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Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the Eel (*Anguilla anguilla*) at the Camargue Nature Reserve, France

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Abstract

Pesticides (organochlorines—OC), polycyclic aromatic hydrocarbons (PAH) and heavy metals are toxic to fish and may be taken in through gills, skin and contaminated foods. Here we measure concentrations of OC, PAH and heavy metals, and their effects in the eel *Anguilla anguilla* from three locations in the Camargue Reserve in southern France. The Camargue Biosphere Reserve is the largest coastal wetland in Western Europe, and *A. anguilla* is a common predator at the top of the food chain. Livers and spleens were analyzed for histopathological, chemical and organo-somatic (HSI and SSI) effects. Gill, liver and spleen samples were collected for histopathological studies. Livers and muscles were sampled for metabolic parameters and persistent organic pollutant analysis. Total lipids were estimated by spectrophotometry and lipid-free residues were used in protein and glycogen analysis. OC pesticides were extracted from lipids of muscles and livers, analyzed by gas chromatography, and PAH from bile were analyzed by fixed wavelength fluorescence spectrofluorimetry. Heavy metals were measured by inductively coupled plasma with optical or with mass spectrometers. High concentrations of contaminants were found in eel tissues. La Capelière had the greatest OC and PAH concentrations; unexpected lesions in gills, livers and spleens were more common at the other sites. Liver and spleen tumors and lipidosis in livers were associated with chronic, and gill lesions with acute exposure. High pesticide and PAH concentrations and lesions in eels from the Camargue reserve demonstrate the contamination of the area. A more complete study in the Camargue reserve is necessary to better understand the impact on wildlife and humans. Also, this study suggests that eel biology must be better understood before continued use of this species as a biomonitor of polluted areas.

Keywords: Biomonitoring; PAH; Organochlorine pesticides; Heavy metals; Camargue reserve; Eels

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1. Introduction

Estuaries and coastal waters are often polluted by pesticides as organochlorine (OC) compounds, polycyclic aromatic hydrocarbons (PAH) and heavy metals. Water pollution and the resulting potential for bioaccumulation of those pollutants in aquatic organisms present a growing risk for wildlife and humans. In the estuary of the Rhone delta, the Camargue Nature Reserve is the largest protected coastal wetland area in Western Europe (Roche et al., 2002a). Despite the fact that this reserve has been registered in the Man and Biosphere Program in 1977, the estuary receives pollutants from a variety of sources (Ramade, 1997). A large portion of applied pesticides does not reach target organisms, including 10–30% of pesticides applied on the ground, and 50–75% of sprayed pesticides. Instead, this portion is carried away into the environment where it may then enter plants and animals (WWF, 1999). Aquatic environments are affected by a complex array of pesticides, with agriculture as the greatest contributor. In the Camargue Reserve, nearby rice production is associated with the largest body of water within the reserve, the Vaccarès Lagoon. Crude oil spills, refinery activities and industrial and urban wastes are important sources of polycyclic aromatic hydrocarbons (PAH) in aquatic ecosystems. PAH are potentially carcinogenic (Shailaja and D'Silva, 2003) and an important concern, because they are ubiquitous contaminants in coastal and freshwater zones (Akaishi et al., 2004). These compounds may induce hepatic lesions, physiological and biochemical disorders in fish. Contaminated fish may then be used to biomonitor the presence and importance of these pollutants. PAH contamination is especially important near industrialized areas (Aas et al., 2000). Finding PAH metabolites in fish bile has proven to be a simple and sensitive method for screening fish for PAH contamination as well as a useful biomarker for assessing exposure to aromatic contaminants in aquatic environments (Leadly et al., 1999; Aas et al., 2000).

Heavy metals often enter the environment through human activities, persist in food webs and accumulate in living organisms. In addition to atmospheric sources (Batty et al., 1996), other potential metal sources (Cu, Cd, Pb and Zn) are nearby rice fields, the Rhône river, the Fos-sur-mer petrochemical complex and waterfowl hunting (Tavecchia et al., 2001). Non-essential heavy metals are usually potent toxins and their bioaccumulation in tissues leads to intoxication, decreased fertility, cellular and tissue damage, cell death and dysfunction of a variety of organs (Oliveira Ribeiro et al., 2000, 2002; Damek-Proprawa and Sawicka-Kapusta, 2003). Essential metals such as copper (Cu), magnesium (Mg), manganese (Mn) and zinc (Zn) have normal physiological regulatory functions (Hogstrand and Haux, 2001), but may also bioaccumulate and reach toxic levels (Rietzler et al., 2001). Effects on the environment of these trace metals, mainly from human discharges are not well known and more information is required to better understand the importance of these pollutants, especially in protected areas.

Here we describe important physiological problems associated with the accumulation of organochlorine pesticides and heavy metals in liver and muscle and of PAH metabolites in eel (*A. anguilla*) bile in the Camargue Biosphere Reserve in France. Also, the effects of field exposure were measured through changes in glycogen, lipid and protein stores (energy reserves) in muscle and liver, which indicate major physiological responses (Smolders et al., 2004).

2. Material and methods

2.1. Study area and organisms

The Camargue Biosphere Reserve is located in the Rhône delta in southern France, covering 13,000 ha, it is between the Northern Vaccarès lagoon and the last undamaged sand dunes on the Mediterranean coast (Ramade, 1999). This reserve is important because it is the largest coastal wetland of Western Europe. The European eel (*A. anguilla*), a catadromous, euryhaline fish, is a common predator at the top of the food web. Living in both sediments and open waters, the eel remains in the wetlands for 9–15 years prior to migration. Eels were collected from three locations in the Vaccarès Lagoon (Fig. 1). Sampling sites were the mouth of the Fumemorte canal (FUM) into which rice fields drain, La Capelière (CAP) in the eastern part of the lagoon, approximately 300 m North of the mouth of the canal, and Mornès peninsula (MOR), at the end of fresh water inputs and the most remote area of the reserve. Thirty-one eels were caught, weighed and measured in October 2003 (Table 1). A condition index (CI) was used as a general health

