Biomonitoring Research Madrid
Spain, 2021

ToxicoWatch Foundation – December 2021

Acknowledgements

Thanks to Zero Waste Europe for making it possible to perform this toxicology research on persistent organic pollutants (POPs) in the environment of Madrid, Spain.

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Abbreviations

Abbreviation	Meaning
APCD	Air Pollution Control Devices
BAT	Best Available Techniques
BEP	Best Environmental Practice
BEQ	Biological Equivalents
BMI	Body Mass Index
dl-PCB	Dioxin-Like Polychlorinated Biphenyls
DR CALUX®	Dioxin Responsive Chemical-Activated LUciferase gene eXpression
dw	Dry Weight
EFSA	European Food and Safety Authority
FITC-T4	Fluorescein IsoThioCyanate L-Thyroxine (T4)
GC-MS	Gas Chromatography Mass Spectrometry GC-MS
GenX	Group of fluorochemicals related to of hexafluoropropylene oxide dimer acid (HFPO-DA)
i-PCB	Indicator Polychlorinated Biphenyl
LB	Lower Bound; results under detection limit are set to zero
LOD	Limit of Detection
LOQ	Limit of Quantification
MB	Middle Bound; values are set as half the detection limit values
MWI	Municipal Waste Incineration
ndl-PCB	Non-Dioxin-Like Polychlorinated Biphenyl (Non-Dioxin-Like PCB)
ng	Nanogram; 10^{-9} gram
OTNOC	Other Than Normal Operating Conditions
PAH	Polycyclic Aromatic Hydrocarbons
PCB	Polychlorinated Biphenyl
PCDD	Polychlorinated Dibenzodioxins
PCDF	Polychlorinated Dibenzofurans
PFAS	Per- and PolyFluoroAlkyl Substances
pg	Picogram; 10^{-12} gram
POP	Persistent Organic Pollutants
RPF	Relative Potency Factors
RvA	Dutch Accreditation Council
SVHC	Substances of Very High Concern
SWI	Solid Waste Incineration
TCDD	2,3,7,8-tetrachloordibenzo-*p*-dioxine
TDI	Tolerable Daily Intake
TEF	Toxic Equivalency Factor
TEQ	Toxic Equivalents
TOF	Total Organic Fluorine
TW	ToxicoWatch
TWI	Tolerable Weekly Intake
UB	Upper Bound (ub), results under detection limit are set as detection limit values.
µg	Microgram 10^{-3} gram
WtE	Waste to Energy (waste incinerator)

Abbreviation	Dioxins, furans (PCDD/F) and dioxin-like PCBs	Toxic equivalency factor
	Congeners	TEF
Dioxins (n=7)		
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin	1
PCDD	1,2,3,7,8-Pentachlorodibenzo-p-dioxin	1
HxCDD1	1,2,3,4,7,8-Hexachlorodibenzo-p-dioxin	0,1
HxCDD2	1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	0,1
HxCDD3	1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	0,1
HpCDD	1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin	0,01
OCDD	Octachlorodibenzo-p-dioxin	0,0003
Furans (n=10)		
TCDF	2,3,7,8-Tetrachlorodibenzofuran	0,1
PCDF1	1,2,3,7,8-Pentachlorodibenzofuran	0,03
PCDF2	2,3,4,7,8-Pentachlorodibenzofuran	0,3
HxCDF1	1,2,3,4,7,8-Hexachlorodibenzofuran	0,1
HxCDF2	1,2,3,6,7,8-Hexachlorodibenzofuran	0,1
HxCDF3	1,2,3,7,8,9-Hexachlorodibenzofuran	0,1
HxCDF4	2,3,4,6,7,8-Hexachlorodibenzofuran	0,1
HPCDF1	1,2,3,4,6,7,8-Heptachlorodibenzofuran	0,01
HPCDF2	1,2,3,4,7,8,9-Heptachlorodibenzofuran	0,01
OCDF	Octachlorodibenzofuran	0,0003
Polychlorinated biphenyl (n=12)		
PCB77	3,3',4,4'-Tetrachlorobiphenyl (#77)	0,0001
PCB81	3,4,4',5-Tetrachlorobiphenyl (#81)	0,0003
PCB126	3,3',4,4',5-Pentachlorobiphenyl (#126)	0,1
PCB169	3,3',4,4',5,5'-Hexachlorobiphenyl (#169)	0,03
PCB105	2,3,3',4,4'-Pentachlorobiphenyl (#105)	0,00003
PCB114	2,3,4,4',5-Pentachlorobiphenyl (#114)	0,00003
PCB118	2,3',4,4',5-Pentachlorobiphenyl (#118)	0,00003
PCB123	2,3,4,4',5-Pentachlorobiphenyl (#123)	0,00003
PCB156	2,3,3',4,4',5-Hexachlorobiphenyl (#156)	0,00003
PCB157	2,3,3',4,4',5'-Hexachlorobiphenyl (#157)	0,00003
PCB167	2,3',4,4',5,5'-Hexachlorobiphenyl (#167)	0,00003
PCB189	2,3,3',4,4',5,5'-Heptachlorobiphenyl (#189)	0,00003

