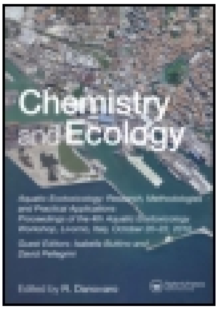

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Flamingo feathers to monitor metal contamination of coastal wetlands: methods and initial results concerning the presence of mercury at six Mediterranean sites

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Flamingo feathers to monitor metal contamination of coastal wetlands: methods and initial results concerning the presence of mercury at six Mediterranean sites

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The feathers of greater flamingo (*Phoenicopterus roseus*) fledglings collected from six colonies (Odiel, Delta Ebre, Camargue, Cagliari, Comacchio and Venezia) of the western Mediterranean Sea were used as biomonitors of metal contamination of coastal wetlands, with a particular focus on mercury. We used single and pooled samples, attempting to minimise the critical factors typical of studies on metals in feathers by: sampling birds of the same age, during the same breeding season; sampling the same type of feather; treating the feathers in the same way and at the same time; and analysing the same digested solution as a single batch and in two different laboratories applying different techniques. Flamingos from Cagliari, a site known for its mercury contamination, had maxima and median values greater than any of those from the other sites in terms of both single and composite samples. There were also high concentrations at the other sites, which in the absence of literature evidence of Hg pollution, suggests either the existence of pollution or that adults are foraging in contaminated wetlands. The flamingo population of Odiel had the lowest median and dispersion of Hg, and is possibly a baseline for the western Mediterranean greater flamingo population.

Keywords: biomonitoring; feathers; mercury; western Mediterranean; flamingos; *Phoenicopterus roseus*

1. Introduction

Mercury (Hg) is a highly toxic element with no known beneficial biological function. It has mutagen and teratogen effects, meaning that its use and release should be reduced, because the difference between tolerable background levels and harmful effects in the environment is exceptionally small [1,2]. For this reason, it is advisable to constantly monitor environmental levels of this pollutant.

Biomonitoring is a widely used method to assess environmental pollution and its impact on biota. Birds are useful biomonitors because they are in a high position in the food chain, are sensitive to environmental changes and are easy to monitor [3,4]. In particular, colonial waterbirds in the

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breeding season tend to forage within a limited range around their colony, while the fledglings usually stay close to the nesting sites for a lengthy period after birth. These facts permit comparisons of responses at sites in natural experiments [5].

In birds, Hg has known toxic effects on embryos and the nervous system, particularly when present in a methylated form (MetHg). It also tends to accumulate in internal tissues [6]. High doses of Hg cause altered behaviour, weight loss, appetite suppression and ataxia, ultimately leading to death. Low dietary intake does not affect adult survival, but reproductive success can be impaired [7]. Birds do, however, seem to avoid some toxic effects by sequestering in the keratin of growing feathers [8,9] large amounts of Hg accumulated since the previous moult (adults) or inherited from the egg (chicks). Accordingly, plumage is a route of excretion instead of a target organ [10,11]. The use of feathers as biomonitors of metal contamination has been recommended by many researchers [3,12,13]. In addition, feathers are relatively easy to collect and the sampling is non-invasive [3,14]. There are two ways of metal loading in to feathers: (1) during feather formation, Hg present in the blood and that mobilised from various tissues (e.g. liver or kidneys) is sequestered in the growing feathers and bound to sulfhydryl groups of keratin [3,12]; and (2) external contamination, such as the deposition of metals on the surface, occurs after feather formation [4,15,16]. Some authors have tried to evaluate the importance of external contamination with respect to the amount of metal sequestered in keratin [4,8,12,17], with indications being that Hg concentrations therein are not at all or are only slightly affected in this way [12,18]. Other factors which might affect the usability of feathers as biomonitors include moulting strategy, the type and position of the feathers [8,18,19] and their colour [20,21]. Many studies have observed differences in Hg concentrations in the feathers of adults and young birds, although for most bird species the mechanisms underlying the Hg levels over time, such as the rate of accumulation and elimination by moulting, continue to be unclear due to the difficulty in determining the age of the adults [3].

To date, flamingos have rarely been included in studies of colonial waterbirds, although they could potentially be good bioindicators of pollution in brackish marshlands, salinas and lagoons, due to their feeding behaviour, their use of sediments and the way the chicks are fed. Like other colonial waterbirds, the greater flamingo (*Phoenicopterus roseus*) tends to feed mainly within a limited range around its colony, although the foraging flights of the adults have only been studied for a few sites [22]. This species feeds by filtering mud, primarily for a wide range of aquatic invertebrates and their eggs and larvae. It occasionally also feeds on the seeds of aquatic plants [22], and seems able to survive when living prey is scarce, even if it only swallows mud that is rich in organic matter. Indeed, even in such conditions, these birds are able to bring up their young successfully. For this species, therefore, and unlike most other birds, mud may be an essential component of its diet [23], although this directly exposes it to the toxic effects caused by contaminated sediment. Until fledgling, flamingo chicks are fed by their parents with only a holocrine secretion [22], but they start to filter mud [24] at about 7 weeks after birth. As a consequence, concentrations of trace elements in the tissues of flamingos may better reflect exposure thereto in feeding areas than is the case with other species, which only absorb trace elements from their prey.

