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TITLE PAGE

Retrospective biomonitoring of mercury and other elements in museum feathers of common kestrel *Falco tinnunculus* using Instrumental Neutron Activation Analysis (INAA)

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ABSTRACT

This study examines the potential to use instrumental neutron activation analysis (INAA) to explore temporal and geographical variation in exposure to heavy metals and other selected elements in common kestrel *Falco tinnunculus* using feathers from a natural history collection. The study gathered samples of two breast feathers from each of 16 adult male kestrel specimens from Naturalis Biodiversity Center, collected in The Netherlands between 1901 and 2001. Feather samples were analysed for more than 50 elements, using INAA at the Reactor Institute Delft. Results (in mg/kg d.w.) were transformed into ratios of mg of element per mm of feather length. The distribution of the mass fractions and ratios was plotted for each element against time and by geographical area. Observed mass fractions and/or ratios are discussed for selected elements (Hg, Cd, Zn, Pt, Pd, Se, Al, Rb, As, Sb, Cr, V, Cl, Br) known to have, at certain concentrations, adverse effects on raptors. Some samples show mass fractions of certain elements (Cr, Cd, Se, As) above levels known to have adverse effects. We conclude that the analysis of museum feathers using INAA provides reference values for concentrations of selected elements, including those of high societal concern such as Hg and Cd, against which to assess concentrations of these elements in feathers of present-day living raptor populations.

KEYWORDS

Raptor, common kestrel, *Falco tinnunculus*, feather, museum specimen, contaminant, exposure, metal, element, INAA

INTRODUCTION

Aim and objectives

The aim of this study was to use instrumental neutron activation analysis (INAA) to explore temporal and geographical variation in exposure to heavy metals and other selected elements in common kestrels *Falco tinnunculus* using feathers from a natural history collection. The study also aims to explore the potential offered by museum bird skin collections for retrospective biomonitoring of heavy metals and other elements using this analytical method.

The study was intended as a pilot with a view to initiating a larger project to examine temporal and geographic variation in exposure to heavy metals and other elements in raptor (and possibly other bird) species using museum specimens, and to compare these with contaminant levels in present-day populations of the same species living in the same geographic regions.

The use of raptors as sentinels of environmental contamination

Birds can be useful sentinels of environmental contamination (e.g. Newton et al. 1993; Rattner 2009). Raptors (birds of prey, owls and scavengers) are especially suitable for monitoring PBT (persistent, bio accumulative, toxic) chemicals (e.g. Sergio et al. 2005, 2006, Movalli et al 2008a), because: (a) they are typically relatively long-lived, apex predators, (b) they effectively integrate contaminant exposure over time (Furness 1993) and over relatively large spatial areas, (c) they are relatively easily studied and captured (especially nestlings) which facilitates collection of samples without sacrificing or harming animals (blood, feather, preen gland oil); and (d) populations can be relatively easily monitored and quantified. These are all criteria identified by the U.S. National Research Council as requirements for sentinel species (NRC 1991). Raptors are also known to have measurable responses to PBT chemicals, ranging from residue accumulation to population decline. Indeed, the current ban under the Stockholm Convention on poly-chlorinated biphenyls (PCBs) and other PBT compounds that are potentially harmful to both people and wildlife has been partly based on exposure and effects data in raptors (Rattner 2009).

Raptor biomonitoring programmes in Europe

An overview of existing raptor contaminant monitoring activities in Europe has recently been published by Gómez-Ramírez et al. (2014). This found that, while there is a large number of biomonitoring programmes using raptors, only a few are established at a national scale, e.g. National Environment Monitoring Programme in Sweden (Helander et al. 2008), the Predatory Bird Monitoring Scheme (PBMS) in the United Kingdom (Walker et al. 2008), the Bird Monitoring Programme in Finland (Koskimies 1989) and the Monitoring Programme for Terrestrial Ecosystems (TOV) in Norway (Gjershaug et al. 2008). In other countries, such as Spain, Germany, Belgium, Italy and The Netherlands, (Gómez-Ramírez et al. 2011; Jaspers et al. 2006; Kenntner et al. 2003; Movalli et al. 2008b; van den Brink et al. 2003) studies are typically limited in spatial extent and/or duration and are rarely repeated (García-Fernández et al. 2008). Overall, there appears to be widespread capability and expertise to use raptors to monitor the effectiveness of EU directives at a pan-European scale. However, existing national and sub-national monitoring initiatives need to be reinforced and coordination at a pan-European scale improved (Movalli et al. 2008a). A paper on the selection of raptor samples for contaminant monitoring has recently been published by Espín et al. (2016a) and a sample monitoring protocol has been developed under the EURAPMON Research Networking Programme (Espín et al. 2016b).

The use of biological collections for retrospective studies

Biological collections in museums can contribute unique and invaluable insights into the study of pathogens, vectors of disease, and environmental contaminants (Suarez and Tsutsui 2004). These collections represent a valuable repository of catalogued samples collected during periods when scientists were not aware about environmental contaminants. Those specimens, which can date back

to the mid-1800s or even earlier, can be useful indicators of environmental contamination during a period of increasing industrialization prior to the 1970s (Campbell and Drevnik 2015).

The preservation techniques used on museum skins is not always known and can contaminate samples. Washing does not always remove this external contamination. However, we used a washing method (Cortes Toro et al 1993, see below) known to be effective for removal of external contamination by heavy metals and other elements, including inorganic Hg that was in the past commonly used to preserve museum skins (however, see discussion on As, below).

The use of museum feathers to establish reference values

Environmental contaminants are chemicals that exist at levels judged to be above those that would normally occur in any particular component of the environment. This raises the question of what is to be considered normal. With most man-made chemicals, such as pesticides, the situation is simple; any detectable level is abnormal as the compounds did not exist in the environment until released by man. On the other hand, chemicals such as metals, polycyclic aromatic hydrocarbons and methyl mercury are naturally occurring and were certainly or probably present in the environment before the appearance of human beings. The concentration of these chemicals varies from place to place and over time. This makes it difficult to judge their normal ranges of concentration (Walker et al. 2006).

One way to help inform these normal ranges, in birds, for naturally occurring chemicals is to conduct work on feathers from museum specimens with a view to establishing retrospective (historical) reference values. Feathers have long been known as a useful matrix for the study of a range of contaminants in birds, including mercury and other metals (e.g. Berg et al. 1966, Scanlon et al. 1980, Movalli 1993, Fasola et al. 1998) and more recently have been used to study organic pollutants such as DDT and metabolites (e.g. Dauwe et al 2005, García-Fernández et al. 2013) and neonicotinoides (Taliensky-Chamudis et al 2017). However, other raptor tissues, when available, may be better matrices for the study of many contaminants (Espín et al 2016a).

Museum collections of raptor skins, at least for certain species, are relatively large, allowing for sufficient sample sizes to test for statistical significance of any differences in contaminant levels observed over time and space. These skins offer certain other advantages in this respect; they are typically well-documented, with data available on the species, sex, maturity, location and date of collection, allowing for interpretation of observed contaminant levels (Thompson et al. 1992). Specimens can date back to the mid-19th century or even earlier, and thus provide data on change in contaminant exposure during a period of increasing industrialisation through to the mid to late 20th century (Campbell and Drevnik 2015).

