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Master in Marine and Coastal Systems
Work performed under the supervision of: Maria João Bebianno

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“Declaração de autoria de trabalho”

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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RESUMO

As aves marinhas são indicadores muito úteis e fiáveis da poluição ambiental e permitem aos investigadores estudar a dinâmica dos contaminantes nos ecossistemas marinhos. Mais especificamente, as aves marinhas podem ser bioindicadores adequados para avaliar as condições ambientais que caracterizam as áreas de estudo. As suas características ecológicas correspondem de forma imediata e intensa às mudanças ambientais e as dietas, dois parâmetros que são fundamentais para atribuí-los como bioindicadores. As espécies de aves marinhas podem ser usadas também como indicadores dos recursos pesqueiros, uma vez que a composição da dieta reflete a disponibilidade de presas e a flutuação da abundancia das mesmas, o que também pode alterar a estratégia de procura de alimento das aves marinhas e seu padrão de distribuição. Adicionalmente, as potenciais flutuações dos stocks haliêuticos podem causar o declínio significativo das populações de aves marinhas, em particular aquelas constituídas por espécies piscívoras generalistas ou especializadas. Como consequência, as aves marinhas são um grupo taxonomicamente variado sendo o indicador mais sensível entre a avifauna para monitorizar as mudanças ambientais causadas por forças naturais e ou atividades humanas. Informações sobre mudanças nos stocks de pescado e atividades piscatórias, como a sobre-exploração das pescarias e o fornecimento de capturas acessórias descartáveis também podem ser obtidas através do estudo do comportamento das aves marinhas, mas requerem conhecimento detalhado da biologia das mesmas e a sua correspondência com as mudanças no fornecimento de alimentos. No entanto, é importante ressaltar que os dados referentes às quantidades e à composição dos itens descartados e potencialmente utilizados como fonte de alimento adicional de aves marinhas, são muito limitados no Mediterrâneo Oriental. Além disso, as aves marinhas fazem parte do topo da cadeia de predadores marinhos, dependendo exclusivamente de ambientes aquáticos em que enfrentam riscos tóxicos por contato externo, por inalação e, particularmente, por ingestão de alimentos e água. Assim, as aves marinhas com uma longevidade longa podem atuar como um bioindicador de poluição química de diferentes origens, uma vez que acumulam uma ampla gama de componentes químicos e outras formas de poluentes marinhos. Consequentemente, essas espécies oferecem oportunidades para detetar e avaliar a longo prazo, efeitos toxicológicos dos poluentes nos ecossistemas marinhos. Por exemplo, uma das suas características ecológicas que pode ser afetada

pela poluição química, é o tamanho da população que varia ligeiramente de ano para ano. As taxas de crescimento dos juvenis, o sucesso da incubação, o sucesso da reprodução e qualquer mudança de comportamento durante o período de incubação podem ser afetados pela contaminação tóxica de poluentes nas áreas de alimentação utilizadas durante a época de reprodução. Muitas espécies de aves marinhas acumulam altas concentrações de contaminantes no organismo. Tendo em consideração que estão no topo da cadeia trófica, as aves marinhas são “monitores” adequadas de poluentes que se amplificam através da cadeia trófica. Além disso, já foi provado que são bioindicadores de poluentes metálicos, utilizados para a determinação da origem do mercúrio em níveis tróficos marinhos. Concentrações de metais são frequentemente relatadas para aves adultas, mas menos frequentemente para juvenis ou crias. No entanto, os juvenis foram propostos como indicadores particularmente úteis para a poluição pois concentram metais durante um período de tempo específico em áreas de alimentação definidas em torno da colônia. As espécies-alvo, nomeadamente a Shearwater de Scopoli (Calonectris diomedea), são aves marinhas migratórias pelágicas, de grande longevidade, com um grau particularmente alto de tenacidade no local e fidelidade ao companheiro. Caracteriza-se como uma espécie deambulante de acordo com sua distribuição pelágica e abrangente. Mais especificamente, os locais de reprodução desta espécie estão localizados na bacia do Mediterrâneo, enquanto os seus terrenos de invernagem estão localizados nas áreas equatoriais pelágica e costeira do Atlântico Este. A dieta inclui principalmente peixes pelágicos e meso pelágicos, lulas, crustáceos e ocasionalmente zooplâncton. Estes organismos marinhos podem ser encontrados em águas rasas e perto de recifes, e constituem a presa mais comum capturada em águas pouco profundas ou perto da superfície do mar. A Grécia hospeda um número significativo de colônias de Shearwater de Scopoli em ilhas e ilhotas desabitadas, espalhadas principalmente no Mar Egeu central e meridional, no Mar Jónico do Sul e do Norte e em torno de Creta. A área de estudo deste trabalho, o complexo da Ilha de Strofades (37 ° 15 'N, 21 ° 00' E) é um grupo remoto de duas pequenas ilhotas baixas e várias rochas, localizadas no Mar Jónico do Sul, 32 milhas náuticas ao sul da Ilha de Zakynthos e 26 milhas náuticas a oeste do Peloponeso. As duas ilhotas principais (Stamfani e Arpyia) são a principal colônia grega da espécie (cerca de 5.550 casais de reprodutores), cobrem uma área de 4 km2 e constituem parte do Parque Nacional Marinho de Zakynthos. Os dados de contaminação por metais em aves marinhas originadas nas Ilhas Strofades são escassos. Um dos objetivos deste estudo será fornecer dados de referencia para os níveis de concentração de metais na Shearwater de Scopoli recolhidos em uma das maiores colônias na Grécia para análises comparativas de diferenças interespecíficas e geográficas em bioacumulação.

No presente estudo, os níveis de cádmio (Cd), chumbo (Pb), cobre (Cu), cobalto (Co), níquel (Ni), manganês (Mn) e zinco (Zn) foram investigados como potenciais fatores de stress químico no sangue de juvenis e adultos Scopoli's Shearwaters no complexo da colonia da ilha Strofades, na Grécia. Foram recolhidas amostras de sangue de juvenis e indivíduos Shearwaters de Scopoli adultos, em 7 épocas de reprodução diferentes entre 2007 e 2014. As amostras foram recolhidas em três sub-colonias da parte oeste, sul e leste da ilha de Stamfani, onde as crias e os reprodutores foram capturados à mão. Foi recolhida uma quantidade de 0,2-0,5 ml de sangue em cada exemplar usando uma seringa com heparina e mantendo em frascos eppendorf com álcool etílico como conservante. Foram recolhidas um total de 238 amostras, das quais 182 eram de juvenis. O processo de análise de metais nas amostras de sangue envolveu a preparação de amostras, digestão e quantificação de cada metal. A digestão foi realizada com placa quente e a quantificação do conteúdo metálico foi realizada por espectroscopia de absorção atômica em forno de grafite. A precisão do método foi verificada analisando duplicados com a mesma quantidade de material de referência certificado de soro sanguíneo. Os brancos de reagente também foram preparados com solução de água Milli-Q acidificada com 0,2% de HNO3 e tratados da mesma maneira para verificar qualquer contaminação. As diferenças estatísticas das concentrações de metal foram investigadas e correlacionadas com idade, gênero e local de nidificação. Entre os metais tóxicos, o cádmio (Cd) variou entre 0,01-0,02 µg /g e chumbo (Pb) entre 0,07-0,53 µg/g, próximo dos padrões europeus. As concentrações de metais essenciais foram geralmente baixas , em comparação com outros estudos. A análise estatística em juvenis e adultos revelou que, em relação à massa corporal, não há relação significativa entre o peso de indivíduos e a concentração de metais. Todos os metais apresentaram uma diferença significativa com os anos. Os resultados estatísticos indicam diferenças entre sexos significativas para Cd, Co, Mn, Pb e Zn para jovens e adultos. O teste de correlação com os locais de nidificação mostra diferenças significativas para a maioria dos metais testados (Cd, Co, Cr, Mn, Ni, Pb e Zn). A Análise

de Componentes Principais (PCA) foi utilizada para detetar as relações entre as concentrações de metal (Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn), ano, sexo e local para aves juvenis e adultas da colônia da Ilha de Strofades. Os resultados da PCA mostraram 34,82% de variância, com PC1 representando (18,76%) e PC2 (16,05%). Os níveis de metais testados não parecem ter efeitos adversos na população estudada, mas a análise da dieta das espécies será útil para uma melhor compreensão da absorção dos metais.

Abstract

Seabirds are very useful and trustful indicators of environmental pollution and allow researchers to study the dynamics of contaminants in marine ecosystems. More specifically, marine birds can be suitable bioindicators to assess the environmental conditions which characterize areas of interest. Their ecological features correspond immediately and intensely to environmental changes and food supplies, two parameters that are fundamental to assign them as bioindicators. Seabird species can be used also as indicators of fishery supplies since diet composition reflects prey availability and abundance fluctuation of prey appearance, which may also alter seabirds foraging strategy and their distribution pattern. Additionally, potential fluctuations of fish stocks may cause significant population decline to seabirds, specifically to those which constitute generalist or specialist piscivorous species . As a consequence, seabirds is a taxonomically varied group which constitutes the most sensitive indicator between avifauna for monitoring environmental changes caused by natural forces and/or human activities . Information regarding fish stock changes and piscatorial activities such as fisheries overexploitation and supply of discarded unsaleable bycatch can also be obtained by studying seabird behavior, but requires detailed knowledge of marine birds biology and their correspondence to food supply changes. However it is important to highlight that data related to the quantities and the composition of items discarded and potentially used as an additional food source for scavenging seabirds, are very limited in the Eastern Mediterranean Sea. Moreover, seabirds constitute top marine predators, depended exclusively on aquatic environments where they face toxic risk by external contact, by inhalation, and particularly by ingestion of food and water. Thus, long-lived seabirds may act as a bio-indicator for chemical pollution of different origin since they accumulate a wide range of chemical components and other forms of marine pollutants Consequently, these species offer opportunities to detect and assess in a long term basis, toxicological effects of pollutants in marine ecosystems. For example, one of their ecological features that could be affected by chemical pollution, is their population size that varies slightly between years. Juvenile growth rates, hatching success, breeding success, and any behavior change during the incubation period, can be affected by toxic pollutant

contamination in the marine foraging areas used during breeding season. Many seabird species accumulate high concentrations of contaminants in their organism. Taking into consideration that are in the highest place of trophic chain, seabirds are suitable “screens” of pollutants that are being amplified via the trophic chain. Also, it has been already proved that they are bioindicators of metal pollutants and used for the determination of origin of mercury in marine trophic levels. Concentrations of metals are often reported for adult birds, but less often for chicks or fledglings. However, juveniles have been proposed as particularly useful indicators for pollution, as they concentrate metals during a specific period of time and from local and definable foraging areas around colony. The target species, namely Scopoli’s Shearwater (*Calonectris diomedea*) is a pelagic, long lived, migratory seabird, with particularly high degree of site tenacity and mate fidelity. It is characterized as a highly vagrant species according to its pelagic and wide-ranging distribution. More specifically, the breeding sites of this species are located in the Mediterranean basin whereas its wintering grounds are located in the pelagic and coastal equatorial areas of the eastern Atlantic. Its diet mainly includes pelagic and mesopelagic fishes, squids, crustaceans and occasionally zooplankton. These marine organisms can be found in shallow waters and near reefs, and constitute the most common prey caught in shallow waters or near the sea surface. Greece hosts a significant number of Scopoli’s Shearwater colonies on uninhabited islets, spread mainly in central and southern Aegean Sea, southern and northern Ionian Sea and around Crete. Our study area, the Strofades Island complex (37° 15’ N, 21° 00’ E) is a remote group of two small low islets and several rocks, located in the southern Ionian Sea, 32 nm south of Zakynthos Island and 26 nm west of the Peloponnese. The two main islets (Stamfani and Arpyia) host the largest greek colony of the species (about 5,550 breeding pairs), cover an area of 4 km^2 and constitute part of the National Marine Park of Zakynthos. Data for metal contamination in seabirds originated from the Strofades Islands are barely exist. One of the aims of this study will be to provide baseline data for metal concentration levels in Scopoli’s Shearwater collected in one of the biggest colonies in Greece for comparative analyses of inter-specific and geographical differences in bioaccumulation.

