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Flagship Species Conservation and Introduced Species Invasion: Toxic Aspects Along Loire River (France)

Charles Lemarchand, René Rosoux, Céline Talon and Philippe Berny

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1. Introduction

In France, the Loire river basin is a unique river system, because of its size, variety of aquatic and wetland habitats and its exceptional diversity of living communities. Succeeding freshwater aquatic biological communities belong to all types of water habitats, from the area of spring stream to estuarine area.

Aside from the great variety and richness of the living communities they host, aquatic ecosystems composing the Loire river system are exposed to many sources of pollution and anthropogenic disturbances affecting water quality and ecosystem functioning throughout the food webs.

Xenobiotic substances in the water are a wide range of contaminants, both organic and metallic and radioactive (nuclear power plants), which focus, by bioaccumulation in the tissues, organs and fat of living beings, and by biomagnification through all trophic levels. This is why some animal species, including predators, can play the role of "biosensors" of contaminants, particularly interesting to study and monitor the health of ecosystems and provide a tool for identification of these elements, then restore water quality and preserve the diversity of environments.

To better understand the pathways of contamination, the phenomena of biomagnification and potential degradation of contaminants during the trophic transfers across the whole Loire river basin, a group of indicator species representing different trophic levels was analyzed. Among them (see figure 1 and table 1 below) are three top-predators, two polyphagous fish (one
sedentary, the other diadromous), three predator and scavenger crayfish species, and a species of benthic bivalve. These invertebrates, accidentally (Corbicula) or voluntarily introduced for aquaculture (crayfish), quickly located in the basin of the Loire River and developed in recent years a marked invasiveness, now causing major imbalances of the whole hydrosystem. Consequences like transmission of pathologies to local species, competition for habitat or resource and direct predation on local or rare species were observed. This selection of bioindicators, or their trophic counterparts in case of absence in pre-selected study sites, should enable to understand the phenomena of water pollution and contamination of food webs by persistent biocides and toxic xenobiotics at different scales of spatial perception from the permanent station (bivalve) to intercontinental vital area (migratory fish-eating birds), through the local scale of a few kilometres (confined fish and / or mammals).

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<th>Fixed Sedentary</th>
<th>Confined / mobile sedentary</th>
<th>Nomads</th>
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<td>Red swamp crayfish</td>
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<td>Orconectes limosus</td>
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Table 1. Indicator species used in this study.

2. Methods

2.1. Study area

Study area corresponded to the whole Loire River and main tributaries catchment in France. 9 study sites were used for the sampling, especially concerning mussels, crayfish or fish (see figure 2). Loire River catchment (117000 km², total length of rivers and tributaries of about 40000 km) is characterized by an important diversity of habitats and species, and is considered as one of the most preserved large hydrosystems in Western Europe. A national and European action plan, “Plan Loire Grandeur Nature”, is running since 1994 to study and conserve this diversity, but also to protect inhabitants from floods and to maintain economic activity.
2.2. Sampling

Concerning rare species like otter or osprey, it is difficult to obtain sufficient sample material from enough individuals to support analysis and statistics. Otter and osprey are fully protected...
by national and international laws, and listed as species of interest by the European Com-
munity (Habitats Directive 92/43/EC, Birds Directive 79/409/CEE. So, for legal and ethical
reasons it was not imaginable to trap or kill otters or ospreys for analyses. To avoid any vital
risk related to handling, capture and bleed of animals were not considered. All operations were
therefore entirely conducted under appropriate authorizations by a non-invasive approach. A
large network, constituted by people in charge of otter and osprey studying and monitoring
in mainland France was built to organize and enhance sampling under the coordination of the
Muséum d’Orléans.

The national agency for game and wildlife (ONCFS), hunting federations (FDC), the national
agency for water and aquatic environments (ONEMA), health centres of the national union
(UFCS) and of the birds protection league (LPO – French representative of Bird Life Interna-
tional), the national research centre on birds population biology (CRBPO, associated with the
French national museum of natural history MNHN), the French Ministry of Environment
(MEEDDM and DREAL Centre), the national agency for forests (ONF), private land owners
and companies, museums, associations (“Loiret Nature Environnement”) and regional
naturalists were contributors for this study. Great cormorant, another protected species in
France, is however concerned by legal shots, in order to limit the fishing impact of the species
in fish farms and ponds. These shot birds were used for toxicological analyses, to avoid
useless destructions.

Concerning otters, only road-traffic killed individuals and those found dead in the wild in
study area were collected. Based on visual observation, carcasses found more than 24h (during
summer) or 48h (during winter) after road collision were considered too degraded and not
taken into account for post-mortem examination and toxicological analyses. Concerning
ospreys, non-hatched eggs and dead young in nests were collected during chicks ringing
operations. As scientists and birdwatchers monitor a majority of osprey nests in continental
France, non-hatched eggs and dead young in nests were reported and sampled as soon as
possible. France is also a major crossing area for migrating osprey from different populations
[1,2]. Due, in one way, to the extreme rarity of this species in continental France (less than one
hundred reproductive individuals), and in an other way that “foreigners” individuals (i.e. born
in neighbour countries, but potentially breeders in France) are able to be found dead within
the national territory (naturally or after illegal shots, electrocution on power cables, or drown
in fish farms), migrating individuals flying towards reproduction areas elsewhere in Europe
(Germany, Great-Britain, Scandinavia) completed sampling.

Other animals studied here (great cormorant, fish, crayfish and mussels) were sampled under
authorizations after legal shots near fisheries or pounds (great cormorants), or using fishing
license (fish and crayfish), near sampling sites where possible.

