OCCURRENCE OF PERSISTENT ORGANIC POLLUTANTS IN SEAGULLS EGGS FROM THE EBRO DELTA (SPAIN)

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Introduction

There has been growing interest about the risks associated with the presence of Persistent Organic Pollutants (POPs) in the environment. POPs are organic compounds that are resistant to degradation through chemical, biological and photolytic processes. Therefore, they are known to be persistent in the environment, to be capable of long-range transport, to bioaccumulate in animal tissue, to biomagnify in food chains and to have potentially significant impacts on the environment.

Birds have the ability to accumulate POPs through the diet and thereafter are transferred to the eggs[1] and this has serious implications at individual or population level. Recent studies in breeding-birds areas have reported unexpected high levels of chemical products used in the past in agriculture (pesticides)[2] and industry (solvents, flame retardants, lubricants, additives)[3]. Therefore, sea birds such as gulls and auks have been recommend as non-invasive biomonitoring matrices by the United Nations Environmental program (UNEP) and the Oslo Paris Convention (OSPAR).

Very little data is available on the distribution and levels of POPs in birds from Spain. Spain is an area highly affected by the historical use of various POPs in both industry and agriculture. Previous studies reported the presence of organochlorinated pesticides in aquatic birds from the Ebro delta and from Doñana[4] and harmful effects were detected in egg shell thickness[2]. Other studies indicate that falcons contain dioxins, furans and polychlorinated biphenyls (PCBs) as a result of a diffuse source of these pollutants [5, 6].

The Ebro delta hosts the most important colony of Audouin seagulls *(Larus audouinii*) and yellow legged gulls (*Larus michahellis*) from southern Europe. This area is also highly impacted by the presence of pesticides, PCBs, dioxins and furans due to the historical use of these compounds and more recently of flame retardants and perfluorinated compounds (PFCs).

The aim of the present study was to determine the occurence of PCBs, short chain chloroparaffins (SCCP) and dioxins and furans in seagull eggs of *Larus michahellis* and the endangered specie *Larus auodouinii*. The first is a common omnivorous species which eats fish, crabs, fish tips and waste. On the other hand, *Larus audouinii* is a protected specie and feeds basically on fish. Both species coexist in the bay of Alfacs, in the Ebro delta. Given these two distinct feeding biology, it was intended to evaluate the source of pollution of these two species and the effects this may produce on egg development.

Materials and methods

The sampled areas are the colonies of yellow-legged seagull (*Larus michahellis*) and audouin gull (*Larus audouinii*) of the Ebro delta, situated in southern Catalonia. This area hosts a population of 40.000 and 70.000 individuals, respectively, and is a crucial area for the breeding and conservation of these species. Twelve eggs were randomly collected from the colony at 3 different sites (36 eggs in total for each colony). Only nests with one egg were sampled, as to ensure that the first egg of the clutch is taken, for comparability purposes.