In the present study levels of cadmium (Cd), lead (Pb), copper (Cu), cobalt (Co), nickel (Ni), manganese (Mn) and zinc (Zn) were investigated as potential chemical stressors in the blood of juveniles and adults Scopoli's Shearwaters in Strofades Island complex colony, in Greece. Blood samples were collected from both juveniles and adult Scopoli's Shearwaters individuals, during 7 different breeding seasons between 2007 and 2014. The samples were collected in three sub-colonies from the western, southern and eastern part of the Stamfani Island, where fledglings and breeders were captured by hand. An amount of 0.2-0.5 ml per blood sample was collected using a heparinsed syringe and kept in eppendorf vials with ethyl alcohol as preservative. A total number of 238 samples were collected, from which 182 were from juveniles. The process of metal analysis of blood samples involved sample preparation, digestion and metal quantification. The digestion was performed using hot plate and the quantification of metal content was performed by Graphite furnace atomic absorption spectroscopy. The accuracy of the method was verified by analyzing duplicates of the same amount of certified reference material of blood serum. Blanks were also prepared with and acidified Milli-Q water solution with 0.2% HNO_3 and treated in the same way to check for any contamination. Statistical differences of metal concentrations were investigated along with relationships with age, gender and nesting site. From toxic metals, cadmium (Cd) ranged between 0.01-0.02 µg/g and lead (Pb) between 0.07-0.53 µg/g, which were close to the respective European standards. Concentrations of essential metals were generally closed to basal levels, compared with other studies. Statistical analysis in juveniles and adults revealed that in relation to body mass there is no significant relationship between the weight of individuals and metals. All metals showed a significant difference with the years. Statistical results indicate significant sexual differences for Cd, Co, Mn, Pb and Zn for both juveniles and adults. Testing for correlation with nesting sites show significant differences for most of the tested metals (Cd, Co, Cr, Mn, Ni, Pb and Zn). Principal Component Analysis (PCA) were used to detect the relationships between metal concentrations (Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn), year, sex and site of for juvenile and adult birds from Strofades Island colony. Results of PCA showed that they represent 34.82% of variance with PC1 representing (18.76%) and PC2 (16.05%). Metal levels tested do not seem to have adverse effects on studied population, but diet analysis of the species will be helpful towards the better understanding of metal uptake.

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1. General Ecology of Seabirds

The interaction between seabirds and marine environment occurs in different levels, from the lowest up to superior levels of the ecosystem. This is One of the appreciable criteria that render them as reliable candidates to monitor the conditions and health of the marine environment. Generally, the ecology of these birds is well known, as well as their characteristics, population numbers and also their colonialist actions and reproductive processes, that constitutes a good occasion for monitoring high numbers of individuals (Piatt, 2007, Furness and Camphuysen, 1997).

More specifically, marine birds can be suitable bioindicators to assess the environmental conditions and characterize the areas of interest. Their characteristics correspond immediately and intensely at environmental changes and food supplies, two parameters that are fundamental to assign them as bioindicators (Sponza et al., 2010). Juvenile growth rates, incubation success, birth success, nesting process and any behavior changes during the incubation period, can be affected by toxic pollutant contamination in the system. Seabird species can be used also as indicators of fishery supplies, by monitoring their searching behavior for preys, because they reflect the prey availability and abundance fluctuation of prey appearance, which influences their diet strategies (Sponza et al., 2010). One of their characteristics that could be affected, is their population size that varies slightly between years.

As an example, disruption of reproduction can occur for European Shag (*Phalacrocorax aristotelis*) in periods of constrained food supply and consequently it abstains from the reproductive process during those years (Furness and Camphuysen, 1997). This disruption can also be observed in seabird populations, when fish stocks are deplete (Piatt, 2007). Additionally, potential fluctuations of fish stocks supplies, can change the number of seabirds (Velarde, 1994), because they have specific diet. As a rule, seabirds are the most sensitive indicators in-between bird species (Furness and Camphuysen, 1997). Moreover, chemical components of the total trophic chain can be taken up from the feeding of seabirds with various levels of the trophic chain. With observations on seabird behavior, their feeding preferences and the characteristics of their population, we can determine if the fish stocks have suffered any fluctuation (Piatt, 2007). Information regarding fish stock changes and piscatorial activities can also be obtained, by studying seabird behavior (Furness and Camphuysen, 1997), but requires in detail knowledge of bird biology and their correspondence to food supply changes. As an example, seabirds that “scan” for finding food, as the Yellow-legged gull (*Larus michahellis*), feeds also on fishes that are rejected by the trawlers, and as a result, piscatorial activity on each area can be evaluated (Gonzalez - Solis, 2003, Ramos et al., 2009). It is important to highlight that the data are related with the quantities and the composition of prey rejects are very limited in Mediterranean Sea. This method of study can lead to a better understanding of way that fishery is affected.

Taking into consideration that are in the highest place of trophic chain, seabirds are suitable “screens” of pollutants that are being amplified via the trophic chain (Furness and Camphuysen, 1997). Also, it has been already proved that they are bioindicators of metal pollutants (eg. mercury) and used for the determination of origin of mercury in marine trophic levels (Furness and Camphuysen, 1997). Seabird feathers are a useful tool to asses air pollution. Also by mercury ingestion they can easily locate the region where the pollution occurred, depended on the type of feathers (the different types of feathers are connected with the seasons and movements of birds between reproductive and not reproductive period) (Furness and Camphuysen, 1997, Arcos et al., 2002, Ramos et al., 2009). Also, monitoring other pollutants can be achieved with the analysis of their concentrations in eggshells.

1.1 Scopoli’s Shearwater

Figure 1: Adult Scopoli’s Shearwater. Photo by the author

The mediterranean Scopoli’s Shearwater, *Calonectris diomedea diomedea (Figure 1)*, constitutes one of the most characteristic types of fauna in the Mediterranean and Greek seas. It is a pelagic, long lived, monogamist, migratory seabird, with particularly high degree of site tenacity (Thibault et al., 1997). It has an

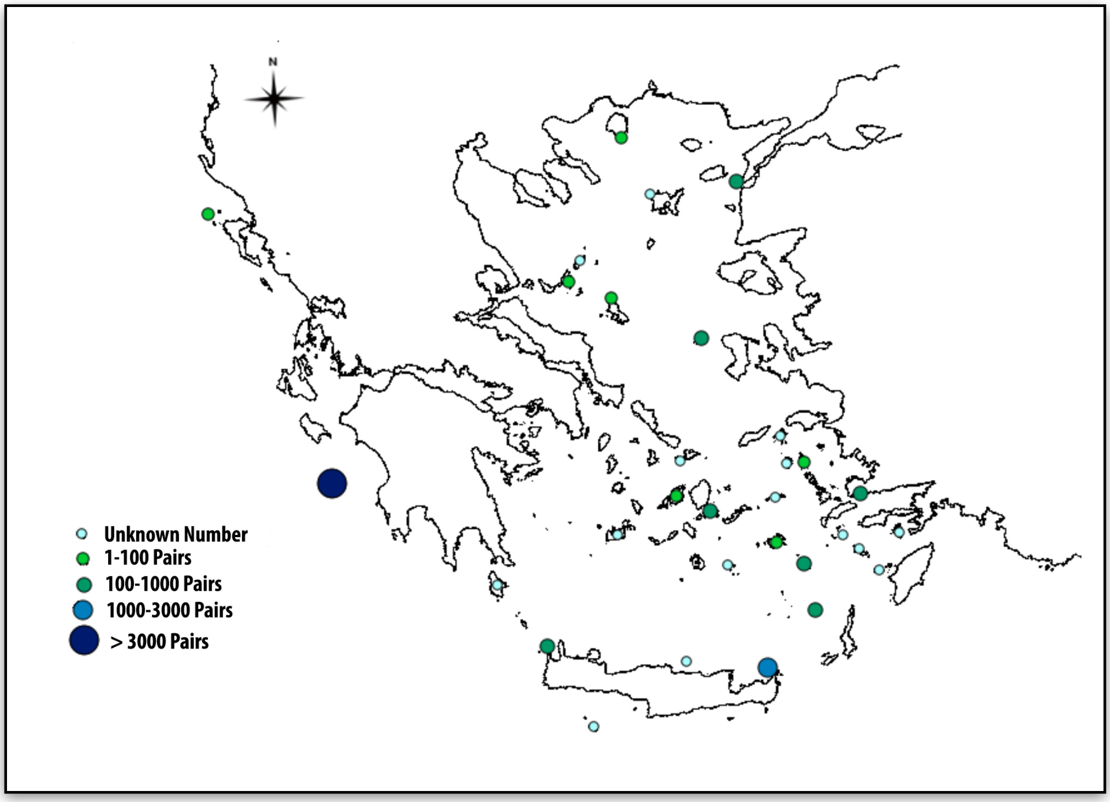

Figure 2: Chart of distribution and size of Scopoli's Shearwater colonies in Aegean and Ionian Sea. *Birdlife International 2004, Handrinos & Akriotis 1997, Ristow et al. 1991, Karris et al. 2010*

aerodynamic form with long and narrow wings. On the upper side, it has a coffee coloration and white on the under part. It's bill is powerful and yellowish. It's easy for someone to confuse them with a young gull but it can be distinguished during flight while the men gull strikes often it's wings and Scopoli's Shearwater can maneuvers easily between the waves without using them. In order to fly comfortably, Scopoli's Shearwater the wind conditions should be higher than calm, which means 20-27 km/h. In total calm conditions it has serious flying problems and strikes his wings to fly. They fly usually low, close to water surface skulking the fishes that are frighten by the boat propellers.

Scopoli's Shearwater's diet is constituted from pelagic and mesopelagic fishes, squids, crustaceans and occasionally zooplankton (Monteiro et al 1996). These and other small fishes that are found in shallow waters and near reefs, are the most common prey and they are caught when they are found in shallow waters or near the sea surface (Granadeiro et al., 1998).

According to Cramp and Simmons (1977) and Belda and Sánchez (2001), Scopoli's Shearwater prey on the day but also in the night or at dawn as in Balearic Islands. In Strofades Islands, Karris et al. (2009 and 2010), found that during the nightly hours (00:00 – 05:00am) the Shearwaters spend most of their time searching for preys, while the morning hours up to midday spend the bigger part for their movings. It was found that a marked Shearwater from Strofades Islands colony, covered a distance of 142,4 km and an area of 580 km^2 within four days, for prey searching (Karris et al., 2010). Studies concluded that, shearwaters can use for their food search an extensive region if the fish stocks are limited close to their colony (Mougin and Jouanin 1977, Karris et al., 2010).

In Greece, Shearwaters are nesting in islands, mainly in central and southern Aegean Sea, southern Ionian Sea and around Crete. Practically, is possible to see them everywhere, because they daily cover long distances and fly far away from their colonies. The European population of Shearwaters is estimated in 270,000 - 290,000 breeding pairs that correspond to 810,000 - 870,000 individuals, by which 234,500 - 243,500 are breeding in Atlantic Ocean and 35,000 - 55,000 in Mediterranean Sea (BirdLife International 2004a). A percentage of 75 - 94% of world distribution of the species is located in European territory, therefore an initial estimate of world population of the species is approximately 900,000 - 1.200,000 individuals. Mediterranean colonies are usually found in inhabited rocky islands and are relatively small (Ristow et al., 1991). Greek population is estimated in 4,400 - 5,000 pairs distributed between 29 colonies in Aegean Sea and Ionian Sea (*Figure 2*) (Birdlife International 2004, Handrinos and Akriotis 1997). New data from Strofades Islands colony increased the Greek population to 8,400 - 10,000 pairs, while only the Strofades Islands colony numbers 5,000 - 6,000 pairs and is the biggest colony of Mediterranean Sea (Karris et al., 2010).

