ORGANOCHLORINE PESTICIDES – A THREAT ON THE HERMAN'S TORTOISE PERPETUATION

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Introduction

Although their synthesis has been considered of a great importance for humankind due to their pesticide action (P. Muller won the Nobel prize in 1948 for medicine for the discovery of DDT insecticide action[1]), subsequent research on organochlorinated compounds as HCHs and DDT and metabolites (e.g. DDE, DDD) has revealed their persistence, bioaccumulation capacity and toxicity (PBT) generating a global concern regarding their effects on living organisms and ecosystems (an overview the harmful effects that persistent organic pollutants may have on biota, their environmental sources and analysis, their environmental trends and processes, their movement through food chains are provided by Jones and de Voogt[2] and Blus[3]).

Hermann's tortoise (*Testudo hermanni boettgeri*) belongs to the Palaearctic tortoises and is present in the South-Western part of Romania, where the submediterranean climate appears[4]. Hermann's tortoise lives in habitats like pasturages, grazing lands, mesophilic oaks rare forests or submediterranean shrubs. Species diet is exclusively herbivorous, sometimes preferring the food represented by the people vegetables gardens. It is a vulnerable species mainly due to the late sexual maturity and slow reproduction rhythm. Habitats destruction and the increase in the number of prey animals (many of the antropophilic) are the main threats on species perpetuation. Hermann's tortoise is a threatened species according to the Romanian legal framework and a strictly protected species according to the Habitats Directive[5].

Very few studies are available in the literature regarding pesticides effects and bioaccumulation on land tortoises (*Testudo* genus mainly), most of the papers dealing with aquatic species. A study performed on a population of *Testudo hermanni* in Southern Greece revealed the effects of some herbicides (2,4-dichlorophenoxyacetic acid (2,4–D), 2,4,5-trichlorophenoxyacetic acid (2,4,5–T)) on the reproductive capacity of the species. The results indicate a higher vulnerability of Hermann's tortoises to the herbicides, which have not been associated with reptile mortality prior to this study[6].

De Solla et al.[7] have determined several pesticides (organochlorine, PCBs, Dibenzodioxin, Furan) in the egg content of snapping turtle (*Chelydra serpentina serpentina*) eggs from St. Lawrence River watershed in the neighborhood of a PCB-contaminated landfill site. They report PCB levels of up to 1% of the egg mass (a maximum of 737.68 ppm) with possible source the aquatic sediments (with PCB concentrations between 2 and 7900 ppm)[8]. The authors do not exclude the possibility that the PCB and organochlorine pesticides to influence the hatching success and the juveniles survivor. PCB concentration in the chorioallantoic membranes of *Caretta Caretta* seem to be higher than in the egg content[9]. Generally, aquatic species tend to get high concentrations of organochlorine compounds because of the large fish component existing in their diet.

Materials and Methods

Samples collection. The egg samples have been collected from the Captive Breeding Centre for Hermann's tortoise, located in Eselnita, Romania, where eggs are artificially incubated in order to increase the percentage of juveniles hatching. We have analysed the eggshell of the fertile eggs from the 2004 clutch. Due to the large numbers of samples, the results are shown as mean values on each considered region (Bazias, Dubova, Svinita-Bostita, Mala – see Fig. 1).

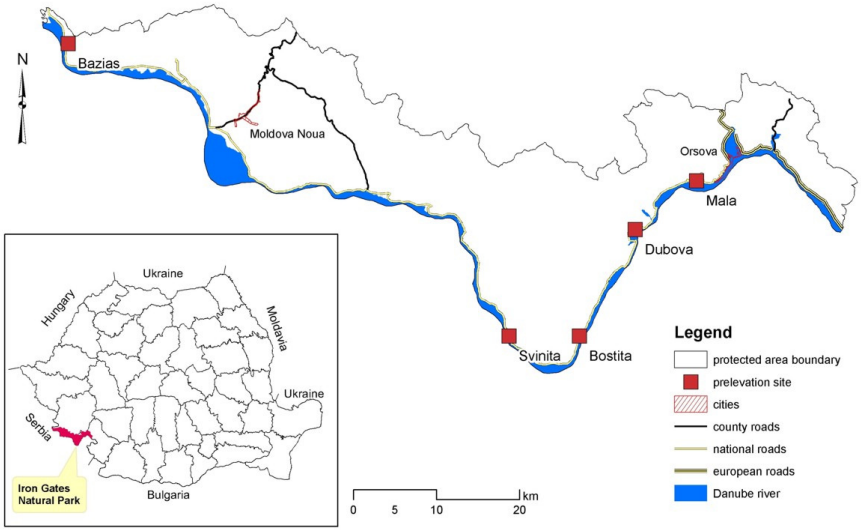

Fig. 1. Location of the samples collection sites.

