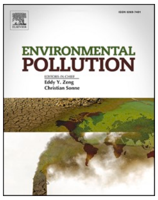
Environmental Pollution 341 (2024) 122903

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



Pesticide concentrations in a threatened freshwater turtle (*Emys orbicularis*): Seasonal and annual variation in the Camargue wetland, France[☆]

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ARTICLE INFO

Handling Editor: Philip N. Smith

Keywords:
Freshwater turtle
Plasma
Bentazone
Water contamination
Liquid chromatography–mass spectrometry

ABSTRACT

Wetlands are among the most threatened ecosystems on the planet and pollution is a major factor causing the decline of wetland biodiversity. Despite the increasing use of pesticides, their fate and effects on freshwater reptiles remain largely unknown. We studied the European pond turtle (*Emys orbicularis*), a long-lived species at risk with a high exposure potential to pesticides. Between 2018 and 2020, we measured 29 pesticides and metabolites in 408 blood samples of turtles from two populations in the Camargue wetland (France). We were able to quantify 24 compounds and at least one pesticide or one degradation product in 62.5% of samples. Pesticide occurrences and concentrations were low, except for a herbicide widely used in rice cultivation and locally detected in water: bentazone that reached high blood concentrations in *E. orbicularis*. The occurrence and the concentration of pesticides in *E. orbicularis* blood depended mainly on the site and the sampling date in relation to pesticide application. Individual characteristics (sex, age, body condition) did not explain the occurrence or the concentration of pesticides found in turtle blood. Assessing the exposure of aquatic wildlife to a cocktail of currently-used pesticides is a first and crucial step before studying their effects at the individual and population levels.

1. Introduction

Freshwater habitats are major biodiversity hotspots (Balian et al., 2008; Reid et al., 2019; Dudgeon, 2019). Freshwater ecosystems represent less than 1% of the Earth surface (Dudgeon et al., 2006; Cazzolla Gatti, 2016), but support at least 125 000 species, including 14% of vertebrates (Balian et al., 2008). Freshwater habitats suffer from multiple anthropogenic pressures, such as overexploitation, habitat destruction, water pollution, flow modification, and climate change (Dudgeon et al., 2006; Cazzolla Gatti, 2016), thus making them among the most threatened ecosystems globally (Revenga et al., 2005). Over the past decades, freshwater biodiversity decline has outpaced terrestrial biodiversity decline, especially for vertebrates (Reid et al., 2019; Dudgeon, 2019; Revenga et al., 2005; Tickner et al., 2020). Despite its potential role in explaining biodiversity declines, water pollution remains poorly studied (O'Brien et al., 2016; Groh et al., 2022). The development and improvement of detection and quantification methods during the last decade, however, now allows to better track the fate of contaminants in the environment and in living organisms (Lazartigues et al., 2011; Hernández et al., 2012; Guibal et al., 2015; Schanzer et al., 2021; Rial-Berriel et al., 2020).

Pesticides are ubiquitous in freshwater environments (Malaj et al., 2014; Boivin and Poulsen, 2017). However, the levels of contamination by currently-used pesticides are poorly documented in free-living

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[☆] This paper has been recommended for acceptance by Philip N. Smith.

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https://doi.org/10.1016/j.envpol.2023.122903
Received 26 July 2023; Received in revised form 19 October 2023; Accepted 7 November 2023

vertebrates (Brühl and Zaller, 2019), despite a growing number of substances being commercialized and increasing application volumes (Bernhardt et al., 2017). Chronic exposure to pesticides can induce a wide range of sublethal effects in non-target species (Köhler and Triebskorn, 2013). For instance, pesticides may induce oxidative stress (Costantini, 2019; Costantini et al., 2014), disrupt the endocrine system (e.g., thyroid hormones (Leemans et al., 2019)), and the reproductive system (Milnes et al., 2006; Barraza et al., 2021; Guillette and Gunderson). In turn, these sub-lethal effects can compromise long-term survival and reproductive output, thus leading to population decline, as previously demonstrated for organochlorine pesticides (Goutte et al., 2015). The contribution of environmental pollutants to biodiversity declines is clearly underestimated (Groh et al., 2022). Most ecotoxicological studies are conducted in the laboratory or in mesocosms on one specific chemical (Bidwell, 2020), without taking into account multiple co-occurring biotic and abiotic stressors and "cocktail" effects of contaminants. Moreover, vertebrate models for aquatic ecotoxicology are generally African clawed frogs *Xenopus laevis* and zebrafish *Danio rerio*, but not reptiles (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2018; Hopkins, 2000; Carvan et al., 2008; Sparling et al., 2010). Reptiles are usually not included in environmental risk assessments (ERA) (Mingo et al., 2017; Ortiz-Santaliestra et al., 2018) and were not integrated in ERA before 2013 in Europe (COST, 2019) even though reptiles are proportionally one of the most threatened groups of vertebrates (Cox et al., 2022). The European Food Safety Authority recently emphasized the need for field studies to assess the risks that contaminants pose to reptiles (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2018).

Several aspects of the biology of reptiles make ecotoxicological studies challenging: they are often elusive and their usually long generation times make it difficult to follow the fate and effects of contaminants over several generations (Hopkins, 2000). Moreover, their slower metabolism compared to endotherms could lead to much different rates of bioaccumulation and metabolization of pollutants (Hopkins, 2000; Hopkins et al., 2005). On the regulatory side, the classification of numerous reptile species as endangered or protected under national and international laws increased the complexity of obtaining the necessary permissions to conduct field or laboratory experiments (Barraza et al., 2021).

Freshwater turtles are key species to assess aquatic reptile contamination with different types of pollutants. As long-lived species with high spatial fidelity (Olivier, 2002; Escoriza et al., 2020; Fay et al., 2023), they can potentially give a comprehensive picture of local contamination because they are exposed to several pollutants in one area over a very long time. Turtles are opportunistic predators and scavengers and, therefore, can have multiple pathways of contamination (Ottonello et al., 2005; Ziane et al., 2020; Ducotterd et al., 2020). Wagner et al. (2015) developed a species specific risk index for pesticide exposure in European protected reptiles and revealed that all evaluated turtles were at high risk of pesticide exposure, including the European pond turtle (*Emys orbicularis*) (Wagner et al., 2015), one of two native freshwater turtles of Western Europe. A few recent ecotoxicological studies have been conducted on *E. orbicularis*, investigating the levels of heavy metals and legacy persistent organic pollutants present in their bodies (Namroodi et al., 2017; Guillot et al., 2018; Beau et al., 2019; Burkart et al., 2021).

