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Trace elements on bird species in the Venetian Lagoon: analysis of non-invasive samples (feathers and egg shells)

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ABSTRACT

The Venetian Lagoon is a natural wetland with an immense biological heritage in which rare and endangered animal and plant species are present. With the advent of industrialization, from the early twentieth century onwards, a series of purely economic choices were conducted which had alarming consequences for the state of the Lagoon. These events led to the establishment of vast areas of high naturalistic and/or landscape value subject to environmental protection on an international level. In these protected areas, ecosystems are monitored using biomonitors, living organisms representative of an area used to measure the concentration of pollutants and the extent of the damage they cause in the environment. Especially birds are considered useful biomonitors because of their visibility, sensitivity to environmental changes and their position on the food web, which makes them suitable to study bioaccumulation. Their ecology, physiology and behaviour have been well studied and they are of interest to the public. Most studies have been conducted on internal tissues, but the number of studies making use of non-destructive matrices (e.g. measuring the concentrations in feathers, feces and eggs) has increased over the years. Especially feathers may be valuable tools to monitor exposure to trace elements as they are excellent indicators of pollutants taken up through diet. This study will focus on the analysis of trace elements in feathers of bird species included in the Birds Directive of the Natura 2000 network, with focus on *Charadrius alexandrinus*. Concentrations obtained from feathers of *C. alexandrinus* are lower than those from feathers of the species used as comparison material and those found by similar studies. Only Hg makes an exception, as it was found in greater quantities in the samples of *C alexandrinus*. The species with the highest concentrations for almost all trace elements are *Thalasseus* s*andvicensis* and *Phalacrocorax carbo sinensis*. The samples of the former have all exceeded the toxicity threshold for Se while, for the latter, values of Pb are 60 times higher than its toxicity threshold. Concentrations found in egg shells are all lower than those found in feathers, except for Ca and Co.

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1) AIM OF THE STUDY

The Venetian Lagoon is one of the largest and most extensive lagoon ecosystems in Europe and in the entire Mediterranean basin.

It is a natural wetland with an immense biological heritage in which rare and endangered animal and plant species are present (Città di Venezia, 2018). Since the times of the Serenissima Republic, the Venetian Lagoon has always been considered a sensitive environment, where fragility and complexity were perceived. With the advent of industrialization, a series of purely economic choices which resulted in a progressive degradation of the ecosystem (Mariutti, 2012). Examples are the fish farms, aimed at lucrative aquaculture and hunting, remediation and the progressive deviation of the original route of rivers and canals in the 19th century and the conversion of some wetlands in industrial areas and agricultural environments in the 20th century (Mariutti, 2012).

These events led to the establishment of vast areas of high naturalistic and/or landscape value subject to environmental protection on an international level. The entire lagoon area has in fact been designated as a World Heritage Site by UNESCO, identified almost entirely as a Site of Community Importance (SCI) and Special Protection Area (SPA) within the Natura 2000 Network by the European Commission and proposed as a Ramsar area "Wetland of International Importance" (Città di Venezia, 2018).

In these protected areas, ecosystems are monitored using bioindicators (or biomonitors), living organisms representative of an area used to measure the exposure to pollutants and the extent of the damage they cause in the environment. Especially birds are considered useful biomonitors because of their visibility, sensitivity to environmental changes and their position on the food web, which makes them suitable to study bioaccumulation and biomagnification. Their ecology, physiology and behaviour have been well studied and they are of interest to the public. Most studies have been conducted on internal tissues, but the number of studies making use of non-destructive methods (like measuring the concentrations in feathers, feces and eggs) has increased

over the years. Especially feathers may be valuable tools to monitor exposure to trace elements as they are excellent indicators of the concentration of pollutants in the body.

This study will focus on the analysis of the body burden of bird species included in the Birds Directive of the Natura 2000 network, such as *Chroicocephalus ridibundus, Egretta garzetta, Ichtyaetus melanocephalus, Phalacrocorax carbo sinensis, Thalasseus sandvicensis, Tyto alba, Phasianus colchicus* and especially *Charadrius alexandrinus*.

Hence, the main questions that this research will try to address are the following:

- What are the concentrations of trace elements in feathers and egg shells of birds included in the Directive 2009/147/EC of Natura 2000 network?
- Does accumulation of trace elements pose a risk for birds of the Venetian Lagoon?

2) INTRODUCTION

- **About trace element pollution**

Human impact on the natural environment has increased in the last century because of population growth and technological development. This has become a relevant issue from an ecological and environmental point of view as the occurrence of pollutants produced by human activity, such as trace elements, poses great risks for all living organisms.

They can accumulate in soils, sediments and in the upper levels of the food webs (in relation to habitat and dietary preferences) at environmentally hazardous levels (Hosseini Alhashemi et al., 2011). Trace elements can be classified as essential and non-essential. Essential elements (i.e. Fe, Co, Cu, Mn, Mo, Zn, Se) are necessary for maintaining the biological processes of humans, animals, plants, and microorganisms. However they can play this important role only if assimilated at low concentrations, since they may be toxic in quantities exceeding metabolic requirements (Singh et al., 2011).

The total concentration of these elements in soil and sediments can be present for a long time into the environment as they can not undergo microbial degradation (Borghesi et al., 2017). However, these remain bioavailable for biota, exposing wildlife to their adverse effects even for many years (Kaur & Khera, 2018).

However, elements that are toxic can turn beneficial under certain conditions (V, W) (Singh et al., 2011) and other ones have unknown biological functions or may exert toxic effects on species, negatively affecting fitness and life-history traits as well as causing diseases (Järup, 2003). The latter is the case of As, Sb, and few others (Cd, Pb, Hg, Ni, Pu, Tl) that are toxic irrespective of quantities, without known beneficial effects on organisms (Borghesi et al., 2017; Hosseini Alhashemi et al., 2011). Contamination by trace elements (for example caused by Zn, Cu, Fe, Ni and Pb) has gotten significant consideration because they can cause serious diseases and genetic disorders like

reproduction failure, kidney toxicity and impair bone development if they are assimilated in high concentrations. (Kaur & Khera, 2018; Ullah et al., 2014).

Trace elements are always present in the environment and can be produced both from anthropogenic and natural activities. Emissions into the environment may occur via a wide range of processes and pathways, including atmospheric emission (during combustion, extraction and processing), surface water (via runoff and releases from storage and transport) and discharges in soils (and hence into groundwaters and crops) (Järup, 2003).

Naturally they are present in very low concentrations and are persistently discharged into the environment from regular sources like rock outcroppings, weathering, vulcanic emissions, forest and prairie fires, natural vegetation and oceanic activities (Kaur & Khera, 2018).

However, it has been demonstrated that metals from anthropogenic inputs tend to be more bioaccessible and then bioavailable than the same elements of natural origins. Trace elements are also present in nature in relation with human activities like industrial manufacturing, combustion products, recreational activities or agricultural run-off (Burger, 1993) and during the last decades industrial effluents, organic fertilizers, wastes, blazing, vehicles and power genesis released into the environment elevated concentrations of toxic elements (Ali et al., 2016). This poses a problem for conservation of terrestrial and acquatic ecosystems (Burger, 1993; Furness & Greenwood, 1996) as it represents a severe risk and threat to the human wellbeing and both fauna and flora through bioaccumulation along the food web (Hofer et al., 2010; Kaur & Khera, 2018; Ullah et al., 2014).

The bioavailability of a trace element is defined as the bioaccessible fraction of the element absorbed in specific conditions within a definite period and subsequently utilized for normal physiological functions (Fairweather-Tait, 1992). For some of the elements this is the incorporation into various metalloproteins, such as Fe in haemoglobin.

Some elements, such as Ca and Mg, have a structural role in bones and teeth. Most elements are cofactors of a wide range of enzyme systems, for example Se in

glutathione peroxidase (Fairweather-Tait, 1992). Trace elements are transferred through the food web from sediments, soils or water to the biota.

Essential and non-essential elements accumulated in soils and sediments can be taken up selectively by plants in response to concentration gradients, with a level of accumulation differing between and within species. For toxic elements such as Cd, Hg and As, a number of biochemical reactions occur in plants and, in an attempt to limit the toxicity effects, they try to store them in the roots and/or shoots and/or leaves, accumulating elements which are strongly poisonous for metabolic activities, photosynthetic functions and water retention of the plant (Peralta-Videa et al., 2009).

Plant species, when stressed by the accumulation of toxic elements in their tissues, have developed a variety of detoxification mechanisms in order to eliminate the biological damage (Hosseini Alhashemi et al., 2011). However, these mechanisms can also make these elements available for the next trophic level, allowing toxic elements enter the food web through herbivory and exacerbating the extent of the ecological deterioration (Baudrot et al., 2018).

Biomagnification is the process by which an element or an organic compound increases its concentration in the tissues of organisms as it travels up the food web. As these trace elements increase progressively in concentration as they pass between trophic levels, organisms in the higher trophic levels become more sensible to bioaccumulation.

The exposure to trace elements occurs not only by direct ingestion of organisms belonging to the lower trophic levels or detritus (as in case of many worms and arthropods), but also through complex interactions between routes of exposure (inhalation, dermal absorption and maternal transfer), intensity (concentration and bioavailability), frequency and duration of the exposure (Baudrot et al., 2018; Thongcharoen et al., 2018).

This kind of contamination can affect populations or single individuals and the food web itself. One of the possible effects is the “trophic cascade” where the food web is disturbed by a change in the abundance of the highest trophic level or in a change in the resources, like the decrease in predator abundance and increase of prey density, with more pressure on prey resources (Baudrot et al., 2018).

- **Birds as biomonitors**

Monitoring environmental contamination and investigating how organisms are affected by trace element uptake has become necessary. A prerequisite for such monitoring is developing reliable methods and strategies to correctly measure trace element exposure, uptake and bioaccumulation (Borghesi et al., 2017, 2016). One of the most relevant tools for accessing exposure to contaminants and also predicting effects is the use of biological indicators as early warning systems for environmental deterioration and as an evaluation tool (Abdullah et al., 2015; Furness & Greenwood, 1996). For this type of monitoring, usually a species of local fauna that is as representative as possible of the area is selected. This is made not only to monitor the health of the species itself but also to quantify the abundance and bioavailability of trace elements, their ultimate effect on the ecosystem's food web and fluctuations in contaminant levels in the environment (Baudrot et al., 2018; Furness & Greenwood, 1996).

When the change in the environment is more complex than the simple presence of pollutants, a suite of indicator species is usually required since the knowledge of their ecology can help to establish and interpret such a system (Furness & Greenwood, 1996). In ecotoxicology, this method is used to identify the effects of different contaminants at various levels of the trophic web (generally aquatic) and to take into account all possible routes of exposure. This allows better analysis of the quality status of an environment.

The data obtained from the use of living organisms to monitor pollution due to trace elements have proved to provide more accurate and promising results than chemical and physical analyzes in terms of bioavailability and biotransference of contaminants. From this kind of data, physiological and behavioral symptoms of induced toxicity can be obrserved as well. (Markowski et al., 2013).

Birds have been recognized since the 1960s as potential bioindicators of environmental pollution and for various types of contaminants, including trace elements (Borghesi et al., 2017). This is because they can be exposed to trace elements both externally, by physical contact, and internally, by consumption of contaminated food (Markowski et al., 2013). Furthermore, the response of some bird species at the top of the food web may point out any change in the lower trophic level (Abdullah et al., 2015).

Most birds occupy high trophic levels in terrestrial and acquatic food webs, and thus are more exposed to the risk of bioaccumulation for a wide range of pollutants, that may influence the health and fitness of individuals (Bustnes et al., 2013). Uptake of contaminated food is the primary route of exposure for birds, although it may occur also through other routes such as dermal contact or inhalation. As diet is the most important pathway for birds, chronic exposure to metals and metalloids through food intake can result in high concentration of these elements in bones, tissues, organs and blood (Ashbaugh et al., 2018). Raptor species are more exposed because of their high trophic position, their scavenging activities, their large spatial living area and long lifespan (Baudrot et al., 2018).

For waterbirds, the ingestion of contaminated food, soil or sediment can provoke acute poisoning, causing death, although most often they are affected by chronic exposure at low concentrations (Borghesi et al., 2016).

Seabirds spend a considerable part of their lives in coastal and marine environments and then are exposed to a wide range of chemicals through ingested food and water. The main factors influencing their vulnerability to bioaccumulation are their trophic position, feeding habit and foraging range (Michelutti et al., 2010). They also represent a biological vector for the transfer of nutrients, organic matter and pollutants from marine to terrestrial habitats and viceversa. Furthermore, the trophic plasticity of some seabirds like gulls allows them to benefit from anthropogenic food resources making them particularly vunlerable to contaminant exposure and accumulation (Signa et al., 2013).

In birds, the bioaccumulation of trace elements can lead to a variety of negative effects on reproduction, such as egg hatchability, hatchlings survival and neurobehavioral development (Hofer et al., 2010). These problems may also include smaller clutch sizes, impaired growth, development and behavioral abnormalities, increased susceptibility to disease or other stresses and disorders in physiological mechanisms (Borghesi et al., 2016). Female birds can also remove excessive concentrations of contaminants via egg laying, threatening the health and survival of the embryo (Ashbaugh et al., 2018; Ohlendorf & Heinz, 1987).

On a broader scale, these effects can greatly affect population dynamics, both in the short and long term (Burger, 1993). The impact may be of particular concern if the populations are in decline, as is the case with many migratory species (Hofer et al., 2010).

Birds at the top of the food chain such as raptors or piscivorous birds have been used intensively in several biomonitoring studies (Abdullah et al., 2015; Burger, 1993; Dauwe et al., 2003; Hosseini Alhashemi et al., 2011; Kushwaha*, 2016; Muralidharan et al., 2004; Signa et al., 2013; Ullah et al., 2014) while comparatively few studies have looked at the effects of metals on passerine species (Dauwe et al., 2002; Hofer et al., 2010; Markowski et al., 2013; Tsipoura et al., 2008).

Levels of trace elements accumulated in bird tissues may give a better picture of hazards due to contaminations than measurements in the physical environment, plants or invertebrates, since birds tend to accumulate these elements to levels much higher than those found in water, air and invertebrates. However, migratory habits can make some bird species less suitable as a biomonitor because individuals can have different migratory patterns, so that it may be difficult to determine the spatial scale they represent. Furthermore, different populations can cross the same site at different times of the year, potentially confusing a monitoring program based on sampling at a site (Furness & Greenwood, 1996).

Given these limitations, the best strategy to be adopted for meaningful and effective monitoring would be the use of resident and nesting species, which do not migrate or are still subject to short-range migration. Another effective way to solve these problems would be to shift attention to the chicks or young birds rather than to adults.

Waterbirds that breed in colonies have been extensively used as sentinels of environmental pollution, in particular for trace elements (Borghesi et al., 2016), as they provide additional advantages as bioindicators of pollution. Easy sampling and limited foraging range around their colony site allows inference about the source of contaminants and dependence on specific habitat and prey resources (Abdullah et al., 2015).

Trace element total concentration (or body burden) can be assessed in birds by analyzing various organs (liver, kidney), tissues (muscle, bone, fat), excrements, eggs and feathers (Burger, 1993; Dauwe et al., 2003; Markowski et al., 2013). Since birds are often protected by environmental laws as bioethical concerns inhibit the killing of free-living organisms for biomonitoring (Furness & Greenwood, 1996), the use of non-invasive biomonitoring tools is preferable and often the only possible solution (Borghesi et al., 2016). Analysis of feather and egg samples of bird species have been documented worldwide over the years (Abdullah et al., 2015; Borghesi et al., 2016; Dauwe et al., 2003; Kaur & Khera, 2018; Markowski et al., 2013; Thongcharoen et al., 2018; Tsipoura et al., 2008).

In birds, deposition of pollutants in feathers and sequestration to their eggs are common routes of elimination for contaminants taken up through food, assimilated through gut and intestine, that reached the blood stream (Kaur & Khera, 2018; Signa et al., 2013).

Egg shells are useful biomonitors because they are vulnerable to the effects of trace metals as non-essential elements are sequestered in the shell while the nutrients are concentrated inside the egg (Hashmi et al., 2015). They can represent also local exposure of the adults that have laid them (Burger, 1993). In fact, egg formation and egg laying were recognized as a means of excreting environmental contaminants by females and results showed a significant relation to our understating of the potential environmental risk (Thongcharoen et al., 2018).

Eggs have a highly consistent composition, sampling takes little time, they are easy to handle and can be taken from the same location each year. Furthermore, eggs from the same brood tend to have similar pollutant levels and reflect element uptake from local diet more than tissues from adult birds. However, dietary intakes of trace elements are not adquately refleted in eggs and usually represent a short period uptake (before the egg is laid) (Furness & Greenwood, 1996).

Ecotoxicological studies in the last three decades have frequently used feathers as monitors of dietary exposure and to assess metal total concentration in birds. Feather analysis has proven to have more advantages as bioindicator of metal exposure not only because they are relatively easy to collect, preserve and transport (Burger, 1993) but also because the concentration of trace elements, at least for some contaminant, may be

higher in feathers than in the other tissues (Borghesi et al., 2017). Feather analysis reflects a few weeks trace element uptake process, namely the three- four weeks needed to completely develop a feather.

Birds are able to eliminate a substantial portion of their body burden of certain trace elements (especially Hg) via their plumage during the molting period: trace elements have affinity for the sulfhydryl rich keratin protein and melanin pigments and usually bind to the protein-molecules in the feather during the short period of feather growth when the feather is connected with the bloodstream through small blood vessels (Burger, 1993; Furness & Greenwood, 1996). Here they accumulate in proportion to blood levels at the time of feather formation, acting as a record of metal levels circulating in the bloodstream (Hofer et al., 2010). After feathers are fully formed, the blood supply to the feathers atrophies, accurately reflecting the body burden during all the stages of feather development (Dauwe et al., 2003). However, the deposition of elements and compounds functionally incorporated in the feather follow a mass-dependent model, while the deposition of non-structural elements (such as contaminants and hormones) occurs incidentally and is time-dependent, making difficult the separation between them (Bortolotti, 2010).

Feather analysis can also be used over several seasons to assess changes in the environment (Burger, 1993) or at different times of the year to assess a change in metal accumulation (Hofer et al., 2010).

- ## The Natura 2000 network

One of the main responses to the current biodiversity crisis has been to plan networks of conservation areas, often designed with the aim of maximizing returns from limited conservation investments while minimizing conflicts with human activities (Maiorano et al., 2007).

Natura 2000 is a network of sites of community interest and of protection areas created by the European Union for the protection and conservation of habitats and plant and animal species.

This network was born during the second half of the 20th century, when a growing awareness of environmental problems and loss of species and habitats began to develop. For this reason, many initiatives and international organizations were created. Their intervention and the decisions made by the conventions that followed (such as the Ramsar and the Berne Conventions) have created, within the European Union, a series of directives and a network of protected areas, which together formed the Natura 2000 network (Evans, 2012). Natura 2000 approaches to conservation and sustainable use in a much wider way, centered on people working with nature and ensuring that the sites are managed ecologically and economically in a sustainable manner (EC, 2018).

Natura 2000 network is based mainly of two directives: the 1979 Bird Directive (79/409 / EEC, then updated in the Directive 2009/147/EC) and the 1992 Habitat Directive (92/43/EEC). The Bird Directive identifies 193 endangered bird species and subspecies for which special protection areas (SPAs) are designated by Member states. These areas are designated according to scientific criteria such as “1% of the population of listed vulnerable species” or “wetlands of international importance for migratory waterfowl”. Based on the information provided, the European Commission determines whether the sites can be integrated into the network for the protection of the species present in them (EC, 2018).

The Habitat Directive aims to protect animals, plants, and habitats identifying sites of community importance (SCIs). The choice of sites is based on scientific criteria to ensure that the natural habitat types listed in the directive's Annex I and the habitats of the species listed in its Annex II are maintained or restored to a favorable conservation status in their natural range. After carrying out an evaluation of each of the habitat types and species occurring in the territory, Member states submit lists of proposed Sites of Community Importance (pSCIs) using Standard Data Forms. These include information such as the size and location, the types of species and/or habitat found on this site. For each biogeographical region, scientific seminars are held to determine which pSCI is best suited for consideration. Once the lists of SCIs have been adopted, Member States must designate them as Special Areas of Conservation (SAC) and take the necessary management or restoration measures to ensure the favorable conservation status of the most threatened sites. Stretching across all 28 EU countries both on land and at sea, the aim of this network is to preserve an extensive range of habitat types and wildlife

species throughout Europe, and ensure the long-term survival of Europe's most valuable and threatened species and habitats, maintaining them at “favorable conservation status” (EC, 2018).

Together, this two Directives form the most ambitious and large-scale initiative undertaken to conserve Europe’s biodiversity and, with a still growing network of sites, Natura 2000 is by far the most important conservation effort being implemented in Europe. With over 27000 sites and covering approximately 18% of the EU’s land area and almost 6% of its marine territory, this is the largest network of protected areas in the world (EEA, 2012; Evans, 2012; Gantioler et al., 2014). This has become important because protected areas provide several ecosystem services and processes that regulate water and air quality, prevent natural hazards and mitigate climate change through storing and sequestering carbon. They also provide cultural services like supporting recreation and tourism and maintaining cultural identity. This results in various benefits for human well-being and habitats sustained within protected areas can increase the resilience of ecosystems to resist and adapt to disturbances even beyond the site level (Gantioler et al., 2014). The data collected for Natura 2000 sites can provide useful information of habitat selection at different spatial scales, as this contains also an extensive description of the site and its ecology (Zub et al., 2018).

However, even though it is the conservation scheme with the best “political” chance of success and there are multiple evidence concerning the positive effects that the directives of the network produce (Hermoso et al., 2018; Kallimanis et al., 2015; Sanderson et al., 2016; Zub et al., 2018), there are still gaps in this system that need to be resolved. From the preliminary findings of an assessment made by the European Commission, more progress and adequate financial mechanisms are needed in the development of conservation measures, even though there have been advances in the implementation of the network (EC, 2016).

Furthermore, the network cannot maintain 44-80% of species in a "favorable conservation status" according to the criteria of the red list of the World Conservation Union (Maiorano et al., 2007). As the decision to designate and implement the Natura 2000 network is due to scientific and conservation goals rather than economic speculation, the benefits and the resources dedicated to conservation demand high costs

of managing and investing in the network. The cost associated with managing Natura 2000, including the management and monitoring actions or the development of management plans, was estimated in 2010 at about €5.8 billion annually (Kettunen et al., 2010). For this reason, several studies and reviews were made to help the network to find more economical funding and show to stakeholders the benefits of the program and reach its financial needs (Gantioler et al., 2014; Hermoso et al., 2018; Maiorano et al., 2007).

Nevertheless, Natura 2000 remains the cornerstone of EU nature conservation policy and its establishment is on an advanced stage, with the coverage provided to areas with high biodiversity still growing (Gantioler et al., 2014; Maiorano et al., 2007).

- **The Venetian Lagoon and avifauna**

The Venetian Lagoon is located northwest of the northern basin of the Adriatic Sea. It has a total surface of about 550 km^2 and is included between the Brenta river to the south and the Sile river to the north. Of the total Lagoon area, about 80% is occupied by water, shallows and saltmarshes, 10% is covered by sandbanks and 5% by islands (ARPAV, 2014). With 100 km of coastline, numerous river courses and irrigation canals, vast brackish environments and various freshwater wetlands, the Venetian territory offers different types of habitats suitable for wild avifauna (Bon et al., 2014). As the analyzed species live mainly in marine/lagoon environments, the description of these habitats will focus on the latter.

The coast was originally occupied by natural areas, consisting of very unstable habitats, in which the combined action of wind, rivers and the sea in relation to the presence of sand generated ecosystems and landscapes in constant evolution. The widespread anthropic intervention in recent years has profoundly changed the morphological aspect of the coastal areas and the dynamics of the natural elements at stake (Bon et al., 2014).

Lagoon surfaces are particularly widespread in the province of Venice, although these have been reduced by land remediation in past centuries. The wide areas of brackish shallow water are delimited towards the sea by sandy shores while the areas lying near the mainland are characterized by salt marshes or fishing farms. (Bon et al., 2014).

The Lagoon has a complex morphological structure, consisting of a dense network of channels that gradually decreases in section landwards. The network of channels conveys the tidal current up to the innermost parts, characterized by a modest hydrodynamism and a reduced water exchange. This type of internal hydrodynamic structure and circulation represent the main feature characterizing the environments and the biological communities within the Lagoon (ARPAV, 2014).

The importance of the Lagoon as a wintering area for tens of thousands waterbirds has long been known. Inventories carried out in 1993-2007 highlight the extreme importance of the Venetian Lagoon for the wintering of waterbirds, not only at national level. Furthermore, in the period 1995-2000 the Venice Lagoon constituted the most important Italian site for the wintering of the aquatic species, with an annual average of 127869 birds (Scarton & Bon, 2009).

Table 1 and the following paragraph give basic informations about the local species that were analyzed for this study. The IUCN Italian Red List classified these species as Not Evaluated, Data Deficient, Least Concern, Near Theratened, Vulnerable, Endangered, Critically Endangered, Extinct in the Wild and Extinct depending on the technical and scientific data collected about their ecological state.

