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Biomonitoring study for the presence of organic micropollutants in raptor eggs by high resolution mass spectrometric techniques.

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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ ΔΙΠΛΩΜΑΤΟΣ ΕΙΔΙΚΕΥΣΗΣ

Μελέτη συστηματικής παρακολούθησης περιβαλλοντικών ρύπων σε αυγά αρπακτικών πτηνών με χρήση τεχνικών φασματομετρίας μάζας υψηλής διακριτικής ικανότητας.

ΑΘΑΝΑΣΟΠΟΥΛΟΥ Ι. ΑΝΤΩΝΙΑ

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ABSTRACT

Over the last decades, thousands of organic environmental contaminants have been released in the aquatic and terrestrial ecosystem, as a consequence of human activities. However, only a few organic micropollutants, termed as priority pollutants (PPs), have been thoroughly studied and are currently included in regulations, due to their persistent, bioaccumulative and toxic (PBT) properties. On the other hand, for the majority of the organic micropollutants, the knowledge regarding their presence in the environment still remains limited and are not currently included in the legislation or regular environmental monitoring programmes. The term “Emerging Contaminants” has been established for these compounds, whereas after their release in the environment, they are distributed in the various compartments in the different ecosystems.

Biomonitoring studies using wildlife are an important source of information for understanding the potential harmful effects of ECs, both in ecological receptors and humans. Raptors play a critical role in research on environmental pollutants, due to their unique characteristics. In addition, eggs have been used commonly as a non-destructive matrix of analysis for environmental pollutants.

The aim of this study is the validation of a generic sample preparation protocol in eggs and the investigation of the occurrence of more than 2,400 ECs in raptor egg samples by liquid (LC) and gas chromatography (GC) coupled with High Resolution Mass Spectrometry (HRMS). In this context, 26 egg samples, gathered from Baden-Württemberg in Germany within 2005 and 2020, were analyzed following generic sample preparation protocols and state-of-the-art HRMS techniques.

The results indicate the presence of several plant protection products (including myclobutanil, propazine, 2,4-dinitrophenol), stimulants (such as anabasine and the TP of nicotine, hydroxy-cotinine), industrial chemicals (including tolytriazole), pharmaceuticals and personal care products (like the parabens methylparaben and ethylparaben) in the tested samples. Additionally, numerous TPs (including nor-tramadol and D, L-N, O-didesmethyl-venlafaxine) were determined in egg samples, emphasizing the power of HRMS techniques in the identification of organic micropollutants. Moreover, 13 Per- and Polyfluoroalkyl Substances (PFAS) were detected in the tested samples, PFOS being the most abundant and frequently detected compound, indicating its persistence and potential harmful effects on human health and the environment.

SUBJECT AREA: Environmental Analytical Chemistry

KEYWORDS: Raptors; Eggs; Emerging contaminants; Priority Pollutants; High Resolution Mass Spectrometry; Biomonitoring.

ΠΕΡΙΛΗΨΗ

Τις τελευταίες δεκαετίες υπάρχουν πολλές ενδείξεις ότι δεκάδες οργανικοί περιβαλλοντικοί ρύποι, έχουν απελευθερωθεί στο υδάτινο και χερσαίο περιβάλλον λόγω των ανθρωπογενών δραστηριοτήτων. Ωστόσο, μόνο μερικοί οργανικοί περιβαλλοντικοί ρύποι, που ονομάζονται ρύποι προτεραιότητας (PPs), έχουν μελετηθεί διεξοδικά και σήμερα συμπεριλαμβάνονται στους κανονισμούς, λόγω των επίμονων και βιοσυσσωρευτικών και τοξικών ιδιοτήτων τους (PBT). Από την άλλη πλευρά, για την πλειοψηφία των οργανικών περιβαλλοντικών ρύπων, η γνώση παραμένει περιορισμένη και δεν συμπεριλαμβάνονται στους ευρωπαϊκούς κανονισμούς. Ο όρος «Αναδυόμενοι ρύποι» έχει καθιερωθεί γι' αυτές τις ενώσεις, ενώ μετά την απελευθέρωση τους κατανέμονται στα διάφορα διαμερίσματα στα διαφορετικά οικοσυστήματα.

Οι μελέτες βιοπαρακολούθησης που χρησιμοποιούν την άγρια ζωή είναι μία σημαντική πηγή πληροφοριών για την κατανόηση των πιθανών επιβλαβών επιπτώσεων των αναδυόμενων ρύπων τόσο στους οικολογικούς υποδοχείς όσο και στους ανθρώπους. Οι κορυφαίοι θηρευτές παίζουν έναν καθοριστικό ρόλο στις μελέτες παρακολούθησης περιβαλλοντικών ρύπων καθώς και σε μελέτες ανάλυσης επικινδυνότητας, λόγω των μοναδικών χαρακτηριστικών που διαθέτουν. Επιπροσθέτως, τα αυγά έχουν χρησιμοποιηθεί ευρέως ως μήτρα ανάλυσης των περιβαλλοντικών ρύπων.

Σε αυτήν την εργασία ειδίκευσης περιγράφεται η επικύρωση ενός γενικευμένου πρωτοκόλλου σε αυγά και η διερεύνηση της ύπαρξης περισσότερων από 2,400 αναδυόμενων ρύπων σε δείγματα αυγών αρπακτικών πτηνών με υγροχρωματογραφία (LC) και αέρια χρωματογραφία (GC) συζευγμένη με φασματομετρία υψηλής διακριτικής ικανότητας (HRMS).Σε αυτή την εργασία ειδίκευσης, 26 δείγματα αυγών κορυφαίων θηρευτών, τα οποία συλλέχθηκαν από το LUBW από διαφορετικά σημεία κατά μήκος της Βάδης-Βυρτεμβέργης της Γερμανίας από το 2005 έως και το 2020, αναλύθηκαν ακολουθώντας γενικευμένα πρωτόκολλα προετοιμασίας και σύγχρονες τεχνικές HRMS.

Τα αποτελέσματα υποδεικνύουν την ύπαρξη πολλών φυτοφαρμάκων (συμπεριλαμβανομένων των myclobutanil, propazine και 2,4-dinitrophenol), διεγερτικών ουσιών (όπως η anabasine και ο μεταβολίτης της νικοτίνης, hydroxyl-cotinine) χημικών βιομηχανιών (tolytriazole), φαρμακευτικών ενώσεων και προϊόντων προσωπικής υγιεινής (όπως τα parabens methylparaben and ethylparaben) στα δείγματα που έχουν αναλυθεί. Επιπροσθέτως, πολλά προϊόντα (βιο)μετατροπής (όπως Nor-tramadol και D, L-N, O-didesmethyl-venlafaxine) ανιχνεύτηκαν σε αυγά αρπακτικών πουλιών, υπογραμμίζοντας τη δύναμη της στοχευμένης ανάλυσης σε συνδυασμό με τη φασματομετρία μάζας υψηλής διακριτικής ικανότητας. Ακόμα 13 PFAS ανιχνεύτηκαν στα δείγματα, με το PFOS να έχει υψηλή συχνότητα εμφάνισης και υψηλά επίπεδα συγκεντρώσεων, υποδεικνύοντας τις πιθανές επιβλαβείς επιδράσεις στην ανθρώπινη υγεία και στο περιβάλλον.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Περιβαλλοντική Αναλυτική Χημεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: Αρπακτικά πτηνά, Αναδυόμενοι ρύποι, Ρύποι Προτεραιότητας, Φασματομετρία Μάζας Υψηλής Διακριτικής Ικανότητας, Βιοπαρακολούθηση.

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PREFACE

This master thesis was performed at the laboratory of Analytical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, under the supervision of Professor Nikolaos S. Thomaidis.

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CHAPTER 1

ENVIRONMENTAL POLLUTANTS

1.1 Introduction

Over the last decades, thousands of organic chemical compounds have been released in the environment and are widespread in wildlife, as a consequence of human activities. Although, few contaminants have been thoroughly studied and are currently included in regulations, for the majority of them, the knowledge still remains limited [1].

1.2 Priority Pollutants (PPs)

The term Priority Pollutants (PPs), has been established for organic contaminants which have persistent, bioaccumulative and toxic (PBT) properties and, thus, have adverse effects for the environment and the humans. For that reason, these compounds are currently included in the legislation and in the monitoring programmes. The U.S. Environmental Protection Agency (EPA) published a list of these toxic chemicals for which the agency intends to promulgate discharge control standards [2].

The term priority pollutants refers to a list of 126 specific pollutants that includes many toxic and bioaccumulative compounds, such as volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorobenzenes, alkylphenols, polybromodiphenylethers (PBDEs) and dioxins [3,4]. In addition, organochlorine pesticides (OCPs) are among the most important persistent organic pollutants (POPs) due to their strong lipophilicity and environmental persistence, and are ubiquitous due to their long-range atmospheric transportation. For instance, the presence of *p,p'*-dichlorodiphenyltrichloroethane (*p,p'*-DDT) and its metabolites' (e.g. 1,1-dichloro-2,2-bis(pchlorophenyl)ethylene (*p,p'*-DDE)) in the environment is of great importance due to their potential harmful effects on both humans and wildlife [5,6].

1.2.1 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs constitute a large group of organic chemical compounds with various structures,which are persistent in the environment. PAHs are deriving from incomplete combustion from either natural sources (such as forest and brush fires, volcanoes) or anthropogenic sources (such as emissions from vehicles and motors and cigarette smoke), or can be formed during biological processes. PAHs are mostly colorless, white, or light yellow solids. PAHs are consisted of two or more benzene rings bonded in linear, cluster, or angular arrangements as it is depicted in **Figure 1** [7].

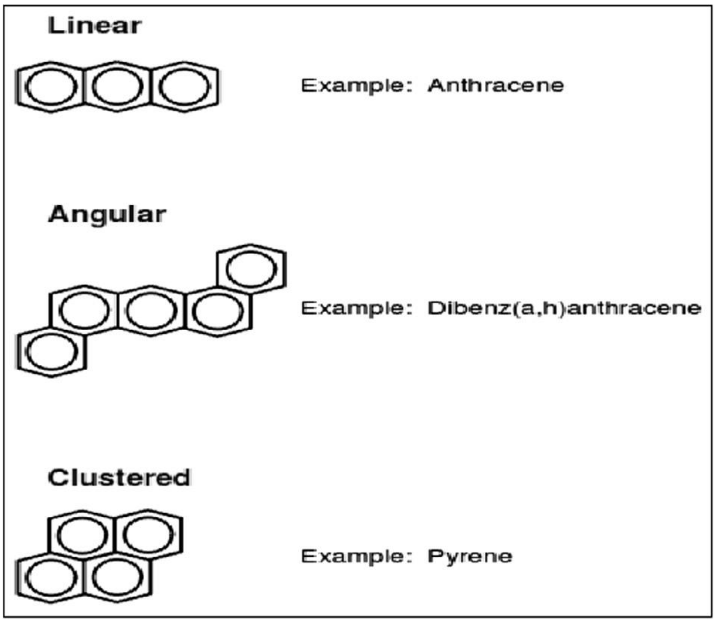
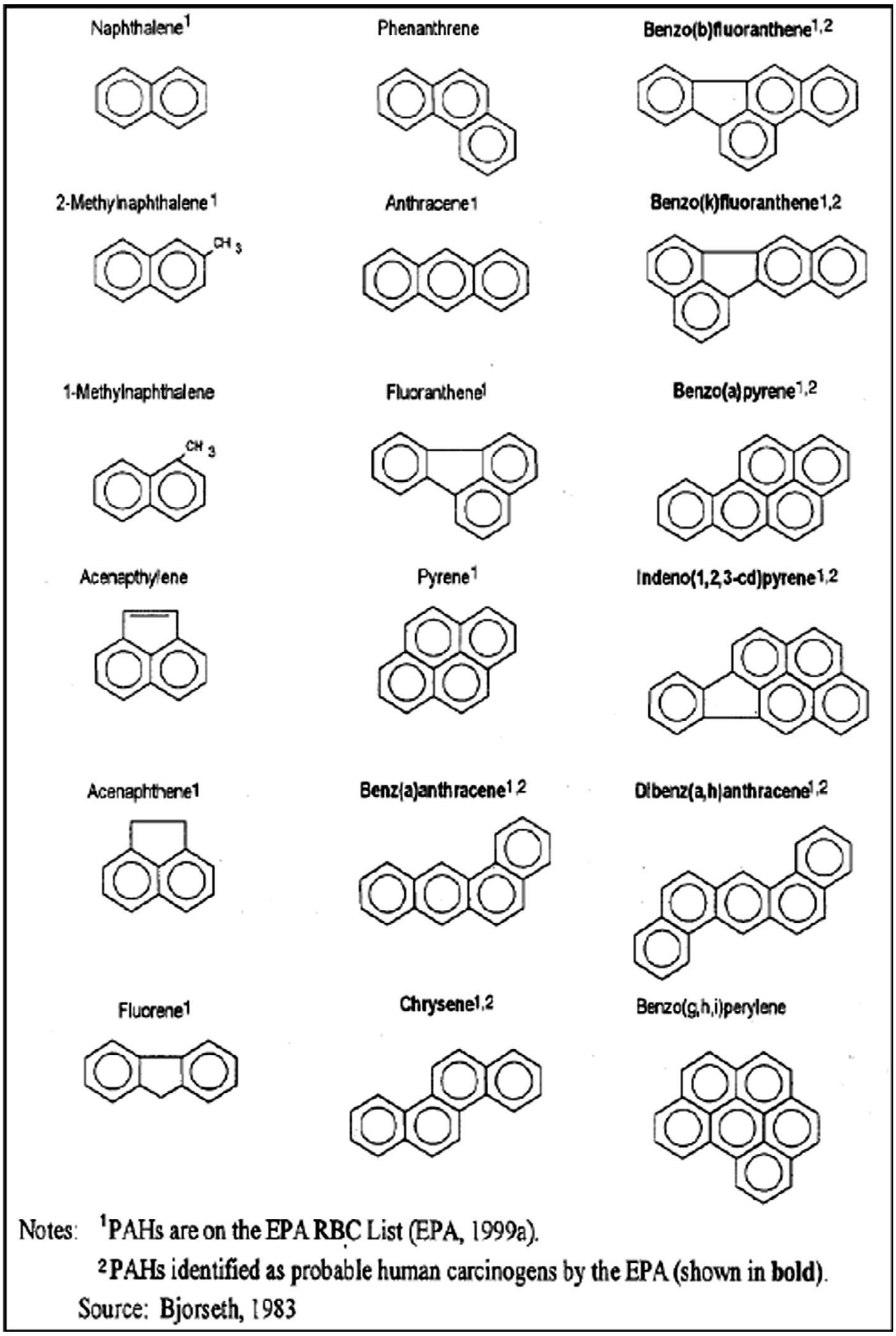


Figure 1: Molecular arrangement of the PAHs [7]**.**

PAHs can be characterized as “small” or “large”, depending on the number of the aromatic rings. The compounds that are often known as “small” PAHs, are having two or more single or fused aromatic rings with a pair of aromatic rings, whereas those compounds containing more than six aromatic rings are known as “large” PAHs. The most commonly analyzed PAHs are depicted in **Figure 2** [7].

PAHs have low solubility in water, are very soluble in organic solvents and highly lipophilic. These compounds have high melting and boiling points and low vapor pressure [7].



Notes: [1]PAHs are on the EPA RBC List (EPA, 1999a).
[2]PAHs identified as probable human carcinogens by the EPA (shown in **bold**).
Source: Bjorseth, 1983

Figure 2: The most commonly analyzed PAHs [7].

The use of PAHs commercially is limited. They are mostly used as intermediaries in pharmaceuticals, photographic products, agricultural products, lubricating materials, thermosetting plastics, and other chemical industries. Furthermore, other PAHs can be contained in asphalt used for the construction of roads or in roofing tar [7].

PAHs have been monitored in aquatic environment since the 1960s. These compounds accumulate in aquatic organisms and as a consequence they end up in organism from different throphic levels through the food webs. In addition, PAHs are frequently measured in the atmosphere for air-quality assesement, in biological tissues for health-effects monitoring, and in foodstuffs for safety reasons [8].

1.2.2 Polychlorinated Biphenyls (PCBs)

PCBs are among a group of man-made chemicals that are known as POPs and they were widely used. In the 1980s these chemicals were banned in many countries. The group of PCBs compromises 209 substances (congeners) with different properties, depending on the number and position of chlorines around the biphenyl ring (**Figure 3**) [9]. These contaminants are mostly present in the form of suspended solid due to the fact that they have less water solubility, low vapor pressure and high octanol-water coefficient [10]. PCBs are lipophilic substances; they degrade very slowly and accumulate in animal tissues via the food chain and, consequently, they are persistent in organisms from various trophic levels [11]. PCBs produce a wide spectrum of adverse effects in animals and humans including skin damage, hepatotoxicity, immunotoxicity, embryotoxicity, teratogenicity and promote tumor growth [12].

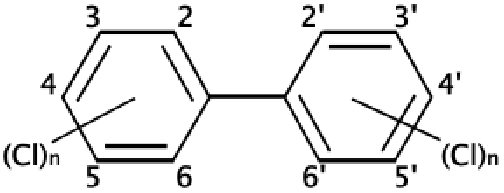


Figure 3: Chemical structure of PCBs (*Source: https://en.wikipedia.org/wiki/Polychlorinated_biphenyl***)**

1.3 Emerging contaminants (ECs)

Although, there are chemicals which are currently included in the current legislation, there is a diverse group of unregulated pollutants known as “emerging contaminants”. The term “Emerging contaminants” (ECs) or “Contaminants of emerging concern” (CECs) refers to chemicals, as well as their metabolites and their transformation products (TPs). ECs are not currently included in the legislation or in regular environmental monitoring programmes, but are investigated for future regulation, due to their frequent detection in the various environmental compartments. According to NORMAN network (Network of reference laboratories, research centers and related organizations for the monitoring of emerging contaminants), (www.norman-network.net , last accessed July 2021) the list of ECs in European aquatic environment include more than 700 chemical compounds and their biotransformation products [(bio)TPs] and have been categorized into more than 20 classes that depends on their use [13,14].

Many chemicals are non-degradable (persistent) contaminants, due to their stability and transportation properties. These compounds can be distributed to the environment through the untreated urban wastewaters and wastewater treatment plants (WWTPs) and they end up into sediment, soil, groundwater and seawater. The sources and the pathways of these ECs can be increasingly associated with industrial, agricultural and municipal activities, transportation and urbanization as well as industrial waste and household sewage. ECs enter the aquatic ecosystem, transported through water and ground in different ecosystems, located far away from the sources of these compounds in the environment [1,10,15]., A schematic overview of the potential sources and the distribution of ECs in the various environmental compartments is depicted in **Figure 4**.

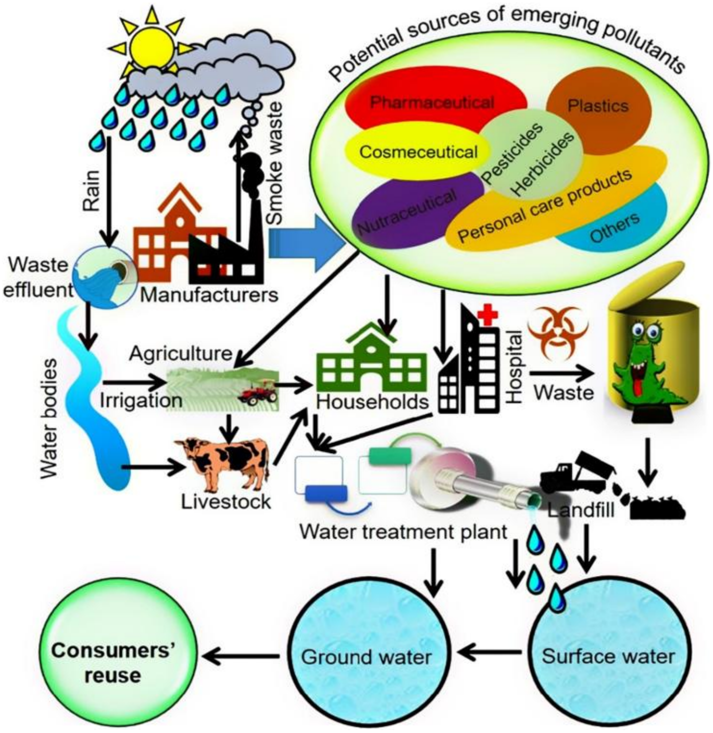


Figure 4: Schematic pathways of some emerging contaminants from sources to receptors[10]

The occurrence and fate of these organic compounds have been thoroughly investigated in the aquatic environment in contrast with the terrestrial ecosystem, in which such knowledge is limited (**Figure 5**) [16].

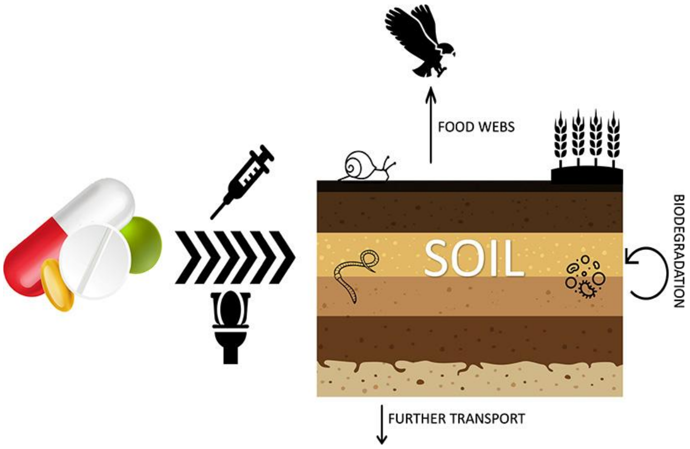


Figure 5: The transportation of ECs in the terrestrial ecosystem [16]**.**

Highly sensitive analytical methodologies have been developed and can be used for the detection of such compounds even in levels of parts per trillion (ppt), even in the most complex environmental matrices [17].

1.3.1 Classes of emerging contaminants

ECs encompass a great variety of compounds such as pharmaceuticals and personal care products (PPCPs), Plant Protection Products (PPPs), drugs of abuse, surfactants, industrial chemicals (such as benzothiazoles and benzotriazoles), polychlorinated naphthalenes (PCNs), Per- and Polyfluoroalkyl substances (PFAS), polychlorinated alkanes (PCAs), polydimethylsiloxanes (PDMSs), synthetic musks, quaternary ammonium compounds (QACs), hormones and steroids, food additives and engineered nano-materials, as well as their(bio)TPs [17]. A general overview is depicted in **Figure 6** below.

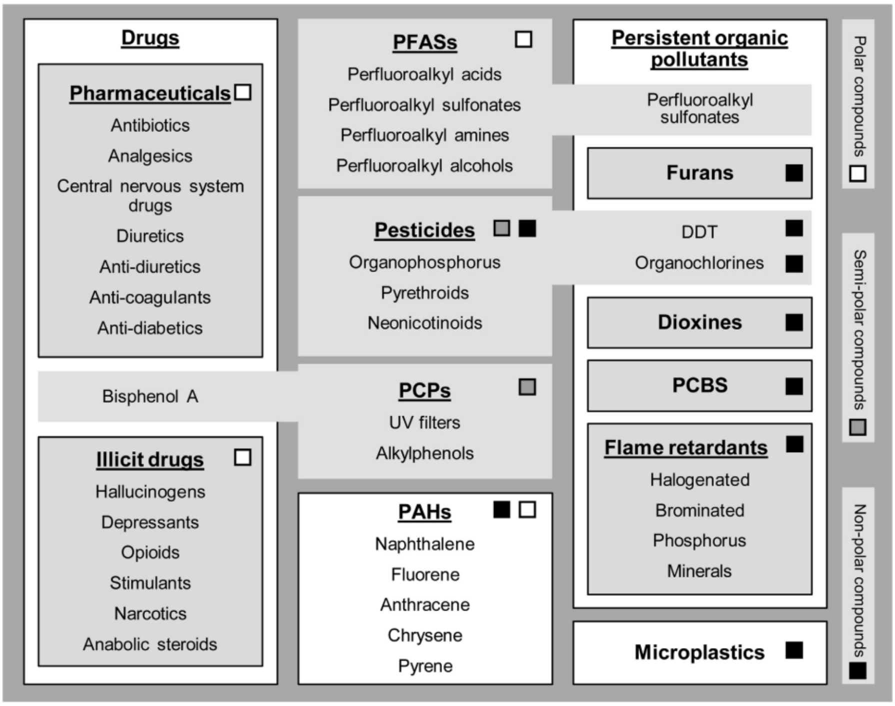


Figure 6: General overview of the micro-pollutants and their polarity [18]**.**

There are some emerging contaminants such as pharmaceuticals and personal care products, flame retardants and synthetic hormones that are considered as endocrine-disrupting compounds (EDCs), because they can alter the normal function of hormones resulting in a variety of health effects. In addition, behavioral and neurotoxic effects have been reported [19].

