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Are the toxic sediments deposited at Flix reservoir affecting the Ebro river biota? Purple heron eggs and nestlings as indicators

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Abstract The Flix reservoir, in the low course of the Ebro River, contains thousands of tons of polluted sediments, accumulated from the activities of a chemical factory. An ongoing project is working toward removing these pollutants. Piscivore birds like the purple heron (*Ardea purpurea*) may be useful bioindicators, so eggs and nestling feathers were sampled during the 2006–2008 breeding seasons at three localities: a reference site situated upstream and two potentially affected by the toxic muds; one at the focal area and one at a distal area, the Ebro Delta. The samples were analyzed for isotopic signatures of ^{15}N and ^{13}C and concentrations of heavy metals and selenium. Baseline nitrogen signatures were higher in riverine sites than in the delta. Nitrogen together with carbon signatures adequately discriminated riverine and deltaic ecosystems. Mercury levels are highly influenced by the polluted sediments at Flix and pose potential risks for the birds, as they are among the highest ever recorded in heron species. Selenium and copper concentrations probably derive from other sources. Except for mercury, heavy metals and selenium levels were below toxic levels. Purple heron eggs and nestling feathers have demonstrated their usefulness as bioindicators for pollution in the river biota; feathers in particular show pollutant impacts on a strict local basis. A long series of study years is necessary in dynamic ecosystems such as this, so continued monitoring of the heron population at Flix is advisable to trace the effects of the toxic muds, particularly during their removal, because of the high levels of mercury detected.

Keywords Purple heron · *Ardea purpurea* · Biomonitoring · Trophic ecology · Stable isotopes · Trace elements

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Introduction

Birds have been widely used as bioindicators of environmental pollution, especially when potential hazards may affect human populations or wildlife. Some of these hazards have occurred on the Iberian Peninsula, including the oil spill in 2002 at the Atlantic north-west after the tanker Prestige wrecked (Moreno et al. 2011; Sanpera et al. 2008), the Aznalcollar mine spill into the wetlands of Doñana National Park in 1998 (Baos et al. 2006; Benito et al. 1999; Gomez et al. 2004; Taggart et al. 2006), and the case that is addressed in this study, in the Flix Reservoir, northeastern Spain.

The Ebro River (Catalonia, NE Spain) discharges into the Mediterranean Sea creating a delta of more than 30,000 ha. The Ebro catchment is the largest river basin in Spain; it covers an area of 85,362 km^2 (http://www.chebro.es), potentially supplies 3 million people, and it contains some heavily industrialized areas. One of these areas is the Flix site, where a chemical industry has been in operation since the early 20th century. This long operational period, along with the construction of a dam next to the plant around 1960, resulted in the accumulation of

200,000–360,000 tons of industrial wastes in the riverbed, occupying an area 700 m in length and 60 m wide. The mixture of heavily polluted sediments is composed of high concentrations of heavy metals (cadmium, arsenic, copper, chromium, selenium, lead and especially mercury), organochlorines (hexachlorobenzene (HCB), pentachlorobenzene, DDTs, polychlorobiphenyls (PCBs), polychloronaphthalenes and polychlorostyrenes) and radioactive ^{210}Pb (Bosch et al. 2009; Fernandez et al. 1999). In addition, the Ebro River is currently affected by different agricultural and industrial activities, with significant impact on the existing biota (Mañosa et al. 2001; Navarro et al. 2010). The pollutants originated at the Flix site are carried downstream by the Ebro River to its delta 90 km away (Llorente et al. 1987; Navarro et al. 2009; Pastor et al. 2004), especially during floods (Vericat and Batalla 2006).

As a consequence, a whole-ecosystem study on the environmental impact of such toxic muds was undertaken to evaluate their effects prior to an ongoing project aimed at removing the polluted sediments; this last initiative may imply a high risk of pollutant dispersal.

Other studies in the Flix reservoir have reported high levels of PCBs in sediments (Fernandez et al. 1999) and effects of pollutants on different sentinel species. Earthworms reached high levels of mercury (Ramos et al. 1999). Zebra mussels (*Dreissena polymorpha*) at this site had the highest levels of Hg and methylmercury ever reported, with mean values 20 times greater than the local background levels (Carrasco et al. 2008). Crayfish (*Procambarus clarkii*) and zebra mussels exhibited high toxic stress levels (high activities and levels of antioxidant enzymes, metallothioneins, lipid peroxidation and DNA strand breaks and decreased levels of glutathione) close to the waste dumps (Faria et al. 2010), even crayfish presented levels of mercury exceeding legal values established by European Union legislation (Suarez-Serrano et al. 2010). Mercury concentrations in tissues of carp (*Cyprinus carpio*) sampled downstream from Flix were one to two orders of magnitude higher than those from carp sampled upstream from Flix (Navarro et al. 2009) and catfish (*Silurus ganis*) exceeded the maximum mercury level recommended for human consumption (Carrasco et al. 2011). Deformities, eroded fins, lesion and tumor anomalies and ectoparasites were clearly more frequent at the impacted area for several fish species (carp, roach: *Rutilus rutilus* and pumpkinseed sunfish: *Lepomis gibbosus*); also a significant lower body condition was detected for these species and bleak (*Alburnus alburnus*), while there was a negative impact on reproductive traits for carp and pumpkinseed (Benejam et al. 2010). The responses to the pollutants were species-specific, and carp had the clearest effects on fitness-related traits at the impacted area, despite also being among the most tolerant to pollution.

Piscivorous birds such as herons (family Ardeidae) are suitable bioindicators of environmental pollution in aquatic systems (Champoux et al. 2006; Connell et al. 2003; De Luca-Abbott et al. 2001; Sakellarides et al. 2006). They are in the upper trophic level of these ecosystems, and consequently they biomagnify and bioaccumulate persistent organic pollutants and some metals (Baker and Sepulveda 2009).

