

A watercolor landscape painting. The top portion is a light blue sky. Below it is a deep blue sea. The middle ground features several large, dark brown, rounded rock formations. The foreground is a mix of green and yellow-green, with scattered dark brown rocks and a path of small red spots leading towards the bottom center.

Dissertation

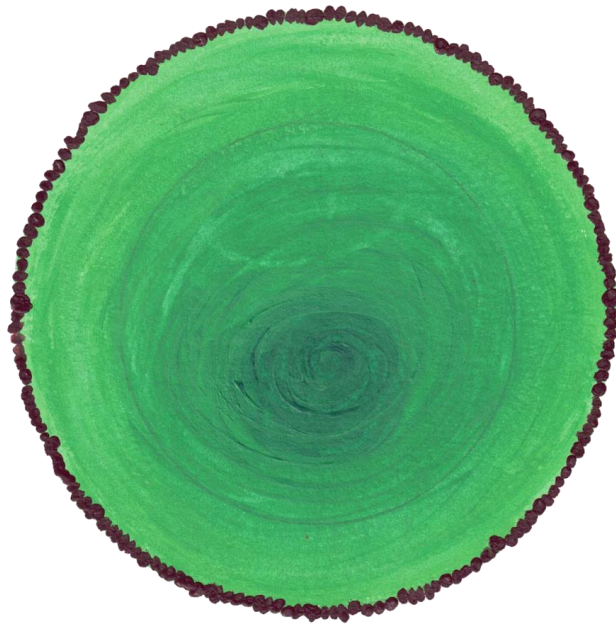
Lucie Michel

**Linking seabird behaviour and environmental toxicology:
An assessment of pollutant exposure, biological matrices
for analysis, and foraging ecology**

Lucie Michel

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Giessen, January 2025

Lucie Michel

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Abstract

Marine ecosystems support human well-being by providing food and helping regulate the climate, but face threats from overexploitation and degradation. Among various threats, pollution has emerged as a major concern. The Mediterranean Sea, in particular, is heavily impacted by pollution, emphasizing the need for comprehensive pollution monitoring. Especially of pollutants, that are subject to bioaccumulation due to their high stability in the environment. Despite this, current efforts to monitor persistent organic pollutants (POPs) lack sufficient geographical coverage, and no standardized evaluation protocols exist for seabirds and marine mammals. Environmental toxicology is a field dedicated to investigating the sources, pathways, and impacts of pollutants as they move through air, water, soil, and living organisms. For lower trophic organisms, pollutant assessment is more straightforward, but grows more complex in highly mobile and long-lived species like seabirds and marine mammals. Despite this, it is crucial to assess pollutants in higher trophic organisms, not only because they play a key role in biodiversity and integrate magnification processes along the food chain, but also because they occupy a similar trophic position to humans as consumers of fish. Scopoli's shearwaters (*Calonectris diomedea*) are long-lived Mediterranean seabirds, adapting their behaviour to seasonal needs but are impacted by anthropogenic stressors and chemical pollution. This PhD thesis focuses on three critical factors that influence the quality and interpretability of pollutant biomonitoring studies in seabirds: (1) development of analytical methods, (2) the choice of tissue matrix, and (3) the foraging ecology of the study species. The goal was to establish baseline data on pollutant levels, diet, and foraging behaviour in Scopoli's shearwaters and to develop effective methods for advancing environmental toxicology research in seabirds, focussing on Scopoli's shearwater as a potential indicator species. A range of methods were employed, including liquid and gas chromatography coupled with mass spectrometry for pollutant analysis, isotope ratio mass spectrometry for stable isotope analysis, DNA metabarcoding for molecular diet analysis, and camera loggers to track foraging behaviour. Bioaccumulative organic legacy and in-use pollutants were detected in all analysed Scopoli's shearwater tissues. Blood values likely reflect local and temporal differences in pollutant burdens. In the study year 2020, isotopic niche was relatively narrow in Scopoli's shearwaters during chick-rearing and pollutant concentrations were lower than during incubation in males. Sample choice depends on the research goal and pollutant type, but blood samples reflect current contamination, support population comparisons and several explanatory variables can be included in routine analyses. Pollutant exposure is likely influenced by foraging strategy and sex. Artisanal fisheries play a role for foraging Scopoli's shearwaters, but distinguishing prey sources (natural vs. discards) is challenging due to size similarities and variable species distribution. This research underscores the importance of interdisciplinary collaboration between analytical chemists and behavioural ecologists to push the boundaries of environmental toxicology.

Zusammenfassung

Marine Ökosysteme tragen zum Wohlbefinden des Menschen bei, indem sie Nahrung liefern und zur Klimaregulierung beitragen. Sie sind jedoch durch Übernutzung und andere anthropogenen Einflüsse bedroht. Eine Herausforderung stellt die Verschmutzung dar und besonders das Mittelmeer ist stark von dem Eintrag von Schadstoffen betroffen. Daher ist die Durchführung von Schadstoff Monitorings von großer Relevanz, insbesondere von Stoffen, die eine hohe Stabilität in der Umwelt aufweisen und daher in Lebewesen bioakkumuliert werden. Derzeitige Schadstoff Monitoring Programme zur Überwachung persistenter organischer Schadstoffe (POPs) sind geographisch unzureichend verteilt und es gibt noch keine standardisierten Bewertungsverfahren für Seevögel und Meeressäuger. Die Umwelttoxikologie untersucht Quellen, Verbreitungswege und Auswirkungen von Schadstoffen die durch Luft, Wasser, Boden und lebende Organismen transportiert werden. Für Lebewesen aus dem unteren Bereich der Nahrungskette ist die Bewertung von Schadstoffen direkter, wird jedoch bei sehr mobilen und langlebigen Arten deutlich komplexer. Dennoch ist die Bewertung von Schadstoffen auf höherer trophischer Stufe von entscheidender Bedeutung, da Spitzenprädatoren nicht nur wichtig für die Biodiversität sind und Prozesse der Anreicherung entlang der Nahrungskette integrieren, sondern auch weil sie als Fischkonsumenten eine ähnliche trophische Position einnehmen wie der Mensch. Gelbschnabel Sturmtaucher (*Calonectris diomedea*) sind langlebige Seevögel des Mittelmeers, die ihr Verhalten saisonalen Bedürfnissen anpassen und durch anthropogene Einflüsse und Schadstoffe beeinträchtigt werden. Diese Dissertation konzentriert sich auf drei entscheidende Faktoren, die die Qualität und Interpretierbarkeit von Biomonitoring-Studien zu Schadstoffen bei Seevögeln beeinflussen: (1) die Entwicklung analytischer Methoden, (2) die Wahl der Gewebe-Matrix und (3) die Ernährungsökologie der untersuchten Art. Ziel dieser Arbeit war es, Grundwerte über Schadstoffbelastung, Ernährung und Nahrungsverhalten in Gelbschnabel Sturmtauchern zu erheben und effektive Methoden für die Weiterentwicklung der Forschung zur Umwelttoxikologie in Seevögeln zu entwickeln, wobei der Gelbschnabel Sturmtaucher als potentielle Indikatorart im Fokus stand. Dazu wurden verschiedene Methoden eingesetzt, darunter Flüssigkeits- und Gaschromatographie in Kombination mit Massenspektrometrie zur Schadstoffanalyse, Isotopenverhältnis-Massenspektrometrie für stabile Isotopenanalysen, DNA-Metabarcoding für molekulare Nahrungsanalysen und Kameralogger zur Untersuchung des Jagdverhaltens. Bioakkumulierte organische Schadstoffe aus chemischen Altlasten und sich in Gebrauch befindenden Substanzen wurden in allen untersuchten Geweben von Gelbschnabel Sturmtauchern nachgewiesen. Die Blutwerte spiegeln vermutlich lokale und zeitliche Unterschiede in der Schadstoffbelastung wider. Im Untersuchungsjahr 2020 war das isotopische Nischenspektrum der Gelbschnabelsturmtaucher während der Brutzeit relativ eng, und die Schadstoffkonzentrationen waren bei Männchen während der Brutpflege geringer als während der Inkubationszeit. Die Wahl des Probenmaterials sollte vom Forschungsziel und vom untersuchten Schadstofftyp abhängig gemacht werden. Blutproben eignen sich gut, um die aktuelle Kontamination darzustellen, sowie für Populationsvergleiche und ermöglichen es mehrere erklärende Variablen in rou-

tinemäßige Analysen einzubeziehen. Die Schadstoffexposition wird vermutlich durch die Nahrungsstrategie und das Geschlecht beeinflusst. Kleinfischerei hat Einfluss auf das Jagdverhalten der Gelbschnabel Sturmtaucher, wobei die Unterscheidung von Beutetieren nach Fischereiabfällen oder natürlich erbeuteten Fischen aufgrund der ähnlichen Größe und variierenden Verbreitung der Arten herausfordernd ist. Diese Forschung betont die Bedeutung der interdisziplinären Zusammenarbeit zwischen analytischen Chemikern und Verhaltensökologen, um die Grenzen der Umwelttoxikologie weiter zu verschieben.

Abbreviations

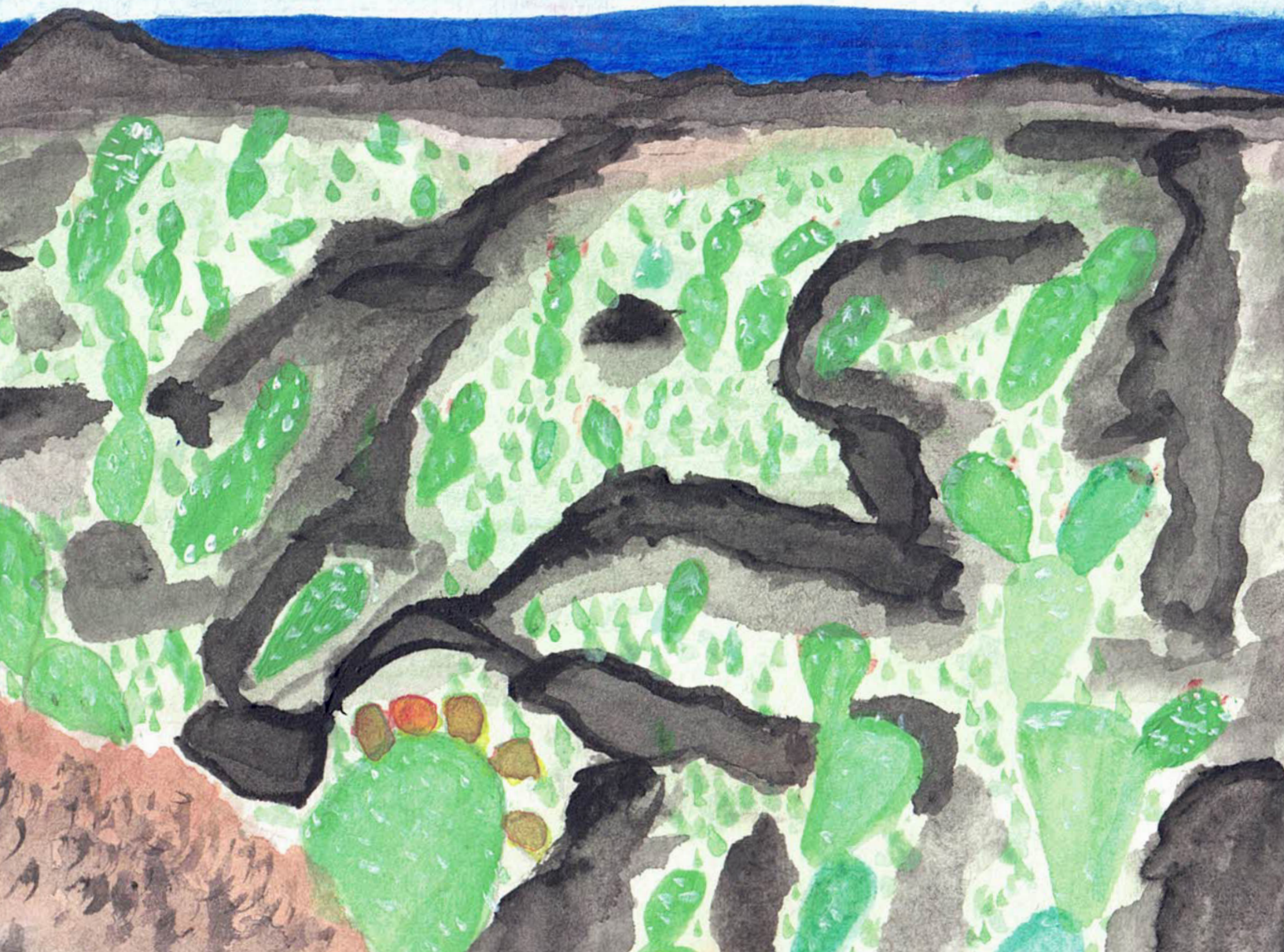
µg	Microgram
µL	Microlitre
2,4'-DDE	1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
4,4'-DDE	1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene
4,4'-DDE-D8	Deuterium labeled 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene
2,4'-DDD	1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane
4,4'-DDD	1,1-dichloro-2,2-bis(4-chlorophenyl)ethane
2,4'-DDT	1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane
4,4'-DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
α-HCH	α-1,2,3,4,5,6-hexachlorocyclohexane
β-HCH	β-1,2,3,4,5,6-hexachlorocyclohexane
γ-HCH	1α,2α,3β,4α,5α,6β-hexachlorocyclohexane (Lindane)
δ-HCH	δ-1,2,3,4,5,6-hexachlorocyclohexane
AD	Adjusted for baseline stable isotope values
BPs	Bisphenols
BzPs	Benzophenones
CAS	Chemical Abstracts Service Registry Number
CEMP	Coordinated environmental monitoring programmes
Cen	Censored Observation
CI	Confidence Intervals
DCM	Dichloromethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichlorethane
DNA	Deoxyribonucleic acid
EI	Electron Ionization
EU	European Union
FO	Frequency of Occurrence
g	Gram
GC-MS	Gas Chromatography coupled with Mass-Spectrometry
HCB	Hexachlorobenzene
HELCOM	Helsinki Commission
Hg	Elemental mercury
IDL	Instrument Detection Limit
IQL	Instrument Quantification Limit
IS	Internal Standard
L	Litre
LCI95	Lower 95 Confidence Interval
LC-MS	Liquid Chromatography coupled with Mass-Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification
MAP	Mediterranean Action Plan
mg	Microgram
MDL	Method Detection Limit
ME	Matrix Effect
MEDPOL	Marine Pollution Assessment and Control Programme
mL	Millilitres
mLOD	Method Limit of Detection
mLOQ	Method Limit of Quantification
MQL	Method Quantification Limit
MSFD	Marine Strategy Framework Directive
<i>m/z</i>	mass-to-charge ratio

ng	Nanogram
NIST	National Institute of Standard and Technology
OCs	Organochlorines
OCPs	Organochlorine pesticides
OPEs	Organophosphate esters
OSPAR	Oslo and Paris Commissions
PAHs	Polyaromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PC	Principle Component
PCA	Principal Component Analysis
PCBs	Polychlorinated biphenyls
PCB 16	2,2',3-Trichlorobiphenyl
PCB 24	2,3,6-Trichlorobiphenyl
PCB 38	3,4,5-Trichlorobiphenyl
PCB 49	2,2',4,5'-Tetrachlorobiphenyl
PCB52	2,2',5,5'-Tetrachlorobiphenyl
PCB 61	2,3,4,5-Tetrachlorobiphenyl
PCB 62	2,3,4,6-Tetrachlorobiphenyl
PCB 65	2,3,5,6-Tetrachlorobiphenyl
PCB 77	3,3',4,4'-Tetrachlorobiphenyl
PCB 85	2,2',3,4,4'-Pentachlorobiphenyl
PCB 99	2,2',4,4',5-Pentachlorobiphenyl
PCB 101	2,2',4,5,5'-Pentachlorobiphenyl
PCB 110	2,3,3',4',6-Pentachlorobiphenyl
PCB 116	2,3,4,5,6-Pentachlorobiphenyl
PCB 118	2,3',4,4',5-Pentachlorobiphenyl
PCB 128	2,2',3,3',4,4'-Hexachlorobiphenyl
PCB 138	2,2',3,4,4',5'-Hexachlorobiphenyl
PCB 146	2,2',3,4',5,5'-Hexachlorobiphenyl
PCB 149	2,2',3,4',5,6-Hexachlorobiphenyl
PCB 153	2,2',4,4',5,5'-Hexachlorobiphenyl
PCB 156	2,3,3',4,4',5-Hexachlorobiphenyl
PCB 167	2,3',4,4',5,5'-Hexachlorobiphenyl
PCB 170	2,2',3,3',4,4',5-Heptachlorobiphenyl
PCB 180	2,2',3,4,4',5,5'-Heptachlorobiphenyl
PCB 183	2,2',3,4,4',5,6-Heptachlorobiphenyl
PCB 189	2,3,3',4,4',5,5'-Heptachlorobiphenyl
PCB 194	2,2',3,3',4,4',5,5'-Octachlorobiphenyl
PCB 73	2,3',5',6-Tetrachlorobiphenyl
PCB 97	2,2',3,4',5'-Pentachlorobiphenyl
PCB 126	3,3',4,4',5-Pentachlorobiphenyl
PCB 151	2,2',3,5,5',6-Hexachlorobiphenyl
PCB 157	2,3,3',4,4',5'-Hexachlorobiphenyl
PCB 200	2,2',3,3',4,5,6,6'-Octachlorobiphenyl
PCDDs	Polychlorinated-p-dioxins
PCDFs	Polychlorinated dibenzofurans
PCN	Polychlorinated Naphthalenes
PFAS	Poly- and perfluoroalkyl substances
PFCs	Perfluorinated carboxylates
PFPeA	Perfluoropentanoic acid
PFHxA	Perfluorohexanoic acid
PFHpA	Perfluoroheptanoic acid
PFOA	Perfluorooctanoic acid
PFOA 13C8	Perfluorooctanoic acid labelled with 13C8
PFNA	Perfluorononanoic acid
PFDA	Perfluorodecanoic acid

	PFUNA	Perfluoroundecanoic acid
	PFDODA	Perfluorododecanoic acid
	PFTriDA	Perfluorotridecanoic acid
	PFTDA	Perfluorotetradecanoic acid
	PFHxDA	Perfluoro-n-hexadecanoic acid
	PFOcDa	Perfluorooctadecaonic acid
	P37DMOA	Perfluoro-3,7-dimethyloctanoic acid
	7H-PFHpA	7H-Dodecafluoroheptanoic acid
PFSAs		Perfluoroalkyl Sulfonates
	PFBS	Perfluorobutanesulfonic acid
	PFPeS	Perfluoropentane sulfonic acid
	PFHxS	Perfluorohexane sulfonic acid
	PFHpS	Perfluoroheptane sulfonic acid
	PFOS	Perfluorooctanosulfonic acid
	PFOS 13C8	Perfluorooctanesulfonate labelled with 13C8
	PFNS	Perfluorononane sulfonic acid
	PFDS	Perfluorododecane sulfonic acid
	PFDODS	Perfluorododecane sulfonic acid
	PFECHS	Perfluoroethylcyclohexane sulfonic acid
FTS		Fluorotelomer Sulfonates
	4:2 FTS	1H,2H-Perfluorohexan sulfonate (4:2)
	6:2 FTS	1H,2H-Perfluorooctane sulfonate (6:2)
	6:2 FTS 13C2	1H,2H-Perfluorooctane sulfonate labelled with 13C2
	8:2 FTS	1H,2H-Perfluorodecan sulfonate (8:2)
	10:2 FTS	1H,2H-Perfluorododecan sulfonate (10:2)
FSA		Fluorosulfonamide
	FOSAA	Perfluoro-1-octanesulfonamidoacetic acid
	MeFOSAA	2-(N-methylPerfluoro-1-octansulfonamido) acetic acid
	PFOSA	Perfluorooctane sulfonamide
	MeFOSA	N-methylPerfluoro-1-octanesulfonamide
	MeFOSE	N-(2-hydroxyethyl)-Nmethylperfluorooctane sulfonamide
	EtFOSE	N-ethyl-N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide
	EtFOSA	N-ethylperfluoro-1-octanesulfonamide
	Gen X	2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)propanoate
	ADONA	Dodecafluoro-3H-4,8-dioxanonanoate
	DecaS	Decasulfonic acid
	9Cl-PF3ONS	9-chlorohexadecafluoro-3-oxanonane-1-sulfonate
	diSAMPAP	Bis[2-(N-ethylperfluorooctane-1-sulfonamido)Ethyl]Phosphate
POPs		Persistent organic pollutants
RBCs		Red Blood Cells
RSD		Relative Standard Deviation
SCSH		Scopoli's shearwater
SD		Standard Deviation
SEAB		Standard Bayesian Ellipse Area
SEAC		Standard Ellipse Area corrected for small sample sizes
SI		Stable Isotope
S/N		Signal to noise ratio
SPE		Solid Phase Extraction
TL		Trophic Level
U		Uncertainty
UCI95		Upper 95% Confidence Interval
UPLC®-MS/MS		Ultra Performance Liquid Chromatography coupled to a Triple Quad- rupole Mass Analyser
UNEP		United Nations Environment Programme
WFD		Water Framework Directive
YESH		Yelkouan shearwater



Synthesis



1. Introduction

1.1 Understanding marine pollution - Its current state and our responsibility

Marine ecosystems are invaluable to humans, providing essential resources such as food and serving as critical component in regulating the global climate (Sitch et al. 2015). Despite their importance, marine resources are being overexploited, and many other human activities are impacting these ecosystems substantially (Halpern et al. 2008). Additionally, pollution and waste from land- and sea-based activities is being transported into the seas via rivers and the atmosphere (Barletta et al. 2019). The main input of chemicals and waste derives from farming (Pandit et al. 2006), urban and industrial waste water (Ahrens and Bundschuh 2014), fuel consumption, spills (Ariese et al. 2005) and incorrect disposal of consumer products (Gola et al. 2021). Some of these chemicals are toxic to marine organisms and some can persist, bioaccumulate and ultimately end up in our bodies by consuming fish and seafood (Burger et al. 2007).

Among the marine regions, the Mediterranean Sea is particularly affected by pollution (Ziveri et al. 2023). Its unique oceanographic features (Tanhua et al. 2013) and high population density favours higher pollutant concentrations. It is a hotspot for climate change with high vulnerability (MedECC 2020) and holds a sensitive balance of biodiversity (Cuttelod et al. 2009). A 2020 report about the pollution state of the Mediterranean Sea pointed out that the main known pollution source is litter (UNEP/MAP 2020) and the risk of plastic exposure to marine organisms is highest (Clark et al. 2023). However specific attention to the evaluation of long-lasting pollutants from unregulated releases in the past (heavy metals, polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), dioxins and furans, polyaromatic hydrocarbons (PAHs) and the recently emerging perfluoroalkyl substances (PFAS) is given. Temporal trends of legacy pollutant concentrations are decreasing, due to regulation efforts. However, the report highlights elevated heavy metal concentrations in mussels and lead, as well as mercury levels in sediment. Additionally, it notes limited data and insufficient geographical coverage on petroleum hydrocarbons and persistent organic pollutants (POPs).

Pollution and its accumulation represent significant yet often underestimated environmental and public health challenges. Monitoring pollutant levels is essential to preserving ecosystems and ensuring human safety. Typically, pollutant monitoring involves analytical assessments of chemicals in certain endpoints such as environmental samples of water and sediment, reflecting local contamination levels. Biomonitoring evaluates bioavailability of pollutants to organisms (mainly using bivalves and fish). Monitoring long-lived species such as seabirds adds another layer of relevance to the study of environmental pollutant levels as they are subject to bioaccumulation. Additionally, their role as top predators allows reflecting pollution dynamics across the food web, including the phenomenon of biomagnification (Burger and Gochfeld 2002). Acting as indicators of marine ecosystem health (Montevecchi 1993, Furness and Camphuysen 1997, Piatt et al. 2007), seabirds not only accumulate contaminants but also act as vectors, transporting pollutants, particularly when forming large aggregations during their breeding periods (Signa et al. 2021). Thus, biomonitoring with seabirds provides critical information about

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pollutant accumulation and magnification, helping to infer risks to human consumers. However, using a highly mobile and long-lived top predator as sample organism for biomonitoring, usually adds complexity to the interpretation of the exposure as pollutant levels in tissues are a function of uptake and absorption (Burger and Gochfeld 2002) and toxicokinetic profiles can differ among tissues. Thus, using seabirds for biomonitoring requires target species- and pollutant type specific considerations during experiment design (Furness and Camphuysen 1997).

1.1.1 Importance of seabirds for the marine ecology, seabird threats and pollution exposure

As top predators, seabirds can influence marine biodiversity by causing trophic cascades and altering prey abundance (Ainley et al. 2015). Additionally, they contribute to the nutrient circulation and energy flow between land and sea (Signa et al. 2021) and the dispersal of genetic material, ensuring gene flow and promoting the colonization of new sites (Viana et al. 2013).

The main threats to seabirds, recently summarized by Phillips et al. (2023), are fishery activities causing high mortality through bycatch and depletion of forage fish through overfishing, predation by synanthropic species such as rats and cats, climate change, disease and pollution. These threats are predominantly of anthropogenic origin or at least significantly intensified by human activities, and they are often interconnected.

Pollution impacting seabirds can be chemical substances, plastic or light. Artificial lights at night emitted from human structures, on the coast (Cianchetti-Benedetti et al. 2018a) and ships at sea affects seabird behaviour as they avoid colony attendance (Austad et al. 2023) and can lead to disorientation, collision (Guilford et al. 2019) or grounding of fledglings (Rodríguez et al. 2015, 2017, Crymble et al. 2020). Plastic pollution can harm seabirds through ingestion (Roman et al. 2019) or entanglement (Ryan 2018). Ingested plastics can also cause accumulation of chemical additives, such as UV-stabilizers or flame retardants (Yamashita et al. 2021). Other chemical pollutants subject to monitoring studies share the property of being persistent in the environment, meaning they are subject to long range transport, resist biological degradation, accumulate within organisms and magnify through the food chain. Metals such as cadmium, lead and mercury play an important role in seabird toxicology (Burger and Gochfeld 2002). Most metals occur naturally in the earth's crust and seawater but human activities such as coal powered electricity generation, industrial processes, mining, and the production and disposal of batteries and paints increase their environmental levels and bioavailability. Elemental mercury (Hg), and its methylated organic form, methylmercury, is a major focus of toxicological studies in seabirds (Provencher et al. 2014, Chastel et al. 2022). Seabirds exhibit some of the highest Hg concentrations observed in wildlife (e.g. mean body feather total Hg concentrations $\geq 20 \mu\text{g/g}$ in albatrosses reported by Cherel et al (2018), liver concentrations exceeding $200 \mu\text{g/g}$ reported by Muirhead and Furness (1988)) The associated health risks of elevated methylmercury to marine organisms range over hormonal changes, motoric impairment to decreased reproduction, while in humans adverse neurodevelopmental effects and psychiatric symptoms caused by long-term exposure are well documented (Driscoll et al. 2013). Organic chemicals include a variety of compounds that are summarized into groups according to their

Synthesis

chemical components and purpose (Fig 1). Some of them are listed as persistent organic pollutants (POPs) in the Stockholm Convention with the aim to eliminate, restrict their production and use, or eliminate their unintentional production (UNEP, 2024). This list comprises toxic chemicals like pesticides, insecticides, fungicides, industrial chemicals, flame retardants, and plastic additives, as well as unintentional byproducts from their production processes, occurring in the environment predominantly as legacy pollutants due to the previous elimination efforts (Table S1). Halogenated compounds form one of the largest groups of environmental pollutants, which are typically lipophilic, semi-volatile, resistant to degradation and persistent in environmental matrices like air, soil or biota (Jones and de Voogt, 1999). Organochlorines - hydrocarbons with a chlorination - encompass organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs) and dioxins (PCDDs) (Fig 1, orange circle). Other halogenated pollutant groups are polybrominated diphenyl ethers (PBDEs) or fluorinated flame retardants. Per- and polyfluorinated alkyl substances (PFAS) are a large group of versatile and persistent industrial chemicals which are categorised as emerging contaminants. Buck et al. (2011) presents a definition and nomenclature of PFAS, which reached scientific consent. Polycyclic aromatic hydrocarbons (PAHs) are molecules formed from planar aromatic rings and primarily derive from combustion of petroleum products but also occur naturally during wildfires or volcanic emissions for example. PAHs are moderately persistent. Their accumulation has been shown in biota, but biomagnification is prevented by metabolization (Nfon et al. 2008, Abdel-Shafy et al. 2016). Other emerging contaminants that hold compounds persistent to biodegradation are, bisphenols used as plastic additives and benzophenones mainly used as UV-filters. Additionally, persistent pharmaceuticals and organophosphate esters (OPEs) are potentially affecting coastal birds and their environment (Dulsat-Masvidal et al. 2023). Phthalates are plastic additives, which are not resistant to biodegradation, but their metabolites can be measured in biological matrices.

Synthesis

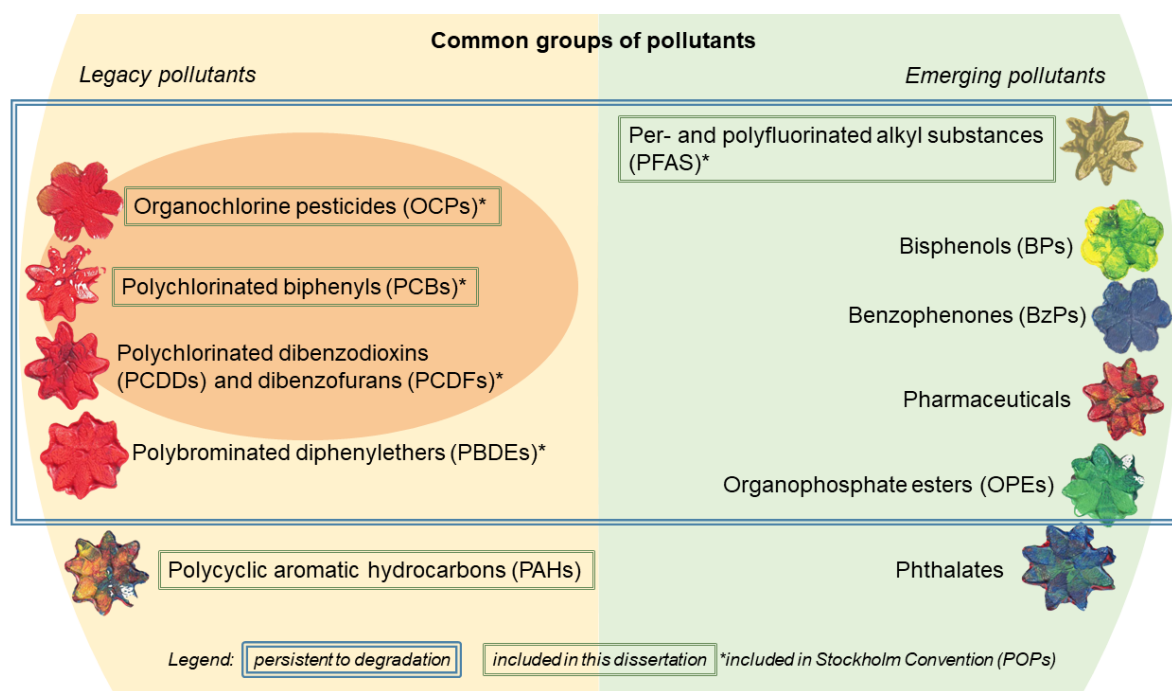


Figure 1 Overview of the common pollutant groups and their generic terms, abbreviation and categorization. Left side: Legacy pollutants are long-lasting toxic chemicals from past human activities that persist in the environment, posing ongoing risks to ecosystems and human health. Right side: Emerging pollutants are chemicals or substances recently detected in the environment that pose or may pose risks to human health or ecosystems, but are not yet fully regulated or understood. Orange circle: summarizes the organochlorines. Legend: Groups that are within the blue double line hold pollutants that are resistant to degradation and persist in the environment, Groups that are outlined with the green line hold pollutants, which are included in this thesis, Groups that are marked with asterisks hold pollutants that are regulated by the Stockholm Convention of persistent organic pollutants. (See table S1 for detailed information on Stockholm Convention POPs)

Mercury (Tartu et al. 2013) and many halogenated organic compounds, in particular long-chained perfluorinated carboxylates (PFCs) (Nøst et al. 2012, Tartu et al. 2014, Blévin et al. 2020), but also phthalates (European Commission, 2021), OPEs (Zhang et al. 2024), Benzophenone UV Filters and Bisphenols (González-Rubio et al. 2020, Kwon et al. 2021) are endocrine disruptors, meaning that they interfere with the normal functioning of the hormonal system. Toxic impacts of OCs on seabirds have been described on reproduction (Bustnes et al. 2003), development (Bustnes et al. 2002) and behaviour (Bustnes et al. 2001). Generally, the observed effects depend on pollutant dose- and species specific behaviour, ranges and life history strategies (Burger and Gochfeld 2002). They vary from acute lethality to sublethal or chronic effects and can cause population decline (Risebrough 1986). Breeding failure associated with OCs or mercury contamination ranges from egg- or embryo malformations (Burger and Gochfeld 2002), over reduced growth and chick survival (Burger and Gochfeld 1997) to impaired courtship behaviour and parental attendance (Fox et al. 1978, McArthur et al. 1983). Mercury contamination had an impact on steroid hormone levels in black legged kittiwakes and individuals with higher mercury levels tended to skip breeding (Tartu et al. 2013). However, associations between pollutant exposure at environmentally relevant concentrations and effects on physiological endpoints are often challenging to detect and disentangle, especially in the case of PFAS (Lopez-Antia et al. 2023). Firstly, this is because results frequently appear contradictory and not all measured variables have direct negative impacts on

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birds. For example, some studies found positive associations between PFAS exposure and body condition (Ask et al. 2021) or body mass (Barghi et al. 2018). Others found positive associations between PFAS exposure and time allocated to egg care (Blévin et al. 2020), as well as telomere dynamics (Sebastiano et al. 2020, Sebastiano et al. 2023) and antioxidant defence in nestlings (Lopez-Antia et al. 2019). Secondly, understanding these associations can be further complicated by their dependence on sex, the antagonistic interactions among contaminants (Blévin et al. 2017, Blévin et al. 2020) and seabirds' exposure to unknown contaminant mixtures (Rochman et al. 2015) and other stressors. Ultimately, shifts in energy and time allocation due to pollutant exposure may lead to imbalances, deficits, or trade-offs in other life-history traits (Blévin et al. 2020).

From a conservational perspective, seabirds are more than indicators of their environment or charismatic flagship species suitable for promoting of environmental awareness. Above all, they are at risk as they are not only affected by any of the above mentioned threats alone, but likely by all of them at the same time or at least a combination of them throughout their lifespan (Dias et al. 2019). Mortality incidents caused by fishery gear, scarcity of food through overexploited fish stocks, increased frequency of extreme weather conditions, as well as predation risk by synanthropic species are a reality and lethal or sublethal pollutant effects are stressors that act on top (Bustnes et al. 2015), especially in the case of unknown pollutant mixtures (Huber et al. 2015, Rochman et al. 2015). Therefore, monitoring of seabird populations is necessary for seabird conservation, for early detection of alarming effects and for fostering of mitigation methods.

1.1.2 Existing European monitoring programmes and coordinated studies on seabirds and pollutants

The Oslo and Paris Commissions (OSPAR) and the Helsinki Commission (HELCOM) are international organisations with a monitoring role focusing on different European marine regions. In 1975, the United Nations Environment Programme (UNEP) launched the Mediterranean Action Plan (MAP) to promote the health of the Mediterranean Sea. As part of this initiative, UNEP established the Marine Pollution Assessment and Control Programme (MED POL), a biomonitoring effort to assess the impact of pollutants on marine organisms. Also population management and monitoring programmes for threatened seabirds, which aim to assess population status number of breeding pairs, breeding success and clutch-size or egg volume were implied (UNEP/MAP 2012). Here, chemical pollution is recognised as a possible threat to Mediterranean seabirds, and shearwaters in specific (UNEP/MAP 2012). A review on the EU Member States' reports on monitoring programmes under the Marine Strategy Framework Directive (MSFD, 2008/56/EC) highlights a need for greater harmonisation and improved synergies, particularly for highly mobile species such as seabirds and transboundary issues such as chemical pollution and marine litter (Joint Research Centre European Commission 2023). OSPAR, which operates in the NE Atlantic agreed on coordinated environmental monitoring programmes (CEMP) which include contaminant monitoring protocols for seabird eggs (OSPAR 2018) and stomachs (ingested plastics) (Kühn et al. 2022). Within the EU water framework directive (WFD) aquatic biology is used as an indicator for the assessment of water bodies. In coastal waters, the WFD considers the three biological

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quality parameters phytoplankton, macrophytes and macrozoobenthos (BMUV/UBA 2022). Under the MSFD, zooplankton, fish, seabirds and marine mammals are added. Within eleven qualitative descriptors for achieving good environmental status, two address the pollution concentrations regarding pollutant limits and effects, which are set by the member states. Guidelines for biological effect studies were subsequently established for OSPAR, HELCOM and MEDPOL. MEDPOL presented a list of publications using a selection of biomarkers to reveal a syndrome characteristic of the animal response to environmental stressors and accumulated pollutants and suggested mussels and fish as target species (UNEP/MAP/MED POL 2007). A review highlights that effect-based methods must allow differentiation of contaminant-related effects from natural processes or other stressors. This requires an understanding of confounding factors, dose-dependency, toxicity mechanisms, and the development of quality assurance and assessment criteria for relevant species (Hylland et al. 2017). Such studies typically rely on controlled laboratory experiments to establish causal links between exposure and effects and understand the underlying mechanisms (Lopez-Antia et al. 2023), which is difficult to align with ethical standards, especially for wildlife. Further reasons why for seabirds and mammals such effect methods / assessment procedures have not been developed and tested for the implementation yet are the high effort to detect effects at environmental concentrations, the difficulty of isolating the effect against other unknown variables and the lack of suitable sample matrices. However, effects of contaminants have been linked to body condition, body mass, hatching success and survival, which are commonly recorded in population monitoring programmes. Furthermore, effects on telomere dynamics, oxidative stress, thyroid hormones, plasmatic cholesterol and triglyceride levels, have been demonstrated, which could be integrated into contaminant monitoring protocols by routine analysis of blood samples.

1.1.3 Seabird behaviour and environmental toxicology: Bridging insights for comprehensive research

The challenges in studying pollutant exposure in seabirds arise from the complexity, seasonality and individuality of seabird behaviour in combination with the interplay of different types of stressors, which calls for integration of knowledge from different disciplines. Environmental toxicology examines the sources, pathways, and impacts of these substances, including how they move through air, water, soil, and living organisms. Behavioural ecology links an animal's behaviour to its environment, resource availability, competition, and predator-prey interactions, providing a deeper understanding of why animals behave the way they do in nature. Behaviours such as feeding, mating, and social interactions, territoriality and parental investment are an adaptive (evolutionary) response to the environment the animal is living in and can be representative of individual and population health. Furthermore, diet and trophic ecology is a key factor influencing pollutant exposure (Mello et al. 2016, Sebastiano et al. 2017). A good understanding of such species-specific characteristics can add the dimension of population health and breeding conditions into the interpretation of pollutant levels, which is vital as impacts of POPs have been shown to increase during adverse environmental conditions (Bustnes et al. 2015).

1.2 Three key factors influencing the quality and informative value of seabird pollutant analyses

This chapter examines three key factors that affect the quality and interpretability of studies on seabird pollutant exposure. These factors form the three pillars of this dissertation, from which the research questions were derived.

1.2.1 Methodology considerations in seabird contaminant analysis

Limitations arise from both fieldwork and laboratory practices. In the field, seabird samples can generally only be obtained in small amounts using minimally invasive methods due to ethical considerations. In the laboratory and data analysis stages, robust sample sizes are needed to ensure sufficient sample preparation and statistical power. Analytical methods must achieve high sensitivity and reproducibility, as environmental pollutants typically occur as mixtures of legacy and emerging contaminants. The complexity of analysing contaminants in biological matrices requires quality control and method validation procedures. For example, guidelines like SANTE 11312/2021v2, originally developed for laboratories involved in the official control of pesticide residues in food and feed across the European Union (EU), have been applied to seabird contaminant monitoring (Oró-Nolla et al. 2024a). Similarly, CEMP guidelines for monitoring contaminants in biota provide technical details for sampling, analysis, quality assurance, and reporting related to contaminants in fish, shellfish, and seabird eggs (OSPAR, 2018).

The analytical method constrains the pollutant spectrum that can be analysed simultaneously. Procedures usually are optimized for specific contaminant classes, but even within these classes, the optimum varies, for example, with chlorination level in PCBs (Wiltschka et al. 2023). Standard analytical workflows for organic contaminants include homogenisation, drying, extraction with organic solvents, clean up, fractionation, and subsequent separation and detection of individual compounds by means of gas chromatography coupled with mass-spectrometry (GC-MS) or liquid chromatography coupled with mass-spectrometry (LC-MS). The principle of combining gas chromatography, which separates the compounds based on their retention time (time of holding on to a stationary phase) and mass spectrometry, which separates the ions by their mass-to-charge ratio (m/z), allows identification and quantification of the detected molecules. For less volatile compounds such as PFAS chromatography using a liquid mobile phase is more suitable than a gaseous phase, which is used for more volatile compounds such as OCs. Optimization is critical at various stages of sample preparation and analysis to obtain balance time and material expenses, sensitivity and resolution. The choice of sample extraction method depends on the stability of the target analyte, the time of processing a desired amount of samples and the technical setup of the chromatographic system. During extraction, compounds are transferred using their chemical and physical properties to move from one phase to another, which can be a solvent or a solid phase. The resolution of the chromatogram can be influenced by parameters like the oven programme, which should be optimized according to temperature at which analytes transition into the

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mobile phase, as well as the material, length and diameter of the stationary phase. During mass spectrometry ionization settings should be adjusted to obtain the most abundant fragments of the mass spectra. Ease and precision of identification and quantification can vary between the analysis technologies used in different laboratories and sample types. For instance, with LC-MS the resulting mass spectra often vary depending on the instrument, due to variations of ionization, mobile, and stationary phase and no general spectral library exists, whereas GC-MS provides reliable matches to general libraries (e.g. NIST Mass Spectral Libraries), which are instrument independent. Additionally, biological samples often contain lipids and proteins, which can cause signal interference and matrix effects that complicate quantification. The choice of internal standard influences the signal interpretability and identification. An optimized set of internal standards should cover the entire span of retention times, closely match the chemical structure of the target analyte, and ideally be isotopically labelled to avoid (m/z) overlap with contaminants present in the sample.

1.2.2 The choice of sample matrix

The choice of sample type depends on the study aim and the toxicokinetics of environmental contaminants in seabirds need to be considered. After ingestion, contaminants get absorbed in the intestine, transported via the bloodstream, and accumulated in internal tissues (e.g. liver, kidney, muscle and fat). Then, these substances are remobilized or redistributed to certain endpoints like feathers, eggs, excreta and preen oil (Carravieri 2014). Different sample types do not equally represent all pollutant classes due to variations in chemical affinity influenced by properties such as solubility, lipophilicity, and stability, which affect absorption potential. The CEMP guidelines (OSPAR 2018) suggest seabird eggs as favoured sample matrix for monitoring of temporal trends and spatial distribution of pollutants. Several studies have shown the applicability of eggs for an array of pollutant types (Barrett et al. 1985, Renzoni et al. 1986, Leat et al. 2011 and 2013, Blévin et al. 2020, Braune et al. 2016 and 2019, Kalia et al. 2021, Colomer-Vidal et al. 2022, Oró-Nolla et al. 2023 and 2024b). However, not in all cases eggs are applicable. Sampling eggs is invasive, especially in threatened species, which lay a single egg per season, like the species from the order tubenoses (Procellariiformes). Also, eggs represent only 50% of the pollutant exposure of breeding seabirds: the exposure of the female, during egg production. Less invasive is the sampling of blood, which represents the current load that is circulating in the body and which can be obtained from targeted, healthy individuals, representative of the entire breeding population. Blood samples reflect relatively recent exposure, because blood cells are continuously being produced and have a lifespan of several weeks (Pérez et al. 2008), but also long-term exposure when contaminants are remobilized (Carravieri 2014). Feathers are least invasive as they can easily be removed from a living bird without causing severe damage, if not vital for flight performance (Jaspers et al. 2007). Moulded feathers can be sampled opportunistically during the respective moulting season even without disturbing or catching the birds. They integrate the pollutant load during the time of feather growth (Peterson et al. 2019), so during the year prior to sampling. Therefore, comparisons of feather concentrations with other tissues are difficult because of this temporal mismatch (Chastel et al. 2022). Further

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complications for interpretation of feather concentrations arise from timing and location differences of moult (Ramos et al. 2009a), differences among feather types (Ackerman et al. 2016), within and among variability of individual feathers (Peterson et al. 2019). However, recommendations on feather sampling design and quality assurance protocols are being developed (García-Fernández et al. 2013, Jaspers et al. 2019). Organs like liver, kidney or brain are important sites for accumulation and excretion, however they can only be obtained from deceased birds (e.g. Lu et al. 2024), which either implies invasive or somehow biased sampling.

Ideally, the distribution and concentrations of various contaminants should be studied across tissue types to better reflect their chemical partitioning, exposure pathways, and excretion mechanisms, encompassing processes such as uptake, current body burden, and elimination. This approach would enable the determination of pollutant-specific profiles and partitioning factors that are broadly applicable to help identify the organs most affected and assess risks and regulatory toxicology of contaminants.

1.2.3 The movement and foraging ecology of the study species

The foraging and movement ecology of seabirds, like the dynamics of the aquatic environment, is defined by flexibility. Foraging strategies are diverse and individual variability still challenges the understanding of seabird ecologists. Intrinsic (age, sex, body condition) and extrinsic factors (wind, and oceanographic features) shape this variability and revealing patterns involves long term datasets derived from ringing and population monitoring. However, for many species such long-term datasets exist. This is the case in the European North Sea (Barrett et al. 1985) and Arctic (Fauchald et al. 2015), the Canadian arctic (Braune et al. 2019) and east coast (Mauck et al. 2018) and the British Antarctic territory (Croxall et al. 1988). At these locations continuous monitoring exists since the 1960ies / 1980ies and already revealed patterns and sensitive markers of seabird vulnerability (Burger and Gochfeld 2002). Rapid and sensitive parameters like breeding success, diet composition or activity budget of breeding adults are useful tools for biomonitoring, since they are directly linked to changes in environmental conditions (Furness and Camphuysen 1997).

Advances in bio-logging technology in the mid-2000s gave way to systematic shearwater tracking studies revealing migration patterns (Yamamoto et al. 2008, Müller et al. 2014, De Felipe et al. 2019) and foraging movements (Magalhães et al. 2008, Paiva et al. 2010, Cecere et al. 2013, Grémillet et al. 2014, Cianchetti-Benedetti et al. 2017), colony attendance (Rubolini et al. 2015), use of wind (De Pascalis et al. 2020), seabird-fisheries interactions (Cianchetti-Benedetti et al. 2018b, Le Bot et al. 2018) and juvenile movements (Raine et al. 2011). Examples of breakthroughs from these types of studies include the discovery of a dual foraging strategy during chick rearing, firstly described in wandering albatrosses (*Diomedea exulans*) (Weimerskirch et al. 1993), later on in shearwaters (Granadeiro et al. 1998) and so far found in 50 seabird species (Phillips et al. 2023). Another discovery was the variation in migration patterns linked to effects of season (breeding vs. nonbreeding periods), breeding stage, breeding status, age, sex and individual specialization (Phillips et al. 2017). Furthermore, foraging areas

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were modelled to predict species distribution and revealed colony specific habitat associations (Péron et al. 2018).

Advances in seabird pollutant biomonitoring have shifted focus from studies linking contaminant levels to observed effects — such as direct mortality and sublethal impacts on development, physiology, and behaviour — to more targeted approaches. Recent research focuses on the role of gene expression (Kreitsberg et al. 2023), improved analytical techniques, and non-invasive sampling methods for large-scale monitoring (Jaspers et al. 2019). Other key developments include risk assessment modelling (Clark et al. 2023), studies on bioavailability (Braune et al. 2019, Kalia et al. 2021, Corsolini et al. 2022), and investigations into pollutant mixtures (Dulsat-Masvidal et al. 2023, Navarro et al. 2023). Research has also explored pollutant accumulation within (Mollier et al. 2024) and across tissues (Lundgren et al. 2024), maternal transfer (Bertolero et al. 2015, Dehnhard et al. 2017, Lopez-Antia et al. 2021, Kuepper et al. 2022), and biomagnification within the food web (Seco et al. 2021). The link between foraging ecology and pollutant exposure has been confirmed to be crucial (Borgå et al. 2005, Leat et al. 2011, Mello et al. 2016, Navarro et al. 2023) as prey depth distribution or shift in diet affect pollutant concentrations (Arcos et al. 2002, Bourgeois et al. 2011). Temporal trends linked to climate change have been addressed (Braune et al. 2019, Elliott et al. 2023, Bustnes et al. 2024). Recently, also in Scopoli's shearwaters from the Mediterranean Sea a long term pollutant monitoring study over 20 years on PFAS has been conducted (Lu et al. 2024).

The focal species of this dissertation was the Scopoli's shearwater, a common and widespread colonial breeding bird in the Mediterranean. Previous research on this species was dedicated to diet, behaviour, breeding phenology, fishery interactions and pollutants. They are surface-feeding foragers and shallow divers with maximum reported dive depths of 5-6 m (Cianchetti-Benedetti et al. 2017, Grémillet et al. 2014). Their diet ranges over zooplankton, crustaceans, and squids, but predominantly consists of small to medium sized fish (Péron et al. 2013, Grémillet et al. 2014, Thabet et al. 2019). These birds also follow fishing vessels and exploit discards and bycatch (Louzao et al. 2011, Cecere et al. 2015, Cianchetti-Benedetti et al. 2018b). Foraging areas are well known and tend to be rather individual according to breeding site and season (Cecere et al. 2013, Cianchetti-Benedetti et al. 2017, Colominas-Cuiró et al. 2022, Morinay et al. 2022). In summary, Scopoli's shearwaters incorporate a wide spectrum of marine organisms in their diet and exploit wide, but relatively distinct marine areas, which vary according to season and breeding stage. The major route of environmental contaminants into the body is via the food (Carravieri 2014), and studying blood stable isotopes can give insight into trophic position ($\delta^{15}\text{N}$) and origin of primary producers ($\delta^{13}\text{C}$) (Hobson et al. 1994). Further, genetic analysis of faecal samples is a prominent tool that has emerged during the last two decades and allows identification of seabird diet on taxonomic level (Barrett et al. 2007, Deagle et al. 2007).

In Mediterranean shearwaters metals, OCs, PFAS, PAHs are the most frequently studied contaminants, but also studies on plastic ingestion also have a high relevance (Codina-García et al. 2013). Most studies so far were carried out in the north and west of the Mediterranean Sea, whereas studies in the

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southern and eastern areas are rare. Generally, shearwaters from the Mediterranean show higher pollutant levels than Atlantic populations (Renzoni et al. 1986, Ramos et al. 2009b, Roscales et al. 2010, Roscales et al. 2011, Escoruela et al. 2018).

