Determinants of Legacy Persistent Organic Pollutant Levels in the European Pond Turtle (Emys orbicularis) in the Camargue Wetland, France

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Running head: Low levels of POPs in *Emys orbicularis* in the Camargue

Determinants of legacy POPs levels in the European pond turtle (*Emys orbicularis*) in the Camargue wetland, France

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Highlights

- We analyzed POPs levels in 174 European pond turtles in the Camargue wetland.
- Only 3 POPs were detected in more than 10% of individual turtles.
- Sex, date, and mass did not explain the occurrence and levels of POPs in turtles.
- The occurrence and levels of PCB 153 increased with turtle age.
- Turtles from the two study sites differed in POP burdens, possibly due to different hydraulic system.

Abstract

Many banned persistent organic pollutants (POPs) remain for decades in the aquatic environment and can have harmful effects on long-lived predators because of their high bioaccumulation and biomagnification potentials. We investigated the occurrence and levels of 18 polychlorinated biphenyls (PCBs) and 16 organochlorine pesticides (OCPs) in European pond turtles (n = 174) from April to July 2018 in the Camargue wetland, France. Although the Camargue was highly contaminated in the last decades, plasma occurrence and levels of POPs were very low: we detected only 3 of the 34 compounds we analyzed in more than 10% of the turtles. POP burdens did not differ between males and females and was uncorrelated with sampling date and body mass. We observed differences in POP burdens between turtles from the two sampling sites. One possible explanation is the agricultural hydraulic system: plasma occurrence and levels were higher for PCB-52 and HCB in turtles captured in drainage channels, but lower for PCB-153 at the site that receives irrigation. Finally, the occurrence and levels of PCB 153 in turtles increased with age, likely because of bioaccumulation and much higher exposure 20-30 years ago than now.

Key words: reptiles, PCB, organochlorine pesticides, delta wetlands

1. Introduction

In Europe, the production and use of persistent organic pollutants (POPs) are prohibited or severely restricted since 2004 through the Stockholm Convention (http://www.pops.int/), but POPs still cause environmental concerns. Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) remain in the environment for decades because of their slow biodegradation and improper handling of contaminated wastes. Levels of POPs in animals increase with age (Vives et al., 2005; Binnington & Wania, 2014) and trophic level (Goutte *et al.*, 2020). Exposure to POPs causes a wide range of adverse effects, including neurotoxicity, endocrine disruption, immune dysfunction, reproductive impairment, and developmental abnormalities, which may ultimately compromise survival and reproductive output and lead to population declines of wild vertebrates (Goutte *et al.*, 2014, 2015; Salice *et al*., 2014).

Pollution, and especially industrial and agricultural discharge, is one of the major threats to freshwater ecosystems (Holt, 2000; Dudgeon *et al.*, 2006). Wetlands support an extremely rich biodiversity, but are among the most transformed and threatened ecosystems of the world (Revenga *et al.*, 2005), experiencing rates of population declines and species extinctions far higher than those in forests, grasslands, and coastal ecosystems (Dudgeon *et al.*, 2006). Deltas are often exposed to high water pollution from intensive agriculture (Kuenzer & Renaud, 2012) and from discharge of contaminants in upstream waters. The Camargue, in the Rhône River delta, is the largest wetland in France and is of international importance under the Ramsar Convention (https://www.ramsar.org/), but it is heavily impacted by

human activities (Cheiron *et al.*, 2018). The contamination of water bodies by agricultural, industrial, and urban discharges has been well studied over the past decades (Comoretto *et al.*, 2007). In particular, levels of PCBs in sediments of the Rhône River increase from upstream to downstream, reaching 417 µg/kg dw for the sum of 7 PCB congeners (28, 52, 101, 118, 138, 153, and 180, Mourier et al. 2014). Mean flux over the 2011–2016 period averaged 14 kg/year for PCB 180 in suspended particulate matter at the outlet of the Rhône River basin (Poulier *et al.*, 2019). Previous studies conducted in the Camargue confirmed that birds (Berny et al., 2002) and fish (Roche et al., 2002, 2003, 2009a) are exposed to OCPs and PCBs.