The research focuses mainly on samples of feathers and egg shells from the species "*Charadrius alexandrinus*" (Kentish plover), a small wader breeding in wetlands and coastal areas of Europe, North-Africa, Middle-East and Central-Asia (Delany et al., 2009).

The trend of the species is strongly negative and its population size is declining all over its distribution range, probably due to habitat loss and fragmentation, increased predation by birds and mammals (taking advantage of human activity) and increased anthropic disturbance of sandy environments for commercial and recreational purposes, (Delany et al., 2009; Scarton, 2017). For these reasons, the species has been listed in the Annex I of the Bird directive and has been included in the IUCN Italian Red List as an endangered species (EN) (IUCN, 2018a; Rondinini et al., 2013).

As a shorebird, *C. alexandrinus* relies on intertidal mudflats (as well as supratidal habitats) for foraging during both the winter and the breeding season, which are only available for a part of the day (Castro et al., 2009). It can be seen along sea coasts in summer (avoiding oceanic coastlines with movements related to the tide and the anthropic disturbance) and in winter it can colonize salt marshes and wetlands (like lakes and lagoons), seasonal watercourses and depressions where salinity mantain a check on local vegetation cover (Bon et al., 2014; Cramp, 1983). Salt pans and fish ponds are used sometimes as supplementary food sources over high tide, but they are also used by some individuals at low tide as an alternative feeding area (Perez-Hurtado et al., 1997)**.** When is in the inland it feeds mainly on insects (like Coleoptera and Diptera) while in brackish and saltwater areas it feeds on polichaetes (Nereidae), small crustaceans (Brachyura and Amphypoda) and small bivalves and molluscs (Gastropoda) (Castro et al., 2009; Cramp, 1983)**.**

Figure 1: Charadrius alexandrinus (from Bon et al., 2014)

In Italy it is a migratory, wintering and nesting species with about 1450 individuals identified in 2007. Its nesting is ascertained in three of the four disjoint portions of the SCI/SPA IT3250023 – "Venice Lido: coastal Habitats", present in the Venetian Lagoon (Pegorer et al., 2015), as well as in the SCI IT3250031 – "Northern Lagoon" and SCI/SPA IT3250003 – "Peninsula of the Cavallino: coastal habitats".

The species settles on the coasts in front of the Venice Lagoon, in the open lagoon and (to a lesser extent) in the valley area and on the coast of Caorle. In the last 20 years, nesting couples were collapsing on the coast and those established on artificial salt marshes were increasing.

The diet of *C. alexandrinus* makes it susceptible to bioaccumulation and biomagnification of toxicants, although if it is not at the top of the food chain. To verify whether the *C. alexandrinus* breeding in the area of the Venice Lagoon are exposed to levels of trace elements that may, directly or indirectly, contribute to the local decline of

the species, feathers were collected from specimens breeding along one of the littoral strips of the lagoon, in the SCI/SPA IT3250003 – "Peninsula of the Cavallino: coastal habitats".

Given the lack of certified reference material suitable for the analysis of feathers, repeated analyses of feather of the following species were used as comparison material during analyses of the samples collected from *C. alexandrinus*:

- *Chroicocephalus ridibundus* (Black-headed Gull): Species of least concern (LC) in the IUCN Italian Red List of Threatened Species (IUCN, 2018b).
  In Italy it is a sedentary nesting species with about 600-1000 pairs estimated in 1998-2000 and a wintering species with about 250000 individuals in 2007 (Bon et al., 2014).

Figure 2: Chroicocephalus ridibundus (from Bon et al., 2014)

  Its diet consists predominantly of aquatic and terrestrial insects, earthworms and marine invertebrates although it may also take fish, agricultural grain and, occasionally, rodents. (IUCN, 2017a).
  In the period 2008-2012 it nested in three quadrants of the Lagoon, two of which in the southern and one in the northern part of lagoon. The former were occupied by colonies of a few couples, rarely above ten. The third quadrant was characterized by some colonies with overall consistencies of more than 100 pairs. Here it feeds on crabs, shrimps, cuttlefish, amphipods, gastropods, polychaetes and insects.
  In winter it is widespread in all the coastal areas and in wetlands. It can be found in urban centers and in open countryside moving also inland. In this season it occupies a wide range of fresh or salt water, coastal or internal, natural or artificial wetlands and even in urban and intensely cultivated areas.
  In the period of nesting, it occupies the coastal brackish environments (salt marshes and sandbanks). Daily commutes between dormitories and feeding areas are typical (Bon et al., 2014).

The samples for this species were collected in the area of Saccagnana, in the municipality of Cavallino-Treporti (Venice), in the SCI IT3250031 – “Northern Lagoon”.

- *Egretta garzetta* (Little egret): Regular wintering and migrating nesting species, considered of least concern (LC) in the IUCN Italian Red List of Threatened Species (Bon et al., 2014; IUCN, 2018c). It inhabits fresh, brackish or saline wetlands and shows a preference for shallow waters in open, unvegetated sites where water levels and dissolved oxygen levels fluctuate daily, tidally or seasonally, and where fish are concentrated in pools or at the water's surface. It is a highly opportunistic feeder, taking mainly small fish as well as amphibians, reptiles and small rodents (IUCN, 2016a).

Figure 3: Egretta garzetta (from Bon et al.,2014)

  In winter it is very common and locally abundant; it uses a very wide typology of environments for the research of food, such as brackish basins, freshwater mirrors, courses of canals and rivers, agricultural areas with sluices, periurban and (in some cases) urban areas.
  The nesting habitat consists of arboreal and/or shrubby marshes located in wetlands such as fishing farms, river courses, flooded senile quarries and abandoned lagoon islands (Bon et al., 2014).
  The samples were collected in the area of Lio Piccolo and Mesole, in the municipality of Cavallino-Treporti (Venice), in the SCI IT3250031 – “Northern Lagoon”.

- *Ichtyaetus melanocephalus* (Mediterranean gull): Migratory regular, estivant and wintering species, considered of least concern (LC) in the IUCN Italian Red List of Threatened Species (Bon et al., 2014; IUCN, 2018d).

  It is an adaptive, opportunistic omnivore that have developed different methods of feeding and have mastered multiple feeding strategies (Milchev et al., 2004). In breeding season it generally feeds on terrestrial and acquatic insects, other times on fish and molluscs (Cramp, 1983). Apparently, it adapts more easily than many other species to new habitats for nesting and wintering (Milchev et al., 2004).

Figure 4: Ichtyaetus melanocephalus (from Bon et al., 2014)

  During the nesting period it feeds mainly on land, chiefly consuming terrestrial and aquatic invertebrates. However, at other seasons, the species frequents shorelines and feeds mainly on marine fish and molluscs. (Milchev et al., 2004). Out of the breeding season, it moves on wider coasts and is not found in inland during wintering (Cramp, 1983).

  The species breeds in lagoons, estuaries and sometimes coastal saltmarsh; often it also breeds inland on large steppe lakes and marshes in warm and dry open lowland areas. It nests near water on flood-lands, fields and grasslands and on wet or dry areas of islands, favoring sparse vegetation but generally avoiding barrens (Cramp, 1983; IUCN, 2018d).

  In Italy, it nests almost exclusively in a few coastal areas of the north Adriatic and Apulia.

  About 2000 couples were estimated at the beginning of the millennium. In Veneto, its reproduction was first established in 1996. After a few years of irregular presence, it is now stably nesting in the Po delta and in the Venice Lagoon. The nesting has been ascertained only in three sites of the Venetian Lagoon: two fishing farms in the northern lagoon (Valle Sacchetta and Valle Paleazza) and a saltmarsh in the southern lagoon. In these colonies it is associated with other

species such as the *C. ridibundus* and *T. sandvicensis*. Crabs, shrimps, cuttlefish, amphipods, gastropods, polychaetes and insects make up its local diet during nesting and fledging period.

In winter the species is widespread along the coastal arch of the province, especially near the harbors of the Venetian Lagoon, in the lagoon of Caorle and in the innermost lagoons, up to the fishing farms (Bon et al., 2014).

The samples for this species were collected in the area of Saccagnana (Valle Sacchetta), in the municipality of Cavallino-Treporti (Venice), in the SCI IT3250031 – "Northern Lagoon".

- *Phasianus colchicus* (Common pheasant): Species non applicable (NA) to the IUCN Italian Red List of Threatened Species (IUCN, 2018f). In Italy it is an introduced species, sedentary and nesting, with a population estimated at 1000-10000 pairs and trends strongly influenced by restocking operations of repopulation (Bon et al., 2014). Where it is introduced it is an opportunistic omnivore, feeding on a diverse range of food, preferring large, energy rich items such as cultivated grains, mast and fruits. It feeds on plant matter such as fruits, seeds, leaves, buds and a small amount of animal matter, for example insects and earthworms (IUCN, 2016b). The species adapts to a wide spectrum of environmental types. Typical of the ecotonal zones, it frequents the margins of the river courses and the agricultural areas, with a preference for steppe environments, rich in bushes and small woods. It settles in agricultural estates dominated by monocultures, interspersed with sluices or uncultivated areas (Bon et al., 2014).

Figure 5: Phasianus colchicus (from Bon et al., 2014)

The samples were collected in the Mira countryside, towards the valleys of the south of the Lagoon of Venice.

- *Phalacrocorax carbo sinensis* (great cormorant): Species of least concern (LC) in the IUCN Italian Red List of Threatened Species (IUCN, 2018e). In Italy it is a sedentary nesting and wintering species with about 3914 nests occupied in 2012 in a total of 48 colonies (Bon et al., 2014).

Figure 6: Phalacrocorax carbo sinensis (from Bon et al., 2014)

It may form large fishing flocks in some areas (IUCN, 2017b).
As a wintering species it is widespread in all the wetlands with greater concentration in the coastal areas. In the Venetian area, it occurs in the internal wetlands with the involvement of all the former flooded quarries, rivers and canals, with presences also in the main urban centers. It frequents fresh and salt water basins, preferably without vegetation, with low or medium depth and generally feeds on flatfish and sole fish, but also on mullets, silversides and gobies. To reproduce it uses areas with dense and often dead (or dying) bushes and trees placed near water (Bon et al., 2014).
The samples were collected in the area of Saccagnana, in the municipality of Cavallino-Treporti (Venice), in the SCI IT3250031 – "Northern Lagoon".

- *Tyto alba* (Barn owl): Species of least concern (LC) in the IUCN Italian Red List of Threatened Species, although a reduction of 50% of its distribution was taken over the last twenty years (Bon et al., 2014; IUCN, 2018h).
It is a sedentary and nesting species, with a population estimated at 6,000-13,000 pairs in Italy. (Bon et al., 2014).
It is found in a great variety of open and semi-open habitats which include forest edges, grassland, scrub, meadows, agricultural fields, and even urban and suburban areas, where it can occupy historic buildings, abandoned warehouses, bridges and viaducts. Barn owl eats a variety of small animals, primarily rodents

(including mice, voles, and shrews). *T. alba* hunts at night like most owls, listening for movement in the undergrowth and swooping down to capture prey (IUCN, 2016d).

Figure 7: Tyto alba (from Bon et al., 2014)

In the last twenty years it has been in strong decline in Northern Italy and it is stable or rising in the South. It prefers the cultivated flat areas, even in an intensive way, where it uses to nest in disused farmhouses and barns. It is sporadic in urban centers, where it can occupy historic buildings, abandoned warehouses, bridges and viaducts (Bon et al., 2014).

The samples for this species were collected in the area of Portegrandi, in the municipality of Quarto d'Altino (Venice).

- *Thalasseus sandvicensis* (Sandwich tern): Vulnerable species (VU) in the IUCN Italian Red List of Threatened Species (IUCN, 2018g). It is a regular migrant, nesting and wintering species (Bon et al., 2014).

  This large tern generally eats small marine fish like clupeids and sandeels, but feeds also on Gadidae, Gobidae, Gasterosteidae, Agonidae, Syngnathidae, Callionymidae and invertebrates (squids, crabs and shrimps). They eat occasionally shrimps and squids (*Crangon* sp. and *Loligo* sp.); polychaete worms are sometimes important in local diets (Annelida). Because they have relatively short foraging ranges, specialized diets and spend a relatively high proportion of the time foraging, they are particularly sensitive to changes in local prey availability. In Europe, they are almost entirely dependent on clupeids (Herring and Sprat), sandeels and Nereis-worms with only occasionally other fish or invertebrate species taken (Courtens et al., 2017; Cramp, 1983).

Figure 8: Thalasseus sandvicensis (from Bon et al., 2014)

It breeds in dense colonies with other terns or *C. ridibundus* and it is gregarious throughout the year, often forming feeding flocks where prey is abundant. It shows a preference for raised, open, unvegetated sand, gravel, mud or bare coral substrates for nesting (IUCN, 2016c). Sandwich tern mainly nests in islands with sandy shores, rocky islets, sand-spits and dunes, beaches and extensive deltas. Outside breeding season, it moves towards warm waters, sometimes traveling near the coast in marine or estuary waters along the coasts (Cramp, 1983). The species responds favourably to habitat management such as vegetation clearance and can be readily attracted to suitable nesting habitats by the use of decoys (IUCN, 2016c).

In Italy, nesting areas are concentrated in the Venice lagoon, in the Emilian Po river delta and in a few Apulian wetlands.

The population was estimated at around 1300 couples in the early 2000s. Established in Venice Lagoon for the first time in 1995, it has nested in recent years with 600-800 couples, concentrated every year in one or three colonies, located exclusively on the salt marshes of the Lagoon. Its local diet consists of anchovies, sprats, sardines, silversides and gobies.

It mainly frequents the marine waters in front of the coasts, the lagoons, the estuaries and the deep freshwater wetlands located in the hinterland (the latter only during migrations and wintering). For the nesting islets, bumps, banks and salt marshes are chosen with varying vegetation cover but always of modest height (Bon et al., 2014).

The samples for this species were collected in the area of Palude Fondello, in the municipality of Chioggia (Venice), in SCI IT3250030 – "mid-lower Venetian Lagoon".

species	english name	italian name	sampling area	foraging behavior	IUCN classification
Charadrius alexandrinus	Kentish Plover	Fratino Eurasiatico	Cavallino-Treporti	invertivorous	EN
Chroicocephalus ridibundus	Black-headed Gull	Gabbiano Comune	Saccagnana	invertivorous	LC
Egretta garzetta (1)	Little Egret	Garzetta	Lio Piccolo	generalist predator	LC
Egretta garzetta (2)	Little Egret	Garzetta	Mesole	generalist predator	LC
Ichtyaetus melanocephalus	Mediterranean gull	Gabbiano Corallino	Saccagnana	invertivorous	LC
Phasianus colchicus	Common Pheasant	Fagiano Comune	Mira	granivorous	NA
Phalacrocorax carbo sinesis	Great Cormorant	Cormorano Comune	Saccagnana	piscivorous	LC
Tyto alba	Common Barn-Owl	Barbagianni	Portegrandi	terrestrtial predator	LC
Thalasseus sandvicensis	Sandwich Tern	Beccapesci	Palude Fondello	piscivorous	VU

Table 1: Description of the species from which the samples used in the study were obtained. For each species Latin name, English name, Italian name, foraging behaviour and IUCN Italian Red List classification are listed. The Little Egret (egretta garzetta) is repeated twice as the samples of the aforesaid species were taken in two different areas.

- **Analyzed trace elements**

Using the mass spectrometry technique, the following were selected as elements of interest for this study:

- Aluminium (Al): it is the most commonly occurring metallic element. It incldes 8% of the earth's crust and is a major component of almost all inorganic soil particles (except quartz sand, chert fragments, and Fe-Mn concretions). It constitutes aluminosilicates, the most common primary and secondary minerals in soils (EPA, 2003a).

  Al content in soils depends on a parent rock and soil type. With the increase of acid rainfalls (and consecutive acidification of surface waters) Al release in bottom sediments and its transition into water has increased. Its presence in the air is dependent on anthropic activities as it is injected into the atmosphere by carbon combustion, motor vehicle exhaust, waste incineration and exhaust gases of metallurgical and cement industry.

  Al can also be released into the air as a result of weathering of rocks (Barabasz et al., 2002).

  Al can be both beneficial and harmful for plants. The benefits consist in stimulation of Fe absorption by root system, increased absorption of P,

prevention of Cu and Mn toxic effects and plant protection against phytopathogenic fungi. On the other hand, it affects plants by decreasing crop production, leading to changes in the morphology of root system, inhibition of its elongative growth, root callosity, reduced number of rootlets, and dying away of growth cone. Al acidified waters are particularly harmful to snails, bivalves and crustaceans as it replaces Ca^{2+} in their bodies (Barabasz et al., 2002).
Toxic potential of dietary Al in healthy animals is low. The chronic toxicity of orally ingested Al is more a function of its disruptive effects on Ca and P homeostasis. Levels of Al higher than 0.1% of the diet may cause decreased growth rates, muscle weakness and disturbances of Ca and P metabolism (Scheuhammer, 1987).
In fish, Al accumulates in gills causing blocks of ion exchange and respiration, while in frogs reproductive processes are disturbed.
In birds, excessive amounts of Al may alter the homeostasis of other elements in the body and cause malfunctions. However, potential toxicity is generally low (Barabasz et al., 2002), since intestinal absorption of orally ingested Al salts is very poor and is almost completely removed from the body by excretion, resulting in little Al retention in kidneys (Scheuhammer, 1987).
The most common way of Al removal for birds is through the excretion system and only a small amount of Al should be stored in the body. Hence, levels of Al above 10 μg/g in bones are considered high and are probably indicative of elevated exposure to Al, or a decreased ability to excrete Al. (de Hamel, 2014; Scheuhammer, 1987).
Al affects egg shells, causing diminished efficacy of Ca absorption and decreased metabolic transformations, resulting in its incorporation into bones (Barabasz et al., 2002). There are no thresholds levels in feathers and eggs associated with detrimental effects in birds.

- Calcium (Ca): it is an alkaline metal, the fifth most abundant element and the third most abundant metal in the Earth's crust by weight. Even if it's a metal element, it is not found in the metallic form in nature as it is very reactive with oxygen and water (Perrone & Monteiro, 2016).

It does occur in various compounds and it makes up 3.64% of all igneous rock (The Environmental Literacy Council, 2015).

Ca is found mostly as limestone, gypsum and fluorite.(Perrone & Monteiro, 2016).

Industries today continue to utilize lime (calcium oxide, CaO) and "slaked" lime (calcium hydroxide, CaOH), which is prepared by heating limestone, in the production of iron as it combines with impurities in the ore separating pure molten iron easily. Ca is also added to metal alloys to improve their properties (The Environmental Literacy Council, 2015).

It plays many key roles in cellular biochemistry and in physiology of living organisms. It influences the excitation of nerves and muscles, ion transport, heart rhythm, blood coagulation, and the permeability of cell membranes (Lanocha-Arendarczyk et al., 2016; The Environmental Literacy Council, 2015).

It is a secondary messenger in signal-transduction pathways and is involved with contraction of muscle cells and neurotransmitters of nervous cells. It is also the cofactor of many enzymes and is essential for the maintenance of the electrical potential difference in cellular membranes (Perrone & Monteiro, 2016).

Ca is a major constituent of bone and teeth, shells of marine organisms, and coral reefs (The Environmental Literacy Council, 2015). 70% of bone is made up of hydroxyapatite, a mineral composed of different Ca salts (Perrone & Monteiro, 2016).

Ca is always present in every plant, as it is essential for their growth. It is contained in soft tissues, fluids and in the structure of every animal's skeleton. Low dietary consumption of Ca leads to lower bone mineral density, rickets and osteoporosis (Lanocha-Arendarczyk et al., 2016).

It is not a toxic element but can influence the toxicity of other elements such as Al, Mg and P (Perrone & Monteiro, 2016).

As for Al, there are no thresholds levels in feathers and eggs associated with detrimental effects in birds.

- Iron (Fe): it is a commonly occurring element, comprising 4.6% of the igneous rocks and 4.4% of sedimentary rocks (EPA, 2003b). It is an essential and

fundamental element for many biological processes such as photosynthesis, respiration, nitrogen fixation, detoxification of reactive species (Khan et al., 2014; Kushwaha*, 2016).

Fe has major scientific and medical interest, but toxicological considerations are important in terms of acute exposures and chronic overload. Fe and its compounds can be present as pollutants in atmosphere and can cause dangerous effects to humans and wildlife (Kushwaha*, 2016).

Fe can occur in either as Fe^{2+} or Fe^{3+}. It occurs predominantly as Fe^{3+} in soils and goethite ($Fe^{3+}O(OH)$) is the predominant mineral form (EPA, 2003b). This oxidation form is predominating in oxygenated waters, but it becomes insoluble after formation of oxyhydroxides (Khan et al., 2014).

Fe^{2+} is thermodynamically unstable in open water and is quickly oxidized to Fe^{3+} (EPA, 2003b; Khan et al., 2014). This form contributes for about 50% of the total dissolved Fe in sea water (Khan et al., 2014).

It enters the aquatic environment from weathering as well as from burning of coke and coal, mineral processing, sewage, industries and iron/steel corrosion (Khan et al., 2014).

Significant amounts of Fe are released into the environment as a result of coal mining. During these operations, pyrite (FeS_2) is exposed to air and water and is oxidized to $FeSO_4$ by bacterial and chemical reactions (EPA, 2003b). This process releases Fe^{2+} that reacts with oxygen to form insoluble Fe^{3+}, which in turn hydrolyzes to form insoluble FeO(OH). This precipitates out in mines and streams acting as reservoirs for Fe^{3+}, which then react with pyrite to generate again soluble Fe^{2+} (EPA, 2003b).

It is a natural component of soils, but its concentration can be influenced by some industries (Kushwaha*, 2016).

Generally, it exists in sea water as Fe^{2+} and Fe^{3+}, which lead to the formation of soluble complexes, colloids and particle phases. Main sources are photoreduction, atmospheric deposition and diffusion from sediments (Khan et al., 2014).

The largest source, by mass, of Fe to the open ocean is windblown soil dust, but it is much less soluble than Fe from other aerosol sources (Mead et al., 2013).

Fe is a critical nutrient for phytoplankton and anthropic inputs like combustion byproducts and biomass burning aerosols can lead to its growth (Mead et al., 2013).

It can have toxic effects dependent on bird taxa.

Fe is not toxic itself, but it can influence the toxicity of other elements. It can enhance the uptake of As^{3+} in some marine organisms (which causes adverse health effects for the organism) and inhibit those of As^{5+} (Khan et al., 2014). Furthermore, Fe can promote the reduction of Cr^{6+} into Cr^{3+} and the same effect was detected for Cd bound to ferric hydroxide, making it less bioavailable for organisms (Khan et al., 2014). An excess of Fe in birds can provoke hemosiderosis which is characterized by its accumulation in body tissues, especially the liver. When liver levels are excessive it may cause icterus, ascites and liver pathologies. Little information is available for Fe concentrations in feather tissues (Dierenfield et al., 1994).

- Cobalt (Co): it is a rare trace metal present in the earth's crust that shares properties with Ni and Fe (EPA, 2005c; Pourret & Faucon, 2018; Ullah et al., 2014).

  Co is a naturally occurring element in air, soil, plants, and water and most of it can be found widely in igneous and sedimentary rocks and minerals, in meteorites (in a few tenths of a percent) and in suspended particulate matter in sea (Khan et al., 2014; Pourret & Faucon, 2018).

  It can be found in nature as Co^{2+} and Co^{3+} (Khan et al., 2014). Its chemistry is dominated by Co^{2+} in the aqueous phase of terrestrial environments (Pourret & Faucon, 2018). Co^{3+} is thermodynamically unstable and insoluble in water and thus it cannot be taken up easily by organisms. It can also replace other trace metals (like Pb and Cd) in many minerals due to its similar geochemical properties (Khan et al., 2014). Its release in the environment occurs via soil and dust, seawater spray, volcanic eruptions, forest fires, and other continental and marine biogenic emissions (EPA, 2005c).

In soils at low pH, Co^{2+} is oxidized to Co^{3+} and often associated with Fe, enhancing leaching and plant uptake. Between pH 6 and 7, Co^{2+} is adsorbed on colloids (EPA, 2005c).

The mean concentration of Co in the open ocean is very low and, when present at low concentrations, it can act as a limiting nutrient for phytoplankton (Khan et al., 2014; Pourret & Faucon, 2018).

The forms in seawater are Co^{2+} and its complexes. The mean concentration of Co in seawater is generally considered to be lower than 1 μg/L and steadily decrease from the surface towards the bottom (Ngoc & Whitehead, 1986). Concentrations in fresh water varies from 0.1 to 10 μg/L (Khan et al., 2014).

Its speciation and mobilization in estuarine and coastal areas is influenced by metal concentration, cation competition, pH, salinity and dissolved oxygen (Khan et al., 2014).

It is associated with Ni, Ag, Pb, Cu, and Fe-Mn ores, where it is obtained as a by-product (Pourret & Faucon, 2018).