All samples were deep-frozen (-40 °C) prior to analyses. For each carcass, a necropsy was
performed, and about 20 g of liver was sampled (preferred to fat because of the very low fat
content of ospreys, especially at the end of spring migration). Sex and weight were determined;
animals were measured and aged according to criteria like total size, teeth development or
plumage characteristics (total and head, body, foot and tail lengths). Non-hatched osprey eggs
were drilled and emptied; eggshell was conserved for future studies on shell thickness. Each
animal is characterised by a specific case-record gathering discovery circumstances, clinical and biometrical data. After necropsies, carcasses were conserved for further showing or collection in museums or, if too degraded, systematically destroyed according to law.

2.3. Organochlorine pesticides analyses

2.0-8.0 g of tissue were sampled and 30 ml of hexane/acetone 75/25 mix was added. Each sample was blended twice with an UltraturraX® (Ika, Werke, Germany) and filtered through a phase separator membrane. The extract was evaporated at 60 °C in a rotary evaporator. The dry extract was dissolved in 10 ml hexane. Two ml of fuming sulphuric acid (SO₃, 7%) were added, and after centrifugation at 4x g, 1 ml of the supernatant was used for OC pesticides quantification by gas chromatography with electron capture detection material. Temperature program and injection conditions are described in [3,4]. Each sample was run in duplicate. Organochlorine pesticides concentrations were calculated by using different mix standards. Recovery level on standard mixtures was always greater than 92%. All standards were purchased from CIL (St Foy la Grande, France), and purity was > 99%. Linearity was determined between 5 and 100 ng.g⁻¹ (r² > 0.99 on standards and spiked samples, 5-point calibration curves). Limits of detection were between 0.5 and 1.0 ng.g⁻¹ lipids for individual PCB congeners. Cod liver oil (BCR349) certified material was used as a regular quality control.

2.4. Organophosphate pesticides analyses

5 g of tissues sample was shaken with 60 ml dichloromethane and 10 g anhydrous sulfate. Mix was then filtered through a Whatman 1 PS membrane, and evaporated under vacuum at 40°C. Dry samples were diluted in 3 ml ethanol, and underwent an ultrasonic step. Extract was then purified with a Sep pack R 300 (Silica Waters, 020810; 500 mg) column conditioned with 2 ml methanol and 2 ml ethanol. 2 ml dichloromethane were used for column elution. Purified samples were dried and diluted with 3 ml dichloromethane. Organophosphate (OP) and 2 carbamates (CA) pesticides (Dichlorvos, Carbofuran, Mevinphos, Phorate, Phorate oxon, Phorate sulfone, Methiocarbe, Terbufos, Diazinon, Disulfoton, Chlorpyriphos methyl, Chlorpyriphos ethyl, Fenitrothion, Pyrimiphos methyl, Malathion, Fenthion, Parathion, Methidathion, Disulfoton sulfoxide, Triazophos) concentrations were determined by GC/MS in SIM mode (OP + carbofuran and methiocarbe). A 5973N MS coupled with a 6890 GC (Agilent®) was used, with a 30m HP5-MS column (0.25 mm ID, 0.25µm thickness). For each samples standard and spiked sample, 2 µL were injected. The temperature program was 100°C (2 min), 55°C/min up to 200 °C (held for 5 min), 50 °C up to 220 °C (held for 3 minutes), followed by 60 °C/min up to 300°C. A final, post-run time of 2 min at 300°C was maintained. Total run time was 13.55 min. Injector was set at 250°C and the He flow was set at 2.5 ml/min.

Each OP or CA was identified based on the following criteria: retention time and 3-4 fragmentation ions with pre-defined relative amounts and 20% variability acceptance for each ion. Linearity was confirmed between 25 and 500 ng.g⁻¹ with 5 point calibration curves and r² >0.99. Recovery was determined between 76% and 104% for all spiked samples and repeatability was considered acceptable with coefficients of variation <15%.
2.5. Pyrethroids pesticides analyses

5 g of tissue sample was shaken in 60 ml ethanol and 10 g anhydrous sulfate, and then filtered through a Whatman 1 PS membrane. Extract was dissolved in 5 ml methanol and underwent a second filtration procedure. Concentrations were determined by GC / ECD and confirmed by GC/MS according to a modified method of the French Food Safety Authority (Anses Met AFSSA). An Agilent GC-ECD 6850 with a 30m HP1 column (0.32 mm ID, 0.25µm film) was used. For each samples standard and spiked sample, 2 µL were injected. The temperature program was common to OC and pyrethroids pesticides (initial temp: 100°C, first ramp 6°C/min up to 220 °C held for 10 min, 2nd ramp 7 °C/min up to 285°C, held for 1 min, total run time 42.29 min) Injector was at 230°C, detector at 300°C. Total He flow was 9 ml/min. Pyrethroids were identified according to their retention times. Linearity was confirmed between 10 and 100 ng g⁻¹ with 5 point calibration curves and r²>0.99. Recovery was determined between 82% and 94% for all spiked samples and repeatability was considered acceptable with coefficients of variation <15%. For all positive samples, a confirmatory analysis was performed with GC/MS in SIM mode. Identification was based on retention times and 3 or 4 ions.