Previous studies using museum raptor specimens

As early as the 1960s, Berg et al. (1966) analysed preserved bird specimens from the Swedish Museum of Natural History and showed that concentrations of accumulated mercury increased during the 1940s and 1950s. There are a few other examples of the use of museum collections for the retrospective analysis of trends in exposure to mercury (e.g. Dietz et al. 2006 for Greenland, Head et al. 2011 for the USA). However, we are not aware of other studies that, like ours, examine exposure to a wide range of metals and other elements using museum collections of raptor skins. This is also the first study reporting on the concentrations of contaminants in feathers of museum kestrels from the Netherlands.

Raptor specimens at Naturalis Biodiversity Center

A number of natural history museums in Europe hold important collections of raptor skins dating from the last 150 years or so. Naturalis Biodiversity Center is the result of a recent merger of the National Museum of Natural History of The Netherlands and the Zoological Museum of the University of Amsterdam and houses a large collection of bird skins. Such collections offer

potential to investigate temporal and geographic variation in the exposure of raptors to contaminants such as Hg, Cd and certain POPs, which are amenable to study using feathers.

Relevance to EU chemicals regulation and to environmental and human health

EU law on human and veterinary medicinal products (EC 2001a, EC 2001b, EC 2004a), industrial chemicals (EC 2006), plant protection products (PPPs) (EC 2009) and biocidal products (EU 2012) as well as aspects of the Water Framework Directive (WFD) (EC 2000), Marine Strategy Framework Directive (MSFD) (EC 2008) and Environmental Liability Directive (EC 2004) aim to prevent and limit negative impacts of chemicals on human health and the environment. These laws are supplemented by global and regional conventions to which the EU, Member States and other European countries are party, such as the Helsinki Convention on the Baltic Sea (1992), OSPAR Convention on the NE Atlantic (1992), Stockholm Convention on persistent organic pollutants (2001) and Minamata Convention on mercury (2013).

A key issue with all such legislative instruments is to determine how effective they are. Measuring the numbers of registrations, authorizations and restrictions on chemicals only provides data on activities undertaken under the auspices of the EU directives. Such measures do not provide information on how *effective* the measures were in achieving mitigation targets; that requires monitoring of contaminants in selected environmental compartments.

Raptors can be particularly useful as sentinels of environmental contamination. The availability of reference values for selected contaminants, derived from museum specimens of a selected raptor species, can enable a better-informed assessment of modern day values observed in the same species, and thus a better-informed assessment of the effectiveness of regulatory controls on these contaminants.

When combined with modern-day monitoring of living raptor populations, historical reference values obtained from museum raptor skins can help to provide early warning of certain legacy and emerging contaminant threats and thereby reduce the costs to environmental and human health that may arise from such contamination (though other tissues such as liver and adipose tissues are more appropriate tissues to use for lipophilic compounds – see Espín et al. 2016a).

MATERIALS AND METHOD

Considerations in selecting the study species

A number of issues should be taken in to account when selecting the species for study. These include:

1. *Residential or migratory species and territorial fidelity.* Some raptor species (or one or the other sex of a species) are migratory and/or occupy very wide-ranging territories. In this case, studies can assess the overall exposure of individuals to contaminants, but it is difficult to make inferences about the geographical source of any contaminant detected. In other raptor species, the male and/or female typically resides in a restricted territory all year round, permitting deductions to be made both about overall exposure and about the geographical source of any contaminant detected.
2. *Number of specimens available in the museum collections.* A statistically valid number of specimens of the selected species (and sex) are required for each time period in order to explore temporal trends. If the intention is also to examine geographical patterns of contamination, then a statistically valid number is required both for each time period and for each geographic area of interest. As museums tend to have higher numbers of specimens for relatively common and widespread species, it is such species for which a statistically valid number of specimens are most likely to be available.
3. *Likely sources of contamination and likelihood of exposure to specific contaminants through the food chain.* Certain raptor species feed predominantly on rodents, and therefore may be

particularly exposed to contaminants that rodents are exposed to, such as fungicide seed dressings and rodenticides. Other raptor species feed predominantly on insect or seed-eating birds, and therefore may be particularly exposed to contaminants that these prey are exposed to, such as insecticides or fungicide seed dressings.

Selection of male kestrels as the species and sex for this study

Taking in to account the above considerations, we chose male kestrels *Falco tinnunculus* as the species and sex for this study for the following reasons:

1. In general, the kestrel in The Netherlands is strongly territorial, depending on availability of food (voles and mice). If food is scarce, females in particular may leave the territory to seek better hunting grounds. Males need less biomass and probably have a greater need to defend their territory, and will eat earthworms in addition to rodents (however, in a period of prolonged snow cover or frozen ground, males also may move from their territory). Moreover, females can excrete metals and other contaminants by egg-laying resulting in greater variability in body burden (Lodenijs and Solonen 2013).
2. The kestrel is a relatively common and widespread species in The Netherlands, both now and historically. The Naturalis Biodiversity Centre holds a relatively large collection of kestrel skins (over 600) dating from 1850 to 1980.
3. The kestrel in The Netherlands feeds predominantly on voles and mice (Goree et al. 1995), as elsewhere, e.g. Norway (Steen et al. 2011), Italy (Casagrande et al. 2008) and Finland (Valkama pers. comm.) but also can eat a lot of earthworms. This means it is particularly exposed to contaminants that these rodents and worms may be exposed to, including fungicide seed dressings, herbicides, insecticides and elements such as mercury, lead and cadmium, from industrial, vehicular and other sources, deposited on vegetation and soil from the atmosphere.
4. The kestrel is widespread and common in much of Europe, and has been relatively frequently studied by ecotoxicologists, providing a relatively large volume of data with which to compare historical contaminant levels.
5. Naturalis receives present-day specimens of dead kestrels, and kestrel nest sites (many use nest boxes in The Netherlands) are relatively accessible for sampling, offering opportunities in the future to collect present-day feather samples and compare present-day contaminant levels with historical levels.

Selection of INAA as the analytical method for this study

In using museum feathers for ecotoxicological studies, the selection of the analytical method needs to take into account the range of contaminants for which feathers are a suitable matrix of study. Feathers are known to be a suitable matrix of study for mercury (Hg) and a range of other heavy metals and other elements, some of which (e.g. Hg) are bound in to the keratin during feather growth, and others of which are deposited in the feather by the blood stream during feather growth but not structurally bound. For those elements that are firmly bound to the keratin (such as Hg), the concentration in the feather will be related to the exposure of the individual and the mass of the feather (mass dependent contaminants), while for those elements that are not structurally bound, the concentration in the feather will be related to the exposure of the individual to the contaminant and the duration of feather growth as reflected by the feather length (time-dependent contaminants). For the former, concentrations are best expressed as mg/kg and for the latter, as mg/kg per mm length of feather (Bortolotti 2010).