We monitored pesticide contamination from 2018 to 2020 in the blood of *E. orbicularis* in the southern French region of the Camargue. The Camargue is the largest wetland in France and is classified as a RAMSAR site. However, water quality is a major issue in this region because of high concentrations of pesticides used in rice cultivation, notably herbicides (Cheiron, 2019; Cheiron and Bricault, 2020; Cheiron and Bricault, 2021). The study was conducted at the Tour du Valat on two populations of *E. orbicularis* monitored by capture-mark-recapture (CMR) since 1997 (Arsovski et al., 2018; Ficheux et al., 2014; Olivier et al., 2010). Our main objectives were.

1. To measure levels of pesticides in water in the main canal draining farmlands of the study area
2. To document levels of pesticides in blood plasma from *E. orbicularis*.
3. To evaluate the temporal and spatial variation of pesticide occurrence and levels in *E. orbicularis* as well as the effects of individual characteristics such as sex, body condition, and age on pesticide burden.

2. Material and methods

2.1. Water sampling and analyses

The study was conducted in Southern France in the Natural Reserve of the Tour du Valat (43°30′N, 4°40′E) located in the Camargue (Fig. 1). Water analyses have been conducted since 2011 by the National Nature Protection Society as part of long-term monitoring of the contamination of the waters that feed into the Camargue Nature Reserve (Cheiron and Bricault, 2021). The sampling site was located at the mouth of the Fumemorte canal, which runs through the Natural Reserve of the Tour du Valat then empties into the Vaccarès lagoon (Fig. 1). In total, 417 pollutants were measured in surface water grab samples collected every month between March and October. These samples were analyzed at a private laboratory approved for water analyses by the French Ministry of Health (CARSO - Laboratoire Santé Environnement Hygiène De Lyon). The list of pesticides and the quantifications methods are presented in Table A1 of the Supplementary Material. We only included pesticides that were also quantified in turtle plasma, which represented 26 compounds.

2.2. Turtle captures and marking

We captured turtles at two nearby sites that differed in their hydrology (Burkart et al., 2021). Esquineau consists of irrigation canals and associated marshes from the Aube de Bouic canal system that comes from the Rhône River (Fig. 1). Thus, this site is less likely to be exposed to pesticides because these canals are used for irrigation, not for drainage. Faïsses consists of drainage canals of the Fumemorte basin with their associated marshes and is thus more likely to be contaminated by pesticides because they drain water from rice fields, among others (Fig. 1). We captured turtles using funnel traps or by hand in canals and marshes during spring and summer of 2018, 2019 and 2020 (2018: from 25 April to 26 July, 2019: from 23 April to 26 July, 2020: from 09 April to 7 August). Funnel traps were checked daily. Captured turtles were taken to the laboratory at the Tour du Valat. Newly captured individuals were uniquely marked by notching the marginal and nuchal scales (Olivier, 2002). Turtles were sexed based on secondary sexual characters (i.e., shape of the plastron and tail, iris color) (Olivier, 2002). We released the turtles the same day at their site of capture. Captures were authorized by the French Departmental Authorities (permits: DREAL Cerfa 13 616–01; N°13-2020-03-27-007).

2.3. Individual characteristics

We weighted turtles with a precision scale (Mettler Toledo PB3001–S) and we measured carapace length with a caliper. Body condition was estimated from the residuals of a linear regression of the log-transformed mass as a function of the log-transformed carapace length, with the addition of sex as a control variable. To determine age, we counted the number of growth rings on the plastron scutes (Castanet, 1988) for individuals less than 5 years old at the time of their first capture. The exact age of individuals that were already adults when they were first captured could not be determined because the growth rings had faded and growth had nearly stopped. Growth rings become difficult to count for older individuals in our study populations (Olivier, 2002). Our study populations have been monitored by capture-mark-recapture

