Journal of Hazardous Materials 487 (2025) 137189

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Shellfish and shorebirds from the East-Asian Australian flyway as bioindicators for unknown per- and polyfluoroalkyl substances using the total oxidizable precursor assay

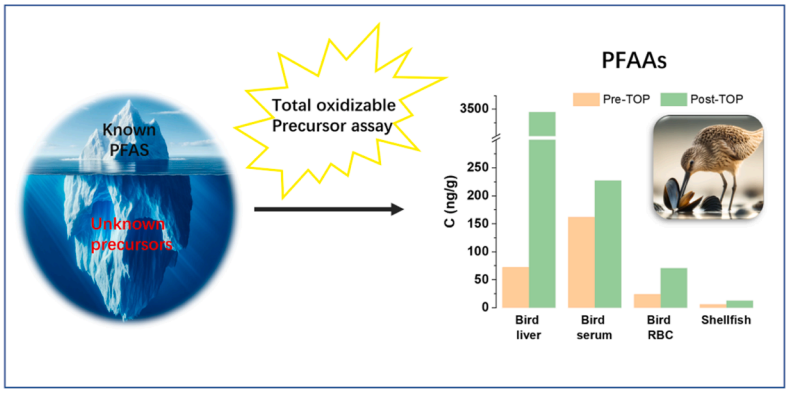
Junjie Zhang [a,*], Lara Cioni [b], Veerle L.B. Jaspers [c], Alexandros G. Asimakopoulos [a], He-Bo Peng [d], Tobias A. Ross [e], Marcel Klaassen [e], Dorte Herzke [b,f,**]

[a] Department of Chemistry, Norwegian University of Science and Technology, Høgskoleringen 5, Trondheim 7491, Norway
[b] NILU, Fram Centre, Tromsø 9296, Norway
[c] Department of Biology, Norwegian University of Science and Technology, Høgskoleringen 5, Trondheim 7491, Norway
[d] Conservation Ecology Group, Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, Groningen 9700 CC the Netherlands
[e] Centre for Integrative Ecology, Deakin University, Geelong 3216, Australia
[f] Norwegian Institute for Public Health, Oslo, Norway

HIGHLIGHTS

- Unknown per- and polyfluoroalkyl substances were found in biota samples.
- Perfluorosulfonic acids were produced in all samples after TOP assay.
- Shorebird serum shows higher levels of perfluorocarboxylic acids than red blood cell.
- Shorebird liver shows the highest levels of perfluoroalkyl acids after TOP assay.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Molluscs
PFAS
PFAS precursors
TOP
Wader birds
Migrating

ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) have gained significant global attention due to their extensive industrial use and harmful effects on various organisms. Among these, perfluoroalkyl acids (PFAAs) are well-studied, but their diverse precursors remain challenging to monitor. The Total Oxidizable Precursor (TOP) assay offers a powerful approach to converting these precursors into detectable PFAAs. In this study, the TOP assay was applied to samples from the East Asian-Australian Flyway, a critical migratory route for millions of shorebirds. Samples included shellfish from China's coastal mudflats, key stopover sites for these birds, and blood and liver samples from shorebirds overwintering in Australia. The results showed a substantial increase in perfluorocarboxylic acids (PFCAs) across all sample types following the TOP assay, with the most significant

* Corresponding author.
** Corresponding author at: NILU, Fram Centre, Tromsø 9296, Norway
E-mail addresses: junjie.zhang@plen.ku.dk (J. Zhang), dhe@nilu.no (D. Herzke).

https://doi.org/10.1016/j.jhazmat.2025.137189
Received 29 August 2024; Received in revised form 17 December 2024; Accepted 9 January 2025
Available online 12 January 2025

increases in shorebird livers (Sum PFCAs increased by 18,156 %). Intriguingly, the assay also revealed unexpected increases in perfluorosulfonic acids (PFSAs), suggesting the presence of unidentified precursors. These findings highlight the need for further research into these unknown precursors, their sources, and their ecological impacts on shorebirds, other wildlife, and potential human exposure. This study also provides crucial insights into the TOP assay's strengths and limitations in studying PFAS precursor dynamics in biological matrices.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) have gained extensive industrial and commercial uses worldwide including fast-food containers, anti-staining fabrics, and fire-suppressing foams [1]. Traditionally, due to their global use, much attention has been placed on the detection of perfluoroalkyl acids (PFAAs) like perfluorocarboxylic acids (PFCAs) and perfluorosulfonic acids (PFSAs). Due to the recent increase in the PFAS diversity used in numerous products and industrial process, there is a growing focus on monitoring a larger part of the big PFAS group [2]. One category is unknown PFAS capable of oxidative transformation, ultimately yielding well known PFAAs as terminal products [3]. These unknown PFAS find application in various sectors such as textiles, cleaning products, pesticides, food packaging, oil, firefighting, and industrial manufacturing. Their annual production volume exceeds that of conventional PFAAs, with many production sites located in China close to the shoreline [1].

Unlike the relatively well-established group of PFAAs, there are hundreds of unknown oxidizable PFAS, many of them with confidential chemical structures [4]. The determination of these diverse compounds poses unique challenges, particularly using traditional mass spectrometry methods such as triple quadrupole mass spectrometry. While high-resolution mass spectrometry can identify a wide array of compounds, the lack of standardized reference materials makes quantification more challenging [5]. To address these difficulties, the Total Oxidizable Precursor (TOP) assay was developed, enabling the semi-quantification of unknown, mostly environmentally stable PFAS, that can be oxidized to known PFAAs under harsh oxidative conditions [6]. Generally, this assay oxidizes unknown or undetected precursors, converting them into quantifiable PFCAs [7]. However, recent research has shown that the TOP assay can also produce PFSAs like perfluorooctanesulfonate (PFOS) when adapted to sample extracts of biological matrices and sediment [8,9], contrary to prior assumptions. This has expanded the utility of the TOP assay to include the analysis of a broader range of PFAA precursors, which can then be detected using triple quadrupole mass spectrometry.

TOP assay applications have been demonstrated in various sample types such as sediment [10], natural water [11–13], wastewater [14], landfill leachate [15], shellfish [16], ducks [17], and human serum [18,19]. Notably, only a minority of these studies are related to biological samples, indicating a need for more investigations in biological matrices. Apart from contaminated areas, previous studies have reported relatively modest increases in PFAA concentrations following the TOP assay for environmental samples. However, simultaneously, recent reports have suggested that the unknown oxidizable PFAS can exhibit higher bioaccumulation factors than short-chain PFAAs [20]. This underscores the importance of conducting additional studies on biological samples to fully understand their potential implications.