1.3 Aims and structure of the thesis

The goal was to study the pollutant exposure of shearwaters in relation to diet, trophic ecology and foraging behaviour. I therefore aimed to 1) establish baseline data on pollutant levels, diet, and foraging behaviour in Scopoli's shearwaters, and to 2) develop effective methods for advancing environmental toxicology and interdisciplinary research in seabirds. I also aimed to link seasonal and prey type specific changes in foraging behaviour and movements to pollutant levels, as accumulated pollutants mainly derive from the diet. The diet, in turn, is linked to foraging behaviour, which varies according to breeding stage and prey type. A further objective was, to lay the groundwork for assessing the Scopoli's shearwater as a potential indicator species for marine pollution and to improve understanding of its ecological role in the marine food web.

This cumulative dissertation is structured in four chapters, which are based on three published peer-reviewed studies and one manuscript, which has been accepted and is currently in press.

Specific aims per chapter:

Chapter 1: Assessing perfluoroalkyl substance pollution in central Mediterranean breeding shearwaters.

- Study the differences in PFAS concentrations and composition between populations
- Identify relationships of PFAS concentrations and trophic markers (stable isotope signatures)
- Investigate the relationship of sex, breeding stage and PFAS exposure
- Explore the potential of Scopoli's shearwaters as biomonitors of PFAS in the marine environment

Chapter 2: Diet of two Mediterranean shearwaters revealed by DNA metabarcoding.

- Describe the diet of two shearwater species with high taxonomic precision, especially for the main prey group fish, which is also targeted by fisheries
- Assess trophic markers (stable isotope signatures) in terms of trophic level and carbon source
- Investigate niche segregation expressed through dietary and isotopic niche
- A broader aim within the scope of this dissertation was to provide valuable context on potential sources of contaminant exposure and the trophic position of seabird prey

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Chapter 3: How shearwaters prey. New insights in foraging behaviour and marine foraging associations using bird-borne video cameras.

- Study detailed foraging behaviour at sea and observe foraging associations and fishery discards consumption by using a new bio logging technology
- A broader aim within the scope of this dissertation was to gain insights that can be of interest for pollution evaluation procedures and risk assessment of this species, as foraging strategy influences exposure to contaminants

Chapter 4: Analysis of organochlorines and polycyclic aromatic hydrocarbons designed for pollutant biomonitoring in three seabird matrices.

- Develop a method to improve comparability of sample types and explore the possibilities of alternative matrices representing different pollutant exposure
- Screen a wide spectrum of contaminants in environmental samples of Scopoli's shearwaters

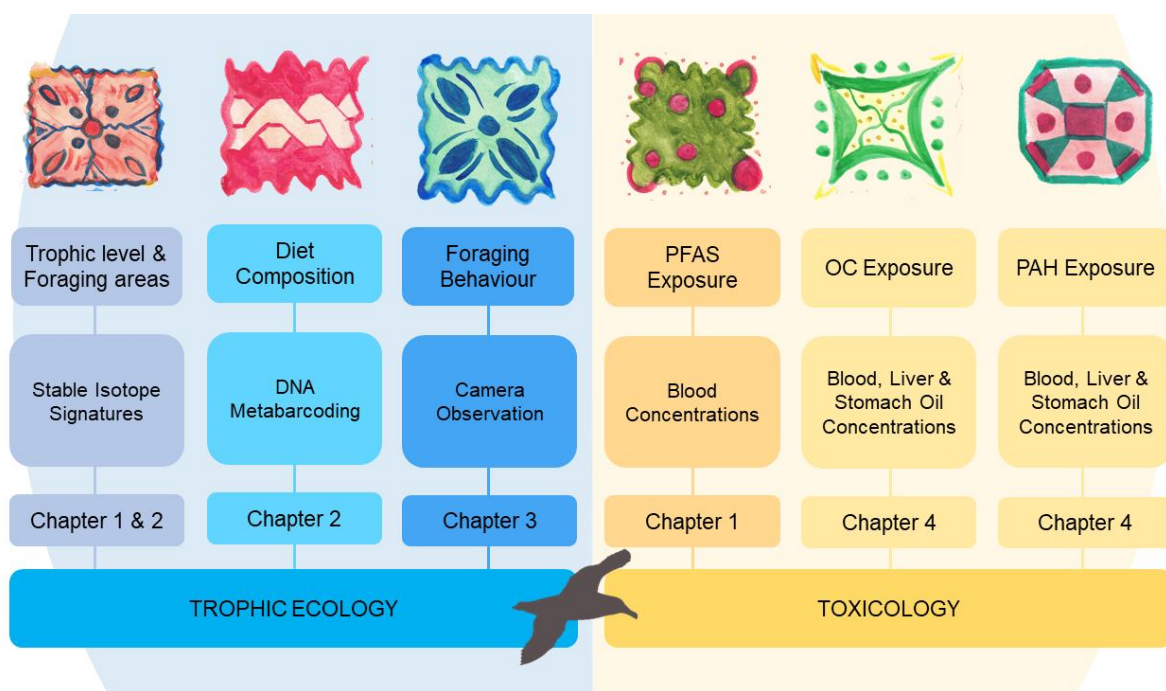


Figure 2 Overview of thesis structure. The top textboxes describe the topics of the research questions investigated in Scopoli's shearwaters (*Calonectris diomedea*), the middle textboxes express the main investigation method used and the dedicated Chapters. The lowest textbox represents the broader research topic to which the chapters can be assigned. PFAS = Per- and polyfluoro alkyl substance, OC = Organochlorine, PAH = Polycyclic aromatic hydrocarbon

2. Chapter outline

Studying the relationship between pollutant exposure, diet, trophic ecology, and foraging behaviour in shearwaters integrates conservation biology, ecotoxicology, and behavioural ecology. It helps to reveal how anthropogenic influences affect marine predators and their ecosystems while informing strategies for reducing pollution and protecting biodiversity.

Chapter 1

Michel, L., Zhang, J., Asimakopoulos A., Austad, M., Bustamante, P., Cecere, J. G., Cianchetti-Benedetti, M., Colominas-Ciuró, R., Dell’Omo, G., De Pascalis, F., Jaspers, V. L. B. & Quillfeldt, P. (2025) Assessing perfluoroalkyl substance pollution in central Mediterranean breeding shearwaters. *Environmental Toxicology and Chemistry*. DOI: 10.1093/etjnl/vgae011

Outline

In the first chapter, we aimed to establish baseline concentrations and profiles of polyfluoroalkyl substances (PFAS) in the blood of Scopoli’s shearwaters. To explore the potential of shearwaters as biomonitors of PFAS in the marine environment, we examined the relationship between PFAS concentrations, foraging areas, and dietary markers of trophic ecology, while also comparing variations across different years and breeding stages. We collected blood samples from 105 breeding Scopoli’s shearwaters across three different Mediterranean colonies in 2020: Linosa (n=52), La Maddalena Archipelago (n=20) and two sampling sites in Malta (n=15). In Linosa, we sampled in May during incubation and in July/August during chick-rearing with an additional 18 samples collected in 2016. PFAS analysis was conducted using Ultra Performance Liquid Chromatography coupled to a Triple Quadrupole Mass Analyser (UPLC®-MS/MS), targeting 39 PFAS congeners. Perfluorooctanesulfonic acid (PFOS), perfluoroundecanoic acid (PFuNA), perfluorotridecanoic acid (PFTriDA), and perfluorododecanoic acid (PFDoDA) were detected in all samples. We revealed regional differences in PFAS concentrations among colonies in the central and southern Mediterranean, as shearwaters from Malta had higher sumPFAS concentrations than birds from Linosa or La Maddalena and PFAS profiles of birds from La Maddalena differed from the ones from Malta. Seasonal differences were also observed, with male shearwaters showing variation in PFAS concentrations between the incubation and chick-rearing stages. While some evidence of PFAS biomagnification through the food web was observed, dietary tracers alone did not fully explain the regional differences in PFAS exposure. This suggests that localized pollutant accumulation plays a significant role. Our results support that Scopoli’s shearwaters could be effective biomonitors of PFAS in remote marine environments, as their blood reflects even small-scale geographical differences in pollutant exposure.

Contribution

Authorship roles: first and corresponding author

Contributor roles: conceptualization, data curation, formal analysis, investigation, resources, writing the original draft

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Chapter 2

Austad, M., Michel, L., Masello, J. F., Cecere, J. G., De Pascalis, F., Bustamante, P., Dell’Omo, G., Griep, S., Quillfeldt, P. Diet of two Mediterranean shearwaters revealed by DNA metabarcoding (**accepted May 2025**)

Outline

This chapter offers insight into the diet of shearwaters aiming to enhance our understanding of their role in the food web of the central Mediterranean Sea, given the uncertainty surrounding their reliance on specific food sources, such as fishery discards. We investigated the diet composition of two shearwaters using genetic tools to identify prey taxa and combined this information with stable isotope analysis to assess their trophic ecology. We sampled blood and dietary remains in three Scopoli’s shearwater populations and one Yelkouan shearwater population. Prey items from 38 families and 21 orders were identified, with small pelagic fish from the orders Clupeiformes and Perciformes constituting the main diet for both species. Certain fish species may have been consumed as fishery discards, as they are unlikely to occur within the dive range of the shearwaters. However, they could also be juvenile, or vertically migrating fish, or driven to the surface during foraging events in association with other marine predators. The dietary composition of the two shearwater species was largely similar, as order level explained only 3% of variation in the diet consumed and genus level 16%. The stable nitrogen isotope marker ($\delta^{15}\text{N}$) also indicated little variation in the trophic level of prey. While both species exhibited overlapping dietary and isotopic niches, Scopoli’s shearwaters displayed a narrower niche space during chick-rearing stage, possibly due to differences in foraging behaviour. These findings highlight the importance of understanding foraging dynamics within the context of resource competition and the increasing pressures on food availability in the Mediterranean.

Contribution

Author role: shared first authorship with M. Austad and corresponding author

Contributor role: conceptualization and design, formal analysis and data curation, fieldwork and sampling, molecular work, writing the original draft

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Chapter 3

Michel, L., Cianchetti-Benedetti, M., Catoni, C., & Dell’Omo, G. (2022). How shearwaters prey. New insights in foraging behaviour and marine foraging associations using bird-borne video cameras. *Marine Biology*, 169, 1-11. DOI: 10.1007/s00227-021-03994-w

Outline

In this chapter, we aimed to investigate at-sea behaviours of breeding Scopoli’s shearwaters during foraging events using lightweight, animal borne video cameras. During chick-rearing stage, shearwaters mostly perform one-day trips in the waters surrounding their breeding sites, which enabled a targeted recording period and fast recapture of the tagged individuals (n=7). In total 19 foraging events were documented and analysed, revealing inter- and intraspecific associations, as well as interactions with fisheries at sea. Shearwaters were observed foraging in association with conspecifics, and the frequency of competitive events increased with the number of birds present in the foraging association. We categorized the observed behaviours, distinguishing between foraging events on natural prey and fishery discards. During natural foraging we observed frequent associations with tunas and sea turtles. Foraging effort was higher for natural prey, as we recorded more time spent, more dives and more competitive events compared to fishery discard foraging. The size and type of prey items were similar across both foraging categories. Our results demonstrate that Scopoli’s shearwaters forage in association with other marine predators and exploit fishery discards. We also showed that in the here observed cases, discards consisted of intact, medium sized fish, implied low foraging effort and originated from small-scale fisheries. Furthermore, the association with predators possibly makes fish available that is usually out of reach of these shallow diving birds. Further, we demonstrated fine-scale behaviours that cannot be recorded with traditional tracking devices, which allow verification if prey was consumed at a foraging site. Foraging associations seem to play a significant role in successfully locating a foraging site and influence foraging success and energy expenses as well.

Contribution

Authorship roles: first and corresponding author

Contributor roles: conception and design, data analysis, fieldwork, writing the original draft

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Chapter 4

Michel, L., Oró-Nolla, B., Dell’Omo, G., Quillfeldt, P., & Lacorte, S. (2024). Analysis of organochlorines and polycyclic aromatic hydrocarbons designed for pollutant biomonitoring in three seabird matrices. *Environmental Science and Pollution Research*, 1-14. DOI: 10.1007/s11356-024-34174-0

Outline

This chapter introduces a method to investigate the accumulation and transfer of a variety of bio-accumulative organic pollutants across different seabird tissues, representing various exposure stages. The aim was to enable comparisons between biological seabird matrices to facilitate studies on pollutant exposure during different life stages. The method was based on GC–Orbitrap–MS analysis, applying a simplified sample treatment involving sample extraction in an ultrasonic bath and purification with Florisil cartridges (5 g, 20 mL). The extraction efficiency was assessed based on recoveries in spiked quality controls and later applied to environmental liver, plasma and stomach oil samples collected from Scopoli’s shearwaters. We targeted 24 organochlorine pesticides, 28 polychlorinated biphenyls and 16 polycyclic aromatic hydrocarbons and yielded satisfactory recoveries (80–120%) in the quality controls at 5 ng for most target analytes. Interfering signal intensification was found for some compounds, which were attributed to matrix effects. 28 of the targeted pollutants were detected in the environmental samples, and composition and detection rate of POPs and PAHs varied among the matrices. Generally, liver had the highest mean sum of organic pollutants (1634 ± 2990 ng/g), followed by stomach oil (233 ± 111 ng/g) and plasma (4.25 ± 4.83 ng/g). The findings suggest that different tissue matrices reflect different types of pollutants, and comparing these matrices offers valuable insights into how pollutants are transferred and retained in marine food webs, especially in top predators like seabirds.

Contribution

Authorship roles: first and corresponding author

Contributor roles: conceptualization, sample collection and preparation, method development and validation, investigation, formal analysis, data curation, writing the original draft

3. Conclusion and Outlook

3.1 Connecting specific findings to broader implications

Scopoli's shearwaters in the central Mediterranean are exposed to a variety of organic contaminants originating from industrial and agricultural activities and incomplete combustion of organic material. Chapters one and four of this thesis established baseline data on pollutant levels. Significant concentrations of PFAS were detected in all of the analysed freeze dried red blood cells, organochlorine pesticides (OCPs), DDT metabolites and PAHs were detected in a subsample of Plasma of the same sample batch. While in liver and stomach oil PCBs were detected, they were below limit of detection in the Plasma samples. These results indicate that the sampling strategy influences the chemicals that can be detected, which when considered during the experiment design, can advance the information value and correct interpretation of toxicological studies.

While Chapter one, investigated trophic ecology in relation to PFAS levels, chapters two and three, were focused specifically on diet and foraging behaviour of shearwaters. Blood concentrations of some PFAS showed a weak positive association of baseline adjusted (AD) $\delta^{15}\text{N}$ pointing to some degree of biomagnification of these compounds, which is supported by previous studies. In shearwaters from Linosa, we found higher PFAS concentrations in males than in females during incubation, which was reflected by higher $\delta^{15}\text{N}$ values in males. However, we found differences in PFAS levels among breeding sites, while there were no differences in stable isotope values after baseline adjustment, which suggests local contamination differences. A comparison with published PFAS concentration measured on Malta reveals contamination hotspots with 7.000 ng/kg in soils in 2019 and 442 ng/L in groundwater in 2021, while in the gulf of Olbia in the north of Sardinia peak PFAS contaminations were measured in biota (1.600 ng/kg to 47.000 ng/kg) in 2021 (Dagorn et al. 2023). In the context of the high concentrations in shearwaters from Malta these results seem to raise more questions than they answer on how water, soil and sedentary biota concentrations might translate into the measured blood concentrations of shearwaters from our study. This example shows how patchy and area / case study restricted contaminant data from soil, water or sedentary biota is.

From this experience, the use of blood samples is suitable for the analysis of a wide range of pollutants, and can simultaneously be used as an indicator of trophic ecology and health / fitness parameters. The sampling procedure for obtaining blood samples proofed to be fast and little invasive, as the handling time was less than 10 minutes and skilled fieldworkers could obtain a small amount of blood from the tarsal vein, without causing any haematoma or injuries. Recent optimizations in GC-MS methods enable determination of contaminants in minimal volumes (100 μL) (Oró-Nolla et al. 2024a, see also Chapter 4). Additionally, this study proofed that analysis of PFAS in freeze-dried blood cells is possible, which does facilitate sample storage and transport; an advantage especially for international collaborations or multi-population studies (Chapter 1). A recent manuscript reviewed the use of Dried

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Blood Spot technology for the determination of environmental contaminants (Samsonova et al. 2022), which could be a promising way to further simplify sampling, storage and transport of blood samples.

A large number of prey taxa, was identified in the diet of Scopoli's shearwaters suggesting generalist and opportunistic foraging strategy. The main prey was clupeids and perch-like fish species, which are predominantly pelagic. Also higher trophic level fish (e.g. tuna) was detected. Interestingly, the variation in (literature) trophic levels of diet taxa, was not reflected in the trophic levels of the shearwaters (calculated from the $\delta^{15}\text{N}$ values). Also the stable isotope signatures of $\delta^{13}\text{C}$ of the shearwaters showed low variation. It's likely that fish from higher trophic levels were consumed as juveniles, which still hold lower $\delta^{15}\text{N}$ values and stay in lower depths, and are less affected by pollutant bioaccumulation. The trophic niche space of Scopoli's shearwaters from Linosa during-chick-rearing was narrower compared to incubation. Indeed, the chick-rearing stage represents a sensitive time, where shearwaters are restricted in terms of foraging area and have to provide for their chicks and themselves in turns. This also suggests that chick-rearing is a period of pronounced vulnerability to local pollutant exposure. Foraging on fishery discards was related to lower foraging effort and lower intraspecific competition, also the species that were consumed as fishery discards were very similar to natural prey (Chapter 3). Despite, this being only a momentary snap-shot of the foraging behaviour, it represents an aspect in estimating the impact of fisheries and discarding on these species. In conclusion, consumption of discards might not necessarily be related to higher pollutant exposure, especially in this area of the Mediterranean, where small artisanal fisheries dominate.

Studies on movement and trophic ecology provide insights into the marine areas utilized by the study species for foraging and wintering. This knowledge enables comparisons of contamination status across different regions (Kunisue et al. 2003). Monitoring of effects on ultimate endpoints like population decline, survival or breeding, often are difficult to link to single effects because the doses at environmental levels are not lethal in most cases. Environmental studies need to consider that multiple stressors can act simultaneously or at different life stages (Chastel et al 2022). It would be highly interesting to model how various contaminants in combination with other stressors may alter population structure over time. Long term monitoring of behavioural variables that could give insight are for example age of first breeding attempt, mate fidelity, longevity, reproductive rate (e.g. skipping breeding seasons). More direct behavioural pattern adjustments could be reflected in parental investment (allocation of brood care vs. self-provisioning) and chick development. Shifting the focus from research on single effects of different contaminants, which can convey confounding messages such as repeated findings of apparent positive effects (Lopez-Antia et al. 2023), to broadening the scope by modelling different health parameters could provide impactful findings that are more actionable for policymakers. For instance, understanding how shifts in energy and time allocation - driven by pollutant exposure, climate change, or other human-induced environmental changes - are linked to imbalances, deficits, or trade-offs in other life-history traits (Blévin et al. 2020) could significantly enhance ecological and policy frameworks.

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3.2 Pushing boundaries – How to overcome current pitfalls, knowledge gaps and limitations?

Based on my experiences during this project I would like to mention some ideas on how to better integrate the existing knowledge aiming to carry out more comprehensive studies that overcome some of the current limitations and pitfalls of seabirds and pollution research.

A. Optimizing comparability

Within this thesis, I have demonstrated some attempts of international frameworks to generate comparable and repeatable results from pollutant biomonitoring (see introduction part 1.1.2). I advocate for advancing this approach by developing guidelines on selecting appropriate sample matrices for different experimental designs. It would be helpful to suggest uniform report styles and establish certified reference materials that match the sample of choice, closely. Furthermore, best practice for sampling and guidelines for streamlining relevant contextual data that consider species specific vulnerabilities would be useful. In addition, statistical handling of contaminant concentrations, which are typically censored by individual detection limits is a non-negligible issue, for which uniformity would be required (Helsel 2011). Moving away from invasive to non-invasive sampling could be facilitated by the development of toxicokinetic databases. Such broadly applicable information that aids in interpreting contaminant concentrations in specific tissues could greatly enhance the ability to classify and compare exposure intensities across different periods or regions.

B. Advanced analytics

Analytical approaches are increasingly shifting towards the principles of green chemistry. Especially developing solvent free extraction methods seems an urgent need, but also widening the scope from targeted to untargeted analysis in retrospect seems very desirable in order to go beyond single use of samples. Furthermore, creating a common ground between the disciplines of analytics and ecology, could be helpful for creating understanding of methodological limitations and how to overcome them.

C. Integrated aspects for policy makers

Future research projects should aim to go beyond informing about threats and pollution levels, focusing instead on developing and testing strategies to reduce pollutants. Furthermore, it should be demonstrated that the current strategy of economical exploitation and pollution of resources to maintain economic growth while fixing the consequences in retrospect is unsustainable. Pollution and the related health risks have negative effects on the economy and the implementation of regulations proves to be difficult and cost intensive as well. A notable example is the PFAS contamination of drinking water in Rastatt, Germany (RP Karlsruhe, August 2018), which highlights the extensive and costly efforts required to remediate such pollution. As long as the current mentality of “profit now and clean up later” will not change, pollutant monitoring will always remain necessary but will lag behind, merely addressing consequences. Therefore, it would be desirable for environmental toxicology to take a proactive role in preventing the future release of potentially harmful pollutants.

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Chapter 1

A watercolor illustration of a coastal landscape. The scene is viewed from an elevated position on a dark brown, textured cliff edge. In the foreground, a body of water with shades of blue and green flows towards the viewer. In the middle ground, a large, dark brown rock formation juts out into the sea. The background shows a vast expanse of blue water meeting a light blue sky at a distant horizon. The overall style is soft and painterly.

Assessing perfluoroalkyl substance pollution in central Mediterranean breeding shearwaters.

Environmental Toxicology

Assessing perfluoroalkyl substance pollution in Central Mediterranean breeding shearwaters

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Abstract

Per- and polyfluoroalkyl substances (PFAS) are synthetic organofluorine compounds used in various products, which are highly durable in the environment and may pose risks to wildlife health. We investigated the blood cell concentrations of PFAS in breeding Scopoli's shearwaters (*Calonectris diomedea*) from three different colonies in the central and southern Mediterranean (Linosa, Malta, and La Maddalena). Shearwaters are flexible, high trophic level foragers, and foraging areas may differ according to sex and breeding stage. We examined inter- and intracolony differences in PFAS blood concentrations and compared them with exploited foraging areas and dietary tracers. Per- and polyfluoroalkyl substances were detected in all samples, with the major congeners detected in descending order being perfluorooctanesulfonic acid (PFOS), perfluoroundecanoic acid (PFuNA), perfluorododecanoic acid (PFDoDA), and perfluorotridecanoic acid (PFTriDA). The mean sum of PFAS during the chick-rearing phase was highest in the birds from Malta (145.1 ng/g dry wt, 95% confidence interval [CI] of the mean 106.8, 183.5) compared with Linosa (91.5 ng/g dry wt, 95% CI 72.9, 110.1) and La Maddalena (84.5 ng/g dry wt, 95% CI 61.7, 107.3), and the PFAS blood composition of shearwaters from La Maddalena and Malta differed. The PFAS concentrations in shearwaters from Linosa were higher during incubation than during chick-rearing, and males had higher PFAS concentrations than females during incubation. Some PFAS were associated with carbon and nitrogen stable isotope values. After baseline adjustment of stable isotope values, no differences were observed for adjusted $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ between the three colonies, suggesting that differences in PFAS levels attributed to diet were minor compared with regional differences. Our study highlights that shearwaters are useful biomonitors of PFAS exposure in remote marine areas.

Keywords: per- and polyfluoroalkyl substances (PFAS), biomonitoring, blood cells, seabirds, stable isotopes

Introduction

Environmental monitoring of per- and polyfluorinated substances (PFAS) and their partially fluorinated precursors is becoming increasingly relevant in ecotoxicology (Cousins et al., 2022). Concern about PFAS is driven by their persistence in the environment, the fast development of new substitutes by industries (Houde et al., 2011), and their toxic effects on living organisms (Sinclair et al., 2020). When released into the environment, their stability and bioaccumulation potential favor long-range transport via water or the atmosphere. Per- and polyfluorinated substances have reached worldwide distribution, being detected even in remote environments (Houde et al., 2006; Sammut et al., 2019; Sun et al., 2023). Marine biota are particularly affected, as the marine ecosystem is a sink and a major distribution pathway for these chemicals (Armitage et al., 2009; Escoruela et al., 2018; Houde et al., 2011) and because of bioaccumulation along the

marine food chain. Perfluoroalkyl acids (PFSAAs), especially perfluorooctane sulfonate (PFOS), are the major contributors of PFAS detected in biota, followed by long-chain perfluoroalkyl carboxylates (PFCAs; Houde et al., 2011). Laboratory experiments have shown toxic effects of PFAS on biota, although toxic effects at environmental concentrations are unlikely to be detected above molecular levels (Jones et al., 2013; Sinclair et al., 2020). The results of field research on bird eggs to determine the effects of PFAS levels on reproduction have been inconsistent (Custer 2021). Some studies have found negative associations between PFOS concentrations and hatching success in tree swallows (Custer et al., 2012, 2014), whereas others on the same species and also on other passerines and seabirds have not (Custer et al., 2019; Groffen et al., 2019; Tartu et al., 2014). This could be partly because PFAS contamination in the environment occurs as an unknown mixture of precursors, degradation products, and co-

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occurring contaminants, which vary according to local sources and transport pathways, and there is limited knowledge of the mechanism or specific PFAS causing impairment. Some adverse effects due to the combination of limited food availability and contaminant exposure have been shown (Keith & Mitchell, 1993), but effects due to PFAS interaction with other compound groups or other natural and anthropogenic stressors are largely unknown (Ahrens & Bundschuh, 2014; Sinclair et al., 2020).

The Mediterranean basin is a marine environment with elevated concentrations of PFAS compared with the Atlantic Ocean (Brumovský et al., 2016; Colomer-Vidal et al., 2022; Escoruela et al., 2018). Notable concentrations of PFAS have been detected in open sea surface and deep water (Brumovský et al., 2016; Schmidt et al., 2019), coastal sediments (León et al., 2020), fish, crustaceans, mollusks (Barhoumi et al., 2022), seabirds (Escoruela et al., 2018), and marine mammals (López-Berenguer et al., 2020). Major pollution input occurs at river mouths and estuaries due to river discharge and through Atlantic inflow in the western Mediterranean (Brumovský et al., 2016; Campo et al., 2015; Pignotti et al., 2017; Schmidt et al., 2019). Most studies on PFAS in higher trophic biota have been carried out in the western Mediterranean and rather close to coastlines. Less is known about the south-central Mediterranean and pelagic zones. Scopoli's shearwaters (*Calonectris diomedea*) breeding in that region could represent the pollution status of these remote areas (Renzoni et al., 1986; Roscales et al., 2010) because they are end consumers in the marine food chain, feeding solely on a marine diet (Grémillet et al., 2014; Michel et al., 2022; Zotier et al., 1999), and cover relatively large marine areas surrounding the colony in search of prey (Cecere et al., 2015), while retaining a degree of fidelity to their foraging areas with little overlap between colonies.

In Mediterranean seabirds, PFAS have been measured in gull eggs (Bertolero et al., 2015; Colomer-Vidal et al., 2022; Parolini et al., 2021) and the blood of shearwaters (Escoruela et al., 2018). Eggs represent the exposure of females during egg production. Blood samples can reveal seasonal and individual differences, which can be caused by differences in physiology or foraging strategy, avoiding potential sex bias. Shearwaters are pelagic foragers and adapt their foraging behavior throughout the breeding season, exploiting different marine areas and prey types (Cecere et al., 2013; Cianchetti-Benedetti et al., 2017; Colominas-Ciuró et al., 2022; Granadeiro et al., 1998; Magalhães et al., 2008; Paiva et al., 2010). Sex differences in foraging strategies and trophic levels are favored by environmental stochasticity (Paiva et al., 2017; Reyes-González et al., 2021) and wind conditions (De Pascalis et al., 2020). Trophic position explained increased concentrations of mercury (Arcos et al., 2002; Gatt et al., 2020; Ramos et al., 2009) in Mediterranean and Atlantic seabirds and persistent organic pollutants in seabirds from Antarctica (Mello et al., 2016). However, for PFAS, this relationship was not clearly confirmed in seabirds.

Aiming to determine environmentally relevant concentrations of PFAS in the seldom studied central and south-central areas of the Mediterranean Sea, we sampled blood from male and female shearwaters from three colonies during breeding. We analyzed 39 PFAS and stable isotopes (SIs) of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$), combined with tracking data from foraging birds. Specifically, we aimed to compare PFAS profiles and concentrations to detect differences between marine foraging areas, sexes, breeding stages, and years with respect to dietary source markers. The overall objective was to investigate the exposure of shearwaters to PFAS in the central Mediterranean Sea and to

uncover possible spatial and sexual patterns during the sensitive breeding phase.

Methods

Sample sites and sample collection

A total of 105 blood samples were collected from breeding Scopoli's shearwaters in three different Mediterranean colonies in 2020: Linosa ($n=52$), in the Pelagie Archipelago, ($35^{\circ}51'33''$ N $12^{\circ}51'34''$ E, Sicily Channel), La Maddalena Archipelago ($n=20$; $41^{\circ}13'60''$ N $9^{\circ}24'0''$ E, Central Tyrrhenian) and two sampling sites in Malta ($n=15$; $35^{\circ}48'37''$ N $14^{\circ}30'17''$ E and $35^{\circ}57'13''$ N $14^{\circ}24'36''$ E, Sicily Channel). Within a large number of monitored nests, known breeders were sampled randomly. In Linosa, we sampled in May during incubation ($n=29$) and in July/August during chick-rearing ($n=23$) and had an additional 18 samples collected in 2016 (See online [supplementary material Table S1](#)). Whole blood in the amount of 0.45 mL was taken from shearwaters with a syringe by puncturing the tarsal vein. At the station, the samples were centrifuged for 10 min at 2,500 rpm, the supernatant plasma was separated, and the samples were stored at -18°C until lyophilization. The blood samples were split up for different analyses, making the freeze-drying step indispensable.

Tracking data

To compare foraging areas used by sampled populations, we used tracking data from global positioning system (GPS)-tagged Scopoli's shearwaters breeding in the three studied colonies. Eight and four randomly chosen known breeders were tagged in Linosa and Malta, respectively, during the breeding season of 2020, coinciding with PFAS sampling, while 15 shearwaters from La Maddalena Archipelago were tracked in 2019. Details on GPS deployment and handling procedures carried out at three colonies are reported in Cecere et al., (2014) and Cianchetti-Benedetti et al., (2017). In Malta, Lotek Pinpoint (~ 3 g) tags and Pathtrack Nanofix (~ 5 g) tags were deployed during late incubation and early chick-rearing. Lotek tags were set to take a position every 90 min and required retrieving the logger for data download, whereas Pathtrack tags were set to take a position every 20 min (but varied depending on solar charging of the internal battery) and data was downloaded via UHF to a base station in the colony.

Molecular sex determination

If unknown, sex was determined by molecular methods. We used a Blood Direct polymerase chain reaction (PCR) Kit (Bio&Sell, Germany) to extract and amplify DNA directly from a minimal amount of blood without prior clean-up. The PCR protocol and program recommended by the manufacturer were used and did not need any adaptations. Correct sex identification was confirmed using samples with known sex. All sexes were determined without doubt except for four samples from Malta for which PCR results were insufficient, reducing the sample size for sex comparisons from 15 to 11.

Chemicals and materials for PFAS analysis

The PFAS analyses were carried out at NTNU Trondheim, Norway. Per- and polyfluoroalkyl substance standards comprised 39 congeners, including 14 PFCAs namely perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTriDA), perfluorotetradecanoic acid (PFTDA),

Table 1. Perfluoroalkyl substances (PFAS) detected in freeze-dried red blood cells of Scopoli's shearwaters (*Calonectris diomedea*) from three locations (Linosa, Malta, La Maddalena) and from both sampling years combined ($n = 105$).

	Mean	SD	Median	Lower 95% CI	Upper 95% CI	Min	Max	% censoring
Sum PFAS	117.31	65.00	99.43	104.73	129.89	17.37	337.45	0
PFOS	61.91	36.54	49.86	54.84	68.99	8.23	185.28	0
PFUnA	18.37	13.21	15.29	15.81	20.92	1.61	70.61	0
PFDoDA	5.38	2.75	4.82	4.85	5.91	0.88	14.50	0
PFTriDA	9.11	5.17	7.44	8.11	10.11	2.14	28.40	0
PFTDA	2.13	1.39	1.90	1.88	2.41	< 0.02	6.24	1
PFNA	5.66	4.57	4.10	4.83	6.60	< 0.10	21.37	3
PFDA	3.08	3.30	2.19	2.48	3.74	< 0.02	19.90	14
PFOSA	4.94	4.19	4.37	4.18	5.78	< 0.02	21.39	17
PFOA	1.84	1.68	1.47	1.53	2.17	< 0.10	8.52	20
10:2 FTS	1.24	1.10	0.92	1.04	1.46	< 0.04	4.27	20
PFHxS	2.75	2.65	2.13	2.26	3.28	< 0.10	10.17	29
PFDS	0.25	0.26	0.19	0.20	0.30	< 0.02	1.38	35
PFHpS	0.12	0.14	NA	0.09	0.14	< 0.04	0.56	73
PFBS	0.40	0.92	NA	0.23	0.59	< 0.04	5.17	78
EtFOSA	0.09	0.18	NA	0.06	0.12	< 0.02	0.99	87
PFNS	0.08	0.12	NA	0.06	0.10	< 0.04	0.62	88
PFECHS	0.04	0.08	NA	0.03	0.06	< 0.02	0.39	89
9CL-PF3ONS	0.04	0.06	NA	0.03	0.05	< 0.50	0.31	89
PFHxDA	0.05	0.06	NA	0.04	0.06	< 0.04	0.36	94
P37DMOA	0.03	0.05	NA	0.02	0.04	< 0.02	0.36	94
PFDoDS	0.03	0.05	NA	< 0.02	0.04	< 0.02	0.37	96
FOSAA	0.02	0.02	NA	< 0.02	0.03	< 0.02	0.19	96
EtFOSE	0.44	0.22	NA	< 0.40	0.48	< 0.40	1.87	97

Note. Expressed in ng/g dry weight ordered by detection rate. In cases with censored data, bootstrapped 95% confidence intervals on the Kaplan-Meier estimate of the mean are given. CI = confidence interval; Min = minimum; Max = maximum; PFOS = perfluorooctanosulfonic acid; PFUnA = perfluoroundecanoic acid; PFDoDA = perfluorododecanoic acid; PFTriDA = perfluorotridecanoic acid; PFTDA = perfluorotetradecanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorododecanoic acid; PFOSA = perfluorooctane sulfonamide; PFOA = perfluorooctanoic acid; 10:2 FTS = 1H,2H-perfluorododecan sulfonate (10:2); PFHxS = perfluorohexane sulfonic acid; PFDS = perfluorododecane sulfonic acid; PFHpS = perfluoroheptane sulfonic acid; PFBS = perfluorobutanesulfonic acid; EtFOSA = N-ethylperfluoro-1-octanesulfonamide; PFNS = perfluorononane sulfonic acid; PFECHS = perfluoroethylcyclohexane sulfonic acid; 9CL-PF3ONS = 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate; PFHxDA = perfluoro-n-hexadecanoic acid; P37DMOA = perfluoro-3,7-dimethylcyclooctanoic acid; PFDoDS = perfluorododecane sulfonic acid; FOSAA = perfluoro-1-octanesulfonamidoacetic acid; EtFOSE = N-ethyl-N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide; NA = not available.

perfluoro-n-hexadecanoic acid (PFHxDA), perfluorooctadecanoic acid (PFOcDa), perfluoro-3,7-dimethylcyclooctanoic acid (P37DMOA), 7H-dodecafluoroheptanoic acid (7H-PFHpA), 9 PFASs namely perfluorobutanesulfonic acid (PFBS), perfluoropentane sulfonic acid (PFPeS), perfluorohexane sulfonic acid (PFHxS), perfluoroheptane sulfonic acid (PFHpS), PFOS, perfluorononane sulfonic acid (PFNS), perfluorododecane sulfonic acid (PFDS), perfluorododecane sulfonic acid (PFDoDS), perfluoroethylcyclohexane sulfonic acid (PFECHS), 4 fluorotelomere sulphonates (FTSs) namely 1H,2H-perfluorohexan sulfonate (4:2) (4:2 FTS), 1H,2H-perfluorooctane sulfonate (6:2) (6:2 FTS), 1H,2H-perfluorodecan sulfonate (8:2) (8:2 FTS), 1H,2H-perfluorododecan sulfonate (10:2) (10:2 FTS), 7 fluorosulphonamides namely perfluoro-1-octanesulfonamidoacetic acid (FOSAA), 2-(N-methylperfluoro-1-octanesulfonamido) acetic acid (MeFOSAA), perfluorooctane sulfonamide (PFOSA), N-methylperfluoro-1-octanesulfonamide (MeFOSA), N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide (MeFOSE), N-ethyl-N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide (EtFOSE), N-ethylperfluoro-1-octanesulfonamide (EtFOSA), and 5 miscellaneous substitute compounds namely 2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)propanoate (Gen X), dodecafluoro-3H-4,8-dioxanonanoate (ADONA), decasulfonic acid (DecaS), 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (9CL-PF3ONS), bis[2-(N-ethylperfluorooctane-1-sulfonamido) ethyl] phosphate (diSAMPAP; Table 1; see online [supplementary material Table S2](#); for supplier and synthesizing details, see online [supplementary material Table S3](#)). Isotopically labelled internal standards perfluoro-n-octanoic acid- $^{13}\text{C}_8$ (99%), perfluoro-1-octanesulfonate- $^{13}\text{C}_8$ (99%) and 6:2 FTS $^{13}\text{C}_2\text{-D}_2$ were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Methanol of liquid chromatography-mass spectrometry grade was purchased from Merck (Darmstadt, Germany).

Ammonium formate ($\geq 98\%$ w/w) was supplied by VWR Chemicals (Trondheim, Norway). Water was purified with a Milli-Q grade system (Q-option, Elga Labwater, Veolia Water Systems LTD, UK) SPE cartridges, HybridSPE (Supelco, Bed wt. 30 mg, 1 mL), were purchased from Sigma-Aldrich (Steinheim, Germany).

Sample extraction for PFAS

Between 30 and 130 mg of lyophilized red blood cells (RBCs) were restored with 300 μL of water and extracted following the protocol described in [Sait et al., \(2023\)](#) with minor modifications. Samples were spiked with 10 μL of 1 mg/L ^{13}C -isotope labelled IS-mixture, and 600 μL of methanol containing 1% ammonium formate (w/v) were added. Extraction was performed by vortexing (30 s), ultrasonication (30 min), and centrifugation (5 min, 3,500 rpm), then the supernatant was passed through Hybrid-SPE cartridges, and the extract was collected and stored in 1 mL amber vials with 150 μL inserts.

Ultra high-performance liquid chromatography-tandem mass spectrometry analysis

Target analytes were determined using an Acquity UPLC I-Class system (Waters, Milford, CT, USA) coupled to a triple quadrupole mass analyzer (QqQ; Xevo TQ-S) with a ZSpray ESI ion source (Waters, Milford, CT, USA). Chromatic separation was performed with a Kinetex C18 (30 \times 2.1 mm, 1.3 μm) connected to a Phenomenex C18 guard column (2.0 \times 2.1 mm). The details of the instrument method are described in [Sait et al., \(2023\)](#).

Quality assurance and quality control of PFAS analysis

Procedural blanks were analyzed to assess any potential background contamination stemming from laboratory materials and

solvents. A comprehensive quality control strategy was used, involving regular injections of solvent blanks and a standard solution at intervals during the analysis. This served to monitor potential issues like cross-contamination, sample carryover, and signal fluctuations and drift. To mitigate any residual effects, the injection needle underwent thorough washing with a mixture solution (methanol-acetonitrile-isopropanol-water, 1:1:1:1) both before and after each injection. For the sake of ensuring precise and accurate measurements, multipoint calibration curves were prepared. These calibration curves encompassed 11 data points, with concentrations spanning from 0.01 to 20 ng/mL⁻¹ in methanol with 1% w/v ammonium formate. The method limits of quantification (LOQs) were estimated from the lowest concentration point in the calibration curve that yielded a positive value while maintaining a linear curve with an R^2 value exceeding 0.99 (Liao et al., 2012; Zhang et al., 2023). The limits of detection (LODs) were estimated as LOQ/3 (Zhang et al., 2023). Method LOQ (mLOQ) and method LOD (mLOD) were estimated from the respective instrumental LOD and instrumental LOQ at a nominal sample weight mass of 0.1 g and ranged from mLOD 0.02–1 ng/g and mLOQ 0.06–3 ng/g. The detailed LODs and LOQs for the target analytes are presented in the online [supplementary material Table S2](#).

During the process of method development and validation in biota samples, a homogeneous pool bird blood sample obtained from three individual birds was prepared. This sample was stored under conditions identical to those of the actual environmental samples. The extraction efficiency of six PFASs, namely PFPeS, PFNS, PFDS, PFDoDS, PFECCHS, and PFOcDA, which were not previously reported in the biota study by Sait et al. (2023), was thoroughly evaluated. The obtained results showed absolute percentage recoveries (\pm relative SD in percent [RSD %]) of 51 \pm 9, 51 \pm 11, 50 \pm 11, 51 \pm 11, 46 \pm 5, and 55 \pm 10, respectively, for a fortification amount of 10 ng. Correspondingly, the relative percentage recoveries (\pm RSD %) for these compounds, when considering C13-PFOS as the internal standard, were as follows: PFPeS (101 \pm 18), PFNS (103 \pm 22), PFDS (100 \pm 21), PFDoDS (103 \pm 22), PFECCHS (93 \pm 9), and PFOcDA (111 \pm 20). This validation process ensures the reliability and accuracy of the developed method for analyzing these specific PFASs in bird blood samples.

Stable isotope analyses and baseline adjustment

The stable isotope (SI) values of carbon and nitrogen were determined in 0.3 mg aliquots of freeze-dried RBC weighed in tin cups at the LIENSs laboratory (France) with a Delta V Plus isotope ratio mass spectrometer with a ConFlo IV interface (Thermo Scientific, Bremen, Germany) and a Flash 2000 elemental analyzer (Thermo Scientific, Milan, Italy). The equipment was calibrated with certified reference materials (See online [supplementary material Section A](#)). A two-point calibration was used with the working standards USGS-61 (caffeine) and USGS-63 (caffeine). Measurements of internal laboratory standards were conducted using acetanilide (Thermo Scientific) and peptone (Sigma-Aldrich) and indicated an analytical precision of <0.15 ‰ for $\delta^{15}\text{N}$ and <0.10 ‰ for $\delta^{13}\text{C}$. Results are expressed in the δ unit notation as deviations from standards (Vienna Pee Dee Belemnite for $\delta^{13}\text{C}$ and N_2 in air for $\delta^{15}\text{N}$) following the formula $\delta^{13}\text{C}$ or $\delta^{15}\text{N} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$, where R is $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$, respectively.

The SI values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ vary in marine areas according to the differences in the basal resources. Although the SI signatures from zooplankton in the Sicily Channel can vary from year to year (Rumolo et al., 2018), the reported gradient of $\delta^{15}\text{N}$ increasing from south to north central Mediterranean remains constant over the years (Campioni et al., 2022; Rumolo et al., 2016).

To correct this latitudinal gradient, we calculated the difference from the two reported means for mesozooplankton from 2011 and 2016 for the main foraging areas of the populations in our study and used it to adjust our SI signatures (See online [supplementary material Table S4](#)). This resulted in a Δ of 1.3 ‰ for $\delta^{15}\text{N}$ between the colonies from the Tyrrhenian Sea and the Sicily Channel and a Δ of 0.2 ‰ for $\delta^{13}\text{C}$.

Data analysis and statistics

Ultra-performance liquid chromatography tandem mass spectrometry data were acquired with MassLynx Ver. 4.1 software, and quantification processing was performed with TargetLynx (Waters, Milford, CT, USA). Excel (Microsoft, 2018) was used to calculate the concentrations of areas detected for the target analytes in the samples in nanograms per gram of dry weight. All statistical analyses were run with R Ver. 4.0.4 (R Core Team 2021). Some PFAS variables contained observations below the LOD/LOQ and thus were left-censored. Compounds that included more than 70% of censored values ($n=26$) were excluded from further analysis, except for PFHpS. The QQplot and cumulative distribution functions showed that most concentrations were not normally distributed, except for PFDoDS, PFECCHS, EtFOSE, 8:2 FTS, P37DMOA, and adjusted $\delta^{13}\text{C}$ ($\text{AD}\delta^{13}\text{C}$) values. Therefore, nonparametric methods have been applied. Summary statistics (mean, SD, median and 95% CI) for compounds with less than 70% censoring ($n=8$) were calculated using the nonparametric Kaplan-Meier method in the NADA package (Ask et al., 2021). Both censored and uncensored PFAS observations were bootstrapped (5,000 repetitions) using the infer package. The data set was examined for differences between the colonies during chick-rearing in 2020, between the incubation and chick-rearing phase in the colony of Linosa in 2020, and between the chick-rearing phases of 2020 and 2016 in Linosa by comparing the 95% CIs, as described by Erickson and Rattner (2020). Differences in PFAS mean concentrations between male and female shearwaters were also examined by comparing their 95% CIs. A Spearman's rank correlation was used to test for the correlation of PFAS and Stable Isotope signatures. Principal component analysis (PCA) was carried out with the 12 PFAS with less than 70% censoring. These had sufficient intercorrelation ($R > 0.3$ and < 0.9 , $p < 0.05$), which was represented by a Kaiser-Meyer-Olkin value between 0.5 and 1 (Tabachnick & Fidell, 2000) and a significant Bartlett test (Field et al., 2012).

Results

Overview of detected PFAS congeners detection rates

The predominant congeners detected in RBC samples ($n=105$) followed the order PFOS > PFuNA > PFDoDA > PFTriDA, and were found in all samples (Table 1). These were followed by PFTDA, PFNA, PFDA, PFOSA, PFOA, and the fluorotelomer sulfonic acid 10:2 FTS, which had $\leq 20\%$ censored observations due to the detection limit. Among these, 10:2 FTS was the only precursor detected. Perfluorohexane sulfonic acid and PFDS were above the detection limit in 71% and 65% of samples, respectively. Perfluoroheptane sulfonic acid, PFBS, EtFOSA, PFNS, PFECCHS, 6:2 FTS, 9CL-PF3ONS, PFHxDA, P37DMOA, PFDoDS, FOSAA, and EtFOSE exhibited more than 70% censored values. Fifteen congeners were not detected, including 4:2 FTS, MeFOSA, 8:2 FTS, MeFOSAA, MeFOSE, GenX, PFPeA, PFHxA, PFHpA, PFOcDA, 7H-PFHpA, PFPeS, ADONA, DecaS, and diSAMPAP. Further analysis included four uncensored PFAS, eight PFAS with less than 70%

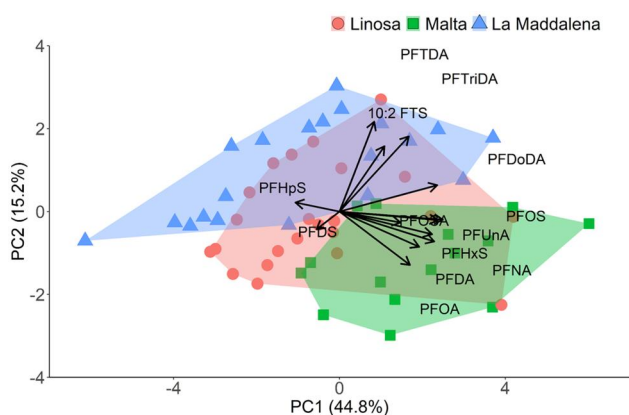


Figure 1. Principal Component Analysis of perfluoroalkyl substances (PFAS) profiles of three studied colonies of Scopoli's shearwaters (*Calonectris diomedea*; $n = 58$) during the chick-rearing phase of the year 2020. PC1 = principal component 1; PC2 = principal component 2; PFOS = perfluorooctanosulfonic acid; PFuNA = perfluoroundecanoic acid; PFDoDA = perfluorododecanoic acid; PFTriDA = perfluorotridecanoic acid; PFTDA = perfluorotetradecanoic acid; PFNA = perfluorononanoic acid; PFOA = perfluorooctanoic acid; PFDA = perfluorodecanoic acid; PFHxS = perfluorohexane sulfonic acid; PFDS = perfluorododecane sulfonic acid; PFHpS = perfluoroheptane sulfonic acid.

censored values, and PFHpS, as it was detected with less than 70% censoring specifically in samples from Malta.

PFAS profile in the different colonies

Several long-chained perfluorinated sulfonic and carboxylic acids were positively correlated (Spearman correlation test; see online [supplementary material Table S5](#)). To convert them into uncorrelated variables (PCs), which explain the maximum variation in the data, we performed a PCA with the 13 PFAS that had less than 70 censoring. Sufficient intercorrelation values were confirmed by an overall sampling adequacy Kaiser-Meyer-Olkin index of 0.82. The PCA revealed three significant components that explained 72.7% of the variance in the data. PC 1 (eigenvalue: 5.83) was determined by six PFAS comprising the intermediate and long-chained carboxylic acids (PFOS, PFuNA, PFDoDA, PFNA, PFDA) and one sulfonate (PFHxS). Principal component 2 (eigenvalue: 1.98) contained four variables which comprised the two long-chained carboxylic acids (PFTDA, PFTriDA), the precursor fluorotelomer sulfonate (10:2 FTS), as well as the 8CF carboxylate PFOA. PC 3 (eigenvalue: 1.12) summarized the perfluoroalkyl sulfonates PFHpS, PFDS, and the fluorosulfonamide PFOSA. The PCA separated the PFAS profiles of the shearwater populations from Malta and La Maddalena mainly along the axis of PC 2 (Figure 1), whereas shearwaters from Linosa had a more homogenous PFAS profile, which overlapped with the hulls of Malta and La Maddalena (Figure 1).

Foraging areas

Birds from La Maddalena foraged in the north Tyrrhenian Sea, along the east coast of Corsica, in the area of the Tuscan archipelago and on the north of Sardinia. These areas were further north than the ones of shearwaters breeding in Linosa and Malta, which showed little overlap. Birds from Linosa exploited the southwestern part of the Sicily channel and foraged in front of the Libyan coast west of Tripoli, whereas the foraging area of birds from Malta expanded more towards the north and east of the Sicily channel, especially along the southeastern Sicilian coast (Figure 2).