For the current study, we selected instrumental neutron activation analysis (INAA) as the analytical method. INAA has proven to be a highly sensitive and versatile method for measuring trace elements in a wide variety of samples (Wainerdi and DuBeau 1963) at microgram levels (Corliss

1963). Almost fifty years ago, Devine and Peterle (1968) demonstrated the utility of neutron-activation analysis in their investigations on populations of Canada Geese *Branta canadensis*. It has been demonstrated that INAA can meet the highest metrological requirements (Greenberg et al. 2011)

INAA has several advantages. First, and critically when dealing with irreplaceable museum specimens, it requires only a very small sample size (we used 2 breast feathers from each specimen; there is no minimum mass for INAA). The removal of feathers from museum skins can have impact on the aesthetic quality and scientific value of these skins and therefore the number sampled from each skin must be kept as low as possible. INAA is moreover non-destructive, so the feather samples may be returned to the museum or used for other analyses. Second, INAA has very low limits of detection and has proven to be a highly sensitive and versatile method for measuring trace elements in a wide variety of samples at microgram levels (Parrish et al. 1983). Third, a single INAA assay can provide concentration values for a large range of elements.

During INAA, the feathers are irradiated with neutrons. These neutrons interact with the nuclei of the atoms in the feathers and in doing so create radioisotopes. Gamma rays, with energies specific to each element, are emitted during the radioactive decay of the feather, and are measured by a spectrometer. Concentrations of numerous elements are measured simultaneously (Bortolotti and Barlow 1986).

The Reactor Institute Delft (RID) at the University of Technology Delft offers several different assays which each detect a different range of elements. For the purposes of this study, we selected the assay which detects the following elements (listed in ascending order in the periodic table): Mg, Al, Si, Cl, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Br, Rb, Sr, Zr, Mo, Pd, Ag, Cd, In, Sn, Sb, I, Cs, Ba, Hf, Ta, W, Re, Pt, Au, Hg, La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Lu, Th, U. This includes a number of elements of particular societal concern, including Hg and Cd.

Collection of museum feather samples

We sampled, in 2013, two breast feathers (total c. 10 mg) from each of 16 male kestrel skins (n=16) dating from the period 1901-2001. *Table 1* provides a list of feather samples with their Naturalis Biodiversity Center specimen ID code, place and date of collection. These data were all available from the Naturalis Biodiversity Center specimen labels.

The collection of each feather was performed following a protocol as follows. To avoid possible variations only one operator collected and processed all samples, in a uniform manner. The operator wore plastic gloves. Individual feathers were isolated and pulled with clean, acid-rinsed, stainless steel forceps. Before taking the measurement of the length and weight of each feather, the operator removed the calamus of each feather⁶ (with scissors) at the point where the first barbs of the vane join at the base of the feather, then the length of each feather was measured (in mm) using a caliper, from the cut end (the base of the vane) to the tip. The feathers were measured flat on a clean table (when necessary placing the feathers under a sterile sheet of glass to flatten them). The feathers were then weighed (in mg) using an electronic balance.

Feathers were placed in labelled polyethylene vials, using forceps. Only one pair of forceps was used to collect the feathers and perform all handling operations. Feather samples were catalogued and stored at room temperature at Naturalis Biodiversity Center until analysis. All the samples were transferred to TU Delft where the analyses were performed.

Cleaning and drying of breast feather samples

⁶ The calamus is removed as this has a different growth rate and therefore could skew the concentration values per mm length of vane (Bortolotti 2010).

In order to remove external contamination from the surface of the feathers, a cleaning process was performed, as recommended for hair samples by the International Atomic Energy Agency (Cortes Toro et al 1993), prior to analytical determination. The feathers were cleaned by adding ultrapure (up) water to the capsule and shaking for five minutes. This process was repeated three times, after which the capsules (with up water) were placed in an ultrasonic bath for 15 minutes. After cleaning, the feathers were dried in a desiccator. The drying process was followed by daily weighing of the capsules, until the mass was stable for two days.

Instrumental neutron activation analysis

The cleaned and dried samples were analysed using INAA at RID. The quality management system of this laboratory is accredited by the Dutch Council for Accreditation for compliance with the ISO/IEC 17025:2005 standard.

Specifically, the samples were irradiated during 2 minutes at a thermal neutron fluence rate of $1.5 \times 10^{13} \text{ cm}^{-2} \text{ s}^{-1}$ and measured, after 1 minute decay time, during 5 minutes at 1 cm distance from a 10% Ge detector. Then, they were irradiated during 5 hours at a thermal neutron fluence rate of $4.6 \times 10^{12} \text{ cm}^{-2} \text{ s}^{-1}$, and measured twice in a well-type Ge detector after 4 and 16 days of decay time, during 1 and 3 hours, respectively. The acquired gamma-ray spectra were analysed using the Apollo software, resulting in a list of mass fractions with associated measurement uncertainties, and detection limits of about 50 elements (Blaauw 1994). The entire analytical method has been validated (Bode and Blaauw 2012).

Counting of fault bars in remiges of the same birds

We analysed fault bars in remiges of the same birds (see *Table 2*). On each feather, 10 growth bars, centred on the point two-thirds of the distance toward the tip of the feather were measured (see Grubb 1989). This analysis was done by R Yosef, who served as a blind test in respect to all other variables measured (e.g. Grubb and Yosef 1994). This measurement was done during a summer school in 2014 on the use of museum specimens for contaminant analysis in raptors.⁷

Growth bars (differently from fault bars) are regular cross-bands on feathers that denote 24h periods of growth (Wood 1950, Grubb 1989). These bars are most commonly seen in the wing and tail feathers, as alternately dark and pale cross bands, rarely in other tracts (Wood 1950). The difference in color between the dark and light bars may be due to a more rapid development of the barbules during the day, hence more of them in a given space, and by a different deposition rate of lipochromic granules (Wood 1950). Since the first definition of ptilochronology (Grubb, 1989) feather growth bar widths have become a standard index of nutritional condition in ornithological research (see Shawkey et al 2003). The width of feather growth bars during each 24 hour period at the time of moult depends on the amount of energy and nutrients invested by an individual into the regeneration process, which at the same time depends on the nutritional condition and quality of an individual (Grubb 1989, 2006). Feather growth as an index of nutritional condition is based on natural selection, which forces birds to regenerate lost feathers as rapidly as possible. Therefore, birds in better nutritional condition regenerate feathers much faster than individuals in poor condition (Grubb 1991; Grubb, 2006). Thus, feather quality is a relatively accurate indicator of an individual's quality.

Fault bars are transparent bands in the feathers of birds produced under stressful and adverse conditions (i.e. Møller et al. 2009). Such bands are narrow malformations in feathers oriented almost perpendicular to the rachis where the feather vein and even the rachis may break. Breaks in the barbs and barbules result in small pieces of the feather vein being lost, while breaks in the rachis result in loss of the distal portion of the feather (e.g. Jovani & Rohwer 2016). Malnutrition was likely the first factor to be related to fault bars (Sebright 1826); a recent review however suggests

⁷ <http://www.eurapmon.net/sm6-naturalis-aug-2014>

that several environmental stressors may result in the development of fault bars in growing feathers (Jovani & Rower 2016).