Only three studies have involved the evaluation of the metal content in the feathers of the greater flamingo, and none of them compared different colonies. Moreover, just one of these studies sampled fledglings, but it did not assess Hg [25]. Cosson et al. measured six trace elements, including Hg, in the feathers of a dozen adult flamingos which were found dead in frozen salt ponds in Camargue [26,27]. More recently, seven adult flamingo corpses found in various Italian wetlands were analysed for Pb, Cd and Hg [28]. Meanwhile, using a sample set obtained from collected and pooled feathers lost by adults near to their breeding location [29], Burger and Gochfeld measured the concentrations of eight metals, including Hg, in the feathers of lesser flamingos (*Phoenicopterus minor*), a closely related species to the greater flamingo, but with slightly different feeding behaviour.

To our knowledge, only one study with the objective of evaluating the effects of local pollution has assessed Hg in the feathers of waterbird chicks living in the Mediterranean area. The squacco heron, *Ardeola ralloides*, an Ardeid, was investigated in the Axios Delta (Greece), with the researchers reporting a wide range of Hg concentrations, with the lowest being 0.36 and the highest 6.54 $\mu g \cdot g^{-1}$ d.w. [30]. On the basis of these results, the pollution by Hg in the Axios Delta was considered by the authors of the study to be high.

Our research evaluated the Hg content in feathers collected from living greater flamingo fledglings at six breeding colonies along the north-western Mediterranean (Figure 1). The entire dataset we obtained is representative of most of the meta-population, relating to 75% of the breeding colonies in the area in 2008. Accordingly, for the first time, a study of Hg in flamingo feathers is based on data collected from more than one breeding site. Moreover, it also assesses the presence of Hg in a greater Flamingo population of a homogeneous age. With our approach, we attempted to minimise the critical factors that are typical of the studies of metals in feathers by: sampling birds of the same age; conducting the study in the same breeding season; sampling the same type of feather from all of the birds; treating the feathers in the same way and at the same time; and analysing the same digested solution as a single batch and in two different laboratories, applying different techniques, as an additional control.

We used both single and pooled samples in order to include all of the sampled sites in this study, with the latter methodological strategy being useful when either resources or samples are limited.

Accordingly, in this article, we discuss the eco-toxicological implications of our Hg results, while also taking into consideration the environmental characteristics of the sites and reporting some of our outcomes about methodological aspects. The implications of using different kinds of samples (single or pooled) are also highlighted.

2. Materials and methods

2.1. *Sampling sites*

In 2008, eight flamingo colonies in the Mediterranean region raised chicks, as reported by the Flamingo Specialist Group [31]. The sampling was performed at the six more western colonies,

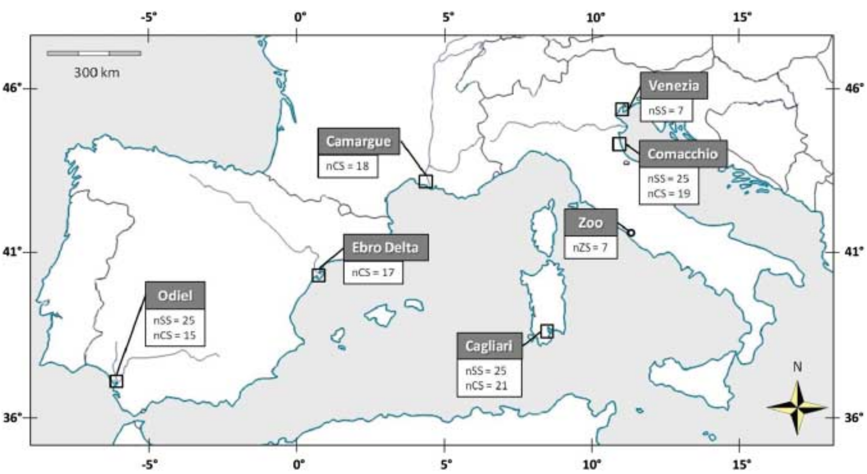

Figure 1. Wild colonies (squares) and captive flock (circle) sampled in July–August 2008. Sampling of wild birds (fledglings) in relation to the 75% of breeding colonies in the area. For Odiel, Cagliari and Comacchio, both single and pooled samples were prepared (nSS, number of single samples; nCS, number of composite samples); for the Ebro Delta, Camargue and the zoo (nZS, number of zoo samples), only CSs were prepared, whereas only single samples were produced for Venezia. This is our entire dataset.

presented herein in the order of west to east (Figure 1). Moreover, some feathers obtained from flamingos in captivity were provided by Bioparco Zoo (for brevity, hereafter referred to as the zoo) (41°55′02″N, 12°29′11″E), which is situated in Rome (Italy). The six colonies were:

(1) Marismas del Odiel (Odiel; 37°14′58″N, 6°59′34″W): estuarine complex at the confluence of the Odiel and Tinto rivers in south-western Spain. It is comprised of intertidal mudflats and saltpans, and is designated as a Biosphere Reserve (1983) and a Ramsar site (1989). After the building of an artificial island in 1989, there was sporadic flamingo breeding success (the most recent being in 1995). In 2008, flamingos returned in large numbers to the artificial island [32], probably as a consequence of there being no breeding opportunity at the Fuente de Piedra Lake (the main Spanish breeding site).
(2) Delta de l'Ebre (Ebro Delta; 40°35′11″N, 0°40′52″E): located on the north-western Mediterranean coast of Spain. Rice fields characterise >60% of the landscape, with natural wetlands covering the remaining area. Flamingos have bred regularly in the Punta de la Banya saltpans since 1993, foraging in coastal lagoons, bays, salt marshes, rice fields and saltworks. Punta de la Banya is a sandy spit connected to the delta plain by a narrow isthmus [33].
(3) Camargue (43°25′56 N, 4°37′37 E): part of the delta of the Rhone River (1450 km^2) and a Biosphere Reserve since 1977 and a Ramsar site since 1986. Flamingos have been breeding here since at least the mid-sixteenth century. After the construction of an artificial island in the late 1960s, breeding became regular at the location from 1976 onwards. It was only in 2007, when no water was pumped into the Fangassier pan, that this bird did not breed in Camargue [34].
(4) Stagno di Cagliari (Cagliari; 39°12′59″N, 9°00′44″E): located in southern Sardinia, this is a complex lagoon system which comprises Macchiareddu industrial saltpans and the Santa Gilla Lagoon. It has been a Ramsar site since 1971. The lagoon is connected to the sea by a harbour and has two major freshwater inflows from the Fluminimannu and Cixerri rivers. Regular breeding by flamingos began at this site in 1993, on the opposite side of the town of Cagliari, on the Stagno di Molentargius. Since 1999, the breeding colony has gradually moved to the Macchiareddu saltpans. Settlement at this new location was followed by a considerable increase in the number of breeding pairs with high rates of productivity [35].
(5) Valli di Comacchio (Comacchio; 44°38′50″N, 12°12′45″E): one of Italy's largest coastal wetlands, the formation of which is related to the late Holocene evolution of the Po River delta area. It has been a Ramsar site since 1981. Once a freshwater wetland, it became brackish in the sixteenth century after connection to the Adriatic Sea. The lagoon now receives freshwater from the Reno River and drainage water from agricultural land. The Comacchio saltpans (industrially active until 1984) are located at the north-western side of the lagoon and have hosted the flamingo breeding colony since 2000. By 2008, the colony had grown to over 1000 pairs [31].
(6) Laguna di Venezia (Venezia; 45°33′53″N, 12°32′01″E): the largest Italian wetland (550 km^2). Flamingos had been attempting to breed in a dammed section (Valle Dogà) of the Northern lagoon since 2007; breeding success was only achieved in 2008 when 22 chicks fledged [36]. Valle Dogà is a private brackish lagoon managed for fish farming and wild-fowling.

2.2. *Sampling procedure*

All of the feathers from the flamingo fledglings were collected between July and August 2008, during the ringing operations coordinated by the Flamingo Specialist Group (FSG). All of the sampled birds were between 5 and 8 weeks old. The feathers were obtained by cutting the distal part of a small number of them from random individuals using stainless steel scissors. We selected the longest scapulars, which were protected from aerial deposition, because they were covered by several others. In addition, some feathers were sampled from captive adult greater flamingos, but the age of these birds was unknown. The sampling was performed either by the authors (in Italy) or by appropriate volunteers (in the other colonies and at the zoo).

The feathers of each specimen were stored separately in plastic bags and kept at room temperature. No freezing is needed to preserve metal concentrations in feathers [3,37].

2.3. *Preparation and analysis of samples*

2.3.1. *Washing and drying*

Following the most commonly used procedures [3,4,18], the feathers were first individually rinsed with distilled water and vigorously washed in deionized water (milli-Q). They were then placed in an acetone solution (1 mol·L^{-1}) and then again in deionized water to remove loosely adherent external contamination [4]. Each feather was gently rubbed to ruffle the barbs during every washing step. The washed feathers were placed into an open case, which was coated with greaseproof paper and divided into compartments to avoid there being any contact between the different specimens and between the container and the samples. They were then dried for 24 h at 40 °C.

Each dried feather was finely ground using stainless steel scissors and gloves, and the dry weight was determined using an electronic 'Sartorius' BP310S balance to the nearest 0.001 g.

2.3.2. *Grouping*

Three distinct sample sets were prepared (Figure 1):

(1) 'Single' samples (SS) from four colonies (Cagliari, Comacchio, Odiel and Venezia), each consisting of a mix of three to four feathers from a single bird and homogenised to minimise the dependence on a single feather [38] or, ultimately, a particular segment thereof.
(2) 'Composite' samples (CS) from five colonies (Cagliari, Camargue, Comacchio, Ebro Delta and Odiel) containing an equal weight of two to three mixed feathers taken from two or three individuals. The composite samples did not include cognate birds, as flamingos lay only one egg [22]. The pooling of samples enabled us to also obtain data from Camargue and the Ebro Delta, since the number of available feathers from these colonies was insufficient to prepare comparable SSs. Conversely, it was impossible to prepare CSs from Venezia due to the low number of specimens;
(3) 'Composite' samples from the zoo (ZS), consisting of seven samples from the feathers of 12 adults living in captivity. The low number of specimens did not permit us to prepare a dataset of independent CSs, while there were not enough feathers to enable them to be used as a SS. When also considering the other uncontrolled variability factors (age of birds, age and type of feathers), we opted for many duplicates, which were prepared by digesting different aliquots of the same pooled sample in order to obtain a very accurate mean value for the flamingos living in unnatural conditions.