In a previous study we showed that purple heron (*Ardea purpurea*) nestlings at Flix showed the highest frequencies of micronuclei in peripheral erythrocytes and reduced blood antioxidant defenses when compared with two other sampling sites, one upstream and another downstream (Quiros et al. 2008). Purple heron eggs showed elevated levels of HCB and PCBs (Barata et al. 2010). Additionally, other bird populations such as terns (family Sternidae) foraging at the Ebro Delta showed the indirect effects of pollutants derived from the Flix reservoir as well as from the intensive agricultural activities taking place in the area (Cotin et al. 2011).

In order to achieve a comprehensive evaluation of the toxic impact at the Ebro River lower course, purple heron eggs and nestlings were used as bioindicators; three sampling sites were chosen according to the breeding colonies of this species in the area of the Ebro River. One of the sites, situated 25 km upstream of the polluted sediments, was designated as reference (l'Aiguabarreig), while the other two, which are potentially affected by the toxic muds, were designated as focal area (Flix) and distal area (the Ebro Delta), situated 90 km downstream. Purple heron colonies were followed and sampled during consecutive years (2006–2008), in order to obtain a comprehensive vision of the highly dynamic ecosystem of the Ebro River, taking into account both trophic ecology and heavy metal exposure.

Heavy metals, when present at high concentrations, are of special concern, as they mainly enter organisms through diet (Burger et al. 1992). However, the exposure of individuals varies according to their trophic habits. Therefore, besides the analysis of contaminants, stable isotopes analysis (SIA) were used to get a better understanding of purple heron trophic ecology (Abdennadher et al. 2011; Nisbet et al. 2002; Ramirez et al. 2011; Sanpera et al. 2007; Tavares et al. 2007). Stable isotope signatures of nitrogen (δ^{15}N) and carbon (δ^{13}C) have been extensively used in studies of bird communities, focusing on their trophic ecology and relationships (Cherel et al. 2008; Cotin et al. 2011; Forero and Hobson 2003; Hobson et al. 1994; Koiadinovic et al. 2008; Moreno et al. 2010). δ^{15}N of tissues reflects the trophic level, with consumer signatures being higher than in their prey (Forero et al. 2005). Information about the source of carbon entering a food web can be obtained from δ^{13}C (Hobson 1999), providing insight about the foraging habitat.

Data obtained from egg samples integrate the adult diet prior to egg laying because herons, as income breeders, obtain most of the materials used in clutch formation from diet (Hobson et al. 2000; Ruiz et al. 1998), while nestling feathers integrate the diet obtained by the adults and consumed by the nestlings during growth. The use of nestlings has two advantages. First, the effect of age-related bioaccumulation is avoided, because the time of exposure has been low and similar for all individuals. Second, because all the chicks are provisioned with prey caught by parents in the surroundings of the colony, the results should reflect only the impact of local pollution.

Here we aim to assess the relevance of the toxic mud in the river contamination processes through a comparative study of purple heron populations breeding at the focal area and two other sites subjected to different contamination pressures. For this purpose, purple heron nestlings and eggs were used as bioindicators and the relationship between trophic level and pollutant concentrations was examined. The first objective was to measure pollutant levels in eggs and nestling feathers in order to establish whether values merit conservational concern, while determining the trophic ecology of these populations, in order to appropriately assess the pollutant exposure. The second objective was to determine which sample type, either eggs or feathers, more accurately reflects such impact or exposure in order to suggest its use as a bioindicator once the toxic muds have been removed. We tested the following main hypothesis: pollutant concentrations and exposure will be higher in herons breeding at Flix and the Ebro Delta than in l'Aiguabarreig after adjusting the pollutant level input by their trophic level. The results may be useful to define conservation policies to be applied in this area, and as both herons and heavy metal pollution are distributed worldwide, results obtained in this study may be used to assess the effects of pollution in other areas of concern.

Materials and methods

Study sites

Purple heron eggs and mantle feathers of nestlings were collected at three selected sites along the Ebro River, NE Spain (see Fig. 1). L'Aiguabarreig site (41°23′N, 00°19′E) is a riverine island called "Illa de los Martinets", located at the confluence of two Ebro tributaries, the Cinca and Segre Rivers. This highly valuable ecological spot and sanctuary for aquatic birds is located upstream from the Flix site (41°14′N, 00°31′E) and therefore unaffected by the Flix factory and its toxic sediments. Despite the industrial activity, Flix dam surroundings have surprisingly become a valuable wetland with a profusion of nesting birds, including herons, storks and marsh harriers. The Ebro Delta (40°42′N, 00°50′E) is one of the largest wetlands in the western Mediterranean region and is home to extensive bird colonies. Occupying an area of 320 km^2, this wetland presents a wide variety of habitats, such as rice fields, farmland, abandoned fields, lagoons, salt marshes and beaches. The coastal lagoons, although connected to the sea and thus expected to hold brackish water, receive considerable freshwater input from the rice fields from spring to autumn, thereby lowering their salinity, which almost reaches that of freshwater during those seasons.

Sampling

Egg sampling was conducted for the breeding seasons of 2006 and 2007 during the laying period, and feather sampling during the breeding seasons of 2006, 2007 and 2008, just 1 week before the estimated peak fledging period. Number of samples was: l'Aiguabarreig (12 eggs collected in 2007; 7 feather samples in 2006, 7 in 2007 and 4 in 2008), Flix (12 eggs collected in 2006, 16 in 2007; 10, 11 and 9 feather samples, respectively) and the Ebro Delta (14 eggs in 2006, 25 in 2007; 4, 16 and 12, feather samples, respectively). Eggs were not collected during 2006 at l'Aiguabarreig due to unusual adverse climatic and river flow conditions which limited the access to the area and nest localization. To avoid pseudo-replication, only one egg or nestling was sampled per nest. Eggs and nestlings were sampled with the permission of the *Serveis de Fauna i Pesca*, *Generalitat de Catalunya* (Spain).