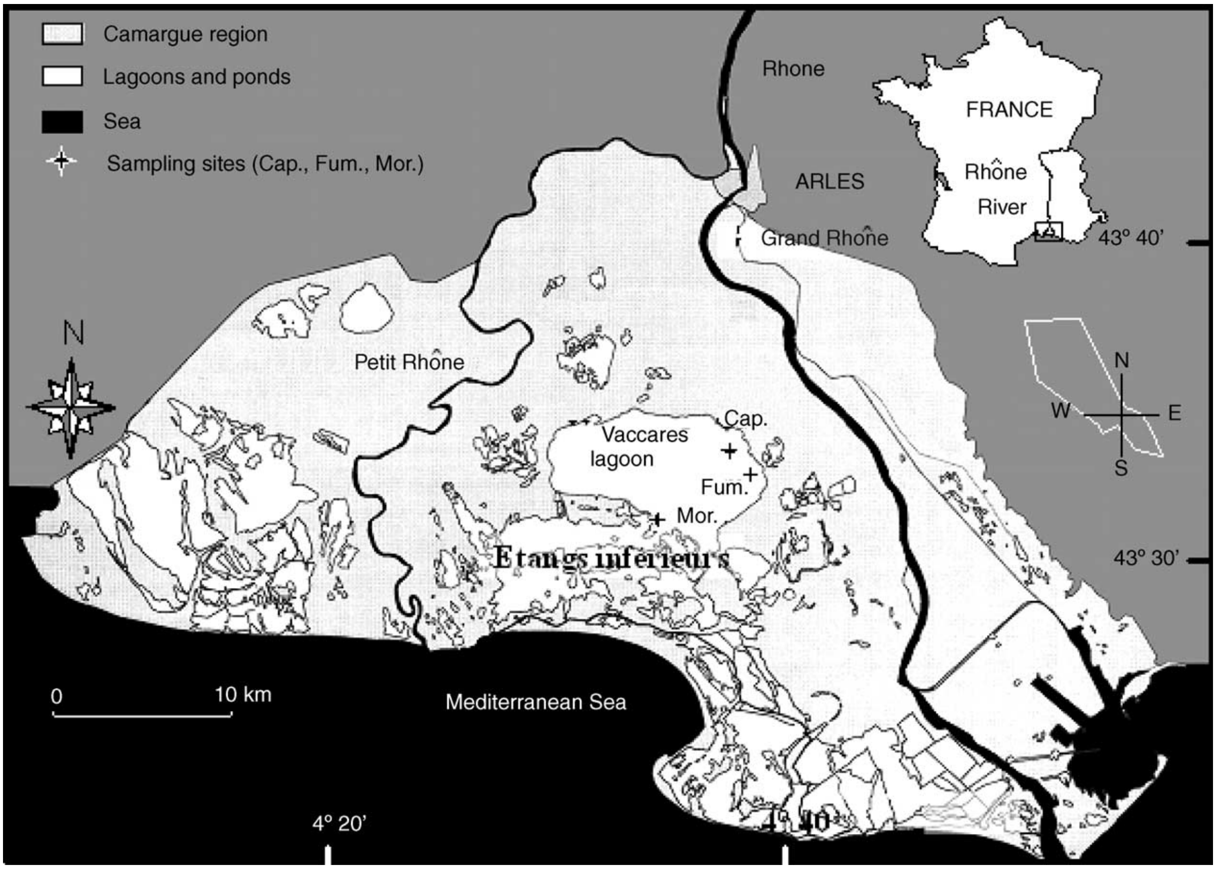

Fig. 1. The Biophere Camargue National Reserve, South of France; Mor, Mornès site; Cap, La Capellière site; Fum, Fumemorte site.

Table 1
Mean length and weight (standard deviation) of *Anguilla anguilla* from Camargue National Reserve—France of individuals samples in this study

	Length (cm)	Weight (g)
Morphological studies		
La Capelière[a,b,c]	42.7 ± 4.3	143.8 ± 35.6
Fumemorte[a,b,c]	63.0 ± 6.0	540.1 ± 147.9
Mornès[b,c]	57.4 ± 5.1	385.7 ± 84.4
Chemical analyses (pesticides, PAH, heavy metals, glycogen, proteins, lipid)		
La Capelière	39.5 ± 2.1	116.35 ± 9.19
Fumemorte	66.0 ± 4.1	558.36 ± 119.49
Mornès	57.4 ± 7.3	383.98 ± 126.14
Indices of biological organization (RHS, RSS)		
La Capelière	40.7 ± 8.8	137.6 ± 100.6
Fumemorte	63.6 ± 5.1	533.3 ± 129.1
Mornès	46.2 ± 11.8	232.8 ± 168.7

[a] Gill.
[b] Liver.
[c] Spleen.

indicator (growth, nutritional state, and energy content) (Sutton et al., 2000; Kleinkauf et al., 2004). Here, the CI was evaluated by means of the weight–length relationship in the reference population ($n > 6000$). It is calculated as the ratio (actual weight/theoretical weight), where the theoretical weight is estimated by the residuals from a logarithmic regression of weight on length. Here, the equation is log (theoretical weight) = 3.304 × log (actual length) − 3.236.

Organs were carefully excised for histopathological and chemical analyses. Livers and spleens were weighed and organo-somatic indices (%) were calculated as follows: HSI: hepatosomatic index [(liver weight/total weight) × 100]; SSI: splenosomatic index [(spleen weight/total weight) × 100].

2.2. Histopathological analyses

A total of 31 immature eels were collected and analyzed (CAP—$n = 9$, FUM—$n = 12$, MOR—$n = 10$; Table 1). Gill, liver and spleen samples were preserved in Alfac fixative solution (16 h), dehydrated in a graded series of ethanol baths and embedded in Paraplast Plus resin (Sigma®). The sliced sections (5 μm) were stained in Haematoxylin/Eosin and observed under a Zeiss microscope (Akaishi et al., 2004).

3. Chemical analyses

3.1. OC, PAH extraction, separation and analytical procedures

Livers and muscles were dissected for metabolic and POP analysis. Water content of liver and muscle was determined by drying at 105 °C for 24 h. Total lipids were determined by weighing after extraction with dichloromethane-methanol solution following the method of Folch et al. (1957). Lipid phosphorus was estimated by spectrophotometry (Fiske and Subbarow, 1925). After lipid extraction, the lipid-free residues were filtered and digested in NaOH solution (1N) for protein and glycogen measurement. Protein was measured following Lowry et al. (1951). Glycogen, after ethanol precipitation, was hydrolyzed by amyloglucamylase and glucose was measured by glucose oxidase method (Hugget and Nixon, 1957) using ABTS as chromogen.

OC were extracted from muscular and hepatic lipids; PAH were extracted from bile. OC and PAH were purified by solid phase extraction (SPE) on florisil (MgO_3Si), an extremely polar, magnesium-loaded silica gel, following the EPA method 3620 (Bond Elut Florisil, 1 g, 200 μm particle size—Varian), first with hexane, to eliminate other lipophilic compounds, then with hexane/di ethyl ether (90/10) for OC clean-up and hexane/dichloromethane (50/50) for PAH.

OCs were analyzed by gas chromatography with AutoSystem XL (Perkin-Elmer), using electron capture detection (^{63}Ni Source) and nitrogen as the carrier gas following an adapted procedure of the EPA Method 8081a. The detection limit ranged from 0.05 to 0.20 g kg^{-1} in fish tissues.

Bile extracts were diluted in methanol (48%) and PAH were detected by fixed wavelength fluorescence on a SFM25 (BioTek) spectrofluorometer following the method described by Aas et al. (2000), using the 16 PAHs considered priority pollutants by the US Environment Protection Agency (US-EPA) as references. The wavelength pairs of excitation/emission of 288/330, 267/309, 334/376, 364/406 and 380/422 nm were used for detection of naphthalene type (2 aromatic rings), phenanthrene type (3 rings), pyrene type (4 rings), benzo(a)pyrene type (5 rings) and benzo(ghi)perylene type (6 rings) PAH, respectively. The concentrations are expressed in terms of concentration in bile.

3.2. Heavy metal analysis

The material used in the digestion process was acid-rinsed. From 80 to 100 μg of muscle and liver tissue samples were placed on Teflon vessels and digested (120 °C, 12 h) with 2.0 ml 70% nitric acid (Baker Instra Analyzed) and 1.0 ml 30% hydrogen peroxide (Baker Instra Analyzed). Fe and Mg concentrations were determined by a Perkin-Elmer OPTIMA-3200RL Inductively Coupled Plasma optical Spectrometer (ICP-OES). Na, Al, Hg, Pb, Cd, As, Cu, Zn, Mn, Ni, Ti, Co, Cr, Rb, Sr and Mo were quantified by a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Two replicate subsamples and a standard reference material (Bovine Liver SRM-1577a) certified by the National Bureau of Standards (NBS) were included in the analyses. To obtain the elevation in the concentration of elements, the mean values from 26 control assays were subtracted from each sample. The advantages of such methods include speed of digestion, lowered risk of contamination and a multielemental quantification of toxic heavy metals (Bordajandi et al., 2003).

