1 Dioxins and related compounds

'Dioxin' is a generic name that hides three families of persistent organic compounds: the polychlorinated dibenzo-*p*-dioxin (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). PCBs have been synthesized and produced worldwide for commercial and industrial purposes. Between 1929 and 1989, an estimated 1.5 million tons of PCBs, marketed under trade names such as Arochlor, Cholphen, Kanechlor, and Phenochlor were produced and used as coolants in transformers and dielectric fluids in capacitors (Jones and de Voogt, 1999). Later, PCBs showed up in lubricant, plasticizer, paint, adhesive and varnish markets (Erickson, 1997). Because of their toxicity, major production was halted in the late 1970s, however their use in Europe will be completely banned only in 2025.

Unlike PCBs, dioxins and furans have never been intentionally manufactured but can be released into the environment from a number of different sources. In fact, PCDDs and PCDFs are generally unwanted by-products, resulting from combustion processes or industrial synthesis of other chlorinated compounds. A well-known example is the formation of 2,3,7,8 TCDD as a by-product that comes from the manufacturing of phenoxy herbicides 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Dioxin and furans are also produced during combustion processes from waste and medical incinerators, metallurgical processes, paper and pulp-bleaching mills, automobile exhausts and forest fires (Olie et al., 1977; Karasek and Hutzinger, 1986; Rappe, 1994; Fiedler, 1996).

1.1 Structures of dioxins, furans and PCBs

All dioxin molecules have in common two benzene rings joined by two oxygen molecules (di-oxin). Varying numbers of chlorine atoms (up to eight) can be located at different positions around this structure, resulting in 75 different structural configurations known as congeners (Figure 1-1). Note the flat, planar structure of this molecule and the symmetrical location of the chlorine atoms at the far ends. PCDFs differ structurally from PCDDs only by a carbon-carbon bond substituting for one of the oxygen bonds. Differing possible arrangements of chlorine atoms around the dibenzofuran molecule gives rise to 135 congeners. Although there are 210 congeners of PCDD/PCDF, only 7 PCDDs and 10 PCDFs which have chlorine substitution in at least all the 2,3,7,8 positions are of concern, owing to their toxicity, stability and persistence in the environment.

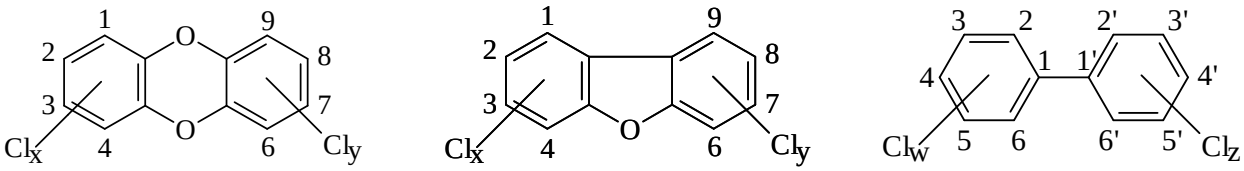

Figure 1-1 : Chemical structure of PCDDs, PCDFs and PCBs

A critical difference between PCBs and the PCDD/Fs is the presence of a free rotation around the carbon 1, 1' bound. The planarity of molecules depends on the chlorine substitution pattern. If no chlorine atoms are present in positions 2,6,2',6' (*ortho* positions), the molecule adopts a planar geometry, similar to the one observed for dioxins and furans. Once chlorine atoms start to be connected in *ortho* positions, steric hindrance drives the molecule to evolve to a non-planar configuration. These substitutional and geometrical parameters are of prime interest since they are closely related to the toxicity of these compounds. Of the 209 PCB congeners, 12 are thought to exhibit 'dioxin-like' toxicity. They are called the non-ortho and mono-ortho PCBs. Other PCB congeners also show evidence of toxicity, although their mechanism of action is different from the dioxin-like congeners.