1.3.2 Pharmaceuticals

The occurrence of pharmaceuticals in the environment is related with their widespread use and consumption. Pharmaceuticals are considered as pseudo-persistent as their release in the environment, from domestic, hospital and industrial waste, is continuous [20]. There are different subclasses of pharmaceuticals, regarding their use and they can be used as human medicine or veterinary drugs. There are a variety of pharmaceuticals but the most common that can be found are analgesics, non-steroidal anti-inflammatory drugs (NSAID), antibiotics, cardiovascular pharmaceuticals (such as *beta*-blockers and diuretics), estrogens and hormonal compounds, antiepileptic drugs and psycho-stimulants [21]. Pharmaceuticals such as antibiotics that are used by humans are excreted with the urine and enter the environment. However, a major part of the substances used as human medicine may be metabolized by liver and as a result will be exposed as metabolites in the environment [22,23]. In addition pharmaceuticals can be transformed by biotic (e.g. microorganisms and wildlife) or abiotic (e.g. ultraviolet, hydrolysis, photolysis) processes and distributed through the different compartments of the environment [24] .

1.3.3 Illicit drugs & Drugs of abuse

The presence of illicit drugs and their metabolites in the environment is a matter of global concern. Illicit drugs are those for which nonmedical use is prohibited by the national or international laws. The classes of illicit drugs include a variety of compounds such as opioids, cocaine, cannabis, amphetamine type substances (ATSs), and ecstasy-group substances. Illicit drugs have been initially identified in aquatic environments in 2004. Recently it’s found in waste water, groundwater and also in surface water [25]. The main transport pathways of illicit drugs and their metabolites into the environment are via human excretion through urine after legal or illegal consumption or direct disposal (accidental or deliberate) of clandestine laboratory wastes into sewage systems [26]. The residues of these substances persisting in consumers’ urine enter the sewage networks with the wastewater and are only partially removed in sewage treatment plants (STPs) [27].

1.3.4 Plant Protection Products (PPPs)

Over the last decades, agricultural activities have greatly increased and as a consequence many of them rely on the use of pesticides. Nevertheless, agricultural areas are not only used for production, but also they are habitats for a variety of plants and animals. Pesticides are chemicals compounds, which are used in order to protect plants and crops against harmful organisms and diseases and to regulate their growth [28]. The class of pesticides includes many chemical compounds such as herbicides, fungicides, insecticides, acaricides, nematicides, molluscicides, bactericides, growth regulators, repellents, rodenticides and biocides. Pesticides enter the aquatic ecosystems via evaporation, spray drift and deposition, and after rain events as runoff and erosion or drainage. In recent years, an increasing global problem has become the use of illegal and counterfeit pesticides and for this reason monitoring data of pesticides in environmental samples are very useful for the review of the authorization and the regulations [28,29].

1.3.5 Per- and Poly- Fluoroalkyl Substances (PFAS)

Per- and Poly- Fluoroalkyl substances (PFAS) are fluorinated synthetic chemicals that do not naturally occur in the environment and are commonly used as surfactants and polymers in for a wide variety of household, industrial and commercial products since 1950s. Several perfluoroalkyl acids (PFAAs), such as perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) are measured and detected in environmental matrices [30–32]. PFCAs and PFSAs have anionic properties that allow them to be water soluble and be absorbed onto soil and sediment at higher chain lengths, giving them the opportunity to widespread at the environment thanks to their greater mobility [32]. PFAS are not readily biodegradable, are persistent and due to their unique physicochemical properties can be transported far away to remote parts and can be reported in air, water, and biota samples worldwide [33].

Among PFASs, that have been detected in the environment, the most common and widespread organic pollutants are perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) for biota and human as well [34]. PFOS and its salt are listed under Annex B of the Stockholm Convention for Persistent Organic Pollutants (POPs) and PFOA and perfluorohexane sulfonate (PFHxS) have been proposed for inclusion. For this reason, industries have replaced these compounds with PFAS that are structurally similar and have not been monitored in the routine or are not listed to the legislation yet [35].

CHAPTER 2

BIOMONITORING STUDIES - LITERATURE REVIEW

2.1 Introduction

Organic micropollutants, afterwards their release in the environment, are distributed in the various environmental compartments in the different ecosystems. Their distribution is depending on their physicochemical properties such as water solubility, polarity and vapor pressure [19].

Biomonitoring studies using wildlife are an important source of information for understanding the potential harmful effects of emerging contaminants, both in ecological receptors and humans. Biota specimens are exposed to these micropollutants via water, sediment and suspended solids and these compounds have the potential to bioconcentrate and biaccumulate through the food webs in apex predators, as it is depicted in **Figure 8** below. Although aquatic food webs have been thoroughly studied, terrestrial food webs represent important gaps in understanding and further investigation is needed. [18,19].

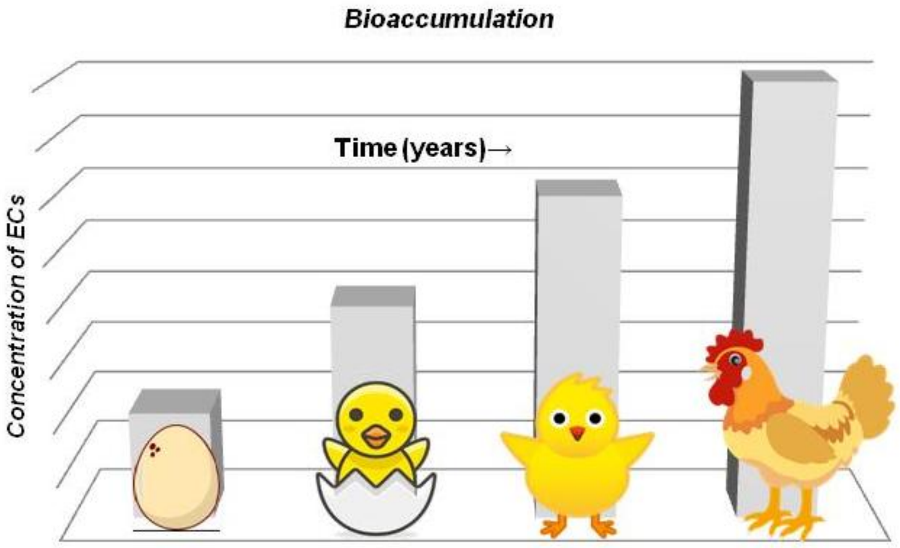


Figure 7: Bioaccumulation of ECs in a trophic level

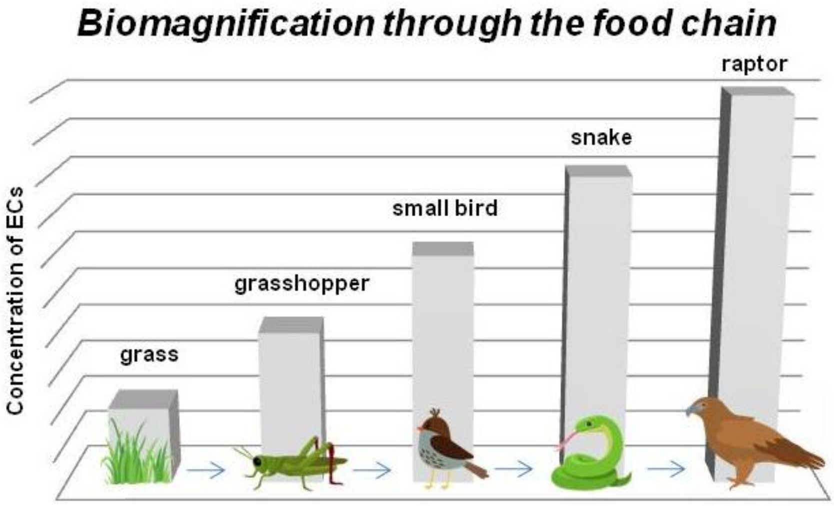


Figure 8: Biomagnification of ECs through the food chain

2.2 Raptors

Raptors are good sentinel of environmental contamination and play a critical role in research on persistent and bioaccumulative environmental contaminants, due to their unique characteristics. As apex predators, they are organisms that occupy the highest trophic positions on their food chains and, thus, accumulate a wide range of contaminants. Moreover, their life expectancy is relatively long, which makes them ideal indicators for compounds that biomagnify and bioaccumulate through food webs. Furthermore, the monitoring of organic micropollutants in raptors can reveal both spatial and temporal trends. Finally, responses of the population of raptor ECs (e.g. population decline due to DDE) are measurable, since their populations can be relatively easily monitored and quantified [36,37].

2.3 Raptors’ eggs

Eggs have been commonly used as a non-destructive matrix of analysis in the monitoring studies for environmental pollutants and non-invasive sampling methods have been employed for their collection. Furthermore, the levels of chemicals in eggs may reflect the exposure of female raptors over time and in relatively large spatial areas. Moreover, eggs reveal potential accumulation of micropollutants and their metabolites, which have been assimilated in previous time periods by breeding. The use of eggs as indicators of contamination is still useful for the extraction of valuable information, even though the levels of contamination may reflect breeding females and not the entire population of raptors such as males and non-breeding birds [38,39].

2.4 Analytical Techniques

2.4.1 Gas chromatography

Gas chromatography (GC) is one of the most widely used techniques for qualitative and quantitative analysis. In gas chromatography, the components of a vaporized sample are separated by being distributed between a mobile gaseous phase and a liquid or a solid stationary phase held in a column. In performing a gas chromatographic separation, the sample is vaporized and injected into the head of a chromatographic column. Elution is brought about by the flow of an inert gaseous mobile phase. In contrast to most other types of chromatography, the mobile phase does not interact with molecules of the analyte. The only function of the mobile phase is to transport the analyte through the column. Column temperature is an important variable that must be controlled. Thus, the column is normally housed in a thermostated oven. The optimum column temperature depends on the boiling point of the sample and the degree of separation required. Roughly, a temperature equal to or slightly above the average boiling point of a sample results in a reasonable elution time. For samples with a broad boiling range, it is often desirable to use temperature programming whereby the column temperature is increased either continuously or in steps as the separation proceeds[40].

There are two types of gas chromatography; gas-solid chromatography (GSC) and gas-liquid (GLC). In GSC, the stationary phase is solid. The retention of analytes is result of adsorption/desorption steps with the solid phase. On the other hand, in GLC, the stationary phase is liquid that is held on a finely-divided inert solid support. The retention of analytes is based on solute partitioning between mobile (gas) and liquid phase. GLC is most common used in sciences and simply referred as gas chromatography.

A GC instrument has simple components. Usually helium, hydrogen or nitrogen gas compressed in cylinders is used as the carrier gas (mobile phase). Flow of the carrier gas into a temperature controlled sample injection is controlled by pressure regulators and gas metering valves. A GC column is attached to the injection port and samples are introduced into the carrier gas steam at a temperature sufficient to insure vaporization of all components. Typically, the sample is introduced with a micro liter syringe which is forced through a rubber spectrum at the injection port. A detector attached directly to the column exit monitors individual sample components as they are eluted from the column. The detector must be insensitive to carrier gas, while detecting sample components that are eluted. A recording of its response with time forms a chromatogram.

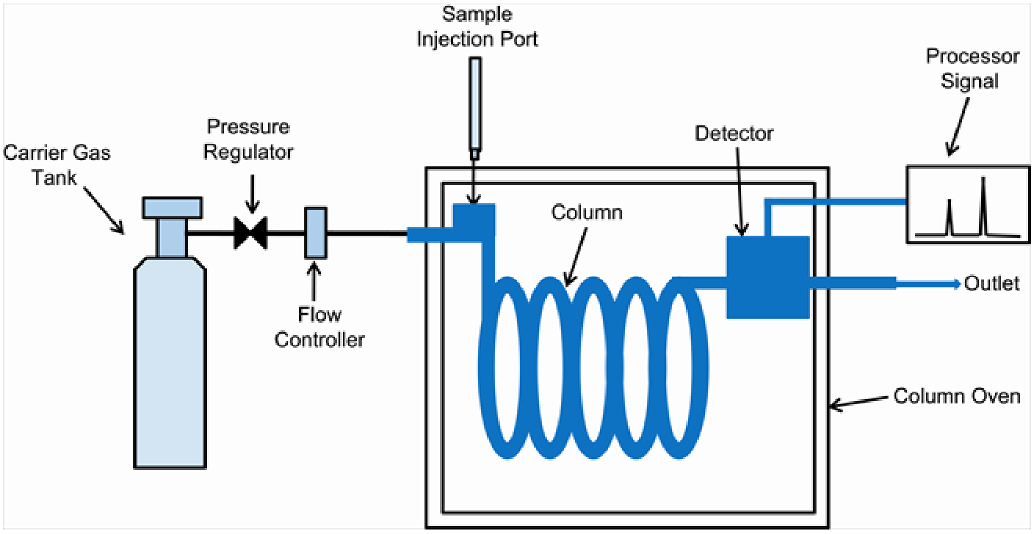


Figure 9: GC Instrumentation (*Source:* https://www.researchgate.net/figure/Schematic-diagram-of-the-main-components-of-a-gas-chromatography-system-Philadelphia_fig3_273134301)

The chromatogram contains the analytical data for the components of a mixture. Qualitative information appears in the characteristics retention time of each component. Quantitative information is contained in peak area [40].

2.4.2 Liquid Chromatography coupled to Mass Spectrometry (LC-MS)

LC-MS is a sophisticated hyphenation of analytical techniques, which enables the determination of organic emerging contaminants in complex environmental matrices. A range of different LC-MS technologies have been put forward in recent years for the analysis of mixtures of many known and unknown compounds at low concentrations in complex matrices[41,42].

2.4.2.1 Reversed Phase Ultra High Performance Liquid Chromatography (RP-UHPLC)

UHPLC uses small-diameter particles in the stationary phase and short columns and provides fast and high resolution separation that increases LC-MS sensitivity and minimizes matrix interference arising from minimal sample preparation [43,44]. UHPLC is commonly performed in reversed-phase (RP) mode, using C_{18} columns. The mobile phase consists of an aqueous and an organic solvent. Methanol and Acetonitrile are commonly used as organic solvents. In some methods, the mobile phase is acidified with small percentages by volume of acetic or formic acid in order to improve ionization of the compounds in the positive ionization mode [45]. Gradient elution programs are preferred for better and faster separations.

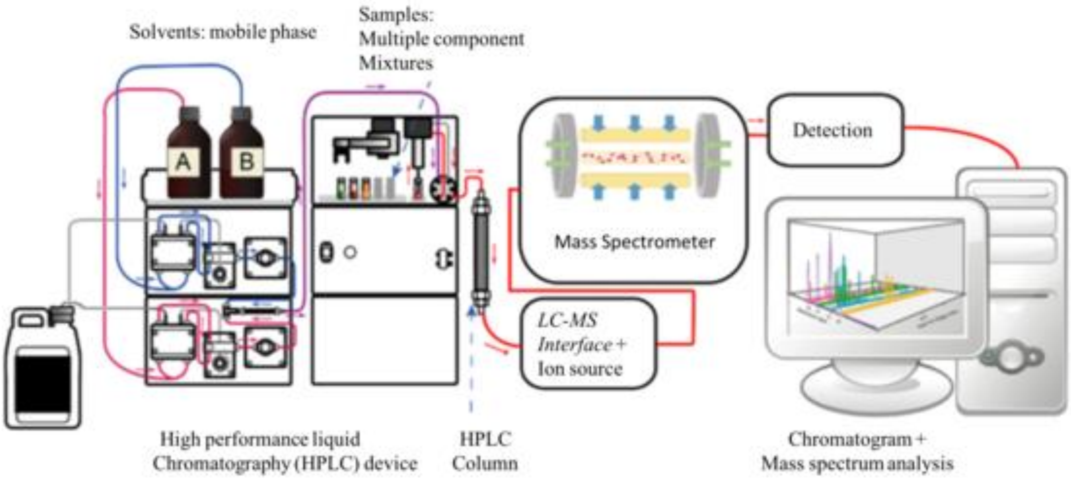


Figure 10: LC Instrumentation (*Source*: https://en.wikipedia.org/wiki/Liquid_chromatography%E2%80%93mass_spectrometry)

2.4.3 High Resolution Mass Spectrometry (HRMS)

Many scientific groups, which are dealing with the determination of organic in biota, develop analytical methods that include liquid chromatography coupled to tandem mass spectrometry using low resolution mass analyzers, usually triple quadrupole (QqQ), because this technique is reliable for qualitative and quantitative determination of selected/known biomarkers.

On the other hand, the use of liquid chromatography coupled to high-resolution mass spectrometry allows the wide-scope screening of parent compounds and (bio)TPs, which may be already known, suspect or unknown. Consequently, it can be used for the determination of the continuous growing and diverse group of ECs [46–48].

Among the possible ionization techniques in LC-MS, electrospray ionization (ESI) is the most widely used, compared with atmospheric pressure chemical ionization (APCI) or the more recent atmospheric pressure photoionization (APPI) [42].

LC-HRMS has an excellent performance on qualitative applications thanks to the high mass accuracy and the selectivity in full-scan acquisition mode that ensure reliable detection and identification, while more and more studies use LC-HRMS for complete analysis, both identification and quantification [46–48]. With full-spectrum accurate-mass data, a theoretically unlimited number of analytes which are present in a sample can be identified, because the acquisitions have been made as “all ions all the time” [41]. The simultaneous determination of a broad number of compounds in one injection, with a corresponding reduction of time and costs, and even when reference standards are not available, make LC-HRMS one of the current trends in environmental analytical chemistry [49]. Moreover, investigation can be performed in a retrospective way in order to detect compounds that initially were not considered, even after years, without additional analysis of the samples. This ability is advantageous, because in some occasions, samples might already have been discarded or the analytes have been degraded [47,48].

Time-of-flight (TOF) is one of the most used HRMS analyzers and it is easily coupled to ultra high performance liquid chromatography (UHPLC). Mass resolution typically ranges from 20,000 up to 80,000 Full Width at Half Maximum (FWHM) and mass accuracy is lower than 2 ppm. Hybrid configurations, such as Quadrupole-Time-of-Flight (Q-TOF), increase the potential of the analyzer for screening purposes and provide relevant structural information by obtaining accurate-mass product-ion spectra after MS/MS experiments [49]. Product-ion spectra can be obtained with either data dependent acquisition or data independent acquisition, where the instrument automatically switches after a full-scan-mode acquisition to a product-ion scan mode as the second scan event in the scan cycles [41].

2.5 Data Acquisition Modes in HRMS

2.5.1 Data Dependent Acquisition (DDA)

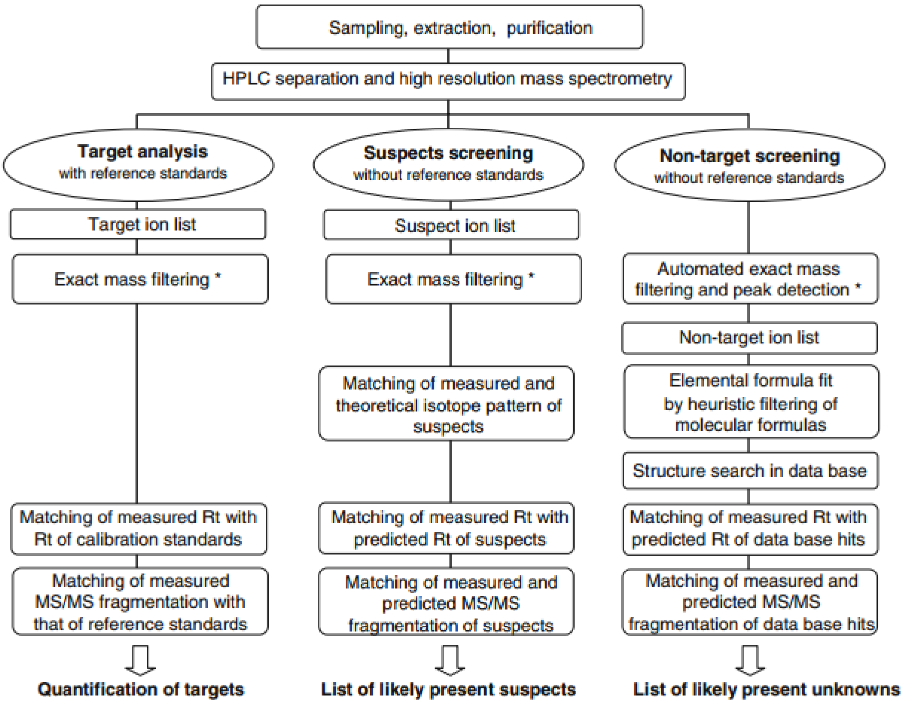
In this acquisition mode, there is firstly a full scan which is defined as the survey scan and data are processed “on-the-fly” to determine the candidates of interest based on predefined selection criteria, such as intensity threshold or suspect inclusion list. If the selection criteria are met, MS/MS analysis is then triggered and MS/MS scans (data-dependent) are performed [42,48]. With this acquisition, ‘clean’ spectra with structural information are obtained in one injection. However, if the number of candidates of interest is big, the number of scans is decreased, so there are less data points that affect the detectability of the chromatographic peak [42].

2.5.2 Data Independent Acquisition (DIA)

With this acquisition, there is no need to pre-select the precursor ion. Full-scan spectra at different collision energies are obtained in one injection. This acquisition provides simultaneously accurate mass data of parent compounds and fragment ions in a single run using two scans, one at low and one at high collision energy. By applying low energy (LE) in the collision cell, no fragmentation is performed. A full-scan spectrum is obtained that provides information for the parent ion (the (de)-protonated molecule) and, in some cases, the adduct ions and the in-source fragments. By applying high energy (HE) in the collision cell, fragmentation is performed and a spectrum similar to MS/MS experiments is obtained. This approach is called all-ions MS/MS, MS^E or bbCID, according to the QTOF manufacturer [42].

2.6 Data treatment

After the sample preparation and the LC-HRMS analysis, raw data can be treated with three different approaches, target, suspect and non-target screening. A systematic workflow for all three approaches is shown in the following **Figure 11**.



*Note that the *m/z* range of the extraction window for the exact mass filtering depends on the mass accuracy and the resolving power of the mass spectrometer used

Figure 11: Systematic workflow for target, suspect and non-target screening by LC-HRMS/MS [42]

2.6.1 Target screening

In this approach, an in house developed database is used for the screening of a large number of compounds. The information included in the database is based on the analysis of the available reference standards[49]. The reference standard in necessary for comparison of the retention time, the MS spectrum profile (precursor ion, adducts, in-source fragments), as well as the MS/MS spectrum (fragment ions and ion ratios) [50]. Quantitation can be performed in full-scan mode, but requires greater effort than in LC-LRMS methods where Single Reaction Monitoring (SRM) mode is used [49,50] .

2.6.2 Suspect screening

In this approach, a list of suspect compounds that are possible to be found in specific samples is built. The screening is based only on the exact m/z of the expected ions, which, in case of the ESI source, are usually the pseudomolecular ions $[M+H]^+$ and $[M-H]^-$, except for some compounds which exclusively show adduct formation. Molecular formula and structure are known, so this information can be efficiently used in the identification and confirmation process[42]. Absence from blank samples, mass accuracy, isotopic pattern retention time prediction, ionization efficiency and information on fragment ions reported in the literature are parameters that can facilitate tentative identification of suspect candidates [42,51].

2.6.3 Non-target screening

In non-target methodologies, samples are searched for compounds without any previous information on them. These unknown compounds are actually new, unexpected or not searched ones in specific samples. Identification is a challenge in this approach, as more than one elemental formula and several plausible structures are obtained for a given unknown compound detected in a sample [47,48]. Except for the elucidation of unknowns, non-target screening is used for the identification of (bio)TPs, arising from in vivo and in vitro experiments, in-silico modeling and degradation laboratory studies [41]. In this case, the number of chemically meaningful structures, which can be assigned to an unknown peak, is limited to structures that show a close relationship with the parent compound and also, an adequate control sample or time series is available [42].

2.7 Confidence in suspect and non-target screening HRMS

The use of High Resolution Mass Spectrometry (HRMS) techniques give the opportunity for the simultaneous determination of a great amount of target, suspect and non-target compounds in full-scan mode within a single analytical run and there is accuracy because of MS. HR-MS techniques are powerful due to their opportunity to detect, identify and confirm a variety of compounds and small molecules, such as emerging contaminants and their transformation products (TPs), if possible. Schymanski et al. (2014) suggested a 5 level system in order to make the communication of identification of ECs and their TPs easier and which is described below and is presented in **Figure 12** [52].

***Level 1:* Confirmed structure** is the ideal situation. The suggested structure is confirmed by the measurement of a reference standard with mass spectrometry (MS), MS/MS and retention time matching.

***Level 2:* Probable structure** marks a proposal for an exact structure using alternative evidence. Level 2a: Library contains conclusive matching literature or library spectrum data and experimental data/information. Level 2b: Diagnostic is preferred when no standard or literature data is available for confirmation and no other structure fits in experimental data.

