UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

STUDY OF OCDD, 1,2,3,4,6,7,8–HPCDD, 1,2,3,6,7,8-HXCDD AND 1,2,3,7,8-PECDD CONGENERS OF DIOXIN ABSORPTION IN POULTRY

Miguel José Sardinha Oliveira Cardo

Orientador: Doutor Fernando Manuel d'Almeida Bernardo

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Segurança Alimentar

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2017

Aos meus pais.

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Aos meus filhos e amigos, por tudo!

Título da Tese:

ESTUDO DE ABSORÇÃO DE CONGÉNERES DE DIOXINAS OCDD,1,2,3,4,6,7,8–HpCDD, 1,2,3,6,7,8-HxCDD e 1,2,3,7,8-PeCDD EM AVES DE CAPOEIRA

Resumo

Os trabalhos experimentais que integram esta tese tiveram como objetivo principal, a integração de quatro linhas de investigação tendo em vista a obtenção de conhecimentos capazes de contribuir para uma caracterização fundamentada do risco e a subsequente gestão de risco nas crises alimentares resultantes da contaminação da cadeia avícola com dioxinas.

Os dados utilizados no desenvolvimento deste trabalho foram recolhidos na sequência de três incidentes de contaminação da cadeia avícola com dioxinas, ocorridos em 2006, 2011e 2016 em Portugal e serviram de base para a realização de uma análise forense para encontrar a fonte de contaminação. Estes trabalhos permitiram identificar claramente, em cenário real, a fonte da contaminação das aves nos incidentes ocorridos em Portugal em 2006, 2011 e 2016, tendo sido possível estabelecer uma clara relação das aparas de madeira como sendo o veículo ambiental das dioxinas. Esta conclusão é baseada na consistência dos resultados obtidos em todos os materiais testados que foram considerados potenciais fontes de contaminação, bem como, pela análise dos perfis da concentração relativa, “impressão digital”, dos 17 congéneres de dioxinas. Pode afirmar-se que as fontes incomuns de contaminação da cadeia alimentar por dioxinas devem ser sempre colocadas em perspetiva quando o incidente não é afiliado nas fontes mais comuns, como a dieta (rações e água).

Nesse contexto, procedeu-se ao desenvolvimento de uma metodologia analítica especialmente adaptada à deteção e quantificação de congéneres de dioxinas em matrizes lenhosas (madeira); ao estudo das contaminações com estes compostos tóxicos no setor do tratamento industrial das madeiras e à avaliação da forma como as referidas contaminações são mantidas e transferidas ao longo da cadeia avícola, quer em termos quantitativos, quer quanto à respectiva especiação química e ainda à forma como é feita a depleção destes contaminantes do organismo em aves de capoeira.

O estudo e caraterização dos contaminantes, nomeadamente, o estudo da influência dos diferentes produtos de tratamento de conservação das madeiras, mais comercializados em Portugal, na contaminação de aparas de madeira de pinho, permitiu reproduzir e estabelecer, em laboratório, uma correlação clara entre os produtos comerciais utilizados no tratamento da madeira e a contaminação das aparas de madeira que foram utilizadas nas camas das aves. O perfil da contaminação das aparas de madeira de pinho tratadas em laboratório encaixa perfeitamente no perfil das camas das aves implicadas nos incidentes de contaminação de aves ocorridos em Portugal.

Surpreendentemente, os tratamentos de superfície das madeiras e os tratamentos de profundidade apresentaram perfis de contaminação muito semelhantes, o que permite considerar que, de forma geral, as camas de aves contaminadas com aparas de madeira tratada apresentarão um perfil semelhante, uma vez que as taxas de retenção dos conservantes na madeira não têm influência no perfil encontrado.

O desenvolvimento da metodologia analítica para pesquisa das dioxinas na madeira utilizada, adaptada do método 1613B da EPA, revelou taxas de recuperação de padrão marcado que variaram entre 71,3% e 86,3%. Estas taxas são consideradas aceitáveis pelo método de referência e satisfazem os critérios estabelecidos na legislação da União Europeia, tanto para géneros alimentícios como para alimentos para animais.

O estudo de avaliação do comportamento da "impressão digital" da contaminação ao longo da cadeia avícola permitiu concluir que, nas amostras de gordura e músculo de galinhas reprodutoras e frangos de engorda, a concentração média relativa do congénere mais clorado OCDD foi, respetivamente, 52% e 53% inferior, comparativamente ao perfil das amostras de aparas de madeira e de ovos de incubação. Por outro lado, a concentração média relativa de 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HxCDD e 1,2,3,4,6,7,8-HpCDD nos perfis das amostras de gordura e músculo de frango e de galinhas reprodutoras foi de 50% a 97% superior quando comparada com os perfis das camas e dos ovos de incubação. Estas conclusões sugerem a existência de uma transformação *in vivo* que promove a descloração dos compostos mais clorados, permitindo aos gestores de risco associar, aos diferentes perfis apresentados pelas aves e pelos ovos, a mesma fonte de contaminação.

Verificou-se um equilíbrio entre a concentração de dioxinas no contaminante ingerido por galinhas (38,85 pg TEQ-OMS/g) e a concentração de dioxina nos ovos (42,25 pg TEQ-OMS/g). O facto de ovos terem concentrações relativas de alguns congéneres mais elevadas do que a gordura das respectivas progenitoras pode decorrer de efeitos associados à fisiologia da postura que conduz a um enorme afluxo de sangue ao ovário e ao oviduto e consequente concentração súbita de nutrientes e dos respectivos contaminantes.

Além deste equilíbrio no teor de contaminação, também existe uma grande semelhança dos perfis da contaminação encontrada pela comparação das concentrações relativas dos diferentes congéneres entre as camas e ovos para incubação (R2 = 0,72 e sd = 0,16) e pela avaliação das representações gráficas dos perfis. Esta semelhança pode ser explicada pelo aumento sérico de uma lipoproteína de muito baixa densidade (VLDLy), de pequeno diâmetro e resistente à hidrólise pelos tecidos extra ováricos, cuja produção é induzida pelos estrogénios produzidos durante a postura de ovos. As partículas VLDLy estão em altas concentrações plasmáticas em galinhas poedeiras que reservam as VLDLy ricas em triglicerol, ligado às dioxinas e furanos, para serem utilizadas nos folículos ováricos em desenvolvimento.

O estudo do incidente ocorrido em Portugal no ano de 2016, numa exploração de patos, revelou a existência de uma redução da contaminação uma vez retirada a fonte de exposição, com um nível de depleção diário médio que variou entre 1,43% e 4,35% pg, evidenciando uma clara redução da contaminação, após retirada a fonte de contaminação. Concluiu-se, no entanto, que os pavilhões onde o período de depleção avaliado foi mais curto, apresentaram percentagens de depleção média diária mais elevadas, evidenciando que a percentagem de depleção vai diminuindo ao longo do tempo, após a retirada do contaminante (ausência de exposição).

Os dados analisados relativamente a uma das crises que envolveu a contaminação de uma grande exploração de galinhas reprodutoras, configuram uma possibilidade de estudo dificilmente repetível em cenário real, da passagem da contaminação “carry-over” do contaminante presente nas respetivas camas constituídas por aparas de madeira, para as galinhas implicadas, para os ovos de incubação e para os pintos nascidos desses ovos. Estas “crises de dioxinas” envolveram, ao longo destes anos, várias dezenas de explorações e milhares de aves de produção, resultando na retirada do mercado e destruição de várias

toneladas de carne de aves, bem como de ovos de incubação. Estes resultados representam um valioso contributo para a avaliação e a gestão de risco.

Palavras-chave: Dioxinas, Impressão digital, Carnes de aves, Depleção, Tratamento de madeiras

Title of the Thesis:

STUDY OF OCDD, 1,2,3,4,6,7,8–HPCDD, 1,2,3,6,7,8-HXCDD AND 1,2,3,7,8-PECDD CONGENERS OF DIOXIN ABSORPTION IN POULTRY

Abstract:

This thesis concerns a study which has integrated four main lines of investigation converging to a main goal – to contribute to the risk management of food crisis resulting from the contamination of the poultry chain with dioxins. Specifically, it includes: the development of a suitable analytical methodology to detect and quantify dioxins congeners in wood matrix; a study of the contaminant of biocides used for industrial wood treatments, the analyses of the pathway on how the contaminations are maintained and transferred throughout the poultry production chain in terms of level of contamination and respective chemical speciation and also depletion of the dioxins from poultry during production.

The study of the dioxin contamination of different industrial wood preservatives, allowed to establish a clear correlation with the contamination profile of poultry and the previous contamination profile incidents found in wood shavings used as poultry litter.

The study of the analytical methodology for wood shavings, adapted from the 1613B EPA method, showed recovery rates of labelled compounds ranging between 71.3% and 86.3%. These rates are considered acceptable, considering the reference method and also the criteria stated in the European Union legislation, for both food and feed.

The study of contamination fingerprint along the poultry chain, allows to establish the relationship with the wood shavings, identified as the source of the dioxins. In muscle samples of poultry, the average concentration of OCDD was about 50% lower when compared to the profile of the wood shaving samples. On the other hand, the average relative concentration of 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD in poultry muscle samples was 50% higher when compared to the litters. These findings suggest the existence of a transformation *in vivo* of the most chlorinated congeners. The study of depletion of dioxins in a holding of ducks presented an average daily depletion rate ranging between 1.43% and 4.35%, showing a clear reduction of contamination after the removal of the source of contamination. The depletion rates are factors that must be taken in consideration to assess risk and, indirectly, helpful to manage risk of dioxins in food.

Keywords: Dioxins, Fingerprint, Poultry meat, Depletion, Wood preservatives

List of Publications

- Cardo, M., Bernardo, F. (2016). Study of depletion and contamination profile of Dioxins in duck intensive production. *Journal of Environmental Protection*, **7**, 2056-2066. http://dx.doi.org/10.4236/jep.2016.713160
- Cardo, M., Nunes, L., Duarte, M., Silva, A., Bernardo, F. (2016). PCDD/F Dioxin Profile of Treated Pinus pinaster Wood. *Journal of Environmental Protection*, **7**, 1971-1979. http://dx.doi.org/10.4236/jep.2016.712153
- Cardo, M., Martins, A., Raminhos, C., Campos, M., Bernardo, F. (2016). Determination of PCDD/F levels in wood shavings used as bedding material for poultry production. *Journal of Environmental Protection*, **7**, 2047-2055. http://dx.doi.org/10.4236/jep.2016.713159
- Cardo, M.O., Castel-Branco, M., Andreozzi, V. and Bernardo, F.A. (2014) Dioxins in the Food Chain: Contamination Fingerprint Analysis in Breeding Hens, Hatching Eggs and Broilers. *Journal of Environmental Protection*, **5**, 1323-1330. http://dx.doi.org/10.4236/jep.2014.513126
- Cardo, M.; Félix, V. Bernardo, F. (2009). Dioxins in poultry products: a case study. *J. Vet. Pharmacol. Therap.*, **32** (Suppl. 1). 95

Oral communications and posters in congresses and scientific meetings

- Oral Communication **-** 11º International Congress of the European Association for Veterinary Pharmacology and Toxicology. Leipzig. Germany (2009), with the work “Dioxins in poultry products: a case study”;
- Poster on “Risk management of Dioxins in poultry products” presented on the “8º Encontro de Química dos Alimentos”, held in Beja, Portugal, from 4 to 7 of March, 2007;

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Abbreviations

ACQ - Alkaline Quaternary Copper

AhR – Aryl hydrocarbon Receptor

ARNT- Aryl hydrocarbon nucleon-transferase

ATSDR – Agency for Toxic Substances and Disease Registry (USA)

CCA - Chromium Copper Arsenate

CCB - Chromated Copper Borate

CCMS - Committee on the Challenges of Modern Society (NATO)

CDC – Centre for Disease Control and Prevention (USA)

CEC - Commission of the European Communities (EU)

CIPR - International Commission for the Protection of the Rhine

ECEH - European Centre for Environment and Health (UNECE/WHO)

EFSA – European Food Safety Agency

EPA – Environment Protection Agency (USA)

EPCEU - European Parliament and the Council of the European Union

EU – European Union

FAO – Food and Agriculture Organization of the United Nations

FPL - Forest Products Laboratory (Forest Service, Department of Agriculture, USA)

HpCDD – Heptachlorodibenzo-p-dioxin

HxCDD - Hexachlorodibenzo-p-dioxin

JECFA – Joint Expert Committee on Food Additives (WHO)

NATO - North Atlantic Treaty Organization

NCEA – National Centre for Environment Assessment (USEPA)

OCDD – Octachlorodibenzo-p-dioxin

OSPAR - Convention for the Protection of the Marine Environment of the North-East Atlantic

PCB – Polychlorinated biphenyl

PCDD – Polychlorinateddibenzo-p-dioxins

PCDF – Polychlorinated dibenzofurans

PCN – Polychloro-naphtalenes

PCP - Pentachlorophenol

PeCDD – Pentachlorodibenzo-p-dioxin

POP – Persistent Organic Pollutants

PVC – Polyvinyl chloride

SCF – Scientific Committee for Food (EU)

sd – Standard deviation

TCDD – Tetrachlorodibenzo-p-dioxin

TDI –Tolerable Daily Intake

TEF – Toxic Equivalency Factor

TEQ – Toxic Equivalents

TWI – Tolerable Weekly Intake

UNECE - United Nations Economic Commission for Europe

UNEP - United Nations Environment Programme

VLDL - Very low-density lipoprotein

VLDLy - Very low-density lipoprotein yolk targeted

WHO – World Health Organization

1. Introduction

In 1990, WHO published data in which it was estimated that more than 90% of human exposure to dioxins is through food. Food of animal origin normally contributes to about 80% of the total population exposure (WHO, 1998).

The evolution of knowledge has led to the definition of strategies and the implementation of measures aimed at reducing environmental contamination by dioxins in order to limit the contamination of feed and food. Legislation has also been developed to establish critical contamination limits for foodstuffs. However, while significant steps have been taken towards reducing exposure, several incidents of contamination of the food and feed chain with dioxins continue to occur.

In Portugal, following the implementation of residue monitoring plans, three incidents of contamination of poultry meat, in 2006, 2011 and 2016, occurred. By the assessment of the contamination profiles in 2006, a link was established with the profile of wood contaminated by chemical substances used in the treatment of wood, which were improperly sent to poultry farms to be used as bedding material for the birds (Cardo *et al.*, 2009).

There are several studies that classify and characterize the profile of the most studied sources of contamination.

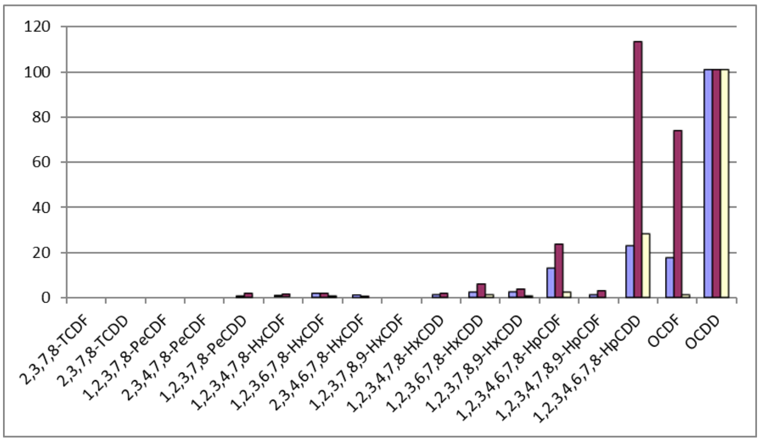

Figure 1 - Contamination profile of PCDDs e PCDFs in treated wood with Pentachlorophenol (Fries, *et al.*, 1996)

During the nineties, a team of researchers established the profile of contamination of dioxins in wood treated with pentachlorophenol (Figure 1) in wood fence posts and walls of wood shelters (Fries *et al*., 1996).

In Portugal in 2011, an association was established between the contamination profile of litters from a breeding hen's holding and the patterns of contamination of poultry meat by dioxins. These litters were made of wood shavings from a given sawmill and had a profile of the contamination that resembled the profile found in wood treated with pentachlorophenol.

1.1 Thesis outline

This thesis is organized in nine chapters. Chapter 2 presents a literature review regarding the subjects covered by this study. Chapters 3, 4, 5 and 6 present the experimental section following four lines of research, which are presented in four scientific articles, published in international peer reviewed scientific journals:

- Study of the adaptation of the analytical methodology for wood, taking into account the current constraints regarding the extraction efficiency. Cardo, M., Martins, A., Raminhos, C., Campos, M., Bernardo, F. (2016). Determination of PCDD/F levels in wood shavings used as bedding material for poultry production. *Journal of Environmental Protection*, **7**, 2047-2055. http://dx.doi.org/10.4236/jep.2016.713159)
- Study and characterization of the contaminant, namely, the influence of the different products of treatment/ preservation of the most marketed wood in Portugal. Cardo, M., Nunes, L., Duarte, M., Silva, A., Bernardo, F. (2016). PCDD/F Dioxin Profile of Treated *Pinus pinaster* Wood. *Journal of Environmental Protection*, **7**, 1971-1979. http://dx.doi.org/10.4236/jep.2016.712153);
- Evaluation study of how the fingerprint of contamination behaves along the poultry production chain. Cardo, M.O., Castel-Branco, M., Andreozzi, V., Bernardo, F.A. (2014). Dioxins in the Food Chain: Contamination Fingerprint Analysis in Breeding Hens, Hatching Eggs and Broilers. *Journal of Environmental Protection*, **5**, 1323-1330. http://dx.doi.org/10.4236/jep.2014.513126);

- Study of the dioxin depletion from poultry organism after removal of the source of contamination. Cardo, M., Bernardo, F. (2016). Study of depletion and contamination profile of dioxins in duck intensive production. *Journal of Environmental Protection*, **7**, 2056-2066. http://dx.doi.org/10.4236/jep.2016.713160).

The result of this research is aiming to contribute to better understand some components of risk assessment (exposure and characterization) and, on this basis, to provide solid elements for a risk management in a food crisis scenario resulting from the contamination of the poultry production chain with dioxins.

1.1.1 Study for the Adaptation of the EPA method 1613b to wood chips

During 2006 and 2011, analytical testing results of poultry meat with high levels of contamination were obtained, with an average of 61.76 pg PCDD/F-TEQ-WHO/g fat in a universe of 10 samples in 2006, and 78.04 of 13 samples of poultry meat in 2011. In these two episodes, the results obtained revealed considerable contaminations of the wood chips used as bedding material in poultry production, indicating that these materials were the most probable source of contamination of the animals.

These results suggested that the wood chips used in poultry litters were obtained from contaminated wood, being the wood preservative treatment the possible source of the dioxins. Treated wood by-products are supposed to be disposed as hazardous residues and recovery is not allowed. In these cases, the treated wood could possibly be improperly disposed and channelled to poultry production as a normal wood by-product.

The lack of a well-established laboratory methodology applied to this specific matrix (wood chips) poses additional challenges to analytical procedures, namely: the uniformity of distribution of the contamination in the matrix, its stability and, above all, the extraction efficiency of the lipophilic compounds that present trace amounts in wood. The study of all these aspects and their influence on the performance of the analytical processes were further investigated in this study.

1.1.2 Study of the contamination profile of wood preservation treatments

A preliminary experimental study was carried out to evaluate whether the contamination profile found in the litters of breeding hens, that caused the contamination of the food chain in 2011, are reproducible, regardless the commercial product used in the pine wood treatment.

The factors to be studied were: Pine wood, the most used in poultry litters in Portugal; The treatments to be applied, being tested four treatments with different preservatives of wood, two treatments of wood anti-sapstain (surface) and two treatments with negative pressure (depth).

The variable response to be studied was the standardized gross concentration of each of the 17 dioxin and furan congeners of each sample (without application of the Toxicity Equivalent Factor values - TEF).

The objective is to characterize the contamination profile of each preservation treatment, in order to enable the identification of the source in a real scenario contamination. However, where a sawmill uses wood, treated in other units using different preservatives, the resulting by-product mixture used as poultry litters might have standard concentrations of each congener of dioxins and furans, provided by each treatment of wood. This is one of the major problems of studies that seek to find the source of an environmental contamination which is, in general, multifactorial.

In the current work, it was essential to ensure that the wood presented no contamination with dioxins and furans prior to the treatments of which they were submitted. However, there are studies that describe the possibility of plants absorbing dioxins produced during forest fires, which may be a problem for the species of wood under examination. In order to reduce any variability in the sampling, this study was developed after the testing results of the batch of wood samples were known. This step could exclude any contaminated wood prior to the experimental treatments.

From the experience gained it was also possible to conclude that, once the wood has been contaminated, its chips have a very similar contamination profile. In three samples of wood shavings provided by the same sawmill, from three different lots, collected from a holding of breeding hens, the results presented a considerable similarity (Figure 2).

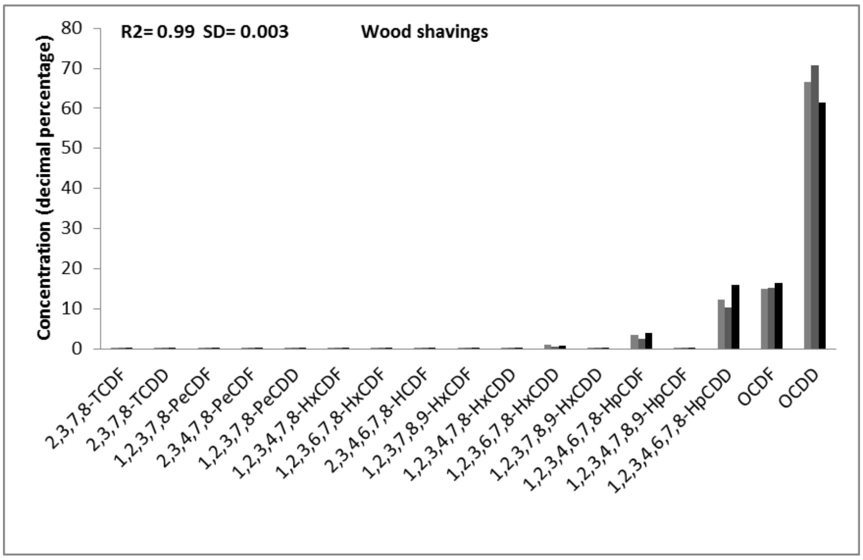

Figure 2 - The results of analyses carried out on poultry litters made of wood chips (Cardo *et al*., 2014).

1.1.3 Study of the contamination profile along the poultry production chain

In 2011, sampling in the origin of the case leading to contingency measures in the crisis management, was carried out in a slaughterhouse, in breeding hens coming from a holding using litters of wood chips provided by a sawmill. Samples were collected from all possible sources of contamination, including feed, water and wood chips from bedding material.

In the same episode, new muscle samples from breeding hens and samples of hatching eggs produced by positive hens, were collected. Broiler farms that received day-hold chicks from the eggs laid by the contaminated breeding hens, were also identified and muscle samples were collected from these broilers.

The contamination profile of these samples was studied and compared to evaluate the behaviour of contamination profiles throughout the poultry production chain, including bedding material, breeding hens, hatching eggs and broilers.

1.1.4 Study of contamination depletion in poultry

The study concerning the depletion of the contamination following the removal of the source (contaminated wood shavings from bedding material), considered the information collected from the incident occurred in Portugal in 2016.

The sampling of meat from a duck farm that revealed the contamination was also performed in a slaughterhouse, as in the previous case (1.1.3). In the investigations carried out, wood shavings were again identified as the source of contamination. In this farm, to study the evolution of the contamination in terms of its levels and profile, the contaminated beds were removed and representative samples of meat were collected in six pens, with a lapse of time of about one month.

I – Part (Literature review)

2. Dioxins and food safety

Dioxins are toxic chemicals that persist in the environment and accumulate in the food chain. Their presence in the environment has declined since the 1970s, following concerted efforts by public authorities and industry (EFSA, 2010).

Dioxins have no technological or other use, but are generated in a number of thermal and industrial processes as unwanted and often unavoidable by-products. In contrast to dioxins, PCBs had widespread use in numerous industrial applications, and were produced in large quantities for several decades with an estimated total world production of 1.2-1.5 million tonnes, until they were banned in most countries by the 1980s (EFSA, 2010).

Dioxins and PCBs are found at low levels in many foods. Long-term exposure to these substances has been shown to cause a range of adverse effects on the nervous, immune and endocrine systems, and impair reproductive function. They may also cause cancer. Their persistence and the fact that they accumulate in the food chain, notably in animal fat, therefore continues to cause some safety concerns (EFSA, 2010).

Dioxins and some PCBs referred to as dioxin-like PCBs (due to their similar toxicological properties) are often considered together within the context of public health. Other PCBs referred to as ‘non dioxin-like PCBs’ have a different mechanism of toxicity but can also cause adverse effects on health.

The word "dioxins" covers a group of polychlorinated organic compounds being 75 dibenzo-p-dioxins (PCDDs) and 135 dibenzofurans (PCDFs), of which 17 are of toxicological concern (CEC, 2001). The most toxic compound is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), classified as a carcinogen in humans by the International Agency for Research on Cancer and other prestigious international organizations (Fiedler *et al.*, 2000).

Dioxins and dioxin-like PCBs are poorly soluble in water. However, they are adsorbed into mineral and organic particles suspended in water (WHO/ FAO, 2012).

The European Union's Scientific Committee for Food (SCF), in agreement with the World Health Organization (WHO), has concluded that dioxins have no carcinogenic effects when exposure is below a certain threshold. Other harmful effects, such as endometriosis, neurotoxic and immunosuppressive effects occur at lower levels and are therefore considered relevant for the determination of tolerable intake (CEC, 2001).

In addition, other polychlorinated biphenyls (PCBs) are chlorinated aromatic hydrocarbons, synthesized by the direct chlorination of biphenyls and theoretically identified as a group of 209 different possible congeners, which can be divided into two groups, according to their toxicological properties. Of these compounds, 12 have dioxin-like toxic properties and are therefore referred to as "dioxin-like PCBs". The remaining PCBs do not exhibit dioxin-like toxicity, having a different toxicological profile. According to the opinion of the Scientific Committee on Food of the European Union, each compound of the dioxin family or dioxin-like PCBs has a different level of toxicity (EC, 2000).

To enable the quantification of the total toxicity of these different related compounds, the concept of Toxicity Equivalence Factors (TEF) was introduced in order to facilitate the risk assessment as well as regulatory control. This means that the analytical result for the 17 dioxin-like compounds and 12 dioxin-like PCBs is expressed in terms of a single quantifiable unit: The Toxic Equivalent Concentration (TEQ) (EC, 2000).

Dioxins and PCBs are extremely resistant to chemical and biological degradation and thus persisting in the environment by its accumulation in the human and animal food chains (CEC, 2001).

More than 90% of the human exposure to dioxins originates from food. Food of animal origin normally accounts for about 80% of such total exposure (EC, 2013). The exposure of animals to dioxins comes essentially from feedingstuffs. Therefore, the contamination of food for human consumption is generally related with the contamination of feedingstuffs. An integrated approach should therefore be followed to reduce the frequency or occurrence of dioxins throughout the food chain, *i.e.*, from raw materials intended to animal feed, through animals for food production, to humans (CEC, 2006). Therefore, feed and in some cases other factors in the production environment, such as soil or litters and air, may be the potential sources of dioxins.

At international level, the European Commission actively participates in a number of relevant international activities, of which the following are particularly worthwhile mentioning (CEC, 2001):

- the 1990 declaration adopted by the North Sea Conference[1] undertaking, *inter alia*, 70 % reductions of chlorinated dioxins;
- the revised Protocol of the Barcelona Convention[2] for the protection of the waters of the Mediterranean from land-based sources, where dioxins are included in the list of substances to be controlled;
- the Joint UNECE/WHO-ECEH Task Force on health aspects of long-range transboundary air pollution organised meetings in order to initiate the preparation of the assessment on health risks of POPs from Long-Range Transboundary Air Pollution[3].

The European Commission is also a contracting party to several conventions with regard to dioxins and PCBs (CEC, 2001):

- The Basel Convention[4] is designed to control the transboundary movements of hazardous waste and their disposal. PCBs and dioxins are classified as hazardous wastes.
- The OSPAR Convention[1] for the protection of the marine environment of the north-east Atlantic agreed in 1998 on the objective to cease emission, discharges and losses of hazardous substances by 2020 in order to achieve, close to zero concentrations of compounds such as dioxins/PCBs in the marine environment.

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[1] The Convention for the Protection of the Marine Environment of the North-East Atlantic or OSPAR convention is the current legislative instrument regulating international cooperation on environmental protection in the North-East Atlantic. The Convention was concluded at Paris on 22 September 1992.

[2] The Convention for Protection of the Mediterranean Sea against Pollution (Barcelona Convention), is a regional convention to prevent pollution from ships, aircraft and land based sources in the Mediterranean Sea. This includes but is not limited to dumping, run-off and discharges. Signers agreed to cooperate and assist in dealing with pollution emergencies, monitoring and scientific research. The convention was adopted on 16 February 1976 and last amended on 10 June 1995.
The Barcelona Convention form part of the United Nations Environment Programme (UNEP).

[3] The Convention on Long-Range Transboundary Air Pollution, often abbreviated as Air Pollution, or OSPAR convention is intended to protect the human environment against air pollution and to gradually reduce and prevent air pollution, including long-range transboundary air pollution. It is implemented by the European Monitoring and Evaluation Programme (EMEP), directed by the United Nations Economic Commission for Europe (UNECE). It was signed in 1979 and entered into force on 1983.

[4] The Basel Convention on the Control of Transboundary Movements of Hazardous Wastes and Their Disposal, is an international treaty that was designed to reduce the movements of hazardous waste between nations, and specifically to prevent transfer of hazardous waste from developed to less developed countries (LDCs). The Convention was opened for signature on 1989, and entered into force on 1992.

- The Convention on the protection of the marine environment of the Baltic Sea Area[5]: the contracting parties declare to prohibit, totally or partially, the use of PCBs in the Baltic Sea and its catchment areas.
- The UNECE POPs Protocol to the Convention on long-range transboundary air pollution[3], signed by the EU in Aarhus in June 1998, aims to control and reduce the emissions of a number of POPs which require the most urgent action, such as dioxins and PCBs.
- The Stockholm Convention (POPs Convention), signed by the EU in May 2001 in Stockholm, aims to reduce the total release of dioxins, furans and PCBs, with the goal of their continuing minimisation and, where feasible, ultimate elimination. In 1995, the Governing Council of the United Nations Environment Programme (UNEP) called for global action to be taken on POPs, to reduce and/or eliminate emissions and discharges of persistent organic pollutants, defined as "chemical substances that persist in the environment for long periods, become widely distributed geographically, bio-accumulate through the food chain, are toxic to humans and animals and pose a risk of causing adverse effects to human health and the environment" (SSC, 2010).

  UNEP promote an Intergovernmental Negotiating Committee to prepare the Stockholm Convention which is a global treaty to protect human health and the environment from persistent organic pollutants (POPs). In implementing the Convention, Governments will take measures to eliminate or reduce the release of POPs into the environment (SSC, 2010). Over 150 countries signed the Convention in 2001 and it entered into force, on 17 May 2004. In March 2016, there were 180 parties to the Convention, (179 states and the European Union). The Stockholm Convention was adopted to EU legislation in Regulation (EC) N. º 850/2004 (EPCEU, 2004). The Stockholm Convention focuses on eliminating or reducing releases of 12 POPs, the so-called "Dirty Dozen". It sets up a system for tackling additional chemicals identified as unacceptably hazardous. It recognizes that a special effort may sometimes be needed to phase out certain chemicals for certain uses and seeks to ensure that this effort is made. It also channels resources into cleaning up the existing stockpiles and dumps of POPs that

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[5] Convention on the Protection of the Marine Environment of the Baltic Sea Area, (Helsinki Convention) is an international convention encompassing various measures for the prevention and elimination of pollution of the Baltic Sea. It was signed in 1992 and entered into force on 2000.

litter the world's landscape. Ultimately, the Convention points the way to a future free of dangerous POPs and promises to reshape our economy's reliance on toxic chemicals.

The Stockholm Convention focus on five essential aims:

- Eliminate dangerous POPs, starting with the 12 worst
- Support the transition to safer alternatives
- Target additional POPs for action
- Clean-up old stockpiles and equipment containing POPs
- Work together for a POPs-free future

At EU level, the SCF adopted, on 30 May 2001, an opinion on the assessment of the risks of dioxins and dioxin-like PCBs in food (EC, 2001). This was based on an update of the new scientific information made available after the adoption of the SCF opinion on this matter in November 2000 (EC, 2000). A Tolerable Weekly Intake (TWI) for dioxins and dioxin-like PCBs of 14 pg TEQ-WHO/kg body weight was established by the Committee. Exposure estimations indicated that a considerable proportion of the European Community population is exposed through the contaminated diet to values above TWI, and certain population groups in several countries may be at increased risk due to specific dietary habits (EC, 2001).