Shearwater is a migratory species. Arrives in Mediterranean Sea on March and leaves on October. Migrates in long distances and most of the individuals cover distances between 15.000 and 35.000 km annually, between two reproductive periods (González-Solís et al., 2007). The migration take place in regions southern from Equator, during the winter period of northern hemisphere. Shearwaters travel using the wind forces, saving great amount of energy (González-Solís et al., 2007). They spend on average 80±17 days, in winter regions and 19±10 and 23±7 days for their travel in fall and in spring respectively, time that is equivalent with an important percentage of their annual cycle. Two of the greatest Shearwater populations, overwinter following the souther Brazil and Benguela currents, an important percentage (29%) of the Mediterranean population (González-Solís et al., 2007).

Figure 3: Pair of Scopoli's Shearwaters in the nest, during

Figure 4: Scopoli's Shearwater chick. Photo by the author

Scopoli's Shearwater is a socially monogamic species. Adults (> 5 years) visit the land only in order to breed in isolated islands, sometimes in dense colonies but more often in smaller and scattered groups. The age of an important percentage species population exceeds 20 years according to banding (marked birds using a unique numbered metal ring) bird trapped in Crete Island, indicate that female individuals live more than males (Ristow et al., 1991, Ristow et al., 1992). They are strictly nocturnal birds in their colonies, with the exception of the Selvagem Grande colony, where they come on land a few hours before sunset (Zino 1971). Also they are nesting in relatively spacious cavities, without any type of defense or natural protection from predators (Catry et al., 2006). Before the breeding period, males play an important role of securing the nest and spending more time keeping it safe (Jouanin et al., 2001), while on the other hand female take the responsibility of the egg production. During the incubation period both males and females spend the same time during the process by changing shifts (*Figure 3*). The complete fledge of the nestlings begins from 8 - 9 of October and lasts until the last week of the same month (Round and Swann 1977), thus the fledging period lasts 83 - 98 days and is relatively short for such size of seabird. The reproductive period begins in March, with the return of adults from the wintering areas in central and southern Atlantic (Ristow et al., 2000, Camphuysen and Van der Meer 2001) to the breeding colonies.

The reproductive success is related with the egg size. Bigger eggs produce nestlings and complete fledgling juveniles (*Figure 4*) more frequent than that the small eggs (Mougin 1998, Ramos et al., 1997). This shows that a connection exists between reproductive characteristics and the physical condition of parents (Ramos et al., 2003). Other important characteristic is the reproductive success it is that most casualties are taking place during the egg phase, with fledge success be always much higher than the hatching success (Ramos et al., 2003). At the end of May, females gives birth an egg which fledges for roughly 52 days. The chick hatches in middle of July and fledging until the end of October when the migration period starts for the wintering areas. The first return of the juveniles in the colony becomes after 4 - 5 years.

The reproduction habitat is the basic parameter that determines the reproductive success. The intraspecific competition for the nesting areas is intense in Shearwaters, as the high levels of daily presence in the nests show, as well as the territory behavior at the period of three months before spawning (Monteiro et al., 1996). The importance of maintenance the nesting place and it's defense, is indicated by the general bad physical condition of adult birds (as it appears from their body mass) in the period before spawning, something that is usual in species that maintain the same place of nesting for years (Monteiro et al., 1996).

1.2 Species Threats

In European level, the species are included in the Part I of European Directive 79/409 for the protection of wild birds, in Part II on Bern Convention on the conservation of European wildlife and natural habitats, while it was also considered as "Vulnerable" according to European threat regime (BirdLife International 2004a). Globally, the species is reported of IUCN[1] as LC[2] (Least Concern).

In national level Shearwater has not been counted in the recent publication of Greek “Red Book”[3] of species because is still maintains rather good populations or even have wide distribution in Greek area and thus they do not appear at the present moment to face problems of extinction (Legakis and Maragkou 2009).

Anselme and Durand (2012) categorized the basic threats for the species in two main levels. That ones that act outside the reproductive areas (colonies) and the in situ threats: In the first category we have those where they have direct or indirect cross-correlation with piscatorial activities that are taking place in the feeding areas and phenomena that are related with climatic changes and pollution cases in the marine ecosystem (Stewart et al., 1997). The second category refers to the in situ threats, that occur in the species colony. The major predators in land are the European wild cats (*Felix silvestris*), Black rats (*Rattus rattus*), European rabbits (*Oryctolagus cuniculus*) and European pine martens (*Martes martes*). Also predating pressure can be applied to eggs, nestlings and also in adults by other bird species such as the Yellow - legged gull (*Larus michahellis*) and the Eurasian eagle - owl (*Bubo bubo*) and Peregrines (*Falco peregrinus*). Notable are also the effects of anthropogenic activities such as public nuisance, the uncontrolled touristic activity and the lights that turned on during evening hours. These have deterrent effects on the adults to visit their nests. Finally, recent studies (Karris et al., 2013, Petrella 2011) that took place in Strofades Islands, showed that a notable threat is the accidental entrapment (by-catch) of Scopoli’s Shearwaters in piscatorial tools, such as trawl line and driftnet.

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[1] IUCN: International Union for Conservation of Nature

[2] LC: In low risk for extinction and is not considered on a red listed category by the IUCN

[3] Red Book of Species: Red Data Book of International Union for Conservation of Nature. List of critically endangered species

2. Study area of Strofades Island complex

Strofades (37°15'10.8"N 21°00’24.2”E) are a complex of two small islands in the ionian Sea, in Greece. They are located between the southern part of Zakynthos Island and the western coasts of Peloponnese (*Figure 5*). They have an extent of 2,6 km^2. The bigger and southerner of the two islands is named Stamfani. It is a rocky island with length about 1 km. The smaller one is called Arpyia. The islands are 27 nautical miles from the southern point of Zakynthos Island and 28 nautical miles from the coasts of Peloponnese.

In the highest part of western nesting site is found a light house that was builded during 20th century and was handled by a lighthouse keeper. Back in time, the islands were drowned in vegetation and the monks that where resided, in order to survive, used the 2/3 of Stamfani Island as farms. They were also breeding ruminant mammals as a food source.

Strofades Islands are enlisted in the Important Bird Areas[4] inventory (IBA) and they have been included in the Corine program as important biotopes and constituting a part

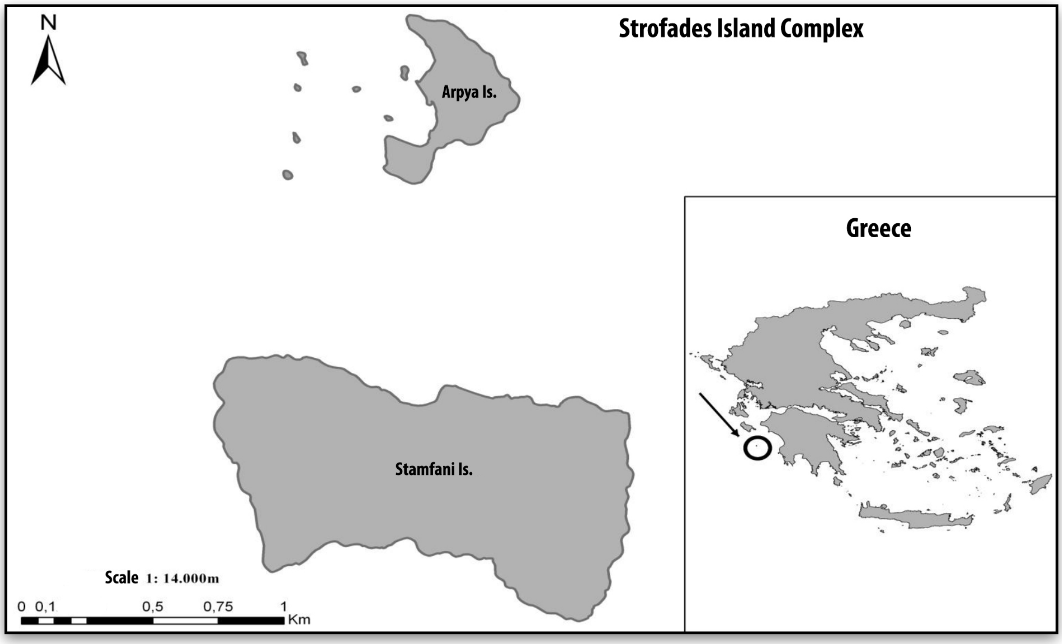

Figure 5: Strofades Island complex location, Southern Ionian Sea, Greece. Map by the author

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[4] Important Bird Area (IBA): Area identified using an internationally agreed set of criteria as being globally important for the conservation of bird populations

of the National Marine Park of Zakynthos Island. These two small islands, that are located in Ionian Sea, as two small dots, and are integral part of the Greek natural and cultural legacy with special interest and needs of management and environmental protection.

The island ecosystem is very rich in species diversity. This can be reflected by the different herpetofauna species, such as Lizards (*Algyroides moreoticuspoy*), Cat snakes (*Telescopus fallax*) and species that are in protection, such as Sea turtles (*Caretta caretta*), Mediterranean seals (*Monachus monachus*) and other marine mammals.

Strofades Islands have a very big importance on bird migration. In spring they stop for resting thousands of migratory birds that come from Africa. They begin to arrive the most interesting birds of Greek avifauna such us the European Bee-eater (*Merops apiaster*), Hoopoe (*Upupa epops*), Red-backed Shrike (*Lanius collurio*) and Nightingales. Until today they have been observed more than 120 species of birds, based on bird banding[5] projects that taking place every Spring in Stamfani island.

If somebody in any case wanted to give at an avifauna species symbolic value for Strofades Islands, this should be the Scopoli's Shearwater (*Calonectris diomedea*), which makes it's nests under dense bushy vegetation near the coasts.

Endemically, Stamfani Island is in the same category as Zakynthos and Cefalonia islands. Also, the islands are connected phytogeographical with Peloponnese. The forest of Strofades Islands, located in Stamfani island, is unique in Greece and perhaps in all Mediterranean Sea. The forestial ecosystem characterized by various bushy sclerophyllous species as Oaks (*Quercus coccifera*), Shrubs (*Pistacia lentiscus*) and olive trees. Is dense in many points and sometimes the sun can not even reach the ground. Arpyia island is covered by maquis vegetation covered by *Pistacia* bushes, Myrtles and Olive trees. Also, more than 300 species of plants are located in the islands, such as the uncommon orchid of Ionian Islands (*Serapias ionica*).

3. Metals and Seabirds

Seabirds are widely used to monitor trace element levels due to their wide distribution and high position in the food web (Piatt et al. 2007). There is evidence that metal concentration levels are increasing in some areas due to human activities (Nriagu 1988; Slemr and Langer 1992; Thompson et al., 1992). Toxic metals, even at relatively low levels, are known to accumulate in many marine animals, including seabirds, although

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[5] Bird Banding: Marking birds by applying a metal ring on their tarsus, with a unique number

they have no known biological function (Thompson 1990).From both a biomonitoring and toxicological viewpoint it is important to understand the physiological processes and dynamics of nonessential metals in the organs and tissues of seabirds (Stewart et al., 1996).

Table 1 presents combined results from various studies, in support of creating the baseline knowledge about the metal concentrations for seabird species. This information will be used as a guideline for an estimation of metal concentrations in different tissues in comparison to the metal concentrations from the derived results of the blood analysis. All the collected information are referring to metal concentrations mean values per grams (dry tissue) or deciliter.

Concentrations of metals are often reported for adult birds, but less often for chicks or fledglings (Walsh 1990). However, juveniles have been proposed as particularly useful indicators for pollution, as they concentrate metals during a specific period of time (i.e. hatching to fledging) and from a local and definable foraging area (Walsh 1990). In addition, only limited data exist concerning metal levels in seabirds from the Strofades Island complex area. One of the aims of this study will be to provide baseline data for metal concentration levels in Scopoli's Shearwater collected in one of the biggest colonies in Greece for comparative analyses of inter-specific and geographical differences in bioaccumulation.

