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Non-invasive monitoring of organohalogen compounds in eggshells and feathers of birds from the Lower Prut Floodplain Natural Park in Romania

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Abstract

The aim of this paper is to quantify the levels of DDT and its main metabolites (DDE, DDD) and also of the HCH congeners in biological samples collected from birds living in the Lower Prut Floodplain Natural Park, Romania. In order to limit the stress on the bird species, a non-invasive approach was performed using chorioallantoic membrane of the eggs and feathers as representative samples. DDTs are the main organohalogen compounds in both categories of samples. Differences appear between species and categories of samples; for *Anas sp.***,** the concentration is up to 10 times higher in eggshells than feathers.

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Keywords: Birds; non-invasive sampling; organochlorine pesticides; DDT congeners; HCH isomers

1. Introduction

Since 1967 when Ratcliffe [1] proved that the disappearance of peregrine falcon (*Falco peregrinus)* in Britain was due to the accumulation of *4,4'*- dichlordiphenyltrichlorethylene (DDE), there has been an increasing concern

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regarding the routes of exposure toward such organochlorine pesticides (OCP) as well as their behaviour in living organisms [2, 3, 4]. Studies of accumulation of various pollutants in birds are very informative about the ecosystem health state as a consequence of their high position in the food chain, long life and high mobility [5, 6, 7, 8]. However, such studies often require invasive methods in order to collect representative samples such as tissues or organs [9, 10]. When it comes to the protected species, such methods are not allowed and, thus, a series of non-invasive methods of sampling were developed, based on analysis of various chemicals in eggs [11] or feathers [12] that can be a direct indicator of the chemical bioaccumulation. These methods are very helpful to avoid a supplementary stress brought onto the population, in addition to the already existing factors that may drastically reduce chances of perpetuation, such as natural disturbances, land-use change, climate change, invasive alien species, overexploitation, pollution, indirect drivers of change or synergistic effect of various drivers [13].

DDE determination in the chorioallantoic membranes using the eggshells available during the hatchling period is a direct indicator of the pollutant level in the egg, through a calculated correlation index [14]. External contamination with organic pollutants via air is probably due to feathers preening with oil from the uropygial gland [15] and has less contribution to the concentration of organic pollutants measured in bird feathers [16].

Despite their use being banned in Romania for the last 30 years, organochlorine pesticides are still present in the ecosystems at levels that might represent a threat to biodiversity. In this context, although Romania has a long history of organochlorine pesticides production, there are only a few studies concerning the bioaccumulation of the halogenated compounds in living organisms, in general [17, 18, 19], and in birds, in particular [20, 21].

Lower Prut Floodplain Natural Park is a newly established protected area overlapping the Romanian side of the Prut River lower sector (Fig. 1). Because it includes habitats and species of a communitarian interest, a surface of 82.47 km^2 has been declared as Natura 2000 Special Protection Area (SPA) [22]. Similar with other Romanian protected areas, the park faces low funding of conservation activities and weakly trained personnel, which often leads to an inefficient conservation of the wildlife resources [23].

This paper describes quantification of some organochlorine pesticides in biological samples (eggshells and feathers) collected through a non-invasive method from birds living within the Lower Prut Floodplain Natural Park in Romania. Thus, the study focused on the following chemicals: *4,4'*– dichlorodiphenyltrichloroethane (DDT) and its main metabolites: *4,4'*- dichlordiphenyltrichlorethylene (DDE), *4,4'*-dichlorodiphenyldichloroethane (DDD) (their sum is expressed as ΣDDT), and fours isomers of hexachlorocyclohexane (HCH) – *alpha, beta, gamma* and *delta* (their sum is expressed as ΣHCH).

2. Experimental

2.1. Reagents and instruments

All reagents (petroleum ether, hexane, ethyl ether) were of chromatographic purity (Sigma-Aldrich). Analytical standards of pesticides (DDT PESTANAL, DDD PESTANAL and DDE PESTANAL and HCH PESTANAL) were purchased from Sigma-Aldrich. Anhydrous sodium sulphate used in the experiments was of analytical reagent grade (Sigma-Aldrich).

