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An overview of detection techniques for monitoring dioxin-like compounds: latest technique trends and their applications

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Abstract

Dioxin-like compounds (DLCs) are considered persistent bioaccumulative toxicants with a number of continuing issues in the field of ecotoxicology and bioassay. In spite of the great need to monitor these compounds, the only analytical technique with sufficient sensitivity and selectivity for determination of DLCs is a combination of high-resolution gas chromatography and high-resolution mass spectrometry. However, these methods require aseptic techniques, long incubation times, and sophisticated technical expertise for getting accurate results. Nowadays, biological techniques such as biomarkers, bioassays, enzyme immunoassays (EIAs), or other methods have been greatly developed as more sensitive, cost-effective and rapid techniques to determine the presence of DLCs in trace levels of environmental and biological samples.
The main aim of this study is to review latest analytical and bioanalytical detection methods (BDMs) for diagnosis and monitoring of DLCs. Likewise, this work characterizes the latest techniques and trends based on their application, advantages and shortcomings for the various BDMs and their differences are also noted.

Key words: Dioxin-like compounds (DLCs), bioanalytical detection methods (BDMs), bioassays, biomarker, enzyme immunoassays (EIAs)

Introduction:

Dioxin-like compounds (DLCs) have been classified as persistent organic pollutants (POPs) by the World Health Organization (WHO) and the US Environmental Protection Agency (USEPA) [1-3] because of their hazardous properties including long lifetimes, global distribution, accumulation and bio-magnification in food chains, and their toxicological effects on humans such as teratogenesis, tumor promotion and modulation of the immune system [4-6].

The global contamination of air, soil and water continues to occur with trace levels of these toxic materials in food such as dairy products, fish, pork and shellfish [7]. Potential human exposure can be detected and monitored by analyzing breast milk, urine, blood, placenta or hair. Similarity in the environment, organisms consumed by humans as well as abiotic matrices (water and sediment) must be examined as sources of exposure [1, 8-12].

In recent decades, a combination of biochemical techniques known as bioanalytical detection methods (BDMs) provides simple and rapid methods to detect DLCs in environmental samples [1, 9-12]. The chemical analytical methods were defined based on gas chromatography (GC) [12], while the biological techniques were developed based on in vivo bioassays or laboratory exposure (whole animal exposures), biomarkers (human effects/ wildlife), organ- or cell-based in vitro bioassays (luciferase, EROD), and protein binding assays (e.g., immunoassays and ligand binding) [8, 13].

Instrumental analytical methods are essential for exact quantification and identification of the individual dioxin compounds, while the in vivo bioassays are frequently applied to predict of whole-organism responses and to assess the

bioavailability [1, 8]. The use of BDM has the high possibility to select between more persistent aryl hydrocarbon receptor agonists and easily biodegradable compounds [8, 13].

Evaluation of environmental contaminates by utilizing bio-detectors such as biomarkers, bioassays, enzyme immunoassays (EIAs), and biosensor is a continuously growing area. This review is an integrated overview based on the available key studies with the following objectives: to summarize a series of critical studies on the evolution of analytical and bioanalytical detection techniques and their applications to provide a better view of the monitoring and diagnosis of DLCs[M1] in environmental and biological samples. To highlight the key findings, the limitations, weaknesses and strengths of the available BMDs methods will be investigated. The chosen methods are based on either the ability of organisms or cells to show special response to DLCs or the ability of biological molecules to detect any specific structural property of dioxin like compounds [27]. General chemical and biological techniques for determination of PBTs are presented in Fig1.

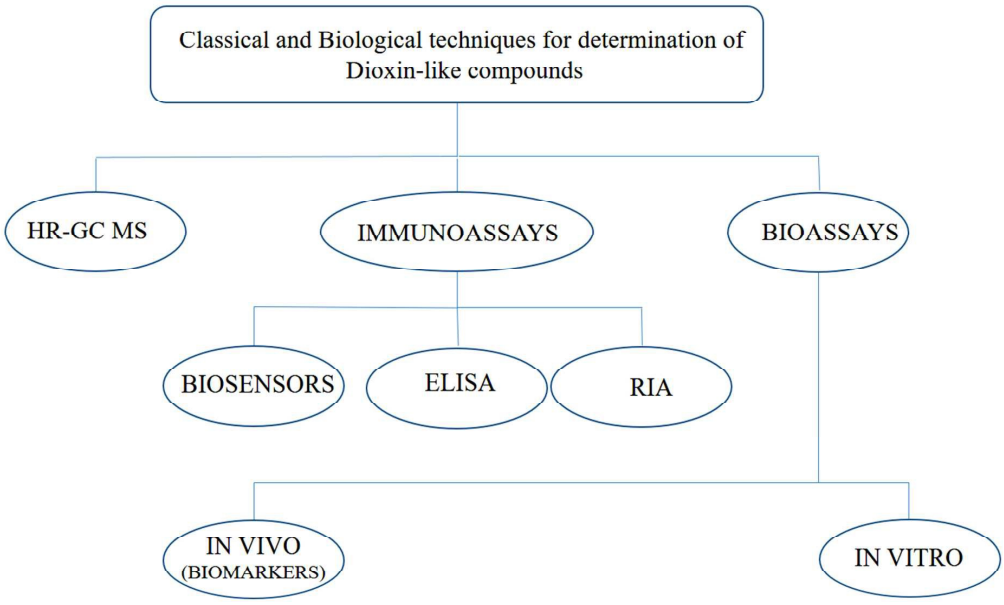
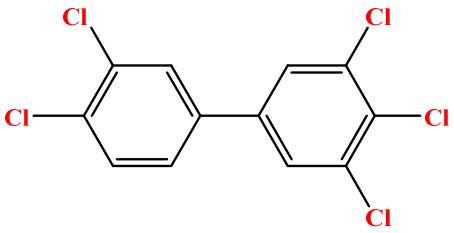


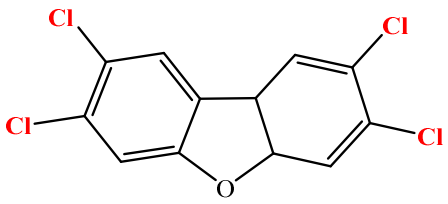
Figure 1. Scheme of chemical and biological techniques for determination of PBTs

1. Chemical structure and mechanism action of DLCs

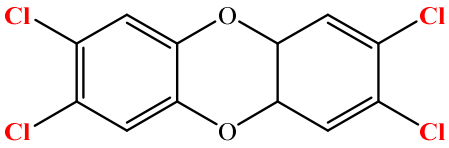
The chemical structure of DLCs is structurally related to halogenated aromatic hydrocarbons (HAHs) having similar mechanism of toxic action [14-16]. Dioxin-like compounds contain 7 of the polychlorinated dibenzodioxins (PCDDs), 10 of the polychlorinated dibenzofurans (PCDFs), and 12 of the polychlorinated biphenyls (PCBs) [17, 18]. The most toxic congener for this class is 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) that is involved in several occupational activities and industrial accidents around the world [7-9]. PCDF and PCDD are by-products of incineration procedures and organic synthesis [4, 19], whereas PCBs are used as coolant fluids and dielectrics in capacitors, electric motors, and transformers [17, 18]. Dioxin like compounds contain two benzene rings linked to two oxygen atoms and include of four to eight chlorine atom substitutions in the 2,3,7 and 8 positions [20]. Chemical basic structures of PCDFs, PCDDs, and a PCB are shown in figure 2 [17, 18].