***Level 3:* Tentative candidate(s)** is the situation where there is not enough information for the exact structure, but there is evidence for possible structure(s).

***Level 4:* Unequivocal molecular formula** refers to the situation where there is not enough evidence to suggest possible structures, but a certain formula is assigned by the spectral information.

***Level 5:* Exact mass (m/z)** is finding out in a sample, but there is no sufficient information to propose even a formula.

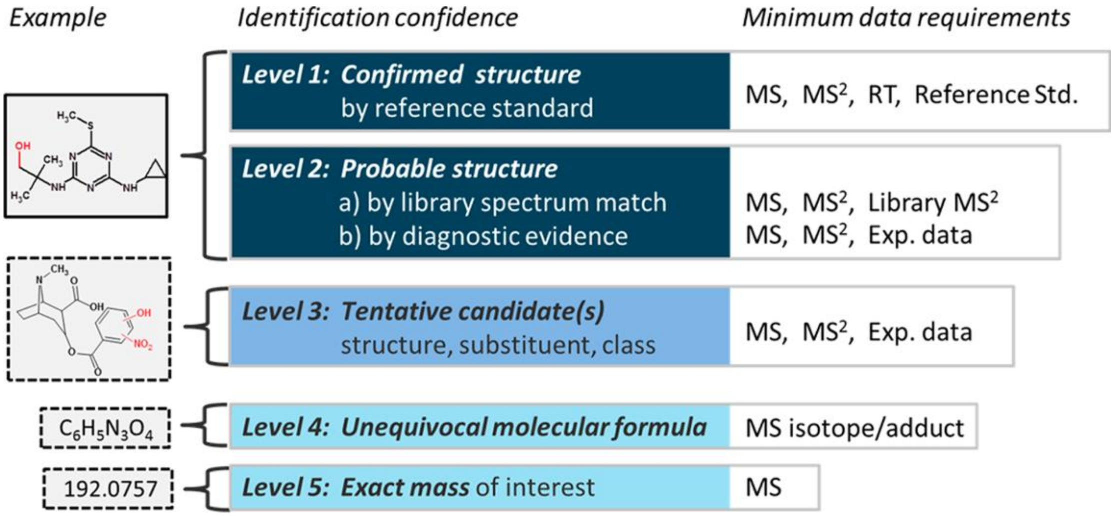


Figure 12: Proposed identification confidence levels in HRMS analysis[52].

2.8 Literature Review

So far, a lot of studies are focusing on the determination of a specific classes of PPs and ECs in eggs [5,6,60–69,11,70–79,53,80,54–59]. In the same time, only a few studies are dealing with the determination of a broad range of micro-pollutants in eggs from different classes, using HRMS [68,80].

An overview of the analytical methodologies that have been establish for the determination of environmental pollutants in eggs from different species is presented below. Details on the species, analytes, extraction and clean-up methods and the analytical techniques that were applied, are presented in **Table 1**.

For the extraction of the micropollutants from the egg matrix, the most used method is ultrasonic extraction [59,61–63,68,69,72,77,78,80]. Other extraction techniques were also reported namely Soxhlet [6,58,59,67,77] and Accelerated Solvent Extraction (ASE) [53,60,62,75,77]. In addition, extraction methods that are referred in **Table 1** are solid-liquid extraction and liquid extraction [5,54,76,77,55,60,62,64–66,70,71] and cold extraction as well [11,79].

Further clean-up after extraction was usually necessary. For the purification of the extracts Solid Phase Extraction (SPE) is mainly used in studies [59,60,62,66,70–72,75,77,78]. The most used sorbent is Oasis WAX [66,70,71]. In addition, silica gel (SiOH) cartridges [62,75], C_{18} cartridge [60], Florisil [77], FL-PR cartridges ([72], Supelclean ENVI-Carb® cartridges [59] and Chromabond HR-XAW SPE cartridges [78] have been used in other studies. In a few studies, for the clean-up of the extracts dispersive SPE (d-SPE) [61,63,64,68,69,72,80] and Gel Permeation Chromatography (GPC) [58,62,67] have been reported as a clean-up step. The use of column chromatography, using silica or alumina as sorbent has also been reported in previous studies [6,58,74,77,79].

For the analysis of the samples gas and liquid chromatographic techniques were used coupled with different detectors. GC was preferred for the determination of specific compounds such as OCPs, PCBs, DDT, DDE, *p,p'*-DDE, hexachlorobenzene (HCB), hexabromocyclododecane (HBCD), PBDEs, Brominated Flame Retardants (BFRs), hexachlorocyclohexane (HCH) and isomers of that such as lindane (γ-HCH) and β-HCH. On the other hand, LC was selected for the determination of fluoroquinolones

(FQs), perfluorochemicals (PFCs) and more specific PFAS, PFAAS, PFCAS, PFOS, BFRs and Alkylphenol polyethoxylates (APEs) as well. Hydrophilic interaction liquid chromatography (HILIC) was used for the determination of coccidiostats [64].

For the analysis of the samples gas and liquid chromatographic techniques were used coupled with different detectors such as ECD [5,11,74,79] and FLD [53,60]. As far as the mass analyzers are concerned, most of the developed methods for the determination of selected environmental pollutants use quadrupole (Q), triple quadrupole (QqQ), quadrupole-ion trap (QIT) and quadrupole-time-of-flight (QToF).

Table 1: Review of egg species, analytes, extraction method, clean-up step and analytical techniques for LC-amenable and GC-amenable compounds

#	*Species*	*Analytes*	*Extraction method*	*Clean-up step*	*Analytical technique*	*Reference*
1	Common kestrel (*Falco tinnunculus*)	OCPs, PCBs, p,p'-DDE	Liquid extraction	Sulfuric acid	GC-ECD	[5]
2	Little owl (*Athene noctua*)	PCBs, PBDEs, HBCD, OCPs, HCB	Soxhlet extraction	Silica column	GC-ECNI-MS GC-EI-MS	[6]
3	Kestrel (*Falco tinnunculus*), Black kite (*Milvus migrans*), Imperial eagle (*Aquila heliaca*), Marsh harrier (*Circus aeruginosus*), Barn owl (*Tyto alba*), Long-eared owl (*Asio otus*), Tawny owl (*Strix aluco*)	PCBs	Cold extraction	-	GC-ECD	[11]
4	Hen	FQs	ASE	-	LC-FLD	[53]
5	Ring-Billed Gull Double Crested Cormorant	PFOS	Liquid extraction	-	HPLC-ES-MS/MS	[54]
6	Laysan and Black-footed (*D. nigripes*) albatrosses	long-chain PFCAs	Liquid extraction	-	HPLC- ESI(-)-MS/MS (MRM)	[55]
	Adelie penguin (*Pygoscelis adeliae*), South polar skua (*Stercorarius maccormicki*)	PFOS				
7	Penguin (*Gentoo*)	PFCs	Ion-pairing liquid extraction	-	HPLC-ESI(-)-MS/MS (MRM)	[56]
8	Peregrine falcons (*Falco peregrinus*)	DDT, organochlorine (CHC) biocides and mercury (Hg), DDE, HCB, PCBs	-	-	-	[57]
9	Peregrine falcon (*Falco peregrinus*)	BFRs	Soxhlet extraction	GPC, silica gel column	GC-EI(+)-HRMS LC-ESI(-)-MS (QqQ) (SRM)	[58]
10	Peregrine falcon (*Falco peregrinus*)	PFASs, PCNs	Ultrasonic extraction, Soxhlet extraction	SPE, multi-layer column	LC-MS/MS (QIT) GC-ECNI-LRMS	[59]
11	Osprey	APEs	ASE	SPE	LC–MS/MS LC–FI	[60]

#	*Species*	*Analytes*	*Extraction method*	*Clean-up step*	*Analytical technique*	*Reference*
12	White-tailed sea eagle (WTSE) (*Haliaeetus albicilla*)	PFAAs, PFOS, PFUnDA	Ultrasonic extraction	d-SPE	UPLC-MS/MS	[61]
13	Caspian tern, Herring gull	PBDEs, non-PBDEs halogenated FRs (NPHFRs), organophosphate esters (OPEs), PFSAs, PFCAs and 5 emerging PFAAs or precursors	ASE, Ultrasonic extraction Solid-liquid extraction	GPC PSA bonded silica SPE	GC-ECNI-MS UPLC-APCI(+)-MS/MS UPLC-MS/MS	[62]
14	Great tits (*Parus major*)	PFAAs: PFSAs & PFCAs	Ultrasonic extraction	d-SPE	UPLC -ES(-)MS	[63]
15	Eggs	Coccidiostats	Solid-liquid extraction	d-SPE	HILIC-MS/MS	[64]
16	Guillemot (*Uria aalge*)	PFOS, PFOA	Solid-liquid extraction		HPLC-ESI-MS/MS	[65]
17	Northern fulmar (*Fulmarus glacialis*), thick-billed murre (*Uria lomvia*), Herring gull (*Larus argentatus*)	fluorotelomer alcohols (6:2 FTOH, 8:2 FTOH and 10:2 FTOH) and polyfluorinated sulfonamides (perfluoro-1-octanesulfonamide (FOSA) and N-methylperfluoro-1-octanesulfonamide (NMeFOSA))	Solid-liquid extraction	SPE	LC-APPI-MS/MS	[66]
18	Hen	HBCD isomers: a-, b-, c-HBCD	Soxhlet	GPC	LC–ESI-MS/MS	[67]
19	Peregrine falcon (*Falco peregrinus)*	PFAS	Ultrasonic extraction	d-SPE	HPLC- ESI-MS/MS HPLC-QTOF-HRMS	[68]
20	Osprey(*Pandion haliaetus*), Tawny owl(*Strix aluco*), Common kestrel (*Falco tinnunculus*)	PFAS	Ultrasonic extraction	d-SPE	UPLC-(ESI-)-MS/MS (MRM)	[69]
21	Herring gull (*Larus argentatus*)	PFSAs; C6, C8, C10 chain lengths	Solid-liquid extraction	SPE	LC-APPI(-)-MS/MS LC-ESI(-)-MS/MS	[70]
22	Herring gull (*Larus argentatus*)	C4-C15 PFSAs and PFCAs, and several precursors	Solid-liquid extraction	SPE	LC-APPI(-)-MS/MS LC-ESI(-)-MS/MS	[71]
23	Chicken	Organophosphate esters (OPEs) and metabolites (mOPEs)	Ultrasonic extraction	SPE d-SPE	LC-ESI-MS/MS (MRM)	[72]

#	*Species*	*Analytes*	*Extraction method*	*Clean-up step*	*Analytical technique*	*Reference*
24	Chicken, duck, and quail	PFOS and PFOA	Simple protein precipitation technique	-	LC-(ESI-)-MS/MS (MRM)	[73]
25	Guillemot (*Uria aalge*) Fulmar (*Fulmarus glacialis*)	PCBs, 4,4′-DDE and β-HCH	Jensen extraction method	Silica gel	GC-ECD GC-ECNI-MS (SIM)	[74]
26	Peregrine Falcon (*Falco peregrinus*)	PBDEs	ASE	SPE	GC-ECNI-MS GC-EI-MS	[75]
27	Guillemot (*Uria aalge*)	PBDEs, HBCD	Liquid extraction	-	GC-ECNI-MS	[76]
28	Yellow-legged gull (*L. michahellis*) Audouin's gull (*L. audouinii*)	Polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs), PBDEs, PCBs, OCs,PFCs and short chain chlorinated paraffins (SCCPs)	Solid-liquid extraction, Soxhlet extraction, ASE, Ultrasonic extraction	Silica gel modified, SPE	UPLC-MS/MS GC-(EI+)-HRMS GC–NCI-MS GC–EI-MS	[77]
29	Great tit (*Parus major*) Blue tit (*Cyanistes caeruleus*)	PFAAs	Ultrasonic extraction	SPE	UPLC-TQD-(ESI-)-MS/MS	[78]
30	Booted eagles (*Hieraaetus pennatus*), Goshawks (*Accipiter gentilis*)	HBC, HCH, lindane (γ-HCH), hexachloro-octahydro-epoxy-dimethanonaphthalene, DDT, DDE, PCBs	Cold extraction	Alumina column chromatography	GC-ECD	[79]
31	Tawny owl (*Strix aluco*)	PFAS	Ultrasonic extraction	d-SPE	HPLC-ESI(-)-QToF-MS	[80]

CHAPTER 3

SCOPE

In recent years, many scientific groups are dealing with the occurrence of PPs and ECs in various environmental matrices. Many of these chemical compounds, through various ways, end up in aquatic and terrestrial ecosystem, and are, thus, detected at various concentrations in environmental samples, like surface waters and aquatic and terrestrial organisms. The detection of emerging contaminants in organisms from the highest trophic levels has raised concerns for their potential bioaccumulation through the food webs.

It is important to mention that the recent advances and improvements in analytical techniques, and especially in high resolution mass spectrometry, have given the opportunity to scientific groups to detect and identify a huge number of chemical compounds, even in complex matrices, like biota. LC- and GC-HRMS data can be used for target, suspect and non-target screening, as well as retrospective screening, years after the treatment of samples without additional analysis of them.

Most of the studies are focused on the determination of selected classes of environmental pollutants in organisms using low resolution mass spectrometric techniques and available reference standards. However, efforts for screening of a wide range of organic micro-pollutants in biota by LC-HRMS and GC-HRMS are very limited.

The aim of this master thesis is the validation of a generic analytical sample preparation protocol for simultaneous determination of more polar, less volatile and thermally unstable emerging contaminants in biota by LC-HRMS and the investigation of the occurrence of more than 2,400 micropollutants in raptor egg samples by liquid (LC) and gas chromatography (GC) coupled with HRMS.

In National and Kapodistrian University of Athens (NKUA), two in-house datasets have been developed, including more than 2,400 contaminants [The LC target list is available as S21 UATHTARGETS in the NORMAN

Suspect List Exchange https://www.norman-network.com/nds/SLE/ (DOI: 10.5281/zenodo.3723478) and the GC target is available as S65 UATHTARGETSGC ((DOI: 10.5281/zenodo.3753372))] used for the wide-scope target analysis, in order to determine PPs and ECs in biota such as eggs that have been collected from Baden-Württemberg, Germany within 2005 and 2020. Eggs' samples were analyzed in collaboration with LUBW. Environment is an example of combining sustainability concerns with protection and conservation of marine resources. The National and Kapodistrian University of Athens, specifically Trace Analysis and Mass Spectrometry (Tr.A.M.S.) group, received 26 raptor eggs from 4 different species, which Peregrine falcon (*Falco peregrinus*), Little owl (*Athene noctua*), Great curlew (*Numenius arquata)* and Eagle owl (*Bubo bubo*) were included.

CHAPTER 4

MATERIALS AND METHODS

4.1 Chemicals and materials

Regarding with the sample preparation, for the extraction using Accelerated Solvent Extraction (ASE), cellulose ASE Extraction filters and Diatomaceous Earth for ASE were obtained from Thermo Scientific (Waltham, Massachusetts, USA). For the clean-up step using Solid Phase Extraction (SPE), the empty solid phase extraction propylene tubes (6mL) and the cartridge 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 the Isolute ENV+ sorbent material and the frits (20μm, 6mL) were purchased from Biotage (Ystrad Mynach, UK). Strata® FL-PR Florisil cartridges ((170 μm, 80 Å), 5 g/ 20 mL, Giga Tubes) where purchased from Phenomenex (Torrance, USA). Regenerated cellulose (RC) syringe filters (diameter 15 mm, pore size 0.2 μm) were obtained from MACHEREY-NAGEL GmbH & Co. KG (Düren, Germany). Regarding the chemicals of the sample preparation; Methanol, Acetonitrile, Isopropanol and Hexane (HPLC grade) were purchased from Fischer Scientific (Loughborough, UK), Ethyl Acetate ≥99.5% (GC) and Dichloromethane were purchased from Sigma Aldrich (Steinheim, Germany), and Formic Acid 98-100% for analysis was purchased from CARLO ERBA Reagents S.A.S. (Barcelona, Spain), while Ammonia solution 25% for analysis was purchased from CHEM-LAB NV (Zadelgem, Belgium).

All the solvents for the chromatographic analysis were hypergrade for LC-MS. Methanol, Acetonitrile were obtained from Merck (Darmstadt, Germany) and Hexane was obtained from Honeywell (New Jersey, USA) and the eluent additives ammonium formate, ammonium acetate and formic acid 99% were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was provided by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA).

The reference standards, which were used for the method validation; Albendazole, Albendazole sulfone, Ambroxol, Amitriptyline, Amlodipine, Amphetamine, Atenolol, Atorvastatin, desethyl-Atrazine, Azithromycin, Benzenesulfonamide, 2-hydroxy-Benzothiazole, 2-amino-Benzothiazole, 2-amino-6-chloro-Benzothiazole, Benzotriazole (BTR), 5-methyl-Benzotriazole, 1-hydroxy-Benzotriazole, 5,6-dimethyl-Benzotriazole, Bisphenol A, Boscalid, Bromazepam, Caffeine, Carbamazepine, Carbendazim, Chloramphenicol, Chlorbufam, Chlorpromazine, Cimaterol, Cimetidine, Ciprofloxacin, Citalopram amide, Citalopram carboxylic acid, Clarithromycin, Clenbuterol, Clofibric acid, Clomipramine, Colchicine, Closantel, Cortisol F, Cortisone E, Danofloxacin, Dapsone, DEET, Decoquinate, Desmethyl-citalopram, Diaveridine, Dibucaine, Diclazuril, Diclofenac, Difloxacin, Dimethoate, Dimetridazole, Diuron, 2-4-Dinitrophenol (DNP), Dorzolamide, Doxepin, Emamectin B1a, Enrofloxacin, Erythromycin, Esmolol, Ethopabate, Etrimfos, Fenbendazole, Fenoxycarb, Fenthion-sulfoxide, Florfenicol, Fluazuron, Flubendazole, Flufenamic acid, Flumequine, Flunixine, Fluoxetine, Furosemide, Gemfibrozil, Hordenine, Hydrochlorothiazide, Imazamox, Imidacloprid, Imipramine, Irgarol, Ketoprofen, Levamisol, Lincomycin, Lorazepam, Lufenuron, Mabuterol, Marbofloxacin, Mebendazole, Meclofenamic Acid, Mefenamic acid, Melatonin, Meloxicam, Metformin, Methomyl, Metolachlor, Metoprolol, Metribuzin, Metronidazole, Myclobutanil, Nalidixic acid, Naproxen, Nicotine, Niflumic acid, Nigericin, Nonylphenol (4-NP), Nor-nicotine, Novobiocin, Olanzapine, Omethoate, Oxamyl, Oxfendazole, Oxprenolol, Paroxetine, Perfluoro butane sulfonic acid (PFBuS), Perfluoro decanoic acid (PFDeA), Perfluoro dodecanoic acid (PFDoA), Perfluoro Heptanoic acid (PFHpA), Perfluoro hexanoic acid (PFHxA), Perfluoro nonanoic acid (PFNA), Perfluoro octanoic acid (PFOA), Perfluoro undecanoic acid (PFUnA), Phenylbutazone, di-n-octyl-Phthalate, diethyl-Phthalate, diphenyl-Phthalate, Prednisolone, Primidone, Progesterone, Prometryn, Propazine, Propoxur, Propranolol, Ractopamine, Ranitidine, Rifaximin, Ronidazole, Salbutamol, Salicylic acid, Sarafloxacin, Sertraline, Siduron, Simvastatin, Spiroxamine, Sulfachloropyridazine, Sulfaclozine, Sulfadimethoxine, Sulfadimidine (Sulfamethazine), Sulfadoxine, Sulfamerazine, Sulfameter

(Sulfumetin), Sulfamethizole, Sulfamethoxazole, Sulfamethoxypyridazine, Sulfamonomethoxine, Sulfamoxole, Sulfapyridine, Sulfaquinoxaline, Sulfathiazole, Sulfisoxazole, Terbutaline, Terbuthylazine, Ternidazole, Theophylline, Thiabendazole, Thiacloprid, Thiamphenicol, Tiamulin, Tiapride, Tilmicosin, Toltrazuril, Toluenesulfonamide, Topiramate, Tramadol, Triamterene, Triclabendazole, Trimethoprim, Valsartan and Venlafaxine, were purchased from Sigma Aldrich (Steinheim, Germany), Toronto Research Chemicals (Ontario, Canada), LGC (Mercatorstrass, Germany), Acros Organics (Morris Plains, NJ) and Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). Standards of Polychlorinated biphenyls (PCBs) and Polycyclic aromatic hydrocarbons (16-PAHs) from Fluka-Sigma-Aldrich (Steinheim, Germany) were used.

Regarding with the internal standards (IS), which were used for the the method validation and analysis, Flunixin-d_3, Meloxicam-d_3, Bisphenol A (BPA)-d_{16}, Diuron-d_6, Atrazine-d_5, Diazepam-d_5 and Amphetamine-d_6 were obtained from Sigma Aldrich (Steinheim, Germany), while Ranitidine-d_6, Fenbendazole-d_3, Cetirizine-d_3, Mefenamic Acid-d_3, Diethyl-phthalate-d_4, Amisulpride-d_5, Sulfadimethoxine-d_6, Sulfamethazine-d_4 and Sulfadiazine-d_4 were obtained from Toronto Research Chemicals (Ontario, Canada).

Internal standards of Chlorfenvinphos-D10, HCH alpha D6, Terbuthylazine D5, Malathion-D7, Dimethoate D6 (O,O-dimethyl D6), Omethoate D6 (O,O-dimethyl D6), Dichlorvos D6, Terbutryn D5(ethyl D5), 2,4´-DDT D8, Chlorpyrifos-D10 (diethyl D10), Simazine D10, Propazine D6 (Isopropyl D6), Trifluralin D14 (dipropyl D14)) were obtained from LGC (Mercatorstrass, Germany). Also, a standard solution of 16 PAHs I.S. (Napthalene-d8, Acenapthylene-d8, Acenapthene-d10, Fluorene-d10, Phenathrene-d10, anthracene-d10, Fluoranthene-d10, Pyrene-d10, Benzo (a) anthracene-d12, Chrysene-d12, Benzo (b) fluoranthene-d12, Benzo (k) fluoranthene-d12, Benzo (a) pyrene-d12, Indeno (1,2,3-c,d) pyrene-d12, Dibenzo (a,h) anthracene-d14, Benzo (g,h,i) perylene-d12) from CPA Chem was used. Working solutions of 1000 mg L-1 were prepared. The working solutions contained all the analytes for the method validation. The solutions stored at -20 °C.

4.2 Sample pretreatment

Raptor eggs (Little owl, Great curlew, Peregrine falcon and Eagle owl) were gathered by LUBW Institute from Baden-Württemberg, Germany within 2005 and 2020 (**Figure 13**). Details for the samples are included in the following **Table 2**. These wet samples were sent in Laboratory of Analytical Chemistry (NKUA) with dry ice, according to the strict protocols.

Figure 13: Sampling location (*Source:* https://www.britannica.com/place/Baden-Wurttemberg , last accessed 15/09/2021)

Table 2: Sample table details and coding

Sample Code	***Species Name***	***Sampling Year***
LUBW 3	Little owl (*Athene noctua*)	2020
LUBW 8	Little owl (*Athene noctua*)	2020
LUBW 12	Little owl (*Athene noctua*)	2020
LUBW 14	Little owl (*Athene noctua*)	2020

Sample Code	***Species Name***	***Sampling Year***
LUBW 18	Little owl (*Athene noctua*)	2020
LUBW 22	Little owl (*Athene noctua*)	2020
LUBW 24	Great curlew (*Numenius arquata)*	2011
LUBW 26	Great curlew (*Numenius arquata)*	2007
LUBW 27	Great curlew (*Numenius arquata)*	2005
LUBW 28	Great curlew (*Numenius arquata)*	2006
LUBW 29	Great curlew (*Numenius arquata)*	2005
LUBW 30	Great curlew (*Numenius arquata)*	2019
LUBW 31	Great curlew (*Numenius arquata)*	2020
LUBW 32	Great curlew (*Numenius arquata)*	2005
LUBW 33	Great curlew (*Numenius arquata)*	2016
LUBW 01	Peregrine falcon (*Falco peregrinus*)	2020
LUBW 04	Peregrine falcon (*Falco peregrinus*)	2020
LUBW 07	Peregrine falcon (*Falco peregrinus*)	2020
LUBW 012	Peregrine falcon (*Falco peregrinus*)	2020
LUBW 013	Peregrine falcon (*Falco peregrinus*)	2020
LUBW 015	Peregrine falcon (*Falco peregrinus*)	2020
LUBW 017	Peregrine falcon (*Falco peregrinus*)	2020
LUBW 019	Peregrine falcon (*Falco peregrinus*)	2020
LUBW 002	Peregrine falcon (*Falco peregrinus*)	2019
LUBW 101	Eagle owl (*Bubo bubo*)	2019
LUBW 102	Eagle owl (*Bubo bubo*)	2020

All samples were lyophilized (temperature: -55°C, vacuum: 5×10^{-2} mbar) before analysis, using the Telstar's Freeze-Dryer LyoQuest (**Figure 14**), before analysis, in order to enhance extraction efficiency, improve the precision and achieve lower detection limits. After lyophilization, the % water content of each sample was calculated and the freeze-dried samples were homogenized using pestle and mortar. After homogenization, the samples were storage in brown glass bottles in the freezer (-80°C) till the analysis (**Figure 15**).