RESULTS

Data and data transformation

Annex 1a and 1b (spreadsheets <*Annex 1a Results & graphs – Mass-dependent 30Apr17.xlsx*> and <*Annex 1b Results & graphs – Time-dependent 30Apr17.xlsx*>) provide the data resulting from the INAA analysis, the transformation of the data and graphs. The following provides an explanation of each tab on the spreadsheet:

- The first tab 'INAA RESULTS' presents the INAA results (mg/kg).
- In Annex 1a, the second tab 'DATA mg/kg d.w.' presents the mass fractions for each element (mg/kg).
- In Annex 1b, the second tab 'RATIOS' calculates the ratios (mg/mm) of element mass per feather length, calculated as: (element mass fraction (mg/kg) x feather mass (kg))/(feather length (mm)). On the results and discussion below, other than for Hg (for which deposition in the feather is surely mass-dependent as Hg binds to the keratin), we use mass of each element per mm of feather length (following Bortolotti, 2010), taking in to account that deposition of many of the elements of interest in feathers may be time-dependent (i.e. it relates to the length of the time period during which the feather is growing and receiving an influx of blood carrying these elements) and not mass dependent (except for those elements that bind structurally to the keratin).
- The third tab 'Specimen AVGs' calculates the average ratio (mg/mm) for each bird, calculated as the average ratio from the two feathers sampled from each bird.
- The fourth tab 'Pivot GEO' calculates the average ratios (mg/mm) for each geographical area (see below for explanation of these areas).
- The fifth tab 'Distr TIME' presents graphs showing the distribution of specimen average ratios over time.
- The sixth tab 'Distr GEO' presents Graphs showing the distribution of the specimen average ratios by geographic area (1 = north, 2 = centre, 3 = south, 4 = inner).

To demonstrate the exploration of possible geographic patterns, data were clustered *a priori* in groups as follows:

1. North (mostly north of 52°15'N) (samples 7,9,10,16)
2. Centre (mostly between 52°9'N and 52°15'N) (samples 4,6,11,14,15)
3. South (close to coast, mostly south of 52°9'N) (samples 1,2,8,13)
4. Inner (east of 4°45'E) (samples 3,5,12)

It should be noted that there is no particular rationale to these geographic groupings in relation to likely contaminant sources. They have been made simply to demonstrate how such geographical groupings can be made and analysed.

Quantitative statistical analysis

Where the INAA results showed a high frequency (>30%) of negative values (i.e. uncertainty largely overlapping with zero (Blaauw, 2016)), the element in question was excluded from quantitative analysis. Such elements (Si, Cr, Ni, Rb, Sr, Zr, Mo, Pd, Ag, In, Ba, Nd, Tb, Lu, W, Re) were examined only for presence/absence with the application of an exact Fisher F test (but no evident temporal or geographic pattern was found).

Moreover, for those elements (Au, Hf, Yb, Ta, Eu, Sm) where all INAA results had a maximum mass fraction <0.10 mg/kg, such very low values didn't result in any meaningful correlative analysis.

Consequently, we carried out quantitative statistical analyses for the remaining 29 elements (i.e. those with no, or $<30\%$ of, negative values): Mg, Al, Cl, K, Ca, Sc, Ti, V, Mn, Fe, Co, Cu, Zn, Ga, As, Se, Br, Cd, Sn, Sb, I, Cs, La, Ce, Dy, Ta, Pt, Hg, Th, U. For those elements, to consider only positive values of mass fraction, any negative values (ranging between -0.03 and 0.00) were transformed to one tenth of the lowest positive value for the relative element.

Time patterns of elements mass fractions were explored with linear and quadratic regression. Geographical patterns were explored with ANOVA. All regressions/differences were considered significant if $p < 0.05$. Statistical analyses were performed with the statistical packages SPSS and Prism.

Temporal and geographical pattern

The pattern of average time dependent ratios (in mg/mm) for each specimen is almost independent of the year of collection. The only significant relations highlighted by the regression analysis were those showing a decrease of As mg/mm over time ($r=0.503$, $p=0.047^*$), and an increase of Se mg/mm over time ($r=0.576$, $p=0.19^*$) (*Figures 1a and 1b*).

To look for significant patterns related to pollution events during the central period, a quadratic regression model was applied, but none of the elements showed a significant curve with such a maximum.

Notably, some of the rarest elements (Zr, In, Nd, Lu) were only detected in 1-2 specimens related to the middle period (1947-1962), altogether in 4 different birds (Spec. 2, 3, 9, 15) (*Table 2*). Possibly due to the small size of the sample, none of these gave a significant result.

Several other rare elements (Ba, La, Ce, Ta) showed exceptionally high mass fraction values in the same period (1947-1952), mostly in the same samples (*Table 2*). For the same period the highest values of cadmium (Cd) mass fractions were detected (*Figure 2*). Ce is a dopant in diesel oil (Costantini et al. 2001) and has been measured also in nail clippings (Gomez Aracena et al. 2006). La is also often linked as an indicator for oil burning processes (eg. Tribalis et al. 2016). The contamination in this period might reflect the aftermath of large-scale pollution during WWII.

The variance analysis highlighted a biased distribution for Selenium Se ($F=4.51$, $p=0.24$, d.f. 3 & 12), with most high values in bird samples collected in the "North" area (*Figure 3a*) and for Mercury Hg ($F=3.47$, $p=0.05$, d.f. 3 & 12), with lower values in the "South" and "Inner" areas (*Figure 3b*). However, as already noted, the geographical areas are arbitrary constructs so this biased distribution should only be considered indicative.

Growth & Fault bars

The average growth rate of each feather based on the growth bars measurement was 2.95 mm/day (± 2.8 SD), ranging from 2.27-3.42.

The number of fault bars was usually 0 (nine cases) or 1 (five cases), but two birds showed respectively 5 and 6 fault bars. The average number of fault bars was consequently 1/feather (± 1.83 SD).

The number of fault bars negatively correlated to the size of growth bars on the feathers ($r=-0.584$, $p < 0.01$), consistent with the fact that fault bars may be interpreted as a negative and growth bars a positive proxy of individual bird health status.

Analysis of variance didn't evidence any temporal or geographical pattern in the growth bar size (respectively $F=0.662$, $P=0.662$ n.s., df 1&2 and $F=0.897$, $p=0.471$ n.s., df 1&3). Application of the

Kruskal Wallis test to the fault bar counts didn't reveal any temporal or geographical pattern (Kruskal Wallis statistic respectively 3.79 with $p=0.285$ n.s., and 1.25 with $p=0.535$ n.s.).

When compared with the measured element mass fractions, growth bar size showed a significant correlation only with the value of K (mg/kg, after log transformation $r=0.61$, $p<0.01$), all other elements ranging between $r=-0.45$ and $r=0.42$ (n.s.). Such a result may possibly be explained by the role of K in proper growth in birds (e.g. NRC 1994).

The possible role of other elements on the residual variability of growth bar size was explored by submitting the data to a stepwise regression analysis. Using time-dependent data, a second element, Ca, was maintained in the model (stepwise regression model: $r=0.685$, $F=5.747$, $p<0.05$; see *Table 3*). Feathers contain CaCO_3 , and thus Ca is needed to grow and maintain good feathers. Ca levels may depend both on Ca intake and Ca metabolism, the latter being affected by chemical pollutants such as DDT. Our data show no decrease of Ca level in the samples during the DDT period (1950s-1960s), so we may argue that growth bar size in these specimens is possibly explained only by nutritional status during the moulting period.

On the other hand, the two cases of high number of fault bars are related to single birds with exceptional mass fraction values of Ca (2663 mg/kg vs a range of 432-1677 for the other 15 birds) or arsenic (460 mg/kg, residual range 7-316).