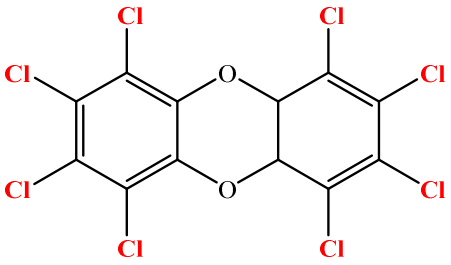
3,3', 4,4', 5-Pentachlorobiphenyl (PeCB)
PCB(126)



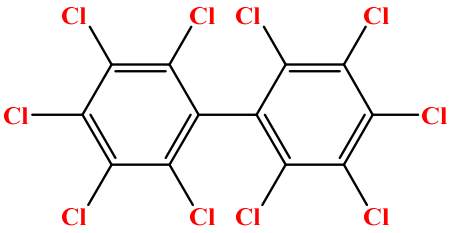
2,3,7,8-Tetrachlorodibenzofuran (TCDF)



2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)



Polychlorinated-dibenzo-*p*-dioxin (PCDDs)



Polychlorinated biphenyls (PCBs)

Figure 2: Chemical structures of dioxin-like compounds

Several molecular mechanisms are reported by which mixtures of dioxin compounds can cause toxicity in humans and wildlife [16, 19, 21]. The most well-known molecular mechanisms of dioxin toxicity including DLCs effect the activation of specific intracellular receptors such as aryl hydrocarbon receptor (AhR), and create a specific complex of atypical enzymes that induce several biological responses such as the induction or repression of gene expression, and causing the disruption and differentiation of tissue, cellular, and biochemical processes [1, 15, 19, 22]. This is known as the canonical mechanism to estimate toxicity of DLCs in bioassay methods [16, 19, 21].

The canonical function of lipophilic agonists is activated after diffusing TCDD into

the membrane of plasma and binding to AhR. The complex of AhR-agonist is activated after releasing the chaperone proteins and binds to the transcription factor AhR nuclear translocator (Arnt) protein. The new complex [AhR-agonist-Arnt] is transferred into the nucleus [16, 19, 21]. In the nucleus, the ligand-AhR-Arnt complex connects to sequences of specific DNA and localized in the promoter sequences of various human genes. This binding leads to transcript adjacent genes P-450 (CYP1B1, CYP1A1and UGT1A1), several enzymes (mixed-function Oxidases) and the AhR-repressor (AhRR) which down-regulates signals of AhR [19, 23, 24]. Finally, a luciferase enzyme will be produced based on luciferin substrate and mRNA translates to catalyze a bioluminescent reaction (Figure 3). Luciferase data is defined based on the relative light unit (RLU) in the exposed cells as the DLCs function has direct effect on the amount of light with defined proportion [16, 19, 25].

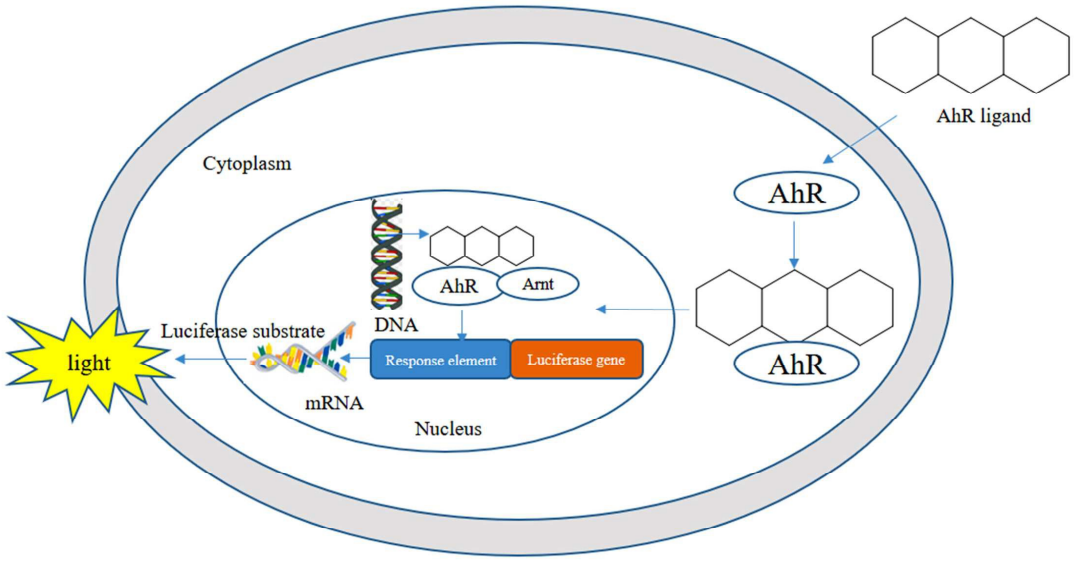


Figure 3: Canonical model of DLCs actions in cells

Thus, total toxicity of dioxin and their relevant congeners is evaluated by the value of the toxic equivalency factors (TEFs). This factor shows the potential of DLCs to induce activation of AhR related to the reference substance [9, 15]. Thus, the TEF

values were estimated experimentally for each DLCs congeners to evaluate the total toxicity of DLCs based on a variety of endpoints, responses and uncertainties in the available data (Table 1) [26-29].

Table 1: Recommended TEFs value for risk assessment of dioxin congeners [22, 28].