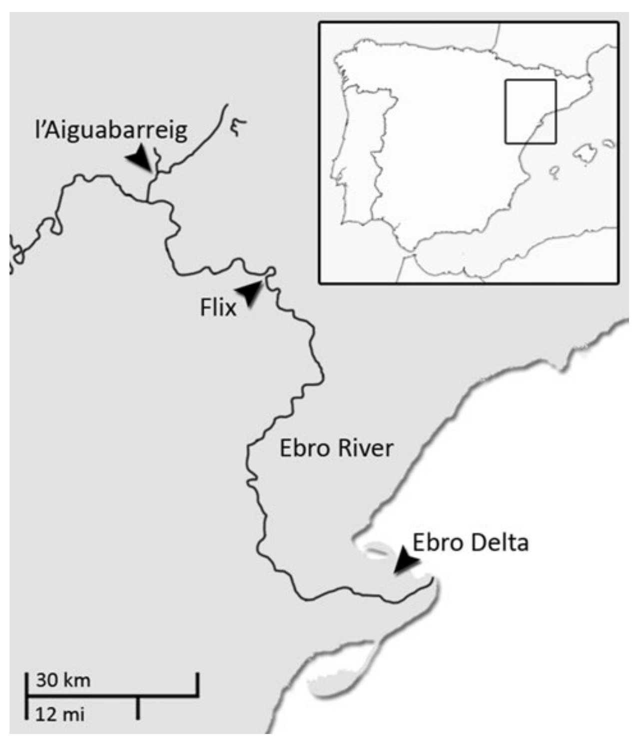

Fig. 1 Map showing the sampling sites

Eggs were labeled and kept refrigerated until reaching the laboratory. Once there, they were kept frozen (−20 °C) until analysis. Egg content was then separated from the egg shell, weighed, and placed into a glass container for freeze-drying. Freeze-dried samples were homogenized and an aliquote was used for trace element determination. A subsample was lipid-extracted for stable isotope analysis using methanol and chloroform, following Folch's method (Folch et al. 1957).

Feather samples were kept in polyethylene bags and frozen until the cleaning process. Once in the laboratory, feathers were cleaned with a 0.1 M NaOH solution and dried for 24 h at 50 °C prior to trace element determination and SIA. Once they were cleaned and dry, in order to homogenize them for SIA, all feathers were ground to an extremely fine powder using an impactor mill (Freezer Mill 6750, Spex CertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature. Additionally, for a better understanding of the trophic ecology of the herons, crayfish and carp from regurgitates obtained during the nestling sampling were also prepared for SIA analysis following the procedure described for egg samples.

Stable isotopes analysis

Sub-samples (ca. 0.36 mg for $\delta^{13}C$ and $\delta^{15}N$) of the homogenized eggs and the ground feathers were placed into tin buckets and crimped for combustion. Isotopic analyses were carried out by EA-IRMS (elemental analysis-isotope ratio mass spectrometry) by means of a Thermo Finnigan Flash 1112 elemental analyzer coupled to a Delta isotope ratio mass spectrometer via a CONFLO III interface.

Stable isotope ratios were expressed in conventional notation as parts per thousand (‰) following the equation: $\delta X = [(\text{R sample}/\text{R standard}) - 1] \times 1{,}000$, where X is ^{15}N or ^{13}C and R is the corresponding $^{15}N/^{14}N$ or $^{13}C/^{12}C$. The standards for ^{15}N and ^{13}C are atmospheric nitrogen and Pee Dee Belemnite, respectively. Precision and accuracy for $\delta^{13}C$ measurements was ≤ 0.1 and ≤ 0.3 ‰ for $\delta^{15}N$. The laboratory applies international standards, which are run for every 12 samples: IAEA CH_7 (87 % of C), IAEA CH_6 (42 % of C) and USGS 24 (100 % of C) for ^{13}C and IAEA N1 and IAEA N2 (with 21 % of N) and IAEA NO_3 (13.8 % of N) for ^{15}N.

Trace elements analysis

Trace metal determination of mercury, selenium, copper, lead, chromium and arsenic (chromium and arsenic only 2007–2008) was carried out by means of ICP-MS Perkin Elmer ELAN 6000.

Before the trace metal determination, homogenized subsamples of freeze-dried eggs and ground feathers (ca. 100 mg) were digested in H_2NO_3 and H_2O_2 in Savilles Teflon digestion vessels for 12 h at 100 °C. Accuracy of analysis was checked by measuring certified reference material (Lobster hepatopancreas Tort-2 and Dogfish liver Dolt-3; National Research Council Canada for eggs and human hair CRM 397, Community Bureau of Reference, Commission of the European Communities in the case of feather samples).

Mean recoveries ranged 96–100 % for total mercury, selenium, copper, lead, chromium and arsenic; and no corrections were done. Values of limit of detection were: 0.1 ng/g for Pb and As, 0.2 for Hg and Cu, 0.5 ng/g for Cr and 1 ng/g for Se. Determinations below such values were set to 'not detected'. All trace elements concentrations were expressed on a dry weight basis (ng/g, i.e., parts per billion).

Trace elements and SIAs were performed at the Serveis Científico-Tècnics (Universitat de Barcelona).

Statistical methods

Values of trace elements concentrations and stable isotope ratios were routinely checked for normal distributions using Kolmogorov–Smirnov and Shapiro–Wilk tests, together with Q–Q plots. Trace elements concentrations showed clear skewed distributions which were normalized by applying a logarithmic transformation. Samples with values under detection limit were assigned with 1/2 of the detection limit value of a trace element when the percentage of detection of that trace element ranged between 50 and 100 %.

When the high number of values below detection limit precludes carrying out comparisons, we looked for differences among localities in the percentage of samples with detected values using Fisher exact test for 2 × 3 tables.

Comparisons among localities and years were carried out using one way analysis of variance and applying the Levene test to check for homoscedasticity. Welch Correction was used accordingly. To test for "a posteriori" pairwise differences we used Tamhane′s or SNK tests. Descriptive statistics and mean differences between groups and their 95 % confidence intervals were used to show the results. Statistical analysis was carried out using PASW Statistics 18.0.

