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Advanced throughput analytical strategies for the comprehensive HRMS screening of organic micropollutants in eggs of different bird species

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HIGHLIGHTS

- 26 eggs of Peregrine falcon, Eurasian curlew, Little and Eagle owl were analyzed.
- 58 organic pollutants were determined by LC- and GC-HRMS wide-scope target analysis.
- Highest frequency and concentration levels were observed for lipophilic compounds.
- PFOS was the most frequent and abundant compound among 13 detected PFAS.
- 50 substances were identified and semi-quantified through suspect screening.

GRAPHICAL ABSTRACT

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ABSTRACT

Raptors are ideal indicators for biomonitoring studies using wildlife in order to assess the environmental pollution in the terrestrial ecosystem, since they are placed in the highest trophic position in the food webs and their life expectancy is relatively long. In this study, 26 eggs of 4 bird species (Peregrine falcon, Eurasian curlew, Little owl and Eagle owl) collected in Germany, were investigated for the presence of persistent organic pollutants (POPs) and thousands of contaminants of emerging concern (CECs). Generic sample preparation protocols were followed for the extraction of the analytes and the purification of the extracts, and the samples were analyzed both by liquid (LC) and gas chromatography (GC) coupled to high resolution mass spectrometry (HRMS), for capturing a wide range of organic micropollutants with different physicochemical properties. State-of-the-art screening methodologies were applied in the acquired HRMS data, including wide-scope target analysis of 2448 known pollutants and suspect screening of over 65,000 environmentally relevant compounds. Overall, 58 pollutants from different chemical classes, such as plant protection products, per- and polyfluoroalkyl substances and medicinal products, as well as their transformation products, were determined through target analysis. Most of the detected compounds were lipophilic (logP>2), although the presence of (semi)polar

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contaminants should not be overlooked, underlying the need for holistic analytical approaches in environmental monitoring studies. p,p'-DDE, PCB 153 and PCB138, PFOS and methylparaben were the most frequently detected compounds. 50 additional substances were identified and semi-quantified through suspect screening workflows, including mainly compounds of industrial use with high production volume.

1. Introduction

Environmental pollution nowadays remains one of the most challenging problems worldwide, since aquatic, terrestrial and atmospheric ecosystems are daily exposed to a diverse and dynamically changing mixture of anthropogenic chemicals. Since these chemicals are distributed in the various environmental compartments and may cause adverse effects in the organisms and, subsequently, in humans, their determination by advanced analytical methodologies is crucial (Zaghloul et al., 2020). Aiming to address the environmental pollution and protect the human health, several EU legislations are currently in force, including policies on Persistent Organic Pollutants (POPs) (European Commision, 2004; European Commission, 2007), plant protection products (European Commmision, 2012) and the REACH Directive (European Commmision, 2006). Moreover, the Stockholm Convention on POPs, a multilateral environmental agreement, focuses on eliminating or restricting the production of POPs and setting up a system for reducing the use of chemicals with hazardous properties. Although the implementation of EU Directives has set Environmental Quality Standards (EQS) for POPs (European Commision, 2013), registered as substances with Persistent, Bioaccumulative and Toxic (PBT) properties, there are thousands of chemicals, which are not systematically monitored and are not included in any current regulation, known as "contaminants of emerging concern" (CECs) (Dulio and Slobodnik, 2009; Sauvé and Desrosiers, 2014; Thomaidis et al., 2012). Monitoring data on both POPs and CECs is a prerequisite for gaining insights on the actual environmental pollution and quality status of the ecosystems. Although data on the biomagnification of POPs in food webs, even after the establishment of mitigation measures, are available (de Wit et al., 2020), information on the presence and behavior of CECs and complex chemical mixtures in the existing literature is scarce. Established methodologies and routine analysis have been developed focusing on specific classes of organic micropollutants and, thus, only a limited number of compounds (i.e. <100) is determined (Badry et al., 2022a). Furthermore, the presence of (bio)transformation products is not evaluated using such targeted analytical methodologies, even if identifying chemical mixtures is critical for accomplishing a thorough risk assessment study (Badry et al., 2022b).

Although the available data on the occurrence, fate, and potential biomagnification of organic micropollutants within the aquatic food webs are plenty, the respective information for the terrestrial ecosystem is limited (Zhang et al., 2021). The chemical analysis of terrestrial and freshwater environmental compartments may provide insights on recent exposure, considering that the transport in these ecosystems is realized at a faster rate in contrast with the marine ecosystem. Therefore, higher intra-year variations in the terrestrial environment have been reported, compared to the marine environment (Bustnes et al., 2015). The presence of organic micropollutants is mainly investigated in abiotic matrices, such as water or sediments (Chiaia-Hernández et al., 2020). On the contrary, data on the occurrence and the possible biomagnification in biota, and especially apex predators, are rare (González-Rubio et al., 2021), although such data may provide actual exposure information, as well as insights into potential bioaccumulative properties of the detected substances (Espín et al., 2016).

The selection of sentinel species is critical, when developing the sampling strategy of a biomonitoring study using wildlife, especially when a species forages on both the aquatic and terrestrial food web or feeds on different trophic levels (Badry et al., 2020). Birds are suitable indicators for assessing local environmental quality, due to their unique biology, habits and physiology (Movalli et al., 2017). Raptors (birds of prey, owls, and scavengers) are sentinel species for biomonitoring studies of organic micropollutants, especially for chemicals with potential of persistent and bioaccumulative properties, due to their unique characteristics (Movalli et al., 2019). Raptors are apex predators with high life expectancy, their population can be monitored and quantified easily, they assimilate the exposure of pollutants over time and extensive areas and are easily captured, which facilitates sampling. Among the potential exposure pathways of raptors to contaminants, such as ingestion, inhalation and dermal contact, diet seems to be the predominant pathway, as they are placed in the upper trophic levels (Herzke et al., 2002). Among raptors, the most commonly used species in biomonitoring studies of organic micropollutants are the white-tailed eagle, the northern goshawk, the peregrine falcon and the tawny owl (Badry et al., 2020). Although there is no optimal all-purpose tissue and the selection of matrix should be decided based on the studied chemicals and the objectives of the study, the most commonly used matrices of analysis of raptor specimens are liver, blood, tissues, feathers, and eggs (Espín et al., 2016).