Measures have been implemented to further reduce environmental contamination caused by the presence and release of dioxins, in order to limit the impact of environmental pollution on feed and food contamination. In October 2001, the European Commission presented a Communication to the Council, the European Parliament and the Economic and Social Committee on a Community strategy on dioxins, furans and polychlorinated biphenyls. The strategy was focused on current and future measures to reduce the release of polychlorinated dibenzodioxins (PCDDs), commonly known as dioxins, polychlorinated dibenzofurans (PCDFs), more commonly referred to as furans, and polychlorinated biphenyls (PCBs) in the environment, thus avoiding harmful effects on the environment and health (CEC, 2001). This strategy consists of two parts, one part containing actions for reducing the presence of dioxins, furans and PCBs in the environment and the other part containing actions for reducing their presence in feed and food and was based on the following facts:

- Bioaccumulation along the trophic chain also occurs from releases from landfills, polluted soils or sediments. The abrupt drop in the "reference levels" in the environment over the last 20 years will probably not be repeated in the coming decades because, despite the efforts made to reduce emissions, there are deposits that will continue to be a continuous source of release;

- Toxic properties appear to have been underestimated, with new epidemiological and toxicological data emerging, especially with regard to endocrine effects, reproduction and neurological development. These data indicated that dioxins and some PCBs have a health impact which is higher than initially assumed, even if at very small doses, particularly in the most vulnerable groups, such as foetuses and infants, directly exposed to maternal levels accumulated;
- Dietary exposure to dioxins and dioxin-like PCBs, of a significant proportion of the European population, exceeds the estimated TWI or TDI (EFSA, 2004):
- The European Union has assumed new obligations by becoming a contracting party to several world conventions on dioxins and PCBs;
- The enlargement of the EU in 2004 probably increased the average exposure in the EU. In fact, the new Member States are likely to produce higher emissions (than the EU past average) due to the diversity of their previous legislation and the large number of obsolete industrial facilities. These countries, formerly referred to as "Eastern Europe", certainly contribute in large part to the total dioxins released into the environment in Europe, which highlights and increases the need to ensure full compliance with the relevant environmental *acquis* in the countries that have recently joined the EU.

The objectives of the European strategy are (CEC, 2001):

- To assess the current state of the environment and ecosystems;
- To reduce human exposure to dioxins and PCBs in the short term and maintain them at safe levels in the medium and long term;
- To reduce the environmental emissions of dioxins and PCBs. The quantitative objective is to reduce levels of human intake to less than 14 pg WHO-TEQ/kg body weight /week.

As food contamination is directly related to feed contamination, an integrated approach is followed to reduce dioxin/PCB incidence all along the food chain, *i.e.*

from feed materials through food-producing animals to humans. Taking measures with regard to feed is therefore a decisive step to reduce human intake. Measures in food and feed solely based on establishing maximum levels would not be sufficiently effective in reducing the level of feed and food contamination, unless the levels were set so low that a large part of the feed and food supply would be declared unfit for animal or human consumption. Besides the important measures to limit the release of dioxins and PCBs into the environment, other measures aiming the reduction of dioxins and dioxin-like PCBs in feed and food, were envisaged to come into application in the course of the year 2002 (CEC 2001).

Under this strategy the legislative measures concerning food and feed would entail the establishment of strict, but feasible Maximum Limits (ML), threshold levels of action for dioxins and PCB’s. These ML would function as an "early warning" mechanism of dioxin and PCB contamination above desirable levels to trigger proactive action by the competent authorities and food operators and target levels over time, so that the exposure of the EU population falls within the acceptable limits recommended by the scientific committees (CEC, 2001).

The strategy also envisaged a set of actions related to the reduction of the presence of dioxins and PCB’s in the environment that had to be identified for the short to medium-term and for the long-term. The short to medium-term actions (5 years) had foreseen measures related to hazard identification, risk assessment, risk management, research, communication to the public and cooperation with third countries and international organisations. The identified long-term actions (10 years) included data collection on the level of dioxin/ PCB contamination in air, water (sediment) and soil in order to be considered and integrated in the global strategy and monitoring and surveillance of the level of dioxin/PCB contamination in air, water (sediment) and soil to enable the identification of measures targeting the sources and improve the consumer protection. Furthermore, this strategy listed high and medium priorities for research in different domains including the transport and final destination of dioxins and PCB’s in the environment (air, terrestrial and aquatic), ecotoxicology and human health, agri food industry, the sources inventory, the analytical aspects, decontamination measures and monitoring (CEC, 2001).

Following the publication of the Community strategy, the European Commission published a Recommendation (CEC, 2002) on reducing the presence of dioxins, furans and PCBs in feed and food. It requires Member States to carry out random monitoring for the presence of dioxins and PCBs and that, in cases of non-compliance and where dioxin levels are above action levels, Member States in cooperation with operators initiate surveys to identify the source of contamination, verify whether dioxin-like PCBs are present and take measures to reduce or eliminate the source of contamination.

Following the publication of the Community strategy on dioxins, furans and polychlorinated biphenyls in 2001, the European Commission published three progress reports, one in 2004, one in 2007 and one in 2010, all related to the progress made in the implementation of the strategy (CEC, 2004; CEC, 2007; CEC, 2010).

In 2006, a new recommendation of the European Commission was issued on reducing the presence of dioxins, furans and PCBs in feed and food, which already included action levels for PCBs, since the Commission already had data on the presence of dioxin-like PCBs in feed and food (CEC, 2006). This recommendation was also repealed twice, one in 2011 to review action levels (CEC, 2011), and another in 2013, to intensify monitoring in certain food (CEC, 2013).

In this context, the European legislation in the safety of food and feed have been developed, notably in the establishment of maximum levels for certain contaminants in foodstuffs (CEC, 2006b) and laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs (EC, 2014).

2.1. Historical note

The first descriptions of toxic effects caused by polychlorinated organic compounds date back to 1947, when abnormally high incidence of cancer was observed in fishermen in Hudson Bay, USA, and its clear correlation with high levels of PCBs in fish fat from that bay was established. Since then, many other episodes have been described, studied and well documented. The most famous were those that occurred in Yusho (Japan, 1968), Yu-Cheng (Taiwan, 1979), Agent orange (Vietnam, 1965-1971), Seveso (Italy, 1976), Sweden (1970), Holland (1989), Brazil (1997-1998), Belgium (1999), Ireland (2008) and Portugal (2006, 2011 and 2016).

In the Yusho incident in 1968, in Fukuoka, Japan, about 1200 persons were affected. In addition to very serious health problems resulting in skin changes (acne, pigmentation and hyperkeratosis), there has been a significant increase in the number of cancer deaths in males, with adverse effects in new-borns: Low weight, very brownish skin, deformities in the gums, being born some already with teeth but with abnormal pigmentation. The food incriminated in this accident was rice oil contaminated with 2 to 3 g of PCB/kg, which on average corresponded to a daily exposure of 633 mg of PCBs, *i.e.*, an exposure about 100,000 times higher than the Tolerable Daily Intake (TDI). This oil was produced by the Japanese company “Kanemi Rice Oil” and contained a pesticide commercially known as “Kaneclhor 400”, which is a commercial blend containing PCBs (WHO, 1993; Penteado and Vaz, 2001; Furen *et al.*, 2005; Charnley and Kimbrough, 2005).

The incident occurred in Yu-Cheng, Taiwan, in 1979, had also its origin in rice oil. The number of victims of this incident was estimated at about 2000. The main signs of intoxication were peri-ocular edema, excessive ocular discharge, delayed reflexes, darkening of the skin, deep deformities in foetuses, intense dental pigmentation in children and increased incidence of cancer. Contaminated rice oil used in cereal flakes contained between 53 and 99 mg / kg of PCBs. These contaminants were introduced into the food chain through the wet oil extraction system. It was estimated that the total accumulated PCBs and PCDFs, *per person*, varied between 700 and 1800 mg. The content of these compounds in the blood of the victims was 50 to 100 µg/liter (WHO, 1993; Penteado and Vaz, 2001).

During the Vietnam War, between 1965 and 1971, the US Army applied about 72 million liters of herbicides contaminated with 2,3,7,8-Tetrachlorodibenzo-p-dioxin as defoliant of the zones near the perimeter of their military bases. The "Orange Agent" was a phenoxy herbicide widely used in these actions. In studies conducted in 1996, 30 years after the incident, levels of 34 pg TCDD/g were found in fish and 82 pg PCDD/g in duck fat. In individuals living close to the military bases, there were levels of 1832 pg TEQ/g in breast milk in 1970, of 103 pg TEQ/g in adipose tissue in 1980 and blood samples taken in 1991/92, showed levels of

33 pg TEQ/g of TCDD, whereas samples collected in inhabitants of northern Vietnam, where "orange agent" was not used, TCDD levels were found below 2.9 pg TEQ/g (Schecter *et al.*, 1995; Gochfeld, 2001; Schecter *et al.*, 2001; Dwernychuk *et al.*, 2002).

In 1976, north of Milan in Seveso, due to an explosion in a reactor of a pesticide factory, dioxins (TCDD) were spreaded throughout the surrounding soils within a range of 6 km. In total, 1800 hectares of land were affected, with the need to displace 730 inhabitants of this region who had been exposed for 15 days. In children descended from these inhabitants, the usual skin signs were detected. Fifteen years later, a significant increase in the incidence of leukaemia and myelomas in women were observed. The other common types of cancer did not increase (Bertazzi *et al.*, 1998).

Since the 1970s, the Baltic Sea is considered to be the most polluted region of Europe with dioxins. The heavy industries of Eastern Europe have dumped effluents for decades without any treatment. The Baltic coastal countries, Sweden and Finland, implemented an ongoing program, monitoring dioxins and PCBs levels in various products of marine origin (fish), wild animals (reindeer, caribou, seals) and food (milk, poultry and eggs), including the milk of lactating women. Fish meal produced from fish caught in highly polluted areas (Baltic, Black Sea and Arctic) may contain significant levels of PCDD/F and PCB and thus contaminating feed (Watterson *et al.*, 1999; Roots *et al.*, 2006, Roots *et al.*, 2007).

In the Netherlands, in 1989, abnormally high levels of dioxins have also been detected in water, soil, pasture and milk. This accident occurred following a fire at the premises of the German chemical industry Sandoz, due to a failure of two incinerators, whose untreated effluents were drained to the Rhine River. Through this river, parts of France and a wide pasture area in the Netherlands, were also contaminated. This incident brought to the discussion the possibility of cross-border contamination (CIPR, 2000).

In Brazil, between 1997 and 1998, in a by-product factory of the citrus juice industry, PCDD/Fs were accidentally introduced into "granulated" peels of these dehydrated fruits. These raw materials were exported to Europe (France, Belgium, the Netherlands and Germany) and used for feeding dairy cows. Following this use, the PCDD/Fs content in cow's milk in Germany, increased on average from a concentration of 0.6 pg TEQ/g fat, recorded in August 1997, to a concentration of 1.4 pg TEQ/g, in March 1998. In April of that same year, the incorporation of this raw material in animal feed was prohibited (Fiedler *et al*., 2000; Malisch, 2000; Pereira, 2004).

The accident in Belgium, in 1999, considered as a recent episode, was caused by a cross-contamination in a tank truck transporting frying oils for refining to be incorporated in feed, which was mixed with the refrigerant liquid containing dioxins in the tank. The oil was inadvertently used and caused contamination of the meat of several animals in many countries where the feed was marketed (Bernard, *et al.*, 2002). As a result of this incident, the affected countries had to adopt exceptional prevention measures (Becerra and Chandran, 2003; Larebeke *et al.*, 2001; Larebeke *et al.*, 2002; Vellinga and Loock, 2002).

France banned all meat of Belgian origin. The Netherlands removed all Belgian meat and closed 350 pig farms. Portugal withdrew from the market 10 tonnes of chicken meat. Greece destroyed 146 tonnes of poultry products. Russia withdrawal 20 tonnes of turkey meat. Egypt has banned all poultry products imported from the European Union. Algeria has adopted the same measures as Egypt. Several countries have prevented imports: France, The Netherlands, Spain, Canada, Austria, South Korea and Cyprus.

In Ireland, during December 2008, following samples collected in pig slaughterhouse under the national residue monitoring plan, results were found with PCB levels above the limit laid down in the EU legislation (CEC, 2006b). The source of contamination of the animals found was feed from a waste recycling plant. There were 17 identified holdings of contaminated pigs, which were responsible for 8% of national slaughters, all of which were slaughtered for destruction. Concerning the meat that was already in the market, the government has faced difficulties to trace back the affected pig meat and therefore ordered the withdrawal of all meat produced in the previous months, despite only 10% would be contaminated. The financial loss to the pig meat sector in Ireland was overwhelming, not only because of the destruction of the commodity, but also because of the image left to the recipients of its exports (JCAFF, 2009). The government provided financial aid to pig farmers following this incident (Kennedy *et al.*, 2009).

In the detected situations of contamination of the food chain in Portugal in the poultry sector, in the years 2006, 2011 and 2016, the source of contamination identified was always dioxins contaminated wood shavings, used as poultry litters. In all episodes, the wood processing plants (sawmills) that supplied the wood shavings and the affected farms, were traced. Environmental authorities have identified problems in the separation of the by-products of treated wood in these plants. The recipient farms were all tested and, in those presenting levels of meat contamination above the established limits, the animals were slaughtered and destroyed (Cardo *et al.*, 2007; Cardo, 2008, Cardo *et al.*, 2009, Cardo *et al.*, 2014).

In the literature consulted, there are many other cases described, smaller in size but also resulting in the release of polychlorinated organic compounds with serious harm to human health and to the environment. Recent history has shown that this type of accidents can also produce serious economic and market impact in countries with high export potential. The government of these countries are highly pressed for the implementation of surveillance and internal monitoring systems of the emissions of these compounds, as well as the control of the different possible and unavoidable ways of importing that chemical hazard, to ensure a reliable control system. In this regard, the collaboration between countries within an international strategy to study the behaviour of dioxins in the atmospheric, terrestrial and aquatic environment, as well as the establishment of models to explain it, is one of the global current priorities (Watterson *et al.*, 1999). The problem related to the existence of chlorinated organic compounds in the environment and in the food chain, is not a problem caused by less developed countries nor resulting from accidents with the visibility of those previously reported. It is rather of a global and permanent nature, as pollutants are generated, disposed of and bioaccumulated daily in hazardous quantities without respecting frontiers.

2.2. Chemical structure of dioxins

Polychlorodibenzo-p-dioxins (PCDDs) and polychlorodibenzofurans (PCDFs), often referred to as "Dioxins", are two classes of aromatic, tricyclic, ether-functional and quasi-planar structure compounds. Benzene rings are attached by two oxygen atoms to unsaturated hydrogen bonds in the benzene nuclei that can be saturated with chlorine atoms (Figures 3 and 4). Derivatives of the PCDDs, whose chlorine substitutions occurred at the tetra-, penta-, hexa-, hepta- and octachlorinated positions, are designated TCDD, PeCDD, HxCDD, HpCDD and OCDD respectively (Assunção and Pesquero, 1999; EC, 2000a; Fiedler *et al.*, 2000).

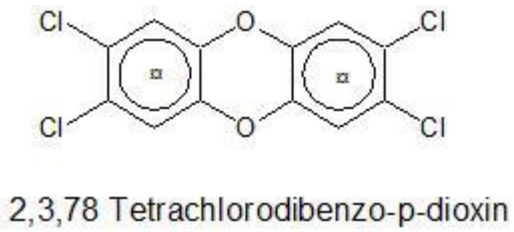

Figure 3 - Structure of a polychlorinated dibenzo-p-dioxin compound (adapted from Huwe, 2002).

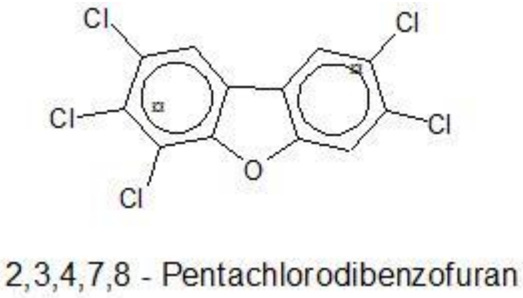

Figure 4 - Structure of a polychlorinated dibenzo-furan compound (adapted from Huwe, 2002).

Depending on the number of chlorine atoms (1 to 8) and on the substitution position, different congeners can be distinguished: 75 for dioxins and 135 for furans (Assunção and Pesquero, 1999; EC, 2000a; Fiedler *et al.*, 2000).

The toxicity of dioxins varies considerably: in particular the congeners in which the substitution occurs at the 2,3,7 and 8 positions are especially important because of their toxicity, stability and persistence. Molecules having a different number of chlorine atoms are called congeners, and those having the same number of chlorine atoms, but in different positions, are called isomers from the same homologue group (Assunção and Pesquero, 1999; Fiedler *et al*., 2000).

Dioxins and furans share similar properties. They have low water solubility and tend to prefer organic phases, such as humus and lipid, to water. Water solubility decreases as the number of chlorine atoms increases. The least soluble congener is OCDD (Mackay *et al.*, 1992).

PCDDs and PCDFs are solid at room temperature and have a rather low volatility. Dispersion in the atmosphere is thus likely to occur mainly in particulate aerosols (WHO Regional Office for Europe, 2000).

Of the 210 congeners theoretically possible, only 17 have considerable toxicity. One of the PCDDs, designated 2,3,7,8-Tetrachlorodibenzo-p-dioxin, is the most toxic synthetic compound known. TCDD induced the appearance of a wide variety of toxicological responses in laboratory animals, namely carcinogenicity, hepatotoxicity, genotoxicity and reproductive toxicity (Assunção and Pesquero, 1999; Bryant *et al*., 2001; Weiss, 2006).

The compounds Polychlorinated biphenyls (PCBs) are also a group of chemical substances that contain chlorine in different positions (Figure 5), and there may be between 1 and 10 chlorine atoms in the molecule (Eurochlor, 2002).

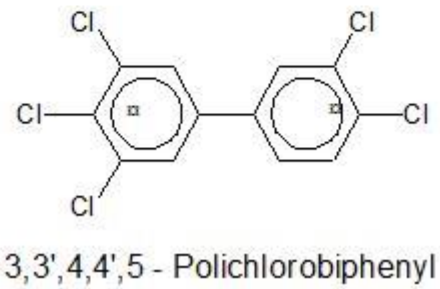

Figure 5 - Structure of a polychlorinated biphenyl (Huwe, 2002).

The viscosity, density and lipidic solubility of PCBs increase, as a function of substitution levels by chlorine atoms, however, their solubility in water is inversely proportional.

2.3. Sources of dioxins

Polychlorinated dibenzo-p-dioxins (PCDD) and dibenzofurans (PCDF) have been demonstrated to occur in nearly all environmental compartments, such as soil, lake and river sediments, and air, in aquatic organisms, plants and human and animal tissues. The majority of the PCDD/F found in the environment today are of anthropogenic origin. To minimize the risk for human health by these highly toxic compounds, it is of great importance to recognize the main sources of PCDD/F and to correlate the environmental occurrence of PCDD/F with these sources (Hagenmaier *et al.*, 1994).

Dioxins are only one amongst several groups of toxic polychlorinated organic substances, as there are a large number of other chemically related compounds that are not dioxins but are also toxic, such as PCDFs, PCBs and PCN (Polychloro-naphthalenes).

Dioxins are contaminants unintentionally generated by activities of the chemical industry of pesticide and herbicide synthesis, or bleach, paper pulp, plastics, especially PVC, solvents, paints and preservatives of wood (pentachlorophenol-PCP) and are also released to the atmosphere following the incineration of municipal, industrial and domestic waste, forest fires and metal smelters at temperatures below 800°C. Dioxins can therefore easily be found in soils, vegetation, the food chain, the animal organism and humans (USEPA, 1994; CEC, 2001).

PCBs, unlike dioxins, are intentionally produced chemicals that were manufactured for decades until they were banned from marketing and use in 1985 due to reproductive toxicity and bioaccumulative effects. Most of these products, characterized by high persistence in biota fat, are found in soils, sediments and in the whole aquatic environment ("historical pollution"). There are two types of use of PCBs (EC, 2000; CEC, 2001):

a. Closed uses: dielectric fluids (insulation) in electrical equipment and hydraulic fluids. In this case, most of the emissions come from leaks, fires, accidents, illegal discharges and irregular eliminations.
b. Open uses: as pesticide additives, flame retardants, sealants, paints, among others. In this case, the main emitting sources are landfill, migration and emissions into the atmosphere from evaporation. Other

less relevant sources are incineration of waste, agricultural use of sewage sludge, combustion of waste oils, as well as PCB reservoirs such as marine and river sediments and port sludge. Their use was due precisely to their intrinsic technical potential, as insulators. The industrial value of PCBs results from their chemical stability, heat resistance, low vapour pressure, high dielectric constant and low acute toxicity. These properties make these compounds particularly useful for the above-described uses and were therefore produced on a large scale. Although they are no longer produced, except under specific conditions, their natural elimination will take a few years because they have very long half-life, they are still used and, in some cases, deposited in "dumps" or landfills or simply abandoned, thus continuing to contaminate the environment (USEPA, 2005; USEPA, 2006).

The chemical stability of PCBs and the fact that they are lipophilic compounds, allow them to persist in the environment and to associate with organic compounds in soil, marine sediments and biological tissues where they can accumulate and enter the food chain. They are distributed in the environment and transported in the atmosphere, being detected in lipid tissues of animals and humans from remote areas (Eurochlor, 2002). The American company "Monsanto Corporation" in 1929, began the production and sale of PCBs to be used as insulators, extremely effective in transformers and electric accumulators, or as hydraulic fluids and for metal finishing. They are also found in electric cables and car components. Only in 1976, the USA Congress banned the production of PCBs. By then, “Monsanto Corporation” had already sold 1.2 million tons of PCBs, however scattered and in unknown locations. Sixty-five percent of them are still used in electrical equipment that is deeply aged, about 20% of them have already been released to the oceans, about 11% are incorporated in the soil and 4% have been incinerated or perhaps degraded (USEPA, 2005; USEPA 2006).

There are several natural sources of dioxin production. For example, prior to the production and use of chlorinated compounds on a large scale, dioxins have been identified in nature, especially in forest soils. The analysis of the profile of the different congeners in the contaminations found and their comparison with the profiles of the contaminations with known sources, reveals that these contaminations cannot be explained by the introduction of the compounds produced by Man and thus confirms the biological formation of these compounds (Rappe, 2003; Otles and Yildiz, 2003). Studies of lake sediments near large industrial sites in the USA, have shown a history of deposition of dioxins and furans. The depositions were quite low until 1920 and had a peak around 1980. U.S. EPA estimates that, between 1985 and 1995, emissions to the environment decreased by about 75 % (USEPA, 1994). These studies may suggest that the deposition of dioxin-like compounds in the environment occurred mostly as a result of industrial activity. The major sources of environmental contamination with dioxins and furans (PCDD/Fs) were grouped into five major groups by USEPA (USEPA, 2005; USEPA, 2006):

A) Combustion - PCDD/Fs are formed in most combustion processes, including the incineration of waste (such as municipal solid waste, sludge, hospital waste and hazardous waste), the burning of various fuels (such as coal, wood and petroleum products), other sources of high temperatures (such as cement kilns) and little or no controlled combustion (such as forest fires, fires, building fires and open-burning);

B) Metal smelters, refineries – PCDD/Fs can be formed during various types of primary or secondary metal operations, including iron smelting, steel production and iron scrap recovery;

C) Chemical industry - PCDD/Fs can be formed as by-products of paper pulp production, chlorinated phenols (such as pentachlorophenol), PCBs, phenolic herbicides and chlorinated aliphatic compounds;

D) Biological and photochemical processes - Recent studies suggest that PCDD/Fs can be formed under certain environmental conditions, such as composting, by the action of microorganisms on chlorinated phenolic compounds. Formation is also reported during the photolysis of highly chlorinated phenolic compounds;

(E) Reservoirs or warehouses - These are materials or sites containing previously formed PCDD/Fs or PCBs from which redistribution and circulation into the environment may be possible. This class includes soils, sediments, biota, water and some anthropogenic materials. The reservoirs become sources from the moment they begin to release into the environment.

USEPA estimates that reducing emissions from identified sources have been responsible for decreasing the levels of PCDD/Fs in the environment in the recent decades. These reductions were imposed by the legislation on emissions from municipal and hospital waste incinerators, changes in the amount of lead in motor fuels, changes in the processes used in pulp mills and reduction in the manufacture of phenolic chlorinated compounds, such as pentachlorophenol and the ban on the use of pesticides such as 2,4,5 -T (2,4,5-Trichlorophenoxyacetic acid, the "orange agent", mentioned above).

At present, major concerns are related to the uncontrolled combustion of domestic waste and domestic fireplaces, which are expected to be the largest source of dioxin emissions into the environment in the future. There are already Danish and Swedish studies on this form of environmental contamination (Lonnermark *et al.*, 2008). The amount of gaseous emissions of PCDD/Fs produced during this combustion process and the amount on the ashes may be reduced according to the temperature of the combustion, the use of inhibitors, the combustion and ash removal conditions, as well as the use of wood not contaminated with paint or wood preservatives (Todd, 2003; Lavric *et al*., 2004).

Concern related to hazardous waste deposits also tends to gain greater prominence within environmental concerns (Todd, 2003).

Recent PCDD/Fs studies on gaseous emissions in several EU Member States, measured as total toxic equivalents, taking into account international toxic equivalency factors (EFT) for PCDD/Fs, assume that the major source of dioxin contamination is due to the gaseous emissions, calculating that they range from approximately 100-1000 g TEQ/year in West Germany, 100-200 g TEQ/year in Sweden, up to 1000 and up to 4000 g TEQ/year in the Netherlands and the UK, respectively. Similar studies on gaseous emissions of dioxins in the USA make a central estimate of 9300 g TEQ/year, which may vary between 3300 and 26000 g TEQ/year (USEPA, 1994).

2.3.1 Combustion and formation of PCDD/Fs

Three hypotheses are described which seek to justify the biosynthesis of dioxins and furans during combustion and their emission through the gaseous effluents from waste incinerators. PCDD/Fs can (Fiedler *et al*., 2000):

(A) Already be present in the waste. Measurements in Germany have been carried out indicating an average concentration of 50 ng TEQ/kg of waste, which is incompletely destroyed or converted during combustion. This source is not relevant in most modern incinerators;

B) Be produced from similar chlorinated precursors (pre-dioxins) such as PCBs, chlorinated phenols and chlorinated benzenes;

C) Be formed again during the process by the pyrolysis of non-similar chemical compounds such as PVC or other chlorinated carbons and/or by the combustion of non-chlorinated organic matter such as polystyrene, cellulose, lignin, carbon and carbon particles in the presence of chlorine donors.

PCDD/Fs are formed during the incomplete combustion of organic matter in the presence of chlorine. During incineration, PCDD/Fs and other polychlorinated compounds such as benzene, phenol, naphthalene and biphenyl, are formed from the carbon that exists in the ashes during the cooling of the exhaust gases and is dependent on several factors such as the carbon morphology, the existence of catalytic ions ($Cu2+$, $Fe3+$, $Pb2+$ and $Zn2+$), oxygen concentration and temperature. The maximum PCDD/Fs formation occurs between 300º and 325º C. The formation of PCDD/Fs below 250º C and above 450º C is negligible (EA, 1999; Meyer *et al*., 2004). Incineration of solid urban and hospital waste is identified as one of the activities that releases larger amounts of dioxins due to the incomplete combustion of these incinerators and the large quantities of PVC (polyvinyl chloride), which are the largest source of chlorine in these incinerators (Otles and Yildiz, 2003). Other sources of dioxins resulting from combustion are, as already mentioned, cement kilns, hazardous waste incinerators, metal smelters, wood combustion and leaded gasoline vehicles (Otles and Yildiz, 2003; Todd, 2003).

Recent studies have shown that domestic combustion of wood treated with wood preservatives significantly increases the formation of dioxins. The formation of *de novo* dioxins during combustion, can occur by two main routes: (i) condensation of chemically similar precursors, such as chlorobenzenes and chlorophenols, or (ii) the combination of carbon, oxygen and chlorine sources in the presence of a metal catalyst.

The most studied catalyst is copper in the form of copper oxide (CuO) or copper chloride (CuCl2). Copper chloride can efficiently chlorinate furans and dioxins at temperatures ranging from 200 to 400ºC and in contrast, copper oxide effectively dechlorinates, the more chlorinated dioxin and furan congeners, by heating under low oxygen conditions (Tame *et al*., 2007). Thus, the amount and distribution of different PCCD/F congeners that are formed during combustion is related to the chlorination and dechlorination reactions that occur (Tame *et al.*, 2007). PCDDs are more likely to result from the condensation or bonding of phenolic compounds and PCDFs result from the chlorination and dechlorination reactions of dibenzofurans (Oh *et al.*, 2004).

There are several studies concerning the formation of dioxins during combustion. The formation of PCDD/Fs in the gaseous emissions is considerable, but is more relevant in reactions that involve catalysts on the surface of the incinerated material during the incineration process (EA, 1999).

In the combustion process, the following PCDD/F emissions should be considered:

- Reactions in the gas phase;
- Non-catalyzed surface reactions;
- Catalyzed surface reactions;
- *de novo* formation.

In reactions in the gaseous phase, which contribute with less than 10% of the total amount of PCDD/F, the dioxins formation probably occurs from short chain chlorinated hydrocarbons. These compounds, by oxidation, undergo an increase in the carbon chain, which form chlorinated aromatic compounds and consequently give rise to chlorinated biphenols. These compounds in contact with hydroxy (OH) groups, release hydrochloric acid (HCl) and form PCDD/F (EA, 1999).

In uncatalyzed surface reactions, the surface appears to function primarily as a site of absorption of PCDD/F precursors and thus concentrates compounds which can react towards PCDD/F formation. The differences between catalyzed and uncatalyzed surface reactions in PCDD/F formation, are difficult to establish and the formation process on uncatalyzed surfaces is not well described in the literature. It is, however, clear that the catalyzed reactions produce more and more diversity of PCDD/F congeners in the different combustion processes (EA, 1999).

In the catalyzed surface reactions, the PCDD/F can be formed from precursor molecules such as chlorophenol, chlorinated benzenes and chlorinated biphenols. This process is well studied and accepted as the major pathway of PCDD/F formation. Precursors are products of incomplete combustion, produced at high temperatures (> 400°C and most effectively at about 750°C). The precursors catalytically react with elements of the ashes to produce PCDD/F and is dependent on the number of precursors and the reaction temperature. Studies show that the formation of PCDD is dependent on the concentration of the precursors, but it was not possible to establish this relation with temperature (EA, 1999).

One of the most important precursor compounds for the formation of PCDD/F is chlorophenol. Two different pathways are described for the formation of PCDDs and PCDFs.

The formation of PCDDs is carried out by the binding of chlorinated phenolic anions to the precursors on the surface of the incinerated materials and the phenolic rings are bonded by oxidation. The role of the catalyst in the oxidation is to transfer electrons that lead to the bonding of the two aromatic rings. In oxidation, there are HCl and Cl release reactions. The process shown in Figure 6 refers to 2,4,6-trichlorophenol, which reacts to form 1,3,7,9-TCDD or 1,3,6,8-TCDD.

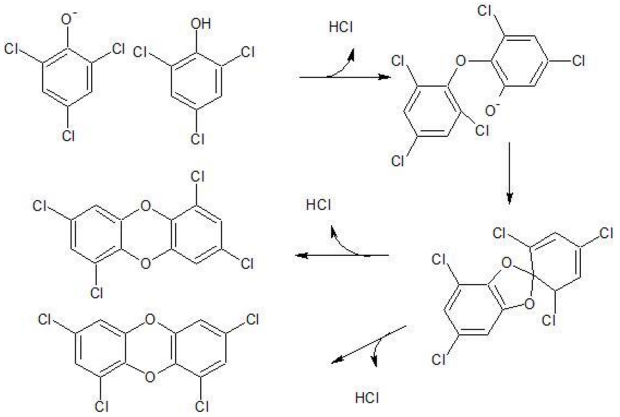

Figure 6 – PCDD formation mechanism (adapted from EA, 1999).

Further precursors, such as chlorobenzene and phenoxyphenols, are mentioned in the furans formation process (Figure 7). The mechanism appears to include a cyclization or ring closure reaction of the Pschorr type (Rotko *et al.*, 2013).

This reaction is catalyzed by a metal and appears to be of particular importance in the incineration of urban solid waste, especially when Cu and Fe metals are present, with Fe having the greatest positive effect on PCDD/F formation (EA, 1999).

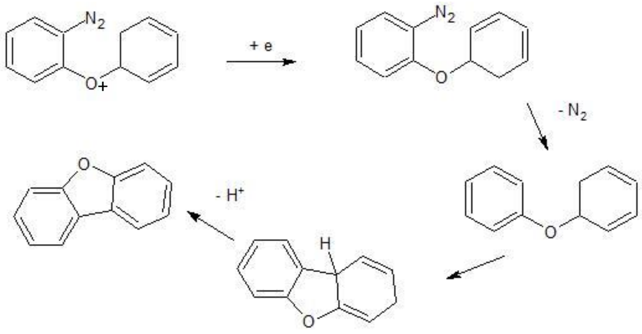

Figure 7 – PCDF formation mechanism (adapted from EA, 1999).

