ALMA MATER STUDIORUM
UNIVERSITA' DI BOLOGNA

FACOLTA' DI SCIENZE MATEMATICHE FISICHE E NATURALI

Corso di laurea magistrale in
SCIENZE PER L'AMBIENTE E IL TERRITORIO

# Metal exposure assessment in Flamingo fledglings (*Phoenicopterus roseus*) from six colonies of the Mediterranean area by feather analysis

Tesi di laurea in: Biogeochimica

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(III sessione)
Anno Accademico 2009/2010

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INTRODUCTORY SECTION

Several boosts drove me to develop a project regarding metal contamination in Great Flamingos (*Phoenicopterus roseus*) breeding in the Mediterranean basin.

First of all, the willingness to perform an interdisciplinary study involving different environmental sciences such as geochemistry, ecotoxicology, and biology. I found metals and some other elements susceptible to be considered the focus of this kind of research, due to their concerns and implications to wildlife: toxicity to certain concentrations or chemical form, bio-accumulation in tissues, essentiality of some elements for metabolic processes, and so on. Metal presence in the environment is a natural condition, and the distribution of them throughout earth crust very irregular depending on geologic and geomorphologic history, so that each area has a peculiar geochemical background which is recorded in soils, waters, sediments but also in plants and animals. However, human activities altered and are modifying these natural geochemical background causing effects on habitat stability, organism health and fitness, including humans and their environment.

The second reason that urged on me to take up this survey directly relates to my advanced knowledge on ornithological arguments, particularly on aquatic birds and their habitats. Some waterbird species consume large amounts of sediment in the course of feeding. For example, in Mallard (*Anas platyrhynchos*) sediment comprised percentages of 3-12% of the material ingested in weight (Connor, 1993; Beyer *et al.*, 1994). This link between birds and sediment includes a lot of environmental implications, worthy to be studied.

Lastly, I was struck by the notice of several Flamingos died in north-eastern Adriatic coastal wetlands in 2006 and 2007, as consequence of lead poisoning caused by shot pellets ingested while feeding.

I realized that this people-fascinating species (included in Annex I of Birds Directive) would be an ideal bioindicator for pollution in coastal wetlands, since his special feeding behaviour causing a link between Flamingos and sediments, probably the closest among waterbirds.

This work arise from a research that is the main part of a biomonitoring project supported by public bodies as Italian National Institute for Environmental Protection and Research of Italy (I.S.P.R.A.), and Province of Ravenna, collaborating in this context with the Interdepartmental Center of Environmental Science Research of Ravenna (C.I.R.S.A.) and the laboratories of Environmental Sciences of Siena University, and Federal Institute for Geosciences and Natural Resources, Hannover (Germany). Field activities involved also the Flamingo Specialist Group (FSG), a global network of Flamingo specialists (both scientists and non-scientists) concerned with the study, monitoring, management and conservation of all Flamingo species. The biomonitoring of metals here presented is based on non-invasive sampling, using feathers as indicators of metal contamination in the population of Flamingos. To do that, analyses were performed with methods assuring low detection limits, as feathers may contain exiguous concentrations of many target elements.

The sites involved in the sampling campaign were in Italy (3 sites), in Spain (2 sites), and in France (1 site). In addition, some specimens from captive Flamingos were included. This means that for the first time, biological samples of Flamingos can be compared among so many locations. Moreover, main part of western breeding population is represented by a sample set for metal analysis, so that the survey has international significance, due to the distribution of Flamingo breeding colonies in the West Mediterranean coastline, but also to the effort turned to sampling campaign.

The fundamental steps of the project include:

1. A review of ecotoxicological researches on birds that used feathers as bioindicator of metal contamination. This step was already completed and the result documented in a thesis presented in 2007 by me.

2. A survey involving a wide number of metal levels in Flamingo feathers collected in several breeding sites, planning a sampling campaign and applying most used analytical methods, in regard of problems surfaced from the study of literature.

3. Initiatives to raise awareness and understanding of wetlands importance and related environmental problems, particularly on passage of metals from abiotic to biotic systems.

As a complementary research, the sampling and analysis of sediments from some critical sampling sites is in due course. Evaluating the chemical results from both biotic and abiotic components will allow a better interpretation of metal concentration anomalies found into the feathers, and prove the passage of certain elements into the metabolism processes of birds.

The objective of point 2 is the subject of the present work, which is structured in a first section that reports some information about the use of bird feathers in biomonitoring surveys, pointing out the advantages and the critical aspects related to the interpretation of metal concentrations obtained from this biogenic material. In the same part of this work are outlined some aspects of feathers and the biology of Great Flamingo.

A second section describes the breeding sites where sampled of birds was performed and methods (sampling, handling of samples and chemical analysis).

Results regarding 41 metals investigated, and a detailed discussion of five metals (arsenic, copper, lead, mercury, zinc), deepening some interpretation problems encountered are included in the third part. Some general considerations are provided about analytical results, and specifically about the five selected metals and a literature-based chemical footprint of the sites.

Final remarks conclude the work.

1. FIRST SECTION

*1.1 Waterbirds and international regulations*

Wetlands support numerous bird species, and belong to the most endangered habitats of Europe. Various agreements and conventions at an international level are in force to reduce the threats to birds. The most important international regulations include the Ramsar Convention and the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). Moreover, inside the Convention on the Conservation of Migratory Species of Wild Animals (Bonn Convention), in 1999 the Agreement on the Conservation of African-Eurasian Migratory (AEWA) entered in force and to date covers 118 countries. It provides for action to be taken by the Range States for migratory waterbirds to which it applies. Italy became a Contracting Party to AEWA on 2006 (fig. 1.1). The European Union (EU), in addition, have further two legal acts: the Wild Birds Directive (2009/147/CE) and the Habitat Directive (92/43/CE). Both acts, together with Bonn Convention are the normative basis for the Nature 2000 system.

It must be remembered that Annex V of Birds Directive states two actions to advance (letters f, g):

- Determining the role of certain species as indicators of pollution
- Studying the adverse effect of chemical pollution on population levels of bird species

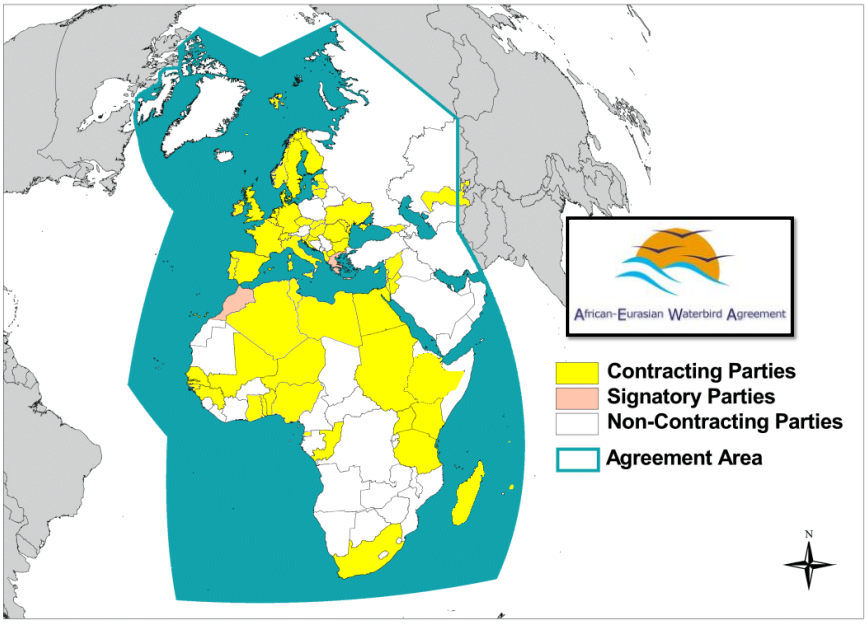


**Fig. 1.1** –AEWA logo and map of contracting (yellow colour) or signatory (pink) Parties into the Agreement Area (blue edge).

*1.2 Biomonitoring using waterbirds*

The main interest of the public and governmental agencies is focused on ecosystem health in terms of potential effects on human health. Concerns for environmental health today includes bioaccumulation in tissues of organisms and associated chronic effects on survival and on reproduction. In concrete terms, scientists try to measure environmental degradation using living organisms. In general, biomonitoring is a relevant and widely used method to assess environmental pollution and its impact on biota and plays a key role in ecological risk assessment. Biomonitoring aims to determine which environmental chemicals organisms have been exposed to and how much of those chemicals actually gets into their bodies. Compared to chemical analysis on abiotic materials, biomonitoring measures the amount of the chemical that actually gets into wildlife or people, instead of the amount they potentially may get into them.

Birds are useful biomonitors because they are high in the food chain, sensitive to environmental changes and easy to be monitored (Burger, 1993; Veerle *et al.,* 2004). On this basis, many waterbirds species are regarded as convenient bioindicators of environmental metal pollution (Scheuhammer, 1989; Kim *et al.,* 1998; Cohen *et al*., 2000). Particularly important in this respect are the species easily recognizable, long-lived, relatively abundant and distributed in a wide geographical range (Furness *et al.,* 1993). Especially, colonial waterbirds, with their central place foraging strategy limiting the activity ranges of nesting adults (fig. 1.2), permit comparisons of responses among sites in natural experiments (Kushlan, 1993). Notwithstanding, recent studies rarely assessed concentrations in feathers of colonial waterbird chicks in order to minimize variability (for example, Fasola *et al.*, 1998; Goutner *et al.*, 2001), and only a part of them compared results among different sites (Burger, 1996; Rumbold *et al.*, 2001; Frederick *et al.*, 2002; Herring *et al.*, 2009).

Heavy metals contamination is a major environmental problem undoubtedly affecting birds as well as other organisms. Contamination of the environment usually results from industrial activities, such as mining and smelting of metalliferous ores, electroplating, gas exhaust, energy and fuel production, fertilizer and pesticide application, and generation of municipal waste. For lead, a further huge source of dispersion in the environment is due to the pellets shot during hunting and shooting (Fisher *et al.*, 2006). As consequence, metals and other exogenous elements enter into the atmosphere, water and soil, changing the background geochemical profiles at different geographical scales. For some pollutants adverse effects on birds are known (for example lead, mercury, cadmium), but for many others less studies were carried out. Besides, some metals (zinc, copper, selenium, and others) are essential elements for metabolism under certain levels,

becoming dangerous when the intake is too high. This double aspect make more difficult to gain knowledge about effects of environmental changes involving this kind of metals.

**Fig. 1.2** – Breeding colony of Kittiwake (*Rissa trydactyla*). According to Kushlan (1993), colonial waterbirds are ideal, among birds, to compare different sites in natural experiments regarding environmental concerns.

Since mid-1960s, the purpose to develop indicators useful as early warnings of environmental quality led many researchers to study the levels of metals in different bird species, in different tissues, excreta, and eggs, in different environmental and biological conditions, and in birds of different genders and ages (Burger, 1993). Birds indeed are often philopatric, returning to the same place to breed (or overwinter) year after year, have a range of diets from herbivorous to omnivorous, are sensitive and exhibit sublethal effects at low enough levels to serve as early warnings, showing toxic effects from a number of pollutants. In addition, the public is interested in birds, in particular in certain "fascinating" species. The importance of public interest is critical because it is required to start and conduct biomonitoring schemes over many years or involving a

wide working group, stimulate allocation of public funds and spur volunteers to cooperate with the survey.

There are some disadvantages in using birds as biomonitors. First, they are mobile, excluding early stages of their life. Using young birds may partially mitigate this inconvenient. Normally, birds are less useful as indicators of point-source pollution (compared for example with sessile invertebrates such as *Mytilus edulis*); however, by selecting accurately the bird species to monitor according to their foraging behavior, types of food and biology, it is possible to take advantage of the ability of birds to integrate exposure over time and space, and consequently target an exposure area of interest (Burger, 1993). Moreover, bird populations may be endangered, making collection of tissues difficult. In that case sampling of feathers and excreta appears the only ethically or lawfully allowed, if technically possible.

All things considered, birds are useful and important tools in assessing both the health of the specific bird population in question, and of the ecosystem in general. Evidently, birds may take part in biomonitoring plans involving different levels of bioindicators and environmental variables in order to reveal different levels of pollutants and to provide different levels of warnings.

### *1.3 Biomonitoring metals using bird feathers*

Presence of feathers is among the characteristics that define the belonging of a living organism to the Class of Birds. Although feather morphology, chemistry, and coloration vary dramatically among species, they are basically keratinaceous structures that provide protection, thermoregulation, enable flight, as well as decoration necessary for species recognition, camouflage, aggression, and mating. Primaries feathers (the longest feathers for flight in the wing) have been extensively studied with the goal to examine undesirable sources of variability in chemical signal, which may limit widespread applicability of the feathers analysis, such as moulting strategy, kind and position of feathers (Furness *et al.,* 1986, Altmeyer *et al.,* 1991; Dauwe *et al.,* 2003), and feather colour (Dmowsky *et al.,* 1984; Gochfeld *et al.,* 1991). The body feathers, on the other hand, are moulted with a random or unpredictable sequence , always allowing the bird to remain nearly completely feathered. There are many more body feathers than flight feathers, and they can be sampled in large number and pooled to provide a better representation than any single flight feather (Burger, 1993).

Regarding the use of feathers in biomonitoring, an important distinction must be made. Feathers can be used as a tool to ascertain levels of exposure for the birds themselves, or they can be used as bioindicators of contamination within food chain and its associated ecosystem. Both goals are important for species conservation, toxicological risk, land management and the public.

Feathers are useful for determining metal bioaccumulation because:

1) Birds deposit in the keratin structure some heavy metals during feather formation
2) Metals deposited in feathers reflect concentrations in blood relatively to the short period of feather growth
3) Feathers are easy to collect even from live birds
4) Collection from live birds can be performed by field assistants
5) Feathers can easily be stored in metal-free containers and do not require refrigeration
6) The metal profiles in feathers are not disrupted by long-term storage at room temperature

The last point is important because it means that feathers can be archived for analysis with other samples at a later time, reducing one source of error (differences in analytical methods) over time (Burger, 1993).

As collecting feathers is a non-destructive sampling method, using feathers as biomonitors of metal contamination has been recommended by many researcher (Goede and De Bruin, 1984; Burger, 1993; Spahn e Sherry, 1999). Sampling can be performed on endangered species and from the same bird over many years.

Indeed, many recent studies applied this invite, even if some warnings arose about difficulty on results interpretation. It is not clear if all metals are deposited in feathers and eventually in which proportion relatively to body burden, and if the metals deposited in feathers may represent recent exposure or mobilization from other tissues, especially in aged birds (Burger, 1993). Not less important, the interpretation of metal concentration in feathers is further complicated by the two ways of metal load in feathers: accumulation from the bloodstream into growing feathers and exogenous contamination contracted after feather formation (Veerle *et al.,* 2004).

More studies on metals in feathers have been done on mercury than on any other metal, followed by lead and cadmium (Burger, 1993). More recent studies confirmed this tendency, further increasing the number of mercury studies compared to other metals. In general, the researchers

evaluate few elements charged with eco-toxicological problems case by case. Actually, very few researchers considered a larger number of elements, and when done, comparisons were carried out with concentrations in other internal tissues or among metals, but they did not compare levels found in feathers with geochemical profiles of breeding, moulting, and feeding sites (often unknown because of mobility of birds).

Recently, Sheifler *et al.* (2006) noticed that few data are available in the literature about the relative importance of external contamination. This is true for most metals. Most researchers consider the atmospheric deposition as the most important source of external contamination, able to resist after many washing procedures (i.e. Janssens *et al.*, 2001), while another contribution may come from the uropygial oil[1], secreted by birds themselves. Instead, it seems useful to better understand the importance of external contamination due to residual particles not moved away by washing procedures. Actually, a single study examined with Scanning Electron Microscope (SEM) the effectiveness of various feathers washing methods, and controlled whether all external particles was removed or not (Weyers *et al.,* 1988). However, they did not perform a chemical characterization of such particles.

Some authors attempted to understand the significance of external contamination compared with the amount of metal sequestered in the keratin especially for mercury. Goede e de Bruin (1984) found on waders that concentration of Hg in the feather did not undergo significant change after formation of the feather and observed a strong correlation between shaft and vane concentrations. They concluded that Hg is deposited in the feather mainly during feather formation. Some birds showed detectable Hg in the uropygial gland, but stability with time of signal in the feather indicated that external contamination due to preening, if present, is not reliable, or was cancelled by washing samples. Regarding zinc, an essential element required for feathering (Sunde, 1972) the authors confirmed that this metal should be carefully controlled by metabolism, at least for shaft. In vanes they found a large variation and supposed that some external contamination occurred in vanes in a short time. On the other hand, for certain samples they observed that a little Zn was lost after washing. It is possible that a little external contamination may be obscured due to the large amount of zinc naturally stored by the bird in

[1] The uropygial gland is the main holocrinc cutaneous gland of birds. It secretes a thick, transparent, complex oil (preening oil) consisting primarily of diester waxes (uropygiols), fats and fatty acids. It performs many functions in the bird including water proofing, and keeping the skin, feathers and bill supple; the oil is said by some to have a function against bacteria, fungi and ectoparasites (Moyer *et al.*, 2003). During preening, a bird transfers this oil to its feathers by rubbing its head and beak against the oil gland and then spreading the oil over the feathers on the rest of the body.

feather structure. The conclusion of Goede and De Bruin (1984) was that also the vane can serve as a monitoring tissue for Zn, if the feathers are sampled soon after growth. They did not get a clear idea about external contamination importance for arsenic, selenium, lead and cadmium. Cadmium was detectable in feathers in few cases, and the other elements indicated some external contamination, so that the researchers suggested to control the following conditions: when the feather was formed and how long this took, and the life history of the bird after formation of the feather. Furness *et al.* (1986) analyzed feathers of nine seabird taxa, and studied the pattern of Hg in the first primaries moulted. They argued that the same pattern found in different species was a prove that Hg concentration in feathers depends on the physiology of moult. In fact, they found a pronounced reduction in Hg level in primary feathers as moult sequence (confirming previous literature) and concluded the observed pattern to be a result of the elimination of accumulated mercury into the feathers. The suggestion of authors was to sample body feathers instead of flight feathers, because the variation depending on moult sequence in body feathers is less conspicuous. A different study was performed by Hahn *et al.* (1993) on Hg, Pb, and Cd, by rinsing the samples only with distilled water (a very soft washing procedure). For each feather they cut out and analyzed microsamples and found a gradient both for Pb and Cd concentrations. Contents of Pb and Cd raised from the feather base to the tip and from the center towards the edge of the vane. In contrast to this, mercury contents was approximately equally high in all parts examined and once again this study confirmed the endogenous incorporation of Hg during feather growth. A good number of metals (twelve) were assessed by Veerle *et al.* (2004) in small passerine feathers. They admitted that for many metals a clear pattern about external versus endogenous contamination is not established and compared metal concentrations in three tail feathers, that were exposed to exogenous contamination for different periods of time. The results indicated that some exogenous contamination was present for most of considered metals (Ag, Al, As, Cd, Co, Cu, Fe, Mn, Pb and Tl) even if vigorous washing of feathers is performed. Thus, levels in regrown (or recently grown) feathers reflect more accurately endogenous deposition of metals in the feather, however exogenous contamination may still occur. For zinc they argued that high deposition rate into the feathers could mask exogenous contamination, so that zinc external contamination in feathers is a less important factor. Finally, regarding Hg, exogenous contamination was declared not important and feathers very suitable biomonitors for mercury contamination, also considering the high body burden of this metal that may be sequestered into feathers during moult (Braune and Gaskin, 1987; Burger, 1993). A lot of metals were analyzed also by Dauwe *et al.* (2003) to assess variation of metals within and among feathers of birds of prey. Molting sequence resulted correlated only with Hg, and feather levels declared adequately reflecting blood levels during

feather growth, with only slightly external contribution. A high capacity of the feather to accumulate copper during feather growth was also verified, however external contamination may occur. For most other metals pattern significant external contamination is likely. The use of bird feathers for the monitoring of cadmium pollution was investigated on Starlings (*Sturnus vulgaris*) by Pilastro *et al.,* (1993). Findings included the confirmation that feathers can accumulate cadmium, but the authors supposed the contamination should be due to intestinal absorption from the food and excretion through the uropygial gland (external contamination). Ek *et al.* (2004) studied Cd, Cu, Pb, Zn and PGE to assess the distribution of internal versus external metal contamination using Laser Ablation-ICP-MS on different species of birds. This study demonstrated that Pb contamination is both external and internal (a relatively high background signal with peaks probably caused by attached particles); Zinc contamination is predominantly internal, according to the authors, and depending on Zn concentration in the diet during feather growth. This is in contradiction with Dauwe *et al.* (2003). Cadmium and copper resulted predominantly internal, with a few externally attached particles containing these metals.

Despite some studies about the significance of external contamination, most of literature existing on bioaccumulation of metals into feathers tends to underestimate this factor. External contamination should be considered a critical aspect that accompany all steps of feathers analysis: especially regarding washing and discussion of analytical data. Probably, a definitive washing method able to remove all exogenous contamination is not improved or even impossible to find, without altering internal mineral stability. Thus, the choice of washing method must be made according to the objectives of the research. Anyway, before interpretation of analytical data, it should be evaluated the washing effectiveness and the kind of external source of contamination. External contamination may be of interest as evidence of exposure to certain pollutants for periods depending upon when the feather was last moulted. However, if the aim is to assess only bioaccumulation levels, external contamination must be strongly minimized, applying vigorous washing procedures. Experiments with washing and metal stability have been conducted with mercury (Appelquist *et al.*, 1984), arsenic, selenium and zinc (Goede et de Bruin, 1984), but the other metals are still unexplored. For mercury, weather conditions should not affect mercury levels (Appelquist *et al.*, 1984). External contamination can occur virtually for all elements from air-borne deposition or from the deposition of contaminants during preening, when birds apply an oily secretion on feathers to gain waterproof (Burger, 1993). In addition, all birds are used to have sand-baths to get rid of parasites, and have several contacts with water each day. Consequently, very adhesive particles of sediment may grip on the surface of the vane or be trapped by the feather structure (Weyers *et al.*, 1988). For this reason the knowledge of geochemical composition

of the sites where birds spent their time, together with the correct choice of the bird species, age of sampled individuals, knowledge of behavior of birds, may help to correctly interpret the results.

In summary, the use of feathers for biomonitoring of metal pollution requires taking in account the following remarks:

a) aims of analyses must be clear: assessing only bioaccumulation in feathers, or acquiring also information about exposure to environmental matrices; in the first case, washing method is strategic and a control of washing effectiveness is recommended; some external contamination can remain, therefore it is strongly recommended to get information about geochemical background of the site of interest;

b) feathers of birds sampled should represent an area as much as possible definable: to assign the analytical response to a geographical context, biology and behavior of birds must be well known, and if possible feathers should be taken from young birds still nursed by parents;

c) factors of variability, for example the age of birds, must be controlled either sampling birds of the same age, either having information about life-history of each bird; regarding variability among feathers, all feathers plucked or cut off should be of the same type;

d) feathers should well represent the individual: pooling of some feathers from a bird is better than sampling of a single feather or even a little part of it.

Only three studies examined metals in feathers of Great Flamingo. Cosson *et al.* (1988) measured some trace elements in feathers from ten adult flamingos died in Camargue because trapped in the frozen salt ponds. Amiard-Triquet *et al.* (1991) choose three greater coverts from 50 young flamingos in the French breeding site. Recently, seven Flamingo carcasses from various Italian wetlands were analyzed for Pb, Cd and Hg (Ancora *et al.,* 2008); in this study, two specimen from Tuscany certainly died because of acute saturnism, having dozens of lead shot in the gizzard and very high lead level in liver and kidney.

*1.4 Feathers and plumages*

Throughout the life of a bird after its hatching event, the skin produces feathers from a determined number of follicles, distributed on the body in patches more o less similarly in all species. The arrangement of feathers in definite areas of the skin is told pterylosis. The term *apterium* is used to indicate a bare space among feather areas in a bird's skin. The pterylosis of a chicken (dorsal and ventral part, excluding the head) is shown in fig. 1.3.

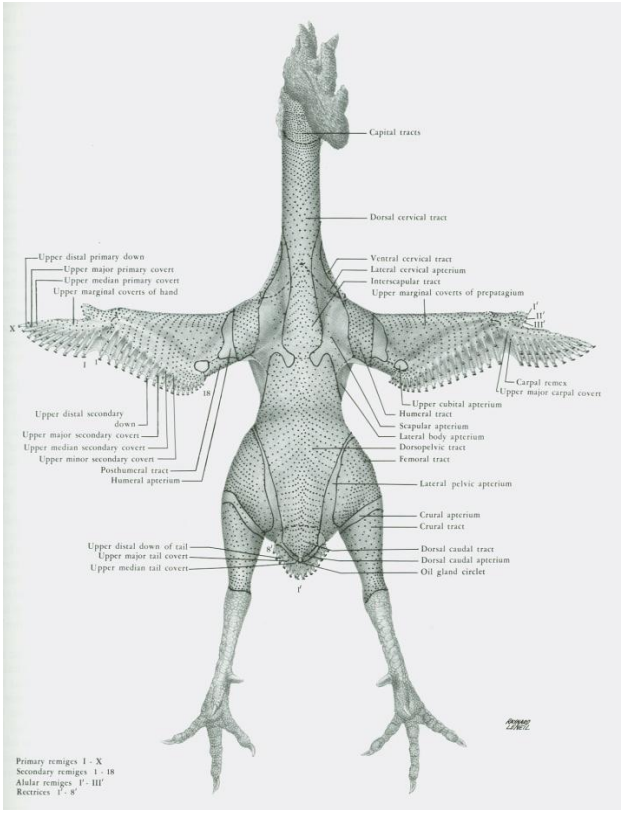


**Fig. 1.3** – Pterylosis of the back of a male of chicken (from Lucas and Stettenheim, 1972)

The first-generation (or natal down) is produced during embryonic life, and is already on the skin at the time of hatching as fluffy down (fig. 1.4a). Within their first few weeks of life birds develop a second-generation (or juvenal feather) grows after hatching and pushes the natal down out of the follicle on its tip (fig. 1.4b). In some species, as flamingos, a second down can replace the natal down before starting the juvenal feathering.


**Fig. 1.4a** – Chick of Montagu's Harrier *Circus pygargus* with natal down (F. Borghesi)
**Fig. 1.4b** – The same bird with juvenal feathers replacing the natal down (F. Borghesi)

The new feather is tightly furled inside a sheath while it forms. As it appears above the skin, it has a long conical shape with a blunt tip. The tip soon dries and flakes off. This allows the feather to begin to emerge and the vane to unfold (fig. 1.5). The ensheathed portion still presents a richly vascularized pulp where keratinization process occurs. The pulp progressively recede. A feather fully grown is entirely free of sheath and has pulp completely resorbed.

**Fig. 1.5** – Duckling of Gadwall (*Anas strepera*). Wing feathers are clearly sheathed, but the sheaths (the azure sticks in this case) are flaking off and the keratinized part of feathers can emerge (F. Borghesi)

After juvenal feathering, some months later, the first moult occurs: the juvenal feather is pushed entirely out of its follicle and is lost, while the tip of the third-generation feather comes out. Moults may involves all o part of the plumage (progressively, sequentially or in many other ways) during a single, periodic event, or may be quite continuous throughout the year. In a typical bird species there is a pre-breeding partial moult, leading to a colorful breeding plumage, and a post-breeding moult, complete in many species. Knowledge of moult strategy in each species is necessary to correctly interpret the levels of metals in feathers and their sources (Burger, 1993).

Structured feathers are generally called body contour feathers, including the soundest and longest wing and tail feathers. They are composed by the *calamus* or quill inserted in the skin, the *rachis* or shaft as prolongation of the *calamus* outward the body, inner and outer vane (which constitutes the main aerial portion of the feather) supported by the *rachis*, several first-level branches fused to the *rachis* (*rami* or barbs), second levels of microscopic branches, of the barbs, named *radii* or barbules (Lucas and Stettenheim, 1972) (fig. 1.6). Barbules have a heavy capacity to hook other barbules thanks to their curved tips, named *hamulii* (fig. 1.7).

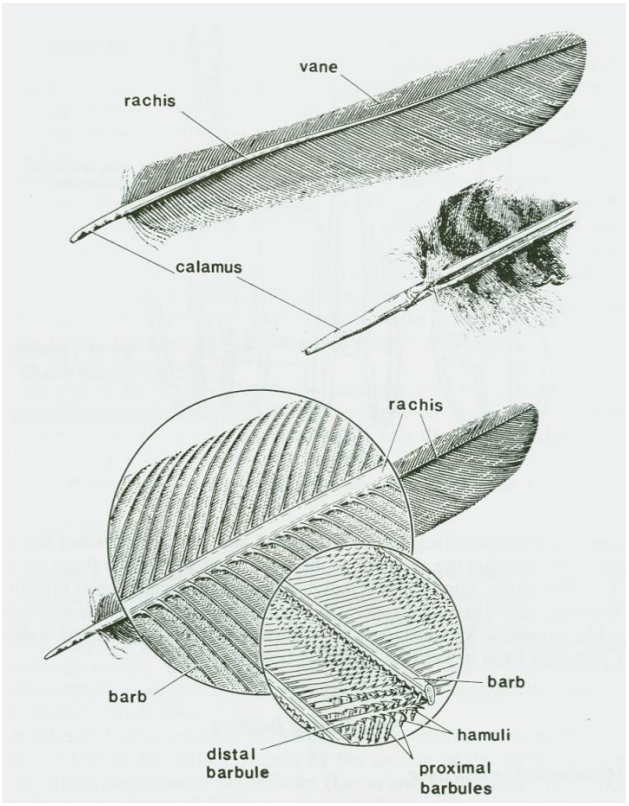


**Fig. 1.6** – Structure of a flight feather (from Van Tyne and Berger, 1971)

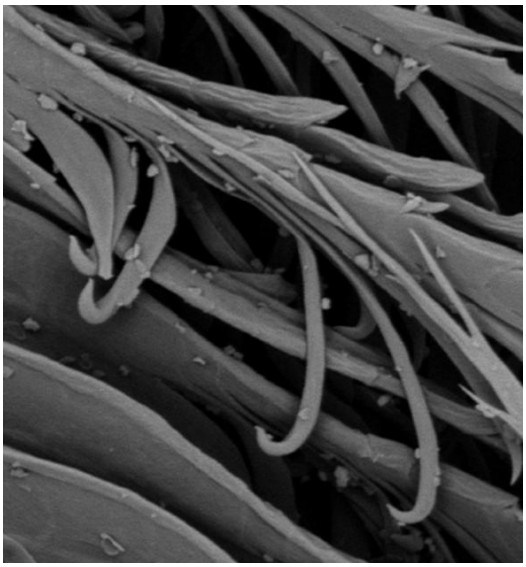

**Fig. 1.7** – Scanning electron micrograph of a contour feather of Flamingo showing hooked distal barbules (*hamulii*) interlocking with the grooved proximal barbules (courtesy of G. Gasparotto, University of Bologna)

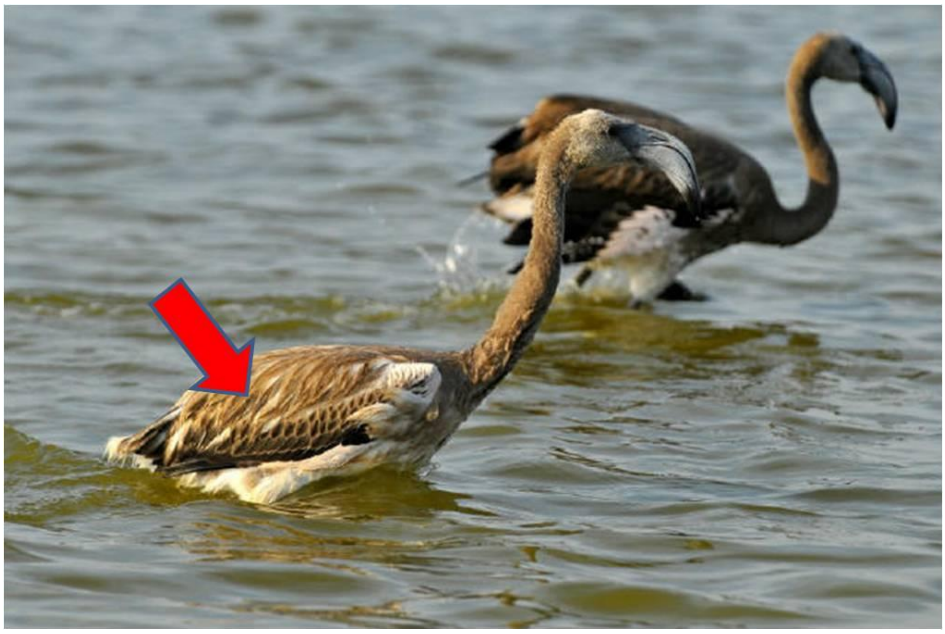

**Fig. 1.8** – Fledgling of Greater Flamingo. Scapular feathers are many, long and widely cover the folded wing (indicated by red arrow in figure) (M.Scarpini)

Depending on the *pterylae* that originate it, the feather assumes different names. Wing feathers are the sum of the flight feathers (remiges) and the wing coverts, the latter with the function to protect the flight feathers. Tail feathers are also considered flight feathers as well as remiges. Between body and wing there are scapular feathers (on the superior part). They are a group of

feathers, few in number, growing in the scapular *apterium*. In most bird species scapulars feathers are very few in number and not of relevant dimension, but in flamingos they are several, long-shaped and well-structured (fig. 1.8).

Recently, the scientists have shown that some important data on the cell structure, modality of keratinization and composition of feathers still remains to be clarified. Keratin refers to a family of fibrous structural proteins. Keratin monomers assemble into bundles to form intermediate filaments, which are tough and insoluble and form strong unmineralized tissues. Like all intermediate filaments, keratin proteins form filamentous polymers in a series of assembly steps beginning with dimerization; dimers assemble into tetramers and octamers and eventually, the current hypothesis holds, into unit-length-filaments (ULF) capable of annealing end-to-end into long filaments. During feather formation the new proteinaceous material is added at the base as the feather elongates. Keratin filaments are abundant in keratinocytes, cells which have undergone keratinization. During the process of epithelial differentiation, cells become cornified as keratin protein is incorporated into longer keratin intermediate filaments. Then, cells undergo a programmed death as they become fully keratinized. As consequence, once fully grown, circulation of blood into the quill stops and all the feather becomes dead material. Two types of keratins are recognized: the α-keratins in the hair, horns, nails, claws and hooves of mammals, and β-keratins, harder, found in nails and in the scales and claws of reptiles, in the feathers, beaks, claws of birds and quills of porcupines. In birds, β-keratin in feathers, beaks and claws is an hydrogen-bonded structure of β-pleated sheets, which are then further twisted and crosslinked by many stabilizing and hardening disulfide bridges, mainly due to amino acid cysteine (Pauling and Corey, 1951; Schor and Krimm, 1961). Extensive disulfide bonding contributes to the insolubility of keratins, except in dissociating or reducing agents. The result is a though matrix, inert, insoluble, amorphous, where disulfide bonds readily may be reduced to sulphydryl groups that become available to bind metals. However, tendency of keratin to incorporate metals is not only related to presence of disulfide bridges, but also to other molecular structures that enter in the feathers, as pigments.

The feathers remain in place for six to twelve months (depending on moult, occurring once or twice a year, except accidental losses) unlike hairs in mammalians, which grow constantly. As consequence, after the few weeks during which the feather is growing, there can be no further deposition of metals from the blood into feathers. Afterwards, metal profile raising should be assigned to external deposition.

*1.5 The Greater Flamingo*

The Greater Flamingo stands among the most popular bird species. The overall number, between 545.000 and 760.000 in the world, seems altogether stable at least in last decades, with fluctuations in most of areas of presence (BirdLife International, 2011). In the Mediterranean region, the presence of two populations was generally recognized (Johnson, 1989). The eastern Mediterranean population might be in contact with Asian range of the species, and was estimated at 60.000 individuals, 300.000 including Asian birds (Wetlands International, 2006). In the West Mediterranean the presence of Flamingos seems to be increased in the last twenty years because new breeding colonies were established in Italy, Spain, and North Africa (Baccetti *et al.,* 2008; Rendon-Martos *et al.,* 2008; Samraoui *et al.,* 2008). Probably it counts about 165.000 individuals (Wetlands International, 2006).

This species, like other flamingos in the world, is very fascinating for birdwatchers and public, and in many cases have been contributing to wetland conservation as flagship. Actually, it is protected throughout much of its range.

The classification of Flamingos in a taxonomic group suffered many controversies. It is still unclear if similarities between Flamingos and other groups of birds derive from a common ancestor or reflect convergent adaptations. Ornithologists in XIX century included Flamingos into Ciconiiformes group (herons, spoonbills, storks, and ibises) because of the external general tracts, then Flamingos passed to Anseriformes group (really, similarities with geese and ducks are numerous), finally they were thought evolved independently from Anatidae and more related to Charadriiformes. At the same time, hypothesis of a separated group Phoenicopteriformes (situated between Ciconiiformes and Anseriformes) have been suggested. DNA hybridization technique recently showed Flamingos more closely related to the Ciconiiformes than to the Anseriformes and not related to the Charadriiformes (Sibley and Ahlquist, 1990). However, more recent studies have changed many times that version: several researchers explored the possibility of a close relationship with Podicipediformes (grebes) (Van Tuinen *et al.*, 2001; Sangster, 2005; Johnson *et al.*, 2006); Fain and Houde (2004) proposed a relation among Flamingos, Strigiiformes (owls), swifts and doves. To date, two classifications are commonly used, though the question has to be better clarified. Johnson and Cézilly (2007) prefer to consider Flamingos as a suborder of the Ciconiiformes; Del Hoyo *et al.* (1992) and others consider them belonging to a separate order between Ciconiiformes and Anseriformes.

Independently by the taxonomic group, until recently, five species of three *genera* were recognized. The Greater Flamingo was described as Old World's subspecies *Phoenicopterus ruber roseus*, whereas the Caribbean Flamingo was told New World's subspecies *ruber*. After experiments on hybridizated DNA, today all species of Flamingo throughout the world are included in a single genus, *Phoenicopterus*, and the Greater and Caribbean Flamingo are split in two species, being the Greater *P. roseus* and the Caribbean *P. ruber* (Johnson and Cézilly, 2007) (fig. 1.9).


**Fig. 1.9 a-b** – Eurasian Greater Flamingo, *P.roseus* [a] (drawing of A.Singer, 1983); Caribbean Flamingo, *P. ruber* [b] (from Collins Birds of the World, 2006).

Considering the purpose of this work, it is convenient to remind some important aspect regarding habitat preferences (particularly during breeding), feeding behavior of adults, and way of raising chicks. Besides, some notes regarding this large wading bird that may be useful to know before enter into the details of biogeochemistry, are also reminded (moult strategy, availability of captive birds, biogeography and movements of wild birds).

**Habitats**

The preferred habitat by Flamingos for foraging is represented by salty coastal areas, which include several possible environmental settings such as salty wetlands, coastal mudflats, river estuaries, lagoons, playas and natural pans when flooded, inland salt lakes, and man-made saltpans (Johnson and Cézilly, 2007). However, flamingos have a feeding behaviour more variable than one may believe: rice field, freshwater marshes, shallow impoundments are often visited (fig. 1.10). Flamingos feed by walking in water up to 80 cm in depth, that means only deep waterbodies

are really precluded. Flamingo presence is very localized, though the number of potentially suitable wetlands is high, because they are strictly gregarious and live in large flocks throughout the year. Therefore, they tend to stand in certain areas until they can find nourishment and disturb is low, deserting the others.

**Fig. 1.10** – Young Greater Flamingos feeding in a partially flooded field in Spain (F. Borghesi)

Nest sites lie in arid or semi-arid regions, where high evaporation and low rainfall increase salinity and keep wetlands shallow. In humid regions, if commercial saltworks are present, Flamingos can breed inside managed or abandoned salinas, that are normally shallow, extensive, and rich in invertebrates. Anyway, islets or sand banks clear of vegetation, and stable year after year, are necessary for the establishment of the breeding colony. Islets completely surrounded by water avoid that terrestrial predators (foxes, stray dogs, and so on) can easily reach the nests. If all conditions are attended, the location can become a permanent nesting site. Otherwise nesting may be opportunistic and infrequent, as it occurs in many places. The breeding success of the colony will be achieved if the nests will be not submerged in the early stages of breeding season, and disturbance by humans and terrestrial predators will be very low. In the western part of breeding range (fig. 1.11) nesting sites are coastal and at sea-level, but in the eastern part some are inland, even at high altitude.

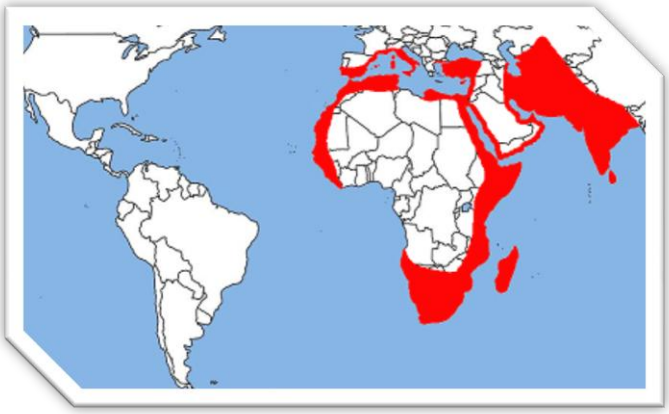

**Fig. 1.11** – Breeding range of the Greater Flamingo (the image is from the Flamingo Resource Centre, FRC, web-based collection of resources and information related to flamingos available to researchers, conservationists, and flamingo keepers - www.flamingoresources.org).

Foraging sites are normally shallow wetlands, brackish or saline, with poor vegetation. Salinity is often several times greater than oceans (up to 200‰ and over). Such condition offers abundant food, but require special adaptations. Flamingos have salt glands located on top of the head in shallow depressions near the eye (Gill, 1995), similarly to seabirds that drink seawater. Salt glands allow excretion of large amounts of ingested salt. Surprisingly, this characteristic of Flamingos has never been studied in detail, even though they forage in some of the most saline habitats used by birds, with highest pH (Johnson and Cézilly, 2007). In order to study physiological treatment of metals adopted by Flamingos, it is necessary to consider that salt and preen (uropygial) glands, feathers, and feces, may have a significant role in excretion of potentially dangerous metals or in regulation of essential metals balance.