PCDD, PCDFs and PCBs are complex mixtures, containing isomers and compounds differing by the number of chlorine atoms attached to the biphenyl group. Altogether there are respectively 75 PCDD, 135 PCDF and 209 PCB congeners representing a sum of 419 congeners (Table 1-1). Among these, 29 are considered to exhibit 'dioxin-like toxicity', resulting from the structural configuration of the chlorine atoms.

Table 1-1 : Number of possible PCDD, PCDF and PCB congeners

Number of chlorine atoms	Number of isomers		
	PCDDs	PCDFs	PCBs
1	2	4	3
2	10	16	12
3	14	28	24
4	22	38	42
5	14	28	46
6	10	16	42
7	2	4	24
8	1	1	12
9	-	-	3
10	-	-	1
Total number of congeners	75	135	209

1.2 Physico-Chemical properties

In general, these compounds have very low water solubility, high octanol-water partition coefficients, high lipophilicity, low vapor pressure, high melting point and tend to bioaccumulate (Rordorf, 1985). In soil, sediment, water and probably air, PCDD/Fs are primarily associated with particulate and organic matter (Walters and Guiseppi-Elle, 1988). They exhibit little potential for significant leaching or volatilization once sorbed to particulate matter but there is a possible dechlorination in function of time.

PCDD/Fs are stable up to 800°C and complete destruction can only occur at temperatures above 1300°C (Addink and Olie, 1995; Huang and Buckens, 1996).

1.3 Mechanism and notion of Toxic Equivalency Factors

The toxicity of dioxins, furans and PCBs are directly related to their chemical structure (2,3,7,8 positions) (Safe, 1986). The 29 dioxin-like congeners are characterized by their ability to bind to the aryl hydrocarbon (AhR) hydroxylase receptor. The activation of the cytosolic Ah receptor by a 'dioxin-like' ligand (i.e. TCDD) leads to a cascade of events as shown in Figure 1-2 (Denison et al., 2004). The TCDD-AhR complex translocates into the nucleus. The complex is released from its associated protein submits and it dimerizes with a resident nuclear protein named Arnt (AhR *nuclear translocator*), wherein it gains ability to bind a specific high affinity sequence of the DNA called DRE (*Dioxin Response Element*) sequence to induce the transcription of target genes (such as the cytochrome P4501A1 (CYP1A1)). The strength of binding by the respective agonists (PCDDs, PCDFs and DL-PCBs) to the Ah receptor correlated with their ability to induce DNA transcription is the foundation of the toxicity equivalence factors (TEFs).