In the *de novo* formation of PCDD/F, the small amount of carbon remaining in the ashes of the material during combustion binds to the catalyst in the ash particles. The chlorine in the metal ligands is transferred to the aromatic carbon rings. It is also possible that the flue gas HCL also serves as an additional chlorine source to the chlorine in the metal ligands. HCl reacts with oxygen in the copper catalysts to form Cl_2 and H_2O. Air oxygen diffuses through the pores of ash particles where it reacts with the carbon that is in contact with the metal ligands containing chlorine. It is the chlorine that enters the substitution reactions for chlorination of the aromatic rings that incorporate the carbon to form the dioxins. Some of the formed PCDD/F diffuses in the gaseous state and the rest remain in the ashes, presenting large amounts of PCDD/F (EA, 1999).

The production of PCDD/F is very dependent on the temperature of the reaction. Larger production occurs at 325°C, which means that dioxins are produced in the colder parts of the combustion (EA, 1999).

The formation of dioxins requires the presence of carbon incorporated into the pores of the ash particles, which means that, in complete combustion, it limits the amount of carbon available for *de novo* formation of dioxins in the ash, after combustion.

The chlorine concentration in the flue gases does not appear to influence the formation of dioxins since the chlorine, in the *de novo* formation of the dioxins, comes from the metal ligands of the pores of the ash particles (EA, 1999).

The *de novo* formation of dioxins seems to favour the formation of PCDF in PCDD debris. This is an important conclusion that shows that in contaminations in which the profile presents a great amount of furans, the formation *de novo* is the explanation (EA, 1999).

The formation of *de novo* does not utilize CO and CO_2 of the combustion gases in the formation of PCDD/F, since this pathway only includes reactions between molecules present in the ashes with air oxygen (EA, 1999).

In high temperature combustion, predominates the catalyst reaction surface pathway and PCDD/F production from precursors compared to *de novo* formation. On the other hand, in low-temperature combustion, the *de novo* production path predominates in relation to the precursor pathways (EA, 1999). The high or low values of PCDD and PCDF indicate whether the reactions were from precursors or via *de novo* formation.

When the combustion temperature drops to about 300°C, PCDF formation predominates in relation to PCDD formation. It is established that, incinerations at higher temperatures for a longer time, result in complete combustion and that, with a rapid decrease in the temperature of the combustion products to shorten the time, the gases remain in the temperature window between 200 and 450°C. Under these circumstances PCDD/F production is very low.

2.3.2 Chemical industry and formation of PCDD/Fs

Dioxins and similar compounds may also be formed as by-products of the chemical industry, such as bleach or the production of other chlorinated compounds, such as pentachlorophenol, PCBs, phenolic herbicides (*e.g.* 2,4,5-trichloro-phenoxyacetic acid), chlorinated benzenes, chlorinated aliphatic compounds, chlorinated catalysts and halogenated diphenyl ethers. Despite the production of intermediate chlorinated phenols, as well as PCBs, has been ceased in the United States and Europe in the 1970s, production continued to occur in other parts of the world until the 1990s, being still allowed for special cases, under limited use, controlled disposal and therefore, these substances may continue to be a source of PCDD/Fs and PCB emissions to the environment (USEPA, 2005; USEPA, 2006).

Processes in the manufacture of industrial chemicals can, theoretically, give rise to persistent organic pollutants (particularly PCDD/Fs and PCB). Most of the processes share common steps, including chlorination of organic or inorganic raw materials, purification of the products, separation of product streams (usually by distillation), destruction of high-molecular-weight side products and recycle of hydrogen chloride. Most of the processes involve a hydrocarbon, saturated or unsaturated, treated with elemental chlorine and, in most processes, also a catalyst (SSCPOP, 2008).

Central to formation of the basic structure of PCDD/Fs and PCB in chemical industry is the presence or generation of aromatic materials. Those materials must, at some point, be chlorinated. PCDD/PCDF formation is favoured in processes where chlorinated phenols are a substrate or phenols are reacted with chlorine (SSCPOP, 2008).

In the 1990s, the contribution of chemical industry to the total emissions of hexachlorobenzene (HCB) worldwide was 33.8% (organic chemical industry 5.8%, other solvent use 0.004% and pesticide use 28%) following non-ferrous metal industry (36%). Fuel combustion (3.7%), iron and steel industry (0.3%) and waste incineration (26%) were other sources identified (Bailey, 2001).

Among these persistent bioaccumulative toxic substances, special attention must be paid to phenolic compounds used as pesticides, because these products may pose a great risk to the environment and to human health.

Several countries have not yet found a safe storage solution for these pesticides. Sometimes they do have little information on existing quantities and even some Member States do not have hazardous waste incinerators for disposal. For example, in Europe, Poland is aware of the existence of about 60,000 tonnes of pesticides under these conditions (about 10,000 in landfills, 25,000 in warehouses and 25,000 in private properties). Bulgaria had about 35 tonnes in 1996. In 1997, Croatia imported more than 503 tonnes of pesticides with persistent bioaccumulative toxicants. Estonia has also a history of importing these products. The situation is probably similar in other Member States of the EU, which should be identified as risk regions. In some of these countries, the concentration of these pesticides has been reduced in the environment, which has not happened in Albania and Romania, where relatively high concentrations of DDT and other chlorinated pesticides in water and sediment, are still present. In Slovakia, the values found show that there is high exposure to hexachlorobenzene from an unknown source, which results in a high concentration of this compound in human tissues. Other countries already prohibit the use of this type of chlorinated pesticides. Hungary was the first country in the world to ban the use of chlorinated pesticides in 1966. The Czech Republic banned it in 1974 followed by Slovakia in 1976. Some African, Asian and South American countries continue to use these products as cheap and efficient pesticides (Holoubek *et al.*, 2001).

The formation of PCDD/Fs resulting from the use of chlorine in the paper pulp bleaching process resulted in the presence of PCDD/Fs in paper and waste water and solid waste from that industry. Sludge from Municipal Wastewater Treatment Plants, also occasionally presents PCDD/Fs (USEPA, 2005).

The persistent and hydrophobic nature of these compounds causes them to accumulate in soil, sediments, organic matter and waste disposal sites. These compounds can be diffused from these sites by various processes, such as through dusts, sediment re-suspension, transport and can be naturally dispersed by volatilization of the sediments. The deposition and accumulation in the leaves of the plants can result in emissions, especially during forest fires or during the course of composting of these plant materials (Watterson *et al.*, 1999; USEPA 2005).

2.4. Congeners Profile in different sources of PCDDs / PCDFs

The analytical profile shows the relative amount of each congener of the PCDD/Fs in a given sample.

The profiles can be useful to identify or eliminate potential sources that contributed to a certain food contamination with PCDD/Fs, to compare different sources of contamination and to predict the quantities of each congener in a process of PCDD/Fs formation (USEPA, 2005). However, to perform chemical fingerprinting there must be a plausible transport pathway from the source to a receptor in order to conclude that a fingerprint is a result of that source.

Once a contamination in food or feed is discovered, it is essential to trace back the origin and discover the source in order to identify potentially contaminated farms, factories, production processes and food products. It has been shown that congener patterns may be very helpful in discovering the source of the contamination. Therefore, it is necessary to collate representative patterns and make them available to risk managers and to the laboratories involved in feed and food control (Hoogenboom *et al.*, 2016).

When comparing patterns of PCDD/Fs, it seems obvious to do this based on the contribution of the individual congeners to the sum of the measured levels. An alternative, often described in literature, is the relative contribution to the TEQ level. The latter approach has some advantages. If the pattern is dominated by the octachlorinated congeners, the other congeners will not really be visible. Furthermore, in the case of elevated levels in food of animal origin, a modified profile is obtained as result of kinetics and metabolism. In these cases, a direct comparison between that pattern and patterns from primary sources may not easily lead to a good fit (Hoogenboom *et al.*, 2016). Studies on the citrus pulp used as feed, following the cow milk incident, have shown that the primary source profile presented very high levels of the most chlorinated furans (OCDF and 1,2,3,4,6,7,8-HPCDF), but these congeners presented rather low transfer rates to milk. In these cases, the pattern based on TEQ contribution clearly gives a much better fit. This can also be overcome by applying transfer rates in the calculations. However, since there is a reasonable correlation between transfer rates and TEFs, a comparison based on TEQ contribution was an appropriate alternative (Hoogenboom *et al.*, 2016).

For tracing sources in feed and food, first, it has to be checked how many congeners are increased in comparison to usual background levels. In most cases, from the 17 PCDD/Fs with 2,3,7,8-substitution routinely determined in feed and food, only a limited number is significantly increased and can be used for source identification. However, also the absence of specific congeners offers important information for identification of the source. The combination may be a suitable starting point for the development of a decision that can assist pointing out the potential source. In addition, also other non-2,3,7,8 substituted congeners may be helpful, but these are not routinely identified and quantified (Hoogenboom *et al.*, 2016).

Hoogenboom *et al.*, (2016), provided a strategy for the identification of sources based on the congener pattern, based on TEQ contribution:

- If the contamination reveals the predominance of PCDFs, the most likely source is PCBs. The pattern may vary depending on the chlorination grade of the original PCB mixture, with high contribution of TCDF in case of a low chlorinated PCBs, and a shift to PeCDFs in case of higher chlorinated PCBs. Since most laboratories routinely analyse for PCBs, it will be easy to check whether this is the source. It is important that information on PCBs is provided in the analytical reports.
- If the contamination reveals the predominance of PCDDs, there are roughly two options. One is the use of contaminated clays, with TEQ based patterns that are in general dominated by PeCDD and to a lesser extent TCDD, HxCDDs (in particular 1,2,3,7,8,9 HxCDD) and HpCDD. On an absolute basis, OCDD is by far the most important congener. However, also incidents with chlorophenols show only PCDDs. Depending on the chlorination grade there is a shift from TCDD, as in the Seveso incident and agent Orange (trichlorophenol, 2,4,5-Trichlorophenoxyacetic acid), to HpCDD, as occurred with materials contaminated with pentachlorophenol. In the Kaolinite incident in Germany during 2010/2011, an intermediate pattern with high contribution of 1,2,3,7,8,9-HxCDD was observed, but also the presence of relative high levels of tetrachlorophenol. Checking for chlorophenols may be an alternative to decide between clay materials and a pattern based on tetrachlorophenols, although the patterns can be distinguished.
- A mixed PCDD/PCDF pattern can be caused by different sources. The pattern dominated by the PeCDD/Fs and to a lesser extent TCDD/Fs points in the direction of burning of chlorinated plastics (PVC). Depending on the materials involved there may be a higher dominance of either the PCDDs or

PCDFs. A mixed pattern is also observed in the case of certain minerals, such as recycled zinc oxide and the so-called sequestered minerals, that were produced by heating minerals in the presence of kelp. These mineral based patterns in general show much higher contributions of the HxCDFs and can as such be distinguished from the patterns caused by burning.

- A fourth category involves profiles containing only one, or maybe two 2,3,7,8-substituted congeners, as in the gelatine incident the Netherlands, in 2006, in which the congener pattern was dominated by 1,2,3,7,8-PeCDD and 2,3,7,8-TCDD, and was not recognized from any previous incident or known dioxin source (Hoogenboom *et al.*, 2007), but also in a more recent incident in Chile with feed contaminated with zinc oxide in 2008. In this episode, 2,3,4,7,8-PeCDF were found as the major congeners in pork samples and contributed about 30% among the congeners (Meekyung *et al.*, 2011).

Usual methods to apply for source identification are the comparison of the homologue profiles, the comparison of the congeners profiles of the 2,3,7,8-substituted PCDD/F and the comparison of the isomer distribution patterns (Hagenmaier *et al.*, 1994).

1. Comparison of the homologue profiles

   Most frequently the profiles of the ten homologue groups of tetra-to-octachlorinated PCDD/F are used to the detection of sources. In these cases, the diagrams are based on the mean values of “n” analytical samples. For the calculation of the arithmetic mean values, all analytical data is normalized to the total sum of PCDD and PCDF= 100. Nevertheless, changes in the homologue profiles can occur temporally and spatially from the source to the sample under consideration, resulting in misinterpretation regarding the source. Therefore, the applicability of the comparison of homologue profiles for source identification in environmental samples is limited.

2. Comparison of the congeners profiles of the 2,3,7,8-substituted PCDD/F

The congeners profile of different thermal processes shows also some variation, however, the differences in the congeners profiles are smaller than that in the homologue profiles. As in the homologue profiles, the congeners profile of chemicals (PCP, PCB and chlorine production) are completely different from those of thermal processes.

3. Comparison of the isomer distribution patterns

The mass fragmentograms obtained in dioxin analysis by GC/MS are such patterns. In some cases, the non-2,3,7,8-substituted isomers are of great importance in characterizing the different sources, *e.g.* 1,2,4,6,8,9-HxCDF and 1,2,3,4,6,8,9-HpCDF to identify PCP as a dioxin source. However, the data of non-2,3,7,8-substituted isomers are not available in many cases because they are not included in routine dioxin analysis reports.

4. Comparison of the profiles of the relative congener concentrations of the 2,3,7,8-substituted PCDD/F

Because of the limitations of the previous methods in source identification, Hagenmaier *et al.*, (1994), have developed a new strategy. The strategy is based on the assumption that the concentration of all isomers changes in the environment within one homologue group, with the same tendency. Therefore, the profiles of relative congener concentrations should be subjected to changes in the environment to a lower degree than the homologue profiles. This new strategy rests on the following calculations:

- The ratio of the concentration of the 2,3,7,8-substituted congeners to the concentration of the corresponding homologue groups;
- The concentration of OCDD and OCDF are divided by the concentration of the 2,3,7,8-substituted PCDD and PCDF, respectively;
- The PCDD/PCDF ratio in form of the quotient of PCDD concentration and the total concentration of PCDD+PCDF.

The application of this procedure to the average source analysis results in a close similarity of the relative congener profiles for all thermal processes. That implies that the isomer distribution patterns for different thermal processes are quite similar and relatively constant. That means that the processes taking place in the air path are homologue-specific but not isomer specific. In contrast to the homologue profiles the relative congener profiles of sewage sludge samples with low PCDD/PCDF concentrations differ significantly from the PCP profile and suggest that the main PCDD/F source of these sludge's is of thermal origin.

Several published studies classify and typify the profile of the most widely studied sources of contamination that risk managers shall bear in mind when assessing a food chain contamination episode (Cleverly *et al.*, 1997; Muto and Sugawara, 2001; Gullett and Touati, 2003; USEPA, 2005; USEPA, 2006).

Track down of dioxin sources requires consistent detection of all congeners. Source identification requires both quantitative data and qualitative information about fingerprint. For inspection and comparison of the profile of the different PCDD/Fs congeners, some observations can be made regarding the data presented in these studies:

a) In combustion emissions it is common that all PCDD/Fs congeners be chlorinated in the 2,3,7,8-positions, although with different relative percentages of total PCDD/Fs (USEPA, 2005);
b) In emissions resulting from combustion, the congener 2,3,7,8-TCDD generally represents 0.1 to 1% of the totals of PCDD/Fs. Exceptions are the fat combustion emissions in boilers, where available data indicates that the 2,3,7,8-TCDD congener generally represents 7% of total PCDD/Fs (USEPA, 2005);
c) It cannot be concluded that the congener octachlorodibenzo-p-dioxin (OCDD) is the dominant congener for all emissions from combustion. OCDD dominates emissions caused by mass combustion, fat burning in boilers, industrial wood combustion, unleaded gasoline combustion, diesel fuel combustion in trucks and sludge incinerators. The dominant congeners for other sources of combustion are 1,2,3,4,6,7,8-HpCDF in mass combustion, hazardous waste incineration and secondary aluminium smelting, OCDF in hospital waste incineration and in coal boilers, the congener 2,3,4,7,8-PeCDF in cement kilns that burn hazardous waste and the congener 2,3,7,8-TCDF in cement kilns which do not burn hazardous waste (USEPA, 2005);

d) In 2008 in Campania, Italy, contaminations with polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) were found in buffalo milk. The study of the congeners distribution showed that the congener profile was characterized by a prevalence of PCDFs in respect of PCDDs, which has suggested that the likely cause of contamination is to be attributed to the illegal burning of waste (Espositoa *et al*., 2009; Espositoa *et al*., 2010);

e) There is a marked difference in the distribution of the PCDD/Fs congeners between the cement kilns that burn hazardous waste and the ones that don't burn hazardous waste. When they do not burn hazardous waste as a combustion supplement, the dominant congeners are 2,3,7,8-TCDF; OCDD; 1,2,3,4,6,7,8-HpCDD and OCDF. When burning hazardous waste, the dominant congeners are 1,2,3,7,8-PeCDF; 2,3,7,8-TCDF; 1,2,3,4,7,8-HxCDF and 1,2,3,4,6,7,8-HpCDD (USEPA, 2005);

f) There are similarities in the profile of penta-chlorophenol (PCP) congeners and gaseous emissions from diesel truck exhausts, emissions from unleaded fuel vehicles and emissions from industrial wood combustion. In these sources, more chlorinated compounds, such as octa and hepta-CDD/CDF, dominate the total emissions and PCDDs have higher total weight than PCDFs (Wunderli *et al.*, 2000; Muto and Sugawara, 2001);

g) After ingestion of wood chips contaminated with PCP, the birds and cattle present contaminations in the muscle in which the OCDD and HpCDD congeners are the dominant ones. However, in the bovine species, unlike birds, the congeners 1,2,3,6,7,8 HxCDD and 1,2,3,4,6,7,8 HpCDF are differentiated, presenting values slightly increased (Fries *et al.*, 2002, Ryan *et al.*, 1985);

h) One conclusion drawn by Fiedler *et al.*, (2000) and Huwe *et al.*, (2000) from different studies is that cattle fed with PCP treated wood chips eliminated large amounts of OCDD (Octachlorodibenzodioxin). The amount excreted was almost four times higher than the amount ingested, which points to the possibility of *in vivo* formation of OCDD from pre-dioxins, *i.e.* chlorinated phenoxyphenois and pentachlorophenol;

i) The incident with citrus pulp from Brazil, in 1998, in which, pellets of feed for dairy cows included lime, have shown that the feed raw material contamination profile presented very high levels of the most chlorinated furans (OCDF and 1,2,3,4,6,7,8-HPCDF), but these congeners presented rather low transfer rates to milk (Hoogenboom *et al.*, 2016);

j) High levels of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) were detected in free-range eggs. The source of dioxins in the poultry farm was pentachlorophenol-treated wood, which was used as structural components in one 40-year-old farm building adapted to a henhouse. The contamination found elevated amounts of OCDD, OCDF, HxCDD, HpCDD and HpCDF (Piskorska-Pliszczynska *et al.*, 2016);

k) The profiles found in litters of wood chips contaminated with treated wood, in the muscle of the breeding hens raised in these litters, in the hatching eggs placed by these contaminated chickens and in the respective chicks born from these eggs showed a coincidence of the peaks of higher relative concentration in the different samples, especially for the congeners 1,2,3,4,6,7,8-HpCDD and OCDD (Cardo *et al.*, 2014);

l) Some studies on dioxin congeners fingerprint analysis, found transformation of the congeners profile from the contamination source to the contaminated organism. Changa *et al.* (2016) observed that OCDD was the dominant PCDD/Fs congener in 54 of the 55 sediment samples and in all water samples, in aquacultures in a place which is right next to an abandoned pentachlorophenol factory. The fish samples showed that unlike the observed in the water and sediment samples, in which OCDD was the major congener, 2,3,4,6,7,8-hexachlorodibenzofuran (HxCDF) predominated in 33 out of 55 fish samples and represented 36.4–74.7% of the total PCDD/Fs in regard to mass concentration;

m) In a study of the poultry production chain contamination with wood shavings from the bedding material in a breeding hens farm which affected the hatching eggs and the offspring, also found transformation of the congeners profile from the contamination source to the contaminated organisms. It was concluded that in the fat and muscle profiles of broilers and breeding hens, the relative average concentration of OCDD was 52% and 53% lower, compared to the profile of the wood shavings and hatching eggs, respectively. On the other hand, the relative average concentration of 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD in the profiles of fat and muscle samples of broiler and breeding hens was 50% - 97% higher when compared to the profiles of the beds and hatching eggs (Cardo *et al.*, 2014);

n) Contaminations may have distinct dioxin sources. Litten and Fowler, (2001), studied suspended solids from the Hudson River and New York/New Jersey harbour waters. The upper site was near to a deactivated facility that manufactured 2,4,5-trichlorophenoxy acetic acid for production of "Agent Orange" where the contamination was dominated by 2,3,7,8-TCDD. The lower site was near a facility incinerating obsolete electrical equipment where the contamination was dominated by 2,3,4,7,8-PeCDF and 1,2,3,4,7,8-HxCDF. The intermediate site had a contamination which was a mixture of both because this site receives water from the upper site by the river current and receives water from the lowest site by the rise of the tide;

o) It should, also, be realized that some congeners with relatively high TEFs may be metabolized by certain species, as is *e.g.* the case for TCDF and 1,2,3,7,8-PeCDF in pigs and cows (Hoogenboom *et al.*, 2016).

Chemical fingerprinting is a well-established technique for distinguishing different sources of contamination, and it is particularly well stablished to work with families of organic compounds, such as PCBs, polycyclic aromatic hydrocarbons, and CDD/CDFs. This is because these classes contain many individual compounds, which together comprise a compositional pattern, also referred to as a "profile," a "signature," or a "fingerprint" and also because the relative concentrations of an individual profile can be used as a marker of the original source of contamination. However, because organic compounds can be transformed in the environment or *in vivo*, segments of the original patterns can be altered. Another complication of these "real world" factors is that multiple sources often mix together and mask the individual signature of a specific source of contamination.

Environmental transformations and mixed sources must be considered when interpreting dioxins contamination fingerprints, especially in fingerprint analysis of samples from soil or sediment, which typically represents many decades of input of CDD/CDFs that may have been chemically transformed during transport from their original source and after deposition and/or mixed with other sources.

Many fingerprint analysis methods are known and they range, from simple profile comparisons of individual samples, to sophisticated multivariate analyses.

Individual profile comparisons can be useful when the profiles are clearly different or similar.

Multivariate statistical analyses such as hierarchical cluster analysis and principal components analysis (PCA) are often used to condense and simplify a complex set of variables (Shields *et al*., 2015; Cardo, 2008).

These widely used and accepted techniques are scientifically defensible, although the underlying mathematics are complex.

There are several chemical fingerprinting methods, Shields, (2015) describe and systematize the most used:

1. Individual Profile Evaluation: The first fingerprinting step, and sometimes the only step, is the evaluation of individual samples by comparison of profiles of the relative concentrations of either the commonly reported seventeen 2,3,7,8-substituted congeners or the 10 homologue classes. Concentrations of CDD/CDFs found in different samples can vary by orders of magnitude, therefore, standardization of the results is necessary so that the congener or homologue profiles from different samples can be compared.
    a. Standardization Methods: Four types of standardization methods are commonly used. Shields (2015) define them as the “2,3,7,8-sum,” “relative homologue,” “relative TEQ,” and “total homologue” methods. Profiles using these four standardization methods in samples of similar sources may have common 2,3,7,8 - sum profiles, but very different profiles can be found using the other standardization methods. Each of these methods provides a different, yet equally valid, view of the relative concentrations of the congeners and homologue classes. The use of multiple standardization methods for both visual comparisons and exploratory data analyses provides a more rigorous analysis than using just one standardization method. The differences of each of these standardization methods are:
        i. “2,3,7,8-Sum” Standardization Method: Each reported 2,3,7,8-substituted congener is divided by the sum of the 2,3,7,8-substituted congeners reported. This is a common standardization method and is similar to dividing each congener by the total CDD/CDFs (USEPA, 2005) and takes advantage of the detail provided in the congener specific results. However, there are two

problems with this method: CDD/CDF profiles can be altered by weathering and bioaccumulation and the profile comparisons can be limited by lack of detection of specific congeners. Another challenge is the problem of viewing the low concentrations that are masked when the relative concentrations of OCDD or others are extremely high. Presenting the relative concentrations on a logarithmic scale allows examination of the pattern of the low concentration congeners. However, if the scale is expanded too much to show the differences in the low percentage congeners, the differences between the major contributing congeners are difficult to detect.

ii. "Relative Homologue" Standardization Method: Each 2,3,7,8-substituted congener is divided by its respective homologue class (*e.g.*, 2,3,7,8-TCDD is divided by the total TCDDs, 1,2,3,4,7,8-HxCDF is divided by the total HxCDFs). OCDD and OCDF are divided by the total 2,3,7,8-substituted dioxins and furans, respectively. This method, somewhat neutralizes the effects of differential weathering and bioaccumulation resulting from the degree of chlorination (*i.e.*, homologue class) among environmental samples (*e.g.*, soil, sediments, and dust). Differences in weathering and bioaccumulation between 2,3,7,8-substituted congeners and non-2,3,7,8-substituted congeners with the same degree of chlorination probably also occur; yet fewer changes are likely within the homologue class than between them.

iii. "Relative TEQ" Standardization Method: The TEQ for each reported 2,3,7,8-substituted congener is divided by the TEQ for the sample. This standardization method takes advantage of the detail provided in the congener-specific results, and it also provides information on which congeners contribute to toxicity. Another advantage of this method is that the dominance of OCDD (often with orders of magnitude greater than some of the lower chlorinated congeners) does not mask the patterns of the low-concentration congeners. However, this standardization method has the same two problems as the "2,3,7,8-sum method", namely, CDD/F profiles of similar environmental media can be altered by weathering and bioaccumulation, and the profile comparisons can be limited by lack of detection of specific congeners.

   iv. "Total Homologue" Standardization Method: Each homologue class is divided by the total CDD/CDFs (*e.g.*, total PeCDFs divided by the total CDD/CDFs). This is a common standardization method and a convenient way of showing gross differences in profiles. However, unlike the congener-specific methods, subtle but potentially important differences among the low-concentration congeners are not presented. Also, unlike the "relative homologue" method, it does not account for any significant dechlorination of environmental samples resulting from weathering or differential uptake by organisms.

2. Exploratory Data Analysis: To complement visual comparisons and/or ratio analyses described above, exploratory data analyses can be used to evaluate sources. Mathematical methods can be used to identify patterns (similarities and differences) in groups of multivariate CDD/CDF congener data. Methods used to assist in source identification of CDD/CDFs include, but are not limited, to double ratio plots, hierarchical cluster analysis, discriminant analysis, principal component analysis (PCA), neural networks, and polytopic vector analysis (PVA).

   Data sets often have a large proportion of undetected congeners. The analyst needs to describe, not only the screening criteria used to include or exclude these data but also, if included, what value was used to represent the estimated concentration. Typically, one-half the detection limit is used, but sometimes, zero, or the full detection limit. The analyst should conduct and describe sensitivity analyses with regard to differences and similarities in the conclusions, depending on the data processing methodology applied.

   Congener profiles can vary significantly depending on the standardization method. The multivariate analyses should be applied to all four common standardization methods if data is available, as sometimes only the 2,3,7,8 congeners are available and no homologue class data is reported, despite sometimes the opposite is true. The results of the exploratory data analysis are more robust if they are consistent across different standardization methods.

2.5. Toxicity of dioxins

The USEPA attributes carcinogenic potential to the complex mixture of dioxin congeners to which humans and animals are exposed through food or air. The tetrachlorinated congener, "2,3,7,8-TCDD" (tetrachloro-dibenzene-p-dioxin) is one of the most toxic substance known, classified as a human carcinogen, based on the evidence from animal and human studies. There is no safety limit, since it is possible to induce cancer in laboratory animals even at very low doses. Other dioxins are classified as potential human carcinogens (McGregor *et al.*, 1998; USEPA, 2004).

Different animal species have a different sensitivity to dioxins, whereas humans, in comparative terms, have an intermediate sensitivity in the spectrum of effects verified in the sequence of exposures to those compounds (USEPA, 2004).

Dioxins are efficiently absorbed in the gastrointestinal tract and accumulate in the lipidic tissues of the body, increasing the risk with prolonged exposure, *i.e.*, with age and with very abrupt weight loss diets, especially in adults and elderly people (Hue *et al.*, 2006; Weiss, 2006). The literature on the toxicokinetics of PCDDs and PCDFs is quite extensive and exposure to PCDDs and PCDFs may result from inhalation of contaminated fly ash, dust and soil or by oral exposure. The uptake following oral exposure of the most toxic PCDDs and PCDFs in experimental animals is generally in the range 50–90%, depending on the vehicle. Uptake of higher chlorinated congeners is much lower, because of lower solubility and larger molecular size. Absorption of more than 90% has been demonstrated in breastfed infants (WHO, 2000). Liver and adipose tissues are the predominant storage sites of absorbed PCDDs and PCDFs. However, the distribution is highly dose-dependent because of induction of specific binding proteins in the liver, and there are large differences in the distribution pattern between various PCDDs and PCDFs (WHO, 2000).

In addition to the carcinogenic potential, a number of other adverse effects on human and animal health are attributed to dioxins, in particular at foetal development level, growth percentile, psychomotor and mental delays and various endocrine disruptions of varying degrees of severity involving, in particular, thyroid and gonads (McGregor *et al.*, 1998).

Depending on the degree of chlorination, 1 to 8 chlorine atoms and their position on the benzene rings, multiple congeners may be formed. The toxicity thereof varies considerably, with particular importance to those in which the 2,3,7 and 8 positions are occupied. However, as already mentioned, of the 210 possible congeners, only 17 are toxicologically worrisome (Assunção and Pesquero, 1999; EC, 2000; Bryant *et al.*, 2001; Weiss, 2006).

PCBs belong to the group of chlorinated hydrocarbons which are synthesized by the direct chlorination of biphenyl. Depending on the number of chlorine atoms, which may be from 1 to 10 and their position on the two rings, it is theoretically possible to have 209 different compounds or congeners, but only 12 have a toxicological action similar to dioxins (EC, 2000b).

From a toxicological point of view, PCBs can be divided into three groups. Non-ortho and mono-ortho have higher toxicological properties similar to dioxins, and di-ortho PCBs are less toxic and have different toxicological properties (EC, 2000b). In PCBs, which do not have any chlorine atoms in the ortho position (Figure 8), the two phenol rings assume a co-planar position and thus, have properties similar to those of dioxins. Non-ortho and mono-ortho PCBs were assigned with toxicity equivalence factor values, TEF (Weiss, 2006).

The exposure assessment should take into account that, although dioxins are more toxic than PCBs, the quantities of PCBs released into the environment are several times higher (EC, 2000).

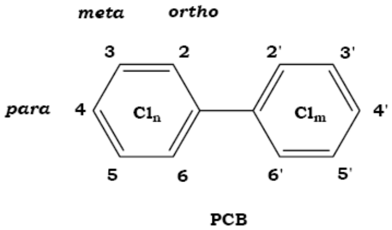

Figure 8 - Molecular structure of PCBs and bonding positions of chlorine atoms (Weiss, 2006).

2.5.1 Toxicity Equivalence Factor and Toxic Equivalent concentration

Each compound in the dioxin family or dioxin-like PCBs has a different level of toxicity. In order to assess the cumulative sum of the toxicities of these different related compounds, the concept of Toxicity Equivalence Factors (TEF) was introduced in order to facilitate risk assessment, as well as regulatory control. TEF values are theoretical values, administrative tools, based on current knowledge that must be revised as new data become known and available (Fiedler *et al.*, 2000).

The transformation of the "gross concentrations" of each congener using the TEF values gives the analytical result for the 17 dioxin-like compounds and 12 dioxin-like PCBs and is expressed in terms of a single quantifiable unit: " TCDD Toxic Equivalent Concentration" (TEQ) (Weiss, 2006).

This conversion is based on the assumption that all dioxin congeners have the same qualitative effect because they bind to the same dioxin receptor in cells but with different intensities. The different intensity is expressed by the TEF and estimated by the relation between the lower toxicity of the respective congener and the toxicity of the 2,3,7,8-TCDD that assumes the theoretical TEF value of 1. Moreover, it is assumed that the toxic effects are not synergistic or antagonistic, but cumulative (EC, 2000b).

TEF estimates the toxicity of dioxin-like compounds relative to the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is assigned a TEF of 1.0 (WHO/FAO, 2012).

Different models of TEF have been developed. At the international level, the most commonly used TEF values are those proposed by NATO Committee on the Challenges of Modern Society (NATO/CCMS) in 1988 and the revision of TEF values for mammals and proposed new values for birds and fish by WHO in 1998. According to the model used, the TEQ values are expressed respectively in I-TEQ (NATO/CCMS) or WHO-TEQ (WHO) (EC, 2000b; Fiedler *et al.*, 2000; Huwe, 2002).

