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Environmental pollution and hunting: exposure of birds to metals in their trophic areas, and of humans to lead in game birds

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"Spesso s'incontra il proprio destino
nella via che s'era presa per evitarlo."

(Jean de La Fontaine)

We often meet our fate in the way
we had taken to shun it."

(Jean de La Fontaine)

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GENERAL ABSTRACT

This doctorate is focused on birds and their relationships with trace elements, including heavy metals of environmental concern. Even though the main subject is biological, large importance in this work has been given to geochemistry, because literature juxtaposing the latter science with the life and ecology of birds is really scarce.

The Mediterranean region is a biodiversity hotspot. Biological richness is well known to be very high, but also, Mediterranean coast hosts every year millions migratory birds, which stop, breed, and winter there. However, Mediterranean countries are among the most influenced in the world by human pressure, in terms of urbanization, industrialization, cultivation, and tourism.

Since anthropogenic inputs are in some conditions capable to change the metal and trace elements (MTEs) environmental levels, and can lead to adverse effects to organisms (poisoning, bioaccumulation, biomagnification), there is a strong impulse by GOs and NGOs operating in the European Union to improve methods and standardization in studies on contaminants involving birds, and, consequently, to gain better understanding of impacts and effects of pollution on the biosphere.

On one hand, birds are potential (and commonly used) biomonitors of change in the environment. Non-invasive sampling of feathers has proven to be a very informative tool to unravel various physiological, ecological and toxicological processes inherent to individuals and populations. On the other hand, there is the need to improve methods in order to avoid biases and, consequently, errors in interpretation of chemical results. It is the case of external contamination (very often overlooked by researchers) on feathers due to the residual presence of lithic particles adhered to the surface.

Relationships between birds and MTEs are the same we know for most organisms: intake of MTEs naturally present in their environment, and assumption of anthropogenic MTEs in polluted feeding areas. Furthermore, birds are exposed to a special source of a very harmful heavy metal: lead (Pb) of ammunition shot by hunters. Pellets and fragments can be (and, surprisingly, are often) assumed by birds from sediments and their preys which have lead pieces embedded in their body.

General aim of this doctorate is to improve the possibility to use birds as biomonitors of metal exposure through non-invasive methods, especially filling some knowledge gaps through a multidisciplinary approach poorly adopted by researchers so far.

In particular, the studies included in this work achieved these objectives:

1) some wetlands in the Northern Adriatic coast (included in the Regional Veneto and Emilia Romagna Po Delta parks), which are very important for colonial waterbirds because they are normally used as feeding and/or breeding areas, have been investigated for their geochemical characteristics and characterized for any future ecotoxicological studies;
2) a new geochemical approach has been explored and proposed for studies on MTE bioaccumulation in feathers, in order to reduce biases caused by environmental (external) contamination. To date, no studies have quantified and corrected for its effect on feather trace element concentration. The model species used in this doctorate for this section is the Greater flamingo (*Phoenicopterus roseus*);
3) new information about type, frequency and distribution of Pb particles embedded in two small-sized bird species killed by hunters has been given. The analyzed species are the European starling (*Sturnus vulgaris*) and the Eurasian woodcock (*Scolopax rusticola*). These data will be useful when the real health risk for consumers (raptors and humans) of this kind of hunted birds will be evaluated.

This work is structured in six scientific articles. For each topic, two research studies have been carried out and reported. The last contribute is a short communication regarding a case of a Peregrine falcon (*Falco peregrinus*) found dead with lead shot in the digestive tract as a consequence of ingestion of birds with embedded lead shot. Four out seven papers have been published between 2013 and 2016 by peer-reviewed international scientific journals. The others have been submitted.

CHAPTER 1 - General introduction and summary

1.1 Environmental pollution, lead ammunition shot by hunters, and birds: the aims of the doctorate

Taking the modern concept of humans as geologic agents introduced by Wilkinson (2005), while discussing the magnitude of the human-induced soil erosion, one can say that a considerable part of anthropogenic environmental alteration regards the geochemistry of sediments. We can, therefore, extend that attribution actually including man among the geochemical agents, being able to change the geochemical profile of the surface sediments of lakes, marshes, lagoons, and coastal environments.

From an environmental point of view, such an alteration may be worrying when the concentrations of one or more pollutant levels in the waterbody or soil increase up to thresholds considered locally or regionally harmful for the ecosystem and its aspects (biodiversity, fitness of organisms, ecosystem amenities).

Metals and many other trace elements (hereafter referred as MTEs) are conservative: bacterial attack substantially does not affect the fate of so-called conservative contaminants in a timescale referable to the target endpoint. However, not all MTEs excesses in the environment are due to the human presence on the Earth. Aluminum (Al), arsenic (As), mercury (Hg), for example, are widely dispersed by natural processes, more than anthropogenic inputs, while cadmium (Cd), lead (Pb), copper (Cu), and zinc (Zn) enrichments in the environment are mostly caused by human activities (Clark, 2001). These are only general examples: each situation has to be assessed for metal contamination by defining a clear hypothesis to be scientifically verified. However, significant amounts of metals have been reaching coastal environments as a consequence of human activities of some kind.

Arsenic, Cd, Pb and Hg are of major concern because they are thought to exert no biological function, but are highly toxic (Hutton, 1987). In addition, the accumulation of As, Cd, Chromium (Cr), Cu, Pb, Hg, Nickel (Ni), Selenium (Se), and Zn through their mutagenic ability can cause DNA damage and carcinogenic effects in the bodies of animals or human beings through food chain (Knasmuller *et al.*, 1998). Despite of the plethora of studies continuously published on these topics, most properties of MTEs, positive or negative, have to be better or newly understood.

Bioaccumulation is a possible effect of a conservative contaminant (Bryan *et al.,* 1979). It is the process that makes an organism reach, through time, the lethal dose of a substance without ever introducing a single lethal dose of it. In other words, bioaccumulation is a process that leads to a higher concentration of a xenobiotic compound in an organism compared to the concentration in the environment in which it lives (water, sediment, soil). When an organism is consumed by its predator, the latter can accumulate in turn a part of the metal burden of the prey. When the prey population is diffusely affected (even at low levels) by metal contamination, the predators, unable to excrete the metal with the same rate of intake, acquire a greater body burden. Biomagnification is such special process of bioaccumulation along the trophic web, simply as an effect of feeding on bioaccumulators. As a consequence, the MTE concentration in tissues of an organism may be higher than the concentration in its food. This is the case of mercury. The biomagnification of methylmercury (the molecular form of mercury metabolized by the animals) is demonstrated (Lindquist *et al.,* 1991; Watras and Bloom, 1992). Also cadmium can be biomagnificated by organisms (Reinfelder and Fisher, 1991), while lead, even though extremely toxic and highly bioaccumulable, is thought not biomagnificable (Hodson *et al.,* 1978). Aquatic invertebrates and fishes are perfect examples of bioaccumulators. Most of waterbirds and humans are good examples of bioaccumulators and top predators.

However, plants and animals evolved in contact with MTEs. On one hand, organisms developed biological processes depending on some essential elements, while, on the other hand, they developed protection mechanisms against the possible toxic effects of MTEs (Kochian *et al.,* 2002; Revathi and Venugopal, 2013). In general, most MTEs are either unreactive and therefore excreted as they are, or regulated to be proficiently used by the organism itself. Some metals are more toxic than others, and even essential metals can become toxic over a very limited range and the quantity that exceeds the natural capacity to excrete tend to be accumulated in tissues.

The Mediterranean basin hosted the development of a number of civilizations over thousands of years. It never stopped to support a high population density (some 455 million inhabitants, at present), a variety of anthropogenic activities, including the industrial development, mining, agriculture, and others. The region also receives a large number of visitors: in 2005, 246 million people – 31% of all international tourists – visited the Mediterranean, particularly its coastal areas (Blue Plan, 2008). Therefore, it should not make surprise if all these factors have generated pollution capable of influencing biogeochemical cycles (physical, chemical, and biological functioning) of the Mediterranean ecosystem. Indeed, pollution is known as one of

the most important problems affecting wildlife conservation in Mediterranean region (Fig. 1.1). The Mediterranean is also characterized by natural processes that can affect the distribution of MTEs (e.g. volcanism), but the evidence of environmental changes provoked by anthropogenic pollution urgently need to be scientifically investigated (Hutzinger, 2005).

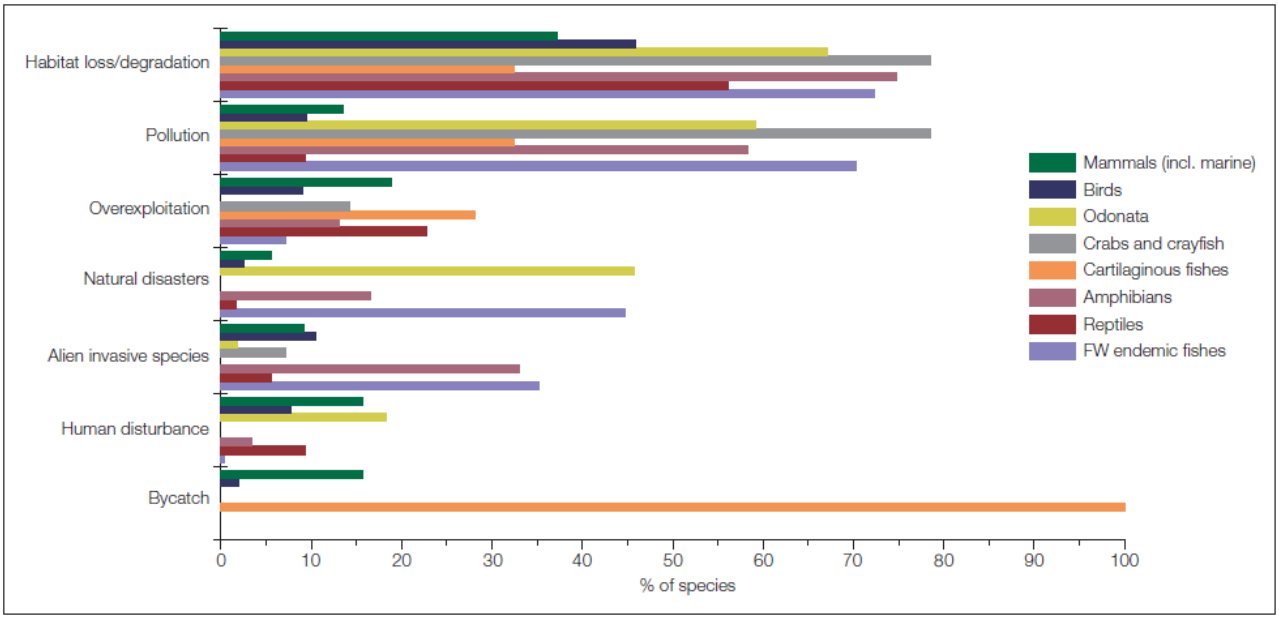

Figure 1.1. Breakdown of the major threats to animals in the Mediterranean. Pollution is known as one of the most important problems affecting wildlife conservation.

The Mediterranean region is a biodiversity hotspot (Cuttelod *et al.*, 2008). Besides this great richness of plants, a high proportion of Mediterranean animals are unique to the region (Mittermeier *et al.,* 2004; Myers *et al.*, 2000). The Mediterranean's importance for wildlife is not limited to the richness or uniqueness of its resident fauna and flora: millions of migratory birds use Mediterranean wetlands and other habitats as stopover, wintering or breeding sites (Cuttelod *et al.*, 2008).

Bird protection has been hardly the most important issue for the European Communities (EC, the predecessor to the European Union), to take on board one of their first pieces of environmental legislation. The concern of the extinction of birds took the first basis in the late nineteenth century in the wake of Rachel Carson's *Silent Spring* (1962). In April 1979, just a few years after the start of an EC environmental policy in 1973, the EC adopted the Council Directive on the Conservation of Wild Birds (79/409/EEC), the so-called "Birds Directive", recently amended and renamed as 09/147/EC. Pollution was the driving force for starting bird conservation, but soon the media in northern Europe blamed the cruel and ruthless hunting practiced in southern Europe,

notably Italy (Meyer, 2013). When the directive was finally enacted, tackling the fierce resistance of French and Italian Governments, the bird protection movement had become pan-European, much like the birds themselves (Meyer, 2013). Notably, Annex V of the Birds Directive states two actions to be advanced: 1) determining the role of certain species as indicators of pollution; 2) studying the adverse effect of chemical pollution on population levels of bird species. Nowadays, OG and NGO are developing sampling protocols in order to improve the quality and standardization of studies on contaminants involving birds (raptors: Espín *et al.*, 2014; seabirds: Borghesi, 2016).

Waterbirds, seabirds and raptors are susceptible to bioaccumulation of a wide range of chemicals, including MTEs, because they are top predators in a wide range of food webs. Such vulnerability makes birds potential indicators of change in the environment and can be used to monitor pollutants that bioaccumulate and/or amplified in concentration up the trophic levels (Burger, 1993; Castro *et al.*, 2011; Carneiro *et al.*, 2015; Furness, 1993). For birds that spend a significant proportion of their life in terrestrial, coastal or marine environments, ingestion of food (including 'false' food, such as plastic items) and water are the main routes of exposure to pollutants (Burger and Gochfeld, 2004; Wilcox *et al.*, 2015). However, birds are not only exposed to diffuse MTE pollution. Hunters shoot millions of birds every year, but most of gunshot miss the target and huge quantities of pellets are dispersed in hunting areas (Bellrose, 1959; Pain, 1991; Andreotti and Borghesi, 2012). As a consequence, hunting represents an important source of lead dispersion and lead shot that lie in soils and wetlands represent in turn a source of lead contamination (and at lesser extent of other MTEs associated to the ammunition, such as barium, antimony, nickel, copper, zinc and others) for years. Flint and Schamber (2010) estimated that Pb pellets in the sediment of wetlands would be available to most species of waterfowl for at least 25 years. Hence, among toxicologically significant Pb sources (e.g., Pb-based paint, mining, smelters, and combustion residue of leaded gasoline; Blus *et al.*, 1999, Church *et al.*, 2006, Beyer *et al.*, 2013), spent ammunition (and lost fishing tackle) are the primary exposure factor for birds in terrestrial and aquatic systems (Haig *et al.*, 2014). Birds in general are directly exposed to plumbism by taking and swallowing lead pellets directly from the floor (Arcangeli *et al.*, 2009, Beintema, 2001; Jenni *et al.*, 2015) or ingesting sediments contaminated by lead pellet undergone to chemical alteration. Furthermore, raptors and scavengers can eat wounded animals carrying embedded lead shot and fragments (another type of direct exposure), or animals with high level of Pb in their flesh (indirect exposure) (Fisher *et al.*, 2006; Haig *et al.*, 2014). Following this

emergency, phasing-out of lead ammunition actually has become one of the goals of UNEP, and especially within the African-Eurasian Waterbirds Agreement, AEWA (Lenten, 2005) and the Convention of Migratory Birds (CMS, 2014). As a consequence, to date this is a topical issue for wildlife management as well as for public health (Andreotti and Borghesi, 2012).

Regarding the opportunity to use birds as bioindicators of pollution in ecotoxicological studies, the colonial nature of many waterbirds and seabirds has several benefits (Kushlan, 1993). Among them, an easy sampling and good quantities of data can be collected from a particular breeding site in a relatively short period of time (Mallory *et al.*, 2006). In addition, since many species return to the same nest and colony sites for years, contaminant loads of individuals can be studied over time (Burger, 1993). Furthermore, during the breeding season birds tend to forage within a limited range around their colony, while the fledglings usually stay close to the nesting sites for a period after birth (Erwin, 1983). These facts allow comparisons of responses at sites in natural experiments (Kushlan, 1993). On the other hand, lead shot ingestion by raptors often involves single adults with common lethal effects (Pain *et al.*, 2009). Humans that usually are game consumers also directly swallows lead fragments remained in meals, but normally with subtle detrimental effects, especially to children and fetuses (CDC, 2005; Kosnett, 2009). Since humans normally cook meat before consuming it, they are exposed to the Pb-contaminated food, passed through high temperature and mixed to acid ingredients capable to increase the dissolution of lead (Mateo *et al.*, 2007; Mateo *et al.*, 2011).

Normally, researchers focus on one or few chemicals in their studies. However, seabirds are endpoints for many elements and substances and there would be need of laboratory studies assessing mixtures of chemicals, but the complexity (and the costs) soon increase, limiting the possibility to explore interactions. Consequently, additive, antagonistic, or synergistic properties of different chemicals are generally unknown (Burger and Gochfeld, 2004). For example, in literature concerning contaminants and seabirds, the effect of persistent organic pollutants or Hg are relatively frequently investigated, but very rarely in combination with HMs (Borghesi, 2015).

The main aim of this doctorate is to give a scientific contribute to improve the possibility to use birds as biomonitors of metal exposure through non-invasive methods. Doing that, an extensive multi-disciplinary approach has been adopted in order to fill some gaps in knowledge about: 1) the abiotic system of several key sites for birds in Northern Adriatic coast, 2) the available methods that can be applied for interpretation of metal concentrations in feathers, 3)

information useful for the evaluation of the potential health risk for consumers—both raptors and humans—eating shot small and medium birds which received poor or no attention so far.

1.2 Biomonitoring metals in the environment by using feathers: the importance to meet biological and geochemical information

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 (Stettenheim, 2006).

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. However, unlike hair, which grows continuously, feathers only grow during a certain period of time (Lucas and Stettenheim, 1972). As a consequence, feather cycles can be divided into two phases: growing phase and resting phase (Lucas and Stettenheim, 1972). In between the two phases there is moulting. 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. Within their first few weeks of life birds develop a second-generation (or juvenal feather) and pushes the natal down out of the follicle on its tip. In some species, as flamingos, a second down can replace the natal down before starting the juvenal feathering (Johnson and Cézilly, 2007).

The new feather is tightly furled inside a sheath while it forms. When a feather grows, blood vessels and nerve fibers enter the ensheathed portion of feather (pulp) where keratinization process occurs and provides nutrition to the growing feather follicles. In this moment, feathers are strictly connected to the bloodstream. The pulp progressively recedes. A feather fully grown is entirely free of sheath and has pulp completely resorbed (Lin *et al.*, 2013). Therefore, when fully grown, feather is a non living tissue. A fully formed feather is 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. 1.2).

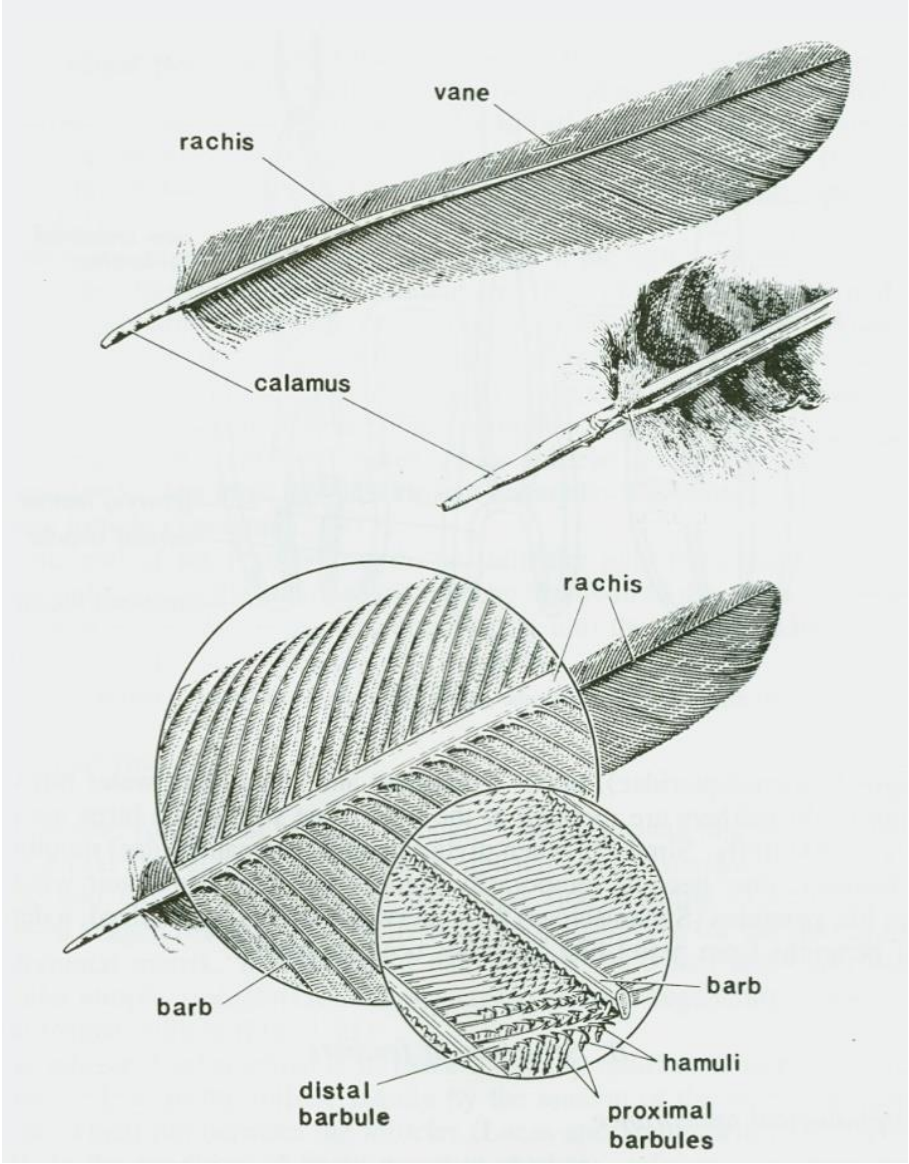

Figure 1.2 – Structure of a flight feather (from Van Tyne and Berger, 1971)

Due to the particular structure and significance of feathers, researchers have tried to use them for many applications, and a comprehensive review of the various uses of feathers in science was published by Smith *et al.*, (2003). Among the uses mentioned by Smith *et al.*, (2003), assessment of metal exposure in birds by using feathers is very frequent in literature, as it was approached for the first time several decades ago, and then explored with greater impulse in 1980s and through following years. The state of the knowledge up to 1991 was outlined and reviewed by Burger (1993). From then on, many studies followed, most of them reporting metal concentrations (interpreted as bioaccumulation) in some feathers, species, and sites.

However, in spite of their attractiveness as a potential bioindicator (feathers can reflect the circulating levels of metals in the bloodstream during feather growth, are easy to collect, easy to store, and represent a non invasive sampling method allowing to gain data from potentially all

species) (Furness, 1993), feathers are not simple to be used in ecotoxicological studies due to, at least, three reasons.

First, it must be taken in account that plumage is composed by different types of feathers: flight feathers (primary, secondary, and tertiary remiges and tail feathers - or rectrices) and body (contour) feathers (all the others). Moulting, occurring once or twice per year (depending on species) can involve all or part of contour and flight feathers, according to the strategy evolved by species, and depending on bird's age. Flight feathers of the wing are normally replaced sequentially, while body feathers and in most cases tail feathers are moulted with an unpredictable sequence always allowing the bird to remain nearly completely feathered. In many species, there is a pre-breeding partial moult, and a post-breeding moult (Howell, 2000). Moulting can be started in a geographic area and completed in another one (Bourgeois and Dromzée, 2014). As strategies in seabirds can be rather variable at species level, knowledge of moult strategy and movements in the year of each species is necessary to select the most convenient target feathers and correctly interpret the levels of metals in feathers and their sources (Burger, 1993; Bourgeois and Dromzée, 2014).

Second, studies assessing metals in bird tissues (including feathers) normally do not contextually investigate the environmental levels of the pollutants in the areas used by birds during feathering. Only recently, this approach has been sometimes undertaken (e.g. Lou *et al.*, 2013). Nevertheless, birds acquire heavy metals through intake of food, drinking, and geophagy (Furness, 1993; Beyer *et al.*, 1999). We can assume that the rate of heavy metal absorption varies depending on species physiology, metal properties, and bio-availability in the environment. And we can be confident that, after absorption, metals circulate in the body, and then can be excreted or can be accumulated in tissues and organs, or sequestered in feathers (Furness *et al.*, 1986). However, studies focused on assimilation and biochemistry of metals in birds are scarce. Despite the high number of studies which assessed concentrations in bird tissues, the number of studies focused on the mechanisms of accumulation and re-mobilization of metals in birds and especially in feathers, in relation to the environmental levels of the pollutants, is so small that the subject can be considered virtually unexplored.

Third, feathers are in contact with airborne pollutants, water, soil and sediment, and uropygial oil (secreted by the uropygial gland and distributed on feathers to make the plumage waterproof) from eruption to loss (Dauwe *et al.*, 2002). This implies that feathers (having an extraordinary entangling structure) carry two kind of information concerning trace elements: a)

the result of bioaccumulation and consequent storage in feathers of part of the elements circulating in blood during feather growth (Burger, 1993); b) external contamination as an effect of the adhesion of various substances onto the feather surface (Ek *et al.*, 2004). For Hg, the external contamination is considered irrelevant if compared to the bioaccumulation (Ek *et al.*, 2004), and many recent studies only assessed this metal (e.g. Borghesi *et al.*, 2011; Rubio *et al.*, 2016). However, the need to biomonitor inorganic pollutants is high worldwide and, despite external contamination represents a confounding factor for most elements, since no washing procedure is able to ensure the total removal of the surface contamination from feathers (Cardiel *et al.*, 2011), studies overlooking this problem are still published with considerable frequency.

All this considered, feathers are still a potential bioindicators, but probably not in any conditions. On one hand, it seems appropriate to reduce as much as possible the external contamination, especially the soil/sediment particles attached and/or entangled in feathers (a thorough washing procedure has to be always carried out, even when only Hg is assessed). On the other hand, it must be taken in account that a residual external contamination remains (Cardiel *et al.*, 2011), and developing methods which allows to validate and better interpret the analytical result is highly desirable. To do this, obtaining trace element concentrations from feathers could be not enough and further information regarding the abiotic component of ecosystem may be essential when we evaluate the exposure of birds to harmful trace elements by using feathers. In fact, the link "sampled bird" – "sampling area" should be always well clear and defined, because the goal of an ecotoxicological study assessing pollutants is both to identify anomalies in the studied organisms and locate the source of the problem (Forbes and Forbes, 1994). Taking birds as biomonitors, that link is not a trivial matter, as birds can fly. Sampling chicks or unfledged birds represents a good choice to achieve this condition. Therefore, geochemical characteristics of the habitat frequented by birds (especially feeding areas) could be used to make more sound the interpretation of the analytical data obtained from feathers, which are likely affected by external contamination despite a thorough washing treatment.

In order to better understand the importance of external contamination caused by the persistence of sediment particles attached on the feather surface, and then develop a method which controls for external contamination, in both studies included in this doctorate assessing many trace elements in feathers (chapters 4 and 5), only fledglings (of Greater Flamingo) have been sampled and geochemical data were obtained by sampling the surroundings of the breeding site. These multidisciplinary studies allowed us to put a light on the recurrent methodological

problem represented by the external contamination, and suggest a possible solution for correctly interpreting data, applicable in many contexts.

Furthermore, two comprehensive geochemical studies (chapters 2 and 3) were carried out in several important feeding and breeding areas for many waterbird species living in the Po Delta area. Only a few of these sites have been geochemically investigated in the past, and environmental information about them are scarce, because they are generally difficult to be acceded. The results of the studies carried out during this doctorate across the wetlands of the Po Delta, may provide a useful basis for future biomonitoring and ecotoxicological studies on trace elements using birds, and especially feathers.

1.3 Embedded lead: game birds are the carriers, whilst raptors (and humans) are the endpoints

Relationships between heavy metals and vertebrates are continuous and incredibly diverse, but sources and pathways seems rather similar for all classes included in this large taxonomic group (Foulkes, 2000; Bhattacharya, 2001). Iron, cobalt, copper, manganese, molybdenum, and zinc are generally required to certain amounts, but all metals are toxic at higher concentrations (some at very low levels, e.g. Pb) and excessive intake can damage the organism (Singh *et al.*, 2011). In birds, metals can be inhaled with air or dust, enter into the body through skin, introduced in the digestive system together with food and water (Furness, 1993). Furthermore, as said in this introductory part (paragraph 1.1), birds (and their consumers, including humans) are probably the most exposed vertebrates to a special source of Pb represented by lead ammunition dispersed in the environment or carried by animals shot by hunters (which are not recovered or escaped after being wounded) (Scheuhammer and Norris, 1996).

Since the invention of firearms, Pb was the metal selected for ammunition, thanks to its ductility and malleability (which make production easy), and high specific weight enhancing the ballistic properties of the shot. However, such properties determine the fate of metal when the shot hits its target. Both Pb pellets (used for hunting to small and medium sized animals) and Pb gunshot (used for hunting to Ungulates) deform and fragment, causing shock and serious wounds to the victim. Furthermore, shot tend to remain in the body of the game more than other types of ammunition (Mori, 1997). Nowadays, Pb is still largely the most used metal for ammunition. In

pellets, a percentage of antimony is added (1,5 – 5%) to make the shot harder, and some arsenic (0.3 – 0.5 ‰) is also added to obtain a spheric shape. Arsenic is not necessary when the pellet is moulded instead of being dripped (De Florentiis, 1987). To date, replacement of Pb shot with alternative ammunition is still occasional (only where hunting with Pb ammunition is banned), and hunters widely prefer to use Pb shot whenever possible. Steel shot is the favourite alternative, mainly due to the relatively low cost (Andreotti and Borghesi, 2012).

Birds can assume Pb of hunting origin through two main ways: primary and secondary. When birds ingest shot pellets dispersed in the environment, assumption is told primary. Secondary assumption occurs when eating contaminated meat (feeding on animals carrying embedded or ingested Pb shot, or having high Pb concentration in their flesh) (Andreotti and Borghesi, 2012). Primary assumption is known since the first half of 1800 and the first strong impulse to the study of the problem is dated back 1950 and continued thereafter, leading to the phasing-out effort in course in Europe and North America (Andreotti and Borghesi, 2012). A wide literature attests that primary ingestion of Pb pellets occurs to a wide number of waterbird species (ducks, geese, swans, waders, flamingos, rails) and to a high percentage of individuals (Mateo *et al.*, 1997), causing the death of millions of birds every year (Beintema, 2001). Ingestion of Pb pellets has been also demonstrated for terrestrial birds such as Galliformes and Columbiformes (Kendall *et al.*, 1996; Larsen, 2006).

However, exposure to secondary assumption is not rare as one could imagine and can affect birds even at population level. Indeed, embedding of Pb shot in living birds is far from uncommon, and several studies demonstrated that a considerable percentage of living medium-sized waterbirds carry embedded Pb pellets in their body (11-43% adults and 2-11% young) (Eisler, 1988; Scheuhammer and Norris, 1996; Miller *et al.*, 2000).

Recently, the dramatic decline of the California Condor (*Gymnogyps californianus*) caused by secondary assumption of spent Pb ammunition (Cade, 2007) confirmed the possibility of an effect at population level for birds of prey (especially scavengers) feeding on hunted animals or their part abandoned by hunters (such as entrails). It is well known that a considerable fragmentation of lead bullet occurs during the impact with flesh and bones, and many large, medium-sized and tiny fragments are retained in the flesh (Cornatzer *et al.*, 2009). In fact, in the last decade, studies assessed the frequency of lead pellets and fragments in wild boar (Dobrowolska and Melosik, 2008) and deer meat (Hunt *et al.*, 2006), and venison (Cornatzer *et al.*, 2009). Copper has been adopted as a good alternative to Pb for bullet, but, again, Pb is still

preferred by most hunters worldwide, and especially for hunting to wild boar in Italy (Andreotti and Borghesi, 2012).

On the contrary, the frequency and type of Pb particles embedded in small birds has been scarcely explored by researchers so far. Small-game hunting (to birds) is traditional in Mediterranean countries, but only little played in most of the northern European countries and North America (Andreotti and Borghesi, 2012). This may explain why knowledge is only partially achieved for small-sized game. The third part of this doctorate is focused on the problem of embedded pellets and their possible fragmentation in small bird game, and the risk of ingestion by consumers (raptors, scavengers, and humans).

Irrespective to the primary or secondary assumption, once ingested and attacked by the gastric acid of birds, part of the metallic lead enters in the blood (Beintema, 2001). If not expelled, a single pellet can be completely dissolved in 2-6 weeks (Plouzeau *et al.*, 2011). Through blood circulation, Pb reaches liver, kidneys, and bones, where it can be accumulated (Scheuhammer, 1987). Depending on the target tissue, Pb can remain some weeks to months (in kidneys, marrow, brain and nervous system), causing immediate toxic effects, or even years (in bones). In the latter case, Pb can be remobilized later and return into the bloodstream, when the body requires higher amounts of calcium. Fetuses and developing organisms are even more prone to the adverse effects of plumbism (Scheuhammer, 1987). Normally, wild birds affected by acute plumbism become predated before death. In some cases, they are able to nestle somewhere and die, being unable to search for food or water. Those birds which survive after an acute episode of plumbism, hardly can find a partner, build a nest, lay eggs, and raise chicks (De Francisco *et al.*, 2003). For birds chronically poisoned by lead, frequency of fatal events (e.g. collisions to vehicles, windows, wires, wind turbines) or predation is higher (Helander *et al.*, 2009).

In humans, until recently, the risk associated to game consumption was considered low, because it was thought that people are able to avoid swallowing of hard fragments (Coburn *et al.*, 2005). Actually, some important studies were carried out on populations of Alaska, Canada, Greenland, and northern Russia. Hunted animals are an important component of diet for these populations and one study showed a correlation between consumption of wild birds and mammals and blood levels of Pb (Nielsen *et al.*, 1998; Bjerregaard *et al.*, 2004; Johansen *et al.*, 2006; Verbrugge *et al.*, 2009). After the ban of lead ammunition in Canada, Pb levels in blood of Inuits (an Eskimo community) decreased about 55% over 12 years (Fontaine *et al.*, 2008). Moreover, a study on native peoples of Canada showed a correlation between the amount of

game consumed by pregnant women and Pb level in the umbilical cord of their babies and Pb levels in blood of breastfed babies (Hanning *et al.*, 2003).

Even though big fragments could be occasionally ingested by humans causing health problems (Gustavsson, 2005), normally they are discharged during preparation of food, or mastication. On the contrary, swallowing of tiny fragments is unavoidable (Scheuhammer *et al.*, 1998; Johansen *et al.*, 2001; Hunt *et al.*, 2006). During the transit through the digestive system, small Pb particles are partially or completely dissolved by gastric acids and very subtle effects can occur. Moreover, big fragments can contaminate the meal during cooking, because subjected to high temperature and improved acidity (when using wine or vinegar). Depending on the recipe, concentrations of Pb in food containing Pb pellets after cooking can be harmful for human health (Mateo *et al.*, 2007; Mateo *et al.*, 2011).

Assimilation of Pb from game was demonstrated on pigs fed with venison cooked by microwave oven (Hunt *et al.*, 2009) and laboratory experiments showed a potential capacity of humans to assimilate Pb from shot (Mateo *et al.*, 2011).

Literature on effect of plumbism derived from diffuse pollution on humas is wide and the social consequences provoked by Pb on humans are of high concern, like the reduction of learning ability (Needleman, 1990; Ma, 1996), up to serious mental retardation (Kosnett, 2009).

Therefore, until phasing out of Pb ammunition is not completely achieved, consuming game involves a risk to health of consumers (humans and animals) and all possible information useful to assess such risk should be scientifically obtained.

The gap in knowledge about embedded lead in small game has been reduced a bit by including two studies on hunted birds. An investigation on a raptor little inclined to feed on carcasses (a Peregrine falcon found dead with pellets in the digestive tract), has been also included in this doctorate as a demonstration of a possible effect of secondary assumption of embedded Pb in prey (chapters 6, 7, and 8).

1.4 Thesis summary

Part 1. Two geochemical studies concerning the geochemical characterization of surface sediments of several Adriatic coastal wetlands used by waterbirds for feeding and/or breeding are reported in chapters 2 and 3. The study area includes historical Po deltaic system areas belonging to both Regional Po Delta Parks (Veneto and Emilia Romagna). The first study consists of an

assessment of the geochemical composition of the surface sediments of 7 wetlands and results have been compared to deep core samples in the area analysed with the same methods. In addition, the influence of anthropogenic activities on the total concentration of metals in sediments have been evaluated by using an index commonly used in literature. The study has been published in the Journal of Geochemical Exploration in 2015. The second study has been submitted on the 30th December 2015 and is to date under review by the same journal. In this study, pseudo-total concentrations of sediments from 4 wetlands in the same area were investigated. Multivariate statistics have been applied to data obtained after aqua regia digestion. With respect to the first study, four out of the 7 sites and 20 further elements were analyzed. Where the real total contents were available from the previous study, a comparison with pseudo-total concentrations was carried out, and reference levels provided by legislation and national guidelines also compared with the results of this study. Citations of these two manuscripts are:

Migani, F., Borghesi, F., Dinelli, E., 2015. *Geochemical characterization of surface sediments from the northern Adriatic wetlands around the Po river delta. Part I: Bulk composition and relation to local background.* Journal of Geochemical Exploration 156, 72-88. *DOI:10.1016/j.gexplo.2015.05.003*

Borghesi, F., Migani, F., Dinelli E., (submitted). *Geochemical characterization of surface sediments from the northern Adriatic wetlands around the Po river delta. Part II: Bulk composition and relation to local background.* Aqua regia *results.* Under revision on Journal of Geochemical Exploration.

Part 2. Feathers are frequently used as a bioindicator of trace element pollution. However, results of most metals, except mercury, are difficult to be interpreted because the presence of external contamination, even after thorough washing. This difficulty is perceivable throughout literature, but rarely researchers tried to overcome the problem. In chapters 4 and 5, two new studies assessing trace elements in feathers by using the Eurasian greater flamingo (*Phoenicopterus roseus*) are presented. A geochemical approach based on geochemical data obtained from sediment samples collected in the flamingo breeding sites has been proposed in order to better understand the raw chemical results. In the first study, whole feathers were used and data analysis were performed by using multivariate statistics. This study has been published by the journal Science of the Total Environment in February 2016. In the second study only shafts were analyzed and a new method has been proposed to tackle the problem of external contamination

and validate the bioaccumulation. This study has been submitted on 24th March 2016 to the journal Methods in Ecology and Evolution. Citations of these two manuscripts are:

Borghesi, F., Migani, F., Andreotti, A., Baccetti, N., Bianchi, N., Birke, M., Dinelli, E., 2016. *Metals and trace elements in feathers: a geochemical approach to avoid misinterpretation of analytical responses.* Science of the Total Environment *544, 476–494*

Borghesi, F., Dinelli, E., Migani, F., Béchet A., Rendón-Martos, M., Amat, J., Sommer S., Gillingham M., (submitted). *Assessing environmental pollution in birds: a new methodological approach for interpreting bioaccumulation of trace elements in feather shafts using geochemical sediment data.* Submitted to Methods in Ecology and Evolution.

Part 3. The problem represented by the embedded lead in game meat is developed in chapters 6, 7, and 8. Two studies (reported in chapters 6 and 7, respectively) are focused on the amount and characteristics of lead fragments embedded in small and medium-sized game birds, respectively the European common starling (*Sturnus vulgaris*) and the Eurasian woodcock (*Scolopax rusticola*). In both studies, already published by scientific journals (European Journal of Wildlife Research in 2013, and Italian Journal of Animal Science in 2016, respectively), an estimation of the embedded lead has been made for edible and non edible parts separately (relate to human consumers), and the risk of a considerable exposure to plumbism raised for all consumers, including scavenger birds and birds of prey, and human consumers. Regarding raptors, a case of intoxication of a Peregrine falcon (*Falco peregrinus*) likely caused by the ingestion of a bird carrying many lead pellets in its body is reported. The latter study is near to be submitted to the Journal of Ornithology in the first days of April, 2016. The citations of the three manuscripts are:

Andreotti, A., Borghesi, F., 2013. *Embedded lead shot in European starlings* Sturnus vulgaris*: an underestimated hazard for humans and birds of prey.* European Journal of Wildlife Research *59, 705-712.*

Andreotti, A., Borghesi, F., Aradis, A., 2016. *Lead ammunition residues in the meat of hunted woodcock: a potential health risk to consumers.* Italian Journal of Animal Science. Doi.: 10.1080/1828051X.2016.1142360

Andreotti, A., Fabbri, I., Menotta, S., Borghesi, F., (submitted*). Embedded lead in game birds: the case of a Peregrine falcon* Falco peregrinus *found dead with lead shot in the digestive tract.* To be submitted to the Journal of Ornithology.

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CHAPTER 2 - Geochemical characterization of surface sediments of some Adriatic coastal wetlands - Part I: bulk composition and relation to local background

2.1 Abstract

The impact on coastal wetlands is a matter of concern worldwide. In the Po Delta (Italian North Adriatic coast) the human presence around wetlands increased considerably in recent years. This study assessed the geochemical composition of the surface sediments of several wetlands included in the historical Po deltaic system, and the influence of anthropogenic activities on the metal levels in the sediments. Eighty-nine samples were collected from 7 sites, and analysed for major and 16 trace elements using X-ray fluorescence comparing the results with background values obtained from bottom cores drilled near the wetlands. The Enrichment Factors (EFs) were calculated to evaluate metal contamination. In general, the sediments resulted a mixture of carbonate and clay, in agreement with the background, except for some organic matter (OM) enrichments and sporadic samples richer in carbonate. The bulk composition is mainly driven by the fine grained fraction. The concentrations of Ba, Cr, Cu, Pb, Rb, Sr, Zn, and V exceeded background values in one or more sites. Copper and Zn enrichments resulted of anthropogenic origin, while high concentrations of Ba, Rb, Sr, and V as an effect of natural processes. Chromium peaks were ascribed to both causes, depending on the site. Lead was generally over the threshold, reaching some unexpected high median EFs and a number of peaks. Lead in many sites, and Cu and Zn enrichments in the most polluted sites are preferably controlled by OM. One of the most polluted sites showed worrisome peaks of Cr, and contamination of Cu and Zn involves the southern lagoons, due to industrial inputs from Ravenna and local settlements. Lead shot widely dispersed in all wetlands but one may explain the highest levels found.

2.2 Introduction

Coastal wetlands represent a critical environment by definition being located at the boundary between continental and marine settings, freshwater and seawater environments with the additional pressure represented by strong anthropogenic activities. Indeed, the human presence around coastal wetlands increased considerably in recent years, and the impact on these environments became a matter of concern (e.g.: Yamamuro and Kanai, 2005). Moreover, coastal wetlands are transitional environments that can accumulate, temporarily or permanently, many

contaminants carried by several pathways (Akoto *et al.*, 2008). Such pollutants, and in particular metals, are not necessarily permanently linked to the sediment, but once adsorbed, can be mobilized by chemical or biological agents within the sedimentary layer or may return in the water column (Salomons and Forstner, 1984). Due to their persistent and bioaccumulative nature in the aquatic ecosystem, trace metals are of major concern (Arnason and Fletcher, 2003). These elements are mostly derived from natural sources, influenced by bedrock geology of the drainage catchment and by weathering processes (Karbassi *et al.*, 2008), but are also introduced to the aquatic environment by a number of anthropogenic processes (Akoto *et al.*, 2008).

Sediments have been widely used as environmental indicators and their ability to trace contamination sources and to monitor pollutants is largely recognized. They play an important role in the assessment of metal contamination in natural waters (Duzzin *et al.*, 1988, Lietz and Galling, 1989, Jha *et al.*, 1990; Pardo *et al.*, 1990; Gonçalves *et al.*, 1991; Huang *et al.*, 1994; Borovec, 1996; Wardas *et al.*, 1996). Indeed, sediments reveal a high capacity to accumulate, register and eventually integrate through the time trace element presence in the water and, therefore, they allow to assess environmental metal levels even if the water concentrations are almost undetectable.

The largest alluvial plain in Italy, namely “Pianura Padana” (Po River Plain) is composed by the alluvial deposits of the Po River, the major river in Italy, and by its tributaries. The whole catchment, except for the mountain area, is characterized by heavy industrialization, extensive agricultural practices, diffuse animal husbandries and high density population. The coastlines north and south of the Po River mouth are also affected by a considerable touristic pressure. The Po delta, although heavily modified by human intervention such as drainage, channelling, embankments and reinforcement of river channels, presents a considerable number of brackish wetlands distributed along a 130 km stretch of the northern Adriatic coast. The natural components of these environments have been affected at various degrees by management practices such as agriculture, fish farming, salt production, and various kinds of recreational activities (e.g. fishing, hunting, tourism), in many cases in combination. Only few of the wetlands included in the Po delta area have been heavily affected by urban and industrial development with significant discharges of nutrients and pollutants (the Pialassa Baiona according to Fabbri *et al.*, 1998, 2000, 2003; Trombini *et al.*, 2003; Covelli *et al.*, 2011; Guerra, 2012), but at present these inputs are less conspicuous.

The literature on the geological setting and palaeogeographic evolution of the area is extensive (Amorosi *et al.*, 1999a, b, 2003, 2004, 2005, 2008; Vincenzi and Stefani, 2005), especially after the recent impulse given by the CARG geological mapping project to scale 1: 50,000 of the Geological Survey of Italy, promoted in the southern Po plain by the Geological Survey of Regione Emilia-Romagna. Also geochemical aspects were investigated and the data on sub-surface soils and deep cores give essential information on the local background levels for selected elements (e.g. Amorosi *et al.*, 2002, 2007, 2008; Curzi *et al.*, 2006; Dinelli *et al.*, 2007, 2012). Conversely, the inorganic geochemistry of the Po delta coastal wetlands has received poor attention, except for the "Pialassa Baiona", a lagoon near the town of Ravenna, historically affected by mercury contamination (e.g.: Fabbri *et al.*, 1998; Trombini *et al.*, 2003; Matteucci *et al.*, 2005; Donnini *et al.*, 2007; Guerra *et al.*, 2009). Consequently, more information on sediment composition is desirable. To improve that knowledge, the inorganic composition of sediments from seven Po delta coastal wetlands has been investigated. The main aims of this study were to gain insight into: 1) the geochemical composition of the surface sediments, including major and trace element concentrations, their distribution, and sources; 2) the influence of anthropogenic activities on the metal levels in the sediments, by using the Enrichment Factor (EF) (Zhang and Liu, 2002; Zhang *et al.*, 2007) having at our disposal a wide dataset relative to the element abundances in bottom sediments sampled around the investigated sites (Amorosi *et al.*, 2002, 2007, 2008; Curzi *et al.*, 2006; Dinelli *et al.*, 2007, 2012; Dinelli, unpublished data).

2.3 Materials and methods

2.3.1 Study area and sampling sites

The seven investigated wetlands are: "Valli di Rosolina" (ROS), "Valle Bertuzzi" (BER), "Valli di Comacchio" (COM), "Vene di Bellocchio" (BEL), "Valle Mandriole" (MAN), "Pialassa della Baiona" (BAI), and "Pialassa dei Piomboni" (PIO) (Fig. 2.1). All the sampling sites are part of the historical Po deltaic system along the stretch of the northern Adriatic coastline between the towns of Ravenna and Chioggia (Bondesan *et al.*, 1995a). At present, they are all included in the Emilia Romagna Po Delta Regional Park, except ROS that is included in the Veneto Po Delta Regional Park. The main characteristics of the sampling sites and the number of samples collected are summarized in Table 2.1.

The evolution of the Po Delta was determined by the combination and the overlap of a set of complex geological, sedimentological and meteorological processes, both terrestrial and coastal

marine, to which the human intervention has been added over the years. The most recent geomorphological evolution was mainly controlled by three concomitant factors: the progressive eastward shift of the coastline, eustatic variations in sea level and subsidence (Ciabatti, 1967; Nelson, 1970; Bondesan *et al.*, 1995a; Simeoni *et al.*, 2000; Simeoni and Corbau, 2009). Recent investigations associated with an extensive drilling campaign promoted by the Geological Survey of Regione Emilia-Romagna as part of the geological mapping project of Italy, have provided more data for a better characterization of the late Quaternary deposits in the area.

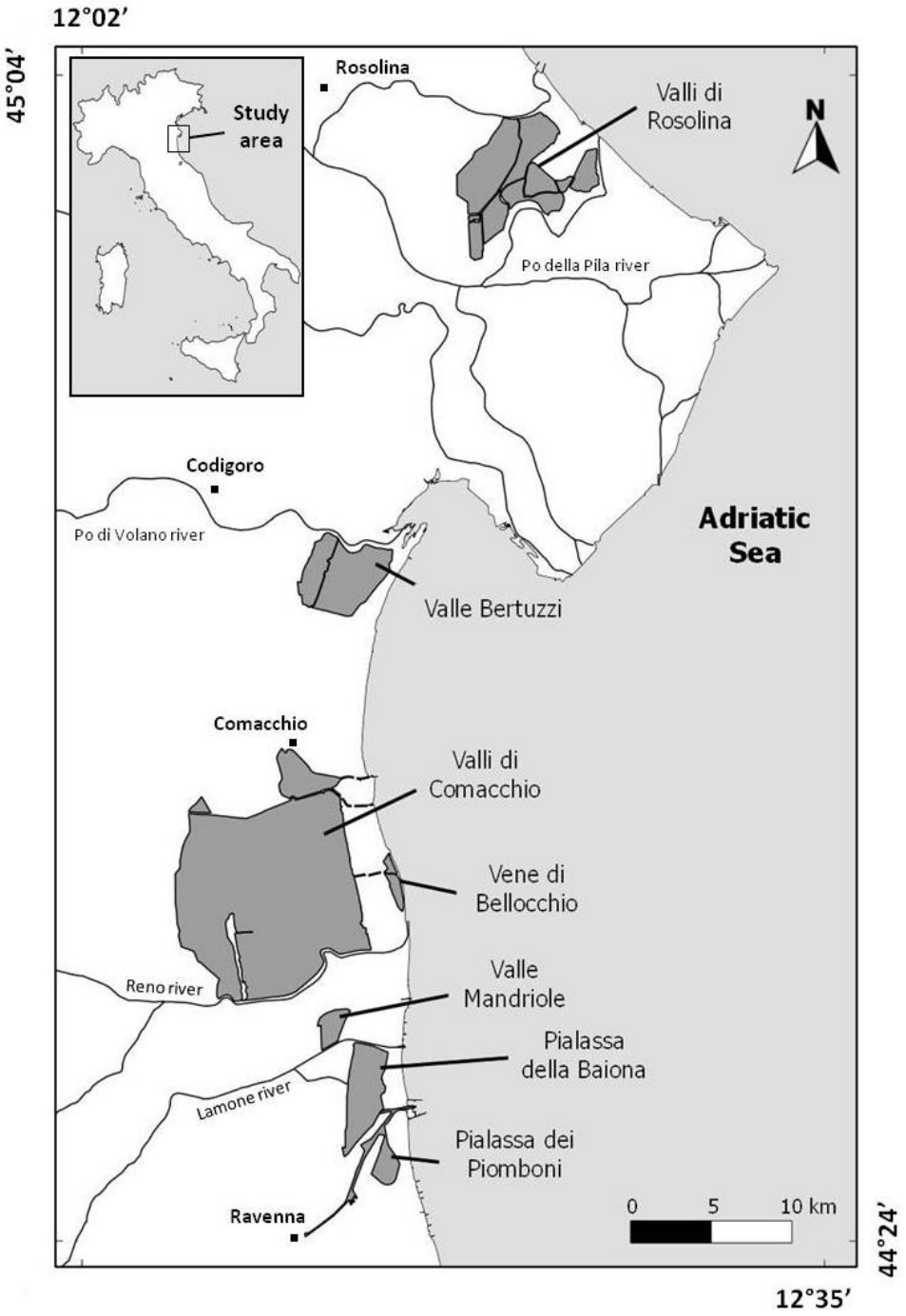

Figure 2.1 – Geographic location and identification of the seven studied coastal lagoons along the north Adriatic coastline.

The recent sediments have a structure closely related to the climatic events that occurred since the last glacial period, known as Würm glaciation (started approximately 75,000 years ago). Above the Last Glacial Maximum (LGM) alluvial deposits, the Ravenna and Comacchio coastal plains, as well as the subsurface of the modern Po Delta, display a common stratigraphic framework: a transgressive-regressive depositional cycle of Holocene age (approximately 30 m thick) made up of retrogradational back-barrier, transgressive shoreline and offshore-transition deposits, overlain by a shallowing-upward succession of prodelta, delta front, delta plain and alluvial plain sediments. This stratigraphic architecture reflects the landward migration of barrier-lagoon-estuary systems, followed by extensive deltaic and coastal plain eastward progradation (e.g. Amorosi *et al.*, 1999; 2007; 2008; Curzi *et al.*, 2006; Stefani and Vincenzi, 2005). The presence of a series of beach ridges of various age is an evidence of the latter process, as shown in Figure 2.2. However, the delta progradation has stopped in the 1950s caused by the progressive reduction of sedimentary inputs related to heavy anthropogenic alteration of rivers and gravel and sand extraction from their bed. This process led to the gradual and still ongoing retreat of the coastline (Dal Cin, 1983; Dal Cin and Simeoni, 1984; Simeoni and Bondensan, 1997; Simeoni *et al.*, 2000). As the coast gradually was moving eastwards, the subsidence gave rise the formation of extensive marshes and lagoons (Caputo *et al.*, 1970; Bondesan and Simeoni, 1983; Bondesan *et al.*, 1995b; CENAS, 1997). Simultaneously, the need to allocate new land for agricultural activities led to the onset of reclamation activities since ancient times, particularly intense from the second half of 1800 until the end of the 1960s, causing the deposition of a fine-grained surface layer where drainage was difficult (Stefani and Vincenzi, 2005).

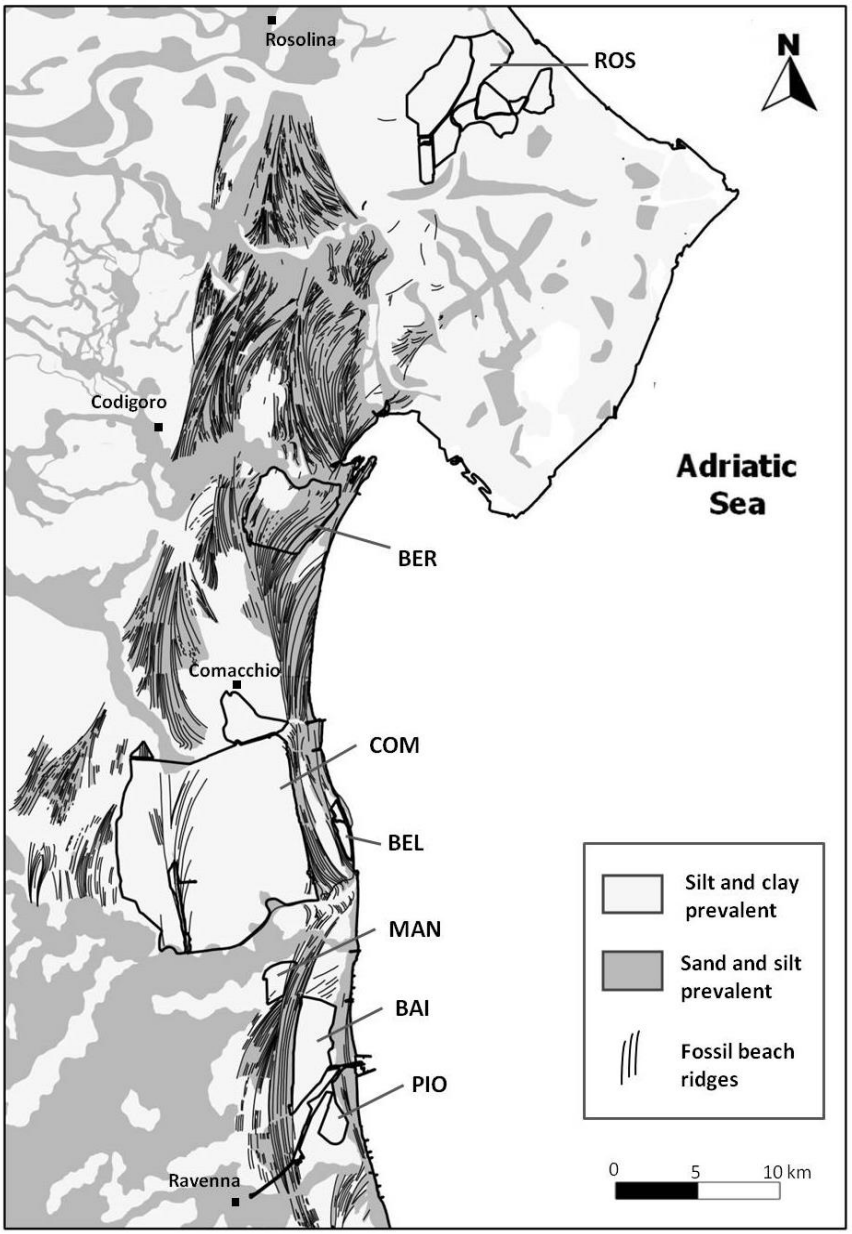

Figure 2.2 - Lithological classification of the areas surrounding the studied wetlands with indication of the preserved beach ridge systems. Redrawn from original data downloaded from Geoportale Regione Emilia-Romagna (http://geo.regione.emilia-romagna.it/geocatalogo/) and Geoportale Regione Veneto (http://idt.regione.veneto.it/app/metacatalog/).