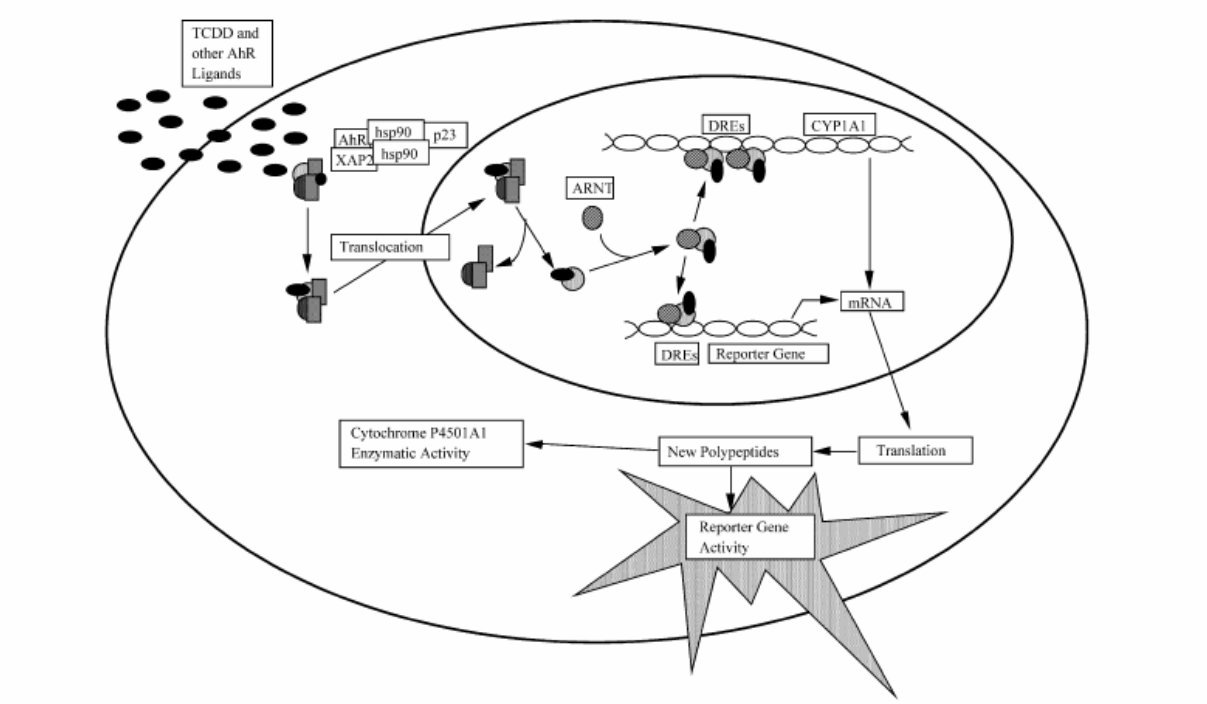

Figure 1-2 : Molecular mechanism of induction of gene expression of TCDD and related AhR agonists in cell bioassays (CALUX) (Denison et al., 2004; Windal et al., 2005)

The complex nature of dioxin, furan and PCB mixtures complicates the risk evaluation for humans. This raises a problem for toxicity assessments where measurements detect various levels of the different PCDD, PCDF and PCB congeners, each of which has a different potential to elicit dioxin-like effects. For this purpose the concept of toxic equivalency factors (TEFs) has been developed and introduced to facilitate risk assessment and regulatory control of exposure to these mixtures (Safe, 1990). The congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is the most toxic congener and is given a TEF of one. Other congeners are given TEFs that are fractions of one (0.00001-0.5), reflecting a pronounced variability in toxicity. TEFs are reassessed frequently as knowledge progresses. Table 1-2 gives consensus TEFs values for humans assigned by the World Health Organization (WHO) in 1998 (Van den Berg et al., 1998). They are re-assessed periodically and the last TEF reassessment was in 2005 (Van den Berg et al., 2005)

Table 1-2 : World Health Organization TEFs for Humans (Van den Berg et al., 1998)

Congeners	WHO TEF	Congeners	WHO TEF
Dioxins		*Non-Ortho PCBs*	
2,3,7,8-TCDD	1	**PCB 77**	0.0001
1,2,3,7,8-PeCDD	1	**PCB 81**	0.0001
1,2,3,4,7,8-HxCDD	0.1	**PCB 126**	0.1
1,2,3,6,7,8-HxCDD	0.1	**PCB 169**	0.01
1,2,3,7,8,9-HxCDD	0.1		
1,2,3,4,6,7,8-HpCDD	0.01		
1,2,3,4,6,7,8,9-OCDD	0.0001		
Furans		*Mono-Ortho PCBs*	
2,3,7,8-TCDF	0.1	**PCB 105**	0.0001
1,2,3,7,8-PeCDF	0.05	**PCB 114**	0.0005
2,3,4,7,8-PeCDF	0.5	**PCB 118**	0.0001
1,2,3,4,7,8-HxCDF	0.1	**PCB 123**	0.0001
1,2,3,6,7,8-HxCDF	0.1	**PCB 156**	0.0005
2,3,4,6,7,8-HxCDF	0.1	**PCB 157**	0.0005
1,2,3,7,8,9-HxCDF	0.1	**PCB 167**	0.00001
1,2,3,4,6,7,8-HpCDF	0.01	**PCB 189**	0.0001
1,2,3,4,7,8,9-HpCDF	0.01		
1,2,3,4,6,7,8,9-OCDF	0.0001		