The toxicity assessment of the different compounds to which these three groups of substances belong (PCDD, PCDF and PCB) and their congeners, are determined by comparison with the toxicity of 2,3,7,8, TCDD, also called “Seveso dioxin” and are expressed in special units of toxic equivalence (Table 1) (Jensen, 2003; Rappe, 2003).

In toxicological concepts, the most toxic congener, 2,3,7,8-TCDD, is referred to as "toxic equivalency factor dioxin" (TEF) = 1.0 (Luscombe, 1999).

Table 1 - Toxic Equivalence Factors (TEF) > 0 of PCDD/Fs e PCBs (1998 – WHO).

Congeners		WHO- TEF
PCDD		
2,3,7,8-TCDD		1
1,2,3,7,8-PeCDD		1
1,2,3,4,7,8-HxCDD		0.1
1,2,3,6,7,8-HxCDD		0.1
1,2,3,7,8,9-HxCDD		0.1
1,2,3,4,6,7,8-HpCDD		0.01
OCDD		0.0001
PCDF		
2,3,7,8-TCDF		0.1
1,2,3,7,8-PeCDF		0.05
2,3,4,7,8-PeCDF		0.5
1,2,3,4,7,8-HxCDF		0.1
1,2,3,6,7,8-HxCDF		0.1
1,2,3,7,8,9-HxCDF		0.1
2,3,4,6,7,8-HxCDF		0.1
1,2,3,4,6,7,8-HpCDF		0.01
1,2,3,4,7,8,9-HpCDF		0.01
OCDF		0.0001
Não-ortho-PCB		
3,3’,4,4’-TCB	(77)	0.0001
3,4,4’,5-TCB	(81)	0.0001
3,3’,4,4’,5-PeCB	(126)	0.1
3,3’,4,4’,5,5’-HxCB	(169)	0.01
Mono-ortho-PCB		
2,3,3’,4,4’-PeCB	(105)	0.0001
2,3,4,4’,5-PeCB	(114)	0.0005
2,3’,4,4’,5-PeCB	(118)	0.0001
2’,3,4,4’,5-PeCB	(123)	0.0001
2,3,3’,4,4’,5-HxCB	(156)	0.0005
2,3,3’,4,4’,5’-HxCB	(157)	0.0005
2,3’,4,4’,5,5’-HxCB	(167)	0.00001
2,3,3’,4,4’,5,5’-HpCB	(189)	0.0001

(...) related compounds that are supposed not to have the same effect as dioxins.

Adapted from Meyer *et al.*, 2004

TEF values assigned to the other congeners are classified in relation to the toxicity of 2,3,7,8-TCDD. To determine the TEQ value of a food or in the atmosphere, the concentration of each congener is multiplied by its TEF value. The total TEQ for a mixture of PCDD/Fs and PCBs can then be established by summing the individual values of the set of congeners (Luscombe, 1999).

In the 1998 review, WHO established the most recommended TEF models for use with mammals and humans and separately developed models for birds and fish because it is not possible to synchronize TEF values between mammals, birds and fish.

Notwithstanding the uncertainties that may compromise the TEF values concept when used for risk analysis purposes (such as non-cumulative interactions, differences in the form of the dose-response curve and the different responses of the species to exposure, prediction of TEQ values), the TEF model, continues to be considered the most appropriate and practical for the risk analysis of compounds with properties similar to those of dioxins (Table 2) (Van den Berg *et al.,* 1998).

Non-dioxin-like PCBs ("classical" or "non-coplanar" PCBs) have a different toxicological profile from dioxins and are therefore not used for the determination of TEQ. These circulate more easily through the muscles and blood, directly affect the nervous system and the brain development. Their concentration in the aquatic biota, namely fish and molluscs, is of several orders of magnitude greater than that of dioxins (CEC, 2001).

In June 2005, a World Health Organization (WHO)-International Programme on Chemical Safety expert meeting was held in Geneva during which the toxic equivalency factors (TEFs) for dioxin-like compounds, including some polychlorinated biphenyls (PCBs), were re-evaluated (Table 3). Changes were decided by the expert panel for 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (TEF=0.3), 1,2,3,7,8-pentachlorodibenzofuran (PeCDF) (TEF = 0.03), octachlorodibenzo-p-dioxin and octachlorodibenzofuran (TEFs = 0.0003), 3,4,4',5-tetrachlorbiphenyl (PCB 81) (TEF = 0.0003), 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) (TEF=0.03), and a single TEF value (0.00003) for all relevant mono-ortho-substituted PCBs.

Table 2 - Toxic Equivalence Factors of PCDD/PCDFs proposed by NATO/CCMS (1988) and by WHO (1998).

Congeners	**I-TEFs (NATO/CCMS)**	**WHO-TEFs**		
		Human/ Mammals	fish	Birds
PCDD				
2,3,7,8-TCDD	1	1	1	1
1,2,3,7,8-PeCDD	0.5	1	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1	0.5	0.05
1,2,3,6,7,8-HxCDD	0.1	0.1	0.01	0.01
1,2,3,7,8,9-HxCDD	0.1	0.1	0.01	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01	0.001	< 0.001
OCDD	0.001	0.0001	< 0.0001	0.0001
PCDF				
2,3,7,8-TCDF	0.1	0.1	0.05	1
1,2,3,7,8-PeCDF	0.05	0.05	0.05	0.1
2,3,4,7,8-PeCDF	0.5	0.5	0.5	1
1,2,3,4,7,8-HxCDF	0.1	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.01	0.01
OCDF	0.001	0.0001	0.0001	0.0001

Adapted from CCME, 2002.

Additivity, an important prerequisite of the TEF concept was again confirmed by results from recent *in vivo* mixture studies. Some experimental evidence shows that non-dioxin-like aryl hydrocarbon receptor agonists/antagonists are able to impact the overall toxic potency of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds, and this needs to be further investigated. Certain individual and groups of compounds were identified for possible future inclusion in the TEF concept, including 3,4,4'-TCB (PCB 37), polybrominated dibenzo-p-dioxins and dibenzofurans, mixed polyhalogenated dibenzo-p-dioxins and dibenzofurans, polyhalogenated naphthalenes, and polybrominated biphenyls. Concern was expressed about direct application of the TEF/total toxic equivalency (TEQ) approach to abiotic matrices, such as soil and sediment, for direct application in human risk assessment. This is problematic, as the present TEF scheme and TEQ methodology are primarily intended for estimating exposure and risks via oral ingestion (*e.g.*, by dietary intake). A number of future approaches to determine alternative or additional TEFs were also identified. These included the use of a probabilistic methodology to determine TEFs that better describe the associated levels of uncertainty and "systemic" TEFs for blood and adipose tissue and TEQ for body burden (Van den Berg *et al.*, 2006).

Table 3 - WHO-TEFs for human risk assessment based on the conclusions of the WHO - 0151 International Programme on Chemical Safety (IPCS) expert meeting, Geneva, June 2005.

Congener	TEF value	Congener	TEF value
Dibenzo-p-dioxins ('PCDDs')		'Dioxin-like' PCBs, Non-ortho PCBs + Mono-ortho PCBs	
2,3,7,8-TCDD	1		
1,2,3,7,8-PeCDD	1	**Non-ortho PCBs**	
1,2,3,4,7,8-HxCDD	0.1	PCB 77	0.0001
1,2,3,6,7,8-HxCDD	0.1	PCB 81	0.0003
1,2,3,7,8,9-HxCDD	0.1	PCB 126	0.1
1,2,3,4,6,7,8-HpCDD	0.01	PCB 169	0.03
OCDD	0.0003		
Dibenzofurans ('PCDFs')		**Mono-ortho PCBs**	
2,3,7,8-TCDF	0.1	PCB 105	0.00003
1,2,3,7,8-PeCDF	0.03	PCB 114	0.00003
2,3,4,7,8-PeCDF	0.3	PCB 118	0.00003
1,2,3,4,7,8-HxCDF	0.1	PCB 123	0.00003
1,2,3,6,7,8-HxCDF	0.1	PCB 156	0.00003
1,2,3,7,8,9-HxCDF	0.1	PCB 157	0.00003
2,3,4,6,7,8-HxCDF	0.1	PCB 167	0.00003
1,2,3,4,6,7,8-HpCDF	0.01	PCB 189	0.00003
1,2,3,4,7,8,9-HpCDF	0.01		
OCDF	0.0003		

The European Commission adopted the result of this re-evaluation in 2012 for calculation of TEQ concentrations. The concentrations of the individual substances in a given sample shall be multiplied by their respective TEF, as established by the

WHO in 2005 (Table 3) and subsequently summed to give the total concentration of dioxin-like compounds expressed as TEQs (EC, 2012).

2.5.2 Tolerable Daily Intake

In 1990, WHO estimated that more than 90% of human exposure to dioxins occurs through diet and mainly food of animal origin, since animals function as recyclers, leading to the accumulation and consequent biomagnification of dioxins in the food chain. Food of animal origin approved for consumption may be responsible for the daily intake of approximately 2 pg TEQ/kg body weight. Other food, particularly those with low lipid content, is less important in terms of daily intake (Fiedler *et al.*, 2000).

In 1998, WHO, the United States Agency for Toxic Substances and Disease Registry (ATSDR), the WHO Joint Expert Committee on Food Additives (JECFA/WHO) and the European Commission's Scientific Committee on Foodstuffs (ECSCF) published an extensive study on the effects of dioxins on human and animal health based on results obtained in animal models. It was recommended that the average daily dose of dioxin exposure should be limited to values within the range of 1 to 4 pg TEQ/Kg of body weight, to ensure that serum levels do not reach troubling values (WHO, 1998; Otles and Yildiz, 2003).

At a meeting in Bilthoven, Holland, in 1990, WHO experts had recommended a tolerable daily intake (TDI) for dioxins and furans of 10 pg TEQ/kg body weight/day (Fiedler *et al.*, 2000). At this time, it would have been agreed that the recommended value should be changed to 1 to 4 pg TEQ/kg/day, after having discussed the results on studies in industrialized countries indicating that the maximum daily dose of PCDD/Fs was estimated at values of 50 to 200 pg TEQ/person/day or 1 to 3 pg TEQ/kg body weight/day for an adult of 60 kg. If dioxin-like PCBs are included in the total intake, total TEQ may be 2 to 3 times higher (Fiedler *et al.*, 2000).

The EU's Scientific Committee for Food (ECSCF) adopted, in 30 May 2001, an opinion on the risk assessment of dioxins and dioxin-like PCBs in food. An acceptable weekly exposure (TWI) was established for dioxins and PCBs in the form of dioxins of 14 pg (WHO-TEQ)/Kg of body weight. This TWI is in line with the tolerable monthly exposure of 70 pg (WHO-TEQ)/kg body weight established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at its fifty-seventh meeting (Rome, 5-14 June 2001). It also corresponds to the lower limit of the TDI range of 1-4 pg WHO-TEQ/kg body weight established by the WHO, following the consultation in 1998. Recent and representative daily exposure data indicate that average daily doses of dioxins and PCBs in the form of dioxins in the EU are between 1 and 3 pg WHO-TEQ/kg of body weight per day, which means that a considerable part of the European population will continue to exceed TWI or TDI (CEC, 2001).

In the USA, based on the 2003 CDC (Centre for Disease Control and Prevention) report, the average level of dioxins in the serum lipids of the population screened by that body would already be below the detection limits of the techniques used. Based on this study, 95% of the monitored population had serum levels of dioxins below 16.8 pg TEQ/g. Compared with previous studies, in which the average serum levels of dioxins in the American population studied in 1970 were close to 80 pg TEQ/g, it was concluded that in 2003, 95% of the US population had serum levels of dioxins four times lower than the levels found in 1970. This data indicates that the effort that has been made to reduce dioxin emissions has produced results, since there is a clear trend of decreasing levels of dioxins in the American population (Otles and Yildiz, 2003).

2.5.3 Dioxin adverse health effects

Most of the available knowledge on the effect of dioxins on health, was obtained from *in vitro* studies (*i.e.* cell culture), from animal testing and from epidemiological studies. However, available data indicate that there is a good correlation between the effects of dioxins in laboratory animals and those described in epidemiological studies in humans (Heuvel and Lucier, 1993; ATSDR, 1998; Grassman *et al.*, 1998).

Various hypotheses have been advanced for the explanation of most or all of the deleterious effects of dioxins and similar compounds on health.

In the mammalian, fish and bird organisms, the dioxin molecules bind to a specific soluble cellular protein Aryl Hydrocarbon Receptor (AhR). Although the mechanism of action is not fully understood, it is known that dioxins can produce changes in the regulation of various genes (expression and/or repression) by altering cellular function. In this way, they interfere with the normal functioning of hormone-producing organs and chemical messengers that interfere with the growth and general regulation of the organism, especially in detoxification mechanisms (WHO-ECEH, 1998; Environment Canada, 2003).

Experiments conducted in recent years have established that the most toxic effects of PCDD/F and some PCB, such as dermal toxicity, immunotoxicity, carcinogenicity and reproductive and developmental toxicity, are mediated by the AhR present in most animal tissues and human (Okey *et al*., 1994; McGregor *et al.*, 1998; EC, 2000b).

Developmental neurotoxicity has been reported in animals and there is evidence of this effect in humans in epidemiological studies in children exposed *in utero* to non-coplanar PCB.

PCB can be grouped into two categories, according to their mechanism of toxicity. Non-coplanar PCBs predominantly have neurotoxic effects. Coplanar PCBs have a dioxin-like effect and also act through the receptor (Ah) and, like PCDD/F, coplanar PCB have a significant number of toxic effects mediated by AhR (CEHPA, 2001).

The ligand (*e.g.* TCDD) enters the cell by passive diffusion through the cell membrane and binds to the Ah receptor (AhR). The binding affinity depends on both the characteristics of the host and the properties of the ligand. After binding of the ligand in the cellular cytosol to the AhR, the TCDD-AhR complex undergoes a transformation/ activation and moves to the nucleus where it interacts with the Aryl hydrocarbon nucleon-transferase (ARNT) protein to form, by phosphorylation, a heterodimer complex linked to specific DNA strands, and may activate the expression or repression of several genes. Binding of the Dioxin-like compounds to the Ah receptor is correlated with the induction of oxidative enzyme systems such as cytochrome P-450-1A1 and cytochrome P-450-1A2. These enzymes belong to a family of 12 cytochrome P-450 isoenzymes, a group of chemically distinct but functionally similar enzymes, found mainly in the endoplasmic reticulum of hepatocytes (Heuvel and Lucier, 1993; Swanson and Bradfield, 1993; Safa, 1998; Sahlberg *et al.,* 2002)

Cytochrome P-450 enzymes are essential for the synthesis of steroids and act on the metabolism of other endogenous compounds. Some of these enzymes are involved in the processes of bio-transformation, conjugation and removal or bio-activation of xenobiotics. Enzyme induction is a biochemical response to exposure to PCDD/F, however enzyme activity does not guarantee that a toxic response is imminent and does not necessarily have any connection with a toxic expression (Environment Canada, 2003).

According to the EPA and to the available scientific data, the interaction of dioxins with the Ah receptor is essential for expression of an adverse effect, with different responses from organisms of the same species and different species related to that receptor binding Ah (US EPA, 2004). For example, this binding to the Ah receptor may explain the fact that the lethal dose of 2,3,7,8-TCDD is 5000 times lower for the guinea pig than for the hamster, the less sensitive species known (Heuvel and Lucier, 1993; Fiedler *et al.*, 2000).

Different dioxin congeners bind to the Ah receptor with different strength giving rise to a change in toxicity. Although there is evidence that the functioning of the Ah receptor is involved in many of the different effects caused by dioxin, there may be effects of TCDD that are not mediated by this receptor. For example, 2,3,7,8-TCDD induces apoptosis in cultures of two human leukemic lymphoblastic T cell lines. However, it was found that the death of these cells was not dependent on the Ah receptor (Hossain, 1998).

Evidence suggests that PCDD/F can contaminate living beings in a variety of ways. Studies indicate that oral absorption may be greater than 50%. However, a study in humans points to an approximate 90% absorption and other studies point to the lack of significant inter-species differences in the gastrointestinal absorption of these compounds in mammals (Muller, 2002).

The dermal absorption of PCDD/F is very slow and dose dependent and most of the administered amount is retained in the layer of the *stratum corneum* of the skin. The percentage of dermal absorption is estimated to range from 10% to 40%, with the absolute amount increasing and the percentage of absorption decreasing with increasing dose (Muller, 2002).

Studies with rodents suggest that trans pulmonary absorption of PCDD/F is almost total (Muller, 2002).

Exposure of foetuses, transplacental, to dioxin-like compounds in maternal blood is also described. In addition, it may be noted that the major source of exposure in the postnatal period is through the ingestion of breast milk (Muller, 2002).

After gastrointestinal absorption, PCDD/F are distributed throughout the lymphatic system. When they enter the bloodstream these compounds are initially found in well-irrigated tissues, but within a few hours the highest concentrations are found in the liver and adipose tissue. The distribution of coplanar PCB is similar to PCDD/F. The half-life for elimination of the various tissues is from hours to weeks in rodents, but in one study in Rhesus monkeys, the half-life for elimination of adipose tissue was about one year. In humans, the half-life for the elimination of adipose tissue and/ or blood is years (Muller, 2002).

PCDD/F are eliminated more in the urine than in the faeces and most are metabolites of the administered compounds (Muller, 2002).

The adverse effects of dioxins are well established in studies with animal experimentation models and highly exposed human populations (Grassman *et al.*, 1998).

The reasons to consider animal models relevant for assessing and predicting human response, are as follows (Grassman *et al.,* 1998):

- Immune, developmental and reproductive responses as well as the carcinogenic responses to dioxins observed in humans also occur in animal models;
- The preponderance of the biochemical effects induced by dioxins, both in animals and in humans, are mediated by the Ah receptor;
- Animal dosage schedules may be wide-ranging so that the range of exposures found in human populations can be examined;
- Quantification of doses based on the internal dose (tissue dose and body load) can be used to compare responses between species, since these

parameters consider the differences between species regarding the clearance rates;

- Biochemical responses to dioxins in animal models show quantitative and qualitative similarity to those observed in human beings.

Occasional exposure to high concentrations of dioxins (acute toxicity) may lead to hepatic impairment, weight loss, and typical skin lesions known as reversible, but often disfiguring "chloracne", characterized by bumps and dark spots on the skin, skin hyperkeratosis and hyperplasia (Luscombe, 1999; Charnley and Kimbrough, 2005).

Longer exposure induces cancer and leads to diminished immune system, nervous and endocrine system changes, reproductive function and physical and mental development (Luscombe, 1999).

The effects of dioxins on human health are therefore very diverse and can be very variable among individuals, suggesting the existence of genetic differences that produce a different response of cells to dioxins (Heuvel and Lucier 1993; Yildiz, 2003).

Studies in Europe and the US show that permanent perinatal exposure to dioxins and PCBs has effects on brain development and thyroid hormone metabolism, causing interference in intelligence and behavioural changes and intellectual and sexual development; in the liver changes in the levels of liver enzymes are noted; in the bone marrow interference in haematopoiesis occurs, in thymus may appear early involution, and pulmonary function may also be affected. An effect also described is the change in the relationship between the birth rates of males and females, with a clear increase in female offspring. This phenomenon was verified after the Seveso accident and in the Yusho and Yucheng accidents, where in the first case the rate was 0.31 (328 boys and 346 girls were born between 1977 and 1996 from potentially exposed parents) when normal is 0.51, *i.e.*, 106/100 (ATSDR, 2000; Fiedler *et al.,* 2000; Mocarelli *et al*., 2000; Okubo *et al.,* 2000; CEHPA, 2001; Yoshimura *et al.,* 2001).

Seventy-one men were studied which, at the time of Seveso's accident in Italy had about six years. After about thirty years of this exposure showed a decrease in the number and motility of spermatozoa (Mocarelli *et al*., 2008).

Other adverse health effects have been described following the accidental exposure of factory workers to large concentrations of dioxins. In these individuals chloracne occurs and there is a small increase in the incidence of cancer (Otles and Yildiz, 2003).

Chronic exposure of animals to dioxins has led to the appearance of several types of cancer, however, the International Agency for Research on Cancer (IARC), which classified TCDD as a human carcinogen, assessed tetrachloro-dibenzodioxin and concluded that this compound did not affect the genetic material and that there was an exposure limit below which the risk of cancer may be negligible (McGregor *et al.,* 1998).

Epidemiological studies with individuals exposed to high levels in certain industries in Germany, the Netherlands and the USA, have concluded that there is an increase in the cancer mortality rate (Fiedler *et al.,* 2000).

In these groups exposed to the same risk factors, average concentrations of 2,3,7,8-TCDD in blood lipids were in the range of 2,000 ng/ kg (with maximum of 32,000 ng/ kg) in the US group. The average concentrations of affected workers in the Dutch group were 1434 ng/ kg (range 301-3,683 ng/ kg). Average values of the order of 1008 ng/ kg were detected in workers with severe chloracne in the BASF accident in Germany and concentrations of the order of 2252 ng/ kg were detected in the Boehringer German workers group (Fiedler *et al.,* 2000).

These concentrations of 2,3,7,8-TCDD found in workers' blood after exposure are comparable to those found in the blood of 2-year-old rats subjected to a carcinogenicity study. In mice exposed to 100 ng of 2,3,7,8-TCDD/ kg body weight/ day, hepatocellular carcinomas and squamous cell carcinomas of the lung, were observed. The concentrations of 2,3,7,8-TCDD found in the blood of these rats were in the range of 5,000-10,000 ng/ kg. These studies reveal that there is a parallelism in the carcinogenic response to 2,3,7,8-TCDD exposure in humans and rats (Fiedler *et al.,* 2000).

Based on the above results, it can be inferred that a residual exposure to 2,3,7,8-TCDD of 2-3 ng/kg, to which human populations are currently exposed, is 100 to 1000 times lower than the inducers doses recorded in rat carcinogenicity studies (Fiedler *et al.,* 2000).

There is, however, some disagreement regarding the linearity of the correlation between the exposure limit and the expression of the carcinogenic processes. USEPA's opinion is based on the linearity of the correlation and thus on the absence of a threshold from which it can be extrapolated that there is a potential risk of cancer. ECSCF and JECFA have concluded that in exposure to dioxins, cancer is an extreme phenomenon. They concluded, in particular with respect to 2,3,7,8-tetrachloro-dibenzodioxin, that this was not a carcinogenic initiation factor and that there was a tolerable threshold of exposure to that compound for all purposes, including cancer, and that the carcinogenicity of the compound would not be linked to mutagenicity or its effect on DNA binding but rather to immunosuppression (Otles and Yildiz, 2003).

One of the most worrying aspects of dioxin toxicity is clearly the extreme sensitivity of the developing foetus and child. Neurological and behavioural effects may persist during school age (CEHPA, 2001).

While the WHO emphasizes that the ultimate goal for human exposure should be less than 1 pg/kg/day, based on lifetime exposure, there is still considerable concern about youth exposure levels. This is of particular concern and is in accordance with available laboratory evidence demonstrating lifelong effects resulting from single low doses at certain critical times such as during gestation (Luscombe, 1999).

The toxic effects in other species show substantial differences, and the toxicological mechanisms are still not well understood (Eisler, 1986; Eisler, 2000) nor well studied:

- Invertebrate organisms: Studies in two species of earthworms showed that they did not express adverse effects after 85 days exposed to an environment highly contaminated with 5 mg/kg of 2,3,7,8-TCDD, but both species died in an environment with 10 mg/kg 2,3,7,8-TCDD. In environments containing concentrations of 50 µg of 2,3,7,8-TCDD, these earthworms accumulated five times the levels of the environment in 7 days. No absorption of dioxins was observed on the surface of the body and there was no degradation of TCDD during digestion, since its excrement showed the absence of mono, di and tri-CDD;

- Aquatic organisms: Sensitive teleost fish species showed reduced growth and necrosis of fins at concentrations below 0.1 ppt of 2,3,7,8-TCDD after a 24 to 96-hour exposure. Concentrations of 1 ppt or higher were eventually fatal. Histopathological and teratogenic effects were reported in very young rainbow trout whose eggs were exposed to concentrations of 10 ppt of 2,3,7,8-TCDD for 96 hours. Some had extensive degeneration and hepatic necrosis, followed by edema and death; the others had teratogenic alterations including operculum and maxillary defects;

- Birds: There are more sensitive species in which the LD_{50} is only 15 µg/kg body weight and other less sensitive ones whose LD_{50} can be 810 µg/kg body weight. Duck (*Anas platyrhynchos*) has an intermediate sensitivity with an LD_{50} of about 108 µg/kg body weight. Signs of intoxication appeared about 7 days after treatment including polydipsia, loss of appetite, hypoactivity, emaciation, weakness, muscle incoordination, hypersensitivity, bruised feathers, tremors, spasms, seizures, immobility and death up to 37 days. At necropsies, the liver was hypertrophied to about twice the normal size and accumulation of fluid in the pericardium and abdominal cavity. Chickens exhibit relative sensitivity to PCDDs with an LD50 of 2,3,7,8-TCDD ranging from 25 to 50 µg/ kg body weight. Chickens fed with 1 to 10 µg/ kg body weight daily for 21 days of 2,3,7,8-TCDD, 1,2,3,7,8,9-hexa CDD or hepta CDD showed signs of edema of the pericardium, subcutaneous and peritoneal, as well as hepatic hypertrophy and necrosis with fat degeneration, ending in death. These signs were observed in Italy in broilers in the case of Seveso in 1976;

- Mammals: Comparative toxicological studies in guinea pig (*Cavia* sp.) and mouse (*Mus* sp.) confirm significant differences in sensitivity and toxic effects between species, for example 2,3,7,8-TCDD produces severe skin lesions (Chloracne) in humans and monkeys, edema in birds and severe liver damage in rats, mice and rabbits. This marked difference is also observable among guinea pigs whose LD_{50} for 2,3,7,8-TCDD is 8400 times lower than that for hamsters (*Cricetus* sp.).

Direct exposure of humans or animals to dioxins, PCDFs or PCBs may occur through inadvertently or accidental liberation of those substances in the atmosphere, although those are not the most frequent way for living beings exposed. Water and food throughout the production chain are the most common vehicles.

2.6. Wood preservatives and the occurrence of PCDD/F and PCBs

Wood is one of the most valuable resources in the world. The world's timber needs will continue to grow because it is an easy material to work with, both simple tools and industrial machinery.

Wood can withstand higher loads compared to iron and may become resistant to most chemicals; it is a good insulator for electricity and temperature differences, being one of the best insulators used in construction. Wood has many useful uses, both in terms of construction and furniture as well as in the paper industry but, it is surprising to know that half the wood produced worldwide is burned. Wood had been the main source of thermic energy until the mid-nineteenth century and its use as a fuel is now more pronounced in developing regions of Africa and South America.

Today, meeting global wood needs has meant increasing production with improved forest management and finding industrial processes that waste less wood, as well as finding new ways to value the by-products of the wood industry and increasing wood durability.

Despite its incomparable characteristics that have made wood, an essential commodity for civilization, it can be damaged by the action of insects and fungi, when they are in environments that allow the access and development of these organisms.

The durability of the wood is variable with the environmental conditions to which they are exposed and with the species of wood. The wood found in the Egyptian pyramids kept in hot and very dry environment, lasted for thousands of years. Under natural conditions, in the temperate climate, there are species that normally last decades, even in contact with the soil but others do not last for ten years (Wilkinson, 1979).

The treatment of wood easily allows to increase its durability by about six times. The techniques of wood preservation must not be considered a novelty, since some of these techniques were already practiced by ancient civilizations. The industrialization of wood treatment began in England around the early 1800s with the treatment of wood beams for railroad growth and telegraph poles (Wilkinson, 1979).

In Portugal, modern timber conservation began later, probably in the early 20th century. The first wood preservative used was "carbolineum" (Europe) or "anthracene oil" (USA), which is an aromatic polycyclic hydrocarbon that has fungicidal action. Subsequently, other treatments were used, such as copper naphthenate, also with fungicide action, and Pentachlorophenol, which is a polychlorinated aromatic compound with disinfectant, fungicide, insecticidal, bactericidal and molluscicidal action. Until the Second World War, copper sulphate treatments were used for railroad and telephone and telegraph poles, obtained in the “Pinhal de Leiria” region, Marinha Grande and Figueira da Foz (Nunes *et al.* 2016).

Later, the treatment of railroad beams with "creosote" began, and around 1952, the treatments with salts mixtures were implemented and vacuum impregnation started to be used (Nunes *et al.*, 2016).

In 1985, there were already 14 companies with 26 vacuum cylinders installed in 17 different locations in Portugal. These companies mainly used mixtures of salts including Chromium Copper Arsenate (CCA). This product was only withdrawn in 2003 because of the possibility of volatilization of arsenic and replaced by CCB, in which arsenic was replaced by boron. Currently, CCB is also no longer used and has been replaced by other formulations that still maintain copper (Nunes *et al.*, 2016).

In Portugal, wood treatment is carried out by 23 companies and is dominated by the production of posts, poles and stakes, with an approximate production of 84300 cubic meters: the most used species to produce those devices is *Pinus pinaster* (Nunes *et al.*, 2016).

Currently, the commercial products used in Portugal are based on Alkaline Quaternary Copper (ACQ). It is a wood preservative, recently introduced in countries in which there is a need for alternatives to Cromated Copper Arsenate (CCA). The products contain copper, a bactericide and fungicide, and a quaternary ammonium compound (quat), which acts as a biocide, increasing the tolerance of treated wood to copper-resistant bacteria and fungi and also acts as an insecticide (USFS, 2006).

The most commonly used commercial products in Portugal are "*Celcure C4*", "*Celcure VS725*", "*Korasit K2*" whose active ingredients are essentially quaternary ammonium salts and copper and "*Tanalith E 8001*", whose active ingredients are propiconazole, tebuconazole, baramine and copper and "*Coprol Premium*", whose active principles are propiconazole and copper. Of the twenty-three existing companies, fifteen use the "*Tanalith E 8001*", two use the "*Celcure C4*" or "*VS725*", four use the "*Korasit*" and two the "*Coprol Premium*". As "*Celcure*" is used by the two largest companies, this product shares with "*Tanalith E*" the leadership of the internal market, with "*Korasit*" and "*Coprol*" having a marginal market share (Nunes *et al.*, 2016).

The log of the trees has two zones that usually have different colours, heartwood and sapwood. The central part of the log is the heartwood, usually darker, and the peripheral part, the sapwood, usually lighter. The sapwood can be defined as the part of the trunk that contains living cells and reserve nutrients, presenting bands whose thickness varies from species to species, with vigour of growth and tree age. The heartwood can be defined as the central part of the trunk that has ceased to have living cells and that the reserve nutrients have been converted into fibres of material resulting from the death of the parenchyma cells and which prevents the circulation of liquids in the cells (Chapelet *et al.*, 1991).

The applicability of preservatives is closely related to the permeability of wood. Some species are poorly permeable to preservative solutions, even using pressure, while other species are very permeable. There are woods that are 10 million times more permeable than others (Wilkinson, 1979).

Despite the complexity of the structure of the wood, in the coniferous trees the water conduction system is very primitive, the preservative solution runs mainly through the tracheids, the most common cells, which form a continuous path to the circulation and a path less important, from radial tracheids to longitudinal tracheids. Some species of conifers contain resin, in these the flow of the solutions can also be produced by the resin channels (Chapelet *et al.*, 1991).

In the hardwood trees (Angiosperms), which are considered more evolved plants, the conduction is carried through specialized cells for the conduction of water, denominated vessel elements, that lead the water that is absorbed by the roots, for the rest of the plant (Chapelet *et al.*, 1991).

2.6.1. Chemical composition of wood preservatives

Wood preservatives are generally classified as being based on the chemical composition of the preservative and the solvent used during the treatment process.

Some preservatives may be water-soluble, *i.e.*, those whose solvent is water. Waterborne preservatives often include some kind of co-solvent such as an amine or ammonia to maintain one or more active principles in the solution.

The preservatives may also be oily or oil soluble, *i.e.* those which are dissolved in a type of organic solvent.

Each solvent has advantages and disadvantages, depending on the objective of the application.

Wood preservatives can also be classified or grouped by type of application or environmental exposure of the wood. Some preservatives have sufficient leaching resistance for situations where wood is directly exposed to soil and water.

2.6.1.1 Oil-born biocides for wood preservation

The most common oil-born preservatives are creosote, pentachlorophenol and copper naphthenate. Occasionally, copper-8 quinolinolate and IPBC (3-Iodo-2-propynyl N-butylcarbamate) are also used for above-ground applications. Conventional oily-type preservatives, such as creosote and pentachlorophenol solutions, have been largely confined to uses that do not involve frequent human contact. The exception is copper naphthenate, which is a more recently developed preservative and has been less widely used. Oily-type preservatives have an oily appearance, oily consistency to the touch, and sometimes have a noticeable odour. The oil or oil solvent, which is used as a carrier, makes the wood less susceptible to cracking (USFS, 2006).

