DISTRIBUTION AND PARTITIONING OF ORGANIC POLLUTANTS AND EFFECTS IN COASTAL BIRDS

Ph. D Thesis of

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“Caminante no hay camino,
se hace camino al andar.”
Antonio Machado

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I. Resum

Les zones costaneres són unes àrees molt sensibles a la contaminació degut a la interacció entre els processos marins i antropogènics. Els emissaris submarins, les desembocadures dels rius, les activitats portuàries, l'abocament de residus, l'aqüicultura, etc. Són algunes de les activitats que poden produir la contaminació de les zones costaneres. Entre d'altres tipus de contaminants, es troben els contaminants orgànics persistents (COPs), aquells compostos orgànics que, en diferent grau, presenten resistència a la fotòlisis, la degradació química i la biològica. Degut a les seves característiques fisico-químiques, els COPs estan distribuïts per tot el món, podent trobar-se fins i tot en zones prístines molt allunyades de la seva font d'emissió original. Aquests contaminants tendeixen a bioacumular-se al llarg de les cadenes tròfiques, de manera que els grans depredadors són més sensibles a aquesta contaminació. Les aus marines estan molt exposades als contaminants orgànics degut als seus hàbits alimentaris i a la seva esperança de vida relativament llarga.

En aquesta tesi s'ha estudiat l'acumulació de vàries famílies de contaminants orgànics en dues espècies de gavina de la Península Ibèrica, el gavià argentat (*Larus michahellis*) i la gavina corsa (*L. audouinii*). El gavià argentat és una gavina de grans dimensions molt comuna a la Península Ibèrica. És una espècie omnívora i oportunista. S'alimenta tant de recursos marins, com terrestres, com depredant sobre altres aus i també freqüenta abocadors i descarts pesquers.
La gavina corsa és una gavina de dimensions mitjanes, endèmica de la regió mediterrània. Fa uns anys estava en situació de risc però el fort creixement poblacional de les últimes dècades l'ha deixat fora de perill sent considerada actualment a nivell internacional com a "quasi amenaçada". És una espècie piscívora però cada vegada explota més descarts pesquers.

En el primer estudi s'analitzen 6 famílies de contaminants orgànics inclosos o sota consideració pel Conveni d'Estocolm (els PCDD/Fs, PBDEs, PCBs, OCs, PFASs i SCCPs) en els ous de les dues espècies de gavina de les respectives colònies del Parc Natural del Delta de l'Ebre. A partir d'aquí els següents treballs es basen en l'estudi de la família dels PFASs en sang i ous d'ambdues espècies i pertanyents a diferents colònies de la Península Ibèrica (Delta de l'Ebre, Illes Medes, Illes Columbretes, Illa de Sa Dragonera, Illa Grosa, Illes Chafarinas, Illes Atlàntiques de Galicia i Ilhas Berlengas), totes en zones d'alt interès ecològic i sota alguna protecció ambiental (parcs naturals, nacionals, reserves marines, reserva de la biosfera, etc.).

A partir dels nivells de PFASs trobats en les diferents matrius (primers ous de la posta, clara i rovell d'ou dels 3 ous de la posta i sang d'exemplars adults) s'estudia l'efecte d'aquests contaminants a nivell físic (paràmetres biomètrics de l'ou, gruix de la closca i índex de dessecació) i hormonal (via l'anàlisi de les hormones esteroidees 17β-estadiol i testosterona). S'estudia també l'acumulació d'aquests contaminants a partir de la dieta (analitzant el seu propi aliment i a partir de l'estudi dels isòtops estables de carboni i nitrogen ($\delta^{13}C$ i $\delta^{15}N$), indicadors

del règim alimentari) i es fa una estima de la depuració dels PFASs en sang i de la seva transferència de femelles a ous.

Els resultats indiquen que els contaminants orgànics són presents en les poblacions de gavià argentat i gavina corsa de la Península Ibèrica, sent els OCs i els PCBs les famílies de compostos amb concentracions més elevades. Entre els PFASs, el compost majoritari és el PFOS. La transferència de contaminants orgànics de mares a cries queda demostrada amb els nivells trobats en els ous d'ambdues espècies. De l'anàlisi de PFASs en sang de mascles i femelles de les dues espècies de gavina s'observa que les femelles descarreguen part dels compostos acumulats als ous presentant així nivells en sang inferior que els mascles. No es troba cap relació entre els nivells de PFASs i els paràmetres biomètrics, com tampoc amb els nivells d'esteroides. Tanmateix, l'estudi de les hormones permet comparar els nivells en ambdues espècies amb altres estudis i discutir la influència de la dieta. L'anàlisi de PFASs en clara i rovell d'ou per separat dels 3 ous de les postes de gavina corsa demostren que aquests compostos tenen més afinitat pels lípids del rovell ja que no es detecten en les clares. La concentració decreixent de PFOS al llarg de la seqüència de posta demostra que les femelles descarreguen la majoria de contaminant en el primer ou però que existeix una bona correlació dels nivells de PFOS entre els 3 ous d'un mateix niu. L'augment de les concentracions d'alguns PFASs en el 3r ou i les diferències en els valors de $\delta^{13}C$ entre el 1r-3r i 2n-3r ou s'atribueixen a l'anomenat "efecte mare" (possible ús de reserves endògenes per a la formació de l'últim ou enlloc de l'energia aportada per la dieta prèvia a la posta).

Aquesta tesi permet augmentar el coneixement sobre l'acumulació de COPs en el gavià argentat i la gavina corsa de la Península Ibèrica, avaluar les diferències entre ambdues espècies i associar-ho a la seva biologia, determinar la distribució geogràfica de COPs utilitzant els ous com a bioindicador de contaminació ambiental i estudiar els processos d'acumulació i transferència de PFASs.

II. Abstract

Coastal areas are very susceptible to contamination due to the interaction of marine and anthropogenic processes. The submarine sewage pipes, river mouths, port activities, waste disposal, aquiculture, etc. are some of the activities that can cause pollution of coastal areas. Among other contaminants, Persistent Organic Pollutants (POPs) are present in coastal areas, organic compounds which, to different degrees, have resistance to photolysis and chemical and biological degradation. According to its physicochemical characteristics, POPs are worldwide distributed and can reach even pristine areas, far away from their sources of original emission. These contaminants tend to bioaccumulate through the food webs, so that predators are more sensitive to this contamination. Seabirds are highly exposed to organic pollutants due to their feeding habits and its relatively long life expectancy.

In this thesis, it has been studied the accumulation of various families of organic pollutants in two species of gulls breeding in the Iberian Peninsula, the yellow-legged gull (*Larus michahellis*) and Audouin‘s gull (*L. audouinii*). Yellow-legged gull is a large gull very common in the Iberian Peninsula. It is an omnivorous and opportunistic species. It feeds both on marine and land resources, preying on other birds and it also frequents dumps and fishery discards. Audouin’s gull is a medium-sized gull, endemic of the Mediterranean region. Few years ago it was endangered but the great population growth in recent decades has kept it out of risk and currently it is considered internationally as "nearly threatened" species. It is a piscivorous species but it is increasingly exploiting more fishery discards and continental preys.

In the first study there are determined 6 families of organic pollutants included or under consideration by the Stockholm Convention (PCDD/Fs, PBDEs, PCBs, OCs, PFASs and SCCP) in eggs of the two gull species from the respective colonies of the Ebro Delta Natural Park. In the following studies, the analyses are based on the study of PFASs family in eggs and blood of both gull species and from different colonies of the Iberian Peninsula (Ebro Delta, Medes Islands, Columbretes Islands, Dragonera Island, Grosa Island, Chafarinas Islands, Atlantic Islands of Galicia and Berlengas Islands). All sampling sites are located in areas of high ecological interest and under any environmental protection (National or Natural Parks, Marine Reserves, Biosphere Reserves, etc.).

From PFASs levels found in the different matrices (first egg of the laying, albumen and yolk of the three eggs of entire clutches and adults’ blood) is studied the effect of these pollutants on physical (biometric parameters of egg, shell thickness and desiccation index) and hormonal level (through the analysis of the steroid hormones: testosterone and 17β-estradiol). It is also assessed the accumulation of these pollutants from the diet of yellow-legged and Audouin’s gull (analyzing their own food and through the study of the stable isotopes of carbon

and nitrogen (δ^{13}C and δ^{15}N), as dietary tracers) and it is estimated the PFASs depuration in blood and its transfer from females to their eggs.

The results indicate that organic pollutants are present in the populations of yellow-legged and Audouin's gull breeding in the Iberian Peninsula colonies, being OCs and PCBs the families of compounds with higher concentrations. Among PFASs, the main compound detected in all samples was PFOS. The transfer of organic contaminants from mother to offspring is demonstrated with the levels found in eggs of both species. Analysis of PFASs in blood of males and females of the two species of gull shows that females discharge a fraction of the compounds accumulated to the eggs, thus females had lower blood PFASs levels than males. There is no relationship between the levels of PFASs and biometrics, nor the levels of steroid. However, the study of hormonal level allows the comparison between the levels in both gull species and with other studies and to discuss the influence of the different diet. The analysis of PFASs in albumen and egg yolk from gull clutches of three eggs suggests that these compounds have more affinity for egg-yolk lipids because they are not detected in albumen. The decreasing concentration of PFOS along the egg sequence shows that females PFOS transfer is higher in the first egg but there is also a good correlation between the levels of PFOS among the 3 eggs from the same clutch. The higher concentrations of some PFASs in the 3rd egg (c), comparing with the 1st (a) and the 2nd (b), and the differences in δ^{13}C values between the a-b and b-c eggs are attributed to the "mother effect" (possible use of endogenous reserves for the formation of the last egg instead of the use of the energy provided by the diet prior to egg laying).

This thesis permits increase the knowledge about the accumulation of POPs in yellow-legged and Auduin's gull from the Iberian Peninsula, to evaluate the differences between the two species and assign it to their biology, determine the geographical distribution of POPs using eggs as a biomarker of environmental contamination and to study the processes of accumulation and transfer of PFASs.

III. List of tables

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V. List of abbreviations

ACN	Acetonitrile
Adj. Sig	Adjusted significance
AG	Audouin's gull
ANSE	*Associació de Naturalistes del Sud-Est*: Naturalist Association of Southeast
ASE	Accelerated Solvent Extraction
BDE 154	2,2',4,4',5,6'-HexaBDE
BDE 183	2,2',3,4,4',5',6-HeptaBDE
BDE 209	2,2',3,4,4',5',6,6'-Deca BDE
B_f	Blood female
BW	Body weight
BWf	Females body weight
BWm	Males body weight
C	Carbon
CAS	Chemical Abstracts Service
C_c	Concentration in crayfish
CE	*Comunidad Europea*: European Community
CEE	*Comunidad Economica Europea*: European Economic Community
Cegg	Concentration in eggs
C_f	Concentration in females
CF_3	3 fluorine atoms and 1 chlorine atom
CFC	Chlorofluorocarbon
C_{fi}	Concentration in fish
C_g	Concentration in gulls
Cl	Chlor
ClO	Chlorine oxide
C_m	Concentration in males
COM	Commission of the European Communities
COP	Conference of the Parties
Corr. Coef.	Correlation coefficient
d	Day
DCM	Dichloromethane
DDE	Dichloro Diphenyldichloro Ethylene
DDT	Dichloro Diphenyl Trichloroethane
DEP	Depuration rate
Di	Desiccation index
DL-PCBs	Dioxine-like PCBs
DOCE	*Diario Oficial de la Comisión Europea*: Official Journal of the European Commission
DOGC	*Diari Oficial de la Generalitat de Catalunya:* Official Journal of Catalonian Government
dw	Dried weight
E_2	17β-estradiol
EDI	Estimated daily intake
Ed.	Editor
Eds.	Editors
EFSA	European Food Safety Authority
ESI	Electrospray interface
et al	*et alii*: and others
eV	Electronvolt
EW	Egg weight
ExCOP	Simultaneous Extraordinary Meetings of the Conferences of the Parties
EYI	Estimated Year Intake

F	Fluor
$F_{x,y}$	F-test (ANOVA)
Fig.	Figure
g	gram
GC-NCI-MS	Gas Chromatography-Negative Chemical Ionisation- Mas Spectometry
GOV	*Acord del Govern*: Government Agreement
H	Hydrogen
ha	Hectare
HBCDs	Hexabromocyclododecanes
HCB	Hexachlorobenzene
HCFC	Hydrochlorofluorocarbon
HCH	*Hexachlorocyclohexane*
HCl	Hydrogen chloride
HF	Hydrogen fluoride
HFC	Hydrofluorocarbon
HRGC	High resolution Gas Chromatography
HRMS	High Resolution Mass Spectometry
HPLC	High Performance Liquid Chromatography
hv	light energy
I	Thickness index
IAEA	International Atomic Energy Agency
IFCS	Intergovernamental Forum of Chemical Safety
IPPC	Integrated Pollution Prevention and Control
Is.	Island
IUCN	International Union for Conservation of Nature
K_d	Daily Constant
K_{dep}	Depuration Constant
kg	Kilogram
km	Kilometer
Kv	Species-specific constant
L	Length
LBS	Land Based Sources
LHC	*Llista d'Hàbitats presents a Catalunya*: List of Habitats in Catalonia
LOD_{inst}	Instrument detection limit
LOD_{method}	Method detection limit
lw	Lipid weight
m	Meter
MA	Massachusetts
MAB	Man And the Biosphere
MB	Mass balance
MDL	Method Detection Limit
M_{egg}	Mass in eggs
min	Minute
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
MRM	Multiple reaction monitoring
N	Nitrogen
n	Sample size
N.A.	Not analyzed
NBS	National Bureau of Standard
N.D.	Not detected
N-E	Nort-East
N.D.	Not detected

ng	Nanograms
NH4OAc	Ammonium acetate
Nº	Number
N-W	North-West
OAPN	*Organismo Autónomo Parques Nacionales*: National Parks Autonomous Agency
O	Atom of oxigen
O_2	Molecule of oxygen
O_3	Ozone
OCs	Organochlorinated compounds
OH	Hydroxyl
OSPAR	Oslo-Paris Convention
P	P-value
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PCAGA	*Plan Coordinado de Actuaciones de la gaviota de Audouin*: Audouin's Gull Performances Coordinated Plan
PCBs	Polychlorinated biphenils
PCDD/Fs	Polychlorinated dibenzo-p-dioxins and furans
P_{cd}	Percentage of Crayfish based Diet
PCDF	Polychlorinated dibenzofurans
PDB	Pee Dee Belemnite
P_{fd}	Percentage of Fish based Diet
PFASs	Perfluoroalkyl and Polyfluoroalkyl Substances
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutane sulfonate
PFCAs	Perfluoroalkylcarboxylic acids
PFCs	Perfluorinated compounds
PFDA	Perfluorodecanoic acid
PFDoA	Perfluorododecanoic acid
PFDS	Perfluorodecane sulfonate
PFHpA	Perfluoroheptanoic acid
PFHxA	Perfluorohexanoic acid
PFHxDA	Perfluorohexadecanoic acid
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFODA	Perfluorooctadecanoic acid
PFOS	Perfluorooctane sulfonate
PFPA	Perfluoropentanoic acid
PFSAs	Perfluoroalkyl sulfonates
PFTE	Polytetrafluoroethylene
PFTeDA	Perfluorotetradecanoic acid
PFTriDA	Perfluorotridecanoic acid
PFUnA	Perfluoroundecanoic acid
pg	Picogram
PhD	Doctor of Philosophy
PLE	Pressuring Liquid Extraction
PNA	*Plan Nacional de Aplicación*: National Plan of Implementation
PNEC	Predicted No Effect Concentration
POPs	Persistent Organic Pollutants
R^2	Regression coefficient
R.D.	*Real Decreto*: Royal Decree
REACH	Registration, Evaluation, Authorisation and Restriction of Chemical substances
RIA	Radioimmunoassay

rpm	Revolutions per minute
RSD	Relative Standard Deviation
S.A.	*Sociedad Anónima*: Corporation
SAICM	Strategic Approach International Chemicals Management
Sbc	Subcolony
SCCPs	Short chain chlorinated paraffins
SD	Standard deviation
S-E	South-East
SE	Standard error
SEM	Standard error of mean
SIA	Stable isotope analysis
Sig.	Significance
SIM	Selected Ion Monitoring
SPE	Solid phase extraction
T	Testosterone
t	permutation test
TDI	Tolerable Daily Intakes
TFA	Trifluoroacetic acid
TQD	Tandem Quadruple Detector
TRA	Transfer capacity
TRV	Toxicity Reference Value
UE	*Unión Europea*: European Union
UK	United Kingdom
UNEP	United Nations Environment Programme
UNESCO	United Nations Educational, Scientific and Cultural Organization
UPLC	Ultra Performance Liquid Chromatography
UPLC-MS/MS	Ultra performance liquid chromatography coupled to tandem mass spectrometry
USA	United States of America
V	Volume
VPDB	Viena Pee Dee Belemnite
vs	Versus
Wd	Width
WHO-TEQ	World health Organisation – Toxic Equivalent
ww	Wet weight
X^{x}_{y}	X-value (Kruskal-Wallis test)
y	Year
yDEP	Depuration rate per year
YLG	Yellow-legged gull
ZEP	*Zona de Especial Protección*: Special Protection Area
ZEPA	*Zona de Especial Protección para las Aves*: Special Protection Area for Birds
ZEPIM	*Zonas Especialmente Protegidas de Importancia por el Mediterraneo*: Specially Protected Areas of Importance for the Mediterranean
α	Confidence interval
δC	Carbon isotope stable proportion
δN	Nitrogen isotope stable proportion
µg	Microgram
µL	Microliter
µm	Micrometer
∑	Sum
ºC	Celsius degree

VI. Thesis layout

The present thesis is organized in seven chapters. The first and the last chapters correspond to the objectives and conclusions, respectively. Chapter 2 is the introduction. Chapters 3, 4, 5, 6 and 7 correspond to different studies carried out regarding the analysis and geographical distribution of contaminants in gulls and to study the species differences on contaminant burden. The corresponding list of references is included in each chapter. The content of the thesis is as follows:

Chapter 1 describe the main and specific objectives of the present thesis.

Chapter 2 comprises the thesis introduction, including background, state of the art, description of the analyzed compounds, the studied species, the study area and the sampling design.

Chapter 3 deals with the impact of Persistent Organic Pollutants (POPs) in the eggs (the first egg of the laying) of two gull species, the opportunist yellow-legged gull (*Larus michahellis*) and the piscivorous Audouin's gull (*Larus audouinii*), from the colonies of the Ebro Delta Natural Park. The studied compounds are included or under consideration by the Stockholm Convention. These are PCDD/Fs (polychlorinated dibenzo-p-dioxins and furans), PBDEs (polybrominated diphenyl ethers), PCBs (polychlorinated biphenils), OCs (organochlorinated compounds), PFCs (perfluorinated compounds, later called PFASs, per- and polyfluoroalkyl substances), SCCPs (short chain chlorinated paraffins). The most ubiquitous families of POPs in the eggs of both gull species are PCBs and OCs and followed by PFCs, PBDEs and SCCPs. The ability of the eggs as to be used as a biomonitoring matrix for these pollutants is discussed. In addition, by determining the levels of pollutants in eggs, it is possible to determine the potential negative impact of POPs on Audouin's gull population, a species considered as *vulnerable*. The contents of this chapter have given rise to the publication of Morales et al. (2012) in the scientific journal Chemosphere 88, 1306–1316. Given the compounds detected in gull eggs from the Ebro delta, their toxicological impact and the little information available in bird eggs compared to other chemical families, it was decided to focus on the accumulation and effects of the PFASs in yellow-legged and Audouin's gull.

Chapter 4 assesses the presence and distribution of PFASs in the eggs (the first egg of the laying) of yellow-legged gull from 8 different colonies of the Iberian Peninsula with high ecological interest (Natural or National Parks, Marine reserves or under other environmental protection). The detection of only compound, PFOS (perfluorooctane sulfonic acid), is discussed together with the different contamination sources of each zone. Individual effects as

well as biometric measurements and eggshell thickness and desiccation indexes are studied. The contents of this chapter have given rise to the publication of Vicente et al. (2012) in the scientific journal Science of the Total Environment 416, 468–475.

Chapter 5 studies the presence of 17 PFASs in eggs of yellow-legged and Audouin's gull which cohabit in the Ebro Delta Natural Park. On the other hand, as PFOS is the main PFASs detected, its transfer from mothers to the eggs is determined and the Estimated Daily Intake, the Depuration rates and the Mass balance of PFOS in yellow-legged and Audouin's gull colonies from the Ebro Delta are evaluated.

Chapter 6 examines the distribution of PFASs in entire clutches (3 eggs) of Audouin's gull breeding in the Ebro Delta. Unlike other chapters, albumen and egg-yolk are analyzed separately. The laying order and mother effect are assessed and the relation with carbon and nitrogen stable isotopes as dietary tracers is studied.

Chapter 7 considers a new approach by determining levels of two steroid hormones, the 17β-estradiol (E_2) and testosterone (T), in the eggs (the first egg of the laying) of yellow-legged gull from 7 Spanish colonies with high ecological interest. In one of the colonies, the Ebro Delta, the two steroids in eggs and blood of the respective progenitors of yellow-legged and Audouin's gull is also studied. The different feeding habits and biology from both species are discussed in order to explain the different hormonal levels. The results encountered are compared to other studies to provide descriptive differences among species and habitats.

Chapter 8 provides some general conclusions of all the studies carried out in this thesis.

Chapter 1. OBJECTIVES

The general aim of the thesis was to study the accumulation and distribution of persistent organic pollutants in coastal birds through the study of two gull species, *Larus michahellis* and *Larus audouinii*, which differ in distribution and ecology. The study site comprised the 8 main gull colonies of the Iberian Peninsula. Taking into account the different anthropogenic pressures in each study area, gull eggs were used as bioindicators of pollution. Within this context, the specific objectives were:

- Design and apply a sampling plan for eggs and blood of Audouin's gull and yellow-legged gull during the breeding period in the different study areas.
- Determine organic pollutants such as PFASs, PCDD/Fs, PBDEs, PCBs, OCs and SCCPs in eggs through the solid-liquid extraction and gas and liquid chromatography coupled to low and high resolution mass spectrometry .
- Determine the distribution in the Iberian Peninsula of the 5 main relevant PFASs using gulls eggs as bioindicator of environmental pollution.
- Study the accumulation of PFASs and its transfer from females to the eggs of two gulls with different ecology, Audouin's gull and yellow-legged gull, which cohabit in the Ebro Delta Natural Park and the effect of the laying order.
- Evaluate the relationship between the accumulation of PFASs through the diet by analyzing their own diet and stable isotope analysis.
- Evaluate the effects of pollution on the levels of estrogen and androgen on the species studied.
- To contribute to the understanding of the ecological status of Parks and Nature reserves as well as increase the knowledge of the species studied.
- Provide information on the levels of organic pollutants included on the Stockholm Convention, as Spain has ratified the agreement.

Chapter 2. INTRODUCTION

2.1 Background and state of the art

2.1.1 Distribution, partitioning and effects of organic pollutants

Organic pollutants can be (i) natural substances where emissions come from oceans, plants foliage, volcanic activity or natural forest combustions (Guenther et al., 1995; El-Shahawi et al., 2010).), (ii) unintentional byproducts of natural or human-induced processes or (iii) specifically synthesized for its use in industrial processes and consumer products (OSPAR, 2009).

Among a large myriad of organic pollutants, *Persistent Organic Pollutants* (POPs) comprise a group of chemicals that have resistance, in a variable range, to photolysis, chemical and biological degradation. Most of them are halogenated compounds with low water solubility (hydrophobic) and high solubility in lipids (lipophylic), what facilitates its bioaccumulation in fatty tissues (Ritter et al., 1995). Different chemical families are classified as POPs and all of them are characterized by their high permanence in the environment, with long half-life in soils, sediments, air or biota. In the aquatic systems and in soils, POPs tend to partition to the solids and organic matter of the soil or sediment, avoiding the aqueous phase, and in organisms, they accumulate in fatty rich tissues which leads to high bioaccumulation rates along the food webs, increasing their concentration in each trophic level (phenomenon called "biomagnification") (Mackay and Fraser, 2000). Under high temperatures, POPs volatilize from soils, vegetation and water bodies and are likely to enter to the gas phase. Their resistance to decomposition and their semi-volatile character enhance transportation for long distances adsorbed to atmospheric particles or by aerosols until they are deposited again on the earth surface, under cold temperature conditions. Furthermore, in rainy areas POPs are deposited to the surface by the phenomena called "wet deposition". Volatilization-deposition cycle can be repeated many times and consequently, these compounds can reach remote areas far away from the original source of contamination (Jones and de Voogt, 1999). This phenomenon is known as "Grasshopper effect" (Gouin et al., 2004). The rate of release from the surface is lower in cold regions such as Poles and high mountains, so at regional or global scale, POPs can potentially migrate from warmer areas to colder areas (Jones and de Voogt, 1999), and thus, are considered global contaminants.

POPs are chlorinated and brominated aromatic compounds and include the families of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs), polybrominated diphenyl ethers (PBDEs), organochlorinated pesticides (OCs), per- and polyfluorinated alkyl substances (PFASs), and paraffines (Jones and de Voogt, 1999), although

other compounds such as phthalates or polycyclic aromatic hydrocarbons (PAHs) are sometimes considered as POPs.

First compounds classified as POPs were the pesticides, like DDT (DDT, l 1,1,1-Trichloro-2,2-bis(4-clorophenyl)-ethane, formerly dichloro-diphenyl-trichloroethane). Its production started around 1920 and once demonstrated its efficiency in agriculture, the production of pesticides increased rapidly. Then started to emerge other chemicals used as surfactants (PFASs), flame retardants (PBDEs), industrial chemicals precursors (PCCD/Fs), agrochemicals (OCs) etc. Between 1960 and 1970 appeared the first hazard evidences of POPs. Since the publication of the book *Silent Spring* on the bird populations decline (Carson, 1962) and the first paper of PCBs in wildlife in the scientific journal of Environmental Pollution in 1970 (Prest et al., 1970), an enormous number of surveys have been conducted in which local and regional contamination by a wide variety of xenobiotic substances has been revealed. The concern of POPs toxicological effects in the environment have led to the control or completely banning of the use of these compounds in many countries (El-Shahawi et al., 2010). Their persistence in the environment due to their physicochemical characteristics leads them reach even human tissues (Jones and de Voogt, 1999)

In terms of toxicology, POPs exposure can cause direct symptoms or health problems throuhout life due to its accumulation. Their toxic effects on wildlife vary according to the species (Stockholm Convention Secretariat, 2012). Some POPs have been identified as endocrine disruptors. Endocrine disruptors can alter the normal function of reproductive and endocrine systems in humans and wildlife. POPs can also cause cancer, births defects, learning difficulties, immunologic dysfunctions and behavioral, neurologic and reproductive problems (El-Shahawi et al., 2010). Some POPs have been detected in mammals breast milk and placenta, what expose them to the offspring. Recently, the presence of certain POP has been associated with reduced immunity in children and simultaneous increase of infections (Roots et al., 2005). Children are more sensible to POPs effects as they are still developing. Apparently, the brain is the most important point of concern as several studies have demonstrated that people exposed to POPs during the childhood have a lower intellectual quotient and less ability to concentrate (Bouwman, 2003; Bolt and Degen, 2002). Most of pesticides and fungicides used in agricultural crops can have lettal effects on birds and little mammals when they eat the harvest. These compounds remain in the soil or reach water systems affecting fish and other biota (Stockholm Convention Secretariat, 2012). Lifestock fed with the treated harvest accumulates these chemicals which can be transferred to humans when they eat its meat. Human exposure to these contaminants is mainly through the diet, but also through the air (for example, some POPs have been detected in the indoor residences). Despite the intake of many POPs in humans are below the limits considered safe by the world health authoritites, their accumulation can cause adverse behavorial changes, reduced reproductive succes (such as heptachlor), photosensitive skin

lesions, colics, ddebilitation, meatabolic disorders (such as HCH), pigmentation of nails and mucous, fatigue, nausea, vomiting (such as PCBs), congenital physical disorders, mental retardation (such as endosulfan), chloracne, inmune and enzime disorders (such as dioxins) (Stockholm Convention Secretariat, 2012).

2.1.2 Legislation

There are some regulatory frameworks regarding the POPs. Considering POPs in coastal areas, which are the subject of this thesis, the main initiatives are listed below.

STOCKHOLM CONVENTION

The Stockholm Convention on POPs in the framework of the United Nations Environment Programme (UNEP) aims to protect human health and environment from the POPs. The ultimate objective is to eliminate or reduce the use of these compounds. The Convention was signed on May 2001 and entered into force on 17 May 2004. Spain signed the ratification on May 2004 and entered into force on 26 August 2004. The Stockholm Convention is the legal international instrument which set the prohibition of the intentional use of POPs mentioned in Annex A, the restrictions of the use of certain POPs, which may have exemptions, (Annex B) and the measures taken to minimize the non intentional emissions of the substances from Annex C.

Since its entry into force, 6 international meetings have been done, *Conference of the Parties* (COP1 in Uruguay on 2005, COP2 in Geneva on 2006, COP3 in Dakar on 2007, COP4 in Geneva on 2009, COP5 in Geneva on 2011 and the recent COP6 in Geneva on May 2013). These meetings establish work plans for those countries that have ratified the Convention. Initially, 12 POPs were included in the Convention (*the dirty dozen*) and divided in 3 categories:

- Pesticides.
- Industrial chemicals.
- By-products.

In subsequent meetings, other compounds have been added in the list. Currently, 179 countries have ratified the Convention and a total of 22 POPs have been included.

Additionally, simultaneous extraordinary meetings of the COPs (ExCOP 2010) to the Basel, Rotterdam (explained later) and Stockholm conventions were held in Bali, Indonesia, from 22 to 24 February 2010. The convening of three independent treaty conferences simultaneously marked a historic departure for international environment governance.

COMPOUND	Nº CAS	PHYTOSANITARY /BIOCIDE	INDUSTRIAL USE	NON INTENCIONAL EMISION
Substances of compulsory compliance				
1. Aldrin	309-00-2	**X**		
2. Chlordane	57-74-9	**X**		
3. Chlordecone	143-50-0	**X**		
4. dichloro-diphenyl-trichloroethane (DDT)	50-29-3	**X**	**X**	
5. Dieldrin	60-57-1	**X**		
6. Endrin	72-20-8	**X**		
7. Heptachlor	76-44-8	**X**		
8. Hexabromobiphenyl	35694-06-5		**X**	
9. Hexachlorobenzene (HCB)	118-74-1	**X**	**X**	**X**
10. Hexachlorocyclohexanes (HCH)		**X**		
α-HCH	319-84-6			
β-HCH	319-85-7			
γ-HCH	58-89-9	**X**		
δ-HCH	319-86-8			
11. Mirex	2385-85-5	**X**	**X**	
12. Polycyclic Aromatic Hydrocarbons (PAHs)			**X**	**X**
13. Polychlorinated biphenyls (PCBs)	1336-36-3		**X**	**X**
14. Polichlorinated dibenzodioxins (PCDDs)				**X**
15. Polichlorinated dibenzofurans (PCDF)				**X**
16. Toxaphene	8001-35-2	**X**		
Other substances considered				
17. Endosulphan	115-29-7	**X**		
18. Dicofol		**X**		
19. Pentachlorophenol	87-86-5	**X**	**X**	
20. Pentabromodiphenyl ether	32534-81-9		**X**	
21. Octabromodiphenil ether	32536-52-0		**X**	
22. Pentachlorobenzene			**X**	
23. Hexachlorobutadiene	87-68-3		**X**	
24. Polychlorinated naphthalenes	-		**X**	
25. Perfluorooctane sulfonate (PFOS)	-		**X**	
26. Short Chain Chlorinated Paraffin	85535-85-9		**X**	

Table 1. POP substances included in Stockholm Convention and in the Regulation and other substances considered in PNA. Source: adaptation from Pla Nacional d'Aplicació del Conveni d'Estocolm i el Reglament 850/2004, sobre Contaminants Orgànics Persistents.

Regulation (CE) 850/2004 on POPs

To carry out the provisions of the Stockholm Convention, the European Union (UE) created the Regulation (CE) 850/2004 on POPs. It was adopted on 26 April 2004, published in the DOCE on 30 April 2004 and on 20 May 2004 it entered into force in all countries from the UE. Article 1 establishes the objective of "protection the human health and the environment against the Persistent Organic Pollutants" considering the "precautionary principle". Therefore, the legal basis of this regulation is established in relation to the protection of the environment in UE included in the article 175.1 of the Treaty Establishing the European Community. The Regulation also tries to ensure coordination and coherence in applying, at the EU level, the

provisions of the Conventions of Rotterdam, Stockholm and Basel, and to participate in the development of the Strategic Approach with regard to International Chemicals Management (Reglament (CE) Nº 850/2004).

Spanish National Plan of Implementation of the Stockholm Convention and the Regulation 850/2004 on POPs (PNA)

Both the Stockholm Convention (article 7) and the Regulation (CE) Nº 850/2004 (article 8) prescribe the obligation to prepare National Plans of Implementation within two years from the entry into force to comply with the provisions of both legal instruments. The process to develop the PNA in Spain started on January 2005 and concluded with the approval by the Council of Ministers on 2 February 2007. PNA was made for the exchange of relevant information between the different sectors and the synthesis of the best available information in Spain and its notification in the Conference of the Parties. PNA objectives were to describe current and planned initiatives to develop and implement an effective strategy that allows the achievement of the European Union purposes of the Stockholm Convention and the Regulation 850/2004 in Spain. Additionally, PNA includes other POP substances also considered priority (Plan Nacional de Aplicación del Convenio de Estocolmo y el Reglamento 850/2004, sobre Contaminantes Orgánicos Persistentes) (table 1).

Other legal instruments

Stockholm Convention is the latest and most ambitious international initiative with the aim to regulate and control POPs. However, this Convention has been preceded from other international and community initiatives.

- Protocol on Persistent Organic Pollutants, from the Geneva Convention on 1979, adopted in Aarhus (Denmark) on 1998.
- International Code of Conduct on the Distribution and Use of Pesticides (1985).
- Basel Convention on Control of Cross-Border Movements of Hazardous Waste and their Disposal (1989).
- Rotterdam Convention on the consent procedure applicable to certain hazardous chemicals and pesticides objective of international trade (1998).
- Convention of Barcelona for the protection of the Mediterranean Sea: Protocol for the Protection of the Mediterranean Sea against pollution caused by activities and land based sources (LBS) and Strategic Action Programme (signed on 1975 and amended on 1995).

- Convention for the protection of the marine environment of the NE Atlantic (OSPAR Convention, 1992).
- London Convention (1972) and the Protocol of 1996 on the prevention of marine pollution by waste spills and other materials.
- Intergovernmental Forum on Chemical Safety (IFCS).
- Aarhus Convention on Access to Information, Public Participation in Decision-making and Access to Justice in Environmental Issues.
- Regulation 1907/2006/CE (Registration, Evaluation, and Authorization of Chemicals (REACH)).
- Council directive 96/61/CE on 24 September 1996 relative to the integrated prevention and control of pollution (IPPC Directive).
- Measures to reduce or eliminate releases resulting from unintentional production of Dioxins, Furans and PCBs (2001).
- Community legislation relating to the presence of POPs in waste water (Directive 86/280/CEE and Water Framework Directive 2000/60/CE).
- Community provisions relating to the presence of POPs in the atmosphere: waste incineration that generates POPs (section D, article 6 of Stockholm Convention).
- International transport of waste containing or contaminated with POPs substances (section 1 D, iv, article 6 of Stockholm Convention), Regulation (CEE) 259/93.
- Identification of contaminated sites (COM (2006) 231, COM (2006) 232, COM (2006) 1165 and COM (2006) 620).
- European Strategy for Environment and Health.

2.1.3 Description of coastal areas

Coastal areas are zones where the interaction of the sea, lakes, estuaries and land processes occur. There is no official definition for coastal areas since its limits are not concrete. A basic definition was given by the Commission on Marine Science, Engineering and Resources of United Stated in 1969 where it was defined that coastal areas were the regions of transition between the land and the sea (Stratton Commission, 1969). On the other hand, the Food and Agriculture Organization of the United Nations defines the coastal areas as the areas of interaction and transition between land and sea or land and large inland lakes, indicating that there are no natural boundaries clearly defining the coastal areas http://www.fao.org/forestry/icam/4302/es/). According to the criteria used, the coastal area is known as coastal area or littoral zone. The term of coastal area is used for legal and administrative criteria assigning a specific geographic area for political and administrative reasons. While littoral zone refers to a geographical area where natural and human phenomena

act (Barragán Muñoz (phD), 2004).

Coastal areas are very important both for ecological and economic reasons, and thus, it is essential to regulate its space and use. In 1988, Spain created the "Ley de Costas 22/1988" and currently it is under supervision with the intention of protecting the coast due to the excessive development and urbanization (Proyecto de Ley 121/000029). The extension of the Spanish littoral is of 504,781 km^2 of land area, of 14,394 km^2 of inland waters and of 7883 km^2 of coastline (Barragán Muñoz, 2004). Due to the increase of anthropogenic activities (both on land, sea and in coast), pollution of coastal areas is a topic of current concern. Agriculture, livestock, industry, urban activities, tourism, fisheries, port facilities, transports, etc. generate wastes which through the water or air are released into the sea where produce an ecological impact in terms of coastal ecosystem level, population or at species level (Stanners i Bourdeau, 1995).

2.1.4 Chemical contamination in birds

Today many species of birds live in a habitat strongly affected by environmental pollution, mainly due to chemicals used in agriculture (pesticides), industry (solvents, flame retardants, additives) or in everyday life (brominated and fluorinated compounds used as additives in many products). Many of these compounds accumulate in birds through ingestion or breathing and can affect their behavior, body condition and reproductive parameters. Among the different families of pollutants, POPs have an important role due to their chemical characteristics, high accumulation potential and high toxicity. The first evidence of the presence of POPs in birds and in their clutches was in 1967 when levels of organochlorine pesticides were associated to the decrease in egg shell thickness of certain birds of prey (Ratcliffe, 1967). Few years later, pesticide residues were detected in eggs (Stickel and Wiemeyer, 1973) and it was suggested that they may cause reproductive effects (Fergin and Schafer, 1977). Another pioneering study in 1978 on Mediterranean gulls showed that birds can also accumulate polychlorinated naphthalenes, PCBs and metals (Vannuchi et al., 1978). These studies have evolved and have revealed over the time that the POPs are distributed globally for all ecosystems, being bird species able to accumulate these compounds and suffer toxic effects. In Canada, very high levels of POPs were detected (35 to 140 mg / kg) and correlated with human activity and in this study it was proposed the gulls as indicators of "local" pollution (Weseloh et al., 1990). In Sweden in 1982 POPs were detected in eggs of white-tailed eagles (*Haliaeetus albicilla*) and it was attributed to the failure of the laying (Helander et al., 1982) and 20 years later it was associated the impact of these pollutants on the development of the egg shell and the reproductive viability (Helander et al., 2002). Numerous effects of POPs on the reproductive potential of fish-eating birds were also reported in the Great Lakes (Giesy et al., 1994) and in

Europe (Bosveld and Van den Berg, 1994). In Greece, PCBs and chlorinated pesticides detected in different species suggested a widespread and diffuse contamination in areas of high ecological interest (Konsantinou et al., 2000; Goutner et al., 2001; Albanis et al., 2003; Antoniadou et al., 2007). In Italy high concentrations of DDTs and PCBs were detected in birds collected near an old factory of DDTs and they were correlated with low levels of steroid hormones and effects of the laying (Cortinovis et al., 2008). The wide distribution of POPs is evidenced when these contaminants are identified in remote areas such as the Arctic (Herzke et al., 2003) and correlated with effects on reproductive behavior and population development (Verreault et al., 2008). It has been detected even "modern" contaminants, such as the flame retardants PBDEs, in Northern Fulmar eggs (*Fulmarus glacial*) (Karlsson et al., 2006).