Since most raptors are protected species, lethal sampling is not legally or ethically authorized (Badry et al., 2020). In this context, the collection of raptor eggs, especially the unhatched ones, is a non-invasive sampling, which is ideal for biomonitoring studies, and especially of vulnerable species, due to the key characteristics of eggs as matrix of analysis (Eriksson et al., 2016; Movalli et al., 2017). Due to their high lipid content, eggs are appropriate indicators for the potential accumulation of lipophilic contaminants, such as polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), organochlorine (OC), pesticides (Elliott and Norstrom, 1998) and other chemicals such as Novel Flame Retardants (NFRs) and Per- and Polyfluorinated Substances (PFAS) (Ahrens et al., 2011; Holmström et al., 2010; Vorkamp et al., 2019). Moreover, eggs can spatiotemporally accumulate contaminants and, thus, can provide insights on long-time exposure in contrast to other type of matrices of analysis (Bourgeon et al., 2012; Morrissey et al., 2010).

For a comprehensive risk assessment of organic micropollutants in wildlife, the development of effective and precise analytical methods is required. Several studies in Europe have so far reported the bioaccumulation of legacy POPs and PFAS in raptor eggs, mainly in peregrine falcon and tawny owl specimens, however, the information on the presence of multi-analyte and multi-class CECs is limited (Oró-Nolla et al., 2021). There are various biomonitoring surveys aiming to the investigation of PFAS concentration levels and temporal trends of PFAS in raptor eggs, such as peregrine falcon eggs gathered from Sweden (Holmström et al., 2010), Greenland (Vorkamp et al., 2019) and tawny owl eggs collected from Norway (Ahrens et al., 2011; Bustnes et al., 2015). Furthermore, Eriksson et al., 2016 analyzed 60 raptor (osprey, common kestrel and tawny owl) eggs from terrestrial and freshwater ecosystems in order to assess the PFAS composition profiles in the different species. The presence of brominated flame retardants in peregrine falcon eggs, gathered from Canada and Spain, has been studied by Guerra et al. (2012), while the temporal trends of polybrominated diphenyl ethers (PBDEs) in American peregrine falcon eggs has been revealed by Chen et al. (2008) and Newsome et al. (2010). In addition, the comprehensive analysis of POPs, such as organochlorine pesticides, PCBs and mercury (Hg), in peregrine falcon eggs, which were found dead in the state of Baden - Württemberg in Germany between 1955 and 2011 was performed by Wegner et al. (2005) and Schwarz et al. (2016). Recent developments in analytical techniques enabled the application of

wide-scope target analysis techniques based on high resolution mass spectrometry (HRMS) coupled to both liquid (LC) and gas chromatography (GC) that allows the simultaneous quantification of a large set of chemicals (i.e. >2400) within each sample (Badry et al., 2022b; Diamanti et al., 2020; Nika et al., 2020). Moreover, HRMS data are accessible anytime for retrospective suspect screening of thousands of chemicals, without the need of additional analysis (Gago-Ferrero et al., 2020).

The overall aim of the current biomonitoring study was the investigation of the presence of thousands of organic micropollutants, including POPs and CECs in eggs of raptors and Eurasian Curlew (*Numenius arquata*, a species threatened with extinction, following state-of-the-art analytical HRMS methodologies. In this context, 26 eggs of 4 bird species were gathered from the state Baden-Württemberg in Germany covering the time period 2005–2020. Generic sample preparation protocols were followed for the extraction of polar and non-polar chemicals and the final extracts were analyzed by liquid (LC) and gas chromatography (GC) coupled to a hybrid high resolution mass analyzer. The HRMS chromatograms were comprehensively treated with two data processing workflows: (a) wide-scope target analysis, using datasets of 2448 known organic micropollutants (including medicinal products, plant protection products, industrial chemicals and their (bio)transformation products) for which reference standards are available, and (b) suspect screening, using the merged NORMAN suspect list of over 65,000 environmentally relevant compounds.

2. Materials and methods

2.1. Chemicals and reagents

All solvents used for the extraction of the analytes were High Performance Liquid Chromatography (HPLC) grade and were obtained from different vendors. Methanol (MeOH), acetonitrile (ACN), isopropanol (iPrOH) and hexane (analytical reagent grade) were purchased from Fischer Scientific (Loughborough, UK), while ethyl acetate ≥99.5% (GC) and dichloromethane (DCM) were purchased from Sigma Aldrich (Steinheim, Germany). Ultrapure water (18.2 MΩ*cm^{-1}) was generated by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). The Cellulose Extraction filters, and Diatomaceous Earth used in Accelerated Solvent Extraction (ASE) were obtained from Thermo Scientific (Waltham, Massachusetts, USA), whereas sodium sulfate (Na_2SO_4) used as sample dispersant was purchased from Sigma Aldrich (Steinheim, Germany). In terms of the purification of the LC extracts using in-house Solid Phase Extraction (SPE) cartridges, empty SPE propylene tubes (6 mL), along with the respective frits (20 μm, 6 mL) were purchased from Phenomenex (Torrance, USA). Regarding the sorbent materials: Sepra ZT (Strata-X), Sepra ZT-WCX (Strata-X-CW) and ZT-WAX (Strata-X-AW) were purchased from Phenomenex (Torrance, USA), while Isolute ENV+ was acquired from Biotage (Ystrad Mynach, UK). For the purification of GC extracts, Strata® FL-PR Florisil cartridges [(170 μm, 80 Å), 5 g/20 mL, Giga Tubes] purchased from Phenomenex (Torrance, USA) were used. Regenerated cellulose (RC) syringe filters (diameter 15 mm, pore size 0.2 μm) were obtained from MACHEREY-NAGEL GmbH & Co. KG (Düren, Germany). Methanol, acetonitrile and isopropanol (LC-MS grade), used during LC-HRMS analysis were obtained from Merck (Darmstadt, Germany), whereas hexane and acetone (for pesticides analysis) were obtained from Honeywell (New Jersey, USA). Ammonium formate, ammonium acetate (LC-MS grade) and formic acid 99% were purchased from Sigma-Aldrich (Steinheim, Germany) while ammonia solution 25% for analysis was purchased from CHEM-LAB NV (Zadelgem, Belgium). The full list of internal standards (IS) and reference standards used in the current biomonitoring study are listed in **Supplementary Information, Section S1**.

2.2. Sampling

Overall, 26 eggs were gathered in the state Baden-Württemberg, Germany, within 2005–2020, and stored in glass bottles. Eggs from 4 different species were collected: Peregrine falcon (*Falco peregrinus*) (PF, n = 9), Eurasian curlew (*Numenius arquata*) (EC, n = 9), Little owl (*Athene noctua*) (LO, n = 6) and Eagle owl (*Bubo bubo*) (EO, n = 2). All samples were lyophilized, homogenized and stored frozen (−80 °C) in amber glass vials before analysis. Sample metadata, including %water content and GIS information, are available in Table S2-1, while the sampling locations are depicted in Fig. 1.