Creosote

Creosote is composed of polycyclic aromatic hydrocarbons or neutral oils: mixtures of anthracene and naphthalene, which make up most of the creosote and are still part of the creosote formation of tar acids (phenols, cresols, xylenols and naphthols - constitute about 15% of creosote) and tar bases (pyridine, quinoline and acridine - constitute about 5% of creosote).

As there are some fungi, crustaceans and termites that are resistant to creosote, reinforced creosote has been developed for the treatment of wood, namely creosote reinforced with at least 2% pentachlorophenol, creosote reinforced with copper (incorporation of copper naphthenate, copper pentachlorophenate or other copper compounds, directly to creosote), Arsenic-reinforced creosote (0.3 to 0.4% arsenic trioxide, As_2O_3). Creosote can also be reinforced with malachite green and tributyltin oxide (bis(tri-n-butyltin)oxide) - TBTO (USFS, 2006; DETF, 2013).

Pentachlorophenol

The active pentachlorophenol (2,3,4,5,6-Pentachlorophenol) principles are chlorinated phenols that are crystalline solids that can be dissolved in different types of organic solvents. The effectiveness of pentachlorophenol (PCP) and the properties of the treated wood are influenced by the properties of the solvent.

This product is obtained by the direct chlorination of the phenol, it is insoluble in water and has acid character.

Due to the acidic character of pentachlorophenol, it can give rise to salts called phenates or phenoxides, when submitted to the action of alkaline hydroxides. But despite its high efficiency as a wood preservative and its insolubility in water, this product should not be used in the marine environment, because it is solubilized by the sodium salt present in sea water.

The molecular formula of pentachlorophenol is C_6Cl_5OH. In commercial form, it contains about 85% PCP, 6% tetrachlorophenols and 6% other types of chlorinated phenols, the remainder being inert materials. When PCP is subjected to the action of sodium hydroxide, it gives sodium pentachlorophenate (PCP-Na), a water-soluble product.

Pentachlorophenol is effective when used in contact with soil, freshwater, or above ground. It is not as effective as when used in sea water. A heavy oil solvent is preferred when the wood is treated to be used in contact with the soil. Wood treated with lighter solvents may be less durable.

The effectiveness of pentachlorophenol is similar to that of creosote for the conservation of hardwood and coniferous trees (USFS, 2006; DETF, 2013).

Carbolineum or Anthracene Oil

Carbolineum is an oily, water-insoluble, flammable, dark brown mixture from coal tar components, smelling of tar. It contains among other things anthracene and phenol.

Because of its rot-resisting and disinfecting effect, Carbolineum was used over many years for the preservation of wooden structures such as railroad ties, telephone poles and cabins.

Carbolineum is an aromatic polycyclic hydrocarbon which has fungicidal action obtained from a fraction of bituminous coal tar at a higher boiling temperature than that used to obtain creosote.

Carbolineum or Anthracene oil is usually applied in the rural environment by brushing, spraying or by immersing the wood (DETF, 2013).

Naphthenate

Naphthenic acids are petroleum derivatives. The most used in wood preservation are copper naphthenate, which has a fungicidal action and is used as an additive to creosote and zinc naphthenate, which is used as a preservative and is very effective against termites.

Copper naphthenate is effective when used in contact with soil, in contact with water, or above ground. It is not suitable for use on wood to be applied in contact with salt water. It is an organometallic compound product of the reaction of copper salts and naphthenic acids (USFS, 2006; DETF, 2013).

Copper-8-quinolinolate

Copper-8 quinolinolate is considered a wood preservation product because it is recognized as an effective fungicidal product and not toxic to mammals. For these reasons, it is accepted in the treatment of wood that can come into direct contact with foodstuffs, such as wooden boxes for transporting fruits and vegetables, among other similar uses.

Copper-8 quinolinolate is effective when used above ground. Its effectiveness is reduced when it is used in direct contact with the soil or with water. Copper-8 quinolinolate is an organometallic compound. Its formula consists of at least 10% copper-8-quinolinolate, 10% nickel-2-ethylhexanoate, and 80% inert ingredients. It is a good preservative for use above ground, as anti-sapstain, to control molds and is also used for treatment of wood under pressure.

The application of copper-8 quinolinolate in wood is normally done in concentrations of 2.5% to 5.0%. However, its application is rather limited due to the high cost of the product and does not justify the use in wood intended for other uses (USFS, 2006, DETF, 2013).

IPBC

The IPBC (3-Iodo-2-propynyl N-butylcarbamate) is not intended for use in contact with soil or on surfaces exposed to the weather. IPBC contains 97 percent butyl 3-iodo-2-propynyl carbamate, which includes a minimum of 43.4% iodine.

The IPBC preservative is a fungicide that, although it appears in oil soluble formulations, can also be used in aqueous formulations.

IPBC is colourless, not an effective insecticide and is generally not used as a single treatment but appears associated with other active principles.

IPBC has relatively low acute toxicity to mammals and has low toxicity to birds, but is highly toxic to fish and aquatic invertebrates.

IPBC has not been used in pressure treatments, so there has been little assessment of the environmental impact of IPBC treated wood (USFS, 2006).

2.6.1.2 Water-soluble wood preservatives

During the last decade, waterborne preservatives have become more widely used. Today the most commonly used preservatives are copper compounds (typically containing ammoniacal copper quaternary compounds or copper azole, whereas sometimes also other copper compounds). Some of the preservatives also contain chromium, boric acid and/or water-based micro emulsions such as azoles or quaternary ammonium compounds (Salminen *et al.*, 2014).

The aqueous preservatives react or precipitate in the treated wood, having a fixation, that allows it to resist leaching. As the aqueous preservatives leave the surface of the wood dry, it can be painted. They are generally used to treat wood used in homes, decks and fences. Aqueous preservatives are mainly used for the treatment of coniferous tree woods (softwood) because they are not effective in protecting wood from hardwoods against deterioration. The treatment of most wood species of hardwood trees is difficult with aqueous preservatives.

These preservatives may increase the risk of corrosion of metals in contact with treated wood used in humid places. All metals should be of galvanized iron, copper, silicon bronze, or stainless steel if the wood is treated with aqueous preservatives containing copper. In these cases, aluminium should not be used.

Aqueous preservatives consist mainly of metal salts and include the arsenic, chromium, copper, zinc and fluorine compounds.

Other metals and anions used with an insecticidal effect, such as mercury, nickel, thallium and cyanide, are not widely used for economic reasons (cost-benefit), lack of efficiency or high toxicity to humans and/or the environment.

Borate-based preservatives are another type of aqueous preservative that does not fix the wood and so leach quickly if exposed to rain or wet soil. Borate treatment is not likely to create corrosion of metals in contact with treated wood (USFS, 2006; DFET, 2013).

Sodium pentachlorophenate and Sodium tribromofenate

Both pentachlorophenol and tribromophenol can be easily transformed into sodium pentachlorophenate (PCP-Na) and sodium tribromofenate (TBP-Na), respectively, when exposed to sodium hydroxide to become water-soluble.

Although none of these preservatives are authorized to treat wood, both provide efficient treatments that meet the essential characteristics of wood preservatives.

The use of TBP-Na was carried out in substitution of sodium pentachlorophenate and, although not having the efficiency of PCP-Na, has been used industrially in the less demanding countries in the quality of wood preservatives due to its low cost in relation to other alternative products.

Chromated copper arsenate (CCA)

Chromated copper arsenate, commonly known as CCA, is marketed in products containing about 19% copper oxide (CuO), with different concentrations of chromium oxide (CrO_3) and arsenic oxide (As_2O_5) being present.

The CCA is suitable for wood used on the surface, in contact with the soil, or in contact with fresh or sea water. CCA-treated wood has dominated the treated wood market since the late 1970s until 2004. The CCA has been voluntarily discontinued by manufacturers for most applications around residential areas where human contact is likely (USFS, 2006 DFET, 2013).

"Wolmanit" CB (CCB)

The Chromated Copper Borate (CCB) is an alternative product to CCA, having the element boron in substitution of the arsenic. This difference in the composition of the product causes a slight loss in resistance to leaching, especially for wood that is not sheltered from the weather or in contact with water or damp soil. However, CCB is very efficient in situations where there are no factors favouring leaching (USFS, 2006; DFET, 2013).

Ammoniacal Copper Arsenate (ACA)

ACA is an effective product against a large number of xylophages fungi and is used in the treatment of wood by impregnation. It consists of arsenic, ammonia and copper hydroxide.

The ammonia present in the formulation of this preservative product opens the cell wall structure, allowing better diffusion of the active ingredients into the cells of the wood.

After treatment, the ammonia evaporates with the drying of the wood, causing precipitation of copper and arsenic elements in the form of copper arsenate (USFS, 2006; DFET, 2013).

Ammoniacal Copper Zinc Arsenate (ACZA)

Ammoniacal Copper Zinc Arsenate (ACZA) contains copper oxide (50%), zinc oxide (25%), and arsenic pentoxide (25%). ACZA is an enhancement of the above formulation, ACA. After treatment, the colour of treated wood varies from olive green to bluish green. The wood may have a slight ammonia odour until completely dried. ACZA is a preservative used to protect wood from deterioration and prevent insect attack in a wide range of exposures and applications (USFS, 2006).

Acid Copper Chromate (ACC)

The acid copper chromate is composed of chromium in the hexavalent form as one of the ingredients. It is a mixture of copper sulphate, sodium dichromate and chromium trioxide marketed under the trademark "CELCURE".

Chromium is included in the formulation of this product to reduce the corrosive effect of copper sulphate to metals and to precipitate copper in the form of insoluble copper chromate (USFS, 2006).

Alkaline Copper Quaternary (ACQ) Compounds

Alkaline Quaternary Copper (ACQ) is one of several wood preservatives that have been developed as alternatives to CCA. The fungicides and insecticides in the ACQ are copper oxide (67%) and a quaternary ammonium compound (quat). Many variations of the ACQ were produced. ACQ Type B (ACQ-B) which is an ammoniacal copper formulation, ACQ type D (ACQ-D) is a copper amine formulation, and ACQ type C (ACQ-C) is an ammoniacal-amine formulation combined with a quaternary ammonium compound.

The stakes treated with these three formulations are protected from fungi and insects when applied in contact with the soil (USFS, 2006; DFET, 2013).

Copper Azoles (CBA-A and CA-B)

Copper azole is another preservative formulation recently developed to address the restrictions applied to CCA. It is composed of copper amine, but with additional biocides, to protect the wood from decay and insect attack. The first formulation of azole copper developed was type A (CBA-A), containing 49% copper, 49% boric acid and 2% tebuconazole. Type A (CA-B) azolic copper does not contain boric acid. It is composed of 96% copper and 4% tebuconazole. Wood treated with this product has a tan-brown colour and little or no odour.

Copper azole is similar to ACQ except that the dissolved copper preservative is boosted by an organic triazole incorporated as an azolic co-biocide such as propiconazole or tebuconazole instead of the quaternary ammonium biocide used in ACQ. This product is effective with smaller retentions than required with ACQ for equivalent performance.

It is widely marketed under the brand name “*Wolmanized*” in North America, and under the “*TANALITH*” brand throughout Europe and other international markets (USFS, 2006; DFET, 2013).

Borates

Boric acid and sodium tetraborate (borax) are not sufficiently soluble to allow adequate treatment of the wood, but higher concentrations can be achieved if the solution is prepared using one part of boric acid and 1.54 parts of tetraborate sodium decahydrate. This saturated solution, if dehydrated, results in the commercial product known worldwide under the trade name "Timbor", considered the main borate preservative. The salt resulting from the dehydration of the solution with both ingredients corresponds to disodium octaborate tetrahydrate or DOT ($Na_2B_8O_{13}.4H_2O$), which has a borate content equivalent to 117.3% of boric acid (H_3BO_3).

Borate compounds are the most commonly used water-based preservatives. Non-fixing preservatives are easily leached. They are used with pressure treatment for wood used in areas with high risk of termites and as surface treatments for a wide range of wood products, such as shelters and the interior of wood structures (FPL, 1999).

Boron has some outstanding performance characteristics, including fungal and insect activity, low toxicity in mammals and a very affordable commercial value. Another advantage of boron is its ability to penetrate into hardwoods that generally resist traditional pressure treatment with other preservatives. Borate treated wood has no colour or odour and can be applied with a brush.

Boron has many potential rural applications, but the chemical is easily released from the wood when exposed to water. It may be an effective treatment for insect protection in areas permanently protected from water (USFS, 2006, DFET, 2013).

2.7. Wood preservation treatments

2.7.1 Pressure treatments

Pressure treatments force the preservative to penetrate the wood under pressure above the atmospheric pressure. Pressure treated wood is more common than all other methods combined and allows better and more uniform penetration of the preservative solution into the wood. In many species, deeper, more uniform penetrations and larger retentions require this method of treatment. Appropriate pressure methods for treating wood with antifungal and pesticides should be used when it is intended for use in situations of high danger of deterioration, namely by contact with soil and water (FPL, 1999).

Aqueous preservatives are generally applied by the full-cell process and preservative retention is controlled by adjusting the concentration of the treatment solution.

The main steps of this method may have several changes, but follows the same principle (FPL, 1999):

i. The wood is closed in the treatment cylinder and vacuum is applied for about half an hour or more to remove air from the cylinder and, as much as possible, of the wood;
ii. Depending on the system, the preservative is admitted into the cylinder without breaking the vacuum, at room temperature or heated;

iii. After the cylinder is full, the pressure is applied until the wood no longer holds preservative or until the required preservative retention is achieved;
iv. When the pressure period is over, the preservative is removed from the cylinder;
v. A short vacuum period is applied to remove excess product from the wood and thus prevent preservative dripping.

2.7.2 Treatments without pressure

There are various types of wood treatment without pressure, which differ greatly in penetration and retention levels, such as thermal process, dual-diffusion vacuum treatment, cold immersion and surface applications.

Looking from the industrial aspect, non-pressure treatments provide a lower durability than the pressure methods.

In industry, the most used methods are described below (FPL, 1999):

a) Immersion of the wood for a few minutes is mainly intended for antifungal treatments, applied after cutting the wood to prevent blueing (sapstain). Blueing is a term used to describe wood that has bluish or gray-black spots on the surface. This colouring is caused by wood fungi. The blueness of the wood is considered a fault that is taken into account during the classification, since this alteration of the colouring of the wood makes it unsuitable for some purposes and thus, less valued. This type of fungus develops on the cells of the wood, but the wood itself is not destroyed as there is no degradation. The discoloration is caused by fungi of the *Ascomycetes* and *Deuteromycetes* groups. There are between 100 and 250 different species of fungi that can cause blueness. The most important species are *Ceratocystis* (*Ascomycetes* group) and *Aureobasidium*, *Alternaria* and *Cladosporium* (*Deuteromycetes* group). The blue coloration can often be the result of mixed colonization’s. The blueness occurs mainly in coniferous wood, and pine wood is particularly susceptible. The risk of blueing can be significantly reduced by the correct choice of cutting season, storage conditions and, above all, by reducing the time between cutting and processing the wood. During the drying of recently cutted wood in the sawmill, the risk of blueness is especially high. Anti-blueing or anti-sapstain products are temporarily effective during the drying phase and prevent wood loss and recovery. Immersion treatment is sufficient to reliably prevent the occurrence of blueness. Other non-pressure methods, such as vacuum treatments for antifungal or anti-bluing and pesticide treatments are also used (USFS, 2006; DFET, 2013).

b) In the vacuum process, the wood is placed in a sealed container (autoclave) and an amount of air is withdrawn, creating a pressure lower than atmospheric pressure in the wood cells. The preservative is inserted into the tank under vacuum, covering the wood, and forces the preservative to penetrate the wood to occupy the space that was reserved for air. The vacuum method is most effective in wood having a high percentage of sapwood relative to the heartwood, such as pine. It is a process widely used in conjunction with the pressure method, since the penetration into the heartwood is much lower than that of the pressure methods, achieving higher level of penetrations and retention, if the wood is dehydrated before (FPL, 1999).

c) The thermal process or hot-cold bath, involves placing wood in a tank of hot preservative oil, followed by immersion in cold preservatives for several hours. The hot bath expands the air inside the wood, forcing the air out of the wood. The wood is then immersed in a cold bath and the heated air retracts, absorbing the preservative. The thermal process is often used in the treatment of posts, with pentachlorophenol or creosote solutions. The retention is greater than in the immersion method (FPL, 1999).

d) Double diffusion, is one of the few processes that uses green or wet wood for the treatment. The wood is successively treated in two solutions of water-based salt, generally one of fluorine and another of copper, which react with each other to form an insoluble compound. The chemicals diffuse into the wood from a high concentration solution to the wood having a low concentration. It produces excellent results on coniferous wood poles, but has not been widely used (FPL, 1999).

II. Part (Experimental work)

Specific objectives

To assess the three episodes of dioxin contamination in the poultry chain occurred in Portugal, by working in each of the "case study" to develop a risk assessment and management methodology on the basis of the available data obtained in real situations, in order to determine:

- the sources and modalities of the contamination of the animals (sources of exposure, including feed, drinking water, poultry atmosphere and litters);

- the role of bedding material made of wood shavings, treated with different preservatives and methods, in the contamination of the poultry litters;

- the development of an appropriate analytical "methodology" that, in the absence of an existing validated reference method, could be better adapted to the reference matrix – the wood chips;

- how the dioxin contamination is transferred throughout the food production chain, *i.e.*, transferred to birds, their meat, possible vertical transmission, eggs, chicks and broilers;

- the dioxins dynamic in the organism of the animals and the physiological mechanisms which could explain the existence of the contaminant in the hatching eggs and in the offspring;

- the evaluation of the contamination fingerprint in the different stages of the poultry production chain and the organic depletion in the live birds;

- in light of the results of this assessment, to put into perspective the way in which the data obtained enables to improve risk management by the application of control procedures and measures that may be appropriate to be implemented throughout the poultry production chain.

3. Determination of PCDD/F Levels in Wood Shavings Used as Bedding Material for Poultry Production

Abstract

During 2006 and 2011, following the implementation of dioxin contamination monitoring in poultry meat, levels higher than legally allowed in meat from poultry slaughtered for human consumption, were found. The wood shavings used as bedding material in the poultry farm showed considerable high contaminations, indicating that these materials were the likely source of contamination of the animals. Wood shavings samples (n=23), used as poultry litters in intensive farms of broilers, were analysed. In both episodes, contamination profiles of higher and lower concentrations, seems to be very similar, being OCDD, OCDF, 1,2,3,4,6,7,8-HpCDD and 1,2,3,4,6,7,8-HpCDF responsible for 97.4% of the total contamination. The present work describes the analytical adapted methodology used and the specific clean up procedures, which revealed that recoveries of 13C12- Labelled compounds added to the wood shavings samples ranged from 71.3% and 86.3%.

Key words: Dioxins, Wood shavings, Analytical method, Food chain.

3.1 INTRODUCTION

Safety of the food chain is periodically challenged due to the occurrence of PCDD, PCDF and PCBs contamination in food.

Some recent cases of contamination of the food chain are known. An accident in Belgium in 1999 with a tanker carrying frying oil for refining and incorporation in feed mixed with the container of coolant fluid containing dioxins. The oil ended up being used inadvertently and caused contamination of meat from different animals in various countries where the feed was marketed (Bernard *et al*., 2002).

In Ireland, in December 2008, following samples taken from a pig slaughterhouse in the national residue monitoring plan, results found PCB levels above the limit laid down in Regulation (EC) N.º 1881/2006 of the Commission of 19 December

2006 (CEC, 2006b). The animal source of contamination found was feed from a waste recycling facility.

During 2006 and 2011, in Portugal, following the implementation of a National Residues Monitoring Plan, the competent authority found residues of dioxin contamination in poultry meat with levels higher than legally allowed in meat from poultry slaughtered for human consumption.

To identify the original source of contamination of these birds, all potential sources of contamination were analysed and the results showed considerable high contaminations of the wood shavings used as bedding material in the poultry farm, indicating that these materials were the likely source of contamination of the animals (Cardo *et al*., 2014)

However, the absence of a well stablished laboratorial methodology applied to that specific matrix (wood shavings) led to the need to adapt the method 1613b (USEPA, 1994).

In this paper it is described the method applied for the determination of PCDD/Fs in this type of matrix and is also done the interpretation of the results, particularly in what regards the source of contamination.

3.2 MATERIALS AND METHODS

3.2.1 Sampling

Sapling procedures, packing, transport and storage were performed by officers from the competent authority, under the scope of the National Residue Monitoring Plan, respecting the official procedures to ensure stability of conditions and integrity of the sample, to avoid causing any change that could affect the level of dioxins. Each sample was individually packed and labelled in opaque polyethylene sample bags, immediately after sampling. Samples of litter were preserved in dry, cool places.

3.2.2 Materials

All chemicals used were residue analysis Pico grade. Native and carbon-13 labelled PCDD and PCDF standards were obtained from Cerilliant, CIL Cambridge

Isotope Laboratories (LGC, Barcelona, Spain) and from Wellington Laboratories (Technospec, Barcelona, Spain). Carbosphere activated carbon, 80-100 mesh, with a surface area of 1000 m2/g was obtained from Altech (I.L.C., Lisbon). The alumina Basic Super I for dioxin analysis was purchased from ICN (Promochem, Barcelona, Spain).

3.2.3 Extraction procedure

The samples of wood shavings were grinded and homogenised, then mixed with sodium sulphate and transferred to a Twisselman extractor. Prior to extraction, samples were fortified with a standard mixture containing 13C12-labeled PCDDs and PCDFs and equilibrated during at least one hour. After that time, a 24 hours' extraction was performed using hexane/dichloromethane mixture (50:50 v/v). The extracts were evaporated to dryness.

3.2.4 Carbon Chromatography

For carbon chromatography were used glass columns with 2 g of Carbosphere. The sample residue was dissolved in dichloromethane and brought onto the top of the Carbosphere column which was placed in a reflux unit and refluxed for 2 h with dichloromethane. This fraction was discarded. The column was rinsed with toluene and refluxed for 1 h with toluene, which was discarded. The PCDD/F fraction was recovered by reverse elution from the column by refluxing with toluene for 24 h. This fraction was carefully evaporated to dryness under a gentle stream of nitrogen.

3.2.5 Alumina Chromatography

The residue was solved in hexane and the mixture was brought onto a column containing acidic silica gel and 5 g alumina, previous washed with hexane. The alumina column was eluted with a mixture of hexane/DCM (97:3 v/v). This eluate was discarded. The PCDDs/PCDFs were eluted using a hexane/ DCM mixture (60:40 v/v).

3.2.6 Silica Chromatography

The residue was solved in hexane and the sample was brought onto a column containing neutral silica, basic silica and two acid silica layers at different concentrations (44% and 22%). The eluate was evaporated to dryness in

Kuderna-Danish concentrators (KD) under a nitrogem blowdown device to be injected in the GC/HRMS.

3.2.7 Instrumental analysis

All analyses were performed by GC–HRMS using a MAT95XL high-resolution mass spectrometer (Finnigan, Bremen, Germany) coupled to Trace GC 2000 gas chromatograph (Thermo Finnigan, Bremen, Germany) equipped with a AS2000 auto-sampler. Gas chromatographic separations were carried out using a DB-5 MS capillary column (60 m x 0,25 mm i.d from J&W Scientific, USA) using helium as carrier gas, with a constant flow at 1 mL/min.

The samples and standards were injected (2 µL) in split less mode (split less time 1 min) at an injector temperature of 280 ºC and at an initial oven temperature of 120 ºC. After 1 min, the temperature was ramped at 25 ºC/min to 200 ºC and then at 3 ºC/min up to 300 ºC. The latter temperature was held during 11 min.

The mass spectrometer was operated in the electron impact ionization mode using selected ion monitoring (SIM). Electron energy was set to 40 eV and the source temperature was set at 250ºC. The MS system was tuned to a resolution of 10 000 (10% valley) and masses issued from FC-43 (Perfluorotributylamine) tuning compound were used as lock mass. In order to stablish the calibration curve for each congener, a set of calibration solutions were injected in every sequence of injection series.

The two most abundant signals of the molecular ion cluster were recorded (from tetra- to octa-chlorodibenzo-p-dioxins and -dibenzofurans). Quantification was performed using internal standards and the isotopic dilution technique. The isotopic ratio between principal and secondary signals, for each congener, was verified using the criteria of ± 15% of the theoretical value. For calculation of the detection limits, a signal-to-noise ratio of 3:1 was applied.

3.2.8 Quality control

Every batch of samples has a procedure blank control and all samples were spike with 13C12- labelled reference compounds. Results were corrected with recovery rate.

3.2.9 Statistical analysis

Analysis of data was carried out in accordance with the methodology of the USA EPA for analysis of contaminants (Russell and Plumb, 2004). This procedure includes the conversion of the congeners concentration into a decimal percentage of the sum of congeners, the construction of the bar plot of the standard concentrations and the use of the square of the Pearson correlation coefficient (r2) (Johnson and Wichern, 2001) as a measure to assess whether the profile of the concentration of congeners in the samples (compared visually on the bar plot) is statistically similar. It is considered that the profiles are similar if the average of r2 is close to 1 and the standard deviation (SD) is next to zero.

3.3 RESULTS

The assessed data from the wood shaving analysis concerns 23 samples, are presented in Table 4.

Table 4 - Results of PCDD/F-WHO-TEQ/g found in wood shavings (pg/g).

Incident	Total Number of Samples (n)	Number of Samples with Conc. (≥2 pg PCDD/F-WHO-TEQ/g)	Number of Samples with Conc. (< 2 pg PCDD/F-WHO-TEQ/g)	Highest concentration (pg PCDD/F-WHO-TEQ/g)	Average concentration (pg PCDD/F-WHO-TEQ/g) (SD)	Average concentration (≥2 pg PCDD/F-WHO-TEQ/g) (SD)	Average concentration (<2 pg PCDD/F-WHO-TEQ/g) (SD)
2006	10	4	6	368	62 (116)	154 (148)	0.47 (0.35)
2011	13	6	7	446	78 (164)	169 (216)	0.42 (0.62)
Total	23	10	13	446	71 (143)	163 (182)	0.44 (0.49)

At that time, the highest levels of dioxins found in the most contaminated wood shavings samples (≥2 pg PCDD/F-WHO-TEQ/g) were 368 pg PCDD/F-WHO-TEQ/g with an average of 154 pg PCDD/F-WHO-TEQ/g in 2006 and 446 pg PCDD/F-WHO-TEQ/g with an average of 169 pg WHO-TEQ/g in 2011.

The contamination profile shows that the total contamination was predominantly constituted by the most chlorinated congeners (97.4%), having OCDD 73,5%, OCDF 8,2%, 1,2,3,4,6,7,8-HpCDD 13% and 1,2,3,4,6,7,8-HpCDF 2,7% (Figure 9).

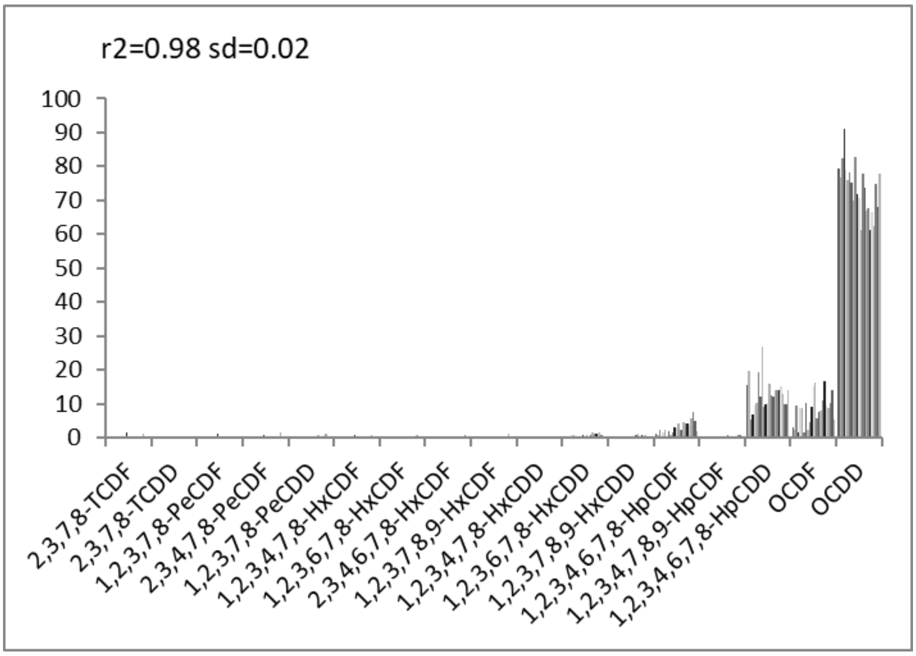

Figure 9 - Relative concentration of the different congeners in all wood shavings samples (contamination profile).

The square of the Pearson correlation coefficient (r2) and standard deviation (sd) of the different congeners relative concentrations found in the wood shavings analysis, is considerable (r2 = 0.98 and sd = 0.02).

Recoveries of 13C12- Labelled compounds added to the wood shavings samples ranged from 71.3% and 86.3% (Table 5).

Table 5 - Recovery rates (R) and Level of Quantification (LQ) by each PCDD/PCDF congener.

	R(%)	S_R(%)	R-2* S_R (%)	R+2* S_R(%)	Average LQ (pg/g)	SD LQ (pg/g)
2378-TCDF	79.3	15.0	49.3	109.3	0.019	0.02
2378-TCDD	84.7	13.1	58.5	110.8	0.013	0.02
12378-PeCDF	85.0	12.1	60.8	109.2	0.028	0.04
23478-PeCDF	84.0	13.0	58.1	109.9	0.028	0.04
12378-PeCDD	86.3	11.5	63.4	109.2	0.04	0.05
123478-HxCDF	85.2	12.8	59.7	110.8	0.041	0.06
123678-HxCDF	79.9	12.8	54.4	105.5	0.041	0.06
234678-HxCDF	80.9	13.3	54.3	107.5	0,043	0.07
123789-HxCDF	84.3	13.9	56.5	112.2	0,043	0.09
123478-HxCDD	85.6	11.2	63.1	108.0	0,059	0.08
123678-HxCDD	81.5	11.2	59.0	104.0	0,05	0.07
123789-HxCDD	81.5	11.2	59.0	104.0	0.05	0.08

1234678-HpCDF	79.3	11.2	56.9	101.8	0.056	0.06
1234789-HpCDF	84.1	12.9	58.4	109.9	0.071	0.09
1234678-HpCDD	83.2	12.0	59.3	107.1	0.09	0.13
OCDF	71.3	14.3	42.8	99.9	0.09	0.14
OCDD	71.3	14.3	42.8	99.9	0.15	0.33

3.4 DISCUSSION

Main differences between used method and USEPA method 1613B

The main steps that have been modified and adapted in the method used for the analysis of wood shavings are resumed in Figure 10, comparatively with the USEPA method 1613B.

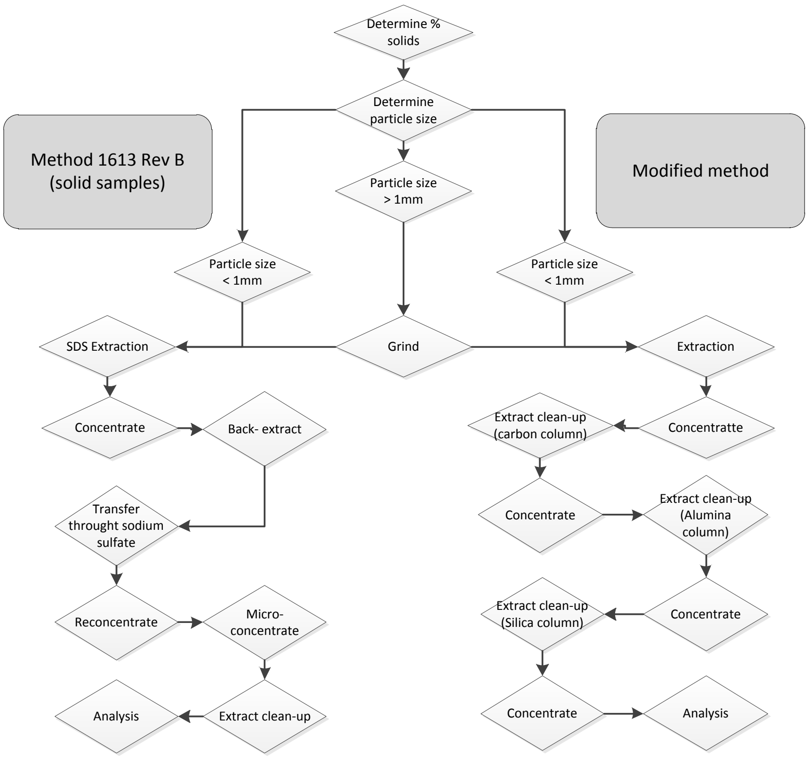

Figure 10 - Flow chart of the USEPA method 1613B and the used method.

The analytical procedure used to quantify PCDD/F on the wood shavings samples in this study was based on the USEPA method 1613B with the follow modifications:

I. Extraction

The reflux was done in one step for a period of 24 hours with hexane/dichloromethane mixture (50:50 v/v) and not the two steps foreseen in the reference method with toluene.