Among the studied sites, BER, COM and MAN represent the residual parts of the submerged lands after these reclaiming actions. A different origin is attributed to the remaining sampling sites ROS, BEL, BAI and PIO), created by the closure of a sea portion between the original coastline and the peninsulas developed at the river mouth as a result of deposition of fluvial sediments (e.g. Reno river for BEL). The latter four lagoons are characterized by a direct connection to the Adriatic Sea and the influence of the tidal oscillations (Tab. 2.1). “Valli di Comacchio” are also connected to the sea by three artificial channels, whereas MAN and BER are

completely isolated from the Adriatic Sea and in this case the water salinity is due only to the saltwater intrusion, a phenomenon that, in recent years, heavily affects the aquifers of the entire deltaic area (COSTA21, 2004; Regione Emilia-Romagna, 2004; Antonellini, 2006, Antonellini *et al.*, 2006; ARPA, 2006). All the seven sites receive freshwater from a regulated system of rivers and channels which drain the surrounding agricultural lands. "Pialassa Baiona" and PIO also receive the effluents from industrial and municipal treatment plants. In the 1960s and 1970s high quantities of chemical compounds were discharged in BAI through the southernmost channel, carrying waste waters from the chemical settlement near the city of Ravenna (e.g.: Anconelli *et al.*, 1980; Angelini and Strumia, 1994; Fabbri *et al.*, 1998, 2000; Miserocchi *et al.*, 1990).

With the exception of BEL and MAN, all the sampling sites are used for human activities, such as fishing or regulated hunting, or both (Tab. 2.1). "Piomboni" is a particular case of an important industrial settlement (harbour) that includes a Special Protected Area (SPA). Actually, the construction of an embankment is in progress in order to separate the two different parts, but at the time of the sampling there was no separation at all. Among the investigated wetlands, COM is relatively the most important site for its extension, whereas PIO, BEL and MAN, are the smallest. The latter two sites although quite small, have been included in this study because of their importance as protected areas intended primarily for biodiversity conservation (Tab. 2.1).

Table 2.1 – The seven study sites ordered from north to south, including some of their geomorphological and management characteristics, and the number of samples collected from (n).

SITE	ID. SITE	EXTENSION (km^2)	TYPE of WETLAND	SEA CONN.	RIVER INPUT	PRESENT USE	SAMPLING	n
Valli di Rosolina	ROS	13.4	brackish lagoon	yes	absent	extensive fishing, regulated hunting	2012	6
Valle Bertuzzi	BER	19.2	brackish lagoon	no	Po di Volano	extensive fishing, regulated hunting	2012	7
Valli di Comacchio	COM	112.3	brackish lagoon	yes	Reno	extensive fishing, regulated hunting	2008-2012	34
Vene di Bellocchio	BEL	1.8	saltmarshes	yes	absent	naturalistic and protected area	2008	4
Valle Mandriole	MAN	2.9	freshwater swamp	no	Lamone	naturalistic and protected area	2013	10
Pialassa della Baiona	BAI	11.5	brackish intertidal lagoon	yes	Lamone	pastime fishing, regulated hunting	2007-2008	22
Pialassa dei Piomboni	PIO	3.1	brackish intertidal lagoon	yes	absent	industrial settlement, extensive fishing, regulated hunting	2013	6

2.3.2 Analytical procedures

2.3.2.1 Sediment sampling and preparation

A total of 89 samples of surface sediments were collected from the seven wetlands (details in Tab. 2.1). Where the depth of water was minor than 60-70 cm, sampling was carried out using a small plastic shovel, whereas a small grab sampler was used in deeper waters. Sediment aliquots were washed with distilled water, then centrifuged to eliminate any excess of salt, removing also coarse remains of animals and plants. After that, samples were dried at 60°C and homogenized by grinding in an agate mortar, for further investigations.

2.3.2.2 Analytical procedures

Major oxide (Al, Ca, Fe, K, Mg, Mn, Na, P, Si, Ti) and trace element (Ba, Ce, Co, Cr, Cu, La, Nb, Ni, Pb, Rb, Sr, V, Y, Zn, Zr, and S) concentrations were determined by X-ray Fluorescence Spectrometry (XRF) at the Department of Biological, Geological and Environmental Sciences (BiGeA – University of Bologna), using a Philips PW 1480 spectrometer equipped with a Rh tube. All analyses were made on pressed powder pellets following matrix correction methods of Franzini *et al.*, (1972, 1975), Leoni and Saitta (1976) and Leoni *et al.*, (1986). International reference materials were used for the calibration and as internal standard to monitor precision and accuracy and to ensure homogeneity in the results between different analytical batches. Estimated precision and accuracy for trace element determination was better than 5%, except for those elements at 10 ppm or lower (10–15%).

The total volatile content (including humidity, organic matter, water incorporated in the lattice of clay minerals, and carbon dioxide in the carbonate minerals) was determined by the weight loss recorded at 950 °C, defined as Loss Of Ignition (LOI). On the majority of samples this was done by thermal analysis using a Setaram TAG24 double furnace apparatus, with simultaneous registration of thermogravimetric (TG), derivative thermogravimetric (DTG) and differential thermal analysis (DTA). A CO_2 atmosphere was used to increase the temperature of carbonate decomposition and the carbonate content was estimated by quantifying the weight loss at temperature higher than 700 °C. Organic matter content has been evaluated considering the weight loss between 110 and 550 °C. On a small number of samples it was not possible to use the instrumentation and it was determined as overall loss after overnight heating at 950°C in platinum crucibles.

2.3.2.3 Statistical analysis

In order to establish inter-element relations, Spearman's correlation coefficients for the major and trace elements were analysed. Statistical analyses (descriptive statistics, Spearman's coefficients, p-values) and graphs were carried out using SPSS 14.0 and GCDkit 2.3 software (Janoušek *et al.*, 2006). Map processing was performed using Quantum Gis 2.2 software downloadable at the QGis website(http://www.qgis.org/it/site/).

2.3.2.4 Evaluation of sediment contamination

A number of methods have been suggested for quantifying metal enrichment in aquatic sediments. The central concept is to produce a numerical result comparing the metal content of each sample with a background level. The choice of the background values is crucial. Many authors have used the average shale values or the average crustal abundance data as reference baselines (Wedepohl, 1995). The best alternative is to compare concentrations between contaminated and uncontaminated sediments, provided that they have similar mineralogy and are texturally comparable (Covelli and Fontolan, 1997; Rubio *et al.*, 2000; Sakan *et al.*, 2009). In the present study, we used reference values calculated from Holocene lagoon deposits sampled from several deep cores drilled in the Po river coastal plain. In addition, these observations will be included in many of the graph presented to facilitate the comparison and the evaluation of the peculiarities of the lagoon sediments.

The Enrichment factor (EF) represents an useful tool in determining the degree of anthropogenic heavy metal pollution and the status of environmental contamination. The EF values were calculated using the formula (2.1) given in Zhang *et al.*, (2007):

$$Ef = \frac{\left(Metal/Al\right)_{sample}}{\left(Metal/Al\right)_{background}} \qquad (2.1)$$

Normalization of element contents against an immobile element is a common practice to accommodate grain size effects and dilution by phases such as quartz and carbonates. Currently, Al is the most frequently used geochemical normalizer in estuarine and coastal sediments, based on the assumption that Al is held exclusively in terrigenous aluminosilicates (Chen and Selvaraj, 2007; Karageorgis *et al.*, 2009; Ho *et al.*, 2010). Values of 0.5-1.5 suggest that the trace metals may be derived entirely from crustal materials or natural weathering processes (Zhang and Liu, 2002),

whereas values greater than 1.5 suggest that a significant portion of trace metal has been delivered from anthropogenic sources (Zhang *et al.*, 2007).

2.4. Results and discussion

2.4.1 Derivation of local background

Taking advantage of extensive geochemical studies carried out in the area it was possible to separate representative samples to be used for the calculation of regional background values. Thanks to the multidisciplinary studies performed, it was possible to separate samples deposited in lagoon environment, thus providing a more sound reference for the studied sites. The chemical analyses were performed using the same analytical technique by the same laboratory so direct comparison is possible. Considering the known geochemical differences related to sediment provenance (Amorosi *et al.*, 2002; Amorosi and Sammartino, 2007; Amorosi, 2012) that identified a major geochemical boundary coincident with the present day Reno river (Fig. 2.1), we calculated two different background values one for the area North of the Reno River (Background N) and one for the area South of it (Background S). For the area north we used 18 samples taken from Amorosi *et al.*, (2002, 2007, 2008), Curzi *et al.*, (2006) and Dinelli *et al.*, (2007) while for the southern area we used 30 samples from Amorosi *et al.*, (2002), Dinelli *et al.*, (2012) and unpublished data from the authors.

As background references we used the 95-percentile of these two anthropogenically non-influenced datasets (Matschullat *et al.*, 1999), and the results are reported in Table 2.2.

The two background show some differences, as concern the major element composition: the Background N has higher SiO_2 and MgO, whereas the Background S has higher Al_2O_3, Fe_2O_3, CaO and K_2O. Well known differences are those concerning Cr and Ni that are the clearest indicators of sediment provenance that are much higher in the Background N. Other elements (V, Zn, Rb, La, Ce, Sr) are higher in the Background S population possibly for the higher content of clay minerals, and also carbonates, as the results suggest. Copper and Pb are slightly higher in the Background S, although less evidently than Zn. The differences for these metals are also confirmed by the 95%-percentile of normalized data for Al_2O_3 content.

In the following elaboration we considered the Background N as reference for the sites ROS, BER, COM, and BEL, while for MAN, BAI, and PIO we used the Background S.

Table 2.2 – Background levels calculated on the element abundances in Holocene lagoon deposits sampled from several deep cores drilled in the Po river coastal plain. For each study sites median (MED), minimum and maximum (MIN, MAX), 95-percentile (95%), and number of samples (n), are reported. In addition, 95%-percentile of Al_2O_3-normalized data used for EF calculations, are given for Cu, Cr, Ni, Pb, and Zn.

	BACKGROUND N (n=18)					BACKGROUND S (n=30)				
	MED	MIN	MAX	95%	95% (Al-n)	MED	MIN	MAX	95%	95% (Al-n)
SiO_2 (wt%)	47.69	43.09	53.58	52.48		41.44	33.85	48.20	43.74	
TiO_2	0.60	0.50	0.67	0.66		0.63	0.50	078	0.74	
Al_2O_3	13.75	11.62	15.21	14.67		13.58	10.18	16.88	16.25	
Fe_2O_3	5.47	4.52	6.24	5.82		6.00	4.29	7.11	6.75	
MnO	0.12	0.09	0.16	0.14		0.13	0.08	0.19	0.17	
MgO	4.29	3.47	5.04	5.03		3.87	3.41	4.28	4.22	
CaO	9.78	6.88	12.28	12.25		12.14	3.72	21.10	16.35	
Na_2O	1.84	1.15	2.26	2.23		1.00	0.59	5.41	1.07	
K_2O	2.27	1.93	2.49	2.42		2.68	1.97	3.37	3.07	
P_2O_5	0.11	0.08	0.19	0.13		0.12	0.06	0.17	0.17	
LOI	13.50	4.81	18.04	18.62		18.12	15.28	21.51	20.50	
V (mg/kg)	96	60	114	110		121	70	158	154	
Cr	169	124	268	223	13.71	131	87	164	163	10.66
Co	17	6	22	21		15	9	25	23	
Ni	118	59	171	154	11.62	84	58	104	100	6.78
Cu	30	12	48	40	2.86	39	23	50	48	3.60
Zn	87	49	98	98	6.82	105	61	134	127	8.50
Rb	119	56	155	139		141	83	205	187	
Sr	271	210	331	309		302	150	479	414	
Y	28	17	43	36		24	12	36	29	
Zr	148	99	206	188		90	60	134	113	
Nb	16	7	22	18		17	3	25	23	
Ba	303	244	411	378		345	280	409	407	
La	27	9	47	33		30	19	44	40	
Ce	56	14	83	65		65	34	92	87	
Pb	17	12	28	25	1.73	20	11	29	27	1.88
S	1340	561	7339	2171		559	29	15218	1900	

2.4.2 Major and trace element abundances in wetland sediments

A summary statistics, subdivided according to sampling site is presented in Table 2.3.

Table 2.3 – Oxides, thermal analysis and trace element results. For each study sites arithmetic mean (M), median (MED), minimum and maximum (MIN, MAX) and number of samples (n), are reported.

	ROS (n=6)				BER (n=7)				COM (n=34)*				BEL (n=4)				MAN (n=10)				BAI (n=22)*				PIO (n=6)			
	M	MED	MIN	MAX	M	MED	MIN	MAX	M	MED	MIN	MAX	M	MED	MIN	MAX	M	MED	MIN	MAX	M	MED	MIN	MAX	M	MED	MIN	MAX
SiO_2 (wt%)	39.1	40.2	31.8	42.0	42.4	42.9	31.0	54.7	39.8	41.6	18.5	66.4	45.9	45.9	45.2	46.7	32.3	33.1	18.7	41.2	41.3	42.4	32.3	48.1	38.2	40.6	28.3	44.9
TiO_2	0.51	0.51	0.43	0.56	0.46	0.43	0.40	0.57	0.50	0.50	0.24	0.67	0.61	0.61	0.60	0.63	0.46	0.46	0.30	0.57	0.53	0.55	0.34	0.62	0.53	0.52	0.48	0.59
Al_2O_3	12.4	12.9	9.9	13.3	10.5	10.4	9.7	11.7	11.0	11.9	5.6	13.8	14.6	14.7	13.6	15.2	10.6	11.0	7.1	12.4	12.4	12.6	10.1	14.0	11.8	11.7	9.5	13.9
Fe_2O_3	4.51	4.72	3.48	4.91	3.77	3.78	3.18	4.24	4.35	4.20	1.34	6.20	5.74	5.76	5.51	5.93	4.32	4.24	3.56	5.10	5.12	5.19	3.83	6.48	4.76	4.75	3.88	5.85
MnO	0.12	0.12	0.11	0.13	0.13	0.13	0.11	0.14	0.12	0.12	0.08	0.24	0.12	0.12	0.09	0.15	0.10	0.10	0.09	0.12	0.11	0.11	0.07	0.18	0.12	0.12	0.11	0.13
MgO	4.38	4.34	4.21	4.59	4.08	4.04	3.63	4.41	3.56	3.76	1.68	4.29	3.6	3.61	3.42	3.78	3.39	3.38	2.49	4.07	3.92	3.99	3.29	4.44	3.59	3.68	2.76	3.99
CaO	12.0	11.4	9.2	18.0	11.3	11.4	9.0	13.3	12.0	8.9	4.8	30.8	8.7	8.6	7.1	10.4	13.0	13.0	9.3	16.3	12.2	12.2	6.1	17.5	17.2	17.2	11.4	25.3
Na_2O	1.75	1.82	1.43	1.96	2.43	2.49	2.01	2.79	2.55	2.40	0.71	8.72	1.20	1.22	1.12	1.24	0.69	0.75	0.42	0.89	1.43	1.13	0.90	4.47	1.34	1.35	0.96	1.57
K_2O	1.97	2.05	1.55	2.10	1.70	1.70	1.50	1.95	1.95	2.02	0.96	2.66	2.50	2.53	2.29	2.66	1.73	1.77	1.09	2.21	2.18	2.19	1.58	2.53	2.01	2.03	1.69	2.28
P_2O_5	0.11	0.10	0.06	0.20	0.14	0.13	0.12	0.17	0.14	0.15	0.06	0.23	0.13	0.13	0.12	0.15	0.21	0.22	0.14	0.32	0.26	0.19	0.13	1.15	0.12	0.13	0.08	0.16
LOI	23.2	22.4	20.2	28.3	23.1	21.0	11.0	34.1	24.1	23.7	8.0	38.8	17.0	16.4	16.1	19.1	33.2	33.4	19.0	56.4	20.5	18.8	13.7	33.6	20.3	19.0	14.5	26.5
H_2O hum	2.11	1.81	1.59	2.93	2.17	1.76	0.45	3.79	2.22	2.31	0.36	5.58	---	---	---	---	3.61	3.27	2.05	6.86	2.42	2.54	0.80	3.99	0.87	0.81	0.09	1.57
OM	6.6	5.8	4.7	10.0	9.2	8.1	2.9	15.7	6.8	7.0	2.0	12.2	---	---	---	---	13.1	11.3	5.8	29.0	5.2	2.5	1.1	29.1	4.1	3.9	1.9	6.5
H_2O clay	2.96	2.89	2.24	3.94	2.70	2.34	1.09	4.66	2.47	2.85	1.01	3.88	---	---	---	---	2.53	2.42	1.12	4.39	5.28	5.84	2.31	6.53	1.44	1.46	0.79	1.82
CO_2	6.4	6.2	3.9	10.2	4.2	4.1	3.0	5.9	2.7	2.7	1.4	4.5	---	---	---	---	11.5	10.3	9.5	16.2	8.8	9.0	1.2	12.3	13.7	12.2	11.4	18.7
V (mg/kg)	94	96	76	102	74	75	57	84	84	88	22	121	119	119	113	125	86	87	64	96	94	94	64	123	105	109	73	131
Cr	149	153	122	165	230	199	112	377	132	123	55	350	128	127	122	137	91	89	47	115	169	137	100	682	130	126	114	157
Co	15	16	10	17	12	12	9	15	10	11	**2**	18	16	17	14	17	10	10	7	15	11	11	6	16	12	12	9	21
Ni	111	113	95	127	131	138	106	146	84	86	27	133	79	79	76	81	70	69	59	77	75	74	51	96	80	84	50	102
Cu	35	34	30	39	25	27	14	41	28	29	3	48	40	40	39	41	30	31	20	38	54	52	21	159	61	71	27	86
Zn	91	94	77	103	85	89	50	122	88	95	20	130	122	122	110	132	124	127	96	146	243	177	67	785	218	227	91	327
Rb	183	190	131	208	128	130	93	170	122	124	**2**	228	148	149	145	150	92	93	50	124	108	110	73	138	111	105	81	159
Sr	799	756	614	1159	693	781	456	882	860	622	**2**	4412	260	253	250	282	341	351	259	417	321	318	221	440	486	418	363	733
Y	23	24	18	27	15	17	8	19	20	21	11	27	28	27	26	33	14	14	2	20	22	23	14	27	15	13	11	25
Zr	139	144	96	163	86	98	17	114	109	121	12	195	125	128	110	133	47	41	6	90	117	111	80	194	50	43	12	92
Nb	20	19	18	24	14	14	10	19	14	14	**2**	25	15	15	13	18	8	8	6	11	12	13	7	16	10	10	7	12
Ba	342	345	285	404	279	282	232	323	257	277	153	363	327	332	267	379	256	252	169	315	280	283	189	329	319	318	274	357
La	26	25	18	33	20	18	11	31	23	23	12	37	31	30	28	36	21	23	8	32	25	24	15	35	26	27	12	37
Ce	43	42	29	55	30	32	17	44	43	44	10	77	63	63	58	69	48	46	34	64	48	49	30	71	70	69	59	88
Pb	45	47	37	51	60	63	29	86	37	28	7	320	29	30	27	31	47	30	16	214	29	29	16	56	18	17	8	33
S	5928	6368	312	8415	9607	10402	5224	12004	6128	6435	**50**	18040	398	245	50	1050	9464	10150	1850	23370	3810	3950	50	8200	7628	7525	5240	9930

Among the chemical parameters are included SiO_2, and Al_2O_3, which are both influenced by the abundance of silicate minerals, although an increase of their ratio is indicative of a relatively coarse grained fraction of the sediment (e.g.: Dinelli *et al.*, 2007). Table 2.3 also reports CaO and volatile contents, the latter expressed cumulatively as LOI, being two further important sediment components. For some of the lagoon samples more details regarding the volatile content are available as a result of the thermal analysis: "H_2O hum" reflects the humidity of the sample, "OM" is indicative of the organic matter content, "H_2O clay" reflects the water adsorbed in clay minerals, and "CO_2" is controlled by the abundance of carbonate minerals (mostly represented by biogenic carbonates).

The major element composition of the whole dataset can be summarized by ternary plots (Fig. 2.3 a, b). Fig. 3a compares the relative proportions of three main inorganic components of the investigated samples: clay fraction (represented by Al_2O_3 and SiO_2), the quartz rich sandy samples (partly represented by the SiO_2 content) and the carbonate content (CaO) either biogenic and detrital.

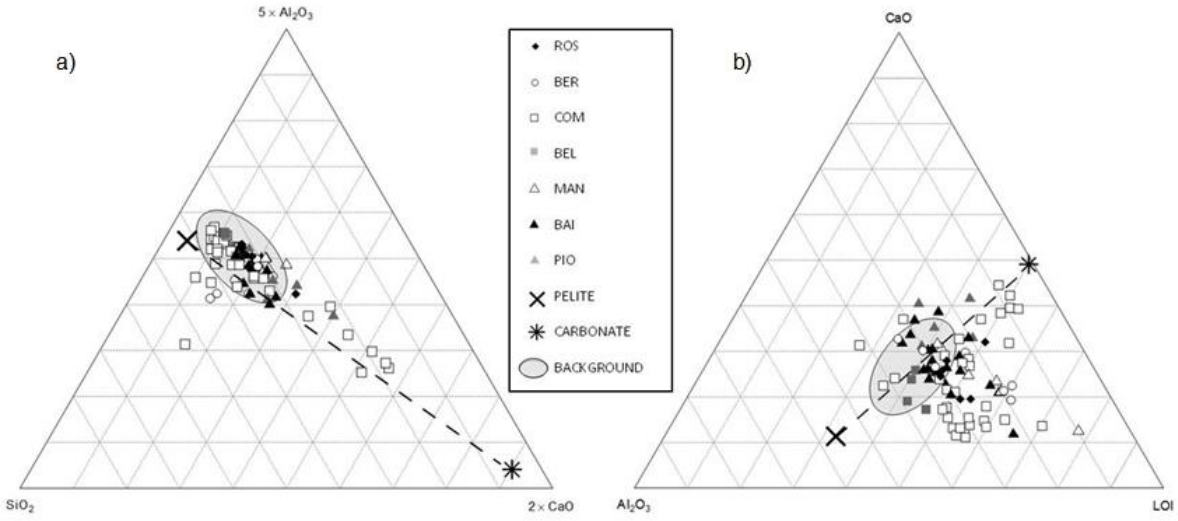

Figure 2.3 (a-b) – Ternary plots illustrating the major element composition of the whole dataset. For comparison, average pelite (cross) and average carbonate (star) (Turekian and Wedephol, 1961) are also plotted in both graphs, and the overall range of the background dataset is represented by an opaque area.

Most samples plot along a straight mixing line between carbonate and clay component, slightly higher in Al content respect to the average pelite, a feature that is shared with background sediments. In fact, more than 75% of our samples is consistent with the background. Few samples, the majority from COM and two from BAI, are richer in carbonates, likely of biogenic origin as testified by field observations. Only three samples from COM and 2 samples from BER have higher proportion of SiO_2, likely related to a coarser grain size of the sample.

The ternary plot in Figure 2.3b considers the volatile content (LOI), and thus can provide indication of the organic matter content, when taking into account the ratio between LOI and CaO of carbonates. Overall, the samples show a rather variable distribution, with a number of samples plotting along the carbonate-clay mixing line, in coincidence with the background sediments, but with approximately half of the analysed samples pointing to the LOI apex. This likely suggest abundance of organic matter and relatively low carbonate. Samples from COM form the largest part of this group, that includes also samples from MAN, BER, and BAI.

By examining the major element distribution in each sampling site (Tab. 2.3), the easternmost site BEL has relatively high SiO_2 and Al_2O_3 levels, low carbonate content, and the lowest LOI median value. The sample number from this site is low, but also the extension of the lagoon is the smallest among the studied sites. The adjacent site COM has similar CaO median value (8.93 wt%), with SiO_2 and Al_2O_3 ratios not significantly different from the other coastal lagoons ROS, BER, BAI and PIO. However, the CaO concentration ranges widely in COM sediments, suggesting a variable distribution of carbonate components within this vast wetland. The MAN sediments have low median concentrations of SiO_2 and Al_2O_3 (33.1 and 11.0 wt%, respectively), and the highest LOI and OM levels (33.4 and 11.3 wt%, respectively). The distribution of Fe_2O_3, TiO_2, MnO, MgO and K_2O in all the sampling sites do not show remarkable differences, with median values very similar in all wetlands.

Regarding the trace elements, relatively high concentrations of Cu in confront of the other sites occur in PIO and BAI, the two wetlands closest to the town of Ravenna (Fig. 2.1 and Tab. 2.3). Compared to the background, BAI median shows less evident difference than PIO, but this site presents the maximum value of Cu throughout all the dataset. Also Zn reaches significantly higher medians compared to the respective background in PIO, BAI and BEL. The Cr and Ni values in the background sediments are generally high especially in the northern area (Tab. 2.2), due to the provenance of the sediments (e.g. Amorosi *et al.*, 2002, 2007, 2008; Amorosi and Sammartino, 2007; Amorosi, 2012; Bianchini *et al.*, 2002, 2012; Curzi *et al.*, 2006; Dinelli *et al.*, 2007). The Cr and Ni concentrations found in the sampling sites confirm these naturally high levels, as for BER sediments in accordance with geographic location and geologic evolution. In BAI, the maximum of 682 mg/Kg Cr is evidently anomalous, but it is a single outlier. As expected from the carbonate content, the Sr medians are generally high in all the northern investigated sites, except in BEL. In aquatic environments, Sr tends to accumulate in some kind of invertebrate shells, which were often very abundant in our samples. It is known that aragonite typically contains about one order

of magnitude more Sr than calcite due to the differences in the crystal lattice structure (Kinsman and Holland, 1969; Tesoriero and Pankow, 1996). Regarding Rb, there is a strong difference between northern and southern sites, the former tending to have medians near or higher and the latter all well lower than the respective background upper limit. Rb tends to accumulate in fine fractions, especially in clay minerals and organic matter (Di Giuseppe *et al.*, 2014) and this may be the reason of such pattern. Cobalt concentrations are similar in all sites and comparable with the background, whereas BEL reports all levels of V relatively high respect the background. Slightly higher levels of Ba than the other sites (within the background upper limit) occur BEL and ROS. A series of trace elements (Y, Nb, La, and Ce) have medians below or around the background. Regarding Pb, medians below the background upper level occur only in PIO. Relatively high concentrations are reported for all the samples from BER and ROS. The remaining sites are quite similar in Pb median levels, but upper limits of the distribution in BAI, MAN and COM are evidently higher than the background. The results from sampling sites regarding S reveal very high concentrations in all the wetlands, except in BEL, but the range in the background datasets is wide, with peaks both for the Northern and the Southern areas.

2.4.3 Inter-element relationship

A correlation matrix for the whole dataset is presented in Table 2.4. The matrix shows that major elements TiO_2, Al_2O_3, K_2O and Fe_2O_3 in the surface sediments were strongly correlated ($r>0.75$, $p<0.01$) and can be related to the presence and abundance of clay minerals. Among trace elements, V and Co were the most correlated to this group of elements ($r>0.6$, $p<0.01$, see also Fig. 2.4a).

Table 2.4 - Correlation matrix between the variables. Bold numbers: correlation significant at the 0.01 level, other numbers: correlation significant at the 0.05 level.

	SiO_2	TiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	P_2O_5	LOI	H_2O hum	OM	H_2O clay	CO_2	V	Cr	Co	Ni	Cu	Zn	Rb	Sr	Y	Zr	Nb	Ba	La	Ce	Pb	S
SiO_2	---																														
TiO_2	**0.546**	---																													
Al_2O_3	**0.609**	**0.749**	---																												
Fe_2O_3	0.360	**0.892**	**0.750**	---																											
MnO					---																										
MgO			0.472			---																									
CaO	-0.457	-0.314	**-0.552**	-0.347			---																								
Na_2O		-0.309		-0.384		0.324	-0.286	---																							
K_2O	**0.708**	**0.862**	**0.880**	**0.827**			**-0.502**		---																						
P_2O_5					-0.314					---																					
LOI	**-0.907**	-0.498	**-0.521**	-0.311					**-0.628**		---																				
H_2O hum	**-0.516**									0.381	**0.701**	---																			
OM	**-0.572**	-0.494							**-0.601**		**0.749**	**0.539**	---																		
H_2O clay											**0.536**	**0.714**		---																	
CO_2	-0.382						**0.724**	**-0.679**							---																
V	0.220	**0.783**	**0.757**	**0.865**		0.273	-0.378	-0.324	**0.717**							---															
Cr	**0.541**	0.436	0.470	0.367		0.455	-0.319		0.466		**-0.505**				**-0.640**	0.427	---														
Co	0.379	**0.714**	**0.671**	**0.705**		**0.502**	-0.384		**0.608**							**0.785**	**0.517**	---													
Ni	0.362	0.358	0.353			**0.528**	-0.392		0.298						**-0.585**	0.376	**0.751**	**0.662**	---												
Cu		0.366	0.328	**0.549**				-0.317	0.303							**0.587**	0.309	0.413		---											
Zn		0.343	0.342	**0.575**				-0.321	0.340	0.458					**0.525**	**0.517**		0.342		**0.753**	---										
Rb	0.344	0.492	**0.651**	0.441		**-0.519**	**-0.572**		**0.546**						**-0.571**	**0.557**	0.490	**0.646**	**0.615**			---									
Sr	-0.387	-0.499	**-0.538**	**-0.659**			0.341	0.434	**-0.614**	.-305	0.327				-0.404	-0.485		-0.283		-0.362	**-0.569**		---								
Y	0.407	**0.640**	**0.660**	**0.679**		0.410	-0.435		**0.629**		-0.353					**0.574**	0.370	**0.683**	0.352	0.304	0.272	**0.556**	-0.334	---							
Zr	0.426	**0.507**	**0.507**	0.445		0.457	-0.410		0.498		-0.349				**-0.532**	0.398	0.500	**0.527**	0.412			**0.632**		**0.719**	---						
Nb		0.416	0.435	0.346		0.446	-0.489		0.391						**-0.587**	0.388	0.477	0.484	**0.574**			**0.810**		**0.518**	**0.704**	---					
Ba	**0.624**	**0.535**	**0.515**	0.378					**0.544**		**-0.657**	**-0.661**		**-0.580**		0.467	0.480	0.484	0.459	0.279		0.401		0.305	0.413	0.337	---				
La	0.279	**0.520**	0.478	0.473					0.449		-0.290					**0.534**	0.290	0.472		0.342		0.331		0.435	0.363		0.437	---			
Ce	0.272	**0.596**	0.484	**0.578**				-0.325	**0.557**		-0.328				**0.571**	**0.558**		0.424		0.327	0.376		-0.380				0.342	0.335	---		
Pb						0.334	-0.394					0.409	**0.659**		**-0.562**		0.414	0.373	**0.534**			**0.530**			0.297	**0.502**				---	
S											0.417		**0.655**						0.274					-0.294						**0.534**	---

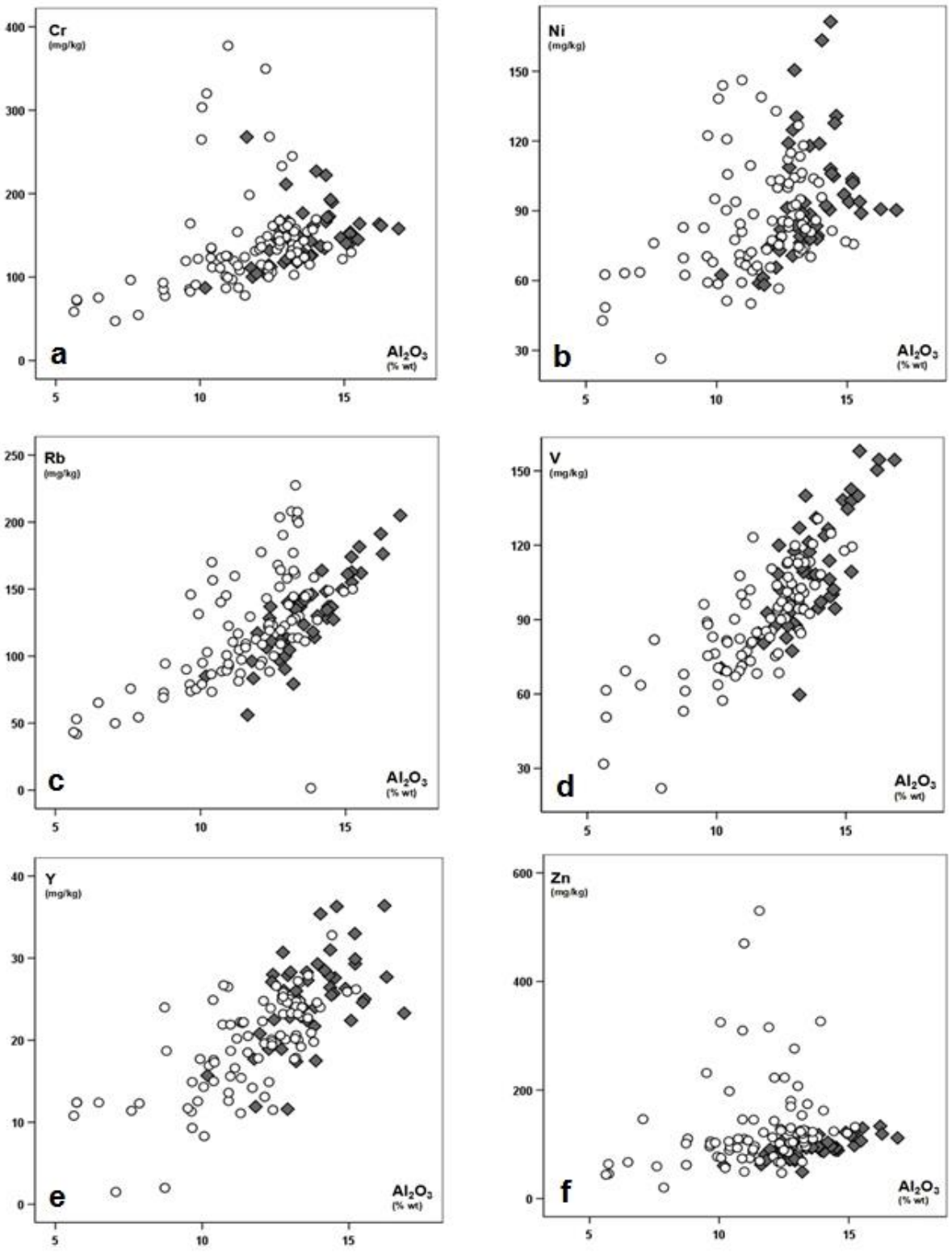

Figure 2.4 (a-b-c-d-e-f) – Relationships between Al_2O_3 and Cr, Ni, Rb, V, Y, Zn. White symbols indicate samples of this study, whereas grey symbols indicate background data.

Correlation levels expressed by V were in general more elevated, with V-Fe_2O_3 the highest ($r=0.87$, $p<0.01$). A positive correlation was also observed between Ce, Cu, Zn, and Y and Fe_2O_3 ($r>0.5$, $p<0.01$). This further supports the importance of the fine grained sediment fraction in

controlling the occurrence and distribution of several elements. The organic matter (OM) seems to exert the most important role in controlling the fate of Pb and S ($r>0.6$, $p<0.01$). Conversely, CaO do not reveal relevant associations with any other element, excepting some inverse significant correlations with Al_2O_3, K_2O, and Rb ($r<-0.5$, $p<0.01$), indicating that the carbonate content might not influence the metal distributions in surface sediments of the investigated wetlands, except for a general dilution. Considering the regional spatial scale of the sampling, the significant positive correlation of SiO_2 with Al_2O_3 and K_2O ($r>0.6$ and $r>0.7$ respectively, $p<0.01$), including in some way TiO_2 ($r>0.5$) suggests a relative high importance of aluminosilicates (mica and feldspars) throughout the studied sites. However, some correlations of SiO_2 with trace elements normally forming heavy minerals were found (Ba: $r=0.63$; Cr: $r=0.54$, $p<0.01$) suggest that there could be also a sorting effect active (Vital and Stattegger, 2000; Garcia *et al.*, 2004), at least locally.

The dominance of aluminosilicates throughout our dataset, can be argued observing the binary diagrams in figure 4, in particular Al_2O_3-V (Fig. 2.4d). Though Al_2O_3 is overall less concentrated than the background, as an effect of dilution caused by enrichments of carbonate, and OM in the wetland surface sediments, the distributions appear consistent with the background for Ni, V, Y. Similarly to the background, Al_2O_3-Ni (Fig. 2.4b), and, in lesser extent, Al_2O_3-Y (Fig. 2.4e) show the most dispersed patterns. For Cr, Rb and Zn, the agreement with the background is observable for most samples, but a small group of samples probably affected by other factors, such us a different provenance of the sediments, hydraulic sorting, and anthropogenic inputs, alter the linearity. The Al_2O_3-Rb graph (Fig. 2.4c) seems to identify two different populations, being a subset clearly Rb-enriched. That subset include all samples from ROS, three out of seven from BER, and many from COM, eventually related to the abundance of muscovite in sediments derived from the Po River.

A more detailed discussion can be made for Cr and Zn. They show significant correlation with other elements (Cr-Ni, $r=0.751$; Cu-Zn, $r=0.753$).

Regarding the strong correlation of Cr-Ni, our results agree with many previous studies focused on the study area (e.g. Amorosi *et al.*, 2002, 2007, 2008; Bianchini *et al.*, 2002, 2012; Curzi *et al.*, 2006; Dinelli *et al.*, 2007). Moreover, Amorosi and Sammartino (2007) detected anomalous high levels of both metals in fluvial, coastal, and deltaic near-surface sediments of the Po coastal plain. Due to its environmental implications, Cr enrichments were studied in depth. In their survey, high Cr near the surface were similar to the Cr enrichments reported from core materials of southern Po plain (Amorosi *et al.*, 2002), in the Po Delta area (Amorosi *et al.*, 2008), from a small

area around the town of Ferrara (Bianchini *et al.*, 2002), and from channel-bed sediments of Po River throughout its catchment (Dinelli and Lucchini, 1999; Vignati *et al.*, 2003). Definitively, the high Cr concentrations related to Po river sediment, indicate a natural source and do not reflect land-use practices or artificial contamination (Amorosi and Sammartino, 2007; Amorosi, 2012). According to Amorosi and Sammartino, the Cr/V ratio in sediments of the southeastern Po plain can be used as a powerful proxy of their Alpine or Apenninic provenance. The two distinct linear trends illustrated by the Authors (Fig. 2.5, grey symbols), correspond to: (a) the Cr-poor lithologies of the north-eastern Apennines and (b) the relatively Cr-rich rocks of the Po River drainage basin. In Figure 2.5, we also plotted the Cr and V concentrations in our samples (white symbols). It is observable that our dataset is quite consistent and coherent with that two-sources model, with a prevalence of samples presumably of Apenninic origin.

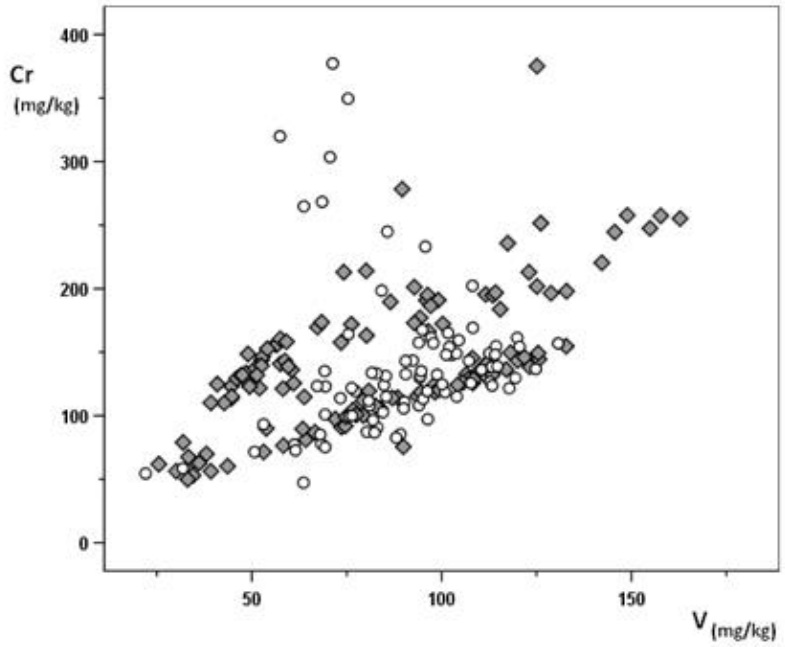

Figure 2.5 – Binary plot of Cr and V according to Amorosi and Sammartino (2007) (grey rhombus) and this study (white circles). Except for few anomalous samples (Cr/V very high), the dataset of this study overlaps the field of Apenninic provenance, with a subset placed in an intermediate position.

In fact, Cr and V concentrations in the majority of our samples plot close to the line of Apenninic provenance (lower Cr). However, a subset seems to be influenced by mixing processes, plotting in an intermediate position between the two provenance fields rather than to be distinctive of Alpine origin. Few evident exceptions to the model are present in our dataset, since 8 samples show Cr/V ratio higher than expected. In particular, Cr-enrichment in 6 samples (3 in BER, 2 in COM and 1 in BAI) is very conspicuous, and for 2 additional COM samples the anomaly is

less remarkable but clear. Local factors, such as enrichment due to a sorting effect in high energy environment could represent a natural origin for these enrichments (Garcia *et al.*, 2004). High energy environment can lead to the deposition of sandy sediments enriched in heavy minerals such as pyroxene and garnet that are common in the sands of the area (Rizzini, 1971; Marchesini *et al.*, 2000) and lead to high chromium concentrations in the sediments (Vital *et al.*, 1999). This is further supported by the weak correlation of Cr with Al_2O_3 and K_2O ($r<0.5$, $p<0.01$), whereas the moderate correlation between Cr and SiO_2 ($r=0.541$, $p<0.01$) suggests that this metal may be mainly associated with the coarse-grained fraction.

If we consider BER and COM, we note that their bottoms are geo-morphologically heterogeneous and include ancient beach ridges systems. Figure 6a presents the relationships between Cr and the SiO_2/Al_2O_3 ratio, used as a grain-size proxy, relative to BER and COM samples. The white squares, indicating the seven Cr-enriched samples from the two sites, have also high SiO_2/Al_2O_3 ratio. This connection between sand contents and Cr-enrichments occurs in well-defined contexts, namely the central area of BER (Fig. 2.6b) and around the northern far end of the "Boscoforte" peninsula in COM (Fig. 2.6c). This result might be interpreted as a consequence of local accumulation of Cr-bearing heavy minerals by wave processes in certain sand structures. This process is localized, since 3 additional samples with high SiO_2/Al_2O_3 ratio are not anomalous for Cr (black symbols in Fig. 2.6a) and are located in different places within the two lagoons (Fig. 2.6b-c). So, the grain-size heterogeneity and sorting effect leading to the local concentration of heavy minerals can represent a likely explanation of the Cr enrichments in those environments, reflecting the characteristic of the ancient depositional environment and not the present day dynamics. Moreover, these sites are far from industrial sites or pollution sources.

The Cr-enrichment that occurs in BAI may be ascribed to sediment contamination, considering that the sample is located in the southernmost part of the site, known as the most polluted portion of the entire lagoon (Soprani *et al.*, 1994; Fabbri *et al.*, 1998, Trombini *et al.*, 2003, and Matteucci *et al.*, 2005), really close to the industrial area of Ravenna. This same site records anomalous levels of other metals (Cu, Zn as discussed later, and Pb) and this further supports a strong anthropogenic impact in this site.

The process that guided the anomalous Cr-enrichment could have involved other heavy minerals as suggested by the relative Ba-enrichment occurring in the same seven samples, that are among the highest within the BER-COM system (Fig. 2.7). Actually Ba can also be related to a coarse feldspar rich fraction since Ba can substitute for K in feldspars and might be representative of a

relatively coarse fraction, whereas Rb is mostly concentrated in the clay fraction, substituting for K in illites (Dypvik and Harris, 2001).

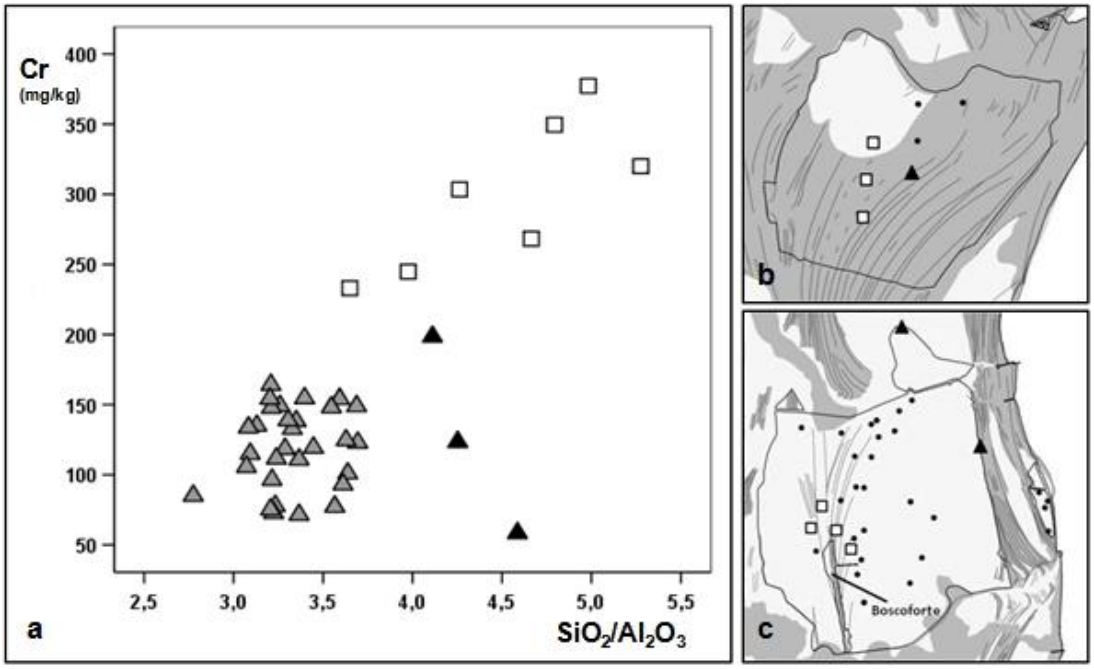

Figure 2.6 (a-b-c) – Relationships between Cr and the SiO_2/Al_2O_3 ratio, grouping BER and COM samples (Fig. 2.6a). White squares indicate the seven Cr-enriched samples, collected in well-defined contexts in BER (Fig. 2.6b) and in COM (Fig. 2.6c). Black triangles identify samples with high SiO_2/Al_2O_3 ratio but not anomalous for Cr, located in different contexts.

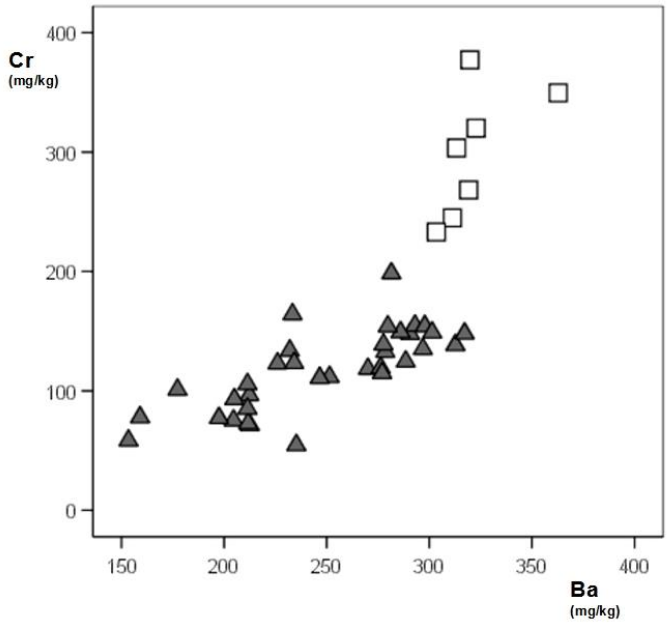

Fig. 2.7 - Relationships between Cr and Ba, grouping BER and COM samples. White squares indicate the same seven Cr-enriched samples presented in Figure 2.6a.

The correlation between Cu and Zn (Tab. 2.4) seems to be driven by some samples with high concentrations of both metals (Fig. 2.8). Observing the Table 2.2 and Table 2.3, it is clear that the background levels of these metals are substantially comparable to the concentrations measured in the majority of sites, with the exception of BAI and PIO, being their distributions evidently dispersed and their medians significantly higher.

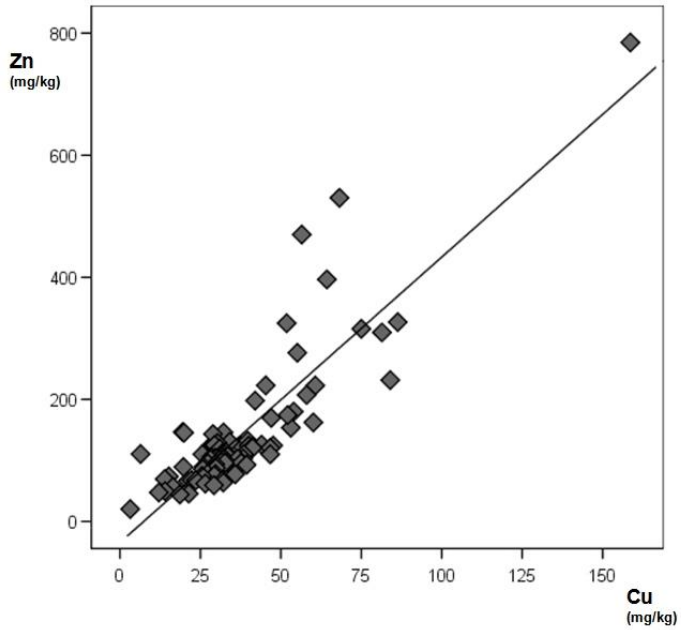

Figure 2.8 – Binary plot Zn-Cu. Few samples with high concentrations of both metals seems to drive the high correlation coefficient reported in table 4.

In order to better interpret this outcome, two subsets are examined separately, the first consisting of ROS, BER, COM, BEL and MAN (S1, n=61) and the second of BAI and PIO (S2, n=28) samples. In fact, the levels of correlation between Cu and Zn result rather different in the two subsets (S1: r=0.50, S2: r=0.85, p <0.01). Furthermore, the Zn levels in S1 are always lower than 150 mg/kg, whereas the subset S2 reaches very high concentrations, with 13 samples of 28 that have Zn levels greater 200 mg/kg. Copper and Zn can be accumulated in basin sediments by water inputs draining agricultural areas (respectively associated with pesticides and soil amendments), as well as from polluted inputs related to port and industrial activities. Although all the investigated sites can be affected by agricultural effluents, Cu and Zn anomalies are almost exclusively restricted to BAI and PIO (Tab. 2.3). Likely, the Cu and Zn enrichments observed in BAI and PIO may be explained by the historical link between both sites and the industrial development of Ravenna. Generally, the behaviour of Cu and Zn in sediment is controlled by the adsorption on

clay minerals, Fe, Mn, Al hydroxides, and organic matter (Mihaljevic, 1999). Our study revealed that Zn tends to associate to Fe_2O_3 in subset S1 ($r=0.65$, $p<0.01$, grey triangles), whereas in S2 it is strongly correlated with LOI ($r=0.84$, $p<0.01$, white squares) (Fig. 2.9 a-b, respectively).

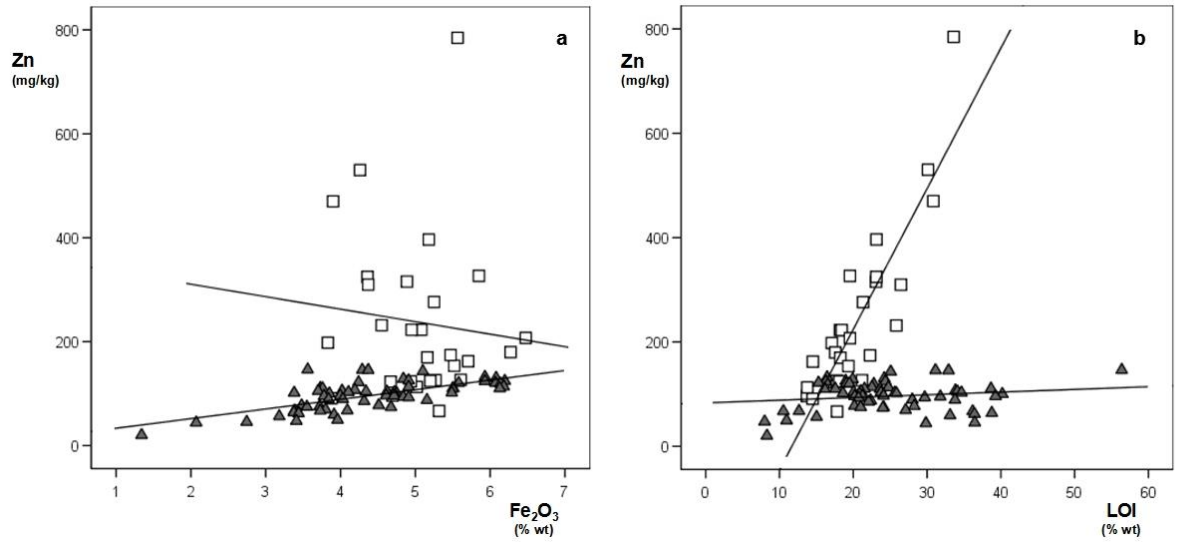

Figure 2.9 - The samples from ROS, BER, COM, BEL and MAN (group S1, n=61, grey triangles) and from BAI and PIO (S2, n=28, white squares) are examined separately for Zn/Fe_2O_3 (a) and Zn/LOI (b) correlations. Zinc is significantly correlated to Fe_2O_3 in S1 subset ($r=0.65$, $p<0.01$), whereas in S2 Zn is strongly correlated with LOI ($r=0.84$, $p<0.01$). The Cu behaviour being very similar.

The Cu behaviour being very similar, is not illustrated here but it is expected given the good correlation between the two elements (Fig. 2.8).

The association of Cu and Zn with the organic matter found in the enriched BAI and PIO samples, supports the hypothesis of an anthropogenic source of these metals in the two southernmost sites. If the input of Cu and Zn would be only from industrial origin, a north-south increasing gradient in BAI would be expected, such as reported for Hg in previous studies (Trombini *et al.*, 2003). In contrast, the irregular distribution of the concentrations in BAI (Fig. 10 a-b), in agreement with Donnini *et al.*, (2007), suggests a plurality of sources, arguably including agricultural effluents and human activities carried out within the wetland, such as the use of antifouling paints for the maintenance of boats and submerged structures (e.g. Turner, 2010; Ytreberg *et al.*, 2010; Takahashi *et al.*, 2012), although the highest values are observed in the southern area (Matteucci *et al.*, 2005; Guerra, 2012). Concerning PIO, the samples richer in Cu and Zn are the nearest to an industrialized bank where the use of paints and galvanization products is huge.