In Spain there have been several studies related to the presence of POPs in birds but there are no studies showing the incidence of PBDEs and PFASs and their effects. A study in 1991 showed the presence of PCBs and chlorinated pesticides in gull eggs from the Ebro Delta, Chafarinas Islands and Cuenca and it was identified PCBs profiles varying with the origin of the sample (González et al., 1991). Subsequent studies suggested gull eggs as a biological indicator of PCBs, HCHs and DDTs but it was indicated the need to collect the first egg of the clutch to avoid variability due to the sampling (Pastor et al., 1995a). Also it has been detected in Spain PCBs, dioxins and furans in gull eggs (Pastor et al., 1995b). Even in apparent pristine areas like the island of Menorca, there was detected DDTs, PCBs, dioxins and furans at concentrations that could induce reduction in the laying, embryonic mortality and deformity in birds (Jiménez et al., 2007).

Per- and polyfluorinated alkyl substances (PFASs) in seabirds

The first study of PFASs in birds was done by Giesy and Kannan on 2001. They analyzed 4 compounds (PFOS, PFHxS, PFOA and a precursor of PFOS, perfluorooctanesulfonamide (PFOSA)) in various tissues of aquatic mammals, birds, fish, turtles and frogs from different areas of North America with varying degree of urbanization. The most ubiquitous compound was PFOS. They studied 11 bird species. PFOS was found in the three tissues analyzed (plasma, egg yolk and liver) at concentration ranges of 1 – 2570, 35 – 320 and 33 – 690 ng/g ww (wet weight), respectively. This study demonstrated that PFOS was widespread in the environment and suggested that it bioaccumulated through the food chain. Shortly afterwards, Kannan and his group (2001) published new results on PFOS levels in 21 species of piscivorous water birds of the United States. In that study, they analyzed liver, kidney, blood and egg yolk. Again, the highest levels of PFOS were found in blood plasma, followed by liver, egg yolks and kidney. Soon after, Giesy et al. (2001) analyzed PFOS, PFHxS, PFOA and PFOSA in 1700 samples of different tissues of aquatic mammals, birds, fish,

amphibians and reptiles from around the world, including the Arctic and Antarctic. PFOS was the most prevalent compound and it was found even in remote regions. Since then, the interest in the study of these compounds in birds and other animals around the world has increased. In addition, the transfer of PFASs from female to eggs has been studied (Gebbink and Letcher, 2012; Gebbink et al., 2009; Verreault et al., 2005), PFASs accumulation potential through diet (Newsted et al., 2007; Gebbink and Letcher, 2012), the depuration kinetics (Newsted et al., 2006; Yoo et al., 2009) and their toxicity and effects (O'Brien et al., 2009; Molina et al., 2006; Hoff et al., 2005).

2.1.5 Stable isotopes

Isotopes are variants of a particular chemical. All isotopes of an element have the same number of protons and electrons but differ in the number of neutrons (Michener and Lajtha (Eds.), 2008). The difference of neutrons does not affect their physical or chemical properties, only its atomic weight, allowing the identification through mass spectrometry (*isotope ratio mass spectrometry* (IRMS)) (Jardine et al., 2003). Stable isotopes (SI) are those considered energetically stable, which do not decay spontaneously and do not undergo radioactive decay (Michener and Lajtha (Eds.), 2008). An isotope tends to be stable when the number of neutrons (T) and protons (Z) is similar ($T/Z \leq 1.5$) (Michener and Lajtha (Eds.), 2008). There are approximately 300 stable isotopes and 1200 radioactive isotopes. Only 21 elements are pure, i.e. have only one stable isotope (Hoefs, 2009).

Geochemists and paleo-oceanographers were the first to use stable isotopes to study the global cycles of elements, past climatic conditions, geology and hydrotherm (Michener and Lajtha (Eds.), 2008). The lack of knowledge in chemistry and the difficult accessibility in stable isotope measurement instruments made that the first studies on ecology using these elements did not begin until some years later (Peterson and Fry, 1987).

The differences in atomic mass as well as in the thermodynamic characteristics cause slight variations in the performance of biochemical processes. These variations favor the lighter or heavier isotope, leading to depletion or enrichment, respectively, of the product relative to the substrate (or consumer relative to its energy resource) (Jardine et al., 2003).

The ratio of stable isotopes is expressed by delta notation (δ) and is measured using the following formula:

$$(1)\quad \delta = ((R_{sample}/R_{standard}) - 1) \times 1000$$

Where δ is the isotope ratio of the sample relative to a standard; R_{sample} and $R_{standard}$ are the fractions of heavy to light isotopes in the sample and the standard, respectively. One is

subtracted from the fraction $R_{sample}/R_{standard}$ so that samples with lower proportion of heavy isotopes than standards have a negative ratio and those with higher proportion have a positive value. δ is given in units per thousand (‰) (Kelly, 2000).

In the present thesis, the stable isotopes of carbon ($^{13}C/^{12}C$; $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$; $\delta^{15}N$) have been determined. SI of carbon and nitrogen have many applications in ecology, one of which is the study of the feeding ecology in vertebrates. They began to be used in ecology in the late 70s and their use in studies on mammals and wild birds has been increasing since then (Kelly, 2000). For carbon, the international standard was a limestone formation of the marine fossil of South California (USA) *Belemnitella americana* (PDB, *Pee Dee Belemnite*) (Craig, 1957). Currently, the PDB is not available, and thus it has defined a new standard reference, Vienna-PDB (VPDB), assigning a fixed value of 1.95 ‰ for calcite 19 from *National Bureau of Standards* (NBS)-19 (Coplen, 1996). The standard for nitrogen is the atmospheric nitrogen, the air (Ehleringer i Rundel, 1989). The relative abundance of stable isotopes of carbon and nitrogen used in ecology is about 98,892% for ^{12}C regarding to 1.108% from ^{13}C and 99.635 % ^{14}N regarding to 0.365% ^{15}N (Michener and Lajtha (Eds.), 2008).

The rate of stable isotopes of a consumer is related to the portion of the diet assimilated. The isotopic fractionation involves alterations in the rates of the tissues of the consumer in relation to the energy resource (Jardine et al., 2003).

From the study of food webs, it has been reported that the carbon isotopic values increase about 1‰ per trophic level, depending on the species (Peterson and Fry, 1987; France and Peters, 1997). Freshwater species tend to be less carbon-enriched (0.2‰) relative to the diet than estuarine species (0.5‰), coastal species (0.8‰) and open sea species (1.1‰) (France and Peters, 1997).

The values of nitrogen increase from 3 to 5 ‰ per trophic level, depending on the species and system (Peterson and Fry, 1987). Consequently, the study of stable isotopes of nitrogen are used to assign the position of the species in the food web (Kelly, 2000) while the SI of carbon are used to discriminate between different ecosystems exploited as food resources, such as terrestrial or marine environments (Peterson and Fry, 1987).

Each corporal tissue has a different *turnover rate* and therefore each tissue reflects dietary integrations over different time periods (Hobson and Bond, 2012). For example, in seabirds, isotopic measurements of bone collagen provide integrated information throughout the life of the individual, those of feathers during the time of its formation (post-breeding molt), those of liver tissues provide integrated information over the previous week and those of muscle of the diet of several months before (Hobson and Bond, 2012). Turnover rate of the heart, brain and blood tissues is about 20 days (Bauchinger and McWilliams, 2009) while SIA in eggs refers to the mother's diet the days before the egg laying, 3 – 5 days in albumen and shell, and about 8 days in egg-yolk (Hobson, 1995).

The study of the diet through the analysis of the eggs is the less invasive method for the species and the easiest sampling (Gloutney and Hobson, 1998). In addition, in many environments, it may be impossible to identify the diet of a species through the direct observation and the analysis of stomach contents is not completely reliable due to the different digestibility of the prey or food consumed (Kelly, 2000).

Among other applications, the analysis of stable isotopes is also used for the study of pollutants. Since δ^{15}N in high trophic level species (predators) represents an integration of trophic levels throughout the life of the organism, it is possible to study the correlations between the burden of δ^{15}N in tissues with the pollutants concentration such as mercury, organochlorines among others (Jardine et al., 2003). SIA has become a valuable tool that when coupled with dietary information helps establish food web interactions and energy flow in various ecosystems. (Jardine et al., 2003).

2.1.6 Seabirds as bioindicators

Bioindicators (or biomarkers) are elements which include biological processes, species or communities used to assess environmental quality, which may be affected over time by anthropogenic disturbances (such as pollution or changes in land uses) or by natural agents (droughts, fires, frosts) (Holt and Miller, 2011). The use of bioindicators started at 60s with pine needles as a biomarker of radioactive contamination (Szepke, R. 1963). Since then, its application has increased in various fields. First using flora as bioindicator of environmental contamination (Chamel et al., 1970; Grodzińska, K. 1977) and then using biota such as amphibians and fish (Birge et al., 1976; Ueno et al., 2004), mollusks (Poirrier and Partridge, 1979; Gordon et al., 1980), nematode (Bongers and Ferris, 1999) etc. in both aquatic and terrestrial systems and also for air quality assessments Bioindicator species are effective because they have a moderate tolerance to environmental variability. A *rare* species, with little tolerance to environmental change is too sensitive or too infrequent to reflect the biotic response to a change (Holt and Miller, 2011). A good sentinel species must meet several criteria (Cunha and Guilhermino, 2006):

a) Easy to identify
b) Broad geographical distribution in the study area.
c) It must be well known, have good knowledge of their ecology, biology and physiology.
d) Abundant and accessible.
e) Reasonably large size.
f) Responsive to pollution but strong enough to survive.
g) Position defined in the food web.
h) Able to reflect local conditions. Have a life cycle restricted in the specific study area.

i) Easy maintenance of the species in the laboratory.

Traditional examples of biomarkers are lichens or bryophytes in terrestrial ecosystems and macroinvertebrates in aquatic systems (Holt and Miller, 2011).

Birds (Kingdom: Animalia, class: aves) is a taxonomic group formed by 9,000 species present in all the world's ecosystems (Burger and Gochfeld, 1996). Depending on the vital strategy, there are bird species resident throughout the year in the same area, or species that migrate in winter, or to reproduce, etc. (Burger and Gochfeld, 1996). Their feeding habits cover also a wide range. There are carnivorous species, herbivorous, piscivorous, omnivorous, scavengers (Burger and Gochfeld, 1996). The behavior of the birds is often related to their dietary practices, the structure of the habitat and other environmental requirements (UNEP, 2004). Thus, birds are a very diverse group and it is easy to find a bioindicator species for every environment and situation. Seabirds (3% of the total birds described (Martínez-Abraín, (phD) 2003)), defined as species that spend much of their lives in coastal areas, are useful as bioindicators of marine environments as they are at the top of web chains and have a long life expectancy which allows them to bioaccumulate contaminants with age (Burger and Gochfeld, 2004). A disadvantage is that in migratory species it is difficult to determine the source of exposure to pollutants (Burger and Gochfeld, 2004). The Oslo-Paris Commission proposed, among other groups, seabirds as good biomarkers of environmental pollution (OSPAR, 1999) and, together with UNEP (*United Nations Environment Programme*), suggest the sampling of eggs as a matrix analysis (OSPAR, 1999; UNEP, 2004).

2.2 Description of the analyzed compounds

This thesis is focused mainly in the analysis of the family of perfluoroalkyl and polyfluoroalkyl substances (PFASs). However, other compounds have been studied but they will be defined in chapter 2 as they were part of the screening study carried out to determine the impact of POPs in gull eggs. These compounds belong to the families of PCDD/Fs, PBDEs, PCBs, OCs and SCCPs. In this thesis we have also studied 2 steroid hormones (17β-estradiol and testosterone) with the aim to evaluate the effect of organic pollutants on the endocrine system. A description of each compound is given below:

2.2.1 Perfluoroalkyl and polyfluoroalkyl substances (PFASs)

PFASs are organic molecules partially o totally fluorinated, where the C-H bond has been substituted by the C-F bond. The fluorine bond provides high resistance to degradation processes and makes them environmentally persistent. The origins of PFASs are in a stain repellent, 3M’s from Scotchgard. This compound was discovered by accident in 1953 working

with an experimental compound where it was spilled on a lab assistant's tennis shoe. The spill kept the spot clean (Renner, 2006). Since then, they have been used the last 6 decades for the manufacturing of industrial products such as additives. These compounds are used mainly to produce polytetrafluoroethylene (PFTE), as well as for the waterproofing of materials in the textile industry, cleaning products, polishes, paintings, varnishes, pesticides, surfactants in the oil industry and mining, etc. All PFASs are synthetic and have surfactant properties.

First PFASs used were formed by chains of carbon atoms and 17 fluorines. This structure was found to be optimal in reducing surface tension (Renner, 2006). The longer the chain C-F, the better surfactant properties but also the more bioaccumulative and toxic. PFASs of 4 carbons or less do not appear to bioaccumulate (Renner, 2006).

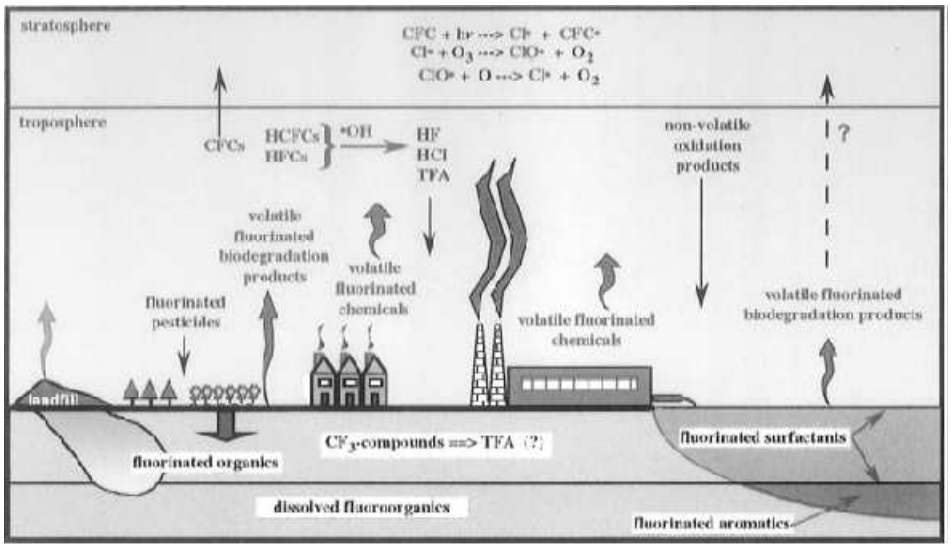

Figure 1. Biogeochemical cycle of fluorinated organic compounds. Source: Key et al., 1997

The physicochemical properties of PFASs vary among compounds; those more soluble remain in the aqueous phase, while the more hydrophobic compounds are accumulated in sediments and/or biota (Giesy et al., 2010). The high stability conferred by the C-F bond make that, historically, PFASs were considered as metabolically inert and non toxic (Sargent and Seffl, 1970). Since the study of Giesy and Kannan (2001) about PFASs in wildlife and the detection of PFOS and PFOA in blood of employees from a fluorochemical manufacturing plant (Olsen et al., 2000), the concern of the threat that these compounds represented for the environment started to rise. At that time, the studies to find other molecules which could be equally effective but less harmful started to emerge. This resulted in the synthesis of short-chain PFASs (Hansen et al., 2001). Over the years and with the knowledge increase, it has been demonstrated that PFASS are biologically active and can cause peroxisomal proliferation and other important alterations in the biochemical processes of organisms (Giesy et al., 2010).

Perfluorosulfonates (PFSAs), such as perfluoroctane sulfonate (PFOS), and perfluorinated carboxylic acids (PFCAs), such as perfluorooctanoic acid (PFOA) are the most

studied compounds (Lindstrom et al., 2011). Both groups are differentiated by the presence of a sulfur atom in their molecular structure. The functional group of each family has its own dynamics when bioaccumlated. PFSAs with the same number of carbons than PFCAs are easier to retain (Conder et al., 2008).

In wildlife, PFOS is the most abundant and it is accumulated basically in the blood and liver, but it is also found in pancreas, testes, kidney, eggs, feathers between other tissues Giesy et al., 2010;). In addition, PFOS is the final degradation product of many PFASs and its concentration in the environment is higher than other compounds of the same family (Giesy and Kanan, 2002). Its presence in the environment may be due to the free release from urban and industrial waste waters, where enter directly into the aquatic system or indirectly through the irrigation system or through the wastewater treatment plants effluents. Another source of environmental PFASs is the use of firefighting foams. Once in the soil, these compounds can reach the vegetation through the water absorption or reenter to the water cycle and discharge to the sea (fig. 1). Once in the sea, PFASs can enter to the food web through the basal trophic levels. Once there, they are available for the marine biota as they biomagnify along the food web. So, top predators have the highest PFASs levels (Linstrom et al., 2011).

For a human toxicological risk assessment, the PFASs concentrations found in food should be in accordance with the respective *tolerable daily intake* (TDI). The European Food Safety Authority (EFSA) published as a reference point of 0.15 μg/kg of body weight (BW) as the TDI for PFOS and 1.5 μg/kg BW for PFOA (Benford et al., 2008).

In this thesis 17 PFASs have been studied, 4 PFSAs (PFOS, perfluorooctane sulfonate; PFHxS, perfluorohexane sulfonate; PFBS, perfluorobutane sulfonate and PFDS, pefluorodecane sulfonate) (fig.2) and 13 PFCAs (PFBA, perfluorobutanoic acid; PFPA, perfluoropentanoic acid; PFHxA, perfluorohexanoic acid; PFHpA, perfluoroheptanoic acid; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; PFUnA, perfluoroundecanoic acid; PFDoA, perfluorododecanoic acid; PFTriDA, perfluorotridecanoic acid; PFTeDA, perfluorotetradecanoic acid; PFHxDA, perfluorohexadecanoic acid and PFODA, perfluorooctadecanoic acid) (fig.3).

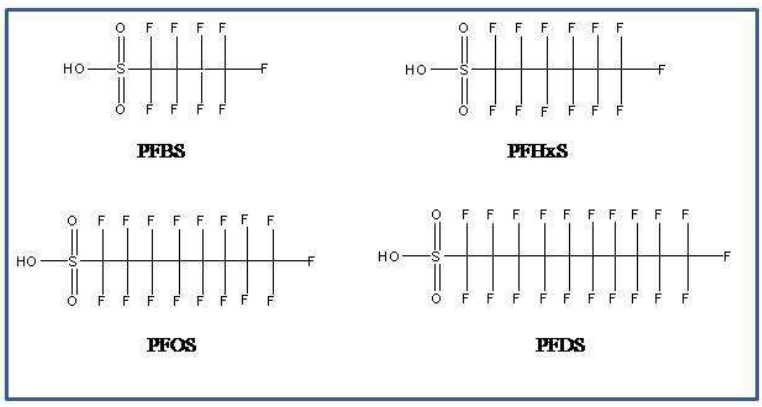

Figure 2. Structures and acronyms of the PFSAs studied

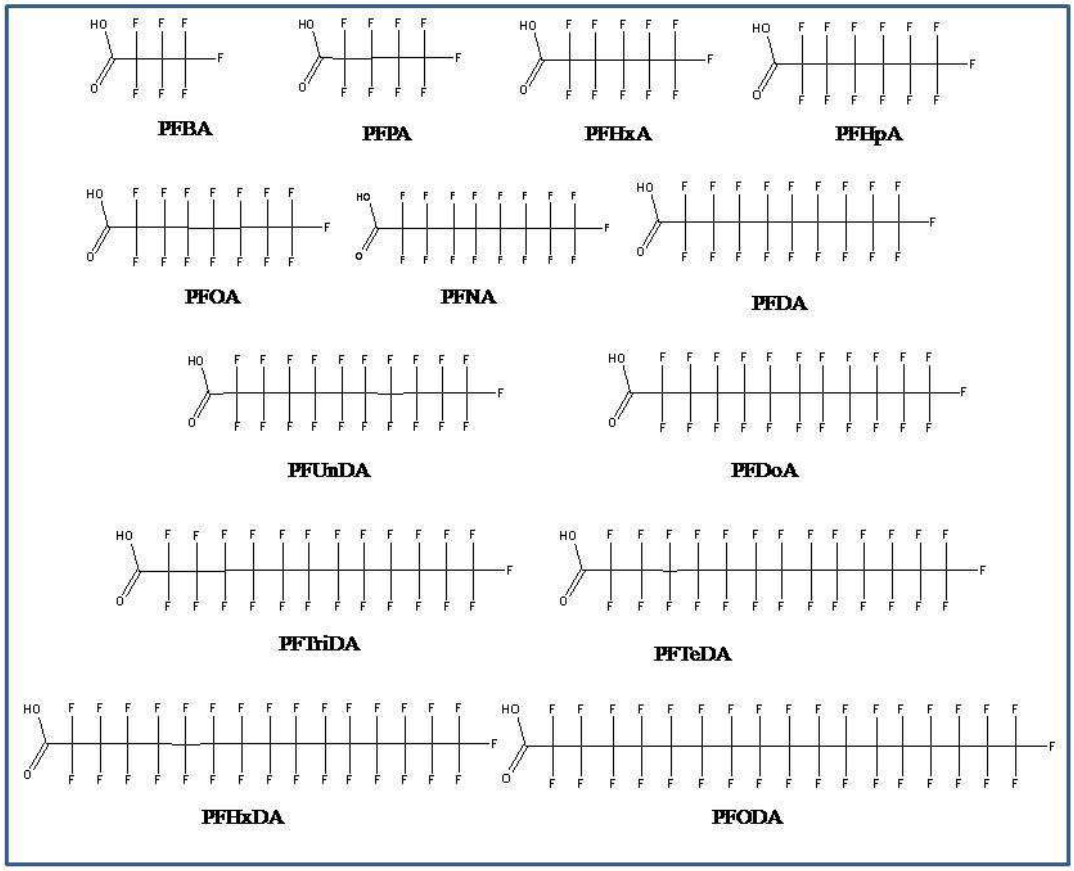

Figure 3. Structures and acronyms of the PFCAs studied.

2.2.2 Steroid hormones

Steroid hormones or steroids are hormones responsible for the regulation of the physiological processes. Hormones are molecules synthesized in certain cells of the organism and directed to cells of other organs with the aim to induce a specific response. However, there exist also hormones that act over the adjacent cells or, even, over the same cell. The receiving cell has a specific membrane protein which binds with the hormone and triggers the signal transduction. The receiving protein of steroid hormones is an intracellular protein located in the cytoplasm. Steroids are lipid molecules and hence, liposoluble, so that they can cross cellular membranes. These hormones bind to specific carrier proteins from blood (Kovacks and Ojeda (Eds.), 2012).

Steroid hormones are classified in 5 groups according to the protein receptors to which they are attached: *glucocorticoids, mineralocorticoids, androgens, estrogens* and *progestogens*. In the present thesis 2 steroids have been studied, 17β-estradiol (E_2) and testosterone (T) (fig. 4). E_2 is an estrogen which, together with estriol and estrone, is the responsible of the females' sexual differentiation. T is an androgen and plays an important role in the development and maintenance of the males' features in vertebrates (Miller, 1988). Both hormones are generated

from the same precursor, cholesterol, which derives from dietary fats or from the own synthesis. Most of T and E_2 are synthesized in the gonads (females' ovaries or males' testes). In the ovaries, the cholesterol is transported to the cellular mitochondria where it starts the transformation's chain until forming androstenedione and testosterone. Androstenedione is another estrogen which is converted to estrone, while T is converted to E_2 through *aromatization*, an enzymatic process. In the testes, E_2 is synthesized in the endoplasmatic reticulum. In contrast with the ovaries where cholesterol is derived from lipoproteins, in the testes most of the cholesterol is from new formation. In the testes also, the first steps for the transformation of cholesterol to other steroids occur in the mitochondria and, the latter, in the endoplasmatic reticulum (Kovacks and Ojeda (Eds.), 2012). In addition, T can differentiate to E_2 through the aromatization process in the brain, cells from adipose tissue and arterial walls (Carere and Balthazart, 2007). Estrone can metabolize to estradiol, reaction which occurs predominantly in the liver. T and E_2 move through the blood plasma bound to proteins, primarily albumin and globulins, and have a lifetime in plasma between 4 and 120 minutes (Kovacks and Ojeda (Eds.), 2012).

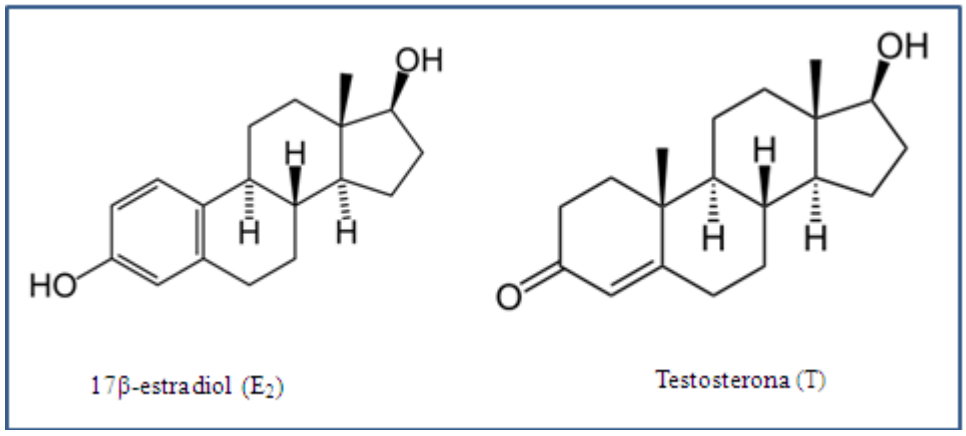

Figure 4. Structures and acronyms of the steroids studied.

2.3 Description of the studied species

In this thesis two gull species have been studied, the yellow-legged gull (*Larus michahellis*) and Audouin's gull (*Larus audouinii*). Both gull species belong to the genus *Larus* (Linnaeus, 1758) (Laridae family, suborder Charadrii, order Charadriiformes (Burger and Gochfeld, 1996)) a taxonomic group widely distributed throughout the world. In many regions of the Iberian Peninsula both species coexist.

As common features (and, in general, from gulls), both species feed in areas near the colony and when they breed, normally in nests made in open areas, chicks leave the nest but, during the first days they still rely on the contribution of food by parents, thus they are semi-precocial birds (Burger and Gochfeld, 1996). Additionally, gulls, and other seabirds are usually

larger than terrestrial birds, less colorful and the sexual dimorphism is less evident (Burger and Gochfeld, 1996).

2.3.1 Yellow-legged gull

- **Phylogeny:** phylogeny of the yellow-legged gull (*Larus michahellis*) has been quite controversial and, still today, there is divergence of views among scientists. *L. michahellis* belongs to the complex *Larus argentatus–cachinnans –fuscus* (Charadriiformes: Laridae). This complex is a classic example of recent speciation in birds (Liebers et al., 2001). Mayr (1940, 1963) proposed a theory to explain the evolution of this group. He named it "The ring species model". According to this hypothesis, an ancestral population of gulls living in the Aralo-Caspian region expanded northward and then spread westward and eastward along the northern coast of Eurasia and into the Mediterranean and to Central Asia (Liebers et al., 2001). The speciation resulted in 30 taxa within the complex. While most of the populations may mate each other, there is a reproductive barrier in N-W of Europe. This barrier to gene flow is evident in the sympatric coexistence (speciation in the same area) of gulls *Larus argentatus* and *L. fuscus* (Liebers et al., 2001). From another chain of taxa distributed in the south appeared *L. michahellis* (Mediterranean Sea), *L. atlantis* (N-E Atlantic), *L. armenicus* (inland lakes of Anatolia, Armenia and Iran), *L. cachinnans* (Black, Caspian and Aral Sea), *L. barabensis* (west of Siberia) and *L. mongolicus* (steppes of Central Asia). In subsequent studies, the chain of southern taxa was classified in only two species *L. armenicus* (monotypic species) and *L. cachinnans* (with the subspecies *L.c.atlantis, michahellis, cachinnans, barabensis, mongolicus*) (Burger and Gochfeld, 1996; Snow and Perrins, 1998). Liebers et al. (2001) develop a new classification after a new analysis. They defined a new division between the Atlantic-Mediterranean clade (*L.atlantis, michahellis, armenicus*) and N-W Paleoartic-Central Asia clade .*cachinnans, barabensis, mongolicus*, *fuscus*) and the isolation of two groups is attributed to the Glacial maximum from 250,000 to 270,000 years ago (Schrag, 2000). Pons et al. (2005), in a mitochondrial marker analysis, classified *L. michahellis* within the group of "white-headed species" together with 22 other *Larus* species. A more recent study suggest that the yellow-legged gull (*L. michahellis*) in the Iberian Peninsula is divided into three subspecies: *L. m. michahellis*, in the Mediterranean; *L. m. lusitanius*, in the Cantabric Sea and the Atlantic coast of Portugal and Galicia; and *L. m. atlantis*, in the Macaronesia and N-W of Africa (Arizaga et al., 2009). This thesis will discuss the *Larus michahellis* as well described by the last official list of Birds in Spain (Gutiérrez et al., 2012).

- **Description:** the yellow-legged gull is a large gull, where females are slightly smaller than males. The maximum wingspan ranges from 120 to 150 cm and weigh between 550 and 1600 g (Olsen and Larsson, 2004). Adults have a gray back and black wing tips with white

spots. They differ from other Laridae because of the yellow coloration of the legs. They have a yellow beak with a red spot and a red orbital ring (fig. 5).

Figure 5. Images of an adult of *Larus michahellis.*

- **Ecology:** most of populations are sedentary, although some individuals migrate to the French Atlantic coast after molting (Martínez-Abraín, (phD) 2003). The breeding season runs from mid-March to April. They form large colonies and each pair usually keep the nest year after year which is placed preferably under bushes or, alternatively, on rocks or sand (Burger and Gochfeld, 1996). They make clutches of 3 eggs of modal size, about 70 – 105 g/egg, with an interval of 1-3 days between the laying of each egg. Egg size decreases within the laying sequence (Rubolini et al., 2006). The egg incubation lasts about 30 days and once hatched, the chicks remain in the nest about 5 - 10 days under the care of parents (Rubolini et al., 2006). It is an omnivorous and opportunist species. But it is also scavenger, feeds on fishing discards, landfill debris or preying on the chicks of other species of seabirds, such as the Audouin's gull (Martínez-Abraín et al., 2003). These feeding habits have allowed the species to expand greatly in recent years to the point to be considered, in some areas, like a plague (Oro and Martínez-Abraín, 2007).

- **Threats and status of the species:** like all seabirds, oil spills are a direct cause of death. Another threat is the plastic particles present in all oceans of the world that can block the digestive tract when are ingested. Through the intake, seabirds can accumulate heavy metals, pesticides and other compounds that can cause malformations or difficulties in the development of the young (Martínez-Abraín, (phD) 2003). In addition, due to its intrusion into human activities and predation on other species of birds, there have been various persecution campaigns (BirdLife International, 2012). Spain and Portugal have carried out several campaigns to control the populations of yellow-legged gull (Oro and Martínez-Abraín, 2007). However this species continues to increase its population size and so the International Union for the Conservation of Nature classifies the species in the status of "least concern" in the IUCN red list (BirdLife International, 2012). It is estimated that in Spain the breeding population of yellow-legged gull in the period 2007-2009 was of 123,900 to 126,499 pairs (Molina (Ed.), 2009) (table 2).

PROVINCE / AUTONOMOUS COMMUNITY	Nº OF PAIRS
Andalusia	**6285 - 6472**
Almeria	974
Cadiz	4514 - 4551
Córdoba	2
Granada	250 - 400
Huelva	373
Malaga	150
Seville	22
Asturias	**4237**
Aragón	**68 - 69**
Huesca	33
Zaragoza	35 - 36
Balearic Islands	**18000**
Canary Islands	**7000**
Cantabria	**1606**
Catalonia	**18966**
Barcelona	55
Girona	9161
Lleida	3
Tarragona	9747
Castilla - La Mancha	**6 - 8**
Ciudad Real	1
Cuenca	1 - 2
Guadalajara	1
Toledo	3

PROVINCE / AUTONOMOUS COMMUNITY	Nº OF PAIRS
Castilla y León	**0**
Ceuta	545
Valencia	**2147 - 2624**
Alicante	1755 - 2134
Castellón	386 - 480
Valencia	6 - 10
Galicia	**45901**
La Coruña	18313
Lugo	3771
Orense	11
Pontevedra	23806
Chafarinas Islands	**5700**
Murcia	**8702 - 10584**
Melilla	**206**
Navarra	**1**
Basque country	**4530**
Guipúzcoa	927
Vizcaya	3603
TOTAL	**123900 - 126499**

Table 2. Breeding population of yellow-legged gull in Spain according to the census of 2007-2009. Source: adaptation of Molina (Ed.), 2009.

2.3.2 Audouin's gull

- Phylogeny: classification of Audouin's gull has not been fully defined. According to an analysis of mitochondrial DNA, this gull is placed in the group of "black-headed species" together with 5 other species (Pons et al., 2005). In this study, only one species from the 6 is classified in the *Larus* genus, while the other 5 (included the Audouin's gull) are categorized in the *Ichthyaetus* genus (*Larus relictus, Ichthyaetus audouinii, I. melanocephalus, I. ichthyaetus, I. hemprichii* and *I. leucophtalamus*) (Pons et al., 2005). The Audouin's gull is the only one that has the white head despite belonging to this group. In the same study, the authors support the theory that the family Laridae constitute a monophyletic group, what means that all come from the same ancestor, while say that *Larus* genus is not monophyletic. Thus, according to Pons et al. (2005), the Laridae would consist of 10 different genus. In a monograph of the species *Larus melanocephalus*, which shares clade with Audouin's gull, the authors declare that the group comes from an ancestor species of the ancient Tethys Sea (García-Barcelona, 2011). Following the discussion over the years about the genus of the group, it was also recommended the creation of the new genus *Ichthyaetus* (García-Barcelona, 2011). So, nowadays, there are two

taxonomic points of view, the one that classifies Audouin's gull within the *Larus* genus and the other one that includes it in the *Ichthyaetus* genus. However, in the last edition of the List of Birds of Spain, it is appointed as *Larus audounii* (Gutiérrez et al., 2012), specific name used in the present thesis.

Figure 6. Images of an adult of *Larus audouinii*.

- Description: is slightly smaller than the yellow-legged gull (*Larus michahellis*). It is about 48 – 52 cm long and 132 – 148 cm wingspan. The beak is red, shorter and more pointed than that of yellow-legged gull and it has a yellow and black spot at the tip. The iris of the eye is black, a feature that distinguishes it from other gulls. It does not have a very pronounced forehead and the neck is sturdy and relatively short. The back is pale gray, which extends to the wings, which have a black spot at the tip. The legs are grayish-green (fig.6). This species does not have much sexual dimorphism unless the female is smaller than the male (Oro, 1998).

- Ecology: it is a semi-pelagic species and usually nests in colonies (Madroño et al. (Eds.), 2004). Generally, they put nests in more or less isolated rocky islets, although the Ebro Delta colony is nested on sandy beaches. Colonies tend to be dense and monospecific, although in some zones it shares territory with the yellow-legged gull (Madroño et al. (Eds.), 2004). Audouin's gull is a piscivorous species specialized in the capture of clupeiforms (such as sardines and anchovies). It practices an active fishing and tends to go out to fish at night (Martínez-Abraín, (phD) 2003). However, its presence is increasingly more frequent in fishing discards (Oro et al., 1999). It is a migratory species, although the exploitation of human resources is making that it becomes more sedentary (Martínez-Abraín, (phD) 2003). Audouin's gull is a monogamous species and its breeding season runs from mid-April to May (Oro, 1998). They reproduce once per year and their clutches are typically of 3 eggs (Oro et al., 1999). Incubation period lasts about 28 days (Oro, 1998). The period of growth of the chicks is about 45 days, after which they are able to fly (Oro, 1998). It is a long-lived species with high survival of adults but with low productivity, except when they feed on fishing discards (Bertolero et al., 2008).

COMMUNITY	COLONY	Nº OF PAIRS	%
Andalusia	Isla de Alboran	526	2.7
Catalonia	Ebro Delta	14177	72.9
Valencia	PN of Albufera de Valencia	88	0.5
	PN Lagunas de La Mata-Torrevieja	450	2.3
	Columbretes Islands	79	0.4
	Benidorm Island	1	0.0
Balearic Islands	Illa de l'Aire (Menorca)	125	0.6
	Illa des Porros (Menorca)	37	0.2
	Cap de Cavalleria (Menorca)	2	0.0
	Sa Dragonera (Mallorca)	100	0.5
	Mola d'Andratx (Mallorca)	30	0.2
	Illot de s'Estopa (Mallorca)	3	0.0
	Illa de Na Guardis (Mallorca)	233	1.2
	Illa de Cabrera	39	0.2
	Illeta de Cala Salada (Eivissa)	245	1.3
	Sa Foradada (Eivissa)	85	0.4
	Illa de s'Espartar (Eivissa)	191	1.0
	Illa de Santa Eulàri (Eivissa)	24	0.1
	Illa d'Espardell (Formentera)	360	1.8
Múrcia	Isla Grosa	582	3.0
	Puerto Mayor	189	1.0
	Salinas de San Pedro	9	0.0
Chafarinas Islands	Chafarinas	1886	9.7
TOTAL		**19461**	**100**

Table 3. Breeding population of Audouin's gull in Spain according to the census of 2007. Source: adaptation of Bertolero et al., 2008.

- Threats and status of the species: it is an endemic species of the Mediterranean region. In the 70s it was declared an endangered species because breeding individuals did not exceed 800 pairs (Genovart et al., 2003). At that time, the largest colony was on Chafarinas Islands (Genovart et al., 2003). In 1981 it was created a new colony, coming from 36 pairs, at the "*Punta de la Banya*", the Ebro Delta. Due to the protection of the place when it was declared Partial Natural Reserve (1986), the colony quickly grew to become by far the most populated (Genovart et al., 2003). Nowadays in Spain, Audouin's gull is present in 5 of the 18 autonomous communities (Andalusia, Catalonia, Valencia, Murcia and Balearic Islands, as well as in Chafarinas Islands) (Bertolero et al., 2008). In Table 3, it is shown the 2007 census of every Spanish region. Currently, 65% of the Audouin's gull world population is comprised in the Ebro Delta colony (about 11,500 pairs), as when the region was protected, became one of the best quality habitat for Laridae in the Mediterranean (Bertolero et al., 2008). The exploitation of fisheries discards has enhanced the expansion of the species, although since 1991 the fishing moratorium coincides with the breeding season of the gull, which can threaten it again (Oro et al., 1999).

