Enzymatic assays for the assessment of toxic effects of halogenated organic contaminants in water and food. A review

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Abstract

Halogenated organic compounds are a particular group of contaminants consisting of a large number of substances, and of great concern due to their persistence in the environment, potential for bioaccumulation and toxicity. Some of these compounds have been classified as persistent organic pollutants (POPs) under The Stockholm Convention and many toxicity assessments have been conducted on them previously. In this work we provide an overview of enzymatic assays used in these studies to establish toxic effects and dose-response relationships. Studies *in vivo* and *in vitro* have been considered with a particular emphasis on the impact of halogenated compounds on the activity of relevant enzymes to the humans and the environment. Most information available in the literature focuses on chlorinated compounds, but brominated and fluorinated molecules are also the target of increasing numbers of studies. The enzymes identified can be classified as enzymes: *i)* the activities of which are affected by the presence of halogenated organic compounds, and *ii)* those involved in their metabolisation/detoxification resulting in increased activities. In both cases the halogen substituent seems to have an important role in the effects observed. Finally, the use of these enzymes in biosensing tools for monitoring of halogenated compounds is described.

Keywords: enzymes; halogenated organic contaminants; toxicity; *in vitro*; *in vivo*; biosensors.

Abbreviations:

1,2,3,7,8-pentachlorodibenzo-p-dioxin (PCDD); 17-hydroxysteroid dehydrogenase 3 (17-HSD3); 2,3,7,8-tetrachlorodibenzofuran (TCDF); 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T); 2,4,6-trichlorophenol (TCP); 2,4-dichlorophenol (2,4-DCPh); 2,4-Dichlorophenoxyacetic acid (2,4-D); 2-chlorophenol (2-CPh); 3,5-diiodo-1-tyrosine (DIT); 3-hydroxysteroid dehydrogenase (3-HSD); 3-iodo-1-tyrosine (MIT); 4-chlorophenol (4-CPh); Acetylcholinesterase enzyme (AChE); Aldose reductase (AR); Androstenedione (DIONE); Aryl hydrocarbon receptors (AhR); Benzoylphenylureas (BPUs); Bovine serum albumin and 5(6)-carboxynaphthofluorescein conjugates (CNF-BSA); Brominated diphenyl ethers (BDEs); Brominated flame retardants (BFRs); Butyrylcholinesterase enzyme (BChE); Catalase (CAT); Carbonic anhydrase (CA); Chemically Activated Luciferase gene eXpression (CALUX); Chlorinated aliphatic hydrocarbons (CAHs); Chlorophenols (CPhs); Commission Regulation (EC); Concentration of contaminant eliciting50% of the maximal response (EC_{50}); Cytochrome P450 family (CYP450); Dehaloperoxidase (DHP); Dichlorodiphenyldichloroethylene (DDE); Dichlorodiphenyltrichloroethane (DDT); Diflubenzuron (DFB); Dioxin-like polychlorinated biphenyls (DL-PCBs); Disinfection byproducts (DBPs); Ethoxyresorufin-O-deethylase (EROD); Ethyl dibromide (EDB); European Food Safety Authority (EFSA); European Union (EU); Glutathione peroxidase (GPX); Glutathione S-transferase (GST); Glyceraldehyde-3-phosphate (GAP); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Gold nanoparticles (Au-NPs); Haloalkane dehalogenase (LinB); Halogenated organic compounds (HOCs); Hexabromocyclododecanes (HBCD); Horseradish peroxidase (HRP); Hydroxylated brominated diphenyl ethers (OH-BDEs); Hydroxysteroid dehydrogenases (HSDs); Iodinated disinfection byproducts (I-DBPs); Iodotyrosine Deiodinase (IYD); Limit of detection (LOD); Maximum rate of the reaction (V_{max}); Michaelis-Menten constant (K_m); Nicotinamide adenine dinucleotides (NADH or NAD^+); Nicotinamide adenine dinucleotide phosphate (NADPH); Organization for Economic Co-operation and Development (OECD); Pentachlorocyclohexane (PCCH); Perfluorinated compounds (PFCs); Perfluorodecanoic acid (PFDA); Perfluorododecanoic acid (PFDoA); Perfluorooctane sulfonamide (PFOSA); Perfluorooctane sulfonate (PFOS);

Perfluorooctanoic acid (PFOA); Perfluorotetradecanoic acid (PFTA); Peroxidase (POD); Persistent environmental pollutants (POPs); Polybrominated biphenyls (PBBs);Polybrominated diphenyl ethers (PBDEs); Polychlorinated biphenyls (PCBs); Polychlorinated dibenzofurans (PCDFs); Polychlorinated dibenzo-p-dioxins (PCBDs); Polyvinyl chloride (PVC); Potassium perfluorooctane sulfonate (PFOSK); Precision-cut liver slices (PCLS); Pregnenolone (PREG); Reactive Oxygen Species (ROS); Residual standard deviation (RSD); Superoxide dismutase (SOD); Tetrabromobisphenol A (TBBPA); Tetrachlorodibenzo-p-dioxin (TCDD); Tolerable weekly intake (TWI); Toxic equivalency factors (TEFs);Triflumuron (TFM);Tyrosinase (TYR); Ultrahigh pressure liquid chromatography and photo-diode array (UPLC-PDA); United States Environmental Protection Agency (US EPA); Uridine 5'-diphospho (UDP)-glucuronosyltransferase (UGT); World Health Organization (WHO);γ-hexachlorocyclohexane dehydrochlorinase (LinA).

1. Introduction

Halogenated organic compounds (HOCs) are organic molecules that contain fluorine, chlorine, bromine or iodine atoms. These chemicals cause adverse effects on a diverse range of plants and animals as well as humans, and are one of the largest groups of environmental chemical pollutants (Farrington, 2014; Häggblom and Bossert, 2005; Kodavanti and Loganathan, 2016). Due to their stability, resisting both chemical and biological degradation, once in the environment most of these compounds have the capacity to be further disseminated. Moreover, HOCs have long-term bioaccumulative characteristics, meaning they are also considered as persistent environmental pollutants (POPs) (Häggblom and Bossert, 2005; Kodavanti and Loganathan, 2016).

Described for the first time described in 1825, the synthesis of benzene hexachloride (lindane), a compound with great insecticide activity, was carried out by Faraday (J. Yang et al., 2019). Since then, the use of these compounds became popular and they were used widely to control insect-borne diseases and increase agricultural productivity in many regions of the world (Kodavanti and Loganathan, 2016; Lipnick and Muir, 2001; Müller, 1942). In the early 1960's, the intense industrial production and the magnified use of new HOCs, together with indiscriminate disposal practices, led to their widespread entry into the environment, causing harmful effects on exposed biota (Häggblom and Bossert, 2005). In addition to anthropogenic causes, generation of such compounds also occurs naturally, with several HOCs having been found in archived whale oil samples from 1921, before industrial production (Teuten and Reddy, 2007).

Many HOCs are accumulated in fatty tissues and undergo biomagnification processes, being integrated in the food chain, causing toxicity and, therefore, a negative impact on animal and human health (Häggblom and Bossert, 2005). Adverse effects on human health and the environment triggered international agreements, such the 1987 Montreal Protocol for volatile compounds that affected the ozone layer (Ozone Secretariat, 2018) and the Stockholm Convention (Convention, 2017). The latter established a regulatory framework for the progressive eradication of POPs. In particular, 12 different types of POPs were identified for elimination and control in more than 150 countries (Kanan and Samara, 2018). Concerns of the scientific community

increased after creation of these international agreements, leading to numerous publications and citations from the early 1990's until the present day (Fig. 1).

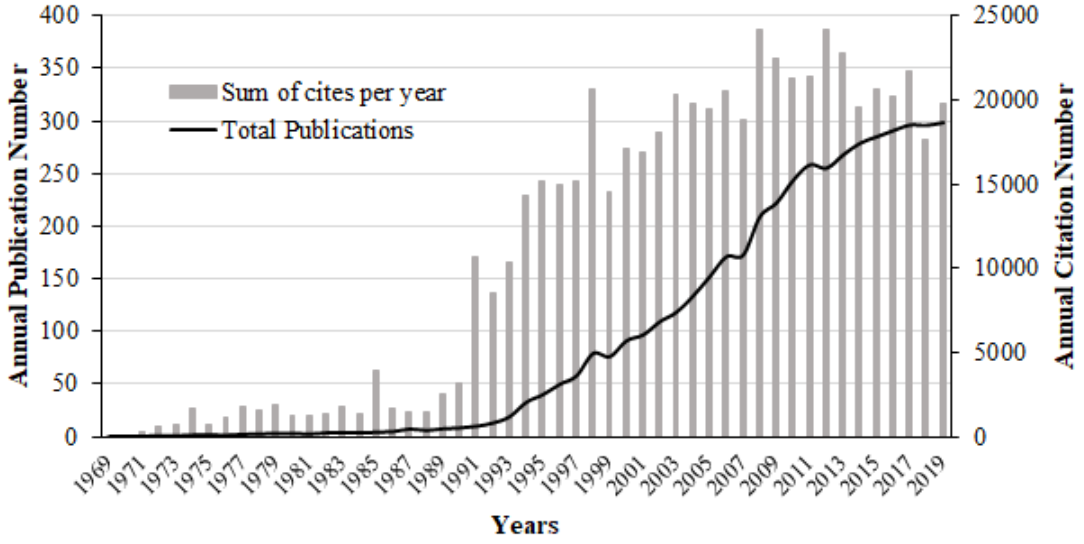

Fig 1. Annual publication and citation numbers from 1969 to 2019 with subject classifications of "toxicology" and "Food Science and Technology" at Web of Science Core Collection (January 21, 2020). (Data originated from the search topics "halogenated contaminants" OR "halogenated contaminant" OR "brominated contaminants" OR "brominated contaminant" OR "fluorinated contaminant" OR "fluorinated contaminants" OR "chlorinated contaminant" OR "chlorinated contaminants" OR "iodinated contaminant" OR "iodinated contaminants", and from commonly used expression as "PFC" OR "PFCs" OR "BFRs" OR "BFR" OR "PFOA" OR "PFOS" OR "CAH" OR "CAHs" OR "PCB" OR "PCBs" OR "PCBDs" OR "PCDFs" OR "DL-PCBs").

Organohalogen pollutants are classified according to the halide anion substitution in the organic core molecule. Organochlorine compounds, the major family of HOCs, can be categorised as chlorinated aliphatic hydrocarbons (CAHs), chlorophenols (CPhs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCBDs) and polychlorinated dibenzofurans (PCDFs). Their broad applicability and versatility led to widespread and improper use as well as inappropriate disposal practices, which accelerated their incorporation into the environment and food products. Since the start of industrial production, this group of highly toxic compounds includes the highest number of compounds restricted by legislation (Kodavanti and Loganathan, 2016; Korrick and Sagiv, 2008; Sawhney and Hankin, 1985). Chlorinated organic compounds

have been associated with type 2 diabetes and obesity (Lee et al., 2014), changes in microbiome (Zhang et al., 2015) and endocrine disruption (Kemsley, 2015; Louis et al., 2013). In 2014, Farrington (2014) described the potential for many thousands of unidentified organochlorides by-products, derived from their production and application globally. In fact, concerns about HOCs also extend to the dissemination and toxic effects of new emerging contaminants, specifically fluorinated (Koponen et al., 2015; Sznajder-Katarzyńska et al., 2019) and brominated organic compounds (Cruz et al., 2019; ten Dam et al., 2012; Zhang et al., 2020).

Perfluoroalkylated compounds (PFCs), are emerging environmental contaminants with amphiphilic properties, meaning they have a wide range of industrial applications such as pesticides or surface coatings (Kodavanti and Loganathan, 2016), and include compounds such as perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorooctylsufonamides and heptadecafluoro-1-decanol. Potential dietary sources for PFCs include seafood and drinking water, as well as nonstick cookware utensils and microwave popcorn bags (Cao et al., 2019; Choi et al., 2018; Kodavanti and Loganathan, 2016). Adverse effects from PFCs exposure (e.g. hepatotoxicity, neoplastic induction, developmental toxicities and fetal defects, immunotoxicity, neurotoxicity and endocrine disruption, are based on laboratory animal models (Lau, 2015; Olsen et al., 2009), but epidemiological reports also suggest bioaccumulation of these compounds (Olsen et al., 1999) and increased risk of cancer (Cao et al., 2018).

Brominated organic compounds are found commonly in fumigants, such as 1,2-dibromoethane or ethyl dibromide (EDB) and brominated flame retardants (BFRs). BFRs are synthetic compounds, more specifically polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), hexabromocyclododecanes (HBCD) and tetrabromobisphenol A (TBBPA), which are used to reduce flammability of industrial and household products (Cruz et al., 2019; Kodavanti and Loganathan, 2016; ten Dam et al., 2012). Due to their toxic effects and potential for bioaccumulation in ecosystems and organisms, including seafood, BFRs are considered as POPs and restricted by legislation (Flórez and Resano, 2013; Richardson and Kimura, 2016; Vandermeersch et al., 2015). Although occasionally human exposure occurs through inhalation of indoor dust and air, BFRs are food contaminants because the primary route of exposure is

dietary (Cao et al., 2019; Cruz et al., 2019; McGrath et al., 2018). Studies on the effects of BFRs in animals revealed an association with thyroid dysfunction, increased risk of cancer, neurotoxicity, and harmful changes in homeostasis, neurobehavioral and reproduction (Kim et al., 2014). Brominated organic compounds, used as disinfection by-products (DBPs) in water treatment processes, are also associated with adverse health impacts, specifically increased risk of carcinogenesis at very low concentrations (Allard et al., 2015; Danchana et al., 2015; Gregory et al., 2011; Maznaya et al., 2019; Richardson and Kimura, 2016; Sun et al., 2013).