Each sample (SS, CS and ZS) weighed between 250 and 400 mg.

2.3.3. *Digestion*

The samples were digested in a 4:1 mixture of 65% HNO_3 and 30% H_2O_2 in a 30-min microwave digestion procedure at between 120 and 180 °C. The clear, orange–yellow or green resulting fluid was diluted by adding deionized water to a final volume of 30 mL.

The samples were randomised before digestion so that each digestion batch included samples from each site and of each type. The randomisation of samples is strongly recommended to reduce the possibility that any time-dependent errors in the laboratory, such as a slow drift from lower to higher reporting levels, might create their own misleading patterns [38]. Three different Certified Reference Materials (CRMs) were included in the digestion batches to enable us to check the accuracy and precision of the laboratories. In particular, these were: Dorm-2 tissue (*Squalus acanthias*) from the National Research Council, Canada; and mussel tissue ERM-CE278 (*Mytilus edulis*) and plankton BCR-414 from the Institute for Reference Materials and Measurements, Geel, Belgium. Several samples from Cagliari and Comacchio were run in duplicate. Duplicates were prepared by digesting separately two different aliquots of the same sample of feathers in order to check for sample homogeneity, rather than to control analytical precision (measured by duplicates of CRMs). Each batch included blanks to check the purity of the chemicals used and for possible contamination.

2.3.4. *Chemical analysis*

Analyses were performed in two different laboratories on aliquots of the same solution. The samples were analysed by both the cold vapour flow injection mercury system (CV-FIMS; Perkin–Elmer 400) at Siena University and atomic fluorescence spectrometry (Instrument PSA 10.035 Millennium Merlin 1631) at the BGR laboratory in Berlin.

2.4. *Data treatment*

When it comes to reporting the Hg results, we set out the following range of values as expressed by the minimum (Min) and maximum (Max), the number of samples (n) and the coefficient of variation (CV), which is the ratio between the standard deviation (SD) and mean in percentages: median and median absolute deviation (MAD); the mean value; the SD; and the standard error (SE). We report the mean and the CV to enable some comparisons to be made with the literature, but we believe that the former is not particularly suitable for describing eco-toxicological data distributions, whereas median values appear to be more appropriate for demonstrating a central tendency [39]. The MAD is defined as the median of the absolute deviations from the median of a univariate dataset. This was preferred to the SD for describing the dispersion of data because it is thought to be a more robust measure of scale, and appears to be more resistant to outliers [40].

The statistical calculations were carried out using the SPSS software v. 14.0®. The analyses performed by the DAS+R module (http://www.statistik.tuwien.ac.at/StatDA/DASplusR/) for the R statistical software (http://www.r-project.org) were also used for an exploratory univariate analysis [39]. The variable in each group was subjected to the Shapiro–Wilk test for normality and to the non-parametric Kruskal–Wallis [41] test by rank to test the equality of population medians among groups, as defined by the sampling site and sample type [42]. The significance level is expressed as $p < 0.05$.

3. Results

3.1. *CRMs and comparisons between the two laboratories and the duplicates*

The Hg results for the CRM (Table 1) correspond with the certified values, albeit with some exceptions depending on the laboratory. Precision (estimated by RE in Table 1) was better overall for the Berlin laboratory, while accuracy was better for the facility in Siena.

Table 1. Certified Reference Materials (CRM) analysed in the two laboratories ($\mu g \cdot g^{-1}$) correspond with the certified values (Cert.v.).

		Hg Siena			Hg Berlin				
	nD	Mean	SE	RE	Mean	SE	RE	Cert. v.	unc.
BCR-14	3	0.27	0.043	16	0.2	0.007	3	0.275	0.016
ERM-E278	4	0.239	0.023	10	0.18	0.009	5	0.196	0.009
Dorm-2	3	4.117	0.116	3	3.938	0.114	3	4.64	0.26

Note: Uncertainties (unc.) are also included. The precision is better for the Berlin laboratory, whereas accuracy is better for Siena (RE, relative error in percentage). The number of duplicates for each CRM is indicated as nD.