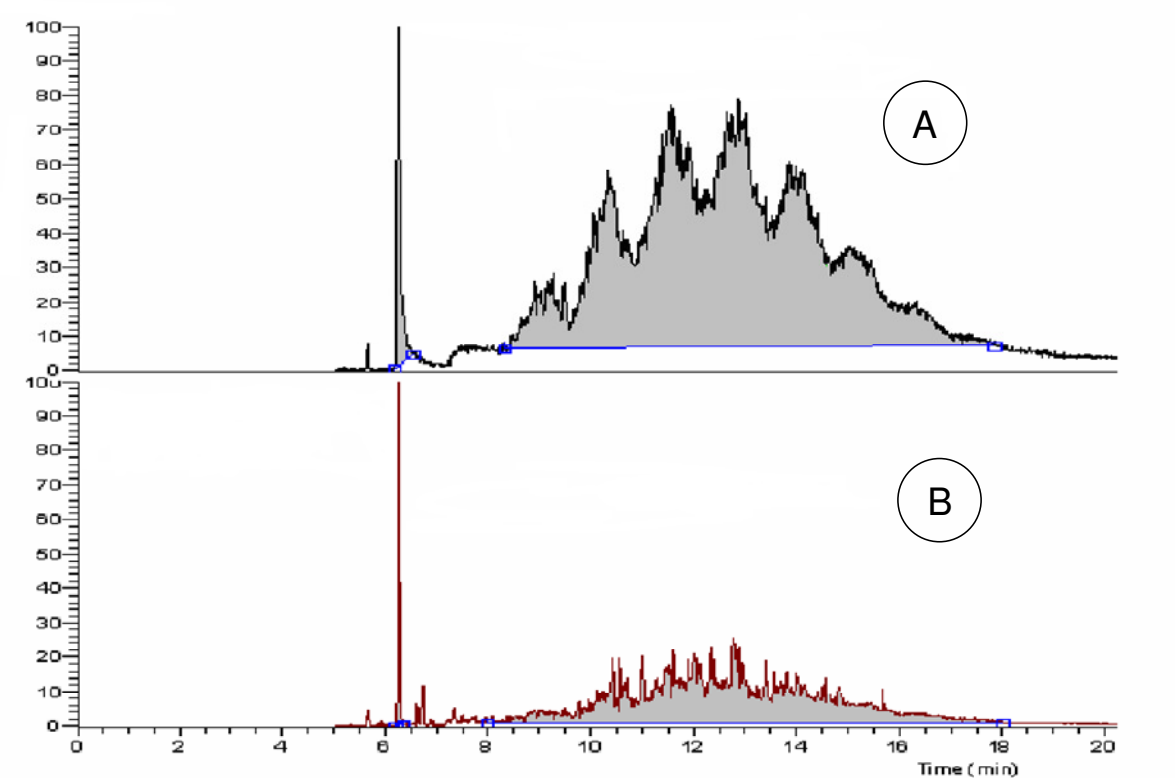
PFCs were wet extracted with solid-liquid extraction using acetonitrile. 1 g of sample was spiked with internal standards (^{13}C-PFOS and ^{13}C-PFOA) at 50 ng/g and the sample was incubated for 18 hours at 7ºC. Afterwards, 9 ml of acetonitrile was added and the sample was thoroughly mixed using a Vortex chemical mixer. The samples were then ultrasonic extracted 3 times for 10 minutes at room temperature and finally centrifuged at 2500 rpm

for 5 minutes. To clean the extracts, 25 mg of activated carbon and 50 µL glacial acetic acid were added, vortexed and centrifuged for 10 minutes at 10,000 rpm. A portion of 350 µl of this extract were resuspended in 150 µl of HPLC water. PFC levels were measured using high performance liquid chromatography coupled to tandem mass spectrometry with an ACQUITY UPLC system (Waters, USA) coupled to a ACQUITY TQD tandem quadrupole mass spectrometer (Waters, USA). An Acquity UPLC BEH C18 Column (1.7 µm particle size, 50 mm x 2.1 mm, Waters, USA) was used for removing any contamination from the mobile phases. The analysis was performed on a LiChroCART HPLC RP-18e column (125 mm x 2 mm i.d., Merck, Germany). The mobile phase was 2 mM NH4OAc (A) / Acetonitrile (B). Gradient elution was 30% B to 90% B in 5 min and to 100% B in 0.1 min and held for 1 min returning to initial conditions in 3 min. PFCs were measured under negative electrospray ionisation using 2 transitions from parent to product ion to identify them[7]. PFC concentrations were calculated using internal standard quantification.

Extraction of PCDD/PCDF were performed following the USEPA 1613 Method for PCDD/PCDFs. 10 g of freeze-dried egg were spiked with 500 pg of mixtures of $^{13}C_{12}$-PCDD/Fs (EPA-1613LCS, Wellington Lab., Guelp, Canada) and then Soxhlet extracted for 24 h with toluene:cyclohexane (1:1, v/v). The extracts were rotary concentrated and kept in the oven overnight (105ºC) for gravimetrical fat determination. Afterwards, fat residues were dissolved in n-hexane. Organic components, fat and other interfering substances were removed by treating the raw extracts with silica gel modified with sulfuric acid (44%). The extracts were concentrated and filtered through a PTFE filter prior to the clean-up. This procedure is based on the use of the Power PrepTM system (FMS Inc., MA, USA) which uses a sequential array of three different Teflon prepacked columns of multilayer silica, alumina and carbon adsorbents, respectively (FMS Inc., Waltham, MA, USA). Finally, the extracts were rotary concentrated and transferred into a vial. The remaining solvent was reduced to dryness under a gentle stream of nitrogen. Instrumental analysis was based on the use of High Resolution Gas Chromatography coupled to High Resolution Mass Spectrometry (HRGC-HRMS). All analyses were performed on a Trace GC ultra gas chromatograph (Thermo Fisher Scientific, Milan, IT) fitted with a 60 m x 0.25 mm i.d. x 0.25 µm film thickness DB-5ms fused silica column (J&W Scientific, CA, USA) coupled to a high resolution mass spectrometer (DFS, Thermo Fisher Scientific, Bremen, Germany) controlled by a Xcalibur data system. Positive electron ionization (EI+) operating in the MID mode at 10,000 resolving power was used. Quantification was carried out by the isotopic dilution method.