Figure 14: Freeze-Dryer, Telstar (Source: *https://www.telstar.com/lab-hospitalsequipment/laboratory-freeze-dryers/lyoquest/*).

Figure 15: Lyophilized and homogenized LUBW samples.

Table 3: Freeze-Drying details

Sample Code	***Mass of wet sample (g)***	***Mass of freeze-dried sample (g)***	***%Water content***
LUBW 3	44.0758	9.5765	78.3
LUBW 8	42.1472	10.7004	74.6
LUBW 12	46.0011	10.0017	78.3
LUBW 14	41.655	8.9966	78.4
LUBW 18	40.2947	8.5183	78.9
LUBW 22	34.693	7.3423	78.8
LUBW 24	42.9043	13.743	68.0
LUBW 26	49.4725	13.4724	72.8
LUBW 27	36.9499	10.0178	72.9
LUBW 28	67.2892	18.7682	72.1
LUBW 29	54.0509	17.0565	68.4
LUBW 30	47.4853	13.3934	71.8
LUBW 31	31.3631	9.7094	69.0
LUBW 32	57.4696	15.7411	72.6
LUBW 33	30.0023	7.8943	73.7
LUBW 01	25.378	4.8068	81.1
LUBW 04	34.9172	6.9949	80.0
LUBW 07	35.1322	11.4645	67.4
LUBW 012	30.3448	6.3434	79.1
LUBW 013	29.7698	6.2632	79.0
LUBW 015	27.239	5.7457	78.9
LUBW 017	21.9985	4.7635	78.3

Sample Code	*Mass of wet sample (g)*	*Mass of freeze-dried sample (g)*	*%Water content*
LUBW 019	26.1897	5.2366	80.0
LUBW 002	25.8047	5.3957	79.1
LUBW 101	54.5468	10.5394	80.7
LUBW 102	31.08	8.1724	73.7

4.3 Method Validation

A representative validation dataset of 80 compounds classified in different chemical classes was used in order to evaluate linearity, accuracy, precision, matrix effects and detectability of the screening method. The compounds of the validation dataset and their fragments in positive and in negative ESI mode are shown in **Table 4**. These selected compounds represented almost all the classes of ECs in the database and had several physicochemical properties, so they eluted all over the chromatogram.

Linearity was studied for each compound by analyzing spiked samples (standard addition curve) in eggs at 7 different concentrations ranging from 0.610 μg/kg w.w. till 24.4μg/kg w.w., as well as standard solutions in the same levels. Using these spiked curves, the limits of detection (LOD) were calculated.

Accuracy was assessed with recovery experiments. Method recovery was calculated by dividing the peak area of the spiked samples by the peak area of the matrix-matched samples at 1.53, 4.58, 9.15 and 15.3 μg/kg w.w. The initial samples were analyzed for determination of the analytes of the validation dataset and if the sample already contained the analyte, its peak area was subtracted from the peak area of the spiked sample and the peak area of the matrix-matched sample. Method recovery was assessed by the following equation:

$$\%\boldsymbol{Recovery} = \frac{(\boldsymbol{Relative\ Area})\boldsymbol{spiked\ sample} - (\boldsymbol{Relative\ Area})\boldsymbol{blank\ sample}}{(\boldsymbol{Relative\ Area})\boldsymbol{matrix\ matched} - (\boldsymbol{Relative\ Area})\boldsymbol{blank\ sample}} \times \mathbf{100}$$

Precision was expressed as method repeatability in 2 spiked samples at 4.58 and 9.15 μg/kg w.w. after the calculation of the factor of matrix effect (FME) by dividing the peak area of matrix-matched samples by the peak area of the standard solutions, matrix effect was assessed by the equation:

$$\%\text{Matrix Effect} = (\text{Matrix Factor} - 1) \times 100.$$

$$\%\boldsymbol{FME} = \frac{(\boldsymbol{Relative\ Area})\boldsymbol{matrix\ matched} - (\boldsymbol{Relative\ Area})\ \boldsymbol{blank\ sample}}{(\boldsymbol{Relative\ Area})\boldsymbol{standard} - (\boldsymbol{Relative\ Area})\ \boldsymbol{solvent}} \times \mathbf{100}$$

Table 4: Validation Dataset

Compound Name	*CAS Number*	*m/z precursor ion*	*Retention time (min)*	*Molecular Formula*	*Fragment 1*	*Fragment 2*	*Fragment 3*	*Fragment 4*
PFOA	335-93-3	412.9653	9.68	C8HF15O2	418.9734	168.9894	218.9862	118.7511
PFBuS	375-73-5	298.9419	6.64	C4F9SO3H	298.9432	79.9574	98.9558	82.9609
PFDeA	335-76-2	512.9589	11.1	C10F19O2H	218.9862	468.9702	268.9830	168.9894
PFOS	2795-39-3	498.9291	10.4	C8F17SO3H	499.0186	168.7352	498.9575	98.7875
PFDoA	307-55-1	612.9526	12.2	C12F23O2H	268.9830	218.9862	568.9627	
PFHpA	375-85-9	362.9685	8.79	C7F13O2H	368.9766	168.9894	112.9856	118.9925
PFHxA	307-24-4	312.9717	7.73	C6F11O2H	368.9766	168.9894	118.9926	268.9833
PFNA	444-03-1	462.9621	10.4	C9F17O2H	218.9862	468.9702	268.9830	168.9892
PFOSA	754-91-6	497.9451	11.6	C8F17SO2NH2	268.9830	218.9862	525.9764	118.9920
PFPeA	5989-64-0	262.9749	6.39	C5F9O2H	268.983	118.9926	196.9834	
PFTrDA	72629-94-8	662.9494	12.6	C13F25O2H	368.9766	318.9787	618.9595	
PFUnA	2058-94-8	562.9557	11.7	C11F21O2H	268.983	218.9862		

Compound Name	***CAS Number***	***m/z precursor ion***	***Retention time (min)***	***Molecular Formula***	***Fragment 1***	***Fragment 2***	***Fragment 3***	***Fragment 4***
5-Me-Benzotriazole	136-85-6	134.0713	5.83	C7H7N3	134.0715	79.0543	77.0386	106.0651
Azoxystrobin	131860-33-8	404.1241	9.02	C22H17N3O5	372.0979	344.1030	329.0800	183.0558
DEET (Diethyltoluamide)	134-62-3	192.1383	8.20	C12H17NO	119.0491	91.0542	109.0648	72.0444
Dimethirimol	5221-53-4	210.1601	7.70	C11H19N3O	140.1070	71.0604		
2.4-Dinitrophenol (DNP)	51-28-5	184.0120	4.50	C6H4N2O5	95.0146	123.0085	109.0174	183.0048
Diuron	330-54-1	233.0243	8.64	C9H10Cl2N2O	72.0444	161.9672	187.9670	159.9715
Imazamox	114311-32-9	306.1448	4.63	C15H19N3O4	306.1445	261.1314	260.1406	264.0979
Imidacloprid	138261-41-3	256.0596	4.76	C9H10ClN5O2	175.0978	209.0589	84.0556	
Metolachlor	51218-45-2	284.1412	10.3	C15H22ClNO2	252.1150	177.1465	284.1415	212.0837
Myclobutanil	88671-89-0	289.1215	9.74	C15H17ClN4	70.0400	125.0153	220.0898	289.1218
Omethoate	1113-02-6	214.0297	3.35	C5H12NO4PS	182.9875	142.9916	127.0145	154.9921
Propazine	139-40-2	230.1167	9.16	C9H16ClN5	230.1162	174.0531	146.0225	188.0702
Propoxur	114-26-1	210.1125	6.94	C11H15NO3	111.0441	153.091	168.0655	93.0339

Compound Name	***CAS Number***	***m/z precursor ion***	***Retention time (min)***	***Molecular Formula***	***Fragment 1***	***Fragment 2***	***Fragment 3***	***Fragment 4***
Siduron	1982-49-6	233.1648	9.37	$C_{14}H_{20}N_2O$				
Terbuthylazine	5915-41-3	230.1167	9.36	$C_9H_{16}ClN_5$	132.0315	138.0764	146.0219	148.0185
Thiabendazole	148-79-8	202.0433	6.15	$C_{10}H_7N_3S$	175.0324	202.0442	131.0599	204.0391
Thiamethoxam	153719-23-4	292.0266	4.25	$C_8H_{10}ClN_5O_3S$	211.0648	181.0542	131.9669	210.0570
Desisopropyl-Atrazine	1007-28-9	174.0541	4.75	$C_5H_8ClN_5$	104.0010	96.0556	132.0323	79.0058
3-4-(dichlorophenyl)-3-methyl urea	3567-62-2	219.0086	8.51	$C_8H_8Cl_2N_2O$				
Methylparaben	99-76-3	151.0401	5.97	$C_8H_8O_3$	46.01225	136.0163	151.0395	151.5412
Melatonin	73-31-4	233.1285	5.36	$C_{13}H_{16}N_2O_2$	174.0913	159.0679	131.0730	143.0730
Flecainide	54143-55-4	415.1451	6.44	$C_{17}H_{20}N_2O_3F_6$	398.1185	98.0964	301.0294	81.0699
Acebutolol	37517-30-9	337.2122	4.78	$C_{18}H_{28}N_2O_4$	116.1070	72.0808	74.0600	98.0964
Atenolol	29122-68-7	267.1703	3.09	$C_{14}H_{22}N_2O_3$	145.0648	190.0863	178.0863	133.0648
Dibucaine	85-79-0	344.2333	8.54	$C_{20}H_{29}N_3O_2$	344.2334	271.1443	74.0964	215.0815
Dorzolamide	120279-96-1	325.0345	3.19	$C_{10}H_{16}N_2O_4S_3$	198.9882	135.0263	151.0212	235.9504

Compound Name	***CAS Number***	***m/z precursor ion***	***Retention time (min)***	***Molecular Formula***	***Fragment 1***	***Fragment 2***	***Fragment 3***	***Fragment 4***
Esmolol	103598-03-4	296.1856	5.18	C16H25N1O4	72.0808	145.0648	74.0600	56.0495
Flufenamic acid	530-78-9		9.31	C14H10F3NO2	280.0591	236.0693	216.0630	234.0536
Hordenine	539-15-1	166.1226	2.28	C10H15NO	121.0648	103.0542	91.0542	93.0699
Ipratropium	22254-24-6	332.2220	4.17	C20H30NO3^1+	124.1121	166.159	290.1751	93.0699
Ketoprofen	22071-15-4	255.1016	8.53	C16H14O3	105.0335	209.0961	95.0491	177.0546
Lamotrigine	84057-84-1	256.0151	5.31	C9H7N5Cl2	210.9824	58.0400	186.9824	220.0384
Lopinavir	192725-17-0	629.3697	11.8	C37H48N4O5	183.1128	429.2537	155.1179	447.2642
Loratadine	79794-75-5	383.1521	11.5	C22H23N2O2Cl1	337.1102	267.0809	259.1356	383.1551
Metoclopramide	364-62-5	300.1473	4.36	C14H22N3O2Cl1	227.0582	184.016	212.0347	300.1479
Mexiletine	31828-71-4	180.1383	5.59	C11H17N1O1	58.0651	121.0648	105.0698	180.1382
Minoxidil	38304-91-5	210.1349	4,88	C9H15N5O1	84.0808	164.0931	110.0587	192.1248
Propafenone	54063-53-5	342.2064	7,81	C21H27N1O3	116.1070	72.0808	98.0964	91.0542
Rivastigmine	123441-03-2	251.1754	4.74	C14H22N2O2	86.0600	58.0287	206.1176	46.0651

Compound Name	***CAS Number***	***m/z precursor ion***	***Retention time (min)***	***Molecular Formula***	***Fragment 1***	***Fragment 2***	***Fragment 3***	***Fragment 4***
Salicylic acid	63-36-5	138.03169	3.58	C7H6O3	93.0346	137.025	138.0287	65.0401
Sulfadiazine	68-35-9	251.0597	3.48	C10H10N4O2S	92.0495	108.0444	96.0556	156.0114
Sulfadimethoxine	122-11-2	311.0809	5.6	C12H14N4O4S	156.0762	218.0235	108.0440	245.1032
Sulfadimidine (Sulfamethazine)	57-68-1	279.0910	4.31	C12H14N4O2S	122.0716	124.0872	126.0663	156.0098
Sulfadoxine	2447-57-6	311.0809	4.75	C12H14N4O4S	108.0444	92.0495	156.0114	140.0455
Sulfamethoxypyridazine	80-35-3	281.0703	4.38	C11H12N4O3S	108.0444	92.0495	156.0114	126.0662
Sulfisoxazole	127-69-5	268.0750	4.71	C11H13N3O3S	92.0498	120.0559	156.0118	113.0709
Terbutaline	23031-25-6	226.1438	3.11	C12H19N1O3	152.0706	125.0597	107.0491	57,0699
Tinidazole	19387-91-8	248.0700	3.86	C8H13N3O4S1	128.0455	286.0258	270.0519	121.0318
Triamterene	396-01-0	254.1149	4.68	C12H11N7	237.0883	212.0931	195.0665	255.1188
Tramadol-O-Desmethylnor	144830-18-2	236.1645	4.28	C14H21NO2	44.0495			
Deacetyl-Diltiazem	42399-40-6	373.1580	6.41	C20H24N2O3S	178.0321	72.0808	150.0372	328.1002
Desalkyl-Flurazepam	2886-65-9	289.0538	8.74	C15H10ClFN2O	140.0262	226.0901	261.0589	

Compound Name	***CAS Number***	***m/z precursor ion***	***Retention time (min)***	***Molecular Formula***	***Fragment 1***	***Fragment 2***	***Fragment 3***	***Fragment 4***
O-Desmethyl-Tramadol	73986-53-5	250.1802	4.06	C15H23N1O2	58.0651			
1-Piperonyl-Piperazine	32231-06-4	221.1285	4.22	C12H16N2O2	135.0441	105.0335	79.0542	
Amphetamine	300-62-9	136.1121	4.16	C9H13N	91.0542	65.0386	119.0859	89.6076
Ritalinic acid	19395-41-6	220.1332	4.61	C13H17N1O2	84.0808	174.1277	56.0495	220.1335
Moclobemide	71320-77-9	269.1051	4.98	C13H17N2O2Cl1	182.0367	138.9945	114.0913	88.0758
Sulpiride	15676-16-1	342.1482	2.94	C15H23N3O4S1	112.1121	214.0169	98.0964	58.0651
Tiapride	51012-32-9	329.1530	3.41	C15H24N2O4S	256.0638	213.0216	74.0964	100.1121
Trazodone	19794-93-5	372.1586	6.48	C19H22N5O1Cl1	176.0818	148.0505	96.0444	237.1153
Citalopram amide	64372-56-1	343.1816	5.15	C20H23FN2O2	237.1074	280.1132	325.1711	222.0839
Citalopram carboxylic acid	NA	344.1656	5.83	C20H22FNO3	281.0967	237.1067	326.1548	222.0832
Desmethyl-Citalopram	97743-99-2	311.1554	6.66	C19H19FN2O	293.1449	262.1027	234.0714	247.0792
Anabasine	40774-73-0	163.1230	2.94	C10H14N2	80.0495	84.0808	120.0808	
Nicotine	54-11-5	163.1230	2.41	C10H14N2	84.0808	80.0495	117.0573	130.0651

Compound Name	***CAS Number***	***m/z precursor ion***	***Retention time (min)***	***Molecular Formula***	***Fragment 1***	***Fragment 2***	***Fragment 3***	***Fragment 4***
Nor-Nicotine	605-89-0	149.1073	3.14	$C_9H_{12}N_2$	130.0645	132.0798	149.1065	106.0651
Irgarol	28159-98-0	254.1434	10.5	$C_{11}H_{19}N_5S$	198.0802	254.1437	255.1457	156.0580
Irgarol-descyclopropyl	NA	214.1121	8.43	$C_8H_{15}N_5S$	158.0492	214.1124	159.0508	143.0258

4.4 Sample preparation

All samples were lyophilized and homogenized before analysis, as referred in previous paragraph . In addition, the concentrations of the analytes can be expressed in both dry weight and wet weight.

Two generic sample preparation protocols per sample were followed. Polar, semi-polar, less volatile and thermally unstable compounds were extracted by the method specific for LC-amenable compounds, whereas different sample preparation method was followed for the extraction of non-polar, volatile and thermostable, GC-amenable compounds.

4.4.1 Extraction of LC-amenable compounds

Samples' weighting was the first step of the protocol; 1g of egg was mixed with anhydrous Sodium Sulfate (Na_2SO_4), which was used as dispersant in order to improve recovery of analytes during or after extraction, with pestle and mortar in a ratio sample: dispersant 1:4. An IS mix solution was spiked in each sample and left in contact with the matrix for at least 30 min prior to the extraction with ASE. Representative compounds from different classes of the LC target list of the NKUA were selected. The conditions which were used for the extraction are summarized in the **Table 5**.

Table 5: Conditions which were used in ASE for LC-amenable compounds

Temperature (°C)	50
Pressure (psi)	1500
Heating Time (s)	300
Static Time (s)	300
# Static cycles	3
Flush Volume (%)	60
Purge Time (s)	180
Extraction Solvent	Methanol:Acetonitrile (2:1)
Total volume of extraction solvents (mL)	50

Before the clean-up step the extract was evaporated till 3-4mL (if the extract was not transparent, it was filtered through filter paper). Then milli-Q water was added till 15mL.

Figure 16: Dionex™ ASE™ 350 Accelerated Solvent Extractor (*source: https://www.thermofisher.com/order/catalog/product/083114*)

In an effort to obtain more clear extracts a defatting step with hexane was used as the first step of sample clean-up. 5mL of n-hexane were added, then the sample was stirred with vortex for 1 minute, it was centrifuged in 4000 rpm for 10 minutes, the two layers were separated, the hexane layer (upper) was rejected and in the water layer milli-Q was added till 50mL. This milli-Q water addition was taken place in order to reduce the percentage of methanol content. To achieve sufficient enrichment for a board range of compounds, SPE with mixed bed multilayer cartridges was used for sample clean-up. These in-house SPE cartridges (**Figure 17**) consisted of 200 mg of Strata-X (polymeric reversed phase sorbent for extraction of neutral and aromatic compounds) and a mixture of 100 mg of Strata-X-AW (weak anion exchanger for extraction of acidic compounds with pKa<5), 100mg of Strata-X-CW (weak cation exchanger for extraction of basic compounds with pKa>8) and 150 mg of Isolute ENV+ (polymeric reversed phase sorbent for extraction of polar compounds). The conditioning of the cartridges was performed with 3mL methanol and 3mL water. The samples were loaded to the SPE cartridges and then they were dried under vacuum at a flow rate of 10mL/min for 0.5 to 1h. The elution of the analytes from the absorbent material was performed by a basic solution (4mL of ethyl acetate: methanol (50:50 v/v) containing 2% ammonia hydroxide (v/v)), followed by an acidic solution (2mL of ethyl acetate: methanol (50:50 v/v) containing 1.7% formic acid (v/v)).

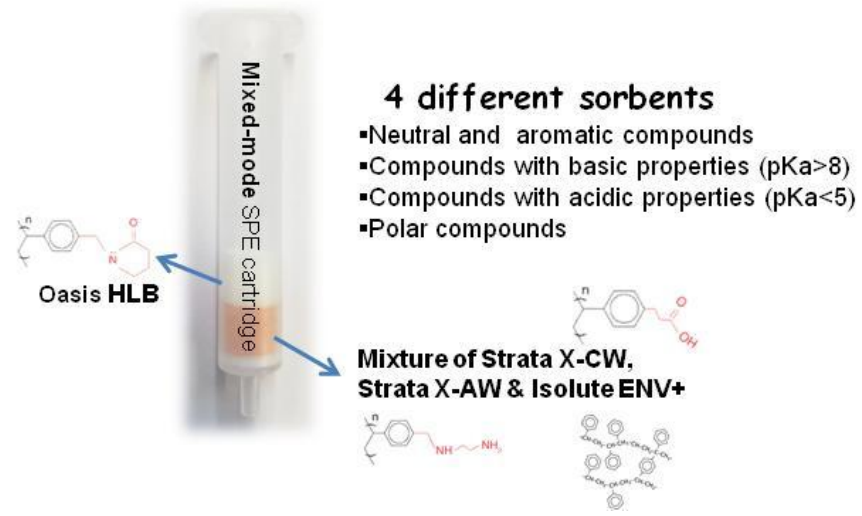


Figure 17: Mixed-mode SPE cartridges.

The extracts were evaporated under a gentle nitrogen stream (40-45°C) till dryness and finally reconstituted to a final volume of 250 μL methanol: water 50:50. Every extract was filtered directly into a 2mL vial using a syringe fitted with a 0.2 μm RC membrane filter in order to remove the solid particles that were still present and may cause blockage of the column filter, and then they were ready for LC-HRMS/MS analysis. After analysis by LC-ESI-QTOF-MS the vials were stored in the freezer at -80°C.

4.4.2 Extraction of GC-amenable compounds

Samples' weighting was the first step of the protocol; 1g of egg was mixed with anhydrous Sodium Sulfate (Na_2SO_4), which was used as dispersant in order to improve recovery of analytes during or after extraction, with pestle and mortar in a ratio sample: dispersant 1:4. An IS mix solution was spiked in each sample and left in contact with the matrix for at least 30 min prior to the extraction with ASE. The conditions which were used for the extraction are summarized in the **Table 6**.

Table 6: Conditions which were used in ASE for GC-amenable compounds

Temperature (°C)	50
Pressure (psi)	1500
Heating Time (s)	300
Static Time (s)	300
# Static cycles	3
Flush Volume (%)	60
Purge Time (s)	180
Extraction Solvent	Hexane:Dichloromethane (2:1)
Total volume of extraction solvents (mL)	50

50 µL of isooctane, used as keeper, were added. Before the clean-up step the extract was evaporated till 10mL (if the extract was not transparent, it was filtered through filter paper).

The extracts were then cleaned-up by SPE. Strata® FL-PR Florisil (**Figure 18**) ((170 µm, 80 Å), 5 g/ 20mL, Giga Tubes, Phenomenex) cartridges were used. The conditioning of the cartridges was performed using 20mL of 10% Isopropanol in dichloromethane, followed by 30mL of hexane. After conditioning, the samples were loaded in the SPE cartridges and the eluent was collected. The elution of the analytes from the adsorbent material was performed using 20 mL of dichloromethane: hexane (50/50 v/v), followed by 20 mL of hexane. The whole extract (cleaned extract and elution solvents) was placed into an evaporation flask. After that 50µL of isooctane was added. The extract was pre-concentrated by rotary evaporation (max. temperature 30 °C) till 10mL. The extract was evaporated using nitrogen stream (max. temperature 30 °C) till dryness and finally reconstituted to a final volume of 250µL of hexane and the reconstituted sample was homogenized using Vortex stirring for 1 min. During the sample preparation 4-fold enrichment of the extracts was achieved. The final extract was filtered through the Regenerated Cellulose (RC) filter (Chromafil - pore size: 0.2 μm; filter diameter: 15 mm), using a syringe, into a 2mL glass vial with an insert placed inside each vial. The vials were pre-labeled with the code of the respective sample. After analysis by GC-APCI-QToF MS the vials were stored in the freezer at -80°C.

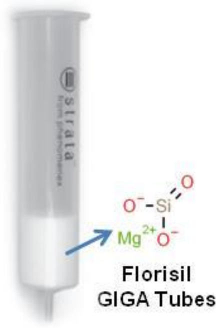


Figure 18: FL-PR Florisil cartridges

4.5 Instrumentation

The samples were analysed by both liquid and gas chromatography hyphenated with a high resolution mass analyser. Detailed information on the instrumental analysis of the RPLC-ESI-QTOF and GC-APCI-QTOF system is provided below in paragraphs 4.5.1 and 4.5.2, respectively.

4.5.1 UHLPC-QTOF-MS

An ultra-High Performance Liquid Chromatography (UHPLC) system (UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany) coupled to a Quadrupole-Time of Flight Mass Spectrometer (QTOF-MS) (Maxis Impact, Bruker Daltonics, Bremen, Germany) (**Figure 19**) was used for the analysis of the samples. The UHPLC apparatus consists of a solvent rack degasser, a binary pump with solvent selection valve (HPG-3400), an auto-sampler and a column. The QTOF-MS apparatus consists of an Electrospray Ionization (ESI) source operating in positive and negative mode.