Freshwater turtles remain poorly studied in ecotoxicology although they can provide useful information on local contamination because of their longevity (Campbell & Campbell, 2002 ; EL Hassani *et al.*, 2019 ; Gaus *et al.*, 2019), high trophic-level, and low dispersal capacity (Ming-ch'eng Adams *et al.*, 2016). Moreover, as ectothermic vertebrates, they have a lower ability to metabolize pollutants than endothermic mammals and birds (de Solla, 2015). The European pond turtle, *Emys orbicularis*, a long-lived (> 40-80 years) opportunistic predator (fish, amphibian, crayfish) and scavenger (Ottonello et al. 2005; Ficetola & De Bernardi, 2006), is facing significant population declines due to multiple environmental alterations, including water pollution (Cheylan 1998). This species is listed as "near threatened" (NT) on the UICN Red List of threatened species in France (REF). Pollution by trace metal elements has been studied in *E. orbicularis* (Namroodi *et al.*, 2017 ; Guillot *et al.*, 2018 ; Beau *et al.*, 2019), but data are lacking on burdens of POPs.

We studied contamination levels in two populations of *E. orbicularis* at the Natural Reserve of the Tour du Valat, in the Camargue, France. Several individuals were of known-age due to capture-mark-recapture (CMR) program, starting more than 20 years ago (Olivier *et al.*, 2010 ; Ficheux *et al.*, 2014 ; Arsovski *et al.*, 2018). The hydraulic system combines irrigation canals originating from the Rhône river and drainage canals, which are exposed to various environmental contaminants from the Rhône river and agricultural plots, especially rice fields (Chauvelon, 1996). In the present study, we assessed recent (2018) levels of OCPs and PCBs in plasma of the European pond turtle (n = 174). We tested the effects of individual traits (mass, sex, and age) on POP burdens. We expected that older and larger individuals should have higher levels of POPs because of bioaccumulation and length of exposure. Moreover, we compared POP burdens of turtles from two populations in different locations in the agricultural hydraulic system (irrigation vs drainage). We expected high levels of OCPs in turtles from the drainage site, as a possible consequence of the remobilization of pesticides from agricultural soils, and high levels of PCBs in turtles from the irrigation channel, as a possible consequence of the historical contamination.

2. Material and methods

2.1. Sampling sites and capture

We conducted the study in the Natural Reserve of the Tour du Valat (43°30' N, 4°40' E, Fig. 1) in France. We captured European pond turtles (n = 174) in canals and marshes from 24 April to 26 July 2018 by hand or with funnel traps (Olivier *et al.*,

2010; Ficheux *et al.*, 2014). We captured turtles at two sites: (1) irrigation canals and their associated marshland (site of Esquineau, n = 126) and (2) drainage canals of the Fumemorte basin and marshes filled with water by these canals (site of Faïsses, n = 48). The sex ratio was the same between the two sites (58.3% females at Faïsses and 60.3% females at Esquineau, Pearson's Chi-squared test, $p = 0.95$).

Individual turtles were identified as part of a long-term CMR program with shallow notches on the marginal and nuchal scales in unique combinations (Olivier, 2002 ; Olivier *et al.*, 2010). Individuals were weighed and sexed by visual observation of the secondary sexual characters. The year of birth can be determined if the first capture occurred within the first 5 years of life by counting the number of growth streaks (Castanet, 1988). This long-term survey starts in 1997, although a few tens of individuals were also marked as adults from 1976. In our data set, 66 of the 174 individuals were of known-aged (5 to 26 year-old, corresponding to a first capture between 1997 and 2018) and some were first captured as adults in 1976, thus being considered as more than 44 year-old. When turtles were captured after their first 5 years of life (N = 108 in the present study), we determined a stage of growth according to growth streaks located on the plastron (Olivier, 2002). By combining estimated age and stage of growth, we then attributed an age class to each turtle: 4 to 8 year-old (N = 20), 9 to 13 year-old (N = 46), 14 to 22 year-old (N = 54), and 23 to 44+ year-old (N = 54). Turtles were released on the same day at their capture site.

2.2. Blood sampling

Plasma is a good matrix to determine individual burdens of POPs because blood sampling is minimally invasive and because circulating levels of POPs in blood are