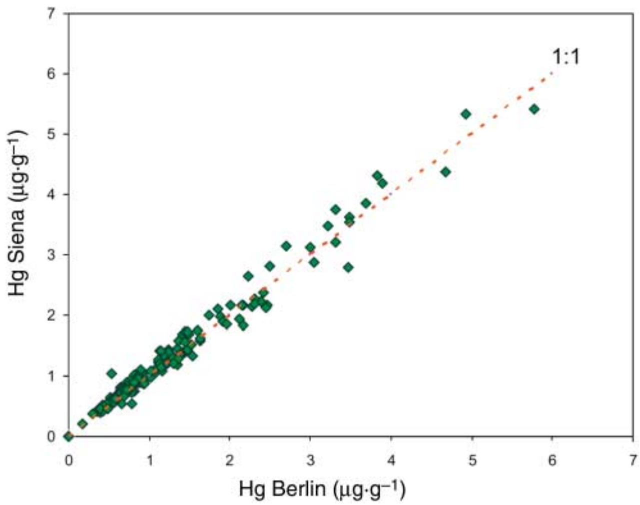

Figure 2. Relationship between the Hg results from the Berlin and Siena laboratories. The good correspondence is testified by the limited dispersion around the 1:1 line, with only a few samples being scattered.

In any event, the results obtained from using the two analytical techniques correspond, as testified by the high Pearson's correlation ($r = 0.99, p < 0.01$) and the limited dispersion around the 1:1 line in Figure 2, with only a few scattered samples. Both analytical methods can thus be proposed and utilised for the analysis of total mercury presence in feathers, given that the quality of the analyses seems to be comparable and of good value. For the sake of simplicity, we have chosen to only report the Berlin data, given its slightly better precision, since this study is mainly focused on comparing concentrations in different greater flamingo populations.

A paired-samples t-test confirmed that the correspondence between the duplicate sets analysed in the Berlin laboratory is high ($t(29) = -0.46, p = 0.65$).

3.2. *Mercury in feathers from different colonies*

Our results, subdivided according to colony and sample type, are set out in Table 2. The flamingos born in Cagliari had the highest median and the widest dispersion for both the SSs and the CSs (Figure 3), as also confirmed by the MAD values. Among the SSs, 36% of the Cagliari birds had higher Hg concentration levels in their feathers than the highest values found in the Comacchio, Odiel and Venezia groups. In direct comparisons, no statistically significant differences were found between Comacchio, Odiel and Venezia (Table 3). Some birds from Comacchio did reveal high values, but the median was lower than in Cagliari ($p < 0.01$) and similar to Odiel ($p > 0.77$). The Odiel values were significantly lower than those from Cagliari ($p < 0.01$). In addition, the feathers from the birds from Cagliari and Venezia were not statistically different ($p > 0.15$).

Table 2. Hg results ($\mu g \cdot g^{-1}$ d.w.) for all samples from the Berlin laboratory.

Site	S.T.	Median	MAD	Mean	SD	SE	Min	Max	n	CV
Cagliari	SS	1.443	1.015	2.092	1.787	0.357	0.306	6.528	25	86
Cagliari	CS	1.478	0.341	1.649	0.818	0.179	0.452	3.485	21	50
Camargue	CS	0.983	0.442	1.167	0.665	0.157	0.446	2.389	18	57
Comacchio	SS	0.658	0.195	0.782	0.515	0.103	0.176	2.462	25	66
Comacchio	CS	1.053	0.372	1.129	0.644	0.148	0.361	2.906	19	57
Ebro	CS	1.246	0.413	1.445	0.643	0.156	0.674	2.702	17	45
Odiel	SS	0.606	0.111	0.706	0.304	0.061	0.379	1.601	25	43
Odiel	CS	0.782	0.082	0.785	0.163	0.042	0.521	1.207	15	21
Venezia	SS	0.940	0.233	0.968	0.324	0.122	0.485	1.373	7	34
Zoo	ZS	–	–	0.734	0.005	0.017	0.679	0.818	7	6

Note: ST, type of sample, CS, composite sample; SS, single sample; ZS, zoo sample. Reported for each site are median and median absolute deviation (MAD), mean values, standard deviation (SD) and standard error (SE); range of values expressed by minimum (Min) and maximum (Max), number of samples (*n*) and coefficient of variation (CV).

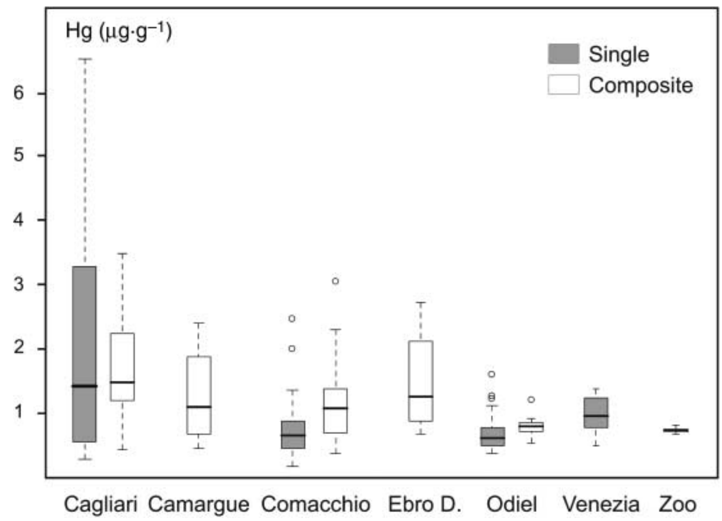

Figure 3. Hg concentrations in flamingo feathers from the different colonies ($\mu g \cdot g^{-1}$ d.w.). The skewed right box-and-whisker plots of Cagliari (SS and CS), Camargue, Comacchio (CS) and the Ebro Delta indicate that some adults breeding in most of colonies feed in polluted wetlands. Hg could be a serious threat to the Cagliari birds, due to its highest median and very dispersed distribution, while Odiel, as well as the zoo birds, could serve as a baseline for Hg in the flamingos growing-up in the Mediterranean area.