II. Concentration

The extracts were concentrated using a rotary evaporator with a recirculating water pump and adapting vacuum to the system, taking care with the speed of evaporation because if it is too fast, part of the analyte may be lost.

The concentration was performed in two steps, warming the water bath and applying different pressures, 550 mbar to evaporate the DCM and 250 mbar to evaporate the n-hexane.

III. Extract clean-up (Carbon column)

A carbon column clean-up was used to remove nonpolar interferences.

Unlike the reference method, the back extract was not performed after the concentration. Instead, the concentrated extract was cleaned-up in a carbon column succeeded by a back flash reflux.

In the adapted method, the column was just pre-eluted with 10 ml of DCM which were discarded. The reference method foresees a pre-elution of the column with 5 ml of toluene followed by 2 mL of methylene chloride: methanol: toluene (15:4:1 v/v), 1 mL of DCM: cyclohexane (1:1 v/v), and 5 mL of hexane.

The sample extract was added to the column, the sample container was rinsed twice with 5 mL portions of DCM applied separately to the column whilst the reference method foresees the same procedure with 1 mL portions of hexane and a final addition of 2 mL of hexane to complete the transfer.

After the addition of the sample, a two hours reflux with 35 mL of DCM was completed and when finished, the carbon columns were washed with 15 ml toluene to drag the excess of DCM, succeeded by a new one-hour reflux of 35 mL of toluene. The reference method foresees an elution with two 3 mL portions of hexane, 2 mL of methylene chloride: cyclohexane (1:1 v/v) and 2 mL methylene chloride: methanol: toluene (15:4:1 v/v).

The column was inverted (back flash), and eluted the PCDDs/PCDFs with 45 mL of toluene during a 24-hour reflux period, whilst the reference method foresees an elution with 20 mL of toluene.

IV. Extract clean-up (Alumina column)

The alumina column was used to remove nonpolar and polar interferences as well as chlorodiphenyl ethers.

The reference method prescribes either the use of acid alumina (6 g acid alumina) or the use of basic alumina (6 g basic alumina). In the methodology used in this study, the column was packed with 5g of basic Alumina (Alumina B- Super 1for dioxin analysis).

The pre-elution of the column followed the reference method.

The concentrated extract solution was diluted in 5 ml hexane and added to the column (no dilution on the reference method). The receiver was rinsed twice, with 2,5 ml portions of hexane (1 ml in the reference method) and apply separately to the column. The interfering compounds were eluted with 20 ml hexane: DCM (97: 3 v/v) (100 ml hexane in the reference method) and the eluate was discarded.

In this methodology, the last elution was completed with 80 ml of a DCM: hexane solution (40:60 v/v) to obtain the final extract which was collected. According to the reference method, the choice of eluting solvents would depend on the choice of alumina (acid or basic), 20 mL DCM: hexane (20:80 v/v) when acid alumina is used or 20 mL DCM: hexane (50:50 v/v) when basic alumina is used.

V. Extract clean-up (Silica gel column)

The extract was concentrated and eluted, after an elution of 1 to 3 ml of hexane (50-100mL hexane in the reference method) through a column of silica filled in the following sequence (glass wool plug into the tapered end of a graduated serological pipet, pack with 1 cm neutral silica, 1 cm basic silica (NaOH), 1 cm acid silica at 44% topped with 1 cm acid silica at 22% and a glass wool plug).

The reference method foresees a different composition of the column which is packed bottom to top with: 1 g silica gel, 4 g basic silica gel, 1 g silica gel, 8 g acid silica gel, 2 g silica gel and 4 g granular anhydrous sodium sulphate taped with glass-wool.

The concentration flask was rinsed twice with 2 ml of hexane (1 mL of hexane in the reference method with an extra elution of 100 mL hexane through the column).

The product of this elution was concentrated and placed in a Kuderna-Danish concentrator (KD) previously prepared with ebullition regulators for injection in the GC/ HRMS.

Level of contamination and congener’s profile

The results revealed average levels of 62 pg PCDD/F-WHO-TEQ/g fat in 2006 and of 78 pg PCDD/F-WHO-TEQ/g fat in 2011, which exceeded the maximum limit allowed by the European legislation for these substances in poultry meat, set at 2 pg PCDD/F-WHO-TEQ/g fat until 2011.

Once applied the statistical methodology, it becomes clear that in both incidents the contamination profiles were very similar (Figure 9).

Risk management concerning the presence of environmental contaminants that may reach the food chain is a challenging task and must always put in perspective some unusual sources of contamination.

In both incidents during 2006 and 2011, the association between the poultry meat contamination and the litters used in the poultry production was established.

The pattern detected in the wood shavings contamination matches the profile found for contaminated technical pentachlorophenol by other authors (Hagenmaier and Brunner, 1987; Fries *et al*., 1996; Cleverly *et al*., 1997). This could suggest that the wood shavings used in the litters were obtained from treated wood, being the wood preservative the possible source (inadequate disposal of wood by-products).

The role of metal catalysts in de novo formation of PCDD/PCDF is described by Tame *et al*. (2017) and cooper is described as having the ability to couple with oxygen, lowering the temperature of exothermic oxidation with chlorination of the carbon. The high chlorination efficiency manifests in the homologues profiles where octa and hepta homologues of dibenzofuran and dibenzo-p-dioxin dominate. Cooper is very often used in commercial industry wood preservative solutions.

Recoveries of 13C12- Labelled compounds added to the wood shavings samples of the modified method are considered as acceptable by the European legislation. The recovery shall be within 60 to 120 % especially for congeners contributing more than 10 % to the TEQ-level for analysis of foodstuffs and feed for confirmatory methods and, for screening methods, the recoveries shall be in the range of 30 to 140 % (EC, 2014; EC, 2014b).

3.5 CONCLUSIONS

In both incidents during 2006 and 2011, contamination profiles of the wood shavings from the poultry litters, in what respect to congeners higher and lower relative concentrations, seems to be very similar (r2 = 0.98 and sd = 0.02), being OCDD, OCDF, 1,2,3,4,6,7,8-HpCDD and 1,2,3,4,6,7,8-HpCDF responsible for 97.4 % of the total contamination.

The method used, with all adaptations described, proved to be accurate and reproducible in the determination of low and high levels of PCDD/PCDF in wood shavings used as litters in poultry production farms. Quality requirements of the reference standards for recoveries, for food and environmental dioxins analysis are met.

Further investigation is still needed to explain the formation of PCDD/F in order to clarify the possible role of wood preservatives in the contamination of the poultry food production chain.

4. PCDD/F Dioxin Profile of Treated *Pinus pinaster* Wood

Abstract

This work describes the treatment of *Pinus pinaster* wood with four different industrial wood preservatives (two anti-bluing or fungicide and two fungicide/ pesticide) and the detection and quantification of the dioxin contamination profile in the wood shavings. The samples were collected from poultry liters during three contamination incidents of poultry meat. Two methods used were, both nonpressure: one by immersing the wood samples in the preservative solution and the other by impregnation of the preservative solution into the wood, with vacuum. It was concluded that there is no difference in terms of contamination profile, caused by the different industrial wood treatment preservative products in study. A clear correlation between the commercial products used in wood treatment and the contamination profile of wood shavings that have been used as bedding material in poultry production was detected.

Key words: Dioxin, Wood treatment, Fingerprint analysis, Food chain.

4.1 Introduction

During 2006, 2011 and 2016, following the implementation of a monitoring plan, contaminations with dioxins in poultry meat were found: the levels of contamination were higher than legally allowed in meat from poultry slaughtered for human consumption (CEC, 2006). To identify the original source of contamination of the birds, all potential sources of contamination were analyzed and the results showed considerable high contamination with dioxins of the wood shavings used as bedding material in the poultry farms, indicating that these materials were the likely source of contamination of the animals (Cardo *et al.*, 2014).

In those incidents of contamination of the food chain with dioxins, the investigations performed revealed that the contaminated wood shavings used as poultry bedding material were delivered by wood industries that illegally disposed wood shavings byproducts produced with treated wood.

In these episodes, contamination profiles of higher and lower concentrations in the poultry muscle and fat, seems to be very similar, being OCDD, OCDF, 1,2,3,4,6,7,8-HpCDD and 1,2,3,4,6,7,8-HpCDF responsible for 97.4 % of the total contamination (Cardo *et al*., 2014).

In Portugal wood treatment is performed by 23 companies and is dominated by the production of poles, beams and poles, with a production of about 84300 cubic meters and the most widely used wood species is the maritime pine (Pinus pinaster) (Nunes *et al*., 2016).

The most widely used commercial products in Portugal are *Celcure C4*, *Celcure VS725*, *Korasit K2* whose active principles are mainly quaternary ammonium salts and copper, the *TANALITH E 8001* whose active principles are propiconazole, tebuconazole, baramina and copper and Coprol Premium whose active ingredients are propiconazole and copper. Of the twenty-three existing companies, fifteen use *TANALITH E 8001*, two use *celcure C4* or *VS725*, four use *Korasit* and two the *Coprol Premium*. As the *Celcure* is used by the two largest companies, this product shares with *TANALITH E* the leadership of the domestic market, representing *Korasit* and *Coprol* a marginal share of the market (Nunes *et al*., 2016).

This work describes the treatment of *Pinus pinaster* wood with four different industrial wood preservatives and the fingerprint analysis of the dioxin contamination profile with the wood shavings samples collected during the food chain contamination incidents. The methods used were both nonpressure, one by immersing the wood samples in the preservative solution and the other by impregnation of the preservative solution with vacuum.

4.2 Materials and methods

1) Wood treatment

The treatment of wood was held at the laboratory of the Center for Structural Behavior of Structures from the Department of structures of the National Civil Engineering Laboratory (LNEC).

For the comparative study of different treatments of wood, a lot of pine wood has been chosen from the same stock and a research analysis for detection and quantification of PCDD/F were made to ensure that the wood batch was not contaminated. The procedure used in the treatment of wood that was described in the Table 7.

Table 6 - Procedure for wood treatment.

Procedure
Choose the wood batch.
A pine batch was chosen with no visible signs of having been burned in forest fires
Selection of samples
The selected samples showed no heartwood in order to use only the sapwood in the treatment and the wood were stored in a room with controlled environmental conditions for dehydration to stabilize the weight.
Marking of samples
The samples were then randomized to be allocated to each treatment and marked with numbering puncture.
Cutting of samples
They were cut into fractions 21,2x4,7x4,7 cm.
Weighing
The weighing of each sample was carried out immediately before treatment and after treatment to calculate the absorption of the solution and retention of the preservative.
Treatment by immersion
The treatment of the samples was performed according to the manufacturer's instructions (time, concentration).
Vacuum treatment
The treatment of the samples was performed according to the manufacturer's instructions (time, concentration) at a negative pressure of 0.92 bar.
Fragmentation of the samples
The samples after dehydration in a controlled atmosphere were fragmented using a chisel and hammer to be received in the mill.
milling
The fragmented samples were grinded into particles having the average size of $1mm^2$. The mill was cleaned of particles with compressed air spray and passed softwood (untreated) between the milling of each sample.
Packaging of samples
The samples were packaged and identified immediately after grinding.

The treatments were performed by immersion and by a vacuum method for comparing the contamination profile of the four different commercial products used for different purposes, two with an anti-bluing industrial product, or fungicidal and other two by depth vacuum impregnation with fungicide and pesticide effect.

Treatment A, for use as an industrial fungicide treatment by immersion at a dilution in water of 7%.

Composition:

- 14.0% trimetilcocoamonia chloride.
- 4.0% sodium tetraborate pentahydrate ($Na_2B_4O_7.5H_2O$); Also called Borax.

Treatment B, for use as an industrial fungicide treatment by immersion at a dilution in water of 1.5 to 3.5%.

Composition:

- 10% Bardap26 (N,N-Didecyl-N-methyl-poly(oxyethyl)ammonium propionate).
- 1.6% DCOIT (4,5-dichloro-2-N-octyl-4-isothiazolin-3-one).
- 2% IPBC (3-iodo-2-carbamate proponyl).
- 0.9% propiconazole ($C_{15}H_{17}Cl_2N_3O_2$).

Treatment C, for use as an industrial fungicide and insecticide treatment by pressure and vacuum in an autoclave to a 2-4% dilution in water.

Composition:

- 4.0% boric acid (H_3BO_3).
- 4.2% Bardap 26 - poly (oxy-1,2-ethanediyl), α- [2- (didecilmetilamónio) ethyl] - ω-hydroxy-propanoate (salt).
- 20% copper (II) carbonate hydroxide, copper (II) 1: 1. Cu (OH) 2; CuCO3.

Treatment D, for use as an industrial fungicide and insecticide for treatment with vacuum and pressure in an autoclave at a dilution of 2% in water.

Composition:

- 14% basic copper carbonate; CuCO3.
- 0.50% didecyldimethylammonium chloride; (N- (3-aminopropyl) -N-dodecilpropano-1,3-diamine) $C_{22}H_{48}ClN$.
- 0.16% propiconazole ($C_{15}H_{17}Cl_2N_3O_2$).
- 0.16% tebuconazole ($C_{16}H_{22}ClN_3O$).

To calculate the absorption of the applied solution, the following formula was used in which (mf) is the final weight; (mi) is the initial weight:

$$Absorption = \frac{mf\,(Kg) - mi(Kg)}{wood\ Volume\ (m^3)}$$

The retention of preservative solution was calculated as below:

$$Retention = \frac{solution\ (\%)}{100} \times Absorption$$

2) Analytical Method

The analytical method used for detection and quantification of dioxin was the USA EPA method 1613 revision B (USEPA, 1994). This method was developed by the Environmental Protection Agency, Science and Technology of the United States for the determination of 2,3,7,8-CDDs/CDFS replaced through octa-chlorination, dibenzo-p-dioxins and dibenzofurans in aqueous matrices, solid or tissue by isotope dilution, followed by capillary column of high resolution gas chromatography (HRGC)—high resolution mass spectrometry (HRMS).

3) Statistical Analysis

Analysis of data was carried out in accordance with the methodology of the USA EPA for analysis of contaminants (Russell and Plumb, 2004). The methodology consists in the conversion of the concentration of the different congeners of each sample to a decimal percentage of the sum of congeners. These standard concentrations in each sample is represented in a bar plot graphic. The square of the Pearson correlation coefficient (r^2) (Johnson and Wichern, 2001) is then used as a measure to assess whether the profile of the concentration of congeners in the samples (compared visually on the bar plot) is statistically similar. It is considered that the profiles are similar if the average of r^2 is close to 1 and the standard deviation (SD) is next to zero.

The same methodology was applied to investigate a possible association between groups of analysis.

4.3 Results

The results showed a negligible contamination of 0.078 ± 0.025 pg PCDD/F-WHO-TEQ/g in the blank wood, without any chemical treatment. Those results were used to correct all the values obtained in the analyzes performed to compare the treatments.

The results obtained during the treatment of wood is presented in Table 8.

Table 7 - Absorption and retention of preservative solution.

Sample	solution	Sample	(mi) (Kg)	(mf) (Kg)	treatment	Conc/ Time	Volume (mm^3)	Liquid absorption (Kg/m3)	Preservative retention (Kg/m3)
MC/1/AZ	A	1A	0.254	0.290	Immersion	7%/ 15 m	468308	78.28	5.48
MC/2/AZ	A	2A	0.293	0.322	Immersion	7%/ 15 m	468308	63.85	4.47
MC/4/AZ	B	1B	0.258	0.289	Immersion	4%/ 15m	468308	65.79	2.63
MC/5/AZ	B	2B	0.266	0.290	Immersion	4%/ 15m	468308	50.12	2.00
MC/7/PR	C	1C	0.243	0.456	Vacuum	4%/ 60 m	468308	455.05	18.20
MC/8/PR	C	2C	0.240	0.403	Vacuum	4%/ 60 m	468308	348.55	13.94
MC710/PR	D	1D	0.276	0.364	Vacuum	2%/ 60 m	468308	187.29	3.75
MC/11/PR	D	2D	0.273	0.394	Vacuum	2%/ 60 m	468308	257.44	5.15

Contamination levels of wood samples subjected to different treatments are presented in the Table 9.

Table 8 - Contamination levels of the different samples/ treatments.

Sample	pg WHO-PCDD/F-TEQ WHO/g
MC/1/AZ	0.17
MC/2/AZ	0.18
MC/4/AZ	0.14
MC/5/AZ	0.14
MC/7/PR	0.34
MC/8/PR	0.29
MC/10/PR	0.27
MC/11/PR	0.20

Analyses for quantification of dioxins and furans in different samples subjected to the four different treatments had a very similar profile with a very high correlation coefficient (R2 = 0.99) and a very low standard deviation (SD = 0.001) (Figure 11). For this reason, it was not made the comparison between treatments.

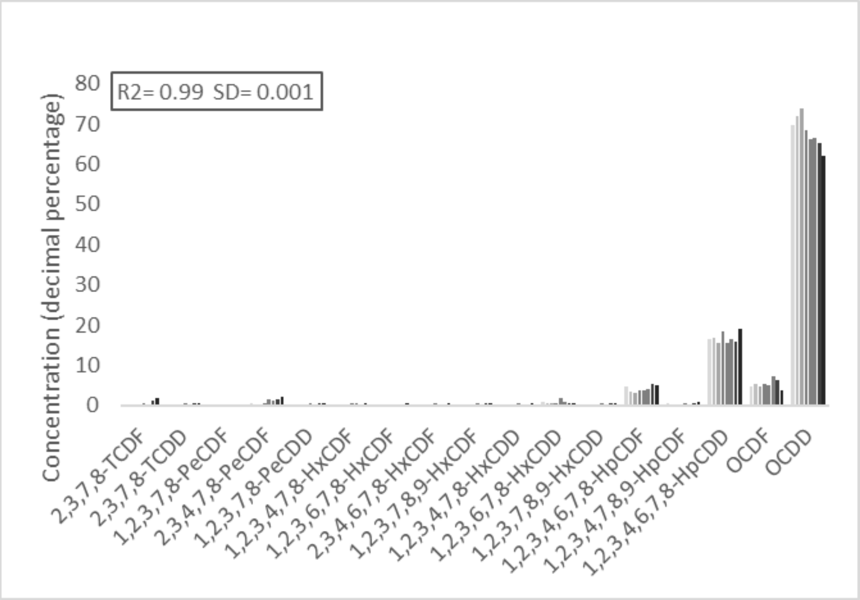

Figure 11 - Profile contamination of samples from different treatments of wood.

Given these results, it is interesting to compare the contamination profile of the total samples taken from the wood shavings used as bedding material during the episodes of poultry dioxins contamination in 2006, 2011 and 2016. For this purpose, only the samples that have showed contamination with substantial amounts, *i.e.* contamination with levels above 2 pg WHO-PCDD/F-TEQ WHO/g, will be used.

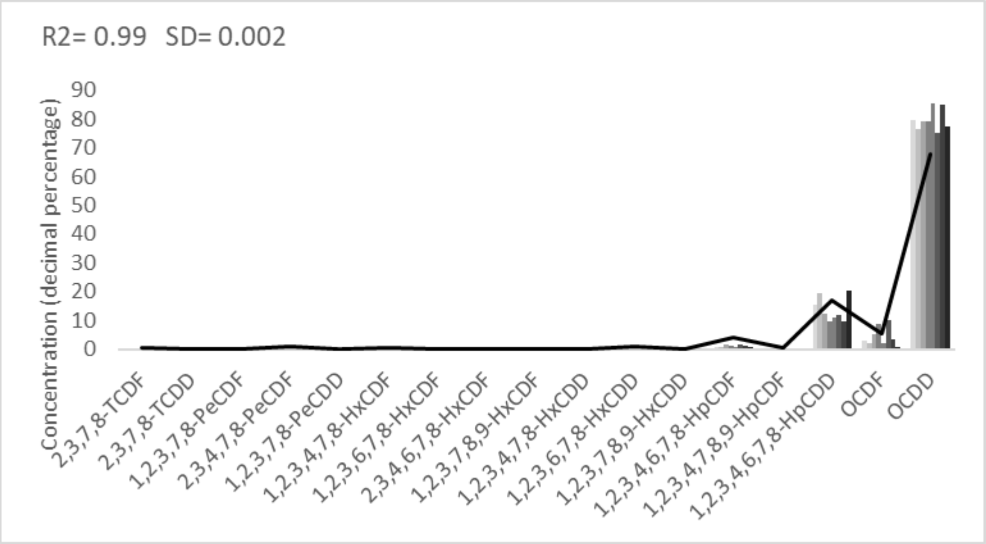

Figure 12 - Fingerprint analysis of treated wood (line) with bedding material incident of 2006.

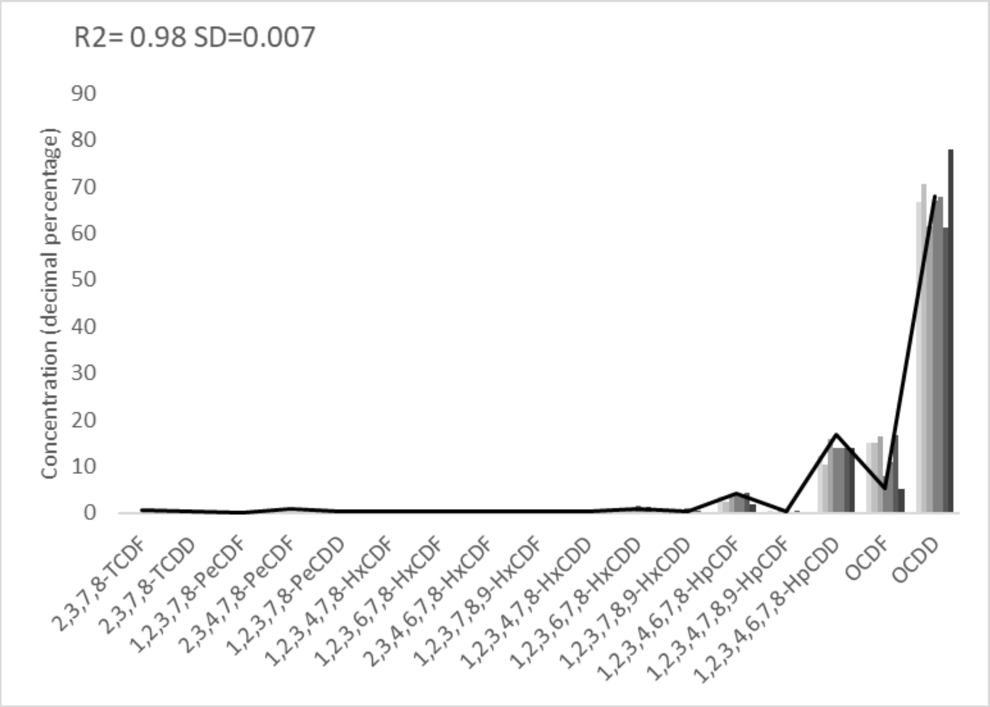

Figure 13 - Fingerprint analysis of treated wood (line) with bedding material incident of 2011.

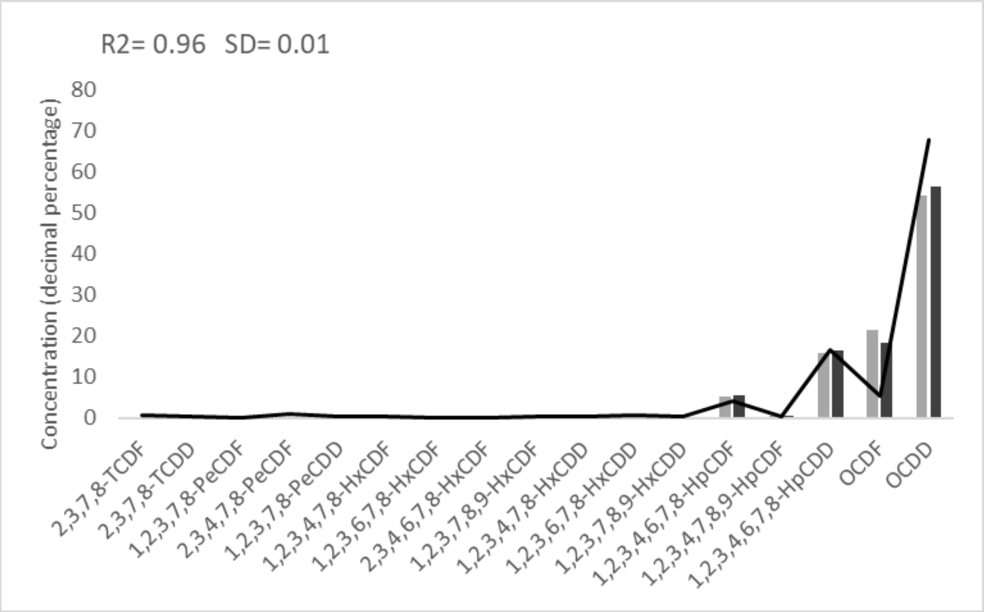

Figure 14 - Fingerprint analysis of treated wood (line) with bedding material incident of 2016.

4.4 Discussion

Several authors describe wood preservative retention comparison studies in different species of wood, with different preservation methods and products with different preservatives.

Table 9 - Comparison of preservative retention levels in different studies.

Author	Wood species	Treatment method	Product	Dimension of samples mm	Volume mm^3	Retention (Kg/m^3)
(Ozdemir *et al*., 2015)	*Pinus sylvestris L.*	Vacuum (45 m)	Tanalith E (2%)	300x100x15	450000	15.39
(Ozdemir *et al*., 2015)	*Pinus sylvestris L.*	Vacuum (45 m)	CCA (2%)	300x100x15	450000	15.67
(Ozdemir *et al*., 2015)	*Pinus sylvestris L.*	Vacuum (45 m)	Bóric acid (1%)	300x100x15	450000	6.92
(Chong, 1977)	*Pinus radiata D.*	Vacuum and pressure	CCA (2%)	4.3x50x25	5375	13.3
(Yildiz *et al*., 2004)	*Pinus sylvestris L.*	Vacuum (60 m)	CCA (2%)	5×10×100	5000	13.24
(Yildiz *et al*., 2004)	*Pinus sylvestris L.*	Vacuum (60 m)	Tanalith E 3491 (2%)	5×10×100	5000	11.64
(Yildiz *et al*., 2004)	*Pinus sylvestris L*	Vacuum (60 m)	ACQ-1900 (2%)	5×10×100	5000	12.99
(Yildiz *et al*., 2004)	*Pinus sylvestris L*	Vacuum (60 m)	Wolmanit CX-8 (2%)	5×10×100	5000	13.04
(Yildiz, 2007)	*Pinus sylvestris L. ssp.*	Vacuum and pressure	Tanalith E 3492 (2.4%)	50x50x100	762000	4.95
(Yildiz, 2007)	*Pinus sylvestris L. ssp.*	Vacuum and pressure	Tanalith E 3492 (2.4%)	50x100x152.4	762000	2.48

Retentions obtained in this laboratory study varied with the concentrations of the solutions used. The vacuum treatment for impregnation of the solution, yielded an average retention of the solute of 16.7 Kg/m3, when a 4% concentration was used in the treatment "C" and an average retention 4.45 kg/m3 when a 2% concentration was used in the treatment D.

The assessment of the wood impregnation studies is quite difficult since the retention levels vary with various factors such as the species of wood, wood moisture content, the volume of samples, the treatment time, pressure used, and the treatment used, *i.e.* if only applies vacuum or if vacuum is alternated with positive pressure. This difference is not as significant in immersion treatments.

The impregnation studies performed by Yildiz *et al*., (2004) compared to this study, exhibited higher retention levels with lower concentrations but with lower volume samples and studies performed by Ozemir *et al*., (2015) showed similar retention levels with similar volume of samples but for less time. Studies by other authors referred in the Table 10, in some cases have higher retentions and other lower, even using vacuum and pressure.

The results of the contamination levels of the treated wood chips showed very low contaminations when compared with the contaminations observed in the incidents occurred in poultry contaminations, probably due to lower retentions comparing to the retentions obtained in industrial conditions.

Contamination profiles of the different treatments used showed a very strong correlation R2 = 0.99 and a standard deviation of 0.001.

The *fingerprint analysis* of the profile of the wood treated in this study, with the profile of the wood shavings from the bedding material implicated in the incidents that took place in Portugal in 2006, 2011 and 2016 with food safety concern (Figures 12-14) showed a very high correlation. R2 = 0.99 and SD = 0.002 compared with the litters tested in 2006, R2 = 0.99 and SD = 0.007 compared with the litters tested in 2011 and R2 = 0.96 and SD = 0.012 compared with the litters tested in 2016.

4.5 Conclusions

The study and characterization of the contaminant, in particular, the study of the influence of different products marketed in Portugal for treatment/preservation of wood, allowed the conclusion that there is no difference in terms of contamination profile, caused by the different industrial wood treatment preservative products.

The study established, at laboratory level, a clear correlation between the commercial products used in wood treatment and the contamination of wood shavings that have been used as bedding material in poultry production. The profile of the contamination of pine wood chips treated in the laboratory fits perfectly into the profile of the wood shavings implicated in the poultry contamination incidents in Portugal.

Surprisingly, the surface treatment of wood and the depth (vacuum) treatments showed very similar contamination profiles, which allows us to consider that, in general, the litters of poultry contaminated with treated wood shavings present a similar profile, since the degree of retention of the preservative in wood does not affect the profile found.

This recurrence of the profile can be important for risk managers because it allows, based on a muscle and fat analysis, immediately associate an equivalent profile to the respective source of contamination without having to waste time and resources to examine all possible sources of contamination.

5. Dioxins in the Food Chain: Contamination Fingerprint Analysis in Breeding Hens, Hatching Eggs and Broilers

Abstract

While routine monitoring poultry meat was obtained from breeding hens, dioxins contaminations were detected in Portugal. Levels of 430.9 pg PCDD/F-WHO-TEQ/g[6] were found, which are higher than the official limits legally allowed for this matrix (1.75 pg PCDD/F-WHO-TEQ/g). To identify the magnitude of the contaminations, 60 samples were collected from poultry farms and different matrices, namely: feed, water, wood shavings from the litters, muscle of the breeding hens, hatching eggs collected in the positive farm and muscle collected from broilers farms supplied by the positive breeding farm. The comparison of the dioxins congeners profiles showed that there was a coincidence of peaks of higher relative concentrations in the wood shavings, with the peaks of the highest relative concentration in the hatching eggs, especially the relative concentrations of the congeners 1,2,3,4,6,7,8-HpCDD and OCDD, which may be explained by the role of VLDLy in the delivery of triglycerides to the oocyte, where they will be used as the energy source for the developing embryo. The comparison of the dioxins congeners profiles of the breeding hens muscle with the poultry muscle, showed a coincidence of peaks of higher relative concentrations in the congeners 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD and OCDD which may indicate a dechlorination pathway "*in vivo*". Results allowed concluding that those wood shavings, improperly used as poultry litters, were certainly the source of contamination of the food chain.

Keywords: Dioxin Fingerprint, Food Chain, Poultry

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[6] The abbreviation "PCDD/F-WHO-TEQ" refers to the toxic equivalence factors (TEF) established for a range of PCDDs and PCDFs by the World Health Organization (WHO).

5.1 Introduction

Food chain safety has been sporadically stressed by the presence of chemical hazards as contaminants of many food matrices. Among these hazards, PCDD, PCDF and PCBs are certainly those of the highest concern because of their high social and economic negative impacts. In the last decade many episodes of food chain contamination with dioxins have been reported in European Union (De Vries, *et al*., 2006; Kennedy, *et al*., 2010). In Portugal, during 2011, an episode of a natural poultry meat contamination with dioxins was also reported (Cardo, *et al*., 2009). Many sources have been incriminated as vehicles for dioxins to food of animal origin: commercial organic feed, noncommercial feedstuffs, soil, plants, worms and insects (De Vries, *et al*., 2006). These are the matrices where original contaminations are systemically searched when some animal production is found carrying higher dioxins contaminations. However, in intensive poultry production, the birds do not assess soil or invertebrates. Some hens are accommodated in cages (laying hens) or inside pavilions without soil contact. And when, in these circumstances, feed is also confirmed as dioxin free, other sources must be searched to manage the risk efficiently. This study concerns to a search performed precisely to determine the source of dioxins contamination found in poultry meat obtained from breeding hens, in its hatching eggs and respective chickens.

5.2 Material and Methods

5.2.1. Sampling

Sampling has been conducted according to following sequence:

- From all possible sources of contamination in the breeding hens positive farm (feed, water and wood chips from litters);
- From hatching eggs laid by the positive breeding hens;
- From the broilers hatched from the eggs laid by the positive breeding hens.

Samples partition was the following: 17 samples of muscle fat were collected from four breeding hen’s new batches of the implicated farm; wood shavings from the bedding material (n = 4), hatching eggs (n = 3), feed (n = 3) and water (n = 3).

From the 20 broiler farms supplied with one day old chicks coming from the positive breeding farm, were also sampled (30 samples: n = 28 broilers muscle fat and n = 2 wood shavings).