Introduction

The complexity of the chemical content of today's household and industrial waste presents a challenge for turning modern waste into energy in (WtE) waste incinerators. Even with the application of the most developed air pollution control devices (APCD), it is still a huge challenge to eliminate the multitude of persistent organic pollutants (POPs) in waste incinerator residues and flue gases. The dynamics of combustion processes and the inevitable emissions of toxic substances of very high concern (SVHC) into the environment is the main topic of ongoing research worldwide. Even in the most remote areas of the world, such as the Arctic (marine environment), toxic chemicals are found, which have been transported huge distances from industry in other parts of the world. Because of the transboundary behavior of persistent organic pollutants, international treaties are required to regulate, mitigate or even eliminate toxic chemical emissions. Loopholes still exist in national and international regulations, resulting in an underestimated registration of persistent organic pollutants. Mandatory measurements for waste incineration relating to toxic pollutants like dioxins are sampled in a very short time frame of 6-12 hours a year in optimal conditions and pre-announced, all according to the EU regulations. These regulations are based on chemical analyses of only a few chlorinated dioxins and furans, while many other POPs remain outside the scope, such as brominated dioxins and PFAS. The limitations of the chemical GC-MS analyses could be overcome with the application of bioassays for measuring POPs even in the flue gases of an incinerator. Continuous monitoring of dioxins and other substances of very high concern in the chimney gives a far more accurate picture of the emission from combustion, especially when it is measured in the event of incomplete combustion as in exceptional operating conditions such as shutdown or start-up.

All over the world, there is growing public awareness and concern over the potentially toxic effects of persistent organic pollutants on human health and the environment. In particular, people living near waste incinerators need to be reassured about their health risks, (short- and long-term exposure to incineration emissions), the safety of such combustion facilities, and compliance with regulations – not only under normal conditions, but also in other than normal operating conditions (OTNOC), such as shut-downs, start-ups, and failures.

ToxicoWatch (TW) aims to function as a bridge between people, science, and government when it comes to dioxins, POPs, and waste incineration. TW performs research on dioxins with a focus on a possible sources like waste incineration emissions by carefully selecting biomarker samples in an area. A sampling with focused matrices like distance, sample location and collecting information about the research area needs to be performed according to the theory of sampling (TOS) with references in the interest of the research. The biomatrices for this study are primarily backyard chicken eggs, pine needles, and mosses. The chemical analyses are expanded with innovative bioassays to investigate a broader spectrum of POPs such as dioxin-like PCBs, other (mixed) halogenated dioxins, PAHs, and PFAS.

This study is part of a Europe-wide biomonitoring research project on POP emissions in possible relation to waste (WtE) incineration. The project is running simultaneously for 2021 and 2022 in three countries: Lithuania, Spain, and the Czech Republic. ToxicoWatch Foundation, based in the Netherlands, is participating as a scientific partner together with three environmental organizations, Ecologists in Action Spain, Hnutí DUHA in Lithuania, and Žiedinė Ekonomika in the Czech Republic, all coordinated by Zero Waste Europe.

The incinerator

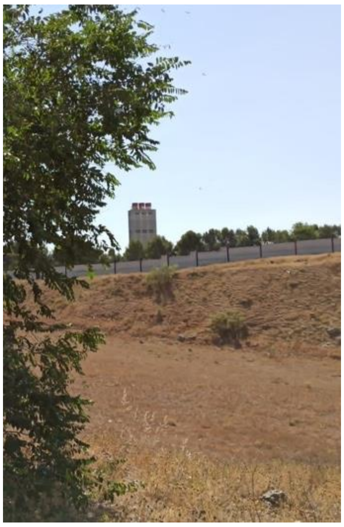


Figure 1: The waste incinerator located at Valdemingómez Technology Park.

In Spain, southwest of Madrid, the Valdemingómez waste-to-energy incinerator (WtE) belongs to Madrid City Council; commissioned in 1996, it currently burns an average of 900–1,000 tons of waste per day with three operating furnaces. Since 2018 Valdemingómez is incinerating more than 300,000 t / year. The energy of this WtE incineration plant of recoverable fraction currently comes from several sorting and recycling plants that process municipal solid waste (MSW): the La Paloma plant, the Las Dehesas plant, and the Las Lomas plant itself. All these plants are located on Valdemingómez Technology Park. The last two plants are a new organic matter treatment and composting plant. These plants mainly recover organic material, plastics, aluminium, and ferrous materials. The rest is what is considered Refuse-Derived Fuel (RDF).