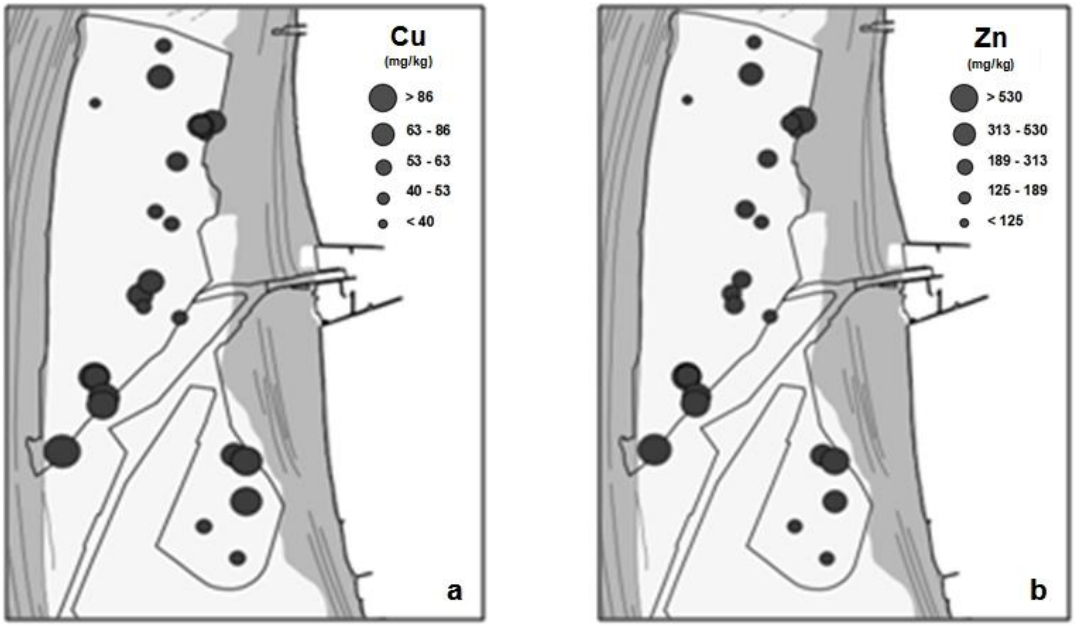

Figure 2.10 (a-b) – Distribution and level of the Cu (10a) and Zn (10b) concentrations in BAI (the largest wetland) and PIO. Highest concentrations in BAI are dispersed, suggesting a plurality of anthropogenic sources.

Hunting to waterfowl has been intense in most of the sampling sites (Tab. 2.1), and relevant amounts of Pb have been discharged as lead pellets. At present, hunting in MAN is not allowed, but in the past this activity have been intensively practiced. The only site not impacted by lead ammunition may be considered BEL. However, all sites have been more or less exposed until 2001 to the atmospheric deposition of tetraethyl-Pb from leaded gasoline. According to Table 2.4, Pb resulted significantly correlated with S and few metals (Ni, Rb, Nb) with correlation coefficient between 0.5 and 0.6 ($p<0.01$). Overall, S resulted as similarly correlated with OM as Pb ($r>0.65$, $p<0.01$), confirming the tendency of Pb to be preferably adsorbed onto organic matter. On the other hand, the moderate association Pb-Rb expectable given the geochemical similarities between the two elements, suggests the possible role of another sediment component, likely clays, in controlling Pb distribution. The inverse correlations Pb-CaO ($r=-394$, $p<0.01$) and Pb-CO_2 ($r=-0.562$, $p<0.01$) suggest that in the study area Pb carbonate may be not important. Therefore, the Pb correlation pattern appears the result of a combination of sources, which caused enrichments both in the organic fraction as well as to the clay fraction throughout the study area, or the mediated result of a different behaviour of Pb site per site. For example, ROS and BER, the northernmost and the most Pb-rich sites (Fig. 2.1, Tab. 2.2), seem to negatively affect the Pb-OM correlation coefficient, being not significant for both sites, analysed singularly or grouped. Furthermore, the overall correlation Pb-Rb is driven by COM and BER ($r=0.674$, $p<0.01$, $n=41$), whereas such correlation is negligible taking the other sites ($r=0.285$, $p<0.05$, $n=48$).

2.4.4 Evaluation of metal pollution

In this section will be considered Cr, Ni, Cu, Zn, and Pb with the boxplot of their EF presented in Figure 2.11, according to the sampling site.

The EF values for all five metals indicate that no significant enrichment has occurred in BEL, which can be essentially considered an unpolluted site. Although the number of samples from BEL is limited to allow a sound interpretation, the natural detrital input could exert presumably the main control on the abundances of metals in this wetland.

The geological origin of Cr and Ni in the sediments of this study, even for some enriched samples, is confirmed by the EF values (Fig. 2.11a-b). Especially Ni clearly derives from natural sources, being EF medians comprised between 0.5 and 1.1. Also for Cr EF medians are included in the same range of Ni, as an indication of general coherence to the natural levels. There are some exceptions, as the binary diagrams in Figures 2.5 and 2.6a could suggest. One out of the four highest Cr-enriched samples from COM have EF greater than 1.5, and the three outliers of BER for Cr have EF values between 1.7 and 2.0. As already discussed in section 2.4.3, these anomalies occur in sites far from industrial areas and their origin could be related to natural processes. The same cannot be said for the three highest EF values in BAI (1.6, 2.5, 5.6), a wetland where effluents from Ravenna industrial areas historically have contaminated the lagoon. These results reveal that point source of Cr could persist in BAI.

The EF values for Zn and, in lesser extent, for Cu confirm the anomalous presence of both metals in PIO and BAI surface sediments (Fig. 2.11c-d), showing various degrees of enrichment and suggesting anthropogenic sources for these metals. Focusing on Cu (Fig. 2.11c), the two southern sites (BAI and PIO) and COM have EF values more dispersed than the other sites, with some significant enrichments. In particular, PIO sediments have median EF value greater than 1.5, while BAI and COM present respectively 4 out of 22 and 3 out 34 samples with EF greater than 1.5. In the remaining sites, Cu is present mainly at natural levels with EF values ranging from 0.5 to 1.3. Zinc enrichment is even stronger both in BAI and PIO (Fig. 2.11d), with median EF values equal to 1.6 and 2.5, respectively, and several very high values, one reaching EF=8. In addition, some EF values for Zn are greater than 1.5 also in BER, COM and MAN samples, and, therefore, an anthropogenic origin of Zn cannot be excluded also there. Since the latter sites are not interested by industrial activities, a plausible explanation for the Zn enrichments could be found in the water inputs draining neighbouring agricultural areas.

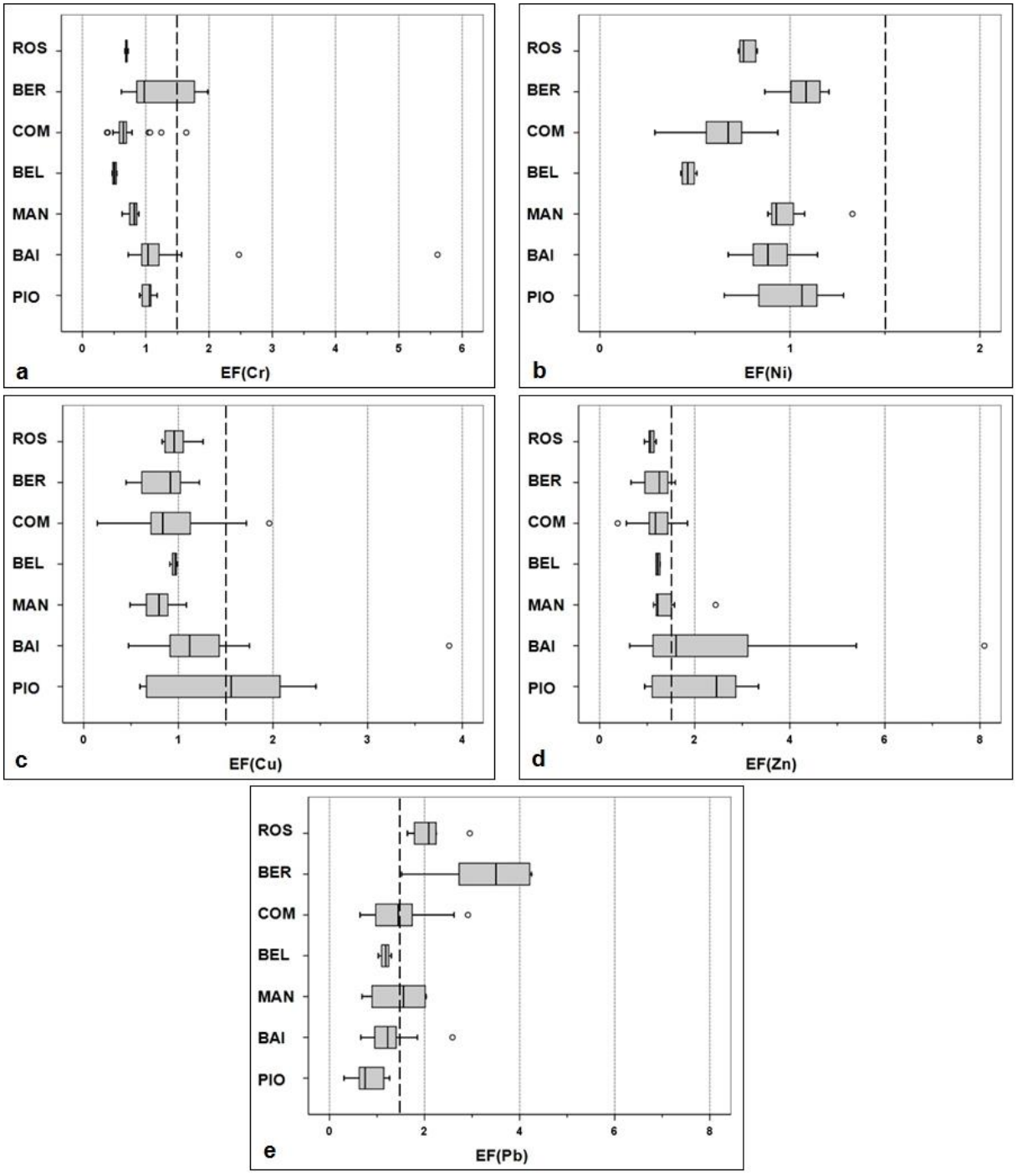

Figure 2.11 (a-b-c-d-e) - Box-plots of the EF values for Cr, Ni, Cu, Zn, and Pb in each sampling site. As a reference threshold for natural/anthropogenic origin of the metals, 1.5 (dashed line) is taken according to Zhang *et al.* (2007).

The box plots of EFs for Pb (Fig. 2.11e) reveal significant enrichments in surface sediments of all the investigated sites, except BEL and PIO. All samples from ROS and BER have EFs for Pb higher than 1.5, with median values equal to 2.1 and 3.5, respectively. Regarding COM, significant enrichments occur in 50% of the surface samples and two samples have EFs greater than 2.5, with the median value around 1.5. A similar pattern occurs in MAN where more than half of the samples shows remarkable enrichments. In BAI, the EFs higher than the threshold account for

about 25%. A general enrichment of Pb in the surface sediments throughout the study area caused by the leaded fuel used until the beginning of this century was expected. However, all sites but BEL have been devoting to hunting, especially exercised from fixed hides that could be thought as point source of kilograms of Pb every hunting season (all the year round in the past). Only considering this type of input, the high number of peaks (BAI), and the surprising high EF medians of BER, ROS, COM, and MAN, could be explained.

2.5. Conclusions

At the current state of knowledge, this study is the most comprehensive geochemical characterization of the surface sediments of the wetlands located along the North Adriatic coast from the town of Ravenna, northward to Chioggia (conventional limits of the historical Po deltaic system).

On the whole, the texture of the seven sites investigated, resulted as a mixing of carbonate and clays with Al content slightly higher than an average pelite. An organic matter fraction is also present and influent, although showing more variability within each site and throughout the area. To the clay fraction can be related a group of major elements, including Ti, Al, Fe and K and a number of trace elements (V, Co, Ce, Cu, Zn, Y). Lead and S are basically controlled by OM, even if the fate of Pb is more site-specific, in relation to the local abundance of this fraction in confront of clays. On the other hand, carbonate influence on the sediment composition resulted overall less important apart for a general dilution and a control on Sr distribution.

Taking advantage of extensive work done in past years it was possible to calculate a regional background for sediments of comparable depositional environment. Two background composition were calculated according to the provenance of the sediments, being different for the area north of the present day course of the Reno River, compared to the southern one. Respect to the background values derived Ba, Cr, Cu, Sr, Pb, Zn, V have been found more concentrated in some sites than the reference. Barium and V anomalies regard sites (ROS and BEL) far from industrial settlements, and are considered the result of local natural processes of enrichment. Strontium resulted clearly more concentrated in all sites, except BEL, likely as effect of the contribution of Sr-rich aragonite of biogenic origin, common in the sediments that was not possible to separate.

Sediments of Po Delta are known to be enriched in Cr (and Ni), due to the geological origin (e.g. Amorosi and Sammartino, 2007). In fact, our analysis outcomes are mainly in agreement with

the literature, and the Enrichment Factors suggested that all Ni origins from crustal matter, and substantially also Cr. However, some anomalous samples from BAI can be likely associated to industrial inputs, given the geographic position and can be of some concern. As a consequence of anthropogenic inputs related to the port and industrial areas of Ravenna, the two lagoons close the town revealed to be evidently polluted by Cu and Zn. These two metals seem to have a different behaviour depending on the origin, tending to associate to OM when the concentration exceeds natural background levels.

Regarding Pb, we expected surface sediment concentrations in average higher than the background as an effect of the exposure to leaded gasoline for many decades. In fact, all sites but BEL and PIO showed EF medians near or higher than the threshold for 'natural' levels. The influence of hunting in the lead input in most of these wetlands may explain some very high EF medians (BER, COM, ROS, MAN), and many peaks (BAI) we found.

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CHAPTER 3 - Geochemical characterization of surface sediments of some Adriatic coastal wetlands - Part II: aqua regia results

3.1 Abstract

Despite the fact that sediment characterization is crucial when assessing environmental quality of aquatic systems, geochemical information of many important wetlands is poor and quality standard levels for sediments are still lacking in regulation frameworks. As a further investigation of a previous study, we investigated the pseudo-total concentrations, after aqua regia digestion, of Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Hg, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Pd, Pt, Rb, S, Sb, Sc, Se, Sn, Sr, Th, Ti, Tl, U, V, Y, Zn, and Zr in sediments from 4 coastal wetlands belonging to the historical Po deltaic system (NE Italy) (Valli di Rosolina, Valle Bertuzzi, Valli di Comacchio, and Pialassa della Baiona).

Multivariate statistics were applied to study relationships among elements and pseudo-total concentrations were compared to various reference information provided by the Italian legislation, environmental guidelines or recent literature, and also real total contents, and background concentrations in order to determine the environmental relevance of the trace elements in the study area. According to the results of this study: 1) shellfishing is legal, and also illegally practiced, in the site the most affected by heavy metal pollution; 2) part of the Pb enrichments in most sites could be originated by lead shot in the sediment; 3) habitat and species conservation in these investigated Special Protected areas should consider geochemical aspects, especially with regard to the management of water levels.

3.2 Introduction

Environmental contamination arising from rapid urbanization, industrial processes, agricultural activities, and domestic waste has become a serious concern worldwide (Lacerda *et al.*, 1988; Li *et al.*, 2007; Tam and Wong, 2000). Tidal marshes are naturally geochemical endpoints, receiving trace elements originating from the weathering of geological formations and then transported along the hydrographic network (Teuchies *et al.*, 2013). Wetlands have long been recognized as an important sink also for anthropogenic inputs of trace elements, especially heavy metals (Bidhendi *et al.*, 2007; Klavins *et al.*, 2000; Rivail Da Silva *et al.*, 1996) and are involved in a variety of physical, chemical, and biological processes that include sedimentation,

setting, adsorption, precipitation, and induced changes in biogeochemical cycles by plants and bacteria (Chandra *et al.*, 2013; Jiao *et al.*, 2014; Xin *et al.*, 2014). Estuarine and coastal sediments are also affected by synergistic combinations of natural environmental changes and anthropogenic activities, and play a key role in controlling the fate of contaminants in the aquatic environment, acting as potential reservoirs of metals and pollutants that can be released to the water column under changing physical and chemical conditions (Karbassi *et al.*, 2007; Opuene *et al.*, 2008; Sundararajan and Natesan, 2010). Sediment pollution by heavy metals is a critical problem in aquatic environment because of their toxicity, persistence and bioaccumulation in the trophic webs (Chapman *et al.*, 1998; Islam and Tanaka, 2004; Machado *et al.*, 2002; Roussiez *et al.*, 2011). As a consequence, most European countries developed normative frameworks listing harmful trace elements. However, legislation normally only includes thresholds (screening values) for agricultural, industrialized and urban soils, and water (Carlon, 2007). Reports and guidelines generally do not provide rigid frameworks for assessment of sediment quality but do identify empirical procedures that can be used to distinguish among natural sedimentary conditions, anthropogenically disturbed sediments (i.e. contamination) and sediment conditions causing adverse effects (i.e., pollution). For this reason, a regulation fixing general quality standard levels for sediments is lacking to date. Nonetheless, in order to manage or translocate dredged port materials, specific guidelines have been drafted for marine and coastal sediments, indicating numerical reference values (e.g. APAT and ICRAM 2007 for Italy). Thus, sediment characterization and monitoring can play a crucial role when assessing impacts on environmental quality of an aquatic system or in any investigative monitoring the fate of pollutants (Brils 2004, 2008; Borghesi *et al.*, 2016), possibly by using the methods provided by legislation.

When working on solid matrices, both the analytical method used and the digestion procedures applied are of high importance. Analysis can be performed in order to evaluate the total element content or, as an alternative, follow partial extractions, with different meaning and purpose according to the reagents and the procedures applied. Possibly, sequential analyses are used in sequence to explore in depth the metal distribution in soils or sediments. Both approaches have their own advantages (Taraškevičius *et al.*, 2012). Geochemical exploration or mapping requires the determination of total concentration (Lis *et al.*, 1999) since these results can be directly linked to the geochemical composition of the substrate or of the sediment. XRF method is accepted in Europe as a valid technique for the determination of metal total contents (ISO/DIS 19258, 2005). On the other hand, the presence of heavy metals in sediments, determined by their

mobile/available concentrations rather their total content, better represents the risks for biota (Ferronato *et al.*, 2013; Salomons and Förstner, 1980). In general, *aqua regia* is accepted as a rapid, reproducible, inexpensive method, capable of quantifying the degree of anthropogenic discharges in trace metal pollution monitoring. In that case, the non lattice held fraction is of prime interest instead of the total metal concentration (Souza *et al.*, 2012). In Italy, *aqua regia* (3:1,v/v, HCl to HNO_3) is suggested as the digestion procedure for monitoring of As, Be, Co, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Sn, Tl, V, and Zn (Italian Legislative Decree 152/06 and subsequent amendments, 2006). Some further elements (Ag, B, Ba, Mo, Te, and U) are considered of environmental concern in other European countries (Carlon, 2007). It must be pointed out that Italian Law determined screening values (SVs) only for soils and water, taking into account only the human health protection concept, omitting any consideration of ecological risk (Carlon, 2007).

As a first step of a comprehensive environmental geochemical study of seven coastal wetlands belonging to the historical Po deltaic system (North Eastern Italy), Migani *et al.*, (2015) determined the total contents of the surface sediments and evaluated the influence of anthropogenic activities on the metal levels. As a second step of the study, pseudo-total concentrations (*aqua regia* extraction) are presented and discussed in this work. The main aims are to: (1) improve the trace element dataset referring to 4 North Adriatic important coastal wetlands forming part of the historical Po delta system, adding data on 20 elements (some of them of environmental concern and near completely unexplored so far in this area): Ag, As, Au, Be, Bi, B, Cd, Cs, Ga, Hg, Li, Mo, Pd, Pt, Sb, Sc, Se, Sn, Tl, and U; (2) understand the relationships between these trace elements and the others, applying multivariate statistics, such as cluster analysis (CA) and principal component analysis (PCA); (3) provide new insights on the environmental relevance of the trace elements in the study area (potential threat to biodiversity and also to human health), comparing pseudo-total concentration to various reference parameters provided by legislation or literature, real total contents of the same samples and background concentrations.

The results of this geochemical study, in its entirety, may have implications for the regional and municipal management for the investigated sites, and are suitable to be used for comparison with future sediment quality data.

3.3 Materials and methods

3.3.1 Study area and sampling sites

The Po delta extends to the easternmost part of the Po River Plain, the largest alluvial plain in Italy, which is characterized by heavy industrialization, extensive agricultural practices, diffuse animal husbandries and high density population. For detailed information on the origin and geomorphological evolution of the study area refer to Migani *et al.*, (2015).

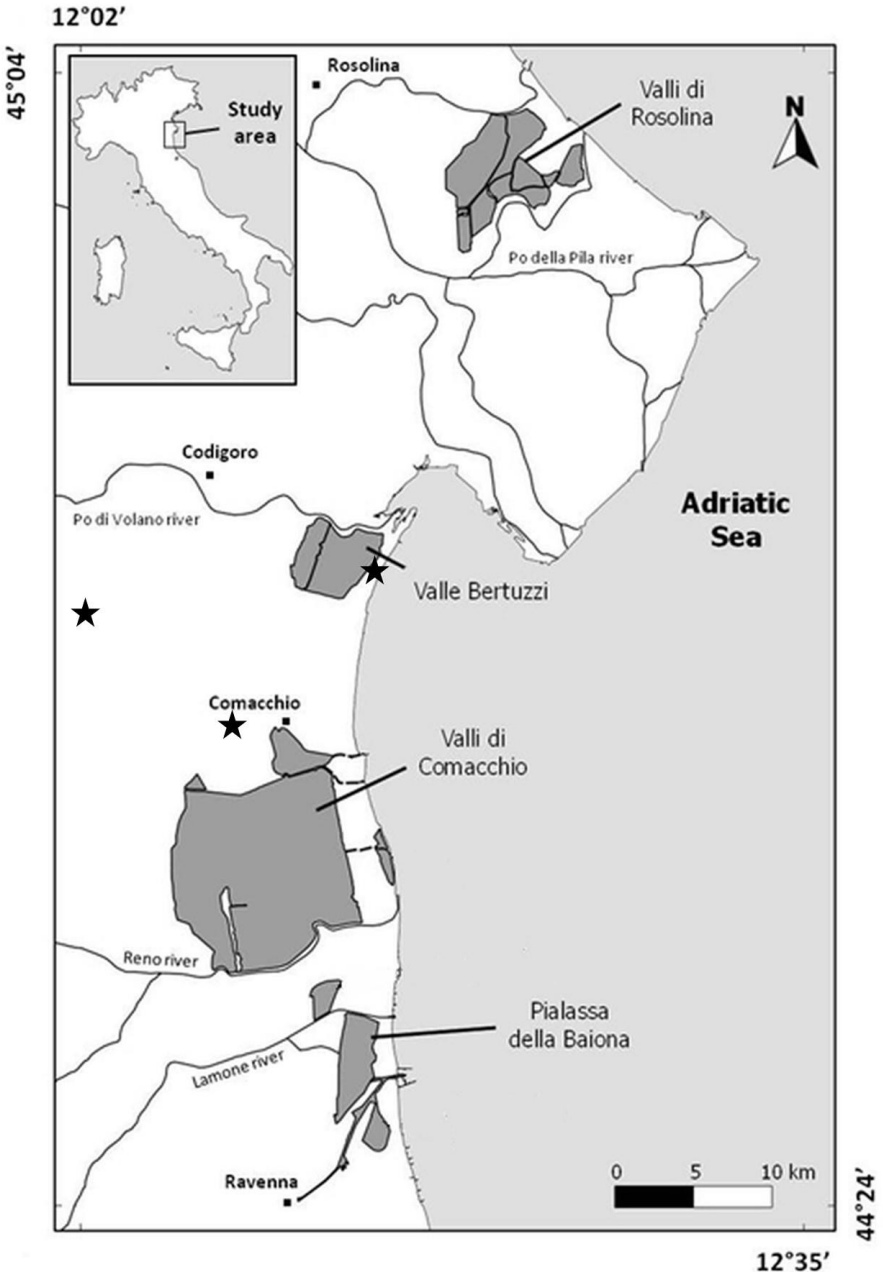

Figure 3.1 – Geographic location and identification of the four studied coastal lagoons along the north Adriatic coastline (dark grey areas) and the three locations where deep cores were drilled for background levels (black stars).

All the four investigated wetlands are part of the historical Po deltaic system along the stretch of the northern Adriatic coastline between the towns of Ravenna and Chioggia (Bondesan *et al.*, 1995). The sites are: "Valli di Rosolina" (ROS), "Valle Bertuzzi" (BER), "Valli di Comacchio" (COM), and "Pialassa della Baiona" (BAI) (Fig. 3.1). At present, they are all included in the Emilia Romagna Po Delta Regional Park, except ROS that is part of the Veneto Po Delta Regional Park. The same wetlands are also included in the European Natura 2000 network, under the European 09/147/EC and 92/43/EEC Directives, as Special Protected Areas (SPA) and/or Special Areas of Conservation (SAC).

The territory surrounding these brackish wetlands along the North Adriatic coast is affected by a considerable touristic pressure and most wetlands are subjected to commercial fishing and shellfishing and used for many recreational activities, such as hunting and fishing. In addition, all main coastal wetlands and lagoons receive effluents from agriculture at different extent and depending on seasonal and climatic conditions. In the past, more than at present, BAI was affected by considerable urban and especially industrial pollution (Fabbri *et al.*, 2001).

The main characteristics of the sampling sites and the number of samples collected are summarized in Table 3.1.

Table 3.1 – The four study sites ordered from north to south, including some of their geomorphological and management characteristics, and the number of samples collected from (n).

SITE	ID. SITE	EXTENSION (km^2)	TYPE of WETLAND	SEA CONN.	RIVER INPUT	PRESENT USE	SAMPLING	n
Valli di Rosolina	ROS	13.4	brackish lagoon	yes	absent	extensive fishing, regulated hunting	2012	6
Valle Bertuzzi	BER	19.2	brackish lagoon	no	Po di Volano	extensive fishing, regulated hunting	2012	7
Valli di Comacchio	COM	112.3	brackish lagoon	yes	Reno	extensive fishing, regulated hunting	2012	9
Pialassa della Baiona	BAI	11.5	brackish intertidal lagoon	yes	Lamone	pastime fishing, regulated hunting	2007-2008	7

Migani *et al.*, (2015) discussed the total concentrations of major (Al, Ca, Fe, K, Mg, Mn, Na, P, Si, Ti) and trace elements (Ba, Ce, Co, Cr, Cu, La, Nb, Ni, Pb, Rb, S, Sr, V, Y, Zn, Zr, and S) obtained by X-ray Fluorescence Spectrometry (XRF) in these and three more wetlands in the area. The influence of anthropogenic activities on the metal levels in the sediments was assessed by using the Enrichment Factor (EF) (Zhang and Liu, 2002; Zhang *et al.*, 2007). The assessment of the anthropogenic metal pollution was possible thanks to the availability of background values (Amorosi *et al.*, 2002, 2007, and 2008; Curzi *et al.*, 2006; Dinelli *et al.*, 2007, 2012; Dinelli, unpublished data). Also for this study, background levels (BKG) were calculated on the element abundances in Holocene lagoon deposits sampled from six deep cores drilled in the Po river coastal plain (Fig. 3.1), by using the same analytical procedures adopted for surface samples.

3.3.2 Analytical procedures

3.3.2.1 Sediment sampling and preparation

A total of 29 samples of surface sediments were collected from the four wetlands (Tab. 3.1). Where the depth of water was lower than 60-70 cm, sampling was carried out using a small plastic shovel, whereas a small grab sampler was used in deeper waters. Sediment aliquots were washed with distilled water, then centrifuged to eliminate any excess of salt, removing also coarse remains of animals and plants. After that, samples were dried at 60°C and homogenized by grinding in an agate mortar, for further investigation. In addition, a small number of borehole samples, in part already published (Amorosi *et al.*, 2002), were submitted for analysis.

3.3.2.2 Analytical procedures

The concentrations of 53 chemical elements (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hf, Hg, In, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Pd, Pt, Rb, Re, S, Sb, Sc, Se, Sn, Sr, Ta, Te, Th, Ti, Tl, U, V, W, Y, Zn, and Zr) were determined by Inductively coupled plasma mass spectrometry (ICP-MS) at the AcmeLabs of Vancouver (Canada). Samples were digested with a modified *aqua regia* solution of equal parts concentrated HCl, HNO_3 and DI-H_2O for one hour in a heating block of hot water bath. Each sample was made up to volume with dilute HCl. To evaluate the analysis quality, an Internal Reference Material (IRM), named DS8, with a composition similar to our sediment samples, was used. Among the analysed elements Ge, Hf, In, Re, Ta, Te and W were below the limit of detection in some or all samples, so they were not taken into account in the statistical analysis of this work. Palladium and Pt were detectable only in sediment from one

sampling site (BAI) and, therefore, they were presented in general results. The results (see table A in Supplementary materials) are in accordance to the expected values for all 46 elements.

Through this analysis, we obtain a pseudo-total concentration of the element, as the *aqua regia* dissolves only a part of the silicates, carbonates, clay minerals and organic matter, thus to integrate the results we considered also some results of the total analyses already published (Migani *et al.*, 2015) (for details see the analytical methods). In particular, Al_2O_3, CaO, Fe_2O_3, SiO_2, H_2O_{hum}, OM, H_2O_{clay} and CO_2, but also the total concentration of selected metals should be the subject of some consideration about their geochemistry.

3.3.2.3 Statistical analysis

Descriptive data analysis (mean, median, minimum, maximum) was used to summarize the variability of data among the investigated sites.

A multivariate statistic approach was used to perform a cluster analysis (CA) and a principal component analysis (PCA, R- and Q-modes) on all the variables considered, in order to identify groups of variables that are correlated and to assist the interpretation of the geochemical data (Reimann *et al.*, 2008; Templ *et al.*, 2008). The CA was performed using the between-groups linkage based on Pearson correlation coefficients. PCA was performed on z-score values of the concentrations of the elements and only the eigenvalues higher than 1 (Kaiser Criterion) and giving a cumulative variance above 89% were retained. The principal components were then rotated using the Varimax rotation with Kaiser normalization.

Statistical analyses (descriptive statistics, CA, and PCA) and graphs were carried out using SPSS 14.0 and GCDkit 2.3 software (Janoušek *et al.*, 2006).

3.4. Results and discussion

3.4.1 Pseudo-total contents in the sediments

Summary statistics, including arithmetic mean (M), median (MED), minimum and maximum (MIN, MAX) and number of samples (n), subdivided according to sampling site, is presented in Table 3.2.

Table 3.2 – Oxides (XRF), volatile components (thermal analysis) and element results (ICP-MS). For each site and background, arithmetic mean (M), median (MED), minimum and maximum (MIN, MAX) and number of samples (n), are reported. For some elements of environmental concern, the chemical basic level (BCL) and the chemical limit level are also reported, as indicated in the national guidelines for the marine sediment handling (APAT-ICRAM, 2007).

	ROS (n=6)				BER (n=7)				COM (n=9)			
	M	MED	MIN	MAX	M	MED	MIN	MAX	M	MED	MIN	MAX
SiO_2 (wt%)	39.08	40.16	31.77	41.98	42.35	42.93	30.99	54.66	47.23	43.95	38.53	58.84
Al_2O_3	12.40	12.93	9.93	13.32	10.49	10.39	9.66	11.71	12.73	12.84	11.18	13.37
Fe_2O_3	4.51	4.72	3.48	4.91	3.77	3.78	3.18	4.24	4.12	4.32	3.39	4.75
CaO	11.99	11.44	9.15	18.02	11.30	11.36	9.03	13.33	8.45	8.20	5.31	14.02
H_2O hum	2.11	1.81	1.59	2.93	2.17	1.76	0.45	3.79	2.22	2.31	0.36	5.58
OM	6.61	5.82	4.68	10.04	9.18	8.10	2.89	15.67	6.77	6.99	2.04	12.18
H_2O clay	2.96	2.89	2.24	3.94	2.70	2.34	1.09	4.66	2.47	2.85	1.01	3.88
CO_2	6.41	6.24	3.89	10.23	4.25	4.12	3.05	5.86	2.70	2.71	1.36	4.52
Ag (mg/kg)	0.076	0.077	0.065	0.083	0.061	0.057	0.031	0.097	0.071	0.088	0.022	0.103
Al	14283	15375	10700	16250	9657	8400	7100	12700	11861	12800	6400	16800
As	8.00	7.15	5.60	14.30	4.72	4.50	3.40	6.20	4.61	4.90	2.60	6.30
Au	0.003	0.002	0.002	0.004	0.002	0.002	0.000	0.003	0.003	0.003	0.001	0.004
B	45.25	38.50	26.50	84.00	54.29	50.00	21.00	87.00	39.50	42.00	20.00	52.00
Ba	96.16	98.80	49.00	138.30	51.66	58.00	25.70	67.60	54.04	47.20	36.40	84.80
Be	0.60	0.70	0.30	0.70	0.43	0.40	0.20	0.60	0.57	0.60	0.30	0.80
Bi	0.34	0.36	0.24	0.41	0.23	0.19	0.11	0.36	0.28	0.33	0.10	0.40
Ca	88775	85275	63900	135500	68507	68500	45200	92950	52789	52789	52789	52789
Cd	0.24	0.24	0.18	0.33	0.31	0.27	0.16	0.51	0.21	0.22	0.07	0.37
Ce	18.42	19.10	15.50	19.95	12.00	12.30	10.20	13.00	15.09	15.70	10.50	18.40
Co	15.06	15.95	11.20	16.55	11.74	9.80	8.20	15.60	10.78	12.05	6.00	14.60
Cr	77.96	80.08	57.30	91.10	137.15	125.30	55.20	214.50	107.11	89.40	46.50	238.85
Cs	1.36	1.33	1.05	1.74	1.04	0.88	0.76	1.50	1.11	1.14	0.73	1.52
Cu	34.30	35.81	27.32	40.53	24.49	21.47	12.56	35.45	29.10	32.54	10.37	41.93
Fe	21958	23350	14800	24450	14671	13100	11600	18800	17450	20100	8900	23600
Ga	4.03	4.35	2.80	4.75	2.81	2.40	2.00	3.80	3.56	4.00	1.90	5.00
Hg	0.060	0.060	0.044	0.076	0.054	0.067	0.018	0.086	0.055	0.050	0.027	0.085
K	3250	3450	2500	3700	2343	1800	1200	3600	3217	3550	1400	4900
La	8.64	8.85	7.70	9.40	5.75	6.00	5.10	6.15	7.08	7.50	5.10	8.50
Li	28.39	30.08	21.00	31.90	18.98	16.10	12.30	25.55	24.82	27.10	13.70	35.50
Mg	15117	15050	14600	15850	11814	9900	8600	15600	11056	12100	5600	15400
Mn	603.83	610.50	512.00	682.00	586.71	448.00	376.00	872.00	392.67	411.00	246.00	532.00
Mo	4.08	4.08	3.10	5.20	6.57	6.29	3.44	8.92	3.71	3.75	2.35	5.29
Na	8764	9085	5695	10790	9776	6880	3640	16100	12118	13940	3720	23440
Nb	0.41	0.42	0.36	0.46	0.43	0.43	0.32	0.51	0.35	0.34	0.24	0.47
Ni	86.13	92.83	59.40	98.30	100.58	100.10	82.40	126.10	77.22	68.70	47.30	122.15
P	406	388	350	500	480	470	400	570	417	440	295	520
Pb	25.14	25.45	19.90	27.45	20.16	21.70	12.80	28.50	27.16	28.20	14.10	37.20
Pd	-	-	BDL	BDL	-	-	BDL	BDL	-	-	BDL	BDL
Pt	-	-	BDL	BDL	-	-	BDL	BDL	-	-	BDL	BDL
Rb	19.98	21.55	13.90	22.40	15.23	12.80	9.80	22.00	20.10	22.20	11.10	26.20
S	9192	9975	6400	10600	7593	7100	3600	11300	7144	8300	2500	10900
Sb	0.55	0.48	0.40	0.77	0.41	0.35	0.25	0.57	0.34	0.36	0.22	0.42
Sc	3.59	3.83	2.40	4.20	2.23	2.00	1.70	2.90	2.97	3.25	1.60	4.00
Se	0.6	0.6	0.3	1.0	0.9	1.0	0.4	1.4	0.7	0.7	0.1	1.2
Sn	1.47	1.40	1.20	2.00	1.96	1.50	0.80	5.00	1.79	1.60	0.70	3.50
Sr	399.96	375.40	314.50	617.30	325.58	347.30	190.60	472.55	356.59	324.30	193.10	679.20
Th	4.78	5.00	3.30	5.55	3.00	3.00	2.50	3.50	4.07	4.20	2.90	5.00
Ti	82	80	50	110	101	85	50	190	73	70	50	100
Tl	0.18	0.18	0.17	0.20	0.15	0.14	0.09	0.22	0.17	0.20	0.08	0.24
U	2.37	2.40	1.75	2.80	2.37	2.40	1.30	3.30	1.66	1.80	0.90	2.20
V	33.42	33.75	28.00	37.00	25.57	21.00	18.00	34.00	26.94	29.50	14.00	36.00
Y	9.31	9.47	7.78	10.14	6.31	6.08	5.03	7.43	7.82	8.16	4.90	9.80
Zn	71.43	75.75	50.50	85.30	62.22	50.80	34.60	94.45	66.92	74.70	32.00	93.30
Zr	1.18	1.25	0.80	1.40	0.64	0.50	0.40	0.95	0.87	0.90	0.40	1.30

Table 3.2 (continued)

	BAI (n=7)				BACKGROUND (n=6)				NATIONAL GUIDELINES	
	M	MED	MIN	MAX	M	MED	MIN	MAX	CBL	CLL
SiO_2 (wt%)	42.16	42.57	37.91	44.82	47.96	47.32	43.43	55.53		
Al_2O_3	12.95	13.19	11.91	13.40	15.03	14.44	13.56	18.23		
Fe_2O_3	5.32	5.32	4.89	5.61	5.82	5.63	5.27	6.88		
CaO	11.34	11.00	9.29	14.36	8.12	8.83	0.81	11.41		
H_2O hum	2.91	2.68	2.09	3.99	-	-	-	-		
OM	2.14	2.01	1.10	3.41	-	-	-	-		
H_2O clay	5.28	5.84	2.31	6.53	-	-	-	-		
CO_2	9.61	9.12	7.43	12.30	-	-	-	-		
Ag (mg/kg)	0.317	0.268	0.062	0.991	0.089	0.093	0.077	0.099		
Al	13257	13900	11000	14100	17933	15900	14400	25600		
As	6.71	6.70	5.90	7.80	7.42	6.50	5.40	10.60	25	32
Au	0.006	0.005	0.001	0.016	0.002	0.002	0.001	0.005		
B	28.00	26.00	22.00	38.00	22.67	19.50	13.00	42.00		
Ba	43.61	41.80	36.50	61.90	65.28	44.80	29.10	141.20		
Be	0.61	0.60	0.40	0.80	0.77	0.70	0.60	1.10		
Bi	0.37	0.35	0.26	0.60	0.38	0.36	0.33	0.46		
Ca	79829	74500	61000	112700	54083	59150	3300	76700		
Cd	0.35	0.33	0.11	0.68	0.14	0.14	0.09	0.23	0.35	0.8
Ce	18.14	17.80	16.80	20.10	24.35	22.05	18.80	33.30		
Co	10.79	10.70	9.90	11.70	17.63	15.30	14.50	25.50		
Cr	56.20	57.50	47.00	70.60	90.48	78.00	69.60	131.50	100	360
Cs	1.26	1.16	1.08	1.57	1.50	1.52	1.23	1.67		
Cu	47.19	49.10	22.43	84.08	37.46	34.31	32.24	53.31	40	52
Fe	22943	23000	20000	25400	26950	25200	23800	34100		
Ga	4.16	4.20	3.90	4.40	5.18	4.70	4.10	7.20		
Hg	5.030	1.893	0.102	25.684	1.799	0.839	0.290	5.469	0.4	0.8
K	3400	3500	2800	3900	3433	3200	3100	4000		
La	8.03	8.00	6.90	9.50	11.55	10.35	8.60	16.20		
Li	27.74	28.00	24.90	30.60	32.40	31.75	27.60	39.40		
Mg	13971	13700	12200	15800	15717	14350	13400	22700		
Mn	546.43	531.00	511.00	644.00	716.67	706.50	253.00	985.00		
Mo	2.33	2.61	0.54	3.34	0.75	0.79	0.50	0.89		
Na	8326	8510	4230	11780	7092	7815	2900	10470		
Nb	0.31	0.29	0.25	0.37	0.32	0.35	0.16	0.44		
Ni	51.09	50.90	46.30	56.50	99.58	88.35	76.30	147.90	70	75
P	699	610	560	1090	425	410	400	470		
Pb	21.41	22.14	11.99	27.92	18.61	18.25	14.57	23.32	40	70
Pd	14.14	10.00	BDL	46	BDL	BDL	BDL	BDL		
Pt	8.14	4.00	BDL	37	BDL	BDL	BDL	BDL		
Rb	23.93	22.40	20.80	29.80	23.85	23.10	21.00	29.00		
S	5800	6000	3400	7200	3067	850	200	8200		
Sb	0.30	0.29	0.19	0.51	0.24	0.22	0.17	0.35		
Sc	3.59	3.60	3.20	4.10	4.80	4.40	4.10	6.40		
Se	0.7	0.7	0.5	1.1	0.30	0.35	0.10	0.40		
Sn	1.71	1.50	0.80	3.20	0.83	0.85	0.60	1.00		
Sr	271.63	274.40	218.00	353.00	158.60	168.80	38.20	233.00		
Th	3.96	4.00	3.50	4.70	6.95	6.45	5.90	8.60		
Ti	84	80	50	150	115.00	125	50	160		
Tl	0.21	0.20	0.14	0.27	0.18	0.17	0.15	0.27		
U	1.24	1.20	1.10	1.50	0.85	0.80	0.70	1.10		
V	31.43	32.00	26.00	35.00	36.83	34.00	31.00	50.00		
Y	9.64	9.63	8.86	10.58	11.36	10.51	9.94	14.29		
Zn	140.73	129.80	58.20	306.60	76.58	70.75	66.70	111.10	100	170
Zr	1.21	1.10	1.00	1.90	1.77	1.75	1.50	2.10		

For all samples, the results on the volatile content are also included (already published in Migani *et al.*, 2015), derived from the interpretation of thermogravimetric (TG), derivative thermogravimetric (DTG) and differential thermal analysis (DTA). These include "H_2O hum" which reflects the humidity of the sample, "OM" as an indication of the organic matter content, "H_2O clay" reflecting the water adsorbed in clay minerals, and "CO_2" mostly deriving from decomposition of aragonite and calcite (controlled by the abundance of carbonate minerals, in particular biogenic carbonates). In addition, some data useful for the definition of the bulk composition of the sediment (SiO_2, Al_2O_3, Fe_2O_3, and CaO) are also included in Table 3.2.

Major element oxides, obtained by XRF, were already summarized in ternary plots and discussed in Migani *et al.*, (2015). It is useful to recall here that more than 75% of our samples were found to be consistent with the background regarding the main composition (carbonatic/Al-rich clayey sediments with few exceptions: some samples from COM and BAI more rich in carbonates possibly of biogenic origin, and some from COM and BER with more SiO_2, indicating a coarser grain size).

Observing Table 3.2, as expected, BER and COM record high concentrations of Cr (ranges: 55-215 mg/kg and 47-239 mg/kg, respectively) and Ni (82-126 mg/kg; 47-122 mg/kg), and comparable with BKG (Cr: 70-132 mg/kg; Ni: 76-148 mg/kg). ROS reports higher contents of Ba (49-138 mg/kg) and Co (11-17 mg/kg), fully explained by the deep core contents (Ba: 29-141 mg/kg; Co: 14.5-25.5 mg/kg). As expected in coastal wetlands, the S concentration is much higher than the BKG (200-8200 mg/kg, median 800 mg/kg) in all sites, with ROS showing the highest median (9975 mg/kg) and BAI the lowest (6000 mg/kg). Evidently, BAI pseudo-total contents are rather different for a number of metals of environmental concern. In this site, medians of Ag (0.3 mg/kg), Au (0.006 mg/kg), Cu (47 mg/kg), Hg (5 mg/kg), and Zn (140 mg/kg) are high if compared to the other sites and BKG (Tab. 2). Interestingly, Pd and Pt, which are detectable in very few cases in our database, give values (and peaks) in BAI (up to 46 µg/kg for Pd and 37 µg/kg for Pt). In addition, an appreciable enrichment of Cd (up to 0.68 mg/kg) and the highest levels of P (up to 1090 mg/kg) are recorded in this site. Even at first glance, it would seem that there is an important anthropogenic influence in the distribution and mobility of metals in BAI.

In order to obtain a better indication of the possible presence of trace metals at contaminant levels, several tools are proposed and discussed here: (a) the statistical parameters derived for Italian agricultural soils according to Cicchella *et al.*, (2015), (b) for those elements analyzed both with *aqua regia* and XRF methods, the mean degree of extractability (DEM)

calculated as the ratio between the two results (Migani *et al.*, 2015), and (c) multivariate statistics (CA and PCA).

3.4.2 Comparison with Italian background/baseline values

The background/baseline values recently published by Cicchella *et al.*, (2015) for agricultural soils (here abbreviated as ASVs) for a large number of elements can give an evaluation of the natural variation, with the advantage of being directly comparable with our data, obtained applying the same digestion procedure in the same laboratory.

Table 3.3 - Synoptic representation of the mean pseudo-total concentrations of trace elements calculated for each site in respect to their best approximation to the 10th, 25th, 50th, 75th, and 90th quantiles (Q10, Q25, Q50, Q75, Q90) as reported by Cicchella *et al.*, (2015) for agricultural soils in Italy.

	ROS	BER	COM	BAI		ROS	BER	COM	BAI
Ag				**	P				
Al					Rb				
As					S	> max	> max	> max	**
Au					Sb				
B	> max	> max	> max	> max	Sc				
Ba					Se		**	*	*
Be					Sn				
Bi					Sr				
Ca					Th				
Cd					Ti				
Ce					Tl				
Co					U				
Cr		*			V				
Cs					Y				
Cu					Zn				**
Fe					Zr				
Ga									
Hg				**m					
K									
La									
Li									
Mg									
Mn									
Mo	> max	> max	> max	**					
Na	> max	**	> max	> max					
Nb									
Ni		*							
Pb									

	Q10
	Q25
	Q50
	Q75
	Q90
*	Q95
**	Q98
> max	> Q100

Being aware that between sediment and agricultural soil data differences could exist in terms of distribution for certain elements (e.g. those involved in biogeochemical processes, those enriched in sea water, etc.), in Table 3.3 we also reported available information about the local

background, and, for some elements of particular environmental concern, the values reported in the guidelines for marine sediments (APAT-ICRAM, 2007).

Table 3.3 provides a synoptic comparison between the mean pseudo-total concentrations of the trace elements calculated for each site in this study and percentiles reported by Cicchella *et al.*, (2015). Depending on the approximated quantile (Q10, Q25, Q50, Q75, Q90), the cells correspondent to each site and metal are differently coloured (darker for more enriched samples). Medium grey corresponds to Q50, while light grey and dark grey to Q25 and Q75, respectively. White cells represent samples comparable to Q10 and the black ones refer to pseudo-totals reaching or higher than Q90. No samples in our dataset resulted lower than Q10. A single star and a double star respectively mark the samples correspondent to the 95^{th} and 98^{th} quantiles (Q95 and Q98), while ">Max" is superimposed in cells related to sites where concentration exceeds the maximum of the reference values.

In the investigated sediments, 21 elements out 44 are comparable with ASVs or even less abundant, while more than the 50% of the analyzed trace elements are enriched if compared to the reference concentrations.

For **Al, As, Ba, Be, Ce, Cs, Fe, Ga, La, Mn, Nb, P, Sc, Th, V, Y, and Zr** no sites report means higher than Q50 and at least two sites are lower than Q50 (Tab. 3.3). In other words, mean concentrations of this large group of elements fall in general in the lower part of the ASVs range. Most of these elements, including REEs (Ce, La, Sc, Y), are normally contained in minerals, such as feldspar and silicates of mafic or felsic rocks, difficult to be dissolved with *aqua regia*. Italian ASVs include soils form Naples and Rome areas, developed on pyroclastic and volcanic rocks, strongly enriched in Al, Be, K, La, Pb, Sn, Th, Tl, U, and V, influencing their overall distribution (Cicchella *et al.*, 2015). Actually, relative higher contents of Ba (median 99 mg/kg), Cs (1.3 mg/kg), Fe (23,350 mg/kg) and La (8.9 mg/kg), as well as Mn (611 mg/kg), which is normally present in dolomite rocks, in ROS (Tab. 2) may be explained by a possible influence of materials from the oriental Alpine area on the sediments of this wetland system. The case of As is particular. A sample from ROS reached a peak of 14.3 mg/kg, though this metalloid of environmental concern is overall low in concentration in our dataset with respect to ASVs, the Italian screening values for soils (20 mg/kg) (Italian Legislative Decree 152/06, 2006), and the baseline chemical level for marine sediments (25 mg/kg) (APAT-ICRAM, 2007).

Lead, Rb, Ti, and Tl means lie around Q50 (22.1 mg/kg, 119 mg/kg, 0.18 mg/kg, respectively) for most or all sites. Rubidium and Ti are not reactive elements in the sediment,

while Pb and Tl are known for their toxicity. During the first phase of this study, Pb was found to be very high locally (greater than 200 mg/kg, Migani *et al.*, 2015). Similarly to the group of elements discussed above, ASVs of Pb and Tl are affected by enrichments in soils sampled in volcanic areas (Cicchella *et al.*, 2015). COM Pb median is comparable to Q75 for pseudo-total contents. The same occurs for the 35% of the samples throughout the whole study area. We do not know the total content of Tl in our samples, but the amount of Tl extracted by *aqua regia* is comparable to the local background (Tab. 3.2).

High concentrations of **B and Na** in our coastal sediment samples in comparison to agricultural samples (Cicchella *et al.*, 2015) were not unexpected (in all sites, both elements are >Max, being 26.9 and 7,237 mg/kg, respectively), because the coastal wetlands and lagoons of this study are subjected to influxes of sea water.

The contents of **Ca, and especially Sr** are much higher than ASVs (Q75, about 80,000 mg/kg, and Q90, 363 mg/kg, respectively). This is the effect of the abundance of aragonite (and calcite) of biogenic origin in the investigated wetlands. **Sulphur** is >Max (5,915 mg/kg) in three sites, with the gradient ROS>BER>COM (Tab. 3.2), and is near Q98 (2,699 mg/kg) in BAI. Tidal salt marshes are normally rich in S (Chou and De Rosa, 2003) where it exists as a variety of species playing a key role, under anoxic conditions due to tidal seawater flooding, in many biogeochemical processes concerning toxic metals (Fabbri *et al.*, 2001; Portnoy, 1999). **Mercury** is on average higher than Q75 (0.057 mg/kg) in ROS, BER, and COM, but are completely off the scale in BAI. The lowest sample of BAI (0.1 mg/kg) is comparable to Q90 (0.085 mg/kg), while the others samples from BAI are much higher than the maximum ASV (0.59 mg/kg). Mercury in BAI also exceeds all quality thresholds for soils (1 mg/kg for non-industrial and 5 mg/kg for industrial) and marine sediments (0.4 mg/kg as baseline and 0.80 mg/kg as limit) (APAT-ICRAM, 2007; Italian Legislative Decree 152/06, 2006). On the one hand, part of this difference may be accounted for by the crucial role of elemental S, organic matter and bioturbation (abundant in wetland sediments and producing methyl-mercury) in Hg mobility in salt marsh sediments (Fabbri *et al.*, 2001; Ravichandran *et al.*, 1998, 1999). On the other hand, Hg accumulation in BAI appear as a non-natural phenomenon. **Tin** is also relatively high in content compared to ASVs, between Q50 (1.03 mg/kg) and Q75 (1.55 mg/kg). Aside from this, high values of Sn are widespread in the investigated area. Most or all samples also exceed the threshold (1 mg/kg) for non-industrial soils (Italian Legislative Decree 152/06, 2006), with means of 1.47 mg/kg ± 0.29 DS of ROS, 1.96 mg/Kg ± 1.45 DS of BER, 1.79 mg/kg ± 0.88 of COM, and 1.71 mg/kg ± 0.75 of BAI, and many peaks up to 3.20

mg/kg (BAI), 3.50 mg/kg (COM), 5.00 mg/kg (BER) (Tab. 3.2). Even though since 2014 (Gazzetta Ufficiale della Repubblica Italiana, 2014) the national thresholds for Sn refers to the "organotin compounds" instead of "tin", the pseudo-total contents of Sn are worthy of attention.

Both **Mo and U** tend to be complexed with fulvic and umic acids and concentrate in the presence of abundant organic matter (Salminen *et al.*, 2005). In this form, they may be easily extracted by *aqua regia.* Especially Mo is actually much higher than ASVs, with contents comparable to Q98 (2.79 g/kg) in BAI, and even higher than the upper limit (3.22 mg/kg) in ROS, BER, and COM. Uranium reaches Q75 (1,62 mg/kg) in ROS, BER, and COM. Pseudo-total contents higher than ASVs are recorded also for Ag (Q75, 0.068 mg/kg, in ROS; Q90, 0.095 mt/kg, in COM; Q98, 0.452 mg/kg, in BAI), Au (Q75, 0.003 mg/kg, in ROS and COM; Q90, 0.0044 mg/kg, in BAI), Cu (Q75, 45.4 mg/kg, in BAI), and Zn (Q75, 75.5 mg/kg, in ROS and COM; Q98, 133 mg/kg, in BAI). Anthropogenic contamination of **Cu and Zn** is well documented for BAI, especially in the southern ponds and channels of the lagoon (Donnini *et al.*, 2007; Matteucci *et al.*, 2005; Migani *et al.*, 2015). Recordings from these sites often exceed indications for marine sediment handling. Respectively, 4 and 6 samples from BAI exceed the baseline concentration levels for Cu and Zn (40 and 100 mg/kg) and one sample exceeds the limit (52 and 170 mg/kg) (APAT-ICRAM, 2007). **Silver and Au** origin were completely unexplored so far in all the investigated areas. In sediments, Ag enrichment is controlled by many factors such as pH, OM, as well as the source material, consisting mainly in sulphide minerals (Ure and Berrow, 1982). At typical environmental pH values, Ag will be adsorbed onto Fe hydroxides, in preference to Cu and Zn (Lottermoser *et al.*, 1999). Soluble complexes of Ag with S are favoured by incomplete oxidation of the sulphide (Webster and Mann, 1984). Silver can also be chelated by humic material (Kabata-Pendias and Pendias, 2001; Mango, 1999).

Enrichments of **Cr and Ni** (total contents) in the investigated sites were presented and discussed in detail in Migani *et al.*, (2015). In respect of ASVs, Cr and Ni seem to be rather mobile, but neither total nor pseudo-total contents of Cr are higher than quality thresholds (APAT-ICRAM 2007; Italian Legislative Decree 152/06, 2006). BAI shows relatively lower values (around Q75, Cr 47.3 and Ni 46.6 mg/kg, instead of Q90, Cr 89.9 and Ni 86.4 mg/mg, or more as in COM, BER, and ROS), because it is more influenced by sediments of Apenninic origin than the other sites (Migani *et al.*, 2015). The potential significant mobility of Cr and Ni found in the investigated wetlands is in line with the findings of Di Giuseppe *et al.*, (2014a) on soils just westwards of COM.