TEF values for individual congeners in combination with their chemical concentration can be used to calculate the total TCDD toxic equivalent concentration (TEQ) contributed by all dioxin-like congeners in the mixture using the following equation assuming dose additivity:

$$TEQ = \sum_i (PCDD_i xTEF_i) + \sum_i (PCDF_i xTEF_i) + \sum_i (PCB_i xTEF_i)$$

Therefore, toxicologists obtained a unique and useful estimation of the toxicity of a sample via the TEQ value. Moreover, it facilitated risk evaluation, regulatory actions and dietary intake assessment for human exposure to those contaminants. However, this approach did not make the analytical chemist's life simpler. Indeed, when a congener-specific physico-chemical analysis is used and validated, analysts have to rule on analytical performances issues such as accuracy (trueness and precision), uncertainty, fitness for purpose, detection and quantification limits expressed here in TEQ. The unit conversion for the evaluation of those parameters is not straightforward and these issues will be addressed in the following chapters.

1.4 Human exposure

The most important route for human exposure to dioxins is food consumption, contributing to 95% of total exposure (Fürst et al., 1990; USEPA report, 1994). Major sources of dioxins for humans are food from terrestrial animal origin and dairy products but also sea products. The pathways from dioxin emission sources to human consumption are illustrated in
Figure 1-3. Dioxins emitted from combustion and industrial sources, or re-entrained from environmental reservoirs, are transported to distant locations through atmospheric or aquatic pathways. The dioxins are deposited on agricultural crops, taken up in the food supply, and then bioaccumulated and biomagnified through the food chain. This is the predominant pathway for human exposure, excluding isolated exposure episodes resulting from industrial or waste disposal accidents (Bernard et al., 1999).

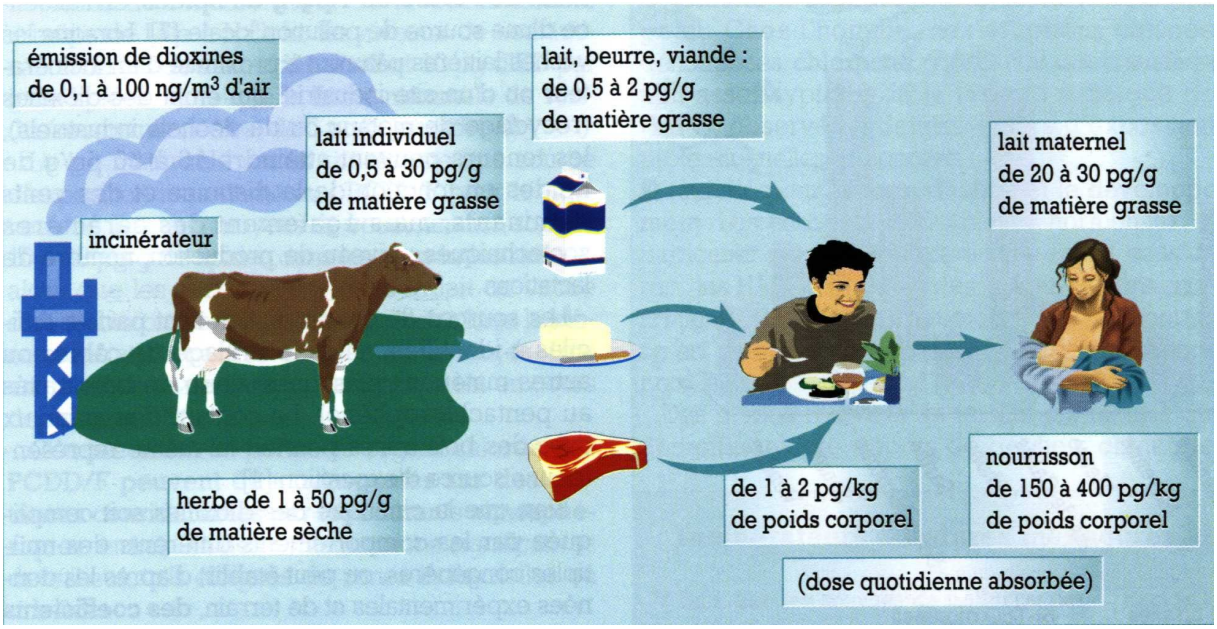

Figure 1-3 : Predominant pathway for human exposure to dioxin.