Shorebirds are under threat worldwide [21] and in particular in the East Asian Australian Flyway (EAAF), where many species are in decline by up to 8 % per year [22–24]. The causes for these population declines can be diverse including habitat loss, habitat alterations, climate change but also pollution along the EAAF, especially in coastal China, has been suggested to play a role [25,26]. The coast of China is the most important replenishing area for shorebirds in the EAAF where they are feeding on the tidal mudflats on a diet of shellfish and other benthic invertebrates. At the same time, this coastline is heavily impacted by mollusc aquaculture [27] and industry, resulting in environmental contamination including PFAS [28]. This study aims to analyze PFAS and their precursors in samples obtained from stopover sites along the entire coast of China (shellfish) and in shorebirds captured at their overwintering site in Australia (shorebird blood and liver). A novel TOP assay method for PFAS precursors will be applied in this study [9], which is designed to oxidize small sample amounts without extensive sample preparation. This comprehensive approach will contribute to a deeper understanding of the prevalence of PFAA precursors in biotic samples along the EAAF and represents a first step in assessing the potential impact of these precursors on shorebirds along the EAAF.

2. Materials and methods

2.1. Chemicals and materials

Methanol (MeOH, LiChrosolv), tert-butyl methyl ether (MTBE, Suprasolv), fuming hydrochloric acid (HCl, p.a. 37 %) and sodium hydroxide (NaOH, EMSURE, ≥ 99.0 %) were obtained from Merck (Darmstadt, Germany). Potassium persulfate ($K_2S_2O_8$, 99.99 %), ammonium acetate (NH_4OAc, LiChropur) and ammonium formate were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonia (NH_3, solution 25 %, AnalaR NORMAPUR) was purchased from VWR (Fontenay-sous-Bois, France). 10 PFCAs perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTriDA), perfluorotetradecanoic acid (PFTDA), and 4 PFSAs PFOS, perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS) and perfluoroheptanesulfonic acid (PFHpS) were purchased from Wellington Laboratories Inc. (Guelph, Ontario, Canada). Perfluorooctane sulfonamide (PFOSA), 1 H,1 H,2 H,2H-perfluorohexanesulphonic acid (4:2 FTS) and perfluorooctane sulfonamidoacetic acid (FOSAA) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). 2-(N-Methylperfluorooctanesulfonamido) acetic acid (MeFOSAA), N-Methylperfluorooctanesulfonamidoethanol (MeFOSE), N-Ethyl-N-(2-hydroxyethyl) perfluorooctylsulphonamide (EtFOSE) were purchased from Chiron AS (Trondheim, Norway). 1 H,1 H,2 H,2H-perfluorooctanesulfonic acid (6:2 FTS), 1 H,1 H,2 H,2H-Perfluorodecanesulfonic acid (8:2 FTS), 1 H,1 H,2 H,2H-Perfluorododecanesulphonic acid (10:2 FTS), isotope labeled PFOA ($^{13}C_8$-PFOA), PFOS ($^{13}C_8$-PFOS) and 6:2 FTS ($^{13}C_2$-D_4-6:2 FTS) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). More details about PFAS can be found in Supplementary Material (Table S1). 15 mL PP tubes were purchased from VWR (Fontenay-sous-Bois, France).

2.2. Sampling

Shellfish (n = 30) samples were collected at 11 important stopover sites for shorebirds along the coast of China, from the southernmost of mainland China- Leizhou Peninsula to the northernmost - Liaodong Bay during the spring migration season for shorebirds in April and May of 2018 and 2019. All sampling locations were located on the bare mudflats, the main foraging area of the shorebirds in each site. At each sampling location, we collected more than 50 g of *Mactra veneriformis*, wrapped in aluminum foil and stored in a zip-lock bag (n > 8). All

samples were placed in a freezer at −20 °C within 12 h.

Bird samples were collected in Australia from three different migratory shorebird species (bar-tailed godwit - *Limosa lapponica*, curlew sandpiper - *Calidris ferruginea* and red-necked stint - *Calidris ruficollis*). These shorebirds use Chinese coastal wetlands as stopover sites during their biannual migrations between their high-latitude breeding grounds and Australian non-breeding grounds. Birds were captured along the coasts of Victoria, Australia, South Australia, and north-Western Australia using cannon-nets, as part of routine monitoring conducted by the Victorian Wader Study Group (VWSG) and Australasian Wader Study Group (AWSG). Blood (~200 μL) was sampled from the brachial veins of shorebirds using Sarstedt Microvette capillary tubes (Nümbrecht, Germany) that contained a clotting agent. Blood was left to clot, then separated into red blood cell (RBC) and serum. Twelve of blood samples were used in this study. Occasionally, cannon-netting may result in bird casualties and it was from these previously frozen birds that liver tissues were sampled (n = 25). Full sample details are presented in Supplementary Material (Table S2), including sample numbers per species and tissue type, locations and date ranges for each species and sample type. All sampling was conducted between December 2012 and February 2020, under the animal ethics and fauna permits relevant to each Australian state jurisdiction held by the VWSG and the AWSG.

2.3. Sample treatment- PFAS extraction

The biological samples were extracted using the method of Sait et al. [29] with minor modifications. Briefly, either ~50 mg of shellfish, ~50 mg of RBC, ~50 mg of bird serum or ~30 mg of bird liver was placed in a 1.5 mL PP tube, after which the internal standards (IS; $^{13}C_8$-PFOA, $^{13}C_8$-PFOS, $^{13}C_2$-D_4-6:2 FTS for shellfish, RBC and serum; $^{13}C_8$-PFOA and $^{13}C_8$-PFOS for liver) mixture was added. The samples were vortex-mixed for 1 minute, followed by 30 min of ultrasonication after adding 0.3 mL of MeOH containing 1 % ammonium formate. After centrifugation for 5 min at 3500 rpm, the supernatant was purified using a Hybrid SPE cartridge which was washed with 1 mL of MeOH. Finally, 150 μL of the resulting extracts were transferred into the injection vial using a 200 μL insert vial. All the extracts were stored at −20 °C until they were analyzed by UPLC-MS/MS.