2.3. Analytical methodology

For the simultaneous extraction of thousands of organic micropollutants covering a wide range of physicochemical properties, two generic sample preparation protocols were followed for polar to semi-polar and non-polar organic micropollutants, based on a previous study (Badry et al., 2022b). Briefly, Accelerated Solvent Extraction (ASE) was used for the extraction of the analytes from the freeze-dried matrices followed by a clean-step using Solid Phase Extraction (SPE) in both protocols. An overview of the sample preparation protocols is depicted in Figure S3-1. The analysis of the final extracts was conducted utilizing two complementary chromatographic techniques (LC and GC) coupled with HRMS. For the determination of polar to semi-polar pollutants an ultra-High Performance Liquid Chromatography (UHPLC) system (UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany) coupled to a Quadrupole-Time of Flight Mass Spectrometer (QTOFMS) (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used. The QTOF-MS apparatus consisted of an Electrospray Ionization (ESI) source operating in positive and negative mode. The non-polar compounds were determined by GC-APCI-QTOF, consisted of a CP-8400 autosampler and a Bruker 450 GC, hyphenated to QTOF-MS with an Atmospheric Pressure Chemical Ionization (APCI) source operating in positive mode. The instrumental analysis parameters are provided in detail in **Section S3.**

The wide-scope target screening was conducted utilizing 3 in-house datasets including 2448 organic micropollutants (for LC-ESI (+)-QTOFMS analysis, LC-ESI(−)-QTOFMS and GC-APCI(+)-QTOFMS). Overall, these databases include 1392 medicinal products (pharmaceutical, veterinary, and illicit drugs) along with their transformation products (TPs), 762 plant protection products and their TPs, more than 200 industrial chemicals -including 56 Per- and Polyfluoroalkyl Substances (PFAS)- and other compounds of different classes such as artificial sweeteners and preservatives. The LC- and GC- amenable organic micropollutants included in the in-house target lists are available as "S21 UATHTARGETS" in the NORMAN Suspect List Exchange https://www.norman-network.com/nds/SLE/(https://doi.org/10.5281/zenodo.3723478) and "S65 UATHTARGETSGC" (https://doi.org/10.5281/zenodo.3753372), respectively.

The data treatment was performed using TASQ Client 2.1 and DataAnalysis 5.1 (Bruker Daltonics, Bremen, Germany) software. The detection of the organic micropollutants was based on strict identification thresholds; mass accuracy (<2 mDa), isotopic fitting (mSigma<100, only for confirmation of positive findings), retention time (<0.20 min) and fragmentation pattern match were applied during the screening process. The quantification of the detected organic micropollutants was performed using the standard addition method and representative structurally related isotope-labeled compounds (Internal Standards, IS) (Gago-Ferrero et al., 2020) and results were expressed in wet weight (w.w.). For the quantification experiments, a pooled sample, composed of the less contaminated individual samples, in terms of the number of the detected compounds and the concentration levels of the detected chemicals, was used. For the detected contaminants, compound-specific LODs/LOQs are provided in Table S4-1. The organic micropollutants that were detected in traces, below the LOQ

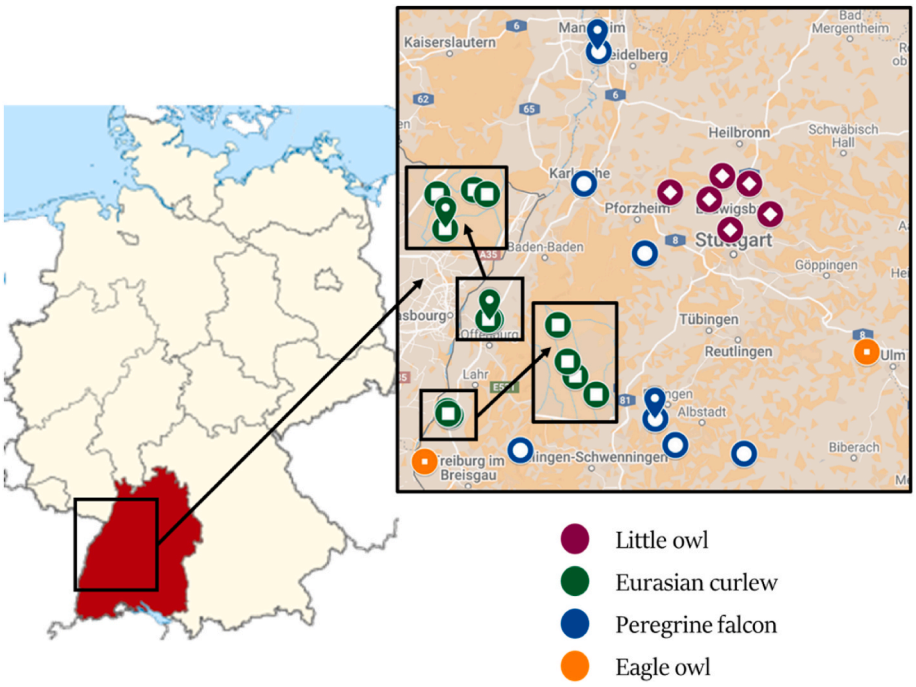

Fig. 1. Sampling locations of the bird eggs samples, used in the current study, in the south Germany (Baden-Württemberg). The pins represent two egg samples, collected from the same sampling location (same coordinates).

(concentration levels between the LOD and LOQ values), were reported as BQL (below the quantification limit). For the statistical treatment of the results, substitution of BQL with LOQ/2 was performed, as indicated by the QA/QC Directive (2009/90/EC).

All the HRMS chromatograms were digitally archived into NORMAN Digital Sample Freezing Platform (DSFP), a cutting-edge chemometric tool developed for investigating the presence of suspect and the identification of unknown compounds in environmental samples (Alygizakis et al., 2019). Suspect screening of more than 65,000 environmentally relevant, included in NORMAN SusDat (https://www.norman-network.com/nds/susdat/), was perform in DSFP. The calibrant masses were used to recalibrate each HRMS chromatogram using HPC fitting algorithm, which is embedded in DataAnalysis 5.1 (Bruker Daltonics, Bremen, Germany) for ensuring mass accuracy below 2 mDa for *m/z* 50-1000. CompassXport 3.0.9.2 (Bruker Daltonics, Bremen, Germany) was used for exporting files in mzML format, and the broadband collision-induced dissociation (bbCID) data were separated in low and high collision energy layer chromatograms, before their upload in the platform, along with their meta-data (instrumental, sample meta-data, matrix-specific meta-data, and retention time of Retention Time Index (RTI) calibrant substances). DSFP has integrated standard operating procedure (SOP) to process the mzML files and all meta-data for the generation of excel-based Data Collection Templates (DCTs), which include condensed information from LC-HRMS files. The substances, included in the DCTs, were evaluated, the false negatives, the target substances and the naturally occurred compounds (e.g. amino acids, vitamins, fatty acids) were removed from the results, before the semi-quantification of the tentatively identified organic micropollutants, using a novel quantitative structure-property relationship (QSPR)-based chemometric tool (Aalizadeh et al., 2022).