Antrhopogenic sources of Co include fossil fuel burning, processing of Co and steel alloys, Cu-Ni smelting and refining, paints, nuclear technology, ceramics, sewage sludge, medicines, foam stabilizer in beer brewing and phosphate fertilizers (EPA, 2005c; Khan et al., 2014; Ullah et al., 2014).

It is an essential element that functions as a component of vitamin B12, which acts as coenzyme in many reactions and is essential to growth and neural function. Although is an essential nutrient, excessive oral doses result in increases in polycythemia, cardiomyopathy, effects on the reproductive system (for males), reduced food intake and growth inhibition (EPA, 2005c). High and frequent Co exposures can cause axonopathy and chronic inhalational intake can lead to chronic respiratory tract disorders. In large doses, it can be carcinogenic (Pourret & Faucon, 2018). Furthermore, it can influence negatively growth rate and Ca-uptake of aquatic herbivores (Khan et al., 2014). No information on adverse or toxic effects of Co on birds has been found in literature.

- Arsenic (As): it is a relatively common element that is present at low concentrations in virtually all environmental matrices (Tchounwou et al., 2012).

It has been used in medicine as a chemotherapeutic, and organoarsenicals were used extensively until about 1945.

It has four valence states (–3, 0, +3, and +5) and, because it is a metalloid, it can be present in both inorganic and organic forms (Finley, 2015). The organic forms are the methylated metabolites CH_5AsO_3 (monomethylarsonic acid - MMA), $C_2H_7AsO_2$ (dimethylarsinic acid - DMA) and C_3H_9AsO (trimethylarsenic acid - TMA). Toxicity of As to aquatic organisms is highest for AsH_3 (gaseous) and progressively lower for AsO_3^{3-}, AsO_4^{3-}, MMA, DMA and complex organoarsenicals such as $C_5H_{11}AsO_2$ (Tchounwou et al., 2012). As^{3-} is generally unstable in air. As^0 is formed by the reduction of As oxides. As^{3+} is produced by smelting operations and is used in synthesizing most arsenicals. This form is generally considered to be more toxic, more soluble, and more mobile. It is oxidized catalytically or biologically (mainly by bacteria) to As^{5+} or orthoarsenic acid (H_3AsO_4). It naturally occurs as sulfides and as a complex with Fe, Co and Ni. Inorganic As is more mobile than the organic form, and thus poses greater problems when leaches into surface waters and groundwater (Eisler, 2000).

As is present in rock and soils associated with igneous and sedimentary rocks, particularly with sulfidic ores.

Large quantities of As are released into the environment as a result of industrial and especially agricultural activities, and these may pose strong ecological hazards (Eisler, 2000). As is used in multiple manufacturing and industrial processes including the production of wood treating chemicals, herbicides, pesticides, desiccants, metal alloys, glass, pharmaceuticals and semi-conductors (EPA, 2005e). It is introduced into the environment through atmospheric deposition of combustion products and through runoff from areas near power plants and nonferrous smelters. Anthropogenic input to the environment is substantial and exceeds that contributed by natural weathering processes by a factor of about 3 (Eisler, 2000).

As levels in soils are normally elevated near arseniferous deposits and in mineralized zones containing Au, Ag and sulfides of Pb and Zn. It is a constituent of numerous minerals and is frequently associated with S frequently

as arsenopyrite (FeAsS). It can be available for plant uptake or reduction by organisms and chemical processes (Eisler, 2000). As occurs in contaminated soils primarily as inorganic As^{5+} and As^{3+} that can be converted in organic forms by microorganisms (EPA, 2005e). Availability or solubility in soils depends on the source (natural or anthropogenic) and the soil's clay content, redox potential, and pH. As can be found in water under oxidizing and reducing conditions and hydrogeological or geochemical conditions will influence its levels.

In water, As occurs in inorganic and organic forms, and in dissolved and gaseous states. Its presence depends on many factors like Eh, pH, organic content, suspended solids, dissolved oxygen, and other variables and exists primarily as a dissolved ionic species (Eisler, 2000).

Wildlife exposure to As occurs through air (emissions from smelters, coal fired power plants, herbicide sprays), water (mine tailings runoff, smelter wastes, natural mineralization), and food (especially seafoods) (Eisler, 2000). It is transfered by plants from the abiotic environment to the biotic one and then taken up by herbivores, which may also serve as food for other animals (Sánchez-Virosta et al., 2015).

Biological processes can methylate inorganic As compounds thus converting them into organo-arsenicals, clearing As from all tissues (except perhaps the thyroid) and greatly reducing toxicity (Eisler, 2000; Finley, 2015).

As metabolism and toxicity for vertebrates vary according to the species and its effects can be altered by physical, chemical and biological modifiers. Negative health effects, for example, can involve respiratory, gastrointestinal and cardiovascular systems and can range from reversible to death.

Signs of inorganic As poisoning in birds (like muscular incoordination, slowness, falling, fluffed feathers, unkempt appearance, loss of righting reflex and seizures) are similar to other toxicants and did not seem to be specific (Eisler, 2000).

The complexity of As toxicity associated with different species highlights the difficulty to determine a reference dose. In general, As concentrations in passerine birds are usually lower than 1 μg/g in unpolluted sites, and lower than 10 μg/g in polluted sites (Sánchez-Virosta et al., 2015). Fate and toxicology of

As in waterbirds are poorly known; as a consequence no theoretical nor empirical adverse-effect threshold is available (Bond & Lavers, 2011; Burger et al., 2007).

- Selenium (Se): it is a metalloid trace element found in air, soil, sediment, and water (EPA, 2007). It is an essential nutrient for most plants and animals but is harmful at slightly higher concentrations. Se constitutes an integral part of the enzyme glutathione peroxidase and may have a role in Vitamin E and the enzyme formic dehydrogenase (Eisler, 2000).
  It exists as Se^{2-}, Se^{0}, Se^{4+} and Se^{6+}. Its speciation in soils is influenced by the chemical and mineralogical composition of the soil, microbial intervention, and the nature of the adsorbing surfaces (EPA, 2007). Inorganic Se exists primarily as Se^{4+} and Se^{6+} in aerated alkaline soils. The former is soluble but can strongly adsorb to soil minerals and organic material, while the latter has high water solubility and inability to adsorb to soil particles. In poorly aerated soils, it predominates as Se^{2-} and Se^{0}, which is virtually insoluble in water (Eisler, 2000; EPA, 2007). Transformations in soils appear to be microbially mediated and fungi, bacteria, and actinomycetes that occur in soils are capable of reducing inorganic Se to elemental forms or organic compounds (EPA, 2007). Metabolism and degradation are significantly modified by interaction with trace metals, agricultural chemicals, microorganisms, and a variety of physicochemical factors (Eisler, 2000).
  In nature Se occurs in the sulfide ores and in rocks, sandstone, shale, carbonates, bedrock, coal oil, and mineral oil. It is released to the environment from natural sources like volcanic eruptions, leaching, rock weathering and biomethylation by plants and bacteria (EPA, 2007).
  Air and surface waters generally contain non-hazardous concentrations of Se and increases are attributed to industrial sources and leaching from seleniferous soils. It is normally present in surface waters at concentrations ranging from 0.1 to 0.3 µg/L. However, at 1 to 5 µg/L, it may biomagnify in aquatic food chains and pose a toxic threat to fish and wildlife (Eisler, 2000).

Anthropogenic releases come from manufacture and production of glass, pigments, rubber, metal alloys, textiles, petroleum, medical therapeutic agents, anti-dandruff shampoos, veterinary medicines, fungicides, gaseous insulators, and photographic emulsions (EPA, 2007).

It bioconcentrates and biomagnifies in aquatic food webs from invertebrates to birds (Rusk & Kirsten, 1991; Saiki et al., 1993). Plants act as primary and secondary medium of biomagnification for wildlife, as Se is an essential element for their growth. In these circumstances, plant Se concentrations may be >1000 μg/g. Toxicity for some plant species is demonstrated by stunted growth, chlorosis, pink leaf veins and pink root tissue (Ashbaugh et al., 2018; Eisler, 2000).

Food is the main source of Se accumulation for birds and other wildlife. High concentrations of Se in foods come from areas naturally high in Se and disposal of sewage sludge or fly ash, mining activity, or emissions from metal smelters (Ohlendorf & Heinz, 1987). Se can protect against the adverse or lethal effects induced by Hg, Cd, As, Tl, Cu, Zn, Ag and various pesticides and this effect has been documented for a wide variety of plant and animal species (Eisler, 2000). Se toxicity has also been reported to be reduced by elevated levels of Pb, Cu, Cd, Ag and As (Ohlendorf & Heinz, 1987).

Se toxicity is most likely to occur in animals grazing on seleniferous forage and acute effects include abnormal posture and movement, diarrhea, labored respiration, abdominal pain, prostration, and death (EPA, 2007).

In birds, dietary concentrations less than 0.3 mg/kg are considered to be below the range for good health and reproduction, while concentrations from 3.0 to 5.0 mg/kg are high and above 5.0 mg/kg are toxic (Ohlendorf & Heinz, 1987).

Both Se^{4+} and Se^{6+} compounds are toxic to birds, but organic Se^{2-} pose the greatest hazard. Selenomethionine has been shown to be highly toxic to birds and seems to be the form most likely to harm wild birds because it results in high bioaccumulation in their eggs (Ohlendorf & Heinz, 1987).

Se induced abnormalities in birds include defects of the eyes, feet or legs, beak, brain and abdomen, liver pathologies and glutathione metabolism. When dietary concentrations are high, bilateral alopecia can be observed as a sign of chronic

selenosis. Reproductive effects of Se include reduced hatchability of eggs and a high incidence of embryo deformities. Excess Se in the diet of laying females can result in the transfer of Se to the eggs or other tissues at harmful levels, affecting egg fertility in some species (Ohlendorf & Heinz, 1987).
Fish-eating birds had the highest concentrations in livers, herbivorous species the lowest and omnivores are intermediate (Eisler, 2000).
Se levels in eggs tend to be usually slightly higher than Hg levels, but (as the latter) eggs can be used to monitor Se pollution in the immediate pre-laying season (Furness & Greenwood, 1996).
Variability of Se concentrations in whole feathers is considerable (Eisler, 2000; Ohlendorf & Heinz, 1987), nevertheless, levels of Se in feathers known to be associated with toxic effects range from 1.8 μg/g to 26 μg/g depending upon species (Burger et al., 2015). Concentrations up to 26 μg/g can have severe adverse effects, depending upon individual chronic exposure (Ashbaugh et al., 2018). The threshold Se concentration considered for feathers is 5 μg/g. (Ashbaugh et al., 2018; St. Clair et al., 2015).

- Molybdenum (Mo): it is a rare element used primarily in manufacture of steel alloys for the aircraft and weapons crafting (Eisler, 2000).
  It is considered an essential or beneficial micronutrient for human, animal and plant health (Eisler, 1989; Smedley & Kinniburgh, 2017; Stafford et al., 2016).
  Mo can participate in a large number of redox reactions for its variable oxidation states and is strongly absorbed by oxides of Al, Fe and Mn. Oxic sediments can be relatively enriched where Mn oxides are present. Mo forms minerals in combination with ore minerals and other elements like S (MoS_2) and Cu (porphyry deposits) thanks to its chalcophile behaviour (Smedley & Kinniburgh, 2017).
  Non-contaminated soils typically have Mo contents less than 10 mg/kg, while in rainwater it is expected to be in sub-μg/L quantities (Smedley & Kinniburgh, 2017).
  It is the most abundant transition metal in oceans, which are supplied by rivers (mean concentration of 1.21 μg/L) that take the element from weathering of

continental material. Open oxic water have Mo concentrations approximating 10 μg/L. Mo in seawater is greater than concentrations required in biological functions and therefore it seems it is not significantly affected by biological activity or biological cycling (Smedley & Kinniburgh, 2017).

Concentrations of Mo in natural fresh surface waters and groundwaters appear to be usually <10 μg/L and often substantially less (Smedley & Kinniburgh, 2017).

Anthropogenic activities that have contributed to environmental contamination include combustion of fossil fuels, and smelting, mining, and milling (Eisler, 2000).

Mo is used as a lubricant additive, catalyst, corrosion inhibitor, and in the manufacture of pigments and ceramics. It is also used in steel alloys and added to cast iron and stainless steel for hardness control and in agriculture to counteract Mo deficiency in crops (Smedley & Kinniburgh, 2017).

It is biologically inactive unless complexed as a cofactor. It acts for the functioning of more than 60 enzymes which catalyze chemical reactions involved in the cycling of N, C, and S (Smedley & Kinniburgh, 2017).

Mo concentrations are usually lower in fish and wildlife than in terrestrial macrophytes. Ruminants are particularly vulnerable to Mo and can suffer from both low and high intakes (Smedley & Kinniburgh, 2017).

Acquatic organisms are comparatively resistant to Mo as adverse effects on growth and survival appear only when Mo conentration in water is over 50 mg/L (Eisler, 1989).

Limited data are available on the effects of Mo on avian species. Since is an essential trace element in birds, the majority of avian studies for the element identified in literature are dietary studies of soluble forms of Mo on domestic species conducted from the 1950s through the 1970s. Birds appear to have a lower biological requirement than most mammalian species, but a higher resistance to excess Mo (Stafford et al., 2016).

In birds, adverse effects on growth, reproduction and survival have been reported at respective dietary concentrations of 200 to 300 mg/kg, 500 mg/kg and at 6000 mg/kg (Eisler, 2000).

In general, its chemistry is complex and inadequately known, and its toxicological properties are related to interactions with Cu and S, although other metals and compounds can make this interrelation unclear (Eisler, 2000).
Mo toxicity on birds is largely secondary to Cu deficiency and S presence, because of the potential formation of thiomolybdates in the stomach which reduce Cu bioavailability. Soluble forms of Mo appear to be more toxic than insoluble forms such as MoS_2 (Stafford et al., 2016). There is a lack of laboratory study relating feather and tissue Mo concentrations with detrimental effects in birds; as a consequence, no toxicity thresholds are available.

- Cadmium (Cd): It is a relatively rare metal which is not biologically essential or beneficial for wildlife. In sufficiently high concentrations, it is toxic to all forms of life, including microorganisms, higher plants, animals, and humans. It is distributed in the earth's crust and in small amounts in zinc ores (Eisler, 2000; Tchounwou et al., 2012).
  Cd occurs in the environment as a divalent metal that is insoluble in water, but its chloride and sulfate salts are freely soluble. If released or deposited on soils, is largely retained in the surface layers to a much lesser extent than most other metals and converted to more insoluble forms, such as $CdCO_3$ and CdS (EPA, 2005b).
  Adsorption and desorption processes are major factors in controlling Cd concentrations in natural waters and tend to influence changes of Cd ions in solution. Its chemical mobility and bioavailability in sediments can be changed by physicochemical conditions (especially pH and redox potential) during dredging and disposal (Eisler, 2000).
  Cd enters in the environment via the atmosphere as particulate matter and is subject to dry and wet deposition. Since it occurs naturally in the earth's crust, Cd may also enter in small amounts from natural weathering of rocks, windblown soil, and volcanoes (EPA, 2005b). However, these sources are minor compared with anthropogenic releases from mining, smelting, fuel combustion, disposal of metal-containing products and application of phosphate fertilizers or

sewage sludges. Concentrations are most likely highest in the localized regions of urban industrialized areas (Eisler, 2000)

Cd is commercially obtained as an industrial by-product of the production of Zn, Cu, and Pb and its major industrial applications include the production of alloys, pigments, and batteries (Eisler, 2000; Tchounwou et al., 2012).

Cd tends to localize in the epithelial membrane while only 0.1-0.5% goes into circulation and in the other tissues. As the dosage increases, a larger percentage is transferred through the epithelial membrane and made available for uptake by other tissues. After reaching organs such as liver and kidney, Cd accumulates over time bound to metallothinein (MT), a low molecular weight, sulphidril-rich protein (Scheuhammer, 1987).

Cd is taken up by plants from soils and translocated with subsequent transfer through the terrestrial food chain (EPA, 2005b). It may biomagnify in terrestrial food webs and tends to accumulate in liver and kidneys. Although it is excreted in urine and feces, Cd tends to increase with the age of the organism and eventually acts as a cumulative poison (Eisler, 2000).

Mammals and birds consistently accumulate Cd mainly through ingestion of contaminated food.

The latter are comparatively resistant to the biocidal properties of Cd. However, detrimental effects in birds were observed at higher concentrations when compared to aquatic biota. Sublethal effects include growth retardation, anemia, renal effects, and testicular damage. In avian tissues Cd tends to accumulate in kidney, liver, brain, bone, and muscle with immature birds more susceptible to toxic effects of Cd than adults. Dietary Cd might have significant reproductive effects as females store of Cd instead of Ca in bones for mobilization during egg production when there is a reduced Ca intake. However, very little Cd is transferred to eggs of birds, regardless of the dietary levels consumed (Eisler, 2000) and in seabird eggs usually the concentrations are lower than 0.7 μg/g (Furness & Greenwood, 1996).

Cd-induced effects associated with oral intake include nephrotoxicity and also possible effects on the liver, reproductive organs, and the hematopoietic, immune, skeletal, and cardiovascular systems (EPA, 2005b)

The critical organ in chronic Cd toxicity is considered to be the kidney, although, in males, the testes may be severely affected by sublethal exposure. With continued exposure, even at low dietary levels, there is an increase in the renal cortex concurrent with an increase in the renal MT concentration (Scheuhammer, 1987).

Bone tissue can be affected by Cd exposure, but the mechanisms by which this occurs are not well understood.

Cd concentration in liver tissue is the best measure as a monitor of total exposure, or as an indicator of the body burden. The liver accumulates approximately half of the body burden of Cd and is generally resistant to the toxic effects of Cd, unlike the kidney where Cd concentrations fall significantly after the production of Cd-induced tubular dysfunction.

Fledgling feathers may be a better indicator of dietary levels since they can accumulate Cd when they grow (Scheuhammer, 1987)**.** Toxic effects of Cd towards seabirds have been observed ar concentrations ranging from 0.1 up to 2 μg/g in feathers (Burger & Gochfeld, 2000).

- Antimony (Sb): it is a semi-metallic element that shares some chemical properties with Pb, As, and Bi. It can exist in a variety of oxidation states (-3, 0, 3, 5) but it is mainly found in two oxidation states, 3 and 5. Sb should exist as Sb^{5+} in oxic systems and as Sb^{3+} in anoxic ones (Filella et al., 2003, 2002).

  Sb is a common component of Pb and Cu alloys and is used in the manufacturing of ceramics and textiles. In the past, it was used in certain therapeutic agents against major tropical diseases, although in recent years, it has been increasingly replaced by other agents (EPA, 2005a). Nowadays, Sb is mostly used in large quantities as a flame retarding additive (Filella et al., 2002). Sb compounds are rather volatile and they are released into the atmosphere during incineration of waste, fossil combustion, and smelting of metals. Sb is one of the elements that show higher enrichments in aerosols over the concentrations expected from sea salt and from crustal sources (Filella et al., 2002).

Typical concentrations in unpolluted acquatic ecosystems are less than 1 µg/L. Concentrations are much higher in Sb-enriched mineralized areas and natural geothermal systems where they can range from 500 mg/L up to 10 wt% (Filella et al., 2003).

However, in the proximity of anthropogenic sources, concentrations can reach up to 100 times natural levels (Filella et al., 2002). Sb^{3+} is oxidised to Sb^{5+} in oxic waters, perhaps through bacterial mediation (Filella et al., 2003).

Sb is a strong chalcophile element and as such it mainly occurs in nature as stibnite and antimonite. Sb concentrations in sediments and soils are of the order of a few µg/g. Higher concentrations are directly related to anthropogenic sources, mainly in proximity to smelting plants.

Both Sb^{3+} and Sb^{5+} ions hydrolyse easily in aqueous solution, thus making it difficult to keep Sb ions stable, except in highly acidic media (Filella et al., 2003).

Sb is present in the aquatic environment as a result of rock weathering, soil runoff and anthropogenic activities.

The distribution and speciation of Sb in freshwater systems have not been extensively studied and range from a few ng/L to a few µg/L depending on location. Sb concentration in oceans is about 200 ng/L and it is not considered to be a highly reactive element (Filella et al., 2002).

Sb has no known biological function and, in high dosages, can be toxic, expressing similar damaging effects to As. Ingested Sb is absorbed slowly and is reported to be a gastrointestinal irritant (Sb^{3+} is absorbed more slowly than Sb^{5+}).

It appears that Sb can be methylated much less rapidly and less extensively than As and that Sb^{3+} compounds are more readily biomethylated than Sb^{5+} compounds (Filella et al., 2003).

There is little evidence of biomagnification of antimony in food chains represented by soil–vegetation– invertebrate–insectivore pathway of grasslands and little indication of significant accumulation by herbivorous mammals despite marked contamination of their diet (Filella et al., 2002).

Trivalent species are reported to be more toxic than pentavalent forms (Filella et al., 2007). High antimony concentrations are generally associated with high arsenic concentrations in sulfide ores.

Observed toxic effects in mammals involve cardiovascular changes which include degeneration of the myocardium, arterial hypotension, heart dysfunction, arrhythmia, and altered electrocardiogram patterns. The mode of action for Sb-induced cardiotoxicity is unknown and no acceptable avian toxicological studies were identified for use in the derivation of a reference dose (EPA, 2005a), nor thresholds for detrimental effects are available.

- Mercury (Hg): is a transition metal element that exists in nature in three forms (elemental, inorganic, and organic), with each having its own toxicity (Tchounwou et al., 2012). It is a mutagen and carcinogen that can cause embryocidal, cytochemical, and histopathological effects. Forms of Hg with relatively low toxicity can be transformed into forms with very high toxicity through biological processes (Eisler, 2000).

  Elemental Hg is a highly volatile liquid at room temperature and is released into the environment as a gas, in lava (from terrestrial and oceanic volcanic activity), in solution, or in particulate form. The atmosphere plays an important role in the mobilization of Hg and is acknowledged as the major source of Hg for watersheds (Eisler, 2000).

  Methylmercury (MeHg +) is the most frequent form in the environment (Tchounwou et al., 2012) and it is produced by the methylation of inorganic Hg present in sediments of fresh and salt water, accumulating in aquatic food chains (Eisler, 2000).

  Additional concentrations that can increase the global input of Hg comes from combustion of fossil fuels, mining and reprocessing of Au, Cu and Pb, runoff from cinnabar mines, wastes from nuclear reactors, chloralkali plants, pharmaceutical and military facilities, incineration of municipal and medical wastes and disposal of batteries and fluorescent lamps (Eisler, 2000).

Sources of exposure to elemental Hg included amalgam, medical instruments, fossil fuel emissions, incandescent lights, batteries and incineration of medical waste.

Hg enters the food web with a mechanism that varies among ecosystems and, as it is ubiquitous and persistent in the environment, exposure to some form of Hg for humans, plants and animals is unavoidable (Rice et al., 2014).

Hg and its compounds have no known normal metabolic function. Its presence in living organisms represents contamination from natural and anthropogenic sources and must be regarded as undesirable and potentially hazardous. MeHg can be bioconcentrated in organisms and biomagnified through food webs, returning directly to humans and other upper trophic level consumers in concentrated form (Eisler, 2000).

Hg enters the acquatic environment through the air-water interface and volcanic and anthropogenic emissions, depositing by precipitation in the ocean. Hg^{2+} tends to settle in the sediment, in the less oxygenated layers. Here, sulphate reducing bacteria manage to uptake Hg into the cells and convert it into monomethylmercury (MMHg), a neurotoxin that moves along the trophic web and becomes highly concentrated in aquatic fauna. This happens because the S^{2-} (a waste product of bacterial sulphate reduction) binds with Hg^{2+} to form HgS, a form that can easily pass through the cell walls. When bacteria take up HgS, they broke the molecule, and then methylates Hg for elimination. If it is not converted to MMHg it can reside in the sediment until it creates a layer that will eventually return to the state of magma and then be emitted by volcanic eruption.

Other factors are able to modify the toxicity of MeHg. For instance, Se can reduce the toxicity of MeHg thanks to the formation of a Hg–Se complex that diverts Hg from sensitive targets and the prevention of oxidative damage by increasing the amount of Se available to the Se-dependent enzyme glutathione peroxidase (Eisler, 2000; Scheuhammer, 1987).

In adult mammals, >90% of the total body burden of inorganic Hg accumulates in the kidneys, whereas, in immature animals, only about 35% of total Hg accumulates in renal tissue with liver and brain taking up proportionately more

of the metal than in the adult. The kidney is also the major reservoir of inorganic Hg in birds.

Significant effects of chronic exposure to inorganic Hg in birds include delayed testicular development, gonadal atresia, reduced number of mating attempts and a depressed fertility of eggs (Scheuhammer, 1987).