The waste incinerator has three lines that can operate simultaneously, with two fluidized bed incinerators, where RDF is combusted. The boiler, where the heat from the combustion gases is recovered for electricity generation, and finally a gas cleaning system. The flue gas cleaning system consists of several phases: removal of coarse particles utilizing a cyclone system; semi-wet absorption, to reduce acid gases (mainly HCl and SO_2) utilizing a lime slurry shower; adsorption with activated carbon, whereby dioxins and furans, among other pollutants, are removed from the gas stream; the bag filter of the waste incineration installation, where the finest particles and the previously used activated carbon are retained; and catalytic reduction of nitrogen oxides. The chimney is 45 metres high, which makes it likely that substances of very high concern (SVHC) could be found in the vicinity. For a long time, this waste incinerator has been encountering opposition from people who are seriously concerned about the health risks resulting from the emission of toxic substances from the WtE incinerator. There are plans to close the incinerator at Valdemingómez in 2025.

Figure 2: Homes of inhabitants (7,283 people) of the Cañada Real Galiana, Madrid (picture Google Earth)

The main populated area near the incinerator is the Cañada Real Galiana. According to data from the Commissioner of the Community of Madrid for this road, 7,283 people live along the entire Cañada Real. The inhabitants of the area often lack basic needs like clean running water and electricity.

Wind direction and depositions

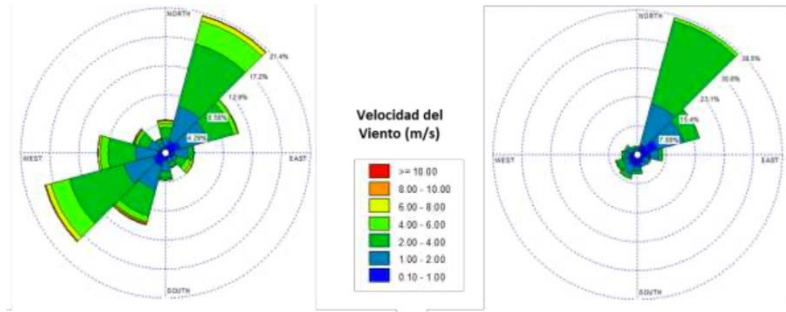


Figure 3: Annual and 2-week wind rose for Madrid, Spain

The annual average wind direction in Madrid is shown in figure 3[1]. This can be used as a model to predict possible depositions by the incinerator. The dominant wind direction is mainly from the South-West and North. The use of a wind rose for modelling deposition emissions from incineration processes is limited. Figure 4 shows on the left the dominant wind direction in Harlingen, the Netherlands (NL), South-West wind from the North Sea. On October 1st 2015, a major malfunction occurred at the WtE waste incineration plant, which was accompanied by prolonged emissions of black clouds that blew in the direction of the UNESCO Wadden Sea on that particular day. The city and region of Harlingen (NL) escaped being hit by an enormous toxic cloud of dioxins. This example of a calamity in a waste incineration process illustrates the limitations of using annual average wind direction "safety models" to determine the load of emission depositions. Dense clouds of emission-loaded dust can and will occur during OTNOC situations like failures, shutdowns, and start-ups. TW studies have learned that in just a few hours emissions of dioxins can emit far more than the annual load of a dioxin model calculated by the regulatory 12 hours (2x 6 hours/year, preannounced) measurement during normal operating conditions. Assuming the emission of dioxins is a discontinued process, calculation with average wind direction and speed is of little importance as large emissions can occur in a very short time frame. Figure 4c shows dioxin-contaminated eggs of a TW research around the WtE waste incinerator in Harlingen (NL).

Wind direction is an indication, but the deposition of emissions can differ completely when OTNOC and other parameters like coastline fumigation along seashores, or mountain ridges and valleys are included, as they should be.

In a very short time, in hours or even minutes, extremely polluted POP clouds of loaded dust can be emitted in whichever wind direction is dominant at that moment. This relativizes the use of average dominant wind directions in calculation models for POP emissions.