Currently, Audouin's gull has not serious threats. The biggest problem is that most of the population is located in one point, the Ebro Delta. Other problems may be the lack of food

resources, its predation by other birds (such as yellow-legged gull) and carnivorous mammals, habitat alterations, levels of environmental pollutants as well as oil spills (Madroño et al. (Eds.), 2004; Martínez-Abraín et al., 2003). Since 1987, Spain has developed a coordinated plan for its conservation and the state and the autonomous community where these species breeds are involved (PCAGA, 1994). In 1998 the Working Group of Audouin's gull supervised by the Spanish Ministry of Environment was formed (Madroño et al. (Eds.), 2004). However, it is still internationally classified as "near threatened" by the IUCN red list (BirdLife International, 2013) and at European level as "localized" (BirdLife International, 2004). On the other hand, it is included in Appendix I of the Directive 79/409/CEE relative to the conservation of wild birds and is in the category of "special interest" in the National Catalogue of Threatened Species. In the Red Book of Vertebrates in Spain on 1992 it was regarded as "rare" (Blanco and González (Eds.), 1992), while in the last Red Book of Birds of Spain it was already classified as "vulnerable" (Madroño et al. (Eds.), 2004). Catalonia (DOGC 4112, 15/04/04) and Valencia (Decree 116/2005, 17 June) made recovery plans where they classified Audouin's gull as "endangered." The Balearic Islands also created a Plan of Official Management (R. D. 439/1990).

2.4 Study areas

In this thesis we studied 8 different areas where colonies of yellow-legged gull and / or Audouin's gull breed (fig. 7). All areas are classified under some form of environmental protection. From all areas, 7 belong to Spain (Ebro Delta, Medes Islands, Columbretes Islands, Dragonera Island, Grosa Island, Chafarinas Islands and the Atlantic Islands of Galicia) and 1 to Portugal (Berlengas Islands).

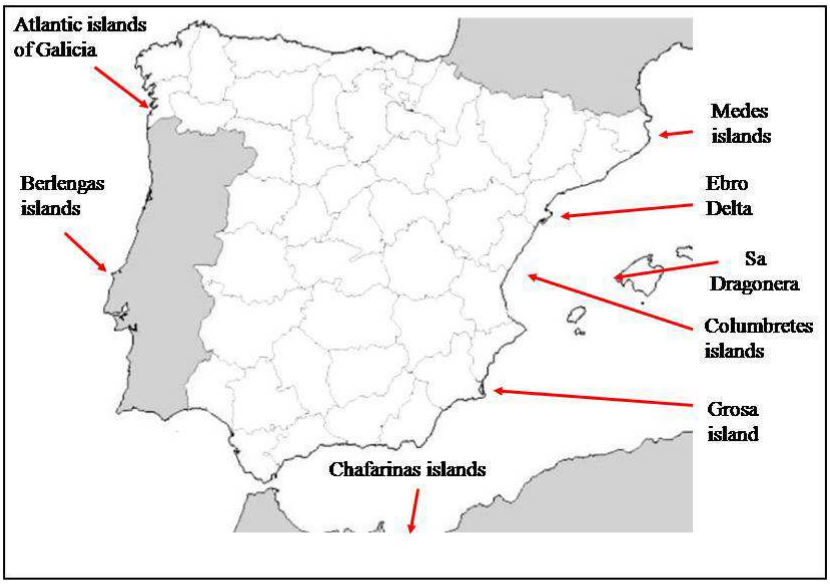

Figure 7. Study areas of this thesis.

2.4.1 Ebro Delta

The Ebro Delta is located in the south of Catalonia, at the mouth of the Ebro River. It is the most important example of recent sedimentary formation in the Iberian Peninsula (less than 6,000 years, during the Holocene) and one of the most important deltas in the Mediterranean (http://www20.gencat.cat/docs). It comprises sandy beaches, dunes and sandy cords, subjected to strong winds and marine dynamics that give its shape changes throughout the year (http://www20.gencat.cat/docs).

It occupies an area of 320 km^2. It has a triangular shape and goes about 20 km into the sea, with one coastal spit in the north, *Punta del Fangar*, and one in the south, *Punta de la Banya* (Mañosa et al., 2001). Punta del Fangar is a floodplain formed only by sand and the surface varies 180,000 and 225,000 m^2 , length ranges between 1800 and 2000 m and the width between 175 and 350 m, depending on the sea level (http://www20.gencat.cat/docs). Punta de la Banya is a peninsula of 2500 ha connected to the rest of the Delta with a sandy spit of 5 km which gives certain isolation.

The maximum altitude of the Delta is about 4 m above sea level, but half of the Delta is in intertidal zone. Its geological and hydrological dynamics is today altered due to the river flood control through dams built along the river and water pipes through the Delta (Mañosa et al., 2001). Nowadays, the Delta landscape is very altered due to the rice crops. The transformation of the habitat by man in the Ebro Delta begins in the sixteenth century, with the first crops of this cereal, but it is during the twentieth century when the agricultural activities increase, covering up to 66% of the delta area (Martínez-Vilalta, 1996) (fig. 8). Besides the intense agricultural activity, there are several areas of high industrial activity upstream of the Ebro River. In the northern part of the basin there are industries such as automotive, textile, food and wood. In the town of Ascó there is a nuclear plant which uses the river water for cooling processes. Around the town of Flix, there is an important chloro-alkali production plant (Ercros, S.A.) which historically produced PCBs and chlorinated pesticides such as DDT. The widespread contamination of these compounds and mercury spill into the Ebro River has been attributed to this plant. Finally, in the banks of the river Cinca, in Monzón (Huesca), a tributary of the River Segre shortly before flowing into the Ebro, there is an industry that manufactures organic pesticides (Montecinca, S.A., Ercros group) (Bosch, (phD) 2009).

The Ebro Delta is considered as the most important wetland in Catalonia. The two coastal spits close two shallow bays of great importance for the production of fish and mollusks which represent a favorable environment for the seabirds feeding and reproduction (Mañosa et al., 2001). In 1962, UNESCO included the Ebro Delta within the Euro-African wetlands group with international interest. Currently, part of the Ebro Delta is classified as a Natural Park. The Park origin is attributed to the popular movement developed in the town of Deltebre on 26 June

Figure 8. Images of the rice crops from the Ebro Delta. From top-left corner to bottom-right: satellite image of Ebro Delta, Ebro Delta crops, first step of irrigated rice crops, growing rice crops.

1983 against a drying project that led to the conversion of a natural pond (*Bassa del Canal Vell*) to a rice field. On August 4, 1983, it was published the Decree of the Ebro Delta Natural Park,which initially only protected natural areas on the left bank of the river. Two years later, Decree 332/1986 extended the protection to the natural areas of the right hemidelta (http://www20.gencat.cat/portal/site/parcsnaturals). The high diversity of fish (50 species) and birds (over 360 species) was determining to include the Ebro Delta to the Special Protection Area for Birds (ZEPA) on 1987 and into the List of Wetlands of International Importance especially for Water birds Habitat (RAMSAR), on March 1993 (http://www20.gencat.cat/portal/site/parcsnaturals). In 1997 the Ebro Delta was included to the Natura 2000 Network as a Site of Community Importance and Special Area of Conservation (http://www20.gencat.cat/portal/site/mediambient). In 2007 the Park was awarded with the certificate of European Charter for Sustainable Tourism by the EUROPARC Federation, an organization that represents Nature Reserves from 32 European countries (http://www.europarc.org). And since last 28th May 2013 the *Terres de l'Ebre* is a Biosphere Reserve designated by the UNESCO Programme Committee *Man and Biosphere* (MAB), which turns Spain as the 2nd country in the world with more number of Biosphere revserves, a total of 45. In 2012 UNESCO rejected the grant of a Biosphere Reserve in Terres de l'Ebre due to the upstream chemical and nuclear activities of the plants of Ascó and Flix. But in the new plan of

2013, the elimination of seven municipalities with high influence of these centrals (Ascó, Vinebre, Garcia, La Torre de l'Espanyol, Riba-roja d'Ebre, La Palma d'Ebre and Flix) allowed the concession.

2.4.2 Medes Islands

The Medes Islands are an uninhabited archipelago located in the Costa Brava, about 1 km from L'Estartit (N-E of Catalonia). The archipelago is composed by 7 islets: Meda gran (18.7 ha), Meda Xica (2.6 ha), Carall Bernat, Tascons Grossos, Medellot, Tascons Petits and Ferranelles (fig. 9) (http://www20.gencat.cat/portal/site/parcsnaturals). The islands show quite asymmetry, being the eastern slopes steep and with vertical cliffs that penetrate up to 50 m under the water, while the western side has gentler slopes.

Medes Islands belong to the Natural Park of Montgrí, Medes Islands and Baix Ter. The scientific interest in the seabed of the Medes already comes back far, but it was not until 1983 when the Government of Catalonia issued the Order to forbid the fishing and the extraction of marine living resources. With the Law 19/1990 of the conservation of the flora and fauna of the Medes, the seabed protection zone was extended. The Decree 328/1992 executed the protection of the area within the Plan for Areas of Outstanding Natural Beauty in 1992 and in 2001 the seabed of the Medes was included in the list of Specially Protected Areas of Importance for the Mediterranean (ZEPIM). The agreement GOV/112/2006 approved its inclusion in the list of Special Protection Areas for Birds (ZEPA) and into the Natura 2000 Network. The Natural Park Montgrí, Medes Islands and Baix Ter was created in May 2010 by the Law 15/2010 (http://www20.gencat.cat/portal/site/parcsnaturals).

Figure 9. Medes Islands. From top-right corner to bottom-left: Meda Gran, Meda Xica and Carall Bernat.

According to the List of Habitats in Catalonia (LHC), the Park includes a total of 25 different habitats. The strong wind, salinity and seabird excretions affect the vegetation of the

islands. The high diversity of marine habitats of the Medes is due to the high productivity of the area, heavily influenced by the contribution of organic matter from the river Ter and the dynamics of winds and currents which benefit the upwelling of deepwater enriched in nutrients. In the whole Park it has been recorded 189 taxa of fauna and the yellow-legged gull is the most abundant bird of Medes Islands forming one of the most populated colonies of the Mediterranean (http://www20.gencat.cat/portal/site/parcsnaturals).

2.4.3 Columbretes Islands

Columbretes are a small archipelago of volcanic origin located about 28 nautical miles (50 km) from the town of Oropesa, Castellón, North of Valencia. The archipelago consists of 4 main islands (Gorssa Islands, Ferrera, Foradada and Carallot) (fig. 10) extending along 9 km and occupying a total of 19 ha, 14 ha of which correspond to the Grossa Island, the largest and the only inhabited. The maximum altitude of Columbretes is 67 m., also in Grossa Island. Both Grossa Island and Carallot have obvious signs of volcanic origin, such as craters and the remains of the central chimney of a volcano (http://www.cma.gva.es).

These islands were formerly called "Islas de las Serpientes" (Snake's Island), name given by the sailors due to the high abundance of this reptile. Until the mid nineteenth century Grossa Island was not colonized, then the lighthouse was built (1860). The Foradada Island hided ships with smuggler activities because they were out of the security services sight. In 1975 the last family of lighthouse-keepers left the island because the lighthouse was automated. The islands were uninhabited until 1987 when the Valencian Government installed the first surveillance guards (http://www.cma.gva.es). Since the late 60s until 1982, Aviation and Navy of the United States and Spain did military practices that damaged a lot the landscape. Those practices together with the over-fishing, led to the protection of the archipelago claiming it Natural Park of Columbretes Islands in 1988 by the Decree 15/1988 of the Valencian Goverment, and Marine Reserve of 5.543 ha in 1990, by the Ministry of Agriculture, Fisheries and Food. With the Law 11/1994, they were reclassified to Natural Reserve by the Valencian Goverment. Today, the Grossa Island is the only one allowed to visit but it is prohibited the collection of minerals, plants or animals (http://www.cma.gva.es). The original flora and fauna of the islands were quite damaged during the period in which they were inhabited since there were introduced domestic animals and a lot of bush flora was used for firewood. The strong isolation of these islands with the mainland led to the formation of different endemisms, emphasizing the plants *Lobularia maritima columbretensis* and *Medicago citrina*, 10 insects and one lizard. Terrestrial fauna is dominated by colonies of nesting seabirds, among which are the Audouin's gull, being Columbretes the only site of Valencia where this species is present (http://www.cma.gva.es).

Figure 10. Columbretes Islands. From top-left corner to bottom-right: Grossa Island, La Foradada, El Carallot and La Farrera.

2.4.4 Sa Dragonera

Dragonera Island is part of the Balearic archipelago and it is located to the west of Mallorca, separated by a small and shallow strait of about 800 m wide and shallow (fig. 11). It is the sixth largest island of the archipelago and it is not inhabited. Administratively, it belongs to the municipality of Andratx, Mallorca. It occupies an area of 288 ha, a maximum length of 3700 m, a maximum width of 780 m and the highest point is 352 m (http://www.conselldemallorca.net/dragonera/). Geologically, it is a continuation of the Serra de Tramuntana (Mallorca) thus presenting a karstic relief. The island is quite rocky and steep, with the S-E hillside slightly sloped and the N-W side has steep cliffs (http://www.conselldemallorca.net/dragonera/).

During the sixteenth century there were built two watch-towers, one of which is not preserved as a lighthouse was built on the same place in 1850. During the early 1900s two lighthouses were built in Sa Dragonera. Around 1975 the lighthouses were automated causing the evacuation of the families who lived there.

In 1974 the company PAMESA bought the island with the purpose of building 1200 housing and a marina with a capacity for 600 boats. But a group of ecologists protest against it and they managed to stop the project by the National Court (1984) and in 1987 the Council of Mallorca bought back the island. In 1995 the Government of Balearic Islands declared Sa

Dragonera together with the Pantaleu and Sa Mitjana islets as Natural Park by Decree 7/1995 (http://www.conselldemallorca.net/dragonera/).

Figure 11. Sa Dragonera.

In Dragonera 361 plant species have been recorded, 18 of which are endemic from the Balearic Islands. Among the fauna, *Puffinus mauretanicus* is the only bird endemic of Balearic Islands, there is also a subspecies of endemic lizard and a snail endemic of the archipelago (http://www.caib.es).

2.4.5 Grosa Island

Grosa Island is located 2.5 km out from the coastal strip that forms the "Mar Menor" and belongs to Murcia (fig. 12). It is an uninhabited volcanic cone with lava soils (andesites) which covers an area of 17.5 ha, has a maximum altitude of 98 m, a length of 600 m and a width of 400 m. The dominant relief is of rocky and steep costs, with a small beach of 50 m long at the west side, protected from easterly winds (http://www.regmurcia.com). The island was a refuge for Berber pirates until the eighteenth century and until 2000 was aimed at military use, when it was agreed to transfer the island to be managed by the then-Department of Agriculture, Water and Environment, in order to take measures for its conservation (http://www.regmurcia.com).

This island is part of the set of "islands and islets of the Mediterranean coast" together with other 17 islands. The whole group was considered Protected Natural Area by the Law 4/1992 of Planning and Protection of the Territory of the Murcia Region (http://www.magrama.gob.es/es/biodiversidad/temas/espacios-protegidos). Subsequently, the Grosa Island, one of the largest in the whole area, was declared as a Wildlife Protection Area by the Law 7/1995 and a Special Protection Area for Birds (ZEPA) for the Autonomous Community of Murcia (79/409/EEC) in 2000. The same year it was included in the Natura 2000 Network as a Site of Community Importance and Special Area of Conservation and designated as Specially Protected Area of Mediterranean Importance for (ZEPIM) under the Barcelona

Convention (Convention for the Protection of the Mediterranean Sea against Pollution), Protocol on Specially Protected Areas and Biological Diversity in the Mediterranean (2001). Since 2005, the Association of Naturalists from South-East (ANSE), together with the Ministry of Environment of Murcia, participates in the project of LIFE - Nature Conservation of *Larus audouinii* in Spain, Grosa island (Murcia). The project aims to strengthen the population of Audouin's gull (*L. audouinii*) in Grosa Island and guaranteeing its preservation, as in this island there is the 3rd largest colony in the world of this species (http://www.asociacionanse.org/proyectos/proyecto-life-naturaleza-conservacion-de-larus-audouinii-en-espana).

Figure 12. Mar Menor and Grosa Island (red circle).

2.4.6 Chafarinas Islands

Chafarinas Islands are a Spanish archipelago of volcanic origin located 1.9 nautical miles (about 3.5 km) from Cape Water (Moroccan coast) and about 27 miles (48.5 km) east of Melilla. The archipelago consists in three islands: Congreso, Isabel II (the only one currently inhabited) and Isla del Rey Francisco (fig.13). The protected zone covers a marine area of 259.86 ha and of 54.6 ha of land. Island of Congreso is the largest (25.6 ha) and the most western, and has a maximum altitude of 137 m. The island of Isabell II occupies about 15.1 ha and the highest point reaches 35 m. El Rey is the most eastern island and the smallest (13.9 ha) and with less altitude (31 m). The islands have a dry thermo-mediterranean climate heavily

influenced by the prevailing winds from the east or west and their morphology is dominated by steep cliffs (http://www.magrama.gob.es/ca/parques-nacionales-oapn). It is a region with fauna interest because, among other species, Audouin's gull forms colonies, and there is the 2nd largest world population of this species. With the Decree 1115/1982 it was declared National Hunting Refuge of Chafarinas Islands (only military personnel have access to them) and in 1989 it was declared as ZEPA by the European Commission. The Autonomous Organization of National Parks (OAPN, Ministry of Environment) is responsible of its regulation and conservation. The meadows of *Posidonia oceanica*, a marine plant of slow growth and very important for the marine ecosystem, helped to include Chafarinas in Annex 1 of the Habitats Directive 92/43/EEC. Since 2006 it is included in the Natura 2000 Network as a Site of Priority Interest. It is important the presence of a subspecies crucifer plant endemic of the island of Congreso (*Brassica fruticulosa djafarensis)* (http://www.magrama.gob.es/ca/parques-nacionales-oapn).

Human history of Chafarinas dates back to the Neolithic by the finding of different archaeological sites. Countless sailors also went to find shelter, but the lack of drinking water always hindered their establishment. As from 1830, coinciding with a French campaign in North Africa, the Spanish government began to have interest in the archipelago, until then "no man's land." In the Council of Ministers of 26th June 1847, it agreed its occupation with several warships from Malaga. On 6th January 1848, the Spanish colonizer landed on Chafarinas, baptized three islands with the current names and built a mooring and water cisterns. At the beginning, the islands were used as an auxiliary prison of that from Melilla, later they were declared Free Port (creating a pleasant urban network) and in 1921 it became a hospital that housed the victims of the campaigns in Africa. In 1965, with the independence of Morocco, the use of the hospital decreased and today the islands have lost their strategic military value, but it is a place of high biological interest (http://www.magrama.gob.es/ca/parques-nacionales-oapn).

Figure 13. Chafarinas Islands. From top to bottom: Rey Francisco, Isabell II and Congreso Sur

2.4.7 Atlantic Islands of Galicia

Atlantic Islands of Galicia are composed by the islands of Cies, Ons, Sálvora and Cortegada and have a total area of 8480 ha (7285 ha marine and 1195 ha land). Cies are 433 ha marine and 2658 ha land, the Ons, 2171 ha marine and 470 land, the Sálvora, 2309 ha marine and 248 land, and the Cortegada, 147.2 ha marine and 43.8 land (http://www.iatlanticas.es).

Cies Islands are an archipelago of three islands (Norte, Sur and Isla do Medio) located in front of the mouth of the Ria de Vigo, Pontevedra, Galicia. They are approximately 14.5 km from the city of Vigo, cover an area of 4.46 km^2, have a maximum length of 7.1 km, a width of 2 km and the highest point in the Norte Island, 197 m. Its geological formation dates back to the late Tertiary and is almost granitic rock. The three Cies islands are mountainous, with a steep west face with cliffs over 100 m and many caves formed by the erosion from the sea and wind. The east side is a little softer and is covered by forests and scrub with some beaches and dunes (http://hoxe.vigo.org).

Cies Islands were formerly called *Islas de los Dioses* (God's Islands) due to their views and biodiversity. During the Middle Ages, they were occupied by various monastic orders and were owned by the Church while suffering attacks by Turkish and Norman pirates and visits from invading armies, such as English army. In the eighteenth century the archipelago was depopulated until the mid-nineteenth century when the first lighthouse was built in the Medio Island. At the end of the 60s, the islands were definitely depopulated remaining only the Park rangers and touristic visitors (http://hoxe.vigo.org).

Ons is an archipelago located in front of the mouth of the Ria de Pontevedra. Ons is the main island of the archipelago, together with Onza and other islets. It belongs to the municipality of Bueu, due to social reasons (not geographical) as the former colonizers came from Bueu, Morrazo region (http://www.magrama.gob.es/es/). Ons length is 5.6 km and its width is of 1.3 km, with a total area of 414 ha. The western coast is irregular and steep while the eastern one is lower and straight where are located the beaches and allowed the human colonization. The maximum altitude is 128 m, where was built the lighthouse. In 1953, 530 people inhabited Ons but the lack of medical services, energy, building material and isolation from the main land led to a decrease of the population and currently there are about 75 inhabitants (http://www.magrama.gob.es/es/). Until 899 the island was property of the kings of Galicia, at that moment it was given to the archbishop of Santiago de Compostela. Thus Ons belonged to the Church until the sixteenth century when it was given to a noble family. After the civil war it was confiscated by the government and in 1983 it was transferred to the Junta de Galicia.

Sálvora island is situated at the mouth of the Ria de Arousa, at 3 km from mainland. It occupies an area of 190 ha and its maximum altitude is 71 m. Its is a rocky island with only 3 sandy beaches (http://www.magrama.gob.es/es/).

The archipelago of Cortegada is at the end of the Ria the Arousa. The main island is Cortegada, distanced from the main land by a channel of 189 m, an important crop of shellfish. It is a flaty island (maximum altitude 22 m) of 54 ha. This orography allows the formation of little lakes. It is of high ecological interest because it has the biggest laurel forest of Europe and a rich seabed.The protection of the natural values of the islands began in 1980 when the archipelago of Cies was declared Natural Park. In 1988, it was awarded with the title of ZEPA. The same award was given also to the neighboring archipelago of Ons in 2001. In 2002 the archipelagos of Cies, Ons, Sálvora and Cortegada were declared Galician Atlantic Islands Maritime-Terrestrial National Park (Law 15/2002) (http://www.iatlanticas.es) (fig. 14).

The marine environment, which occupies an 85% of the whole area of the Park, is of great importance. The Gulf Stream runs up to the Galician coast bringing warm water from the Caribbean which softens the climate of the region. During the summer, the prevailing continental winds moves surface water outward from the Rias which allows the rise of cold water what causes a local phenomenon of upwelling of nutrients. The presence of tides has also a great importance on the organisms living in the littoral zone (http://www.iatlanticas.es).

Figure 14. Galician Atlantic Islands Maritime-Terrestrial National Park. From top-left corner to bottom-right: Cies, Cortegada, Sálvora and Ons.

Quaternary glaciations with the consequent increase of sea level 8000-9000 years ago led to the isolation of the archipelago allowing the generation of endemic species, among which is interesting to enhance a subspecies of lizard and a bush. A threat to the conservation of the original natural values of the islands is the invasive species such as the American mink (*Neovison vison*) and domestic cat (Felis silvestris catus) (http://www.medioruralemar.xunta.es).

2.4.8 Berlengas Islands

Berlengas Islands are an archipelago located 5.7 nautical miles (10 km) of Cape Carvoeiro, central Portugal. Numerous islands and islets make up the archipelago, which is divided into three zones: the Berlenga, the Estelas and the Farilhões-Forcadas (fig. 15). The total extension of the protected area is about 9560 ha (104 ha land and 9456 ha marine) (http://portal.icn.pt). The Berlenga consists of a main island, which gives the name, and different islets and rocks. The maximum length is about 1500 m and the width, 800 m. The highest point of the island is less than 90 m (Amado, 2007). The Estelas is an archipelago located less than 2 km from the Berlenga, with a maximum altitude of 45 m, in the largest island. The Farilhões-Forcadas are two groups of islets located about 12 km northeast of Berlenga. The relief is steeper, reaching a maximum height of 94 m (Amado, 2007). Berlenga and Estelas are composed by igneous rocks (red granites) while Farilhões-Forcadas by metamorphic rocks. It is noteworthy the deformations of rocks caused by the interaction of tectonic faults that produced some caves and other natural cavities. Climate is markedly oceanic dominated by strong winds and Atlantic influences (http://portal.icn.pt).

Figure 15. Berlengas Islands. From top-left corner to bottom-right: Estelas, Farilhões-Forcadas and Berlengas

It could be considered as the first protected area in the world since in 1465 the King Alfonso V of Portugal banned the hunting. But it was not until 1981 when it was officially declared Natural Reserve of Berlengas with the Decree-Law n. º 264/81. From 1998, with the commemoration of the International Year of the Oceans, its protection was reinforced and expanded throughout the archipelago. In 1999 it was awarded with the title of Special Protection Area (ZEP) in order to protect wild birds and their habitats (Directive 79/409/EEC). Subsequently, the archipelago of Berlengas became part of the Natura 2000 Network, giving importance to terrestrial flora and other fauna, particularly those from the marine area (http://portal.icn.pt).

The separation of the archipelago from the continent, which occurred during the Jurassic, facilitated the emergence of several endemic species. Among the 100 plant species of Berlengas, three are endemic. The strong population increase of the gull *Larus argentatus* has led to decrease the endemic lizard *Podarcis berlengesis* due to its predation (Amado, 2007).

2.5 Sampling design

The sampling strategy is a very important point when designing a study because it is the basis of all the work. The sampling design should take into account the objectives of the investigation and ensure a great representation of the study area, which also allows a good statistical analysis to achieve a good level of confidence (OSPAR, 1999). For environmental pollution studies, a sample size (number of samples) representative of the target population must be selected which allows the sampling to be repeated annually. According Guidelines for Monitoring Contaminants in Biota (OSPAR, 1999), the sampling of seabird eggs should reflect the most important breeding areas of a given species. An effective sampling of eggs must be done during the first laying period of the year and must result in a minimum of 10 eggs randomly collected from 10 nests (one egg per clutch) as soon as possible once the laying has started (between the 1st and the 5th day of incubation) to ensure they are fresh (OSPAR, 1999). To avoid repeating nests, making the mistake of taking the 2nd egg, nests already sampled must be tagged. Once collected, the egg should be labeled with a non-toxic and water-resistant marker (pencil, for example) and later should be transported quickly to the laboratory to avoid deterioration (OSPAR, 1999). Once in the lab, each egg must be weighed and measured before opening and afterwards, the egg content must be weighted. Embryonated eggs must be rejected (OSPAR, 1999).

To reduce the impact of sampling on the population studied, the sampling time should be as short as possible and choose species, which if it is possible, make replacement clutches. The Guidance for Global Monitoring Programme for Persistent Organic Pollutant (UNEP,

2004) recommended the sampling of birds that breed in colonies, as well as a minimum of 12 eggs taken from different nests in the same colony.

Considering these two guidelines (OSPAR, 1999 and UNEP, 2004) we designed our sampling. In this thesis there are 8 areas of study, two target species of gulls (yellow-legged gull and Audouin's gull) and two matrices (eggs and blood) which were sampled in two different periods (2009 and 2010). The sampling period was always during the breeding season of the species.

In 2009 two sampling campaigns were undertaken. On one hand, the first egg was (because that is where the female transfers the maximum amount of pollutants (Pastor et al., 1995a)). On the other hand, the whole clutch was collected (3 eggs) because we wanted to study the transfer of pollutant along the laying sequence. The studied colonies were divided into three subzones (subcolonies) to ensure the representation of the potential variability and 12 first-eggs were randomly collected, as long as it was possible. Thus, in general, we had a total of 36 eggs (12 per subcolony) per species and colony. Following the protocols, eggs were labeled with a pencil, placed in egg-boxes and then stored in refrigerators. In the campaign of 2010, the sampling of Audouin's gull eggs was reduced to 7 eggs per subcolony to minimize the impact of sampling on the species, since the year before it was found that the variability of pollutant levels was very low within a colony.

For blood sampling, those nests where eggs were taken were tagged to capture the adults and collect the blood. Adults were captured by trap-boxes. The traps were placed in the morning to catch one parent while the other was searching food and at evening to capture the other parent.

For every sampling the authorisation from National or Natural Parks was requested and researchers and / or guards with great knowledge of the area aided in the collection of samples. The sampling was only for scientific reasons and the sampling was done in a way that produced the least disturbance of the species.

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Chapter 3. Persistent Organic Pollutants in gull eggs of two species (*Larus michahellis* and *Larus audouinii*) from the Ebro Delta Natural Park

3.1 Abstract

The aim of this study was to determine the impact of priority and emerging Persistent Organic Pollutants (POPs) in gull eggs from two species, the scavenger *Larus michahellis* and the protected species, *Larus audouinii*. These two species share habitat in the Natural Park of the Ebro Delta (Catalonia, Spain). Compounds studied are included or under consideration in the Stockholm Convention and comprise polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs), polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), organochlorinated compounds (OCs), perfluorinated compounds (PFCs) and short chain chlorinated paraffins (SCCPs). Four methods based in selective extraction and gas or liquid chromatography coupled to mass spectrometry were used and quality parameters are provided. OC pesticides and marker PCBs were the most abundant chemical families detected in eggs from the two species, followed by PFCs, PBDEs (especially BDE 209) and SCCPs. Dioxin-like PCBs and PCDD/Fs were also detected in all samples. The overall widespread presence of POPs is discussed in terms of feeding habits, bird ecology and anthropogenic pressures in the protected Ebro Delta breeding area.

3.2 Introduction

Several studies in bird-breeding areas have reported unexpected high levels of Persistent Organic Pollutants (POPs) in bird eggs. Birds have the ability to accumulate POPs through the diet and are thereafter transferred to the eggs (Bustnes et al., 2008a,b). The presence of POPs in birds was first evidenced in 1973 when organochlorinated pesticides (OC) were detected in birds and in their layout (Stickel et al., 1973) and it was suggested that they could induce reproductive effects (Fergin and Schafer, 1977). Another study performed in 1978 in Mediterranean gulls demonstrated that birds accumulated polychlorinated naphthalenes, polychlorinated biphenyls (PCBs) and metals (Vannuchi et al., 1978). In Sweden, 1982, POPs were detected in Bald Eagle (*Haliaeetus albicilla*) and they were the cause of the failure of the clutches (Helander et al., 1982) and 20 years later, the effect of these contaminants on the development of the egg shell and reproductive availability were evidenced (Helander et al., 2002). In Canada, dichlorodiphenyldichloroethylene (DDE), PCBs and mirex were detected at

60000–69600000 pg/g ww (wet weight) in herring gulls (*Larus argentatus*), with the highest levels attributed to highly contaminated sites (Weseloh et al., 1990). In the Great Lakes of the United States, PCBs, polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) were identified in aquatic birds (Kannan et al., 2001) at 7.4–97 pg/g ww. In Greece, several PCB congeners and OC pesticides were detected in bird species attributed to a generalized and diffuse contamination in areas of ecological interest (Konstantinou et al., 2000; Albanis et al., 2003). In Italy, DDTs and PCBs were identified at high concentrations in birds collected close to a DDT producing factory and were correlated to low levels of steroidal hormones and negative effects in the offsprings (Cortinovis et al., 2008). New generation of POPs, such as perfluorinated compounds (PFCs), used as water repellents in many industrial and domestic appliances, have also been detected in liver of Great Cormorant (Phalacrocorax carbo) at 1873000–2249000 pg/g (Kannan et al., 2002). On the other hand, polybromodiphenyl ethers (PBDEs), used as flame retardants, have been detected in gannet (*Morus bassanus*) from United Kingdom during the period 1977–2007 (Crosse et al., 2012). The pervasive distribution of POPs is evidenced when these compounds are detected in remote areas like the Arctic (Herzke et al., 2003), as for PBDEs in eggs of Fulmar Boreal (*Fulmarus glacialis*) (Verreault et al., 2005; Karlsson et al., 2006) or PFCs in several Arctic birds (Haukås et al., 2007).

Spain is a country where OC pesticides have been historically used in agriculture and on the other hand, PCBs, OCs and PBDEs have been produced for decades until prohibition. In addition, primary and secondary sectors are or have been responsible for the emission and spreading of POPs in the environment. González et al. report DDE and PCBs in 3 gull species from the Ebro Delta, Chafarinas Islands and Cuenca at levels up to 15480000 and 20500000 pg/g, respectively (Gonzalez et al., 1991). In Flamingos (*Phoenicopterus roseus*) from Doñana National Park, PCBs were detected at 528000 pg/g ww and among OC pesticides, 4,40- DDE was found at 721000 pg/g ww and residues of hexachlorocyclohexane isomers, hexachlorobenzene, aldrin, heptachlor, and heptachlor-epoxide were detected at much lower concentrations (Guitart et al., 2005). Also from Doñana, PCBs and DDTs were detected in red kite (*Milvus milvus*) at levels associated with reproductive impairment, reduced hatching success, embryo mortality, and deformities (Gómara et al., 2008). PCDD/Fs and ortho-PCBs leveled up to 20000 pg/g ww and 3335160 pg/g ww, respectively, in peregrine falcon (*Falco peregrinus*) from central Spain (Merino et al., 2005). In relation to emerging POPs, BDE 209 was detected in eggs from two colonies of white stork (*Ciconia ciconia*) in Spain at mean levels of 1640 pg/g ww (Muñoz-Arnanz et al., 2011) and more recently, PFCs have been identified in the 8 main gull colonies from the Spain and Portugal at levels up to 54000 pg/g ww, and differences among Mediterranean and Atlantic colonies have been evidenced (Vicente et al., 2012).

POPs may affect bird condition, behavior, reproductive success and may lead to serious consequences for the development of the chicks, reinforcing the necessity of a better knowledge on the occurrence of POPs in sensitive areas which are refuges for numerous wildlife bird species. In addition to that, the Stockholm Convention has proposed bird eggs to assess the levels of POPs (Reference C.N., 2001). Among bird species, in this study we propose the use of gull eggs as bioindicators of POPs, in particular of yellow-legged gull (*Larus michahellis*) and the endangered and protected species Audouin gull (*Larus audouini*). Gulls are widespread in Europe, Northern Africa and Asia and are suitable for being used as indicators or biomonitors of environmental pollution (Weseloh et al., 1990). Namely, they live up to 30 years and they lay their eggs in a specific colony throughout their life. *L. michahellis* have scavenger habits based on rubbish tips, fish discards, robbing catches from smaller seabirds or eating smaller animals like pigeons. On the other hand, *L. audouinii* feeds exclusively on pelagic fish. Due to their increasing population, collection of eggs represent a noninvasive sampling protocol and of high simplicity, due to the large colonies settled in many coastal areas.

Table 4. Summary of the quality parameters obtained for each chemical class of POPs. (n.a. = not analysed).

Compound	Repeatability	Recovery ± STD	MDL (ww)
PFCs			
PFBS	14	96 ± 3	130 pg/g
PFHxS	11	83 ± 3	170 pg/g
PFOS	7.0	94 ± 4	220 pg/g
PFOA	7.2	107 ± 9	110 pg/g
PFNA	10	115 ± 9	130 pg/g
PCDD/F			
2,3,7,8-TCDF	1.5	67 ± 9	0.013 pg/g
1,2,3,7,8-PeCDF	1.4	69 ± 5	0.063 pg/g
2,3,4,7,8-PeCDF	1.1	68 ± 2	0.063 pg/g
1,2,3,4,7,8-HxCDF	2.8	65 ± 2	0.063 pg/g
1,2,3,6,7,8-HxCDF	2.2	70 ± 1	0.063 pg/g
2,3,4,6,7,8-HxCDF	1.1	61 ± 1	0.063 pg/g
1,2,3,7,8,9-HxCDF	1.3	74 ± 1	0.063 pg/g
1,2,3,4,6,7,8-HpCDF	2.8	78 ± 6	0.063 pg/g
1,2,3,4,7,8,9-HpCDF	0.6	70 ± 9	0.063 pg/g
OCDF	2.5	n.a.	0.125 pg/g
2,3,7,8-TCDD	0.7	69 ± 8	0.013 pg/g
1,2,3,7,8-PeCDD	1.6	71 ± 4	0.063 pg/g
1,2,3,4,7,8-HxCDD	1.9	75 ± 2	0.063 pg/g
1,2,3,6,7,8-HxCDD	0.7	76 ± 2	0.063 pg/g
1,2,3,7,8,9-HxCDD	3.0	n.a.	0.063 pg/g
1,2,3,4,6,7,8-HpCDD	1.5	76 ± 13	0.063 pg/g
OCDD	0.2	56 ± 17	0.125 pg/g
DL-PCBs			
PCB-81	0.8	60 ± 4	0.10 pg/g
PCB-77	2.7	68 ± 5	1.57 pg/g
PCB-123	1.7	69 ± 7	0.24 pg/g
PCB-118	2.6	72 ± 3	12.2 pg/g
PCB-114	0.4	80 ± 2	0.34 pg/g
PCB-105	1.7	83 ± 4	2.99 pg/g
PCB-126	0.6	65 ± 8	0.04 pg/g
PCB-167	0.8	75 ± 1	0.74 pg/g
PCB-156	3.3	78 ± 6	1.66 pg/g
PCB-157	0.8	82 ± 5	0.21 pg/g
PCB-169	3.3	80 ± 7	0.02 pg/g
PCB-189	2.2	79 ± 8	0.21 pg/g

Compound	Repeatability	Recovery ± STD	MDL (ww)
Marker-PCBs			
PCB-28	8.2	82 ± 4	0.29 pg/g
PCB-52	10	88 ± 9	0.34 pg/g
PCB-101	7.0	82 ± 8	0.31 pg/g
PCB-118	10	83 ± 7	0.72 pg/g
PCB-138	9.3	85 ± 6	0.34 pg/g
PCB-153	11	86 ± 4	0.34 pg/g
PCB-180	6.2	101 ± 5	0.29 pg/g
SCCPs	6.2	97 ± 5	0.48 ng/g
PBDEs			
BDE 28	3.4	116 ± 13	9.60 pg/g
BDE 47	4.7	111 ± 9	7.68 pg/g
BDE 99	5.2	98 ± 5	11.3 pg/g
BDE 100	2.2	107 ± 2	3.6 pg/g
BDE 153	3.3	86 ± 5	3.12 pg/g
BDE 154	3.8	91 ± 5	2.88 pg/g
BDE 183	5.6	53 ± 5	4.56 pg/g
BDE 209	10	46 ± 2	120 pg/g
OC pesticides			
Aldrin	6.7	75 ± 9	0.50 pg/g
Dieldrin	10	40 ± 5	0.40 pg/g
Endrin	6.4	73 ± 11	0.80 pg/g
α-Endosulfan	9.7	69 ± 8	2.04 pg/g
β-Endosulfan	10	65 ± 9	1.27 pg/g
Endosulfan-sulfate	13	71 ± 18	1.29 pg/g
α –chlordane	8	68 ± 5	0.80 pg/g
γ-chlordane	9	73 ± 8	0.80 pg/g
Oxychlordane	5	83 ± 7	0.30 pg/g
Cis-nonachlor	12	67 ± 10	9.20 pg/g
Trans-nonachlor	8	79 ± 9	9.20 pg/g
Mirex	5	85 ± 3	5.0 pg/g
Hexachlorobencene	4.6	94 ± 7	0.22 pg/g
Heptachlor	6.3	91 ± 6	0.26 pg/g
2,4-DDT	5.2	91 ± 12	0.43 pg/g
4,4'-DDT	5.8	96 ± 6	0.38 pg/g
2,4-DDD	7.7	88 ± 11	0.43 pg/g
4,4'-DDD	7.1	82 ± 10	0.48 pg/g
2,4-DDE	4.5	87 ± 13	0.17 pg/g
4,4'-DDE	6.9	87 ± 8	0.24 pg/g
α –HCH	7.8	82 ± 12	0.26 pg/g
β –HCH	12	76 ± 13	1.01 pg/g
δ-HCH	11	83 ± 11	1.92 pg/g
γ-HCH	13	84 ± 7	0.98 pg/g

The aim of the study was to evaluate the impact of 6 POP families in eggs of *L. michahellis* and *L. auodouinii*, which coexist in the Punta de la Banya Peninsula, in the Ebro Delta Natural Park (Catalonia, NE Spain). The POPs considered in this study include priority and emerging substances listed or under consideration in the Stockholm Convention (PFCs, PCBs, SCCPs, OC pesticides, PBDEs, PCDD/Fs). The presence and distribution of POPs is discussed and compared to previous studies carried out in other areas or bird species and discussed in terms of bird ecology and anthropogenic pressures.