To understand the possible role of single elements in determining the appearance of a single fault bar, the mg/kg values of the five birds showing one bar have been compared with the nine values of the birds showing no bars with Kolmogorov-Smirnov test for small samples. The only element showing a statistically significant difference is As ($Z=-2.2$, $p=0.029$) but the effect is doubtful, as As mass fraction is significantly influenced by year (mg/kg= $5241.3-2.63*\text{year}$; $r=0.549*$). The Kolmogorov-Smirnov test performed on the residuals after the regression is not significant ($Z=0.33$, $p=0.797^{\text{ns}}$). Cu and Se approach the significance threshold (respectively $Z=-1.93$, $p=0.060$ and $Z=-1.80$, $p=0.83$), suggesting a possible detectable difference with a bigger sample size.

A cumulative effect of combined elements has been tested by means of discriminant analysis, looking for the combination of elements able to separate birds carrying a fault bar from birds with unmarked feathers. A supervised analysis including only Cu and Se identifies a discriminant function correctly classifying 12 out of 14 birds (85.7%), with lower Cu and higher Se, or both, in presence of a fault bar. An unsupervised analysis with a stepwise variable selection, considering all 29 selected elements but As, only included Ti and Se, correctly classifying 100% of the birds (*Table 4, Figures 4a and 4b*).

While Selenium (Se) is a metalloid trace element that birds need in small amounts for good health, high concentration of Selenium in birds tissues may result in reproductive failure and adult mortality (Ohlendorf et al 1987). Our results suggest that relatively higher values of Selenium, as revealed by analysis of feathers, may influence the sensitivity of feather follicles to acute stress during feather growth (Jovani and Rohwer 2016), and possibly overall bird condition, either alone or in combination with other microelements. However, our values for Se do not exceed 2 mg/kg d.w. (3×10^{-7} mg/mm). This lies within the range of 1-4 mg/kg for the background concentration of Se in feathers (with 10% moisture content), according to Ohlendorf & Heinz (2009). An Se concentration of 5 mg/kg has been identified as a threshold warranting further study (USDI, 1998).

DISCUSSION

The uptake mechanisms for metals in feathers are: inhalation of airborne particles; dry or wet deposition; contamination through direct contact with the environment (water, soil or vegetation); preening; and ingestion of contaminated prey. Most raptors ingest the feathers, furs, and bones of their prey and discard excess material as pellets (Jensen et al. 2002). Metals can be lost from

feathers through precipitation, bathing in water, and rubbing (Goede and de Bruin 1986; Weyers et al. 1988).

Unlike many organic chemicals, metals cannot be easily metabolized into less toxic compounds. Metal pollution affects wildlife indirectly by altering habitat, food chains, community structure and between-species ecological interactions (Koivula and Eeva 2010) but also with direct effects such as morphological or neurological changes, developmental instability during ontogeny and alteration in biochemical processes (e.g. changes in enzyme activities and free radical levels) (Scheuhammer 1987).

Metals are very reactive and thus toxic to many organisms when interfering with metabolism and important biochemical reactions (e.g. Scheuhammer 1987). The concentration of a given metal in the body depends on the level and duration of metal exposure. The toxicity can be related to the isotope forms of metals as well as the interactions with other xenobiotics (Gochfeld and Burger 1987; Koivula and Eeva 2010).

There is little information available about toxic threshold values of essential and trace elements in bird feathers (Theuerkauf et al. 2015).

For some legacy contaminants such as Hg, a known neurotoxin, there is already a global concern as shown by the recent Minamata Convention on Mercury, a global treaty to protect human health and the environment from the adverse effects of this metal. However, other metals and elements are already, or may prove to be, of societal concern. This study aims to provide data also on these other elements.

Discussion of observed mass fractions of selected elements potentially toxic to raptors

The small number of samples ($n = 16$) for this study means that we have only 3-7 samples per time period and only 3-5 samples per geographic area. This allows us only to look for some broad, obvious pattern. We make some observations here on values observed for some selected elements of potential toxicity to raptors. Some of these elements (e.g. Hg, Cd) produce chronic effects on biota and are considered hormone disruptors and immunosuppressive agents, as well as causing adverse effects on the nervous and reproductive systems.

Mercury

Our Hg values range from 0.4 to 4.5 mg/kg d.w. According to Eisler (1987), Hg concentrations of 5–40 mg/kg in bird feathers are associated with adverse effects, including impaired reproduction (Lodenius and Solonen 2013). Burger and Gochfeld (2000b) similarly considered Hg levels in feathers greater than 5 mg/kg to be associated with adverse reproductive effects in birds. Concentrations of 15 mg/kg Hg may be required for adverse effects in some predatory birds (Spry and Wiener 1991; Wiener and Spry 1996; Burger & Gochfeld 2009). Feather Hg concentrations of 10–15 mg/kg are thought to be harmful to bald eagles *Haliaeetus leucocephalus* (Cristol et al. 2012). However, Bowerman et al. (1994) instead affirmed that Hg (max. 66 mg/kg in feathers) did not affect reproduction of bald eagle in the Great Lakes region in North America. Our Hg values are below those associated with adverse effects.

Feathers are an excellent resource for retrospective analysis of Hg (Head et al. 2011). Hg is incorporated into the feather at the time of formation where it remains stably bound to keratin and is thus physiologically unavailable (Appelquist et al. 1984). More than 70% of a bird's total body burden of Hg can be found in the feathers, but this percentage can vary across species and with the degree of exposure (Braune and Gaskin 1987; Honda et al. 1986). More than 90% of total feather Hg exists as methyl-Hg (MeHg), the form that is bioaccumulative and bioavailable in aquatic food chains (Thompson and Furness 1989).

Feather Hg concentrations reflect an integration of Hg found in the blood at the time of feather formation (due to recent dietary intake) and Hg in the muscle (accumulated over a longer time

period) (Evers et al. 2005). Levels of Hg and other contaminants in bird feathers are strongly correlated to those in bird muscle or liver (e.g. Thompson et al. 1991; Bearhop et al. 2000) suggesting that the analysis of feathers is a viable alternative to estimate the overall contamination status of the birds.

Feathers are an effective and commonly used indicator of overall MeHg body burden. There is a direct relationship between concentration of MeHg in feathers and body burden of MeHg during the time of feather growth. However, feather type (e.g. flight versus body) and species' moult patterns affect this relationship (Bond et al. 2015). Once grown, feathers are inert, and the Hg bound within the feather is stable (Appelquist et al. 1984). It is therefore possible to examine temporal trends in feather MeHg (and therefore MeHg body burden) using dated museum specimens, allowing retrospective analyses (Bond et al. 2015).

All feathers could possibly be used to screen at high contamination sites, but when specific information on internal tissue concentrations is required, body feathers are probably the most appropriate. Body contour feathers have been shown to be most representative of total body MeHg burden (e.g. Doi & Fukuyama 1983, Furness 1986, Movalli 2000, Vo et al. 2011).

