^{-1} of cadmium.

Cadmium causes histopathological changes in the liver, kidney, gill, spleen, and bone marrow, as well as hypocalcemia and hypoglycemia, and inhibits the intake of Ca^{2+} through gills, therefore affecting plasma ion composition and osmoregulation (27). It has also been reported that cadmium has hepatotoxic effects and causes hepatic dysfunctions and increases in certain enzyme activities (14), as well as histopathological lesions in the liver and necrosis in hepatocytes (45, 47). Furthermore, a study on *Bufo arenarum* revealed that cadmium accumulates particularly in the liver (48). Much like in other sources, our study also detected serious deformations in the hepatic and renal tubules. The damage observed in liver cross-sections, necrosis, lesions, and haemorrhages verify that cadmium primarily targets the liver. Effects on kidneys include epithelial cell deformations in the pronephric tubules and enlargement of the tubular lumen. Similar results were reported for *Rana ridibunda* in a study that revealed progressive nephropathy and glomerulonephropathy as a result of cadmium exposure (14).

In conclusion, even though the histopathological findings of our study support the results of previous studies, its main contribution is that it is the first to report results for *P. variabilis*, which has shown to be a very good indicator species for aquatic ecosystems. Our LC_{50} values were lower than those reported by other investigators. The mechanisms and biological route of cadmium, with special reference to amphibians, has not yet been fully clarified and further detailed acute, sublethal, and chronic toxicity studies are necessary.

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Sažetak

Akutni toksični učinci kadmija na ličinke zelene žabe, *Pseudepidalea variabilis* (Pallas, 1769) (Amphibia: Anura)

Štetni utjecaji kadmija na okoliš i njegovo nakupljanje u prirodi posljednjih su godina poprimili zabrinjavajuće razmjere. U okviru ove studije istražili smo akutne toksične učinke kadmija na morfologiju i histologiju ličinaka zelene žabe, *Pseudepidalea variabilis* (Pallas, 1769). Embriji žabe dobiveni su od jedinki koje su u fazi ampleksusa prikupljene u prirodi. U laboratorijskim uvjetima embriji su bili držani do razvojnog stadija 26, kada su izloženi kadmiju u koncentracijama od 0, 1, 5, 10, 25 i 50 μg L^{-1} tijekom 96 sati. U pokusu smo odredili sljedeće letalne koncentracije kadmija: LC_{10} 26,98 μg L^{-1}, LC_{50} 35,35 μg L^{-1} i LC_{90} 46,31 μg L^{-1}. Izloženost kadmiju u koncentracijama većim od 1 μg L^{-1} negativno je utjecala na veličinu tijela ličinaka. Histološke analize upućuju na sljepljivanje škržnih listića, krvarenja u jetrima, pojavu edema u trbušnoj šupljini i deformacije pronefričkih kanalića (pri koncentracijama većim od 10 μg L^{-1}). Dobiveni rezultati pokazali su da je zelena žaba vrlo osjetljiva na kadmij, na što upućuje vrijednost LC_{50} koja je u našem pokusu bila niža od vrijednosti zabilježenih u drugim istraživanjima. Prema tome, ta se vrsta može smatrati pouzdanim pokazateljem okolišnog stresa u slatkovodnim ekosustavima.

KLJUČNE RIJEČI: *embriotoksičnost; histopatologija; letalne koncentracije; teški metal*

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