Magnesium and Co pseudo-total contents in ROS are considerably high if compared to ASVs (Q90, 22.7 mg/kg and Q75, 16.0 mg/kg, respectively for Mg and Co). Cobalt (together Cr and Ni) is indicative of mafic rocks as geogenic source, and together with Mg higher values in ROS, may indicate the Eastern Alpine contribution in the composition of these sediments.

Pseudo-total contents of **Se** (>Q90 in BER, COM, and BAI, Q90 in ROS) are considerably high in all the investigated sites. Selenium seems to be much more available in this area than in most of the other parts of Italy (Cicchella *et al.*, 2015). All sites record means comparable to Q90 (0.66 mg/kg). COM and BAI reach Q95 (0.70 mg/kg) and BER even Q98 (0.85 mg/kg). For the wetlands investigated in this study, no information was available on this element in surface sediments so far. Median values in each site are definitely higher than the background (Tab. 3.2). Selenium is mobile as anionic forms, and tends to concentrate in organic-rich sediments and be adsorbed on the surface of Fe and Mn hydroxides (Howard, 1977). It is an important essential element at low levels, but if up-taken in excess, it can also be toxic (Mertz, 1987).

Lastly, five elements (Bi, Cd, K, Li, and Sb) are comprised between Q50 and Q75 of ASVs: **Cd**, Q75 (0.34 mg/kg in BAI) and **Sb** (Q75, 0.55 mg/kg, in ROS) are of environmental concern and included in the list of harmful elements for Italian soils. Both metals will be discussed more in detail in section 3.4.6. **Bismuth** has low mobility under most environmental conditions and it is relatively rare in the Earth's crust (Burford *et al.*, 2011). However, it is apparently more abundant in ROS and BAI in respect of ASVs (Q75, 0.40 mg/kg) than in COM and especially BER (Tab. 3). Both K and Li are higher than ASVs in ROS, COM, and BAI (Q75, K 2999 mg/kg, Li 27.6 mg/kg).

3.4.3 Extractability by aqua regia compared to total content

With respect to real total contents, the pseudo-total metal concentration obtained after *aqua regia* digestion, suggests a different pollution hazard (Ferronato *et al.*, 2015). In order to have an indication of the potential hazard represented by certain toxic trace element in the study area, it could be useful to calculate the extractability degrees, which are reported in Table 3.4 for 28 elements.

Taraškevičius *et al*. (2013) provided an estimation of the values of the degree of extractability (DET) of many harmful elements listed in normative documents of various countries. In this study, soil samples from Europe were analyzed by a large number of different laboratories for both real total and *aqua regia* contents. The degree of extractability calculated on pseudo-total and total contents obtained in our study for Ba, Co, Cr, Cu, Mn, Mo, Ni, Pb, V (abbreviated DEMs), have been compared with DETs, which can be considered a good reference, taking into account

that the matrix and the specificity of soil composition (clay content, volatile compound, major element contents) can influence the degree of extractability (Taraškevičius *et al.*, 2013). In descending order, DETs were: Cu (91%), Mn (89%), Zn (89.5%), Ni (88%), Co (86%), Mo (82%), Pb (79%), V (61%), Cr (56%), and Ba (20.5%).

In this study, DEMs for these elements are: Cu (106%; 100-109%), Co (103%; 99-113%), Zn (80%; 75-89%), Ni (76%; 70-79%), Pb (63%; 33-91%), Mn (58%; 53-66%), Cr (53%; 46-61%), V (33%; 30-36%), Mo (31%; 20-46%), and Ba (20%; 17-28%).

Copper was expected to be highly dissolved by *aqua regia*. In all the study sites, Cu is even coincident with the correspondent total contents. Surprisingly, also pseudo-total contents of **Co** are practically identical to total contents, and therefore higher than DET. Despite this, it is comparable in all sites with the background values (Migani *et al.*, 2015 and Tab. 3.2 in this study). Divalent cobalt is bioaccessible when it is bound to humic and fulvic acids and inorganic colloids (McBride, 1994; Qian *et al.*, 1998), and the full extractability of Co *aqua regia* may suggests a possible good bioavailability of this essential metal for life. In according to Taraškevičius *et al.*, (2013), the extractability of **Zn** is very high in BAI (89%), while it seems slightly less extractable in ROS, BER, and COM (75-79%). On the contrary, **Ni** has less extractable degree than DET throughout the study area, but it is much more extractable than **Cr**, which in turn is slightly less extracted than the predicted DET. The case of **Pb** is unique, because in ROS, COM, and especially BER it seems less mobile in respect of DET (DEMs: 57% and 67%, and 35%, respectively), but a much higher extractability has been found in BAI (91%). As shown in Figure 3.2, *aqua regia* Pb data show a definitely lower variability than XRF, especially among BER samples.

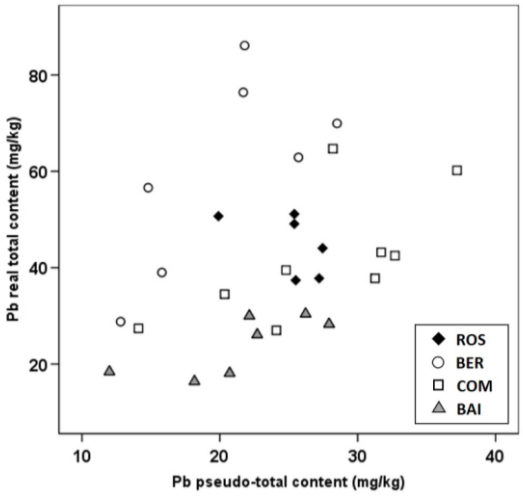

Figure 3.2 – Relationship between Pb pseudo-total and real total contents. No significant correlation resulted. The different colours and symbols indicate the belonging of each sample to its sampling site.

Manganese and **V** reveal limited mobility in the wetlands included in this study in relation to the respective DETs. Even more evident is the limited mobility of **Mo,** with an expected DET of 82%, but showing DEMs very low in BAI (20%) and not much higher in the other sites (30-46%). In BER, COM, and BAI, **Ba** shows DEM (17-20%) rather similar to DET, although it seems to be more extractable in ROS (28%).

Table 3.4 - Mean values of degree of extractability (DEM) of *aqua regia* contents to real totals for each sampling site.

	Degree of extractability (DEM)			
	ROS	BER	COM	BAI
Al	0.21	0.18	0.18	0.19
Ba	0.28	0.19	0.18	0.17
Ca	1.05	0.85	0.87	0.98
Ce	0.45	0.45	0.36	0.34
Co	1.00	1.02	0.99	1.13
Cr	0.52	0.61	0.53	0.46
Cu	1.00	1.04	1.09	1.09
Fe	0.68	0.56	0.59	0.62
K	0.19	0.17	0.18	0.17
La	0.35	0.33	0.29	0.37
Mg	0.57	0.48	0.46	0.58
Mn	0.66	0.59	0.53	0.58
Mo	0.33	0.46	0.30	0.20
Na	0.65	0.53	0.62	1.12
Nb	0.02	0.03	0.02	0.02
Ni	0.77	0.77	0.79	0.70
Pb	0.57	0.35	0.67	0.91
P	1.05	0.80	0.58	1.03
Rb	0.11	0.12	0.11	0.21
S	1.27	0.78	0.94	1.33
Sc	0.24	0.24	0.26	0.60
Sr	0.50	0.47	0.49	0.98
Th	0.38	0.21	0.28	0.52
Ti	0.03	0.04	0.02	0.03
V	0.36	0.34	0.30	0.34
Y	0.42	0.43	0.40	0.46
Zn	0.78	0.75	0.79	0.89
Zr	0.009	0.009	0.006	0.012

We analyzed both pseudo-total and real total contents of further elements, not reported in Taraskevicius *et al.*, (2013). In the investigated Po Delta sites, **Al** (DEMs: 18-21%), **K** (17-19%), **Nb** (2-3%), **Rb** (11-21%), **Ti** (2-4%), and **Zr** (1%) are very little dissolved by *aqua regia* digestion. Also **Ce** (34-45%), **La** (29-37%), **Y** (40-46%) are not much extracted. Likely, all these elements in sediments are preferably firmly bound in clays or immobilized in insoluble minerals. The same could be supposed for **Sc** and **Th**, but their degree of extractability in BAI is higher (60% and 52%, respectively). **Calcium** and **S** pseudo-total and total contents are highly extracted in all sites (Ca: 85-105%; S: 78-127%). In BER, Ca and S seems to be relatively slightly less extractable. **Sodium** and

P are more variable among sites (Na: 53-112%; P: 58-105%), with the highest extractability of Na in BAI, and of P in ROS and BAI. **Fe**, **Mg**, and **Sr** pseudo-total contents are about half of the correspondent total, with the exception of Sr in BAI (98%).

We could not calculate a degree of extractability for Ag, As, Au, B, Be, Bi, Cd, Cs, Ga, Hg, Li, Sb, Se, Sn, Tl, and U because data of these elements are available for this study only as a result of *aqua regia* method. According to Taraškevičius *et al.*, (2013), Cd and Hg may represent 95% of the total, As 80%, B, Be, Sb and Sn about 50%, while U only 35%.

3.4.4 Inter-element relationship – cluster analysis (CA)

An R-mode cluster analysis including pseudo-total contents of all elements, parameters from thermal analysis, and selected major oxides obtained by XRF (Migani *et al.*, 2015) was performed using the between-groups linkage and Pearson correlation coefficients, as suggested in literature for graphically highlighted correlations between variables (Facchinelli *et al.*, 2001).

Four clusters well connected in a wide group (Group A), and a rather isolated small group of a few elements, named Group B, have been defined (Fig. 3.3). Group A can be subdivided into:

Group A

Cluster 1 is composed by 3 sub-groups of elements that can be generally related to clay minerals, although with variable degree of correlation (Al, Fe, Li, Ga, Sc, K, Rb; Cs, Mg, V, Ce, La, Th, Y, Zr, Be). In turn, these sub-groups are correlated with Al_2O_3 and Fe_2O_3 obtained by XRF analysis (Migani *et al.*, 2015).

Cluster 2 includes P and some other elements (Ag, Au, Bi, Cd, Cu, Hg, Tl, and Zn) together with the percentage of the water incorporated in clayey minerals (H_2O_{min}) (equivalent to the weight loss by the sample at 550-650°C). These metals are also related to clays (Fig. 3.3), probably reflecting chemical interaction (adsorbing processes or simply the affinity for the fine fraction of the sediment), but kept distinct in a different cluster, possibly for the effect of markedly different spatial distribution. A main factor, independent from geochemical features, may influence the fate of Ag, Au, Bi, Cd, Cu, Hg, Tl, and Zn in the investigated area. Cluster 2 includes elements of anthropogenic origin such as Cu, Hg, and Zn (Migani *et al.*, 2015; Miserocchi *et al.*, 1993), and some metals almost unexplored in the study area (Ag, Au, Bi, Cd, Tl) that might be locally affected by contamination. A relation with the organic matter of anthropic origin may be supposed by the presence in this group of P.

Cluster 3 clearly accounts for elements mainly related to the fraction of the sediment strongly controlled by organic matter and the chemistry of S: Ba, Co, Mn, Mo, Nb, Pb, Se, Sn, and U. The presence in this cluster of H_2O_{hum} (the percentage of weight loss at 200°C) may also explain B and Na in this group. Niobium is little studied in sediments (Salminen *et al.*, 2005), but it seems that the rather negligible amount of Nb extractable by *aqua regia* in this study (Tab. 3.4) is influenced by OM in these wetlands, as suggested by Scheib *et al.*, (2012) for european soils.

Cluster 4 represent carbonates (CaO, Ca, Sr, and CO_2, corresponding to the weight loss 650-950°C). In this cluster are surprisingly also grouped As and Sb, although with weak correlation. The presence of both metalloids in cluster 4, known to have little relation with carbonates (Wilson *et al.*, 2010) is unclear. In fact, As and Sb are normally associated to hydrous Fe and Mn oxides, clays and organic matter rich in sulphides and phosphates (Filella *et al.*, 2002, 2003; Ure and Berrow 1982; Wilson *et al.*, 2010). However, the cluster distance of the pair Arsenic and Sb from the group of variables characterizing carbonates is rather high.

Group B only includes Cr, Ni, Ti and SiO_2. This likely indicates that the pseudo-total concentration of these metals in the sediment fraction is mainly controlled by silicates rather than by any other geochemical parameter, including clays. Therefore, it can be supposed that there is a major control of coarse silicatic fraction on the mobility of Cr, Ni, and Ti.

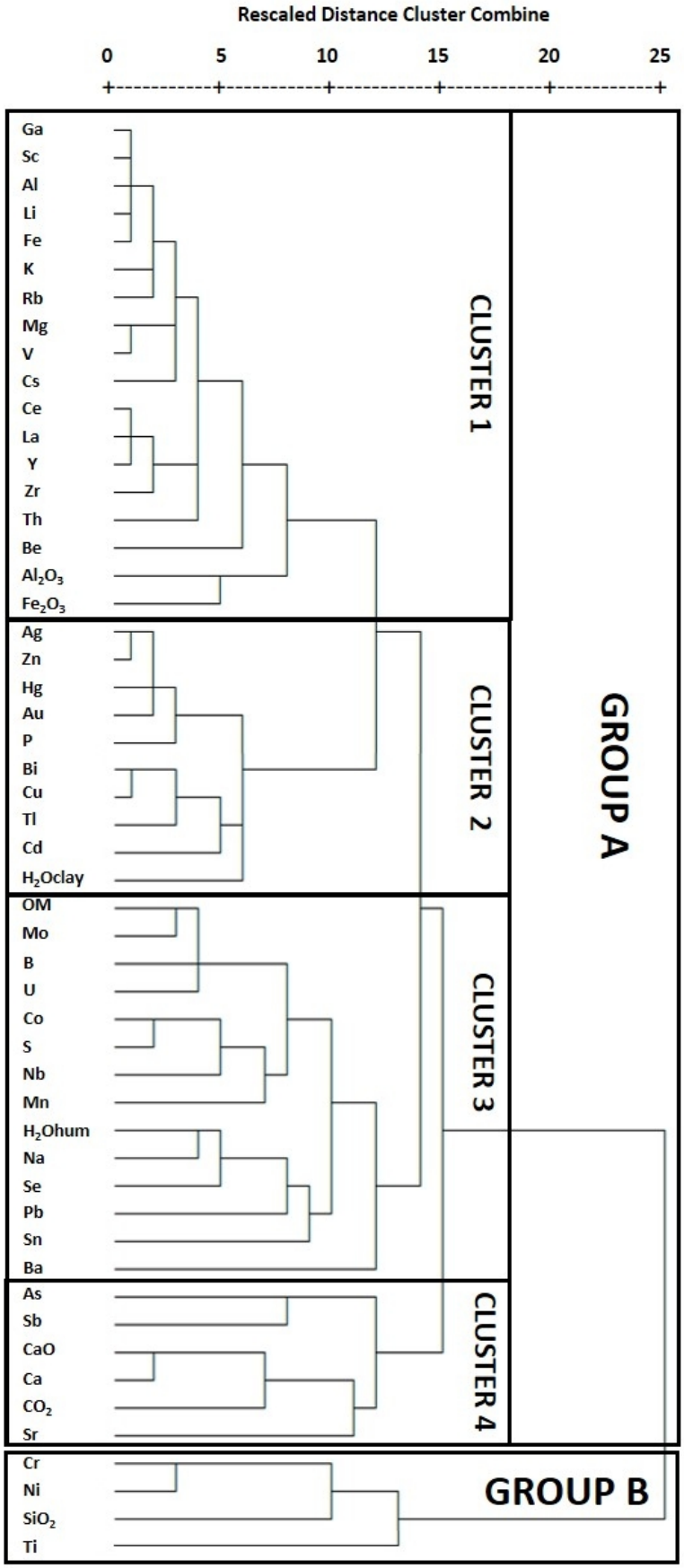

Figure 3.3. Cluster dendrogram illustrating the basic scheme of correlations among major oxides obtained by XRF (Migani *et al.*, 2015), pseudo-total contents of trace elements, and parameters emerged by the thermal analysis.

3.4.5 Principal component analysis (PCA)

For this study, a CA appeared a suitable exploratory tool aimed to unravel the complexity of a large number of variables and many novel geochemical data. As a simplification, the cluster analysis assigned each element to its own group with a limited possibility of highlighting some complex relationships. In addition, the graphical representation (dendrogram) providing a scheme of the correlations, allowed a first good understanding of the multivariate behavior of the dataset (Templ *et al.*, 2008). As a positive consequence, the PCA of variables (R-mode), assisted by PCA of events (Q-mode) will be interpreted more efficiently.

PC analysis with Varimax rotation was applied and the results are given in Table 3.5. Six factors were selected with a cumulative variance of 89.8% for the sediment samples (Tab. 3.5). Loadings are hereafter abbreviated with L.

PC1: Fe_2O_3, Al, Be, Ce, Co, Cs, Fe, Ga, K, La, Li, Mg, Rb, Sc, Th, V, Y, Zr (L >0.7); Al_2O_3, H_2O_{hum}, As, Ba, Bi, Cu, Mn, Pb, S, Tl (0.7>L>0.4); Cr (L<-0.4). PC1 accounts for 34.4% of the total variance. It represents elements of similar origin, controlled by clay minerals, oxides and secondary species involving Fe, Mn, and Al. Iron and Al seem to strongly control the contents of Be, Ce, Co, Cs, Ga, K, La, Li, Mg, Rb, Sc, Th, V, Y, Zr and at some extent As, Ba, Bi, Cu, Mn, Pb, and Ti. Sulphur may have a part in the processes of mineralization of some elements of this group. Lithogenic processes during weathering of natural parent materials may be the key factor of this group. Even if a clay component, Silica has negative load in this group, indicating that in the study area it also represents coarser sediment fractions, as reported in Migani *et al.*, (2015). As a result of PC1, Cr seems therefore less controlled by clays than coarse textured fractions.

PC2: OM, B, Mo, Nb, Se, S, U (L>0.7); H_2O_{hum}, Cd, Co, Mg, Mn, Na, Ni, Sb, Sn, V (0.4<L<0.7); Al_2O_3, SiO_2 (L<-0.4). PC2 accounts for 19.5% of the total variance. It is dominated by the role of organic matter and S on the fate of a number of elements such as Mo, Nb, Se, U, Cd, Co, Mn, Ni, Sb, Sn, and V. The redundancy of Co, Mn, and V (included both in PC1 and PC2) may indicate the very high tendency of these elements to be controlled both by various geochemical factors such as clayey materials (PC1) and the fine fraction rich in organic matter and likely colloidal particles. The inclusion of Nb in a group controlled by OM, as shown by CA, is confirmed with PCA. The occurrence of Na, B, Mg in this factor might depend on the influence of seawater entrained in the sediments.

PC3: Ag, Au, Cd, Cu, Hg, P, Zn (L>0.7); H_2O_{min}, CO_2, Bi, Sn, Tl (0.4<L<0.7).

PC3 accounts for 13.3% of variance. It presents high positive loadings of Ag, Au, Cd, Cu, Hg, P, and Zn. Bi, Sn and Tl are also included in this group. This group of metals could be dominated by anthropogenic factors. The presence of H_2O_{min} may reveal a relation with hydrated silicates more than OM, but the simultaneous presence of CO_2 is confusing. The importance of this group in terms of localization and environmental issues will be discussed after.

PC4: CaO, Ca (L>0.7); CO2, As, Sb, Sr (0.4<L<0.7); SiO2, Cr, Ni (L<-0.4). PC4 accounts for 9.2% of variance. It clearly represents carbonates, not a significant substrate for accumulation processes involving most of the elements in this study. As occurred in cluster analysis, a surprising association with As and Sb can be observed also in the PCA results. Interestingly, SiO2, Cr, and Ni are grouped here with negative moderate loadings. All considered, this factor seems to define a different fine sediment fraction from those controlled by the elements included in PC1 and PC2, instead of a true relationship between carbonates and two elements typically present in the environment as anionic mobile species.

PC5: Na, Pb, Sr (0.4<L<0.7); Ti (L<-0.7). PC5 accounts for 5.6% of variance. It is a not very clear component, as it includes the small amount of Ti, that has been dissolved by *aqua regia,* with a high negative loading value (-0.74), and Na, Pb, and Sr with moderate loading, in turn included with some importance in PC2, PC1 and PC4, respectively.

PC6: Ba, Ni, Sb (0.4<L<0.7) ; H_2O_{min} (<-0.4). PC6 accounts for 4.7% of variance. Once again, a rather unclear component, including Sb, together with Ba and Ni. The negative loading of H_2O_{min} might indicate a different fate for part of the content of these elements, not involving hydration processes of clays. As the investigated areas have long been used for hunting, it is worth considering that Ba and Sb are major primer elements in the gunshot residues together with Pb (Dalby *et al.*, 2010). PC6 scores neatly separates BAI (negative scores from -1.70 to -1.20, except one sample with positive score as high as 1.34) from ROS (positive scores from +0.56 to +1.80). Samples from BER and COM are mixed and distributed from -0.86 to +0.55, with an exception in COM (+1.08). A relationship with Pb is not evident in any site, but a positive correlation (r_S=0.73, $p<0.01$) between PC6 scores and extractability degree of Pb can be observed taking ROS and BER samples (Fig. 3.4a).

Table 3.5 – The rotated (Varimax) component matrix for major oxides obtained by XRF (Migani *et al.*, 2015), pseudo-total contents of trace elements, and parameters emerged by the thermal analysis. Eigenvalues and proportion of variance explained are presented. Loadings lower than |0.40| are omitted.

Element	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Communalities
Al_2O_3	0.661	-0.533					0.947
CaO				0.925			0.944
Fe_2O_3	0.775						0.898
SiO_2		-0.627		-0.593			0.974
H_2O hum.	0.500	0.528					0.877
OM		0.932					0.956
H_2O clay			0.634			-0.438	0.813
CO_2			0.427				0.904
Ag			0.968				0.981
Al	0.920						0.968
As	0.567			0.422			0.660
Au			0.905				0.890
B		0.876					0.889
Ba	0.436					0.553	0.619
Be	0.804						0.796
Bi	0.672		0.650				0.978
Ca				0.913			0.963
Cd		0.553	0.786				0.959
Ce	0.885						0.973
Co	0.702	0.613					0.945
Cr	-0.607			-0.537			0.868
Cs	0.815						0.820
Cu	0.476		0.845				0.980
Fe	0.943						0.973
Ga	0.924						0.973
Hg			0.921				0.919
K	0.818						0.957
La	0.871						0.946
Li	0.927						0.981
Mg	0.804	0.441					0.970
Mn	0.483	0.654					0.883
Mo		0.897					0.913
Na		0.669			0.558		0.884
Nb		0.845					0.846
Ni		0.442		-0.447		0.516	0.912
P			0.870				0.928
Pb	0.508				0.598		0.896
Rb	0.865						0.888
S	0.484	0.754					0.922
Sb		0.516		0.521		0.486	0.788
Sc	0.974						0.988
Se		0.765					0.810
Sn		0.622	0.449				0.639
Sr				0.691	0.463		0.852
Th	0.892						0.947
Ti					-0.741		0.730
Tl	0.566		0.577				0.815
U		0.818					0.886
V	0.842	0.443					0.977
Y	0.907						0.968
Zn			0.957				0.993
Zr	0.916						0.898
Eigenvalues	22.60	9.46	6.27	4.35	2.29	1.74	
Total variance %	34.41	19.53	16.32	9.24	5.65	4.65	
Cumulative var.%	34.41	53.94	70.26	79.50	85.15	89.80	

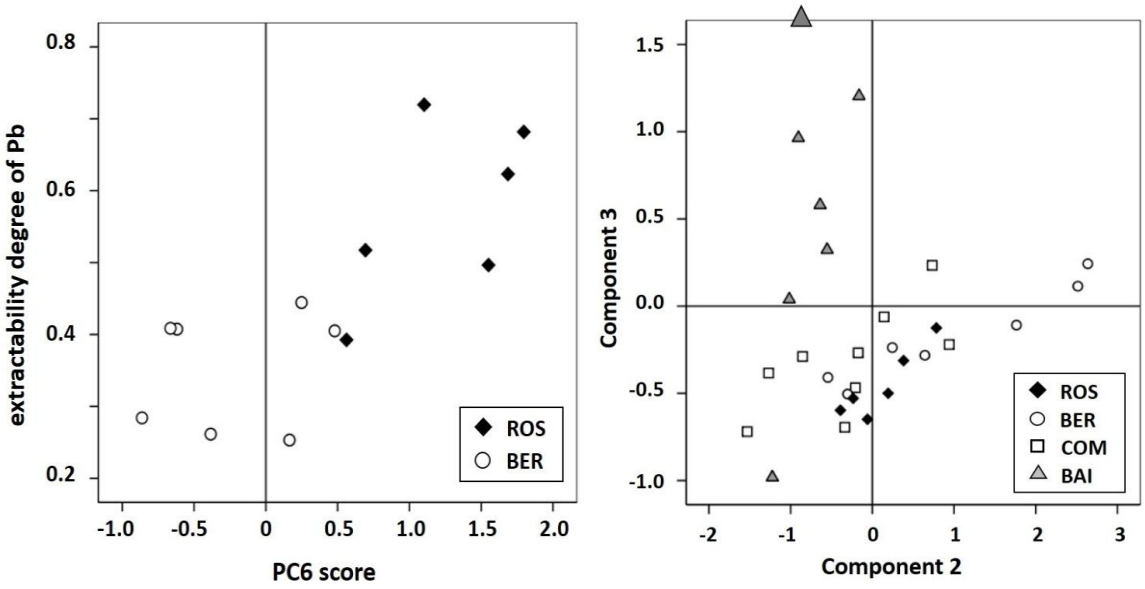

Figure 3.4a – Relationship between PC6 scores and extractability degree of Pb (ROS and BER samples). PC6 include Ba and Sb, two major components of primer and powder of the cartridges used for hunting. The extractability degree is defined as the ratio between the Pb pseudo-total concentrations obtained in this study and total concentrations from Migani *et al.*, (2015).

Figure 3.4b – Score plots illustrating events (samples) in the rotated space, obtained by Q-mode PCA and combining PC2/PC3. PC2 include elements essentially controlled by organic matter (OM). PC3 groups some harmful heavy metals suspected to be of anthropogenic origin. A sample from BAI with an off the scale score is plotted as a larger triangle on the upper limit of PC3 scale.

The relationship between As and Sb first shown by CA, seems to be more complex according to PCA. In fact, after this analysis, Sb appears more ubiquitous (included in PC2, PC4, and PC6 with moderate loadings), but definitely less dependent on Fe compounds and silicate substrates (PC1) than expected and when compared to As. On the other hand, Mn is associated to As in PC1 and to Sb in PC2 and may play a role for absorbing both metalloids in a oxidized complex. The As seems insensitive to OM content, while Sb likely binds on such substrate to some extent, according to Wilson *et al.* (2010).

Principal component analysis shows Sn in two separate behaviours. The first may be more influenced by the reactions mediated by S and OM, while a different fate may be common to many harmful metals of probable anthropomorphic origin. Therefore, the presence of organotin species or other chemical species of mobile Sn is plausible.

According to the CA results, Pb showed a general tendency to belong to the group including organic matter and S, and also a less expected association to Se and Na. According to the PCA results, an association to some extent with Fe and Mn oxides (probably as secondary species) may be proposed.

Considering that XRF revealed considerable enrichments of Cr in samples characterized by coarse texture (Migani *et al.*, 2015), the negative loading of Cr in PC1 (clays and fine mineral fraction) and PC4 (carbonates) is not surprising. There is also a mutual relation with SiO_2 (negative loading of both Cr and Ni in PC4). A different fate of Ni is highlighted by its inclusion in PC2. This may explain the higher extractability of Ni in respect of Cr.

Among the elements grouped in PC3, Cu also shows more tendency to associate also to clays or, more likely, to Fe and Mn compounds, while Cd, Hg, and Zn are strongly and uniquely present in PC3. With the aid of Q-mode PCA, the great importance of BAI in determining the PC3 can be appreciated (Fig. 3.4b). Conversely, organic matter (here represented by PC2), as well as other geochemical factors not shown here, seems to have a small role in controlling polluting heavy metals in this site. Likely, major spatial importance factors influence the local distribution of the contaminants, while, in the other sites, a clear dependence of pseudo-total concentrations from OM abundance emerges (Fig. 3.4b). In addition, Figure 3.4b shows the negligible influence of carbonates in the accumulation processes of the harmful elements occurring in BAI.

3.4.6 Considerations on elements of environmental concern: Ag, As, Be, Cd, Co, Cr, Cu, Hg, Ni, Pb, Sb, Se, Sn, Tl, V, and Zn.

In this paragraph, all trace elements of relevant environmental concern in accordance with Italian law are summarized and discussed in relation to the outcomes of this comprehensive environmental geochemical study. Silver has also been included, because it is listed among the harmful elements for soils in some European countries (Lithuania, Slovakia, The Netherlands).

Migani *et al.*, (2015) found Cr, Cu, Pb, V, and Zn to be locally or generally more concentrated than the reference background levels (total contents). By calculating Enrichment Factors (Zhang *et al.*, 2007), they proved the natural origin of Cr, Ni and V in all the studied sites. This may be the case also for Be and most of Co, following the indication of CA and PCA in this study. However, Co, even though derived from natural sedimentary processes, is bioaccessible when it is bound to humic and fulvic acids and inorganic colloids as Co^{2+} (McBride, 1994; Qian *et al.*, 1998). The full extractability of Co resulted with *aqua regia,* together with the moderate association with OM highlighted by our study, may suggest a possible good bioavailability of this essential metal for life.

As commented before (see par. 3.4.2), **Cr** and **Ni**, are naturally abundant in the investigated area. However, both metals are of environmental concern and included in the national guidelines for handling of marine sediments (APAT-ICRAM, 2007). Many samples in our study from BER and

COM have concentrations of Cr and Ni definitely higher than the chemical basic level for politic sediments and concerning Ni, ROS, BER, and COM concentrations are even higher than the chemical limit level (Tab. 3.2). Di Giuseppe *et al.*, (2014b) found Ni invariably more mobile in respect to Cr and therefore thought more phytoavailable, and a similar result was reported for one of the studied lagoons (Baiona, Donnini *et al.*, 2007). In the present study we can observe Ni more extractable than Cr (Tab. 3.4) and this may confirm the higher mobility of Ni in this area.

Apart from metals of natural origin, in the part I of this geochemical investigation (Migani *et al.*, 2015) a high degree of pollution was pointed out for Cu and Zn in the southern wetlands (including BAI). In addition, Pb, which was evaluated on the limit between natural and anthropogenic origin in most sites, showed strong anomalies in ROS, BER, and COM, known to have been used for hunting for a long time (Migani *et al.*, 2015). Critical pollution levels involving at least Cu, Hg, and Zn, are well known for BAI (Donnini *et al.*, 2007; Matteucci *et al.*, 2005; Miserocchi *et al.*, 1993). Our results demonstrated that a number of elements including Ag, Au, Bi, Cd, Cu, Hg, Sn, Tl, and Zn is mainly driven by BAI. This appears to be a very indicative result of probable anthropogenic relevance (industrial and agricultural inputs) and our study demonstrated that besides Cu, Hg, and Zn, also Ag and Tl, at least two metals harmful for biota deserve attention in BAI: **Ag** and **Tl** (Fig. 3.5a and 3.6f).

Silver is biologically active, highly toxic to fish and many micro-organisms, but thought relatively harmless to higher life forms, including humans (Edwards *et al.*, 1995). However, Ag uptake can compete with metabolic processes involving Cu and Se in humans (Mertz, 1987). Thallium, although rather rare in the Earth's crust, is extremely toxic (similarly to Cd, Hg, and Pb) (Borgmann *et al.*, 1998).

Copper and **Zn** boxplots showed in Figures 3.5f and 3.6h confirm the known anomalies (Migani *et al.*, 2015) improving the information about their relative high mobility in BAI.

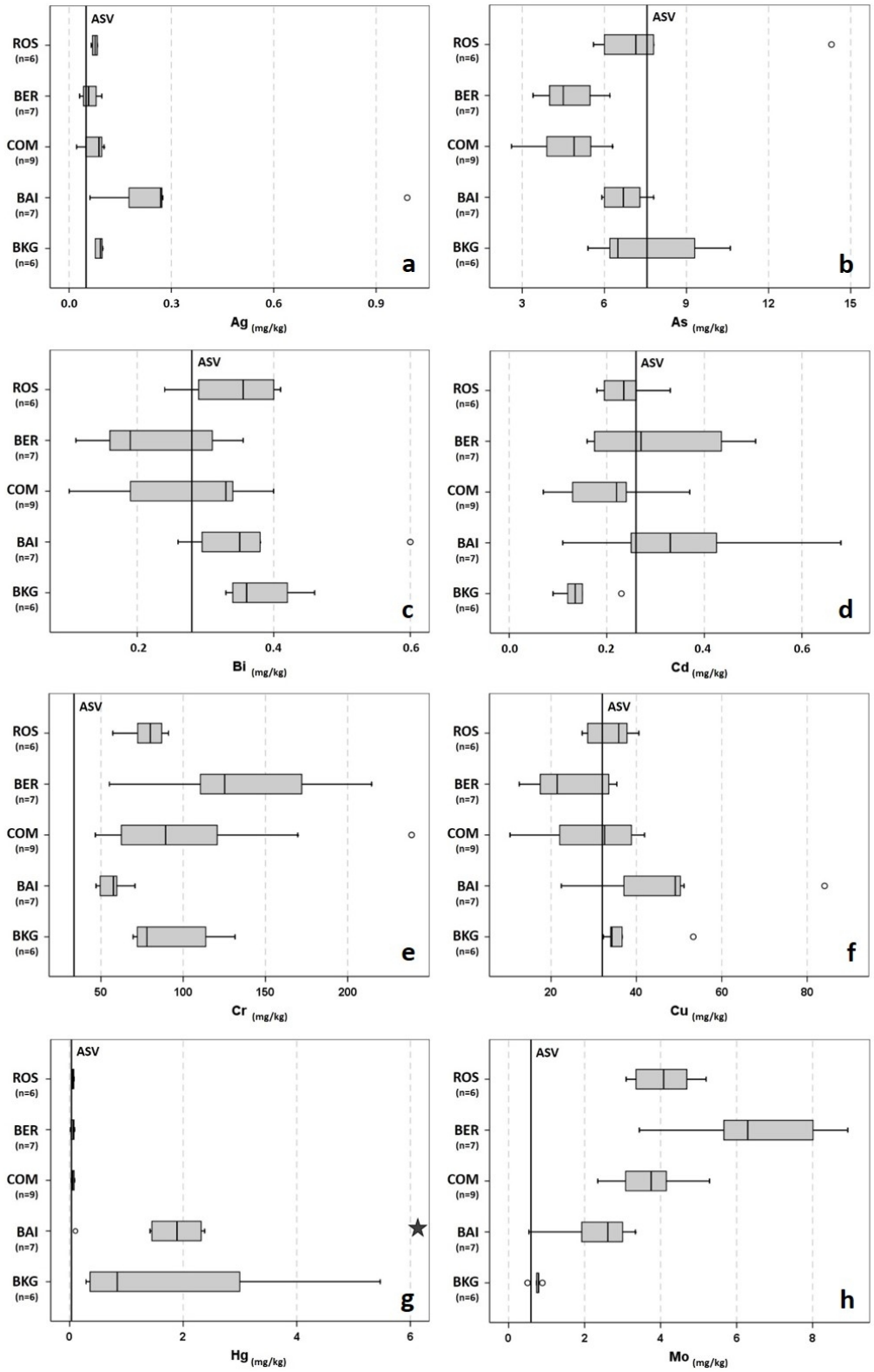

Figure 3.5 (a,b,c,d,e,f,g,h) - Box-plots of the pseudo-total concentrations of Ag, As, Bi, Cd, Cr, Cu, Hg, and Mo in each sampling site. As a reference threshold for Italian agricultural soils (ASV), according to Cicchella *et al.*, (2015), solid line is added.

Natural levels of **Hg** have been assessed by Miserocchi *et al.*, in 1993 for BAI (about 0.1 mg/kg). This information is still not available for ROS, BER, and COM, but we can speculate that a background level for these sites is not much different. In addition, ASVs never exceeded 0.6 mg/kg for Hg in Cicchella *et al.*, (2015). The waste discharges containing Hg ceased 30 years ago in BAI (Matteucci *et al.*, 2005), but our data confirm that Hg-enriched sediments are still widespread throughout the lagoon with scattered exceptions, and extremely high peaks remain (Fig. 3.5g).

In this subgroup of pollutants **Sn** does not seems site-dependent. However, its anthropogenic nature may be supposed, because the overall higher pseudo-total contents in respect of the reference values used for this study: thresholds from laws and guidelines and ASVs (see Fig. 3.6e and paragraph 3.4.3). Pseudo-total contents of Sn in our study do not seem to be much associated to SnS_2, insoluble under reducing conditions, and soluble Sn bounded to Fe and Al hydroxides, as expected (Salminen *et al.*, 2005). Its association with OM is more evident. The Sn propriety to form complexes and compounds with organic substances is used by humans for producing anti-fouling paints for ships and agricultural pesticides of particular concern (Fent, 1996). Therefore, high values of extractable Sn in sediments rich in organic matter (source of S) and characterized by frequent redox processes must be taken into consideration as an indication of potential hazard.

Also **As** and **Sb** may derive from human impact on the surrounding lands, but this has not been highlighted by clustering or PCA as for metals. With regard to some peculiarities, Sb and As can show similar chemical behaviour. For example, they are renowned for a similar mechanisms of negative bioactivity (Burford *et al.*, 2011). Interestingly, they have been grouped together with multivariate analyses. The historical use of As for poisoning and as a main component of some pesticides induced the production of a rich literature regarding this metalloid. In this regard, the very high concentration found in one sample from ROS (14.3 mg/kg) (Fig. 3.5b) should be considered with attention, because As is essential for some organisms, including humans, but is toxic depending on its abundance, valency and speciation, regardless of the natural or anthropogenic origin (Bora *et al.*, 2013).

On the contrary, **Sb** has received scanty attention by researchers until recently (Filella *et al.*, 2003). Yet, the high toxicity of Sb is recognized nowadays, and various key aspects of the environmental behavior of Sb summarized by Filella *et al.*, (2009) still remain unclear. Among the investigated sites, Sb was lower than ASVs only in BAI (Fig. 3.6c). In sediments, both As and Sb show affinity for Fe and Mn oxyhydroxides (Wilson *et al.*, 2010), while organic matter should preferably influence Sb fate in sediments (Filella *et al.*, 2002, 2003). The association between Sb

and OM emerged from results, it is less important for As. Traditionally, Sb was used in lead-antimony alloys, for example to make harder lead ammunition for hunting (Andreotti and Borghesi, 2012), but nowadays it is released in the environment mainly as a component of flame retardants (Touval, 2000; Zanetti *et al.*, 2002). Some of the Sb extracted by *aqua regia* is related to Ba and Ni, in according to the PCA outcomes and the possibility that the association Ba-Sb can be in relation with the historical use of these wetlands for hunting should be further investigated.

A wide literature puts **Cd** among the most studied toxic metals because it is classified as a human carcinogen. Moreover, the human body has limited capacity to respond to cadmium exposure, as the metal cannot undergo metabolic degradation to less toxic species and is poorly excreted (Sarkar *et al.*, 2013). We found relatively high levels of Cd (higher than ASVs) and dispersed distribution in BER and BAI (Fig. 3.6d) as a corroboration of some Cd contamination in those areas.

No information was available on **Se** in this geographical area before this study. We showed relatively high concentrations of this element when compared to ASVs. Although Se may exist in sediments in four oxidation states, under normal conditions, Se IV (Selenite) and Se VI (Selenate) are dominant and controlled by many factors such as pH, redox conditions and organic matter content (Eich-Greatorex *et al.*, 2007; Mayland *et al.*, 1991). Selenite is sorbed more strongly than selenate onto clays but more weakly than many other anions (Barrow and Whelan, 1989). Organic selenium species are more volatile (Mayland *et al.*, 1991). Considering the extraction method used and the substantial bioavailability of both selenite and selenium (Barceloux, 1999), the high pseudo-total contents recorded in this study may also indicate a higher potential bioavailability in these transitional environments. Selenium is an essential component to protect organisms from oxidative degradation processes, but aquatic organisms (e.g., bivalves), and plants, can accumulate and magnify Se in the food chain (Barceloux, 1999; Eich-Greatorex, 2007). Positive or negative ecological effects provoked by such concentrations in the Po Delta are far from being understood, because no ecotoxicological study has been carried out in this area involving this element. In the presence of high contents of Hg (as in BAI) and other metals, bioavailable Se can play a role in contrasting their toxic and detrimental effects (Yang *et al.*, 2008). Anthropogenic inputs involving Se are possible given the difference observed with local background data, although no sample exceeded the screening values for soils following the Italian law (Fig. 3.6d).

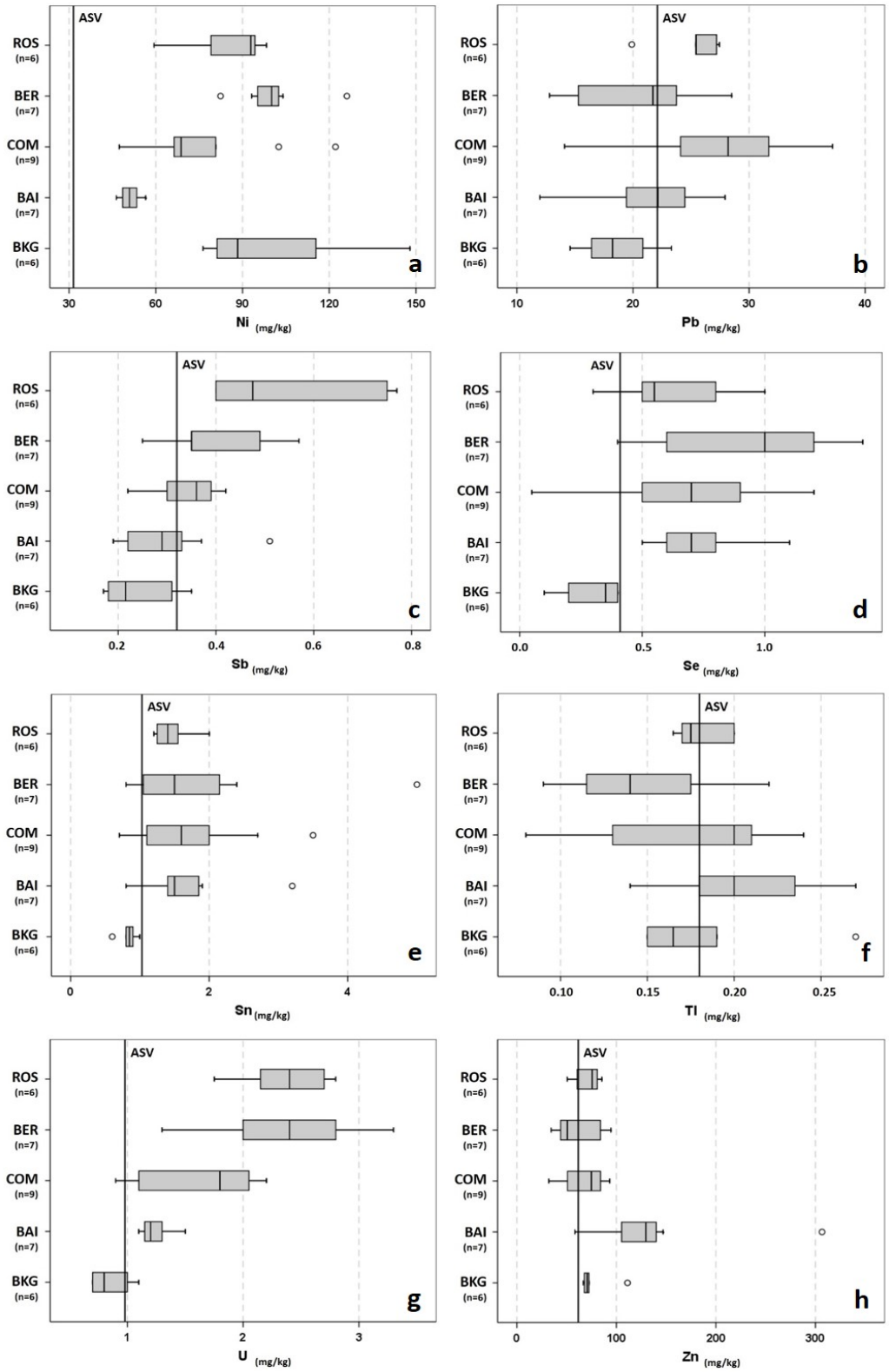

Figure 3.6 (a,b,c,d,e,f,g,h) - Box-plots of the pseudo-total concentrations of Ni, Pb, Sb, Se, Sn, Tl, U, and Zn in each sampling site. As a reference threshold for Italian agricultural soils (ASV), according to Cicchella *et al.*, (2015), solid line is added.

Lead is widespread in soils and sediments as a consequence of the prolonged use of tetraethyl-Pb in fuels until recently. The investigated sediments are not an exception and Pb total concentrations were found to be relatively high for all the samples from BER and ROS and many from BAI and COM in comparison with the background (Migani *et al.*, 2015). In addition, wildfowling (hunting of ducks from fixed blinds) has been conducted in the sampling sites for as long as a century. Only recently, the hunting period has been reduced to the winter months and the number of game birds to be shot limited. No estimation of annual discharge or number of lead pellets in the surface sediments is available for the investigated sites. However, there are studies in some Italian wetlands similarly used for hunting (Bianchi *et al.*, 2011; Tinarelli and Tirelli, 1999), which have estimated zero to 3,000,000 lead pellet per hectare, corresponding to 0-404 kg/ha of Pb (Bianchi *et al.*, 2011). An impact of hunting, occurred by the discharge of large quantities of metallic Pb in the form of lead pellets in front of historical hides was supposed in all four sites of this study. It is arguable that, if metallic Pb fragments lie in the surficial layer of the sediments (Andreotti and Borghesi, 2012), this kind of Pb may emerge by XRF with scattered peaks, conversely lacking when samples are dissolved by *aqua regia*. In other words, we can expect that peaks due to lead ammunition in XRF results will be less perceivable in pseudo-total results and as a consequence the extractability degree will vary a lot sample-by-sample. Figure 3.2 seems to confirm this hypothesis. In fact, no correlation exists between pseudo-total and total contents of the same samples, and ROS and BER Pb pseudo-total concentrations are fully comparable with the other two sites. Furthermore, a general enrichment caused by secondary Pb products coming from the slowly weathering process involving such metallic Pb will be added to historical tetraethyl-Pb pollution and background values. As an indication of that, the 35% of the samples throughout the whole study area is higher than ASVs for Pb (Fig. 3.6b).

3.5 Conclusions

In order to complete a comprehensive geochemical study on several coastal wetlands of the Po Delta area (Italy), an *aqua regia* digestion has been applied.

Environmental geochemical information has been built-up integrating, for each element analyzed several data from literature such as reference values for Italian agricultural soils (ASVs) (Cicchella *et al.*, 2015) and total concentrations and thermal analysis obtained on the same samples (Migani *et al.*, 2015). In addition, we calculated the degree of extractability (pseudo-total contents / total contents). Concentration limit values provided by Italian legislation or suggested

by national guidelines have been used to identify anomalies. In some cases, background local values were useful to support or discuss such indications. In some cases, when the law and guidelines did not include elements, background values and AVSs have been used as a reference. Two statistical multivariate methods, in the present case cluster analysis (CA) and principal component analysis (PCA), were used to explore relationships among elements.

As final remarks at the end of this study, we can conclude that:

- 23 out of 44 elements show pseudo-total contents relatively higher than ASVs. Boron, Ca, Cr, Mg, Mo, Na, Ni, S, Se, Sn, and Sr were the more abundant in all sites, whereas Ag, Au, Hg, and Zn, especially in BAI, were not. Arsenic is in general relatively low, but one sample in ROS reached a very high concentration and should be taken into consideration and further investigated.
- The extraction of Co, Cu, Pb (in BAI), Ca, and S by *aqua regia* has been nearly complete. Also Zn was highly extractable, especially in BAI. On the contrary, Mn, Mo, Pb (in ROS) and V seem less dissolved by *aqua regia* than expected. Elements associated to clay minerals are much lower in pseudo-total contents than expected, but REEs (Sc, Y, Ce, La) emerged as slightly more mobile, following this gradient: Sc>Th>Y>Ce>La>Al>K>Rb>Ti>Nb>Zr. Actually, Sc and Th would be between La and Al only considering three sites, but the BAI's higher degree of extractability of Sc and Th increases this parameter for both. Iron and Mn should significantly participate in the pseudo-total contents as hydroxides and colloids influencing the fate of many other elements, and probably for this reason they are slightly more extractable. The findings regarding Pb, taking the insights in the present study and the total concentration peaks observed in the phase I (Migani *et al.*, 2015), lead us to suppose that there is an impact of historical hunting on the accumulation of Pb in most of the investigated wetlands.
- Hg in BAI is proved to be higher than all reference values both for surface and subsurface soils (non-industrial) and marine and coastal sediments. In one sample, the concentration was 5-fold the limit for industrial soils. Also Sn exceeded the limits (in all sites), but the nature (organic or inorganic) of this element in the studied area is not known to date.
- A group of elements likely influenced by anthropogenic activities (Ag, Au, Cd, Cu, Hg, Zn) and P substantially identify one particular site: BAI. While such elements in ROS, BER and COM seems to be controlled by the availability of organic matter in the substrate, for BAI the accumulation processes appear to be independent from geochemical aspects.

Arguably, harmful inorganic contaminants in BAI are spatially distributed following hydrological dynamics and the position of points of source.

For the purpose of managing these wetlands, some important considerations are:

- in spite of the evident high levels of pollution involving many contaminants, part of BAI is currently available for legally licenced commercial shellfishing and recreational fishing. Such activities should be considered like 'agricultural harvesting' potentially impacting on consumers' health. To make matters worse, the illegal collection of mussels is obvious and continuous (Borghesi, pers. obs.);
- our study suggests that in the studied wetlands, and especially in BER, there may exist sources of slow-releasing Pb, which are compatible with high concentrations of lead shot in the sediment;
- starting from the geochemical evidence after this study, further investigations should be conducted to improve the knowledge of the geochemical mechanisms capable of influencing the mobility of harmful elements in these wetlands. In fact, some actions taken to favor the biodiversity and habitat conservation, such us drying ponds or changing chemical parameters (e.g. salinity, pH) can temporarily change the redox condition of the surface layer in polluted sectors, making mobile metals which were previously immobilized in minerals.

3.6 Acknowledgements

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CHAPTER 4 - Metals and trace elements in feathers: a geochemical approach to avoid misinterpretation of analytical responses

4.1 Abstract

Assessing trace metal pollution using feathers has long attracted the attention of ecotoxicologists as a cost-effective and non-invasive biomonitoring method. In order to interpret the concentrations in feathers considering the external contamination due to lithic residue particles, we adopted a novel geochemical approach. We analysed 58 element concentrations in feathers of wild Eurasian Greater Flamingo *Phoenicopterus roseus* fledglings, from 4 colonies in Western Europe (Spain, France, Sardinia, and North-eastern Italy) and one group of adults from zoo. In addition, 53 elements were assessed in soil collected close to the nesting islets. This enabled to compare a wide selection of metals among the colonies, highlighting environmental anomalies and tackling possible causes of misinterpretation of feather results. Most trace elements in feathers (Al, Ce, Co, Cs, Fe, Ga, Li, Mn, Nb, Pb, Rb, Ti, V, Zr, and REEs) were of external origin. Some elements could be constitutive (Cu, Zn) or significantly bioaccumulated (Hg, Se) in flamingos. For As, Cr, and to a lesser extent Pb, it seems that bioaccumulation potentially could be revealed by highly exposed birds, provided feathers are well cleaned. This comprehensive study provides a new dataset and confirms that Hg has been accumulated in feathers in all sites to some extent, with particular concern for the Sardinian colony, which should be studied further including Cr. The Spanish colony appears critical for As pollution and should be urgently investigated in depth. Feathers collected from North-eastern Italy were the hardest to clean, but our methods allowed biological interpretation of Cr and Pb. Our study highlights the importance of external contamination when analysing trace elements in feathers and advances methodological recommendations in order to reduce the presence of residual particles carrying elements of external origin. Geochemical data, when available, can represent a valuable tool for a correct interpretation of the analytical results.

4.2 Introduction

As a consequence of human activities, coastal aquatic ecosystems are now polluted by a large diversity of contaminants which may have a considerable impact on the ecohealth of these habitats (Calow, 1993; Halpern *et al.*, 2008). However, trace element inputs may derive also from natural processes such as rock weathering, volcanism, and water discharge (Garrett, 2000).

Many elements (e.g. Fe, Co, Cu, Mn, Mo, Zn) are essential to life, but toxic in excessive quantities (Singh *et al.*, 2011). Similarly, Se exerts a protective action against Hg in the marine environment, but Hg can biomagnify in the food chain with detrimental effects on organisms (Beijer and Jernelöv, 1978; Cuvin-Aralar and Furness, 1991). Other elements that are normally toxic, turn beneficial under certain conditions (e.g. V, W, and even Cd) (Lane *et al.*, 2005; Singh *et al.*, 2011). On the other hand, As, Sb, and few others (e.g. Pb, Hg, Ni, Pu, Tl) seem to be toxic irrespective of quantities, without known beneficial effects on organisms (Hargreaves *et al.*, 2010). Most of Rare Earth Elements (REEs) have been so far thought to be non-essential, but toxicological effects in humans are demonstrated for some (Pałasz and Czekaj, 2000). The accumulation of persistent contaminants in sediments exposes wildlife living in coastal wetlands to the adverse effects of such pollutants (Furness and Rainbow, 1990) even for many years after the polluting event (Hill *et al.*, 2010). All this considered, many researchers point out that urgent and persistent monitoring studies on biogeochemical behaviour of practically all trace elements should be undertaken.

In waterbirds, the ingestion of food, soil or sediment is the most important intake pathway (Beyer *et al.*, 1994; Burger and Gochfeld, 2002). Acute poisoning can occur, causing death of fully developed birds (Pain and Rattner, 1988), but more often chronic exposure at low concentrations occurs, especially in long-lived birds (Scheuhammer, 1987). Possible consequences are impaired growth, development, reproduction, behaviour, resistance to diseases and disorder in other physiological mechanisms contributing to population decline (Burger, 1993; Dauwe *et al.*, 2005; Eeva *et al.*, 1995; Frederick and Jayasena, 2011; Gangoso *et al.*, 2009; Koller, 1980; Nam and Lee, 2006; Scheuhammer, 1987; Scheuhammer and Norris, 1996; Snoeijs *et al.*, 2004; Talloen *et al.*, 2008).

Waterbirds have been extensively used as sentinels of environmental pollution, in particular for trace elements (e.g. Burger and Gochfeld, 2004; Furness, 1993; Lewis and Furness, 1991; Nygard *et al.*, 2001; Peakall, 1992; Sanpera *et al.*, 2007, 2000; Tavares *et al.*, 2009; Walsh, 1990). Colonial species are considered more suitable for that purpose (Fox and Weseloh, 1987). The fidelity to the breeding sites and relative abundance in a limited area imply several advantages (Kushlan, 1993). On the other hand, colonial waterbirds are often protected by environmental legislation and therefore the use of a non-invasive technique is preferable or the only possible solutions.

Potentially, feathers are good bioindicators since they are simple to collect, store and use (Furness, 1993; García-Fernández *et al.*, 2013). Birds sequester some metals in growing feathers proportionally to blood levels (Barbieri *et al.*, 2009; Burger and Gochfeld, 2009a; Golden *et al.*, 2003) and a relatively high proportion of the body burden of certain metals is stored in feathers (e.g. Hg, Furness *et al.*, 1986; As, Smith *et al.*, 2008).