The biokinetics and toxicology of MeHg in birds have been more studied than those of inorganic Hg due to the greater toxicity and bioaccumulation of the methylated form (Wolfe et al., 1998). In fact, some birds poisoned by inorganic Hg recovered after treatment was withdrawn, but chicks that were fed MeHg later developed toxic signs and usually died, even if the treated feed was removed (Eisler, 2000). In birds, MeHg is almost fully absorbed, more slowly metabolised than other organomercurials and has a lower excretion rate (so its biological half-life is relatively long). Because of its chemical stability and lipophilicity, MeHg penetrates the blood-brain barrier, affecting the central nervous system and producing brain lesions. In birds, spinal cord degeneration may be the most characteristic effect of MeHg toxicity. MeHg poisoning is characterised by reduced food intake, weight loss, progressive weakness in wings and legs, calmness, withdrawal, hyporeactivity, hypoactivity, eyelid drooping and muscular incoordination. Once such symptoms occur, death is a virtual certainty even with the removal of the source of exposure (Eisler, 2000).

Reproduction is one of the most sensitive toxicological responses, with the major toxic result of MeHg ingestion being decreased hatchability egg shell thinning, reduced clutch size, aberrant behavior and impaired hearing of juveniles and early embryonic mortality (Scheuhammer, 1987; Wolfe et al., 1998).

Hg concentrations in tissues and feathers are highest in fish-eating species and those that eat benthic invertebrates. Hg residues are usually highest in kidney and liver, but total Hg contents are significantly modified by diet, availability, and migratory patterns (Eisler, 2000).

Egg laying is a relevant route for reducing the female's body burden (especially the first egg) as Hg levels decline with laying sequence in gulls and terns (Eisler, 2000). However, for some species, Hg concentrations in eggs is small compared

to the female's body burden, therefore its removal through egg laying has been considered negligible (Furness & Greenwood, 1996). In eggs, Hg accumulates in the albumen portion in a dose-dependent fashion and in response to increasing dietary levels of MeHg and rarely exceeds 0.5 µg/g. They are a particularly useful means for monitoring Hg pollution in the vicinity of a breeding colony in the immediate pre-laying season (Furness & Greenwood, 1996).

It has been shown experimentally that feathers incorporate Hg in a dose dependent manner and they may provide a convenient substrate for monitoring increased dietary exposure to MeHg in birds (Furness & Greenwood, 1996; Scheuhammer, 1987). Contamination of feathers by Hg after their formation is probably minimal as it does not concentrate in the uropygial gland or in the secreted oils. In free-living bird species, it has been estimated that up to 60%-70% of the total body burden of Hg may be present in the plumage (Honda et al., 1986a, 1986b). Thus, the Hg content of a feather should be a reasonably good indicator of dietary and/or tissue Hg levels extant during the growth of the feather (Scheuhammer, 1987).

For birds, levels of total Hg concentrations associated with adverse reproductive effects are 5 µg/g in feathers (Eisler, 2000; Hargreaves et al., 2010).

- Lead (Pb): is a naturally occurring trace element present in air, soil, sediment, and water. When absorbed in excessive amounts, it is a mutagen and teratogen with carcinogenic properties (Eisler, 2000; EPA, 2005d)

  Although Pb occurs naturally in the environment, its chemistry is complex. In nature, Pb occurs mainly as Pb^{2+} but under strong oxidizing conditions it can be oxidized to Pb^{4+}, of which only simple compounds are stable. Organic compounds are more toxic than inorganic ones (Eisler, 2000).

  In water, Pb is precipitated as carbonates or hydroxides and is more soluble and bioavailable at low pH, low organic content, low concentrations of suspended sediments and low concentrations of Ca, Fe, Mn Zn and Cd salts. Pb compounds tend to concentrate in the water surface microlayer, especially when surface organic materials are present in thin films (Eisler, 2000).

In the sediments, Pb is mobilized and released when the pH decreases suddenly or ionic composition changes.(Eisler, 2000)

When released to soil as halides, hydroxides, oxides, carbonates, or sulfates, Pb is relatively immobile and persistent and normally converts from soluble compounds to insoluble $PbSO_4$ or $Pb_3(PO_4)_2$. It forms complexes with organic matter and clay minerals, fixating and limiting its transfer to aquatic systems. However, leaching of Pb can be rapid for soils from highly contaminated sites (EPA, 2005d).

Pb enters the atmosphere mainly through smelter, vehicle emissions and anthropogenic activities like fossil fuels burning, mining, and manufacturing increase its release in high concentrations (Eisler, 2000; Tchounwou et al., 2012).

Pb has many different industrial, agricultural and domestic applications. It is used in the production of batteries, ammunitions, metal products, and devices to shield X-rays (Tchounwou et al., 2012).

Pb is not considered essential nor beneficial for plants, birds or mammals and the effects caused by Pb poisoning are several (EPA, 2005d). Continuous exposure to low concentrations of Pb may result in reproduction impairment and liver and thyroid malfunctions. Pb modifies the function and structure of kidney, bone, the central nervous system and the hematopoietic system and produces adverse biochemical, histopathological, neuropsychological, fetotoxic, teratogenic, and reproductive effects (Eisler, 2000). Its bioavailability is strongly influenced by chemical speciation, but it is toxic in most of its chemical forms. It can be incorporated into the body by inhalation, ingestion, dermal absorption, and transfer to the fetus. Younger, immature organisms are more susceptible to Pb than adults (Eisler, 2000).

Absorbed Pb accumulates primarily in bones of mammals and birds; among soft tissues, kidneys accumulate the highest concentrations (Eisler, 2000)**.** Clinical signs of toxicity found in domestic animals are encephalopathy and gastrointestinal malfunction. Behavorial signs of poisoning include anxiety, hyperexcitability and possible violent behaviour. Locomotor alterations range from ataxia and lack of coordination to the rigidity of all the posterior muscles to

compulsive hypermotility. Pb can interfere with the synthesis of heme, altering enzime conentration in urine and blood. Other signs of poisoning include fatigue, anorexia, loss of weight, decreased milk production, paraplegia, mortality and interference with resistance to infectious diseases (EPA, 2005d). Birds subjected to Pb intoxication suffer from medullary bone formation and destruction associated with storage and liberation of Ca during egg shell formation. Affected birds struggle to move, avoid other birds and become increasingly susceptible to predation (Eisler, 2000). Female birds accumulation is generally greater than males and laying females accumulated 4-5 times more Pb in bones than non-layers. This greater deposition may be related to the increased turnover of skeletal Ca necessitated by egg shell formation during reproduction (Scheuhammer, 1987).

The best index of life-long exposure to Pb is bone tissue since it has a high affinity for it and, once bound, Pb is generally non-mobilisable.

Pb transfer to eggs is generally low, with concentrations of less than 0.4 μg/g in seabird eggs (Furness & Greenwood, 1996).

Pb concentrations can be measured in feathers, but interpretation is difficult as they can be affected by external contamination from secretive products and exposure to the environment (Burger & Gochfeld, 2000).

The avian wildlife concentration limit for Pb in feathers is 4 μg/g (Burger & Gochfeld, 2000; Hargreaves et al., 2010).

3) MATERIALS AND METHODS

- **Study area and sample collection**

Figure 9 shows all the areas in which samples were collected for this study. Egg shells and feathers of *C. alexandrinus* and of most of the considered reference species were collected in the Cavallino-Treporti peninsula, the northernmost littoral strip separating the Venice Lagoon from Adriatic Sea.

Along the 13 km of the coast of the peninsula a fragmented system of natural and restored dunes is present, covering an area of about 350,000 m^2 (Cecconi & Nascimbeni, 1997). This area was chosen to collect feathers and egg shells of *C. alexandrinus* as a small population nests here regularly on drift lines (habitat code 1210 - Annual vegetation of drift lines, according to the Directive 92/43/EEC), embryonic and shifting dunes (habitat codes 2110 - Embryonic shifting dunes and 2120 - Shifting dunes along the shoreline with *Ammophila arenaria* (white dunes)), and partially also on the semifixed dunes (habitat codes 2230 - Malcolmietalia dune grasslands and 2130* - Fixed coastal dunes with herbaceous vegetation (grey dunes)). Here, *C. alexandrinus* feeds on invertebrate communities in the shallows of the northern basin of the Lagoon, on the shores and on drift material on the beach.

Samples of the species *C. ridibundus*, *I. melanocephalus*, *P. carbo sinensis* and *E. garzetta* were collected in the SCI IT3250031 "Upper Lagoon of Venice" (habitat codes 1150* - Coastal lagoons and 1140 - Emerging muddy or sandy expanses during low tide), in the northern area of the Cavallino-Treporti peninsula. The samples were collected from dead adults (*P. carbo sinensis*, *E. garzetta*), and dead youngs (*C. ridibundus* and *I. melanocephalus*). The same type of habitat was found in the area of Portegrandi, during the collection of feathers from a dead adult of *T. alba*. Samples from an adult *T. sandvicensis* were collected around Palude Fondello, an area near Chioggia included in the SCI IT3250030 (Habitat codes 1150 * - Coastal lagoons, 1420 - Mediterranean halophyte and termoatlantic prairies and fruticetes (*Sarcocornetea fruticosi*), 1140 - muddy or sandy expanses emerging at low tide, 1510 * - Mediterranean salted steppes (*Limonietalia*), 1410 - Mediterranean inundated pastures

(*Juncetalia maritimi*), 1320 - Spartina meadows (*Spartinion maritimae*) and 1310 - Pioneer vegetation in *Salicornia* and other annual species of muddy and sandy areas). For the area of Mira, where samples of *P. colchicus* were collected, the habitat codes were not specified since it is not considered a protected area.

Within the Lagoon, trace elements concentrations in shallow sediments are well known for As, Ag, Cd, Hg, Cu, Zn, Pb and V, but data are still lacking for Se, Rb, Cs and rare-earth elements. There is not recent data for sediments of the littoral strips (Donazzolo et al., 1981; Giusti & Zhang, 2002; Zonta et al., 2007).

In comparison with the industrial district and the urban areas, shallows and mudflats of the northern basin of the Lagoon have low concentrations of metals (Picone et al., 2017). However, recent studies in the area highlighted that shallows far from the urban centres and the industrial settlement (which are important for waterbird foraging) may suffer from biomagnification of trace elements and consequently cause toxic effects through aquatic invertebrates (Dominik et al., 2014; Picone et al., 2017, 2016).

Samples of feathers and egg shells were collected for each species in the period between March 2017 and August 2018.

Tail feathers were collected and analyzed for each individual. For the deceased subjects (*P. carbo sinensis*, *E. garzetta*, *C. ridibundus* and *I. melanocephalus*) more than one feather was collected as more sampling material was present. For *C. alexandrinus*, tail feathers of 8 females and 5 males were collected.

For egg shells, 7 samples from hatched eggs of *C. alexandrinus* were collected from different nests.

Figure 9: Collection areas of the various feather and egg samples analyzed in this study. Samples of the species Chroicocephalus ridibundus, Ichtyaetus melanocephalus and Phalacrocorax carbo sinensis were collected in the same area. The coordinates of samples of Phasianus colchicus are not present as they have not been collected.

- **Instrumentation**

The elemental analysis was carried out using inorganic mass spectrometry, using the Inductively Coupled Plasma Mass Spectometry (or ICP-MS) technique.

The mass spectrometer used in this analysis is an instrument that separates and classifies the atomic and molecular gaseous ions according to their mass/charge ratio and is based on the physical principles of separation through the use of electric and/or magnetic fields.

In mass spectrometry, for elemental analysis the plasma (ICP-MS) is used as ionization system. Compared to other techniques of elementary determination, this results to include more elements, it has a very wide linear dynamic range, a shorter analysis time and superior performances with regard to the limits of detection. In inorganic mass spectrometry, we refer to the fact that the samples are nebulized, ionized in an argon plasma at high temperatures and then analyzed on the basis of their mass/charge ratio.

The parts that make up a mass spectrometer with inductively coupled plasma source are:

- Sample introduction system: Liquid samples are introduced using a nebulizer that creates an aerosol. The sample is pumped into the nebulizer where the liquid is transformed into small droplets thanks to the action of a gas flow (generally argon). Then a spray chamber goes to select the droplets that are formed by passing only the smallest ones. It is a crucial component since it has a relevant effect on transport efficiency, precision, and washout. Larger droplets are removed by condensation on the walls of the chamber. The finest aerosol is conveyed towards the torch and then towards the plasma where it is desolvated, brought to the gaseous state, atomised and finally ionized. The selection is due to the geometry of the room as the spray chamber comes in different types with different perfomances and applications (Scott double-pass, cyclonic, etc.).
- Torch: Structure responsible for the formation of plasma. It is made up of concentric quartz tubes at the end of which there is a copper coil in tension which gives rise to an oscillating magnetic field produced by a generator generally at 27.4 MHz. To start the ionization, an electric shock is applied to gas (generally argon), causing it to reach temperatures between 6000 and 10000 K. The plasma will be maintained by the magnetic field which generates a continuous movement of charged particles, which collide against other neutral particles ionizing them.
- Interface: The purpose of this component is to connect the plasma system, at ambient pressure, with the mass spectrometer, where the pressure is much lower (1×10^{-5} bar). The system consists of two perforated metal coaxial cones which are used to introduce ions to the analyzer. To maintain the pressure and ensure that there is not an excessive loss of ions, it is necessary to adequately size the

holes of the cones. The first cone is a copper sampler cone on which a pierced nickel tip (1 mm diameter) is melted. This cone allows the ions of the central plasma area to pass. Being therefore very close to the plasma, it is cooled together with the copper coil and the spray chamber. Immediately after the ions have passed through this cone, pass into the second and smaller one called skimmer (0.4 mm) where a further selection takes place. After the sample had passed the cones, inside the high vacuum region, the electrostatic lenses collate the ions, towards the analyzer.

- Quadrupole analyzer: It separates the ions according to the mass/charge ratio and conveys them to the detector. The flow of ions is conducted inside four conductor bars by means of electromagnetic lenses and is influenced by the oscillating magnetic field applied to the pairs of diagonally opposite bars. This field makes the ions move with a certain sinusoidal type trajectory, crossing only the quadrupole with certain ions with a certain mass. The frequency of the oscillating field is varied to select the various ions present on the basis of the mass/charge ratio.
- Detector: consists of a conversion cathode in which collide ions (and emit a proportional number of electrons) and an electro-multiplier that amplifies the electrons produced and provides a measurable electrical signal. The intensity of the generated current is proportional to the quantity of ions arriving at the detector.

The instrument used for sample analysis was an Agilent 7500a (Agilent Technologies Inc. Santa Clara, California).

Mineralization of samples is carried out using a microwave oven (Ethos1-Milestone S.r.l, Italy). This method is preferred because it allows to digest the sample faster and allows a high control on the reaction.

For sample dilution, standards preparation and material cleaning, ultrapure water was used (18.2 MΩ cm, Elga PURELAB Ultra AN MK2), in order to obtain appropriate detection limits and avoid external contamination.

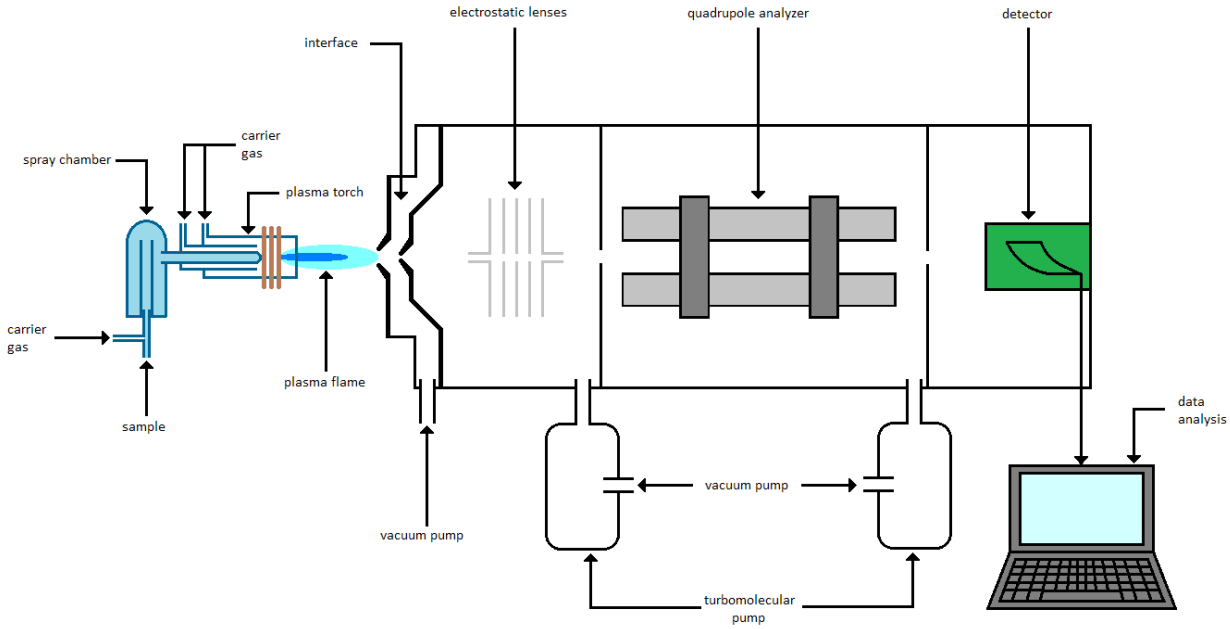

Figure 10: General scheme of operation of an Inductively Coupled Plasma Mass Spectrometer (ICP-MS) used for the analysis of trace elements in this study

- **Sample treatment and analysis**

In order to be analyzed through ICP-MS, the samples need to be mineralized through wet acid digestion.

In chemistry, mineralization is the process that allows the complete dissolution of the sample (solid) through strong acids, bringing the elements in solution and allowing their analysis.

The sample must undergo this process and can not be introduced into the mass spectrometer before this step. It is necessary because it destroys the organic part of a complex matrix to allow the extraction of the inorganic analyte (taking it from a solid matrix to a liquid one) and allows the control of its concentration in the samples.

Furthermore, with mineralization the trace elements that are contained in the samples can be more easily detected by the ICP-MS, improving the accuracy of the analysis.

Samples are placed inside quartz and TFM containers (called vessels). It is a microwave-transparent polymeric material which allows direct heating of the sample.

TFM is widely used because is resistant to high temperatures (up to 300 °C) and has good chemical inertia.

The containers have a relief valve, which prevents the internal pressure from becoming too high. A probe inside one of the vessels ensures a constant internal temperature monitoring.

“Vessel-inside-vessel” technology was used for this analysis. This was developed by Milestone in the late 90s and uses a smaller secondary vessel to contain the sample while the primary vessel contains the digestion reagents required to achieve accurate temperature monitoring. This configuration reduces the amount of acid required for digestion to near stoichiometric quantities, diminishing the dilution factor and increasing the detection limit. The use of this technology helps to control exothermal reactions by providing a heat sink for the energy liberated during oxidization. Quartz is the ultimate vessel material for ultra-trace analysis, since it can be cleaned more effectively, and has lower background levels than extra high purity TFM (Milestone, 2018).

Before the introduction of the sample, each vessel was first subjected to washing cycles in microwave oven with super-pure nitric acid and subsequently with hydrogen peroxide and ultrapure water with a ratio of 1: 4.5. A last washing cycle was done by placing quartz containers, each containing 1:9 ultrapure nitric acid, inside the vessels.

Samples were cleaned with a 1M solution of acetone and rinsed several times with ultrapure water, to remove any external source of contamination.

One feather for specimen (~10 mg) was analyzed. Due to their nature and composition, fragmentation of feather samples can lead to loss of material and thus was avoided; the only exception was the feather of *P. carbo sinesis*. For egg shells, they were analyzed about 900 mg per sample.

Samples of feathers and egg shells were placed in the quartz containers and then inside the TFM vessels. Together with the sample, ultrapure hydrogen peroxide and ultrapure nitric acid in proportion 2:8 were added in the quartz containers.

Outside the quartz vessels, ultrapure hydrogen peroxide and ultrapure water in proportion 1: 4,5 were added.

Ultrapure nitric acid (Plasma Pure Plus, SCP Science) and ultrapure hydrogen peroxide (ROMIL Ltd.) were used.

Subsequently, the samples were mineralized through a program that reaches temperatures above 150°C. For each digestion cycle, the blank solutions were randomized to avoid different conditions of digestion in the vessels.

At the end of digestion, the content of each vessel was diluted 1:6 (feathers) and 1:10 (eggs) with ultrapure water, weighed and stored at -20 °C until analysis with ICP-MS.

The entire sample preparation was carried out accurately to minimize any sources of contamination.

For the quantitative analysis, a calibration curve is used to correlate the concentration of the analyte in solution (mapped on the x-axis) with the response signal of the instrument (mapped on the y-axis).

For the trace elements of interest, a set of external standard solutions of differing concentrations was analyzed in ICP-MS and a calibration curve, that covers a range of concentrations that includes the concentration of analyte in the sample, was obtained. A multielementary standard IMS2 and a Hg standard were employed (ULTRA Scientific); since Hg is volatile, standards were freshly made prior any subset of analysis. The approach is to measure the signal intensity of an element in an unknown solution (sample) and reference it to the signal intensity of the same element in another solution, whose elemental concentrations are known (external standards).

An internal standard (Rh 10 μg/L) is analyzed simultaneously with standards and samples. As it shares a similar behavior, atomic mass and ionization potential with the analyte, using an internal standard is essential to verify if there have been internal signal drifts (caused for example by the matrix effect). Internal standards are elements that are absent in the samples and follow the same analysis process of the sample, providing a signal that can be distinguished from the signal of the analyte. Any factor that affects the analyte signal will also affect the signal of the internal standard to the same degree. Hence, the ratio of the two signals will exhibit less variability.

Suggested elements to use as internal standards are Y, Ge, Rh or In and Re or Pt. Rh is especially preferred in ICP-MS as it exhibits excellent inter-element chemical compatibility.

Standard deviation (SD), relative standard deviation percentage (RSD%) and standard error percentage (SE%) were evaluated ofr any measurement; in all the specimens RSD% was < 10% and SE% was < 5%.

- **Statistical analysis**

For the statistical analysis, a one-way Analysis Of Variance (ANOVA) was performed. Variance is a measure of dispersion of data around the arithmetic mean (how data are far from the arithmetic mean):

$$var(y_1, \dots, y_n) = \frac{1}{n}\sum_{i=1}^{n}(y_i - \bar{y})^2$$

The purpose of this analysis is to determine whether (for a specific variable) the arithmetic means of groups of samples are statistically different or not within a specific level of confidence (H_0 hypothesis). This hypothesis can be confirmed or rejected using the relationship between the sum of squares between the groups (SSB) and the sum of squares within the groups (SSW) according to the following equation:

$$F = \frac{SSB/(p-1)}{SSW/(n-p)}$$

$$SSB = \sum_{i=1}^{p} n_i(\bar{y}_i - \bar{y})^2 \qquad SSW = \sum_{i=1}^{p}\sum_{j=1}^{n}(y_{ij} - \bar{y}_i)^2$$

[with n= “number of samples” and p= “groups in which samples are divided”]

The following parameters can also be derived from the total sum of squares (SST) according to the equation:

$$\mathrm{SST} = SSB + SSW$$

[with $SST = \sum_{i=1}^{p}\sum_{j=1}^{n}(y_{ij} - \bar{y})^2$]

Using the critical values of F distribution table and from the relationship between the degrees of freedom from the numerator (p - 1) and those from the denominator (n - p), a number (F_{crit}) can be obtained below which the hypothesis H_0 is confirmed with a significance value α. If $F \leq F_{crit}$ then H_0 is confirmed, otherwise it is rejected.

One way ANOVA was used to assess whether possible gender-related differences in trace element deposition may occur in *C. alexandrinus*.

4) RESULTS

- ***Charadrius alexandrinus* (feathers)**

  - Al: Samples have a mean concentration of 59.1 µg/g. Samples from males have a mean value of 62.1µg/g, while females have a mean concentration of 57.2 µg/g. Some individuals make an exception, with values of 101.8 µg/g from CA-m4, 83.6 µg/g from CA-m5 and 82.5 µg/g from CA-f4 higher than the global mean and values of 27.2 µg/g from CA- m2 and 31.1 µg/g from CA-m3 lower than the global mean. ANOVA analysis did not evidence any difference between sexes, as H_0 hypothesis was accepted (F= 0.013; F_{crit}= 4.844; p= 0.912).

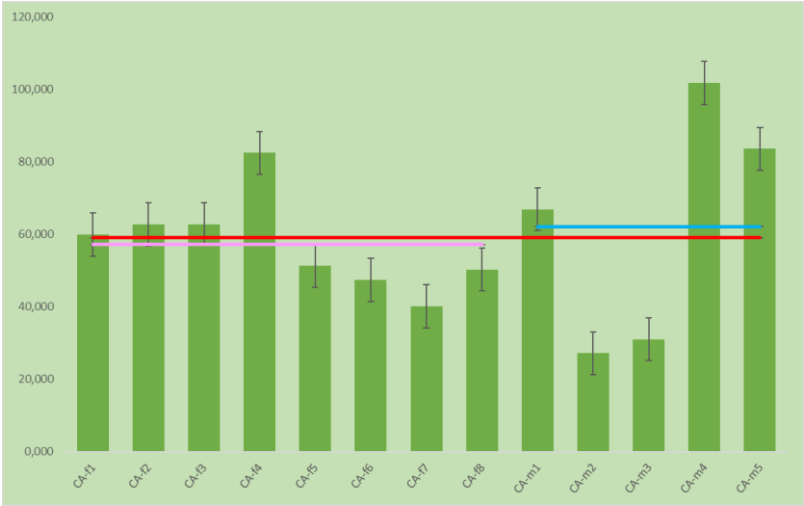

Figure 11.1: Al concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

  - Ca: The mean concentration is 512 µg/g, with males reaching a mean concentration of 517.3 µg/g and females have a mean value of 508.7 µg/g. CA-m4 (776.3 µg/g), CA-f1 (625.0 µg/g) and CA-f5 (654.2 µg/g) are higher than the global mean while CA-m3 (307.0 µg/g) and CA-f3 (362.2 µg/g) are lower. ANOVA analysis did not evidence differences between males and females, as H_0 hypothesis was accepted (F= 0.001; F_{crit}= 4.844; p= 0.975).

