DECHLORANE PLUS AND RELATED COMPOUNDS IN GULL EGGS (*Larus michahellis*) FROM SPANISH NATURAL PARKS

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Introduction

Dechlorane plus (DP, $C_{18}H_{12}Cl_{12}$), Dechloranes 602 (Dec 602; $C_{14}H_4Cl_{12}O$), 603 (Dec 603; $C_{17}H_8Cl_{12}$) and 604 (Dec 604; $C_{13}H_4$ Br_4Cl_6) have been used since the 1960s as additive halogenated flame retardants in many consumer products such as wire coatings, furniture, or electrical hard plastic connectors in televisions and computers[1]. Their production has become important and increased since the 1970s because they have been used as substitute for Mirex after its banning[2]. Although DP has been less widely used than some brominated flame retardants, such as polybrominated diphenyl ethers (PBDEs), it has been categorised by the US EPA as a high production volume (HPV) substance in USA[3]. Although DP and DP analogues (Dec 602, 603 and 604) have been used for nearly 50 years, there was no evidence of their presence in the environment until a few years ago[2,4]. Currently, the number of scientific publications regarding to dechloranes is increasing, although is still limited compared to other flame retardants. Since 2006 several studies have reported the occurrence of DP in different environmental matrices, such as air, water bodies and biota [5-7], most of them focused on DP and their stereoisomers, *syn*-DP and *anti*-DP, while information about DP analogues is still scarce. Moreover, most of the studies are done near to manufacturing areas and, consequently, concentration levels are higher than those expected in others less exposed sites. Therefore there is a need to dispose reliable information about the environmental occurrence, fate and behaviour of all these halogenated flame retardants. Marine birds are well known as appropriate organisms for bio-monitoring purposes because of the high trophic position of these birds in the food chain, which leads to the bioaccumulation of persistent organic pollutants (POPs) that are present in the environment. Studies in bird-breeding areas of special protection have reported unexpected high levels of POPs[7-10]. Recently, the occurrence of DP and related compounds in eggs of seabirds has been reported[11,12]. These findings reinforce the necessity of a better knowledge of the presence of halogenated compounds on sensitive areas which are refuges for numerous wildlife bird species. At least within species, POP levels in eggs reflect the contaminant burden of the female at the time of egg laying, especially the uptake of contaminants from food recently ingested around the colony, although some contaminants may derive from the previously accumulated levels in the adipose tissue.

The aim of this work is to investigate the occurrence of DP and DP analogues in eggs of yellow-legged gull (*Larus michaellis*) f) as bioindicators of environmental pollution from areas of special protection. The study includes four Spanish sites located at Atlantic Islands of Galicia National Park, the Cabrera Archipelago National Park, Ebro Delta Natural Park and the National Hunting Refuge of Chafarinas Islands. For this purpose, a fast and simple selective pressurised liquid extraction method in combination with gas chromatography-ion trap mass spectrometry operating in negative ion chemical ionisation (GC-NICI-MS) has been developed and validated, and has been applied to the analysis of the target compounds in gull egg samples. The results and conclusion of the evaluation study is presented here.

Materials and methods

Chemicals

Standard solutions of *syn*-DP and *anti*-DP, Cl10-DP (decachloropentacyclooctadecadiene) and Cl11-DP (undecachloropentacyclooctadecadiene), at 50 µg/ml in toluene and $^{13}C_{12}$-BDE 77 and $^{13}C_{12}$-BDE 138 (MBDE-MXFR) at 2000 ng/µl, used as surrogate standards, were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). Dec 602, Dec 603 and Dec 604 were supplied from Toronto Chemical Research Inc. (Toronto, Canada). A standard solution of CB 209 at 100 ng/µl was supplied from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Florisil, activated at 650ºc for 8h and stored at 150ºC before use, silica, anhydrous Na_2SO_4 and sulphuric acid were purchased from Merck (Darmstadt, Germany). Solvents such as hexane, dichloromethane,

and acetone were obtained from Sigma-Aldrich (St. Louis, MO, USA), and isooctane was purchased from Riedel-de Haën (Darmstadt, Germany).