**Feeding behavior**

The food resources of many wetlands where Flamingos breed are insufficient to satisfy the needs of all the bird in the colony and of the numerous non-breeding flamingos gravitating around the colony, throughout the breeding season. As a consequence, many individuals will forage in other wetlands within a determinate home-range (Johnson and Cézilly, 2007). In Italy studies are in progress to precisely define breeding home-ranges (Baccetti, pers. comm.). Some calculations of foraging flights have been made in France, Spain, Tunisia and Turkey, using different methods, among them resightings of birds banded with PVC rings, dye-marked flamingos, or satellite tracking. In Camargue (Southern France), a study confirmed that flamingos breeding at the Étang du Fangassier feed throughout the Rhône delta, some birds reaching lagoons up to 70 km west of the colony. Flamingos breeding in Fuente de Piedra Lake (in the South of Spain) move considerably

far away, because the surrounding wetlands, placed 10-20 km around the colony, can support small numbers of birds. Many birds forage in the marshes and saltpans between River Guadalquivir and Cadiz (that are 140-200 km from Fuente de Piedra). In Tunisia, an aerial survey allowed to observe a large number of birds foraging in the same wetland hosting the chicks, but the missing number of the parents were found feeding up to 80 km distant. Observations in Turkey confirmed a possible home range around 100 km, covered to reach a very extensive saltpan (Johnson and Cézilly, 2007). Substantially, most flamingos raising chicks seem to forage close to the colony, or find suitable feeding areas up to 50-100 km distant, in the case of food scarcity in relation to the number of breeding pairs. Exceptionally, birds breeding in remote wetlands can move away up to 200 km to forage.

Although Flamingos are predominantly predators of invertebrates, similarly to herbivorous animals, they must spend considerable time feeding, filtering water and loose sediment. In polluted areas, the highly frequent contact and ingestion of sediment during feeding expose Flamingos to metal intake much more than the bird species collecting merely plants and preys from environment. The anatomy of the flamingo's bill and tongue is specialized for the particular feeding method adopted by all Flamingo species (Jenkin, 1957; Zweers *et al.*, 1995) (fig. 1.12).

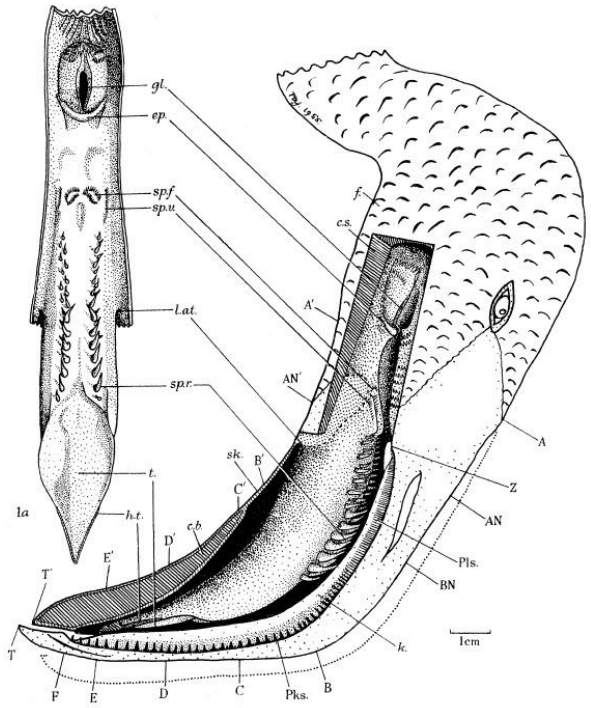


**Fig. 1.12 -** Bill anatomy of flamingos (from Jenkin, 1957). It is important to note the big, strong tongue (t.), and the filtering lamellae (sp.r.).

Strong muscles at the base of the tongue allow the flamingo to apply it in a rapid back movement creating suction. The frequency of this piston-like movement is four time per second. The bill is hold slightly open (about 5 mm at the tip) and the inner surface of the upper mandible is lined with plates of *lamellae* directed inwards and backwards towards the tongue. Lamellae retain the food when the water is expelled near the base of the bill. When flamingos feed on mud, the process is apparently reversed: the outer lamellae prevent the coarser particles from entering the bill while the finer mud is swallowed and the organic content extracted. The inner lamellae are spaced approximately 0.5 mm apart, so particles ranging in diameter from 0.5 mm to 4-6 mm are taken (Del Hoyo *et al.,* 1992). Flamingos are able to feed in water only a few millimeters in depth and in water deep 50 cm, up to 80 in some circumstances, and typically take food in the mud at the bottom of the water. They are able to choose the size of the organisms they swallow, but not the type (Jenkin, 1957). Primarily, the Greater Flamingo eat small aquatic invertebrates: the range of species is very wide. It will take advantage of any abundance or population explosion of invertebrates (for example brine shrimp *Artemia salina*). Flamingos feed also on the seeds of aquatic plants, cultivated rice, and all organic content of the mud they decide to swallow. During breeding season many birds also feed in brackish or fresh water where brine shrimps are absent (Johnson and Cézilly, 2007). The rock particles greater than 0.5 mm may be kept into the stomach with some mud, as “grit” with the function to grind the food.

**Fig. 1.13 -** Flamingos feeding in typical packed flocks near the breeding site (A. De Faveri). Walking fledglings (grey birds), still unable to fly, are grouped in nurseries (crèches), and closely surrounded by the adults (white-pinkish birds).

Flamingos forage in flocks even of thousands of birds (fig. 1.13), but little is known about the way they distribute themselves over foraging grounds (Johnson and Cézilly, 2007). Due to the gregarious behaviour, it is reasonable to suppose that large portions of the colony may be simultaneously subject to the same threats, if the selected feeding area is polluted. On the other hands, flocks of the same colony feeding in different areas may encounter metal profiles in the sediments totally different. It is also reasonable that any day, a portion of birds could change flock, so that the homogeneity of the metal intake of each birds, day by day, may depend on the fidelity to the foraging site of each individual.

**Breeding and raising chicks**

Breeding ecology of the Great Flamingo is well known, as well as biology. Since 1983 in Camargue, and since 1986 in the Fuente de Piedra Lake, an enormous quantity of observations and recordings have been carried out at close range.

**Fig. 1.14 –** A little portion of the breeding colony in Comacchio saltpans, Italy (G. Arveda).

Nests are very dense on the muddy islets or banks, and the birds tightly packed (fig. 1.14). The total number of birds that attempt to breed each year might be determined by water levels (and hence the available surface of the islands and the food availability in the surrounding wetlands).

Flamingos lay one single egg. Usually, synchrony in egg-laying amongst a certain number of pairs occurs, and birds which lay before the first main wave of breeders are likely to have a low breeding success (Johnson and Cézilly, 2007). The result is a high homogeneity regarding the age of the chicks, even if birds that failed first attempt may make a second (rarely a third) laying. The nest is a truncated, cone-shaped mound of mud or sand, on top of which the egg is laid and the chick stay until it is able to walk. The material is taken from the close surroundings of the nest and scraped up with bill movements by sitting flamingos. No materials are carried to the nest by them. The shape of the mound is also adjusted using legs (Johnson and Cézilly, 2007). Therefore, Flamingo chicks, during early stage of their life, are continuatively in direct contact with the sediment used for building the nest.

After about 29 days of incubation, Flamingo chicks hatch. They are semi-precocial (able to walk in few days, but in need be fed for a long time) and clad in white down. At one week of age they are able to easily stand up. Chicks at 7-10 days begin to wander away from the nest. A secondary darker down replace the first between 3 and 7 weeks after hatching (Berry and Berry, 1976). Chicks aged about two weeks form a nursery (crèche) walking around the birth site (fig. 1.15). The crèche remains until young flamingos become able to fly (about 80 days in Camargue as reported by Johnson and Cézilly, 2007). However, flying young do not leave the crèche immediately.


**Fig. 1.15 a-b –** Crèche of flamingo chicks surrounded by several adults close to the nesting site [a] and an adult looking after the nursery including chicks aged 4-5 weeks [b]. They show the dark secondary down and the growing first feathers (M. Zenatello [a], G. Arveda [b]).

In most bird species, food for chicks normally is collected and carried by parents, in some cases after a pre-digestion (that means the food will be regurgitated by parents). Flamingos adopt a feeding method unique among birds, with some similarities with that used by pigeons, doves, and parrots during the first days of life of their chicks. Flamingos (of both sexes) are able to secrete the crop liquid to feed its chick (fig. 1.16). The liquid is a deep red secretion from the upper digestive

tract, containing about 8% protein, 0,2% glucose and 18% fat, rich in carotenoids and blood cells (Lang, 1963; Fisher, 1972). The red coloration is due to carotenoids and blood (Bartlett in: Studer-Thiersch, 1966) and the secretion is the sole source of nutrition practically until fledging. Independent feeding does not start before 7 weeks after birth, because formation of lamellae are not completed, but chicks may show filter behaviour (Zweers *et al.*, 1995) and start their direct intake of sediment.

**Fig. 1.16 –** Adult feeding a chick. A secretion drip from the parent bill in to the mandible of the chick (G. Arveda).

Growth of young flamingos take long time, up to 18 months or more (Studer-Thiersch, 1986). Chicks growth rates vary considerably from one site to another and from one year to another (Johnson and Cézilly, 2007). This factors prevent an accurate estimate of the age of chick based on the measurements of tarsus or other bare parts (without considering sexual dimorphism, significant in the Greater Flamingo). However, during banding operations, tarsus and weight of fledglings are measured, in order to have a rough idea of the birth date.

**Feathers and moult**

Both males and females acquire adult plumage by several years. At the very earliest it is acquired at 30 months, but most birds not until they are 4 years of age (Johnson *et al.*, 1993). Even some 10 years old individuals were found with retained traces of immature plumage (Jonhson and Cézilly, 2007). Thus, it is not easy to surely assign an age to one feathers in adult flamingos. Just before fledging, juvenile feathers on the wing (flight feathers and wing coverts) are fully or near fully grown, and the brown-grey down (the second plumage grown after hatching) is completely worn. The adults seems be able to choose the moult strategy, thought complex and variable on the basis of certain conditions, anyway the first young feathers remain unmoulted at least for several months (Cramp and Simmons, 1977).

**Captive flamingos**

Flamingos are extremely popular zoo animals. The total number of captive flamingos (of different species) registered with the International Species Information (ISIS) was 14324 in 2008. Many flamingos are also held by non-ISIS-registered zoos and by private breeders. On the other hand, reproduction of Flamingos in captivity is not easy at all, and captive flocks composed by young birds of the same age, or chicks, are very rare. Although captive flamingos can be very useful tools for illustrating problems and potential solutions in the ecosystem of their wild counterparts, studies that involves measuring a physical or behavioural parameters are not simple to perform (King, 2008). So, no data about metal concentration in feathers of young lamingos born in a zoo are available.

**Western metapopulation of Flamingos**

Breeding sites of the five countries including the most of the western metapopulation (France, Spain, Italy, Turkey, and Mauritania) are in active salinas for the 36% and generally the existence of colonies depends on water management and appropriate conditions (Béchet *et al.,* 2006). It is supposed that the distribution of breeding flamingos through the nesting sites are despotic, age-related, and depending on the degree of saturation of largest, stable colonies. In fact, younger birds tend to breed at small and recently-established colonies (Rendon *et al.*, 2001). Therefore, smallest and new colonies could allow early recruitment into the overall breeding population.

Since 1990s, it is known that French, Spanish, and Italian populations are not closed, because important exchanges among colonies of breeding flamingos banded as chicks have been observed. The Flamingo network aims to study environmental and individual factors influencing juvenile and adult dispersal in order to provide sound conservation planning at an appropriate scale (Béchet *et*

*al.*, 2006). To approach any conservation issue at the appropriate geographical scale, metapopulation theory should be useful, because difficulties encountered by some colonies can be compensated by the demographic connections among colonies (Esler, 2000). On the other hand, problems suffered by a subset of a metapopulation might be exported in virtue of the exchanges among colonies, affecting the whole population with problems before thought only local.

The Greater Flamingo is a partially migratory bird, at times nomadic. Flamingos born in France, Spain, and Italy were observed to breed at other colonies in the western Mediterranean, and similarly, to switch from one colony to another afterward (Balkiz *et al.,* 2007). Recoveries of Iranian Flamingos at the farthest northwest in France and at the farthest southeast in Asia occurred in the past, but the nature of these movements was not known. The degree of connectivity with the eastern Mediterranean was unclear until some years of banding were gone in the always growing Comacchio breeding colony, and after two banding campaign in the largest Turkish colony in 2003 and 2005. Analysis of resightings proved that the western Mediterranean metapopulation extends over the Turkish colony faced on the Mediterranean Sea, with directional dispersal from west to east. On the other hand, flamingos from Turkey have been proven to move outside the distribution range of the western Mediterranean metapopulation (Balkiz *et al.*, 2007). This bring into question the hypothesis of a lack of strong interaction between western Mediterranean and Southwest Asia (Johnson, 1989), and one can argue there are a sufficient exchange rate (even very low) to homogenize the gene pool at a larger geographical scale (Mills and Allendorf, 1996).

2 SECOND SECTION

*2.1 The sampling sites*

An inventory of the more important Greater Flamingo breeding sites up to year 2000 were made by Johnson and Cézilly (2007). Half of the world's Flamingo breeding sites are situated in the Mediterranean region. Western Mediterranean breeding sites lies both in Europe and in North Africa. Relatively to the European sites, where at least one breeding attempt has been successful since 1950, the largest colonies include: Salin de Giraud saltpans, Camargue (France) and Laguna de Fuente de Piedra, in Malaga Province (Southern Spain) (fig. 2.1).


**Fig. 2.1** – West European breeding sites of Greater Flamingo. The two main sites (yellow), the other important sites (white), and the newest or sporadic (pink) (based on Johnson and Cézilly, 2007)

In Spain, other significant breeding sites are recognized. Three are in the Eastern coast: Salinas de La Trinitat-Punta de la Banya (Ebro delta), Santa Pola saltpans and Pantano de El Hondo (Alicante); Doñana, Huelva-Sevilla (far South), actually lies in the Atlantic coast, just beyond the Straits of Gibraltar. There is no lack of other sporadic Spanish establishments. In Italy, there are stable colonies in Cagliari lagoons (Sardinia), in Margherita di Savoia saltpans (Apulia), and Comacchio saltpans (Emilia Romagna). New successfully attempts were recorded in northern part of Venice

lagoon (Veneto) and in Diaccia Botrona salt marsh (Tuscany), the latter with only two chicks fledged (Baccetti *et al.*, 2008). Orbetello (Tuscany) hosted another single case of breeding in Italy in 1995 (fig. 2.1).

In 2008, nine colonies in the Mediterranean region raised chicks, as reported by the Flamingo Specialist Group (FSG) (Childress *et al.,* 2008). Relatively to the whole population of Europe and North Africa, more than 37800 pairs of Greater Flamingos bred in 2008, with 11330 chicks fledged. Fewer chicks and fewer pairs attempting to breed resulted in that year than in the five previous years, despite the increasing number of sites used (fig. 2.2, from Béchet and Germain, 2009).

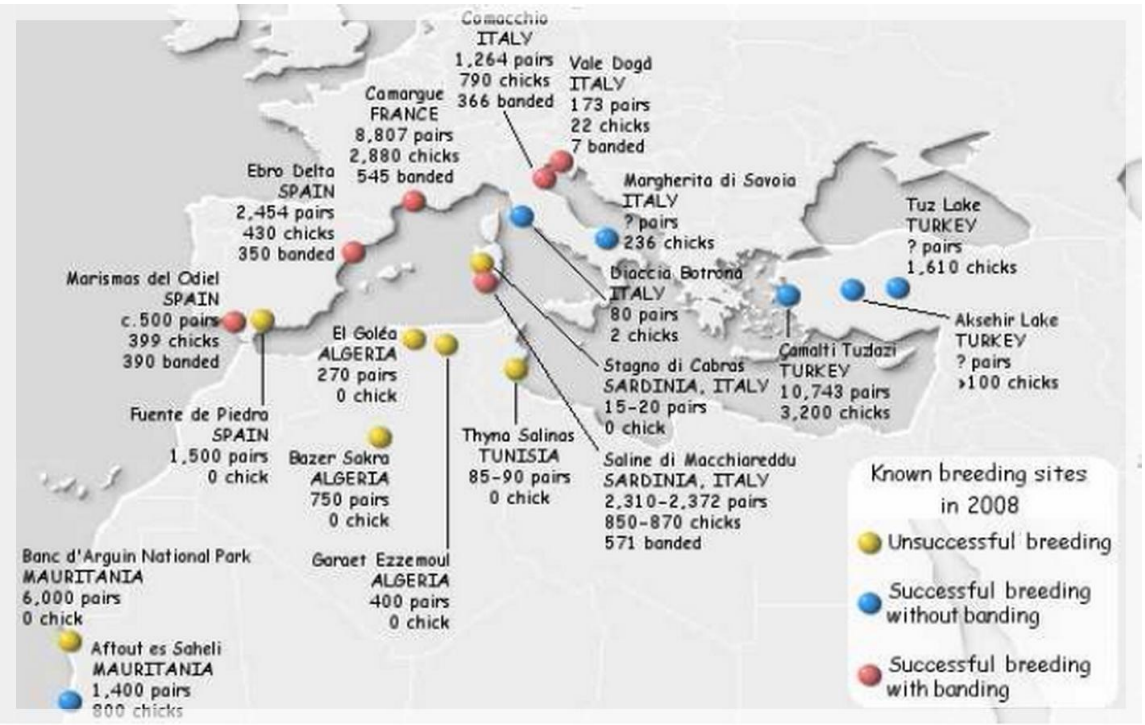


**Fig. 2.2** – Map of Greater Flamingo breeding and banding in 2008. The sites are indicated by dots (yellow = breeding attempted but failed; blue = successful breeding but no banding performed; red = chicks raised and banded. For each site, number of pairs, total of chicks raised, and of fledglings banded are reported (map included by Béchet and Germain in a Flamingo Specialist Group report).

In six of these, Doñana (Odiel marshes), Ebro Delta, Camargue, Cagliari, Comacchio, and Venice, banding of fledglings was carried out by researchers and volunteers under their respective national ringing schemes and the FSG' supervision. Sampling of feathers for metal assessment was

performed in all six sites, here described in geographical order (from west to east), while samples from captive birds have been obtained from Bioparco Zoo (Rome) (fig. 2.3).

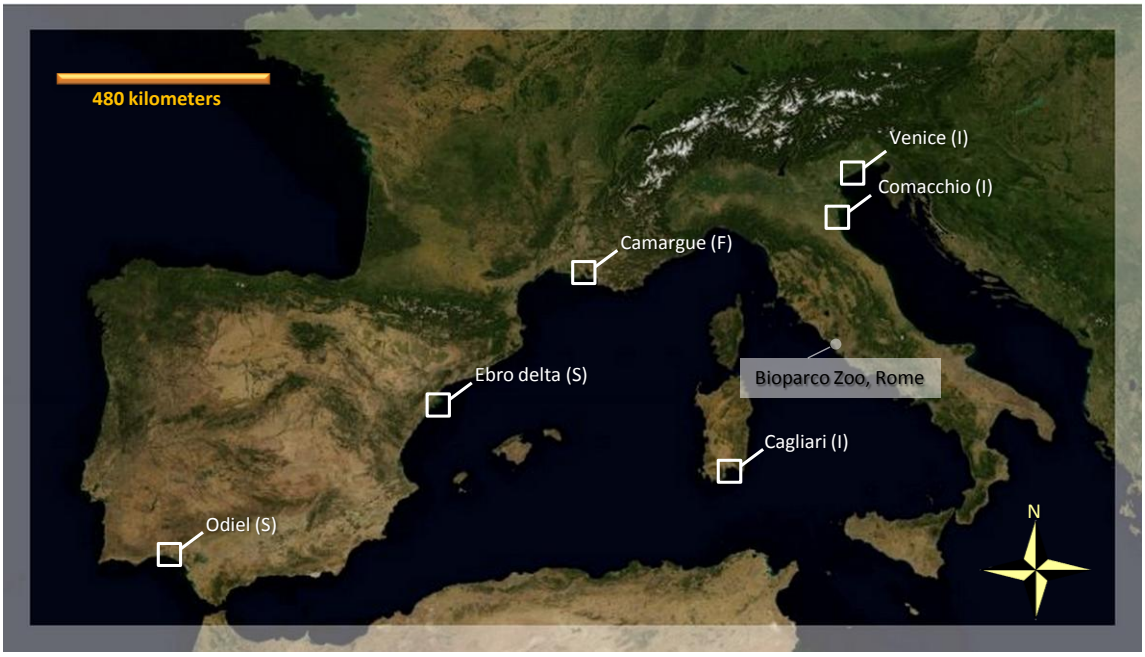


**Fig. 2.3** – Sampling sites in 2008: wild colonies (white labels and squares) and captive flock (black label on white background, and white dot).

*Marismas del Odiel*: estuarine complex at the confluence of the Odiel and Tinto rivers in south-western Spain (fig. 2.4). It comprises intertidal mudflats and saltpans and it is a Biosphere Reserve (1983) and Ramsar site (1989). In spite of its importance for nature conservation, Odiel salt marshes are greatly affected by pollution from the Odiel river. In fact, acid mine drainage originating from several minesites of the Iberian Pyrite Belt (IBP) and derived from mine waste oxidation cause water acidification generate pollution. IPB is a very big system of sulphide mines that have been worked for at least 4500 years, and intensively since the mid-19$^{th}$ century. Odiel river and Tinto river drain basin sedimentary formations and the so-called sedimentary Vulcan complex. Then both rivers flow into Rìa of Huelva estuary, where they release a significant contaminant load, because of the sharp change of pH and salinity (Nieto *et al.,* 2007). In addition, from 1966 fertilizers and paper industries, and copper foundries were established in the area of Huelva, enhancing the dispersion of pollutants from mining sources into the estuary (Grande *et al.,* 2000), and contributing to make this system one of the most polluted, and studied, in western Europe. Several studies assessed metals in sediments of the river and estuary system, but fewer

researches have been carried out on sediments of contiguous shallow wetlands. Among them, Borrego *et al.* (2002) examined a number of sediment samples throughout the estuary and related wetlands, and found all sectors enriched in anthropogenic metallic contributions. They supposed that high Fe, Cu, Zn, Pb and Ba were due to Fe-rich acid waters and sediments carried by the rivers, while phosphates, As, Hg and U originated from industrial effluents and redistributed all over the system by tidal low energy streams. Sediments from the streams flowing through Odiel marshes were analyzed by Morillo *et al.* (2008). According to their interpretation As, Cd, Cu, Pb, and Zn should be higher than background values, and capable to produce noxious effects on aquatic organisms in most of Odiel salt marshes; Cr, Mn, and Ni levels did not seem cause concern about possible sources of pollution. Cd and Zn were found the most mobile among the elements studied while Cr, Fe, Ni, and As strongly linked to the sediments. Hg was not analysed. No information is available for salinas ponds.


**Fig. 2.4** – Location of the breeding colony in Odiel marshes. The artificial islet lies just in the Salinas (yellow dot).

After the building of an artificial island in 1989, sporadic breeding success of Flamingos occurred (the last once in 1995). After many years, in 2008 the birds returned in large numbers on the artificial island (Rendon-Martos *et al.,* 2008), probably as consequence of no breeding opportunity in Fuente de Piedra lagoon. The islet lies within the Doñana Nature Reserve and National Park (1969), therefore into the Biosphere Reserve, and the Ramsar site.

*Delta of Ebro*: located in the north-western Mediterranean coast of Spain, this wetland system is at the mouth of the Ebro River, the largest river in Spain. It covers an area of 330 $km^2$, with a triangular shape, going some 20 km into the sea, with one coastal spit in the north and one in the south. These spits close two shallow bays (fig. 2.5). The Ebre Delta Natural Park covers about 7300 ha of upland and 470 ha of marine area. Punta de la Banya is an area with special protection (Integral Reserve) of 2500 ha formed by the southern sandy spit, and connected to the delta plain by a narrow isthmus. Salt marshes and dunes cover most of the area that includes 780 ha of the commercial Trinitat salt pans (Curcó *et al.,* 2009). The river system receives heavy metal contamination deriving from various anthropogenic sources, such as urban, industrial and agricultural activities. Chemical industries are located near the Flix Reservoir (95 km upstream from the delta) that discharged into the lower Ebro River a considerable load of heavy metals (Gomez-Gutierrez *et al.,* 2006). Lower course tributaries, joining the Ebro on the left side, add pollution in the closing section of Ebro, before debouching into the Sea (Ramos *et al.,* 1999). In the delta area rice fields are widely distributed (over 60% of the surface). It is an activity that extensively uses different types of pesticides containing many heavy metals, and fertilizers rich in Cd and Zn. Important natural wetlands cover the remaining area but the wide use of herbicides in rice fields heavily impacted biota and wildlife (Manosa *et al.,* 2001). Assessing metal levels in crayfish, a very recent survey, identified two important sources of heavy metal pollution in the lower Ebro river and delta (Suarez-Serrano *et al.,* 2010). The researchers found highest Hg and Pb concentrations in crayfish near the contaminated sediments at Flix Reservoir (point source). The bioaccumulation of Cu, Zn, Cr, and As were related to the intense rice cultivation (As levels were very high, but it is unknown if a natural source of As exist in the area). Pb levels in crayfish were thought also resulting from the extensive hunting in wetlands and rice fields (Mateo *et al.,* 1997). Additional source of contamination might derive from accumulation of lead shot pellets. Guitart *et al.* (1998) estimated 4-9 metric tons of lead shot pellets spread every year over the rice fields and lagoons of the Ebro Delta as a consequence of waterfowl shooting.

Flamingos regularly breed in the Punta de la Banya saltpans almost continuously since 1993, foraging in coastal lagoons, bays, salt marshes, rice fields and saltworks. Census of Flamingos (and other bird species) are bimonthly conducted by the park staff in several bays and coastal lagoons of the delta system, and, obviously, in the saltworks of the Punta de la Banya. Banding of fledglings began in 2004, with the same procedure used in the other sites of western Europe. Flamingos cause some impact to the rice fields, because they tend to visit the same preferred fields many times. To discourage nocturnal visits by flamingos, the park organizes spring scaring campaigns in

April-June when rice plants are small and Flamingos are preparing to breed (Curcó *et al.*, 2009). Saltpans are privately owned, within a natural park and Ramsar site.


**Fig. 2.5** – Location of the breeding colony in Ebro delta. The islet lies in the salinas de la Trinitat (yellow dot).

*Camargue*: it constitutes that part of the Rhône river delta enclosed by the Petit Rhône to the west and by the Grand Rhône to the east, and the Mediterranean Sea at south (fig. 2.6). It is essentially modeled by fluvial ramblings and ramifications, and by marine deposits. Camargue covers 1450 $km^2$ (it is the largest delta of European Mediterranean), declared Biosphere Reserve since 1977 and Ramsar site since 1986. In its southern part the abundance of brackish lagoons and saltpans (soils are usually submitted to hydromorphy and salinisation by evaporation of shallow groundwater which leads to salt precipitation) makes it suitable for numerous waterbirds in all seasons. Important economic activities in the Camargue include bull breeding, rice cultivation, salt production and tourism. Although the Rhône River does not flow directly through the central Camargue, it affects its northern part through the pumping of water via a canal network used to sustain hunting or agricultural activities. Indeed, during the last 30 years, a large area of Camargue has been fitted for rice cultivation in order to limit evaporation and salt precipitation by large inputs of fresh waters. To date, upland soils developed on the fluvial deposits of an ancient Rhône branch are mostly cultivated. In the north-west domain of Étang des Vaccarès (the largest lagoon

located in the middle of Camargue), for examples, rice was cultivated for 21 years between 1975 and 2003, whereas fennel and wheat were cultivated the rest of the time. Soils in that area have a silty-sandy composition and the main minerals are quartz, calcite, K-feldspar and mica. The clay fraction contains illite, chlorite, smectite and mixed layer of smectite–illite (Desplanques *et al.,* 2006). More in general, soils of Camargue region are silt-clay and sandy, with high pH (7 to 8) and dominated by fine particles, rich in limestone and poor in organic matter. Oxides of Fe and Mn are naturally abundant in the sediment and are susceptible of dissolution or deposition depending on changing of environmental conditions, as periodic flooding and drying of paddy fields may determine (Sonney *et al.*, 2005). High mercury concentrations were registered in suspended sediments of the Rhône River (Cossa and Martin, 1991; Santiago *et al.*, 1994) related to fluvial sediment transport, but no other data are available for Hg in the lagoon sediments. Yet, the "Etude hydraulique et hydrobiologique des canaux de Camargue: Etat des lieux – diagnostic" (2004) states that main sources of pollution derive from the water flowing into the Rhône, as well as the drainage waters from rice fields. Regarding Pb and saturnism in birds, one study investigated the density of lead shot pellets in 14 Camargue marshes, and depth distribution were controlled in five of these. Densities resulted variable depending marsh type and high, the maximum being near two million/ha of shots. A two-years monitoring on experimentally seeding of pellets showed that over 97% of shot remained in the first 6 cm of sediment (thus completely available to be swallowed by waterbirds during feeding) (Pain, 1991).

A recent study sampled some sediment cores from the early tract of the two Rhône arms, before entering in the Camargue wetlands and from the Têt river, a sub-coastal river to the west of Petit Rhône. Metals assessed were Al, Fe, Mn, Mg, Cd, Co, Cr, Cu, Hg, Ni, Pb, Sc, and Zn along each core. Chromium, Cobalt and Nickel resulted of lithogenic origin, but most top-soil slightly enriched in metals: Pb > Cd > Hg > Cu > Zn. Anthropic contribution resulted declining since the late of 1960s, except for Hg that started to decline since the late of 1980s, nevertheless the load of metals during last decades seems to be low compared with most of rivers draining anthropized areas (Ferrand, 2010).

Flamingos have bred here since at least mid-16th century. An important conservation program in late 1960s included the construction of an island upon which Flamingos could breed. Gradually, the island has been fully colonized and breeding became regular since 1976. Only in 2007, when no water was pumped into the Fangassier pan, Flamingos did not breed in Camargue (Béchet *et al.,* 2009). The Étang du Fangassier is owned by the Compagnie des Salins du Midi et des Saline de

l'Est (Salins). It lies within the Camargue Regional Park (1970), consequently included in the Ramsar site (and UNESCO Man and the Biosphere Program).


**Fig. 2.6** – Location of the breeding colony in Camargue. The islet lies in the Étang du Fangassier (yellow dot).

*Stagno di Cagliari*: located in southern Sardinia, it consists of a lagoon system of about 15 $km^2$, that comprises Macchiareddu industrial saltpans and the Santa Gilla lagoon (fig. 2.7). Ramsar site since 1971. The lagoon is connected to the sea through a harbor and has two major freshwater inflows from the Fluminimannu and Cixerri rivers. Santa Gilla lagoon is considered an important source of quality fish and shellfish in Sardinia, but it has been exposed to the discharge of industrial wastes containing mercury, lead and zinc since mid-1960s. In particular, for inorganic Hg, it has been estimated about 26 tons released from a chloralkali plant in the 1960s and 1970s, mainly accumulated in the western side of the wetland (Cottiglia *et al.*, 1977; Contu *et al.*, 1985) and mostly bound to the organic matter or in the sulphide form (Contu *et al.*, 1984). A detailed analysis of the Hg distribution into the area was carried out by Degetto (1986). Other metals were assessed in the same survey and the researcher demonstrated that high concentrations of Hg, Pb, Zn, and to a lesser extent Cr, were present in some sediment layers. Hg was found to be almost entirely confined in a surface layer (< 10 cm) in front of the on-shore industrial area (hundreds of ppm in many points, mean about 20 ppm d.w., whereas 1 ppm was the mean value in the remaining lagoon). On the other hand, the eastern side was mostly affected by the discharge of

untreated municipal sewage from Cagliari. Degetto *et al.* (1997) reported a general improvement of the situation of the lagoon after a restoration project started in 1986, and ended in 1992. The project led to modify all the hydrodynamics of the lagoon, in order to facilitate the daily exchange of water with the sea, to dredge about 6 millions $m^3$ of sediments, and to redirect municipal sewage into the Cagliari harbour. However, the new levels measured after the conclusion of the project showed concentrations still high in a central-western sector of the lagoon. As consequence of the dredging operations, a new well-defined stratification was soon observed (Degetto *et al,.* 1992), inducing particular concern for Pb and Zn, apparently more mobile respect to the previous conditions.


**Fig. 2.7** – Location of the breeding colony in Cagliari wetlands. At present, the colony breeds onto the abandoned dykes of the Macchiareddu salt pans.

Regular breeding of Flamingos began at the opposite side of Cagliari city, in the Stagno di Molentargius in 1993 (Schenk *et al.*, 1995), after few failed attempts recorded in the site in 1975, 1980 and 1992, and in Saline di Macchiareddu in 1979, 1981, and 1982 (Brichetti *et al.*, 1992). Since 1999 the breeding colony gradually moved to Macchiareddu saltpans. The settlement in the new site was followed by a considerable increase of the number of breeding pairs with high productivity. Breeding success has always been high, between 61% and 94%, except in three years of total abandonment of the colony, the last in 2010 (Nissardi *et al.,* 2009; Nissardi pers. comm.).

In 1997 the Sardinian colony have been involved in the Greater Flamingo banding program, but some discontinuity occurred depending on fund availability. Up to 2007, 1837 individuals banded between 1997 and 2006 were controlled. Notwithstanding the reading effort is presumably lower than in European countries, more than 30% of resightings was coming from North Africa (from France 24%, Tunisia 21%, Spain 15%, North Adriatic wetlands 10%, Algeria 8%, others sites 17%, and Sardinia about 60%). It was ascertained that Sardinian breeding flamingos during the breeding periods attends mainly Cagliari wetlands, but use for feeding also other wetlands within a radius of 100 km from Cagliari (Nissardi *et al.*, 2009).

*Comacchio lagoon*: one of largest coastal wetlands of Italy, whose formation is related to the late Holocene evolution of the Po river delta area (fig. 2.8). Ramsar site since 1981. Once a freshwater wetland, it became brackish in the 16$^{th}$ century after linking to the Adriatic sea. The lagoon now receives freshwater from the Reno river, which flows from the Apennines and runs along the southern side of the lagoon in the lower tract, and drainage water from agricultural lands. It is known that near-surface (1 m deep) soils surrounding the wetland are characterized by high Cr and Ni concentrations (Amorosi and Sammartino, 2007) that are recorded in the area from 10000 years (Amorosi *et al*., 2002) that can be referred to natural inputs related to sediment provenance and not to anthropogenic contamination. Literature on metals in top sediments of Comacchio lagoon is poor. A biogeochemical characterization of Valle Spavola and Fattibello (the portion of 7.3 km$^2$ of the lagoon most close to the town of Comacchio) was carried out by dense sampling and analysis of superficial sediment in 1997-98 (Frascari *et al.,* 2002), but this area and Flamingos are poorly associated, even if the breeding colony is not far. Water exchanges with salt pans and the main lagoon are indirect or absent. Anyway, results of the study are of interest for the mineralogical composition assessed. Carbonatic component (50% in superficial samples in Fattibello) may be of two types: terrigenous calcite and dolomite, and biogenic Mg-calcite and aragonite. Aragonite derives from mollusk shells, while Mg-calcite is higher in correspondence of autochthonous constructor Anellidae *Ficopomatus enigmaticus* colonies. A siliceous component is also present in addition to the carbonaceous component, consisting of quartz, plagioclases, feldspars and clayey minerals (illites, chlorites and serpentine). Frascari *et al.* (2002) did not analyzed metals because the aim of his study was to understanding the euthrophication problems.

Comacchio saltpans (industrially active till 1984), lie in the north-west of the lagoon and host the Flamingo breeding colony since the year 2000, when few pair fledged some chicks, after an unsuccessfully attempt in the autumn before. In 2008 the number of fledgings reached 1264 (Albanese *et al.,* 2009), and increased again to reach 1822 in 2010. The origin of flamingos

breeding in Comacchio salt pans tends to be represented with the same pattern in the last years. Two-third of banded birds resulted born in Comacchio, about one-third in France. Only few individuals came from Orbetello, Sardinia, Spain, and Turkey (Arveda *et al*., 2009). In 2010, during the early stages of the breeding period, some volunteers monitored for banded flamingos the main potential feeding areas throughout the subcoastal wetland system from Cervia salt pans (50 km south of Comacchio) to Rosolina marshes (50 km to the north). Aerial photographs were taken, and sightings of banded flamingos were made on the colony. The data are to be analyzed to better understand foraging distribution of flamingos breeding in Comacchio lagoon. The nesting dykes and islets are in a Government property (Azienda Autonoma dei Monopoli di Stato) and lie within the Po Delta Park of Emilia Romagna (1983), into the Ramsar site.


**Fig. 2.8** – Location of the breeding colony in Comacchio lagoon. The colony breeds onto the residual banks of abandoned saltpans (yellow dot).

*Lagoon of Venice*: the largest wetland in Italy, extending more than 50.000 ha. The Lagoon of Venice is a coastal lagoon (fig. 2.9). It represents a type of wetland rare and unique, sufficient to satisfy the first criterion for the designation of wetland of international importance (Ramsar). Its origins is similar to others in the Mediterranean, but the subsequent natural evolution and human impact differentiated that wetland from other sites. Stratigraphical studies demonstrated that, at least since the upper Pleistocene, a variety of different systems of wetlands developed in this area (Smart and Viñals, 2004). Brenta and Piave rivers, of Alpine origin, are the major providers of

water and sediments, and play an essential part in the formation of the lagoon. For effect of tidal action on river contribution, a lot of different geomorphologies and water bodies with every possible variety of salinity may coexist within the lagoon boundaries. The lagoon has been affected, over the last century, by the industrial growth of the area of Porto Marghera (facing the west-central lagoon), with pollutant effects on sediments (often with inputs of heavy metals) and water pollution mainly in the central-northern part of the lagoon (Apitz *et al.,* 2007, 2009). Furthermore, many of the rivers incoming from the Alps carry polluted sediments and waters into the lagoon (Smart and Viñals, 2004). Geology, hydrology, geochemistry, and many other environmental aspects have been well studied in the lagoon (Osservatorio naturalistico della laguna di Venezia – Comune di Venezia 2006). On the basis of the inlet flows, the basin volume and the recycle of the lagoon waters, the average renewal time of the waters is two days (Frignani *et al.*, 1997). Regarding grain-size of sediments, the pattern in the lagoon changes through the time. Recent observations noticed a prevalence of silts and clayey-silt in the central-northern lagoon, and silty-sands and sandy-silts in the southern. In the lagoon of Venice, dolomites and silicates show an opposite gradient: high quantities of dolomites are recently found in the northern sector (except along the boundary with mainland were silicates are more abundant perhaps as consequence of Sile river influence) and at south-west of Venice; high presence of silicates is found in the south of the lagoon. Among the pollutants metals affecting the lagoon, As is the most significant. It is relatively abundant in the alluvial deposits of Brenta river, but main inputs also derived from Porto Marghera industries and affect channels. Mercury shows the highest levels close to the industrialized area in the centre of the lagoon. Significant high levels are also present in the northern lagoon, but the source is unknown. Among other considerable inputs from Porto Marghera, zinc must be considered since the presence of an important metallurgic plant. Similarly to other metals (Cu, Pb, Cd, Hg) the distribution of Zn throughout the lagoon is changing and concentration are increasing in the northern and southern lagoon, whereas in front of Porto Marghera levels seem to be decreasing. Nickel seems of natural origin and shows a more random distribution (Oss. Nat. della laguna di Venezia – Com. di Venezia, 2006). Frignani *et al.* (1997) and Bellucci *et al.* (2002) assessed metals in the northern and southern lagoon sectors respectively. Bellucci *et al.* (2002) also determined metals in the channels of industrial area. Frignani *et al.* (1997)found the sediments heterogeneous in composition and pollutant contamination at north of Venice. Zinc had by far the greatest concentrations, especially in the sediments which are heavily polluted by the waste effluents from Porto Marghera. Copper, Ni and Mn seem to derive from the sediments of Dese river. Manganese followed rather closely the pattern of silicate constituents. Chromium seemed to have neither a clear pattern, nor a prevailing

input. It must be underlined that all reported information does not cover the whole Venice lagoon. Unlike the other sectors, practically none geochemical information are similarly available for the private northern "Valli" (Dogà, Dragojesolo, Grassabò), just the venetian wetlands most frequented by birds, including Flamingos, and less linked to the hydrological and tidal dynamics of the whole lagoon.

Flamingos were attempting breeding in a dammed section (Valle Dragojesolo, 1200 ha) of the northern lagoon in 2007: eggs laid were abandoned because of human disturbance. A colony tried again in 2008 in a remote part of Valle Dogà (1980 ha), situated just 5 km at the northwest of Valle Dragojesolo. That time, breeding success was achieved on a islet of about 80 $m^2$, and 22 chicks fledged. The local property owners allowed ringing of young birds, but only seven were banded, being the others already able to fly away. Among adults observed on the small colony, nine individuals ringed as chicks at Comacchio, three from the Camargue and just one from Sardinia were recorded, all aged two to five years (Baccetti *et al.,* 2008). Valle Dogà is a private brackish lagoon managed for fish farming and wildfowling included in the Ramsar area.

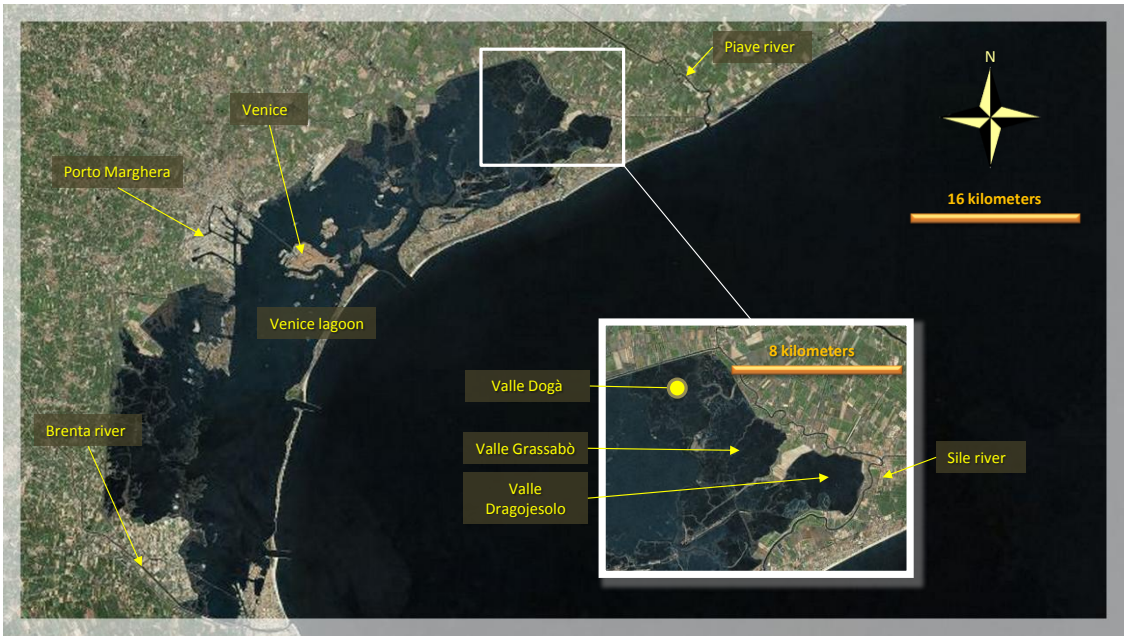


**Fig. 2.9** – Location of the breeding colony in Venice lagoon. The colony established its nests in a small bank in a private marsh in the far north (yellow dot in zoomed section).