Lead (Pb)

Is one of the toxic metals that can be found and affect the biological processes of the species, which no any known biological requirement in avian species. Pb can be found in the Earth's crust, but in relatively low concentrations. During the Industrial Revolution, the use of Pb in many different cases, such as batteries, fossil fuels and smelters, resulted the disturbance of Pb-biogeochemical cycle (Eisler 1988, Von Schirnding and Fuggle 1996, de Villiers et al., 2010). Environmental broadcasting of Pb residues poses a hazard for wildlife species (Eisler 1988, Franson and Pain 2011). Birds can be contaminated by Pb by the inhalation and ingestion and is possible to have particles of Pb on the feathers, as are spending most of their lives at the breeding grounds

(Gonzalez-Solis et al., 2007). The deposition of contaminants may be particularly important in procellariiform species, because they show long breeding seasons and relatively short wintering periods (Thompson et al., 1999, Monteiro et al., 1996). The effects of Pb in avian species can damage the hematopoietic system and also the vascular, nervous, renal, or reproductive system (Kendall and Scanlon 1982, Lumeij 1985, Burger and Gochfeld 1985, Scheuhammer 1987, Blus et al., 1991, Bakalli et al., 1995, Jose Rodriguez et al., 2010). Franson and Pain in a study of 2011, divided Pb exposure and effects in four different levels. The background level, subclinical level, clinical level and severe clinical level. Background exposure conferring to concentrations in wider environments far away from emission sources. The other three

Table 1: Comparative table of different seabird species, metal concentration levels and type of sampled tissue. By the author

Species	Tissue	Cd	Pb	Cu	Hg	Zn	Co	Ni	Mn	Area	Reference
Calonectris diomedea	**Kidney (μg/g)**	11.28 ±8.74		21.16 ±7.99		110.68 ±32.35				Azores, Portugal	Stewart et al. 1997
	Kidney (μg/g)	9.31 ±10.07		12.59 ±3.54		114.94 ±24.39				Faila, Pico Is., Portugal	Stewart et al. 1996
	Liver (μg/g)	3.03 ±1.72		18.86 ±5.29		198.86 ±5.29				Azores, Portugal	Stewart et al. 1997
	Liver (μg/g)	2.03 ±2.78		13.29 ±7.38		176.43 ±48.61				Faila, Pico Is., Portugal	Stewart et al. 1996
Phoebetria fusca	**Blood sample (μg/g)**	0.187 ±0.15	0.034 ±0.022							Marion Is., South Africa	Summers et al. 2014
Phoebetria palpebrata	**Blood sample (μg/g)**	0.04 ±0.02	0.05 ±0.007							Marion Is., South Africa	Summers et al. 2014
Diomedea exulans	**Blood sample (μg/g)**	0.304 ±0.122	0.138 ±0.151							Marion Is., South Africa	Summers et al. 2014
Oceanodroma leucorhoa	**Liver (μg/g)**			20.7 ±1.49		155 ±12.6			15.8 ±2.27	Gull Is., Canadian Atlantic Coast	Elliot et al. 1992
	Liver (μg/g)			20.7 ±2.81		173 ±40.3			16.0 ±1.00	Kent Is., Canadian Atlantic Coast	Elliot et al. 1992
	Liver (μg/g)			17.8 ±2.03		138 ±14.6			17.3 ±1.37	Ile Is., Canadian Atlantic Coast	Elliot et al. 1992
Fratercula arctica	**Liver (μg/g)**			15.3 ±0.69		99.5 ±15.9			8.87 ±1.55	Gull Is., Canadian Atlantic Coast	Elliot et al. 1992
	Liver (μg/g)			23.3 ±3.13		91.1 ±8.94			11.3 ±1.3	Ile Is., Canadian Atlantic Coast	Elliot et al. 1992
Larus fuscus	**Kidney (μg/g)**	28.86 ±18.57		13.51 ±3.95	1.84 ±1.19	118.98 ±33.41				Lancashire, England	Stewart et al. 1996
	Liver (μg/g)	2.83 ±1.71		12.56 ±3.43	1.55 ±1.09	61.21 ±19.65				Lancashire, England	Stewart et al. 1996
Puffinus carneipes	**Feathers (ng/g)**	288 ±816	515 ±367	18382 ±3053		92244 ±33945	257 ±102	2649 ±3593	2198 ±0.56	W. Australia	Bond et al. 2010
Uria aalge	**Liver (μg/g)**	1.98 ±0.56		19.9 ±0.8		80.4 ±7.0					

levels represent elevated or above background exposure. Subclinical levels can cause physiological effects but can not reduce the normal biological functions (Franson and Pain 2011).

Cadmium (Cd)

Cadmium (Cd) is also a toxic metal with no known biological requirement in birds. During the Industrial Revolution, anthropogenic activities (smelters, fossil fuel uses, fertilizers, production of batteries and plastics) altered the natural distribution of Cd (Eisler 1985, De Villiers et al., 2010). Cd is less volatile than Pb, so atmospheric transport is not of great concern. Fluvial transport is more important and sets a hazard to aquatic wildlife species (Eisler 1985, Furness 1996, Wayland and Scheuhammer 2011). Birds can easily contaminated with Cd by ingestion, than inhalation, and it is also possible to have particles on the feathers (Eisler 1985, Furness 1996).

There are other metals that can be found and monitored in birds, such as Copper (Cu), Chromium (Cr), Cobalt (Co), Nickel (Ni), Manganese (Mn) and Zinc (Zn).These are essential elements, but may also become toxic, if present in excessively high concentrations, as they are accumulated in tissues (Elliott and Scheuhammer, 1997). Metal particles can enter on each organism by digestion and transported through the food chain. Studies show that seabirds can increase their body metal concentrations levels by digesting specific pelagic fish species (Karris et al., 2016).

This study examines metals, with no any biological requirement in seabird organisms, that can be toxic in low concentrations (Pb, Cd) and essential metals that can be toxic if present high concentrations (Cu, Cr, Co, Ni, Mn and Zn). All the previously mentioned, can be transported through the trophic levels and depending their concentration levels, to evaluate the environmental state.

Many studies resulted in that blood can be used to examine recent exposure to metals in humans and birds (Subramanian and Meranger 1981, Garcá- Fernández et al., 1996, Tirelli et al., 1996, Benito et al., 1999, Van Wyk et al., 2001, Wayland et al., 2001, Wilson et al., 2004, Bazzi et al., 2008). In birds, Pb and Cd are absorbed into the bloodstream and quickly end up into soft tissues including liver, kidney, bone, and growing feathers. Also, an exposure by ingestion can give elevated levels in tissues and blood (Samuel et al., 1992, Franson and Pain 2011, Wayland and Scheuhammer 2011). In 2001 Van Wyk and et al, found similar mean Pb concentrations between blood, brain, liver and muscle tissue, by examining African vultures. They concluded that blood can be a trustworthy indicator of body charge by metals. Blood sampling can not cause death and it's possible to be applied on live animals. It has to be noted that blood sampling is not affecting any of the bird's vital functions and is not causing any damage to its organs. Feathers can be contaminated by external sources, unlike blood sampling. These are the reasons why blood sampling is a successful method to monitor toxic metals in species.

4. Material and Methods

4.1 Field Sampling

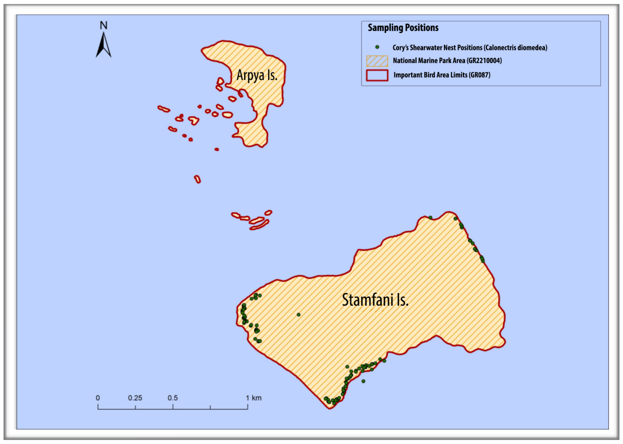

Figure 6: Position of the sampled nests of Stamfani Island perimeter. Map by D. Portolou

Juvenile and adult Scopoli's Shearwaters were sampled between 2007 and 2014 from the breeding colony of Strofades Islands in southern Ionian Sea, Greece. The species colony at the study area hosts about 5,500 breeding pairs (Karris et al. 2017) and constitutes the largest colony in eastern Mediterranean. Sampling took place at Stamfani Island during the end of the fledging period (end of September-mid of October) in order to sample fledglings as well as adults. Capturing burrowing seabirds is a challenging task. They attack in order to defend their nests. Their nests are under boulders and in very deep holes, so a hand made catch pole was the best solution. Blood samples were collected in three sub-colonies from the western, southern and eastern part of the island

(Figure 6), where fledglings, breeders and prospectors were captured by hand. Permissions for the fieldwork were provided by the local authorities e.g. management body of National Marine Park of Zakynthos, National Coastguard Authorities and Metropolis of Zakynthos.

At a first stage, morphometric and weight data were collected per individual. Blood sampling was followed by using the main wing vein, while the shearwater was securely held. The handler had to extent the wing to expose the main vein for bleeding by the sampler. An amount of 0.2-0.5 ml per blood sample was collected using a heparinsed syringe and kept in eppendorf vials (1.5 ml) with ethyl alcohol as preservative. A total number of 238 samples were collected, from which 182 were from juveniles. After each sampling, shearwaters were placed back in their nests. The collected samples were placed in boxes with ice and transported at -8C^{o} to the laboratory and stored at -20C^{o} in the lab of TEI of Ionian Islands. In September 2016 they were transported to the University of Algarve in the same conditions until further analysis.

4.2 Preparation of samples and laboratory analysis

The process of metal analysis of blood samples involved sample preparation, digestion and metal quantification. The digestion was performed using hot plate and the quantification of metal content was performed by Graphite furnace atomic absorption spectroscopy for the metals: Cd, Co, Cr, Cu, Mn, Ni, Pb and flame atomic adsorption spectroscopy for Zn.

The analysis of metals was performed at the chemical laboratory (LaQ) in the University of Algarve.

4.2.1 Preparation and acid digestion of samples

The total amount of blood samples were transferred into polypropylene digestion tubes (50 ml) previously dried at 40 C^{o} for 24h until constant weight and the weight noted. The tubes containing the blood samples were then placed in a hot plate (set at 40 C^{o} for 45

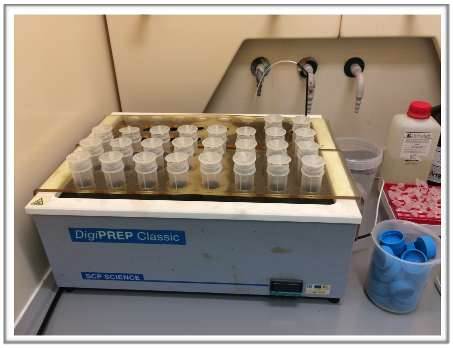

Figure 7: Digesting the samples at 60 C^o

minutes, to avoid destroying any bounds of blood proteins) to evaporate ethyl alcohol (that was used for field preservation and the dry weight of the blood samples were noted. Then 1 mL of concentrated nitric acid (HNO_3) p.a. was added to each sample that were digested at 60 C^o for more than one hour (Figure 7).