3.3 Materials and methods

3.3.1 Chemicals and Standards

The chemical contaminants analyzed in this study are listed in Table 4, grouped by methods used. *n*-hexane, dichloromethane (DCM), acetone for pesticide residue analysis and sulfuric acid (analytical reagent grade, 95–97%) were purchased from Merck (Darmstadt, Germany). Florisil (0.150–0.250 mm) for residue analysis and silica gel (0.063–0.2 mm) were also from Merck. Anhydrous sodium sulfate was supplied by Panreac (Barcelona, Spain). All adsorbents were activated at 650ºC overnight and stored at 120ºC before use.

3.3.2 Sample collection and preparation

Eggs of yellow-legged gull (*Larus michahellis*) and Audouin's gull (*Larus audouinii*) were collected at the beginning of the breeding season in the Punta de la Banya, Ebro Delta Natural Park (southern Catalonia 40º37'N, 00º35'E), in 2010. The Ebro Delta is an area of intense agricultural activity with 21.000 ha of rice cultivation and receives upstream waters that flow close to a chloro-alkali plant (Lacorte et al., 2006; Bosch et al., 2009). However, the Punta de la Banya is not directly impacted by these activities but is characterized by dunes with psammophylous vegetation, saltmarshes or dishes in the saltworks. This site hosted a population of 9.744 yellow-legged gull and 14.177 Audouin's gull breeding pairs in the 2007 census (Molina et al., 2009), and is a crucial area for the breeding and conservation of the threatened Audouin's gull. Subcolonies of each species were basically monospecific (Oro et al., 2009).

The sampling method is based on the guidelines of UNEP and OSPAR. For yellow-legged gull, 12 eggs were randomly collected from 3 subcolonies (36 eggs in total) on 5-6th of April 2010. For Audouin gull, because its condition as protected species, 7 eggs from 3 subcolonies (21 eggs) were collected on 25th of April 2010. Mean distance between yellow-legged gull subcolonies was 3315 m (SD = 706 m) and of 3050 m (SD = 960 m) between

Audouin's gull subcolonies. To ensure that the eggs were fresh and to avoid pseudoreplication, only the first egg of each nest was sampled (i.e. nest with only one egg). The eggs were transported to the laboratory as soon as possible and were then further processed as discussed below.

3.3.3 Analytical methods

A schematic overview of the sample preparation, extraction and analysis is given in Figure 16. Twelve (*L. michahellis*) or seven eggs *(L. audouinii*) from each subcolony were pooled. Of each sample, one fraction was used as wet sample for the analysis of PFCs, while the other fraction was freeze-dried for the analysis of the other POPs. Eggs represent a complex matrix due to the high amount of lipoproteins and other potential interfering compounds. Therefore, for the detection of the various POPs, mass spectrometric based methods using Selected Ion Monitoring acquisition mode was always performed to eliminate potential sample interferents and to provide selective identification and quantification of POPs, as suggested for Stockholm Convention POPs (de Boer et al., 2008).

3.3.3.1 Extraction and analysis of PFCs (Method 1)

Native compounds of perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorohexane sulfonic acid (PFHxS) and perfluorobutane sulfonic acid (PFBS) and surrogate standards perfluoro-n-(1,2,3,4-$^{13}C_4$) octanoic acid (m-PFOA) and sodium perfluoro-1-(1,2,3,4-$^{13}C_{4)}$ octanesulfonate (m-PFOS) were supplied by Wellington Laboratories (Ontario, Canada). Stock standard solutions were prepared in acetonitrile at a concentration of 5,000 ng/ml and were stored at -18ºC. The calibration curve was performed over the concentration range of 1.25 – 100 ng/ml.

The PFCs were solid-liquid extracted following the method of Fernández-Sanjuan et al., 2010. Briefly, 1 g of whole egg was spiked with 100,000 pg/g of ^{13}C-PFOS and ^{13}C-PFOA and after incubation for 18 hours at 7ºC, 9 ml of acetonitrile were added, the sample was vortexed and ultrasonic extracted 3 times for 10 minutes at room temperature. Afterwards the samples were centrifuged at 2500 rpm for 5 minutes and the supernatant was transferred to a new vial and evaporated to dryness. One ml of acetonitrile was added to the dried extracts, followed by 10 minutes incubation in the ultrasonic bath. To clean the extracts, approximately 25 mg of activated carbon and 50 µl glacial acetic acid were added, vortexed for 1 minute, and centrifuged for 10 minutes at 10,000 rpm. One ml of the supernatant was transferred to a clean micro vial, evaporated and reconstituted with 350 µl of acetonitrile and 150 µl of HPLC water. PFCs were measured using with an ACQUITY UPLC system (Waters, USA) connected to a

ACQUITY TQD tandem quadrupole mass spectrometer (Waters, USA). An Acquity UPLC BEH C18 Column (1.7 µm particle size, 50 mm x 2.1 mm, Waters, USA) was used as mobile phase residue trap to remove any contamination from the mobile phases. The analysis was performed on a LiChroCART HPLC RP-18e column (5 µm particle size, 125 mm x 2 mm i.d., Merck, Germany) at a flow rate of 0.4 ml/min. Ten µl were injected. The mobile phase was 2 mM NH_4OAc (A) / acetonitrile (B) and the gradient and ionization and acquisition conditions used are as indicated in Fernández-Sanjuan et al. (Fernández-Sanjuan., 2010). The PFC concentrations were calculated using ^{13}C-PFOS as surrogate standard for the perfluoro sulfonates and ^{13}C-PFOA for the perfluoro carboxylates.

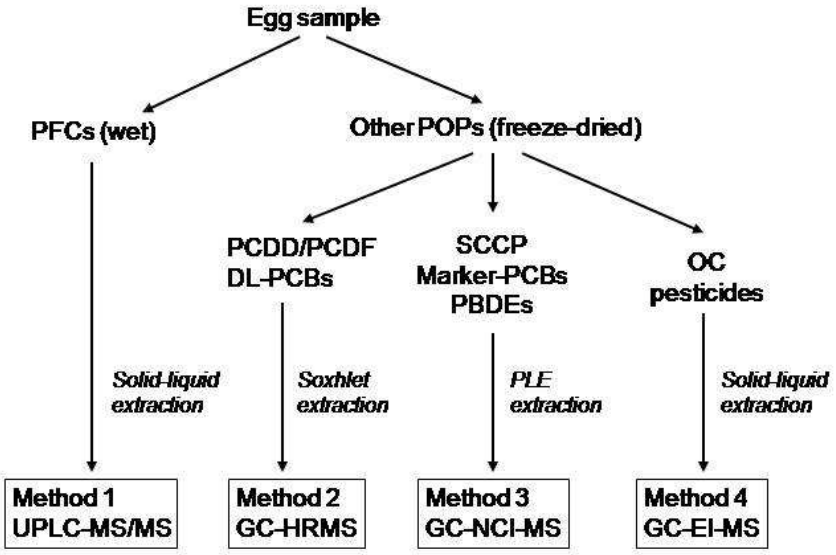

Figure 16. Schematic overview of the sample preparation, extraction and analysis for 6 chemical families of POPs.

3.3.3.2 Extraction and analysis of dioxins, furans and dioxin like PCBs (Method 2)

Extraction of PCDD/Fs and DL-PCBs (Table 4) were performed following the USEPA 1613 Method for PCDD/Fs and USEPA Method 1688 for DL-PCBs. PCDD/Fs and DL-PCBs were analyzed only in subcolony 1 for both *L. michahellis* and *L. audouinii*. Ten g of freeze-dried egg were spiked with 500 pg of mixtures of ^{13}C12-PCDD/Fs (EPA-1613LCS, Wellington Lab., Guelp, Canada) and 500 pg of ^{13}C12-DL-PCBs containing 2,3',4',5 –tetrachlorobiphenyl (PCB-70), 2,3,3',5,5'–pentachlorobiphenyl (PCB-111) and 2,2',3,3',4,4',5-heptachlorobiphenyl (PCB-170) (WP-LCS, Wellington Lab., Guelp, Canada) and then Soxhlet extracted for 24 h with toluene:cyclohexane (1:1). The extracts were rotary concentrated. Afterwards, fat residues were dissolved in n-hexane. Organic components, fat and other interfering substances were removed by treating the raw extracts with silica gel modified with sulfuric acid (44%). The extracts were concentrated and filtered through a PTFE filter prior to the clean-up. This procedure is based on the use of the Power PrepTM system (FMS Inc., MA,

USA) which uses a sequential array of three different Teflon prepacked columns of multilayer silica, alumina and carbon adsorbents, respectively (FMS Inc., Waltham, MA, USA). Finally, the extracts were rotary concentrated and transferred into a vial. The remaining solvent was reduced to dryness under a gentle stream of nitrogen to 10 µl by and 1000 pg and 500 pg of labeled PCDD/Fs and DL-PCBs were added, respectively (EPA-1613ISS and WP-ISS, Wellington Laboratories Inc., Guelph, Ontario, Canada). Instrumental analysis was based on the use of High Resolution Gas Chromatography coupled to High Resolution Mass Spectrometry (HRGC-HRMS). All analyses were performed on a Trace GC ultra gas chromatograph (Thermo Fisher Scientific, Milan, IT) fitted with a 60 m x 0.25 mm i.d. x 0.25 µm film thickness DB-5ms fused silica column (J&W Scientific, CA, USA) coupled to a high resolution mass spectrometer (DFS, Thermo Fisher Scientific, Bremen, Germany) controlled by a Xcalibur data system. Positive electron ionization (EI+) operating in the MID mode at 10,000 resolving power was used. Quantification was carried out by the isotopic dilution method. Calibration was performed from 0.5 to 1,000 ng/ml for PCDF, from 0.5 to 500 ng/ml for PCDDs (1.0 – 2,000 ng/ml for OCDF/D) and 0.1-200 ng/ml for DL-PCBs.

3.3.3.3 Extraction and analysis of SCCPs, marker PCBs and PBDEs (Method 3)

Short-chain chlorinated paraffin (SCCP C_{10} - C_{13}, 63% Cl) at 100 µg/ml in cyclohexane was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Individual PCB congeners, 28, 52, 101, 118, 138, 153 and 180, at >99% purity were supplied by Promochem GmbH (Wesel, Germany). A stock standard solution mixture of the seven PCB congeners at 300 ng/ml was prepared by weight in isooctane from the individual standard solutions. PBDEs congeners 28, 47, 99, 100, 153, 154 and 209 were purchased from Wellington Lab., Guelp, Canada. Seven calibration standard solutions of SCCPs (between 0.5 and 30 µg/ml), PCBs (from 0.5 to 200 ng/ml) and PBDEs (0.5 to 500 ng/ml) were prepared by dilution of the primary standard solutions. [$^{13}C_{12}$]-PCB-30 and [$^{13}C_6$]-hexachlorobenzene (Dr. Ehrenstorfer GmbH) and BDE 77 (Wellington) were used as surrogate internal standard, while [$^{13}C_{12}$]-PCB-209 (Dr. Eherenstorfer GmbH) was used as injection standard for quantification purposes.

For the analysis of SCCPs, PCBs and PBDEs in gull egg samples, a simultaneous extraction and clean-up method based on pressurized liquid extraction (PLE) ASE 100 Accelerated Solvent Extractor System (Dionex, Sunnyvale, CA, USA) was used. Briefly, 1 g of the freeze dried egg samples was spiked with appropriate amounts of $^{13}C_6$-hexachlorobenzene, $^{13}C_{12}$-PCB-30 and BDE 77 and was kept overnight at room temperature to equilibrate. The sample was then mixed with 3 g of anhydrous sodium sulfate and loaded into a 34 ml PLE extraction cell on top of 16 g of silica modified with sulfuric acid (20%, w/w), which was used as fat retainer. Extraction was performed at 100ºC with a mixture of n-hexane:dichloromethane

1:1 (v/v) working at a constant pressure of 1500 psi, a flush volume of 60% and a purge time of 90 s. Three static extraction cycles of 5 min each were applied to achieve the maximum recovery of the analytes. The extract was then rotary evaporated to approximately 1 ml after addition of 100 µl of iso-octane as a keeper and it was fractionated on 10 g of Florisil activated at 350ºC for 12h. Two fractions were obtained using (F1) 40 ml of n-hexane and 20 ml of a n-hexane:dichloromethane mixture 85:15 (v/v), and (F2) 60 ml of a n-hexane:dichloromethane 1:1 (v/v). Fraction 1 contained the PCB and PBDE congeners, while the SCCPs were eluted in fraction 2. All fractions were rotary concentrated and the final volume was adjusted to 50 µl after adding an adequate amount of $^{13}C_{12}$-PCB209 used as internal standard.

Extracts were then analyzed by GC-NCI-MS in 3 independent runs (one per chemical family) to obtain high selectivity and eliminate potential interferences among halogenated compound (Santos and Galceran, 2001). GC-NCI-MS analysis was carried out on a Trace GC 2000 series gas chromatograph (ThermoFinnigan) equipped with an AS2000 autosampler and coupled to a GCQ/Polaris ion trap mass spectrometer (ThermoFinnigan, Austin, TX, USA). MS operating conditions were the following: electron ionization mode using automatic gain control (AGC) with electron energy of 70 eV and an emission current of 250 µA. The transfer line and ion source temperatures were kept at 270ºC and 200ºC, respectively. The electron multiplier voltage was set to 1450 V (10^5 gain) by automatic tuning. Methane was used as moderated gas at a pressure of 1.8×10^{-4} mTorr (reading on the ion gauge).

The chromatographic separation of SCCPs was performed using a 15 m × 0.25 mm I.D., 0.25 mm of film thickness DB-5MS (5% phenyl, 95% methyl polysiloxane) fused-silica capillary column (J&W Scientific, Folsom, USA). The oven temperature was programmed from 90ºC (held for 1 min) to 300ºC at 25ºC min^{-1} (held for 5 min). Helium was used as carrier gas at a constant flow rate of 1 ml/min. One-µL of samples and standards was injected in splitless injection mode (1 min) at an injector temperature of 280ºC. The MS acquisition method was time programmed in two segments. In the first segment, the internal standard $^{13}C_6$-hexachlorobenzene was detected by monitoring the *m*/*z* 286–296 region at 0.64 s per scan, whereas SCCPs were monitored in the second segment by scanning the range *m*/*z* 70–75 region ($[HCl_2]^-$ and $[Cl_2]^{-\bullet}$ cluster ions) at 0.63 s per scan. Quantification of SCCPs was performed as the sum of total area below the elution profile of SCCPs.

For marker PCB, chromatographic separation was performed on a 30 m x 0.25 mm I.D., 0.25 µm film thickness DB-5MS fused-silica capillary column (Agilent J&W, Folsom, CA, USA). In addition, confirmation of the marker PCB congeners was carried out using a DB-17 (50% phenyl-, 50% methylpolysiloxane), 30 m x 0.25 mm I.D., 0.25 µm film thickness fused-silica capillary column (J&W Scientific). The oven program for both DB-5MS and DB-17 capillary columns was: 90ºC (held for 1 min) to 180ºC at 15ºC/min and to 300ºC at 2.5ºC/min

and 1 µl was injected. Xcalibur version 1.2 software was used for data acquisition and processing of the results.

For PBDEs, the same column as for SCCP was used with the temperature program from 120ºC (held for 1 min) to 205ºC at 8ºC/min and to 310ºC (held for 10 min) at 6ºC/min. The total run time was of 39 min. Two µl of sample were injected using the purge splitless injection at a temperature of 300ºC with a pulse pressure of 15 psi, a pulse time of 2 min, purge flow of 50 ml/min and purge time of 1.5 min. Acquisition was performed in time scheduled Selected Ion Monitoring (SIM) using the bromine ion (m/z 79/81) as quantification ion and minor ions at m/z 161 $[HBr_2]^-$ and specific $[M\text{-}HBr_2]^-$ depending on the bromination level of each PBDE as identification ions.

3.3.3.4 Extraction and analysis of OC pesticides (Method 4)

A separate extraction was needed for OC pesticides to recover "drin" pesticides since they are degraded when using sulfuric acid in the clean-up process of SCCP, PCBs and PBDEs. Standards were purchased as DDT mix 164 or individual standards at 10 or 100 ng/ml in isooctane, all from Dr. Ehrenstorfer (Germany) (Table 4). Amounts of 100,000 pg of the surrogate standards $^{13}C_{12}$-DDT and endosulfan d_4 for OC pesticides were added to 1 g of freeze-dried homogenized sample in a glass centrifugation tube and incubated for 18 hours at 4ºC. Ultrasonic extraction (5 min) was performed using 20 ml of hexane:dichloromethane (1:1). This process was repeated 3 times, with vortexing between extractions. All aliquots were combined and were centrifuged for 5 min at 4000 rpm. The supernatant was recovered and the extract was evaporated to 2 ml at 35ºC under a nitrogen stream. Cleanup of the extracts was done with solid phase extraction cartridges with 10 g Florisil (Waters, USA). The cartridge was washed with 30 ml hexane:dichloromethane mixture and then 2 ml of the extract was loaded onto the column. Elution was done with 25 ml hexane:dichloromethane mixture. After elution the purified extract was evaporated to 250 µl under N_2 and stored at -20ºC until further analysis. Calibration was performed from 10 to 1500 ng/ml. For the analysis of OC pesticides, a GC-EI-MS Agilent 6890 gas chromatograph connected to an Agilent 5973 Network mass spectrometer (Santa Clara, CA, USA) equipped with a 30 m x 0.25 mm I.D., 0.25 µm film thickness DB-5 capillary column, (J&W Scientific, USA) was used at the following chromatographic conditions: injector temperature of 250ºC; column program of 60ºC (1 min), 80 to 220ºC (10ºC/min), 220 to 300ºC (8ºC/min, kept for 10 min). Helium was used as carrier gas at 104 kPa. The splitless mode was used for injection of 2 µl with the valve opened for 2 sec. Acquisition was performed in Selected Ion Monitoring (SIM) using the base peak and 2 other most intense ions.

3.3.4 Quality Control / Quality Assurance

For each method, quality control analysis was performed using chicken (*Gallus gallus*) eggs bought from the supermarket. Pooled chicken eggs (n=6) were spiked with 100,000 pg/g for PFCs, 5,000 pg/g for OC pesticides and 500 pg/g for PBDEs, PCBs and SCCP, based on data available in the literature, and extracted according to the methods described above. Table 4 reports the quality parameters using the 4 independent analytical methods. The recovery rates for the PFCs were between 83 and 115 % with a relative standard deviation (RSD) lower than

Table 5. Levels (pg/g ww) of each compound in the pooled eggs from 3 subcolonies of *L. michahellis* and *L. audouinii* collected from the Ebro Delta, in 2010.

Compound	*L. michahellis*			*L. audouinii*		
	Area 1	**Area 2**	**Area 3**	**Area 1**	**Area 2**	**Area 3**
PFCs						
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFHxS	630	520	360	790	480	450
PFOS	86700	88900	49500	82300	62100	88200
PFOA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFNA	1970	1880	1260	1170	1310	1360
Marker-PCBs						
PCB-28	1113	1247	2254	1330	1407	578
PCB-52	1547	1932	6527	1572	1791	987
PCB-101	1135	3528	3925	1200	4222	683
PCB-118	36468	31002	36508	37225	20720	14094
PCB-138	161999	94207	106793	77579	85017	68321
PCB-153	291252	353034	380645	428905	364872	167137
PCB-180	225147	234551	253152	222331	202903	96355
ΣMarker-PCB	718661	719502	789805	770142	680932	348155
PBDE						
BDE 28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BDE 47	506	447	849	789	771	1060
BDE 99	773	559	747	388	426	1352
BDE 100	121	159	58	140	184	179
BDE 153	436	122	561	621	439	692
BDE 154	20	52	79	112	89	146
BDE 183	64	33	115	104	76	117
BDE 209	38515	37204	33534	31027	40801	28307
ΣPBDE	40435	38576	35943	33181	42786	31853
SCCPs	2870	4233	6504	6430	7609	5054
OC pesticides						
Aldrin	n.d	n.d	n.d	n.d	n.d	n.d
Dieldrin	n.d	n.d	n.d	n.d	n.d	n.d
Endrin	980	1145	1390	1004	1230	1757
α-Endosulfan	n.d	n.d	n.d	n.d	n.d	n.d
β-Endosulfan	865720	695212	1268648	1258453	987256	1128628
Endosulfan-sulfate	n.d	n.d	n.d	n.d	n.d	n.d
α –chlordane	78	59	83	94	79	105
γ-chlordane	325	568	784	1250	1012	1478
Oxychlordane	1923	1420	1430	2015	1506	2516
Cis-nonachlor	n.d	n.d	n.d	n.d	n.d	n.d
Trans-nonachlor	3622	1831	3227	1194	2701	1960
Mirex	11331	12593	11483	14657	12287	17236
Hexachlorobencene	1176	1535	2215	2004	1316	1381
Heptachlor	n.d	n.d	n.d	n.d	n.d	n.d
2,4-DDT	n.d	n.d	n.d	0	n.d	n.d
4,4'-DDT	956	880	1232	1886	1384	1834
2,4-DDD	97	79	120	170	153	190
4,4'-DDD	n.d	n.d	n.d	n.d	n.d	n.d
2,4-DDE	20.4	15.8	11.7	21.6	50.2	86.3
4,4'-DDE	19317	22012	27080	21285	18409	11541
α –HCH	n.d	n.d	n.d	n.d	n.d	n.d
β –HCH	n.d	n.d	n.d	n.d	n.d	n.d
δ-HCH	n.d	n.d	18	n.d	n.d	n.d
γ-HCH	628	732	358	703	873	491

n.d = not detected

9%. Using the labeled ^{13}C surrogate standard, PCDD/Fs were recovered between 61 and 78% (except OCDD). DL-PCBs were extracted with the same method and the recovery of the $^{13}C_{12}$-PCBs were between 60 and 83%. For PCDDs, PCDFs and DL-PCBs, the USEPA methods (1613 and 1688) establish recoveries of the labeled surrogate standards between 70 and 130%. SCCP were recovered in a 97% with a RSD of 6%. In this case, external standard quantification was performed. For marker PCBs, recoveries were between 82 and 101% with RSD values ranging from 4 to 9%, indicating no interferences from other halogenated compounds. The recovery rates for the PBDEs were between 53 and 116% with a RSD of 2-13%, although BDE 209 was only recovered in 46%. OC pesticides were recovered in between 65 and 96%, except for dieldrin whose average recovery rates were of 40%. Given that eggs contain a high fat content which can interfere with the determination of the analytes, the use of surrogate standards was mandatory to control extraction efficiency in each sample and ensure accurate quantification.

In addition, unspiked chicken eggs and blank samples with no matrix were used to evaluate background contamination. Method Detection Limits (MDLs) were calculated as the amount of analyte that produced a signal-to-noise ratio (S/N) of 3:1 from the spiked samples. All compounds were detected at the low pg/g-ww level (Table 4), showing the effectiveness and sensitivity of the MS methods developed for the analysis of priority and emerging POPs in bird's eggs. The lipid content was analyzed gravimetrically and was of 8.2% and 6.9% for *L. michahellis* and *L. audouini*, respectively and the water content was of 76.3 and 77%. For comparability purposes, the concentration of target compounds is given on a wet weight (ww) basis.

3.4 Results and Discussion

3.4.1 Species related differences on the levels of POPs in gull eggs from the Ebro Delta

The accumulation of POPs in birds depends on the habitat, environmental and specie-related factors. Feeding habits have been identified as the main source of contamination in birds and stable isotope signatures suggested that gulls from marine colonies are exposed to contaminants via marine prey although exposure scenarios are colony-specific (Gebbink et al., 2011). Because POPs are accumulated life-long and are ovo-deposited at each breeding season, gull eggs have become an excellent indicator of pollution. Target compounds were detected in eggs of both *L. michahellis* and *L. audouinii* from each subcolony in the Punta de la Banya (Tables 5 and 6). Figure 17 shows that in both gull species, the concentration was OC pesticides > marker PCBs > PFCs > mono-ortho PCBs > PBDEs > SCCP > non-ortho PCBs > PCDDs > PCDFs. Previous studies also report PCBs and DDTs as the dominant residues in

herring gull eggs from both Lake Ontario and Green Bay (Norstrom and Hebert, 2006). In another study, PCB and 4,4'-DDE constituted 90% of the contaminants found in liver of glaucous gull (*Larus hyperboreus*) from Bjørnøya (74°N, 19°E) and Longyearbyen (Herzke et al., 2003). Considering the mean values for the 3 subcolonies, for ΣOC pesticides, ΣPFCs and SCCP, levels in *L. audouinii* were higher than in *L. michahellis*. On the contrary, ΣPBDEs and marker ΣPCBs were slightly higher in *L. michahellis.* (Figure 17). Considering the levels of mono-ortho PCBs, non-ortho PCBs and marker PCBs from subcolony 1, in all cases *L. audouinii* contained higher levels compared to *L. michahellis*, and this corresponds to a similar accumulation rate for all PCB congeners. Taking into consideration that both species share habitat and have similar biology, this differential POPs levels are basically attributed to the feeding habits. *L.audouinii* feeds exclusively on pelagic fish or fish tips although can also feed on crabs. On the other hand, *L.michahellis* has a much wider habitat, is an opportunistic species and feeds on fish tips but also on waste and predates chicks and eggs. The higher dispersion in OC pesticides, PFCs, and SCCP concentrations in the 3 subcolonies of *L. michahellis* (CV from 29 to 40%) may reflect the opportunistic feeding habits. For marker PCBs and PBDEs, theslight variation within the 3 subcolonies of *L. michahellis* suggesta specific food (e.g. fish) as a source of contamination. Variability of concentrations among the 3 subcolonies of *L. audouinii* was also observed for marker PCBs, but this was due that subcolony 3 had much lower levels than the other subcolonies. Bird's age distribution and settlement within each colony may explain the subtle differences, although impossible to discern.

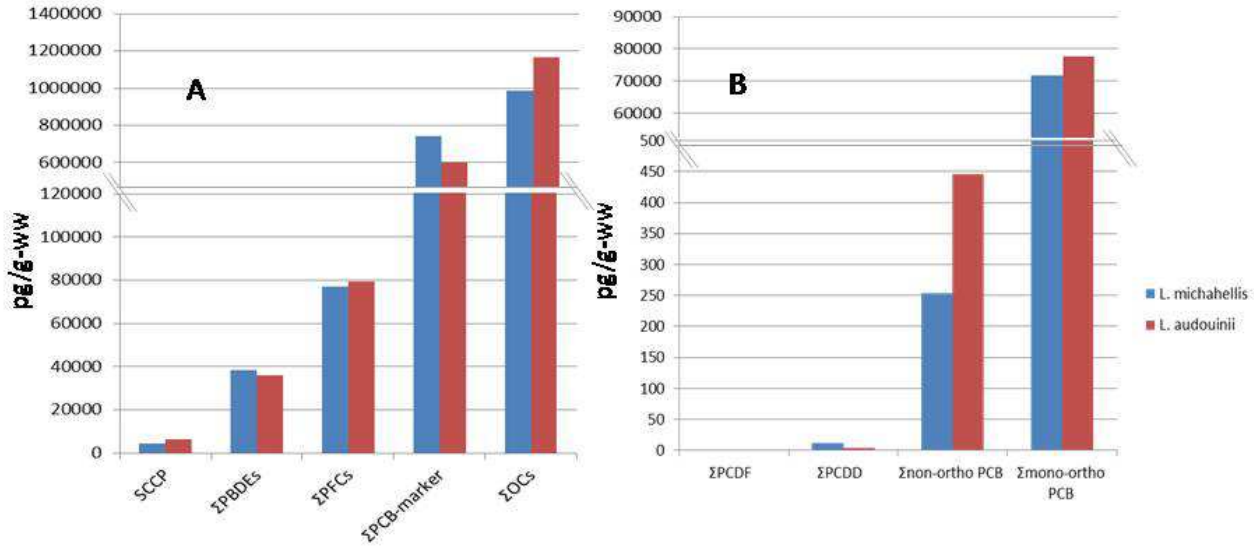

Figure 17. Comparison among *L. michahellis* and *L. audouini* on the total concentration of each chemical family, with (A) pg/g of OC pesticides, PCBs, PFCs, PBDEs and SCCP and (B) DL-PCBs, PCDFs, and PCDDs.

3.4.2 Perfluorinated compounds

Similar to Gebbink et al., PFOS was the main PFC detected (Gebbink et al., 2011), followed by PFNA and PFHxS. Mean ΣPFCs were detected at 77,240 ± 29 pg/g-ww (mean from the 3 subcolonies) in *L. michahellis* and 79,387 ± 17 pg/g-ww in *L. audouinii* (figure 17). A previous study carried out in 2009 in the same colony of *L. michahellis* showed mean concentrations of 43,000 pg/g-ww (Vicente et al., 2012). These levels are low when compared to eggs of *L. argentatus* from North America containing 484,000 – 507,000 pg/g-ww (Gebbink et al., 2009) or *Larids* eggs from the Great Lakes and the St. Lawrence River area, with 299,000-486,000 pg/g-ww, with the highest PCF egg concentrations in urbanized areas (Gebbink et al., 2011). Other studies show PFOS as the predominant PFC since it was detected in 95% of the livers analyzed of birds from Japan and Korea at levels of 650,000 pg/g-ww while PFOA and PFHxS were found in 5-10% of the samples analyzed at 21,000 and 34,000 pg/g-ww (Kannan et al., 2001). In contrast to that, the Punta de la Banya in the Ebro Delta is a Natural Park where no direct industrial nor agricultural activities are carried out.

3.4.3 PCDD/Fs and DL-PCBs

Levels of PCDD/Fs and DL-PCBs are reported in Table 6. PCDD/Fs, expressed as the sum of the seventeen toxic congeners, were detected in both species at concentrations of 5.8 and 13.2 pg/g-ww in *L. audouinii* and *L. michahellis*, respectively. In terms of total WHO-TEQ_{2005} concentrations, the levels of PCDD/Fs were 0.7 and 1.0 pg WHO-TEQ_{2005}/g-ww in *L. michahellis* and *L. audouinii*, respectively. These values expressed as wet weight, correspond to lipid weight values of 8.5 and 14.5 pg WHO-TEQ_{2005}/g-lw, both levels being above the maximum established at the European Regulation (2.5 pg WHO-TEQ/g lw for PCDD/Fs) if gull eggs were considered as food (Commission Regulation (EU) No 1259/2011). For DL-PCBs, concentrations expressed as the sum of the twelve dioxin like congeners were 71,972 and 77,989 pg/g-ww in *L. michahellis* and *L. audouinii*, respectively. In terms of total WHO-TEQ_{2005} concentrations, the levels were 22.8 and 36.0 pg WHO-TEQ_{2005}/g-ww respectively, which corresponds to lipid weight values of 278 and 522 pg WHO-TEQ_{2005}/g-lw in *L. michahellis* and *L. audouinii*, respectively. Taking all these into account, when DL-PCBs are included in the total WHO-TEQ (PCDD/Fs and DL-PCBs), in both species, these values reach up to 100 times higher than the maximum established at the European Regulation for the sum of the two families of compounds (5.0 pg WHO-TEQ/g lw for PCDD/Fs+DL-PCBs) (Commission Regulation (EU) No 1259/2011).

Generally, the PCDD/Fs levels found in this study are consistent with data published in the literature for similar gull species. For instance, previous studies report PCDD/Fs between

12-64 pg/g-ww and 3.4-30 pg/g-ww in herring gulls from the Great Lakes collected in 1998 (Giesy and Kannan, 2001). In terms of dry weight, PCDD/Fs levels in *L. audouinii* eggs from Ebro Delta collected in 1992 were 140.67 ± 30.50 pg/g-dw (Pastor et al., 1995). These concentrations are slightly higher than those found in the present study (55.8 pg/g-dw for *L. michahellis* and 25.2 pg/g-dw for *L. audouinii*). If the results are expressed in terms of lipid weight, they are comparable to PCDD/Fs concentrations reported for *L. crassirostris* eggs from Hokkaido (Japan) (between 22.9 and 120.2 pg/g-lw) (Choi et al., 2001) and for *P. eburnean* eggs from Canadian Artic (79 pg/g-lw)(Braune et al., 2007). For DL-PCBs, in particular non-ortho PCBs, similar values than those obtained in this study have been reported. Non-ortho PCB levels were 2,221 pg/g-lw in *P. eburnea* eggs from Canadian Artic (Braune et al., 2007) and between 2,455 and 7,434 pg/g-lw in *L. crassirostris* eggs from Hokkaido (Japan) (Choi et al., 2001). Samples of *L. audouinii* eggs from Ebro Delta showed total concentrations of non-ortho PCBs of 4,300 pg/g-dw (Pastor et al., 1995), which is as the case of PCDD/Fs values, are slightly higher than those found in this study. Therefore if the results from the present study are compared with the data previously reported by Pastor et al., 1995 in *L. audouinii* eggs from Ebro Delta, a decrease in PCDD/Fs and non-ortho PCB levels is observed. This decrease could be explained by the ban of PCB manufacturing in 1979 and the enforcement of measures to minimize PCDD/Fs sources in order to reduce the levels in the environment (e.g. Directive 2010/75/EU, which includes the maximum level for PCDD/Fs emissions from incineration plants).

3.4.4 Marker PCBs

Marker PCBs were detected from 1,113 pg/g-ww (PCB 28) to 380,645 pg/g-ww (PCB 153) in eggs of *L. michahellis* and from 578 (PCB 28) to 428,905 pg/g-ww (PCB 153) in *L. audouinii*. While homogeneous concentration were found among the 3 subcolonies of *L. michahellis*, in *L. audouinii*, subcolony 3 showed the lowest levels, attributed maybe to age differences. For both species, PCB 153 was the predominant congener detected, followed by PCB 180, PCB 138 and PCB 118, while PCBs 101, 152 and 28 were found at one or two orders of magnitude lesser concentration (Table 5). Previous studies in Audouin gull of the Ebro Delta report PCBs 138 as the most prominent congener, followed by, in decreasing order, by PCB 153, 180, 170, 101, 151, and 194, with the highest levels in Ebro Delta, followed by Chafarinas and Cuenca (González et al., 1991). Marker PCB congeners were previously identified in *L. audouinii* at ΣPCBs 10,300,000 ± 7 μg/g-dw with levels decreasing from the first to the third eggs (Pastor et al. 1995, b). In terms of dry weight, comparing this concentration from 1995 with the ΣPCB_7 of 3,133,565 pg/g-dw and 2,607,578 pg/g-dw in *L. michahellis* and *L. audouinii*, respectively from 2010, it can be shown that the levels have decreased slightly within

15 years, following the same behavior as observed for PCDD/Fs and DL-PCBs. In other studies from Spain, ΣPCBs in booted eagle (*Hieraaetus pennatus*) and goshawk (*Accipiter gentilis*) were of between 34,100–270,000 pg/g-ww and from non detected to 43,500 pg/g-ww, respectively (Martinez-Lopez et al., 2007). Similarly, PCBs were found in all the 12 bird species sampled in the Campania region, Southern Italy, at levels ranging between 6,000 and 8,431,000 pg/g-ww and congeners 180, 153, 138, and 118 predominated and contributed to more than 98% of the ΣPCB_7 and were significantly higher in omnivorous birds than in carnivorous ($p < 0.01$) (Naso et al., 2008).

Table 6. Levels (pg/g ww) of PCDD/Fs and DL-PCBs of *L. michahellis* and *L. audouinii* collected from the Ebro Delta, in 2010 (subcolony 1).

