UNIVERSITÀ DEGLI STUDI DI MILANO
School of Veterinary Medicine
PhD course in Veterinary and Animal Sciences
Class XXXII

A survey on environmental pollutants, drug and metal residues in different foods of animal origin and the related risk.

Federica Ceriani

Tutor: Prof. Francesco Arioli.
Coordinator: Prof. Valeria Grieco.

ACADEMIC YEAR 2018/2019

"Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less"

Marie Curie

ABSTRACT

The presence of xenobiotic residues, both drugs and environmental contaminants, in food is a cause for concern and therefore the European Authorities issue reports or laws in order to propose monitoring plans, Health-based Guidance Values (HBGV) and maximum residue levels or maximum levels (MRLs and MLs).

Based on these considerations, this doctoral thesis studies the presence of residues in different foods of animal origin, aimed at a characterization of the risk for the consumer.

Firstly, we studied seafood, which is an excellent source of nutrients, with important human health benefits. We focused on mussels and clams, filter feeders animals, suitable bio indicator organisms due to their bioaccumulation ability of a wide range of environmental pollutants. In the first research study, we evaluated the Italian consumer risk related to metal exposition through molluscs, on the basis on the MLs stated by the European Union, where available, or, otherwise, based on the HBGV stated by EFSA. About our results, regarding the human metal exposure, we conclude that there is a low risk for the average consumer; however, high percentile consumers, may be subjected to skin lesions, and lung, skin and bladder cancer due to high intake of As, while Ni sensitive individuals can undergo allergic dermatitis due to constant Ni presence in the studied molluscs. Subsequently, we focused on most consumed fish like salmon, tuna which consumption has consistently risen. In the second study about salmon, the aim was to investigate the presence of persistent organic pollutants (POPs) and antimicrobials in wild and farmed salmons from different geographic areas. Farmed salmons showed slightly higher presence of environmental contaminants than wild ones, likely due to the decreased possibility of a constant exposition. Antibiotics were seldom found only in farmed salmon. Risk related to organophosphate compounds (Ops), polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs) and organochlorine pesticides (OCPs) deriving from salmon intake were of is very low concern, while the presence of polybromodiphenyl ether (PBDE99) and perfluorooctanoic acid (PFOA), is a cause for a bit higher concern. The substantial lack of data about the detected antibiotics in salmon did not allow an extrapolation from MRLs of terrestrial animals and a risk characterization

In the third work on tuna, a long-living fish with high biomagnification ability, we studied the presence of metals with high toxicological importance for public health (Hg, Pb, Cd, As, Cr, Ni).

One hundred thirty-one samples were analysed. One red tuna from the Adriatic Sea and 11 yellow tunas exceeded Pb maximum levels (MLs); three red tunas from different Mediterranean sub-areas exceeded Hg MLs. The evaluation of cumulative effects indicated that only a negligible health hazard could derive from the ingestion of tuna, for both average and high consumers. The risk of carcinogenicity from Cr is still under debate at the concentrations detectable in food.

In these two works, we confirm a low risk, related to the studied compounds, for average consumer health due to fish consumption.

The regular consumption of meat and meat products provides a significant intake of proteins and essential micronutrients. Pork meat, for example, is used in many countries to produce derivative products (hams and cured meats) with high qualitative value. Also, game animal meat consumption, though being a niche product, is constantly increasing and hunters, their families and persons closely associated with them can be regarded as a high consumption subpopulation. Furthermore, game animals are a suitable indicator about environmental pollutant such as PCBs, PBDEs, PAHs and brominated flame retardants (BFRs). In the fourth work we studied the occurrence of PBDEs and perfluoroalkyl substances (PFASs) from eight EU Member States (Austria, Denmark, French, Germany, Holland, Italy, Poland and Spain). The European Commission has not stated maximum limits (MLs) for some environmental pollutants such as polybrominated diphenyl ether PBDEs and PFASs; no perfluoroalkyl substances were detected, except PFOA, in only one Austrian sample. PBDEs were detected in three out of 77 samples: the one coming from Germany showed the presence of all congeners analyzed the ones from Netherland and Italy, respectively PBDE 153 and PBDE 100. The results show that the analyzed samples do not pose a risk for human beings about PFASs and PBDEs. A following report from EFSA, requires a new attention on PFAS, with HBGV being drastically reduced. In the fifth work we studied four different animal species (chamois, roe deer, red deer and wild boar) that have different nutrition habits. Game animals are a suitable sentinel species to have a picture of the environment. Muscle samples from seventy-nine animals were collected during the hunting season in a Northern Italy mountain area. No PBDEs were found in the samples. OCPs, OPs and PCBs were detected in almost all samples at different concentration ranges, showing higher frequency in ungulate species than in wild boar. PFAs were found only in wild boar. Anthracene and benzopyrene, among PAHs, were found only in chamois at low concentrations. A low risk for consumers can be indicated due to the frequent detection of

contaminants at trace levels, to the scarce prevalence of high concentrations of some contaminants and to the low consumption of game animal meat.

An important topic in the researches carried out in my doctorate was the investigation of POPs in organic honey. However, even if organic beekeeping excludes (or restrictively allows) the use drugs or pesticides many pollutants may contaminate bee matrices, comprising bee, honey and pollen. Therefore, the focus was the investigation of a broad spectrum of analytes, pesticides, persistent organic pollutants and antibiotics in organic honeys collected in different productive areas with different agricultural, zootechnical or anthropic impact to verify the potential transfer of xenobiotics into supply chain from different sources than beekeeping practices. The presence of several compounds, such as PCBs, PBDE and PAHs was confirmed, not only in proximity to highly urbanised centres, where the concentrations were higher, but in all environment contexts, confirming the theory that these are ubiquitous contaminants. No antibiotics were found in samples analysed suggesting that presence of antibiotics is from beekeeping practices.

The analytes in the different matrices required different approaches for sample pretreatment, extraction, clean up and fractionation before the analysis with liquid chromatography–tandem mass spectrometry (LC-MS/MS) or – gas mass spectrometry (GC-MS/MS). The approach of analytical-instrumental nature has provided for the optimisation of instrumental performances as well as of the steps of sample pretreatment, in order to achieve good levels of sensitivity, specificity and robustness of the method to then make considerations of qualitative, quantitative and statistical nature. The trials planning, optimisation and validation of the methods were performed according to Commission SANTE/10553/2018 (SANTE 2018).

The results of this manuscript suggest that there is a low risk for the average consumer health. Environmental concentrations of persistent organochlorine compounds have been decreasing over the past two decades, and this correlates with remarkable advances in the detection of exceedingly low levels of these compounds in human populations and the improvement of European control. PCBs still are present in environment due to their industrial source even if their use was banned in many industries application. Regarding emerging compounds, PFAs still need to be concern due to their wide use and their possible toxicological role. Recently European commission decreased the HBGVs for these classes to safeguard human health. Antibiotics still are a matter of concern and need a close control to ensure human safety and decrease antimicrobial resistance.

RIASSUNTO

La presenza di residui chimici negli alimenti, costituiti sia da composti farmaceutici che da contaminati ambientali, è un argomento di crescente interesse e preoccupazione per la sanità pubblica.

L'Unione Europea periodicamente sancisce leggi e rapporti aggiornati, con lo scopo di condurre piani di monitoraggio e linee guida sull'impiego di tali composti a livello industriale, agricolo e terapeutico e livelli massimi residuali (LMR e ML) negli alimenti, al fine di prevenire il rischio per il consumatore.

Sulla base di queste considerazioni, il presente elaborato ha lo scopo di studiare la presenza di residui chimici in diversi alimenti di origine animale, al fine di caratterizzare il rischio per il consumatore.

In primo luogo, ci siamo focalizzati sullo studio di alimenti provenienti dal settore ittico, che è un'ottima fonte di nutrienti, con importanti benefici per la salute umana.

Ci siamo concentrati su cozze e vongole, animali filtratori e bioindicatori adatti per le loro caratteristiche a bioaccumulare un'ampia gamma di inquinanti ambientali.

Lo scopo del primo elaborato è stato quello di valutare il rischio per il consumatore dei principali metalli (Hg, Cd, Pb, Ni, Cre As), attraverso il consumo di molluschi sulla base dei limiti massimi dichiarati dall'Unione Europea o, ove disponibili, sulla base dei valori soglia dichiarati dall'EFSA. Dal primo lavoro è emerso che vi è un basso rischio per il consumatore medio; tuttavia, i consumatori ai percentili superiori, possono essere soggetti a lesioni cutane e/o neoplasie polmonari, cutanee e vescicali per l'elevata assunzione di As. Soggetti Ni sensibili, possono invece essere soggetti a dermatiti allergiche.

Il secondo lavoro di ricerca si è invece concentrato sulla ricerca dei medesimi metalli nel tonno, il cui consumo è in aumento secondo i dati della commissione europea, per le sue capacità di bioaccumulo. Sono stati, così, analizzati 131 campioni provenienti da diverse zone FAO. Dai risultati, è emerso che solo un tonno rosso, proveniente dal mare Adriatico e 11 tonni gialli hanno superato i livelli massimi residuali di Pb; tre tonni rossi provenienti da diverse sottozone del

Mediterraneo hanno superato i livelli massimi consentiti per il mercurio. La valutazione degli effetti tossicologici cumulativi ha indicato un rischio trascurabile sia per i medi che alti consumatori.

L'obiettivo del terzo studio è stato quello di studiare la presenza di inquinanti organici persistenti e di antimicrobici nei salmoni selvatici e di allevamento di diverse aree geografiche. I salmoni d'allevamento hanno mostrato una presenza di contaminanti ambientali superiore a quelli selvatici, probabilmente a causa di un maggiore impatto demografico. Il rischio legato ai composti organofosforati, agli idrocarburi policiclici aromatici, ai policlorobifenili e ai pesticidi organoclorurati derivanti dall'assunzione di salmone si è rilevato molto basso, mentre la presenza di polobromodifenilietere congenere 99 (PBDE99) e acido perfluoroottanoico (PFOA) suscita maggiore preoccupazione. Gli antibiotici sono stati riscontrati con bassa frequenza solo nel salmone allevato.

Da questi lavori, sulla base dei dati ottenuti, possiamo confermare che vi è basso rischio per il consumatore medio

Il consumo regolare di carne e prodotti a base di carne fornisce un significativo apporto di proteine e micronutrienti essenziali. La carne suina, ad esempio, è impiegata in molti paesi per produrre prodotti derivati (prosciutti e salumi) ad alto valore qualitativo. Anche il consumo di carne di selvaggina, pur essendo un prodotto di nicchia, è in costante aumento e i cacciatori, le loro famiglie e le persone a loro strettamente legate possono essere considerati una sottopopolazione ad alto consumo. Nel quarto lavoro abbiamo cosi studiato la presenza di polibromodifenilietere e sostanze perfluoroalchiliche provenienti da otto Stati membri dell'UE (Austria, Danimarca, Francia, Germania, Olanda, Italia, Polonia e Spagna). La commissione Europea non ha definito limiti massimi per tali composti e dai nostri risultati non sono state rilevate sostanze perfluoroalchiliche ad eccezione dell'acido perfluoroottanoico in un solo campione austriaco. I polibromodifenilietere sono stati rilevati solo in 3 dei 77 campioni investigati. I risultati mostrano che i campioni analizzati non rappresentano un rischio per il consumatore. Recentemente una successiva relazione EFSA ha richiesto di porre maggiore attenzione sulla presenza dei perfluoroalchilici, i cui valori soglia sono stati ridotti drasticamente per il loro rischio tossicologico.

Nel quinto lavoro ci siamo focalizzati su quattro diverse specie selvatiche (camoscio, capriolo, capriolo, cervo e cinghiale) con abitudini alimentari differenti. Gli animali selvatici sono considerati

specie sentinelle e quindi ottimi indicatori ambientali. Campioni muscolari di settantanove animali sono stati raccolti durante la stagione venatoria in una zona montana dell'Italia settentrionale. Nei campioni non sono stati trovati polibromodifenilieteri. Al contrario i pesticidi organoclorurati e organofosforati e i policlorobifenili sono stati rilevati in quasi tutti i campioni a diversi intervalli di concentrazione, mostrando una frequenza maggiore nelle specie di ungulati rispetto al cinghiale. I PFA ,invece, sono stati riscontrati solo nei cinghiali. Tra gli idrocarburi, antracene e benzopirene, sono stati trovati solo nel camoscio a basse concentrazioni. Possiamo nuovamente concludere che per il frequente ritrovamento a basse concentrazioni dei contaminati, ad eccezione di singoli composti riscontrati ad alte concentrazioni, e del basso consumo di carne di selvaggina rapportata ad altre tipologie di carne, vi è un basso rischio per il consumatore italiano.

Un ulteriore tema di crescente interesse per la sanità pubblica è stato lo studio dei contaminati ambientali persistenti nel miele, in particolare il miele biologico. Infatti, nonostante l'apicoltura biologica escluda (o consenta in modo restrittivo) l'impiego di farmaci o pesticidi, molti inquinanti possono contaminare api, miele e polline. Pertanto, l'attenzione si è concentrata sullo studio di un ampio spettro di analiti quali, pesticidi, inquinanti organici persistenti e antibiotici in mieli organici raccolti in diverse aree produttive con diverso impatto agricolo, zootecnico o antropico per verificare il potenziale trasferimento di xenobiotici nella catena di approvvigionamento da fonti diverse rispetto alle pratiche apistiche. È stata confermata la presenza di diversi composti, come policlorobifenili, i polibromodifenilietere e gli idrocarburi policiclici aromatici non solo nelle arnie in prossimità di centri altamente urbanizzati, dove le concentrazioni erano più elevate, ma in tutti i contesti ambientali, confermando la possibilità di trasferimento da fonti ambientali e l'ubiquità di tali composti. Il mancato ritrovamento di antibiotici nei campioni analizzati esclude la possibilità di trasferimento accidentale delle molecole dall'ambiente in cui sono posizionate le arnie.

Per ottenere una così ampia e diversificata ricerca, ogni lavoro è stato approciato in modo differente per il pretrattamento dei campioni, l'ottimizzazione del metodo analitico, l'estrazione degli analiti e il loro successivo clean up prima dell'analisi con cromatografia liquida in spettrometria di massa tandem (LC-MS/MS) o gas spettrometria (GC-MS/MS). L'approccio di natura analitico-strumentale ha richiesto per ogni ricerca un'accurata e ampia ricerca per ottenere l'ottimizzazione delle prestazioni strumentali e delle fasi di pretrattamento dei campioni, al fine di raggiungere buoni livelli di sensibilità, specificità e robustezza dei metodi analitici impiegati per poi fare

considerazioni di natura qualitativa, quantitativa e statistica. La pianificazione delle prove, l'ottimizzazione e la convalida dei metodi sono state eseguite secondo la Commissione SANTE/10553/2018 (SANTE 2018).

I risultati di questo lavoro suggeriscono che il rischio per la salute media dei consumatori è basso. Le concentrazioni ambientali dei composti organoclorurati persistenti sembrano diminuite negli ultimi due decenni, probabilmente grazie ai progressi nella rilevazione analitica e al miglioramento dei controlli europei. I PCB sono ancora presenti nell'ambiente a causa del loro ampio impiego a livello industriale nel secolo scorso e delle loro peculiarità chimico fisiche, anche se il loro uso, oggi, è stato vietato in molte applicazioni. Per quanto riguarda i composti emergenti, i PFAs destano preoccupazione a causa del loro ampio uso e del loro possibile ruolo tossicologico. Recentemente la Commissione Europea ha infatti drasticamente diminuito i livelli soglia per queste classi per salvaguardare la salute umana. Gli antibiotici sono ancora motivo di preoccupazione e necessitano di uno stretto controllo per garantire la sicurezza umana e ridurre le resistenze, tema tuttora più che attuale.

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CHAPTER 1

Introduction

1. INTRODUCTION

1.1. Food safety and toxicological risk

At the beginning of this century, the scientific community began to consider life sciences, both regarding human and animals, and environmental sciences under the general concept of "One Health". This approach, however, was not completly new: infact already in 1821, the term “zoonosis” had been coined to define a contagious pathology transferable from animals to human and vice versa. Nevertheless, human medicine and veterinary medicine were considered two separate disciplines in the whole 20th century (CDC, 2016) even if, meantime, their interaction grew until, in recent years, many factors changed the global view of the public health, and the scientific word understood that it is impossible to deal separately human health, animal health and environment.

In this new global view, the scientific world became conscious that, the close interactions among humans, animals and environment may cause damages to biota or to abiota resulting in e.g. infectious illnesses or environmental impairments and exposition to different sustances dangerous for human health. In other words, a sustainable development depends on the health and well-being between humans, animals and the ecosystems in which they coexist (Rapport et al., 1998).

In the period after the Second World War the rapid industrialization and economic improvement, deeply changed the world and life style, and in different industrial and agricultural sectors, several chemicals were indiscriminately used to improve the final product. The massive production of chemical compounds like e.g. plasticisers, pesticides, diatermic and dieletric substances and petrochemicals caused their wide distribution in the ecosystem, worsening the quality of soil, air, water and food (El-Shahawi et al., 2010). The resultant toxicological risk was realized in the following decades.

For a better comprehension a few cases are below reported as examples: in 1960 in the lake Michigan district the wide use of polychlorinated biphenyls (PCBs) caused the infertility of mink females due to the increasing industrialization and 1970 in the same area Polybrominated biphenyls (PBBs), manufactured as flame retardants, entered in food chain through livestock feed (Longnecker et al., 1997) in 1990 in Mediterrean sea a viral epidemic caused the mortality of more

than eleven hundred dolphins: researcher studied their tissues and observed the presence of PCBs at concentrations two or three times higher than healthy dolphins.

Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs, PCDFs) and PCBs were detected, in the United States at the last of the eighties of the last century, in blood samples of people professionally exposed; in the sixties and seventies, organclhorine compunds were already detected in blood samples of population in United States and the occurrence of kidney cancer was correlated to the high presence of PCBs (Schecter et al., 1995; Shalat et al., 1989).

The exposure to chemical compounds is usually caused by mixtures of different substances or congeners that can share some toxicological modes of action and effects, evocking dose addition. The Total Dose must be so considered, i.e. the sum of the doses of each toxic agent with similar effects in humans and animals exposed for long time and at high levels.

Foodstuff contamination remains one of the main sources of exposure and could pose a threat for human health and for the environment (Vogt et al., 2012).

Consumer's food safety awareness is rising both in developed and developing countries, buf if the microbiological risk has higher importance in developing countries, due to the poor hygiene and lack of knowledge on food trail safety, the risk regarding chemical compounds is still a problem also for developed country (Odeyemi et al., 2019).

In the European Union (EU), consumer protection is a matter of extreme importance and European Commission and European Food Safety Authority (EFSA) safeguard against the harmful effects of chemical residue establishing safe levels by making Official Controls on food and asking to indipendent researchers to monitor the presence of this compounds in food and in environment to characterize the risk. The chemical compounds can occur in food as *residues*, defined, according Codex Alimentarius Commission, as "Residues of veterinary drugs include the parent compounds and/or their metabolites in any edible portion of the animal product and include residues of associated impurities of the veterinary drug concerned" (FAO, 2010). A generic and clearer definition could be: "*small amounts of a xenobiotic or its metabolites present in animal tissues or found in their products, capable of determining toxicological effects against the consumer*" (De Brabander et al., 2007). One of the main purposes of the global health organisations is to manage and reduce the risk deriving from exposure to residues (Zinsstag et al., 2011).

According to Food and Agriculture Organization (FAO) of the United Nations (UN), Food Security exist when*: "all people, always, have physical, social and economic access to sufficient, safe and*

nutritious food which meets their dietary needs and food preferences for an active and healthy life. Household food security is the application of this concept to the family level, with individuals within households as the focus of concern" (FAO, 2003). The base of this concept is that food security must include the very important aspect of food safety and ultimately "Risk assessment, Risk management and Risk communication" (WHO, 2010). To improve the knowledge regarding chemical compounds in environment and the human exposure, one challenge for the scientists is to improve their knowledge on the occurrence of chemical compounds in food in order to evaluate toxicological risk. This manuscript aims to give a contribution on the presence of different categories of chemical compounds eventually present in the most consumed foods and collect usuful data to improve the global information regarding chemical compounds in food.

1.2 Environmental behaviour and toxicological properties.

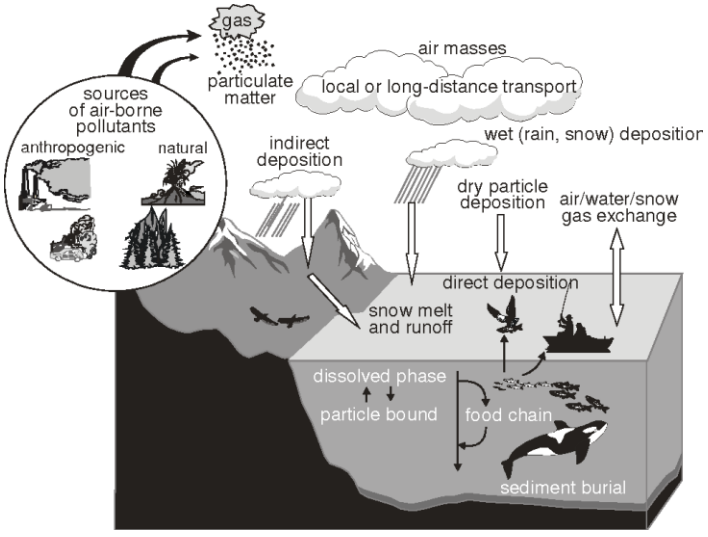
The last period of the twentieth century showed a global flow in awakening against the problem of environmental pollution, that is any physical, chemical and biological alteration of the quality of the environment (Wasi et al., 2013). Chemical pollutants are compounds present in environment at higher concentrations than natural ones. From the environment, most compounds are transferred to food, inducing oral exposure in the consumers and eventually evoking toxicological effects (Chen et al., 2019) In table 1 a survey of these compounds is reported.

Table 1: Molecules that can occurr as residues in products of animal origin

• **Drugs**	Antibiotics, hormones and others
• **Insecticides, Fungicides**	Organochlorine and organophosphorate insecticides
• **Packaging material**	Plastics
• **Environmental pollutants**	Halogenated compounds (Polychlorinated biphenyl, Polychlorinated dibenzofurans, Polychlorinated dibenzodioxins) Micotoxins Metals Not Halogenetic compounds (polyfluoroalkyl substances)
• **Food additives**	e.g. nitrate and nitrite compounds

One important aspect is their environmental fate: most of these compounds are known in literature as *persistent organic pollutant* (POPs), i.e. contaminants that, due to their chemical and physical properties persist for long time in the environment and pose major and increasing threats to human health and the environment itself. This definition, stated by the Stockolm Convention, was adopted by a Conference of Plenipotentiaries held from 22 to 23 May 2001 in Stockholm and entered into force on 17 May 2004. The Convention covers 23 priority pollutants that are produced intentionally and unintentionally (for example from sources such as waste incinerators). The purpose of the Convention is to minimise and eliminate the production, import and exportation of persistent pollutants. Signatory Countries should develop action plans to reach this aim and use alternative materials (European Commission 2006/507). POPs can enter in the air phase; both by evaporation or bound to particulate matter, and through atmosphere they can reach very long distance from their original source before being re-deposited (Galiulin et al., 2002) (Figure 1).

Figure 1: POPs environmental fate



Moreover, the combination of resistance to metabolism and lipophilicity properties lead to accumulation and biomagnification in tissues of animals at the highest trophic level, including humans. The most important classes of halogenated aromatics compounds include PCBs,

polychlorinated dibenzo-p-dioxins and-furans (PCDD/Fs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCs) (e.g. Dyphenylaliphatic compounds like DDT and its metabolites, aryl hydrocarbons like lindane and cyclodienes like chlordane), most of brominated flame retardants and some perfluoroalkil substances (PFAs).

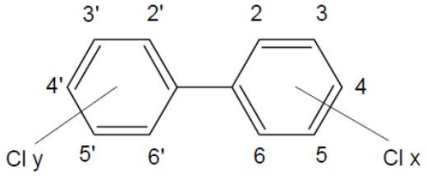
POPs are introduced in air, soil and sediment of surface water, from agricultural sewage sludge, petroleum refining, chemical and synthetic industries, textile processing source (Gupta and Ahmad, 2012; Pi et al., 2018).Therefore, farm and grazing animals are exposed to POPs throught feed, water and soil contamination.

Many of these compounds can interfere with the endocrine system in human and animals, causing toxic effects and thereby are called "endocrine disruptors". Their toxicity regards e.g. reproductive system dysfunction, suppression of the immune system, damage to the thyroid, cancer and several other effects (Chen et al., 2019). They can behave like the hormones or influence the hormone levels of the organism and thus have effects on human health and on organisms present in the environment, especially in the critical stages of development. Perhaps the most familiar and well-characterised example of such an interaction is binding of a substance to a hormone receptor, e.g. the oestrogen receptor (ER). Such substances may exhibit agonist or antagonist activity in relation to the receptor, depending on the nature of its interaction with the ligand-binding site of the receptor but there are several ways with these substances can interact with the organism. In March 2013 EFSA asked to scientific community to collect information to provide the risk linked with these substances and in May 2018, EFSA elaborated an opinion on the hazard of endocrine disruptors (EFSA, 2013) that, according to World Health Organization (WHO) International Programme on Chemical Safety (IPCS) (WHO/IPCS), are so defined: "*An endocrine distruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations*" (WHO,2002).

1.3 Polychlorinated Biphenyls

Polychlorinated biphenyls (PCB) are synthesised by catalysed chlorination of biphenyl. Depending on the number of chlorine atoms (1-10) and their position at the two rings, 209 different compounds, PCB congeners, are possible (Figure 2).

Figure 2: Structural formula of PCBs.



PCBs production started in the 1920s and they were largely used world-wide in industry and commercial applications (repellents, carbonless copy paper, plasticizers in paints, sealants, plastics and rubber products etc), due to their physical and chemical properties, such as non-flammability, chemical stability, high boiling point, low heat conductivity and high dielectric constants. They were identified as environmental contaminants at the end of the 1960s. It is estimated that more than 1 million tons of technical PCBs mixture were used in to the industry until the 1980s, when the manifacture, processing and distribution of PCB has been prohibited in almost all industrial countries (EFSA, 2005). However, they are still released in environment due to the incorrect discarding of waste, the illicit use and their application in old electrical equipment and hydraulic system.

Polychlorinated biphenyls can be divided into two groups according to their toxicological properties. One group, consisting of 12 congeners, shows toxicological properties like dioxins (effects on liver, thyroid, immune function, reproduction and behaviour), therefore named “dioxin-like PCB” (DL-PCB). They are able to bind to the AH receptor (Aryl Hydrocarbon Receptor) located in the cytoplasm, thus causing hepatotoxicity, in particular hyperplasia, vacuolization and increased triglyceride contents and enzyme activity, thyrotoxicity through the bound to the hormone receptor, affecting thyroid hormone status by inhibiting the binding of T4 to transthyretin which is an important transport protein for both T4 and T3 (Chauhan et al., 2000), immunodepression as well as alteration of reproduction and behaviour. Moreover, they can increase the transcription of genes that encode for different biotransformative enzymes such as cytochrome P450.

The second group, mentioned to as “non-dioxin-like PCB” (NDL-PCB), have a partially different toxicological profile, with effects on the developing nervous system and neurotransmitter function, infact they can interfere with intracellular sequestration of calcium and increased activation of protein kinase C (PKC), thereby altering intracellular signal transduction pathways (Kodavanti and

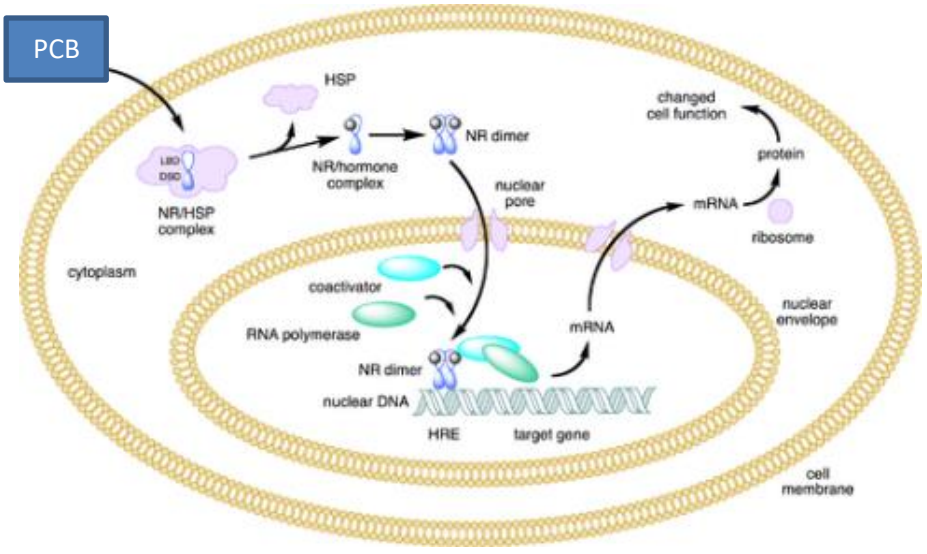
Tilson, 1997). Morover, NDL-PCBs can induce cellular apoptosis, increase in reactive oxygen species, and alterations of the levels of dopamine and acetylcholine. NDL-PCBs may also interfere with the binding of testosterone with the androgen receptor (Schrader and Cooke, 2003). Furthermore, NDL-PCB can induce a UDP-glucuronosyltransferase which can enhance the elimination of T4 from the circulation via glucuronidation (Hood and Klaassen, 2000). PCBs are able to cause immunological effects, as morphological changes in organs related to the immune system, as well as functional impairment of humoral- and cell-mediated immune responses immune defects included decreases in thymic weight, reduced B cell numbers, reduced cytotoxic T-lymphocyte response, and reductions in plaque forming cell response and IgM. PCB 153 can produce modification on DNA bases. Infact Robertson and Gupta (2000) showed that metabolism of PCB, generates electrophilic metabolites and reactive oxygen species that can damage DNA.

However, it is difficult to characterize the different toxicological profiles of NDL-PCBs and DL-PCBs, which are always present together and in different proportions in mixtures, as the first ones have a very low toxicity compared to the second ones and this difference does not permit to describe an exact picture of the toxicity, in qualitative and quantitative terms, of NDL-PCBs (EFSA, 2018); however they were classified by IARC (1987, 2016) in Group 2A (probably carcinogenic to humans), based on limited evidence in humans and adequate in animals. No published peer reviewed data are available on the carcinogenic potency of single congeners and it is difficult to carachterize this non-dioxin like congener toxicity because mixtures contain both, as described above. Inhibition of cellular communication, inhibition of apoptosis together with induction of oxidative stress, are mechanisms which may be of relevance for PCB-related tumour promotion (Worner and Schrenk, 1996; Bohnenberger et al., 2001).

The kinetics behaviour of the PCB congeners in the organism is influenced by their lipophilic nature and the number of chlorine atoms. PCBs with highly chlorinated atoms have longest half lives and therefore the greatest accumulation (PCB 138, PCB 153, PCB 170, and PCB 180). They are usually adsorbed from gastrointestinal tract (Aoky, 2001), but also inhalations represent a source of exposure (Currado and Harrad, 1997; Alcock 1998). In humans, PCBs are well absorbed and subsequently distributed to liver and fat tissues, in which there is the major accumulation. The structure of PCBs and the chlorination degree defines the rates of PCB elimination that is *via* urine, faeces and milk. Breast milk represents a source of exposure for infants (EFSA, 2005).

The PCB-AhR binding causes the release of the inhibitory regulatory protein Hsp90 from AhR. At this point the complex constituted by PCB-AhR migrates in the nucleus, where it binds to the factor ARNT (Ah Receptor Nuclear Translocator) and induces the transcription of specific genes called DRE (Dioxin Responsive Elements), which are involved in the mechanisms of differentiation and cell division, in the metabolism of some hormones such as thyroid and some growth factors. The AhR receptor has an important role in the nervous, reproductive and immune system (Figure 3)

Figure 3: PCB-AhR binding causes the release of the inhibitory regulatory protein.



NDL-PCBs can alterate the calcium (Ca^{++}) homeostasis and induce cellular apoptosis and thyroid hormone (TH) homeostasis (Th levels and transport). They are similar in agonist interaction with the hepatic nuclear receptors. Morover, they can alterate reproductive system (Dingemans et al., 2016).

To easily describe the PCB presence in a food matrix, EFSA selected six individual congeners, called “indicator-PCBs” (PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB153) as they represent about 50% of the total PCBs in mixtures (EFSA, 2005). Clearly speaking, the analysis of a matrix can be previously made for the presence of NDL-PCBs, that, for their abundancy are more easily detectable than DL-PCBs. Their eventual presence in a food matrix is therefore the indicator of a presence of the DL-PCBs that, due to the high toxicity, requires at this stage a specific analysis. The Control Organs simultaneously carry out the analysis of NDL-PCBs, DL-PCBs and dioxins,

because European regulations stated Maximum Levels (MLs) in food for all these categories of substances (European Commission 1881/2006; European Commission 1259/2011).

Occurrence and exposure assessment

On the request made by the European Union, in accordance with Regulation (EC) No. 178/2002 (European Commission, 178/2002), EFSA elaborated a scientific opinion concerning the risk for animal and human health related to the presence of dioxins and DL-PCBs in feed and food. So, in 1997 started a program to monitor the occurrence of dioxins and PCBs in food and in environment. In almost all cases, the determinations of PCBs were limited to the indicator PCBs.
Based on toxicity studies and available datas found in literature, EFSA 2018 reported that the main critical effect was on semen quality, following pre- and postnatal exposure. Therefore, it was estimated that daily exposure in adolescents and adults should be below 0.25 pg TEQ/kg bw/day. The CONTAM Panel established a Tolerable weekly intake (TWI) of 2 pg TEQ/kg bw/week.

1.4. Organoclhorine and organophoshorate insecticides

Insecticides (figure 4, figure 5) are a wide group of subtances, very different each other in their chemical structure and physical charachteristic.

Figure 4: Structural formula of DDT (Organoclhorine compounds)

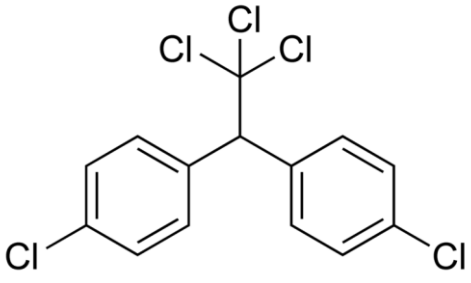
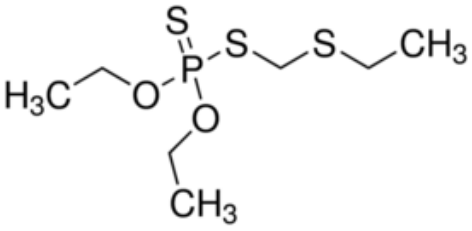


Figure 5: Structural formula of phorate (organophoshorate compounds)



They were introduced into the agriculture in the first half of the last century to destroy or control any harmful organism (including micro-organisms and weeds), or to prevent their damage during production, processing, storage, transport and marketing of crops and food (for humans and animals). They include different classes depending on their structure and target, but, in this manuscript, we focus on two classes of insecticides: organochlorine and organophosphorus compounds (OPs). Organochlorine (OC) (figure 5) are an heteregoneous group of compounds that belong to three chemical classes with structures related to dichlorodiphenylethane (DDT), to cyclohexane, the most important compound being hexachlorocyclohexane (HCH), a mixture of various isomers (α, β, lindane) and the cyclodiens (aldrin, dieldrin, endrin). The most well-known and widely used compound was DDT, which is a mixture of various isomers (e.g. pp'-DDT; op'-DDT, etc), still present in environment. Organochlorines are compounds with low volatility, lipophilic and high chemical stability as well as slow biodegradation rate that makes them persistent in the environment. Morover, by evaporation they can pass into the atmosphere and reach long distance from the place of release, as well as through soil erosion they can spread into the aquatic environment contaminating it (Naso et al., 2005; Muccio et al., 2002; Binelli et al., 2004).