All heavy metal analyses were carried out at the Spectroscopy Service of the University of Barcelona, Spain.

3.3. Statistical procedures

Intersite variability was compared using one-way analysis of variance (ANOVA) followed by Scheffé's and Bonferroni/Dunn post hoc tests and paired comparisons were used to check differences between muscular and hepatic concentrations. Pearson correlations between contamination level and biological data were calculated. All tests were regarded as statistically significant when $p < 0.05$.

3.4. Chemicals

Reference organochlorines were purchased from CIL Cluzeau (Ste-Foy-la-Grande, France) and the 16 EPA priority PAH were purchased from LGC Promochem (Molsheim, France).

4. Results

4.1. Pesticide OC in muscle and liver

Bioaccumulation of OC was greatest in individuals collected in CAP and MOR for both liver ($p = 0.003$ and 0.014, respectively) and muscle tissues ($p = 0.015$ and 0.006, respectively; Table 2).

The greatest levels of OC were not found in individuals living in the Fumemorte, which should be the most contaminated with pesticides ($p < 0.016$). The most common organochlorine pesticide found in muscle tissue was an endosulfan metabolite. Livers showed highest bioaccumulation of lindane and its isomers ($\sum$HCH) followed by δ chlordane and β endosulfan, fish muscles and liver had high concentrations of lindane and its δ isomer. Very high levels of γ chlordane in muscle were found in eels from the Vaccarès lagoon and the FUM canal. Moreover, the

Table 2
Mean concentrations (deviation error) of different analyzed OC in muscle and liver of eels (*A. anguilla*) from two sites in the Vaccarès lagoon (La Capelière and Mornès) and at the mouth of the main rice-field canal (Fumemorte)

	Muscular OC impregnation (ng g^{-1} dry weight)			Hepatic OC impregnation (ng g^{-1} dry weight)		
	La Capelière ($n = 9$)	Fumemorte ($n = 9$)	Mornès ($n = 8$)	La Capelière ($n = 9$)	Fumemorte ($n = 9$)	Mornès ($n = 8$)
Banned or restricted pesticides						
α HCH	11.4 ± 2.3	14.6 ± 2.9	15.6 ± 2.4	9.5 ± 1.7	1.5 ± 0.9 a,b	30.5 ± 11.8
β + γ HCH	293.1 ± 41.2	165.5 ± 20.7 b	120.3 ± 34.7 c	112.9 ± 64.9	80.9 ± 28.6	24.5 ± 7.1
δ HCH	415.0 ± 49.2	420.9 ± 157.8	578.3 ± 93.9	22.0 ± 6.7	65.9 ± 45.6	11.1 ± 2.6
Aldrine	53.9 ± 6.5	44.0 ± 8.1	78.0 ± 18.7	33.2 ± 8.5	20.5 ± 6.7	45.1 ± 13.5
Dieldrine	40.5 ± 9.0	26.4 ± 5.1	40.5 ± 7.7	27.8 ± 5.5	19.5 ± 9.4 a	55.5 ± 9.2 c
Endrine	40.9 ± 9.8	47.9 ± 15.6	73.0 ± 23.0	52.0 ± 6.8	2.6 ± 1.7 a,b	42.0 ± 10.4
Endrine aldehyde	120.1 ± 36.2	85.8 ± 43.8	60.4 ± 22.5	58.2 ± 24.9	29.2 ± 10.7	80.2 ± 30.5
α Chlordane	14.2 ± 4.6	51.7 ± 13.6 a,b	14.2 ± 4.1	29.7 ± 18.2	15.7 ± 8.8	30.5 ± 10.0
γ Chlordane	399.2 ± 78.1	189.4 ± 60.3 b	334.8 ± 35.0	283.9 ± 85.1	29.5 ± 12.1 a,b	184.7 ± 36.2
Heptachlor	4.2 ± 2.5	2.6 ± 2.6 a	40.1 ± 18.5	26.7 ± 5.0	15.5 ± 4.8	34.1 ± 9.3
Heptachlor epoxide	48.2 ± 10.1	56.6 ± 16.4	25.9 ± 7.7	25.9 ± 7.7	55.6 ± 19.7 a	117.6 ± 13.5 c
Used pesticides						
Fipronil	100.5 ± 50.6	72.8 ± 15.3	75.8 ± 14.1	140.5 ± 25.8	48.1 ± 28.8 b	98.3 ± 14.1
α Endosulfan	55.7 ± 8.6	130.2 ± 27.7 b	110.7 ± 46.1	82.3 ± 21.1	29.0 ± 8.8 a,b	61.1 ± 7.2
β Endosulfan	92.8 ± 20.5	90.7 ± 33.6	112.6 ± 21.8	104.5 ± 21.2	42.5 ± 17.2 a,b	185.2 ± 32.2
Endosulfan sulfate	857.6 ± 267.2	37.6 ± 13.8 a,b	1099.4 ± 135.7	23.0 ± 11.1	44.9 ± 18.9	27.8 ± 11.3

Letters indicate differences ($p < 0.05$). (a) Eels from Fumemorte vs. Mornès; (b) Eels from Fumemorte vs. La Capelière; (c) Eels from La Capelière vs. Mornès.

chlordane accumulation was increased in eels coming from CAP ($p = 0.001$ CAP versus FUM). Concentrations of the banned substances, aldrin, dieldrin and endrin, were relatively stable in muscle and liver of individuals from the Vaccarès Lagoon. Eels coming from the FUM showed a lower hepatic concentration of dieldrin ($p = 0.006$) and endrin ($p < 0.0004$) (Table 2).

A higher concentration of γ chlordane occurred in the liver of the individuals from the lagoon sites, following the same standard distribution as that described for muscles, when compared to FUM ($p < 0.03$). The compound currently used in rice-field (i.e., fipronil) showed similar concentrations in muscles and in livers, while endosulfan (isomers plus metabolites), suspected to be sprayed in the nearby areas, was more concentrated in livers than muscles of eels from all three sites ($p < 0.05$; Table 2).

4.2. Bile PAH

Total PAH in bile of *A. anguilla* does not show the same distribution patterns as pesticides; the concentration observed in CAP was higher than that in FUM ($p = 0.008$) and MOR ($p = 0.004$) (Fig. 2). PAH found in bile had the highest concentration in the youngest eels from CAP (yellow versus juvenile $p = 0.0017$). The most abundant metabolite was pyrene and other hydrocarbons with four aromatic rings. Pyrene is frequently used as a marker of the total PAH contamination in fish. Naphthalene and 3-ring PAHs such as phenanthrene were also abundant in the youngest eels from CAP ($p < 0.004$). The other analyzed compounds with five and six aromatic rings were less common (Table 3).