Congeners	*Mammal	Birds	Fish
Chlorinated dibenzo-p-dioxins			
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PeCDD	1	1	1
1,2,3,4,7,8-HxCDD	0.1	0.05	0.5
1,2,3,6,7,8-HxCDD	0.1	0.01	0.01
1,2,3,7,8,9-HxCDD	0.1	0.1	0.01
1,2,3,4,6,7,8-HpCDD	0.01	<0.001	0.001
OCDD	0.0003	0.0001	<0.0001
Chlorinated dibenzofurans			
2,3,7,8-TCDF	0.1	1	0.05
1,2,3,7,8-PeCDF	0.03	0.1	0.05
2,3,4,7,8-PeCDF	0.3	1	0.5
1,2,3,4,7,8-HxCDF	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.01
OCDF	0.0003	0.0001	<0.0001
Non-ortho–substituted PCBs			
3,3',4,4'-TCBd	0.0001	0.05	0.0001
3,4,4',5-TCB	0.0003	0.1	0.0005
3,3',4,4',5-PeCB	0.1	0.1	0.005
3,3',4,4',5,5'-HxCB	0.03	0.001	0.00005
Mono-ortho PCBs			
2,3,3',4,4'-PeCB	0.00003	0.0001	<0.000005
2,3,4,4',5-PeCB	0.00003	0.0001	<0.000005
2,3',4,4',5-PeCB	0.00003	0.00001	<0.000005
2',3,4,4',5-PeCB	0.00003	0.00001	<0.000005
2,3,3',4,4',5-HxCB	0.00003	0.0001	<0.000005
2,3,3',4,4',5'-HxCB	0.00003	0.0001	<0.000005
2,3',4,4',5,5'-HxCB	0.00003	0.00001	<0.000005
2,3,3',4,4',5,5'-HeCB	0.00003	0.00001	<0.000005

Whole activation of AhR receptors is expressed as TCDD toxic equivalent (TEQ) based on the AhR signaling assessment [15, 16]. In complex mixtures, the TEQ is

estimated based on multiplying the individual concentration of dioxin congeners by their respective TEF [26, 28].

Recently, 7 dioxin-like compounds (2,3,7,8-TCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PCDD, 2,3,4,7,8-PCDF, PCB-77, PCB-126, and PCB-169) have been identified as key indicator congeners for most of the total TEQ (93%, n = 132) in calculated plasma samples or human blood [30]. This is a relatively inexpensive quick screening method while, it is essential to make stable patterns of key indicators to be present in the selected matrix. The total organic halogen (TOX) value is another possible method to predict a TEQ value based on electrochemical titration as reported in relevant studies of fly ash and combustion gas of incinerators [1, 8, 30].

In complex mixtures, the TEQ is estimated by summing the TEQs of all congeners [31]. The more details about AhR- signaling pathways and mechanism actions of DLCs were recently discussed by Tavakoly Sany et al. [18, 19]. A comparison of TEQ values (pg/g lipid weight) in agency food standards and selected foods is summarized in Table 2.

Table 2: A comparison of TEQs value (pg/g lipid weight) in agency food standards and selected foods [32-34]

Mean PCDD/PCDDF									
Exposure Pathway	Australia	New Zealand	USA	North America	Europe	Asia	Netherlands	UK	*EU standard
Poultry	0.02-0.53	0.037-0.29	0.10-5.17	0.03-3.9	0.6-0.9	0.67	1.06	0.13-0.18	1.75
Fish	1.56-3.04	0.33-0.41	2.45-21.1	0.033-0.53	0.01-8.9	0.002-10.2	0.181	1.06	3.5
Beef	0.0006-0.24	0-0.11	0.89-2.86	0.5-4.1	0.6-1	1.0	0.82	0.41-0.42	2.5
Pork	0.05-0.22	0-0.20	0.64-3.97	0.6-23	0.2-1.4	0.8	0.24	-	1
Lamb	0.004-0.25	0-0.07	-	-	-	-	-	-	2.5
Eggs	0.013-0.42	0.017-0.12	0.8	0.44-0.3	0.5-2.7	-	1.52	0.24	2.5
Bread/cereals	0.00039-0.021	0.0012-0.0059	-	-	0.019	-	-	0.18-0.2	0.03
Milk	0.04-0.23	0.019-0.16	0.98	0.3-0.9	0.3-2.5	0.3-1.8	0.57	0.46-0.47	2.5
Fruit/ vegetables	0.000023-0.013	0.0012-0.0016	-	-	0.029	-	-	-	0.75
Mean PCB									
Poultry	0.18-0.24	0.018-0.14	0.29	0.3	0.59-0.7		1.72	0.47-0.53	1.25
Fish	9.46-9.5	0.77	30	0.11-0.289	0.03-9	0.004-2.0	0.412	3.57	3
Beef	0.03-0.11	0.0036-0.092	0.49	0.5	0.914	-	1.24	0.25-0.31	1.5
Pork	0.04-0.073	0.15-0.434	0.06	0.02-1.7	0.09-0.815		0.23	-	0.25
Lamb	0.02-0.06	0.01-0.045	-	-	-	-	-	-	1.5
Eggs	0.04-0.11	0.05-0.11	0.87	0.0299	0.2-0.6		0.87	0.11-0.20	2.5
Bread/cereals	0.0003-0.005	0.00099-0.004	_	-	0.11	-	-	0.06-0.15	0.01
Milk	0.04-0.11	0.027-0.15	0.59	0.5	0.2-1.8	-	0.69	0.34-0.43	3
Fruit/ vegetables Soil ingestion	0.00006-0.0016 -	0.0012-0.00258	-	-	0.03-0.125		-	-	0.5

2. Classical Analytical Method

Extensive databases are reported to detect chemical contaminants based on several analytical methods. The dioxin level in food samples is usually very low (ppt level) and separation requires complicated physicochemical techniques such as high-resolution gas chromatography tandem mass spectrometry (HRGC-MS). Until about 25 years ago, the HRGC-HRMS or gas chromatograph electron capture detector (ECD), was the only method for the diagnosis of dioxin congeners in human tissues, fish, and adipose tissue [35, 36]. This technique focuses on the quantification and separation of DLCs from matrices on the basis of differences in their polarities, molecular size, charges, redox potentials, and mass.

HRGC-HRMS was first used in the 1970s to detect TCDD in tissue samples from Vietnam exposed to Agent Orange [37] and has since been considered the gold standard to measure all DLCs [38, 39]. Now, most dioxin laboratories worldwide such as the US Air Force, CDC, and the WHO have routinely applied [1, 40] gas chromatography to estimate concentrations of DLCs in a variety of environmental media (water and sediment) and human tissue [1, 8, 12]. The advantages of this method are the pattern and congener specificity, the structure conformation, and the calculation of the TEQ (toxic equivalents) by the international standardization and TEF-concept. The disadvantages are that not all standards of individual DLCs are available, the potential loss in specificity, and their information is not enough to assess the potential interactions and potential biological activity of the toxic chemical contaminants in animals [1, 36]. Likewise, a typical dioxin analysis with this method is usually time-consuming (about 2 weeks) and very expensive (≈$1000 per sample) [8, 30]. All the samples must be extracted using organic solvents and spiked with isotopically labelled internal standards before clean-up procedures. The clean-up process for PCDDs, PCDFs and PCBs is complex since samples are usually associated with problems such as the presence of unwanted contaminants that are present in much higher concentrations than the analytes. These contaminants sometimes cause the signal of dioxin to be completely hidden or give false results on the analytical equipment used [41].

Besides HRGC-MS, there are a variety of other spectroscopic methods in combination with chemometric analysis data which widely applies for quality control of food compositions. Fluorescence spectroscopy is one of these techniques which is mostly used for biological and food samples due to its sensitivity and high selectivity.

The relative concentration of analytes will be obtained by using the PARallel FACtor (PARFAC) algorithm. By resolving the complex fluorescence landscapes into excitation and emission of special fluorophores [42, 43].