149 significantly correlated to concentrations of POPs stored in fat (Keller *et al.*, 2004 ;
150 Dabrowska *et al.*, 2006), liver, kidney, and muscle tissues (Van de Merwe *et al.*, 2010).
151 We collected blood samples (2 ml, always less than 1% of the turtle body mass) from
152 the dorsal coccygeal vein (Innis & Knotek, 2020; Keller *et al.*, 2004) with a Terumo
153 Company® syringe (Somerset, USA) pre-impregnated with heparin to prevent blood
154 clotting during collection and equipped with a 25G needle (Franklin Lakes, USA).
155 We then centrifuged the samples to separate the plasma from the red blood cells. We
156 collected blood samples from 174 individuals (126 from Esquineau and 48 from
157 Faïsses), 66 individuals were of known age ranging from 5 to 31 years old (32 females
158 and 19 males from Esquineau; 9 females and 6 males from Faïsses). Samples were
159 stored at −18 °C until analysis in the UMR 7619 METIS of Sorbonne Université,
160 France.
161
162 **2.3. Chemical analyses**
163 We determined the levels of 17 OCPs (p,p′-DDT and metabolites (p,p′-DDE and p,p′-
164 DDD); pentachlorobenzene (PeCB); hexachlorobenzene (HCB);
165 pentachloronitrobenzene (Quintozene); four isomers of hexachlorocyclohexane (α-,
166 β-, γ- (lindane), δ-HCH); aldrin; endrin; isodrin; telodrin (Isonbenzan); heptachlor;
167 heptachlor epoxide; heptachlor endo-epoxide), 7 marker PCBs (M-PCBs, IUPAC
168 numbers # 28, 52, 101, 118, 138, 153 and 180), and 12 dioxin-like PCBs (DL-PCBs,
169 IUPAC numbers # 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189) in the 174
170 plasma samples.
171 Samples were processed through Solid Phase Extraction (SPE), using hexane:
172 dichloromethane, 9:1, using a validated protocol (Tapie et al. 2011) that was adapted

for plasma samples (see Supplementary Material for a detailed description of the procedure and method validations). PCBs and OCPs were analyzed using an Agilent 7890 A gas chromatograph (GC) coupled to a 7000 B triple quadrupole mass spectrometer system (MS/MS) (Agilent Technologies, Les Ulis, France). Recovery rates (% RR) of compounds were assessed on replicate plasma samples of European pond turtles (n = 4) with spiked solutions (100 ng of each compound). The repeatability of the method was assessed in terms of relative standard deviation (% RSD) of the recovery. RR and RSD are provided in Table S2 (Supplementary Material). Recovery rates were not satisfactory (< 75% or > 125%) for isodrin, endrin, heptachlor epoxid, heptachlor-endo-epoxide, p,p'-DDD, PCB-77, PCB-189 (Table S2) and they were not further considered.

2.4. Statistical analyses

We performed statistical analyses with R software version 3.3.2 (R Core Team, 2016). Only POPs detected (i.e. > LOQ) in at least 10% of the samples were included in the statistical analyses. Since age and weight were positively correlated ($t = 3.65$, $df = 64$, $p = 0.0005$) and females were significantly heavier than males ($W = 474$, $p < 0.0001$), body mass, sex and age were tested separately..

We used generalized linear models (GLM) with a binomial distribution and a logit link function to test for the effects of sex, site, the interaction sex * site, sampling date, and age (or body mass) on contaminant occurrence. For each model, we used a backward elimination to progressively remove non-significant terms ($p > 0.05$). For all analyses, model specification and validation were based on residual analysis.

196 The effects of sex, sampling site, date, and age (or body mass) on pollutant
197 concentrations were tested by considering left-censored data, i.e. pollutant levels
198 below the LOQ. To do so, group comparisons and linear regressions were performed
199 using Peto-Prentice tests and tobit models respectively, with the function cendiff of
200 the NADA package and the function tobit of the AER R-package.
201
202 ## 3. Results
203
204 ### 3.1. POP levels and occurrences
205 In 37 of the 174 samples, all contaminant levels were below the limit of
206 quantification, which represented 10.4% of turtles sampled in Faïsses and 24.4% of
207 turtles sampled in Esquineau. Eleven compounds were never detected in the plasma
208 samples: PeCB, Lindane, Quintozene, Heptachlor, and the dioxin-like PCBs: PCB-
209 118, -105, -114, -126, -156, -157 and -167. The following seven compounds were
210 detected at least in one sample from Esquineau, but not in samples from Faïsses:
211 alpha-HCH, 44'-DDT and PCBs 28, 101, 123, 169 (Table 1). Overall, the contaminant
212 concentrations in the plasma samples were very low (Table 1).
213
214 ### 3.2. Influence of individual traits, sampling site, and date on POP occurrences and
215 levels
216 Only three POPs (HCB, PCB-52 and -153) had substantial occurrence, being detected
217 in more than 10% of individuals (Table 1). Plasma occurrences of PCB-52 and HCB
218 were significantly higher in turtles captured at Faïsses compared to Esquineau, while

219 the occurrence of PCB-153 was higher in turtles captured at Esquineau (Tables 1 and
220 2). HCB occurrence slightly increased with sampling date (Table 2). Sex, mass, and
221 age class did not explain POP occurrence in turtles, except for PCB-153 whose
222 detection frequency was higher in older individuals (Table 2).