For the analysis of SCCPs and PCBs in gull egg samples, pressurized liquid extraction (PLE) was performed using an ASE 100 Accelerated Solvent Extractor System (Dionex, Sunnyvale, CA, USA). For sample treatment, 1 g of the freeze dried egg samples was spiked with $^{13}C_6$-hexachlorobenzene and $^{13}C_{12}$-PCB-30 and the sample was then mixed with 3 g of anhydrous sodium sulphate and loaded into a 34 ml PLE extraction cell on top of 16 g of silica modified with sulphuric acid (20%, w/w), which was used as fat retainer. The extraction cell was then placed in the ASE 100 system and the egg sample was extracted at 100ºC with a mixture of n-hexane:dichloromethane 1:1 (v/v) working at a constant pressure of 1500 psi, a flush volume of 60% and a purge time of 90 s. Three static extraction cycles of 5 min each were applied to achieve the maximum recovery of the analytes (>90%). The extract was then rotary evaporated to approximately 1 ml after addition of 100 µl of iso-octane as a keeper and it was fractionated on 10 g of Florisil activated at 350ºC for 12h. Two fractions were obtained using (F1) 40 ml of n-hexane and 20 ml of a n-hexane:dichloromethane mixture 85:15 (v/v), and (F2) 60 ml of a n-hexane:dichloromethane 1:1 (v/v). Fraction 1 contains the PCB, while the SCCPs were eluted in the fraction 2. All fractions were rotary concentrated to 50 µl and were analyzed by GC-NICI-MS with a Trace GC 2000 series gas chromatograph (ThermoFinnigan) equipped with an AS2000 autosampler and coupled to a GCQ/Polaris ion trap mass spectrometer (ThermoFinnigan, Austin, TX, USA). The chromatographic separation of these compounds was performed using a DB-5MS of 15 m x 0.25 mm I.D., 0.25 µm for SCCP and of 30 m for PCBs. Methane was used as moderated gas at a pressure of $1.8 \times 10{-}4$ mTorr (reading on the ion gauge). Quantification of SCCPs was performed as the sum of total area below the elution profile of SCCPs (Figure 1). PCB determinate was based on the quantification of the individual PCB congeners: CB28, 52, 101, 118, 138, 153, and 180.

Figure 1. GC-NCI-MS chromatogram of a (a) SCCP solution at 15 µg/ml and (b) gull egg.