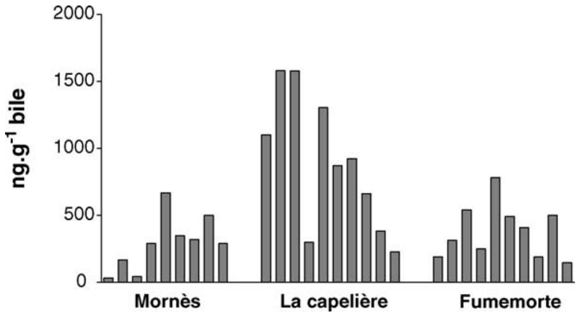

Fig. 2. Total PAH distribution in bile from the eel *Anguilla anguilla* from three sites in the Camargue Reserve.

4.3. Heavy metals in muscle and liver

The highest concentration of metals (Al, Hg, Ti, Zn, As and Mg; Table 4) were found in CAP with a significant difference in Co concentration between MOR and CAP ($p < 0.001$). Muscle and liver distribution of metals is provided in Table 4. FUM and CAP differed in Hg ($p = 0.006$), Cu ($p = 0.012$) and Co ($p < 0.001$). At MOR, Hg levels were significantly higher ($p < 0.0001$) and Co levels significantly lower ($p < 0.001$) than at FUM. The highest concentrations of the analyzed metals (Al, Hg, Cu, Ti, Zn, Mn, Ni, Mg and Fe) were found in livers. Metal-enrichment factors in livers were around 50 times greater than those in muscle for Fe, 20 times greater for Mn and Mo, 10 times greater for Ni and Co, and three times greater for Na and Zn. Hg and Cr showed similar concentrations in both tissues. The liver was the most important target organ for heavy metals and only Sr and As showed highest values in muscle (three and two times greater than in liver, respectively). Eels from FUM showed the lowest concentrations of

Table 3
Mean concentration (±S.E.M.) of total PAH in bile of eel (*A. anguilla*) from two sites in the Vaccarès lagoon (La Capelière and Mornès) and at the mouth of the main rice-field canal (Fumemorte)

	Bile PAH concentration (ng g^{-1} bile)		
	La Capelière (n =10)	Fumemorte (n = 10)	Mornès (n = 9)
2 Aromatic rings (like Naphtalene)	223.8 ± 38.8	84.9 ± 12.8 a	70.1 ± 17.7 b
3 Aromatic rings (like Phenanthrene)	221.6 ± 44.2	132.4 ± 29.1	70.0 ± 18.3 b
4 Aromatic rings (like Pyrene)	311.0 ± 53.4	110.6 ± 15.3 a	104.7 ± 22.7 b
5 Aromatic rings (like Benzo[a]pyrene)	92.9 ± 19.1	36.6 ± 5.5 a	34.0 ± 7.2 b
6 Aromatic rings (like Benzo[ghi]perylene)	44.0 ± 9.5	17.6 ± 2.6 a	17.1 ± 3.3 c,b

Letters indicate differences ($p < 0.05$). (a) Eels from Fumemorte vs. La Capelière; (b) Eels from La Capelière vs. Mornès.

Table 4
Mean concentrations (± standard error) of metals in muscle and liver of eels (*A. anguilla*) from two sites in the Vaccarès lagoon (La Capelière and Mornès) and at the mouth of the main rice-field canal (Fumemorte)

	Muscular metal concentration (μg g^{-1} dry weight)			Hepatic metal concentration (μg g^{-1} dry weight)		
	La Capelière ($n = 9$)	Fumemorte ($n = 9$)	Mornès ($n = 8$)	La Capelière ($n = 10$)	Fumemorte ($n = 9$)	Mornès ($n = 8$)
Na	1249 ± 121	1017 ± 58	1280 ± 114	4100 ± 797	3151 ± 144	2897 ± 406
Al	50.3 ± 10.1	47.2 ± 5.1	16.0 ± 3.2 a,c	85.0 ± 27.0	48.3 ± 11.0	124 ± 25
Zn	55.4 ± 3.4	57.9 ± 4.0	57.1 ± 4.5	206 ± 108	215 ± 17	201 ± 40
Rb	2.53 ± 0.36	1.98 ± 0.28	2.29 ± 0.37	2.03 ± 0.15	1.44 ± 0.21 a	1.74 ± 0.34
Sr	2.30 ± 0.73	1.62 ± 0.59	1.79 ± 0.48	0.77 ± 0.15	0.43 ± 0.05 b	0.64 ± 0.15
Cu	0.43 ± 0.07	0.19 ± 0.04 b	0.30 ± 0.06	72.3 ± 45.1	76.5 ± 10.2	59.9 ± 18.0
Mn	0.32 ± 0.11	0.11 ± 0.03	0.23 ± 0.08	7.55 ± 2.71	7.56 ± 0.44	7.74 ± 1.44
Ti	46.3 ± 4.3	43.2 ± 3.0	47.0 ± 3.3	70.2 ± 13.0	53.2 ± 1.6	62.4 ± 2.8
Hg	0.16 ± 0.02 b,c	0.44 ± 0.06	0.61 ± 0.08 a	0.32 ± 0.20	0.42 ± 0.05	0.76 ± 0.14
Mo	0.04 ± 0.00	0.06 ± 0.03	0.03 ± 0.01	0.93 ± 0.19	0.76 ± 0.02	0.76 ± 0.02
As	2.46 ± 0.73	0.17 ± 0.01	4.67 ± 1.99	1.27 ± 0.31	0.10 ± 0.02 a,b	1.28 ± 0.74
Cr	1.46 ± 0.07	2.48 ± 1.17	1.59 ± 0.13	1.51 ± 0.32	1.05 ± 0.06	1.36 ± 0.20
Ni	0.83 ± 0.40	0.16 ± 0.10	0.13 ± 0.05	7.74 ± 1.90	6.01 ± 0.37	3.66 ± 0.99
Co	0.06 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.48 ± 0.12	0.29 ± 0.05	0.16 ± 0.04
Cd	–	–	–	0.13 ± 0.04	0.23 ± 0.06	0.44 ± 0.17
Pb	0.79 ± 0.58	0.24 ± 0.05	0.21 ± 0.02	0.64 ± 0.36	0.38 ± 0.10	–
Fe	14.5 ± 5.0	17.7 ± 8.5	12.5 ± 1.6	801 ± 280	1166 ± 83	1175 ± 395
Mg	637 ± 77	539 ± 39	685 ± 77	646 ± 123	481 ± 11	583 ± 42

Letters indicates differences ($p < 0.05$); (a) Eels from Fumemorte vs. Mornès; (b) Eels from Fumemorte vs. La Capelière; (c) Eels from La Capelière vs. Mornès.

Rb (FUM versus MOR $p = 0.017$), Sr (FUM versus CAP $p = 0.028$) and As (FUM versus CAP $p = 0.0009$ and FUM versus MOR $p = 0.043$).

4.4. Histopathology

A. anguilla had several interesting microscopic features including the occurrence of necrotic areas, neoplasias, and abundant hepatic lipid accumulation among others (Table 5). Microscopic analyses in gills showed occasional lesions in individuals from FUM (aneurisms and secondary lamellar fusion, Fig. 3B and E). Internal and external parasites were only observed in individuals from FUM, the site connecting the Vaccarès Lagoon with the rice fields (Fig. 3C and D, Table 5).