Figure 19: UHPLC-QTOF-MS, Maxis Impact, Bruker Daltonics (source: *http://www.directindustry.com/prod/bruker-daltonics/product-30029-991983.html***).**

In the analysis, two separate reversed-phase chromatographic runs were performed for positive and negative ESI mode. An Acclaim RSLC 120 C_{18} column (2.1 × 100 mm, 2.2 μm) (Dionex Bonded Silica Products, Thermo Scientific, Dreieich, Germany), preceded by an ACQUITY UPLC BEH C_{18} 1.7 μm guard column of the same packaging material (VanGuard Pre-column, Waters, Dublin, Ireland), and thermostated at 30°C, was used. In the positive ESI mode, the aqueous mobile phase consisted of 90% H_2O, 10% CH_3OH, 5mM $HCOONH_4$, 0.01% HCOOH and the organic mobile phase consisted of CH_3OH, 5mM $HCOONH_4$, 0.01% HCOOH. In the negative ESI mode, the aqueous mobile phase consisted of 90% H_2O, 10% CH_3OH, 5mM CH_3COONH_4 and the organic mobile phase consisted of CH_3OH, 5mM CH_3COONH_4. The gradient elution program was the same for both ionization modes and applied changes in mobile phase and in flow rate. It started with 1.0% of organic phase (flow rate 0.200mL/min) for 1 min, increasing to 39.0% by 3 min (flow rate 0.200mL/min), and then 99.9% (flow rate 0.400mL/min) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.480mL/min) and then initial conditions were restored within 0.1min, kept for 3 min and then the flow rate decreased to 0.200mL/min for last minute. The gradient elution program which is used in the chromatographic analysis is also presented in the following **Table 7**. The injection volume was set to 5μL.

The operating parameters of the ESI interface were the following: capillary voltage 2500 V for positive and 3000 V for negative mode, end plate offset 500 V, nebulizer pressure (N2) 2.0 bar, drying gas (N2) 8.0 L/min, drying temperature 200°C.

Data were acquires through a Data Independent Acquisition (DIA) scan mode, called broad-band Collision Induced Dissociation (bbCID), which provided both MS and MS/MS spectra simultaneously using two different collision energies with a scan rate of 2 Hz and a mass range of 50-1000 Da. Low collision energy (4eV) provided a full scan spectrum (MS) and high collision energy (25eV) provided a spectrum where all ions were fragmented (bbCID MS/MS).

Table 7: The gradient elution program of LC-HRMS analysis

Time (min)	***Flow Rate (mL/min)***	***Aqueous Solvent (%)***	***Organic Solvent (%)***
0	0.2	99.0	1.0
1	0.2	99.0	1.0
3	0.2	61.0	39.0
14	0.4	0.1	99.9
16	0.48	0.1	99.9
16.1	0.48	99.0	1.0
19.1	0.2	99.0	1.0
20	0.2	99.0	1.0

An external calibration of the QTOF mass spectrometer was performed with a sodium formate solution before analysis. Also, a calibrant injection was performed automatically at the beginning of each run and the segment of 0.1-0.25 min was used for the internal calibration. The calibration solution of sodium formate consisted of 10mM sodium formate clusters in a mixture of water:isopropanol 1:1. The theoretical exact masses of calibration ions with formulas $Na(NaCOOH)_{1-14}$ in the range of 50-1000 Da were used for calibration. The instrument provided a typical resolving power of 36,000-40,000 during calibration.

Bruker's software that was used for raw data analysis was Data Analysis 5.1 and TASQ Client 2.1.

4.5.2 GC-APCI-QTOF-MS

The analysis of the egg samples was carried out utilizing a GC-APCI-QTOF-MS system (**Figure 20**). The GC-APCI-QTOF system consisted of a Bruker 450 GC, a CP-8400 Autosampler and a hybrid quadrupole time of flight mass spectrometer (QTOF-MS) (Maxis Impact, Bruker Daltonics).

GC was operated in splitless injection mode (Restec Split liner w/Glass Frit (4mm × 6.3 ×78.5() and the splitless purge valve was activated 1 min after injection. The injection volume was 1 µL. A Restek Rxi-5Sil MS column of 30 m (0.25 mm i.d. × 0.25 µm film thickness) was used with Helium as carrier gas in a constant flow of 1.5mL min^{-1}.

The GC oven was programmed as follow: 55°C initial hold for 3 min, increase at a rate of 15°C min^{-1} to 180°C, then increase with a step of 6.5°C min^{-1} to 280°C and hold for 5 min followed by an increase of 10°C min^{-1} to 300°C and hold for 5.28 min. the temperature of splitless injector port, GC-MS transfer line and MS source was maintained at 280, 290 and 250°C, respectively.

The QTOF mass spectrometer was calibrated with Perfluorotributylamine (FC43) prior to each injection.

The operating parameters of APCI interface were: capillary voltage, 5000 V; corona voltage, 2000 V; end plate offset, 500 V; nebulizer, 3.5 bar; drying gas, 1.5 L min−1. The QToF MS system operated in two different acquisition modes. In broadband collision-induced dissociation (bbCID) a Data Independent Acquisition (DIA) mode, in which two sequential full scan events are triggered. The first scan at low collision energy (4eV) results in a MS full scan over the range of m/z 50–1000. The second scan at high collision energy (25eV) results in a MS/MS all ion fragment mode also in the range of m/z 50–1000. In Data Dependent Acquisition (DDA) mode, the first scan at low collision energy (4eV) results in a MS full scan over the range of m/z 50–1000, whereas the second scan results in a full scan MS spectra and in MS/MS spectra of the 5 most abundant ions per MS scan in a single run in the range of m/z 50–1000. The scan rate was 8 Hz per cycle.

Bruker’s software that was used for raw data analysis was DataAnalysis 5.1 and TASQ Client 2.1.

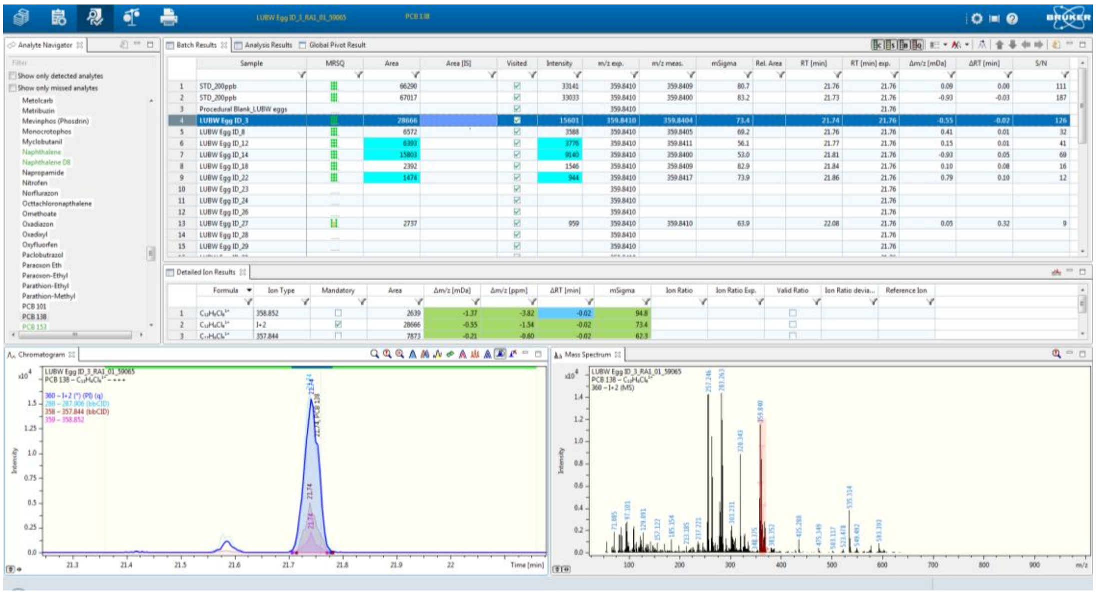
Figure 20: GC-QTOF-MS, Maxis Impact, Bruker Daltonics.

4.6 Target screening for the determination of environmental contaminants

Two in-house databases developed in NKUA of more than 2,400 ECs and PPs and their TPs were used for the target screening of the biota (raptor eggs) samples. The databases contained compounds from different classes; personal care products, steroids & hormones, pharmaceuticals (>450), antibiotics (>50), illicit drugs and new psychoactive substances (>500), industrial chemicals (>100), pesticides (>900), sweeteners, surfactants, biocides, PCBs, PAHs, OCs as well as their (bio) TPs. The databases contained information for the precursor ions, retention time, adducts (adduct ions), in-source fragments and bbCID MS/MS fragments, as well as identifiers for the compounds (CAS number, InChi). This information was acquired from the analysis of the standard solutions of these compounds, which were available in the laboratory, with the bbCID method, or was part of the manufacturer's database, Bruker's ToxScreener 2.1, which was built with the same bbCID method.

The raw data were processed with Bruker's TASQ Client 2.1 and Data Analysis 5.1 (Bruker Daltonics, Bremen, Germany). The TASQ method in TASQ Client 2.1 (**Figure 21**) created in all samples the Extracted Ion Chromatogram (EIC) of the precursor ion of the compounds included in the database with a mass error window of ±0.005Da.

Every peak, which was detected for a target compound was evaluated according to strict identification thresholds that were set to the method and after manual inspection. The screening parameters that were set to method were an area threshold of 1000 counts and an intensity threshold of 500 counts. The first identification criterion was the mass accuracy, which refers to the difference between the accurate mass (measured) and the exact mass (theoretical) and is expressed in mDa or ppm. The threshold for mass accuracy was 2mDa (or 5pppm). The second one was the retention time shift (threshold; 0.2 min), which refers to the difference between the measured retention time and the one that is recorded to the database. Moreover, the isotopic fitting, which refers to the correlation between the theoretical and the experimental isotopic pattern, was evaluated. Its calculation is based on the standard deviation of the masses and the intensities for all isotopic peaks and is expressed by the mSigma value. Lower mSigma value indicates better isotopic fitting. The mSigma threshold was set to 200. However, this value was only considered as a positive confirmation and not for rejecting peaks, because strong matrix effects combined with low concentration levels of analytes may affect the isotopic pattern results and give a bad mSigma value, although the compound may be present. Apart from the precursor ion, the presence of additional characteristic ions for each compound (qualifier ions) was considered as an additional identification criterion. In order to confirm the screening results, bbCID MS/MS fragments were examined, as well as adducts and in-source fragments in full scan MS.



	Sample	MRSQ	Area	Area [IS]	Visited	Intensity	m/z exp.	m/z meas.	mSigma	Rel. Area	RT [min]	RT [min] exp.	Δm/z [mDa]	ΔRT [min]	S/N
1	STD_200ppb	▦	66290		☑	33141	359.8410	359.8409	80.7		21.76	21.76	0.09	0.00	111
2	STD_200ppb	▦	67017		☑	33033	359.8410	359.8400	83.2		21.73	21.76	-0.93	-0.03	187
3	Procedural Blank_LUBW eggs				☑		359.8410					21.76			
4	LUBW Egg ID_3	▦	28666		☑	15601	359.8410	359.8404	73.4		21.74	21.76	-0.55	-0.02	126
5	LUBW Egg ID_8	▦	6572		☑	3588	359.8410	359.8405	69.2		21.76	21.76	0.41	0.01	32
6	LUBW Egg ID_12	▦	6393		☑	3776	359.8410	359.8411	56.1		21.77	21.76	0.15	0.01	41
7	LUBW Egg ID_14	▦	15803		☑	9140	359.8410	359.8400	53.0		21.81	21.76	-0.93	0.05	69
8	LUBW Egg ID_18	▦	2392		☑	1546	359.8410	359.8409	82.9		21.84	21.76	0.10	0.08	16
9	LUBW Egg ID_22	▦	1474		☑	944	359.8410	359.8417	73.9		21.86	21.76	0.79	0.10	12
10	LUBW Egg ID_23				☑		359.8410					21.76			
11	LUBW Egg ID_24				☑		359.8410					21.76			
12	LUBW Egg ID_26				☑		359.8410					21.76			
13	LUBW Egg ID_27	▦	2737		☑	959	359.8410	359.8410	63.9		22.08	21.76	0.05	0.32	9
14	LUBW Egg ID_28				☑		359.8410					21.76			
15	LUBW Egg ID_29				☑		359.8410					21.76			

	Formula	Ion Type	Mandatory	Area	Δm/z [mDa]	Δm/z [ppm]	ΔRT [min]	mSigma	Ion Ratio	Ion Ratio Exp.	Valid Ratio	Ion Ratio devia...	Reference Ion
1	$C_{12}H_4Cl_6^{1+}$	358.852	☐	2639	-1.37	-3.82	-0.02	94.8			☐		
2	$C_{12}H_4Cl_6^{1+}$	I+2	☑	28666	-0.55	-1.54	-0.02	73.4			☐		
3	$C_{12}H_4Cl_6^{1+}$	357.844	☐	7873	-0.21	-0.60	-0.02	62.3			☐		

Figure 21: Data Treatment Interface- TASQ Client 2.1 (Bruker Daltonics)

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Method validation results

As mentioned in chapter 4.5, for the evaluation of linearity, recovery, repeatability, matrix effects and detectability of the generic developed protocol, a representative validation dataset of 80 compounds with different physicochemical properties from different classes of ECs and PPs was used.

5.2 Internal Standard

The Internal Standard (IS) method was used to improve the accuracy of quantification. Different IS were used for each analyte. As internal standards, deuterium labeled standards were used such as Flunixin –d3, Diurion-d6, Diazepam-d5, Cetirizine-d8, Sulfadimidine-d4, Amilsupride-d5, Amphetamine-d6, Sulfadiazine-d4 and Atrazine-d5. Concerning the linearity evaluation, the correlation coefficient of each analyte’s spiked curve (0.610, 1.53, 3.05, 4.58, 9.15, 15.3, 24.4 μg/kg w.w.) was compared. In first occasion for not using internal standard, the absolute areas was used and in the occasion of using an IS, the relative areas of analytes was used. The relative area was calculated by the below equation.

$$\boldsymbol{Realative\ Area = \frac{Absolute\ Area\ of\ Analyte}{Area\ of\ IS}}$$

It was observed that for all the analytes the correlation coefficient was improved by the use of IS. **Figures 22** and **23** illustrate the regression results for Myclobutanil, with and without the use of IS.

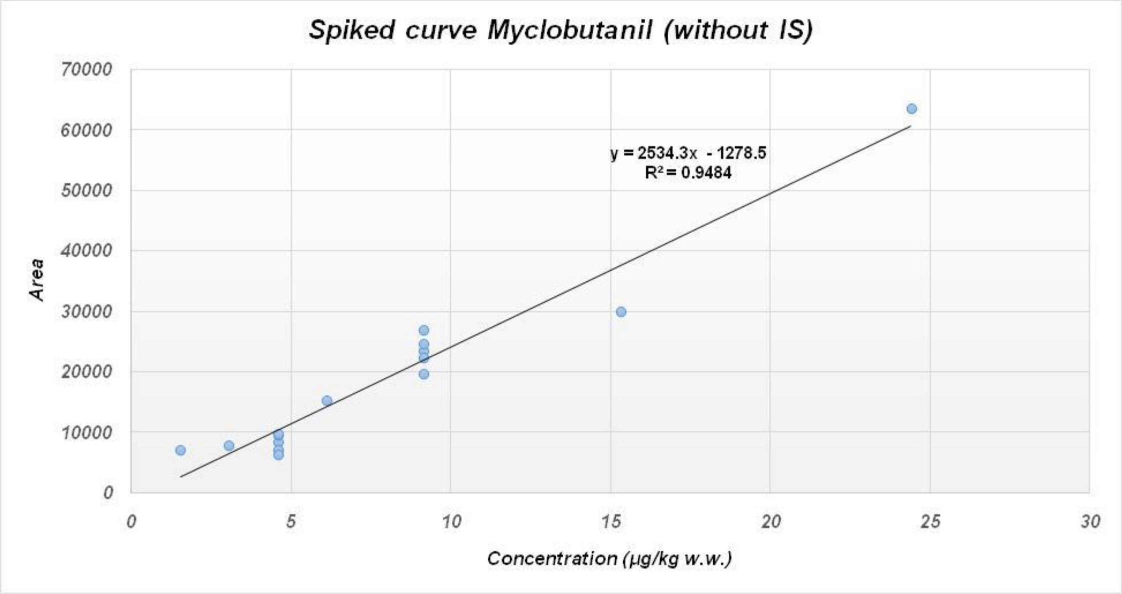


Figure 22: Spiked curve of Myclobutanil without IS

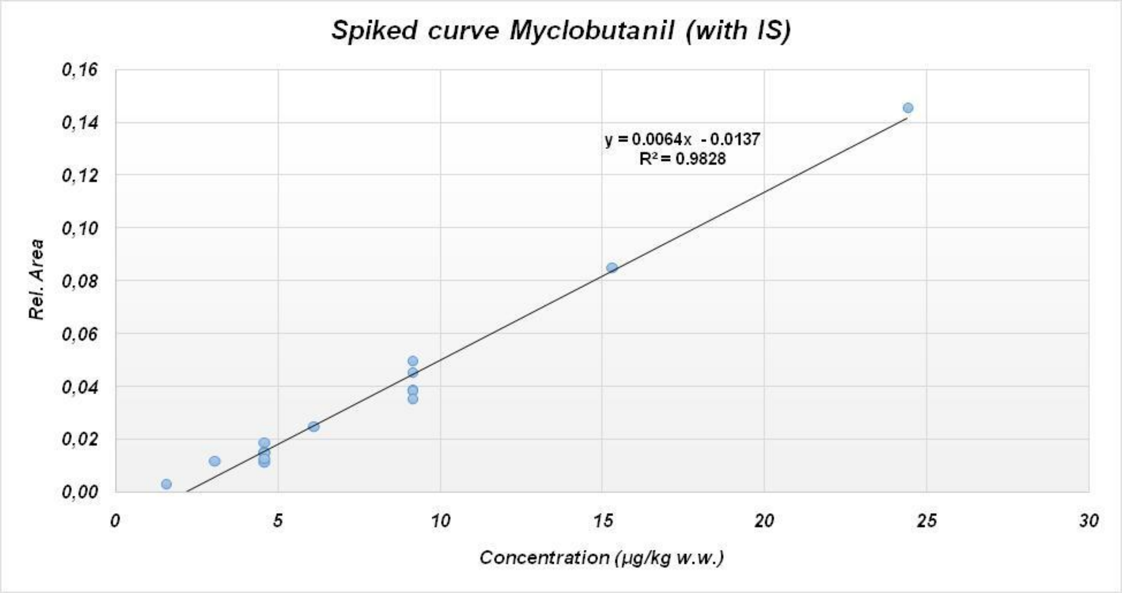


Figure 23: Spiked curve of Myclobutanil with Diuron-d6 (IS)

5.3 Linearity

Linearity of the method was studied for each compound by analyzing spiked samples in seven different concentration levels (0.61, 1.53, 3.05, 4.58, 9.15, 15.3, 24.4 µg/kg w.w.). The regression lines were determined by the least squares method, and were of the form:

$$\boldsymbol{y} = (\boldsymbol{a} \pm \boldsymbol{Sa}) * \boldsymbol{C} + (\boldsymbol{b} \pm \boldsymbol{Sb})$$

in which:

y= Relative peak area of each analyte

a: slope

b: intercept

C: concentration of analyte

Sa: Standard deviation of intercept

Sb: Standard deviation of slope

The correlation coefficients (R^2) for all spiked curves were also calculated. The values of all the above parameters of each compound are presented in **Table 8** for eggs.

5.4 Limits of detection (LODs) and Limits of Quantification (LOQs)

The method Limits of Detection (LODs) and Limits of Quantification (LOQs) were calculated for the method from the data of the spiked curves and are presented in **Table 8** in eggs. The highest LOD concentration was 7.67 μg/kg w.w. As it is illustrated in the Chart below in **Figure 24**, for the majority of the compounds (75%), the LOD was less than 2μg/kg w.w. and only 2.5% was higher than 6 μg/kg w.w.

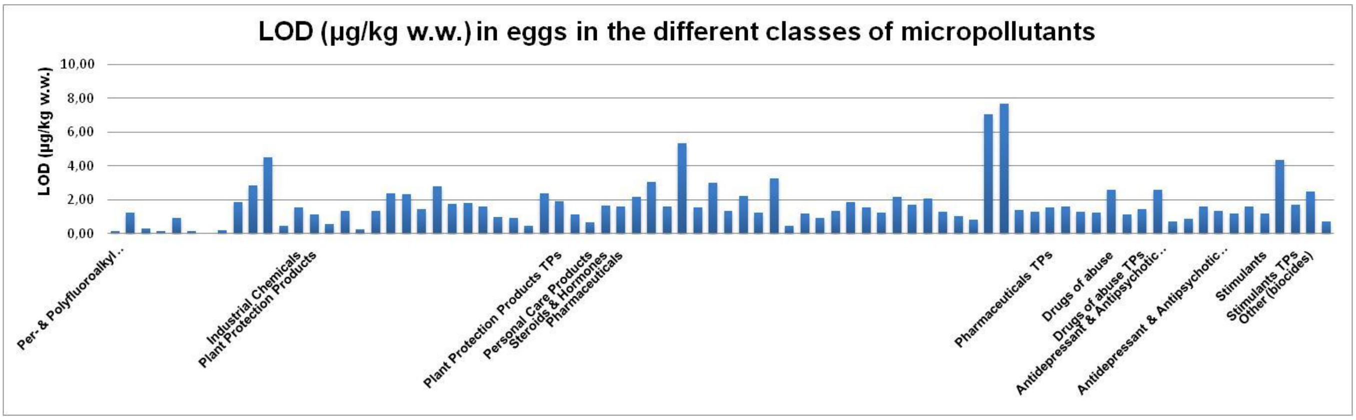


Figure 24: LOD (μg/kg w.w.) in eggs in the different classes of micropollutants

In the Box-Plot diagram below (**Figure 25**), a comparison of the LODs in eggs in the different classes of micropollutants is presented. The median value of LOD was aproximately 1.3μg/kg w.w. in the different classes. The LODs of PFAS and Stimulants & TPs are more variable than the other classes.