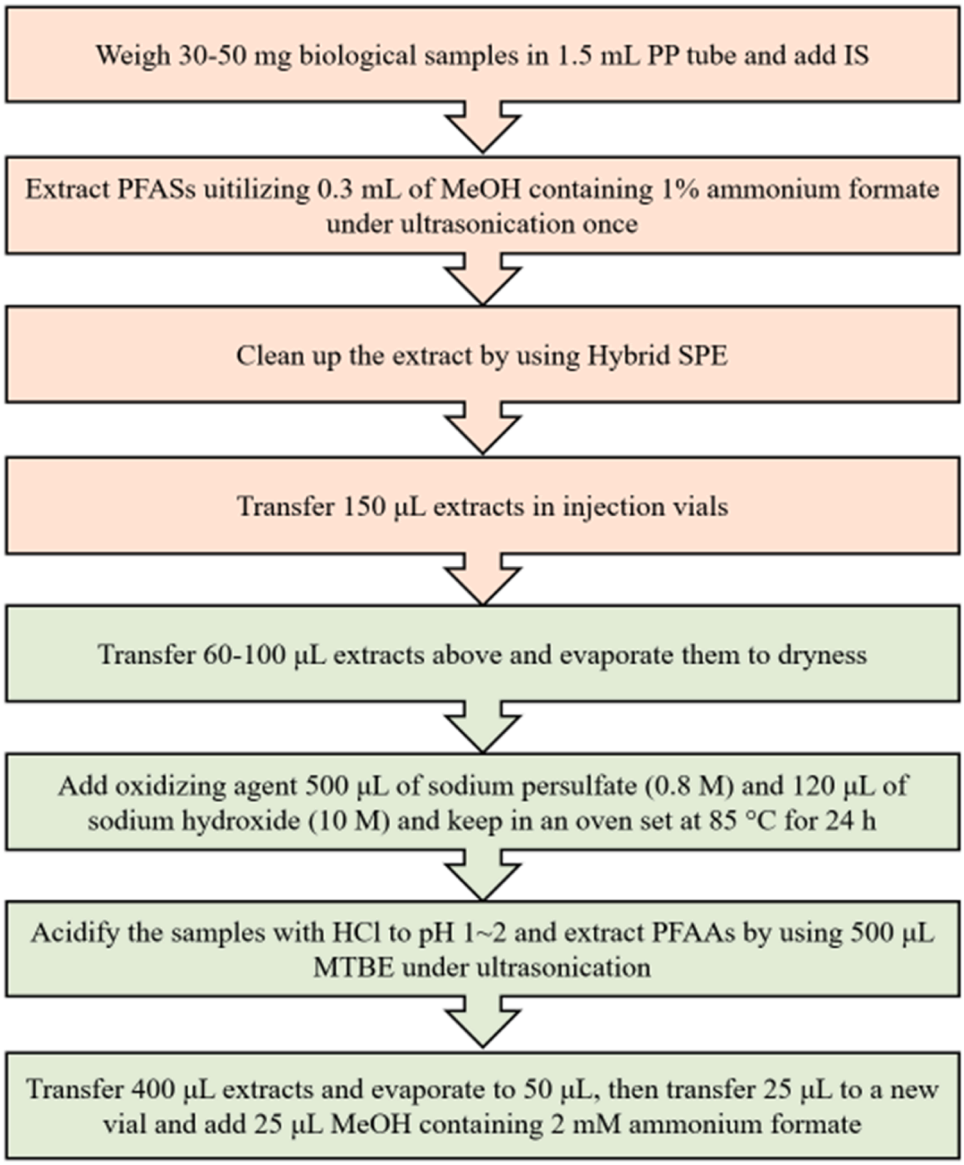


Fig. 1. The workflow of the sample preparation for PFASs extraction and TOP assay.

2.4. TOP assay

The TOP assay method used in this study was adapted from the method developed by Cioni et al. [9]. To summarize, 60–100 μL of the extracts obtained from the aforementioned samples were employed for the TOP assay. Before oxidation, ~100 μL of the extracts underwent an evaporation process to dryness, removing any residual methanol. Then, 500 μL of sodium persulfate (0.8 M) and 120 μL of sodium hydroxide (10 M) were added. The vials containing the samples and reagents were securely capped, subjected to vortex mixing, and subsequently placed in an oven set at 85 °C for a duration of 24 h. Following oxidation, the samples were acidified by adding 50 μL of concentrated HCl, achieving a pH range of 1–2 in each batch. Liquid-liquid extraction with MTBE was then employed. This involved adding 500 μL of MTBE to the samples, which were further subjected to vortex mixing and sonication for 10 min. Afterward, 400 μL of the organic phase was transferred to 2 mL glass vials. Subsequently, 50 μL of a 2 % ammonia solution in MeOH was added. The vials were left uncapped for approximately 2 h to allow for the evaporation of MTBE until the remaining volume reached 50 μL. Then 25 μL was transferred into a new insert vial and 25 μL MeOH containing 2 mM ammonium formate was added. A brief workflow for both PFAS extraction and TOP assay is presented in Fig. 1.

2.5. Instrumental analysis

The PFAS, including isotopically labeled compounds, were separated using a Kinetex C18 column (30 × 2.1 mm, 1.3 μm, 100 Å, Phenomenex) that was serially connected to a Phenomenex guard column (C18, 10 × 2.1 mm). This chromatographic setup was integrated into the ACQUITY UPLC system, which encompasses a binary pump, a sample manager, and a column manager (Waters, Milford, MA, USA). The detection was carried out using the Xevo TQ-S triple-quadrupole mass spectrometer in negative multiple reaction monitoring (MRM) mode (Waters, Milford, MA, USA). For the specifics of the mobile phase gradient, please refer to Table S3 in Supplementary material for comprehensive details. The flow rate employed during the analysis was set at 0.25 mL per minute, and each sample injection volume was 4 μL.

2.6. QA/QC

For each type of sample, two procedural blanks were meticulously executed to evaluate and address any potential contamination that might occur during the sample pretreatment. The recoveries of most of the target compounds across different extraction methods (pre-TOP and post-TOP) ranged from 60 % to 120 %, as detailed in Table S4&S5. To ensure the accuracy of the analysis, instrument blanks and residuals were scrutinized by periodically injecting pure MeOH every 20 injections. In preparation for and following each injection, the injection needle was meticulously cleansed with a specially formulated mixture solution comprising of MeOH, ACN (acetonitrile), IPA (isopropyl alcohol), and water in a 1:1:1:1 ratio. This rigorous cleaning regimen was enacted to minimize the likelihood of any residual effects that might influence subsequent analyses. The determination of PFAS concentrations in the samples relied on wide-ranging calibration curves spanning from 0.01 to 20 ng/mL. To establish the instrumental limits of quantification (iLOQs) for each target analyte, criteria such as achieving a signal-to-noise ratio greater than 10 and considering the lowest concentration from the calibration curves were employed (Table S6). Subsequently, the method limits of quantification (mLOQs) were computed by incorporating the iLOQs, the final reconsitution volume of the extraction method, and the average sample weight. These rigorous quality control measures were essential to ensure the precision and reliability of the results. In addition, it must be noticed that the IS $^{13}C_2$-D_4-6:2 FTS (chemical structure shown in Fig. S1) can be oxidized to PFPeA and PFHxA [9]. The yield rates of $^{13}C_2$-D_4-6:2 FTS to PFPeA (1.4 ± 0.12 ng) and PFHxA (1.5 ± 0.13 ng) in blank samples were 23.8 ± 2 % and 22.1 ± 1.8 %, respectively. For shellfish, RBC and serum, the final concentrations of PFPeA and PFHxA after TOP assay were corrected accordingly. No carbon labeled 6:2 FTS was added to liver samples as an internal standard, avoiding any unintended contribution to the formed PFCAs.

2.7. Data analysis

The instrument data were acquired and recorded using MassLynx 4.1, with subsequent data integration facilitated through TargetLynx. To evaluate the significance of differences between the datasets of PFAAs concentrations before and after TOP assay, a Paired *t*-test was applied, and results were considered significant when $p < 0.05$. Data < mLOD were substituted with 1/2 the mLOD. Since the moisture content of shellfish was not determined, the approximate moisture of these samples was obtained from literature. The moisture content in soft tissues of mollusks is approximately 80 % [30]. All sample concentrations are presented in terms of wet weight (w.w.).

3. Results and discussion

3.1. PFCAs increment after TOP assay

After the TOP assay, the concentrations of Sum PFCAs increased in all samples, but with varying degree (Fig. 2).