Results

Descriptive statistics (mean, standard deviation, minimum and maximum) for stable isotopes are presented in Table 1. Trace element descriptive statistics (geometric mean and 95 % CI) are presented in Table 2 for feathers and in Table 3 for eggs. One of the eggs sampled during 2007 at

Table 1 Descriptive statistics of stable isotopes signatures in feathers and eggs of purple heron nestlings (*Ardea purpurea*) from the three sites and years

	Site	Year	Sample									
			Feather					Egg				
			N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
$\delta^{15}N$ (%)	Aiguabarreig	2006	7	18.5	0.9	16.7	19.6					
		2007	7	18.9	1.6	15.8	20.5	12	19.0	2.3	14.7	21.5
		2008	4	19.2	1.9	16.4	20.3					
	Flix	2006	10	22.3	0.7	20.8	23.2	12	20.5	1.3	18.1	22.7
		2007	11	21.6	1.2	19.0	22.8	15	19.8	2.1	16.1	22.3
		2008	9	21.6	0.5	20.6	22.2					
	Ebro Delta	2006	4	13.9	1.4	12.3	15.5	14	13.9	1.5	11.8	17.1
		2007	16	13.9	0.7	12.8	15.6	25	13.3	1.0	11.6	15.7
		2008	12	14.4	1.0	13.0	15.7					
$\delta^{13}C$ (%)	Aiguabarreig	2006	7	−25.6	0.9	−27.0	−24.3					
		2007	7	−27.5	0.5	−28.3	−27.0	12	−27.2	1.5	−29.9	−25.2
		2008	4	−27.4	0.9	−28.7	−26.8					
	Flix	2006	10	−25.8	0.8	−27.1	−24.4	12	−27.5	0.9	−28.9	−25.9
		2007	11	−27.0	0.7	−28.1	−25.9	16	−28.3	0.9	−29.2	−25.8
		2008	9	−28.6	0.4	−29.3	−28.1					
	Ebro Delta	2006	4	−24.0	1.8	−26.2	−22.2	14	−24.9	1.0	−26.3	−23.2
		2007	16	−24.1	0.9	−25.0	−22.1	25	−26.3	1.5	−30.4	−23.1
		2008	12	−22.6	3.2	−25.6	−16.9					

Flix site presented a nitrogen value out of range and was excluded from those analyses involving $\delta^{15}N$.

Stable isotopes of prey (crayfish and carp samples) are shown in the scatterplot of $\delta^{15}N$ and $\delta^{13}C$ in Fig. 2.

Feather and eggs samples

With regard to $\delta^{15}N$ no significant interaction between locality and year was detected. Significant differences were found among localities for both feathers ($F_{2,77} = 445$, $p < 0.001$) and eggs ($F_{2,75} = 146$, $p < 0.001$), but not among years. In feather samples, Flix presented the higher values, followed by l'Aiguabarreig, and with the lowest values, the Ebro Delta, while for eggs l'Aiguabarreig and Flix had the highest values (with no significant differences between l'Aiguabarreig and Flix) and the Ebro Delta the lowest (Fig. 3).

A significant interaction between locality and year was found in carbon signatures of feathers (see Fig. 3); although localities always ranked in the same order, magnitude of differences among them varies with year. Significant differences were found among localities in all years ($F_{2,18} = 3.9$, $p = 0.038$; $F_{2,31} = 69.4$, $p < 0.001$; $F_{2,22} = 19.5$, $p < 0.001$, for 2006, 2007 and 2008 respectively). No interaction was found in egg samples, but significant differences were found among localities ($F_{2,75} = 28$, $p < 0.001$) and between years ($F_{1,75} = 14.3$, $p < 0.001$). The Ebro Delta was the locality with the highest values for both eggs and feathers, while l'Aiguabarreig and Flix showed the lowest (with no significant differences between l'Aiguabarreig and Flix). Signatures were higher in 2006 than 2007 for egg samples.

Mercury showed a significant interaction between locality and year in feathers and significant differences were found among localities in all years ($F_{2,18} = 33.7$, $p < 0.001$, $F_{2,31} = 54.1$, $p < 0.001$, $F_{2,22} = 15.5$, $p < 0.001$, for 2006, 2007 and 2008 respectively). Post hoc comparisons showed that Flix was the locality with the highest levels and l'Aiguabarreig and the Ebro Delta did not differed significantly. Egg samples did not show an interaction, but significant differences among localities ($F_{2,75} = 14.8$, $p < 0.001$) and between years ($F_{1,75} = 20$, $p < 0.001$) (Fig. 4). In eggs, Flix again showed the highest values, followed by the Ebro Delta, with the lowest values in l'Aiguabarreig. Levels were higher in 2006 than in 2007.

Selenium levels showed significant differences among localities (Feather: $F_{2,77} = 22.9$, $p < 0.001$; Egg: $F_{2,75} = 10.9$, $p < 0.001$) and in egg samples also between years ($F_{1,75} = 6.3$, $p = 0.014$) (Fig. 4). No interaction among site and year was found in feather or egg samples. For feather samples, Flix was the site with the highest values, followed by l'Aiguabarreig and the Ebro Delta with the

Table 2 Descriptive statistics of trace elements expressed in ng/g in feathers of purple heron nestlings (*Ardea purpurea*) from the three sites and years