Contrary to previously mentioned HOCs, iodinated organic compounds are still not of concern, despite data released during the last decade suggesting they also have harmful effects on the environment and health. Iodide is present naturally in ground water, but concentrations are incremented by anthropogenic activities, including improper disposal of iodinated wastes, and release of iodide-containing pharmaceuticals. These increased environmental concentrations are usually linked to undesirable effects in wastewater treatment plants generating iodinated disinfection by-products (I-DBPs) (Dong et al., 2019; Duirk et al., 2011; Hapeshi et al., 2013; Kormos et al., 2010). For example, the use of oxidizing agents like chlorine, chloramine, ozone (Tian et al., 2013; Weinberg et al., 2002) or potassium permanganate (Ye et al., 2012), promotes oxidation of iodide anion to hypoiodous acid, which acts as a precursor, reacting with organic matter to generate I-DBPs. Such compounds are incorporated in drinking water at concentrations from ng/L to μg/L and ingested by humans directly (drinking water) or indirectly (cooking water) (Dong et al., 2019), alongside iodinated table salt, making calculation of exposure problematic (Becalski et al., 2006; Pan et al., 2016). Recently reviewed by Dong et al. (2019), iodine-containing compounds can be present as iodinated methanes, iodo-acids, iodo-haloacetamides, iodo-haloacetonitriles, iodo-haloaldehydes and iodo-phenols. Although more than 700 DBPs have been characterised chemically, determination of concentrations and study of their toxicological effects are limited by a lack of chemical standards. Notwithstanding, some of these iodinated compounds have been associated with strong cytotoxic and genotoxic effects in Chinese hamster ovary cell-lines (Duirk et al., 2011; Liberatore et al., 2017; Plewa et al., 2004; Richardson et al., 2008), *Sprague-Dawley* rat whole embryo culture (Hunter and Tugman, 1995), *platynereis*

dumerilii embryos (Pan et al., 2016; Yang and Zhang, 2013) and *Salmonella typhimurium* cultures (Plewa et al., 2004). Their widespread presence in water systems and subsequent exposure to them, demands development of robust analytical methodologies for iodinated compounds as well as deeper knowledge about their potentially harmful effects.

After more than four decades of banning or restricting severely the use and production of HOCs, their concentrations in the environment have declined, but in some cases relatively slowly. Their persistence, widespread occurrence and variety of harmful effects, are a real threat to life on earth, with new compounds still being discovered and integrated into the food chain (Farrington, 2014; Häggblom and Bossert, 2005; Kodavanti and Loganathan, 2016).

The presence of HOCs have been correlated with alterations in the activities of several enzymes. Some are susceptible to inhibition by HOCs, while others metabolise these compounds. By determining the Michaelis-Menten constant (K_m) to assess the affinities of enzymes for their substrates and calculating maximal rate of enzymatic catalysis (V_{max}), enzymatic kinetics can be used to measure the impacts of different molecules in biochemical reactions (Engelking and Engelking, 2015). Inhibitor compounds can act through competitive, non-competitive, uncompetitive, or mixed inhibition mechanisms, altering natural K_m and V_{max} values. Moreover, such processes may be reversible or irreversible depending on the non-permanent or permanent binding of inhibitor molecules to the enzyme structure (Amine et al., 2015, 2006; Shuler and Kargi, 2002). In the case of reversible inhibitors their affinity, K_i, is calculated in order to classify them as strong or weak inhibitors (Bhagavan, 2002).

Among the different groups of enzymes analysed to study the effects of HOCs, the cytochrome P450 family (CYP450) is the most recurrent. CYP450 is responsible for oxidative metabolism of many endogenous (steroids, fatty acids) and exogenous (drugs, xenobiotics) substrates. For example, the activities of CYP1A1, 1A2, 2A1, and 1B1 are induced by dioxins and coplanar PCBs (Carpenter, 2006; Cheung et al., 2002; Stagg et al., 2016). CYP450s are iron-containing hemoproteins, responsible for first phase of xenobiotic oxygenation, facilitating conjugation to an endogenous substrate. Such conjugation reactions, carried out in a second phase by endogenous antioxidant enzymes, convert xenobiotics to more hydrophilic compounds, allowing

detoxification and, ultimately, excretion. However, these elimination mechanisms can result in activation of highly reactive or more toxic species, as well as damaging intermediates (Halliwell and Gutteridge, 1999; Stagg et al., 2016). The altered antioxidant response can induce the activity of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPX) and NADHDT-diaphorase, which as endogenous antioxidant enzymes, are the first-line defence against free radicals and free radical-generating agents (Halliwell and Gutteridge, 1999; Lemaire et al., 2011; Stagg et al., 2016). In addition, acetylcholinesterase enzyme (AChE) activity can also be correlated indirectly with the presence of HOCs. This enzyme catalyses hydrolysis of the neurotransmitter acetylcholine to choline and reacts irreversibly with some HOCs, especially organophosphorus insecticides (Colovic et al., 2013). The anticholinergic activity of HOCs, has also been observed for butyrylcholinesterase enzyme (BChE), compromising the butyrylcholine hydrolysis and affecting the neurotransmission processes (Bayrak et al., 2019, 2017; Massoulié et al., 1993; Öztaskın et al., 2017; Rezai et al., 2018).

The biological activity of some synthetic and natural HOCs, has also been exploited for development new drugs used as anti-epileptics and for the treatment of diabetes, edema and glaucoma (Bayrak et al., 2019; Boztas et al., 2019; Kocyigit et al., 2017; Oktay et al., 2017; Taslimi et al., 2018). This activity is associated with the inhibition of specific metabolic enzymes, namely carbonic anhydrases (CAs) and their isoforms, which are involved in reversible conversion of carbon dioxide to bicarbonate (Imtaiyaz Hassan et al., 2013), aldose reductase (AR), an oxidative stress inducer, and digestive enzymes, such as α-amylase and α-glycosidase (Taslimi et al., 2018; Öztaşkın et al., 2019).

The response of enzymes, like tyrosinase (TYR), are also of interest to food, agriculture, pharmaceutical industries, as well as in environmental applications (Agarwal et al., 2014; Sambasiva Rao et al., 2013). TYR has an important role in melanin synthesis, catalysing monooxygenation of phenols (monophenolase or cresolase activity), and oxidation of ortho-diphenols (diphenolase or catecholase activity) (Agarwal et al., 2014; Sambasiva Rao et al., 2013; Seo et al., 2003). Both pathways can be affected by halogenated phenols and pesticides resulting in inhibition of TYR activity. Interestingly, rather than being inhibited, TYR can metabolise halo-

monophenols like 4-chlorophenol (Anh et al., 2004). Like TYR, the iodotyrosine deiodinase (IYD) has L-tyrosine-based activity, but it also catalyses reductive dehalogenation of halogenated tyrosines to 3-iodo-L-tyrosine. IYD has also been described to act on brominated and chlorinated tyrosine. Inhibition of IYD activity has been associated with HOCs, suggesting these compounds might disturb thyroid homeostasis, opening the way for its use as a biomarker in toxicological studies, or a bioreceptor in biosensing strategies (Shimizu et al., 2013).

Despite the inhibition potential of HOCs, some enzyme groups are able to metabolise these pollutants to less halogenated products, taking part in detoxification of these compounds. For instance, naturally occurring γ-hexachlorocyclohexane dehydrochlorinase (LinA) and haloalkane dehalogenase (LinB) catalyse cleavage of carbon-halogen bonds (Häggblom and Bossert, 2005; Olaniran and Igbinosa, 2011). LinA catalyses dehydrochlorination of halophenols, such as lindane, generating less chlorinated products that can be dechlorinated hydrolytically by LinB (Longoria et al., 2008; Szatkowski et al., 2011; Wannstedt et al., 1990). LinB has also been described as having catalytic activity against some CAHs (Campbell et al., 2006; Hutter et al., 1995; Mazurenko et al., 2018; Shahar et al., 2019a, 2019b). Also recognised as having high detoxification potential, heme-containing enzymes, like dehaloperoxidase (DHP) and horseradish peroxidase (HRP), catalyse oxidative dehalogenation of halophenols to their corresponding quinones (Longoria et al., 2008; Wannstedt et al., 1990), but can also be inhibited by several HOCs, revealing the complex biological interactions of such compounds (Szatkowski et al., 2011).

With particular focus on fluorinated, chlorinated and brominated organic compounds, this review aims to describe the state-of-the-art on exposure, bioaccumulation, and toxicity of HOCs as well as bioassays *in vitro* and *ex vivo*. Moreover, this paper aims to review thoroughly enzymes induced or otherwise affected by exposure of organisms to HOCs, highlighting them as biomarkers for contamination and biomagnification within the trophic chain. Growing concerns about toxic effects on human health have resulted in increased demand for low-cost, rapid, selective, sensitive and ready-to-use analytical tools for detection of these POPs determination in

the environment as well as foods. Enzymes for their application in biosensing approaches for the analysis of HOCs in environmental and food samples are also included.

2. Fluorinated organic compounds

Fluorinated organic compounds are an emerging class of global environmental pollutants. They are characterised by strong and stable carbon-fluorine bonds, and great potential for bioaccumulation in the environment and human body (Kodavanti and Loganathan, 2016; Koponen et al., 2015). PFOS and PFOA were the most frequently used PFCs until 2001 when the lead manufacturing company in PFCs stopped PFOS production, making PFOA the most common (Zhao et al., 2017). PFOS is still the most common product formed from breakdown of fluorinated commercial products (Qu et al., 2010), making control of both, PFOS and PFOA, in environmental matrices necessary, as well as a better understanding of their toxic effects in living organisms. Exposure to PFOS and PFOA has been associated with hepatic, immunological, reproductive, neurobehavioral, reproductive and hormonal toxicities, as well as genotoxic and carcinogenic potential (Squadrone et al., 2015). The presence of PFOS and PFOA has been detected in environmental and biological samples globally, with evidence of bioaccumulation at higher trophic levels. In fact, these compounds have been identified in human samples such as blood, serum and milk (Guerranti et al., 2013) at different regional concentrations. For example, in the US, concentrations up to 656 µg/L for PFOS and 88 mg/L for PFOA have been detected, while in Europe concentrations ranged from 1 to 116 µg/l for PFOS and from 0.5 to 40 µg/L for PFOA (Fromme et al., 2009).

Dietary intake is the major exposure route for PFCs in human populations (Koponen et al., 2015) and thus, the European Commission has published the proposal for a directive 2017/0332 setting limits at 0.4 µg/L for PFOS and 4 µg/L for PFOA in drinking water. European Food Safety Authority (EFSA) agreed that such compounds can be harmful and listed sources of exposure: fish and other seafood, meat and meat products, and eggs and egg products for PFOS; and milk and dairy products, drinking water and fish and other seafood for PFOA (Knutsen et al., 2018).

Tolerable weekly intakes (TWI) of 13 and 6 ng/kg body weight (bw) have been established for PFOS and PFOA, respectively.

Besides PFCs, commercial fluorinated insecticides, such as benzoylphenylureas (BPUs) and their derivatives, are also widely used sources of HOCs in the environment. Triflumuron (TFM), diflubenzuron (DFB) and novaluron are common BPUs, and studies have shown these compounds are toxic for aquatic organisms and they bioaccumulate (up to 160 times) from water (Pereira Maduenho and Martinez, 2008; Santorum et al., 2019; Timoumi et al., 2019). Furthermore, DFB metabolites, such as 4-chloroaniline, are more toxic to fish than the parent compound and are classified as probable carcinogens. The accumulation and fate of PFCs and fluorinated insecticides in the trophic chain can be harmful to living organisms, including humans and it is important that more is known about their toxicity and potential risks for human health.

2.1. Studies *in vivo* on fluorinated organic compounds

Toxicological studies *in vivo* are performed under specific exposure conditions using whole animals or organisms. Different types of toxic effects are examined, such as acute, subacute and chronic toxicities, among others. Additionally, these studies allow critical concentrations definition, identifying their specific effects and bioaccumulation potential (Zeng et al., 2019). The adverse effects of PFCs, altered enzymatic activities, are shown in Table 1.

Because of its small size, sensitivity to different compounds and short lifespan allowing multi-generational studies to be performed, *Daphnia magna* is an often-used model organism to assess toxicities of environmental contaminants. Exposure of *D. magna* to PFOS and PFOA causes physiological and metabolic changes including decreased body weight and offspring numbers, and function of some enzymes, such as AChE, GST, SOD and CAT (Jeong et al., 2016; H. B. Yang et al., 2019). Chronic and acute exposures increase AChE activity at concentrations ranging from 100 to 1000 µg/L (PFOA) and from 1 to 10 µg/L (PFOS). Difference in binding affinities, higher in the case of PFOS, agrees with the toxicities found for both compounds (H. B. Yang et al., 2019). Alteration in GST, SOD and CAT activities, associated with antioxidant defence systems, cause also concerns around the presence of potential xenobiotics. Yang et al. (2019),

following OECD Test no. 202 (OECD, 2004), showed that CAT and GST activities were inhibited at 14 mg/L PFOS or higher, while SOD and CAT were inhibited by PFOA concentrations at 20 mg/L and higher. Considering the complexes formed by these enzymes and PFCs, similar binding modes were found for SOD and GST regardless of the PFCs, but CAT was more sensitive to PFOS than PFOA suggesting different mechanism of interaction, SOD was more sensitive to PFOA than PFOS and GST was the less sensitive to both PFCs.

Exposure of *D. magna* to PFOS and PFOA was also tested by Liang et al. (2017) using OECD guidelines (OECD, 2004). After 10 days of exposure GST activity was increased at 4 mg/L of PFOS but inhibited at higher concentrations. This effect, contradictory effects at high and low doses, is known as hormesis and has been described for many drugs and xenobiotics (Bhakta-Guha and Efferth, 2015). Regarding CAT, activity decreased significantly only at 16 mg/L PFOS. After 21 days of exposure, a decrease in GST activity was observed, demonstrating that *D. magna* was unable to detoxify PFOS and avoid oxidative stress. Further exploration with CAT also indicated hormesis with activities higher at 4-8 mg/L PFOS, but inhibited at 16 mg/L. This suggests induction of CAT to metabolise PFCs and reduce oxidative stress, but when concentration increases the endogenous antioxidant system is overwhelmed. AChE was also affected by exposure to PFCs; activity decreased at lower concentrations but increased at higher concentrations to ensure neural signal transmission. At concentrations higher than 16 mg/L, tolerance of the organism was exceeded, and *D. Magna* died. (Liang et al., 2017).