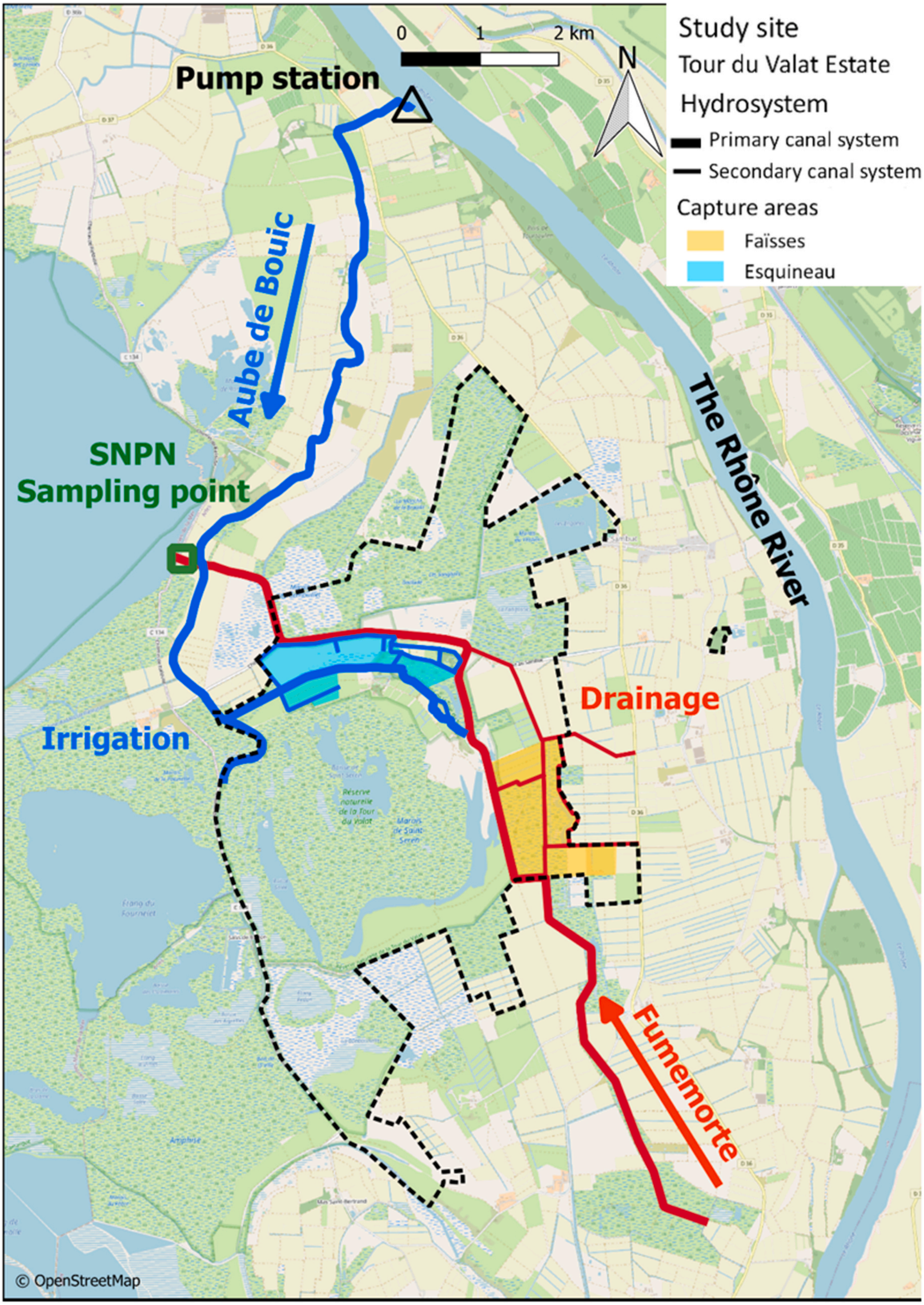

Fig. 1. Capture areas of two populations of *E. orbicularis* in the Natural Reserve of the Tour du Valat, Camargue, France (blue area: Esquineau, irrigation canals and marshes, orange: Faïsses, drain canals and marshes, green square: water sampling point in the Fumemorte Canal). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

for over 40 years, the first individuals were marked in 1976 (Olivier, 2002). This long-term monitoring allowed us to know the exact age of most individuals in these two populations.

2.4. Blood sampling

We collected 1.5 mL (always less than 1% of the individual body mass) of blood from the dorsal coccygeal vein using a previously heparinized Terumo syringe and a 25G needle. Captured individuals were selected for this procedure if their mass was over 300 g. The samples were then centrifuged to separate plasma from hematocytes and were stored at −18 °C until analysis in the UMR 7619 METIS of Sorbonne University. This procedure was authorized by an Ethics Committee (permit: APAFIS 17899–201 812 022 345 423), following the EU Directive 2010/63/EU for animal experiments.

Blood is a good tissue to measure pesticide levels non-lethally (Keller et al., 2004a; Pacyna-Kuchta, 2023; Keller et al., 2004b). *E. orbicularis* is a protected species under French law, thus only minimally invasive procedures are allowed. Blood also allows to quantify circulating levels of pesticides and thus of levels to which organs such as the liver, kidneys, and brain are exposed (Keller et al., 2004b).

2.5. Pesticide analyses in plasma

We determined the levels of 29 pesticides or their metabolites in the 408 plasma samples: 22 herbicides (atrazine, diethylatrazine, deisopropylatrazine, hydroxyatrazine, bentazone, chlortoluron, diflufenican, isoproturon, metazachlor, S-metolachlor (and metabolites ESA et OXA), nicosulfuron, oxadiazon, pendimethaline, penoxsulam, propanil (and metabolite 3,4 dichloroaniline), prosulfuron, terbuthylazine, desethyterbuthylazine, simazine), 4 insecticides (imidacloprid and metabolites olefin and urea; tebufenozide), 2 fongicides (carbendazim, tebuconazole), and 1 algicide (, irgarol). These target compounds were chosen based on their local use and on pesticides levels detected in the water of the Fumemorte canal since 2011 b y the National Nature Protection Society (SNPN). Among the parent compounds, five of them (atrazine, carbendazim, isoproturon, propanil and simazine) were banned in France before our study (respectively in 2004, 2009, 2017, 2008, and 2004).

Pesticide extraction and purification were performed based on a protocol developed to determine herbicide concentrations in human serum with a multi-residues analysis (Dong et al., 2014). After being thawed and vortexed for 5 sec, we collected 100 μL of plasma, added 10 μL of a mix of 14 internal standards (atrazine ^{13}C, bentazone D6, carbendazim D3, diethylatrazine ^{13}C, diflufenican D3, hydroxyatrazine ^{13}C, imidacloprid D4, isoproturon D6, metolachlore D6, nicosulfuron D6, tebuconazole D6, terbutylazine D5, 5 ng.μL^{-1}, Sigma Aldrich). After one night at 4 °C, we added 200 μL of acetonitrile, centrifuged (6000 rpm; 3 min), transferred the supernatant in nylon filter tubes (porosity 0.2 μm, VWR, Fontenay sous Bois) and centrifuged again (6000 rpm; 1 min). The extracts were then transferred in 2 mL glass vials with 400 μL inserts. Nylon filter tubes were rinsed with 75 μL of acetonitrile and then transferred in the vials. Extracts were stored at −18 °C. Chemicals were identified by an Agilent 1200 liquid chromatograph (LC) interfaced to a 6410 B quadrupole mass spectrometer system (LC: 1200; MS/MS: 6410 B; Agilent Technologies, Les Ulis) with a Zorbax Eclipse Plus C18 column (2.1 × 150 mm; 3.5 μm; Agilent Technologies, Les Ulis).

We took 10 μL of each thawed vial content and injected them in the LC device. The mobile phase is defined by a gradient with an acetonitrile +0.1% formic acid solvent and an EMG +0.1% formic acid solvent. Mass acquisition was done in Dynamic Multiple Reaction Monitoring (dMRM, see Supplementary Material). The quantification is then performed with an internal standard calibration scale with Mass Hunter software (Agilent). The methodological validation was based on the yield and quantification limits (LOQ) of these chemical analyses (See Supplementary Material for quality controls). Occurrence of a compound was defined as its detection above the LOQ. Overall pesticide occurrence was defined as the detection of at least one compound above its LOQ in the plasma.