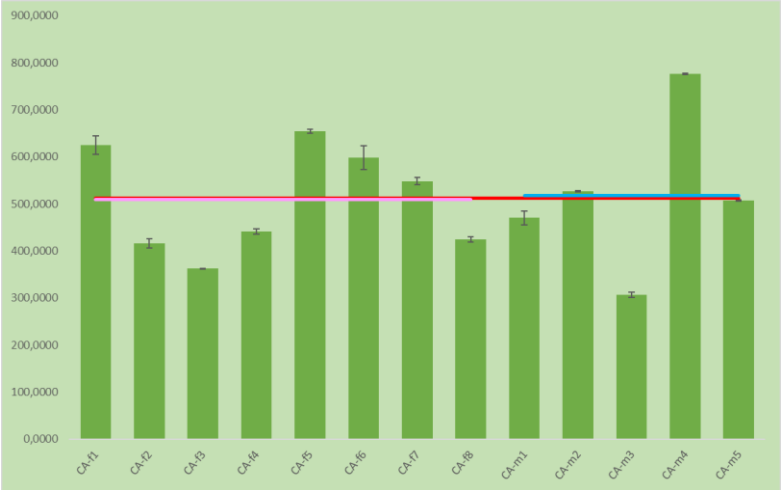

Figure 11.2: Ca concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- Fe: The global mean is 183.6 µg/g. Males have an average concentration of 184.6 µg/g and females have a mean of 183.0 µg/g. Exceptions are individuals CA-m4, CA-m5 and CA-f3 with concentrations of 246.3 µg/g, 255.4 µg/g and 265.8 µg/g respectively and CA- m2, CA-m3 and CA-f5 with concentrations of 124.2 µg/g, 87.6 µg/g and 129.7 µg/g respectively. Since H_0 hypothesis was accepted, ANOVA analysis showed no difference between sexes (F= 0.047; F_{crit}= 4.844; p= 0.832).

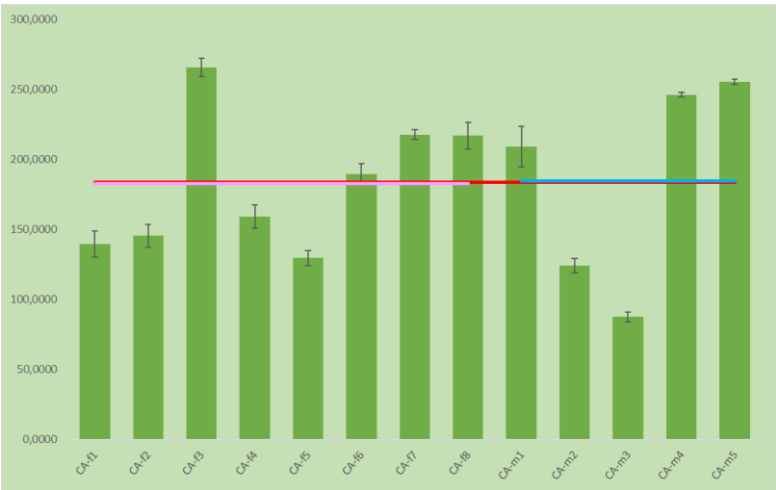

Figure 11.3: Fe concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- Co: The samples have a mean concentration of 48 µg/kg. Samples from males have a mean of 51 µg/kg and females have a mean of 46 µg/kg. Individuals CA-f1, CA-m1 and CA-m4 have higher concentrations than the global mean (86 µg/kg, 88 µg/kg and 79 µg/kg, respectively) and CA- f6, CA-f7, CA-f8 and CA-m5 are lower than the global mean (15 µg/kg, 23 µg/kg, 8 µg/kg and 11 µg/kg). ANOVA analysis did not evidenced differences between males and females, as H_0 hypothesis was accepted (F= 0.059; F_{crit}= 4.844; p= 0.813).

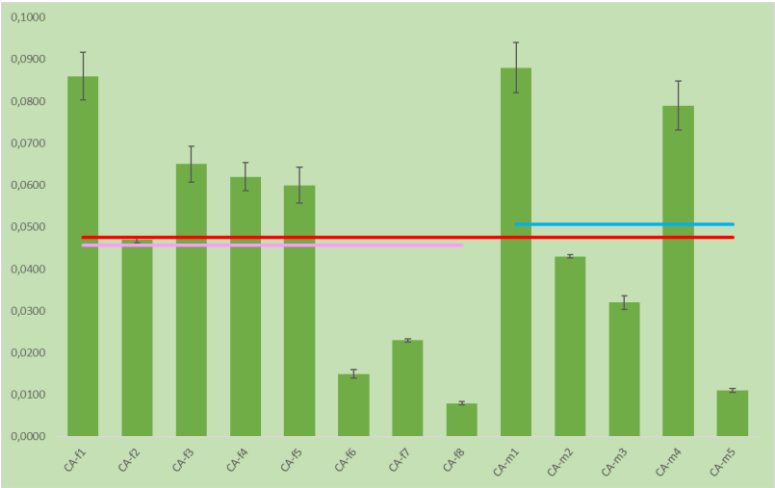

Figure 11.4: Co concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- As: The global mean value is 219 µg/kg, while samples from males and females have a mean concentration of 101 µg/kg and 293 µg/kg respectively. Females CA-f3 and CA-f5 reach the concentrations of 720 µg/kg and 475 µg/kg, respectively, whilst individuals CA- f1, CA-m2, CA-m4 and CA-m5 have the lowest concentrations (79 µg/kg, 97 µg/kg, 60 µg/kg and 11 µg/kg). It was the only element in which ANOVA analysis indicated a difference between males and females, since H_0 hypothesis was rejected (F = 5.326; F_{crit}= 4.844; p= 0.041).

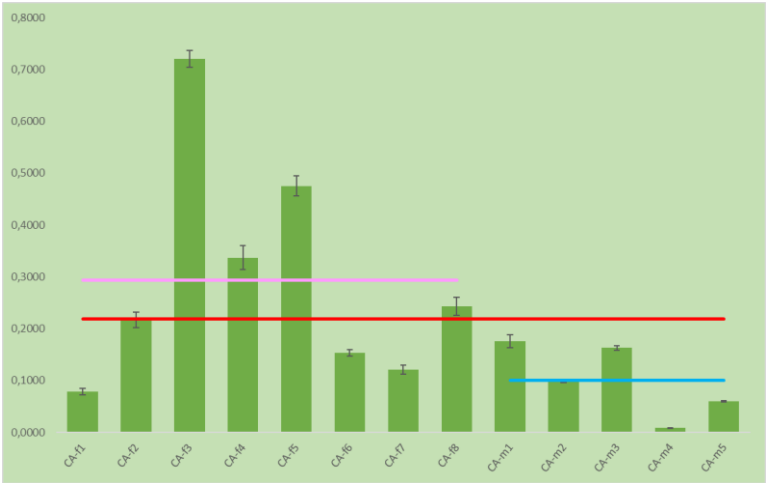

Figure 11.5: As concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- Se: Samples mean concentration is 3490 µg/kg. Males have a mean value of 3942 µg/kg and females have a mean of 3207 µg/kg. Individuals CA-f2 (5966 µg/kg), CA-f3 (4856 µg/kg), CA-f4 (5150 µg/kg) and CA-m1 (4817 µg/kg) make an exception, showing concentrations higher than the global mean, while CA- f5 (1976 µg/kg), CA-f6 (873 µg/kg), CA-f7 (1994 µg/kg) and CA-f8 (1435 µg/kg) are lower than the global mean. ANOVA analysis did not show any difference between sexes, as H_0 hypothesis was accepted ($F= 1.396$; $F_{crit}= 4.844$; $p= 0.262$).

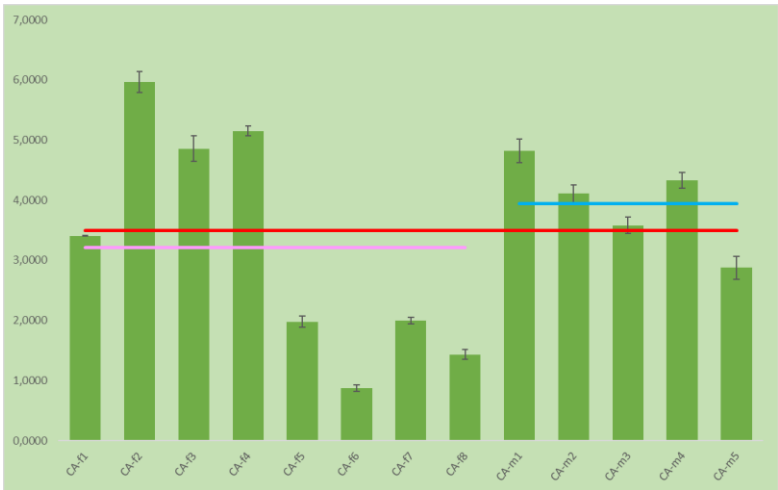

Figure 11.6: Se concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- Mo: The global mean concentration is 170 µg/kg. Females have a mean concentration of 162 µg/kg while males have a mean value of 183 µg/kg. Values of 308 µg/kg from CA-f1, 253 µg/kg from CA-f3, 258 µg/kg from CA-m2 and 279 µg/kg from CA-m1 are higher than the global mean and values of 87 µg/kg from CA- f6, 65 µg/kg from CA-m5, 70 µg/kg from CA-f7 and 106 µg/kg from CA-f8 are lower than the global mean. Since H_0 hypothesis was accepted, ANOVA analysis did not evidence any difference between males and females ($F= 0.137$; $F_{crit}= 4.844$; $p= 0.718$).

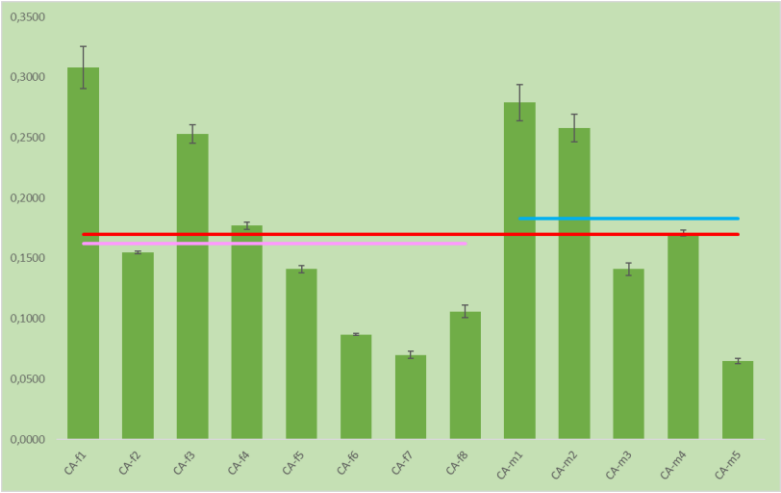

Figure 11.7: Mo concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- Cd: The global mean is 202 µg/kg. Males have an average concentration of 176 µg/kg, females have a mean of 218 µg/kg. CA-f3 have the highest concentration with 563 µg/kg while CA- f5, CA-m5, CA-f7, CA-m4 and CA-f8 have the lowest with 68 µg/kg, 38 µg/kg, 13 µg/kg, 48 µg/kg and 42 µg/kg respectively. ANOVA analysis did not show differences between males and females, as H_0 hypothesis was accepted ($F= 0.002$; $F_{crit}= 4.844$; $p= 0.963$).

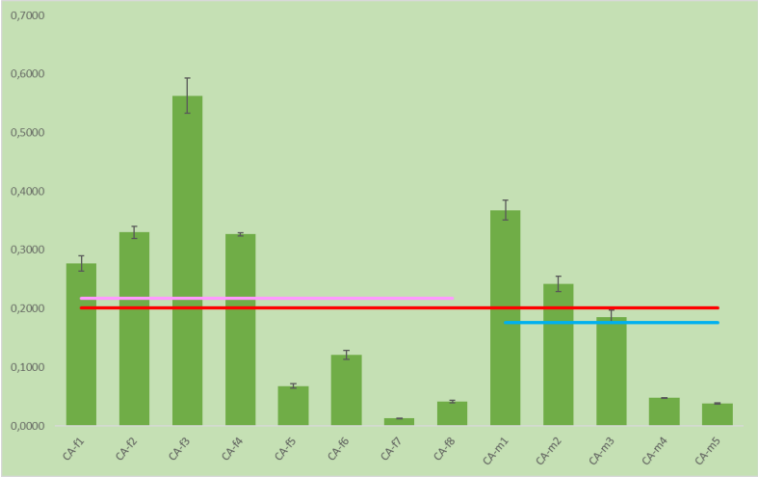

Figure 11.8: Cd concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- Sb: The mean concentration is 100 µg/kg, males reach a mean of 97 µg/kg and females have a mean of 101 µg/kg. Some samples make an exception, with the value of 137 µg/kg from CA-f3, 137 µg/kg from CA-f2 and 129 µg/kg from CA-f1 higher than the global mean and values of 28 µg/kg from CA- f5 and 57 µg/kg from CA-m4 lower than the global mean. ANOVA analysis did not show any difference between sexes, as H_0 hypothesis was accepted ($F= 0.005$; $F_{crit}= 4.844$; $p= 0.946$).

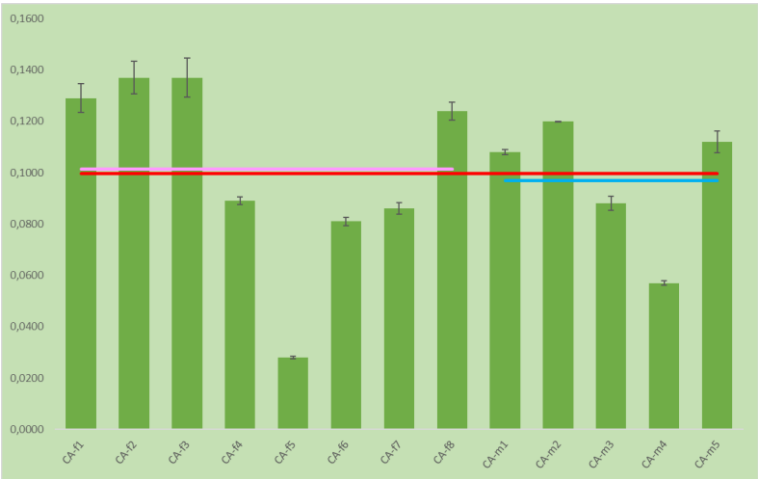

Figure 11.9: Sb concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- Hg: The global mean concentration is 8710 µg/kg. Females have a mean concentration of 8397 µg/kg, males have a mean concentration of 9211 µg/kg. Exeptions are CA-f2 with a value of 13.4 µg/g, CA- f1 with 4282 µg/kg and CA-m5 with 2802 µg/kg. Since H_0 hypothesis was accepted, ANOVA analysis did not evidence any difference between males and females ($F= 0.034$; $F_{crit}= 4.844$; $p= 0.857$).

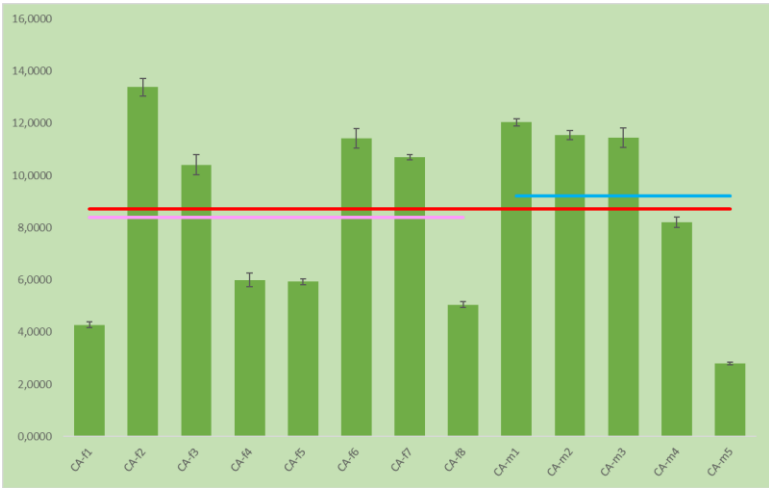

Figure 11.10: Hg concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- Pb: Samples have a mean concentration of 816 µg/kg. Samples from males have a mean value of 1055 µg/kg, while females have a mean concentration of 667 µg/kg. CA-m4 have a concentration higher than the global mean (1878 µg/kg) while CA-f8 have a lower concentration than the global mean (139 µg/kg) ANOVA analysis did not evidence differences between males and females, as H_0 hypothesis was accepted ($F= 2.522$; $F_{crit}= 4.844$; $p= 0.141$).

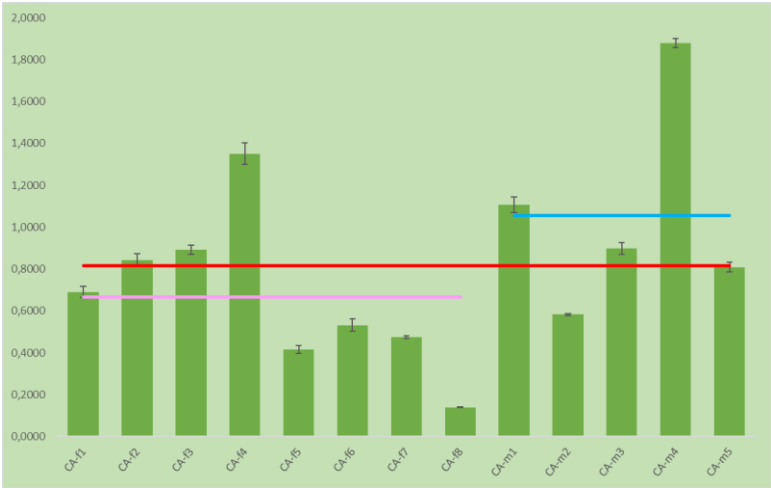

Figure 11.11: Pb concentrations in feathers of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean, while the pink and blue line indicate the arithmetic mean for females and males respectively.

- ***Charadrius alexandrinus* (egg shells)**

  - Al: the analyzed egg shells have a mean concentration of 21.3 µg/g. Values consistently higher than the mean were observed in samples Nest 1, Nest 2 (32.3 µg/g and 32.2 µg/g), and lower in samples Nest 5 and Nest 6 (with concentrations of 9.0 µg/g and 13.6 µg/g respectively).

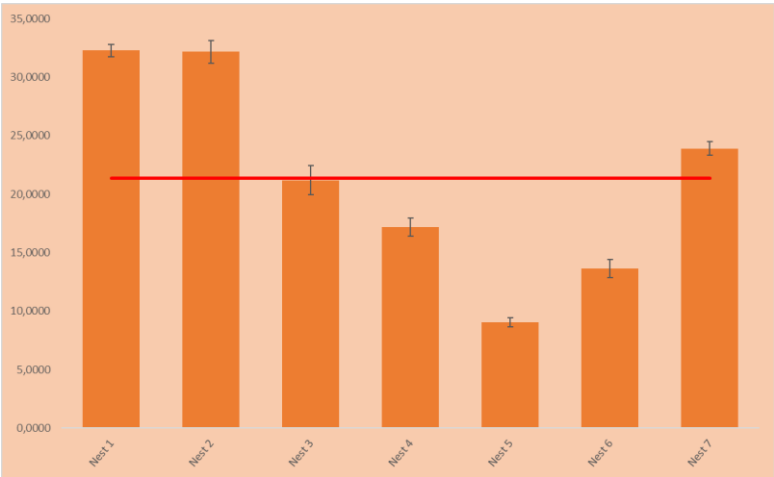

Figure 12.1: Al concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

  - Ca: As eggs are made mainly of this element, concentrations of this element are very high, with a mean of 106,721 µg/g. Sample Nest 6 has a higher

concentration than the global mean (138,966 µg/g) while Nest 3 has a lower concentration compared to the mean (72,752 µg/g).

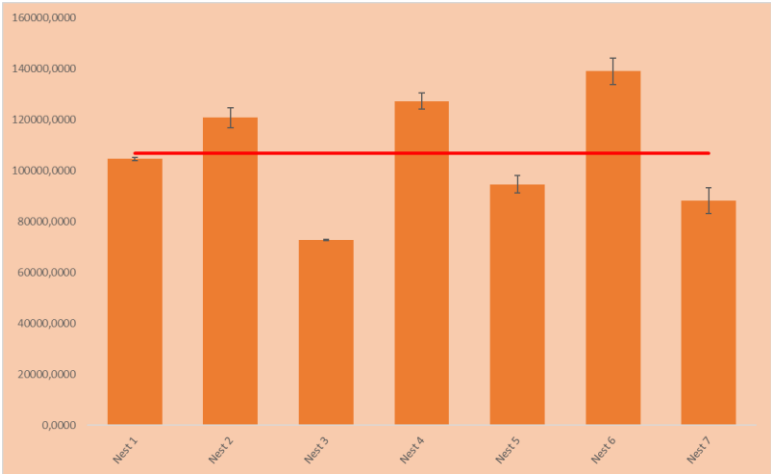

Figure 12.2: Ca concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- Fe: The global mean concentration is 198.9 µg/g. Nest 6 with 400.7 µg/g showed the highest concentration while Nest 5 has the lowest with 55.3 µg/g.

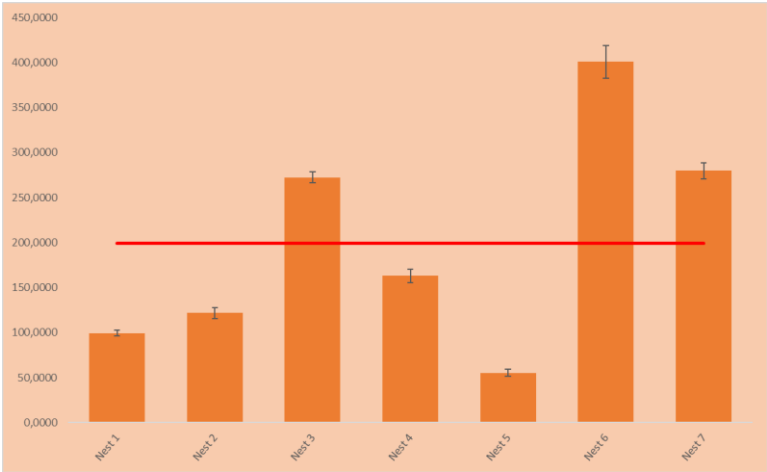

Figure 12.3: Fe concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- Co: The overall mean concentration is 852 µg/kg. Values higher than the mean were observed in Nest 3 with 1452 µg/kg and Nest 5 with a concentration of 337 µg/kg.

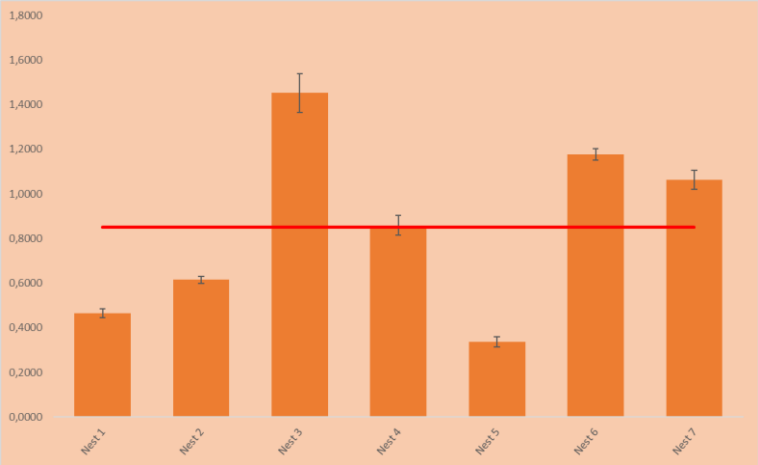

Figure 12.4: Co concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- As: The samples have a global mean of 283 µg/kg. Nest 3 makes an exception, having a concentration of 1366 µg/kg, which greatly exceeds the values of the other samples. Nest 1 (36 µg/kg), Nest 2 (42 µg/kg), Nest 4 (39 µg/kg) and Nest 5 (21 µg/kg), have lower concentrations compared to the mean.