Studies with wheat (*Triticum aestivum*) showed induction of chlorophyll and soluble protein synthesis, also stimulating growth after exposure to PFOS at concentrations below 10 mg/L (Qu et al., 2010). Higher concentrations exerted elongation and biomass inhibition of roots and leaves as well as increased permeability of wheat root cells and antioxidant potential, because of induction of SOD and peroxidase (POD) activities. Under stress, plants trigger induction of SOD and CAT, to reduce reactive oxygen species (ROS) such as superoxide ($^{\cdot}O_2^-$), hydroxyl ($^{\cdot}OH$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and nitric oxide ($^{\cdot}NO$) (Wu and Tiedemann, 2002). ROS are necessary in living organisms and produced during common metabolisation reactions, but in case of imbalance between ROS and antioxidant defenses damages can occur (Gulcin 2020).

Furthermore, stimulated by the enzymatic products of SOD, POD eliminated excess of H_2O_2 and ensured correct cellular function (Bowler et al., 1992). Nevertheless, when exposed to 200 mg/L PFOS, wheat displayed significant inhibition of SOD and POD activities, affecting antioxidant defence system (Qu et al., 2010).

Earthworms (*Eisenia fetida*) are used commonly as standard biological indicators for soil pollution and have been also used to assess the toxicity of fluorinated organic compounds. For example, Zhao et al. (2020) studied the effects of perfluorooctane sulfonamide (PFOSA) and its biotransformation in earthworm. Worms were exposed to sand treated with 1.07 nmol/g PFOSA, which is the most important intermediary of PFOS precursors causing its accumulation in the environment and biota (Bizkarguenaga et al., 2016; Brandsma et al., 2011; Mejia Avendano and Liu, 2015). CYP450 and GST activities in earthworms increased in response to PFOSA exposure, up to six days, but then decreased. The induced activities of these enzymes confirm their involvement in PFOSA metabolism, contrary to POD activity, which did not change (Zhao et al., 2020).

Other fluorinated compounds also affect enzyme activities in living organisms. The insecticide DFB has been associated with changes in GST, CAT and AChE activities in freshwater fish, namely *Prochilodus lineatus*. Pereira Maduenho and Martinez (2008) observed that antioxidant defences were activated in presence of DFB and, more specifically, CAT and GST activities increased after 96 hours of exposure to 25 mg/L DFB in water. In contrast, AChE exhibited lower activity suggesting DFB is a potent inhibitor of muscle AChE.

The effects of fluorinated contaminants have been demonstrated in different species and could be extrapolated to humans. Changes observed in enzyme activities, impact organisms' metabolism, producing disorders and pathologies at low concentrations and mortality at high doses. Thus, the use and widespread distribution of these hazardous contaminants must, therefore, be controlled to avoid harmful effects in the environment and humans, particularly through dietary exposure.

2.2. Studies *ex vivo* and *in vitro* on fluorinated organic compounds

Studies *in vivo* identified enzymes with an important role in the metabolism of fluorinated xenobiotics, and suggested others which activities are significantly inhibited by these pollutants. Studies *in vitro* have been developed to better understand the interaction of contaminants with enzymes and their role in normal metabolism, where fluctuations in enzymes activities can be also a useful tool to monitor and measure the presence of contaminants.

As stated previously, fluorinated organic contaminants are associated with altered CYP450 activities *in vivo*. However, as shown in Table 1, evidence has also been described for bioassays *in vitro*. Narimatsu et al. (2011) characterised the inhibition caused by PFOS on the activity of different microsomal fractions of human CYP450, as well as in recombinant CYP450s. The authors studied both, inhibition and inactivation processes, promoted during exposure to 0, 10, 15, 20 and 30 µM PFOS. In the inhibition assays, PFOS concentrations were incubated simultaneously with the substrate molecules while, in inactivation studies, PFOS was preincubated with the CYP450s in the presence of NADPH (nicotinamide adenine dinucleotide phosphate), before addition of the substrates. Among the various CYP450-mediated reactions, diclofenac (NSAID drug) metabolisation by CYP2C9 was inhibited the most (K_i= 36 nM), followed by CYP2C8 (K_i= 4 µM) > CYP2A6 > CYP2B6 > CYP2C19 and CYP3A4 (K_i= 35-40 µM). Although IC_{50} values in inhibition and inactivation assays were different, this trend was repeated. For instance, the IC50 values of CYP2C9 inhibition and inactivation assays, were 0.04 nM and 0.064 nM, respectively. CYP2C8 also showed the same response, with an inhibition IC_{50} of 5.09 µM, and inactivation IC_{50} of 5.91 µM. The reactions were followed by measuring substrates or products of the reaction by HPLC and UV or fluorescence detector. CYP2C9 metabolises different medicines and endogenous compounds, apart from diclofenac 4-´-hydroxylation. Considering that the PFOS K_i value for diclofenac 4´-hydroxylation was considerably low, oxidation of several endogenous compounds in human body, might be impacted by PFOS bioaccumulation, affecting the normal physiological processes. So, inhibition of these enzymes, especially the CYP2C9, could indicate the presence of PFOS in the exposure media.

In the case of other fluorinated compounds, such as triflumuron (TFM), activities of CYP450s *in vitro* increased due to their contribution to TFM metabolisation. TFM is a benzoyl-phenyl-urea

insecticide that is commonly used for many fruits and vegetables due to its low mammalian toxicity. Timoumi et al. (2019) observed that different cDNA encoding recombinant human CYP450 in *Escherichia coli*, were affected by TFM to different extent. TFM biotransformation by various CYP450s was performed using HPLC-DAD (high pressure liquid chromatography with diode array detector) at concentrations between 1 and 150 µM. Taking into account K_m values, the authors concluded that the order of affinity was 3A4>> 2C9 > 2C8 > 2A6 > 1A2 > 2B6 > 2D6 > 2C19 > 2C18 > 1A1 at lower TFM concentrations; and 1A2 >> 2C9 = 3A4 = 2A6 > 2C19 > 2B6 = 2C8 > 2D6 at higher TFM concentrations.

UDP-glucuronosyltransferase (UGT) plays an important role in the metabolism of a considerable number of drugs (15% drugs of the market). Directly or after initial oxidative metabolism by CYP450 enzymes (Seo et al., 2014), UGT also metabolises bilirubin, oestrogen, thyroid hormones and serotonin, among others (Y. Z. Liu et al., 2019). Likewise, hydroxysteroid dehydrogenases have a role in conversion of cholesterol to testosterone along with CYP450 enzymes. 3-Hydroxysteroid dehydrogenase (3-HSD) catalyses transformation of pregnenolone (PREG) into progesterone in presence of NAD^+ while 17-hydroxysteroid dehydrogenase 3 (17-HSD3) converts androstenedione (DIONE) to testosterone in presence of NADPH (Zhao et al., 2010). Activities of 11 isoforms of recombinant human UGT, were tested *in vitro* in presence of 14 different PCFs (0.5-100 µM). Enzymatic reactions with substrates were monitored using UPLC-PDA (ultrahigh pressure liquid chromatography and photo-diode array) separation and detection system. Inhibition of their activities was observed at the highest concentration of PCFs (100 µM) and PFOA, perfluorodecanoic acid (PFDA), perfluorododecanoic acid (PFDoA), perfluorotetradecanoic acid (PFTA) and PFOS exerted the strongest inhibition (K_i=1-20 µmol/L) (Y. Z. Liu et al., 2019). Another study examined inhibition of 3-HSD and 17-HSD3 activities by five PFCs at 0.1 to 1000 µM using microsomes containing HSD extracted from rat and human testicles (Zhao et al., 2010). In order to follow the reaction, the steroids were separated chromatographically on thin layer plates and measured using scanning radiometer. Even though there were differences between both species (rat vs human HSD), PFOS, PFOA and potassium perfluorooctane sulfonate (PFOSK) were potent inhibitors of 3-HSD and 17-HSD. While human

3-HSD was inhibited by PFCs at concentrations above 250 μM, the rat enzyme was inhibited at 2-55 μM. In the case of 17-HSD3, the human enzyme was inhibited at concentrations higher than 0.1 μM for PFOS, PFOA or PFOSK, but the rat enzyme was inhibited by 20 μM PFOA and higher than 250 μM PFOS and PFOSK. Since these enzymes are vital for transformation of endogenous substances or/and xenobiotics, it is clear that PFCs can produce toxicities by disrupting reactions catalysed by UGT, 3-HSD and 17-HSD3 and, therefore, changes in the activities of these enzymes can be exploited to monitor the presence of PFCs.

Like studies *in vivo*, catalase (CAT) was inhibited by PFOS *in vitro* at high concentrations, greater than 10 μM. However, PFOA had no significant effect on CAT activity (Xu et al., 2018). The authors also studied binding interactions between both PFCs and CAT and, noting decreased α-helix and increased β-sheets contents in the enzyme structure, inhibition by PFOS could be explained by the bind of the fluorinated compounds close to the enzyme's active site, while the binding site for PFOA was on the surface of the enzyme. Thus, PFOS can disrupt one of the primary endogenous antioxidant enzymes leading to alterations because of the accumulated reactive oxygen species (ROS).

Aside from PFCs and TFM, other fluorinated compounds can affect enzymatic activities. AChE is inhibited by some fluorinated pesticides such as novaluron and DFB, though the inhibition potential is low. These pollutants have low degradation rates in environment and are, as a consequence, available for bioaccumulation. Fluorinated pesticides can induce pathologies due to inhibition of enzymes like AChE, resulting in cholinergic hyperstimulation caused by accumulation of acetylcholine in the synaptic cleft. De Souza et al. (2018) exposed oyster viscera to 0.001-10 mM of DFB and to 0.00025-2.5 mM of novaluron to assess the endogenous AChE activity determining IC_{20} values of 0.091 mM for novaluron and 1.35 mM for DBF. However, K_i values could not be calculated because of the weak inhibition potentials. Compared with studies *in vivo* (Pereira Maduenho and Martinez, 2008), this study *in vitro* generated results suggesting low inhibition potential of AChE activity by DBF.

3. Chlorinated organic compounds

Organochlorinated compounds were widespread as pesticides, pharmaceuticals, refrigerants, plastics and cleaning agents, among others (Farrington, 2014). Accidental spills and leaks, indiscriminate disposal practices and recurrent use as biocides have resulted in increased dissemination of chlorinated organic compounds into the environment, which are nowadays associated with ground and surface water contamination. These compounds can also appear as undesirable by-products from chlorine disinfection of water and bleaching of paper pulp (Häggblom and Bossert, 2005). These compounds can be generated through geogenic sources, such forest fires and volcanic emissions; and biological processes where the halide is introduced naturally as part of the carbon cycle (Farrington, 2014; Häggblom and Bossert, 2005; Wannstedt et al., 1990).

Chlorinated compounds are present in diverse forms, occurring as both aliphatic and aromatic hydrocarbons with at least one covalently bonded chlorine atom (Zimmermann et al., 2020). Chlorinated aliphatic hydrocarbons (CAHs) are a diverse group of compounds that are, in the main, used as industrial solvents, intermediaries in the synthesis of other chemicals, and cleaning and degreasing products (Field and Sierra-Alvarez, 2004; Zimmermann et al., 2020). CAHs are short hydrocarbon chains containing at least one covalently bonded chlorine atom, having apolar nature, low flammability and subject to rapid evaporation (Zimmermann et al., 2020). They can be categorised as chlorinated methanes, chlorinated ethanes, chlorinated ethenes, chlorinated fluorocarbons, chlorinated acetic acids, chlorinated propanoid compounds and chloro-1,3-butadienes (Field and Sierra-Alvarez, 2004). Although some microorganisms are able to metabolise CAHs, they can be trapped in soils exhibiting a marked environmental persistence over periods of decades (Leisinger, 1996; Rockne and Reddy, 2003). Bioaccumulation of CAHs has also been reported and trace concentrations of CAHs have been found in species like Pacific Walrus (*Odobenus rosmarus divergens*) (Seagars and Garlich-Miller, 2001). CAHs contamination is associated with anthropogenic activities, specifically, improper handling and release into the ecosystem, resulting in toxic and carcinogenic exposures (Guha et al., 2012; Leisinger, 1996; Lu et al., 2015).

As the simplest aromatic hydrocarbons category, chlorophenols (CPhs) have widespread occurrence throughout the environment and are found as contaminants in water, sludge products, waste disposal sites, and municipal landfills. CPhs are present as mono-, di-, tri-, tetra-, penta-chlorophenols and lindane as hexachlorophenol. There are also 19 known congeners, such as 2-chlorophenol (2-CPh), 2,4-dichlorophenol (2,4-DCPh), 2,4,6- trichlorophenol (TCP) and pentachlorophenol. The chlorination of a benzene ring provides this group with broad-spectrum antimicrobial, biocide, wood preservative, and pharmaceutical properties, and they can also be used as dyes (Olaniran and Igbinosa, 2011; Oluwasanu, 2018; Pera-Titus et al., 2004). CPhs are generated from biodegradation of pesticides and herbicides, or from incineration of municipal wastes (Olaniran and Igbinosa, 2011). As CAHs, CPhs persist in the environment and are resistant to biodegradation. Moreover, their lipophilicity, provided by the extent of chlorination, permits bioaccumulation in aquatic organisms and, ultimately, incorporation into the trophic chain. Main source of exposure are topic exposure, inhalation or oral ingestion, and are recognized as having carcinogenic potential and mutagenic activity, thus, considered harmful to human health (Olaniran and Igbinosa, 2011; Pera-Titus et al., 2004).