A *Shimadzu* 2010 gas-chromatograph equipped with *AOC-20 Series* autosampler, with an electron capture detector and splitless injector was used. A Rtx-CL Pesticides column (Lenght: 30 m; diameter: 0.25 mm) was used. The carrier gas was He 6.0. The volume of injection varied between 5-10 μL. The operational parameters: a) Temperature: *Column***:** 120 ^oC, *Detector***:** 310 ^oC, *Injector***:** 250 ^oC; b) pressure: *column 0.8 kPa; detector1.5 kPa*; c) Starting temperature: 30 ^oC, temperature gradient: 9.5 ^oC/min, 5 minutes stationary temperature.

2.2. Procedures

Samples collection. 17 eggshells containing chorioallantoic membrane (CAM) from 6 bird species – European starling (*Sturnus vulgaris),* mallard duck (*Anas platyrhynchos),* coot (*Fulica atra),* collared-dove (*Streptopelia decaocto),* herring gull (*Larus argentatus)* and European roller (*Coracias garrulus)* were collected during the hatchling season of 2008. 23 feathers (FEA) from 12 species - greylag goose *(Anser anser),* bean goose (*Anser fabalis),* mallard duck *(Anas platyrhynchos),* garganey *(Anas querquedula),*white stork *(Ciconia ciconia),* black-

headed gull (*Larus ridibundus)*, herring gull (*Larus argentatus)*, ring-necked pheasant (*Phasianus colchicus)*, common wood pigeon (*Columba palumbus)*, common tern *(Sterna hirundo)*, mute swan (*Cygnus olor)* and ruff *(Philomachus pugnax*) were collected between 2008 and 2009.

Species were chosen depending on samples availability, dietary habits and their suitability for biomonitoring programs. The European starling is frequently used in biomonitoring surveys due to species etology: populations are easily established as they nest in any conditions, eggs are easy to collect, its diet consists mainly soil invertebrates, which is an important source of organochlorine pesticides [24, 25]. Coots and gulls are offering information on broader areas in aquatic habitats [9], while the white stork is a top predator whose feeding habits depend on the foraging area and thus is highly exposed to pollutants accumulation [26].

The samples (see Table 1, where n - number of samples; LOQ - limit of quantification, SD - standard deviation) were collected from eight locations within the Lower Prut Floodplain Natural Park – Maicaşu (MAI), Vlăşcuţa Lake (VLAS), Maţa-Rădeanu Ponds Complex (MRAD), Pochina Lake (POC), Valeni (VAL), Cotul Chiului (COC), Şovârca (SOV), Vlădeşti Lake (VLAD) (Fig. 1).

The eggshells were collected from the bird nests and close vicinity and stored in sterile bags Whirl Pak (NASCO, Fort Wilkinson, WI). The feathers were collected from the bird nests areas and also from feeding areas and sealed in paper envelopes, with the exception of those coming from *Phasianus colchicus* that were collected from two freshly hunted individuals. All samples were frozen after collection, prior to analysis.

Samples processing. For the feathers samples, only the quill was analysed, as this is the feather part in direct contact with the blood flow, while the vane is more exposed to external contamination and thus correlation with internal organs and blood is weaker [27]. For each analysis – chorioallantoid membrane and feather – a quantity of 0.5 – 2.0 g was well grinded in a pestle and introduced in a closed vial. 10 mL of petroleum ether (boiling point 40-60 ^{0}C) were added and the vial was stirred for 15 – 20 minutes and then left to rest overnight. The sample was separated by decantation, filtered on anhydrous sodium sulfate and the filtrate was collected in another vial. The extraction procedure was repeated three times, the filtrates being collected in the same vial. The filtrate volume was measured and further cleaned on fluorisyl column (80-100 mesh), as it follows: the column was washed with 50 mL