2.6. Herbicides analyses

2 g of tissue sample was shaken during 5 minutes in 8 ml acetone, and then centrifuged at 4x g; supernatant was placed in separate tubes, and this extraction was performed twice. Samples were evaporated under nitrogen, and dry extract was dissolved in 1 ml aceton/methanol (50:50) solution. Extract was then purified with a SPE C18 500 mg column conditioned with 2 ml acetone and 2 ml methanol. Column was vacuum dried and purified samples were diluted in 3 ml acetone. After drying under nitrogen, samples were diluted in 1 ml methanol. Herbicides (Trifluraline, Atrazine, Simazine, Terbutylazine, Diuron, Alachlor, Metolachlor, Cyanazine, Epoxiconazole) concentrations were determined by GC/MS spectrometry. A 5973N MS coupled with a 6890 GC (Agilent®) was used, with a 30m HP5-MS column (0.25 mm ID, 0.25µm thickness). For each samples standard and spiked sample, 2 µL were injected. The temperature program was 85°C held 1 min, followed by 6°C/min up to 170°C (held for 12 min), then followed by 20°C/min up to 280°C, held for 4.33 min (total run time 37 min). Injector was at 250°C and in the splitless mode. Each herbicide was identified based on the following criteria: retention time and 3-4 fragmentation ions with pre-defined relative amounts and 20% variability acceptance for each ion. Linearity was confirmed between 100 and 500 ng.g⁻¹ with 5 point calibration curves and r²>0.99. Recovery was determined between 67% and 98% for all spiked samples and repeatability was considered acceptable with coefficients of variation <15%.

2.7. Anticoagulant rodenticides

Analyses for anticoagulant rodenticides (8 compounds marketed in France: bromadiolone, chlorophacinone, difenacoum, difethialone, warfarin, coumatetralyl, brodifacoum, flocoumafen) contamination were completed using high-performance liquid chromatography according to [5,6]. Briefly, 1.0-2.0 g of liver were used. All solvents and reagents used were of the highest purity available. 50 µl of each sample or standard solution were injected in a C18
column (10 nm pores, 5 µm granule size), 250 x 4 mm (Chromcart Nucleosil, Macherey-Nagel, Strasbourg, France). The HPLC system used was made of an isocratic pump (L6000), an automatic sampler (AS2000), and a fluorimetric detector (F1000). Specific software (D7000 HSM) was used for data acquisition (Merck, Nogent-sur-Marne, France). Linearity was determined on standards and spiked liver samples between 0.05 and 1 mg.kg$^{-1}$ ($r^2$>0.99%). Percentage of recovery varied between 80.1% and 89.2% for bromadiolone, chlorophacinone and difenacoum (the three most common rodenticides in France). Limits of detection were 0.02 mg.kg$^{-1}$ for all anticoagulants tested.

2.8. Calculation methods and statistical analysis

Geometric means of $p,p'$-DDE, $p,p'$-DDD and $p,p'$-DDT were added to calculate the sum of DDTs ($\Sigma$ DDTs). Geometric means of lindane, endosulfan, DDE, DDD, DDT, heptachlor, heptachlor epoxide, aldrin and methoxychlor were summed to provide the sum of pesticide concentrations ($\Sigma$ Pesticides). All these were chosen by the National Veterinary School of Lyon (VetAgro Sup Campus Vétérinaire de Lyon, France) standard protocol [7,3,4,8]. The Mann-Whitney test was used to compare two independent samples, Kruskall-Wallis for $k$ comparisons, Spearman correlation rank test to quantify associations between two variables. Statistics were performed using R. [9].

3. Results

3.1. Contamination of osprey

3.1.1. Organochlorine pesticides

Contamination results of osprey harvested in France by organochlorine pesticides are presented in Table 2 and figure 4. Organochlorine pesticides were detected in 20 samples out of 27 (74%) who have undergone analysis. Among the compounds tested, only residues of DDT (mainly $p,p'$-DDE) and methoxychlor were detected, with one single case of contamination with lindane, though forbidden to use since 1998. DDE was detected in 12 samples (44%), including 5 eggs from different nests in France. DDE concentrations, averaging 0.92 mg.kg$^{-1}$, remained generally low (between 0.1 and 8.2 mg.kg$^{-1}$ fresh weight, this extreme value being high, however), and overall comparable to levels found in the literature concerning other prosperous population [10,11]. On unhatched eggs, the maximum concentration of DDE was 4.6 mg.kg$^{-1}$, which is slightly above the threshold of 4.5 mg kg$^{-1}$, beyond which the shell of the egg, fragile, can break under the weight of the incubating adult [11,12]. This egg was not damaged, but these significant results suggest a relatively recent use of DDT in the basin of the Loire River, probably after its ban in France (1973). Indeed, even if the species is a migratory one, contamination of osprey eggs was documented as more indicative of contamination of breeding then wintering areas [13].
Methoxychlor was meanwhile detected in 8 samples, or 27%, again with relatively low concentrations. The average concentrations of methoxychlor reached 0.04 mg kg$^{-1}$, significantly lower than the values found in the literature [14]. In France, no significant changes in concentrations of organochlorine pesticides were observed, depending on the sex or age of ospreys, this result can be explained by the small total sample size. Aldrin, heptachlor and endosulfan were never detected in the tissues analyzed ospreys, and lindane only once, in contrast to what was observed in previous studies conducted overseas [11,15,16,17]. This difference could be explained by greater exposure of ospreys to contaminants in America, where the species declined but not disappeared, than in continental France, where ospreys disappeared after direct destruction and before massive use of pesticides in the environment. So accumulation pattern of organochlorine was probably different among ospreys from America, which were heavily affected and locally literally decimated by the accumulation of pesticides. The ban of DDT and other organochlorine pesticides, has led to a slow decline in levels observed in tissues of osprey to the current situation which has seen numbers recover. In France, organochlorine pesticides were banned and declined in the environment decades before the return of the species, resulting in a lower and declining exposure and accumulation.