2.4. QA/QC

A thorough quality assurance and quality control (QA/QC) protocol was applied during the sample preparation and instrumental analysis to minimize and evaluate potential losses of analytes during extraction, to trace any potential laboratory contamination and to assure the good performance of instrumental analysis. A mix of isotopically labeled compounds was added into each sample prior to extraction to assure satisfactory recovery of the target compounds. Moreover, spiked samples of three concentration levels, with a mix of known CECs and POPs, were also analyzed in each batch of samples. A procedural blank (reagent blank) was prepared to assess any external contamination with every batch of samples, which might have been brought in during the sample preparation of the extracts and analysis. A mix of known analytes (RTI calibrant substances) was used to assess the stability of retention time during instrumental analysis. Two mixtures were prepared for LC-HRMS analysis (for positive and negative ESI detection modes), containing 18 compounds each (Aalizadeh et al., 2021), while for GC-HRMS a mix of n-alkanes was used, composed of 16 compounds from n-pentadecane to n-triacontane. In addition, solvent blanks using MeOH: H_2O (50:50 v/v) for LC and hexane for GC were injected between samples to monitor carryover and background contamination. A QC sample (standard solution of a mix of contaminants) was injected after every 10 injections in order to check instrumental drift, ensure good operation and high sensitivity of the instrument.

3. Results

3.1. Wide-scope target analysis results

Overall, 58 organic micropollutants were determined through LC- and GC-HRMS wide-scope target analysis in the 26 tested eggs samples. The wide-scope target analysis results are summarized in Table S4-2. The detected organic micropollutants were classified in 6 different chemical classes, based on their main use. The majority of the detected compounds were plant protection products (PPPs) and TPs (28%, n = 16), and per- and polyfluoroalkyl substances (PFAS) (22%, n = 13), as depicted in Fig. 2, whereas the contribution of the rest classes was as follows: medicinal products (MPs) and TPs (21%, n = 12), industrial chemicals (19%, n = 11), personal care products (PCPs) (7%, n = 4), tobacco related CECs and TPs (3%, n = 2). The dominance of PPPs could be linked to the dietary habits of the species used in the current study, which breed mainly in agricultural habitat and, thus, are known as both

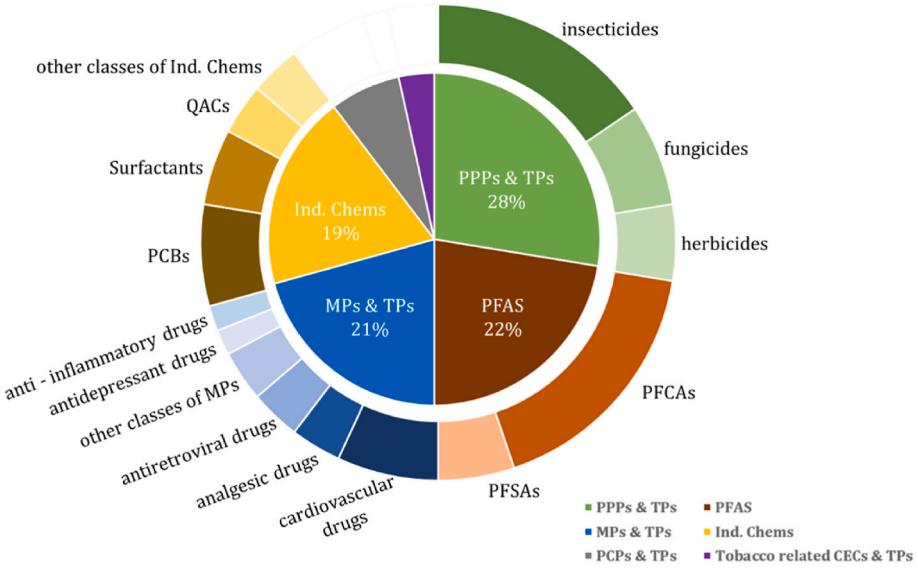

Fig. 2. Classification of the detected compounds based on their main use. Abbreviations: PPPs: Plant Protection Products, PFAS: Per- and Polyfluoroalkyl Substances, MPs: Medicinal Products, Ind. Chems: Industrial Chemicals, PCPs: Personal Care Products, PFCAs: Per fluorinated carboxylic acids, PFASs: Per fluorinated sulfonic acids, TPs: Transformation Products.

insectivorous and herbivorous species (Badry et al., 2020; Gómez-Ramírez et al., 2012). Due to the ubiquitous presence of PFAS in the environment, their PBT properties and the potential harmful effects in the human health, PFAS are the most studied class of organic micropollutants in raptor specimens (González-Rubio et al., 2021). 5 organic micropollutants were the most frequently detected in the tested egg samples, being present in more than half of the analyzed samples. The majority of these chemicals are known for their PBT properties. The highest % frequency of appearance (%FoA) was observed for p,p'-DDE (%FoA: 100), a breakdown product of the organochlorine pesticide DDT, whereas the polychlorinated biphenyl congeners PCB 153 and PCB 138 (%FoA: 54 and 69, respectively) and the perfluorooctanesulfonic acid (PFOS) (%FoA: 88) have also been determined in the majority of the tested egg samples (Figure S4-1). Moreover, the known for its ubiquitous presence in the environment personal care product methylparaben was present in 24 samples (%FoA: 92).

The majority of the determined chemicals through wide-scope target analysis were lipophilic, as depicted in Fig. 3 (Table S4-2). Furthermore, among the detected organic micropollutants the highest %FoA and maximum detected concentrations, were observed for chemicals with high logP>2 (strong lipophilic character), reinforcing the statement that mainly lipophilic compounds are accumulated in eggs. However, based on the results, the presence of (semi)polar organic micropollutants should not be neglected and therefore a holistic analytical approach for the determination of contaminants with a broad range of physico-chemical properties is required to reveal the overall chemical profile of organic micropollutants in birds.