2.6. Statistical analyses

We conducted all statistical analyses with R software (v4.1.1). We used GLMMs (Generalized Linear Mixed Models) with individual identity as a random effect to control for repeated sampling of individuals. We tested the effects of year, site, sampling date (Julian day), sex, body condition, and the interaction site × year and sampling date × year on (A) the detection of at least one compound above the LOQ, hereafter defined as overall pesticide occurrence (see above, binomial GLMMs with a logit link), (B) the detection of bentazone, i.e. bentazone occurrence (binomial with a logit link), and (C) bentazone concentration (Gaussian with an identity link) only in samples with values higher than the LOQ ($N = 149$). Bentazone levels were log-transformed to best fit the model assumptions. Because age and body condition were strongly correlated, we also conducted the same GLMMs by testing the effect of age on the subset of known-aged individuals ($n = 209$ samples when testing pesticides or bentazone occurrence and $n = 56$ when testing bentazone concentration). The *lme4* package (Bates et al., 2022) was used and model assumptions were checked by examining residual plots. Models were progressively reduced through stepwise elimination of non-significant variables ($p > 0.05$). Pairwise comparisons were conducted using Tukey post hoc tests in the *emmeans* package (Lenth et al., 2023).

3. Results

3.1. Pesticides levels in the Fumemorte canal

Among pesticides examined both in the water and in the plasma, only 35% (9/26) were detected above their LOQ in the water in 2018 and 23% (6/26) were quantified in 2019 and 2020.3,4 dichloroaniline, bentazone, oxadiazon, penoxsulam, tebuconazole and tebufenozide were quantified every year. The sum of all the pesticide concentrations increased between April and May, except for 2020 when they started increasing in August (Fig. 2, Table A2 Supplementary Material). Bentazone was by far the most prevalent pesticide and was responsible for the high peaks observed every year between 16 497 and 20 660 ng.L^{-1} (Fig. 2). However, the bentazone peak in 2019 was later in the season compared to 2018 and 2020, in early July (Fig. 2).

3.2. Pesticides in plasma samples of E. orbicularis

We sampled 257 individuals, corresponding to 408 blood samples (some individuals being sampled several years, $n = 116$). We obtained 255 blood samples from Esquineau and 153 from Faïsses. Among the 116 individuals sampled several years, only one male switched from Esquineau in 2018 to Faïsses in 2020. There was no difference in sex ratio between sites (61.4% females at Faïsses and 59.6% females at Esquineau, Pearson's chi-squared test, $p = 0.794$). We knew the age of 127 among the 257 turtles (mean = 15.6 years, sd = 8.8, see Table A8, Supplementary Material). Data on individual characteristics are summarized in the Supplementary Material (Table A8).

A majority of plasma samples (62.5%, 255/408) were contaminated by at least one pesticide or one degradation product. Among the 29 compounds analyzed, 24 were detected at least in one sample, and 11 only in Esquineau (Table A1). The frequency (% of values above the LOQ) of each compound was low (less than 10%), except for the herbicide bentazone, which was detected in 36.5% of plasma samples. The number of compounds detected varied between years: 24, 12, and 8 compounds were detected in 2018, 2019, and 2020, respectively.

a) Overall pesticide occurrence

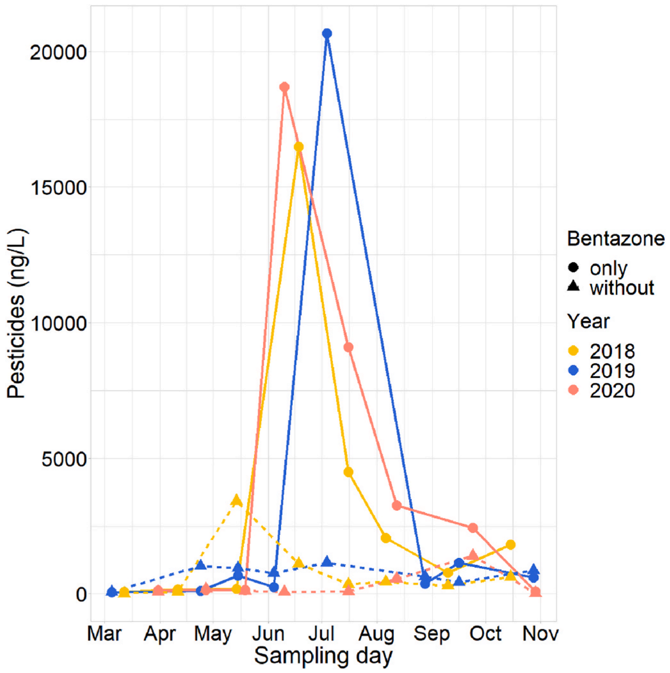

Fig. 2. Sum of the pesticide levels detected in water of the Fumemorte canal (SNPN), France, according to sampling date in 2018 (yellow), 2019 (blue), and 2020 (red), (dotted lines and triangle dots: sum of the pesticide levels excluding bentazone, solid lines and circle dots: bentazone levels only). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The probability to detect at least one compound in a sample tended to decrease during the season (i.e., with the date of capture) in 2018, but to increase in 2019 and in 2020 (Table 1; Fig. 3). Samples from Faïsses were also more frequently contaminated than samples from Esquineau, regardless of the year (Table 1; Fig. 3, overall pesticide occurrence of 80.3% and 40%, respectively). We did not find any effects of body condition or sex on overall pesticide occurrence. We found similar results when considering only known age individuals (Table 1). Age did not have any effect on the probability to detect a compound in the plasma (Table 1).

b) Bentazone occurrence

Bentazone was more frequently detected in samples from 2018 and from 2020 than in samples from 2019 (Table 1; Fig. 4). Bentazone was also more frequently detected in samples from Faïsses than in samples from Esquineau (Fig. 4). We did not find an effect of sampling date, but the interaction between year and sampling date was nearly significant (Table 1). We found qualitatively similar results for the subset of turtles of known age. The effect of individual characteristics, including age, remained non-significant (Table 1).

c) Bentazone concentrations

Bentazone concentrations were higher in samples from 2018 and from 2020 than in samples from 2019 (Table 1; Fig. 5). Bentazone concentrations were also higher in samples from Faïsses than in samples from Esquineau (Table 1; Fig. 5). Bentazone concentrations increased during the season in 2018 and in 2020, but they were stable in 2019. Body condition and sex did not affect plasma bentazone concentrations. We found qualitatively similar results for the subset of known age turtles (Table 1).

Table 1
Effects of environmental and individual variables on the occurrence and levels of pesticides in the blood of *E. orbicularis* living in the Camargue wetland, France from 2018 to 2020.