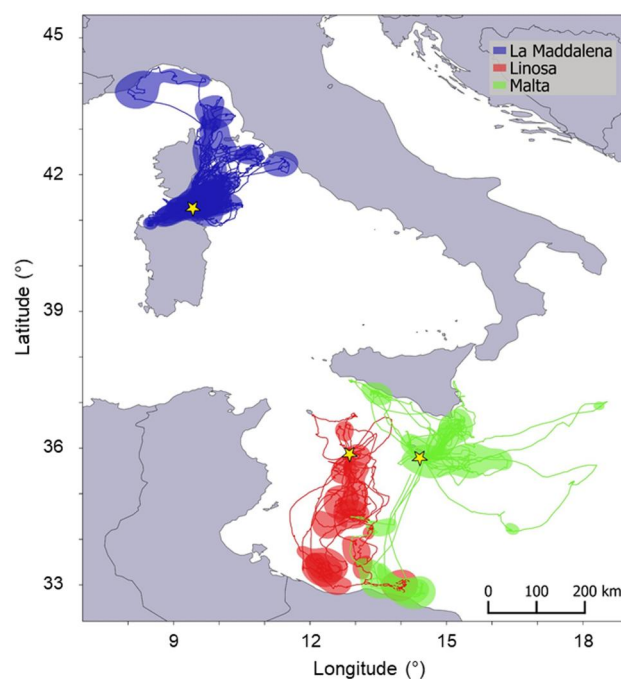


Figure 2. Foraging movements of Scopoli's shearwaters (*Calonectris diomedea*) tracked during the summer of 2020 (Linosa, Malta) and 2019 (La Maddalena) and 50% utilization distributions.

Between colony differences in PFAS during the chick-rearing phase 2020

Among the three colonies, PFAS concentrations and profiles differed (Figure 3); complete summary statistics per colony are shown in the online [supplementary Tables S6a and S6b](#). Birds from Malta had the highest concentrations of the sum PFAS, Linosa and La Maddalena birds showed similar concentrations (95% CI Linosa 75.69–110.30, Malta 114.13–183.57, La Maddalena 65.08–106.15). We observe the same pattern for PFOS (95% CI Linosa 40.98–61.99, Malta 65.12–105.60, La Maddalena 32.49–56.33), PFNA (95% CI Linosa 2.38–4.38, Malta 5.65–9.50, La Maddalena 2.35–4.19), PFDA (95% CI Linosa 0.78–1.89, Malta 3.31–5.44, La Maddalena 0.67–1.87), and PFHxS (95% CI Linosa 1.14–2.80, Malta 3.13–5.13, La Maddalena 1.51–3.27). In La Maddalena birds, PFuNA concentrations were the lowest (95% CI Linosa 11.54–16.19, Malta 14.68–27.40, La Maddalena 6.16–10.87), whereas PFTriDA were higher than in Linosa birds (95% CI Linosa 5.32–9.03, Malta 6.85–11.09, La Maddalena 9.31–14.59), and PFTDA were highest compared with birds from Linosa and Malta (95% CI Linosa 1.53–2.50, Malta 0.98–1.66, La Maddalena 2.69–4.16).

In Malta, 95% CIs of males and females generally overlapped during the chick-rearing phase; however, the upper limits for all compounds were higher in females (See online [supplementary material Table S6b](#)). In La Maddalena sum PFAS (95% CI females 51.65–89.51, males 69.32–145.85), PFOS (95% CI females 25.16–43.14, males 36.64–83.17), PFuNA (95% CI females 4.76–8.93, males 6.45–15.51), PFDoDA (95% CI females 2.48–4.21, males 3.32–7.06), PFTriDA (95% CI females 7.41–13.17, males 10.00–18.61), and PFDS (95% CI females 0.07–0.19, males, 0.13–0.36). Concentrations tended to be slightly higher in males than in females as their 95% CIs showed little overlap. In Linosa, PFTriDA differed between the sexes during chick-rearing, as males had higher concentrations than females (95% CI females 3.99–6.10, males 6.69–14.03), also the 95% CIs of PFDoDA (95% CI females 3.53–5.61, males 4.51–7.92) and 10:2 FTS (95% CI females 0.64–1.53, males 1.78–1.33) showed little overlap.

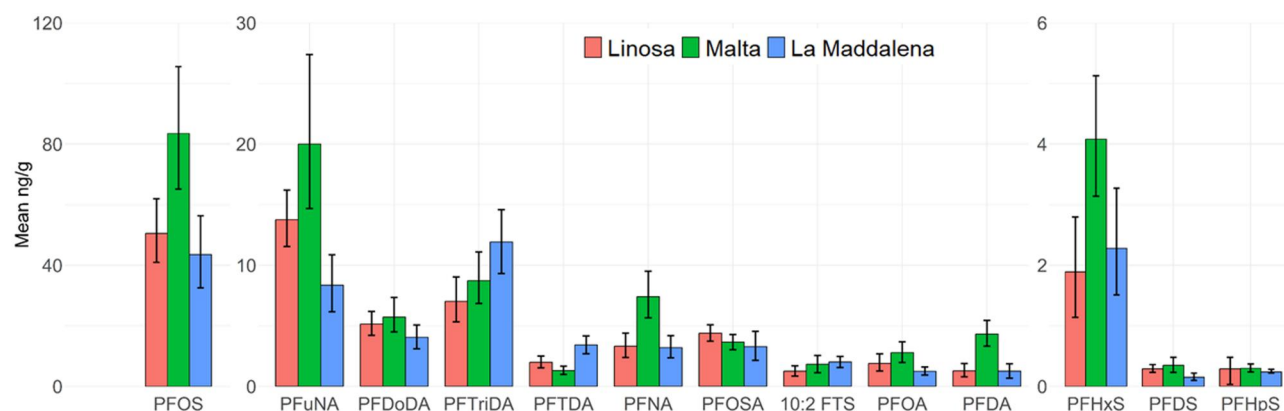


Figure 3. Mean concentrations in ng/g dry weight of all perfluoroalkyl substances (PFAS) with less than 70% censoring in red blood cells from the three Scopoli's shearwater (*Calonectris diomedea*) colonies during the chick-rearing phase in the year 2020. Whiskers show 95% confidence intervals of the mean. PFOS = perfluorooctanosulfonic acid; PFuNA = perfluoroundecanoic acid; PFDODA = perfluorododecanoic acid; PFTrIDA = perfluorotridecanoic acid; PFTDA = perfluorotetradecanoic acid; PFNA = perfluorononanoic acid; PFOSA = perfluorooctane sulfonamide; 10:2 FTS = 1H,2H-perfluorododecan sulfonate (10:2); PFOA = perfluorooctanoic acid; PFDA = perfluorodecanoic acid; PFHxS = perfluorohexane sulfonic acid; PFDS = perfluorodecane sulfonic acid; PFHpS = perfluoroheptane sulfonic acid.

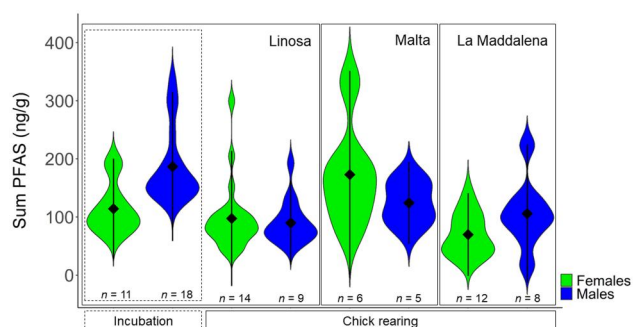


Figure 4. Sum perfluoroalkyl substances (PFAS) distribution in female and male Scopoli's shearwaters (*Calonectris diomedea*) from Linosa, Malta and La Maddalena in the year 2020, during chick-rearing and incubation. Continuous lines mark samples taken during the chick-rearing phase and dashed line marks samples taken during incubation. Black rhombs show means and vertical lines show standard deviations. Sample size (n) is given under each violin plot.

Within colony differences in PFAS according to breeding phase and sex in Linosa

No differences in PFAS concentrations between the two years in Linosa were detected, except that PFHpS was not detected in 2016. In Linosa birds, the sum of PFAS concentrations during the incubation phase was higher than during the chick-rearing phase (95% CI incubation 137.19–184.57, chick-rearing 75.69–110.30; Figure 4). This difference was primarily due to males having higher concentrations than females during incubation (95% CI females 92.00–139.52, males 160.69–218.38). Later, during the chick-rearing phase, the sum concentrations in males decreased to levels similar to those of females (95% CI females 66.74–111.96, males 76.98–126.58). In Linosa females, the sum PFAS concentrations remained consistent between the incubation and chick-rearing phases. However, specific PFAS congeners, such as PFOA (95% CI incubation 2.02–4.32, chick-rearing 1.19–2.82), showed a slight decrease during chick-rearing compared to incubation. Others, including PFuNA (95% CI incubation 15.93–26.22, chick-rearing 10.09–15.82), PFNA (95% CI incubation 5.63–10.67, chick-rearing 2.09–4.73), PFDA (95% CI incubation 2.59–5.41, chick-rearing 0.57–2.09), PFOSA (95% CI incubation 8.67–14.06, chick-rearing 3.61–5.48), and PFHxS (95% CI incubation 2.94–5.59,

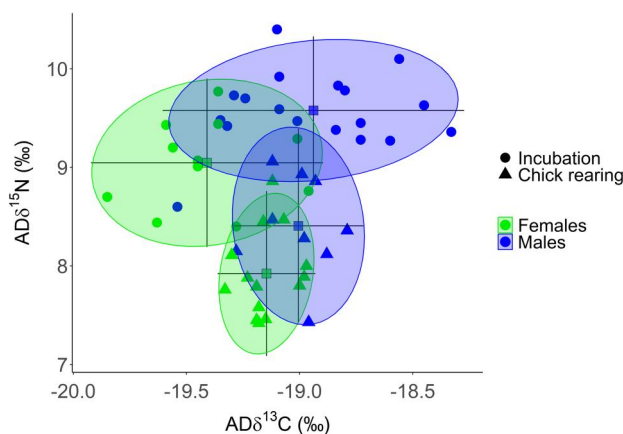


Figure 5. Adjusted stable isotope values for carbon and nitrogen in red blood cells from Scopoli's shearwaters (*Calonectris diomedea*) in Linosa ($n = 52$) plotted for sex and breeding phase in the year 2020. AD $\delta^{13}C$ = adjusted stable isotope ratio of carbon; AD $\delta^{15}N$ = adjusted stable isotope ratio of nitrogen. The ellipses were set to cover 80% of the points, assuming a multivariate normal distribution, while the error bars were 1.96 times the standard deviation.

chick-rearing 1.49–4.47), decreased considerably. The only compound that increased during chick-rearing in females was 10:2 FTS (95% CI incubation 0.28–0.61, chick-rearing 0.64–1.53).

SI signatures of carbon and nitrogen SI signature differences between colonies during chick-rearing phase 2020

After correcting the SI values for the baseline difference with the means of mesozooplankton from the Tyrrhenian Sea and the Pelagic Islands (Campioni et al., 2022), no differences in adjusted $\delta^{15}N$ (AD $\delta^{15}N$) between colonies were observed (95% CI Linosa 7.91–8.32, Malta 7.69–8.29, La Maddalena 8.01–8.58). Adjusted $\delta^{13}C$ (AD $\delta^{13}C$) values were similar between the three colonies (95% CI Linosa –19.15 to –19.03, Malta –19.23 to –19.02, La Maddalena –19.12 to –19.00). Although showing low correlation coefficients (range of $\rho = 0.19$ –0.38; range of p -values = < 0.001 –0.04) most long-chained perfluorinated carboxylic acids (PFuNA, PFDODA, PFTrIDA, PFTDA, PFNA, PFDA) and some sulfonic acids (PFOS and PFHxS) were positively correlated with the

AD $\delta^{15}\text{N}$ signatures (See online [supplementary material Table S5](#)). With similarly low correlation coefficients (range of $\rho = -0.19$ to -0.25 , range of p -values = < 0.01 – < 0.05) PFuNA, PFOSA, PFOA and correlated negatively with AD $\delta^{13}\text{C}$, whereas PFTriDA ($\rho = 0.19$, p -value = 0.04) and PFTDA ($\rho = 0.22$, p -value = 0.02) correlated positively with AD $\delta^{13}\text{C}$ (See online [supplementary material Table S5](#)).

SI signature differences according to year, breeding phase and sex in Linosa birds

The AD $\delta^{15}\text{N}$ signatures were higher in the chick-rearing phase of 2016 than in 2020 (95% CI 2016 8.73–9.21, 2020 7.91–8.32), similarly AD $\delta^{13}\text{C}$ values (95% CI 2016 –19.01 to 18.69, 2020, –19.15 to 19.03). In both years, males showed higher AD $\delta^{15}\text{N}$ and AD $\delta^{13}\text{C}$ values than females (See online [supplementary material Table S6b](#)). The AD $\delta^{15}\text{N}$ values were higher during incubation than during chick-rearing in Linosa in both sexes (Figure 5; See online [supplementary material Table S6b](#)). The AD $\delta^{13}\text{C}$ signatures stayed constant over the breeding period in males (95% CI incubation –19.09 to 18.79, chick-rearing –19.10 to 18.92; Figure 5), whereas those of females tended to increase during chick-rearing (95% CI incubation, –19.55 to 19.25; chick-rearing, –19.20 to 19.09; Figure 5). In Malta and La Maddalena, no sex difference was found in the SI values during chick-rearing (See online [supplementary material Table S6b](#)).

Discussion

How measured PFAS concentrations and composition compare with previous studies

To our knowledge, this is the first report of PFAS concentrations in lyophilized RBCs of seabirds. Because PFAS are associated with blood proteins, most other studies have used whole blood or plasma. A study on blood cells of Australian shorebirds reported significant variations in PFOS concentrations, with levels being lower in natural wetlands (median 14 ng/g wet wt, range: < 0.01 –379 ng/g wet wt) and higher near a water treatment plant (median 52 ng/g wet wt, range: < 0.01 –1280 ng/g wet wt; Ross et al., 2023). In comparison, our results for PFOS are similar, with a median of 50 ng/g dry weight (Table 1) but a narrower range of 8–185 ng/g dry weight (See online [supplementary material Table S6b](#)). However, it is important to consider that there is a dilution factor of approximately 70% from dry weight to wet weight concentration. The water content in the whole blood of water birds ranges between 79.1% (Eagles-Smith et al., 2008) and 86% (Dulsat-Masvidal et al., 2023). Because RBCs are the remaining “pellet” after centrifugation and is thicker than plasma, its moisture content is expected to be slightly lower. A determination of moisture content in human RBCs showed 71.4%, so we estimate a moisture content of 70% for simplicity.

Reported mean (\pm SE) PFOS concentrations in whole blood from incubating shearwaters were 42 ± 11 ng/mL at a more polluted site in the western Mediterranean and 12 ± 2 ng/mL at a colony in the southern Mediterranean (Escoruela et al., 2018). When comparing whole blood to RBC concentrations, two factors must be considered: (1) a dilution factor, as concentrations in human whole blood are about half those in plasma (Ehresman et al., 2007; Hanssen et al., 2013), and (2) differences in PFAS partitioning due to their chemical properties, as plasma from herring gulls had four times the PFAS concentrations compared to RBCs (Gebink & Letcher, 2012). Although lyophilized RBCs are not frequently used in biomonitoring studies, the PFAS concentrations found in our samples demonstrate that PFAS can be reliably measured in lyophilized RBCs, with a remarkably low sample volume required.

In accordance with other seabird studies, the most abundant PFAS detected was PFOS, followed by the long-chained carboxylates with nine fluorinated carbons and upwards. This is consistent with the known behavior of longer-chain PFAS in biota, as they have more bioaccumulation potential than shorter-chain PFAS (Conder et al., 2008; Sturm & Ahrens, 2010; Szabo et al., 2021). Sulfonates detected had shorter carbon lengths, which is well in line with the literature, as they are more bioaccumulative than PFCAs of the same fluorinated carbon length (Conder et al., 2008).

How observed PFAS patterns and regional and seasonal differences relate to diet and tracking data

Distribution of PFAS in seawater has been reported to be largely homogenous and dominated by PFHxA, PFHpA, PFOA, PFHxS, and PFOS in the western Mediterranean (Brumovský et al., 2016), whereas in higher trophic biota, different compositions and concentrations have been observed, suggesting differences in bioaccumulation potential as well as geographical and species-specific differences (Colomer-Vidal et al., 2022; Escoruela et al., 2018). This study confirms these findings, because it reports regional differences in PFAS patterns and concentrations in shearwaters and in addition, seasonal changes in PFAS blood levels. In birds from La Maddalena, mean PFTDA levels were at a higher concentration than in those from Malta and Linosa, whereas PFuNA levels were lower and mean PFTriDA levels were higher in La Maddalena birds than in Linosa birds. Birds from La Maddalena tend to forage closer to the coastlines of Sardinia and Corsica. This has been shown by the GPS device deployments in 2019 used for our study and confirmed by tracking data from the same colony in 2013 and 2018 (De Pascalis et al., 2020; Morinay et al., 2022), suggesting that likely when birds in our study were sampled in 2020, they used the same areas (Figure 2). In contrast, birds from Linosa are more pelagic foragers and birds from Malta forage both in coastal and pelagic areas (Figure 2).

During the chick-rearing phase, birds from Malta had the highest PFAS concentrations, with mean sum PFAS being more than 50% higher than birds from Linosa and La Maddalena. The baseline-adjusted SI values did not show any differences between the colonies. We, therefore, assume that differences in PFAS exposure are more likely to be related to the marine areas used for foraging than to dietary habits. Because the PFAS burden of Malta birds was significantly higher than that of birds from Linosa and La Maddalena, potential point sources could lead locally to a higher exposure of prey fish of similar trophic position. Malta is not known for a high level of industrialization involving PFAS manufactures (Sammut et al., 2019). Still, according to the recently published map of PFAS contamination in Europe (Dagorn et al., 2023), several PFAS contamination sites and hot-spots are located on Malta as well as on the south and east coast of Sicily, where shearwaters breeding on Malta forage (Figure 2). The sampled colonies on Malta are located in the immediate vicinity of an industrial site with a resident chemical industry and a former military airport. Studies on PFAS in the Maltese environment found that groundwater, surface water, and soil were contaminated with PFAS, and suggested that atmospheric transport but also disposal of imported contaminated materials contributed to Malta's PFAS burden (Sammut et al., 2017, 2019).

In shearwaters from Linosa, we found higher PFAS concentrations in males than in females during incubation, which was reflected by higher AD $\delta^{15}\text{N}$ values in males. A weak positive association of AD $\delta^{15}\text{N}$ and some PFAS points to some degree of bioaccumulation of these compounds, which is supported by

previous studies (Barghi et al., 2018; Ng & Hungerbühler 2014). The differences reported in the present study between males and females could be due to PFAS excretion during egg-laying, as discussed for mercury (Becker 1992; Lewis et al., 1993; Ramos et al., 2009) and organochlorine pollutants (Dehnhard et al., 2017; van den Brink et al., 1998) and suggested for PFAS as well (Bertolero et al., 2015; Hitchcock et al., 2019; Jouanneau et al., 2021; Leat et al., 2013). Also, sexual segregation of foraging areas, which has been reported during years with variable environmental conditions (Paiva et al., 2017), could have contributed to lower PFAS concentrations in females. In both sexes, $\text{AD}\delta^{15}\text{N}$ values and PFAS concentrations during the incubation phase were higher than during the following chick-rearing phase. This finding is supported by results from Costantini et al., (2017) and indicates a diet based on higher trophic-level prey during incubation. However, foraging areas vary according to the breeding phase. During incubation, breeders mostly perform several day trips south to coastal areas of Libya in the area of Tripoli (Cecere et al., 2013; Cianchetti-Benedetti et al., 2017; Colominas-Ciuró et al., 2022), whereas during chick-rearing, they alternate short and long trips (Cecere et al., 2014), remaining more often in the pelagic areas close to the colony. Thus, PFAS levels in males and females converged again during chick-rearing, pointing towards the dynamics of concentrations in blood, affected by diet, egg laying, and fasting (Mollier et al., 2024).

Generally, breeding shearwaters have long fasting periods during incubation with lower activity rates, decreased energy expenditure (Cianchetti-Benedetti et al., 2017), metabolism, and stress (Colominas-Ciuró et al., 2022). This could influence the remobilization, excretion and metabolism of tissue-accumulated substances, as shown for lipophilic persistent organic pollutants (Bustnes et al., 2012) and PFOS, PFTrIDA, and PFuNA (Mollier et al., 2024). Also, it may explain the overall higher blood concentrations during incubation. The positive and negative associations with some PFAS and the $\text{AD}\delta^{13}\text{C}$ indicator for pelagic or littoral feeding support that the foraging area and proximity to the coast may determine different PFAS profiles. However, the associations were weak, and a previous study on other persistent organic pollutant classes in birds from Linosa found no relation between the SI signatures and the contaminant concentrations measured (Costantini et al., 2017).

Conclusion

Through the use of a multicolony approach, we successfully showed the variation in PFAS concentrations in a top predator foraging in different areas of the Mediterranean Sea and concluded that the contamination status of the environment surrounding the colony seems to be the primary determinant of PFAS concentrations. This supports that shearwaters can be used as PFAS biomonitors of remote marine environments, because even relatively small-scale geographical differences can be detected. Because PFAS concentrations have been associated with effects on reproductive parameters, morphometrics, metabolic processes, and physiological markers, especially in juvenile and breeding female seabirds (Blévin et al., 2017; Robuck et al., 2020; Sebastiano et al., 2023; Szabo et al., 2021), follow-up studies assessing possible risks to shearwaters in these life stages, critical for healthy population development, are advisable.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

Data, associated metadata, and calculation tools not presented are available from the corresponding author (lucie.michel@bio.uni-giessen.de)

Author contributions

Lucie Michel (Conceptualization, Data curation, Formal analysis, Investigation, Resources, Writing—original draft) Junjie Zhang (Methodology, Supervision, Validation, Writing—review & editing) Alexandros Asimakopoulos (Conceptualization, Methodology, Supervision, Validation, Writing—review & editing) Martin Austad (Resources, Writing—review & editing) Paco Bustamante (Project administration, Writing—review & editing) Jacopo Cecere (Resources, Writing—review & editing) Marco Cianchetti-Benedetti (Resources, Writing—review & editing) Roger Colominas-Ciuró (Resources, Writing—review & editing) Giacomo Dell’Omo (Resources, Writing—review & editing) Federico De Pascalis (Resources, Writing—review & editing) Veerle Jaspers (Conceptualization, Supervision, Writing—review & editing) Petra Quillfeldt (Conceptualization, Project administration, Supervision, Writing—review & editing)

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Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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SUPPORTING INFORMATION

Assessing perfluoroalkyl substance pollution in central Mediterranean breeding shearwaters

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A. Certified reference materials for SI Analysis

$\delta^{13}\text{C}$: USGS-24, IAEA-CH6, IAEA-600, USGS-61, USGS-62, USGS-63, USGS-41a.

$\delta^{15}\text{N}$: IAEA-N2, IAEA-NO-3, IAEA-600, USGS-61, USGS-62, USGS-63, USGS-41a.

Table S1 Overview of collection location and time of Scopoli's shearwater (*Calonectris diomedea*) freeze-dried red blood cell samples analysed for PFAS and stable isotope values.

Colony	Year	Breeding Phase	N Females	N Males	N NA	N Total
Linosa	2020	Incubation	11	18	0	29
Linosa	2020	Chick-rearing	14	9	0	23
Linosa	2016	Chick-rearing	8	7	3	18
Malta	2020	Chick-rearing	6	5	4	15
La Maddalena	2020	Chick-rearing	12	8	0	20

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Table S2 Retention time (RT) and relative retention time (RRT), internal Standards (IS) used for quantification of each target analyte in blood of Scopoli's shearwaters (*Calonectris diomedea*) and limits of detection (LOD) and quantification (LOQ) in ng/ml. LOQ was set at the lowest valid point in the calibration row. LOD was estimated as LOQ/3. mLOQ were estimated from the respective instrumental LOD and instrumental LOQ at a nominal sample weight mass of 0.1 g.

Target	RT (min)	RRT	IS	LOD [ng/mL]	LOQ [ng/mL]	mLOD[ng/g]	mLOQ[ng/g]
PFPeA	1.03	0.73	PFOA- ¹³ C ₈	0.020	0.05	0.10	0.30
PFBS	1.06	0.69	PFOS- ¹³ C ₈	0.007	0.02	0.04	0.12
7H-PFHpA	1.09	0.77	PFOA- ¹³ C ₈	0.020	0.05	0.10	0.30
4:2 FTS	1.16	0.82	6:2 FTS- ¹³ C ₂	0.020	0.05	0.10	0.30
PFHxA	1.17	0.83	PFOA- ¹³ C ₈	0.030	0.10	0.20	0.60
GenX	1.19	0.77	PFOS- ¹³ C ₈	0.070	0.20	0.40	1.20
PFPeS	1.19	0.78	PFOS- ¹³ C ₈	0.020	0.05	0.10	0.30
PFHpA	1.29	0.91	PFOA- ¹³ C ₈	0.007	0.02	0.04	0.12
PFHxS	1.30	0.84	PFOS- ¹³ C ₈	0.020	0.05	0.10	0.30
ADONA	1.30	0.92	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
PFECHS	1.40	0.91	PFOS- ¹³ C ₈	0.003	0.01	0.02	0.06
6:2 FTS	1.41	1.00	6:2 FTS- ¹³ C ₂	0.007	0.02	0.04	0.12
PFOA	1.41	1.00	PFOA- ¹³ C ₈	0.020	0.05	0.10	0.30
PFHpS	1.41	0.92	PFOS- ¹³ C ₈	0.007	0.02	0.04	0.12
PFNA	1.54	1.09	PFOA- ¹³ C ₈	0.020	0.05	0.10	0.30
PFOS	1.54	1.00	PFOS- ¹³ C ₈	0.020	0.05	0.10	0.30
P37DMOA	1.61	1.14	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
9CI-PF3ONS	1.61	1.14	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
FOSAA	1.67	1.18	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
PFNS	1.67	1.09	PFOS- ¹³ C ₈	0.007	0.02	0.04	0.12
PFDA	1.68	1.19	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
8:2 FTS	1.68	1.19	6:2 FTS- ¹³ C ₂	0.020	0.05	0.10	0.30
MeFOSAA	1.75	1.24	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
PFDS	1.82	1.18	PFOS- ¹³ C ₈	0.003	0.01	0.02	0.06
PFUnA	1.83	1.30	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
PFDoDA	1.98	1.40	PFOA- ¹³ C ₈	0.020	0.05	0.10	0.30
10:2 FTS	1.98	1.40	6:2 FTS- ¹³ C ₂	0.007	0.02	0.04	0.12
PFOSA	1.98	1.40	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
PFDoDS	2.12	1.38	PFOS- ¹³ C ₈	0.003	0.01	0.02	0.06
PFTriDA	2.13	1.51	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
SAMPAP	2.19	1.55	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
PFTDA	2.29	1.62	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
MeFOSA	2.37	1.68	PFOA- ¹³ C ₈	0.007	0.02	0.04	0.12
MeFOSE	2.38	1.69	PFOA- ¹³ C ₈	0.300	0.50	1.00	3.00
EtFOSE	2.54	1.80	PFOA- ¹³ C ₈	0.070	0.20	0.40	1.20
EtFOSA	2.55	1.81	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
PFHxDA	2.67	1.89	PFOA- ¹³ C ₈	0.007	0.02	0.04	0.12
PFOcDA	3.14	2.23	PFOA- ¹³ C ₈	0.003	0.01	0.02	0.06
diSAMPAP	3.46	2.45	PFOA- ¹³ C ₈	0.007	0.02	0.04	0.12

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Table S3 List of PFAS target analytes with molecular structures, CAS number, formulae, molecular mass and supply information.

Abbreviation	Full Name	CAS	Formula	Molecular mass	Supplier
<i>Perfluorocarboxylic Acids (PFCAs)</i>					
PFPeA	Perfluoropentanoic acid	2706-90-3	C ₅ HF ₉ O ₂	264.1	Sigma Aldrich (97%, neat)
PFHxA	Perfluorohexanoic acid	307-24-4	C ₆ HF ₁₁ O ₂	314.1	Sigma Aldrich (97%, neat)
PFHpA	Perfluoroheptanoic acid	375-85-9	C ₇ HF ₁₃ O ₂	364	Sigma Aldrich (99%, neat)
PFOA	Perfluorooctanoic acid	335-67-1	C ₈ HF ₁₅ O ₂	414.1	Sigma Aldrich (96%, neat)
PFNA	Perfluorononanoic acid	375-95-1	C ₉ HF ₁₇ O ₂	464.1	Sigma Aldrich (97%, neat)
PFDA	Perfluorodecanoic acid	335-76-2	C ₁₀ HF ₁₉ O ₂	514.1	Sigma Aldrich (98%, neat)
PFUnA	Perfluoroundecanoic acid	2058-94-8	C ₁₁ HF ₂₁ O ₂	564.1	Sigma Aldrich (95%, neat)
PFDoDA	Perfluorododecanoic acid	307-55-1	C ₁₂ HF ₂₃ O ₂	614.1	Sigma Aldrich (97%, neat)
PFTriDA	Perfluorotridecanoic acid	72629-94-8	C ₁₃ HF ₂₅ O ₂	664.1	Sigma Aldrich (97%, neat)
PFTDA	Perfluorotetradecanoic acid	376-06-7	C ₁₄ HF ₂₇ O ₂	714.1	Sigma Aldrich (96%, neat)
PFHxDA	Perfluoro-n-hexadecanoic acid	67905-19-5	C ₁₆ HF ₃₁ O ₂	814.1	Chiron (50 µg/mL, methanol)
PFOcDa	Perfluorooctadecaonic acid	16517-11-6	C ₁₈ HF ₃₅ O ₂	914.1	Wellington Laboratories (2000 ng/mL, solution MIX methanol) PFCAMXC
P37DMOA	Perfluoro-3,7-dimethyloctanoic acid	172155-07-6	C ₁₀ HF ₁₉ O ₂	514	Wellington Laboratories (50 µg/mL, methanol)
7H-PFHpA	7H-Dodecafluoroheptanoic acid	1546-95-8	C ₇ H ₂ F ₁₂ O ₂	346.1	Sigma Aldrich (95%, neat)

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Perfluoroalkyl Sulfonates (PFASs)

PFBS	Perfluorobutanesulfonic acid	375-73-5	C ₄ F ₉ SO ₃	299.1	Cambridge Isotope Laboratories (50 µg/mL, methanol)*
PFPeS	Perfluoropentane sulfonic acid	2706-91-4	C ₅ HF ₁₁ O ₃ S	350.11	Wellington Laboratories (2000 ng/mL, solution MIX methanol) PFCAMXC*
PFHxS	Perfluorohexane sulfonic acid	355-46-4	C ₆ F ₁₃ O ₃ S	399.1	Sigma Aldrich (98%)
PFHpS	Perfluoroheptane sulfonic acid	146689-46-5	C ₇ F ₁₅ O ₃ S	449.1	Cambridge Isotope Laboratories (50 µg/mL, methanol)
PFOS	Perfluorooctanosulfonic acid	1763-23-1	C ₈ F ₁₇ O ₃ S	499.1	Cambridge Isotope Laboratories (50 µg/mL, methanol)
PFNS	Perfluorononane sulfonic acid	68259-12-1	C ₉ H ₂ F ₁₆ O ₃ S	550.14	Sigma Aldrich (98%)
PFDS	Perfluorodecane sulfonic acid	335-77-3	C ₁₀ HF ₂₁ O ₃ S	550.14	Wellington Laboratories (2000 ng/mL, solution MIX methanol) PFCAMXC*
PFDoDS	Perfluorododecane sulfonic acid	79780-39-5	C ₁₂ H ₂ F ₂₄ O ₃ S	550.14	Wellington Laboratories (2000 ng/mL, solution MIX methanol) PFCAMXC*
PFECHS	Perfluoroethylcyclohexane sulfonic acid	335-24-0	C ₈ H ₂ F ₁₅ O ₃ S	550.14	Wellington Laboratories (50 µg/mL, methanol)*

Fluorotelomer Sulfonates (FTS)

4:2 FTS	1H,2H-Perfluorohexan sulfonate (4:2)	757124-72-4	C ₆ H ₅ F ₉ O ₃ S	328.2	Cambridge Isotope Laboratories (50 µg/mL, methanol)
6:2 FTS	1H,2H-Perfluorooctane sulfonate (6:2)	27619-97-2	C ₈ H ₅ F ₁₃ O ₃ S	428.2	Cambridge Isotope Laboratories (50 µg/mL, methanol)
8:2 FTS	1H,2H-Perfluorodecan sulfonate (8:2)	39108-34-4	C ₁₀ H ₅ F ₁₇ O ₃ S	528.2	Cambridge Isotope Laboratories (50 µg/mL, methanol)
10:2 FTS	1H,2H-Perfluorododecan sulfonate (10:2)	120226-60-0	C ₁₂ H ₅ F ₂₁ O ₃ S	628.2	Cambridge Isotope Laboratories (50 µg/mL, methanol)

Fluorosulfonamide (FSA)

FOSAA	Perfluoro-1-octanesulfonamidoacetic acid	2806-24-8	C ₁₀ H ₄ F ₁₇ NO ₄ S	557.2	Chiron (50 µg/mL, methanol)
MeFOSAA	2-(N-methylperfluoro-1-octanesulfonamido)acetic acid	2355-31-9	C ₁₁ H ₆ F ₁₇ NO ₄ S	571.2	Chiron (50 µg/mL, methanol)
PFOSA	Perfluorooctane sulfonamide	754-91-6	C ₈ H ₂ F ₁₇ NO ₂ S	499.1	Sigma Aldrich (99%)
MeFOSA	N-methylperfluoro-1-octanesulfonamide	31506-32-8	C ₉ H ₄ F ₁₇ NO ₂ S	513.2	Chiron (50 µg/mL, acetonitrile)
MeFOSE	N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide	24448-09-7	C ₁₁ H ₈ F ₁₇ NO ₃ S	557.23	Chiron (50 µg/mL, acetonitrile)

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EtFOSE	N-ethyl-N-(2-hydroxyethyl)-N-methylperfluorooctane sulfonamide	1691-99-2	$C_{12}H_{10}F_{17}NO_3S$	571.25	Chiron (50 µg/mL, acetonitrile)
EtFOSA	N-ethylperfluoro-1-octanesulfonamide	4151-50-2	$C_{10}H_6F_{17}NO_2S$	527.2	Chiron (neat)
Miscellaneous					
Gen X	2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)propanoate	62037-80-3	$C_6H_4F_{11}NO_3$	347.1	Accu Standard
ADONA	Dodecafluoro-3H-4,8-dioxanonoate	958445-44-8	$C_7H_5F_{12}NO_4$	395.1	Wellington Laboratories (50 µg/mL, methanol)*
DecaS	Decasulfonic acid	13419-61-9	$C_{10}H_{21}O_3S$	244.33	Sigma Aldrich (99%)*
9Cl-PF3ONS	9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	73606-19-6	$C_8ClF_{16}KO_4S$	570.7	Wellington Laboratories (50 µg/mL, methanol)*
diSAMPAP	Bis[2-(N-ethylperfluorooctane-1-sulfonamido)ethyl] phosphate	30381-98-7	$C_{24}H_{22}F_{34}N_3O_8P_2S_2$	1221.5	Wellington Laboratories (50 µg/mL, methanol)*
Internal Standards					
PFOA $^{13}C_8$	Perfluorooctanoic acid - $^{13}C_8$, 99%		$^{13}C_8HF_{15}O_2$	422.1	Cambridge Isotope Laboratories (50 µg/mL, methanol)
PFOS $^{13}C_8$	Perfluorooctanesulfonate- $^{13}C_8$, 99%		$^{13}C_8F_{17}O_3S$	507.1	Cambridge Isotope Laboratories (50 µg/mL, methanol)
6:2 FTS $^{13}C_2$	1H,2H-Perfluorooctane sulfonate- $^{13}C_2$, 99%		$^{13}C_2C_6D_4F_{13}NaO_3S$	456.16	Cambridge Isotope Laboratories (50 µg/mL, methanol)

*Standards were in salt form but assumed neat when calculating calibration curves. This yielded an error due to the wrong molar mass being used. However, the resulting error was smaller than the overall method error, so it was assumed insignificant

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Table S4 Baseline correction of stable isotopes calculated from the rounded difference in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ baseline values of zooplankton along the latitudinal gradient reported by Rumolo et al. 2016 and Campioni et al. 2022. To adjust the SI values measured in La Maddalena birds, the average of the difference (Δ) of the zooplankton values from the Tyrrhenian Sea (representing La Maddalena) and the Sicily Channel (representing Linosa and Malta) measured in 2011 and 2016, were subtracted from the values measured in La Maddalena shearwaters in this study.

Sample location	Sample type	Mean $\delta^{13}\text{C}$ (‰)	Mean $\delta^{15}\text{N}$ (‰)	Sampling Year	Source
Tyrrhenian Sea	Zooplankton	-21.10	5.10	2011	Rumolo 2016
Sicily Channel	Zooplankton	-21.40	3.80	2011	Rumolo 2016
Δ Tyrrhenian Sea – Sicily Channel 2011		0.30	1.30	2011	
Tyrrhenian Sea	Zooplankton	-23.30	3.40	2016	Campioni 2020
Sicily Channel	Zooplankton	-23.40	2.10	2016	Campioni 2020
Δ Tyrrhenian Sea – Sicily Channel 2016	Zooplankton	0.20	1.30	2016	
Δ Mean (2011, 2016)	Zooplankton	0.25	1.30		
La Maddalena	RBC SSH	-18.80	9.60	2020	This study
Δ adjusted La Maddalena	RBC SSH	-19.05	8.30	2020	This study
Linosa	RBC SSH	-19.10	8.20	2020	This study
Malta	RBC SSH	-19.10	8.00	2020	This study

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Table S5 Correlation coefficients (rho) (method = spearman) and p-values (p < 0.05, ** p < 0.01, *** p < 0.001) for the PFAS with less than 70% censoring, adjusted stable isotope signatures for carbon and nitrogen for all red blood cells (n=105).

	PFOS	PFUnA	PFDoDA	PFTriDA	PFTDA	PFNA	PFOSA	10:2 FTS	PFOA	PFDA	PFHxS	PFDS	PFHpS	AD $\delta^{13}C$	AD $\delta^{15}N$	Σ PFAS
PFOS	1.00															
PFUnA	0.82***	1.00														
PFDoDA	0.77***	0.82***	1.00													
PFTriDA	0.46***	0.44***	0.71***	1.00												
PFTDA	0.19*	0.20	0.51***	0.74***	1.00											
PFNA	0.81***	0.80***	0.64***	0.34***	0.10	1.00										
PFOSA	0.31**	0.47***	0.36***	0.10	0.10	0.42***	1.00									
10:2 FTS	0.21*	0.10	0.35***	0.50***	0.42***	0.00	0.00	1.00								
PFOA	0.47***	0.49***	0.42***	0.20	0.10	0.62***	0.53***	0.10	1.00							
PFDA	0.68***	0.74***	0.57***	0.37***	0.00	0.77***	0.39***	0.00	0.52***	1.00						
PFHxS	0.66***	0.54***	0.45***	0.28**	0.10	0.77***	0.27**	0.00	0.64***	0.67***	1.00					
PFDS	0.51***	0.52***	0.57***	0.32**	0.19*	0.36***	0.10	0.24*	0.27**	0.31**	0.32**	1.00				
PFHpS	0.45***	0.35***	0.27**	0.22*	0.10	0.45***	0.10	0.20	0.34***	0.45***	0.44***	0.26*	1.00			
AD $\delta^{13}C$	-0.12	-0.19*	-0.06	0.19*	0.22*	-0.15	-0.22*	0.13	-0.25*	-0.12	-0.05	-0.02	-0.07	1.00		-0.13
AD $\delta^{15}N$	0.24*	0.38***	0.29***	0.34***	0.19*	0.26*	0.18	-0.10	-0.04	0.29***	0.19*	0.18	-0.05	0.29**	1.00	0.30**

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Table S6a Scopoli's shearwater (*Calonectris diomedea*) blood concentrations of PFAS (ng/g) and adjusted stable isotope ratios in the incubation (INC) and chick-rearing (CR) phase of 2020 in three different colonies and in the chick-rearing phase of 2016 in Linosa*. SD = standard deviation; CI = confidence interval of the mean, censoring = percentage of censored values.

PFAS	Colony	Year	Phase	Mean	SD	Median	lower 95% CI of mean	upper 95% CI of mean	Min	Max	cen- soring
SUMPFAS	Linosa	2016	CR	95.37	49.17	86.06	75.33	123.11	47.61	299.93	0
	Linosa	2020	INC	159.57	64.76	148.28	137.19	183.57	65.93	337.45	0
	Linosa	2020	CR	91.57	41.33	78.62	75.69	110.30	41.00	204.73	0
	Malta	2020	CR	144.96	64.63	128.92	114.13	183.10	74.13	333.12	0
	La Maddalena	2020	CR	84.71	46.56	81.84	65.08	106.15	17.37	223.91	0
PFOS	Linosa	2016	CR	48.87	24.94	44.69	38.60	62.59	18.49	145.97	0
	Linosa	2020	INC	80.52	37.83	78.28	67.33	94.71	31.63	183.78	0
	Linosa	2020	CR	50.55	24.75	42.96	40.98	61.99	21.86	125.55	0
	Malta	2020	CR	83.48	38.09	73.07	65.12	105.60	43.76	185.28	0
	La Maddalena	2020	CR	43.56	26.89	40.74	32.49	56.33	8.23	136.15	0
PFuNA	Linosa	2016	CR	16.91	11.47	14.87	12.35	23.52	6.35	66.40	0
	Linosa	2020	INC	29.00	13.80	26.16	24.19	34.26	8.89	70.61	0
	Linosa	2020	CR	13.76	5.58	12.89	11.54	16.19	5.49	26.20	0
	Malta	2020	CR	20.00	11.54	17.55	14.68	27.40	7.27	61.06	0
	La Maddalena	2020	CR	8.36	5.41	7.44	6.16	10.87	1.61	22.60	0
PFDoDA	Linosa	2016	CR	4.93	2.32	4.60	3.99	6.22	2.25	14.48	0
	Linosa	2020	INC	6.61	2.82	6.34	5.63	7.63	2.16	13.58	0
	Linosa	2020	CR	5.14	2.39	4.59	4.21	6.19	1.92	11.38	0
	Malta	2020	CR	5.72	2.62	5.21	4.49	7.34	2.66	14.50	0
	La Maddalena	2020	CR	4.04	2.21	3.93	3.11	5.07	0.88	10.84	0
PFTriDA	Linosa	2016	CR	8.29	3.07	7.77	6.95	9.87	3.86	18.60	0
	Linosa	2020	INC	9.57	5.35	8.28	7.78	11.57	3.09	28.40	0
	Linosa	2020	CR	7.01	4.46	5.45	5.32	9.03	2.14	22.25	0
	Malta	2020	CR	8.72	3.98	7.52	6.85	11.09	4.47	20.44	0
	La Maddalena	2020	CR	11.92	5.99	11.70	9.31	14.59	2.65	22.90	0
PFTDA	Linosa	2016	CR	1.75	0.84	2.00	1.34	2.13	<0.02	2.78	0
	Linosa	2020	INC	2.03	1.27	1.88	1.58	2.50	0.14	5.75	0
	Linosa	2020	CR	1.99	1.16	1.81	1.53	2.50	0.38	4.88	0
	Malta	2020	CR	1.30	0.66	1.16	0.98	1.66	0.25	2.70	0
	La Maddalena	2020	CR	3.42	1.68	3.51	2.69	4.16	0.97	6.24	0
PFNA	Linosa	2016	CR	3.87	3.08	2.82	2.66	5.58	<0.10	16.51	5
	Linosa	2020	INC	9.55	4.96	8.52	7.82	11.42	3.12	21.37	0
	Linosa	2020	CR	3.31	2.40	2.47	2.38	4.38	<0.10	9.58	9
	Malta	2020	CR	7.40	3.62	6.49	5.65	9.50	3.19	16.10	0
	La Maddalena	2020	CR	3.19	2.13	2.47	2.35	4.19	0.83	9.19	0
PFDA	Linosa	2016	CR	2.18	1.96	1.30	1.35	3.21	<0.02	9.09	11
	Linosa	2020	INC	5.87	4.07	5.44	4.48	7.49	0.58	19.90	0
	Linosa	2020	CR	1.28	1.29	0.75	0.78	1.89	<0.02	6.23	26
	Malta	2020	CR	4.32	2.07	3.68	3.31	5.44	2.03	8.33	0
	La Maddalena	2020	CR	1.25	1.22	0.78	0.67	1.87	<0.02	4.06	35
PFOSA	Linosa	2016	CR	4.49	3.55	2.66	2.83	6.35	<0.02	12.20	39
	Linosa	2020	INC	8.60	4.58	7.06	7.02	10.40	<0.02	21.39	7
	Linosa	2020	CR	4.38	1.55	4.06	3.73	5.08	<0.02	7.08	26
	Malta	2020	CR	3.65	1.19	3.69	3.04	4.28	<0.02	6.16	7
	La Maddalena	2020	CR	3.28	2.60	2.64	2.15	4.54	0.40	11.16	15
10:2 FTS	Linosa	2016	CR	1.18	0.64	0.95	0.89	1.50	<0.04	2.39	11
	Linosa	2020	INC	0.66	0.47	0.51	0.49	0.84	<0.04	2.03	30
	Linosa	2020	CR	1.25	0.98	0.97	0.85	1.70	<0.04	3.23	28
	Malta	2020	CR	1.83	1.39	1.69	1.12	2.56	<0.04	4.27	13
	La Maddalena	2020	CR	2.01	1.03	1.95	1.55	2.47	<0.04	3.67	10
PFOA	Linosa	2016	CR	1.52	0.81	1.15	1.15	1.95	<0.10	4.05	39
	Linosa	2020	INC	2.50	1.66	2.02	1.93	3.16	<0.10	8.52	30
	Linosa	2020	CR	1.89	1.65	1.33	1.26	2.68	<0.10	7.43	7
	Malta	2020	CR	2.78	1.62	2.52	1.97	3.68	<0.10	6.38	7
	La Maddalena	2020	CR	1.24	0.77	0.90	0.93	1.61	<0.10	3.19	25
PFHxS	Linosa	2016	CR	2.67	1.39	1.99	1.77	3.99	<0.10	8.03	67
	Linosa	2020	INC	4.36	2.28	3.62	3.54	5.19	<0.10	8.33	57
	Linosa	2020	CR	1.89	1.67	1.23	1.14	2.80	<0.10	7.95	4
	Malta	2020	CR	4.08	1.92	3.65	3.13	5.13	<0.10	8.95	13
	La Maddalena	2020	CR	2.28	2.01	1.44	1.51	3.27	<0.10	9.69	20

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PFAS	Colony	Year	Phase	Mean	SD	Median	lower 95% CI of mean	upper 95% CI of mean	Min	Max	cen- soring
PFDS	Linosa	2016	CR	0.33	0.21	0.25	0.24	0.46	<0.02	1.01	39
	Linosa	2020	INC	0.35	0.28	0.25	0.26	0.46	<0.02	1.38	35
	Linosa	2020	CR	0.29	0.15	0.23	0.23	0.35	<0.02	0.67	27
	Malta	2020	CR	0.35	0.23	0.29	0.24	0.49	<0.02	0.84	27
	La Maddalena	2020	CR	0.15	0.11	0.09	0.10	0.22	<0.02	0.41	50
PFHpS	Linosa	2016	CR	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	100
	Linosa	2020	INC	0.26	0.08	0.22	0.22	0.31	<0.04	0.49	91
	Linosa	2020	CR	0.29	0.03	NA	<0.04	0.48	<0.04	0.48	65
	Malta	2020	CR	0.30	0.11	0.26	0.24	0.37	<0.04	0.54	33
	La Maddalena	2020	CR	0.24	0.07	0.22	0.22	0.28	<0.04	0.56	70
AD δ 13C	Linosa	2016	CR	-18.85	0.35	-18.84	-19.01	-18.69	-19.33	-18.31	0
	Linosa	2020	INC	-19.12	0.38	-19.16	-19.26	-18.98	-19.85	-18.33	0
	Linosa	2020	CR	-19.09	0.14	-19.11	-19.15	-19.03	-19.33	-18.79	0
	Malta	2020	CR	-19.14	0.20	-19.19	-19.23	-19.02	-19.38	-18.57	0
	La Maddalena	2020	CR	-19.06	0.14	-19.05	-19.12	-19.00	-19.29	-18.8	0
AD δ 15N	Linosa	2016	CR	8.96	0.52	8.84	8.73	9.21	8.16	9.83	0
	Linosa	2020	INC	9.38	0.46	9.42	9.20	9.54	8.4	10.4	0
	Linosa	2020	CR	8.11	0.49	8.07	7.91	8.32	7.42	9.06	0
	Malta	2020	CR	7.97	0.59	7.80	7.69	8.29	7.36	9.27	0
	La Maddalena	2020	CR	8.30	0.64	8.39	8.01	8.58	6.73	9.6	0

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Table S6b Female and male Scopoli's shearwater (*Calonectris diomedea*) blood concentrations of PFAS (ng/g) and adjusted stable isotope ratios in the incubation (INC) and chick-rearing (CR) phase of 2020 in three different colonies and in the chick-rearing phase of 2016 in Linosa*. SD = standard deviation; CI = confidence interval of the mean, censoring = percentage of censored values.