Polychlorinated biphenyls (PCBs), widely used as insulating fluids in transformers and capacitors, and hydraulic systems as well as flame retardants, inks and surface coating, have a biphenyl core, on which numerous chlorine substitutions can be made. There are 209 possible PCBs congeners (George et al., 1988). Chlorination of biphenyl rings generates mixtures of different compounds, chlorine contents and purities, usually identified by commercial names like Chloretol (United States) and Phenochlor (France), among others. Like CPhs, the position and number of chlorine substitutions affect their lipophilic character and toxicity, with higher bioaccumulation potential found for the more substituted biphenyls.

Toxic effects of PCBs were rapidly noticed during the first decade of commercialization, with reports of poising by inhalation or dermal contact during manufacturing, but also among electricians (George et al., 1988; Kodavanti and Loganathan, 2016). Extensive dissemination of PCBs in several ecosystems, was detected through bioaccumulation in seabirds and their eggs, and in tissues of animal and vegetal species used for human consumption (Farrington, 2014).

These molecules undergo biomagnification, increasing their concentration as they pass through each trophic level (Sawhney and Hankin, 1985). PCBs are easily distributed among air, water and soil, bioaccumulating in plankton, bivalve molluscs, fish, reptiles, marine mammals, birds and terrestrial animals (Kodavanti and Loganathan, 2016). Food poising episodes in humans and domestic animals, resulted in cessation of PCBs production and sales after 1971 as well as regulation of PCBs levels in food products (Farrington, 2014; George et al., 1988). Studies have demonstrated that contaminated fish can be a key source of PCBs, but other foods like plants, meat, and dairy products are also sources of human PCBs exposure (Korrick and Sagiv, 2008; Sawhney and Hankin, 1985). Poisoning with PCBs is associated with neurobehavioral effects, hypothyroidism, infertility, and reproductive system disorders, hypertension and cardiovascular diseases, liver diseases, asthma arthritis, diabetes, and low birth weights (Carpenter, 2006). Maximum concentrations of PCBs in food products were established in European Union (EU) by Commission Regulation (EC) No. 1881/2006 of 19 December 2006 and with subsequent amendments (Commission recomendation No. 2014/663/EU, 2014; Commission Regulation (EC) No. 1881/2006, 2006; Commission Regulation (EU) No. 1067/2013, 2013; Commission Regulation (EU) No. 1259/2011, 2011; Commission regulation (EU) No. 277/2012, 2012).

Polychlorinated dibenzo-p-dioxins (PCBDs) and polychlorinated dibenzofurans (PCDFs) are highly toxic molecules that have never been produced intentionally, arising as by-products of various industrial processes (Kodavanti and Loganathan, 2016; Lipnick and Muir, 2001). Commonly known as dioxins and furans, PCDBs and PCDFs present some similarities with PCBs congeners, which has resulted in the tendency to include both as subgroups of chlorinated biphenyls (Reiner et al., 2006). Considering the amount of chlorine substitutions there are 75 and 135 probable chlorinated dioxins or dibenzofurans, respectively, including some of the most toxic chemical substrates (Kanan and Samara, 2018; Kodavanti and Loganathan, 2016; Lipnick and Muir, 2001). PCBs congeners that only contain chlorine in the meta and para positions, assume planar configurations, acquiring dioxin-like activity and are known as dioxin-like polychlorinated biphenyls (DL-PCBs) (Carpenter, 2006). These three categories (PCDBs, PCDFs and DL-PCBs) have been associated with extreme persistence in the environment, high toxicities, and increased

risk of cancer (Kanan and Samara, 2018). Their toxic potency, is commonly measured in relation to toxic equivalency factors (TEFs), comparing it with the toxicity or biological endpoints of TCDD concentrations (Kanan and Samara, 2018; Lipnick and Muir, 2001)

Since early 1900s through to the 1970s, these compounds have shown a marked presence in the environment, indicating their correlation with anthropogenic activities. Besides indiscriminate disposal practices and the use of chlorinated pesticides, industrial activities like thermal and metal processing operations also generate PCBD/Fs. Such compounds are easily dispersed into the air and easily transported to distant sites (Kanan and Samara, 2018; Kumar et al., 2013). Moreover, processes involving combustion, like those in diesel vehicles, industrial activities (e.g. pulp and paper mills), reservoir processes (e.g. landfill burning), and other incineration sources, such as burning of sewage sludge, forest fires and of municipal and medical wastes, are prone to generate dioxins that are released into the environment (Kanan and Samara, 2018; Urban et al., 2014). Indeed, Kanan and Samara (2018) reviewed worldwide sources of PCDBs, PCDFs and DL-PCBs and concluded that combustion processes and industry are major sources of these compounds. They are easily adsorbed by soils and plants and integrate readily into the trophic chain, bioaccumulating in adipose tissues until consumed by humans in contaminated food products (Kumar et al., 2013).

Consumption of foods containing PCDBs, PCDFs or DL-PCBs, has been associated with toxic effects such as infertility, endocrine disruption, and modulation of sex and thyroid hormones (Kumar et al., 2013). Consequently, maximum levels for dioxins and furans in foodstuffs, recently reviewed by Hädrich and co-workers (2018), have been established in the EU by Commission Regulation (EC) No. 1881/2006 of 19 December 2006 and the subsequent amendments (Commission recomendation No. 2014/663/EU, 2014; Commission Regulation (EC) No. 1881/2006, 2006; Commission Regulation (EU) No. 1067/2013, 2013; Commission Regulation (EU) No. 1259/2011, 2011; Commission regulation (EU) No. 277/2012, 2012).

3.1. Studies *in vivo* on chlorinated organic compounds

Toxic effects of some chlorinated compounds are so evident that some species can be used as bioindicators (Table 2). Insects, for example, are the main link between producers (plants) and secondary consumers, and the initial step for the transportation and bioaccumulation of organic pollutants in the biosphere (Rosenberg et al., 1986). Yu Liu and co-workers (2018) reported the effects of dichlorodiphenyltrichloroethane (DDT) and PCBs in dragonfly, moth, grasshopper and litchi stinkbugs. They found different contamination patterns and marked biomagnification of chlorinated compounds in moths and grasshopper larvae, and a strong positive correlation for bioaccumulation in dragonfly larvae. They conclude that mechanisms regulating organic pollutants in insects during metamorphosis were common to all the species studied.

Several examples of bioaccumulation and transference to higher trophic levels can be found for chlorinated organic compounds. The Yangtze River Delta (China) has been exposed to several soil and groundwater contamination incidents, leading to accumulation of CAHs (Lu et al., 2015). Six species (amphibians, fish and birds) from the sites were sampled and screened for chlorinated contaminants. High concentrations of CAHs and 20 % of total PCBs were found in tissues from short-tailed mamushi snake (*Gloydius brevicaudus*), Peregrine falcon (*Falco peregrinus*) and Asiatic toad (*Bufo gargarizans*). However, all the species analysed exhibited concentrations of several other chlorinated chemicals including Dechlorane 602, DDTs, chlordane and heptachlor, among others. These findings suggest extensive contamination of wildlife and transport to higher trophic levels (Zhou et al., 2016).

Besides the importance of transportation and/or bioaccumulation to higher trophic levels, chlorinated compounds have been associated with the alteration of biochemical processes in organisms, resulting in modified metabolomic patterns and differential expression of key enzymes during exposure. Some investigations have concentrated on the use of antioxidant responses as biomarker for pollution. For instance, Cheng et al. (2002) evaluated the effects of chlorinated pesticides and PCBs in marine mussels (*Perna viridis*), specifically activities of antioxidant enzymes. The antioxidant responses were assessed in mussel gills and hepatic tissues after a 30 days exposure to water from polluted sites in Hong Kong. Moreover, concentrations of chlorinated pesticides and PCBs were also assessed in the tissues and correlated with endogenous

antioxidant systems. The authors found increased GST and peroxidase activities; other antioxidant enzymes presenting no alteration in their activities. The presence of PCBs was associated with GST induced activity, but the apparent lack of correlation between pollutant concentrations and other antioxidant enzymes was related to inhibitory effects of these POPs. Cheng et al. (2002) found increased lipidic peroxidation promoted by chlorinated pesticides, which, in turn, was related to the oxidative damage of DNA bases, especially in mitochondria. All the effects showed that animals were under oxidative stress, requiring more defences against free radicals.

The use of mussels and oysters as model organisms, allows the effects of POPs to be directly related to food contamination, but also, the use of these and other animals as geographic sentinels for monitoring chemicals of environmental concern (Farrington, 2014). Ferreira et al. (2005) correlated tissue concentrations of DDT and PCBs with antioxidant defence systems of two fish species, namely mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*), collected from contaminated sites in Douro estuary in Portugal. Liver SOD and CAT exhibited increased activity in mullet, indicating exposure to the pollutants enhanced ROS and therefore, oxidative stress. After 1 month of depuration in filtered seawater, mullet livers showed decreased activities for these enzymes. For flounders, the antioxidant response was not consistent, since the animals did not eat during the experiments. However, the authors found increased oxidized lipids and proteins in both species, suggesting the presence of pollutants induced oxidative stress responses. In a different study, Ferreira et al. (2004) found that accumulation of DDT was the highest in flounder liver, while PCBs accumulated preferentially in mullet muscle. Bioaccumulation of contaminants in these species, indicated that both fish are suitable sentinels for monitoring pollution in southern European estuaries (Ferreira et al., 2004).

Gendron et al. (1997) evaluated the effects of organochlorine pesticides and PCBs in aquatic salamander (*Necturus maculosus*). The animals, sampled at nine sites along the St. Lawrence and Ottawa rivers (CA), showed that concentrations of several PCBs congeners and chlorinated pesticides (e.g. chlordane, dieldrin and DDT) were bioaccumulating in the amphibians' gonads. Moreover, amounts of the bioaccumulated contaminants could be related to the extent of

contamination at various sites. Animals exposed to higher levels of contamination exhibited stress responses due to disruption along the hypothalamic–pituitary–interrenal axis. Liver glycogen reserves were also depleted in salamanders collected from the most contaminant sites, indicating involvement of these chemicals in the corticosterone path for glycogen replenishment (Gendron et al., 1997).

In addition to endocrine function, it has been shown that PCBs affect many, if not all, aspects of liver function and that different contaminants induced distinct liver CYP450 isoenzymes. Despite very few studies in humans, Fitzgerald et al. (2005) investigated associations between PCBs and the CYP450 activities *in vivo* among Native Americans. In this study, it was found that consumption of contaminated fish from the St. Lawrence River led to bioaccumulation of PCBs at concentrations up to 0.408 ± 0.064 ng/mL in serum, concomitant with an induced activity of the CYP1A2. An important relation between serum levels of PCB-153, PCB-170 and PCB-180, and induced CYP1A2 activity was found in volunteers, suggesting early biological effect of such exposure (Fitzgerald et al., 2005).

The strong potential for magnification along the trophic chain and the negative effects known and/or suspected of chlorinated compounds in human health, call for improved knowledge about their toxicological effects. Bioassays have been proposed as efficient tools for screening organochlorine compounds in food products, as defined by Commission regulation (EU) No 2017/644, for methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs (Commission regulation (EU) No. 2017/644, 2017).

3.2. Studies *ex vivo* and *in vitro* on chlorinated organic compounds

The study of chlorinated contaminants *in vivo* arises from the need to predict, rapidly and consistently, impacts of these compounds in organisms and populations. As summarized in Table 2, use of microsomes isolated from livers of animals in contact with toxic compounds (Ferreira et al., 2004), cell lines (Brack et al., 2002; Petrulis et al., 2001; Tom et al., 2001; Whyte et al., 1998) and whole-tissues (Lemaire et al., 2011), have been proposed as quicker ways of investigating toxicity. Isolated enzymes have also been exploited for *in vitro* inhibition studies by chlorinated

compounds. CAs isozymes isolated from human erythrocytes, demonstrated to be strongly inhibited by synthetic chlorophenols derivatives, generating Ki values of 23.27±0.68 nM and 10.64 ± 4.01 nM for hCA I and hCA II, respectively. The inhibition observed for these synthetic chlorophenols has been pointed as potential therapeutic for epilepsy or cerebral edema (Oktay et al., 2017).

A recent work described use of oyster (*Crassostrea rhizophorae)* gills and viscera extracts, to assess endogenous AChE activity, as a specific biomarker for toxicity caused by chlorinated organophosphorus pesticides (de Souza et al., 2018). The authors evaluated catalytic efficiency of the extracts *in vitro* for hydrolysis of acetylcholine, obtaining K_M values of 1.32±0.20 mM and 0.43±0.12 mM in gills and viscera, respectively. AChE from viscera extracts was inhibited strongly (K_i values of 8.96 µM and 157 µM for dichlorvos and chlorpyrifos, respectively). Moreover, the authors proved the suitability of oyster extracts for pesticides biomonitoring (1 µM - 10 mM) in estuaries. Extrapolation of these results indicated that oyster exposure to these compounds, might affect life cycle of these molluscs with a direct impact on their populations.