Organochlorine insecticides were widley used from the fourties and the seventies of the last century, when their use was prohibited, and they were replaced by organophosphorus compounds and carbamates. Unfortunately, the restrictions and bans have not prevented the occurrence of pollution events as for example in 1996 in Piedmont, where there was a serious environmental pollution from DDT due to the accidental discharge of waste in the river Toce, reported also by national press (La Repubblica, 1996). Morover in 2006, the World Health Organization and the U.S. Agency for International Development endorsed indoor DDT spraying to control malaria, and therefore DDT continues to be used for malaria control in several African and Asian countries (Sougoufara et al., 2017).

The toxic action of insecticides should be directed exclusively towards the target species, but most of these compounds do not have a high specificity, so toxicity can occur in many living beings (Nebbia, 2009). Morover, as for non-insecticide organochlorine, they are often a mixture of congeners of the same group. So, it is hard to distinguish the specific health effects. However, it is possible to confirm that the main toxicological effect of OCs is on nervous system. It has been observed that long OCs (DDT) exposure at high doses causes human ataxia, paraestesia, dizziness, nausea, vomiting, tremors and lethargy. They can cause a decrease in rest potential with a consequent increase in nervus excitability; inhibit ATP-ases and inhibit the release of neuotransmitters. Many organochlorines are endocrine disruptors and they are associated with reproductive effects, embriotoxicity and immunological problem (Longnecker et al., 1997).

In 1991, the International Agency for Research on Cancer (IARC) rated OCs and DDT as “possibly carcinogenic to humans (Group 2B)” (IARC 1991).

This rating was largely based on the induction of liver tumors in experimental animal studies that reported significant increases in hepatomas (neoplastic liver cell tumors) and researcher observed an association between OCs and tumors like breast cancer, non-Hodgkin's lymphoma, pancreatic cancer or leukemia and Hodgkin's disease but the datas are still inconclusive (Eskenazi et al., 2009) (Longnecker et al., 1997).

The organochlorines, infact, are quickley absorbed into the organism and after binding to serum lipoproteins, are transported to lipid-rich tissues such as liver, kidney and nerve tissue. In the body, they are slowly biotransformed and in humans the half-life can be up to 3 years for DDT.

The organophosphorus (figure 6) and carbamate insecticides are represented by a wide variety of chemical structures having different chemical and physical properties. The group includes esters of phosphoric, phosphonic, phosphamidic, phosphorotionic, and other phosphorous-based acids. They are acetylcholinesterase inhibitors (AChE) that is a neurotrasmettitor involved in the transmission of nerve impulses to effector cells at cholinergic, synaptic, and neuromuscular junctions.

Their acute toxicity is caused by the inhibition of acetylcholinesterase in the synaptic space of both muscaric and nicotinic receptors in the parasympatic system, and in the neromusclar junction and have a role also in the central nervous system: muscarinic effects appear firstly and include: myosis, sweating, scialorrhea, increase in peristalsis, abdominal pain, vomiting and diarrhea, bronchoconstriction and increase in bronchial secretions; nicotinic effects are tachycardia, muscle fasciculations, tremors and muscle paralysis; central effects are restlessness, excitement, asthenia,

respiratory center depression, convulsions. Exitus can be lethal and occurs for respiratory failure due to bronchoconstriction and hypersecretion (muscarinic), paralysis of respiratory muscles (nicotinic), and depression of respiratory centers (central). In several epidemiological studies, the association between OP exposure and neurobehavioral effects and delayed neurologic diseases such as Parkinson's disease has been observed (Colosio et al., 2003), (Manthripragada et al., 2010), (Rohlman et al., 2011). In addition, OPs may contribute to birth deficits, childhood brain tumors, leukemia and lymphomas and may also act as liver and respiratory system toxicants (Koureas et al., 2012).

Acetylcholinesterase inhibitors are not persistent in environments and do not cause significant bioaccumulation phenomena. They may persist for a few hours or weeks depending on their specific and soil characteristics. This results in minimal contamination of water resources and soils.

Regulatory status

Pesticide residues resulting from the use of plant protection products on crops or food products that are used for food or feed production may pose a risk factor for public health. For this reason, European Commission defines rules for the active substances used in agriculture. To ensure the consumer protection, EC defined the Maximum Levels (MLs) that are the highest concentration of a pesticide tolerated in food or feed when pesticides are applied correctly (Good Agricultural Practice). They are established with Regulation (EC) No 396/2005 (EC, 2005). European Commission set Maximum Residues Levels (MRLs) for more than 500 pesticides in over 370 food products and a default MRL of 0.01 mg kg^{-1}, a level equal to the limit of quantification (LOQ) achievable with analytical methods, is applicable for pesticides not explicitly mentioned in the ML legislation. Regulation (EC) No 396/2005 imposes on Member States the obligation to carry out controls to ensure that food on the market is compliant with the legal limits.

Occurrence and exposure

About organochlorine insecticides, being POPs and therefore banned in developed Countries, the risk for biota comes from the environmental pollution occurred in the last century.

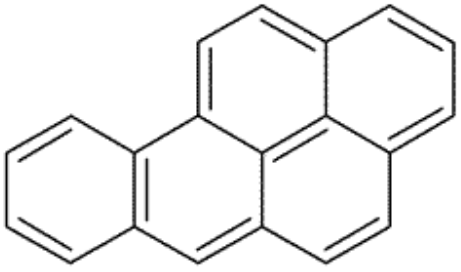
The major problem, for acetylcholinesterase inihibitors, regards the misuse of insectidices for plant protection, for wich agriculture ministry fixed planned checks on fruit and vegetables to avoid consumer risk. Studies conducted from 1985 until now, showed the high occurrence of diazinion,

demeton, chlorpyrifos, phorate that are among the most employed and for wich, EC defined a directive on the consumer risk (European Commission 1107/2009). Lastly, even non-food crops, like floriculture, indirectly contribute to the exposure of animals and humans to organophosphorous insecticides, which through the soil and therefore water can be transferred to them.

1.5. Polycyclic aromatic hydrocarbon

Polycyclic aromatic hydrocarbons (PAHs) constitute a wide class of chemical compounds. The structure of these compounds is the presence of two or more benzene rings joined together (Figure 6).

Figure 6: Chemical structure of benzopyrene.



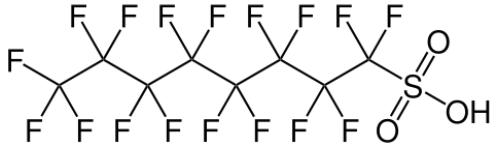
The term PAH identifies compounds containing only carbon and hydrogen atoms and their chemical properties are different on the base on the number of rings and molecular wheight. PAHs are stable compounds, although after photodecomposition in the presence of air and light give rise to many oxidation products, especially quinones and endoperoxides. They are produced from combustion processes, industrial processes like production of alluminum, iron, and steel, electricity generation systems, petroleum products, pyrolysis of organic material containing carbon, such as coal, wood, waste, forest fires, tobacco smoke, and volcanoes which can be a natural source with an impact on the environment. Due to the several sources, they are ubiquitous compounds. PAHs are always present in complex mixtures of different compounds and so they are found in different environment compartments (air, water, soil.). Due to their structure, the major route of exposure is

air, infact their presence as air pollutants represents an important health problem because many of them have proved to be carcinogens in laboratory animals. PAHs can contaminate food through environment and during production process. In unprocessed foods, the presence of PAHs is essentially due to environmental contamination: deposition of particulate matter atmospheric (e.g. on wheat, fruit and vegetables), absorption from contaminated soil (e.g. potatoes), uptake by contaminated river and sea water (e.g. shellfish, fish and crustaceans). Common sources of PAHs in processed food are heat treatments (grilling, baking and frying) and some manufacturing processes as drying through the combustion fumes (e.g. in the case of vegetable oils) and the food smoking processes with the traditional methods. Regards their toxicological profile, their cancerogenity properties is correlated to their chemical structure, infact they must have at least four condensed rings. The condensation of the rings decreases, in fact, their aromaticity and makes metabolic reactions of epoxydation easier with formation of compounds with carcinogenicity properties. It must be underlining that dihydrodiolepoxides of PAHs are the actual carcinogenetic agents (EFSA, 2008a).

2.1. Emerging contaminants

2.1.1 Perfluoroalkyl substances

Figure 7 Structural formula of perfluorooctane sulfonate as an example of perfluoroalkyl susbtances



Perfluoroalkyl substances (PFAS) are a group of fluorinated compounds, with high thermal and chemical inertness due to their structure which consist of an anionic site bound to a lipophylic chain: they therefore are generally hydrophobic but also lipophobic and consequently do not bioaccumulate in fatty tissues differently from other persistent halogenated compounds (Figure 7).

Perfluoroalkyloctan sulphonic acid (PFOS) and perfluoroctanoic acid (PFOA), the two usually investigated PFAS, and other "perfluorinated compounds have been widely used in industrial and consumer applications including stain- and water-resistant coatings for fabrics and carpets, oil-resistant coatings for paper products approved for food contact, fire-fighting foams, mining and oil well surfactants, floor polishes, and insecticide formulations" (EFSA 2008b). Because of their extensive use they are widely found in the environment and, although their production dates to the the fifties, they are commonly considered emerging contaminants. In fact, in the last years, the threshod doses have been reviewed regarding the "end points" subsequently considered.

In 2008 EFSA CONTAM Panel established a Tolerable daily intake (TDI) for PFOS of 150 ng/kg b.w. derived from a subchronic study in Cynomolgus monkeys.

In subacute and chronic studies toxicity liver developmental toxicity was observed. Other sensitive effects were changes in thyroid hormones and high-density lipoprotein (HDL) levels in rats and monkeys. PFOS moreover induced liver tumours in rats, probably due to a non-genotoxic mode of action. For PFOA, the CONTAM Panel established a TDI of 1.5 μg/kg b.w.

However, the epidemiological studies on human, made on professional exposure, were ambiguous and often did not agree with animal studies.

In the years 2005-2013, the joint "C8 Science Panel" between Du Pont (E.I. du Pont de Nemours and Company) and plaintiffs regarding releases of C8 from the Washington Works plant in Parkersburg, West Virginia carried out exposure and health studies in the Mid-Ohio Valley communities, which had been potentially affected by the releases of PFOA (or C8) emitted since the 1950s. Many articles have been published and for six disease categories, the Science Panel concluded that there was a Probable Link to C8 exposure: diagnosed high cholesterol, ulcerative colitis, thyroid disease, testicular cancer, kidney cancer, and pregnancy-induced hypertension.

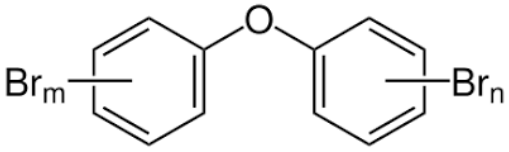
Based on the collected data, in 2016 the US Agency for the register of toxic substances and diseases (ATSDR) indicated guide values of 30 (PFOS) and 20 (PFOA) ng kg^{-1} b.w./day as 'Reference Dose' (RFD), for the whole population included most vulnerable groups. These values were supported by experimental data with fetal developmental toxicity in laboratory animals exposed during pregnancy (preterm and underweight births, ossification defects) and with a reduced seroconversion in following vaccination.

In 2016, the Netherlands National Institute for Public Health and the Environment extrapolated, from liver toxicity observed in studies in laboratory animals, a PFOA TDI of 12.5 ng kg^{-1} b. w. /

day. In 2018, EFSA elaborated a new opinion, on European Commission request, on the risk to human health related to the presence of PFOS and PFOA in food. For PFOS, the increase in serum total cholesterol in adults, and the decrease in antibody response at vaccination in children were identified as the critical effects. For PFOA, the increase in serum total cholesterol was the critical effect. CONTAM Panel established a tolerable weekly intake (TWI) of 13 ng kg^{-1} body weight (b.w.) per week for PFOS and 6 ng kg^{-1} b.w. per week for PFOA. For both compounds, exposure of a considerable proportion of the population exceeds the proposed TWIs (EFSA 2018).

2.1.2 Polybrominated diphenyl ethers

Figure 8: Polybrominated diphenyl ethers



Polybrominated Diphenyl Ethers (PBDEs) are a class of brominated hydrocarbons with a basic structure consisting of two phenyl rings linked by an oxygen atom. There are 209 possible compounds, commonly referred to as PBDE congeners, which differ in the number and position of the bromine atoms in the two phenyl rings (Figure 8). They are commonly named as Brominated flame retardants; in fact, the weak carbon-bromine bond is thermally-labile, and the fire heat releases bromine radicals that intercept carbon radicals to decrease flame, so reducing heat and carbon monoxide production (EFSA 2011).

Since the 1960s PBDEs were used as additives to retard fire and flames in several commercial and household products. The major commercial PBDE mixtures were penta-, octa-, and deca-bromodiphenyl ethers, related to the bromine atoms present in the molecule. DecaBDE's main use was for electronic enclosures, such as television cabinets. OctaBDE was largely used in plastics for business equipment. PentaBDE was principally used in foam for cushioning in fabri (Hooper K, McDonald, 2000; ATSDR 2017).

Exposition to PBDEs occurs from food, air, water or soil. The higher-brominated PBDEs, that are, analogously to PCBs, the less toxic, generally are taken through the respiratory system, mainly in

the form of house dust while the lower-brominated congeners penta- and tetra- PBDEs exposure is mainly oral, i.e. through food. Toxicity of PBDEs is also affected by their kinetics: decaBDE has an apparent half-time of 15 days, while lower-brominated PBDEs have apparent half-times as high as 94 days. Thus, lower brominated PBDEs accumulate and persist in body fat o a very higher degree than decaPBDE also accumulates in body fat, but to a lesser degree (ATSDR 2017; EFSA 2011).

Litlle is known about the health effects of PBDEs in human, as most of the information regards their toxicity in studies on laboratory animals even if recent studies have evaluated associated PBDE concentrations in human tissues like blood or breast milk and health effects. Neurobehavioral changes and damage to the reproductive systems of adult rats and mice orally exposed to small amounts of lower-brominated PBDEs during early development was observed. Altered neurobehavior was also observed in rats and mice that ingested higher doses of decaBDE during early development. Subchronic studies on adult rats and mice demonstrated thyroid and liver effects for ingestion o moderate doses of lower-brominated PBDEs. Some PBDEs might impair the immune system. Evidence from human studies suggest a possible association between PBDE exposure and altered neurodevelopment. DecaBDE chronic assumption evoked toxic effects in the pancreas (diabetes), nervous system, immune system, and reproductive system (ATSDR 2017). Epidemiological studies on humans did not demostate association between PBDEs and an augmented risk ofnon-Hodgkin lymphoma, pancreatic testicular and breast cancer (EFSA 2011).

The International Agency for Research on Cancer (IARC) classifies PBDE as a Group 3 carcinogen (not classifiable as to its carcinogenicity to humans) based on inadequate evidence of carcinogenicity in humans and inadequate or limited evidence in experimental animals (IARC 2018a).

To protect health and the environment, EU has adopted legislation aimed at reducing or ending the sale and use of some BFRs. Directive 2003/11/EC (European Commission 2003) of the European Parliament and of the Council Council Directive 76/769/EEC (European Commission 1976) relating to restrictions on the marketing and use of certain dangerous substances and preparations prohibits the sale of two commercial mixtures of PBDEs, known as pentaBDE and octaBDE, in concentrations above 0.1% by mass.

From July 2006, in accordance with Directive 2002/95/EC (European Commission 2002) of the European Parliament and of the Council on the restriction of the use of certain hazardous substances in electrical and electronic equipment, all electrical and electronic equipment can no longer contain

PBDE, in any concentration. In July 2008 also a third PBDE mixture, the decaBDE, which had originally been exempted from the restrictions, was banned by the European Court of Justice (EFSA, 2008).

The critical endpoint identified by EFSA Contam Panel is the effect on neurodevelopment. The Panel provided the related benchmark doses (BMDs), based on animal experiments, and their corresponding lower 95 % confidence limit for a benchmark response of 10 %, $BMDL_{10s}$, for some PBDE congeners: PBDE-47 (309 μg/kg b.w.); PBDE-99 (12 μg/kg b.w.); PBDE-153 (83 μg/kg b.w.) and PBDE-209 (1,700 μg/kg b.w). However, the kinetics of PBDE congeners in animals and humans differ considerably and cause severe uncertainties, therefore it was considered inappropriate to establish health-based guidance values. The Panel used a margin of exposure (MOE) approach for the health risk assessment using the estimated body burden in animals as starting point (EFSA, 2011). The risk charachterization on PBDEs exposure with food in the following presented papers is similarly approached.

3.1 Metals and metalloids

Metals and metalloids are ubiquitous and non-biodegradable environmental chemicals.

Due to anthropogenic activities, their concentration in the environment is generally increasing and the biomagnification in the food chain is a major issue (Wu et al., 2016; Alloway B.J. 2013).

The presence of these chemicals in the environment and in food can cause, on exposed humans and animals, several toxic effects such as cancers, birth and immune system defects, mental retardation, behavioral abnormalities, immunotoxicity, low fertility, altered sex hormone balance, altered metabolism and specific organ dysfunctions (Wu et al., 2016). It is now widely aknowledged that environmental contaminats toxicity must be considered not only as single chemicals but as mixtures (Wang and Fowler, 2008). The cumulative toxicity of metals and metalloids, below described and then considered in the works presented in Chapter 3, is therefore always accounted.

3.1.2 Arsenic

Arsenic is a metalloid with two oxidation states: As^{3+} up to ten times more toxic than As^{5+}. It is abundant in the soil and is present in almost all plant and animal tissues. A large amount of As, equal to an estimated quantity of 40000 tons, is introduced in the environment every year both for

rock erosion and anthropic activities, like electronic, varnish or heavy industry and carbon combustion. In water and soil, bacteria can oxidate, reduce methylate and demethylate As, that is found in food in its organic or inorganic form.

Organic arsenic species, particularly arsenobetaine, are the most common forms in seafood, while in foods of terrestrial origin the predominant arsenic forms are iAs, both As5+ and As3+, through grain-based processed products, and single methylated arsenic species (methylarsonate, methylarsenite and dimethylarsinate (DMA). Arsenic enters the food chain mainly through contaminated water and soil (Francesconi, 2010). Due to its fast toxicokinetics, organic arsenic has a very low toxicity. As^{3+} binds to sulphydril grup of enzymes of the tricarboxylic acid cycle and therefore tissues with high oxidative requirements such as intestinal epithelium, kidney, liver skin and lung are the most affected. As $^{5+}$ can substitute for phosphate, decoupling the oxidative phosphorylation in oxidative phosphorylation. Among products of animal origin, the most significant contribution is provided by the consumption of seafood, in which over 90% of As is in organic form, therefore not very toxic. In molluscs and shellfish, the accumulation is higher than other seafood. Currently no maximum limits have been set for As in food, however the relatively high concentrations of the metalloid in molluscs and crustaceans have contributed to raise the attention in the EU. In 2009, in fact, EFSA (EFSA 2009a) re-evaluated the provisional tolerable weekly intake (PTWI) of As of 15 mg kg^{-1} body weight, and based on epidemiological studies suggested a range, for a 0.1% increased incidence ($BMDL_{01}$) of skin lesions and cancer of the lung, skin and bladder, between 0.3 and 8 mg kg^{-1} b.w. day^{-1} (EFSA 2009a).

3.1.3 Cadmium

Cadmium contaminates the environment through natural occurrence and anthropic, both industrial and agricultural, activities. Cadmium is poorly absorbed by humans and animals after dietary exposure (less than 10%) but is concentrated in the kidney tubules and to a lesser degree in the liver, with a very long biological half-life ranging in the humans 10 to 30 years. Cadmium induces the synthesis of metallothionein, to which binds in many tissues and in the form of complex methallothionein-cadmium, accumulates in the kidney. In this organ, especially to the proximal tubular cells, after metabolism it is released in its free form and explicates its major action and is therefore nephrotoxic (Osweiler G.D.1996). Bone demineralisation, both through adirect effect or indirectly caused by renal dysfunction, is another important effect. If the exposure is chronic and at

high levels the tubular damage can worsen to renal failure. The International Agency for Research on Cancer (1993) have classified cadmium in Group 1 (human carcinogen) (IARC 1993). Epidemiological studies associate with cadmium the increase of risk of cancer to the lung, endometrium, bladder, and breast (EFSA 2009b).

3.1.4 Chromium

Chromium is a trace element found in nature in different states of oxidation; the most interesting forms under toxicological aspects are the trivalent form Cr (III) and the hexavalent Cr (VI) form. The second form is scarcely present in food, due to the reduction activty of most foods, and is reduced in gastrointestinal tract, but is largley persent in soil and water. The sources of Cr mainly occur in environment from industrial processes, use of fossil fuels, metal refining, textile and cement industries. In respect to other metals, Cr have an important role in glucose and lipid metabolism, and even if high concentrations are toxic for human health. Its mode of action is not well known.

In human hepatic, renal alterations and haemolytic anemia have been described due to chronic exposure to trivalent chromium; Cr (VI) is almost exclusively present in drinking water and is classified by the International Agency for Research on Cancer (IARC 2018b) as carcinogenic to humans (Group 1). EFSA suggested a tolerable daily intake (TDI) for Cr (III) of 300 mg kg^{-1} body weight (EFSA 2014).

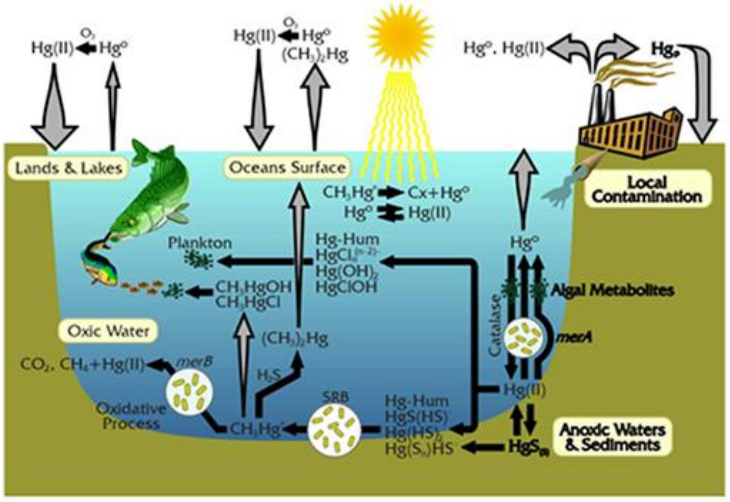
3.1.5 Mercury

Mercury is a metal immitted into the environment from both natural and anthropogenic sources (electrical installations, industry and chemical factory). Natural sources are degassing of the earth's crust, emissions from volcanoes and evaporation from water. Once released, mercury undergoes different transformations in atmosphere, ocean and land (figure 9). The three chemical forms of mercury are elemental mercury (Hg^0), inorganic mercury and organic mercury. The most frequent form of organic mercury in food chain is methylmercury. The critical target for toxicity of inorganic mercury is the kidney. Other targets include liver, nervous system, and immune system, reproductive and developmental systems.

The EFSA Contam Panel established a tolerable weekly intake (TWI) for methylmercury of 1.3 µg/kg b.w., expressed as mercury (EFSA 2012). Unborn children are the most vulnerable group for

developmental effects of methylmercury exposure, and pregnant women can be present in the group of high and frequent fish consumers. The most important critical effect of Hg are nervous system dysfunctions like tremors, irritability, memory problems, changes in vision and hearing. EU has set maximum levels (MLs) for Hg (EC 1881/2006) in foods.

Figure 9: Mercury cycle

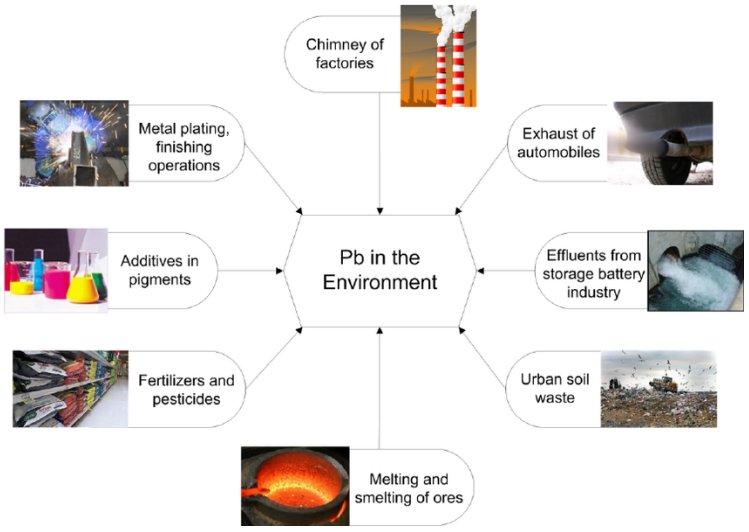


Sources: Mason, Robert P, W.F. Fitzgerald, and François MM Morel. 1994 .François M. M. Morel Albert G. Blanke, Professor of Geosciences Department of Geosciences, Princeton University.https://morel.princeton.edu/research/mercury-cycling-and-methylation.

3.1.6 Lead

Lead is present in the earth's crust and enters in the composition of over 200 minerals that are naturally present in the soil (figure 10). Its concentration varies greatly depending on areas, whether they are urban or rural, due to some human activities such as foundries, pigments for paints, metalmeccahinche industries, and production of ammunitions and glazes for ceramics (Nebbia, 2008). The water systems favour its diffusion in the soil where it tends to be located mostly at the level of the roots of the plants. Aquatic and terrestrial fauna has the same ability to bioconcentrate lead at different levels and, among the terrestrial animals, the most exposed are herbivores due to their grazing behaviours. Human exposure is mainly via food and water, as well as air, dust and soil. Cereal and cereal products are the products responsible of most of the dietary lead exposure. In the organism lead tend to bioaccumulate in bones and children are more exposed than adults, for wich house dust and soil can be an important source of exposure (EFSA 2010).

Figure 10: Lead comtamination



Sources: Lead sources in the environment (adapted from Sharma and Dubey 2005)

The critical effects of lead are developmental neurotoxicity in young children and cardiovascular effects and nephrotoxicity in adult. In fact, the 95th percentile lower confidence limit of the benchmark dose (BMD) of 1 % extra risk ($BMDL_{01}$) for developmental neurotoxicity is 0.50 µg/kg b.w. per day, for systolic blood pressure (SBP) is 1.50 µg/kg b.w. per day, while, accounting for an extra risk of 10% the BMDL for chronic kidney disease (CKD) is 0.63 µg/kg b.w. per day. Based on these data EFSA considers no longer appropriate the current PTWI of 25 µg/kg b.w. The International Agency for Research on Cancer classified inorganic lead as probably carcinogenic to humans (Group 2A) in 2006. Maximum levels (MLs) for Pb in food are set in the Commission Regulation (EC) No 1881/2006 (EFSA, 2010).

3.1.7 Nickel

Ni is a widespread component of Earth's surface. Its presence in food chain and drinking water is determined by both natural and anthropogenic factors. There are no MLs for Ni in food. A tolerable daily intake of 2.8 µg Ni/kg body weight (b.w.) per day was choosen as health-based exposure level. The critical effects reported after Ni exposures are respectively dermatitis (SDC) in Ni sensitive individuals and reproductive and developmental toxicity after chronic exposure.

Ni and Ni compounds have been classified by IARC (2012) as human carcinogens causing cancers of the lung, nasal cavity and paranasal sinuses after inhalation. Moreover, it is reported that individuals sensitised to nickel, after dermal contanct or oral ingestion, may develop eczematous flare-up reactions in the skin (systemic contact dermatitis, SCD (EFSA, 2015).

4.1. Antibiotics

Antimicrobials, like antibiotics, are substances able to kill micro-organisms or to hamper their growth and proliferation. The term "antibiotic" was invented by Selman Waksman, who discovered the antibiotic streptomycin, but the era of antibiotics began in the 1940s, with the introduction of penicillin that has been recognised as one of the greatest advances in therapeutic medicine, discovered by Alexander Fleming, Professor of Bacteriology at St. Mary's Hospital in London, in 1928. Antibiotics are compounds produced by bacteria and fungi which can kill, or inhibiting, competing microbial species. In 1945, Fleming, Florey and Chain were awarded the Nobel Prize for "the discovery of penicillin and its curative effect in various infectious diseases" (the Nobel Prize in Physiology or Medicine, 1945).

Unfortunatley, the influence of antibiotics is now declining due to the progressive rise of resistance, and this phenomenon is observed among for all antimicrobial drugs. The wide use and misuse of antibiotics, infact, were the causes of the growth and spread of micro-organisms that are resistant to their action, resulting in loss of treatment efficacy and serious risks to public health (Lobanovska et al., 2017). In literature there are increase reports of bacterial species which are resistant to all know antibiotics and a well-known example of a bacterium that has developed the ability to resist multiple antibiotics is Meticillin-resistant Staphylococcus aureus (MRSA). Resistant bacteria can spread through many pathways. When antimicrobial resistance occurs in zoonotic bacteria in animals and food, it can also compromise the effectiveness of the treatment of infectious diseases in humans. In the field of food safety, policy makers must protect consumers from risks related to the food chain and implement the best control measures to reduce those risks. Data released today by the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) reveal that antimicrobials used to treat diseases that can be transmitted between animals and humans, such as campylobacteriosis and salmonellosis, are losing their effectiveness. Vytenis Andriukaitis, European Commissioner for Health and Food Safety, said: *"The report*

released today should ring – again – alarm bells. It shows that we are entering a world where more and more common infections become difficult – or even sometimes impossible – to treat. Let's make sure that we increasingly act all together, in every country and across the public health, animal health and environment sectors under the One Health approach." The joint report, which presents the data collected from 28 EU Member States from humans, pigs and calves under one year of age, confirms the rise in antibiotic resistance already identified in previous years. The EU has strictly regulated controls on the use of antibiotics and other veterinary drugs, particularly in food animal species, by issuing several Regulations and Directives. Council Regulation 2377/90/EEC (EC 2377/90), established maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin and Council Directive 96/23/EC (EC 96/23) contains guidelines for controlling veterinary drug residues in animals and their products with detailed procedures for EU Member States to set up national monitoring plans, including details on sampling procedures. In the specific case, the Criteria to define the performance of analytical methods and the interpretation of results have been established in the Commission Decision 2002/657/CE (CE 2002/657).

Antimicrobial Resistance (AMR) is a global health security threat that requires intensive cross-sectional action by governments and society. Surveillance that generates reliable data is the essential basis of sound global strategies and public health actions to contain AMR and is urgently needed around the world. In June 2017, the European Commission adopted a unified EU Health Action Plan against Antimicrobial Resistance, calling for effective action and recognising that it must be addressed in terms of both human health and animal health and the environment. The prudent use of antimicrobials is essential to limit the emergence and spread of antibiotic-resistant bacteria in humans and animals.

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CHAPTER 2

Aims

The increasing globalization, begun in the last century, has been associated with a growing release of chemical pollutants, as widley described above, whose toxicological effects are often not fully defined. The European Union and European Safe Authority defined safe levels and recommended to monitor their occurrence in food asking to collect more information in order to safeguard human health against their toxicological effects. On the light of the different topics discuss in the introduction, the specific aim of this research study was to focus the attention on the presence of categories of chemical compounds considered of emerging interest for public health (e.g. environmental pollutants like, PBDEs, PAHs, perfluoroalkyl substances (PFASs) and categories with better-known toxicity (e.g. metals, PCBs, dioxins) in most consumed foods (fish and meat) or in niche foods (meat from game animals) in order to improve information in literature and characterize the risk.

In this view the estimate of human exposure and the improvement of information regarding the occurrence of chemical compounds in environment and consequently in food were done.

CHAPTER 3

Research papers

All papers were reported keeping the reference style indicated by the guidelines of each Journal.

3.1. Mussels and clams from the italian fish market is there a human Exposition risk to metals and arsenic?

Published in: Chemosphere. Volume 194, 2018, Pages 644-649

L.M. Chiesa [a], **F. Ceriani** [a], M. Caligara [b], D. Di Candia [c], R. Malandra [d], S. Panseri [a, *], F. Arioli [a]

[a] *Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria, 10, 20133 Milan, Italy*

[b] *Department of Biomedical Sciences for Health, University of Milan, Via L. Mangiagalli, 37, 20133 Milan, Italy*

[c] *Department of Biomedical, Surgical and Dental Sciences, University of Milan, Via L. Mangiagalli, 37, 20133, Milan, Italy.*

Keywords: Arsenic, ICP-MS, Mussels, Clams, Health risk.

ABSTRACT:

Seafood is associated with many beneficial effects on human health. However, the overall level of con- taminants in biota has increased over the last two centuries and seafood is one of the sources of oral exposition to contaminants. Therefore, this work aimed to evaluate cadmium, lead, mercury, arsenic, chromium and nickel presence in mussels and clams, from the Italian market, and the associated risk. The samples were from five different FAO areas. Analyses were carried out using inductively-coupled plasms-mass spectrometry. The sample concentrations were below the maximum levels stated by Commission Regulation (EC) 1881/2006, except one mussel sample, which was non-compliant for cad- mium (2.13 ± 0.20 mg kg^{-1}). For arsenic, nickel and chromium, maximum levels are not stated by the European Union. In this study, arsenic ranged from 1.29 to 13.35 mg kg^{-1} and nickel ranged from <LOQ 3.98 mg kg^{-1}, except one sample, whose nickel concentration was 21.70 mg kg-1. Chromium was found only in 15 samples, with a maximum concentration of 2.81 ± 0.27 mg kg^{-1}, in one clam sample. Our results indicate that the average Italian consumption of molluscs, does not pose a risk for consumers, except nickel, which can cause allergic dermatitis in nickel-sensitive individuals. However a particular concern is caused by the exposition to As of the 95th percentile consumers: the Hazard Index for skin lesions, was >1, and

BMDL10 for lung bladder and skin cancer in all mussel samples was overcome, in the 100% and 25% of mussel and clam samples, respectively.

1. Introduction

Seafood is an excellent source of nutrients including proteins, vitamins (A, D and B12), minerals and fatty acids, with important human health benefits. Epidemiological and clinical evidence suggests that consumption of high levels of n-3 fatty acids eicosa- pentaenoic acid and docosahexaenoic acid, from one or two fish- based meals per week, induces protective effects against coronary heart disease, with a 36% decrease in the risk of coronary death and 17% decrease in total mortality. Seafood is also associated with beneficial effects on neurologic development in toddlers and chil- dren, such as improved visual acuity, raised mental processing scores and language comprehension (Mozaffarian and Rimm, 2006; EFSA, 2014a). However, over the last two centuries, the level of contaminants, heavy metals and chemical compounds, has increased in the environment and, subsequently, in biota, due to anthropogenic activity, such as industrial activities, mining and agriculture. Some metals, such as lead (Pb) and mercury (Hg) have no biological roles (Sarmiento et al., 2011) and their presence needs to be monitored to prevent and minimise the potential health risks associated with seafood consumption. In many instances, animals near the top of the food chain, such as carnivorous fish species, are most affected by biomagnification of xenobiotics, building up greater and or more dangerous amounts of toxic materials than animals lower down the food chain. Herbivorous species, like mussels and clams, are filter-feeders, bioaccumulating a wide range of environmental pollutants in their tissues and, thus, can be used as suitable bioindicator organisms to monitor trace metal pollution in marine environments (Langston and Spence, 1995). In the Eu- ropean Union (EU), the breeding of bivalves provides the laying of juvenile bivalves in the open sea and their permanence for about 2 years until adult stage (Baylon, 1990). After collection, the animals are microbiologically controlled, before their marketing. If they do not meet the microbiological criteria after collecting, they must stay in a depuration place, provided with periodically replaced clean water, in order to reduce the microbial load (EU, 2004). In this context, it could be supposed that they can release several chem- icals, comprising metals.

One fundamental feature of metals is their ability to persist in the environment (Scientific Committee of Problems of the Environment (SCOPE, 1987). Consequently, they can be taken up by

marine organisms due to their presence in water and sediments and they can be biomagnified in superior trophic animals including humans (Kim and Lee, 2010). The metal contamination of seafood is a globally recognised public health risk (Lozano et al., 2010; Squadrone et al., 2016). Long-term exposure and/or high concen- trations of metals, can cause adverse effects on human health, such as skin diseases, acute and chronic intoxication, nervous system, blood and gastrointestinal dysfunctions, respiratory problems, as well as mutagenic and cancerogenic effects (Martin and Griswold, 2009).