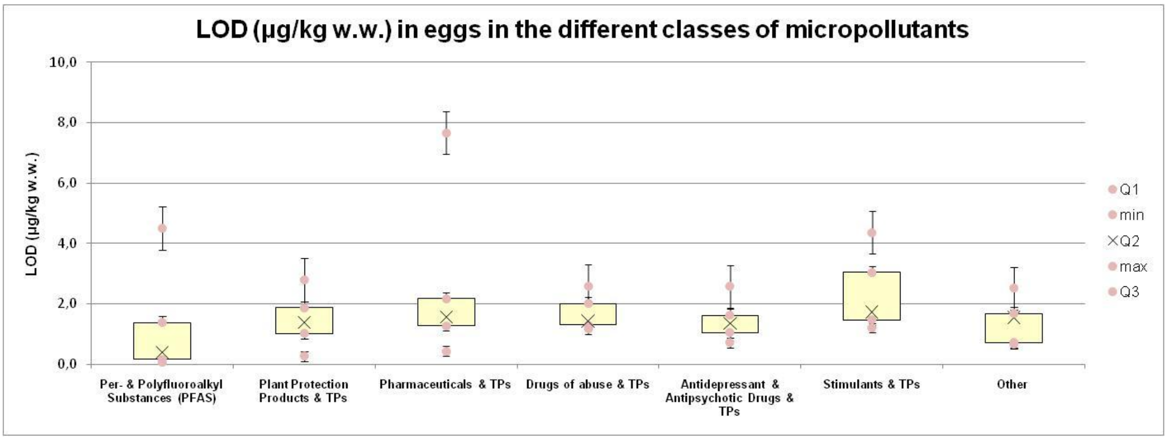


Figure 25: Box-Plot of the LODs (μg/kg w.w.) in eggs in the different classes of micropollutants

Table 8: Validation results in eggs – Linearity: Slope, intercept and correlation coefficient, Detectability: LODs

Class	*Compound*	*Internal Standard*	*a*	*Sa*	*b*	*Sb*	*R^2*	*Method LOD μg/kg w.w.*	*Method LOQ μg/kg w.w.*
Per- & Polyfluoroalkyl Substances (PFAS)	***Perfluorooctanoic acid (PFOA)***	Flunixin-d3	0.00342	0.00014	-0.103	0.022	0.98	0.156	0.468
	Perfluorobutanesulfonate (PFBuS)	Flunixin-d3	0.00820	0.00033	-0.179	0.050	0.97	1.22	3.68
	Perfluorodecanoic acid (PFDeA)	Flunixin-d3	0.00327	0.00018	-0.146	0.027	0.96	0.287	0.860
	Perfluorooctanesulfonic acid (PFOS)	Flunixin-d3	0.00338	0.00017	-0.168	0.027	0.97	0.156	0.468
	Perfluorododecanoic acid (PFDoA)	Flunixin-d3	0.00158	0.00011	-0.091	0.017	0.94	0.906	2.72
	Perfluoroheptanoic acid (PFHpA)	Flunixin-d3	0.00469	0.00025	-0.077	0.038	0.96	0.120	0.360
	Perfluorohexanoic acid (PFHxA)	Flunixin-d3	0.00313	0.00018	-0.109	0.028	0.96	0.0563	0.169
	Perfluorononanoic acid (PFNA)	Flunixin-d3	0.002291	0.000095	-0.084	0.014	0.97	0.198	0.595
	Perfluorooctanesulfonamide (PFOSA)	Flunixin-d3	0.00573	0.00034	-0.245	0.053	0.96	1.88	5.68
	Perfluoropentanoic acid (PFPeA)	Flunixin-d3	0.00224	0.00020	-0.074	0.032	0.89	2.84	8.60
	Perfluorotridecanoic acid (PFTrDA)	Flunixin-d3	0.000791	0.000025	-0.0516	0.0046	0.99	4.50	13.5
	Perfluoroundecanoic acid (PFUnA)	Flunixin-d3	0.00215	0.00014	-0.104	0.021	0.93	0.483	1.45

Class	Compound	Internal Standard	a	Sa	b	Sb	R^2	Method LOD µg/kg w.w.	Method LOQ µg/kg w.w.
Industrial Chemicals	***Benzotriazole- 5-Me***	Diuron-d6	0.001192	0.000055	-0.0274	0.0085	0.97	1.55	4.64
Plant Protection Products	***Azoxystrobin***	Diazepam-d5	0.00515	0.00019	-0.131	0.028	0.98	1.11	3.36
	DEET (Diethyltoluamide)	Cetirizine-d8	0.00909	0.00035	-0.238	0.053	0.98	0.567	1.70
	Dimethirimol	Diuron-d6	0.00297	0.00013	-0.089	0.020	0.97	1.33	4.04
	Dinitrophenol-2-4- (DNP)	Flunixin-d3	0.00353	0.00026	-0.200	0.042	0.93	0.261	0.783
	Diuron	Diuron-d6	0.000748	0.000032	-0.0192	0.0050	0.97	1.33	4.04
	Imazamox	Sulfadimidine-d4	0.00649	0.00051	0.053	0.077	0.91	2.39	7.23
	Imidacloprid	Sulfadimidine-d4	0.00249	0.00019	0.017	0.029	0.91	2.31	7.00
	Metolachlor	Diuron-d6	0.001219	0.000057	-0.0075	0.0087	0.99_8	1.44	4.35
	Myclobutanil	Diuron-d6	0.000389	0.000014	-0.0137	0.0023	0.98	2.80	8.39
	Omethoate	Amisulpride-d5	0.000788	0.000045	-0.0204	0.0068	0.95	1.73	5.25
	Propazine	Atrazine-d5	0.001498	0.000065	-0.0751	0.0098	0.97	1.79	5.36
	Propoxur	Diuron-d6	0.000558	0.000028	-0.0182	0.0045	0.97	1.61	4.88

Class	***Compound***	***Internal Standard***	***a***	***Sa***	***b***	***Sb***	***R^2***	***Method LOD µg/kg w.w.***	***Method LOQ µg/kg w.w.***
	Siduron	Diazepam-d5	0.00939	0.00031	-0.187	0.046	0.98	0.982	2.98
	Terbuthylazine	Atrazine-d5	0.00142	0.000044	-0.0130	0.0066	0.98	0.941	2.85
	Thiabendazole	Sulfadimidine-d4	0.0165	0.0011	-0.11	0.17	0.94	0.469	1.14
	Thiamethoxam	Sulfadimidine-d4	0.00340	0.00027	-0.038	0.040	0.91	2.37	7.20
Plant Protection Products TPs	***Atrazine-desisopropyl***	Atrazine-d5	0.000906	0.000055	0.0114	0.0085	0.95	1.90	5.75
	(3,4-dichlorophenyl)-3-methyl urea	Diuron-d6	0.000343	0.000013	-0.0053	0.0019	0.98	1.13	3.41
Personal Care Products	***Methylparaben***	Flunixin-d3	0.00208	0.00012	-0.196	0.019	0.96	0.671	2.01
Steroids & Hormones	***Melatonin***	Sulfadimidine-d4	0.00707	0.00039	-0.095	0.059	0.95	1.68	5.08
Pharmaceuticals	***(±)-Flecainide***	Diuron-d6	0.00248	0.00013	-0.092	0.020	0.96	1.62	4.92
	Acebutolol	Sulfadimidine-d4	0.02320	0.0017	-0.33	0.25	0.92	2.20	6.65
	Atenolol	Sulfadiazine-d4	0.0241	0.0025	-0.41	0.39	0.87	3.07	9.20
	Dibucaine	Diuron-d6	0.000950	0.000050	-0.0274	0.0076	0.96	1.62	4.91
	Dorzolamide	Sulfadiazine-d4	0.00114	0.00015	-0.028	0.030	0.89	5.34	16.2

Class	*Compound*	*Internal Standard*	*a*	*Sa*	*b*	*Sb*	*R^2*	*Method LOD µg/kg w.w.*	*Method LOQ µg/kg w.w.*
	Esmolol	Amphetamine-d6	0.00965	0.00050	-0.127	0.075	0.96	1.56	4.74
	Flufenamic acid	Flunixin-d3	0.00255	0.00025	-0.002	0.038	0.88	3.01	9.13
	Hordenine	Amisulpride-d5	0.00365	0.00016	-0.061	0.024	0.97	1.34	4.05
	Ipratropium	Sulfadimidine-d4	0.00542	0.00039	-0.063	0.060	0.93	2.22	6.73
	Ketoprofen	Diuron-d6	0.000568	0.000023	-0.0128	0.0035	0.98	1.23	3.73
	Lamotrigine	Diuron-d6	0.000753	0.000080	-0.025	0.012	0.86	3.29	9.96
	Lopinavir	Cetirizine-d8	0.00730	0.00031	-0.080	0.046	0.97	0.433	1.33
	Loratadine	Diuron-d6	0.000443	0.000017	-0.0092	0.0026	0.98	1.19	3.61
	Metoclopramide	Amisulpride-d5	0.001794	0.000056	-0.0312	0.0084	0.98	0.945	2.86
	Mexiletine	Amphetamine-d6	0.00433	0.00019	-0.029	0.029	0.97	1.35	4.10
	Minoxidil	Sulfadimidine-d4	0.01120	0.00070	0.02	0.10	0.94	1.87	5.68
	Propafenone	Diuron-d6	0.001043	0.000054	-0.0338	0.0081	0.96	1.57	4.77
	Rivastigmine	Amphetamine-d6	0.02289	0.00094	-0.59	0.14	0.97	1.24	3.77

Class	***Compound***	***Internal Standard***	***a***	***Sa***	***b***	***Sb***	***R^2***	***Method LOD µg/kg w.w.***	***Method LOQ µg/kg w.w.***
	Salicylic acid	Flunixin-d3	0.00200	0.00014	-0.185	0.021	0.94	2.15	6.52
	Sulfadiazine	Sulfadiazine-d4	0.00388	0.00020	-0.100	0.033	0.97	1.72	5.21
	Sulfadimethoxine	Sulfadimidine-d4	0.00288	0.00017	-0.045	0.029	0.96	2.06	6.23
	Sulfadimidine/ Sulfamethazine	Sulfadimidine-d4	0.00422	0.00017	-0.090	0.027	0.98	1.30	3.95
	Sulfadoxine	Sulfadimidine-d4	0.00621	0.00022	-0.046	0.032	0.98	1.05	3.19
	Sulfamethoxypyridazine	Sulfadimidine-d4	0.002973	0.000075	-0.070	0.012	0.99	0.840	2.55
	Sulfisoxazole	Sulfadimidine-d4	0.00090	0.00017	0.013	0.032	0.82	7.07	21.4
	Terbutaline	Sulfadimidine-d4	0.00167	0.00032	0.023	0.064	0.80	7.67	23.2
	Tinidazole	Amisulpride-d5	0.000428	0.000020	-0.0058	0.0030	0.97	1.39	4.22
	Triamterene	Amphetamine-d6	0.00459	0.00019	-0.123	0.029	0.97	1.29	3.90
Pharmaceuticals TPs	***Tramadol-O-Desmethylnor/ N-O-disdesmethyltramadol***	Amphetamine-d6	0.00809	0.00042	-0.274	0.063	0.96	1.56	4.71
	Desacetyldiltiazem	Cetirizine-d8	0.00432	0.00022	-0.162	0.035	0.96	1.62	4.90
	Desalkylflurazepam	Diuron-d6	0.000423	0.000018	-0.0105	0.0027	0.97	1.27	3.85

Class	*Compound*	*Internal Standard*	*a*	*Sa*	*b*	*Sb*	*R^2*	*Method LOD µg/kg w.w.*	*Method LOQ µg/kg w.w.*
	Tramadol-O-Desmethyl	Amisulpride-d5	0.00338	0.00014	-0.080	0.021	0.97	1.24	3.75
Drugs of abuse	***1-Piperonylpiperazine***	Amphetamine-d6	0.00231	0.00017	-0.054	0.030	0.94	2.59	7.85
	Amphetamine	Amphetamine-d6	0.00561	0.00021	-0.033	0.032	0.98	1.16	3.50
Drugs of abuse TPs	***Ritalinic acid***	Amphetamine-d6	0.00389	0.00018	-0.072	0.028	0.97	1.43	4.33
Antidepressant & Antipsychotic Drugs	***Moclobemide***	Sulfadimidine-d4	0.0129	0.0011	-0.12	0.16	0.90	2.57	7.80
	Sulpiride	Amisulpride-d5	0.003104	0.000073	-0.017	0.011	0.99	0.711	2.15
	Tiapride	Amisulpride-d5	0.00469	0.00014	-0.079	0.020	0.99	0.876	2.65
	Trazodone	Diuron-d6	0.000839	0.000043	-0.0339	0.0068	0.97	1.62	4.92
Antidepressant & Antipsychotic Drugs TPs	***Citalopram amide***	Amphetamine-d6	0.01826	0.00082	-0.54	0.12	0.97	1.35	4.10
	Citalopram carboxylic acid	Amphetamine-d6	0.0264	0.0011	-0.68	0.16	0.97	1.21	3.66
	Desmethylcitalopram	Amphetamine-d6	0.00811	0.00043	-0.276	0.064	0.96	1.60	4.85
Stimulants	***Anabasine***	Sulfadiazine-d4	0.0380	0.0031	-0.29	0.47	0.90	1.20	3.61
	Nicotine	Sulfadiazine-d4	0.00674	0.00098	-0.10	0.15	0.82	4.36	13.2

Class	***Compound***	***Internal Standard***	***a***	***Sa***	***b***	***Sb***	***R^2***	***Method LOD µg/kg w.w.***	***Method LOQ µg/kg w.w.***
Stimulants TPs	***Nor-Nicotine***	Cetirizine-d8	0.00380	0.00021	-0.201	0.033	0.96	1.73	5.23
Other (Biocides)	***Irgarol***	Atrazine-d5	0.001176	0.000093	0.001	0.015	0.93	2.51	7.61
	M1 (Irgarol-descyclopropyl)	Atrazine-d5	0.00669	0.00016	-0.109	0.024	0.99	0.711	2.15

5.5 Recovery

Recovery experiments were performed at 4 different levels 1.53, 4.58, 9.15, 15.3 μg/kg w.w.). The majority of the analytes had satisfactory recoveries between 60-120% as it is showed in the diagram in **Figure 26** and in the Box-Plot in **Figure 27**.

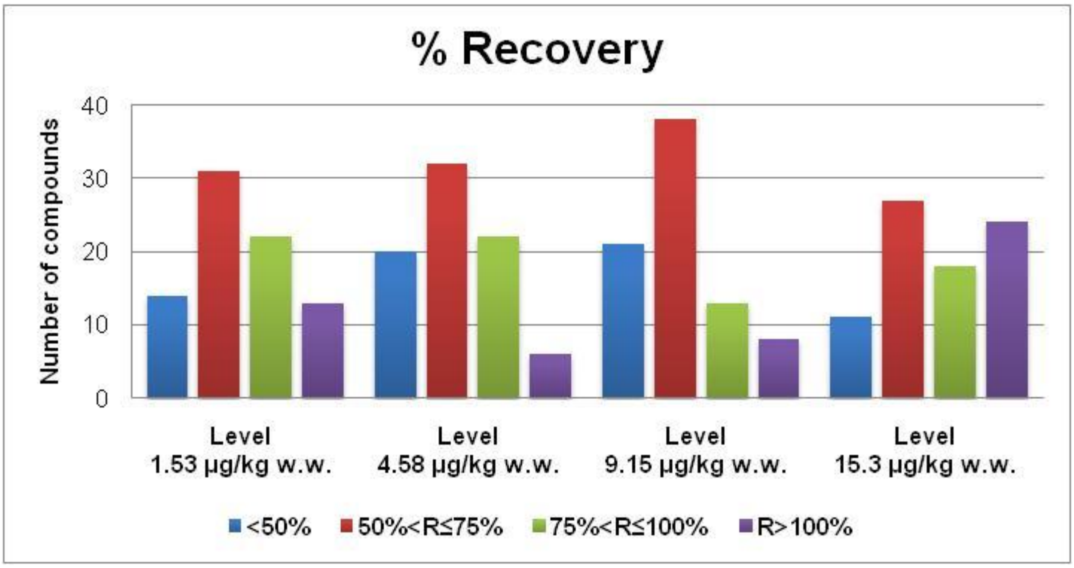


Figure 26: %Recovery in different levels

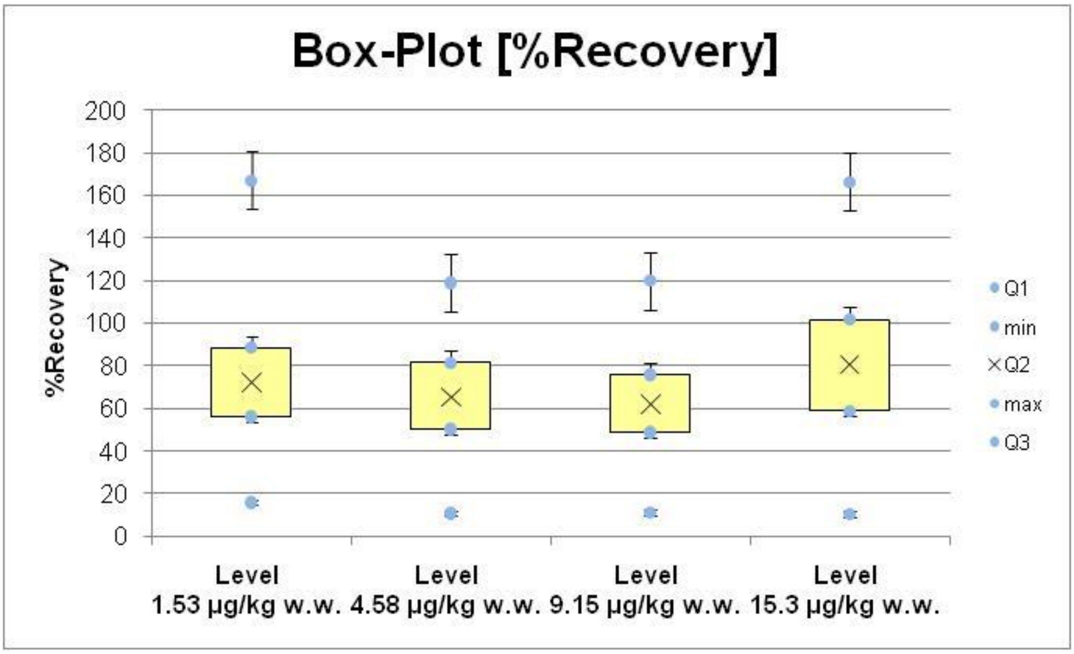


Figure 27: Box-Plot of %Recovery of the method in 4 different levels of concentration

5.6 Matrix effect

The results of %FME are summarized in **Figure 28** for concentration levels 1.53, 4.58, 9.15 and 15.3 µg/kg w.w. For the majority of the compounds the FME was less than 20% in each level.

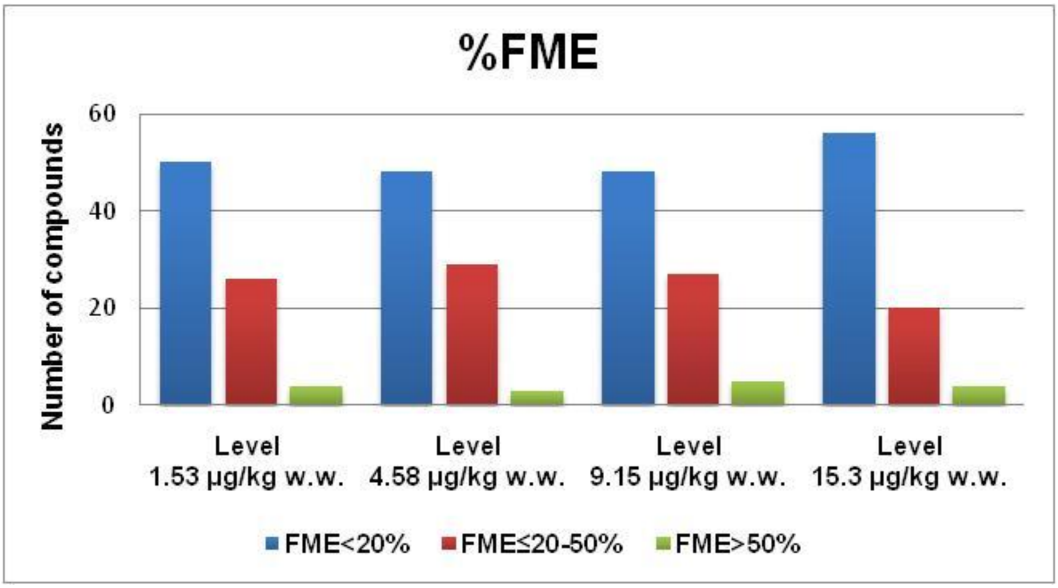


Figure 28: %Factor of Matrix Effect (%FME) in different levels

5.7 Repeatability

Method repeatability experiments were performed at 2 different levels of concentration (4.58 and 9.15 µg/kg w.w.) in 6 spiked samples in each level. The repeatability of the method was below 20% for the majority of the tested analytes, as it is illustrated in the following chart in **Figure 29** and in the Box-Plot in **Figure 30**.

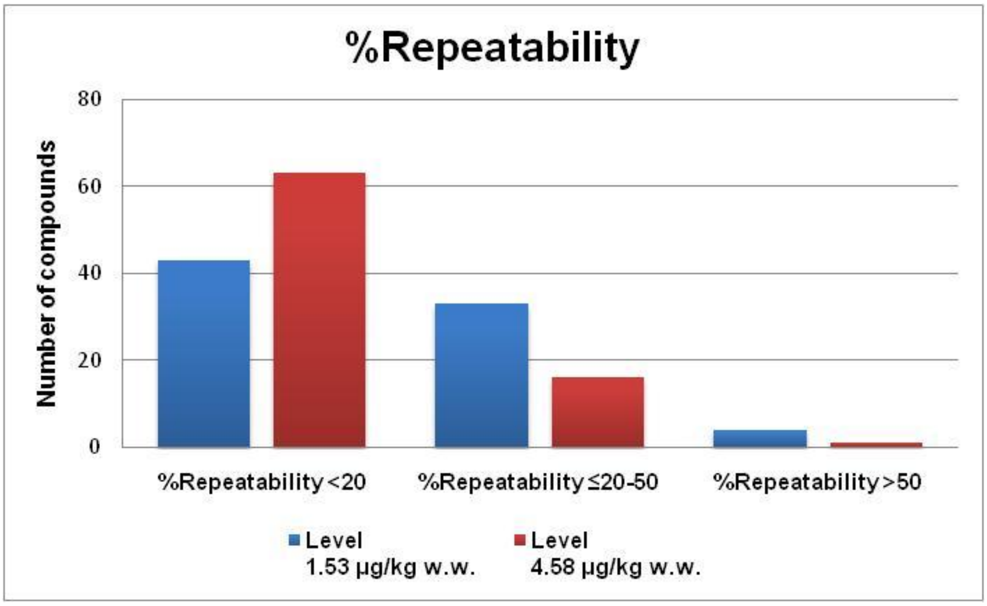


Figure 29: %Repeatability of the method in 2 different levels of concentration

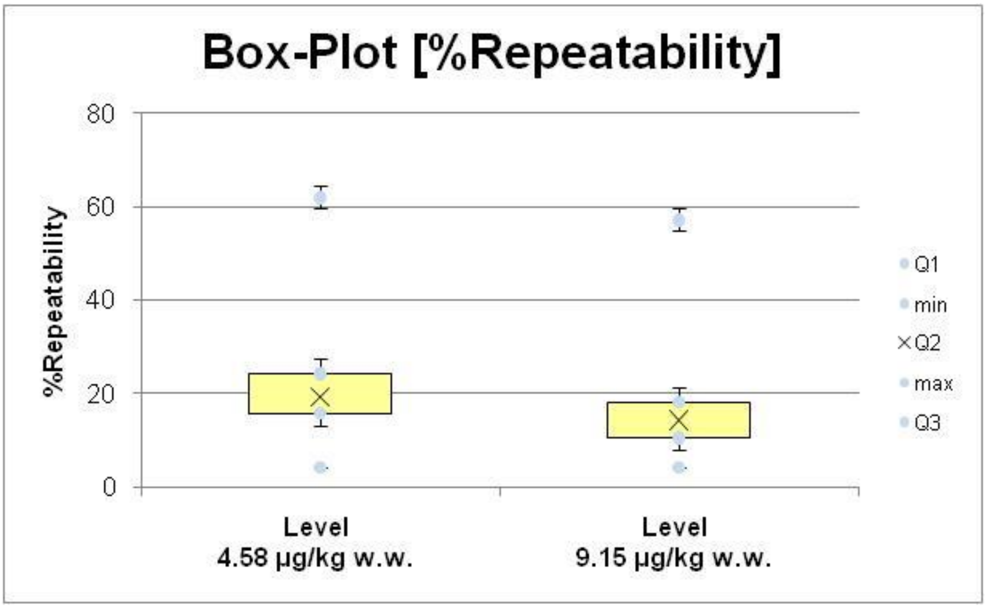


Figure 30: Box-Plot of the %Repeatability of the method in 2 different levels of concentration

The validation results for %Repeatability, %Recovery, %Factor of Matrix Effect are presented in **Table 9**.