PFAS	Colony	Year	Phase	Mean	SD	Median	Females				Males				Max	cen-soring			
							upper	lower	95% CI	95% CI	upper	lower	95% CI	95% CI					
SUI/PFAS	Linosa	2016	CR	119.26	60.42	94.91	86.93	172.50	79.08	299.93	0	79.48	25.17	75.85	61.69	99.60	49.90	125.72	0
	Linosa	2020	INC	114.11	39.85	102.79	92.00	139.52	65.93	197.50	0	187.39	61.09	163.03	160.69	218.38	121.89	337.45	0
	Linosa	2020	CR	86.86	41.60	76.31	66.74	111.96	41.00	204.73	0	98.30	36.40	84.43	76.98	126.58	66.48	192.95	0
PFOS	Malta	2020	CR	173.77	77.44	162.39	116.16	243.01	74.13	333.12	0	124.30	30.68	124.37	96.78	152.46	84.08	161.73	0
	La Maddalena	2020	CR	69.97	33.79	62.48	51.65	89.51	33.53	141.01	0	106.48	52.96	101.96	69.33	145.85	17.37	223.91	0
	Linosa	2016	CR	61.77	28.62	50.41	46.03	86.93	43.19	145.97	0	41.02	14.72	38.31	30.70	52.74	24.05	67.34	0
	Linosa	2020	INC	50.92	21.16	43.57	39.39	65.13	31.63	103.73	0	98.60	34.10	84.94	84.04	116.03	64.68	183.78	0
	Linosa	2020	CR	49.67	26.01	43.06	37.22	65.41	21.86	125.55	0	51.62	20.21	43.86	39.86	67.38	35.93	105.31	0
	Malta	2020	CR	97.16	42.60	91.29	66.57	135.02	43.76	185.28	0	71.94	19.89	71.10	54.27	90.08	46.15	99.87	0
PFUNA	La Maddalena	2020	CR	33.82	16.16	30.38	25.16	43.14	15.55	68.21	0	58.03	32.46	53.09	36.64	83.17	8.23	136.15	0
	Linosa	2016	CR	22.71	14.28	16.69	15.35	35.51	14.12	66.40	0	12.13	5.03	11.39	8.59	16.16	6.35	21.68	0
	Linosa	2020	INC	20.77	8.57	19.13	15.93	26.22	8.89	34.35	0	34.06	13.95	29.26	28.04	41.17	16.26	70.61	0
	Linosa	2020	CR	12.83	5.42	11.76	10.09	15.82	5.49	22.17	0	15.14	5.32	14.27	11.77	18.98	7.34	26.20	0
	Malta	2020	CR	26.56	15.56	23.49	15.63	41.20	7.27	61.06	0	15.89	4.42	15.73	12.07	19.79	10.06	22.81	0
	La Maddalena	2020	CR	6.74	3.66	5.92	4.76	8.93	2.71	14.49	0	10.74	6.51	9.20	6.45	15.51	1.61	22.60	0
PFDoDA	Linosa	2016	CR	5.88	2.82	4.85	4.38	8.44	3.94	14.48	0	4.14	1.35	4.18	3.13	5.19	2.25	6.35	0
	Linosa	2020	INC	4.48	1.66	4.24	3.54	5.52	2.16	7.57	0	7.91	2.56	7.64	6.76	9.16	3.72	13.58	0
	Linosa	2020	CR	4.50	1.96	4.02	3.53	5.61	1.92	8.67	0	6.09	2.57	5.38	4.51	7.92	2.55	11.38	0
	Malta	2020	CR	6.92	3.67	6.33	4.21	10.23	2.66	14.50	0	4.96	0.97	5.13	3.88	5.81	3.00	6.23	0
	La Maddalena	2020	CR	3.32	1.54	3.10	2.48	4.21	1.57	5.51	0	5.10	2.58	4.83	3.32	7.06	0.88	10.84	0
	Linosa	2016	CR	8.14	3.94	7.05	5.76	11.36	3.86	18.60	0	8.07	1.84	8.06	6.63	9.55	4.17	11.62	0
PFTrIDA	Linosa	2020	INC	5.62	1.97	5.10	4.51	6.83	3.09	9.40	0	11.99	5.30	11.72	9.69	14.69	4.34	28.40	0
	Linosa	2020	CR	4.99	1.96	4.86	3.99	6.10	2.14	10.36	0	10.07	5.36	9.59	6.69	14.03	2.95	22.25	0
	Malta	2020	CR	10.88	5.16	10.20	6.86	15.25	4.47	20.44	0	8.38	2.00	8.35	6.59	10.17	5.92	11.21	0
	La Maddalena	2020	CR	10.14	5.08	9.21	7.41	13.17	4.53	20.74	0	14.53	6.12	15.17	10.00	18.61	2.65	22.90	0
	Linosa	2016	CR	1.67	0.68	1.75	1.18	2.12	0.40	2.56	0	1.84	0.72	2.04	1.28	2.35	0.68	2.65	0
	Linosa	2020	INC	1.20	0.56	1.15	0.86	1.53	0.22	2.10	0	2.53	1.31	2.55	1.92	3.16	0.14	5.75	0
PFTDA	Linosa	2020	CR	1.76	0.79	1.73	1.34	2.18	0.68	3.12	0	2.33	1.50	2.10	1.36	3.36	0.38	4.88	0
	Malta	2020	CR	1.66	0.82	1.73	0.94	2.29	0.25	2.70	0	1.08	0.35	1.01	0.81	1.46	0.66	1.76	0
	La Maddalena	2020	CR	3.04	1.74	2.78	2.08	4.06	0.97	6.24	0	3.98	1.33	4.17	2.95	4.84	1.01	5.74	0
	Linosa	2016	CR	4.84	4.19	3.14	2.40	8.33	1.77	16.51	0	79.48	25.17	75.85	2.44	3.66	1.62	4.18	0
	Linosa	2020	INC	8.04	4.27	7.37	5.63	10.67	3.12	17.06	0	10.46	5.06	9.02	8.32	12.98	5.01	21.37	0
	Linosa	2020	CR	3.30	2.45	2.14	2.09	4.73	<0.10	9.58	7	3.38	2.03	2.76	2.21	5.12	1.54	9.55	11
PFDA	Malta	2020	CR	8.52	3.75	7.70	5.81	11.74	4.52	16.10	0	6.48	1.92	6.82	4.51	8.11	3.19	8.77	0
	La Maddalena	2020	CR	2.81	1.89	2.24	1.89	4.13	0.98	9.19	0	3.75	2.12	3.36	2.36	5.35	0.83	8.13	0
	Linosa	2016	CR	2.39	2.53	1.31	0.91	4.46	0.62	9.09	0	1.72	0.77	1.46	1.17	2.36	<0.02	3.26	14
	Linosa	2020	INC	4.03	2.36	4.04	2.59	5.41	0.58	7.48	0	7.01	4.43	6.18	5.10	9.26	1.07	19.90	0
	Linosa	2020	CR	1.17	1.35	0.58	0.57	2.09	<0.02	6.23	29	1.47	1.00	1.31	0.79	2.34	0.37	3.00	23
	Malta	2020	CR	5.20	2.18	5.38	3.45	6.91	2.03	8.09	0	4.27	1.99	3.78	2.70	6.40	2.26	8.33	0
La Maddalena	2020	CR	1.43	1.03	0.99	0.88	2.09	<0.02	3.77	25	1.31	1.26	0.54	0.22	2.86	<0.02	4.06	50	

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PFAS	Colony	Year	Phase	Females						Males									
				Mean	SD	Median	lower 95% CI	upper 95% CI	Min	Max	cen-soring	Mean	SD	Median	lower 95% CI	upper 95% CI	Min	Max	cen-soring
PFOSA	Linosa	2016	CR	7.58	2.83	6.31	4.63	11.07	<0.02	12.10	38	3.95	2.17	2.98	2.49	6.17	<0.02	9.03	29
	Linosa	2020	INC	11.26	4.49	10.46	8.67	14.06	5.95	21.39	0	6.98	3.61	5.84	5.48	9.06	<0.02	21.09	11
	Linosa	2020	CR	4.52	1.67	4.03	3.61	5.48	<0.02	7.08	21	4.51	0.98	4.15	3.80	5.35	<0.02	6.76	34
	Malta	2020	CR	3.93	1.27	3.94	2.89	5.03	1.78	6.16	0	3.44	1.06	3.27	2.52	4.44	2.27	5.24	0
10:2 FTS	La Maddalena	2020	CR	3.54	2.76	2.56	2.07	5.35	<0.02	11.16	8	3.09	1.87	2.46	1.40	4.78	<0.02	11.16	25
	Linosa	2016	CR	1.33	0.63	1.10	0.89	1.82	<0.04	2.39	13	1.17	0.64	1.02	0.68	1.72	<0.04	2.24	14
	Linosa	2020	INC	0.43	0.22	0.37	0.28	0.61	<0.04	1.08	36	0.82	0.50	0.71	0.58	1.09	<0.04	2.03	22
	Linosa	2020	CR	1.06	0.76	0.76	0.64	1.53	<0.04	2.25	29	1.77	0.98	1.52	0.99	2.77	<0.04	3.23	34
PFOA	Malta	2020	CR	2.23	1.32	2.31	1.14	3.29	0.36	4.26	0	1.25	0.70	1.12	0.42	2.00	<0.04	2.16	20
	La Maddalena	2020	CR	1.82	0.83	1.72	1.32	2.30	<0.04	11.16	8	2.37	1.10	2.19	1.46	3.18	<0.04	3.67	12
	Linosa	2016	CR	1.85	0.89	1.37	1.29	2.69	<0.10	4.05	37	1.48	0.66	1.20	1.00	2.19	<0.10	2.89	29
	Linosa	2020	INC	3.05	1.94	2.51	2.02	4.32	1.00	8.52	0	2.16	1.31	1.78	1.58	2.83	<0.10	5.36	11
PFHxS	Linosa	2020	CR	1.86	1.36	1.38	1.19	2.82	<0.10	6.81	29	2.04	1.77	1.29	1.09	3.52	<0.10	7.43	34
	Malta	2020	CR	3.04	1.44	3.08	1.87	4.18	0.83	5.42	0	2.55	1.02	2.39	1.71	3.59	1.40	4.57	0
	La Maddalena	2020	CR	1.19	0.69	0.90	0.83	1.66	<0.10	3.19	17	1.44	0.75	1.03	0.91	2.29	<0.10	0.85	37
	Linosa	2016	CR	5.38	0.00	NA	0.00	8.03	<0.10	8.03	87	2.50	0.75	2.21	1.74	3.45	<0.10	3.89	43
PFDS	Linosa	2020	INC	4.21	2.14	3.67	2.94	5.59	<0.10	8.26	9	4.46	2.31	3.80	3.42	5.58	1.64	8.33	0
	Linosa	2020	CR	2.30	1.64	1.52	1.49	4.47	<0.10	7.95	65	1.91	1.29	1.41	0.94	3.12	<0.10	5.63	45
	Malta	2020	CR	4.62	1.26	4.15	3.31	5.77	<0.10	6.20	17	3.48	1.28	3.22	2.46	4.75	2.01	5.89	0
	La Maddalena	2020	CR	2.10	2.10	1.23	1.13	3.64	<0.10	9.69	25	2.57	1.48	2.08	1.51	3.74	<0.10	5.00	13
PFHpS	Linosa	2016	CR	0.50	0.24	0.37	0.35	0.75	<0.02	1.01	38	0.26	0.08	0.23	0.19	0.34	<0.02	0.41	29
	Linosa	2020	INC	0.23	0.06	0.20	0.19	0.29	<0.02	0.38	55	0.43	0.32	0.34	0.30	0.61	<0.02	1.38	11
	Linosa	2020	CR	0.26	0.14	0.19	0.19	0.35	<0.02	0.67	42	0.34	0.12	0.32	0.25	0.44	<0.02	0.54	22
	Malta	2020	CR	0.40	0.24	0.37	0.22	0.61	0.13	0.84	0	0.33	0.16	0.16	<0.02	0.68	<0.02	0.68	60
ADδ13C	La Maddalena	2020	CR	0.12	0.04	0.09	0.07	0.19	<0.02	0.22	67	0.23	0.13	0.17	0.13	0.36	<0.02	0.41	25
	Linosa	2016	CR	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	100	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	100
	Linosa	2020	INC	0.27	0.05	0.23	<0.04	0.40	<0.04	0.40	73	0.27	0.08	0.23	0.22	0.33	<0.04	0.49	61
	Linosa	2020	CR	0.17	<0.04	NA	<0.04	0.27	<0.04	0.27	93	0.31	<0.04	NA	<0.04	0.48	<0.04	0.48	89
ADδ15N	Malta	2020	CR	0.32	0.08	0.27	0.26	0.40	<0.04	0.49	17	0.32	<0.04	NA	<0.04	0.47	<0.04	0.47	80
	La Maddalena	2020	CR	0.25	0.08	0.22	0.22	0.31	<0.04	0.56	58	0.18	<0.04	NA	<0.04	0.27	<0.04	0.27	87
	Linosa	2016	CR	-19.17	0.17	-19.23	-19.28	-19.03	-19.32	-18.87	0	-18.53	0.18	-18.50	-18.68	-18.40	-18.81	-18.33	0
	Linosa	2020	INC	-19.41	0.24	-19.43	-19.55	-19.25	-19.76	-19.01	0	-18.94	0.33	-18.94	-19.09	-18.79	-19.47	-18.39	0
ADδ13C	Linosa	2020	CR	-19.15	0.10	-19.16	-19.20	-19.09	-19.31	-18.98	0	-19.01	0.13	-18.99	-19.10	-18.92	-19.22	-18.83	0
	Malta	2020	CR	-19.17	0.20	-19.21	-19.31	-18.98	-19.36	-18.87	0	-19.18	0.03	-19.18	-19.21	-19.16	-19.21	-19.15	0
	La Maddalena	2020	CR	-19.07	0.15	-19.05	-19.16	-18.98	-19.28	-18.85	0	-19.04	0.13	-19.05	-19.13	-18.95	-19.21	-18.86	0
	Linosa	2016	CR	8.54	0.22	8.52	8.39	8.70	8.25	8.89	0	9.42	0.42	9.56	9.08	9.72	8.77	9.80	0
ADδ15N	Linosa	2020	INC	9.05	0.41	9.07	8.80	9.29	8.44	9.65	0	9.58	0.36	9.55	9.40	9.75	8.84	10.27	0
	Linosa	2020	CR	7.92	0.40	7.85	7.71	8.15	7.44	8.70	0	8.41	0.46	8.41	8.08	8.71	7.67	9.00	0
	Malta	2020	CR	7.93	0.51	7.82	7.58	8.42	7.46	8.70	0	8.05	0.72	7.91	7.43	8.75	7.40	9.97	0
	La Maddalena	2020	CR	8.16	0.62	8.29	7.77	8.49	6.97	8.91	0	8.52	0.60	8.53	8.09	8.95	7.63	9.36	0

*The data have been bootstrapped (5,000 replicates), and the mean of the descriptive statistics is presented (mean, standard deviation (SD), median, 95% confidence interval of the mean, minimum and maximum). Some PFAS were left-censored, and the values presented are estimated based on the Kaplan-Meier technique.



Chapter 2

An abstract painting of a landscape. The top portion is a light blue sky. Below it is a dark, almost black mountain peak. The slopes of the mountain are rendered in shades of brown and reddish-brown. The foreground is a mix of green and yellow-green, suggesting a field or grassy area. The overall style is expressive and textured, with visible brushstrokes.

**Diet of two Mediterranean shearwaters revealed
by DNA metabarcoding.**

Diet of two Mediterranean shearwaters revealed by DNA metabarcoding

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Abstract

Information on seabird diet is key to understanding their ecological role in the marine food web. The Mediterranean Sea is a biodiversity hotspot that is experiencing a series of growing threats, including overfishing and climate change. The Scopoli's (*Calonectris diomedea*) and Yelkouan shearwaters (*Puffinus yelkouan*), two marine predators in the region, are expected to have a piscivorous diet and exploit fishery discards, but their exact reliance on different resources is still unclear. We sampled four populations in the central Mediterranean Sea and used a combination of DNA metabarcoding and stable isotopes to compare their diets and assess trophic niches. We found prey items from 38 families belonging to 21 orders. Clupeiformes and Perciformes were the main prey groups identified in both shearwater species. In fact, diet composition largely overlapped and differed by only 3% variation in the diet consumed at order level and 16% at genus level, despite sampling different populations. The results suggest high overlap of dietary and isotopic niches, while Yelkouan shearwaters occupied a wider niche space overall. Certain taxa were potentially derived from discards but are also available naturally as juvenile fish or in foraging associations with marine megafauna such as predatory fish and turtles. These findings highlight the strong dietary overlap and ecological similarities between Scopoli's and Yelkouan shearwaters, emphasising the importance of understanding their foraging dynamics in the context of resource competition and the increasing pressures on Mediterranean marine ecosystems.

Keywords: stable isotopes, trophic level, niche, foraging ecology, *Puffinus yelkouan*, *Calonectris diomedea*

Introduction

Seabirds hold a significant function at the top of the marine food chain and are widely recognised as sensitive indicators of the marine ecosystem (Furness & Camphuysen 1997). The study of seabird diets provides fundamental context for their ecology and the use of seabirds as indicators within ecosystem management (Lewison et al. 2012; Carreiro et al. 2023). The Mediterranean Sea is a hotspot of marine biodiversity, which is threatened by intense anthropogenic pressures (Colloca et al. 2017; Piroddi et al. 2020; Clark et al. 2023). Its unique oligotrophic and heterogeneous oceanographic features include a summer thermocline that affects productivity (Zotier et al. 1999). Therefore, it is especially interesting to reveal the diet and trophic niches of marine predators in the region.

The Scopoli's shearwater (*Calonectris diomedea*) is a common and widely distributed species breeding in the Mediterranean Sea, while the smaller Yelkouan shearwater (*Puffinus yelkouan*) is distributed mainly in the central and eastern areas of the basin. The two species belong to the family *Procellariidae* and while Scopoli's shearwaters generally forage at or close to the surface (maximum dive depth 5.4 m) (Grémillet et al. 2014; Cianchetti-Benedetti et al. 2017), Yelkouan shearwaters regularly dive deeper (maximum dive depth 30.2 m) (Péron et al. 2013). The two species display high trophic plasticity, with a diet ranging from zooplankton to a variety of small to medium sized pelagic fish, which is often their main prey, but also crustaceans and squids (Péron et al. 2013; Afán et al. 2014; Grémillet et al. 2014; Thabet et al. 2019). Moreover, foraging shearwaters are often associated with other marine megafauna which either drive (predatory fish) or attract (turtles) fish to the surface (Michel et al. 2022). In fact, the decline of Atlantic bluefin tuna (*Thunnus thynnus*) has been suggested as a reason for Yelkouan shearwaters from Sardinia to repeatedly travel to distant foraging areas, rather than foraging in waters closer to their colonies (Pezzo et al. 2021).

In addition to natural foraging, shearwaters in the Mediterranean interact with fishing vessels, feeding on bait and discards (Arcos and Oro 2002; Karris et al. 2018; Michel et al. 2022). In Scopoli's shearwaters, Cianchetti-Benedetti et al. (2018) reported that 40% of tracked individuals interacted with fisheries, while Michel et al. (2022) observed such interactions on 16% of trips. The level of interaction between Yelkouan shearwaters and fisheries has not been quantified yet (Bicknell et al. 2013). However, interaction is expected due to documented accidental bycatch (e.g. Cortés et al. 2018). While fisheries do to some extent offer an alternative food source through the supply of discards (Arcos and Oro 2002; Church et al. 2019), overall intensive fishery activities are depleting resources available to seabirds (Grémillet et al. 2018). The reduction in prey is further aggravated by climate change, which impacts prey distribution, seasonality (mismatch in timing of prey availability and seabird breeding) and size (Baudron et al. 2014; Pistorius et al. 2022). Due to the impact of variation in food resources on seabird demography (Louzao et al. 2006), identification of diet and comparison within predatory guilds is an imperative but challenging aspect to seabird conservation (Barrett et al. 2007; Deagle et al. 2010; Carreiro et al. 2023). The pressure on Mediterranean marine food webs is particularly marked, both through overfishing (Colloca et al. 2017) and climate change (Schickele et al. 2021). When competitors with shared limited

resources coexist in a community, one or more dimensions within their niche is often adjusted resulting in niche segregation (Hutchinson 1957, 1959). Petalas et al. (2024) review niche segregation in sympatric seabirds and present three niche spaces, each with multiple dimensions: dietary, isotopic and spatial, urging research to include measurements within more than one niche space. There is potential competition for resources between Scopoli's and Yelkouan shearwaters because their breeding seasons and foraging areas overlap (Cecere et al. 2013; Cianchetti-Benedetti et al. 2018, Gatt et al. 2019). Based on studies on their foraging behaviour (Péron et al. 2013; Grémillet et al. 2014; Cianchetti-Benedetti et al. 2017), there might be a segregation due to dive capacity.

In addition to stable isotope analysis (SIA), genetic analysis of faecal samples is a prominent tool that has emerged during the last two decades and allows for improved identification of seabird diet (Barrett et al. 2007; Deagle et al. 2007). Primers specific to prey groups are applied within PCR assays to amplify prey DNA from dietary samples. The resulting amplicons are sequenced and matched with genetic reference libraries to provide a relatively detailed taxonomic and semi-quantitative assessment of prey composition. DNA analysis of faecal samples can give a detailed picture of the prey taxa consumed within the previous days (Deagle et al. 2010). On the other hand, SIA can reflect a longer period and a broader picture on trophic status and preferred foraging areas (inshore vs. offshore) (Hobson et al. 1994).

In this study, we firstly aimed to describe the diet of Scopoli's and Yelkouan shearwaters in the Central Mediterranean through DNA metabarcoding of faecal and regurgitate samples. We aimed to discuss these results in relation to previous studies on the shearwater species' diet and methodological limitations. By using a fish-specific primer, we aimed to increase the taxonomic precision for this prey type specifically and discuss if fishery discards could be facilitating access to certain taxa. We then aimed to investigate potential niche segregation within dietary items and blood stable isotope values of these two shearwaters. We expected overlap and a similar overall diet composition due to similar trophic level values found in previous studies applying stable isotope analysis, but variations in specific prey species due to different foraging behaviour. We also expected isotopic niche segregation in breeding stages that temporally overlap in the two species, driven by potential competition.

Methods

Sampling procedures

The breeding seasons of the two shearwater species are offset by approximately two months. On Malta, the Yelkouan shearwater generally lays its single egg at the end of February until mid-March, hatching occurs between end of April and mid-May, and fledging takes place by early July. The Scopoli's shearwater lays the egg in the second half of May, hatching occurs mid-July and fledging takes place in October. Hence, incubation of the Scopoli's shearwater is concurrent to the chick-rearing of Yelkouan shearwaters. Sampling occurred during both incubation and chick-rearing of the two shearwater species.

We sampled Scopoli's shearwaters at three colonies in the Central Mediterranean, Linosa (35.865, 12.865), Malta (35.900, 14.400) and La Maddalena (41.230, 9.410), while all Yelkouan shearwater samples were collected on Malta (Table 1; Fig. S1). We collected faecal samples with cloacal swabs and opportunistically when defecation occurred during handling. In the case of Scopoli's shearwaters, we collected samples from only adults. For Yelkouan shearwaters, we collected samples from both adults and from nestlings/fledglings. We also collected fresh faeces from burrow substrate from locations with only Yelkouan shearwaters nesting, but could not determine whether these samples were from adults or chicks. Furthermore, we opportunistically collected regurgitate samples during capture-mark-recapture sessions from adult Yelkouan shearwaters arriving at the colony during the chick-rearing period. Faecal samples were stored in 96% ethanol while regurgitate samples were stored frozen at -18°C.

Additionally, we collected blood samples for stable isotope analysis from adults of both species (Table 1). Blood from both shearwaters was collected during their respective incubation and chick-rearing stage. The blood was drawn from the tarsal vein, using a syringe and 25-gauge needle in the case of Scopoli's -, and 27-gauge needle in the case of Yelkouan shearwaters. Later at the station, we centrifuged the blood to separate plasma from the red blood cells (RBC) and both fractions were stored at -18°C.

Molecular diet analysis

We extracted DNA from 206 samples in total using the QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Germany) and followed manufacturer protocols. For large samples, we weighed-in between 180 and 220 mg (n=21), but in most cases the entire sample, including the swab, was extracted and no sample weight could be obtained (n=185). Similar to Masello et al. (2023), we added 2-3 bashing beads (ZR Bashing Bead™ 2.0 mm, Zymo Research, USA) to each sample to ensure proper homogenization during mixing in a Disruptor Genie™ (Scientific Industries SITM, USA).

We mixed each regurgitate sample from 2021 in an open tray using a spatula, and took out a small proportion from the homogenous paste for DNA extraction. In between samples, the tray and tools were cleaned with chlorine bleach and 96% ethanol. For the 2019 and 2020 samples, we took out a small proportion of the homogenous paste without any mixing. DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen), same as for the faecal samples. We measured nucleic acid concentration with a ThermoScientific Nanodrop 2000 micro-volume UV-VIS spectrometer for all samples (Thermo Fisher Scientific, USA) and when concentration was over 100 ng/μl, dilution by a factor of 20 was carried out prior to PCR amplification.

In order to identify prey to the family level, we applied the Metazoa primer pair (mlCOI-intF/jgHCO2198; Leray et al. 2013) in PCR amplifications on all samples (Table S1). PCR amplification and sequencing preparation was carried out in a separate lab-room to DNA extractions and under a UV clean bench (Clever Scientific, UK). Samples with a positive PCR result were analysed further with a primer specific to fish (12S, Xavier et al. 2018). On Yelkouan shearwater samples only, we applied a third primer specific to cephalopods (16S, Berry et al. 2017). We carried out an index PCR following the procedure described in Masello et al. (2023) on samples with a positive PCR result. In

total, we sent 68 samples from Scopoli's shearwater and 92 samples from Yelkouan shearwaters for sequencing (Table 1), together with negative controls for each primer per study species. Sequencing was carried out on a MiSeq desktop sequencer (Illumina, USA) at SEQ-IT GmbH & Co. KG, Kaiserslautern, Germany.

Table 1 Number of dietary samples sequenced and blood samples collected from Scopoli's shearwaters (*Calonectris diomedea*) and Yelkouan shearwaters (*Puffinus yelkouan*) per colony, breeding stage, year and age. For faecal & regurgitate samples, numbers of sequenced samples and number of positive samples for prey DNA as per sequencing are given.

Species	Colony	Year	Breeding Stage	Age	Sample type	N	N (positive for prey DNA)
Scopoli's shearwater	La Maddalena	2020	Chick-rearing	Adult	Faecal	22	15
				Adult	Red blood cells	22	—
	Linosa	2021	Chick-rearing	Adult	Red blood cells	33	—
				2020	Incubation	Adult	Faecal
		2020	Chick-rearing	Adult	Red blood cells	31	—
				Adult	Faecal	23	7
				Adult	Red blood cells	24	—
	Malta	2020	Chick-rearing	Adult	Faecal	8	5
				Adult	Red blood cells	15	—
Yelkouan shearwater	Malta	2021	Incubation	Adult	Red blood cells	6	—
			Incubation & Chick-rearing	Adult	Faecal	23	15
			Chick-rearing	Chicks	Faecal	34	12
		2020	Chick-rearing	Un-known	Faecal (Burrows)	11	7
				Adult	Regurgitate	10	10
				Adult	Red blood cells	30	—
				Adult	Regurgitate	6	6
	2019	Chick-rearing	Adult	Red blood cells	11	—	
			Adult	Regurgitate	5	5	

An additional three samples from Yelkouan shearwaters were sequenced but did not give any yields and were omitted from the table, resulting in a total of 89 and 68 successfully sequenced samples for Yelkouan and Scopoli's shearwater. N positive to prey DNA shows the number of sequenced samples which yielded prey DNA. Blood (RBC, centrifuged blood cell component) was analysed for stable isotope values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

Bioinformatics

Molecular operational taxonomic units (MOTUs) were identified using a custom workflow implemented in GALAXY (Masello et al. 2021, The Galaxy Community 2022), which consisted of the following steps: 1) sequence quality assessment with FASTQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>; accessed on 12 Nov 2024), 2) adapter and quality trimming of the paired-end reads with TRIMMOMATIC (minimum quality score of 20 over a sliding window of 4 base pairs; Bolger et al. 2014), 3) merging of the overlapping paired-end read pairs using FLASH (Magoč and Salzberg 2011), 4) conversion of sequence files to FASTA with the FASTX-Toolkit (https://github.com/agordon/fastx_toolkit; accessed on 12 Nov 2024), 5) extraction of amplicons from the FASTA files in MOTHUR (Schloss et al. 2009), 6) removing identical replicates (dereplicate, plus strand), 7) detecting and removing chimeric sequences (de novo, minimal abundance ratio of parent vs. chimera 2, 'no' vote pseudo count 1.4, 'no' vote weight 8, minimum number of differences in segment 3, minimum divergence from closest parent 0.8, minimum score 0.28), and 8) clustering sequences into MOTUs, rejecting if identity was lower than 0.97, with VSEARCH (Rognes et al. 2016). To reduce index assignment errors and thus cross contamination from other samples in the batch, we applied a filtering step for index reads with a quality score threshold of 26 as recommended by Wright & Vetsigian (2016). Finally, using the BLASTn algorithm (Altschul et al. 1990), MOTU sequences were matched to reference sequences in the National Center for Biotechnology Information (NCBI) GenBank nucleotide database, using a cut-off of 90% minimum sequence identity and a maximum e-value of 0.00001.

For the taxonomic assignments, we used the percentage similarity of the query and the reference sequences, maintaining a BLASTn assignment match greater than 98%, and a minimum sequence length of 190 base pairs, as short fragments are less likely to contain trustworthy taxonomic information (Deagle et al. 2009; Vesterinen et al. 2013). MOTUs were assigned to the species level only in cases where the BLASTn assignment match was > 99%, and all retained hits of a MOTU, with the same quality criteria (sequence identity and length), corresponded to the same species. Otherwise, we assigned the MOTU to the lowest common taxonomic level (e.g. genus or family; Kleinschmidt et al. 2019). If two species resulted in the same match percentage, we assigned the next common taxonomic level. Consequently, in the case of the Metazoa primer, only family level assignments were possible. Of the identified MOTUs we only retained those that occur in the Mediterranean Sea at their assigned taxonomic level. The raw data set included a wide range of non-specific contaminant DNA (e.g., human, bacterial) that could be excluded as potential prey taxa. Based on previous literature fish, cephalopods, marine crustaceans and pelagic tunicates were considered potential prey (Sarà 1993; Bourgeois et al. 2011; McInnes et al. 2017a; Thabet et al. 2019).

Sequencing success and positive samples

A total of 251 and 120 MOTUs were obtained for Yelkouan and Scopoli's shearwaters respectively, with several identified as non-prey with an especially high number of sequences arising from the study species DNA (Procellariidae, Table 2). The yields of prey DNA varied with collection method in respect to non-

prey DNA, with higher yields for opportunistic regurgitate and faecal samples and the lowest yields from cloacal swabs (Fig. S2). Samples were retained for further analysis if any number of reads from prey MOTUs were detected, but excluded samples with non-Prey DNA only. Moreover, prey MOTUs were retained in any sample on the condition that the number of reads was larger than twice than any number of reads for specific MOTUs detected in controls. We then converted the number of reads to presence/absence values (1/0) for each MOTU. Prey MOTUs were identified in 31 Scopoli's shearwater samples and in 55 Yelkouan shearwater samples and we used these sample numbers to calculate % frequency of occurrence per MOTU (Barrett et al. 2007) for each shearwater species respectively.

Table 2: Sequencing results measured as the percentage of non-prey DNA sequences and prey DNA (fish, cephalopods, marine crustaceans and pelagic tunicates) for Scopoli's (*Calonectris diomedea*) and Yelkouan (*Puffinus yelkouan*) shearwaters. Valid sequences are those belonging to Molecular operational taxonomic units with BLASTn assignment match greater than 98%, a minimum sequence length of 190 base pairs and more than twice the number of reads found in controls. Within range sequences refer to taxa occurring within the Mediterranean range.

	Scopoli's shearwater		Yelkouan shearwater	
	total sequences	% of total sequences	total sequences	% of total sequences
Non-prey DNA	505090	97.86	569880	73.36
of which				
Procellariidae	490788	95.09	560998	72.22
Prey DNA	11041	2.14	206910	26.64
of which				
valid	8041	1.56	165766	21.34
within range	7987	1.55	139520	17.96

Stable isotope analyses and baseline correction

The stable isotope (SI) values of carbon and nitrogen were measured on ~0.3 mg aliquots of dried red blood cells (RBC) weighed in tin capsules. The analysis was conducted at the LIENSs laboratory (France) using a Delta V Plus isotope ratio mass spectrometer equipped with a Conflo IV interface (Thermo Scientific, Bremen, Germany) and a Flash 2000 elemental analyser (Thermo Scientific, Milan, Italy). Calibration was performed using certified reference materials (details in Supplementary Information, Section A), with a two-point calibration method employing the working standards USGS-61 (caffeine) and USGS-63 (caffeine). Internal laboratory standards, acetanilide (Thermo Scientific) and peptone (Sigma-Aldrich) were analysed together with the samples, yielding an analytical precision of <0.15 ‰ for $\delta^{15}\text{N}$ and <0.10 ‰ for $\delta^{13}\text{C}$. Results are reported in δ units, representing deviations from standards (Vienna Pee Dee Belemnite for $\delta^{13}\text{C}$ and atmospheric N_2 for $\delta^{15}\text{N}$), using the formula:

$$\delta^{15}\text{N} \text{ or } \delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$$

where R is $^{15}\text{N}/^{14}\text{N}$ or $^{13}\text{C}/^{12}\text{C}$, respectively. Fluctuation of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ SI values in marine environments occur due to differences in baseline resources along a latitudinal gradient (Rumolo et al. 2016, Campioni et al. 2022). To account for this latitudinal gradient, we calculated the difference between the

two reported mesozooplankton means from the Tyrrhenian Sea and the Sicily Channel, the primary foraging areas of the populations in our study, applying this adjustment to our SI values as previously described in Michel et al. (2025).

Statistical analysis

We tested for differences in community composition using permutation tests in the R package *vegan* (Oksanen et al. 2022). To visualise differences in diet composition between shearwater species, colonies and sample types, we employed non-metric multidimensional scaling (NMDS; Kruskal 1964) on presence/absence data with the ‘metaMDS’ function in the same package. NMDS ordinated the data and reduces information down to two dimensions for easier visualisation and interpretation. The ‘metaMDS’ function allowed us to assess how well the two-dimensional configuration matched the original data using a stress parameter, where values < 0.05 indicate excellent agreement, < 0.1 very good, and < 0.2 a good representation. In our tests, the stress values were below 0.2, indicating a good fit. To improve visualisation in NMDS plots we also used the function ‘ordiellipse’ to obtain the centroid per shearwater species and ‘ordihull’ to create polygons around the points per species. We further used permutational multivariate analysis of variance (PERMANOVA) in the function ‘adonis’ to assess the variation in diet between shearwater species, colonies and sample types and present the test statistics. Finally, in order to assess how well our sample sizes captured the diversity of prey, we plotted rarefaction curves for MOTUs for each shearwater species using the function ‘rarecurve’ in package *vegan* (Oksanen et al. 2022). To calculate the trophic level, we followed the framework by Weiss et al. (2009) and the same base values as those used by Péron et al. (2013) and Thabet et al. (2019) in studies from the central Mediterranean:

$$TL = TL_{\text{copepods}} + 1 + (\delta^{15}\text{N}_S - \delta^{15}\text{N}_{\text{copepods}} - \Delta d_{\text{RBC}}) / 3.4$$

where TL_{copepods} is the estimated TL of copepods (2.0), $\delta^{15}\text{N}_S$ is the value in respective shearwaters; $\delta^{15}\text{N}_{\text{copepods}}$ is the mean nitrogen isotope value of copepods in the western Mediterranean Sea (2.8 ‰, Cardona et al. 2012); and Δd_{RBC} is the discrimination factor for blood (2.6 ‰, mean from seabirds summarised in Thabet et al. (2019)) and 3.4 ‰ is an assumed constant trophic enrichment factor (Post 2002). While different diet-tissue discrimination factors are found between for example blood and feathers, whole blood and the blood cell component of blood have close to identical isotopic signatures (Cherel et al. 2014). To test whether trophic level differed between years, species, site and breeding stages we used separate Generalised Linear models (GLM) for each categorical predictor variable. To test breeding stage and site by species we constructed composite categorical variables (e.g. “SCSH_Incubation”). To measure isotopic niche segregation, we used the package *SIBER* (Jackson and Parnell 2023) to calculate niche width, as Standard Ellipse Areas, of each shearwater species based on the adjusted isotope values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Since the breeding stages of the two shearwaters are temporally staggered, we specifically compared the Scopoli’s shearwater incubation period with the Yelkouan shearwater chick-

rearing period (temporally overlapping) and the chick-rearing periods of both species (temporally distinct). We estimated the Standard Bayesian Ellipse Area (SEA_B) (20,000 iterations, burn-in 1000, thin 10) in addition to Standard Ellipse Area corrected for small sample sizes (SEA_C) (Jackson et al. 2011). With 4000 posteriors, we expressed overlap between the ellipses as the proportion of non-overlapping area of the two ellipses. We carried out all statistical analyses within the R environment (v. 4.2.3 - 4.4.1) (R Core Team 2024).

Results

Prey composition of Scopoli's and Yelkouan shearwaters

Both shearwater species fed on fish, crustaceans and cephalopods, while in one Yelkouan shearwater sample we also found a pelagic tunicate (Table 3). In total, we found prey items from 37 families which belong to 21 orders (Table 3, Table S2).

We found low variation between the diet consumed by Scopoli's and Yelkouan shearwaters. With MOTUs at order level, shearwater species explained only 3% variation in the diet consumed (NMDS $F=2.937$, $R^2=0.034$, $P=0.016$; Fig. 1) and at genus level shearwater species explained 16% of the fish diet consumed (NMDS $F=10.649$, $R^2=0.162$, $P=0.001$; Fig. 2, Fig. S3). Diet at order level was largely overlapping across sample types (NMDS $F=1.556$, $R^2=0.054$, $P=0.113$, Fig. S4) and colonies (NMDS $F=2.956$, $R^2=0.066$, $P=0.006$; Fig. S5) for both shearwater species. In Yelkouan shearwaters diet overlapped for the two age classes sampled in that shearwater species (NMDS $F=2.281$, $R^2=0.084$, $P=0.061$, Fig. S6).

Overall, Perciformes contributed greatly to the diet of both shearwaters. The main differences between the consumed prey were that Scopoli's shearwaters consumed a higher proportion of Clupeiformes, while Syngnathiformes were detected more frequently in Yelkouan shearwaters (Table 3, Fig. 3). At genus level, depth-ranges of prey were obtained from fishbase.se (Froese & Pauly 2024) (Fig. 4). Moreover, at this taxonomic resolution, it is evident that *Trachurus* had a high FO in both species, but was more prevalent in the Yelkouan shearwater diet. Both shearwaters preyed similarly on *Engraulis*, while more Scopoli's shearwater individuals consumed *Sardinella*, *Auxis*, *Phycis* and *Thunnus*, and more individuals of the Yelkouan shearwater consumed *Macroramphosus*, *Mullus* and *Scomber* (Table 3, Fig. 4, Fig. S7). Rarefaction curves demonstrate that there might be higher diversity than what our sample sizes were able to capture for both shearwater species (Figs. S8 and S9).

Table 3: Frequency of occurrence (%FO) for the prey consumed by Scopoli's shearwaters (N=31) and Yelkouan shearwaters (N=55) in the central Mediterranean as identified by DNA metabarcoding of faecal and regurgitate samples. The primers used were Metazoa COI (M, Leray et al. 2013), Fish 12S (F, Xavier et al. 2018) and Cephalopoda 16S rRNA (C, Berry et al. 2017).

Phylum	Class	Order	Family	Species	Common Name	Primer(s)	%FO SCSH	%FO YESH													
Arthropoda	Branchiopoda	Diplostraca	Podonidae	<i>Evadne spinifera</i>		M	-	4													
						C	-	2													
Mollusca	Malacostraca	Decapoda	Benthescymidae	<i>Gennadas elegans</i>	Graceful blunt-tail prawn	M, C	10	13													
			Luciferidae			C	-	2													
			Munidae	M	-	2															
			Euphausiidae	M	10	9															
			Argonautidae	M	-	4															
	Cephalopoda	Octopoda		Argonautidae	<i>Argonauta argo</i>	argonauts	M, C	-	9												
				M			-	5													
				Tremoctopodidae	<i>Tremoctopus violaceus</i>	Greater argonaut blanket octopods Blanket octopus	C	-	4												
				M			-	5													
				C			-	4													
Chordata						M	6	5													
						Ommastrephidae	flying squids	M	3	5											
						Onychoteuthidae		M	3	-											
						Doliolidae	pelagic tunicates	M	-	2											
						Atherinidae	Sand smelt	F	3	-											
						Aulopiformes	Aulopiformes		aulopiforms	M, F	13	13									
									greeneyes	M	13	11									
									Shortnose greeneye	F	3	4									
						Batrachoidiformes	Beloniformes					M	-	2							
												Synodontidae	lizardfishes	M	-	2					
												Batrachoididae		M, F	6	7					
												Belonidae	Exocoetidae		<i>Belone belone</i>	Garfish	F	6	-		
																	M, F	-	7		
												Clupeiformes	Clupeidae					M, F, C	48	36	
																		herrings	M	26	20
																		sardinellas	F, C	23	11
																		European pilchard	F, C	-	7
																		anchovies	M	32	33
Gadiformes						F	23	22													
						European anchovy	F	3	-												
						cods	M, F, C	19	18												
						rocklings	M	-	2												
						hakes	M	6	15												
Lophiiformes						F	-	7													
						Greater forkbeard	F, C	13	2												
						Blackbellied angler	F	3	-												
						grey mullets	F	-	2												

Phylum	Class	Order	Family	Species	Common Name	Primer(s)	%FO SCSH	%FO YESH
	Myctophiformes		Myctophidae		lanternfishes	M, F	3	7
	Osmeriformes		Argentinidae	<i>Ceratopsopelus maderensis</i>	Horned lanternfish	F	-	7
	Perciformes			<i>Argentina sphyraena</i>	Argentine perch-like fish	C	-	4
			Carangidae	<i>Trachurus</i> sp.	saurels	M, F, C	61	85
				<i>Trachurus trachurus</i>	Atlantic horse mackerel	M	26	75
				<i>Caranx crysos</i>	Blue runner	F, C	16	60
			Centrolophidae		medusafishes	C	-	2
			Gobiidae		gobies	F	-	9
				<i>Lesueurigobius suerii</i>	Lesueur's goby	F	-	2
			Mullidae	<i>Mullus surmuletus</i>	goat fishes	M	3	24
			Nomeidae		Red mullet	F	-	20
			Scombridae		driftfishes	M, C	-	11
				<i>Auxis rochei</i>	mackerels and tunas	M	45	29
				<i>Scomber</i> sp.	Bullet tuna	F	35	2
				<i>Thunnus</i> sp.	mackerels	F	6	18
				<i>Thunnus thynnus</i>	albacores & tunas	F	13	2
			Sparidae		Atlantic bluefin tuna	F	13	-
				<i>Boops boops</i>	porgies	M, F, C	6	33
				<i>Dentex maroccanus</i>	Bogue	C	-	2
				<i>Pagellus acarne</i>	Morocco dentex	C	-	2
				<i>Pagellus bogaraveo</i>	Axillary seabream	F	-	4
				<i>Pagellus erythrinus</i>	Blacksport seabream	F	-	2
				<i>Spicara maena</i>	Common pandora	F	-	4
				<i>Spicara smaris</i>	Blotched picarel	C	-	2
				<i>Spondyliosoma cantharus</i>	Picarel	C	-	4
					Black seabream	F	-	4
			Trichiuridae		cutlassfishes	M	3	4
			Citharidae		cithartids	M	-	2
			Sebastidae		rockfishes	M	-	4
				<i>Helicolenus</i> sp.	rockfishes	F	-	2
			Sternoptychidae		hatchetfishes	M	-	4
				<i>Maurolicus muelleri</i>	Silvery lightfish	F	-	2
			Centriscidae		snipefishes	M	3	31
				<i>Macroramphosus</i> sp.	snipefish	F	3	25
				<i>Macroramphosus scolopax</i>	Longspine snipefish	C	-	7

% Frequency of occurrence was calculated for as the number of samples in which each specific prey MOTU was detected from the total of samples with at least one positive detection of a prey MOTU (SCSH = 31; YESH = 55)

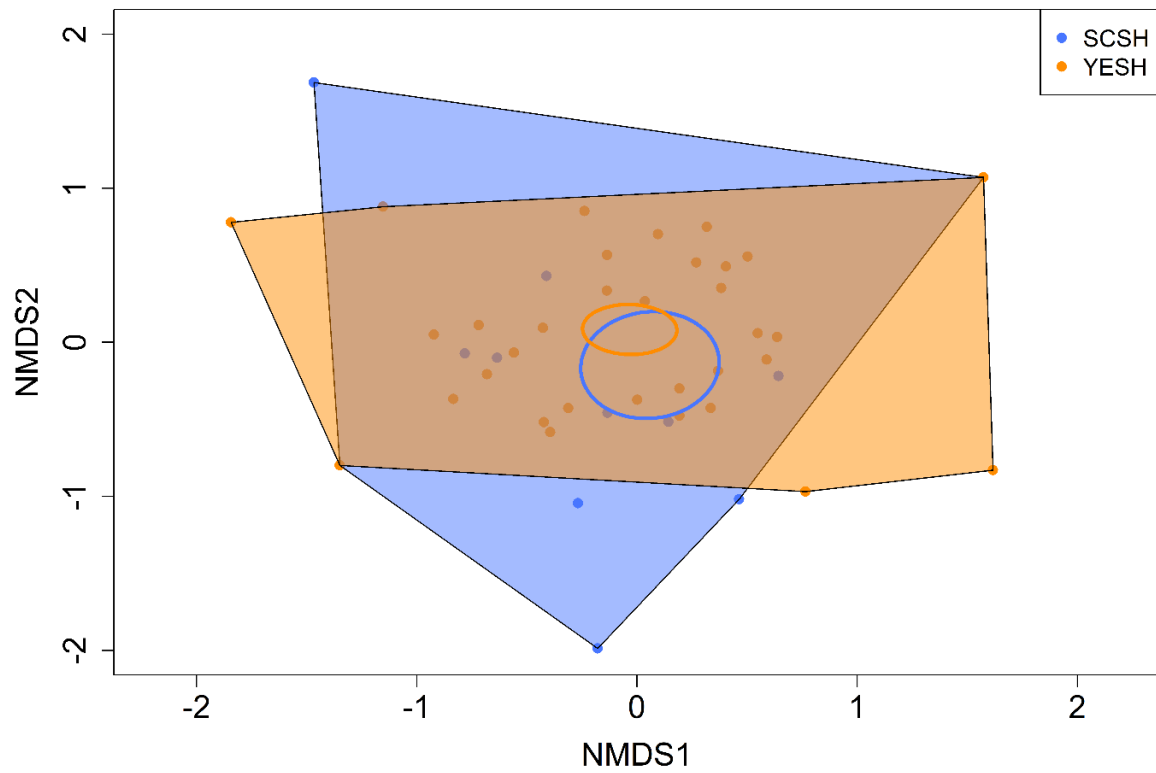


Figure 1 Diet composition on order level of Scopoli's (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) shearwaters from the central Mediterranean. Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. NMDS stress level was 0.115.

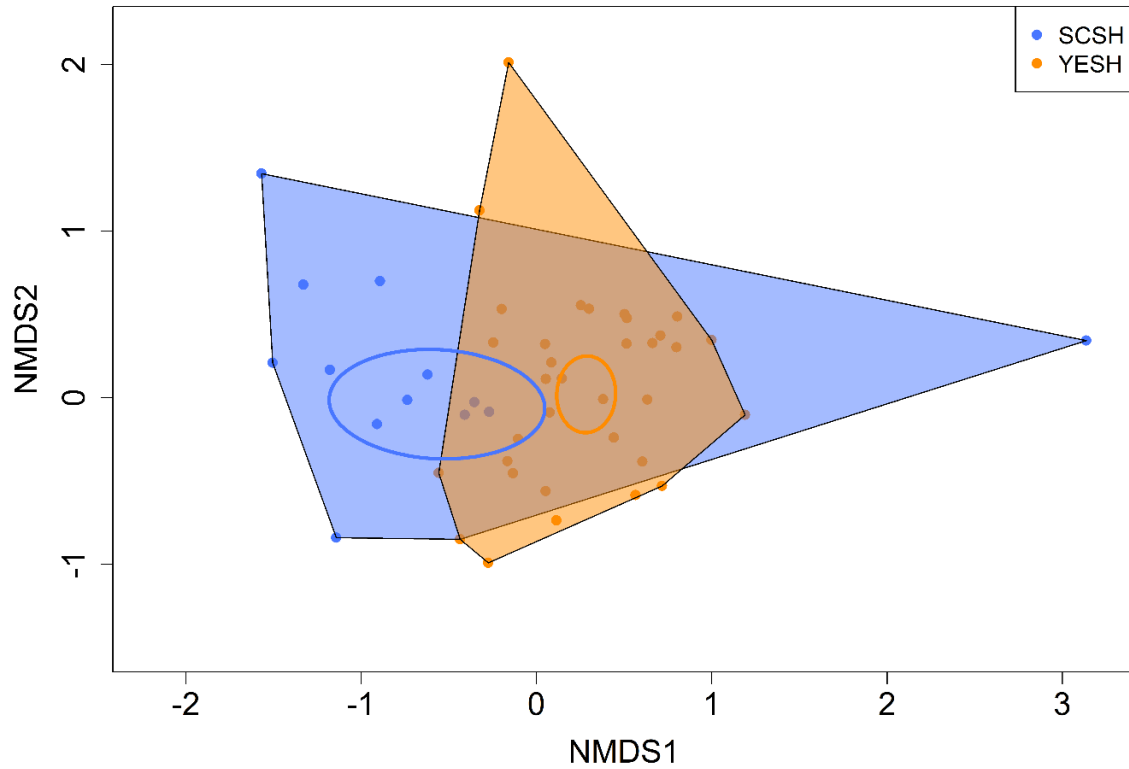


Figure 2 Diet composition on genus level for fish prey consumed by Scopoli's (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) shearwaters from the central Mediterranean. Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. The NMDS stress level was 0.112.

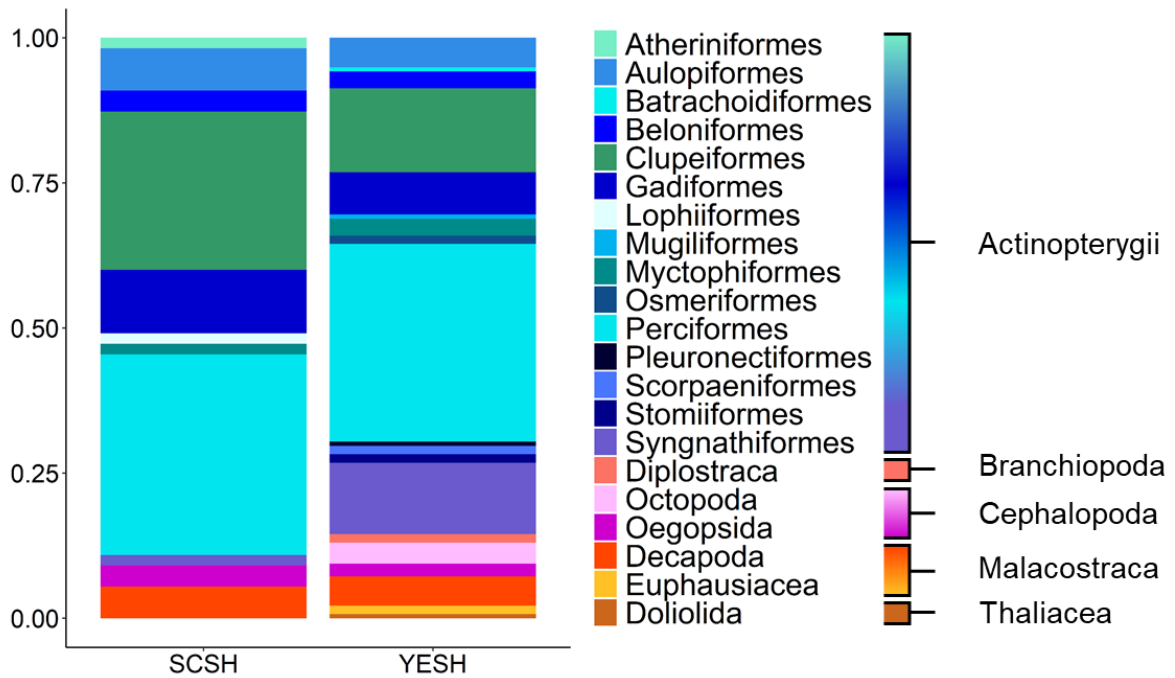


Figure 3 Diet Composition of Scopoli's (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) shearwaters from the central Mediterranean on the level of taxonomic order. The percent stacked barplot is showing the percentage contribution of each dietary item to the whole. The taxonomic order of dietary items is listed alphabetically and separated by class.