3.1.1. *Polychlorinated biphenyls (PCBs)*

Non-dioxin-like PCBs, regulated under the Stockholm Convention, were frequently detected in the tested eggs. Four, out of the seven congeners, included in the target list, PCB 28, 138, 153 and 180 were present in the tested samples and their %FoA, as well as cumulative concentrations are illustrated in Fig. 4. PCB 138 and 153 were the most frequently determined compounds among PCBs (n = 18 and 14, respectively), while the rest PCBs presented %FoA less than 25. The

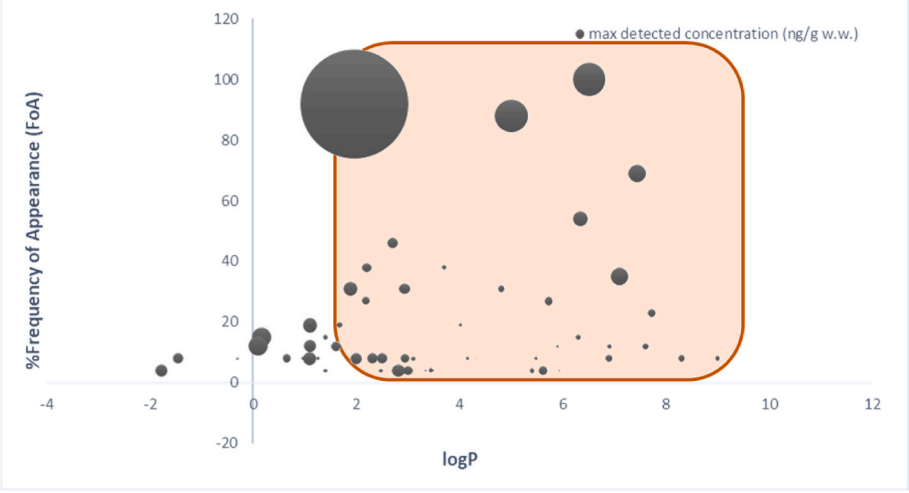

Fig. 3. The lipophilicity (logP) of the determined organic micropollutants through wide-scope target analysis is summarized in the figure. The most frequently detected compounds, along with the highest maximum concentrations were observed for micropollutants with high lipophilic character (log P > 2, marked spot in the figure).

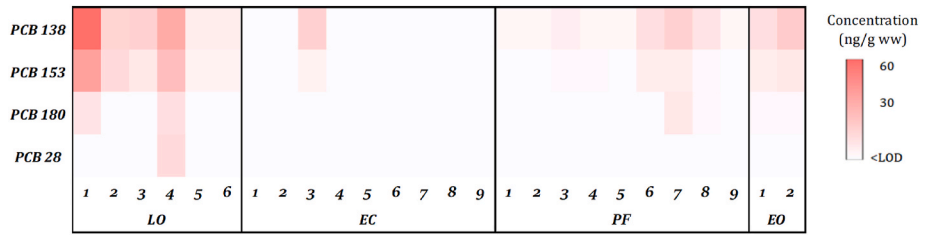

Fig. 4. Detection of non-dioxin-like PCBs in bird eggs. The concentration levels are visualized as heatmap. White tiles in the heatmap refer to concentrations below the limit of detection (LOD).

same detection trend was also observed in a previous study, analyzing eggs from different raptor and owl species collected in Czech Republic, where PCB 153 was the most abundant followed by PCB congener 138 and 180 (Kubistova et al., 2003). PCB 153 has also been reported in common guillemot (*Uria aalge*) eggs collected in Northern Europe (Jörundsdóttir et al., 2009). The higher levels of high-chlorinated congeners compared to low-chlorinated compounds could be explained by the tendency of the former to accumulate in the body. The sample with the highest total concentration of PCBs (90.2 ng/g w.w.) was the LO1 egg, gathered from Affalterbach in 2020, while the total mean concentration of PCBs was 20.3 ng/g w.w. Generally, the eggs of little owl species presented significantly higher cumulative levels for PCBs compared to those of Peregrine falcon and Eagle owl, while only one out of nine eggs of Eurasian Curlew was contaminated by PCBs, as depicted in Fig. 4. However, it should be noted that Little owl, Peregrine falcon and Eagle owl eggs were recent samples, collected within 2019–2020, while the sampling of Eurasian curlew eggs covers the period from 2005 to 2019, therefore the sampling time may have affected the detection trend of PCBs.

3.1.2. Plant protection products (PPPs) and TPs

Thirteen plant protection products (PPPs) and their TPs were detected in the tested samples. Among them, two organochlorine pesticides (OCPs) and three carbamates were determined through wide-scope target analysis. OCPs are continuously monitored due to their known persistence in the environment, whereas monitoring data on their presence in apex predators, and specifically in raptor eggs, are of high importance due to their bioaccumulative properties and lipophilicity (Buck et al., 2020). The degradation product of both p,p'-DDT and Dicofol, p,p'-DDE was the most ubiquitous among the detected contaminants, being present in all tested samples, collected from 2005 to 2020. Although its parent compound, p,p'-DDT, has been banned in Europe since 1972 and was not detected in the monitored egg samples (LOD: 0.907 ng/g w.w.), p,p'-DDE was present in high concentration levels, reaching 163 ng/g w.w. in an Eagle owl egg, collected in 2020, reflecting the historical use of DDT-related compounds. The dominance of p,p'-DDE in the overall DDT-compounds profile has already been reported in previous studies (Sun et al., 2020), whereas the high detection frequency and the concentration levels are in accordance with those reported the literature (Mora et al., 2011; Potter et al., 2009; Strause et al., 2007). Furthermore, Hexachlorobenzene, included in Directive (2013)/39/EU, was detected in all Little owl eggs and in one Eagle owl egg, at a mean concentration of 8.07 ng/g w.w., which is below the Environmental Quality Standard (EQS) of 10 ng/g set for biota (fish). Three carbamate insecticides, isoprocarb, promecarb and 2,3,5-trimethacarb, were detected in two individual egg samples from Peregrine falcon and Little owl specimens. Raptors are incidentally exposed in the carbamate insecticides, due to their use as antiparasitic drugs in livestock, and the monitoring of these compounds is crucial due to their lethal properties (Espín et al., 2016). The insect repellent DEET, presented high FoA of 27% in the tested samples, with mean concentration at 3.92 ng/g w.w. This finding is in agreement with the study of Wicke et al. conducted in Germany in 2014–2015, where the occurrence of 106 micropollutants in urban stormwater runoff of Germany was investigated (Wicke et al., 2021). DEET was among the 11 detected plant protection products, being present in the samples of all different tested species and was detected in more than half of the analyzed samples (FoA: 52%). Phthalamic acid, the transformation product of folpet and phosmet was detected in three samples with a mean concentration below the method LOQ (29.8 ng/g w.w.). Phosmet which is a broad-spectrum insecticide, has been recently banned in EU through the Regulation (EU) 2022/94, where the renewal of this active ingredient was not approved. 2,4-Dinitrophenol (DNP), which is used both as an herbicide and a fungicide, was detected only in one out of four investigated species (Eurasian curlew), with high frequency. The concentration levels (mean concentration: 2.67 ng/g w.w.) were comparable with the reported levels in common guillemot (Uria aalge) eggs collected in Ireland and Wales (2.2 ng/g w.w.) (Power et al., 2021). Propazine was detected only in one sample at below LOQ (5.36 ng/g w.w.) levels and was the only representative of chloro-s-triazine group which is considered to have endocrine-disrupting properties by the U.S. Environmental Protection Agency. The highest cumulative levels of PPPs were measured in PF4 (107 ng/g w.w., 7 compounds) and LO3 (98.6 ng/g w.w., 9 compounds), which were both collected from areas with extended agricultural activity, indicating the high exposure of the respective birds in plant protection products.