*2.2 Sampling*

The feathers were collected in 2008, in all six active colonies east of Turkey, with the exception of the breeding colony of Margherita di Savoia salt pans (Southern Italy), where sampling conditions were not suitable. All feathers of flamingo fledglings were collected during the ringing operations coordinated by the Flamingo Specialist Group (FSG) (fig. 2.3, §2.1).

Since longtime Flamingos are banded throughout the world. Some techniques exist for capturing adults able to fly, but the risk of injury to the birds is high, therefore generally unacceptable to researchers and conservationists (Johnson and Cézilly, 2007). Most flamingos are captured as fledglings, when they are able to move together in a crèche and are no more vulnerable to predation by gulls. In this context, the manipulation of birds during banding operations is a favourable occasion for sampling.

**Fig. 2.10** – The corrall were flamingo fledglings will be herded at the final step of the capture (A. Berbash)

To capture part of the crèche, flamingo fledglings are herded into a corral by a number of ornithologists and volunteers organized in teams (up to 200 people in France, about 100-120 in Sardinia and Comacchio). The capture happens surrounding the crèche and slowly seeing to it the

entrance of the corral (fig. 2.10). The door will be closed when the wanted number of fledglings is reached. In that moment, the count of the total number of fledglings (including the captured ones and those let be free) is made. Then the teams in charge of banding and measuring are quickly formed and a sort of efficient "assembly line" starts, while the handlers assigned to the corral pass the chicks to the porters for processing. Within the space of a couple of hours 400-600 fledglings are banded with a stainless-steel ring and a PVC leg-band, measured, and released (fig. 2.11). Since the liberation is sequential as the birds are banded, the best moment to sample feathers was just before release.


**Fig. 2.11 a-c** – Three moments of the banding operations. The handler passes a flamingo from the corral to a volunteer [a]. A team quickly performs the banding procedure applying PVC and metal rings, and taking measurements [b]. Immediately after, a volunteer releases a banded bird [c] (G. Arveda)

All captured chicks were 5 to 8 weeks old, but the sampling was made from the best feathered birds, therefore is presumably that most of sampled birds were 7 to 8 weeks old. The feathers were sampled by cutting the distal part of a small number of feathers from random individuals, using stainless steel scissors (fig. 2.12). Longest scapulars were selected, according to a sampling protocol previously transmitted to all site representatives (fig. 2.13), protected from aerial deposition because covered by several others. Scapulars feathers are a group of long, quite narrow feathers situated at the base of the upper wing. The vane is rather stiff, less rigid than primaries, secondaries and greater coverts, but not so fluffy as body feathers. 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 (Appelquist et al. 1984, Burger 1993).


**Fig. 2.12** – Sampling of a scapular feather on a young flamingo just before the release. For each individual 2-6 feathers were collected (A. Andreotti)


**Fig. 2.13** – Each colony sampled referring to the same graphical and point by point instructions. Here the illustration showing the requested result of cutting (F. Borghesi)

**Tab. 2.1** - Number of samples for each breeding site

| Site | Country | SS[1] | CS[1] |
|---|---|---|---|
| Cagliari | Italy | 25 | 21 |
| Camargue | France | - | 19 |
| Comacchio | Italy | 25 | 19 |
| Ebro | Spain | - | 17 |
| Odiel | Spain | 25 | 15 |
| Venice | Italy | 7 | - |
| *Captivity* | *Italy* | - | *7* |

[1] SS = Single samples; CS = Composite samples

*2.3 Sample preparation*

**Washing and drying**

The feathers were first individually rinsed with distilled running water and vigorously washed in deionized water (milli-Q), then in acetone 1mol/L, and again in deionized water to remove loosely adherent external contamination. Each feather was accurately caressed to ruffle the barbs during every washing step (fig. 2.14). Overall, washing procedure lasted 4-5 minutes for each sample. The washed feathers were placed into an open case coated with oven paper and divided into compartments to avoid contacts among different specimens and with the container and dried for 24h at 40°C (fig. 2.15).

This method follows the most common procedures used by researchers in order to meet the removal of exogenous contamination with the need to preserve metal profiles into the feathers (Burger, 1993; Gochfeld *et al.*, 1996; Dauwe *et al.,* 2003; Veerle *et al.,* 2004). Of course, there are also other approaches that span from analyze the sample "as it is" (e.g. Spahn and Sherring 1999; Herring *et al.*, 2009), or rinsing it 2-4 times with only deionized water (Hahn *et al.*, 1993; Pilastro *et al.*, 1993). The reason for avoiding use of strong reagents is that they could remove also some metals contained in keratin, and hence bioaccumulated (Edwards and Smith, 1984; Burger, 1993), except for mercury which is not affected by a variety of treatments (Applequist *et al.*, 1984). Actually, only few studies applied aggressive passages in ultrasonic baths using some chemicals,

such as non ionic surfactants, organic solvents, or a combination of both (e.g. Altmeyer *et al.*, 1991; Weyers *et al.*, 1988; Furness *et al.*, 1986), or other peculiar reagents (e.g. 0.25M NaOH, Sheifler *et al.,* 2006, or commercial surfactant detergent, Muralidharan *et al.,* 2004). Leonzio *et al.* (2009) avoided chemicals: they simply brushed dry feathers and cleaned gross particulate with an air jet. However, none of these methods assure the complete elimination of external contamination.

**Fig. 2.14** – A washing step. The feather, immersed in acetone is caressed again with fingers and brush. At this step gloves are needed (F. Borghesi)

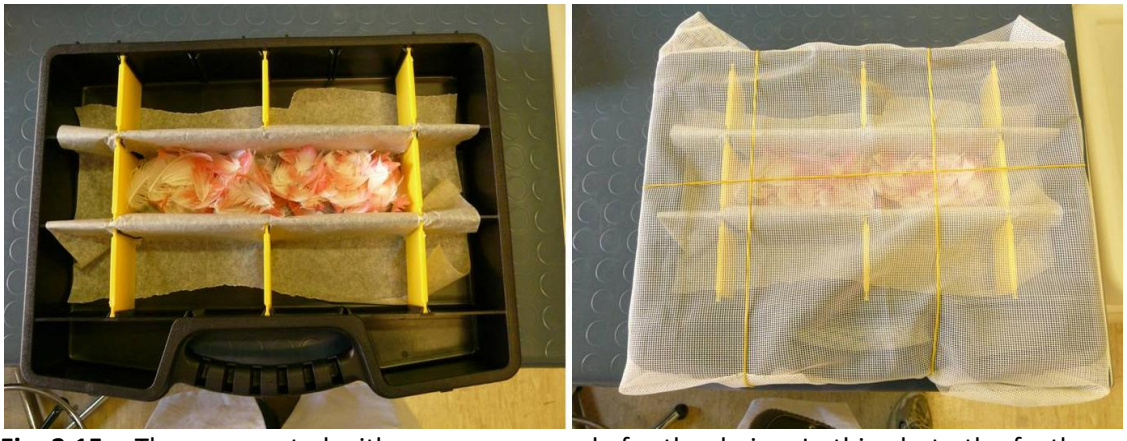

**Fig. 2.15** – The case coated with oven paper ready for the drying. In this photo the feathers of captive birds. The case could be subdivided to host up to 20 samples per time (F. Borghesi)

**Effectiveness of cleaning**

To verify the effectiveness of the cleaning procedure, selected feathers from different colonies were observed by scanning electron microscopy (SEM) using the SEM Philips 515, equipped with an energy-dispersive spectrometer (EDAX 9100) of the Department of Earth and Geo-Environmental Sciences of the University of Bologna.

Fragments of feathers were fixed on stabs, coated with gold (fig 2.16) and observed to verify the density and type of eventual dust fragments not removed by washing.

**Fig. 2.16** – Some feather segments were coated with gold and observed by SEM to verify eventual particles not removed by washing (F. Borghesi).

**Choice, snipping, and weighting of feathers**

Snipping a lot of feathers may take much time, hence a pre-weighting was performed to discharge the samples too small, to join the low-weighted samples in CS, and to put aside the feathers in surplus. The minimum weight fixed for each samples was approximately 260 mg, hypothetically corresponding to 2-4 longest scapular feathers, including vane and shaft, and excluding the quill. The number of feathers necessary to reach the minimum weight depends on the point of cutting individuated by the operator during the sampling from the birds. Weight limit was determined in order to represent each feather and each bird and avoid dependence on a single feather or a small part of it, as a significant variability in metal concentration among feathers may occur (Altmeyer *et al.*, 1991), as well as metals in feathers are not distributed homogeneously (Dauwe *et al.*, 2003).

Each sample was finely cut using stainless steel scissors and gloves and final dry weight was determined, directly into the Teflon® vessels for microwave digestion, using an electronic "Sartorius" BP310S balance to the nearest 0.001 g (fig. 2.17). Each sample was between 250 and 400 mg, and derived from 3-4 feathers of one bird in the case of SS and 2-3 feathers of 2-3 individuals in the case of CS.

**Fig. 2.17** – The grounded feathers are weighted directly in the Teflon vessel, ready for the acid digestion process (F. Borghesi)

**Sample digestion**

The samples were digested in the laboratory of the Department of Environmental Sciences (University of Siena) where similar works were recently performed (Leonzio *et al.*, 2009). Samples were digested in a 4:1 mixture of 65% $HNO_3$ and 30% $H_2O_2$ with a 30 minutes long microwave digestion procedure. The microwave program was well tried out with feathers by the laboratory and consisted in a temperature pathway reaching 120°C in 5 minutes, then holding this heat for few minutes, before a new rapid increase to the final 180°C until the end of the program (fig. 2.19). Each digestion batch hosted 24 Teflon® vessels and included two blank samples containing only the acid mixture to check the purity of chemicals used and possible contaminations (fig. 2.18). Certified samples were prepared in the same way. Calibration solutions were included: three samples composed by remnant feathers, and three composed by bovine muscle were prepared as above. A standard solution containing Cd, Cr, Co, Hg, Mo, Ni, Pb, and V, was added to them to three levels of concentration. Samples were randomized before digestion so that each digestion batch included samples from each site and of both type. Some of the sample were run in duplicate

in order to check for sample homogeneity. The clear, orange-yellow or green resulting fluid was diluted by adding deionized water to a final volume of 30 ml (fig. 2.20).

**Fig. 2.18** – A batch of 24 teflon vessels prepared for the digestion cycle (F. Borghesi)

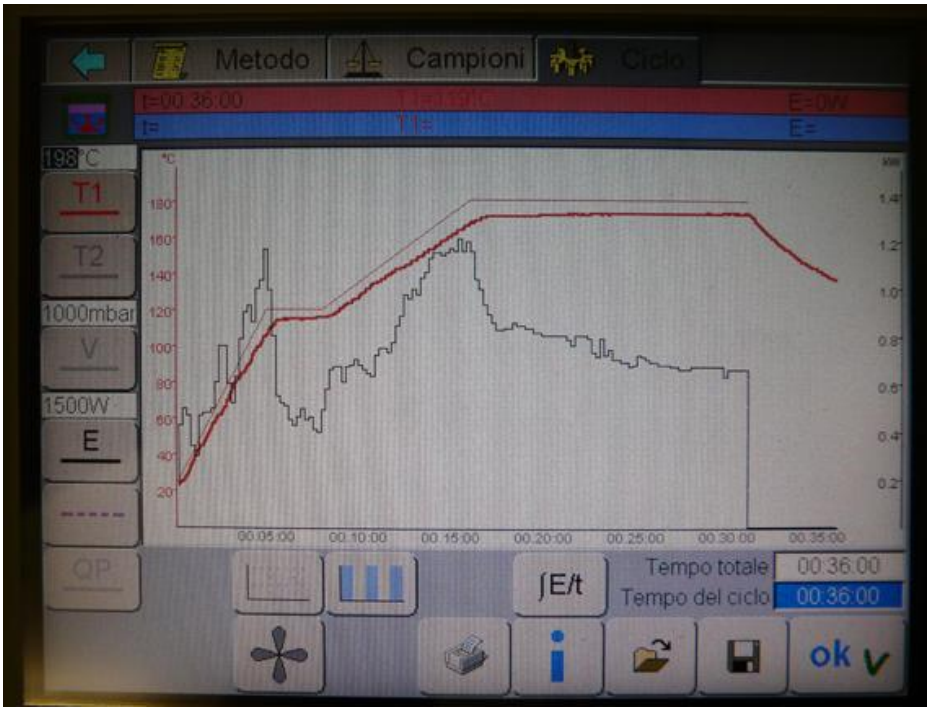


**Fig. 2.19** – The screen of the microwave software interface, after the digestion cycle. The narrow red line is the planned way of the temperature, the thick red line the real. The black line shows the power supply managed by the software (F. Borghesi)

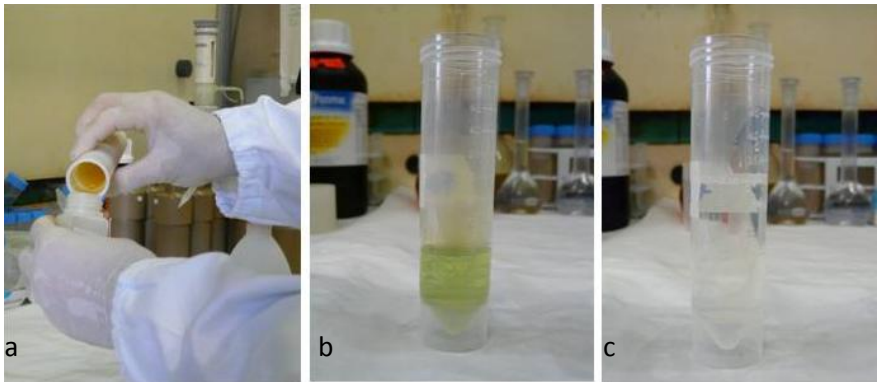


**Fig. 2.20 a-c** – Recovery of the digested sample [a], a digested sample before dilution [b], and a blank sample after dilution with deionized water to 30 ml [c] (F. Borghesi)

The method applied is comparable to other widely applied in the literature, although with slightly different strength and proportions among reagents (e.g. 1:1 mixture of 70% $HNO_3$ and 30% $H_2O_2$, Blust *et al.*, 1988; Eens *et al.,* 1998; Dauwe *et al.*, 2000; Janssens *et al.*, 2001). Somebody used modified procedures (Connell *et al.*, 2002, following Zhou and Liu, 1997, used a 5:2 mixture of 65% $HNO_3$ and 100% Milli-Q water following addition of $H_2O_2$ during digestion). Recent studies applied some other method on feathers, as a 5:2 mixture of concentrated $H_2SO_4$ and $HNO_3$ (Herring *et al.*, 2009) or simply pure $HNO_3$ (Lucia *et al.,* 2010).

*2.4 Chemical analysis*

**Analytical methods**

The cooperation given by BGR laboratory of Berlin (Germany), below shorten BER, allowed the analysis of a wide number of elements. Department of Environmental Sciences of Siena University (SIE) also offered its collaboration and was able to analyze mercury, lead and cadmium. In this positive scenario, it was possible, on one hand to obtain a rare huge database regarding metals in feather of flamingos, on the other hand, comparable results from two different laboratories and between different analytical instrumentation for Cd, Hg, and Pb. Figure 2.21 shows an overall visual synthesis of all analyzed elements.

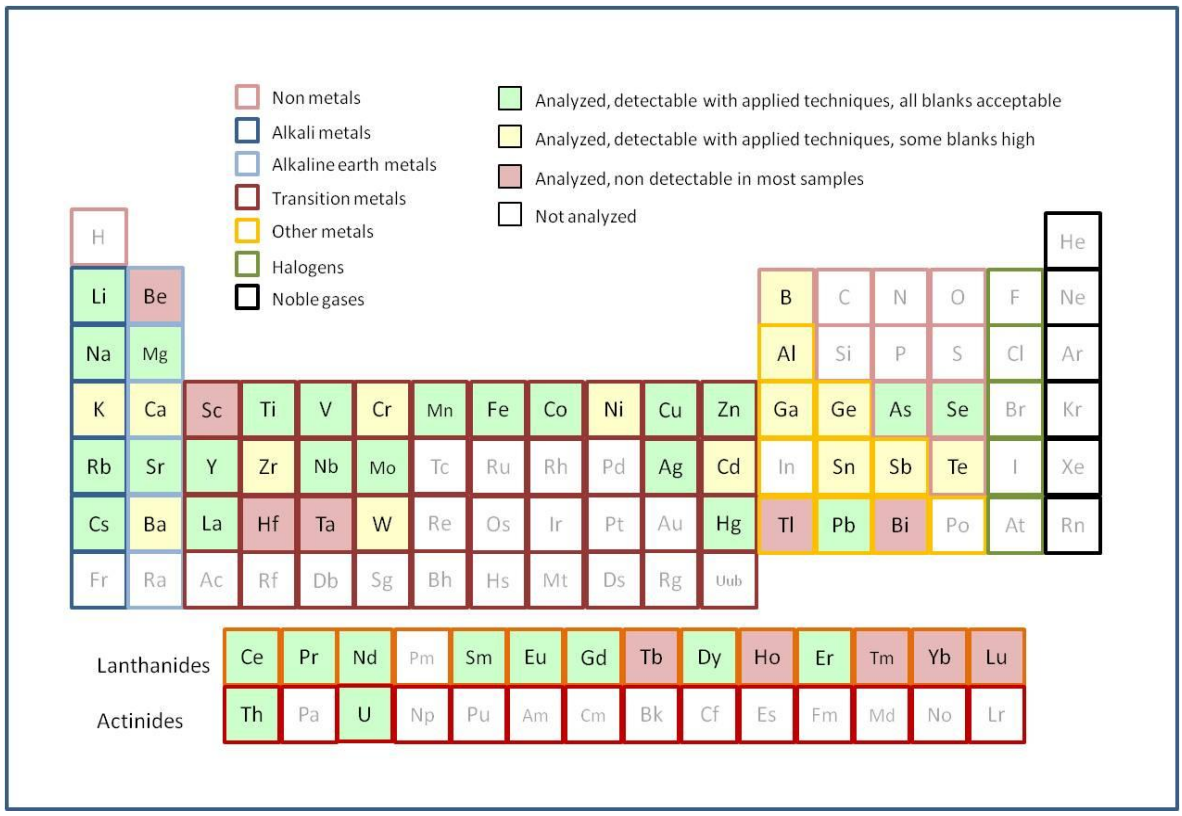


**Fig. 2.21** – A synoptic table of all elements. Green filled squares relate to elements resulted detectable in feathers with applied techniques. Yellow squares refer to elements detected and measured which blanks samples resulted high in value. Elements in red squares resulted under the detection limits or strictly close. White squares are the element not analyzed at all. The colour of the frame indicate the type of element (F. Borghesi)

Mercury was analysed by cold vapour flow injection mercury system (CV-FIMS; Perkin Elmer® 400) at Siena University and atomic fluorescence spectrometry (AFS; Instrument PSA 10.035 Millennium Merlin® 1631) at BGR laboratory in Germany. Both analyses were performed on aliquots of the same solution. Details of technical characteristics are resumed in Appendix A.

The analysis of other elements (Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Ho, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, Pb, Pr, Rb, Sb, Sc, Se, Sm, Sn, Sr, Ta, Tb, Te, Th, Ti, Tl, Tm, U, V, W, Y, Yb, Zn, Zr) was performed by inductively coupled plasma quadrupole-based mass spectrometry (ICP-QMS) on the same solutions carried out in the BGR laboratories. As the best method to analyze Al, Ca, Fe, K, Mg, and Na should be inductively coupled plasma atomic emission spectrometry (ICP-AES), 50 extraction were checked by both kind of instruments showing good accordance. The Cd and Pb were also analysed by Graphite Furnace Atomic Absorption

Spectrometry (GFAAS) at the University of Siena. Details of technical characteristics are resumed in Appendix A.

A reference table of detection limits for GFAAS, ICP-AES, and ICP-QMS is reported in Appendix B.

**Quality control**

Certified Reference Materials were also analyzed. It was impossible to find certified materials of feathers. Some certified human hairs are to be found, but not tested for a good number of metals. Normally, human hairs are prepared to search organic compounds and only few metals. Some samples of Certified Materials (about 250-260 mg each one) were randomly inserted among samples of feather for digestion and analysis. It was possible to get three kind of certified materials:

- DORM-2.

  Dogfish (*Squalus acanthias*) muscle and liver. In tab. 2.2 comparisons among certified values and laboratory results are reported. The uncertainties represent 95 percent tolerance limits for an individual sub-sample of 250 mg or greater. DORM-2 was tested for homogeneity at the National Research Council (NRC) in Ottawa, Canada, and prepared following the advice of the NRC Committee on Marine Analytical Chemistry. The results for the Certified Reference Materials (samples of 259-362 mg) were in agreement with certified values as reported in table 2.2 for most elements, except for aluminium, chromium, and tin. The value obtained for lead from both laboratories is very different from CRM value. Calculating related errors, Berlin laboratory resulted more precise for Cd, Siena for Pb, and both very precise for Hg. Regarding the accuracy, Siena was more close to CRM values for all three elements. Total mercury tended to be underestimated by both laboratories.

**Tab. 2.2** - Results for the DORM-2 Certified Reference Materials (CRM) analyzed in the two laboratories (mean±SD; relative error RE). Certified values (C.V.) and uncertainty (unc.) are also included. Concentration are expressed as mg/kg.

| Element | C.V. ± unc. | Berlin | | Siena | |
|---|---|---|---|---|---|
| | | Mean ± SD | RE (%) | Mean ± SD | RE (%) |
| Aluminium | 10.9 ± 1.7 | 30.0 ± 3.5 | | | |
| Arsenic | 18.0 ± 1.1 | 17.7 ± 0.6 | | | |
| Cadmium | 0.043 ± 0.008 | 0.053 ± 0.007 | 13.2 | 0.048 ± 0.011 | 22.9 |
| Cobalt | 0.182 ± 0.031 | 0.160 ± 0.011 | | | |
| Chromium | 34.7 ± 5.5 | 27.8 ± 2.75 | | | |
| Copper | 2.34 ± 0.16 | 2.05 ± 0.08 | | | |
| Iron | 142 ± 10 | 138 ± 8.74 | | | |
| Lead | 0.065 ± 0.007 | 0.24 ± 0.06 | 25.0 | 0.228 ± 0.027 | 11.8 |
| Manganese | 3.66 ± 0.34 | 3.27 ± 0.190 | | | |
| Mercury | 4.64 ± 0.26 | 3.938 ± 0.114 | 2.9 | 4.117 ± 0.116 | 2.8 |
| Nickel | 19.4 ± 3.1 | 14.2[2] ± 1.27[2] | | | |
| Selenium | 1.40 ± 0.09 | 1.46 ± 0.06 | | | |
| Silver | 0.041 ± 0.013 | 0.035 ± 0.004 | | | |
| Thallium[1] | (0.004) | 0.004 ± 0.001 | | | |
| Tin[1] | (0.023) | 0.11 ± 0.002 | | | |
| Zinc | 25.6 ± 2.3 | 27.2 ± 0.61 | | | |

[1]Information value only
[2]One lecture of Nickel failed, therefore here is not included in this value

- ERM®-CE278.

  Mussel tissue (*Mytilus edulis*). The certified uncertainty is the half-width of the 95 percent confidence interval of unweighted means of 4 to 11 data sets.

  European Reference Material ERM®-CE278 was originally certified as BCR-278R. It was produced and certified under the responsibility of the Institute for Reference Materials and Measurements (IRMM), Geel (BE). The amount suggested to be used for each sample is 400 mg. The results for mussel tissue Certified Reference Materials are strongly in agreement with certified values as reported in tab. 2.3 for all elements, Even for chromium (Al and Sn are not certified in ERM®-CE278). Related errors resulted evidently lower in BGR laboratory results for Cd and Hg, while comparable for Pb. Cd and Pb means are more accurate in Berlin results, but Hg underestimated, as noticed for DORM-2. On the

other hand, Siena reported low values for Cd and Pb, and high value for Hg. Altogether the according with CRM may be considered very good.

**Tab. 2.3** - Results for the ERM®-CE278 Certified Reference Materials (CRM) analyzed in the two laboratories (mean±SD; relative error RE). Certified values (C.V.) and uncertainty (unc.) are also included. Concentration are expressed as mg/kg.

| Element | CRM ± unc. | Berlin | | Siena | |
|---|---|---|---|---|---|
| | | Mean ± SD | RE (%) | Mean ± SD | RE (%) |
| Arsenic | 6.07 ± 0.13 | 6.28 ± 0.17 | | | |
| Cadmium | 0.348 ± 0.007 | 0.358 ± 0.012 | 3.4 | 0.283 ± 0.033 | 11.7 |
| Chromium | 0.78 ± 0.06 | 0.83 ± 0.19 | | | |
| Copper | 9.45 ± 0.13 | 9.47 ± 0.34 | | | |
| Lead | 2.00 ± 0.04 | 2.00 ± 0.05 | 2.5 | 1.884 ± 0.056 | 3.0 |
| Manganese | 7.69 ± 0.23 | 7.96 ± 0.45 | | | |
| Mercury | 0.196 ± 0.009 | 0.180 ± 0.009 | 5.0 | 0.239 ± 0.023 | 9.6 |
| Selenium | 1.84 ± 0.10 | 1.82 ± 0.06 | | | |
| Zinc | 83.1 ± 1.7 | 85.5 ± 1.05 | | | |

- BCR-414.

  It consists of a powder of freeze-dried plankton. This CRM provides certified means of 11 metals, with uncertainty is the half-width of the 95 percent confidence intervals. Unweighted means were obtained from 4 to 17 data sets. For other 6 metals, an indicative value is reported, with uncertainty given as standard deviation. The minimum amount to be used for each sample is fixed in 100 mg. This material has been certified by BCR (Community Bureau of Reference). The certificate has been revised under the responsibility of IRMM. The method applied in BGR laboratories tended to underestimate all the values of this plankton Certified Reference Materials. As, Cd, Co, Cr, Cu, Fe, Pb, and Mo results were low but acceptable, whereas Hg, Mn, Ni, Sc, Se, Sr, V, and Zn were slightly out of range (tab. 2.4). Siena Hg mean is very good, but this laboratory underestimated Cd and Pb similarly to Berlin. Potassium in Berlin samples provided a result 1000 times higher than the reference value. Related errors resulted once again lower in BGR laboratory. Evidently, metals in this CRM are not easy to extract and detect even using different methods. Only CV-FIMS seems able to detect all mercury but with a humble precision.

In general, no clear overestimate resulted from all analytical results performed in both laboratories and using different analytical methods. The metal concentrations regarding feathers here presented may be considered of good precision and accuracy, if anything for many metals slightly conservative.

**Tab. 2.4** - Results for the BCR-414 Certified Reference Materials (CRM) analyzed in the two laboratories (mean±SD; relative error RE). Certified values (C.V.) and uncertainty (unc.) are also included. Concentration are expressed as mg/kg.

| Element | C.V. ± unc. | Berlin | | Siena | |
|---|---|---|---|---|---|
| | | Mean ± SD | RE (%) | Mean ± SD | RE (%) |
| Arsenic | 6.82 ± 0.28 | 6.40 ± 0.20 | | | |
| Cadmium | 0.383 ± 0.014 | 0.349 ± 0.022 | 6.3 | 0.261 ± 0.029 | 11.1 |
| Cobalt[1] | 1.43 ± 0.06 | 1.29 ± 0.04 | | | |
| Chromium | 23.8 ± 1.2 | 20.9 ± 1.50 | | | |
| Copper | 29.5 ± 1.3 | 27.1 ± 1.63 | | | |
| Iron[1] | 1850 ± 190 | 1780 ± 50 | | | |
| Lead | 3.97 ± 0.19 | 3.65 ± 0.39 | 10.7 | 3.68 ± 0.60 | 16.3 |
| Manganese | 299 ± 13 | 256 ± 15 | | | |
| Mercury | 0.276 ± 0.018 | 0.200 ± 0.007 | 2.9 | 0.270 ± 0.043 | 15.9 |
| Molybdenum[1] | 1.35 ± 0.20 | 1.22 ± 0.05 | | | |
| Nickel | 18.8 ± 0.8 | 13.8 ± 2.52 | | | |
| Potassium[1] | 7.550 ± 0.17 | 6794 ± 0.49 | | | |
| Scandium[1] | 0.54 ± 0.02 | 0.35 ± 0.04 | | | |
| Selenium | 1.75 ± 0.10 | 1.54 ± 0.06 | | | |
| Strontium[1] | 261 ± 25 | 229 ± 4.16 | | | |
| Vanadium | 8.10 ± 0.18 | 7.06 ± 0.44 | | | |
| Zinc | 111.6 ± 2.5 | 100.0 ± 2.60 | | | |

[1]Metal provided with a CRM indicative value and standard deviation instead of half-width of the 95 percent confidence intervals.

*2.5 Statistical methods*

In this study, for each variable are shown arithmetic medians, median absolute deviations (MAD), means, standard deviations (SD), minimum and maximum (min, max), coefficient of variation (CV), and number of samples (n), subdivided according to colony and sample type. All sample duplicates were included in the working database.

MAD is defined as the median of the absolute deviations from the median of a univariate dataset:

$$MAD = median_i\left(\left|X_i - median_j\left(X_j\right)\right|\right)$$

It was preferred to SD to describe the dispersion of data, because it is thought a more robust estimator of scale, and appears more resistant to outlier values (Huber, 1981).

Coefficient of variation is the ratio between the SD and mean (in percentage). Mean and CV are reported to allow some comparisons with literature, but mean is not very suitable to describe this kind of ecotoxicological data distributions, whereas median values appear more appropriate to show central tendency (Reimann *et al.*, 2008).

Correlations among variables were analysed by using the determination coefficient ($R^2$) in a linear regression model. The distributions of variables for each colony and sample types are illustrated by the cumulative frequency distribution, which is a plot of the number of observations falling in or below an interval. The cumulative curves were preferred to other graphical methods, because they describe data dispersion and allow an easy identification of possible subpopulation within each colony.

The variables in each group were subjected to the Shapiro–Wilk test for normality. Nonparametric Kruskal–Wallis test by ranks was used to test equality of population medians among groups defined by the sampling site and sample type (Helsel and Hirsch, 1992). Statistical calculations and analyses were performed using MS Excel® and the DAS+R module.

# 3 THIRD SECTION

## *3.1 Comparison between laboratories*

A comparison among 229 results obtained from the two laboratories (BER and SIE) was possible relatively to three metals: Cd, Hg, and Pb. Some considerations have been formulated about other elements in relation to Certified Reference Materials in the paragraph 2.4.

The agreement among the results is very good for Hg, as testified by the limited dispersion around the 1:1 line in figure 3.1, with only few samples scattered. A very slight tendency to return lower values may be observed for BER results at the low and medium concentrations, but, on the whole, the alignment along the line of one-to-one is very good. One sample evidently reveals an error having about 5 mg/kg in BER data set and 0.5 mg/kg in SIE. It is a sample from Camargue (white diamond in the fig. 3.1), to handle with attention when Hg discussion will be made. A Sardinian composite sample differs among the to labs for 1 full ppm (3.49 mg/kg vs 2.47 mg/kg, BER vs SIE). This sample (yellow diamond), was split before digestion and replicated in both labs, and returned 3.10 mg/kg from SIE and 3.45 from BER. In spite of a quite wide variability, it may be considered anyway. Rather distant from each other are a Sardinian single sample and one SS from Comacchio. The Sardinian (red diamond) have a duplicated too, that confirm a concentration for this sample around 0.5 mg/kg. For the latter scattering sample (green diamond) a repetition is not available.

This result, supported by the good correspondence to CRM values, suggests that both analytical methods (CV-FIMS used by SIE; AFS by BER) are suitable for analysis of total mercury in feathers. The quality of analyses is quite comparable and good.

So good one-to-one correspondence does not occur for Pb. As shown in fig. 3.2, most of data lie under the 1:1 line. This means that tendentially SIE underestimate Pb concentrations in feathers respect to BER. This become more evident over 1 mg/kg, but it seems true already at lower levels of Pb. Only six samples obviously except from this “rule”. They are samples where SIE result is evidently higher than BER. The six samples are spacing throughout all the data interval, and refer to samples very different from each other.

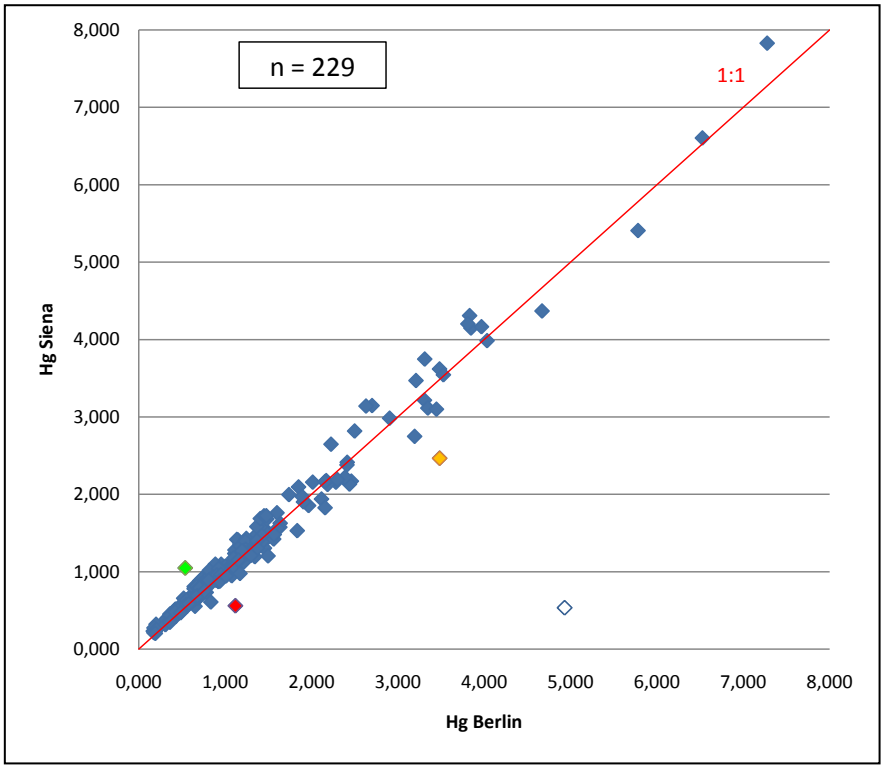


**Fig. 3.1** – Relationship between Hg results from Berlin and Siena laboratories. Red line show 1:1 correspondence. Data are plotted with blu diamonds, while four scattering data are highlighted by different colour (n=229).

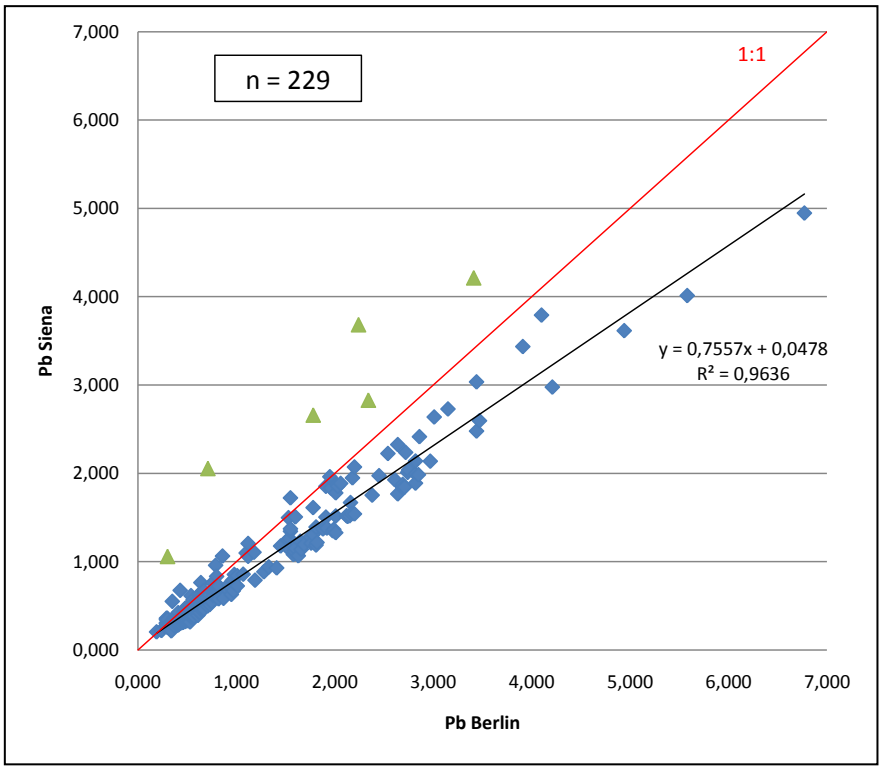


**Fig. 3.2** – Relationship between Pb results from Berlin and Siena laboratories. Red line show 1:1 correspondence. Data are plotted with blu diamonds, while six scattering data are highlighted by green colour (n=229). The interpolation line is calculated without the six outliers.

They are (from the lowest to the highest concentration):

- a SS from Cagliari
- a CS from Odiel
- a SS from Venice
- two SS from Comacchio
- a plankton Reference Material (BCR-414)

The expected value of the CRM was 2.00 ± 0.04 mg/kg, while SIE reported for this sample 4.213 mg/kg. This lead to believe that also the other five samples are definitely overestimated.

If that six samples are excluded, the interpolation line of all data show a very high correlation between the two labs. A slight divergence for high concentrations (over 2 mg/kg) can be observed.

In definitive, both analytical methods (GFAAS used by SIE; ICP-QMS by BER) are suitable to analyze Pb in feathers, but with some warnings for GFAAS. Both methods returned good estimates on CRM, with SIE a little lower, so that an overall underestimate of GFAAS of Pb in feathers may be possible. However, results from both methods may be taken in account as conservative. In addition, GFAAS shows some times sporadic anomalous results. This suggests to prepare an adequate number of repetitions (of the same digested solution) in order to test time by time the performances of the instrument. ICP-QMS is very sensitive and reliable.

Considering Cd, a more complicated situation appears. Apparently, any type of correlation between the two laboratories happens (fig. 3.3). Actually, at least three subsets may be individuated. In correspondence with the range of concentrations between 0.300 and 0.400 mg/kg, eight analytical results do not differ a lot between the two labs. Seven of them are CRM. As observed for Pb, in this case BER tends to return values somewhat higher than SIE. This group contains also one flamingo sample, an adult found dead, not discussed here.

Another group with good one-to-one correspondence lie at very low concentrations. This group of samples is plotted apart in fig. 3.4. There is a high correlation and a good 1:1 correspondence relatively to this large subset (n=186). On the other hand, in the same interval, for 33 samples, that is where SIE results are higher than BER, no correlation can be found (fig. 3.4).

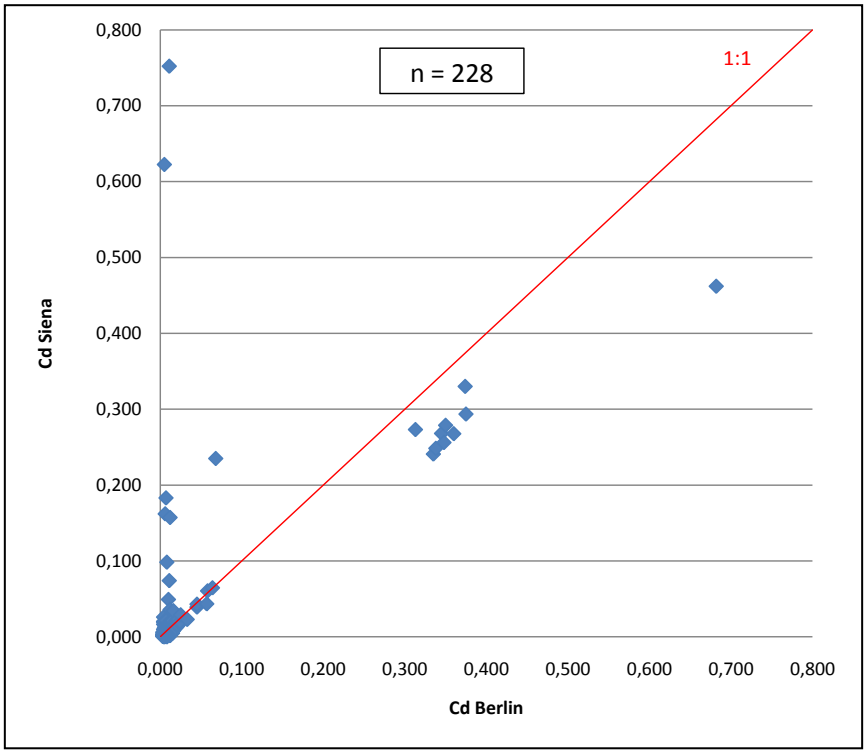


**Fig. 3.3** – Relationship between Cd results from Berlin and Siena laboratories. Red line show 1:1 correspondence. Data are plotted with blu diamonds (n = 228). The interpolation line is calculated without the six outliers.

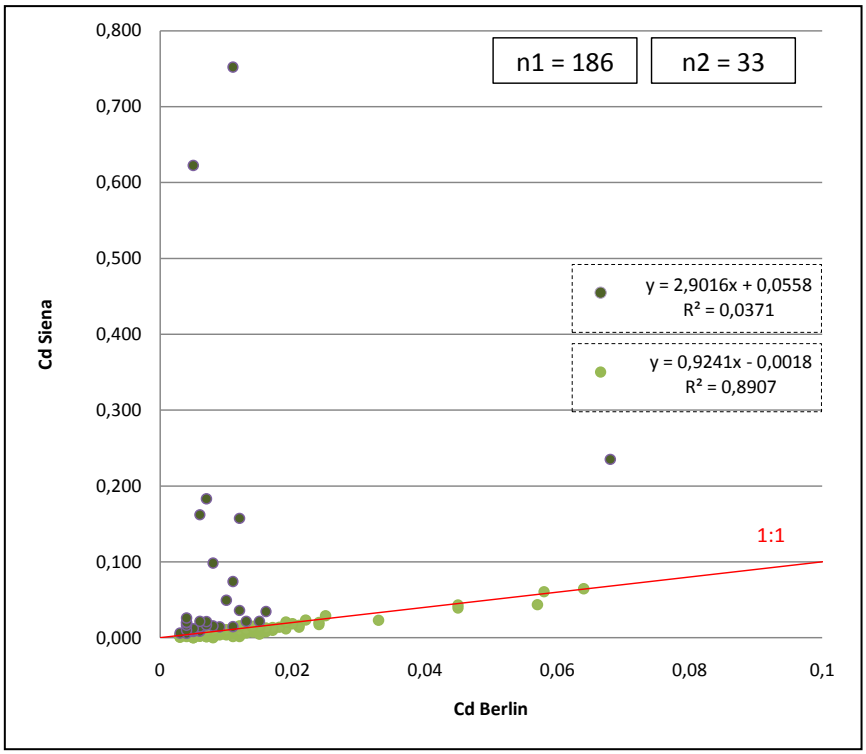


**Fig. 3.4** – Relationship between samples below 0.08 mg/kg Cd from BER and SIE labs. Data are plotted with violet dots (BER result higher than SIE) and green dots (viceversa). The regression equations and determination coefficients are calculated for the two groups of samples. Red line show 1:1 correspondence.