223 Levels of HCB and PCB-52 were significantly higher in turtles from Faïsses,
224 while PCB-153 levels tended to be higher in turtles from Esquineau (Tables 1 and 3).
225 Moreover, levels of PCB-153 were lower in the youngest turtles (Table 3, Fig. 2). Sex,
226 mass, and date did not explain variation in levels of POPs among turtles (Table 3).

227

228 ## 4. Discussion

229

230 The aim of this study was to characterize legacy levels of POPs (OCPs and PCBs) in
231 the plasma of European pond turtles in the Camargue, France, and to determine
232 whether these levels were a function of individual traits and habitat types. Plasma
233 levels of POPs were low and often below limits of quantification (from 0.2 to 3.1
234 ng/mL). We found significant differences in burdens of POPs between turtles from
235 the two sampling sites and these differences could be attributed to the hydraulic
236 system (drainage/irrigation). The occurrence and levels of PCB-153 were higher in
237 older turtles.

238 The Rhône River has been historically contaminated, leading to an
239 accumulation of POPs in sediments downstream (Mourier et al. 2014). Previous
240 studies conducted on several animal taxa in the Camargue confirmed high exposure
241 during the last decades, with high concentrations of PCBs in muscles of eels (*Anguilla*
242 *anguilla*) fished between 1997 and 2000 (Roche *et al.*, 2004), but also in eggs of little

egrets (*Egretta garzetta*) collected in 1996 (Berny *et al.*, 2002). We detected low levels of POPs in the pond turtle in Camargue, which could indicate a decline in legacy POP exposure for wild species in the Camargue. Although a longitudinal study of the same species at the same locations would be required to test this hypothesis, decreasing PCB concentrations have been observed in the Rhône River sediments over the last decades (Liber *et al.*, 2019) and water analyses conducted by the Nature Protection National Society (SNPN) have not detected PCBs and OCPs in the Fumemorte canal since 2011 (Cheiron, 2019). Moreover, organochlorine contamination across food webs tended to diminish in the Vaccarès lagoon (Roche *et al.*, 2009a), the largest water body of the Camargue Biosphere Reserve. Exotic red swamp crayfish (*Procambarus clarkii*), the main prey of European pond turtles in the Camargue (Ottonello *et al.,* 2005), were not contaminated by POPs in 2019 (i.e. < LOQ, with LOQ ranging from 0.9. to 1.98 ng/g dw, unpublished data).

One of the first studies looking at the plasma concentration of POPs in turtles documented high PCBs and OCPs levels in snapping turtles (*Chelydra serpentina*), in Ontario, Canada and sampled in 2001–2004 (Letcher *et al.*, 2015): plasma concentrations of OCPs (sum of 17 contaminants) ranged from 0.2 to 236 ng/g w.w. and the most abundant pesticide was p,p'-DDE (mean ± SE: 27 ± 6 ng/g w.w.). In our study, plasma concentrations of POPs (sum of 16 contaminants) were much lower, ranging from 0 to 2 ng/g w.w and p,p'-DDE levels didn't exceed 2.3 ng/g ww. Snapping turtles are freshwater turtles with similar feeding habits than European pond turtle: they also consume plant and animal matter, including aquatic invertebrates, fish, frogs, reptiles (Ernst et al. 1994).Other studies of turtles

documented plasma concentrations of POPs comparable to those in our study; for instance, for PCB 153 and HCB in another freshwater species, the Western pond turtle (*Actinemys marmorata*) in Sequoia National Park, USA, in 2012 (Meyer *et al.*, 2016), as well as in marine turtle, such as Loggerhead sea turtles (*Caretta caretta*) in the Eastern Atlantic Ocean in 2011-2012 (Bucchia *et al.*, 2015), and in Green turtles (*Chelonia mydas*) and Hawksbill turtles (*Eretmochelys imbricata*) in Cape Verde between 2009 and 2011 (Camacho *et al.*, 2014).