	Year	Site											
		l'Aiguabarreig				Flix				Ebro Delta			
		%	Mean	95 % CI		%	Mean	95 % CI		%	Mean	95 % CI	
[Hg]	2006	100	1501.0b	1087.4	2072.0	100	7377.9a	5186.1	10496.1	100	2407.0b	1944.1	2980.1
	2007	100	1548.4b	1160.5	2065.9	100	6614.7a	5081.2	8610.8	100	1395.7b	1106.8	1760.0
	2008	100	1469.1b	1277.2	1690.0	100	3781.4a	2555.1	5596.3	100	1801.3b	1562.8	2076.3
[Se]	2006	100	1811.9B	1542.8	2127.9	100	2609.2A	2311.2	2945.7	75	1289.9C	624.6	2663.7
	2007	86	1654.6	1122.9	2437.9	100	2364.4	2047.9	2729.7	81	1244.3	1007.7	1536.4
	2008	100	2214.6	2065.4	2374.6	100	2238.9	1723.6	2908.3	100	1705.8	1479.1	1967.3
[Cu]	2006*	100	8638.4	7658.0	9744.4	100	8076.5	7373.7	8846.2	100	11282.6	9618.3	13234.9
	2007**	100	5698.3	4585.4	7081.1	100	6070.0	5478.3	6725.6	100	5849.4	5385.7	6353.0
	2008**	100	5921.9	5395.0	6500.2	100	5704.8	4698.3	6927.0	100	7766.2	6033.2	9996.9
[Pb]	2006	100	1349.6	1009.7	1804.0	100	1622.0	1383.3	1901.9	100	2038.2	1476.3	2813.9
	2007	0				9				0			
	2008	25				56	54.8	33.3	90.2	75	76.1	50.1	115.8
[Cr]	2007*	100	1603.8B	1511.4	1701.9	100	1736.5A	1619.9	1861.5	100	1541.2B	1447.0	1641.4
	2008**	100	1961.2	1848.5	2080.8	100	2059.0	1961.4	2161.5	100	2055.8	1956.2	2160.5
[As]	2007	29				27				88	157.3	138.5	178.7
	2008	100	69.8B	48.1	101.3	100	68.0B	52.2	88.8	100	148.0A	117.7	186.1

Asterisks and capital letters show significant differences among years or among localities respectively, in the case of no interaction between both factors. Minuscule are used to show significant differences between localities for a particular year when interaction was detected

Table 3 Descriptive statistics of trace elements expressed in ng/g in eggs of purple heron (*Ardea purpurea*) from the three sites and years

	Year	Site											
		l'Aiguabarreig				Flix				Ebro Delta			
		%	Mean	95 % CI		%	Mean	95 % CI		%	Mean	95 % CI	
[Hg]	2006*					100	1007.9A	703.4	1444.2	100	636.0B	513.2	788.6
	2007**	100	159.5C	100.6	253.0	100	579.8A	366.0	918.3	100	257.6B	195.5	339.5
[Se]	2006*					100	3808.6A	3293.7	4404.1	100	2937.5B	2495.0	3458.5
	2007**	100	3468.0A	3114.0	3862.3	100	3398.8A	3123.6	3698.2	100	2690.7 B	2451.5	2953.4
[Cu]	2006					100	5211.5a	4743.4	5725.7	100	4713.3a	4363.0	5091.8
	2007	100	3721.5c	3261.5	4246.4	100	4022.1b	3686.8	4387.9	100	4342.1a	4087.3	4612.8
[Cr]	2007	100	2529.1	2453.4	2607.1	100	2425.2	2244.5	2620.4	100	2599.3	2527.5	2673.1
[As]	2007	83	127.8B	115.1	141.9	69	130.6B	103.3	112.9	100	167.8A	151.6	185.6

Asterisks and capital letters show significant differences among years or among localities respectively, in the case of no interaction between both factors. Minuscule are used to show significant differences between localities for a particular year when interaction was detected

lowest values. In eggs l'Aiguabarreig and Flix had the highest values (with no significant differences between them), while Ebro Delta the lowest, and levels during 2006 were higher than in 2007.

Concerning copper levels, a significant interaction between locality and year was found in eggs. Significant differences were found among localities ($F_{2,75} = 4$, $p = 0.022$) and between years ($F_{2,75} = 23.3$, $p < 0.001$) in feathers, while in eggs differences were found only in 2007 ($F_{1,24} = 3.3$, $p = 0.082$; $F_{2,50} = 3.6$, $p = 0.034$, for 2006 and 2007 respectively). In feather samples, pairwise tests failed to find significant differences, and levels during 2006 were higher than those detected in 2007 and 2008. For eggs, differences found in 2007 showed the Ebro Delta as the site with the highest levels and l'Aiguabarreig as the one with the lowest, while Flix presented intermediate values.

Chromium levels did not show an interaction between locality and year, but showed significant differences among localities ($F_{2,55} = 3.27$, $p = 0.045$) and years ($F_{1,55} = 87.19$, $p < 0.001$) in feathers. For feather samples, Flix was

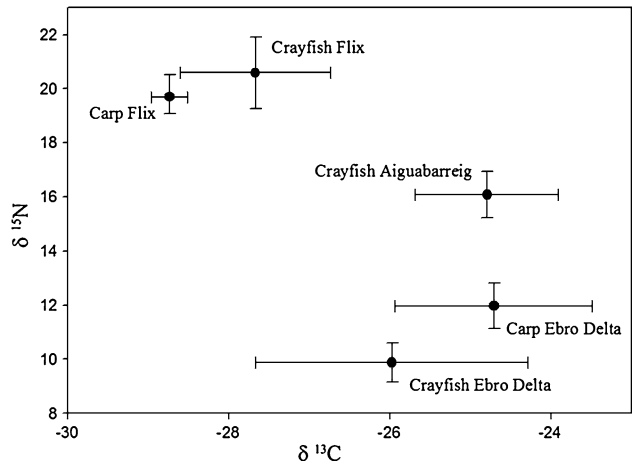

Fig. 2 Plots of δ^{15}N and δ^{13}C signatures indicating the mean position of the crayfish and carp sampled of the three sampled sites (two for carp). *Circles* indicate mean value, *lines* their corresponding 95 % confidence intervals

the site with the highest values, while l'Aiguabarreig and the Ebro Delta presented lower ones (with no significant differences between l'Aiguabarreig and the Ebro Delta). Also, levels were higher during 2008 than during 2007. Significant differences were not found in egg samples ($F_{2,50} = 2.59$, $p = 0.085$).

Arsenic levels showed significant differences among localities (Feather$_{2008}$: $F_{2,22} = 15.9$, $p < 0.001$; Egg$_{2007}$: $F_{2,50} = 11.16$, $p < 0.001$) for both sample types. For both feather and eggs samples, the Ebro Delta was the site with the highest values, l'Aiguabarreig and Flix had lower ones (with no significant differences between them). In 2007 percentage of feather samples above detection limit were compared, resulting in a significant difference among localities (Fisher exact test, $p = 0.0014$), showing in the Ebro Delta higher percentages of detection.