4.2.2 Quantification

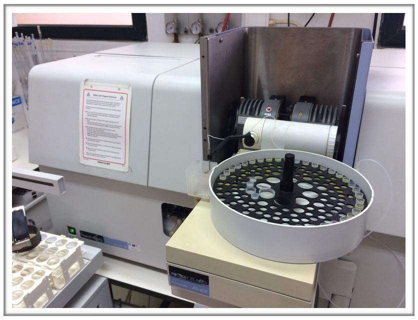

Figure 8: Graphite furnace atomic absorption spectroscopy method

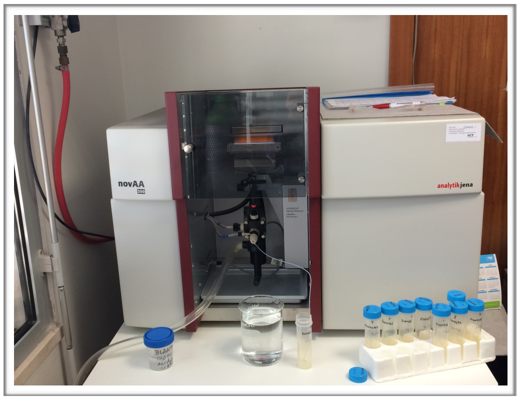

Figure 9: Flame atomic adsorption spectroscopy to determine Zn concentrations

After the digestion of samples, tubes were diluted up to a total volume of 5 ml by adding 0.2% nitric acid solution (HNO_3 - Milli Q Water). 44 samples were handled at each time. The accuracy of the method was verified by analyzing duplicates of the same amount of certified reference material of blood serum (SeronormTM Trace Elements Whole Blood L-1, Sero). Blanks were also prepared with and acidified Milli-Q water solution with 0.2% nitric acid (HNO_3) and treated in the same way to check for any contamination.. Thereafter, 1ml of each sample was transferred trace metal free tubes for using graphite furnace atomic absorption spectroscopy (AAnalyst 800 - THGA Furnace) (Figure 8). Blanks that were placed between every 10 samples and the detection limit of each metal was calculated from the instrument detection limit (IDL: Cd 0.0116µg/l, Co 0.2416 µg/l, Cr 0.1996µg/l, Cu 0.2430µg/l, Mn 0.0585µg/l, Ni 0.2098µg/l, Pb 0.3975µg/l, Zn 2.7820µg/l). To calculate metal concentration a calibration curve using commercial standard of metals for atomic absorption measurements (Merck CertiPUR Inorganic Mixes) was used. The Analytik Jena nov AA 350 flame atomic adsorption spectroscopy was used for the analysis of Zn (Figure 9), using internal standards at the beginning and the end of the procedure.

4.3 Statistical Analysis

The statistical analyses of the data were conducted using SPSS statistical package (IBM Company, Chicago, IL, USA) and Gretl statistical software. Means, standard deviations, medians, and ranges of metal concentrations in blood samples were calculated per sub-colony and per breeding year. All data were checked for normal distribution and if necessary, transformed using logarithm scale to obtain normal distribution. More specifically we used a set of four tests (Doornik-Hansen, Shapiro-Wilk, Lilliefors and Jarque-Bera) so as to check normality of metal concentrations in total as well as per breeding season, and to proceed (if this was necessary) to log-transformation of data to meet model requirements. Additionally, pairwise correlation coefficients (Pearson's product-moment correlation) was employed in order to obtain relevant correlation matrix and identify possible relationships between selected variables. One-way ANOVA tests were used to determine significant differences of metal concentrations between sub-colonies and breeding years. Concentrations of the metals were also compared between different age classes and genders. A Principal Component Analysis was also used to relate metal concentration patterns in the blood samples from different sub-colonies. Sex determination was based on a polymerase chain reaction-based methodology combined with morphometric variables (Karris et al. 2013). Variables are presented as mean ± standard deviation (SD), and significance level was set to $p < 0.05$.

Table 2: Total number of blood samples per year, detectable metal, age and sex of Scopoli's Shearwater (*Calonectris diomedea*) on Strofades Island group, (Ionian Sea, Western Greece)

	Cd				Co				Cr				Cu				Mn				Ni				Pb				Zn			
	Juv		Ad		Juv		Ad		Juv		Ad		Juv		Ad		Juv		Ad		Juv		Ad		Juv		Ad		Juv		Ad	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
2007	6	1	2	2	10	10	2	2	11	11	2	2	11	11	3	2	11	11	3	2	8	9	2	2	4	1	0	1	5	7	2	0
2008	5	3	0	1	6	5	4	1	21	14	6	2	21	14	6	2	11	22	4	1	12	5	3	1	11	8	3	3	0	3	1	0
2009	7	3	1	0	5	4	0	0	15	21	1	0	15	21	1	0	15	21	1	0	9	15	0	0	9	8	0	0	15	21	1	0
2010	6	8	1	0	8	7	1	0	11	9	2	0	11	9	2	0	11	9	2	0	8	8	2	0	3	3	1	0	7	6	2	0
2011	8	8	0	0	2	5	0	0	11	11	0	0	11	11	0	0	11	11	0	0	7	9	0	0	7	7	0	0	3	5	0	0
2012	3	12	1	1	2	7	0	1	6	18	1	1	6	18	1	1	6	18	1	1	4	16	1	1	2	5	1	0	4	14	1	1
2014	2	7	17	9	0	3	12	6	7	16	12	7	7	16	17	9	7	16	17	10	7	13	9	5	1	5	2	2	6	12	17	10

5. Results

5.1 General results

Table 2 shows the total number of samples of juveniles and adults per year, season and sex used for metal analysis.

Normality test for all variables resulted that non of them was normal distributed ($p<0.05$) and thus data were transformed using logarithmic scale (log10) to obtain normal distribution.

In Table 3 median and interquartile range (25% - 75%) of metal concentrations in blood from all Scopoli's Shearwater samples are presented. Among the non-essential metals, Pb concentrations range between 0.07-0.53 µg/g, followed by Cd (0.01-0.02 µg/g). Among the essential metals, concentrations of Zn were between 8.84-39.35 µg/g, followed by Cu (1.52-4.99 µg/g), Cr (0.23-1.81 µg/g), Ni (0.17-0.82 µg/g), Mn (0.10-0.44 µg/g) and Co (0.07-0.26 µg/g).

Table 3: Median and interquartil range (25% - 75%), of metal concentrations (µg/g) in blood samples of Scopoli's Shearwater (*Calonectris diomedea*) from Strofades Island complex in Greece.

	Blood concentrations (µg/g)	
Element	Median	Interquartil range (25%-75%)
Cd	0.01	0.01 - 0.02
Co	0.14	0.07 - 0.26
Cr	0.87	0.23 - 1.81
Cu	2.97	1.52 - 4.99
Mn	0.23	0.10 - 0.44
Ni	0.45	0.17 - 0.82
Pb	0.20	0.07 - 0.53
Zn	23.31	8.84 - 39.35

Statistical analysis (correlation matrix with the total amount of data) of metal concentrations in juveniles and adults revealed that in relation to body mass there is no significant relationship between the weight of individuals and metal concentrations and for this reason metal concentrations could be compared between, years, sites and sex.

5.2 Variation of metal concentrations

Metal concentrations of Scopoli's Shearwater blood samples during seven breeding seasons (2007-2012 and 2014), are presented in Figure 10. All metals showed a significant difference with the years. More specific, analysis of variance (one way ANOVA) indicated that Cd show a significant difference on 2008 (Figure 10 - I) (one way ANOVA, $p < 0.05$). The different racial groups present differentiation in levels in Co for the studied years (Figure 10 - K): F(6,76)=5.2341, $p < 0.05$). 2008 was the year that Co showed a significant difference ($p < 0.05$). Cr, like cobalt, present different levels for the studied years: F(6,184)=10.711, $p < 0.05$ and showed also a significant difference for 2008 (Figure 10 - L). The same for Cu (Figure 10 - M), were present significant different levels for the years: F(6,179)=13.141, $p < 0.05$ and resulted a significant difference for 2008 ($p < 0.05$). The same statistical test resulted significant differences for Mn: (F(6,181)=4.3671, $p < 0.05$) (Figure 10 - N) with the highest levels on 2008, 2012 and 2014. For Ni (Figure 10 - O) and Pb (Figure 10 - P), one way ANOVA resulted also significant difference on 2008: F(6.120)=3.3464, $p < 0.05$ and F(6.72)=5.9136, $p < 0.05$) respectively. Lastly, ANOVA test showed that the significant different levels of Zn (Figure 10 - Q) with the years were with 2008, 2009 and 2014: F(6.133)=9.0067, p=0.00 ($p < 0.05$). Highest concentrations were in 2008 for all metals (Cd, Co, Cr, Cu and Pb (Figure 10 - I, K, L, M, O, P)), except for Zn, were the highest concentration was in 2009, followed by 2014 and 2008 and Mn, were the highest concentration was in 2014.

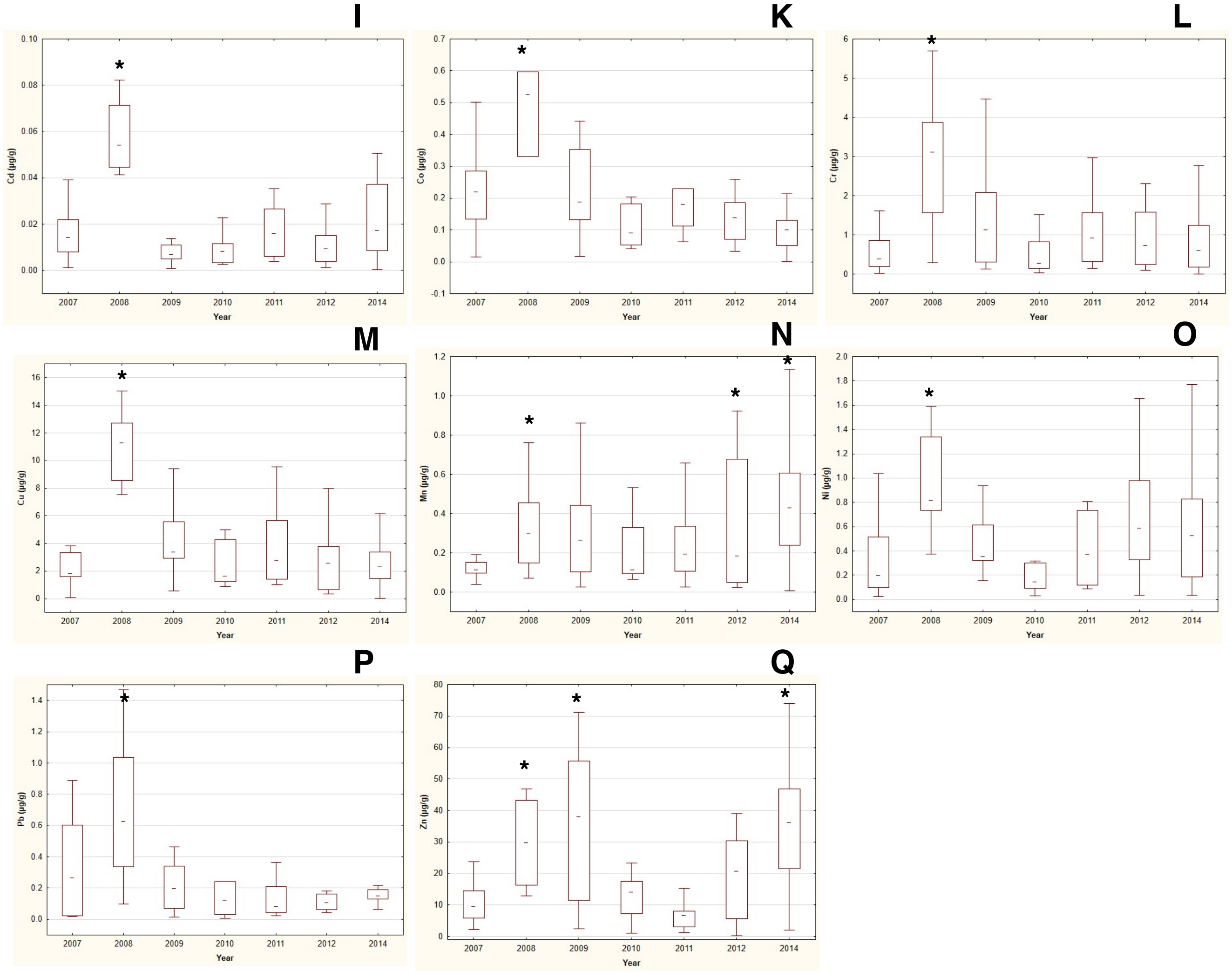

Figure 10: Box plots for metal concentrations (Cd (A1), Co (A2), Cr (A3), Cu (A4), Mn (A5), Ni (A6), Pb (A7), Zn (A8)) in blood samples of juveniles and adults Scopoli's Shearwaters during seven breeding seasons (2007-2012 & 2014). The star (*) indicates the year with statistically significance differences resulted by T-test ($p<0.05$).