Samples processing. Sample processing was done as following: the egg was weighed and then was smashed into a capsule, weighed again and the difference between the two weights was made, in order to determine the weight of each component – core and eggshells. The core – albumen and yolk – were put in stove at 40 ^{0}C. The sample was then evaporated, until a paste was obtained. Parts of this paste were then used for analysis.

For each analysis – core and eggshell – a quantity of 1 – 2 g was taken, well grinded in a pestle, and then introduces in a vial hermetically closed and 10 mL petroleum ether were added (Merck petroleum ether, boiling point 40-60 ^{0}C). The vial was stirred well, for 15 – 20 minutes and left overnight. The sample was separated by decantation, filtered on anhydrous sodium sulfate and the filtrate was collected in another vial. The extraction was repeated three times, the filtrates being collected in the same vial. The filtrate volume was measured and then was chromatographied on fluorisyl column (80-100 mesh), for purification, as it follows: the column was washed with 50 mL hexane (4 mL/min), then the sample was passed through the column with a rate flow of 3 mL/min. The column was eluted with 50 mL hexane and the first fraction was collected (non-polar compounds). Then, another 75 mL hexane containing 5% ethylic ether were passed, and the second fraction was collected (organochlorine pesticides). The column was finally washed with another 75 mL hexane with 50% ethylic ether content, in order to remove any remained polar compound. The eluted fractions were evaporated and then dissolved in 1 mL petroleum ether and hexane and a volume of 5 – 10 µL was injected to the chromatographic column. A pesticide standard was also injected into the column.

Method of analysis. The organochlorine pesticides that were extracted from the samples were determined by electron capture detector gas chromatography (GC - ECD). There were determined the following analytes: hexachlorcyclohexane (HCH), as its isomers alpha, beta, gamma, delta and p,p' – dichlordiphenyltrichlorethane (DDT) as its isomers *p,p'*- dichlordiphenyltrichlorethylene (DDE) (o + p), 2,2-bis-(*p*-chlorphenyl)-1,1-dichlorethane (DDD) (op + pp) şi DDT (op + pp). The detection limit was of 0.1 ng/g.

A CARLO ERBA gas-chromatograph was used, equipped with a packed column, splitless injector and Electron Capture Detector. The mobile phase was N_2. The operational parameters are shown in Table 1.

Table 1. Operational parameters of the instruments

Pressure:	**Temperature:**		**Column**
Column: 0,8 kPa	**Column:** 215 ^{0}C	(Starting temperature 30 ^{0}C, temperature gradient – 9,5 ^{0}C/min, 5 minutes stationary temperature)	Glass (L = 1,85 D. I. 3 mm), package 1,5% OV17; 1,95%% QF-1, bonded on Chromosorb PAW-DMCS 0,125 - 0,150 mm.
Detector: 1,5 kPa	**Detector:** 275 ^{0}C		
	Injector: 225 ^{0}C		

Results and Discussion

The concentrations of organochlorine compounds are shown in Table 2. The first aspect to be noticed is that DDD has not been detected in any of the samples. It is possible to notice that the concentrations are quite similar between regions, in terms of total amount of HCH (total-HCH, the difference between the regions with maximum and minimum concentration is lower than 10%) and total amount of DDT (DDT-total, the difference between maximum and minimum is lower than 20%). Also, similar values are registered in terms of the percentage of each compound in the total amount of organochlorine compounds which might suggest that the contamination sources are similar, but differences might appear in the different exposure of the individuals. Mala region has registered the highest average values for both total-HCH and total-DDT, being the closest to a human settlement.

Table 2. Concentration of organochlorine compounds in the eggshell (in ppb)

	Bazias	**Dubova**	**Svinita - Bostita**	**Mala**
alfa-HCH	4.803217	3.6382	3.404014	2.273425
beta-HCH	36.22858	41.832571	42.2414	44.34075
gamma-HCH	0.60855	0.2401286	0.132929	0.164375
delta-HCH	1.127333	0.3924857	0.010557	0.017
total-HCH	**42.76768**	**46.103386**	**45.7889**	**46.79555**
DDE o+p	24.126	27.36	32.75029	34.7685
DDD op+pp	SLD	SLD	SLD	SLD
DDT op+pp	48.06635	50.980871	50.88806	51.31013
DDT-total	**72.19235**	**78.340871**	**83.63834**	**86.07863**
DDT/DDE	**2.142998**	**2.1342059**	**1.660541**	**1.597446**
OC-total	**114.96**	**124.44426**	**129.4272**	**132.8742**
% alfa	4.178162	2.923558	2.63006	1.710961
% beta	31.51407	33.61551	32.63718	33.37048
% gama	0.529358	0.1929607	0.102705	0.123707
% delta	0.980631	0.3153908	0.008157	0.012794
% DDE	20.98642	21.985747	25.30401	26.16648
% DDT	41.81136	40.966833	39.31789	38.61557

The DDT/DDE report is larger that 1 in all cases, which might suggest that although the use of pesticides has been officially banned in the '80, important amounts of obsolete pesticides exists in private households and are occasionally used.