In recent decades, several biological assays have been developed as alternatives to analytical analyses such as immunoassays using chemical-specific antibodies, biosensors, and biomarkers.

3. Modern Bioassay Screening Methods

A stated goal of research to improve the safety of food and seafood is to promote an economical set of inspection and monitoring activities that will decrease the exposure of consumers to hazardous materials. In recent decades, simple and rapid analyses have been developed to screen potential of dangerous contaminants in food products and environmental samples [30, 44]. Indirect techniques based on biological evaluation are frequently employed as powerful alternatives to analytical methods [1, 30, 44]. The biological assays are rapid and low cost methods in comparison with chemical analyses and their mechanism is defined based on their toxicological specificity, which refers to the relationship between the toxic potential and assay response [30, 45, 46]. Bioassay screening methods are divided to in vivo bioassays (biomarkers and laboratory bioassays) and in vitro bioassays [8].

These bio-analytical tests might bridge the gap between effect and cause. Some advantages of the bio-analytical methods include [30, 45]: (1) short procedure time and a fast estimation of the total potency of aryl hydrocarbon receptor (AhR) agonists, (2) high sensitivity to detect lower concentrations at pictogram level, (3) low cost, (4) the ability to predict the results of bioassays in terms of magnitude of effect,

but not the total spectrum of action, (5) their mechanism defined based on their toxicological specificity, which refers to the relationship between the toxic potential and assay response, (6) improved reliability, accuracy, and scientific concepts for the quantitative evaluation of ecological and human health risks.

The bioanalytical methods have the following disadvantages [8, 45]: (1) questions about the degree of reliability (the relationship between chemical information and bioassay information) (2) the limitation of date validation for different complex matrices of some biomarkers/bioassays, due to their new development, (3) lack of cross-validation studies between different biomarkers/bioassays, (3) limited inter laboratory cross-validation studies using similar technology, (4) lack of international and national round robin studies in various complex matrices which have not been performed, (5) limited predictive battery from a toxicological point of view due to the need for in vivo–in vitro extrapolation, (7) international evaluated quality criteria.

The ability of several key biological molecules such as receptors, antibodies and enzymes are evaluated in biological methods to detect specific responses of cells or organisms to DLCs, or to recognize the chemical structure of a dioxin component. There are more reports and reviews about bioassays and biomarkers of dioxin and dioxin-like compounds before 2001 [10, 30, 37, 40]**,** while in recent years, there is not a complete overview of the available methods for detection of dioxin compounds. In this review, more effort has been made to present the current methods of dioxin detection. General bio-assay approaches for determination of halogenated aromatic hydrocarbons are presented in Table 3.

Table 3. Commonly used bio-assay approaches for environmental monitoring of dioxin-like compounds and their characteristics.

Methods	Advantages	Disadvantages	Applications	Reference
In vivo biomarkers	Most defensible screening method with most published data for wildlife due to uncertainties of the vitro screen in toxicokinetics and bioavailability.	Time-consuming, requiring high doses, competitive inhibition occurred, ethical critical issues, costly method.	All types of biological responses to dioxin-like compounds in plants, wildlife and human.	[8], [28]
In vivo laboratory bioassays	direct relationship between in vivo bioassays and endpoints of concern which cause to estimate the integrated responses at the whole-animal levels	Expensive and time-consuming	Death and reproductive abnormalities in mink, death of trout fry, death of fish early life stages, development deformities in domestic chicken.	[28], [47], [48]
In vitro bioassays	Gives an integrated estimation of the biologically active contaminates in various environmental medias, inexpensive and rapid method	There is no distinguishing between receptor antagonists and receptor agonists.	High potential application to monitor marine and coastal sources, including seafood.	[49], [8]
Receptor-binding assays (Ah receptor)	Used to estimate the ability of dioxin mixtures or compounds to compete with dioxin analogs (radiolabeled) or TCDD for binding to the Ah receptor	Some problems in part of quality control of the gel retardation methods make this method unreliable and complementary DNA (cDNA) microarray chips cannot analyze the activity of genes responsible for DLCs.	Pharmacokinetic studies.	[50], [51], [1], [8], [51]
Enzyme inhibition assays	-	Is a poor method for detection of dioxin-like compounds due to its poor limit of detection	Marine and freshwater	[52], [53]
DNA binding assays (DRE binding gel)	A quite sensitive method to detect the presence of Ah receptor agonists	Sometimes provides false positive data and there are some problems in part of quality control of gel retardation methods which makes it	Biological samples	[54] ,[55], [56]

		unreliable.		
Cell culture Bioassay (CYP1A induction)	This technique has been promoted including measurement of immune-detectable CYP1A protein and messenger RNA to provide more reliable measurement of CYP1A-inducing potency in in vitro bioassays based on the reporter gene systems.	Some polyaromatic hydrocarbons (PAHs) and HAHs, at high doses, are able to inhibit induction of CYP1A activities and their results are not able to reflect the level of induced CYP1A proteins.	Environmental samples	[57], [57], [58]
Reporter gene assays (DRE-luciferase construct)	strong positive correlation with in vivo assays, high metabolic capacity, long incubation time (72 h), bioassay quality: CV 29–38%	Time-consuming method which many chemical contaminates (PCB) are involved and cause to inhibit EROD activity leading to a lower induction, faster and less expensive alternatives required for HTPS having narrow linear working range than in vitro luciferase assays, sensitive to oxidative stress, low mRNA and enzyme stability and in vivo season-dependent fluctuations in educability	Testing of the biological relevant sum of TEQ	[59], [60], [61], [62], [63]
Biosensors (for PCBs)	interesting techniques due to their low cost of energy, rapid, less use of chemical reagents, on-site analysis, minimal waste production, miniaturization and the possibility to combine with other multiplex technologies, selectivity, sensitivity, limit of stability and detection advantages	The biosensors are used for monitoring of DLCs contaminations in food products, however have been applied for environmental monitoring	Environmental purposes, health and medical care	[64], [65], [66], [67]
Immonuassays (for PCBs or PCDDs)	simplicity, speed, low cost and parallel processing of many samples, potential field use as well as easy automatization	the presence of cross-reacting compounds, costly development, and nonspecific interferences	Detection of DLCs in food products	[67], [68], [69], [70]

3.1. In vivo biomarkers

Biomarker methods are able to detect characteristics of biological changes (physiological, biochemical) due to the presence of dioxin like compounds. This method is commonly used based on biological effects and their specific reactions which can be a complementary method [44, 71]. Here, the term 'in vivo biomarker' refers to biological changes due to the 'natural' exposure to pollutants in environment [8, 72]. Several researchers have found strong relationships between exposure to specific classes of environmental contaminants and in vivo biomarker responses [8, 30, 44]. Several biomarkers (like DNA adducts and enzymes) have been introduced for the determination of dioxin compound levels in all target biological tissues such as molecules, cells, organs, individuals, populations and ecosystems.