5.3 Age and sex differences

Figure 11 presents the distribution of metals (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn) in blood of adult and juvenile Scopoli's Shearwaters according to sex. Statistical results indicate significant sexual differences for Cd, (Figure 11 A'), Co (Figure 11 B'), Mn (Figure 11 E'), Pb (Figure 11 G') and Zn (Figure 11 H') for both juveniles and adults, (T-test, $p < 0.05$). Regarding Cr (Figure 11 C') significant differences exist on gender for adults and juveniles, with the higher concentrations on male juveniles and female adult birds. For Cu (Figure 11 D') only juveniles show significantly difference with slightly higher concentrations on males (t-test, $p < 0.05$). Ni (Figure 11 F') levels were also significantly different with gender for juveniles (t-test, $p < 0.05$) and adults (t-test, $p < 0.05$), were metal concentrations in female juveniles were higher than in males while in the adults it was the opposite with metal levels in male adults were higher than females. Statistical results are shown on annex table (P1).

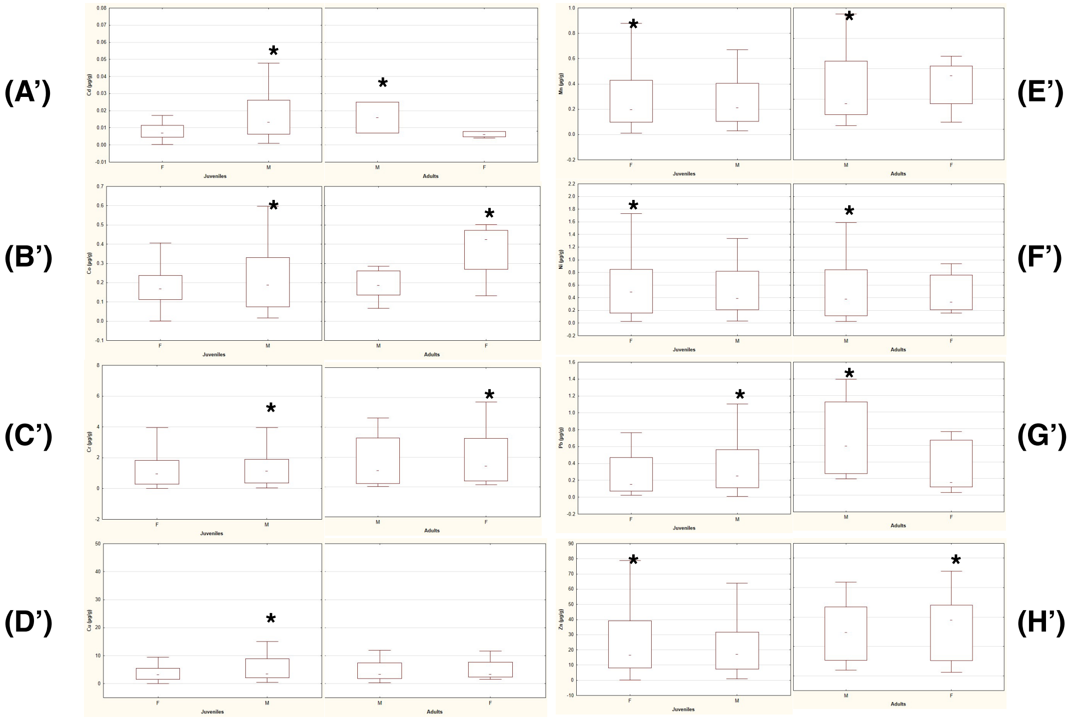

Figure 11: Metal concentrations of Cd (A'), Co (B'), Cr (C'), Cu (D'), Mn (E'), Ni (F'), Pb (G'), in blood samples of Scopoli's Shearwaters per gender during seven breeding seasons (2007-2012 & 2014). (*) indicate different differences. ($p < 0.05$).

5.4 Spatial variation of metal concentrations

Figure 12 presents the distribution of metal concentrations (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn) in Scopoli's Shearwater blood samples between the two breeding sites/sub-colonies on Stamfani Island. The results showed significant differences for most of the tested metals (Cd (Figure 12 A), Co (Figure 12 B), Cr (Figure 12 C), Mn (Figure 12 E), Ni (Figure 12 F), Pb (Figure 12 G), Zn (Figure 12 H) for nesting sites (t-test, $p < 0.05$)). The only exception was for Cu (Figure 12 D), were no statistical significant differences were found (t-test, $p > 0.05$). Concentrations of Cd were higher on the western part for juvenile and adults. Co concentrations were also higher on western area, with much higher levels at West than South for adults. On the opposite, Cr and Cu concentrations were higher in southern sub-colony. For Mn, concentrations were similar among sites, but higher in adults from the western part. Levels of Ni, Pb and Zn were higher in the southern sub-colony for juveniles, but much higher on adult birds that breed on the western part. A table with the results of the T-test (P2) is given in Annex session.

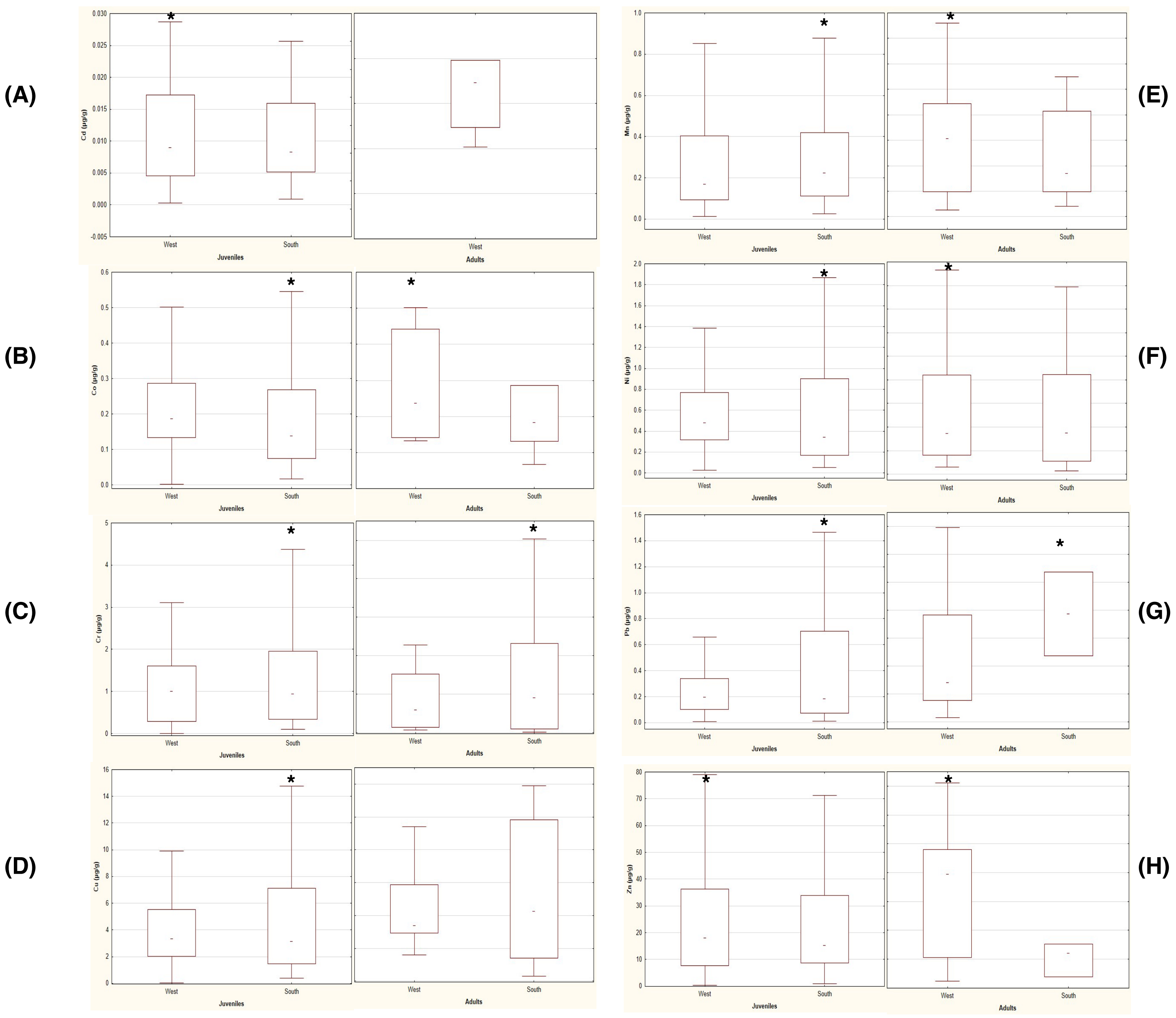

Figure 12: Metal concentrations (Cd (A), Co (B), Cr (C), Cu (D), Mn (E), Ni (F), Pb (G), in blood samples of Scopoli's Shearwaters per nesting site, during seven breeding seasons (2007-2012 & 2014). The star (*) indicates significant differences ($p<0.05$).

5.5 Principal Component Analysis

Principal Component Analysis (PCA) were used to detect the relationships between metal concentrations (Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn), year, sex and site of for juvenile and adult birds from Strofades Island colony. Results of PCA showed that they represent 34.82% of variance with PC1 representing (18.76%) and PC2 (16.05%). Figure 13, shows the distribution of metal levels, according to the two principal components (Table 4 explains the symbols that appear on Figure 13). PC1 (18.76%), which is the most important component, indicates a negative relationship between Pb and Cr with the rest of the metals. PC2 (16.05%) indicates that the distribution of Cd and Zn is opposite (in the negative part of PC2) to Co, Cr, Cu, Mn, Ni and Pb. Furthermore, PC1 relates strongly with Mn (r=0.554) and Zn (r=0.420) (Table A.1 in Annex) and PC2 with Cr (r=0.599) (Table A.1 in Annex). PCA indicated that in 2007, Zn and Co had negative distribution, based on PC1 (38.90%) and Cd, Cr, Cu, Mn, Ni, and Pb have a positive distribution, opposite to Co and Zn. The non-essential metals are negatively related with Zn and Co, with a small differentiation with the rest of the metals (see A1 in Annex). In most of the individuals Zn and Co are the dominant metals and the distribution of these metals is negatively related with the levels of the rest metals. Based on the second principal component (17.46%), the essential metals (Zn, Co, Ni and Mn) are negatively correlated with the non-essential metals (Pb, Cd).

In 2008, based on the PC1 (19.96%), Pb and Cr have a negative relationship with the rest of the metals. The PC2 (16.80%) describes the negatively relationship of Cu and Co with Pb, Cr, Zn, Mn and Cd (see A2 in Annex). Also PC2 describes the differences between age in which Juveniles had highest relationship with Cu and Co, than adults.

For the year 2009, the results from PC1 (33.23%) show that the non-essential metals (Pb and Cd) have a negative relationship with the rest of the metals. Juveniles had higher correlation of Pb and Cd, based on PC1, than adults. Also male individuals from Western part of the island had highest relationship with Pb. On the other hand, males and females are highly related with Ni, Cr, Co, Cu (see A3 in Annex). The results from PC2 (20.80%) show a high correlation of Cd and Mn. For Zn, Co and Co the relationship was very small. Cu and Pb levels were highly related with PC2 and negatively related with Cd and Mn (A3 in Annex).

PC1 (44.48%) for 2010, shows that Zn and Cd levels are opposite with the rest of the tested metals. Generally from all the individuals, Zn and Cd showed to have the highest response. Juveniles were highly related with Zn and Cd, based on PC1, than with adults (see A4 in Annex). PC2 (19.01%) show that Mn, Cu and Cr had lower relationships and opposite of Zn and Cd. On PC2 the relationship with Ni, Co and Pb were higher.

In 2011 Zn was the only metal that had a negative relationship compared with the other metals, based on PC1 (34.14%). Juveniles had higher relationship with Zn and Cu than adults. Females from western and southern area were related more with Zn (see A5 in Annex). PC2 (18.39%) resulted a very small relationship between Cr, Cu, Zn and Mn. Higher relationships were with Cd, Co (positive related) and Ni, Pd (negative related)

2012 was the year with the least of differentiation based on PC1 (40.38%). Most of the juveniles did not show any relationship with the metals. Females were the individuals with the highest relationship with all the metals (see A6 in Annex). PC2 (16.12%) resulted in a negative and strong relationship between Cr, Cu, Pb and positive relationship with Zn and Co. Cd, Ni and Mn had lower relationship with PC2.

