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Shell of Snail *Helix aspersa maxima* (Helicidae) as a Protection of Bioaccumulation Toxic Sodium Fluoride in Soft Tissue*

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The aim of this work was to determine the extent of bioaccumulation of sodium fluorides in tissues of snails under strictly controlled conditions, and also to determine resistance and tolerance to sodium fluoride load in these organisms. The study was performed on snails removed from aestivation. Quantitation of fluoride levels was done in soft tissues (foot, hepatopancreas) and shells of mature snails. Results show that long exposure to sodium fluoride pollution at a low level results in accumulation principally in the soft tissues of the snails. Because of the possibility of fluoride accumulation in the foot, the number of snails used for culinary purposes must be controlled, as it can potentially cause chronic toxemia caused by this trace element. Results also show that the shells of snails offer protection against the bioaccumulation of toxic fluoride in the soft tissue. The *Helix aspersa maxima* snail is characterised by high resistance and tolerance to fluoride load. Fluoride levels in soft tissues of the snail cannot serve as an indicator for biomonitoring purposes. In contrast, levels in the shell rose significantly with the concentration of fluoride and can be used in biomonitoring of sodium fluoride pollution.

Key words: Fluoride, shell, snail, bioaccumulation, monitoring.

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Fluorine is a compound of over 80 minerals. Fluoride compounds reach the atmosphere through volcanic activity, forest fires and man's industrial activities. This compound is widely distributed in earth's crust and is released into the air, soil and water by a great variety of industrial, agricultural and other activities in a number of geographical locations (CHLUBEK 2003).

The toxicity of fluorine has been well documented (JĘDRZEJCZUK & MILEWICZ 1996; MILLER 1997; MINTA *et al.* 1994). Fluoride in food plays a contributory role in endemic fluorosis (DESAI & DESAI 1998). In recent years, attention has been directed to the inhibitory properties of fluoride on the metabolic pathways of glycolysis under aerobic and anaerobic conditions (KĘDRYNA *et al.* 1993). Studies by GUMIŃSKA *et al*. (1991) have revealed that fluorides exert a synergistic effect manifested by decreased levels of ATP, and studies *in vitro* (human blood) have shown that fluorides are capable of depleting the ATP pool. RAĆ *et al.* 2005 have shown that AEC (adenylate energy charge) in snail muscle is reduced in parallel to an increasing exposure to fluoride. Contamination of the environment with fluorides is one of the most worrying consequences of civilization in view of the toxic effect of this element on plants, animals and humans. Exposure of vertebrates to low levels of fluoride, if sufficiently long, results in the accumulation of fluoride, ultimately associated with musculo-skeletal symptoms and metabolic disorders (MACHOY *et al.* 1995; ZAKRZEWSKA 1995). Molluscs are generally recognized as convenient bioindicators of environmental contamination (DWOJAK & ZAKRZEWSKA 1998; JURKIEWICZ-KARNKOWSKA 1998). Untill now, the majority of papers concerning the monitoring of heavy metals in the ecosystem have been carried out on molluscs which have been collected in rural areas. In the present study, snails from a controlled snailculture (from Zootechnics Institute in Balice), where age, type of provender, pollution and others parameters could be precisely controlled.

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With this information it may be judged how resistant snails are to certain pollution, and to what extent they accumulate and eliminate the harmful compounds. The aim of this work was to determine the extent of bioaccumulation of fluorides in tissues of *Helix aspersa maxima* under strictly controlled conditions. This approach may verify the hypothesis that snails are suitable for the monitoring of environmental hazards. The aim of this work was also to determine the resistance and tolerance of snails to fluoride load. This investigation complements previous knowledge on *Helix aspersa maxima*, which as an dietary element, must fulfil the requirements of healthy food.

Material and Methods

The study was performed on *Helix aspersa maxima* snails removed from aestivation. Snails were given standard pulverized food (recommended by the Institute of Zootechnics in Balice) supplemented with varying doses of sodium fluoride in two different groups (Table 1). The first group was subjected to a long culture time and low fluoride dose in food, while the second group had a short culture time and high fluoride dose in food, e.g. A_1 is a control group and A_2 possessed 1.5 mgF/kg in food during 109 days, B_3 possessed 665 mgF/kg in food during 40 days, etc. Sodium fluoride was used, which is a highly water-soluble compound.