A. Overall pesticide occurrence	All (408 samples)			Known-aged (209 samples)		
	chi^2 (	df	p value	chi^2	df	p value
year	**19.644**	**2**	**< 0.001**	**8.202**	**2**	**0.017**
site	**39.489**	**1**	**< 0.001**	**19.291**	**1**	**< 0.001**
sampling date	**6.417**	**1**	**0.011**	**4.522**	**1**	**0.033**
year x sampling date	**12.751**	**2**	**0.002**	**9.567**	**2**	**0.008**
year x site	4.579	2	0.101	1.147	2	0.564
body condition	1.823	1	0.177	_	_	_
sex	0.134	1	0.715	0.002	1	0.962
age	_	_	_	<0.001	1	0.986
B. Bentazone occurrence	**All (408 samples)**			**Known-aged (209 samples)**		
	chi^2	**df**	**p value**	**chi^2**	**df**	**p value**
year	**15.322**	**2**	**< 0.001**	**9.834**	**2**	**0.007**
site	**34.380**	**1**	**< 0.001**	**17.631**	**1**	**< 0.001**
sampling date	3.542	1	0.060	0.001	1	0.975
year x sampling date	5.952	2	0.051	3.902	2	0.142
year x site	2.683	2	0.261	1.851	2	0.396
body condition	0.017	1	0.896	_	_	_
sex	0.173	1	0.677	0.021	1	0.886
age	_	_	_	0.565	1	0.452
C. Bentazone levels	**All (149 samples)**			**Known-aged (56 samples)**		
	chi^2	**df**	**p value**	**chi^2**	**df**	**p value**
year	**23.239**	**2**	**< 0.001**	**12.240**	**2**	**0.002**
site	**88.659**	**1**	**< 0.001**	**25.476**	**1**	**< 0.001**
sampling date	**25.323**	**1**	**< 0.001**	**16.606**	**1**	**< 0.001**
year x sampling date	**14.444**	**2**	**< 0.001**	**8.817**	**2**	**0.012**
year x site	3.114	2	0.211	1.511	2	0.470
body condition	0.033	1	0.855	_	_	_
sex	0.996	1	0.318	0.0003	1	0.986
age	_	_	_	0.625	1	0.429

Footnotes: Results of generalized linear mixed models (GLMM) for Pesticide and Bentazone occurrence and general linear mixed models (LMM) for B Table 1 entazone levels on a subset of samples with quantifiable levels of bentazone, with log-transformed data. Known-aged: subset of individuals of known age.

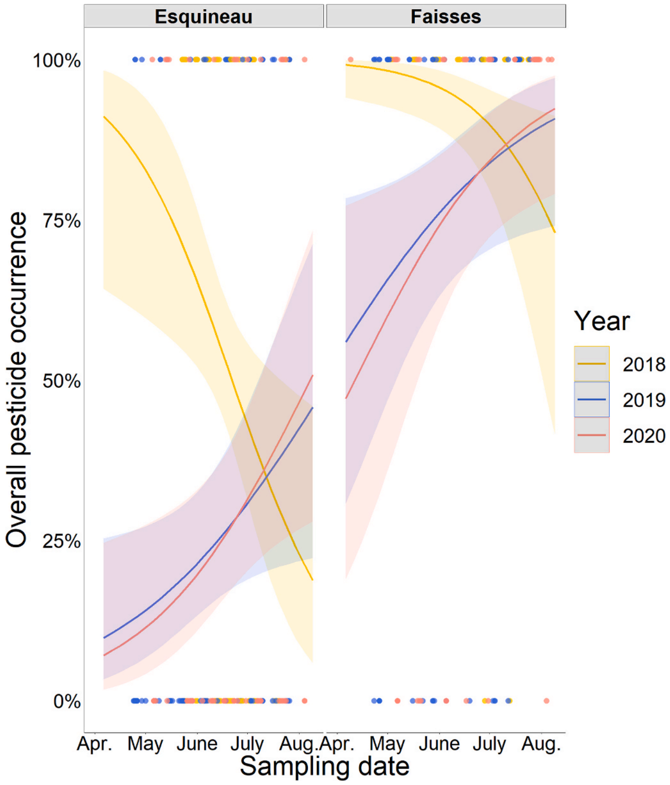

Fig. 3. Occurrence of total pesticide detected in blood plasma of *E. orbicularis* of two populations in the Camargue, France, according to the sampling date, the year (2018: yellow; 2019: blue; 2020: red), and the sampling site (dots: data, lines: predictions of the mixed-effect model including the sampling site and the interaction of sampling date and the year, R^2 conditional = 0.44). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

We performed paired tests to determine the consistency of bentazone concentrations in turtles sampled in two or three years. Turtles sampled both in 2018 and in 2019 (n = 44) exhibited significantly lower bentazone levels in 2019 than in 2018 (Fig. 6A; paired *t*-test: t = 2.142, df = 21, p = 0.044). Turtles sampled in both 2019 and in 2020 (n = 58) exhibited significantly higher bentazone levels in 2020 than in 2019 (Fig. 6B; paired *t*-test: t = −3.144, df = 28, p = 0.004). Bentazone levels did not differ between years in turtles sampled both in 2018 and in 2020 (n = 60, Fig. 6C; paired *t*-test: t = −1.909, df = 29, p = 0.066). One individual was sampled in Esquineau in 2018 (0.00 ng/g) and in Faisses in 2020 (78.92 ng/g). Turtles sampled in all three years exhibited consistent bentazone concentrations (Fig. 6D; ANOVA, F (102) = 1.815, p = 0.168).

4. Discussion

Studies on currently used pesticides quantification (as well as pesticide metabolites) in wild animals remain scarce, especially in reptiles. We measured the plasma levels of 29 pesticides or degradation products in two populations of *E. orbicularis* living in the Camargue, a French wetland with high pesticide application. We detected 24 compounds and, generally, pesticide occurrences and bentazone levels were linked to the site and date of capture. We did not find any effect of age or sex for pesticide occurrence and bentazone levels. Overall, these results strongly suggest that blood contamination is linked to seasonal pesticide application.