Finally, for 2014, PC1 (23.98%) resulted that Pb and Cd were stronger related with each other and negatively related with Cu, Mn and Zn. The rest of the metals (essential metals) were positively related and opposite of the non-essential metals., Ni and Cr had different response with the rest of the metals. Correlations between Zn, Co and Mn were higher in juveniles. Females from the western part of the island were related with the non- essential metals and Cr (see A7 in Annex). In PC2 (21.50%), only Cr has an opposite relationship with the other metals.

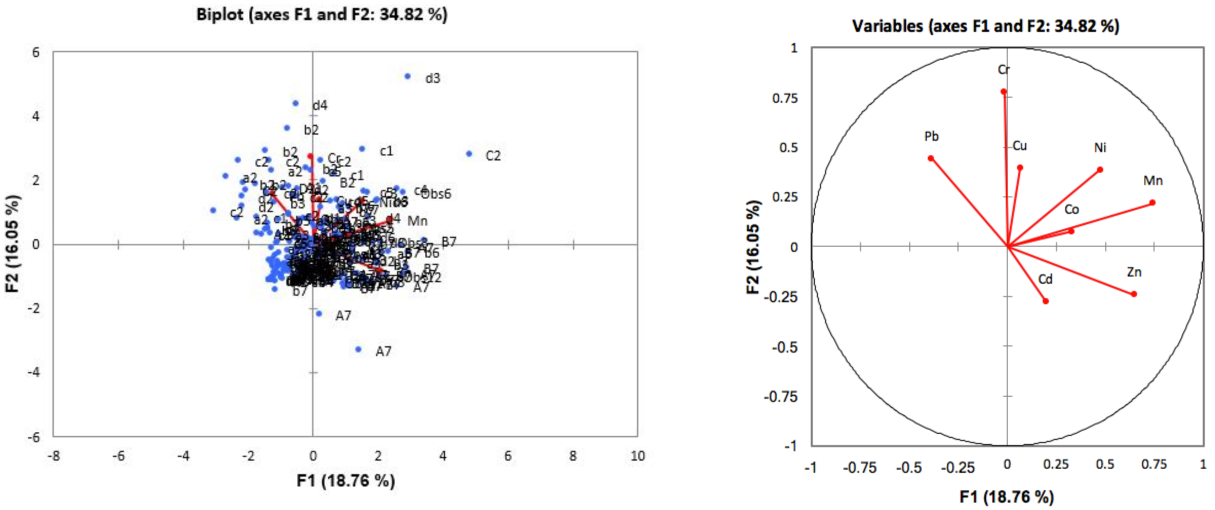

Figure 13: Result of principal component analysis. xx’ axis shows component 1 and yy’ axis shows component 2. The red arrows indicate the distribution of the tested metals. The blue dots indicate the distribution of the sampled birds on the according on the two components. Explanation of blue dot labels are given on table 3.

Table 4: Explanation of blue dots labels on principal component analysis results

Year	Males/West	Females/West	Males/South	Females/South
2007	A1,a1	B1,b1	C1,c1	D1,d1
2008	A2,a2	B2,b2	C2,c2	D2,d2
2009	A3,a3	B3,b3	C3,c3	D3,d3
2010	A4,a4	B4,b4	C4,c4	D4,d4
2011	A5,a5	B5,b5	C5,c5	D5,d5
2012	A6,a6	B6,b6	C6,c6	D6,d6
2014	A7,a7	B7,b7	C7,c7	D7,d7

* Capital letters referring to adults birds and lowercase to juveniles

6. Discussion

Marine birds as top consumers are particularly vulnerable by spatially and temporally defined threats such as marine pollution. They are exposed to a wide range of chemicals and other forms of marine pollution because they depend exclusively on the marine environment where they face toxic risk by external contact, inhalation, and particularly ingestion of food and water.

Scopoli's shearwater is a long-lived migratory pelagic Procellariiform species with high rates of nest tenacity and mate fidelity, and exploits persistent productive marine areas. It is a surface feeder, consuming pelagic fish mainly, at least during incubation and the chick-rearing period (Afán et al. 2014). Recent findings have shown that this seabird adopts a dual strategy, feeding on epipelagic shelf prey in shallow waters during short trips and on oceanic prey items, normally associated with different water masses, during long trips (Cecere et al. 2013). Shearwaters feed on a range of different fish but also on specific cephalopods such as European squids (Alonso et al. 2012, Neves et al. 2012). According on the results of Karris et al. (2015), bottom trawler fishery operations in the study area provide significant amounts of benthopelagic prey species to shearwaters during their pre-laying period in spring. This alternative food supply can be characterized as normally unavailable due to the foraging ecology of Scopoli's shearwater and, as a consequence, may affect the population dynamics of local colonies and specifically the Strofades population. Analysis of metals (Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn) in the blood (Table 3) showed that concentrations were in general lower compared with metal levels in blood of other similar seabird species (Table 1). No significant relationship was detected between the weight of individuals and metal levels (correlation matrix with the total amount of samples), for adult and juveniles, which allowed us to compare metals levels with years, age of the birds and breeding site. This is in contrast with other studies on Shearwaters (Chihiro et al. 2017) were a strong correlation of body weight with cadmium was found. This was the result that chicks mainly eat small fish and later change their diet to squids or to other organisms containing high Cd levels so they accumulate more of this metal, because of bigger prey. According with recent studies for morphometrics and sex dimorphism on Scopoli's Shearwater on Strofades colony (Karris et al. 2013), the morphometrics is not changing from juveniles to adults. Combining the information of these two studies we can assume that Scopoli's Shearwaters does not change their diet as they grow and they eat the same species of fishes and squids.

PCA analysis resulted A positive relationship of Cd and Zn, was observed supported by PCA analysis (Figure 13) which was consistent with previous reports (Chihiro et al. 2017). These metals are expected to interact with a detoxification system, such as metallothionein (MT), a low molecular weight cysteine-rich protein involved in the homeostasis of essential metals, such as Zn. The synthesis of MT can be induced by several metals, such as Cd, Zn and Cu. Zn is essential for the synthesis of MT. When Cd is taken up, Zn in MT is replaced with Cd to achieve detoxification. It was reported that Cd and MT concentrations are positively related in many seabirds (Eliot et al. 1992; Stewart et al. 1996). High accumulation of Cd induces the synthesis of.

According to the present results (Table 3), blood levels of cadmium in Scopoli's Shearwater are similar to levels from other blood seabird species. Concentrations of cadmium varied in from 0.01-0.02 µg/g, compared with other seabirds such as in Marion Island, South Africa (0.04 µg/g - 0.304 µg/g) (Summers et al. 2014). However, cadmium concentrations on Strofades colony were found in low values if we compare with maximum levels set out by the European community, for foodstuff contaminants (0.1 mg/g) (European Commission Regulation, 2006). Differences in cadmium levels, compared with other studies, can be related with various factors such as the region, the productivity of the marine ecosystem, the season of sampling, but also the use of different analytical processes used, the sampling method and also the environmental contamination (Galitsopoulou, 2014).

Another aspect of the current study was the variation of metal levels throughout different breeding seasons. Cadmium concentrations were higher in 2008, in relation with other years (Figure 1). Sex differentiation of cadmium concentrations were also detected. The males had higher cadmium concentrations, in relation with females, for both juveniles and adults and juvenile birds showed significant higher cadmium levels than adults

(Figure 2A. 11A) indicating that cadmium accumulation depends on the age and gender. There is a possibility that the food are different between males and females, and it causes the differences of metal accumulation (Chihiro et al. 2017). Pawel et al (2000) showed that juvenile Black-headed Gulls had always higher cadmium levels compared with adults. Zn levels were also significantly higher (Figure 11 - H') in juveniles.

The western part of Strofades Island had higher cadmium concentrations (Figure 12 - A). This may be the result of different foraging distribution pattern between birds that breed in different sub areas of Stamfani Island, but this has to be proved by using remote sensing techniques on adult birds that breed in the western part that seem to use different feeding areas than the birds of southern part.

The number of samples was relatively similar, except from the Eastern region, where the ground morphology difficult the sampling process and the amount of samples were very small and were not taken into account on the data analysis. According to previous studies, in the Adriatic sea no relation between cadmium levels and the sampling area was detected (Desideri et al., 2010). In contrast Pastor et al. (1994) in western Mediterranean, found that cadmium concentration was influenced by foraging area and that increased concentrations were observed in coastal piscatorial areas with significant industrial activity and assembled urban population. Different factors that may influence metal concentrations in feeding areas, like the existence of river flows and also incidents of direct industrial rejections within urban waste, without any actions of correct environmental management (Topcuoğlu et al., 2002). Another important parameter that can have an impact on the metal concentrations is the season of foraging activity by the adults-breeders. Food provision to nestlings begins from mid July just after egg hatching, up to few days before fledgling (late September-early October).

The months with increased cadmium concentrations in ichthyofauna in Eastern Mediterranean are September, October and November (Galitsopoulou, 2014). The effect of seasonal foraging activity in the accumulation of cadmium is attributed, mainly on physiologic differentiations of fish catches, throughout year (Özden, 2013.

There is a substantial body of literature describing the effects of lead contamination on seabird behavior and physiology (Burger and Gochfeld 2000). Lead is an anthropogenic contaminant that has no biological function and can be transported by great distances atmospherically (Nriagu 1989 and Burger 1993).

Concentrations of lead in the blood of the birds range between 0.07-0.53 µg/g, comparatively with other seabird species colonies, such as in Marion Island (0.034 µg/g - 0.138 µg/g) (Summers et al. 2014). The levels of lead concentration were in some cases higher than maximum levels set out by the European community, for foodstuff contaminants (0.3 mg/g) (European Commission Regulation, 2006), that are expressed in Table 3 on results. Levels from other studies of different marine species in the same region (Galitsopoulou, 2014) show that Pb levels didn't exceed 0.297 µg/g of sample. It is a possibility for an indication for contamination.

Lead concentrations were higher than cadmium. This observation is in agreement with previous results of related seabirds spread around different colonies, where lead concentrations showed significant differences in comparison with cadmium (Summers et al.2014, Bond et al., 2010). Statistical analysis indicate significant higher levels of lead for the breeding season of 2008. In contrast with cadmium concentration, lead was higher in birds from the southern sector. This may be explained by possible difference in foraging distribution pattern in comparison with those of the western sector. Higher concentration of lead was also found on juveniles compared to adults of southern sector. This can result the assumption that birds breeding on the southern sector are searching for food in more lead contaminated areas that birds form the western sector.

Essential elements are present in all living organisms and necessary for regulation of body functions and are usually maintained by homeostasis mechanisms, which act in the removal of excess and maintenance of normal body concentrations (Kim and Oh, 2013). Not significant correlation levels of essential metals with body mass may reflect that Shearwaters, when compared with other birds, may use less energy by keeping lower metabolic rates (Colwell, 2010) and ingest lower prey mass. Kim et al. (1996) resulted that essential metal that their levels are not correlated with body mass reflected by low pollution levels (Cr, Co, Cu, Mn, Ni, Zn) in their habitats.

Concentrations of cobalt, chromium, copper, manganese and nickel (Table 3) were much lower compared to other colonies of related seabird species (Table 1). 2008 was the year with significant higher levels for these metals (Figure 10 K - Figure10 O), with an exception of Mn that showed high values also on 2012 and 2014 (Figure 10 - N). Differences for all these metals were found between age and gender (Figure 11 B’ - F’) and also between the different breeding sectors (Figure 12 B F). This can be a result of the different metal levels on feeding areas.

Zinc levels (8.84-39.35 µg/g) were low compared to other seabird species and also from shearwaters, breeding in different colonies (Table 1) (Stewart et al., 1996; Stewart et al., 1997; Eliot et al., 1992; Bond et al., 2010). High zinc concentrations are probably due to metabolic requirements during the process of fledging (Pawel et al. 2000). It has been shown experimentally with poultry that high zinc levels are needed for feather growth, and indeed deficiency in zinc causes a frayed feather condition (Sunde 1972) and as was expected, juveniles showed significant higher levels of zinc, compared with adult birds (Figure 11 - H’). There were differences on Zn levels between birds from the two sub-colonies of Stamfani Island, were the birds from the western sector have significant higher concentrations for juvenile and also adult birds (Figure 12 - H). We assumed that these metal variations could be attributed to different foraging distribution pattern followed by breeders of the two sub-colonies during the whole breeding season. Consequently, the separated feeding areas that birds may use for minimizing intraspecific competition for food resources are possibly characterized by different contamination regime. This assumption have to be verified with further tracking studies on breeders foraging flights combined with metal concentration analysis on their diet items captured within their main foraging areas.