Samples

Eggs of yellow-legged gull (*Laurus michahellis*) were collected at the beginning of the breeding season from four colonies located at the Atlantic Islands of Galicia National Park (Galicia, north-west of Spain), the Cabrera Archipelago National Park (Balearic Islands, east of Spain), Ebro Delta Natural Park (Catalonia, Est of Spain) and the National Hunting Refuge of Chafarinas Islands (south of Spain), during the time period 2010-2011. The sampling method is based on the guidelines of UNEP and OSPAR. For each colony and year, 12 eggs were randomly collected from three sub-colonies (36 eggs in total per colony and year). To ensure that the reproductive potential of each nest was maintained, only the first egg of each nest was sampled. The eggs were transported to the laboratory in a cool box and the eggs of each sub-colony were then pooled, freeze-dried and stored at -20ºC before analysis.

Sample treatment

The analysis of egg samples was performed by selective pressurised liquid extraction (S-PLE) using an ASE 100 Accelerated solvent extractor (Dionex, Sunnyvally, CA, USA). Before extraction, a portion of 0.5 g of dry weight sample was spiked with appropriate amounts of the surrogate internal standards, $^{13}C_{12}$-BDE 77 and $^{13}C_{12}$-BDE 138, and was kept overnight to equilibrate. The sample was then mixed with 2g of anhydrous sodium sulphate and loaded into a 34 ml PLE extraction cell on top of 20 g of acidified silica (H_2SO_4, 44% w/w) used as lipid retainer. Samples were extracted at 100ºC and 1500 psi with a mixture of n-hexane:dichloromethane (1:1, v/v) as extraction solvent, applying 3 static cycles of 5 min each and a flush volume of 60%. The extract was then rotary evaporated to *ca.* 1ml and fractionated onto a Florisil column (5g). The target compounds were eluted using with 20 ml of n-hexane and 40 ml of a solvent mixture of hexane-dichloromethane (85:15, v/v). The extracts were combined and rotary evaporated and then concentrated under a gentle stream of nitrogen up to 50 µl. The final extract was analysed by GC-MS working at negative ion chemical ionisation (NICI) mode after addition of an appropriate amount of CB-209 used as syringe internal standard. The lipid content of egg samples was determined by PLE (without sorbent for lipid removal) of an additional sub-sample and further gravimetric measurements.

GC-MS analysis

The analysis of DPs and related compounds was performed on a Trace GC 2000 gas chromatograph Trace GC 2000 series gas chromatograph equipped with an AS2000 autosampler (Thermo Fisher Scientific, Milan, Italy) and coupled with a Thermo DSQII quadrupole mass spectrometer. The chromatographic separation was performed on a DB-5ms (Agilent-J&W Scientific, Folsom, USA) fused-silica capillary column (15 m x 0.25 mm I.D., 0.25 µm of film). The oven temperature was programmed from 100ºC (held for 2 min) to 200ºC at 20ºC/min (held for 1 min) and then to 280ºC at 10ºC/min, and finally to 310ºC at 15ºC/min (held for 10 min). Helium was used as carrier gas at a flow of 1.5 ml/min. Injector temperature was kept at 260ºC and 2 µl of samples and standards were injected in splitless injection mode (1 min). The MS operating conditions were the following: NICI mode using methane as moderating gas at 2 ml/min with electron energy of 120 eV and an emission current of 250 µA. The transfer line and ion source temperatures were kept at 280ºC and 150ºC, respectively. For MS acquisition, selected ion monitoring (SIM) mode was employed at a dwell time of 80 ms and a delay time of 20 ms. A set of eight calibration solutions containing the target compounds at concentrations ranging from 1 to 250 pg/µl, $^{13}C_{12}$-BDE 77 and 138 at 15.0 pg/µl and CB-209 at 5.0 pg/µl were used for quantification purposes.