In shellfish samples, the concentrations of Sum PFCAs after the TOP assay (ranging from 0.83 to 75 ng/g, with a median of 6.5 ng/g) were significantly higher than those before the TOP assay (<0.036–28 ng/g, median: 3 ng/g) ($p < 0.05$) (Fig. 2a). The two compounds that exhibited the most substantial increases were PFHpA (median concentrations, before: <0.036 ng/g, after: 0.11 ng/g) and PFOA (before: <0.14 ng/g, after: 3.3 ng/g).

In bird blood samples, the concentrations of Sum PFCAs were significantly higher after the TOP assay compared to before the TOP assay in both RBC (before: 0.56–22 ng/g, median: 2.4 ng/g; after: 0.37–249 ng/g, median: 10 ng/g) and serum (before: 1.1–249 ng/g, median: 9 ng/g; after: 4.4–355 ng/g, median: 33 ng/g) (Table S7) ($p < 0.05$). The most notable changes were observed for PFPeA, PFHxA, and PFOA (Fig. 2b&2c). Before the TOP assay, both PFPeA and PFHxA were below the limit of detection (LOD), while after the TOP assay they were detected in three samples in RBC and five samples in serum at the highest concentrations of 135 ng/g (PFPeA in RBC) and 220 ng/g (PFPeA in serum). Before the TOP assay, only one sample in both RBC and serum contained PFOA, while after the TOP assay, PFOA could be detected in 9 RBC samples and in 11 serum samples, with the highest concentrations reaching 11 ng/g in red blood cells and 22 ng/g in serum.

In bird liver the concentrations of Sum PFCAs increased from < 0.072–126 ng/g (median: 2 ng/g) to 421–8268 ng/g (median: 3172 ng/g) after the TOP assay (Table S7). Notably, significant concentration increases were mainly observed for PFPeA (median concentration, before: <0.72 ng/g; after: 181 ng/g), PFHxA (before: <0.14 ng/g; after: 428 ng/g), PFHpA (before: <1.4 ng/g; after: 915 ng/g), PFOA (before: <0.14 ng/g; after: 1177 ng/g), PFNA (before: <0.14 ng/g; after: 293 ng/g), PFDA (before: <0.072 ng/g; after: 135 ng/g), and PFUnA (before: <0.072 ng/g; after: 14 ng/g) (Fig. 2d).

3.2. PFSA increment after TOP assay

Although it was previously assumed that the TOP assay only results in the conversion into PFCAs, also some increases in PFSAs were observed (Table S8). Taking a closer look at liver samples, the concentration of SumPFSAs was in the range of 2.8–312 ng/g (median: 32 ng/g) before the TOP assay. After the assay, the concentrations increased, with a range of 26–313 ng/g (median: 108 ng/g), and the highest

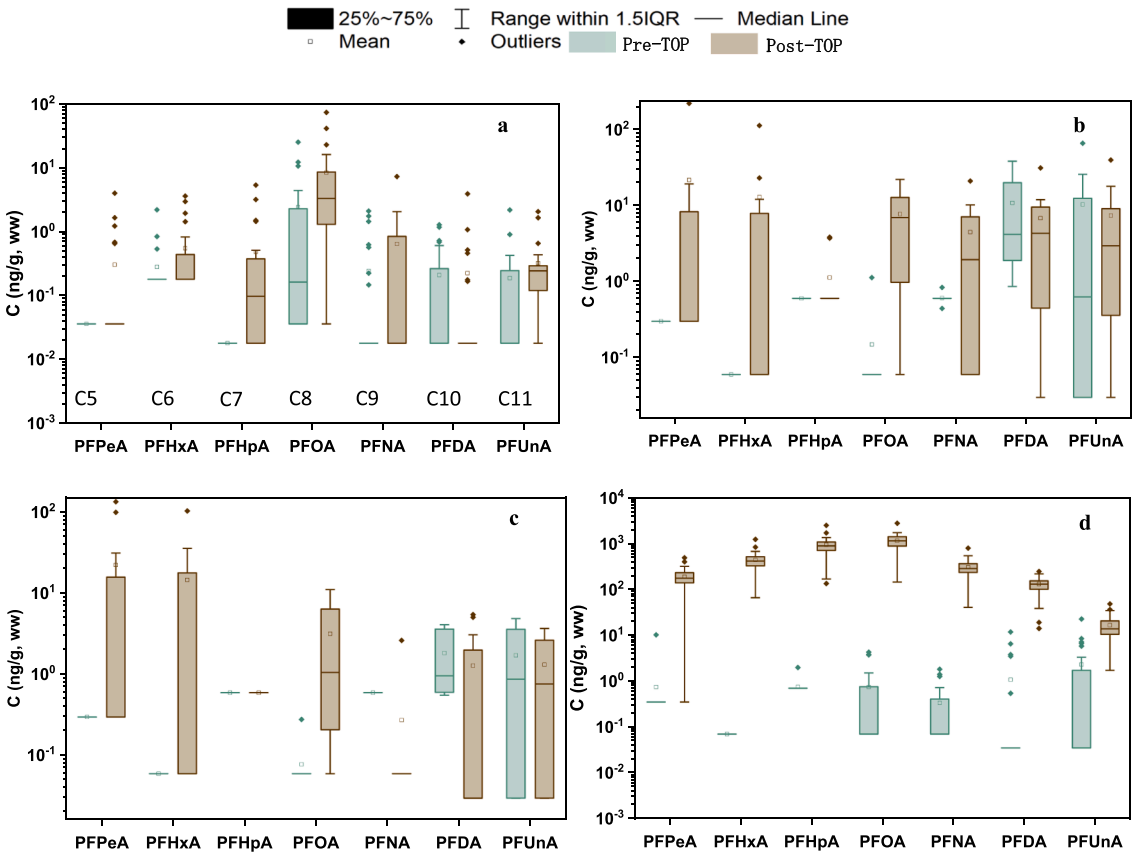


Fig. 2. Distribution of PFCAs concentrations before and after TOP assay in shellfish (a, n = 30), shorebird serum (b, n = 12), shorebird red blood cell (c, n = 12) and shorebird liver (d, n = 25).

increase was observed for PFBS concentrations after the TOP assay (median: 85 ng/g) compared to those before the assay (median: 9.9 ng/g) ($p < 0.05$) (Fig. 3). In addition to liver samples, slight increases in PFSAs were also observed in shellfish, RBC, and serum (Fig. 3). These results are in line with previous studies, which have reported similar findings. For instance, a study on textiles [8] documented an increase in the detection frequencies (DF) and concentrations of PFBS and PFHxS after the TOP assay. The pre-TOP assay data showed 50 samples with PFBS above the detection limit while post-TOP had 130 samples, with concentrations ranging from n.d. (not detected)-1.63 μg/m^2 before the assay and n.d.–12.1 μg/m^2 after the assay [8]. A similar trend was observed for PFHxS (pre-TOP: 58/160 in DF, n.d.–0.833 μg/m^2; post-TOP: 141/160 in DF, n.d.–2.84 μg/m^2) [8]. Another study reported that the precursors MeFOSAA and EtFOSAA spiked in human serum samples could be converted into PFOA (74 %), PFOS (17.8 %), PFHpA (3.3 %), and PFHxA (2.2 %) by the TOP assay [9]. These studies collectively suggest that precursors containing sulfonate groups can undergo conversion not only into PFCAs but also into PFSAs when subjected to the TOP assay. One plausible explanation is that precursors such as perfluorobutane sulfonamide may not be solely oxidized into PFBA but may also undergo hydrolysis, leading to the formation of PFBS [31]. This finding suggests the need for further research on the behavior of PFAA precursors during the TOP assay.