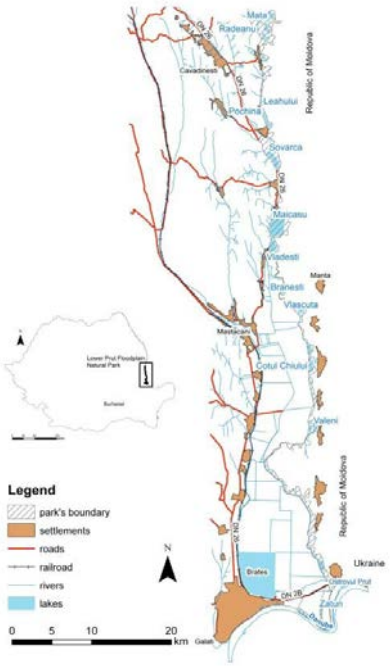

Fig. 1. Lower Prut Floodplain Natural Park location.

Table 1. OCP concentration (ng/g wet weight) in chorioallantoic membranes and feathers of birds.

Species	Sample type	Feeding behaviour	DDE mean±SD (min - max)	DDD mean±SD (min - max)	DDT mean±SD (min - max)	Alpha HCH mean±SD (min - max)	Beta HCH mean±SD (min - max)	Gamma HCH mean±SD (min - max)	Delta HCH mean±SD (min - max)
Sturnus vulgaris	*CAM (n=4)*	Omnivore	181.09±166.56 (41.33 – 412.36)	43.29±35.73 (21.18 – 96.69)	76.35±60.86 (39.87 – 167.35)	1.19±0.66 (0.76 – 2.16)	4.65±4.58 (1.27 – 11.06)	27.43±33.26 (6.94 – 77.04)	3.67±3.33 (0.17 – 7.99)
Anas platyrhynchos	*CAM (n=4)*	Herbivore	124.51±133.75 (18.41 – 315.48)	39.86±22.70 (19.69 – 69.26)	87.62±46.43 (41.07 – 151.80)	3.89±3.53 (1.40 – 9.00)	8.60±11.33 (0.39 – 25.36)	26.95±20.45 (8.70 – 55.48)	3.32±1.17 (2.53 – 5.05)
Fulica atra	*CAM (n=3)*	Herbivore	22.41±25.86 (6.36 – 52.24)	14.34±17.74 (3.11 – 34.79)	38.14±47.26 (10.45 – 92.70)	0.97±1.56 (0.06 – 2.78)	1.78±3.04 (0.02 – 5.29)	10.69±13.84 (2.35 – 26.67)	1.64±2.84 (<LOQ – 4.92)
Streptopelia decaocto	*CAM (n=1)*	Granivore	81.97	35.71	64.88	2.39	6.11	13.8	6.9
Larus argentatus	*CAM (n=2)*	Omnivore	7.94±2.29 (6.32 – 9.56)	4.73±0.74 (4.21 – 5.26)	13.81±1.85 (12.50 – 15.12)	0.04±0.02 (0.03 – 0.05)	0.04±0.02 (0.02 – 0.05)	3.54±1.45 (2.52 – 4.56)	<LOQ (LOQ – 0.01)
Coracias garrulus	*CAM (n=3)*	Insectivore	7.90±1.92 (6.25 – 10.00)	3.88±0.55 (3.25 – 4.23)	11.18±1.04 (10.22 – 12.28)	0.07±0.01 (0.06 – 0.09)	0.05±0.03 (0.02 – 0.08)	4.25±1.11 (3.10 – 5.32)	<LOQ (<LOQ – 0.01)
Anser fabalis	*FEA (n=1)*	Herbivore	12.2	2.1	21.0	0.6	0.9	5.3	0.2
Anser anser	*FEA (n=1)*	Herbivore	9.8	6.2	11.5	0.1	0.1	8.3	<LOQ
Anas platyrhynchos	*FEA (n=7)*	Herbivore	11.31±2.58 (7.24 – 15.06)	6.61±2.01 (4.22 – 9.35)	16.60±4.50 (10.33 – 25.03)	0.54±0.40 (0.12 – 1.09)	0.41±0.57 (0.02 – 1.26)	6.51±2.48 (3.15 – 10.31)	0.17±0.16 (0.02 – 0.43)
Anas querquedula	*FEA (n=1)*	Herbivore	12.8	5.1	12.2	0.2	0.1	5.3	0.2
Ciconia ciconia	*FEA (n=2)*	Omnivore	10.64±3.54 (8.14 – 13.15)	6.33±2.60 (4.49 – 8.17)	20.73±2.30 (19.10 – 22.35)	1.73±1.35 (0.78 – 2.68)	0.16±0.01 (0.15 – 0.17)	4.89±2.05 (3.44 – 6.34)	0.33±0.27 (0.14 – 0.52)
Larus ridibundus	*FEA (n=3)*	Omnivore	9.72±4.01 (6.45 – 14.19)	5.56±3.24 (3.21 – 9.25)	12.99±0.76 (12.32 – 13.82)	0.97±0.16 (0.80 – 1.12)	0.09±0.09 (0.03 – 0.20)	6.25±1.09 (5.13 – 7.32)	0.13±0.10 (0.06 – 0.25)
Larus argentatus	*FEA (n=2)*	Omnivore	12.89±2.17 (11.35 – 14.42)	6.10±4.91 (2.63 – 9.58)	18.69±0.90 (18.05 – 19.33)	0.61±0.38 (0.34 – 0.88)	0.07±0.07 (0.03 – 0.12)	4.98±2.36 (3.32 – 6.65)	0.19±0.19 (0.06 – 0.32)
Phasianus colchicus	*FEA (n=2)*	Granivore	12.54±1.55 (11.44 – 13.63)	6.00±0.63 (5.55 – 6.45)	17.67±1.82 (16.35 – 18.92)	0.43±0.29 (0.22 – 0.63)	0.07±0.01 (0.06 – 0.08)	6.54±1.29 (5.63 – 7.45)	0.42±0.51 (0.07 – 0.78)
Columba palumbus	*FEA (n=1)*	Granivore	12.6	4.3	12. 6	0.1	0.1	5.6	0.1
Sterna hirundo	*FEA (n=1)*	Piscivore	10.0	4.1	18.1	0.3	0.1	3.3	<LOQ
Cygnus olor	*FEA (n=1)*	Herbivore	10.3	5.3	12.6	0.2	0.1	5.3	0.1
Philomachus pugnax	*FEA (n=1)*	Aquatic invertebrates	14.5	3.6	11.7	0.6	<LOQ	8.3	0.4

