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Original Article

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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 δ15N and δ13C 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 (PFSAs), 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 ($\delta^{13}C$) and nitrogen ($\delta^{15}N$), 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°51′33″ N 12°51′34″ E, Sicily Channel), La Maddalena Archipelago ($n = 20$; 41°13′60″ N 9°24′0″ E, Central Tyrrhenian) and two sampling sites in Malta ($n = 15$; 35°48′37″ N 14°30′17″ E and 35°57′13″ N 14°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 adaptions. 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 = perfluorodecanoic acid; PFOSA = perfluorooctane sulfonamide; PFOA = perfluorooctanoic acid; 10:2 FTS = 1H,2H-perfluorododecan sulfonate (10:2); PFHxS = perfluorohexane sulfonic acid; PFDS = perfluorodecane 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-dimethyloctanoic 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), perflurooctadecaonic acid (PFOcDa), perfluoro-3,7-dimethyloctanoic acid (P37DMOA), 7H-dodecafluoroheptanoic acid (7H-PFHpA), 9 PFSAs namely perfluorobutanesulfonic acid (PFBS), perfluoropentane sulfonic acid (PFPeS), perfluorohexane sulfonic acid (PFHxS), perfluoroheptane sulfonic acid (PFHpS), PFOS, perfluorononane sulfonic acid (PFNS), perfluorodecane 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-octanesulfonamido-acetic acid (FOSAA), 2-(N-methylperfluoro-1-octansulfonamido) 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 sulphonamide (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-$^{13}C_8$ (99%), perfluoro-1-octanesulfonate-$^{13}C_8$ (99%) and 6:2 FTS $^{13}C_2$-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 (≥ 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 × 2.1 mm, 1.3 µm) connected to a Phenomenex C18 guard column (2.0 × 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 PFSAs, namely PFPeS, PFNS, PFDS, PFDoDS, PFECHS, 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 (± relative SD in percent [RSD %]) of 51 ± 9, 51 ± 11, 50 ± 11, 51 ± 11, 46 ± 5, and 55 ± 10, respectively, for a fortification amount of 10 ng. Correspondingly, the relative percentage recoveries (± RSD %) for these compounds, when considering C13-PFOS as the internal standard, were as follows: PFPeS (101 ± 18), PFNS (103 ± 22), PFDS (100 ± 21), PFDoDS (103 ± 22), PFECHS (93 ± 9), and PFOcDA (111 ± 20). This validation process ensures the reliability and accuracy of the developed method for analyzing these specific PFSAs 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 $\delta^{15}N$ and < 0.10 ‰for $\delta^{13}C$. Results are expressed in the δ unit notation as deviations from standards (Vienna Pee Dee Belemnite for $\delta^{13}C$ and N_2 in air for $\delta^{15}N$) following the formula $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 10^3$, where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively.

The SI values of $\delta^{13}C$ and $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ between the colonies from the Tyrrhenian Sea and the Sicily Channel and a Δ of 0.2 ‰ for $\delta^{13}C$.

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, PFECHS, EtFOSE, 8:2 FTS, P37DMOA, and adjusted δ13C (AD$\delta^{13}C$) 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 ≤ 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, PFECHS, 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%