For Cd in feathers, GFAAS does not seem reliable enough. More samples showed low values, less than 0.7 mg/kg, and in this range too many values scattered. It is presumably that Cd concentrations in feathers are too close the detection limits for graphite furnace. A better behaviour is recordable for concentrations among 0.3 and 0.4 mg/kg. The series of results obtained in BER seems is sensitive and less prone to produce "inconsistent" data. A CRM containing 0.043 ± 0.008 mg/kg was available and both labs returned similar values. Thus anomalous results of SIE may be considered random events, not easy to control. ICP-QMS might be the unique technique to use for Cd in feathers.

*3.2 Elements in flamingo feathers: the whole dataset*

Fifty-eight chemical elements were analyzed (see § 2.4, fig. 2.21). Results of almost elements with detectable concentrations are graphically presented in fig. 3.5 (SS) and fig. 3.6 (CS) ranked by medians. Descriptive statistics are reported in tab. 3.1 (SS) and tab. 3.2 (CS). Having many missing data, or heavy overlap with blanks results, Bi, Hf, Ho, Lu, Sc, Ta, Tb, Tl, Tm, even if analyzed, are not reported. A group of elements with detectable levels in feathers were unfortunately affected by high levels in blank samples that masked the true concentration values, and consequently were also excluded from the graphical representations. They were: B, Ba, Ca, Cd, Ge, Sb, Sn, Te. A different choice was made for Al, Be, and W, which also showed high blanks, but apparently no appreciable adding effects on flamingo feathers concentrations were observed. Thus, results of Al, Be, and W are presented anyway, warning about the anomaly on blank values. All data refer to BER laboratory.

Regarding SS (Cagliari, Comacchio, Odiel, Venice), median values of almost elements were lower than corresponding mean values (fig. 3.5). This indicates that distributions are asymmetric, and characterized by high density of values in the lower range of the dataset, and a smaller group of individuals with high values. This tendency is more pronounced for Al, As, Co, Cs, Ga, Hg, Mn, Pb, Rb, V, but appreciable also for Ce, Cr, Dy, Er, Fe, Gd, Nb, Nd, Ni, Pr, Sm, Ti, Y. The reason is due to the occurrence of a well defined group of samples with very different concentration. Except As (Odiel), Hg (Cagliari), Pb and Cr (Comacchio and Venice), the distinct group in the upper range for all these elements is due solely to Comacchio colony. On the other hand, Na, Mg, K, Zn, Sr, Cu, Se, and U had median and mean very close, thus a more symmetric distribution.

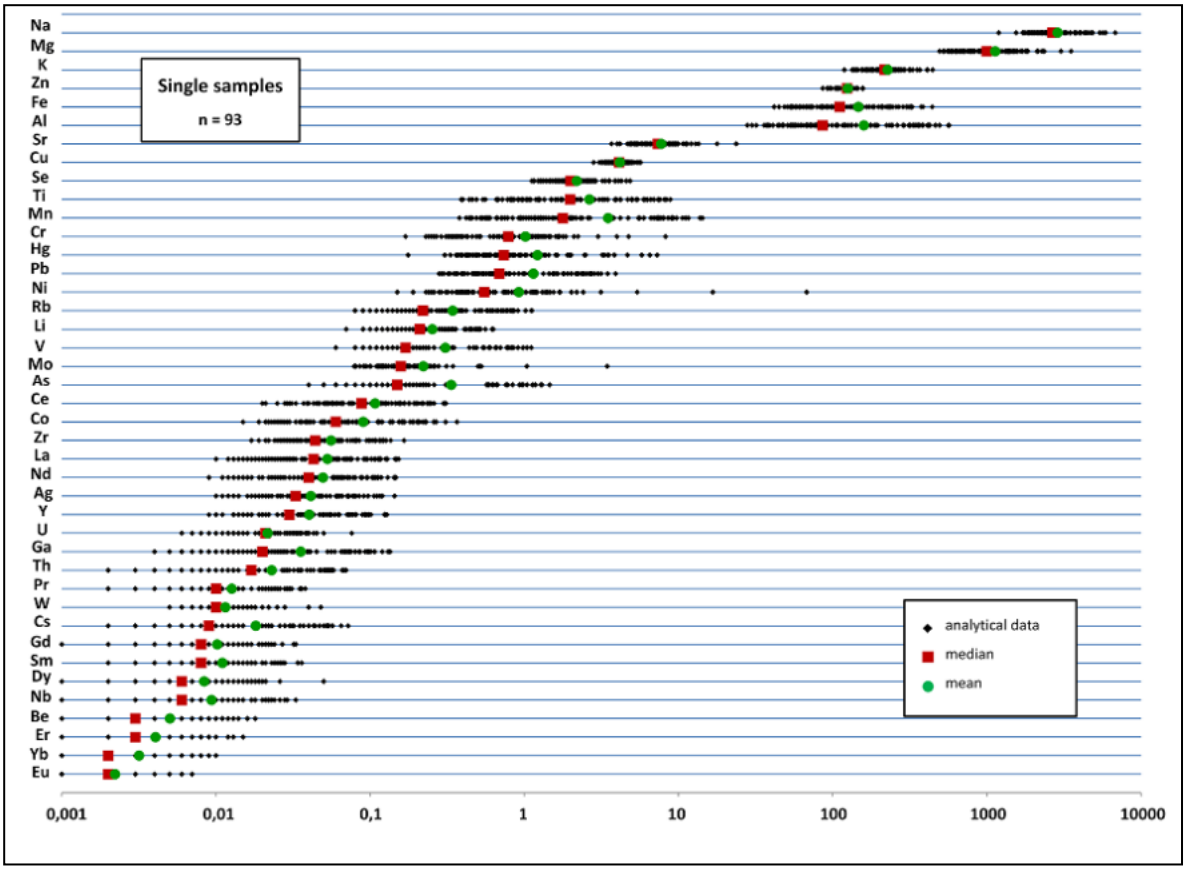


**Fig. 3.5** – Overall diagram of detectable elements (analytical data are black rhombs) in SS, ranked by medians. Median (red square) and mean (green dot) are plotted for each element. Concentrations are expressed in mg/kg d.w. and reported in a log scale.

Na, Mg, K, Zn, Fe, Al have central values over one hundred mg/kg. Na, Mg, and K are essential elements very abundant in all organisms. The high concentrations and the homogeneous distribution, especially for Na and Mg, indicate their importance in the feathers' composition. Al is known as a metal without important biological functions, but it resulted high in flamingo feathers (average 159 mg/kg Al), and dispersed. It is not commonly analyzed, but recently Lucia *et al.* (2010) reported Al data in unwashed feathers of geese and waders. They reported means of 226 mg/kg, 107 mg/kg, and 96 mg/kg in different species. The Al levels found in flamingos seem rather high, but within the cited range, if considering the different sample treatment and the much higher number of specimens in the present work. This might indicate some external contamination by clays not fully removed by our washing procedure. This problem perhaps partially affected also Fe, whose distribution is similar to Al, even if it is an important blood component and surely enters in keratin structure during feather growth. Differently from Na, Mg and K, where the eventual contribution from soil particles seem less relevant compared to the endogenous accumulation, Al and Fe may be mainly exogenous. In case of Zn, a very compact distribution and a perfect

accordance between median and mean is observed, however this element is fully discussed in paragraph 3.9.

Within the range 1-10 ppm, there are five elements: Sr, Cu, Se, Ti, Mn (fig. 3.5). For Sr and Cu the accordance between median and mean is good and both display low dispersion. Not many data are available for Strontium in feathers in literature, but it is known that it is a non-nutrient trace element that tends to follow calcium during nutrient uptake, internal distribution, and excretion within organisms (Blum *et al.*, 2000). The levels and the distribution of Sr in Flamingo feathers may be a result of some physiological regulation, but reliable Ca results are not available, to compare these elements between them. Extensive is literature on Copper, even in Greater Flamingo, and will be discussed in detail in § 3.6. Selenium is an essential element for animals and humans, but some human activities (burning of fossil fuels, industrial copper refining, production of glass and electronics, fertilizers) are responsible for the redistribution of selenium in the environment (IPCS, 1987). In this study Se resulted the 9$^{th}$ element in median value (median 2.0 mg/kg, mean 2.2 mg/kg, min 1.1 mg/kg, max 4.9 mg/kg), data very similar to those reported by Cosson *et al.* (1988) in covered part of primary flight feathers of adult flamingos in Camargue (mean 2.7 mg/kg, min 1.3 mg/kg, max 5.6 mg/kg). The same authors reported higher values in the exposed part of the same feathers (mean 5.0 mg/kg, min 2.4 mg/kg, max 12.0 mg/kg) and related it to some kind external contamination in adult birds. On the other hand, Amiard-Triquet *et al.* (1991) found very low levels of Se in Flamingo fledglings in Camargue (mean < 1 mg/kg, min < 0.4 mg/kg, max 2.3 mg/kg) and they did not observed any difference between outer and inner barbs of newly growth greater wing coverts. Burger (1996) reported that 42 studies on birds found 0.3 - 54 mg/kg d.w. of Se in feathers, with median value of 2.2 mg/kg. In conclusion, our results for Se are comparable to literature data even if appreciable higher concentrations and a more dispersed distribution were observed for Cagliari samples compared to other sites. It is likely that feathers could have considerable levels of Se of endogenous origin, considering the low dispersion of the concentrations and the good accordance between median and mean. Titanium and Manganese were also relatively high in flamingo feathers. Both metals showed very dispersed data and irregular distributions, with definitely higher levels in Comacchio samples. Titanium medians were similar in the other colonies, while Mn was higher in Odiel than Cagliari and Venice. Same considerations made for Al applies for Ti, while Manganese is also an essential element. In flamingo feathers was found a Mn median value of 1.8 mg/kg (min 0.4, max 14.3). The median value of Mn calculated on 19 studies on bird feathers was 3.4 mg/kg, with a huge variability (Burger, 1996). Concluding, a significant external contamination by soil particles seemed to be likely for Mn and Ti.

Cr, Hg, Pb, and Ni formed a group of metals with median value between 0.5 and 0.8 mg/kg (fig. 3.5). Mercury and Pb are the most frequently assessed metals in feathers, therefore they will be discussed in detail in § 3.8 and § 3.7. All metals in this group are non-essential and toxic at low concentrations. Top sediments and soils of Comacchio lagoon surroundings are naturally enriched in Cr and Ni (Amorosi and Sammartino, 2007) and Comacchio bird feathers drove the central values of Cr and Ni towards 0.8 and 0.55 mg/kg respectively, whereas they would be half in value considering only Cagliari and Odiel datasets. Venice showed Cr high values, but unexpectedly very low concentrations of Ni, whereas Comacchio was high for both. Many high outliers are also present for both metals, especially for Ni. Compared to median values of Cr reported by Burger (1996) (8.1 mg/kg considering 17 studies) Cr in flamingo feathers appears rather low.

Medians of five elements lie in the range 0.15 - 0.22 mg/kg: Rb, Li, V, Mo, As (fig. 3.5). Rubidium, V, and As show medians very different from means and are dispersed. Lithium and Mo show quite comparable median and mean. Lithium, as essential element, is to date not clear, but recently some biological function were supposed (Ermidou-Pollet and Pollet, 2006). However, even if the biological importance were true, the biological requirements of Li is thought very low, thus it reasonable that concentrations found in feathers might be due mainly to exogenous contributions, though the origin of Li in feathers is to be verified. Molybdenum is an essential trace element for several enzymes in mammals (Moura and Xavier, 1978), but that function is little explored in birds. Molybdenum results are particular, since most of samples from all sites shows Mo concentration in a short range, but few high outliers caused a remarkable divergence between median and mean. One colony (Odiel) stand out for Mo lowest levels and lowest dispersion. Arsenic is considered an important element in toxicology, poisonous for life. Sometimes of natural origin, in many cases it is an anthropogenic pollutant, and will be discussed in §3.5.

The remaining 21 elements have a median concentration lower than 0.1 mg/kg (fig. 3.5). Among them there are all the 11 rare earth elements (REE) detectable in flamingo feathers. All REEs show an invariant pattern: Comacchio with the definitely higher concentrations, Venice with the lower, Cagliari and Odiel in the middle. Cerium, Er, Gd, and Sm are slightly lower in Cagliari than Odiel, while Dy, La, Nd, Pr, Y, and Yb seem more similar among the two sites (tab. 3.1). The mean concentrations of lanthanides (15 of the 17 elements named rare earth) in the earth's crust are comparable to those of life-important elements like cobalt and selenium. For some, biological assumption and some toxicological effects are demonstrated in humans (Pałasz and Czekaj, 2000). In particular, the researchers reported cytophysiological effects or enzymes interferences for Dy, Eu, Gd, La, Pr and Tb probably due to some chemical analogies with Ca. It is impossible to

understand if flamingos can intake REEs, perform some biological treatment of them, and somehow put them in feathers. More information about sediments of nesting sites will helps to solve this point. At this moment, the most plausible hypothesis is that REEs in feathers derive from soil particles captured by barbs and barbules, and not washed away.

Non-REEs in this wide group of not much concentrated elements are Ag, Be, Co, Cs, Ga, Nb, Th, U, W, and Zr (fig. 3.5). All these elements are rather dispersed in asymmetric way, except U and W. Silver singularly shows a reversed pattern compared to other elements: it is more concentrated in Odiel flamingo feathers, and virtually absent in Comacchio. Cagliari and Venice samples have similar low levels between the two extreme groups. Cesium, Ga, and Zr had patterns identical to REE, with Odiel and Cagliari quite similar. Cobalt is apparently similar due to much higher concentrations in Comacchio birds, but Odiel and Cagliari are sharply different, the latter more similar to Venice at the lowest levels. Regarding Nb and Th, the ranking is different, resulting Odiel lower than Cagliari, with Venice and Comacchio extremely low and high respectively. For this two elements without known biological functions, some Cagliari samples group with Comacchio ones at high concentrations (tab. 3.1).

Regarding CS, Venice samples were not available, while samples from Camargue and Ebro delta were added to the series of data. In general, all points discussed for SS are confirmed for CS. However, Ebro delta flamingo feathers revealed anomalies for some elements, which explains some difference in distribution (fig. 3.6).

The first group of constitutive elements (Na, Mg, K) does not change the general distribution compared to SS, even considering Ebro and Camargue (tables 3.1 and 3.2). On the contrary, median of Mg is higher in CS. Al, Fe, Zn in CS are not very different from SS. Zinc is discussed in detail in § 3.9.

In the second group (Sr, Cu, Se, Ti, Mn) some differences appear. High Strontium outliers present in SS disappear (fig. 3.6), reducing overall variability, while the central values in CS increase. Homogeneity of Cu and Se distributions resulted greatly consolidated in CS (Cu is discussed in § 3.6). Selenium median in CS is definitely lower than in SS, due to the addition of Camargue and Ebro delta data (tab. 3.2). The smaller variability of Ti and Mn in CS depend mostly on average levels of this metal both in Ebro and Camargue (tab. 3.2). In the third group (Cr, Hg, Pb, and Ni), Chromium and Nickel partially lose SS outliers. Actually, a contraction of the range is appreciable in CS for Cr and Ni, but Ni is so high in Ebro delta that a drop of the median is observed only for Cr, whereas median of Ni is higher in CS than in SS. The effect of Ebro and Camargue on Hg is clear (tab. 3.2). Both sites showed dispersed and medium to -high Hg levels. However, Hg resulted

unexpectedly higher in CS series for all sites, but Cagliari, so that median value in CS was definitely higher than SS (tables 3.1 and 3.2). Mercury resulted much less variable in CS (Hg is discussed in detail in § 3.8). Lead seems to behave as expected for median, a little lower in CS. On the other hand, distribution results more dispersed in CS. The reason of this was quite complex and fully examined in § 3.7.

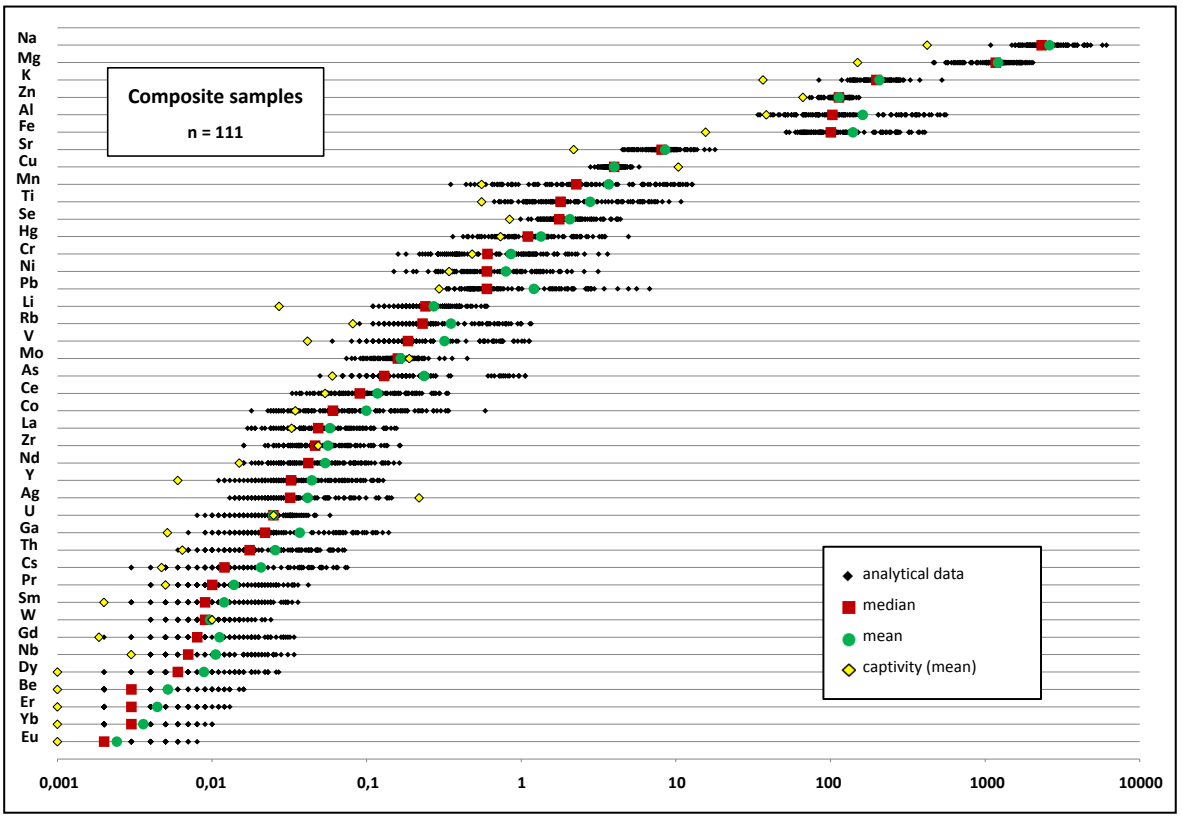


**Fig. 3.6** – Overall diagram of detectable elements (analytical data are black rhombs) in CS. Median (red square) and mean (green dot) are plotted for each element. Means of captive birds are also plotted (yellow diamonds). Concentrations are reported in a log scale (mg/kg d.w.).

The fourth group, including Li, Rb, V, Mo, As, fell in the range 0.13–0.24 mg/kg, similar to SS. Lithium, Rb, and V in CS resulted unchanged regarding dispersion of data, but medians increased in CS, because those metals are high in Camargue and Ebro (tab. 3.2). The considerable number of outliers for Mo in SS disappeared in CS (fig. 3.6) and central values became concordant. Arsenic resulted on average 3 times higher in Ebro delta than in Camargue birds (tab. 3.2). The two sites compensated, thus SS and CS pattern did not differ significantly. As is discussed in detail in § 3.5.

Concerning the remaining 21 elements, no appreciable anomalies are inserted by Camargue and Ebro delta datasets (tab. 3.2). Only Cs, has higher median value. The tendency of U to have median equal to mean is confirmed in CS.

**Tab. 3.1** – Descriptive statistics regarding SS. On the rows there are the 41 elements presented, on the columns the sites, and for each site, Median±Median Absolute Deviations (M±MAD), mean±Standard Deviation (m±SD), minimum and maximum (min-max) are reported. Also overall parameters of all colonies are presented.

| | Cagliari | | | Comacchio | | | Odiel | | | Venice | | | ALL COLONIES | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max |
| **Na** | 3761±610 | 3857±1099 | 1866-6805 | 2356±316 | 2287±412 | 1196-2910 | 2528±228 | 2475±360 | 1710-3012 | 2618±245 | 2758±609 | 1923-3798 | 2662±391 | 2879±985 | 1196-6805 |
| **Mg** | 1487±172 | 1677±544 | 1077-3516 | 1017±151 | 1070±276 | 631-1781 | 669±102 | 674±119 | 494-940 | 717±118 | 712±122 | 568-885 | 998±334 | 1132±540 | 494-3516 |
| **K** | 226±41 | 254±81 | 119-445 | 226±29 | 230±43 | 134-314 | 210±37 | 205±41 | 140-272 | 158±22 | 181±45 | 136-245 | 217±36 | 227±61 | 119-445 |
| **Zn** | 109±10.5 | 114±15.1 | 86.2-157.0 | 126.0±8.0 | 127.2±13.5 | 103.0-156.0 | 131.0±12.0 | 130.9±16.1 | 92.2-158.0 | 147.0±7.0 | 147.0±7.7 | 137.0-158.0 | 124.0±13.0 | 125.4±17.0 | 86.2-158.0 |
| **Fe** | 69.8±18 | 78.4±29.8 | 41.7-145.0 | 237.0±55.0 | 253±75 | 136.0-442.0 | 106.0±15.9 | 117.3±29.2 | 84.4-186.0 | 79.0±11.6 | 75.9±15.8 | 53.8-100.7 | 111.0±43.8 | 146.7±91.0 | 41.7-442.0 |
| **Al** | 53.5±8.5 | 57.3±17.8 | 32.0-105.0 | 333.0±85.0 | 332.3±107.3 | 176.0-569.0 | 91.0±19.0 | 99.8±29.0 | 62.0-155.0 | 36.0±8.0 | 42.6±16.0 | 28.0-71.0 | 86.0±42.0 | 159.3±139.9 | 28.0-569.0 |
| **Sr** | 7.75±1.1 | 8.65±3.73 | 5.66-23.70 | 9.09±0.83 | 9.02±1.67 | 5.38-13.50 | 5.20±0.27 | 5.22±0.71 | 3.68-6.95 | 7.35±1.03 | 7.04±1.28 | 5.29-8.60 | 7.35±1.57 | 7.73±2.85 | 3.68-23.70 |
| **Cu** | 3.81±0.41 | 3.95±0.6 | 3.07-5.62 | 4.12±0.38 | 4.12±0.53 | 2.82-5.22 | 4.48±0.46 | 4.35±0.59 | 3.31-5.68 | 4.62±0.59 | 4.65±0.66 | 3.77-5.45 | 4.12±0.42 | 4.17±0.61 | 2.82-5.68 |
| **Se** | 2.76±0.66 | 2.9±0.91 | 1.23-4.85 | 1.59±0.21 | 1.65±0.34 | 1.12-2.49 | 1.92±0.23 | 2.03±0.41 | 1.15-2.76 | 2.13±0.17 | 2.14±0.38 | 1.67-2.85 | 2.00±0.41 | 2.19±0.79 | 1.12-4.85 |
| **Ti** | 1.07±0.37 | 1.43±0.85 | 0.40-3.21 | 4.99±1.62 | 5.09±2.03 | 1.98-8.86 | 1.50±0.42 | 1.66±0.58 | 0.96-3.06 | 0.49±0.07 | 0.55±0.15 | 0.39-0.83 | 1.98±1.07 | 2.65±2.18 | 0.39-8.86 |
| **Mn** | 0.71±0.22 | 0.78±0.35 | 0.38-2.02 | 7.64±1.60 | 7.98±2.75 | 3.63-14.30 | 1.96±0.39 | 1.93±0.59 | 1.03-3.81 | 0.95±0.22 | 0.90±0.26 | 0.56-1.17 | 1.78±1.12 | 3.50±3.60 | 0.38-14.30 |
| **Cr** | 0.43±0.19 | 0.91±1.50 | 0.17-8.24 | 1.18±0.30 | 1.41±0.87 | 0.63-4.75 | 0.40±0.07 | 0.51±0.24 | 0.32-1.26 | 1.60±0.18 | 1.54±0.36 | 1.09-2.10 | 0.79±0.41 | 1.02±1.06 | 0.17-8.24 |
| **Hg** | 1.30±0.87 | 2.19±1.95 | 0.31-7.28 | 0.66±0.20 | 0.75±0.47 | 0.18-2.46 | 0.61±0.11 | 0.71±0.30 | 0.38-1.60 | 0.94±0.23 | 0.97±0.32 | 0.48-1.37 | 0.74±0.29 | 1.22±1.33 | 0.18-7.28 |
| **Pb** | 0.46±0.09 | 0.49±0.20 | 0.28-1.33 | 1.91±0.43 | 1.97±0.76 | 0.79-3.91 | 0.62±0.09 | 0.64±0.16 | 0.39-1.10 | 1.81±0.36 | 2.12±0.59 | 1.45-3.15 | 0.69±0.29 | 1.15±0.86 | 0.28-3.91 |
| **Ni** | 0.41±0.14 | 0.49±0.23 | 0.19-0.94 | 1.15±0.31 | 1.76±2.9 | 0.55-16.70 | 0.40±0.14 | 0.58±0.56 | 0.19-2.41 | 0.29±0.08 | 0.32±0.12 | 0.20-0.52 | 0.55±0.27 | 0.92±1.79 | 0.19-16.70 |
| **Rb** | 0.14±0.03 | 0.15±0.05 | 0.10-0.30 | 0.66±0.15 | 0.67±0.21 | 0.37-1.12 | 0.21±0.03 | 0.23±0.06 | 0.13-0.42 | 0.12±0.03 | 0.11±0.03 | 0.08-0.16 | 0.22±0.10 | 0.34±0.27 | 0.08-1.12 |
| **Li** | 0.21±0.05 | 0.23±0.08 | 0.14-0.48 | 0.36±0.10 | 0.4±0.12 | 0.22-0.63 | 0.12±0.01 | 0.14±0.04 | 0.10-0.25 | 0.10±0.01 | 0.11±0.03 | 0.07-0.16 | 0.21±0.08 | 0.25±0.14 | 0.07-0.63 |
| **V** | 0.12±0.04 | 0.14±0.07 | 0.06-0.34 | 0.66±0.14 | 0.65±0.21 | 0.29-1.11 | 0.15±0.04 | 0.15±0.05 | 0.09-0.24 | 0.08±0.02 | 0.09±0.04 | 0.06-0.16 | 0.17±0.08 | 0.31±0.28 | 0.06-1.11 |
| **Mo** | 0.16±0.02 | 0.19±0.08 | 0.12-0.51 | 0.20±0.03 | 0.34±0.6 | 0.12-3.45 | 0.11±0.02 | 0.14±0.12 | 0.08-0.53 | 0.14±0.02 | 0.15±0.02 | 0.12-0.18 | 0.16±0.04 | 0.22±0.36 | 0.08-3.45 |
| **As** | 0.11±0.04 | 0.11±0.05 | 0.04-0.24 | 0.15±0.02 | 0.16±0.05 | 0.10-0.31 | 0.84±0.22 | 0.90±0.27 | 0.57-1.46 | 0.07±0.01 | 0.06±0.01 | 0.05-0.08 | 0.15±0.07 | 0.34±0.37 | 0.04-1.46 |
| **Ce** | 0.06±0.023 | 0.068±0.038 | 0.02-0.156 | 0.180±0.053 | 0.185±0.065 | 0.078-0.310 | 0.075±0.021 | 0.081±0.031 | 0.040-0.158 | 0.033±0.008 | 0.035±0.012 | 0.021-0.052 | 0.088±0.042 | 0.108±0.072 | 0.020-0.310 |
| **Co** | 0.029±0.006 | 0.032±0.011 | 0.015-0.073 | 0.167±0.051 | 0.179±0.070 | 0.072-0.366 | 0.063±0.009 | 0.066±0.015 | 0.043-0.113 | 0.030±0.005 | 0.033±0.006 | 0.025-0.040 | 0.060±0.031 | 0.090±0.077 | 0.015-0.366 |
| **Zr** | 0.032±0.007 | 0.037±0.013 | 0.021-0.075 | 0.082±0.032 | 0.087±0.034 | 0.033-0.166 | 0.048±0.012 | 0.049±0.017 | 0.028-0.095 | 0.022±0.004 | 0.022±0.004 | 0.017-0.027 | 0.044±0.016 | 0.056±0.033 | 0.017-0.166 |
| **La** | 0.030±0.012 | 0.035±0.019 | 0.010-0.075 | 0.091±0.024 | 0.090±0.032 | 0.038-0.153 | 0.036±0.010 | 0.038±0.015 | 0.019-0.076 | 0.019±0.006 | 0.019±0.006 | 0.012-0.027 | 0.043±0.020 | 0.053±0.035 | 0.010-0.153 |
| **Nd** | 0.024±0.009 | 0.029±0.017 | 0.009-0.065 | 0.087±0.022 | 0.088±0.031 | 0.037-0.147 | 0.034±0.010 | 0.037±0.015 | 0.017-0.074 | 0.012±0.003 | 0.014±0.005 | 0.009-0.022 | 0.040±0.021 | 0.050±0.035 | 0.009-0.147 |
| **Ag** | 0.033±0.007 | 0.036±0.018 | 0.017-0.111 | 0.021±0.006 | 0.024±0.019 | 0.012-0.120 | 0.071±0.011 | 0.073±0.024 | 0.040-0.144 | 0.036±0.007 | 0.031±0.014 | 0.010-0.043 | 0.033±0.014 | 0.041±0.028 | 0.010-0.144 |
| **Y** | 0.021±0.007 | 0.023±0.013 | 0.009-0.056 | 0.073±0.020 | 0.075±0.026 | 0.034-0.128 | 0.025±0.008 | 0.025±0.010 | 0.013-0.047 | 0.015±0.004 | 0.014±0.004 | 0.009-0.019 | 0.030±0.015 | 0.040±0.030 | 0.009-0.128 |
| **U** | 0.026±0.006 | 0.030±0.012 | 0.018-0.076 | 0.025±0.006 | 0.025±0.008 | 0.009-0.040 | 0.011±0.003 | 0.011±0.004 | 0.006-0.020 | 0.009±0.002 | 0.010±0.002 | 0.006-0.013 | 0.021±0.009 | 0.022±0.012 | 0.006-0.076 |
| **Ga** | 0.012±0.004 | 0.014±0.007 | 0.005-0.031 | 0.074±0.019 | 0.076±0.027 | 0.039-0.135 | 0.018±0.004 | 0.019±0.006 | 0.011-0.032 | 0.006±0.002 | 0.007±0.003 | 0.004-0.013 | 0.020±0.011 | 0.035±0.033 | 0.004-0.135 |
| **Th** | 0.013±0.006 | 0.019±0.015 | 0.004-0.057 | 0.039±0.011 | 0.041±0.014 | 0.018-0.070 | 0.010±0.003 | 0.011±0.005 | 0.006-0.022 | 0.003±0.001 | 0.003±0.002 | 0.002-0.006 | 0.017±0.010 | 0.023±0.018 | 0.002-0.070 |
| **Pr** | 0.007±0.003 | 0.008±0.004 | 0.002-0.017 | 0.021±0.006 | 0.022±0.008 | 0.009-0.038 | 0.009±0.003 | 0.009±0.004 | 0.005-0.019 | 0.003±0.001 | 0.004±0.002 | 0.002-0.006 | 0.010±0.005 | 0.013±0.009 | 0.002-0.038 |
| **W** | 0.009±0.002 | 0.010±0.003 | 0.006-0.018 | 0.014±0.002 | 0.015±0.006 | 0.007-0.040 | 0.008±0.001 | 0.010±0.008 | 0.006-0.048 | 0.006±0.001 | 0.006±0.001 | 0.005-0.008 | 0.010±0.003 | 0.011±0.007 | 0.005-0.048 |
| **Cs** | 0.005±0.002 | 0.006±0.003 | 0.002-0.014 | 0.040±0.010 | 0.042±0.014 | 0.020-0.072 | 0.007±0.002 | 0.008±0.003 | 0.005-0.015 | 0.003±0.001 | 0.003±0.002 | 0.002-0.006 | 0.009±0.005 | 0.018±0.019 | 0.002-0.072 |
| **Gd** | 0.005±0.002 | 0.005±0.003 | 0.002-0.012 | 0.019±0.005 | 0.019±0.007 | 0.009-0.033 | 0.007±0.002 | 0.007±0.003 | 0.003-0.014 | 0.003±0.001 | 0.003±0.001 | 0.001-0.005 | 0.008±0.004 | 0.010±0.008 | 0.001-0.033 |
| **Sm** | 0.005±0.002 | 0.006±0.004 | 0.002-0.014 | 0.020±0.005 | 0.020±0.007 | 0.008-0.036 | 0.008±0.002 | 0.008±0.003 | 0.004-0.016 | 0.003±0.001 | 0.003±0.001 | 0.002-0.004 | 0.008±0.005 | 0.011±0.008 | 0.002-0.036 |
| **Dy** | 0.004±0.002 | 0.004±0.002 | 0.002-0.010 | 0.015±0.004 | 0.016±0.008 | 0.007-0.050 | 0.005±0.002 | 0.005±0.002 | 0.003-0.011 | 0.002±0.001 | 0.002±0.001 | 0.001-0.003 | 0.006±0.003 | 0.008±0.007 | 0.001-0.050 |
| **Nb** | 0.005±0.002 | 0.007±0.005 | 0.002-0.018 | 0.018±0.005 | 0.017±0.007 | 0.006-0.033 | 0.004±0.001 | 0.004±0.001 | 0.003-0.007 | 0.002±0.001 | 0.002±0.001 | 0.001-0.003 | 0.006±0.003 | 0.009±0.008 | 0.001-0.033 |
| **Be** | 0.002±0.001 | 0.002±0.001 | 0.001-0.005 | 0.010±0.003 | 0.010±0.004 | 0.005-0.018 | 0.003±0.001 | 0.003±0.001 | 0.001-0.005 | 0.001±0.000 | 0.001±0.000 | 0.001-0.002 | 0.003±0.002 | 0.005±0.004 | 0.001-0.018 |
| **Er** | 0.002±0.001 | 0.002±0.001 | 0.001-0.006 | 0.007±0.002 | 0.007±0.003 | 0.003-0.015 | 0.003±0.001 | 0.003±0.001 | 0.001-0.005 | 0.001±0.000 | 0.001±0.000 | 0.001-0.002 | 0.003±0.002 | 0.004±0.003 | 0.001-0.015 |
| **Yb** | 0.002±0.001 | 0.002±0.001 | 0.001-0.005 | 0.006±0.002 | 0.006±0.002 | 0.003-0.010 | 0.002±0.001 | 0.002±0.001 | 0.001-0.004 | 0.001±0.000 | 0.001±0.000 | 0.001-0.001 | 0.002±0.001 | 0.003±0.002 | 0.001-0.010 |
| **Eu** | 0.001±0.000 | 0.001±0.001 | 0.000-0.002 | 0.004±0.001 | 0.004±0.001 | 0.002-0.007 | 0.001±0.000 | 0.002±0.001 | 0.001-0.004 | 0.001±0.000 | 0.001±0.001 | 0.000-0.001 | 0.002±0.001 | 0.002±0.002 | 0.000-0.007 |

**Tab. 3.2** – Descriptive statistics regarding CS. On the rows there are the 41 elements presented, on the columns the sites, and for each site, Median±Median Absolute Deviations (M±MAD), mean±Standard Deviation (m±SD), minimum and maximum (min-max) are reported. Also overall parameters of all colonies and the mean found in captive birds are presented.