Induction of the cytochrome P450 1A gene (CYP1A) has commonly been applied as a biomarker of exposure to dioxin compounds in marine mammals, fishes and humans [8, 73]. This method is estimated based on the determination of immune-detectable CYP1A proteins or messenger RNA [30, 74]. This induction occurs following the binding of dioxin and dioxin like compounds to the AhR (aryl hydrocarbon receptor). Induction of CYP1A is parallel with the gene expression (due to AhR-dependent changes). This gene is responsible for some forms of dioxin toxicity. The biomarker studies show significant positive correlations between toxicity of dioxin congeners and induction of CYP1A [75, 76]. Moreover, the experimental analysis shows that presence of AhR ligands is significantly correlated with the CYP1A induction in vertebrate animals [44, 76]. As with other biomarkers, the efficiency of CYP1A induction to indicate dioxin exposure is limited by the

biological specificity of the response [8, 77]. Based on previously reported bioassay studies, CYP1A induction is able to provide relevant information of dioxin exposure in most vertebrates such as cartilaginous and bony fish, reptiles, amphibians, birds and mammals because these animals have a dioxin-responsive AhR/CYP1A system (a functional AhR-CYP1A pathway) to possess the appropriate response mechanism [45, 74, 75].

Several studies show that the CYP1A induction is not an appropriate biomarker in aquatic invertebrates and early vertebrates because these organisms ancestors diverged prior to the evolution of a dioxin-responsive AhR pathway [8, 71, 72, 74]. Moreover, the presence of AhR homologs for binding to typical AhR ligands or TCDD has not been confirmed in invertebrate [8, 78]. Another main point is that CYP1A induction cannot be suitable for in vivo biomarkers when the normal response of a species is to develop dioxin resistance through genetic adaptation or physiological acclimation. In such cases, the use of CYP1A induction as a biomarker to indicate exposure causes a high rate of wrong negative results [8].

Other biomarkers for dioxin-like compounds are the induction of aryl hydrocarbon hydroxylase (AHH), ethoxyresorufin-O-deethylase (EROD), hepatic acetanilide-4-hydroxilase, chinon-oxidoreductase, aldehyde-dehydrogenase, glutathione-S -transferases, Phase II drug-metabolizing enzymes like glucuronosyl transferases, and the accumulation of hepatic porphyrin, as well as the reduction of plasma thyroid hormone and levels of hepatic vitamin A [28, 76]. Dioxin-like compounds also cause the production of chloracne in rabbit pinna and pericardial edema in newborn chicken which are considered as biomarkers in intact animals [1, 28].

In general, in vivo biomarkers are considered as defensible screening methods for wildlife, because of uncertainties of the vitro screen in toxicokinetics and bioavailability [30]. In vivo bioassay testing has the following disadvantages such as; being time-consuming, requiring high doses, the occurrence of competitive inhibition, critical ethical issues, costly methods due to requiring invasive or euthanasia surgical equipment for animals [28, 30]. Likewise, in vivo testing incorporates various main reactions, which are limited in in-vitro assays. These reactions include metabolism, pharmacokinetics, and interactions with the transport of proteins and multiple bindings, which effect target tissues [44, 76].

3.2 In vivo laboratory bioassays

In vivo bioassays include the experimental exposure of laboratory animals to mixtures of contaminated materials [30]. In this assay the relative exposure levels, the potential health effects in environmental mixtures, the species responsiveness and bioavailability of contaminates are assessed by determining: thymus weight reduction (immune toxicity and thymic atrophy), the liver size (hepatotoxicity), reproductive toxicity (malformations, number of offspring, irregular cycles), wasting syndrome (progressive loss of weight until death), and EROD/AHH [30, 45]. Investigators have estimated dioxin activities and their toxicity in the early-life stage of several organisms by exposing bird or fish eggs, and other wildlife tissues [79-81]; For example: development deformities in domestic chicken (5.8 ppt in eggs), death of trout fry (40 ppt TCDD in eggs) [79, 81], abnormalities in reproductive organs and death in mink (1000 ppt body burden, 5–10 ppt in food) can be mentioned [8, 30].

The main advantage of this bioassay is the estimation of integrated responses at the whole-animal level because of the direct relationship between in vivo bioassays and endpoints of concern, including development and reproductive effects or cancer in exposed organisms. However, this method is expensive and many time is required to be studied on whole animals [82, 83].

3.3 In vitro bioassays

In vitro bioassays utilizing cellular extract or cultural cells are being developed to detect the presence of chemical pollutants. In vitro bioassays consist of DNA-binding assays, receptor-binding assays, native responses in cell culture, reporter gene assays, and changes in gene expression or enzyme inhibition assays in cultured cells [30, 45]. In vitro bioassays based on biomarker responses are able to make an integrated estimation of the biologically active contaminates in various environmental medias and have several advantages in comparison with other methods discussed above. Such bioassays are inexpensive and rapid methods for the specific analysis of adopted hazards and critical control points, which have a high potential application to monitor marine and coastal sources, including seafood [8, 45].

3.3.1 Receptor binding

Various types of in vitro bioassays are developed to monitor dioxin like compounds. The in vitro assay is also used to estimate the ability of dioxin mixtures or compounds to compete with dioxin analogs (radiolabeled) or TCDD for binding to the Ah receptor. This is known as a competitive binding method by using [^{3}H]TCDD and [^{125}I]2-iodo-7,8-dibromodibenzo-p-dioxin binding agents [84, 85]. One of the big disadvantages of such in vitro bioassays is that there is no distinguishing between

receptor antagonists (compounds that bind to the receptor but do not activate transcription) and receptor agonists (compounds that either bind or activate transcription) [1, 84].

3.3.2. DNA-binding bioassay

The DNA-binding bioassay is based on the ability of dioxin compounds to activate or transform AhR receptors into DNA-binding forms when the receptor has suitable ligands. DNA binding and receptor transformations are estimated by the electrophoretic mobility shift (gel shift) techniques based on the detection of specific protein (AhR)-DNA complexes and their mobility during electrophoresis [54, 55], which is called GRAB (gel retardation of AhR DNA binding) bioassay [55, 56]. This system is known as a quite sensitive method to detect the presence of Ah receptor agonists and biological effects of active congeners at the AhR level [30, 54-56]. Unfortunately, these assays do not necessarily distinguish receptor antagonists and AhR agonists and providing false positive data [86]. Thus, it may not be practical to detect the synergistic effect of natural dioxin and DLCs in environmental samples [30, 56, 86]. Another problem is in the quality control of the gel retardation methods which make them unreliable. Facing these difficulties, as a screening technology for DLCs, the complementary DNA (cDNA) microarray chips cannot analyze the activity of genes responsible for DLCs [30, 55].