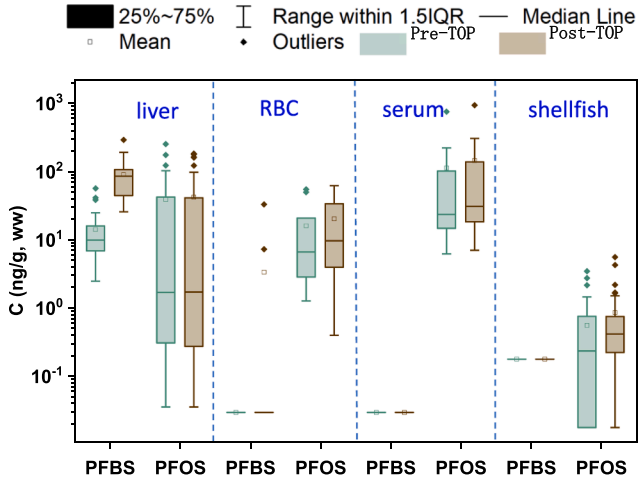


Fig. 3. Concentrations of PFBS and PFOS before and after TOP assay in shorebird liver (n = 25), RBC (n = 12), serum (n = 12), and shellfish (n = 30).

3.3. Fate of known oxidizable PFAS

Prior to the TOP assay, 4:2 FTS, 6:2 FTS, 8:2 FTS, 10:2 FTS, PFOSA, FOSAA, MeFOSAA, EtFOSE, MeFOSE, were detected across different sample types. In shellfish, RBC, serum and liver samples, the sum concentrations of these precursors varied (Fig. 4a). Shellfish had concentrations ranging from < 0.036–13 ng/g (with a DF of 29/30). RBC and serum had concentrations ranging from < 0.06–25 ng/g (DF 11/12) and from < 0.06–70 ng/g (DF 11/12). Liver samples exhibited concentrations from < 0.072–143 ng/g (DF 14/25) (Table S8). The concentrations of precursors after the TOP assay were observed to be lower than their pre-assay levels. The concentration in shellfish decreased to < 0.036–2.3 ng/g (DF 16/30), with 4:2 FTS and EtFOSE being the main precursors decreasing. 6:2 FTS was only found in 2 RBC and 1 serum after TOP assay, 8:2 FTS, PFOSA and MeFOSAA were all oxidized (Table S10). EtFOSE was the main oxidized precursor in liver (Table S8). This intriguing pattern strongly suggests that the precursors examined in

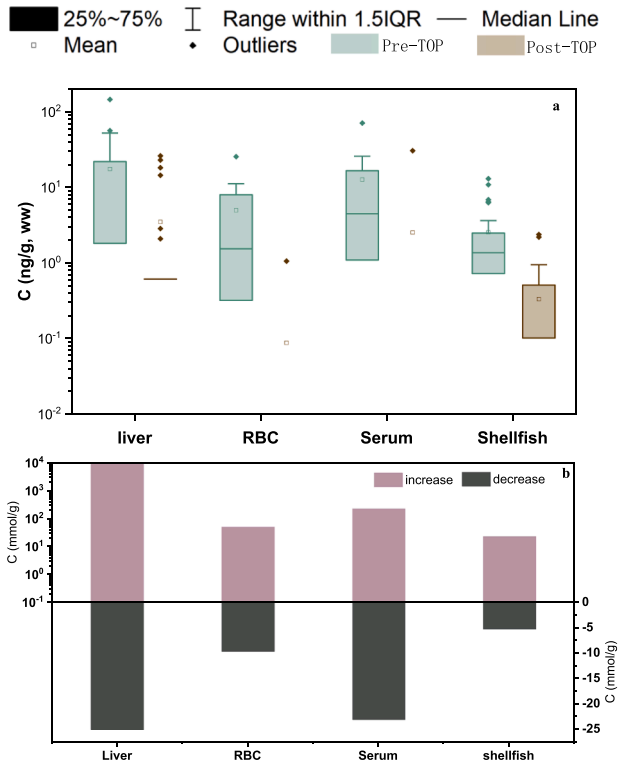


Fig. 4. Concentrations of precursors before and after TOP assay (a) and comparison of increase in PFAAs and decrease in precursors (b) in shorebird liver (n = 25), RBC (n = 12), serum (n = 12), and shellfish (n = 30).

this study can indeed be oxidized by the TOP assay. However, it's important to note that the oxidation process is not always entirely complete. For example, the concentration of PFOSA was < 0.036–7.5 ng/g (DF 14/30) before the TOP assay and was < 0.036–2.2 ng/g (DF 10/30) after the TOP assay in shellfish samples (Table S8). This observation suggests that the TOP assay is a semi-quantitative technique, providing an interesting avenue for future research.

The changes in PFAS before and after TOPA were quantified in millimoles per gram (mmol/g) because of their different molecular weights. The positive mean changes in PFAAs concentrations were 23 mmol/g in shellfish, 163 mmol/g in RBC, 225 mmol/g in serum, and an extreme value of 8936 mmol/g in shorebird liver (Fig. 4b). The 1 mmol precursors tested in this study can be converted to the same or fewer than 1 mmol PFAAs (Table S11) [32]. Hence, the relative mean changes in precursor concentrations were comparatively lower than for PFAAs. Shellfish showed declines of precursor concentrations of 5.2 mmol/g, RBC 9.6 mmol/g, serum 23 mmol/g, and the liver 25 mmol/g and the concentrations of PFCAs converted approximately from these precursors were 2.5, 7.8, 20 and 23 mmol/g (Table S12). This leads to a compelling conclusion: substantial amounts of unknown oxidizable PFAS are present in these samples, particularly in shorebird liver. The existence of these unidentified precursors underscores the complexity of the PFAS family and suggests the existence of a complex network of chemical and biological processes yet to be unraveled.

3.4. Comparison of different sample types in this study and other studies

The assessment of ΔPFAAs across various sample types reveals fascinating insights into the relationships of precursors among different sample matrices and the presence of both known and unknown oxidizable PFAS. Principal component analysis based on ΔPFAAs indicated that shellfish are part of the shorebirds' diet, while the distribution of precursors in liver and blood showed distinct patterns (Fig. 5a).