In particular, Pb can cause severe brain and kidney damage, and in pregnant women, may cause miscarriage. Hg can cause nervous system dysfunctions like tremors, irritability, memory problems, changes in vision and hearing. Cadmium (Cd) can cause kidney diseases and respiratory problems. Inorganic arsenic (As) has been linked to cancer of the skin, lungs and bladder. Chromium (Cr) can cause breathing problems, cough, asthma, allergic reactions and chronic exposure could cause liver and kidney cancer, particularly linked to Cr (VI) (Martin and Griswold, 2009). Nickel (Ni) could cause allergic reactions and long-term exposure could result in reproductive diseases. Moreover, it is genotoxic, with cancerogenic, immunotoxic, hepatotoxic, neurotoxic, and nephrotoxic effects only after inhalation (EFSA, 2015; Das et al., 2008). The EC has set maximum levels (MLs) for Cd (1 mg kg^{-1}) and Pb (1.5 mg kg^{-1}) in bivalve molluscs, and for Hg (0.5 mg kg^{-1}) in seafood (EU, 2006). No MLs have yet been established for As, Ni, and Cr by the EU. In 2009, however, the European Food Safety Agency (EFSA, 2009a) concluded that the provisional tolerable weekly intake (PTWI) of As at 15 mg kg^{-1} body weight, recommended by the Joint Food and Agricultural Organisation/World Health Orga- nisation (FAO/WHO) Expert Committee on Food Additives (JECFA) was no more applicable. Instead, based on epidemiological studies on exposure to As through water and food, the EFSA suggested a range, rather than a single reference point that included the benchmark dose lower confidence limit for a 0.1% increased inci- dence (BMDL01) of skin lesions and cancer of the lung, skin and bladder, between 0.3 and 8 mg kg^{-1}body weight day^{-1} (EFSA, 2009a,b).

Although Cr has no defined MRLs, in 2014, the EFSA suggested a tolerable daily intake (TDI) for Cr(III) of 300 mg kg^{-1}body weight, which was based on the lack of adverse effects and carcinogenicity in mice and rats, and for the inadequate information about repro- ductive and developmental toxicity. Cr(VI) is classified by the In- ternational Agency for Research on Cancer

(IARC) as carcinogenic to humans (Group 1). However, it is scarcely present in food, which is mostly a reductive medium and is largely reduced in the gastro-
intestinal tract. Therefore, the EFSA concluded that the oral expo- sure to Cr(VI), for the European population is of low concern for health of all age groups but of potential concern only for high consumers of drinking or bottled water, for the oxidising agents present, in the groups “Infants”, “Toddlers” and “Other children” (EFSA , 2014b). In 2015, the EFSA set a TDI for Ni at 2.8 mg kg-1 body weight, obtained from studies on the dose response curve of the incidence of litters with a post-implantation loss in rats. EFSA, also, consid- ered, the possibility of eczematous and allergic reactions elicited by acute oral exposure and reported a BMDL10 of 1.1 mg Ni kg-1 body weight, with a margin of exposure (MOE) of 10 or higher, ac- counting for the variability of immune response in nickel-sensitised individuals (EFSA, 2015).

According to the 2016 European Market Observatory for Fish- eries and Aquaculture Products (EUMOFA) report, the consumption of seafood products has been constantly increasing in the last few years, with mussels among the major species. In 2014, 1.27 kg mussels per person per year and 0.33 kg clams per person per year, was recorded. In the same year, the EU production of bivalves increased 14%, reaching 609,600 tons, mainly due to an increase in mussels farmed in Spain, the major producer, followed by France and Italy, where bivalve production represents 70% of all fishery national production (EUMOFA, 2016).

In the current study, inductively coupled plasma-mass spec- trometry (ICP-MS) was used to quantitate the six mentioned metal contaminants (As, Cd, Cr, Hg, Pb and Ni), in mussels and clams obtained from the Italian fish market, and evaluate the Italian consumer's risk, on the basis on the MRLs stated by the EC, where available, or, otherwise, based on the tolerable intakes recom- mended by the EFSA.

2. Materials and methods

2.1. Chemicals and reagents

Nitric acid (HNO3, 69% Hiperpur Solution) and hydrogen peroxide (H2O2, 30% Hiperpur Solution) were from Panreac Qui- mica SLU (Castellar del Valle´s, Barcelona, Spain). Purified water was obtained through a Milli-Q system (Millipore, Merck KGaA, Darm- stadt, Germany).

2.2. Standards

Pb, 1000 mg mL^{-1} (0.10% w/v), (Pb metal in 2% HNO3); Hg, 1000 mg mL^{-1} (0.10% w/v), (Hg metal in 2% HNO3); Cd, 1000 mg mL^{-1} (0.10% w/v), (Cd metal in 2% HNO3); As, 1000 mg mL^{-1} (As metal in 2% HNO3); Ni, 1000 mg mL^{-1} (0.10% w/v), (Ni metal in 2% HNO3); Cr, 1000 mg mL^{-1} (0.10% w/v), (Cr metal in 2% HNO3), and the internal standard Yttrium (Y), at 1000 mg mL^{-1} (0.10% w/v), (Y metal in 2% HNO3), were all Baker Instra-Analysed® Reagents (JT Baker, Deventer, Holland).

2.3. Sample collection

A total of 50 mussel (Mytilus galloprovincialis, Mytilus edulis, Mytilus chilensis) and 40 clam (Venerupis philippinarum, Perna canaliculus, Tapes decussatus, Tapes semidecussatus, Meretrix mere- trix, Meretrix lyrata) samples were collected from June 2016 until February 2017, at the wholesale Milan fish market, which supplies the whole country. Their origin was randomly chosen, in order to simulate the mollusc meal consumption of an Italian consumer. The samples were from five different FAO areas (Chile; Thailand and Vietnam; New Zealand; North Spain, North France and Portugal; and the Mediterranean Sea). Each sample was prepared from 50 or more molluscs, aged approximately 2 years and with sizes ranging from 35 to 60 mm for mussels and 22e25 mm for clams. The edible part was finely dispersed with an Ultraturrax (IKA®-Werke GmbH and Co. KG, Staufen, Germany) at 13500 rpm for 2 min. All samples were stored at —20 °C, until analysis.

2.4. Sample preparation

A 0.5 ± 0.05 g part of each pooled sample was digested with 5 mL HNO3 (65% aqueous solution) and 0.5 mL H2O2 (30% aqueous solution), in closed Teflon vessels. For the mineralisation, the samples were placed in 3M-TFM® vessels and microwave-digested (Milestone Inc., Shelton, CT, USA), at high temperature (200 ○C) for

30 min. A blank solution was prepared, adding only the HNO3/H2O2 (5 mL/0.5 mL) mixture, in one vessel. After mineralisation, the samples and the blank solution, were brought to a volume of 50 mL, with ultrapure water. Then, 1 mL of each solution was placed in a 15-mL vial and brought to 10 mL with ultrapure water

2.5. ICP-MS analyses

The inductively coupled plasma mass spectrometer (Agilent 7500ce, Agilent Technologies, Santa Clara, CA, USA) used, was equipped with a Cetac ASX-510 auto-sampler (Thermo Fisher Scientific, San Jose, CA, USA). Argon and helium were used pure, at 99.999%.

2.6. Quality control

Quality control, expressed as trueness, was performed by ana- lysing the Certificated Reference Material ERM-CE278k mussel tissue (European Commission's Joint Research Centre, Institute for Reference Materials and Measurements, Geel, Belgium) digested with the same protocol of the samples. The following parameters were also considered, based on Commission Regulation (EC) No 333/2007 (EU, 2007): limit of detection (LOD), defined as three times the standard deviation (SD) of the noise from six different sample blanks; limit of quantification (LOQ), defined as ten times the SD of the noise from six different sample blanks; repeatability, in terms of relative standard deviation (RSD) of measurements made on twelve blank samples spiked with 1 mg kg-1 of each metal, respectively. Uncertainty, expressed as expanded measurement uncertainty (U) was evaluated on repeatability data: unless other- wise stated, results are reported as concentration ± U. Recovery was not calculated, due to the lack of extraction procedures, as stated by the above-mentioned Regulation (EU, 2007).

2.7. Statistical analysis

The descriptive statistics was performed using Microsoft Excel® 2013.

Results and discussion

Mussel and clam samples were analysed for the presence of Cd, Pb and Hg, for which the EU set MLs (EU, 2006), and As, Cr and Ni, for which only the EFSA tolerable intakes or threshold doses are reported (EFSA, 2009a, 2014a, 2015). Tables 1 and 2 summarise the data (mg kg^{-1} wet weight) as the mean ± SD, as well as the 25th, 50th (median), 75th and 100th (maximum concentration) per-

centiles in mussels and clams, respectively. Trueness ranged from 102.3 to 105.3%. The LODs were 0.001 mg kg^{-1}, for Cd and Hg, and 0.005 mg kg^{-1} for the remaining metals. The LOQ was 0.01 mg kg^{-1}, for all metals. These analytical limits allowed us to assess the risk based on the MLs and threshold doses. Repeatability, expressed as RSDs, ranged from 2.5 to 6.2%. Uncertainty, related to a 1.0 mg kg^{-1} spike of each metal in 12 blank samples, was between 0.05 and 0.12 mg kg^{-1}.

3.1. Metals with MLs: Cd, Hg and Pb

Cd was detected in all samples analysed, except one. The Cd concentration ranged between 0.08 and 2.13 mg kg^{-1}in mussels and < LOQ-0.90 mg kg^{-1}in clams. All concentrations were lower than the ML set by EC 1881/2006, of 1 mg kg^{-1}1, except one mussel sample from North Spain (2.13 ± 0.10 mg kg^{-1}).

Hg was detected in all samples analysed but was always detected below the ML (0.5 mg kg^{-1}). An overview of mercury studies performed in the Mediterranean Sea region, found that although atmospheric concentrations of Hg species at several coastal sites in the Mediterranean Sea Basin were significantly higher than those recorded at several other coastal sites distributed across North Europe, Hg water levels were comparable to other oceans, reported to represent a geological source for Hg (Kotnik et al., 2014).

Due to its unique chemical-physical properties, Pb persists in the environment for a long time (Orescanin et al., 2006). The con- centrations of this metal were, however, lower than the ML statedby the EU (1.5 mg kg^{-1}), both in mussels (<LOQ-0.79 mg kg^{-1}) and in clams (<LOQ- 0.93 mg kg^{-1}).

Table 1: Concentrations (mg kg-1), expressed as means±SDs and percentiles of the six analysed metals in mussel samples; n ¼ number of positive samples. Means and SDs are calculated only on positive samples.

	Pb	Hg	Cd	As	Ni	Cr
n	48	50	50	50	50	6
Mean±SD	0.23±21	0.05±0.07	0.29±0.27	5.04±2.34	0.96±0.88	0.051±0.14
25th percentile	0.09	0.02	0.14	3.45	0.32	0.00
Median	0.17	0.04	0.23	4.76	0.57	0.00
75th percentile	0.31	0.06	0.38	5.94	1.54	0.00
Maximum concentration	0.79	0.16	2.13	13.36	3.98	0.64

Table 2: Concentrations (mg kg^{-1}), expressed as means±SDs and percentiles of the six analysed metals in clam samples; n ¼ number of positive samples. Means and SDs are calculated only on positive samples.

	Pb	Hg	Cd	As	Ni	Cr
n	40	39	36	39	39	9
Mean ±SD	1.23±2.35	0.05±0.07	0.31±0.27	4.86±2.33	1.23±2.35	0.16±0.48
25th percentile	0.09	0.019	0.14	3.278	0.33	0.000
Median	0.17	0.03	0.24	4.68	0.62	0.000
75th percentile	0.31	0.04	0.41	5.81	1.64	0.000
Maximum Concentration	0.93	0.53	0.90	12.65	21.70	2.82

3.2. Metals without MLs: As, Cr and Ni

The toxic effects of As, are produced mostly by its inorganic forms. This study analysed the total As i.e. the sum of organic and inorganic As. The concentration ranged from <LOQ 13.36 mg kg^{-1} in mussels and from <LOQ 12.65 mg kg^{-1} in clams. The highest concentrations were found in one mussel sample from the Italian coast of the South Adriatic Sea and in one clam sample from Portugal. Even if the EU has not stated MRLs for As, the report titled "Dietary exposure to inorganic As in the European population" (EFSA, 2014a), provides some interesting observations. After withdrawing the PTWI of 15 mg kg^{-1} body weight, in 2011, the JECFA indicated "a BMDL for a 0.5% increased incidence of lung cancer, of 3.0 mg kg^{-1} body weight per day" (JECFA, 2011). Previ- ously, the EFSA (2009a) had indicated "a $BMDL_{01}$ between 0.3 and 8 mg kg^{-1} body weight per day for an increased risk of 1% of cancer of the lung, skin and bladder, as well as skin lesions". In the report, the EFSA cared to recommend the speciation of As present in food samples. Yet, although As was detected in 97.4% of almost 104,000 food samples, the data collected to calculate human exposition, only reported the total As concentration (EFSA, 2009a). Moreover, the data from the scientific literature do not allow to accurately calculate the amount of inorganic As from total As in seafood (Francesconi, 2010). This is because the relationship is variable between species and the content of inorganic As is inversely pro- portional to the total As content. For instance, in 175 Norwegian blue mussel samples, Sloth and Julshamn (2008), found that the inorganic/organic As ratio was usually less than 10%, except in a few mussels, in which inorganic As represented up to 42% of the total. Thus, to assess the risk associated with As present in the analysed molluscs, the current study applied a very conservative approach, by supposing that the normal amount of inorganic As in mussels and clams is the 42% of the total and considering the maximum concentrations detected in mussels and clams, as if they were usual concentrations. The inorganic As concentration should, therefore, be 5.61 mg kg^{-1} in mussels and 5.31 mg kg^{-1} in clams. Accounting for the abovementioned annual consumption of molluscs, an adult consumer, with a body weight of 70 kg, should, therefore, consume (5.61 X 1.27) (365 X 70) $^{-1}$= 0.28 µg (kg body weight day) $^{-1}$ of As from mussels and (5.31 X 0.33) X (365 X 70) $^{-1}$ = 0.07 µg (kg body weight day^{-1} As from clams, which are below the BMDL10 value of 0.3 mg kg body $weight^{-1}$. Cr was found in 12% of the mussel samples and 22% of the clam samples, with maximum concentrations of 0.64 ± 0.03 and 2.82 ± 0.13 mg kg^{-1}, respectively. When based on a 300^{-g} fish meal day^{-1},

which, according to the European Agency for the Evaluation of Medicinal Products (EMEA), is an "arbitrarily high consumption value to ensure the protection of the majority of the consumers" (EMEA, 2001), these concentrations of edible tissue of mussels or clams, would provide a Cr intake of 0.19 and 0.85 mg day^{-1}, respectively. These values are significantly lower (110 and 25 times, respectively) than 21 mg day^{-1}. Cr intake for an adult person of 70 kg, calculated from the TDI value of 300 mg Cr (III) kg^{-1} body weight per day. As stated by EFSA the toxic relevance of CR(VI) is negligible in food, therefore was not considered (EFSA, 2014b).

Ni was detected in all samples analysed. The concentration range was 0.12- 3.98 and 0.097 - 21.70 mg kg^{-1} in mussels and clams, respectively. Thus, a consumer, eating the more contaminated mussels or clams, would potentially be exposed to (3.98X 1.27) $(70X\ 365)^{-1}$= 0.2 µg (kg body weight $day)^{-1}$ or (21.70 X0.33)X$(70\ 365)^{-1}$ = 0.27 µg (kg body weight $day)^{-1}$, respectively, amounts of Ni, which are lower than the TDI of 2.8 µg kg^{-1} body weight. The acute toxicity of Ni is, however, important, as the most frequent adverse effect in the population is allergic dermatitis. Systemic contact dermatitis, elicited by Ni oral ingestion via food and drugs in nickel-sensitive individuals, consists of generalised eczematous flare-up reactions. A single meal (0.3 kg) of the most contaminated mussels and clams, should provide to a consumer weighing 70 kg, (3.98 × 0.3/ 70) ¼ = 17 and (21.70 × 0.3/70) ¼ = 93 µg kg^{-1} body weight, respectively, an amount much higher than the BMDL10 of 1.1 mg kg-1 body weight. It is of concern, however, that the MOE, when the less contaminated mussels and clams are considered, is lower than the minimum recommended value of 10: a meal should supply 0.51 and 0.42 mg Ni kg^{-1} body weight, providing MOE values of 2.2 and 2.6 (Yoshihisa and Shimizu, 2012; EFSA, 2015).

3.3. Evaluation of the Hazard Index

The studied elements in mussels and clams are actually a mixture of chemicals that can share some common toxicological effects, so evoking a dose addition: the Total Dose, i.e. the dose of each toxic agent with similar effects. As far as the cancer risk, oral intake with food has a major role only for arsenic on lung, skin and bladder. Lung cancer is a toxicological effect of exposition only through inhalation of Cd and Ni, similarly to bladder cancer for Cd (EFSA, 2009a, b, 2015). As the cancer risk by mollusc intake is induced only by As, just the non-carcinogenic effects where considered. The Hazard Index (HI) was therefore calculated as

HI=$\Sigma^{n}_{1=6}$ *Estimated Intake*$_i$/ R*f*V$_i$, where, RfVi is the Reference Value, representing the human daily intake of the chemical i [e.g., TDI or BMDL] and the Estimated Intake, evaluated from the above mentioned annual consumptions, is in same units as the RfVi (Teuschler, 2013) The considered R*f*V$_i$ where the reference doses indicated by EFSA (2009a,b, 2010a,b, 2014b, 2015), eventually recalculated on a daily basis. To maintain a very conservative approach, only the highest concentrations of each element in mussels and clams were accounted for. The Target Hazard Quotients (THQ), i.e. the ratio of the potential exposure to the sub- stance and the level at which no adverse effects are expected were then evaluated as THQ daily intake/RfV for each com- pound, for the different end points indicated by EFSA. The results are shown in Table 3.

Table 3: Hypothetic target hazard quotients and hazard indexes for the estimated daily exposure to the studied elements via mussels and clams at the highest concentrations detected. All the reference values are by EFSA. TWIs are recalculated and expressed on a daily basis. As is reported as inorganic Arsenic and only Cr(III) is considered

RfV	Element	Mussels Clams		Target hazard quotients: MUSSELS-CLAMS (reference values - mg kg^{-1} day^{-1})			
		Daily exposure (mg kg^{-1})		Skin	Reproduction/Development neurotoxicity	Developmental Blood pressure	Kidney
$BMDL_{10}$	Pb	0.04	0.01		0.08-0.02 (0.5)	0.03-0.007 (1.5)	0.06-0.016 (0.63)
TWI	Hg	0.01	0.01		0.06-0.06 (0.18)		
TWI	Cd	0.11	0.01				0.29-0.03 (0.38)
$BMDL_{10}$	As	0.28	0.07		0.93-0.23		
TDI	Ni	0.20	0.28		0.07-0.10 (2.8)		
TDI	Cr	0.03	0.04		0.0001-0.0001 (300)		
HAZARD INDEX				0.93-0.23	0.0701-0.1001	0.14-0.08	0.35-0.046

All the HIs were lower than one, indicating that a negligible health hazard could derive from the ingestion of mussels. A study conducted by Leclercq et al. (2009), shows that in 2005e2006 the mean individual daily consumption for Italian adult population was about 40 g day^{-1} of fish and, for the group belonging to the 95th percentile about 145 g $day^{-1.}$. Assuming that the variation between groups in mollusc consumption is similar, the THQ values should be multiplied by (145/40) 3.65. Accounting for this consumption, all mussel samples and the 25% of clam samples showed a HI > 1 for skin lesions in high per- centiles consumers while all samples (except one) showed a HI value < 0.1 for Cd and Pb merged dose (Table 4).

Table 4: Hazard indexes for the estimated daily exposure to the studied elements via mussels and clams of the 95 percentile fish consumers

	Hazard index for 95 percentile consumers	○SKIN	Reproduction/development	Developmental neurotoxicity	Blood pressure	Kidney
		As	Ni+ Cr	Pb + Hg	Pb	Pb + Cd
MUSSELS	Minimum	1.24	0	0.01	0.00	0.06
	Median	2.86	0.03	0.05	0.03	0.02
	Maximum	8.08	0.26	0.34	0.10	1.14
	Percent of values>	100%	-	-	-	2%
CLAMS	Minimum	0.00	0.00	0.00	0.00	0.00
	Median	0.07	0.03	0.02	0.004	0.05
	Maximum	1.98	0.36	0.17	0.29	0.13
	Percent of values > 1	25%	-	-	-	-

Discussion

Metal contamination in mussels and clams that are currently present in the Italian market, provide reasons for low concern for average consumers. According to the EU MLs or the EFSA threshold doses, Pb and As do not pose a risk to public health (EU, 2006; EFSA, 2014a). Cr showed concentrations that elicit oral exposure sub- stantially lower than the TDI. (EFSA, 2014b). Hg was present in only one clam sample, at a concentration higher than the maximum level, but compliant accounting for the uncertainty (0.53 ± 0.04 mg kg^{-1}). Considering that the chronic toxicity was evaluated in establishing the MRL, this concentration could not be considered a risk for the consumer. A similar consid- eration could be made for Cd, detected in one mussel sample, at a concentration of about twice the MRL. In this case, too, because the chronic toxicity studies were accounted for in establishing an MRL, the risk can be considered very low (EU, 2006). High percentiles consumers are a cause of concern due to chronic oral exposition to As: in fact all mussel samples and 10 clam samples showed a HI > 1 for skin lesions. It must be highlighted that this consumer group is furthermore subject to lung, skin and bladder cancer risk, as the BMDL10 value of 0.3 mg kg^{-1} is overcame in all the samples (EFSA, 2009a). Ni demands particular consideration, too. No chronic toxicity risk was indicated by our study. Systemic contact dermatitis, a manifestation of acute Ni toxicity, is the most prevalent effect in the population. Based on our results, the Ni content of all the mollusc samples analysed, could cause the allergic reaction in Ni-sensitive individuals, thus, representing a concern.

5. Conclusion

Finally, this survey on the metal contents in mussels and clams from the Italian market, shows a very low risk for the average consumer health, when the chronic toxicity is considered. High percentile consumers, however, are subject to risk skin lesions, and lung, skin and bladder cancer due to high intake of As. The occur- rence of acute toxicity, in the form of allergic dermatitis in sensitive subjects, due to constant Ni presence in the studied molluscs, must, however, be highlighted. Further studies are ongoing to widen the sample number, in order to compare adequate numbers of samples from the different FAO areas.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgement

This work is dedicated to our good friend and colleague Marina Caligara, who passed away before its publication. Federica Ceriani is the recipient of a Ph.D. fellowship in Veterinary and Animal Science in the Laboratory of Veterinary Toxicology at the University of Milan.

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3.2. Exposition to metals and arsenic from yellow and red tuna consumption.

Published in: *Food Additives & Contaminants Part A, Volume 36, 2019, Pages 1228-1235.*

Luca Maria Chiesa[1], **Federica Ceriani[1]**, Antonio Procopio[2], Sonia Bonacci[2], Renato Malandra[3], Sara Panseri[1*], Francesco Arioli[1].

[1] *Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy*

[2] *Dipartimento di Scienze della Salute, Università Magna Græcia, Germaneto, Catanzaro, Italy*

2 ATS Milano-Città Metropolitana, Director of Veterinary Unit, Milan, Italy

* Corresponding author: Dr. Sara Panseri, Phone: 0250317931; Fax: 00390250317941. E-mail: sara.panseri@unimi.it. Address: Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy.

KEYWORDS Tuna; arsenic; metals; risk; hazard index.

ABSTRACT

Tuna is one of the most frequently consumed fish and, as a predator, can biomagnify pollutants. Metal and other elements pollution is an important worldwide concern. Based on these considerations, the aim of this work was to investigate the occurrence of As, Cd, Cr, Ni, Hg and Pb in tuna coming from different FAO areas to evaluate human exposure. The analysis was performed on muscle tissues through a quadrupole inductively coupled mass spectrometry. One hundred thirty-one samples were analysed. One red tuna from the Adriatic Sea and 11 yellow tunas exceeded Pb maximum levels (MLs) with a concentration ranging 0.31–0.86 mg kg^{-1}; three red tunas from different Mediterranean sub-areas exceeded Hg MLs, with a concentration range 1.19 to 1.80 mg kg^{-1}. All the Hazard Indexes (HIs) were lower than one, indicating that only a negligible health hazard could derive from the ingestion of tuna, for both average and high consumers. The risk of carcinogenicity from Cr is still under debate at the concentrations detectable in food.

1. Introduction

Fish is an excellent source of high-value protein rich in essential amino acids and micro and macro elements, and has an advantageous fatty acid profile, resulting from the content of essential

polyunsaturated fatty acids, known to support good health (Usydus et al. 2009). Tuna is one of the most frequently consumed and commercially attractive fish worldwide (Ikem and Egiebor 2005). Tuna, as a predator, is a high-performance fish with very high metabolism rates; thus, having high food intake rates, it increases the accumulation of pollutants (Voegborlo et al. 1999). Pollution by metal and other elements in fish is an important word wide concern due to the health risk associated with fish consumption and diet is the main route of exposure. Many metals naturally occurring in the environment, including copper, iron, manganese, nickel, and zinc have important biological roles. However, a significant number of metals, like cadmium, lead, and mercury have no biological roles, but have highly toxic properties when consumed by animals, including humans, and are classified as toxic metals (Chen et al. 2016). The World Health Organization lists cadmium, lead, and mercury in its list of top ten chemicals of major public health concern (WHO 2016) and exposure to these metals has been linked to numerous neurodevelopmental and neurodegenerative disorders in humans (Von Stackelberg et al. 2015). Inorganic arsenic (As) has been linked to increased risk of cancer of the skin, lungs and bladder, and skin lesions. Other symptoms associated with chronic arsenic exposure are peripheral neuropathy, encephalopathy, hepatomegaly, bone marrow depression, diabetes and renal function impairment (EFSA 2009a). Inorganic arsenic was the first element to be identified as a human carcinogen and the IARC allocated it to Group 1 (IARC 2012a). Cadmium (Cd) can damage kidneys and cause poor reproductive capacity, hypertension, and hepatic dysfunction (Abou-Arab et al. 1996). The kidney is the critical target organ for dietary exposure to cadmium and renal damage is characterised by cadmium accumulation in convoluted proximal tubules (EFSA 2009b). Data on exposure to Cd have also been associated with an increased risk of lung cancer through inhalation by workers or smokers, and bladder, endometrium, testicular, pancreatic and gall bladder cancer (Huff et al. 2007; EFSA 2009b). No sure causal association between Cd oral exposition and cancer is currently available (European Commission (EC) 2007) even if some recent data seem to indicate an association with cancer at low dietary exposures (Åkesson et al. 2014). However, studies on dietary exposure to Cd did not show an increase of incidence of total or specific cancers in 90,000 Japanese of both sexes (Sawada et al. 2012), and of breast cancer in 30,000 U.S. postmenopausal women (Adams et al. 2012). Consequently, even if IARC (IARC 2012b) allocates Cd in Group 1, information regarding the carcinogenetic effect of Cd is still incomplete for risk assessment by oral intake. Mercury (Hg) has been associated with neurotoxicity, ototoxicity, tremors, irritability, memory problems, changes in

vision and hearing. Moreover, it has been associated with developmental toxicity and cardiovascular disease (EFSA 2012). The critical target for acute toxicity of mercury is the kidney followed by the liver, nervous system, immune system, reproductive and developmental systems. Nickel (Ni) is classified by IARC (IARC 2012a) as a human carcinogen causing cancers of the lung, nasal cavity and paranasal sinuses only after inhalation and studies on animals did not give any evidence of oral carcinogenicity. Oral absorption of Ni can elicit eczematous flare-up reactions in the skin in Ni-sensitised individuals (EFSA 2015). Some other metals (e.g. chromium) cause nephropathy, anuria, neurotoxicity and embryotoxicity (EFSA 2014b). Lead (Pb) causes nervous dysfunction, kidney damage and chronic toxicity, poor reproductive capacity, hypertension, tumours, hepatic dysfunction and may cause miscarriage in pregnant women (EFSA 2010). The risk from exposure to As and metals requires further comment. The European Commission (European Commission (EC) No 1881/2006 2006) set maximum levels (MLs) for Cd (0.10 mg kg^{-1}), Pb (0.30 mg kg^{-1}) and for Hg (0.1 mg kg^{-1}) in tuna. No MLs have yet been established by the European Union for As, Cr and Ni (EU). EFSA established a BMDL01 for As between 0.3 and 8 µg/kg b.w. day^{-1} for an increased risk of cancer to lung, skin and bladder, and skin lesions (EFSA 2009a). Cd is a primary toxic on the kidney and may cause renal dysfunction (EFSA 2009b). The CONTAM panel defined a tolerable weekly intake (TWI) of 2.5 µg kg^{-1} b.w. In 2014, EFSA suggested a tolerable daily intake (TDI) for Cr (III) of 300 µg kg^{-1} body weight, which was based on reproduction and developmental toxicity reported in some studies and from a long-term study on rats of the US National Toxicology Programme (NTP) (NTP 2010). Cr (VI), classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1), is not present in food, considered a strong reducing medium (EFSA 2014b). Hg TWI (1.3 µg g^{-1}) expressed as total Hg is derived from neurodevelopmental toxicity (EFSA 2012). The TDI for Ni is 2.8 µg kg^{-1} body weight, a value derived from studies about the incidence of litters with post-implantation loss in rats. Considered the possibility of eczematous and allergic reactions elicited by acute oral exposure a BMDL10 of 1.1 µg Ni kg^{-1} body weight, with a margin of exposure (MOE) of 10 or higher, accounting for the variability of the immune response in nickel-sensitised individuals is also stated (EFSA 2015). The critical effects of Pb are developmental neurotoxicity in infants and children (BMDL01 = 0.50 µg kg-1 day^{-1}), cardiovascular effects and prevalence of chronic kidney disease (CKD) in adults (BMDL01 = 1.50 µg $kg^{-1}day^{-1}$and BMDL10 = 0.63 µg kg^{-1} day^{-1}, respectively) (EFSA 2010). Infants (aged 0–3 years) are more exposed than children (5–10 years) and adults since

Pb is better absorbed in growth plates than bone tissues. The International Agency for Research on Cancer classified inorganic lead as probably carcinogenic to humans (Group 2A) in 2006, for limited evidence of carcinogenicity in humans and sufficient evidence in animals (NTP 2004; IARC 2006). However, since the doses used to induce tumours in rats are very high compared to human intake, EFSA considered human exposure to lead through food unlikely to represent a significant cancer risk (EFSA 2010). Several studies are present in the literature on the presence of metals in fish (Table 1). The aim of this work is to investigate the occurrence of As, Cd, Cr, Ni, Hg, Pb in tuna coming from different Fishing Areas (FAO) to evaluate human intake.

Table 1: Concentration of As and metals in tuna from literature analysed by ICP-MS. (a: average concentration; b: median concentration)

Reference	Element	Area	Concentration range ($\mu g\ g^{-1}$ w.w.)
Voegborlo et al. 1999	Hg	Libya	0.20-0.66
	Cd		0.09-0.32
	Pb		0.18-0.40
Storelli and Marcotrigiano, 2001	Hg	Mediterranean Area	0.07-4.26
Storelli et al. 2005	As	Mediterranean Area	1.62-5.01
	Cd		0.01-0.04
	Hg		0.13-0.35
	Pb		0.07-0.18
Licata et al. 2005	Cd	Sicily	n.d.-0.26
	Hg		2.45-4.21
	Pb		n.d.-0.24
Storelli et al. 2010	Cd	Mediterranean Area	n.d.-0.03
	Hg		0.07-1.76
	Pb		n.d.-0.33
Guérin et al. 2011	Cr	France	0.22[a]
	Ni		0.34[a]
	Pb		0.011[a]
Mol 2011	Cd	Turkey	0.01-0.02
	Hg		0.06-0.30
	Pb		0.09-0.45
Olmedo et al., 2013	As	Spain	0.033[b]
	Cd		0.008[b]
	Hg		0.00[b]
	Pb		0.004[b]

2. Materials and methods

2.1. Chemicals and reagents

Nitric acid (HNO3, ≥69.0%, Trace SELECT) and hydrogen peroxide (H2O2 ≥ 30% Trace SELECT Ultra) were purchased from Fluka analytical (Germany). Hydrochloric acid Superpure was purchased from Carlo Erba. Purified water was obtained through a Milli-Q Integral 5 system (Millipore, Merck KGaA, Darmstadt, Germany).

2.2. Standards

Two multielement standards solution: IV-ICPMS -71A containing 10 μg ml^{-1} of arsenic (As), aluminium (Al), cadmium (Cd), chromium (Cr), manganese (Mg), nickel (Ni), lead (Pb), zinc (Zn), and CMS-1 containing 10 μg ml^{-1} of yttrium (Y), were purchased from Inorganic Ventures (Christiansburg, Virginia, USA). Mercury standard solution containing 1000 mg L^{-1} was purchased from Fluka. IV-ICPMS-71A and mercury standard solutions were used daily to prepare calibration standards in 2% HNO3/HCl (1:1). Standard 100 μg L^{-1} yttrium solution was prepared daily and added to all samples as internal standard, to verify changes in instrumental sensitivity.

2.3. Sample collection

A total of 131 of red tuna (Thunnus thynnus) and yellow tuna (Thunnus albacares) samples were collected from March 2017 until October 2017, at the wholesale Milan fish market, which supplies the whole country. Their origin was chosen randomly with the aim of simulating the tuna consumption of an Italian consumer. The FAO areas are shown in Table 2. The edible part was finely dispersed with an Ultraturrax (IKA®-Werke GmbH and Co. KG, Staufen, Germany) at 13500 rpm for 2 min. All samples were stored at −20°C, until analysis.

Table 2. Tuna sample details: number of samples, species, FAO area, country of origin

Species	FAO Area	Country	Total
Red Tuna *(Thunnus thynnus)*	27	Spain VIIIC-	3
		North Spain	2
	37	Sicily-	8
		Adriatic Sea	4
Yellow Tuna *(Thunnus albacares)*	51	Maldive- Indian Ocean	15
	57	Maldive-Sri Lanka-Indian Ocean	47
	71	Pacific Ocean	52
Total			**131**

2.4. Sample preparation

The sample preparation was carried out using an Anton Paar Multiwave 3000 digestion system equipped with a XF100 rotor. To decontaminate PTFE vessels, a cleaning procedure was carried out by adding 4 ml of HNO3 and 4 ml of H2O in each vessel, in the following conditions: 1100 W for 15 min. After cleaning, vessels were rinsed with ultrapure water and dried. Aliquots of 0.5 g of each homogenised sample were weighted directly into the PTFE vessel of the microwave system. The digestion was performed by adding 1 ml of H2O, 4 ml of HNO3, 0.5 ml of HCl and 0.5 ml of H2O2. The operating conditions used for the microwave digestion were 800 W over 15 min and held at this power for 30 min. After digestion, samples were quantitatively transferred to a graduated polypropylene test tube and diluted with ultrapure water to 50 ml. The analytical batch consisted of a set of calibration standard, samples, and a minimum of three procedural blanks. A midrange calibration standard was analysed after each batch of 15 samples to verify instrumental drift throughout the run. Seven-point calibration curves covering the range 0.01–100 μg L^{-1} were used for quantitative analysis. Standard solutions were prepared by diluting the multielement solutions.

2.5. ICP-MS analyses

The analysis was performed by a quadrupole inductively coupled mass spectrometry, X Series 2 (Thermo Scientific, Waltham, MA, USA), equipped with a collision cell incorporating kinetic energy

discrimination which efficiently eliminates matrix, argon and based spectral interferences using reaction gases He/H2 (97:3). The sample solutions were pumped by a peristaltic pump from tubes arranged on CETAC ASX-520 auto-sampler (Thermo Scientific, Omaha, NE, USA). Argon and He/H2 (9:7) mixture were used pure, at 99.999%. Instrument sensitivity, resolution and mass calibration were optimised daily with the tuning solution (Multielement Tune A, containing 10 μg L^{-1} of Ba, Be, Bi, Ce, Co, In, Li, Ni, Pb, U in 2% HNO3, to maximise ion signals and to minimise interferences effects due to high oxide levels (CeO+ /Ce+ < 2%) and doubly charged ions (Ba2+/Ba+ < 3%). In order to verify the robustness of the analytical method, Yttrium was added as internal standard and analysed with the run. Sample data were qualified following the Internal Standard Recovery method, and required to be within a 80–120% limit.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad InStat version 3.00, GraphPad Software, San Diego California USA. The comparison between FAO areas was made through the Kruskal–Wallis Test (Nonparametric ANOVA) for samples with nonGaussian distribution and Dunn's Multiple Comparisons Test when a significant difference was found. P was set at 0.05.