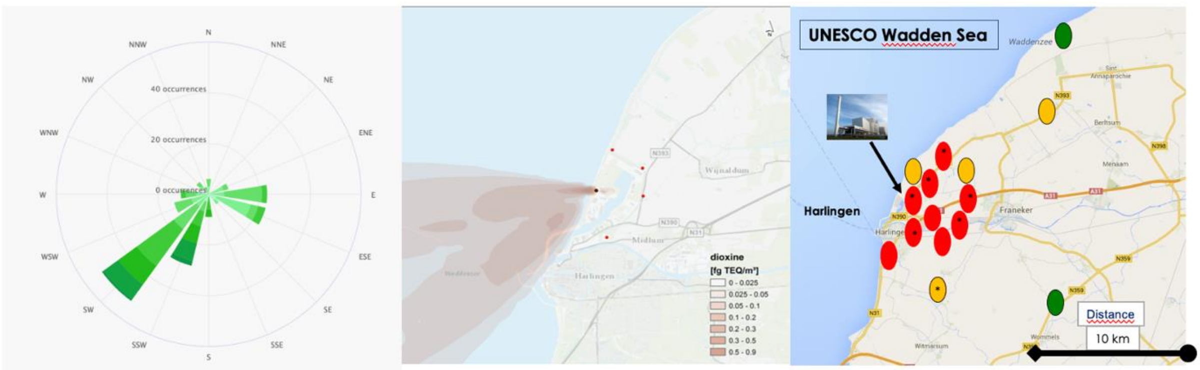


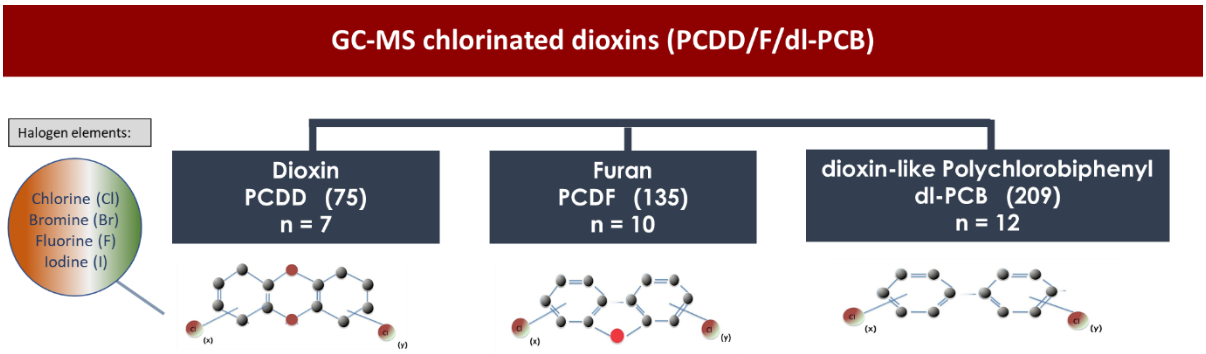
Figure 4: Wind rose for Harlingen (a), dioxin cloud during calamity, 2015 (b), contaminated eggs, Harlingen (c)

[1] *studio de evaluación de la incidencia en la salud de las emisiones procedentes del parque tecnológico de Valdemingómez, Madrid 2019*, page 165

Dioxins

Dioxins and furans are classified as highly toxic chemicals that have a serious effect on human health, causing cancer, diabetes, neurotoxicity, immunotoxicity, and chloracne. The emission of dioxins by incinerators was discovered in 1977 in the Netherlands[2]. Although dioxins also can be formed by volcanic eruptions, forest fires, or other natural events, the anthropogenic origin of dioxin is a far more than the natural source. Major sources of atmospheric dioxins (PCDD/Fs) include stationary emissions, especially from various types of incinerators, including secondary aluminum smelters, sinter plants, small-scale municipal solid waste incinerators (MSWI), medical waste incinerators (MWI), electric-arc furnaces, industrial waste incinerators, cement kilns, and crematoria. At the Stockholm Convention in 2004, 184 nations agreed to do their utmost to reduce the emissions of dioxins and other unintentionally produced organic pollutants. To achieve the goal of the Convention, Parties are required to implement the Best Available Techniques (BAT) and apply the Best Environmental Practices (BEP)[3].

The term 'dioxin' refers to three groups of substances: polychlorinated dibenzo-p-dioxins, (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (dl-PCBs). Figure 5 provides a schematic view where the black balls represent carbon atoms, the red oxygen, and the orange chlorine atoms (these can be substituted by other halogenated elements, like bromine, fluorine and iodine to form dioxins). The possible combinations with chlorine atoms (congeners) are 75 for dioxins (PCDDs), 135 for furans (PCDFs), and 217 PCBs congeners. Of these chlorinated congeners, 29 are found to be toxic and therefore regulated in EU; 7 PCDDs, 10 PCDFs, and 12 dl-PCBs. Only chlorinated dioxins and furans (PCDD/F) are regulated by EU for emissions of persistent organic pollutants (POPs) from waste incinerators. Dioxin-like polychlorinated biphenyls, brominated and mixed halogenated dioxins, all substances with dioxin-like properties, are (still) not regulated in the EU[4].



Congeners of chlorinated compounds (chemical GC-MS analysis)

	Dioxins, furans (PCDD/F) and dioxin-like PCBs	
Abbreviation	Congeners	TEF
Dioxins (n=7)		
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin	1
PCDD	1,2,3,7,8-Pentachlorodibenzo-p-dioxin	1
HxCDD1	1,2,3,4,7,8-Hexachlorodibenzo-p-dioxin	0,1
HxCDD2	1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	0,1
HxCDD3	1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	0,1
HpCDD	1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin	0,01
OCDD	Octachlorodibenzo-p-dioxin	0,0003

Furans (n=10)		
TCDF	2,3,7,8-Tetrachlorodibenzofuran	0,1
PCDF1	1,2,3,7,8-Pentachlorodibenzofuran	0,03
PCDF2	2,3,4,7,8-Pentachlorodibenzofuran	0,3
HxCDF1	1,2,3,4,7,8-Hexachlorodibenzofuran	0,1
HxCDF2	1,2,3,6,7,8-Hexachlorodibenzofuran	0,1
HxCDF3	1,2,3,7,8,9-Hexachlorodibenzofuran	0,1
HxCDF4	2,3,4,6,7,8-Hexachlorodibenzofuran	0,1
HPCDF1	1,2,3,4,6,7,8-Heptachlorodibenzofuran	0,01
HPCDF2	1,2,3,4,7,8,9-Heptachlorodibenzofuran	0,01
OCDF	Octachlorodibenzofuran	0,0003