| | Cagliari | | | Camargue | | | Comacchio | | | Ebro | | | Odiel | | | ALL COLONIES | | | Captivity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max | M±MAD | m±SD | min-max | m |
| Na | 3094±414 | 3045±570 | 1868-3950 | 1948±229 | 2047±410 | 1087-2947 | 2081±210 | 2030±299 | 1490-2653 | 4332±1406 | 3671±1386 | 1914-6091 | 2450±206 | 2409±315 | 1812-2890 | 2320±406 | 2610±898 | 1087-6091 | 422 |
| Mg | 1356±183 | 1393±215 | 1054-1813 | 1318±213 | 1308±295 | 566-1843 | 1037±122 | 1028±209 | 571-1503 | 1685±141 | 1629±304 | 878-2037 | 639±64 | 639±97 | 463-824 | 1220±262 | 1214±379 | 463-2037 | 150 |
| K | 199±26 | 199±37 | 135-269 | 168±23 | 173±44 | 84-276 | 211±17 | 212±30 | 154-274 | 266±84 | 246±109 | 118-526 | 213±23 | 213±35 | 165-285 | 200±30 | 207±57 | 84-526 | 37 |
| Zn | 111.0±7.0 | 112.6±11.8 | 96.8-152.0 | 89.8±6.2 | 91.8±11.4 | 73.3-115.0 | 120.5±7.5 | 122.4±12.4 | 98.0-153.0 | 112.0±7.0 | 111.5±7.8 | 99.4-122.0 | 133.0±6.0 | 127.2±12.2 | 103.0-141.0 | 115.0±10.0 | 113.5±16.0 | 73.3-153.0 | 66.3 |
| Al | 54.5±12.0 | 59.2±17.8 | 34.0-104.0 | 114.0±20.0 | 119.9±39.0 | 69.0-218.0 | 346.0±59.0 | 351.8±92.9 | 204.0-558.0 | 96.0±16.0 | 97.1±26.4 | 58.0-167.0 | 119.0±19.0 | 111.1±24.1 | 66.0-142.0 | 108.0±42.0 | 161.5±129.6 | 34.0-558.0 | 38.4 |
| Fe | 74.1±10.6 | 81.0±20.8 | 52.0-137.0 | 77.9±10.9 | 85.4±25.0 | 51.8-148.0 | 263.5±45.5 | 264.9±62.9 | 165.0-407.0 | 96.8±11.9 | 100.6±21.0 | 75.6-148.0 | 124.0±7.0 | 120.6±19.8 | 78.2-151.0 | 106.0±34.9 | 139.8±85.9 | 51.8-407.0 | 15.6 |
| Sr | 7.31±0.78 | 7.64±1.23 | 5.61-10.20 | 8.05±1.03 | 8.02±1.56 | 4.85-11.40 | 9.27±0.73 | 9.08±1.17 | 6.97-11.60 | 12.50±0.80 | 12.71±2.36 | 8.92-17.90 | 5.23±0.32 | 5.21±0.42 | 4.52-5.93 | 8.28±1.55 | 8.54±2.56 | 4.52-17.90 | 2.18 |
| Cu | 3.69±0.24 | 3.74±0.38 | 3.16-4.73 | 4.68±0.41 | 4.66±0.48 | 3.91-5.78 | 4.08±0.28 | 4.01±0.42 | 3.12-5.06 | 3.52±0.14 | 3.47±0.31 | 2.80-3.90 | 4.19±0.36 | 4.20±0.39 | 3.66-4.74 | 3.94±0.40 | 3.99±0.55 | 2.80-5.78 | 10.37 |
| Mn | 0.73±0.15 | 0.78±0.30 | 0.35-1.84 | 2.65±0.61 | 2.67±0.99 | 1.23-5.29 | 8.08±1.84 | 8.40±2.33 | 4.22-12.80 | 2.87±0.64 | 3.15±1.05 | 1.84-6.24 | 2.07±0.26 | 1.93±0.38 | 1.20-2.46 | 2.37±1.50 | 3.68±3.29 | 0.35-12.80 | 0.55 |
| Ti | 1.69±0.54 | 1.97±1.00 | 0.78-4.91 | 1.34±0.27 | 1.50±0.65 | 0.67-2.84 | 5.77±1.36 | 5.70±1.82 | 2.91-10.80 | 1.10±0.14 | 1.15±0.27 | 0.72-1.80 | 2.25±0.33 | 2.12±0.49 | 1.08-2.83 | 1.95±0.86 | 2.79±2.12 | 0.67-10.80 | 0.55 |
| Se | 2.84±0.43 | 3.00±0.74 | 1.76-4.38 | 1.62±0.21 | 1.62±0.31 | 1.11-2.15 | 1.60±0.14 | 1.57±0.17 | 1.32-1.99 | 1.74±0.29 | 1.80±0.47 | 0.99-3.00 | 1.97±0.11 | 2.02±0.21 | 1.58-2.35 | 1.81±0.34 | 2.06±0.74 | 0.99-4.38 | 0.84 |
| Hg | 1.47±0.34 | 1.65±0.87 | 0.42-3.48 | 1.08±0.44 | 1.40±1.07 | 0.45-4.93 | 1.12±0.36 | 1.22±0.68 | 0.36-3.20 | 1.25±0.41 | 1.44±0.64 | 0.67-2.70 | 0.78±0.08 | 0.78±0.16 | 0.52-1.21 | 1.18±0.40 | 1.34±0.81 | 0.36-4.93 | 0.73 |
| Cr | 0.44±0.21 | 0.58±0.43 | 0.16-2.00 | 0.39±0.08 | 0.42±0.10 | 0.26-0.61 | 1.13±0.13 | 1.21±0.33 | 0.72-2.13 | 1.25±0.32 | 1.63±0.88 | 0.60-3.62 | 0.38±0.02 | 0.38±0.05 | 0.29-0.49 | 0.69±0.35 | 0.86±0.64 | 0.16-3.62 | 0.48 |
| Ni | 0.48±0.19 | 0.57±0.36 | 0.15-1.75 | 0.54±0.11 | 0.58±0.22 | 0.30-1.25 | 1.00±0.21 | 1.09±0.29 | 0.56-1.78 | 1.16±0.72 | 1.26±0.88 | 0.35-3.14 | 0.35±0.02 | 0.41±0.16 | 0.28-0.83 | 0.64±0.20 | 0.80±0.53 | 0.15-3.14 | 0.34 |
| Pb | 0.47±0.05 | 0.47±0.09 | 0.33-0.71 | 0.54±0.07 | 0.57±0.15 | 0.34-0.97 | 1.87±0.29 | 1.97±0.53 | 0.96-2.97 | 2.17±1.69 | 2.34±2.06 | 0.40-6.77 | 0.63±0.09 | 0.68±0.13 | 0.49-0.95 | 0.63±0.29 | 1.21±1.15 | 0.33-6.77 | 0.29 |
| Li | 0.20±0.02 | 0.20±0.03 | 0.13-0.27 | 0.25±0.04 | 0.27±0.06 | 0.20-0.40 | 0.39±0.05 | 0.40±0.09 | 0.25-0.60 | 0.28±0.04 | 0.27±0.05 | 0.16-0.34 | 0.16±0.02 | 0.15±0.03 | 0.11-0.19 | 0.25±0.07 | 0.27±0.11 | 0.11-0.60 | 0.03 |
| Rb | 0.16±0.03 | 0.16±0.05 | 0.09-0.29 | 0.21±0.04 | 0.23±0.08 | 0.15-0.43 | 0.71±0.11 | 0.74±0.19 | 0.43-1.16 | 0.22±0.02 | 0.22±0.04 | 0.15-0.31 | 0.27±0.01 | 0.26±0.03 | 0.19-0.32 | 0.23±0.07 | 0.35±0.26 | 0.09-1.16 | 0.08 |
| V | 0.14±0.03 | 0.15±0.06 | 0.06-0.34 | 0.18±0.03 | 0.18±0.07 | 0.09-0.35 | 0.68±0.11 | 0.71±0.20 | 0.38-1.13 | 0.20±0.04 | 0.21±0.06 | 0.14-0.36 | 0.18±0.03 | 0.17±0.04 | 0.09-0.22 | 0.19±0.06 | 0.32±0.27 | 0.06-1.13 | 0.04 |
| Mo | 0.17±0.02 | 0.19±0.07 | 0.12-0.45 | 0.14±0.02 | 0.14±0.03 | 0.10-0.20 | 0.20±0.02 | 0.20±0.03 | 0.12-0.25 | 0.15±0.01 | 0.14±0.02 | 0.11-0.19 | 0.10±0.01 | 0.10±0.02 | 0.07-0.13 | 0.16±0.03 | 0.17±0.06 | 0.07-0.45 | 0.19 |
| As | 0.10±0.02 | 0.10±0.03 | 0.05-0.18 | 0.09±0.01 | 0.09±0.02 | 0.06-0.12 | 0.17±0.03 | 0.17±0.05 | 0.11-0.34 | 0.25±0.03 | 0.24±0.04 | 0.18-0.35 | 0.79±0.10 | 0.82±0.14 | 0.61-1.06 | 0.14±0.05 | 0.24±0.25 | 0.05-1.06 | 0.06 |
| Ce | 0.067±0.019 | 0.081±0.040 | 0.035-0.203 | 0.075±0.019 | 0.084±0.038 | 0.033-0.186 | 0.197±0.036 | 0.209±0.060 | 0.112-0.336 | 0.076±0.020 | 0.081±0.029 | 0.045-0.165 | 0.100±0.020 | 0.093±0.024 | 0.051-0.127 | 0.097±0.038 | 0.118±0.070 | 0.033-0.336 | 0.054 |
| Co | 0.032±0.006 | 0.034±0.010 | 0.018-0.063 | 0.078±0.018 | 0.103±0.120 | 0.035-0.585 | 0.177±0.036 | 0.201±0.068 | 0.091-0.339 | 0.052±0.010 | 0.060±0.038 | 0.031-0.184 | 0.072±0.009 | 0.071±0.013 | 0.044-0.091 | 0.065±0.030 | 0.100±0.090 | 0.018-0.585 | 0.035 |
| La | 0.036±0.009 | 0.040±0.019 | 0.018-0.099 | 0.040±0.009 | 0.043±0.019 | 0.017-0.094 | 0.095±0.018 | 0.100±0.028 | 0.054-0.156 | 0.039±0.009 | 0.042±0.015 | 0.024-0.082 | 0.047±0.008 | 0.044±0.011 | 0.025-0.061 | 0.048±0.015 | 0.058±0.033 | 0.017-0.156 | 0.033 |
| Zr | 0.033±0.006 | 0.037±0.014 | 0.016-0.070 | 0.036±0.007 | 0.038±0.012 | 0.023-0.063 | 0.086±0.016 | 0.095±0.030 | 0.040-0.165 | 0.041±0.006 | 0.042±0.009 | 0.026-0.059 | 0.056±0.010 | 0.057±0.012 | 0.040-0.075 | 0.046±0.017 | 0.056±0.031 | 0.016-0.165 | 0.049 |
| Nd | 0.029±0.008 | 0.034±0.017 | 0.015-0.084 | 0.037±0.009 | 0.040±0.019 | 0.015-0.091 | 0.093±0.015 | 0.099±0.028 | 0.059-0.163 | 0.033±0.007 | 0.035±0.012 | 0.020-0.072 | 0.046±0.009 | 0.043±0.011 | 0.023-0.060 | 0.043±0.017 | 0.054±0.034 | 0.015-0.163 | 0.015 |
| Y | 0.025±0.008 | 0.028±0.014 | 0.011-0.073 | 0.032±0.008 | 0.035±0.015 | 0.014-0.077 | 0.078±0.015 | 0.083±0.023 | 0.047-0.128 | 0.027±0.005 | 0.029±0.008 | 0.019-0.051 | 0.031±0.006 | 0.029±0.007 | 0.016-0.039 | 0.033±0.011 | 0.044±0.028 | 0.011-0.128 | 0.006 |
| Ag | 0.038±0.007 | 0.041±0.016 | 0.018-0.089 | 0.028±0.004 | 0.031±0.014 | 0.015-0.072 | 0.020±0.004 | 0.022±0.007 | 0.013-0.037 | 0.033±0.013 | 0.042±0.030 | 0.014-0.140 | 0.090±0.029 | 0.096±0.031 | 0.054-0.145 | 0.031±0.012 | 0.042±0.030 | 0.013-0.145 | 0.218 |
| U | 0.032±0.006 | 0.031±0.008 | 0.016-0.058 | 0.026±0.006 | 0.027±0.008 | 0.013-0.042 | 0.026±0.004 | 0.028±0.008 | 0.011-0.046 | 0.019±0.003 | 0.019±0.005 | 0.011-0.029 | 0.012±0.001 | 0.012±0.002 | 0.008-0.015 | 0.024±0.007 | 0.025±0.010 | 0.008-0.058 | 0.025 |
| Ga | 0.015±0.004 | 0.016±0.007 | 0.007-0.035 | 0.024±0.005 | 0.025±0.009 | 0.013-0.049 | 0.081±0.017 | 0.082±0.024 | 0.045-0.139 | 0.019±0.003 | 0.020±0.006 | 0.010-0.037 | 0.022±0.004 | 0.022±0.004 | 0.014-0.029 | 0.023±0.008 | 0.037±0.031 | 0.007-0.139 | 0.005 |
| Th | 0.020±0.007 | 0.025±0.014 | 0.007-0.065 | 0.015±0.004 | 0.015±0.007 | 0.006-0.036 | 0.044±0.007 | 0.046±0.012 | 0.026-0.072 | 0.012±0.002 | 0.014±0.006 | 0.007-0.031 | 0.014±0.003 | 0.013±0.004 | 0.007-0.020 | 0.018±0.008 | 0.026±0.017 | 0.006-0.072 | 0.006 |
| Cs | 0.006±0.002 | 0.007±0.004 | 0.003-0.018 | 0.016±0.004 | 0.017±0.007 | 0.007-0.037 | 0.044±0.008 | 0.046±0.013 | 0.025-0.075 | 0.012±0.003 | 0.013±0.005 | 0.007-0.025 | 0.010±0.002 | 0.009±0.002 | 0.005-0.013 | 0.012±0.006 | 0.021±0.018 | 0.003-0.075 | 0.005 |
| Pr | 0.008±0.002 | 0.009±0.005 | 0.004-0.023 | 0.009±0.002 | 0.010±0.005 | 0.004-0.023 | 0.024±0.004 | 0.025±0.007 | 0.013-0.042 | 0.009±0.002 | 0.009±0.003 | 0.005-0.019 | 0.012±0.002 | 0.011±0.003 | 0.006-0.015 | 0.011±0.004 | 0.014±0.008 | 0.004-0.042 | 0.005 |
| Sm | 0.006±0.002 | 0.007±0.003 | 0.003-0.017 | 0.008±0.002 | 0.009±0.004 | 0.003-0.020 | 0.022±0.004 | 0.023±0.006 | 0.013-0.036 | 0.007±0.001 | 0.008±0.003 | 0.004-0.016 | 0.009±0.002 | 0.009±0.002 | 0.005-0.013 | 0.009±0.003 | 0.012±0.008 | 0.003-0.036 | 0.002 |
| W | 0.009±0.002 | 0.009±0.003 | 0.004-0.018 | 0.007±0.001 | 0.008±0.003 | 0.004-0.015 | 0.013±0.002 | 0.013±0.003 | 0.007-0.022 | 0.008±0.002 | 0.010±0.005 | 0.005-0.024 | 0.006±0.001 | 0.006±0.001 | 0.004-0.009 | 0.009±0.002 | 0.010±0.004 | 0.004-0.024 | 0.010 |
| Gd | 0.005±0.001 | 0.006±0.003 | 0.002-0.015 | 0.008±0.002 | 0.008±0.004 | 0.003-0.020 | 0.020±0.004 | 0.022±0.006 | 0.013-0.034 | 0.006±0.001 | 0.007±0.002 | 0.005-0.014 | 0.009±0.002 | 0.008±0.002 | 0.005-0.012 | 0.008±0.003 | 0.011±0.008 | 0.002-0.034 | 0.002 |
| Nb | 0.009±0.003 | 0.011±0.006 | 0.004-0.026 | 0.005±0.001 | 0.006±0.003 | 0.003-0.013 | 0.019±0.004 | 0.020±0.006 | 0.010-0.034 | 0.004±0.001 | 0.004±0.001 | 0.003-0.007 | 0.005±0.001 | 0.005±0.001 | 0.003-0.007 | 0.007±0.003 | 0.011±0.007 | 0.003-0.034 | 0.003 |
| Dy | 0.005±0.002 | 0.005±0.003 | 0.002-0.014 | 0.006±0.001 | 0.007±0.003 | 0.003-0.015 | 0.016±0.003 | 0.017±0.005 | 0.010-0.027 | 0.005±0.001 | 0.005±0.002 | 0.003-0.010 | 0.007±0.001 | 0.006±0.002 | 0.003-0.009 | 0.007±0.003 | 0.009±0.006 | 0.002-0.027 | 0.001 |
| Be | 0.002±0.000 | 0.002±0.001 | 0.001-0.005 | 0.004±0.001 | 0.004±0.001 | 0.002-0.007 | 0.010±0.002 | 0.011±0.003 | 0.006-0.016 | 0.003±0.000 | 0.004±0.001 | 0.002-0.007 | 0.003±0.001 | 0.003±0.001 | 0.002-0.004 | 0.004±0.002 | 0.005±0.004 | 0.001-0.016 | 0.001 |
| Er | 0.003±0.001 | 0.003±0.002 | 0.001-0.008 | 0.003±0.001 | 0.003±0.002 | 0.001-0.007 | 0.008±0.001 | 0.008±0.002 | 0.005-0.013 | 0.003±0.001 | 0.003±0.001 | 0.002-0.005 | 0.003±0.001 | 0.003±0.001 | 0.002-0.004 | 0.003±0.001 | 0.004±0.003 | 0.001-0.013 | 0.001 |
| Yb | 0.002±0.001 | 0.003±0.001 | 0.001-0.007 | 0.002±0.000 | 0.003±0.001 | 0.001-0.006 | 0.006±0.001 | 0.007±0.002 | 0.004-0.010 | 0.002±0.000 | 0.002±0.001 | 0.001-0.004 | 0.003±0.000 | 0.002±0.001 | 0.001-0.003 | 0.003±0.001 | 0.004±0.002 | 0.001-0.010 | 0.001 |
| Eu | 0.001±0.000 | 0.001±0.001 | 0.000-0.003 | 0.002±0.001 | 0.002±0.001 | 0.001-0.004 | 0.005±0.001 | 0.005±0.001 | 0.003-0.008 | 0.002±0.001 | 0.002±0.001 | 0.001-0.003 | 0.002±0.000 | 0.002±0.001 | 0.001-0.003 | 0.002±0.001 | 0.002±0.002 | 0.000-0.008 | 0.001 |

*3.3 Concentrations in captive bird feathers*

By analyzing the feathers collected by the operators of the Bioparco of Rome on 12 adult flamingos, an overall mean was obtained for each element presented here, because it was not possible to obtain a median value from the feathers sampled on captive birds (see §2.2).

Referring to figure 3.6, the mean of captive birds (CM, from now on) resulted obviously lower than the mean of wild birds (WM) for almost elements (35 on 41). Really, for 19 elements, CM resulted definitely outside the lower limit of the wild birds data range.

Also Zinc in captive birds is lower than wild birds, being the CM equal to 66.3 mg/kg (tab. 3.2). Zinc is thought an essential element for feather structure, therefore such a low value may indicate a slight deficiency of this metals during the moulting period, or a different accumulation rate in adult birds compared to growing chicks. A preliminary study on some captive flamingo fed with cereals and dog croquettes (very probably enriched in essential elements) revealed 129.6 mg/kg and 94.3 mg/kg in wing and body feathers respectively (Migani, 2009), more close to the WM found here (113.5 mg/kg Zn) excluding bioparco birds.

Evident exceptions to the pattern described above regard Ag, Ce, Co, Cr, Cu, Hg, La, Mo, U, W, Zr. Only Ag and Cu resulted much higher in CM than in WM and could be easily explained if the zoo management make use of ionizers or mineral sanitizers for pools (at this moment this information is not available). Ionizers are devices designed for swimming pools, provided with electrodes that supply a stream of copper and silver ions to the water. The copper ions function as an algaecide and the silver ions function as a bactericide. In order to understand if the high concentrations of Cu and Ag found on captive birds are bioaccumulated or derive from the ions strongly adhered to the feather surface, further experiments are needed.

Cerium, Co, Cr, La, and Hg resulted relatively high in captive birds, but CM were evidently lower than WM. A group including Mo, U, W, Zr shows about the same mean in captive and wild birds, Mo even slightly higher in captive birds. Bioparco zoo is placed in the center of Rome: According to geological knowledge on the Roman soils (De Vivo *et al.*, 2008), their feathers are characterized by high values of U and Zr. In addition, Bioparco birds are rich in Mo and W (and Cr, though less evident). Thorium is considered as a marker for the main bedrock lithologies occurring in the Roman–Neapolitan area, and REEs, Al, Bi, Hf, Ti, Tl, have been found in soil (De Vivo *et al.*, 2008). In contrast, flamingos of the Bioparco are scarce in Th, and REEs seem less important in captive flamingo feathers than expected, except for Ce and La, having values within the range of wild birds. In particular, Gd, Nd, Pr, Sm resulted very low in feathers, and Dy, Er, Eu, and Yb virtually

absent. Surprisingly, Co (essential trace element) and Hg (non essential, toxic element) resulted somewhat high in feathers from the zoo, but no explanations can be formulated with the few available data.

*3.4 Considerations about single and composite samples*

To evaluate how medians and variability change using CS instead of SS, exclusively Cagliari, Comacchio, and Odiel samples were used. A comparison between SS and CS medians is shown in fig. 3.7, by plotting the ratios between composite samples median (MCS) and single samples median (MSS) for each element. Uncertainty was calculated with the following formula:

$$UNC = \left(\frac{CS\ MAD}{MCS} + \frac{SS\ MAD}{MSS}\right) \cdot \frac{MCS}{MSS}$$

where MCS is the median of composite samples of a certain element and CS MAD its median absolute deviation, MSS and SS MAD the same for single samples, and UNC the uncertainty of the ratio MCS/MSS.

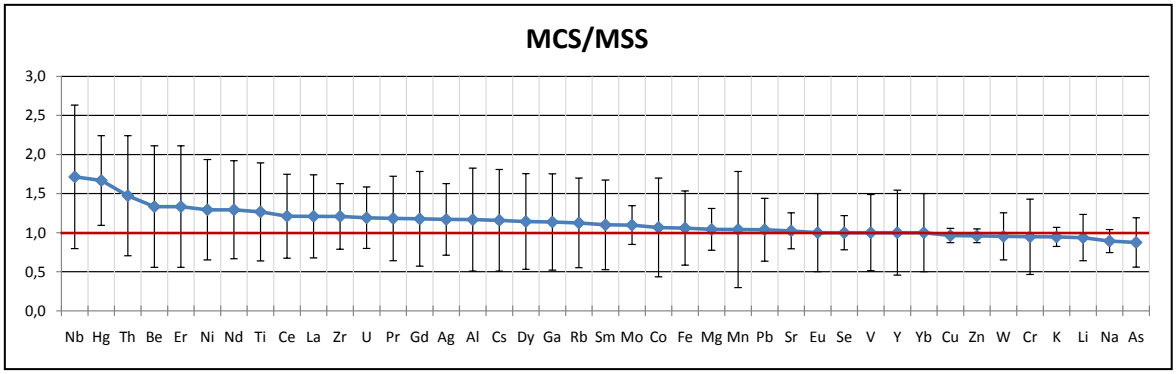


**Fig. 3.7** – CS and SS median ratios (MCS and MSS respectively) are reported on the vertical axis (MCS/MSS), for the 41 elements discussed (x-axis). Red line indicates the perfect agreement of both parameters. Uncertainty are expressed as vertical error bars (see text for calculation method). Only Cagliari, Comacchio, and Odiel data are considered.

Practically all elements (40 on 41) tendentially show the same median value in SS and CS. Except Mercury, no medians appear higher in CS. No elements have lower median in CS, only MCS/MSS resulted on the boundary of significant difference.

The case of mercury is extraordinary, being median in CS 1,7 times higher than SS, and tolerance bar does not cross the 1:1 line. This difference will be statistically tested and discussed in the Hg dedicated section (§ 3.8).

Regarding dispersion of data, a comparison between SS and CS medians is shown in fig. 3.8. Only Zr seems more dispersed in CS than SS. A group of eight elements is practically on the red line (La, Sm, Mg, V, Yb, Nb, Cs, Ag). The other elements result less dispersed in CS, with Ce, Th, Fe, Mn, Nd, and Pr that are definitely reduced in spread by using CS.

In theory, CS should better represent the contamination level of each population, coping in a better way with individual variations than SS. On the other hand, SS allow to properly describe the real extent of the true profile of the sample set, although the central values may be affected by few extreme measures. The results confirmed that outliers and extreme values of datasets were limited by CS, but the range remained highly representative. Concerning the medians, used in this work as central value of the populations, unexpectedly in CS it was higher than in SS, in particular for some important elements, such as Hg, Ni, Ti, and many others.

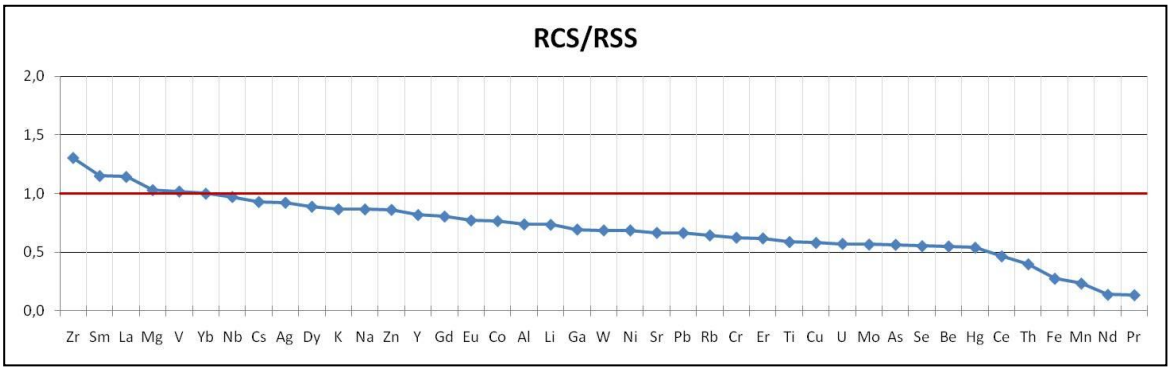


**Fig. 3.8** – Max/min ratios were calculated for CS (RCS) and SS (RSS) for each element. The RCS/RSS ratios are plotted for the 41 elements discussed here. Red line indicates the perfect comparability between CS and SS ranges. Only Cagliari, Comacchio, and Odiel data are considered.

*3.5 Arsenic*

Arsenic levels in feathers result low and homogenous in all sites, but Odiel. Arsenic was the 20$^{th}$ element found in order of median concentration, which was 0.15 ± 0.07 in SS and 0.14 ± 0.05 mg/kg in CS (tab. 3.3). Median Absolute Deviation (MAD), less sensitive to strong anomalies than Standard Deviation (SD), indicates that substantially As distribution is rather compact in the interval 0.05 - 0.25 mg/kg. The Coefficient of Variation (CV), namely the ratio between SD and mean expressed as a percentage, is very different considering all sites (111%) or each single site (18-45%), revealing that some important anomalies are present into the whole dataset.

**Tab. 3.3** – Arsenic levels (mg/kg, dry weight) on Flamingo feathers (SS and CS). Median, Median Absolute Deviation (MAD), mean, Standard Deviation (SD), minimum (min) and maximum (max), Coefficient of Variation (CV%), and number of samples (n) are presented for each site and for the whole dataset. All colonies parameters relate to wild birds only.

| ARSENIC (single samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 0.11 | 0.04 | 0.11 | 0.05 | 0.04 | 0.24 | 45 | 30 |
| **Comacchio** | 0.15 | 0.02 | 0.16 | 0.05 | 0.10 | 0.31 | 30 | 31 |
| **Odiel** | 0.84 | 0.22 | 0.90 | 0.27 | 0.57 | 1.46 | 30 | 25 |
| **Venice** | 0.07 | 0.01 | 0.06 | 0.01 | 0.05 | 0.08 | 18 | 7 |
| **ALL COLONIES** | **0.15** | **0.07** | **0.34** | **0.37** | **0.04** | **1.46** | **111** | **93** |

| ARSENIC (composite samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 0.10 | 0.02 | 0.10 | 0.03 | 0.05 | 0.18 | 32 | 30 |
| **Camargue** | 0.09 | 0.01 | 0.09 | 0.02 | 0.06 | 0.12 | 19 | 19 |
| **Comacchio** | 0.17 | 0.03 | 0.17 | 0.05 | 0.11 | 0.34 | 28 | 30 |
| **Ebro Delta** | 0.25 | 0.03 | 0.24 | 0.04 | 0.18 | 0.35 | 17 | 17 |
| **Odiel** | 0.79 | 0.10 | 0.82 | 0.14 | 0.61 | 1.06 | 18 | 15 |
| **Captivity** | --- | --- | 0.06 | --- | 0.06 | 0.06 | --- | 7 |
| **ALL COLONIES** | **0.14** | **0.05** | **0.24** | **0.25** | **0.05** | **1.06** | **104** | **111** |

It is clear from tab. 3.3 that the Odiel population has very different results compared to other sites. Median of Odiel SS is 7.6 to 12 times higher than other sites, and 3.2 to 8.8 times for CS. No overlap exist between Odiel data and other colonies (fig. 3.9 and fig. 3.10). Cumulative curve of Odiel SS (fig. 3.9) shows that 9 samples are, even if high in value, distributed quite regularly in the lower part of the range (0.57 - 0.67 mg/kg As) the remaining subset, starting from 0.76, clearly reflect the presence of anomalies also within the Odiel population (fig. 3.9).

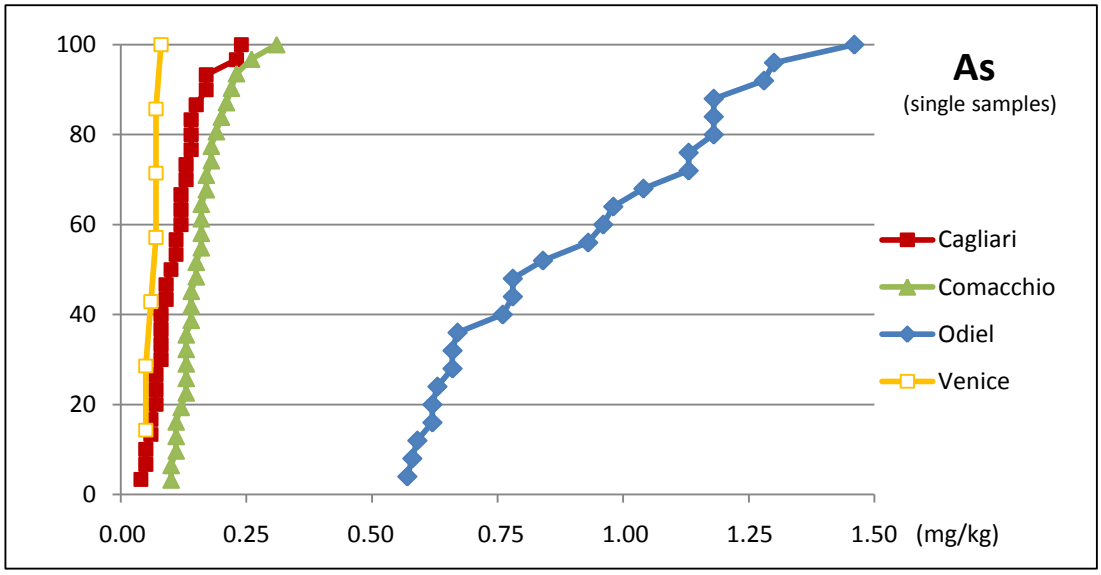


**Fig. 3.9** – Cumulative curves of Arsenic concentrations in SS for Cagliari, Comacchio, Odiel, and Venice.

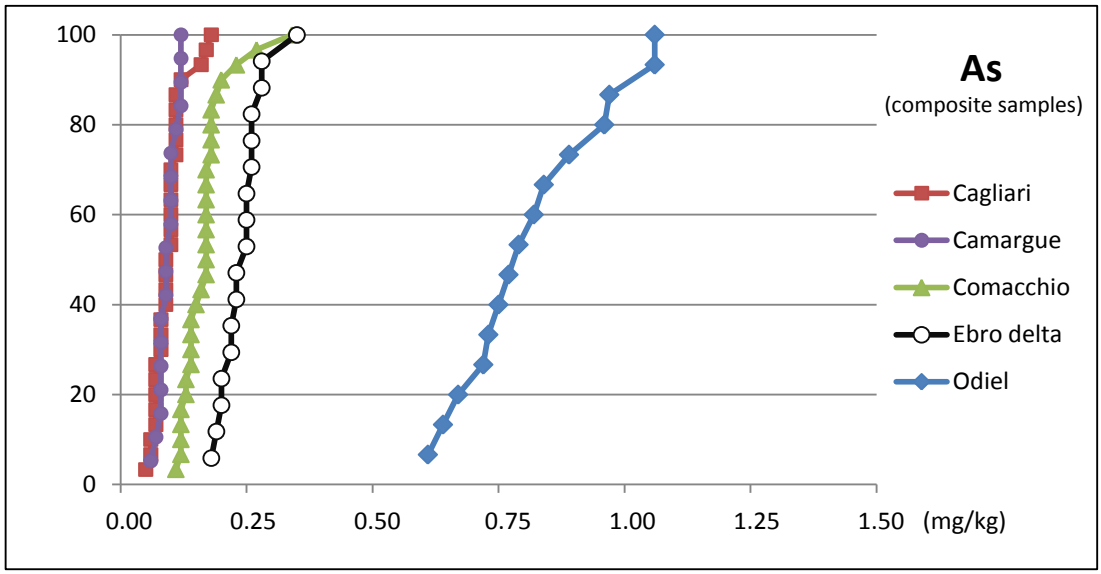


**Fig. 3.10** – Cumulative curves of Arsenic concentrations in CS for Cagliari, Camargue, Comacchio, Ebro delta, and Odiel.

On the other hand, Cagliari, Camargue, and Venice Flamingos probably represent populations with background levels of Arsenic in feathers, judging from the concentrations and the overlap in cumulative curves (fig. 3.9, 3.10).

Kruskal-Wallis test confirmed that Cagliari and Camargue are very similar for As (tab. 3.4), whereas all other sites result significantly different (tab 3.4 and 3.5). Arsenic levels in Venice samples are the lowest in a narrow interval below 0.09 mg/kg As. Comacchio, and more evidently Ebro delta, seem slightly higher.

**Tab.** 3.4 – Pairwise comparison between sites by using Kruskal-Wallis test performed on SS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was < 0.0001.

| ARSENIC (single samples) | | | | |
|---|---|---|---|---|
| | **Cagliari** | **Comacchio** | **Odiel** | **Venice** |
| **Cagliari** | ----- | | | |
| **Comacchio** | < 0.0001 | ----- | | |
| **Odiel** | < 0.0001 | < 0.0001 | ----- | |
| **Venice** | 0.0085 | < 0.0001 | < 0.0001 | ----- |

**Tab.** 3.5 – Pairwise comparison between sites by using Kruskal-Wallis test performed on CS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was < 0.0001.

| ARSENIC (composite samples) | | | | | |
|---|---|---|---|---|---|
| | **Cagliari** | **Camargue** | **Comacchio** | **Ebro** | **Odiel** |
| **Cagliari** | ---- | | | | |
| **Camargue** | NS | ---- | | | |
| **Comacchio** | < 0.0001 | < 0.0001 | ---- | | |
| **Ebro** | < 0.0001 | < 0.0001 | < 0.0001 | ---- | |
| **Odiel** | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | ---- |

In literature there are no data regarding As in Greater Flamingo feathers, but this element was measured in pooled feather samples of adult Lesser Flamingo. The mean concentration resulted 0.98 ± 0.30 mg/Kg. This value was thought by the authors low, compared to other bird species (Burger and Gochfeld, 2001), while compared to our results it is similar to Odiel birds, the highest population.

The lack of correlation with any other element suggests that, although As concentrations in feathers might by affected by external deposition (Goede and de Bruin, 1984), As in young Flamingo feathers is mainly bioaccumulated. If this is the case, As levels found in Odiel flamingos could be of some concern and further assessment on As bioaccumulation in trophic chain would be advisable. In fact, high concentrations of As in the environment is a threat for wildlife causing decrease in growth rates in waterbirds (Camardese *et al.*, 1990), and alteration of behaviour of ducklings (Whitworth *et al.*, 1991).

Actually the Odiel marshes are known for high concentrations of many metals (in particular As, Cd, Cu, Pb, Zn), and for arsenic much higher that all the other sites. Although strongly linked to the sediments (Morillo *et al.*, 2008), the lack of any correlation with other elements in feathers would suggest some kind of bioaccumulation. Rice fields in Ebro Delta cover a large area and are extensively visited by Flamingos to forage. Some relation between Arsenic in crayfish and rice cultivation was found in Ebro Delta (Suarez-Serrano, 2010), but it is unclear if natural sources of As exist in the area or high As concentrations in the environment are a result of chemicals used in agriculture practices. A quantification of endogenous and exogenous As in feathers would be helpful to confirm this conclusion, for example performing a speciation analysis by using X-ray Absorption Spectroscopy (XAS) (Smith *et al.*, 2008).

For Cagliari and Comacchio, available feathers were enough in weight to create double samples from some birds (SS) or groups of birds (CS). Duplicated samples were analyzed together with all other samples. Then, correlation among duplicated samples was tested in order to control the homogeneity within them (fig. 3.11 and fig. 3.12). In fig. 3.11 also Certified Reference Materials (BCR-414 and ERM-278) are plotted, and indicate a good homogeneity of samples for As in Cagliari and Comacchio. A plot without CRM is shown to better look at the distribution of data (fig. 3.12).

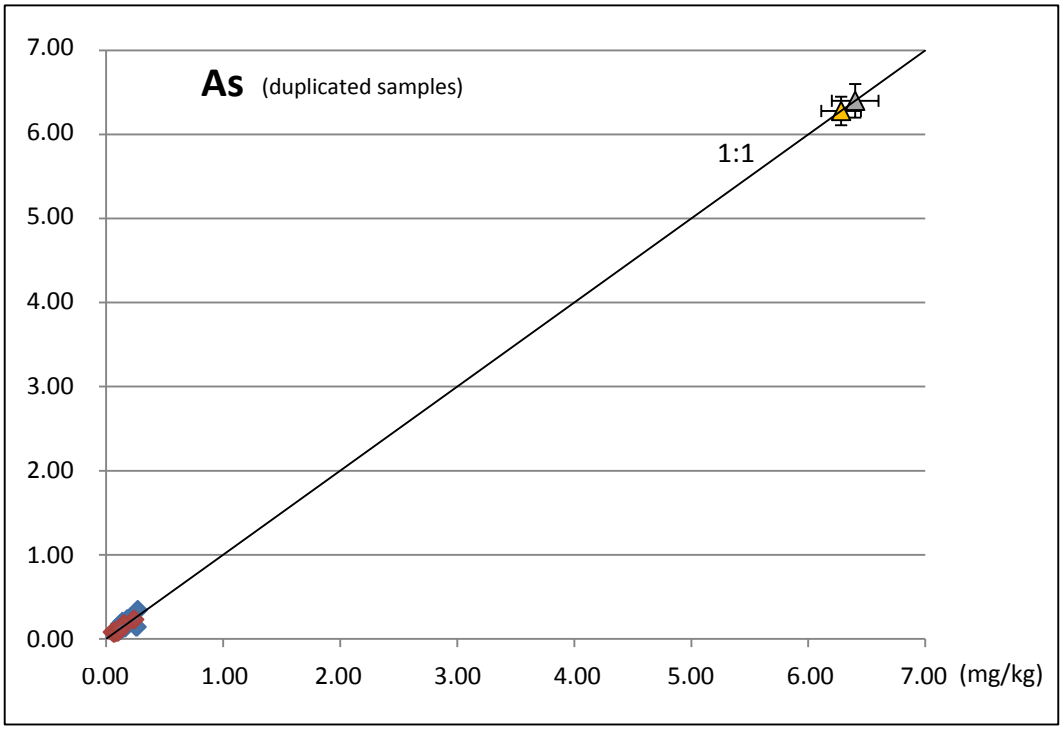


**Fig. 3.11** – Scatter plot of As levels (mg/kg, d.w.) in duplicated samples of Comacchio (COM) and Cagliari (CAG). The black line show 1:1 correspondence. Certified Reference Materials (BCR-414 and ERM-278) results, with error bars (1σ), are plotted with grey and orange triangles respectively.

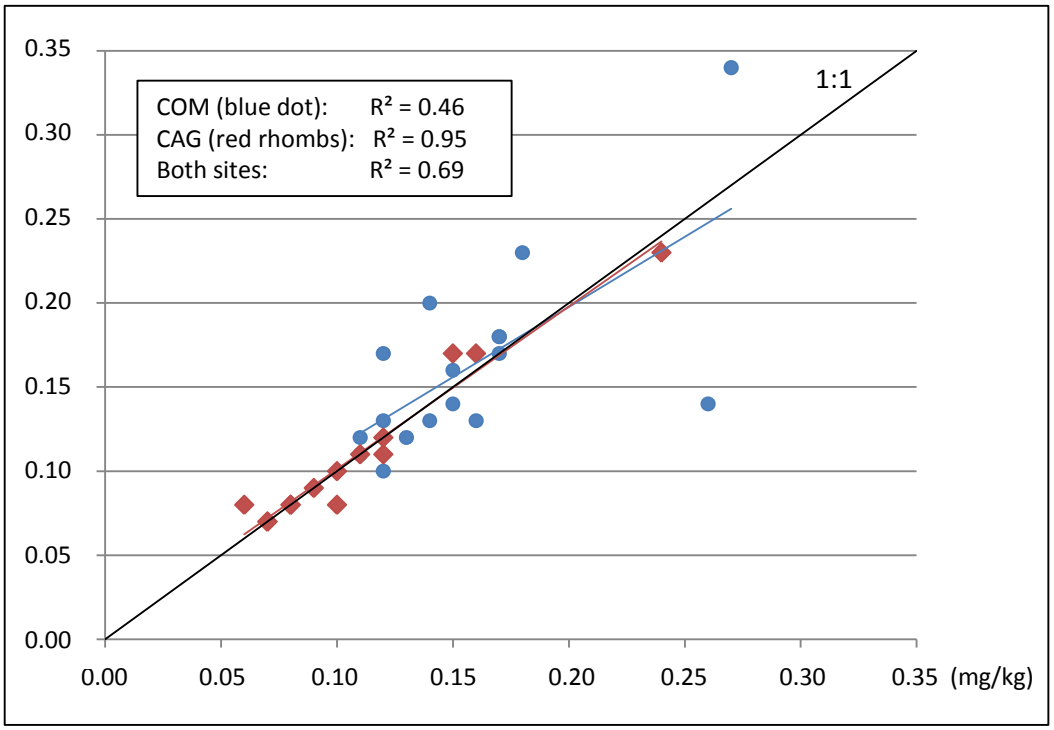


**Fig. 3.12** – Scatter plot of As levels (mg/kg, d.w.) in duplicated samples of Comacchio (COM) and Cagliari (CAG). The black line show 1:1 correspondence. Blue line and red line interpolate COM and CAG samples respectively. Determination coefficient ($R^2$) for each site and for both sites is reported.

Correlation of Cagliari duplicated samples resulted very high ($R^2$ = 0.95) (fig. 3.12). On the other hand, Comacchio display lower correlation ($R^2$ = 0.46), possibly linked to some kind of external contamination. However overall correlation was clear ($R^2$ = 0.69) and for most samples, if external contamination exist, it is likely due to an homogeneous factor, like preening. Actually, some researchers postulated that external contamination of As on feathers would be caused by preening (uropygial) oil (Goede and de Bruin 1984). External contamination of feathers by uropygial oil would indicate that some biological treatment of the pollutant occurred, differently from exogenous contamination by soil particles or air-borne pollution.

No significant differences in median values were observed by using SS or CS in Cagliari, Comacchio, and Odiel (tab. 3.6). However, CS reduced the occurrence of very high concentrations, making Odiel range 0.61 – 1.06 mg/kg, instead of 0.57 - 1.46 mg/kg in SS. Thus, to compare several sites, CS seem powerful in improving anomalous distributions, maintaining median values representative. On the other hand, in order to look in depth within anomalous populations such as Odiel, SS are more able to represent the true pattern of concentration.

**Tab.** 3.6 – Results of the Kruskal–Wallis test performed on CS and SS of flamingo feathers collected from Cagliari, Comacchio, and Odiel. The table reports the As median (mg/kg) for each type and p-values for significant differences.

| **ARSENIC** | | | |
|---|---|---|---|
| | SS | CS | p-value |
| **Cagliari** | 0.11 | 0.10 | NS |
| **Comacchio** | 0.15 | 0.17 | NS |
| **Odiel** | 0.84 | 0.79 | NS |

*3.6 Copper*

Copper was the 8$^{th}$ element found in order of median concentration, which are 4.12 ± 0.42 in SS and 3.90 ± 0.40 mg/kg in CS (tab. 3.7). Median Absolute Deviation (MAD), and CV indicate that copper concentrations are quite constant in the whole database. Median was not available for captive birds, but certainly they report a different level of Cu, having mean 2.6 times greater than wild colonies (see paragraph 3.3). All samples from wild Flamingos are comprised in a narrow

interval (2.82 – 5.68 mg/kg in SS; 2.80 – 5.78 mg/kg in CS). Besides, the overall Coefficient of Variation (CV) in SS (15%) is the same of CV of the sites taken individually (13-15%). In CS, the sites have even lower CV (9-10%), but overall CV is a little higher (14%). This is controlled by the Camargue subset which is generally enriched in Cu.

**Tab. 3.7** - Copper levels (mg/kg, dry weight) on Flamingo feathers (single samples and composite samples). Median, Median Absolute Deviation (MAD), mean, Standard Deviation (SD), minimum (min) and maximum (max), Coefficient of Variation (CV%), and number of samples (n) are presented for each site and for the whole dataset. All colonies parameters relate to wild birds only.

| COPPER (single samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 3.81 | 0.41 | 3.95 | 0.60 | 3.07 | 5.62 | 15 | 30 |
| **Comacchio** | 4.12 | 0.38 | 4.12 | 0.53 | 2.82 | 5.22 | 13 | 31 |
| **Odiel** | 4.48 | 0.46 | 4.35 | 0.59 | 3.31 | 5.68 | 14 | 25 |
| **Venice** | 4.62 | 0.59 | 4.65 | 0.66 | 3.77 | 5.45 | 14 | 7 |
| **ALL COLONIES** | **4.12** | **0.42** | **4.17** | **0.61** | **2.82** | **5.68** | **15** | **93** |

| COPPER (composite samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 3.69 | 0.24 | 3.74 | 0.38 | 3.16 | 4.73 | 10 | 30 |
| **Camargue** | 4.68 | 0.41 | 4.66 | 0.48 | 3.91 | 5.78 | 10 | 19 |
| **Comacchio** | 4.08 | 0.28 | 4.01 | 0.42 | 3.12 | 5.06 | 10 | 30 |
| **Ebro Delta** | 3.52 | 0.14 | 3.47 | 0.31 | 2.80 | 3.90 | 9 | 17 |
| **Odiel** | 4.19 | 0.36 | 4.20 | 0.39 | 3.66 | 4.74 | 9 | 15 |
| **Captivity** | --- | --- | 10.37 | --- | 10.20 | 10.80 | --- | 7 |
| **ALL COLONIES** | **3.94** | **0.40** | **3.99** | **0.55** | **2.80** | **5.78** | **14** | **111** |

Looking at cumulative curves, some particularities within sites can be observed in the pattern of the distributions (fig. 3.13 and fig. 3.14). In SS, Odiel reveals two distinct groups of samples with a discontinuity around the median value (fig. 3.13). In Cagliari and Comacchio such discontinuity is less visible, but still detectable, and data were more regularly distributed in the lower half range rather than in the upper. This pattern is still appreciable practically for all sites also in CS (Comacchio held on the identical pattern in both type of samples), but clearly evident only in the Carmargue series (fig. 3.14). Both in SS and CS, 1 to 4 samples in each sites are definitely higher,

determining an extension of the data range, otherwise even narrower. Only considering the lower part of the curves, Greater Flamingos chicks seem to have copper background levels in feathers in the range 3.2 - 4.3 mg/kg Cu.