3. Results and discussion

Table 3 shows the data relative to metal concentration in tuna tissues and the differences between the various sampling areas. The Mediterranean Sea samples show the maximum concentrations for As (5.53 mg kg^{-1}), Cd (0.034 mg kg^{-1}), Cr (0.216 mg kg^{-1}), Hg (1.80 mg kg^{-1}) and Ni (0.319 mg kg^{-1}). Based on the results reported in Table 3 the estimated daily intakes (EDI) with tuna of an average European consumer, for any considered element, were calculated as: EDI = [(highest value between mean and median concentration in tuna) x annual tuna intake]/ (365 days x 60 kg body weight). The estimated per capita consumption in the EU in 2015 was 2.77 kg tuna (EUMOFA 2017). Arsenic is present predominantly in the organic forms of arsenobetaine, arsenocholine, monomethylarsonic acid and dimethylarsinic acid.

Table 3: Mean ± SD, Minimum, Median and Maximum concentration of each metal and comparison from different FAO zones. Concentrations expressed as mg kg^{-1} muscle

		FAO 27 N=5	FAO 37 N=12	FAO 51 N=15	FAO 57 N=47	FAO 71 N=52	TOTAL N=133
As	Mean±SD	0.93±0.30	2.29±1.63	1.06±0.48	1.28±0.53	1.02±0.39	1.23±0.73
	Minimum	0.55	0.34	0.37	0.44	0.56	0.34
	Median	0.85	2.41	0.90	1.15	1.00	1.06
	Maximum	1.33	5.52	2.01	3.11	1.72	5.52
Cd	Mean±SD	0.017±0.003	0.018±0.007	0.017±0.005	0.013±0.005	0.014±0.004	0.014±0.005
	Minimum	0.015	0.008	0.008	0.007	0.006	0.006
	Median	0.016	0.017	0.017	0.011	0.014	0.014
	Maximum	0.023	0.034	0.026	0.025	0.028	0.034
Cr	Mean±SD	0.039±0.027	0.037±0.024	0.050±0.045	0.042±0.033 (a)	0.053±0.029	0.047±0.03
	Minimum	0.010	0.014	0.016	0.012	0.015	0.010
	Median	0.038	0.032	0.030	0.031	0.045	0.037
	Maximum	0.083	0.101	0.167	0.216	0.150	0.216
Hg	Mean±SD	0.36±0.08	0.72±0.49	0.25±0.09	0.12±0.10 (b)	0.07±0.02 (c)	0.18±0.24
	Minimum	0.24	0.091	0.033	0.041	0.053	0.033
	Median	0.37	0.55	0.27	0.083	0.065	0.086
	Maximum	0.45	1.80	0.43	0.41	0.11	1.80
Ni	Mean±SD	0.014±0.011	0.056±0.092	0.020±0.014	0.030±0.043	0.040±0.043	0.035±0.047
	Minimum	0.007	0.004	0.005	0.004	0.006	0.004
	Median	0.011	0.015	0.015	0.018	0.024	0.018
	Maximum	0.033	0.31	0.049	0.29	0.23	0.31
Pb	Mean±SD	0.048±0.014	0.087±0.099	0.094±0.090	0.089±0.098 (d)	0.18±0.14	0.12±0.12
	Minimum	0.034	0.034	0.030	0.008	0.017	0.008
	Median	0.044	0.056	0.059	0.059	0.18	0.07
	Maximum	0.070	0.39	0.39	0.44	0.86	0.86

The toxicity of As compounds depends on the chemical form: inorganic As is much more toxic than the organic form (Hindmarsh and McCurdy 1986; Sirot et al. 2009). According to EFSA (2014a), fish and other seafood represent a problem when trying to derive the amount of inorganic arsenic from total arsenic because the ratio may depend on the seafood type (Cullen and Reimer 1989; EFSA 2009a), and the relative proportion of inorganic arsenic tends to decrease as the total arsenic content increases. In tuna, arsenobetaine is the dominating arsenic compound (Larsen et al. 1993) while toxic inorganic arsenic is present at lower concentrations. The CONTAM panel considered the average amount of inorganic arsenic in fish to be 0.1–3.5%. With a conservative approach, we decided to consider inorganic arsenic as 10% of the total arsenic and the highest of the mean and median values, 1.24 and 1.06 mg kg^{-1}, respectively, of total arsenic in tuna. Applying the above formula, the EDI of As is 0.016 µg kg-1 day-1 which is 19 times lower than EFSA BMDL05 related to cancer of the skin, lungs and bladder, and skin lesions (0.3 µg kg^{-1}) (EFSA 2014a). Cd was detected in all samples analysed, with a concentration range 0.006–0.034 mg kg^{-1}, always lower than MLs set by European Commission

1881/2006. Median and mean had the same value of 0.014 mg kg^{-1}, and EDI of Cd calculated as above would be 0.0017 µg kg^{-1} b.w. This value is 210 times lower than TWI of 2.5 µg kg-1 b.w., considered on a daily base (i.e. divided by seven). Cr was in almost all samples investigated with a concentration range 0.01–0.22 mg kg^{-1}. The EDI calculated on the mean (0.047 µg g^{-1}), would provide a Cr intake of 0.0089 µg kg-1 day^{-1}, about 33700 times lower than TDI (300 µg kg-1 day^{-1}). Cr VI has not been evaluated due to its very low presence in the food (EFSA 2014b). The presence of mercury in tuna requires further comment. Areas 57 and 71 show significantly lower concentrations than other zones; the Mediterranean Sea (Area 37), however, while not showing differences between zones 27 and 51, provided the highest concentration by far of Hg and the highest median. This fact is likely to be due to the different number of samples from different areas. Three red tuna samples from the Mediterranean Sea, respectively, from Sicily (1.29 mg kg^{-1}), the Adriatic Sea (1.19 mg kg^{-1}) and Cyprus (1.80 mg kg^{-1}), exceeded the MLs set by the EU at 1 mg kg^{-1}. Our results agree with other studies conducted on tuna in the Mediterranean region where the concentrations of Hg were 0.12–3.23 mg kg^{-1} (Storelli and Marcotrigiano 2001) and 0.49–1.81 mg kg^{-1} (Srebocan et al. 2007). In tuna muscle tissues organic Hg is between 75%-100% of total Hg (Storelli et al. 2005). Based on the worst hypothesis, i.e. Hg was totally methylmercury, the EDI calculated on the mean (0.18 µg g^{-1}) content, would provide a Hg intake of 0.0021 µg $kg^{-1}day^{-1}$, about 88 times lower than TWI (1.3 µg g^{-1}) expressed as total Hg. Ni was detected in all tuna samples. The concentration range was 0.008–0.86 mg kg^{-1}. Considering the mean value, an average consumer would be exposed to 0.0042 µg kg^{-1} day^{-1}that is about 670 times lower than the TDI value of 2.8 µg kg^{-1}body weight, calculated by EFSA (2015). The acute toxicity of Ni plays a major role, as systemic contact dermatitis is a frequent adverse effect in nickel-sensitive individuals exposed to this metal through food. A 300 g serving of the most contaminated tuna would supply (0.31 x 0.3/60) = 1.6 µg kg^{-1} body weight to a 60 kg consumer, that is an amount higher than the BMDL10 of 1.1 µg kg^{-1}body weight. Considering the recommended minimum value of 10 for the margin of exposure (MOE), the value considered of no concern would be 0.11 µg kg^{-1}body weight. The calculated concentration of Ni in tuna in a single meal that should not lead to the limit being exceeded is 0.022 mg kg^{-1}muscle; 85% of our samples had a Ni concentration higher than this calculated value (EFSA 2015). A risk of contact dermatitis through tuna intake is therefore present, even if lower than that observed in mussel and clams (Chiesa et al. 2018). Food is the main route of exposure of humans to Pb and cereal products contribute most to dietary exposure. Eleven analysed samples exceeded the

MLs (0.30 mg kg $^{-1}$) with a range concentration 0.30–0.86 mg kg^{-1}. Ten of them were yellow tunas from Pacific FAO areas 57 and 71, while the one presenting the lower concentration was a red tuna from Sicily. The comparison between zones showed a significant difference between FAO areas 57 and 71, which has a statistical significance due to similar numbers in the groups (Tables 1 and 3). The mean Pb concentration found in tuna samples was 0.12 mg kg^{-1}. An average consumer would, therefore, take in 0.014 µg kg-1 day^{-1}, an amount 45 times lower than the lower reference point referred to adults (BMDL10 = 0.63 µg kg^{-1}day^{-1}). A study (Teuschler 2013) on Italian food consumption patterns in the '90s reported that fish consumption by children is about 65% that of adult consumers. If the weight of 16 kg for a 4-year-old child and the above-reported intake are accounted for, daily intake results in 0.037 µg kg-1 day^{-1}, a value 13 times lower than the BMDL01 value of 0.50 µg kg^{-1}day^{-1}for developmental neurotoxicity in infants and children. This value could pose some concern if lower ages, and the intake of other foods are considered. In fact, cereals and cereal-based products, potatoes, leafy vegetables and tap water are the main contributors to Pb exposition (EFSA 2010) and tuna seems to contribute significantly to the health-based guidance value.

3.1. Evaluation of the hazard index

Metals and As in tuna can share some toxicological effects and evoke a dose addition that results in a Total Dose, i.e. the dose of each toxic agent with similar effects. Therefore, we calculated the Hazard Index (HI), i.e. the sum of more than one Target Hazard Quotients (THQ) shared by the different chemicals studied. Firstly, the THQ, i.e. the ratio of the estimated exposure to each substance and the level with no adverse effects, were evaluated as THQ = daily intake/RfV for each compound, for the different critical effects specified by EFSA. To calculate the HI, the following equation was used: HI = Σi=6 Estimated intakei /RFVi, where, RfVi is the Reference Value, that is the human daily intake of the substance i [TDI, TWI or BMDL] and the Estimated Intake, evaluated from annual consumption, is in the same units as the RfVi (Teuschler 2013). The RfVi were the reference doses indicated by EFSA (2009a, 2009b, 2010, 2012, 2015), reconsidered on a daily base when necessary. All the HIs were much lower than one, indicating that only a negligible health hazard could derive from the ingestion of tuna, at least for the chemicals studied (Table 4).

Table 4 Hypothetic target hazard quotient (THQ) and hazard index (HI) values for estimated daily exposure to the studied elements via tuna at mean concentrations detected. All the reference values (RfV) are by EFSA. TWIs are recalculated and expressed on a daily base. As is reported as inorganic Arsenic and only Cr(III) is considered. EDI is the estimated daily intake.

RfV		**BMDL**	**TWI**	**TDI**	**TWI**	**TDI**	**BMDL**		
Element		**As**	**Cd**	**Cr**	**Hg**	**Ni**	**Pb**	**HI**	**HI**
EDI ($\mu g\ kg^{-1}$)		0.016	0.0017	0.0089	0.0021	0.0042	0.014	Average consumers	95% consumers
THQ (RfV $\mu g\ kg^{-1}\ day^{-1}$)	**Skin/lung/bladder cancer; skin lesions**	0.053 (0.3)						0.053	0.19
	Reproduction/ Development			0.000030 (300)		0.0015 (2.8)		0.0015	0.0055
	Developmental neurotoxicity				0.011 (0.19)		0.028 (0.5)	0.039	0.14
	Blood pressure				0.011 (0.19)		0.0093 (1.5)	0.020	0.073
	Kidney		0.0048 (0.36)				0.022 (0.63)	0.027	0.099

The HI for the 95th percentile consumers was also calculated based on previous studies (Leclercq et al. 2009; Chiesa et al. 2018), that indicated an estimated fish intake ratio of 3.65 for higher versus average consumers. Accounting for this value an EDI for the higher consumers was calculated and shown in Table 4. For this group of population, too, tuna is a negligible source of exposure for the chemicals studied. Finally, this work shows low risks for the health of average consumers. All the HIs were much lower than one, indicating that only a negligible health risk could derive with the intake of tuna from the chemicals studied. There is some concern about a cancer risk evoked by Pb and Cd. The evidence for the carcinogenicity of Pb in humans, and human exposure through food are not however considered sufficient to represent a risk (EFSA 2015). IARC (2012a) states that the evidence of Cd as a human carcinogen is sufficient, based on professional exposure and lung cancer but not all recent data associate Cd and cancer at low dietary intakes (Åkesson et al. 2014). The health risk assessment should, therefore, consider Cd for this toxic effect, if more unambiguous data become available.

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3.3 Risk characterisation from the presence of environmental contaminants and antibiotic residues in wild and farmed salmon from different FAO zones

Published in: Food Additives & Contaminants Part A, Volume36 (1), 2019, Pages 152-162.

Luca Maria Chiesa [a], Maria Nobile [a], **Federica Ceriani** [a], Renato Malandra [b], Francesco Arioli [a] Sara Panseri [a]*.
[a] *Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy*
[b] *ATS Milano-Città metropolitana, Director of Veterinary Unit, Via Celoria 10, 20133 Milan, Italy*

[*] Corresponding author: Sara Panseri, Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy. Phone: 0250317931; Fax: 00390250317941; E-mail: sara.panseri@unimi.it

Abstract

Salmon consumption is increasing year by year. Salmon aquaculture is the fastest growing food production system in the world, and often uses feed mixed with antibiotics or other drugs. Feed can be also contaminated by environmental contaminants like persistent organic pollutants and organophosphorus pesticides that usually accumulate in fatty tissue, or emerging contaminants such as perfluoroalkyl substances (PFASs), that instead bioaccumulate in protein tissues. Therefore, there is the need to investigate the presence of antibiotics and environmental contaminants, with multi-class and multi-residue liquid chromatography-high resolution mass spectrometry and gas chromatography tandem mass spectrometry methods to monitor a broad spectrum of residues comparing between wild and farmed salmons. The presence of residues was encountered at a concentration range of 0.35–51.52 ng g^{-1} for antibiotics only in farmed salmon, 0.19–34.51 ng g^{-1} for PFASs and 0.26–9.01 ng g^{-1} for (polybrominated diphenyl ethers) PBDEs, and 0.19–5.91 ng g^{-1} for organochlorine pesticides with higher frequencies and concentrations in farmed fish. Finally, the risk deriving from salmon intake is low, being of minor concern only for PBDE 99 and perfluorooctanoic acid.

Keywords: POPs, PFASs, organophosphorus pesticides, antibiotics, salmon, toxicological risk.

1. Introduction

The consumption of salmon has risen consistently at global level. The global salmon supply covers both wild and farmed species. The intensive practices of aquaculture, adopted to meet the growing demand for fish (especially salmon, sea bream and sea bass), have already shown for some years the evidence of treatments with feed mixed with antibiotics or other veterinary drugs to prevent the spread of infections (Dickson 2014). In this context, one of the main sources of consumer exposure to pesticides and antibiotics is representing by food route. These molecules enter into the food chain as they are used for therapeutic, prophylactic or illicit purposes or for their accumulation in the environment especially in breeding contexts and in this specific case in aquaculture. In Europe, maximum residue limits (MRLs) have been established for various classes of antibiotics (European Commission 1990) and they were banned as growth promoters since the 1990s, due to the possibilities of transmission of antibiotic resistance to human pathogens. On the other hand, food and particularly fish, can be also contaminated by environmental contaminants like persistent organic pollutants (POPs), that usually accumulate in fatty tissue, or new environmental contaminants, as well as PFASs, that instead prevail in protein fraction as also highlighted by previous researches conducted on eels and mussels (Chiesa et al. 2018c, 2018d).

The attention must be focalized toward flame retardants (BFRs) because from 1980 their use rapidly increased in industrial field. These compounds tend to persistent in air, soil and water compartments for long time. Their potential to bioaccumulate, allows them to partition in the fatty tissues of living organisms, bioconcentrate, and finally biomagnify in the animals at the higher trophic levels, including humans, possibly causing, through chronic exposition, endocrine disruption and cancer (Wania and Mackay 1995). In 2003 European Commission (EC) fixed limits on the use of these compounds in the industry due to their toxicological risks. The legislation about their residue in food has not been enacted yet, due to the low data present in literature and so EC, in 2014, indicated to conduct more studies in order to collect further data about their presence in foods to better delucidate the consumer exposure risks (European Commission 2014). Bioaccumulation can occur through two forms: long-term, low-level contamination resulting from gradual diffusion of persistent chemicals through the environment, and relatively shorter term, higher level contamination caused by industrial accidents and waste disposal. The sources that lead to the presence of xenobiotics in food are numerous and difficult to

identify or exclude from our daily environment.

PFASs have aroused in these last year's great scientific interest especially concern the public health implications. Moreover, as stated by European Food Safety Authority (EFSA) the importance to monitorate these chemicals is crucial not only for food safety but great effort must be paid requests to set up and validate adequate and sensible analytical methods. Fish seems to be an important source of human exposure to PFASs, although the data might be influenced by results of studies in relatively polluted areas, which is likely to over-estimate exposure from commonly consumed fish (EFSA 2008).

In addition, very few and partial research are present in literature about contaminants and antibiotics residues in both farmed and wild fish species. These works furnished only a partial overview since they are focused on the detection of one antibiotic or a few classes of antibiotics and contaminants, considering as target fish different types of seafood, with no particular focus on salmon. Table 1 is a summary of the state of art about literature works regarding the analytes of interest in the edible salmon matrix. Based on the above-mentioned considerations, the aim of the present work is to investigate the presence of antibiotics and POPs, implementing the control framework on salmon, by developing multiclass and multiresidual HPLC-HRMS and GC-MS/MS methods to monitor a broad spectrum of residues then evaluating xenobiotic distribution in wild and farmed salmons from different geographic areas. This could add knowledge toward consumer's food safety and the possible exposure to contaminants via fish consumption.

Table 1. Summary of the state of art about the analytes of interest in the edible salmon matrix.

Reference	Investigated Compounds	Matrix	Analytical technique	LOD-LOQ CCβ-CCα (ng g^{-1})	Min and Max concentration range (ng g^{-1})
Antibiotics					
Pleasance et al. (1992)	Macrolides	**Salmon**	HPLC-ESI-MS/MS	0.5-1 (LOD)	No application
Rambla-Alegre et al (2010)	Quinolones	Gilthead **Salmon** Trout Sea bass Mussel Prawn Turbot	MLC	1-7 (LOD) 5-30 (LOQ)	Not found
Dickson et al. (2014)	Macrolides Lincosamides	**Salmon** Shrimp Tilapia	HPLC-MS/MS	0.5-3 (LOQ) 0.5-5 (LOD)	No application
Fedorova et al. (2014)	Tetracyclines β-lactams Quinolones Sulfonamides Phenicols Macrolides Lincosamides Diaminopyrimidines	**Salmon** Squid Sea bream Mussels Pangasius Mackerel Seawolf Sprat Carp Tuna Trout Herring Char Cod Tilapia Shark Crayfish Perch Shrimp	HPLC-MS/MS	0.062-4.6 (LOQ)	2.9-25
Done et al. (2015)	Tetracyclines Quinolones Sulfonamides Macrolides Cephalosporins Diaminopyrimidines	Shrimp Tilapia Trout **Salmon** Catfish Swai	HPLC-MS/MS	0.1-25.5 fw (LOD)	0.3- 112.5
Kim et al. (2017)	Quinolones Sulfonamides Macrolides Lincosamides Diaminopyrimidines	Rockfish Mullet Sea bream	HPLC-ESI-MS/MS	0.209-0.53 (MDL)	nd-3.16 (muscle)
Jia et.al. (2018)	Sulfonamides	**Salmon**	UHPLC/ESI Q-Orbitrap	0.07-2.33 (CCα) 0.04-1.34 (CCβ)	1.39-31.2

PFASs					
Kannan et al. (2005)	PFOS PFHS PFOA PFOSA	**Salmon** Whitefish Carp Trout	HPLC-ESI/MS/MS	7.5-75 (LOQ)	32-173
Berger et al. (2009)	PFHxS PFOS PFOSA PFOA PFNA PFDcA PFUnA PFDoA PFTriA PFTeA PFPeDA	Perch **Salmon** Trout Burbot Whitefish	HPLC/HRMS	0.02-0.30 (LOD)	0.05-10.1
Chang et al. (2012)	PFHxA PFOA PFNA PFDA PFUndA PFDoDA PFHxS PFOS PFOSA N-MeFOSA	Water Milk **Salmon** Beef Liver	UHPLC-MS/MS	0.12-26.3 (LOD) 0.54-65.6 (LOQ)	0.05-23.5
Rasmussen et al. (2017)	Element BFR PCB PFOS PFOA PFNA PFOSA	**Salmon** Halibut Prawns	HPLC-MS/MS	0.4-0.5 (LOD)	0.2-0.6 (PFC analysed only in halibut)
PCBs, PBDEs, OCPs					
Jacobs et al. (2002)	PCBs PBDEs DDT	**Salmon** Aquafeed Fish oil	GC/MS	0.1-0.5 lipid (LOD)	1-460 lipid
Johnson et al. (2007)	PAHs PCBs DDT	**Salmon**	GC/MS	0.16-4.2 lipid (LOD)	1300-27000 lipid
Kelly et al. (2008)	PCBs PCDDs PCDFs OCPs	**Salmon** Aquafeed	GC/MS	0.00015-0.030 lipid (LOD)	0.002-756 lipid
Shaw et al. (2008)	PBDEs	**Salmon**	GC-MS	0.001-0.010 ww (LOD)	0.4-1.4 ww
Cullon et al. (2009)	PCBs PCDDs PCDFs DDT	**Salmon**	GC/MS	-	9.07-56.09 ww
Montory et al. (2010)	PCBs PBDEs	**Salmon**	GC-MS	0.010 ww (LOD)	0.27-78 ww
Norli et al. (2011)	OCPs PCBs	**Salmon** Tilapia Carp Catfish	GC-MS	1-10 ww (LOQ)	0.7-149.9 ww
Vuorinen et al. (2014)	PCBs PBDEs PCDDs	**Salmon**	GC-MS	0.01–0.6 (LOQ)	0.001-110 ww

PCDFs	

n.d: not detected

1. Material and methods

1.1. Chemicals and reagents

All solvents, formic acid (98–100%), acetic acid (99.9%), sodium acetate, 25% ammonia solution, ammonium formate, trichloroacetic acid (TCA), and disodium hydrogen phosphate dihydrate, citric acid monohydrate and EDTA, to prepare EDTA-McIlvaine buffer solution, pH 4, were purchased from Merck KGaA, Darmstadt, Germany. Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The extraction cartridges Oasis WAX 3 mL, 60 mg for PFASs and Oasis HLB 3 mL, 60 mg for antibiotics were provided by Waters (Milford, MA, USA). SupelTM QuE Citrate (EN) tubes for POP extraction and SupelTM QuE-ZSEP (EN) tubes for the clean-up step were from Supelco (Sigma Aldrich, St.Louis, MO, USA).

Mixtures of non dioxin like-polychlorinated biphenyl (ndl-PCB) congeners (PCB 28; 52; 101; 138; 153 and 180) and PBDE congeners (PBDE 28; 33; 47; 99; 100; 153 and 154), PCB 209, internal standard (IS) for PCBs, and 3-fluoro-2,2,4,4,6- pentabromodiphenyl ether (FBDE), IS for flame retardants, were from AccuStandard (New Haven, USA). A mixture of 19 organochlorine pesticides (OCs) (aldrin, α-1,2,3,4,5,6-Hexachlorocyclohexane (α-HCH); hexachlorobenzene; β-1,2,3,4,5,6-Hexachlorocyclohexane (β-BHC); 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene (DDE), 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene, 4,4′-DDE; 1,1,1-Trichloro-2,2-bis[4-chlorophenyl]ethane 2,40-DDT; 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane 4,40-DDD; 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethan 4,40-DDT; endosulphan I; endosulphan II; endosulphan sulphate; endrin; heptachlor; heptachlor epoxide; lindane and trans chlordane) was purchased from Restek (Bellefonte, PA, USA). Organophosphorous pesticide (OP) standards of chlorpyriphos diazinon, disulphoton, ethoprophos, fenthion, mevinphos, methyl parathion and phorate were purchased from Sigma-Aldrich, St Louis, Mo, USA. Florisil (100e200 96 mesh) was provided by Promochem (Wesel, Germany). 4-nonylphenol (IS for OCs and OPs) was purchased from Merck (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

PFASs analysed by HPLC-HRMS system were both sulphonates and carboxylates: perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutane sulphonic acid (PFBS), perfluoroheptanoic acid (PFHpA), PFOA, perfluorohexane sulphonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), PFOS, perfluorododecanoic acid (PFDoA), perfluoroundecanoic acid (PFUnDA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA), and perfluorooctadecanoic acid (PFODA). All these compounds and the two ^{13}C-labeled internal standards (ISs) perfluoro-[1,2,3,4,5-^{13}C5]nonanoic acid (MPFNA) and perfluoro-[1,2,3,4-^{13}C4]octanesulfonic acid (MPFOS) were purchased from Chemical Research 2000 Srl (Rome, Italy).

Antimicrobial agents analised by HPLC-HRMS system: amoxicillin, ampicillin, benzylpenicillin, cefquinome, ceftiofur, cefalexin, ciprofloxacin, chloramfenicol, chlortetracycline, cloxacillin, danofloxacin, dicloxacillin, dimetridazole, doxycycline, enrofloxacin, florfenicol, florfenicol amine, flumequine, furaltadone, furazolidone, lincomycin, lomefloxacin, marbofloxacin, nalidixic acid, nitrofurazone, oxolinic acid, oxytetracycline, ronidazole, spyramicin, sulfadiazine, sulfathiazole, sulfadimethoxine, sulfadimidine, sulfamerazine, tetracycline, thiamphenicol, tiamulin, tilmicosine, tinidazole, trimethoprim, tylosin and enrofloxacin-d5 as the internal standards (IS) were purchased from Merck.

2.2 Standard solutions

The working solutions of POPs were prepared daily in hexane from stock solutions (10 µg mL^{-1}) stored at -20 °C. The working solutions of antibiotics and PFASs were prepared daily in methanol from stock solutions (1 µg mL^{-1}).

2.3. Sample collection

To carry out a survey that is representative of the situation of contamination by emerging contaminants, it has been decided to evaluate and examine several samples in relation to the species and types available on the market. We collected 66 samples of wild and farmed salmon from five geographical areas and three different FAO zones (Table 2): 25 farmed *Salmo salar* from Norway (FAO 27), 17 farmed *Salmo salar* from Scotland and 2 wild *Salmo salar* from Scotland- North East Atlantic (FAO 27), 14 wild *Oncorhynchus nerka* or *Sockeye salmon* from Canada (FAO 67), 5 wild *Oncorhynchus*

kisutch and 2 wild *Oncorhynchus keta* from USA- Pacific Ocean (FAO 77). In particular both medium (average weight 5.20 ± 0.57 Kg) and small size (average weight 3.11 ± 0.34 Kg) were considered. All samples were collected at the fish market of Milan (Italy) and the dead fish were transported to the laboratory where it was taken the abdominal (rich in deposit fat) and the dorsal part (part more muscular).

Table 2. Salmon sample details: number of samples, species, FAO area, country of origin and type.

Species	**FAO Area**	**Country**	**Farmed**	**Wild**	**Total**
Salmo salar	27	Norway	25	-	25
		Scotland	17	2	19
Red salmon (Oncorhynchus nerka)	67	Canada	-	15	15
Silver salmon (Oncorhynchus kisutch)	67	USA - Pacific	-	5	5
Keta salmon (Oncorhynchus keta)	77	USA - Pacific	-	2	2
Total			42	24	**66**

2.4. Sample extraction and purification protocol for antibiotics

The sample extraction and clean-up procedure carried out for antibiotics in 1 g samples of salmon muscle is the same reported in our previous works (Chiesa et al. 2017, 2018a and 2018b).

2.5. Sample extraction and purification protocol for PFASs

The pretreatment and extraction of 2 g sample for PFASs was the same described in our previous works (Chiesa et al. 2018c, 2018d).

2.6. Sample extraction for pesticides

The extraction of pesticides and contaminants was performed on 1 g samples using the QuEChERS method described in Chiesa et al. (2018d).

2.7. LC-HRMS Orbitrap analyses

HPLC- Q-Exactive Orbitrap equipped with a heated electrospray ionisation (HESI) source was used for detection and quantification of antibiotics and PFASs. HPLC analysis was performed by an HPLC system (Surveyor MS quaternary pump, Thermo Fisher Scientific, San Jose, CA, USA). A Synergi Hydro-RP reverse-phase HPLC column (150 × 2.0 mm, i.d. 4 μm), with a C18 guard column (4 × 3.0 mm; Phenomenex, Torrance, CA, USA) was used. The mobile phase used for the antibiotics separation consisted of a binary mixture of solvents A (aqueous HCOOH 0.1%) and B (MeOH), instead for PFASs were used C (aqueous NH_4COOH 20 mM) and B (MeOH). The two gradients and MS parameters were described in our previous works (Chiesa et al. 2018a, 2018c).

The full scan (FS) acquisition was combined with a data-independent acquisition (DIA) strategy, providing the MS^2 spectra for a confirmatory response.

Detection of the analytes was based on the calculated exact mass of the protonated/deprotonated molecular ions, and at least one specific and typical fragment. Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to acquire and elaborate data.

2.8. GC-MS/MS analyses

A GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) in electron ionization (EI) mode was used for the detection and quantification of pesticides and POPs in salmon samples. A fused-silica capillary column Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, Bellefonte, PA, USA) was used. The oven temperature program and all operation parameters were the same of our previous work (Chiesa et al. 2018d). The QqQ mass spectrometer was operated in selected reaction monitoring mode (SRM) detecting two-three transitions per analyte. The Xcalibur processing and instrument control software program and Trace Finder 3.0 for data analysis and reporting (Thermo Fisher Scientific) were used.

2.9. Validation on the basis of the different residues

Antibiotic validation was carried out following the Commission Decision guidelines 657/2002/CE (European Commission 2002). For POPs, including PFASs, validation was performed following the

European Commission (2015) SANTE/2015 guidelines. All the validation parameters and the assessment of validation protocols are already described in our previous works (Chiesa et al. 2018a, 2018d).

3. Results and discussion

3.1. Validation parameters

The methods showed high specificity and selectivity. The mean recoveries ranged between 70 and 120%, indicating the efficiency of the three extraction protocols, as reported in our previous works (Chiesa et al., 2018a, 2018d). Matrix validation curves demonstrated a good linearity with a good fit ($R^2 > 0.99$) for all compounds. Repeatability expressed as coefficient of variation (CV %), was lower or equal to 20 % for all analytes. Our satisfactory results showed high method sensitivity for the selected compounds both for LC-HRMS and GC-MS/MS analyses, with CCα (from 0.51 to 5.76 ng g^{-1} wet weight) and CCβ (from 0.65 to 5.93 ng g^{-1} wet weight) for antibiotics and LOQs (from 5.00 to 20.00 pg g^{-1} wet weight for PFASs and from 0.50 to 5.00 ng g^{-} wet weight for all contaminants and pesticides) well lower than the MRLs, where they are set.

3.2. Application of the methods

Table 3 shows the compounds detected in the collected salmon samples. About pesticides, the most have been found in farmed salmon than wild salmon (Table 3).

Table 3. Detected antibiotic and contaminant residues in farmed and wild salmon samples. Concentrations expressed as median, 1st and 3rd quartiles, median, minimum and maximum values are in ng g-1; % = prevalence of detected compound.

Detected compound	**Farmed N=42**					**Wild N=24**				
	%	**1st quartile**	**median**	**3rd quartile**	**min – max**	**%**	**1st quartile**	**median**	**3rd quartile**	**min – max**
PCB (ICES-6)										
PCB101	2	0	0	0	0.58	4	0	0	0	0.38
OCPs										
Aldrin	40	0	0	1.75	1.36-3.12	29	0	0	1.70	1.69-3.01
Endosulfan II	5	0	0	0	0.19-0.29	8	0	0	0	0.54-1.03

Endosulfan sulfate	45	0	0	1.80	0.30-2.31	1330	0	0	0	1.29-1.88
HCB	79	0.72	0.44	1.17	0.27-5.91	17	0	0	0	0.14-4.41
PBDEs										
PBDE 28	11	0	0	0	0.54-1.12	4	0	0	0	1.71
PBDE 33	4	0	0	0	0.71	8	0	0	0	1.44-1.65
PBDE 47	7	0	0	0	0.70-8.31			n.d.		
PBDE 99	29	0	0	0	0.41-6.01	29	0	0	0.68	0.65-9.01
PBDE 100			n.d.			4	0	0	0	0.76
PBDE 153	11	0	0	0	0.26-1.72			n.d.		
PBDE 154	11	0	0	0	0.62-0.77	8	0	0	0	0.56-0.83
PFASs										
PFBA	29	0	0	1.25	0.62-19.22	29	0	0	0.28	0.19-34.51
PFOA	48	0	0	2.30	0.83-8.52	13	0	0	0	0.86-2.91
PFOS			n.d.			4	0	0	0	1.77
Antibiotics										
Fenbendazole	26	0	0	2.64	1.06-51.52			n.d.		
Doxycycline	5	0	0	0	0.35-0.63			n.d.		
Nalidixic acid	5	0	0	0	0.57-0.87			n.d.		

n.d. =not detected

No OPs and polycyclic aromatic hydrocarbons (PAHs) were found although OPs are the most frequently used group of insecticides all over the world (Lavado and Schlenk 2011). They are one of the most common classes involved in poisoning because of the inhibition of acetyl-cholinesterase and even if, they are less persistent in the environment than organochlorine pesticides, they can also reach the food chain and may represent risk to human health (Lavado and Schlen 2011).

The same consideration must to be done about PAHs. Food can be contaminated by environmental PAHs that are present in air, soil or water, by industrial food processing methods (e.g. heating, drying and smoking processes) and by home food preparation. Few works in literature reported their presence in salmon (Table 1).

About PCBs, only PCB 101 has been detected in one farmed sample coming from Norway (0.58 ng g^{-1}). These results appear different from other studies present in literature (Jiacobs, Covaci, and Schepens 2002; Hites et al. 2004) where PCBs were detected with higher frequencies (Table 1). Regarding OCs, only 4 congeners were found out of all investigated compounds (Aldrin, Endosulfan II, Endosulfan sulfate, Hexachlorbenzene) (Table 3). Even if no MRLs are stated by EU for these pesticides, the conservative concentration of 0.01 mg kg^{-1} is set by Regulation (EC) 396/2005 (European Parliament 2005) as a default value in the absence of MRLs. The salmon samples never exceeded this limit; however, some consideration could be done about this result. Farmed salmon results more contaminated than wild salmon (Table 3). No DDD was found in this work and this result is different than literature where DDD and its congeners were the most common compounds detected (Table 1). The wild salmon migrates between freshwater and seawater during its life cycle. The spawning and juvenile rearing takes place in rivers and streams, and the main growth takes place in the sea. The juveniles spend 1–4 years in freshwater, migrate to the sea where they spend 1–3 winters and then return to their natal (parent) rivers or streams to spawn (Mills 1989). This makes them great swimmers and less exposed to a constant source of environmental pollutants. Farmed salmon, instead, lives in aquaculture under human control, closer to the coast and more exposed to pollution. It is to be underlined that farmed salmon has higher lipid content than wild salmon, making it more subjected to bioaccumulation to POPs, as it has been reported in a study by Hamilton et al. (2004) due to total lipid levels of 16.6% versus 6.6%. The highest detection has been found in European Countries (Norway and Scotland) then Pacific Countries. This result only partially agree with Hites et al. (2004), which concluded that levels of contaminants are higher in farmed salmon than wild and higher in European country than America.