Polychlorinated biphenyl (n=12)		
PCB77	3,3',4,4'-Tetrachlorobiphenyl (#77)	0,0001
PCB81	3,4,4',5-Tetrachlorobiphenyl (#81)	0,0003
PCB126	3,3',4,4',5-Pentachlorobiphenyl (#126)	0,1
PCB169	3,3',4,4',5,5'-Hexachlorobiphenyl (#169)	0,03
PCB105	2,3,3',4,4'-Pentachlorobiphenyl (#105)	0,00003
PCB114	2,3,4,4',5-Pentachlorobiphenyl (#114)	0,00003
PCB118	2,3',4,4',5-Pentachlorobiphenyl (#118)	0,00003
PCB123	2,3,4,4',5-Pentachlorobiphenyl (#123)	0,00003
PCB156	2,3,3',4,4',5-Hexachlorobiphenyl (#156)	0,00003
PCB157	2,3,3',4,4',5'-Hexachlorobiphenyl (#157)	0,00003
PCB167	2,3',4,4',5,5'-Hexachlorobiphenyl (#167)	0,00003
PCB189	2,3,3',4,4',5,5'-Heptachlorobiphenyl (#189)	0,00003

Figure 5: Schematic overview of dioxins (PCDD/F/dl-PCB), © ToxicoWatch

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2 *Olie K. , Vermeulen P.L., Hutzinqer O. (1977). Chemosphere No. 8, po 455 - 459, 1977.*
3 *Guidelines on Best Available Techniques and Provisional Guidance on Best Environmental Practices relevant to Article 5 and Annex C of the Stockholm Convention on Persistent Organic Pollutants (2008). Stockholm Convention on Persistent Organic Pollutants.*
4 *C. Budin et al. (2020). Chemosphere 251, 126579*

The EU sets limits of 2.5 pg TEQ/g fat for PCDD/F and of 5.0 pg TEQ/g fat for the sum of dioxin (PCDD/F/dl-PCB) for eggs. An EU action limit is set on 1.75 pg TEQ/g fat for PCDD/F and dl-PCB in eggs, see figure 6. For bioassay DR CALUX the EU limits are 1.7 pg BEQ/g fat (eggs) and 3.3. pg BEQ/g fat (eggs) for the sum of dioxins (PCDD/F/dl-PCB), see figure 7.

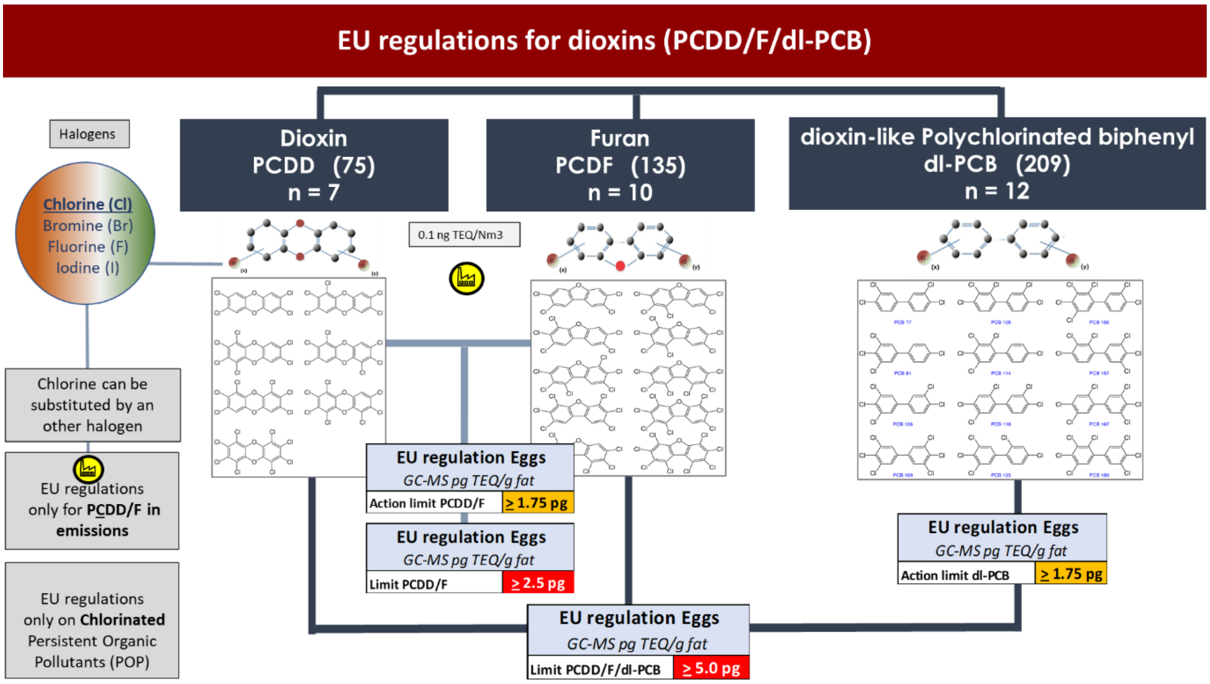

Figure 6: EU regulations for dioxins (PCDD/F/dl-PCB), ©ToxicoWatch

Figure 7 displays the difference between the chemical analysis with GC-MS and the bioassay DR CALUX. GC-MS analyse specific compounds, while DR CALUX measures the total toxic effect of a mixture of dioxin-like activity.