3.3.3. Cell culture bioassays

Cell culture bioassays (e.g., EROD, P450HRGS, CALUX) have been applied by several researchers to evaluate dioxin contamination in various environmental samples because the results of this assays have close correlation with the results provided by chemical analysis of dioxins congeners [45, 87, 88]. Cell culture

bioassays are the most sensitive method based on the specific responses, and intergrade all possible and active interactions of dioxin compounds in a complex mixture. This is a main advantage of the AhR-based bioassays because of direct determination of the total average of TEQ for dioxin, which can be applied as the relative potencies (REP) and measured in case of risk assessment [45, 58].

In vitro bioassays employ different sample incubations (e.g. possible test strategy for detection of DLCs and PAHs: 4–6 h; in vitro luciferase bioassays: 4–48 h; EROD (ethoxyresorufin-O-deethylase) bioassays: 24–72 h) to select more persistent AhR agonists and easily biodegradable compounds, while the incubation time for most persistent DLCs are determined at 24–48 [30, 45, 89]. In addition, cell culture bioassays determine the level of dioxin-induced gene expression by employing a native response including CYP1A induction and by increasing an artificial construct expression consisting luciferase or other reporter genes under the control of particular regulatory parameters, which are able to respond to dioxin mixtures or compounds [30, 45].

The application of CYP1A induction in in vitro bioassays is known as an integrated method to measure DLCs, which was first explained more than 20 years ago [90-92]. Since that time, various studies added more improvements in sensitivity and speed by using fluorescent plate readers and multi-well plates [74, 92]. These techniques have been promoted to include measurement of immune-detectable CYP1A protein and messenger RNA, which provides more reliable measurement of CYP1A-inducing potency in in vitro bioassays based on the reporter gene systems [8, 90, 91].

In this system, luciferase (as reporter gene) is inserted into a plasmid, which is controlled by dioxin-responsive enhancer elements (DREs). When a sensitive luminometer is applied, cells expressing luciferase offer a construction around 3 to

10 fold [8] higher sensitivity than cells expressing using the native CYP1A response [8, 90]. Likewise, an investigator reported some pitfalls relevant to the catalytic determination of CYP1A activities due to some biphasic dose-response curves which have frequently been found in estimating CYP1A induction activities of cultured cells [8, 74, 92]. Also, they have recently recorded that some poly aromatic hydrocarbons (PAHs) and HAHs, at high doses, are able to inhibit induction of CYP1A activities. Thus, their results are not able to reflect the level of induced CYP1A proteins [8, 57, 93].

A: AHH/EROD bioassay

The EROD assay is more generally applied to estimate the binding of the DLCs to the AhR and the subsequent induction of CYP1A related de-ethylation of 7-ethoxyresorufin [30, 45]. In this method, activities of several CYPs are estimated based on the different substrates, for CYP1A2 (methoxyresorufin-O-demethylase; MROD), for CYP1A1 (EROD), and for CYP2B1 (pentoxyresorufin-O-depenthylase; PROD)[45]. Likewise, in this bioassay several cell lines are used such as the chicken embryo hepatocytes, rat H4II cell line, Hepa 1 (mouse), human hepatoma Hep G2 and Hep 3, GPC16 (guinea pig) and fish cell lines like PLHC-1, RTL-W1, or RTG-W1[30, 45, 76].

The main advantages of this bioassay are: highly published data, estimation of the total biological relevant TEQ of the persistent class of AhR active compounds, strong positive correlation with in vivo assays, analysis of the catalytic activity of CYP1A1 which present more effects on wildlife/human than luciferase induction or immunoassay, 24h/72h kinetic for distinguishing between unstable/stable agonists high metabolic capacity, long incubation time (72 h), bioassay quality: CV 29–38% and distinguishing among AhR antagonists/ agonists [8, 94].

Some drawbacks were reported by researchers including; more time-consuming, species-specific, many chemical contaminates are suitable substrates for P450 and can inhibit EROD activity leading to a lower induction, faster and less expensive alternatives required for HTPS [8, 30], more narrow linear working range than in vitro luciferase assays, sensitive to oxidative stress, low mRNA and enzyme stability and in vivo season-dependent fluctuations in educability [45, 94].

B: In vitro luciferase bioassays (CALUX or P450HRGS)

Recombinant cell lines are provided by stable or transient transfection of several type cells with reporter genes under transcriptional control of the dioxin response element (DRE)[59]. Firefly luciferase (luc) was considered as the most common reporter genes. These newly recombinant cell lines still include the full machinery, involved in the mode of action of DLCs [59, 60]. Moreover, a DNA-construct has combined the cells consisting of DREs from a wide type of species (rat, mouse, human) connected to firefly luciferase genes. They are able to quantify all type compounds that activate the AhR to produce the luminescent enzyme luciferase. Some advantages of this production are (1) the resistance of the firefly luciferase is higher than CYP and (2) this enzyme has more copies of the main vectors in the cell line than the natural P450 enzyme or P450HRGS (P450 human reporter gene system). The cellular response can be estimated by using appropriate reagents (e.g., ATP and the substrate luciferin) and an automated luminometer applied to quantify the produced bioluminescence emission [30, 60]. The production of luciferase is an easy mechanism to estimate the amount of AhR binding (a subordinate of the amount of agonist in the sample). This bioassay method are applied for a wide type of recombinant cell lines such as from guinea pig, rainbow trout, human, rat or mouse hepatoma cells based on the affinity

and the concentrations of the chemicals applied to the cells [59, 95, 96].

The analyzed results obtained from PCB, PCDF, and PCDD congeners, confirm that the amount of the relative potencies (REP) to induce the chemical-activated luciferase gene expression activity (CALUX) in mouse and rat were significantly correlated with the reported amount of TEF. While for some PCB congeners, the amount of REP tested by the human cell lines (P450HRGS) showed significant differences [60, 96]. Several complex mixtures of DLCs were significantly correlated to TEQ and TEF values tested by the CALUX bioassay rather than by the EROD bioassay. The specificity of the bioassays is a difficult issue for this type of assays. False-positive results would make the test acceptable, while false-negative results might be unreliable to some extent in bio-monitoring work. False-negative results were presented from EROD assays, because the reaction of CYP1A with a number of compounds (benzimidazole drugs at low concentrations; PCBs with high concentrations) is inalterably inhibited [45, 60, 96].

The main advantage of choosing an encoded reporter gene for an enzyme could be that so far no inhibitors are reported and the luciferase induction can only occur via the AhR. The in vitro luciferase assays directly effect on AhR-mediation, because these bioassays respond to any compound able to bind to the AhR. Thus, these assays are considered fast methods (EROD assay: usually 24–72 h; in vitro luciferase: 4–48 h), with a high linear working range, based on the instrument used (fluorometer and luminometer), and lower sensitivity to interferences [60, 96]. Thus, recombinant cells show high dynamic ranges, selectivity, and sensitivity than wild type cells. The luciferase bioassays also have the ability to estimate the presence of either bio-stable DLCs or biodegradable PAHs based on test procedures at both 6 and 16 h (P450HRGS) or 6- and 24/48 h (CALUX). This bioassays are performed without a

different cleanup process because the luciferase and the metabolism of most the PAHs are produced after 24 h [96-98].