The use of bioanalytical methods for screening PCDD/Fs and DL-PCBs in food samples is considered by EU legislation (Comission Regulation (EU) No. 589/2014, 2014; Commission regulation (EU) No. 709/2014, 2014). One of the cell-based bioassays used frequently for determination of tetrachlorodibenzo-p-dioxin (TCDD) and similar halogenated aromatic hydrocarbons, is the Chemically Activated Luciferase gene eXpression bioassay (CALUX) (Denison et al., 2004; Fochi et al., 2008). The use of recombinant rat (H4IIe) or mouse (Hepa1c17) hepatoma cell lines, integrating an aryl hydrocarbon receptors (AhR)-responsive luciferase reporter gene, permits the screening of these contaminants. The exposure of these cells to halogenated contaminants activates the AhR signaling pathway, which, in turn, induces expression of the luciferase enzyme. This enzyme activity can be monitored via a bioluminescent reaction and correlated with the presence of contaminants. The AhR signalling pathway also regulates expression of endogenous proteins, having been studied extensively to assess induction of xenobiotic metabolising enzymes activities (Denison et al., 2004; Hädrich et al., 2018). The AhR regulation of CYP1A1 induction, is used widely to measure effects of toxic compounds in

cell lines. The presence of toxicants induces enzyme expression, leading to increased CYP1A1 activities in cultures. The enzymatic activities can be accessed by means of the ethoxyresorufin-*O*-deethylase (EROD) fluorometric assay, where the enzyme deethylates the substrate, 7-ethoxyresorufin, to a fluorescent product, resorufin (Stagg et al., 2016). In a work by Petrulis et al. (Petrulis et al., 2001), toxic effects of four PCB congeners and TCDD were screened in primary *Sprague-Dawley* rat hepatocytes cell lines. The cell cultures were also subjected to binary mixtures of TCDD and PCBs in a 24 h exposure assay and competition for the binding sites in the binary mixtures was also evaluated. At 10 pM TCDD, AhR occupancy was 50%, affording 30-40% EROD maximal induction, while from 100 pM to 1 nM AhR occupancy was 90%, achieving a near maximal response of EROD activity. PCB 153 failed to activate the AhR signalling pathway, showing no EROD activity. However, in binary mixtures with non-saturating TCDD concentrations (10 pM), PCBs 77, 156 and 169 induced EROD activity. This behaviour indicated binding of PCBs congeners to the free AhR at the lower TCDD concentration, activating the CYP1A1 signalling pathway and reflecting higher EROD activity. When subjected to PCBs and saturating TCDD concentrations, the cell-line exhibited PCB dose-dependent EROD activity. At high concentrations, dioxin binds to available AhR, inducing the CYP1A1 expression, which is inhibited by the excess PCBs. PCBs competed with 7-ethoxyresorufin for CYP1A1; K_i values were 0.05, 3.0, 0.68 and 0.17 μM for PCBs 77, 153, 156 and 169, respectively. Based on these K_i values, PCB 77 was the most potent inhibitor of CYP1A1 activity, followed by PCB 169 and PCB 156, and PCB 153 was the weakest inhibitor. The authors showed that binary mixtures of PCBs and dibenzo-p-dioxins, promoted CYP1A1 expression but also inhibited activity, making EROD assay unsuitable for screening dioxin contamination using the toxic equivalent factor approach (Petrulis et al., 2001).

Induction of EROD activity by chlorinated hydrocarbons was also studied in three different rainbow trout (*Oncorhynchus mykiss*) pituitary cell lines (Tom et al., 2001). Results were similar to those using rat hepatocytes, with CYP1A1 activity induced by TCDD. In this work, the isolated effects of 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PCDD), PCB-126 and 2,3,7,8-tetrachlorodibenzofuran (TCDF), were also related to induced EROD activity in the cell lines. By

defining the potency as the concentration eliciting 50% of the maximal response (EC_{50}) at 48h, the authors rated TCDD, PCDD, TCDF and PCB-126, at respectively 17.4, 21.4, 57.9 and 469.1 pM, as the most to less potent inducers. Moreover, there was a clear correlation found for the induced CYP1A1 activity between toxicant concentrations and incubation times and, to ensure reproducible results, exposure times must be controlled rigorously. These data allowed species that are difficult to maintain in laboratory to be studied, such as the rainbow trout, albeit the effects are limited to a specific cell types (Tom et al., 2001).

To avoid limitations associated with cell lines, Lemaire et al. (2011) proposed the use of whole tissues, such as precision-cut liver slices (PCLS), to investigate the impact of PCB 126 on CYP1A-mediated metabolism and oxidative stress response. Using salmon (*Salmo salar*) as an animal model, the authors found that PCB 126 concentrations, 2 nM to 200 nM, induced CYP1A expression and, consequently, activation of EROD signalling. However, the antioxidant response was low at higher PCB 126 concentrations, suggesting the need for longer incubation times to generate oxidative stress in the tissues (Lemaire et al., 2011). As an alternative approach to measure cellular toxicity of chlorinated compounds Levy et al. (2018) used. a bioassay based on cryo-preserved H4IIE cell lines to determine dioxins and DL-PCBs, through EROD assay method. This bioassay allowed determination of TCDD standards in the femtogram range, and was applied successfully in several types of matrices, namely milk, eggs, mollusc, fish, poultry, meat, liver, oil and guar gum. Despite apparently good results and the easy-to-use characteristics of the assay, cryo-preserved H4IIE cells were susceptible to antagonism and cytotoxicity effects of sample extracts, and the assay to false results.

4. Brominated organic compounds

Despite being prohibited, brominated organic compounds are found in environmental samples and all along the trophic chain suggesting their bioaccumulation is a real problem and a risk for humans (Vandermeersch et al., 2015). BFRs have penetrated the environment as a result of release during their use or when they burnt (Kowalski and Płaszczyk, 2017; Zhihua et al., 2018) and. studies on the effects of BFRs in animals described toxic adverse effects (Kim et al., 2014).

Brominated fumigants, such ethyl dibromide (EDB), were used as scavengers of lead in gasoline, insecticides, nematicides, and miticides, but in 1984 their use was banned by the US EPA and a maximum contaminant level in drinking water (0.05 mg/L) established, due to suspected adverse effects on the endocrine system, some organs such as liver and kidney, and carcinogenic effects (Reardon et al., 2009). EDB is easily absorbed by beeswax and honey, so its control is carried out routinely in this food (Tananaki et al., 2005).

4.1. Studies *in vivo* on brominated organic compounds

Toxicities of brominated compounds have been demonstrated using studies *in vivo* in different organisms (Table 3). BDE-47, BDE-209 and TBBPA have shown the greatest effects in growth and development, and, for this reason, effects on enzyme activities have been performed mostly in presence of these compounds (Darnerud et al., 2001; Feng et al., 2013; Yanhua Liu et al., 2018; Qiu et al., 2018; Ronisz et al., 2004; Sarkar et al., 2016; Sun et al., 2019; Tang et al., 2018; Xiong et al., 2018; Yu et al., 2018; Zhao et al., 2019). Large and small organisms have been used to explore their impact on enzymes, particularly those responsible for controlling oxidative stress caused by contaminants (SOD, GST, CAT, POD etc.) (Tarrahi et al., 2018).

Due to their simple structure, obvious physiological metabolic characteristics, and sensitivity to environmental pollutants etc. mosses are commonly used for atmospheric biomonitoring (Di Palma et al., 2017). Three different moss species (*H. plumaeform*e, *T.cymbifolium* and *P. cuspidatum*) were exposed to BDE-47 and BDE-209 and increased SOD activity was observed in response to BDE-47at 0.049 mg/L or higher concentrations. On the other hand, SOD activities in *H. plumaeforme* and *T. cymbifolium* were significantly induced by BDE-209 at 0.024 mg/L μM or higher concentrations while, in case of *P. cuspidatum* no change was observed. A similar correlation was observed for POD activity in *H. plumaeforme* and *P. cuspidatum* which was abruptly increased in presence of BDE-47 and BDE-209 at 0.24 mg/L or higher concentrations. *T.cymbifolium* POD activity was affected by 0.24 mg/L or more BDE-47, but no change was observed at difference concentrations of BDE-209. Similarities between SOD and POD suggest they work together to reduce harmful effects caused by ROS in *H. plumaeforme* and *T.cymbifoliu*

(Zhao et al., 2019). In the case of CAT, highest activities in *H. plumaeforme* and *T.cymbifolium* were detected at 0.024 mg/L BDE-47, decreasing at higher concentrations. Similar behaviour was observed for BDE-209, at lower concentrations activities increased but, when concentrations were above 0.048 mg/L, activities dropped. Furthermore, *P.cuspidatum* CAT activity was inhibited at BDE-47 concentrations above 0.024 mg/L. These differences among species can be attributed to differences in biological characteristics and detoxification mechanisms.

Lemna minor is a model aquatic plant used for ecotoxicological research and environmental monitoring (Zezulka et al., 2013). Like mosses, this aquatic plant is also affected by BDEs. Apart from effects on growth, physiological functions, and mortality, *L. minor* SOD and POD activities increased at 0-10 mg/L BDE-209 and BDE-47 but decreased at higher concentrations. It indicates that, at low concentrations, the enzymes work to reduce ROS but, at higher concentrations, their activities are inhibited, perhaps through denaturation of the enzyme. However, CAT activity was always higher than in controls at different concentrations of BDE-209 (Qiu et al., 2018; Sun et al., 2019).

When *D. Magna* was exposed to BDE-47 and BDE-209, CAT and GST activities were altered. While CAT activity increased at all tested concentrations of BDE-47 (0.5-8 μg/L) and BDE-209 (0-400 μg/L), GST activity significantly decreased at 5 μg/L BDE-47 and 25 μg/L BDE-209. This study also showed that AChE was negatively affected by BDE-47 and BDE-209(Xiong et al., 2018).

Toxicity and adverse effects have also been studied in higher organisms. *Carassius auratus* was affected by exposure to BFRs; SOD and CAT activities were inhibited at 10 and/or 100 mg/kg of BDE-209, TBBPA and HBCD (Feng et al., 2013). In the case of female rats, CAT and SOD activities were measured in presence of TBBPA, and differences were found between enzyme activities in livers and kidneys. CAT and SOD activities in kidney were inhibited at 50 mg/kg TBBPA while, in liver, no changes were observed (Yu et al., 2018).

All studies showed that BFRs affected enzymes involved in reduction of oxidative stress, irrespective of the organism. The responses of each organism differed in presence of different concentrations and/or different compounds.

4.2. Studies *ex vivo* and *in vitro* on brominated organic compounds

Several works confirm that some enzyme activities change when they are exposed to different brominated compounds *in vitro* (Table 3). EROD activity changed in presence of BDE-47, BDE-99, BDE-153, BDE-200, HBCD and TBBPA. Ronisz et al. (2004) published evidence that TBBPA inhibited EROD activity, but HBCD had no effect at 2000 mg/L or 10 g/L. In contrast, Wang et al. (2018) reported an increased EROD activity at 0.001-10 µg/L TBBPA and 0.001- 0.1 µg/L BDE-209, at concentrations lower or higher than this activity did not change. HBCD inhibited EROD activity at all concentrations tested. In the case of BDE-47 the activity did not change. The assays were performed in buffer so differences between the two studies could be caused by the origins of the enzymes, trout and tilapia liver, respectively. Furthermore, BDE-47, BDE-99, BDE-205 and PBB-153 produced similar effects on EROD activity in trout liver enzymes according to Nakari and Pessala (2005). Activity increased up to a given concentration, but above this concentration activity decreased, and, in some cases, inhibition was observed. Although the concentrations tested were different (0-44 µg/L BDE-47, 0-50 µg/L BDE-99, 0-219 µg/L BDE-205 and 0-264 PBB-153), the same pattern was repeated.

Apart from EROD, oxidoreductases such as catalase, dehydrogenase and polyphenol oxidase and urease soil enzymes have also been exposed to 10 µg/g TBBPA and BDE-209 (Yu et al., 2017). Enzymatic activities in soil are related to many biochemical processes (Yang et al., 2006) and, consequently, they are common indicators of adverse effects of some contaminants in soil quality (Matyja et al., 2016). The effects of BFRs together with heavy metals, such as Pb and Cd, have been studied because previously information indicated that the combination of these chemicals could have different ecological effects due to changes in mobility or bioavailability. Catalase activity was assessed spectrophotometrically, and results showed that TBBPA and BDE-209, inhibited urease significantly and, in presence of Pb and Cd, inhibition was even greater. In contrast, dehydrogenase activity was induced by BDE-209 but, when BDE-209, TBBPA, Pb and Cd were altogether inhibition became significant. The same behaviour was observed for polyphenol oxidase, where mixtures of compounds produced enzyme inhibition but,

independently, CAT activity was slightly induced. The authors concluded that BDE-209 exhibited higher eco-toxicological potential for soil enzymes than TBBPA and demonstrated that inhibition was promoted by BDE-209, TBBPA, Cd and Pb (Yu et al., 2017).

The human hepatoblastoma cell line-HepG2 (often used model to assess BDEs toxicity) has been exposed to BDE-47 (2, 10 and 50 μM) and its metabolites, by Tang (2018). DNA damage, cell cycle dysregulation, cell apoptosis, ROS generation and SOD and GST were studied and the authors determined that BDE-47, BDE-28 and BDE-7 induced SOD and GST activities at 2 μM but at higher concentrations (50 μM) SOD and GST activities decreased rapidly. These results confirmed that these enzymes are important in reducing oxidative stress and organic toxins, at low concentration, but at high concentrations inhibition or saturation occurs.

Iodotyrosine deiodinase (IYD) is a dehalogenase involved in deiodination of two compounds released with thyroid hormones, 3-iodo-1-tyrosine (MIT) and 3,5-diiodo-1-tyrosine (DIT). Deiodination produces iodide and tyrosine, which can be reused in thyroid hormone synthesis. Low IYD activity can reduce iodide retention leading to hypothyroidism (Shimizu et al., 2013). Apart from iodide dehalogenation IYD can react with 3-bromo and 3-chloro-1-tyrosine, but not 3-fluoro-1-tyrosine. Consequently, concentrations of thyroid hormone can be altered affecting the metabolism.

Shimizu et al. (2013) studied the activity of IYD *in vitro* in presence of 44 halogenated compounds, 15 of which were BDEs or their metabolites, using recombinant human IYD expressed in a microsomal fraction from HEK-293 T cells. Parent BDEs, such as BDE-47, BDE-99 and BDE-100. or methylated metabolites did not induce inhibition. In contrast, some hydroxylated metabolites, such as 2-OH-BDE-28, 2-OH-BDE-15, 4-OH-BDE-17, 4-OH-BDE-49, 4-OH-BDE-90 and 4-OH-BDE-42, inhibited the enzyme at 100 μM. The authors concluded that BDEs with hydroxyl group at the 2- or 4- position in the phenyl ring were inhibitors of IYD. The inhibition potential of some natural and synthetic brominated compounds against metabolic enzymes has also been proposed for therapeutic applications. Novel bromophenols derivatives yield nano- and micromolar inhibition levels of CAs isozymes activities. Despite the different results described in literature, some authors point out the dependence of CA inhibition on the

number of bromine substitutions in the molecules (Bayrak et al., 2019, 2017; Boztas et al., 2019; Boztaş et al., 2015; Çetinkaya et al., 2014; Göksu et al., 2016; Kocyigit et al., 2017; Taslimi et al., 2016). These bromophenols also demonstrate a strong anticholinergic potential against AChE and BChE, achieving nanomolar inhibition levels. However, the inhibition mechanisms of both enzymes have been related with the presence of methoxyl, methylsulfonyl and hydroxyl moieties, rather than to the presence of the halide (Bayrak et al., 2019, 2017; Boztas et al., 2019; Öztaşkin et al., 2015; Öztaskın et al., 2017; Öztaşkın et al., 2019; Rezai et al., 2018). Nano- and micromolar concentration levels of bromophenols are described for the inhibition of key enzymes in clinical course of diabetes mellitus. Activities of α-amylase, α-glycosidase and AR are strongly inhibited by the candidate molecules, however, the involvement of the halide moiety in the biocatalysts function is not clear (Öztaşkın et al., 2019; Taslimi et al., 2018).