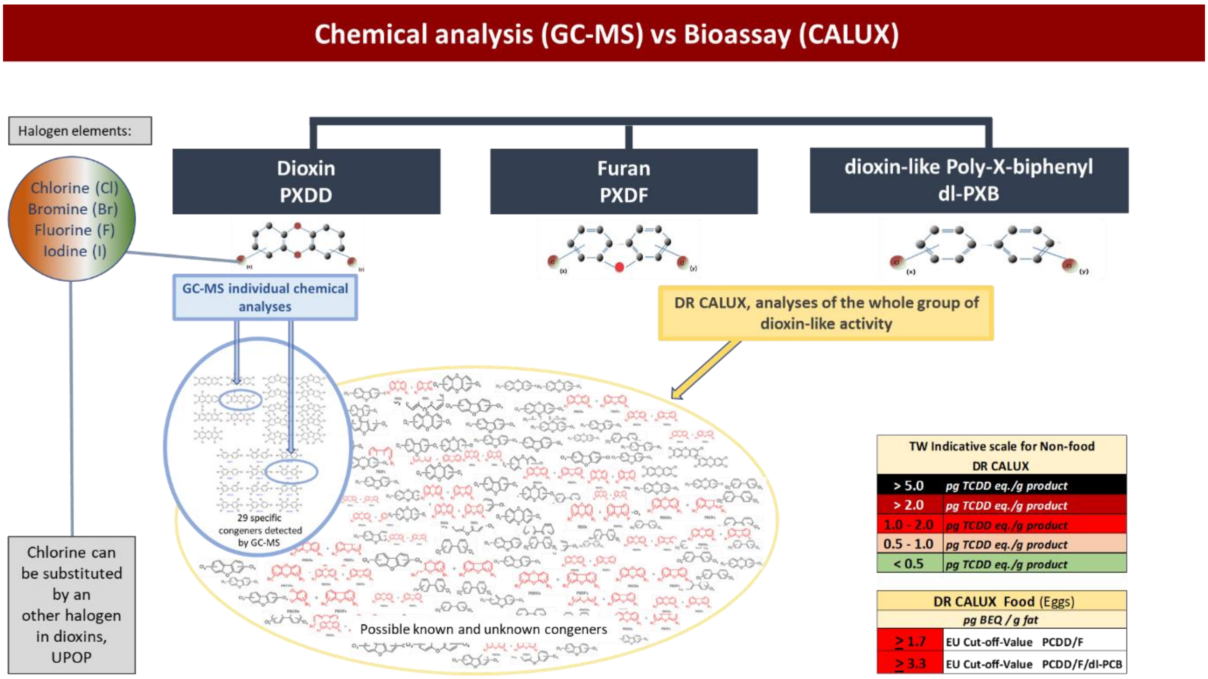

Figure 7: Chemical GC-MS analysis of dioxins (PCDD/F/dl-PCB) vs bioassay DR CALUX analysis, ©ToxicoWatch

Emissions of waste incineration

In this biomonitoring research the focus will be on persistent organic pollutants (POPs) like PCDD/F, PXDD/F, PAH and PFAS. See red clouds in Figure 8. A central question in this research is whether waste incineration is a solution for waste disposal and energy production, when there is an unintentionally production and emissions of POPs, such as dioxins (PCDD/F/dl-PCB). Figure 8 shows the quantities of emissions per 100,000 tonnes of waste. This figure, is made up the configuration of the WtE waste incinerator REC in Harlingen, the Netherlands with the specific configuration of Air Pollution Control Devices (APCD) and specific waste input. A big difference in volume of mega-tonnage CO_2 and the relative tiny amount of the extreme toxic of dioxins, expressed in milligrams.

Although this research is mainly focus on the emissions of substances by air, which is only a small amount of the toxic substances, the main output are the incinerator residues, like fly and bottom ash. The processing, storage and sustainable application of toxic incineration residues is an environmental risk[5]. For more sustainability and a healthy environment the focus need to be on more recycling of waste materials. Important in this context, the production of non-toxic material in order to prevent (unknown) toxic recycling and with that to prevent a possible toxic greenwashed recycling waste tsunami in the future.