Information on lead levels in feathers is shown in Table 2; due to the highly variable percentage of detected samples, running adequate statistical analysis was not possible, so quantitative differences among sites or years are not given. Nevertheless, we were able to compare percentage of feather samples with quantifiable values among localities, and we found that in 2007 there was not significant differences (Fisher exact test, $p = 0.54$) whereas significant difference arises in 2008 (Fisher exact test, $p = 0.031$) samples from l'Aiguabarreig presenting a lower percentage of detection. Lead was not detected in eggs samples.

Comparison between feather and egg signatures and trace element levels

For such comparisons, only data from 2007 are taken into account, as both feather and eggs samples were collected at all sites only during that year.

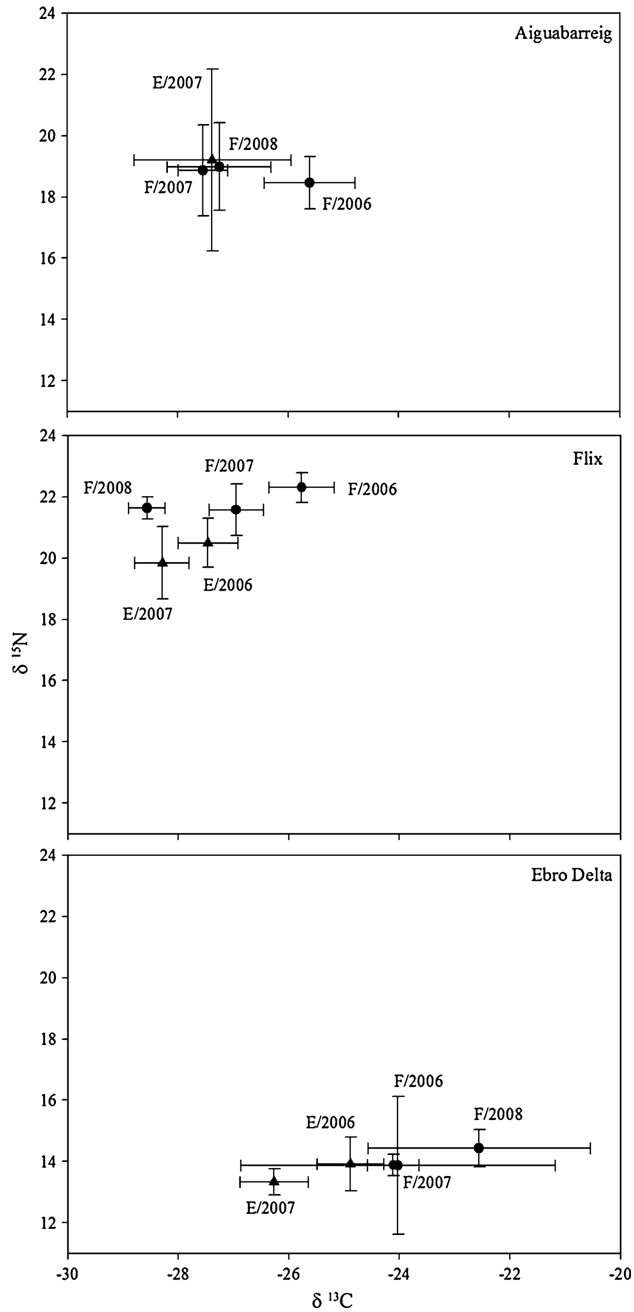

Fig. 3 Plots of δ^{15}N and δ^{13}C signatures indicating the mean position of the purple heron nestlings and eggs of the three sampled sites in each year. *Circles* (*feathers* F) and *triangles* (*eggs* E) indicate mean value, and *lines* their corresponding 95 % confidence intervals

With regard to mercury, selenium and copper levels, no interaction between site and sample type was detected, and significant differences were found among sample types (Hg: $F_{1,83} = 205.3$, $p < 0.001$; Se: $F_{1,83} = 107.9$, $p < 0.001$; Cu: $F_{1,83} = 93.8$, $p < 0.001$). Chromium levels presented interaction between site and sample type, and significant differences were found among sample types in all sites (l'Aiguabarreig: $F_{1,17} = 312.7$ $p < 0.001$; Flix:

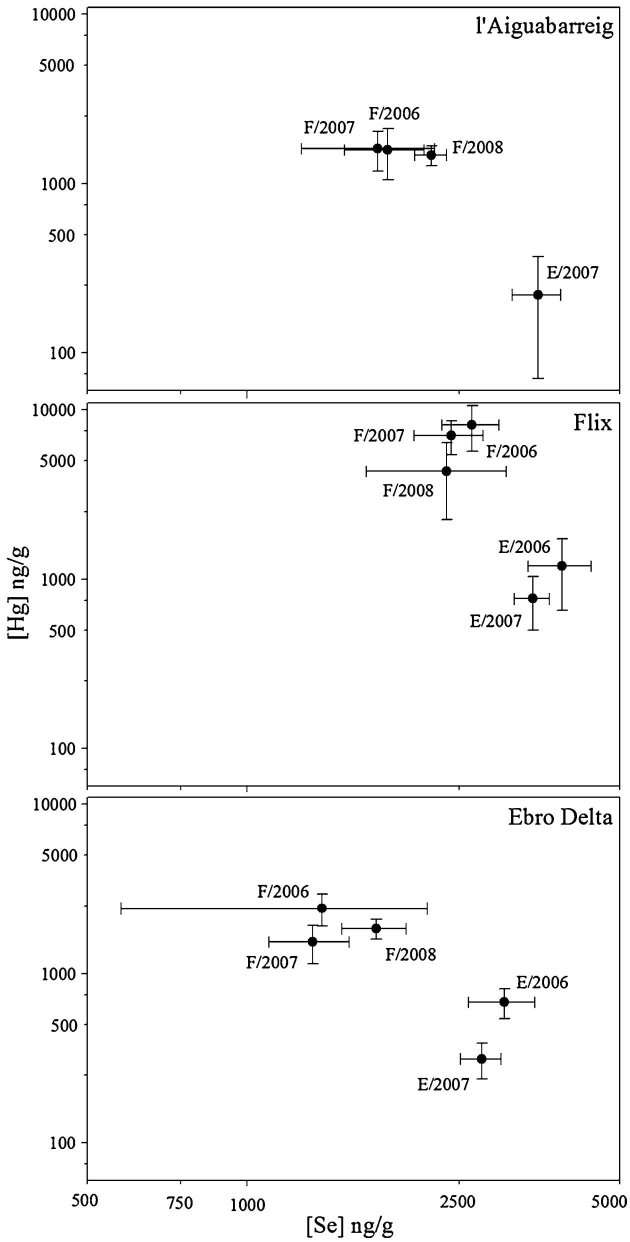

Fig. 4 Plots of mercury and selenium concentrations (given on a logarithmic scale) indicating the mean position of the purple heron nestlings and eggs of the three sampled sites in each year. *Circles* (*feathers* F) and triangles (*eggs* E) indicate mean value, and *lines* their corresponding 95 % confidence intervals

$F_{1,25} = 42.93$, $p < 0.001$; Delta: $F_{1,39} = 324.52$, $p < 0.001$). Metal levels followed the same pattern in all sites, such that mercury and copper levels were higher in feather than in egg samples, while selenium and chromium levels presented the opposite pattern. Arsenic levels were equivalent in both sample types. Arsenic levels were not compared due to the small percentage of detected samples in feather.

Regarding nitrogen signatures, differences were found among sample types ($F_{1,82} = 5.3$, $p = 0.024$). Nitrogen signatures followed the same pattern as mercury and copper, being higher in feather than in egg samples. On the other hand, carbon signatures presented a significant interaction between locality and sample type. Significant differences were found between sample type in Flix and Ebro Delta ($^{13}C_{Flix}$: $F_{1,25} = 18.4$, $p < 0.001$, $\delta^{13}C_{Delta}$: $F_{1,39} = 27.8$, $p < 0.001$) but were not found at l'Aiguabarreig ($\delta^{13}C$ $_{l'Aiguabarreig}$: $F_{1,17} = 0.3$, $p = 0.623$). Signatures were higher in feather samples at Flix and the Ebro Delta.

Discussion

Nitrogen signatures are used to estimate trophic level, although the ratios can be affected by several factors, such as the food-chain length in a given ecosystem or by environmental differences in the baseline (Cabana and Rasmussen 1994). The latter seems to be the case of this study, as the outstanding differences found between the riverine localities (Flix and l'Aiguabarreig) and the Ebro Delta apparently relied on baseline differences, likely caused by the eutrophication of the river. Both riverine sites are located at reservoirs, with marked eutrophication indicated also by the higher $\delta^{15}N$ of the main purple heron prey (see Fig. 2). A similar situation was observed in little egret (*Egretta garzetta*) from Chikly island (Abdennadher et al. 2011), exposed to eutrophication and food-web enrichment in nitrogen rich sewage. On the other hand, purple heron $\delta^{15}N$ signatures from the Ebro Delta resemble those of the freshwater tern species that inhabit this area (Cotin et al. 2011), where the continuous water flow into rice fields and lakes probably prevents eutrophication. The possibility that the $\delta^{15}N$ differences are caused by changes in diet is ruled out as regurgitates from all localities presented the same prey species, mainly crayfish and carp (J. Cotin, personal observation). Therefore, although $\delta^{15}N$ signatures reflect trophic level, the changes observed among the different localities are based on differences in the baseline. Carbon signatures maintain the same pattern between sites each year, although slight differences can be observed between years. The values reflect riverine and deltaic habitats, being around −24 ‰ for the Ebro Delta freshwater habitats (Cotin et al. 2011). Observed changes between years in riverine localities are probably due to the highly dynamic nature of the Ebro River. Annual changes at the Ebro Delta are probably related to a partial shift to more brackish feeding areas, such as the coastal lagoons, as habitats influenced by the sea present a higher isotopic ratio than freshwater habitats (Michener and Schell 1994). This fact is reflected in the higher variability of carbon signatures found in the Ebro Delta. The combined use of nitrogen and carbon signatures helps to discriminate well enough riverine and deltaic ecosystems (high nitrification in the river and lower carbon signatures in the delta).

When comparing egg and nestling feather samples, Burger et al. (2009) found no differences in their usefulness as bioindicators of contamination in herons, although other studies found nestling feathers more representative of regional pollution, as egg samples may be influenced to a different extent by the female body burden accumulated during the wintering or migration period (DesGranges et al. 2009; Hughes et al. 1997). Nevertheless, as the purple heron is considered an income breeder (Hobson et al. 2000; Ruiz et al. 1998) differences between egg and feather samples are probably due to routing. Moreover, feathers reflect the narrower diet offered to the chicks by their parents and, in a small and unknown percentage, the transfer of some of the female's burden into the egg. Accordingly, both samples would be reliable bioindicators, although the lower variability shown by the isotopic data and the higher levels observed in most of the trace elements analysed (except for chromium, but especially selenium, as this element is appreciably transferred to eggs (Focardi et al. 1988; Sell 1977) suggest that feather samples may be a more accurate bioindicator. Also, from a conservational perspective, sampling nestling feathers is more respectful to heron populations, provided that sampling protocols follow certain conditions while entering heron colonies, which are highly sensitive to human disturbance.

Although some differences were found in chromium and arsenic, levels are very similar in all sites. Chromium levels detected at Flix could be slightly influenced by the toxic muds and arsenic probably is higher at the Ebro Delta due to a partial use of foraging habitats influenced by the sea, as it has been seen in other species inhabiting this area (unpublished data). Arsenic and chromium levels are below toxic levels and within those reported for several bird species, including herons (Burger and Gochfeld 2009; Padula et al. 2010), and lead levels are below those causing adverse reproductive effects (around 4,000 ng/g in feathers reported by (Burger and Gochfeld 2000). Although hunting activities using lead shot are high at the Ebro Delta, herons are generally not at risk from this source, as they do not normally ingest lead pellets.