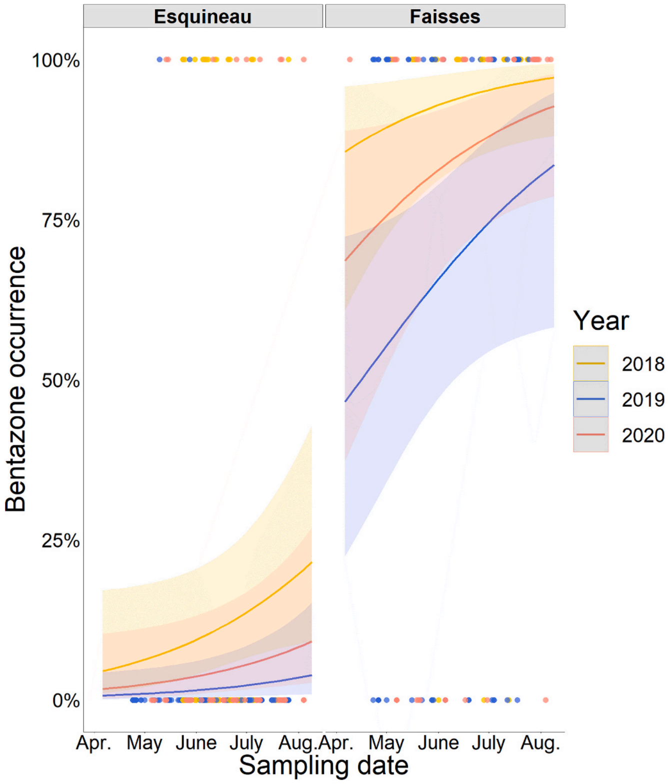

Fig. 4. Occurrence of bentazone detected in blood plasma of *E. orbicularis* of two populations in the Camargue, France, from 2018 to 2020, according to the sampling date, the year (2018: yellow; 2019: blue; 2020: red) and the sampling site (dots: data, lines: predictions of the mixed-effect model including the sampling site, the sampling date and the year, R^2 conditional = 0.71). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.1. *Evidence of contamination and exposure pathways*

Our main result is that 82.8% of the pesticides or degradation products were detected in blood samples. With the exception of bentazone, compounds reached a quantifiable level in very few samples, however. Several of these compounds were also detected between 2018 and 2020 (n = 10) in water by the SNPN at the mouth of the Fumemorte canal, less than 5 km away from the most distant point of turtle capture. Thus, polluted water is likely to be a major pathway of contamination for freshwater reptiles. Yet, some of the pesticides we detected in turtle blood samples were not detected in water analyzes run by the SNPN (n = 7). The dilution of the compounds along the waters of the Fumemorte to the collection point several kilometers downstream could decrease the probability of detection. Moreover, there are multiple exposure pathways for turtles (diffusion and ingestion of water, but also pulmonary respiration or ingestion of soil particles and contaminated prey). Some studies found substantial levels of bentazone in sediments (Calvo et al., 2021; Peris et al., 2022), even if the calculated sediment-water distribution coefficient was low (Log Kd = 0.15) (Peris et al., 2022). Even if contaminants are not detected in the main drainage canal, exposure and contamination can occur in the surrounding hydraulic system. The potential other exposure pathways (air, sediments) also need to be taken into consideration, notably sediments.

4.2. *Site and seasonal variation in pesticides*

Site of sampling was a major predictor of pesticide burden. Overall pesticide and bentazone occurrences as well as bentazone levels were significantly higher in Faïsses than in Esquineau, regardless of the year.

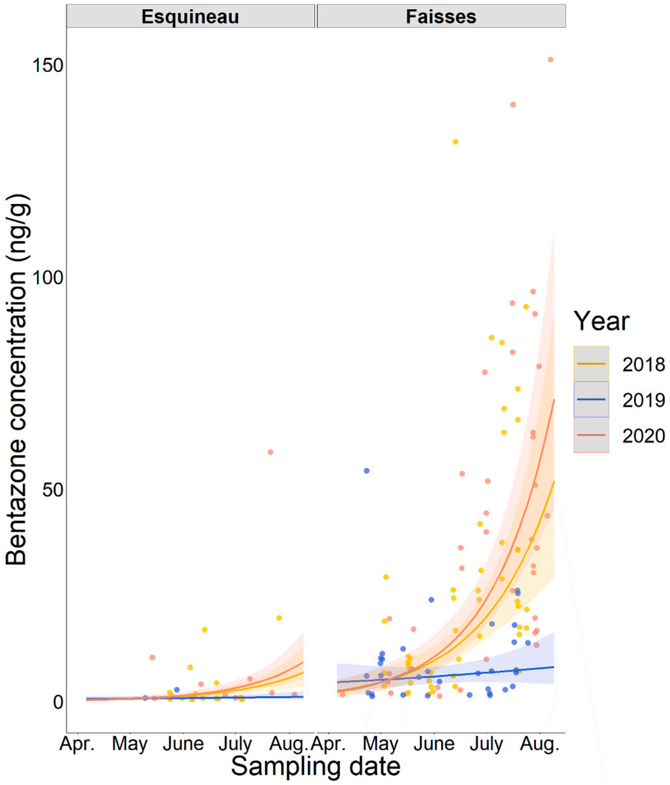

Fig. 5. Bentazone levels (ng/g) detected in blood plasma of *E. orbicularis* of two populations in the Camargue, France, from 2018 to 2020, according to the sampling date, the year (2018: yellow; 2019: blue; 2020: red) and the sampling site (dots: data, lines: predictions of the mixed-effect model including the sampling site and the interaction of sampling date and the year, R^2 conditional = 0.72). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

However, pesticide diversity was higher in Esquineau. Of the 11 compounds detected in Esquineau, but not in Faïsses, seven were only detected in 2018. As more compounds were detected in 2018, it is likely that it was particular regarding pesticide use, runoff, or resolubilization. Pesticide runoff could have happened upstream from the Esquineau canal system, leading us to detect several chemicals, including compounds not detected in the drainage canal system flowing through the Fumemorte canal. This situation has been documented in the Camargue in a study of pesticide runoff on a rice farm. One of the four pesticides quantified was already detected in irrigation canals before pesticide spreading started on the farm (Comoretto et al., 2007). However, we should note that twice as many samples were taken from Esquineau in 2018 compared to Faïsses, increasing the probability of detection for uncommon compounds. The higher frequency of contamination occurrences in Faïsses than in Esquineau was mainly driven by bentazone (Table A1). Faïsses comprises drainage canals and associated marshes, explaining why bentazone, a widely and heavily used pesticide in the Camargue (Cheiron, 2019; Cheiron and Bricault, 2021; Comoretto et al., 2007), appears with higher frequency and levels in samples from this site. Keeping in mind the limitations imposed by the lack of replication of our sampling sites (only one for each hydrology type), our results suggest an effect of fine scale hydrology as well as the agricultural practices in the immediate surroundings on pesticide contamination in *E. orbicularis.*