In terms of occurrence of POPs, our results in European pond turtles were low compared to other studies (Bucchia et al., 2015 ; Meyer et al., 2016). The occurrences of dioxin-like PCBs in 8% of European pond turtles, as well as p,p'-DDE in 2% of individuals were much lower than those found in plasma of Loggerhead sea turtles that ranged from 63% in the Atlantic Ocean to 100% in the Adriatic Sea for dioxin-like PCBs and 100 % for p,p'-DDE (Bucchia *et al.*, 2015).

Despite low detection frequencies and levels, we found differences in POP burdens between turtles from the two sampling sites. Turtles in Faïsses, a site receiving water from drainage canals, exhibited significantly higher occurrence and levels of HCB compared to turtles from Esquineau, likely because of the remobilisation of trapped OCPs in soils. On the other hand, the occurrence and levels of PCB 153 were higher in turtles from Esquineau, a site with irrigation channels. A previous study has documented higher concentrations of PCBs in the aquatic fauna in irrigation canals compared to the drainage canals downstream of rice fields (Roche *et al.*, 2009b). We also found that concentrations of PCB-52 were higher in turtles at Faïsses, however, which may be due to a lower sedimentation rate of PCB-52

289 associated with its lower molecular weight and a greater solubility due to a lower
290 level of chlorination compared to PCB-153 (Gong *et al.*, 1998 ; Alkhatib & Weigand,
291 2002). The processes of deposition and release of PCBs from the irrigation part to the
292 drainage part of the canals need to be further studied. In addition, other populations
293 of pond turtles in the two types of hydraulic systems should be studied to confirm
294 this pattern.

295 The occurrence and levels of PCB-153 were lower in the youngest individuals
296 (4 to 8 year old), which could be attributed to a shorter exposure period. PCB-153 is a
297 recalcitrant chemical with a high hydrophobicity (high octanol-water partition
298 coefficient), high resistance to metabolic transformation, and slow respiratory
299 elimination through air-exhalation (high octanol-air partition coefficient). PCB-153 is
300 also more prone to bioaccumulation than many other PCB congeners (Kelly *et al.*
301 2007), especially in long-lived air-breathing predatory species (Rowe, 2008). It is also
302 possible that there is a dietary shift during growth in pond turtles leading to the
303 consumption of higher trophic-level prey with age, and these higher trophic level
304 prey would be more PCB contaminated due to biomagnification. This hypothesis,
305 however, is not supported by dietary studies of *E. orbicularis* in Camargue), showing
306 a shift to a more herbivorous diet with age, using prey items identification in faecal
307 samples (Ottonello *et al.*, 2005) or no difference in the proportion of plants,
308 invertebrates and vertebrates, using metabarcoding analyses (Duccotterd et al.
309 2020).)

310 We did not detect differences in POP loads between male and female pond
311 turtles, despite males often being more contaminated than females in other turtle

312 species (Guirlet *et al.*, 2010 ; Bangma *et al.*, 2019; Lambiase e*t al.*, 2021) due to a
313 transfer of pollutants from the mother to the eggs through vitellogenesis (Moss *et al.*,
314 2009 ; de Solla, 2010).
315
316

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are indebted to the many people who participated in the turtle captures since 1997.

We thank the French Ministry of Environment for giving us the permission to capture European pond turtles.

Funding

This work was funded by the Water agency Rhône Méditerrannée Corse and the Fondation de la Tour du Valat.

Author contributions

AO, LB, CLG and NM conducted the field work and collected the blood samples. LB and FA conducted the laboratory work to prepare plasma samples for analysis with gas chromatography and tandem mass spectrometry. LB, OL, AG, AO, GBD, and MV conceived and coordinated the study, participated in data analysis, and in writing the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

340 **Ethical consideration**

341 This study was performed in accordance with laws relative to capture, transport, and
342 experiments on *E. orbicularis* (DREAL permit # CERFA_13616-01) and all procedures
343 were approved by an independent ethical committee (APAFIS#17899-
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525 **Figure captions**

526 **Figure 1:** Sampling locations for European pond turtles in the regional nature reserve
527 of Tour du Valat, Camargue, France. The two capture locations are Esquineau (in
528 blue), irrigated with water pumped from the Rhône River, and Faïsses (in orange),
529 consisting of drainage canals of agricultural parcels.

530

531 **Figure 2:** Increase in plasma PCB 153 (ng/mL) levels in European pond turtles with
532 age class (4 to 8, 9 to 13, 14 to 22, 23 to higher than 44 years old). Each point
533 represents an individual. Red and green squares correspond to mean and median
534 levels for age class, respectively. Age class was estimated according to growth
535 streaks located on the plastron (see methods).