Livers were the most damaged organs. In *A. anguilla*, the morphological characteristics of liver is as described for other teleost fish (Rabitto et al., 2005), where hepatocytes surround vessels and typical hepatic cells with rounded nuclei, evident nucleoli and delimited cytoplasm are observed (Fig. 4A). Necrosis (Fig. 4D) was found in all sites and in almost all specimens (Table 5). The presence of prenecrotic areas, described here as foci of red and white blood cells (Fig. 4B and C), showed a similar distribution and incidence as described above for necrotic areas (Table 5). Apoptosis was more common in individuals from MOR and FUM (Table 5) than in those from CAP. This type of cell death is characterized by intense chromatin stain concentrating close to the nuclear envelope, and by the typical nuclear shape (Fig. 4E and F). Livers of some individuals from MOR and FUM showed different forms of preneoplasic foci (Fig. 5). These foci are classically recognized as an early stage of tumour development. A rounded and nonencapsulated tumour presenting a completely differentiated and visible mass of cells was observed in liver of two individuals from FUM (Fig. 6A). Differentiated and amorphous cells including numerous nuclei (Fig. 6B) are diagnostic for typical neoplasic areas. Lipidosis was common, with the greatest incidence in FUM followed by MOR (Table 5). Individuals with greater lipidosis also had, in the hepatic cytoplasm, variable amounts of clear, round, well-demarcated vacuoles and, in some cells, multiple cytoplasmic vacuoles (Fig. 6C and D; Table 5). Spleens had lesions that have not been previously described. This organ is important for blood cell development

Table 5
Histopathological findings in gills, livers and spleens of *A. anguilla* from Camargue National Reserve—France

	La Capelière	Fumemorte	Mornès
Gills			
Lamellar fusion	0(7)	6(7)	na
External parasite and aneurisms	0(7)	3(7)	na
Internal parasites	0(7)	3(7)	na
Liver			
Pre necrosis area	5(9)	11(12)	7(10)
Necrosis area	8(9)	11(12)	8(10)
Lipid intracellular accumulation	1(9)	11(12)	5(10)
Cell death like apoptosis	1(9)	7(12)	5(10)
Preneoplasic foci	0(9)	2(12)	2(10)
Tumor	0(9)	2(12)	0(10)
Melanomacrophage centers	1(9)	4(12)	4(10)
Spleen			
Free melanomacrophages	9(9)	12(12)	10(10)
Melanomacrophages centers	9(9)	12(12)	10(10)
Necrosis area	5(9)	7(12)	1(10)
Pre necrosis area	3(9)	7(12)	1(10)
Tumor	0(9)	2(12)	0(10)

na: Not analyzed.

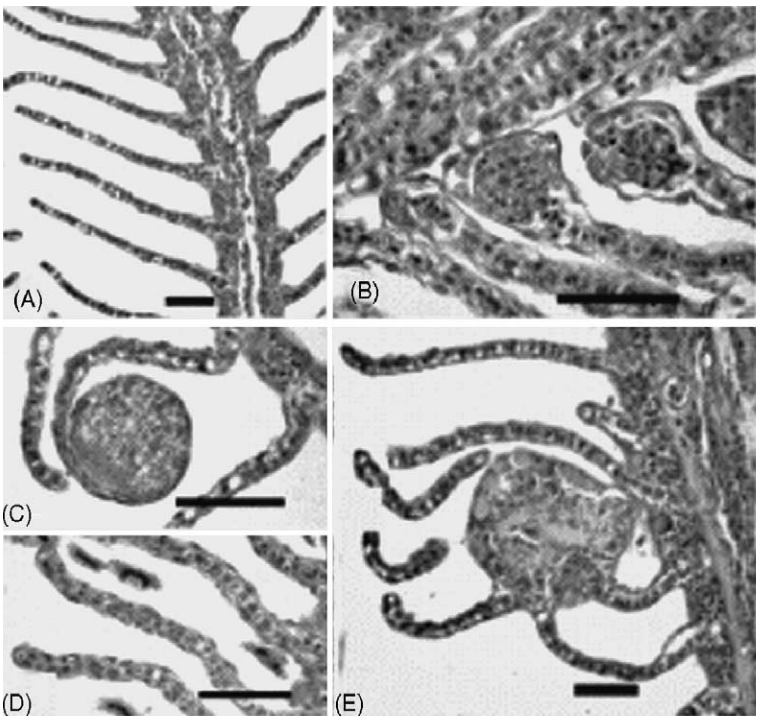

Fig. 3. Cross section of gills in *A. anguilla* from Camargue Reserve. A. Primary and secondary lamellas: La Capelière site. B. Aneurisms in secondary lamellae extremity: Fumemorte site. C. Internal parasite within secondary lamellae: Fumemorte site. D. External parasites in gills: Fumemorte site. E. Fusion among secondary lamellas: Fumemorte site. Scale bar 50 μm, Hematoxylin and Eosin stain.

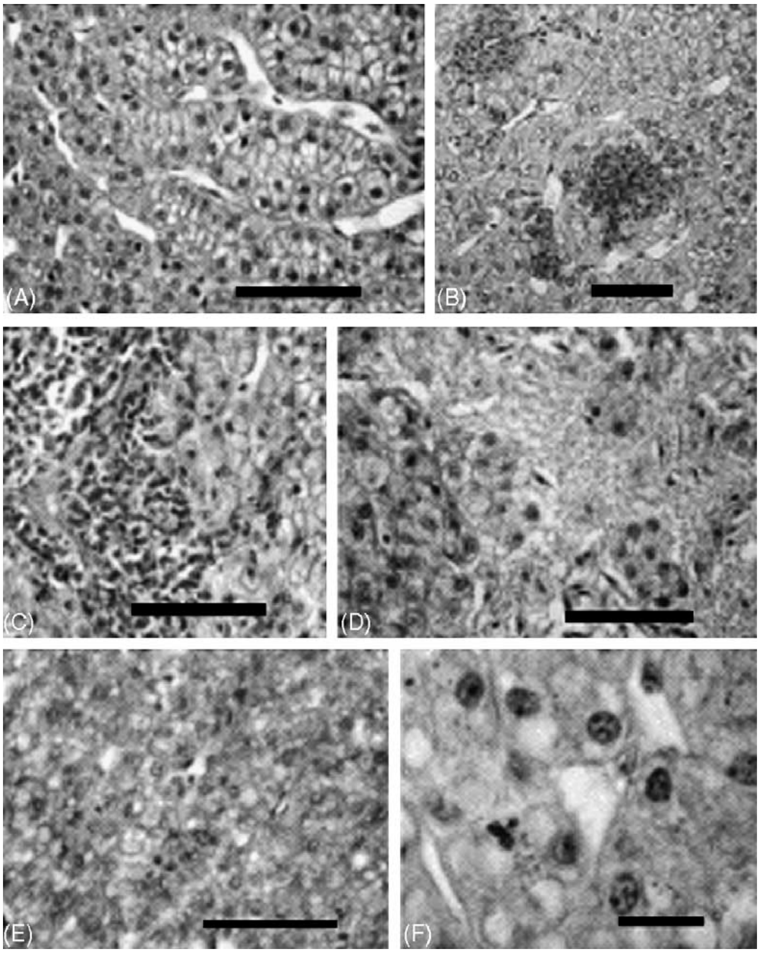

Fig. 4. Cross section of the liver of *A. anguilla* from Camargue Reserve. A. Hepatocytes and sinusoids in non affected tissue: La Capelière site. B. Red blood cells accumulation: Mornès site. C. White blood cells accumulation: Fumemorte site. D. Necrosis area: Mornès site. E. Apoptosis in a very affected tissue: Fumemorte site. Scale bar 50 μm. F. Figure of apoptosis: Mornés site. Scale bar 10 μm, hematoxylin and eosin stain.

and replacement, and is characterized by a large number of melano-macrophage and melano-macrophage centres (Fig. 7A and C, respectively). Necrosis and pre-necrosis were observed in individuals from all studied sites, but the highest incidence was in individuals from FUM (Table 5 and Fig. 7C). Tumours were also found in spleens of individuals from FUM, which also had the highest incidence of lesions. Spleen tumours were not necessarily found in the same individuals as liver tumours, and so FUM had a 30% tumour incidence rate (Table 5). Spleen tumours, similar to those in livers, were not encapsulated and contained a large mass of differentiated cells, and within which was an active and encapsulated melano-macrophages centre (Fig. 7D).