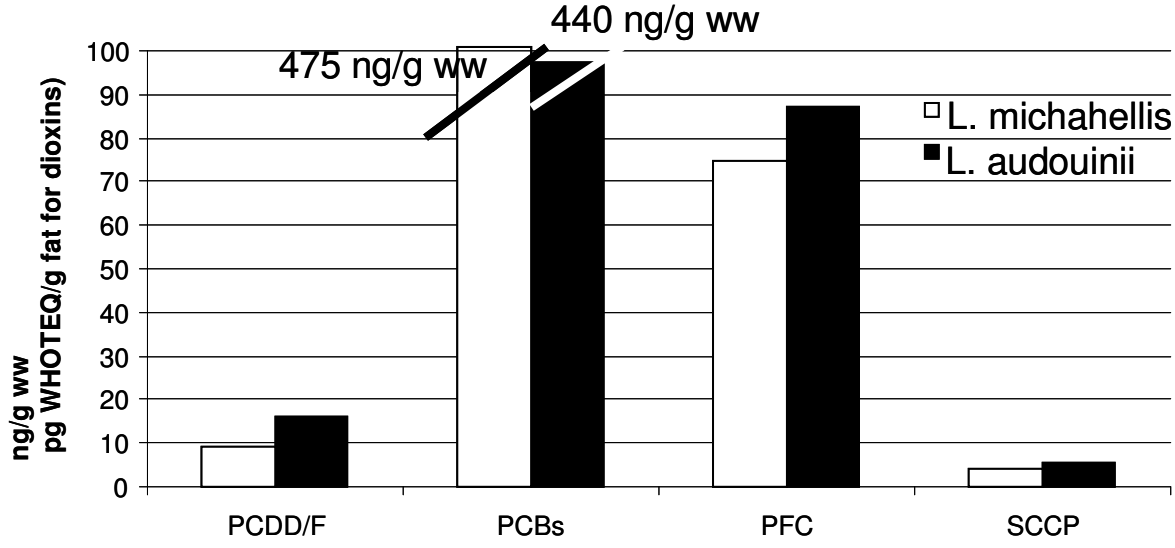


Results and discussion:

Seagull eggs collected in 3 areas of the Ebro delta colony presented all target compounds. Among PFCs, PFOS was the only compound detected at 45.1 ± 4 µg/g-ww (mean of 3 pools of 12 eggs each collected in 3 parts within the colony) in *Larus michahellis* and 63 ± 4 µg/g-ww in *Larus audouini*. PCDDs and PCDFs were detected in *Larus michahellis* at 9.08 pg WHO-TEQ/g lw, representing levels higher than the ones legislated in chicken eggs (3 pg TEQ/g lw). In *Larus audouinii*, 15.97 pg WHO-TEQ/g lw were found. Previous studies reported mean concentration for PCDDs and PCDFs of 126.66 ± 26.70 pg/g-dw and 14.01 ± 3.67 pg/g-dw, respectively, from Audouin seagull (*Larus audouinii*) from the Ebro collected in 1992[8]. Marker PCBs were in *Larus michahellis* from 0.124 ± 0.9 ng/g-ww (PCB 28) to 229 ± 98 ng/g-ww (PCB 153) with differences among the eggs collected from the 3 areas of the colony given their natural distribution according to birds' age. The ΣPCBs were of 475 ng/g-ww and similar levels were found in Larus *audouinii* (440 ± 209 ng/g-ww). Marker PCB congeners were previously identified in *Larus audouinii* at ΣPCBs 2 ± 0,7 µg/g-dw with levels decreasing from the first to the third eggs[9]. In other studies also from Spain, total PCBs (ng/g wet wt) in booted eagle (*Hieraaetus pennatus*) and goshawk (*Accipiter gentilis*), PCBs were of between 34.1–270 ng/g-ww and from non detected - 43.5 ng/g-ww[10]. SCCP were detected at 3.9 ± 1.6 ng/g-ww and 5.5 ± 1.6-ww in *Larus michahellis* and *Larus audouinii*, respectively. It is the first time these compounds are identified in seagull eggs of these 2 species from the Ebro delta. SCCP are additives of high resistance used as cutting fluids, plasticizers, paints, flame retardants, polyesters and polyolefin, etc. and their presence in seagull eggs indicate that they are dispersed in the environment and are accumulated in birds and transferred to the egg. They are known to trigger effects towards aquatic organisms and are considered carcinogenic in humans (group 2b).

Figure 2 shows the concentration of each chemical family in the 2 species monitored. The slight higher levels detected in the protected species (*Larus audouinii*) is attributed basically to feeding habits. This species feeds exclusively on fish or when fish tips are rare due to fishing bans, on crabs. On the other hand, *Larus michahellis* feeds on waste or fish tips. Feeding habits have, thus, an important role in the accumulation of POPs, given that the 2 species coexist in the same area. Finally, eggshell parameters were evaluated to determine whether the presence of this cocktail of chemicals can affect the egg developmental stage. No correlation was observed between the levels of contaminants and egg shell parameters. However, other effects towards the species cannot be disregarded given the overall high concentration of the POPs detected.

Figure 2. Comparative levels of total PCDD/F, PCBs, PFC and SCCP in seagull eggs of 2 species (*L. michahellis and L. audouinii*).



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