3.1.3. Medicinal products (MPs) and TPs

The analysis results revealed the presence of twelve MPs and TPs in the tested samples. Eggs of Little owl species were proven to be more contaminated by MPs, considering their high detection rate. Overall, the %FoA of MPs ranged from 4 to 31%, except for D,L-N,O-Didesmethylvenlafaxine, which was detected in twelve samples (FoA: 46%). Four cardiovascular beta-blockers (atenolol, sotalol, carteolol and betaxolol) were among the MPs determined in the tested egg samples. Among them, atenolol was the most frequent and most abundant compound of MPs with 15% FoA and mean concentration at 27.7 ng/g w.w. Two antiretroviral drugs, darunavir and lopinavir, represent the class of antiretroviral drugs in the list of detected compounds. Lopinavir was detected only in one sample (LO4) at below LOQ levels (1.33 ng/g w.w.), however, Darunavir presented a FoA of 31%, as being present only in Little owl and Eurasian curlew eggs. The opioid analgesic drug Nalbuphine was detected in four eggs with a mean concentration below LOQ (2.11 ng/g w.w.), while the main metabolite of tramadol, N-desmethyltramadol was determined in eight samples, including eggs of all species, reaching a maximum concentration at 18.4 ng/g w.w. (in EO2). Moreover, the ephedrine derivative diocethedrin, the antiepileptic drug pregabalin and N-acetyl mesalazine (metabolite of the anti-inflammatory drug mesalazine), were detected with low frequency only in eggs of selected species; Peregrine falcon, Eurasian curlew and Little owl, respectively. Although medicinal products, including human and veterinary drugs, are produced in high volumes and are widely used, only a limited number of studies has focused on the investigation of their presence in raptors. Two recent studies revealed the presence of several medicinal products in wild bird livers gathered the Canary Islands and Northern Germany, respectively (Badry et al., 2022b; Rial-Berriel et al.,

2021). To the authors knowledge, this is the first biomonitoring study revealing the presence of medicinal products in eggs of different species of birds of prey.

3.1.4. Personal care products (PCPs)

Three parabens (methyl-, ethyl- and propyl-paraben), which are used as preservative ingredients in cosmetic, personal hygiene products, food products and pharmaceutical, as well as, octocrylene and 1,2-benzisothiazolinone, used as ingredients in sunscreens, liquid hand soaps and cosmetics, were present in the tested egg samples. Methylparaben presented high frequency of detection (FoA: 92%), as it was present in 24 out of 26 analyzed samples, and was the most abundant compound of this study, reaching up to 1760 ng/g w.w. (mean concentration at 218.5 g/g w.w.). In a study conducted in the United States, Xue et al. reported the occurrence of methylparaben in herring gull eggs, collected in 1988 and in loon eggs at a mean concentration of 4.63 and 3.80 g/g w.w., respectively (Xue and Kannan, 2016), which are two-fold lower that the levels reported in this study. Moreover, Power et al. revealed the presence of 16.1 ng/g w.w. of methylparaben in a common guillemot egg, collected from Aughris Head, Ireland (Power et al., 2021). The different concentration levels of previous studies may be attributed to the different habitat and trophic position of the studied organisms. Methylparaben and propylparaben are the most known parabens and are often used in combination due to their synergistic antimicrobial action (Xue and Kannan, 2016). However, their detection trend in this study does not support this statement. Both propylparaben and ethylparaben were detected only in one sample (EC3), whereas methylparaben, was the only PCP detected in Eagle owl eggs, at considerably lower concentration levels (mean concentration at 219 ng/g w.w.), compared to the rest investigated species. The different detection profile may be linked to the tissue analyzed or the sensitivity (LODs) of the methods compared. Both octocrylene and 1,2-benzisothiazolinone, presented higher frequency of detection in Little owl eggs and their maximum detected concentration in this study was at 45.6 and 32.2 ng/g w.w., respectively. Octocrylene is a highly lipophilic compound, stable and resistant to sunlight degradation and is widely used as UV filter. In 2013, Gago-Ferrero et al., conducted an innovative study for the occurrence of UV filters in marine mammals, where they examined the presence of UV filters in tissue liver of Franciscana dolphin (*Pontoporia blainvillei*) and among the determined compounds, octocrylene was the most frequent