Metal accumulation in feathers may vary in relation to trophic levels, foods, and moult (Braune, 1987; Dauwe *et al.*, 2003; Nygard *et al.*, 2001; Stewart *et al.*, 1999, 1997). Within species, age (Burger, 1995; Burger and Gochfeld, 2000), reproductive stage (Wayland *et al.*, 2005), location, physiological condition, and clutch (Rumbold *et al.*, 2001) can also have an effect. However, a lot of previous investigations on pollutants in feathers were conducted on adults of unknown age, foraging areas, moulting stage and migratory status patterns.

Chick feathers appear more suitable for assessing metal bioaccumulation because the weight of variability factors such as age, moult, and exposition to atmospheric deposition is minimized (Burger, 1993). However, relatively few studies have focused on chicks comparing different colonies. Such studies mainly referred to herons and ibises (Abdennadher *et al.*, 2011; Abdullah *et al.*, 2015; Burger, 2013; Clarckson *et al.*, 2012; Cotìn *et al.*, 2012; Golden *et al.*, 2003; Goutner *et al.*, 2001; Herring et al, 2009; Kim and Oh, 2014a; Rubio *et al.*, in press; Rumbold *et al.*, 2001; Sepulveda *et al.*, 1999; Spahn and Sherry, 1999), terns and gulls (Burger, 1996; García-Tarrasón *et al.*, 2013; Kim and Oh, 2014b; Ramos *et al.*, 2013; Sanpera *et al.*, 2007), and pelagic birds (Blévin *et al.*, 2012) and focused especially on Hg.

Feathers are subjected to external contamination to some extent (Ek *et al.*, 2004; Fasola *et al.*, 1998; Hahn *et al.*, 1993; Hollamby *et al.*, 2006; Valladares *et al.*, 2010). It can occur from direct atmospheric deposition, contact with soil, dust or water, or from the deposition of contaminants on feathers during preening (Dauwe *et al.*, 2002). Waterfowls and seabirds may also secrete metals through salt gland and embrocate them on their feathers (Dmowski, 1999). The extent of the external contamination is element-related (Jaspers *et al.*, 2004): for Hg, it is considered negligible (e.g. Appelquist *et al.*, 1984; Dauwe *et al.*, 2003; Dmowski, 1999; Ek *et al.*, 2004; Goede and De Bruin, 1984), but for most elements this is not true or not clear (Cardiel *et al.*, 2011; Dmowski, 1999).

Nevertheless, very few studies have highlighted the problem of external contamination (Scheifler *et al.*, 2006) or aimed to solve it, and therefore the analytical results cannot always can be easily interpreted, with the possible exception of Hg (Dauwe *et al.*, 2003). In most studies,

feathers were washed before analysis. A thorough alternate washing with deionized water and acetone was frequently adopted (e.g. Ansara-Ross *et al.*, 2013; Carvalho *et al.*, 2013; Costa *et al.*, 2013). In some studies, instead of acetone, other solutions based on NaOH (e.g. Cotìn *et al.*, 2012; Ramos *et al.*, 2013), or a chloroform-methanol mixture were applied (e.g. Lucia *et al.*, 2013). These methods were occasionally followed by an ultrasonic bath. Less frequently, feathers were simply rinsed with deionized water (Markowski *et al.*, 2013), sometimes preceded by a washing with a mild-metal free detergent (Bryan *et al.*, 2012). In some cases, feathers were not washed at all (Abdennadher *et al.*, 2011; Kim and Oh, 2014a, 2014b). In any case, it was demonstrated that no washing procedure is able to ensure the total removal of the surface contamination from feathers (Cardiel *et al.*, 2011; Espín *et al.*, 2014).

The Greater flamingo (*Phoenicopterus roseus*) is a potential sentinel of the health of coastal ecosystems since it is large, wide-ranging, abundant, long-living, as well as easily observed and monitored. The breeding ecology of the Greater flamingo is well known, as well as its biology (Johnson and Cézilly, 2007), as is desirable when a sound interpretation of the contamination level in the study areas is the main objective (Burger and Gochfeld, 2009a, 2009b). This species feeds in flock, on small benthonic invertebrates and occasionally seeds of aquatic plants by filtering mud, typically in brackish wetlands and saltpans. During filtering, particles ranging in diameter from 0.5 mm to 4-6 mm are swallowed (Del Hoyo *et al.*, 1992). Unlike most birds, Greater flamingos can ingest mud as an essential component of their food, indeed they may be able to bring up their chick whilst feeding solely on mud rich in organic matter (Jenkin, 1957). Flamingos produce a secretion from their upper digestive tract rich in proteins, fat, carotenoids and blood cells (Fisher, 1972; Lang, 1963). Such liquid represents the sole source of nutrition for chicks, practically until independent feeding, which does not start before seven weeks after birth (Zweers *et al.*, 1995). All chicks in the colony, therefore, eat a similar kind of food, whose trace metal load must be dependent on blood levels of their parents. Breeding flamingos may feed in the vicinity of the breeding site (e.g. Camargue, France), but daily flights of up to 200 km are possible in Spain (e.g. Fuente de Piedra in Spain), depending on food availability in the area (Johnson and Cézilly, 2007).

Only five studies on trace elements used Greater flamingo feathers. Amiard-Triquet *et al.*, (1991) measured trace elements in fledglings in Camargue, while two other studies in the same area studied a small group of adults found dead (Cosson *et al.*, 1988a, 1988b). More recently, 7 carcasses found in various Italian wetlands were analysed for Pb, Cd and Hg (Ancora *et al.*, 2008). Finally, Hg was assessed in chicks from several colonies in the Western Mediterranean (Borghesi *et*

al., 2011). Part of the data reported in the latter paper has been included in the statistical analysis of this work.

We investigated the exposure to trace elements of the Greater flamingo analyzing fledgling feathers from 4 colonies in Western Europe. One group of captive adults was used as a control. Considering the inherent problem of this sampling matrix on the presence of a certain amount of unavoidable external contamination (Espín *et al.*, 2014; Cardiel *et al.*, 2011), we included geochemical information regarding the soils and sediments close to the nesting sites. We worked with very extensive sets of elements both for feathers and soils that allowed the application of a novel geochemical approach to interpret the data. Combining univariate and multivariate statistics, we were able to identify general trends and local signals concerning many elements of environmental concern and improve the information on the external contamination in feathers.

4.3 Material and methods

4.3.1 Study sites

Sampling was carried out in 2008, when eight colonies, all in the western Mediterranean region, raised chicks (Childress *et al.*, 2008). In this study, samples from four breeding sites have been included: Étang du Fangassier, Camargue (Southern France) (CAM); Marismas del Odiel, Huelva (South Western Spain) (ODI); Comacchio saltpans in Comacchio Lagoon (North Eastern Italy) (COM); Macchiareddu Salinas in Santa Gilla Lagoon, Cagliari (Sardinia, Italy) (CAG) (Fig. 4.1).

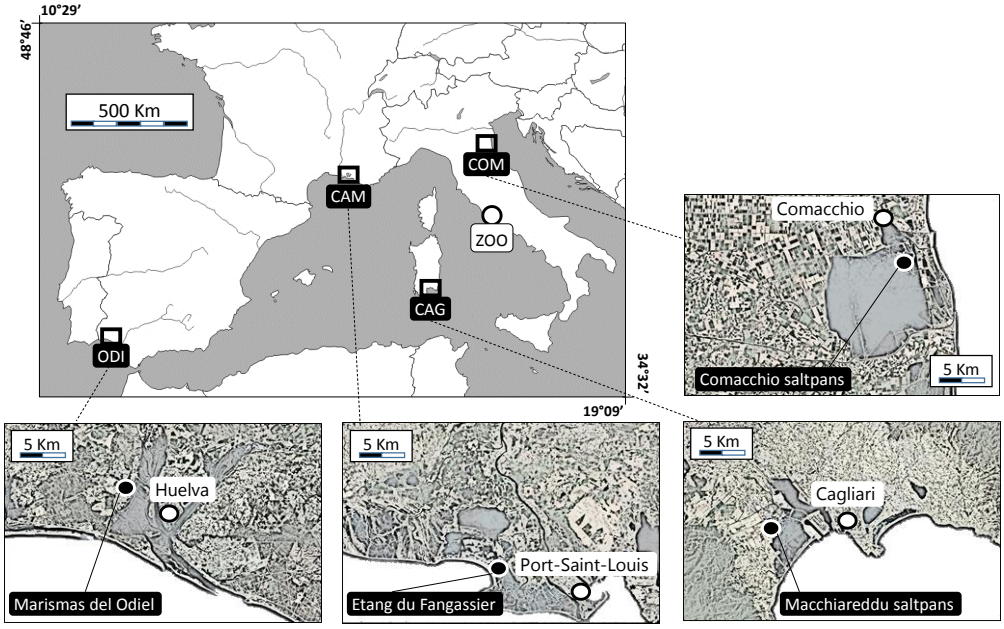

Figure 4.1 – Sampling sites. Small maps refer to wild colonies. From West to East: ODI = Marismas del Odiel, Spain; CAM = Camargue, France; CAG = Cagliari, Sardinia, Italy; COM = Comacchio, Italy; ZOO = Rome Bioparco Zoo, Italy (captive birds).

Huelva surroundings have been the subject of a number of geochemical studies, due to the important inputs of acid and metal rich mine waters from the Iberian Pyrite Belt (Guillén *et al.*, 2011). As a consequence, As, Cd, Cu, Pb and Zn in Odiel sediments are known to be higher than background values (Morillo *et al.*, 2008). Regarding the Camargue, geochemical literature is scarce, with only a few studies published focusing on Rhone River arms upstream from the coastal marshes (Cossa and Martin, 1991; Santiago *et al.*, 1994). Santa Gilla Lagoon is a part of the Stagno di Cagliari, which includes Macchiareddu industrial saltpans. The lagoon is connected to the sea and receives freshwater inflows from two small rivers. Santa Gilla lagoon has been polluted by industrial wastes containing Hg, Pb and Zn (Contu *et al.*, 1985). The Comacchio Lagoon is a brackish wetland, but receives also freshwater from a river and drainage waters from agricultural lands. There are geochemical studies in the areas surrounding the Comacchio Lagoon focused on sediment provenance (Amorosi *et al.*, 2002; Amorosi and Sammartino, 2007; Curzi *et al.*, 2006) and recently it was included into a comprehensive survey aimed at evaluating the contamination level of sediments in the Northern Adriatic coastal wetlands. As a consequence of geological origin, Comacchio sediments are naturally enriched in Cr and Ni, but Cu, Pb, and Zn anomalies were reported which are likely to be of anthropogenic origin (Migani *et al.*, 2015).

4.3.2 Sampling, sample preparation and analysis of sediments

From each site, 6 surface sediment samples were collected (*n*=24), in close proximity to the 4 islets on which flamingos bred in 2008: three samples from nests (consisting of a truncated cone of muddy sediment, 30-60 cm high), and three samples of mud from the wetland floor 3, 6, and 9 meters off the islet respectively. Samples were grabbed (just after the breeding season) by using a small plastic shovel, and stored in plastic boxes, then frozen at -20°C. Before digestion process, sediment aliquots were washed with distilled water, then centrifuged to eliminate any excess of salt, removing also coarse remains of animals and plants. After that, samples were dried at 60°C and homogenized by grinding in an agate mortar. The powders (15g for each sample) were sent to ACMELabs (Canada) for analysis. A modified Aqua Regia solution (1 part concentrated hydrochloric acid to 1 part nitric acid to 1 part deionized water) was added to each sample and digested at 90° for 1 h. After cooling the solution was made up to final volume of 300 ml with 5% HCl. The concentrations of 53 chemical elements (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hf, Hg, In, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Pd, Pt, Rb, Re, S, Sb, Sc, Se, Sn, Sr, Ta, Te, Th, Ti, Tl, U, V, W, Y, Zn, Zr) were determined by a Perkin Elmer Elan 6000 inductively coupled

plasma mass spectrometer (ICP-MS). To evaluate the analysis quality, an ACME's in-house Reference Material of soil, named DS8, was used.

4.3.3 Sampling, sample preparation and analysis of feathers

Every year, a number of flamingo colonies are subjected to ringing operations coordinated by the Flamingo Specialist Group (FSG). We collected our samples from chicks caught for ringing, therefore no bird was caught specifically for this study. A small number of scapular feathers was sampled from random individuals 5 to 8 weeks old. The sampling from fledglings during ringing operations met the need to collect many individuals in a short time to reduce the disturbance on breeding birds (Furness and Camphuysen, 1997). The feathers were stored in plastic bags and kept at room temperature. To limit the analytical effort, but to represent each colony well, composite samples (*n*=74) were obtained by pooling an equal weight of feathers taken from 3 individuals. We assumed that 250 - 400 mg obtained from 3 whole feathers avoided dependence on a single feather. Therefore, each pooled sample was a composition of 9 feathers from 3 different flamingos, each sampled only once for this study. In addition, a group of 7 samples was prepared by using a pool of feathers of 12 adult flamingos living in captivity in a single corral (Bioparco Zoo, Rome, ZOO) (Fig. 4.1). In such captive condition, it was not possible to assign the feathers to each individual, therefore all adult feathers were thoroughly mixed and 7 samples prepared from this mixture, in order to obtain a mean value for flamingos living in unnatural conditions. The number of samples is reported in Table 4.1.

Table 4.1 – The number of samples from each sampling site. Each sample is composed of three specimens, pooling three feathers from each one, except for ZOO samples (each sample is obtained from the same mix of feathers of 12 birds).

Site abbreviation	Site name	Number of pooled feather samples
CAG	Macchiareddu saltpans (Cagliari, Sardinia, Italy)	21
CAM	Étang du Fangassier (Camargue, Southern France)	19
COM	Comacchio saltpans (North Eastern Italy)	19
ODI	Odiel marshlands (Huelva, Southern Spain)	15
ZOO	Bioparco di Roma (Roma, Central Italy)	7

Following the most frequently adopted washing method in literature (Espín *et al.*, 2014 and e.g. Jaspers *et al.*, 2004), all the feathers were first individually rinsed with distilled water and vigorously washed in deionized water (milli-Q). They were then placed in an acetone solution (1 mol·L−1) and then again in deionized water to remove loosely adherent external contamination. In order to enhance the removal of particles, each feather was gently rubbed to ruffle the barbs during every washing step. The washed feathers were placed into an open case, which was coated

with greaseproof paper and divided into compartments to avoid there being any contact between the different specimens and between the container and the samples. They were then dried for 24 h at 40 ◦C. Each dried feather was finely ground using stainless steel scissors and gloves, and the dry weight was determined using an electronic 'Sartorius' BP310S balance to the nearest 0.001 g. Chemical analysis was performed at the BGR laboratory in Germany by using atomic fluorescence spectrometry (Instrument PSA 10.035 Millennium Merlin 1631) for Hg and by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or inductively coupled plasma quadrupole-based mass spectrometry (ICP-QMS) for the other elements. Blanks to check possible contaminations and three Certified Reference Materials (CRMs) were included in batches sent for analysis (DORM-2, ERM-CE278, and BCR-414). For feather samples, 58 chemical elements were analysed (Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Hg, 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). The CRM results included Ag, Al, As, Cd, Co, Cr, Cu, Fe, Hg, K, Mn, Mo, Ni, Pb, Sc, Se, Sn, Sr, Tl, V, and Zn, and were in agreement with certified values.

4.3.4 Data treatment

Inter-element relations were analysed by using Spearman's correlation coefficients for major and trace elements in sediments and nonparametric statistical methods were applied since distributions failed the normality test. To compare the concentrations found in captive flamingos with the values found in wild birds, the 95% confidence interval of each distribution was calculated as 95th percentile (not reported). Moreover, concentrations were compared between sites both for sediments and feathers, and between nest and wet sediments within sites by using the non-parametric Mann-Whitney test (Reimann *et al.*, 2008). The significance level considered is $p < 0.05$. An in-depth investigation of feather results was made through Factor Analysis (FA), in order to clarify the complex multivariate relationships among elements. This statistical method allowed us to reason on a lower number of variables identifying factors driving the distribution of the samples in the multidimensional space formed by all analyzed elements (Joliffe, 1986). The factors worked as tools suitable for the interpretation of the origin of most elements. Before running the FA, natural log of each data value was calculated. The factors were extracted as principal components and then rotated according to the Varimax criterion.

All statistical analyses and graphs were carried out using SPSS 14.0 software. Maps were produced using Quantum Gis 2.2 software downloadable at http://www.qgis.org/it/site/. Sampling site acronyms have been overlapped on a void map of Mediterranean Sea (downloaded at http://d-maps.com/carte.php?num_car=3138&lang=en), while toponyms and sampling points have been overlapped on satellite images of each site, downloaded from National Geographic

MapMachine (http://maps.nationalgeographic.com), after photo editing performed with Photofiltre 7.1.1. software.

4.4 Results and discussion

4.4.1 Geochemical data on sediments sampled from flamingo breeding sites.

Table 4.2 reports means (m) and standard deviations (SD) of each group of 6 samples per site (3 nest and 3 wetland sediment samples) and significant differences between nest material and wet sediments sampled close to the islet ($p<0.05$). Also significant differences between sites are reported, both for nest and wet sediments ($p<0.05$). Germanium, Hf, In, Pd, Pt, Re, S, Ta, Te, and W were excluded from the present work since they were below the limit of detection in most or all sediment samples. Here below, abbreviations used in table 4.2 are reported:

NEST = samples collected from flamingo's nests

WET = samples collected from the wetland floor (3, 6, and 9 meters off the islet)

*MW-test columns report only significant differences. Sites not significantly different from any other are not indicated in the sequences.

Most of the elements are significantly higher in ODI wet sediments, but Cd in this site is the lowest both in nest and wet sediments. Odiel can also be identified for negligible presence of Ca and Sr in comparison to the other sites (with CAM nests significantly richer in both elements), but higher levels of As, Au, Ce, Cs, Cu, La, Ti, V, Zn, Zr, and many other elements such as Bi, Co, Fe, Ga, Hg, K, Li, P, Pb, Rb, Sb, Sc, Sn, Th (especially in wet sediments), and Mo (especially in nests). No important anomalies are reported in CAM for metals: only Ag and Ba are significantly higher than other sites in wet sediments. Noticeably, CAG reports higher enrichments in nest material of Ag, Ba, Hg, and Pb, and in both types of sample Cd is more concentrated than other sites. Comacchio samples are significantly richer in Al (wet sediments), B, Cr and Rb (nests), Mg and Ni (both). Scattered Sr-rich samples are present in all sites but ODI. Many significant differences have been found between nest material and wet sediment samples, especially in CAG and ODI. Almost invariably, wet sediments in ODI are richer in trace elements than nests (except Cs). The opposite trend characterizes CAM samples. The sign of the differences in CAM is more variable: As, Ca, Hg, Sb, and Se are higher in nests; Bi, Mo, Na, Tl, and Zr in wet samples. Interestingly, COM shows similar concentrations in nests and wet sediments for most samples, but Pb is significantly more concentrated in nests, while U and Mo in wet.

Evident metal pollution in ODI samples is not surprising, as it is well known that the Odiel and Tinto rivers release a significant load of contaminants associated to acid mine drainage originating from the Iberian Pyrite Belt (Borrego *et al.*, 2002; Nieto *et al.*, 2007). In addition, from 1966 paper industries and copper foundries were established in the Huelva area, enhancing the dispersion of pollutants (Grande *et al.*, 2000) and contributing to make this system one of the

most polluted, and studied, in Western Europe. According to Morillo *et al.*, (2008), As, Cd, Cu, Pb, and Zn in streams flowing through Odiel marshes could be capable of producing noxious effects on aquatic organisms. Cadmium and Zn were found to be the most mobile among the elements studied while Cr, Fe, Ni, and As are strongly linked to the sediments. Cossa *et al.*, (2001) investigated the distribution of Hg into the Tinto-Odiel Estuary, Rio Huelva and Cadiz Gulf finding concentrations higher than those recorded in the pre-industrial age. Nelson and Lamothe (1993) described an enrichment of Sb (150-300 mg/kg) in respect of background values (1-5 mg/kg) in the estuarine system of South Western Spain, attributable to mining.

In the Camargue geochemical studies are scarce, especially in the coastal ponds, but a recent investigation sampled some sediment cores from the early tract of the two Rhône arms and from a sub-coastal river. Chromium, Co and Ni are of lithogenic origin, but most top-soils were slightly enriched in metals: Pb>Cd>Hg>Cu>Zn. Nevertheless, the load of metals during the last decades seems to be low compared with most of draining areas of rivers impacted by human activities (Ferrand, 2010). This seems to be confirmed by our dataset, except for unexpected relatively high levels of Ag.

Santa Gilla Lagoon (CAG) has been exposed to the discharge of industrial wastes containing Cr, Hg, Pb and Zn since the mid-1960s (Degetto, 1986). Degetto *et al.*, (1997) reported a general improvement of the situation of the lagoon after a restoration project started in 1986. However, the concentrations measured in a central-western sector of the lagoon were still high (Degetto *et al.*, 1992). Recently, high Hg levels were found in flamingo fledgling feathers (Borghesi *et al.*, 2011). The present study reveals relatively high concentrations of Ag, Ba, Bi, and, as expected, Hg, Pb, and Zn in Macchiareddu saltpans.

Anomalies of Cr and Ni in COM sediments were also expected, as near-surface soils surrounding the wetland are characterized by high concentrations of these metals (Amorosi and Sammartino, 2007). Enrichments of Cr and Ni are thought to be natural inputs related to sediment provenance (Amorosi *et al.*, 2002; Amorosi, 2012; Migani *et al.*, 2015; Borghesi *et al.*, submitted).

Geochemical data from Rome Zoo are not available, but the area is well known to present strong anomalies of Zr, U, and W in soils, and, to some extent of Hg, Ce, Co, and La (Cicchella *et al.*, 2015; Salminen *et al.*, 2005).

A correlation matrix including the subset of 31 elements available both from sediments and feathers is presented as a useful tool to identify the associations between metals in the sediments (Tab. 4.3).

Table 4.2 – Descriptive statistics of element concentrations in sediments for each sampling site. Significant differences between nest soil and wet sediment are highlighted by grey background. Differences between sites are summarized in the last two columns (for nest and wet, respectively). Single capital letters A, B, C, D are used for brevity, and correspondent to CAG, CAM, COM, and ODI, respectively. Italic font: elements not available for feathers.

	A		B		C		D		MW test (p<0.05)	MW test (p<0.05)
N=6	CAG		CAM		COM		ODI			
N=3	NEST	WET	NEST	WET	NEST	WET	NEST	WET	NEST*	WET*
(mg/kg)	m±SD	m±SD	m±SD	m±SD	m±SD	m±SD	m±SD	m±SD		
Ag	0.164±0.026	0.049±0.021	0.110±0.011	0.114±0.014	0.082±0.017	0.079±0.038	0.031±0.010	0.045±0.018	A>B=C>D	B>C>A=D
Al	13213±2462	6267±4960	8000±265	8783±1894	18300±2651	13733±5631	17850±1906	18700±1992	A=C=D>B	C>D>A=B
As	7.29±0.13	1.87±1.45	10.42±0.78	7.52±1.15	7.20±1.83	4.83±1.36	33.25±1.38	106.02±22.64	D>B>A=C	D>B=C>A
Au	0.002±0.001	0.002±0.001	0.002±0.001	0.002±0.001	0.003±0.001	0.003±0.001	0.004±0.001	0.006±0.001	D>A=B	D>A=B=C
B	61.9±10.6	36.0±28.2	45.5±4.0	38.3±1.5	76.0±8.7	61.3±19.1	61.0±4.0	60.7±9.9	C>A=D>B	C=D>B
Ba	127.0±41.7	32.4±12.1	58.7±2.8	63.1±11.2	45.3±6.2	46.0±6.6	26.3±1.7	24.6±0.6	A>B>C>D	B>C>A=D
Be	0.74±0.030	0.37±0.31	0.57±0.06	0.70±.017	0.83±0.12	0.67±0.31	0.73±0.06	0.83±0.21	C=D>B	A=B=C=D
Bi	0.60±0.16	0.24±0.10	0.18±0.02	0.22±0.01	0.44±0.03	0.34±0.18	0.55±0.03	0.83±0.03	A=D>C>B	D>A=B=C
Ca	84300±14214	126717±43402	158517±2253	137767±16461	36633±16936	89467±71095	1833±586	3567±1193	B>A=C>D	A=B=C>D
Cd	0.31±0.10	0.27±0.01	0.17±0.02	0.19±0.02	0.14±0.06	0.20±0.02	0.08±0.04	0.08±0.07	A>B=C>D	A>B=C>D
Ce	24.7±4.0	8.2±6.5	11.8±1.5	13.3±1.6	21.3±3.2	17.8±5.7	37.2±0.5	36.8±5.4	D>A=C>B	D>A=B=C
Co	7.4±0.4	3.1±2.5	6.7±0.8	8.5±2.1	15.1±1.7	11.5±3.9	11.4±0.1	15.1±5.2	C>D>A=B	D>B=C>A
Cr	17.2±2.3	7.2±5.4	15.6±1.00	16.9±3.5	75.6±11.5	58.0±24.1	37.1±3.2	38.9±3.4	C>D>A=B	C=D>B>A
Cs	1.53±0.24	0.87±0.59	1.03±0.14	1.17±0.15	1.43±0.25	1.11±0.20	2.13±0.04	1.66±0.24	D>A=C>B	D>B=C>A
Cu	20.7±1.2	9.0±4.7	19.6±1.9	16.9±2.5	33.8±3.9	31.6±9.5	70.6±4.8	111.2±69.5	D>C>A=B	D>C>B>A
Fe	17263±2311	7350±5706	13467±1457	15483±3018	28533±2113	20633±7068	27767±1258	39417±1575	C=D>A>B	D>B=C>A
Ga	3.79±0.51	1.80±1.35	2.35±0.13	2.48±0.43	5.52±0.55	3.90±1.61	5.72±0.24	6.17±0.55	C=D>A>B	D>A=B=C
Hg	0.13±0.05	0.05±0.03	0.05±0.02	0.02±0.01	0.06±0.01	0.06±0.03	0.04±0.01	0.15±0.02	A>B=C=D	D>B=C>A
K	23538±6557	22800±6393	18033±1050	16117±4000	25817±1581	18567±7223	27050±786	27433±1429	A=C=D>B	D>B=C
La	12.5±2.6	4.1±3.2	5.7±0.6	6.2±1.0	9.6±1.7	8.0±2.7	17.5±0.8	15.9±2.2	D>A>C>B	D>A=B=C
Li	19.9±3.1	9.2±7.2	17.4±0.8	20.6±3.4	40.4±5.7	29.6±12.3	35.1±2.4	42.6±1.5	C=D>A=B	D>B=C>A
Mg	8163±1359	3067±2538	8600±1054	9183±1125	17633±1750	15433±1537	7317±592	8867±1332	C>A=B=D	C>B=D>A
Mn	680±188	133±148	501±35	604±75	573±93	903±585	552±27	695±228	A=B=C=D	B=C=D>A
Mo	1.0±1.1	3.3±1.9	0.3±0.1	0.5±0.1	0.9±0.1	2.4±1.0	2.3±0.2	3.9±1.7	D>A=C>B	A=C=D>B
Na	4209±126	357±172	4793±1221	15700±1223	8375±4454	7633±246	1967±304	9702±2502	B=C>D>A	B>D>C>A
Nb	0.50±0.08	0.23±0.14	0.40±0.01	0.31±0.06	0.43±0.09	0.38±0.18	0.20±0.05	0.25±0.05	A=B=C>D	A=B=C=D
Ni	20.1±2.6	8.4±4.5	20.5±2.9	22.9±4.2	78.0±9.4	63.2±27.3	21.4±1.1	24.3±1.1	C>A=B=D	C>B=D>A
P	1598±561	202±148	3902±371	555±100	983±381	587±156	620±52	950±223	B>A>C=D	D>B=C>A
Pb	68.2±12.8	31.9±19.8	20.3±1.4	18.7±2.9	51.4±10.4	28.1±5.9	45.9±2.6	79.9±2.7	A>C=D>B	D>A=B=C
Rb	25.4±5.6	14.4±11.8	13.1±0.8	13.6±1.9	31.8±6.0	23.2±9.7	39.3±1.3	35.7±2.9	C>D>A>B	D>C>A=B
Sb	0.72±0.13	0.37±0.23	0.43±0.07	0.27±0.06	0.43±0.16	0.33±0.17	1.05±0.07	1.33±0.17	A=D>B=C	D>A=B=C
Sc	2.8±0.4	1.3±0.9	2.1±0.2	2.7±0.4	4.8±0.9	3.4±1.1	4.1±0.2	4.8±0.3	C=D>A>B	D>A=B=C
Se	0.95±0.10	0.38±0.18	0.97±0.12	0.37±0.18	0.73±0.21	0.77±0.38	0.42±0.13	0.62±0.09	A=B>C=D	A=B=C=D
Sn	1.10±0.25	0.52±0.35	0.65±0.15	0.55±0.05	1.38±0.19	1.27±0.25	1.38±0.13	2.43±0.84	A=C=D>B	D>C>A=B
Sr	494±54	786±197	699±62	626±126	212±84	696±577	29±8	43±10	B>A>C>D	A=B=C>D
Th	4.3±0.5	2.2±1.9	2.2±0.3	2.9±0.5	5.2±1.5	4.0±1.1	5.7±0.4	5.1±0.6	C=D>A>B	D>A=B=C
Ti	0.007±0.001	0.006±0.004	0.005±0.001	0.005±0.001	0.006±0.001	0.006±0.001	0.016±0.002	0.015±0.002	D>A=B=C	D>A=B=C
Tl	0.16±0.02	0.09±0.04	0.09±0.01	0.13±0.02	0.15±0.01	0.14±0.05	0.12±0.01	0.13±0.02	A=C>D>B	A=B=C=D
U	0.93±0.06	1.10±0.59	0.62±0.03	0.73±0.12	0.88±0.20	1.63±0.40	0.92±0.03	1.60±0.44	A=C=D>B	C=D>A=B
V	24.0±2.3	10.8±5.5	14.5±1.3	16.8±3.5	36.5±4.8	28.3±9.1	44.2±1.8	53.8±4.3	D>C>A>B	D>A=B=C
Y	9.4±1.0	3.6±2.9	7.2±0.7	8.4±1.2	11.3±1.6	10.1±2.0	8.2±0.4	11.5±0.5	A=C>B=D	B=C=D>A
Zn	107±18	52±29	61±6	54±7	108±4	77±44	166±2	323±140	D>A=C>B	D>A=B=C
Zr	2.3±1.4	1.9±0.6	0.6±0.1	1.3±0.3	1.5±0.1	1.0±0.4	4.2±1.2	3.8±1.2	D>A=C>B	D>A=B=C

Table 4.3 – Significant Spearman's correlations between trace elements in sediments (p<0.01, two tails). All sites are included in the analysis. Strong correlations (rS>|0.70|) are highlighted using bold font.

	Ag	Al	As	Ce	Co	Cr	Cs	Cu	Fe	Ga	Hg	K	La	Li	Mg	Mn	Mo	Na	Nb	Ni	Pb	Rb	Se	Sr	Th	Ti	U	V	Y	Zn	Zr
Ag	---																														
Al		---																													
As			---																												
Ce		**0.857**	0.628	---																											
Co		**0.795**	0.519	0.560	---																										
Cr		**0.741**			**0.795**	---																									
Cs		**0.820**		**0.914**			---																								
Cu		0.549	**0.834**	0.633	**0.726**			---																							
Fe		**0.899**	**0.749**	**0.829**	**0.856**	0.655	0.699	**0.732**	---																						
Ga		**0.986**	0.557	**0.897**	**0.801**	0.699	**0.827**	0.629	**0.939**	---																					
Hg				0.536							---																				
K		0.695		**0.711**			0.684		0.545	**0.703**	0.601	---																			
La		**0.836**	0.551	**0.988**			**0.933**	0.568	**0.759**	**0.861**	0.555	**0.730**	---																		
Li		**0.923**	0.559	**0.729**	**0.929**	**0.826**	0.656	0.648	**0.953**	**0.932**			0.663	---																	
Mg					0.695	**0.870**								0.619	---																
Mn																---															
Mo	-0.639																---														
Na																		---													
Nb	**0.832**																-0.569		---												
Ni		0.531			0.656	**0.940**								0.625	**0.931**					---											
Pb		0.630	0.526	**0.733**			0.591		0.580	0.654	**0.874**	**0.772**	**0.749**								---										
Rb		**0.957**		**0.920**	0.674	0.586	**0.905**	0.573	**0.836**	**0.962**		**0.798**	**0.913**	**0.826**							0.684	---									
Se	0.674										0.533								**0.766**				---								
Sr		**-0.859**	-0.556	**-0.846**	-0.683		**-0.791**	-0.624	**-0.836**	**-0.898**		**-0.759**	**-0.816**	**-0.814**							-0.606	**-0.880**		---							
Th		**0.962**		**0.862**	0.692	0.656	**0.890**		**0.814**	**0.940**		**0.705**	**0.860**	**0.838**							0.610	**0.964**		**-0.828**	---						
Ti		0.660	0.693	**0.878**			**0.844**	0.628	**0.702**	**0.723**		0.612	**0.865**	0.565							0.585	**0.783**		-0.685	0.686	---					
U																	**0.703**										---				
V		**0.927**	**0.714**	**0.906**	**0.792**	0.624	**0.782**	**0.729**	**0.972**	**0.968**		0.627	**0.850**	**0.926**							0.613	**0.911**		**-0.875**	**0.868**	**0.786**		---			
Y		**0.841**		0.633	**0.812**	**0.726**	0.580		**0.813**	**0.802**			0.598	**0.842**	**0.732**					0.640		0.695		-0.616	**0.786**			**0.744**	---		
Zn		0.616	**0.873**	**0.713**	0.682			**0.956**	**0.771**	0.688	0.523	0.524	0.659	0.646							0.654	0.643		-0.687	0.519	0.655		**0.770**		---	
Zr		0.554	0.600	**0.757**			0.678	**0.708**	0.564	0.613		0.653	**0.755**								0.584	**0.704**		-0.655	0.561	**0.746**		0.659		**0.750**	---

Considering all 4 sites, there are a wide number of significant correlations ($p<0.01$), and many of them strongly positive ($rS>0.70$). Most of the positive correlations involve Al, which is highly correlated to Ce, Co, Cs, Fe, Ga, La, Li, Rb, Th, V and Y ($rS=0.80$ or more). There are also the correlations between Al and Cr ($rS>0.70$), Pb, K, Ti, Zn ($rS>0.60$). This was not unexpected as sampling sites are more or less rich in fine-grained sediments to which many elements of this group are typically associated. Within the group of elements correlated to Al, Cr shows a very high correlation with Ni, in turn highly correlated to Mg, whereas Pb is associated also to Hg, Ti, and Zr. At least two other groups of elements (some of them of environmental concern) are highlighted by the matrix in Table 4.3. They are 1) Ag, Se, and Nb; 2) As, Cu, Fe, and Zn. Negative strong correlations involve Sr, in particular with Al, Ce, Cs, Fe, Ga, K, La, Li, Rb, Th, and V. In aquatic environments, many invertebrates produce aragonite shells, rich in Sr due to the particular structure of this crystal (Kinsman and Holland, 1969; Tesoriero and Pankow, 1996). Aragonite is normally poor in metal contents in respect to silicate minerals, and this may determine the observed pattern. Furthermore, particularly low levels of Ca and Sr found in ODI samples may indicate a locally scarce presence of carbonates of biogenic origin. Titanium seems to have affinity both with Al-group and As-group. Weaker or no correlations are shown by a few elements, such as Mn, Mo, Na and U.

4.4.2 Metal concentrations in feathers

Detectable concentrations in feathers are graphically presented in Figure 4.2, with all 4 colonies pooled together. The elements are ranked by medians with the mean also plotted. Descriptive statistics for each sample site are reported in table 4. Twenty elements have not been used for this paper. Among them, Be, Bi, Eu, Hf, Ho, Lu, Sc, Ta, Tb, Tl, Tm, and Yb, had mostly missing data. Some other elements (B, Ba, Ca, Cd, Ge, Sb, Sn, Te) were affected by high levels in blank samples to the point that we preferred to exclude them from this study.

Median values of almost all elements are lower than corresponding mean values (Fig. 4.2). This tendency is more pronounced for Al, Fe, Ti, Mn, Pb, Cr, Rb, V, As, Co, Ag, Ga, and Cs. More symmetric distributions have been found for Na, Mg, K, Zn, Sr, Cu, and Se (Fig. 4.2). In particular,

Na, K, Zn, Sr, Cu, and Se show a very compact distribution and good accordance between medians and means.

A group of elements (Na, Mg, K, Al, Zn and Fe) have central values higher than 100 mg/kg with Na and Mg concentrations one order of magnitude up. In the range 1-10 mg/kg, there are 5 elements: Sr, Cu, Ti, Mn, and Se. All but Ti are known to be essential elements with biological functions at low concentrations. Mercury, Pb, Ni, and Cr, four non-essential elements and hazardous for the health, show medians of around 1 mg/kg or slightly below. Medians of 7 elements lie between 0.07 and 0.24 mg/kg: Rb, Li, V, Mo, As, Ce, and Co. This group includes essential elements (Li, V, Mo, and Co), one REE (namely Ce), and an important toxic element (As). A group of enriched samples completely separated from the others is evident for As. The remaining 18 elements have medians well below 0.05 mg/kg, including the other 8 REEs (La, Nd, Y, Pr, Sm, Gd, Dy, and Er). All the elements, REEs included, in this wide group are invariably dispersed (especially Ga) with a high density of values in the upper part. Only Zr is an exception.

Figure 4.2 also allows us to compare the data collected from wild birds with the average value obtained by captive adult flamingos. Captive bird means (CM) are evidently lower than the mean values of wild birds (WM) for 31 out of 38 elements. For 20 of these (Na, Mg, K, Al, Zn, Fe, Sr, Ti, Se, Pb, Rb, Li, V, Nd, Y, Ga, Th, Sm, Gd, Dy), CM are lower than the 95% CI of wild data. Within the range of wild bird concentrations (5^{th}-95^{th} percentile) are Mn, Hg, Ni, Cr, As, Ce, Co, La, Cs, Pr, Nb, (CM<WM), and Mo, Zr, U, W (CM approximately equal to WM). Evident exceptions are represented by Ag and Cu, which are so high in captive birds that they are out of range of the entire wild bird dataset. The overdispersion of high density of values in the upper part of many element distributions in feathers (Fig. 4.2) indicates a number of enriched samples (internally or externally). The relatively homogeneous distribution of some elements (Na, Mg, K, Zn, Cu, Sr, and Se) could suggest that they play an important role in the feathers' composition, but without further information this cannot be considered more than a clue.

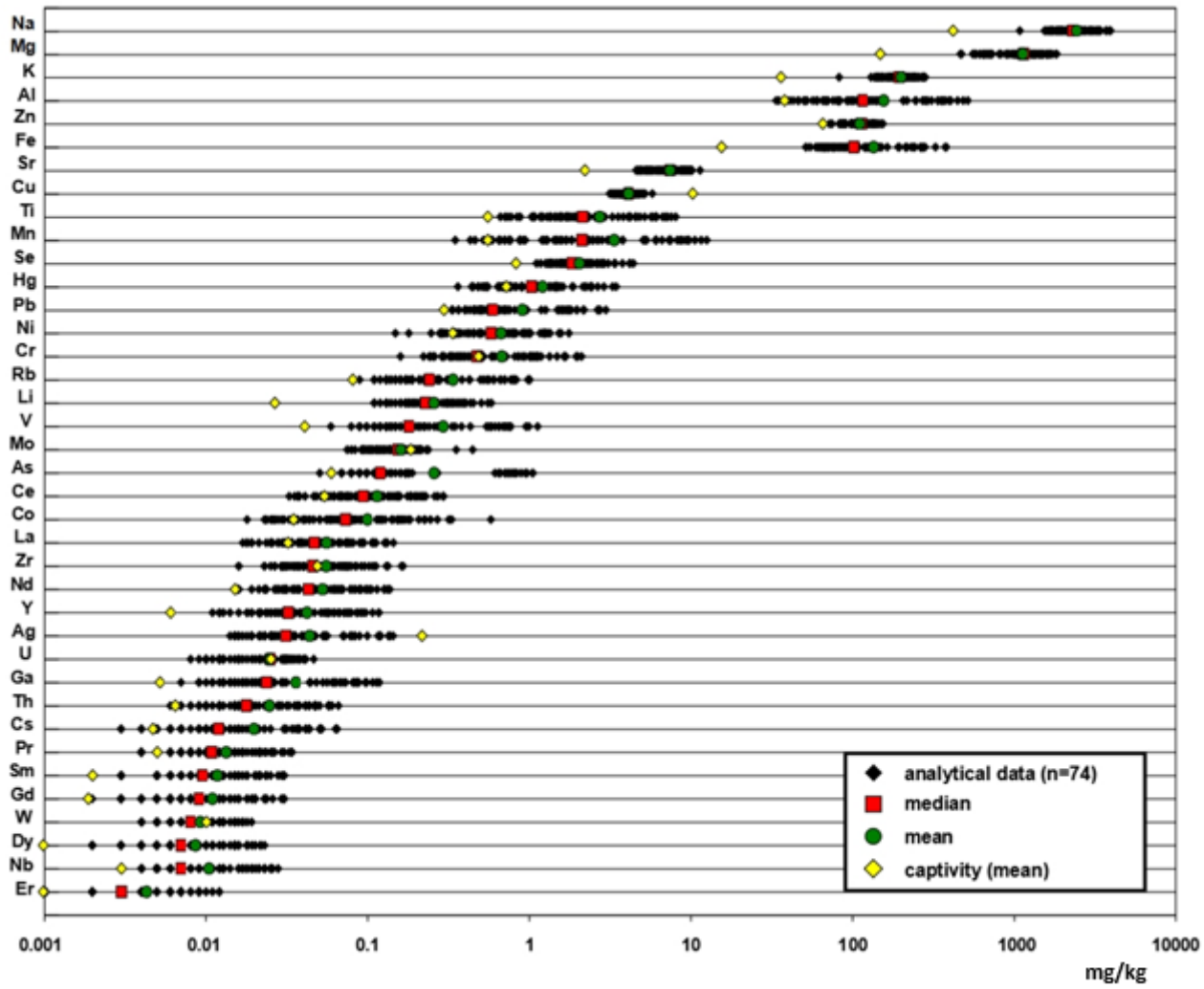

Figure 4.2 – Overall plot of all Flamingo feather concentrations graphically represented by using logarithmic scale (black rhombus). Mean (circle) and median (square) are also plotted, as well as the reference of captive bird (yellow rhombus). Elements are in order of concentration expressed as mg/kg.

Table 4.4 reports medians (M) and median absolute deviations (MAD), means (m) and standard deviations (SD), and ranges of each group of samples per site. Significant differences between sites are also reported ($p<0.05$). For most elements COM shows the highest concentrations. Exceptions are Ag and Na, which are higher in ODI, Co in CAM, and Cu, Er, K, Rb, Sr, Y, and Zr in CAG. Noticeably, feathers from ODI show the lowest levels of Fe and La, very concentrated in sediments from this site (Tab. 4.2).

Table 4.4 – Descriptive statistics of element concentrations in flamingo feathers for each sampling site. Median (M) and median absolute deviation (MAD), mean (m) and standard deviation (SD), range (min-max) are reported. Significant differences between sites are summarized in the last column. Single capital letters A, B, C, D are used for brevity, and correspondent to CAG, CAM, COM, and ODI, respectively.

	A		B		C		D		ZOO (n=7)	MW test (p<0.05)
	CAG (n=21)		CAM (n=19)		COM (n=19)		ODI (n=15)			
(mg/kg)	M±MAD	m±SD	M±MAD	m±SD	M±MAD	m±SD	M±MAD	m±SD	m	
Ag	0.036±0.007	0.040±0.015	0.028±0.004	0.031±0.014	0.019±0.004	0.021±0.007	0.090±0.029	0.096±0.031	0.218	D>A>B>C
	Range (Ra): 0.018-0.082		**Ra:** 0.015-0.072		**Ra:** 0.014-0.036		**Ra:** 0.054-0.145			
Al	53±10	58±17	114±20	120±39	339±61	343±84	119±19	111±24	38	C>B=D>A
	Ra: 34-100		**Ra:** 69-218		**Ra:** 204-511		**Ra:** 66-142			
As	0.100±0.010	0.096±0.031	0.090±0.010	0.094±0.018	0.170±0.020	0.161±0.035	0.790±0.100	0.819±0.143	0.060	C>D>A=B
	Ra: 0.050-0.180		**Ra:** 0.060-0.120		**Ra:** 0.110-0.270		**Ra:** 0.610-1.060			
Ce	0.067±0.019	0.076±0.034	0.075±0.019	0.084±0.038	0.196±0.035	0.201±0.053	0.100±0.020	0.093±0.024	0.054	C>A=B=D
	Ra: 0.035-0.153		**Ra:** 0.033-0.186		**Ra:** 0.112-0.297		**Ra:** 0.051-0.127			
Co	0.031±0.005	0.032±0.008	0.078±0.018	0.103±0.120	0.175±0.030	0.194±0.063	0.072±0.009	0.071±0.013	0.035	B>D>A=C
	Ra: 0.018-0.046		**Ra:** 0.035-0.585		**Ra:** 0.118-0.333		**Ra:** 0.044-0.091			
Cr	0.59±0.30	0.64±0.48	0.39±0.08	0.42±0.10	1.12±0.19	1.24±0.38	0.38±0.02	0.38±0.05	0.481	C>A=B=D
	Ra: 0.160-2.000		**Ra:** 0.260-0.610		**Ra:** 0.720-2.130		**Ra:** 0.290-0.490			
Cs	0.006±0.002	0.007±0.003	0.016±0.004	0.017±0.007	0.043±0.008	0.045±0.012	0.010±0.002	0.009±0.002	0.005	C>B=D>A
	Ra: 0.003-0.015		**Ra:** 0.007-0.037		**Ra:** 0.025-0.065		**Ra:** 0.005-0.013			
Cu	3.7±0.28	3.76±0.40	4.68±0.41	4.66±0.48	4.09±0.24	3.95±0.36	4.19±0.36	4.20±0.39	10.371	A>B=C=D
	Ra: 3.240-4.730		**Ra:** 3.910-5.780		**Ra:** 3.120-4.400		**Ra:** 3.660-4.740			
Dy	0.004±0.002	0.005±0.002	0.006±0.001	0.007±0.003	0.016±0.003	0.016±0.004	0.007±0.001	0.006±0.002	0.001	C>A=B=D
	Ra: 0.002-0.011		**Ra:** 0.003-0.015		**Ra:** 0.010-0.023		**Ra:** 0.003-0.009			
Er	0.002±0.001	0.003±0.001	0.003±0.001	0.003±0.002	0.008±0.001	0.008±0.002	0.003±0.001	0.003±0.001	0.001	A=B>C>D
	Ra: 0.001-0.006		**Ra:** 0.001-0.007		**Ra:** 0.005-0.012		**Ra:** 0.002-0.004			
Fe	73.4±10.6	78.4±19.3	77.9±10.9	85.4±25.0	263.0±32	255.3±55.6	124.0±7.0	120.6±19.8	15.6	C>A>B>D
	Ra: 52-126		**Ra:** 51.8-148.0		**Ra:** 165.0-376.0		**Ra:** 78.2-151.0			
Ga	0.014±0.004	0.016±0.007	0.024±0.005	0.025±0.009	0.081±0.017	0.080±0.021	0.022±0.004	0.022±0.004	0.005	C>A>B=D
	Ra: 0.007-0.035		**Ra:** 0.013-0.049		**Ra:** 0.045-0.117		**Ra:** 0.014-0.029			
Gd	0.005±0.002	0.006±0.003	0.008±0.002	0.008±0.004	0.020±0.004	0.021±0.005	0.009±0.002	0.008±0.002	0.002	C>A=B>D
	Ra: 0.002-0.012		**Ra:** 0.003-0.020		**Ra:** 0.013-0.031		**Ra:** 0.005-0.012			
Hg	1.478±0.302	1.649±0.818	0.885±0.400	1.167±0.665	1.053±0.372	1.129±0.644	0.782±0.082	0.785±0.163	0.734	C>A=B=D
	Ra: 0.452-3.485		**Ra:** 0.446-2.389		**Ra:** 0.361-2.906		**Ra:** 0.520-1.207			
K	199±25	202±38	168±23	173±44	201±15	208±30	213±23	213±35	37	A>D>B=C
	Ra: 141-269		**Ra:** 84-276		**Ra:** 154-274		**Ra:** 165-285			
La	0.034±0.008	0.038±0.017	0.040±0.009	0.043±0.019	0.094±0.018	0.098±0.025	0.047±0.008	0.044±0.011	0.033	C>A=B>D
	Ra: 0.018-0.077		**Ra:** 0.017-0.094		**Ra:** 0.054-0144		**Ra:** 0.025-0.061			
Li	0.200±0.020	0.203±0.035	0.250±0.040	0.270±0.062	0.390±0.050	0.395±0.083	0.160±0.020	0.152±0.027	0.027	C>D>A=B
	Ra: 0.130-0.270		**Ra:** 0.200-0.400		**Ra:** 0.250-0.580		**Ra:** 0.110-0.190			
Mg	1379±214	1402±221	1318±213	1308±295	1019±121	1015±199	639±64	639±97	150	C>B=D>A
	Ra: 1054-1813		**Ra:** 566-1843		**Ra:** 667-1503		**Ra:** 463-824			
Mn	0.720±0.150	0.746±0.240	2.650±0.610	2.667±0.989	7.470±1.400	8.107±2.133	2.070±0.260	1.935±0.382	0.554	C>A=B=D
	Ra: 0.350-1.290		**Ra:** 1.230-5.290		**Ra:** 5.010-12.700		**Ra:** 1.200-2.460			
Mo	0.169±0.017	0.189±0.075	0.139±0.017	0.143±0.026	0.194±0.019	0.193±0.025	0.099±0.008	0.100±0.015	0.189	C>D>A=B
	Ra: 0.119-0.447		**Ra:** 0.096-0.198		**Ra:** 0.146-0.235		**Ra:** 0.074-0.134			
Na	3106±333	3084±530	1948±229	2047±410	2120±201	2037±320	2450±206	2409±315	422	D>C>A=B
	Ra: 2097-3950		**Ra:** 1087-2947		**Ra:** 1560-2653		1812-2890			
Nb	0.009±0.003	0.010±0.005	0.005±0.001	0.006±0.003	0.019±0.004	0.019±0.005	0.005±0.001	0.005±0.001	0.003	C>B=D>A
	Ra: 0.004-0.026		**Ra:** 0.003-0.013		**Ra:** 0.010-0.028		**Ra:** 0.003-0.007			
Nd	0.028±0.008	0.032±0.014	0.037±0.009	0.040±0.019	0.092±0.015	0.095±0.024	0.046±0.009	0.043±0.011	0.015	C>B>D>A
	Ra: 0.015-0.065		**Ra:** 0.015-0.091		**Ra:** 0.063-0.137		**Ra:** 0.023-0.060			
Ni	0.520±0.230	0.581±0.384	0.540±0.110	0.581±0.217	0.990±0.190	1.058±0.255	0.350±0.020	0.409±0.162	0.341	C>A=B=D
	Ra: 0.150-1.750		**Ra:** 0.300-1.250		**Ra:** 0.670-1.600		**Ra:** 0.280-0.830			
Pb	0.47±0.03	0.46±0.08	0.54±0.07	0.57±0.15	1.76±0.23	1.93±0.51	0.63±0.09	0.68±0.13	0.294	C>D>A=B
	Ra: 0.330-0.650		**Ra:** 0.340-0.970		**Ra:** 1.190-2.970		**Ra:** 0.490-0.950			
Pr	0.008±0.002	0.009±0.004	0.009±0.002	0.010±0.005	0.024±0.004	0.024±0.006	0.012±0.002	0.011±0.003	0.005	C>A=B=D
	Ra: 0.004-0.018		**Ra:** 0.004-0.023		**Ra:** 0.013-0.034		**Ra:** 0.006-0.015			
Rb	0.150±0.020	0.158±0.043	0.210±0.040	0.233±0.076	0.700±0.100	0.710±0.170	0.270±0.010	0.261±0.033	0.081	A=C=D>B
	Ra: 0.090-0.260		**Ra:** 0.150-0.430		**Ra:** 0.430-1.000		**Ra:** 0.190-0.320			
Se	2.830±0.420	2.975±0.707	1.620±0.210	1.622±0.309	1.540±0.140	1.539±0.182	1.970±0.110	2.022±0.209	0.840	C>B>A>D
	Ra: 1.850-4.380		**Ra:** 1.110-2.150		**Ra:** 1.320-1.990		**Ra:** 1.580-2.350			
Sm	0.006±0.002	0.007±0.003	0.008±0.002	0.009±0.004	0.021±0.004	0.022±0.005	0.009±0.002	0.009±0.002	0.002	C>B>D>A
	Ra: 0.003-0.014		**Ra:** 0.003-0.020		**Ra:** 0.013-0.031		**Ra:** 0.005-0.013			
Sr	7.29±0.730	7.51±1.17	8.05±1.030	8.02±1.56	9.260±0.740	8.873±1.060	5.230±0.320	5.206±0.418	2.183	A>D>B=C
	Ra: 5.61-10.20		**Ra:** 4.85-11.40		**Ra:** 7.070-10.100		**Ra:** 4.520-5.930			
Th	0.019±0.007	0.023±0.013	0.015±0.004	0.015±0.007	0.044±0.006	0.045±0.011	0.014±0.003	0.013±0.004	0.006	C>A=B=D
	Ra: 0.007-0.059		**Ra:** 0.006-0.036		**Ra:** 0.026-0.066		**Ra:** 0.007-0.020			
Ti	1.660±0.440	1.855±0.963	1.340±0.270	1.503±0.652	5.690±1.290	5.467±1.493	2.250±0.330	2.117±0.485	0.554	C>D>B>A
	Ra: 0.780-4.910		**Ra:** 0.670-2.840		**Ra:** 2.910-8.170		**Ra:** 1.080-2.830			
U	0.031±0.005	0.030±0.006	0.026±0.006	0.027±0.008	0.024±0.003	0.027±0.007	0.012±0.001	0.012±0.002	0.025	C>A=B>D
	Ra: 0.016-0.040		**Ra:** 0.013-0.042		**Ra:** 0.016-0.046		**Ra:** 0.008-0.015			
V	0.140±0.030	0.141±0.050	0.180±0.030	0.185±0.070	0.660±0.090	0.687±0.187	0.180±0.030	0.168±0.038	0.041	C>A=B=D
	Ra: 0.060-0.260		**Ra:** 0.090-0.350		**Ra:** 0.380-1.130		**Ra:** 0.090-0.220			
W	0.010±0.002	0.009±0.003	0.007±0.001	0.008±0.003	0.013±0.002	0.013±0.003	0.006±0.001	0.006±0.001	0.010	C>A>B=D
	Ra: 0.004-0.018		**Ra:** 0.004-0.015		**Ra:** 0.008-0.019		**Ra:** 0.004-0.009			

Table 4.4 – (continued).