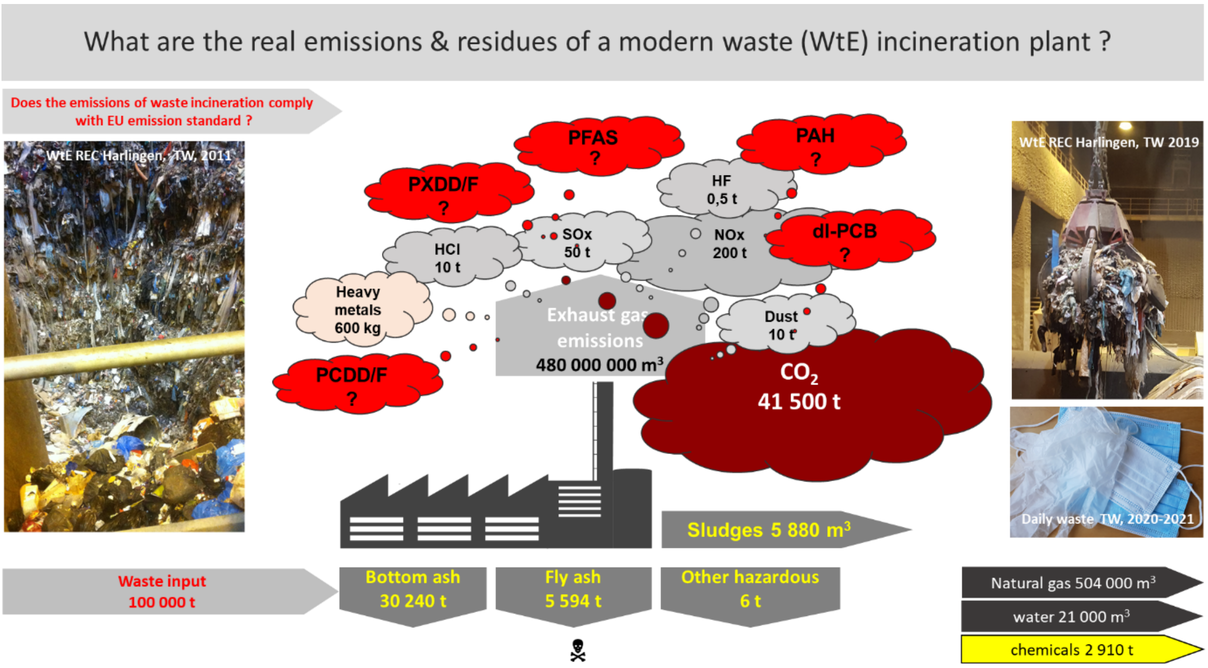

Figure 8: What are the real emissions of WtE incineration? © TW

[5] *ToxicoWatch (2020). The hidden impacts of incineration residues, Zero Waste Europe*

Polychlorinated biphenyl (PCB)

Polychlorinated biphenyls (PCBs) are chemicals that were widely used in industrial processes from the 1930s until the late 1970s. PCBs were used extensively in many industrial applications, including fire-resistant transformers and insulating condensers. The substances were used as heat exchanger fluids, and in aluminum, copper, iron, and steel manufacturing processing. PCBs were also used as plasticizers, in natural and synthetic rubber products, as adhesives, insulating materials, flame retardant, lubricants in the treatment of wood, clothes, paper, and asbestos, chemical stabilizers in paints, pigments, and as dispersing agents in formulations of aluminum oxide. PCBs were added in small quantities to inks, plastics, paints, sealants, adhesives, and dye solvents for carbonless paper. Although their production ended in 1979, huge amounts of PCBs are still in the environment[6].

From a toxicological point of view, there is a significant difference between dioxin-like PCBs and non-dioxin-like PCBs. Polychlorinated biphenyl congeners without chlorines in the ortho positions are called "coplanar" because the two phenyl rings can assume a planar state. This subgroup of 12 PCB congeners (non-ortho or mono-ortho chlorine substituted) with at least four chlorine substituents easily adopt a coplanar structure with toxicological properties similar to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), see Figure 9. This subgroup is termed dioxin-like PCBs (dl-PCBs) and are referred to as the 12 dioxin-like PCBs, see also Figure 5, 6. Due to their lipophilic properties and poor degradation, PCDD/Fs and dl-PCBs accumulate in the food chain and are persistent in the environment. Prevention or reduction of human exposure is best performed by source-directed measures, i.e., strict control of industrial processes to reduce the formation of dioxins. The greatest uncertainty with PCB and incinerator emissions lies in the composition of waste content and the distribution of PCB between air and waste. A TW study revealed that 10% of the emissions in the flue gases of an incinerator chimney were dioxin-like PCBs (dl-PCBs)[7]. However, in biomatrices around the incinerator, including eggs, milk and vegetation, the contribution of the TEQ dl-PCB is often more than 50%. More research is needed to confirm a direct relation to the emissions from a waste incinerator. PCB 126 was particularly dominant in all biomatrix samples.