Additionally, two positive samples of wood shavings were also tested for detection and quantification of pentachlorophenol - a wood preservative-using extraction with acetone/10% H_2SO_4 under reflux, clean-up with celite/H_2SO_4 column.

The sample storage and transportation were made according good laboratory practices, to ensure sample stability and integrity, avoiding any change that could affect the reliability of the analytical procedure. Each sample was individually identified and packed immediately after collection.

Litter samples were preserved in dry and dark conditions in a cool place, while muscle samples were frozen.

5.2.2. Analytical Method

The analytical method used for detection and quantification of dioxin was the USA EPA method 1613 revision B (USEPA, 1994). This method was developed by the Environmental Protection Agency, Science and Technology of the United States for the determination of 2,3,7,8-CDDs/CDFS replaced through octa-chlorination, dibenzo-p-dio- xins and dibenzofurans in aqueous matrices, solid or tissue by isotope dilution, followed by capillary column of high resolution gas chromatography (HRGC) - high resolution, mass spectrometry (HRMS).

5.2.3. Statistical Analysis

Analysis of data was carried out in accordance with the methodology of the USA EPA, for analysis of contaminants (Russell and Plumb, 2004). The methodology is summarized in four basic steps.

Step One: Standardization - the concentration of congeners observed for each sample is standardized. The constituents of each sample are converted to a decimal percentage of the sum of congeners.

Step Two: Construction of the bar plot of the standard concentrations - graphic representation of the standard concentration of the different congeners for each sample. The Y-axis of the bar plot represents the relative amount of each congener in the sample (standard concentration) and in the X-axis it is represented the identification of the 17 different congeners. This chart will give an indication of the congeners that are present in greater concentration and if that pattern is maintained for the different samples.

Step Three: Statistical assessment of pattern reproducibility - this step concerns the used of the square of the Pearson correlation coefficient (r^2) as a measure to assess whether the profile of the concentration of congeners in the samples (compared visually on the bar plot) is statistically similar. The Pearson correlation coefficient (r^2) measures the degree and direction of the correlation (positive and negative) between two variables of metric scale (Johnson and Wichern, 2001).

Thus, a table is generated with the values of r^2, in which each sample is compared with all others. If all values of the square of the Pearson correlation coefficient are near 1, it can be stated that the pattern of the samples, compared in pairs, is very similar.

In order to assess the similarity of the concentration of congeners profile in various samples, it was used the global average comparison which is defined as the average values of r^2 found. It is considered that the profiles are similar if the average of r^2 is close to 1 and the standard deviation (SD) is next to zero.

Step Four: Evaluation of the source of contamination - once established the congener's profile of the dioxins contaminations in each group of samples (litters, breeding hens, hatching eggs and broilers), the same methodology of the previous steps was applied to investigate a possible association between them.

5.3 Results

A total of 17 samples were found positive in the 60 samples collected: 3 wood shavings, 4 breeding hens muscle fat, 3 hatching eggs and 7 broilers muscle fat. All samples of feed and drinking water were negative.

The higher WHO-TEQ levels of PCDDs and PCDFs contaminations were detected in breeding hens muscle fat (430.9 pg WHO-TEQ/g) with an average of 193.66 pg WHO-TEQ/g. The higher level of dioxins found in the positive wood shavings samples was 65.94 pg WHO-TEQ/g with an average of 38.85 pg WHO-TEQ/g.

The higher level of dioxins found in the positive hatching eggs samples was 61.33 pg WHO-TEQ/g with an average of 42.25 pg WHO-TEQ/g. The higher level of dioxins found in the positive broiler muscle fat samples was 8.59 pg WHO-TEQ/g with an average of 4.67 pg WHO-TEQ/g (Figure 15).

The two wood shavings samples tested for detection and quantification of pentachlorophenol, revealed a concentration of 1.1 and 2.0 mg/kg of the product.

The square of the Pearson correlation coefficient (r^2) and standard deviation (sd) of the different congeners relative concentrations within each group of analysis is considerable: r^2 = 0.99 and sd = 0.01 for the litters, r^2 = 0.75 and sd = 0.15 for the breeding hens, r^2 = 1 and sd = 0 for the hatching eggs and r^2 = 0.83 and sd = 0.11 for the broilers.

The square of the Pearson correlation coefficient (r^2) and standard deviation (sd) of the different congeners relative concentrations between each group of analysis is not so strong:

r2 = 0.54 and sd = 0.16 for the wood shavings versus breeding hens (Figure 16);

r2 = 0.58 and sd = 0.02 for the breeding hens versus hatching eggs (Figure 17);

r2 = 0.72 and sd = 0.16 for the hatching eggs versus broilers (Figure 18).

The square of the Pearson correlation coefficient (r^2) and standard deviation (sd) of the different congeners relative concentrations between breeding hens and broilers muscle fat (r2 = 0.8 and sd = 0.12) is very high (Figure 19), as well as between litters and hatching eggs (r2 = 0.72 and sd = 0.16) (Figure 20).

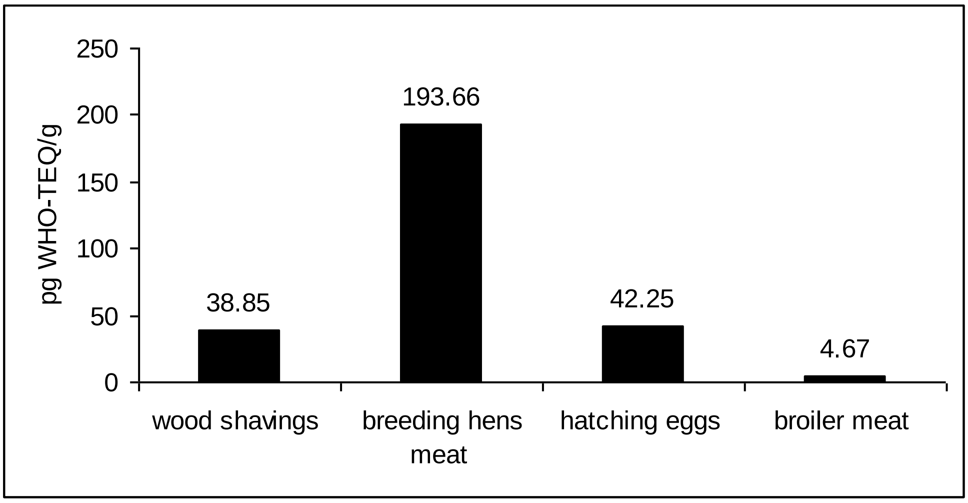

Figure 15 - Average Levels of dioxins found in positive samples.

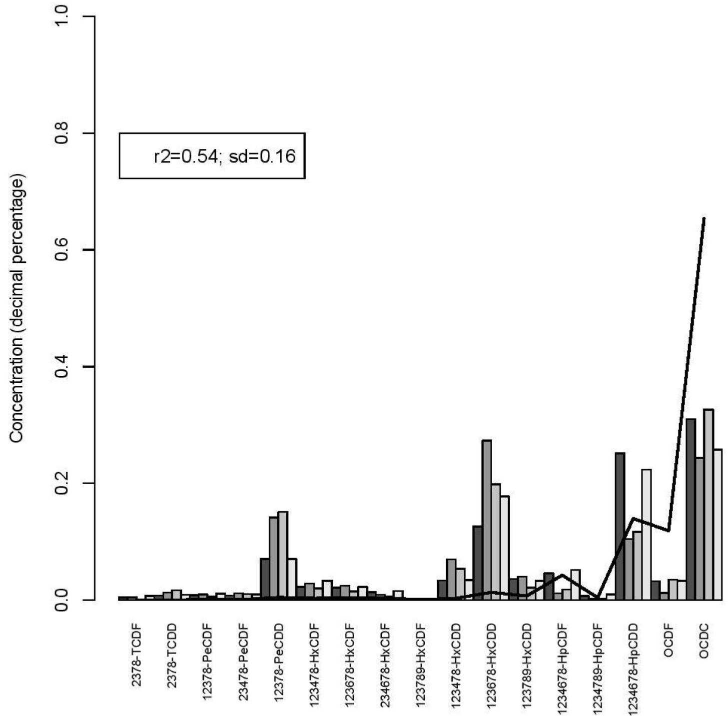

Figure 16 - Wood shavings from bedding material (line) Vs Breeding hens.

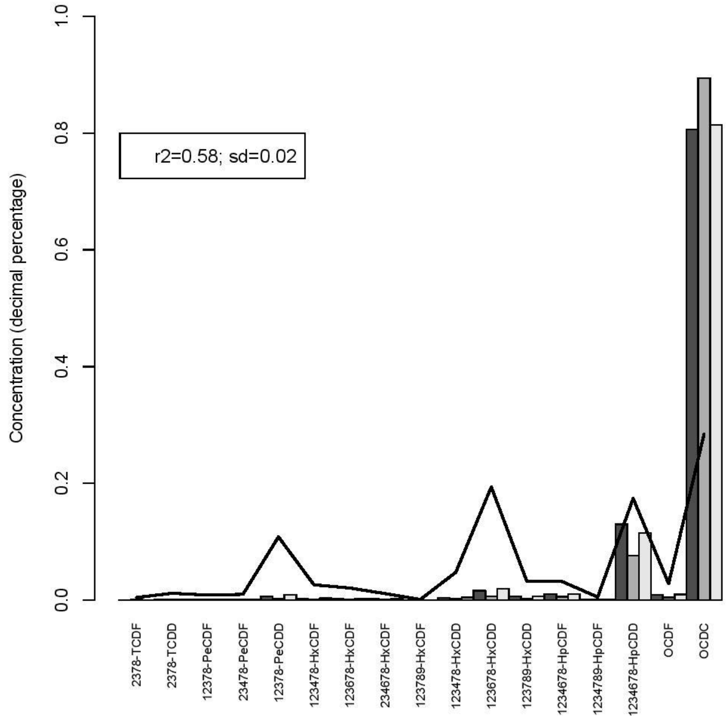

Figure 17 - Breeding hens (line) Vs Hatching eggs.

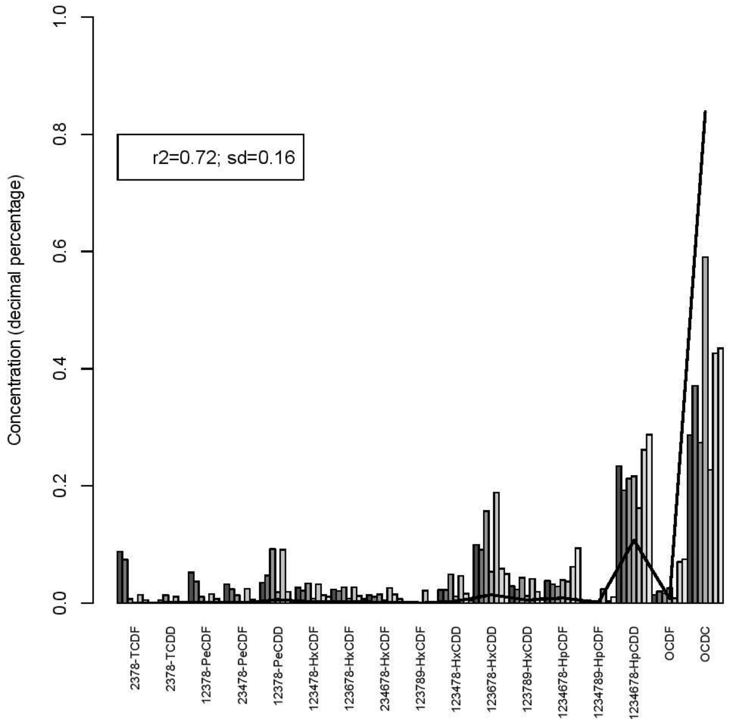

Figure 18 - Hatching eggs (line) Vs Broilers.

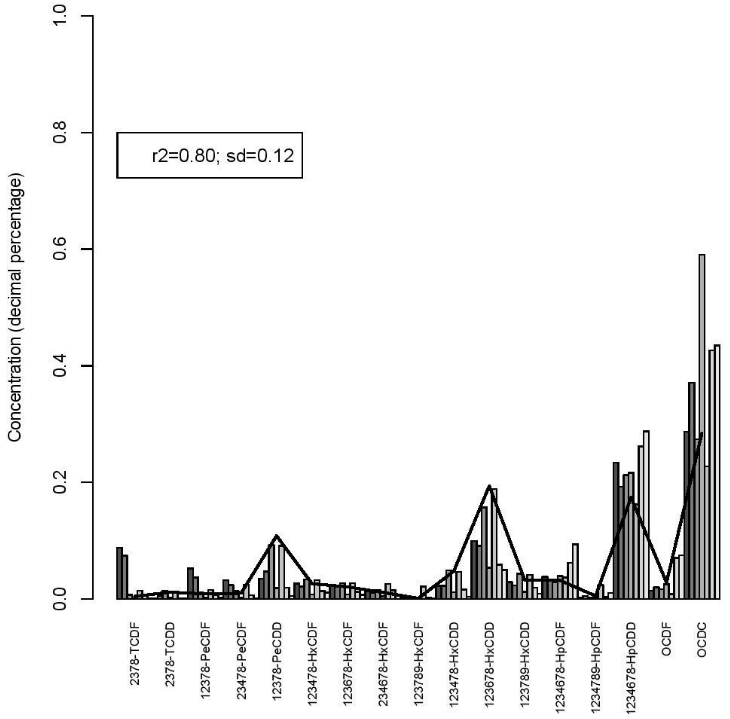

Figure 19 - Breeding hens (line) Vs Broilers.

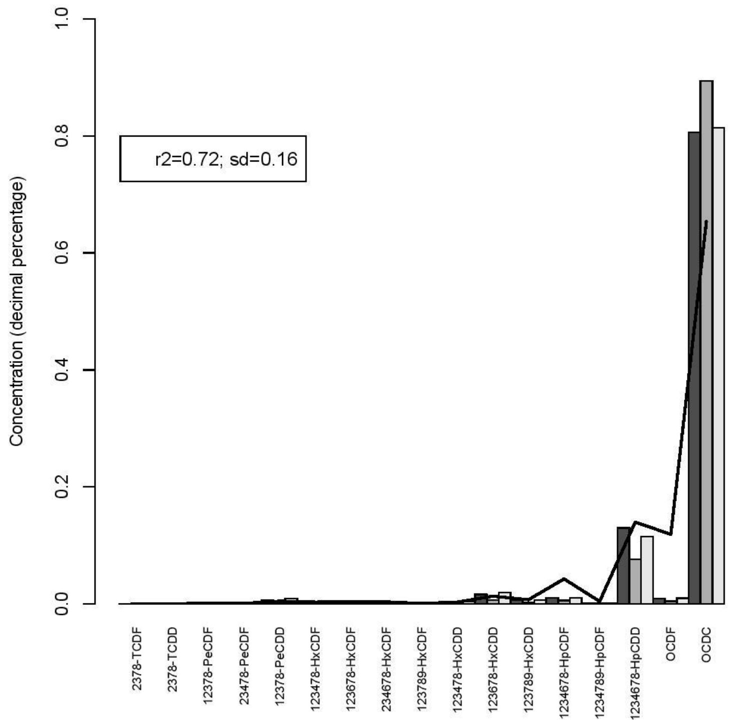

Figure 20 - Wood shavings from bedding material (line) Vs Hatching eggs.

5.4 Discussion

The results set showed considerable high contaminations of the wood shavings, indicating that these materials are the likely source of contamination of the animals.

During the earlier stages of its development, chicks have as habit to eat some particles of bedding materials. That behaviour is hazardous potentiated due to the fact that they select some special fragments with darker points, because they mimic the image of the small arthropods that are usually eaten in natural conditions (instinctive behaviour). Some wood by-product, obtained from partial burned pines, frequently have these different colour points.

The pattern detected in the wood shavings matches with the profile found for contaminated technical pentachlorophenol by other authors (Hagenmaier and Brunner, 1987; Fries, *et al*., 1996; Cleverly, *et al*., 1997); this suggests that the wood shavings used in the litters were obtained from treated wood, being the wood preservative the possible source.

The assessment carried out between the profiles found in each of the wood shavings samples and the muscle fat of the breeding hens reared on those litters; the hatching eggs laid by those contaminated hens and the respective hatched chicks showed a coincidence of peaks of higher relative concentration in its samples, concerning especially to the relative concentrations of the congeners 1,2,3,4,6,7,8-HpCDD and OCDD.

Graphic representation of the relative concentrations of the different dioxin congeners in each matrices had different comparative expressions. For example, in the broilers and breeding hens muscle fat profiles, the average relative concentration of OCDD was 52% and 53% lower, comparatively to the wood shavings and hatching eggs profile, respectively (Salvantem, *et al*., 2007). On the other hand, the average relative concentration of 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD in the broilers and breeding hens muscle fat profiles was 50% - 97% higher when compared with the litters and hatching eggs profiles.

The figures suggest that, probably, there was some factor that may affect the consistency in quantitative terms of the relative concentration of congeners found in wood shavings and in poultry muscle fat. Many explanations can be pointed to these facts, like: an “*in vivo*”, metabolic pathway (dechlorination of the most chlorinated congeners); a differentiation on the lipophilic affinity of each congener; variations on the affinity to the Ah receptor and selective faecal excretion by the birds, also equivalent to a lower absorptive capacity (Lynam, *et al*., 1998; Huwe, *et al*., 2000; Pirard and Pauw, 2004).

It was verified an equilibrium between the dioxin concentration in the contaminant ingested by chickens (38.85 pg WHO-TEQ/g) and the dioxin concentration in the eggs (42.25 pg WHO-TEQ/g), which is confirmed by other study (De Vries, *et al*., 2006; Petreas, *et al*., 1991).

The fact that the square of the Pearson correlation coefficient (r2) of the different congeners relative concentrations between litters and hatching eggs (r2 = 0.72 and sd = 0.16) and the graphic representations of the profiles was so consistent, may be explained by the estrogens produced during egg production which stimulate the liver to produce the egg-yolk targeted, very-low density lipoprotein (VLDLy). This oestrogen-dependent shift in VLDL synthesis from the production of generic VLDL, which ranges in size from 30 to >200 nm, to smaller, yolk-targeted VLDL, which ranges in diameter from 15 to 55 nm represents a dramatic shift in lipid metabolism associated with changes in the composition and structure of VLDL in egg-producing females. Whereas the role of generic VLDL is to transport triglycerides throughout the body for tissue utilization or storage in adipose tissue, the function of VLDLy is to deliver triglycerides to the oocyte, where they will be used as the energy source for the developing embryo. The smaller diameter of VLDLy is thought to be critical for enabling the particles to pass through the pores in the granulosa basal lamina of the ovary, allowing them access to the developing ovarian follicles. VLDLy particles are in high plasma concentrations in laying hens and are resistant to hydrolysis by extra-ovarian tissues which preserves the triglycerol-rich VLDLy for uptake by the developing ovarian follicles (Salvantem, *et al*., 2007).

5.5 Conclusions

This study clearly identified the source of the poultry contamination, attending to the relationship established with an environmental vehicle of dioxins (wood shavings). This achievement is based on the consistence of the results obtained with all the tested materials regarded as potential source and the fingerprint analysis of the relative concentration of the 17 dioxin congeners.

It can be stated that unusual sources of food chain contamination with dioxins must be always put in perspective when the incident is not affiliated in the most common sources.

6. Study of Depletion and Contamination Profile of Dioxins in Duck Intensive Production

Abstract

During 2016, following the implementation of a dioxin contamination monitoring in poultry meat (ducks) in a slaughterhouse, a positive sample was found. The investigation identified the wood shavings used as bedding material in the duck farm as the possible source of contamination of these animals. In this episode, contamination profiles of higher and lower concentrations, seems to be very similar, being OCDD, OCDF and 1,2,3,4,6,7,8-HpCDD responsible for 74,4% of the total contamination of those animals. The study revealed a reduction of poultry contamination after the removal of the source of contamination, with an average daily depletion percentage level ranging between 1.43% and 4.35%.

6.1 Introduction

Safety of the food chain is periodically challenged due to the occurrence of PCDD, PCDF and PCBs contamination in food. During the monitoring of dioxin in 2016, in a duck slaughterhouse, higher levels than legally allowed in poultry meat for human consumption were found. The identified source of contamination of the ducks was the wood shavings used as bedding material in the poultry farm, which presented considerable high levels of PCDD/F, indicating that these materials were the likely source of contamination of the animals.

Relevant dioxin depletion studies in cow milk have been conducted notably by McLachlan and Richter, (1998), Fries *et al*., (1999), Malisch, (2000) and other in pigs and broilers by Hoogenboom, *et al*., (2004), Pirard and Pauw, (2005), but evidence on the depletion rates of dioxins in poultry is yet to be well established. Furthermore, studies on the transformation "*in vivo*" of the contamination profile during the life period in poultry highlight the need to deepen the study in this field.

This paper addresses the study of the average depletion rates of PCDD/PCDF in ducks and of the contamination profile of dioxins. The results may provide a strong contribution for risk management in food safety incidents due to dioxin contamination in the poultry food production chain.

6.2 Materials and methods

a. Sampling

In the implicated farm, representative samples of muscle and fat from the ducks were performed with composite samples of muscle and fat of ten ducks for each pen with 3450 ducks. The first and the second samples were collected in each pen with a difference of approximately one month to access the progress of both, the contamination level and the contamination profile.

Sampling has been conducted according to following sequence:

- Two samples of muscle and fat of ducks in the slaughterhouse
- Ten samples from the ducks breed in the 10 existing pens in the farm (n= 10);
- Two samples of wood shavings (n=2) tested for detection and quantification of PCDD/PCDF.
- Seven samples of muscle and fat in the six positive pens, one in each pen and two samples in pen number 9 (n=7).

The bedding material (wood shavings) of the positive pens were changed to rice husk nearly 15 days before each first sample collection, except for pens 1 and 8, in which the change was made a week later in pen 1 and on the same day, in the case of the pen 8.

The sample storage and transport were made according to Good Laboratory Practices to ensure sample stability and integrity, avoiding any change that could affect the reliability of the analytical procedure. Each sample was individually identified and packed immediately after collection. Litter samples were preserved in dry and dark conditions in a cool place, while muscle samples were frozen.

b. Analytical Method

The analytical method used for detection and quantification of dioxin was the USA EPA method 1613 revision B (USEPA, 1994). This method was developed by the Environmental Protection Agency, Science and Technology of the United States for the determination of 2,3,7,8-CDDs/CDFS replaced through octa-chlorination, dibenzo-p-dioxins and dibenzofurans in aqueous matrices, solid or tissue by isotope dilution, followed by capillary column of high resolution gas chromatography (HRGC)—high resolution mass spectrometry (HRMS).

c. Statistical Analysis

Analysis of data was carried out in accordance with the methodology of the USA EPA, for analysis of contaminants (Russell and Plumb, 2004). The methodology is summarized in four basic steps.

Step One: Standardization

The concentration of congeners observed for each sample is standardized. The constituents of each sample are converted to a decimal percentage of the sum of congeners.

Step Two: Construction of the bar plot of the standard concentrations

Graphic representation of the standard concentration of the different congeners for each sample. The Y-axis of the bar plot represents the relative amount of each congener in the sample (standard concentration) and in the X-axis it is represented the identification of the 17 different congeners. This chart will give an indication of the congeners that are present in greater concentration and if that pattern is maintained for the different samples.

Step Three: Statistical assessment of pattern reproducibility

This step concerns the used of the square of the Pearson correlation coefficient (r2) as a measure to assess whether the profile of the concentration of congeners in the samples (compared visually on the bar plot) is statistically similar. The Pearson correlation coefficient (r) measures the degree and direction of the correlation, positive and negative, between two variables of metric scale (Johnson and Wichern, 2001). Thus, a table is generated with the values of r2, in which each sample is compared with all others. If all values of the square of the Pearson correlation coefficient are near 1, it can be stated that the pattern of the samples, compared in pairs, is very similar. In order to assess the similarity of the concentration of congeners profile in various samples, it was used the global average comparison which is defined as the average values of r2 found. It is considered that the profiles are similar if the average of r2 is close to 1 and the standard deviation (SD) is next to zero.

Step Four: Evaluation of the source of contamination

Once established the congener's profile of the dioxins contaminations in each group of samples, the same methodology of the previous steps was applied to investigate a possible association between those groups.

6.3 Results

The results revealed levels with an average of 18 pg PCDD/F-WHO-TEQ/g fat in the first samples and an average of 8 pg PCDD/F-WHO-TEQ/g fat in the second samples (Table 11).

Table 10 - Comparison of the level of contamination in samples collected over time.

Pen	Number of ducks	First sample pg PCDD/F-TEQ-OMS/g	Ducks age. First sample	Second sample pg PCDD/F-TEQ-OMS/g	Ducks age. Second sample	Depletion in days	Average depletion/ day pg PCDD/F-TEQ-OMS/g	Average depletion/ day (%)
1	3450	17.1	40	18.7	75	35	-0.05	-2.86
2	3450	9.05	70	0.76	95	25	0.33	4.00
3	3450	3.23	42	0.92	67	25	0.09	4.00
8	3450	47.1	47	31.7	81	34	0.45	2.94
9	3450	15.4	40	9.45	63	23	0.26	4.35
9	3450	9.45	63	1.29	110	47	0.17	2.13
9	3450	15.4	40	1.29	110	70	0.20	1.43
10	3450	24.1	117	1.30	159	42	0.54	2.38

The evolution of the contamination profile of the ducks in the six pens, after the removal of the source of contamination is shown in Figures 21, 22, 23, 24, 25 and 26.

The Square Pearson Correlation Coefficient within each group ranged from 0,70 to 0,99.

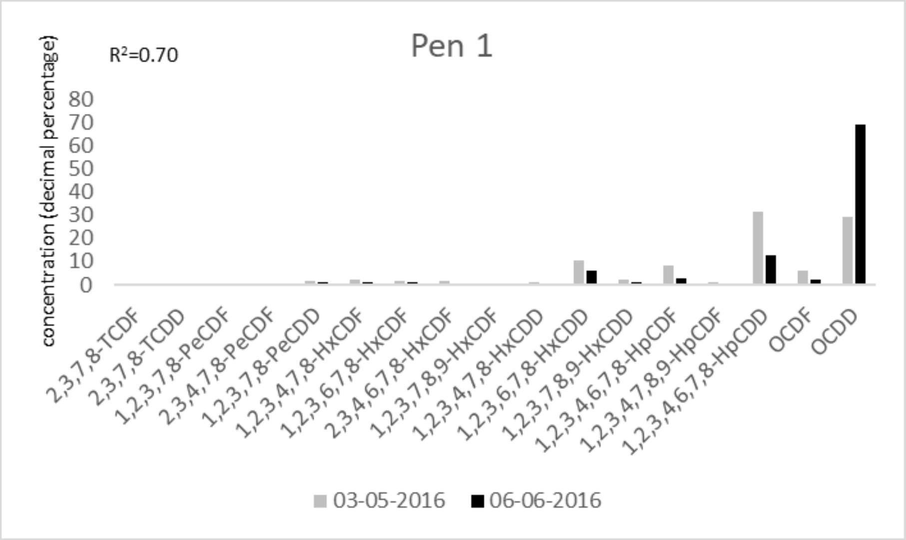

Figure 21 - Assessment of contamination profile in pen 1.

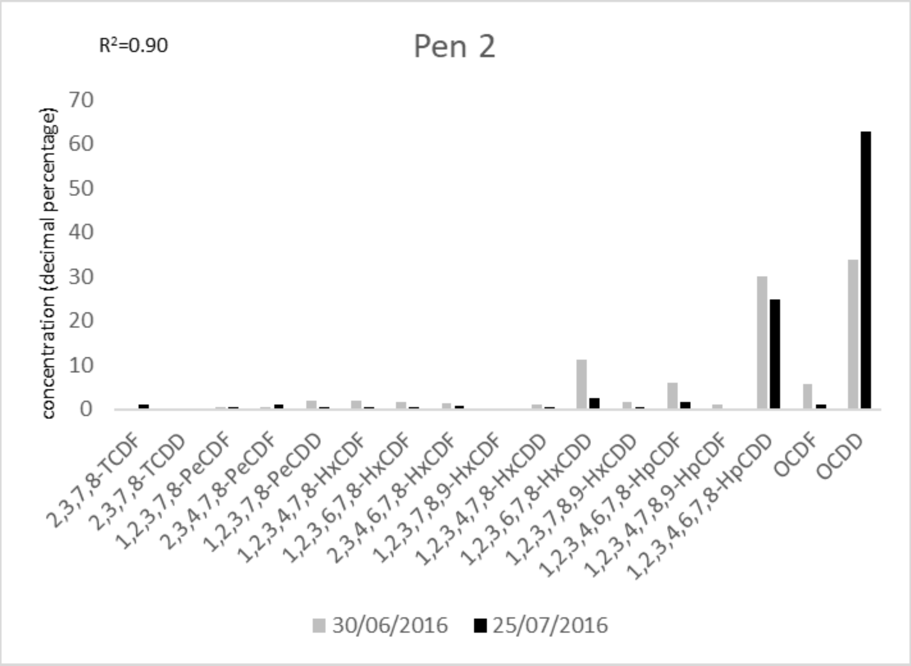

Figure 22 - Assessment of contamination profile in pen 2.

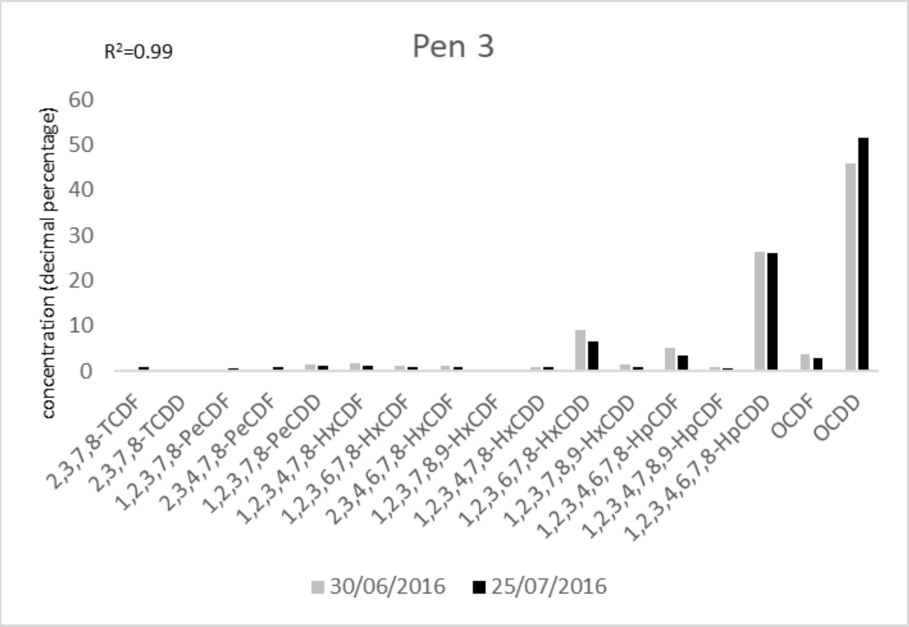

Figure 23 - Assessment of contamination profile in pen 3.

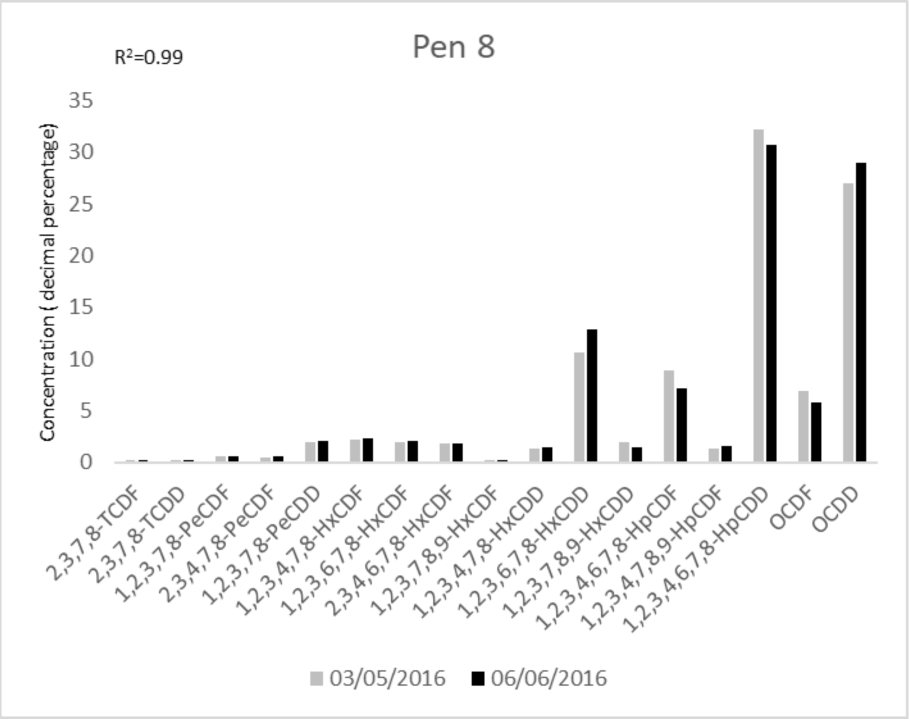

Figure 24 - Assessment of contamination profile in pen 8.