hexane (4 mL/min), then the sample was loaded onto the column with a flow rate 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% diethyl ether were passed, and the second fraction was collected (organochlorine pesticides). The column was finally washed with another 75 mL hexane with 50% diethyl ether content in order to remove any remained polar compound. The eluted fractions were evaporated to residue, dissolved in 1 mL hexane and a volume of 5 – 10 μL was injected to the chromatographic column. The pesticide standard was also injected into the column.

3. Results and discussions

3.1. Concentrations profile of OCP in biota samples

Table 1 shows that the DDT congeners are the prevalent contributors to the total organochlorine content in both categories of the analysed samples (CAMs and feathers). The concentration ratio of DDT and its metabolites provides information about the time line when the parent compound (DDT) has entered the system [28].

The OCP concentration levels in bird eggs and tissues differ among the species [21, 29, 30, 31] due to different feeding behaviour, capacity to metabolise primary pollutants (in our case DDT) or food availability [32]. DDT half-live is estimated to be approximately 20 years in the moderate climate of Romania [33], which means that a ratio higher than 1 would suggest that the birds have been recently exposed to DDT. For the CAM samples, the ratio is lower or close to 1. Moreover, for starling, the metabolisation process is advanced, concentration of metabolites being much higher than of the original compound.

The situation is similar for the feathers samples, only 1/3 of the samples slightly exceeding 1, but with the highest value of 1.51. Notably, all the three DDT congeners were identified in all samples indicating that both metabolic mechanisms (aerobic and anaerobic) are present [4].

From the HCH isomers, *gamma*-HCH was the main congener in all samples, which indicates that lindane has been used for pest control purposes (containing about 96% *gamma*-HCH), instead of technical HCH, which contains a maximum of 10-12% of the *gamma* isomer. The presence of *alpha*-, *beta*- and *delta*- congeners of HCH in feathers is accidental and at very low concentration levels. The percentage of the *gamma*-HCH from the ΣHCH in feathers is higher than 75% in nearly all samples and over 90% in one third of them.