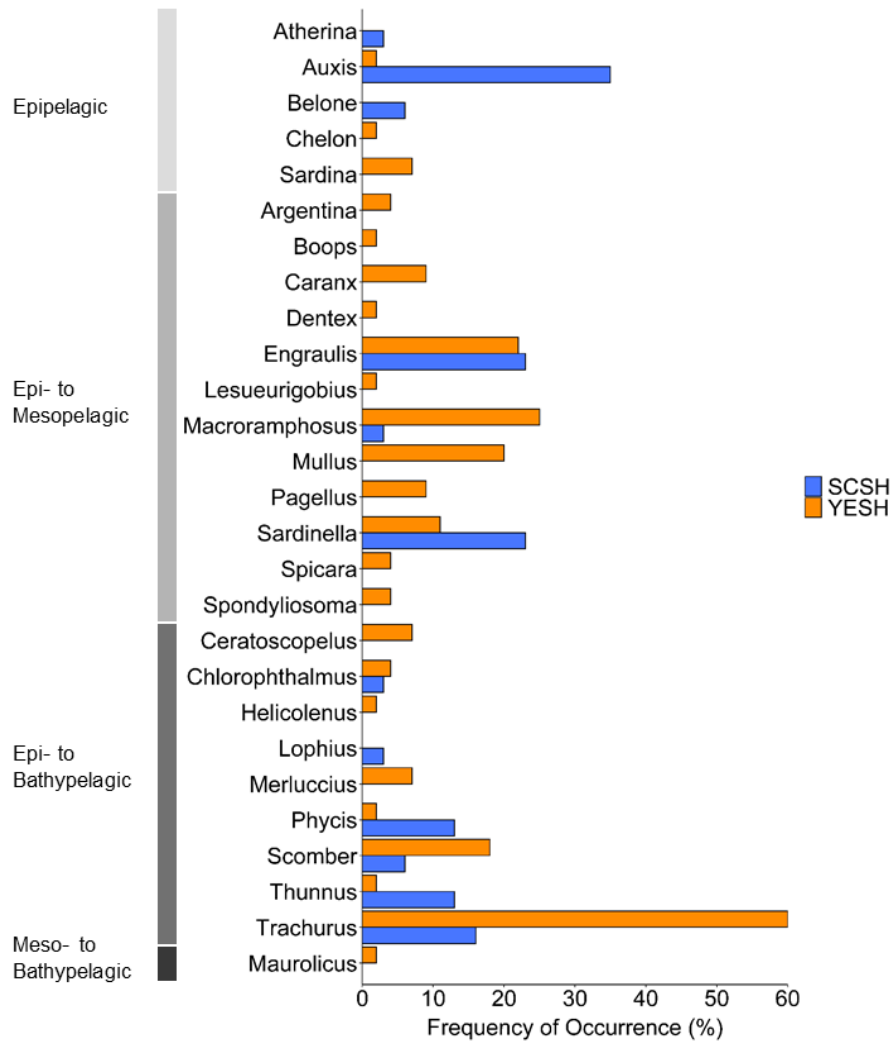


Figure 4 Composition and frequency of occurrence (%) of fish genera in the diet of Scopoli's shearwaters (SCSH, in blue) (*Calonectris diomedea*) and Yelkouan shearwaters (YESH, in orange) (*Puffinus yelkouan*) from the central Mediterranean revealed by DNA Metabarcoding of faecal and regurgitate samples. We categorised the fish genera into their pelagic zone according to the depth-range information from fishbase.se. (Froese & Pauly 2024). If several species within a genus were geographically relevant prey, we adapted the range to include the minimum and maximum depth range for all relevant species. We then assigned a pelagic zone, where a depth range between 0-200 m falls in the category epipelagic, 200-700 m mesopelagic, 700-1000 m bathypelagic. The order of the fish genera represents their preferred pelagic zone where depth increases from top to bottom.

Trophic ecology

The means of adjusted $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for both shearwaters and years showed little variation, and high overlap between the species (Table 4, Fig. 5). The trophic level calculated for both Scopoli's and Yelkouan shearwaters were very similar (Table 4), with no significant differences detected by year, species or site using GLMs. This was also the case when calculating trophic level per breeding stage (Table S3), but the trophic level was higher for the incubation period in Scopoli's shearwaters (GLM parameter estimate=0.345 \pm 0.122, $P=0.050$).

Niche width was narrowest in Scopoli's shearwaters during the chick-rearing period (SEA_B mean=0.372 95% CI=0.296 - 0.449) and widest in Yelkouan shearwaters during the chick-rearing period (SEA_B mean =0.821 95% CI=0.576 - 1.08) (Table S3, Figs. S10 and S11). The overlap between Scopoli's and Yelkouan shearwater SEA_B in their respective chick-rearing periods was higher than the overlap between incubating Scopoli's shearwaters and chick-rearing Yelkouan shearwaters (Fig. S12).

Table 4 Mean stable isotope values of $\delta^{13}C$ and $\delta^{15}N$ (\pm SD) from blood cells in Scopoli's shearwater and Yelkouan shearwater collected from adult shearwaters during the breeding seasons of 2020 and 2021 (N = Number of samples). $\delta^{13}C$ and $\delta^{15}N$ values from Scopoli's shearwaters sampled at La Maddalena were adjusted for latitudinal gradient by subtracting 0.25 and 1.3 respectively. Trophic level (TL) was calculated using the formula and base values presented in Thabet et al. 2019).

	year	N	mean AD $\delta^{13}C$	SD	mean AD $\delta^{15}N$	SD	TL
Scopoli's shearwater	2020	92	-19.10	0.26	8.57	0.80	3.93
	2021	33	-18.94	0.27	8.35	0.62	3.87
Yelkouan shearwater	2020	11	-19.22	0.21	7.87	0.53	3.73
	2021	36	-19.16	0.56	8.78	0.44	3.99

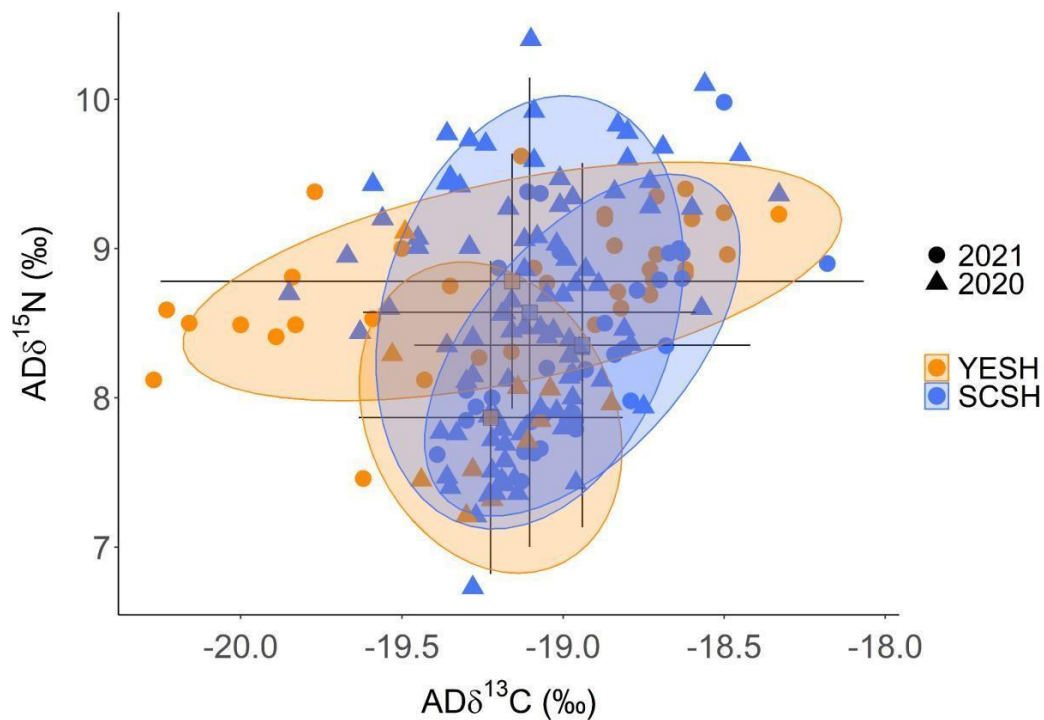


Figure 5 Stable isotope values for $\delta^{13}C$ and $\delta^{15}N$ in red blood cells of Scopoli's shearwaters (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) in the sampling years 2020 and 2021. Values are adjusted for a latitudinal gradient. The ellipses were set to cover 80 % of the points, assuming a multivariate normal distribution, while the error bars were 1.96 times the standard deviation.

Discussion

Diet in comparison to previous studies

With this study, we assessed the diet of two shearwater species endemic to the Mediterranean basin, focusing on individuals breeding and foraging in the central Mediterranean. Both species fed on several prey taxa, suggesting generalist and opportunistic foraging. These results are in line with previous work based on stable isotope analysis (Péron et al. 2013; Grémillet et al. 2014, Thabet et al. 2019), but through the application of DNA metabarcoding, we achieved a higher taxonomic resolution, especially for fish prey. Clupeiformes were one of the main fish groups consumed by both shearwaters and match previous studies utilising visual analysis of stomach content and regurgitates (Sarà 1993; Bourgeois et al. 2011), but we found lower FO for *Sardina pilchardus*, while similar FO for *Engraulis* sp. and also *Scomber* sp. (Perciformes). In addition to previously described fish prey, we identified *Trachurus* sp. (60% FO), *Macroramphosus* sp. (25% FO) and *Mullus surmuletus* (20% FO) among others in Yelkouan shearwater diet and *Auxis rochei* (35% FO) and *Thunnus thynnus* (13% FO) in Scopoli's shearwater diet.

We identified four Cephalopod families, all at 3-5% FO, which is in line with previous indications that cephalopods are present in the diets of both shearwater species (Lago and Metzger 2020), but are secondary in occurrence compared to pelagic fish (Thabet et al. 2019). However, in the stomach contents of Yelkouan shearwaters breeding on islands in SE France and sampled in 2004 to 2007, cephalopod beaks were completely absent (Bourgeois et al. 2011). In both Scopoli's and Yelkouan shearwaters, we found low contributions of crustaceans to the diet, but amplification with only one universal primer might not be suitable to entirely detect marine invertebrates in faecal samples (Leite et al. 2021; Zhao et al. 2021; de Leeuw et al. 2024). Decapoda and Euphausiacea, identified by metabarcoding in our study, were also found in the stomach contents of Yelkouan shearwaters, albeit at decreasing abundance from the pre-laying to the chick-rearing period (Bourgeois et al. 2011). In Scopoli's shearwaters from Zembra, Tunisia, however, using stable isotope mixing models, Thabet et al. (2019) determined crustaceans to make up around half or even the entirety of the adult diet during chick-rearing with variations between years.

Application of DNA metabarcoding

Through the application of DNA metabarcoding, we have broadened the knowledge of prey consumed by shearwaters in the Mediterranean. However, faecal samples of shearwaters, especially those collected by cloacal swabs, yielded low amounts of prey DNA, possibly due to effects of varying digestion during different breeding-stages (McInnes et al. 2017a; Komura et al. 2018). Adult shearwaters revert between short and long foraging trips that are chick- and self-provisioning, respectively, and during the short trips have been shown to use endogenous lipid reserves and limit digestion (Cherel et al. 2005). Moreover, during incubation birds fast at the nest for several days, resulting in decreased body mass (Colominas-Ciuró et al. 2022) and decreased amounts of food DNA in faecal samples (McInnes et al. 2017a). This

can result in a high proportion of samples that do not yield prey DNA, driving up sequencing costs without adding to the dataset unless screened at the initial PCR stage (Deagle et al. 2007).

To avoid sequencing samples with non-target DNA only, initial PCR amplification should not be carried using a single universal primer pair, or include a blocking oligonucleotide primer for the specific host species (Vestheim & Jarman 2008; Deagle et al. 2010) and inspection of differential amplicon length (Kleinschmidt et al. 2019). The use of blocking primers can increase the amplification of rare prey DNA found at lower concentrations compared to host DNA (Vestheim & Jarman 2008), but should be considered with caution since they can affect the amplification of prey DNA too (McInnes et al. 2017a). Moreover, future studies can focus on obtaining samples large enough to extract the non-uric acid portion from samples (McInnes et al. 2017a), and consider using additional DNA extraction kits such as the Invitrogen PureLink Microbiome Purification kit (de Leeuw et al. 2024). However, even low prey DNA yields provide evidence of the prey taxa in the diet, albeit at underestimated dietary diversity (Figs. S8 and S9). The regurgitates collected opportunistically in this study gave proportionally higher prey DNA yields compared to faecal samples, and extraction could probably be improved further following other protocols (see Nimz et al. 2022).

DNA metabarcoding can be further subject to bias caused by different prey digestibility (Deagle et al. 2010; Pompanon et al. 2012) or secondary predation (Nimz et al. 2022; Marcuk et al. 2024). Therefore, final interpretations must account for the plausibility of the results in the context of known aspects of the food chain. Nevertheless, metabarcoding allows for identification of ingested items and offal as well as spawn or juvenile prey that are generally underrepresented in studies of hard parts such as otoliths, vertebrae and cephalopod beaks (Alonso et al. 2014; McInnes et al. 2017b). On the other hand, identified diet taxa without associated morphological information, poses difficulties in interpreting how prey items were foraged (Komura et al. 2018). In the absence of morphological information in dietary samples (i.e. faecal samples), we recommend including isotopic analysis as done in the current study.

Fish composition and SI values

Fish species that are usually found at greater depths can become accessible to shallow divers due to vertical migration (Olivar et al. 2012), larval development in surface waters (D'Onghia et al. 2006), secondary predation (Nimz et al. 2022), fishery discards (Karris et al. 2018) or predator assemblies that drive prey fish to the surface (Michel et al. 2022). The identified depth ranges of the detected fish genera revealed that all fish genera consumed by shearwaters in our study, with one exception (*Maurolicus*), can occur in the epipelagic zone, meaning we found no obvious evidence of discard consumption.

Molecular analysis identified some taxa that preferentially occur at great depths, usually out of reach of both shearwaters (e.g. *Macroramphosus*, *Chlorophthalmus*, *Phycis*). However, low variation in $\delta^{13}\text{C}$ values found in blood samples does not indicate a prevalence of demersal fishes, as $\delta^{13}\text{C}$ is expected to increase in benthic species compared to pelagic ones (Hobson et al. 1994). Also, trophic levels of both shearwater species showed low variation even though they consumed some high trophic level taxa (e.g.

Thunnus, TL: 4.5; *Auxis* TL: 4.4; *Merluccius* 4.4; *Lophius* TL: 4.4; Froese and Pauly 2024). Due to enrichment of ^{15}N up the food chain (Post et al. 2002), we would expect a larger difference between the TL of shearwaters and their prey if shearwaters consumed these fish as adults (including offal). One explanation could be that these taxa were consumed as spawn or juveniles, which hold a lower trophic level and can occur closer to the surface than adults (D'Onghia et al. 2006, Laiz-Carrión et al. 2015). Generally, SI values increase with body size of fish (Estrada et al. 2005; Hirsch and Christiansen 2010; Fanelli et al. 2018). Consumption of juvenile fish does not exclude that fish were consumed as discards, because a majority of fishery discards is made up of undersized commercial species (Karris et al. 2018). Another compatible explanation is that the quantity of prey consumed from fishery discards is low compared to other prey and while they are identified in DNA analysis, contribute less to the trophic status. Indeed, in shearwaters during the chick-rearing period, more natural foraging events were observed than foraging on discards (Michel et al. 2022), and foraging on discards might not be efficient overall (Cianchetti-Benedetti et al. 2018). However, our results are presence/absence observations and do not determine quantities in the sample.

Even though high overlap between the years 2020 and 2021 was found we still expect that seasonal variation in consumed prey species is likely to occur due to dynamics of the prey stocks (Romero et al. 2021; Thabet et al 2019; Neves et al. 2012) and altered foraging behaviour during breeding (Cecere et al. 2013, 2014). Scopoli's shearwaters were sampled at three different breeding sites, and the SI values among colonies from the central Mediterranean did not differ after baseline adjustment, which supports our results on similar diet composition (Fig. S5). Trophic levels are also within estimates of Scopoli's shearwaters from Zembra (north Tunisia) during the chick-rearing period in 2016 (Thabet et al. 2019). However, unlike Campioni et al. (2022) who measured isotopic variations in feathers grown in the chick-rearing period of 2015, we did not find higher $\delta^{15}\text{N}$ in Linosa compared to La Maddalena.

Dietary and isotopic niche space in the two shearwater species

Albeit sampling from different populations, we found a high overlap in the diet and trophic level calculated from stable isotope values in the two shearwater species. The slight variation in the fish taxa consumed can be attributed to differences in foraging behaviour particularly a higher diving capacity in Yelkouan shearwaters (Péron et al. 2013; Grémillet et al. 2014; Cianchetti-Benedetti et al. 2017). Another reason for slight differences can be seasonal changes in fish communities from the earlier breeding season of the Yelkouan to the Scopoli's shearwater, which can be driven by the intensification of the summer thermocline (Zotier et al. 1999). Although diet across sample types, colonies and age groups was similar, unequal sample size and a third primer applied to Yelkouan shearwater samples could also contribute to the variation. Despite possible methodological effects on the DNA metabarcoding results, the widest isotopic niche width was identified in chick-rearing adult Yelkouan shearwaters supporting their broader diet.

Niche segregation appears to be higher during the breeding stages that are simultaneous, while there is more overlap in niche space during the chick-rearing periods. The overlap in isotopic niche space occupied by two species during their chick-rearing periods could indicate that their staggered breeding seasons are a strategy to avoid competition. However, it is important to note that our sampling included individuals from diverse populations, meaning that some of the observed differences may stem from variations in prey availability across different seascapes.

Conclusion

Clupeiformes and Perciformes represent the main groups of fish found at high FO in the current and in previous diet studies of two shearwater species, while other taxa such as *Macroramphosus* sp. might reflect opportunistic foraging to cope with changing marine ecosystems (Romero et al. 2021). We did not detect any demersal fish species which were definitely consumed as offal from fisheries by the study species. However, forage fish such as Clupeiformes can be consumed as discards from trawling operations (Arcos and Oro 2002; Karris et al. 2018) and while discards might sustain scavenging seabird populations on the short-term (Genovart et al. 2016; Sherley et al. 2020), unsustainable fishing practices are depleting fish stocks including Clupeids and *Trachurus* sp. in the central Mediterranean region (Hattab et al. 2013; FAO 2023). Irrespective of whether pelagic species are consumed by natural predation or as discards, the breeding performance and abundance of shearwaters in the Mediterranean follows fish stocks, especially small pelagic species (Louzao et al. 2006, Martín et al. 2019), which in turn require ecosystem-based management (Colloca et al. 2017; Piroddi et al. 2020). Hence, we advocate for increased spatio-temporal or gear selectivity to avoid immature or spawning individuals (Tsagarakis et al. 2014; Basilone et al. 2021), and protection of spawning and nursery areas from bottom-trawling (Piroddi et al. 2020). Moreover, management of overfishing should especially be focussed in areas identified as important foraging areas for shearwaters taking into consideration population specific spatial structure and temporal heterogeneity (Morinay et al. 2022). Future research should further disentangle the relationship between fishery activity, seabird diet composition and demographic traits, important in regard to policy directed at discard bans (Bicknell et al. 2013; Genovart et al. 2016). Our results indicate a narrower niche space in chick-rearing Scopoli's shearwaters making them potentially more sensitive to further reductions in forage fish availability. Yelkouan shearwaters have a wider niche space, potentially due higher diving capability but further studies should investigate the cost of this behaviour. Ultimately, our findings underscore the complexity of shearwater diets but lay the foundation for further comparisons between seasons and seascapes in the region.

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Statements

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Authorship contribution

Martin Austad, Lucie Michel and Petra Quillfeldt contributed to the study's conceptualization and design. Formal analysis and data curation were carried out by Martin Austad, Lucie Michel and Juan F. Masello. Martin Austad, Lucie Michel, Giacomo Dell’Omo, Federico De Pascalis and Jacopo G. Cecere carried out the fieldwork. Martin Austad and Lucie Michel contributed equally to writing the original draft, which was reviewed and edited by all co-authors. Investigations were led by Martin Austad and Lucie Michel. All authors read, commented and approved the final manuscript. Parts of the funding were acquired by Martin Austad. Supervision and project administration were carried out by Petra Quillfeldt. Paco Bustamante contributed also in the project administration.

Ethics declarations

Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article

Ethics approval

In Malta, all handling and sampling of shearwaters were carried out under permits from the Environment & Resources Authority (ERA) and the Wild Birds Regulation Unit (WBRU), Malta. In Linosa fieldwork was carried out according to the national legislation under the permit n. 2452 issued the Regional authorities (Regione Siciliana, Assessorato Regionale dell’Agricoltura, dello Sviluppo Rurale e della Pesca Mediterranea, Dipartimento Regionale dello Sviluppo Rurale e Territoriale) on February 1st, 2018. The fieldwork was carried out with moral responsibility for the animals, with the final goal to preserve and enhance the future survival of that species and to provide knowledge vital to their conservation. No specimens were collected for this study.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability statement

Until acceptance all data is available on request from the corresponding author. Genetic and Isotope Data will be made available on GENBANK: <https://www.ncbi.nlm.nih.gov/genbank> (NGS: Sequence Read Archive) and Zenodo, <https://zenodo.org>, respectively.

Electronic Supplementary material to:

Diet of two Mediterranean shearwaters revealed by DNA metabarcoding

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ESM: Diet of two sympatric Mediterranean shearwaters revealed by DNA metabarcoding

Table of content:

Figure S1	Colony locations, marked with yellow stars, in the Central Mediterranean where dietary and blood samples were collected from Scopoli's shearwaters (La Maddalena, Linosa and Malta) and Yelkouan shearwaters (Malta). Foraging movements are shown as gps-tracks (solid lines) and associated 50% utilisation distribution areas (polygons). Scopoli's shearwaters were tagged during chick-rearing in 2019 (La Maddalena), incubation and chick rearing in 2020 (Malta) and chick rearing in 2020 (Linosa) (Michel et al. 2025 ¹), while Yelkouan shearwaters were tagged during incubation and chick-rearing in 2019 and 2020 (Malta; unpublished data found on https://data.seabirdtracking.org as datasets 1935, 2220, 2221 and 2222). Foraging movements are shown for visual reference of foraging areas only and are not necessarily from the same individuals sampled for dietary and isotopic analysis.
Table S1	Primers used in this study for the detection of prey species in faecal and regurgitate samples from Scopoli's and Yelkouan shearwaters.
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B.	Complimentary results to diet and trophic analysis
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Table S2	Best blast results for each detected Molecular taxonomic unit (MOTU) and corresponding accession number, the sequence length, E-value and the bit-score from Scopoli's shearwater (<i>Calonectris diomedea</i>) and Yelkouan shearwater (<i>Puffinus yelkouan</i>).
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Figure S4	Diet composition at order level of Scopoli's (<i>Calonectris diomedea</i>) (SCSH) and Yelkouan (<i>Puffinus yelkouan</i>) (YESH) shearwaters from the central Mediterranean, presented by sample type (regurgitates in purple ellipse and hull; faecal samples by cloacal swabs in black; opportunistic faecal samples during handling in grey and faecal samples collected from burrow substrate in red. Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. The NMDS stress level was 0.110.

¹ Michel L, Zhang J, Asmiakopoulos A, Austad M, Bustamante P, Cecere JG, Cianchetti-Benedetti M, Colominas-Ciuró R, Dell'Omo G, De Pascalis F, Jaspers VLB, Quillfeldt P (2025) Assessing perfluoroalkyl substance pollution in central Mediterranean breeding shearwaters. *Environ Toxicol Chem* 44:420-431. <https://doi.org/10.1093/etjnl/vgae011>

ESM: Diet of two sympatric Mediterranean shearwaters revealed by DNA metabarcoding

Figure S5	Diet composition on order level of Scopoli's (<i>Calonectris diomedea</i>) (SCSH) and Yelkouan (<i>Puffinus yelkouan</i>) (YESH) shearwaters from three central Mediterranean colonies: La Maddalena, Linosa, Malta. Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. NMDS stress value: 0.112.
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Table S3	Mean bulk stable isotope values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ($\pm\text{SE}$) from blood cells in Scopoli's shearwater and Yelkouan shearwater collected from adult shearwaters during the breeding seasons of 2020 and 2021 (N = Number of samples). Values are presented separately for the three colonies sampled and for incubation (INC) and the chick-rearing period (CHR). $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from Scopoli's shearwaters sampled at La Maddalena were adjusted for latitudinal gradient by subtracting 0.25 and 1.3 respectively. Trophic level (TL) was calculated using the same formula and base values. Isotopic niches per shearwater species and breeding stage were measured as Bayesian Standard Ellipse Areas (SEA_B , mean with 95% credible intervals) and sample size-corrected Standard Ellipse Areas (SEA_c)
Figure S10	Stable isotope values for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in red blood cells of Scopoli's shearwaters (SCSH) and Yelkouan (YESH) during chick-rearing and incubation periods. Values are adjusted for a latitudinal gradient. The ellipses were set to cover 80 % of the points, assuming a multivariate normal distribution, while the error bars were 1.96 times the standard deviation.
Figure S11	Standard Bayesian Ellipse Area (SEA_B) for the chick-rearing and incubation periods of Scopoli's- (SCSH) and Yelkouan shearwaters (N=94; 31; 41, 6 respectively) from three central Mediterranean colonies. Adjusted $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from blood were used to calculate niche metrics. Dots represent the mode and boxes present the 50%, 75% and 95% credible intervals. Red crosses represent the Standard Ellipse Area (SEA_c) estimated with maximum-likelihood.
Figure S12	Standard Bayesian Ellipse Area (SEA_B) overlap, expressed as a proportion of the non-overlapping area, between chick rearing Scopoli's shearwaters and chick-rearing Yelkouan shearwaters (non-temporally simultaneous breeding stages) and incubating Scopoli's shearwaters and chick-rearing Yelkouan shearwaters (temporally simultaneous breeding stages). Adjusted $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from blood were used to calculate niche metrics

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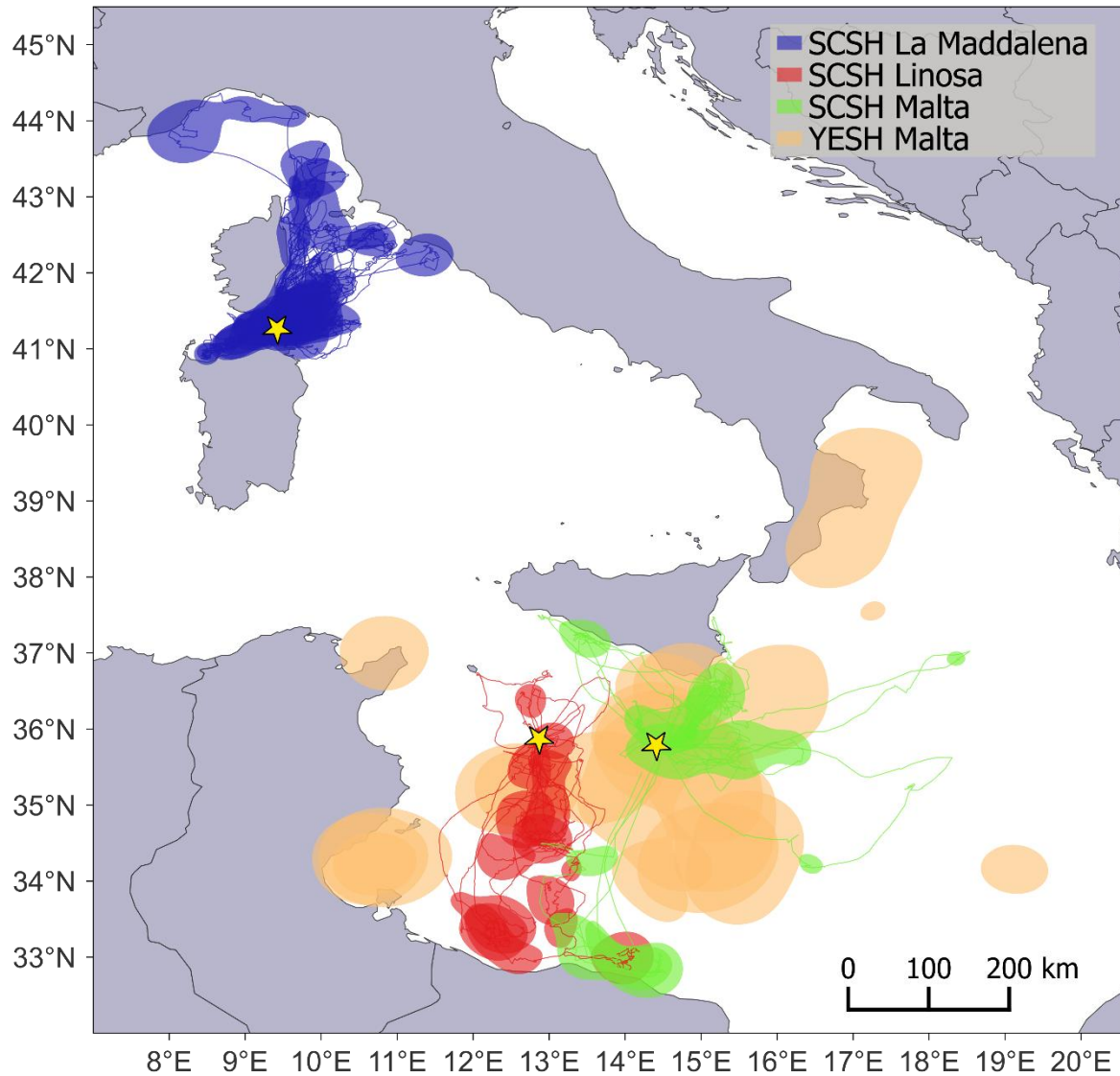


Figure S1 Colony locations, marked with yellow stars, in the Central Mediterranean where dietary and blood samples were collected from Scopoli's shearwaters (La Maddalena, Linosa and Malta) and Yelkouan shearwaters (Malta). Foraging movements are shown as gps-tracks (solid lines) and associated 50% utilisation distribution areas (polygons). Scopoli's shearwaters were tagged during chick-rearing in 2019 (La Maddalena), incubation and chick rearing in 2020 (Malta) and chick rearing in 2020 (Linosa) (Michel et al. 2025²), while Yelkouan shearwaters were tagged during incubation and chick-rearing in 2019 and 2020 (Malta; unpublished data found on <https://data.seabirdtracking.org> as datasets 1935, 2220, 2221 and 2222). Foraging movements are shown for visual reference of foraging areas only and are not necessarily from the same individuals sampled for dietary and isotopic analysis.

² Michel L, Zhang J, Asmiakopoulos A, Austad M, Bustamante P, Cecere JG, Cianchetti-Benedetti M, Colominas-Ciuró R, Dell'Omo G, De Pascalis F, Jaspers VLB, Quillfeldt P (2025) Assessing perfluoroalkyl substance pollution in central Mediterranean breeding shearwaters. *Environ Toxicol Chem* 44:420-431. <https://doi.org/10.1093/etjnl/vgae011>

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Table S1. Primers used in this study for the detection of prey species in faecal and regurgitate samples from Scopoli's and Yelkouan shearwaters.

Prey Target	Gene	Primer name	Sequence 5'-3'	Annealing temperature (°C)	Amplicon size (bp)	Reference
Metazoa	COI	mCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC	48	313	Leray et al. 2013
		jgHCO2198	TAIACYTCIGGRTGICCRARAAYCA			
Osteichthyes	12S	FishF1	CGGTAAAACCTCGTGCC	56	~300	Xavier et al. 2018
		FishR1	CCGCCAAGTCCTTTGGG			
Cephalopoda	16S rRNA	Ceph16S1_F	GACGAGAAGACCCCTADTGAGC	55	~200	Berry et al. 2017
		Ceph16SR_Short	CCAACATCGAGGTCGCAATC			

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A. Certified reference materials for SI analysis

$\delta^{13}\text{C}$: USGS-24, IAEA-CH6, IAEA-600, USGS-61, USGS-62, USGS-63, USGS-41a.

$\delta^{15}\text{N}$: IAEA-N2, IAEA-NO-3, IAEA-600, USGS-61, USGS-62, USGS-63, USGS-41a.

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B. Complimentary results to diet and trophic analysis

In Yelkouan shearwaters we used different collection methods for diet samples and compare the number of sequences respective to prey vs non-prey DNA outputs from Illumina sequencing. For faecal samples we used cloacal swabs (N=44), defecation into a cup container (N=8) or opportunistic collection while handling shearwaters (N=29), but also collection of fresh faeces from burrow substrate without knowing which individual bird the sample was from (N=15). We also collected regurgitate samples opportunistically (N=34).

Of these samples, we obtained DNA sequence data for 89 samples as follows: 38 cloacal swabs, 1 faecal sample in a cup container, 11 faecal samples from burrow substrate, 18 faecal samples collected during handling and finally 21 regurgitate samples. Prey sequence yields were highest for regurgitate samples, followed by faecal samples collected opportunistically during handling and by those collected from the burrow substrate (Fig. S1). Cloacal swabs had high non-prey sequence yields, especially sequences from the birds themselves and low prey sequence yields (Fig. S1). Due to only one sample being successfully sequenced following cup collection we do not present it in the graph, but it yielded 8% prey sequences and 92% non-prey sequences.

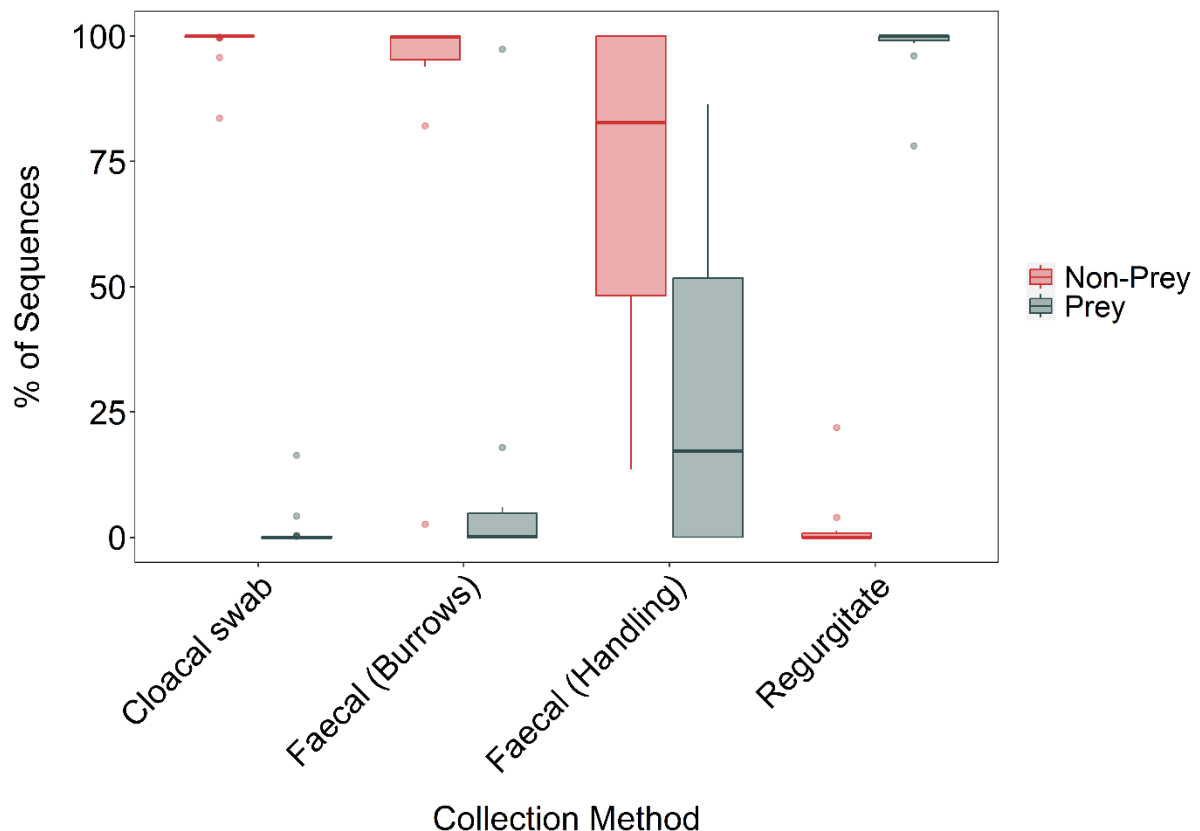


Figure S2 Percentage of non-prey and prey sequences for Yelkouan shearwaters from Illumina sequencing using one universal primer and two specific primers to Fish and Cephalopods respectively. Sequence yields are shown separately for faecal samples collected by cloacal swabs (N=38), collection of faecal samples from burrow substrate (N=11), faecal samples collected opportunistically during handling of shearwaters (N=18) and regurgitate samples also collected opportunistically during handling (N= 21). Fish, cephalopods, marine crustaceans and pelagic tunicates were considered prey, while sequences belonging to any other organism, including DNA from the shearwaters themselves, were considered non-prey

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Table S2 Best blast results for each detected Molecular taxonomic unit (MOTU) and corresponding accession number, the sequence length, E-value and the bit-score from Scopoli's shearwater (*Calonectris diomedea*) and Yelkouan shearwater (*Puffinus yelkouan*).

Order	Family	Species	Accession Number	Ident % (blast)	Sequence length	E-value	Bit-score
Diplostraca	Podonidae	<i>Evadne spinifera</i>	KT208687.1	99.0	313	1.39E-155	562
			AY075071.1	100.0	202	3.85E-99	374
Decapoda	Benthescymidae	<i>Gennadas elegans</i>	MF496986.1	100.0	217	1.92E-107	401
			MW124894.1	99.0	314	1.39E-155	562
	Munididae	Euphausiidae	KU521508.1	98.8	336	1.06E-166	599
			MW505644.1	100.0	285	5.06E-145	527
Octopoda	Argonautidae	<i>Argonauta argo</i>	ON367817.1	98.5	336	4.92E-165	593
			ON367817.1	100.0	193	3.67E-94	357
	Tremoctopodidae	<i>Tremoctopus violaceus</i>	MW351787.1	99.1	325	2.96E-162	584
			MT271737.1	100.0	209	5.15E-103	387
Oegopsida	Ommastrephidae		MF980597.1	100.0	313	1.38E-160	579
			MT223433.2	99.7	321	2.29E-163	588
Doliolida	Doliolidae		OP437494.1	99.4	322	2.96E-162	584
			GQ352660.1	98.9	266	1.42E-129	475
Atheriniformes	Atherinidae	<i>Atherina</i> sp.					
Aulopiformes	Chlorophthalmidae	<i>Chlorophthalmus agassizi</i>	EU366553.1	98.8	339	2.27E-168	604
			AP002918.1	100.0	273	1.86E-138	505
Batrachoidiformes	Synodontidae		KY176664.1	99.7	313	6.41E-159	573
			MN022258.1	98.2	331	4.96E-160	577
Beloniformes	Belontiidae	<i>Belone belone</i>	MW995336.1	100.0	260	3.06E-131	481
			KU360728.1	100.0	268	1.10E-135	496
Clupeiformes	Clupeidae	<i>Sardinella</i> sp.	LC543936.1	100.0	325	2.93E-167	601
			KR056136.1	100.0	266	1.41E-134	492
			MH329246.1	100.0	272	6.67E-138	503

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Order	Family	Species	Accession Number	Ident % (blast)	Sequence length	E-value	Bit-score
	Engraulidae		MT410920.1	99.7	339	2.26E-173	621
Gadiformes		<i>Engraulis</i> sp.	MT410920.1	98.9	272	6.72E-133	486
		<i>Engraulis encrasicolus</i>	MW818368.1	99.0	203	3.29E-96	364
Lophiiformes	Lotidae		OK148206.1	99.1	324	1.06E-161	582
	Merlucciidae		MH194428.1	99.4	326	1.77E-164	592
Mugiliformes	Phycidae	<i>Merluccius merluccius</i>	FR751402.1	100.0	268	1.10E-135	496
	Lophiidae	<i>Phycis blennoides</i>	MT410873.1	100.0	262	2.32E-132	484
Myctophiformes	Mugilidae	<i>Lophius budegassa</i>	MT410891.1	100.0	268	1.10E-135	496
	Myctophidae	<i>Chelon</i> sp.	KF374961.1	100.0	272	6.67E-138	503
			OK354000.1	99.1	321	4.96E-160	577
Osmeriformes		<i>Ceratoscopelus maderensis</i>	ON000320.1	100.0	271	2.39E-137	501
		<i>Argentina sphyraena</i>	K1128703.1	100.0	265	5.04E-134	490
Perciformes			KY176677.1	99.7	311	8.29E-158	569
			AP003091.1	99.6	269	1.43E-134	492
	Carangidae	<i>Trachurus</i> sp.	LR991635.1	100.0	230	1.22E-114	425
		<i>Trachurus trachurus</i>	C_057648.1	99.3	268	2.38E-132	484
		<i>Caranx crysos</i>	AB752307.1	98.1	270	1.87E-128	472
	Centrolophidae		HQ909498.1	98.2	330	4.96E-160	577
	Gobiidae		EF218625.1	100.0	267	3.93E-135	494
		<i>Lesueurigobius suerii</i>	OK148208.1	100.0	327	2.27E-168	604
	Mullidae		C_052759.1	100.0	268	1.10E-135	496
		<i>Mullus surmuletus</i>	KC015306.1	100.0	310	6.41E-159	573
	Nomeidae		OK501307.1	99.7	337	2.90E-172	617
	Scombridae		MK548578.1	100.0	268	1.10E-135	496
		<i>Auxis rochei</i>	OK501307.1	100.0	269	3.07E-136	497
		<i>Scomber</i> sp.	MT410869.1	100.0	267	3.93E-135	494
		<i>Thunnus</i> sp.	MT410869.1	100.0	266	1.41E-134	492
		<i>Thunnus thynnus</i>	MT410869.1	100.0	266	1.41E-134	492
	Sparidae		KY438842.1	100.0	321	4.92E-165	593
		<i>Boops boops</i>	KU510485.1	100.0	266	1.41E-134	492

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Order	Family	Species	Accession Number	Ident % (blast)	Sequence length	E-value	Bit-score
		<i>Dentex maroccanus</i>	EU410413.1	100.0	265	5.04E-134	490
		<i>Pagellus</i> sp.					
		<i>Pagellus acarne</i>	C_037505.1	100.0	268	1.10E-135	496
		<i>Pagellus bogaraveo</i>	AB305023.1	100.0	270	8.56E-137	499
		<i>Pagellus erythrinus</i>	C_037732.1	99.6	269	1.43E-134	492
		<i>Spicara</i> sp.					
		<i>Spicara maena</i>	OM935682.1	100.0	267	3.93E-135	494
		<i>Spicara smaris</i>	KF796615.1	100.0	266	1.41E-134	492
		<i>Spondylisoma cantharus</i>	MW818245.1	100.0	200	6.97E-98	370
	Trichiuridae		OP367787.1	100.0	330	4.89E-170	610
Pleuronectiformes	Citharidae		KM538291.1	99.7	313	6.41E-159	573
Scorpaeniformes	Sebastidae		HM007747.1	99.1	322	1.37E-160	579
		<i>Helicolenus</i> sp.	LC732519.1	100.0	267	3.93E-135	494
Stomiiformes	Sternoptychidae		AP012963.1	98.8	339	2.27E-168	604
		<i>Maurolicus muelleri</i>	AP012963.1	100.0	265	5.04E-134	490
Syngnathiformes	Centriscidae		MH378490.1	100.0	254	7.31E-128	470
		<i>Macroramphosus</i> sp.	LC738811.1	100.0	268	1.10E-135	496
		<i>Macroramphosus scolopax</i>	AB854118.1	100.0	264	1.81E-133	488

Table S2 provides a summary for each MOTU. A more detailed overview including the number of reads for each sample per MOTU and the corresponding Accession Numbers, Ident % (blast), sequences length, E-value and Bit-Score is provided in the excel tables "TableS2_R2_YESH" for Yelkouan shearwater samples and "TableS2_R2_SCSH" for Scopoli's shearwater samples.

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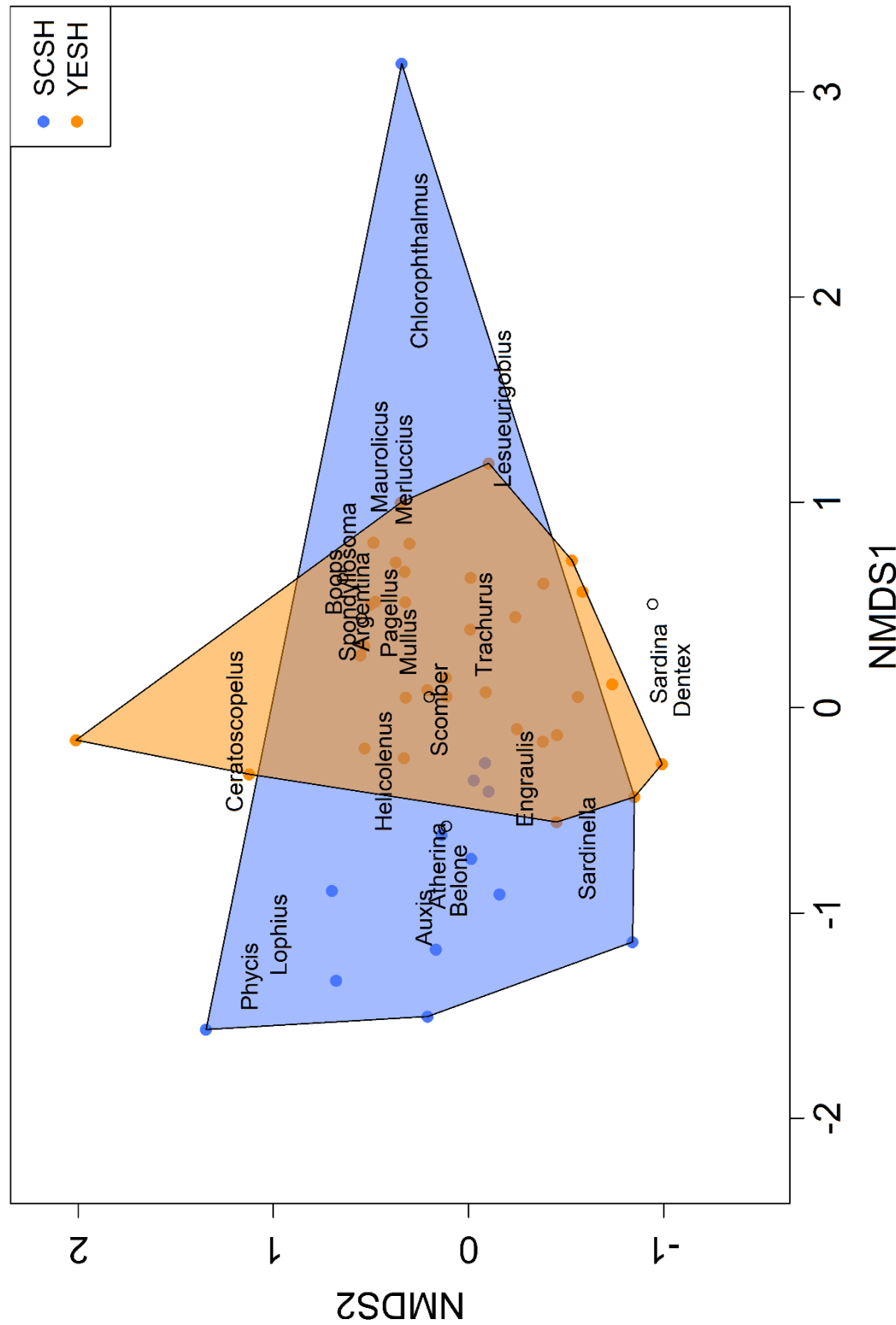


Figure S3 Diet composition on genus level (names displayed) for fish prey consumed by Scopoli's *Calonectris diomedea* (SCSH) and Yeikouan (*Puffinus yeikouan*) (YESH) shearwaters from the central Mediterranean. Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. The NMDS stress level was 0.112.

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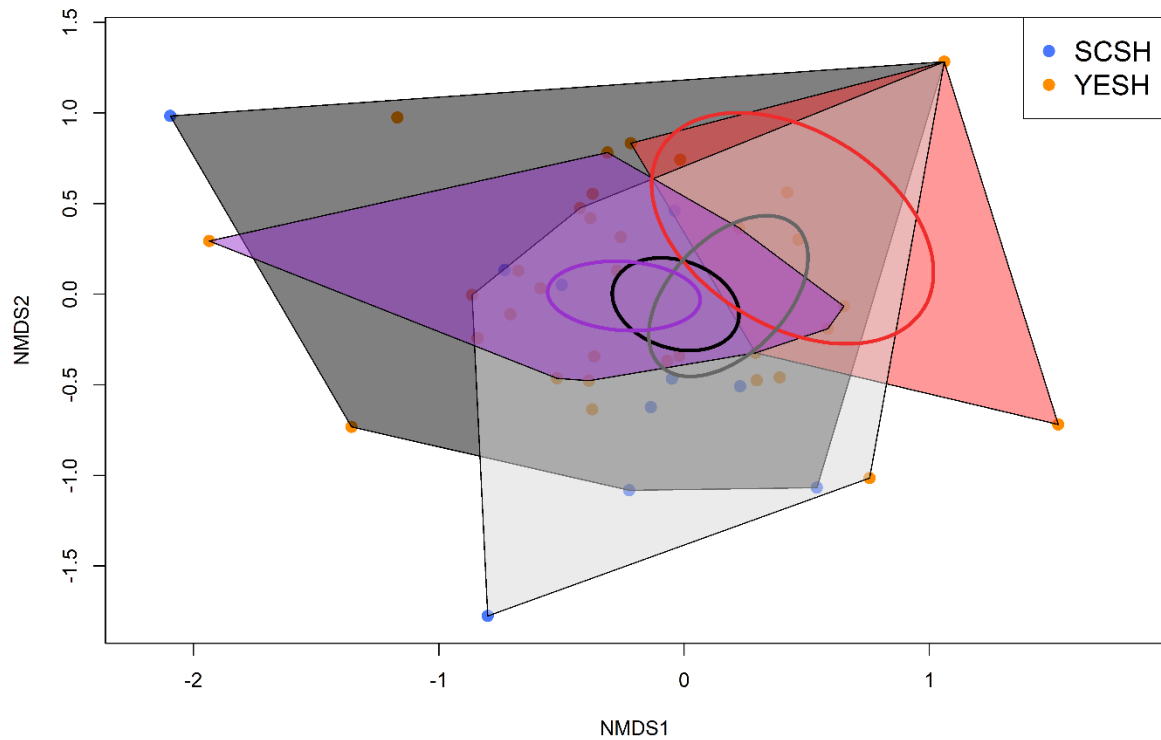


Figure S4 Diet composition at order level of Scopoli's (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) shearwaters from the central Mediterranean, presented by sample type (regurgitates in purple ellipse and hull; faecal samples by cloacal swabs in black; opportunistic faecal samples during handling in grey and faecal samples collected from burrow substrate in red). Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. The NMDS stress level was 0.110.