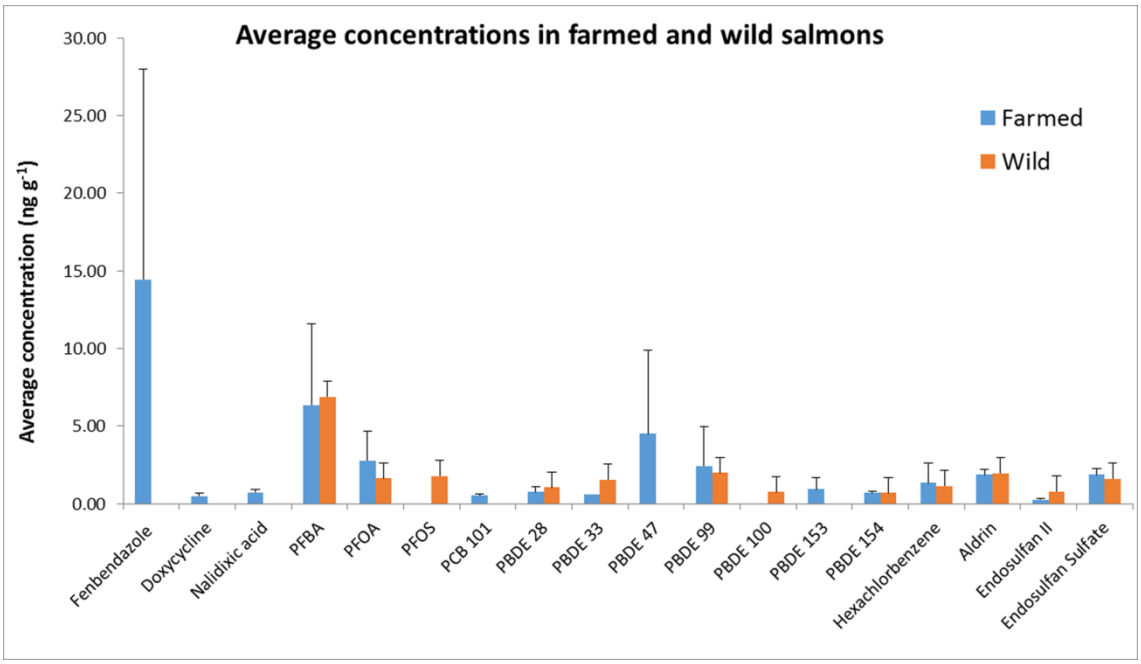
In this study, PBDEs were detected more frequently in farmed than wild salmons; the most recurrent congeners was PBDE 99, whose presence was however equal in farmed and wild salmons (Table 3). PBDEs are associated with liver, thyroid, reproductive and nervous system toxicity, (EFSA 2011). Based on the composition of PBDE mixtures, occurrence in the environment and in food, EFSA considers eight PBDE congeners of primary interest for dietary exposure and, among these, only -47, -99, and -153 show significant toxicity data derived from epidemiological data on human exposition and studies on behavior in mice. Due to the different kinetics between human and mice and to the body burden of these bioaccumulabile compounds, an approach based on the margin of exposure (MOE) was

used, concluding that a MOE larger than 2.5 between animal and human could indicate the absence of health concern. The mean chronic dietary exposure for average European consumers is 1.91 ng kg^{-1} b.w. day^{-1} (maximum upper bound) for BDE-47, 0.65 ng kg^{-1} b.w. day^{-1} for PBDE-99 and 0.42 ng kg^{-1} b.w. day^{-1} for PBDE-153. All the MOEs are larger than 2.5 so not representing a risk concern (EFSA 2011). The annual per capita salmon consumption reported by EUMOFA (European Commission 2017), is 2.17 kg corresponding to a daily intake of 5.95 g. The median concentrations we detected were always zero, so the mean values found in salmon are here considered: 140 ng kg^{-1} w.w., 440 ng kg^{-1} w.w. and 40 ng kg^{-1} w.w. for PBDE 47, 99 and 153 respectively, calculated both on farmed and wild. Under these considerations, an average consumer of 70 kg should be exposed to the three PBDE through salmon intake to: 140 x 0.00595/70=0.012 ng kg^{-1} day^{-1}, 440 x 0.00595/70= 0.037 ng kg^{-1} day^{-1} and 40 x 0.00595/70= 0.0034 ng kg^{-1} day^{-1}, respectively. The contribution of fish to alimentary exposition of PBDE-47 is about 80%, while 30% is the contribution of PBDE-99, while it is not specified the contribution for PBDE-153 (EFSA 2011). As salmon represents about the 9% of the annual consumption of fish (European Commision, 2017), the values considered safe by EFSA are to be recalculated on salmon contribution, i.e. 0.8 x 0.09= 0.07 for PBDE 47, 0.3 x 0.09 = 0.03 for PBDE 99, that are 7% and 3%, respectively of the total safe value. The recalculated safe values will be 1.91 x 0.07= 0.13 ng kg^{-1} day^{-1} (PBDE 47), and 0.65 x 0.03=0.02 ng kg^{-1} day^{-1} (PBDE 99). The safe value for PBDE 47 is still higher of salmon contribution while PBDE 99 presents a matter of concern.

As regards PFASs, only PFBA, PFOA and PFOS were detected. In particular PFBA was detected both in farmed and wild salmons (Figure 1), with a same incidence and similar concentrations detected in the second and third quartiles (Table 3). The highest concentration (34.51 ng g^{-1}) was related to one wild sample of Canada of little size (3.3 kg). The presence of the short-chain PFASs was already verified also in eels of Lake Garda (Chiesa et al., 2018c) and mussels and clams of different FAO zones (Chiesa et al. 2018d) due to the ineffectiveness of wastewater treatment and drinking water treatment plants at removing smallest PFASs.

PFOA was the compound found with the highest frequency in the farmed samples, at concentrations slightly higher than the wild ones (Figure 1).

Figure 1. Histogram of average concentrations (ng g^{-1}) of detected antibiotics, PFASs and environmental contaminants in farmed and wild salmons.



Only one wild samples of North-Atlantic Scotland showed PFOS at the concentration of 1.77 ng g^{-1}. If we compare our results to the literature works, we found only three PFASs in our real samples, even if our analytical limits were lower than others (Table 1). In accordance with Chang et al. (2012), the levels and the positive rates of PFOS were lower than that of PFOA, which might be attributed to recent restrictions and bans of the use of PFOS (European Commission 2007). Instead, their average concentration of PFOA was higher than ours. Our concentration of PFOA and PFOS were also well lower than those found in the liver of Chinook salmon, from Michigan waters (USA), found by Kannan et al. (2005). Our PFOA concentrations were higher than those reported from Berger et al. (2009) for salmons from Lake Vättern and the Baltic Sea.

For PFASs no MRLs are stated by European Union. The threshold doses, however, have been recently dramatically diminished. In 2015 the US Agency for the Register of Toxic Substances and Diseases (ATSDR) recommended a 'Reference Dose' (RfD) of 30 ng kg bw^{-1} day^{-1} for PFOS and 20 for PFOA ng kg bw^{-1} day^{-1}. In 2017, the Dutch National Institute for Public Health and the Environment (RIVM), stated a TDI of 12.5 ng kg bw^{-1} day^{-1} for PFOA (RIVM 2017). The TDI from RIVM is 120 times lower than the previous EFSA PFOA TDI = 1.5 µg kg^{-1} b.w. day^{-1} and substantially in line with the ATSDR RfD.

As above stated the salmon daily intake is 5.95 g and is the 9% of the total seafood consumed per capita, while contribution of fish consumption to PFASs exposure is the 10% of the threshold limit (RIVM 2017). This means that salmon alone contributes for 0.9%. Accounting for a chronic consumption and with a conservative approach, the highest value between mean and median was considered: 2.51 ng g^{-1} of PFOA resulting in a daily intake of 14.93 ng, that, for a person of 70 kg is 0.21 ng kg^{-1} day^{-1}. This value is much lower than the RIVM TDI, but if we consider only the contribution of salmon, it results to give a significant contribution to the human toxicological risk. The RIVM TDI should therefore multiplied by 0.009 to evaluate human toxicological risk limit deriving from PFOA in salmon. The Quality Standard for salmon should therefore be 0.11 ng g^{-1}, a value lower than the mean value we detected, meaning that the contribution of salmon to PFOA exposure is higher than it should be. An analogous calculation for PFOS, accounting for the ATSDR RfD (in absence of a RIVM TDI) gives an exposition of PFOS through salmon meals of 0.15 ng kg^{-1} $day^{-1}$and a contribution of salmon to RfD of 0.27 ng kg^{-1} $day^{-1,}$ thus not giving a cause for concern as regards PFOS.

As regard antibiotics, few traces of nalidixic acid and doxycycline were found in farmed salmons. These few detections might confirm the previous statement on the possibility of antibiotic dilution in the open sea (Chiesa et al. 2018a).

On the other hand, the anthelmintic fenbendazole was found with high incidence and high concentrations (if compared with the other compounds) only in farmed salmon, too (Table 3, Figure 1). On this regard, Kim et al. (2017) confirmed that the findings of this molecule were associated with the usage patterns of pharmaceuticals in terrestrial and aquaculture areas. Currently the MRLs for fenbendazole are established for all ruminants, porcine, equidae and recommended for chicken tissues. However, no information on the applicability of the analytical method to fish was available and therefore extrapolation of MRLs to fish are not recommended (EMA/CVMP/914694/2011 2013).

Conflict of Interest

No potential conflict of interest was reported by the authors.

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3.4 Levels and distribution of PBDEs and PFASs in pork from different European countries.

Published in: Food Additives & Contaminants: Part A, 2018, Volume 35, pages 2414-2423.

Luca Maria Chiesa[a], Shih-Kuo Lin[a,b], **Federica Ceriani**[a], Sara Panseri[a] and Francesco Arioli[a]

[a] Department of Health, Animal Science and Food Safety, University of Milan, Italy.

[b] Council of Agriculture, Executive Yuan, Bureau of Animal and Plant Inspection and Quarantine, Taipei City, Taiwan.

Keywords: GC-MS/MS, LC-HRMS, PBDEs, PFASs, Pork, food safety

Abstract

Meat and meat products are included in a great number of human diets. However, the great consumption of meat needs to be controlled for the presence of traces of contaminants. The European Commission has not stated maximum limits (MLs) for some environmental pollutants such as the perfluoroalkyl substances (PFASs) and polybrominated diphenyl ether (PBDEs); the European Food Safety Authority (EFSA) Scientific Panel has recommended that more occurrence data for PFASs in food should be collected to improve the accuracy of future exposure calculations. Therefore, the distribution of PFASs and PBDEs traces from eight EU Member States were investigated through liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) and Gas Chromatography-Mass Spectrometry (GC-MS/MS). No perfluoroalkyl substances were detected, except PFOA, in only one Austrian sample at the concentration of 0.531 ng g^{-1}. PBDEs were detected in three out of 77 samples: the one came from Germany showed the presence of all congeners analyzed in the concentration range 0.53-0.77 ng g^{-1}, the ones from Netherland and Italy, respectively PBDE 153 (0.53 ng g^{-1}) and PBDE 100 (0.62 ng g^{-1}). The results show that the analyzed samples do not pose a risk for human beings about PFASs and PBDEs. Further studies are needed to keep monitoring their presence in foodstuff, as it has been suggested by European Commission.

1. Introduction

Generally, food of animal origin, plays an important role in determining the exposure of human beings to contaminants of chemical origin (Vogt et al.,2012). The PFASs and PBDE contamination in food are a global issue of environmental pollution. PBDEs are one class of brominated flame retardants (BFRs) that can be released from manufacturing commercial products (e.g. acrylonitrile-butadiene-styrene and polystyrene plastics, polyurethane foams), packaging materials, electronic devices, as computers or televisions. PBDEs can be released into the air, water, and soil at places where they are produced or used, but they have very low water solubility, and when these substances are released to water, they typically bind to sediment (ATSDR, 2011). These substances generally bind strongly to soil particles, and therefore, do not move easily through soil layers (Routti et al., 2015).

Perfluoroalkyl substances, such as perfluorooctane sulfonate (PFOS), represent a class of compounds showing high thermal, chemical, and biological inertness. Their application began in the early 1950s and, due to their widespread use, they are globally found in the environment, both in animals and in humans (Routti et al., 2015). Many countries, e.g., the Germany, French, Denmark and Spain, reported the results of PFASs analysis from human serum samples (Ingelido et al., 2010) and other animals (Chiesa et al., 2018), where they found very low concertation with the average about 15 pg g^{-1} in pork and higher in fish where they reach 45 ng g^{-1} (Table 1). The highest concentrations are found near densely inhabited areas due to discharge of industrial and municipal wastewater and fire-fighting operations (Lindstrom et al., 2011; Zacs et al., 2016). On the basis of their properties, the EFSA proposed tolerable daily intake (TDI) levels for PFOA (1,500 ng kg^{-1} body weight per day) and PFOS (150 ng kg^{-1} body weight per day) (EFSA, 2012) due to their adverse effects in experimental animals and due to dietary exposure has been suggested as the main exposure route to PFASs.

Most of information regarding toxicity of PBDEs and their metabolites is from animal studies that show developmental neurotoxicity and endocrine disruption (Costa et al., 2007; Darnerud et al., 2008). In one study has been shown the effects of PBDEs in humans.

Table 1. The literature data on PFOA, PFOS and PBDE.

Investigated Compounds	Author	Analytical technique	Sample matrix	Producing area	Concentration or range
PFOA	Ingelido et al. 2010	HPLC-MS	Human serum	Italy	5.77 ng g^{-1} h.w.
	Noorlander et al. 2011	LC-MS/MS (ESI)	Pork	Netherland	Average 15 pg g^{-1} w.w.
	Domingo et al. 2012	UPLC-MS/MS	Meat, meat products	Spain	<300 pg g^{-1} f.w.
.	Guerranti et al. 2013	HPLC-MS/MS	Pork	Italy	n.d. <LOD w.w.
PFOS	Noorlander et al. 2011	LC-MS/MS (ESI)	Pork	Netherland	14 pg g^{-1} w.w.
	Domingo et al. 2012	UPLC-MS/MS	Meat, meat products	Spain	34 pg g^{-1} f.w.
	Guerranti et al. 2013	HPLC-MS/MS	Pork	Italy	0.74 ng g^{-1} w.w.
PBDE	Bocio et al. 2003	GC/MS	Pork, pork products	Spain	172 ng g^{-1} w.w.
	Perelló et al. 2009	HRGC/HRMS	Loin of pork	Spain	7.05 ng kg^{-1} f.w.
	Törnkvist et al. 2011	GC-MS/MS	Meat	Sweden	0.023 ng g^{-1} f.w.
	Gong et al. 2014	GC/MS	Pork	China	0.32173±0.75326 ng g^{-1} w.w.

Analytical technique: f.w.: fresh weight; h.w.: whole weight; w.w.: wet weight; n.d.: not detected.

The authors detected four congeners (PBDEs 47, 99,100, 153) in greater than 97% of woman serum samples analysed and found significant decreases in fecundability associated with PBDE exposure in women (Harley et al., 2010). The EFSA Panel on Contaminants in the Food Chain (EFSA, 2011) considers eight PBDE congeners to be of primary interest: PBDE-28, -47, -99, -100, -153, -154, -183 and -209 and the levels of PBDE-209 were the highest in almost all the food categories studied. In 2008, the United States Environmental Protection Agency (EPA, 2009) issued health assessments of four individual PBDE congeners, PBDE-47, -153, -99 and -209, within its Integrated Risk Information System (IRIS) program. The dominant food category that is exposed to PBDE, is food with high fat

content, because there is a relationship between the PBDEs levels and the fat content (EFSA, 2011). In 2012–13 an US meat and poultry (beef, pork, chicken, turkey) study has been reported that the mean summed concentrations of seven PBDE congeners from beef, pork, chicken and turkey were 0.40, 0.36, 0.19, and 0.76 ng g^{-1} lipid weight (lw), suggested that the US consumer daily intake of PBDEs from meat and poultry was 6.42 ng day^{-1} (Lupton SJ and Hakk H. 2017).

Meat and meat products are included in a great number of human diets. Their regular consumption means a significant intake of proteins and essential micronutrients. In addition, pork meat is used in many countries to produce derivative products (hams and cured meats) with high qualitative value and relative recognition as Protected Designation of Origin (PDO) and the Protected Geographical Indication (PGI) products.

However, the great consumption of pork meat (Table 2) needs to be controlled for the presence of residues. EU has not stated Maximum Limits (MLs) for PBDEs and PFASs; the EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) recommended that more occurrence data for PFASs in food should be collected to improve the accuracy of future exposure calculations (EFSA,2008). Subsequently, the European Commission issued the Commission Recommendation 2010/161/EU on the monitoring of PFASs in food in the Member States (EFSA,2012). It's, therefore important, give information on the presence of these pollutants in food, mainly in those products whose consumption is highest (Table 2). EFSA's CONTAM Panel acknowledged that there were significant data gaps on issues such as the contribution of different foodstuffs, among which pork, to human exposure and that further research and data collection would be necessary (EFSA,2008).

Table 2. Intake of pork meat of European countries (g/capita/day) and fresh water fish.

Region	Pork intake: year 2000	Pork intake: year 2013	Freshwater Fish intake 2000	Freshwater Fish intake 2013
European Union	113	107	6	10
Austria	165	144	5	11
Denmark	74	68	8	2
France	103	91	9	12
Germany	145	142	6	12
Netherland	149	100	5	8
Italy	103	110	5	8
Poland	131	127	4	5
Spain	175	134	5	11

Data sources: Food Supply - Livestock and Fish Primary Equivalent, provided by Food and Agriculture Organization of the United Nations (FAO).

Toxicological studies show that PFOS and PFOA are adsorbed after oral exposure and primarily accumulate in the serum, kidney and liver (EFSA,2008). Perfluoroalkyls tend to remain in the body unchanged for long periods. It takes approximately 4 years for the level to halve, so the constant exposure could increase the levels in the organism resulting in adverse overcome (ATSDR, 2009). People could be exposed to PBDEs in a wide variety of ways, including foods or dusts/soils, air, or through skin contact. The toxicokinetic of PBDEs depends on the number and position of the bromine atoms: the more toxic congeners are the lower brominated PBDEs, due their ability to bioaccumulate, mainly in body fat, consequently decabromodiphenyl ether is expected to be less toxic than lower-brominated PBDEs. Nowadays, the effects of PBDEs are not all well established and it is not known if PBDEs are carcinogens to human. However, the International Agency for Research on Cancer (IARC)

has classified PBDE as a Group 3 carcinogen based on inadequate evidence of carcinogenicity in humans and inadequate or limited evidence in experimental animals (ATSDR, 2011). So, based on the EFSA recommendation, in this paper we investigated the presence of PFASs through LC-HRMS and PBDE through GC-MS/MS in pork samples from eight EU Member States, to improve the knowledge on data gap of these compounds in literature.

2. Materials and Methods

2.1. Chemicals and reagents

The ^{13}C-labeled PFOS (MPFOS) and ^{13}C-labeled Perfluorononanoic acid (MPFNA), which were used as the internal standard (IS) in this study, and the 17 PFASs derivatives the Perfluorobutyric acid (PFBA), Perfluoropentanoic acid (PFPeA), (Perfluorobutane sulfonate acid), Perfluorohexanoic acid (PFHxA), Perfluoroheptanoic acid (PFHpA), Perfluorooctanoic acid (PFOA), Perfluorononanoic acid (PFNA), Perfluorodecanoic acid (PFDA), Perfluorooctane sulfonic acid (PFOS), Perfluoroundecanoic acid (PFUdA), Perfluorododecanoic acid (PFDoA), Perfluorodecane sulfonic acid (PFDS), Perfluorotridecanoic acid (PFTrDA), Perfluorotetradecanoic acid (PFTeDA), Perfluorohexadecanoic acid (PFHxDA), Perfluorooctadecanoic acid (PFODA), Perfluorobutane sulfonate acid (PFBS) and Perfluorohexane sulfonic acid (PFHxS), which were used for standard curve constructions, were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Mixtures of polybrominated diphenyl ether (PBDE) congeners (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154) and 3-fluoro-2,2',4,4',6-pentabromodiphenyl ether (FBDE) as IS for PBDEs were purchased from AccuStandard (New Haven, USA). All standard purity was greater than 98%. Hexane and acetone (special grade for pesticide residue analysis (Pestanal) were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA). Each solvent is in HPLC or analytical grade. Purified water was supplied from Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The solid phase extraction cartridges (Oasis WAX 3 mL, 60 mg) were bought from Waters^{TM} (Milford, MA, USA). The ammonium formate, sodium acetate, acetic acid (99.9%) and 25% ammonia solution were purchased from Fluka. QuEChERS materials for the extraction were obtained from Supelco (SigmaeAldrich, St.Louis, MO, USA); Supel^{TM} QuE Citrate (EN) tubes, containing Sodium Citrate tribasic dihydrate and Sodium Citrate dibasic sesquihydrate. Magnesium Sulphate and Sodium Chloride were used for the extraction. Supel^{TM} QuE-Z SEP (EN) tubes were used for the clean-up step.

2.2. Standard solutions

To make the stock solution, each of 17 standard PFASs compounds were prepared for 1 mg mL^{-1} concentration in methanol and store at -20 °C. The working solutions which were diluted from the stock solution at concentrations of 10 ng mL^{-1} and 100 ng mL^{-1} in methanol were freshly prepared before use and store at 4 °C.

Working solutions of PBDEs were prepared by diluting the stock solution in hexane for pesticides and then stored at -20 °C. An uncontaminated meat sample (previously checked for the presence of PBDEs and considered blank with a concentration of compounds less than limit of detection (LOD) used as control was selected for all procedure's optimization steps. For meat fortification, 1.0 g of the control sample was spiked by adding an appropriate volume of the standard working solution to cover the concentration range from 0.5 to 10 ng g^{-1} (five calibration points: 0.5, 1, 2, 5, 10 ng g^{-1}) for PBDEs in relation to literature to realise the matrix-matched calibration curves.

2.3. Sample collection

The muscle samples belonged to pigs from the food chain weighing 130 to 160 kg, and, to minimize the damage to the carcass, the used muscles were obliquus internus abdominis and obliquus externus abdominis. Seventy-seven frozen samples from eight different European countries (Austria, Denmark, French, Germany, Holland, Italy, Poland and Spain) were collected. The samples were homogenized and then stored in -20 °C refrigerator; they were defrosted before being analyzed. The date of sample collection was from Dec 5th, 2016 to May 5th, 2017.

2.4. Sample extraction of PFASs

Weight 1.0 g of homogenized sample into a 15-mL polypropylene screw-cap centrifuge tube. Add 50µL of internal standard solution (which contains 100 ng mL^{-1} MPFNA and 100 ng mL^{-1} MPFOS in methanol) into the tube, to proceed a final concentration of 5 ng mL^{-1} over the matrix. Shake the tube by hand to mix it with the sample matrix. Add 10 mL of acetonitrile, vortex for 1 minute, then put the tube into the water tank with ultrasonication for 30 minutes in room temperature. Ultrasonicated samples were centrifuged at 4,612×g, 4 °C, for 10 minutes. Transfer all supernatant liquid into the evaporation flask and dried it with rotary vacuum evaporator at 35 °C. Add 10 mL of Milli-Q water into flask and resuspend the analyte by vortex for 10 seconds. Load the re-suspended liquid into

Waters^{TM} WAX SPE cartridge, which was previously conditioned with 3 mL of 0.05mL mL^{-1} NH_4OH in methanol, followed with 3 mL of methanol, and 3 mL of Milli-Q water. After sample liquid running out through the cartridge, flush the cartridge through 3mL of 25mM acetate buffer pH 4.5 to release proteins and lipids from cartridge, followed with 2 mL of methanol. Elute the cartridge with 3 mL of 0.05mL mL^{-1} NH_4OH in methanol and transfer the eluted liquid into evaporation flask then dried it with rotary vacuum evaporator at 35 °C. The dried analyte was solved with 100 µL of methanol:ammonium formate 20 mM (10:90 v/v) to reconstruct the final volume. Transfer liquids into a screw vial and perform for the analysis with LC-HRMS.

For the estimation of recovery ratio, use blank pork samples of 1.0 g, divided into group A and B. In Group A, spike into matrix with 50 µL of internal standard solution (which contains 100 ng mL^{-1} MPFNA and 100 ng mL^{-1} MPFOS in methanol) into the tube, to proceed a final concentration of 5 ng mL^{-1}, and with 10 µL of 17 PFASs mixture (each single compound contains 100 ng mL^{-1}) to proceed a final concentration of 1 ng mL^{-1}, then run the extraction procedure. In the Group B, spike the internal standard solution of 50 µL into matrix, then run the extraction procedure. While solid phase extraction finished, spike the 10 µL of 17 PFASs mixture into eluted liquid. Use LC-HRMS to determine concentration of each PFASs then calculate the ratio of same PFASs between Group A and B.

For coefficient of variation of intra-day (repeatability), and inter-day (reproducibility) evaluation, use blank pork samples of 1.0 g, spike into matrix with 50 µL of same internal standard solution into the tube, to proceed a final concentration of 5 ng mL^{-1}, and 10 µL of 17 PFASs mixture (each single compound contains 100 ng mL^{-1}) to proceed a final concentration of 1 ng mL^{-1}, then run the extraction procedure. Use LC-HRMS to determine the concentration of each PFASs from each tube and calculate the value of each PFASs for the coefficient of variation of intra-day (repeatability) and inter-day (reproducibility).

2.5. Sample extraction of PBDEs

The extraction of PBDEs was performed using the QuEChERS (quick, easy, cheap, effective, rugged and safe) method. Briefly, 1.0 g of sample was homogenized and transferred to a QuEChERS extraction tube, then a solution containing the ISs (FBDE) was added to the sample to a final concentration of 100 ng g^{-1}. 10 mL of acetonitrile were added as extraction solvent; the tube was shaken for 1 min using a vortex and centrifuged for 10 min at 4,612×g at 4 °C. Later, the supernatant

was transferred to a QuEChERS clean-up tube, shaken and centrifuged at the same conditions described above. The extract was collected, divided into two aliquots and dried under vacuum in a centrifugal evaporator at a temperature of 35 °C. The residue was dissolved in 200 µL hexane for the analysis by GC-MS/MS.

2.6. LC-HRMS Orbitrap analyses

The LC-HRMS analysis was performed by an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), composed with a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with a 20-µL loop. Chromatographic separation was carried out using a Synergi Hydro RP reverse-phase HPLC column (150 x 2.0 mm, internal diameter 4 µm), with a C18 guard column (4 x 3.0 mm; Phenomenex, Torrance, CA, USA). To minimize PFASs background contamination in the system, use stainless steel column tubes and peeks. Moreover, since PFOA and PFOS were always present in the blank of the chromatographic system, we mounted a small Megabond WR C18 column (5 cm x 4.6 mm, i.d. 10 µm) between pump and injector to delay our analytes of two minutes than those already present in the system.

The mobile phase used for the gradient consisted of a programmed mixture of solvents A (aqueous ammonium formate 20 mM), and B (Methanol). The elution started with 10% B, which increased to 40% in 4 min. Subsequently, the mobile phase B was gradually increased to 95% at the 12^{th} minute, which remained constant up to the 18^{th} minute. The initial conditions were reached at the 20^{th} minute, with an equilibration time of 7 min. The run was performed at flowrate of 0.3 mL min^{-1}.

The detector was a Thermo Q-Exactive Plus (Thermo Scientific, San Jose,CA, USA), equipped with an heated electrospray ionization (HESI) source. Capillary temperature and vaporizer temperature were set at 330 °C and 280 °C, while the electrospray voltage was set at 3.50 kV operating in negative mode. Sheath and auxiliary gas (nitrogen) were set at 35 and 15 arbitrary units, with S lens RF level of 60. Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HPLC-HRMS system. The exact mass of the compounds was calculated using Qualbrowser program in Xcalibur 3.0 software. Instrument calibration was done every analytical session with a direct infusion of a LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA).

The Full scan (FS) acquisition was combined with an Independent Data Acquisition (DIA) mode, providing the MS2 spectra for confirmatory response, based on an inclusion list.

The resolving power of FS was set at 70,000 FWHM. In consideration of molecular weight to our compound list, a scan range of m/z 200–950 was chosen; the automatic gain control (AGC) was set at 1 x 10^6 and the maximum injection time was 200 ms. The DIA segment operated in negative mode at 35,000 FWHM.

Detection of analytes was based on retention time of target compounds, on calculated exact mass of the deprotonated molecular ions, and at least one specific and typical fragment (Table 3). The formula of the compounds, with the exact theoretical mass of the parents and the diagnostic transition used to confirm the different PFASs are reported in Table 3. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

Table 3 Formula, exact theoretical mass of the parents, diagnostic transitions and validation parameters of the selected PFASs. The electrospray ionization (ESI) is set as negative.

Compound*	Name	Formula	Exact mass [m/z]	Transition [m/z]	LOD (pg g^{-1})	LOQ (pg g^{-1})	Recovery (%)	intra-day CV (%) (n=5)	inter-day CV (%) (n=7)
PFBA	Perfluorobutyric acid	$C_4HF_7O_2$	212.9792	168.98836	10	30	99	6	20
PFPeA	Perfluoropentanoic acid	$C_5HF_9O_2$	262.97601	218.98560	10	30	104	15	14
PFBS	Perfluorobutane sulfonate acid	$C_4F_9HO_3S$	298.94299	98.95434	5	15	119	19	20
PFHxA	Perfluorohexanoic acid	$C_6HF_{11}O_2$	312.97281	268.98288	10	30	112	11	15
PFHpA	Perfluoroheptanoic acid	$C_7HF_{13}O_2$	362.96962	318.97949	5	15	109	7	10
PFHxS	Perfluorohexane sulfonic acid	$C_6F_{13}HO_3S$	398.9366	98.95437	5	15	101	19	20
PFOA	Perfluorooctanoic acid	$C_8HF_{15}O_2$	412.96643	368.97681	8	24	114	8	11
PFNA	Perfluorononanoic acid	$C_9HF_{17}O_2$	462.96323	418.97385	20	60	110	8	11
PFOS	Perfluorooctane sulfonic acid	$C_8F_{17}HO_3S$	498.93022	79.95598	10	30	84	13	17
PFDA	Perfluorodecanoic acid	$C_{10}HF_{19}O_2$	512.96004	468.97064	28	84	87	5	9

PFUdA	Perfluoroundecanoic acid	$C_{11}HF_{21}O_2$	562.95684	518.96729	30	90	87	13	20
PFDS	Perfluorodecane sulfonic acid	$C_{10}F_{21}HO_3S$	598.92383	79.95593	50	150	81	10	15
PFDoA	Perfluorododecanoic acid	$C_{12}HF_{23}O_2$	612.95365	568.96436	5	15	80	12	20
PFTrDA	Perfluorotridecanoic acid	$C_{13}HF_{25}O_2$	662.95046	618.96094	30	90	80	8	16
PFTeDA	Perfluorotetradecanoic acid	$C_{14}HF_{27}O_2$	712.94726	668.95795	50	150	83	10	15
PFHxDA	Perfluorohexadecanoic acid	$C_{16}HF_{31}O_2$	812.94088	768.95093	50	150	80	9	13
PFODA	Perfluorooctadecanoic acid	$C_{18}HF_{35}O_2$	912.93449	868.94507	50	150	80	16	20

*= reported in alphabetic order

2.7. GC-MS/MS analyses

Triple quadrupole mass spectrometry (QqQ) in electron ionization (EI) mode was used for the simultaneous detection and quantification of PBDE in meat samples.

A GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) was used to confirm and quantify residues in meat samples by using a fused-silica capillary column Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, Bellefonte, PA, USA). The oven temperature program was as follows: initial temperature of 80 °C, held for 3 min, and increased to 170 °C at 10 °C min^{-1}; then, increased from 170 °C to 190 °C at 3°C min^{-1}, and raised to 240 °C at 2 °C min^{-1}, before being ramped to 280 °C at 3 °C min^{-1} and finally from 280 °C to 310 °C at 10°C min^{-1} and held at this temperature for 5 min. The carrier gas (Helium, purity higher than 99.999%) was in constant flow mode at 1.0 ml min^{-1}. A volume of 1 μL was injected using a programmed temperature vaporiser injector (PTV) in splitless mode with a 1-min splitless period and the following inlet temperature programme: 80 °C (0.05 min), 14.5 °C s^{-1} to 200 °C (1 min) and 4.5 °C s^{-1} to 320 °C (12 min – cleaning phase). A baffle liner (2 mm × 2.75 mm × 120 mm, Siltek-deactivated; Thermo Fisher Scientific) was used. The transfer line was maintained at 270 °C and the ion source at 250 °C. The electron energy and emission current were set to 70 eV and 50 μA, respectively. The scan time was 0.3 s and the peak width of both quadrupoles was 0.7 Da full widths at half maximum. Argon was used as a

collision cell gas at a pressure of 1.5 mTorr. The QqQ mass spectrometer was operated in selected reaction monitoring mode (SRM) detecting two-three transitions per analyte. Identification of PBDEs was carried out by comparing sample peak relative retention times with those obtained for standards under the same conditions and the MS/MS fragmentation spectra obtained for each compound.

The Xcalibur^{TM} processing and instrument control software program and Trace Finder 3.0 for data analysis and reporting (Thermo Fisher Scientific) were used.

2.8. Analytical performances and method validation

The validation was carried out following the European Commission SANTE/2015 guideline (SANTE, 2015). SANTE/2015 has been superseded by SANTE/2017 (SANTE, 2017). For the PFASs, the method showed high specificity, without interference signals close to the retention time of the analytes, and consequently showed a high signal-to-noise (S/N) ratio in presence of analytes even at concentrations in the order of pg g^{-1}. Selectivity demonstrated a good compliance with the relative retention times for each analyte, which in our case were within 2.5% tolerance, with a S/N ratio greater than 3 when compared with the standard solution mix, both in FS and MS2 chromatograms. Moreover, diagnostic fragments showed an ion ratio within the recommended tolerances (European Commission, 2002).

The mean recoveries for all analytes ranged between 80 and 117%, indicating the efficiency of the extraction protocol.

By searching the S/N ratio of each sample, the LOD values of 17 PFASs were from 5 pg g^{-1} to 50 pg g^{-1}, the limit of quantification (LOQ) values were from 15 pg g^{-1} to 150 pg g^{-1}.

Matrix validation curves were linear over the working range demonstrating a good fit for all analytes with an R^2 value greater than 0.99. Precision in terms of intra- and inter-day repeatability (Thompson et al., 2010) was calculated using one-way analysis of variance ANOVA, expressed as coefficients of variation (CVs), and was below 19 and 21%, respectively.

About PBDE, the selectivity of the method was evaluated by injecting extracted blank meat samples. The absence of interferences was proved by the lack of peaks with a S/N ratio higher than 3 at the retention times of the target compounds. Pork samples, previously analysed and checked for the absence of all PBDEs, were used as control samples during the optimisation and validation procedure.

For the LOQ of the methods, we used the lowest validated spiked level meeting the requirements of recovery within the range of 70–120% and an RSD less than or equal to 20%, as defined by the European Commission (EC, 2002). Finally, the extraction methods were also evaluated for their repeatability, linearity and recovery. Recoveries were calculated at LOQ for all compounds. (Table 4). The repeatability as CV% was calculated by analysing six replicates at the same fortification level.

Table 4. The retention times (Tr), precursor ions (m/z), product ions (m/z), Collision Energy (V), Recovery (%), LOQ (ng g^{-1}) of investigated polybrominated diphenyl ethers (PBDE).