The experiment was started with 25 snails per group. During the experiment some of the snails died. Only live specimens were used for chemical analyses, (various of number of sample in Table 1), all of the same size and age. The effect of dose on tissue levels of fluoride were studied in the first and second part of the experiment (culture A and B, ref. Table 2 and 3). A control culture without fluoride supplementation was run in parallel. Prior to collection of material for chemical analysis, the snails were kept without food for 48 hours and then killed by freezing in liquid nitrogen. Quantitation of fluoride levels was performed using soft tissues (foot, hepatopancreas) and shells of mature snails. Fluoride determinations using an ion-selective electrode (in shells) were done according to the method of LASSOCIŃSKA *et al.* (1982), as modified for shells by the authors. Purified and degreased shells were pulverized with a ball mill (KM-1) and transferred to plastic test tubes. Samples weighing approximately 40 mg were then placed in polyethylene test tubes, covered with 1 mL of 2M $HClO_4$ (for dissolution of shells and freeing of fluoride ion) and left at 70°C in a thermomixer (type 5437 from Eppendorf) for several hours (until complete dissolution of shells). The tubes were brought to room temperature and 4 mL of 1M trisodium citrate (Al and Fe complexing compound, displacing F^- from complexes with these metals) was added. Next, 1 mL was transferred to polyethylene tubes and mixed with 1 mL TISAB (Total Ionic Strength Adjustment Buffer, pH 5.5) using a plastic stirrer. The fluoride concentration was read with an OP 262 apparatus (Radelkis, Budapest, Hungary). Fluoride content was calculated based on the weight of the pulverized sample. Fluoride measurements using gas chromatography (in hepatopancreas and foot) were done according to the method of DURDA *et al.* (1986) and MIKOŁAJEK *et al.* (1996), with some modifications to account for material specificity. Samples stored at -20°C were cleaned of other tissues, thawed, dried at 80°C and homogenized with a KM-1 ball mill. Approximately 20 mg of homogenate was dissolved in 0.5 mL 25% HCl in sealed Eppendorf tubes at 95°C for 24 hours. The temperature was next reduced to ambient, one drop of hydrogen peroxide was added and the tubes were left for 12 hours. Extraction was done with 0.5 mL of a benzene, trimethylchlorosilane and isopentane (internal standard) mixture by

Table 1

Details of the experimental protocol

Culture group	Number of samples	Culture duration	Concentration of fluoride ions in food (mg/kg)			
A	A_1 = 13	109 days	A_1	A_2	A_3	
	A_2 = 12		control*	1.5**	150	
	A_3 = 16					
B	B_1 = 10	40 days	B_1	B_2	B_3	B_4
	B_2 = 23		control*	133	665	1330
	B_3 = 14					
	B_4 = 22					

* concentration in tap water was 0.15 mg F^-/L

** maximum allowable concentration of fluoride in tap water in Poland

shaking for 20 min. at 4°C. For this aim, an Eppendorf shaker was placed in an ice bath and set at maximum power. Tubes were centrifuged at 20000xg and 4°C for 6 min. The supernatant was withdrawn and transferred to a CHROM 5 (Laboratorni Pristroje Praha) gas chromatograph equipped with a 2 m × 4 mm i.d. column filled with 20% modified silicone oil on Chromosorb P (DC200/50). Oven, injector and FID detector temperatures were 85°C, 220°C, and 270°C, respectively. Nitrogen served as a carrier gas flowing at 20 mL/min. Aliquots of 1 mL were injected every 20 min and the detector signal was sent to an IBM-PC class computer operating with dedicated software from Medson (Poznań, Poland). Concentrations of fluoride were adjusted for mass of sample taken for analysis.

Results and Discussion

Statistical analysis was done with Statistica 5.1PL software using non-parametric tests: Kruskal-Wallis Test, Mann-Whitney U Test and Spearman Rank Correlation Coefficient. The results of part A (Table 2) show that exposure to the maximum allowable concentration in water according to Polish standards was not accompanied by a statistically significant increase in fluoride concentrations in soft tissues. Such increases were noted when the fluoride load was approximately 100 times higher. Accumulation was greatest in the foot. Statistically significant increases in fluoride content of the shell were noted with the smallest dose of fluoride but accumulation was least as compared with the soft tissues studied. It seems that long exposure to fluoride pollution at a low level results in accumulation principally in the soft tissues of snails. Because of the possibility of fluoride accumulation in the foot, the number of snails used for culinary purposes must be controlled, as it can potentially cause chronic toxemia caused by this trace element.