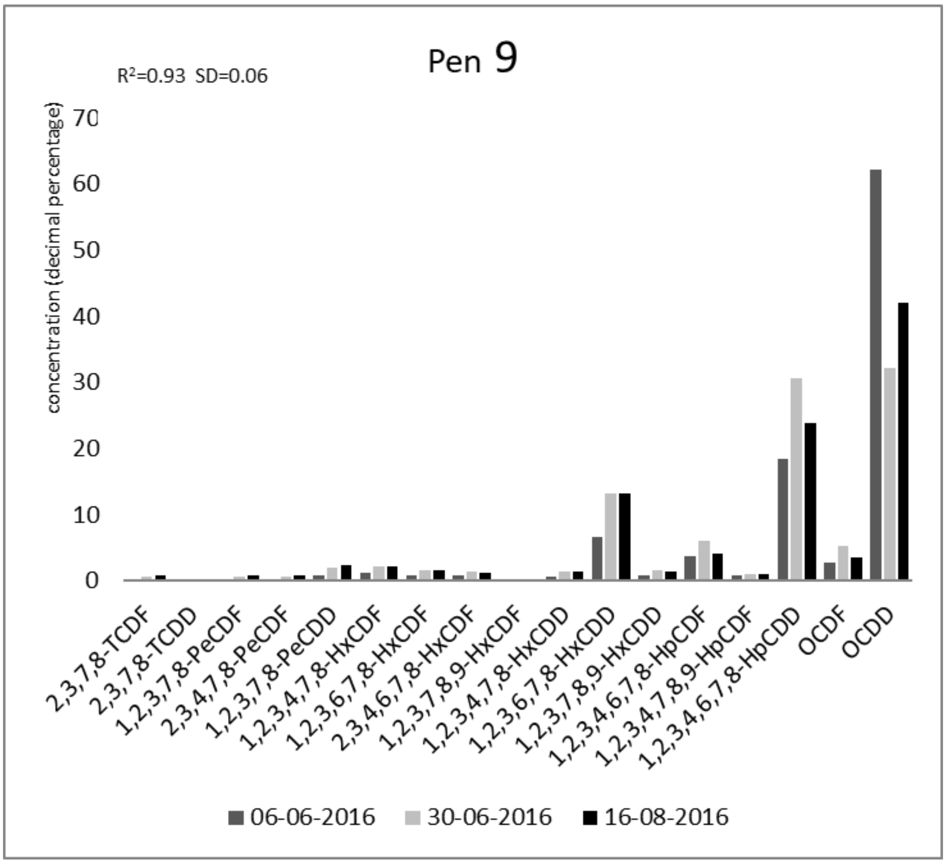

Figure 25 - Assessment of contamination profile in pen 9.

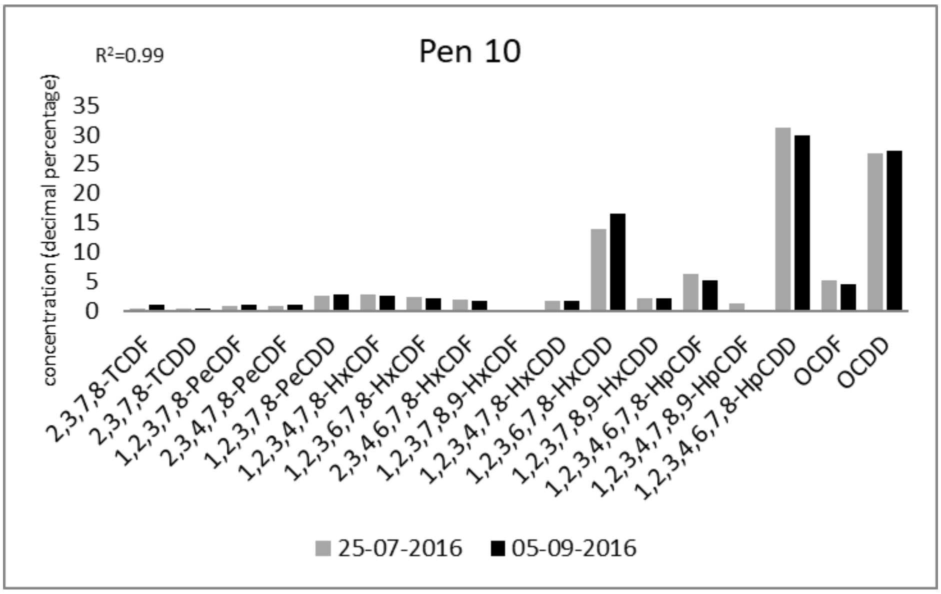

Figure 26 - Assessment of contamination profile in pen 10.

The profile of the samples collected in the bedding material (wood shavings) in the affected farm is presented in Figure 27. The level of contamination of the wood shavings was 239 and 39.7 pg PCDD/F -TEQ-WHO/g and the Square of the Pearson Coefficient was $R^2=0.99$.

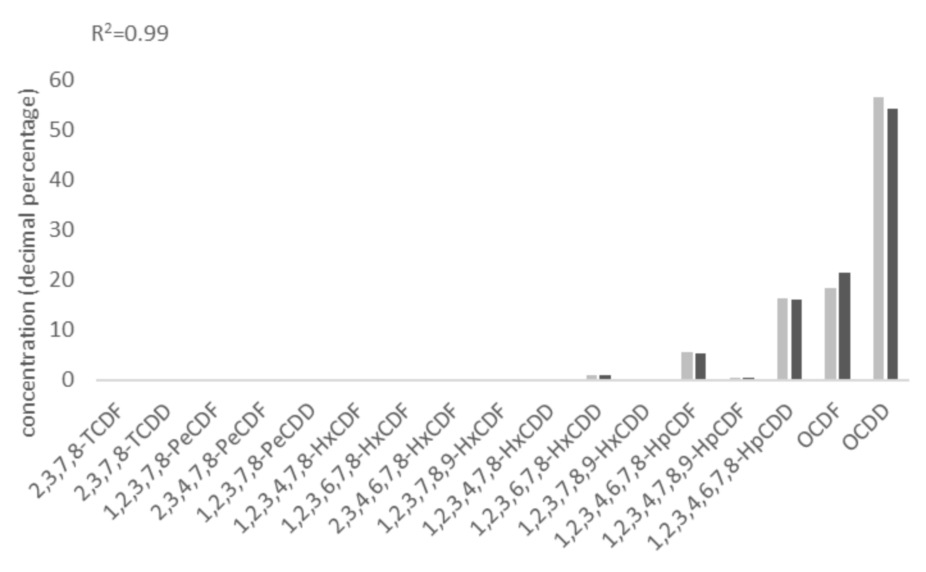

Figure 27 – Wood shavings contamination profile.

The comparison of the profile of the average contamination of the bedding material and all the samples collected from muscle and fat of the different pens with ducks is presented in Figure 28.

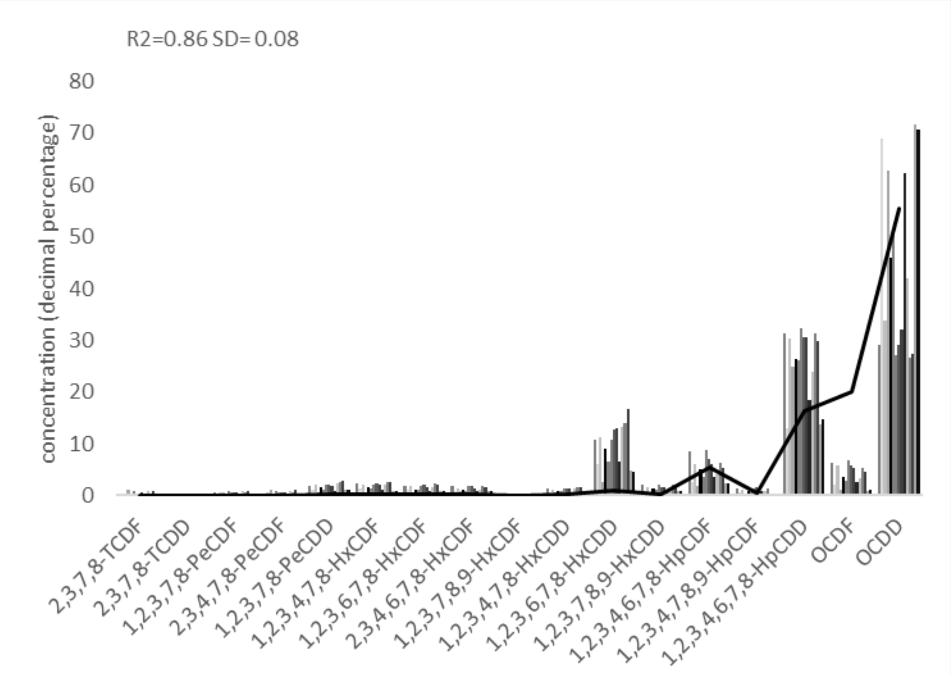

Figure 28 – Comparison wood shavings from bedding material (line) and muscle and fat of ducks.

6.4 Discussion

The contamination levels found in the poultry meat exceeded the maximum limit allowed by the European legislation for these substances in poultry meat, set at 1,75 pg PCDD/F-WHO-TEQ/g fat (CEC, 2006b).

The study of the incident occurred during 2016 in the duck production holding revealed a reduction of contamination after the removal of the source of contamination, with an average daily depletion percentage level ranging between 1.43% and 4.35%.

Hoogenboom, *et al*., (2004) found depletion rates of 4,7% in broilers in a tree weeks' elapse period, however the contamination profile was significantly different, being the least chlorinated congeners the predominant in the overall contamination. The author states that the least chlorinated congeners decrease more rapidly than the higher chlorinated congeners.

Several factors may contribute to influence the depletion level. This number of variables in addition to the few results in each affected pen did not allow the confirmation of what was previously expected, *i.e.* that the depletion level is influenced by the level of initial contamination, by the age of the ducks, that is, with the growth rate (dilution effect), by weight of the ducks and by the elapsed time.

The variables, except for the initial contamination, showed very similar quantitative values:

- The first analysis in all pens were made in ducks in the same production stage, the last third of fattening, *i.e.*, during the finishing phase, shortly before the expected date of slaughter, which is the period when they show lower growth rates;

- The second analysis in the positive pens were made, with one exception, not later than 110 days of live, despite the decision to extend the life of animals with the expectation that their contamination would be reduce after changing the bedding material. However, with the exception of four samples, the life time could not be further extended due to the fact that slaughter of ducks after 90 days is not advantageous from a commercial point of view. Furthermore, there was the need to ensure that there were no welfare problems by overpopulation of the pens, i.e.

as the ducks grow older, the vital free space for each one gets tighter, with losses in animal health and mortality. These conditions did not allow extending the depletion time in at least some pens.

The depletion rate (Average daily depletion percentage) has a negative association with the depletion period (Figure 29). In the case of pen 1, in which the bedding material was replaced by rice husk later, one week after the first sample, the depletion rate showed negative values.

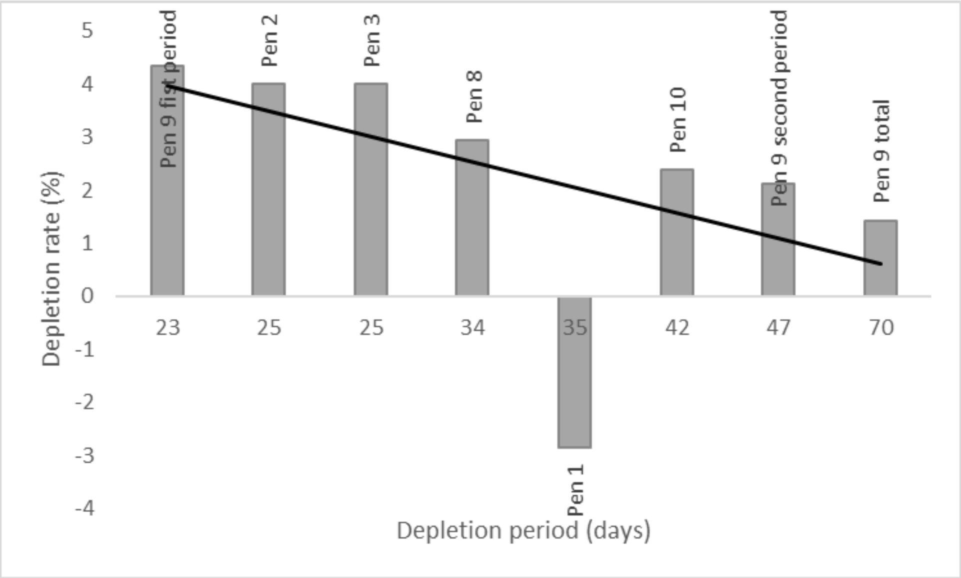

Figure 29 – Depletion rate variation with the depletion period in days.

In some pens, there was an increase of the most chlorinated congeners (pen 1, 2 and 3), in another pen, precisely the opposite happened (pen 9) and yet, there were two other pens in which the profile has remained unchanged (pen 8 and pen 10). This lack of consistency in the behavior of the congener's relative concentration does not allow drawing up conclusions concerning the profiles in the different pens.

The pattern detected in the wood shavings matches with the profile found in contaminated wood shavings obtained from treated wood by other authors (Hagenmaier and Brunner, 1987; Fries, *et al*., 1996), this suggests that the wood shavings used in the litters were obtained from treated wood, being the wood preservative the possible source.

The assessment carried out between the profiles found in the wood shavings samples and the muscle fat of the ducks reared on those litters showed a coincidence of peaks of higher relative concentration in each samples. These results concern in particular the relative concentrations of the congeners 1,2,3,4,6,7,8-HpCDD and OCDD (Cardo, *et al*., 2014). Graphic representation of the relative concentrations of the different dioxin congeners in each matrix had different comparative expressions. For example, in the duck muscle fat profiles, the average relative concentration of OCDD and OCDF was respectively 18% and 81% lower, comparatively to the wood shavings. On the other hand, the average relative concentration of 1,2,3,6,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD in the duck muscle fat profiles was respectively 89,4% and 35,4% higher when compared with the litters.

In the wood shavings, the concentration of the three most chlorinated congeners (1,2,3,4,6,7,8-HpCDD, OCDF and OCDD) represents 91,6% of the total concentration of the 17 congeners, while, in the muscle and fat of the contaminated duck analysis it represents only 74,4%.

The figures suggest that, probably, there are some factors which may affect the consistency in quantitative terms of the relative concentration of congeners found in wood shavings and in duck muscle fat. Several explanations could justify this lack of consistency: A dechlorination of the most chlorinated congeners due to an "*in vivo*" metabolic pathway; a differentiation on the lipophilic affinity of each congener; variations on the affinity to the Ah receptor and selective faecal excretion by the birds, also equivalent to a lower absorptive capacity (Lynam, *et al*., 1998; Pirard and Pauw, 2004).

6.5 Conclusions

This study clearly identified the source of the duck farm contamination, taking into account the relationship established with an environmental vehicle of dioxins (wood shavings). This achievement is based on the consistency of the results obtained with all the tested materials regarded as potential contamination source and the fingerprint analysis of the relative concentration of the 17 dioxin congeners.

This study was developed in a real scenario, involving 20 000 animals and subjected to legal constraints. These are related to animal welfare (excessive weight, husbandry area and animal density) and the deadlines imposed on official services to take action against the results found, as well as economic interests of the producer involved in the incident, limiting the depletion times applied in each pen and the number of analyzes performed. Despite these constraints, it was concluded that this study in a duck farm, revealed a reduction of contamination in muscle and fat of the ducks after the removing the source of contamination, with an average daily depletion percentage level (depletion rate) ranging between 1.43% and 4.35%. These results do not allow to establish a fixed depletion rate for ducks, since it was not possible to establish a pattern associated with age or initial contamination level. Furthermore, it was concluded that pens with shorter estimated depletion period, showed higher average daily depletion percentage. This fact indicates that the average daily depletion percentage decreases over time after the removal of the contamination source.

7. Global Discussion

The current study was carried out using real data collected during risk management contingency measures, following the contamination of the poultry production chain with dioxins occurred in Portugal in 2006, 2011 and 2016.

The field study included hundreds of analysis for detection and quantification of dioxins, collected in dozens of affected poultry farms (breeding and fattening), in egg incubation centres and in slaughterhouses. In all three incidents, thousands of poultry were slaughtered and destroyed and thousands of kilograms of poultry meat were withdrawn from the market. These crises had a substantial financial impact in the poultry sector, especially on the affected farms, but there was no abnormal impact on consumption at market level.

The results obtained in this study, provided the answers to better understand and explain the contamination of the animals by an uncommon and rarely described route. They will contribute to the evaluation and improvement of the crisis response capacity in cases of contamination of the food chain, notably with litters made of treated wood shavings.

A reflection on the results obtained allows mentioning, with respect to the adaptation of the analysis method 1613 revision B that, the recovery rates of $^{13}C_{12}$-labelled compounds added to the samples of wood chips varied between 71.3% and 86.3%. These rates are considered acceptable by both the 1613 B method (EPA, 1994) and the criteria for food and feed laid down in European Union legislation.

According with the EU criteria for food and feed, the recovery shall be within 60% to 120%, especially for congeners contributing more than 10% to the TEQ-level for analysis of foodstuffs and feed for confirmatory methods and, for screening methods, the recoveries shall be in the range of 30% to 140% (EC, 2014; EC, 2014b).

The contamination profile detected in the woodchips of the bedding material corresponds to the profile of technical pentachlorophenol contaminated by dioxins found by other authors (Hagenmaier *et al.*, 1987; Grozwalski and Gorski, 1990; Fries *et al.*, 1996; Cleverly *et al.*, 1997; Johnson *et al.*, 2001). This profile could suggest that the wood chips used in the bedding material were obtained from wood treated with pentachlorophenol. Pentachlorophenol contains dangerous impurities, namely up to 0,1% of polychlorodibenzodioxins and 1 to 5% of polychlorinated phenoxyphenois. However, the use of this substance for the treatment of wood has been subject to several restrictions in Europe since 1991, but there are some derogations for its use in the industrial treatment of wood (Directive 91/173/EEC of 21 March). Nevertheless, wood treated with this product may not be used inside buildings, for decorative purposes or not, for whatever purpose (housing, work, leisure) or in the manufacture of containers or packaging that may come in contact with other materials or products intended for human and/or animal consumption.

This has raised new possibilities, such as the possibility that industrial products for the treatment of wood (pesticides and fungicides) currently in use may also be contaminated, possibly during the manufacturing process, as pesticides can contain impurities of PCDD/Fs, and their precursors, as a result of various manufacturing processes and conditions (Holt *et al.*, 2012).

The levels of contamination of treated wood will thus be dependent on the concentration of PCDD/Fs in the preservative solution and on the amount of product that is retained by the wood in the treatment process.

Several authors describe studies comparing retention of wood preservatives in wood obtained from different species, with different conservation methods and with different preservatives.

The retentions obtained in the laboratory in the present study varied with the concentrations of the solutions used. With impregnation treatment of the solution with vacuum, an average solute retention of 16.7 kg/m^3 was obtained when a concentration of 4% in the treatment C was used and an average retention of 4.45 kg/m^3 with a concentration of 2% in treatment D.

Comparison of wood impregnation studies is quite difficult since retention levels vary with a number of factors, such as wood species, wood moisture content, volume of tested wood pieces, treatment time, pressure used, and the treatment itself, that is, if only vacuum is applied or the vacuum is switched with positive pressure. This difference is not as substantial in immersion treatments.

The impregnation studies performed by Yildiz *et al*., (2004), compared to the values obtained in the present study, presented higher retention levels with lower concentrations, but with samples with a much lower volume. The studies performed by Ozemir *et al*., (2015) showed equivalent retention levels, with specimens of similar volume, but for less time. The studies performed by other authors, in some cases, have larger retentions and other smaller ones, even using vacuum and pressure. In industrial conditions the retentions are substantially higher.

The results of the contamination levels of the treated wood chips presented very low contaminations when compared to the contaminations of the wood chips, observed in the incidents of poultry contaminations, probably due to lower retentions compared to the retentions obtained under industrial conditions.

The contamination profiles of the different treatments used, showed a very strong correlation R^2=0.99 and a standard deviation of 0.001, so there was no significant difference, in terms of profile, of the contamination caused by the different wood treatment products.

The comparison of the profile of the wood treated in the present study, with the profile of the litters of wood chips responsible for the contamination of the poultry chain in the incidents occurred in Portugal in 2006, 2011 and in 2016, also showed a very high correlation. R^2=0.99 and SD=0.002 compared to the beds analysed in 2006, R^2=0.99 and SD=0.007 compared to the beds analysed in 2011 and R^2=0.96 and SD=0.012 compared to the beds analysed in 2016.

This recurrence of the profile may be important for risk managers because it allows, on the basis of a sample of poultry meat, to immediately associate a profile equivalent to its source of contamination without having to waste time and resources analysing all possible sources of contamination.

The set of results obtained during incidents of contamination of the poultry production chain in 2006, 2011 and 2016, showed considerable contamination of the wood chips, indicating that these materials are the probable source of contamination of the animals.

During the early stages of their development, chicks have a habit of eating some particles from bedding materials, contaminating themselves through the digestive tract in addition to contamination by inhalation and skin contact. This behaviour potentiates the danger of contamination, since the chicks select some special fragments with darker points, because they look like small arthropods that are normally consumed under natural conditions (instinctive behaviour). Furthermore, some by-products of wood, obtained from partially burned pine, treated or painted wood, often have different coloured particles.

The results of analysis carried out in Portugal show that levels of average contamination with dioxins (pg TEQ-WHO/g) are decreasing along the poultry production chain (193.7 pg TEQ-WHO/g for breeding hens, 42.3 pg TEQ-WHO/g for hatching eggs and 4.7 pg TEQ-WHO/g for broilers).

The evaluation performed between the profiles found in the wood shavings samples of the litters, in the muscle/fat of breeding hens reared on those beds and in the hatching eggs laid by these contaminated chickens and their respective day-hold chicks, showed a coincidence of the peaks with the highest relative concentration in their samples, especially for the relative concentrations of the congeners 1,2,3,4,6,7,8-HpPCDD and OCDD.

The graphical representation of the relative concentrations of the different dioxin congeners, in each of the matrices, had different comparative expressions. For example, in the fat and muscle profiles of broilers and breeding hens, the relative average concentration of OCDD was 52% and 53% lower, compared to the profile of the wood chips and hatching eggs, respectively (Salvantem *et al*., 2007). On the other hand, the relative average concentration of 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD in the profiles of fat and muscle samples of broiler and breeding hens was 50% to 97% higher, when compared to the profiles of the beds and hatching eggs.

The numbers suggest that there is probably a factor that may affect the consistency, in quantitative terms, of the relative concentration of congeners found in wood chips and in poultry muscle. Many explanations can be pointed out for these facts, such as: an *in vivo* metabolic pathway that allows dechlorination of the most chlorinated congeners; a differentiation in the lipophilic affinity of each congener; variations in the affinity for the receptor "Ah" and the selective faecal excretion by birds, also equivalent to a lower absorption capacity (Lynam *et al*., 1998; Pirad and paw, 2004).

A balance between the concentration of dioxins in the contaminant ingested by chickens (38.85 pg TEQ-WHO/g) and the concentration of dioxin in the eggs (42.25 pg TEQ-WHO/g) was verified, which is confirmed by other studies (De Vries *et al.*, 2006; Petreas *et al.*, 1991).

The fact that the square of the Pearson correlation coefficient (r^2) of the comparison of the relative concentrations of the different congeners between bedding material and hatching eggs (R^2=0.72 and sd=0.16) and the graphical representations of the profiles are so consistent, can be explained by the oestrogens that is produced by the hens during laying of eggs that stimulate the liver to produce a very low density lipoprotein (VLDLy) intended for the production of yolk (egg yolk targeted). This shift from the synthesis of generic VLDL to oestrogen-dependent VLDL synthesis, translates into a variation in lipoprotein diameter which ranges from 30 to> 200 nm in size to a lower VLDLy, ranging from 15 to 55 nm in diameter. This change represents a dramatic change in the lipid metabolism of females in posture associated with changes in the composition and structure of VLDL. While the role of generic VLDL is the transport of triglycerides throughout the body for use in tissues or storage in adipose tissue, the function of VLDLy is to deliver triglycerides to the oocyte where they will be used as energy source for the developing embryo. The smaller diameter of VLDLy is thought to be critical to allow particles to pass through the pores of the basal lamina of granulosa of the ovary, allowing them access to the developing ovarian follicles. VLDLy particles are at high plasma concentrations in laying hens and are resistant to hydrolysis by extra ovarian tissues that reserve the triglycerol-rich VLDLy for use in developing ovarian follicles (Salvantem *et al.*, 2007).

The study of the duck farm in Portugal in 2016 revealed a reduction in contamination, once the source of contamination was withdrawn, with an average daily depletion level ranging from 0.093 to 0.453 pg PCDD/F-TEQ-WHO/g, which is equivalent to 1.43% to 4.35% of the initial contamination.

Another study, in which poultry was intentionally fattened with contaminated feed during 6 weeks, conclude that there was no decrease in dioxin residues in broiler chicken's edible tissues after a 2 or 4 weeks' withdrawal period (Iben *et al.*, 2003).

Depletion of PCDD and PCDF in eggs obtained from laying hens reared on woodchips treated with pentachlorophenol with a contamination of 40 pg WHO-

TEQ/g was studied by Fochi *et al.,* (2006). They found a regression line for the half-life of the different dioxin congeners in the posture, finding values of the order of 4 weeks for the mean depletion of the different congeners in the eggs. The authors considered that the pathway of exposure through dermal absorption and inhalation, besides feeding, cannot be excluded. This differs from previous studies that found values of 1.5 weeks for depletion after one week of exposure to contaminated feed and of 7 weeks for flocks reared on soil naturally contaminated with PCDD / PCDF (Fochi *et al.*, 2006).

The scarcity of results and the number of variables did not allow us to confirm what was expected, that is, that the level of depletion is influenced by the level of the initial contamination, with the age of the ducks, that is to say, with the rate of growth (effect of dilution) and with the time elapsed.

The variables, except for the initial contamination, showed very similar quantitative values:

- The first analysis was carried out on ducks in the same age group, in the last third of the fattening period, that is, in the finishing phase, shortly before the date of slaughter, which is the period when lower growth rates are registered;
- The fact that the owner of the farm decided to extend the life of the animals expecting that the contamination would be reduced. However, the duck slaughtering after 80 days would not be commercially advantageous and the need to ensure that there were no welfare problems by overcrowding of pavilions, did not allow prolonging the depletion time in, at least, some pavilions.

The analysis of the profiles in the different pens also did not allow conclusions to be drawn, since in some of the pens there seems to have been an increase in the more chlorinated congeners (Pavilions 1,2 and 3) and in another pavilion the opposite happened (Pavilion 9) and there were still two pavilions in which the profile remained unchanged (Pavilions 8 and 10).

These results lead to the conclusion that it was not possible to establish a standard associated to the age or initial contamination level because no average fixed depletion rate for ducks could be fixed. It could be concluded that, the pens where the evaluated depletion period was shorter, presented higher percentages of daily depletion, indicating that the average daily depletion percentage decreases over time, after removal of the source of contamination.

8. Conclusions

The first incident of contamination of the poultry production chain in Portugal pointed out a different source of contamination from the usual ones (feed and water), revealing that the contamination was introduced into the food chain indirectly through poultry beds that allowed prolonged exposure of poultry flocks to dioxins.

The samples collected from this matrix revealed considerable contamination levels. However, knowing that wood does not have much fat and that dioxins are preferentially bound to fat in nature, the extraction efficiency of the reference analytical method was questioned.

The fact that further investigations identified wood chips as the source of contamination and that this matrix had very low levels of fat, obliged, in order to assess the real importance of these contamination routes, to improve the method of analysis, in particular, to increase the capacity of extraction, as analytical data must be reliable. Chemical fingerprinting analysis methods, are dependent on the good quality of analytical chemistry data and are thus, vulnerable to data quality problems. Analytical data for the same compounds, but from different laboratories or derived by different methods, can introduce uncertainty in the comparison of the results. The frequency of “non-detects” in the congeners results and how the reporting limit is handled, can sometimes be critical to the data analyses and can potentially bias fingerprinting analysis results, particularly when compounds, such as CDD/CDFs, occur at extremely low concentrations.

The new method resulted from this adaptation, proved to be quite efficient in extracting and achieving marked pattern recovery rates required both by the reference method for the development of adaptations and by the European legislation applicable to dioxin detection and quantification methods for PCDD/Fs and PCBs, in both food and feed.

The fact that the analysis of wood chips during the three incidents of contamination of the food chain always revealed the same contamination profile, led to the need to evaluate the possible existence of different contamination profiles in the most used wood treatment products in Portugal.

The results of the comparison of four commercial solutions (two anti-sapstain and two pesticides of depth penetration) showed that the contamination profile of the different treatments showed a fairly high correlation (R^2 = 0.99 and SD = 0.001). It is concluded that there is no difference in the wood contamination profile, regardless to the commercial product used. This profile also showed a very high correlation when compared to the profiles of contamination of the wood chips found in the incidents of 2006, 2011 and 2016. However, the values of the contamination levels found in the treated wood in the laboratory were substantially lower than the contamination levels of the poultry litters found during such incidents.

The retention rates of the preservative solution in the laboratory are less significant than those obtained in the industrial treatments and consequently the contamination levels of the wood were expected to be lower, although it has been verified that the retentions have no influence on the resulting congeners profile, which remained unchanged.

These results suggest that the PCDD/Fs present in the contamination of the treated wood result from the process of manufacturing the pesticides and not from the wood treatment process. For this reason, treated wood must be disposed off as hazardous waste and its recovery, prohibited.

The misuse of hazardous wood by-products in poultry production has resulted in the absorption of dioxins, either by ingestion of some of the chips, especially in the early stages of growth, or by absorption through inhalation or contact with contaminated beds.

The constancy and strong correlation of the contamination profiles observed in the wood treated with different wood preservatives was not reproducible in the contaminated meat and egg samples. This may suggest that the biotic PCDD/Fs distribution is more complex than the abiotic PCDD/Fs distribution.

The contamination fingerprint of the bedding material has undergone surprising changes when passed to the birds, *i.e.*, there is a maintenance of the profile between the contaminated litters and the eggs and there is a change in the profile in the live birds, in the breeding hens, as in the meat of the broilers. These data suggest an *in vivo* transformation of dioxins and furans by dechlorination of the most chlorinated congeners.

Poultry contamination has also been studied, not only in terms of contamination levels but also in their profiles. This evaluation allowed to conclude that the contamination levels of the birds decreases as the time passes after the removal of the source of contamination (wood chips). This study was limited by additional constraints related to animal welfare and other legal provisions to which official services are subjected to.

It was found that the possibilities of contamination of the food chain can come from unexpected and sometimes multifactorial sources. This study contributed with new factors to be considered in a reflection of the control measures – under risk management – applicable in the poultry industry.

Based on these conclusions, it may be considered a review of the measures applicable to risk management, namely regarding the actions of the different entities involved and the responsibility given to the different stakeholders in the agro-food production chain.

The wood industry, notably the 23 companies, involved in the wood treatment in Portugal and the hundreds of sawmills that import, process, distribute and apply treated wood in the country, often do not understand the impact of their activities as suppliers of production factors in the animal food production chain. The separation of by-products from wood in the sawmills and wood industries, working on treated wood, must be very careful and rigorous and the by-products of treated wood should be sent as hazardous waste for incineration. Such awareness is essential, as the recovery of hazardous by-products to poultry farming is cost-effective and their disposal, as hazardous waste, is very costly.

Environmental authorities have been accompanying and collaborating with the veterinary health authority in the management of these crises. Their more frequent and targeted surveillance actions may be essential in the prevention and punishment of offenders by the application of penalties or sanctions, with dissuasive capacity to maintain and perpetuate illicit acts, would be determinant.

Poultry producers are dependent on the quality of the wood shavings provided by the wood industry. Other poultry bedding materials, such as rice husk and shredded straw, have been used, without great success, because they are not as good in terms of handling and maintaining the good condition of the birds' paws and are not available in quantities that can meet the needs of the national poultry production. Livestock producers will have to carry out random analysis of the wood shavings batches supplied and request a declaration that will hold the supplier accountable, stating that the consignment of wood chips delivered does not contain treated wood chips.

In recent incidents, the slaughtering and destruction of animals and the withdrawal from the market of meat have represented high losses to the poultry production companies targeted, some of which have been bankrupt, as a result of these episodes.

Veterinary health authorities monitor the presence of dioxins annually in poultry products and are responsible for the management of crises resulting from contamination of the food chain. In light of the significant number of incidents of contamination of the poultry chain with dioxins and the associated risks, the possibility for intensifying the sampling for dioxins in poultry meat and table eggs should be assessed and considered accordingly.

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