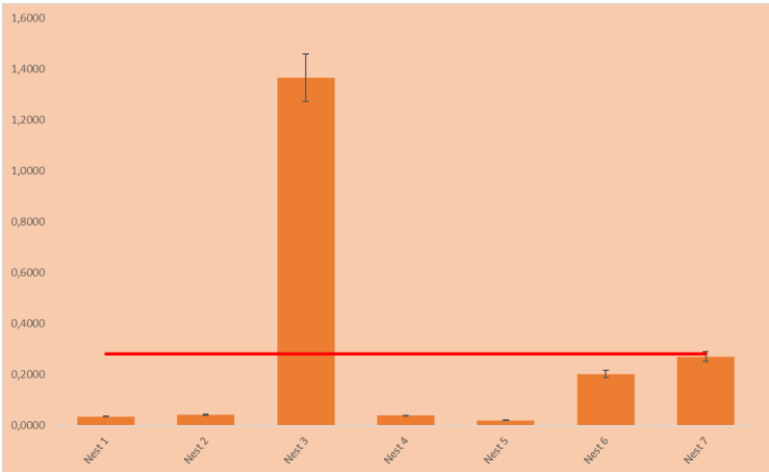

Figure 12.5: As concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- Se: The global mean concentration is 850 µg/kg. Nest 6 and Nest 7 have higher concentrations than the global mean, both with 1.4 µg/g. The lowest value comes from Nest 3 with 513 µg/kg.

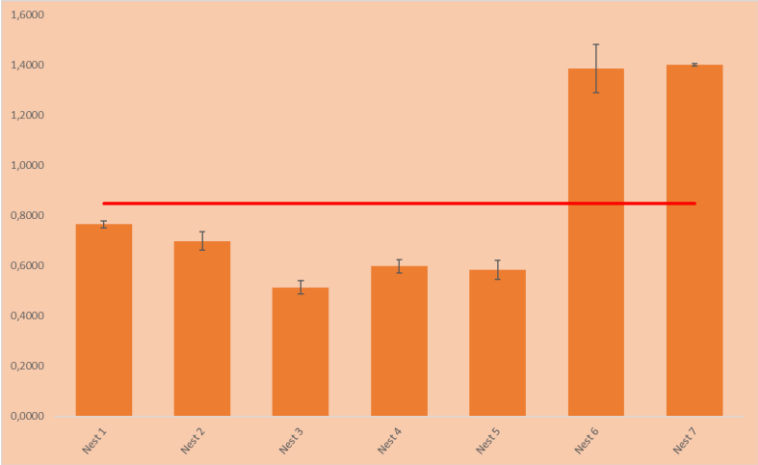

Figure 12.6: Se concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- Mo: The global mean for the element is 34 µg/kg. Some samples make an exception, with Nest 6 having a concentration higher than the general mean (101 µg/kg) and Nest 2, Nest 4 and Nest 5 having concentrations lower than the mean (20 µg/kg, 3 µg/kg and 4 µg/kg respectively).

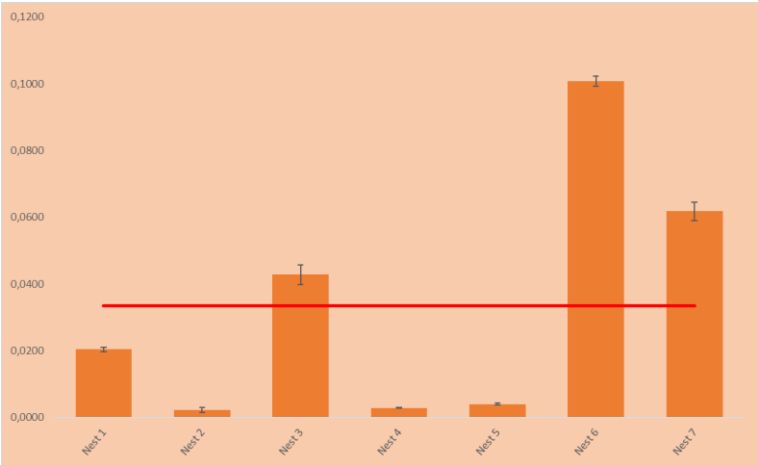

Figure 12.7: Mo concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- Cd: For this element, the mean concentration is 21 µg/kg. Nest 7 exceeds this value with a concentration 41 µg/kg, while Nest 3 has the lower concentration of 11 µg/kg.

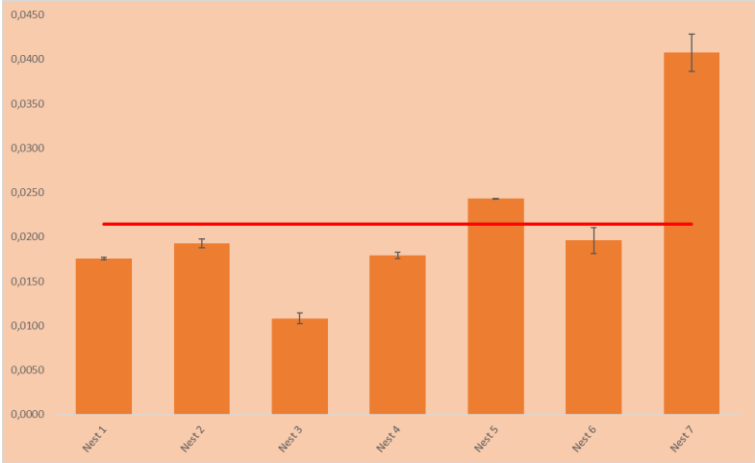

Figure 12.8: Cd concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- Sb: Mean concentration for the element is 4 µg/kg. Nest 7 with 12 µg/kg is higher than this mean while Nest 2 is lower with 0.8 µg/kg.

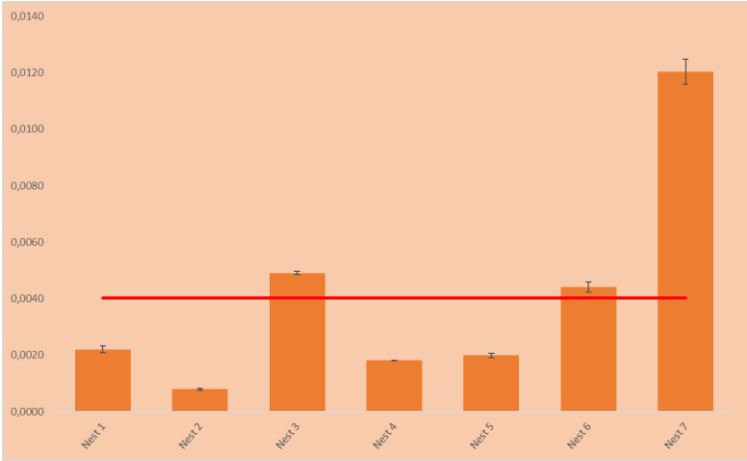

Figure 12.9: Sb concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- Hg: The samples have a mean concentration of 183 µg/kg. Nest 5 and Nest 6 have higher concentrations (370 µg/kg and 441 µg/kg respectively), Nest 1 and Nest 2 have the lowest (29 µg/kg and 19 µg/kg respectively).

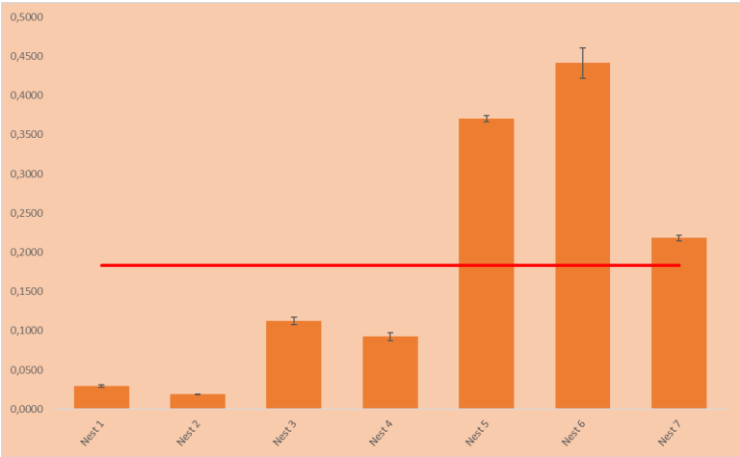

Figure 12.10: Hg concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- Pb: The global mean concentration is 24 µg/kg. Nest 7 (63 µg/kg) exceeds the mean while Nest 3 and Nest 4 share the lowest value of 8 µg/kg.

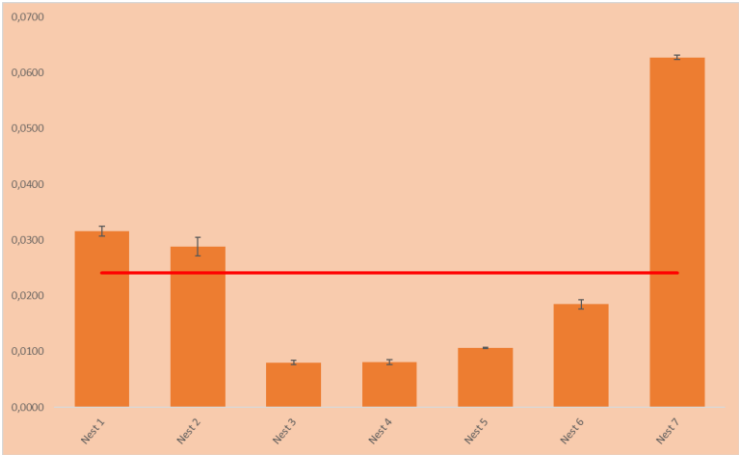

Figure 12.11: Pb concentrations in egg shells of C. alexandrinus. The values are expressed in µg/g. The red line indicates the global arithmetic mean.

- **Other species (feathers)**

In general, as the samples come from different species, it is noted that concentrations have really wide ranges.

- Al: The species with the highest concentrations are *T. sandvicensis* (TS) and *P. carbo sinensis* (PCS) both with piscivorous diet. For PCS, the mean is 1121.9 μg/g, and the samples do not show variability; in contrast, for TS, a great variability was observed (2459.7 μg/g, 1579.2 μg/g and 858.4 μg/g, respectively). The lower deposits come from *P. colchinus* (PC) and *C. ridibundus* (CR), birds with mixed diet but not apical in the respective trophic webs. For CR the mean is 42.7 μg/g, whilst for PC, the mean is 50.6 μg/g; in both cases samples do not show much variability. As concern *E. garzetta* (EG), the mean is 228.8 μg/g, but the samples do show some variability, since an individual showed a value of 98.3 μg/g. For *I. melanocephalus* (IM), the mean is 342.7 μg/g, and the samples show great variability between themselves (607.7 μg/g and 77.7 μg/g). In *T. Alba*, the mean Al deposited is 267.6 μg/g. The deposit gradient between species can be summarized as follows: TS > PCS > IM > TA > EG > CR > PC.

- Ca: Three groups may be identified on the basis of mean Ca content in the feathers. TS (1500.6 μg/g), PCS (1354.6 μg/g) and IM (1401.8 μg/g) are the species characterized by higher deposit for this element. There is no relevant connection between these species, with the exception of TS and PCS which are both piscivorous. The lowest deposits come from the mostly granivorous PC (158 μg/g). Intermediate values were observed for CR (514.4 μg/g), EG (598.8 μg/g) and TA (654.9 μg/g).

- Fe: a deposit gradient between species can be noticed (TS > PCS > CR > TA = IM > PC > EG). The species with the highest concentrations of the element are TS (1273.1 μg/g) and PCS (879.4 μg/g), both species with prevalence of piscivorous diet. The lowest concentrations are from EG (173 μg/g) and PC (269.7 μg/g). In intermediate position are IM (313 μg/g), TA (330.6 μg/g) and CR (411 μg/g). For this latter, a relevant variability between feathers has been observed.

- Co: There is a marked difference between piscivorous species and mixed-trophic and generalist species. Highest Co concentrations come from TS (578 μg/kg) and PCS (1106 μg/kg), whilst the lowest concentrations come from CR (81

µg/kg), EG (74 µg/kg), IM (79 µg/kg) and PC (68 µg/kg). There is low variability among feathers of the same species, with exception of PCS.

- As: a deposit gradient between species is visible. It can be summarized as IM > TS > CR > PCS > TA > EG > PC. The species with the highest concentrations are *TS* (1379 µg/kg) and *IM* (2039 µg/kg). The lowest concentrations come from EG (129 µg/kg) and PC (80 µg/kg). Within-species variability s generally low, except for the 2 gull species (IM and CR) and cormorants (PCS). For *T. Alba*, the mean is 606 µg/g. Since waterbirds tends to have As concentrations higher than terrestrial (PC and TA) and terrestrial/aquatic species (EG), accumulation seems to be linked to the aquatic food web.

- Se: there is a deposit gradient between species (PC > TS > CR > TA > EG > PCS > IM). *P. colchicus* (6440 µg/kg), *T. sandvicensis* (6020 µg/kg) and *C. ridibundus* (3631 µg/kg) are the species with the highest concentrations. There is no relevant connection between these species, highligthing that Se uptake is not dependent upon aquatic environment. The samples show great variability between themselves (5562 µg/kg, 6271 µg/kg, 1652 µg/kg and 1039 µg/kg). The lowest concentrations come from the piscivorous and mixotrophic species PCS (1193 µg/kg) and IM (544 µg/kg). For EG and IM the mean values are 1601 µg/kg and 544 µg/kg, respectively. The samples do not show much variability.

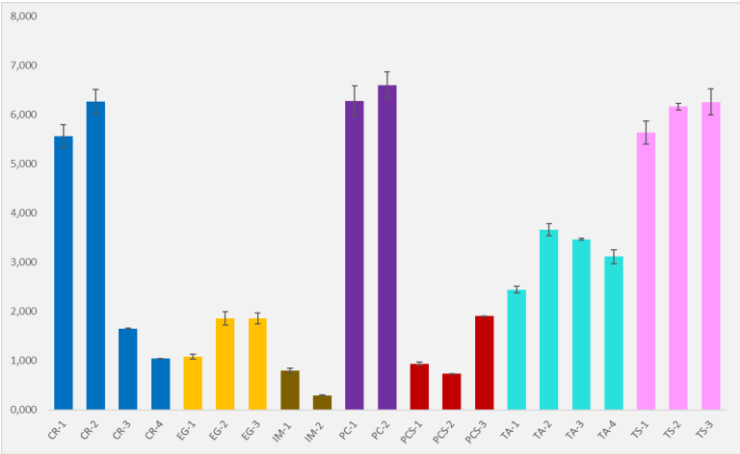

Figure 13.1: Se concentrations in feathers of various species analyzed in this study. The values are expressed in µg/g. Names of each sample correspond to the Latin name of the species to which they belong (CR = C. ridibundus; EG = E. garzetta; IM = I. melanocephalus; PC = P. colchicus; PCS = P. carbo sinensis; TA = T. alba; TS = T. sandvicensis).

- Mo: a deposit gradient between species can be noticed (TA > IM > TS > CR > PC > PCS > EG). The highest concentrations come from TA (773 µg/kg) and IM (736 µg/kg). The smaller deposits are from EG (73 µg/kg), although this species has carnivorous habits and may be considered apical to its trophic network. For *C. ridibundus*, the mean is 591 µg/kg. For *P. colchicus*, the mean is 516 µg/kg. For *P. carbo sinensis*, the mean is 343 µg/kg.

- Cd: a deposit gradient between species is clearly discernible. It can be summarized as follows: TS > PC > CR = IM > PCS > TA = EG. The species with the highest concentrations are TS (525 µg/kg) and PC (330 µg/kg). Lowest concentrations come from EG (65 µg/kg) and TA (74 µg/kg), although they may act as predators, in the respective trophic web. The 2 gull species showed intermediate concentrations of Cd (IM = 196 µg/kg; CR = 221 µg/kg) and both evidenced a relevant variability among individuals.

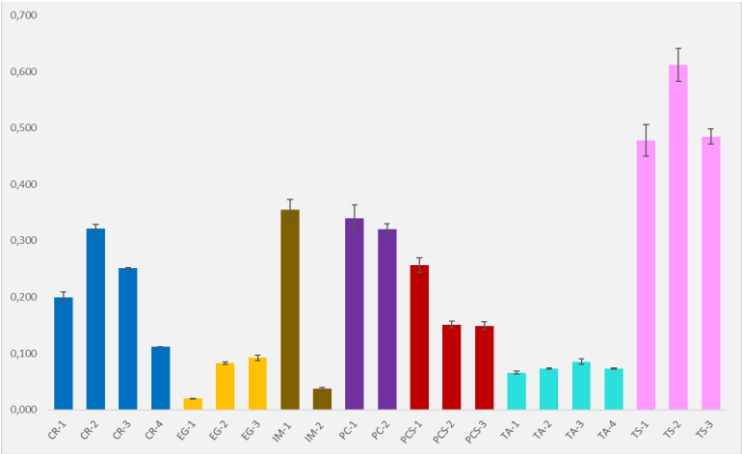

Figure 13.2: Cd concentrations in feathers of various species analyzed in this study. The values are expressed in µg/g. Names of each sample correspond to the Latin name of the species to which they belong (CR = C. ridibundus; EG = E. garzetta; IM = I. melanocephalus; PC = P. colchicus; PCS = P. carbo sinensis; TA = T. alba; TS = T. sandvicensis).

- Sb: there is a deposit gradient between species (TS > CR > TA > PCS > PC > EG > IM). TS (725 µg/kg) and CR (651 µg/kg) have the highest concentrations, whilst the lowest were observed in EG (84 µg/kg) and IM (24 µg/kg). As concern the other species, for PC the mean is 107 µg/kg, for PCS is 417 µg/kg. and for TA the mean is 487 µg/kg.

- Hg: an evident deposit gradient between species can be noticed (CR > IM > EG > TS > PCS > PC > TA). The species with the highest concentrations are the 2 gulls with mostly invertivorous feeding habits CR (4310 µg/kg) and IM (2044 µg/kg). The lowest concentrations were observed in the "terrestrial" species PC (203 µg/kg) and TA (95 µg/kg), although the latter can be considered to be apical to its trophic network.

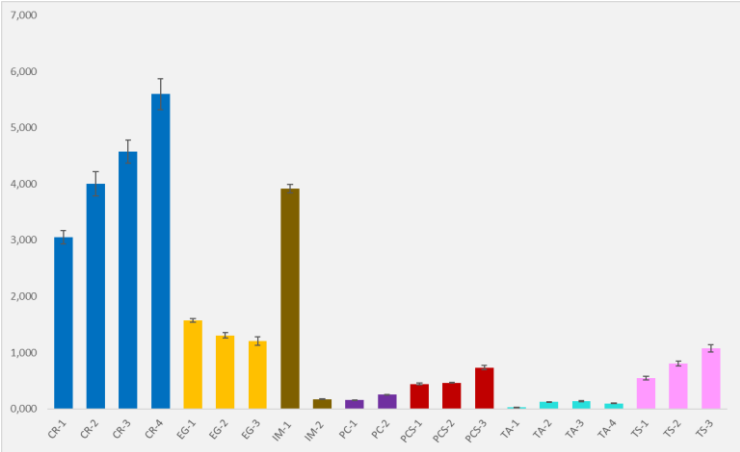

Figure 13.3: Hg concentrations in feathers of various species analyzed in this study. The values are expressed in µg/g. Names of each sample correspond to the Latin name of the species to which they belong (CR = C. ridibundus; EG = E. garzetta; IM = I. melanocephalus; PC = P. colchicus; PCS = P. carbo sinensis; TA = T. alba; TS = T. sandvicensis).

- Pb: The highest concentration for this element was found in the piscivorous PCS (181 µg/kg); it was at least 2 orders of magnitude higher than those observed in the other species, since the second highest value was observed in TS with 2.6 µg/kg. The lowest concentration was measured in PC (0.3 µg/kg). The gulls CR and IM, showed intermediate concentrations with mean values of 0.4 µg/kg in CR and 1.1 µg/kg. For *EG*, the mean is 0.7 µg/kg, whilst for TA the mean is 1.9 µg/kg.

5) DISCUSSION

- **Comparison among feathers**

Concentrations identified in the samples of this study are thought to reflect 2–3 weeks of dietary exposure. Samples of *C. alexandrinus* are compared with other species analysed in this study and with the results obtained in other studies on shorebirds (in particular those belonging to genus *Charadrius*, *Calidris* and *Pluvialis*) in wetland areas. Table 2 shows a comparison between the arithmetic means of these values, showing information about the corresponding species.

For Al, the mean concentration in feathers of *C. alexandrinus* (59.1 μg/g) is lower than values from other species, with the exception of *P. colchicus* (50.6 μg/g) and *C. ridibundus* (42.7 μg/g). Even concentrations found in other studies (Lucia et al., 2010) are higher than those found in feathers of *C. alexandrinus*. One sample of *C. alexandrinus* makes an exception since it has a value in between those found in *Calidris canutus* (107 μg/g) and in *P. squatarola* (96 μg/g) from Lucia et al. (2010). The highest concentrations come from the species *T. sandvicensis* (1632 μg/g) and *P. carbo sinensis* (1121 μg/g), both piscivorous species.

The mean concentration of Ca in feathers of *C. alexandrinus* (512 μg/g) is lower than values from other species, with the sole exception of *P. colchicus* (158 μg/g). Information obtained from a previous thesis show that the average concentration of the element for the species *Anas crecca* is 631 μg/g, which is higher than the value found in *C. alexandrinus*. However, two samples make an exception, since their concentrations are higher than this value. The highest concentrations found for the element come from the species *I. melanocephalus* (invertivorous) with 1401 μg/g, *T. sandvicensis* (piscivorous) with 1500 μg/g and *P. carbo sinensis* (piscivorous) with 1354 μg/g.

For Fe, the mean concentration in feathers of *C. alexandrinus* (183.6 μg/g) is lower than values from other species, with the sole exception of *E. garzetta* (173 μg/g). This concentration is higher than the one found in *C. canutus* (160 μg/g) from Lucia et al. (2012) but lower than the one found in *C. alpina* (262 μg/g) from the same study. Five samples of *C. alexandrinus* exceed their mean concentration with one sample having an

higher concentration than those found for *C. alpina* in Lucia et al. (2012). The highest concentrations come from the species *T. sandvicensis* (1273 μg/g) and *P. carbo sinensis* (879.4 μg/g).

For Co, the mean concentration in feathers of *C. alexandrinus* (48 μg/kg) is lower than all values from other species. Even concentrations found in other studies (Lucia et al., 2014) are higher than those found in feathers of *C. alexandrinus*, since the lower value found in literature is 140 μg/kg for *C. alpina* (Lucia et al., 2014). The highest concentrations come from *P. carbo sinensis* (piscivorous) with 1106 μg/kg, *T. sandvicensis* (piscivorous) with 578 μg/kg and *T. alba* (predator) with 196 μg/kg.

For Al, Ca, Fe and Co the exposure of *C. alexandrinus* seems to be negligible, irrespective of the fact that thresholds for detrimental effects are lacking for feathers.

The mean concentration of As in feathers of *C. alexandrinus* (219 μg/kg) is lower than values observed in the other analyzed species, except for *P. colchicus* (80 μg/kg) and *E. garzetta* (129 μg/kg). Concentrations found in other studies (Lucia et al., 2010, 2014, 2012) are all higher than those found in feathers of *C. alexandrinus*, with the lower value found in literature being 1190 μg/kg (*C. canutus*) from Lucia et al. (2012). Mean concentrations of *I. melanocephalus* (2039 μg/kg) and *T. sandvicensis* (1379 μg/kg) are the highest concentrations found in this study. Thus, for As, the exposure of *C. alexandrinus* is higher than that of birds more closely related to the terrestrial habitat, but lower as compared with other waders and seabirds. In any case, due to the lack of a toxicity threshold, it is not possible to determine if exposure to As may pose a risk for *C. alexandrinus*.

For Se, the mean concentration in feathers of *C. alexandrinus* (3490 μg/kg) is higher than most of the other analyzed species, except for *P. colchicus* (6440 μg/kg), *T. sandvicensis* (6020 μg/kg) and *C. ridibundus* (3631 μg/kg). Concentrations found in other studies concerning waders (Lucia et al., 2014) are usually higher than this mean from *C. alexandrinus*, with the exception of the value found for *Charadrius nivosus* in Oklahoma (2700 μg/kg) in Ashbaugh et al. (2018) and for *P. squatarola* (1900 μg/kg) in Lucia et al. (2010). Nevertheless, it should be noted that *C. alexandrinus*, as most of the other waders, tends to have higher Se concentration in the feathers as compared with other waterbirds. Moreover, four specimens of *C. alexandrinus* have concentrations in

between those found in *C.nivosus* in Texas (6450 μg/kg) from Ashbaugh et al. (2018) and in *C. alpina* (4700 μg/kg) from St. Clair et al. (2015) and two showed concentrations in between this last value and the one found in *C.nivosus* in New Mexico (4090 μg/kg) from Ashbaugh et al. (2018). Even though the mean concentration is lower than the threshold value of 5000 (Ashbaugh et al., 2018; Burger et al., 2015; St. Clair et al., 2015)., two samples of *C. alexandrinus* exceed this values, evidencing a possible exposure to deleterious levels of Se for part of the local population. Similarly, also *P. carbo sinensis* (6440 μg/kg) and *T. sandvicensis* (6020 μg/kg) mean Se concentrations exceed the toxicity threshold of 5 μg/g (Ashbaugh et al., 2018; Burger et al., 2015; St. Clair et al., 2015).