4.5. Available energy reserves (proteins, glycogen, lipid)

A. anguilla at the end of summer are normally fat. Indeed, neutral lipids represent up to 83% of total lipids in liver and 97% in muscle, repectively. Yet, eels from FUM contained an excess of lipids in muscles and livers, essentially due to the accumulation of neutral lipids ($p = 0.01$). The sampling sites

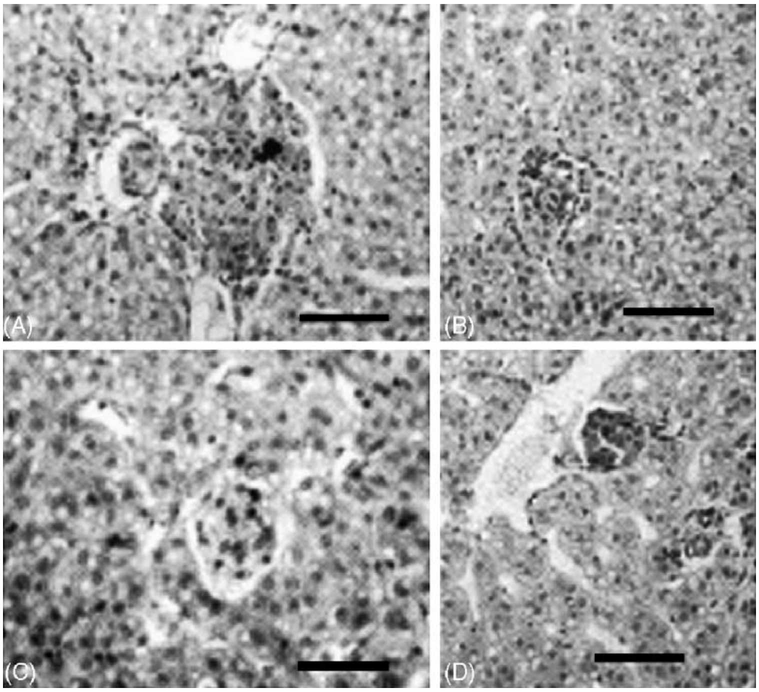

Fig. 5. Cross section of the liver of *A. anguilla* from Camargue reserve. Different preneoplasic foci are shown. A and B: Mornès site; B and D: Fumemorte site. Scale bar 50 μm, hematoxylin and eosin stain.

Table 6
Overview of different endpoints describing the effects of field exposure on conditions status and physiological energetic of eel (*A. anguilla*) from two sites in the Vaccarès lagoon (La Capelière and Mornès) and at the mouth of the main rice-field canal (Fumemorte)

	La Capelière	Fumemorte	Mornès
Hepatic components			
Protein	296.0 ± 18.1 (9)	257.6 ± 9.8 (10) a	336.3 ± 15.6(10)
Glycogen	68.2 ± 20.8 (9)	43.1 ± 11.3(10)	57.1 ± 17.6 (10)
Total lipids	329.3 ± 24.3 (9) b	432.5 ± 30.4 (10) a	335.6 ± 31.8 (10)
Neutral lipids	280.5 ± 25.2 (9)	381.6 ± 30.8 (10) a	281.4 ± 34.9 (10)
Phospholipids	48.8 ± 3.7 (9)	51.0 ± 4.5(10)	54.2 ± 5.3 (10)
Muscular components			
Protein	343.0 ± 28.1 (10)	355.4 ± 26.2 (10)	394.3 ± 39.9 (9)
Glycogen	0.558 ± 0.175 (10)	0.619 ± 0.181 (10) a	0.147 ± 0.070 (9)
Total lipids	380.2 ± 57.2 (10)	510.2 ± 46.2 (10) a	330.1 ± 32.7 (9)
Neutral lipids	370.1 ± 57.3 (10)	499.9 ± 46.0 (10) a	316.2 ± 33.5 (9)
Phospholipids	10.1 ± 0.9 (10) c	10.3 ± 0.7 (10) a	13.9 ± 1.0 (9)
Condition status			
CI	1.05 ± 0.04 (10)	0.93 ± 0.03 (10) b	0.99 ± 0.04 (10)
HIS	1.56 ± 0.12 (10)	1.16 ± 0.09 (10)	1.46 ± 0.09 (10)
SSI	0.096 ± 0.007 (10)	0.106 ± 0.013 (10)	0.106 ± 0.013 (10)

Letters indicate differences ($p < 0.05$). (a) Eels from Fumemorte vs. Mornès; (b) Eels from Fumemorte vs. La Capelière; (c) Eels from La Capelière vs. Mornès; CI, condition index; HIS, hepatic somatic index; SSI, spleen somatic index, number of individuals analyzed in parentheses.

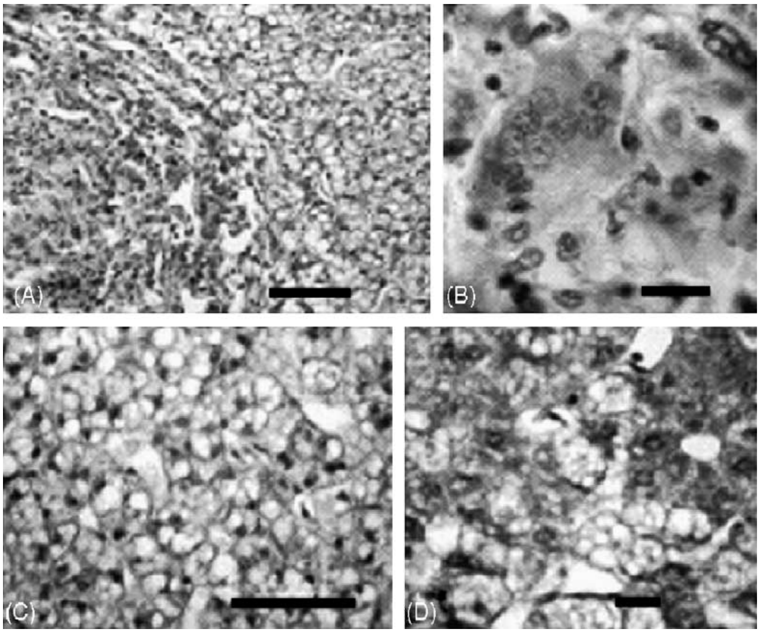

Fig. 6. Cross section of the liver *of A. anguilla* from Camargue Reserve. A. Tumor showing a large neoplasic area: Fumemorte site. B. Detail showing a multinuclear neoplasic cell: Fumemorte site. D. High cellular lipid accumulation showing many vesicles: Fumemorte site. A and C scale bar 50 μm; B and D scale bar 10 μm, hematoxylin and eosin stain.