Considering the natural expansion of the species in France [18] and reproductive success of most couples, organochlorine pesticides do not appear to threaten the osprey as breeding species in France in the short term, or affect the stability of European populations. Some relatively high observed values, however, does not rule out any risk for individual survival, and it should be noted that the relatively small sample size and lack of perspective, given the
recent nature of the toxicological study of case, require some caution and further research programs will give more relevance to these results.

3.1.2. Ospreys contamination by organophosphate pesticides, carbamates and pyrethroids

Among the highly toxic cholinesterase inhibitors measured in this study (mevinphos, phorate, malathion, phorate sulfone, chlorpyrifos-ethyl, parathion, fenitrothion, methiocarb, methidathion, disulfoton sulfone and triazophos) several were quantified in tissue ospreys (see Table 2 and figure 4). Contamination of ospreys by organophosphate pesticides appeared weak and dispersed, only a few individuals being affected. Organophosphate pesticides were detected only in adult and subadult tissues, and never in juveniles or eggs (see Table 1.1). For contaminated individuals, differences by age, gender or geographical origin of birds is not significant. No individuals from the breeding population of mainland France analyzed here have revealed contamination by organophosphates. Triazophos, disulfoton sulfone and mevinphos were the most frequently detected compounds in Osprey. As described above, the recovered ospreys were generally in good physical condition and had normal values of total weight and overweight, they showed no clinical signs manifest organophosphate poisoning during the review post mortem, such as diarrhea, pulmonary edema, or tighten clows. In addition, some of them were recovered during migration, where energy reserves are mobilized and may lead to phenomena of acute intoxication, and no evidence of this type has been observed, the causes of death. Measured concentrations remained well below thresholds for toxic doses of cholinesterase inhibitors (5 to 10 mg.kg\(^{-1}\) ww) and did not constitute a lethal agent for individuals.

Carbamate pesticides were not detected in tissues of osprey in this study (see Table 2 and figure 4), in contrast to recent work on other raptors in France, such as the red kite (Milvus milvus) [19], showing a wide poisoning of wildlife by these elements. It can be assumed that the diet of the osprey, specialized and based on consumption of fish has little exposure to the accumulation of carbamates compared to other species, especially opportunistic scavengers like kite. Given these observations about the osprey and the evolution of the regulation of
organophosphate and carbamate pesticides, the measured concentrations of the first, relatively small and not likely to increase very slightly, are not likely to pose a constitute a threat to the conservation of the species.

Pyrethroids have not been detected (see Table 2 and figure 4), in any Osprey analyzed in this study. Given the extreme rarity of investigation of these compounds in the literature on wildlife, further studies are probably needed to really set the overall contamination of aquatic systems by pyrethroids, despite weaker toxicity compared to organophosphates and carbamates for mammals and wild birds [20,21].

3.1.3. Contamination by herbicides

The results for herbicides analyzed in this study are shown in Table 2 and figure 4. As observed for organochlorine pesticides, herbicide contamination appeared weak and undiversified. 10 Osprey showed detectable concentrations of herbicides. Terbuthylazine, cyanazine and alachlor were the only herbicides measured, with low concentrations (from 0.01 to 0.28 mg.kg\(^{-1}\)) and the observed variations according to age, sex or geographical origins of birds were not statistically significant. Only one individual from the breeding population of mainland France showed a measurable concentration of herbicides. As in the case of organochlorine pesticides and carbamates, herbicide concentrations remained low, and probably have very little impact on the conservation of the species, but the total sample size is too small for a definitive conclusion. Moreover, insofar as herbicides are rarely analyzed in comparable studies of the literature, very few items are available for comparison [21].

3.1.4. Contamination by anticoagulant rodenticides

Anticoagulants have not been detected in tissues of osprey in this study (see Table 2 and figure 4). These results can probably be easily linked to the diet of strictly fish-eating species [22], limiting exposure to elements mainly used against the proliferation of rodents that ospreys do not capture. But given that in one hand, the possibility for the Osprey to capture and eat rodents for periods where fishing is difficult or even impossible [23] and on the other hand the regular presence of anticoagulants residues in various environmental compartments (including aquatic ones), highlighted by the decreasing thresholds of analytical detection, the risk of poisoned ospreys by anticoagulants can not be totally excluded.

\[
\begin{array}{c|c|c|c|c|c|c|c}
\text{Pesticides} & \text{Total} & \text{Eggs} & \text{Juveniles} & \text{Subadults} & \text{Adults} & \text{Males} & \text{Females} \\
\hline
\text{OC Pesticides} & 0.96 & 1.38 & 0.11 & <d.l. & 1.61 & 1.23 & 0.28 \\
\text{OP Pesticides} & 0.33 & <d.l. & 0.56 & <d.l. & 0.80 & 0.66 & 0.58 \\
\text{Herbicides} & 0.36 & 0.04 & 0.24 & <d.l. & 0.99 & 0.29 & 1.21 \\
\text{Carbamates} & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. \\
\text{Pyrethroids} & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. \\
\text{Anticoagulants} & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. & <d.l. \\
\end{array}
\]

Table 2. Data concerning osprey intoxication by pesticides (mg.kg\(^{-1}\); n.a.: not analyzed; d. l.: detection limit)
3.2. Contamination of European otters

3.2.1. Contamination by organochlorine pesticides

3.2.1.1. Upper part of the basin

The results concerning contamination of otters from upstream Loire river basin (Limousin and Auvergne regions) by organochlorine pesticides are shown in Table 3 and figure 6 below. Organochlorine pesticides were detected in all individuals analyzed, the amount of pesticides reaching a maximum of 9.4 mg.kg\(^{-1}\) lipid weight. Residues of DDT, DDE mainly constitute the dominant share (70-90%) of the total pesticides detected in the tissues of otters. DDT was detected in the tissues of both from the basin of the river Allier individuals, suggesting recent use of the pesticide, after legal prohibition in France, dating back to 1973. As observed in the preliminary study of otter scats [3], lindane is the most abundant organochlorine pesticide most frequently detected after the DDTs. The concentrations of aldrin and heptachlor remained weak, endosulfan and methoxychlor were never observed in the tissues. Concentrations of organochlorine pesticides measured in the liver of individuals were significantly higher (p<0.05) than those measured in spraints otters from the same population [3], suggesting a low elimination of these toxic compounds via the general metabolism. These results also emphasize the need to complement the study of otter scats by their tissues when they are available, in addition to a wider range of measurement of contaminants [24,25].