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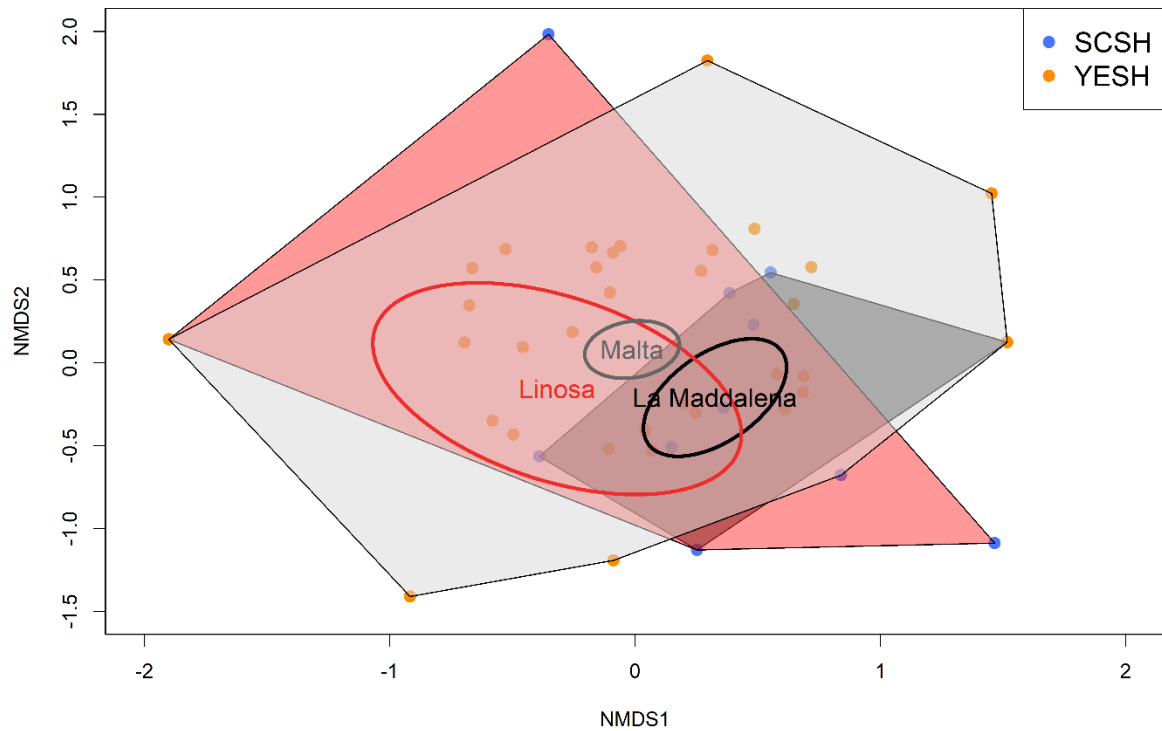


Figure S5 Diet composition on order level of Scopoli's (*Calonectris diomedea*) (SCSH) and Yelkouan (*Puffinus yelkouan*) (YESH) shearwaters from three central Mediterranean colonies: La Maddalena, Linosa, Malta. Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. NMDS stress value was 0.112.

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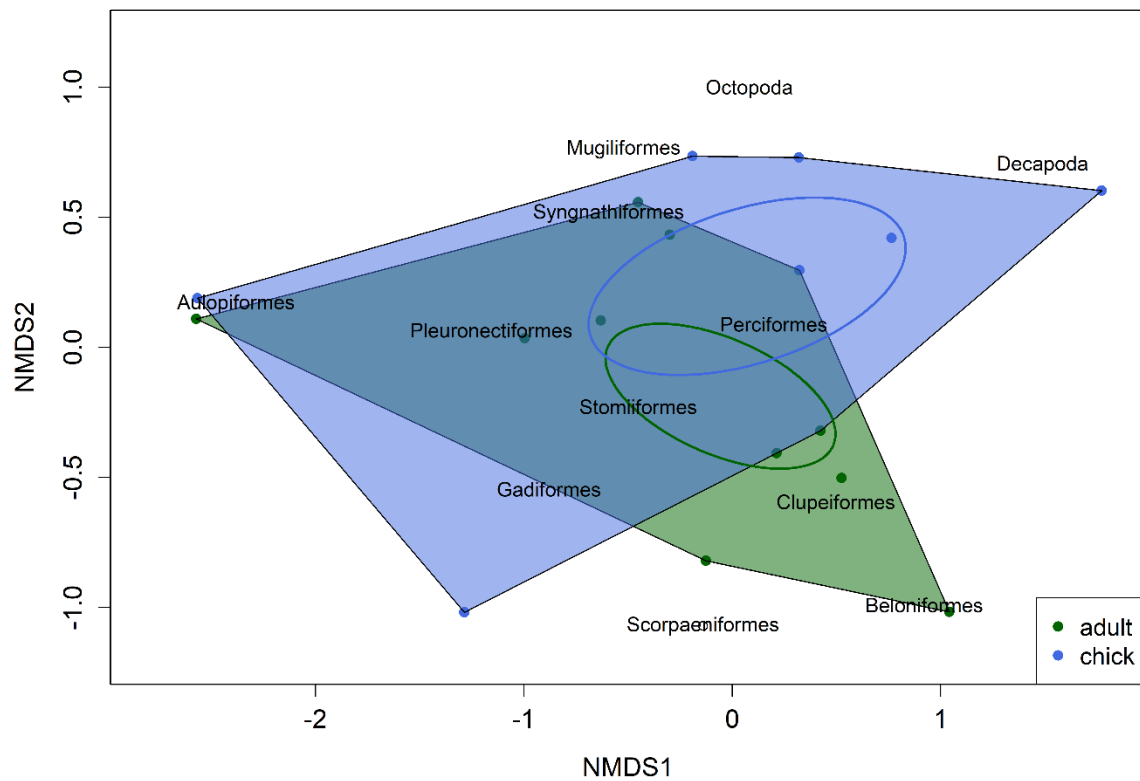


Figure S6 Yelkouan shearwater (*Puffinus yelkouan*) diet composition on the order level, comparing diet in faecal samples from adults (N=15) and chicks (N=12). Non-metric multidimensional scaling (NMDS) was used to condense multidimensional information into two dimensions. NMDS stress level was 0.034.

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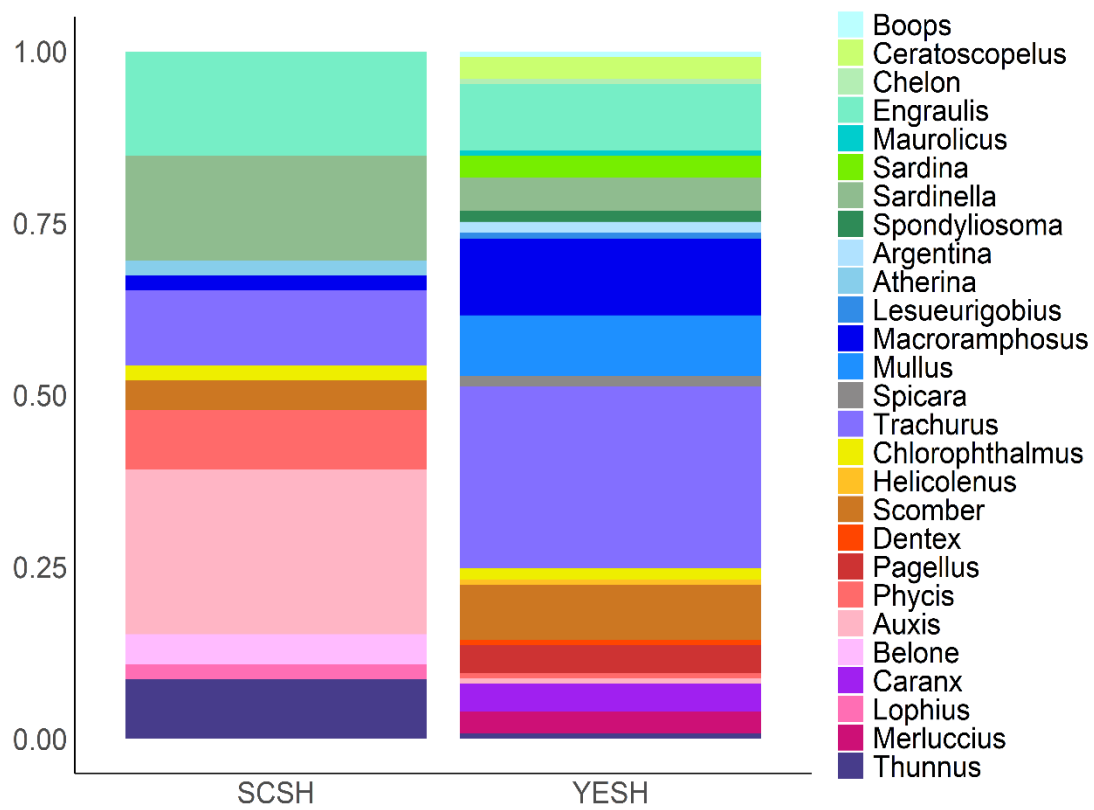


Figure S7 Composition of diet fish genera of Scopoli's (SCSH) and Yelkouan shearwaters (YESH) from the central Mediterranean. (The legend order and colour scheme represent the trophic level from top: low to down: high according to fishbase.se)

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Rarefaction curves were created using the function 'rarecurve' in package vegan for both Scopoli's and Yelkouan shearwaters separately on the order level (Fig. S3) and at the genus level (Fig. S4).

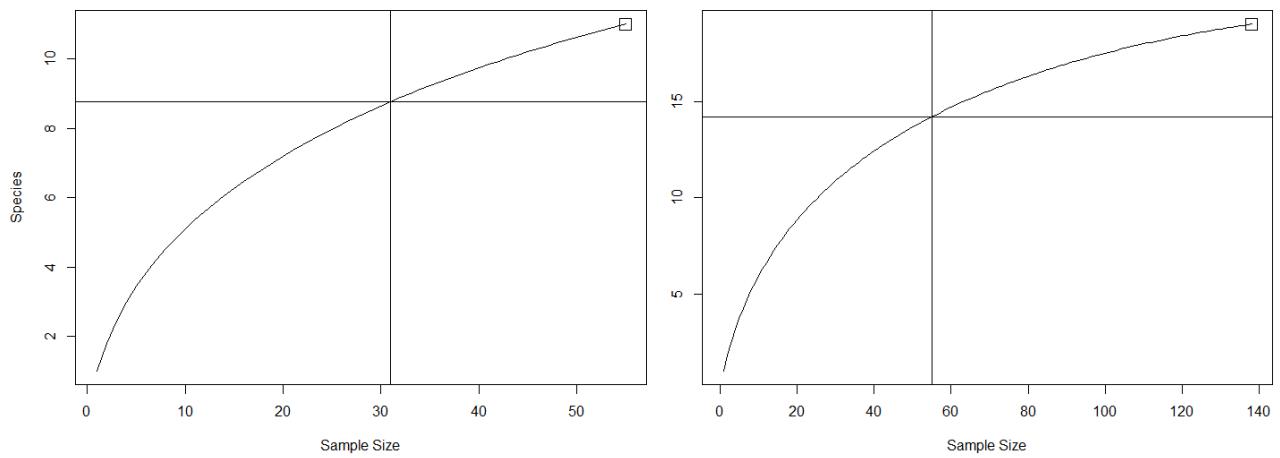


Figure S8 Rarefaction curves for diversity in diet (MOTUs at order level) identified for Scopoli's shearwaters (left) and Yelkouan Shearwaters (right) with the respective sample sizes (samples positive to prey MOTUs) marked in vertical lines.

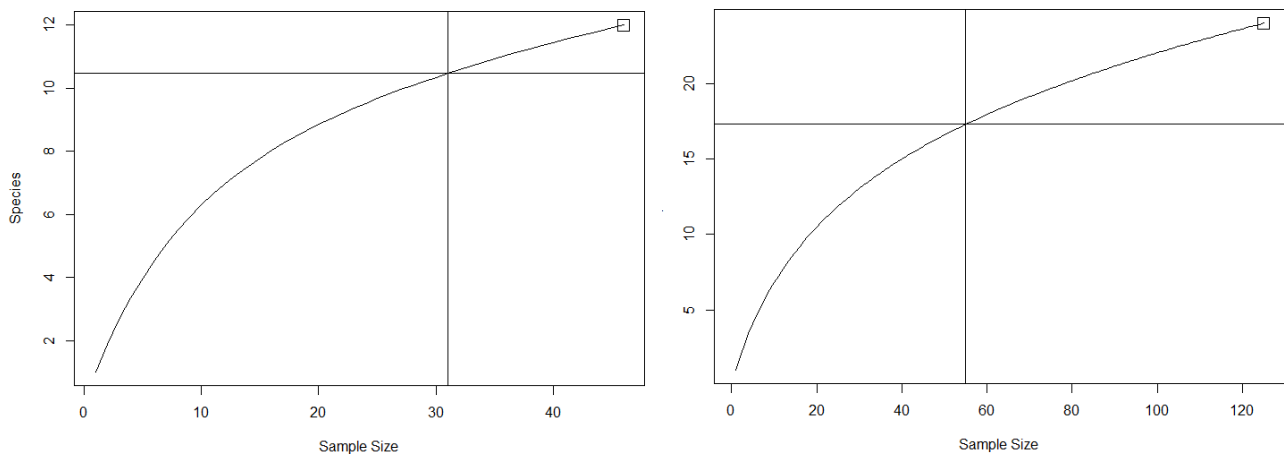


Figure S9 Rarefaction curves for diversity in fish diet (MOTUs at genus level) identified for Scopoli's shearwaters (left) and Yelkouan Shearwaters (right) with the respective sample sizes (samples positive to prey MOTUs) marked in vertical lines.

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Table S3 Mean bulk stable isotope values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ($\pm\text{SD}$) from blood cells in Scopoli's shearwater and Yelkouan shearwater collected from adult shearwaters during the breeding seasons of 2020 and 2021 (N = Number of samples). Values are presented separately for the three colonies sampled and for incubation (INC) and the chick-rearing period (CHR). $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from Scopoli's shearwaters sampled at La Maddalena were adjusted for latitudinal gradient by subtracting 0.25 and 1.3 respectively. Trophic level (TL) was calculated using the same formula and base values. Isotopic niches per shearwater species and breeding stage were measured as sample size-corrected Standard Ellipse Areas (SEA_c) and Bayesian Standard Ellipse Areas (SEA_B, mean with 95% credible intervals).

Year	Colony	Species	Breeding Stage	N	mean $\text{AD}\delta^{13}\text{C}$	SD	mean $\text{AD}\delta^{15}\text{N}$	SD	TL	SEA	SEA _c	SEA _B
2020	Linosa	SCSH	INC	31	-19.12	0.393	9.37	0.469	4.17	0.514	0.532	0.537 [0.356 - 0.738]
2020	Linosa	SCSH	CHR	24	-19.09	0.140	8.16	0.557	3.81	0.368	0.372	0.372 [0.296 - 0.449]
2020	Malta	SCSH	CHR	15	-19.14	0.218	7.97	0.621	3.76			
2020	La Maddalena	SCSH	CHR	22	-19.07	0.155	8.31	0.639	3.86			
2021	Linosa	SCSH	CHR	33	-18.94	0.266	8.35	0.622	3.87			
2021	Malta	YESH	INC	6	-19.89	0.251	8.55	0.310	3.93	0.101	0.126	0.191 [0.0584 - 0.364]
2020	Malta	YESH	CHR	11	-19.22	0.209	7.87	0.535	3.73	0.794	0.814	0.821 [0.576 - 1.08]
2021	Malta	YESH	CHR	30	-19.01	0.408	8.83	0.447	4.01			

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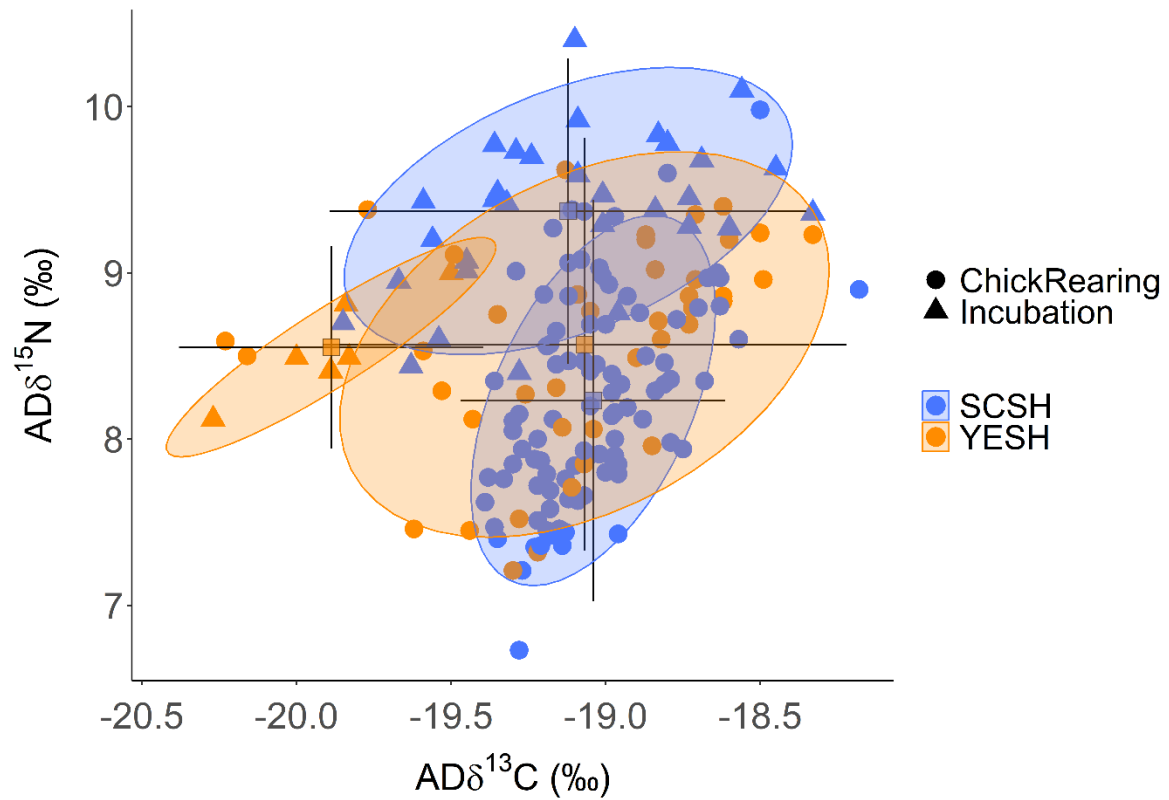


Figure S10 Stable isotope values for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in red blood cells of Scopoli's shearwaters (SCSH) and Yelkouan (YESH) during chick-rearing and incubation periods. Values are adjusted for a latitudinal gradient. The ellipses were set to cover 80 % of the points, assuming a multivariate normal distribution, while the error bars were 1.96 times the standard deviation.

ESM: Diet of two sympatric Mediterranean shearwaters revealed by DNA metabarcoding

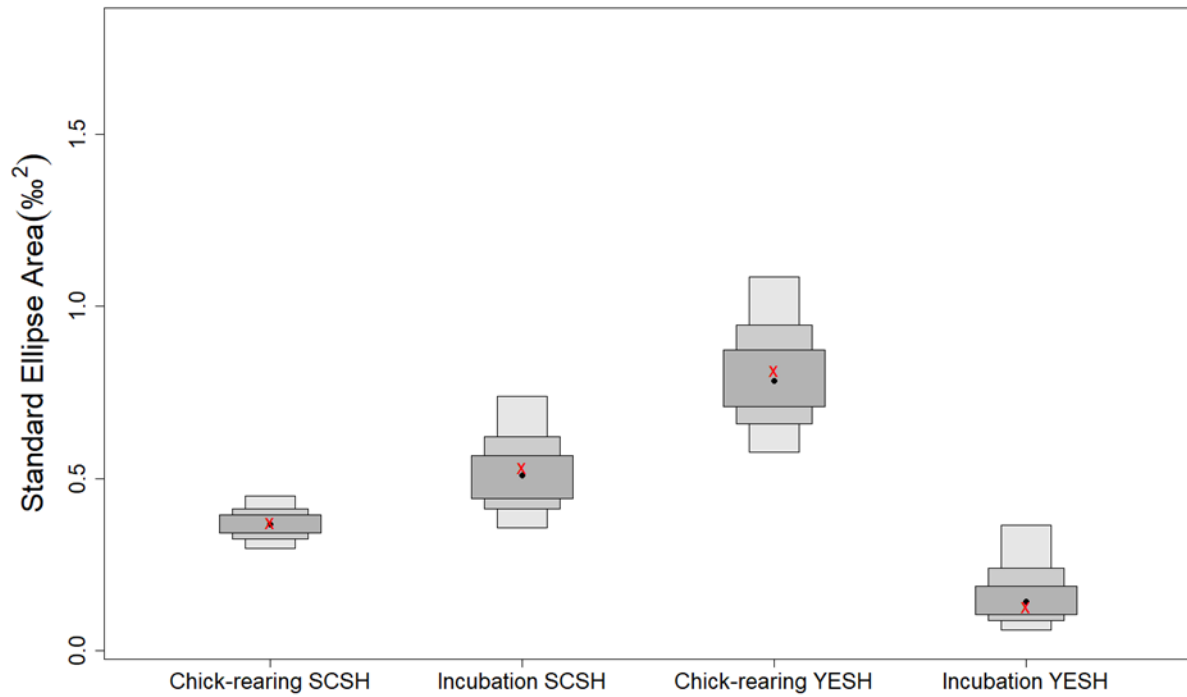


Figure S11 Standard Bayesian Ellipse Area (SEA_B) for the chick-rearing and incubation periods of Scopoli's- (SCSH) and Yelkouan shearwaters (N=94; 31; 41, 6 respectively) from three central Mediterranean colonies. Adjusted $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from blood were used to calculate niche metrics. Dots represent the mode and boxes present the 50%, 75% and 95% credible intervals. Red crosses represent the Standard Ellipse Area (SEA_c) estimated with maximum-likelihood.

ESM: Diet of two sympatric Mediterranean shearwaters revealed by DNA metabarcoding

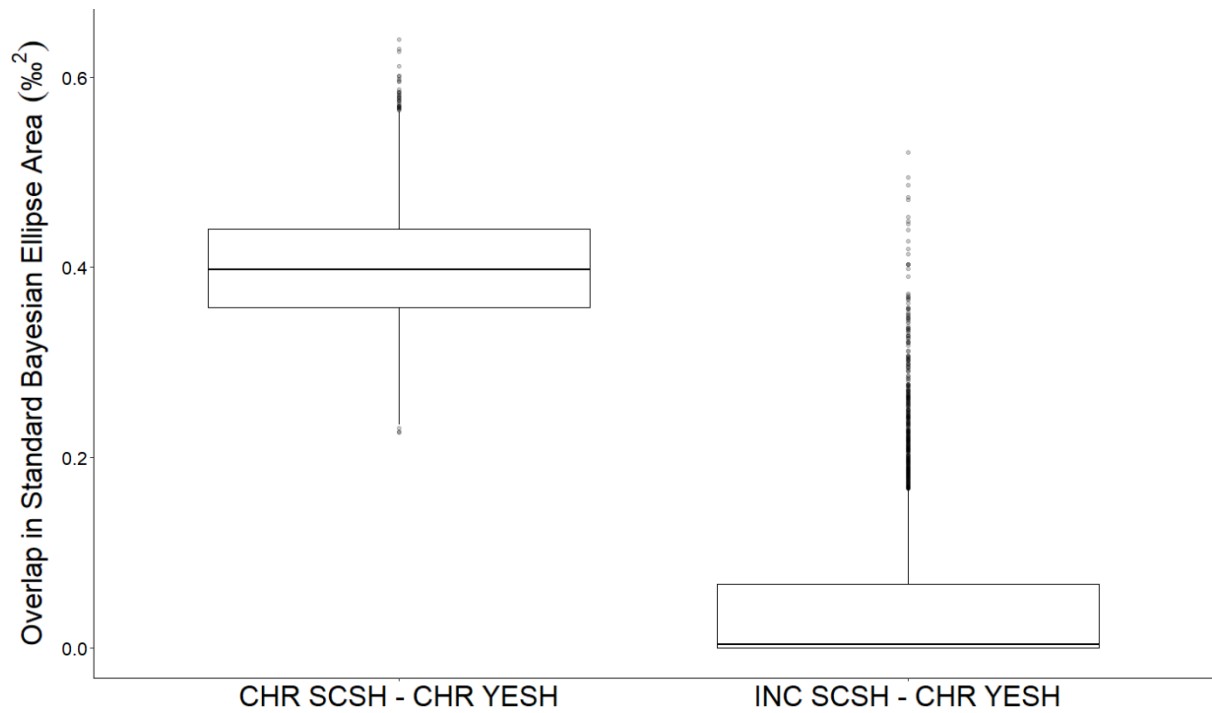


Figure S12 Standard Bayesian Ellipse Area (SEA_B) overlap, expressed as a proportion of the non-overlapping area, between chick rearing Scopoli's shearwaters and chick-rearing Yelkouan shearwaters (non-temporally simultaneous breeding stages) and incubating Scopoli's shearwaters and chick-rearing Yelkouan shearwaters (temporally simultaneous breeding stages). Adjusted $\delta^{13}C$ and $\delta^{15}N$ values from blood were used to calculate niche metrics.



Chapter 3

A watercolor illustration of a coastal landscape. The sky is a light, pale blue. The water is a vibrant, textured blue. Several dark brown, elongated landmasses or islands are scattered across the water. The foreground shows a dark, textured brown area, possibly a beach or a rocky shore.

**How shearwaters prey.
New insights in foraging behaviour and marine foraging
associations using bird-borne video cameras.**



How shearwaters prey. New insights in foraging behaviour and marine foraging associations using bird-borne video cameras

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Abstract

Conventional bio-logging techniques used for ethological studies of seabirds have their limitations when studying detailed behaviours at sea. This study uses animal-borne video cameras to reveal fine-scale behaviours, associations with conspecifics and other species and interactions with fishery vessels during foraging of a Mediterranean seabird. The study was conducted on Scopoli's shearwaters (*Calonectris diomedea*) breeding in Linosa island (35°51'33" N; 12°51'34" E) during summer 2020. Foraging events were video recorded from a seabirds' view with lightweight cameras attached to the birds' back. Foraging always occurred in association with other shearwaters. Competitive events between shearwaters were observed, and their frequency was positively correlated to the number of birds in the foraging aggregation. Associations with tunas and sea turtles have been frequent observations at natural foraging sites. During foraging events, video recordings allowed observations of fine-scale behaviours, which would have remained unnoticed with conventional tracking devices. Foraging events could be categorised by prey type into “natural prey” and “fishery discards”. Analysis of the video footage suggests behavioural differences between the two prey type categories. Those differences suggest that the foraging effort between natural prey and fishery discards consumption can vary, which adds new arguments to the discussion about energy trade-offs and choice of foraging strategy. These observations highlight the importance of combining tracking technologies to obtain a complete picture of the at-sea behaviours of seabirds, which is essential for understanding the impact of foraging strategies and seabird-fishery interactions.

Keywords At-sea behaviour · Marine associations · Animal-borne video cameras · Foraging ecology · Seabird-fishery interactions

Introduction

Understanding patterns in seabirds' foraging behaviour can help identify critical factors for successful foraging, breeding and life-history traits (Davoren et al. 2003; Weimerskirch 2007) in marine habitats. So does understanding the role of social interactions (Jones et al. 2018). Observations of foraging behaviours can give information about preferred prey type (Elliott et al. 2008) and accessibility limitations such

as depth (Burger 2001) and are relevant for evaluating the costs and benefits of foraging because behavioural factors like flapping, landing, and take-off can influence foraging effort (Shaffer et al. 2001). Furthermore, it is crucial to comprehend the natural foraging ecology of seabirds to describe the impact of human activities such as exploitation by fisheries on marine ecosystems (Furness et al. 2007; Lescroël et al. 2016; Le Bot et al. 2018).

Scopoli's shearwaters (*Calonectris diomedea*) are known to be flexible foragers that forage mainly at the sea surface (Zootier et al. 1999; Cianchetti-Benedetti et al. 2017). They cover vast areas of the open sea on the search for prey, but little is known about how these shearwaters locate prey, forage and how they associate with conspecifics or other species. Previous studies found that Scopoli's shearwaters interact with fishing vessels (Cecere et al. 2015; Cianchetti-Benedetti et al. 2018; Karris et al. 2018), but their behaviour in the vicinity of fishing vessels or consuming discards

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has rarely been described in detail. Costs and benefits of foraging strategies can vary according to the circumstances encountered (e.g., level of intraspecific competition (Lewis et al. 2001), type of prey consumed (van Donk et al. 2019). On the one hand, consumption of discards can be disadvantageous and lead to reduced reproductive success if the energy spent on consuming discards cannot be compensated by the nutritional quality of the discarded food source and natural stocks are depleted (Grémillet et al. 2008; Le Bot et al. 2019). On the other hand, discards can represent an important food source as they provide seabirds with resources (benthic or large fish) that cannot be obtained in different ways (Furness 2003). However, interactions with fisheries can be fatal for seabirds because of the risk of by-catching related to fishing gear like hooks from long-line fishing and nets from gillnetting (Karris et al. 2013; Dimech et al. 2009; Cortés et al. 2018). This study aimed to observe foraging associations and fine-scale foraging behaviours of a Mediterranean seabird using bird-borne video cameras and see how they vary according to prey type.

Remote sensing techniques have gained importance for seabirds' behavioural research and comprise a refined and sophisticated set of technologies (Wilson et al. 2002; Ropert-Coudert 2005; Yoda et al. 2019), but fine-scale behaviours during foraging and circumstances that can influence the behaviours are difficult to identify and verify. Bird-borne video cameras allow direct observations, snapshots from the seabird's life, revealing new and unexpected behaviours. So far, this technique was used for land (Bluff and Rutz 2008; Rutz and Troscianko 2013) and marine birds (Takahashi et al. 2004; Watanuki et al. 2008; Sakamoto et al. 2009; Grémillet et al. 2010; Votier et al. 2013; Tremblay et al. 2014) but rarely for small-medium sized seabirds (Yoda 2019) due to the weight constriction and battery duration of video cameras.

The study site, Linosa island, located in the Sicily Channel in the central Mediterranean Sea, is important for demersal fisheries (Russo et al. 2014) and small artisanal fisheries (Lleonart and Maynou 2003; Kelleher 2005). The Scopoli's shearwater is a colonial breeding burrow-nester that raises a single chick per breeding season. Partners share their parental duties. After chick hatching in mid-July, adults forage close to the colony and return to feed their chick at night frequently (Cianchetti-Benedetti et al. 2017). Its natural diet includes a wide range of medium to small-sized fish, squid, and eventually zooplankton (Grémillet et al. 2014). Fishery discards are suggested to be part of its diet as well (Cianchetti-Benedetti et al. 2018; Karris et al. 2018; Cecere et al. 2015). The recorded video material of this study provides exclusive insights into the at-sea behaviour of Mediterranean shearwaters, first-time proof of foraging associations with sea turtles, and firsthand evidence for discard consumption and seabird-fishery interactions.

Methods

Data collection

The study was carried out in the colony of Scopoli's shearwaters of Linosa island (35°51'33" N; 12°51'34" E) during the breeding season 2020. Linosa hosts one of the biggest European colonies of Scopoli's shearwaters with 10,000 estimated couples (Baccetti et al. 2009). Seven birds were equipped with miniaturised video cameras (Technosmart Europe S.r.l., Rome, IT) during the early chick-rearing period (20th July to 10th August 2020). The cameras with a resolution of 720 P weighed 21 g and recorded continuously for 5 h approximately. They were set to start recording at 12 AM (local time) to cover the peak times of foraging activity (Cianchetti-Benedetti et al. 2017). Only birds weighing more than 710 g (i.e., males) were selected to ensure that the device did not exceed 3% of the bodyweight of birds. Camera deployment occurred at night when the birds visited the colony. Birds were captured in their nest, and the camera was attached to the back feathers using marine waterproof Tesa@tape (Wilson and Wilson 1989). The attachment position ensured a frontal view of a 45° angle which included the bird's head. The deployment procedures did not take longer than 5 min, and the bird was released inside the nest afterwards. In six out of seven cases, the birds were recaptured in the night following the first capture. Indeed, during this phase, the shearwaters in Linosa perform mainly one-day foraging trips (Cianchetti-Benedetti et al. 2017). In one case, the camera was recovered after seven days because the bird probably completed a long foraging trip. None of the selected birds deserted the nest after the camera deployment, and their chicks fledged successfully at the end of the breeding season.

Data analysis

Different behaviours related to foraging situations were identified by inspecting the video recordings: foraging events, competitive behaviour, and association with other species. Foraging events were defined as the approach and sojourn at a foraging assembly. Landing at a foraging site was considered the start of a foraging event (Video S1). When the bird moved away from the assembly (to perform other behaviours, like flying, preening, or sleeping), it was considered the end of the foraging event. Sequences of foraging events that were interrupted by flying off for more than 0.5 min or were interrupted by cleaning behaviour or resting for more than 1 min were counted as separate foraging events. To estimate the number of shearwaters

present at the moment of the foraging attempt, a sequence of three aerial frames was taken right before landing on the foraging spot, and all birds in those frames were counted. The mean number of counted birds in the three shots was used to represent the number of birds that participated in the foraging bout and, therefore, competed for prey at the same moment. When other species were associated with the foraging events, these were, in general, fish or turtles. There were no other bird species. Unfortunately, it was not always possible to determine prey species in the pictures because of the low resolution of the video. A dive was defined when the bird immersed its body totally underwater, which was identified by underwater images (Fig. 1a) and Video S2). As the camera was attached to the dorsal feathers, video recordings provided reliable proof that the body was immersed in the water. A prey was considered "natural" when it was captured during feeding without anthropogenic influence (Video S2). In these cases, the prey was alive and actively swimming in small schools. Conversely, the prey was considered fishery discard when it could be identified as anthropogenic in origin. This was the case when fish was damaged or floating lifeless, scattered on or below the water surface (Fig. 1b and Video S3) and when foraging occurred with a fishing vessel or gear insight.

Competitive events included all observations which originated from direct competition with other shearwaters (Fig. 1c, d). Bill fights, competition for the same fish, getting pushed out from the assembly, or having to fly off, were counted as competitive events. No direct competition with other species was observed.

The relationship between the number of competitive events and the number of birds associated with the foraging event was tested with a linear regression using a GLMM of the Poisson family (log-link) for count data. A random effect for bird ID was included to account for the multiple measurements of the same individuals. The model included the fixed effect "mean number of birds in feeding aggregation" as explanatory variables. The GLMM mentioned above was also used to test for behavioural differences (duration of foraging, number of dives performed, number of competitive events) between prey type categories "natural prey" and "fishery discards". Because of too few data points, the latter models did not converge when integrating a random factor to control for repeated measurements. For this reason, the data will be quantitatively described only. Model diagnostics were performed with the package DHARMA. All statistical analyses were run with R version 4.0.4 (R Core Team 2021).

Results

In total, 34 h (min 2:15, max 5:43 per individual) of video material from seven birds were recorded. Individuals foraged on either natural prey or fishery discards but never both during the same foraging trip. Overall, 19 foraging events were filmed, of which three (16%) were assigned to fishery discards, whereas 16 (84%) occurred on natural prey (Table 1). At natural foraging sites, birds were often associated with loggerhead sea turtles (*Carretta caretta*) (Video S1 and S4) and bluefin-tunas (*Thynnus thynnus*) (Video S4). Within 16 natural foraging events, shearwaters

Fig. 1 **a** Diving shearwaters with the body completely immersed in the water. In front the head of the filming shearwater is visible **b** Discard foraging event, this fish is floating lifeless, vertical to the water surface. In the context of the video, more scattered fish carcasses are visible **c** Example of a competitive event; the filming bird gets attacked with the bill by conspecific at foraging event **d** Example of birds repositioning themselves at the centre of foraging activity by flying off and landing.

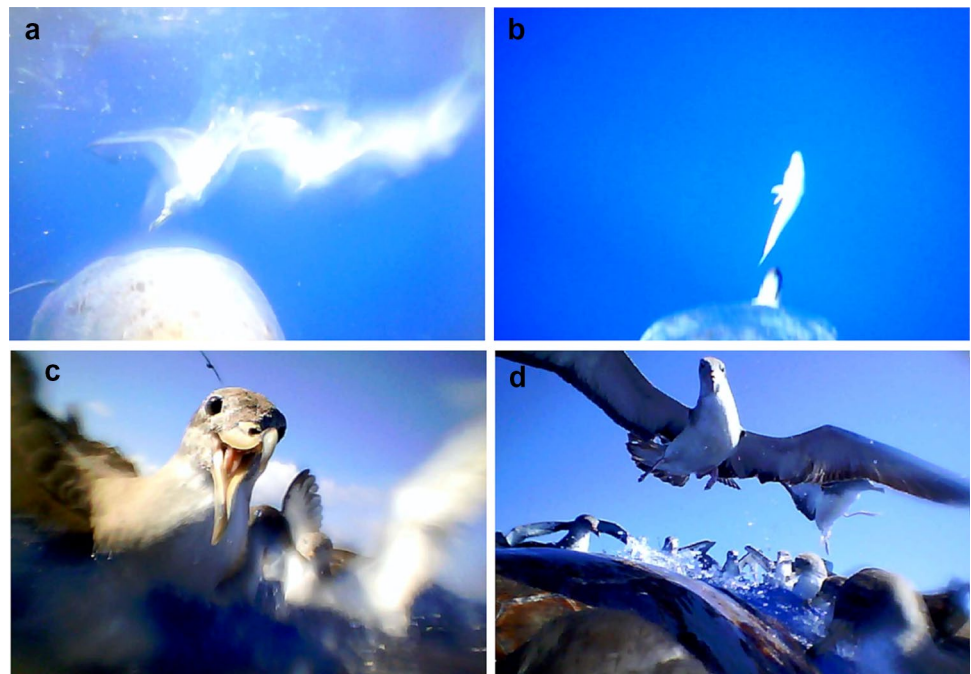
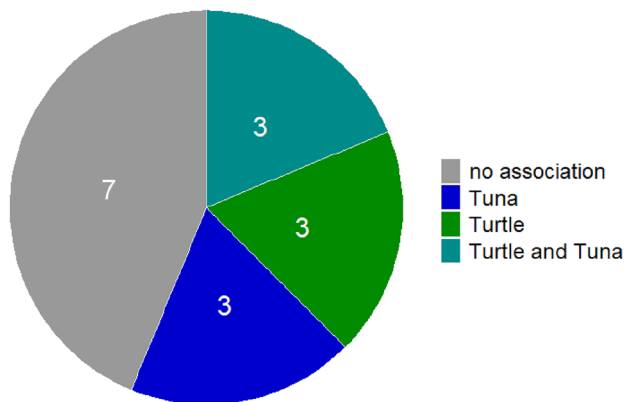


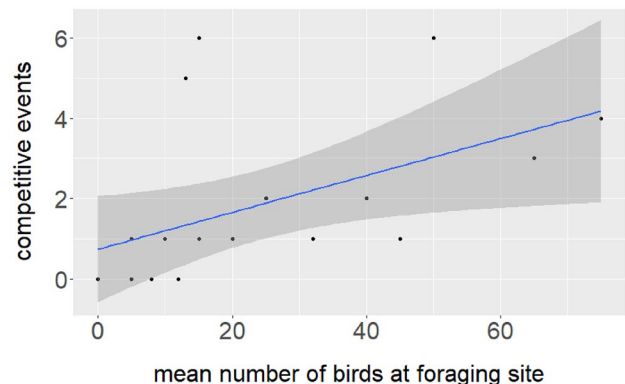
Table 1 This table compares the observed behaviours of individuals during foraging

Bird ID	Dives		Time at foraging site (S)		Competitive events		N foraging events	
	Natural	Discards	Natural	Discards	Natural	Discards	Natural	Discards
1	23	0	990	0	8	0	3	0
2	0	0	0	400	0	0	0	2
3	24	0	1,110	0	5	0	2	0
4	0	5	0	375	0	2	0	1
5	7	0	678	0	8	0	3	0
6	9	0	964	0	1	0	3	0
7	26	0	443	0	11	0	5	0
Σ	89	5	4,185	775	33	2	16	3
MEAN (SD)	12.7 (10.6)	0.7 (1.7)	597.9 (430.2)	110.7 (175.2)	5.5 (3.9)	0.3 (0.7)	2.3 (1.7)	0.4 (0.7)

For each of the two prey type categories ("natural" referring to natural prey consumption and "discards" referring to the consumption of fishery discards), the number of dives, the time spent at the foraging sites in seconds, the number of observed competitive events and the total number of foraging events per animal are displayed. Totals are highlighted in bold, and means per bird with standard deviation in brackets are given at the bottom part of the table

**Fig. 2** Distribution of interspecific interactions during natural foraging events ($n=16$)

were associated with turtles in three events, with tunas in three events, and with both tunas and turtles in three other events (Fig. 2). At discard sites, no association with other animals was observed. Birds competed with their conspecifics at foraging sites (Fig. 1c, d). The number of competitive interactions was positively correlated to the number of birds in the aggregation at the moment of the foraging attempt (GLMM: $\chi^2 = 6.43$, p -value = 0.011, $R^2(m) = 0.32$, $R^2(c) = 0.50$). Variance in the random factor "bird ID" was $\text{Var} = 0.27 \pm 0.52$ (Fig. 3). Interestingly, a tendency for differences in foraging strategy according to the type of prey was observed (Fig. 4). Shearwaters tended to spend more time foraging when preying on natural prey than on discards (Fig. 4a). Also, shearwaters that fed on natural prey tended to dive more frequently than those that fed on discards (Fig. 4b).

**Fig. 3** Scatterplot showing a positive correlation between competitive events and the number of birds present at a foraging aggregation at the moment of foraging attempt ($n=19$)

Discussion

Our study revealed the at-sea behaviour of a medium-size seabird using miniaturised video cameras. To the best of the author's knowledge, this is the first time extended video recordings and complete foraging events, from prey location to active feeding, have been documented for Scopoli's shearwaters. Video recordings revealed fine-scale foraging behaviours, intra-, and interspecific associations, and point to differences in foraging effort depending on prey type consumed.

Consistent observations in Scopoli's shearwaters' foraging behaviour were that: (1) not a single bird foraged alone, without conspecifics in the vicinity, and (2) foraging events were diverse and always occurred opportunistically. These included feeding on discards with and without fishery

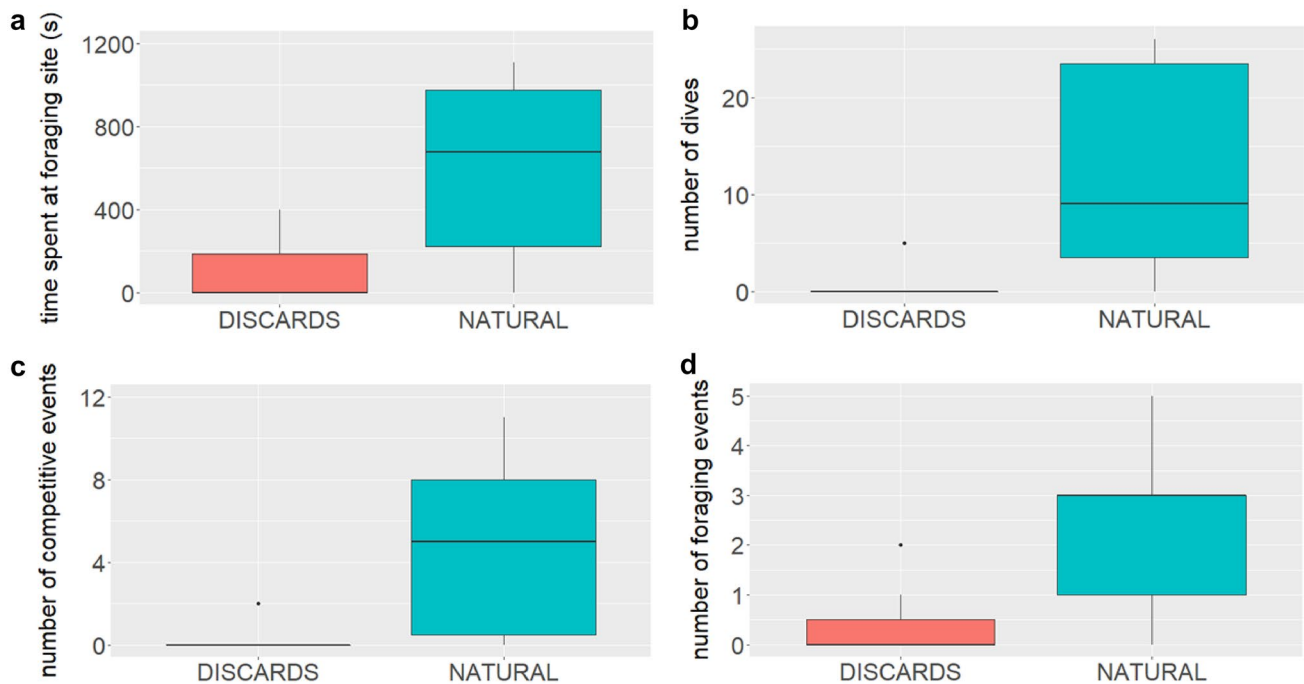


Fig. 4 Plots of **a** the duration spent at a foraging site in seconds **b** the number of dives observed **c** the number of competitive events observed **d** the number of foraging events assigned to each prey type

(natural prey and fishery discards). These boxplots visualise the data presented in Table 1

vessels in sight, feeding in association with hunting tunas, feeding in association with buoyant turtles. Feeding took place close to the surface, and shallow dives prevailed. Foraging events were observed more frequently with natural prey than with discards. This study collected data only on male shearwaters weighing > 700 g to ensure that the devices did not exceed 3% of the body weight. Therefore, no sex comparisons could be made.

Competition

Competitive events increased with the number of birds at foraging sites which could lower the chance of catching prey depending on its abundance. Competitive events tended to be more frequent at natural foraging sites (Fig. 4c). This resulted most likely from the circumstances at the natural foraging sites. There were well-defined aggregation cores where foraging activities were concentrated (Fig. 5 left, Video S1, S4). Here, shearwaters mainly sat on the water surface, dipping their head repeatedly into the water without immersing the whole body (Video S1). These head dips might have served to observe or catch prey right beneath them. Eventually, birds in the aggregation spread their wings to keep other individuals at a distance and defend their spot (Fig. 5 left). Repositioning by flying up and landing again at the centre of the foraging activity occurred repeatedly. While at discarding sites, preys were more scattered on and beneath

the water surface (Fig. 5 right), there was no defined core or centre of foraging activity and therefore less competition. Due to the fragmentary character of the videos, interpretations must be made with caution; there might have been temporal mismatches between the peak activity at a foraging site and when the equipped bird arrived there. It is also possible that bigger fishing vessels attract more birds resulting in higher competition at discarding. Other studies reported high competition around fishing vessels (Arcos et al. 2001; Karris et al. 2018).

Foraging associations

Shearwaters frequently foraged in association with other marine species like Bluefin tunas and Loggerhead turtles (Fig. 2). Marine predators benefit from these interactions because they increase their foraging success (Veit and Harrision 2017). In the Mediterranean, the association of seabirds with subsurface predators seems to be a common phenomenon and previously has been reported for shearwaters (Arcos et al. 2008; Monteiro et al. 1996). Tunas that hunted from below and pushed fish into a dense ball formation close to the surface were observed (Fig. 5 bottom left). Although Audouin's gulls and terns have been associated with hunting tunas at Ebro delta (Oro 1995) and Northern gannets were almost always accompanied (93% of cases) by other scavenging birds (Votier et al. 2013), no

Observation of differences between foraging on natural prey and fishery discards

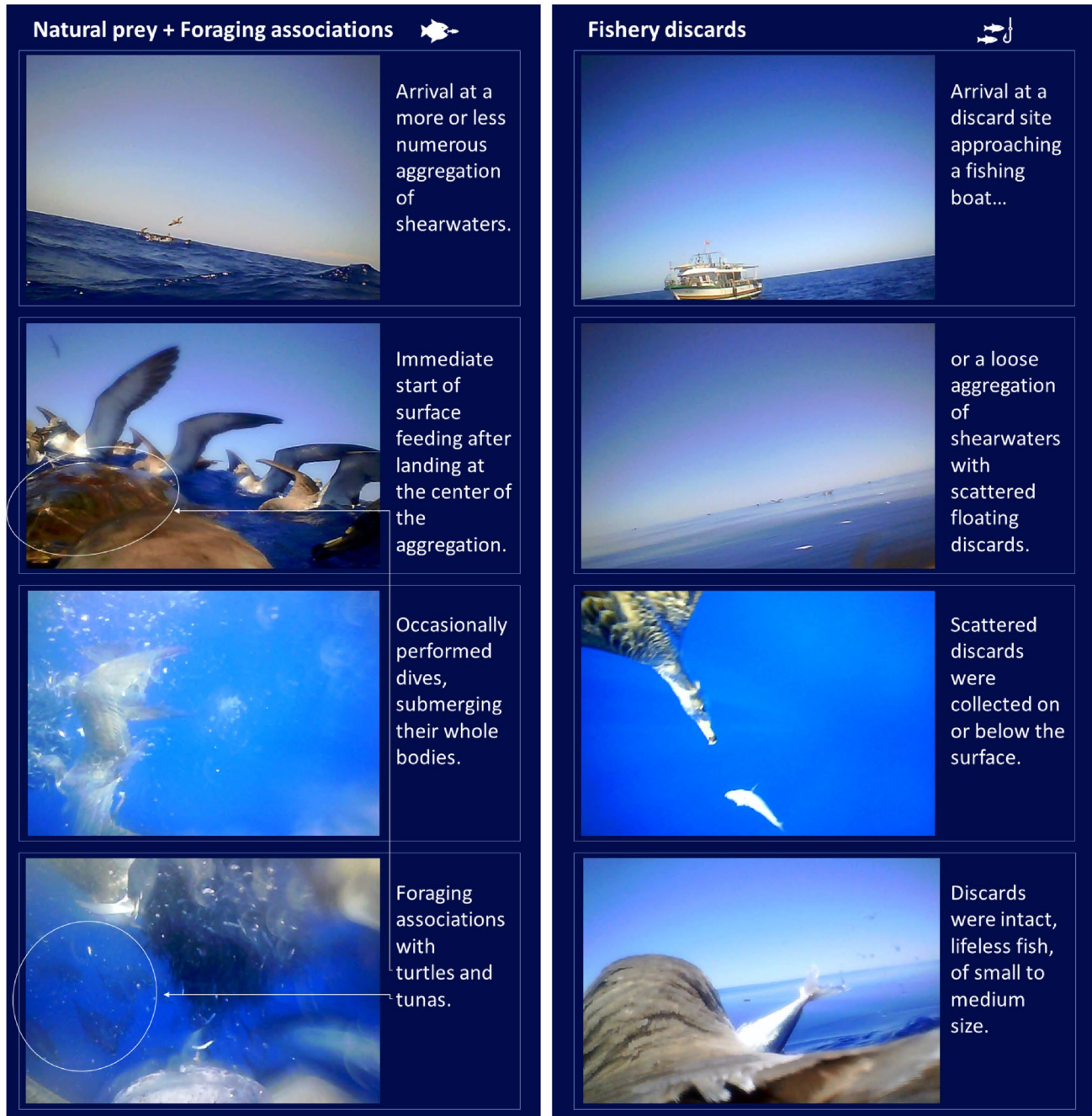


Fig. 5 Confrontation of the main observed differences during natural prey (left) and fishery discards (right) consumption supported by screenshots from the video material. Each picture highlights a distinctive feature of the respective foraging strategy and is shortly

explained in the text next to it. Particularly addressed are differences during the approach at the foraging site, in foraging behaviours, prey, and foraging associations. Associated tunas and turtles are indicated with white arrows and circles

other bird species has been observed in this study, which might be explained by the large distance to the coast or the relatively small size of the aggregations. However, it is possible that due to the restricted view of the camera,

other species or associated tunas/turtles were present but not visible.