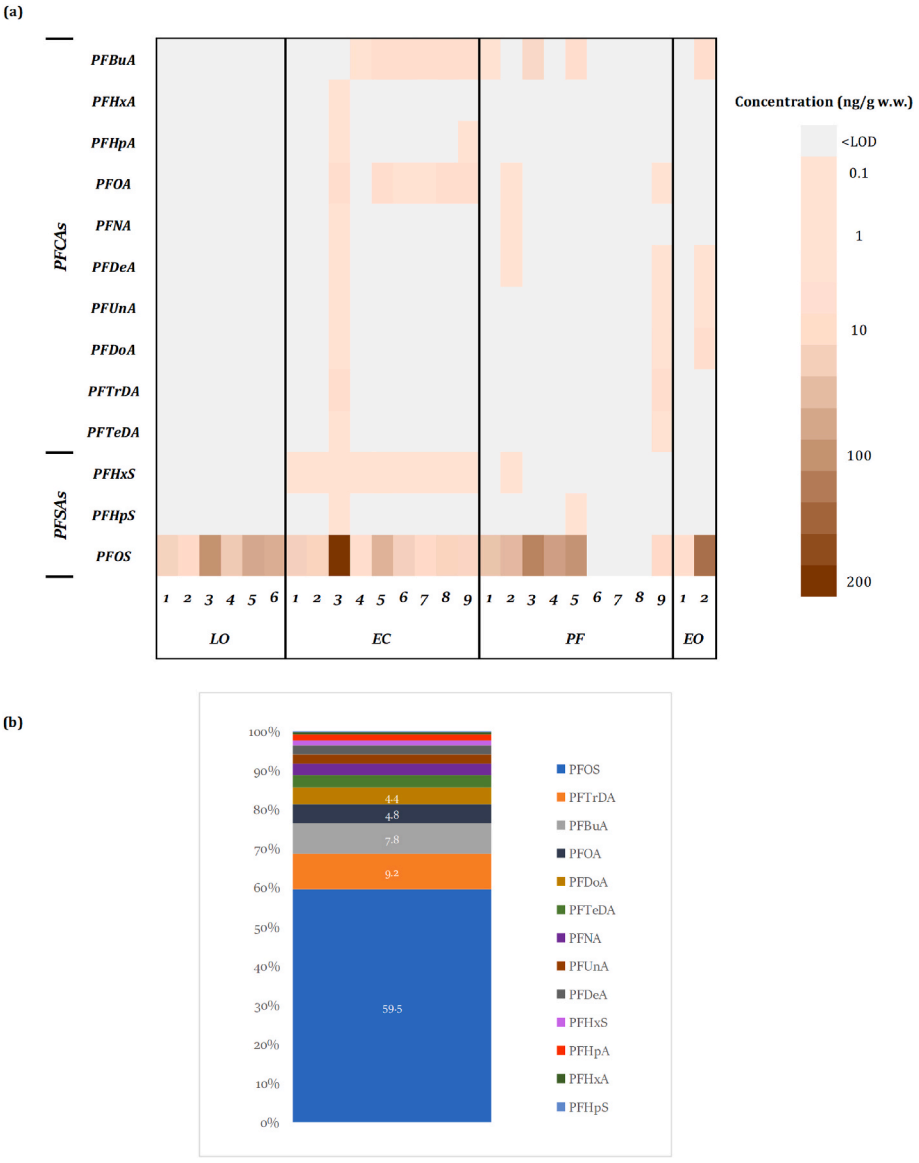

Fig. 5. (**a**) Concentration levels (ng/g w.w.) of PFAS in the bird eggs used in the current study provided as heatmap. Grey tiles in the heatmap refer to concentrations below the limit of detection (LOD). (**b**) Percentage of individual PFAS concentrations are depicted as stacked bar plots.

compound detected in the tested samples (21 out of 56, 38% frequency of detection) with concentrations range from 89 to 782 ng/g lipid weight (Gago-Ferrero et al., 2013). In another study, in 2015, Gago-Ferrero et al., reported the occurrence of octocrylene as the most frequent compound at maximum concentration approximately at 30 ng/g dry weight in fish samples collected from Spanish rivers (Gago-Ferrero et al., 2015).

3.1.5. Tobacco related compounds and TPs

The tobacco alkaloid anabasine and the metabolite of nicotine, hydroxy-cotinine were both detected in two out of twenty-six tested samples (only in eggs of Eurasian Curlews and Eagle owls). Hydroxy-cotinine was detected at a maximum concentration of 17.0 ng/g w.w., which is in agreement with the concentration levels (10.7 ng/g w.w.) of a study conducted with common guillemot (*Uria aalge*) eggs from Ireland (Power et al., 2021), while anabasine was present only at below LOQ levels (3.61 ng/g w.w.).

3.1.6. Per- and polyfluoroalkyl substances (PFAS)

The wide-scope target analysis results revealed the presence of thirteen PFAS (out of 56 that are included in the database), including ten Perfluoroalkyl Carboxylic Acids (PFCAs) and three perfluoroalkyl sulphonic acids (PFSAs), in the tested biota samples. Fig. 5 illustrates (a) the detected concentration levels in the samples as a heat map and (b) the distribution profile of PFAS. Perfluorooctanesulfonic acid (PFOS) was the dominant compound of this class, as it was detected at a mean concentration up to two orders of magnitude higher than the rest PFAS (43.8 ng/g w.w.) and presented high detection frequency of 88%. From a statistical point of view, PFOS ranked third when sorting the compounds of this study by both %FoA and maximum detected concentration. The detected concentration levels for PFOS, exceeded the Environmental Quality Standards (EQS) set by Directive (2019)/39/EU at 9.1 ng/g w.w. for fish, in twenty-two out of twenty-three samples. PFOS has already been reported in the literature as a predominant compound in raptor eggs. In a recent study, Eriksson et al. analyzed eggs of three different raptor species collected in Sweden within 1997–2014, and PFOS was the most abundant compound with mean concentrations at 70, 7.9 and 3.8 ng/g w.w. in eggs of osprey, tawny owls and common kestrel, respectively (Eriksson et al., 2016). In the present study, apart from PFOS, the PFSAs Perfluorohexanesulfonic acid (PFHxS) and Perfluoroheptanesulfonic acid (PFHpS) were found in ten and two samples, respectively, at concentration levels below 3.5 ng/g w.w. Regarding previously reported results for PFCAs, Groffen et al. conducted a study in eggs of great tits (*Parus major*), collected near a fluorochemical factory and in three other areas in Antwerp, Belgium and their results indicated the presence of 12 PFAS (4 PFSAs and 8 PFCAs). The concentrations of PFSAs were among the highest ever reported in eggs with median concentrations of 10,380 (extrapolated), 99.3 and 47.7 ng/g w.w. for PFOS, PFHxS and perfluorodecane sulfonic acid (PFDS), respectively (Groffen et al., 2017). In our study, Perfluorobutanoic acid (PFBuA) and Perfluorooctanoic acid (PFOA), presented high frequency of detection >30% (38 and 31%, respectively), among PFCAs, and were the most abundant compounds, reaching concentrations up to 11.7 and 6.43 ng/g w.w., respectively. The detected levels for PFOA are in agreement with those reported by Groffen et al. at a median concentration of 19.8 ng/g w.w., mentioning that this value is the highest ever reported in bird eggs (Groffen et al., 2017). Moreover, in a previous study analyzing eggs of peregrine falcons from Sweden, Perfluoroundecanoic acid (PFUdA) and Perfluorotridecanoic acid (PFTrDA) were detected at a mean concentration of 4.2 and 7.2 ng/g w.w., respectively (Holmström et al., 2010), which are in line with the results of our study. The highest cumulative concentration of PFAS (187 ng/g w.w.), was measured in eggs of Eurasian curlew, collected from Rust, a rural area in Germany, in 2005 (EC3 sample). Moreover, it should be noted, that EC3 is among the oldest samples of this study and therefore the detection of lower concentrations in more recent samples may be attributed to the continuously increasing production and application of new PFAS over the last decades, which are used as replacement compounds of the legacy PFOS and PFOA, included in the Stockholm Convention for Persistent Organic Pollutants (Androulakakis et al., 2022). All screened PFAS were below LOD in three out of nine analyzed eggs of Peregrine falcon species (PF6, 7 and 8 samples). Eurasian curlew species, as migratory birds, spend their winter in areas close to the estuaries of big, and probably contaminated, rivers of the Iberian Peninsula (Boschert, 1992), whereas Peregrine falcons are resident birds.