Figure 5. European otter (Lutra lutra). Photo C. Lemarchand.

These results emphasize the gradual decrease in concentrations of organochlorine pesticides in the environment after their ban. Females appeared to be more contaminated than males (p <0.05) by pesticides. So despite this significant contamination, prosperity observed population should be related to the availability of vacant habitats, like suggested above concerning osprey. Several studies [24,26,27] have already reported high concentrations of organochlorines in
increasing otter populations, a good reproduction pattern in unsaturated habitats offsetting losses due to intoxication [3,28,29].

3.2.1.2. Lower part of the basin

Results concerning the contamination of otters from the downstream part of the basin by organochlorine pesticides are presented in table 3 and figure 6 below, challenged prospects for comparison with data from the upstream, above. For this part of the basin, organochlorine pesticides were detected in all individuals, but concentrations remained under 0.5 mg.kg\(^{-1}\) lipid weight, significantly lower than in the upstream areas (p <0.05). Residues of DDTs remained the most abundant elements. Unlike the upstream, the parent compound (DDT as itself) was not detected. Other pesticides remained at very low levels (mainly traces of lindane), or were not detectable. The variations with sex or age of the contamination of otters from the downstream part of the basin remained weak and insignificant. These data suggest a significantly lower contamination of this area and local food webs by organochlorine pesticides. It therefore appears that the contamination by organochlorine do not pose an immediate threat to the conservation of the species in the basin of the Loire, as noted elsewhere in Europe [4].

3.2.2. Contamination by organophosphate pesticides, herbicides, carbamates and pyrethroids

Of all the compounds tested, none of organophosphate pesticides, herbicides, carbamate pesticides or pyrethroids has been detected in tissues of otters, whether from the upstream or downstream or marsh western basin of the Loire river (see table 3 and figure 6). As the analysis has been carried out in the same series as those for other species in this study (including the
osprey), any risk of an experimental bias detection can be excluded here. If the absence of carbamates and pyrethroids was observed for all three super-predators (see above and below) and seems to suggest a robust lack of accumulation of these compounds in aquatic food webs, however the absence of organophosphorus pesticides and herbicides in this population of otters seems surprising, since their presence (even if weak and dispersed) in both studied birds also highlights their environmental persistence and ability accumulative in food webs to higher levels. However, the bibliographic data for the European otter are rare on these compounds for possible comparison, and testing hypotheses to try to explain these results.

3.2.3. Contamination by anticoagulant rodenticides

Of all the otters from the Loire River basin (all periods and all collection sites combined) analyzed for their potential contamination with anticoagulants residues, only two individuals involved bromadiolone concentrations that reached 0.40 and 0.85 mg.kg\(^{-1}\) fresh weight respectively (see table 3 and figure 6; [4]). Chlorophacinone and difenacoum (often used in France) were never been found in any of the otters analyzed. These results highlight the risk of poisoning non-targeted species during rodent control campaigns [6]. The two individuals concerned were males from the same sector of the Allier River, where the band of riparian vegetation and the banks have long been treated with bait (carrots or apples arranged on floating rafts) poisoned with anticoagulants for the removal of raccoons and muskrats. If these treatments “air” are banned in France since 2006, the practice remains active locally, and the use of anticoagulants in the form of buried wheat grains poisoned bromadiolone, especially against the proliferation of land voles (Arvicola scherman) can result for the otter by secondary poisoning due to predation on non-target rodents such as amphibian voles (Arvicola sapidus, now a protected species in France).

Due to the absence of clinical evidence of intoxication anticoagulants such as severe anemia or bleeding, and the relatively small number of individuals involved, anticoagulants do not seem to pose a threat to the conservation of the otter, especially as current practices no longer allow the use of anticoagulants in the aquatic area. However, lowering the thresholds regular analytical detection puts increasingly highlight the significant environmental release anticoagulants, and some care must be set in the future about their illicit use and monitoring.

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>Total</th>
<th>Juveniles</th>
<th>Subadults</th>
<th>Adults</th>
<th>Old</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organochlorine</td>
<td>1.83</td>
<td>1.63</td>
<td>1.92</td>
<td>2.01</td>
<td>1.77</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Organophosphate</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
</tr>
<tr>
<td>Herbicides</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
</tr>
<tr>
<td>Carbamates</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
</tr>
<tr>
<td>Pyrethroids</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
<td>&lt; d.l.</td>
</tr>
<tr>
<td>Anticoagulants</td>
<td>0.62</td>
<td>n.a.</td>
<td>0.62</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.62</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Table 3. Data concerning otter intoxication by pesticides (mg.kg\(^{-1}\); n.a.: not analyzed; d. l.: detection limit)
3.3. Contamination of great cormorants