Locating foraging sites

It has been suggested that seabirds locate prey in the vast pelagic environment by search patterns (Viswanathan et al. 1996) which can be influenced by spatial memory (Goyert 2015), olfactory (Nevitt and Bonadonna 2005) and visual stimuli (Martin and Prince 2001). These latter include search/recognition of fishing vessels or inter and intraspecific predator assemblies (Tremblay et al. 2014; Votier et al. 2013; Sakamoto et al. 2009). All foraging events in this study were preceded by approaching an aggregation of conspecifics. In no case, shearwaters foraged without conspecifics insight. Video recordings showed that birds flew for some time (30 min to 4 h), likely searching for aggregations of conspecifics until they approached a spot with other foraging shearwaters.

Floating turtles: a consistent observation

Associations with turtles that are floating on the surface have been a consistent observation. There was evidence for a minimum of three turtles floating simultaneously on the water surface in proximity. Shearwaters assembled around them and fed on fish that aggregated below the turtles (Fig. 6, Video S4). It was not clear whether turtles attracted the fish like other buoyant objects in pelagic waters, if they preyed on the fish themselves or if they were injured and preyed upon. It has been reported that Balearic shearwaters fed on fish associated with buoyant objects (Arcos et al. 2000), but this is the first time that feeding associations with shearwaters and turtles have been described. Turtles are frequently injured by boats in the Italian waters, some get stranded, and others float (Casale et al. 2010). It was not clear from the video recordings whether turtles that were floating were injured or healthy, nor whether they were foraging.

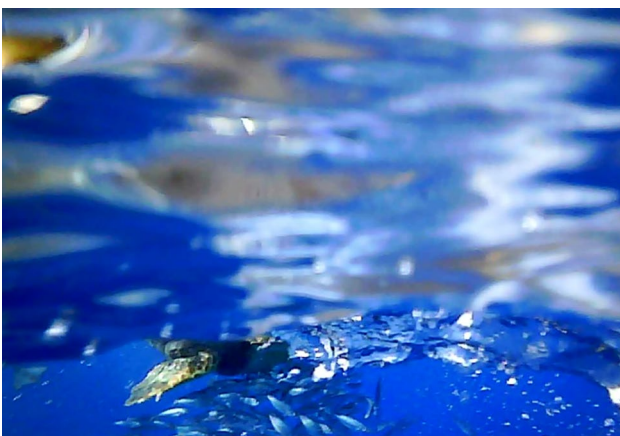


Fig. 6 Underwater view of *C. caretta* that is floating on the surface and fish aggregation underneath it

Loggerhead turtles are generalist and versatile predators of slow-moving or sessile prey (Tomas et al. 2001). Even though fish represented the most important prey of *C. caretta* in the western Mediterranean, the authors believe that this high rate of fish in the diet originated from discarded by-catch (Tomas et al. 2001). Observations by remotely operated vehicles in the Northwest Atlantic showed that Loggerhead turtles tended to remain within the near-surface and surface zones of the water column through much of the footage (Patel et al. 2016). However, they did not specify whether turtles were floating at the surface.

Differences in prey type and foraging effort

The here observed shearwaters feeding on natural prey tended to spend more time at the foraging sites, dive more, and engage in more competitive events than the birds feeding on fishery discards. Individual differences in foraging strategies within seabird populations have been described in previous studies (Votier et al. 2010; Patrick et al. 2015) and emerged in this study as well. Although the number of individuals studied here was low, this was reflected by individuals feeding either on natural prey or on fishery discards but in no case on both of them. These observed differences could be due to the individual preferences of the study subjects and are therefore not universally valid. There is evidence for personality influencing the foraging strategy of seabirds (Patrick and Weimerskirch et al. 2014).

Nevertheless, the video material documents constant distinct characteristics of the circumstances at the foraging site (Fig. 5), which likely cause the observed behavioural differences. The higher amount of time spent at natural foraging sites coupled with the higher frequency of dives indicate that feeding on natural prey can be more time and energy-demanding for shearwaters than feeding on discards. Still, natural prey consumption was more common than discard consumption in this study (Fig. 4d). It is possible that locating discarding sites would have involved longer search and coverage of larger distances, as reported by Cianchetti-Benedetti et al. 2018. These longer trips are avoided during the early chick-rearing period in which shearwaters usually perform short foraging trips close to the colony (Cianchetti-Benedetti et al. 2017; Paiva et al. 2010; Granadeiro et al. 1998; Magalhães et al. 2008). In this study, one-day trips prevailed, which might have had a strong influence on the foraging strategy.

Discard consumption has been suggested to be sub-optimal for breeding gannets (Grémillet et al. 2008). Cape gannets (*Morus capensis*) that foraged on discards increased their foraging effort (Pichegru et al. 2007), and Northern gannets (*Morus bassanus*) had lower body condition (Le Bot et al. 2019). This occurred in populations breeding in areas with depleted fish stocks, and discards

were mixed of offal and whole fish. By contrast, in the present study, all exploited discards represented an easy and energetically efficient prey, as they consisted of intact fish, which was collected within a shorter time and with less diving effort than natural prey (Fig. 4, Video S3). Factors like the availability of natural prey, the individual energetical demands according to the life-history stage, and the nutritional quality of fishery discards should be considered when discussing the benefits and costs of foraging strategies.

Seabird-fisheries interactions have been studied extensively for many species and marine habitats (Le Bot et al. 2018). In the Sicily Channel, about 40% of breeding Scopoli's shearwaters tagged with GPS interacted with fishery vessels (Cianchetti-Benedetti et al. 2018). A study that deployed cameras and GPS on Northern gannets reported a 42% association with fishing vessels (Votier et al. 2013). In the present study, ships were encountered in two cases representing 28% of the sampled birds, with foraging occurring only in one case. Additionally, one discard site was visited, but no vessel was in sight. There might have been a time shift here since the discards were discharged and when the shearwater arrived at the foraging site. These observations highlight the importance of combining technologies to detect foraging when studying seabird-fisheries interactions and quantify discard consumption. Additionally, GPS and accelerometer data can help to quantify energy effort invested into the respective foraging strategies. Paired with environmental data, they can be a powerful tool to study the impact of fishery overexploitation and discarding on seabirds.

Dives

Dive events in which their whole body was underwater were observed infrequently and mostly lasted fractions of seconds. Dives started from sitting on the surface or, less frequently, from plunging into the water from flight. Accordingly, a recent study found a high proportion of shallow and quick dives in Scopoli's shearwaters as 50% of the dives occurred in the first 0.5 m of depth and 78% were less than 1 m deep and lasted < 2 s (Cianchetti-Benedetti et al. 2017). Shallow dives were recorded both during foraging at natural and discard sites. Although Scopoli's shearwaters can dive to 5–6 m or deeper (Cianchetti-Benedetti et al. 2017; Grémillet et al. 2014), the videos document that they extensively exploited food on or right beneath the water surface, which might make them less prone to access deeper and more agile prey and therefore, depend on foraging associations with predators that push the fish to the surface.

Conclusions

Video recordings allowed to observe fine-scale foraging behaviours that cannot be recorded with traditional tracking devices. They can verify if the prey was consumed at a foraging site and give an additional measure of energy expenses that have to be invested into successful foraging. Additionally, they allowed the observation of intra- and interspecific associations, which seem to play a significant role in successfully locating a foraging site and influence foraging success and energy expenses as well. The differences in fine-scale behaviours between natural prey and fishery discard consumption should be verified and considered in future studies investigating the impact of fishery activities on seabirds.

Although this study observed a low proportion of discard consumption compared to previous publications, concluding that they are an unimportant food source for these shearwaters would be inadequate. Assessing the impact of fisheries waste is not trivial, and it likely needs to be determined by a situation-based approach, as also suggested by Clark et al. (2020). It has been reported that there are great individual differences in foraging strategies within seabird populations (Patrick et al. 2015; Votier et al. 2010). Therefore, the importance of fishery discards consumption might vary on the individual level. Moreover, it can vary from year to year since fish stocks of the central Mediterranean are subject to fluctuations (Patti et al. 2004; Arcos et al. 2008). The discards observed in this study were intact. They implied low hunting effort, which could balance any energetical deficit from long searches, meaning that shearwaters might profit opportunistically from high-quality fishery discards, especially during the energy-demanding reproductive period in which they cover less area in search of prey. In this area of the Mediterranean, discards might be a less reliable food source since fishery fleets consist mainly of small artisanal boats, which produce small amounts of discards (Colloca et al. 2017; Tsagarakis et al. 2014), whose impact can vary in different situations.

In future research, bird-borne camera observations should be coupled with accelerometers and GPS. Supported with data about the position of the foraging sites and movement patterns at-sea behaviour, marine associations, and impact of fishery discards consumption on seabirds can be studied in greater detail. This knowledge can be valuable to determine comprehensive conservation measures.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00227-021-03994-w>.

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Author contributions All authors contributed to the study's conception and design. LM and MC-B analysed the data. LM, MC-B and GDO carried out the fieldwork. LM drafted the manuscript, which was commented on by all co-authors. All authors read and approved the final manuscript. All authors gave final approval for publication.

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Availability of data and material The datasets used during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Additional declarations for articles in life science journals that report the results of studies involving humans and/or animals Not applicable.

Ethics approval All fieldwork was carried out according to the national legislation. The research was carried out with moral responsibility for the animals, with the final goal to preserve and enhance the future survival of that species and to provide knowledge vital to their conservation. No specimens were collected for this study. Birds were removed from their nest, weighed, and measured. Then, the logger was attached to the bird with tape, and it was returned to the nest burrows within 5 min of initial capture. The logger was recovered, and the tape was removed after 1 (in one case 7) days. We avoided any disturbance to the nest during the first days of the life of chicks. No nest desertion was observed due to our operations, and all chicks fledged successfully. The study was carried out under the permit n. 2452 issued by the Regional authorities (*Regione Siciliana, Assessorato Regionale dell'Agricoltura, dello Sviluppo Rurale e della Pesca Mediterranea, Dipartimento Regionale dello Sviluppo Rurale e Territoriale*) on February 1st, 2018.

This research complies with the IUCN Policy Statement on Research Involving Species at Risk of Extinction (Scopoli's shearwater *Calonectris diomedea* is classified as "Least concern" in 2018).

Consent to participate. Not applicable.

Consent for publication. Not applicable.

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Chapter 4

A watercolor illustration of a coastal landscape. The top portion shows a bright blue sea meeting a white, overcast sky. Below the sea, there are several large, brown, rounded hills or landmasses. The foreground consists of a green field with some brown patches, suggesting rocks or soil. The overall style is soft and painterly.

Analysis of organochlorines and polycyclic aromatic hydrocarbons designed for pollutant biomonitoring in three seabird matrices.



Analysis of organochlorines and polycyclic aromatic hydrocarbons designed for pollutant biomonitoring in three seabird matrices

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Abstract

Pollutant biomonitoring demands analytical methods to cover a wide range of target compounds, work with minimal sample amounts, and apply least invasive and reproducible sampling procedures. We developed a method to analyse 68 bioaccumulative organic pollutants in three seabird matrices: plasma, liver, and stomach oil, representing different exposure phases. Extraction efficiency was assessed based on recoveries of spiked surrogate samples, then the method was applied to environmental samples collected from Scopoli's shearwater (*Calonectris diomedea*). Extraction was performed in an ultrasonic bath, purification with Florisil cartridges (5 g, 20 mL), and analysis by GC–Orbitrap–MS. Quality controls at 5 ng yielded satisfactory recoveries (80–120%) although signal intensification was found for some compounds. The method permitted the detection of 28 targeted pollutants in the environmental samples. The mean sum of organic pollutants was 4.25 ± 4.83 ng/g in plasma, 1634 ± 2990 ng/g in liver, and 233 ± 111 ng/g in stomach oil (all wet weight). Pollutant profiles varied among the matrices, although 4,4'-DDE was the dominant compound overall. This method is useful for pollutant biomonitoring in seabirds and discusses the interest of analysing different matrices.

Keywords POPs · Biomonitoring · Shearwaters · Biological matrices · High-resolution mass spectrometry · Seabirds

Introduction

Biomonitoring with wild birds is important for observing the current environmental burden and temporal trends of bioaccumulative legacy and emerging contaminants (Becker 2003; Pacyna-Kuchta 2023). Such programs allow for the detection of potential acute and chronic toxic effects and may serve as a basis for the regulation of harmful groups of chemicals (Gómez-Ramírez et al. 2014; Espín et al. 2016; Badry et al. 2022; Kreitsberg et al. 2023). Seabirds can serve as indicators for the health of the marine environment (Parsons et al. 2008) and often have been used to assess

persistent organic pollutants (POPs) in ecotoxicological studies (Walker 1992).

Organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs), summarised in the following as persistent organic pollutants (POPs), are listed in the Stockholm Convention, a treaty that aims at the elimination or unintentional production of POPs. The greater use of OCPs in agriculture and PCBs for industrial processes led to global pollution of the environment like soil, water, and air and due to their persistence and bioaccumulation potential, ultimately wildlife, as well (Pattnaik et al. 2020). Literature reports a wide range of adverse effects of POPs on birds by interfering with their endocrine, immune, and neural system; reproduction; and development and growth (Hao et al. 2021). Polycyclic aromatic hydrocarbons (PAHs) are formed as by-products of combustion and burning processes of both anthropogenic and natural origin. Certain types of PAHs are subject to monitoring and restrictions by international authorities due to their known carcinogenic and mutagenic properties (Jinadasa et al. 2020). PAHs can be found in air, water, dust, and soil in concentrations that may pose a risk to living organisms. These can enter the body through inhalation, ingestion, and dermal contact. Both POPs and

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PAHs caused severe toxic effects in seabirds in the past (Burger and Gochfeld 2002). As a consequence of their banning or regulation, long-term studies record decreasing POP and PAH concentrations in seabirds (Rigét et al. 2010, Braune et al. 2007, Bianchini et al. 2022, Elliott et al. 2023). However, due to their persistence and bioaccumulation potential, these compounds are still detected in seabirds worldwide (Costantini et al. 2017; Lewis et al. 2020, 2022; Yamashita et al. 2021), and while legacy POPs are decreasing, total POP concentrations tend to increase due to the accumulation of emerging POPs (Helgason et al. 2008; Jang et al. 2022). Other studies report recent increases in legacy pesticides and stable PCB concentrations in seabirds from the Arctic (Bustnes et al. 2024). Monitoring of legacy POPs continues to be relevant in the light of climate change. Changes in the bioavailability of legacy POPs have been reported due to altered transport and exposure pathways (Braune et al. 2019; Kalia et al. 2021, Corsolini et al. 2022), and increased effective temperatures have been associated with increased OC levels in the blood (Bustnes et al. 2024). Chronic pollutant exposure can affect expression of genes linked to pollutant metabolism and physiological processes (Kreitsberg et al. 2023). It can cause hormone disruption and ultimately lead to behavioural changes that can lower the adaption potential to environmental stochasticity (Esparza et al. 2022) and allocation of parental investment/self-provisioning (Blévin et al. 2020). Exposure of biota to pollution in the environment usually occurs as a complex mixture of different kinds of pollutants (Rochman et al. 2015, Suaria et al. 2016; Gkotsis et al. 2022; Dulsat-Masvidal et al. 2023; Navarro et al. 2023) which in combination can potentially pose threats to seabird populations (Lavers et al. 2014; Hao et al. 2021). Therefore, analytical methods that can cover multiple pollutant classes, targeting both legacy and emerging POPs, are required in biomonitoring studies.

Monitoring pollutants in birds poses challenges to the analytical protocol, as samples of wild birds can be obtained only in limited amounts (Warner et al. 2018). The methods must be highly sensitive and specific while covering a large spectrum of target compounds. A widely used methods for monitoring organochlorine compounds and PAHs are gas chromatography coupled to mass spectrometry (GC–MS). The choice of extraction method depends on the matrix type but usually involves liquid extraction with a non-polar solvent (Moradi et al. 2023). Fatty matrices are sometimes saponified before extraction, but also a solid-phase extraction can be used for the clean-up (JEFCA 2006).

We developed a method based on gas chromatography coupled to Orbitrap mass spectrometry (GC–Orbitrap–MS) to analyse an array of POPs and PAHs in three seabird matrices that represent different exposure stages. Plasma

reflects the current body burden, while the liver reflects the accumulated burden of the main detox organ, and stomach oil reflects the uptake by the diet and chick exposure. In this study, we present and evaluate the method's performance using three tissues and the results of its application to environmental seabird samples. Scopoli's shearwaters (*Calonectris diomedea*) hold a high trophic level in the marine food chain, exposing them to bioaccumulative contaminants. They have been used as biomonitors for various pollutants in the Mediterranean Sea, such as metals (Renzoni et al. 1986; Ramos et al. 2009); POPs (Roscales et al. 2010); per- and polyfluoroalkyl substances (PFAS) (Escoruela et al. 2018); and PAHs (Roscales et al. 2011). Differences in pollutant concentrations between shearwater populations have been detected, which likely reflect the pollution status of the environment. We used samples of shearwaters breeding on a remote island in the central Mediterranean to test whether our method is sensitive enough to detect targeted bioaccumulative pollutants in samples with expected low to medium concentration levels (Costantini et al. 2017).

In this study, we aimed to facilitate direct comparisons of pollutant concentrations across different exposure stages of a seabird with special interest in the current body burden of rearing adults and fledglings; firstly, the pollutants transferred to the chicks by the stomach oil which is used to feed the chicks. To this end, we employed a single protocol to measure a comprehensive array of target POPs in three seabird matrices.

Methods

Environmental sample collection

Field site and study species

The fieldwork was conducted in the Scopoli's shearwater colony of Linosa Island (35° 51' 33" N; 12° 51' 34" E) located in the Sicily Channel. Between February and October, this small island is the breeding ground of an important population of Scopoli's shearwaters (Müller et al. 2014; Péron and Grémillet 2013), with 10,000 breeding pairs (Baccetti et al. 2009). Breeding birds lay a single egg per season, and both parents are involved in brood care (Hamer et al. 1987). These long-lived birds (Péron and Grémillet 2013) exhibit a remarkable breeding site and mate fidelity (Mougin et al. 2000). They are top predators foraging exclusively on a marine diet (Zotier et al. 1999; Grémillet et al. 2014; Michel et al. 2022), and foraging areas are mainly pelagic, extending along the western part of the Sicily Channel south to the Libyan coast (Cecere et al. 2013, Colominas-Ciurò et al. 2022).

Seabird sample collection and preparation

A total of 0.45 mL of whole blood was collected from adult shearwaters ($n=11$) in July 2020 and from fledglings ($n=10$) in October 2019 with a syringe by puncturing the tarsal vein. At the station, the samples were centrifuged for 10 min at 2500 rpm, the supernatant plasma was separated, and the samples were stored at $-18\text{ }^{\circ}\text{C}$ until analysis. Liver samples ($n=10$) were obtained from birds that were killed by a feral dog in May 2021. The carcasses were collected after the incident was discovered and stored at $-18\text{ }^{\circ}\text{C}$ until dissection. In the laboratory, liver samples were freeze-dried and ground in mortars cleaned with acetone and methanol. The water content was determined by weighing before and after the freeze-drying process. The stomach oil of six chicks was sampled in August 2020. A soft plastic tube attached to a 10 mL syringe was gently inserted into the pharynx of a well-fed chick. When the tube reached the end of the oesophagus, 2 mL of stomach oil was extracted by carefully pulling the syringe. Sampled chicks were checked upon on the next days in order to make sure they continued to be well nourished by their parents.

Pollutant analysis

Chemicals and materials used

Analytical standards comprised 24 organochlorine pesticides, 28 polychlorinated biphenyls (PCBs), and 16 polycyclic aromatic hydrocarbons (PAHs). A working solution containing all target compounds was prepared at $0.2\text{ ng}/\mu\text{L}$ in isooctane. We selected six PCB congeners unlikely to be detected in seabird tissues as internal standards (IS) for PCBs and used labelled IS for PAHs and OCPs. Complete compound names, formulae, CAS identifiers, and supplier details are provided in Table S1. Hexane, ethyl acetate, dichloromethane (DCM), acetone, and isooctane were purchased from Merck (Darmstadt, Germany). For the cleanup, Mega Bond Elut–Florasil cartridges (5 g, 20 mL, Agilent Bond Elut) were purchased from Agilent Technologies (Santa Clara, CA, USA).

Sample extraction

One hundred microliter plasma, 50 mg liver, and $100\text{ }\mu\text{L}$ stomach oil were spiked with 5 ng of the IS, and 1 mL of hexane-to-dichloromethane (1:1,v/v) was added. The samples were then vortexed for 1 min and sonicated for 10 min in an ultrasonic bath for three consecutive times, followed by centrifugation (3500 rpm; 10 min). The supernatant was cleaned up by passing it through 5-g Florasil cartridges. Conditioning of the cartridges and elution was performed using 10 mL and 24 mL of hexane-to-dichloromethane (1:1),

respectively. The extracts were evaporated under a gentle stream of nitrogen to nearly 1 mL using a TurboVap®, transferred to amber chromatographic vials, and further evaporated until near dryness using $400\text{ }\mu\text{L}$ of isooctane as keeper. Samples were stored at $-21\text{ }^{\circ}\text{C}$ until analysis. This extraction condition was compared to three other methods and considered adequate for seabird blood (Oró-Nolla et al. 2024).

GC–MS–Orbitrap analysis and data processing

The analysis was performed using a TRACE 130 GC coupled to a Hybrid Quadrupole–Orbitrap™ Mass Spectrometer (GC–Orbitrap–MS) with an HCD (higher energy collision-induced dissociation) and a TriPlus™ RSH Autosampler with a hot split/splitless injector with a single taper liner ($78.5\text{ mm}\times 4\text{ mm ID}$) from Thermo Fisher Scientific (Waltham, MA, USA) and operated with an electron ionization (EI) source at 70 eV. A volume of $2\text{ }\mu\text{L}$ was injected using a splitless time of 1.5 min at $300\text{ }^{\circ}\text{C}$. Helium (99.999%) was used as carrier gas at a constant flow rate of $1.0\text{ mL}/\text{min}$. A Phenomenex ZEBRON B-5MS (Torrance, CA, USA) fused silica column of 60 m length \times 0.25 mm inner diameter \times $0.25\text{ }\mu\text{m}$ film thickness was employed. The oven program was set to $60\text{ }^{\circ}\text{C}$, held for 3 min, and then increased to $120\text{ }^{\circ}\text{C}$ at $30\text{ }^{\circ}\text{C}/\text{min}$ and to $320\text{ }^{\circ}\text{C}$ at $6\text{ }^{\circ}\text{C}/\text{min}$ (held for 35 min). The system was calibrated using a perfluorotributylamine calibration solution (FC 43, CAS 311–89-7) to achieve an accuracy of $<0.5\text{ ppm}$. During the measurement, internal mass calibration was performed using five background ions from the column bleed as lock mass ($\text{C}_3\text{H}_9\text{Si}^+$, m/z 73.04680; $\text{C}_3\text{H}_9\text{O}_2\text{Si}_2^+$, m/z 133.01356; $\text{C}_5\text{H}_{15}\text{O}_3\text{Si}_3^+$, m/z 207.03235; $\text{C}_7\text{H}_{21}\text{O}_4\text{Si}_4^+$, m/z 281.05114; $\text{C}_9\text{H}_{27}\text{O}_5\text{Si}_5^+$, m/z 355.06993) with a mass extraction window of $\pm 5\text{ ppm}$. Full scan acquisition was employed at a mass range (m/z) of 70–1000 with a resolving power of 60,000 Full Width at Half Maximum (FWHM), measured at 200 m/z .

The GC–MS data was acquired using Xcalibur 4.4 (Thermo Fischer Scientific), and quantification processing was performed with Trace Finder 5.1 EFS (Thermo Fischer Scientific). Identification was carried out at the specific retention time of each compound and by searching for the exact mass of molecular ions plus specific fragment ions (Table S2) in the chromatogram. The congener identity of PCBs with the same chlorine number was determined by elution order with respect to the IS PCB of the same chlorination level. Quantification was performed using IS calibration and the IS used for each analyte is specified in Table 1. A response factor for each point of the calibration row was calculated. In some quality controls (which are marked with asterisks in Table 2), the signal was re-quantified with external calibration, meaning that we used the uncorrected signal

Table 1 Quality parameters for 68 target pollutants. Retention time (in minutes), IS used for correction, main quantification ion (theoretical), mass error of the quantification ion, mean response factor of the calibration curve \pm relative standard deviation, R^2 , linearity range within the calibration curve (ng/mL), repeatability and reproducibility (both at 5 absolute ng; $n=4$) and instrumental detection limits (IDL) given in pg injected

Compound	RT (min)	IS	Qlon (m/z)	Qion error (ppm)	Response factor \pm RSD%	R^2	Linearity range (ng/mL)	Repeatability (RSD%)	Reproducibility (RSD%)	IDL (pg injected)
Naphthalene	10.54	Naphtalene D8	128.0620	-0.70	0.94 \pm 16.8	0.9985	1-300	0.36	1.30	0.02
Acenaphthene	15.85	Acenaphthene D10	153.0697	-1.24	0.80 \pm 10.2	0.9974	1-120	1.35	10.2	0.02
Acenaphthylene	15.96	Acenaphthene D10	152.0620	-0.46	0.59 \pm 20.9	0.9969	1-300	1.89	7.00	0.00
Fluorene	17.75	Acenaphthene D10	165.0698	-0.61	0.71 \pm 15.1	0.9982	1-120	1.50	3.67	0.04
α -HCH	19.72	Pentachlorobenzene ¹³ C ₆	180.9373	-0.17	0.50 \pm 17.7	0.9993	1-300	1.89	2.74	0.04
Hexachlorobenzene	19.78	Pentachlorobenzene ¹³ C ₆	283.8096	-0.11	0.53 \pm 14.9	0.9975	1-200	0.84	1.29	0.03
β -HCH	20.90	Pentachlorobenzene ¹³ C ₆	180.9373	0.06	0.44 \pm 17.9	0.9937	2-300	11.8	22.2	0.07
γ -HCH	21.02	Pentachlorobenzene ¹³ C ₆	180.9373	<0.01	0.46 \pm 63.0	0.9983	2-300	11.7	15.3	0.03
Phenanthrene	21.43	Phenanthrene D10	178.0777	0.11	0.88 \pm 8.3	0.9982	1-120	1.34	1.16	0.01
PCB 24	21.46	PCB 73	255.9607	-0.47	1.54 \pm 10.6	0.9990	2-120	0.66	2.58	0.01
Anthracene	21.63	Phenanthrene D10	178.0777	0.11	0.83 \pm 7.4	0.9985	1-120	1.20	9.43	0.01
PCB 16	21.73	PCB 73	255.9607	-0.31	0.44 \pm 12.1	0.9980	2-120	2.78	5.02	0.02
δ -HCH	22.10	Pentachlorobenzene ¹³ C ₆	180.9373	0.07	0.37 \pm 47.9	0.9986	2-300	1.65	4.20	0.01
Heptachlor	23.21	PCB 73	271.8098	-0.11	0.18 \pm 7.1	0.9991	2-120	2.11	8.99	0.05
PCB 52	23.86	PCB 73	291.9190	0.17	1.04 \pm 13.8	0.9907	1-120	1.82	2.55	0.02
PCB 49	23.99	PCB 73	291.9190	0.07	0.97 \pm 13.2	0.9985	1-120	1.55	2.55	0.03
PCB 38	24.12	PCB 73	255.9613	1.76	1.44 \pm 10.1	0.9968	1-120	0.63	2.46	0.01
PCB 62	24.13	PCB 73	291.9190	0.17	1.09 \pm 14.8	0.9980	1-120	2.30	7.58	0.02
PCB 65	24.18	PCB 73	291.9190	0.07	1.16 \pm 17.0	0.9937	1-120	2.26	5.38	0.02
Aldrin	24.41	PCB 73	262.8560	-2.05	0.28 \pm 11.2	0.9987	1-120	1.41	5.89	0.01
Isodrin	25.40	PCB 73	192.9373	-0.41	0.24 \pm 10.4	0.9970	1-120	2.93	10.0	0.02
PCB 61	25.66	PCB 73	291.9190	0.17	1.30 \pm 12.0	0.9968	1-200	1.81	1.46	0.02
Heptachloropoxide	25.71	PCB 73	352.8436	-0.34	0.15 \pm 6.7	0.9971	2-120	1.55	1.25	0.01
Oxychloridane	25.84	PCB 73	252.8955	-0.20	0.09 \pm 5.9	0.9986	2-120	1.81	3.41	0.03
Fluoranthene	26.14	Phenanthrene D10	202.0777	0.10	0.98 \pm 9.2	0.9976	1-120	1.56	5.77	0.06
2,4'-DDE	26.46	4,4'-DDE-D8	245.9998	-0.12	7.99 \pm 11.8	0.9951	1-120	2.60	2.55	0.02
trans-Chlordane	26.49	4,4'-DDE-D8	372.8254	0.05	0.69 \pm 13.4	0.9989	2-300	3.56	5.52	0.01
α -Endosulfan	26.49	4,4'-DDE-D8	236.8409	0.38	0.35 \pm 19.7	0.9979	2-300	2.70	4.54	<0.005
cis-Chlordane	26.49	4,4'-DDE-D8	372.8254	-0.15	0.60 \pm 35.0	0.9983	1-300	3.73	17.7	<0.005
PCB 101	26.64	PCB 97	325.8798	-0.15	1.57 \pm 13.2	0.9986	1-120	2.18	1.18	0.01
PCB 99	26.79	PCB 97	325.8798	-0.11	1.64 \pm 15.8	0.9951	1-200	2.85	3.86	0.01
Pyrene	27.02	Phenanthrene D10	202.0776	-0.49	1.00 \pm 12.7	0.9967	1-120	1.03	6.46	0.01
PCB 116	27.42	PCB 97	325.8804	1.69	1.76 \pm 16.0	0.9969	1-200	1.43	2.57	<0.005

Table 1 (continued)

Compound	RT (min)	IS	QIon (m/z)	Qion error (ppm)	Response factor ± RSD%	R ²	Linearity range (ng/mL)	Repeatability (RSD%)	Reproducibility (RSD%)	IDL (pg injected)
4,4'-DDE	27.52	4,4'-DDE-D8	245.9998	-0.20	5.16 ± 14.2	0.9982	1-200	2.80	2.35	0.02
PCB 85	27.59	PCB 97	325.8804	1.60	1.02 ± 15.3	0.9975	1-120	2.68	3.20	0.02
PCB 110	27.75	PCB 97	325.8804	1.78	2.12 ± 13.1	0.9933	1-120	1.53	3.51	0.01
Dieldrin	27.77	PCB 151	262.8564	-0.53	0.24 ± 11.7	0.9983	2-120	3.16	5.58	0.03
2,4'-DDD	27.79	4,4'-DDE-D8	235.0076	-0.17	7.59 ± 16.2	0.9989	1-300	2.49	3.59	0.01
PCB 77	27.91	PCB 151	291.9190	-0.14	3.36 ± 11.5	0.9988	1-200	2.29	3.07	0.02
PCB 149	28.45	PCB 151	359.8409	-0.19	1.60 ± 11.6	0.9971	1-120	1.90	1.01	0.01
Endrin	28.46	PCB 151	262.8566	0.34	0.23 ± 18.4	0.9986	3-300	7.26	33.9	<0.005
β-Endosulfan	28.85	4,4'-DDE-D8	236.8409	0.38	0.17 ± 18.7	0.9995	3-300	8.93	12.2	<0.005
2,4'-DDT	28.94	4,4'-DDE-D8	235.0076	-0.13	5.90 ± 10.6	0.9975	2-300	3.14	19.4	0.02
PCB 146	29.06	PCB 126	289.9032	1.11	0.77 ± 19.9	0.9964	1-200	2.61	8.44	0.01
4,4'-DDD	29.15	4,4'-DDE-D8	235.0077	0.30	2.96 ± 10.8	0.9998	1-300	3.14	19.4	0.02
PCB 153	29.26	PCB 126	359.8410	0.08	0.81 ± 10.3	0.9935	2-200	2.86	9.34	0.01
PCB 118	29.42	PCB 126	325.8804	1.60	1.67 ± 13.1	0.9956	1-120	1.72	5.14	0.01
4,4'-DDT	30.05	4,4'-DDE-D8	235.0077	0.04	0.89 ± 6.6	0.9962	2-120	2.18	33.9	0.01
PCB 138	30.05	PCB 126	359.8410	-0.17	0.84 ± 13.2	0.9959	1-120	1.95	3.06	0.01
PCB 187	30.50	PCB 126	393.8024	1.32	0.54 ± 15.1	0.9927	2-300	3.50	3.04	<0.005
PCB 183	30.67	PCB 126	393.8024	1.17	0.60 ± 14.1	0.9960	2-200	1.41	0.79	0.02
PCB 128	30.90	PCB 126	359.8410	-0.08	0.78 ± 13.1	0.9978	2-120	3.47	3.31	0.01
PCB 167	31.00	PCB 126	359.8410	<0.01	1.02 ± 16.6	0.9969	2-200	1.51	2.12	0.01
PCB 156	31.67	PCB 157	359.8410	<0.01	1.33 ± 14.0	0.9975	2-120	0.83	2.03	0.01
Methoxychlor	31.70	PCB 157	227.1068	0.45	0.53 ± 10.3	0.9964	2-120	3.98	24.4	<0.005
1,2-Benzanthracene	31.83	Chrysene D12	228.0934	-0.18	0.66 ± 18.3	0.9976	1-300	2.33	9.09	0.01
Chrysene	31.96	Chrysene D12	228.0935	0.13	0.62 ± 18.0	0.9982	1-300	2.30	15.5	0.01
PCB 180	32.10	PCB 157	393.8024	1.32	0.57 ± 17.6	0.9972	2-120	2.58	1.00	0.01
PCB 170	32.94	PCB 200	393.8024	1.17	0.83 ± 7.7	0.9978	2-120	2.02	2.61	0.01
Mirex	33.39	PCB 200	271.8098	-0.52	0.72 ± 8.0	0.9998	3-120	1.68	3.32	0.01
PCB 189	33.86	PCB 200	393.8024	1.24	0.95 ± 12.7	0.9938	3-120	0.91	8.46	0.03
PCB 194	34.80	PCB 200	429.7605	2.07	0.46 ± 17.4	0.9931	3-120	1.61	8.18	0.04
Benzo[b]fluoranthene	35.86	PCB 200	252.0935	0.04	4.38 ± 11.5	0.9961	1-120	2.93	10.5	0.01
Benzo[k]fluoranthene	35.95	PCB 200	252.0935	0.16	5.54 ± 8.47	0.9995	1-300	4.37	17.7	0.01
Benzo[a]pyrene	35.95	PCB 200	252.0935	-0.04	4.10 ± 4.4	0.9989	2-300	3.91	11.4	<0.005
Indeno[1,2,3-cd]pyrene	40.68	PCB 200	276.0936	-0.43	1.44 ± 22.3	0.9972	3-300	2.84	33.6	0.02
Dibenz[a,h]anthracene	40.77	PCB 200	278.1093	0.83	1.17 ± 31.4	0.9976	3-300	7.46	40.2	0.01
Benzo[g,h,i]perylene	41.63	PCB 200	276.0936	0.69	1.57 ± 7.5	0.9973	3-300	3.78	41.8	0.02

of the sample multiplied by the absolute ng of the spike level (5 ng) divided by the signal of the calibration curve because the signal was very intense. The accuracy of the single mass measurement was described as the mass measurement error (Δm_i , in ppm). This was calculated as the difference between the theoretical mass and the experimental mass divided by the theoretical mass and multiplied by 10^6 as described by Oró et al. (2023).

Quality assurance and quality controls

To determine the recovery of our method, we used spiked samples of human plasma and food-grade chicken liver. As we did not have a comparable matrix, which would be expected to contain low levels of organochlorine contaminants for stomach oil, we used a pooled sample of shearwater stomach oil. Four replicates of each matrix (plasma, liver, stomach oil) were spiked with 5 ng of the analytical standard mixture (Table S1) and 5 ng of the IS and extracted as indicated before. Potential background contamination from laboratory materials or solvents was assessed by analysing procedural blanks. To determine the initial contamination load of the spiked samples, we measured one matrix blank per sample type. We monitored potential sample carryovers or signal fluctuations and drift by performing repeated injections of solvent blanks and standard solutions. Repeatability was calculated as the relative standard deviation of five consecutive replicates of a standard at a concentration of 20 ng/mL was calculated. Instrumental reproducibility was determined by calculating the relative standard deviation of four replicates measured along the sequence. The calibration curve consisted of ten points, with vial concentrations ranging from 1 to 300 ng/mL. The limits of detection and quantification of the instrument (IDL, IQL) were determined as three and ten times the concentration of the lowest calibration point divided by the signal-to-noise ratio of the instrument. The method limits of detection (MDL) for each matrix were determined as three times the measured concentration of a spiked sample divided by the signal-to-noise ratio of the instrument. Matrix effects (MEs) were calculated for the three matrices to evaluate the method performance. MEs greater than 20% are considered weak, between 20 and 50% medium and those greater than or equal to 50% have strong effects (Li et al. 2016). The performance of the analytical procedure was evaluated by calculating the method uncertainty (U) for the three matrices as reported in Oró-Nolla et al. (2023). U is expressed as a percentage, and values below 50% indicate satisfactory robustness and reliability of the method. The mean recovery percentages of the spiked samples and MEs as well as MDLs in ng/g (w.w.) are presented based on the four repetitions for each matrix. The results are reported in ng/g wet weight (w.w.) as plasma and stomach oil were extracted as liquids and liver a freeze-dried

powder. The mean moisture content of livers was determined as $32.5 \pm 1.3\%$ by weighing the sample before and after the freeze-drying process and later converted to wet weight using this factor.

Results and discussion

Quality parameters and recoveries

Table 1 shows the instrumental and methodological quality parameters. Mass errors fluctuated negatively and positively around the mean of 0.18 ± 0.75 ppm with only aldrin and PCB 194 exhibiting a higher mass error of $-2.05/2.07$ ppm, respectively. The linearity of the ten-point calibration curve, which covered concentrations between 1 and 300 ng/mL, was not always obtained in the higher calibration points. To achieve R^2 values greater than 0.99 and a low relative standard deviation of the mean response factor for all analytes, we excluded some of the higher calibration concentrations. As a result, the linearity range varies from compound to compound but still covers two orders of magnitude, as presented in Table 1. The relative standard deviation of the mean response factor was well below 20% (mean $13.1 \pm 3.99\%$) for all compounds except for two HCH derivatives (δ -, γ -HCH), cis-chlordane, and dibenz[a,h]anthracene. The repeatability of the method was well below 10% (mean $2.44 \pm 1.49\%$) for all compounds except for β -HCH and γ -HCH, which was 12%. Reproducibility for most compounds was well below 20% (mean $6.23 \pm 5.21\%$). Exceptions were observed for 3 PAHs (indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene, benzo[g,h,i]perylene), OCPs (endrin, 4,4'-DDT, methoxychlor), which showed some variations along the sequence. IDL are reported in absolute pg injected (injection volume 2 μ L) and ranged between <0.005 and 0.07.

All 68 targeted compounds were recovered in the spiked plasma, while acenaphthylene was not detectable in stomach oil, and heptachlor was not detectable in the liver. Sixty-five out of 68 targeted compounds in human plasma and 60 in chicken liver were within the recovery range of 80–120%. In pooled shearwater stomach oil, 56 compounds were in the range of 60–120%. The extraction RSD was $>20\%$ in two compounds in plasma (δ -, and γ -HCH) and liver (γ -HCH and endrin) and in ten compounds in oil (β -HCH, γ -HCH, aldrin, β -endosulfan, endrin, 4,4'-DDT, methoxychlor, PCB 180, PCB 194 and benzo[g,h,i]perylene). We found signal intensification in plasma for seven OCPs (β -HCH, δ -HCH, γ -HCH, 4,4'-DDT, methoxychlor, 2,4'-DDD, β -endosulfan), in the liver for three OCPs (4,4'-DDT, methoxychlor, 2,4'-DDD) and stomach oil only for the two DDT metabolites (4,4'-DDT, 2,4'-DDD). In these cases, we re-calculated the extraction efficiency by external calibration to obtain more realistic recoveries. With this re-quantification, only

Table 2 Extraction efficiency assessed on the basis of recoveries in % of the spiked quality controls (5 absolute ng; $n=4$), method detection limits (MDL) in ng/g w.w. and expanded uncertainty (U) for plasma, liver and stomach oil, respectively

Compound	Plasma			Liver			Stomach oil		
	Recovery \pm RSD (%)	MDL (ng/g w.w.)	U (%)	Recovery \pm RSD (%)	MDL (ng/g w.w.)	U (%)	Recovery \pm SD (%)	MDL (ng/g w.w.)	U (%)
Naphthalene	83 \pm 5.9	0.43	29	105 \pm 8.1	1.60	19	90 \pm 10	0.95	33
Acenaphthene	91 \pm 5.9	0.79	23	79 \pm 13	0.32	17	63 \pm 11	0.15	78
Acenaphthylene	81 \pm 6.4	1.16	42	87 \pm 12	4.67	21	n.d	0.63	133
Fluorene	99 \pm 9.4	0.84	25	102 \pm 7.6	2.18	16	97 \pm 8.3	1.66	22
α -HCH	95 \pm 14	1.30	39	90 \pm 8.9	5.02	40	83 \pm 9.9	3.38	42
Hexachlorobenzene	97 \pm 13	1.51	35	84 \pm 6.0	3.05	11	93 \pm 6.5	2.35	36
β -HCH*	74 \pm 15	0.94	70	36 \pm 13	4.32	27	25 \pm 23	0.33	158
γ -HCH*	94 \pm 26	0.94	69	55 \pm 23	3.53	35	58 \pm 25	3.46	78
Phenanthrene	94 \pm 2.9	0.32	15	96 \pm 6.2	0.95	12	88 \pm 13	0.54	40
PCB 24	87 \pm 13	0.32	42	85 \pm 11	1.02	15	62 \pm 18	0.59	81
Anthracene	99 \pm 4.3	0.39	8	95 \pm 8.2	1.28	13	83 \pm 14	0.75	44
PCB 16	105 \pm 3.0	1.13	9	92 \pm 13	3.57	15	95 \pm 6.1	3.26	12
Heptachlor	98 \pm 9.3	0.49	25	n.d	0.16	32	96 \pm 5.4	1.10	16
δ -HCH*	135 \pm 32	1.52	264	45 \pm 18	4.36	37	43 \pm 4.0	2.64	121
PCB 52	92 \pm 10	1.03	31	80 \pm 14	3.47	10	86 \pm 6.0	3.14	33
PCB 49	94 \pm 12	1.12	34	87 \pm 13	3.47	11	92 \pm 13	2.48	29
PCB 38	98 \pm 10	0.35	28	94 \pm 16	1.16	11	97 \pm 7.5	1.16	20
PCB 62	95 \pm 9.9	0.91	27	83 \pm 15	2.64	11	90 \pm 11	2.06	35
PCB 65	92 \pm 8.3	0.90	26	86 \pm 15	2.54	14	93 \pm 4.5	2.09	18
Aldrin	86 \pm 13	0.26	87	100 \pm 8.2	1.07	8	21 \pm 62	0.24	230
Isodrin	93 \pm 11	0.41	31	83 \pm 16	1.40	12	82 \pm 10	0.85	44
PCB 61	82 \pm 16	0.84	53	89 \pm 18	2.66	11	93 \pm 12	2.50	33
Heptachlorepoxyde	98 \pm 9.0	0.49	16	83 \pm 8.0	1.23	9	64 \pm 14	0.64	65
Oxychlorane	84 \pm 9.9	0.45	40	88 \pm 15	1.30	20	63 \pm 13	0.55	80
Fluoranthene	96 \pm 4.5	0.22	14	66 \pm 7.4	0.46	13	84 \pm 12	0.46	31
2,4'-DDE	95 \pm 10	0.45	29	84 \pm 5.3	1.28	15	79 \pm 2.3	0.83	42
trans-Chlordane	94 \pm 5.4	0.49	18	75 \pm 11	1.09	19	98 \pm 13	1.04	35
α -Endosulfan	100 \pm 10	0.53	27	92 \pm 14	1.28	30	87 \pm 10	0.96	36
PCB 101	94 \pm 13	0.61	26	73 \pm 9.3	2.57	17	89 \pm 13	1.78	40
PCB 99	100 \pm 6.7	0.68	18	94 \pm 14	3.13	26	85 \pm 14	1.93	46
cis-Chlordane	88 \pm 15	0.46	46	91 \pm 19	1.05	24	90 \pm 11	0.71	36
Pyrene	101 \pm 7.4	0.22	19	92 \pm 5.9	0.72	11	70 \pm 11	0.40	60
PCB 116	78 \pm 7.1	0.56	46	90 \pm 14	1.65	24	82 \pm 17	1.23	48
4,4'-DDE	91 \pm 10	0.41	32	100 \pm 3.0	1.49	12	69 \pm 17	0.58	73
PCB 85	90 \pm 12	1.41	37	96 \pm 8.2	3.39	17	99 \pm 8.9	2.94	24
PCB 110	94 \pm 9.8	0.63	28	88 \pm 14	1.96	15	95 \pm 12	1.59	32
Dieldrin	114 \pm 5.8	0.61	33	112 \pm 15	1.88	32	73 \pm 21	0.79	72
2,4'-DDD*	57 \pm 8.7	0.32	88	51 \pm 8.8	0.78	8	47 \pm 14	0.40	116
PCB 77	88 \pm 16	0.88	39	75 \pm 10	2.31	21	79 \pm 14	2.39	55
PCB 149	87 \pm 16	1.36	48	100 \pm 14	2.06	32	95 \pm 12	2.29	22
Endrin	85 \pm 14	0.17	46	61 \pm 27	0.27	57	53 \pm 62	0.13	117
β -Endosulfan*	82 \pm 22	0.50	72	43 \pm 6.0	0.73	15	9 \pm 24	0.07	193
2,4'-DDT	94 \pm 5.5	0.44	18	100 \pm 7.5	1.59	14	88 \pm 3.3	0.93	26
PCB 146	76 \pm 16	1.41	61	87 \pm 11	2.53	19	100 \pm 13	3.21	35
4,4'-DDD	94 \pm 12	0.38	34	102 \pm 14	1.61	29	97 \pm 8.5	1.02	23
PCB 153	92 \pm 17	4.10	47	104 \pm 7.6	2.56	15	53 \pm 10	0.77	98

Table 2 (continued)

Compound	Plasma			Liver			Stomach oil		
	Recovery \pm RSD (%)	MDL (ng/g w.w.)	U (%)	Recovery \pm RSD (%)	MDL (ng/g w.w.)	U (%)	Recovery \pm SD (%)	MDL (ng/g w.w.)	U (%)
PCB 118	95 \pm 11	0.55	31	99 \pm 8.8	1.78	19	94 \pm 9.8	2.23	28
4,4'-DDT*	64 \pm 14	0.26	107	112 \pm 17	1.91	35	25 \pm 30	0.29	158
PCB 138	82 \pm 12	1.08	45	83 \pm 9.9	1.95	19	69 \pm 3.6	2.03	63
PCB 187	84 \pm 18	1.44	54	111 \pm 9.2	6.44	15	98 \pm 7.1	3.37	19
PCB 183	87 \pm 16	1.56	46	93 \pm 18	6.94	13	83 \pm 14	3.90	48
PCB 128	75 \pm 13	1.32	58	75 \pm 7.9	2.22	14	84 \pm 6.1	3.14	36
PCB 167	82 \pm 16	1.08	45	101 \pm 4.9	2.38	16	103 \pm 5.7	3.52	31
PCB 156	86 \pm 12	1.11	41	112 \pm 6.8	2.31	14	98 \pm 9.3	3.91	25
Methoxychlor*	74 \pm 19	0.42	69	110 \pm 10	1.92	23	19 \pm 85	0.05	243
1,2-Benzanthracene	99 \pm 8.7	0.26	17	93 \pm 6.4	1.05	11	63 \pm 7.0	0.77	68
Chrysene	102 \pm 8.5	0.29	21	86 \pm 9.2	1.23	13	88 \pm 6.1	1.03	27
PCB 180	99 \pm 16	1.96	42	107 \pm 16	7.26	15	84 \pm 31	3.35	59
PCB 170	93 \pm 13	1.72	36	80 \pm 14	5.71	18	82 \pm 18	3.64	23
Mirex	87 \pm 17	0.35	50	88 \pm 14	1.74	29	87 \pm 14	1.14	44
PCB 189	86 \pm 16	1.96	48	94 \pm 13	9.38	25	67 \pm 22	4.79	83
PCB 194	76 \pm 17	1.82	64	93 \pm 15	4.41	15	42 \pm 61	3.16	191
Benzo[b]fluoranthene	97 \pm 5.2	0.24	15	84 \pm 12	0.97	30	90 \pm 10	2.91	33
Benzo[k]fluoranthene	101 \pm 3.7	0.24	10	82 \pm 12	0.92	27	83 \pm 14	2.89	49
Benz[a]pyrene	97 \pm 7.9	0.29	22	85 \pm 5.9	0.99	13	77 \pm 18	1.07	63
Indeno[1,2,3-cd]pyrene	89 \pm 14	0.43	35	81 \pm 6.7	4.41	16	83 \pm 11	3.04	43
Dibenz[a,h]anthracene	100 \pm 11	0.98	30	86 \pm 18	3.41	29	68 \pm 22	3.10	98
Benzo[g,h,i]perylene	92 \pm 10	0.51	31	104 \pm 7.8	5.47	18	65 \pm 34	2.11	104

*Quantified with external calibration using the signal of the calibration curve at 20 ng/mL, due to signal intensification

δ -HCH in plasma recovered over 120%. We attribute these variations to matrix effects (Table S3), as we already subtracted potential contamination of the matrix or procedural blanks. Table S4 in the supporting info compares the recoveries with internal and external standard quantification. δ -HCH in plasma showed the strongest intensification, a high deviation of the repetition (32%) and high U value (264%), and a strongly positive matrix effect value (127%). A compound may have interfered with the m/z of δ -HCH. Generally, matrix effects were strongly positive in β -HCH, δ -HCH, and methoxychlor in human plasma, in 4,4'-DDT, methoxychlor in chicken liver, and consistently negative in shearwater stomach oil (Table S3). With the re-quantification by external calibration of stomach oil quality controls, 2,4'-DDD showed realistic recovery and RSD of 47 \pm 14%, but 4,4'-DDT had a very low recovery of 25 \pm 30%. Some other OCPs (endrin, aldrin, β -endosulfan, methoxychlor) and higher chlorinated PCBs were not recovered well (PCB 194) or had high RSD (PCB 180) in stomach oil and dibenz[a,h]anthracene had slightly higher and not fully satisfactory RSD, U, ME values. MDL were mostly below 1 ng/g or in

the low ng/g range (Table 2) which compared to studies that used whole blood is a little higher (Dulsat-Masvidal et al. 2023; Campioni et al. 2024). MDL varies according to the matrix and extraction method used, for example, MDL for PAHs was higher in the raptor liver than in blood (Morin-Crini et al. 2022). MDL for POPs in seabird eggs was 1–1.12 ng/g (Elliott et al. 2023), and PAHs in loon plasma was 5 ng/g a similar range to ours (Paruk et al. 2016).