In general, the advantage of in vitro luciferase/reporter gene assays are: covering the limitations of the EROD bioassay (no inhibition; wider working range, faster assay); same results to in vitro–in vivo EROD-REPs assay; testing of the biological relevant sum of TEQ; the ability of distinguish between antagonist and agonist as well as between unstable and stable AhR agonists; bioassay quality: CV 29%; species- and tissue-specific; HTPS possible; providing the choice of reporter gene and answering to main biological effects (protein binding, membrane passage) [45, 96]. Likewise, the following drawbacks were reported for this assay as well: stable transfected cells and luminometer are necessary; missing of possible tissue factors due to the transformation into a recombinant cell; outer signal pathways; stability of luciferase; inducing for any compound able to bind to the AhR, lack of higher TEQs to cleanup (e.g., in blood) [8, 30, 96].

C: Chemical-activated fluorescent protein expression (CAFLUX)

The CAFLUX assay employs the EGFP gene (enhanced green fluorescent protein) as a reporter gene to active AhR instead of the firefly luciferase gene used in the CALUX assay [96, 99]. EGFP is a type of protein derived from *Aequoria victoria* (jellyfish) carrying a cyclic tripeptide acting as a fluorophore [30].

The advantages of the CAFLUX compared to the CALUX assay are the easy of measurement, low cost, and lack of requirement for reagent addition [45, 99]. In addition, there is no expensive luminometer or substrate needed and only a standard fluorometer is used to measure the expression of the EGFP gene. The CAFLUX

bioassay also shows the same sensitivity as the CALUX bioassay, which has a detection limit, was less than 1 pM of TCDD. Furthermore, this method is considered a nondestructive bioassay, which allows the researcher to follow the gene expression in real-time [56, 100]. In the CAFLUX assay, persistent and non-persistent AhR agonists produce a cumulative signal. If the level of AhR agonists becomes low, this could be an advantage because AhR agonists can be detected by recurrent exposure of CAFLUX cells [94, 100].

The main disadvantage of this method is it's extreme sensitivity to low concentrations of non-persistent agonists. Therefore, it is difficult to test only the persistent class of DLCs [30, 45]. Likewise, in this bioassay the background fluorescence of cells is increased because of the high stability of EGF-protein, which leads to limit the dynamic range over time of usage. The parallel CAFLUX/CALUX bioassay would provide separate determination of the non-persistent/ persistent class of AhR-active compounds. However, in this bioassay, the available data set would not allow to use this bioassay as a monitoring method [30, 45, 60].

3.4 Immunoassay

Immunoassay methods are considered to be successful methods, which employ antibodies to detect specific congeners of dioxin compounds. The most common immunoassays are radioimmunoassay (RIA), fluorescence immunoassay (FI), and the enzyme-linked immunoassay (ELISA) [8, 30, 56].

The first study to analyze DLCs by applying immunoassays was performed based on the RIA method. Polyclonal antibodies, used in this method, made it more time-consuming compared to other methods. Later, Stanker and his coworkers (1987) developed a new and efficient method known as Mab-based ELISAs (DD3) [30]. The

selectivity of this method was the same as the RIA method. The commercial ELISA kits are also produced for rapid screening of DLCs in environmental samples such as dioxin RISC kit products, SDI from Envirogard, DD3 from Millipore, RaPID from BioScan Screening Systems and DF1 high performance dioxin/furan EIA from CAPE Technologies [101]. In addition, some immunoassays methods were developed to test PCBs in seafood and food based on an optical spectrophotometer methods [56, 96].

Biologically, the ELISAs use the ability of specific antibodies (biological molecules) to select and reverse bind to organic compounds. The immune system of all mammals produce antibodies for the purpose of self-protection against foreign compounds. This property leads to increase the immune ability of animals. Therefore, antibodies are considered as highly specific reagents to identify the analyte. The labeled ligands or the coating antigens are other key biological molecules in most environmental ELISA's kits. In these kits several molecules (e.g the binder molecules, the target analyte, coating antigen or an excess amount of labeled analyte) are allowed to react until an equilibrium was reached. The ligand molecules are able to label with an enzyme, a fluorescent molecule, and a radioactive tracer, so that the fraction of these molecules that have been bound can be measured [56, 102].

The free analyte is separated from the amount of labeled analytes and the bound phase, or binder molecules are estimated. The reaction of an enzyme linked antigen or antibody (ELISAs) and radioactive tracer (RIA) are used to perform the final detection. Over three decades, these methods have been applied in a wide range of array tests in the medical field for detection of various analytes from therapeutic drugs and hormones with excellent reliability [30, 56, 102]. In comparison to cell-based bioassays, the main advantages of immunoassay are the simplicity, speed, low

cost and parallel processing of many samples, potential field use as well as easy automatization. Although, some disadvantages such as the presence of cross-reacting compounds, costly development, and nonspecific interferences are presented [8, 56, 96]. In general, RIA and ELISAs methods are easy and quick competitive immunoassays. But, the detection of analytes and reagents usually compete for a limited supply of the binding reagent which leads to an inverse dose–response with the high variation in the low concentration of analyte [56, 103]. The sensitivity of these assays is strongly dependent on the detection avidity of the reagent for the binder compared to the analyte. Also, only one selected analyte is detected in these bioassays [60, 96, 102]. In the past, the ELISAs assay has not been used widely due to the high sensitively of mass spectrometer methods and the importance of the cleanup process. But, by developing new polyclonal antibodies (Pabs) in these assays, the critical selective extraction and solvent exchange to an assay-friendly solvent (e.g methanol and DMSO which were sensitive for TCDD), this method became more popular [30, 56].

3.5. Sensor technology

Recently, sensor techniques are gaining more interest because of several advantages of these methods in comparison to biological assays and conventional chemical analysis, for example, cost-effectiveness, simplicity, and the possibility for on-site and real-time analysis [104-106]. The biological and physical sensors (biosensors) are considered two promising technologies that could be applied for estimation of DLCs throughout environmental samples and the food chains involving wide types of samples such as air, soil, water, food, and animal tissues. In this section, the major focus is on biosensor techniques but some physical sensors and combinations of both

techniques are briefly described.

3.5.1 Physical Sensors

Although physical sensors have been commercially established and successfully used for various industrial purposes, limited information has been reported about the application of physical sensors to detect DLCs. Recently, the successful application of carbon nanotube techniques offers the possibility for detection of DLCs [107, 108]. Other physical sensors reported as detection elements to estimate DLCs, are lasers induced fluorescence (LIF) sensors which work based on fluorescence enhancement and quenching effects [104, 109], nanostructured-based surface-enhanced Raman scattering spectroscopy [64, 110], porous anodic alumina (PAA) based capacitive sensors [64], and the use of surface photo voltage [104]. The main advantages of these types of sensors are high specificity and sensitivity. However, these sensors are not able to detect the level of biological toxicity. In general, no physical sensor techniques have been commercially developed to detect DLCs in environmental samples and food chains and these type of sensors are still in the developmental phase [64, 104].