Food surveys in industrialized countries show a daily intake of PCDD/Fs in the order of 1-3 pg I-TEQ/ Kg body weight for a 60 kg adult (Beck et al., 1992; Theelen et al., 1993; Schecter et al., 1994; Jimenez et al., 1996; Malisch, 1998; Becher et al., 1998). In Belgium, similar values were reported for PCDD/Fs (1 pg WHO-TEQ/kg b.w) (Focant et al., 2002).
If dioxin-like PCBs are also included, the daily total TEQ intake can be higher by a factor 2 to 3 (Liem et al., 2000; Focant et al., 2002). Compared to adults, the daily intake of PCDD/Fs and PCBs for breast fed babies is one or two orders of magnitude higher but nevertheless the benefit of breast feeding has been demonstrated for years and is still greatly recommended.

The latest WHO studies showed higher mean levels of PCDD/Fs and PCBs in breast milk in industrialized countries (10-35 pg WHO-TEQ/g milk fat) compared to lower levels in developing countries (<10 pg WHO-TEQ/g milk fat). But the last rounds of the WHO studies point out a general decrease of the human body burden for the listed POPs. Exposure to other emerging persistent contaminants, such as brominated flame retardants, is increasing but is out of the scope of the present work (Rahman et al., 2001, Solomon and Weiss, 2002).

1.5 European dioxin legislation in food and feed

Extensive European Community legislation has been adopted after the contamination episodes and the following increased awareness of food issues. In particular the fat contamination crisis of the Belgian food supply, also called the 'chicken gate'[1], highlighted the absence of community legislation in this field. This absence greatly increased the difficulty of management of such problems at community level. Although some national legislation was already implemented in few EU countries like Germany, there was really a need for harmonized limits.

The measures consisted on a Community strategy for dioxins, furans and PCBs focused on two distinct aspects:

1 From the environment side, on current and future measures to reduce the release of those contaminants into the environment.

2 From the food and feed safety side, the means to further decrease the presence of dioxin in foodstuffs and feedingstuffs. The aim of this strategy is to bring the majority of the European population below the tolerable weekly intake (TWI) of 14 pg-TEQ/kg b.w. per week, recommended by the Scientific Committee for Food (SCF, 2000).

This incident and many others from a recent past (citrus pulp pellets, kaolinic clay, cholin chloride, dried bakery waste, and more recently waste fat from gelatin production) showed

[1] ***What happened in Belgium in springtime 1999?***
In Belgium, when this incident became public at the end of May 1999, it suddenly turned into an important public health crisis with far going economic (more than 1 billion €) and political consequences. A few weeks later, the attention was drawn to the fact that the dioxin crisis was due to PCB contamination and the dioxin crisis became a PCB accident (Bernard et Al., 1999, Bernard et al., 2002, Saegerman et al., 2002). A mixture of PCBs contaminated with dioxin (mainly furans congeners) were added, by accident or by criminal negligence, to a stock of recycled fat used in the production of animal feeds. Although first symptoms of dioxin poisoning in poultry were noticed in February 1999, the source was only discovered in May 1999 and the contamination spread out through more than 2500 farms in less than three months. Large PCB monitoring programs were launched to solve the problems and a dedicated crisis cell within the Ministry of Agriculture was set up. Afterwards, a bank of data (CONSUM) was created for the monitoring of the food chain as a tool for 'early warning contamination' in order to trigger rapid actions. Then, CONSUM has been completely integrated into the federal agency for food security (AFSCA). The crisis also put forward the controversy related to PCBs, dioxins and furans toxicity. At the end of 1999, two risk assessments studies performed by different experts led to divergent conclusions. One led to the conclusions that there was no reason for concern (Bernard et al., 1999); the other one claimed, with the same data available, a cancer risk of 40 to 8000 cases (van Larebeke et al.). Again, the public was completely disappointed by these contradictories information.