Inorganic Hg (IHg) compounds (usually HgCl_2) were used up to the 1930s to preserve museum bird skins and this can affect the observed value of Hg using INAA (which does not distinguish organic from IHg). Head et al. (2011) analysed feather samples in museums in the US and found no feather sample after 1936 had evidence of IHg contamination, but several prior to 1936 did show evidence of contamination. They concluded that one may assume that all Hg found after the 1930s is organic. Bond et al. (2016) affirmed that MeHg is the only appropriate measure of a birds' Hg body burden when analysing museum specimens with unknown preservation histories (Hogstad et al. 2002).

However, Strekopytov et al (2017) note that mercury treatments were used in the US up to the 1980s. They examined Hg in kestrel feathers from four specimens (collected between 1872-1954) from the Natural History Museum in London and observed highly elevated values (4409 to 26960 ppm). They conclude that any value over 1000 ppm is likely to relate to museum preservative treatment.

IHg preservation does not interfere with the selective measurement of MeHg in feathers. While INAA measures only total Hg, it is non-destructive and the proportions of IHg and MeHg can be calculated through subsequent GC-MS analysis. In any case, our samples did not show unusually high levels of Hg for those samples dating from before 1940. It seems safe to assume that the Hg observed is therefore organic and not derived from preservation methods. It is probable that Hg was not used in preservation of these museum samples (van der Mije, pers. comm.). Mashroofeh et al (2015), analysing Hg content of feathers from Iranian museums, assumed 85-90% of Hg in feathers to be MeHg and therefore analysed for total Hg.

MeHg concentrations in biota have increased due to anthropogenic Hg in many regions. Museum feathers from an endangered seabird from the North Pacific (the black-footed albatross *Phoebastria nigripes*) showed a sharp increase in MeHg concentrations after 1940 (Vo et al. 2011). Dietz et al. (2009) analysed museum samples from marine-food webs in the Arctic and observed that Hg concentrations in marine mammals and birds of prey began to rise in the mid- to late 19th century and accelerated in the 20th century. The authors attributed >90% of this rise to anthropogenic Hg sources (Sunderland & Selin 2013).

Hg is probably the metal of most concern for adverse biological effects (Walker et al. 2001) and some recent studies have documented increasing levels of Hg in biota, especially in the Arctic (Mallory and Braune 2012). In Sweden, rather constant levels of Hg have been found in birds of prey during the period 1840–1940. After the introduction of alkyl Hg compounds for seed dressing the concentrations increased significantly amounting to at least 10–20 times the previous level and decreased sharply after the Hg ban in 1966 (Berg et al. 1966; Westermarck et al. 1975; Johnels et al.

1979; Wallin 1984). In Finland, Hg levels in feathers of Eurasian sparrowhawks *Accipiter nisus* increased sharply in the 1960s and decreased thereafter slowly. In the Eurasian kestrel, Hg levels were high already in the 1950s but afterwards showed a similar decrease to that in the Eurasian sparrowhawk (Lodenius and Solonen 2013). In Central Norway, Bustnes et al. (2013) observed no temporal changes in Hg levels in tawny owl *Strix aluco* tail-feathers (n = 633) collected annually between 1986 and 2005.

Published results show rather variable concentrations of Hg in feathers for different species of birds of prey (see table 1 in the review of Lodenius and Solonen 2013). In terrestrial food chains Hg concentrations are often lower and Denneman and Douben (1993) concluded that barn owls from a polluted area in the Netherlands are not adversely affected by heavy metals (Lodenius and Solonen 2013).

Selenium

Our samples show an increase in Se values over time, with values reaching almost 2 mg/kg d.w. (3.01×10^{-7} mg/mm) in some feathers, levels associated with sub-lethal adverse effects in raptors. Burger and Gochfeld (2009) reported that for Se, feather levels of 3.8 to 26 mg/kg (depending upon species) result in mortality (converted after Burger 1993) and of 1.8 mg/kg result in sublethal adverse effects (after Heinz 1996). Our samples also show that feathers with higher levels of both Se and Ti, or with higher levels of Se and lower levels of Cu, are more likely to exhibit a fault bar, representing metabolic stress.

Ansara-Ross et al. (2013) reported selenium levels in owl breast feathers lower than those reported in feathers from the laggar falcon (0.95–5.2 mg/kg dw) (Movalli 2000) and bald eagles (0.8–3.2 mg/kg dw) (Bowerman et al. 1994).

The interrelationship of Hg and Se is complex, as each is known to ameliorate or decrease the toxic effect of the other (Falnoga et al. 2006; Komsta-Szumaska et al. 1983). Se binds and inactivates Hg (Fenstad et al 2017). For this reason, some authors have suggested that the molar ratio of Se to Hg may be an indicator of the potential protective effect of Se on Hg, with a ratio somewhere above 1:1 being protective (Ralston, 2008, 2009; Ralston et al., 2008 Burger et al. 2013). While Se acts as an antagonist to methyl Hg, there are indications that the amounts of Se in birds often are insufficient to bind all body Hg (Odsjö et al. 2004; Lodenius and Solonen 2013). In our samples, we found no correlation between Hg mass fractions and Se ratios ($r=0.245$, $p=0.360$ n.s.).

Cadmium

Cd values for some feathers exceed 1 mg/kg d.w. (3×10^{-7} mg/mm). Such levels may be associated with adverse effects (Burger and Gochfeld 2009). The highest levels of Cd were observed for the central time period 1947-1956 (*Figure 2*).

Cd is not a nutritionally essential element for animals (Furness 1996). In addition to being toxic above certain levels, Cd may induce deficiencies of essential elements through competition at active sites in biologically important molecules (Walker et al. 1996). Cd causes sub-lethal behavioural effects at lower concentrations than lead and mercury (Eisler 1985), but feather levels known to cause adverse effects in the birds themselves have not been determined from laboratory studies. However, conversion factors developed from Burger (1993) suggest that feather levels that are associated with adverse effects would range from 0.1 mg/kg (shearwaters) to 2 mg/kg (terns) (Burger and Gochfeld 2009). In birds, chronic exposure to Cd reduces reproductive success, e.g. by reducing Ca intake and egg production as well as increased susceptibility to stresses, diseases and oxidative and histopathological damage (Koivula & Eeva 2010).

Jager et al. (1996) analysed buzzards found dead in the Netherlands and found that most non-urban parts of the country were moderately contaminated with Cd, Cu and Pb; the North-South gradient in aerosol deposition and soil content of heavy metals (especially Cd and Pb) was reflected in the

common buzzard *Buteo buteo*. Hontelez et al. (1992) reported that several regions of The Netherlands near zinc smelters or dumping-grounds were polluted by Cd, Zn, Pb, Cu and analysis of soil, invertebrates and small mammals had shown a considerable contamination of Cd and Pb in particular areas (eg. Ma et al. 1991). We found a highly significant correlation between Cd and Zn levels ($r = 0.754$, $p = 0.0007$) suggesting that these two elements may have come from zinc smelters.

Zinc

We found Zn levels in the range 122 to 423 mg/kg d.w. (1.76 to 5.83×10^{-5} mg/mm). Ansara-Ross et al. (2013) found Zn levels in breast feathers of the African grass owl *Tyto capensis* and barn owl *T. alba* in South Africa in the range 16.9-103 and 6.11-34.1 mg/kg d.w. respectively. Movalli (2000) found Zn in the range 70-151 mg/kg in feathers of the laggar falcon *Falco biarmicus jugger* in Pakistan.