5. Enzymatic biosensors for HOCs determination

Taking advantage of the altered enzyme activities *in vivo*, biosensing strategies have been developed to determine organohalogen concentrations in different sample types. Some biocatalysts use these chemicals as enzymatic substrates and are an efficient mean to measure their concentrations (Bidmanova et al., 2016; Shahar et al., 2019a; Wu, 2011). Other enzymes exhibit inhibited activity in presence of halogenated compounds, decreasing conversion rates of natural substrates. Diverse strategies presented by several authors show promising applicability for the determination of halogenated compounds in food and environmental samples. To screen the enzymatic activity, numerous analytical techniques are usually utilized downstream the biochemical reaction. As summarized in Table 4, biosensing strategies, based on chromatographic (Bidmanova et al., 2016; Brack et al., 2002; Shimizu et al., 2013), electrochemical (Anu Prathap et al., 2012; Besombes et al., 1995; Kaur et al., 2016; Kim et al., 2008; McArdle and Persaud, 1993; Miao et al., 2016; Nomngongo et al., 2012; Vidal et al., 2008; Wu, 2011; Xia et al., 2015; Xu et al., 2019), potentiometric (Hutter et al., 1995), conductimetric (Anh et al., 2004), spectrophotometric (de Souza et al., 2018; Hipólito-Moreno et al., 1998; Q. Liu et al., 2019; Mazurenko et al., 2018; McArdle and Persaud, 1993; Shahar et al., 2019a; Silva et al., 2015) and

fluorescence spectroscopic methods (Bidmanova et al., 2016, 2010; Campbell et al., 2006; Mazurenko et al., 2018; Sok and Fragoso, 2018) can be found in literature.

Wu (2011), for example, proposed a biosensor for determination of chlorinated pesticides, based on direct electrochemistry of house fly cytochrome 6A1 (CYP6A1), an isoenzyme from the CYP450 family. The biocatalyst, confined to the surface of an electrode, exhibited two voltammetric signals, corresponding to Fe^{II}/Fe^{III} redox couple from the enzyme heme group. The electrochemistry of CYP6A1 proved to drive epoxidation of aldrin to dieldrin and heptachlor to heptachlor epoxide, enhancing the registered signals. The immobilized enzyme exhibited affinity for these substrates, presenting apparent Michaelis Menten constants (K_M^{app}) of 27.26 mg/L for aldrin, and 16.13 mg/L for heptachlor. The amperometric calibration of CYP6A1-based biosensors revealed analytical ranges from 3.31 mg/L to 16.57 mg/L for aldrin, and 3.33 mg/L to 17.40 mg/L for heptachlor. Despite good reproducibility, defined in terms of residual standard deviation (RSD) of 3.5%, and satisfactory activity maintained after 10 days the limit of detection (LOD) for aldrin was one hundred times greater than values suggested by the World Health Organization (WHO), meaning preconcentration was required for real sample analysis (Wu, 2011).

In dehydrochlorination reactions catalysed for Lin B and Lin A, halogen anions and protons are released from the halogenated molecules, varying the pH allowing, therefore, a simplified monitoring of their activities (Longoria et al., 2008; Szatkowski et al., 2011; Wannstedt et al., 1990). Bidmanova et al. (2010) presented a LinB-based fibre-optic biosensor for determination of 3-chloro-2-(chloromethyl)-1-propene. The biosensor, based on co-immobilization of LinB and bovine serum albumin tagged with 5(6)-carboxynaphthofluorescein conjugates (CNF-BSA), allowed release of protons to be monitored, which were correlated with HOCs concentrations. The biosensor exhibited linear dependence with 3-chloro-2-(chloromethyl)-1-propene concentrations in the range from 0 and 100 mg/L, reaching a LOD of 1.75 mg/L.

The same research group (Bidmanova et al., 2016) described a fluorescence-based biosensor for screening and monitoring chlorinated pollutants under field conditions. An identical strategy, based on the fluorescence response of CNF-BSA conjugates, allowed the determination of

analytes by fluorometry. The authors attempted two biosensor variations, one using LinA and LinB co-immobilized, and other using LinA alone to improve sensitivity. However, the response was faster and more reproducible for lindane determination using LinA alone, allowing calibrations up to 100 mg/L (LOD of 12.1 mg/L). Nevertheless, LinB enzyme proved suitable to determine 3-chloro-2-(chloromethyl)-1-propene and 1,2-dichloroethane with LODs of 1.4 mg/L and 4.6 mg/L, respectively, in water samples. The LinB system was also tested to detect 1,2,3-trichloropropane, but the biosensor was unresponsive (Bidmanova et al., 2016). More recently, Mazurenko et al, (2018), presented a pocket-sized fluorometric assay based on LinA activity, achieving an analytical range for lindane from 2.91 mg/L to 87.25 mg/L. The biosensor responded in 15 minutes and was suitable for surface and groundwater samples analysis. The authors also studied a fluorometric system using LinB, obtaining an analytical range from 1.43 mg/L to 114.41 mg/L for determination of bis(2-chloroethyl) ether.

Alternative approaches to determine 1,2-dichloroethane using LinB, have been reported by Shahar et al. (2019b). The biosensor was fabricated by incorporating LinB and a proton sensitive chromoionophore indicator dye. The colour shift, measured with an optic-fibre reflectance spectrophotometric method, allowed CAH to be determined in the concentration range from 5 mg/L to 60 mg/L (LOD 1 mg/L). RSD values of 3.4-4.3 % validated the reproducibility of this device, which provided a response within six minutes, for analysis *in situ* of river, tap and bottled waters without pretreatment. The same group reported a reflectometric biosensor based on a similar strategy, immobilizing LinB and a chromoionophore in polyacrylate microspheres (Shahar et al., 2019a). LinB catalysed dehalogenation of 1,2-dichloroethane, promoting the colour change from violet to blue, which was measured by reflectance spectrophotometry. The authors reported a reduced linear range up to 30 mg/L, but better sensitivity (LOD 0.3 mg/L). Moreover, this biosensor was faster (2 minutes) and gave a stable response over six days of storage (Shahar et al., 2019a).

Whole-cells biosensors are another strategy for detecting HOCs. Hutter et al. (1995) developed a bioassay based in *Rhodococcus* strains to determine CAHs. Small alginate gel beads containing the bacterial cells expressing LinB, were added to samples and released chloride anions were

measured by a chloride ion-selective electrode. With an analysis time of 5 minutes, the authors attained a LOD as low as 10 µg/L and an RSD of 5.5% for 1-chlorobutane. On the other hand, Campbel et al. (2006) took advantage of a fibre optic fluorimeter to explore the activity of LinB expressed in *Xanthobacter autotrophicus*, a soil bacterium. The authors measured 1,2-dichloroethane, by immobilizing bacterial cells on the tip of the optic fibre using calcium alginate. This small fluoresceinamide-based biosensor worked as a pH optode achieving an LOD of 11 mg/L and a linear range up to 65 mg/L. Moreover, the determination of 1,2-dichloroethane was performed in 8-10 minutes, exhibiting an RSD less than 9%. Prathap and co-workers (2012), used *Escherichia coli* cells overexpressing LinA as bioreceptors to determine lindane and pentachlorocyclohexane (PCCH). By immobilizing cells on to an electrode surface, the acidic pH generated during dehalogenation reactions altered surface conductivity, which can be monitored using pulsed amperometry. This strategy allowed analysis in the part-per-trillion range, with 60-100 s measurements. The device had 15 days shelf-life and a LOD of 2 ng/L; RSD values ranged between 1 – 2%. Activity of LinA overexpressed in *E.coli* showed good selectivity for lindane and PCCH but did not respond to aliphatic or aromatic degradation products of the pesticide; or to other commonly used pesticides such as DDT or Dichlorodiphenyldichloroethylene (DDE). Application of this microbial biosensor to real samples required strict control of the samples pH to minimise errors (Anu Prathap et al., 2012).

Based on LinB activity, an optical biosensor has been constructed to detect EDB in water samples (Bidmanova et al., 2016; Reardon et al., 2009). The biosensor was based on two-layer detection system, using a pH-sensitive fluorophore immobilised on the tip of the optical fibre, with the enzyme immobilised over this first layer. EDB was metabolised by LinB, releasing bromide anions and protons. The protons were detected by increasing the immobilised fluorophore fluorescence intensity. Reardon et al. (2009) tested a biosensor for EDB in the range from 1 µg/L to 10 mg/L, achieving a linear response between 1 and 10 µg/L and a LOD of 1 µg/L. Bidmanova et al. (2016) built a biosensor to monitor several hazardous halogenated compounds based on LinA activity. The γ-hexachlorocyclohexane dehydrochlorinase was immobilised to the fibre to detect EDB between 4 mg/L and 25 mg/L, but also, 1,2,3-trichloropropane, 1,2-di-chloroethane,

3-chloro-2-(chloromethyl)-1-propene and γ-hexa-chlorocyclohexane, achieving LODs of 2.4, 1.4, 2.7, 1.4 and 12.1 mg/L, respectively.

Taking advantage of detoxicant properties of DHP and HRP, Szatkowski and co-workers (2011) studied the role of these peroxidase enzymes in the metabolism of 4-chlorophenol (4-CPh) and TCP. DHP activity was dependent on the extent of halogenation, specifically active for trihalophenols, moderately active towards dihalophenols and inactive for monohalophenols. Despite similarities between both enzymes, DHP activity was inhibited by 0.96 g/L 4-CPh, while HRP formed stable enzyme-substrate complexes, catalysing dehalogenation of the phenolic molecules. On the other hand, both enzymes catalysed dehalogenation of TCP, with DHP removing all the three chlorine atoms from the molecule structure. Despite the recognised detoxicant potentials reported for DHP and HRP (Longoria et al., 2008; Szatkowski et al., 2011; Wannstedt et al., 1990), only HRP has been used in biosensing strategies. Although HRP present catalytic activity for certain halo-monophenols (Szatkowski et al., 2011), the majority of biosensor approaches are based the inhibition of its activity. For instance, Nomngongo et al. (2012) reported a HRP-based amperometric biosensor for determination of PCB-1, PCB-28 PCB-100, BDE-100 and PBB-1 in wastes water from landfill leachates. Polyaniline-mediated HRP reduction of H_2O_2, was inhibited via a non-competitive inhibition mechanism in presence of the three PCB congeners, allowing device calibration for each contaminant. Amperometric responses were linear in the ranges 0.93 to 18.9 µg/L, 0.73 to 15.7 µg/L,0.93 to 27.1 µg/L, 0.862 to 13.3 µg/L and 0.422 to 25.6 µg/L; and LODs were 0.022 µg/L, 0.016 µg/L, 0.019 µg/L, 0.018 µg/L and 0.014 µg/L; for respectively PCB-1, PCB-28PCB-100, PBB-1 and BDE-100. The biosensor exhibits a rapid and sensitive analysis, with suitable linear ranges and low LODs for selected HOCs. It required only short and easy sample preparation, fitting the requirements for screening methods to detect these halogenated compounds.

Inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the broad-spectrum fungicide chlorothalonil, was used to its determination in cucumber samples (Q. Liu et al., 2019). GAPDH is NAD-dependent for oxidative phosphorylation of glyceraldehyde-3-phosphate (GAP), generating NADH which, in presence of phenazine methosulfate, converts the

nitrotetrazolium blue chloride into formazan. This reaction can be monitored spectrophotometrically using the colour shifting from yellow to purple. Since chlorothalonil inhibited GAPDH activity, the device was useful to detect the presence of chlorothalonil with a dynamic range from 0.13 mg/L to 2.66 mg/L. Moreover, the biosensor was sensitive enough to allow determination of 0.27 mg/L of the fungicide by eye, exhibiting good recovery values and selectivity in cucumber samples (Q. Liu et al., 2019).

Xu et al. (2019) designed a voltammetric biosensor for chlorpyrifos, immobilising AChE on electrodeposited gold nanoparticles. A linear concentration range between 0.10 ng/L and 1 µg/L; and a LOD of 2.06×10^{-3} ng/L were achieved in cabbage, and tap, purified, river and lake waters samples. This device exhibited an RSD of 3.74% and shelf-life of 28 days. Also based on AChE inhibition, Kaur et al. (2016) proposed an electrochemical biosensor for the determination of chlorpyrifos-methyl in spiked lettuce. Using voltammetric analysis, the biosensor recovered 95-97% of spiked pesticide concentrations, within an analytical linear range from 1 ng/L to 50 µg/L. Several examples of AChE inhibition-based biosensors can be found in literature for chlorpyrifos determination in food products including cabbage, lettuce or orange juice (Kim et al., 2018; Miao et al., 2016; Silva et al., 2015; Xia et al., 2015). The applicability of AChE-based biosensors for determination of chlorinated organophosphorus pesticides seems to be limited to single use devices, since these toxicants inhibit enzyme activity irreversibly. Some authors have suggested the use of regenerating agents to reactivate the biosensors for subsequent measurements, but this approach increased the cost of such devices.