Table 3. Pairwise comparison of medians between sites by using the Kruska—Wallis test performed on the single sample ($p < 0.05$).

	Cagliari	Comacchio	Odiel	Venezia
Cagliari	–			
Comacchio	<0.01	–		
Odiel	<0.01	NS	–	
Venezia	NS	NS	NS	–

Note: Comacchio and Odiel had similar results, and were less contaminated than Cagliari. Venezia could not be differentiated from the other sites. NS, not significant.

Table 4. Pairwise comparison between sites by using the Kruskal–Wallis test performed on the composite samples ($p < 0.05$).

	Cagliari	Camargue	Comacchio	Ebro	Odiel
Cagliari	–				
Camargue	NS	–			
Comacchio	<0.05	NS	–		
Ebro	NS	*0.06*	*0.06*	–	
Odiel	<0.001	NS	NS	<0.001	–

Note: As in the single samples, the Cagliari Hg is higher than that of Odiel and Comacchio, but is not different from that of Camargue and the Ebro Delta. There is some concern about the Ebro Delta, which is more similar to Cagliari than Camargue or Comacchio (nearly significant limits are reported in italics), and higher than Odiel. NS < not significant.

Table 5. Kruskal–Wallis test performed on the composite sample (CS) and single sample (SS) medians ($\mu g \cdot g^{-1}$) from the Berlin laboratory ($p < 0.05$).

	CS	SS	p-value
Cagliari			
Hg median	1.478	1.443	0.90
Comacchio			
Hg median	1.053	0.658	0.02
Odiel			
Hg median	0.782	0.606	0.04

Note The differences resulted in less dispersed distributions (Comacchio and Odiel), with the CSs being slightly higher than the SSs.

When it comes to the CSs (Table 4), the feathers from Odiel revealed concentrations of Hg which were lower than in those obtained from the birds at Cagliari ($p < 0.001$) and Ebro ($p < 0.001$), but there were no differences between Odiel and Comacchio and Odiel and Camargue. In turn, the levels of Hg in the Comacchio results were lower than those from Cagliari ($p < 0.05$), but the difference between Comacchio and Ebro was on the edge of significance ($p = 0.06$), as was also the case for the difference between Camargue and Ebro. The MAD was relatively high in the Camargue dataset, while the variability in the values from Odiel was also the lowest for the CSs.

The ZSs had a mean value of 0.734 $\mu g \cdot g^{-1}$, while the means from the wild colonies (CSs) were close to or higher than 1 $\mu g \cdot g^{-1}$, except in the case of Odiel, where the values were 0.785 ± 0.042 (mean $\pm$ SE, $\mu g \cdot g^{-1}$).

3.3. *Single versus composite samples*

A comparison between the CSs and the SSs can be seen in Table 5. Significant differences were found in the medians of the Comacchio and Odiel CSs and SSs, with the median values of the former being higher than those of the latter. The CS and SS results in Cagliari were not, however, statistically different.

4. Discussion

4.1. *Comparison between the CSs and the SSs*

In theory, the CSs should represent the Hg contamination levels of each population as effectively as the SSs do, although some variations arising from the use of the former may be masked. Indeed, when the population under analysis is too large, and the handling of the samples becomes complex or expensive, CSs may provide a more economical alternative to the traditional sampling methods [43]. When interpreting CSs, empirical comparisons can be made using deviations between the central values thereof and a known reference range for healthy individuals [43]. However, SSs permit the proper description of the real extent of the true profile of the sample set, although the central values may be affected by a few extreme measures. As expected, we appreciated that there would be a substantial contraction of the range in the Cagliari and Odiel CSs, but this difference in variability was not seen in Comacchio. However, all three of the CS datasets had a higher minimum than those from the SSs. The CSs and the SSs did not always produce the same median values. There was statistical equality of the medians when the SS data was highly dispersed (Cagliari), but not so when the datasets were somewhat compact (Comacchio and Odiel), producing a CS median that was slightly higher than expected. This result confirmed the findings in other studies, in which slight differences between CS and SS midpoints were ascribed to a 'statistical artefact' due to the larger number of individuals in the pooled samples and the smaller error term of the CSs [43]. However, Kruskal–Wallis tests between colonies revealed the same significant differences in the CSs and SSs. Accordingly, for this purpose, both kinds of samples seem to be effective.