that the contamination of feeds is a major route of livestock exposure to dioxins. And because dioxin-like compounds pass through various trophic levels of human food chains into humans, the idea of controlling the early upstream food chain by setting strict limits in animal feedingstuffs was an excellent proposal. For this purpose, valuable information on the occurrence of PCDD/Fs and D-L PCBs in food collected from EU members food contamination data were used to elaborate a database. Only dioxin and furan data were available at that time in various matrices compared to scarce information for DL-PCBs. Enough data was collected to establish, in a first stage, regulatory levels.

Verstraete (Verstraete, 2002) presented the legislative measures in feedingstuffs and foodstuffs as three pillars:

- ✓ *Strict but feasible maximum levels in various food and feed matrices*
- ✓ *Action levels, acting as a tool for "early warning" of dioxin contaminations above background levels in food and feed*
- ✓ *Target levels to be achieved in food and feed items in order to bring the population below the TWI.*

Maximum and target levels have already been set and have been applicable since July 1, 2002 (Council Directive 2001/102/EC and Council Regulation n°2375/2001) while target levels will be established by the end of 2008. Maximum and action levels are expressed in TEQ resulting in the sum of the only seventeen 2,3,7,8 PCDD/F congeners using the WHO TEF values. However, the approach is pro-active and from a toxicological point of view it was intended to incorporate the 12 DL-PCBs in maximum levels once more comprehensive data on background levels will be available. This has been done by the end of 2006 (Commission Directive 2006/13/EC and Commission Regulation n° 2006/199). In some cases (e.g. fish samples), the levels of DL-PCBs can lead to unrealistic maximum levels since DL-PCBs may contribute to more than 80% of the TEQ. Separate maximum levels for dioxins/furans on one side and DL-PCBs on the other side can then be considered during a transitional period. In order to ensure a smooth switchover, the existing levels for the sum of the 17 PCDD/Fs continue to be applied, in addition to the newly set maximum levels for the sum of the 29 PCDD/Fs and DL-PCBs. The separate maximum levels remain applicable until the end of 2008. This point of view is indeed tenable as commonly sources of contamination are different.

The action levels were established by reducing the maximum levels by at least 30% with the aim of triggering actions on identification of sources and pathways of dioxin contamination.

Measures to reduce or to prevent such contamination should then be enforced. This entails permanent monitoring programs to detect the presence of those contaminants in food and feed inside the European market. In order to facilitate free trade of goods, harmonization of acceptance criteria for dioxin analysis is needed (Malisch et al., 2001). Measures have been taken and analytical requirements for dioxin analysis in food and feed have been adopted (Directives 2002/69/EC and 2002/70/EC) and; recently, been revised in the Commission Regulation 1883/2006 by the end of 2006. It is a continuous revision process as knowledge progress.

The main consequences that follow from these legislative measures led to the set-up of large monitoring programs of the food chain. To cope with the great number of samples statistically required for monitoring, the strategy involves the use of cost-effective screening methods (capillary gas chromatography in combination with low resolution mass spectrometry, i.e. GC/LRMS, or AhR based bioassays for TEQ determinations), characterized by a high sample throughput, and the gold standard gas GC/HRMS reference method, used to bear out their presence. Food and feed samples generally show background levels and hence the monitoring strategy promote the use of screening methods. They are used to sieve large numbers of samples for potential non-compliant results but in addition they should be characterized by false negative rates of less than 1%. The positive or non-complaint samples need to be confirmed afterwards by the reference GC/HRMS method.