The data has been statistically tested for normality, distribution with Skewness test and the results are shown in Table 3.

Table 3. Statistical analysis of the obtained data.

	Mean	**Confidence limit**		**Range**	**Variance**	**Standard deviation**	**Standard error**	**Skewness test**
		–95%	**+95%**					
Alpha–HCH	3.52971	1.87922	5.18020	2.52979	1.07588	1.037246	0.518623	0.04894
Beta –HCH	41.16083	35.64424	46.67741	8.11217	12.01927	3.466882	1.733441	-1.38018
Gamma-HCH	0.28650	-0.06257	0.63556	0.47562	0.04812	0.219367	0.109683	1.75950
Delta-HCH	0.38684	-0.44848	1.22216	1.11678	0.27558	0.524955	0.262477	1.39245
Total-HCH	45.36388	42.52968	48.19808	4.02787	3.17248	1.781144	0.890572	-1.66156
DDE o+p	29.75120	21.98139	37.52100	10.64250	23.84284	4.882913	2.441457	-0.21984
DDT op+pp	50.31135	47.91245	52.71025	3.24378	2.27281	1.507584	0.753792	-1.91191
DDT-total	80.06255	70.25859	89.86650	13.88628	37.96123	6.161268	3.080634	-0.65308

Hatching efficiency. In order to evaluate a possible influence of the organochlorine pesticides on the hatching efficiency, we have considered all the eggs artificially incubated in the Captive Breeding Centre from Eselnita

and their evolution during their presence within the Centre.

- **Bazias Group –** out of 50 incubated eggs, 43 were fertile eggs and 7 were infertile. Out of 43 fertile eggs, 39 hatched. There were 4 dead juveniles, in advanced Yintema[10] developing stage. One of the four juveniles had a very big vitelus. Out of the 39 hatched eggs, 5 had exoskeleton disorders.
- **Bostita Group -** out of 67 incubated eggs, 58 were fertile eggs. Out of 58 fertile eggs, 2 died when hatched. Out of the 56 hatched eggs, 9 had exoskeleton disorders.
- **Ciucaru Mare Group -** out of 70 incubated eggs, 51 were fertile eggs and 19 were infertile. Out of 51 fertile eggs, 40 hatched. There were 9 dead juveniles, in advanced Yintema developing stage. Out of the 40 hatched eggs, 3 had exoskeleton disorders, 3 died when hatched (unknown causes).
- **Mala-Eselnita Group -** out of 128 incubated eggs, 101 were fertile eggs and 27 were infertile. Out of 101 fertile eggs, 21 died when hatched (unknown causes) or inside the egg, in advanced Yintema developing stage. Out of the remained 80 hatched eggs, 12 had exoskeleton disorders.
- **Bucovat-Tarovat Group -** out of 111 incubated eggs, 86 were fertile eggs and 25 were infertile. Out of 86 fertile eggs, 9 died when hatched or inside the egg, in advanced Yintema developing stage. Out of the remained 77 hatched eggs, 16 had exoskeleton disorders.

Therefore, the overall number of incubated eggs during 2002-2005 was 546, the overall number of living juveniles 380, out of which 80 showed different disorders. In addition, there were another 120 non-hatched eggs, with the juveniles in different Yntema developing stages. The remaining were infertile eggs. The incubation average time was 56 days (maximum 66 days, minimum 50 days). The average hatch was of 5.39 eggs per tortoise. No information is available in the literature related to the lethal concentration for *Testudo hermanni* so a comparison was not possible.

Although we cannot affirm that the organochlorine pesticides are the cause of the large percentage of infertile eggs or juveniles that did not developed correctly, the important concentrations of HCH and its isomers and ∑DDT (sum of DDT, DDE and DDD) quantified within the eggshell samples of Hermann's tortoise (*Testudo hermanni boettgeri*) are likely to represent a threat to the perpetuation of the species.

Acknowledgements

The work has been partially funded by the CNCSIS project *Organic and Inorganic Pollutants Bioaccumulation in Species Protected by the Romanian and International Legislation*. The authors would also like to thank Prof. Maria Patroescu and the CCMESI team for the support in obtaining the samples within the Captive Breeding Centre for Hermann's tortoise.

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