3.3.1. Organochlorine pesticides

The results of contamination of cormorants by organochlorine pesticides are shown in Figure 8 below. OC Pesticides were detected in all individuals for all study sites. The observed values were generally low, less than 0.1 mg.kg⁻¹ of fresh weight, less than those observed for ospreys and otter (see above; [4,8]). The most abundant organochlorine pesticides are DDE, metoxychlor and lindane. The set of the species logically reflects the overall trend of contamination of watersheds. The measured differences in the contamination of cormorants by pesticides according to the study site, the subspecies (P. c. carbo or P. c. sinensis), age or sex of the birds were found non-significant (see figure 4.1). The different species of cormorants were often used as models for studies in toxicology [30,31,32,33]. The concentrations of organochlorine pesticides identified by these authors in different tissues of great cormorants were generally higher than those observed in the basin of the Loire River, with no impact on populations, such as cases of direct mortality or reduced expansion of the population. Following these authors, and given the strong dynamic of great cormorants population observed in France, organochlorine compounds are probably not, for the moment, a direct threat to the species.

Figure 7. Great cormorant (Phalacrocorax carbo). Photo C. Lemarchand.
3.3.2. Contamination by organophosphate pesticides, carbamates and pyrethroids

As in the case of the osprey, carbamates and pyrethroids pesticides were not detected in tissues of cormorants in this study. In this case also, the diet of cormorants, based on aquatic prey (fish and crayfish) probably explains the low or the absence of transfer of carbamates to the predator and seems to confirm the very low accumulation of pyrethroids in aquatic systems, at least in predators and top predators food compartments [20,21]. It is likely that these values situated below the detection limits of current analytical methods and not likely to increase in the future won’t constitute a threat to the conservation of the species. However, given the relatively recent nature of the use of certain compounds (including pyrethroids) and the lack of experience and comparable data in the literature, research of pyrethroids and carbamates in cormorant tissues should be continued in order to determine the actual level of environmental contamination, or to assess the risk of interaction with other potentially toxic elements.

3.3.3. Contamination by herbicides

Contamination of cormorants by herbicides appeared weak and concerned a small number of compounds. Of all the individuals analyzed, 40% showed contamination by herbicides, alachlor and metolachlor were the only two compounds detected. The fungicide Epoxyconazol was also detected several times. Contamination by these three elements appeared on the completeness of the study sites, and values remained low, usually less than 0.1 mg.kg⁻¹. The
differences between sites, sex, age or subspecies remained insignificant. Alachlor and epoxy-
conazol were also detected in osprey, other fish-eating bird, with orders of comparable size. Other herbicides detected in osprey (see above) were not, however, found in the tissues of the great cormorant, this may be related to differences in diet (and thus accumulation through food) between the two species or varying metabolic capabilities from one species to another. These low concentrations of herbicides probably have no or very low impact on the conservation of the species, especially in view of the expansion of cormorant species noted in recent decades. However, as in the case of the osprey, the lack of experience or comparable studies of these compounds in the literature limit any definitive conclusion [21].

3.3.4. Contamination of cormorants by anticoagulant rodenticides

Anticoagulants rodenticides were not detected in the tissues of great cormorants in this study. Like underlined for the osprey, these results may be related to the diet of this almost strictly fish-eating species [34], limiting exposure to the accumulation of anticoagulants. However, as noted for the osprey, the regular presence of anticoagulant residues in various trophic ranks, highlighted by the decreasing analytical detection limits can, do not exclude the possibility of contamination, and anticoagulants should be at least sporadically sought in future toxicological studies on the species.

3.4. Contamination of fish

3.4.1. Contamination by organochlorine pesticides

Among the contaminants of this family that have been researched in fish tissues (both species studied here) from Loire river basin, only three of them were found: DDTs (mainly DDE), endosulfan sulfate and lindane. DDE was only found in the downstream half of the basin, in chub and mullet tissues, upstream does not reveal any trace of contamination. The concentrations found when the molecule was detected were relatively high ranging from 0,4 to 1,6 mg.kg\(^{-1}\) BW. Contamination of fish by DDTs (like those observed in the cormorant, osprey and otter) therefore indicates recent use of this compound, however banned since 1973 in France. Endosulfan was found on the same sites as DDT, with values ranging between 0,17 and 0,26 mg.kg\(^{-1}\) ww. It therefore appears that the contamination of the Loire river basin with endosulfan sulfate is generally low to negligible, which may reflect an improvement over a previous situation where the compound was regularly detected in the environment, although no studies about it is available in the literature on the basin [35]. Lindane was the most prevalent organochlorine in geographical terms, and showed the highest concentrations, ranging from 0,04 to 3,42 mg.kg\(^{-1}\) fresh weight. Its distribution within the basin, however, is heterogeneous, no significant difference between species, or sampling sites, or particular geographical gradient could be underlined. Contamination of the Loire river basin by lindane is comprehensive and relatively high despite the former prohibition of this compound, and it affects all trophic compartments, since apart from fish, lindane was also found in significant quantities in the tissues of otter [4].
3.4.2. Contamination of fish by organophosphate pesticides

The detection of organophosphate in tissues of fish (whatever the species considered) is very punctual. The results demonstrate the absence of contamination gradient along the river. Compounds belonging to this family are banned from use in the European Union since 2000. They are recognized as non-persistent as quickly degraded in the environment (less than 4 weeks for most) and metabolized by organisms [35,36]. Finally, the fish are very sensitive to organophosphate acting rather a mode of acute toxicity. In other words, the fish exposed to organophosphate die very quickly, organophosphates and disappear in aquatic food webs and chronic contamination is difficult to detect.