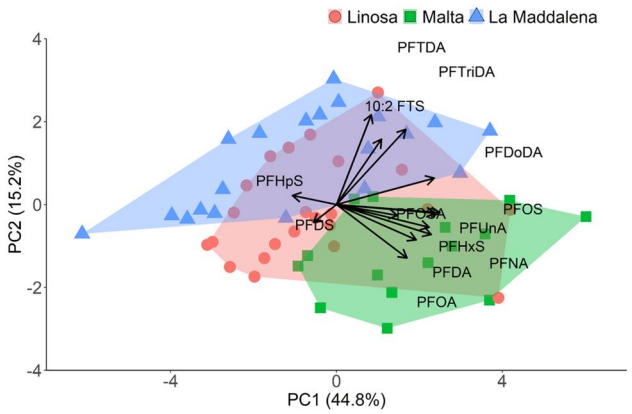

Figure 1. Principal Component Analysis of perfluoroalkyl substances (PFAS) profiles of three studied colonies of Scopoli's shearwaters (*Calonectris diomedea*; $n = 58$) during the chickrearing phase of the year 2020. PC1 = principal component 1; PC2 = principal component 2; PFOS = perfluorooctanosulfonic acid; PFuNA = perfluoroundecanoic acid; PFDoDA = perfluorododecanoic acid; PFTriDA = perfluorotridecanoic acid; PFTDA = perfluorotetradecanoic acid; PFNA = perfluorononanoic acid; PFOSA = perfluorooctane sulfonamide; 10:2 FTS = 1H,2H-p erfluorododecan sulfonate (10:2); PFOA = perfluorooctanoic acid; PFDA = perfluorodecanoic acid; PFHxS = perfluorohexane sulfonic acid; PFDS = perfluorodecane 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).

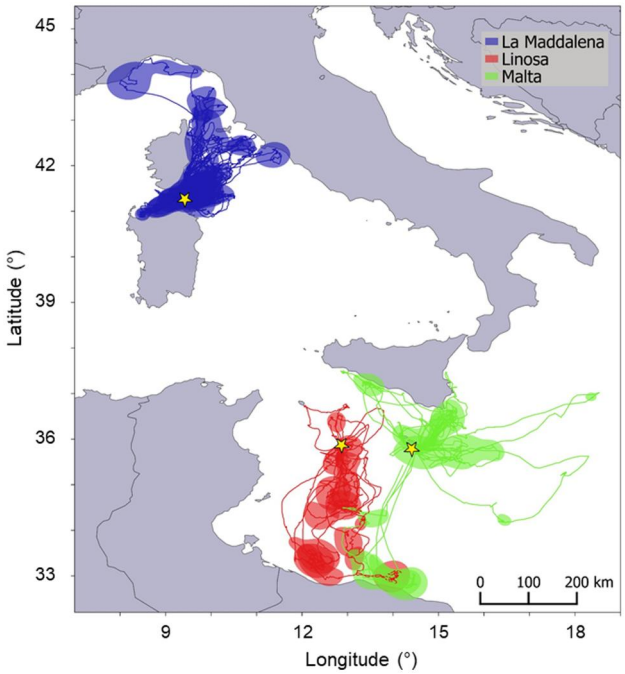

Figure 2. Foraging movements of Scopoli's shearwaters (*Calonectris diomedea*) tracked during the summer of 2020 (Linosa, Malta) and 2019 (La Maddalena) and 50% utilization distributions.

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.