Zn is a trace element essential for proper body function (Movalli 2000) and is thought also to provide protection against the renal toxicity of Cd (e.g. Hutton 1981). Most animal species are tolerant of excessive intake of Zn (Goede 1985).

Platinum and Palladium

We found levels of Pt and Pd ranging from zero to 0.65 and 1.7 mg/kg d.w. (5.86×10^{-8} and 1.31×10^{-7} mg/mm) respectively, reaching similar levels at the upper end of this range to those found by Jensen et al. (2002) in Sweden. Jensen et al. (2002) found concentrations ranged from 0.3 to 1.8 mg/kg for Pt in three raptor species in Sweden and concluded that there is evidence for increasing platinum group element (PGE) concentrations from 1917 to 1999 in two of the species studied, the peregrine falcon *Falco peregrinus* and Eurasian sparrowhawk, reflecting the introduction of automobile catalyst exhaust systems. They also affirmed that PGE contamination of feathers is predominantly external in the form of nanometer-sized particles externally attached to the feather.

Until recently, it was believed that PGEs (Platinum group elements) are relatively inert, but it has now been shown that these metals undergo environmental transformations into more reactive species (Lustig et al. 1996, 1997, 1998; Rauch and Morrison 2000). The toxicity of Pt has been investigated in detail (Lindell 1997), but less is known about Pd and Rh. Recently, Pd has been increasingly favoured over Pt as the catalytic metal for oxidation in automobile catalysts (e.g. Palacios et al. 2000a, 2000b). This is a concern because it has been shown that Pd is more mobile in the environment than either Pt or Rh (e.g. Jarvis et al. 2001; Moldovan et al. 2001).

Aluminium

We found indications for Al levels in the range of 100 to 800 mg/kg d.w. (6.42×10^{-6} to 1.06×10^{-4} mg/mm). However, the related NAA signal can only be selectively assigned to Al if also the amounts of Si and P have been measured. The latter (P) can't be done by INAA and the Si amount is also difficult to measure in feathers. However, both interferences are only relevant for very high mass fraction ratios of P vs. Al and Si vs. Al. It has been assumed that for our study, the interferences can, for a first estimate, be neglected.

Ansara-Ross et al. (2013) found Al concentrations in South African owl feathers similar to *T. alba* and *A. noctua* from Belgium (13.7–49.5 mg/kg d.w.) (Dauwe et al. 2003). Bustnes et al. (2013) analysed different elements in tail feathers (only the lower shaft of the feather was used) of tawny owls in Central Norway from 1986 to 2005. In their samples Al showed decreasing levels until the mid-1990s, but a considerable increase afterwards (concentrations up to 146.1 mg/kg dry weight). They found this pattern of changes in Al concentrations difficult to interpret. Two strong drivers for increasing Al concentrations in Norwegian soil have been identified; sea salt deposition on land and high production of dissolved organic carbon (Lange et al. 2006). Events with high deposition of sea salt in soil in Norway are related to weather patterns such as storms, and possibly precipitation and

snow melt may lead to changes in the soil pH which again may lead to release of Al (Hindar et al. 2004; Lange et al. 2006). It is thus possible that variation in weather may have increased the availability of Al to voles and thence to owls since the mid-1990s.

Al has a ubiquitous natural distribution and use in cooking ware, cans, medications, food processing, and water purification ensure considerable human exposure. It is generally held that Al does not have a biological function. A potential role for Al in the pathogenesis of Alzheimer's disease has been postulated (Markesbery et al. 1981).

INAA is a sensitive method that requires no sample dissolution, chemical processing, or separations. Hence, the potential for reagent and laboratory contamination is minimal compared to other analytical techniques used for determining trace element content. This is especially important when dealing with an element such as Al, which is abundant in most laboratory ware and in common dust. V is a good indicator for oil burning, and both Al and V are common components of dust (Bortolotti and Barlow 1985) so it's important to use an effective method of laundering to remove external depositions of Al and V from feathers. Nuclear reaction interferences do exist, but with this technique they are precisely evaluated by independent determination of the interfering elements in the same sample (Markesbery et al. 1981).

According to some authors (eg. Steinnes 2009), for some elements such as Al and Rb, increased pH in the soil, for example through changes in precipitation will increase the release to the food chain (Nyholm and Tyler, 2000; Steinnes 2009; Bustnes and al. 2013).

Increased atmospheric deposition of sulphuric and nitrogenous compounds has been demonstrated in The Netherlands (e.g. Berendse and Aerts 1987; Roelofs et al. 1996), which alters the chemical status of the soil by increasing nitrogen availability, inducing soil acidification and mobilising toxic metal ions (e. g. Houdijk et al. 1993; De Graaf et al. 1998, Roem and Berendse 2000).

Rubidium

We found Rb levels from 0 to 1.975 mg/kg d.w. (6.79×10^{-8} to 1.21×10^{-7} mg/mm). This compares with values of 0.018 to 1.24 mg/kg in tawny owl 1986-2005 in the area surrounding Trondheim, Norway (Bustnes et al. 2013).

Rb is a metal that occurs naturally in soil and with high mobility thus readily available for plant uptake (Nygård et al. 2012). In Norway in the same species the same authors found that Rb increased by 61% over the 20 year period and, as Nyholm and Tyler (2000) found that low pH in forest soil increases uptake of Rb in plants, which is then propagated through the food web, these authors affirmed that both increased Al and Rb in tawny owl feathers may be an indication of lower pH values in the soil in the owl habitats.

Arsenic

Our samples show relatively high values for As for samples for the period from 1900-1930 (14.4 to 596.9 mg/mm d.w., 4.35×10^{-6} to 6.3×10^{-5} mg/mm), which may presumably be attributed to the use of arsenic in the early decades of the 20th century to treat and preserve museum specimens. Indeed, Desjardins (2016) using an XRF analyser found high levels of As in a range of mounted specimens and skins of various taxa in the collection of Naturalis (of 69 specimens examines, 33 had As levels over 1000 ppm). It is possible that relatively high levels of Ag and Au in these same samples is due to the presence of impurities in the arsenic compounds used to treat and preserve these feathers.

As, especially in its inorganic forms, can cause the death of an individual, produce sublethal effects and affect reproduction on birds. It accumulates mostly in body tissues such as liver and kidney, in particular in top predators (e.g. barn owl, sparrowhawk, kestrel) (Koivula and Eeva 2010).

Differences between species in accumulation of toxic elements have been explained by diet (Bezel' et al. 1994). Erry et al. (1999) suggested that As burdens can vary between raptor species according to variation in their diet and variation in the ability to metabolise and excrete As.

Antimony

Our levels are in the range 0 to 11.06 mg/kg d.w. (0 to 8.55×10^{-7} mg/mm). Ansara-Ross et al. (2013) found Sb levels in breast feathers of African grass owl and barn owl in South Africa in the range 0 - 0.724 and 0 - 1.29 mg/kg dw respectively. In The Netherlands, Denneman and Douben (1993) found a level of 22.1 mg/kg dw in barn owl feathers from a contaminated site and a level of 0.34 mg/kg dw for a relatively uncontaminated site.