The date of capture influenced overall pesticide occurrence and this effect depended on the year. The probability of a plasma sample to be contaminated increased over the season in 2019 and 2020, whereas it decreased in 2018. This decline in 2018 supports the hypothesis of a particular event of contamination in early season, especially at Esquineau, where several compounds were detected during that year only. Water quality data from the SNPN also revealed a peak of total pesticide levels every year, occurring between June and July depending on the year. This is consistent with pesticide application in agricultural fields during late spring-early summer and whose drainage waters flow to the mouth of the Fumemorte canal. A similar result was found in blackbirds where azole fungicides blood concentrations increased during late spring/early summer (Angelier et al., 2023), as well as in bumble bees where differences in pesticide frequency detection across seasons followed local pesticide applications (Botías et al., 2017). Pesticide pollution in the waters of the Camargue has peaks matching the application and drainage of rice fields (Cheiron and Bricault, 2021). Contamination of blood samples did not follow the peak observed in water, with instead a decrease in occurrence later in the season. This could be explained by the fact that water analyses in the Fumemorte mouth did not necessarily reflect the local water contamination. As previously indicated, turtles can be contaminated through multiple pathways, including exposure by ingestion (prey or sediments) and by respiration (atmospheric levels). In addition, despite a high site fidelity, *E. orbicularis* still have home range of several hectares (Cadi et al.) and can therefore spend time in the vicinity of agricultural field and be exposed to pesticides before and after the peak in water releases. Another non-mutually exclusive hypothesis is that individuals could metabolize pesticides at different rates. These results on the effects of year and of date of sampling, support the argument that this species responds rapidly to environmental pesticide contamination, at least for the molecules analyzed.

4.3. *Influence of individual characteristics*

Individual characteristics (age, sex, and body condition) had no effect on the occurrence and levels of pesticides in turtle blood. Although higher contamination levels should be expected in older individuals as a result of bioaccumulation, there was no age-related contamination for these pesticides in turtles from 4 to 42 years old. This could be explained by high metabolization and excretion rates of these compounds. Little is known about pesticide metabolization and excretion in reptiles, especially for hydrophilic compounds. A study on an Asian lizard species showed a fast metabolization for imidacloprid, the second most quantified pesticide in our study (Wang et al., 2019). We may also have had limited power to detect an effect of age because all the compounds (except bentazone) were detected in a small proportion of individuals (less than 5%) and turtle age was known for a subset of 209 samples. Maternal transfer of contaminants has been confirmed in several reptiles, including turtles (Barraza et al., 2021). Currently used pesticides are usually less lipophilic than Persistant Organic Pollutants which could explain why we found no sex differences in our study. We did not find an impact of pesticide exposure on body condition. Although the relationship between body condition and contaminants has been extensively studied, the direction of the effect remains unclear and variable between contaminants (Mingo et al., 2017; Brodeur et al., 2011; Esther et al., 2022; Bellot et al., 2022).

4.4. *Bentazone occurrence and levels*

Bentazone was by far the most common pesticide in water analyzes performed by SNPN. Environmental standards were never exceeded (highest concentration quantified in the three years: 20 660 ng.L^{-1}) because the bentazone Environmental Quality Standard, Predicted No Effect Concentration, and Maximum Allowed Concentration are high (70 000, 23 000, and 450 000 ng.L^{-1}, respectively). Bentazone is banned for rice cultivation in France, but benefited from a derogation in 2018 and in 2020, but not in 2019 (Cheiron and Bricault, 2021). It is usually spread in flooded fields after the emergence of rice to control weed development. This leads to important run-off in the ditches and massive spill when rice fields are drained into canals (Cheiron, 2019; Cheiron,