3.1.7. Industrial chemicals

Apart from PCBs and PFAS, six additional compounds of industrial use were detected in the tested samples. Three long-chain methyl-alkyl amines (N-methyldodecylamine, N,N-dimethyldodecylamine and N,N-dimethyltetradecylamine) were found with FoA up to 12%, at concentration levels up to 6.64 ng/g w.w., whereas the sub-class of Quaternary Ammonium Compounds (QACs) is represented in this study findings by benzododecinium (benzyl-dimethyl-dodecylammonium) and didecyldimethylammonium (DADMAC (C10:C10)). The highest %FoA (19%), was calculated for didecyldimethylammonium, which was detected in 5 egg samples at trace levels (<LOQ: 4.00 ng/g w.w.). Moreover, tolytriazole (the chemichal mixtures of 4- and 5- methyl benzotrizole) which is commonly used in runway deicer, circulating water cooling systems, automotive coolants, brake and hydraulic fluids, metalworking fluids, was detected only in EC3 at below LOQ levels (4.64 ng/g w.w.). All the aforementioned industrial chemicals were detected in EC3 sample, collected from Rust, a rural area in Germany, in 2005. However, as previously mentioned, the high content of chemicals in Eurasian curlew species, may be linked to their migratory habits, as they pass the winter in the estuaries of big rivers of the Iberian Peninsula, which probably highly contaminated with several classes of pollutants (Boschert, 1992).

3.2. Suspect screening results

In total, 50 additional substances were identified and semi-quantified through suspect screening workflows. The results are summarized in Table S4-2 and visualized as a heatmap in Figure S4-2. 44 compounds were tentatively identified at level of confidence 3 (Schymanski et al., 2014), indicating that isomeric structures are also possible for these chemicals, whereas for 6 compounds a probable structure was proposed by library spectrum match (level 2A). The majority of the identified substances were classified as industrial chemicals, including high production chemicals such as plasticizers, cosmetic's ingredients, flame retardants etc. 20 industrial chemicals were registered in the ECHA database, indicating that they are produced or imported in Europe in more than 1 ton per year. The presence of 6 highly produced (>10 tonnage) industrial chemicals (acetyl tributyl; peroxydicarbonic acid, C, C′-bis[4-(1,1-dimethylethyl)cyclohexyl] ester; 2-hydroxy-5-nonyl-benzaldehyde; undec-10-enal; cyclopropanecarboxylic acid, 2-[1-(3, 3-dimethylcyclohexyl)ethoxy]-2-methylpropyl ester and methyl 9-decenoate) among the identified substances in the bird egg samples may be of high concern, due to their continuous release in the environment. The identified in level 2A substance, 2,6-Dimethylaniline, included in the list with Persistent, (very) Mobile and Toxic (PMT/vPvM) REACH substances (https://zenodo.org/record/6482414), provided by the German Environment Agency (UBA, Umweltbundesamt), was identified in 72% of the tested samples, in estimated concentration levels ranging from 9 to 897 ng/g w.w., indicating that such monitoring data can support the in-silico data, predicting the potential PBT properties and support environmental risk assessment. Furthermore, 9 substances, classified as pharmaceuticals, were tentatively identified, among them the nonsteroidal anti-inflammatory drug Ibuprofen, as well as the antibiotic 4-hydroxy-2(1H)-Quinolinone. The presence of the antiviral drug telbivudine has been previously reported in fish samples, gathered from Dniester River, Ukraine (Diamanti et al., 2020).

4. Conclusions and future perspectives

The current biomonitoring study provided evidence that different bird species from the state Baden-Württemberg, Germany are exposed to a "cocktail" of organic micropollutants. The wide-scope target analysis of more than 2,400 chemicals revealed the presence of 58 organic micropollutants, including their (bio)transformation products, classified in different chemical classes, whereas the suspect screening of more than 65,000 chemicals resulted in the identification of additional 50 chemicals. The source of transformation products in biota is very challenging, since the transformation may have occurred as a result of in-vivo metabolism (as a consequence of phase I and phase II reactions after the parent molecule is introduced into the organism) or due to bioaccumulation from environmental uptake (as a result of abiotic process, such as hydrolysis and/or photodegradation in the natural environment or during water-treatment processes (e.g., chlorination, ozonation and advanced oxidation) (Bletsou et al., 2015; Suman et al., 2022). The organic micropollutants, determined through wide-scope target analysis, were mainly plant protection products, followed by per- and polyfluoroalkyl substances (PFAS) and medicinal products, indicating that the terrestrial organisms are daily exposed to a diverse group of chemicals and, thus, the systematic monitoring of a large number of chemicals is really urgent for the better chemicals management. Among the detected compounds, the highest concentration levels and the most frequently determined compounds had a strong lipophilic character, suggesting that the egg is an ideal matrix of analysis for the monitoring of lipophilic compounds, whereas the detection of more polar compounds underlines that a holistic analytical approach by fully exploiting the capabilities of HRMS is crucial for demonstrating the current state of pollution in the environment. Furthermore, compounds included in the Stockholm Convention, were also detected in the tested egg samples, confirming their persistency and bioaccumulative properties in biota, despite their restriction by EU regulation. The compounds, which were tentatively identified through suspect screening workflows, were mainly industrial chemicals and medicinal products. All acquired HRMS data are digitally stored and are available for future retrospective screening of any additional pollutant of interest. Overall, based on the current study outcomes, an untargeted monitoring of chemicals using HRMS techniques is required to reveal the chemical fingerprint of the environmental pollution in the organisms of the upper trophic levels.

In the future, the current study could be extended including samples from different states of Germany to draw safe conclusions on the spatial distribution, potential sources of pollution and accumulation patterns of organic micropollutants in birds of prey. Moreover, time-series samples could reveal time-trends of chemicals' emission in the environment and their uptake by biota species. Such exposure data, supported by risk assessment results can provide insights on the possibly adverse effects of the chemicals on the terrestrial species, and in particular on apex predators which can act as a proxy for humans. This strategy can help the assessment of better chemicals' management through the implication of effective mitigation measures.

Author contributions

Georgios Gkotsis: Conceptualization, Investigation, Formal analysis (HRMS analysis), Data Curation, Writing - Original Draft and Review & Editing; **Maria-Christina Nika**: Project administration, Investigation, Data Curation, Writing - Review & Editing; **Antonia I. Athanasopoulou:** Investigation, Formal analysis (target screening), Writing - Original Draft; **Konstantinos Vasilatos:** Formal analysis (sample preparation), Writing - Original Draft; **Nikiforos Alygizakis**: Investigation, Formal analysis (suspect screening), Writing - Review & Editing; **Martin Boschert:** Resources (provision of samples), Writing - Review & Editing; **Raphaela Osterauer:** Resources (provision of samples), Writing - Review & Editing, Funding acquisition; **Kai-Achim Höpker:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition; **Nikolaos S. Thomaidis**: Conceptualization, Writing - Review & Editing, Supervision, Resources (instrumentation, analytical methodologies, reference standards).

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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