4.2. *The role of the greater flamingo as a biomonitor for mercury*

4.2.1. *Single samples*

The main evidence obtained from the SS results is that the Cagliari Hg concentrations were significantly higher than those obtained from Comacchio and Odiel. The levels from Venezia, meanwhile, could not be differentiated from the other colonies. Cagliari had very dispersed results, with box plots skewed to the right, while the other sites had more centred distributions, with only a few samples out of the high-end of the scale in Comacchio and Odiel (Figure 3). Only the Cagliari flamingos had Hg results which were $>3.0\ \mu g \cdot g^{-1}$ d.w. Indeed, in this colony, 32% of the birds had Hg concentrations which were above this threshold, while 8% even had levels $>5.0\ \mu g \cdot g^{-1}$. These critical values were proposed by Connell et al. [44], who studied Hg contamination in several egreteries by using the feathers of adult Ardeids. The authors argued that no adverse effects would be expected with Hg values $<3.0\ \mu g \cdot g^{-1}$ d.w., while reproductive and behavioural effects are likely with levels $>5.0\ \mu g \cdot g^{-1}$. No eco-toxicological Hg levels of reference are available for flamingos. It does, however, seem to be relevant that the upper limit of the Hg concentrations found in the SSs from the Cagliari flamingos and the heron chicks in Axios Delta [30] are the same, at $6.5\ \mu g \cdot g^{-1}$ d.w. As the sampled birds were alive and apparently in good health in both studies, two possible interpretations can be suggested: the chicks of the greater flamingo, like the squacco herons, can tolerate up to $6.5\ \mu g \cdot g^{-1}$ of mercury without evident adverse effects, while if Hg levels are above this value in their feathers it is unlikely that the birds can survive; alternatively, it is possible to see the feathers as a 'box' that is able to contain a particular maximum concentration of Hg, in this case of $6.5\ \mu g \cdot g^{-1}$, in relation to the rate of growth and available bonds of this feathers. Our findings in the Cagliari birds can be regarded as representative of a polluted site. Accordingly, we cannot exclude the possibility that fledglings with Hg concentrations $>6.5\ \mu g \cdot g^{-1}$ may be dead or unable to reach the corral, where they become trapped. Of the sites investigated herein, Cagliari was the only one affected by known past mercury contamination [45]. Indeed, the Santa Gilla Lagoon has been exposed to the discharge of industrial waste containing Hg since the mid-1960s. Degetto et al. [45] reported a general improvement in the circumstances of the lagoon after a restoration project ended in 1992. However, with the major differences to the other sites, our results suggest that the fledglings living there are still exposed to high Hg concentrations, which are a potential threat to them. However, at least 56% of the SSs from Cagliari contained Hg levels which were much $<3.0\ \mu g \cdot g^{-1}$. Foraging flights in respect of the Sardinian colony have not been well researched, but it is known that greater flamingos can feed in wetlands as far away from their nests as 70–80 km (Camargue, Tunisia), or exceptionally up to 200 km (southern Spain) [22]. It is, therefore, very unlikely that the flamingos nesting near Cagliari could reach feeding areas outside Sardinia, although some of them may avoid local pollution by also feeding in the less contaminated wetlands located on the south-western and south-eastern coasts of the island (50–90 km).

Conversely, the areas of Comacchio, Odiel and Venezia do not cause any particular concern, with all of the results from the samples obtained at these locations being $<3.0\,\mu g \cdot g^{-1}$ and not statistically different. The Venezia median, however, is slightly higher than that of Comacchio and Odiel, possibly due to an exposition to Hg. The nesting site of this newly established colony is close to the heavily polluted area of Porto Marghera, which has discharged pollutants mainly in the central–northern part of the lagoon [46,47]. Comacchio and Odiel are not directly affected by Hg sources near to the breeding sites, even though accessibility to certain occasional feeding areas may explain the slight skewing to the right of the whiskers in the box-plots. Very high levels of anthropogenically derived Hg are known to be present in Pialassa Baiona [48], a coastal lagoon situated <20 km south-east of the Comacchio colony. Flamingos visit this site irregularly and in small numbers, as is the case for other coastal wetlands in the Po River delta area. Meanwhile, during the breeding season, they mainly tend to feed in the Comacchio Lagoon, where there are no industrial settlements. The Odiel salt marshes are affected by contaminated effluents from nearby mining areas and are thus heavily polluted by various metals [49]. High levels of Hg were recently documented in the soil of an island adjacent to the breeding area, due to ancient human activities [50], but the influence of this point-like-source on the Hg intake of fledglings growing in the Odiel marshes seems to be limited or absent.

4.2.2. *Composite samples*

Odiel is the wild colony in our study, with the lowest Hg levels being found in the feathers of the birds located there. Given the low CS median, the very small degree of variability, and the similarity with captive birds ($0.785 \pm 0{,}042$ vs $0.734 \pm 0{,}017\,\mu g \cdot g^{-1}$ d.w., mean $\pm$ SE), the Odiel levels can be regarded as a baseline for the presence of Hg in the feathers of flamingo fledglings growing in the western Mediterranean area. When compared with adult lesser flamingo pooled samples with a dry weight of $0.077 \pm 0.027\,\mu g \cdot g^{-1}$ (mean $\pm$ SE) [29], the Odiel results were roughly 10 times higher. The difference is probably due to the different feeding behaviour, because lesser flamingos mainly filter blue–green algae from the surface of the water or benthic diatoms from the sediment [51]. In addition, they live in regions with low urban and industrial pressure and low background levels of contaminants in the soil.