The complexity of the studied matrices favours deviations in the desired recovery and increased RSD for certain OCPs and PAHs. In biological matrices, lipids and other constituents such as salts, hormones, or proteins may generate interferences leading to signal enhancement or suppression which varies unpredictably with matrix type (Kim et al. 2016). Moradi et al. (2023) report differences in recovery and RSD between plasma and serum for acenaphthylene, anthracene, fluorene, and phenanthrene. Morin-Cringi et al. (2022) report higher RSD for phenanthrene, benzo(k)fluoranthene, and dibenz[a,h]anthracene in blood and fluoranthene, pyrene, and benzo[g,h,i]perylene in the liver. Good recoveries were achieved for PAHs in avian blood cells and plasma

(60.0 to 92.4% for BCs and from 52.5 to 109% for plasma) using QuEChERS extraction followed by phospholipid solid-phase extraction clean-up and HPLC analysis although naphthalene, acenaphthylene, and fluoranthene were not available in the matrix spike samples of plasma (Provatas et al. 2015). The same study reported higher MDL in plasma than blood cells (4.14–12.4 ng/g for BCs and from 7.70 to 41.6 ng/g for plasma). Especially in the case of stomach oil, impurities hamper satisfactory recoveries as shown in our results. Zhao et al. (2013) analysed soybean oil applying a similar extraction/clean-up method (LLE with acetonitrile/acetone 60/40 v/v and 20 mL SPE cartridges) and HPLC analysis and reported similar recoveries (60–77%) for chrysene, benzo-(b)fluoranthene, benzo(a)perylene, benzo(k)fluoranthene, dibenz[a,h]anthracene, benzo[g,h,i]perylene, and indeno[1,2,3-cd]pyrene. A previous study on vegetable oils (Wu 2012) achieved good recoveries and repeatability when washing their samples twice with 50 mL of heated 4% saline solution. Zhang et al. (2017) achieved good recoveries for PAHs in vegetable oil by using magnetic dispersive solid-phase extraction, which also helped to minimize lipid impurities. Referring to the matrix effect levels mentioned by Li et al. (2016), most compounds in this study showed strong matrix effects above $\pm 50\%$ in all matrices, which is expected for biological matrices (Oró-Nolla et al. 2023). Matrix-matched calibration may help to reduce the overestimation of the signal (Kim et al. 2016). However, the use of IS for correction would normally compensate for the matrix effects as we achieved satisfactory recoveries in most target compounds.

For acenaphthene (0.50–1.1 ng/mL), hexachlorobenzene (< 0.01 –0.50 ng/mL), phenanthrene (1.10–2.00 ng/mL), fluoranthene (< 0.01 –0.40 ng/mL), pyrene (0–1.00 ng/mL), and methoxychlor (0.30–4.30 ng/mL), low background contamination was detected in the procedural blank vials, and mean peak areas of the blanks were subtracted from the areas of the sample before quantification. Background levels of some POPs were detected in the matrix blanks of stomach oil, as pooled environmental samples were used (Table S5). In these cases, the contribution of detected compounds was subtracted before the recovery determination. Among matrices, the lowest uncertainty and matrix effects were observed in the liver, followed by plasma, and the highest in stomach oil (Table 2; Table S3). As the liver was the only matrix processed as a dry powder, it can be assumed that the water content of the sample may influence solvent accessibility, and extraction efficiency is improved. According to the analytical quality control and method validation procedures for food and feed proposed by the European Union (Pihlström et al. 2021), the U value should be below 50% to maintain interlaboratory standards. In this study, we obtained U below 50% for 54 (plasma), 67 (liver), and 41 (stomach oil) of our target compounds. The threshold of

50% has been established for food and feed not necessarily for complex biological matrices like the ones used in this study. Therefore, we believe that the reported values for U are acceptable.

Pollutants detected in shearwaters

In shearwaters, we detected concentrations above MDL for 28 out of our 68 targeted compounds. These consisted of four PAHs, seven OCPs, and 17 PCBs which are listed in Table 3. Among the three seabird matrices in this study, liver samples exhibited the highest pollutant concentrations, followed by stomach oil and plasma. The dominant compound in all three matrices was 4,4'-DDE (Table 3), detected in all liver and stomach oil samples and only in six out of 21 of the plasma samples. Generally, the concentrations deviated greatly from sample to sample. In the liver, there was one sample with higher concentrations compared to the others, resulting in high standard deviations (Table 3). This sample could be suitable for further retrospective non-targeted analyses. The composition and detection rate of POPs and PAHs varied among the matrices (Fig. 1), while in plasma, PCBs were mainly below the detection limit PAHs phenanthrene, fluoranthene, and pyrene were prevalent, and methoxychlor was detected in most of the samples. Plasma concentrations of 4,4'-DDE were lower but comparable to previously measured blood concentrations in this colony (Costantini et al. 2017). Interestingly, previous studies found higher concentrations of \sum PCBs than \sum DDT metabolites in the blood and liver of Linosan shearwaters (Renzoni et al. 1986; Costantini et al. 2017). Also in Bermuda petrels (Campioni et al. 2024) and giant petrels (Roscales et al. 2016), the blood concentrations of single indicator PCBs were similar or slightly higher than 4,4'-DDE. Even though the quality parameters for 4,4'-DDE are good, it cannot be excluded that some matrix effects that we faced in the quality controls of other DDT metabolites are causing the high concentrations. However, the concentration of 4,4'-DDE was high relatively in all three matrices. Commonly, 4,4'-DDE is the most prevalent of the DDT metabolites in wild birds, and this was the case in previous studies in the blood of other Mediterranean shearwaters (Roscales et al. 2010; Costantini et al. 2017), and temporal shifts of pollutant profiles are possible as Arctic eiders showed increasing trends of 4,4'-DDE blood concentrations while PCBs remained stable over a time period between 2007 and 2017 (Bustnes et al. 2024). The liver had a high prevalence and concentrations of PCB congeners (#153, #138, #187, #183, #128, #170, and #180), and the OCP mirex was detected in five out of the ten samples. Generally, the liver samples showed a dominance of higher chlorinated biphenyls, such as hexa-, hepta-, and octachlorinated

Table 3 Mean \pm standard deviation (SD), lower (LCL₉₅) and upper (UCL₉₅) 95% confidence intervals, minimum (min) and maximum (max) concentrations and percent of censoring (Cen) of detected compounds in native samples of plasma ($n=21$), liver ($n=10$) and stomach oil ($n=6$) of Scopoli's Shearwaters (*Calonectris diomedea*), expressed in ng/g w.w. Estimates of the shape and scale parameters (mean, SD and 95 confidence interval of the mean) for censored observations were calculated using the non-parametric Kaplan–Meier method from the R package EnvStats

	Plasma										Liver										Stomach Oil									
	Mean (ng/g w.w.) \pm SD	LCL ₉₅	UCL ₉₅	Min	Max	Cen	Mean (ng/g w.w.) \pm SD	LCL ₉₅	UCL ₉₅	Min	Max	Cen	Mean (ng/g w.w.) \pm SD	LCL ₉₅	UCL ₉₅	Min	Max	Cen	Mean (ng/g w.w.) \pm SD	LCL ₉₅	UCL ₉₅	Min	Max	Cen						
	<MDL	NA	NA	0.00	1.53	100	4.66	NA	NA	0.32	21.4	71	2.86 \pm 2.81	1.36	4.36	0.95	9.71	40	2.96	NA	NA	3.08	4.66	90	2.96	NA	NA	0.00	0.54	100
Hexachlorobenzene	2.23 \pm 4.72	0.70	3.80	0.32	21.4	71	2.86 \pm 2.81	1.36	4.36	0.95	9.71	40	<MDL	NA	NA	1.28	1.77	90	<MDL	NA	NA	1.28	1.77	90	<MDL	NA	NA	0.00	0.75	100
Phenanthrene	0.40 \pm 0.03	NA	NA	0.00	0.84	100	3.08	NA	NA	0.00	3.08	90	<MDL	NA	NA	0.00	3.08	90	<MDL	NA	NA	0.00	3.08	90	<MDL	NA	NA	0.00	2.50	100
Anthracene	<MDL	NA	NA	0.22	0.76	81	2.53 \pm 1.14	1.89	3.3	1.56	5.44	0	<MDL	NA	NA	3.13	42.8	60	<MDL	NA	NA	3.13	42.8	60	<MDL	NA	NA	0.00	0.46	100
PCB 61	0.25 \pm 0.12	0.21	0.29	0.00	0.68	100	8.05 \pm 11.7	1.74	14.3	3.13	42.8	60	<MDL	NA	NA	1.04	4.94	0	<MDL	NA	NA	1.04	4.94	0	<MDL	NA	NA	0.00	1.93	100
Fluoranthene	<MDL	NA	NA	0.22	0.65	52	2.03 \pm 1.14	1.41	2.83	1.04	4.94	0	<MDL	NA	NA	115	7207	0	<MDL	138	397	123	507	0	<MDL	NA	NA	0.00	0.40	100
PCB 99	0.31 \pm 0.12	0.27	0.35	0.41	14.9	71	1090 \pm 2182	291	2513	3.39	8.34	90	<MDL	NA	NA	1.96	4.07	90	<MDL	NA	NA	1.96	4.07	90	<MDL	NA	NA	0.00	2.94	100
Pyrene	3.00 \pm 5.21	1.60	4.40	0.00	0.63	100	4.07	NA	NA	0.00	0.63	100	4.07	NA	NA	2.06	2.73	90	<MDL	NA	NA	2.06	2.73	90	<MDL	NA	NA	0.00	1.59	100
4,4'-DDE	<MDL	NA	NA	0.00	1.36	100	2.73	NA	NA	0.00	1.36	100	2.73	NA	NA	1.59	1.72	90	<MDL	NA	NA	1.59	1.72	90	<MDL	NA	NA	0.00	2.29	100
PCB 85	<MDL	NA	NA	0.00	0.44	100	1.72	NA	NA	0.00	0.44	100	1.72	NA	NA	2.53	66.7	40	<MDL	NA	NA	2.53	66.7	40	<MDL	NA	NA	0.93	1.22	83
PCB 110	<MDL	NA	NA	0.00	1.41	100	14.5 \pm 19.5	3.72	25.3	0.00	1.61	100	1.62 \pm 0.97	1.18	2.06	0.00	1.61	100	<MDL	1.18	2.06	0.00	1.61	100	<MDL	1.18	2.06	0.00	3.21	100
PCB 149	<MDL	NA	NA	0.00	0.38	100	<MDL	NA	NA	0.00	0.38	100	<MDL	NA	NA	12.7	355	0	<MDL	26.7	44.1	24.3	47.1	0	<MDL	NA	NA	0.00	2.23	100
2,4'-DDT	<MDL	NA	NA	0.00	4.10	100	86.6 \pm 108	33.6	176	1.78	16.4	90	<MDL	NA	NA	1.78	16.4	90	<MDL	26.7	44.1	24.3	47.1	0	<MDL	NA	NA	0.00	0.29	100
PCB 146	<MDL	NA	NA	0.26	3.68	90	<MDL	NA	NA	0.26	3.68	90	<MDL	NA	NA	1.91	1.91	100	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	2.23	100
PCB 146	<MDL	NA	NA	0.00	1.08	100	48.5 \pm 67.4	20.0	93.1	9.59	230	0	8.13 \pm 4.70	3.78	15.5	2.03	15.7	17	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	0.29	100
4,4'-DDD	<MDL	NA	NA	0.00	1.44	100	45.2 \pm 68.8	3.54	86.9	6.44	244	20	<MDL	NA	NA	6.44	244	20	<MDL	26.7	44.1	24.3	47.1	0	<MDL	NA	NA	0.00	3.37	100
PCB 153	<MDL	NA	NA	0.00	1.56	100	92.1 \pm 129	14.4	169	6.94	415	20	4.53 \pm 0.90	4.28	4.77	3.90	6.00	67	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.37	100
PCB 118	0.51 \pm 0.8	0.39	0.62	0.00	1.32	100	31.0 \pm 51.9	3.57	58.5	2.22	171	50	<MDL	NA	NA	2.22	171	50	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	6.00	67
4,4'-DDT*	<MDL	NA	NA	0.00	1.08	100	4.39 \pm 3.73	2.61	6.20	2.28	14.5	60	<MDL	NA	NA	2.28	14.5	60	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.14	100
PCB 138	<MDL	NA	NA	0.00	1.11	100	18.8 \pm 24.2	9.78	27.7	2.31	79.6	60	<MDL	NA	NA	2.31	79.6	60	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.52	100
PCB 187	<MDL	NA	NA	0.42	3.14	57	5.66	NA	NA	0.42	3.14	57	5.66	NA	NA	2.84	5.66	90	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.91	100
PCB 183	<MDL	NA	NA	0.00	1.12	100	0.88 \pm 0.77	0.63	1.12	0.42	3.14	57	5.66	NA	NA	2.84	5.66	90	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.91	100
PCB 128	<MDL	NA	NA	0.00	1.08	100	4.39 \pm 3.73	2.61	6.20	2.28	14.5	60	<MDL	NA	NA	2.28	14.5	60	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.52	100
PCB 156	<MDL	NA	NA	0.00	1.11	100	18.8 \pm 24.2	9.78	27.7	2.31	79.6	60	<MDL	NA	NA	2.31	79.6	60	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.91	100
Methoxychlor*	0.88 \pm 0.77	0.63	1.12	0.42	3.14	57	5.66	NA	NA	0.42	3.14	57	5.66	NA	NA	2.84	5.66	90	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	9.05	33
PCB 180	<MDL	NA	NA	0.00	1.96	100	142 \pm 146	59.6	283	21.0	447	0	<MDL	NA	NA	21.0	447	0	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.35	100
PCB 170	<MDL	NA	NA	0.00	1.72	100	34.0 \pm 39.6	11.5	56.5	5.71	129	30	<MDL	NA	NA	5.71	129	30	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.64	100
Mirex	2.28	NA	NA	0.35	2.38	95	38.3 \pm 46.4	19.1	57.6	1.74	119	50	<MDL	NA	NA	1.74	119	50	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	1.14	100
PCB 194	<MDL	NA	NA	0.00	1.82	100	43.4 \pm 56.4	16.8	69.9	4.41	175	50	<MDL	NA	NA	4.41	175	50	<MDL	1.18	2.06	0.00	1.61	100	<MDL	NA	NA	0.00	3.61	100

*4,4'-DDT and methoxychlor showed signal intensification in liver quality controls

Fig. 1 The percentage composition of all analysed pollutant classes in the environmental samples from Scopoli's shearwaters (*Calonectris diomedea*). Plasma ($n=21$), liver ($n=10$), stomach oil ($n=6$). Only concentrations above MDL are included

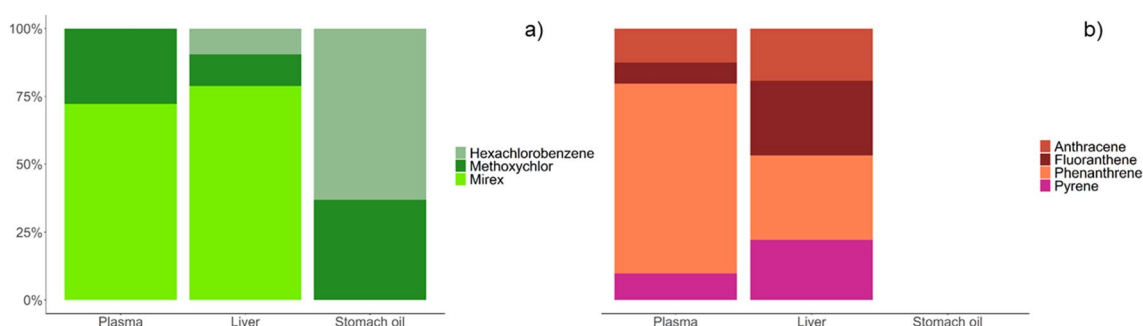
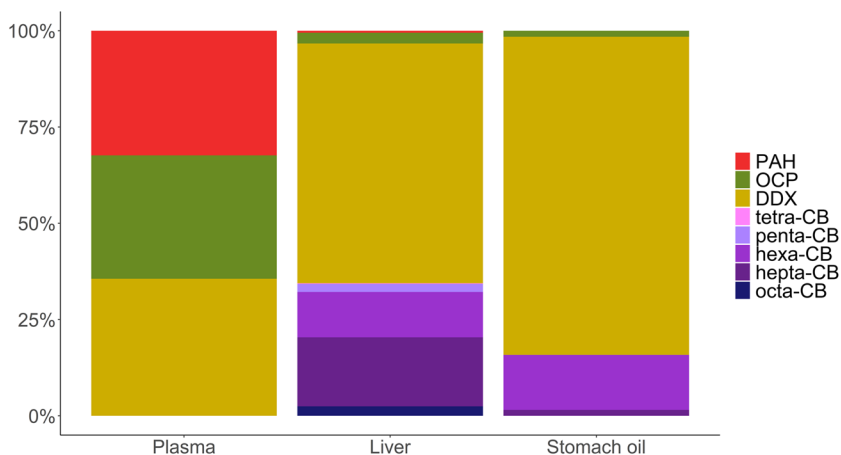


Fig. 2 The percentage composition of **a)** PAHs and **b)** OCPs (excluding DDXs) in the environmental samples from Scopoli's shearwaters (*Calonectris diomedea*). Plasma ($n=21$), liver ($n=10$), stomach oil ($n=6$). Only concentrations above MDL are included

biphenyls, compared to tetra- and pentachloro biphenyls (Fig. 1). The main contributor to OCPs after 4,4'-DDE in the liver was mirex (Fig. 2a). The proportion of PAHs in liver differed slightly from plasma, but phenanthrene contributed to the profile in both matrices (Fig. 2b). In stomach oil, PCB #138 was detected in five out of six samples, and methoxychlor was most prevalent after 4,4'-DDE. Interestingly, in stomach oil, we did not detect any PAHs (Figs. 1 and 2b).

Conclusion

This study presents a simple and direct method designed to detect POPs and PAHs in small volumes of three seabird matrices, which pose challenges to the analytical

set-up due to their complex composition. We were able to recover 68 targeted compounds in spiked plasma and 67 compounds in liver and stomach oil. In few cases, recoveries were high due to matrix effects. Sufficiently good uncertainty values indicate the excellent performance of the extraction and analytical protocol. In adult and juvenile shearwaters from a remote Mediterranean colony, we detected 4 POPs (range 0.45–14.9 ng/g) and 4 PAHs (range 0.24–21.4 ng/g) in Plasma; 22 POPs (range 1.72–7207 ng/g) and 4 PAHs (range 1.04–9.71 ng/g) in liver; 8 POPs (range 0.17–507 ng/g), and no PAHs in stomach oil. This indicates bioaccumulation along all life stages and especially the exposure of young chicks through their first food source: stomach oil. Differences in pollutant patterns in the matrices were observed, which provides insight into specific bioaccumulation patterns related to exposure scenarios.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11356-024-34174-0>.

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Author contribution LM, PQ, and SL contributed to the study's conceptualization. PQ carried out project administration and supervision together with SL and BON, who supervised the analytical work. LM and GDO provided resources for this study. LM, BON, and SL developed the methodology and validation. LM carried out the investigation, formal analysis, data curation, and wrote the original draft of the manuscript, which was reviewed and edited by all co-authors. All authors read and approved the final manuscript. All authors gave final approval for publication.

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Data availability Data, associated metadata, and calculation tools not presented are available from the corresponding author (lucie.michel@bio.uni-giessen.de).

Declarations

Ethics approval All fieldwork was carried out according to the national legislation under the permit n. 2452 issued the Regional authorities (Regione Siciliana, Assessorato Regionale dell'Agricoltura, dello Sviluppo Rurale e della Pesca Mediterranea, Dipartimento Regionale dello Sviluppo Rurale e Territoriale) on February 1st, 2018. This research complies with the IUCN Policy Statement on Research Involving Species at Risk of Extinction (Scopoli's shearwater *Calonectris diomedea* is classified as "Least concern" in 2018). The fieldwork was carried out with moral responsibility for the animals, with the final goal to preserve and enhance the future survival of that species and to provide knowledge vital to their conservation.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication The participants have consented to the submission to the journal and publication of their data.

Conflict of interest The authors declare no competing interests.

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Supporting Information

Title of the manuscript

Analysis of organochlorines and polycyclic aromatic hydrocarbons designed for pollutant biomonitoring in three seabird matrices

Journal name

Environmental Science and Pollution Research

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Chapter 4

Table S1 Names, compound class, formulae, CAS-Numbers and supplier details of native and internal standards used in this study

Target analytes				
Name	Compound class	Formula	CAS	Supplier and packaging unit
2,4'-DDE	DDX	C ₁₄ H ₈ Cl ₄	3424-82-6	AccuStandard, (New Haven, CT, USA) 10 ng/μL in Toluene
4,4'-DDE	DDX	C ₁₄ H ₈ Cl ₄	72-55-9	
2,4'-DDD	DDX	C ₁₄ H ₁₀ Cl ₄	53-19-0	
4,4'-DDD	DDX	C ₁₄ H ₁₀ Cl ₄	72-54-8	
2,4'-DDT	DDX	C ₁₄ H ₉ Cl ₅	789-02-6	
4,4'-DDT	DDX	C ₁₄ H ₉ Cl ₅	50-29-3	
Aldrin	OCP	C ₁₂ H ₈ Cl ₆	309-00-2	
α-HCH	OCP	C ₆ H ₆ Cl ₆	319-84-6	
β-HCH	OCP	C ₆ H ₆ Cl ₆	319-85-7	
δ-HCH	OCP	C ₆ H ₆ Cl ₆	319-86-8	
γ-HCH	OCP	C ₆ H ₆ Cl ₆	608-73-1	
Dieldrin	OCP	C ₁₂ H ₈ Cl ₆ O	60-57-1	
α -endosulfan	OCP	C ₉ H ₆ Cl ₆ O ₃ S	33213-65-9	
β-endosulfan	OCP	C ₉ H ₆ Cl ₆ O ₃ S	959-98-8	
Endrin	OCP	C ₁₂ H ₈ Cl ₆ O	72-20-8	
Heptachlor	OCP	C ₁₀ H ₅ Cl ₇	76-44-8	
Hexachlorobenzene	OCP	C ₆ Cl ₆	118-74-1	
Isodrin	OCP	C ₁₂ H ₈ Cl ₆	465-73-6	
Methoxychlor	OCP	C ₁₆ H ₁₅ Cl ₃ O ₂	72-43-5	
Mirex	OCP	C ₁₀ Cl ₁₂	2385-85-5	
Oxychlorane	OCP	C ₁₀ H ₄ Cl ₈ O	26940-75-0	
PCB 16	PCB	C ₁₂ H ₇ Cl ₃	38444-78-9	LGC Limited (Teddington, UK) 10 mg neat
PCB 24	PCB	C ₁₂ H ₇ Cl ₃	55702-45-9	LGC Limited (Teddington, UK) 10 mg neat
PCB 38	PCB	C ₁₂ H ₇ Cl ₃	53555-66-1	LGC Limited (Teddington, UK) 10 mg neat
PCB 49	PCB	C ₁₂ H ₆ Cl ₄	41464-40-8	LGC Limited (Teddington, UK) 20 mg neat
PCB 52	PCB	C ₁₂ H ₆ Cl ₄	35693-99-3	LGC Limited (Teddington, UK) 10 mg neat
PCB 61	PCB	C ₁₂ H ₆ Cl ₄	33284-53-6	LGC Limited (Teddington, UK) 10 mg neat
PCB 62	PCB	C ₁₂ H ₆ Cl ₄	54230-22-7	LGC Limited (Teddington, UK)
PCB 65	PCB	C ₁₂ H ₆ Cl ₄	33284-54-7	LGC Limited (Teddington, UK)
PCB 77	PCB	C ₁₂ H ₆ Cl ₄	32598-13-3	LGC Limited (Teddington, UK) 25 mg neat

Chapter 4

Target analytes				
Name	Compound class	Formula	CAS	Supplier and packaging unit
PCB 85	PCB	C ₁₂ H ₅ Cl ₅	65510-45-4	LGC Limited (Teddington, UK) 10 mg neat
PCB 99	PCB	C ₁₂ H ₅ Cl ₅	38380-01-7	LGC Limited (Teddington, UK) 5 mg neat
PCB 101	PCB	C ₁₂ H ₅ Cl ₅	37680-73-2	LGC Limited (Teddington, UK) 10 mg neat
PCB 110	PCB	C ₁₂ H ₅ Cl ₅	38380-03-9	LGC Limited (Teddington, UK) 5 mg neat
PCB 116	PCB	C ₁₂ H ₅ Cl ₅	18259-05-7	LGC Limited (Teddington, UK) 10 mg neat
PCB 118	PCB	C ₁₂ H ₅ Cl ₅	31508-00-6	LGC Limited (Teddington, UK) 10 mg neat
PCB 128	PCB	C ₁₂ H ₄ Cl ₆	38380-07-3	LGC Limited (Teddington, UK) 25 mg neat
PCB 138	PCB	C ₁₂ H ₄ Cl ₆	35065-28-2	LGC Limited (Teddington, UK) 10 mg neat
PCB 146	PCB	C ₁₂ H ₄ Cl ₆	51908-16-8	LGC Limited (Teddington, UK) 5 mg neat
PCB 149	PCB	C ₁₂ H ₄ Cl ₆	38380-04-0	LGC Limited (Teddington, UK) 5 mg neat
PCB 153	PCB	C ₁₂ H ₄ Cl ₆	35065-27-1	LGC Limited (Teddington, UK) 10 mg neat
PCB 156	PCB	C ₁₂ H ₄ Cl ₆	38380-08-4	LGC Limited (Teddington, UK) 10 mg neat
PCB 167	PCB	C ₁₂ H ₄ Cl ₆	52663-72-6	LGC Limited (Teddington, UK) 10 mg neat
PCB 170	PCB	C ₁₂ H ₃ Cl ₇	35065-30-6	LGC Limited (Teddington, UK) 5 mg neat
PCB 180	PCB	C ₁₂ H ₃ Cl ₇	35065-29-3	LGC Limited (Teddington, UK) 5 mg neat
PCB 183	PCB	C ₁₂ H ₃ Cl ₇	52663-69-1	LGC Limited (Teddington, UK) 5 mg neat
PCB 189	PCB	C ₁₂ H ₃ Cl ₇	39635-31-9	LGC Limited (Teddington, UK) 5 mg neat
PCB 194	PCB	C ₁₂ H ₂ Cl ₈	35694-08-7	LGC Limited (Teddington, UK) 5 mg neat
Naphthalene	PAH	C ₁₀ H ₈	91-20-3	AccuStandard, (New Haven, CT, USA) 200 ng/μL in Toluene
Acenaphthylene	PAH	C ₁₂ H ₈	208-96-8	
Acenaphthene	PAH	C ₁₂ H ₁₀	83-32-9	
Fluorene	PAH	C ₁₃ H ₁₀	86-73-7	
Phenanthrene	PAH	C ₁₄ H ₁₀	85-01-8	
Anthracene	PAH	C ₁₄ H ₁₀	120-12-7	
Fluoranthene	PAH	C ₁₆ H ₁₀	206-44-0	
Pyrene	PAH	C ₁₆ H ₁₀	129-00-0	
1,2-benzanthracene	PAH	C ₁₈ H ₁₂	56-55-3	
Chrysene	PAH	C ₁₈ H ₁₂	218-01-9	
Benzo[b]fluoranthene	PAH	C ₂₀ H ₁₂	205-99-2	
Benzo[k]fluoranthene	PAH	C ₂₀ H ₁₂	207-08-9	
Benz[a]pyrene	PAH	C ₂₀ H ₁₂	50-32-8	
Indeno[1,2,3-cd]pyrene	PAH	C ₂₂ H ₁₂	193-39-5	

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Target analytes				
Name	Compound class	Formula	CAS	Supplier and packaging unit
Dibenz[a,h]anthracene	PAH	C ₂₂ H ₁₄	53-70-3	
Benzo[g,h,i]perylene	PAH	C ₂₂ H ₁₂	191-24-2	
Internal standards				
Pentachlorobenzene ¹³ C ⁶	OCP	C ₆ HCl ₅	-	AccuStandard, (New Haven, CT, USA) 200 ng/μL in Toluene
Naphthalene D8	PAH	C ₁₀ H ₈	1146-65-2	Sigma-Aldrich 4000 ng/μL
Acenaphthene D10	PAH	C ₁₂ H ₁₀	15067-26-2	
Phenanthrene D10	PAH	C ₁₄ H ₁₀	1517-22-2	
Chrysene D12	PAH	C ₁₈ H ₁₂	1719-03-5	
Perylene D12	PAH	C ₂₀ H ₁₂	1520-96-3	
4,4'-DDE-D8	DDX	C ₁₄ H ₈ Cl ₄	93952-19-3	LGC Limited (Teddington, UK) 10 mg neat
PCB 73	PCB	C ₁₂ H ₆ Cl ₄	74338-23-1	LGC Limited (Teddington, UK) 10 mg neat
PCB 97	PCB	C ₁₂ H ₅ Cl ₅	41464-51-1	LGC Limited (Teddington, UK) 10 mg neat
PCB 126	PCB	C ₁₂ H ₅ Cl ₅	57465-28-8	LGC Limited (Teddington, UK) 10 mg neat
PCB 151	PCB	C ₁₂ H ₄ Cl ₆	52663-63-5	LGC Limited (Teddington, UK) 5 mg neat
PCB 157	PCB	C ₁₂ H ₄ Cl ₆	69782-90-7	LGC Limited (Teddington, UK) 10 mg neat
PCB 200	PCB	C ₁₂ H ₂ Cl ₈	52663-73-7	LGC Limited (Teddington, UK) 5 mg neat

Table S2 Additional quality parameters for 68 target POPs in elution order. Second and third diagnostic ions in m/z, slope, stable parameter (b) and R² for the calibration curve

Compound	Ion 2 [m/z]	Ion 3 [m/z]	Slope	b	R ²
Naphthalene	129.0654	102.0464	0.89	-0.04	0.9985
Acenaphthene	154.0777	152.0620	0.81	-0.03	0.9974
Acenaphthylene	153.0654	126.0464	0.80	-0.22	0.9969
Fluorene	166.0777	163.0542	0.58	0.06	0.9982
α-HCH	218.9110	108.9606	0.66	-0.10	0.9993
Hexachlorobenzene	285.8066	281.8125	0.46	0.02	0.9975
β-HCH	182.9343	218.9110	1.49	-0.65	0.9937
δ-HCH	218.9110	108.9606	0.88	-0.27	0.9983
Phenanthrene	152.0620	179.0810	0.90	-0.04	0.9982
PCB 24	186.0230	257.9578	1.36	0.05	0.9990
Anthracene	176.0620	152.0620	0.84	-0.03	0.9985

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Compound	Ion 2 [m/z]	Ion 3 [m/z]	Slope	b	R ²
PCB 16	220.9919	186.0230	0.42	0.01	0.9980
δ-HCH	182.9343	218.9110	0.65	-0.16	0.9986
Heptachlor	269.8125	100.0074	0.17	0.00	0.9991
PCB 52	291.9188	289.9218	1.21	-0.17	0.9907
PCB 49	219.98411	291.9188	0.84	0.04	0.9985
PCB 38	186.0230	257.9578	1.46	-0.08	0.9968
PCB 62	289.9218	219.9841	0.95	0.04	0.9980
PCB 65	289.9218	219.9841	0.94	0.10	0.9937
Aldrin	66.9990	263.4950	0.25	0.01	0.9987
Isodrin	194.9343	262.8564	0.25	-0.01	0.9970
PCB 61	289.9218	219.9841	1.09	0.06	0.9968
Heptachlorepoxyde	354.8407	236.8407	0.15	0.00	0.9971
Oxychlordane	186.9107	115.0000	0.09	0.00	0.9986
Fluoranthene	200.0620	101.0385	0.95	-0.01	0.9976
2,4'-DDE	247.9968	317.9345	8.86	-0.85	0.9951
trans-Chlordane	374.8224	376.8195	0.85	-0.14	0.9989
α-Endosulfan	259.9841	169.9684	0.49	-0.11	0.9979
cis-Chlordane	376.8195	374.8224	0.48	-0.05	0.9983
PCB 101	253.9451	327.8798	1.42	0.06	0.9986
PCB 99	253.9451	327.8798	2.00	0.10	0.9951
Pyrene	200.0620	201.0698	0.87	0.05	0.9967
PCB 116	253.9451	327.8798	1.47	0.09	0.9969
4,4'-DDE	247.9968	317.9345	4.46	0.20	0.9982
PCB 85	253.9451	323.9870	1.04	0.04	0.9975
PCB 110	327.8769	323.9870	2.34	0.09	0.9933
Dieldrin	79.0450	81.0334	0.26	-0.01	0.9983
2,4'-DDD	237.0046	165.0698	9.87	-0.04	0.9989
PCB 77	289.9218	219.9841	2.93	0.12	0.9988
PCB 149	361.9789	289.9032	1.62	0.07	0.9971
Endrin	242.9529	280.9266	0.23	-0.01	0.9986
β-Endosulfan	169.9684	192.9373	0.22	-0.02	0.9995
2,4'-DDT	237.0046	165.0698	10.1	-0.09	0.9975
PCB 146	361.9789	289.9032	0.65	0.04	0.9964
4,4'-DDD	165.0698	199.0309	3.32	-0.05	0.9998

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Compound	Ion 2 [m/z]	Ion 3 [m/z]	Slope	b	R ²
PCB 153	361.9789	144.9032	0.83	0.02	0.9935
PCB 118	327.8769	323.9870	1.82	-0.15	0.9956
4,4'-DDT	165.0698	245.9997	0.93	-0.03	0.9962
PCB 138	361.9789	289.9032	0.72	0.13	0.9959
PCB 187	395.7989	324.0000	0.45	0.03	0.9927
PCB 183	395.7989	324.0000	0.50	0.02	0.9960
PCB 128	361.9789	144.9032	0.76	-0.03	0.9978
PCB 167	361.9789	289.9032	0.82	0.05	0.9969
PCB 156	361.9789	289.9032	1.17	0.05	0.9975
Methoxychlor	228.1101	152.0620	0.50	-0.02	0.9964
1,2-Benzanthracene	101.0385	226.0304	0.88	0.02	0.9976
Chrysene	226.0777	229.0967	0.81	0.02	0.9982
PCB 180	395.7989	397.8976	0.45	0.03	0.9972
PCB 170	395.7989	324.0000	0.77	0.01	0.9978
Mirex	236.8407	269.8125	0.70	-0.01	0.9998
PCB 189	395.7989	324.0000	0.87	0.05	0.9938
PCB 194	427.7629	431.0320	0.49	0.09	0.9931
Benzo[b]fluoranthene	250.0777	253.0967	4.47	-0.31	0.9961
Benzo[k]fluoranthene	250.0777	253.0967	4.91	-15.0	0.9995
Benz[a]pyrene	250.0777	253.0967	3.99	-10.8	0.9989
Indeno[1,2,3-cd]pyrene	274.0777	277.0967	1.86	-5.71	0.9972
Dibenz[a,h]anthracene	276.0933	274.0777	1.58	-4.16	0.9976
Benzo[g,h,i]perylene	274.0777	277.0967	1.78	-4.56	0.9973

Table S3 Matrix effects (ME) for each target compound

Compound	Plasma ME [%]	Liver ME [%]	Stomach oil ME [%]
Naphthalene	-45	-32	-19
Acenaphthene	-58	-65	-53
Acenaphthylene	-56	-60	-75
Fluorene	-53	-33	-43
α-HCH	-61	-60	-54
Hexachlorobenzene	-70	-68	-47
β-HCH	2	-64	-77
γ-HCH	-5	-54	-30
Phenanthrene	-44	-20	-38
PCB 24	-58	-40	-78
Anthracene	-49	-30	-54

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Compound	Plasma ME [%]	Liver ME [%]	Stomach oil ME [%]
PCB 16	-47	-29	-63
Heptachlor	-55	-93	-63
δ-HCH	127	-55	-57
PCB 52	-61	-34	-62
PCB 49	-63	-34	-63
PCB 38	-61	-26	-61
PCB 62	-63	-35	-67
PCB 65	-66	-38	-66
Aldrin	-68	-36	-92
Isodrin	-65	-36	-74
PCB 61	-70	-32	-65
Heptachlorepoxyde	-49	-44	-76
Oxychlorane	-51	-43	-85
Fluoranthene	-50	-57	-56
2,4'-DDE	-63	-67	-78
trans-Chlordane	-53	-68	-73
α-Endosulfan	-49	-61	-72
PCB 101	-54	-66	-76
PCB 99	-64	-62	-83
cis-Chlordane	-69	-58	-73
Pyrene	-48	-42	-63
PCB 116	-72	-65	-77
4,4'-DDE	-69	-60	-48
PCB 85	-69	-63	-76
PCB 110	-66	-61	-76
Dieldrin	-54	-64	-78
2,4'-DDD	-43	-49	-59
PCB 77	-64	-75	-82
PCB 149	-71	-67	-75
Endrin	-61	-79	-88
β-Endosulfan	-2	-54	-94
2,4'-DDT	-64	-58	-75
PCB 146	-72	-49	-68
4,4'-DDD	-64	-57	-69
PCB 153	-73	-50	-66
PCB 118	-65	-42	-67
4,4'-DDT	-11	101	-56
PCB 138	-75	-60	-67
PCB 187	-72	-35	-65
PCB 183	-71	-36	-79
PCB 128	-70	-53	-73
PCB 167	-71	-38	-65
PCB 156	-71	-50	-69
Methoxychlor	18	87	-56
1,2-Benzanthracene	-42	-29	-67
Chrysene	-40	-38	-52
PCB 180	-69	-52	-59

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Compound	Plasma ME [%]	Liver ME [%]	Stomach oil ME [%]
PCB 170	-66	-52	-78
Mirex	-71	-51	-78
PCB 189	-72	-51	-84
PCB 194	-80	-57	-86
Benzo[b]fluoranthene	-64	-60	-81
Benzo[k]fluoranthene	-52	-51	-76
Benzo[a]pyrene	-57	-54	-80
Indeno[1,2,3-cd]pyrene	-59	-56	-86
Dibenz[a,h]anthracene	-61	-58	-91
Benzo[g,h,i]perylene	-58	-43	-91

Table S4 Summary table listing and categorizing issues that occurred in the spiked quality controls and how we resolved them using external calibration and explained them using uncertainty and matrix effect values.

Target compound	issue in repetitions	quality control	signal intensified?	requantified with calibration	external with	signal remained after ext cal?	intensified	Uncertainty	Matrix effects
β-HCH	high RSD in repetitions	stomach oil qc	yes, in plasma	yes	no	no	< 50 in plasma and stomach oil	small positive Matrix effect in plasma (2)	
γ-HCH	high RSD in repetitions	plasma, liver and stomach oil qc	yes, in plasma	yes	no	no	< 50 in plasma and stomach oil	very low negative effect in plasma (-5)	
δ-HCH	high RSD in repetitions	plasma qc	yes, in plasma	yes	yes, in plasma	yes, in plasma	high uncertainty in plasma 264	high positive matrix effect in plasma (127)	
Aldrin	high RSD in repetitions	stomach oil qc	no	no	no	no	< 50 in plasma and stomach oil	strong negative effect in stomach oil (-92)	
2,4'-DDD	high RSD in repetitions	liver and stomach oil qc	yes, in liver and stomach oil	yes	no	no	< 50 in plasma and stomach oil	negative effects in all matrices	
Endrin	high RSD in repetitions	liver and stomach oil qc	no	no	no	no	< 50 in liver and stomach oil	negative effect in stomach oil (-88)	
β-endosulfan	high RSD in repetitions	stomach oil qc	yes, in plasma	yes	no	no	< 50 in plasma and stomach oil	very low negative effect in plasma (-2), strong negative effect in stomach oil (-94)	
4,4'-DDT	high RSD in repetitions	stomach oil qc	yes, in plasma and stomach oil	yes	yes, in liver	yes, in liver	< 50 in plasma and stomach oil	strong positive effect in liver (101)	
Methoxychlor	high RSD in repetitions	stomach oil qc	yes, in plasma and liver	yes	yes, in liver	yes, in liver	< 50 in plasma and stomach oil	positive effect in plasma (18) and strong positive effect in liver (87)	
PCB 180	high RSD in repetitions	stomach oil qc	no	no	no	no	< 50 in stomach oil	negative effects in all matrices	
PCB 194	high RSD in repetitions	stomach oil qc	no	no	no	no	< 50 in plasma and stomach oil	negative effects in all matrices	
Benzo(<i>b,h,i</i>)perylene	high RSD in repetitions	stomach oil qc	no	no	no	no	< 50 in stomach oil	strong negative effect in stomach oil (-91)	

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Table S5 Compounds with peak areas and vial concentrations [ng/mL] in matrix blanks of pooled stomach oil, which were subtracted from the quality control samples

Compound	peak area	concentration in ng/mL in vial
Hexachlorobenzene	709108	2.2
PCB 99	99574	0.2
4,4'-DDE	8632109	5.8
PCB 149	165210	0.7
2,4'-DDT	402218	0.2
PCB 146	113739	0.5
PCB 153	1873288	7.4
PCB 118	646218	1.2
4,4'-DDT	118220	0.5
PCB 138	1478138	5.7
PCB 187	185708	1.1
PCB 180	671862	5.2
PCB 170	34334	0.3
PCB 189	19575	0.1

Appendix

Supporting material

Upon publication of this thesis all raw data will be uploaded to open online data repositories: Genetic Data will be made available on GENBANK: <https://www.ncbi.nlm.nih.gov/genbank> (NGS: Sequence Read Archive) and Isotope Data on Zenodo, <https://zenodo.org>.

Until then the publications, individual supporting information and data files of each chapter are saved in a shared folder at JLUbox: <https://jlubox.uni-giessen.de/getlink/fiBawMkRwvGr5xgE1K6qMrSj/>

Appendix

Table S1 Overview of compounds currently listed in the Stockholm Convention of persistent organic pollutants (POPs). Full names, common abbreviation, Annex listed, pollutant category and specification about the (past) use and chemical group adapted from Carravieri 2014, Sah & Joshi 2011, and the website of the Stockholm Convention (www.pops.int). Measures according to Annexes: Annex A: Elimination, Annex B: Restriction, Annex C: unintentional production. Grey backgrounds indicates that the pollutant was included into this thesis.

Name	Abbreviation	Annex	Category	Application specification (adapted from Carravieri 2014 and pops.int)	
Initial POPs					
			Pesticide	Industrial Chemical	By-products
Aldrin		A	+	Applied to soils to kill pest insects; aldrin is readily and rapidly converted to dieldrin in the environment.	
Chlordane		A	+	Broad-spectrum insecticide. Technical chlordane is a mixture of chlordane, heptachlor, nonachlor and related compounds.	
Dieldrin		A	+	Used principally to control termites and textile pests, but also against insect-borne diseases and insects living in agricultural soils. Dieldrine derives also from aldrin transformation.	
Endrin		A	+	Applied on leaves of crops such as cotton and grains. Also used to control rodents.	
Heptachlor		A	+	Primarily used to kill soil insects and termites, but also crop pests and malaria-carrying mosquitoes.	
Mirex		A	+	Primarily used as an insecticide against ants and termites. Also used as a fire retardant in plastics, rubber, and electrical goods.	
Toxaphene		A	+	Insecticide used on cotton, cereal grains, fruits, nuts, and vegetables. Also used to control ticks and mites in livestock.	
Hexachlorobenzene	HCB	A,C	+	Primarily used in agriculture as a fungicide for the protection of wheat and other cereals. Also a by-product of the manufacture of certain industrial chemicals and exists as an impurity in several pesticide formulations.	+
Polychlorinated biphenyls	PCBs	A,C	+	There are 209 different congeners. PCBs were used in industry as heat exchange fluids, in electric transformers and capacitors, and as additives in paint, carbonless copy paper, and plastics.	+

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Name	Abbreviation	Annex	Pesticide	Industrial Chemical	By-pro-ducts	Category	Application specification (adapted from Carravieri 2014 and pops.int)
Dichlorodiphenyl-tri-chloroethane	DDT	B	+				Widely used during World War II to protect soldiers and civilians from diseases spread by insects (mainly typhus). Agricultural applications included mainly cotton. Still applied against mosquitoes in several countries to control malaria. Metabolites of biological and ecological relevance are 2,4' - and 4,4' -DDT; 2,4' - and 4,4' -DDD; and 2,4' - and 4,4' -DDE.
Polychlorinated dibenzo-p-dioxins	PCDDs	C			+		There are 75 different congeners. PCDDs are produced unintentionally due to incomplete combustion (wood, fossil fuel, waste, etc.), and during the manufacture of pesticides and other chlorinated substances.
Polychlorinated dibenzofurans	PCDFs	C			+		There are 135 different congeners. PCDFs are produced unintentionally from many of the same processes that produce PCDDs, to which they are structurally similar.
New POPs							
Alpha Hexachlorocyclohexane	α -HCH	A	+			+	α -HCH was used as pesticide, but is also by-products of lindane.
Beta Hexachlorocyclohexane	β -HCH	A	+			+	β -HCH was used as pesticide, but is also by-products of lindane.
Chlordecone		A	+				Mainly used as an agriculture insecticide, chemically related to mirex
Decabromodiphenyl ether (commercial mixture)	DecaBDE	A		+			DecaBDE has been mainly used as an additive flame retardant in plastics, polymers, composites, textiles, adhesives, sealants, coatings and inks. DecaBDE containing plastics are used in housings of computers and TVs, wires and cables, pipes and carpets, commercial textiles, mainly for public buildings and transport, and in textiles for domestic furniture.
Dechlorane Plus	DDC-CO	A	+			+	Used as substitute for mirex and flame retardant
Dicofol		A	+				Dicofol is an organochlorine miticidal pesticide that has been used in agriculture to control mites on a variety of field crops, fruits, vegetables, ornamentals, cotton, tea.
Endosulfan		A	+				Insecticide and acaricide used on a variety of food and non-food crops. It is the most recently added POPs to the Stockholm Convention and is still widely used in China and India and other developing countries.

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Name	Abbreviation	Annex	Pesticide Category	Industrial Chemical	By-product	Application specification (adapted from Carravieri 2014 and pops.int)
Hexabromobiphenyl	HBB	A		+		Mainly used as flame retardant, especially in the 1970s.
Hexabromocyclododecan	HBCDD	A		+		HBCDD is used as a flame retardant additive, with the intent of delaying ignition and slowing subsequent fire growth during the service life of vehicles, buildings or articles, as well as while materials are stored.
Hexa- and Heptabromo-diphenyl ether		A		+		Mainly used as flame retardant. They may be converted to lower, and possibly more toxic, PBDE congeners by debromination.
Hexachlorobutadiene	HCBD	A,C		+	+	HCBD is unintentionally formed and released from the production of certain chlorinated hydrocarbons, magnesium, polyvinyl chloride, ethylene dichloride and vinyl chloride monomer and incineration of acetylene, chlorine residues caused by poor abatement control. Previously, it was intentionally produced or used in the production of lubricants, as a solvent, a heat transfer liquid and hydraulic liquid, yet HCBD is not known to be currently intentionally produced or used.
Lindane	γ -HCH	A		+		γ -HCH (lindane) was primarily a broad-spectrum insecticide for soil and wood treatments. It is still used in human pharmaceuticals for control of head lice and scabies.
Methoxychlor		A		+		Methoxychlor is an organochlorine pesticide originally developed as a replacement for DDT. Methoxychlor has been used as an insecticide combating a wide range of pests.
Pentachlorobenzene	PeCB	A,C		+	+	Used in many applications: as a fungicide, a flame retardant and as a chemical intermediate. Also produced unintentionally during combustion and industrial processes. Also present as impurities in solvents and pesticides.
Pentachlorophenol and its salts and esters	PCP	A		+		PCP has been used as herbicide, insecticide, fungicide, algacide, disinfectant and as an ingredient in antifouling paint. Some applications were in agricultural seeds, leather, wood preservation, cooling tower water, rope and paper mill system. Chlorinated contaminants including hexachlorobenzene, pentachlorobenzene, and dioxins and furans are produced during the manufacturing process.

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Name	Abbreviation	Annex	Pesticide	Industrial Chemical	By-pro-ducts	Category	Application specification (adapted from Carravieri 2014 and pops.int)
Perfluorohexane sulfonic acid (PFHxS), its salts and PFHxS-related compounds	PFHxS	A		+			PFHxS, its salts and related substances have unique properties with a high resistance to friction, heat, chemical agents, low surface energy and used as water, grease, oil and soil repellent. It is widely utilized in a variety of consumer goods such as carpets, leather, apparel, textiles, firefighting foam, papermaking, printing inks, sealants, non-stick cookware.
Perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride	PFOS, PFOS-F	B		+			Widespread applications: electric and electronic parts, firefighting foam, photo imaging, hydraulic fluids and textiles. PFOS is also an unintended degradation product of related anthropogenic chemicals, and still produced in several countries. These compounds have a hydrophilic character, in contrast to most other POPs.
Perfluorooctanoic acid (PFOA), its salts and PFOA-related compounds	PFOA	A		+	+		PFOA, its salts and PFOA-related compounds are used widely in the production of fluoroelastomers and fluoropolymers, for the production of non-stick kitchen ware, food processing equipment. PFOA has been detected in industrial waste, stain resistant carpets, carpet cleaning liquids, house dust, microwave popcorn bags, water, food, and Teflon. Unintentional formation of PFOA is created from inadequate incineration of fluoropolymers from municipal solid waste incineration with inappropriate incineration or open burning facilities at moderate temperatures.
Polychlorinated naphthalenes	PCNs	A,C		+	+		PCNs have historically been used in many applications including: use as wood preservative, as additive to paints and engine oils, and for cable insulation and in capacitors. PCNs are unintentionally generated during high-temperature industrial processes in the presence of chlorine. Of the known releases, combustion (primarily waste incineration) is considered the most significant current source.
Short-chain chlorinated paraffins	SCCPs	A					Short-chain chlorinated paraffins (SCCPs) have been used as softeners in plastics, paints, coatings and sealants, as flame retardants in rubber, plastics and textiles as well as an extreme pressure lubricant in metal working fluids

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Name	Abbreviation	Annex	Pesticide	Industrial Chemical	By-products	Category	Application specification (adapted from Carravieri 2014 and pops.int)
Tetrabromodiphenyl ether and pentabromodiphenyl ether	c-tetraBDE, c-pentaBDE						Commercial pentabromodiphenyl ether (c-pentaBDE) is a mixture of brominated flame retardants (BFRs), mainly isomers of pentabromodiphenyl ether and tetrabromodiphenyl ether. Brominated flame retardants are a group of brominated organic substances that inhibit or suppress combustion in organic material. C-pentaBDE is or has been used almost exclusively in the manufacture of flexible polyurethane (PUR) foam for furniture and upholstery in homes and vehicles, packaging, and non-foamed PUR in casings and electronic equipment (EE). They are also used to some extent in specialized applications in textiles and in industry.
UV-328		A				+	UV-328 is a phenolic benzotriazole that is used as a UV absorber to protect surfaces against discoloration and degradation under UV/sunlight. UV-328 has wide range of applications, but its main uses are in paints and coatings, and as an additive in a wide variety of plastics, including in the non-food contact layer of food packaging. In the automobile sector, UV-328 is used in paints, coatings and sealants, as well as in liquid crystal panels and meters mounted on vehicles, and resin for interior and exterior parts of vehicles. In food packaging, it is used as an additive in plastics, printing ink and adhesives.

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