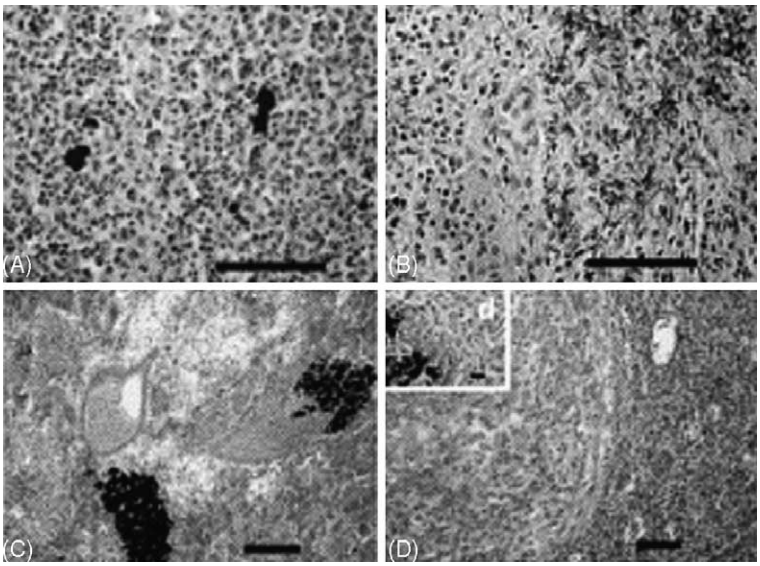

Fig. 7. Cross section of the spleen of *A. anguilla* from Camargue Reserve. A. Melanomacrophage: La Capelière site. B. Differentiated tissue: Mornès site. C. Melanomacrophage center and necrosis areas: Fumemorte site. D. Tumor showing a large neoplasic area. Inset: a melanomacrophage within the tumor (d): Fumemorte site. Scale bar 50 μm, hematoxylin and eosin stain.

usually did not influence structural components (proteins and phospholipids), but FUM had lower values for hepatic proteins ($p = 0.0004$) and muscular phospholipids than MOR ($p = 0.008$). Interestingly, higher glycogen levels occurred in the muscle of eels from FUM than those from the other sampling sites ($p < 0.05$; Table 6).

4.6. Linking levels of biological organisation (CI, SSI, HSI)

The morphometric index is a quite interesting indicator for disturbances of feeding in the fish. The condition indices (CI) of sampled eels were compared with a normal population (more than 6000 individuals) from different Camargue ecosystems. For species that store energy reserves hepatically, the hepatic somatic index (HSI) is useful in ecotoxicological investigations. The HSI and the spleen somatic index (SSI), a marker of the spleen response, are indicators of physiological status. Here, while no inter-site difference was observed for HSI and SSI, eels from FUM had CI significantly lower than 1 ($p = 0.02$), indicating a typical weight deficit (Table 6).

5. Discussion

Organochlorine (OC) compounds, such as pesticides, may influence fish development, reproduction and behaviour (Sapozhnikova et al., 2004). While depuration of endosulfan is considered rapid (Novak and Ahmad, 1989), its muscle and liver concentrations at the Camargue Reserve were much greater than those found in eels from the Netherlands (Van der Oost et al., 1996) and in sea cod from the White Sea, Russia (Muir et al., 2003). Also, these concentrations were greater than those established as the oral reference dose by the Integrated Risk Information System of the EPA (IRIS, 2000). Although lindane has been banned in France since 1998, a high concentration was found here. Hence, this is a very persistent pesticide. The Maximum Residues Limits (LMR) of lindane estimated by the Food and Agriculture Organization of the United Nations (FAO, 1999) is $2\,mg\,kg^{-1}$ of animal flesh. In eels from Vaccarès, 5 years since the banning of lindane, the $\sum$HCH burden is $62 \pm 6\,ng\,g^{-1}$ wet weight. Although under the allowed limit, this concentration emphasizes the persistence of such compounds.

Chlordane had greater concentrations in muscle than in liver. This chemical was banned in Europe (1970) and the USA (1988), but persists for a very long time in the environment, remaining in soils for over 20 years. Furthermore, it may travel long distances. Here, chlordane concentration in the eels sampled was greater than that found in eels from Poland and the Baltic Sea (Karl et al., 1998), in fishes from Australia (Connell et al., 2002) and from the White Sea in Russia (Muir et al., 2003).

Heptachlor was banned in Europe (1970) and the USA (1974), but again, this chemical and its principal metabolite (heptachlor epoxide) persist and may remain in soils and surface waters for many years, and bioaccumulate in fish. In eels from Camargue, $\sum$heptachlor had greater liver than muscle concentrations (Jabber et al., 2001; Das et al., 2002; Sapozhnikova et al., 2004) for unknown reasons.

Although dieldrin may be used as a pesticide, here it was assumed to be a metabolite of aldrin. In soil, water and living organisms, aldrin is rapidly epoxidized to dieldrin. Muscle dieldrin concentrations from the Camargue Reserve were greater than those in fish from Egypt (Nemr and Abd-Allah, 2004), Australia (Connell et al., 2002), India (Jabber et al., 2001) and the USA (Sapozhnikova et al., 2004), but lower than those from Britain (Yamaguchi et al., 2003). Endrin has not been produced or sold in France officially since the restriction of its use in the seventies; nevertheless this compound with its breakdown product, endrin aldehyde, is still found in aquatic organisms. Contrasting with data presented here, endrin had greater liver than muscle concentrations in two fish species (Sapozhnikova et al., 2004).

Fipronil is in a class of insecticides known as phenylpyrazoles that are potent inhibitors of the gamma-aminobutyric acid (GABA)-gated chloride channel (Ohi et al., 2004). Although fipronil experimentally bioaccumulates in fish muscles, with subsequent depuration after 14 days (EPA, 2004), no literature data were available to compare with the high levels found here in the Camargue Reserve.

No studies were found in the literature on (Sr, Co, Mo, and other elements) levels in European eels, but the results obtained are in accordance with metal concentrations in other fish species (Agusa et al., 2004).

Nonessential metal concentrations at the Camargue Reserve (Cd, Hg, Pb and As) are similar to (Linde et al., 1999), lower than (Hendriks and Pieters, 1993; Usero et al., 2003) and greater than (Mason and Barak, 1990) those reported in other fish species in studies in polluted locations. While Linde et al. (1999) reported higher concentrations of cadmium in eels than in other animals, fish in general accumulate very little of this heavy metal (Calamari et al., 1982). Here, cadmium was not found in any significant level. Mercury, on the other hand, a very toxic pollutant, was found in high concentrations and may have come from atmospheric transportation from other areas. Mercury levels exceeded 0.30 mg/kg, the maximum allowable concentration under E.C. Directives (Mason and Barak, 1990). For the other elements no high levels were measured. The large intraspecific variation found here was similar to that from other European regions and may be explained by uptake, age, sex and individual metabolic response to detoxification (Nendza et al., 1997; Van der Oost et al., 1998; Verweij et al., 2004; Vives et al., 2005).

Using morphological characteristics of target organs as biomarkers is very useful in biomonitoring programmes and has been recently recommended by STAP/GEF (2003) for monitoring persistent organic pollutants (POPs) in aquatic ecosystems. Microscopic damage to gills, livers and spleens has been recently reported in other fish species (Dutta and Meijer, 2003; Marty et al., 2003; Akaishi et al., 2004; Brown and Steinert, 2004). Because gills are in contact with water and are exposed to dissolved contaminants and to trophic contamination, fusion of lamellae and aneurysms (from FUM) suggests an acute exposure to contaminants, while gill parasites on individuals from the same site means compromised immune system. Hence, gill damage described here shows both acute and long-term effects of contaminants in *A. anguilla*.