Table 9: Validation results in eggs- Repeatability, Recovery, and Factor of Matrix Effect

Compound	***% Repeatability***		***%Recovery***				***%FME***			
	Level 4.58 µg/kg w.w.	***Level 9.15 µg/kg w.w.***	***Level 1.53 µg/kg w.w.***	***Level 4.58 µg/kg w.w.***	***Level 9.15 µg/kg w.w.***	***Level 15.3 µg/kg w.w.***	***Level 1.53 µg/kg w.w.***	***Level 4.58 µg/kg w.w.***	***Level 9.15 µg/kg w.w.***	***Level 15.3 µg/kg w.w.***
Perfluorooctanoic acid (PFOA)	16	10	89	66	88	113	18	19	20	14
Perfluorobutanesulfonate (PFBuS)	24	8	69	73	63	115	13	13	15	9
Perfluorodecanoic acid (PFDeA)	4.2	4.3	72	46	62	81	11	15	14	12
Perfluorooctanesulfonic acid (PFOS)	4	4	72	76	75	81	11	15	14	12
Perfluorododecanoic acid (PFDoA)	7.7	7.6	57	44	43	46	12	17	17	16
Perfluoroheptanoic acid (PFHpA)	23	14	75	75	74	72	22	25	24	18
Perfluorohexanoic acid (PFHxA)	16	15	77	75	76	72	16	22	27	15
Perfluorononanoic acid (PFNA)	18	6	73	42	52	88	16	22	17	13
Perfluorooctanesulfonamide (PFOSA)	8.2	11	80	80	85	89	44	87	75	77
Perfluoropentanoic acid (PFPeA)	43	31	85	96	72	166	13	13	14	9.0

Compound	**% Repeatability**		**%Recovery**				**%FME**			
	Level 4.58 µg/kg w.w.	**Level 9.15 µg/kg w.w.**	**Level 1.53 µg/kg w.w.**	**Level 4.58 µg/kg w.w.**	**Level 9.15 µg/kg w.w.**	**Level 15.3 µg/kg w.w.**	**Level 1.53 µg/kg w.w.**	**Level 4.58 µg/kg w.w.**	**Level 9.15 µg/kg w.w.**	**Level 15.3 µg/kg w.w.**
Perfluorotridecanoic acid (PFTrDA)	14	6	22	11	29	30	14	20	10	12
Perfluoroundecanoic acid (PFUnA)	22	9	69	24	34	48	12	20	14	12
5-Me-Benzotriazole	8.3	10	72	86	75	114	18	18	19	17
Azoxystrobin	19	10	56	71	69	93	10	11	14	10
DEET (Diethyltoluamide)	18	11	61	61	62	69	12	14	19	16
Dimethirimol	26	13	54	51	53	52	8.7	9.2	11	8.2
2.4-Dinitrophenol (DNP)	18	17	97	54	75	105	38	49	45	42
Diuron	21	11	92	80	91	95	49	48	46	43
Imazamox	28	14	62	64	65	76	35	37	34	37
Imidacloprid	31	17	75	60	72	81	25	26	33	32
Metolachlor	37	28	16	15	14	16	10	11	10	9

Compound	% Repeatability		%Recovery				%FME			
	Level 4.58 µg/kg w.w.	Level 9.15 µg/kg w.w.	Level 1.53 µg/kg w.w.	Level 4.58 µg/kg w.w.	Level 9.15 µg/kg w.w.	Level 15.3 µg/kg w.w.	Level 1.53 µg/kg w.w.	Level 4.58 µg/kg w.w.	Level 9.15 µg/kg w.w.	Level 15.3 µg/kg w.w.
Myclobutanil	19	14	67	67	63	70	55	49	52	48
Omethoate	12	24	56	38	30	56	56	59	58	57
Propazine	58	17	25	23	24	32	6.0	8.0	7.0	9.0
Propoxur	26	18	88	86	97	89	6.9	7.1	6.2	6.7
Siduron	15	7.9	95	94	92	101	13	12	19	14
Terbuthylazine	12	10	61	51	52	56	12	11	15	12
Thiabendazole	25	18	65	56	58	59	22	22	19	17
Thiamethoxam	31	18	43	49	48	51	13	13	12	12
Atrazine-desisopropyl	20	18	102	101	113	101	18	15	22	18
(3,4-dichlorophenyl)-3-methyl urea	16	14	103	90	96	103	8.6	9.8	10	9.5
Methylparaben	52	48	108	113	106	120	4.0	2.4	3.0	4.0

Compound	% Repeatability		%Recovery				%FME			
	Level 4.58 µg/kg w.w.	Level 9.15 µg/kg w.w.	Level 1.53 µg/kg w.w.	Level 4.58 µg/kg w.w.	Level 9.15 µg/kg w.w.	Level 15.3 µg/kg w.w.	Level 1.53 µg/kg w.w.	Level 4.58 µg/kg w.w.	Level 9.15 µg/kg w.w.	Level 15.3 µg/kg w.w.
Melatonin	14	22	83	58	50	60	8.0	7.5	7.9	5.5
(±)-Flecainide	32	18	41	57	37	87	4.8	7.6	11	7.4
Acebutolol	21	18	46	43	45	62	25	28	26	28
Atenolol	46	23	30	48	52	70	27	24	23	20
Dibucaine	21	22	39	31	29	36	9.6	11	13	9.0
Dorzolamide	18	15	82	79	75	89	35	39	41	27
Esmolol	16	16	118	115	120	119	63	49	62	38
Flufenamic acid	18	14	104	62	54	89	26	23	21	13
Hordenine	22	18	83	88	59	114	18	18	15	14
Ipratropium	26	18	52	50	55	64	31	38	42	25
Ketoprofen	18	12	69	77	76	103	15	16	12	12

Compound	**% Repeatability**		**%Recovery**				**%FME**			
	Level 4.58 µg/kg w.w.	**Level 9.15 µg/kg w.w.**	**Level 1.53 µg/kg w.w.**	**Level 4.58 µg/kg w.w.**	**Level 9.15 µg/kg w.w.**	**Level 15.3 µg/kg w.w.**	**Level 1.53 µg/kg w.w.**	**Level 4.58 µg/kg w.w.**	**Level 9.15 µg/kg w.w.**	**Level 15.3 µg/kg w.w.**
Lamotrigine	5.3	14	82	99	86	81	21	25	22	23
Lopinavir	13	13	100	107	102	110	43	45	48	46
Loratadine	7.1	15	30	22	17	23	9.9	11	12	9.2
Metoclopramide	19	6	70	83	69	111	13	10	13	8.0
Mexiletine	17	12	69	66	66	67	20	18	16	17
Minoxidil	16	22	84	58	47	44	19	12	14	12
Propafenone	21	19	55	53	56	63	28	21	15	24
Rivastigmine	20	13	89	66	50	95	26	32	23	24
Salicylic acid	62	57	106	95	102	111	5.0	7.0	6.0	4.0
Sulfadiazine	17	13	59	53	55	59	11	12	14	9
Sulfadimethoxine	14	17	66	68	62	57	14	11	12	13

Compound	**% Repeatability**		**%Recovery**				**%FME**			
	Level 4.58 µg/kg w.w.	***Level 9.15 µg/kg w.w.***	***Level 1.53 µg/kg w.w.***	***Level 4.58 µg/kg w.w.***	***Level 9.15 µg/kg w.w.***	***Level 15.3 µg/kg w.w.***	***Level 1.53 µg/kg w.w.***	***Level 4.58 µg/kg w.w.***	***Level 9.15 µg/kg w.w.***	***Level 15.3 µg/kg w.w.***
Sulfadimidine/ Sulfamethazine	19	10	60	59	58	61	12	13	10	11
Sulfadoxine	11	12	90	59	68	67	12	14	13	12
Sulfamethoxypyridazine	8.9	7.8	72	66	62	65	10	10	11	8.0
Sulfisoxazole	7.1	25	58	56	57	53	14	12	13	8.0
Terbutaline	32	24	35	27	31	37	12	11	12	10
Tinidazole	21	11	100	81	82	92	49	50	48	50
Triamterene	22	12	167	97	95	114	17	22	25	20
Tramadol-O-Desmethylnor /N-O-disdesmethyltramadol	23	15	122	82	102	115	36	39	37	34
Desacetyldiltiazem	33	16	57	42	49	55	6.0	7.0	12	7.0
Desalkylflurazepam	16	15	84	84	85	109	14	13	16	9.0
O-Desmethyl-Tramadol	22	10	90	75	81	100	6.0	7.0	13	7.0

Compound	**% Repeatability**		**%Recovery**				**%FME**			
	Level 4.58 µg/kg w.w.	***Level 9.15 µg/kg w.w.***	***Level 1.53 µg/kg w.w.***	***Level 4.58 µg/kg w.w.***	***Level 9.15 µg/kg w.w.***	***Level 15.3 µg/kg w.w.***	***Level 1.53 µg/kg w.w.***	***Level 4.58 µg/kg w.w.***	***Level 9.15 µg/kg w.w.***	***Level 15.3 µg/kg w.w.***
1-Piperonylpiperazine	19	25	64	65	43	70	38	27	37	23
Amphetamine	17	12	75	74	52	86	18	15	20	10
Ritalinic acid	22	16	107	114	103	136	12	10	19	10
Moclobemide	32	28	53	50	55	63	21	19	33	16
Sulpiride	16	6	85	86	53	98	9.0	10	15	8.0
Tiapride	16	7	85	81	75	111	12	12	17	9.0
Trazodone	26	14	55	56	51	56	50	64	57	62
Citalopram amide	23	10	142	92	67	116	41	45	45	35
Citalopram carboxylic acid	23	13	102	78	45	102	24	29	32	25
Desmethylcitalopram	16	22	81	58	37	83	29	29	32	27
Anabasine	24	16	35	50	29	59	41	33	37	29

Compound	***% Repeatability***		***%Recovery***				***%FME***			
	Level 4.58 µg/kg w.w.	***Level 9.15 µg/kg w.w.***	***Level 1.53 µg/kg w.w.***	***Level 4.58 µg/kg w.w.***	***Level 9.15 µg/kg w.w.***	***Level 15.3 µg/kg w.w.***	***Level 1.53 µg/kg w.w.***	***Level 4.58 µg/kg w.w.***	***Level 9.15 µg/kg w.w.***	***Level 15.3 µg/kg w.w.***
Nicotine	37	49	18	23	11	10	20	15	29	16
Nor-Nicotine	55	25	45	22	32	73	6.0	6.0	12	6.0
Irgarol	20	30	38	29	35	22	11	14	15	12
M1 (Irgarol-descyclopropyl)	13	4.3	112	119	111	119	13	14	18	13

5.8 Target screening results

As mentioned in the chapter 3, 26 samples of raptor eggs were gathered from different locations across Baden-Württemberg, Germany. In total, 58 compounds were detected in the samples from 8 different classes. The highest percentage of the detected compounds were Plant protection products and their TPs (28%), followed by Per- and Poly- fluoroalkyl substances (PFAS) (22%) and Pharmaceuticals and their TPs (19%). The classes of the detected compounds are presented in the **Figure 31**, as well as in the **Table 10**. Selected classes of the detected contaminants will be referred and analyzed in the following paragraphs. All the analytes that were detected are presented in **Table 11**.

Table 10: Classes of detected compounds

Classes of compounds	# of detected compounds	% detected compounds
Plant Protection Products and TPs	16	28
Personal Care Products	4	7
Industrial chemicals	10	17
Per- and Poly- Fluoroalkyl Substances (PFAS)	13	22
Antidepressant & Antipsychotic drug TPs	1	2
Stimulants & TPs	2	3
Pharmaceuticals & TPs	11	19
Preservatives	1	2
Total	*58*	*100*

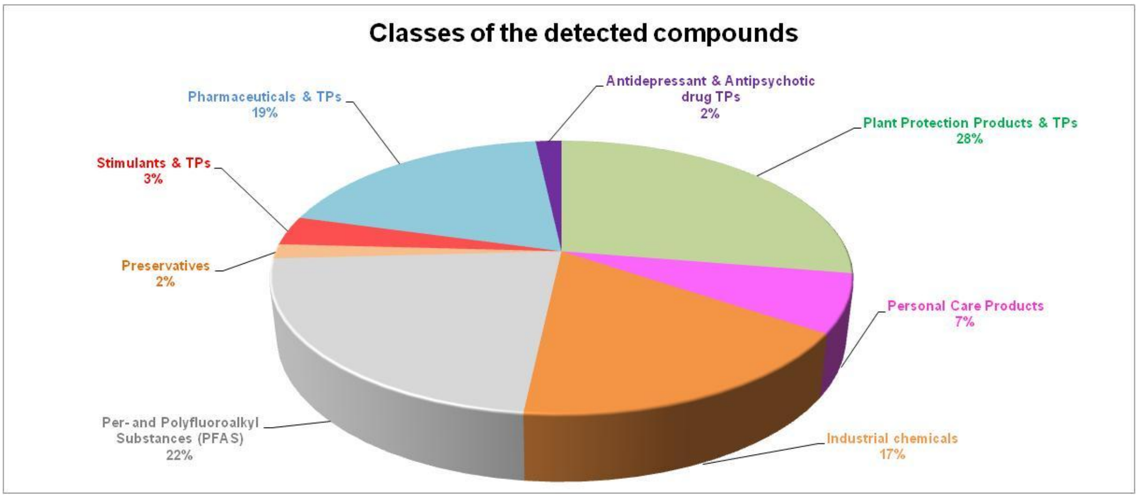


Figure 31: % detected compounds of different classes

Table 11: Results of wide-scope target screening

Analyte	Species																										LOD (μg/kg w.w.)	LOQ (μg/kg w.w.)
	Little owl						Great curlew									Peregrine falcon									Eagle owl			
	Sampling Years																											
	‘20	‘20	‘20	‘20	‘20	‘20	‘11	‘07	‘05	‘06	‘05	‘19	‘20	‘05	‘16	‘20	‘20	‘20	‘20	‘20	‘20	‘20	‘20	‘19	‘19	‘20		
	Sample ID																											
	3	**8**	**12**	**14**	**18**	**22**	**24**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**01**	**04**	**07**	**012**	**013**	**015**	**017**	**019**	**002**	**101**	**102**		
Benzododecinium (Benzyl-dimethyl-dodecylammonium)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2.86	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.832	2.50
Didecyldimethylammonium (DADMAC (C10:C10))	<LOD	<LOD	<LOD	BQL	BQL	BQL	<LOD	BQL	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.33	4.00
N,N-Dimethyldodecylamine	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	1.37	<LOD	1.22	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.270	0.810
N,N-Dimethyltetradecylamine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3.83	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	6.64	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.667	2.00
N-Methyldodecylamine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.97	5.90
PCB 138	49.4	13.9	14.6	29.2	5.75	5.57	<LOD	<LOD	14.6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	BQL	5.06	BQL	BQL	10.1	14.5	8.65	BQL	10.0	16.9	1.55	4.64
PCB 153	32.5	11.3	7.25	22.0	3.70	3.96	<LOD	<LOD	4.21	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	BQL	<LOD	5.5	5.8	BQL	<LOD	5.39	7.19	0.856	2.57
PCB 180	8.27	<LOD	<LOD	9.60	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	7.64	BQL	<LOD	BQL	BQL	1.30	3.90
PCB 28	<LOD	<LOD	<LOD	11.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.37	4.11
Tolytriazole (4-Me & 5-Me Benzotriazole)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.55	4.64
Atenolol	<LOD	<LOD	52.7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	48.8	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	3.07	9.20
Betaxolol	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	24.3	3.12	9.35
Carteolol	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	25.9	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	2.92	8.75

Analyte	**Species**																										***LOD*** ***(µg/kg w.w.)***	***LOQ*** ***(µg/kg w.w.)***
	Little owl						**Great curlew**									**Peregrine falcon**									**Eagle owl**			
	Sampling Years																											
	‘20	‘20	‘20	‘20	‘20	‘20	‘11	‘07	‘05	‘06	‘05	‘19	‘20	‘05	‘16	‘20	‘20	‘20	‘20	‘20	‘20	‘20	‘20	‘19	‘19	‘20		
	Sample ID																											
	3	***8***	***12***	***14***	***18***	***22***	***24***	***26***	***27***	***28***	***29***	***30***	***31***	***32***	***33***	***01***	***04***	***07***	***012***	***013***	***015***	***017***	***019***	***002***	***101***	***102***		
Darunavir	BQL	<LOD	BQL	BQL	17.8	11.4	28.6	26.9	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3.47	10.4
Dioxethedrin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	10.4	<LOD	<LOD	<LOD	2.67	<LOD	<LOD	<LOD	<LOD	0.685	2.05
Ethylparaben	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.43	4.30
Lopinavir	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.443	1.33
Methylparaben	147	101	1005	36.3	156	304	172	51.8	5.30	165	32.8	17.9	127	18.2	18.8	3.22	17.4	893	1760	62.4	28.5	<LOD	<LOD	23.7	48.5	48.8	0.671	2.01
Nalbuphine	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	3.72	BQL	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.703	2.11
N-Acetyl mesalazine	<LOD	<LOD	BQL	<LOD	BQL	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	9.38	28.1
Octocrylene	<LOD	<LOD	17.9	16.3	28.4	24.8	19.0	37.3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	18.9	BQL	45.6	<LOD	<LOD	<LOD	4.53	13.6
Pregabalin	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	25.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	7.64	22.9
Propylparaben	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2.36	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.192	0.576
Sotalol	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	22.1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	4.80	14.4
Nor-Tramadol (Tramadol-N-desmethyl)	<LOD	<LOD	3.21	BQL	2.08	4.62	<LOD	1.73	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	8.32	<LOD	<LOD	6.83	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	18.4	0.474	1.42
D L-N O-Didesmethyl-Venlafaxine	BQL	10.6	13.0	BQL	16.8	8.16	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	8.38	BQL	BQL	<LOD	BQL	BQL	<LOD	<LOD	1.34	4.03
PFDeA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2.21	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	3.32	0.287	0.860
PFDoA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3.26	<LOD	5.14	0.906	2.72

Analyte	**Species**																										LOD (µg/kg w.w.)	LOQ (µg/kg w.w.)
	Little owl						**Great curlew**									**Peregrine falcon**									**Eagle owl**			
	Sampling Years																											
	‘20	‘20	‘20	‘20	‘20	‘20	‘11	‘07	‘05	‘06	‘05	‘19	‘20	‘05	‘16	‘20	‘20	‘20	‘20	‘20	‘20	‘20	‘20	‘19	‘19	‘20		
	Sample ID																											
	3	***8***	***12***	***14***	***18***	***22***	***24***	***26***	***27***	***28***	***29***	***30***	***31***	***32***	***33***	***01***	***04***	***07***	***012***	***013***	***015***	***017***	***019***	***002***	***101***	***102***		
PFHpA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.611	<LOD	<LOD	<LOD	<LOD	<LOD	1.44	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.120	0.360
PFHps	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.118	0.355
PFHxA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.507	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0563	0.169
PFHxS	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	BQL	3.03	BQL	2.20	BQL	BQL	1.21	1.45	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.293	0.878
PFNA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2.44	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.88	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.198	0.595
PFOA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	5.69	<LOD	5.14	2.29	2.29	5.70	6.43	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	0.156	0.468
PFOS	18.1	9.73	80.6	26.6	59.6	55.9	18.6	17.1	162	6.08	49.8	19.6	9.04	16.8	15.3	32.2	44.0	95.1	67.0	78.4	<LOD	<LOD	<LOD	10.6	4.41	111	0.156	0.468
PFTeDA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3.38	<LOD	<LOD	0.730	2.19
PFTrDA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	4.50	13.5
PFUnA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.74	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	3.06	0.483	1.45
Perfluorobutanoic acid	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3.90	6.00	6.33	5.49	6.30	5.09	BQL	<LOD	11.7	<LOD	5.55	<LOD	<LOD	<LOD	<LOD	<LOD	5.57	1.11	3.33
DEET (Diethyltoluamide)	<LOD	<LOD	2.24	BQL	<LOD	<LOD	3.36	<LOD	BQL	<LOD	<LOD	<LOD	9.28	<LOD	<LOD	<LOD	<LOD	<LOD	3.08	<LOD	<LOD	<LOD	<LOD	7.81	<LOD	<LOD	0.567	1.70
2.4-Dinitrophenol (DNP)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3.72	<LOD	1.18	<LOD	<LOD	1.94	1.88	4.62	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.261	0.783
Formetanate	<LOD	<LOD	1.85	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.613	1.84
Isoprocarb	<LOD	<LOD	10.4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	16.3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.44	4.33

Analyte	Species																										LOD (µg/kg w.w.)	LOQ (µg/kg w.w.)
	Little owl						Great curlew									Peregrine falcon									Eagle owl			
	Sampling Years																											
	‘20	‘20	‘20	‘20	‘20	‘20	‘11	‘07	‘05	‘06	‘05	‘19	‘20	‘05	‘16	‘20	‘20	‘20	‘20	‘20	‘20	‘20	‘20	‘19	‘19	‘20		
	Sample ID																											
	3	**8**	**12**	**14**	**18**	**22**	**24**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**01**	**04**	**07**	**012**	**013**	**015**	**017**	**019**	**002**	**101**	**102**		
Myclobutanil	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	12.7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	2.80	8.39
Phthalamic acid	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	55.5	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	9.92	29.8
Picaridin (Icaridin)	<LOD	<LOD	13.4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	18.4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2.56	7.67
Promecarb	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2.10	6.30
Propazine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.79	5.36
Schradan	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.593	1.78
Tebutame	<LOD	<LOD	<LOD	<LOD	<LOD	13.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.96	5.87
Thiabendazole	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.469	1.41
Trietazine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.423	1.27
2.3.5-Trimethacarb	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	17.0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	4.36	13.1
4.4-DDE	19.5	152	29.5	11.8	17.1	18.7	16.5	138	57.9	113	15.8	91.1	9.93	BQL	BQL	4.05	6.75	27.9	41.0	31.2	37.1	18.4	14.9	41.7	19.1	163	1.10	3.31
Hexachlorobenzene	9.84	10.3	10.6	7.49	9.29	8.04	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	0.583	1.75
1,2-Benzisothiazolinone	27.8	32.2	21.8	<LOD	BQL	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	6.30	18.9
Anabasine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	1.20	3.61
Hydroxy-Cotinine	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	17.0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	BQL	5.03	15.1

In the **Figure 32**, the frequency of appearance of each compound is presented. The most frequent compound that was detected in all egg samples was *p,p'*- DDE followed by Methylparaben and PFOS. 4,4-DDE (*p,p'*-DDE) and Methylparaben were detected in significantly high concentrations in the tested samples with a mean concentration 74,3 and 210 μg/kg w.w., respectively. The highest number of plant protection products (PPPs) and their TPs was noticed in the little owl sample 12, whereas in the Great curlew sample 27, 7 industrial chemicals were detected (mainly Surfactants and QACs). Among the analyzed raptor species, the highest number of pharmaceuticals, personal care products and their TPs was observed in the Little owl specimens.

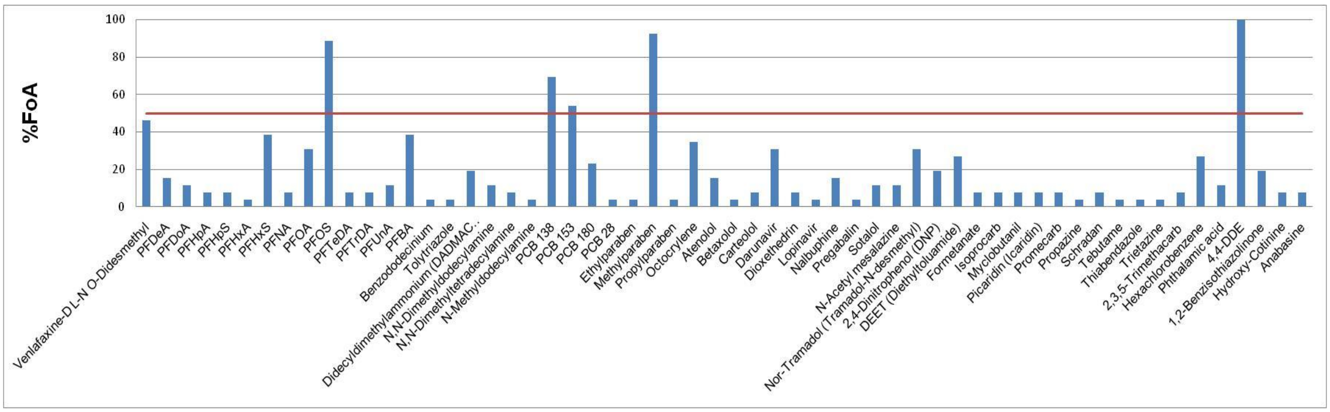


Figure 32: Frequency of Appearance

In total, 4 Polychlorinated Biphenyls (PCBs) were detected in the tested samples. The sample with the highest total concentration of PCBs (90.2 μg/kg w.w.) was the egg sample 3 (Little owl, sampling year: 2020). While the sample, in which the 4 PCBs were determined, was the egg sample 14 (Little owl, sampling year: 2020). In Great curlew specimens the PCBs were not detected, with the exception of the sample 27, in which 2 PCBs congeners (138, 153) were determined with a total concentration of 18.8 μg/kg w.w. in the Box-plot in **Figure 33** it is obvious that the variation of concentration in Little owl samples was higher than the other two species.