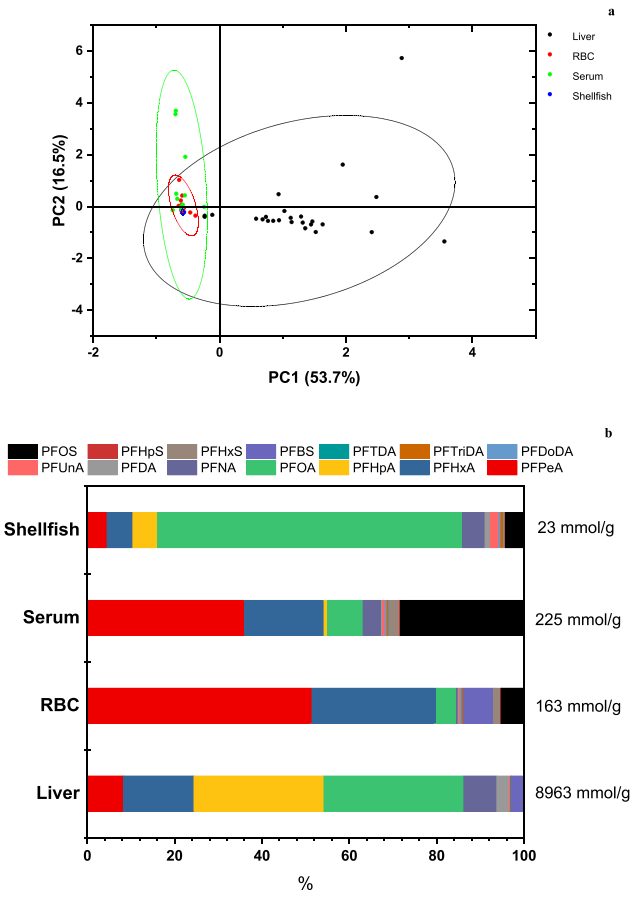


Fig. 5. Principle component analysis (a) and composition (b) of ΔPFAAs (in mmol/g) after TOP assay in shellfish (n = 30), shorebird serum (n = 12), RBC (n = 12), and liver (n = 25).

Shorebirds consume a variety of foods such as shellfish and seaworms [33], contributing to the ingestion of PFAA precursors. The differences in ΔPFAA distribution are attributed to (1) the fact that liver and blood samples are not from the same birds, and (2) variations in PFAS distribution between liver and blood, since precursor metabolization to PFCAs and PFSAs happens in the liver [34]. In detail, the highest ΔPFAAs were observed in liver samples, followed by serum, RBC and shellfish (Fig. 5b). This suggests an efficient biological uptake from prey followed by possible accumulation and/or biotransformation of precursors and oxidizable PFAS in bird liver. To support this notion, a previous study noted significantly higher bioaccumulation factors for certain precursors like MeFOSAA, EtFOSAA, and PFOSA compared to PFCAs (<C9) and PFSAs (<C8) in freshwater mussels [20]. The liver, rich in blood and proteins, is acknowledged as the main target organ for PFAS accumulation, likely also accumulating higher concentrations of other unknown PFAS. This finding underlines the role of bird liver as a potential hotspot for precursor accumulation and/or potentially high metabolic transformation of different PFAS. Moreover, the majorly formed PFAAs differed across sample types. In shellfish, PFOA emerged as the primarily formed PFAAs. Serum samples displayed a prevalence in increase of PFPeA, PFHxA, and PFOS, while in liver samples mostly PFOA and PFHpA were formed. This intriguing variation strongly suggests that different types of precursors are present in the different compartments due to differing toxicokinetics and exposures. Some precursors are known to yield various oxidized products [32]. For instance, MeFOSA's primary oxidized product is PFOA (84 %), EtFOSAA undergoes conversion, mainly leading to PFOA (>60 %), while 10:2 FTS

can be oxidized into PFHxA (6 %), PFHpA (14 %), PFOA (16 %), PFNA (29 %), and PFDA (28 %) [32] after the TOP assay [35].

Comparing the relative ΔPFCAs (%) in our study with those reported in other relevant studies offers valuable insights into the significance and uniqueness of our findings. In our investigation, the ΔSumPFCAs was 182 % in shellfish, 583 % in RBC, 122 % in serum, and a staggering 18,1 % in liver (Fig. 6). Notably, our liver samples exhibited even higher ΔPFCAs (%) after TOP than samples collected from aqueous film-forming foam contaminated areas [36], for which the ΔsumPFCAs was 11,15 % in deep soil and 5063 % in surface soils (Fig. 6). In comparison, bream collected from three major streams in Germany (Rhine, Elbe, Danube) and wild boar collected from three German areas had a ΔPFCAs (%) in liver of 696 % [16] and 127 % [37] respectively, which are much lower than the bird liver in this study. This discrepancy could be attributed to the mobility and habitat of the species studied, as well as possible difference in species-specific bioaccumulation and biotransformation capacity. The freshwater bream and the wild boar, characterized by limited movement, exhibited relatively low ΔPFCAs (%) possibly due to their more localized exposure to PFAS precursors. In contrast, the migratory shorebirds here sampled, known for their extensive travel, have greater opportunities for ingesting PFAAs precursors along the highly polluted East-Asian coast [28,38]. Lastly, the absence of the formation of PFAAs with a longer perfluorinated carbon chain length than PFOS in the present study points consequently to the absence of precursors and/or unknown oxidizable PFAS with perfluorinated carbon chain lengths longer than 8 carbons.

4. Conclusion

It is essential to acknowledge the limitations of this study. While this research employed a non-direct TOP assay, which has its advantages, it's noteworthy that alternative methodologies, such as direct TOP, have been explored in prior studies [39]. For instance, research has compared the results of oxidizing extracts with those of the whole samples in soil and reported that the concentration of PFHxA increased in the whole samples following oxidation, whereas no significant differences were noted for PFOA. The choice of assay type should be informed by various factors, including sample volume and the amount of material available, which can limit the application of direct TOP. In addition, it's worth noting that this study did not encompass the full range of short-chain PFAAs, including compounds like perfluorobutanoic acid (C4) and perfluoropropionic acid (C3). These short-chain PFAAs can also potentially originate from oxidized precursors. Consequently, there is a need for further exploration and comprehensive studies to gain a more comprehensive understanding of the relationship between precursors and PFAAs in biological matrices, especially in (bird) liver.