3.5.2 Biosensors

Currently, biosensor techniques are quite interesting due to their several advantages such as low cost of energy, rapid analysis, less use of chemical reagents, on-site analysis, minimal waste production, miniaturization and the possibility to combine with other multiplex technologies [66, 111]. Nowadays, biosensor methods are successfully being applied for environmental purposes, and in health and medical care because of the same requirements such as selectivity, sensitivity, stability and limit of detection. However, significant differences were observed in different matrices

between food samples as compared to those met in the health and medical fields which results in the need for additional extraction, preparation and cleanup procedures [64, 66, 104].

The biosensor has been defined by Thevenot *et al* as "a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with a transduction element" [112]. Biosensors include two major components: the transducer, which converts the event into an electronic signal and the bio-recognition element, which detects the specific contaminants. Theoretically, biosensor techniques are categorized based on their bio-recognition elements being applied. These elements consist of DNA, antibodies, enzymes, biological receptors, and whole cells. Some of these biological elements have been studied for measuring DLCs [67, 104].

Biosensors could be a promising technology for monitoring and surveillance of DLCs contamination in food products because of their high throughputs, real-time determination, reliability, and accuracy. These advantages are useful for making management decisions to prevent chemical contamination and improve public health and safety. Although, various types of biosensors have been reported in environmental monitoring, they still need further validations and optimizations [66, 67, 104, 112].

A. Immunosensors

Antibody-based biosensors (immunosensors) are more versatile compared to other biosensors. Antibodies may be selected as monoclonal, polyclonal or recombinant, depending on the production method that is applied and selective properties needed

[67, 113]. One of the best characteristics of immunosensors is their high selectivity towards potential complementary antigens (in our case individual dioxins like compounds). The applications of this type of sensor to detect DLCs in food products have been successful so far [68, 114]. However, some limitations have been reported when this method was applied to real samples, as they may incorrectly bind to other chemical contaminants that have similar chemical structures with antigens [104, 110]. Other research reported the successful application of a preliminary disposable electrochemical immunosensor to detect non-dioxin-like PCBs in adipose tissue, meat extracts, and ruminant milk. An electrochemical signal was used as a transducer and an accelerated solvent extractor (ASE) was applied to extract the sample. Their results showed higher reproducibility and sensitivity of the sensing element to the specific screened antigen (PCB 28) compared to other congeners. However, a highly effective extraction method (solid-phase extraction) was required to obtain purified extracts to probe the sensor [68, 114].

In addition, immunosensors have also been successfully developed based on quartz crystal microbalance as a transducer to detect DLCs in food samples. A high correlation was found between the golden standard and chemical analysis with a lower detection limit which was 1 ppt. However, clean up and extraction processes still needed more effort and these procedures play a major role in the precision and accuracy of any method for detection of DLCs [69, 106].

B: Whole Cell-Based Biosensors

In biosensor techniques, animal tissue or whole cells are also used as a sensing element [70]. Genetically, whole cell-based biosensors have been used for the direct detection of organophosphorus pesticides in water samples [115]. However, little scientific information regarding potential cell-based biosensors for detection of DLCs

are available. Recently, a whole cell-based biosensor has been developed to detect PCB contamination in sediment or soil samples. The *Pseudomonas sp* gram negative bacteria was used as a biological recognition element according to the optical detection. This species is able to oxidize PCB compounds, which results in the production of yellow meta ring-fission metabolites that can be estimated by an optical transducer through the absorption spectra [70, 115].

The advantages of a whole cell-based biosensor for sediment or soil samples are its simple measurement, extraction and preparation techniques and low-cost instruments. A drawback was reported such as presence of other yellow metabolites from unidentified factors, which could prevent the precision and accuracy of the measurement [70, 107, 115].

C. Biomimetic Based Biosensors

This type of sensor was developed based on a sensing element, which was synthesized from a mimic of a natural bio-receptor, such as an enzyme or antibody, that can be applied as a biological recognition element for biosensor technology purposes [64, 67].

Antibodies, applied as biosensors, can be changed when exposed to chemical reagents during clean up and extraction procedures because of reduction in sensitivity of the bioassay. Thus, synthetic peptides (as a detector) are used as alternatives to solve this limitation in soil samples [42]. On-head technology is used to improve the sensitivity of these synthetic peptides and dioxin concentrations were estimated based on measuring fluorescence intensity, which decreased when dioxin concentrations increased (detection limit was 0.2 ng TCDD/mL).

However, this still needed clean up and extraction procedures prior to

evaluation. Oligopeptides were synthesized to mimic AhR binding sites and immobilized onto a gold surface to detect DLCs in food samples such as milk, eggs and chicken. The range of detection of dioxin mixtures, TCDD, and PCBs was from 1 to 10 ppb, 1 to 5 ppb and 1 to 20 ppb, respectively with a variation of coefficient less than 15% [104, 109]. Two clean up methods were applied after extraction; First one based on the acid/base silica, alumina and carbon, and second one based on acid/base liquid/liquid partitioning (simplification). The outputs of two different clean up methods were the same and biomimetic receptors have shown high potential to detect DLCs in food matrices. However, the final results required more efforts to establish a wide commercial application of this method [70, 96, 104].

4. Conclusion

This research provides insight into the limitations and potential of different techniques to detect the presence of DLCs in the food products and environmental samples. Since DLCs are strongly toxic and ubiquitously present in environmental samples and food products, effective monitoring has to be used as an early warning system to prevent exposure of humans and animals to these toxic chemicals. On-site and real-time monitoring of DLCs is essential to make correct management decisions. Although, chemical analytical methods are the gold standards for analyses, but need sophisticated and expensive instruments and facilities, well-trained operators and costly reagents.

Bioanalytical methods have a very high potential to assess the effects of chemicals on environmental samples and food products . However, more effort must be done if bioassay techniques are to transition from being research tools to being widely used analytical techniques, which promote chemical analysis. In order to make this

transition, the bioassay methods must be clearly described and must meet commonly accepted performance frameworks.

The approval of bioassay-monitoring tests by several agencies is currently in progress (in some high-profile programs) to increase their credibility. These improvements of biochemical technologies leads to encourage new users to become involved. It is essential to promote an understanding of bioassay monitoring techniques within the comprehensive analytical community, including knowledge of their shortcomings and benefits. Likewise, interpretation of results from any single biochemical and chemical analysis technology must take into account their limitations as well. In conclusion, there is an increasing consensus that a battery of in vivo and in vitro bioassays is needed to widely assess the effects of DLCs in complex mixtures.

Sensor technology might offer promising tools to detect of DLCs in food products and environment samples, although these techniques are still under development because different biological recognition elements provide different limitations and advantages. Thus, sensor technology requires further standardization and optimization in order to allow their application to detect levels of contaminants in environmental samples and food products.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

These authors contributed equally to this work. All authors read and approved the final manuscript

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