Y	0.024±0.006 0.026±0.012 **Ra:** 0.011-0.056	0.032±0.008 0.035±0.015 **Ra:** 0.014-0.077	0.075±0.013 0.080±0.021 **Ra:** 0.047-0.118	0.031±0.006 0.029±0.007 **Ra:** 0.016-0.039	0.006	A=B=C>D
Zn	111±7 113±13 **Ra:** 97-152	90±6 92±11 **Ra:** 73-115	118±8 121±14 **Ra:** 98-153	133±6 127±12 **Ra:** 103-141	66	C>A>B=D
Zr	0.032±0.005 0.036±0.013 **Ra:** 0.016-0.066	0.036±0.007 0.038±0.012 **Ra:** 0.023-0.063	0.085±0.013 0.096±0.033 **Ra:** 0.040-0.165	0.056±0.010 0.057±0.012 **Ra:** 0.040-0.075	0.049	C=D>A>B

4.4.3 Bioaccumulation or external contamination: interpretation of the analytical results in feathers

According to Ek *et al.*, (2004), the residual exogenous contamination capable of masking internal signals of certain trace elements could primarily depend on lithic residual particles firmly adhered to the feather structure. Indeed, taking into consideration the preliminary findings of Weyers *et al.*, (1988), we performed a number of observations with Scanning Electron Microscope (SEM) of some flamingo feather fragments that revealed the presence of residual specks even after thorough washing. The characterization, performed by Energy Dispersive X-Ray *Analysis* (*EDX*), revealed that most residues were consisting of organic dust, probably originated by the feather itself after the preparation, but also lithic fragments were still present, determined as clays and carbonates (unpublished data). In light of that, the commonly used procedures to clean feathers involving deionized water and organic solvents, successfully remove most of surface atmospheric deposition, loosely attached particles and fatty compounds including preening oil (Burger, 1993; Hahn et al, 1993; Weyers *et al.*, 1988), but some soil particles remain.

In their ecotoxicological study, Cardiel *et al.*, (2011) included both a target metal and a metal considered scarcely absorbed at the intestinal level, indicating that if a correlation between the two metals is found in feathers, it should be considered an effect of external contamination rather than bioaccumulation. However, geochemical processes are complex and the associations among trace elements may substantially change at local scale. In our study, we included a large number of elements, geochemical local data, and the feathers were sampled from young birds of known origin, which may carry on their feathers only particles from local soils and have had only a short time to accumulate on feathers an important load of atmospheric deposition.

When attributable to external contamination, strong correlations found in feathers should be correspondent to the correlations found in the sediment sampled within the area frequented by the unfledged birds. Conversely, in the presence of prevalent endogenous bioaccumulation, one can expect that the elements well correlated in the sediments will not be as much correlated

in the feathers, due to the different bioavailability and metabolic ways proper to each element and each group of organisms.

Considering the sediments, correlation coefficients (Tab. 4.3) higher than $rS>0.970$ ($p<0.01$) for all sampling sites can be legitimately considered a sound indication of an association that we should not find in feathers due to the different bioavailability and metabolic ways of flamingo fledglings proper to each element. This is the case of Al-Ga, Fe-V, and Ce-La, represented in all sampling sites with a very similar ratio (Fig. 4.3 a,b,c). On the other hand, for certain elements very strong correlations may exist at site scale, even if the overall correlation coefficient, though significant, is not among the highest. The case of Al-Cr seems indicative, having an overall coefficient equal to 0.741 ($p<0.01$), but coefficients in each site between 0.974 and 0.998 ($p<0.01$), due to a site-specific ratio Al:Cr (Fig. 4.3d).

Simply observing that similar high correlations are present in feathers (Fig. 4.4 a and c), Al, Ce, La, and Ga can be considered of external origin in flamingo feathers, and therefore taken as useful "tools" to assess the prevalent external contribution of other elements having a not so across-the-sites clear pattern.

We are confident that also Fe and V (Fig. 4.4b) can be considered mainly of external origin for CAG, CAM, and COM but they should be handled with caution relatively to ODI.

The case of Cr is less simple (Fig. 4.3d). In all sites it is associated to Al in sediments, with a site-specific ratio depending on the lithological composition of sediments of each site, eventually altered by anthropogenic inputs. Figure 4.4d shows that the correlation Al-Cr in feathers tends to weaken or disappear in all sites. This case does not offer clear evidence of external contamination, and further analysis must be carried out in order to interpret Cr and many other elements.

In this regard, a Factor Analysis (FA) was applied to the analytical results from feathers (Tab. 4.5). Five factors have eigenvalues >1 and were selected, explaining for 89 % of the total variance.

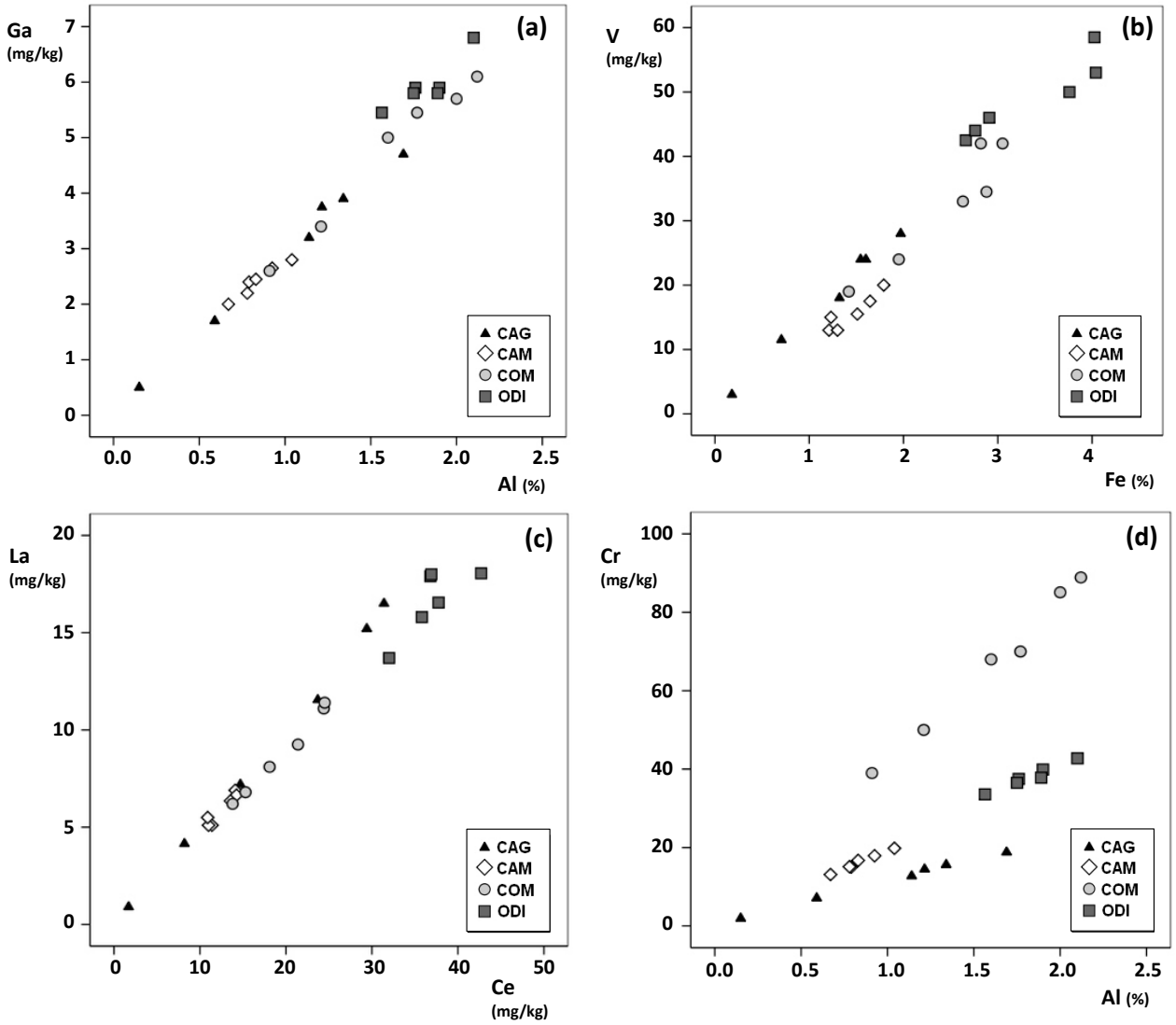

Figure 4.3 a,b,c,d – Ga-Al, V-Fe, and La-Ce are pairs of elements strongly correlated in sediments (r_S>0.970, p<0.01) considering all sites. Even though the sample sizes at site scale are small (n=6), Al and Cr are evidently correlated but at site scale (r_S between 0.974 and 0.998, p<0.01).

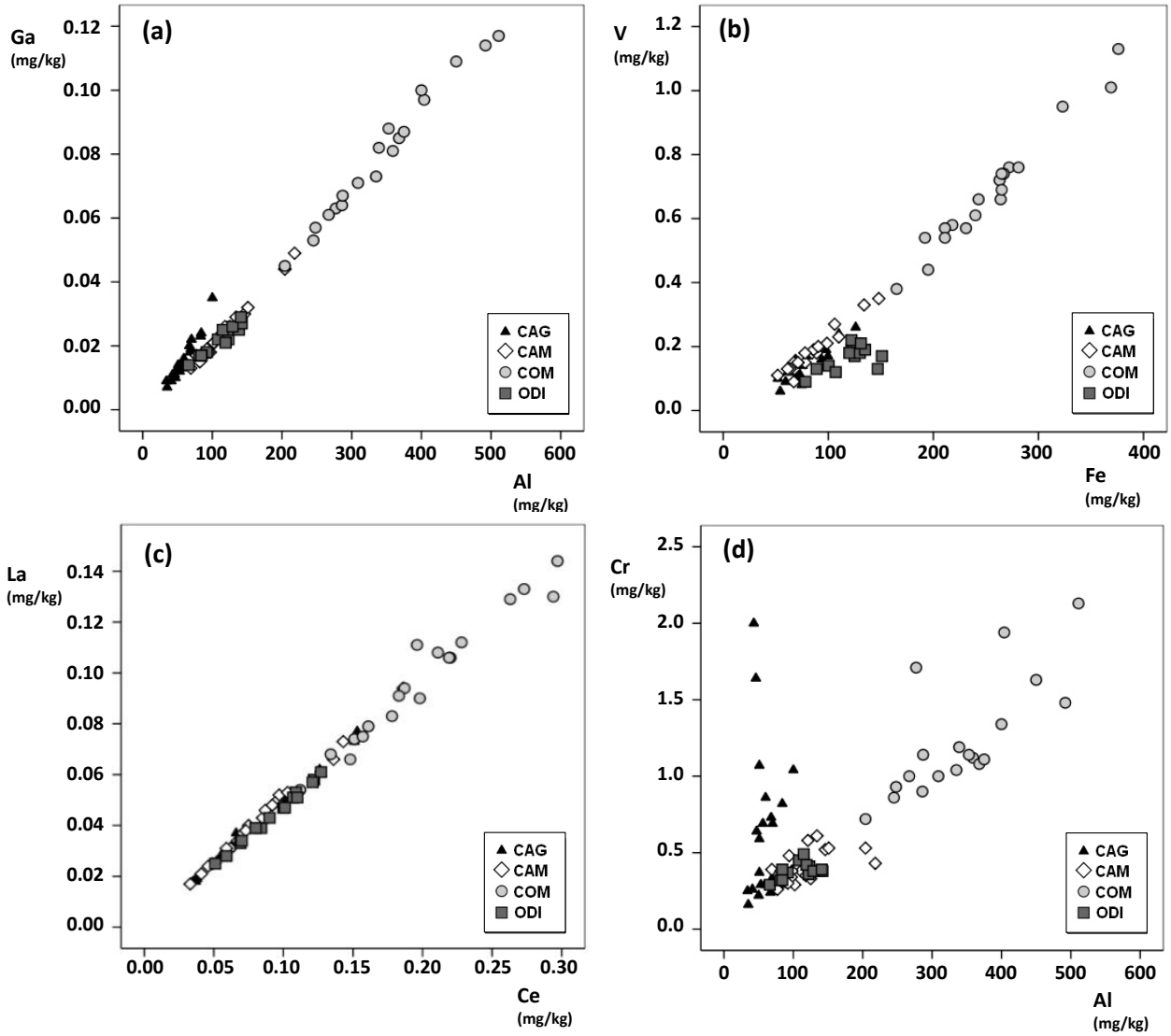

Figure 4.4 a,b,c,d – Ga-Al and La-Ce are pairs of elements highly correlated also in feathers from all sampled sites. Vanadium and Fe are similarly highly correlated except in ODI. Aluminum and Cr show clear anomalies in CAG and COM.

Table 4.5 - The five significant factors resulted from the factor analysis (eigenvalues greater than 1). Rotated factor loadings (those lower than 0.50 have been suppressed), eigenvalues and proportion of variance explained are presented.

Element	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Communality
Ag		-0.707				0.571
Al	0.915					0.960
As		-0.866				0.893
Ce	0.986					0.944
Co	0.790					0.812
Cr	0.575					0.629
Cs	0.893					0.974
Cu					-0.836	0.350
Dy	0.976					0.958
Er	0.964					0.935
Fe	0.924					0.954
Ga	0.939					0.975
Gd	0.978					0.975
Hg			-0.515			0.252
K				0.954		0.534
La	0.982					0.940
Li	0.700	0.638				0.913
Mg		0.890				0.820
Mn	0.858					0.926
Mo		0.794				0.785
Na				0.722		0.785
Nb	0.793					0.900
Nd	0.989					0.955
Ni	0.567					0.617
Pb	0.888					0.885
Pr	0.986					0.945
Rb	0.928					0.952
Se	-0.513		-0.573			0.706
Sm	0.985					0.964
Sr		0.849				0.825
Th	0.848					0.888
Ti	0.930					0.935
U		0.875				0.875
V	0.936					0.974
W						0.562
Y	0.963					0.970
Zn				0.754		0.790
Zr	0.920					0.879
Eigenvalues	23.11	5.22	3.184	1.33	1.20	
Total variance %	55.88	15.69	6.41	6.40	5.18	
Cumulative var.%	55.88	71.57	77.99	84.38	89.57	

Factor 1: (Al, Ce, Co, Cs, Dy, Er, Fe, Ga, Gd, La, Li, Mn, Nb, Nd, Pb, Pr, Rb, Sm, Th, Ti, V, Y, and Zr). This factor accounts for 55.88% of the model variance. It is very probable that it defines the wide group of elements characterizing the residual sediment particles more abundant in the feathers and only partially removed after the washing process. The group includes Al, Ce, La, Ga with a very high positive loading (> +0.90) and many other elements with high or very high positive loadings (> +0.70). Chromium and Ni show moderate positive loadings (> +0.50) indicating that they could contribute to the external contamination to some extent. The information given by factor 1 is consistent with the correlation matrix (Tab. 4.3), due to the grouping of Al, Fe, Ce, Cr, Ga, La, and V. The inclusion of Dy, Er, Gd, Nd, Pr, and Sm to this factor (concentrations not available for sediments) with loadings greater than 0.96, leads us to consider these REEs as good indicators of external contamination. Factor 1 also includes Pb with a high positive (> +0.80), and Se with a moderate negative loading (< -0.50) but these elements, as for Cr, may hide peculiarities that will be discussed later.

Factor 2: (Ag, As, Li, Mg, Mo, Sr, and U). Factor 2 accounts for 15.69% of the total variance. Silver and As enter into factor 2 with high negative loadings (< -0.70), and Mg, Mo, Sr, and U with high positive loadings (> +0.70). In sediments, Mo is the only element significantly positively correlated to U ($rS = 0.703$, $p>0.01$), Ag is negatively correlated to Mo ($rS = -0.639$, $p<0.01$). Silver and Mo are also correlated to Nb ($rS = 0.832$ and $rS = 0.569$, $p<0.01$), but with opposite sign. Strontium is negatively correlated to most of the elements, with the exceptions of Ag, Mg, Mo, U, and some others not grouped in factor 2, not correlated at all (Tab. 4.3). Moreover, factor 2 includes As and Li, in sediments correlated respectively to some and most of metals correlated to Al (which are grouped in factor 1). Lithium appears in factors 1 and 2 with a comparable loading. It is difficult to better define Factor 2, because it probably includes elements with a different fate in feathers, but it cannot be excluded that As, Li, Mg, and Sr could be passed through biological processes. Conversely, it is recommended to consider the bioaccumulation of Ag, Mo, and U in feathers very cautiously.

Factor 3: (Hg and Se). It explains 6.41% of the variance. Mercury and Se appear in this factor with moderate negative loadings (< -0.50). A rather low correlation coefficient was found between Hg and Se ($rS = 0.533$, $p<0.01$), while Se is strongly correlated to Nb in sediments ($rS=0.77$, $p<0.01$), which is missing in factor 3, probably due to a divergent fate in feathers. The FA indicates that Se in flamingos could follow Hg in metabolic processes as a protective response according to Yang *et al.*, (2008).

Factor 4: (K, Na, and Zn). Very high positive loadings are related to K, Na, and Zn (> +0.70), accounting for the 6.40% of the variance in this factor. In sediments, K is well correlated to Al, Ce, Cs, Ga, La, Pb, Rb, Th, and Zr (rS> 0.65, p<0.01) due to the tendency of K to be rich in the fine-grained fraction of sediments. In factor 4, none of these elements follows K. However, Na and K are the most abundant elements in salt and their association in factor 4 may raise suspicions of external contamination. Zinc is known as a constitutive element in feathers (Jaspers *et al.*, 2004) and in factor 4 is not followed by other elements correlated to it in sediment (Cu, As, Fe, Ce, V, and many other elements included in factor 1). This outcome allows to conclude that Zn in feathers prevalently originated from internal input.

Factor 5: (Cu). This factor accounts for 5.18 % of the total variance. It is practically dominated by Cu, with a very high negative loading (-0.84). The natural levels of Cu in coastal wetlands are often altered by human activities, especially by agricultural and industrial inputs. Therefore, it is desirable to understand if the possible presence of external contamination of Cu may mask the internal accumulation or not. An in-depth analysis of this element site per site will be developed in the next paragraph.

4.4.4 Assessing metal pollution by using flamingo feathers

In this paragraph we discuss the insights regarding the sampled flamingo colonies from a point of view of the trace elements, taking in account all the information deduced in this work.

Only two studies on flamingo fledglings are available, one dates back more than two decades ago (Amiard-Triquet *et al.*, 1991), and the second, concerning Hg, analysed data from this survey (Borghesi *et al.*, 2011). Recently, a comparative study used feathers of Little Egret (*Egretta garzetta*) chicks, including a colony breeding in the confluence of Odiel and Tinto rivers (Rubio *et al.*, in press).

Comparisons between feathers from wild birds and captive birds in this study are not supported by sound statistics, due to the different sampling methods and the different sample features (age, condition, nourishment). However, our data from a zoo are useful to highlight the tendency of captive birds to have a metal burden in feathers lower than wild birds, despite the expectations based on the literature stating that adult birds normally show a higher metal accumulation than fledglings (Burger, 1993). Results on captive birds approximate to a “fingerprint” of the geochemical environment in which they live, at least for Ce, Co, La, Hg, U, W, and Zr. However, there are two clear exceptions concerning Ag, and Cu, which are higher in

captive birds and out of the range of the entire wild bird dataset (Fig. 4.2). A tentative explanation of this may be the modern use of Ag and Cu as bactericide and algaecide for pools.

4.4.4.1 Aluminum, Iron, Lithium, Manganese, REEs, and others.

The FA performed on feathers grouped Al with most of the elements already correlated in sediments (Tab. 4.3 and Tab. 4.5). The overall mean in flamingos (154 mg/kg) is within the range of the concentrations reported in unwashed feathers in a recent study on three waterbird species (Lucia *et al.*, 2010). Since it is a metal poorly treated by metabolism (Cardiel *et al.*, 2011) and very abundant in sediments (up to 18,700 ± 1992 mg/kg in ODI), these high burdens suggest that it is difficult to remove clay by using the washing procedures suggested in literature. All this considered, Al can be actually considered one of the key elements for checking external contamination in feather studies through a geochemical approach.

The same may be said for Fe and V associated to Al in clays ($rS = 0.899$ and $rS = 0.927$ in our study, respectively, $p<0.01$) as well as in feathers (Tab. 4.5). Iron is an important blood component and likely enters in keratin structure, but external contamination of Fe could be dominant, as a consequence of the high concentration of Fe in sediment (up to 39,417 ± 1575 mg/kg in ODI) and the capacity (shared with Mn) to form secondary products such as oxides and hydroxides in the fine-grained fraction of sediments. For similar reasons, Li concentrations found in feathers might also be mainly exogenous. However, the biochemical pathways of Li in birds (and feathers) are poorly understood and some biological functions of Li were recently suggested (Ermidou-Pollet and Pollet, 2006). Lanthanides (representing 15 out of 17 REEs) are not thought to be very relevant in biological studies, but the alteration of natural levels of REEs, already in progress, is almost unexplored (Kulaksiz and Bau, 2013). In addition, cytophysiological effects or enzymes interferences for Dy, Eu, Gd, La, Pr and Tb in humans are reported (Pałasz and Czekaj, 2000). This study indicates that REEs found in feathers derive from soil particles captured by barbs and barbules. In wild Greater Flamingos, the concentration of Al, Ce, Co, Cs, Dy, Er, Fe, Ga, Gd, La, Li, Mn, Nb, Nd, Pb, Pr, Rb, Sm, Th, Ti, V, Y, and Zr are thought to be the result of external contamination and whole feathers showing higher levels of these elements may simply be the result of the fact that it is difficult to clean lithic particles (e.g. COM feather samples in this study).

4.4.4.2 Mercury and Selenium.

An association between Hg and Se exists both in sediments even though not strong (rS = 0.533, p<0.01) and in feathers (group 3 in FA, Tab. 4.5). However, Se is also strongly correlated to Nb in sediments (rS = 0.766, p<0.01) but their fate substantially diverge in feathers (Nb in group 1 in FA). Some kind of Se-Hg relationship is expected in exposed organisms to Hg, because the latter seems to be more selenophilic than thiophilic in organisms, and Se is supposed to have some detoxifying effect on Hg (Melnik *et al.*, 2010). Mediterranean waterbirds may be considerably exposed to Hg, due to the intense Hg pollution from natural and anthropogenic sources occurring in the Mediterranean basin (Žagar *et al.*, 2014). In fact, a recent detailed study compared Hg levels in feathers of several Flamingo colonies in West Mediterranean area (including data of the present study), and reported more or less important anomalies in about half of the specimens (Fig. 4.5a), especially from CAG, but, unexpectedly, ODI and captive birds had low levels of Hg (Borghesi *et al.*, 2011). Considering that both CAG and ODI sediments show high Hg anomalies (m=0.13 ± 0.05 mg/kg and m=0.15 ± 0.02 mg/kg, respectively), low concentration of Hg in feathers from ODI (M=0.782 ± 0.082 mg/kg) may indicate that the Huelva district might play a minor role as a feeding area for fledglings' parents during breeding season. However, also Rubio *et al.*, (in press) assessed low Hg in Little Egret chick feathers in Odiel marshes and this coincidence may suggest the hypothesis of a low bioavailability of Hg in this heavily polluted area. Selenium in wild flamingo feathers is definitely higher than in captive birds (Fig. 4.5b). Selenium may be present in feathers as external contamination, but all clues (FA outcomes, the rather compact and symmetric distribution in feathers, similar levels in sediments from all sites not corresponding to similar levels in feathers) seems to indicate a relevant internal contribution, at least in Greater flamingos.

4.4.4.3 Arsenic.

Table 4.3 shows significant high correlations between As and Cu (rS=0.834), Fe (rS=0.749), V (rS=0.714), and Zn (0.873) in sediments. To some extent, As is also associated to elements biologically much less active such as Ce (rS=0.628), Ti (rS=0.693), and Zr (rS=0.600). Conversely, we found no correlations in feathers between As and such metals (p<0.01). In our study, sediments and nests (m=106.02±22.64 mg/kg and 35.25±1.38 mg/kg) and feathers (M=0.79±0.10 mg/kg) in ODI samples are extraordinarily rich in comparison to the other sites (Tab. 4.2 and Fig. 4.5c). Arsenic compounds can bond to the sulphur-rich proteins contained in feathers (Murphy *et al.*,

1990) and these results are in disagreement with the hypothesis of an external prevalence of As for any site. However, external contamination of As in feathers cannot be excluded (Goede and de Bruin, 1984), since the fine-grained particles of sediments containing As are very difficult to remove (Smith *et al.*, 2008). In fact, As is strongly adsorbed by sediments, especially in association with organic matter, Fe and Mn oxides (Gorny *et al.*, 2015). This metalloid is well known to be hazardous for life (Eisler, 1988) and excessive intake of As can cause acute and chronic toxicity to organisms ingesting sediment (Bhattacharya *et al.*, 2007; Sánchez-Virosta *et al.*, 2015). Despite this, As is not often included in ecotoxicological studies based on feathers compared to other conservative pollutants such as Hg, Pb, and Cd. In the literature, there are no data regarding As in Greater flamingo feathers, but this element was measured in pooled feather samples of adult Lesser flamingo (*Phoeniconaias minor*). Burger and Gochfeld (2001) without investigating the internal or external origin of As, found a mean concentration of 0.98 ± 0.30 mg/kg, considered by the authors as rather low. However, that value is comparable to the concentrations in feathers from ODI, ranging 0.61 - 1.06 mg/kg (Tab. 4), where flamingos do not only seem highly exposed to As, but it is also very likely that an important dose is taken up and bioaccumulated.

4.4.4.4 Zinc.

As for many other metals, ODI sediments are strongly enriched in Zn, reaching a mean of 323 ± 140 mg/kg in wetland samples. Camargue soil samples have the lowest concentrations (61 ± 6 and 54 ± 7 mg/kg in nests and sediments, respectively), while CAG and COM have intermediate and very similar levels (just above 100 mg/kg). In wetland sediments, Zn of anthropogenic origin tends to be enriched in the fine-grained fraction (Foster and Charlesworth, 1996; Migani *et al.*, 2015; Borghesi *et al.*, submitted). In our study, Zn appears independent from all other elements in feathers, in particular from elements determined as external contaminants (FA include Zn in factor 4 together with K and Na), therefore the internal signal seems prevailing. It has long been known that Zn is the most important metal required for feather formation (Honda *et al.*, 1986; Sunde, 1972), but excessive intake of this metal represents a potential risk for wildlife health (Sundaresan *et al.*, 2008). Even though it is well known that Zn is regulated by birds during keratinization processes (Burger, 1993), each site shows a specific distribution pattern (Fig. 4.5d). In particular, ODI and COM shows rather irregular and relatively higher distributions (with ODI more rich in Zn than CAG, $p<0.01$, and CAM significantly lower than the other sites, $p<0.001$). This pattern seems to correspond to the exposure levels of birds. Captive birds are rather low in Zn, probably as a

consequence of the artificial food provided to them by the zoo, which is not adequately rich in this essential trace element. Unpublished data of the authors revealed that captive flamingos fed also with dry dog food enriched in essential elements showed Zn values in feathers comparable with the results of this study on wild birds. Zinc is often analyzed in feather studies. In Camargue Amiard-Triquet *et al.*, (1991) found mean concentrations (91.8 ± 11.4 mg/kg) in Flamingo's greater coverts very similar to the scapular feathers we analyzed in the same site (M=90 ± 6 mg/kg). However, their distribution was wider (range: 48.3 – 158.1 mg/kg), embracing an interval that includes both captive and wild birds of this study (Fig. 4.5d).

4.4.4.5 Strontium.

In sediments, Sr reveals a strong negative correlation practically with all elements typically linked to clays (e.g., Al, Ce, Cs, Fe, Ga, K, La, Li, Rb, Th, and V, $rS>0.70$, $p<0.01$). Strontium concentrations are highly variable in sediments (Tab. 4.2), being up to two orders of magnitude lower in ODI samples (as well as Ca) than the other sites, but in feathers it is one of the elements showing the most compact distribution (Fig. 4.2). In some sites (especially CAM, but also CAG and COM) Sr concentrations in feathers appeared positively correlated to elements that are anticorrelated to Sr in sediments (Tab. 4.6), suggesting internal accumulation. Strontium is rich in shell invertebrates, which are a large part of the flamingo diet and it is possible that part of Sr in excess is stored in feathers as a consequence of some physiological regulation. Not many data are available for Sr in the literature on feathers, but it is known that it is a non-essential trace element that tends to follow Ca during nutrient uptake, internal distribution, and excretion within organisms (Blum *et al.*, 2000). Nevertheless, factor analysis gave contradictory results regarding its importance as a constitutive essential element for flamingo feathers, therefore, the fate of Sr in flamingo feathers remains unclear and needs to be assessed by improving preparation methods and carrying out further studies.

Table 4.6 – Significant positive correlations in feathers between Sr and the elements that are anticorrelated to Sr in sediments ($p<0.01$).

	Al	*Ce*	*Cs*	*Fe*	*Ga*	*K*	*La*	*Li*	*Rb*	*Th*	*V*
CAG								*0.67*			
CAM	*0.80*	*0.81*	*0.80*	*0.86*	*0.77*		*0.81*	*0.89*	*0.84*	*0.76*	*0.85*
COM				*0.59*							*0.62*
ODI											

4.4.4.6 Silver.

In sediments, Ag has relatively high concentrations in CAG nests (0.164 ± 0.026 mg/kg) and, with some surprise, in all CAM samples (0.110 ± 0.011, nests; 0.114 ± 0.014 wetland floor). Correlations ($p<0.01$) are few and particular: positive with Nb ($rS = 0.832$) and Se ($rS = 0.674$), and negative with Mo ($rS = -0.639$). In feathers, Ag singularly shows a very different pattern with respect to sediments and, in general, to many other elements: it is more concentrated in ODI, and low in CAM and COM samples (Tab. 4.4). In addition, FA includes Ag in a miscellaneous group in which the negative relationship Ag-Mo is confirmed, but As appears with the same sign of Ag, and Li, Mg, Sr, and U with the sign of Mo. On the other hand, Nb and Se in feathers have a completely different fate than Ag. In captive birds, it is the only metal, along with Cu, definitely higher than wild flamingos from all sampling sites, likely as an effect of the use of chemicals to sanitize the cages. Silver in waterbird chicks is practically unexplored to date, and regarding the internal or external origin, it remains one of the most unsolved elements, together with Mg, Mo, Na, Sr, and U.

4.4.4.7 Chromium.

Chromium was rarely assessed in feathers and, when included in biomonitoring surveys, it was poorly or not discussed at all (e.g. Abbasi *et al.*, 2015). In our case, in sediment Cr is evidently correlated to Al ($rS=0.741$), but with a site-specific ratio (Fig. 3d). In feathers, the Al-Cr correlation is lost in CAG and ODI, but it remains strong in COM ($rS=0.74$, $p<0.01$). For CAM, a correlation remains, but weaker ($rS=0.50$, $p=0.03$). The FA results for Cr are not very useful since Cr is represented with similar coefficients in all five factors (Tab. 4.5). However, as showed in Figure 4.4d, a number of specimens from CAG (50%) seem to be heavily enriched in Cr (with respect to Al), whereas Cr in ODI is unexpectedly low and seems insensitive to Al variation. Despite the strong Al-Cr correlation in COM, there are three samples evidently enriched in Cr also in this colony. Cr-enrichments in CAG feathers are observable when higher than 0.50 mg/kg, and in COM only when higher than 1.50 mg/kg (Fig. 4.4d). The site-specific threshold is likely determined by the degree of external contamination, higher in the latter subset. The external contamination of Cr seems to be comparable with the internal signal in magnitude, and only peaks could be detected, depending on the geochemical characteristics of the investigated site and the effectiveness of the washing method.

4.4.4.8 Copper.

In the present study, Cu was moderately correlated in sediments with many elements, but a correlation coefficient higher than 0.90 is found only with Zn (Tab. 4.3). This correlation completely disappears in feathers, but the prevalent internal origin of Zn is sufficient to alter the Zn:Cu ratio in feathers impeding interpretation of Cu in this way. Despite the differences observed through sites for Cu in sediments, with ODI levels 3- to 10-fold the other sites (Tab. 4.2), the results in wild bird feathers are quite similar and homogeneous in all sites, even more clearly than Zn (Fig. 4.5d and Fig. 4.5e), as expected for an essential metal well regulated by metabolism (Solonen *et al.*, 1999). Conversely, flamingos from a zoo show higher values of Cu, but we think this may be caused by some kind of non-natural contamination, frequent nearby human settlements or provided as supplement in the artificial diet. Copper comes out from FA as a single-metal factor (Tab. 4.5). A wider literature assessing Cu concentration in feathers exists. According to Honda *et al.*, (1986), Cu (with Zn) participates in keratinization processes, although the suspicion of a prevalent external contamination remained in some studies on passerines in polluted areas (Jaspers *et al.*, 2004, Leonzio *et al.*, 2009). However, evidence that Cu contamination could be predominantly internal in feathers even in presence of some Cu-containing particles has been reported (Ek *et al.*, 2004). Useful information to interpret Cu results is at site level. In CAG sediments, Cu-Co, Cu-Fe, and Cu-V seem the most useful metal associations ($rS > 0.95$, $p<0.01$). As shown in Table 4.7, flamingo feathers from CAG preserve the very high correlations between Co, Fe, and V, whilst the correlations with Cu are lost. For CAM, Fe and V can be used for the same purpose, revealing the same evidence (Tab. 4.7).

Table 4.7 - Significant correlations in CAG and CAM feathers between metals indicators of external contamination and Cu. Correlations with Cu pointed out in sediments disappear in feathers ($p<0.01$).

	CAG (n=21)				CAM (n=19)			
	Cu	Co	Fe	V	Cu	Co	Fe	V
Cu	---				---			
Co	NS	---			NS	---		
Fe	NS	0.78	---		NS	NS	---	
V	NS	0.90	0.86	---	NS	NS	0.97	---

Therefore, Cu in flamingo feathers mainly derives from internal bioaccumulation. Cu levels of this study agree with those of Wenzel *et al.*, (1996) concerning the possible limited capacity of feathers to be a sensitive biomonitor for variations in Cu environmental levels, but relatively high levels of Cu in CAM should be further investigated. Copper was analyzed in flamingo fledglings in

Camargue two decades ago and has very similar results to this study (Amiard-Triquet *et al.*, 1991: 3.75 ± 1.03 mg/kg and 4.31 ± 1.64 mg/kg, different portions of greater coverts; this study: from M=3.7 ± 0.28 mg/kg in CAG to 4.68 ± 0.41 mg/kg in CAM).

4.4.4.9 Lead.

Some Pb peaks has been found in sediments near the ODI islet (m=79.9 ± 2.7 mg/kg) and in nest materials of CAG (m=68.2 ± 12.8 mg/kg) and COM (m=51.4 ± 10.4 mg/kg). The lowest concentrations within the whole dataset are recorded in CAM (m=18.7 ± 2.9 mg/kg in sediment). The correlation matrix (Tab. 4.3) reports Pb-Ce, Pb-Hg, Pb-K, and Pb-La as the strongest ones ($rS>0.70$, $p<0.01$). Lead is moderately negatively correlated to Sr ($rS= -0.61$, $p<0.01$). The significant high correlations Pb-Ce and Pb-La recorded in feathers (considering each and all sites: $rS>0.62$, and $rS>0.89$, respectively), seem to confirm the prevalent external origin of Pb in Flamingo feathers, as well as the grouping of Pb together with elements pointed as external contaminants (Tab. 4.5). At site level, three colonies (CAG, CAM, and ODI) show values not far from Pb measured in captive birds, while feathers from COM appear Pb-enriched (Fig. 4.5f). Lead is a very important contaminant, included in almost all studies of metals in feathers. The huge variability of Pb concentrations reported in previous studies is very difficult to interpret owing to the presence of external contamination which may mask internal, less conspicuous, signals (Ek *et al.*, 2004; Fasola *et al.*, 1998; Weyers *et al.*, 1988). In addition, Pb is largely excreted by feces (Leonzio *et al.*, 2009; Scheuhammer, 1987), perhaps accumulated in the supra-orbital 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). However, experimental tests supported the capability of feathers to bioaccumulate Pb (Dauwe *et al.*, 2002), and endogenous lead levels in nestling feathers tend to increase significantly near metallurgic factories (Dauwe *et al.*, 2000; Eens *et al.*, 1999; Janssens *et al.*, 2001; Nam *et al.*, 2004) and urban areas (Scheifler *et al.*, 2006). Researchers have long debated on the Pb contamination from the use of Pb-additives in gasoline and paints (Pokras and Kneeland, 2009), but at present, after the ban of leaded fuels in most countries, the main concern on Pb contamination in birds is related to Pb ammunition (ingested or embedded in the body) (Andreotti and Borghesi, 2013; Pain, 1996). The problem of spent ammunition ingestion may be seriously acute in marshlands (De Francisco, 2003), and especially in Europe (Pain, 1992). Very high Pb shot pellet densities have been found in wetlands surrounding the study areas (up to 148 pellets/m^2 in SW Spain: Mateo *et al.*, 2007; 199 pellets/m^2 in Camargue: Pain, 1991; 71

pellets/m^2 in the Po Delta, Italy: Tinarelli and Tirelli, 1999). Similarly, elevated ingestion rates of Pb pellets have been ascertained in many waterbirds (Mateo *et al.*, 2007; Tavecchia *et al.*, 2001; Guillemain *et al.*, 2007). Flamingos can ingest Pb pellets too (Camargue: Bayle *et al.*, 1986; SW Spain: Ramo *et al.*, 1991; Mateo *et al.*, 1997; Po Delta, Italy: Arcangeli *et al.*, 2007). They are particularly exposed given their habit of treading when feeding: with their feet they excavate craters in soft sediments, probably unearthing Pb pellets (Mateo *et al.*, 2007). Furthermore, in NE Italy flamingos are used to feeding on the seeds dispensed by hunters around their hunting posts to attract wild ducks (Arcangeli *et al.*, 2007). Thus they pasture in places where most shot pellets fall down and accumulate on the sediment.

Amiard-Triquet's (1991) reported Pb levels in flamingo fledgling feathers from Camargue well beyond 3 mg/kg, a value even higher than COM. In general, applying the washing procedure described for this study, the magnitude of the exogenous contamination of Pb seems to mostly mask the internal bioaccumulation level, at least in COM. As tested by Cardiel *et al.*, (2011) and recommended by experts in monitoring contaminants in raptors from Europe (Espín *et al.*, 2014), it is possible that a washing with water, acetone, 2% nitric acid and again water is the best washing process to remove Pb external contamination. However, four COM samples show evident peaks whose origin cannot be clarified (Fig. 4.5f).

4.5 Conclusions and recommendations

The analytical results of many ecotoxicological studies on the bioaccumulation of metals trace elements in feathers are difficult to interpret due to the external contamination.

The geochemical approach of our study confirms that most of elements present in sediments, even in scarce concentration, cause external contamination in whole-feathers. This important drawback occurs even when taking into account many variability factors and when feathers are thoroughly washed. According to our results, the external input of Al, Ce, Co, Cs, Dy, Er, Fe, Ga, Gd, La, Li, Mn, Nb, Nd, Pb, Pr, Rb, Sm, Th, Ti, V, Y, and Zr is capable of masking the bioaccumulation signal and to date nothing can be said about their bioaccumulation in feathers.

For Cu, Zn, and likely Se, we found evidence that they could be constitutive or bioaccumulated. Regarding Hg, feathers could be an efficient way to excrete it from internal organs (Ackerman *et al.*, 2011). Flamingos could accumulate these elements in feathers in such a high concentration that the contribution of residual external dirt was negligible after washing.

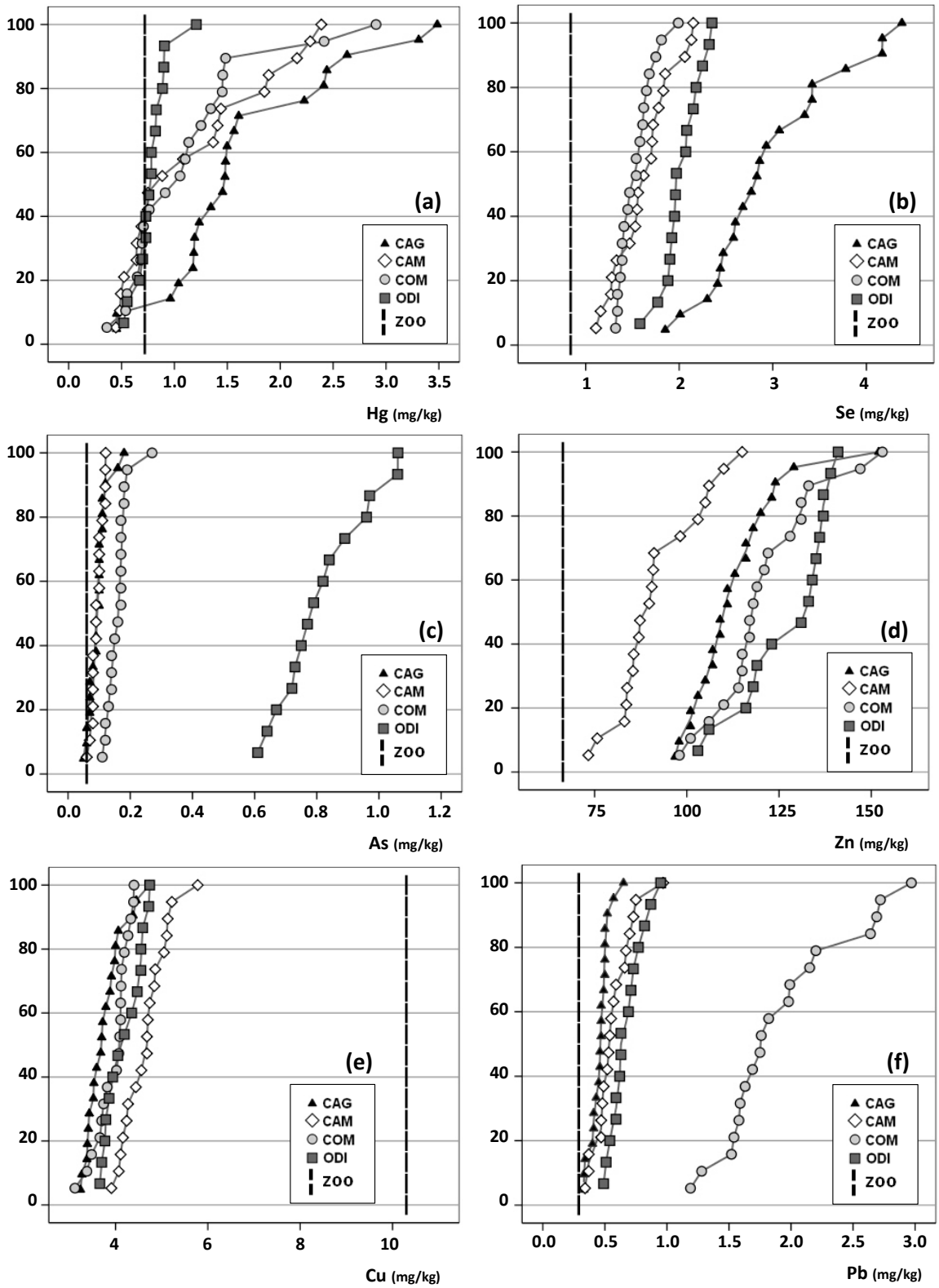

Fig. 4.5a-b-c-d-e-f – Cumulative curves of Hg, Se, As, Zn, Cu, and Pb in feathers for each flamingo colony. The average level of captive birds is represented by a vertical dashed line.

Arsenic, Cr, and in some way Pb could reveal anomalies due to elevated intake of bioavailable forms of these contaminants, but sampling and washing methods should be improved in order to obtain cleaner feathers, or, alternatively, feathers may be used to monitor environmental exposure instead of bioaccumulation, bearing in mind that, conversely, perceptible bioaccumulation may confuse the pattern. Further in depth analyses on these elements are still required.

After our study, the fate in feathers of Ag, K, Mg, Mo, Na, Sr, and U remains completely obscure.

Feathers can be confirmed as an important tool for non-destructive biomonitoring, but more attention is required on the validation of the methods. Surveys including birds of unknown origin, age, moulting quarters, and other uncontrolled variability factors, should be avoided. When such surveys are carried out, their results need to be treated with caution. As already suggested in previous studies (see Cardiel *et al.*, 2011), the use of only shafts deprived of vanes should be preferred to reduce the external contamination, but it is still poorly explored and the shaft is not always available.

As a main indication, feather studies should be limited to birds whose feather dirt is associable to the sampling site (like chicks or fledglings) and a geochemistry approach may be helpful in studies concerning contaminants on birds referable to a precise area. This may not be the case of fledged or adult birds, given the impossibility of knowing their movements in previous months (with some exceptions). Moreover, washing methods may be improved upon.

Regarding our dataset on Greater flamingos breeding in four Western Europe sites, we can draw some conclusions on potential concerns involving this species and its habitats, in respect of the environmental exposure to trace elements:

- Confirming Borghesi *et al.*, (2011) outcomes, Hg has been accumulated in feathers to some extent in all sites. In particular, there is a need to be concerned about CAG, where the anomalous high levels of Hg affect all the sampled birds.
- Clear Cr anomalies in many CAG samples suggest the need of further studies in this area.
- ODI appears as a critical site for As. Arsenic effecting the birds breeding in ODI should be urgently investigated further.
- Substantially, COM feathers were rather inscrutable for all but a few elements, because feathers were harder to clean in comparison to all the other sampling colonies, due to the

clay composition of the sediment. However, findings suggest that Cr and Pb bioaccumulation are worth further investigation.

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CHAPTER 5 - Assessing environmental pollution in birds: a new methodological approach for interpreting bioaccumulation of trace elements in feather shafts using geochemical sediment data

5.1 Abstract

1. Environmental trace element composition can have an important impact on ecosystem and population health as well individual fitness. Therefore, carefully assessing bioaccumulation of trace elements is central to studies investigating the ecological and evolutionary impact of pollution. Colonial birds are important bioindicators since non-invasive sampling can easily be achieved through the sampling of chick feathers, which controls for some confounding factors of variability (age and environmental heterogeneity). However, external contamination (ExCo) of feathers is difficult to remove even after washing and has frequently been overlooked in the literature.
2. We developed a new method to reliably interpret bioaccumulation of 14 trace elements (As, Cd, Cr, Cu, Hg, La, Ni, Pb, Se, Sn, and Zn) in feathers using chicks of a colonial species: the Greater Flamingo, *Phoenicopterus roseus*. First, only shafts were used to remove ExCo retained in vanes. Second, we applied a thorough washing procedure. Third, we applied a new analytical method to control for ExCo, which assumes that ExCo is mainly due to adhered sediment particles and will have similar trace element concentrations to sediment geochemical composition of sampling sites. We validated this new methodology by comparing trace element composition and particle composition (by scanning electron microscopy and mass-spectrometry) of washed and unwashed feathers.
3. The washing procedure removed 99% of K indicating that most of the salt ExCo was removed. Scanning electron microscopy and mass-spectrometry revealed that some sediment particles remained after washing, especially clays which are likely to severely bias bioaccumulation interpretation. We successfully controlled for ExCo by calculating the ratio of ExCo due to sediment using the geochemical fingerprint of sediment samples. Our methodology leads to conservative estimates of bioaccumulation for As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Sn, and Zn.
4. We have validated a new more reliable method of analysing trace element concentrations in feathers, which effectively controls for ExCo, if geochemical sediment data can be meaningfully compared to ExCo of feathers. We have demonstrated that overlooking ExCo

leads to potentially erroneous conclusions and we urge that the method applied in this study be considered in future studies.

5.2 Introduction

Most metals and trace elements are omnipresent in the environment as a consequence of natural processes and anthropogenic activities. Some of them play an essential role in biological functioning (e.g. metabolism, neuronal functions). However, other elements (e.g. mercury, lead, cadmium, arsenic, etc.; Kabata-Pendias & Pendias, 2001) may also exert detrimental, toxic effects on species if they accumulate in the food chain (Amaral *et al.,* 2006) which will negatively affect fitness and life history traits of plants and animals, as well as cause diseases in wildlife and humans (Nriagu, 1989; Järup, 2003).

During the last centuries, the anthropogenic exposure level of trace elements has hugely increased after the industrialization era, especially in wetlands, which, in many cases, act as geochemical endpoints and tend to accumulate pollution (Reddy & DeLaune, 2008). The total concentration of metals in soil and sediments persists for a long time because they do not undergo microbial degradation (Kirpichtchikova *et al.,* 2006). It has been demonstrated that metals from anthropogenic inputs are often weakly associated to the finest fraction of the top layers of sediment and organic matter (e.g. Salomons & Förstner, 1984; Palanques *et al.,* 1995; Migani *et al.,* 2015) and consequently tend to be much more bioavailable and bioaccumulable than the same elements of natural origin (Bryan *et al.,* 1979; Di Giuseppe *et al.,* 2014). Monitoring environmental metal contamination and investigating how organisms are affected by the excess of trace element intake or, more generally, the alteration of the natural geochemical profile is of central importance in evolutionary ecology and human and wildlife health. A prerequisite for such monitoring is to develop reliable methods to correctly measure metal exposure, intake, and bioaccumulation.

For several decades, birds have proven to be valuable biomonitors for various types of pollutants, including metals (Furness & Greenwood, 1993). Ecotoxicological studies in the last three decades have frequently used feathers in order to assess metal accumulation in birds and feather analysis has proven to be a very informative tool to unravel various physiological, ecological and toxicological processes inherent to individuals and populations (Burger, 1993; Smith *et al.,* 2003; Tsipoura *et al.,* 2008). An important advantage of feathers with respect to blood metal concentration is that feathers are relatively easy to collect, preserve, and transport and sampling

is virtually harmless to birds (Burger, 1993). Moreover, metal accumulation in feathers generally represents a longer-term contamination process, while levels in blood represent a recent contamination directly associated with feeding (Carvalho *et al.,* 2013). Since concentration levels in feathers reflects the body accumulation during the entire time of feather development, potential age biases can be circumvented by restricting the analyses to chick feathers. However, external contamination (ExCo) has always challenged researchers and has often been overlooked (but see: Hahn *et al.,* 1993; Fasola *et al.,* 1998; Ek *et al.,* 2004; Hollamby *et al.,* 2006; Valladares *et al.,* 2010; Borghesi *et al.,* 2016). External contamination is defined as the part of the concentration that is not attributable to bioaccumulation in the keratin structure (i.e. metals stored during feather growth as an effect of internal bioaccumulation and metabolic processes, hereafter referred to as bioaccumulation for brevity). ExCo is normally attributed to atmospheric dust, water, or deposition of contaminants on feathers during preening (Dmowski & Dmowski, 1999; Dauwe *et al.,* 2002; Jaspers *et al.,* 2004). However, a recent study on the Greater Flamingo-*Phoenicopterus roseus* pointed out the major importance of sediment particles in complicating the interpretation of analytical results (Borghesi *et al.,* 2016). Most previous field studies have tried to remove ExCo through washing, however to date no washing procedure is completely effective in ensuring the total removal of ExCo from feathers (Cardiel *et al.,* 2011; Espín *et al.,* 2014). Furthermore, so far no studies have tried to quantify the magnitude of ExCo and to consequently validate the bioaccumulation data of trace elements. To continue to use feathers as indicators of bioaccumulation of trace elements, it is important to improve the methodology by reducing the relevance of ExCo, and at the same time, find new methods for estimating more accurate data about bioaccumulated concentrations.

In our study, we adopted five measures for that purpose: 1) we used only shafts, because feathers deprived of vanes capture dirt less efficiently (Cardiel *et al.,* 2011); 2) we sampled chicks, which avoids variability due to age; furthermore chicks have sediment particles of proven origin entangled in their plumage; 3) we used local geochemical information from sediments collected around nesting islets, in order to compare the local geochemical fingerprint to the element ratios in feathers (Borghesi *et al.,* 2016); 4) we chose an extensive set of elements (14), including some which are supposed to have little or no bioaccumulation and are useful to check for ExCo in the investigated sites as they are indicators of clays (i.e. Al and La) and other fine fractions of the sediment such as oxides and hydroxides (i.e. Fe), and salt (i.e. K). The comparison between sediment and feather concentrations has been performed by adopting a new method capable of

estimating the relative importance of ExCo for each element and to correct the analytical result for ExCo. The aim of this study is to validate this new method.

In order to achieve this goal, we used the Greater Flamingo as a model species. The ecology and biology of this species are well known due to long term studies (Johnson & Cézilly, 2007), a major advantage for an ecotoxicological study. The Greater Flamingo has a large breeding range including many important Mediterranean wetlands (Balkız *et al.,* 2007), feeds mainly on small benthonic invertebrates by filtering sediments of brackish wetlands and saltpans. During feeding it can ingest a considerable quantity of sediments from which the organic matter contained therein is digested as a component of diet (Jenkin, 1957). Their particular feeding behaviour leads flamingos to be directly exposed to polluted sediments. In addition, flamingos feed their chicks with a liquid secreted from the upper digestive tract, rich in proteins, fat, carotenoids, blood cells, and, as a consequence, with part of the pollutants previously bioaccumulated and metabolized (Lang, 1963; Fisher, 1972). All of these reasons make greater flamingo chicks a good choice among birds as an environmental indicator of the effect of trace element accumulation in Mediterranean wetlands (Borghesi *et al.,* 2011, 2016). However, from the age of 3 weeks old, chicks form a large crèche in the muddy and brackish wetland near the vicinity of the breeding island (Johnson & Cézilly, 2007), leading to high exposure to local environmental elements. Therefore, as highlighted by Borghesi *et al.,* (2016), ExCo can dominate trace element concentration of greater flamingo chick feathers.

5.2 Methods

5.2.1 Sample collection

All of the feathers from flamingo chicks were collected between July and August 2014, during the ringing operations in three breeding colonies of the western Mediterranean: Aigues-Mortes (AIG), southern France (N 43° 33', E 4° 11'); Fuente de Piedra (FDP), southern Spain (37° 06'N, 04° 45'W) and the heavily polluted Odiel marshes (ODI) (Guillén *et al.* 2011), southern Spain (37º 17' N, 06º 55' W) (Fig. 5.1). All of the sampled birds were between 5 and 8 weeks old (Johnson & Cézilly, 2007). Ten feathers were obtained by cutting the distal part from random individuals using stainless steel scissors. We selected the longest internal scapulars that were protected from aerial deposition (Borghesi *et al.* 2011, 2016). Feathers were kept in envelopes at room temperature until analysis. In addition, for each sampling site we collected seven sediment samples of 200-500g within and on the reeve of the water body where the breeding islet was

situated. Each of the 21 sediment samples was kept in plastic containers in dry room temperature conditions prior to analysis.

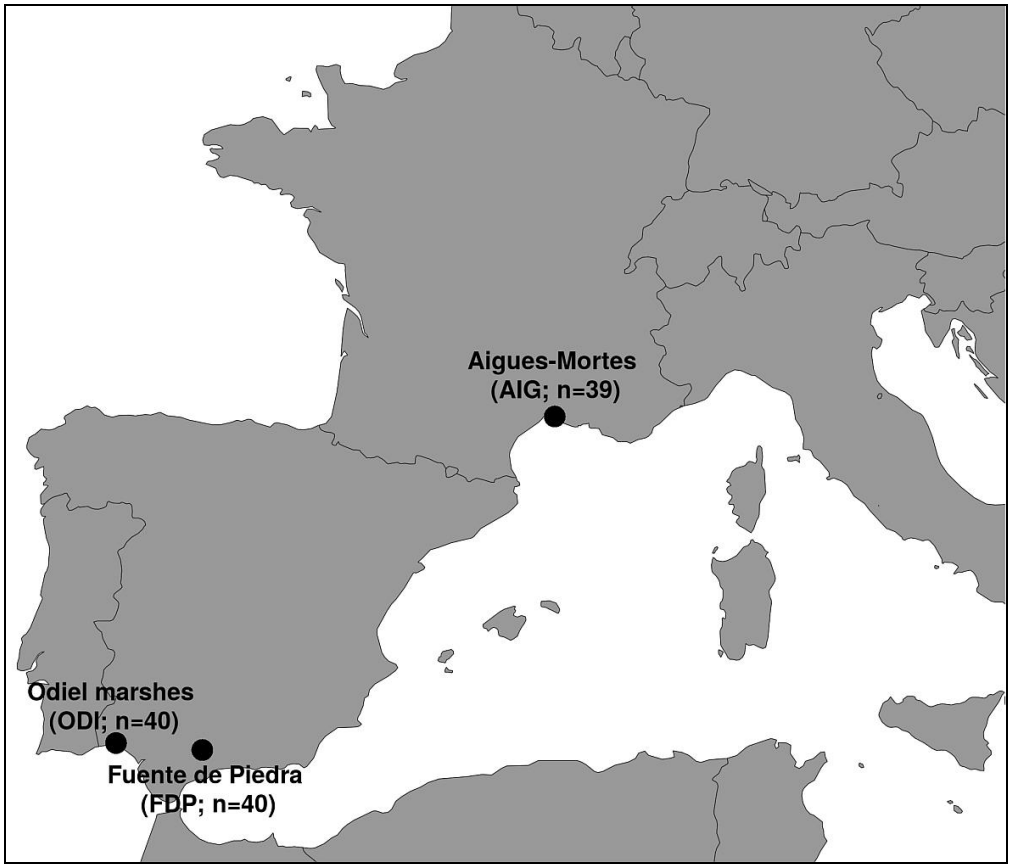

Figure 5.1– Map showing the location of the three breeding colonies sampled for greater flamingo chick feathers and sediment. Sample sizes of washed feather shafts are in brackets

5.2.2 Sample preparation and analysis

We chose to analyse 14 elements in both sediments and feathers. Eleven elements were chosen because of environmental concern: As, Cd, Cr, Cu, Hg, La, Ni, Pb, Se, Sn, and Zn (ATSDR 1994; Hamasaki *et al.* 1995; Hamilton, 2004; Cempel & Nikel, 2006; Stern, 2010; Tchounwou *et al.* 2012; Walters *et al.* 2014; Herrmann *et al.* 2016). Aluminium, Fe and K were chosen as indicators of clay and the finest fraction of sediment (Leeder, 1982).