Table 2

Median concentrations of fluoride (mg/kg dry mass) in tissues of snail

Fluoride dose (mg/kg)	Shell	Hepato-pancreas	Foot
control (n=13)*	7.02	34.79	173.88
1.5 (n=12)	9.35***	40.84	172.69
150 (n=16)	58.22**	201.58**	1627.27**

* n – number of samples
** level of significance P<0.0001 (Mann-Whitney U-test) as compared with controls
*** level of significance P<0.005 (Mann-Whitney U-test) as compared with controls

The results of part B (Table 3) show that short exposure to a very high concentration of fluoride results in accumulation principally in shells. Statistically significant increases in fluoride content of the tissues were noted with all doses of fluoride, but accumulation was greatest in the shell and least in soft tissues. This shows that the shell protects against the bioaccumulation of toxic fluoride in soft tissue and may be used as an indicator for biomonitoring, in contrast to fluoride levels in soft tissues.

Table 3

Median concentrations of fluoride (mg/kg dry mass) in tissues of snail

Fluoride dose (mg/kg)	Shell	Hepato-pancreas	Foot
control (n=10)*	64.80	13.02	7.92
133 (n=23)	638.18**	88.91**	36.42**
665 (n=14)	1680.66**	102.99**	182.49**
1330 (n=22)	1137.50**	240.07**	145.85**

* n - number of sample
**level of significance P<0.0001 (Mann–Whitney U-test) as compared with controls

The monitoring of fluoride levels in the environment is necessary in view of its toxicity. Correlations between the content of fluoride in soil and tissues of snails (*Helix pomatia*, *Arion rufus*) have been reported (VOGEL *et al.* 1989). Several authors found correlations between fluoride levels in the environment and snail shells (DWOJAK & MACHOY 2000) in which fluoride is deposited in the form of calcium fluoride (VOGEL *et al.* 1989). VOGEL (1989) observed that tissue concentrations of fluoride in snails depend on the distance from the emitter and that these molluscs are capable of achieving tissue levels between 207 and 808 μg/g with maximum concentrations in the intestines and shell and minimum in reproductive organs (VOGEL & OTTOW 1991). The results of this study (culture group A) indicate that there is no significant accumulation of fluoride in soft tissues when snails were exposed to the maximum allowable concentration in drinking water (1.5 mg F^-/L). Significant differences as compared with controls were observed with exposure to a hundred-fold higher concentration. During chronic exposure to fluoride, the process of accumulation was most evident in the foot. Accumulation in the shell was significantly increased with the lowest concentration of fluoride, but levels remained below those in the foot or hepatopancreas. It can be inferred that accumulation of fluoride in soft tissues is not a suitable indicator for biomonitoring purposes due

to low sensitivity. Shells seem to be more suited for this aim as revealed in experiments with *Helix pomatia* (DWOJAK & ZAKRZEWSKA 1998). For many years molluscs have been used as bioindicators of environmental pollution (DWOJAK & MACHOY 2000; JURKIEWICZ-KARNKOWSKA 1994; RAĆ 2003). Many mollusc species with wide ranges, high densities and the ability to withstand high fluoride content in organs realize the fundamental criterions of good bioindicators. This seems controversial because (acc. to our research) a hundredfold surplus of permissible concentration of fluoride (part A) in drinking water causes an inconsiderable increase of F^- in organs. Therefore, the monitoring of environmental pollution using snails as indicators may be difficult, even if pollution levels are dangerous for human beings. Industrial pollution of the environment with fluorides and other substances has been on the rise in recent years. The content of fluoride in some industrialized areas can be as high as 1000 mg/kg food (ŻYLUK & MACHOY 1988), causing non-specific symptoms of fluorosis, such as loss of appetite and weight, reduced mobility, and cahexia in extreme cases (ŻYLUK & MACHOY 1988; ZAK- RZEWSKA 1995). Exposure of snails to 1330 mg F^-/L (part B) was associated with reduced mobility and decreased consumption of food. The latter effect could be behind lower intake of fluoride and reduced accumulation of this element in tissues. *Helix aspersa maxima* seems to be characterised by high resistance and tolerance to fluoride load. In conclusion, the shell of *Helix aspersa maxima* protects against the bioaccumulation of toxic fluoride in soft tissue, although long-term exposure to fluoride pollution results principally in accumulation in soft tissues of snails (especially in foot). The shell is best suited as an indicator for biomonitoring of fluoride pollution. The snail has a very high resistance and tolerance to fluoride load, and as an element of some diets, may not meet the requirements of healthy food.

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