Compound	*L. michahellis*	*L. audouinii*
PCDD/F		
2,3,7,8-TCDF	0.04	0.1
1,2,3,7,8-PeCDF	n.d.	n.d.
2,3,4,7,8-PeCDF	0.2	0.5
1,2,3,4,7,8-HxCDF	0.1	0.5
1,2,3,6,7,8-HxCDF	0.3	0.2
2,3,4,6,7,8-HxCDF	0.3	0.3
1,2,3,7,8,9-HxCDF	n.d.	n.d.
1,2,3,4,6,7,8-HpCDF	0.4	0.07
1,2,3,4,7,8,9-HpCDF	0.09	0.08
OCDF	0.2	n.d.
2,3,7,8-TCDD	0.2	0.3
1,2,3,7,8-PeCDD	0.3	0.4
1,2,3,4,7,8-HxCDD	n.d.	n.d.
1,2,3,6,7,8-HxCDD	0.3	0.4
1,2,3,7,8,9-HxCDD	0.08	0.1
1,2,3,4,6,7,8-HpCDD	0.9	0.4
OCDD	9.7	2.4
ΣPCDD/F pg/g-ww	13.2	5.8
DL-PCB		
PCB-105	8513	10984
PCB-114	554	728
PCB-118	36468	37225
PCB-123	525	777
PCB-156	13623	12808
PCB-157	2699	3036
PCB-167	5177	7983
PCB-189	4158	4001
Σmono-ortho PCB pg/g-ww	71718	77543
PCB-77	19.8	72
PCB-81	3.4	4
PCB-126	196	323
PCB-169	35	47
Σnon-ortho PCB pg/g-ww	254	446
WHO-TEQ_{2005} pg/g ww	**(Upperbound values)**	
PCDD/Fs	0.7	1.0
DL-PCBs	22.8	36.0
PCDD/F+DL-PCB	23.5	37.1
Fat (%)	8.2	6.9
Moisture (%)	76.3	77.0

n.d. = not detected

3.4.5 PBDEs

Individual PBDEs were detected in *L. michahellis* and *L. audouinii* at levels between 20 (BDE 154) and 38,515 pg/g-ww (BDE 209) and 75.9 (BDE 183) and 40,801 pg/g-ww (BDE 209), respectively (Table 5). A clear predominance of BDE 209 was found in both species,

accounting for more than 89% of the total PBDE burden (Table 5). This ubiquity of BDE 209 in eggs has seldom been found. In a previous study, out of 39 PBDE congeners monitored in pooled eggs samples of herring gull (*Larus argentatus*) from seven colonies spanning the Laurentian Great Lakes over the period 1982-2006, BDE209 concentrations ranged from 4,500 to 20,000 pg/g-ww, somehow lower than in the present study, but constituted 0.6-4.5% of ∑39 PBDE. In that study, the major BDE congeners identified were BDE-47, -99, -100 at $\Sigma PBDE_3$ 186,000 – 498,000 pg/g-ww (Gauthier et al., 2008). Differences on the PBDE profile among colonies of the United States and Spain can be attributed to the use of PBDE formulations. While in the United States penta BDE formulations were widely used, in Europe deca-BDE formulations prevailed and have been used for a longer time, until their prohibition in 2000 and 2003, for penta and deca-formulations, respectively (Directive Directive 2003/11/CE and European Court of Justice 2008-04-01). In another study, the temporal trends (1983-2003) of PBDEs and hexabromocyclododecanes (HBCDs) were evaluated in eggs of herring gulls (*Larus argentatus*), Atlantic puffins (*Fratercula arctica*), and black-legged kittiwakes (*Rissa tridactyla*) in North Norway, and it was found that PBDE concentrations increased between 1983 and 1993 and then leveled out, and that BDE 209 ranged from non-detectable to parts per billion (Helgason et al., 2009). Other studies report PBDE levels in great blue heron eggs and herring gull eggs from the Great Lakes at 70,000-1,377,000 pg/g-ww (Champoux et al., 2010). Similarly, BDE 209 was found in white stork eggs from two colonies in Spain at average concentrations of 9,080 pg/g-ww for the urban/industrial colony and 1,640 pg/g-ww for the rural colony, and BDE 209 accounted for 38-44% of the total PBDEs (Muñoz-Arnanz et al., 2011).

3.4.6 SCCP

SCCP were detected at 4,536±40 pg/g-ww in *L. michahellis* and 6,364±20 pg/g-ww in *L. audouinii*, and it is the first time these compounds are identified in gull eggs from the Ebro Delta and to our knowledge from other bird eggs from other parts of the world. There is only one study from Campbell who report non-quantifiable levels of SCCP in eggs from varies bird species (Campbell et al., 1980). Figure 18 shows the GC-NCI-MS chromatogram of a standard solution and an egg sample where the characteristic profile of SSCP can be observed. As other types of POPs, gulls can accumulate SCCP through the diet and transfer them to the egg. SCCP have previously been detected in sediments of the Catalan coast and therefore, are likely to be detected in biota. SCCP are additives of high resistance used as cutting fluids, plasticizers, paints, flame retardants, polyesters and polyolefin, etc. They are of concern because they are persistent, have been found in remote areas such as the Arctic, and could accumulate to levels

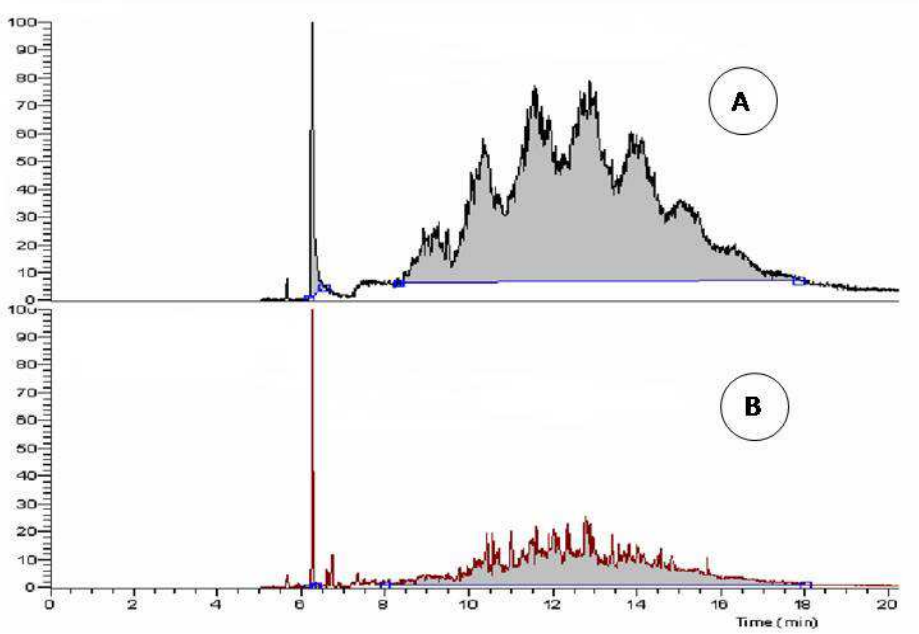

Figure 18. GC-NCI-MS chromatogram of a (a) SCCP solution at 15 µg/ml and (b) gull egg.

that are toxic to fish and other aquatic organisms. They are known to trigger effects towards aquatic organisms and are considered carcinogenic in humans (group 2b). Santos et al., reviews the presence and accumulation of SCCP in sediments and biota and highlights that even its ubiquitous presence, there are very few studies that report the levels and impact in environmental samples (Santos et al., 2006). This is in line with recent decisions from the Stockholm Convention who, given the lack of information on its environmental and health effects and trends in the levels in the environment, decided to postpone any decision-making and revise the risk profile in relation to SCCP.

3.4.7 Organochlorinated pesticides

OC pesticides accounted for the main POPs detected in gull eggs, with slightly higher mean concentration in *L. audouini* compared to *L. michahellis* (Figure 17), although this variation was compound specific. Very homogeneous concentrations were observed between the levels of OC pesticides in each of the 3 subcolonies within each species, indicating that within a colony, exposure pathways and accumulation follow a yearly accumulation pattern (gulls lay 3 eggs each year). Endosulfan, DDTs and mirex were the most abundant compounds. Residues of these compounds have been previously identified along the Catalan coast (Sánchez-Ávila et al., 2010). β-endosulfan was detected at the highest concentrations, with mean values of 943,193 ± 31 in *L. michahellis* and 1,124,779± 12 pg/g-ww in *L. audouinii*. Technical endosulfan is a 7:3 mixture of α- and β-endosulfan which are conformational isomers. α -endosulfan is more thermodynamically stable than the β isomer, but it is more volatile and less

water-soluble, and has a relatively high vapor pressure and Henry's Law constant, which together produce a greater rate of disappearance. The higher water solubility of β –endosulfan may explain its presence in water, fish and finally its high bioaccumulation in gull eggs. ΣDDTs were detected in *L. michahellis* at 23,940 ± 20 pg/g-ww and at 19,003 ± 21 pg/g-ww in *L. audouinii*, much lower than the levels reported in Audouin gull from the Ebro Delta in the 1992 breeding season (Pastor et al., 1995). 4, 4'-DDT was detected at 1,023 ± 18 pg/g-ww in *L. michahellis* and at 1,701 ± 29 g/g-ww in *L. audoinii*, 22 and 10 times in average lower concentration than 4,4'-DDE, respectively. These differences in DDE/DDT ratios between the 2 species may suggest a higher metabolization rate in *L. michahellis*. The prevalence of 4,4′-DDE was also found in bird eggs from different species from south Italy, where it was detected from 4 to 4,504,000 pg/g-ww and the highest 4,4′-DDE levels were in carnivorous species, followed by omnivorous and insectivorous ones (Naso et al., 2003).

Mirex is one of the "dirty dozen" according to the Stockholm Convention and its production and use is banned because if can bioaccumulate at levels that impair harmful effects. Mirex was detected at 11,802 ± 6 pg/g-ww in *L. michahellis* and at 14,726 ± 17 in *L. audouinii*. As other types of OC pesticides, mirex may come from direct contamination as a result of its use in agriculture. The slight higher levels of mirex in *L. audouinii* may reflect the feeding habits of the gull based in marine fish. This same behavior was found for endrin and chlordanes, except for trans-nonachlor which was present at higher concentrations in *L. michahellis*. Trans-nonachlor is a bioaccumulating component of the pesticide chlordane and was found at higher concentration than the parent compound. Finally, hexachlorobencene and γ-HCH were detected at similar concentrations in both gulls. The Ebro Delta is highly agricultural and rice cultivation is spread over the whole area and until prohibited in Spain, OC pesticides have been widely used. In addition to that, Ebro river waters do contain OC pesticides due upstream transport from an organohalogenated producing factory (Fernández et al., 2000).

The effects POPs can cause at the different stages of bird development, colony and population survival are largely unknown. Bustnes et al. indicate that when the concentrations of PFCs and OCs pesticides are of similar magnitude in a gull population, OCs are more likely to cause adverse ecological effects (Bustnes et al., 2008). Synergic or antagonist effects can occur, with interactions with the different contaminants. Considering the 2 gull species, one representing a plague (*L. michahellis*) with no risk of survival and another one representing a protected and endangered species (*L. audouinii*), it is of great interest to integrate toxicological analysis to determine the potential targets and effects. In addition, given that all POPs were detected in gull eggs at relevant concentrations, it is also of interest to determine the long term concentrations of legacy and emerging pollutants to determine if actions to restrict the use and emissions of several toxic and bioaccululative contaminants are effective in protected breeding areas.

3.5 Conclusions

It is well known that birds accumulate POPs and that these compounds are transferred to the eggs, triggering possible deleterious effects to the development of the egg, the chick or of the colony well- being. Gulls, because of their biology and feeding ecology, accumulate contaminants which are released through the egg at each breeding season. Therefore, eggs become a suitable matrix for the biomonitoring of POPs and permit to evaluate the contamination impact within an area. In this study, we have determined priory and emerging POPs in seagull eggs of 2 species to increase the knowledge on their presence in the Ebro Delta Natural Park, an ecosystem historically impacted by the use and spills of pesticides, PCBs and other contaminants. Paradoxically, the protected species *L. audouinii* had the highest levels of several POPs, compared to *L. michahellis*, a scavenger specie. It is early to evaluate the effects this cocktail of contaminants may produce to gulls, but considering the neurotoxicity, carcinogenicity and endocrine disruption effects of POPs, actions should be launched to evaluate the sources of pollution and take actions to minimize their impact upon birds, especially in protected species.

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Chapter 4. Distribution of perfluorinated compounds in yellow-legged Gull eggs (*Larus michahellis*) from the Iberian Peninsula

4.1 Abstract

This study is aimed to evaluate the presence and distribution of Perfluorinated Compounds (PFCs) in Yellow-legged gull eggs (*Larus michahellis*) collected from 8 National or Natural Parks from the Iberian Peninsula. In each colony, 12 eggs were randomly collected and pooled from 3 areas of the colony and analyzed using liquid-solid extraction and liquid chromatography coupled to tandem mass spectrometry. Perfluorooctanate sulfonate (PFOS) was the only compound detected in the eggs and its presence was higher in the colonies situated in NE Iberian Peninsula due to the more industrial and mass urbanization in this area compared to the SW Mediterranean or Atlantic colonies. Accordingly, the Medes site, followed by the Ebro Delta and Columbretes, all situated in the NW Mediterranean coast, contained the highest PFOS levels (40.5-54.0 ng/g ww (wet weight)). In all other colonies, PFOS was detected at levels of 10.1-18.6 ng/g ww. Egg shell biometry was studied and it was found that the presence of PFOS did not affect the development of the egg.

4.2 Introduction

Perfluorinated Compounds (PFCs) have aroused concern because of their increasing distribution in the environment and their still unknown effects on biota. PFCs, such as perfluorinated sulfonates and perfluoro carboxylic acids, are a group of chemicals used as anti-adherent in pans, as waterproof and breathable material (e.g. type Gore - tex ®) and as surfactants in many industrial products (inks, paints, flame retardants, foams, etc.). Although their use is currently regulated (UNEP, 2009), residues have been found in many biological matrices due to their high accumulation potential (Giesy and Kannan, 2001).

Birds are very sensitive to chemical pollution because of their dietary habits and their relatively long life (Furness and Camphusyen, 1997). In a pioneer study performed in 2001, Giesy and Kannan reported the presence of perfluorooctane sulfonate (PFOS), a highly bioaccumulative compound, in the blood, plasma and egg yolk of several bird species across USA at levels up to 2220 ng/mL (plasma of Bald eagles, *Haliaeetus leucocephalus*) (Kannan et al., 2001). In another study PFOS was detected in liver of the Great cormorant (*Phalacrocorax carbo*) at 1873 to 2249 ng/g, and it was associated to industrialization of the sampled area (Kannan et al., 2002). The environmental prevalence of PFCs is demonstrated when Haukås detected PFOS in the liver of the Arctic species Black guillemot (Cepphus grylle) at a level of.

13.5 ± 2.8 ng/g ww and in the liver of Glaucous gull (*Larus hyperboreus*) at 65.8 ± 22.4 ng/g ww (Haukås et al., 2007). Whole eggs represent a non-invasive matrix to monitor the presence of contaminants and can be used as indicators of pollution. Spatial studies have been carried out to evaluate the occurrence of PFCs in bird eggs in Common shag (*Phalacrocorax aristotelis*) from Norway (Herzke et al., 2009), in Cormorant (*Phalacrocorax carbo)* from Germany (Rüdel et al., 2011), in Egrets (*Egretta garcetta*) from China (Wang et al., 2008) and Korea (Yoo et al., 2008) and several bird species from North America (Custer et al., 2009; 2010; Rattner et al., 2004; Gebbink et al., 2009; 2011). All these studies show that, despite the species analyzed and the geographical settlement, PFCs are ubiquitous contaminants ranging from a few to thousands ng/g-ww and prove the suitability of bird eggs as indicators of PFCs pollution. In addition to spatial studies, temporal trends have been carried out using bird eggs. In 2 colonies from northern Norway, PFCs concentrations in Herring gull (*Larus argentatus*) eggs showed a nearly 2-fold significant increase from 1983 to 1993, followed by a leveling off in 2003 (Verreault et al., 2007). Temporal trends of PFCs using Swedish Peregrine Falcon (*Falco peregrinus*) eggs revealed a high increase of PFOS over the period of 1974 to 1984, remaining constant thereafter (Holmström et al., 2010). The accumulation of PFCs in birds can have serious implications as these compounds are transferred from the female bird to her eggs during laying period and this may lead to toxicological effects at the developing, individual or population level (Pusch et al., 2005; Verboven et al., 2008).

The Iberian Peninsula is an area highly affected by the historical and present use of organohalogenated compounds in both industry and agriculture, and has lead to their accumulation in biota. A study performed in 1991 identified the presence and profiles of PCBs and organochlorinated pesticides in larid (*Larus michahellis, Larus audouinii* and *Gelochelidon nilotica*) eggs from Cuenca, Ebro Delta and Chafarinas islands (Gonzalez et al., 1991). Other studies proposed gull eggs as biological indicator of PCBs, dioxins and furans (Pastor et al., 1995a) and hexachlorocyclohexane and DDTs (Pastor et al., 1995b) and suggested the need to collect the first egg to avoid variability in the levels due to the sampling procedure. A sampling conducted in 1996 in Doñana showed that Greater flamingo eggs (*Phoenicopterus roseus*) contained traces of PCBs and chlorinated pesticides at generally moderate levels, not affecting the colony wellbeing (Guitart et al., 2005). Also in Doñana, dioxins and furans were detected in Black kite (*Milvus migrans*) at levels exceeding the toxicity thresholds according the No Observed Effect Level (NOEL) (Gómara et al., 2008). Even in pristine areas like the island of Menorca, DDTs, PCBs, dioxins and furans were detected at concentrations that could reduce offspring, embryonic mortality and deformity in two raptor species (*Pandion haliaetus* and *Milvus milvus*) (Jiménez et al., 2007).

Unlike other persistent organic pollutants (POPs), no data is available on the occurrence and distribution of PFCs in birds from the Iberian Peninsula. The objective of the present study was to determine the geographical distribution of 5 environmental relevant PFCs using gull eggs as biomarkers of pollution. Yellow-legged gull (*Larus michahellis*) represents a common and widespread species with a unique habitat. The collection of eggs does not cause any effect on their already vast populations. In addition, gull eggs have been recently proposed as a non-invasive biomonitoring tool for environmental pollutants (Stockholm Convention, United Nations Environmental Programme (UNEP) and the Oslo Paris Convention (OSPAR)). The sampled areas were 8 main gull colonies of the Iberian Peninsula and represented Mediterranean and Atlantic environments. The potential risks of PFCs on eggshell thickness and egg size is discussed, as described earlier for DDTs (Lundholm, 1997; Ratcliffe, 1967; Verboven et al., 2008).

Table 7. Description of the colonies sampled, ordered from northeast to northwest of the Iberian Peninsula.

Colony	Category	Coordinates		Distance from mainland (km)	Altitude in the middle (m)	Feeding habits (chicks)	Reference
Medes	Marine reserve	3º13'E	42º02'N	0.87	63	Refuse tips, pelagic and benthonic preys	Ramos et al., 2009
Ebro Delta	Natural park	0º40'E	40º35'N	0	0	Pelagic preys, crops and terrestrial preys and refuse tips	Ramos et al., 2009
Columbretes	Nature reserve	0º41'E	39º54'N	53.78	not available	Most pelagic, but also brackish and freshwater preys	Ramos et al., 2009
Dragonera	Natural park	2º18'E	39º35'N	0.94	175	Increasing trophic spectrum and refuse tips feeding	Bermejo et al., 2009
Grosa	Natural park	0º42' W	37º43'N	1.57	34	Refuse tips, brackish, freshwater and pelagic preys	Ramos et al., 2009
Chafarinas, Rey	Hunting ground	2º25' W	35º10'N	3.3	73	Most pelagic preys, but also benthic, terrestrial preys and refuse tips	González-Solís et al., 1997
Chafarinas, Congreso sur		2º26' W	35º10'N	6.67	6		
Berlengas	Nature reserve	9º30' W	39º24'N	10.32	74	Most refuse tips	Personal communication
Cies	National park	8º54' W	42º13'N	2.54	70	Most pelagic preys, but also benthic preys and refuse tips	Moreno et al., 2010

4.3 Materials and methods

4.3.1 Chemicals and reagents

Native compounds of perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), perfluorobutane sulfonate (PFBS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) were supplied by Wellington Laboratories (Ontario, Canada). Stock standard solutions were prepared in acetonitrile at a concentration of 5 ng/µL and were

stored at -18ºC. Perfluoro-n-(1,2,3,4-$^{13}C_4$) octanoic acid (m-PFOA) and sodium perfluoro-1-(1,2,3,4-$^{13}C_4$) octanesulfonate (m-PFOS), also from Wellington Laboratories, were used as internal standards. HPLC grade water and acetonitrile were supplied by Merck (Darmstadt, Germany) and glacial acetic acid from Panreac (Barcelona, Spain).

4.3.2 Sampling areas and preparation for analysis

Fresh eggs of Yellow-legged gull (*Larus michahellis*) were collected from 8 National or Natural Parks of the Iberian Peninsula in March-May 2009 to determine the occurrence of PFCs (Figure 19). These sites represent the most important gull colonies of the Iberian Peninsula (Bermejo, 2009). These included the northern Mediterranean colonies (Medes Islands, Ebro Delta and Columbretes Islands), the southern Mediterranean (Dragonera Island, Grosa Island and Chafarinas Islands) and the Atlantic (Berlengas Islands and Cies Islands). The western Mediterranean was split into northern and southern areas because hydrographical data indicate the existence of two major units influenced respectively by the Algerian current and the Northern current (Millot 1987, Millot 1999). The limit between currents varies seasonally and annually (Pinot et al., 2002), but the 37.8 isohaline, as established in MEDATLAS II climatology (http://www.ifremer.fr/sismer/program/medar/), seems to be a convenient frontier between the two regions (Figure 19).

Figure. 19. Map showing the Yellow-legged gull colonies that were sampled in March–May 2009. In each colony, 36 eggs were collected, divided in 12 eggs in 3 subcolonies of each colony. The dashed line shows the limit between the north and south basins in the western Mediterranean.

At each colony, 36 eggs were randomly collected in 3 different subcolonies (12 eggs in total per subcolony). This sampling approach is proposed by UNEP and OSPAR guidelines since it covers the geographical variability of the eggs within each colony and does not cause any effect in the colony. Eggs were halved and one half was used for individual analysis and

another half was pooled with the other 12 eggs of each subcolony. As a result, a total of 3 pooled samples were analyzed per colony, except in Columbretes islands and Dragonera, where only 12 eggs were found and so one pooled sample was analyzed. In Berlengas islands, 4 pooled samples were analyzed. The PFCs were analyzed in individual and pooled egg samples to validate the sampling procedure (Figure 20). The first egg was sampled since it represents the maximum pollutant transfer levels from female to eggs, as demonstrated for PCBs, dioxins and furans (Pastor et al., 1995b), and for comparison among different colonies. Embryonated eggs were not used.

4.3.3 Egg and eggshell parameters

Eggs were transported inside cool boxes to the laboratory. The following egg parameters were measured before being opened: (i) the length and width and (ii) the weight of the whole egg. Afterwards, eggshells were dried at room temperature for one month and then the following was measured: (iii) the weight of the dried shell and (iv) the eggshell thickness. The eggshell thickness was measured at three points around the equator, using a Ratio digital calliper with a precision of ± 0.01 mm.

The eggshell thickness index (I) values were calculated for all the eggs according to the method given by Ratcliffe (1967).

The egg volume was calculated using the formula $V = Kv * L * Wd^2$ given by Hoyt (1979), where Kv is a species-specific constant whose value is 0.000477 for *Larus michahellis* (Oro, 2008), L = length (mm) and Wd = width (mm) of the egg.

Desiccation index (Di) was calculated for each egg as a measure of functional quality according to the formula given by Helander (Helander et al., 2002). Di expresses the mean density of the entire egg content at the time of laying because the egg immediately begins to lose weight by diffusion of water vapour. A low Di value indicates desiccated eggs.

4.3.4 Extraction

The PFCs were solid-liquid extracted from wet samples using acetonitrile, based on the method of Meyer (Meyer et al., 2009). About 1 g of sample was weighted in polypropylene tubes and internal standards (m-PFOS and m-PFOA) were added at a concentration of 100 ng/g ww and incubated for 18 hours at 4ºC. Nine ml of acetonitrile were then added and the sample was thoroughly mixed using a vortex mixer. Samples were then extracted in an ultrasonic bath for 10 minutes at room temperature (3 times). Between each period of 10 minutes, the samples were thoroughly mixed. Afterwards the samples were centrifuged at 2,500 rpm for 5 minutes. The supernatant was transferred to a new vial and evaporated to dryness. Then 1 mL of

acetonitrile was added to the dried sample and incubated for 10 minutes in the ultrasonic bath. The samples were purified by adding 25 mg of activated carbon and 50 µL of glacial acetic acid and were vigorously mixed for 1 minute. Afterwards, the samples were centrifuged for 10 minutes at 10,000 rpm. One mL of the supernatant was transferred to a clean micro vial and 350 µL were diluted with 150 µL of HPLC water and analyzed.

4.3.5 Instrumental analysis

The PFCs were measured using ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). The system consisted of Acquity Ultra performance LC system (Waters, USA) connected to a Triple Quadruple Detector Acquity Ultra performs LC. An Acquity UPLC BEH C_{18} column (1.7 µm particle size, 50 mm x 2.1 mm, Waters, USA) was used as mobile phase residue trap to remove any contamination from the mobile phases. Ten µL of extract were injected. The analysis was performed on a LiChroCART HPLC RP-18e column (125 mm x 2 mm x 5 µm particle size, Merck, Germany). The mobile phase consisted of 2 mM NH_4OAc (A)/ acetonitrile (B). Gradient elution was starting from 70% A and 30% B, increased to 90% B in 5 minutes and to 100% B in 0.10 min and held for 1 min, at a flow rate of 0.4 mL/min. The chromatographic time was of 6 min. Then, initial conditions were regained in 1 min and the system was stabilized for 3 min at initial conditions. The various PFCs were measured under negative electrospray ionisation. The 2 transitions from parent to product ion used to identify each compound as well as the dwell time, cone voltage, collision energy and retention times are summarised in Fernández-Sanjuan et al. (2010). To identify the target compounds, the retention time and these 2 transitions were used. Internal standard quantification was performed using m-PFOS to quantify PFOS, PFHxS and PFBS and m-PFOA to quantify PFOA and PFNA.

4.3.6 Quality Control parameters

The samples were extracted and analysed in batches together with a procedural blank to control any external contamination during the whole analytical process. A five point calibration curve was built over a concentration range of 1.25 to 50 ng/mL. The samples and quality controls were spiked with the internal standards at a concentration of 100 ng/g-ww. Recovery studies were performed twice by two operators and in duplicate using chicken eggs spiked with native compounds at concentrations of 10, 100 and 250 ng/g ww. Instrument detection limits (LOD_{inst}) were calculated using the lowest concentration standard solution at 1.25 ng/mL for each compound and were calculated using three times the value of the signal-to-noise ratio (the ratio between the peak intensity and the noise one minute after the peak signal). Method

detection limits (LOD_{method}) were calculated in the same way, using spiked chicken eggs at 10 ng/g ww.

4.4 Results and discussion

4.4.1 Sampling and method performance

The first step was to test the performance of the sampling procedure. Figure 20 compares the PFOS values from analyzing 12 individual eggs with the PFOS concentration from the pooled sample (n = 12) from Yellow-legged gull eggs collected from 3 subcolonies of the Ebro Delta site. We compared each pooled sample (i.e. single observation) with the mean of the individual eggs of each subcolony by a modified *t*-test. In all cases the pooled samples did not differ from the individual egg samples (subcolony 1: $t_{10} = 1.691$ $p = 0.122$; subcolony 2: $t_{10} = 2.199$ $p = 0.052$; subcolony 3: $t_{11} = 1.356$ $p = 0.202$; Figure 20). On the other hand, the PFOS concentration from the individual egg did not differ between the three subcolonies ($F_{2,31} = 0.848$, $p = 0.437$). This indicates the very low variability of PFOS within the individuals of a colony and therefore, the sampling of 36 eggs is indicative of the pollution level within the whole colony.

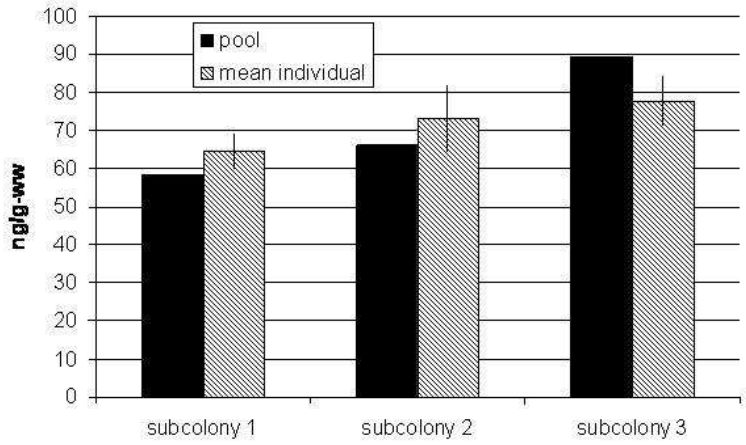

Figure 20. Graph comparing the PFOS levels in the pooled mixture of 12 eggs and the mean concentration and relative standard deviation in individual Yellow-legged gull eggs (n=12) for the 3 subcolonies in the Ebro Delta.

Regarding the analytical performance, good resolution and selectivity were obtained indicating that lipids were totally removed during the extraction step and no analyte suppression was observed. In addition, the solvent trap installed prior to the analytical column avoided any external contamination from the HPLC system and only traces of PFNA were detected below the LOD. Acquisition was performed in a 6 min chromatogram, which permitted a high sample throughput and solvent saving. The method was particularly repetitive (<14%, n=18 injections during 5 days). Table 8 provides the quality parameters of the method used. As regards to the

extraction efficiency, by extracting 1 g of chicken eggs samples, PFCs recoveries of between 86 ± 1 and 127 ± 4% at 10 ng/g ww spiking level, between 84 ± 3 and 125 ± 10% at 100 ng/g ww spiking level and from 82 ± 3 to 116 ± 9% at 250 ng/g ww spiking level, Recoveries were performed by 2 independent chemists and results were equal, indicating the robustness of the method. m-PFOS and m-PFOA were used to evaluate the extraction efficiency in Yellow-legged gull egg samples and recoveries were of 92 ± 7% and 85 ± 10%, respectively. Chicken eggs used for spiking experiments did not have any PFC contribution. Another important issue is the sensitivity of the method. The LOD obtained were between 0.09 and 0.30 ng/g ww, similar to those calculated for Zebra mussels (*Dreissena polymorpha)* (Fernández-Sanjuan et al., 2010). Overall, the method proved highly robust, repetitive and selective for spiked chicken samples and thus, able to be used for monitoring PFCs in the eggs of Yellow-legged gull.

Table 8. Quality parameters of the method such as response factor (F), regression coefficient (R^2), repetitivity, recovery at 10, 100 and 250 ng/g, limits of detection, LOD ng/g ww and blank levels.

Compound	F (slope)	R^2	Repetitivity %	%R**			LOD (ng/g-ww)	Blank levels*
				Low (10 ng/g)	Medium (100 ng/g)	High (250 ng/g)		
PFBS	2.44	0.997	14	107 ± 15 93 ± 1	89 ± 12 96 ± 3	97 ± 12 93 ± 4	0.10	n.d.
PFHxS	5.2	0.996	11	105 ± 13 86 ± 1	85 ± 10 84 ± 3	94 ± 10 82 ± 3	0.20	n.d.
PFOA	3.52	0.984	7	108 ± 7 116 ± 5	110 ± 7 107 ± 9	102 ± 4 108 ± 5	0.09	n.d.
PFNA	1	0.988	10	118 ± 9 127 ± 4	125 ± 10 115 ± 9	116 ± 9 116 ± 4	0.13	< LOD
PFOS	1.83	0.999	7	118 ± 11 105 ± 4	92 ± 11 94 ± 4	99 ± 2 104 ± 3	0.30	n.d.

n.d. = not detected. * Blank levels were measured with chicken eggs spiked with the internal standards. ** The percentage recoveries in spiked chicken samples were performed by two operators and in duplicate.

4.4.2 Spatial distribution of PFCs in Yellow-legged gull eggs

Among the 5 PFCs studied, PFOS was the only compound detected in Yellow-legged gull eggs from all colonies at mean levels from 10.1 to 54.0 ng/g ww. This is in accordance to previous studies where PFOS accounted for >90% of perfluorinated sulfonates in Herring gull eggs (*Larus argentatus*) across the Laurential Great Lakes of North America (Gebbink et al., 2011) and was the predominant perfluorinated alkyl substance in Falcon eggs (*Falco peregrinus*) (Holmström et al., 2010). As regards to perfluorocarboxylic acids, PFOA and PFNA were not detected although longer chain length PFCAs (C10-C15) should be explored as they have been reported in eggs from gull species and other wild birds at higher concentrations

compared to the PFOA and PFNA (Lofstrand et al., 2008; Gebbink et al., 2009; Holmstrom et al., 2010).

From a north to south and east to west distribution, Figure 21 shows the concentration of PFOS in gull eggs from each colony in the Iberian Peninsula, where each value is the average concentration of 12 pooled eggs in 3 subcolonies collected at each colony. The presence of PFOS in Yellow-legged gull eggs was colony dependant and differed among them (Figure 21; $F_{2,17} = 63.42$, $p < 0.001$). Differences were found between northern Iberian Peninsula colonies (Medes Islands, Ebro Delta and Columbretes), that contained the highest PFOS levels, and the southern Mediterranean colonies (Dragonera, Grosa island and Chafarinas) and the Atlantic colonies (Berlengas and Cies) (Tukey's HSD test, $p < 0.001$). Among the southern Mediterranean and Atlantic areas, no differences were found (Tukey's HSD test, $p = 0.371$).

Seawater pollution, feeding ecology and distance to land play an important role in the accumulation of PFCs (Schiavone et al., 2009). In addition to that, we found the PFOS exposure scenarios were colony-specific and responded to emission and environmental pressures.

Considering seawater pollution, the Mediterranean is an enclosed basin that has limited exchange of deep water with outer oceans and where the water circulation is dominated by salinity and temperature differences rather than winds. Such geographic and climatological configuration make the basin a sink of pollutants, derived from river discharges, wastewater discharges through treated (emmisaries) or untreated wastewaters, and run-off (Sánchez-Avila et al., 2009). As a result, pollution in this region has been extremely high in recent years. The United Nations Environment Programme has estimated that 650 million tons of sewage, 129,000 tons of mineral oil, 60,000 tons of mercury, 3,800 tons of lead and 36,000 tons of phosphates are dumped into the Mediterranean each year (www.wissenschaftsparlament.eu). As regards to PFCs, Sánchez-Ávila et al. (2010) has estimated a daily input of 190 g of PFCs to the NW Mediterranean based on 8 wastewater discharge emmisaries and 6 Catalan rivers and Loos et al. (2008) estimated a mass load contribution around 300 g PFOA per hour or 2.6 tons per year to the Adriatic Sea. Differing from those conditions, the Atlantic coast is characterized by a more severe climate which is influenced by the temperatures of the surface waters, water currents and winds. Because of the ocean's great extension and the important oceanic currents, PFC pollution in the Atlantic coast is lower than in the Mediterranean (Yamashita et al., 2005; Gómez et al., 2011). Differences among northern Mediterranean colonies (Medes, Ebre and Columbretes) with southern Mediterranean (Dragonera, Grosa Island and Chafarinas) and Atlantic (Berlengas and Cies) might be in part due to differences in waterbodies generated by sea currents. The Catalan-balearic sea in Northern Mediterranean carries northern cold waters from the Gulf of Lion southward along the continental slope in the Balearic Sea. This current bifurcates in the northern end of the Eivissa Channel. On the other hand, the SW Mediterranean is connected by a stream of Atlantic water that flows eastward from the Strait of Gibraltar to the

Sicilian channel through the Algerian current (Millot, 1999). Temporary eddies leave the Algerian current and supply the Balearic Islands with Atlantic water (López-Jurado 1990, Millot 1999), thereby expanding northward the influence of Atlantic water. Maybe this high influence of the Atlantic water in the southern Mediterranean reduces the concentration of PFOS in Dragonera, Grosa Island and Chafarinas (Figure 19). However, more studies are needed to evaluate the concentration of PFOS within the Mediterranean.

In addition to sea currents, anthropogenic pressures play an important role in the accumulation of PFCs in Yellow-legged gull eggs, as suggested by Gebbink et al. (2009). Within the Iberian Peninsula, northern Mediterranean colonies (Figure 19) which have the highest PFOS levels are settled in areas characterized by a dynamic industrial sector with textile, metalurgic and chemical factories as the main activities and also represent a highly populated area. On the other hand, Dragonera, Grosa Island, Chafarinas, Berlengas and Cies do not receive direct industrial and urban discharges, but rather agricultural run-off. Similar to this study, Giesy and Kannan (2001) detected lower PFCs levels in wildlife from remote areas (Arctic and the North Pacific Oceans) than in urban and industrial regions. In the USA, Gebbink et al., (2009) found that the accumulation of PFCs in Herring gull eggs (*Larus argentatus*) was highly lake- and/or colony-dependant, showing higher concentrations in eggs from colonies in close proximity to highly urbanized and industrialized sites in Lakes Erie and Ontario.

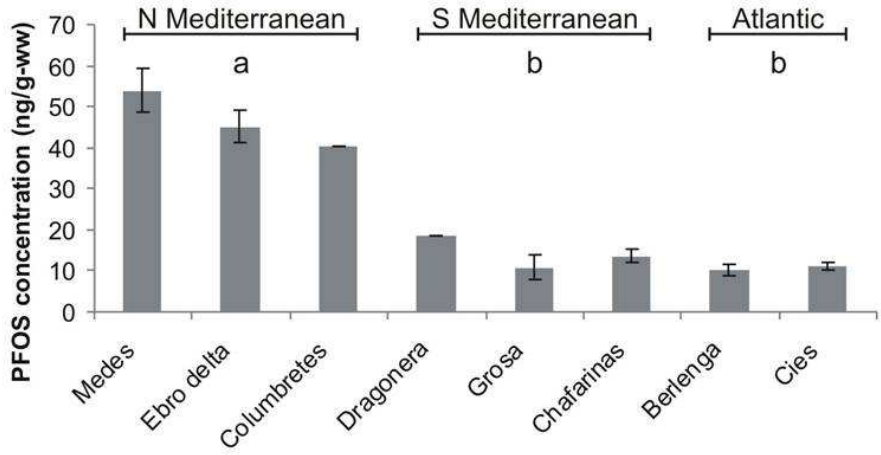

Figure 21. Mean concentration of PFOS in each colony for the three marine areas of the Iberian Peninsula. Each measurement corresponds to 3 pooled samples (n=12 eggs each) of the 3 sites of each colony, except for Dragonera and Columbretes where only 12 eggs were obtained, and Berlengas which measurement corresponds to 4 pooled samples.

Feeding habits account for the main input and accumulation of PFCs in birds. Gebbink et al. (2011) studied dietary tracers [δ15N and δ13C stable isotopes (SIs)] and revealed that PFCs exposure is colony dependant. Dietary tracers are useful to determine the feeding habits of each colony. Yellow-legged gulls from the Iberian Peninsula feed basically on both aquatic (marine and freshwater) and terrestrial prey, including fish discards and refuse tips, but when

the colonies are close or in the mainland, waste becomes and important food source. Ramos et al. (2009) studied the feeding habits of Yellow-legged gulls chicks in the western Mediterranean colonies and found that younger chicks fed on invertebrates while older chicks used refuse dumps as well as fishery discards. On the other hand, *Larus audouinii* and *Larus cachinnans* from Chafarinas feed on pelagic and benthic preys, but also on fish, and on terrestrial preys and refuse tips (González-Solís et al., 1997). SI signatures suggested that gulls from most marine colony sites are exposed to PFCs via marine prey. On the other hand, for the freshwater sites, egg SIs suggested both aquatic and terrestrial prey consumption as the source for PFC exposure depending on the colony and differences between marine and terrestrial biota were observed (Holmström et al., 2010).