5.2.2.1 Sediments

Digestion and trace analysis of sediment samples was carried out by AcmeLabs, Vancouver (Canada). Samples were digested with a modified *aqua regia* solution of equal parts concentrated in HCl, HNO_3 and DI-H_2O for one hour in a heating block within a hot water bath. Each sample volume was equalised with diluted HCl. This mild extraction method was selected because it was the most comparable to those used to dissolve feathers (and possibly the residual sediment

particles on feathers). The concentrations of 64 chemical elements (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Ga, Gd, Ge, Hf, Hg, Ho, In, K, La, Li, Lu, Mg, Mn, Mo, Na, Nb, Nd, Ni, P, Pd, Pr, Pt, Rb, Re, S, Sb, Sc, Se, Sm, Sn, Sr, Ta, Tb, Te, Th, Ti, Tl, Tm, U, V, W, Y, Yb, Zn, Zr) were determined by Inductively coupled plasma mass spectrometry (ICP-MS). To evaluate the analysis quality, an Internal Reference Material (IRM), named DS10, with a composition similar to our sediment samples, was used. Only the 14 trace elements analysed in feathers are considered in this study (Al, As, Cd, Cr, Cu, Fe, Hg, K, La, Ni, Pb, Se, Sn, and Zn). As for feathers, concentrations in sediments are expressed in mg/Kg.

5.2.2.2 Feathers

Vanes were manually separated from shafts by keeping fingers of one hand on the feather tip and then detaching each vane by pulling from the top to bottom with the other hand. Subsequently, the 2 mm distal portion (which still had some tiny barbs) was cut off. This method allowed us to obtain rachides completely deprived of barbs and the cuticle connecting barbs to the shaft. From each specimen, five rachides were prepared, in order to reduce variability between feathers and obtain enough feather weight per sample.

Thirty-nine samples in AIG and 40 samples in FDP and ODI (in total 119 samples, corresponding to 595 rachides) were thoroughly washed through three steps by sequentially using acetone, Triton X(™) detergent, and deionized water. During each step, washing sonication was performed for 20 minutes. After washing, feathers were dried in a dry box at room temperature. From this point forward we now refer to the latter feather samples as “washed feathers”.

In order, to test the effect of washing on feather trace element composition, we duplicated 10 individuals from each site (30 of the 119 individuals in total). For this treatment, five rachides from each individual (a total of 150 rachides) of similar weight were directly sent to the digestion process described below (i.e. no washing procedure was performed prior to digestion). From this point forward we now refer to the latter feather samples as “unwashed feathers”.

All feather shafts (washed and unwashed; 0.329–0.127 g dry weight of shaft from each sample) were digested and analysed at the Trace Element Analysis Core Laboratory of Dartmouth College, Hanover, NH, USA. Digestion was carried out in 0.5 ml acid mixture (9:1 HNO_3:HCl) and then diluted to a final volume of 10 ml with deionized water in polypropylene tubes. Digestion was performed with open polypropylene vessels in a microwave digester reaching a temperature of 105°C. Total concentration of 14 trace elements (Al, As, Cd, Cr, Cu, Fe, Hg, K, La, Ni, Pb, Se, Sn, and Zn) were measured by Agilent 8800 ICP-MS. QA/QC was evaluated by adding oyster, tomato, and

hair Standard Certified Materials (NIST 1566b, 1573a, NIES #13) in batches. Recoveries ranged between 74 and 122 % of the certified values with exceptions for Al, Cr, and occasionally Ni, which had lower recoveries. In addition, three fortified blanks, 8 sample duplicates, and 7 spiked duplicates were also ran and recovery percentages ranged between 84 and 132%. Concentrations in feathers are expressed in mg/Kg dry weight (dw) and no corrections for recoveries were made. Detection limits were calculated as three times the standard deviation of the calibration blank, and based on a sample weight of 100 mg.

5.2.3 Examination of feather with Scanning Electron Microscopes (SEM)

In order to make morphological observations of external particles and possibly infer the nature of external contaminants, shaft segments 1 cm long have been scanned with a Jeol JSM-5400 Multi-Purpose Digital Scanning Electron Microscopes (SEM) equipped with WDS and EDS Systems at University of Bologna, Department of Biological, Geological, and Environmental Sciences (BiGeA). Six feather shafts were selected from each site (18 feather shafts in total), and prepared for SEM without any washing treatment (i.e. the same treatment as unwashed feathers). In addition, 3 feather segments from ODI were scanned after a thorough cleaning procedure with tap water, a commercial detergent, and acetone (i.e. substantially the same treatment as washed feathers but without sonication).

5.2.4 Correction of element concentrations feathers for environmental contamination

Feathers, even washed, retain a certain quantity of sediment (see results). For the sake of argument, if we assume that all bioaccumulation is masked by ExCo, analytical results from chick feathers should tend to represent the geochemical characteristics of local sediments instead of the actual assimilation and accumulation in keratin structure of trace elements. If so, the relative abundances of elements in sediment and feathers should be similar. In contrast, if elements are mostly bioaccumulated then they should be in a higher concentration than expected if the chemical fingerprint of feathers is only determined by ExCo. Using the 14 elements analysed in this study, and investigating the ratios between concentration in feathers and sediment, we can check which elements in feathers are clearly enriched with respect to expected ExCo concentrations.

By investigating sediment element concentration, we were able to infer what the predicted concentration of feather elements would be if ExCo was 100% (predicted external contamination; PExCo) for each element. A previous study (Cardiel *et al.,* 2011) has suggested that Al is a good indicator of ExCo because it is known to be scarcely metabolized by birds (Beyer *et al.* 1999) and it

is a main component of clays and hydroxides (Moore & Reynolds, 1989). However, we found that Al is extracted in smaller concentrations by the acid digestion step than most of the other elements. As a consequence, a certain amount of ExCo of elements will be overlooked when using Al as the geochemical reference. In contrast Fe is well recovered by the methods applied in this study, it is reported to be only negligibly bioaccumulated in shafts of seabirds (Howell *et al.,* 2012) and it represents a wider gamma of compounds in sediments (Reddy & DeLaune, 2008). Finally, it is important to note that a small amount of Fe maybe bioaccumulated, which means that we are actually using a conservative approach and may be slightly overestimating ExCo. For sound biological interpretation the latter is highly preferable than ignoring ExCo and reporting highly inflated bioaccumulated values. For all of the aforementioned reasons we chose to infer feather PExCo using Fe. We calculated the PExCo of feathers as:

$$ExCoF_i = \frac{PExCo_i}{w_i}$$

where x_i is the concentration of Fe in the feather sample *i* and y_j is the concentration of the element studied in sediment at the breeding colony *j* and z_j is concentration of Fe in sediment at breeding colony *j*. From PExCo, we can deduce the proportion of element concentration found in feather that is due to ExCo (external contamination factor; ExCoF):

$$ExCoF_i = \frac{PExCo_i}{w_i}$$

where w_i is the element concentration of interest of the feather sample *i*. Using these two simple equations we estimated, for each feather, the proportion of ExCo for each element within each breeding colony site. For pedagogical reasons, we also applied the above equations to median feather concentrations for each breeding site and 95% quantiles (i.e. x_i and w_i are median values or 95% quantiles of each element for each breeding site instead of for each individual feather). For each feather, we were then able to correct element concentration for ExCo by using the following formula:

$$corrected\, w_i = w_i - (ExCoF_i * w_i)$$

5.2.5 Statistics

All statistics were carried out in R version 3.2.4 (R Core Team, 2016). To investigate the effect of washing of feathers on element concentrations we applied a paired Wilcoxon-Pratt signed-rank test (Pratt, 1959) between element concentrations for feathers that were not washed and for feathers that were washed (n = 30). We calculated r as a measure of effect size which is the z-value divided by the square root of the sample size (in our case 30; Pallant, 2007). An r value between 0.1-0.3 is considered as small, a value between 0.3-0.5 to be medium and finally any value above 0.5 is considered as large. Median differences between washed feathers and unwashed feathers as well as associated 95% confidence intervals were also reported.

To investigate the effect of correcting ExCo on element concentration of feather shaft we calculated the mean difference in feather concentration between raw element concentration of feather shaft and element concentration of feather shaft corrected for ExCo (n = 119) and the associated Cohen's D (Cohen, 1988) (note that applying a paired Wilcoxon-Pratt signed-rank test here always yielded a significant result since ExCo correction always reduces concentration of elements, however this does not allow us to assess whether ExCo correction had a negligible or strong effect). Since many element concentrations were not normally distributed we calculated 95% confidence intervals by bootstrapping (1000 bootstraps) as recommend by Nakagawa and Cuthill (2007) using the boot package implemented in R (Canty & Ripley, 2015). A Cohen's D of below 0.2 is considered as negligible, between 0.2-0.5 small, between 0.5-0.8 medium and larger than 0.8 as large (Nakagawa & Cuthill, 2007). We therefore considered that ExCo correction to have an appreciable effect on element concentration when Cohen's D was equal to or greater than 0.2.

5.3 Results

5.3.1 The effect of washing feathers

The washing procedure significantly reduced trace element concentration for 12 of the 14 elements analysed in feathers: Al, As, Cd, Cr, Cu, Fe, K, La, Ni, Pb, Se, and Zn (Fig. 5.2). The effect was strong (r > 0.500) for Al, As, Cu, Cd, Cr, Fe, K, La, Ni, and Zn. A medium effect (r > 0.300) was observed for Se and Pb. For Sn and Hg washing did not significantly reduce trace composition (Fig. 5.2).

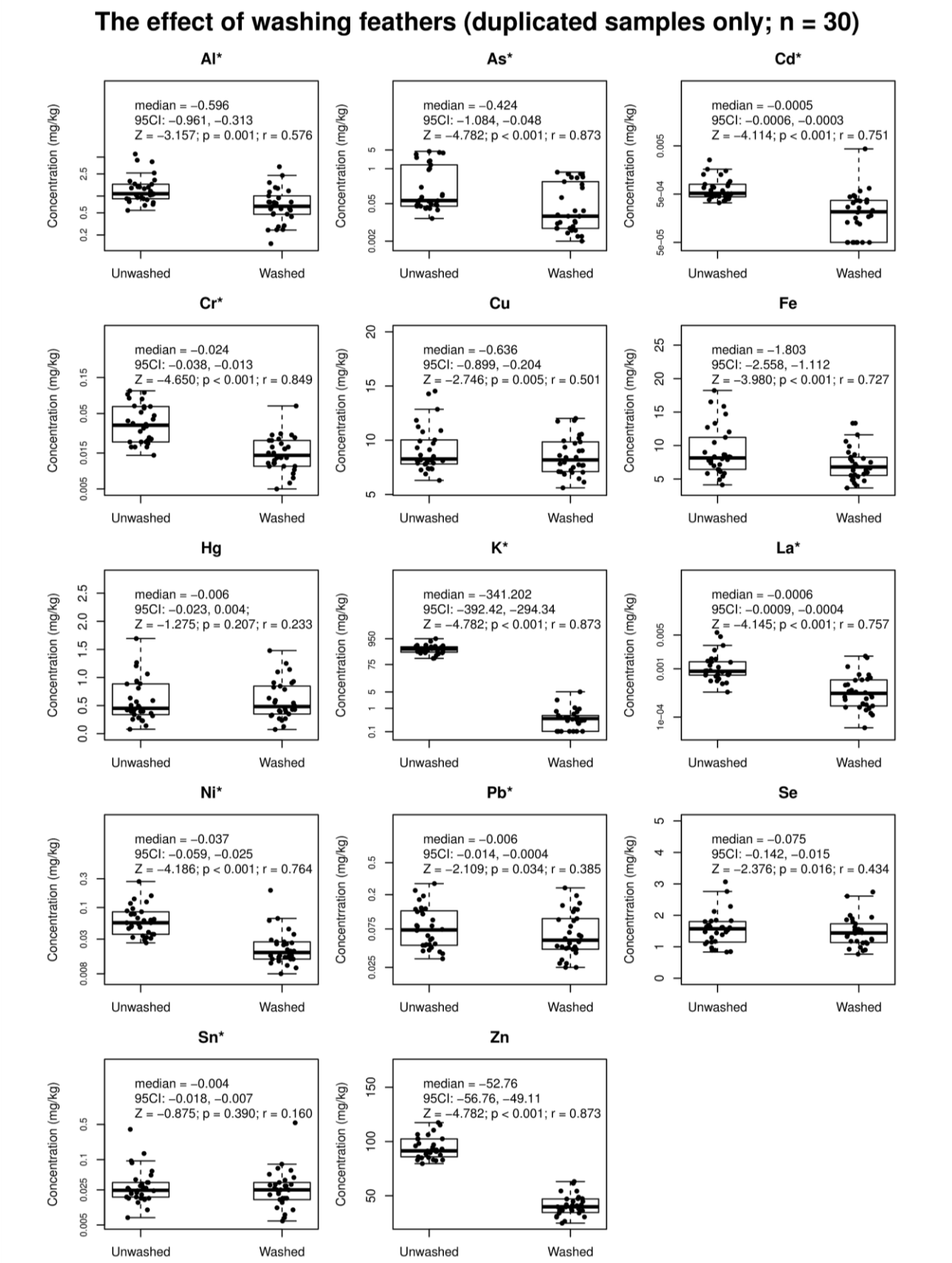

Figure 5.2 - Boxplot of paired unwashed and washed shaft feathers for the 14 elements investigated (*n* = 30). Median difference between washed and unwashed feathers, 95% confidence intervals, the z-value and p-value of the paired Wilcoxon-Pratt signed-rank test and the effect size *r* are shown within the boxplots of each element. Elements with a * were plotted on the log scale (but were not log transformed prior to analysis)

5.3.2 Examination of feathers with Scanning Electron Microscopes (SEM)

SEM examination of 18 unwashed shaft segments of 1 cm revealed a large diversity of particles which densely covered the feathers. A quantitative count of external particles was not possible due to their abundance and complexity. A large number of particles (>200) were found, most of them predominantly composed of sulphur (S) associated with other elements. We concluded that these particles were probably mostly from organic matter derived from feathers, which were discarded from further analysis. Of the remaining particles, one to six putative external contaminants per segment were thoroughly examined for their dimension, shape and chemical composition (for the most abundant elements only according to instrumental limitations). This resulted in a total of 66 lithic particles analysed for their element composition by SEM.

The analysed particles tended to range from less than 1 to 30 µm in all segments, although on rare occasions, they measured up to 100 µm. Particles appeared as amorphous terrigenous aggregations (Fig. 5.3a), definite solid crystals (Fig. 5.3b), piles of stacked sheets (Fig. 5.3c), electrostatically adhered soft objects, or a combination of the aforementioned.

By observing the spectrum, a classification of the geological nature of each x-rayed particle has been provided. As shown in Table 5.1, a variety of Na and Mg salts emerged as the most abundant components of particles in all sites. In salts, K was detectable only in AIG samples. Occasionally, Ca was appreciably present in FDP salts. In all sites, clay particles were often associated with salt particles.

Aluminium was a common element in clays in all sites, but the composition of other elements changed according to sites. Potassium was detected in clay particles investigated in AIG, whereas clays from FDP and ODI showed heterogeneous composition, being either calcic, sodic, or potassic. Noticeably, Mg was detectable in clays only in ODI samples (7 out 8), which were very variable in their overall composition and in some cases particularly rich in Fe, Ti, Cr and potentially many other metals.

Hydroxides were present in particles from all sites, but were not very frequent. They appeared as Al-hydroxides, Mn was detectable in one particle from FDP. Minerals such as quartz, mica, chlorite, muscovite and gypsum were occasionally found in ODI samples, while Ca-carbonates were found in FDP. Four particles (2 in FDP and 2 in ODI samples) were apparently composed uniquely of Al. This may be due to the use of metallic tools, such as scissors and tweezers. A few lithic particles containing Cl or Ca remained undetermined.

Table 5.1 - Summary of element composition for each particle analysed by SEM showing the number of particles with a certain element composition and its geological interpretation within each site: Aigues-Mortes (AIG), Fuente de Piedra (FDP) and Odiel marshes (ODI).

Site	Number of particles	Element composition	Geological interpretation
AIG	5	Na, Mg, Cl	Salt crystals
	2	Na, Cl	Salt crystals
	2	Mg, Cl	Salt crystals
	2	Na, K, Cl	Salt crystals
	1	N	Organic matter
	1	K, Cl	Salt crystals
	1	Al, O	Al hydroxide
	1	Al	Aluminum
	1	Al, Si, K, Ca	Clay
	1	Al, Si, K	Clay
	1	Mg, Cl, Al	Salt and Al hydroxide/oxide
	1	Si	Quartz
FDP	3	Na, Mg, Cl	Salt crystal
	2	Cl	Chloride
	1	Mg, Na, Ca, Cl	Salt crystals
	1	Mg, Cl	Salt crystals
	1	Na, Cl	Salt crystal
	1	Mg, Cl, Al, Si, Ca, Fe	Salt and clay
	1	K, Ca, Mg, Cl, Al, Si	Salt and clay
	1	Mg, Cl, Al, O	Salt and Al oxide/hydroxide
	1	Mg, Cl, Al	Salt crystal and aluminum
	1	Na, Mg, Cl, Al	Salt crystal and aluminum
	1	Na, Cl, Ca, C, O	Salt and carbonate
	1	Na, Al, Si	Clay
	1	Ca, C, O, Mn	Carbonate and Mn oxide hydroxide
ODI	9	Na, Cl	Salt crystals
	4	Mg, Cl	Salt crystal
	2	Na, Mg, Cl	Salt crystal
	2	Mg, Al, K, Ti, Fe, Si	Clay (mica)
	2	Si	Quartz
	2	Ca, S, O	Gypsum
	2	Al	Aluminum
	1	Mg, Al, Fe, Si	Clay (phyllosilicates)
	1	Na, Mg, Al, K, Cl, Si	Clay (phyllosilicates)
	1	Na, Mg, Al, K, Fe, Si	Clay (phyllosilicates)
	1	Al, K, Fe, Si	Clay (phyllosilicates)
	1	Al, Fe, Cr, Ca, Al, Si	Clay
	1	Mg, Al, Si	Clay
	1	Cl	Chloride
	1	Ca	Calcium oxide

In addition to the 18 unwashed shafts, three different shaft segments from ODI (the site where external contaminants are more likely to be rich in trace elements) were analysed by SEM, which were submitted to a washing procedure with water and detergents and then rinsed under running water. Much less lithic particles were visibly found, but some scattered particles were still present. In general, they were less frequent, smaller and seemed less complex in shape. At least 5 lithic objects were found and have been classified as sodium chloride crystals (2), carbonatic mineral (2), metallic aluminum (1).

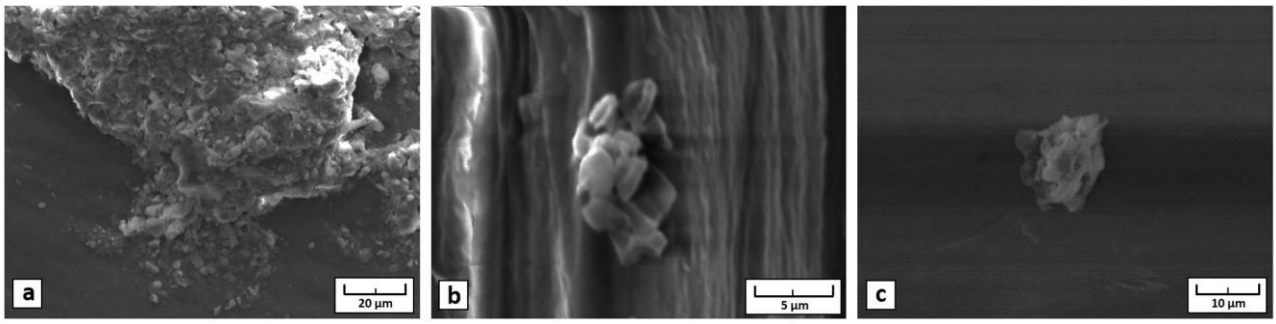

Figure 5.3 (a, b, c) – Electron microscopy pictures of three typical examples of three types of particles found in unwashed feather shafts: a. amorphous terrigenous aggregations; b. definite solid crystals; and, c. piles of stacked sheets.

5.3.3 Using geochemical data to assess the importance of ExCo on shaft trace element concentration

We found strong variation between elements of the importance of ExCo on trace element concentration in feathers. For Cu, Hg, Se, and Zn we found a median ExCoF lower than 0.5% in all the investigated sites and the 95th percentile (95ile) ranged between 0.7% and 0.2% indicating that Cu, Hg, Se, Zn are clearly bioaccumulated in feathers and dominate ExCo (Tab. 5.2). In contrast, for Al, K, and La median ExCoF were much higher than 100% (Tab. 5.2) suggesting that ExCo dominates any bioaccumulation for these elements. The ExCo was less clear cut for the other elements (As, Cd, Cr, Ni, Pb, and Sn; Tab. 5.2). Among these elements, Sn seems to be mostly bioaccumulated, with little variation between sites and median ExCoF ranging between 2-5% (Tab. 5.2). For As, Cd, Cr, Ni, and Pb, the ExCoF was more variable between the sampling sites. Arsenic had ExCoF of 14% (95ile = 3-45%) in AIG, ExCoF of 38% (95ile = 18-100%) in FDP, and only a ExCoF of 3% (95ile = 2-8%) in ODI (Tab. 5.2). There was a lower variation of the effect of ExCo for Pb which has a ExCoF of 12% (95ile = 7-19%) in AIG, ExCoF of 22% (95ile = 14-36%) in FDP and a ExCoF of 10% (95ile = 5-15%) in ODI (Tab. 5.2). For Cd, Cr, and Ni, there was strong variation of the effect of ExCo on trace element concentrations within site, although there was little variation between sites (Tab. 5.2). For Cd, we calculated a median ExCoF of 21%, 32% and 35% in ODI, AIG and FDP respectively (Tab. 5.2). For Ni, median ExCoF ranged between 33-61% (AIG>FDP>ODI), with 95ile within site ranging between 17-99%, 13-100%, 12-50% in AIG, FDP, and ODI, respectively. We found the strongest variation in ExCoF for Cr, where in all sites the 95ile range reached 100% (Tab. 5.2). AIG and ODI had a median ExCoF of around 60% with 95ile ranging from 12 to 100%, while FDP had the highest ExCo for this element (median ExCoF=96%, 95ile = 25-100%; Tab. 5.2).

Table 5.2 - Summary statistics for each samplings site showing median concentration of washed feathers prior to ExCo correction (Feather), median sediment concentration (Sediment), the predicted concentration if feather concentration is entirely due to external contamination (PExCo, see methods for calculation formula), the percentage of feather median concentration explained by external contamination (ExCo) and 95% quantiles (ExCoQ). Elements are ordered according to ExCo within each site. Iron (Fe) is highlithed in bold since this element was used as the reference for PExCo, ExCo and ExCoQ calculations and feather Fe concentration was assumed a priori to be 100% ExCo.

Elements	Feather (mg/kg)	Sediment (mg/kg)	PEC (mg/kg)	ExCo (%)	ExCoQ (±95%ile)
a. Aigues-Mortes (AIG; *n* = 29)					
Se	1.747	0.050	0.0001	0.003	0.002-0.007
Hg	0.471	0.120	0.0001	0.027	0.010-0.083
Cu	9.946	3.840	0.0041	0.042	0.032-0.051
Zn	43.31	19.70	0.0212	0.049	0.034-0.077
Sn	0.023	1.100	0.0012	5.250	2.005-13.17
Pb	0.063	7.220	0.0078	12.31	6.581-19.42
As	0.016	2.100	0.0023	14.08	2.689-45.18
Cd	0.000	0.050	0.0001	31.98	5.378-107.6
Ni	0.016	9.100	0.0098	60.69	17.17-98.87
Cr	0.014	8.600	0.0093	64.66	15.95-280.3
Fe	**7.422**	**6900**	**7.4218**	**100**	**NA**
Al	0.416	3600	3.8722	930.2	492-2361.1
K	0.103	1000	1.0756	1044.3	107.2-1044.3
La	0.0002	2.900	0.0031	1341.5	312-3119.3
b. Fuente de Piedra (FDP; *n* = 30)					
Hg	0.544	0.021	0.00001	0.003	0.001-0.007
Se	1.597	0.600	0.0004	0.024	0.015-0.042
Zn	40.48	19.60	0.0127	0.031	0.024-0.058
Cu	7.225	13.74	0.0089	0.124	0.102-0.234
Sn	0.025	1.200	0.0008	3.106	0.114-10.687
Pb	0.041	13.76	0.0089	22.09	13.87-36.37
Cd	0.0001	0.070	0.0000	35.32	6.644-86.36
As	0.005	3.100	0.0020	37.96	17.84-100.77
Ni	0.018	13.40	0.0087	47.50	13.32-113.88
Cr	0.011	16.00	0.0104	96.16	25.37-231.16
Fe	**6.306**	**9700**	**6.3063**	**100**	**NA**
K	0.290	4200	2.7306	942.3	122.0-2651.0
La	0.0003	4.600	0.0030	1167.2	399.8-2265.6
Al	0.599	13400	8.7118	1454.3	506.8-2810.3
c. Odiel (ODI; *n* = 30)					
Se	1.403	0.500	0.0001	0.006	0.004-0.009
Hg	0.347	0.168	0.00003	0.008	0.002-0.022
Zn	38.65	563.2	0.0962	0.249	0.158-0.380
Cu	8.509	247.1	0.0422	0.496	0.334-0.665
Sn	0.025	2.400	0.0004	1.640	0.079-4.390
As	0.499	91.20	0.0156	3.123	1.912-8.032
Pb	0.148	83.82	0.0143	9.676	5.043-15.06
Cd	0.000	0.240	0.00004	21.22	7.483-82.01
Ni	0.016	30.90	0.0053	32.73	12.00-48.88
Cr	0.011	45.30	0.0077	70.36	12.29-161.24
Fe	**7.193**	**42100**	**7.1930**	**100**	**NA**
K	0.314	7000	1.1960	380.9	57.36-1161.2
La	0.000	15.00	0.0026	553.4	140.0-1447.9
Al	0.621	23900	4.0834	657.6	160.4-1403.2

5.3.4 Using geochemical data to correct for external contamination of feathers

Correcting each individual sample mirrored median ExCoF results (Fig. 5.4). Prior to ExCoF correction Al and La could erroneously be interpreted as bioaccumulated (Fig. 5.4). However, ExCoF correction revealed that actually Al and La concentrations in feather is likely to be entirely due to external contamination and bioaccumulation is either highly unlikely or below instrumental detection limits (Fig. 5.4). Similarly, prior to ExCoF correction, a high number of feathers revealed bioaccumulation of K (Fig. 5.4). However, after ExCoF correction, only 6 feathers out of 119 revealed concentrations above instrumental detection limits (> 0.103 mg/kg) suggesting very small bioaccumulation of this element (Fig. 5.4). Of the remaining elements ExCoF had an appreciable effect (Cohen's D > 0.200) on element concentration for Cr, Ni, and Pb (Fig. 5.4). However, ExCoF correction had a negligible effect (Fig. 5.4; Cohen's D < 0.200) on the concentration of bioaccumulation for As, Cd, Cu, Hg, Se, Sn and Zn (Fig. 5.4).

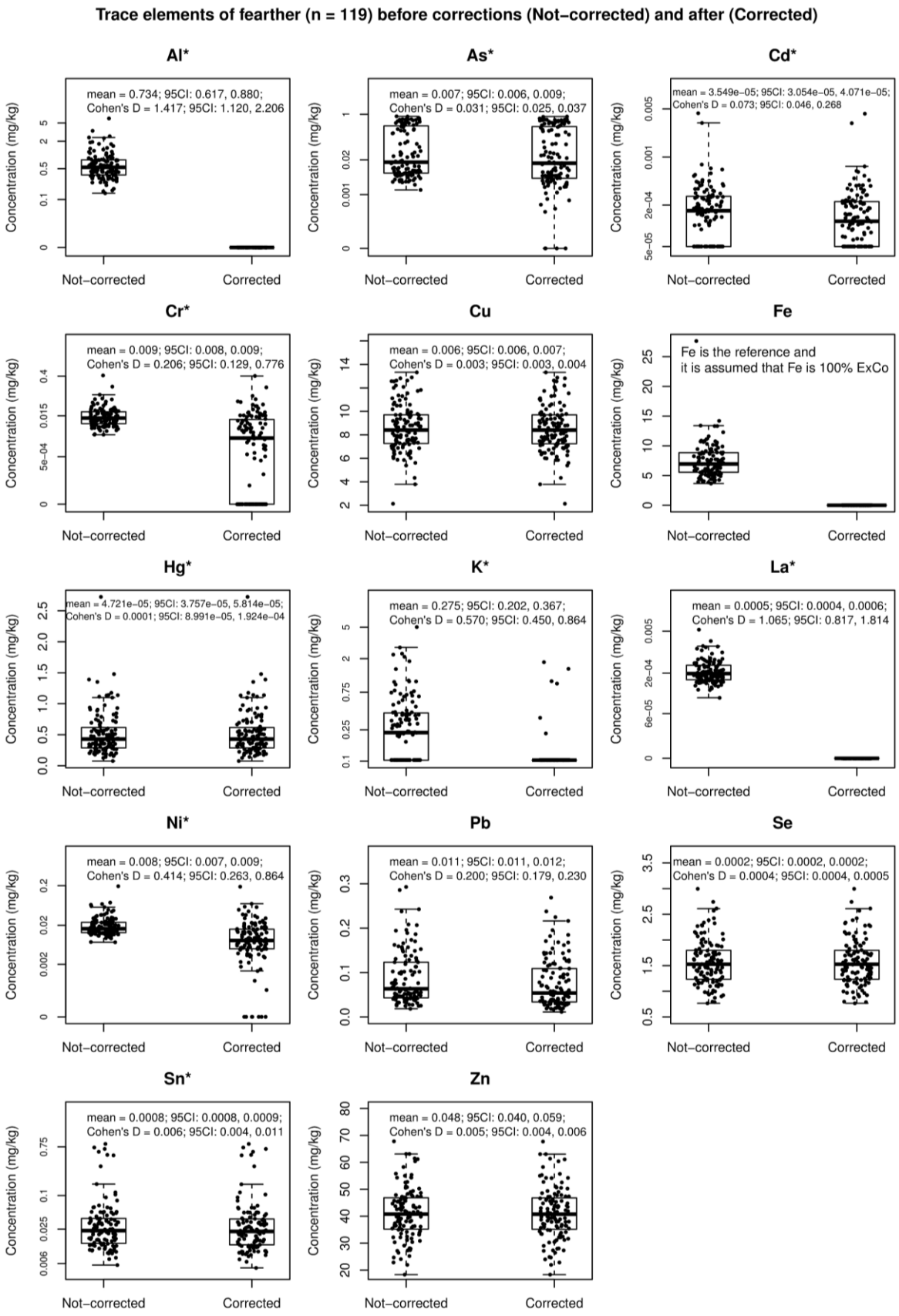

Figure 5.4 - Boxplot of paired shaft feathers not corrected for ExCo and corrected for ExCo for the 14 elements investigated (*n* = 119). Mean difference and Cohen's D between not corrected and corrected concentrations and associated 95% confidence intervals (calculated by bootstrap, *n* = 1000) are shown within the boxplots of each element. Elements with a * were plotted on the log scale (but were not log transformed prior to analysis).

5.4 Discussion

Our results show that our novel methodological approach efficiently dealt with external contamination found in feather shafts and significantly changed interpretation of feather element concentration. We sampled chicks which allowed us to control for the effect of age on bioaccumulation and the shorter time of exposure to external environmental agents than adults (Burger, 1993). Prior to analysis, we took two methodological measures to minimise ExCo and unreliable biological interpretations. First, unlike most studies in feathers, in this study we removed the vanes in order to limit the tendency of feathers to entangle dirt among barbs (Cardiel *et al.,* 2011). Furthermore, vane and shaft sequester metals differently (Bortolotti, 2010; Howell *et al.,* 2012), which may confuse biological interpretation if analysed together. Scanning electron microscopy highlighted that a huge quantity of lithic particles and salt crystals are trapped in unwashed feathers, even when deprived of vanes. Therefore, our second measure was to wash shafts, combining the most common methods applied in the literature to date (Ansara-Ross *et al.* 2013; Costa *et al.* 2013; Carvalho *et al.* 2013; Rubio *et al.* 2016) with a prolonged ultrasonic bath treatment (Weyers *et al.,* 1988). SEM observation also revealed that some ExCo remained in washed feathers and that ExCo cannot be ignored prior to data analysis. We successfully controlled for the remaining ExCo by calculating the ratio of ExCo due to sediment using the geochemical fingerprint of sediment samples. Our methodology allowed us to have conservative estimates of 10 bioaccumulated elements (As, Cd, Cr, Cu, Hg, Ni, Pb, Se, Sn, and Zn).

5.4.1 The effect of washing

After washing, 99% of K was removed (the concentration of K went from 137-951 mg/kg in unwashed shafts to <0.22-5.05 mg/kg in washed shafts), a much higher percentage than any of the other elements analysed in this study. Since K is a dominant element in salt, we can conclude that the washing effect was near complete in removing salt, which is likely to be a dominant residue in coastal bird feathers. We note however, that despite the effectiveness of washing, some K remained (< 0.22 - 5.05 mg/kg), suggesting that either some residual ExCo remained (rare small salt crystals were observed even in washed shafts by SEM), or some bioaccumulation or both.

The washing treatment of feathers also significantly reduced the concentration of 11 of the remaining 14 elements, with only Sn and Hg not significantly reduced. For Sn either trace element concentration from environmental contaminants was negligible relative to the concentration from bioaccumulation, or the efficiency of the washing procedure was lower than for the other trace

elements. Like most elements, very little is known about the characteristics of Hg ExCo, however feather concentration of Hg is considered to be a good indicator of bioaccumulation irrespective of washing procedures (Jaspers *et al.,* 2004; Pedro *et al.,* 2015). Previous studies have shown that Hg levels in feathers are highly correlated with Hg concentration in the diet (Lewis & Furness, 1991, 1993; Hahn *et al.,* 1993; Monteiro & Furness, 1995) and in internal tissues (Thompson *et al.,* 1991) even when potential ExCo is ignored. Furthermore, Hg concentration in feathers is stable over time under various experimental environmental treatments suggesting that ExCo has little effect on this element (Appelquist *et al.,* 1984). Our study is therefore consistent with the literature that ExCo of Hg is irrelevant regardless of the washing treatment.

Observing shafts by SEM demonstrated that unwashed feathers are very rich in lithic particles and are likely to be the main contributors of ExCo in feathers. Most lithic particles are salt crystals, clays and other fine residuals which can be removed in part by washing (Font *et al.,* 2007). In fact, SEM observations on some ODI washed samples revealed that since these lithic particles are electrostatic and very small (typically 1-30 µm) some ExCo remain, even after the thorough washing treatment. ExCo of lithic particles from salt crystals is essentially made of Na, Mg, and K chlorides and Ca and Mg carbonates. However, we believe that any remaining ExCo by salt is likely to have a negligible effect on metal concentration because K is hundreds of times more concentrated in salt than Cu, Cr, and Zn (1,800-3,900 mg/Kg of K, 0-1.2 mg/Kg of Cu, 12-14 mg/Kg of Cr, and 7.4-7.5 mg/Kg of Zn in two collected and analysed samples of salt from Aigues-Mortes water; see dryad data: “Dryad hyperlink if accepted”). In contrast, terrigenous particles, such as clays, hydroxides and organic matter contain higher concentrations of metals, and the presence of a few of these lithic particles in a feather sample is sufficient to mask bioaccumulation for some elements (Borghesi *et al.,* 2016). Therefore, further analytical methods are necessary to soundly interpret feather data.

5.4.2 Assessing the importance of ExCo on shaft trace element concentration

We found strong variation between elements on the importance of ExCo on trace element concentration in feathers. On the one hand, ExCo had a negligible effect on trace element concentrations for some elements (median ExCoF less than 0.5% for Cu, Hg, Se, and Zn; and around 5% for Sn), while on the other hand, ExCo dominated element concentrations for Al, K, and La (median ExCoF more than 100%). The latter is consistent with the hypothesis that residual ExCo after washing is essentially made of clays. Indeed, K is incorporated in the structure of certain clay

minerals such as illite, and commonly adsorbed on the surfaces of many others (Salminen, 2005), and clays have the capability of adsorbing rare earth elements (REEs) released/dissolved during weathering, with La being one of the most abundant REEs (Moldoveanu & Papangelakis, 2012). Aluminum, K, and La concentrations are therefore good signals of residual ExCo and should only be used as controls of ExCo in trace element studies in feathers. Regarding the remaining five elements (As, Cd, Cr, Ni, and Pb), ExCo had a more nuanced effect on trace element concentration (depending on the element and the site), and the use of these elements in feathers to infer bioaccumulation needs some ExCoF corrections in order to avoid inflated interpretation of bioaccumulated concentrations.

5.4.3 Correcting for the effect ExCo on shaft trace element concentration

By calculating an ExCoF for each individual sample we were able to correct concentration values for ExCo for each sample by subtracting from the element concentration the proportion of element concentration that was estimated to be due to ExCo. Of the ten elements of environmental concern analysed in this study (As, Cd, Cr, Cr, Cu, Hg, Ni, Pb, Se, Sn and Zn), ExCoF correction for Cr, Ni, and Pb did appreciably change mean concentrations (Figure 4). This may have important consequences when investigating the relationship between element concentration and other variables (such as body condition or fitness traits), including differences in bioaccumulation between sites which is beyond the scope of this study. The use of feathers as a bioindicator of Cr pollution seems to be the most problematic among the metals investigated in this study. Many samples had low levels of Cr and bioaccumulation concentration was similar or smaller to ExCo concentration. Therefore, even small amounts of ExCo completely masked bioaccumulation (especially in AIG, where metal concentration in sediments is the lowest). However, after ExCo correction this study shows that some bioaccumulation of Cr is detectable.

Trace element concentration in sediment at ODI was higher for 13 out of 14 elements (the exception was Se which was similar in ODI and FDP, and lower in AIG). These results are consistent with the extensive literature which demonstrates that ODI is one of the most polluted estuarine areas in the world (Guillén *et al.,* 2011). However, following careful consideration of ExCo, appreciably higher trace element concentrations in feathers in ODI were only found for As and Pb. Although detailed interpretation of bioaccumulation and differences between sites is beyond the scope of this article, the latter suggests that there are important differences in how chicks

metabolise each element during feather development and that not all trace elements in feathers are reliable environmental bioindicators.

In conclusion, as pointed out by previous studies, without careful consideration of ExCo, conclusions about the validity of the concentration of element bioaccumulation in feather shafts are unreliable. We have developed a new more reliable method of analysing trace element concentrations in feathers which effectively controls for ExCo. Many studies continue to overlook ExCo leading to potentially erroneous conclusions and we urge that methods applied in this study be considered in future studies investigating feather trace element concentrations.

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CHAPTER 6 - Assessing the lead pellets and fragments in game birds: the case of the European starling (*Sturnus vulgaris*).

6.1 Abstract

The poisoning of wild animals by lead (Pb) ammunition fired by hunters has been known for many decades, especially in the case of waterbirds. More recently, it has been demonstrated that raptors are also exposed to the risk of plumbism when feeding on unretrieved quarry that was wounded or killed by hunters. Further studies reveal that even humans can be subject to a significant Pb dose while consuming game animals killed by traditional ammunition. Given the relevance of this issue, several pieces of research have been carried out to assess frequency, dimension, and the number of Pb fragments embedded in the carcasses of ungulates, partridges, ducks, and other birds to evaluate the risk related to the consumption of game meat. In spite of their great importance as quarry species across southern Europe, until now, no data have been available on small passerines. To assess the quantity and type of Pb embedded in songbirds, we x-rayed 196 starlings shot in Italy and found Pb pellets and/or visible fragments in 118 carcasses (60.2 %). We counted 128 shotgun pellets in 85 carcasses. In 28 birds, we detected both whole pellets and lead fragments; in 33, we found only small fragments. By excising and weighing a sample of 20 shotgun pellets (diameter 1.35–1.99 mm), we calculated a Pb load of 3.75 g in the whole sample of 196 starlings, corresponding to an average of 27.32 mg/100 g of body weight. This is a conservative estimation, because fragments were not considered. Compared to game birds of a larger size, the starlings in our study had a lower amount of embedded Pb, but the shot pellets and fragments embedded in their tissueswere abundant and tiny. Given the results of previous studies, the quantity and level of fragmentation suggest that the risk of Pb poisoning cannot be ruled out for humans and birds of prey consuming the meat fromsongbirds killedwith traditional ammunition.

6.2 Introduction

Lead (Pb) is a toxic metal whose effects on living organisms are well known. In mammals and birds, Pb poisoning (plumbism) particularly affects digestive functions, the nervous system, kidney clearance, reproductive performances, the production of red blood cells, and blood pressure. In the worst cases, organisms are induced into a coma and die. Sometimes, permanent

neurological and behavioral alterations may persist even if the critical phase has been overcome (Damstra, 1977; Eisler 1988; Ma, 1996; Pain, 1996; De Francisco *et al.*, 2003).

Generally, prolonged exposure to low doses of Pb does not cause evident clinical symptoms. Chronic poisoning is thus much more difficult to detect (Landrigan and Todd, 1994). In human fetuses and children, even very low exposure hampers the development of the nervous system, raising a social alarm (Needleman *et al.*, 1990; CDC, 2005; EFSA CONTAM, 2010). Given the relevance of this issue, the safe threshold of blood Pb levels (BLL) in humans has been progressively lowered to 10 μg/dl by health authorities of many countries. Moreover, new data are now suggesting a further reduction in the BLL (Canfield *et al.*, 2003; CDC, 2005; Chandramouli *et al.*, 2009; EFSA CONTAM, 2010). At the same time, several measures have been implemented to reduce the risk of poisoning, such as prohibiting the use of Pb in many products like toys, domestic paint, welding materials, or gasoline (Pokras and Kneeland, 2009).

The poisoning of wild animals by Pb ammunition fired by hunters has been known for many decades, particularly in the case of waterbirds (Bellrose, 1959). Ducks, geese, swans, and shorebirds swallow shot lying in superficial sediment, either taking pellets instead of seed or ingesting them as grit to facilitate the grinding of food in their muscular gizzard. Annually, the number of waterbirds affected by this kind of intoxication reaches several millions worldwide (Beintema, 2001).

Terrestrial birds also frequently ingest spent ammunition, as more recently highlighted by a number of authors. Partridges, pheasants, doves, and other granivorous birds peck at the shot in soil for the same reasons as waterbirds (Kendall *et al.*, 1996; Schultz *et al.*, 2002; Potts, 2005; Fisher *et al.*, 2006; Kreager *et al.*, 2008; Thomas *et al.*, 2009). Furthermore, raptors are exposed to the risk of plumbism when feeding on unretrieved quarry that has been wounded or killed by hunters; unwittingly, they consume the Pb embedded in the flesh, swallowing shot pellets or bullet fragments (Pain *et al.*, 1997; Miller *et al.*, 2000; Kenntner *et al.*, 2001; Donázar *et al.*, 2002; García- Fernández *et al.*, 2005; Krone *et al.*, 2006; Cade, 2007; Kenntner *et al.*, 2007; Pain *et al.*, 2007; Komosa and Kitowski, 2008; Gangoso *et al.*, 2009; Helander *et al.*, 2009; Hernández and Margalida, 2009; Krone *et al.*, 2009).

Even humans can ingest a significant Pb dose while consuming game killed by traditional ammunition (Nielsen *et al.*, 1998; Bjerregaard *et al.*, 2004; Johansen *et al.*, 2006; Verbrugge *et al.*, 2009). This toxic metal can be easily ingested and absorbed for two main reasons: (1) bullets and pellets tend to fragment in the body of game animals, producing a large amount of particles that

are too small to be detected and removed during food preparation and mastication (Scheuhammer *et al.*, 1998; Johansen *et al.*, 2001, 2006; Cornatzer *et al.*, 2009; Hunt *et al.*, 2009) and (2) during cooking, the high temperature facilitates the solubilization of Pb particles in the flesh, particularly in the presence of acid ingredients (wine or vinegar) (Mateo *et al.*, 2007, 2011).

A recent statement by the European Food Safety Authority (EFSA) suggests that the Pb intake that is related to the consumption of game meat is not negligible when it comes to human health. At present, the EFSA is considering whether the threshold for the consumption of Pb through diet set by the Joint FAO/WHO Expert Committee on Food Additives in 1986, which corresponds to a provisional tolerable weekly intake of 25 µg/kg of body weight, is still appropriate. Such an approach is supported by the lack of evidence available with respect to a number of critical endpoints, including developmental neurotoxicity in young children and cardiovascular effects and nephrotoxicity in adults (EFSA CONTAM, 2010).

Recently, several studies have been carried out on bullet fragmentation in the carcasses of ungulates to assess the risk of Pb ingestion for raptors and humans, with the results revealing high rates of flesh contamination (Hunt *et al.*, 2006; Dobrowolska and Melosik, 2008; Cornatzer *et al.*, 2009; Hunt *et al.*, 2009; Krone *et al.*, 2009; Tsuji *et al.*, 2009). Some authors have also demonstrated that shotgun pellets may produce Pb fragments when they hit the body of medium-sized bird species such as seabirds, ducks, woodcocks, grouses, pheasants, and partridges (Frank, 1986; Scheuhammer *et al.*, 1998; Johansen *et al.*, 2001, 2004; Pain *et al.*, 2010; Mateo *et al.*, 2011). Information is not yet available on small passerines despite their great importance as quarry species across Europe, especially in the Mediterranean region.

According to Annex II/B of the EU Directive 2009/147/CE, seven species of small passerine (belonging to the Alaudidae, Turdidae, and Sturnidae families) can be hunted within union borders. Game bag statistics are not available on a continental scale, but we can estimate that at least several tens of millions of birds are annually harvested bearing in mind the number of hunters devoted to traditional songbird shooting and the high amount of birds killed pro capita. For instance, in the Brescia district (northern Italy) alone, about 30,000 hunters shoot an average of 870,000 thrushes each year (Andreotti *et al.*, 2010). Such figures suggest that small passerines shot with traditional ammunition may be a non-negligible source of Pb poisoning for both raptors feeding on unretrieved birds and the people eating traditional dishes based on songbird meat.

In this study, we focused our attention on the European starling, Sturnus vulgaris, a widespread species hunted in Portugal, Spain, France, Greece, Malta, Hungary, Romania, Bulgaria,

and Cyprus. In addition, in many other countries not listed above, starlings are shot to prevent crop damage, especially to cherries, grapes, and olives. Using radiographs, we recorded (1) the amount of embedded shotgun pellets and (2) the fragmentation frequency in young starlings killed in Italy to prevent damage to crops. Our findings allow us to evaluate the potential health hazard for consumers—both raptors and humans—eating shot small passerines.

6.3 Methods

We x-rayed 196 starlings shot in two Italian districts (Piacenza and Bologna) and 9 found dead from other causes near Bologna (controls) (Fig. 6.1).

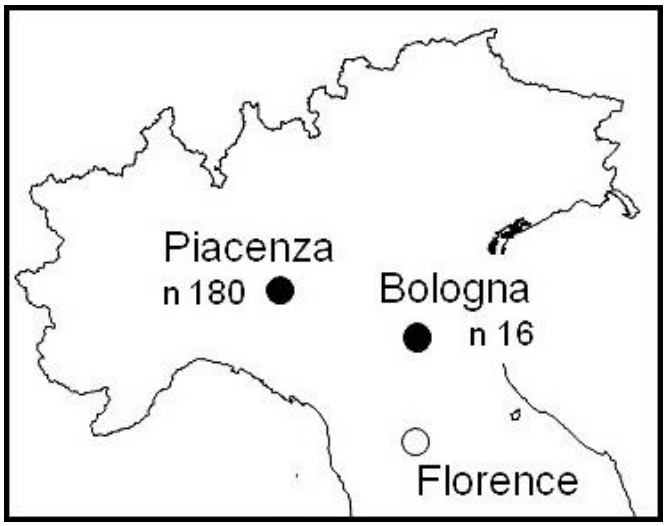

Figure 6.1 - Location of the districts where the starlings were collected. 180 shot birds originated from the Piacenza district (breeding seasons 2005 and 2006) and 16 from the Bologna district (2005). The nine birds that died from other causes (controls) were collected near to Bologna in 2011.

No birds were expressly killed for this study. The shot starlings were killed by local authorities to prevent crop damage in compliance with directive 2009/147/EC. These birds were shot using the same rifles and Pb ammunition normally used by hunters. Our controls died by accident and were collected after their death. All of the birds were juveniles in their first plumage and died before the onset of the complete postnatal molt, which normally starts in the second month of life (our own data). After death, the birds were frozen whole and stored at −20 °C. X-ray photographs (35×40 cm) were taken grouping together up to eight thawed birds. If necessary, the radiography was repeated to clarify any doubts. We ideally subdivided the body of each bird into six sectors so that the anatomical parts normally consumed by humans or raptors could be easily treated separately (Fig. 6.2).

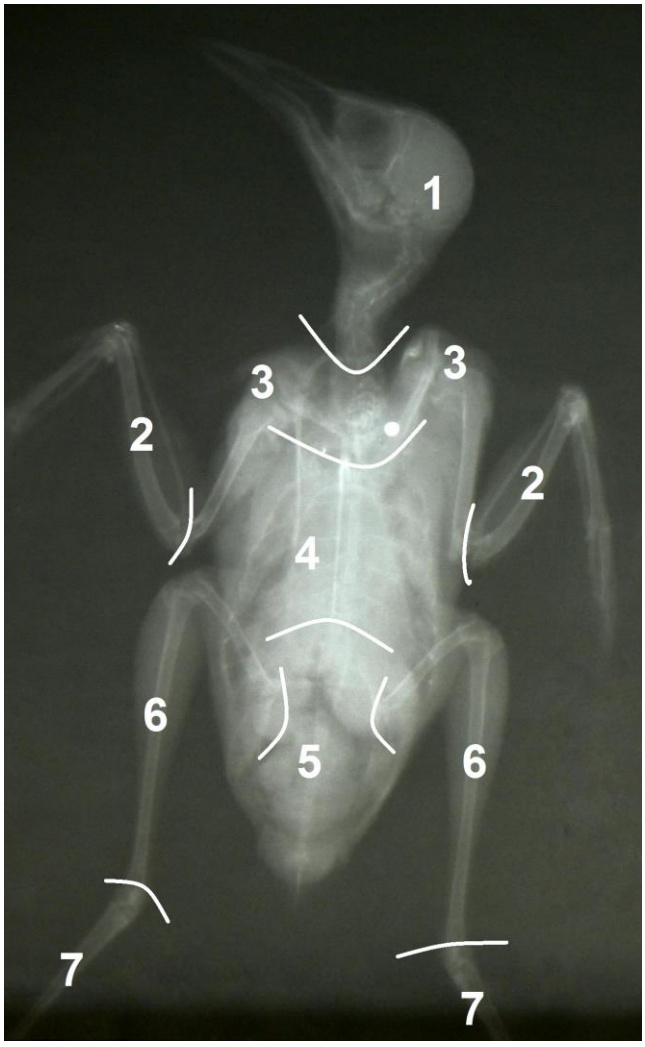

Figure 6.2 - X-ray photograph of a starling, showing the body sectors where the position of each shot pellet and fragment was annotated. 1: head and neck; 2: wings; 3: humerus and pectoral girdle; 4: thorax; 5: abdomen; 6: femur and tibiotarsus; 7: tarsus and metatarsus. A whole pellet is visible in Sector 3.

We regarded Sectors 3, 4, 5, and 6 as corresponding to edible parts, i.e., those most commonly eaten by humans and raptors.

Upon examining the radiographs, we counted the number of whole shot pellets embedded in each sector. Thereafter, we assessed the frequency of Pb fragmentation, recording the clusters of radiodense particles or single macrofragments using a hand lens. Fragments were classified as “macro” when exceeding 0.5 mm and “micro” when they were smaller. Normally, microfragments are not easily detectable to the naked eye on x-ray photographs (Hunt *et al.*, 2006). The fragments were scored independently of their dimensions as follows: 0=none visible; 1=1–2 macrofragments; 2=2–4 microfragments; and 3=>4 fragments.

To evaluate the quantity of the embedded Pb and the proportion of the pellet mass fragmented into small particles, we excised 20 shotgun pellets from 20 different starlings. These pellets were

accurately washed, dried, and weighed by means of a Sartorius Research R200 D Electronic Semi-Microbalance (accuracy d=0.01 mg). The diameter of each pellet was calculated as the mean value of three different measures obtained using a digital caliper (Wiha DigiMax, d=0.05 mm).

6.4 Results

Ammunition residues were found in 118 of the 196 carcasses (60.2 %). In 57 (29.0 %) and 33 (16.8 %) respectively, we observed only whole pellets or fragments, while in 28 (14.3 %) we found both. Pellets and fragments were embedded in all body sectors (Tab. 6.1), but with significant differences in frequency ($\chi 2=128.92$, df=6, $p < 0.001$). Neither pellets nor Pb particles were found in the nine starlings found dead from other causes. In the anatomical regions most commonly eaten by humans or raptors, we found Pb residues in 99 cases (50.5 %).

6.4.1 Shotgun pellets

Radiographs revealed 128 pellets in 85 starlings (mean=0.65, SD=0.95, range=0–4, n=196).Most of the pellets (53.9 %) were located in Sectors 3 and 4, while only a small fraction (11.7 %) was found in the head, neck, and the distal parts of the wings and legs (Tab. 6.1). In the edible parts, we counted 113 pieces of shot, corresponding to 88.3 % of the total amount.

6.4.2 Fragments

A minimum of 82 fragmentation centers were detected in 61 starlings (mean=0.42, SD=0.73, range=0–4, n=196). Fragment clusters were particularly concentrated (64.6 %) in the wings and the pectoral girdle (Tab. 6.1). In the edible parts, we estimated 54 zones with Pb particles, representing 65.8 % of the detected centers of fragmentation. In most cases, the fragments were assigned to Class 2 (48.8 %); it was only in the wings that there were 1–2 large particles (Class 1) instead of several small fragments (Tab. 6.2).