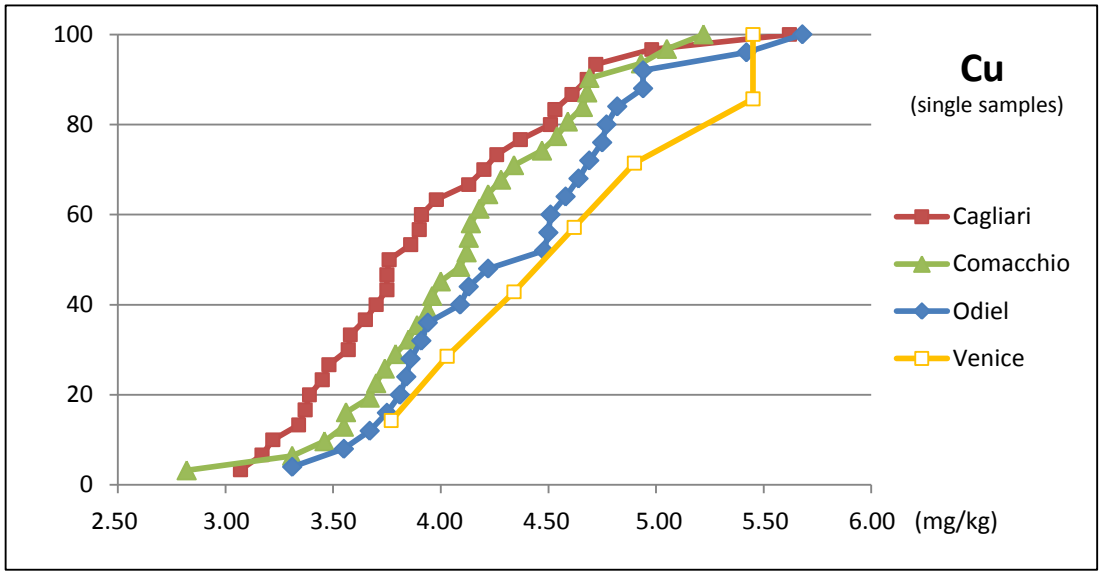


**Fig. 3.13** – Cumulative curves of Copper concentrations in SS for Cagliari, Comacchio, Odiel, and Venice.

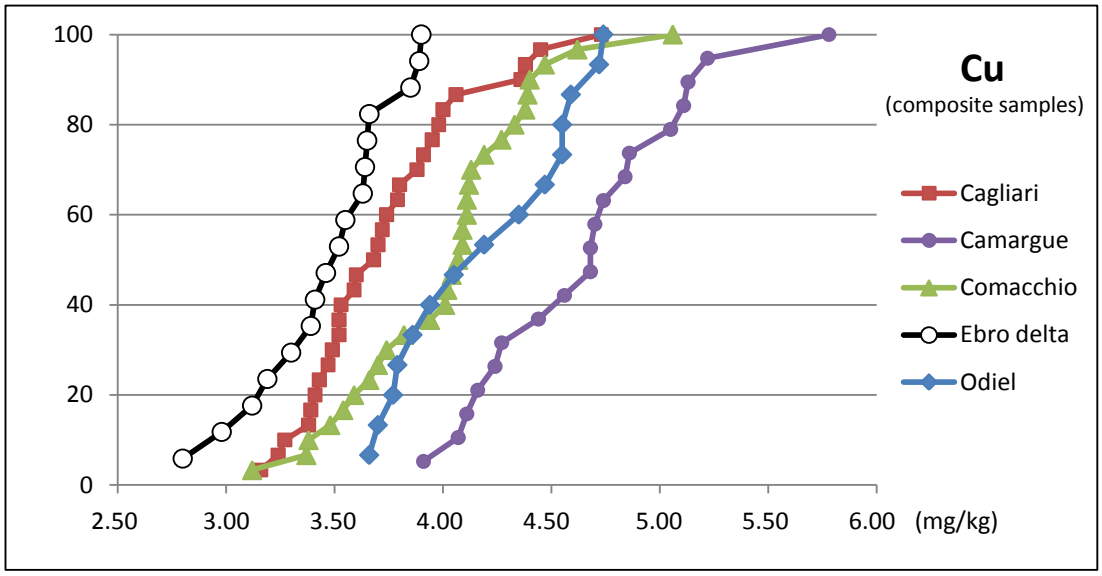


**Fig. 3.14** – Cumulative curves of Copper concentrations in CS for Cagliari, Camargue, Comacchio, Ebro delta, and Odiel.

Kruskal-Wallis test on SS confirmed that Cagliari is different from Odiel and Venice, whereas other sites do not result significantly different (tab. 3.8). Regarding CS, Comacchio and Odiel result similar (tab. 3.9) although with slightly different distribution (fig. 3.14).

**Tab.** 3.8 – Pairwise comparison between sites by using Kruskal-Wallis test performed on SS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was 0.016.

| COPPER (single samples) | | | | |
|---|---|---|---|---|
| | **Cagliari** | **Comacchio** | **Odiel** | **Venice** |
| **Cagliari** | ----- | | | |
| **Comacchio** | NS | ----- | | |
| **Odiel** | 0.013 | NS | ----- | |
| **Venice** | 0.015 | NS | NS | ----- |

**Tab.** 3.9 – Pairwise comparison between sites by using Kruskal-Wallis test performed on CS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was < 0.0001.

| COPPER (composite samples) | | | | | |
|---|---|---|---|---|---|
| | **Cagliari** | **Camargue** | **Comacchio** | **Ebro** | **Odiel** |
| **Cagliari** | ---- | | | | |
| **Camargue** | < 0.0001 | ---- | | | |
| **Comacchio** | 0.006 | < 0.0001 | ---- | | |
| **Ebro** | 0.037 | < 0.0001 | < 0.0001 | ---- | |
| **Odiel** | 0.001 | 0.006 | NS | < 0.0001 | ---- |

According to Honda *et al.* (1986) copper (with zinc) participates in keratinization processes, thus Cu (and Zn) is metabolically regulated. For this reason, Cu levels are relatively constant between species (Solonen *et al.,* 1999). In feather studies, Cu is often analyzed, and some data are available even for Flamingos. Burger (1993) reviewed many studies of metals in feathers, and reported Cu levels in the 7 mg/kg range. Values below 7 mg/kg in birds may occur mainly in unpolluted sites

(Parker, 1985; Solonen *et al.,* 1999). Kim *et al.* (1996) pointed out that it is relatively easy to find in adult seabirds levels greater than 15 mg/kg (Kim *et al.*, 1996), and Wenzel *et al.* (1996) showed as Cu in belly feathers of Kittiwake (a gull species) chicks started from 4 mg/kg at birth and progressively increased to approximately 12 mg/kg after three weeks, with a similar increase in liver and kidney. Very high values (up to 40 mg/kg and over), likely due to exogenous contamination, may be found on small birds living close to cultivated areas where copper is widely used as a fungicide (Leonzio *et al.*, 2009). Copper and other metals were analyzed in Greater Flamingo fledglings in Camargue (Amiard-Triquet *et al.*, 1991). They sampled some greater coverts (GC, relatively small feathers covering flight feathers in the wing) and outer and inner web (namely, the two parts of the vane, separated by the rachis) were separately analyzed after a washing procedure comparable to that of this work. In the French study, mean levels resulted very similar to overall means of this study, with an appreciable wider range in their data. Considering mean of the Camargue subset only, the results found (4.66 ± 0.48 mg/kg) are fully included in the outer GC result, but slightly higher than inner GC. The Cu levels of the French study are reported in tab. 3.10.

**Tab.** 3.10 – Copper levels found in Camargue, according to Cosson *et al.* (1988) on adult Flamingos variously aged, and Amiard-Triquet *et al.* (1991) on Flamingo fledglings. Mean, SD, CV, min and max are reported for inner and outer part of wing greater coverts (GC) relatively to the first study, and for inner and outer web of wing primary feathers (PP). Outer part of feathers was thought more exposed to external contamination than inner part.

| | Mean | SD | CV | Min | Max | Age | Source |
|---|---|---|---|---|---|---|---|
| **Outer GC1** | 4.31 | 1.64 | 38,1% | 1.91 | 7.43 | ≈ 2 months | Amiard-Triquet *et al.* (1991) |
| **Inner GC1** | 3.75 | 1.03 | 27,5% | 1.90 | 7.06 | ≈ 2 months | Amiard-Triquet *et al.* (1991) |
| **Outer PP2** | 7.9 | 3.2 | 40.5% | 4.7 | 16.7 | Adults | Cosson *et al.* (1988) |
| **Inner PP2** | 3.2 | 1.0 | 31.3% | 1.8 | 5.2 | Adults | Cosson *et al.* (1988) |

Before, Cosson *et al.* (1988) studied metal levels, including Cu, in feathers of 12 flamingos found dead during a severe winter in Camargue. The age of birds was much variable. Outer web and inner web of primary feathers (PP) were analyzed for copper, and results were nonconforming, suggesting external contamination (tab. 3.10). Inner PP, less exposed, showed mean (3.2 ± 1.0 mg/kg) comparable with our results, indeed slightly lower, while outer PP were definitely higher

(mean 7.9 ± 3.2 mg/kg) with wider variability, and maximum concentration about 3 times the maximum levels found in SS.

No significant correlations exist between Cu and any other element. This suggests that external contamination caused by residual soil particles, if present, unlikely modified the magnitude of endogenous signal. To verify whether even the more enriched samples are not altered by external contamination, a control on the samples showing highest concentrations was performed. A dataset with the sixteen highest samples selected from all sites and of both types (SS and CS) has been created, and correlations among all metals were tested. Good correlation was observed with elements known for little or absent biological function, that led to believe the likely presence of exogenous lithic fragments (fig. 3.15), and yet, copper in feathers was not significantly correlated neither to that elements, neither to the others (fig. 3.16).

It seems that external contamination of Cu in Flamingo fledgling is irrelevant compared to the bioaccumulation rate. In literature, contrasting results about exogenous and endogenous origin of Cu in feathers can be found. Copper investigated along unwashed feather shafts of different species by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was found predominantly of internal origin, with a few externally attached particles (Ek *et al.*, 2004). On the other hand, an experiment carried out on passerines in a polluted area induced the researchers to conclude that Cu in feathers was partially due to exogenous contamination (Veerle *et al.,* 2004).

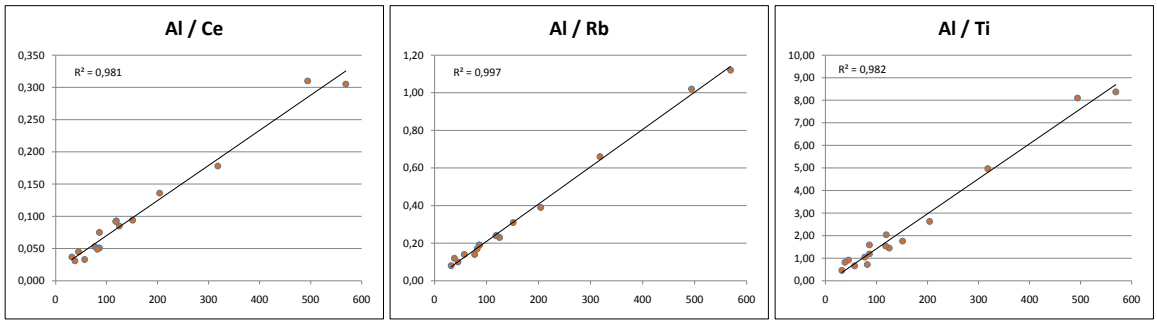


**Fig. 3.15** – Scatter plots of 16 samples (SS and CS) with high Cu from different sites. Given as an example here are illustrated some plots concerning Al (x-axis) against Ce, Rb, and Ti. These elements are thought with little or none biological functions. Determination coefficients ($R^2$) were very high, indicating possible exogenous contamination by soil particles. Concentrations are expressed as mg/kg d.w.

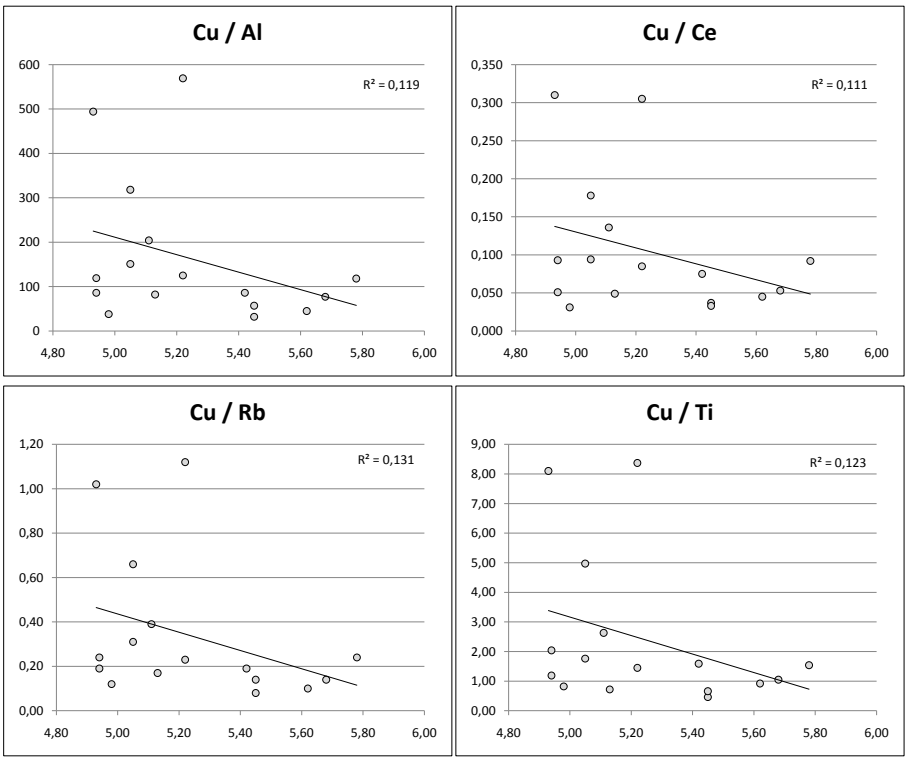


**Fig. 3.16** – Scatter plots of 16 samples (SS and CS) with high Cu from different sites. Copper levels (x-axis) are compared to the elements shown in fig. 3.15 (Al, Ce, Rb, Ti, known for little or none biological functions). Moreover, no correlations were found among Cu and any other elements. Determination coefficients ($R^2$) are reported. Concentrations are expressed as mg/kg d.w.

As consequence of the supposed endogenous origin of Cu in Flamingo feathers, duplicated samples are expected to be highly homogeneous. On the contrary, low correlation is found ($R^2$ = 0.33) (fig. 3.18). Cagliari and Comacchio result similar for tendency and correlation level ($R^2$ = 0.25, $R^2$ = 0.37 respectively). According to Ek *et al.* (2004), Cu signal along the feather might vary as effect of Cu levels in the diet during feather growth (Ek *et al.*, 2004), and concentrations in growing feathers of chicks follows the accumulation of copper in internal tissues with age (Wenzel *et al.*, 1996), therefore it is no more surprisingly that two pieces randomly taken from the same feather contained similar or slight different amounts of Cu.

Observing the scatter plot of duplicated samples (Cagliari and Comacchio), together with Certified Reference Materials (Dorm-2 and ERM-278) (fig. 3.17), the samples appear slightly dispersed in the same way for both sites. Actually, correlations are low, overall as well as each site (fig. 3.18).

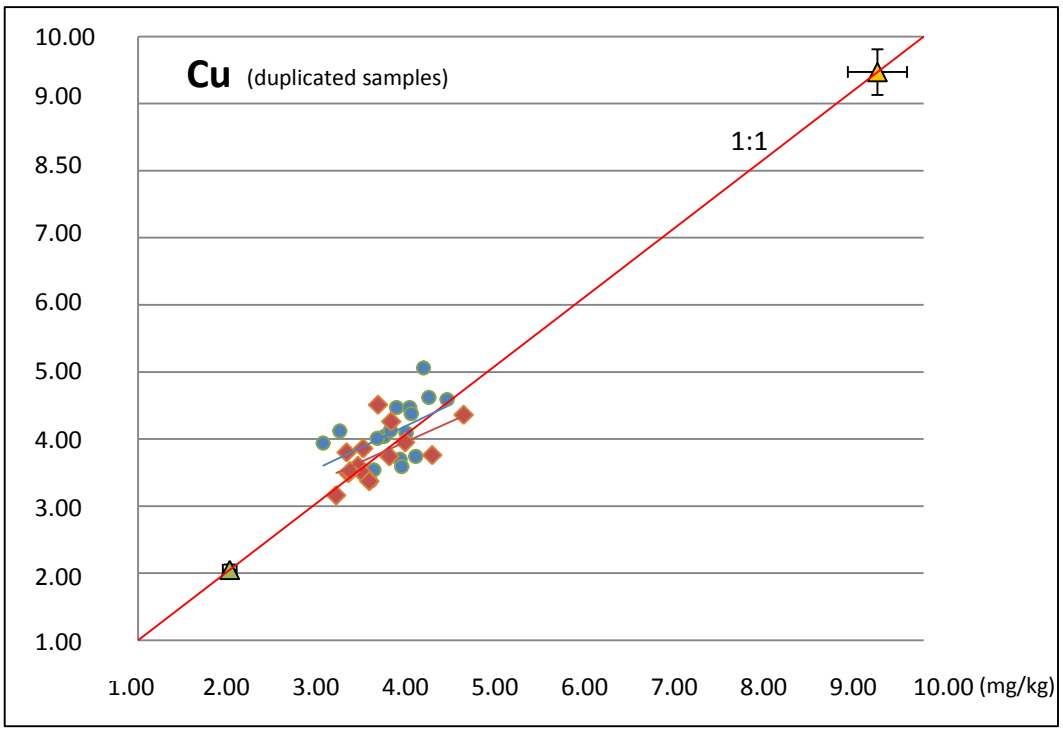


**Fig. 3.17** – Scatter plot of Cu levels (mg/kg, d.w.) in duplicated samples of Comacchio (COM) and Cagliari (CAG). The black line show 1:1 correspondence. Certified Reference Materials results with error bars (1σ), are plotted with green and orange triangles.

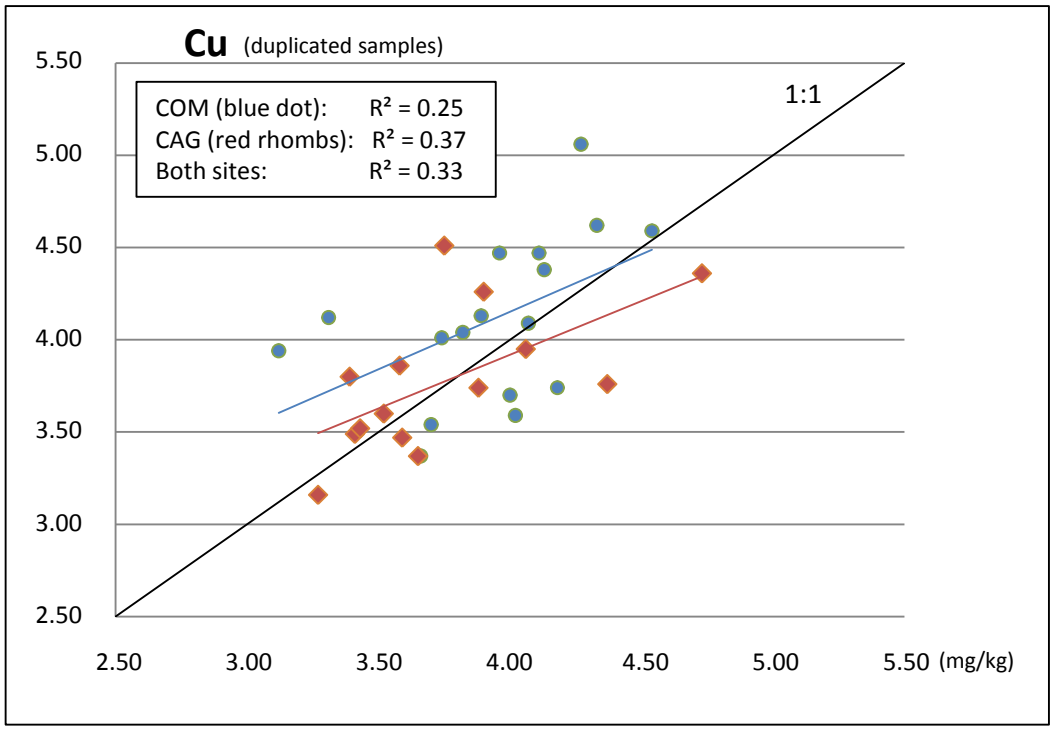


**Fig. 3.18** – Scatter plot of Cu level (mg/kg, d.w.) in duplicated samples of Comacchio (COM) and Cagliari (CAG). The black line show 1:1 correspondence. Blue line and red line interpolate COM and CAG samples respectively. Determination coefficients ($R^2$) for each site and for both sites are reported.

The dynamics of copper movement within the body of birds is largely unknown. Its potential as a bioindicator of environmental pollution or of internal levels has been for long time largely unexplored (Burger, 1993), and not many studies have been carried on in recent years in that way. However, is opinion of some researchers that scarce variability among populations, an effect of metabolic regulation, may limit the potential for detecting variations in environmental levels (Wenzel *et al.,* 1996). Results from Flamingo fledglings only partly seem to confirm this doubt, because Odiel and Camargue pattern appear not so much regular, apparently not caused by exogenous contamination. This could mean that some kind of environmental anomaly is recorded by feathers from Odiel and Camargue. It is known that Huelva estuary is affected by Cu pollution from two sources: copper foundries and waters coming from mines (Grande *et al.,* 2000; Morillo *et al.,* 2008). Camargue has top-soils only slightly enriched in Cu from anthropic origin (Ferrand, 2010). Odiel Flamingo colony showed the most evident discontinuity in SS, and the French colony showed Cu levels significantly higher than other sites. This results suggests to further monitor this metal in those environmental contexts.

No significant differences in median values were observed by using SS or CS in Cagliari, Comacchio, and Odiel (tab. 3.11). Composite samples partly softened irregularities in some distributions (Odiel, Cagliari) respect to SS. As copper could be less sensitive to environmental changes than other metals, the use of SS to detect anomalies within a population seems better.

**Tab.** 3.11 – Results of the Kruskal–Wallis test performed on CS and SS of flamingo feathers collected from Cagliari, Comacchio, and Odiel. The table reports the Cu median (mg/kg) for each type and p-values for significant differences.

| **COPPER** | | | |
|---|---|---|---|
| | SS | CS | p-value |
| **Cagliari** | 3.81 | 3.69 | NS |
| **Comacchio** | 4.12 | 4.08 | NS |
| **Odiel** | 4.48 | 4.19 | NS |

*3.7 Lead*

Lead was the 14th element found in order of median concentration, which was 0.69 ± 0.29 mg/kg in SS and 0.63 ± 0.29 mg/kg in CS (tab. 3.12).

**Tab. 3.12** – Lead levels (mg/kg, dry weight) on Flamingo feathers (single samples and composite samples). Median, Median Absolute Deviation (MAD), mean, Standard Deviation (SD), minimum (min) and maximum (max), Coefficient of Variation (CV%), and number of samples (n) are presented for each site and for the whole dataset. All colonies parameters relate to wild birds only.

| LEAD (single samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 0.46 | 0.09 | 0.49 | 0.20 | 0.28 | 1.33 | 41 | 30 |
| **Comacchio** | 1.91 | 0.43 | 1.97 | 0.76 | 0.79 | 3.91 | 38 | 31 |
| **Odiel** | 0.62 | 0.09 | 0.64 | 0.16 | 0.39 | 1.10 | 25 | 25 |
| **Venice** | 1.81 | 0.36 | 2.12 | 0.59 | 1.45 | 3.15 | 28 | 7 |
| **ALL COLONIES** | **0.69** | **0.29** | **1.15** | **0.86** | **0.28** | **3.91** | **75** | **93** |

| LEAD (composite samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 0.47 | 0.05 | 0.47 | 0.09 | 0.33 | 0.71 | 18 | 30 |
| **Camargue** | 0.54 | 0.07 | 0.57 | 0.15 | 0.34 | 0.97 | 27 | 19 |
| **Comacchio** | 1.87 | 0.29 | 1.97 | 0.53 | 0.96 | 2.97 | 27 | 30 |
| **Ebro Delta** | 2.17 | 1.69 | 2.34 | 2.06 | 0.40 | 6.77 | 88 | 17 |
| **Odiel** | 0.63 | 0.09 | 0.68 | 0.13 | 0.49 | 0.95 | 20 | 15 |
| **Captivity** | --- | --- | 0.29 | --- | 0.29 | 0.30 | --- | 7 |
| **ALL COLONIES** | **0.63** | **0.29** | **1.21** | **1.15** | **0.33** | **6.77** | **95** | **111** |

Very little variability (compare MAD, SD, and CV) characterizes some sites (Cagliari, Camargue, Odiel), whereas the remaining (Comacchio, Ebro delta, and Venice) result in various ways uneven. Except an outlier in the Cagliari series, all samples in homogeneous populations are included in a narrow interval, indicatively below 1 mg/kg (0.28–1.10 mg/kg in SS; 0.33–0.97 mg/kg in CS). Comacchio and Venice are more dispersed (0.79–3.91 mg/kg in SS; 0.96–2.97 mg/kg in CS), and Ebro delta shows the widest range of values (0.40–6.77 mg/kg, CV = 88%), with a very peculiar pattern. For this reasons, the overall Coefficient of Variation (CV) in SS (75%) is higher than the sites taken individually (25-41%), and the CV in CS (95%) is completely determined by the high

variability of Ebro delta (88%). Composite samples of Cagliari, Comacchio, and Odiel show a more compact range than SS.

As shown by cumulative curves (fig. 3.19 and fig. 3.20), when series have relatively high levels of Pb, different subpopulations are recognizable. This is true for SS and CS. In SS, Comacchio reveals at least three distinct groups of samples, and a couple of specimens apart, having over 3.4 mg/kg. None of these groups is well below 1 mg/kg. Venice samples overlap the median-upper range of Comacchio. In Comacchio CS, the group highest in Pb is still clear, but the discontinuity between the lowest and the median group is no more appreciable. Also in Ebro delta three distinct sub-populations are recognizable, but differently from Comacchio pattern, the group lower in Pb is superimposed to the lowest range of Cagliari, Camargue and Odiel. Besides two intermediate values, the series of the remaining 9 samples of Ebro delta starts over 2.5 mg/kg (similarly to the highest group of Comacchio) and extends up to about 7 mg/kg, well over all sites (fig. 3.20). In particular, the highest five samples of Ebro delta seem very highly contaminated. Cagliari, Camargue, and Odiel could represent the Pb background levels in feathers for Greater Flamingos chicks (0.3–0.9 mg/kg).

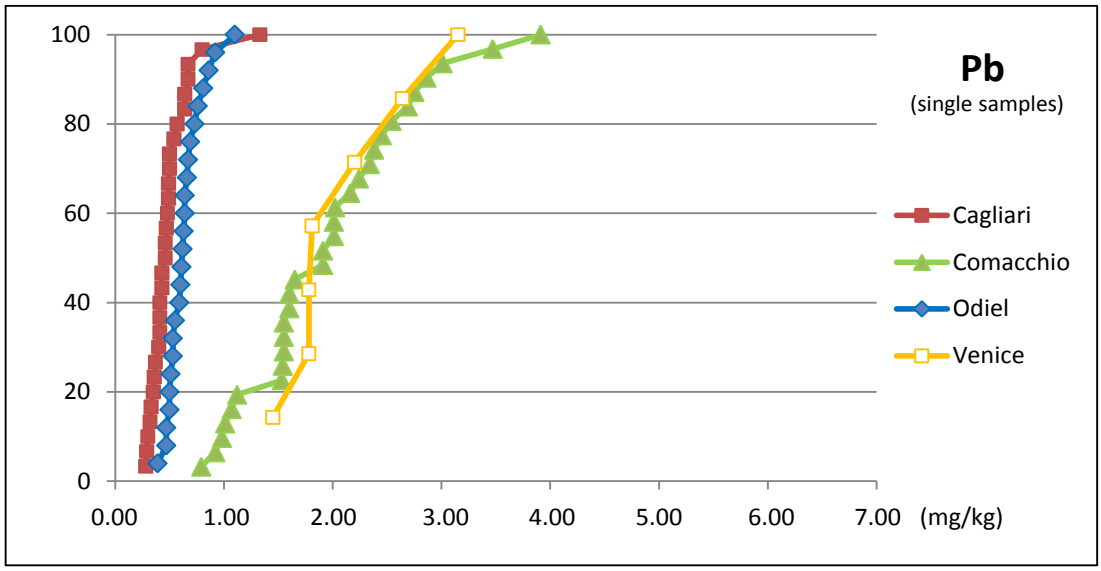


**Fig. 3.19** – Cumulative curves of lead concentrations in SS for Cagliari, Comacchio, Odiel, and Venice.

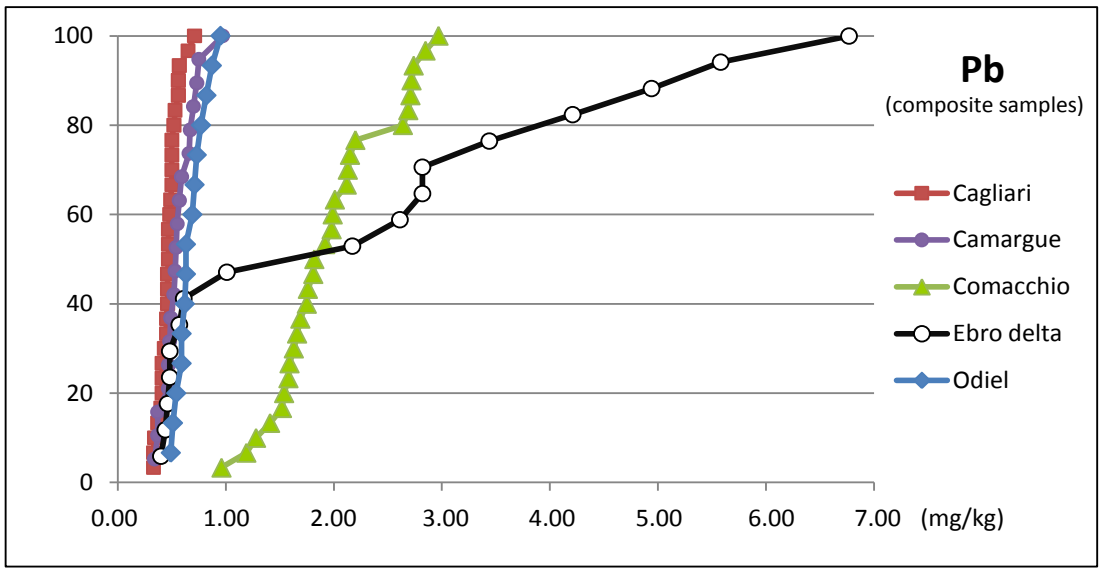


**Fig. 3.20** – Cumulative curves of lead concentrations in CS for Cagliari, Camargue, Comacchio, Ebro delta, and Odiel.

Kruskal-Wallis test on SS confirmed that only Comacchio and Venice are similar (tab. 3.13). Regarding CS, Comacchio and Ebro delta series resulted not significantly different, as well as Ebro delta and Odiel (tab. 3.14). Looking at the cumulative curves (fig. 3.20), the latter similarity was less expected, and probably due to the 40% of overlapping of respective ranges. As this overlap regards also Camargue and Sardinia, just only one sample of Ebro (1.01 mg/kg Pb), closer to Odiel median rather than other medians, controls the statistical similarity with Odiel, and the difference with Cagliari and Camargue.

**Tab.** 3.13 – Pairwise comparison between sites by using Kruskal-Wallis test performed on SS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was < 0.0001.

| **LEAD (single samples)** | | | | |
|---|---|---|---|---|
| | **Cagliari** | **Comacchio** | **Odiel** | **Venice** |
| **Cagliari** | ----- | | | |
| **Comacchio** | < 0.0001 | ----- | | |
| **Odiel** | < 0.001 | < 0.0001 | ----- | |
| **Venice** | < 0.0001 | NS | < 0.0001 | ----- |

**Tab.** 3.14 – Pairwise comparison between sites by using Kruskal-Wallis test performed on CS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was < 0.0001.

| LEAD (composite samples) | | | | | |
|---|---|---|---|---|---|
| | **Cagliari** | **Camargue** | **Comacchio** | **Ebro** | **Odiel** |
| **Cagliari** | ---- | | | | |
| **Camargue** | 0.012 | ---- | | | |
| **Comacchio** | < 0.0001 | < 0.0001 | ---- | | |
| **Ebro** | < 0.001 | 0.021 | NS | ---- | |
| **Odiel** | < 0.0001 | 0.026 | < 0.0001 | NS | ---- |

Lead is considered an highly toxic elements with no biological functions. Concerning birds, Heinz *et al.* (1999) demonstrated that the ingestion of lead-contaminated sediments from a heavily polluted industrial area causes the accumulation of lead in tissues and harmful effects in Mallards (*Anas platyrhynchos*), including death. Actually, the most concern for waterbirds regards ingestion of spent shotgun lead pellets while feeding (Bellrose, 1959; Pain, 1996). There is no doubt that millions of birds die annually worldwide from lead poisoning ("saturnism") (in the U.S.A., around three millions), this problem being most acute in marshlands (De Francisco, 2003). There are two types of lead poisoning: acute, when it appears in birds ingesting a large amount (over 6 pellets for a duck-sized bird) in a short time, causing their death in a few days; and chronic, when the birds consume only a small number of pellets or a relatively longer period of time elapses, and the birds gradually weaken and usually die of starvation (Scheuhammer, 1991; Pain, 1992). As Flamingos constantly stir up the sediments and filter the mud in search of food (invertebrates, seaweed, seeds, etc.), it is likely that they can swallow large amounts of sediment during feeding, and ingestion of lead shot is demonstrated. Occasionally, sporadic or massive mortalities due to acute saturnism were documented in Camargue (Bayle *et al.*, 1986), south-western Spain (Ramo *et al.*, 1991), in Po delta, Italy (Arcangeli *et al.*, 2007), and Tuscany (Ancora *et al.*, 2008). In the New World, the problem was verified on the closely related Caribbean Flamingo (Schmitz *et al.*, 1990). However, on the whole, the Mediterranean area is the one most affected by the problem of saturnism in birds (Pain, 1992).

Lead is largely excreted by feces (Scheuhammer, 1987), perhaps accumulated in the supra-orbital nasal glands (salt glands) in ducks and seabirds (Buggiani and Rindi, 1980; Howarth *et al.*, 1982), and seems to be higher in internal tissues than in eggs and feathers (Ek *et al.*, 2004). Lead concentrations between 0.5 and 4 mg/kg are common for birds (Pb is the second metal more studied in feathers, after mercury), but in literature, much higher levels were reported for heavily polluted areas (Burger, 1996). Substantially, a huge variability of lead levels in feathers were reported in numerous studies. The interpretation of lead levels is always very difficult, because of possible important external contamination (Goede and de Bruin, 1984; Weyers *et al.*, 1988). In the uropygial gland Pb was detected in very low concentrations compared to levels in feathers of waders, but some external contamination during preening, still reflecting the bird's exposure to this element, was not excluded (Goede and de Bruin, 1984). However, endogenous lead levels in nestling feathers tend to increase significantly near metallurgic factories (Eens *et al.*, 1998; Dauwe *et al.*, 2000; Janssens *et al.*, 2001; Nam *et al.*, 2004) and urban areas (Sheifler *et al.*, 2006).

Lead levels found on adult Lesser Flamingos in a unpolluted coastline in Namibia (pooled samples of 2-3 birds, mean 0.39 ± 0.07 mg/kg) (Burger and Gochfeld, 2001), are consistent with Cagliari (0.47 ± 0.09 mg/kg) and so different from concentrations of Camargue (0.57 ± 0.15), that are the lower in our study.

Lead in Greater Flamingo was analyzed in the study carried out by Amiard-Triquet *et al.* (1991) in Camargue. Their results (tab. 3.15) were particularly distant from Camargue mean in our study (0.57 ± 0.15 mg/kg), and high (means well over 3 mg/kg) if compared to all sites (except Ebro delta). Means and range of values in the French study are more similar to Delta Ebro than the other sites.

Cosson *et al.* (1988) found lower Pb levels in feathers of 12 flamingos found dead during a severe winter in Camargue (tab. 3.15), than their colleagues did on Flamingo fledglings. The age and the different origin of birds caused much larger variability in their samples, and external contamination in outer PP was clear. Results regarding outer PP are difficult to compare to our results, while Pb levels in inner PP have some features reminding Comacchio for range (0.36–3.78 mg/kg in Cosson et al.; 0.79–3.91 mg/kg in Comacchio).

**Tab.** 3.15 – Lead levels found in Camargue, according to Cosson *et al.* (1988) on adult Flamingos variously aged, and Amiard-Triquet *et al.* (1991) on Flamingo fledglings. Mean, SD, CV, min and max are reported for inner and outer part of wing greater coverts (GC) relatively to the first study, and for inner and outer web of wing primary feathers (PP) in the second. Outer part of feathers was thought more exposed to external contamination than inner part.

| | Mean | SD | CV | Min | Max | Age | Source |
|---|---|---|---|---|---|---|---|
| **Outer GC** | 3.59 | 1.21 | 33,7% | 0.64 | 6.34 | ≈ 2 months | Amiard-Triquet *et al.* (1991) |
| **Inner GC** | 3.25 | 1.11 | 34,2% | 0.50 | 5.95 | ≈ 2 months | Amiard-Triquet *et al.* (1991) |
| **Outer PP** | 2.81 | 2.34 | 83.3% | 0.91 | 9.67 | Adults | Cosson *et al.* (1988) |
| **Inner PP** | 1.12 | 0.91 | 81.3% | 0.36 | 3.78 | Adults | Cosson *et al.* (1988) |

More recently, Ancora *et al.* (2008) analyzed primary wing feathers from some adults dead in different Italian wetlands. Some of them were poisoned by lead pellets. The two flamingos dead by saturnism reported 1.66 and 1.61 mg/kg Pb in feathers. Three other individuals were under detection limit for Pb and the remaining had concentrations of 0.43 and 1.41 mg/kg. Washing was the same of our study. but Lead concentrations in these adults were rather low, and similarly to Cosson *et al.* (1988) data, the various origin of the specimens make difficult any reliable comparison with our results.

In our study, involving several sites and a wide number of elements, more than one clue led to suppose a possible widespread exogenous contamination in feathers, at least for almost specimens. First, all elements were tested for correlations with Pb, and good correlation were verified with elements with no important biological functions. An overall scatter plot between Aluminium and Samarium in all sites is shown as an example of the strong link between REEs and other elements considered to be of exogenous lithic origin in feathers (fig. 3.21). Very high correlations Al/Sm are clear in all sites, inducing to believe that some terrigenous residuals are present practically in all feathers.

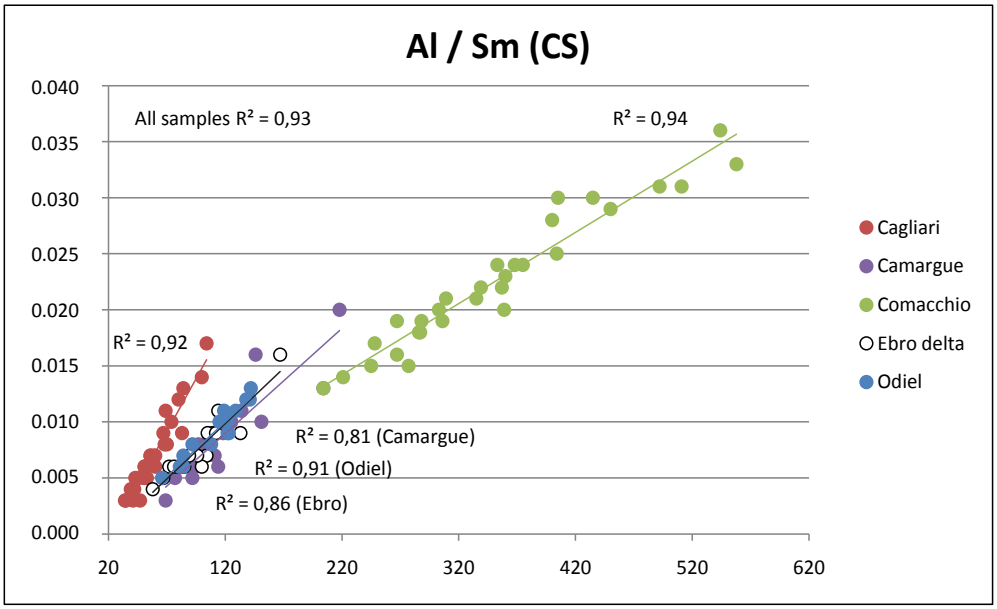


**Fig. 3.21** – Scatter plots between Al (x-axis) and Sm (y-axis) for all sites (CS) as an example of the link between REEs and other elements with no known biological functions in feathers. Determination coefficients ($R^2$) are reported for each site and the whole dataset. Concentrations are expressed as mg/kg d.w.

Furthermore, figure 3.21 highlights that a clear correlation is true also taking the whole dataset, despite the different slope of each site interpolation lines. In particular, Camargue, Ebro delta, and Odiel seem to have similar kind of sediment (Ebro and Odiel interpolation lines are perfectly superimposed), whereas Cagliari and Comacchio appear rather different. These difference could reflect different geochemical signatures of the lithic exogenous material

Generally, Pb in feathers is somewhat correlated with a lot of elements (such as Al, Be, Cs, Fe, Ga, Mn, Rb, V) and REEs in all sites, but not at the same level, and Ebro delta is a clear exception. Concerning REEs, here only the correlation of Pb and Sm is shown (fig. 3.22), as representative, but the results are the same taking any REEs. For each sites, further two scatter plot are presented, including the highest Pb-correlated elements in feathers (fig. 3.22).

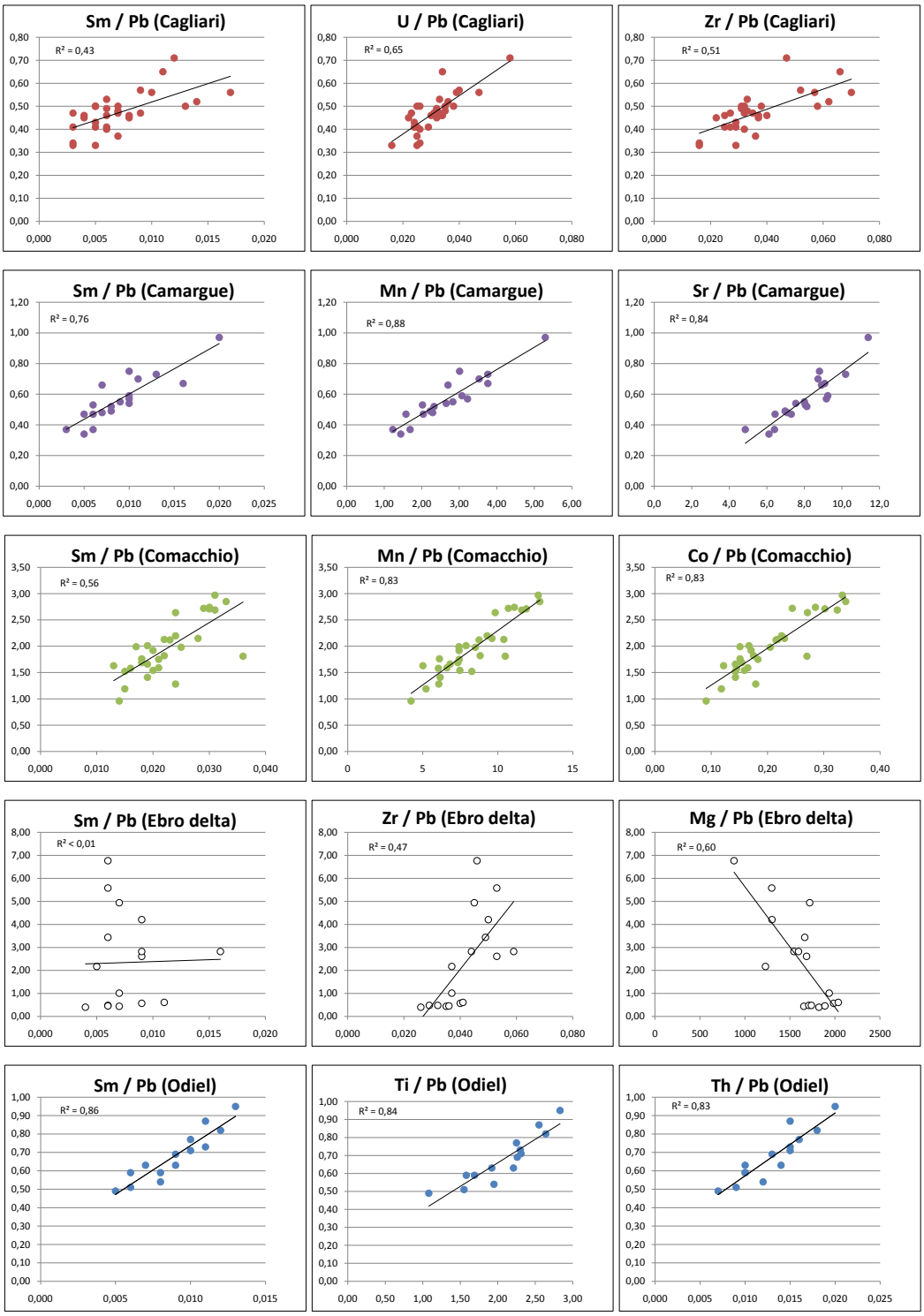


**Fig. 3.22** – Scatter plots between Pb and Sm (as representative of Pb/REEs associations) and, for each site, between Pb (vertical axis) and the two elements highest correlated to (CS). Determination coefficients ($R^2$) are reported. Concentrations are expressed as mg/kg d.w.