Chromium

Cr values for 14 of the 32 feathers are above 3 mg/kg d.w. (5×10^{-7} mg/mm) with a highest value 20.65 mg/kg d.w. (2.3×10^{-6} mg/mm). Levels above 2.8 mg/kg may be associated with adverse effects (Burger and Gochfeld 2000). High amounts of Cr in a diet have been shown to cause altered growth patterns and reduction in survival, e.g. in the offspring of American black duck *Anas rubripes* (Eisler 2000).

Vanadium

Our values for V were in the range of 0.086 to 3.3 mg/kg d.w. (2.1×10^{-8} to 3.87×10^{-7} mg/mm). Bond and Lavers (2011) found Vanadium levels of 0.21-0.92 mg/kg in breast feathers of flesh-footed shearwaters *Puffinus carneipes*. Ansara-Ross et al. (2013) found 0.14 mg/kg d.w. in breast feathers of South African owls.

Little is known about the toxicity of V in birds but histopathological changes in the intestine, kidney, liver and heart have been detected in mallards and Canada geese. Blood chemistry changes, intestinal haemorrhage, indications of hepatic oxidative stress, liver lesions and kidney congestion have been shown in chronic feeding trials. Even low levels of V can diminish egg production and cause alteration in metabolic rates (laying hens and mallards) (Koivula and Eeva 2010).

Chlorine, Bromine

Haskins et al. (2013) using INAA found significant Cl and Br concentrations in feather samples from a turkey vulture *Cathartes aura* in Canada, being 1,600–7200 and 11–80 mg/kg, respectively (Haskins et al. 2013). Our levels were comparable to these levels, in the range 500 to 2760 mg/kg d.w. (1.5×10^{-5} to 3×10^{-4} mg/mm) and 3.6-160 mg/kg d.w. (5.14×10^{-7} to 1.73×10^{-5} mg/mm) for Cl and Br respectively.

INAA is a convenient and quick method, and indeed one of the very few analytical methods currently available for measuring the total amounts of Cl, Br and I. This characteristic has been used to assess simultaneously the amounts of extractable organochlorinated (EOCl), extractable organobrominated (EOBr) and extractable organoiodinated compounds (EOI) in one extraction (e.g. Xu et al. 2011).

CONCLUSIONS

This is the first study in Europe to use museum feather samples to analyse such an extensive range of elements, including many elements for which there are no or few published data. The study demonstrates the potential to exploit the raptor collection at Naturalis Biodiversity Center and other similar collections worldwide to explore temporal and geographic variation in levels of selected elements, including some important contaminants such as Hg and Cd. It has provided for the first time data that can contribute towards the development of reference values for contaminants in a raptor species in the Netherlands. It has demonstrated, *inter alia*, that:

- *Mass fraction values (mg/kg) and ratios (mg/mm) can be obtained for selected elements (including elements of high societal concern, such as Hg, Cd and Cs) using INAA with very small samples (2 breast feathers), such that sampling does not harm the aesthetic or research value of the sampled specimens.*
- *INAA analysis can detect very low mass fractions of elements in 1-2 breast feathers and can provide values for a wide range of elements in a single assay.*
- *Data from this study suggest that there are mass fractions and ratios of some elements (e.g. Cr, Cd, Se) for certain dates and locations that are at levels likely to have had adverse effects on kestrel health and reproduction.*
- *It would be possible, given a larger, statistically valid sample size, to examine temporal patterns for the mass fraction values (mg/kg) and ratios (mg/mm) of selected elements in a given geographical area (e.g. the whole of Netherlands, or selected regions within The Netherlands).*
- *The analysis of museum feather specimens using INAA can provide reference values for mass fractions (mg/kg) and/or ratios (mg/mm) of selected elements (including elements of high societal concern such as Hg and Cd), against which to assess the mass fractions of these same elements in present-day living raptor populations.*
- *Such contaminant studies are best suited to common, widely distributed raptor species which meet the following criteria: species (and sex) that is resident and with high territorial fidelity (as opposed to migratory or vagrant) such that any contaminants found may reasonably be assumed to reflect exposure in the location from which the specimen was originally collected; relatively common and widely distributed species with a large number of specimens in the museum collection, allowing for statistically robust sampling for selected time periods and/or selected geographical areas; likely dietary exposure to a range of contaminants of societal concern; species that is widespread in Europe and relatively frequently studied, providing data with which to compare results; possibility of sampling present-day populations to compare present-day with historical contaminant levels.*

In conclusion, this study has shown that INAA, using very small feather samples from museum specimens, delivers data on element mass fractions (mg/kg) and ratios (mg/mm) for a wide range of elements in a single assay, allowing the testing of various hypotheses related to patterns of contamination in time and space, and providing reference values against which to assess contaminant levels in present-day living raptor populations. This can have great relevance for the monitoring of elements of high societal concern, including mercury and cadmium.

Enhanced collaboration between ecotoxicologists and museums (and, indeed, environmental specimen banks and other collections) on contaminants in birds offers an important new direction for research using these collections (Movalli et al in press). With planning, use of birds (and indeed other higher taxa) as biomonitors can be coupled with standard museum processing and preservation to simultaneously achieve very different scientific gains (Rocque and Winker 2005). Increasingly refined analytical abilities will continue to enhance the usefulness of museum specimens for contaminant studies as new techniques such as INAA reduce the amount of sample required for analyses.

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ANNEX 1a

INAA RESULTS, DATA TRANSFORMATION AND GRAPHS FOR MASS-DEPENDENT ELEMENT DEPOSITION IN FEATHERS

See spreadsheet <Annex 1b Results & Graphs - Mass-dependent FINAL 30Apr17.xls>

Explanation of spreadsheet data and graphs

- **Tab 'INAA RESULTS'**: INAA results (mg/kg)
- **TAB 'DATA mg per kg d.w.'**: data (mg/kg)
- **TAB 'Specimen AVGs'**: average mass fraction (mg/kg) for each bird, calculated as the average ratio for the two feathers sampled from each bird
- **TAB 'Pivot GEO'**: Average mass fraction (mg/kg) for each geographical area
- **TAB 'Distr TIME'**: Graphs showing the distribution of specimen average mass fractions over time
- **TAB 'Distr GEO'**: Graphs showing the distribution of the specimen average mass fractions by geographic area (1 = north, 2 = centre, 3 = south, 4 = inner)

ANNEX 1b

INAA RESULTS, DATA TRANSFORMATION AND GRAPHS FOR TIME-DEPENDENT ELEMENT DEPOSITION IN FEATHERS

See spreadsheet <Annex 1b Results & Graphs - Time-dependent FINAL 30Apr17.xls>

Explanation of spreadsheet data and graphs

- **Tab 'INAA RESULTS'**: INAA results (mg/kg)
- **TAB 'RATIOS'**: ratios (mg/mm) of element mass per feather length, calculated as: $(\text{element mass mg/kg} \times \text{feather mass kg}) / (\text{feather length mm})$
- **TAB 'Specimen AVGs'**: average ratio (mg/mm) for each bird, calculated as the average ratio for the two feathers sampled from each bird
- **TAB 'Pivot GEO'**: Average ratios (mg/mm) for each geographical area
- **TAB 'Distr TIME'**: Graphs showing the distribution of specimen average ratios over time
- **TAB 'Distr GEO'**: Graphs showing the distribution of the specimen average ratios by geographic area (1 = north, 2 = centre, 3 = south, 4 = inner)