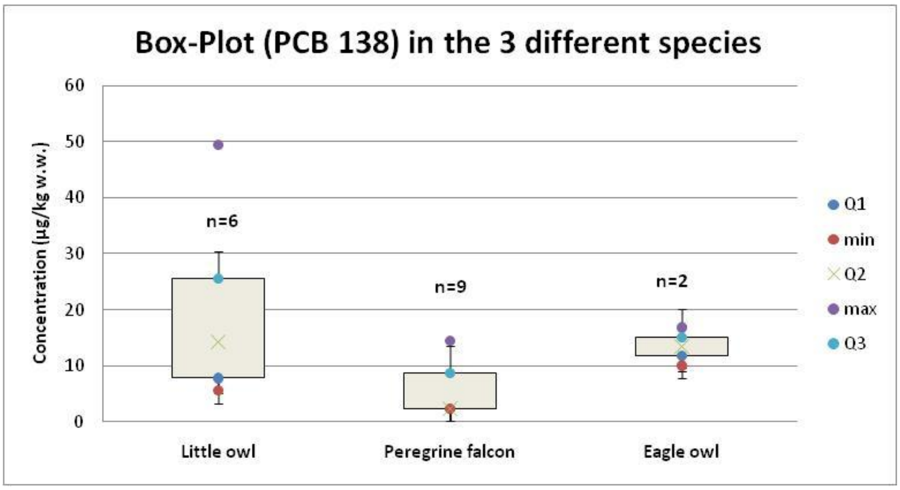


Figure 33: Box-Plot of PCB 138

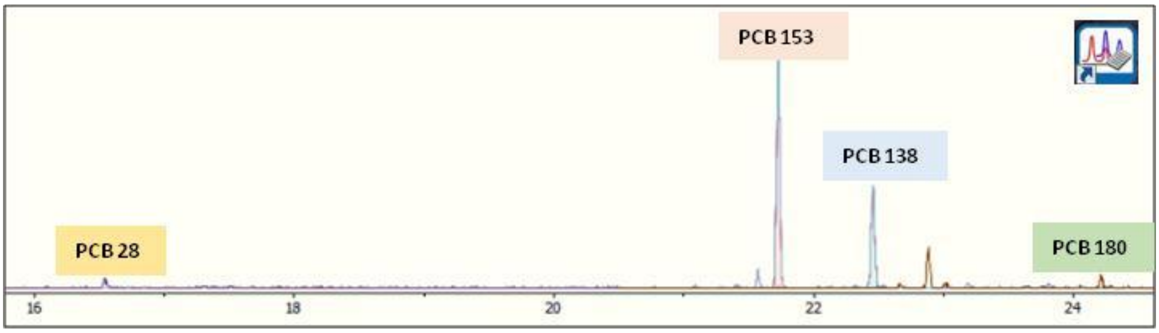


Figure 34: EIC of the detected PCBs in the little owl sample 14

In total, 13 per- and poly- fluoroalkyl substances (PFAS) were determined in the tested samples. Most of them are PFCAS (77%-10 substances) (PFTrDA, PFNA, PFOA, PFBA, PFTeDA, PFHxA, PFHpA, PFUnA, PFDoA, PFDeA) and the rest of them are PFSAS (PFOS, PFHxS, PFHpS). PFOS (**Figure 35**) was the most abundant detected PFAS, which was present in the 88% of the tested samples in concentrations ranging from 4.41 to 162 μg/kg w.w., indicating that the aforementioned compound is ubiquitous in the terrestrial ecosystem and the organisms of the higher trophic levels (raptors).

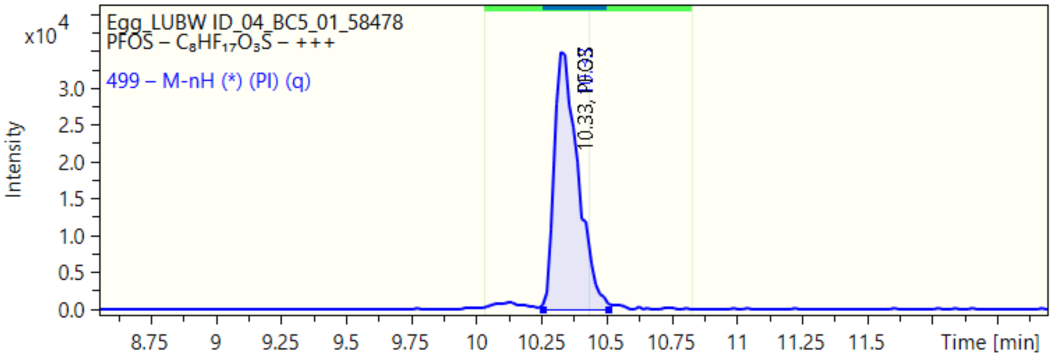


Figure 35: Extracted ion chromatogram of PFOS

The highest total concentration of PFAS (187 µg/kg w.w.), along with the highest number (12) of the detected PFAS were observed in the egg sample 27 (Great curlew, 2005). The cumulative concentration in each sample is illustrated in **Figure 36**. It is obvious that in the majority of the samples the total concentration was less than 50 µg/kg w.w. In the following Box-plot in **Figure 37** it can be remarked that the variation of concentration between the 4 different species was lower in Great curlew species. Although the median observed concentration was lower in this species it is obvious an outlier.

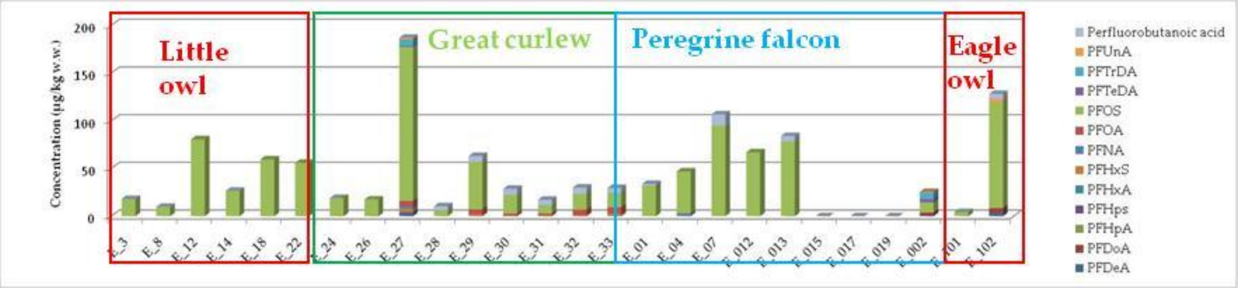


Figure 36: Total concentration of PFAS in each tested sample

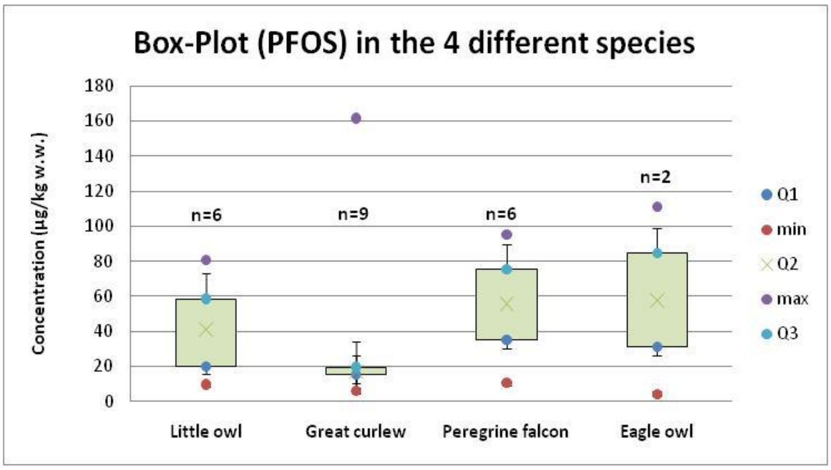


Figure 37: Box-plot of PFOS in the 4 different species

As far as the plant protection products are concerned, 13 different PPPs and their TPs were detected. The most frequent and most abundant among them was *p,p'*-DDE which is a transformation product of DDT. In addition, Hexachlorobenzene (**Figure 38**), which is a POP, was detected in all Little owl samples and in one Eagle owl. Apart from *p,p'*-DDE, the second most abundant PPP was Phthalamic acid, which is a TP as well.

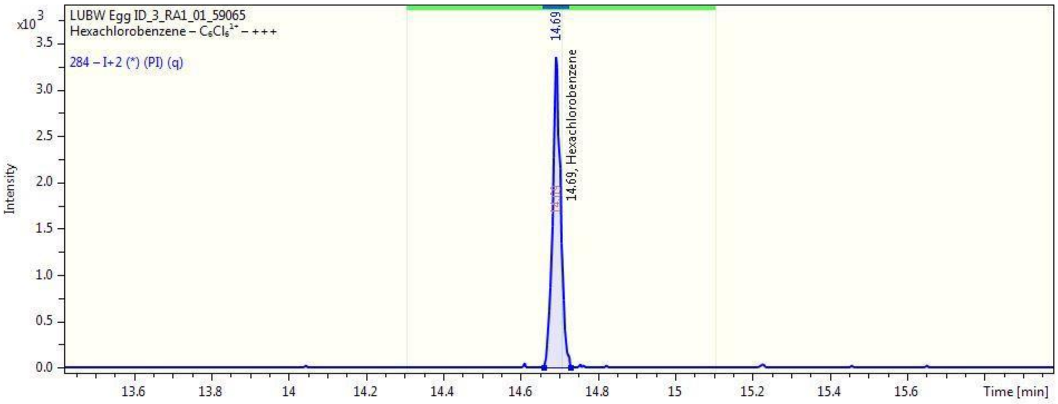


Figure 38: Extracted ion chromatogram of Hexachlorobenzene

The half of the PPPs that were detected in the tested samples, were insecticides as it is depicted in the chart below in the **Figure 39**, and it may be related with the dietary habits of raptor birds, which are known as insectivorous and herbivorous as well.

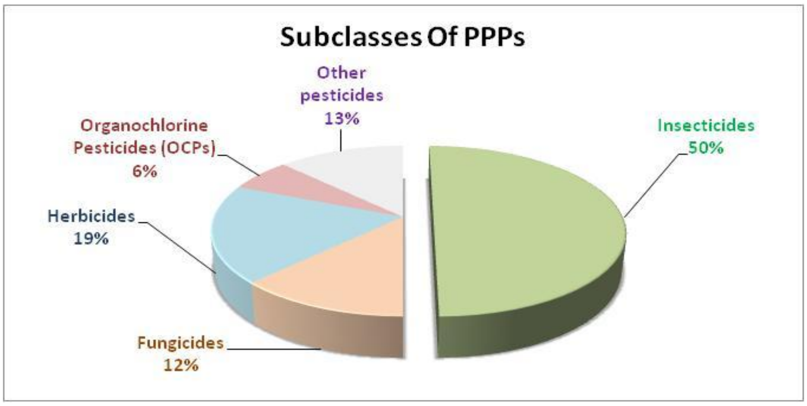


Figure 39: Subclasses of Plant Protection Products

Figure 40 demonstrates the total concentration of PPPS in each tested sample and in the majority of the samples the total concentration was less than 50μg/kg w.w.

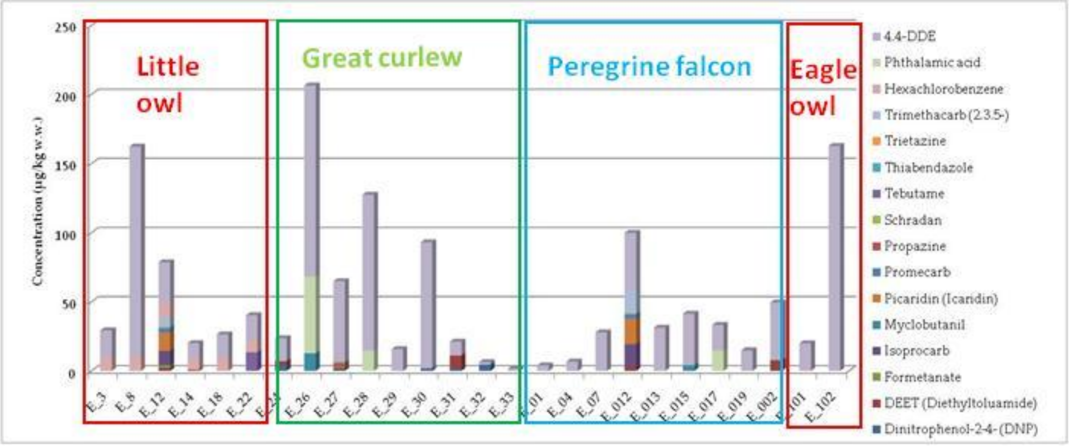


Figure 40: Total concentration of Plant Protection Products in each tested sample

In total, 11 different pharmaceuticals and TPs were determined in the tested egg samples. The most frequent detected pharmaceuticals were Darunavir and Nor-Tramadol, whereas the predominant pharmaceutical was the beta-blocker Atenolol. Darunavir was detected in Little owl samples and Great curlew samples as depicted in the chart in **Figure 41**. In the majority of the tested samples the total concentration was less than 20 μg/kg w.w.

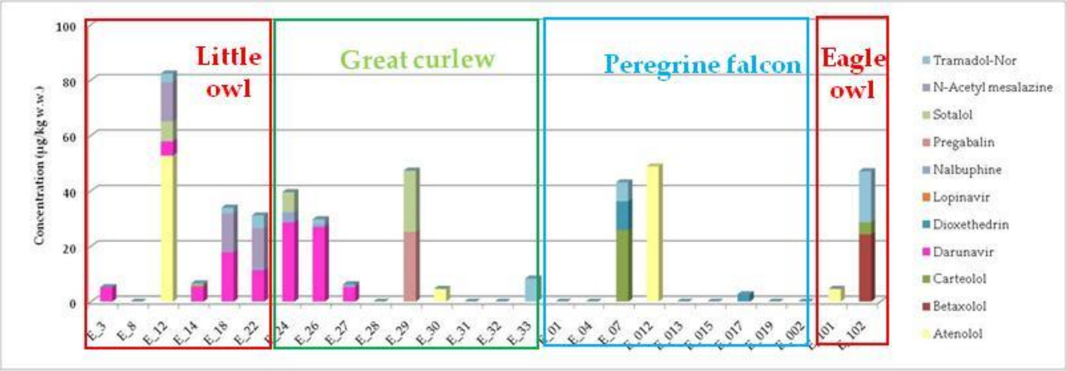


Figure 41: Total concentration of Pharmaceuticals in each tested sample

Approximately 46% of them were Beta-adrenergic agonists and it is depicted in the chart below in the **Figure 42**.

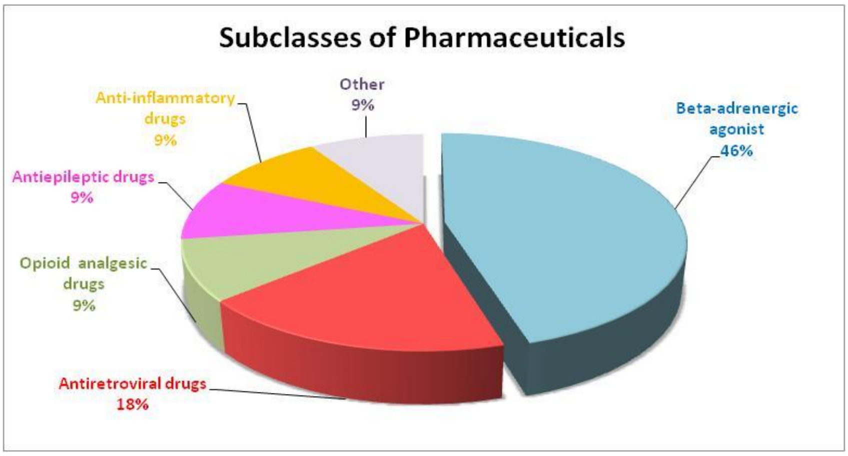


Figure 42: Subclasses of Pharmaseuticals and their TPs

In total, 4 different Personal Care Products (PCPs) were determined in the analyzed samples, including methylparaben, ethylparaben, octocrylene and propylparaben. Methylparaben (**Figure 43**) was the most frequent and the most abundant among them. It is necessary to underline the fact that Methylaparaben was the most abundant compound among the 58 detected analytes, in concentration of 1760 μg/kg w.w. Methylparaben has been considered as Endocrine disrupting chemical (EDC). The mean concentration of methylparaben was higher in Little owl, in contrast with the rest tested species (**Figure 44**).

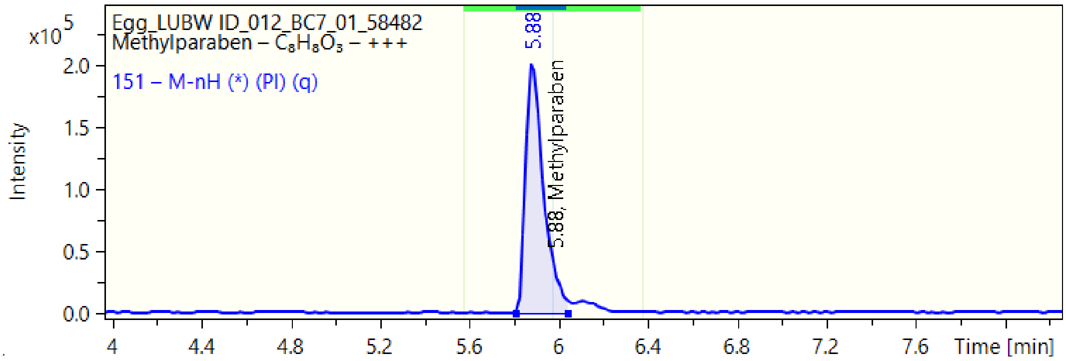


Figure 43: Extracted ion chromatogram of Methylparaben

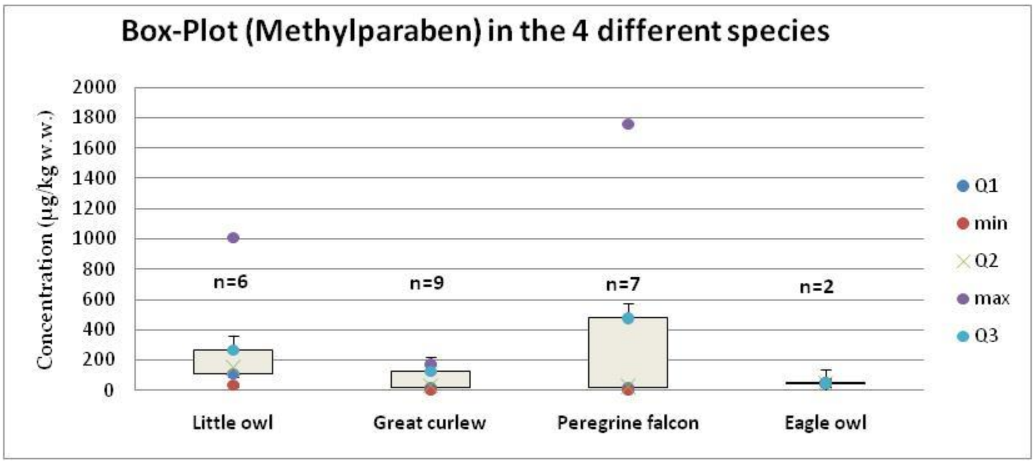


Figure 44: Box-Plot of concentration of Methylparaben in the 4 different species

Furthermore, six (bio) transformation products [(bio)TPs] of ECs were detected in the tested samples, such as the TP of the antipsychotic drug Venlafaxine, D L-N O-Didesmethyl-Venlafaxine, and the TP of the anagelsic drug Tramadol, Nor-Tramadol, underlining the advantages of the HRMS techniques in the identification of organic micropollutants.

The sample with the highest number of detected contaminants (35) was the egg sample 27 (Great curlew, sampling year: 2005), while the sample with the lowest number of the detected contaminants (8) was the sample 01 (Peregrine falcon, sampling year: 2020).

The highest total contaminants concentration (1.99 mg/kg w.w.) was detected in the egg sample 012 (Peregrine falcon, sampling year: 2020), whereas the less total contaminants concentration was detected in the egg sample 01 (Peregrine falcon, sampling year: 2020) reaching up to 0.43 mg/kg w.w.

These results are in line with the already existing literature. Previous studies indicated the occurrence of *p,p'*-DDE, HBC, PFAS, PCBs and Methylapraben as it is already mentioned in chapter 2.8. For example, Xue et al. 2016 reported the occurrence of Methylparaben in eggs and the mean concentration was 4.63 μg/kg w.w. in herring gull eggs from 1988 and 3.80 μg/kg w.w. in loon eggs [81]. In 2010, Holmoström et al. reported a mean concentration 83 μg/kg w.w. of PFOS in samples from 2006, which was the most predominant compound among PFAS, in terrestrial biota with the analysis of Peregrine falcon (*Falco peregrinus*) eggs collected from 1974 to 2007 across Sweden and in our study the mean concentration of PFOS was 43.8 μg/kg w.w. [68]. Significant differences in PFOS concentrations in Swedish osprey (*Pandion haliaetus*) eggs within the periods 1997–2001 (103 μg/kg w.w.), 2008–2009 (64 μg/kg w.w.) and 2013 (70 μg/kg w.w.) were observed by Eriksson et al. (2016) [69].

CHAPTER 6

CONCLUSIONS & FUTURE PERSPECTIVES

Environmental organic micropollutants end up to the aquatic and terrestrial ecosystem through various pathways, enter the food webs and, thus, they are bioaccumulated through the food chains to apex predators. Apex predators are useful as indicators for environmental contamination, due to their unique characteristics. The analysis of raptors' eggs gives the opportunity to examine the presence of organic micropollutants with potential persistent bioaccumulative and toxic (PBT) properties in the terrestrial ecosystem.

The validation of a developed protocol and the evaluation of the linearity, the recovery, the repeatability, the matrix effect and the detectability, using a representative validation dataset of emerging contaminants with different physicochemical properties from a wide variety of classes of micropollutants were carried out. The quality parameters that were analyzed were satisfactory, as it is referred to a wide-scope method.

Wide-scope target screening using two databases with more than 2,400 emerging contaminants, priority pollutants, as well as their (bio)transformation products, was applied based on specific identification criteria; mass accuracy, retention time, isotopic pattern and MS/MS information, were attributed in order to facilitate confidence.

In total, 58 compounds were detected in the 26 tested raptor egg samples from 8 different classes. Plant Protection Products and their TPs was the most frequent detected class. Methylparaben was the most abundant compound among all. In addition, *p,p'*-DDE was detected in all egg samples and Methylparaben, PFOS and PCB congener 138 were detected with high detection frequency.

To conclude, this biomonitoring study reveal the presence of micropollutants in raptor eggs which were collected across Baden-Württemberg in Germany. However we may consider that the analyzed samples of the current study are limited and additional samples are needed to draw confident conclusions.

It is important to note that our samples were collected in a limited area in Germany. Therefore, this study does not provide information on general concentrations in the German environment and future studies should take this into account. Consequently, sampling from different locations across Germany and different countries will give the opportunity to compare the results and estimate the total environmental pollution and the global distribution of chemicals. In addition, it is proposed the analysis of different matrices of raptor specimens, such as feathers and eggshells, in order to compare the concentration levels in the different parts of an organism. Moreover, the analysis of more samples and from different species is needed in order to compare the concentrations and to examine if the same compounds are detected in different species or it is related to the species, their habitat and their eating habits. Additionally, the analysis of the prey of apex predators and organisms of different trophic levels is needed in order to study the biomagnification through the food chain. Furthermore, HILIC should be used for the identification of more polar compounds as a complementary chromatographic technique to RP. Finally, application of Suspect and Non-target screening workflows is crucial for the identification of more organic micropollutants.

ABBREVIATIONS-ACRONYMS

PPs	Priority Pollutants
EPA	Environmental Protection Agency
POPs	Persistent Organic Pollutants
VOCs	Volatile organic compounds
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PBDEs	Polybromodiphenylethers
OCs	Organochlorine compounds
***p,p'*-DDT**	p,p'-dichlorodiphenyltrichloroethane
***p,p'*-DDE**	1,1-dichloro-2,2-bis(pchlorophenyl)ethylene
ECs	Emerging contaminants
CECs	Contaminants of emerging concern
TPs	Transformation products
PBT	Persistent Bioaccumulative Toxic
WWTPs	Wastewater treatment plants
PPCPs	Pharmaceuticals and personal care products
PPPs	Plant Protection Products
PCNs	Polychlorinated naphthalenes
PFASs	Per- and Polyfluoroalkyl substances
PCAs	Polychlorinated alkanes
PDMSs	Polydimethylsiloxanes
QACs	Quaternary ammonium compounds
EDCs	Endocrine-disrupting compounds
NSAID	Non-steroidal anti-inflammatory drugs
PFAAs	Perfluoroalcyl acids
PFCAs	Perfluoroalkyl carboxylic acids
PFSAs	Perfluoroalkyl sulfonic acids
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PFHxS	Perfluorohexane sulfonate
GC	Gas chromatography
GSC	Gas-solid chromatography

GLC	Gas-liquid chromatography
LC-MS	Liquid chromatography coupled to Mass Spectrometry
RP-UHPLC	Reversed Phase Ultra High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
QqQ	Triple quadrupole
ESI	Electrospray ionization
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
TOF	Time-of-flight
Q-TOF	Quadrupole-Time-of-Flight
FWHM	Full Width at Half Maximum
DDA	Data Dependent Acquisition
DIA	Data Independent Acquisition
bbCID	Broad-band Collision Induced Dissociation
HE	High energy
LE	Low energy
SRM	Single Reaction Monitoring
ASE	Accelerated Solvent Extraction
SPE	Solid Phase Extraction
d-SPE	dispersive SPE
GPC	Gel Permeation Chromatography
HCB	Hexachlorobenzene
HBCD	Hexabromocyclododecane
BFRs	Brominated Flame Retardants
HCH	hexachlorocyclohexane
Q	Quadrupole
QIT	Quadrupole-ion trap
APEs	Alkylphenol polyethoxylates
HILIC	Hydrophilic interaction liquid chromatography
IS	Internal standards
NKUA	National Kapodistrian University Of Athens
TrAMS	Trace Analysis Mass Spectrometry
EIC	Extracted Ion Chromatogram

LOD	Limit of Detection
LOQ	Limit of Quantification
FME	Factor of Matrix Effect

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