Table 6.1 - Distribution of whole pellets and fragments in different body sectors

	1. head neck	**2. wings**	**3. humerus pectoral girdle**	**4. thorax**	**5. abdomen**	**6. femur tibiotarsus**	**7. tarsus metatarsus**	**TOT**
pellets	6	7	32	37	27	17	2	**128**
fragments	1	26	27	7	4	16	1	**82**

Table 6.2 - Distribution of fragment classes in different body sectors. Fragment scores: 0 = none visible; 1 = 1-2 macro fragments; 2 = 2-4 micro fragments; 3 = >4 fragments

	1. head neck	2. wings	3. humerus pectoral girdle	4. thorax	5. abdomen	6. femur tibiotarsus	7. tarsus metatarsus	TOT
score 1	0	14	7	2	2	4	0	**29**
score 2	1	11	14	3	0	10	1	**40**
score 3	0	1	6	2	2	2	0	**13**

6.4.3 Estimation of the embedded amount of Pb

The mean diameter of the excised pellets was 1.65 mm (SD=0.15, range=1.35–1.99, n=20). The total weight was 585.6 mg (mean=29.28 mg, SD=6.76, range=16.29–44.25, n=20), enabling us to infer that all of the pellets detected by radiography weighed 3.75 g, corresponding to 19.1 mg of Pb per bird on average (16.8 mg if considering only the 113 pellets embedded in the edible parts). Taking into account the 196 shot starlings, we estimate the Pb load due to the pellets to be 27.32 mg/100 g of body weight, assuming a rough weight of 70 g per bird (Spina and Licheri 2003).

The pellet weight revealed a bimodal distribution, suggesting that two different sizes of ammunition were used in starling control interventions (No. 10, diameter= 1.9 mm, weight=40 mg; No. 11, diameter=1.7 mm, weight=29 mg) (Fig. 6.3). The ratio between Nos. 10 and 11 pellets in our sample was approximately 45:55. Therefore, we can hypothesize that the initial mass of all of the excised pellets was 679 mg, which implies an erosion of 13.7 %. This rough estimation gives an idea of the magnitude of the loss of fragments following firing and the impact on the body.

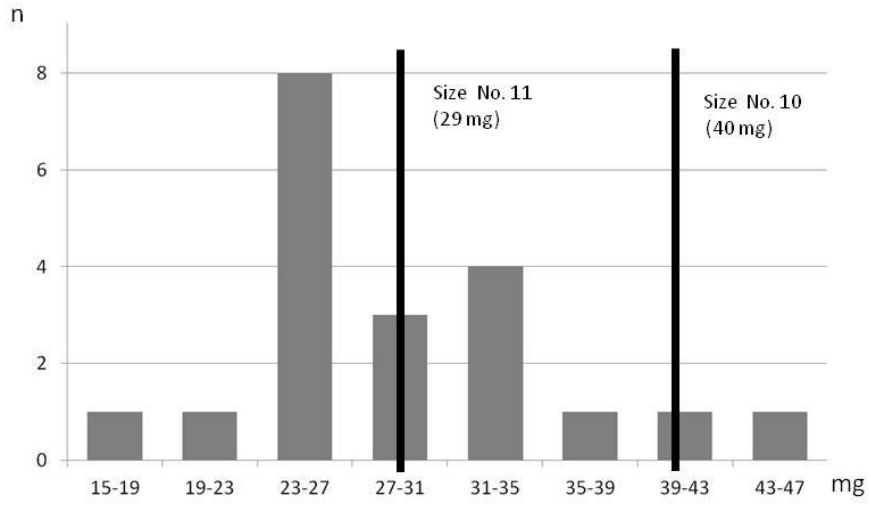

Figure 6. 3 - Weight frequencies of pellets excised from starling carcasses (n = 20). Black lines show the size of commercialised gun pellets.

6.5 Discussion

The Pb quantities found in this study can be regarded as a conservative estimate of the Pb burden embedded in small passerines harvested during the hunting season. In game carcasses, Pb particles may derive from both lethal shot and old wounds that have subsequently healed. In some quarry species, the number of healthy birds with embedded pellets may be unexpectedly high (up to 43 % in waterbirds) and increases with age (Eisler, 1988; Scheuhammer and Norris, 1996; Falk *et al.*, 2006). The fact that all of the starlings examined in this study were in the first few weeks of life allows us to be reasonably sure that the Pb derives exclusively from the ammunition used to kill them. In addition, the age of the birds may affect Pb quantity and the frequency of fragmentation, given the structural characteristics of the skeletal system; newly fledged Passerines do not have fully formed bones, for instance, skull ossification in most young starlings is not completed until autumn (Winkler, 1979; Svensson, 1992), and it may be the case that pellets consequently pass straight through the body more easily at that age. We may thus suppose that Pb content would be higher in the starlings killed during the hunting season in autumn and winter, when adults represent a relevant fraction of the population and juveniles are older and have a more formed skeleton.

Furthermore, our calculation of the Pb load is conservative because we only took whole pellets into account. This decision was made given the impossibility of obtaining a sound estimate of the mass of the embedded fragments using the methods adopted in our study. To get around this problem, we tried to determine the loss of the initial mass of pellets by comparing the weights of the excised pellets to those produced by ammunition factories. The loss of 13.7 % according to our calculation is highly informative about the underestimation of the total Pb load, but we avoided using this finding because it is biased by flaws. Firstly, we do not know which types of cartridge were actually used to kill the starlings. Therefore, our assumptions about the original size of the shotgun pellets are hypothetical. Secondly, when a cartridge is fired, part of the Pb is dispersed as a powder due to both the pellets in the shot colliding with each other and the pellets being abraded on the choke of the gun as they travel through the barrel. This lost powder at firing, before the pellet reaches the quarry, introduces an element of overestimation, even if studies on soil contamination in shooting ranges suggest the relatively low relevance of this type of dispersion (Craig *et al.*, 2002). Finally, in our calculations, the loss was solely referred to those pellets stopped by bodies, causing an underestimation. In fact, we verified that a conspicuous

fraction of the starlings (16.8 %) contained fragments but no pellets, revealing that an unknown number of pellets must have passed through the starlings, leaving Pb particles in the tissues.

Despite the small dimension and the slender structure of songbirds, that might suggest that a rather high rate of shot passes through the body, the mean number of gun pellets per mass unit in our study is greater than the mean found in other studies on larger game birds (Tab. 6.3). Nevertheless, the overall Pb mass is lower in Passerines given the considerably smaller pellet size (Tab. 6.3).

Table 6.3 - Pb contamination in different game bird species; mean body weights and pellet sizes were taken from standard ornithological texts and from hunting sources, respectively.

	n birds	n pellets/bird	reference for n of shot	body weight (g)	references for weight	n pellets/100g	shot size	Pb mass (mg)/100g
Starling	196	0.65	this study	70	Spina and Licheri 2003	0.93	10-11	27
Red-legged Partridge	64	3.67	Mateo *et al.*, 2011	500	Cramp 1980	0.73	5-6	95
Thick-billed Murre	50	3.7	Johansen *et al.*, 2001	900	Cramp 1989	0.41	4	72
Common Eider	25	10.4	Johansen *et al.*, 2004	2000	Cramp and Simmons 1977	0.52	0-6	114

6.5.1 Hazard for humans

A small game meal is generally based on 150–200 g of meat per person (Johansen *et al.*, 2001; Pain *et al.*, 2010). We can therefore assume that a single portion comprises at least four/five oven-ready starlings. Our study reveals a high probability that at least one bird in a meal could be contaminated by whole pellets or fragments. On the basis of our results, we can expect a load of 65–85 mg of Pb in each portion, which is a small quantity if compared with that found in larger game birds (Tab. 6.3). Nevertheless, cooking such quantities of Pb is no less dangerous for human health. Indeed, Mateo *et al.*, (2011) observed that the concentration of bioaccessibile Pb in cooked redlegged partridge meat was significantly related to the presence of x-ray visible pellets and/or fragments, irrespective of their dimension and number. Similarly, previous studies have demonstrated that Pb contamination in game meat is more closely associated with the presence of small particles scattered throughout tissues, rather than whole pellets (Scheuhammer *et al.*, 1998; Johansen *et al.*, 2001). This suggests that the toxicity of Pb particles is more related to the

surface area than to their mass, since the larger surface of the pellets allows the acid to dissolve the Pb faster during cooking or digestion (Hunt *et al.*, 2006). Therefore, considering the frequency of whole pellets and fragments recorded in our study, it can be inferred that the people who eat songbirds are no less exposed to plumbism than those consuming partridges or seabirds, even though a three/fourfold lower amount of total Pb was found (Table. 3). The ingestion of a dose of Pb estimated in a single meal based on shot starlings represents a major risk, particularly for infants and children given the detrimental effects on central nervous system development (CDC, 2005; EFSA CONTAM, 2010).

The type of ammunition used to hunt passerines has another implication for human health. Pellets are very small in size and are therefore hard to detect and remove during food preparation. Even when we excised pellets from the carcasses, we had difficulty in finding them, despite knowing their exact position in the body thanks to x-rays. Accordingly, most of the pellets embedded in the flesh tend to remain during cooking, leaking Pb into the meal. In addition, the majority of fragments found were very tiny and too small and scattered to be detected and discarded by a consumer.

6.5.2 Hazard for birds of prey.

In general, birds of prey are exposed to the risk of Pb ingestion when feeding on unretrieved game carcasses. However, the anatomical location of embedded shot in game birds may influence the availability of Pb for foraging raptors. Miller *et al.*, (2000) reported that only 73 % of the pellets embedded in waterfowl were available to bald eagles Haliaeetus leucocephalus. These authors observed that nonedible prey remains were mainly represented by feathers and bones from the wings, pelvis, and vertebral column. In our analysis, we estimated that over 88 % of pellets and 65 % of fragments were embedded in parts of carcasses accessible to raptors, and about half of the starlings had whole pellets and/or fragments in anatomical parts more accessible to raptors. This figure suggests that birds of prey eating unretrieved small passerines are actually exposed to the ingestion of Pb. Therefore, it is not surprising that even species that feed mainly on songbirds (e.g., sparrowhawks Accipiter nisus and common kestrels Falco tinnunculus) may have high Pb concentrations in their bones (Komosa and Kitowski, 2008), especially if wintering in Mediterranean countries, where small passerines are intensively hunted.

Several species of raptor have been experimentally induced to ingest Pb shot, but these kinds of study did not provide enough data to highlight a clear correlation between the number of

swallowed shot pellets and the effects on birds. A dose–response relationship seems to be mediated by several parameters such as age, physical and environmental conditions, and diet (see Kendall *et al.*, 1996 for a review). However, Hoffman *et al.*, (1985) noted that the nestling growth of the American kestrel Falco sparverius was impaired after only 4 days of treatment with a 125-mg/kg dose of metallic Pb powder, which was expressly used to simulate the form of Pb encountered in Pb shot poisoning. Since that dose is comparable to the mean mass embedded in the edible parts of a single starling (16.8 mg), a potential hazard for raptors feeding on unretrieved songbirds can be expected, especially for the species that are smaller in size.

However, in order to carry out a complete risk assessment related to the use of Pb ammunition, it is necessary to know how frequently a bird of prey can feed on unretrieved shot passerines. Unfortunately, at present, this information is completely lacking in Italy. Given the popularity of songbird hunting in most Mediterranean countries, the collection of precise bag statistics and knowledge of the frequency of crippled birds not retrieved by hunters would be desirable.

With respect to game birds of a larger size, the small passerines in our study had a lower mass of embedded Pb, but shot pellets and fragments embedded in their tissues were more abundant and tiny. The quantity and, particularly, the level of fragmentation of Pb embedded in young starlings shot in May–June to prevent damage to cherries suggest that the risk of Pb poisoning cannot be ruled out for humans and birds of prey consuming meat from songbirds killed with traditional ammunition. On the basis that small passerine hunting is a widespread practice in the Mediterranean region, a rapid transition to alternative ammunition is recommended.

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CHAPTER 7 - Assessing the lead pellets and fragments in game birds: the case of the Eurasian woodcock (*Scolopax rusticola*)

7.1 Abstract

Wild meat often retains metallic particles originating from the ammunition fired by hunters. Since ammunition are traditionally lead(Pb)-based, the consumption of game meat may entail the ingestion of Pb embedded in tissues. To assess the related risks to human health, information is needed on the number, dimension and spatial distribution of Pb particles embedded in popular quarry species. In this study we focused on the Eurasian woodcock (*Scolopax rusticola*), a medium sized bird intensively hunted across its range. We X-rayed 59 carcasses of woodcock shot by Italian hunters in Ukraine. Adults and juveniles were examined separately, since adults may retain lead fragments in old wounds originating in previous hunting seasons. To check the ammunition types and evaluate the mean weight of the embedded gunshot, we excised a sample of 62 whole pellets from 20 birds.

Ammunition residues were found in 57 of the 59 woodcock (96.6 %). Radiographs revealed 215 whole pellets and 125 fragmentation centres in 51 (mean = 3.64) and in 48 birds (mean = 2.14) respectively. Most fragmentation centres (75.7 %) contained tiny particles (< 1 mm). The overall estimated Pb load ranged from 45 to 52 mg/100g wet weight, most of which (84.6%) in edible parts. No differences were found between adults and juveniles.

The number of embedded pellets per unit of body mass (1.21/100 g of body weight) was higher in comparison with other bird species and also with woodcock shot in the UK, presumably owing to the hunting methods adopted by Italian hunters.

The quantity and characteristics of ammunition residues we found suggest that game meat consumers are exposed to a relevant Pb assumption.

7.2 Introduction

Lead (Pb) is a toxic element whose effects on human health are well known (Landrigan and Todd, 1994). In the last decades we have become aware that adverse consequences to organisms may arise even at very low doses, when clinical symptoms are not evident. Many epidemiological studies revealed that low Pb exposures (<10 µg/dL in the bloodstream) are especially detrimental to foetuses and children, hampering the development of the nervous system and causing

permanent negative effects on cognitive function and behaviour (Canfield *et al.,* 2003; CDC, 2005; Lanphear *et al.,* 2005; Chandramouli *et al.,* 2009). In adults, Pb affects information processing and short-term verbal memory, causing psychiatric symptoms and impairment of manual dexterity (Weisskopf *et al.,* 2007). Furthermore, relatively low exposures among adults have been associated with elevated systolic blood pressure, increased risk of myocardial and stroke mortality, cancer and nephropathies (Menke *et al.,* 2006; Schober *et al.,* 2006; EFSA CONTAM, 2010; Huang *et al.,* 2013).

These findings prompted international health authorities to reject a safe threshold for Pb exposure as inappropriate and to advocate strongly for reduced intake of Pb as far as possible especially for the most sensitive categories (infants, children and pregnant women) (EFSA CONTAM, 2010; JECFA, 2010).

Since ingestion is considered the major source of exposure to Pb in developed countries, the intake of Pb for the population of Europe was estimated by analyzing Pb contamination in all food categories (EFSA, 2012). According to EFSA (2012), Pb levels in food have decreased recently, but the consumption of some food categories may remain a cause of concern. Particularly high concentrations were recorded in wild boar and pheasant meat, well above the maximum levels admitted by the Commission Regulation No 1881/2006 for the categories “Meat (excluding offal) of bovine animals, sheep, pig and poultry” (max Pb level: 100 µg/kg wet weight) and “Offal of bovine animals, sheep, pig and poultry” (max Pb level: 500 µg/kg wet weight) (Commission of the European Communities, 2006). The EFSA food category “boar (wild pig)” meat gave a mean of 1,143 µg/kg, 100 fold higher than “pork/piglet” meat (mean value 11 µg/kg). Furthermore, some boar samples peaked at 232,000 µg/kg. Similarly, “pheasant” meat reached Pb concentrations 28 fold higher than “chickens” meat (344 µg/kg, *versus* 12 µg/kg). Similar differences between game and domestic animals were found in the UK (Pain *et al.,* 2010).

These results are due to the use of Pb ammunition for hunting game animals. High Pb values have been frequently found both in venison and game birds shot by hunters and intended for human consumption (Tsuji *et al.,* 1999; Johansen *et al.,* 2004; Cornatzer *et al.,* 2009; Pain *et al.,* 2010). Soft tissues of large mammals often retain a high number of fragments up to more than 45 cm away from the wound channel, as a result of the frangibility of the various kinds of rifle bullet. In most cases fragments are too small and are hardly ever detected or removed during food preparation (Hunt *et al.,* 2006; Tsuji *et al.,* 2009; Hunt *et al.,* 2009; Grund *et al.,* 2010; Knott *et al.,* 2010; Lindboe *et al.,* 2012). Contamination is even higher in birds, because they are generally

killed by several small gunshot pellets instead of a single bullet. Normally, pellets are so small that it is difficult to remove them from the flesh even if they remain whole. Furthermore, they do tend to fragment, creating a large amount of microscopic splinters and particles (Scheuhammer *et al.,* 1998; Mateo *et al.,* 2011; Andreotti and Borghesi, 2013).

The typical practices employed to prepare game meat for consumption (dressing, marinating and cooking in wine, vinegar or in other acidic conditions) facilitate the conversion of metallic Pb into organic compounds, which are more easily absorbed by the digestive system (Mateo *et al.,* 2007; Hunt *et al.,* 2009; Mateo *et al.,* 2011). Several studies have revealed a significant association between Pb blood levels and wild game consumption in human populations, not only in arctic regions where wild game is a significant part of the diet (Hanning *et al.,* 2003; Bjerregaard *et al.,* 2004; Johansen *et al.,* 2006) but also at mid latitude regions (Iqbal *et al.,* 2009). Such evidence has encouraged researchers and several national authorities for health and food safety to assess the risk of Pb poisoning through the consumption of game meat. The results of these assessments concluded that the risks posed by Pb ammunition on human health are not negligible and appropriate measures are needed to minimize those risks (AESAN, 2012; Green and Pain, 2012; VKM, 2013; Green and Pain, 2015).

In Europe, a rough estimation of the human population exposed to Pb through the consumption of game meat can be obtained through analysis of the number of hunters and their relatives. According to the European Federation of Associations for Hunting and Conservation, in Europe there are seven million hunters, without including Russia and Turkey. Therefore, it can be assumed that several tens of millions of Europeans, corresponding to approximately 2-4% of the whole population, more or less regularly eat game meat. The amount of birds shot annually in Europe is estimated to be 101 million (Hirschfeld and Heyd, 2005).

To assess the risk to the health of wild meat consumers it is important to know the quantity and characteristics of the Pb embedded in the flesh of the most popular quarry species. In this study we focused on Pb contamination in the Eurasian woodcock (*Scolopax rusticola*), a medium sized bird intensively hunted across its range. We examined adults and juveniles separately, since adults may retain lead fragments in old wounds originating in previous hunting seasons (Falk *et al.,* 2006; Newth *et al.,* 2011; Holm and Madsen, 2013). According to Annex II/A of the EU Directive 2009/147/CE, the woodcock can be hunted in all the States within the Union borders (European Parliament and Council of the European Union, 2010). Furthermore, the species is intensively shot in the Balkans and in the European countries of the former USSR, both by resident and foreign

hunters. Given the popularity of woodcock hunting in Europe, the consumption of its meat is widespread and considerable, but unfortunately only rough estimates are available on the number of birds annually killed on the continent. Ferrand and Gossmann (2009a) proposed an approximate figure of 3-4 million individuals, while Hirschfeld and Heyd (2005) suggested a minimum of 2,730,125 woodcock shot per year in the EC, Switzerland and Norway.

Specific objectives of our study were: i) to estimate the frequency of both whole pellets and fragments in shot woodcock; ii) to assess if any difference exists in pellet and fragment frequency between adults and juveniles; iii) to estimate the Pb burden embedded in the edible parts, and iv) to evaluate implications for the human health.

7.3 Materials and methods

We examined a sample of 59 individuals (28 juveniles and 31 adults) selected from a stock of 485 Eurasian woodcock shot by Italian hunters during a hunting trip in Ukraine in October 2011 and seized by the Italian Custom Agency because they were imported without complying to sanitary regulations. No bird was expressly killed for this study. The birds were frozen whole and stored at -20° C. On analysis the woodcock were thawed, aged by plumage and moult status (Ferrand and Gossmann, 2009b) and weighed to the nearest gram with a Pesola spring balance. Our sample of juveniles and adults was selected with their body weights within a range of $\pm$ 15% the average weight of the whole seized stock. We considered both age and weight because 1) old birds may have embedded pellets remaining from healed wounds in previous hunting seasons (Falk *et al.,* 2006; Newth *et al.,* 2011) and 2) weight is a reliable measure of body mass, that in turn affects the number of embedded pellets (Pain *et al.,* 2010).

We X-rayed the birds with digital radiography equipment (Kodak DirectView CR 800 System, and Kodak DirectView CR cassette 35 x 43 cm with a matrix size of 2048 x 2500). Metal particles are easy to distinguish from bone and grit because they are clearly more radio-opaque (Knott *et al.,* 2010). Radiographs were examined at full size to detect whole pellets and large fragments. Micro fragments were counted by zooming in to 150%. We considered as “large” those fragments exceeding 0.5 mm in diameter (Hunt *et al.,* 2006).

Following Andreotti and Borghesi (2013), we subdivided the body of each bird into seven sectors so that the anatomical parts normally consumed by humans could be easily considered separately (Fig. 7.1). We regarded Sectors 3, 4, 5 and 6 as edible, i.e. those most commonly eaten by humans.

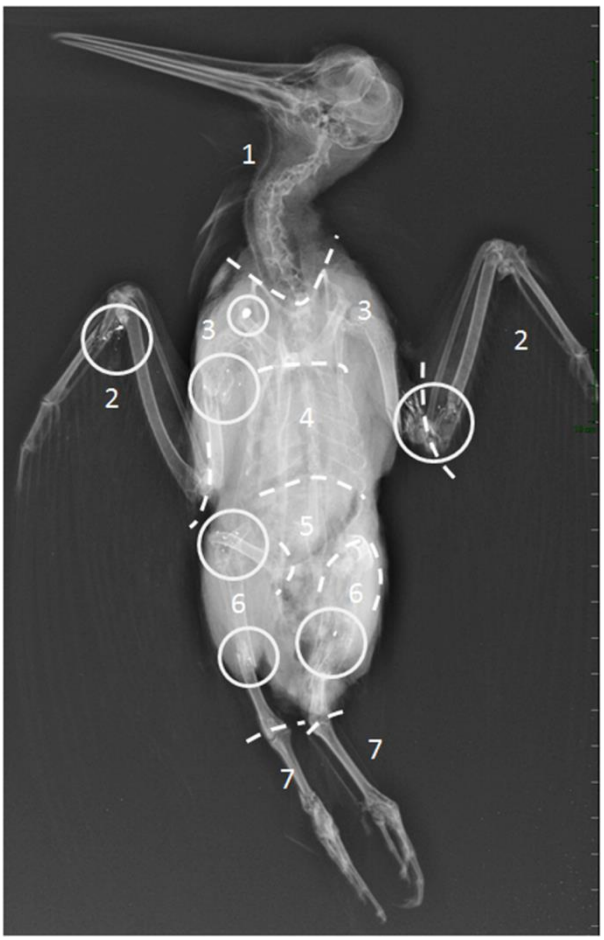

Figure 7.1 - X-ray photograph of a woodcock, showing the body sectors where the position of each shot pellet and fragment was noted. 1 head and neck, 2 wings, 3 humerus and pectoral girdle, 4 thorax, 5 abdomen, 6 femur and tibiotarsus, 7 tarsus and metatarsus. A whole pellet and six fragmentation centres can be recognised inside the grey circles.

We counted the number of whole shot pellets, single macro fragments and clusters of radiodense particles (fragmentation centres - FC) embedded in each body sector. The fragments were scored as follows: 0 = none visible; 1 = 1-2 macro fragments; 2 = 2-4 micro fragments; and 3 = > 4 fragments, regardless of their size. We tested whether the number of pellets and fragments observed in the body varied among age classes and body sectors with a chi-square test of independence performed using the R software (R Core Team, 2013).

To check the ammunition types used by hunters, we performed a necropsy on 20 woodcock and excised 62 whole pellets (2-4 from each bird). These pellets were accurately washed, dried, and weighed by means of a Sartorius Analytical balance (accuracy d = 0.1 mg). Furthermore, each pellet was examined for colour, form and size, and tested to see whether they were attracted by a bar magnet.

To evaluate the Pb quantity embedded in our sample, we multiplied the number of whole pellets by the mean weight of the excised pellets. Since most pellets lose fragments under the impact, this estimation was repeated considering the mean weight of the heaviest pellets from each woodcock, except in the case of 3 woodcock with pellets of different sizes, for which the mean weight of the excised pellets was used. Finally, we related the estimated amounts of Pb to the overall weight of the whole woodcock sample to obtain the Pb burden in 100g wet weight.

7.4 Results and discussion

Ammunition residues were found in 57 of the 59 carcasses (96.6%). We observed only whole pellets or fragments in 9 (15.8%) and 6 (10.5%) carcasses respectively, while in 42 carcasses (73.7%) we found both. Pellets and fragments were embedded in all body sectors (Tab. 7.1 a,b), but with significant differences in frequency (pellets: chi-square value = 77.17, df = 6, $P < 0.001$; fragments: chi-square value = 79.9, df = 6, $P < 0.001$). In the anatomical regions most commonly eaten by humans, we found Pb residues in 47 carcasses (79.7%). No significant difference was detected between age classes in frequencies of whole shot and fragments (pellets: chi-square value = 10.34, df = 6, P = 0.11; fragments: chi-square value = 7.68, df = 6, P = 0.26).

Shot gun pellets - Radiographs revealed 215 pellets in 51 woodcock (mean = 3.64, SD = 3.97, range = 0-17, n = 59). Most of the pellets (n = 131, 60.9%) were located in Sectors 3 and 4 while only a small fraction (n = 15, 7%) was found in the distal parts of the wings and legs (Sector 2 and 7; Tab. 7.1 a,b). In the edible sectors, we counted 182 pieces of shot, corresponding to 84.6% of the total amount (mean = 3.08, SD = 3.64, range = 0-17, n = 59). In 3 adults we detected pellets of different size by examining the radiographs. We ascertained that they were whole pellets of different dimensions by examining them after excision. During the necropsy we did not find any evidence of connective tissue encapsulation.

Table 7.1(a) - Distribution of pellets in different body sectors of juveniles and adults of Eurasian woodcock shot during hunting activity. Significant differences were found in frequency in different body sectors (chi-square value = 77.17, df = 6, P < 0.001). No significant difference was detected between age classes (chi-square value = 10.34, df = 6, P = 0.11).

	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	TOT	Mean	SD
Adults (n=31)	9	9	27	29	22	5	1	102	3.29	3.54
Juveniles (n=28)	9	3	33	42	13	11	2	113	4.04	4.43
TOT	18	12	60	71	35	16	3	215	3.64	3.97

[a]head neck; [b]wings; [c]humerus pectoral girdle; [d]thorax; [e]abdomen; [f]femur tibiotarsus; [g]tarsus metatarsus

Table 7.1(b) - Distribution of fragments in different body sectors of juveniles and adults of Eurasian woodcock shot during hunting activity. Significant differences were found in frequency in different body sectors (chi-square value=79.9, df = 6, P < 0.001). No significant difference was detected between age classes (chi-square value = 7.68, df = 6, P = 0.26).

	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	TOT	Mean	SD
Adults (n=31)	15	22	9	1	2	5	5	59	1.90	1.99
Juveniles (n=28)	6	26	15	3	1	8	7	66	2.36	2.54
TOT	21	48	24	4	3	13	12	125	2.14	2.27

[a]head neck;[b]wings;[c]humerus pectoral girdle; [d]thorax; [e]abdomen; [f]femur tibiotarsus; [g]tarsus metatarsus

Table 7.2(a) - Distribution of different fragment classes in adults of Eurasian woodcock shot during hunting activity (score 0 = none visible; score 1 = 1-2 macro fragments; score 2 = 2-4 micro fragments; score 3 = > 4 fragments, regardless of their size).

	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	TOT
score 1	1	2	1	0	1	0	3	8
score 2	13	13	2	1	1	4	2	36
score 3	1	7	6	0	0	1	0	15
TOT	15	22	9	1	2	5	5	59

[a]head neck; [b]wings; [c]humerus pectoral girdle; [d]thorax; [e]abdomen; [f]femur tibiotarsus; [g]tarsus metatarsus

Table 7.2(b) - Distribution of different fragment classes in juveniles of Eurasian woodcock shot during hunting activity (score 0 = none visible; score 1 = 1-2 macro fragments; score 2 = 2-4 micro fragments; score 3 = > 4 fragments, regardless of their size).

	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	TOT
score 1	0	4	2	1	1	1	1	10
score 2	3	9	6	2	0	3	3	26
score 3	3	13	7	0	0	4	3	30
TOT	6	26	15	3	1	8	7	66

[a]head neck; [b]wings; [c]humerus pectoral girdle; [d]thorax; [e]abdomen; [f]femur tibiotarsus; [g]tarsus metatarsus

Fragments - A minimum of 125 FC were detected in 48 woodcock (mean = 2.14, SD = 2.28, range = 0-10, n = 59). Fragmentation centres were concentrated (n = 93, 74.4%) in the head, neck, wings and the pectoral girdle (Tab. 7.1b). In the edible sectors, we estimated 44 FC, representing 35.2% of the detected FC (mean = 0.75, SD = 1.32, range = 0-6, n = 59). In most cases, the fragments were assigned to Score 2 (n= 62, 49.6%) or Score 3 (n = 36%), revealing a prevalence of small tiny particles (Tab. 7.2a,b).

Pellet characteristics - All pellets were non-magnetic, dark, dull and deformed, and therefore were assumed to be Pb. Overall they weighed 2,319.8 mg (mean = 37.4 mg, SD = 16.7, range = 13.1–76.7, n = 62). When taking the heaviest pellets from each necropsied woodcock, we obtained a mean weight of 43.8 mg (SD = 16.7, range = 20.0-76,7 n = 20).

Woodcock weights - The mean weight of the whole stock of seized woodcock did not differ significantly from the examined sample (whole stock: mean = 313.8 g, SD = 30.1, n = 403; examined woodcock: 304.0 g, SD = 1.7, n = 59; t = -1.94, P= 0.05). The mean weights of adults and juveniles did not differ (juveniles: mean = 304.0 g, SD = 15.4, n = 28; adults: mean = 300.4 g, SD = 19.5, n = 31; t-test = -0.18 P=0.40).

Pb mass - On the basis of both the mean weight of the excised pellets and the heaviest pellet subset we estimated the Pb burden embedded in woodcock ranging from 45 to 52 mg/100g wet weight.

7.4.1 Comparison with previous studies

Our study revealed that the majority of the X-rayed carcasses contained visible particles of metallic Pb deriving from spent ammunition. In comparison with data collected in previous studies

on different bird species, woodcock had a higher rate of embedded whole pellets per unit of body mass, *i.e*. 1.21 pellets/100 g of body weight, *vs*. 0.93 in European starlings (Andreotti and Borghesi, 2013), 0.73 in red legged partridges (Mateo *et al.,* 2011), 0.52 in common eiders (Johansen *et al.,* 2004) and 0.41 in thick-billed murres (Johansen *et al.,* 2001). Moreover, the frequency of pellets counted in our study is relatively high when compared to the figures reported by Pain *et al.,* (2010) for the Eurasian woodcock hunted in the UK. The authors X-rayed oven-ready carcasses (without feathers, viscera and heads) and recorded numbers of pellets and large radio-dense fragments. They found a mean of 1 pellet per woodcock, a low value with respect to the mean of 3.1 detected in our study considering edible sectors only.

These discrepancies in pellet frequency may be due to different hunting methods adopted with regard to the target species and traditional hunting practices. The low shot resistance of woodcock leads many hunters to prefer cartridges with numerous pellets of smaller size than those used to shoot game birds with a similar body mass. In addition, the hunting technique may influence the firing distance, which affects the spread pattern of projectiles hitting the prey.

For example, in the UK woodcock are often flushed towards the guns by a line of "beaters" walking through the wood, while Italian hunters prefer to walk behind a pointing dog, shooting the woodcock at a shorter distance over the dog's point (Spanò and Fadat, 2014). Hence, the quantity of Pb embedded in quarry bird species appears to be related not only to the species, but also to the hunting technique. These results should be borne in mind when assessing Pb contamination levels in game meat from different countries.

The concomitant presence of pellets of different size embedded in 3 adults might be explained by either the use of cartridges loaded with mixed grains or the existence of old wounds originating in previous hunting seasons. Since only slight changes occur in tissues surrounding Pb gunshot embedded in old wounds (Sanderson *et al.,* 1998), the circumstance that we did not observe connective tissue encapsulation does not allow us to exclude the presence of pellets from previous hunting seasons.

We did not find a higher frequency of whole pellets in adults when compared to juveniles, as found in long-lived species such as sea ducks and geese that can accumulate pellets in subsequent hunting seasons (Falk *et al.,* 2006; Newth *et al.,* 2011; Holm and Madsen, 2013). This result might be influenced by two factors: i) woodcock have little or no resilience to the shot and hardly ever survive when injured; ii) adults are more cautious than juveniles and flush earlier when stalked leading to them being shot at longer distances by a wider pellet spread. This latter factor is

relevant in assessing the Pb load in woodcock meals because juveniles are generally far more abundant than adults in hunting bags (more than 65% juveniles in France, Boidot and Aurousseau, 2013; 81.1% juveniles in our sample).

If we compare data on fragments recorded in starlings in a study carried out with a similar methodology (Andreotti and Borghesi, 2013), we observe a higher density of FC in woodcock (0.72 FC *per* 100 g of body weight, *versus* 0.60 in starlings), even if the fragmentation rate of pellets, calculated as the ratio FC/whole pellets, appears to be slightly lower in woodcock than in starlings (0.58 *versus* 0.64). The difference in the fragmentation rate might be related to a higher frequency of pellets that have passed through the starlings leaving Pb particles in the tissues, but have not remained in the carcass due to the smaller body mass of this species. Fragment distribution in woodcock differs significantly from starlings when considering all body sectors (chi-square value = 27.7 df = 6 $P < 0.0001$), but there is no difference when taking into account the edible parts only (chi-square value = 0.4 df = 3 $P = 0.9$).

Levels of Pb contamination in woodcock meat found in our study are much higher than those reported for venison examined by X-ray. As far as we know, there is only one estimate of the total mass of bullet fragments left in carcasses of deer (Knott *et al.,* 2010). This corresponds to 1.6 mg/100g wet weight, 30-fold lower than the estimates of Pb embedded in woodcock. Pb concentrations determined through chemical analysis confirm a higher burden of Pb in birds than in ungulates, but these differences are less relevant. For example, in partridges values have been found 2-8 fold higher than in red deer or wild boar (Mateo *et al.,* 2011; Sevillano *et al.,* 2011). This discrepancy can be explained considering that in birds there is a relatively higher amount of large Pb particles (essentially whole pellets) that are generally removed before performing chemical analyses.

7.4.2 Implication for the human health

Our findings reveal that woodcock meat derived from animals shot by traditional Pb ammunition retains a considerable quantity of metallic Pb in the form of both large particles (i.e. macro fragments and whole pellets) and tiny fragments. Even when substantial pieces of Pb are removed from game meat and the bioavailability of the metallic Pb is low, the presence of Pb is still significant, especially where tiny fragments are spread widely. Chemical analyses have found high levels of Pb contamination in game tissues samples of quarry species where Pb particles were detected by X-ray (Tsuji *et al.,* 1999; Johansen *et al.,* 2001; Tsuji *et al.,* 2009; Pain *et al.,* 2010;

Mateo *et al.,* 2011). Furthermore, embedded Pb can be easily absorbed through the intestine by game meat consumers, given the amount of tiny fragments, easily attacked by the gastric acid, and the leaching processes during cooking treatments (Mateo *et al.,* 2007; Hunt *et al.,* 2009; Mateo *et al.,* 2011). Hence, regular woodcock meat consumers are exposed to real health risks.

Hunters are not usually woodcock specialists, killing both ungulates and small game during the same hunting season. This implies that people consuming woodcock often eat other game birds, small mammals and ungulates. It has been found that frequent consumption of big game meat alone is enough to expose the hunter population to the risk of Pb poisoning (Sevillano Morales *et al.,* 2011; Lindboe *et al.,* 2012). Consumption of contaminated woodcock meat will increase the intake of Pb in game consumers already exposed to the risk of Pb poisoning. In Italy, more than 230,000 ungulates are estimated to be shot annually, producing 6828.7 tons of game meat (Ramanzin *et al.,* 2010), in addition to at least 17 million birds (Hirschfeld and Heyd, 2005) and an unknown number of hares and rabbits. Since the use of lead ammunition is banned exclusively in wetlands of Special Protection Areas and in some hunting districts exclusively for the hunting of cloven hoofed games, only a negligible fraction of game birds and mammals is killed with lead-free shot or bullets. This means that the 750,000 licensed hunters in Italy and their relatives consume approximately tens of millions of meals derived from ungulates and no less than 5 million meals from game birds, killed with Pb ammunitions.

The high frequency of Pb pieces and, in particular, of tiny fragments recorded in this study reveals that precautions during the preparation for consumption of shot game birds are not enough to guarantee Pb-free meat. The removal of whole pellets before cooking is not easily achieved, given their small size and abundance, and does not prevent the ingestion of a significant quantity of the smallest particles. Additionally, Pb residues are almost uniformly distributed in game bird carcasses, making removal more difficult. It is a different case to ungulates, where fragments of ammunition are concentrated in a radial zone around the wound channel, allowing a small concentrated area of carcass to be removed and disposed of. The distribution of Pb in bird carcasses makes it impossible to define handling procedures which reduce the risk for game bird consumers. The use of Pb-free pellets is the only effective solution to avoid the consumption of Pb when eating shot small game. It could also reduce the Pb pollution in the environment. Positive effects have been observed in wildlife and ecosystems, where lead ammunition has been banned (Mateo *et al.,* 2014).

7.5 Conclusions

The considerable amount of embedded particles of metallic Pb found in our study suggests that a significant risk to human health is associated with the frequent consumption of woodcock killed using traditional Italian hunting techniques. Therefore, a risk assessment is recommended.
To reduce the hazard of Pb poisoning on regular woodcock consumer and more in general on game meat consumers, actions should be promoted to raise awareness of the risks among exposed groups (hunters, gamekeeper, etc.). A mandatory Pb-free certification mark could be used to provide a guarantee of safety for game meat consumers. At the same time, the adoption of a total ban for the use of lead ammunition in both aquatic and terrestrial ecosystems is strongly recommended (Group of Scientists, 2014).

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CHAPTER 8 - Embedded lead in game birds: the case of a Peregrine Falcon (*Falco peregrinus*) found dead with lead shot in the digestive tract

8.1 Abstract

Lead (Pb) ammunition represents a source of intoxication for raptors eating game. Even if the Peregrine Falcon commonly preys on game birds, only a few cases of Pb poisoning have been recorded up to now. We document an adult female found dead with many Pb shot in the digestive tract**.** Concentrations in heart blood clot (0.10 mg/kg) suggest that Pb shot raised Pb blood levels, while the low values in liver, kidney and bone lead us to exclude that poisoning was the ultimate cause of death. The increase of blood Pb levels might have impaired the coordination, provoking a fatal collision

8.2 Introduction

Lead (Pb) ammunition represents a relevant source of intoxication for birds. In wetlands waterbirds may ingest Pb shot lying in the surface sediments, when seeking grit or mistaking them for seeds (Beintema, 2001). Similarly, granivorous birds may face the same fate in areas where hunting is intensively practiced. Even species with specialized feeding habits, such as woodcock, may pick up and swallow shot dispersed by hunters (Pain *et al.,* 2009).

Scavenging birds ingest Pb when eating carcasses of unretrieved game or gut piles left by stalkers in the field after hunting (Pain *et al.,* 2009). The contamination of game flesh and viscera occurs either when Pb bullets or shot are used. Bullets tend to split into many fragments when they impact bones or other body structures, spreading through the tissues in a radius of several decimeters around the wound channel (Grund *et al.,* 2010). Fragmentation occurs also when Pb shot hit gamebirds, such as ducks, partridges, waders and small passerines (Andreotti *et al.,* 2016). Even raptors feeding exclusively on live prey are exposed to Pb ingestion. Several studies revealed that a relevant number of individuals hit by firearms do survive, retaining embedded Pb particles in their body (Guillemain *et al.,* 2007). The likelihood of poisoning for a bird of prey varies according to the proportion of game in the diet, the size of game taken, the season, and the local hunting intensity (Pain *et al.,* 1993).

The Peregrine Falcon (*Falco peregrinus*) is one of the raptors less inclined to feed on carcasses. Its diet is composed almost entirely of small to large-sized birds (from 10 g to 2 kg) caught on the fly (Cramp and Simmons, 1980). Though this species commonly preys on game birds, such as pigeons, doves, partridges, ducks and thrushes, only a few cases of Pb poisoning in peregrines have been collected up to now. The ingestion of Pb shot was ascertained in a unique case in Spain, through the analysis of regurgitated pellets (only 1 pellet with Pb shot was found out of 117, Mateo *et al.,* 2007). Furthermore, 1 bird out of 9 was found exceeding the threshold value of bone Pb > 10 ppm dry weight (d.w.) (Mateo *et al.,* 2003). In the UK elevated Pb concentrations (> 6 ppm d.w.) were detected in the liver of 4 out of 26 individuals examined by Pain *et al.,* (1995). The latter authors concluded that a Peregrine with Pb liver concentrations > 20 ppm d.w. died as a consequence of exposure to Pb shot embedded in its prey. We document a case of an adult female Peregrine Falcon found dead with many Pb shot in the digestive tract**.**

8.3 Methods

The bird was recovered on February 7, 2015 at the foot of a road lighting pole, in an intensively cultivated area of the Po Plain, near Faenza (Ravenna District, north east Italy). It was examined externally, weighed to the nearest gram, X-rayed with an analog radiography equipment, and autopsied. Since the radiography revealed shotgun pellets in the digestive tract (Fig. 8.1), the content of crop, gizzard and intestine was inspected. Most of the pellets were extracted, thoroughly washed, dried, and weighed by means of a Sartorius Analytical balance (accuracy d = 0.1 mg). Then they were examined for colour, form and size, and tested to see whether they were attracted by a bar magnet. Liver, kidneys, heart, abdominal fat and bones (coracoids and clavicles) were excised, stored at -20°C and subsequently thawed and analysed. The post-mortem heart blood clot was extracted to estimate Pb levels in the bloodstream. Pb concentrations were measured by means of a Inductively Coupled Plasma Mass Spectrometer (ICP-MS). High purity deionized water was obtained by Evoqua water technologies (Barbsbuttel DE); nitric acid was from J.T. Baker (Center Valley PA, USA); hydrochloric acid was from Sigma (St. Luis MO, USA). Each sample (about 3 g) was homogenized, and added to 10 mL of nitric acid in a screw cap polypropylene 50 mL sample tube (Digi-Tubes SCP Science); samples were placed in a Digi-Prep system (SCP Science) at 75 °C overnight. Then, after cooling, samples were diluted to 200 mL with an acid solution and analyzed by ICP/ MS (7700 Agilent Technologies USA) with an ASX-500 CETAC Autosampler (Cetac Technologies Omaha NE, USA). Results were expressed as wet

weight (w.w.). According to Pain *et al.* (1995), we adopt 1 ppm w.w. = 3.5 ppm d.w. to compare our data with the literature.

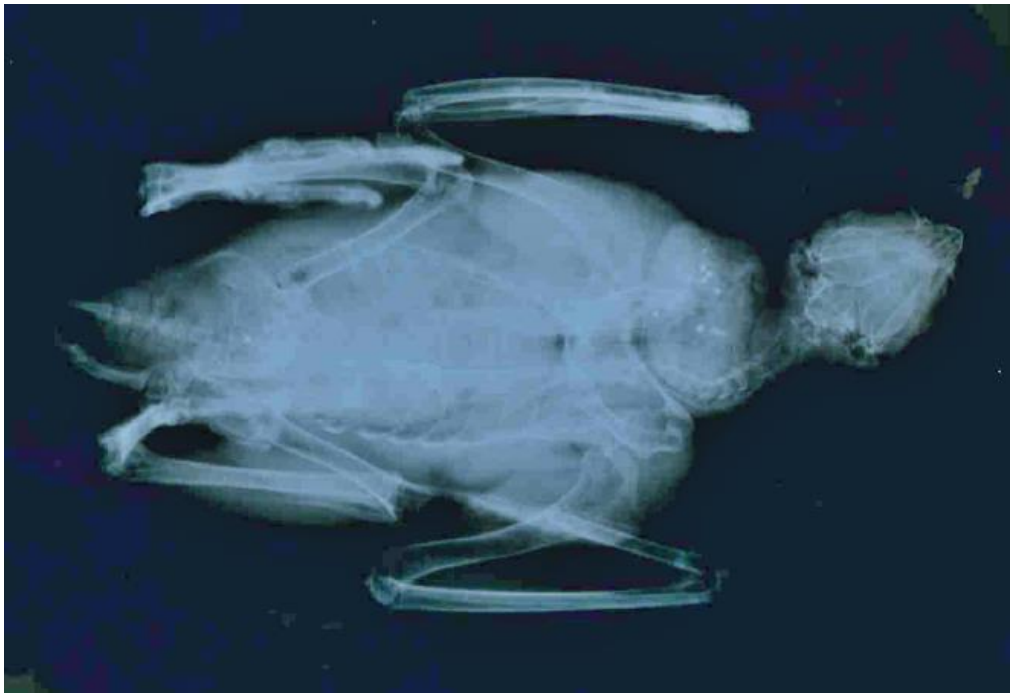

Figure 8.1 - X-ray photograph of the Peregrine falcon, showing the Pb shot in the digestive tract

8.4 Results and discussion

External examination revealed good body condition and plumage status. Neither injuries nor lesions were observed, except for an eye hematoma. The bird weighed 1,080 g, within the range reported for adult female peregrines (Cramp and Simmons, 1980). X-rays allowed us to identify 7 pellets in the upper part of the digestive tract and 1 in the cloaca (Fig. 8.1). During necropsy, we excised 6 pellets from the crop and 1 from the stomach. All the pellets had similar characteristics and size (mean weight = 31.0 mg, SD = 4.1, range = 27.0-38.3) and were considered to be composed by Pb upon appearance.

In the crop we found many remains of bones, muscular tissues, feathers and about 10 sunflower seeds. It was possible to identify a bird tongue, an esophagus, some groups of feathers not completely grown and a tract of intestine. All the feathers and anatomical parts were attributed to feral pigeon *Columba livia.* In the gizzard we identified a leg of a feral pigeon, a leg of a European starling *Sturnus vulgaris*, feathers of different size and colour, fragments of bones from birds of different size, cervical vertebrae of a large bird, muscular tissues and 12 sunflower seeds.

Presumably, the starling was the Peregrine penultimate meal and the feral pigeon the last one. The 7 pellets mixed with the ingesta were likely embedded in the pigeon, whereas that one in the cloaca was swallowed along with the starling. Pellets were undersized for pigeon (No. 10, commercial weight CW = 40 mg, instead of No. 8, CW = 70 mg, or 7, CW = 90 mg, normally used to control feral pigeons). This could have allowed the pigeon to survive until being caught by the falcon.

Pb tissue concentrations were 0.10 mg/kg in heart blood clot, 0.086 mg/kg in liver, 0.198 mg/kg in kidney, 0.141 mg/kg in abdominal fat and 3.379 mg/kg in bone (w.w.). In the heart blood clot Pb levels were well above the values reported by Carpenter *et al.* (2003) for 2 turkey vultures *Cathartes aura* fed with uncontaminated food (0.00 and 0.02 ppm w.w.), but below the concentrations measured in 5 vultures experimentally dosed for many weeks with Pb shot (6.00-29.56 ppm w.w. in 3 birds dead or with severe clinical signs and 1.12-1.87 ppm w.w. in 2 birds which survived and euthanized at the end of the study). Pb concentrations in liver, kidney and bone reflect background contamination (Pain *et al.*, 1995, 2007 and 2009; Carpenter *et al.*, 2003; Mateo *et al.*, 2003).

Pb concentrations in blood, liver, kidney and bone provide information on the timing of Pb exposure. Pb levels in the bloodstream increase within 24 hours following absorption (Hoffman *et al.*, 1981), but tend to decrease rapidly (half-life of 14 days in the California Condor, Fry *et al.*, 2009). Liver and kidney retain Pb for weeks to months (Pain *et al.*, 2009). In bones Pb is stored for years, thus reflecting lifetime chronic exposure (Mateo *et al.*, 2003; Pain *et al.*, 2005 and 2007). Measured tissue concentrations in the Peregrine suggest that the ingestion of Pb shot caused a visible increase in Pb blood levels, but it was too recent to allow a relevant absorption in soft tissues and bones. Thus the ultimate cause of death for the bird cannot be attributed to acute Pb poisoning. Nevertheless, the rapid increase of Pb levels in the bloodstream might have impaired coordination and dexterity of this fast-flying predator, and might have caused a fatal collision (suggested by the cerebral hemorrhage). Indeed, a significant association was found between sub-acute Pb concentration and the proportion of birds recovered with injuries by trauma and/or electrocution (Berny *et al.*, 2015).

Both species preyed on by the Peregrine analysed in this study cannot be hunted in Italy, but are intensively shot all year round to prevent damage to crops. In the Ravenna District in 2014 and 2015 feral pigeons were legally shot (in derogation regime) without any bag limit, whereas a quota of one thousand individuals per year was fixed for the starling. To prevent Pb poisoning in

raptors, a ban on the use of Pb shot - extended to hunting and pest control operations - is strongly recommended.

8.5 Acknoledgements

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9 - General conclusions

The main aim of this thesis was to provide new data, methodological tools, and insights, to improve the possibility to use birds as biomonitors of metal exposure.

As a work definitely structured in three parts, each one focused on a specific discipline (geochemistry of sediments, biomonitoring, risk assessment), this doctorate thesis has specific conclusions and recommendations that have been exposed in each chapter. However, it can be convenient for the reader to find here a summary of the main outcomes of each research carried out during the whole period of study, before focusing on a general comment about the overall work done.

In the first part, high attention has been paid to gain more data regarding the abiotic environment of habitats (coastal brackish wetlands) used by colonial waterbirds of high ecological importance (and therefore focus of many conservation efforts). Waterbirds show a strong relationship with the mud and are therefore especially exposed to pollution, where anthropogenic inputs have altered the natural levels of metals and trace elements (MTEs) in sediments. The Po Delta area is very rich of brackish wetlands and lagoons which represent crucial feeding sites for millions migratory birds and hosts many breeding colonies of waterbirds each year. Its relevance as an ornithological hotspot is worldwide. Despite this, a number of that wetlands were completely unexplored under a geochemical point of view, because private and therefore closed to people (including researchers). Conversely, a few wetlands have been studied in deep in the last two decades and information about their sediment features and quality are known for some elements. However, information about many trace elements was scarce even for the better studied wetlands and comparisons with the less studied ones had never or little been explored before this work. The first geochemical study of this doctorate allowed to obtain geochemical data in 7 Po Delta sites: Valli di Rosolina (ROS), Valle Bertuzzi (BER), Valli di Comacchio (COM), Vene di Bellocchio (BEL), Valle Mandriole (MAN), Pialassa della Baiona (BAI), and Pialassa dei Piomboni (PIO). A second study was focused on a selection of 4 sites: ROS, BER, COM, and BAI. After these studies, which consist to date in the most comprehensive geochemical characterization of the surface sediments of the wetlands located along the North Adriatic coast from the town of Ravenna, northward to Chioggia, we know the total concentrations of 26 trace elements: Al, Ba, Ca, Ce, Co, Cr, Cu, Fe, K, La, Mg, Mn, Na, Nb, Ni, P, Pb, Rb, S, Si, Sr, Ti, V, Y, Zn, and Zr, determined by X-ray fluorescence spectrometry (XRF) and pseudo-total concentrations of 46 elements: Ag, Al, As, Au,

B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hf, Hg, In, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Pd, Pt, Rb, Re, S, Sb, Sc, Se, Sn, Sr, Ta, Te, Th, Ti, Tl, U, V, W, Y, Zn, and Zr, determined by inductively coupled plasma mass spectrometry (ICP-MS) after *aqua regia* digestion. Moreover, by using some available background values and other tools, high levels of Cr and Ni have been explained to be of natural origin (in agreement with literature for some sites), whereas others elements (e.g. Ag, Bi, Cd, Cu, Hg, and Zn in BAI) are of anthropogenic origin and environmental concern. These results should be taken into account by managers when taking some actions in order to favor the biodiversity and habitat conservation, such us drying ponds or changing chemical parameters capable to influencing the mobility of harmful elements, or allowing activities such as shellfish. Eventually, some Pb anomalies have been supposed to be caused by the high presence of lead ammunition in the sediment, as an effect of about a century of waterfowl hunting.

In the second part of the thesis, the need to develop reliable methods to correctly measure metal exposure, intake, and bioaccumulation when undertaking biomonitoring surveys has been considered. Birds offer the possibility to assess MTE bioaccumulation by using a unique non-invasive method: assessing concentrations in feathers. This attractive opportunity has proven to be a very informative tool to unravel various physiological, ecological and toxicological processes inherent to individuals and populations. However, as highlighted in the first biogeochemical study of this doctorate, through the multivariate analysis carried out on 48 element concentrations in flamingo feathers from several colonies in the Mediterranean, and analyzing data on about 50 elements obtained from sediment samples collected near the breeding sites, environmental contamination (ExCo) of feathers has been proved to create an important bias, particularly due to the adhered sediment particles. The first study here reported has raised the considerable problem of ExCo, whereas the second study (again on flamingos) presents and validates a new method for correcting for ExCo during the analysis of feather trace elements. Also, the second study confirmed that ExCo is negligible for Cu, Hg, and Zn, and drawn the same conclusion for Se and Sn. On the contrary As, Cd, Cr, Ni, and Pb, even if bioaccumulated at some extent, need correction for ExCo in order to correctly interpret the concentrations as an effect of bioaccumulation. Aluminum, K and La emerged as not or negligibly bioaccumulated elements. The method has been developed by testing the washing effect, observing by SEM the nature of external particles, and analyzing the geochemical fingerprint of each sampling site. The problem of ExCo in feathers was not new in literature, of course, but in this doctorate it has been explored for a higher number of elements

and supported by a sound geochemical approach never applied in previous studies. The proposed method can be applied to any species of birds for which environmental contamination can be traced to local geochemical sediment data. However, the use of feathers deprived of vane instead of whole feathers (in other words only the rachis), should reduce the amount of ExCo, and has been suggested as a good choice for future ecotoxicological studies. As a general suggestion, the approach of using geochemical data to quantify environmental contamination may also appeal to investigators using non-avian model organisms (e.g. plants).

The third part is focused on a particular source of Pb exposure which unites birds and humans: game birds shot by hunters using Pb ammunition. Mammals and birds, when shot, often retain in their body some Pb pieces. When consumers eat so-killed animals, they often swallow Pb fragments, and can be victim of plumbism, a form of acute/chronical poisoning caused by the toxic effect of Pb. As a demonstration of a possible consequence, a case of a Peregrine falcon found dead with Pb pellets embedded in its last meal has been reported as a last scientific contribute of this thesis. This kind of assumption is called 'secondary' (being 'primary' assumption when birds pick up Pb pellets directly from the soil or sediment) and especially affects raptors, scavengers, and humans. Through two different studies, the frequency, type, and mass of Pb has been calculated or estimated (depending on the type of particle) in two small-sized bird species (European starling and Eurasian woodcock) by using a number of radiographies and excising pellets from some carcasses. Small birds like Passerines were not considered in previous studies because they are of minor interest as game in most countries. On the contrary, in Mediterranean countries, hunting to small bird species (20-300 g) is traditional. A considerable amount of embedded particles of metallic Pb has been found in most of carcasses investigated in both studies, suggesting that a significant risk for human health is associated with the frequent consumption of small game birds. Raptors and scavengers eating unretrieved small game are also exposed to plumbism being Pb fragment abundant and tiny even in hunted Passerines.

The main objectives of this doctorate have been achieved filling some gaps in literature regarding the knowledge of important bird areas, the improvement of methodologies frequently adopted for biomonitor pollution by using birds as indicators, the completion of a cognitive framework regarding a problem involving bird and human health only recently raised as prior for international fundamental agreements such as the Convention of Conservation of Migratory Species (CMS) of UNEP.