TYR inhibition by chlorinated phenols and pesticides has also been widely employed to determine both groups of molecules. Vidal and co-workers (2008) proposed an amperometric biosensor based on the reversible competitive inhibition of TYR activity by atrazine and dichlorvos (pesticides). The authors used different quinonic derivatives, such as 1,2-naphthoquinone and 1,2-naphthoquinone-4-sulfonic, which were co-immobilized with the enzyme, achieving a reagent-less biosensor. Applying -600 mV, o-diol substrates were released from the quinonic derivatives and thereafter oxidized by TYR, generating a current. In presence of chlorinated compounds, TYR activity was inhibited with current values proportional to inhibitor concentrations. With an

RSD value of 11.23 %, the TYR amperometric biosensor permited determination of atrazine and dichlorvos within concentration ranges from 0.17 mg/L to 2.16 mg/L, presenting LODs of 12.94 µg/L and 15.47 µg/L, respectively. Near 100% accuracy was achieved for both chlorinated species in spiked river and irrigation water samples.

Different strategy based on TYR immobilisation on a carbon electrode, by means of polypyrrole film, allowed determination of 3,4-dichlorophenol, chloroisopropylphenylcarbamate, 3-chloroaniline and atrazine. The distinct affinity of substrates and inhibitors for TYR, resulted in different conditions to determine each of the chlorinated molecules. The quasi-reversible competitive inhibition of TYR catalysed catechol oxidation to quinone, allowing determination of 0.07 mg/L to 81.50 mg/L 3,4-dichlorophenol. On the other hand, the use of dopamine as a substrate was more sensitive for determination of competitive inhibitors like atrazine, 3-chloroaniline and chloroisopropylphenyl carbamate, determining 0.86 mg/L, 0.26 mg/L and 0.43 mg/L, respectively (Besombes et al., 1995). McArdle and Persaud (1993) also reported construction of an amperometric TYR-based biosensor for atrazine. Using two different configurations, the authors could detect 107.84 mg/L atrazine in a stirred batch, achieving greater sensitivities in a flow-through system (21.57 mg/L). The herbicide resulted in a reversible competitive inhibition of TYR activity for catechol violet, requiring 15 minutes for analysis. The biosensor was suitable for artificial seawater analysis, achieving satisfactory analytical ranges and good stability over 20 days.

Electrochemical monitoring of chlorinated species in filtered water samples using TYR has also been reported (Kim et al., 2008). Using gold nanoparticles (Au-NPs) for enzyme immobilisation, the authors reported electrochemical monitoring of TYR activity for catechol oxidation, inhibited by 2,4-dichlorophenoxyacetic acid (2,4-D) and atrazine. Both molecules were determined successfully in the dynamic range from 0.1×10^{-5} to 0.5×10^{-3} µg/mL, with a response time of only 3-5 minutes. LOD values for atrazine and 2,4-D were 0.35 µg/mL and 0.55 µg/mL, respectively. Moreover, stability of the biosensor was such that TYR activity was about 90% of the initial activity after 10 days (Kim et al., 2008).

TYR activity is also amenable to conductimetric detection of atrazine and diuron (Anh et al., 2004). The biochemical reaction of crosslinked TYR and its substrates resulted in increased conductance. Interestingly, the authors reported the use of 4-chlorophenol as TYR substrate, showing the potential of this device to determine chlorinated phenol by enzymatic catalysis. With a response time of 1-5 minutes, the conductimetric biosensor detected concentrations as low as 1 µg/L diuron and atrazine, exhibiting linear dynamic ranges of 2.3 µg/L to 2330 µg/L and 2.15 µg/L to 2150 µg/L, respectively. The device presents an RSD value of 5% and was stable for 23 days. Inhibition by atrazine metabolites was also examined in this work, demonstrating the applicability of TYR-based systems for determination of desisopropylatrazine and desethylatrazine (Anh et al., 2004).

Spectrophotometric analysis of TYR inhibition by atrazine, has also been proposed as for corn samples analysis (Hipólito-Moreno et al., 1998). Biocatalytic oxidation of catechol to 1,2-benzoquinone was exploited in a non-aqueous flow-injection system, providing a LOD of 0.5 mg/L and a linear range from 1 mg/L up to 7 mg/L. The device returned suitable RSD (7%) and accuracy, recovering near 100% of the spiked atrazine concentration from corn samples.

Diphenolase activity of TYR for L-Dopa oxidation, proved to be suitable for determination of chlorinated species. Vibol and Fragoso (2018) reported spectrophotometric and fluorescence methods for determination of the effects of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-D on TYR activity. Based on TYR fluorescence intrinsic signal, the authors reported that both molecules exhibited strong inhibitory effects. Both compounds compete with L-Dopa for the active centre of the enzyme, exhibiting a linear proportionality up to 4.42 mg/L for 2,4-D, and 5.12 mg/L for 2,4,5-T. Inhibition constants (K_i) were 10.22±0.26 mg/L, indicating that 2,4,5-T had greater inhibitory potential for TYR than 2,4-D (17.02±0.88 mg/L). This work was focused on the interaction of both herbicides with the TYR structure, but also showed the potential of the system to be applied for monitoring these halogenated pollutants.

6. Conclusions

Since the early 1960's, production and use of halogenated organic compounds, along with improper disposal practices, has caused their widespread presence and persistence in the environment and wildlife. Incorporation of HOCs in the trophic chain is a real threat to human health, which has led to international laws governing the use of HOCs. There is a growing demand to generate more and better knowledge about the effects of HOCs and promote an increased number of investigations exploring their toxicity. Possibly due to the higher number of molecules and their extended uses, chlorinated organic compounds are the major focus of research, with a broad range of literature describing their toxicity, environmental dispersion, and presence in different samples. PCDBs, PCDFs and DL-PCBs are considered the most toxic compounds in this group, with several authors relating their presence in the organisms with severe symptoms of toxicity. However, newly emergent HOCs, like PFOS, PFOAS and the BFRs, have been more prominent in recent years, with studies on their toxicological potency and presence in products for human consumption. PFCs are associated with adverse effects on hormonal, neurological and reproductive regulation, while brominated organic compounds are suspicious to damage endocrine system and vital organs including the kidneys and liver. Both groups of compounds have been related to cancer episodes. The environmental presence of molecules from these groups have been confirmed by several authors as well as in several species of animals and plants, some of them used directly as food products. Good examples can be found for seafood, which is consumed without further processing.

Despite the emergent worry about HOCs, iodinated compounds are still largely ignored by the scientific community. These compounds are suspicious to be extremely toxic at very low concentrations and have the most facilitated route for ingestion by humans via tap water. Iodinated organic compounds are the least studied HOCs, but should demand the attention of the scientific community, since there is a gap in understanding of their toxic effects, structures, and detection.

Several authors have studied the effects of HOCs in flora and fauna from contaminated sites, finding evidence of toxic effects in different populations as well as bioaccumulation processes. The suitability of bioassays *in vivo* and *ex vivo* to assess toxicological effects is undeniable but associated with ethical issues. In most cases, these methods require animal sacrifice for analysis

of tissues and are complicated and time-consuming. The demand for simpler, faster, but still sensitive techniques to screen HOCs toxicity has led to the development of whole-cell bioassays. Cell-lines can mimic effects *in vivo* and offer an alternative for species difficult to maintain under laboratory conditions. However, their use restricts results to one type of cell, missing the totality of organisms' responses.

Even with different protocols to evaluate the toxicity of HOCs, a consensus for their involvement in alteration of expression, regulation and activity of enzymes exists. Fluorinated, chlorinated and brominated organic compounds affect the activities of enzymes involved in antioxidant systems, such as CAT, POD and SOD. Additionally, these POPs affect enzymes involved in metabolism of endogenous and exogenous compounds. Organochloride compounds, and some brominated molecules, cause altered activities of CYP450 enzymes, leading to disturbed EROD activities. Moreover, these compounds not only alter antioxidant and AChE activities but also activities of other different enzymes.

Standard GC-MS methods, covered by EU legislation (Commission Regulation (EU) No. 589/2014, 2014; Commission regulation (EU) No. 709/2014, 2014) and used by several authors (Andersson et al., 2019; Fan et al., 2017; Pereira et al., 2017; Schmidt and Göen, 2017; Wannstedt et al., 1990), offer sensitive limits of detection and accuracy for the identification and determination of HOCs. However, chromatographic strategies are limited by cost, analysis time and complexity, meaning they have limited applicability as routine methodologies. Cell-line based bioassays, covered by the same legislation, are valid alternatives for detection and toxicity of HOCs, but require specific cell-lines, complex protocols and cell culture facilities. Moreover, the biological complexity of whole-cell systems and, consequently, the potential for uncontrolled parameters, make use of these bioassays prone to false negative results, especially since HOCs can induce and inhibit enzymatic activities depending on their concentrations. Enzymatic biosensors have, therefore, been suggested as alternative detection tools for screening these POPs. Based on altered enzymes activity, several authors developed biosensing strategies, providing selective, low-cost, rapid, simple and sensitive tools (Chobtang et al., 2011). Despite some good results, however, the vast majority of biosensing approaches still lack the necessary sensitivities

and limits of detection demanded by international regulations. Moreover, the applicability of the proposed devices is still limited to laboratory facilities and relatively clean samples, requiring laborious extraction steps and prohibiting their use in analysis of raw food products *in situ*.

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Table 1. Effects of fluorinated organic compounds on enzymatic activities *in vivo*, *ex vivo* and *in vitro*.

Organism/Cell-line	Compounds	Exposure concentrations (mol /L)	Affected enzymes	Enzymatic activity effect	References
in vivo					
Daphnia magna	PFOS	$2.0 \times 10^{-9} – 2.0 \times 10^{-5}$	Acetylcholinesterase	Induction	(Jeong et al., 2016)
			Glutathione S-transferase	Induction	
		$1{,}6 \times 10^{-5} – 1.2 \times 10^{-4}$	Acetylcholinesterase	Inhibition	(Yang et al., 2019)
			Superoxide Dismutase	Inhibition	
			Catalase	Inhibition	
			Glutathione S-transferase	Inhibition	
		$2.0 \times 10^{-6} – 3.2 \times 10^{-5}$	Acetylcholinesterase	Hormesis behaviour	(Liang et al., 2017)
			Catalase	Hormesis behaviour	
			Glutathione S-transferase	Hormesis behaviour	
	PFOA	$1{,}6 \times 10^{-6} – 4.0 \times 10^{-4}$	Acetylcholinesterase	Inhibition	(Yang et al., 2019)
			Superoxide Dismutase	Inhibition	
			Catalase	Inhibition	
			Glutathione S-transferase	Inhibition	
Wheat seedlings *(Triticum aestivum)*	PFOS	$2.0 \times 10^{-7} – 2.0 \times 10^{-5}$	Superoxide Dismutase	Induction	(Qu et al., 2010)
	PFOS	$2.0 \times 10^{-7} – 4.0 \times 10^{-4}$	Peroxidase	Inhibition	
Earthworm *Eisenia fetida*	PFOSA	$0.0 – 2.0 \times 10^{-5}$	CYP450s	Induction	(Zhao et al., 2020)
			Glutathione S-transferase	Induction	
Freshwater fish *(Prochilodus lineatus)*	DBF	8.1×10^{-5}	Catalase	Induction	(Pereira Maduenho and Martinez, 2008)
			Acetylcholinesterase	Induction	
			Glutathione S-transferase	Inhibition	
ex vivo* and *in vitro					
Insect cell microsomes	PFOS	$1.0 \times 10^{-6} – 3.0 \times 10^{-5}$	CYP450s	Inhibition	(Narimatsu et al., 2011)

Escherichia cells	Triflumuron	$1.0\times10^{-6} – 1.5\times10^{-4}$	CYP450s	Catalysis	(Timoumi et al., 2019)
Insect cells	14 PFSAs	$0{,}5\times10^{-7} – 1.0\times10^{-4}$	UGT-glucuronosyltransferases	Inhibition	(Y. Z. Liu et al., 2019)
Human testicle microsomes	PFOS, PFOA	$1.0\times10^{-7} – 1.0\times10^{-3}$	Hydroxisteroid dehydrogenase	Inhibition	(Zhao et al., 2010)
Oysters´viscera and gills	Diflubenzuron	$1.0\times10^{-6} – 1.0\times10^{-2}$	Acetylcholinesterase	Inhibition	(de Souza et al., 2018)
	Novaluron	$2.5\times10^{-6} – 2.5\times10^{-3}$	Acetylcholinesterase	Inhibition	
Bovine liver	PFOA	$0.0 – 3.0\times10^{-5}$	Catalase	Not affected	(Xu et al., 2018)
	PFOS	$0.0 – 3.0\times10^{-5}$	Catalase	Inhibition	

Table 2. Effects of chlorinated organic compounds on enzyme activity *in vivo*, *ex vivo* and *in vitro*.