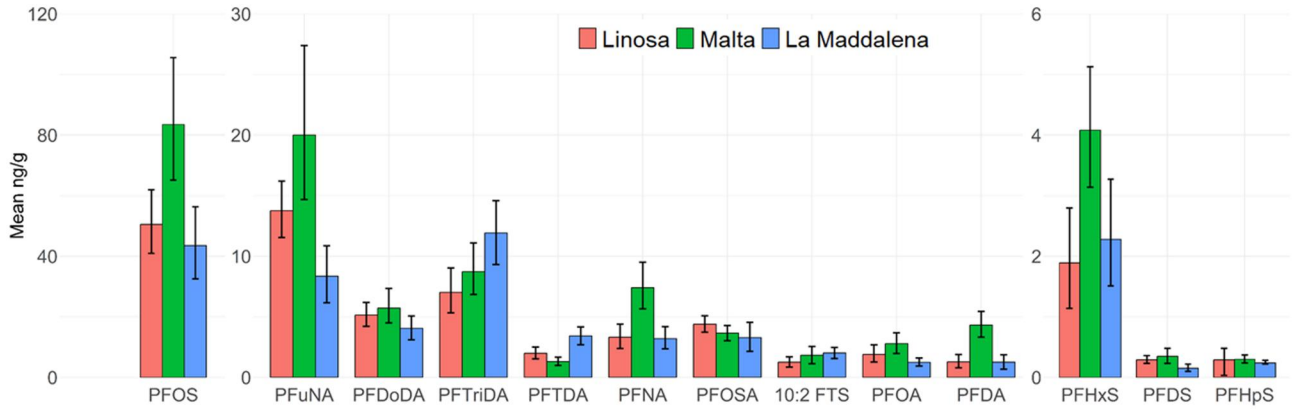

Figure 3. Mean concentrations in ng/g dry weight of all perfluoroalkyl substances (PFAS) with less than 70% censoring in red blood cells from the three Scopoli's shearwater (Calonectris diomedea) colonies during the chick-rearing phase in the year 2020. Whiskers show 95% confidence intervals of the mean. 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.

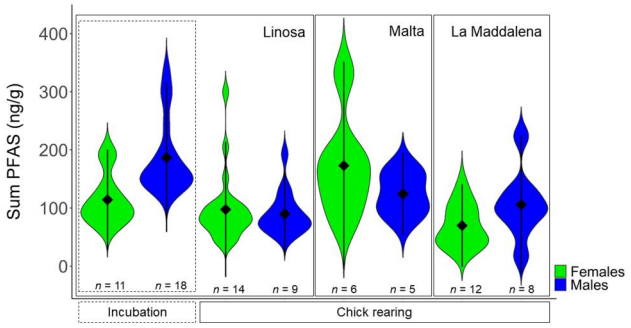

Figure 4. Sum perfluoroalkyl substances (PFAS) distribution in female and male Scopoli's shearwaters (*Calonectris diomedea*) from Linosa, Malta and La Maddalena in the year 2020, during chick-rearing and incubation. Continuous lines mark samples taken during the chick-rearing phase and dashed line marks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

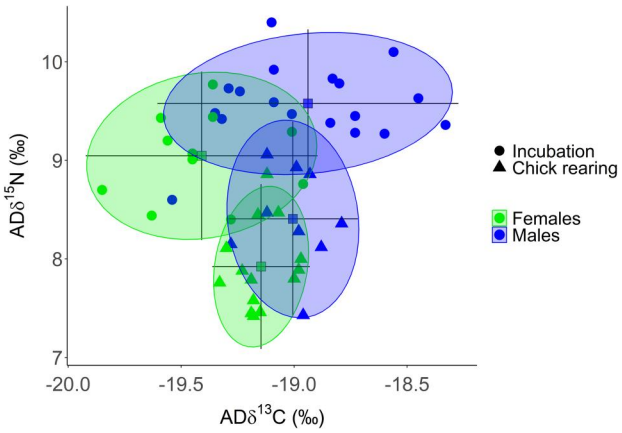

Figure 5. Adjusted stable isotope values for carbon and nitrogen in red blood cells from Scopoli's shearwaters (*Calonectris diomedea*) in Linosa ($n = 52$) plotted for sex and breeding phase in the year 2020. ADδ13C = adjusted stable isotope ratio of carbon; ADδ15N = adjusted stable isotope ratio of 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.

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, 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

ADδ^{15}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 ADδ^{13}C, whereas PFTriDA (rho = 0.19, *p*-value = 0.04) and PFTDA (rho = 0.22, *p*-value = 0.02) correlated positively with ADδ^{13}C (See online supplementary material Table S5).

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

The ADδ15N 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 ADδ13C values (95% CI 2016 –19.01 to 18.69, 2020, –19.15 to 19.03). In both years, males showed higher ADδ15N and ADδ13C values than females (See online supplementary material Table S6b). The ADδ15N values were higher during incubation than during chick-rearing in Linosa in both sexes (Figure 5; See online supplementary material Table S6b). The ADδ13C 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 (± 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 (Gebbink & 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 hotspots 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 ADδ^{15}N values in males. A weak positive association of ADδ^{15}N and some PFAS points to some degree of biomagnification 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, ADδ^{15}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 ADδ^{13}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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