Finally, distance to mainland is another factor that influences feeding ecology. Among Yellow-legged gull colonies studied, Columbretes is the most separated colony from mainland (≈54 km, Table 7) which cause a fish-based diet rather than waste-based (Duhem et al., 2005; Ramos et al., 2009). The high PFOS levels in this colony (40.5 ng/g ww) were attributed to its geographical location and to the specific food habits, since Columbretes is a Natural Park with no anthropogenic pressure.

Table 9 gives evidence of the worldwide spread distribution of PFCs by comparing PFOS concentration in eggs of several bird species, including the present results of Yellow-legged gull from the Iberian Peninsula. Levels of the present study were similar or lower than most of the results found in literature, most of them referring to the northern hemisphere. The lowest PFOS concentration was detected in two Antarctic penguin species (*Pygoscelis adeliae* and *Pygoscelis papua*) (Schiavone et al., 2009) that presented 2-3 orders of magnitude lower concentration than in other sites, including the Norwegian Arctic (Verreault et al., 2005). Overall, interspecies ecological and feeding habits play an important role in PFC accumulation in birds and therefore bird eggs become a very useful matrix to determine the impact of pollutants within an area.

4.4.3 Effects of PFOS on egg parameters

It is recognized that some anthropogenic substances released into the environment can affect avian egg production. Exposure of female birds to DDTs and PCBs, for example, is known to reduce eggshell thickness and egg size (Lundholm, 1997; Ratcliffe, 1967; Verboven et al., 2008). Other studies indicated that high levels of hexachlorobenzene, oxyclordane, DDE, and PCBs correlate with a worse condition of gull chicks (Bustnes et al., 2000, 2008). A decrease in shell thickness due to DDT exposure has been described as the major cause of population decline in raptors from Spain (Martinez-Lopez et al., 2007). Also, effects in embryonic development have been described (Cortinovis et al., 2008; Hurk et al., 2007;

Verboven et al., 2008) as well as at population and community level (González-Solís et al., 2002). Therefore, risk assessment tools have been proposed to determine the effects of the POPs in birds (Strause et al., 2007).

Table 9. Levels of PFOS in eggs of several bird species, ordered according to the sample location.

Specie	Common name	Location	Levels (ng/g ww)	Reference
Falco peregrinus	Swedish peregrine falcon	Sweden	40 – 220	Holmström et al., 2010
Phalacrocorax aristotelis *Somateria mollissima*	Common shag Common eider	Norway	28.9 14.6 – 29	Herzke et al., 2009
Larus hyperboreus	Glaucous gull	Norwegian Arctic	51.7 – 196	Verreault et al., 2005
Phalacrocorax carbo *Corvus frugilegus*	Cormorant Rook	Germany	14 – 1451 0.6 – 16.5	Rüdel et al., 2011
Uria aalge	Guillemot	North western Europe	3.2 – 760	Löfstrand et al., 2008
Nycticorax nycticorax *Ardea alba,* *Egretta garzetta*	Night heron Great egret Little egret	South China	22.6 – 343 14.4 – 27.7 31.6 – 87.2	Wang et al., 2008
Egretta garzetta	Little egret	Korea	30.4 – 1205	Yoo et al., 2008
Ardea herodias	Great blue heron	Minnesota	34 – 1848	Custer et al., 2010
Ardea herodias	Great blue heron	North America	45.9 - 9453	Custer et al., 2009
Pandion haliaetus	Osprey	North America	115 – 291	Rattner et al., 2004
Phalacrocorax auritus *Larus argentatus*	Double-crested Cormorant European Herring gull	North America	170 73	Giesy and Kannan, 2001
Larus delawarensis	Ring-billed gull	North America	67	Kannan et al., 2001
Larus argentatus	European Herring gull	North America	484 – 507	Gebbink et al., 2009
Larus glaucescens *L. californicus* *L. delawarensis* *L. argentatus*	White-headed gull California gull Ring-billed gull European Herring gull	Canada	7.15 – 599	Gebbink et al., 2011
Pygoscelis papua Pygoscelis adeliae	Gentoo Penguin Adélie Penguin	Antarctica	0.29 0.38	Schiavone et al., 2009
Larus michahellis	Yellow-legged gull	Iberian Peninsula	10.1 – 54.0	Present study

As regards to PFCs, little is known on the effects they may cause on birds. Newsted et al. (2006, 2007) reported that PFCs induce liver weight gain in the Mallard (*Anas platyrhynchos*) and the Northern Bobwhite (*Bobwhite virginianus*), a decrease in body weight

and a decrease in the length of testes. In field studies, Hoff et al. (2005) indicated that there is a significant increase in the activity of alanine aminotransferase, which is a biomarker of liver damage. Also, a decrease in serum cholesterol and triglycerides levels were correlated with increased levels of PFOS, which suggests that PFOS affect the metabolism of lipids in the exposed organisms. In addition, negative association between concentrations of PFOS in eggs and hatching success was reported at PFOS concentrations as low as 150 ng/g ww (Custer, 2012). Finally, impaired hatching success and sublethal toxicological effects from PFOS exposure in the Swedish Peregrine falcon (*Falco peregrinus*) were not ruled out (Holmström et al., 2010). Newsted et al. (2005) described Toxicity Reference Values (TRVs) and Predicted No Effect Concentrations (PNECs) for PFOS based on two avian species (*Anas platyrhynchos* and *Colinus virginianus*). For PFOS, TRV for eggs was of 1.7 μg /mL and PNEC was of 1.0 μg/mL. However, an uncertainty value of 6 was used in order to protect other avian species because the analisys was carried out on only two bird species (Newsted et al., 2005).

In this study, differences among egg parameters (Table 10) were evaluated to determine the potential effects of PFOS during egg development. In a first instance, differences among colonies were significant for all egg measurements (length: $F_{7,233} = 6.28$, $p < 0.001$; width: $F_{7,233} = 13.21$, $p < 0.001$; weight: $F_{7,233} = 2.67$, $p = 0.011$; volume: $F_{7,233} = 15.66$, $p < 0.001$; thickness: $F_{6,186} = 14.61$, $p < 0.001$; eggshell weight: $F_{6,186} = 7.89$, $p < 0.001$), as well for desiccation index ($F_{6,186} = 11.83$, $p < 0.001$), but not for eggshell thickness index ($F_{6,186} = 1.94$, $p = 0.077$). Thickness, dried thickness weight and the two indexes were not available for the eggs from Berlengas islands because these parameters were not measured when sampling. Finally, there was no significant correlation between the eggshell thickness index or the desiccation index and the PFOS concentration (Spearman rank correlation, both $p > 0.05$), indicating that the levels of PFOS in Yellow-legged gull do not affect shell parameters and thus, have not an impact at this stage.

4.5 Conclusions

Gull eggs have been proposed as a biomonitoring matrix to determine the presence of contaminants (Stockholm Convention, United Nations Environmental Programme (UNEP) and the Oslo Paris Convention (OSPAR)). Our target species, the Yellow-legged gull, is omnivorous and also an opportunistic species, sedentary within each colony and very common along the Iberian Peninsula coast. Its characteristic biology makes this species interesting as pollution indicator. PFOS was the only compound detected in whole egg samples and its concentration was higher in the most industrialized sites of the Catalan coast (Medes, Ebro and Columbretes) compared to the southern Mediterranean (Dragonera, Mar Menor and Chafarinas) or Atlantic

Table 10. Egg characterization from each colony.

Colony	Weight (g)		Length (mm)		Width (mm)		Thickness (mm)		Dried eggshell weight (g)		Eggshell thickness index I = Wt(mg)/L (mm) *Wd(mm)		Volume (mL) V = 0.000477 * L (mm) * Wd² (mm)		Desiccation index Di = (Wt egg (g) - Wtshell (g))/V (mL)	
	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM
Medes Is.	91.2	0.9	71.2	0.4	49.7	0.3	0.368	0.005	6.2	0.1	1.74	0.02	84.0	1.2	1.015	0.0071
Ebro Delta	89.7	1.3	70.7	0.5	48.8	0.3	0.385	0.005	6.2	0.1	1.78	0.02	80.4	1.2	1.039	0.002
Columbretes	85.5	3.1	69.9	0.9	48.1	0.6	0.377	0.006	6.1	0.3	1.81	0.07	77.4	2.9	1.026	0.012
Dragonera	85.6	2.5	69.0	0.9	47.2	0.6	0.454	0.014	5.5	0.2	1.67	0.04	73.7	2.2	1.092	0.021
Grosa	89.2	1.3	70.3	0.6	48.8	0.3	0.370	0.005	6.1	0.1	1.77	0.03	80.1	1.3	1.038	0.004
Chafarinas	82.7	1.3	69.2	0.6	48.7	0.2	0.395	0.008	6.0	0.11	1.78	0.02	78.3	1.2	0.982	0.010
Berlengas	82.8	0.8	68.4	0.3	47.7	0.2	NA	NA	NA	NA	NA	NA	74.2	0.7	NA	NA
Cies	77.6	1.1	67.7	0.4	46.3	0.3	0.368	0.005	5.5	0.1	1.74	0.02	69.4	1.0	1.039	0.006

n.a. = not analysed

colonies (Berlengas and Cies). Nonetheless, the concentrations detected did not induce any negative effect upon egg shell parameters. Except for eggshell thickness index, significant differences were found for egg weight, length, width, eggshell weight, volume and Dessication index between colonies. However, negative effects at other stages of development or in the long term cannot be ruled out given the high accumulation potential of PFC in Yellow-legged gull eggs.

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Chapter 5. Accumulation and maternal transfer of perfluoroaklyl sulfonates and carboxylates in yello-legged (*Larus michahellis*) and Audouin's gull (*Larus audouinii*) from the Ebro Delta Natural Park

5.1 Abstract

This study was aimed to characterize the presence of 17 perfluoroalkyl and polyfluoroalkyl substances (PFASs) in eggs of yellow-legged gull (YLG) and the protected species Audouin's gull (AG), which cohabit in the Ebro Delta Natural Park (Catalonia, Spain). Perfluorooctane sulfonic acid (PFOS) accounted for 56% in YLG eggs and 54% in AG eggs of the ΣPFAS and the mean concentration in eggs were of 110.2 ± 0.67 and 99.3 ± 12.70 ng/g wet weight (ww) in YLG and AG, respectively. Other compounds detected were perfluoroalkylcarboxylic acids and perfluoroalkylsulfonates from 6 to 14 fluorine chain. The Estimated Daily Intake through diet (fish and crayfish), depuration rates and transfer capacity from blood to eggs was studied for PFOS, the main compound detected. EDI was of 118.9 ± 31.7 ng/d in AG females, 127.8 ± 36.2 ng/d in AG males, 158.5 ± 42.3 in YLG females and 170.4 ± 48.3 ng/d in YLG males. PFOS levels in blood werehigher in males than females(61.4 ± 25.6 and 60.6 ± 25.2 ng/g ww in YLG and AG males, and 27.3 ± 14.2 and 25.2 ± 12.6 ng/g ww in YLG and AG females), with little differences among species. The lower levels in females are attributed to the annual release of PFOS to eggs during the laying period. AG females released 5544 ± 1568 ng/egg and YLG released 6715 ± 2676 ng/egg. According to these concentrations, the maternal transfer of PFOS from female's blood to eggs was estimated.

5.2 Introduction

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) have become persistent organic pollutants (POPs) widely spread in the environment due to its use over the last 60 years in the industry (Giesy and Kannan, 2002). Seabirds, as top predator animals, are very sensitive to chemical pollution because of their feeding habits and their relatively long life (Furness and Camphuysen, 1997), which lead to the bioaccumulation of PFASs (Kannan et al., 2005) and transfer directly to their offspring via eggs (Pusch et al., 2005). The occurrence of PFASs in bird tissues and eggs has been reported worldwide. Gebbink et al. (2011) found levels between 7.8 and 486 ng ΣPFAS/ g wet weight (ww) in eggs of differents *Larids* from 15 colonies across Canada; Haukås et al. (2007) detected 8.49 – 225 ng PFOS/g ww in livers of *Larus hyperboreus*

from the Barents sea; Herzke et al. (2009) detected a median of 29 ng PFOS/g ww in eggs of *Phalacrocorax aristotelis* and from 14.6 to 32 ng PFOS/g ww in eggs of *Somateria mollissima* in Norway; Rüdel et al. (2011) found levels between 30 and 150 ng PFOS/g in eggs of *Larus argentatus* from North and Baltic Sea; Wang et al. (2008) detected 14.4 – 343 ng PFOS/g in egg of 3 waterbird species from South China. Because PFOS is the main compound detected in biota, it is bioaccumulative, toxic and widespread, the Stockholm convention has taken initiatives to control its environmental presence and there is a high interest in estimating the sources of pollution ants and the impact on wildlife.

Our target species, yellow-legged gull (YLG, *Larus michahellis*) and Audouin's gull (AG, *Larus audouinii*) cohabit in the Ebro Delta Natural Park, in Catalonia. The former is a resident species in the Ebro Delta and the latter is mainly a breeding species with a reduced wintering population. These two gull species are fish and seafood feeders (Moreno et al., 2010, Sanpera et al., 2007, Navarro et al., 2010). AG is an endemic species from the Mediterranean region included on the IUCN 2012 Red List as Near Threatened (BirdLife International, 2012) and classified as Vulnerable by the Spanish Ministry of Environment. Although Audouin's gull is considered an adapted specialist in the capture of clupeiforms (such as sardines and anchovies), it can also exploit fishery discards and eventually crayfish from the riverine environment (Navarro et al., 2010, Oro and Ruiz, 1997). AG is threatened by different factors, among others, the reduction of small-pelagic fish stocks by the trawling fisheries, the chemical contamination and the competition and predation from YLG. On the other hand, YLG is the Western Mediterranean relative of the herring gull (*Larus argentatus*), a common gull whose population has increased dramatically during the lasts decades due to its intrusion in human activities where high amounts of food are available (Bosch et al., 1994). YLG is an opportunistic feeder that also exploits fishing discards, refuses from landfills and nestlings of other seabirds (Duhem et al., 2005).

The Ebro delta is an area of high ecological interest. Early in 1962, the UNESCO declared this area an Euro-African wetland of international interest and latter in 2013 a Biosphere Reserve. Furthermore, the Ebro Delta was proclaimed Natural Park in 1983. However, the area is highly affected by human activities as in a radius up to 70 km there are two nuclear stations (Ascó and Vandellòs) and a chloro-alkali industry. An intensive agricultural activity of rice cultivation is also developed in the zone. These activities are the main responsible of the pollution impact in the area (Barata et al., 2010, Lacorte et al., 2006). In a previous study, 7 chemical families of POPs were detected in high concentrations in gull eggs inhabiting in the Ebro Delta (Morales et al., 2012), suggesting that the activities carried out in the area affect residential wildlife. In addition, Vicente et al. (2012) found that the colony of the Ebro Delta was one of the most impacted by PFOS among 7 main colonies of the Iberian Peninsula, with levels between 40 and 50 ng PFOS/g ww in eggs of *Larus michahellis.*

Because gulls inhabiting in the Ebro Delta Natural Park are highly affected by chemical pollution, the aim of the present study was: (i) to characterize the presence of 17 PFAS in homogenized pooled eggs of *L. michahellis* and the protected species *L. audouinii*; (ii) to estimate the net annual accumulation of PFOS, the main compound detected, by determining the Estimated Year Intake (EYI); and (iii) to determine the maternal transfer from females' blood to the eggs. Overall, this study pretends to quantify the mass balance of PFOS, one of the most bioaccumulative and toxic PFAS in gulls.

5.3 Materials and methods

5.3.1 Chemicals and reagents

A mixture of native perfluoroalkylcarboxylic acids (PFCAs) (perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA) and perfluorooctadecanoic acid (PFODA)) and native perfluoroalkylsulfonates (PFSAs) (perfluorobutane sulfonic acid (PFBS), perfluorohexane sulfonic acid (PFHxS), perfluorooctane sulfonic acid (PFOS) and perfluorodecane sulfonic acid (PFDS)) was supplied by Wellington Laboratories (Ontario, Canada). Stock standard solutions were prepared in acetonitrile at a concentration of 5 ng/µl for all native compounds and were stored at -18ºC. Perfluoro-n-(1,2,3,4-$^{13}C_4$) octanoic acid (m-PFOA) and sodium perfluoro-1-(1,2,3,4-$^{13}C_4$) octane sulfonic acid (m-PFOS), also from Wellington Laboratories, were used as surrogate standards. HPLC grade water and acetonitrile were supplied by Merck (Darmstadt, Germany) and glacial acetic acid from Panreac (Barcelona, Spain).

5.3.2 Sampling

Yellow-legged gull and Audouin's gull fresh eggs and blood were collected from the Ebro Delta in March-May 2009. Each colony was sampled in 3 subcolonies to have a significant sampling coverage (fig. 22). A non invasive procedure was used by sampling the first egg of each nest as it represents the maximum pollutants transfer level from female to eggs and permits comparisson among subcolonies (Vicente et al., 2012). Twelve random nests were sampled per subcolony, except for YLG subcolonies 1 and 2, where only 11 nests were sampled. Each nest was tagged and incubating gulls (parents) of the same nests were captured using drop traps and tent spring traps. The traps were placed in the morning to capture one of the parents and again at

dawn, to capture the other. This was done since at the laying period one of the parent birds hatches the nest while the other is feeding. Blood was obtained by puncturing the brachial vein using a heparinized syringe and was placed in an eppendorf with 0.05 mL of heparine, transported refrigerated to the laboratory and kept at -20ºC until analysis. A subsample of blood was stored in 99% ethanol to sex the gulls by DNA-analysis. According to their plumage, all individuals were older than 4 years old and, therefore, in reproductive age. From most nests, the blood from the male and the female was collected, but in others only one of the two partners was captured. Adults of both species and eggs were weighted (table 11 and 14). Besides, 5 sardines (22.7 ± 11.8 g of body weight (BW)), 5 anchovies (11.9 ± 5.9 g of BW) and 8 crayfishes (9.02 ± 3.9 g of BW) (table 11) were sampled from the area to determine the PFASs content in the main gull's food. Fish and crayfish were transported refrigerated and in the same day, whole fish were homogenized whereas the shell of the crayfish was emptied and only the flesh, including the head, was analyzed. After collection, eggs were kept at ambient temperature and rapidly transported to the laboratory. Immediately after, whole eggs content was homogenized with a mixer. Individual eggs were halved, homogenized and one portion was used for individual analysis and the other was used to make a pooled sample from each subcolony (n = 3 samples per colony). Pooled samples were used to characterize the 17 PFASs and individual eggs were used to study the accumulation potential and maternal transfer of PFOS. Samples were frozen at -18ºC until analysis.

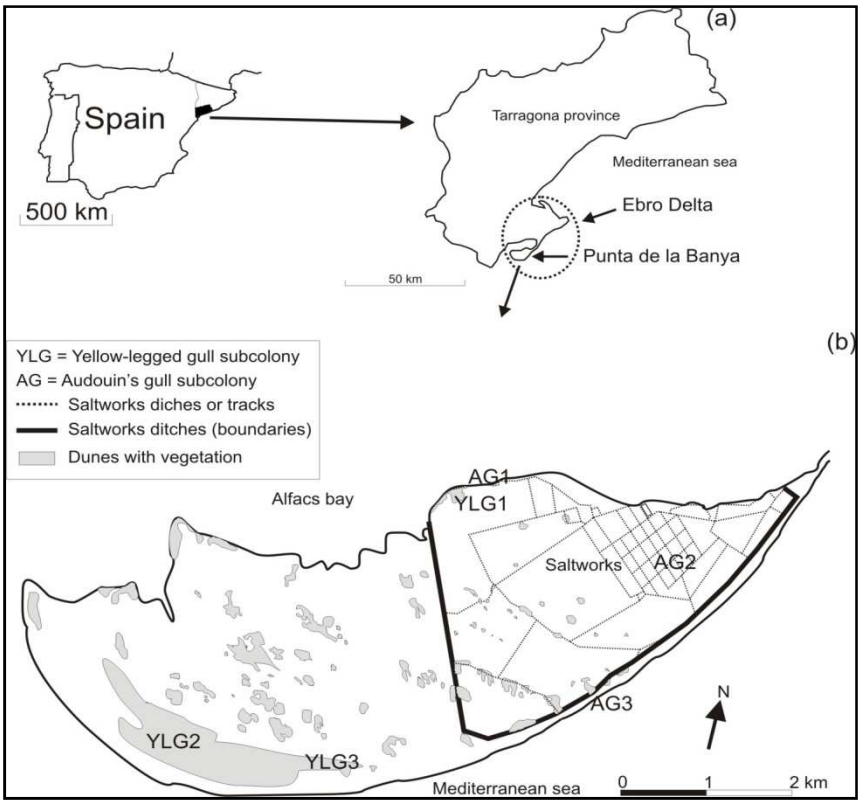

Figure 22. Map showing the Audouin's and yellow-legged gull colonies that were sampled in March-April 2009 in Punta de la Banya, Ebro Delta Natural Park, Catalonia (NE Spain).

5.3.3 Extraction and analysis of PFASs

PFASs were solid-liquid extracted from homogenized wet samples using acetonitrile, adapted from the method of Fernández-Sanjuan et al. (2010) and Vicente et al. (2012). Briefly, about 1 g of eggs was weighted in polypropylene tubes and internal standards (m-PFOS and m-PFOA) were added at a concentration of 100 ng/g, and incubated for 18 hours at 4ºC. For the blood samples, 300 µL were analyzed. In all cases, 9 mL of acetonitrile were added and the samples were thoroughly mixed using a vortex mixer. Samples were extracted in an ultrasonic bath for 10 min at room temperature. This procedure (vortexing and ultrasonic extraction) was repeated 3 times without changing the solvent. Afterwards, the samples were centrifuged at 2.500 rpm for 5 min. The supernatant was transferred to a new vial and evaporated to dryness. Then, 1 mL of acetonitrile was added to the dried sample and incubated for 10 min in the ultrasonic bath. The samples were purified by adding 25 mg of activated carbon and 50 µL of glacial acetic acid and were vigorously mixed for 1 minute. Afterwards, the samples were centrifuged for 10 min at 10.000 rpm. The supernatant was transferred to a clean micro vial, and 250 µL of this were diluted with 250 µL of water with 10 mM ammonium acetate buffer of mobile phase. Sardines, anchovies and crayfishes were also extracted using the same protocol as that one used for the eggs.

PFASs were measured using an Acquity Ultra Performance Liquid Chromatography system connected to a Triple Quadruple Mass Spectrometry Detector (Waters, USA). An XBridge C_{18} column (3.5 µm particle size, 50 mm x 4.6 mm, Waters, USA) was used as mobile phase residue trap to remove any contamination from the mobile phases. The analysis was performed on an Acquity UPLC BEH C_{18} column (1.7 µm particle size, 100 mm x 2.1 mm, Waters, USA). Five µL of extract were injected. The mobile phase consisted of (A) 10 mM ammonium acetate/methanol:(B) acetonitrile (80 : 20) with 10 mM ammonium acetate. Gradient elution started from 50% A and 50% B, held for 3 min and increased to 100% B in 7 min and held for 2 min, at a flow rate of 0.3 mL/min. Then, initial conditions were regained in 1 min and the system was stabilized for 2 min at initial conditions. The various PFASs were measured under negative electrospray ionisation using 2 transitions from parent to product ion to identify each compound except for PFBA and PFPA where only one transition was used. Internal standard quantification was performed using m-PFOS to quantify PFBS, PFHxS, PFOS and PFDS and m-PFOA to quantify PFBA, PFPA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTriDA, PFTeDA, PFHxDA and PFODA. The recoveries of target compounds in chicken eggs spiked at 100 ng/g ww were between 67 ± 3 and 96 ± 5 %, except for PFODA which was recovered in 38 ± 5%. Limits of Detection (LOD) calculated using a signal to noise ratio of 3 were between 0.07 and 0.4 ng/g ww, except for PFODA which had a LOD of 1.34 ng/g ww.

Table 11. PFOS concentration (range and mean ± standard deviation (SD)) in blood and eggs of Audouin (AG) and yellow-legged gulls (YLG) and in gulls' diet (fish: anchovies and sardines, and crayfish) from the Ebro Delta Natural Park and sample size (N).

SAMPLE	BW mean ± SD (g)	PFOS range (ng/g ww)	PFOS mean± SD (ng/g ww)	N
AG female	526 ± 39.3	11.2 - 50.8	25.2 ± 12.6	12
AG male	617 ± 38.2	25.9 - 101.4	60.6 ± 20.8	15
YLG female	882 ± 43.2	8.42 - 58.8	27.3 ± 14.2	17
YLG male	1104 ± 58.6	26.1 - 134	61.4 ± 25.6	18
AG eggs	67.2 ± 3.7	50.8 - 149	82.5 ± 22.9	36
YLG eggs	89.9 ± 7	31.8 - 140.3	74.7 ± 29.2	34
anchovies	22.7 ± 11.8	0.54 - 1.14	0.83 ± 0.25	5
sardines	11.9 ± 5.9	0.73 - 1.49	1.09 ± 0.31	5
Total fish	17.3 ± 8.8	0.54 - 1.49	0.96 ± 0.30	10
Crayfish	9.02 ± 3.9	0.23 - 0.63	0.42 ± 0.14	8

5.3.4 Data Analysis

Descriptive statistical analysis was performed for blood and eggs of the 2 species. Blood concentrations were converted to ng/g units approaching gulls' blood density to humans' blood density (1.06 g/mL) (Cutnell and Johnson, 1998), in order to compare blood levels with egg levels. Differences between PFOS concentrations in eggs from both gull species, in blood of different sexes within each species and in blood between species were evaluated by analysis of variance (ANOVA one way). All data were tested for normality and homoscedasticity and no transformation was necessary because they meet ANOVA assumptions. Data were analyzed with the statistical software package MATLAB for Windows (version 7.0). Mean ± SD are presented.

5.3.5 Description of the calculations used

To determine the accumulation and mass transfer of PFOS (the main compound detected) from gulls to eggs, the following equations were applied:

$$MB\ (ng/year) = EYI - yDEP - M_{egg}\ (\text{only for females}) \qquad \text{Eq. 1.}$$

Where,

- MB is the PFOS Mass Balance in gulls (ng/y);
- EYI is the Estimated Year Intake of PFOS (ng/y);
- yDEP is the depuration rate from blood (ng/y);

- M_{egg} is mass of PFOS in the eggs (as gulls lay once per year and we consider only one egg per laying) (ng/y);

EYI was calculated as the annual Estimated Daily Intake (EDI) based on Average Daily Intake from Newsted et al. (2007) (table 13). YLG is a sedentary species and spend the whole year in the Ebro Delta colony. AG is a migratory bird although the increasing exploitation of human resources is becoming more sedentary (Martínez-Abraín, (phD) 2003). Considering that (i) both gulls spend the whole year in Ebro Delta and that fish and crayfish is the main feed in gulls from the Ebro Delta; (ii) that the daily water consumption is negligible and (ii) that landfills feeding contribution is impossible to quantify, but may contribute, at least for YLG, to PFOS accumulation (Becker et al., 2001), the following equation was used and then it was calculated per year to find EYI (ng/y):

$$EDI\ (ng/d) = P_{fd}C_fK_d + P_{cd}C_cK_d \qquad \text{Eq. 2}$$

where**,**

- P_{fd} (*Percentage of fish based diet*) corresponds to the fish (sum of anchovies and sardines) percentage of the total diet (69% for females and 80% for males) (Navarro et al., 2010);
- C_f (*Concentration in fish*) is PFOS mean concentration in sardines and anchovies (ng/g);
- P_{cd} (*Percentage of crayfish based diet*) corresponds to crayfishes percentage of the total diet (31% for females and 20% for males) (Navarro et al., 2010);
- C_c (*Concentration in crayfish*) is the PFOS mean concentration in crayfish (ng/g).
- K_d (*Daily Constant*) is the daily food intake. As daily energy expenditure varies across activities and seasons (Schreiber and Burger (Eds.), 2002) and the breeding season is a time of high energy demands, then K_d is maximum at this time (Ridgway, 2010). We used 150 g food/day for AG and 200 g food/day for YLG (communication of Biological Station from Ebro Delta Natural Park).

DEP represents the amount of PFOS eliminated from the gulls' blood to other organs (Yoo et al., 2009). DEP was calculated with the following equation and then it was calculated per year (yDEP (ng/y)) (table 12):

$$DEP\ (ng/d) = K_{dep} * C_g * B_g, \qquad \text{Eq. 3}$$

where,

- K_{dep} is the Depuration Constant (d^{-1});
- C_g (*Concentration in gulls*) corresponds to PFOS mean blood concentration in males (C_m) or females (C_{fm}) (ng/g);

- B_g *(Blood of gulls*) is the mean weight of blood in males (B_m) or females (B_{fm}) (assuming that blood weight represents the 10% of the total gull's body weight (BW), as in domestic fowl was assed as 9-10 % (Heald and Badman, 1963)). Specifically, males were generally heavier than females, and YLG individuals were heavier than AG. The mean BW of AG was 617 ± 38 g in males and 526 ± 39 g in females and for YLG, 1104 ± 58 g in males and 882 ± 43 g in females (table 11).

Furthermore, the transfer capacity (TRA) was studied considering only those nests where blood from the 2 parents and the first egg were collected (10 eggs for AG nests and 6 for YLG) (table 15). We used blood concentration of PFOS to analyse TRA because: (i) liver is the main corporal tissue responsible of the egg formation by synthesizing the yolk-precursors (Gebbink and Letcher, 2012), which are then secreted in the blood (Vézina et al., 2003). Changes in blood composition are reflected in egg formation, and (ii) PFOS concentrations in liver and blood are highly correlated (Dauwe et al., 2007). On the other hand, we assume that females PFOS levels before laying were the same than those for the male of the same nest as we sampled the blood after the laying period and it is impossible to know their levels before laying. The following equation was used:

$$TRA\ (\%) = (M_{egg}/(C_m * B_{fm})) * 100 \qquad \text{Eq. 4}$$

where,

- M_{egg} (*Mass in eggs*) is the absolute mass of PFOS in the corresponding egg (ng);

- C_m (*Concentration in males*) is the PFOS concentration in the blood of the males corresponding to each egg (ng/g);

- B_{fm} (*Blood female*) is the specific female blood weight (10% of the females BW) corresponding to each egg (g);

5.4 Results and Discussion

5.4.1 PFAS concentrations in pooled eggs

Figure 23 shows the mean levels of PFASs detected in YLG and AG pooled eggs. PFOS levels in eggs (C_{egg}) and the corresponding egg weights (EW) are indicated in table 14. Compounds not detected were PFBA, PFPA, PFHxA, PFHpA, PFHxDA and PFBS, which are mainly short chain perfluorinated carboxylic acids or sulfonates which are less bioaccumulative (Renner, 2006). PFOS was the main compound detected at mean concentrations in eggs of 99.3 ± 12.7 and 110.2 ± 0.67 ng/g wet weight (ww) in AG and YLG, respectively (table 11), as

observed in earlier studies (Vicente et al.,2012). PFOS represented the 56 % of ΣPFAS in YLG and the 54% in AG eggs. Following, PFTriDA was detected at 25.9 ± 4.06 ng/g ww in AG and at 28.5 ± 1.6 ng/g ww in YLG and PFUnA at 25.7 ± 3.08 ng/g ww in AG and at 26.5 ± 1.5 ng/g ww in YLG. Other compounds were detected between 1.35 ± 0.03 and 8.73 ± 1.66 ng/g ww in AG and between 1.44 ± 0.06 and 6.69 ± 0.01 ng/g ww in YLG, being PFDS the compound detected at the lowest concentration in both species. ΣPFAS in AG was of 183.9 ng/g ww and in YLG of 194.8 ng/g ww, suggesting an overall widespread distribution of these compounds in gull species cohabiting in the Ebro Delta Natural Park. For both species, there was very little variability among the concentrations obtained from the 3 subcolonies, indicating that PFASs accumulation is very similar in all individuals and that they are equally affected by the presence of PFASs. There were no significant differences in the mean levels of all PFASs detected in both species ($F_{1,18}$ = 0.005, P = 0.94).

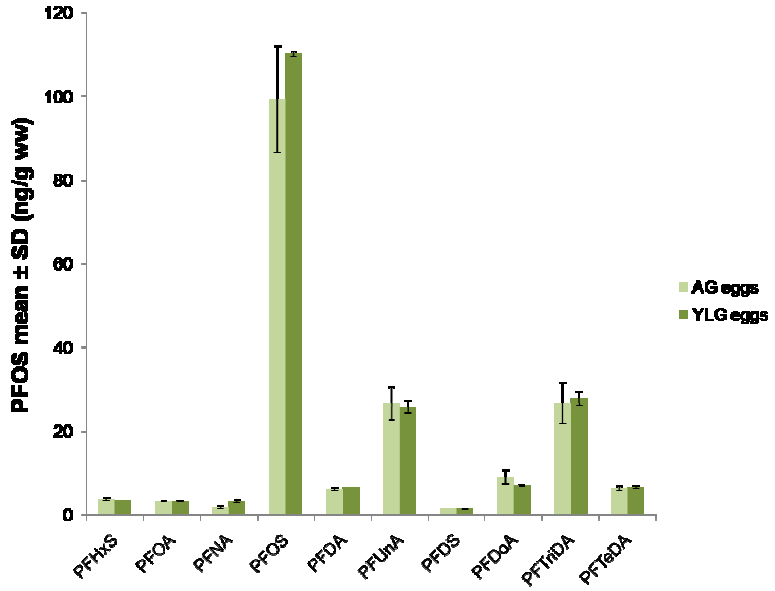

Figure 23. PFASs concentration (ng/g ww) in Audouin's (AG) and yellow-legged (YLG) pooled eggs from the Ebro Delta Natural Park.

Gebbink and Letcher (2012) studied PFASs distribution among different tissues (liver, brain, muscle, and adipose), plasma/red blood cells and whole egg clutches (yolk and albumen) of female herring gulls (*Larus argentatus*) from Great Lakes of North America. They analyzed 4 PFSAs (PFBS, PFHxS, PFOS and PFDS), 9 PFCAs (PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTriA and PFTeA) and several precursors (FTUCA, FTOH, PFOSA and NMe-FOSA). In eggs, ∑PFSAs was almost three times the amount of ∑PFCAs, which were only detected in yolk. Among PFSAs, PFOS was the most ubiquitous compound followed by PFDS and PFHxS. PFBS, the shorter chain of PFSAs, was not detected. Considering a 35% of yolk in eggs (Ricklefs, 1977) of precocial birds (as gulls are semi-precocial birds (Tjørve et al., 2009)), Gebbink's results were converted to ng /g of egg in order to compare with the results

found in the present study. Gebbink and Letcher (2012) detected 90.3 ± 13.6 ng/g egg of ∑PFSAs, which is similar to the results herein presented in eggs of YLG and AG from the Ebro Delta Gebbink and Letcher (2012) also showed that PFOS concentration in plasma and red blood cells were the highest among all PFASs analyzed. In other studies of PFASs in Norwegian glaucous gull and Swedish guillemot, PFOS, PFUnA and PFTriA were the main compounds detected in various tissues (Holmström and Berger, 2008; Verreault et al., 2005) which is in accordance with the present study.

Because PFOS was the compound detected at the highest concentration in eggs from the 3 subcolonies of both species, this compound was selected to study the accumulation potential and maternal transfer from blood of AG and YLG to eggs.

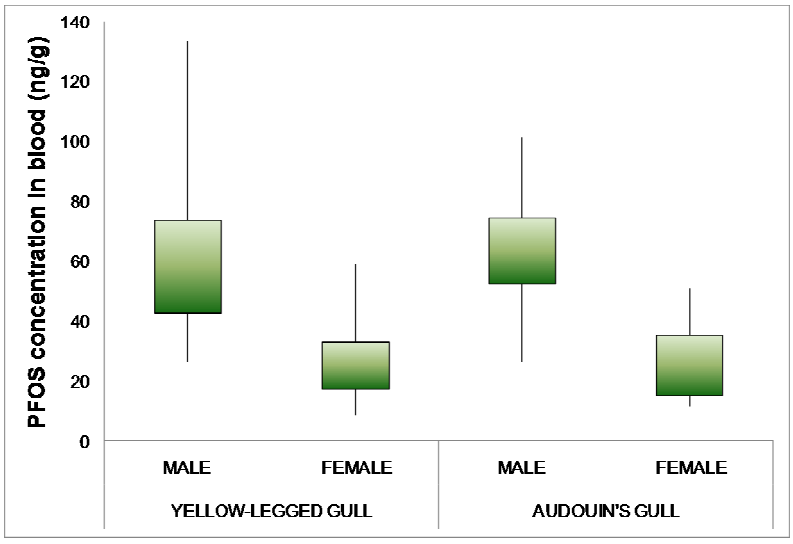

Figure 24. PFOS concentration in blood for both species and sexes and represented by a box plot (quartile 75%) and minimum and maximum levels found.

5.4.2 Estimated Daily Intake

PFOS was analyzed in main gull diet to assess the EDI. PFOS mean levels in sardines were 1.09 ± 0.31 ng/g ww (n = 5), 0.83 ± 0.25 ng/g ww in anchovies (n = 5), and considering all fish, the mean concentration was 0.96 ± 0.30 ng/g ww (n = 10) (table 11). PFOS mean concentration in crayfish was of 0.42 ± 0.14 ng/g ww (n=8) (table 11).

Considering AG females, the EDI was 118.9 ± 31.7 ng/day (d):

$EDI_{AGf} = P_{fd}K_dC_f + P_{cd}K_dC_c$ = (69/100)*150 g*(0.96 ± 0.3 ng/g) + (31/100)*150 g*(0.42 ± 0.14 ng/g) = (99.4 ± 31.05) + (19.5 ± 6.51) = 118.9 ± sqrt $((31.05)^2 + (6.51)^2)$ = 118.9 ± 31.7 ng/d.

Doing the same calculations for AG males, EDI was of 127.8 ± 36.2 ng/d. For YLG females, EDI was of 158.5 ± 42.3 ng /d and for YLG males, EDI was of 170.4 ± 48.3 ng/d, assuming they eat the same food as AG but a higher amount per day (200 g instead of 150 g).

For the following calculation of mass balance (MB), the Estimated Year Intake (EYI) was calculated by multipliying EDI x 365 days (table 13).

5.4.3 PFOS concentrations in blood

PFOS levels in blood (C_m for males and C_{fm} for females)and body weights of gulls (BW_m and BW_{fm} for males and females, respesctively) are indicated in table 14. In AG blood, PFOS ranged between 25.9 ng/g and 101.4 ng/g (mean 60.6 ± 20.8 ng/g) in males and between 11.2 ng/g and 50.8 ng/g (mean 25.2 ± 12.6 ng/g) in females (table 11). In YLG blood, PFOS ranged between 26.1 ng/g and 134 ng/g (mean 61.4 ± 25.6 ng/g) in males and between 8.42 ng/g and 58.8 ng/g (mean 27.3 ± 14.2 ng/g) in females (table 11). The variability on the PFOS concentration in blood is attributed to food and age differences among individuals. In both species there were no significant differences between PFOS concentrations in blood among subcolonies for each sex (AG males: $F_{2,12} = 0.06$, $P = 0.94$; AG females: $F_{1,10} = 0.40$, $P = 0.54$; YLG males: $F_{2,15} = 1.01$, $P = 0.38$; YLG females: $F_{2,14} = 0.18$, $P = 0.83$). PFOS levels in males were significantly greater than in females, around two times higher ($F_{1,56} = 45.847$, $P < 0.001$; fig. 24), but no differences were found between species ($F_{1,56} = 0.065$, $P = 0.799$) or the interaction of both factors (sex*species $F_{1,56} = 0.025$, $P = 0.874$). Although there may be other depuration mechanisms, the differences in PFOS content in males and females in both species suggest that female birds mainly reduce their PFOS burden by transferring them into eggs, as previously suggested (Duhem et al., 2005, Verboven et al., 2009).