Livers are useful to describe and document the effects of pollutants (Oliveira Ribeiro et al., 2002; Bondy et al., 2003; Damek-Proprawa and Sawicka-Kapusta, 2003; Padros et al., 2003; Akaishi et al., 2004; Brown and Steinert, 2004). Necrosis in livers from the Camargue reserve is not necessarily due to specific pollutants since little evidence links damage to specific organic or inorganic compounds (Chang et al., 1998; Rabitto et al., 2005). Necrosis is strongly associated with oxidative stress where lipid peroxidation is a clear source of membrane bilayer susceptibility (Li et al., 2000; Avci et al., 2005). Pollutants (pesticides—Azzalis et al., 1995, heavy metals—Stohs and Bagghi, 1995, PAH—Ibuki and Goto, 2002) are associated with increased free radical concentrations within the cytosol. These oxidative forms may increase programmed cell death or disturbed cell homeostasis and cellular necrosis. Also, prenecrotic areas suggest another necrosis event where the invasion of blood cells in the tissue is an evidence of cell injury. Individuals with high incidence of necrosis also displayed prenecrosis, strongly suggesting a continuous exposure to the related xenobiotic compounds present in the environment.

Apoptosis is due to a complex pathway in which biochemical mechanisms are responsible for activation of key events. For example, induction of apoptosis via the release of cytochrome *c* due to the effects of heavy metals in mitochondria is well known (Araragi et al., 2003) as are BCL_2 inhibition and disturbances of intracellular Ca^{2+} concentrations (Sallas and Buchiel, 1998). Lindane also increases intracellular Ca^{2+} concentration (Duchiron et al., 2002), which may be associated with cell death. This would partially explain the toxicity of this insecticide to fish (Betoulle et al., 2000). Also, chlordane (Ogata et al., 1989; ATSDR, 1994), lindane (Azzalis et al., 1995, EPA) and endrin (Bagchi et al., 2002) apparently induced cell death in livers of fish and mammals. Although the mechanisms are still unclear, apparently PAH can induce apoptosis in fish (Holladay et al., 1998; Mann et al., 1999; Buchiel and Luster, 2001; Weber and Janz, 2001). Lipidosis in hepatocytes of *Sebastes* spp. was associated with PAH residues in bile (Marty et al., 2003). While lipid accumulation may be normal physiological storage, it may also be a mechanism for defence against liposoluble contaminants (Biagianti-Risbourg et al., 1997). Lipidosis in eels from FUM and MOR are the result of cellular effects due to the high incidence of hepatocyte death by apoptosis and associated necrosis. Three of the greatest pesticide concentrations at the Camargue reserve may cause neoplasias: chlordane (NCI, 1977), heptachlor (Akay and Alp, 1981) and lindane (Wolff et al., 1987). Otherwise, some heavy metals and PAH are also considered potentially carcinogenic agents (Bal and Kasparzak, 2002; Shailaja and D'Silva, 2003). PAH induce the formation of mixed function oxygenase (MFO) in the fish liver, with side effects due to the formation of highly carcinogenic intermediates (Shailaja and D'Silva, 2003). Tumours found in livers and spleens of the eel are results of long-term exposure to a combination of potentially carcinogenic pollutants (Roche et al., 2002b). This damage is described in the literature as strong evidence for BaP toxicity, but in this study may also be due to pesticides and heavy metals. Liver neoplasia and PAH exposure are linked (Myers et al., 1998), making this finding relevant for use in environmental risk assessment. In fish the spleen is important for immune system development along with production and replacement of blood cells. Macrophage accumulation in melanomacrophage centres (MMC) is common in fish spleens and may be found in kidneys and livers (Leknes, 2001; Rabitto et al., 2005). Macrophage aggregates in fish livers have been associated with crude oil exposure (Marty et al., 2003) and macrophage activation has been described to cells exposed to lindane (Duchiron et al., 2002). Hence, macrophage accumulation is a potentially useful biomarker.

Glycogen as a biomarker may be ambiguous. POP contamination may lead to glycogen depletion, which is a consequence of failure to feed and a decrease in gluconeogenesis due to modified gluconeogenic enzyme activities (Viluksela et al., 1999). Glycogen and lipid depletion and gut and liver ultrastructural changes occur at low doses of endosulfan in carp (Braunbeck and Appelbaum, 1999). Conversely, chronically exposed organisms may develop physiological tolerance to pollutants, thereby not showing a response to pollutants (Thomas et al., 1999). Indeed, elevated glycogenolysis occurs following acute chemical stress and compensatory process developed during chronic exposure in *Cyprinus carpio* (Oruc and Uumlner, 1998) and other fishes species from Camargue Reserve (Roche et al., 2002b). Here, excess glycogen was concomitant with pathology.

Morphological findings described here are corroborated by field studies of environmental contaminant exposure (Malins et al., 1987). Decreased prevalence of lesions occurs following reduction in chemical contaminant inputs (Moore et al., 1996). Here, lesions found in target organs surprisingly showed that Vaccarès Lagoon presented distinct impact levels of persistent contaminants. Also, we show that the use of bioindicators without prior knowledge of the intrinsic tolerance of the organisms to different classes of contaminants could cause erroneous diagnoses. For example, *A. anguilla* from CAP (smaller individuals) had greater concentration of PAH in bile and higher pesticide concentrations in muscles and livers than fishes from other sites. Otherwise, individuals from MOR, supposedly a non-polluted site, had high pesticide concentrations but low PAH concentrations in the bile. Surprisingly, fishes from FUM, which links the Camargue reserve with the rice-fields, had lower liver and muscle pesticide concentrations and lower PAH concentrations in the bile than fishes from other sites.

6. Conclusion

This study found pathological concentrations of organochlorine pesticides and heavy metals in muscles and livers and PAH in the bile of *A. Anguilla* from the Biosphere Camargue Reserve. The relative ecological risks of these toxicants in these waterways were evaluated. For OC and PAH, it should be noted that their occurrence in the eels from the Camargue Reserve indicates their ubiquitous presence in this environment. Habitat, physiologic factors, lipid content, geographic origin and feeding behaviour are all important aspects that explain pollutant storage and elimination from these animals.

Histopathological lesions are indicative of damage at the tissue and organ level indicating that the contaminants have chronic effects. In addition, the links between the pollutants described here and histopathological lesions are clear, due to the presence of tumours in both livers and spleens.

In aquatic ecosystems, fishes are located at the highest level of the biomagnification process for xenobiotics. PAH, organochlorine pesticides and some heavy metals have been considered to be xenobiotic models for monitoring because of their ubiquity in the environment, their persistence, their bioaccumulative properties and their potential for toxicity. We have showed that the Vaccarès Lagoon, the largest waterbody in the Camargue Nature Reserve, is contaminated by persistent pollutants from multiple sources. These compounds are brought to the site via irrigation channels but also by atmospheric transfer, which explains the low amplitude of inter-site variations. If sustained in the future, this pollution could really endanger the health of the whole wetland ecosystems and affect the populations of piscivorous birds, major component of its protected biodiversity.

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