In Cagliari, correlation of Pb with REEs is apparent although not as good as in other sites. Highest correlations, but not much more evident than Pb/REEs, are to U and Zr. It is likely that Pb in many Sardinian samples is associated to granites or porphyries containing U, and therefore originated by external contamination. However, the origin of Pb in a small part of specimens remains unexplained.

Camargue shows good correlation with Sm, but even higher correlations ($R^2$ around 0.80 and over) were verified with some others REEs, Mn, Sr, U, Fe and many other elements. Also for this site, Pb presence in feathers appears to be controlled by soil particles in most of samples.

Comacchio correlations are verified in confront to Sm and all REEs, but they are not very high, being $R^2$ included between 0.52 and 0.60. Lead highest correlations are found with Mn and Co, but $R^2$ over 0.70 result also comparing Pb to U (but not Zr), Ni (but not Cr), and Mg. Not all Comacchio samples are exhaustively explained by external contamination due to residual particles, as shown in fig. 3.23. This diagram reports all Comacchio samples on horizontal axis. For each element (Gd, Mg, Mn, Pb, and U), the sum of all concentrations was calculated, and the relative concentration within each sample (RC) respect to that sum is plotted on vertical axis. RC was calculated as follows:

$$RC_{e,i} = \frac{c_{e,i}}{\sum_i c_{e,i}} \cdot 100$$

where *e* is the considered element and $c_{e,i}$ is the concentration of *e* in the sample $i^{th}$.

From sample 22 to 31 (the highest in Pb levels) the association to REE and Mn (Cobalt showed similar pattern to Mn) is very poor, except sample 26. In this group, Uranium goes with some samples, but with a pattern unclear, and Mg appears randomly distributed. On the opposite side, samples 1 to 4 are closely related to Mn, U, and Gd, with Mg variable again. Magnesium, and the other considered metals, become regularly associated to Pb levels in a large intermediate group samples (7 to 21, with very few exceptions). Respect to the populations detected by means of cumulative curves within Comacchio colony (fig. 3.20), this group involves the population showing intermediate Pb levels and the low part of the highest (fig. 3.23). In light of the observations shown after this analysis, the origin of Pb in samples 22, 27, 30, and 31, and in general in samples with the highest levels remains to be solved. A role of Mg to associate Pb in feathers in most Comacchio birds with intermediate Pb levels seem significant, as well as REEs, Mn and U. It is

unknown if these samples have similar contributes of endogenous and exogenous Pb: if this were the case, Mg might participate to the storing of Pb in keratin.

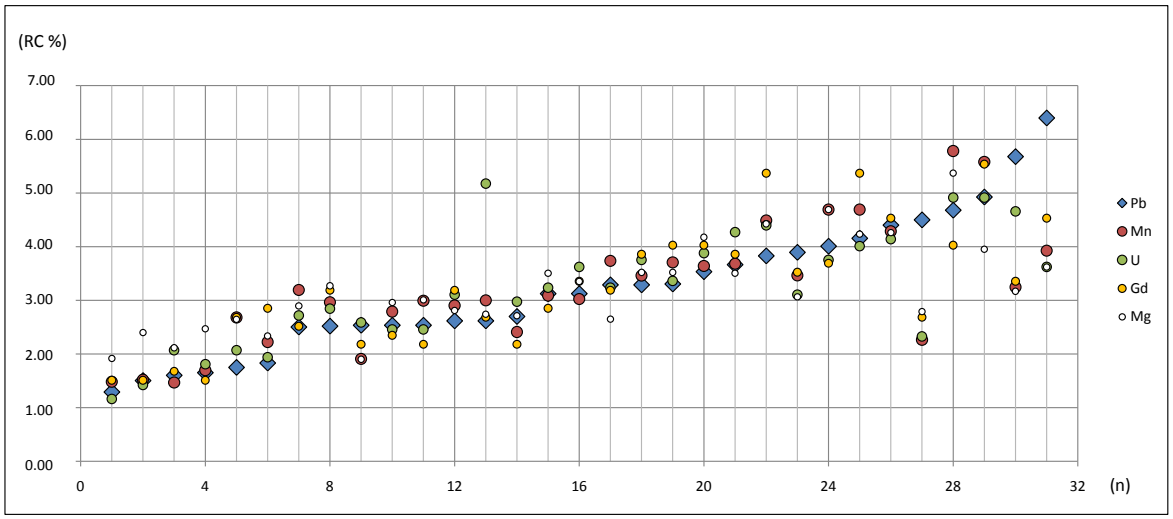


**Fig. 3.23** – Standardized Pb concentrations of SS are plotted against Mn, U, Gd (as example of REE), and Mg. On the horizontal axis, is reported the series of samples in the SS dataset. The percentage levels of the elements, respect to the summation of all concentrations, are plotted on vertical axis for each sample.

In Ebro delta Pb behaves in a more complicated way, and needs an in-depth analysis within the dataset. Lead does not show any correlation with REEs and generally with any other elements. Only a slight correlation ($R^2$ = 0.52) can be found with Zr. It is the only site where slight negative correlations were observed (to Li, Mg, Na, and Ni, with $R^2$ between 0.53 and 0.60, Mg in fig. 3.22). In all correlations tested in Ebro delta using the whole dataset, a peculiar pattern comes out: the presence of two distinct populations (except two doubtful samples) is clear in Pb/Cr and Pb/Ni scatter plots (fig. 3.24), and it is difficult to interpret without observing in depth the two population.

Lead of the two groups (1 = Pb higher than 2 mg/kg; 2 = Pb lower than 1 mg/kg) was compared to all elements separately. The two doubtful samples were not considered, because of their changeable behaviour. Group 2, even if with small number of samples, show high correlations to REEs, Al, Rb, Sr, Th, and most of all V ($R^2$ = 0.85) (fig. 3.25). This subset is similar to other sites, with Pb in feathers highly correlated to elements unlikely metabolized by Flamingos. On the other hand, group 1 is not correlated to any elements, Rb/Pb is reported as an example (fig. 3.25). Only a slight negative correlation ($R^2$ = 0.38, becoming $R^2$ = 0.59 removing a scattering sample) can be

found with Cu (fig. 3.25). According to the findings concerning some Comacchio SS, it can not be excluded that the specimens highest in Pb reflect endogenous high contamination, and that some antagonism may occur with an essential trace element such as copper. Evidently, this hypothesis must be verified through further studies.

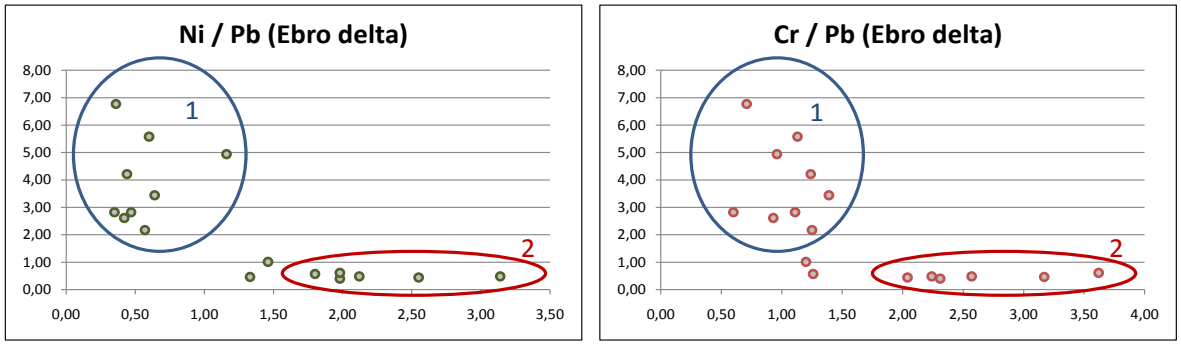


**Fig. 3.24** – Scatter plots Cr/Pb and Ni/Pb in Ebro delta. Vertical axis refers to Pb. Two distinct groups of samples are appreciable, except two doubtful specimens, more close to group 2 in Ni/Pb, vice versa to group 1 in Cr/Pb. Determination coefficients ($R^2$) are reported. Concentrations are expressed as mg/kg d.w.

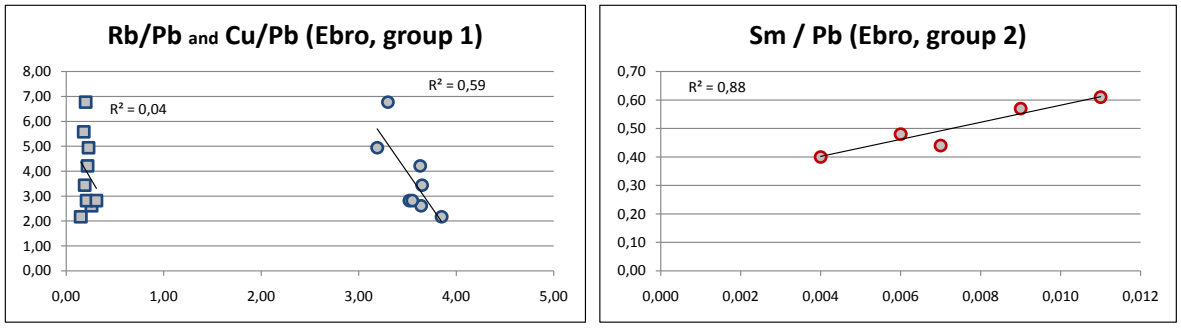


**Fig. 3.25** – Scatter plots of Ebro delta samples, grouped by Pb level (reported on y-axis). Group 1, showing no correlations to Rb/Pb (as well as all elements), includes samples higher than 2 mg/kg Pb, while group 2, highly correlated to REEs, Al, Rb, Sr, Th, and V, lies below 1 mg/kg. Two intermediate samples are not included. Determination coefficients ($R^2$) are reported. Concentrations are expressed as mg/kg d.w.

Lastly, lead in Odiel colony is clearly highly associated to REEs, except for one sample, scattering in all plots, then removed from this analysis. Determination coefficient is over 0.85 for Pb versus Sm (fig. 3.22), and it is over 0.80 for Ce, Gd, La, Nd, and Pr. Odiel is particular for Fe, not correlated to Pb and REEs at all, whereas in all other sites Fe is well associated to presence of REEs and

sometimes sensitive to Pb. Instead, Pb is most correlated to Ti and Th in Odiel (fig. 3.22). This coexistence of Pb, Th and Ti is characteristic of this colony, because Pb follows both elements only in Odiel, whereas Th and Ti are correlated in all sites. Concluding, in most Odiel samples is confirmed a contribution of external Pb contamination capable to mask the bioaccumulated level.

Burger (1993), to biomonitor lead using feathers, recommended to collect feathers soon after molt, either from fledglings. She also believed that external contamination effects could be mitigated by washing feathers and controlled collecting feathers from birds in the same population living in the same place, taking note of age of feather. In our study those suggestions have been followed, but external contamination seems to affect Pb concentrations in Flamingo fledglings feathers. Applying the washing procedure described in chapter 2.3, the magnitude of exogenous contribution in our study tended to mask endogenous accumulation, according to Denneman and Douben (1993). However, Ek *et al.* 2004, investigating Pb along unwashed feather shafts of different species by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) found Pb of external origin, with attached particles of high concentration, but also an internal signal. In Ebro delta, and in a lesser measure Comacchio, the origin of Pb in some samples with high levels remains unclear, and a response of the feathers to elevated intake cannot be excluded. If this would be the case, a possible antagonism with Cu seems possible. This disagrees with Dauwe *et al.* (2003), which observed zinc levels less concentrated in regrown feathers in lead-treated small birds. However, metabolic synergies and antagonisms involving trace metals could be different species by species.

Duplicated samples resulted indeed very dishomogeneous. Comacchio data generally scatter outside the error interval of CRM, more than Cagliari (fig. 3.26). The determination coefficient of duplicated samples wholly taken is low, but not very indicative, as within sites no correlation exists ($R^2$< 0.01 CAG, $R^2$=0.03 COM). This agrees with Dauwe *et al.* (2003), that found lead concentrations significantly different among different segments of Tawny Owl (*Strix aluco*) and Sparrowhawk (*Accipiter nisus*) washed (with acetone) feathers. The lack of any correspondence in Pb levels in samples obtained from different parts of the same feathers is the logical result when the metal concentrations is mainly due to lithic particles caught by feather structure. Duplicated samples gives the further feeling that a new washing method, able to remove lithic fragments, is needed when the aim is to understand the bioaccumulation of Pb in feathers.

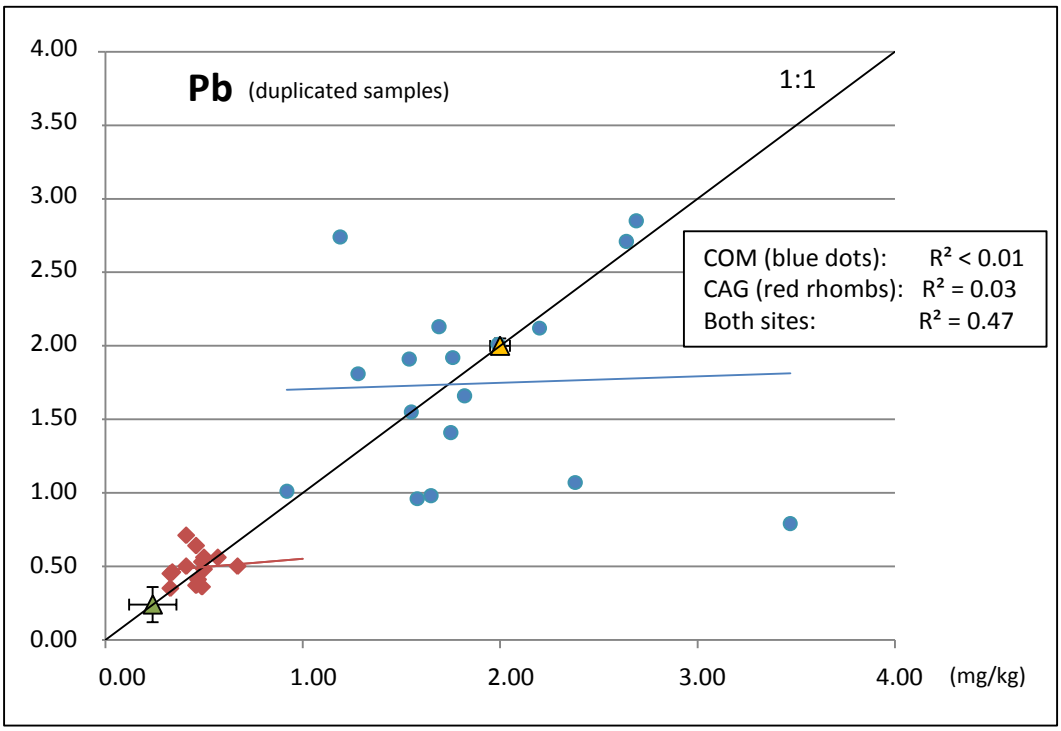


**Fig. 3.26** – Scatter plot of Pb levels (mg/kg, d.w.) in duplicated samples of Comacchio (COM) and Cagliari (CAG). The red line show 1:1 correspondence. Certified Reference Materials (Dorm-2 and ERM-278) results, with error bars (1σ), are plotted with green and orange triangles respectively. Red line and blue line interpolate CAG and COM samples respectively. Determination coefficients ($R^2$) for each site and for both sites are reported.

No significant differences in median values were observed by using SS or CS in Cagliari, Comacchio, and Odiel (tab. 3.16). The use of SS or CS to compare Pb in feathers of different colonies seems indifferent, but this conclusion should be verified when the concentrations in feathers will be only endogenous.

**Tab.** 3.16 – Results of the Kruskal–Wallis test performed on CS and SS of flamingo feathers collected from Cagliari, Comacchio, and Odiel. The table reports the Pb median (mg/kg) for each type and p-values for significant differences.

| **LEAD** | | | |
|---|---|---|---|
| | SS | CS | p-value |
| **Cagliari** | 0.46 | 0.47 | NS |
| **Comacchio** | 1.91 | 1.87 | NS |
| **Odiel** | 0.62 | 0.63 | NS |

*3.8 Mercury*

In the ranking by median values of the elements found in Flamingo feathers, Mercury comes immediately before Lead, being the 13th. Median concentration in SS is 0.74 ± 0.29, while in CS is higher (1.18 ± 0.40 mg/kg, tab. 3.17). Median Absolute Deviation (MAD) and CV vary considerably among sites, so that the measures of variability for all colonies are rather high (even CV = 109% in SS).

**Tab. 3.17** - Mercury levels (mg/kg, dry weight) on Flamingo feathers (single samples and composite samples). Median, Median Absolute Deviation (MAD), mean, Standard Deviation (SD), minimum (min) and maximum (max), Coefficient of Variation (CV%), and number of samples (n) are presented for each site and for the whole dataset. All colonies parameters relate to wild birds only.

| MERCURY (single samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 1.30 | 0.87 | 2.19 | 1.95 | 0.31 | 7.28 | 89 | 30 |
| **Comacchio** | 0.66 | 0.20 | 0.75 | 0.47 | 0.18 | 2.46 | 63 | 31 |
| **Odiel** | 0.61 | 0.11 | 0.71 | 0.30 | 0.38 | 1.60 | 43 | 25 |
| **Venice** | 0.94 | 0.23 | 0.97 | 0.32 | 0.48 | 1.37 | 33 | 7 |
| **ALL COLONIES** | **0.74** | **0.29** | **1.22** | **1.33** | **0.18** | **7.28** | **109** | **93** |

| MERCURY (composite samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 1.47 | 0.34 | 1.65 | 0.87 | 0.42 | 3.48 | 53 | 30 |
| **Camargue** | 0.98 | 0.44 | 1.20 | 0.66 | 0.45 | 2.39 | 55 | 18 |
| **Comacchio** | 1.12 | 0.36 | 1.22 | 0.68 | 0.36 | 3.20 | 56 | 30 |
| **Ebro Delta** | 1.25 | 0.41 | 1.44 | 0.64 | 0.67 | 2.70 | 44 | 17 |
| **Odiel** | 0.78 | 0.08 | 0.78 | 0.16 | 0.52 | 1.21 | 21 | 15 |
| **Captivity** | --- | --- | 0.73 | --- | 0.68 | 0.82 | --- | 7 |
| **ALL COLONIES** | **1.18** | **0.40** | **1.31** | **0.73** | **0.36** | **3.48** | **56** | **111** |

In extremely low value of Comacchio (below 0.2 mg/kg) and the very high concentrations found in Cagliari (up to 7.28 mg/kg) control the extent of the overall range of Hg data. Cumulative MAD is slightly affected by Sardinian outliers in SS and its low value indicates that most of birds show similar Hg concentrations; on the contrary, high SD is due to the anomalous population of Cagliari.

In CS, where Cagliari pattern is apparently not so strong as in SS, without comparable highest concentrations, MAD proportionally follows the increase of median value (the MAD/median ratio do not vary), while cumulative CV becomes more similar to CV of each site.

In this context, Odiel distinguish itself for the lowest Hg levels and a clear homogeneity of the population both in SS and CS (tab. 3.17, fig. 3.27 and 3.28). Only four samples from this site are slightly higher (a little over 1 mg/kg). Also Venice and Comacchio SS samples appear rather homogenous, with Venice slightly higher, and Comacchio with only few samples rich in Hg (> 1.50 mg/kg). The situation is more complicated in the cumulative curves of CS (fig. 3.28): except Odiel, all sites show one or more distinct sub-populations in the upper part of range, whereas the lower part of Camargue, Comacchio, Ebro delta, and Odiel datasets are homogenous, overlapping, and included in a narrow interval (0.36 – 0.93 mg/kg Hg). We suppose that this range could represent background Hg values in Flamingo fledglings in the Mediterranean area. Cagliari has very few samples in such low interval of CS, but also in Sardinian colony a population showing basic concentrations is advisable in SS (0.31 – 0.70 mg/kg, n = 9). Observing Cagliari SS samples with Hg higher than 1 mg/kg (fig. 3.27), an intermediate rather homogenous group of 6 specimens is easily detectable, then followed by several samples included in a wide, higher interval (1.4 – 7.3 mg/kg Hg, n = 15). The latter includes, in the lowest part, only two samples from Comacchio and one from Odiel, and no other.

In addition to Cagliari, CS curves (fig. 3.28) reveals that Hg obvious anomalies are present also in Camargue (even removing one outlier probably affected by analytical error, §3.1) and Ebro delta, and even Comacchio seems to show a discontinuity in the CS series, in correspondence or just below the median value. Such discontinuity is only barely noticeable in SS.

Captive birds show mean value comparable to Odiel, the apparently less contaminated wild colony (tab 3.17, CS). Birds of Bioparco zoo may be thought low contaminated by means of the 0.73 mg/kg Hg in feathers, and considering that adults accumulate metals during about one year (i.e. the time elapsing between two subsequent moults), while Flamingo chicks segregate into the feathers the metal loaded in tissues provided by parents in a considerably shorter period (about one month).

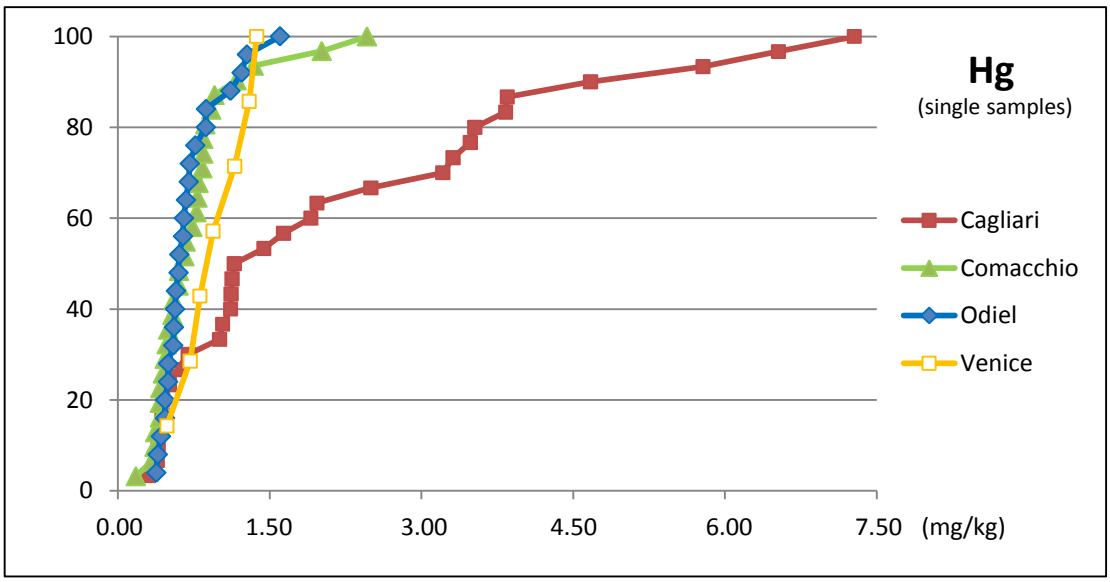


**Fig. 3.27** – Cumulative curves of Mercury concentrations in SS for Cagliari, Comacchio, Odiel, and Venice.

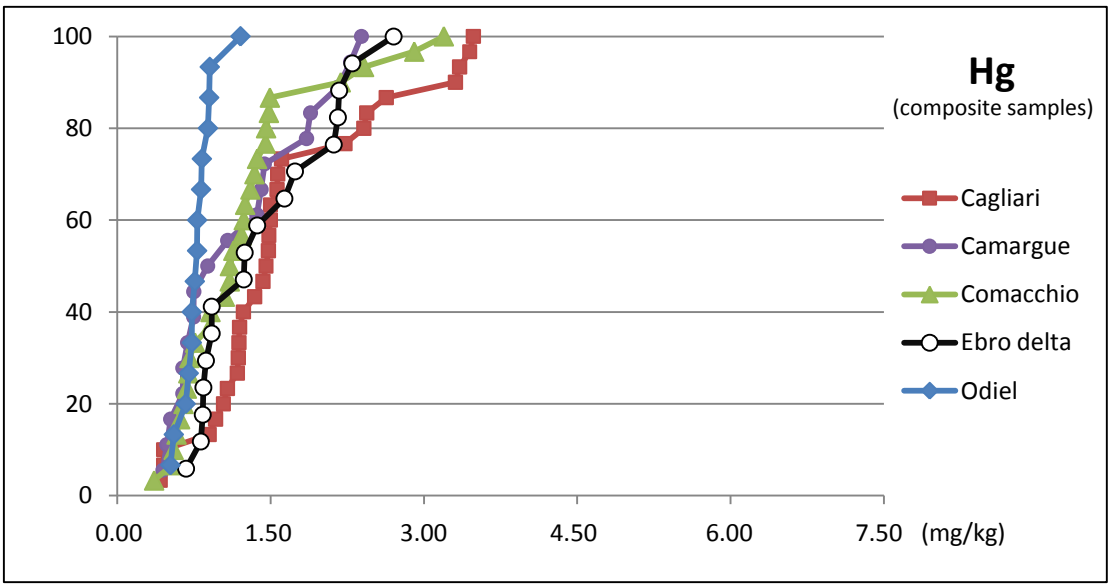


**Fig. 3.28** – Cumulative curves of Mercury concentrations in CS for Cagliari, Camargue, Comacchio, Ebro delta, and Odiel.

Kruskal-Wallis test on SS confirmed that Cagliari is statistically different from Comacchio and Odiel, while Cagliari and Comacchio do not differ from Venice. Odiel is similar to Comacchio and differs from Venice (tab. 3.18). Regarding CS, Odiel is statistically different from all sites, but Cagliari. Camargue, Comacchio, and Ebro delta results not different in the pairwise comparison. Cagliari is still different from Comacchio, but similar to Camargue and Ebro delta (tab. 3.19).

**Tab.** 3.18 – Pairwise comparison between sites by using Kruskal-Wallis test performed on SS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was 0.002.

| MERCURY (single samples) | | | | |
|---|---|---|---|---|
| | **Cagliari** | **Comacchio** | **Odiel** | **Venice** |
| **Cagliari** | ----- | | | |
| **Comacchio** | 0.001 | ----- | | |
| **Odiel** | 0.003 | NS | ----- | |
| **Venice** | NS | NS | 0.038 | ----- |

**Tab.** 3.19 – Pairwise comparison between sites by using Kruskal-Wallis test performed on CS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was 0.001.

| MERCURY (composite samples) | | | | | |
|---|---|---|---|---|---|
| | **Cagliari** | **Camargue** | **Comacchio** | **Ebro** | **Odiel** |
| **Cagliari** | ---- | | | | |
| **Camargue** | NS | ---- | | | |
| **Comacchio** | 0.022 | NS | ---- | | |
| **Ebro** | NS | NS | NS | ---- | |
| **Odiel** | < 0.0001 | NS | 0.025 | < 0.0001 | ---- |

Hg is known for its toxicity on embryo and nervous system of birds, particularly when present in a methylated form (MetHg), and tends to accumulate in internal tissues. Birds seem to avoid some toxic effects by sequestering large proportions of Hg body burden in keratin of growing feathers (Furness *et al.*, 1986). Therefore, plumage is considered a route of excretion instead of a target organ (Thompson and Furness, 1989; Wolfe *et al.,* 1998). Mercury is largely the most investigated metal in feathers from a quantity of bird species throughout the world (Burger, 1993). Despite the additional studies carried out in the two last decades, Hg in Flamingo feathers have been poorly assessed up to date and did not involved fledglings or chicks. On seabirds, two studies supported

the suitability of fledglings feathers to represent Hg environmental anomalies in the vicinity of the nest, since most of mercury inherited from egg by embryo is sequestered in first down plumage and therefore eliminated before the starting of feather growing process (Furness *et al.,* 1986, Stewart *et al,.* 1997).

In general, trophic level determine mercury bioaccumulation in birds, and feather concentrations as consequence (Stewart *et al.*, 1997; Hahn *et al.*, 1993). Ardeids, the most investigated colonial waterbirds, lie at a upper level than Flamingos, as they mainly feed on fishes and other vertebrates. Two colonies of Great Egret (*Casmerodius albus*) in Everglades (Florida, U.S.), showed 3.2 ± 1.36 to 4.4 ± 1.19 µg/g d.w. (mean ± SD) in scapulars of chicks. This heron is rather generalist, eating large invertebrates, fishes, amphibians, and varying diet accordingly to food availability (Rumbold *et al.*, 2001). These data are higher than all groups of Flamingos we studied except Cagliari. According to their higher position into the trophic chain, Squacco Heron (*Ardeola ralloides*) chicks in Axios Delta (Greece) show relatively high means, (2.79 ± 1.36 and 3.40 ± 1.34 mg/kg d.w. in two different years), well over all Flamingo colonies, but lower than Cagliari most Hg-rich samples. However, Goutner *et al.* (2001) reported also concentrations spread in a wide range (0.36–6.20 mg/kg), similar to Cagliari Flamingo SS. As the authors, on the basis of their results on Hg, considered Axios Delta polluted, we suppose Flamingos born in Sardinia definitively contaminated.

Burger and Gochfeld (2001) found 0.077 ± 0.027 µg/g d.w. (mean ± SD) on pooled samples obtained from primaries of adult Lesser Flamingos in Namibia. We never found so low concentrations in any SS or CS and so little variation. Even in the least contaminated samples (from Odiel and captivity), Hg concentrations are about 10 times higher than in Lesser Flamingos. Our highest CS means (from Cagliari) are about 20 times higher. Even though the Lesser Flamingo has a considerably different feeding behaviour than the Greater, mainly filtering blue-green algae from the surface of water or benthic diatoms from sediment (Del Hoyo *et al.*, 1992), the negligible feather concentrations found by researchers state that mercury in feathers is virtually absent even in adult birds, in regions with low urban and industrial development and low background levels in soils.

Recently, Hg values in Greater Flamingo feathers were assessed by Ancora *et al.* (2008), referring to dead adults. They report data from four individuals, three of them around 1 mg/kg (d.w.), that we can consider adherent to background levels for Mediterranean wild Flamingos, and one with 2.36 mg/kg Hg, evidently higher but not interpretable without information about age and moulting site.

Adult Greater Flamingos examined by Cosson *et al.* (1988b) showed Hg levels in primaries between 0.17 and 5.49 mg/kg (d.w.), mean values 1.70 mg/kg related to covered webs and 1.92 mg/kg to exposed webs. Standard deviation was 1.36 in both cases (tab. 3.20). These means are higher and the dispersions larger than the Camargue CS we analyzed. This is not unexpected, being the group sampled by French researchers disparate in age (1,5 to over 25 years) and probably in provenance.

**Tab.** 3.20 – Mercury levels found in Camargue, according to Cosson *et al.* (1988) on adult Flamingos variously aged. Mean, SD, CV, min and max are reported for inner and outer web of wing primary feathers (PP). Outer part of feathers was thought more exposed to external contamination than inner part.

| | Mean | SD | CV | Min | Max | Age |
|---|---|---|---|---|---|---|
| Outer PP | 1.92 | 1.36 | 70.8% | 0.28 | 5.41 | Adults |
| Inner PP | 1.70 | 1.36 | 80.0% | 0.17 | 5.49 | Adults |

No obvious positive correlations can be found between Hg and any other element, so that Hg levels in feathers are independent from any other elements. Only a very faint correlation to K ($R^2$ = 0.43) is shown by Delta Ebro (fig. 3.29). This Spanish site show moderate negative correlations between Hg and some elements (included REEs), removing the two samples most rich in Mercury. In particular, determination coefficients are around 0.65 against Dy and Y, and also Al, Cs, and Mn (fig. 3.29). This results, found in only one site and definitely contradicted by other colonies, are hard to explain. As $R^2$ is low, a random factor could be likely.

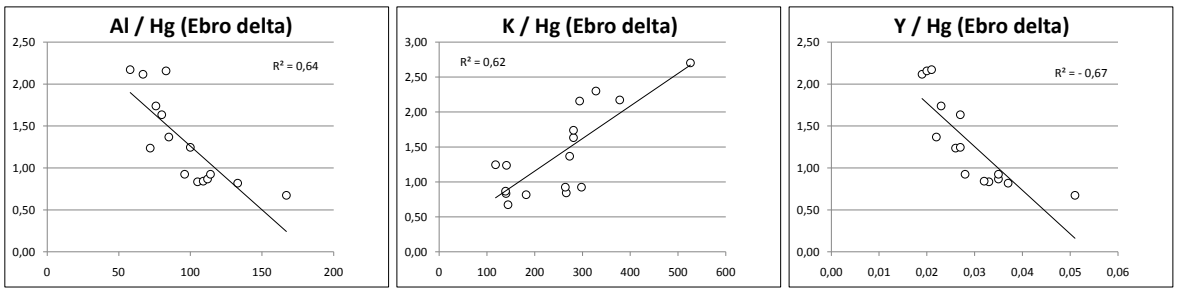


**Fig. 3.29** – Scatter plots of Ebro delta CS. Correlations Al/Hg and Y/Hg are calculated removing the 2 samples highest in Hg. Determination coefficients ($R^2$) are reported. Concentrations are expressed as mg/kg d.w.

The lack of clear correlations with any other element suggests that external contamination of Hg in Flamingo feathers is irrelevant, according to almost researchers. Though metal levels in feathers may increase with time as a result of exogenous deposition even in young birds (Amiard-Triquet *et al.*, 1991), this process seems to be less conspicuous for Hg than other metals (Veerle *et al.*, 2004).

Duplicated samples show near perfect homogeneity (fig. 3.30). Only very few samples of Cagliari scatter a little away from 1:1 line, and outside the tolerance of Certified Reference Materials (Dorm-2 and ERM-278).

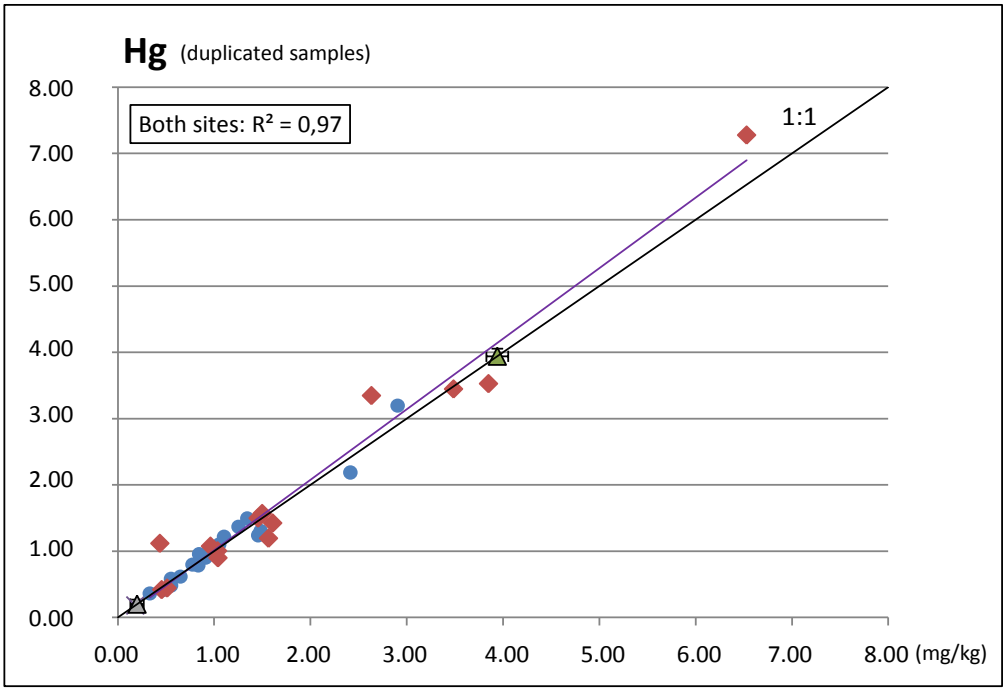


**Fig. 3.30** – Scatter plot of Hg levels (mg/kg, d.w.) in duplicated samples of Comacchio (COM) and Cagliari (CAG). The black line show 1:1 correspondence. Certified Reference Materials results with error bars (1σ), are plotted with green (Dorm-2) and grey triangles (BCR-414).

No sign lead to have doubts about the endogenous origin of Hg in feathers, according to a large number of studies that approached the question. Altmeyer *et al.* (1991) observed identical Hg concentrations in upper and lower section of vanes in all primary wing feathers from a raptor species. They verified the same result between vanes and shafts. Other researches noticed Hg constantly higher in vane than in shaft but no significant differences were recorded (Goede and de

Bruin, 1984; Honda *et al.*, 1986; Dauwe *et al.*, 2003). More recently, on raptors, five different segments of tail feathers were compared for Hg content (Dauwe *et al.*, 2003) and once again no significant differences were found. On the contrary, different feathers from one single bird can show different levels of Hg, in relation to time of moult (Furness *et al.*, 1986; Hahn *et al.*, 1993). In our survey, this factor was controlled by sampling young birds in the first plumage, all grown at the same time. In general, it was ascertained that feathers are very suitable biomonitors for mercury contamination (Veerle *et al.*, 2004), and especially older nestlings are suitable indicator organisms for local Hg contamination (Wenzel *et al.*, 1996). We demonstrated that this belief can be applied to assess Hg bioavailability also in Flamingo colonies.

The results of our study indicate that Hg level in Flamingo fledglings feathers can be very low, presumably where sediments are not polluted by this harmful metal. However, most of sites recorded more or less important anomalies in about half of specimens, probably determined by relatively high levels in areas used by Flamingo parents while rising chicks. Among the sites we investigated, Cagliari area is known to have been affected in the past by heavy Hg contamination, and the problem might be not completely solved, despite decontamination works attempted in the early 1990s (Degetto *et al.*, 1997). The Delta of Ebro and Camargue colonies deserve further investigation because several CS display high Hg concentrations, but no single samples were available for an in-depth analysis within the two populations. However, one can hypothesize that one or more wetlands reached by some adults during feeding activity are polluted with relevant Hg concentrations. Perhaps Comacchio breeding birds feed in at least two areas differently rich in Hg, but they do not arouse special concern for raising chicks. If heavy polluted sites exist around Comacchio colony, they are visited only by few Flamingo during the breeding period.

Unexpected significant differences in median values by using SS or CS are shown in Odiel, and especially in Comacchio (tab. 3.21). The causes of these differences do not seem to be the same. The high homogeneity of the two compared Odiel distributions facilitated the positive response of the statistical test, and the p-value of Odiel test resulted just below the significance boundary. Differently, Comacchio shows non homogeneous pattern more clearly in CS than SS. Median value is actually higher in Comacchio CS, and the statistical difference obvious.

**Tab.** 3.21 – Results of the Kruskal–Wallis test performed on CS and SS of flamingo feathers collected from Cagliari, Comacchio, and Odiel. The table reports the Hg median (mg/kg) for each type and p-values for significant differences.

| MERCURY | | | |
|---|---|---|---|
| | SS | CS | p-value |
| **Cagliari** | 1.30 | 1.47 | NS |
| **Comacchio** | 0.66 | 1.12 | < 0.001 |
| **Odiel** | 0.61 | 0.78 | 0.04 |

*3.9 Zinc*

Zinc is highly concentrated in Flamingo feathers analyzed in this study. According to median ranking it was the 4$^{th}$ element after Na, Mg, and K (three elements surely involved in a number of metabolic processes), though Al and Fe have mean concentrations a little higher.

Zinc medians are in the order of one hundred mg/kg (124 ± 13 mg/kg, SS; 115 ± 10 mg/kg, CS) (tab. 3.22). Among all assessed elements, CV is the lowest (14%), only Copper resulted similar. In fact, the interval including all samples in each site is very narrow (fig. 3.31 and 3.32). However, Camargue seems rather different, with only the 30% of specimens (the highest in Zn), overlapping the range of the other sites. For this reason, the French colony influences the overall parameters of variability in CS, otherwise even smaller. Also captive birds give strange, and unexpected, results , because mean value appears definitely low (66.3 mg/kg, against 91.8 ± 11.4 mg/kg Zn of Camargue).

**Tab. 3.22** - Zinc levels (mg/kg, dry weight) on Flamingo feathers (single samples and composite samples). Median, Median Absolute Deviation (MAD), mean, Standard Deviation (SD), minimum (min) and maximum (max), Coefficient of Variation (CV%), and number of samples (n) are presented for each site and for the whole dataset. All colonies parameters relate to wild birds only.

| ZINC (single samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 109.0 | 10.5 | 114.0 | 15.1 | 86.2 | 157.0 | 13 | 30 |
| **Comacchio** | 126.0 | 8.0 | 127.2 | 13.5 | 103.0 | 156.0 | 11 | 31 |
| **Odiel** | 131.0 | 12.0 | 130.9 | 16.1 | 92.2 | 158.0 | 12 | 25 |
| **Venice** | 147.0 | 7.0 | 147.0 | 7.7 | 137.0 | 158.0 | 5 | 7 |
| **ALL COLONIES** | **124.0** | **13.0** | **125.4** | **17.0** | **86.2** | **158.0** | **14** | **93** |

| ZINC (composite samples) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | median | MAD | mean | SD | min | max | CV (%) | n |
| **Cagliari** | 111.0 | 7.0 | 112.6 | 11.8 | 96.8 | 152.0 | 10 | 30 |
| **Camargue** | 89.8 | 6.2 | 91.8 | 11.4 | 73.3 | 115.0 | 12 | 19 |
| **Comacchio** | 120.5 | 7.5 | 122.4 | 12.4 | 98.0 | 153.0 | 10 | 30 |
| **Ebro Delta** | 112.0 | 7.0 | 111.5 | 7.8 | 99.4 | 122.0 | 7 | 17 |
| **Odiel** | 133.0 | 6.0 | 127.2 | 12.2 | 103.0 | 141.0 | 10 | 15 |
| **Captivity** | --- | --- | 66.3 | --- | 65.2 | 66.7 | --- | 7 |
| **ALL COLONIES** | **115.0** | **10.0** | **113.5** | **16.0** | **73.3** | **153.0** | **14** | **111** |

Despite the great homogeneity of data wholly taken, only Ebro delta and Venice show populations really homogeneous (fig. 3.32; fig. 3.31). Considering the cumulative curves of the other sites, each of them show a typical pattern (fig. 3.32; fig. 3.31). Cagliari, Comacchio, Odiel (SS), and Camargue (CS) show small tails in the low- and high-range and a large intermediate group.