3.4.3. Contamination of fish by herbicides

So similar to organophosphates, herbicides are not found at all sites. The three compounds detected throughout the watershed were Atrazine, the Terbuthylazine and Linuron. Atrazine was the most regularly detected compound in samples, values ranging between 45 mg.kg⁻¹ ww in chub from upstream areas and 210 mg.kg⁻¹ ww in both chub and mullet from the downstream part of the basin the Loire, intermediate areas reached 183 mg.kg⁻¹ ww. It may be suspected a concentration gradient of Atrazine in the tissues of fish along the Loire River, which proves coherent with the gradient use of this compound in agriculture [35].

3.5. Contamination of molluscs

3.5.1. Contamination by organochlorine pesticides

Table 4 below summarizes the data for contamination of mussels from the Loire basin. Asatic clam were not found in the downstream site (number 9). Of the eight OC pesticides analyzed, only lindane was detected consistently in the tissues of mussels, DDE is also common, this compound is indeed detected in the majority of sites. DDT was found in the Loire basin, but more rarely in Asiatic clams from the upstream part of the basin and from the area situated close to the estuary. In mussels from the central part of the basin, concentrations were higher. The measured concentrations of DDT are low but as in the case of other species studied, this detection still suggests a relatively recent use of DDT,
widely after the ban of this compound. For lindane and DDE concentrations were comparable across all sites sampled and are not significantly different. There are no significant differences between sites and between mussel species. Lindane is mostly met with 0.51 mg / kg lipid compound and then the DDE with 0.36 mg / kg lipid concentrations found in other European rivers [37,38,39].

Figure 10. Asiatic clam (Corbicula fluminea). Photo C. Lemarchand.

<table>
<thead>
<tr>
<th>Sampling Site</th>
<th>Species</th>
<th>Lindane (min-max)</th>
<th>DDE (min-max)</th>
<th>DDT (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Corbicula fl.</td>
<td>0.2 (0-0.3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Corbicula fl.</td>
<td>0.72 (0.4-1.1)</td>
<td>0.4 (0-0.9)</td>
<td>0.006 (0-0.03)</td>
</tr>
<tr>
<td>3</td>
<td>Corbicula fl.</td>
<td>0.1 (0-0.2)</td>
<td>0.533 (0.4-0.7)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Corbicula fl.</td>
<td>0.8 (0.7-0.9)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Corbicula fl.</td>
<td>0.52 (0.4-0.7)</td>
<td>0.6 (0.4-0.9)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Corbicula fl.</td>
<td>0.52 (0.3-0.7)</td>
<td>0.1 (0-0.3)</td>
<td>0.024 (0-0.1)</td>
</tr>
<tr>
<td>7</td>
<td>Corbicula fl.</td>
<td>0.41 (0.3-0.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Corbicula fl.</td>
<td>0.67 (0.5-0.8)</td>
<td>0.29</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Table 4. Mean concentrations (min-max) of OC pesticides in mussels from the Loire river basin (mg kg⁻¹ lipid)
3.5.2. Contamination of molluscs by organophosphate pesticides, pyrethroids and herbicides

None of these compounds was detected in the tissues of Asiatic clams in the samples, regardless of the site in question. So it seems that the specific accumulation of these compounds modalities globally rare remaining in the tissues of species analyzed here, also check for molluscs, without, however, we can attest to the lack of toxicological effects induced due the lack of experience and references on this topic.

3.6. Contamination of crayfish

3.6.1. Contamination by organochlorine pesticides

Results of crayfish contamination by organochlorine pesticides are shown in Table 5 below. Of the eight OC pesticides analyzed, only DDE was found in the whole homogenates of crayfish sampled (see table 5). DDE contamination therefore concerns all introduced crayfish species and all sites. DDT as itself was also detected twice, and values have been added to those of DDE (constituting DDTs).

![Figure 11. From left to right: signal crayfish (Pacifastacus leniusculus), spiny-cheek crayfish (Orconectes limosus) and Red swamp crayfish (Procambarus clarkii). Photos C. Lemarchand](http://dx.doi.org/10.5772/57176)
Other organochlorine pesticides, including lindane and endosulfan were not found in crayfish tissues, whatever the species and location. The concentrations of total OC pesticides, in that case DDTs (0.13 mg kg\(^{-1}\) of fat on average) are not significantly different from one site to another and appear to be higher than those found elsewhere in literature. Indeed, in the Meuse River, the tissues of *Orconectes limosus* had rates of DDTs ranging from 0.553 to 4.278 ng kg\(^{-1}\) dry weight [40]. However, the values recorded here are remote toxic levels can kill 50% of crayfish in 24 hours (DDT = 0.588 mg kg\(^{-1}\)) established by Huner & Bar in 1991 (in [41]). Like molluscs, statistical analyzes conducted to compare the observed values within the different species apart from the effect of the sampling site, did not reveal any significant differences.

3.6.2. Contamination by other pesticides analyzed

As we observed for shellfish, organophosphate pesticides, herbicides and pyrethroids were not found in crayfish tissues, whatever the site or species considered. As suggested for other species of this study (see above), it remains difficult to certify the absence of toxicological effects induced given the lack of experience and references on this topic.