Quality Control

A daily isomer-specific GC tests to check the separation, sensitivity and calibration of the instrumental method were carried out. Procedural blanks covering both the instrumental and the methods were routinely performed during the analysis to avoid cross-contamination. A freeze-dried hen egg with no detectable amounts of DPs spiked at concentrations near of the limits of quantification was used as quality control sample. Limits of detection and quantification, precision (RSD% <15%) and linearity were routinely checked to assure the quality of the results. Recoveries for $^{13}C_{12}$ BDE 77 and $^{13}C_{12}$BDE138 in the analysed samples were 90 ± 7% and 92 ± 9%, respectively. All gull egg samples were analysed in duplicate.

Results and discussion

To simplify sample handling and to reduce the extraction time for the analysis of DP and related compounds in egg samples, we examined the capability of PLE to simultaneously perform in-cell extraction and clean-up. Initial experiments were conducted to evaluate several sorbents as lipid retainer. Florisil and modified silica (H_2SO_4, 44% w/w) were tested as trap sorbents for in-cell PLE clean up. For modified silica, 20 g of sorbent into a PLE cell of 34 ml were enough to obtain clean extracts, while for Florisil 35-40 g were required (100 ml cell) for achieving similar results. For the selective PLE extraction of the target compounds, the highest recoveries were obtained using a mixture of n-hexane:dichloromethane 1:1 (v/v) for modified silica and 70:30 (v/v) for Florisil. Additional PLE operating parameters, such as extraction temperature, number and time of static cycles and flash volume were also optimised to obtain maximum recovery of the analytes with minimum presence of matrix-interfering compounds. After evaluating both sorbents, silica modified with sulphuric acid (44%, w/w) was selected as lipid retainer and the highest efficiency in the PLE extraction of the target compounds was obtained at 100ºC with n-hexane:dichloromethane 1:1 (v/v), three cycles of 5 min each and 60% of flush volume, at constant pressure of 1500 psi. The method provides recoveries higher than 90% for all the compounds and the extraction/clean-up procedure was accomplished in approximately 20 min with a solvent consumption of 100 ml. Therefore, silica gel modified with H_2SO_4 (44%, w/w) is proposed as fat retainer for the simultaneous extraction and clean-up of PDs in egg samples.

The developed method was applied to the analysis of Dechlorane Plus, isomers *syn*- and *anti*-, Dechlorane 602, 603 and 604, and the two dechlorinated products, Cl10-DP and Cl11-DP, in gull eggs of protected areas from Spain. Table 1 shows the mean concentrations (average of three sub-colonies from each site) found of DPs and related compounds in eggs of yellow-legged gull in the natural areas studied during the period 2010-2011.

Table 1. Mean concentrations (±sd)[a] of DPs and related compounds (pg/g wet weight) found in eggs of yellow-legged gull from the four Spanish Natural Parks.

	Atlantic Islands of Galicia		Cabrera Archipelago		Ebro Delta		Chafarinas Islands	
Compound	2010	2011	2010	2011	2010	2011	2010	2011
syn-DP	190 ± 15	203 ± 38	138 ± 14	125 ± 8	65 ± 13	58 ± 2	59 ± 8	64 ± 4
anti-DP	462 ± 19	568 ± 121	374 ± 82	291 ± 6	121 ± 36	101 ± 4	123 ± 16	130 ± 6
Total DPs	652 ± 34	771 ± 159	512 ± 96	416 ± 14	186 ± 50	159 ± 6	182 ± 24	194 ±1 0
f_{anti}	0.71	0.74	0.73	0.70	0.65	0.63	0.67	0.67
Dec 602	2042 ± 304	2103 ± 158	615 ± 23	1873 ± 66	452 ±3 5	1072 ± 124	1031 ± 59	1647 ± 104
Dec 603	1548 ± 139	1823 ± 681	638 ± 87	1409 ± 22	135 ± 47	880 ± 70	641 ± 112	951±59
Dec 604	38 ± 5	55 ± 4	11 ± 1	37 ± 2	14 ± 2	15 ± 1	19 ± 3	25±3
Cl10-DP	< mLOD[b]	< mLOD[b]	< mLOD[b]	< mLOD[b]	< mLOD[b]	< mLOD[b]	< mLOD[b]	< mLOD[b]
Cl11-DP	< mLOD[c]	10 ± 1	< mLOD[c]	7 ± 1	< mLOD[c]	< mLOD[c]	< mLOD[c]	< mLOD[c]