For Mo, the mean concentration in feathers of *C. alexandrinus* (170 μg/kg) is lower than most of the other analyzed species, with the exception of *E. garzetta* (73 μg/kg). Comparison values could not be identified due to lack of element-related studies. The highest concentrations from this study come from the species *T. alba* (predator) with 773 μg/kg, *T. sandvicensis* (piscivorous) with 595 μg/kg and *I. melanocephalus* (invertivorous) with 736 μg/kg.

The mean concentration of Cd in feathers of *C. alexandrinus* (202 μg/kg) is higher than those measured in *T. alba, I. melanocephalus, E. garzetta* and *P. carbo sinensis*, but lower than that measured in *T. sandvicensis* (525 μg/kg), *P. colchicus* (330 μg/kg) and *C. ridibundus* (221 μg/kg). Moreover, *C. alexandrinus* has a higher concentration than the one found in *P. apricaria* (100 μg/kg) by Lucia et al. (2010) but lower than that found in *C. mongolus* (380 μg/kg) from Kim & Koo (2008). One specimen of *C. alexandrinus* has a Cd concentration in between those found for the same species (910 μg/kg) and for *C. tenuirostris* (500 μg/kg) from Kim & Koo. The mean concentration of *T. sandvicensis* (525 μg/kg) is the highest found in this study. No sample in this study reached or exceeded the toxicity threshold of 2000 μg/kg (Burger & Gochfeld, 2000); nevertheless, since effects in waterbirds may occur at level of 100 μg/kg, it cannot be excluded that local species could be exposed to detrimental Cd concentrations.

The mean concentration of Sb in feathers of *C. alexandrinus* (100 μg/kg) is lower than most of the species, except for *E. garzetta* (84 μg/kg) and *I. maelanocephalus* (24 μg/kg). Comparison values could not be identified due to lack of element-related

studies. The highest concentrations come from the species *T. Sandvicensis* (piscivorous) with 725 μg/kg and *C. ridibundus* (invertivorous) with 651 μg/kg.

Concentrations of Hg found in *C. alexandrinus* were almost all superior not only to samples from other species, but also to the general toxicity threshold for feathers (Burger et al., 2015; Hargreaves et al., 2010; St. Clair et al., 2015), with a mean concentration of 8710 μg/kg. It also exceeds all comparison values found for the element in literature, since the maximum concentration found for *Charadriidae* is 5500 μg/kg in *C. alpina* (Goede et al., 1989). These data evidenced that for *C. alexandrinus* the risk due to exposure to Hg is significant and severe and should be taken into consideration also for the conservation of the species at local and national level.

For Pb, the mean concentration in feathers of *C. alexandrinus* (816 μg/kg) is lower than most of the species, except for *E. garzetta* (656 μg/kg), *C. ridibundus* (433 μg/kg) and *P. colchicus* (287 μg/kg). Concentration from *C. alexandrinus* is lower than the comparison values found in literature, with the exception of the one found in *C. canutus* (770 μg/kg) from Lucia et al. (2012). Mean value is also lower than the concentration observed in *P. apricaria* (2700 μg/kg) by Lucia et al.(2010) and in *C. alpina* (1120 μg/kg) by Lucia et al. (2014). The highest concentrations come from the species *P. carbo sinensis* (piscivorous), which reaches a concentration of 180.9 μg/g, exceeding the toxicity threshold of 4 μg/g (Ashbaugh et al., 2018; Hargreaves et al., 2010; St. Clair et al., 2015).

Regarding the statistical analysis, significant differences between males and females are considered only for As. This suggests that the causes are not related to the contamination of the site, rather in foraging differences between the two sexes or in differences in metabolism. A similar result was found in Lucia et al. (2010), as only As was influenced by sex and the species *Anser anser* showed higher As in the liver and in the feathers compared to the male birds. However, this species can not be related to those considered in this study as it belongs to a different genus which also has a different type of diet.

- **Comparison between egg shells and feathers (females)**

Only few studies related to the trace element analysis have been found on this matrix, so there are comparison values only for Co, Se, Cd, Hg and Pb (Lam et al., 2005, 2004).

For Co, six egg shell samples (Nest 3, Nest 6, Nest 7, Nest 4, Nest 2 and Nest 1) exceed values found in other studies, surpassing the concentration of 0.356 μg/g found in the study performed by Lam et al. (2004). Levels in egg shells are higher than those in feathers, with a maximum value of 1,452 μg/g (Nest 3). For feathers it is 0.086 μg/g (CA-f1).

Se concentrations detected in egg shells are all lower than those found in other studies (Lam et al., 2005, 2004), which arrive to a maximum concentration of 15.58 μg/g.

For Cd, concentrations in egg shell samples are all lower than concentrations found in feathers. All samples exceed the values found in other studies (Lam et al., 2005, 2004). There is limited evidence that Cd concentrations in egg shells may be high and the possible use of them to monitor Cd levels has not yet been explored (Furness & Greenwood, 1996).

Five egg shell samples (Nest 6, Nest 5, Nest 7, Nest 3 and Nest 4) exceed Hg concentrations found in other studies (Lam et al., 2004), which reach the concentration of 0.071 μg/g. Two samples (Nest 1 and Nest 2) make an exception as both are in between 0.056 μg/g and 0.004 μg/g (Lam et al., 2005, 2004). No egg shell sample exceed the threshold of 0.5 μg/g. However, this value takes into account the whole egg and the threshold has been rarely exceeded in literature (Furness & Greenwood, 1996).

For Pb, only one egg shell sample (Nest 7) places in between the highest concentrations found in literature, which are 0.152 μg/g and 0.06 μg/g (Lam et al., 2005, 2004). One samples is in between 0.06 μg/g and 0.03 μg/g (Lam et al., 2005, 2004) while the other samples are lower than this last concentration. No egg shell sample reaches the value of 0.4 μg/g identified in the study by Furness & Greenwood (1996).

6) CONCLUSIONS

In general, concentrations obtained from feathers of *C. alexandrinus* are lower than those from feathers of the species used as comparison material and those found by similar studies. Only Hg makes an exception, as they are found in greater quantities in the samples of *C alexandrinus* feathers and largely exceeded the other values of concentration.

These values also exceeded the toxicity thresholds considered for feathers, making clear that the conditions of this species in the study area are compromised by the presence of this element, whose impact in the food web and whose presence in sediments and fish fauna have already been proved in recent studies in the Venetian Lagoon (Dominik et al., 2014; Picone et al., 2017, 2016; Zonta et al., 2018).

A hypothesis is that preys of *C. alexandrinus*, including polychaetes, amphipods, small bivalves and other molluscs, are Hg accumulators in the benthic food web of shallows and canals of the Lagoon, and their body burden is transferred in *C. alexandrinus* when they are preyed. Furthermore, a couple of feather samples of *C. alexandrinus* (CA-f2 and CA-f4) have exceeded the toxicity threshold for Se while two more (CA-f3 and CA-m1) are very close to this value, making it plausible that the element is bioavailable in their feeding grounds, and deserves carefull monitoring (together with Hg) in the future.

Speaking of the other species analyzed in this study, the highest concentrations for almost all the elements have been identified mainly in feathers collected from piscivorous *T. sandvicensis* and *P. carbo sinensis*. The samples of the former have all exceeded the toxicity threshold for Se while, for the latter, values of Pb are 60 times higher than its toxicity threshold. Samples for these species come from different areas respectively. However, they are considered piscivorous species and they both winter and reproduce (especially *T. sandvicensis*) in the Venetian lagoon. Furthermore, SCIs IT3250031 and IT3250030 share the same habitat codes and are included in the same SPA (IT3250046 “Venice Lagoon”).

This indicates not only that the cause of these high concentrations may be the aquatic and lagoon environment, but the extent of this cause covers both sides of the Venetian Lagoon (from Chioggia to Cavallino-Treporti). Another hypothesis that should be considered for these values in terns and cormorants is that most of the concentrations found in these species have been accumulated outside the Venetian Lagoon, in places likely to be contaminated by these elements. This can be considered a risk factor as it would lead to an increase in average concentrations of these elements in the Lagoon and therefore their bioavailability for the trophic web, increasing the risk of biomagnification.

For what can be seen from the comparison between egg shells and feathers, the concentrations found in egg shells are all much inferior to those found in feathers. The only elements that are beyond these considerations are Co and Ca. For the former, the reference values found in the study of Lam et al. (2004) were exceeded by most egg shell samples. However, no correspondence of these concentrations with harmful effects has been proven. For these reasons, further research must be taken for this element (we exclude Ca for reasons already mentioned) before taking into account these values and determine their effects, as no comparison values were found for insufficient documentation related to the topic.

Given the following conclusions, it is necessary to manage the bioavailability of the most dangerous elements for these species (in particular Hg) not only for the health of the most sensitive ornithic species such as *C. alexandrinus* and *T. sandvicensis* (considered respectively endangered and vulnerable species in the IUCN Italian Red List of Threatened Species), but also to improve the conditions of an area protected by the Natura 2000 network.

matrix	source	species	n	Al	Ca	Fe	Co	As	Se	Mo	Cd	Sb	Hg	Pb
FEATHERS	THIS STUDY	*Charadrius alexandrinus*	5	62,14 ± 23,4 (male)	517,32 ± 14,6 (male)	184,55 ± 18,2 (male)	0,05 ± 28,6 (male)	0,1 ± 31 (male)	3,94 ± 8,4 (male)	0,18 ± 21,4 (male)	0,18 ± 35,1 (male)	0,1 ± 11,7 (male)	9,21 ± 18,9 (male)	1,06 ± 21,1 (male)
			8	57,16 ± 8 (female)	508,68 ± 7,7 (female)	183,03 ± 9,2 (female)	0,05 ± 21,4 (female)	0,29 ± 25,8 (female)	3,21 ± 21,1 (female)	0,16 ± 18 (female)	0,22 ± 30,8 (female)	0,1 ± 13,2 (female)	8,4 ± 14,5 (female)	0,67 ± 19,5 (female)
		Tyto alba	4	268 ± 32,4	655 ± 23,4	331 ± 15,9	0,2 ± 38,5	0,61 ± 29,1	3,17 ± 8,4	0,77 ± 7,4	0,07 ± 5,3	0,49 ± 33,6	0,1 ± 25,5	0,99 ± 34,6
		Chroicocephalus ridibundus	4	42,74 ± 12,2	514 ± 40	411 ± 58,2	0,08 ± 38,7	0,92 ± 33,9	3,6 ± 36,7	0,59 ± 9,7	0,22 ± 20	0,65 ± 14,4	4,31 ± 12,4	0,43 ± 47,3
		Egretta garzetta	3	229 ± 30	599 ± 19,2	173 ± 20,4	0,07 ± 30,8	0,13 ± 3,6	1,6 ± 16,2	0,07 ± 25,1	0,07 ± 34,8	0,08 ± 27,6	1,36 ± 8,1	0,66 ± 24,8
		Ichtyaetus melanocephalus	2	343 ± 77,3	1402 ± 77,5	313 ± 65,7	0,08 ± 78,1	2,04 ± 77,1	0,54 ± 46	0,74 ± 9,8	0,2 ± 81,1	0,02 ± 12,5	2,04 ± 91,6	1,09 ± 77,4
		Phalacrocorax carbo sinensis	3	1122 ± 30,4	1355 ± 20,8	879 ± 27,1	1,11 ± 25,2	0,86 ± 24,9	1,19 ± 30,4	0,34 ± 28,2	0,19 ± 19,2	0,42 ± 45,1	0,55 ± 17,3	181 ± 19,1
		Phasianus colchicus	2	50,57 ± 23,1	158 ± 27,2	270 ± 16,4	0,07 ± 32,4	0,08 ± 39,6	6,44 ± 2,5	0,52 ± 19,2	0,33 ± 3	0,11 ± 41,8	0,2 ± 24,1	0,29 ± 11,5
		Thalasseus sandvicensis	3	1632 ± 28,4	1500 ± 25,3	1273 ± 30,5	0,58 ± 28,3	1,38 ± 42,7	6,02 ± 3,2	0,6 ± 24,6	0,52 ± 8,3	0,73 ± 19,9	0,81 ± 18,8	2,59 ± 10,1
	KIM & KOO (2008)	*Charadrius alexandrinus*	5	-	-	-	-	-	-	-	0,91	-	-	9,84
		Charadrius mongolus	2	-	-	-	-	-	-	-	0,38	-	-	20,7
		Calidris alpina	6	-	-	-	-	-	-	-	1,13	-	-	14,8
		Calidris tenuirostris	10	-	-	-	-	-	-	-	0,5	-	-	20,8
	LUCIA ET AL (2010)	*Calidris canutus*	3	107 ± 50,1	-	-	-	1,2	8,7	-	0,06 ± 0,01	-	2,09 ± 0,6	3,31 ± 1,22
		Pluvialis squatarola	8	96 ± 44,6	-	-	-	2,5	1,9	-	0,09 ± 0,07	-	1,18 ± 0,44	6,52 ± 9,93
		Pluvialis apricaria	1	-	-	-	-	-	-	-	0,1	-	-	2,7
	LUCIA ET AL (2012)	*Calidris canutus*	15	-	-	160 ± 16	-	1,19 ± 35,6	3,7 ± 14,7	-	0,03 ± 43	-	1,26 ± 21,5	0,77 ± 12,7
	LUCIA ET AL (2014)	*Calidris alpina*	28	-	-	262 ± 358	0,14 ± 0,15	2,46 ± 2,24	9 ± 4,6	-	0,03 ± 0,02	-	1,83 ± 1,48	1,12 ± 0,89
	ST CLAIR ET AL (2015)	*Calidris alpina pacifica*	59	-	-	-	-	-	4,7 ± 0,5 / 10,4 ± 0,9	-	-	-	2,6 ± 0,2	0,9 ± 0,1
	ASHBAUGH ET AL (2018)	*Charadrius nivosus*	30	-	-	-	-	-	6,45 ± 1,23 (Texas)	-	-	-	-	-
			36	-	-	-	-	-	2,7 ± 0,22 (Oklahoma)	-	-	-	-	-
			7	-	-	-	-	-	4,09 ± 1,67 (New Mexico)	-	-	-	-	-
EGGS	THIS STUDY	*Charadrius alexandrinus*	7	21,34 ± 15,7 (eggs)	106721 ± 8,3 (eggs)	198,9 ± 23,3 (eggs)	0,9 ± 17,9 (eggs)	0,28 ± 65,2 (eggs)	0,85 ± 16,9 (eggs)	0,03 ± 42 (eggs)	0,02 ± 16,5 (eggs)	0,004 ± 35,9 (eggs)	0,18 ± 34,4 (eggs)	0,02 ± 30,8 (eggs)
	LEM ET AL. (2004)	*Nycticorax nycticorax*	9	-	-	-	0,36 ± 0,02	-	8,16 ± 0,2	-	0,008 ± 0,002	-	0,06 ± 0,01	0,03 ± 0,01
		Egretta garzetta	9	-	-	-	0,36 ± 0,04	-	7,59 ± 0,7	-	0,006 ± 0,002	-	0,07 ± 0,03	0,15 ± 0,2
	LEM ET AL. (2005)	*Nycticorax nycticorax*	9	-	-	-	0,36 ± 0,02	-	8,16 ± 0,2	-	0,008 ± 0,002	-	0,06 ± 0,01	0,03 ± 0,01
		Egretta garzetta	9	-	-	-	0,36 ± 0,04	-	7,59 ± 0,7	-	0,006 ± 0,002	-	0,07 ± 0,03	0,15 ± 0,2
		Sterna anaethetus	9	-	-	-	0,002 ± 0,001	-	15,58 ± 1,9	-	0,002 ± 0,001	-	0,004	0,06 ± 0,04

Table 2: Comparison of the arithmetic means (with SE) of the samples (feathers and eggs) analyzed this study with the values found in studies on similar species. The values are expressed in µg/g. SE for the values analyzed in this study are related to the distance of each individual value with respect to the arithmetic mean reported in the table. Arithmetic means from Lucia et al 2012 and Lucia et al. 2014 reported the standard deviation (SD) instead of SE.

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APPENDIX

CASE OF STUDY: ENVIRONMENTAL POLLUTION IN THE AREA OF KEMPEN~BROEK (NL)

During a period of study abroad at Utrecht University, I had the opportunity to carry out a research project with the supervision of prof. dr. Martin Wassen focused on the determination of a possible trace metal pollution present in the area of Kempen~Broek, a natural park of 25000 hectares across the borders of Dutch and Belgian Limburg and North Brabant (Netherlands). Since the area in question presents various SCIs and SPAs included in the Natura 2000 network, it can be considered of similar importance to the Venetian Lagoon and this inferential study can be a preliminary assessment and starting point for a more in-depth analysis using the methods explained in this thesis.

- ## Introduction

Border Park Kempen~Broek is characterized by a chain of nature reserves and is located on the territory of the municipalities of Cranendonck, Nederweert and Weert in the Netherlands and Bocholt, Bree, Kinrooi and Maaseik in Belgium (Grenspark Kempen~Broek, 2014).

Originally it was a drenched area, where the seepage water from the Kempens Plateau comes to the surface and where several streams flow from the source on the Kempens Plateau to the Maas. As a result, the area once consisted of extensive marshes that lay between higher and dry sands (Natuurmonumenten, 2018).

Man has been exploiting the landscape for the past centuries in many ways and at different stages. In 1839 the area became a border region between Belgium and the Netherlands, in which people from both countries drove trade. This was not always legal and smuggling there was rampant. Bit by bit the area was mined for agriculture but later on parts were left to their fate.

This created a particularly varied mosaic of landscapes: marshes, drift dunes, forests, heathlands, stream valleys and agricultural areas (Grenspark Kempen~Broek, 2014).

Furthermore, it is possible to notice the presence of different environments and landscapes such as swamp forests, open water and streams, but also pieces of heather, hayfields and grasslands.

In the central, wettest part and in the stream valleys the various nature reserves are still present. In here it is possible to observe a great variety of local fauna: several areas are grazed by Tauros cattle and, in addition to dragonfly and red deer, these are also depicted as a symbol of the border park (Natuurmonumenten, 2018).

Various sub-areas are protected as a Natura 2000 site in Europe: the habitat directive areas Abeek with adjacent wetlands, Itterbeek with Brand, Jagersborg and Schootsheide and Bergerven and the birds directive area Hamonterheide, Hageven, Buitenheide, Stramprooierbroek and Mariahof.

Several managers are now trying to forge these areas together into an uninterrupted, cross-border green north-south axis. This green axis is embedded in a 'shell' of cultural landscapes, some of which have retained their small-scale character while others have been intensively cultivated and arranged to meet the requirements of large-scale, contemporary agriculture (Grenspark Kempen~Broek, 2014). Around 40 parties work together to maintain, expand and experience this region with its natural identity and natural values across the border. In this way they want to ensure that future generations like to stay, visit and enjoy this area.

In the proximity of the natural park, metal smelting activities (in particular Zn) have been carried out over the years. In fact, about 20 kilometers away from Kempen~Broek, a factory that produces Zn is present since 1973. The process used in this factory produces an insoluble ammonium salt compound, called jarosite [$KFe^{+3}{}_3(SO_4)_2(OH)_6$], that can no longer be processed and needs to be stored in special basins (Digibron, 1982).

The company that owns this factory is called Budelco and is known as one of the largest producers of chemical waste in the Netherlands. The company produces 120000 tonnes of jarosite annually, which contains high concentrations of elements such as Zn, Cd, Pb and As. This has been stored since 1973 and all these years the company has promised to look for processing possibilities, without concrete result (Calje, 1990).

The Kempen area has previously suffered from Cd and Zn pollution in the soil and the organization Natuurmonumenten has pointed out that the accumulation of trace elements in the upper soil layer in nature reserves is much greater than, for example, in agricultural land (Copius Peereboom-Stegeman & Copius Peereboom, 1989; Sterk, 1990). As the factory is located near the studied area, it can be considered a potential source of pollutants and, therefore, a danger for the conservation of the natural park.

The environmental science group of the Copernicus institute has access to a station in Kempen~Broek managed by Natuurmonumenten.

This organization manages very diverse nature reserves in this area, such as the Laurabossen, the Wijffelterbroek and the Kettingdijk. The latter are remnants of an extensive pelt marsh where special plants such as narrow prickly fern, snake root and ordinary solomon's seal grow and rare animals such as alpine salamander are present (Natuurmonumenten, 2018). Natuurmonumenten still manages a few other nature reserves, such as Budelerbergen and the Loozerheide and Weerter, where the station is located.

Regular inventories of breeding birds have been collected from this station. These showed an abundant presence of birds in the area. However, the data have not been analyzed in relation to environmental quality. This is essential to understand the current situation of the area and could lead to considering the presence of a possible variation of environmental conditions that has led to a change in the population size of local breeding birds over the years.

The aim of this study is to carry out a preliminary analysis of these environmental conditions through the inventories of breeding birds collected in the station and considering the main trends of local bird populations.

These have been compared with those at national level to highlight the difference between trends of local bird population and their national average.

Assuming that the possible decrease in local bird populations can be caused by the waste elements produced by the factory, a further comparison of the inventories is carried out with trends of provincial areas in which trace element pollution is likely to be present.

As the area in question has already been subject to conservation activities in the past and there is a potential source of pollution nearby, further contamination would undermine its already precarious stability.

Furthermore, analysis of the individual species present in the inventories from a foraging behavior point of view is taken to underline the health status of the most sensitive species.

- ## Materials and methods

To analyze the breeding bird communities of the area to outline a possible anthropic impact, it is necessary to gather inventories of the different species present in the area and process them in order to be compared with national inventories. This comparison and the analysis of the trends obtained from the inventories can outline the growth or decrease of the different communities over time. This can be used to notice possible deviations from their standard trend and to formulate hypotheses regarding the possible causes.

As these deviations can be generated by various phenomena such as climate change or land-use, we must analyze these inventories carefully in order to exclude other causes and focus on the possible pollution of the area. To be able to associate it more effectively with this, it is necessary to make a further comparison between the trends of Kempen~Broek inventories and those collected in other areas of the Netherlands where environmental pollution is more likely to be present. The similarities that can be found by comparing these trends can lead trace elements and waste release to have a greater impact than the other possible causes of deviation.

To understand the sensibility of local bird species to these trace elements, a specific analysis must be carried out. From the study of the different characteristics of these species (in the research only foraging behavior is considered) we may identify and outline which species are more affected to the causes of population decrease.

By comparing the trends developed from the data collected at the station with those describing trends at national level, a gradual decrease in the populations of breeding birds in the area over the years should be identified.

Coincidences and similarities with trends found in provincial trends should lead us to hypothesize the presence of environmental pollution as a probable factor of interference for this species in this area.

From the division and subsequent comparison of the different breeding bird species present in the inventories according to their foraging behavior, some species (like insectivorous and piscivorous ones) should be considered as the most sensitive to anthropic impact.

Hence, it is possible to show that these species can potentially assimilate the source of pollution (probably produced and released by the factory) in much higher quantity than others because of their sensitivity and position in the trophic web.

Data on the inventories of breeding birds in Kempen~Broek were collected by Natuurmonumenten. These were made through observations done by volunteers, ecologists working for the organization and ecologists hired by the same. As most of the observations belong to the species-groups plants and birds, the latter were extracted from those.

Bird data is collected according to the Dutch Breeding Bird Monitoring Program (BMP), started from the collaboration between Statistics Nederlands and Sovon and where all common and scarce breeding birds in the Netherlands are covered.

Between March and July all plots are visited 5-10 times. Size of study plots, as well as exact number, timing and duration of visits, depend on habitat type and species coverage. All birds with territory- or nest-indicative behavior are recorded on field maps and, at the end of the season, species-specific interpretation criteria are used to determine the number of territories per species. After a first check by the project coordinator at Sovon, Statistics Netherlands performs standardized checks using computer routines to detect possible errors (van Turnhout et al., 2008). The results of this analysis are called territoria, points where the bird intend to reproduce but it is not required that the birds really did reproduce.

The program consists of five modules, focused on either all species or specified groups or habitats: rare species, birds of prey, pasture and field birds, special types and all types (Vergeer et al., 2016). Two of these modules were used to collect data for Kempen~Broek:

- BMP-A: Inventory of all species present in a counting area of 10 to 250 ha. It becomes at least seven (bird-poor area) to twelve times (very bird-rich area) completed, usually around sunrise and at least once at night.
- BMP-B (or BSP): Inventory of several dozen mainly scarce species in a counting area of 30 to 300 ha. The counting area is completed at least six (bird-poor) to ten times (bird-rich) in full. This happens especially in the early morning, but also at other times of the day.

Natuurmonumenten provided these inventories in two different formats: as a shapefile (GIS), in which the geographical position of each territoria is recorded for each year of observation, and as an excel document, in which the counts for each species are present for each year of observation.

The former is used in this research to find a possible change in the geographical distribution of each species in the area. The procedure is performed using the QGIS program.

The latter is used to outline the change in size of the population of each species per year and comparing them with trends at the national level and provincial level.