Compound	Name	Formula	Mass [m/z]	Tr minute	Precursor ion [m/z]	Product ion [m/z]	Collision energy (V)	LOQ ng g^{-1}	Recovery (%)	Intra-day CV (%) n=6	inter-day CV (%) n=6
PBDE 28	2,4,4'-Tribromodiphenyl ether	$C_{12}H_7Br_3O$	406.9	32.35	248	139	30	0.5	88	4	8
					246	**139**	30				
					408	246	10				
PBDE 33	2,3',4'-Tribromodiphenyl ether	$C_{12}H_7Br_3O$	406.9	31.95	246	139	30	0.5	89	4	10
					248	**139**	30				
					406	246	10				
PBDE 47	2,2',4,4'-Tetrabromodiphenyl ether	$C_{12}H_6Br_4O$	485.8	38.52	326	217	30	0.5	91	3	7
					328	219	30				
					482	**326**	20				
PBDE 99	2,2',4,4',5-Pentabromodiphenyl ether	$C_{12}H_5Br_5O$	564.7	41.27	404	297	30	0.5	89	1	5
					406	297	30				
					563	**404**	20				
PBDE 100	2,2',4,4',6-Pentabromodiphenyl ether	$C_{12}H_5Br_5O$	564.7	42.01	404	297	30	0.5	90	7	10
					406	297	30				
					564	**404**	10				
PBDE 153	2,2',4,4',5,5'-Hexabromodiphenyl ether	$C_{12}H_4Br_6O$	643.6	43.70	482	324	30	0.5	93	3	6
					484	**377**	30				
					642	482	20				
PBDE 154	2,2',4,4',5,6'-Hexabromodiphenyl ether	$C_{12}H_4Br_6O$	643.6	44.91	**484**	**324**	30	0.5	92	3	2
					486	326	30				
					644	484	20				

*=The precursor ion and product ion value reported in **bold** indicates the diagnostic transition.

3. Results and discussion

3.1. Method validation parameters

The methods showed high specificity, without any interference close to the retention time of each compound, and consequently a S/N ratio great than or equal to 3 in the presence of analytes was confirmed, even at the lowest detectable concentration demonstrating good selectivity. Matrix validation curves show good linearity over the working range with a good fit (R^2 greater than 0.99) for all compounds. The mean recoveries (from 80 to 119%), with the other validation parameters, are reported in Tables 1 and Table 2. The CVs % are below 19% and 21%, satisfying the criteria required by the European Commission (EC, 2002) and specified by Thompson et al, (2010). Regarding the LOD and LOQ for PFASs and for PBDEs, our satisfactory results show high method sensitivity for the selected compounds both for LC-HRMS and GC-MS/MS analyses.

3.2. Application to pork samples

Overall results in terms of number detected, concentration levels and distribution of contaminant residues in the pork samples investigated are summarised in Table 5.

Table 5. Quantification results of chemical residues from different production areas.

Production area	**N° of Sample**	**Analyte detected**	**Concentration (ng g^{-1} fresh weight)**
Austria	7	PFOA (n=1)	0.53
Denmark	8	n.d.	-
France	8	n.d.	-

		PBDE 28		0.57
		PBDE 33		0.73
		PBDE 47		0.60
Germany	10	PBDE 99	(n=1)	0.74
		PBDE 100		0.77
		PBDE 153		0.70
		PBDE 154		0.53
Netherland	8	PBDE 153	(n=1)	0.53
Italy	20	PBDE 100	(n=1)	0.62
Poland	8	n.d.		-
Spain	8	n.d.		-

n.d.=Not detected

Based on results of 77 samples, only PFOA was detected in an Austrian sample with the concentration of 0.531 ng g^{-1}. PBDEs were detected in three out of 77 samples; only one, coming from Germany, showed the presence of all congeners analysed with the range concentration from 0.53 to 0.77 ng g^{-1}. In the other two samples, coming from Netherland and Italy, only one congener was detected, respectively PBDE 153 (0.53 ng g^{-1}) and PBDE 100 (0.62 ng g^{-1}).

Based on our results some consideration could be made. EU has not stated MLs for PBDEs in food, due to the risk characterization has not been defined about PBDEs in people, thought recent studies have evaluated associations between PBDE concentrations in human tissues (e.g., blood, human milk) and health effects (immunological, reproductive, developmental, genotoxic and carcinogenic effects) (ATSDR, 2011). Comparing our results on literature, in this work has been found very low concentration than other study about the presence of PBDEs in pork meat coming from Spain (109 ng g^{-1}) (Bocio et al., 2003), Catalonia (32.3 ng g^{-1}) (Perellò et al., 2009); Sweden (63.6 ng kg^{-1}) (Domingo et al., 2004) and (8.074 ng g^{-1}) China (Gong et al., 2014).

Due to their lipophilicity, Törnkvist et al., 2011 have shown that the highest contributors to the total of PBDEs intake were fish (39%) and dairy products (31%), followed by meat (17%). Vouriner et al.

2012, studied the biomagnification of PBDEs in Atlantic salmon from three areas of the Baltic Sea and they demonstrated that PBDE accumulation is dependent on both age and fat content.

Humans can be exposed to PBDEs in a wide variety of ways. The main routes of exposure is from the contaminated foods, environment (air, soils) and skin contact with contaminated products. Several studies indicate that infants and toddlers have higher exposures to PBDEs compared to children or adults, due to their smaller weight and their frequent skin contact with the floor dust (ATSDR, 2011). Information on PBDE dietary intake is very scarce in literature. It is also important to note that we analysed fresh meat, whereas preparation and different cooking methods can influence the levels of contaminants and so also consumers exposure. It has been observed that during the cooking process PBDE losses were higher than other POPs probably to lipid remove during the process. (Perello et al.,2009). Pork is widely used into the market, mostly due to its products. In almost all the places that we have included in our paper we have distinctive derived products in which the amount of PBDEs could be increase/reduce to the industrial processing method (ATSDR, 2011). On the base on this consideration we could suppose that the human intake, on the base on our results, don't pose a risk for human beings. It reasonable defined that a risk could be present due to the long exposure to this compound.

About PFOA we could do a similar consideration. PFOA are a class of chemical compounds that due to their chemical structure, are very stable in the environment and resistant to biodegradation and hydrolysis (ATSDR, 2009). In living organisms, perfluoroalkyls, unlike PBDEs, bind to protein albumin in blood, liver, and eggs, but do not accumulate in fat tissue. Due to their hydrolytic properties are more present in water environment and tend to be much present in fish than other products.

PFOS in our samples did not appear concerning, in fact it was found to be predominant compound in fish samples (Chiesa et al., 2018; Squadrone et al., 2015, Guerranti et al., 2013), although other studies have found low concentrations in pork (15 pg g^{-1}; 0.74 ng g^{-1}) (Guerranti et al., 2013; Noorlander et al., 2011).

The PFOA concentrations was found in only one sample coming from Austria (0.531 ng g^{-1}). Our results provide reasons for low concern. Based on what has been reported in literature, our concentration looks under than other study made in Italy (less than 500 pg g^{-1}) (Guerranti et al., 2013); Belgium and Spain (55 pg g^{-1}) (Corneli et al., 2012; Ericson et al., 2008); and Norway (15 pg g^{-1})

(Haug., 2010). The Highest concentration has been found in Fat fish (1.678 pg g^{-1}) (Berger et al., 2009). EFSA Scientific Panel on Contaminants in the Food Chain recommended that more occurrence data for PFASs in food should be collected to improve the accuracy of future exposure calculations (EFSA, 2008). Subsequently, the European Commission issued the Commission Recommendation 2010/161/EU on the monitoring of PFASs in food in the Member States (EFSA, 2012). This paper gives a contribute about the knowledge of their presence in foodstuff. On the bases of these results, there is no risk for human beings, but further studies are needed to keep monitoring their presence in foodstuff, as it has been suggested by EU.

Conflicts of Interest Statement

No potential conflict of interest was reported by the authors.

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3.5. Presence of organic halogenated compounds, organophosphorus insecticides and polycyclic aromatic hydrocarbons in meat of different game animal species from an Italian subalpine area.

Published in, Food Additives & Contaminants Part A, Volume 36, 2019, Pages 1244-1252.

Francesco Arioli [a ¥,] **Federica Ceriani[a¥],** Maria Nobile [a ¥], Roberto Viganò [b], Martina Besozzi [b], Sara Panseri [a*], Luca Maria Chiesa [a]

[a] Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy.

[b] Studio Associato ALPVET, Consulenza veterinaria faunistica e ambientale, Busto Arsizio (Va).

* **Corresponding author**: Sara Panseri, Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy. Phone: 0250317931; Fax: 00390250317941; E-mail: sara.panseri@unimi.it

¥: These authors equally contributed to the work.

Keywords: game animals, non-dioxin-like polychlorobiphenyls, organochlorine insecticides, organophosphorus insecticides, polybromodiphenyl ethers, perfluoroalkyl substances, polycyclic aromatic hydrocarbons.

Research Highlights:

- NDL PCBs, PBDEs, PFASs, PAHs, insecticide presence was monitored in game animals
- The muscle of red deer, roe deer, chamois and wild boar from Piedmont was analysed
- An endemic presence of PCBs, organochlorine and organophosphorus pesticides was found
- Generally, the highest prevalence of quantifiable contaminants was found in red deer.
- The highest concentrations of NDL PCBs and phorate were detected in wild boar.

Abstract

The exposure to several compounds such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), organophosphorus pesticides (OPs), polycyclic aromatic hydrocarbon (PAHs), perfluoroalkyl substances (PFASs) and polybrominated diphenyl ethers (PBDEs) is a public health issue. The European Union (EU) recommended that its member states monitor the presence of emerging contaminants, like PBDEs and PFASs, in food and in the environment to obtain an accurate estimation of exposure. The tissues of wild animals exposed to these compounds can represent a suitable indicator of environmental pollution. The aim of this work is to evaluate: i) the occurrence of PCBs, PBDEs, PFASs, PAHs, OCPs and OPs in four game animals' meat (chamois, red deer, wild boar and roe deer); ii) interspecies differences and iii) human exposure. Muscle samples from seventy-nine animals were collected during the hunting season in a Northern Italy mountain area at altitudes ranging from 300 to 2500 meters above sea level. The analyses were performed with gas chromatography-mass spectrometry (GC-MS/MS) and ultra-performance liquid chromatography - tandem mass spectrometry (UHPLC-MS/MS). No PBDEs were found in the samples. OCPs, OPs and PCBs were detected in almost all samples at different concentration ranges, showing higher frequency in ungulate species than in wild boar. PFAs were found only in wild boar. Anthracene and benzopyrene, among PAHs, were found only in chamois at low concentrations. The lack of an accurate pattern of exposure as well as variable consumption by hunters does not allow accurate risk characterisation. However, a low risk for consumers can be indicated due to the frequent detection of contaminants at trace levels, the scarce prevalence of high concentrations of some contaminants and the low consumption of game animal meat. In conclusion, the organisation of a control plan on residues in game animals would be advisable.

1. Introduction

The exposure to polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), organophosphorus pesticides (OPs), polycyclic aromatic hydrocarbons (PAHs) perfluoroalkyl substances (PFASs) and polybrominated diphenyl ethers (PBDEs) is a major issue for public health. Recently, the European Union (EU) recommended that Member States monitor the presence of emerging contaminants like PBDEs and PFASs in food and in the environment to obtain an accurate estimation of exposure (European Commission 2014; EFSA 2018a). PBDEs reduce the flammability of combustible materials and are used in a wide range of products (electronics, vehicles, construction materials, etc.).

Directive 2003/11/EC (European Commission 2003) revising Directive 76/769/EEC on the marketing and use of certain dangerous substances, forbade preparation and sale of two commercial mixtures of PBDEs, known as penta BDE and octa BDE, in concentrations higher than 0,1 % by mass. Since July 2006, under Directive 2002/95/EC, all electrical and electronic equipment may no longer contain PBBs and PBDEs, at any concentration, other than deca PBDEs. PFASs have been used since the 1950s to make oil- and water-proof paper and textiles, in food packaging and fire-fighting foams (Paul et al. 2008). Some OPs, like e.g. chloripyrifos and ethoprophos, are still approved for use on plants in the EU (European Commission 2005b). In the past, compounds like PCBs and OCPs were widely used in industry or agriculture. They tend to be transported by atmospheric circulation, thus contaminating areas far from the place of emission (Wania and Mackay 1996). Although several compounds have been banned in Italy since the 1970s, some are still present in the environment and bioaccumulate in fatty tissues (Vallack and Giordano 1998). PBDE toxicity includes neurotoxicity and endocrine disruption (Costa et al. 2007; EFSA 2011); eight of them are considered of primary interest by the EU and relevant toxicological data are available on four of them (BDE-47; BDE-99; BDE-153 and BDE-209): the main targets are the liver, thyroid hormone, and the reproductive and nervous system. Moreover, PBDEs cause DNA damage through the induction of reactive oxygen species (EFSA 2011). Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are involved in hepatotoxicity, developmental toxicity, immunotoxicity and reproductive and hormonal effects. (EFSA 2018a); dioxin-like PCBs (DL-PCBs) and non-dioxin-like PCBs (NDL-PCBs) elicit various toxicological profiles, with neurological, neuroendocrine, endocrine and immunological effects (EFSA, 2005). However, no health-based guidance values for humans could be defined for NDL-PCBs because they usually occur

with the more toxic DL-PCBs, muddling the interpretation of their effects. OCPs and OPs are involved in neurological and endocrine effects (Ray, 2010; Richardson, 2010), as shown by studies on laboratory animals. For NDL-PCBs, the EU defined Maximum Levels (MLs) at 40 ng g^{-1} fat for bovines, sheep, poultry and pigs (European Commission 2011). Regarding PBDEs, EFSA (2011) fixed the following lower benchmark doses (BMDLs): BDE-47, 309 μg/kg b.w.; BDE-99, 12 μg/kg b.w.; BDE-153, 83 μg/kg b.w.; BDE-209, 1,700 μg/kg b.w. based on neurodevelopment as a critical endpoint (EFSA 2011).

Very recently, EFSA proposed the tolerable weekly intake (TWI) as 13 ng/kg body weight (b.w.) per week for PFOS and 6 ng/kg b.w. per week for PFOA based on increase in total serum cholesterol as an endpoint (EFSA 2018a).

Wild animals can be exposed to the above mentioned compounds and the levels their tissues represent a suitable indicator of environmental pollution. Several studies are present in literature regarding the presence of these compounds in game animals (Table 1).

Finally, even if game animal consumption in Italy is very low, the apparent intake of game meat being 0.1-0.3 kg *per capita* per year, the availability of wild meat has been increasing in the noughties (Ramanzin et al. 2010). Moreover, game animal meat is usually eaten by a small portion of the population, like hunters and closely linked people, who could be more exposed to the associated dietary risk: it is in fact estimated that game animal consumption in some regions rises to 1.0-4.0 Kg *per capita* per year (Ramanzin et al. 2010). In this study, the occurrence of persistent, bioaccumulative, toxic (PBTs) substances and OPs was evaluated in four game animals (chamois, red deer, wild boar and roe deer).

Table 1: Concentration of environmental compounds from literature analysed by GC-MS/MS and LC-HRMS.

Reference	Element	Area	organs	instruments	concentrations
Riebe et al. 2016	PFOA PFNA PFOS PFBS	Germany, Austria	Chamois (Liver)	LC–MS/MS	<LOQ 1.9[a] µg g^{-1} w.w. 2.4[a] µg g^{-1} w.w. <LOQ
Szymczyk-Kobrzyńska et al. 2003	DDD DDT γ-HCH PCBs	Poland	red deer (perirenal fat)	GC-MS/MS	traces 8.2[b] µg kg^{-1} 0.6[b] µg kg^{-1} 23.7[b] µg kg^{-1}
Naso et al. 2004	OCPs PCBs	Centre of Italy	Roe deer (liver and perirenal fat)	GC-MS/MS	<LOQ <LOQ- 414.5 ng g^{-1} l.w.
Falk et al. 2012	PFOS PFNA PFDA PFOA	Germany	Roe deer (Liver)	LC–MS/MS	6.3[a] µg kg^{-1} w.w. 1.2 [a] µg kg^{-1} w.w. 0.3 [a] µg kg^{-1} w.w. 0.5 [a] µg kg^{-1} w.w.
Guitart et al. 1999	HCB PCBS pp DDE pp'DDT Aldrin Heptachlor-epox.	Spain	Chamois	GC-MS/MS	6.61-79.05 ng g^{-1} w.w. 2.87-30.36 ng g^{-1} w.w. 0.79- 6.42 ng g^{-1} w.w. N.D.- 1.81 ng g^{-1} w.w. N.D.- 0.14 ng g^{-1} w.w. N.D.- 1.23 ng g^{-1} w.w.

a: median concentration; b: average concentration; w.w.: wet weight; l.w.: lipid weight

2. Materials and methods

2.1. Chemicals and reagents

All solvents, formic acid (98–100%), acetic acid (99.9%), sodium acetate, 25% ammonia solution, ammonium formate, trichloroacetic acid (TCA), and disodium hydrogen phosphate dihydrate, citric acid monohydrate and EDTA, to prepare EDTA-McIlvaine buffer solution, pH 4, were purchased from Merck KGaA, Darmstadt, Germany. Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The extraction cartridges Oasis WAX 3 mL, 60 mg for PFASs were provided by Waters (Milford, MA, USA). SupelTM QuE Citrate (EN) tubes for POP extraction and SupelTM QuE-ZSEP (EN) tubes for the clean-up step were from Supelco (Sigma Aldrich, St.Louis, MO, USA). Mixtures of non-dioxin-like polychlorinated biphenyl (ndl-PCB) congeners (PCB 28; 52; 101; 138; 153 and 180) and PBDE congeners (PBDE 28; 33; 47; 99; 100; 153 and 154), PCB 209, internal standard (IS) for PCBs, and 3-fluoro-2,2,4,4,6- pentabromodiphenyl ether (FBDE), IS for flame retardants, were from AccuStandard (New Haven, USA). Organochlorine pesticides (OCPs) (aldrin, α-1,2,3,4,5,6-Hexachlorocyclohexane (α-HCH); hexachlorobenzene (HCB); β-1,2,3,4,5,6-Hexachlorocyclohexane (β-HCH); 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene, 4,4′-DDE;1,1,1-Trichloro-2,2-bis[4-chlorophenyl]ethane 2,40-DDT; 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane 4,40-DDD; 1,1-Dichloro-2,2-bis(4-chlorophenyl)ethan 4,40-DDT; endosulphan I; endosulphan II; endosulphan sulphate; endrin; heptachlor; heptachlor epoxide; lindane and trans chlordane) were purchased from Restek (Bellefonte, PA, USA). Organophosphorous pesticide (OPs) standards of chlorpyriphos diazinon, disulfoton, ethoprophos, mevinphos and phorate were purchased from Sigma-Aldrich, St Louis, Mo, USA. 4-nonylphenol (IS for OCs and OPs) was purchased from Merck (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). PFASs analysed by HPLC-HRMS system were both sulphonates and carboxylates: perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorobutane sulphonic acid (PFBS), perfluoroheptanoic acid (PFHpA), PFOA, perfluorohexane sulphonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), PFOS, perfluorododecanoic acid (PFDoA), perfluoroundecanoic acid (PFUnDA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), perfluorohexadecanoic acid (PFHxDA), and perfluorooctadecanoic acid (PFODA). All these compounds and the two ^{13}C-

labeled internal standards (ISs) perfluoro-[1,2,3,4,5-^{13}C5] nonanoic acid (MPFNA) and perfluoro-[1,2,3,4-^{13}C4] octanesulfonic acid (MPFOS) were purchased from Chemical Research 2000 Srl (Rome, Italy).

1.2. Analytical Standard Solutions

The working solutions of analytes were prepared daily in hexane from stock solutions (10 µg mL^{-1}) stored at -20 °C.

1.3. Investigated species

The investigated species were chamois *(Rupicapra rupicapra)*, red deer *(Cervus elaphus)*, roe deer *(Capreolus capreolus)* and wild boar (*Sus scrofa*), chosen for their habitats, characterized by different altitudes. In the Alps, chamois live at 1000-2800 m above sea level, in both woody and rocky areas. Red deer live in deciduous and mixed forests at 500-1500 m above sea level. Roe deer live in mixed forests with dense undergrowth and in agriculture areas (700-1200 m above sea level). They are strict herbivores and eat grass, herbs, berries, mushrooms and shoots, blackberries and buds of deciduous and coniferous trees. Wild boars occupy the same geographical areas as red deer and are widespread (0-900 m above sea level). Wild boars are omnivorous and search for food burrowing into the ground. Their diet consists of herbs, tubers, mushrooms, corn, grain cereals, potatoes, insects, snails, amphibians, reptiles and small rodents. Muscle samples from hunted animals, precisely twelve roe deer, twenty-four chamois, twenty-three red deer and twenty wild boars, were collected in the Province of Verbano-Cusio-Ossola located in North East Piedmont, a region of North West Italy. The muscles chosen were diaphragm and *M. obliquus externus abdominis* and *M. rectus abdominis* with the aim of not damaging the carcass during the inspection visit.

1.4. Extraction procedures

1.4.1. Pesticides, PCBs and PBDEs extraction

Sample extraction was described in our previous work (Chiesa et al. 2018a) using the QuEChERS Technique. Briefly, 1 g of sample was spiked with the IS solution (4-nonylphenol, PCB 209 and FBDE) to a final concentration of 50, 25, 20 ng g^{-1} and then transferred to a QuEChERS extraction tube. Ten milliliters of hexane/acetone (4:1) were added as extraction solvent; the tube was shaken for 1 min and centrifuged for 10 min at 2500×g at 4°C. Later, the supernatant was transferred to a

QuEChERS clean up tube Z-SEP and centrifuged in the same condition described above. The extract was dried under vacuum in a centrifugal evaporator at a temperature of 40°C and then dissolved in 1 mL of hexane for GC-MS/MS analysis.

1.4.2. PFASs

The pre-treatment and extraction protocol for PFASs was the same as that described in our previous works (Chiesa et al. 2018b). 2 g of sample was spiked with the two internal standards at a concentration of 5 ng mL^{-1}. Protein precipitation and analyte extraction were carried out by addition of 10 mL of acetonitrile, stirring and sonication for 15 min. After centrifugation (2500×g, 4°C for 10 min), the supernatant was evaporated in a rotary vacuum evaporator at 35°C and then suspended in 10 mL of water, ready for the SPE extraction using the Oasis WAX Cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL of 0.5% ammonium hydroxide in methanol, 3 mL of methanol, and 3 mL of Milli-Q water. The sample was loaded, washed with 3 mL of 25 mM acetate buffer pH 4.5 followed by 2 mL of methanol.

Finally, the compounds were eluted using 3 mL of 0.5% ammonium hydroxide in methanol. The eluate was dried in a rotary vacuum evaporator at 35°C and suspended in 100 µL of methanol: ammonium formate 20 mM (10:90 v/v).

1.5. Instrumental analyses

1.6. GC-MS/MS analyses

Triple quadrupole mass spectrometry (QqQ) in electron ionization (EI) mode was used for the simultaneous detection and quantification of pesticides and POPs in meat samples. The mass condition was the same as that of our previous work (Chiesa et al. 2018a). A GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) was used to confirm and quantify residues by using a fused-silica capillary column Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., 0.25 µm film thickness, Restek, Bellefonte, PA, USA). The oven temperature program and all operation parameters are reported in the work mentioned before. The QqQ mass spectrometer was operated in selected reaction monitoring mode (SRM) detecting two-three transitions per analyte. Identification of POPs was carried out by

comparing sample peak relative retention times with those obtained for standards under the same conditions and the MS/MS fragmentation spectra obtained for each compound.

The XcaliburTM processing and instrument control software program and Trace Finder 3.0 for data analysis and reporting (Thermo Fisher Scientific) were used.

1.7. LC-HRMS Orbitrap analyses

LC-Orbitrap was used for detection and quantification of PFASs. HPLC analysis was performed by an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), equipped with a Surveyor MS quaternary pump and degasser, a Surveyor AS autosampler and column oven, and a Rheodyne valve with a 20-µL loop. The analytes were chromatographically separated, using a Synergi Hydro-RP reverse-phase HPLC column (150 × 2.0 mm, i.d. 4 µm), with a C18 guard column (4 × 3.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase used for the PFAs separation consisted of a binary mixture of solvents C (aqueous NH_4COOH 20 mM) and B (MeOH). The two gradients were described in our previous works (Chiesa et al. 2018b).

The detector was a Thermo Q-Exactive Plus Orbitrap (Thermo Scientific, San Jose, CA, USA), equipped with a heated electrospray ionisation (HESI) source. Capillary and vaporiser temperatures were set at 320 and 280°C, respectively, while the electrospray voltage was set at 3.00 kV, operating in negative mode for PFASs. Nitrogen as sheath and auxiliary gas was set at 35 and 15 arbitrary units, respectively. The full scan (FS) acquisition was combined with a data-independent acquisition (DIA) strategy, providing the MS^2 spectra for a confirmatory response.

Detection of the analytes was based on the calculated exact mass of the protonated/deprotonated molecular ions, and at least one specific and typical fragment. Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HPLC-HRMS system, to calculate the exact mass of the compounds and to acquire and elaborate data.

1.8. Method validation

Validation was performed following the SANTE/2017 guidelines (European Commission 2017). All the validation parameters and the assessment of validation protocols have already been described in our previous works (Chiesa et al. 2018a, 2018b).

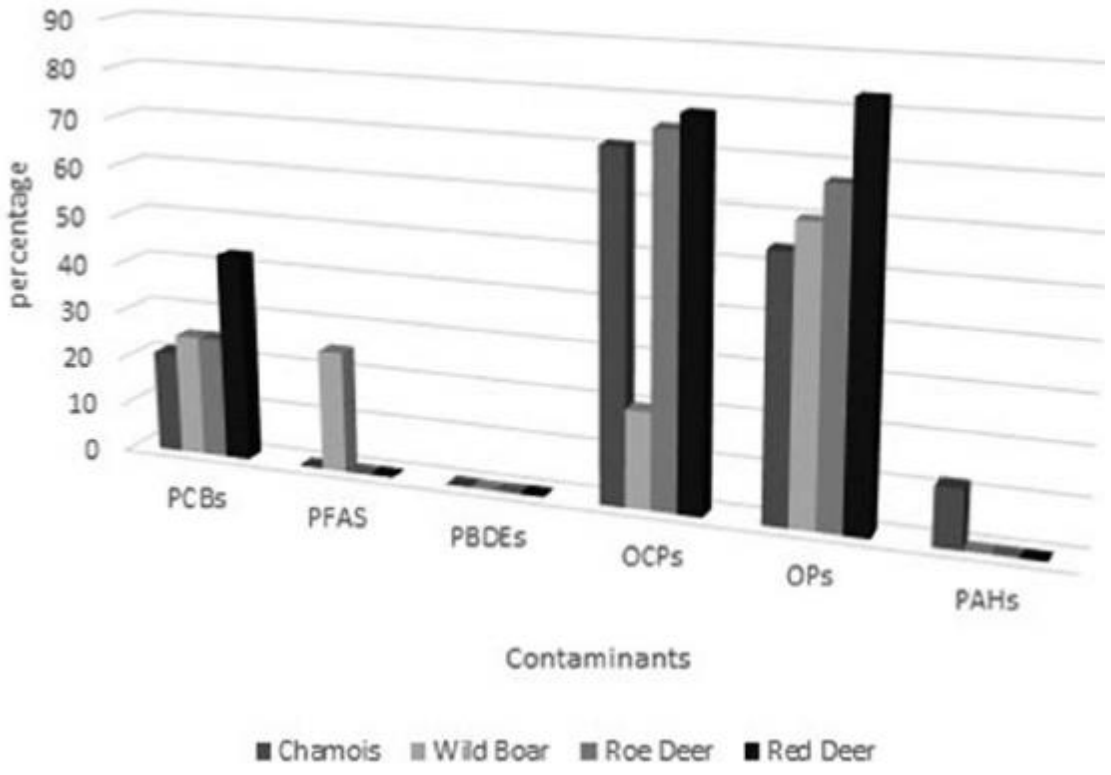
2. Results and discussion

Limits of Quantification (LOQs) were in the range 15.0-150 pg g^{-1} for PFASs, 0.50 ng g^{-1} for PCBs, PBDEs and PAHs, 1.0 ng g^{-1} for organochlorine and organophosphorus pesticides. All the validation parameters satisfied the SANTE/2017 guidelines. The results concerning NDL-PCB are summarized in Table 2. As the PCB MLs concentrations for farm animals are expressed in ng g^{-1} lipid weight (l.w.) (European Commission 2011) we adopted this formula also for wild animals. At least one PCB congener was quantified in 32% of the samples, with higher prevalence in red deer than other species (Table 2, Figure 1). 68% of the samples showed traces of PCBs under the limit of quantification. All the six NDL-PCB indicators were found in all species with a frequency PCB 138> PCB 153> PCB 180>PCB 52>PCB 101>PCB 28. Their presence was reported in other studies conducted in Italy (Naso et al. 2004; Turrio-Baldassarri et al. 2009) and in Europe (Mateo et al. 2012; Romanić et al. 2012). Red deer muscle had more frequent quantifiable concentrations of PCBs than other investigated species. However, the highest total concentration of the six NDL-PCBs was found in wild boar (674 ng/g l.w), that is an animal at higher trophic levels, therefore less representative as a sentinel species compared to herbivorous ungulates (Niewiadowska et al. 2013). An interesting aspect could be the evaluation of a possible relationship between the presence and concentration of PCBs, and age and sex of animals. However, some considerations have to be made in advance: firstly, the ages were only estimated, because they were hunted animals that lived wild; secondly, even considering age ranges, the groups had no homogeneous numbers; thirdly, the quantifiable concentrations of PCBs, i.e. those higher than the LOQ, were scarce. Although all these premises made statistical analysis impossible, a consideration can be made. Among the juvenile animals, aged less than one year, the frequency of PCB detection was one out of four roe deers, one out of two chamois and zero out of two wild boars. No samples were collected from young red deers. Therefore, PCBs were quantifiable in 37% of young animals, a percentage comparable to the 32% calculated on all the animals. Therefore, although bioaccumulation of lipophilic substances takes place over time, transfer from mother to young animal via milk is an important cause of exposure to PCBs in lactating animals (EFSA 2018b).

Table 2 PCBs in wild animal samples. Concentrations expressed as mean ±SD, in ng g^{-1}; N= number of animals; n = quantifiable samples. Values are expressed in lipid weight.

	Chamois N=24			Wild boar N=20			Roe deer N=12			Red deer N=23		
	nd	<LOQ	mean ±SD (n)	nd	<LOQ	mean ±SD (n)	nd	<LOQ	mean ±SD (n)	nd	<LOQ	mean ±SD (n)
PCB 28	0	22	42.9 (1)	0	18	24.5±0.2 (2)	0	12	-	0	22	38.18 (1)
PCB 52	0	18	51.4±11.3 (3)	0	17	10.8±3.5 (3)	0	12	-	0	18	40.8±9.7 (5)
PCB 101	0	24	-	0	19	31.4 (1)	0	12	-	0	21	58.0±26.1 (2)
PCB 138	0	20	63.6±24.6 (4)	0	15	190±143 (5)	0	10	52.9±29.9 (2)	0	18	77.1±49.0 (5)
PCB 153	0	21	79.2±17.8 (3)	0	16	190±140 (4)	0	12	-	0	18	84.9±65.7 (5)
PCB 180	0	24	-	0	19	6.8 (1)	0	12	-	0	21	158±129 (2)

Figure 1 Percentage of muscle samples of the four game animal species with quantified concentrations of the compounds studied.



Finally, PCB presence in mountain areas where anthropic activities are scarce is due to their chemical and physical features. Tremolada et al. (2015) described the seasonal trend of PCBs in air and soil in high-altitude mountains in the Italian Alps. According to their research, PCBs follow the seasonal trend of the mountain climate, tending to be transported in the atmosphere during the warm season and deposited to the soil during the cold season. They concluded that PCBs can be present also in high-altitude mountains. Table 3 summarizes all the results regarding other contaminants; data are expressed in ng g^{-1} wet weight (w.w.). No PBDEs were quantified in muscles, with only few samples showing the presence of PBDE 154 under the LOQ. As regards PFASs, we found only PFOS in 25% of wild boar samples. Their concentration range (0.83-2.90 ng g^{-1}) was lower than the concentration range found in the muscle (<LOQ-28.6 ng g^{-1}) of wild boars of Hesse, Germany (Stahl et al. 2012). Regarding PAHs, only anthracene and benzopyrene at low concentrations were found in chamois. The presence of OCPs and OPs in the meat of wild terrestrial animals is regulated by Regulation N° 396/2005 of the Parliament and of the Council (European Commission 2005a) with successive amendments reported in the EU pesticide database (European Commission 2005b). The value of all MRLs is 0.01 mg kg^{-1} w.w. except DDT and metabolites whose MRL is 0.05 mg kg^{-1} and ethoprophos for which MRLs are not stated in food of animal origin. The use of detected pesticides is banned in the European Union (European Commission 2005b).

Table 3 Detected contaminants in wild animal samples. Concentrations expressed as mean ±SD, in ng g-1; N= number of animals; n = quantifiable samples. Values are expressed in wet weight.

	Chamois N=24			**Wild boar N=20**			**Roe deer N=12**			**Red deer N=23**		
	n d	**<LOQ**	**mean ±SD (n)**	**n d**	**<LOQ**	**mean ±SD (n)**	**n d**	**<LOQ**	**mean ±SD (n)**	**n d**	**<LOQ**	**mean ±SD (n)**
PBDE154	21	3	-	20	0	-	10	2	-	21	2	-
PFOS	24	0	-	15	0	1.44±0.86 (5)	12	0	-	23	0	-
Antracene	23	0	1,54 (1)	20	0	-	12	0	-	23	0	-
Benzopyrene	21	1	1.49±0.04 (2)	19	1	-	12	0	-	23	0	-
Aldrin	0	15	2.62±5.03 (9)	0	19	0.04 (1)	0	5	3.43±3.84 (7)	0	15	61.4±179 (8)
α HCH	0	22	1.63±3.89 (2)	0	20	-	0	12	-	0	14	4.19±12.9 (9)

β HCH	0	21	0.24±0.4 (3)	0	20	-	0	12	-	0	21	0.09±0.31 (2)
Endrin	0	24	-	0	20	-	12	0	-	0	23	-
Endosulfan S	0	24	-	0	20	-	0	9	0.38±0.8 9 (3)	0	20	13.50±44.6 (3)
Endosulfan I	0	24	-	0	20	-	12	0	-	0	21	0.04±0.14 (2)
HCB	0	14	1.91±1.6 4 (10)	0	19	0.06 (1)	0	11	5.77 (1)	0	11	10.18±25.5 5 (12)
Lindane	0	16	1.07±1.0 5 (8)	0	19	0.03 (1)	0	12	-	0	21	0.71±3.30 (2)
pp DDD	0	23	0.66 (1)	0	19	2.99 (1)	0	12	-	0	23	-
op DDT	0	24	-	0	20	-	0	12	-	0	19	0.23±0.77 (4)
pp DDT	0	24	-	0	18	1.89±2.22 (2)	0	12	-	0	22	3.88 (1)
pp DDE	0	24	-	0	17	10.38±10.9 1 (3)	0	12	-	0	22	0.48 (1)
Ethoprofhos	21	0	1.71±2.4 3 (3)	6	12	0.12±0.33 (2)	10	2	-	15	2	2.50±1.92 (6)
Diazinon	0	24	-	0	18	0.28±0.77 (2)	1	10	1.76 (1)	2	21	-
Disulfoton	0	17	0.82±1.4 9 (7)	0	17	0.89±2.85 (3)	0	9	0.81±1.5 4 (3)	1	14	1.74±3.0 (8)
Phorate	0	16	7.25±11. 1 (8)	0	9	33.4±86.9 (11)	2	2	17.1±15. 3 (8)	0	11	7.75±8.8 (12)

Quantifiable OCPs were found in 58% of the samples analysed with the highest prevalence in roe deer, followed by red deer, chamois and wild boar. α-HCH, aldrin, HCB, endosulfan sulfate and DDT were detected with higher frequency and/or higher concentration. One out of 24 chamois samples (19.24 µg g^{-1}), six out of 23 red deer samples (38.22- 880 µg g^{-1}) exceeded the Aldrin MRL value, while two roe deer showed a value just above the MRL only if analytical error is not considered. Regarding α- and β-HCH, only one out of 24 chamois samples (11.7 µg g^{-1}) and two out of 23 red deer samples (29.2 µg $g^{-1}$and 56.5 µg g^{-1}) showed higher concentrations of α-HCH, while β-HCH was always below the limit. The samples were always compliant for endrin. As regards endosulfan and endosulfan sulfate, the limits were exceeded only for the latter for two out of 23 red deer samples (149 and 161 µg g^{-1}). Only

one out of 24 red deer samples exceeded the lindane MRL (15.9 µg g^{-1}). HCB was introduced as a fungicide and was banned in the European Union in 1981. However, even afterwards, HCB continued to be used in industrial and chemical manufacturing and released into the environment during incineration, as a by-product and in several pesticide formulations (EFSA 2006); only in red deer the MRL was exceeded in four out of 23 samples with values ranging from 17.15 µg g^{-1} to 121 µg g^{-1}. DDT and its metabolites were found to be below the LOQ in almost all samples. When quantifiable, values of DDT and metabolites were roughly comparable in wild boar and red deer. Quantification of OPs was possible in 66 % of the samples tested. Residues were found in all species. In particular, 67 % of the concentrations higher than LOQ were found in roe deer, 54 % in chamois, 83 % in red deer and 60 % in wild boar. All species showed a high contamination of phorate and disulfoton. As the persistence of these compounds is not high (Ragnarsdottir 2000) their presence could be due to current use in agricultural areas, although most compounds, such as phorate, demeton, diazinon and disulfoton are not approved in Europe (European Commission 2005a). The area, in fact, is close to agriculture zones and in a previous study conducted on honey produced in the same area, a high presence of OPs like demeton, disulfoton and menvinphos was found, (Chiesa et al. 2018c), evidence of illicit use of these compounds.