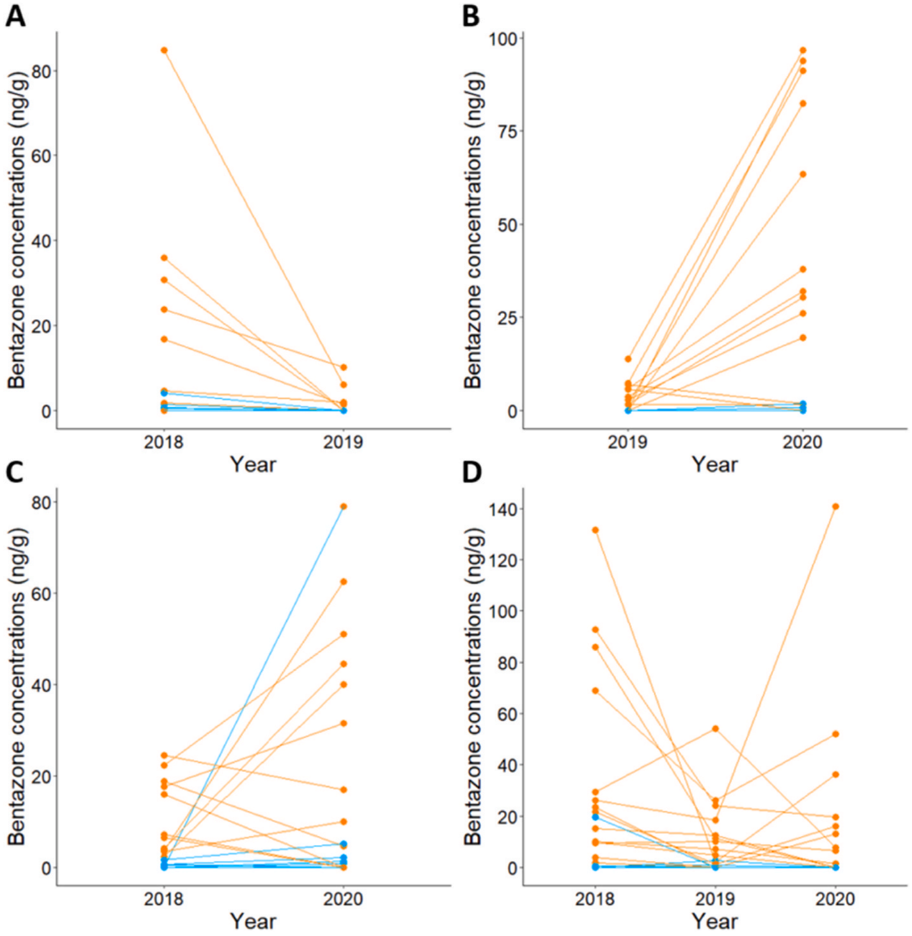

Fig. 6. Bentazone concentrations (ng/g) detected in blood plasma of *E. orbicularis* of two populations in the Camargue, France, from 2018 to 2020, in individuals sampled for two (A, B, C) or three (D) years (blue: samples from Esquineau, orange: samples from Faïsses). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2019; Cheiron and Bricault, 2021; Al Housari et al., 2011). In the Camargue, application generally occurs in mid-May, but bentazone was present at low levels starting in April, which could explain why we did not observe an effect of sampling date on bentazone occurrence. In 2020, water analyzes showed a slower decrease of the Fumemorte canal contamination during the summer months, possibly leading to a longer exposure period for turtles. A previous study in the Rhône River delta indicated that bentazone levels increased in the ditch outlets with a 1–2 week lag compared to the date of pesticide application in the fields (Comoretto et al., 2007). Consequently, another hypothesis is that turtles that live in the vicinity of rice fields could have been contaminated earlier in the season and thus have had a low, but detectable, level of bentazone. Bentazone blood levels, however, seemed to follow the increase in the water levels in 2018 and in 2020. For 2019, the peak of bentazone in the water later in the season could explain why we did not find an increase in blood bentazone levels that year. Interestingly, we were able to quantify similar amounts of bentazone in the water in 2019 compared to 2018 and 2020 even though this molecule was not authorized on rice that year. The crop rotation system of the rice in the Camargue includes alfalfa, on which bentazone is authorized, which could explain these results (Cheiron and Bricault, 2021). Our results suggest that the amount of pesticide spread can influence the contamination of wild species. It should be noted that the water sampling site is located downstream from the Faisses site and, thus, pesticide levels in the water at the sampling site may be higher than the levels in the small drainage canals where the turtles live. Several studies also documented considerable contamination by bentazone in the Ebro Delta, another large Mediterranean Delta with intensive rice cultivation (Kuster et al., 2008; Barbieri et al., 2021; Matamoros et al., 2020). Peaks also occurred in drainage systems between June and July, and concentrations were similar to ours. Several studies calculated the risk quotient of bentazone in Spanish rivers and concluded that this herbicide presented a high risk for aquatic ecosystems because of its high concentrations (Peris et al., 2022; Palma et al., 2021). Bentazone could thus represent a worrisome hazard for freshwater species in numerous wetlands, as it is classified as “harmful to aquatic life with long lasting effects” under European Regulation (n°1272/2008).

We observed strong annual variation in contamination at the individual level. Turtles sampled during two consecutive years (2018 and 2019 or 2019 and 2020) exhibited lower bentazone levels in 2019 than in 2018 or 2020. Interestingly, an individual without detectable levels of bentazone in Esquineau in 2018 was captured in Faisses in 2020 with one of the highest bentazone concentrations we measured. Keeping in mind that this was a single observation, it also suggests that bentazone contamination is linked to environmental exposure rather than individual susceptibility. Even more tellingly, individuals sampled during the three years and with bentazone levels above the LOQ in 2018 had lower levels in 2019 then again higher levels in 2020. These results suggest the absence of bioaccumulation and the rapid excretion of bentazone in turtles. In addition, bentazone has a low octanol-water partition coefficient (Log Kow comprised between −0.55 and 0.77 depending on pH), a bioaccumulation factor ranging from 1.4 to 19 depending on the species (Environmental Quality Standard Proposal 25 057-89-0, Ineris, 2009), and a metabolization estimated to 10% in rats

(European Food Safety Authority, 2015). Little to no information is available on metabolization and excretion rates in reptiles, but toxicokinetics studies showed that excretion is rapid in mammals (European Food Safety Authority, 2015). An *in situ* study in catfishes living in rice field did not detect bentazone in fish organs despite water concentrations similar to what we found in our study, and again consistent with the findings of toxicological studies (Fantón et al., 2021). Rodrigues et al. (2018) quantified bentazone (among other pesticides) in a Portuguese estuary, in water and in a bivalve species, and found low to moderate bioaccumulation (Rodrigues et al., 2018). Hence, our transversal study (known-age individuals) indicated no age-related contamination by bentazone and our longitudinal study (annual variation in the same individuals) confirmed this. This low accumulation potential is consistent with the EFSA assessment of the substance (European Food Safety Authority, 2015) and suggests that high plasma bentazone levels quantified in *E. orbicularis* reflect transient environmental exposure of major concern. Future studies should examine the potential detrimental effects of bentazone, a widely used herbicide in the Camargue and in many agricultural areas worldwide.

5. Conclusion

Overall, there was a diversity of pesticides detected in the blood of *E. orbicularis,* although each compound was found in only a few individuals, with the exception of bentazone. Despite a lack of spatial replication, the main factor explaining differences in pesticide and bentazone occurrences and levels was the sampling site, probably due to differences in hydrology (i.e., the site of Faisses receiving drainage waters from agricultural fields). We also found effects of the year and of the date of sampling, indicating a rapid response of *E. orbicularis* to environmental contamination. Our work calls attention to the potential detrimental effects of bentazone, a widely used herbicide in the Camargue and in many agricultural areas worldwide.

CRediT authorship contribution statement

Leslie-Anne Merleau: Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Olivier Lourdais:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Anthony Olivier:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Marion Vittecoq:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Gabriel Blouin-Demers:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. **Fabrice Alliot:** Formal analysis, Investigation, Resources, Writing – review & editing, Supervision. **Louisiane Burkart:** Formal analysis, Investigation, Writing – review & editing. **Yvann Foucault:** Formal analysis, Investigation, Writing – review & editing. **Carole Leray:** Investigation, Resources, Writing – review & editing. **Emmanuelle Migne:** Investigation, Resources, Writing – review & editing. **Aurélie Goutte:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marion Vittecoq reports financial support was provided by Rhone Mediterranee Corsica Region Water Agency. Olivier Lourdais reports financial support was provided by French Biodiversity Office.

Data availability

Data will be made available on request.

Acknowledgement

The present work was funded by the Water Agency Rhône Méditerranée Corse, the Fondation de la Tour du Valat, the Office Français de la Biodiversité, Plan Ecophyto II under the project “CISTOX” OFB.21.09.42, and the doctoral school of the Ecole Pratique des Hautes Etudes (ED 472). We thank the French Ministry of Environment for giving permission to capture European pond turtles and the Société Nationale de Protection de la Nature for sharing their data on the contamination of the Fumemorte canal. We are also indebted to Michaël Guillon for the mapmaking of the capture areas and to Nicolas Martin, Clément le Gac, Aésane Méric and Louis Spinner for the capture and the blood sampling of the turtles.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122903.

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