3.6.3. Comparison between crayfish, mussels and other species contamination

Comparison of concentrations of toxic elements studied here can assess the overall contamination of lower trophic level organisms, although their ecology and mobility are different, used to estimate the potential transfer to higher trophic compartments. Such an approach can only, however, be indicative, in the absence of local and complete knowledge of the diet of different predator species. Concerning organochlorine pesticides, comparison clearly shows a significant difference in contamination between molluscs and crayfish, the latter being much less contaminated (p <0.05). Ecology and nutrition mode of filtering molluscs probably explain this higher accumulation of organochlorine pesticides, compared to crayfish, more mobile and whose diet is more diverse and variable during their development cycle. Organophosphate pesticides, herbicides and pyrethroids were not found in the tissues of crayfish or mussels, as in the case of shellfish. The latter two were sparsely and irregularly noted in the tissues of fish, cormorants and otters (see above). This failure to detect organophosphate pesticides in species of the Loire River basin was also observed by [42], the short half-life of organophosphate limiting their entry into aquatic food webs via fish or invertebrates (in [42]). But this does not mean that these substances do not have any impact on the environment as aquatic ecosystems may be affected in the short term before these substances are degraded into non-toxic products [42]. Pyrethroids are little persistent compounds in the environment, and do not seem to bioconcentrate or biomagnify in organisms [43], although they are very toxic to fish and vertebrates [44]. The failure to find any molecule of the family of pyrethroids in our analysis would reinforce the idea that these compounds do not accumulate in food webs (see above). The absence of herbicides in the tissues of our specimen could be explained by a particular physico-chemical behaviour (biodegradability) or a very low absorbance of these molecules by fat tissue. The lack of perspective and tracks from similar work, however, requires some caution with respect to any final conclusion.
Table 5. Mean concentrations and range of organochlorine pesticides in crayfish from Loire River basin (mg/kg lipid weight)

<table>
<thead>
<tr>
<th>Study site</th>
<th>Species</th>
<th>DDTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pacifastacus leniusculus</td>
<td>0.09 (0-0.24)</td>
</tr>
<tr>
<td>2</td>
<td>Orconectes limosus</td>
<td>0.30 (0.05-0.8)</td>
</tr>
<tr>
<td>3</td>
<td>Orconectes limosus</td>
<td>0.07 (0-0.2)</td>
</tr>
<tr>
<td>4</td>
<td>Pacifastacus leniusculus</td>
<td>0.06 (0-0.19)</td>
</tr>
<tr>
<td>5</td>
<td>Orconectes limosus</td>
<td>0.04 (0-0.09)</td>
</tr>
<tr>
<td>6</td>
<td>Orconectes limosus</td>
<td>0.21 (0-0.8)</td>
</tr>
<tr>
<td>7</td>
<td>Orconectes limosus</td>
<td>0.06 (0-0.15)</td>
</tr>
<tr>
<td>8</td>
<td>Orconectes limosus</td>
<td>0.07 (0-0.19)</td>
</tr>
<tr>
<td>9</td>
<td>Procambarus clarkii</td>
<td>0.07 (0-0.26)</td>
</tr>
</tbody>
</table>

4. Conclusion

This study, conducted during three years on Loire River basin has, on the one hand, confirmed preliminary data on some species, and on the other hand, very significantly supplemented the knowledge of the contamination of several species from different trophic levels. Thus, for the European otter, the results of contamination in Loire basin completed those acquired in other regions of France, and now cover a sample of individuals and geographical area of very important interest, at the international scale. Data on the great cormorants and ospreys are especially rare in Europe, and this work is therefore a standard concerning Western Europe knowledge of the contamination of osprey. Similarly, the results for the freshwater bivalves and crayfish are likely to improve the understanding of the toxicity of environmental contaminants on invasive species development.

Among the main results of this work, one should consider the universal nature of the contamination: no individual of any species from the whole basin appeared free of xenobiotics. Of the 54 elements systematically analyzed, organochlorine pesticides, were found most frequently. Work to identify and address sources of pollution will therefore affect the entire watershed, not just the most contaminated sites already known.

The results have often revealed a significant inter-and intra-specific contamination, which seems logical in view of the diversity of selected species, habitats and local diet variability. Despite this variability, trends can be highlighted: for example, the magnitudes of the main contaminants are the same for the three species of top predators (otters, osprey, cormorants), which highlights even for migratory species the existence of widespread contamination of all trophic compartments, and a non-linear but actual flow of contaminants.

The “modern” pesticides, i.e. those placed on the market after the progressive ban of organochlorine very persistent pesticides appeared much rarer than the latter in top predators. Thus,
organophosphates, carbamates, pyrethroids, herbicides were scarce in ospreys and virtually absent in otters and cormorants. Lower toxicity and persistence of these compounds could be suggested, limiting their accumulation, but caution must be observed in this interpretation, since their relatively recent introduction into the aquatic environment have delayed their integration into trophic webs to top predators. It is therefore appropriate to continue standard measures of these pesticides in aquatic species, to confirm or refute this hypothesis.

None of the species studied here seems threatened with extinction in the short term due to contamination by xenobiotics. These observations are an improvement from the perspective of conservation of heritage species such as otters and osprey, which were directly threatened with extinction due to contamination by organochlorine pesticides 20 or 30 years ago. However, questions remain, particularly for species facing multiple causes regression in which contamination may be an aggravating factor, as European eel (Anguilla anguilla) or pearl mussel (Mararitifera margaritifera). The accumulation of some elements in the tissues of fish may result in restrictions on fishing activity (highly developed in France) detrimental to the economy, both for commercial fishing for recreational fishing. Improvement of water quality, must therefore take into account all the species, and not rely on requirements of the jewels of biodiversity. The gradual decrease of concentrations of certain elements (including organochlorine pesticides) in the tissues of the studied species reflects the positive effects of the discharge control or the prohibition of compounds. Finally, regular analyses of xenobiotic (pesticides but also metals and PCBs), pharmaceutical compounds or drug residues in various compartments of wildlife will provide information on the level of contamination in real time, but also the possible impact of unforeseen events or following measures controlling the flow of pollutions.

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This section was written in French to avoid useless translations of institutions or structures/person names.

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