Table 12. Depuration rate per day (DEP) and per year (yDEP) in Audouin's gull (AG) and yellow-legged gull (YLG) males and females, using the 2 values of PFOS half-life, 6.86 d and 125 d (Yoo et al., 2009, Newsted et al., 2006). Calculations are done using the concentration of PFOS found in blood of males for males DEP and also for females DEP.

SAMPLE	$DEP_{6,86}$ (ng/d)	DEP_{125} (ng/d)	$yDEP_{6,86}$ (ng/y)	$yDEP_{125}$ (ng/y)
AG female	322.1 ± 8,27	17.54 ± 0.45	117569.4 ± 3020.7	6402.3 ± 164.5
AG male	377.8 ± 8.04	20.57 ± 0.44	137909.3 ± 2936.2	7509.9 ± 159.9
YLG female	546.7 ± 11.18	29.77 ± 0.61	199537.9 ± 4080.2	10865.16 ± 222.2
YLG male	684.3 ± 15.16	37.26 ± 0.82	249761.7 ± 5534.7	13600.9 ± 301.4

5.4.4 Depuration rates

PFOS depuration varies with species and depends on the age of the individual. Juvenile birds show a faster PFOS depuration than adults (Yoo et al., 2009). Depuration rate is also dose-dependent. Individuals exposed to higher amounts of PFOS experiment faster depuration rates

(Newsted et al., 2006, Yoo et al., 2009). PFASs in blood may enter to the enterohepatic recirculation. Enterohepatic recirculation is referred to the circulation of biliary acids and other substances from the liver to the bile, followed by entry into the small intestine, absorption by the enterocyte and transported back to the liver. Once in the liver, they can be recirculated or excreted by the urine (Yoo et al., 2009). As the depuration constants have not been defined for gulls, the PFOS depuration constants were estimated according to the PFOS half-life ($t_{1/2}$) of 6.86 days (d) for juvenile mallards (*Anas platyrhynchos*) (Newted et al., 2006) and 125 d calculated for juvenile chickens (*Gallus gallus domesticus*) (Yoo et al., 2009). Considering a first order kinetics ($t_{1/2}$= ln2/K), the depuration constants K_{dep125} and $K_{dep6.86}$ corresponded to 0.0055 d^{-1} and 0.101 d^{-1}, respectively.

We calculated the depuration rates for both sexes using the PFOS concentration found in blood of males from both gull species (table 12). While PFOS values may not vary in males due to the egg laying, the best DEP approach for females would be with PFOS concentration in its blood before the laying. As it is impossible to have these values, we used males' PFOS values to calculate females DEP trying to approaching reality. Table 12 show the daily depuration rates (DEP) and the annual depuration rates (yDEP) considering a half-life of 125 d and half-life of 6.86 d and PFOS levels in blood of males and/or females for both species.

Table 13. PFOS Estimated Year Intake (EYI), depuration rate per year (yDEP) (using the 2 values of PFOS half-life found in the literature, 6.86 d and 125 d (Yoo et al., 2009, Newsted et al., 2006), mass of PFOS in eggs (M_{egg}) and PFOS Mass Balance (MB) calculated using $yDEP_{125}$ (as $y\ DEP_{6.86}$ is higher than EYI) in Audouin's gull (AG) and yellow-legged gull (YLG) males and females. Calculations are done using the concentration of PFOS found in blood of males for males DEP and also for females DEP.

SAMPLE	EYI (ng/y)	$yDEP_{6.86}$ (ng/y)	$yDEP_{125}$ (ng/y)	M_{egg} (ng/y)	MB (ng/y)
AG female	43394.85 ± 11579.7	117569.4 ± 3020.7	6402.3 ± 164.5	5544 ± 1568.8	31448.6 ± 11686.6
AG male	46647 ± 13229.1	137909.3 ± 2936.2	7509.9 ± 159.9	0	39137.1 ± 13230.1
YLG female	57859.8 ± 15439.5	199537.9 ± 4080.2	10865.16 ± 222.2	6715.5 ± 2676.6	40278.3 ± 15671.4
YLG male	62196 ± 17638.8	249761.7 ± 5534.7	13600.9 ± 301.4	0	48595.1 ± 17641.4

Using the PFOS half-life in blood of 6.86 d, the depuration rate (DEP) is higher than that one found using the half-life of 125 d. As it is well established that PFOS is accumulated in birds, and according to our results, it is apparent that the value of Kdep corresponding to the half-life ($t_{1/2}$) of 125 d calculated for juvenile chickens (*Gallus gallus domesticus*) explain much better the accumulation of PFOS in gulls than the half-life value of 6.86 d for juvenile mallards (*Anas platyrhynchos*) (Newted et al., 2006) because PFOS would be completely eliminated from the blood when using the half-life of 6.86 (table 13). DEP_{125} in AG and YLG males were 20.57 ± 0.44 ng/d and 37.26 ± 0.82 ng/d respectively. In AG females, DEP_{125} was of 17.54 ± 0.45 ng/d (using PFOS concentration in males' blood) and in YLG females was 29.77 ± 0.61 (table 12).

5.4.5 Transfer capacity to eggs

Once accumulated, PFOS tend to associate to very low density lipoproteins (VLDL) and other egg-yolk proteins synthesized in the adults' liver and these complexes are transferred via blood to the ovary and then reach the eggs (Yoo et al., 2009). PFOS mean concentrations in individuals eggs were of 82.5 ± 22.9 and 74.7 ± 29.2 ng/g (ww) in AG and YLG, respectively (table 11). In AG eggs, PFOS ranged from 50.8 to 149 ng/g ww (table 11). Among the eggs of the 3 AG subcolonies, there were no significant differences between PFOS mean concentrations ($F_{2,33}$ = 2.15, P = 0.13). Somehow lower levels were detected in YLG eggs (fig. 25), where PFOS ranged between 31.8 ng/g ww and 140.3 ng/g ww (table 11). Again, among the 3 YLG subcolonies, there were no significant differences between PFOS mean concentrations in eggs ($F_{2,31}$ = 0.85, P = 0.44). The slightly difference between PFOS concentrations in eggs of both species was not statistical significant ($F_{1,68}$ = 3.91, P = 0.052).

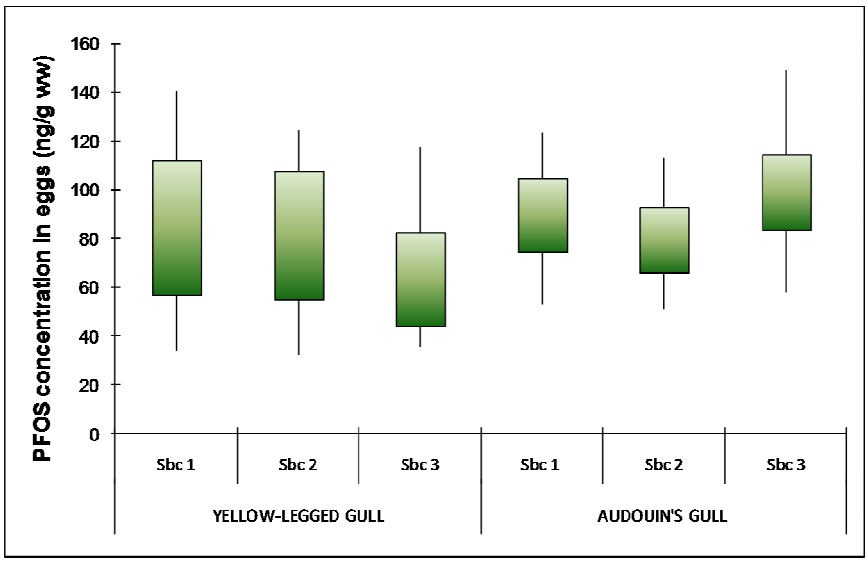

Figure 25. Box plot (quartile 75%) of PFOS concentration in eggs for both species in the three subcolonies (Sbc) sampled and minimum and maximum levels found. Each subcolony corresponds to the measurement of 11 (YLG Sbc1 and Sbc 2) or 12 (the other subcolonies) individual eggs.

Assuming that (i) both species spend all the year in Ebro Delta and they feed on similar food, (ii) that PFOS burden in blood is constant along the year and (iii) that females have the same blood concentration than males from the same nest before laying, the PFOS transfer from mother's blood to the eggs was studied.

Taking into account those nests where blood from both parents and the first egg were sampled, the mean transfer percentage was 190 ± 75% in AG species and 119 ± 17% in YLG (table 15). The transfer values higher than 100% suggest that blood is not the only tissue responsible of transferring PFOS to the eggs. This is in agreement with (Dauwe et al., 2007) that indicates that liver is the main organ responsible for the egg formation.

Table 14. Individual values of PFOS concentrations in blood and eggs, weights of females, males and eggs of Audouin's gull (AG) and yellow-legged gull (YLG). PFOS concentration in males blood (C_m), males body weight (BW_m), PFOS concentration in females blood (C_{fm}), females body weight (BW_{fm}), PFOS concentration in eggs (C_{egg}), egg weight (EW. **NA**: not analyzed

SPS	C_m (ng/g)	BW_m (g)	C_{fm} (ng/g)	BW_{fm} (g)	C_{egg} (ng/g ww)	EW (g)
	54.5	630	15.2	535	56.2	64.1
	NA	NA	35.1	600	123.3	69.3
	40.0	615	NA	NA	93.8	NA
	101.4	670	NA	NA	111.5	70.3
	NA	NA	35.4	500	87.3	64.7
	71.6	640	17.5	490	108.1	64.5
	83.7	630	11.3	465	52.5	68.2
	61.6	610	NA	NA	86.8	58.5
AG	26.7	620	50.8	520	71.0	65.6
	61.3	615	NA	NA	54.6	69.4
	56.4	615	NA	NA	60.1	69.1
	83.5	500	21.7	565	114.9	63.7
	26.0	640	14.8	570	58.0	72.5
	50.4	630	24.1	495	84.6	68.0
	59.7	595	15.3	525	63.6	73.6
	55.3	595	42.2	545	88.7	65.6
	77.4	NA	18.6	545	86.9	68.6
Mean	60.6	614.6	25.2	529.6	82.5	67.2
SD	20.9	38.3	12.7	38.5	22.9	3.8

SPS	C_m (ng/g)	BW_m (g)	C_{fm} (ng/g)	BW_{fm} (g)	C_{egg} (ng/g ww)	EW (g)
	40.3	1130	NA	NA	72.5	98.0
	NA	NA	44.4	850	140.3	91.0
	NA	NA	58.8	880	73.6	85.6
	NA	NA	20.6	810	108.6	84.1
	NA	NA	15.5	850	64.7	90.0
	27.3	1150	NA	NA	33.9	104.4
	NA	NA	33.1	860	67.3	87.3
	NA	NA	17.6	840	48.8	67.3
	57.4	1090	12.0	850	48.3	94.7
	73.0	1040	NA	NA	130.7	98.5
	133.7	1220	NA	NA	116.7	86.9
	NA	NA	31.0	910	40.6	95.9
	59.1	1150	NA	NA	110.9	91.8
YLG	71.7	1070	NA	NA	63.0	81.1
	NA	NA	8.4	880	69.4	102.1
	84.0	1080	NA	NA	73.9	90.5
	26.1	1080	NA	NA	73.7	96.8
	35.3	1170	28.6	870	47.5	77.5
	74.0	1070	17.4	970	84.2	97.1
	61.2	1050	26.1	860	88.0	82.2
	NA	NA	51.5	920	65.0	87.1
	76.0	1080	35.0	950	117.7	84.9
	41.7	1040	29.6	910	50.4	86.3
	NA	NA	23.5	930	44.0	92.0
	48.0	1050	NA	NA	60.1	90.5
	69.2	1050	NA	NA	82.1	91.6
	NA	NA	10.8	850	40.5	92.3
Mean	61.1	1095.0	27.3	881.8	74.7	89.9
SD	26.5	53.3	14.2	43.2	29.2	7.8

NA: not analysed

5.4.6 Mass balance of PFOS in gulls

The mass balance accounts for the annual net accumulation of PFOS considering EYI, elimination through depuration (yDEP) and eggs' transference along the year (M_{egg}). If $K_{dep6.86}$ is used, the mass balance of PFOS is negative, which means that PFOS depuration from blood is faster than PFOS intake and then PFOS would not be accumulated.

As it is impossible to analyze the blood from females before laying (non-invasive capture is impossible), MB was calculated for females using the PFOS values in males' blood (table 13). So, as it is shown in table 13, MB in AG females would be 31449 ± 11686 ng/y, in AG males, 39137 ± 13230 ng/y; in YLG females, 40278 ± 15671 ng/y; and in YLG males, 48595 ± 17641 ng/y.

Table 15. PFOS transfer capacity in AG (n = 10) and YLG (n = 6) considering those nests where both parents and egg were found. Mass of PFOS of each egg (M_{egg}), PFOS concentration in males' blood (assuming females before laying have similar values than males) corresponding to each egg (C_m), specific females blood weight corresponding to each egg (B_{fm}) and percentage of PFOS transferred from females to their eggs (TRA).

SPS	M_{egg} (ng)	C_m (ng/g)	C_{fm} (g)	TRA (%)
YLG	4574.4	57.4	85	93.8
	3680.0	35.3	87	119.9
	8171.1	74.0	97	113.8
	7229.9	61.2	86	137.3
	9994.5	76.0	95	138.4
	4349.9	41.7	91	114.6
Mean	6333.3	57.6	90.2	119.7
SD	2516.1	16.6	5.0	16.7

SPS	M_{egg} (ng)	C_m (ng/g)	C_{fm} (g)	TRA (%)
AG	3601.1	54.5	53.5	123.5
	6972.8	71.6	49.0	198.8
	3583.6	83.7	46.5	92.1
	4657.0	26.7	52.0	335.4
	7321.1	83.5	56.5	155.2
	4204.9	26.0	57.0	284.2
	5751.1	50.4	49.5	230.5
	4683.0	59.7	52.5	149.5
	5815.5	55.3	54.5	192.9
	5958.7	77.4	54.5	141.2
Mean	5254.9	58.9	52.6	190.3
SD	1316.2	21.0	3.4	75.3

The high values of EDI compared to the PFOS blood depuration (DEP), suggests that a portion of the PFOS assimilated by the diet are accumulated in blood. This is in consonance with other studies where PFOS was found in other tissues (such as liver, blood, brain, kidney, pancreas and muscles) (Giesy et al., 2010, Gebbink and Letcher, 2012). Calculations were done

considering one egg per clutch. In fact, our target species have a modal clutch size of 3 eggs, so females eliminate more PFOS than those we estimated. According to these estimations, YLG accumulates more PFOS than AG because they eat 200 g/food per day while AG eats 150 g/food·d. Higher ingestions leads to higher PFOS intakes and consequently to higher PFOS accumulation. In both species, males accumulate more PFOS than females as the later eliminate part of its PFOS body burden through maternal transfer to the eggs.

5.5 Conclusions

It has been demonstrated that PFOS is bioaccumulates in gulls through the diet and that a low depuration rate leads to a annual net accumulation of PFOS. In each laying period, females transfer PFOS to the offspring via eggs. However this transfer is not enough to counteract the PFOS intake. In this process, blood transfer nutrients from the liver during egg formation and PFOS can be remobilized from the liver or other tissues to the eggs. The levels of PFOS in eggs were very similar along the colony and among the 2 species. This suggests that the gulls are equally impacted by PFOS pollution and that similar feeding habits contribute to EDI. Finally, it is largely unknown the effects that PFOS and other Persistent Organic Pollutants may cause to the egg development and chick survival, this topic calls for further studies where toxicological endpoints should be studied both at individual and population level, especially for endangered species.

5.6 Acknowledgements

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Chapter 6. Perfluoroalkyl and polyfluoroalkyl substances in entire clutches of Audouin's gulls from the Ebro Delta

6.1 Abstract

The aim of the present study was to determine the distribution of per- and polyfluoroalkyl substances (PFASs) in three-egg clutches of Audouin's gull (*Larus audouinii*) breeding in Ebro Delta's colony, according to the laying order. 5 PFASs were analysed in 30 eggs (yolk and albumen separately). Carbon and nitrogen stable isotopes were measured as dietary tracers. PFASs were not detected in albumen. In egg yolks, PFOS was the main PFAS detected followed by PFNA, PFHxS and PFOA. Mean ΣPFAS for a-eggs was of 236.5 ± 57.5 ng/g yolk wet weight (ww), for b-eggs was of 140.5 ± 56.2 ng/g yolk ww and for c-eggs, 133.7 ± 54.8 ng/g yolk ww. PFOS concentration decreased according to the laying order of the eggs, showing significant differences between consecutive eggs. Good correlation (r_s^2 = 0.7 – 0.9) was observed for PFOS concentration within the eggs from the same clutch. No relationship was found between PFOS levels and stable isotopes signatures.

6.2 Introduction

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) are chemicals used since the 50s in the industry as surface-active agents in various applications, e.g. in textiles products, metal plating, food packaging, fire fighting foams, floor polishes, denture cleansers, shampoos, coatings, in the photographic industry, and in hydraulic fluids (Clara et al., 2009). The continued disposal and release of PFASs to the environment cause the contamination of sea waters and oceans (Sánchez-Avila et al., 2010; Gómez et al., 2011, Yamashita et al., 2008) entailing their accumulation in the marine fauna (Bossi et al., 2008) and its bioconcentration along the food web (Tomy et al., 2004). PFASs bioaccumulation is directly related with the fluorinated carbon chain length. The longer the chain, the higher the bioaccumulation potential (Martin et al., 2003; Giesy and Kanan, 2002). Tomy et al. (2004) found that fluorinated organic compounds are biogmagnified along the Arctic marine food web. Nakata et al. (2006) determined perfluorooctane sulfonic acid (PFOS) in the coastal food chain of the Ariake Sea, Japan and its results suggested a biomagnification of PFOS between each trophic level. PFOS is known as the most bioacumulative and persistent PFAS (Conder et al., 2008) and for this reason, in 2009, the Stockholm Convention declared PFOS as a persistent organic pollutant (POPs) whose emissions should be minimized and monitoring and biomonitoring data on their sources, accumulation and fate in the environment should be promoted (UNEP, 2009).

Seabirds are highly exposed to environmental contamination because of their ecological habits, high trophic position in the marine food webs and relative long life (Furness et al., 1997). PFASs accumulated in birds can be transferred to the offspring via eggs (Vicente et al., in preparation) and cause toxicological effects at the individual and population level (Verboven, 2008). In income breeders, like gulls, egg composition reflects the assimilated diet of the females before laying (Hobson, 1995). Therefore, bird eggs are good bioindicators of environmental pollution, as it has been reported before for a myriad of contaminants (Braune, 2007; Pereira et al., 2009).

Our target species is the Audouin's gull (*Larus audouinii*), a medium-sized gull endemic of the Mediterranean and the western coast of Saharan Africa. It was included in the "IUCN Red List of Threatened Species" (BirdLife International. 2012) and in the Threat category in the list of the Ministry of Environment of Spain (Madroño, 2004). The most important threat comes from the concentration around 79% of the world population in only one breeding colony, the Ebro Delta, in Catalonia (Bertolero et al., 2008). The Ebro delta has been declared Euro-african wetland of international interest in 1962, Natural Park in 1983, Special Protection Area for Birds, ZEPA, in 1987, RAMSAR site in 1993 and recently, Biosphere Reserve by the UNESCO (May 2013). The Audouin's gull is originally a piscivorous species consuming mainly clupeiforms (Oro and Ruiz, 1997), but it can also exploit fishery discards and freshwater preys (i.e. american crayfish, *Procamburus clarkii*) (Pedrocchi et al., 2002).

As food is the main uptake pathway of PFAS, the study of the feeding habits is of high importance to determine exposure and accumulation of contaminants to birds. Feeding habits can be studied by conventional methods, such as behavioural observations or the analysis of stomach contents or regurgitates, but these have the disadvantages of giving information of a specific diet moment and imply sampling difficulties (González-Solís et al., 1997). Stable isotope analysis (SIA) is used as dietary tracer and provides information of the species trophic position. They are useful also for relating the animals' pollutants burden with their feeding habits (Ramos et al., 2013; Sanpera et al., 2007; Ramos et al., 2009). In previous studies it was observed that gulls from the Ebro delta accumulate high amounts of Persistent Organic Pollutants (POPs) and that audouins' gull eggs had higher levels of some contaminants in comparison to yellow legged gull, fact that was attributed to the fish based diet (Morales et al., 2012). In addition, it was found that accumulation of PFAS in eggs from the Ebro colony was amongst the highest in the Iberian Peninsula (Vicente et al., 2012), and thus, indicates that gulls from the Ebro Delta are highly exposed to contaminants due to the widespread contamination of the area. However, while it is well established that pollutants are transferred to eggs, little is known on the overall release to entire clutches and the amount of PFAs transferred according to the laying sequence, and how diet is involved in this process.

The aim of this study was to evaluate the distribution of 5 main PFASs (perfluorooctane sulfonic acid (PFOS), perfluorohexane sulfonic acid (PFHxS), perfluorobutane sulfonic acid (PFBS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA)) in entire three-egg clutches of *L. audouinii*, a species with conservation interest in Ebro Delta. Yolk and albumen were analysed separately to determine the distribution of PFASs in the egg and according to its laying order. The correlation between PFAS concentration in eggs of the same clutch was also investigated. Stable isotopic tracers' analysis was done to study the relationship between PFASs and feeding habits of *L. audouinii*. $\delta^{15}N$ was used to assess the trophic position and $\delta^{13}C$ to categorize the Audouin's gull dietary exploitation of marine or continental/freshwater resources.

6.3 Materials and methods

6.3.1 Standards and Chemicals

Native compounds of PFOS, PFNA, PFHxS and PFBS were supplied by Wellington Laboratories (Ontario, Canada). Stock standard solutions were prepared in acetonitrile at a concentration of 5 ng/µl for all native compounds and were stored at -18ºC. Perfluoro-n-(1,2,3,4-$^{13}C_4$) octanesulfonic acid (m-PFOA) and sodium perfluoro-1-(1,2,3,4-$^{13}C_4$) octanesulfonate (m-PFOS), also from Wellington Laboratories, were used as surrogate standards. HPLC grade water and acetonitrile were supplied by Merck (Darmstadt, Germany), glacial acetic acid from Panreac (Barcelona, Spain) and activated carbon from Supelco (Bellefonte, Pennsylvania, USA).

6.3.2 Sampling

The work was conducted at the Punta de la Banya colony (Ebro Delta Natural Park, NE Iberian Penninsula) during the reproductive season of 2009. During the peak egg-laying period of Audouin's gull (late April), nests were tagged when the first egg was laid. Inspections were conducted every two days (thus avoiding daily annoyances) until clutch completion to establish the final clutch size and laying order (a-, b-, c-egg). Ten three-egg clutches were selected and freshly laid eggs were sampled and replaced with dummy eggs. The 30 eggs were marked, placed under refrigeration and transported to the laboratory. The field work took place from 04/24 to 05/10.

6.3.3 PFASs extraction and analysis

Eggs were measured and weighted and albumen and yolk separated. Each matrix was weighted and homogenized. From each sample, different aliquots were kept in glass tubes at -23ºC until analysis.

PFASs were solid-liquid extracted from homogenized albumen and yolk wet samples. Briefly, about 1 g of sample was weighted in polypropylene tubes and internal standards (m-PFOS and m-PFOA) were added at a concentration of 20 ng/g and incubated for 18 hours at 4ºC. Nine mL of acetonitrile were added and samples were then extracted in an ultrasonic bath for 10 min at room temperature (3 times). Afterwards, the samples were centrifuged at 2.500 rpm for 5 min. The supernatant was transferred to a new vial and evaporated to dryness. Then 1.5 mL of acetonitrile was added to the dried extract and incubated for 10 min in the ultrasonic bath. Albumen samples were purified by adding 25 mg of activated carbon, while yolk samples with 50 mg (because of the higher amount of fats) and 50 µL of glacial acetic acid and were vigorously mixed for 1 minute. Subsequently, the samples were centrifuged at 10.000 rpm for 10 min. The supernatant was transferred to a clean micro vial and evaporated to dryness. Finally, samples were resuspended with 150 µL of ACN and diluted with 350 µL of HPLC water.

PFASs were measured using an Acquity Ultra Performance Liquid Chromatography system connected to a Triple Quadruple Mass Spectrometric Detector (Waters, USA). An Acquity UPLC BEH C_{18} column (1.7 µm particle size, 50 mm x 2.1 mm, Waters, USA) was used as mobile phase residue trap to remove any contamination from the mobile phases. Ten µL of extract were injected. The analysis was performed on a Symmetry column (150 mm x 3.5 µm x 2.1 particle size, Waters, USA). The mobile phase consisted of water with 2 mM NH_4OAc (A)/ Acetonitrile (B). Gradient elution started at 70% A and 30% B, increased to 100% B in 5.10 min and held for 3.9 min, at a flow rate of 0.4 mL/min. The chromatographic time was of 9 min. Then, initial conditions were regained in 1 min and the system was stabilized for 3 min at initial conditions. The various PFASs were measured under negative electrospray ionisation using 2 transitions from parent to product ion to identify each compound following the method used by Fernández-Sanjuan et al. (2010). To identify the target compounds, the retention time and 2 transitions were used. Internal standard quantification was performed using m-PFOS to quantify PFOS, PFNA, PFBS and m-PFOA to quantify PFOAs and PFHxS. Quality parameters and performance of the method are described in a previous study (Vicente et al., 2012).

6.3.4 Stable isotopes analysis

Yolk subsamples were subjected to lipid extraction by successive rinsing in a chloroform and methanol solution (2:1), following the protocol of Folch et al. (1957), in order to reduce variability due to differences in lipid content among tissues (DeNiro and Epstein,1981). Yolk and albumen subsamples were lyophilised and ground to a powder. Then, 0.35-0.38 mg of yolk and albumen from each subsample were placed into tin capsules for stable carbon and nitrogen isotope ratio determination. Isotopic analyses were carried out at the Scientific and Technical Services of the University of Barcelona (Spain) by means of a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLOIII interface (Thermo Finnigan MAT, Bremen, Germany), with International Atomic Energy Agency (IAEA) standards being applied every 12 samples to calibrate the system. Stable isotope ratios were expressed in the standard δ notation relative to Vienna Pee Dee Belemnite ($\delta^{13}C$) and atmospheric N_2 ($\delta^{15}N$). Replicate assays of standards indicated analytical measurement errors of ±0.1‰ and ±0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

6.3.5 Data Analysis

Descriptive statistics and graphics were done with the statistical software package PASW Statistics 18 (2009). Intracluch differences in PFASs were evaluated using nonparametric Anova for repeated measurements (Friedman test) followed by a pairwise comparison using Wilcoxon sign ranked tests, with p-values adjusted for multiplicity. The study of the correlations between eggs from same clutch was assessed by the Spearman's rank correlation coefficient. Also, rank correlation was used to assess the relationship between PFASs and stable isotope signatures.

6.4 Results and discussion

6.4.1 Partitioning of PFASs in yolk and albumen

PFASs were only detected in yolk samples. Based on chicken (*Gallus gallus domesticus*) egg composition, yolk is about 47.5% of water, 33% of lipids, 17.4 % of proteins and other molecules, and albumen composition is about 88.5% of water, 10.5% of proteins and other molecules (Burley and Vadehra, 1989). Therefore, the yolk is richer in terms of lipids and proteins. PFASs have proteinophilic nature (Giesy and Kannan, 2002) which contributes to the tendency of yolks to accumulate PFASs. In addition, Newsted et al. (2007) found that PFOS

was associated with very low density lipoproteins (VLDL) in the yolk of mallards and quails. This explains that PFAS were not detected in egg albumen, which is in accordance to previous study where PFAS were neither detected in albumen of *Larus argentatus* eggs (Gebbink and Letcher, 2012).

6.4.2 PFASs concentration in yolks from entire clutches

Table 16. PFOS, PFNA, PFHxS and PFOA levels along the egg laying sequence in each clutch (ng/g ww) of *L. audouinii* egg yolks from Ebro Delta Natural Park. PFBS was not detected.

Clutch	egg	PFOS	PFNA	PFHxS	PFOA
1	a-	190.6	5.8	ND	35.7
	b-	71.9	ND	ND	10.5
	c-	58.2	ND	ND	24.4
2	a-	227.5	7.8	ND	ND
	b-	149.2	ND	ND	ND
	c-	67.6	ND	ND	ND
3	a-	132.6	ND	ND	ND
	b-	105.9	ND	ND	ND
	c-	197.1	ND	ND	ND
4	a-	159.3	ND	ND	ND
	b-	54.3	ND	ND	ND
	c-	53.6	ND	ND	ND
5	a-	267.6	ND	4.1	ND
	b-	160.2	ND	2.5	ND
	c-	101.5	ND	3.2	ND
6	a-	121.8	5.0	1.5	ND
	b-	84.6	ND	ND	ND
	c-	65.9	ND	6.1	ND
7	a-	208.3	ND	2.8	ND
	b-	132.8	ND	ND	ND
	c-	95.6	ND	ND	ND
8	a-	145.3	ND	ND	ND
	b-	68.4	ND	ND	ND
	c-	52.9	ND	ND	ND
9	a-	281.4	4.6	ND	ND
	b-	183.1	4.3	ND	ND
	c-	148.5	4.6	ND	ND
10	a-	188.8	3.9	4.1	ND
	b-	132.3	ND	20.3	ND
	c-	165.1	3.6	ND	ND

ND: not detected

Table 16 shows the concentration of PFASs in yolk samples. ΣPFAS mean ± SD for a- eggs was 236.5 ± 57.5 ng/g yolk wet weight (ww), for the b- was 140.5 ± 56.2 ng/g yolk ww and for the c-, 133.7 ± 54.8 ng/g yolk ww. Among the studied compounds, PFOS was found in all samples. Its contribution to the ΣPFAS was 81% for the a- eggs, 82% for the b- eggs and

71% for the c- ones. In bird eggs, the prevalence of PFOS over other PFASs has been previously demonstrated (Verreault et al. 2005; Gebbink et al., 2011; Vicente et al, 2012).

PFOS values ranged from 121.8 to 281.4 ng/g yolk ww in a-eggs, from 54.3 to 183.1 ng/g ww in b-eggs and from 52.9 to 197.1 ng/g ww in c-eggs. PFNA (3.6 – 7.8 ng/g ww) and PFHxS (1.5 – 20.3 ng/g ww) were found in 8 eggs (mostly the first eggs). PFOA was detected only in one clutch (3 eggs) (10.5 – 35.7 ng/g ww) and PFBS was not detected (table 16). PFOS appeared in the highest level in the first egg and the concentration decreased according to laying order (Figure 26). The mean values and standard deviation of PFOS for the 10 clutches of 3 eggs in order of laying were 192.3 ± 54.7, 114.3 ± 43.7 and 100.6 ± 52 ng/g yolk ww for a, b and c eggs, respectively.

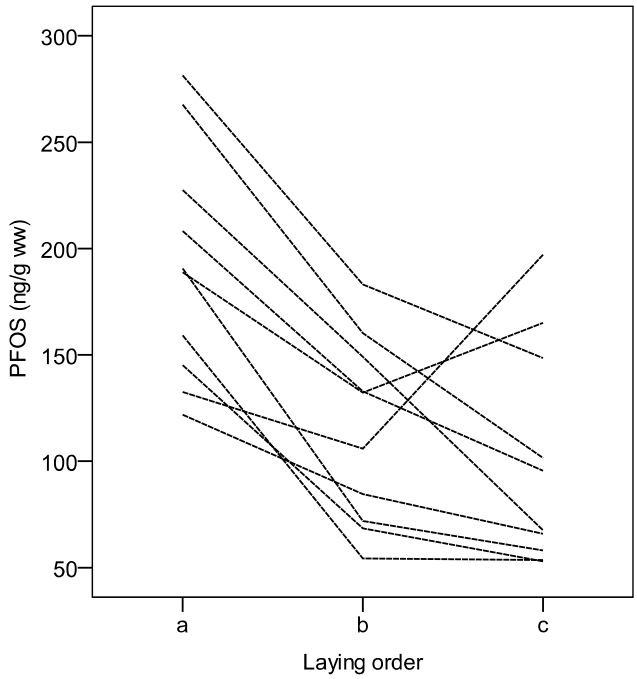

Figure 26. PFOS concentration in egg yolk (ng/g ww) of Audouin's gull according to the egg laying order ((first egg (a), second (b) and third (c)) per each clutch (N = 10).

Unlike PFOS, the low detection of the other 4 compounds indicates their lower presence in the environment and wildlife (Kannan et al., 2002) and their lower accumulation potential. Conder et al. (2008) reported that sulfonated PFASs (such as PFOS, PFHxS and PFBS) are more bioaccumulative than the carboxylated ones (such as PFOA and PFNA) and that PFASs with less than 7 fluorinated carbons are not considered bioaccumulative. Vicente et al. (in preparation) analysed the same 5 PFASs in Audouin's and yellow-legged gull (*Larus michahellis*) first eggs. PFOS was the only compound detected (PFOS mean ± SD for *L. audouinii* was 82.5 ± 22.9 ng/g whole egg ww). As yolk and albumen were not analyzed separately, to compare previous results to these from the present study we converted PFOS levels in egg-yolk to ng/g whole egg, considering a 35 % of yolk in eggs (Ricklefs, 1977) of precocial birds (as *L. audouinii* is a semi-precocial bird (Tjørve et al., 2009). Once converted, a

mean ± SD of 67.3 ± 19.1 ng PFOS/g egg ww in the 1st eggs was found, which is slightly lower than those detected in Audouin's gulls collected in 2009 (Vicente et al., in preparation).

The variability of PFOS concentrations found among clutches could be explained by several reasons. On one hand, the concentration of PFOS in gulls' eggs depends on the location of the gull colony (Vicente et al., 2012). If the female has been feeding on fishing discards, continental preys, landfills or other organisms the days before laying, the concentration of PFASs may change. Arcos and Oro (2002) demonstrated that fish from the fishing discards, mainly demersal species, contained double concentration of mercury than the epipelagic fish (the natural preys of gulls). On the other hand, Larids are income breeders (Hobson, 1995), they generally use exogenous resources for the egg formation. However, endogenous reserves could be needed for the formation of the eggs (normally in the latter eggs) which may alter the egg composition and pollutants burden (Verreault et al., 2006). Age also plays an important role in the bioaccumulation of PFASs (Yoo et al., 2009).

Table 17. Wilcoxon signed-rank test parameters for PFOS comparisons along egg sequence (first egg (a), second (b) and third (c)). Statistical test, standard error (SE), statistical test deviation and significance (Sig.)

Egg sequence	Statistical test	SE	Deviation	Sig.
a – b	1.1	0.447	2.46	0.042
a – c	1.9	0.447	4.249	<0.001
b – c	0.8	0.447	1.789	0.221

6.4.3 Effect of the egg laying order on the PFOS discharge

With the aim to analyze the relation between the egg laying order and PFOS levels, 2 non-parametric tests were used (Friedman and Wilcoxon test). For PFOS, differences between egg order (a-, b- and c- eggs) were significant (Friedman test: $P < 0.001$). Wilcoxon signed-rank test showed that the a-eggs presented higher amount of PFOS than the b-eggs ($P < 0.001$) and than the c-eggs ($P = 0.042$), but no significant differences were found when comparing the b-eggs and c-eggs ($P = 0.22$) (Table 17). Taking into account the ΣPFOS discharged in the whole clutch, the mean percentage of transfer per egg is shown in table 18. PFOS were discharged in 48 ± 9% in the first egg, 27 ± 4 % in the second and 24 ± 9% in the third. PFOS concentration decreased along the egg sequence.

Pastor et al. (1995) determined the levels of certain organohalogenated compounds (such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and hexachlorobenzene (HCB) in 56 eggs belonging to 26 clutches (of 1, 2, 3 or 4 eggs) of Audouin's gull from Ebro delta. When comparing clutches of 2 eggs with clutches of 3, they

found similar trends among the a- and b-eggs for the 2-egg clutches and among the b- and c-eggs, for the three-egg clutches. Three-egg clutches are formed by those females well fed, under better conditions, while females in lower conditions have not enough energy and lay 1 or 2 eggs per clutch (Pastor et al., 1995). This indicated a marked "mother effect" on clutch size. In fact, those mothers under better food conditions use the energy from the food gathered during the laying period to form the a- and the b-egg, while the c-egg should be formed using endogenous reserves. Those females under lower food conditions may already need the use of endogenous reserves to form the 2nd egg. Therefore, the concentration of organohalogenated compounds showed differences between the eggs according to the order of laying but also in relation to the clutch size. In addition, Morera et al. (1997) in the Ebro Delta and Sanpera et al. (2000) in Chafarinas islands (Spain) reported that there was a correlation between the eggs of Audouin's gull and the mercury concentration, decreasing according to the egg laying sequence. Furthermore, Verreault et al. (2006) showed that organohalogen contaminants are retained in the female in a selective way which depends on the compounds' chain length, hydrophobicity, persistence, recalcitrant characteristics, etc. As a result, more bioaccumulative compounds (like PFNA because has more fluorinated carbons than the other 4 PFASs) will be less transferred to the eggs. Concentrations in the first eggs reflect the chemicals intake with the food recently ingested by the female, but some compounds can be derived from the life accumulation, especially those in the latter eggs (Verreault et al., 2006, Becker et al., 2001, Braune and Norstrom, 2009). Consequently, if females have to mobilise endogenous reserves to complete the clutch because of the lack of energetic resources, probably they would use them for the formation of the third egg. So, the variability along the laying sequence of the clutch could be explained by the "mother effect", depending on the origin of the pollutant. This could explain the increase of PFOS, PFNA, PFHxS and PFOA in the third eggs of some clutches (table 16).

Table 18. PFOS transfer percentage (%) along the egg sequence of each clutch; mean and standard deviation (SD).

clutch	a-egg	b-egg	c-egg
1	59.4	22.4	18.1
2	51.2	33.6	15.2
3	30.4	24.3	45.2
4	59.6	20.3	20.1
5	50.6	30.3	19.2
6	44.7	31.1	24.2
7	47.7	30.4	21.9
8	54.5	25.6	19.9
9	45.9	29.9	24.2
10	38.8	27.2	33.9
Mean ± SD	*48.3* ± 9.0	*27.5* ± 4.2	*24.2 ± 8.9*

6.4.4 Pollutant correlation between eggs from the same clutch

In order to examine the correlation between PFASs concentrations in the eggs from the same clutch, Spearman's rank correlation coefficient and Pearson product-moment correlation coefficient were used. Both tests were compared to better discuss the findings.