Regarding copper, shifts in concentrations among years could reflect the dynamics of the river. This metal seems to be equally available in all the habitats, and year to year variations could be explained by the use of copper sulphate in agriculture. Levels reported are far below toxic levels (Attia et al. 2011).

Eggs are good bioindicators of selenium (Ohlendorf et al. 2011) and levels are higher at riverine sites than at the Ebro Delta. Therefore, the river is probably affected by other sources of selenium rather than the polluted sediments. Although feathers are poor indicators of this pollutant (Ohlendorf and Heinz 2011), the higher levels found at Flix seems to indicate a slight effect from this site on herons.

Avian embryos are very sensitive to the toxic effects of selenium, which is reflected by reduced hatchability of fertile eggs and teratogenic development of embryos (Janz et al. 2010.). Although the threshold at which negative impacts occur in birds is widely disputed, Ohlendorf and Heinz (2011) recommended levels higher than 12 mg/kg dry weight as a concentration associated with elevated probability for reduced egg hatchability in sensitive and moderately sensitive species and levels lower than 3.0 mg/kg as a mean concentration for background conditions. Our values don't reach that threshold, but are in all cases above the range of those reported in little egret eggs and feathers in Pakistan (Boncompagni et al. 2003), feathers of black-crowned night heron (*Nycticorax nycticorax*) in USA, (Golden et al. 2003) breast feathers from several heron species from Hong Kong and Szechuan (Burger and Gochfeld 1993) and among the highest ever reported for a heron species, although a negative impact cannot be certain, as thresholds levels in feathers are not clear (Ohlendorf and Heinz 2011).

Concerning mercury, Flix presented the highest concentrations among the studied sites, meaning that the amount of mercury leaching from the toxic muds into the Ebro River ecosystem is high and continous at Flix Reservoir, as the concentrations are stable through the years even in a highly dynamic ecosystem as the Ebro River, as also shown in a study conducted with zebra mussel (Carrasco et al. 2008). The only change in this pattern was during 2008, in which some of the individuals from Flix site may have been feeding upstream, away from the main mercury input, as reflected by the lower levels in nestling feathers. Levels detected at l'Aiguabarreig are much higher than the ones expected for a 'reference' site, so this area must also be affected by other sources of mercury. Even the Ebro Delta, situated 90 km from the Flix site, shows intermediate levels that may be explained by the sediments being carried downstream. Purple heron show relatively high concentrations at this site, as other bird species foraging at the same area have already shown (Cotin et al. 2011).

The high mercury levels detected at Flix are of special concern, with purple heron nestlings having values as high as 13,600 ng/g in feathers, which is within the alerting range, as concentrations of mercury between 5,000 and 15,000 ng/g in feathers have been related to adverse effects on growth and reproduction in birds (Eisler 1987). In fact, adverse effects have already been pointed out in previous studies for this breeding population, which in part may be due to the fact that heron embryos have been reported to be highly sensitive to mercury (Heinz et al. 2009). The elevated levels of micronuclei in peripheral erythrocytes (Quiros et al. 2008) and the reduced blood antioxidant defenses (Barata et al. 2010) are some of the physiological stress responses that these birds have developed to pollution. Also, other species in this ecosystem, including zebra mussels, crayfish and several fish species, have shown effects from this pollutant (Benejam et al. 2010; Faria et al. 2010; Navarro et al. 2009; Suarez-Serrano et al. 2010).

Finally, it should be noted that mercury levels found in nestling feathers at Flix are far higher than the ones found for little egret in Pakistan (Boncompagni et al. 2003) and Hong Kong (Connell et al. 2002) or several heron species from China (Burger and Gochfeld 1993) and even higher than those reported for little egret and night heron feathers in the Axios Delta, Greece (Goutner and Furness 1997) and northern Italy (Fasola et al. 1998). Higher levels have been found in heron species in the Everglades, USA (Frederick et al. 2004), but feathers were taken from adult birds conserved in museums, which probably reflect bioaccumulation. High values of mercury in blood were found for some years in the Carson River (Nevada) for two heron species, snowy egret (*Egretta thula*) and black-crowned night heron (Henny et al. 2007), but to our knowledge, mercury levels detected in purple heron at the Flix site are the highest ever reported in nestlings feathers of a heron species.

Conclusions

- Nitrogen signatures reflect trophic level, but differences among localities are greatly influenced by baseline values, being higher in the riverine sites than in the delta. Nitrogen together with carbon signatures adequately distinguish riverine and deltaic ecosystems (high nitrification in the river and lower carbon signatures in the delta).
- Eggs and nestling feathers of purple herons have demonstrated their usefulness as bioindicators for trace element pollution in the river biota. Nestling feathers, which are grown from dietary items provided by parents in the surroundings of the colony, show pollutant impacts on a local basis. Moreover, since chicks belong to a homogeneous age-class, the effects of age-related bioaccumulation on pollutant levels are expected to be negligible.
- Eggs and nestling feathers of purple herons have demonstrated their usefulness as bioindicators for trace element pollution in the river biota. Nestling feathers, which are grown from dietary items provided by parents in the surroundings of the colony, show pollutant impacts on a local basis. Moreover, since chicks belong to a homogeneous age-class, the effects of age-related bioaccumulation on pollutant levels are expected to be negligible.
- Mercury is highly influenced by the polluted sediments at Flix reservoir, while selenium and especially copper concentrations probably derive from other sources.
- Although most of the trace element content of purple heron samples are below alerting values, the high mercury levels from the Flix site pose potential risks for these birds and are among the highest ever reported.
- From our study it is apparent that in highly dynamic ecosystems as the Ebro River and its Delta, a series of study years is necessary to properly understand pollutant spatio-temporal changes, so a long-term monitoring programme of the purple heron population at Flix is recommended to advise the effect of the toxic muds, particularly during and after their removal which could result in a increased bioavailability of mercury for river biota.

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