The concentration of all the analysed pesticides in both categories of samples is lower than the guidelines reported to determine eggshell thinning and reference values in feather that would lead to bird mortality [34].

3.2. Concentration of OCP in the chorioallantoic membranes of bird eggs

The highest mean concentrations for DDE and DDD were found in samples collected from *Sturnus vulgaris* (Fig. 2); in addition, the highest concentrations for all the three DDT congeners was also found for an individual of *Sturnus vulgaris* (Table 1).

Its feeding typology (omnivore species) suggests that the main source of contamination is the forest and agricultural ecosystems near the river rather than the aquatic ecosystem. For such species, there are significant differences between samples from the same region, situation recorded by Reynolds et al. [35] in Alabama and Colorado, where large variability appeared even between eggs from the same clutch. The same study recorded much higher values of DDE in eggshells of *Sturnus vulgaris*, a mean concentration of 700±60 ng/g and a maximum of 4360 ng/g. Eens et al. (2013) reports DDE as dominating the OCPs profiles in European starling (percentages varying from 60 to 99% of total sum of OCPs), which is also the case in our results.

Important concentrations of DDTs (with the highest mean concentration for DDT, 87.6 ng/g, and particular concentrations between 41.1 ng/g and 151.8 ng/g) (Table 1) were determined in eggshells of *Anas sp.* (mainly *Anas platyynchos*) but these values are however lower than those obtained by Aurigi et al. [20] in the Danube Delta (Eastern Romania, where Danube River meets the Black Sea). Similarly, the results obtained for individuals *Fulica atra* from the Lower Prut Floodplain Natural Park (60.5 ng/g DDT + DDE) are much lower than those reported in the same study (average values of 1264.11 ng/g of DDT + DDE in the Danube Delta), suggesting that the human impact on the birds living in the Danube Delta is much greater than the one in the Prut River region. On the other hand,

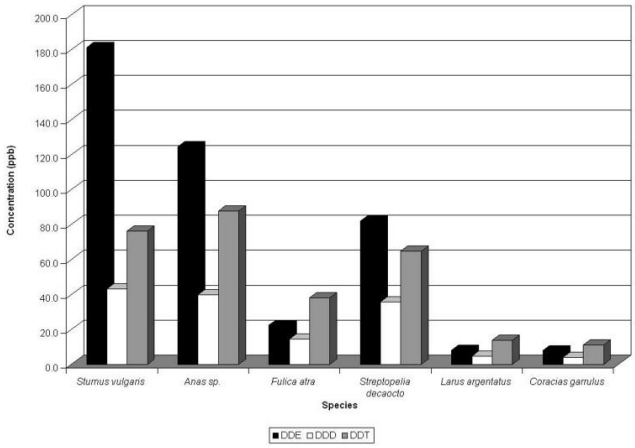

Fig. 2. DDTs concentration in the chorioallantoic membranes depending on the species.

considering the time passed between the two studies the OCP values have had lowered due to degradation by aquatic microorganisms or accumulation in living organisms. A similar decline from one decade to another was revealed in the USA [36] between the 1970's, 1988 and 1994 (use of DDT was banned in the USA in 1972, earlier than in Romania - 1985).

All CAM samples contained detectable concentrations of DDD, although at lower levels than DDT and DDE. The mean concentrations for DDD ranged between 3.9 ng/g in *Coracias garrulus* and 43.3 ng/g in *Sturnus vulgaris.*

As previously mentioned in section 3.1., *alpha-*, *beta-* and *delta-* congeners of HCH appear accidentally for all species under study (Fig. 3).