Finally, due to the non-homogeneous food habits of individual hunters compared to hunted animals, it is difficult to perform a risk characterization. Therefore, a mere qualitative description can be made: firstly, all quantified PCBs samples exceeded the MRL except one; the mean concentrations were 67.9, 138, 135 and 306 ng g^{-1} respectively in roe deer, chamois, red deer and wild boar, indicating a possible matter of concern about exposure to PCBs. PBDEs were always below the LOQ. As regards PFOSs, considering a tolerable weekly intake (TWI) of 13 ng kg^{-1} (EFSA 2018a), and a consumption of 200 g per day of wild boar meat, with an unreal and very conservative approach, the highest value found would lead to exposure about 190 times lower than the indicated TWI.

The higher values of the four PAHs in our samples would produce an exposure by far lower than the calculated exposure considered safe by EFSA (2008) for average and high European consumers. Moreover, the margins of exposure (MOEs) resulting from $BMDL_{10}$ values derived from carcinogenicity studies (Culp et al. 1998) were 17500 and 9900, for average and high European

consumers. The concentrations of the PAHs found in our samples indicate an even lower exposure, therefore a very low risk.

The meat of red deer poses some causes of concern because OCPs were generally present in this species at higher concentrations and frequencies than other species, except for DDT and metabolites, similarly present in wild boar.

OPs deserve a separate consideration, because since they are not very persistent in the environment, their presence in the meat of animals means that illicit use still occurs. Moreover, an appreciable difference between species does not appear. In fact, seven roe deer, eight chamois, six red deer and three wild boars showed concentrations above the MRL, ranging from 15.0 to 40.0 ng g^{-1} casually distributed between species. In this case, keeping in mind the above statement about hunters' eating habits, the high number of non-compliant samples suggests a particular caution in the consumption of this meat, e.g. a non-frequent intake.

As a conclusion, in the light of the results of this work, for the majority of the contaminants investigated, the consumption of game meat is not a cause for concern due to the scarce presence of high concentration residues and to its low consumption, even by hunters or closely linked people. An endemic presence of contaminants is highlighted, and the detection of OPs needs particular attention due to their low persistence, that indicates illicit use. Our data suggest that a residue control plan would be necessary on game animals.

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3.6. Distribution of POPs, pesticides and antibiotic residues in organic honeys from different production areas.

Published in, Food Additives & Contaminants Part A, Volume 35, 2018, Pages 1340-1355.

Luca Maria Chiesa, Sara Panseri*, Maria Nobile, **Federica Ceriani** & Francesco Arioli.

Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy.
* Corresponding author: Dr. Sara Panseri, Phone: 0250317931; Fax: 00390250317941. E-mail: sara.panseri@unimi.it. Address: Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy.

Keywords: organic honey; pesticides; antibiotics; triple quadrupole mass spectrometry (GCMS/ MS); LC-Orbitrap; food safety.

Abstract

Demand for honey is increasing, especially if it is organic and if its nutritional properties are linked to untreated environments in order to guarantee quality for health. Sources of contamination of honey can be divided into environmental and apicultural Therefore, the distribution of POPs (persistent organic pollutants), pesticides and antibiotic residues from geographical areas with different contamination sources (high anthropic impact, intensive farming, husbandry and low anthropic impact) was investigated in order to confirm the potential transfer of xenobiotics into the supply chain and to give beekeepers tools for the selection of areas dedicated to organic production. The presence of PCBs, PBDE and PAHs was confirmed, not only in proximity to highly urbanised centres, where the concentrations were higher, but in all environment contexts, confirming their ubiquity. No antibiotics or neonicotinoids were detected in 95 organic honeys, demonstrating the absence of apicultural treatments and consequently the good quality of honey of different areas. These results are important due to the undefined regulatory European situation on honey antibiotic limits.

1. Introduction

Honey is a natural food product, made of nectar, secretions of livings parts of plants or excretions of insects sucking on the living parts of plants, which Apis mellifera bees collect, transform by combining

with specific substances and deposit in honeycombs (Wilczynska et al., 2007; Panseri al., 2014). Honey is generally considered as a natural and healthy product. In these last years, much consumer interest regarding honey and its derived products is oriented towards organic foods (Berrie et al., 2005). Regarding this, the European Commission establishes that the qualification of organic honey and other beekeeping products is closely bound to the characteristics of hive treatments as well as the quality of the environment (Blasco et al., 2004). The Council Regulation

1804/1999 EC is very restrictive with regard to the production of organic honey in terms of the origin of bees, siting of the apiaries, feed, disease prevention and veterinary treatments (Malhat et al., 2015). In addition, the use of allopathic chemically synthesized medicinal products for preventive treatments in organic beekeeping is prohibited, since these fat-soluble and non-volatile compounds can accumulate in the stored honey, where they are able to migrate from the wax comb (Panseri et al., 2014). Safety of food and feed is one of the main objectives in consumer health policy. Maintaining a high level of protection in this contest remains crucial not only for public health, but also to preserve consumer confidence in food. Bees and bee products can be contaminated by several sources. Contamination may derive either from the environment or inadequate beekeeping practices (Baggio et al., 2009).

Contaminants of key importance for the above products include antibiotics. Like all living organisms, honey bees can suffer from pests and diseases. Bee diseases caused by microorganisms such as American and European foulbrood, and nosemosis can be cured by anti-infectious agents (Baggio et al., 2009). The presence of antibiotic residuals in food products constitutes an important health risk as it is associated with the increased microbial resistance to antibiotics. The antibiotics and chemotherapeutics of interest in apiculture include tetracyclines (oxytetracycline), aminoglycosides (streptomycin), sulfonamides (sulfamethazine, sulfathiazole, sulfadiazine, sulfamethoxazole, sulfamerazine, sulfadimethoxine), macrolides (tylosin, erythromycin), neonicotinoids derivates and they are systematically used for the treatment of infections in humans and animals (Balsco et al., 2003). Furthermore, they are classified based on their chemical structure r their mechanism of action (Lambert et a., 2013). At present, no MRLs have been established for antibiotics and sulfonamides in honey (Commission Regulation (EU) No 37/2010, 2010 and amendments), meaning that the use of antibiotics in beekeeping is not permitted in the EU. However, anti-infectious agents could be used in the EU in apiculture based on the “cascade” system as described in Article 11 of Directive 2001/82/EC (2001) of

the European Parliament and of the Council, as amended by Directive 2004/28/EC (2004) of the European Parliament and of the Council. The cascade system is open to all animal species, including honey bees (Anonymous, 2016a, 2017). In the EU the absence of MRLs for residues of antibiotics and chemotherapeutics in honey resulted for a long period in a zero-tolerance for antibiotic residues in honey. In some EU Member States (Belgium, France, UK) action limits, recommended target concentrations, nonconformity, or tolerance levels were applied (Leidoux et al., 2012). However, in 2012, it was decided to restrict zero-tolerance to residues of non-allowed substances while residues of allowed substances should be judged based on scientific risk assessment (Anonymous, 2016b, 2016c). This last decision makes honey trade more complex, and there are signs that not all European Member States are respecting this decision and that in some countries a zero-tolerance for residues of antibiotics and chemotherapeutics is still applied. Recently in Italy the National residue control Plan implemented detection limits of 5 μg kg-1for sulfamides, tetracyclines and macrolides and 1.3-1.6 μg kg-1 for aminoglycosides confirmatory methods (Italian Ministry of Health, 2017). The recent unrest on the neonicotinoids resulted in the proposal of the European Commission to restrict the use of clothianidin, imidacloprid and thiamethoxam for seed treatment, soil application and foliar treatment on bee-attractive plants and cereals (EFSA 2013a; EFSA 2013b and EFSA 2013c). Therefore, in organic honey production, direct pollution by beekeeping practices as well as indirect contamination from the environment must be prevented. Many pollutants in the environment may contaminate bee matrices, comprising bee, honey and pollen. Environmental pollutants include pesticides (Marini et al., 2012), heavy metals (Wilczynska et al., 2007), bacteria and radioactive materials (Debayle et al., 2012). Honeybees are able to cover a wide area and come into contact with contaminated food sources, such as pollen, nectar and water during foraging. Therefore, honeybees and beehive products are considered potential indicators for environmental biomonitoring (Malhat et al., 2015; He et al., 2015). As an example, organophosphorus pesticides (OPs) represent important environmental and food contamination sources, as they are widely used in agriculture for the control and protection of crop-eating insects. Previous research has highlighted that the contamination of honey is strictly related to the environmental context as well. In particular several pesticides used for crop protection are as a consequence able to contaminate honey representing a potential risk for food safety (Kaufmann et al., 2012). At present few data are available on multiresidue screening of xenobiotics examining the relation between a production context in which intensive farms are present and their potential risk of

honey contamination with antibiotic substances. This study could be considered the continuation of our previous work as regard the analysis of POPs in honey samples (Chiesa et al., 2016a). In the previous paper, honey samples were extracted by a different technique and collected from the North and South of Italy considering only the industrialised area and the agricultural one. In this new study, the main focus was the investigation of a broader spectrum of analytes, pesticides, persistent organic pollutants and antibiotics in organic honeys collected from different productive areas according to their characteristics (area with an agricultural, zootechnical or anthropic impact) to confirm the potential transfer of xenobiotics into supply chain from different sources than beekeeping practices. This approach could be particularly useful to give the beekeepers tools for the selection of areas dedicated to production in particular for the organic ones. Lastly, this paper presents a rapid, accurate and sensitive method to evaluate multiple antibiotic substances by using LC Orbitrap approach.

2. Materials and methods

2.1.Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100%) was obtained from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO, USA). Ammonium formate, trichloroacetic acid (TCA) crystals and the ingredients required to prepare EDTA-McIlvaine buffer solution, pH 4 (disodium hydrogen phosphate dihydrate, citric acid monohydrate and EDTA) were purchased from Fluka. Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The extraction cartridges (Oasis HLB 3 mL, 60 mg) used for antibiotics and neonicotinoids were provided by Waters (Milford, MA, USA). QuEChERS materials for the extraction of POPs and polar pesticides were obtained from Supelco (SigmaeAldrich, St.Louis, MO, USA); SupelTM QuE Citrate (EN) tubes, containing sodium citrate tribasic dihydrate and sodium citrate dibasic sesquihydrate. Magnesium sulphate and sodium chloride were used for the extraction. SupelTM QuE-PSA (EN) tubes were used for the clean-up step. Mixtures of polychlorinated biphenyl (PCB) congeners (PCB 28; PCB 52; PCB 101; PCB 138; PCB 153 and PCB 180) and polybrominated diphenyl ether (PBDE) congeners (PBDE 28; PBDE 33; PBDE 47; PBDE 99; PBDE 100; PBDE 153 and PBDE 154), PCB 209 as internal standard (IS) for PCBs, and 3-

fluoro-2,2,4,4,6- pentabromodiphenyl ether (FBDE) as IS for PBDEs, were purchased from AccuStandard (New Haven, USA). Organochlorine pesticides (OCPs) (α-HCH; β-BHC; aldrin; pp'-DDD; pp'-DDE; op'-DDT; pp'-DDT; endosulphan I; endosulphan II, endosulphan sulphate, endrin; heptachlor; heptachlor epoxide; hexachlorobenzene; lindane; trans chlordane) was purchased from Restek (Bellefonte, PA, USA). Organophosphorus pesticide (OPs) standards of chlorpyriphos, demeton, disulfoton, ethoprophos, mevinphos, phoratediazinon, were purchased from Sigma-Aldrich (St Louis, Mo, USA). Florisil (100e200 96 mesh) was provided by 6 Promochem (Wesel, Germany). Hexane, acetone, ethyl acetate (special grade for pesticide residue analysis (Pestanal)) and 4-nonylphenol (IS for OCPs and OPs) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Polar pesticides: atrazine, azinphos-ethyl, azinphos-methyl, azoxystrobin, benalaxyl, bitertanol, bupirimate, buprofezin, cadusafos, chlorfenvinphos, cyproconazole, cyprodinil, ethoxyquin, fenamiphos, fenarimol, fludioxonil, flusilazole, furalaxyl, kresoxim-methyl, malathion, metalaxyl, methidathion, oxadixyl, paraoxon-methyl, phosalone, piperonyl butoxide, pyrazophos, pirimicarb, pirimiphos-methyl, pirimiphos-ethyl, profenophos, propachlor, propargite, quinalphos, simazine, tetrachlorvinphos, tetraconazole, triazophos, and 4- nonylphenol, used as internal standard, were also purchased from Fluka. Antibiotics and neonicotinoids: actamiprid, amoxicillin, ampicillin, benzyl penicillin, cefquinome, ceftiofur, cefalexin, ciprofloxacin, chloramphenicol, chlortetracycline, clothianidin, cloxacillin, danofloxacin, dicloxacillin, dimetridazole, dinotefuran, doxycycline, enrofloxacin, florfenicol, florfenicol amine, flumequine, furaltadone, furazolidone, imidacloprid, lincomycin, lomefloxacin, marbofloxacin, nalidixic acid, nytempiram, nitrofurazone, oxolinic acid, oxytetracycline, ronidazole, spectinomycin, spiramycin, sulfadiazine, sulfathiazole, sulfadimethoxine, sulfadimidine, sulfamerazine, tetracycline, thiacloprid, thiamethoxam, thiamphenicol, tiamulin, tilmicosine, tinidazole, trimethoprim, tylosin and enrofloxacin d5 as the internal standard (IS) were purchased from Fluka. The purity of all standards was > 98%.

2.2.Standard solutions

Working solutions of POPs were prepared by diluting the stock solution in hexane for pesticides and then stored at -20 °C. Mixed compound calibration solution, in hexane, was prepared daily from the stock solutions (10 μg mL-1) and the proper volume was used as a spiking solution as well. For each

LC-HRMS standard, stock solutions were prepared (1 mg mL-1) in methanol and kept at -20 °C. Working solutions at 10 and 100 ng mL-1, were prepared daily. Each working solution was maintained at 4 °C during the method validation procedure.

2.3. Sample collection

Sample collection was conducted to obtain representative samples from different environmental and anthropogenic contexts, as summarized in Table 1. The investigated areas were identified as follows: High anthropic impact (HA): includes honey samples of *Tilia europaea* botanical origin from beehives located close to large inhabited centres, with an important industrial activity, presence of motorway junctions and high population density. Low anthropic impact (LA): these areas are characterized by the presence of woodland vegetation, the absence of large industrial activities and the presence of modest road networks; the botanical origin was *Castanea sativa Miller.* Intensive farming area (IF): this group includes samples of Tilia europaea botanical origin from areas where the main activity identified was cultivation. In particular, cereal crops, orchards, vineyards and greenhouses were highlighted. Intensive husbandry area (IH): these are territories in which the presence of cattle farms, chickens, horses and also a fish farm emerged; the sample botanical origin was both Castanea sativa Miller and Tilia europaea. Farming and husbandry areas (FH): this category includes areas with intermediate characteristics compared to the previous two. Due to the simultaneous presence of large cultivated areas and breeding farms, mainly of cattle, it was not possible to assign the samples to one of the other groups. It was therefore decided to form an area that can be considered polyvalent depending on the results obtained. Also, in this case, the sample botanical origin was both *Castanea sativa Miller* and *Tilia europaea*. Moreover 18 organic samples, purchased from the market were included in our application.

Table 1 Origins of 95 honey samples from different production areas. **(C)** = High anthropogenic impact; **(F) =** Free, low anthropogenic impact; **(A)** = Agriculture areas; **(Z)=** Farming area; **(AZ)=** Agriculture and farming areas.

Sample No.	Sample's identification	Origin Information	Area characteristic in relation to its potential pesticides sources
18	-	Market	EU Produced (variable Pesticides, antibiotic sources)
15	C	North Italy[a-b]	Industrialized area (PCBs,OCs source)
12	F	North Italy (Novara-V.C.O.)[c]	Free Area (no presence of industries or agricultural intensive systems; absence of pesticides and antibiotics)
10	Z	North Italy (Novara)[d]	Farming Area(Antibiotic source)
25	AZ	North Italy[e]	Farming and agriculture area (Antibiotics, OCs source)
15	A	Centro of Italy[f]	Agriculture Area (OCs source)

a= Lombardia (Rho, north west); b= Piemonte (Novara, north west); c= Piemonte (Novara; Stresa Verbano-Cusio-Ossola (VCO); d= Piemonte (North west); e= Piemonte (Alessandria-Asti-Torino); f= Emilia Romagna (Modena).

2.4. Sample extraction for antibiotics and neonicotinoids

The sample treatment clean-up carried out for antibiotics and neonicotinoids in honey was similar to the method adopted in our previous works on antibiotics in different matrices (Chiesa et al., 2015, 2016, 2017, 2018; Pastorelli et al., 2005) with some modifications, as well as an increased number of analysed compounds. Briefly, an aliquot (1 g) of honey was spiked with the IS (enrofloxacin-d5) at a final 5 ng mL-1 and 100 µl of 20% TCA for protein precipitation and 5 mL McIlvaine buffer (pH 4.0) were added for the extraction. The samples were vortexed and sonicated for 15 min. For honey the defatting steps applied in the previous mentioned studies were excluded. After centrifugation (2500 x g, 4 °C, 10 min), the supernatant was transferred to a clean polytetrafluoroethylene centrifuge and

purified by SPE Oasis HLB cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL methanol and 3 mL Milli-Q water. The samples were loaded, and then washed with 2 x 3 mL methanol: water (5:95 v/v). Finally, the analytes were eluted with 5 mL methanol and collected in a 15-mL glass tube. The eluate was evaporated in a rotary vacuum evaporator at 40 °C. The dried extract was reconstituted in 200 μL methanol:water (10:90 v/v), and then transferred to an auto-sampler vial. The injection volume was 10 μL.

2.5. Sample extraction for pesticides

The extraction of pesticides and contaminants was performed using the QuEChERS method. The ASE extraction (Chiesa et al., 2016a) was changed with this new quickly, easy and cheap strategy, in order to combine the extraction of polar pesticides and POPs. In the meantime we obtained and confirmed the same validation parameters of the previous work about POPs. Briefly, two grams of sample were homogenized and transferred to a QuEChERS extraction tube, then a solution containing the ISs (4-nonylphenol, PCB 209 and FBDE) was added to the sample to a final concentration of 100 ng g-1. Ten millilitres of acetonitrile were added as extraction solvent; the tube was vortex-mixed for 1 min and centrifuged for 10 min at 2500×g at 4°C. Later, the supernatant was transferred to a QuEChERS clean up tube, shaken and centrifuged under the same conditions described above. The extract was collected, divided into two aliquots and dried under vacuum in a centrifugal evaporator at a temperature of 35°C. The residue was dissolved in 200 μL methanol:ammonium formate 10 mM (10:90 v/v) for the analysis by LC-HRMS and in 200 μL hexane for GC-MS/MS analysis. For honey fortification, 2 g of the control sample was spiked by adding an appropriate volume of the standard working solution to cover the concentration range from 1 to 100 ng g-1 for PCBs, from 0.5 to 10 ng g-1 for PBDEs, and from 5 to 100 ng g-1 for OCs and OPs, and also in relation to pesticide MRLs when available in order to obtain matrix-matched calibration curves.

2.6. LC-HRMS Orbitrap analyses

LC-Orbitrap was used for the simultaneous detection and quantification of polar pesticides and antibiotic in honey samples. HPLC analysis was performed by an HPLC system (Thermo Fisher

Scientific, San Jose, CA, USA), equipped with a Surveyor MS quaternary pump and degasser, a Surveyor AS autosampler and column oven, and a Rheodyne valve with a 20-μL loop. The analytes were chromatographically separated, using a Synergi Hydro-RP reverse-phase HPLC column (150 × 2.0 mm, i.d. 4 μm), with a C18 guard column (4 × 3.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase used for the antibiotics and neonicotinoids separation gradient consisted of a binary mixture of solvents A (aqueous HCOOH 0.1%) and B (MeOH). The elution started with 5% B, which increased to 95% in 10 min and remained constant up to the 14th min. The initial conditions were obtained again at the 17th min, with an equilibration time of 8 min. The run was performed at 0.3 mL min-1 for a total of 25 min. The mobile phase used for polar pesticides was a binary combination of solvents C (aqueous ammonium formate 10 mM) and B (MeOH). The elution started with 10% B, which increased to 95% after 13 min and remained constant up to the 20th min. The initial conditions were obtained again at the 22nd min, with an equilibration time of 6 min. The run was performed at 0.3 mL min-1 for a total of 28 min. The detector was a Thermo Q-Exactive Plus Orbitrap (Thermo Scientific, San Jose, CA,

USA), equipped with a heated electrospray ionisation (HESI) source. Capillary and vaporiser temperatures were set at 320 and 280°C, respectively, while the electrospray voltage was set at 3.00 kV, operating in both positive and negative mode. Nitrogen as sheath and auxiliary gas was set at 35 and 15 arbitrary units, respectively. Instrument calibration was performed every analytical session, using LTQ Velos ESI negative and positive ion calibration solutions (Pierce Biotechnology Inc., Rockford, IL, USA). The full scan (FS) acquisition was combined with a data-independent acquisition (DIA) strategy, providing the MS2 spectra for a confirmatory response. For antibiotics and neonicotinoids, the FS resolution was 70,000 FWHM. On the basis of the compound list, a scan range of 120–1000 m/z was chosen; the automatic gain control (AGC) was set at 1 x 106, and the maximum injection time was 150 ms. The DIA segment operated in both positive and negative mode at 17,500 FWHM. The AGC target was set to 2 x 104, with 100 ms maximum injection time. A loop count of 20 was set for the positive ion mode, 10 for the negative. The precursor ions are filtered by the quadrupole, which operates at an isolation window of 1 m/z. Fragmentation of the precursors was optimised with two-step normalised collision energy (25 and 40 eV). For polar pesticides, the FS resolution was 70,000 FWHM. On the basis of the compound list, a scan range of 180–600 m/z was chosen; the automatic gain control (AGC) was set at 1 x 106, and the maximum injection time was 200

ms. The DIA segment operated in both positive and negative mode at 17,500 FWHM. The AGC target was set at 2 x 104, with 100 ms maximum injection time. A loop count of 2 was set for the positive ion mode, 1 for the negative. We set an isolation window of 1 m/z. Fragmentation of the precursors was optimised with three-step normalised collision energy (10, 40, and 60 eV). Detection of the analytes was based on the calculated exact mass of the protonated/deprotonated molecular ions, and at least one specific and typical fragment. The formula of the selected compounds, the exact theoretical mass of the parents and the diagnostic transition used to confirm the studied antibiotics and neonicotinoids are reported in Tables 2 and 3, respectively. Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HPLC-HRMS system, to calculate the exact mass of the compounds and to acquire and elaborate data.

2.7. GC-MS/MS analyses

Triple quadrupole mass spectrometry (QqQ) in electron ionization (EI) mode was used for the simultaneous detection and quantification of pesticides and POPs in honey samples. The mass condition was the same of our previous work (Chiesa et al., 2016a). A GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) was used to confirm and quantify residues in honey samples by using a fusedsilica capillary column Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, Bellefonte, PA, USA). The oven temperature program was as follows: initial temperature of 80°C, held for 3 min, and increased to 170°C at 10°C min-1; then, increased from 170°C to 190°C at 3°C min-1, and raised to 240°C at 2°C min^{-1}, before being ramped to 280°C at 3°C min-1 and finally from 280°C to 310°C at 10°C min-1 and held at this temperature

for 5 min. The carrier gas (helium, purity higher than 99.999%) was in constant flow mode at 1.0 ml min-1. A volume of 1 μL was injected using a programmed temperature vaporiser injector (PTV) in splitless mode with a 1-min splitless period and the following inlet temperature programme: 80°C (0.05 min), 14.5°C s-1 to 200°C (1 min) and 4.5°C s-1 to 320°C (12 min – cleaning phase). A baffle liner (2 mm × 2.75 mm × 120 mm, Siltek-deactivated; Thermo Fisher Scientific) was used. The transfer line was maintained at 270°C and the ion source at 250°C. The electron energy and emission current were set to 70 eV and 50 μA, respectively. The scan time was 0.3 s and the peak width of both quadrupoles

was 0.7 Da full widths at half maximum. Argon was used as a collision cell gas at a pressure of 1.5 mTorr. The QqQ mass spectrometer was operated in selected reaction monitoring mode (SRM) detecting two-three transitions per analyte. Identification of POPs was carried out by comparing sample peak relative retention times with those obtained for standards under the same conditions and the MS/MS fragmentation spectra obtained for each compound. The XcaliburTM processing and instrument control software program and Trace Finder 3.0 for data analysis and reporting (Thermo Fisher Scientific) were used.

2.8. Analytical performances and method validation

For antibiotics and neonicotinoids, validation was carried out following the Commission Decision guidelines 657/2002/CE (European Commission 2002, 2008), by spiking the samples with all the selected analytes, in six replicates, at three concentration levels, for three different days resulting in three analytical series (matrix validation curves). The three concentration levels (C0, 2C0, 3C0), previously planned according to the minimum concentration detectable with our instrumentation (C0) were 0.5, 1 and 1.5 ng g-1 for all analytes and 3, 6, 9 ng g-1 for amoxicillin and benzyl penicillin. Instrumental linearity was assessed through six-point calibration curves, constructed in the solvent containing a fixed amount of the IS (5 ng mL-1), with the initial analyte concentration corresponding to the minimum detectable concentration for each analyte up to 100 ng mL^{-1}. Detection limits CCα and detection capabilities CCβ were calculated using the three-validation series following the Document SANCO/2004/2726-revision 4 (European Commission 2008). Intraday and inter-day repeatability (Thompson 2000), representing precision, were calculated using one-way analysis of variance (ANOVA) expressed as CV % (coefficient of variation). Robustness of the method was evaluated using the fractional factorial design of Youden (European Commission, 2002). Matrix effect was estimated according to the protocol described by Matuszewski et al. (2003). For polar pesticides and POPs, validation had already been carried out following the European Commission (2015) SANTE/2015 guideline and reported in a previous work (Chiesa et al., 2016a). The selectivity of the method was evaluated by injecting extracted blank honey samples.

The absence of interferences was proved by the lack of peaks with a signal-to-noise ratio higher than 3 at the retention times of the target compounds. Honey samples, previously analysed and checked for

the absence of all polar pesticides and POPs, were used as control samples during the optimisation and validation procedure. For the limit of quantification (LOQ) of the methods, we used the lowest validated spiked level meeting the requirements of recovery within the range of 70–120% and an RSD ≤ 20%, as defined by the European Commission (2015). Finally, the extraction methods were also evaluated for their repeatability, linearity and recovery. Recoveries were calculated at LOQ for all compounds. The repeatability as CV% was calculated by analysing six replicates at the same fortification level.

2.9. Statistical analysis

As residue concentrations in honey did not follow a normal distribution, checked through the Kolmogorov–Smirnov test, the non-parametric Kruskal–Wallis ANOVA test was used to evaluate the differences of contaminants in samples among the productive areas investigated. The level of significance was set at $P \leq 0.05$ throughout this study. Data were analysed using SPSS 15.0 software (SPSS, Inc., Illinois, USA). In addition, it must be pointed out that, for the calculations, ½ LOD was used for compounds with a concentration lower than LOD.

3. Results and discussion

3.1. Method validation parameters

The methods showed high specificity, without any interference close to the retention time of each compound, and consequently a S/N ratio ≥3 in the presence of analytes was confirmed, even at the lowest detectable concentration demonstrating good selectivity. Matrix validation curves showed good linearity over the working range with a good fit ($R2 > 0.99$) for all compounds. The mean recoveries (from 82 to 120%), with the other validation parameters, are reported in Tables 2 and 3. The CVs % were lower than 20% for all analytes, satisfying the criteria required by the European Commission (2015) and specified by Thompson (2000). Regarding the CCα and CCβ for antibiotics and neonicotinoids, and LOQs for POPs, our satisfactory results showed high method sensitivity for the selected compounds both for LC-HRMS and GC-MS/MS analyses. Method ruggedness was good for all compounds. A modest matrix effect was found, with values ranging from 84□116% for the selected analytes in honey samples.

Table 2. Formula, exact theoretical mass of the parents, diagnostic transition and validation parameters of the selected antibiotics and neonicotinoids.

Compound[a]	Class	Formula	Exact mass [m/z]	Main fragment [m/z]	Ion	CCα (ng g^{-1})	CCβ (ng g^{-1})	Recovery %	intra-day CV (%)	inter-day CV (%)
Actamiprid	neonicotinoid	C10H11ClN4	223.07450	126.01052	(+)	0.51	0.75	90	14	17
Amoxicillin	penicillin	C16H19N3O5S	366.11182	114.00109	(+)	3.15	3.58	84	13	16
Ampicillin	penicillin	C16H19N3O4S	350.11690	106.06545	(+)	0.56	0.79	83	13	15
Benzylpenicillin	penicillin	C16H18N2O4S	335.10600	176.06030	(+)	3.10	3.45	113	14	18
Cefalexin	cephalosporin	C16H17N3O4S	348.10125	158.02704	(+)	0.54	0.74	87	12	17
Cefquinome	cephalosporin	C23H24N6O5S2	529.13224	134.09634	(+)	0.58	0.81	97	13	16
Ceftiofur	cephalosporin	C19H17N5O7S3	524.03629	126.01212	(+)	0.52	0.77	95	16	18
Chloramphenicol	amphenicol	C11H12Cl2N2O5	321.00505	257.03409	(-)	0.54	0.82	95	12	15
Chlortetracycline	tetracycline	C22H23ClN2O8	479.12157	444.08377	(+)	0.55	0.85	102	11	14
Ciprofloxacin	fluoroquinolone	C17H18FN3O3	332.14050	288.15005	(+)	0.51	0.81	112	10	12
Clothianidin	neonicotinoid	C6H8ClN5O2S	250.01600	169.05406	(+)	0.57	0.89	86	10	13
Danofloxacin	fluoroquinolone	C19H20FN3O3	358.15615	314.16579	(+)	0.55	0.90	93	11	12
Dimetridazole	nitroimidazole	C5H7N3O2	142.06110	112.06335	(+)	0.61	0.98	89	13	15
Dinotefuran	neonicotinoid	C7H14N4O3	203.11387	129.08963	(+)	0.59	0.92	90	12	14
Doxycycline	tetracycline	C22H24N2O8	445.16054	410.12305	(+)	0.55	0.90	106	11	14
Enrofloxacin	fluoroquinolone	C19H22FN3O3	360.17180	316.18188	(+)	0.52	0.81	113	9	11
Florfenicol	amphenicol	C12H14Cl2FNO4S	355.99319	185.02769	(-)	0.54	0.83	95	11	14
Florfenicol amine	amphenicol	C10H14FNO3S	248.07512	130.06515	(+)	0.57	0.88	96	10	15
Flumequine	quinolone	C14H12FNO3	262.0874	244.07686	(+)	0.62	0.96	86	14	16
Furaltadone	nitrofuran	C13H16N4O6	325.11426	100.07608	(+)	0.53	0.87	90	14	18
Furazolidone	nitrofuran	C8H7N3O5	226.04585	95.03703	(+)	0.57	0.90	87	11	13
Imidacloprid	neonicotinoid	C9H10ClN5O2	256.05958	209.05874	(+)	0.55	0.80	87	11	15
Lincomycin	lincosamide	C18H34N2O6S	407.22103	126.12775	(+)	0.55	0.79	90	10	12
Lomefloxacin	fluoroquinolone	C17H19F2N3O3	352.14672	265.11438	(+)	0.51	0.77	120	9	11
Marbofloxacin	fluoroquinolone	C17H19FN4O4	363.14631	320.10410	(+)	0.53	0.75	110	9	10
Nalidixic acid	quinolone	C12H12N2O3	233.09207	205.06041	(+)	0.52	0.72	96	12	17
Nytempiram	neonicotinoid	C11H15ClN4O2	271.09563	225.10242	(+)	0.57	0.85	88	14	16
Nitrofurazone	nitrofuran	C6H6N4O4	199.04618	152.96921	(+)	0.57	0.91	90	14	18
Oxolinic acid	quinolone	C13H11NO5	262.07100	244.06044	(+)	0.54	0.78	98	13	19

Oxytetracycline	tetracycline	C22H24N2O9	461.15546	426.11816	(+)	0.51	0.73	107	14	15
Ronidazole	nitroimidazole	C6H8N4O4	201.06183	140.04529	(+)	0.60	0.94	85	12	14
Spyramicin *	macrolide	C43H74N2O14	422.26428	174.11231	(+)	0.58	0.91	91	15	19
Sulfadiazine	sulfonamide	C10H10N4O2S	251.05972	156.01120	(+)	0.59	0.96	83	14	16
Sulfadimethoxine	sulfonamide	C12H14N4O4S	311.08085	156.07666	(+)	0.62	0.99	82	14	15
Sulfadimidine	sulfonamide	C12H14N4O2S	279.09102	149.02325	(+)	0.61	0.94	82	13	17
Sulfamerazine	sulfonamide	C11H12N4O2S	265.07537	156.01135	(+)	0.57	0.79	87	12	13
Sulfathiazole	sulfonamide	C9H9N3O2S2	256.02089	156.01120	(+)	0.54	0.77	82	13	19
Tetracycline	tetracycline	C22H24N2O8	445.16054	410.12305	(+)	0.54	0.74	116	11	16
Thiacloprid	neonicotinoid	C10H9ClN4S	253.03092	126.01062	(+)	0.58	0.81	84	13	16
Thiamethoxam	neonicotinoid	C8H10ClN5O3S	292.02656	211.06470	(+)	0.55	0.80	96	13	14
Thiamphenicol	amphenicol	C12H15Cl2NO5S	353.99752	185.02805	(-)	0.56	0.81	88	9	11
Tiamulin	diterpene	C28H47NO4S	494.32986	192.10501	(+)	0.64	0.96	114	12	15
Tilmicosine *	macrolide	C46H80N2O13	435.2903	174.11232	(+)	0.65	0.92	97	14	19
Tinidazole	nitroimidazole	C8H13N3O4S	248.06995	121.03193	(+)	0.59	0.97	90	13	18
Trimethoprim	sulfonamide	C14H18N4O3	291.14517	245.10294	(+)	0.53	0.76	104	8	12
Tylosin	macrolide	C46H77NO17	916.52643	174.11229	(+)	0.55	0.78	94	10	12
Enrofloxacin-d5	IS	C19D5H17FN3O3	365.20318	321.21289	(+)	-	-	-	-	-

*in these cases the double charged species $[M+2H]^{+2}$ were considered; [a]= reported in alphabetic order

Table 3. Formula, exact theoretical mass of the parents, diagnostic transition and validation parameters of the selected polar pesticides.