Organism	Compounds	Exposure concentrations (mol /L)	Affected enzymes	Enzymatic activity effect	References
in vivo					
Human	PCB-153, PCB-170, PCB-180	0-7.5×10^{-9} (in serum)	Cyp1A2 (N-demethylation)	Induction	(Fitzgerald et al., 2005)
Marine mussels (*Perna viridis*)	PCBs and chlorinated pesticides	Contaminated water of Hong Kong	Glutathione S-transferase	Induction	(Cheung et al., 2002)
			Glutathione Peroxidase	Induction	
			Superoxide Dismutase	Not affected	
			Catalase	Not affected	
			Glutathione Reductase	Not affected	
			NADPH DT-diaphorase	Not affected	
Acuatic salamander (*Necturus maculosus*)	PCBs and chlorinated pesticides	Contaminated water of St. Lawrence and Ottawa rivers	Hypothalamo–pituitary–interrenal (HPI) activity	Inhibition	(Gendron et al., 1997)
Mullet (*Mugil cephalus*) and Flounder (*Platichthys flesus*)	DDT, PCBs	Contaminated water in Douro estuary (Portugal)	Superoxide Dismutase	Induction	(Ferreira et al., 2005)
			Catalase	Induction	
ex vivo* and *in vitro					
Rainbow trout pituitary (*Oncorhynchus mykiss*) Cell lines	TCDD, PCDD, TCDF	1×10^{-11} – 1×10^{-9}	CYP450 (EROD)	Induction	(Tom et al., 2001)
	PCB-126	1×10^{-10} – 5×10^{-9}	CYP450 (EROD)	Induction	
Salmon (*Salmo salar*) Precision-cut liver slices	PCB-126	2.0×10^{-9} – 2.0×10^{-6}	Cyp1A (EROD)	Induction	(Lemaire et al., 2011)
H4IIE cell-lines	Dioxins, TCDD, DL-PCB	0.0 - 1.1×10^{-6}	Cyp1A (EROD)	Induction	(Levy et al., 2018)
Primary Rat hepatocytes (*Sprague-Dawley* rats)	TCDD	1.0×10^{-10} – 1.0 ×10^{-5}	CYP450 (EROD)	Induction	(Petrulis et al., 2001)

Oyster (*Crassostrea rhizophorae*)	Dichlorvos, chlorpyrifos	1.0×10^{-6} – 1.0×10^{-2}	Acetylcholinesterase	Inhibition	(de Souza et al., 2018)
Human erythrocytes	3-chloro-1-substituted aryl pyrrolidine-2,5-diones derivatives	2.3×10^{-8} – 3.7×10^{-8}	Carbonic anhydrase I	Inhibition	(Oktay et al., 2017)
		1.1×10^{-8} – 3.2×10^{-8}	Carbonic anhydrase II	Inhibition	

Table 3. Effect of brominated organic compounds on enzyme activity *in vivo*, *ex vivo* and *in vitro*.

Organism	Compounds	Exposure concentrations (mol /L)	Affected enzymes	Enzymatic activity effect	References
in vivo					
Moss *(Hypnum plumaeforme, Thuidium cymbifolium and Plagiomnium cuspidatum)*	BDE-209, BDE-47	$0.0 – 5\times10^{-6}$	Superoxide Dismutase	Depends on species	(Zhao et al., 2019)
			Catalase	Depends on species	
			Peroxidase	Depends on species	
			Superoxide Dismutase	Depends on species	
			Peroxidase	Depends on species	
			Catalase	Hormesis behaviour	
Duckweed *(Lemna minor L.)*	BDE-209	$0.0 – 2.1\times10^{-5}$	Peroxidase	Hormesis behaviour	(Sun et al., 2019)
			Superoxide Dismutase	Hormesis behaviour	
			Catalase	Induction	
	BDE-47	$1.0\times10^{-5} – 2.1\times10^{-5}$	Peroxidase	Hormesis behaviour	(Qiu et al., 2018)
			Superoxide Dismutase	Hormesis behaviour	
			Catalase	Hormesis behaviour	
Daphnia Magna	BDE-209	$0.0 – 4.2\times10^{-7}$	Acetylcholinesterase	Inhibition	(Xiong et al., 2018)
			Glutathione S-transferase	Inhibition	
			Catalase	Induction	
	BDE-47	$1.0\times10^{-9} – 1.7\times10^{-8}$	Acetylcholinesterase	Inhibition	
			Glutathione S-transferase	Inhibition	
			Catalase	Induction	
Goldfish (*Carassius auratus)*	BDE-209, TBBPA, HBCD	$1.0\times10^{-5} – 1.0\times10^{-4}$	Superoxide Dismutase	Inhibition	(Feng et al., 2013)
			Catalase	Inhibition	
			Glutathione peroxidase	Inhibition	
***Sprague-Dawley* rats (Female)**	TBBPA	9.2×10^{-5}	Superoxide Dismutase	Inhibition	(Yu et al., 2018)
			Catalase	Inhibition	

ex vivo* and *in vitro					
Rainbow trout (*Oncorhynchus mykiss*) and feral eelpout (*Zoarces viviparus*)	HBCD	3.1×10^{-3}	CYP450 (EROD)	Not affected	(Ronisz et al., 2004)
	TBBPA	3.7×10^{-3}	CYP450 (EROD)	Inhibition	
Saccharomyces cerevisiae	BDE-47	$0.0 – 9.0\times10^{-8}$	CYP450 (EROD)	Inhibition	(Nakari and Pessala, 2005)
	BDE-99	$0.0 – 8.9\times10^{-8}$	CYP450 (EROD)	Inhibition	
	BDE-205	$0.0 – 2.7\times10^{-7}$	CYP450 (EROD)	Inhibition	
	PBB-153	$0.0 – 4.2\times10^{-8}$	CYP450 (EROD)	Inhibition	
Liver from *Mossambica tilapia*	BDE-47	$2.1\times10^{-13} – 2.1\times10^{-7}$	CYP450 (EROD)	Catalysis & Inhibition	(Wang et al., 2018)
	BDE-209	$1.0\times10^{-13} – 1.0\times10^{-7}$	CYP450 (EROD)	Catalysis & Inhibition	
	HBCD	$1.6\times10^{-13} – 1.6\times10^{-7}$	CYP450 (EROD)	Catalysis & Inhibition	
	TBBPA	$1.8\times10^{-13} – 1.8\times10^{-7}$	CYP450 (EROD)	Catalysis & Inhibition	
Enzymatic activity bioavailable in soil	BDE-209	1.0×10^{-5}	Catalase	Catalysis	(Yu et al., 2017)
			Dehydrogenase	Catalysis	
			Urease	Inhibition	
			Polyphenol oxidase	Inhibition	
	TBBPA	1.8×10^{-5}	Catalase	Catalysis	
			Dehydrogenase	Catalysis	
			Urease	Inhibition	
			Polyphenol oxidase	Inhibition	
Microssomal fraction from HEK-293 T cells were	PBDEs and their metabolites	$1.0\times10^{-7} – 1.0\times10^{-3}$	Iodotyrosine Deiodinase	Inhibition	(Shimizu et al., 2013)
HepG2 cells	BDE-7, BDE-28, BDE-47	$2.0\times10^{-6} – 1.0\times10^{-5}$	Superoxide Dismutase	Hormesis behaviour	(Tang et al., 2018)
			Glutathione S-transferase	Hormesis behaviour	

Electric Eel (*Electrophorus electricus*)	Bromophenol derivatives with cyclopropyl moiety	1.6×10^{-7} – 9.2×10^{-7}	Acetylcholinesterase	Inhibition	(Boztaş et al., 2019)
Human erythrocytes	Benzyl(methyl)sulfane derivative bromophenols	4.2×10^{-8} – 2.3×10^{-7}	Carbonic anhydrases	Inhibition	(Bayrak et al., 2019)
		8.4×10^{-10} – 1.5×10^{-8}	Acetylcholinesterase	Inhibition	
		9.3×10^{-10} – 1.9×10^{-8}	Butyrylcholinesterase	Inhibition	
	Dimethoxy-bromophenol derivatives incorporating cyclopropane moieties	5.4×10^{-10} – 5.0×10^{-5}	Carbonic anhydrases	Inhibition	(Boztaş et al., 2015)
	Brominated diphenylmethanone	2.2×10^{-6} – 2.9×10^{-4}	Carbonic anhydrases	Inhibition	(Çetinkaya et al., 2014)
	9,10-Dibromo-N-aryl-9,10-dihydro-9,10-[3,4]epipyrrolo- anthracene-12,14-diones	6.9×10^{-8} – 5.0×10^{-5}	Carbonic anhydrases	Inhibition	(Göksu et al., 2016)
	Tetrabromo chalcone derivatives	8.2×10^{-9} – 2.1×10^{-8}	Carbonic anhydrases	Inhibition	(Kocyigit et al., 2017)
	Bromophenols	1.9×10^{-9} – 5.0×10^{-9}	Carbonic anhydrases	Inhibition	(Taslimi et al., 2016)
	Brominated diarylmethanones	2.8×10^{-9} – 5.8×10^{-9}	Acetylcholinesterase	Inhibition	(Öztaşkin et al., 2015)
	Brominated diarylmethanones and methylated bromophenols	4.3×10^{-9} – 1.3×10^{-7}	Carbonic anhydrases	Inhibition	(Öztaşkin et al., 2017)
		4.6×10^{-9} – 3.8×10^{-8}	Acetylcholinesterase	Inhibition	
		7.4×10^{-9} – 2.9×10^{-8}	Butyrylcholinesterase	Inhibition	
ND	1-(4,5-dihydroxybenzyl)pyrrolidin-2-one derivative bromophenols	2.6×10^{-9} – 1.6×10^{-8}	Acetylcholinesterase	Inhibition	(Rezai et al., 2018)
		1.3×10^{-8} – 5.4×10^{-8}	Butyrylcholinesterase	Inhibition	
		8.9×10^{-9} – 1.5×10^{-8}	Acetylcholinesterase	Inhibition	

	Bromophenols and methoxylated bromophenols derivatives	4.3×10^{-9} – 4.4×10^{-8}	α-glycosidase	Inhibition	(Öztaşkin et al., 2019)
Porcine pancreas	Bromophenols	1.4×10^{-8} – 4.4×10^{-8}	α-glycosidase	Inhibition	(Taslimi et al., 2018)
Cow Liver (*Bos taurus*)		9.0×10^{-8} – 1.1×10^{-6}	Aldose reductase	Inhibition	
Saccharomyces cerevisiae		3.8×10^{-9} – 2.9×10^{-8}	α-amylase	Inhibition	

ND – Not described

Table 4. Enzymatic biosensors for the determination of HOCs based on enzyme catalytic activity and enzyme inhibition.

Enzymes	Compounds	Matrices	Techniques	Limit of detection	References
Catalytic					
Cytochromes P450	Aldrin, Heptachlor	Buffer	Amperometry	2.9 -3.4 µg/mL	(Wu, 2011)
γ-hexa-chlorocyclohexane dehydrochlorinase	γ-hexachlorocyclohexane (lindane)	Surface waters and groundwater	Fluorescence	12.1 µg/mL	(Bidmanova et al., 2016)
				2.9 µg/mL	(Mazurenko et al., 2018)
Haloalkane dehalogenase	3-chloro-2-(chloromethyl)-1-propene	Buffer	Fluorescence	1.75 µg/mL	(Bidmanova et al., 2010)
	1,2-dichloroethane	Water	Reflectometry	0.3 µg/mL	(Shahar et al., 2019)
		Surface waters and groundwater	Fluorescence	2.7 µg/mL	(Bidmanova et al., 2016)
	1,2,3-trichloropropane	Surface waters and groundwater	Fluorescence	1.4 µg/mL	(Bidmanova et al., 2016)
	Bis(2-chloroethyl) ether	Surface waters and groundwater	Fluorescence	1.4 µg/mL	(Mazurenko et al., 2018)
	Ethyl dibromide (EDB)	Water	Fluorescence	25 µg/mL	(Bidmanova et al., 2010)
		Water	Fluorescence	1 ng/mL	(Reardon et al., 2009)
Inhibition					
Glyceraldehyde-3-phosphatase	Chlorothalonil	Buffer	Spectrophotometry	13 ng/mL	(Q. Liu et al., 2019)
Tyrosinase	Atrazine	Buffer	Amperometry	0.86 µg/mL	(Besombes et al., 1995)
			Spectrophotometric	32 µg/mL [a]	(McArdle and Persaud, 1993)
			Amperometry (Hydrodynamic cell)	1.1 µg/mL [a]	(McArdle and Persaud, 1993)
			Amperometry (Flow-through system)	2.15 µg/mL [a]	(McArdle and Persaud, 1993)

			Conductometry	1 ng/mL	(Anh et al., 2004)
		Corn	Spectrophotometry	50 ng/kg	(Hipólito-Moreno et al., 1998)
		Filtered river water	Amperometry	0.35×10^{-6} µg/mL	(Kim et al., 2008)
		River and irrigation waters	Amperometry	13 ng/mL	(Vidal et al., 2008)
	2,4-Dichlorophenoxyacetic acid	Filtered river water	Amperometry	0.55×10^{-6} µg/mL	(Kim et al., 2008)
		Buffer	Fluorescence	11 µg/mL [a]	(Sok and Fragoso, 2018)
	2,4,5-Trichlorophenoxyacetic acid	Buffer	Fluorescence	13 µg/mL [a]	(Sok and Fragoso, 2018)
	3,4-dichlorophenol	Buffer	Amperometry	65 ng/mL	(Besombes et al., 1995)
	Chloroisopropyl phenyl carbamate	Buffer	Amperometry	0.43 µg/mL	(Besombes et al., 1995)
	3-chloroaniline	Buffer	Amperometry	0.26 µg/mL	(Besombes et al., 1995)
	Diuron	Buffer	Conductometry	1 ng/mL	(Anh et al., 2004)
	Dichlorvos	River and irrigation waters	Amperometry	15.5 ng/mL	(Vidal et al., 2008)
Horseradish Peroxidase	PCB-1, PCB-28, PCB-101, BDE-100, PBB-1	Water wastes from landfill leachates	Amperometry	14-22 ng/L	(Nomngongo et al., 2012)
Acetylcholinesterase	Chlorpyrifos-methyl	Lettuce	Differential pulse voltammetry	1 ng/kg	(Kaur et al., 2016)
	Chlorpyrifos	Orange juice	Spectrophotometry	3.5 ng/mL	(Silva et al., 2015)
		Buffer	Cyclic Voltammetry	1.1 ng/mL	(Xia et al., 2015)
		Buffer	Spectrophotometry	5.3 µg/mL	(de Souza et al., 2018)
		Cabbage	Electrochemiluminescence	28 ng/kg	(Miao et al., 2016)
		Water	Differential pulse voltammetry	2.06×10^{-6} ng/mL	(Xu et al., 2019)

	Dichlorvos	Orange juice	Spectrophotometry	0.022 ng/mL	(Silva et al., 2015)
	Dichlorvos	Buffer	Spectrophotometry	37.5 ng/mL	(de Souza et al., 2018)
	Diflubenzuron	Buffer	Spectrophotometry	419 µg/mL	(de Souza et al., 2018)
	Novaluron	Buffer	Spectrophotometry	3.9 µg/mL	

[a] minimum concentration tested