The findings of this study underscore the significance of the TOP assay, particularly when dealing with biological samples. While the TOP assay was introduced in 2012 [6], its application has been somewhat limited. One contributing factor to this limited use is the historical perception that the increase in perfluoroalkyl acids (PFAAs) following the TOP assay is not substantial in environmental samples like surface water and soil. However, the notable rise in precursor concentrations observed in this study suggests the need for a reevaluation of the assay's significance in biological matrices. The substantial precursor concentrations in biological samples raise a red flag, with respect to migrating birds acting as biovectors of a broad range of PFAS as well as indicating the potential for higher exposure risks in wildlife, but also for human exposure by e.g. shellfish consumption. This is especially concerning because some precursors can undergo biotransformation [40], converting into PFAAs, and liver is the vital organ to metabolize pollutants [41], which might further elevate the associated exposure risks. Recent studies have indicated that PFAS concentrations were generally low in shorebirds in Australia [25], even at a wastewater treatment plant [26], but these may be underestimations since TOP assays were not performed in these studies and the total amount of PFAS shorebirds are exposed to

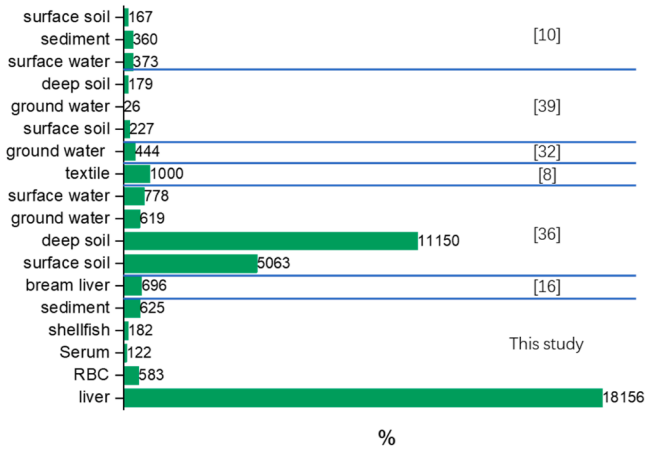


Fig. 6. Comparison of ΔPFCAs (%) after TOP assay in this study with earlier published literature.

may be much higher. Thus, our findings emphasize the necessity of more extensive investigations into the implications of the TOP assay, particularly in biota such as declining populations of shorebirds.

Environmental implication

Per- and polyfluoroalkyl substances (PFAS) are widely used in industry and pose risks to various organisms. The total oxidizable precursor (TOP) assay is an effective method for converting PFAS precursors into detectable perfluoroalkyl acids (PFAAs). This study applied the TOP assay to samples from the East Asian-Australian Flyway, including shellfish from China and shorebird tissues from Australia. Results showed significant increases in perfluorocarboxylic acids (PFCAs), particularly in shorebird livers, indicating numerous unidentified precursors. These findings highlight the need for further research into these precursors, their sources, and their ecological impacts on wildlife and humans.

CRediT authorship contribution statement

Lara Cioni: Writing – review & editing, Methodology. **Junjie Zhang:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **He-Bo Peng:** Writing – review & editing, Resources. **Tobias A. Ross:** Writing – review & editing, Resources. **Marcel Klaassen:** Writing – review & editing, Resources. **Dorte Herzke:** Writing – review & editing, Supervision. **Veerle L. B. Jaspers:** Writing – review & editing, Project administration. **Alexandros G. Asimakopoulos:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This project was funded by the Norwegian Research Council (NRC) project COAST IMPACT 302205/SHJ (to Veerle L. B. Jaspers) and by the Australian Research Council (ARC) Discovery Project Grant DP190101861 (to Marcel Klaassen) and the EU ITN project PERFORCE3 grant nr 860665 (to Lara Cioni and Dorte Herzke). PFAS extraction and determination before and after TOP assay were done in EnviroChemistry Lab at NTNU and the TOP assay in the Organic Lab at NILU, respectively. Bird samples were obtained with the assistance of the Global Flyway Network (GFN), Australasian Wader Studies Group (AWSG) and

Victorian Wader Study group (VWSG).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.137189.

Data availability

Data will be made available on request.

References

[1] Evich, M.G., Davis, M.J.B., McCord, J.P., Acrey, B., Awkerman, J.A., Knappe, D.R. U., et al., 2022. Per- and polyfluoroalkyl substances in the environment. Science 375, eabg9065.

[2] E.M. Sunderland, R. Lohmann, Evaluating the importance of precursor transport and transformation for groundwater contamination with PFAS, in, 2022.

[3] Ateia, M., Chiang, D., Cashman, M., Acheson, C., 2023. Total oxidizable precursor (TOP) assay-best practices, capabilities and limitations for pfas site investigation and remediation. Environ Sci Technol Lett 10, 292–301.

[4] Wang, Z., DeWitt, J.C., Higgins, C.P., Cousins, I.T., 2017. A never-ending story of per- and polyfluoroalkyl substances (PFASs)? Environ Sci Technol 51, 2508–2518.

[5] Shojaei, M., Kumar, N., Guelfo, J.L., 2022. An integrated approach for determination of total per- and polyfluoroalkyl substances (PFAS). Environ Sci Technol 56, 14517–14527.

[6] Houtz, E.F., Sedlak, D.L., 2012. Oxidative conversion as a means of detecting precursors to perfluoroalkyl acids in urban runoff. Environ Sci Technol 46, 9342–9349.

[7] Chiang, D., 2019. What Total Oxidizable Precursor Assay Can and Cannot Tell Us about Precursors of Perfluoroalkyl Acids. CDM Smith.

[8] Zhu, H., Kannan, K., 2020. Total oxidizable precursor assay in the determination of perfluoroalkyl acids in textiles collected from the United States. Environ Pollut 265, 114940.

[9] Cioni, L., Nikiforov, V., Coelho, A., Sandanger, T.M., Herzke, D., 2022. Total oxidizable precursors assay for PFAS in human serum. Environ Int 170, 107656.

[10] Meng, Y., Yao, Y., Chen, H., Li, Q., Sun, H., 2021. Legacy and emerging per- and polyfluoroalkyl substances (PFASs) in Dagang Oilfield: multimedia distribution and contributions of unknown precursors. J Hazard Mater 412, 125177.

[11] Chen, H., Zhang, L., Li, M., Yao, Y., Zhao, Z., Munoz, G., et al., 2019. Per- and polyfluoroalkyl substances (PFASs) in precipitation from mainland China: contributions of unknown precursors and short-chain (C2C3) perfluoroalkyl carboxylic acids. Water Res 153, 169–177.

[12] McGuire, M.E., Schaefer, C., Richards, T., Backe, W.J., Field, J.A., Houtz, E., et al., 2014. Evidence of remediation-induced alteration of subsurface poly- and perfluoroalkyl substance distribution at a former firefighter training area. Environ Sci Technol 48, 6644–6652.

[13] Gockener, B., Fliedner, A., Rudel, H., Badry, A., Koschorreck, J., 2022. Long-term trends of per- and polyfluoroalkyl substances (PFAS) in suspended particular matter from german rivers using the direct total oxidizable precursor (dTOP) assay. Environ Sci Technol 56, 208–217.

[14] Houtz, E.F., Sutton, R., Park, J.S., Sedlak, M., 2016. Poly- and perfluoroalkyl substances in wastewater: significance of unknown precursors, manufacturing shifts, and likely AFFF impacts. Water Res 95, 142–149.

[15] Wang, B., Yao, Y., Chen, H., Chang, S., Tian, Y., Sun, H., 2020. Per- and polyfluoroalkyl substances and the contribution of unknown precursors and short-chain (C2-C3) perfluoroalkyl carboxylic acids at solid waste disposal facilities. Sci Total Environ 705, 135832.

[16] Göckener, B., Fliedner, A., Rüdel, H., Fettig, I., Koschorreck, J., 2021. Exploring unknown per- and polyfluoroalkyl substances in the German environment – the total oxidizable precursor assay as helpful tool in research and regulation. Sci Total Environ 782.