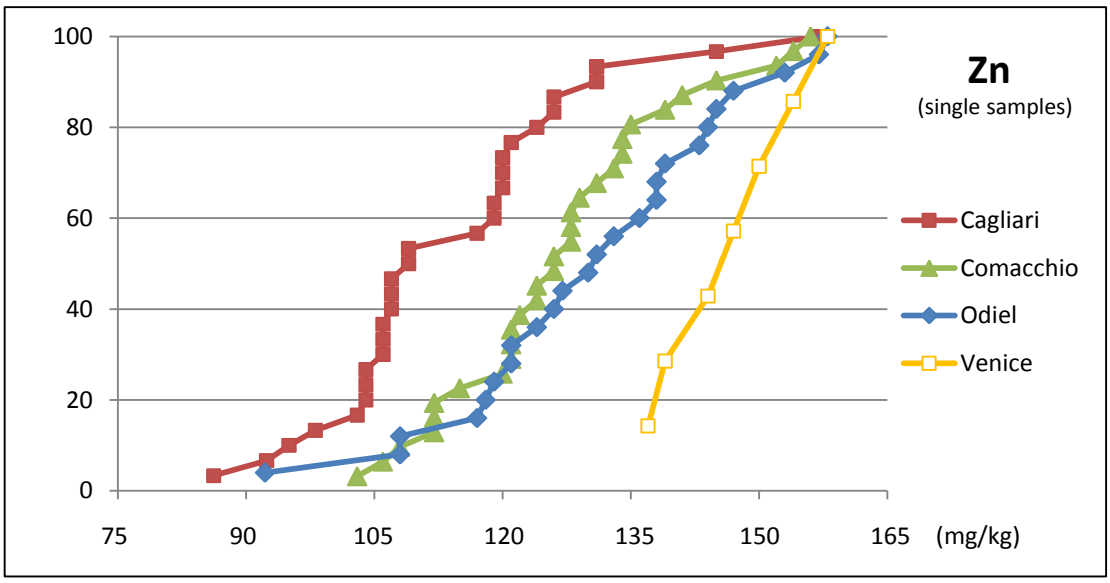


**Fig. 3.31** – Cumulative curves of Zinc concentrations in SS for Cagliari, Comacchio, Odiel, and Venice.

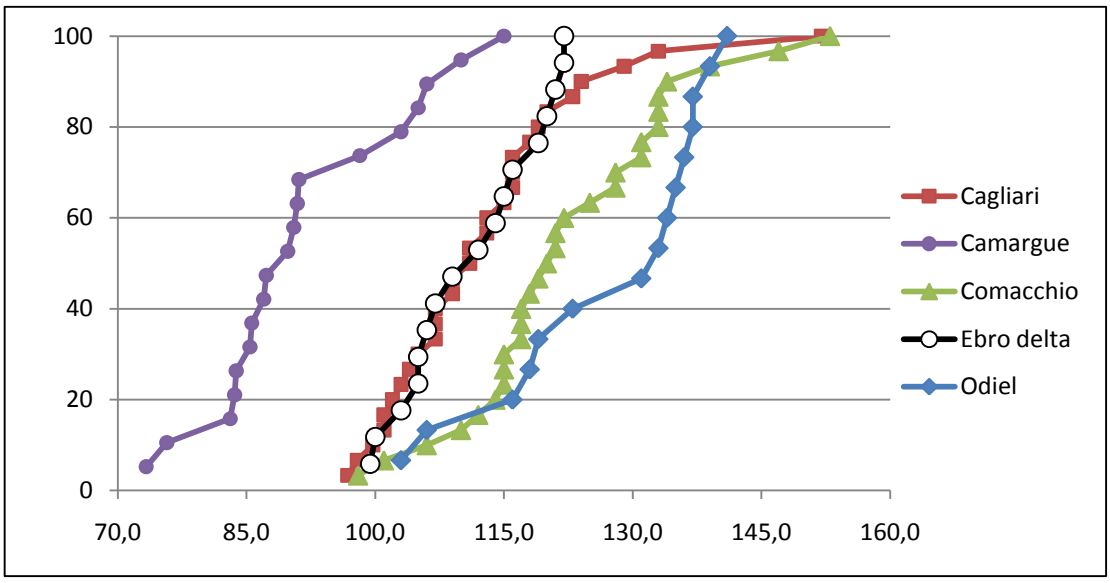


**Fig. 3.32** – Cumulative curves of Zinc concentrations in CS for Cagliari, Camargue, Comacchio, Ebro delta, and Odiel.

The intermediate group is continuously distributed (Comacchio and Odiel SS, fig. 3.31) or splitted in sub-populations (Cagliari SS, fig. 3.31); Comacchio, Camargue, Odiel CS, fig. 3.32). The sub-populations in Cagliari SS disappears in CS (except for the highest anomalies), and on the other hand those of Comacchio and Odiel become more clear (fig. 3.32).

Kruskal-Wallis test on SS finds statistical differences among all sites, except Comacchio and Odiel. (tab. 3.23). Regarding CS, Comacchio and Odiel similarity is confirmed, as well as Cagliari and Ebro look alike (tab. 3.24). The difference between Camargue and the other sites is obvious.

**Tab.** 3.23 – Pairwise comparison between sites by using Kruskal-Wallis test performed on SS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was < 0.0001.

| ZINC (single samples) | | | | |
|---|---|---|---|---|
| | **Cagliari** | **Comacchio** | **Odiel** | **Venice** |
| **Cagliari** | ----- | | | |
| **Comacchio** | < 0.001 | ----- | | |
| **Odiel** | < 0.001 | NS | ----- | |
| **Venice** | < 0.001 | 0.001 | 0.011 | ----- |

**Tab.** 3.24 – Pairwise comparison between sites by using Kruskal-Wallis test performed on CS. P-values for significant differences are reported ($p<0.05$). The p-value resulted from of comparison among all four sites was < 0.0001.

| ZINC (composite samples) | | | | | |
|---|---|---|---|---|---|
| | **Cagliari** | **Camargue** | **Comacchio** | **Ebro** | **Odiel** |
| **Cagliari** | ---- | | | | |
| **Camargue** | < 0.0001 | ---- | | | |
| **Comacchio** | 0.001 | < 0.0001 | ---- | | |
| **Ebro** | NS | < 0.0001 | 0.003 | ---- | |
| **Odiel** | < 0.001 | < 0.0001 | NS | 0.001 | ---- |

Since long time, Zinc is known as an essential element required for feather formation (Sunde, 1972). Honda *et al.* (1986) confirmed that zinc participates in keratinization processes. It is thought that Zinc, as well as Mn, may have an effect of pigmentation. Therefore, these metals would be more abundant in eumelanin-rich feathers (grey, blackish and black feathers), and in dependence of diet content of these ions during feather growth (Niecke *et al.*, 1999). Anyway, zinc accumulation in feathers is supposed internally well regulated (Burger, 1993). However, Zinc is also dispersed in the environment as a consequence of human industrial activities, and it is proved that high doses of Zn in birds could induce decrease of appetite and/or digestibility of prey,

therefore explaining decreased body weight (Sundaresan *et al.*, 2008). On the other hand, exposure to high levels of nonessential elements increases the levels of zinc-binding proteins, and may alters physiological uptake and accumulation of zinc (Elliott and Scheuhammer, 1997; Heinz *et al.*, 1999). At least cadmium and lead (Dauwe *et al.*, 2002) could influence the metabolism of zinc, but it remains to be demonstrated whether lowered levels of zinc accessible for accumulation in feathers may occur as a consequence (Dauwe *et al.*, 2000).

Zinc is often analyzed in feather studies, and researches on Flamingo carried out in Camargue already cited in previous paragraphs, reported some data about this element. Regarding non-Flamingo species, Wenzel *et al.* (1996) showed as Zn (and Cu, §3.6) in Kittiwake chicks were increasing in tissues and belly feathers when the chick grew older. The authors found in feathers an increase from 79 mg/kg at birth to approximately 116 mg/kg after three weeks, with a similar increase in liver. The authors argued that levels of this metabolically regulated metal are not only influenced by environmental contamination but also by physiological demands. However, in contrast to mercury, less than 20% of the total body content were incorporated in newly formed feathers (Honda *et al.*, 1986), so that no effective elimination of zinc was observed.

Little Egret (*Egretta garzetta*), a species belonging to Ardeids family, showed values ranging from 77.5 to 134.5 mg/kg in different breeding sites in Hong Kong (Connell *et al.*, 2002). In different kind of feathers of three heron species in India, zinc levels were comprised in the interval 101-196 mg/kg (Muralidharan *et al.*, 2004). These values were in the same range reported for different species of raptors living in Finnish unpolluted sites (Solonen *et al.*, 1999).

Concerning Flamingo fledglings studied by Amiard-Triquet *et al.* (1991) in Camargue (tab. 3.25), inner and outer GC segments showed mean concentrations very similar to scapular feathers we analyzed in the same site (91.8 ± 11.4 mg/kg), therefore in average depleted in Zn compared to other colonies. The range of their dataset were wider (48.3–158.1 mg/kg Zn), including some concentration levels even below captive birds mean (that is 66.3 mg/kg), and some others near the highest we found in all sites. Comparing the results of both studies, a smaller availability of this metal seems plausible for Camargue birds, or a concealed biochemical factor could limit the Zn metabolism processes involving Flamingo chicks feathering.

Cosson *et al.* (1988) verified that in adult Flamingos, external contamination in exposed part of feathers is likely for Zn. Actually the inner part, less exposed, showed very low concentrations (66.1 ± 19.7 mg/kg) more similar to the adult captive flamingos than the wild ones (tab. 3.25; tab. 3.22). One may suppose than in adults Zn request for feathers may be normally lower than young

Flamingos, but the hypothesis that this essential ion was just enough or deficient in moulting captive birds, as well as in the dead birds studied in Camargue, remains a sound possibility. Regarding outer part of PP in Cosson *et al.* (1988), some birds were certainly contaminated by external deposition, because of the heavy contrast with inner part. This difference was not appreciable in fledglings feathers analyzed by Amiard-Triquet *et al.* (1991).

**Tab.** 3.25 – Zinc levels found in Camargue, according to Cosson *et al.* (1988) on adult Flamingos variously aged, and Amiard-Triquet *et al.* (1991) on Flamingo fledglings. Mean, SD, CV, min and max are reported for inner and outer part of wing greater coverts (GC) relatively to the first study, and for inner and outer web of wing primary feathers (PP) in the second. Outer part of feathers was thought more exposed to external contamination than inner part.

| | Mean | SD | CV | Min | Max | Age | Source |
|---|---|---|---|---|---|---|---|
| Outer GC | 85.0 | 16.1 | 18.9% | 51.4 | 134.5 | ≈ 2 months | Amiard-Triquet *et al.*, 1991 |
| Inner GC | 90.5 | 17.7 | 19.6% | 48.3 | 158.1 | ≈ 2 months | Amiard-Triquet *et al.*, 1991 |
| Outer PP | 101.1 | 40.2 | 39.8% | 44.5 | 189.8 | Adults | Cosson *et al.*, 1988 |
| Inner PP | 66.1 | 19.7 | 29.8% | 37.7 | 105.5 | Adults | Cosson *et al.*, 1988 |

Considering the whole dataset, Zinc resulted poorly correlated to other elements. In particular, in Ebro delta, the most homogeneous population, Zinc is absolutely insensitive to the variations of any other elements. Except Camargue, only a very slight correlation can be observed against Selenium, but determination coefficients ($R^2$) do not exceed 0.30. However, in Camargue, where Zn/Se $R^2$ is irrelevant, could exist some association between Zn-K and Zn-Na ($R^2$=0.63 and $R^2$=0.58 respectively). Determination coefficients rise when the three samples most rich in Zn are removed (becoming $R^2$=0.71 and $R^2$=0.66 respectively) (fig. 3.33). However high correlation between K and Na is common: Ebro delta shows undoubtedly the highest ($R^2$=0.98), followed by Cagliari ($R^2$=0.80), and Camargue ($R^2$=0.78). In Odiel it is weaker ($R^2$=0.63) and in Comacchio it do not exist at all. However, the Zn-K-Na link is characteristic of Camargue only. Apart these contrasting results, Zinc appears really independent from other elements in feathers, in particular non biologic elements, therefore a significant external contamination is unlikely.

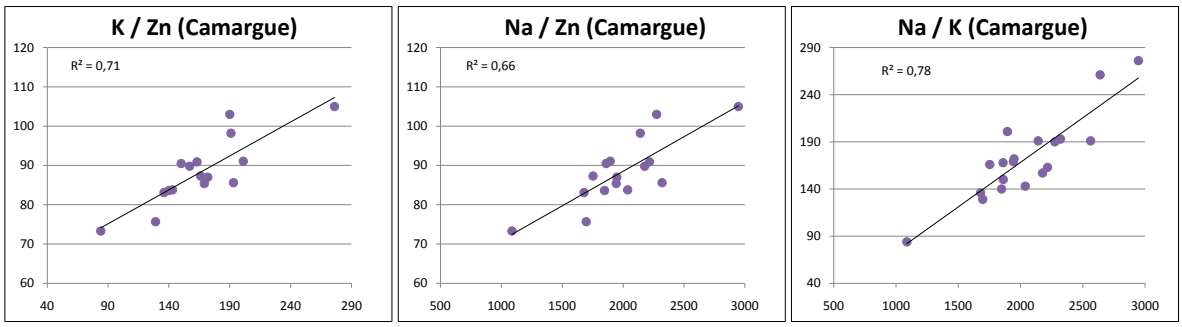


**Fig. 3.33** – Scatter plots of Camargue CS. Correlations K/Zn and Na/Zn are calculated removing the two samples highest in Zn. Determination coefficients ($R^2$) are reported. Concentrations are expressed as mg/kg d.w.

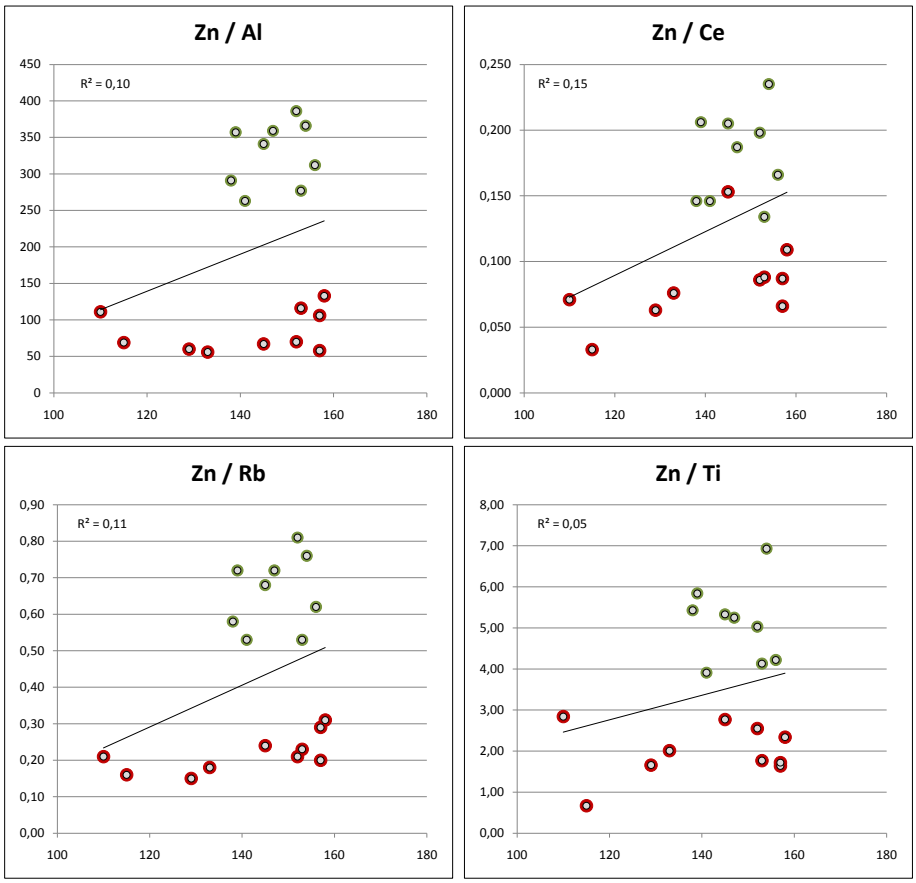


**Fig. 3.34** – Scatter plots of 19 samples (SS and CS) with high Zn from different sites. Zinc levels are compared to Al, Ce, Rb, Ti, known for little or none biological functions. Moreover, no correlations were found among Zn and any other elements, even considering Comacchio samples (green edged dots), and other samples (red edged dots) separately. Determination coefficients ($R^2$) are reported. Concentrations are expressed as mg/kg d.w.

In-depth control to exclude the hypothesis of external contamination on specimens high in Zn, was performed by selecting the most concentrated samples (both SS and CS) and comparing them to four elements thought non bioavailable (as made for Cu, §3.6). Zinc resulted once again not correlated, even considering the Comacchio samples (supposed externally contaminated by REEs and other not very mobile elements) apart (fig. 3.34).

In literature, Zinc is generally thought not much affected by external contamination, if feathers are collected shortly after formation (Goede and de Bruin, 1984). However, Dauwe *et al.* (2003) suspected a contribution from external deposition of Zn, because of the higher values observed in more exposed segments of feathers. LA-ICP-MS analyses along shafts (Ek *et al*., 2004), showed no evidence of any zinc-rich particles. Variations seen by the researchers along the shaft were thought depending on Zn concentration in the diet during feather growth, in according to Veerle *et al.* (2004). External contamination of Zn, if present in Ek *et al.* study, should be thought originated from high air-born pollution, but it is unlikely: even near highly polluted sites, zinc levels in passerines may be lower than in unpolluted sites (Dauwe *et al.,* 2000) and similar among feathers exposed for different periods (Veerle *et al*., 2004); besides, no correlation was found between vane and uropygial gland Zn concentrations (Goede and de Bruin, 1984), therefore even contamination caused by preening oil uniformly smeared on the feathers was not proved. It is likely that the high deposition rate of zinc into the feathers could mask the negligible exogenous contamination of zinc (Veerle *et al.*, 2004).

Duplicated samples are contrasting between Cagliari and Comacchio (fig. 3.35). Regression line fits very well Sardinian samples ($R^2$ = 0.79), whereas determination coefficient for Comacchio is not high ($R^2$ = 0.46). However, homogeneity of duplicated samples wholly taken is good ($R^2$ = 0.70). Certified Reference Materials (ERM-278 and BCR-414) plotted in fig. ZNR-CRM, show a small tolerance, crossed by few samples of Cagliari and most of Comacchio. The poor correspondence levels in Comacchio duplicated samples might suggest some external contamination, but it was excluded by the above-mentioned considerations. Rather, we are given to believe that, where variability of Zn in feathers was not clearly homogeneous, the availability of this essential metal for keratinisation was more irregular during feathering.

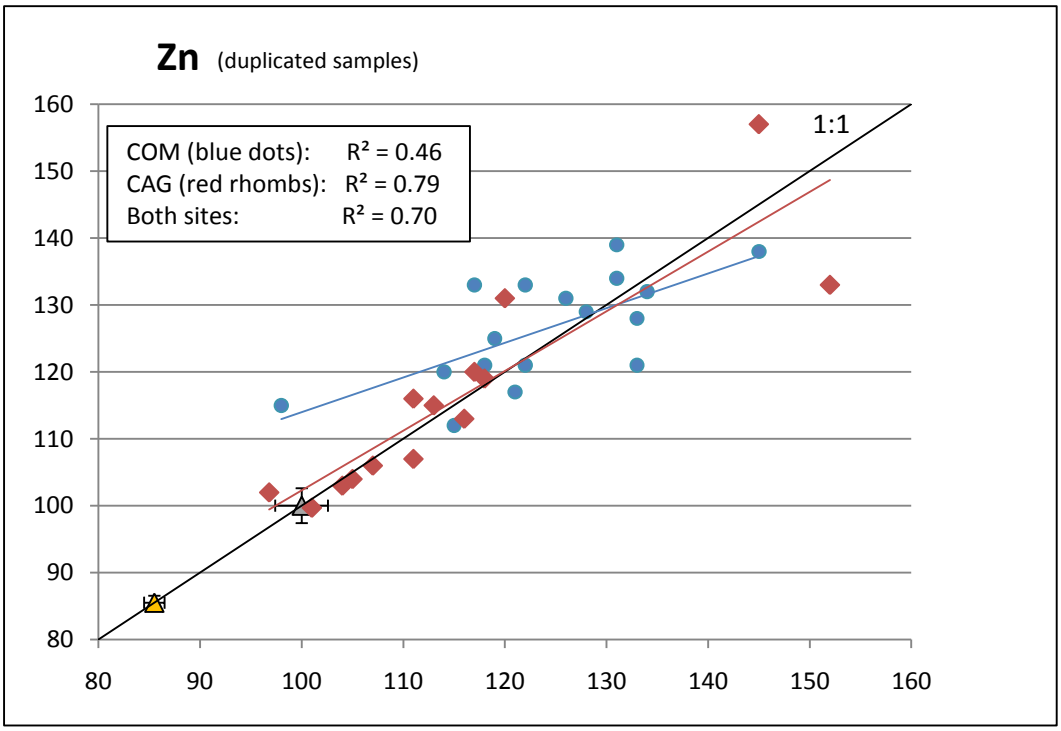


**Fig. 3.35** – Scatter plot of Zn levels (mg/kg, d.w.) in duplicated samples of Comacchio (COM) and Cagliari (CAG). The black line show 1:1 correspondence. Blue line and red line interpolate COM and CAG samples respectively. Certified Reference Materials results with error bars (1σ), are plotted with grey (BCR-414) and orange (ERM-278) triangles. Determination coefficient ($R^2$) for each site and for both sites is reported.

Similar zinc levels in different populations (Ranta *et al.*, 1978) and species (Solonen *et al.*, 1999; Ek *et al.*, 2004) limits the potential for detecting variations in environmental levels (Wenzel *et al.*, 1996). However, our opinion is that Zinc concentrations unduly low should cause concern as well as levels too high. In this work some anomalies were noticed: Camargue is definitely lower in Zn than other sites, in addition to some obvious high values and low values in several datasets. Odiel river and linked marshes are polluted by several metals, and Zinc is one (Borrego *et al.*, 2002; Morillo *et al.*, 2008). Cumulative curves show Zinc in feathers from this site generally more concentrated than others, suggesting high availability of this metal, even if no statistical difference is found between Odiel and Comacchio. On the other hand, in Ebro delta rice cultivation is widespread, and pesticide commonly used (containing Zn and other metals). However, Flamingo fledgling of Ebro delta do not show any anomalous levels of Zn in feathers. Some Zn enrichments in sediment of rivers draining the Camargue region were verified by Ferrante (2010), but the accessibility to is unlikely, judging by low Zn in feathers from France. Zinc of anthropogenic origin was found also in Cagliari area (Degetto, 1986). Sardinian flamingos not always show homogenous

levels in feathers, but the range of values appears normal. Finally, Zinc has been dispersed into Venice lagoon by metallurgic plant of Porto Marghera. We do not know how much and where such pollution affects Venice lagoon. Venice concentrations in feathers are uniformly included in a range relatively high.

No significant differences in median values were observed by using SS or CS in Cagliari, Comacchio, and Odiel (tab. 3.26). Curiously, Comacchio and Odiel show more irregularities in the distribution pattern in CS than SS, while, as expected, Cagliari population becomes more homogenous by using CS. As argued, Zinc is well regulated by birds and therefore not much sensitive to environmental changes than other metals, but it remains unclear if SS and CS can be used without distinction. Probably the choice of the type of sampling depends on the aim of the survey.

**Tab.** 3.26 – Results of the Kruskal–Wallis test performed on CS and SS of flamingo feathers collected from Cagliari, Comacchio, and Odiel. The table reports the Zn median (mg/kg) for each type and p-values for significant differences.

| **ZINC** | | | |
|---|---|---|---|
| | SS | CS | p-value |
| **Cagliari** | 109.0 | 111.0 | NS |
| **Comacchio** | 126.0 | 120.5 | NS |
| **Odiel** | 131.0 | 133.0 | NS |

# 4 FINAL REMARKS

This study intended to biomonitor, through a non-invasive sampling method on birds, the passage into the trophic chain of a large number of elements accumulated in sediments of Mediterranean coastal wetlands. After a review of the argument, based on the scientific literature of the last two decades, we decided that feathers were the preferable tissue to sample and analyze, especially from a protected species as Greater Flamingo. Feathers are dead tissue when fully grown, but during formation they are in contact with blood from which they receive essential elements and possibly other compounds capable to enter into keratin structure.

Greater Flamingo proved to be an ideal species for this survey for a lot of reasons:

1) Flamingos are colonial waterbirds: they breeds in large number, often returning in the same nesting site year by year (coastal salt pans or salt marshes in the Mediterranean area); during chick raising the home range of adults is relatively limited, including brackish wetlands they visit daily, while flightless chicks stay long time (up to 2-3 months) in the surrounding of the islet where they born;
2) adult Flamingos feed their chicks pouring a secretion (a liquid rich in carotenoids and red blood cells) into the chick's bill: the substances swallowed by chicks in the first stage are controlled by adult metabolism, theoretically similar in all Flamingos;
3) each year, most of the countries hosting breeding colonies organizes and coordinates, through a network of ornithologists and volunteers, banding campaigns: part of flamingo fledglings are captured, marked with plastic and metal rings, measured, and released. In these contexts feather sampling is relatively easy to perform;
4) Flamingos are omnivorous, but in the breeding period they are relatively high in the trophic chain, feeding mainly on invertebrates;
5) through their particular feeding behavior, Flamingos swallow considerable amounts of sediment together with food, exposing themselves to pollutants present in the environment: for this reason they rather differ from other animals that assume hazardous compounds only through their preys.

Feathers are relatively simple to be sampled, stored, and prepared for analysis, but interpretation is non always easy. Age of birds, feather colour, type of feather, bird species, and sample treatment, are variability factors possibly influencing analytical results. However, the most frequent uncertainties in the interpretation of results regarded the impossibility to distinguish

between endogenous contribution (from blood) and external contamination (air-borne pollution, uropygial oil smeared during preening, or soil particles adhered to feathers).

To control general variability factors:

1) we focused the survey on one only species;

2) only birds of the same age were sampled;

3) the same type of feather was collected from each bird;

To limit external contamination:

4) sampled birds were 7-8 weeks old fledglings: their feathers were still growing at the sampling time, thus exposed to exogenous contributions for a brief time;

5) sampled scapular feathers were not expected as exposed to dust as other flight and body feathers, because covered by a lot of others;

6) washing and digestion methods followed those widely used in literature, in order to remove, before analysis, dust and loosely adherent particles, and extract for analyses, amounts of metals proportionately comparable to other similar studies.

Sampling was carried out in 2008, in six colonies of the Mediterranean area (Ebro delta and Odiel marshes, Spain; Camargue, France; Cagliari, Comacchio, and Venice, Italy). Of the 58 elements analyzed, we obtained data of: Ag, Al, As, Be, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Hg, K, La, Li, Mg, Mn, Mo, Na, Nb, Nd, Ni, Pb, Pr, Rb, Se, Sm, Sr, Th, Ti, U, V, W, Y, Yb, Zn, Zr. Five of them were studied in detail (As, Cu, Hg, Pb, and Zn). Two kind of samples were prepared (single samples, one specimen corresponding to one bird; composite samples, one specimen corresponding to 2-3 birds). Single and composite samples (SS and CS) were prepared for Cagliari, Comacchio, and Odiel, while for Venice only SS were available. For Camargue and Ebro delta it was not possible to prepare SS, therefore only CS were analyzed. The different responses of the two kind of samples were compared in order to obtain useful indications on the use of one type or the other in future studies.

In general SS and CS showed the same median value for all elements, with an exception (Hg). Ranges were practically invariable for all elements regarding whole datasets, but some difference could be recorded taking only some sites alone. For the elements studied in detail the pattern of the distributions sometimes changed, in dependence of element and site. A mean concentration

was also obtained from pooled samples of captive Flamingos (adults) kept in Bioparco Zoo (Rome, Italy).

Quality control included the analysis of three Certified Reference Materials and comparing the Cd, Hg, and Pb results between two labs (Siena, Italy and Berlin, Germany). Very good accordance was verified for Hg, even if analytical instruments were different (CV-FIMS, Siena; AFS, Berlin). Some problems occurred for Cd and Pb in Siena results (GFAAS) indicating that Cd levels were at detection limit for the instrument, and Pb was subject to some interferences. More reliable and very sensitive resulted ICP-QMS (Berlin).

Several checks (direct and indirect) were done to identify external contamination:

1) direct: occurrence and type of particles present on some feather fragments by SEM-EDAX observation. No previous studies, except Weyers *et al.* (1988), carried out this check;

2) indirect: correlations among all the studied elements with focus on the relation with some elements with no biological function known (e.g. Al, REEs, Ti). High correlations among not much mobile elements were shown practically in all feathers, indicating that they can be useful guide to identify (and with further studies, quantify) the presence of lithic residuals. Concerning metal assessment in feathers, this approach is completely original and showed that As, Cu, Hg, and Zn are not correlated to any elements. It is very likely they are mainly bioaccumulated in Flamingo fledgling feathers;

3) indirect: duplicated single and composite samples from Cagliari and Comacchio were correlated to test the homogeneity of concentrations in feathers. The rationale is that if some soil particles rich in metals are still present, a wide difference between duplicated samples is expected between replicates of the same sample. This resulted very obvious for lead. Not high correlation were found for arsenic and zinc in Comacchio (Cagliari was highly homogeneous). Copper was not highly correlated in both sites, whereas Hg was very homogeneous. As external contamination of copper and zinc was demonstrated to be irrelevant compared to endogenous accumulation, the imperfect correspondence of duplicated samples might be due to day-by-day availability of these essential elements for feather keratinization.

However, important information were obtained from this study:

1) arsenic was high in Odiel flamingo feathers, as expected, because of the pollution from mines and industries involving that site is well known. As arsenic compounds are thought

hazardous for life, this result is of some concern, and As bioavailability and effects on wildlife in Odiel wetlands should be further investigated. Cagliari and Camargue results seem indicate the background levels of this element in Flamingo feathers. Comacchio showed slightly higher levels, but without concern. Ebro delta series was higher than other sites (except Odiel) and this is might be due to the extensive use of pesticides in rice fields, often visited by feeding flamingos;

2) copper in feathers seems less sensitive to environmental variations, because it could be well regulated by the metabolism. However this aspect has not been investigated in depth, and our results are not decisive: on one hand show little differences among almost sites (but well detectable at least in Camargue), on the other hand reveal irregularities in Camargue and Odiel distributions of data. Copper pollution is well known for Odiel estuary, and, in recent studies Camargue top soil were found enriched in copper of anthropic origin, therefore it seems appropriate to make some warnings regarding effects of copper on aquatic organisms in these sites;

3) mercury pollution was only partially removed from Cagliari wetlands in the 1990s, and it is still bioavailable for birds. This is evident comparing our Hg results among Flamingo colonies, and even to Hg levels found birds species higher in the trophic chain as Ardeids. Mercury can be virtually absent in feathers of birds living in unpolluted areas, but we found anomalous high values in about half samples of each site. This suggests that feeding areas affected by some Hg pollution, situated not far from the colony, are visited by at least a part of adults during breeding period in most of sites;

4) lead in feathers resulted, according to most researchers, mainly of external origin. We concluded that endogenous signal in Flamingos is nearly always masked by lead remained on the feather surface even after vigorous wash. This inconvenient does not allow neither to compare the bioavailability level of lead different Flamingo colonies, neither if this metal is normally stored in Flamingo feathers. Lead shots had been dispersing by hunters in wetlands since about hundred years and they are available to be swallowed by waders, ducks and flamingos in huge quantities. Acute poisoning by lead shot in Flamingos occurred in various Mediterranean wetlands, the most recent in Northern Adriatic coastal marshes. It must be noticed that the correlation among Pb and elements with no relevant biological function was highest in samples with low Pb levels (all Camargue and Odiel samples, and part of Cagliari, Comacchio, and Ebro delta ones), whereas the good

correlation disappeared for Pb-rich specimens (most of Comacchio and Ebro delta high Pb-concentrated samples). Actually, this result is unexpected, because it would be logical the contrary. The lead origin of these enriched samples remains unclear, and it could be solved only comparing this results to other environmental variables;

5) zinc is known as the most important metal in feathering processes and it resulted high and homogenous also in Flamingo feathers. However, zinc pollution occurs for sure in Odiel and Venice lagoon, and it is possible in Camargue and Ebro delta. As an essential element, metabolism of zinc can be altered by other undesirable metals, therefore anomalous low levels should be concern as well as high ones. Consequently, feathers concentrations too low could indicate zinc deficiency or some kind of environmental problem contrasting with the biochemistry of birds. In the light of that, Camargue (and captive birds) appears anomalously low in zinc.

Captive birds were included in this research in order to have a reference concentration level in non-wild Flamingos. We had only a mean value of 12 birds (without a uncertainty parameter). Only Ag and Cu resulted higher in captive birds than wild birds, probably due to the use of these metals as algicidae (Cu) and bactericidae (Ag). If they are bioaccumulated or simply deposited on feather surface should be cleared by further investigations. Molybdenum, U, W and Zr concentrations were comparable to wild birds. Uranium and Zr may be due to attached particles of soil (roman area is relatively enriched by these element), while the presence of Mo and W remains unclear.

The survey took advantage from the establishment of an efficient working group and international cooperation allowed sampling in so much sites. Washing and cutting of feathers were performed in the C.I.R.S.A. and I.S.P.R.A. laboratories. Sample digestion was carried out at Environmental Sciences Department of Siena University. Chemical analyses of all elements were performed in Berlin laboratory of the Federal Institute for Geosciences and Natural Resources, Hannover (Germany), meanwhile Cd, Hg, and Pb were analyzed also at Siena University. This work was supported by Province of Ravenna and I.S.P.R.A.

APPENDIX A

Techical characteristics of analytical instruments used.

***Atomic fluorescence spectrometry (AFS)***

Atomic fluorescence spectrometry is a sensitive (0,2 μg/L for Hg), method of trace analysis. In this method, a sample solution is atomized in a cell, the resulting atoms are illuminated with a light source, and a fraction of the atomic fluorescence, resulting when a portion of these excited atoms undergo radiational deactivation and emit radiation toward the detection device, is measured (Winefordner and Elser, 1971).

***Cold vapour with Flow Injection Mercury System (CV-FIMS)***

The PerkinElmer® Flow Injection Mercury System (FIMS) is a compact mercury analyzer coupled to cold vapor atomic absorption measurement. It allows the determination of sub-ppb concentrations of mercury, and limits interferences, since the analyte is separated from the matrix. Theoretically, the FIMS provides mercury detection limits of less than 0,005 μg/L. The FIMS uses peristaltic pumps to deliver the carrier and reductant streams and for waste removal. The sample loop is filled with an exact volume of sample, then it is introduced into the carrier stream and transported to the mixing section for reaction of reduction from $Hg^{2+}$ to $Hg^{0}$. The resulting reaction mixture is then carried to a gas/liquid separator where the gaseous elemental mercury is liberated and, after passing through a polytetrafluoroethylene (PTFE) filter, is transported to the absorption quartz cell by a carrier gas. In my case 500 μL of digested solution were load in the sample loop and transported by a 3% solution of HCl towards the reduction process. The carrier gas was nitrogen.

***Inductively Coupled Plasma (ICP) atomic emission spectrometry (AES) and quadrupole mass spectrometry (QMS).***

The inductively coupled plasma (ICP) is a high temperature source primarily used for generating atomic vapor from an aqueous sample. Observing the atomic and ionic emission lines emitted by the sample atoms in the high temperature plasma can determine the elemental composition of the sample (in the AES instrumentation). A plasma is highly ionized, electrically conducting gas. Upon ionization and collision processes the electrons produce argon ions and more electrons in a cascade manner. No external source of electrons is required. The magnetic field couples vast amounts of energy into the argon gas inside the induction coil, so that the argon plasma may reach

a temperature between 6,000 and 8,000 K. Because of the high temperature or excitation energy in the ICP, the 75 most metallic elements in the periodic table can be determined with high accuracy (ppb concentrations for most of them). The sample is first subjected to a nebulization. Then water is driven off, and remaining solid and liquid portions are converted to gases. Later, gas phase bonds are broken, and only atoms are present. At this point, atoms gain energy from collisions and emit light of a characteristic wavelength. Finally, a grating dispersers light that is quantitatively measured. ICP-AES offers the possibility to simultaneously analyze several elements with the same sample (1-60 elements in one minute), but it needs of relatively high quantities of sample. Detection limits are not the lowest, being of the order of 1-10 ppb.

ICP-QMS uses a plasma of the same type as in ICP-AES, but here it is used to ionize the atoms of the elements, i.e. to make them electrically charged. The charged particles (ions) are then separated by mass in a mass spectrometer. This allows the different elements in a sample to be separated and their concentrations determined. The core of the ICP-MS system is the interface through which ions from the plasma enter the high-vacuum chamber of the mass spectrometer. ICP-QMS combines the advantages of ICP (simple and rapid sample handling) and mass spectrometry (high sensitivity) in a multi-element technique (all elements in one minute or less) . Detection limits are generally much lower than in ICP-AES: typically 1-10 ppt; certain elements can be detected at the ng/L level or lower in very small aqueous solutions.

***Graphite furnace atomic absorption spectrometry (GFAAS)***

In Graphite furnace atomic absorption spectrometry (GFAAS) free atoms will absorb light at frequencies or wavelengths characteristic of the element of interest. Within certain limits, the amount of light absorbed can be linearly correlated to the concentration of analyte present. Free atoms of most elements can be produced from samples by the application of high temperatures. In GFAAS a small graphite tube is electrically heated to a temperature up to 3000°C to generate the cloud of atoms. The samples are deposited in the tube, where are vaporized and atomized. The high performance of the method consists in high sensitivity and low detection limits (usually in the sub-ppb range, a factor of up to 1000x compared to flame AAS), because of the higher atom density and longer residence time in the tube. GFAAS is suitable for some elements, but time expensive, since it is able to analyze one element at time with a sample throughput of 3-4 minutes each one. The method requires very small quantities of samples.

APPENDIX B

Detection limits of the analytical instruments regarding the 58 analyzed elements, according to Thermo Elemental (2001).

| Element | GFAAS | ICP-AES (Radial) | ICP-AES (Axial) | ICP-QMS |
|---|---|---|---|---|
| | (ppb) | (ppb) | (ppb) | (ppt) |
| Ag | 0.05 | 2 | 0.5 | 0.01-0.1 |
| Al | 0.25 | 6 | 1.5 | 0.1-10 |
| As | 0.33 | 12 | 2 | 1-10 |
| B | 43 | 0.5 | 0.2 | 10-100 |
| Ba | 0.4 | 0.2 | 0.04 | 0.01-0.1 |
| Be | 0.025 | 0.2 | 0.06 | 0.1-1 |
| Bi | 0.3 | 18 | 2 | 0.01-0.1 |
| Ca | 0.04 | 0.03 | 0.03 | 1-100 |
| Cd | 0.02 | 1 | 0.1 | 0.01-0.1 |
| Ce | | 8 | | 0.01-0.1 |
| Co | 0.5 | 2 | 0.5 | 0.1-1 |
| Cr | 0.025 | 2 | 0.4 | 0.1-1 |
| Cs | 0.3 | 3200 | | 0.01-0.1 |
| Cu | 0.07 | 2 | 0.3 | 0.1-1 |
| Dy | 1.8 | 0.3 | | 0.01-0.1 |
| Er | 3.8 | 0.7 | | 0.01-0.1 |
| Eu | 0.8 | 0.3 | | 0.01-0.1 |
| Fe | 0.06 | 1 | 0.3 | 0.1-100 |
| Ga | 23 | 7 | | 0.1-10 |
| Gd | | 3 | | 0.01-0.1 |
| Ge | 0.5 | 10 | | 1-10 |
| Hf | | 4 | | 0.01-0.1 |
| Ho | | 0.5 | | 0.01-0.1 |
| K | 0.02 | 6.5 | 0.5 | 0.1-100 |
| La | | 0.02 | | 0.01-0.1 |
| Li | 0.1 | 1 | | 0.01-1 |
| Lu | | 0.05 | | 0.01-0.1 |
| Mg | 0.01 | 0.1 | 0.03 | 0.1-1 |
| Mn | 0.03 | 0.3 | 0.05 | 0.1-1 |

| Element | GFAAS | ICP-AES (Radial) | ICP-AES (Axial) | ICP-QMS |
|---|---|---|---|---|
| | (ppb) | (ppb) | (ppb) | (ppt) |
| Mo | 0.14 | 4 | 0.5 | 0.01-0.1 |
| Na | 0.05 | 1 | 0.2 | 0.1-100 |
| Nb | | 4 | | 0.01-0.1 |
| Nd | | 2 | | 0.01-0.1 |
| Ni | 0.24 | 6 | 0.4 | 0.1-10 |
| Pb | 0.04 | 14 | 1 | 0.01-0.1 |
| Pr | | 0.8 | | 0.01-0.1 |
| Rb | 0.06 | 35 | | 0.01-0.1 |
| Sb | 0.35 | 18 | 2 | 0.01-0.1 |
| Sc | | 0.2 | 0.05 | 1-10 |
| Se | 0.65 | 20 | 5 | 1-100 |
| Sm | | 7 | | 0.01-0.1 |
| Sn | 0.6 | 0.1 | 0.01 | 0.01-0.1 |
| Sr | 0.1 | 0.1 | 0.01 | 0.01-0.1 |
| Ta | | 9 | | 0.01-0.1 |
| Tb | 0.2 | 5 | | 0.01-0.1 |
| Te | 0.5 | 27 | | 1-10 |
| Th | | 17 | | 0.01-0.1 |
| Ti | 1.6 | 0.6 | 0.09 | 0.1-1 |
| Tl | 0.75 | 16 | 3 | 0.01-0.1 |
| Tm | | 1.5 | | 0.01-0.1 |
| U | | 3.5 | 0.4 | 0.01-0.1 |
| V | 0.7 | 2 | 0.5 | 0.01-10 |
| W | | 17 | | 0.01-0.1 |
| Y | | 0.2 | | 0.01-0.1 |
| Yb | 0.15 | 0.3 | | 0.01-0.1 |
| Zn | 0.0075 | 1 | 0.06 | 0.1-10 |
| Zr | | 0.8 | | 0.01-0.1 |

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