Correlation between PFOS concentration in eggs within the same clutch was found. The coefficients with Spearman's rank correlation were 0.939 between the first and second egg ($\alpha < 0.001$), 0.733 between the first and the third ($\alpha = 0.016$) and 0.855 between the second and the third ($\alpha = 0.002$). The coefficients with Pearson product-moment correlation were 0.927 between the first and second egg ($\alpha < 0.001$), 0.675 between the first and the third ($\alpha = 0.032$) and 0.780 between the second and the third ($\alpha = 0.008$) (Table 19). Minimal differences were found between the 2 tests and, in both cases, tests pointed to a strong association among the 3 eggs of the same clutch, indicating that those nests with high PFOS level in the first egg will have also high levels in the second and also in the third.

Table 19. Spearman's rank correlation coefficient and Pearson product-moment correlation coefficient parameters bettwen eggs of the same clutch (first a-, second b- and third c-eggs). Correlation coefficient (Corr. Coef.) and significance (Sig. (bilateral)).

Egg sequence	Spearman			Pearson		
	Corr. Coef.	Sig. (bilateral)	N	Corr. Coef.	Sig. (bilateral)	N
a- *vs* b-	0.94	<0.001	10	0.93	<0.001	10
a- *vs* c-	0.73	0.016	10	0.67	0.032	10
b- *vs* c-	0.86	0.002	10	0.78	0.008	10

To our knowledge, the present work is the first study analysing PFASs correlations along the laying sequence of eggs from *L. audouinii*. Recently, Gebbink and Letcher (2012) studied maternal PFASs transfer in Great Lakes herring gulls, but they did not distinguish among the eggs along the laying sequence. Pastor et al. (1995) suggested that to evaluate the impact of organochlorine pollutants in the environment, it is appropriated to use the first egg, as it is the less influenced by the "mother effect". But they proposed the analysis of the third egg in order to study the adults' charge of pollutants as the last egg is the most influenced by the endogenous reserves. For PFOS, the sampling of the third egg could be suitable as there exists a strong correlation between the first, second and third egg. However, for the other 4 PFASs, which have been detected mostly in the first eggs, the use of the third egg solely would suppose an underestimation of the concentration of PFAS and therefore, the impact and distribution could not be properly assessed. So, we propose the analysis of the first egg as (i) it contains the highest levels of PFASs; (ii) it is easy to sample as long as the laying period is properly

surveyed; (ii) it is possible to compare the concentration of pollutants among eggs of different colonies and (iv) it does not affect the wellbeing of the population, which is specially relevant for protected species, as only one egg is sampled.

Table 20. Nitrogen and carbon isotope signatures of Audouin's gull egg yolks (‰).

clutch	egg	$\delta^{15}N$	$\delta^{13}C$
	a	11.48	-18.99
1	**b**	12.10	-18.54
	c	11.50	-18.88
	a	11.93	-18.37
2	**b**	11.60	-18.06
	c	11.66	-18.59
	a	11.56	-18.81
3	**b**	11.65	-18.38
	c	11.69	-19.61
	a	12.06	-18.83
4	**b**	12.28	-18.25
	c	12.12	-19.69
	a	11.88	-18.57
5	**b**	12.28	-18.74
	c	12.11	-18.47
	a	12.32	-19.42
6	**b**	12.45	-19.51
	c	12.22	-20.00
	a	12.08	-18.37
7	**b**	12.12	-18.97
	c	12.33	-18.77
	a	11.82	-18.26
8	**b**	11.80	-18.64
	c	11.79	-18.81
	a	12.22	-19.05
9	**b**	12.21	-18.61
	c	12.35	-19.80
	a	12.31	-18.47
10	**b**	12.30	-18.99
	c	12.31	-19.48

6.4.5 Stable isotopes signatures

Considering all eggs, values of $\delta^{13}C$ in yolks ranged between -18.06 and -20 ‰ (with a mean and a standard deviation of -18.86 ± 0.51 ‰) and $\delta^{15}N$ values ranged from 11.48 to 12.45 ‰ (12.02 ± 0.29 ‰) (table 20). Significant differences were found for $\delta^{13}C$ between the egg laying order ($F_{2,27}$=4.29, *P*=0.02). To investigate which of the 3 categories was the responsible, post-hoc comparisons were performed comparing a- *vs* b- eggs, b- *vs* c-eggs and a- *vs* c-eggs. Significant differences were found between b- *vs* c-eggs and a- *vs* c-eggs ($F_{1,18}$=5.93, *P*=0.02 and $F_{1,18}$=5.44, *P*=0.03, respectively). Mean and standard deviation of $\delta^{13}C$ values for the 1st, 2nd and 3rd eggs were -18.71 ± 0.37, -18.67 ± 0.41 and -19.21 ± 0.56‰, respectively. $\delta^{13}C$ differentiates feeding strategies (Gebbink et al., 2011a) and high $\delta^{13}C$ values indicates marine prey consumption while low values of $\delta^{13}C$ are influenced by continental preys and refuse tips (Muñoz-Arnanz et al., 2012). These results and the higher amounts of PFASs found in some c-eggs support the idea that *L. audouinii* females could be using endogenous reserves for the formation of the latter eggs as $\delta^{13}C$ values are lower in the c-eggs (Pastor et al., 1995, Verreault et al., 2006). No significant differences were found for $\delta^{15}N$ between the 3 categories of eggs, as $\delta^{15}N$ indicates the relative trophic position of the species (Gebbink et al., 2011a).

Spearman's rank correlation coefficient was done according to the laying sequence in order to search for any association between PFOS values and stable isotopes signatures, but no significant correlation was found. PFOS levels reflect the bioaccumulation potential while SIA provides information of the recently assimilated diet. The lack of correlation between these 2 factors may come because bioaccumulation (which depends on the age) and biomagnification (which depends on the food web) are independent. Gebbink et al. (2011b) also reported minimal relationships between PFASs concentrations and carbon and nitrogen stable isotopes levels in *Larus argentatus* eggs in a long term study from Laurentian Great Lakes, which indicates the complexity of aquatic and terrestrial food as a source of PFASs to gulls. This supports the idea that *L. Audouinii* is amplifying their feeding habits exploiting increasingly other food resources such as continental preys or refuse dumps (Pedrocchi et al., 2002).

6.5 Conclusions

This study demonstrates that PFASs have more affinity for egg-yolk proteins than for albumen proteins. PFOS was the most ubiquitous. Differences of PFOS concentration among the 3 eggs within a clutch were found and an important laying order effect existed in terms of PFOS transfer, being the first egg the one with the highest levels. The strong correlation of PFOS between the 3 eggs of the same clutch and the prevalence of the other 4 PFASs in the first

egg suggest that the sampling of the first egg of *L.audouinii* is appropriate for biomonitoring studies using bird eggs as indicators of environmental pollution.

Stable isotopes signatures support that *L.audouinii* may use endogenous reserves for the formation of latter eggs which also concords with the differential pattern of PFASs in the c-egg indicating a "mother effect". The lack of correlation between PFAS and stable isotopes signatures indicates the complexity of the PFASs exposure to Audouin's gulls and the expanding feeding habits of this species. Future studies with bigger sample size and an extended temporal sampling are needed to understand the dynamics of PFASs in gulls.

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Chapter 7. 17β-estradiol and testosterone in blood and eggs of *Larus michahellis* and *Larus audouinii* from the Iberian Peninsula

7.1 Abstract

The aim of this study was to characterize the levels of 2 steroid hormones, 17β-estradiol (E_2) and testosterone (T), in eggs of yellow-legged gull (*Larus michahellis*, YLG) from 7 colonies from the Iberian Peninsula (Ebro Delta Natural Park, Medes Islands, Columbretes Is., Dragonera Is., Grosa Is., Chafarinas Is. and Atlantic Is.). T levels in YLG eggs ranged from 4.57 to 17.28 ng/g wet weight (ww) and significant differences were found between Atlantic and Mediterranean colonies. On the other hand, E_2 in YLG eggs ranged between 0.05 – 0.21 ng/g ww and no differences were observed among colonies. To determine species related differences, E_2 and T were also analyzed in eggs of Audouin's gull (*Larus audouinii*, AG) from the Ebro Delta Natural Park, where both species cohabit. T levels in AG eggs ranged from 3.41 to 4.46 ng/g ww and E_2 in AG eggs were 1.25 – 2.58 ng/g ww, and significant differences were found among species.

Finally, steroid levels were analysed in blood of AG and YLG progenitors of eggs from the Ebro delta with the aim to study the transfer of steroid hormones from female to the eggs. Steroid blood levels were 0.17 – 1.93 ng T/mL ww and 7.43 – 314.2 ng E_2/mL ww in YLG and 0.02 – 0.63 ng T/mL ww and 0.03 – 0.08 ng E_2/mL ww in AG. Significant differences were found for T and E_2 in blood comparing both species. Such differences are associated with the different feeding habits and metabolism. No differences were found between sexes within each species.

7.2 Introduction

17β-estradiol (E_2) and testosterone (T) are two steroid hormones which regulate physiological processes related with the reproductive system. E_2 is an estrogen which, together with estriol and estrone, is involved in females' sexual differentiation, whereas T is an androgen and has an important role in the development and maintenance of males' characteristics in vertebrates (Miller, 1988). Both hormones are synthesised in females' ovaries and in males' testes, were T is differentiated to E_2 via aromatization (Kovacs and Ojeda, 2012). Contrary to mammals, in birds the homogametic sex is the male, while female phenotype is forced by the estrogen action (Balthazart et al., 1995). It has been demonstrated that E_2 synthesis is not

exclusive of gonads, where it is transported via blood to the target cell and it acts as an endocrine factor (Simpson and Davis, 2001). Such E_2 production needs external androgenic precursors, so circulating T levels plays an important role as E_2 substrate (Simpson and Davis, 2001). The secretion of these estrogens decrease with advanced aging (Labrie et al., 1997).

Circulating sexual hormone levels in males and females birds may change due to environmental conditions, breeding season, prelaying and laying period and feeling of threat (Hau et al, 2000; Tarlow et al., 2003; Ros et al., 2002) but they are also related with age class, infections and social stimuli (Madsen et al., 2007). Furthermore, there are certain environmental contaminants (such as polychlorinated biphenyls (PCBs), Polybrominated diphenyl ethers (PBDEs), dichlorodiphenyltrichloroethane (DDT), between others) whose presence in biota is associated with endocrine disruption as they can mimic or block the action of steroid hormones (Verreault et al., 2006) by causing a direct interaction with hormone receptors, alterations in receptor affinity, modification of transport proteins or interference with biosynthesis or decomposition of the steroids (Giesy et al., 2003). Steroid hormones are important regulators of the prenatal maternal effects (Groothius et al., 2005) and their presence in the egg-yolk can have significant effects on the offspring phenotype (William et al, 2004).

Yellow-legged gull (*Larus michahellis*, YLG) is a common and large gull present in the Iberian Peninsula. YLG has an omnivorous diet but often scavenges on refuse tips (Duhem et al., 2005). Due to their opportunistic feeding habits, YLG population has increased severally over the last decades (Prunier, 2003). YLG is widespread in the Iberian Peninsula and the colony in the Ebro Delta Natural Park is one of the most populated (Molina, 2009).

Audouin's gull (*Larus audouinii*, AG) is a medium size gull, endemic of the Mediterranean area. At present, this species is expanding but it is still delicate and it was categorized as Near Threatened species on the IUCN 2012 Red List (IUCN, 2012) and still classified as Vulnerable on 2004 by The Spanish Environmental Ministry (Madroño et al., 2004). Nowadays the Ebro Delta colony is the biggest breeding population of AG in the world (65%), where they cohabit with YLG. AG is a piscivorous species, considered a specialist in the capture of clupeiforms (pelagic fishes), but they can also feed on fishery discards and terrestrial preys (Oro and Ruiz, 1997; Navarro et al., 2010).

The aim of the present study was to evaluate the burden of two sexual hormones in gulls eggs and blood. In a first step, E_2 and T were analyzed in YLG from 7 colonies of high ecological interest of the Iberian Peninsula. In a second step, both steroids were analysed in AG eggs from Ebro delta and differences between both species were evaluated.

7.3 Materials and methods

7.3.1 Sampling

Yellow-legged gull (YLG) fresh eggs were collected from 7 National or Natural Parks: Medes Islands, Ebro Delta, Columbretes Islands, Dragonera Island, Grosa Island, Chafarinas Islands and Atlantic Islands (fig. 27). Sampling was performed in March-May of 2009. These sites respresent the most important gull colonies of the Iberian Peninsula. In each colony, 36 eggs were randomly collected in 3 subcolonies (12 eggs per subcolony). Only the first egg was taken in order to compare the levels among colonies. Once in the lab, eggs were opened and the content was homogenized. The 12 eggs of each subcolony were pooled so 3 pooled samples were analysed per colony (except in Colombrets and Dragonera Is., where only 12 eggs were found and so only one pooled sample was analysed). Afterwards, egg pools were freeze-dried.

Figure 27. Map showing the 6 colonies sampled from the Iberian Peninsula.

YLG and Audouin's gull (AG) fresh eggs and blood were collected from the Ebro Delta Natural Park. A total on 36 eggs (12 per 3 subcolonies) per species where collected and they were processed as depicted before. Adults' blood from both species (YLG and AG) was taken from those nests where the eggs were found. From some nests we could get the blood from the male and the female, but in the others we could not capture both members and we only had blood from one of the parents. From YLG, blood was taken from 17 males and 19 females (6 pairs of them sharing the same nest) and from AG, 15 males and 12 females (9 from the same nest). Blood was obtained by puncturing the brachial vein using a heparinized syringe and was placed in an eppendorf with 0.05 mL of heparine. Blood was mixed with heparin to avoid coagulation and kept frozen until extraction.

7.3.2 Extraction and analysis of steroids

T and E_2 were determined by radioimmunoassay (RIA), performing the methodology described by Morcillo et al. (1999). Briefly, about 60 mg of freeze-dried egg samples were mixed with 1 mL of ethanol in a glass tube and then extracted 3 times with ethyl acetate in an ultrasonic bath at room temperature (10 min) followed by a centrifugation at 3000 rpm (10 min). After evaporation to dryness, 1 mL of methanol:water (4:1) was added and samples were immersed in an ultrasonic bath (10 min). Then, a delipidation with 2.5 mL of petroleum ether was done and samples were evaporated to dryness. 4 mL of milli-Q water were added to the dry residue and incubated in an ultrasonic bath. Afterwards, a C18 SPE cartridge (Isolute, International Sorbent Technology, Mid Glamorgan, UK; 1 g, 6 mL) connected to a vacuum system was activated with 4 mL methanol and 8 mL milli-Q water. To purify the samples, Cartridges NH_2 (Sep-Pack® Plus; Waters, Milford, MA, USA) were fitted on the C18 cartridges and the C18-NH_2 complexes were washed with 8 mL of hexane and subsequently, steroids were eluted with 9 mL of diclhoromethane:methanol (7:3). This fraction was collected and evaporated to dryness. Dry egg extract was resuspended in 900 µL of 50 mM potassium phosphate buffer pH 7.6, with 0.1 % of gelatine (Gelatin electrphoresis reagent Type A; SIGMA code G-8150).

For blood analysis, 350 µL of sample were vigorously mixed with 500 µL of ethyl acetate and centrifuged at 10000 rpm (5 min, 3 times). After that, samples were evaporated to dryness and stored at -20ºC. Afterwards, dry blood samples were resuspended in 500 µL of the same potassium phosphate buffer as indicated above.

Finally, egg and blood extracts were sonicated (10 min). 100 µL of sample were assayed for E_2 and T using the commercial RIA Kits with iodinated hormones and specific antibodies (RADIM TESTOSTERONE RIA Kit code KS24CT and RADIM 17β-estradiol RIA Kit code KS25CT). Standard curves with the steroids dissolved in the same phosphate buffer were performed in each run.

In order to confirm the high levels of 17β-estradiol in YLG blood (both males and females), 3 samples (re-diluted 1:3 with acetonitrile) of each species (one with the highest levels of E_2, one of the middle and one the lowest levels) were reanalysed using combined HPLC coupled to tandem Mass Spectrometry (UPLC-MS/MS) with an electrospray interface (ESI). The analytical system consisted of an Acquity Ultra Performance LC system connected to a Triple Quadruple Detector (TQ Detector, Waters, USA). E_2 was detected using multiple reaction monitoring (MRM) under negative ESI, according to the following conditions: Parent ion was 269 m/z; daughters were 183, 145, 143 m/z and; cone and collision voltages were in the range of 65-70 V and 40-50 eV for each daughter. The analysis was performed on a LiChroCART HPLC RP-18e column (125 mm × 2 mm × 5 µm particle size, Merck, Germany)

and the flow rate was set at 0.2 mL/min. Injection volume was of 10 µL and the autosampler tray was maintained at 20 ºC. The mobile phase consisted of acetonitrile (A) / water (B) and the gradient elution started from 30% A and 70% B, increased to 90% A in 5 min and was held for 3 min. Initial conditions were reached in 1 min, and the system was stabilized for 4 min. E2 was quantified by integrating the area of the peak using the external calibration curve of the authentic standard.

7.3.3 Data analysis

Descriptive statistical analysis was performed for blood and eggs of the 2 species. Differences between E_2 and T concentrations in eggs from all colonies of YLG were evaluated by the non parametric test Kruskal-Wallis and between regions (Atlantic *vs* Mediterranean) by one-way ANOVA (to avoid pseudoreplication the colony factor was included in the error term). Differences in steroid levels of eggs from both gull species were evaluated by a randomization test with software Resampling (http://www.uvm.edu/~dhowell/) and differences in blood between the species and between sexes were evaluated by analysis of variance (two-way ANOVA). Data were analyzed with the statistical software package MATLAB for Windows (version 7.0) and R, version 2.11.1 (R Development Core Team, 2010). Values are presented as Means ± SEM.

7.4 Results

7.4.1 Egg steroid concentration

In all colonies, T levels in YLG eggs ranged between 4.57 and 17.28 ng/g wet weight (ww) (table 21). No significant differences were found between T in eggs from all colonies (Kruskal-Wallis χ^2_6 = 12.366, P = 0.054). However, when comparing the eggs from Atlantic (Atlantic Islands) *versus* Mediterranean colonies (Medes Islands, Ebro Delta, Columbretes Islands, Dragonera Island, Grossa Island and Chafarinas Islands) significant differences for T were found ($F_{1,14}$ = 9.431, P = 0.008; Table 21). These differences are caused by the highest concentration detected in one of the subcolonies of Atlantic Is. (17.28 ng/g ww) (table 21). E_2 levels in YLG eggs ranged between 0.05 and 0.21 ng/g ww (table 21). No significant differences were found for E_2 considering eggs from all colonies (Kruskal-Wallis χ^2_6 = 8.953, P = 0.176) neither Atlantic *versus* Mediterranean colonies ($F_{1,13}$ = 0.004, P = 0.954; Table 21).

Table 21. Testosterone and 17β-estradiol levels (ng/g wet weight (ww)) in *Larus michahellis* and *L. audouinii* pooled eggs from seven colonies of National or Natural Parks of the Iberian Peninsula. Each colony was divided in 3 subcolonies

	***Larus michahellis* (ng/ g ww)**							***L. audouinii* (ng/ g ww)**
	Medes Is.	**Columbretes Is.**	**Dragonera Is.**	**Grossa Is.**	**Chafarinas Is.**	**Cies Is.**	**Ebro Delta**	**Ebro Delta**
	14.50	7.81	9.86	11.75	5.56	17.28	7.03	3.41
T	8.13			7.89	5.49	9.15	6.97	4.62
	9.17			11.40	4.58	10.89	4.83	4.50
	0.061	0.079	0.098	0.100	0.049	0.090	0.118	1.536
E2	NA			0.096	0.054	0.097	0.097	1.256
	0.099			0.098	0.074	0.148	0.214	2.587

N.A., not analysed.

7.4.2 Egg and blood steroid concentration in Ebro Delta

T levels in eggs from AG were 3.41, 4.49 and 4.62 ng/g ww for each subcolony (table 21). T levels in YLG eggs from the Ebro Delta were 4.83, 6.97 and 7.03 ng/g ww in each subcolony. Significant differences were not found between T levels of YLG and AG eggs from Ebro Delta (permutation test t = -0.027, P = 1). E_2 levels in AG eggs were of 1.25, 1.54 and 2.58 ng/g ww and in YLG eggs were of 0.097, 0.118 and 0.214 ng/g ww (table 21). Significant differences were not found between E_2 levels in eggs from both target species from Ebro Delta (permutation test t = 0.768, P = 0.593).

Table 22. Testosterone and 17β-estradiol levels (mean ± sem) in *Larus michahellis* and *L. audouinii* adults' blood (ng/mL wet weight (ww)) from Ebro Delta Natural Park.

Steroid	***L. michahellis* (ng/ mL ww)**		***L. audouinii* (ng/ mL ww)**	
	Males	**Females**	**Males**	**Females**
T	0.59 ± 0.14	0.48 ± 0.07	0.12 ± 0.04	0.05 ± 0.01
E2	91.8 ± 15.7	117.7 ± 18.9	0.06 ± 0.004	0.06 ± 0.005

Blood was only sampled in YLG and AG males and females from the Ebro Delta (Table 22). T levels were significantly greater in YLG than in AG (ANOVA $F_{1,62}$ = 26.015, P < 0.001) (fig.28). However, no differences were found between sexes (ANOVA $F_{1,62}$ =1.015, P = 0.318) or the interaction of sex and species (ANOVA $F_{1,62}$ = 0.050, P = 0.824) (fig.28). E_2 levels show a similar pattern than T levels, being significantly greater in YLG than in AG (ANOVA $F_{1,62}$ = 57.686, P < 0.001) and no differences were found between sexes (ANOVA $F_{1,62}$ = 0.840, P = 0.363) or the interaction of sex and species (ANOVA $F_{1,62}$ = 0.699, P = 0.406) (fig. 29).

E_2 levels in blood samples were validated by UPLC-MS/MS. The 3 samples analysed from AG blood were not detected and the three from YLG were in the same range than values found by RIA (table 23).

Table 23. Comparison of 17β-estradiol concentrations in 3 samples of *Larus micahellis*' blood (ng/mL wet weight (ww)) from Ebro Delta using RIA and UPLC MS/MS methods.

Sample	RIA	UPLC MS/MS
1	7.43	53
2	169.73	240
3	95.12	139

7.5 Discussion and conclusions

The significantly high levels of T in the eggs from YLG of the Atlantic colony (Atlantic Is.) could be explained due to specific geographical differences in diet among colonies and individuals. Differences in feeding habits are found between YLG individuals from the Atlantic Is. colony (Moreno et al., 2010). Atlantic Is. are situated in an upwelling area so high levels of primary production and abundant prey resource are characteristic. In the Mediterranean colonies, YLG tend to feed in food derived from human activities, such as garbage and fishery discards (Ramos et al., 2009). In recent studies, it has been shown that the type of diet can alter animal steroids levels (Ruhlen et al., 2011; Ploumidou et al., 2010). In addition, Verboven et al. (2005) proved that a correlation exists between the nestling environment and the hormone content of eggs.

No statistical differences were found in T neither in E_2 levels between YLG and AG eggs. As it is known, females transfer specific components to the eggs (such as antibodies, steroid hormones and carotenoids) which influence the chicks' growth and survival. The transfer of maternal T enhances the development and competitive ability of the offspring (Verboven et al., 2005). The amount of hormones transferred varies among females depending on social and non-social variables (such as food availability, mate attractiveness, laying date, sexual competition and maternal parasite) (Carere and Balthazart, 2007). Hormone levels in birds' eggs may vary depending on the order of laying, food availability, station and quality of the male and social interactions (Engelhardt et al., 2009).

Different levels of T and E_2 were found in egg yolk of an insectivorous bird, *Parus major* (18 ng T/g ww and 8 ng E_2/g ww) (Groothius et al., 2005) which comparing to our values, considering a 35 % of yolk in egg (Ricklefs, 1977), are in an intermediate burden position (conversion of their yolk values to egg values: 6.3 ng T/g egg and 2.8 ng E_2/g egg ww).

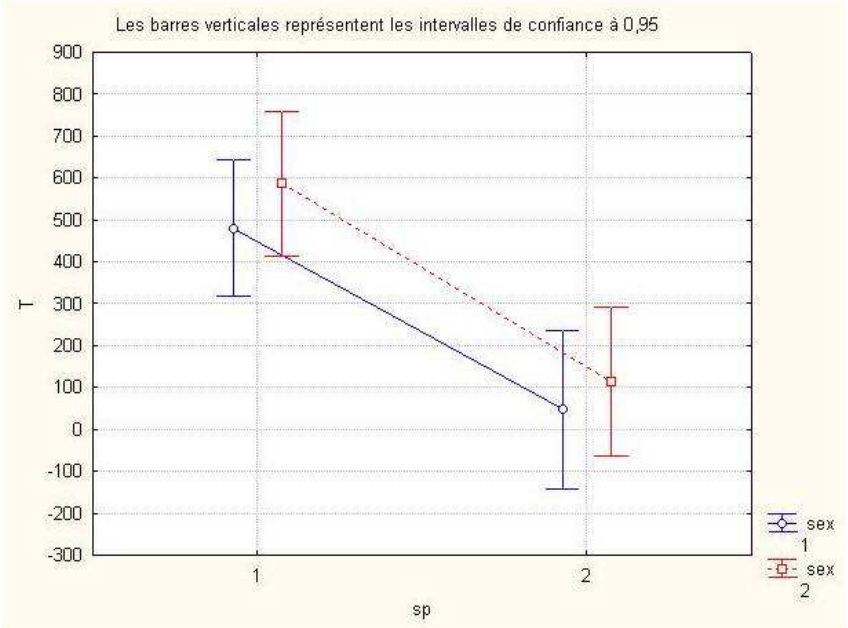

Figure 28. T levels (ng/mL ww) in blood of YLG (n = 17) and AG (n = 15) males (blue) and females (red) (YLG, n = 19; AG, n = 12) from the Ebro Delta Natural Park.

Levels between 7.9 and 11.4 ng T/g ww and 0.7 – 1.09 ng E_2/g ww were described for the piscivorous bird *Podiceps cristatus* (considering a 75% of water content in bird eggs) (Cortinovis et al., 2008). Similar steroid levels have been reported in eggs of *L. hyperboreus* (2.79 – 9.3 ng T/g ww and 0.04 – 0.2 ng E_2/g ww; piscivorous) (Verboven et al., 2008), *L. ridibundus* (0.16 – 0.39 ng T/g ww; omnivorous and opportunistic) (Eising et al., 2003) and *L. fuscus* (2.74 – 4.10 ng T+DHT (dihidrotestosterona)/g ww; omnivorous and opportunistic) (Royle et al., 2001).

Few differences were found between T levels in blood for both species while E_2 levels were higher in YLG. The blood analysis by UPLC-MS/MS confirms the high values of E_2 in YLG blood levels and the theory that the RIA method is more sensible than the UPLC MS/MS for the analysis of estrogens (Faupel-Badger et al., 2010). For both hormones, YLG blood presented significant higher levels than AG, but E_2 concentration in YLG blood was remarkably higher than in AG blood.

Compared to our results, slightly higher levels of T (0.37 - 1.09 ng/mL) were found in plasma of *Fregata magnificiens* males (a pelagic piscivorous species) (Madsen et al., 2007). Levels of T reported by Williams et al. (2004) in the plasma for the insectivorous bird *Sturnus vulgaris* (0.09 – 0.38 ng/mL) were similar than the levels found in the present study for YLG. Whereas E_2 levels in plasma of *S. vulgaris* (0.05 – 0.20 ng/mL) (Williams et al., 2004) were much lower. In tropical birds, E_2 levels in plasma are nearly baseline levels and do not show any seasonal variation, while T, despite presenting low levels (maximum of 1.57 ng/mL in males),

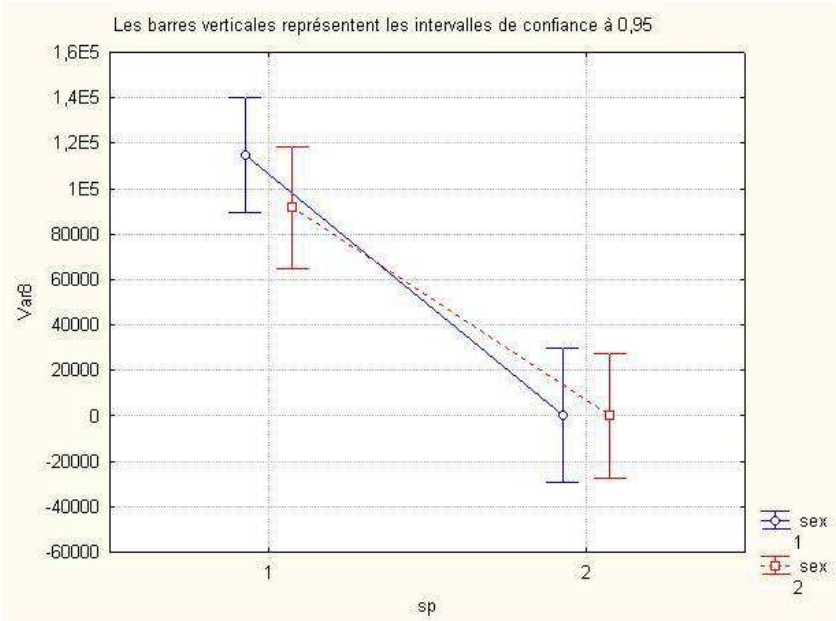

Figure 29. E_2 levels (ng/mL ww) in blood of YLG (n = 17) and AG (n = 15) males (blue) and females (red) (YLG, n = 19; AG, n = 12) from the Ebro Delta Natural Park.

are more oscillating along the year (Hau et al., 2000; Wikelski et al., 2000). Shah et al. (2011), also found lower E_2 levels in plasma of developing *Sturnus vulgaris* (insectivorous) with no significant difference between sexes (0.003 – 0.006 ng E_2/mL in females and 0 – 0.011 ng E_2/mL in males). Furthermore, in a study with *Sula granti* (feeder of marine fish and invertebrates), levels between 0.05 and 0.2 ng T/mL in plasma were described, while E_2 was not detected (Tarlow et al., 2003). To our knowledge, the highest T and E_2 levels in avian plasma reported before was for *Spheniscus humboldti*, a seabird also feeder on pelagic fishes (maximum T concentration in males: 3.32 ng/mL, in females: 1.15 ng/mL; maximum E_2 concentration in males: 1.12 ng/mL and in females: 1.27 ng/mL). These hormonal levels suffer an oscillation along the year showing highest values during the breeding season and minimal throughout moulting (Otsuka et al., 1998). This variation was already observed in *L. occidentallis wymani* (Wingfield et al., 1980 and 1982).

T levels can fluctuate also as a result of blood parasite infection (Madsen et al., 2007). For example, Valkiūnas (2005) indicated that haemosporidian (protozoan parasites) infections may be related to gonadal hormones. Williams et al. (2004) also found that levels of steroids vary during the laying period, with E_2 suffering more fluctuation than T. At the beginning of the laying period, E_2 level increases rapidly, while it decreases linearly during later stages of the laying cycle (Williams et al., 2004). T regulates also avian behaviour (song and aggression) during the breeding season (Hau et al., 2000). Maximum levels of T in plasma of seabirds have been described in prelaying period and thereafter it decreases until breeding time. In *Larus occidentalis*, females and males presented similar levels of T, as they share the territorial defence (Lormeé et al., 2000). Ros et al. (2002) also demonstrated that the level of T in blood of

L. ridibundus is dependant of the threat condition (both adults like chicks). In addition, Tarlow et al. (2003) found that T levels in *Sula granti* chicks were correlated with the adults' visits, being lower in those chicks with more parental care. So, T fluctuation is not exclusive of the sexual context.

Most of studies are based on T levels but little is known about the relation of E_2 levels and behaviour or feeding habits. Our results of T and E_2 in eggs show similar levels than those found in previous studies for different avian species. T levels in blood are also comparable for both target species. But, despite E_2 blood levels of AG are similar to other species, YLG show the highest levels. Schlinger et al. (1992) found that E_2 could increase in females with aggressive or sexual behaviour. In other studies, a relation in the hormones burden with estrogenic impact of certain organic and inorganic compounds has been observed (such as phenols, DDT, PCB, phthalates, *brominated flame retardants* (BFRs), PBDEs), which are also found in birds' diet (Jobling et al., 1995; Giesy et al., 2003; Verboven et al., 2008). They can reach the animals and bioaccumulate along the food web (Tomy et al., 2004; Morales et al., 2012). Verreault et al. (2006) investigated the relationship between organohalogen contaminants and reproductive hormones in *Larus hyperboreus* plasma and they found a correlation among the pollutants and progesterone in males. No such correlation was found for females neither for any compound with T. However, Verreault et al. (2006) did not detect E_2 in *L. hyperboreus* plasma. Sanderson et al. (2002) studied the action of certain pesticides on the aromatase activity that catalyses the transformation of T to E_2. They found that while dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE) or imazalil, among other compounds, decreased or inhibited the aromatase activity, other pesticides such as vinclozolin or atrazine increased its activity (Sanderson et al., 2002). Furthermore, Cantón et al. (2005) showed that most of the brominated flame retardants analysed (such as 4-bromophenol (4BP) and tetrabromobisphenol A (TBA)) inhibited the aromatase, while tribromophenol (TBP) induced its activity.

As mentioned before, AG is a piscivorous species while YLG is an omnivorous one, feeding also from landfills (Duhem et al., 2005). Munilla et al. (1997) reported that YLG diet in Galicia (north-west of the Iberian Peninsula) was composed of 40.1% of rubbish, 36.4% of *Polybius henslowii* (a species of marine crab) followed by pelagic and batipelagic fishes. So, YLG is more exposed to those endocrine disrupting compounds found in the environment. Considering the different diet of the target species and the disrupting endocrine action of certain anthropogenic compounds, the steroid levels were compared with the levels of perfluoroalkyl and polyfluoroalkyl substances (PFASs) analysed in the same samples and reported in a previous study by the same group (Vicente et al., in preparation) but no relation was found. Such comparison excludes PFASs as potential endocrine disruptor in AG and YLG.

Our results, in first term, describe the levels of E_2 and T in eggs of YLG and AG from the Iberian Peninsula and adults' blood from both species from the Ebro Delta colony. The high levels found for E_2 in the blood from YLG males and females may be associated to their opportunistic feeding habits that expose YLG individuals to endocrine disrupting compounds between others. Further studies examining the steroids biological mechanisms are required.

7.6 Acknowledgements

The Natural and National Parks studied authorized this scientific study. Jordi Ruiz from the Servei de Protecció i Gestió de la Fauna (Catalan Government) and Javier Zapata from the Ministry of Environment in Spain are acknowledged for sampling permission. This study was financed by the Ministry of Education of Spain through a bilateral program between Spain and Portugal and by the Ministry of Environment, project [2009/038].

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Chapter 8. General conclusions

The studies performed in this thesis have given rise to the following conclusions:

1. Yellow-legged and Audouin's gull accumulate contaminants due to their feeding habits and are transferred to their eggs. Thus, eggs become excellent bioindicators of environmental pollution.
2. The transfer of pollutants to the offspring via laying eggs reduces the concentration of these compounds in females' blood.
3. Among the different families of POPs analyzed in yellow-legged and Audouin's gull eggs, OCs and markers PCBs are the most ubiquitous compounds detected followed by PFASs, PBDEs and SCCPs.
4. PFASs are widespread in eggs of gulls from breeding colonies with high ecological interest of the Iberian Peninsula. PFOS is the most abundant compound showing levels between 10.1 to 110.2 ng/g in eggs. This is attributed to the high production of PFOS for industrial products and consumer goods, the consequent release to the environment and the high accumulation potential.
5. Using eggs of yellow-legged gull as biomonitoring matrix, PFOS concentration is higher in the most industrialized sites of the Catalan coast (Medes, Ebro Delta and Columbretes) compared to the southern Mediterranean (Dragonera, Grosa Island and Chafarinas) or Atlantic colonies (Berlengas and Atlantic islands of Galicia).
6. Egg and eggshell parameters are significantly different among all colonies studied, but they are not correlated with PFOS levels which mean that PFOS does not affect shell formation. Such differences in shell biometry are attributed to the specific characteristics of each sampling zone.
7. PFOS is the most abundant PFASs both in blood and eggs from yellow-legged and Audouin's gull from the Ebro Delta Natural Park. The slight differences in pollutants levels between the two gull species from the colonies cohabiting in the Ebro Delta are associated to different feeding habits. PFOS intake via diet is higher than the sum of PFOS depuration rate and its excretion via eggs which leads to an accumulation of PFOS in yellow-legged and Audouin's gull adults
8. PFASs have more affinity for egg-yolk proteins than for proteins from albumen, as PFASs were not detected in eggs' albumen of yellow-legged and Audoui'n gull. In egg-yolks, PFOS was the main PFAS detected followed by PFNA, PFHxS and PFOA.
9. Within a clutch of Audouin's gull colony from the Ebro Delta, PFOS concentration decreased according to the laying order of the eggs, showing significant differences

between each category of eggs (a-, b- and c-eggs). Furthermore, good correlation was observed for PFOS concentration within the 3 eggs from the same clutch.

10. A “mother effect” was found in Audouin’s gull eggs. The higher concentration of some PFASs in the 3rd egg (c-) of the clutch and the significant differences of carbon stable isotopes values found between b- *vs* c-eggs and a- *vs* c- eggs is attributed to the fact that *L. audouinii* females could use endogenous reserves for the formation of the last egg. The formation of the a- and the b- eggs is associated with the energy taken from the food gathered the days before the laying.
11. 17β-estradiol and testosterone levels were analysed in blood and eggs of yellow-legged and Audouin’s gull. The two steroids levels are significantly different in both species, but no differences were found between sexes. No relation was found between PFASs and steroid levels. The higher values of 17β-estradiol in blood of yellow-legged compared to Audouin’s gull blood are associated to different feeding habits, behavior and intrinsic biology.

Future recommendations:

- Further studies with extended temporal sampling are required to evaluate the effects that organic pollutants may produce in gulls and to understand the PFASs dynamics in gulls and the accumulation along the food webs. Studies of pollution in top predators, such as gulls, leads to evaluate the transfer of pollutants along the food web and therefore to assess the status and impact of environmental pollution in the ecosystems.

APPENDIX A. Other articles

- Gómez, C., Vicente, J., Echavarri-Erasun, B., Porte, C., Lacorte, S. 2011. Occurrence of perfluorinated compounds in water, sediment and mussels from the Cantabrian Sea (North Spain). Marine pollution bulletin 62(5): 948-955.

- Sánchez-Avila, J., Fernandez-Sanjuan, M., Vicente, J., Lacorte, S. 2011. Development of a multi-residue method for the determination of organic micropollutants in water, sediment and mussels using gas chromatography–tandem mass spectrometry. Journal of Chromatography A 1218(38): 6799-6811.

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