When it comes to Cagliari, the CS results confirm the previously described outcome obtained using the SSs: it has the highest median and the most dispersed data, although the dispersion is reduced by the pooling effect. Moreover, the flamingos breeding in the Ebro Delta seem to be exposed to Hg to some extent, given the similarity with Cagliari in the data distribution. Limited information is available about Hg distribution in the environment of the Ebro Delta, even though there are industrial activities discharging waste along the watershed, along with sources of pollution represented by the lower course tributaries (Siurana and Segre rivers) [52]. Because several of our flamingo chicks had high Hg concentrations in their feathers, we can hypothesise that one or more of the wetlands reached by some of the adults during feeding activities are polluted with relevant Hg concentrations, and further investigation is thus recommended.

Camargue was not statistically different from any of the other sites studied, including Cagliari. This implies that most of the birds from Camargue should be not contaminated, due to the location's similarity to Odiel. However, the presence of some samples with high levels of Hg concentration and a distribution of data that is similar to the Ebro Delta suggest that some individuals from Camargue may be in touch with important Hg sources. Greater flamingo adults examined by Cosson et al. [27] had Hg levels in their primaries of between 0.17 and 5.49 $\mu g \cdot g^{-1}$ d.w., with mean values of $1.70 \pm 1.36\,\mu g \cdot g^{-1}$ (mean ± SD) related to the covered vane and of $1.92 \pm 1.36\,\mu g \cdot g^{-1}$ (mean ± SD) to the exposed vane. These means seem to be higher and the dispersions greater than in the Camargue CSs we analysed ($1.167 \pm 0.665\,\mu g \cdot g^{-1}$, mean ± SD). This is not unexpected given that the group sampled by the French researchers was disparate in age (1.5 to more than 25 years) and probably also in provenance. In the Camargue region, high Hg concentrations related to fluvial sediment transport were registered in the suspended sediments of the Rhone River [53], but no other data are available concerning Hg in lagoon sediments. Accordingly, additional investigations are also recommended for this site.

In Comacchio, the dataset pattern is similar to that of Camargue and the Ebro Delta, including in relation to the prevailing low concentrations of Hg and the few samples that contain high levels of this contaminant. One isolated CS had an Hg value of $2.906\,\mu g \cdot g^{-1}$, which is very close to the $3.0\,\mu g \cdot g^{-1}$ threshold. However, Comacchio can be distinguished from Camargue and the Ebro Delta, and is also statistically different from Cagliari ($p < 0.05$). The reason for these high values has already been outlined.

4.2.3. *Zoo samples*

The variability parameters of this subset are not strictly comparable with the variability in wild colonies. However, we can advance some arguments using the mean values. Captive birds have a different level of mercury content (mean $0.734\,\mu g \cdot g^{-1}$ dry weight) compared with free-living adult lesser flamingos [29]. However, the amount of Hg in the feathers of the zoo birds is lower when compared with the other results concerning the greater flamingo adults [27,28]. In addition, comparison with the Odiel CSs (discussed above) strengthens the assumption that the Hg levels found in the greater flamingos living in Bioparco Zoo in Rome are similar to the Hg baseline levels for Mediterranean free-living flamingos, making them suitable for use as a reference point for a low-contaminated population like the Odiel wild birds.

5. Conclusions

This study reveals that in the Mediterranean area, Hg concentrations in the feathers of fledgling greater flamingos can be low where the sediments at their nesting and feeding locations are not polluted by this harmful metal. The Odiel colony, along with the captive birds in Bioparco Zoo, seem to be representative of such a condition, and thus also seem to be suitable for use as a reference point for birds with lows levels of Hg contamination living in the Mediterranean basin.

Conversely, most of the investigated sites recorded more or less important anomalies in about half of the specimens, and one can hypothesise that one or more of the wetlands reached by some adults during their feeding activities are polluted with relevant levels of Hg in the sediment. Of these colonies, Cagliari showed the highest Hg concentrations in the feathers, with several samples being higher than the reference values indicated by Connell et al. [44]. This result is unsurprising, because the Cagliari area is known to have been affected in the past by heavy Hg contamination, which is still present despite decontamination work carried out in the early 1990s [45].

When it comes to Camargue, Comacchio and the Ebro Delta colonies, there are no documented particular concerns about Hg in the vicinity of the relevant nesting sites, but some fledglings nevertheless had high levels of Hg in their feathers. This may be explained if heavily polluted sites located around the colony are visited by just a few flamingos during the breeding period. In order to better understand the eco-toxicological issues of these colonies, further monitoring campaigns on flamingos and their feeding areas are required, as are assessments of the levels of Hg and the mapping of foraging flights.

To date, Venezia is an occasional breeding site, and this initial investigation on only a few birds suggests that there has been some appreciable intake of Hg by the chicks.

We also compared pooled (CS) and individual samples (SS). For the extremely dispersed distributions, the median values cannot produce identical results, as verified in the Cagliari dataset. However, statistical comparisons among the colonies produced the same outcomes, and in the case of limited resources, the use of CSs could be a valid alternative with which to obtain quicker and less expensive responses.

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