[17] Guckert, M., Rupp, J., Nurenberg, G., Nodler, K., Koschorreck, J., Berger, U., et al., 2023. Differences in the internal PFAS patterns of herbivores, omnivores and carnivores - lessons learned from target screening and the total oxidizable precursor assay. Sci Total Environ 875, 162361.

[18] AC, M.F.C., Cioni, L., Van Dreunen, W., Berg, V., Rylander, C., Urbarova, I., et al., 2023. Legacy perfluoroalkyl acids and their oxidizable precursors in plasma samples of Norwegian women. Environ Int 178, 108026.

[19] Cioni, L., Plassmann, M., Benskin, J.P., Coelho, A., Nost, T.H., Rylander, C., et al., 2023. Fluorine mass balance, including total fluorine, extractable organic fluorine, oxidizable precursors, and target per- and polyfluoroalkyl substances, in pooled human serum from the Tromso population in 1986, 2007, and 2015. Environ Sci Technol.

[20] Pickard, H.M., Ruyle, B.J., Thackray, C.P., Chovancova, A., Dassuncao, C., Becanova, J., et al., 2022. PFAS and precursor bioaccumulation in freshwater recreational fish: implications for fish advisories. Environ Sci Technol 56, 15573–15583.

[21] Ma, Y., Choi, C.-Y., Thomas, A., Gibson, L., 2022. Review of contaminant levels and effects in shorebirds: knowledge gaps and conservation priorities. Ecotoxicol Environ Saf 242.

[22] Studds, C.E., Kendall, B.E., Murray, N.J., Wilson, H.B., Rogers, D.I., Clemens, R.S., et al., 2017. Rapid population decline in migratory shorebirds relying on Yellow Sea tidal mudflats as stopover sites. Nat Commun 8, 14895.

[23] Clemens, R., Rogers, D.I., Hansen, B.D., Gosbell, K., Minton, C.D.T., Straw, P., et al., 2016. Continental-scale decreases in shorebird populations in Australia. Emu - Austral Ornithol 116, 119–135.

[24] Murray, N.J., Marra, P.P., Fuller, R.A., Clemens, R.S., Dhanjal-Adams, K., Gosbell, K.B., et al., 2017. The large-scale drivers of population declines in a long-distance migratory shorebird. Ecography 41, 867–876.

[25] Ross, T.A., Zhang, J., Chiang, C.-Y., Choi, C.-Y., Lai, Y.-C., Asimakopoulos, A.G., et al., 2024. Running the gauntlet; flyway-wide patterns of pollutant exposure in blood of migratory shorebirds. Environ Res 246.

[26] Ross, T.A., Zhang, J., Wille, M., Ciesielski, T.M., Asimakopoulos, A.G., Lemesle, P., et al., 2023. Assessment of contaminants, health and survival of migratory shorebirds in natural versus artificial wetlands - the potential of wastewater treatment plants as alternative habitats. Sci Total Environ 904, 166309.

[27] Peng, H.B., Chan, Y.C., Compton, T.J., Cheng, X.F., Melville, D.S., Zhang, S.D., et al., 2021. Mollusc aquaculture homogenizes intertidal soft-sediment communities along the 18,400 km long coastline of China. Divers Distrib 27, 1553–1567.

[28] Wang, J., Shen, C., Zhang, J., Lou, G., Shan, S., Zhao, Y., et al., 2024. Per- and polyfluoroalkyl substances (PFASs) in Chinese surface water: temporal trends and geographical distribution. Sci Total Environ 915, 170127.

[29] Sait, S.T.L., Rino, S.F., Gonzalez, S.V., Pastukhov, M.V., Poletaeva, V.I., Farkas, J., et al., 2023. Occurrence and tissue distribution of 33 legacy and novel per- and polyfluoroalkyl substances (PFASs) in Baikal seals (Phoca sibirica). Sci Total Environ 889, 164096.

[30] Yang, R., Zhou, Q., Liu, J., Jiang, G., 2006. Butyltins compounds in molluscs from Chinese Bohai coastal waters. Food Chem 97, 637–643.

[31] L. Pontus, Total oxidizable precursor assay: Applications and limitations. A study on the occurrence of perfluoroalkyl and polyfluoroalkyl substances (PFASs) in Chinese Wastewater Treatment Plants, in, Orebro University, 2020.

[32] Martin, D., Munoz, G., Mejia-Avendano, S., Duy, S.V., Yao, Y., Volchek, K., et al., 2019. Zwitterionic, cationic, and anionic perfluoroalkyl and polyfluoroalkyl substances integrated into total oxidizable precursor assay of contaminated groundwater. Talanta 195, 533–542.

[33] Chan, Y.-C., Peng, H.-B., Han, Y.-X., Chung, S.S.-W., Li, J., Zhang, L., et al., 2019. Conserving unprotected important coastal habitats in the Yellow Sea: shorebird occurrence, distribution and food resources at Lianyungang. Glob Ecol Conserv 20.

[34] Robuck, A.R., McCord, J.P., Strynar, M.J., Cantwell, M.G., Wiley, D.N., Lohmann, R., 2021. Tissue-specific distribution of legacy and novel per- and polyfluoroalkyl substances in juvenile seabirds. Environ Sci Technol Lett 8, 457–462.

[35] Gockener, B., Eichhorn, M., Lammer, R., Kotthoff, M., Kowalczyk, J., Numata, J., et al., 2020. Transfer of Per- and Polyfluoroalkyl substances (PFAS) from feed into the eggs of laying hens. Part 1: analytical results including a modified total oxidizable precursor assay. J Agric Food Chem 68, 12527–12538.

[36] Casson, R., Chiang, S.-Y.D., 2018. Integrating total oxidizable precursor assay data to evaluate fate and transport of PFASs. Remediat J 28, 71–87.

[37] Rupp, J., Guckert, M., Berger, U., Drost, W., Mader, A., Nodler, K., et al., 2023. Comprehensive target analysis and TOP assay of per- and polyfluoroalkyl substances (PFAS) in wild boar livers indicate contamination hot-spots in the environment. Sci Total Environ 871, 162028.

[38] Singh, K., Kumar, N., Kumar Yadav, A., Singh, R., Kumar, K., 2023. Per-and polyfluoroalkyl substances (PFAS) as a health hazard: current state of knowledge and strategies in environmental settings across Asia and future perspectives. Chem Eng J 475.

[39] Houtz, E.F., Higgins, C.P., Field, J.A., Sedlak, D.L., 2013. Persistence of perfluoroalkyl acid precursors in AFFF-impacted groundwater and soil. Environ Sci Technol 47, 8187–8195.

[40] Nilsson, H., Karrman, A., Rotander, A., van Bavel, B., Lindstrom, G., Westberg, H., 2013. Biotransformation of fluorotelomer compound to perfluorocarboxylates in humans. Environ Int 51, 8–12.

[41] Zaefarian, F., Abdollahi, M.R., Cowieson, A., Ravindran, V., 2019. Avian Liver: the forgotten organ. Animals 9.