Compound	Formula	Exact mass [m/z]	Main fragment [m/z]	Ion	LOQ (ng g^{-1})	Recovery %	intra-day CV%	inter-day CV%
Atrazin	C8H14ClN5	216.10105	174.05385	(+)	8.80	87	12	16
Azinphos-ethyl	C12H16N3O3PS2	346.04435	114.96143	(+)	8.91	89	13	15
Azinphos-methyl	C10H12N3O3PS2	318.01305	142.99245	(+)	8.15	83	14	17
Azoxystrobin	C22H17N3O5	404.1241	372.09729	(+)	8.05	90	12	17
Benalaxyl	C20H23NO3	326.17507	148.11185	(+)	8.11	91	11	15
Bitertanol	C20H23N3O2	338.18630	70.04069	(+)	7.92	93	10	14
bupirimate	C13H24N4O3S	317.16419	108.01172	(+)	8.95	90	14	17
Buprofezin	C16H23N3OS	306.16346	201.10551	(+)	7.70	95	12	16
Cadusafos	C10H23O2PS2	271.09498	158.96980	(+)	9.05	88	14	19
Chlorfenvinphos	C12H14Cl3O4P	358.97681	155.04663	(+)	9.11	85	13	18
Cyproconazol	C15H18ClN3O	292.12112	70.04073	(+)	9.24	87	11	15

Cyprodinil	C14H15N3	226.13387	108.08103	(+)	9.52	82	14	19
Ethoxyquin	C14H19NO	218.15394	190.12244	(+)	8.87	85	13	17
Fenamiphos	C13H22NO3PS	304.11308	217.00816	(+)	8.23	91	13	15
Fenarimol	C17H12Cl2N2O	331.03994	81.04534	(+)	9.54	85	14	19
Fludioxonil*	C12H6F2N2O2	266.07356	227.04482	(+)	9.05	84	14	18
Flusilazole	C16H15F2N3Si	316.10761	165.06987	(+)	9.15	82	15	19
Furalaxyl	C17H19NO4	302.13868	95.01640	(+)	7.78	87	11	14
Kresoxim-methyl	C18H19NO4	314.13868	222.09219	(+)	8.00	89	12	15
Malathion	C10H19O6PS2	331.04334	99.00809	(+)	7.65	93	11	14
Metalaxyl	C15H21NO4	280.15433	220.13306	(+)	7.50	96	9	12
Methidathion	C6H11N2O4PS3	302.96913	145.00656	(+)	8.47	92	10	13
Oxadixyl	C14H18N2O4	279.13393	219.11262	(+)	8.85	91	11	16
Paraoxon-methyl	C8H10NO6P	248.03185	234.02864	(+)	9.80	88	13	17
Phosalone	C12H15ClNO4PS2	367.99414	182.00029	(+)	9.56	85	14	19
Piperonyl butoxide*	C19H30O5	356.24315	177.09122	(+)	7.93	92	12	15
Pirimicarb	C11H18N4O2	239.15025	72.04513	(+)	7.67	95	11	14
Pirimiphos-ethyl	C13H24N3O3PS	334.13488	198.1058	(+)	7.15	98	10	13
Pirimiphos-methyl	C11H20N3O3PS	306.10358	108.05595	(+)	7.38	97	11	14
Profenophos	C11H15BrClO3PS	372.94242	344.91083	(+)	8.10	90	13	18
Propachlor	C11H14ClNO	212.08367	170.03662	(+)	7.07	96	11	14
Propargite *	C19H26O4S	368.18901	231.17419	(+)	8.22	91	13	16
Pyrazophos	C14H20N3O5PS	374.0934	194.55950	(+)	7.29	95	10	13
Quinalphos	C12H15N2O3PS	299.06138	147.05527	(+)	8.89	84	15	19
Simazine	C7H12ClN5	202.0854	132.03226	(+)	9.03	85	14	19
Tetrachlorvinphos	C10H9Cl4O4P	364.90653	127.01553	(+)	8.55	87	12	16
Tetraconazole	C13H11Cl2F4N3O	372.02881	91.05791	(+)	9.33	83	14	18
Triazophos	C12H16N3O3PS	314.07228	162.06616	(+)	8.15	89	14	17
IS: 4-nonylphenol	C15H24O	219.17544	133.06580	(-)				

* The $[M+NH_4]^+$ adduct were considered.

3.2. Application to organic honey samples

Overall results in terms of number detected, concentration levels and distribution of contaminant residues and antibiotics in the organic honey samples investigated are summarised in Table 4 and Figure 1. A total ion current (GC-MS/MS) chromatograms of blank honey samples spiked with investigated compounds and a naturally contaminated sample are shown in figures 2 and 3.

Table 4. Distribution of contaminant residues in 77 organic honeys from different productive areas. Concentration are expressed as single value, when only one positive was detected, or mean ± sd, ng

	High anthropization (N^a=15)		Intensive farming (N^a =15)		Intensive farming /husbandry (N^a =25)		Intensive husbandry (N^a =10)		Low anthropization (N^a =12)		MRL
		n^b		n^b		n^a		n^b		n^b	ng g^{-1}
PCB (ICES-6)											
PCB 28	5.33	1	35.1	1	nd	-	nd	-	19.2±14	4	-
PCB 52	127 [1]	1	93.8 ± 20.1	2	nd	-	nd	-	35.5±31.7	4	-
PCB 101	5.15	1	22.4	1	nd	-	nd	-	80.2 ±105	4	-
PCB 138	105	1	Nd	-	nd	-	nd	-	77.3 ±57.0	4	-
PCB 153	118	1	Nd	-	nd	-	nd	-	61.6 ± 26.9	4	-
PCB 180	7.56	1	Nd	-	nd	-	nd	-	5.40 ± 0.66	3	-
PBDEs											
PBDE 28	4.06	1	Nd	-	nd	-	nd	-	2.35 ± 1.20	2	-
PBDE 33	Nd	-	Nd	-	nd	-	nd	-	0.80	1	-
PBDE 47	5.90	1	Nd	-	nd	-	nd	-	Nd	-	-
PBDE 99	1.61	1	Nd	-	3.12	1	nd	-	0.88	1	-
PBDE 100	Nd	-	Nd	-	7.98	1	nd	-	1.31	1	-
PBDE 153	36.9	1	Nd	-	Nd	-	nd	-	62.7 ± 68.1	2	-
PBDE 154	Nd	-	Nd	-	Nd	-	nd	-	0.99	1	-
OCs											
α HCH	Nd	-	7.78	1	Nd	-	nd	-	Nd	-	-
β BHC	Nd	-	Nd	-	Nd	-	nd	-	Nd	-	-
Hexachlorbenzene	Nd	-	69.7	1	Nd	-	nd	-	Nd	-	10
Lindane	Nd	-	11.9±7.73	2	Nd	-	nd	-	Nd	-	10
Heptachlor	Nd	-	51.0	1	Nd	-	nd	-	Nd	-	10
Aldrin	Nd	-	59.9	1	Nd	-	nd	-	Nd	-	10
Heptachlor epoxide	Nd	-	53.5	1	Nd	-	nd	-	Nd	-	10
Trans chlordane	Nd	-	-	-	Nd	-	nd	-	Nd	-	10
Endosulfan I	Nd	-	Nd	-	Nd	-	nd	-	Nd	-	10
Endosulfan II	Nd	-	20.6	1	Nd	-	nd	-	Nd	-	10
pp' DDE	Nd	-	60.6	1	Nd	-	nd	-	5.30	1	50

Endosulfan Sulfate	Nd	-	nd	-	Nd	-	nd	-	Nd	-	10
Endrin	Nd	-	104	1	Nd	-	nd	-	Nd	-	10
op' DDT	Nd	-	Nd	-	Nd	-	nd	-	Nd	-	50
pp' DDD	Nd	-	78.4	1	Nd	-	nd	-	23.0	1	50
pp' DDT	Nd	-	10.5	1	Nd	-	nd	-	Nd	-	50
PAHs											
Chrysene	Nd	-	Nd	-	Nd	-	nd	-	6.32	1	-
Antracene	Nd	-	Nd	-	Nd	-	nd	-	51.5 ±45.2	2	-
Benzofluoranthene	Nd	-	Nd	-	Nd	-	nd	-	85.7± 103	2	-
Benzopyrene	42.7	1	Nd	-	Nd	-	nd	-	94.0 ± 137	3	-
OPs											
Ethoprophos	Nd	-	Nd	-	Nd	-	nd	-	Nd	-	-
Phorate	Nd	-	39.8	1	Nd	-	nd	-	Nd	-	10
Demeton	Nd	-	344	1	Nd	-	nd	-	Nd	-	10
Diazinon	Nd	-	22.9	1	Nd	-	nd	-	Nd	-	10
Disulfoton	Nd	-	50.9	1	Nd	-	nd	-	2.05	1	10
Chlorpyrifos	Nd	-	28.4	1	Nd	-	nd	-	Nd	-	50
Mevinphos	9.92 ± 10.1	14	27 ± 61	11	1.65± 0.64	25	nd	-	3.60 ± 1.35	10	-
Antibiotics											
Amoxicillin	nd	-	nd	-	nd	-	nd	-	Nd	-	-
Ampicillin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Benzylpenicillin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Cefalexin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Cefquinome	nd	-	nd	-	nd	-	nd	-	nd	-	-
Ceftiofur	nd	-	nd	-	nd	-	nd	-	nd	-	-
Chloramphenicol	nd	-	nd	-	nd	-	nd	-	nd	-	-
Chlortetracycline	nd	-	nd	-	nd	-	nd	-	nd	-	-
Ciprofloxacin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Danofloxacin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Dinotefuran	nd	-	nd	-	nd	-	nd	-	nd	-	-
Doxycycline	nd	-	nd	-	nd	-	nd	-	nd	-	-
Enrofloxacin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Florfenicol	nd	-	nd	-	nd	-	nd	-	nd	-	-
Florfenicol amine	nd	-	nd	-	nd	-	nd	-	nd	-	-
Flumequine	nd	-	nd	-	nd	-	nd	-	nd	-	-
Furaltadone	nd	-	nd	-	nd	-	nd	-	nd	-	-

Furazolidone	nd	-	nd	-	nd	-	nd	-	nd	-	-
Lincomycin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Lomefloxacin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Marbofloxacin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Nalidixic acid	nd	-	nd	-	nd	-	nd	-	nd	-	-
Nitrofurazone	nd	-	nd	-	nd	-	nd	-	nd	-	-
Oxolinic acid	nd	-	nd	-	nd	-	nd	-	nd	-	-
Oxytetracycline	nd	-	nd	-	nd	-	nd	-	nd	-	-
Ronidazole	nd	-	nd	-	nd	-	nd	-	nd	-	-
Spyramicin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Sulfadiazine	nd	-	nd	-	nd	-	nd	-	nd	-	-
Sulfadimethoxine	nd	-	nd	-	nd	-	nd	-	nd	-	-
Sulfadimidine	nd	-	nd	-	nd	-	nd	-	nd	-	-
Sulfamerazine	nd	-	nd	-	nd	-	nd	-	nd	-	-
Sulfathiazole	nd	-	nd	-	nd	-	nd	-	nd	-	-
Tetracycline	nd	-	nd	-	nd	-	nd	-	nd	-	-
Thiamphenicol	nd	-	nd	-	nd	-	nd	-	nd	-	-
Tiamulin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Tilmicosine	nd	-	nd	-	nd	-	nd	-	nd	-	-
Tinidazole	nd	-	nd	-	nd	-	nd	-	nd	-	-
Trimethoprim	nd	-	nd	-	nd	-	nd	-	nd	-	-
Tylosin	nd	-	nd	-	nd	-	nd	-	nd	-	-
Neonicotinoids											
Actamiprid	nd	-	nd	-	nd	-	nd	-	nd	-	-
Clothianidin	nd	-	nd	-	nd	-	nd	-	nd	-	50
Dimetridazole	nd	-	nd	-	nd	-	nd	-	nd	-	-
Imidacloprid	nd	-	nd	-	nd	-	nd	-	nd	-	50
Nytempiram	nd	-	nd	-	nd	-	nd	-	nd	-	-
Thiacloprid	nd	-	nd	-	nd	-	nd	-	nd	-	200
Thiamethoxam	nd	-	nd	-	nd	-	nd	-	nd	-	50
Polar pesticides											
Atrazin	nd	-	nd	-	nd	-	nd	-	nd	-	50
Azinphos-ethyl	nd	-	nd	-	nd	-	nd	-	nd	-	-
Azinphos-methyl	nd	-	nd	-	nd	-	nd	-	nd	-	-
Azoxystrobin	nd	-	nd	-	nd	-	nd	-	nd	-	50
Benalaxyl	nd	-	nd	-	nd	-	nd	-	nd	-	-

Bitertanol	nd	-	nd	-	nd	-	nd	-	nd	-	50
bupirimate	nd	-	nd	-	nd	-	nd	-	nd	-	50
Buprofezin	nd	-	nd	-	nd	-	nd	-	nd	-	50
Cadusafos	nd	-	nd	-	nd	-	nd	-	nd	-	10
Chlorfenvinphos	nd	-	nd	-	nd	-	nd	-	nd	-	10
Cyproconazol	nd	-	nd	-	nd	-	nd	-	nd	-	50
Cyprodinil	nd	-	nd	-	nd	-	nd	-	nd	-	50
Ethoxyquin	nd	-	nd	-	nd	-	nd	-	nd	-	50
Fenamiphos	nd	-	nd	-	nd	-	nd	-	nd	-	10
Fenarimol	nd	-	nd	-	nd	-	nd	-	nd	-	50
Fludioxonil	nd	-	nd	-	nd	-	nd	-	nd	-	50
Flusilazole	nd	-	nd	-	nd	-	nd	-	nd	-	50
Furalaxyl	nd	-	nd	-	nd	-	nd	-	nd	-	-
Kresoxim-methyl	nd	-	nd	-	nd	-	nd	-	nd	-	-
Malathion	nd	-	nd	-	nd	-	nd	-	nd	-	50
Metalaxyl	nd	-	nd	-	nd	-	nd	-	nd	-	50
Methidathion	nd	-	nd	-	nd	-	nd	-	nd	-	20
Oxadixyl	nd	-	nd	-	nd	-	nd	-	nd	-	10
Paraoxon-methyl	nd	-	nd	-	nd	-	nd	-	nd	-	10
Phosalone	nd	-	nd	-	nd	-	nd	-	nd	-	10
Piperonyl butoxide	nd	-	nd	-	nd	-	nd	-	nd	-	-
Pirimicarb	nd	-	nd	-	nd	-	nd	-	nd	-	50
Pirimiphos-ethyl	nd	-	nd	-	nd	-	nd	-	nd	-	-
Pirimiphos-methyl	nd	-	nd	-	nd	-	nd	-	nd	-	50
Profenophos	nd	-	nd	-	nd	-	nd	-	nd	-	50
Propachlor	nd	-	nd	-	nd	-	nd	-	nd	-	20
Propargite	nd	-	nd	-	nd	-	nd	-	nd	-	50
Pyrazophos	nd	-	nd	-	nd	-	nd	-	nd	-	50
Quinalphos	nd	-	nd	-	nd	-	nd	-	nd	-	50
Simazine	nd	-	nd	-	nd	-	nd	-	nd	-	10
Tetrachlorvinphos	nd	-	nd	-	nd	-	nd	-	nd	-	-
Tetraconazole	nd	-	nd	-	nd	-	nd	-	nd	-	20
Triazophos	nd	-	nd	-	nd	-	nd	-	nd	-	50

nd = not detected; a = standard deviation; b = detection frequency

Fig 1. Detection frequency of xenobiotic compounds in organic honeys from different productive areas

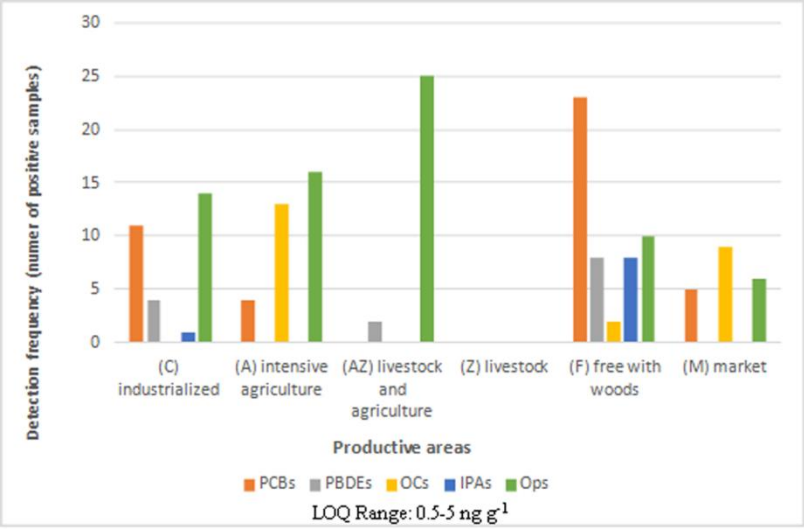


Fig 2. Total ion current (GC-MS/MS) chromatogram of blank organic honey samples spiked with some investigated compounds.

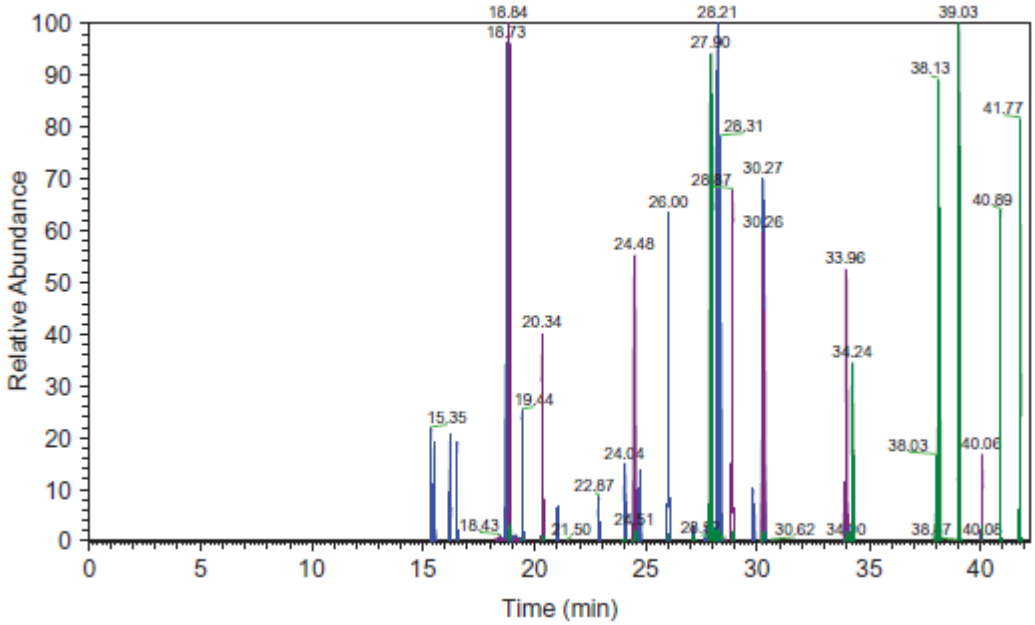
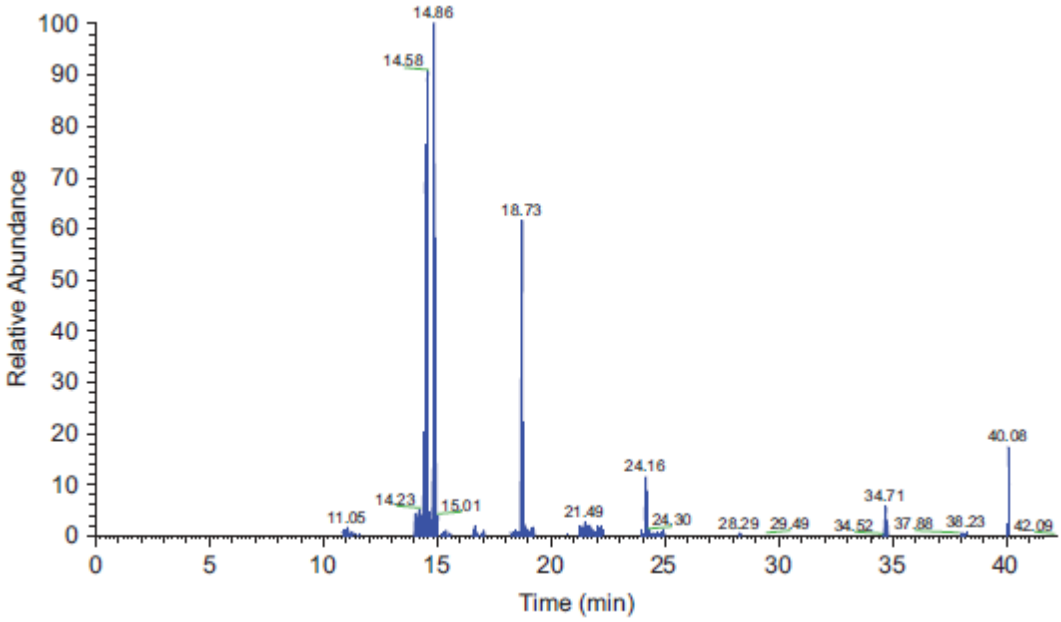


Fig 3. Total ion current (GC-MS/MS) chromatogram of organic honey sample naturally contaminated with some contaminats



Among the POPs, the six PCBs examined were found in 3 of 5 areas (HA, IF, LA) with relatively low detection frequencies ranging from 6.7% to 33.3 %. However, this aspect requires particular consideration, primarily because in two areas - HA and LA - all 6 congeners were found. In particular, area HA includes two zones in the province of Milan (Lombardy) and one in the province of Novara (Piedmont). All these zones are characterized by significant industrial activities and a strong urbanization impact, also with the presence of important highway junctions nearby. Area LA was chosen as a less densely populated representative area characterized mainly by woodland vegetation, with small roads, except for some motorways at a minimum distance of 3 km. In the two zones of this area, we found 4 out of 12 samples with concentration range 4.39-234 ng g^{-1}. Their presence in this apparently unspoiled area confirmed the theory that nowadays PCBs are ubiquitous contaminants, as reported in others works (Chiesa et al. 2016a; Panseri et al., 2014; Tette et al., 2016; Herrera et al.,2005). Their presence in the environment can be regardless of their proximity to industrial contexts and therefore of their territorial origin (Chiesa et al. 2016a; Blasco et al., 2004). A similar consideration has to be made regarding area IF, located in Emilia Romagna, characterised by intensive agriculture. Despite the fact that the hives were located close to fields, woods, vineyards and orchards, one sample

showed the presence of PCBs 28, 52, 101 and one sample of the congener 52 (Table 4). Considering honey taken from the market, of the 18 analysed samples only one from Hungary showed the presence of 5 of the 6 congeners investigated, with concentration range 10.5 (PCB 52)-44.1 (PCB 28) ng g-1. Overall data concerning PCBs showed that there are no significant differences in concentrations among the investigated areas and therefore PCB contamination is not influenced by the sample's source. A similar phenomenon was observed for brominated flame retardants (BFRs). This research showed that they were present in 3 out of 5 areas (HA, LA, FH) and absent in the market samples. Regarding FH area, PBDE congeners 99 and 100 were found in just one sample at the concentrations of 3.12 ng g -1 and 7.98 ng g -1, respectively. The PBDE concentrations regarding areas HA and LA appear more noteworthy. Four congeners were detected in one sample in the HA area from the zone of Milan and 6 congeners were detected in two samples from Novara and VCO, with maximum concentrations higher for one order of magnitude. In particular PBDE 153 showed a concentration higher than area HA, 111 ng g -1 versus 36.9 ng g -1. The scarce literature regarding BFR monitoring plans in honey, confirms their presence in the environment. Our results agree with a previous study that shows a higher concentration in the LA area characterised by woodlands than in area HA with industrial activities (Chiesa et al. 2016a). About polycyclic aromatic hydrocarbons (PAHs), our work shows that samples showing high concentrations of the 4 polycyclic aromatic hydrocarbons belonged to areas HA and, mostly, LA. In this last area benzofluoranthene and anthracene were found in 2 samples with the highest

concentrations of 158 ng g -1 and 83.5 ng g -1. Benzopyrene, on the other hand, was the only hydrocarbon present in both HA and LA areas, quantified at 42.7 ng g -1 and 252 ng g -1, respectively. Their presence is probably due to the fact that they are a category of chemicals that derives from various processes, both natural, such as forest fires, and industrial, such as combustion processes at high temperatures, so the environment and conditions probably have significant roles in the presence of this class of pollutants. OCPs were only found in 2 areas. Residues of pp'DDE and pp'DDD in a single sample were found with a concentration of 5.39 ng g -1 and 24.0 ng g -1, respectively, in area LA. The presence of organochlorine pesticide residues in eastern Piedmont has already been demonstrated by Chiesa et al (2014) at concentrations of pp'DDE and pp'DDD of 8.8 ng g -1and 2 ng g -1. Although the use of OCPs was banned in the 1970s, their presence in the environment is due to their chemical properties, since their high stability, low volatility and lipophilic nature ensure the tendency to persist

in the environment and accumulate in foods. In one sample of area IF, dedicated to agricultural production with orchards, cereal crops and vineyards, almost all OCP compounds were detected at concentrations ranging from 7.80 ng g-1 of α HCH to 103 ng g-1 of endrin. Other OCPs were only sporadically detected. As regards commercial honeys, three of the 18 samples analysed were found to have different OCPs at varying concentrations. One sample from Hungary showed the presence of α HCH (8.38 ng g-1), lindane (16.9 ng g-1), aldrin (172 ng g-1, and endrin (99.9 ng g-1); the second, from Ukraine, showed a concentration of lindane (116 ng g-1), heptachlor (5.93 ng g-1), aldrin (196 ng g-1), pp'DDE (9.22 ng g-1) and endosulfan II (415 ng g-1). The third, a mixture of honeys of different origins, in particular Moldovan-Bulgarian and Argentinean, showed a concentration of heptachlor (8.54 ng g-1), aldrin (110 ng g-1), endosulfan sulfate (635 ng g -1), endosulfan II (38.3 ng g-1) and pp'DDD (6.94 ng g -1). Particular attention must be given to OPs. Demeton, diazinon, and disulfoton were found in just one sample from the intensive cultivation area at concentration higher than the EU maximum level (ML) of 0.01 mg kg^{-1}, together with chlorpyrifos at permitted concentration. The honey from the market showed the presence of demeton, disulfoton and chlorpyrifos. In particular, Disulfotonwas detected in 2 samples, coming from Ukraine and Hungary, with the same concentrations of 65 ng g-1, exceeding the MRLs. Mevinphos has been found in almost all samples from 4 of 5 areas at higher concentrations and detection frequencies if compared to other substances, even if its use is not permitted. Mevinphos was used in the past mainly for vineyard pest defence and its chemical properties allow it to persist in the environment (EPA 1996). Particularly striking are the high frequencies in area FH, characterized by the presence of husbandry farms and large vineyards. Regarding its toxicity, Mevinphos is active by contact inhalation and ingestion. The biochemical mechanism of Mevinphos acute toxicity is through inhibition of acetylcholinesterase, causing nerve paralysis, with possible consequences like convulsions, coma, pulmonary oedema, muscle paralysis and death by respiratory blockage (EPA 1994). However, it has no Maximum Residual Limits for honeys, while a MRL for agricultural products has been set at 0.01 mg kg -1. The presence of mevinphos could be traced back to its past use and environmental persistence, as Panseri et al (2014) demonstrated. As regards the analysis of antibiotics, neonicotinoids and polar pesticides, no residues were found in organic honey samples. Some conclusions can be made: the sources of honey contamination can be divided into environmental (heavy metals such as lead, radioactive isotopes, organic pollutants, pesticides, pathogenic bacteria and genetically modified organisms) and apicultural

(acaricides, antibiotics etc.). In the first case the contaminants can reach the beehive: through air and water by means of bees, which can transport them directly into the colony; through the air, water and soil reaching plants, which can then pass them on through nectar and honeydew. In the case of apicultural techniques contaminants are due to beekeepers' use of antibiotics directly on the beehive for the control of bacterial honeybee diseases (Bogdanov 2006). Indeed, it can be said that for POPs the environmental contamination and transport of the substances through bees is effective and demonstrated with the detected residues in honeys. In the case of antibiotics, the environmental context near agricultural or livestock areas has not shown any contamination or possible transfer of antibiotics to honeys. On the other hand, when antibiotics were reported in the literature (Table 5), authors always refer to contamination which was strictly related to beekeeping practices. So, on the basis of our application, the contamination of antibiotics appears to be merely linked to beekeeping techniques. In fact, as regards the organic honeys examined in this study, the absence of treatment directly on the hives was ascertained, confirming the suitability of our analytical methods and consequently the good and effective quality of our organic honey samples collected in different areas. Finally, even a negative result appears important due to the undefined regulatory European situation regarding limits on antibiotics in honey. In conclusion, the optimised analytical method was applied to survey contaminant residues through samples produced in different areas characterized by different contamination sources. The determination of residues in the environment and food is important in order to avoid human exposure by dietary intake. Based on our results, it can be confirmed that honey is an optimal matrix for environmental pollutant research and bees are a suitable bioindicator to survey environmental pollution. The results have shown some peculiarities. In particular, OCs and Ops were found in the farming area, as we expected, confirming that the contamination could be linked to the area. The husbandry area have been shown to be free of any kind of contamination. The presence of several compounds, such as PCBs, PBDE and PAHs was confirmed, not only in proximity to highly urbanised centres, where the concentrations were higher, but in all environment contexts, confirming the theory that nowadays PCBs are ubiquitous contaminants. Particular concern regards honey samples coming from area LA, chosen as an area hypothetically free from contaminants and where a lower concentration of contaminants was expected; the contamination was instead evident, and the area was mostly polluted by PCBs, PBDEs and PAHs, suggesting industrial sources. They may be present in the environment regardless of proximity to industrial regions. The situation was totally different for

antibiotic residues that were not found in any organic honeys regardless of the production area. This phenomenon could provide interesting evidence of the close relationship between the presence of antibiotics and its respective provenance from beekeeping practices rather than from areas also involved in intensive breeding contexts. This kind of approach is important in order to indicate that beekeepers select untreated production areas, suitable for organic honey, especially when its demand is increasing for its nutritional properties, and to guarantee quality for health.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgements

This work was partially funded according to the project: "Distribution and risk assessment of xenobiotics in different foods of animal origin and their role for food safety" - Piano di Sostegno alla Ricerca 2017 - Linea 2 – Azione A

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CHAPTER 4

Summary & Conclusion

SUMMARY

Food contamination is an issue of major concern for human health and for the environment. The presence of drug residues and contaminats in food is often regulated and human exposition is control by European Organisms. This doctoral dissertation is a part of a larger project aims to carry out a survey on the safety of food of animal origins. With this purpose we focused the attention on some of the most consumed (fish, meat) and food with scarce literature (game meat). Food of increasing importance, like honey, was also investigated related to environmental contaminants for which European Commission (EC) asked to improve the data to collect more information and evaluate the human risk. In this work, the papers published on international scientific journal are exposed.

Two studies focus on the occurrence of cadmium, lead, mercury, arsenic, chromium and nickel in 50 mussel, 40 clam and 130 tuna samples available to the Italian consumers, and the risk associated to the consumption of this seafood. Analyses were carried out using inductively-coupled plasms-mass spectrometry. For the first three metals only one mussel sample was non-compliant for cadmium. For arsenic, nickel and chromium, maximum levels are not stated by the European Union. A risk characteritazion demonstrated that the average Italian consumption of molluscs, does not pose a risk for consumers, except nickel, which can cause allergic dermatitis in nickel-sensitive individuals. A risk for large consumers comes from Arsenic presence in mussels and, to a lesser degree in clams. One red tuna and 11 yellow tunas exceeded lead maximum levels. Three red tunas exceeded mercury maximum levels. The risk characterization showed that only a negligible health hazard could derive from the ingestion of tuna, for both average and high consumers.

Another paper on seafood focus on the presence of organochlorine and organophosphorous compounds, polybromodiphenylethers, perfluoroalkyl substances and antibiotics in both acquaculture and wild salmon. Polychlorobiphenyl 101 and polybromodiphenylethers congeners 28, 33, 47 and 100, 153, 154 were seldom observed; congener 99 was detected in 33% of the samples. Pentafluorobenzoic acid and perfluorooctanoic acid were detected about in 30 % of the samples. Aldrin and Endosulfan sulphate were often detected (about 30%-35% of the samples) while hexaclhorobenzene was detected in about 80% of farmed and only in 20% of wild salmons. As far as antibiotics only fenbendazole was frequently detected; doxiclyne and nalidixic acid were found in 5% of only farmed salmon. The risk

deriving from salmon intake is low for all the substances analysed, being of minor concern only for PBDE 99. However, after the 2018 EFSA revision of perfluorooctanoic acid tolerable weekly intake the risk from perfluoroalkyl substances results to be high for the level for serum cholesterol, adopted as the new end point (this is the conclusion different from what reported in the paper, written before the 2018 EFSA report was published).

The presence of perfluoroalkyl substances and polybrominated diphenyl ethers was investigated in a paper related to pork meat coming from eight European countries. No perfluoroalkyl substances were detected, except perfluorooctanoic acid, in only one Austrian sample. polybrominated diphenyl ethers were detected in three out of 77 samples: the one from Germany showed the presence of all congeners analyzed, the ones from Netherland and Italy, respectively PBDE 153 and PBDE 100. The charactherization of the risk resulted in no concern for human health.

A further paper dealt with the occurrence of polychlorinated biphenyls, organochlorine pesticides, organophosphorus pesticides, polycyclic aromatic hydrocarbon, perfluoroalkyl substances and polybrominated diphenyl ethers in muscle from chamois, red deer, wild boar and roe deer. Muscle samples from seventy-nine animals were collected during the hunting season in Northern Italy mountain areas. No polybrominated diphenyl ethers were found in the samples. organochlorine pesticides, organophosphorus pesticides and polychlorinated biphenyls were detected in almost all samples at different concentration ranges, showing higher frequency in ungulate species than in wild boar. perfluoroalkyl substances were found only in wild boar. Anthracene and benzopyrene were found only in chamois at low concentrations. The lack of hunter consumption estimates does not allow accurate risk characterisation.

The final work regarded a food of raising importance for human consumption: honey. Therefore, the distribution of persistent organic pollutants, pesticides and antibiotic residues from different geographical areas was investigated to confirm the potential transfer of xenobiotics into this food and to give beekeepers tools for the selection of areas dedicated to organic production correlalted to their antrhopization and industrialization. The presence of polychlorinated biphenyls, polybrominated diphenyl ethers and polycyclic aromatic hydrocarbon was confirmed, not only in proximity to highly urbanised centres, where the concentrations were higher, but in all environment contexts, confirming

their ubiquity. No antibiotics or neonicotinoids were detected in 95 organic honeys, demonstrating the absence of apicultural treatments and consequently the good quality of honey of different areas.

The analytes in the different matrices required different methods for sample pretreatment, extraction and clean up before the analysis with liquid chromatography–tandem mass spectrometry (LC-MS/MS) or – gas mass spectrometry (GC-MS/MS). The approach of analytical part has required the optimisation of instrumental performances as well as all steps of sample pretreatment, to reach good levels of sensitivity, specificity and robustness of the method to then make qualitative and quantitative considerations. The planning, optimisation and validation was performed according to Commission SANTE/11945/2015 and SANTE/10553/2017 document.

In conclusion we presented data about the occurrence of many persistent organic pollutants and antibiotic residues in different food of animal origin coming from different areas so contributed to improve the knowledge regarding contamination in food.

The results of this manuscript suggest that there is a low risk for the average consumer health. Environmental concentrations of persistent organochlorine compounds have been decreasing over the past two decades, and this could be correlate with remarkable advances in the detection of low levels of these compounds in human populations and the improvement of European control. PCBs still are present in environment due to their wide consumption in the past and their chemical and physical properties even if their use was banned in many industries application. Regarding emerging compounds, PFAs still need to be concern due to their wide use and their possible toxicological role. Antibiotics still are a matter of concern and need a close control to ensure human safety and decrease antimicrobial resistance.

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Aknowledgements:

- I would like to thank my research group whom I have shared these trhee years of my life and which has allowed the success of these researches.
- Special thanks to my tutor who has helped, supported, guided and taught me over the past three years. *Thank you*.
- Thanks to those who have shared with me these three years for the laughter, the moments of discouragement and support that have helped me go ahead.

"***Coming togeheter is a beginning,***
Staying togeheter is progress,
And working togeheter is success."
-Henry Ford.