[a](n=3 sub-colonies, each sub-colony was analysed by duplicate)
[b] Method detection limits of Cl10-DP: 1.5 pg/g ww; [c] Method detection limits of Cl11-DP: 1.9 pg/g ww

Among the target compounds, Dechlorane 602 and 603 were the most abundant DPs in all samples and ranging between 452±35 pg/g w/w for Ebro Delta and 2103±158 pg/g w/w for Atlantic Islands in 2010. Similar values were observed for 2010 and 2011, except for D602 and D603, that showed an increasing trend on the concentration with the time. These results are consistent with the high chlorination of these compounds and indicate their persistence and bioaccumulation in biota at the top of the food web.

Total DP concentrations, sum of *syn*- and *anti*-isomers, ranged from 118± to 771± pg/g w/w. Among the areas studied, the Atlantic Islands of Galicia and Cabrera Archipelago showed the highest DP levels, followed by Chafarinas Islands and Ebro Delta. These values are similar to those in a previous work by Muñoz-Arnaz et al.[11] for eggs of two gull species (*Larus michahellis and Larus audouinii*) collected from Chafarines Islands in 2007 (average 290 pg/g ww, ranging from 39.5-433 pg/g ww), demonstrating that the concentration of DP in this area remains nearly constant from 2007 due to a diffuse inputs of DP to the marine environment. Comparing the levels found in the present study with those obtained in eggs of herring gulls from Laurentian Great Lakes (1500-4500 pg/g ww), differences of one order of magnitude lower are observed. Several factors may contribute

to explain these differences, although the influence of manufacturing plants or different feeding habits of the species could be the most likely reasons. To study the possible DP stereoselective enrichment in the egg samples, the *anti*-isomer fractional abundance (f_{anti} =[*anti*-DP]/([*anti*-DP]+[*syn*-DP])) was determined (Table 1). As can be see, the mean f_{anti} values was 0.69±0.04 and ranged from 0.63 to 0.74. These results are consistent with values of f_{anti} reported by Wang et al. (0.59-0.8) and Hoh et al. $(0.75\text{-}0.80)^2$ for technical DP. It seems that there is essentially no stereoselective enrichment of *syn*- or *anti*- isomer in the present gull eggs regardless of the colony and year. Regarding to the dechlorinated DP compounds, Cl10-DP and Cl11-DP, concentration levels lower than de detection limits were obtained, except in Atlantic Islands of Galicia and Cabrera Archipelago where Cl11-DP were detected at very low concentrations (10±1 pg/g ww and 7±1 pg/g ww, respectively) in egg samples from 2011.

The findings of this study confirm the presence of DP and related compound in eggs of yellow-legged gull from different Spanish protected areas, indicating diffuse but also constant inputs of these contaminants by the effect of anthropogenic activities developed near of these natural sites. The differences observed on the concentrations of the studied areas could be attributed to the different feeding habits, seasonal variation of food composition, age and condition of the birds. Gull eggs have shown to be a suitable matrix for the biomonitoring of these compounds and they permit to evaluate the contamination impact of a local area. In addition, collection of eggs is a relatively non-invasive technique that can minimize the adverse effects on the bird community. It is early to evaluate the effects that these contaminants may produce to the gulls, but considering the toxic and harmful effects of detected PD and related compounds, additional studies should be launched to assess the sources and fate of these pollutants and to take actions to minimize their impact upon birds, especially for protected species.

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