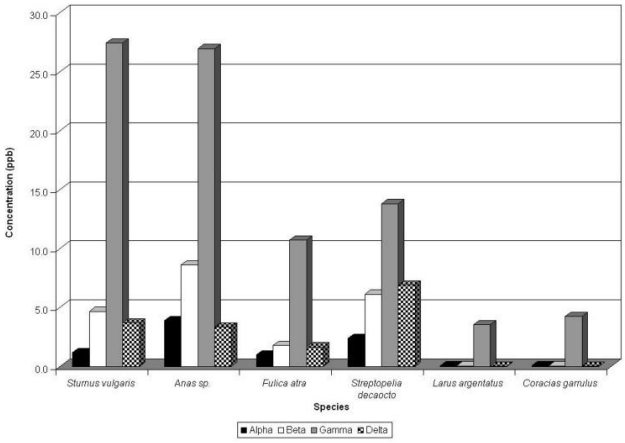

Fig. 3. HCH concentration in the chorioallantoic membranes depending on the species.

The mean concentrations of *gama*-HCH ranged between 3.5 ng/g in *Larus argentatus* and 27.4 ng/g in *Sturnus vulgaris*, whilst the highest average value of ΣHCH was 42.8 ng/g in *Anas sp.* A relatively high concentration (13.8 ng/g) was found for *Streptopelia decaocto*, which is a granivore species. This could be explained by the fact that seeds were often treated with lindane prior to their storage as a fungi control measure [37].

3.3. Concentration of OCP in bird feathers

While in CAM samples DDE is the main pollutant, the situation is opposite in feathers. Thus, DDT is dominant in all the species, with two exceptions, probably due to a preferential excretion compared to its DDE metabolite. The exception was *Philomacus pugnax*, were DDE had shown the highest concentration of all three DDT congeners (14.6 ng/g of DDE) and *Columba palumbus*, where DDT and DDE average concentrations are equal to 12.6 ng/g (Fig. 4).

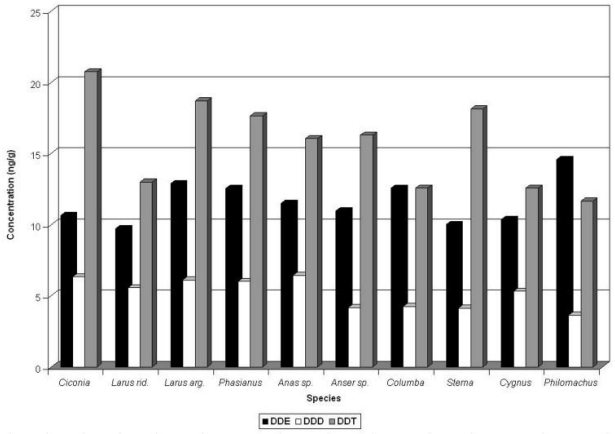

Fig. 4. DDTs concentration in feathers depending on the species.

The average concentration for DDT was highest in *Ciconia ciconia*, 20.7 ng/g, whilst the lowest was found for *Philomachus pugnax*. Concentration of DDTs are similar with those reported by de la Casa-Resino et al. [38] in blood samples of white storks from Spanish colonies.

DDD is also present in all feathers samples, but the average concentrations range is much narrower than for the CAM samples: 3.7 ng/g for *Philomacus pugnax* and 6.4 ng/g in *Anas sp.* Since the same species exhibits the highest DDE and lowest DDD concentrations, it is possible that one metabolite concentration increases on the other metabolite expense.

All DDTs concentration levels are lower than in CAM samples, the particular values ranging between: 6.5 (*Larus ridibundus*) and 15.1 ng/g (*Anas platyrhynchos*) for DDE, 2.1 (*Anser fabalis*) and 9.6 ng/g (*Larus argentatus*) for DDD, whilst both lowest and highest particular concentrations for DDT are in feathers of *Anas platyrhynchos*: 10.3 ng/g and 25.0 ng/g respectively (*Anas platyrhynchos*).

For the HCH congeners, the values are similar with those found for the CAM samples. However, *gamma*-HCH seems to be present at elevated levels of concentration (Fig. 5). For the other congeners, with one exception *(*for *Ciconia ciconia*), all values were lower than 1.0 ng/g. The lower concentrations of OCP found in feathers of *Larus argentatus* are similar to those of Jaspers et al. [16] where ΣDDT and ΣHCH concentrations were found to be 12 ng/g and 1.1 ng/g, respectively.