Further work on Scopoli’s Shearwater diet would be required for a more detailed analysis to explain the metal concentrations found in the present study. Also, further studies on chick’s diet including analysis of regurgitations could give more information on the dynamics and accumulation of metals in these shearwaters. This, in turn, would allow a greater insight into the baseline levels of metals which in a relatively unpolluted environment such as the remote Strofades Island complex.

7. Conclusions

We examined a variety of metals in blood of juvenile and adults Scopoli's Shearwaters birds from the Southern Ionian Sea breeding area. The current blood metal levels indicate than in general, shearwaters are not at great risk, although our toxicological knowledge regarding many elements is insufficient to draw firm conclusions. Scopoli's Shearwaters did not have high blood concentrations of Cd and Pb and also of non toxic metals. Further research is needed to determine the contribution of contaminants in the organs and do a correlation with feeding areas.

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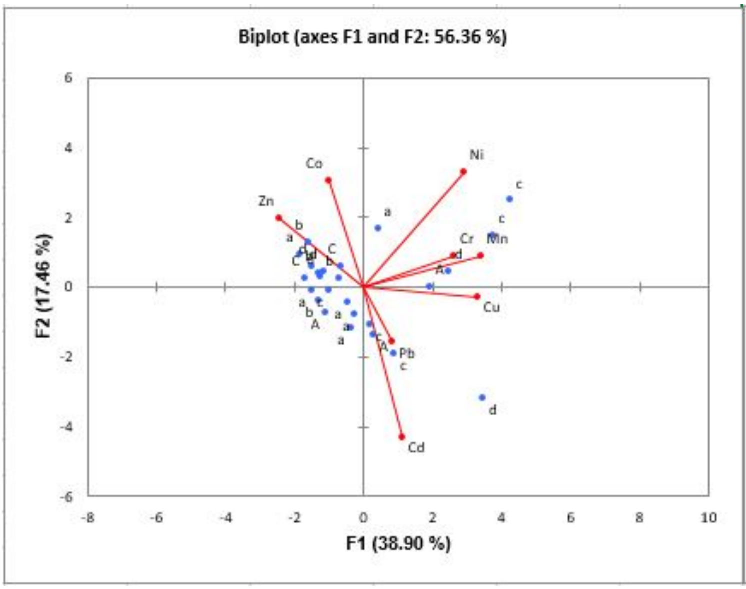
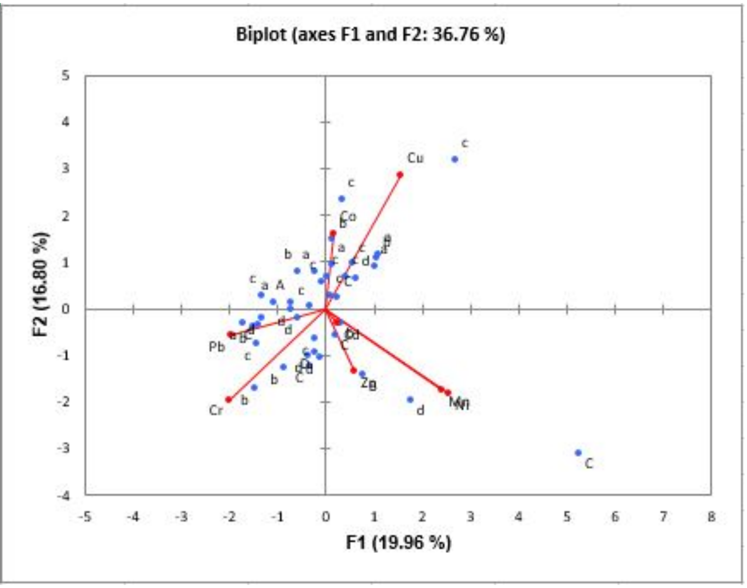
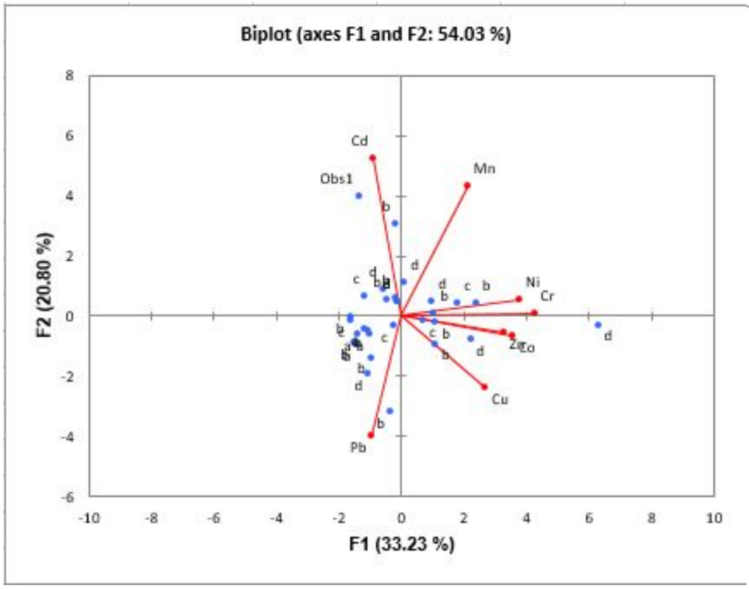
ANNEX



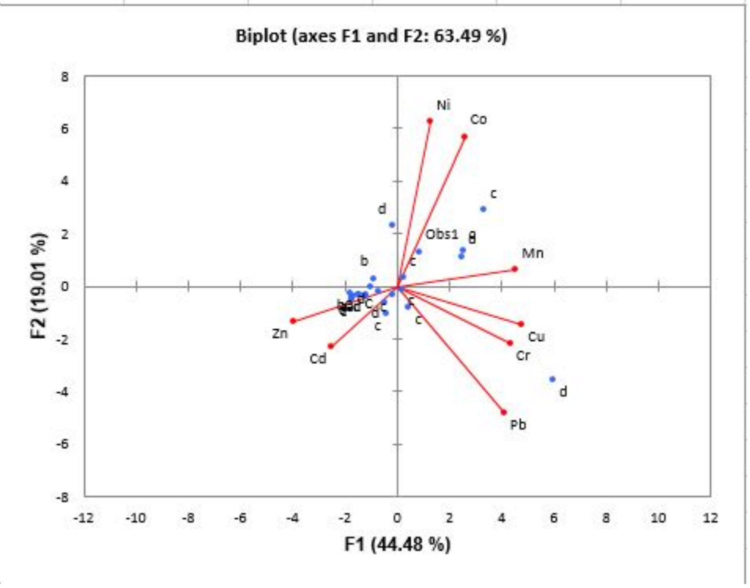
A1: Result of principal component analysis for 2007. The blue dots indicate the distribution of the sampled birds on the according on the two components. Explanation of blue dot labels are given on table 3.



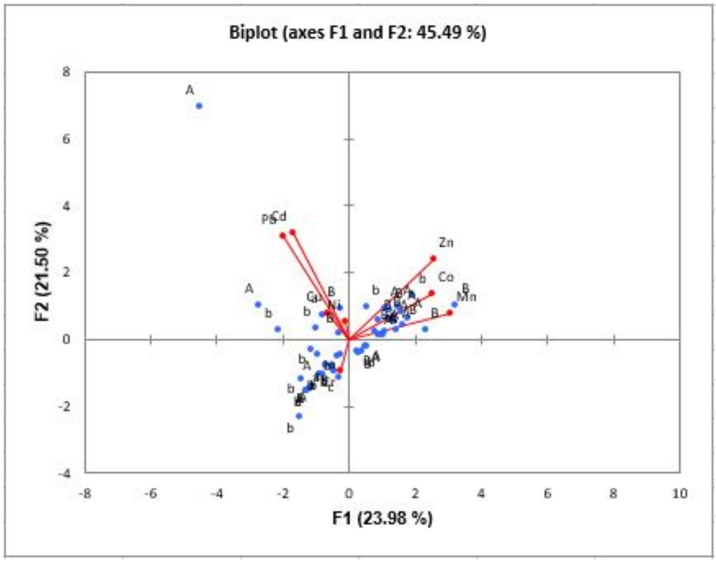
A2: Result of principal component analysis for 2008. The blue dots indicate the distribution of the sampled birds on the according on the two components. Explanation of blue dot labels are given on table 3.



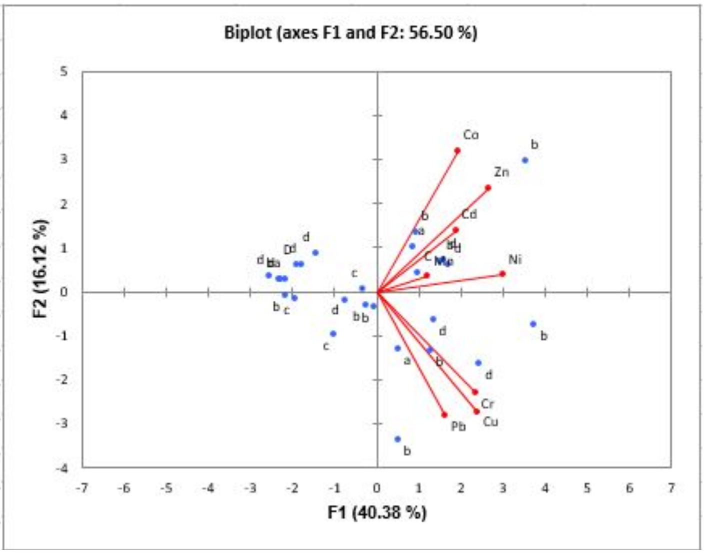
A3: Result of principal component analysis for 2009. The blue dots indicate the distribution of the sampled birds on the according on the two components. Explanation of blue dot labels are given on table 3.



A4: Result of principal component analysis for 2010. The blue dots indicate the distribution of the sampled birds on the according on the two components. Explanation of blue dot labels are given on table 3.



A7: Result of principal component analysis for 2014. The blue dots indicate the distribution of the sampled birds on the according on the two components. Explanation of blue dot labels are given on table 3.



A6: Result of principal component analysis for 2012. The blue dots indicate the distribution of the sampled birds on the according on the two components. Explanation of blue dot labels are given on table 3.

Table A.1: Squared cosines between the observation vectors and the factor axes. Values in bold correspond for each variable to the factor for which the squared cosine is the largest.

Metal	PC1	PC1
Cd	0.039	0.076
Co	0.110	0.006
Cr	0.000	**0.599**
Cu	0.004	0.155
Mn	**0.554**	0.047
NI	0.225	0.148
Pb	0.148	0.195
Zn	**0.420**	0.059

P1: Resulted p-values from t-test for gender on juvenile and adult Scopoli's Shearwaters

Metal	Juveniles	Adults
Cd	0.0001	0.000
Co	0.0002	0.000
Cr	0.031	0.012
Cu	0.000	0.297
Mn	0.0002	0.005
Ni	0.0000	0.001
Pb	0.001	0.008
Zn	0.002	0.0003

P2: Resulted p-values from t-test for the different nesting sites on juvenile and adult Scopoli's Shearwaters

Juveniles

Metal	West	South
Cd	0.000	0.001
Co	0.000	0.000
Cr	0.019	0.000
Cu	0.002	0.142
Mn	0.001	0.001
Ni	0.001	0.002
Pb	0.002	0.001
Zn	0.001	0.002

Adults

Metal	West	South
Cd	0.001	0.001
Co	0.002	0.025
Cr	0.002	0.002
Cu	0.320	0.224
Mn	0.000	0.001
Ni	0.005	0.049
Pb	0.000	0.049
Zn	0.000	0.000