To be able to derive the best trends possible from these data, the TRIM program is used to calculate missing counts using estimation and produce annual indices using the Loglinear Poisson regression (GLM) (Pannekoek & Strien, 2005).

The national and provincial inventories are provided by Sovon Dutch Centre for Field Ornithology (Sovon & CBS, 2017). This private organization coordinates the monitoring of wild bird populations in the Netherlands and carries out research on the ecology and demography of bird populations.

The data consists in a set of indexes based on observations of more than 8000 volunteers and trained field biologists. The method and the procedure used to collect

these observations are the same used in the inventories provided by Natuurmonumenten. The indexes are calculated from the results of the Meetnet Breeding Birds and show developments in breeding bird stocks since 1990. One of the years (usually the first) of the series is set at 100, so the figures indicate the development compared to this so-called base year. The assessment of the trend is based on that of all monitoring projects that are carried out within the framework of the Network Ecological Monitoring. The trends were calculated using the TRIM program.

To be able to compare them with national and provincial indexes, data on breeding birds collected in Kempen~Broek have been selected and isolated from other species. Table A-1 shows the species selected from the inventories that were analyzed in this study. Then, territoria for each species were sorted by year and observation site and processed through the TRIM program to obtain the missing counts.

Using the same procedure used for national inventories, the indexes for each species were calculated by the total sum of all sites for a year divided by the total of the first year of the set, which is set at 100. In this way, each year is indicating the development of the total population of a species compared to the base year (usually the first year available).

The shapefile provided has undergone similar processing: using the QGIS program, unnecessary data has been removed and sorted by species. Later, the territoria of each species were classified by year of observation to identify a possible shift of the stock over time. A soil cover map was provided by Natuurmonumenten to improve the observation and to observe other useful details related to the position of the species in their study environment.

species	Dutch name	English name	foraging behavior
Accipiter gentilis	Havik	Northern Goshawk	predator
Aegithalos caudatus	Staartmees	Long-tailed Tit	insectivorous/vegetarian
Buteo buteo	Buizerd	Common Buzzard	predator
Caprimulgus europaeus	Nachtzwaluw	European Nightjar	insectivorous/vegetarian
Certhia brachydactyla	Boomkruiper	Short-toed Treecreeper	insectivorous
Columba oenas	Holenduif	Stock Dove	vegetarian
Columba palumbus	Houtduif	Common Wood Pigeon	vegetarian
Cuculus canorus	Koekoek	Common Cuckoo	insectivorous/vegetarian
Cyanistes caeruleus	Pimpelmees	Blue Tit	insectivorous/vegetarian
Dendrocopos major	Grote bonte specht	Great Spotted Woodpecker	insectivorous
Dendrocopos minor	Kleine bonte specht	Lesser Spotted Woodpecker	insectivorous
Dryocopus martius	Zwarte specht	Black Woodpecker	insectivorous
Erithacus rubecula	Roodborst	European Robin	insectivorous
Falco tinnunculus	Torenvalk	Common Kestrel	predator
Ficedula hypoleuca	Bonte vliegenvanger	European Pied Flycatcher	insectivorous
Gallinula chloropus	Waterhoen	Common Moorhen	piscivorous
Garrulus glandarius	Gaai	Eurasian Jay	omnivorous
Lophophanus cristatus	Kuifmees	European Crested Tit	insectivorous/vegetarian
Lullula arborea	Boomleeuwerik	Woodlark	insectivorous/vegetarian
Motacilla alba	Witte kwikstaart	White Wagtail	insectivorous
Muscicapa striata	Grauwe vliegenvanger	Spotted Flycatcher	insectivorous
Oriolus oriolus	Wielewaal	Eurasian Golden Oriole	insectivorous
Parus major	Koolmees	Great Tit	insectivorous/vegetarian
Phylloscopus collybita	Tjiftjaf	Common Chiffchaff	insectivorous
Phylloscopus trochilus	Fitis	Willow Warbler	insectivorous
Picus viridis	Groene specht	European Green Woodpecker	insectivorous
Poecile montanus	Matkop	Willow Tit	insectivorous/vegetarian
Scolopax rusticola	Houtsnip	Eurasian Woodcock	piscivorous
Sitta europaea	Boomklever	Eurasian Nuthatch	insectivorous
Strix aluco	Bosuil	Tawny Owl	predator
Troglodytes troglodytes	Winterkoning	Winter Wren	insectivorous
Turdus merula	Merel	Common Blackbird	insectivorous
Turdus philomelos	Zanglijster	Song Thrush	insectivorous
Turdus viscivorus	Grote lijster	Mistle Thrush	insectivorous
Vanellus vanellus	Kievit	Northern Lapwing	insectivorous

Table A-1: Latin name, Dutch name, English name and foraging behavior of the selected species of breeding birds that were analyzed in the study. Foraging behavior categorized as insectivorous/vegetarian usually refers to birds that during the breeding season feed their chicks with insects, spiders, etc. and outside the breeding season usually eat seeds and the like. Many of these species migrate to more southern locations after the breeding season

- **Results and discussion**

As an example, Figure A-1 shows three distribution maps obtained from the elaboration of the inventories obtained by Kempen~Broek through the QGIS program.

From these it is possible to notice that all the species are concentrated in specific areas: Laurabossen, Wijffelterbroek, the area that includes Stramproyse Heide and Areven and Tungelerwallen.

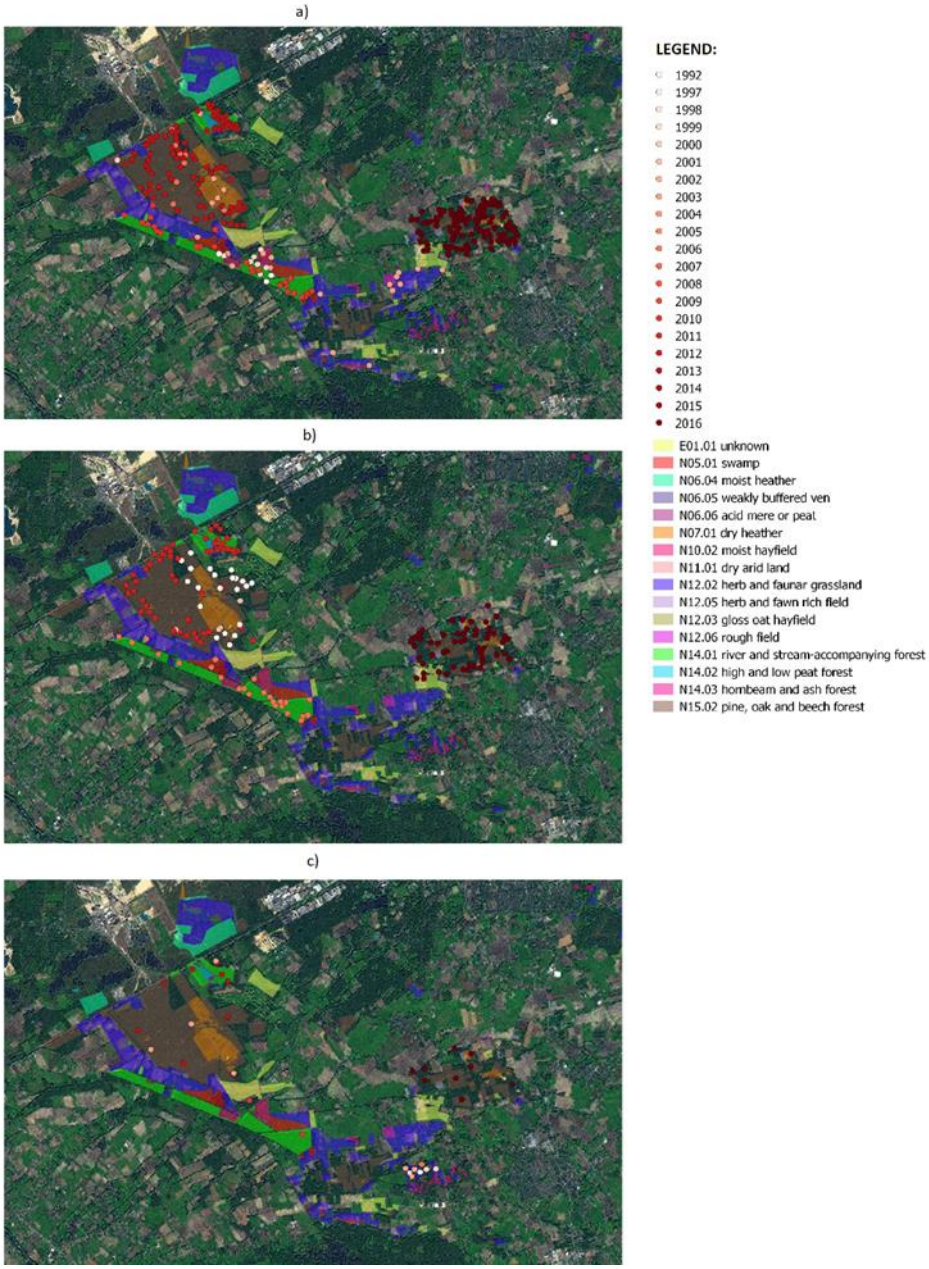

Figure A-1: GIS data describing the distribution of populations of Erithacus rubecula (a), Columba palumbus (b) and Garrulus glandarius (c) in the area of Kempen~Broek from 1992 to 2016.

Laurabossen is an area in the western part of Kempen~Broek, where pines used to be grown for the Lauramines that once existed in South Limburg. This woodland is characterized by the presence of pine, oak and beech, but also contains dry heather and herbaceous meadows.

Wijffelterbroek and the area in-between with Laurabossen are located in the southwestern part of the park and here different types of habitats are present: river and stream-accompanying forests, swamps, wet hayfields and prairies.

The area of Stramproyse Heide / Areven is located in the southern part of the park and basically consists of a forest area with pine trees but also grasslands, herb/fawn rich fields and a hornbeam/ash forest.

Tungelerwallen is a former sand and heath area mainly consisting of heather and coniferous forests and is located in the eastern part of Kempen~Broek.

From the analysis of the movement pattern of bird populations and the changes in distribution between years, it has been possible to observe a general shift of most of them from the south area, where the areas of Stramproyse Heide and Areven are present, to the western area of the park (Laurabossen) in the time period that goes from 1992 to 2008 and since circa from 2012 to 2016 heading east to the area of Tungelerwallen.

The species *C. palumbus*, *C. caeruleus*, *D. minor*, *E. rubecula*, *G. glandarius*, *P. major*, *P. collybita*, *P. trochilus*, *S. aluco*, *T. troglodytes*, *T. merula*, *T. philomelos* and *V. vanellus* show clearly such a movement pattern. For some species, like *A. gentilis*, *D. martius* or *L. arborea*, this tendency cannot be seen clearly, since the number of territories is not sufficient for a clear interpretation of the movement of the bird population. Few other species (like *C. europaeus*, *F. hypoleuca*, *G. chloropus* and *M. alba*) do not show this kind of movement and apparently prefer to stay mainly in the western Laurabossen area.

The movement of the species from the southern to the western part of the park can generally be observed from 1992/1998 to 2008 while the shift from the west to the east areas is observed from 2012 to 2016.

Figure A-2 shows the results obtained by comparing national trends with trends obtained from Kempen~Broek inventories. From these we can clearly see a general decrease in the population size in most of the bird species.

Some of these species show an exponential growth of the population up to extreme levels. For this reason, these species cannot be considered for the analysis, because their tendency is not realistic or because the data present for these species are not sufficient for adequate normalization.

Other species follow the national trend (*F. tinnunculus*) or show a steady growth of the population (*S. europaea*, *S. rusticola*, *P. trochilus*, *G. glandarius*, *A. caudatus*).

To determine if some form of environmental pollution is present in the area, it was necessary to search for inventories of the most affected areas in the Netherlands. For this reason, the provinces of Noord-Holland and Limburg were selected.

According to Ilyin et al. (2014), Noord-Holland appears to be the province that contributes most to pollution in a national scale through iron and steel processing and has the highest air concentration of these elements of the Netherlands. Furthermore, the maximum total deposition is noted for the Noord-Holland province that is characterized by the highest deposition flux from national sources, caused by large emission sources located in this province and accounting for about 60% of total national emission of lead in the Netherlands.

The province of Limburg was considered not only because it is the area in which Kempen~Broek and Weert are located, but also because, as stated by Ilyin et al. (2014), the southern area of the Netherlands (which also includes the provinces of Noord-Brabant, Zuid-Holland and Utrecht) is more polluted than the northern part (where Noord-Holland is present). This is due to the fact that the southern area is more subject to resuspension of these elements, especially in border areas such as Limburg. In fact, Limburg is located in the Dutch / Belgian border, considered in the EMEP/MSC-E report (Ilyin et al., 2014) as the area with greater foreign contribution as regards this type of pollution (where it exceeds 80%).

The results obtained by comparing Kempen~Broek trends with those in these areas are shown in Figure A-3. From this it is possible to observe that some of the insectivorous species (*D. minor*, *D. martius* and *S. europaea*) have a tendency similar to that from Noord-Holland but for most of the other species it shows a decreasing trend compared to the provincial trend. Only *C. europaeus* appears to have a trend similar to the one from Limburg while the other species are always decreasing in comparison to this. A particular “sensitive-to-pollution” group of species is the birds of prey, with 2 species out of 4 (*F. tinnunculus* and *S. aluco*) showing similarities to Noord-Holland, one (*A. gentilis*) similar to Limburg and one (*B. buteo*) decreasing compared to both provincial trends.

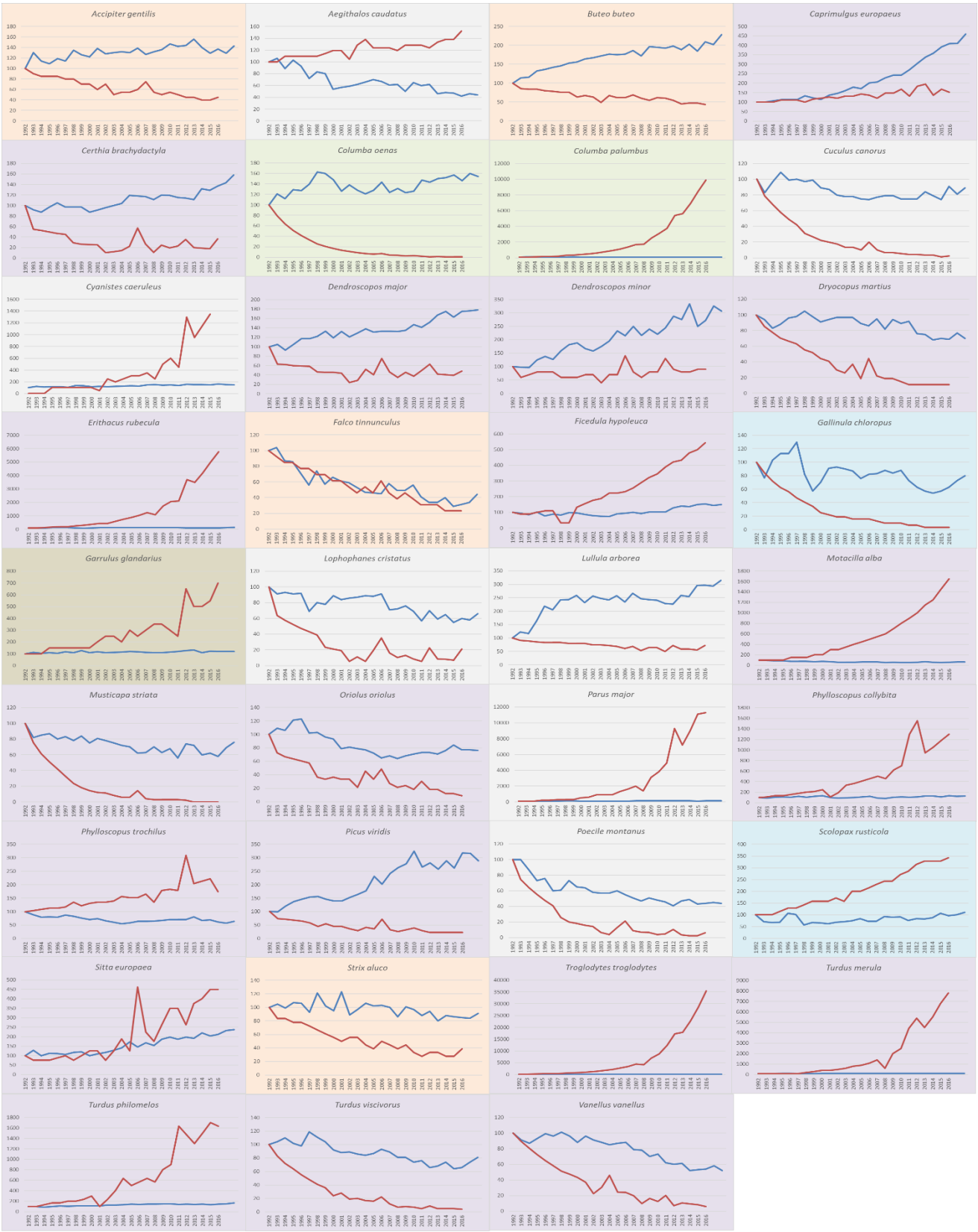

Figure A-2: Comparison between national trends (blue line) and trends taken from Kempen~Broek (red line) for every species of breeding bird considered. According to their foraging behavior, the species are divided in birds of prey (orange background), insectivorous (violet background), herbivorous (green background), piscivorous (light blue background), insectivorous/vegetarian (grey background) and omnivorous (brown background). See table A-1 for classification.

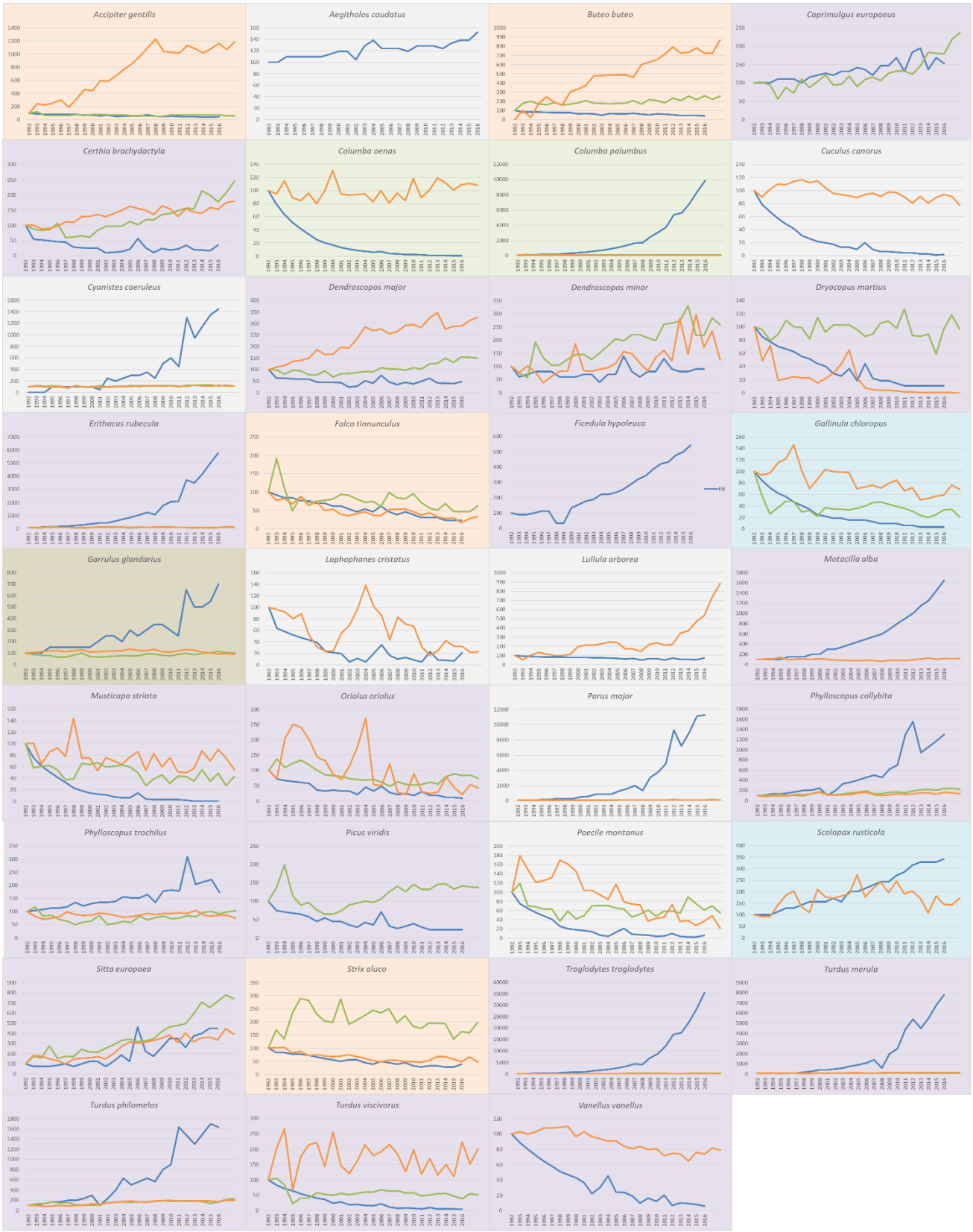

Figure A-3: comparison between the trends from the provinces of Noord-Holland (orange line) and Limburg (green line) and the trends taken from Kempen~Broek (blue line). According to their foraging behavior, the species are divided in birds of prey (orange background), insectivorous (violet background), herbivorous (green background), piscivorous (light blue background), insectivorous/vegetarian (grey background) and omnivorous (brown background). See table A-1 for classification. Not every province trend is compared as they are not provided for every species.

- ## Conclusions and recommendations

Focusing on the data provided and the results obtained from this study, a decrease in most of the breeding bird populations compared to national trends was observed. This allows us to notice that there is an interference factor that is influencing Kempen~Broek to the point of pushing these populations to reduce their density and size.

After the comparison with the provinces of Noord-Holland and Limburg and the discovery of some similarities in the trends of some bird populations, it was possible to state that there is a high probability that this may be caused by environmental pollution of the area.

By dividing the breeding bird species by their different foraging behavior, it has been possible to identify two groups of species more sensitive to this kind of pollution than the others: the insectivorous species and the birds of prey. This is in line with the concept of biomagnification, as these species are higher in the trophic web and therefore much more sensitive to pollutants than other species.

From a geographical point of view, it was possible to obtain information regarding the general movement of the species. Many species showed a trend of moving breeding areas from south to west (from 1992 to 2008) and then from west to east (from 2012 to 2016). The cause of this movement pattern is not certain. It might be just an effect of counting areas that may be different between years because of a certain focus of the volunteers doing the counts (Gaby Bollen - personal communication, 12 June 2018). Still, it is possible that during these periods of time the same factor that affect the population growth must have pushed the bird populations to move to other areas.

The elaboration of the inventories and shapefiles provided by Natuurmonumenten can be considered reliable but not certain, as the data were incomplete both from a temporal and a geographical point of view. In the inventories, the counts of the territories were missing in some years and for this reason it was not possible to obtain a precise progression but only a statistically more probable one, by means of the TRIM program.

The same thing can be said regarding the shapefile, as the observation points are located only in certain areas during certain time periods. This did not allow for a total overview of the area and does not allow to see a real shift in population but only to hypothesize it.

However, the results obtained have allowed us to look at the environmental situation of the area, indicating the possibility that the decrease of bird populations is probably caused by pollution. Despite the incompleteness of the data provided, these results still followed the hypotheses made for this study, showing a general decrease in the population size over the years and similarities with provincial trends in which the presence of environmental pollution is highly probable. The most sensitive species obtained from the analyzes are the *A. gentilis* (predator), *B. buteo* (predator), *C. brachidactyla* (insectivorous), *D. major* (insectivorous), *D. minor* (insectivorous), *D. martius* (insectivorous), *F. tinnunculus* (predator), *M. striata* (insectivorous), *O. oriolus* (insectivorous), *P. viridis* (insectivorous), *S. europaea* (insectivorous), *S. aluco* (predator), *T. viscivorus* (insectivorous) and *V. vanellus* (insectivorous). This seems reliable from a scientific point of view as these species feed on organisms that can first come into contact with pollutants.

As this type of study has never been performed in Kempen~Broek before, the conclusions drawn from this research can lead to a greater awareness of the environmental quality of Kempen~Broek and can start more specific activities of rehabilitation and conservation of local fauna and flora. However, it is important to quantify the degree of pollution and the possible causes in a more concrete way. This can be done by a chemical analysis of organs such as feathers, eggs and feces that have been shown to be bioaccumulators of pollutants. To obtain the best performance for this type of analysis, a technique based on the use of mass spectrometry, such as ICP-MS, is recommended as it is very sensitive and able to determine the different substances present in very low concentrations (up to μg/g).

From this conclcusions, the following questions need to be answered through more in-depth research:

- How can the movement pattern of breeding bird populations over the years highlight a possible environmental pollution in their nesting areas?
- Are there any correlations between changes in the size of the bird population in a specific area and the possible presence of trace elements in it?
- What are the links between the foraging behavior of breeding birds and their sensitivity to trace element pollution?