Concentration of the ΣDDT analysed in birds from the Lower Prut Floodplain Natural Park range between 20.8 ng/g and 492.0 ng/g in CAM, respectively 26.5 ng/g and 43.6 ng/g in feathers. Similarly, concentration of ΣHCH determined in birds from the Lower Prut Floodplain Natural Park are distributed between 2.4 ng/g and 98.3 ng/g in CAM, respectively 3.4 ng/g and 11.3 ng/g in feathers.

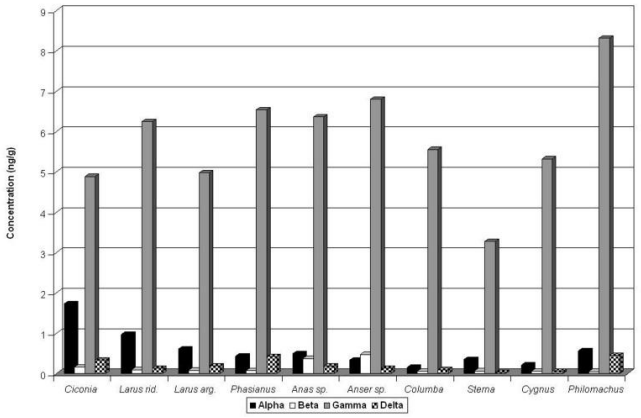

Fig. 5. HCH isomers concentration in feathers depending on the species.

3.4. Comparison between levels of OCP in the choriollantoic membranes and feathers

It can be observed (Table 1) that the concentrations are higher in CAM compared to feathers, suggesting that the maternal transfer of pollutants to embryo is predominant compared to the excretion through feathers and preen oil [15].

For *Anas sp.* and *Larus argentatus* it was possible to compare values in eggshells and feathers (Table 2). For *Anas sp.***,** the concentration is up to 10 times higher in eggshells, whilst for *Larus argentatus*, feathers display higher concentrations of organochlorine pesticides. A similar situation was registered by Becker and Sperveslage [39], who studied the level of a series of organochlorine pesticides in eggs and chicks from the same nest of *Larus argentatus* and found that the DDTs concentration is higher in the chicks than in the eggs. Rajeai et al. [40] report comparable values for all the parameters in Table 2 for mallard ducks from the coast of Caspian Sea.

Table 2. Comparative values of OCP in eggshells and feathers. Values are given in ng/g (wet weight).

Anas sp.	Eggshell	Feathers	***Larus argentatus***	Eggshell	Feathers
DDE	124.5	11.5	DDE	7.9	12.9
DDD	39.9	6.4	DDD	4.7	6.1
DDT	87.6	16.0	DDT	13.8	18.7
Gamma-HCH	27.0	6.4	Gamma-HCH	3.5	5.0
ΣHCH	42.8	34.0	ΣHCH	3.6	7.4

4. Conclusions

Chemical analysis on eggshells and feathers of birds from the Lower Prut Floodplain Natural Park revealed the presence of organochlorine pesticides (DDT and its metabolites and HCH congeners). The DDE and DDT are the dominant compounds in all samples, despite ban on use of DDT in Romania more than 20 years ago. From the HCH congeners, only gamma isomer exhibits significant levels, its contribution to the sum of HCH isomers ranging between 50% and 98.83%. However, the levels of concentrations do not emphasise a more recent contamination of the aquatic ecosystems with organochlorine pesticides, but accumulation of obsolete residues from the period they were used on larger scale. Moreover, the level of halogenated compounds does not represent an immediate threat to biodiversity.

The analysed samples confirm the usefulness of non-invasive sampling for biological monitoring purposes. Still, a larger number of samples should be obtained and correlations with pollutants concentration in other biological

samples (blood, tissues) should be performed in future studies.

The comparison with the results of organochlorine pesticides quantification in bird samples from the Danube Delta led to the conclusion that the human insertion within the Lower Prut Floodplain is much more reduced compared to the input of pollutants that the Danube River brings from all over Europe within the Danube Delta.

Acknowledgements

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