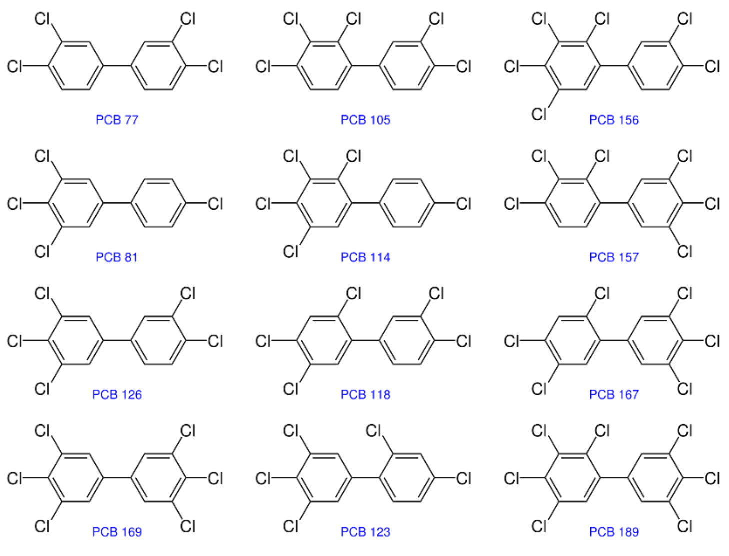

Figure 9:dioxin-like PCB (dl-PCB) congeners

[6] *Petrlík J., Arkenbout A. (2019) Dioxins – The old dirty (dozen) guys are still with us www.researchgate.net/publication/332877688*
[7] *Toxicowatch (November 2018). Hidden Emissions: A story from the Netherlands, a case study, Zero Waste Europe, https://zerowasteeurope.eu/wp-content/uploads/2018/11/NetherlandsCS-FNL.pdf*

Polycyclic aromatic hydrocarbon (PAH)

Polycyclic aromatic hydrocarbon (PAH) represent a class of ubiquitously occurring environmental compounds that are implicated in a wide range of toxicological effects. This class of compounds is known by their carcinogenic, mutagenic, and teratogenic properties. PAH leads to the development of a variety of disorders affecting all body systems as well as causing skin cancer and other skin diseases in animals and humans.

The PAHs with more than four (4) benzene rings have the most carcinogenic activity. PAH is able to reduce the effectiveness of measles vaccination through immunotoxicity to innate and adaptive immune cells[8]. Routine measurement of PAH contamination generally involves chemical analytical analysis of a selected group of representatives. The United States Environmental Protection Agency (EPA) and the European Commission (EU) classify 16 PAHs as priority pollutants (EPA-16): naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene (B[a]P), indeno[1,2,3-c,d]pyrene, benzo[g,h,i]perylene, and dibenz[a,h]anthracene, see Figure 10. However, this will result in an underestimation of the PAH in a sample[9]. PAHs form a very large group of several tens of thousands (>10.000) of compounds when taking into account the attaching with halogens, hydroxyl or when a nitrogen atom can be in the place of a carbon atom in the ring. In this research a bioassay (PAH CALUX) analysis method is used to measure the total toxic effect of all toxic PAH in a sample. When measuring with a chemical (GC-MS) analysis on a pure sample with known PAH individual congeners, like benzo[a]pyrene, the results with a bioassay (PAH CALUX) analysis, are the same in measured values if the Relative Potency Factor (RPF) are taken into account. In environmental samples, like in this research, high levels of PAH are found, because the bioassay measures the total toxic effect of all present PAH in the sample. The results of a PAH CALUX analysis will be expressed in equivalent benzo[a]pyrene, a class 1B carcinogen.

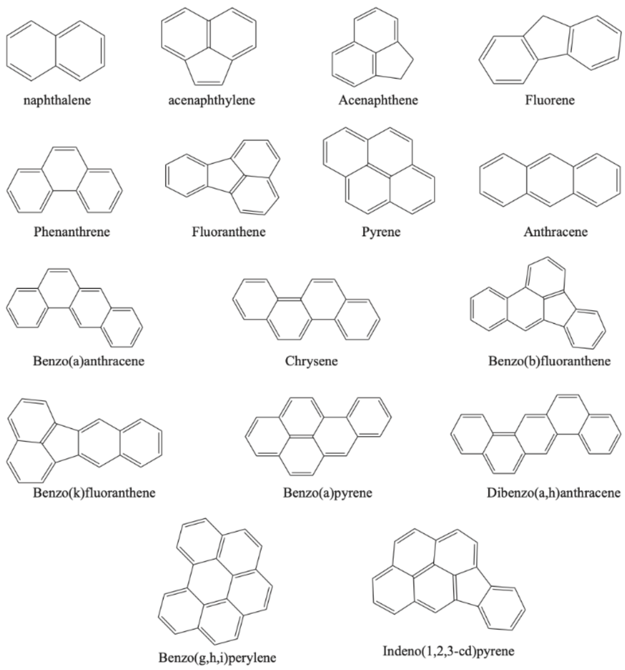

Figure 10: Molecular structures of the most common PAHs (Hussain 2018)

[8] Ruri Vivian Nilamsari et al. 2020. Polycyclic Aromatic Hydrocarbons (PAH) Reduces the Effectiveness of Measles Vaccination Through Immunotoxicity to Innate and Adaptive Immune Cells. Research J. Pharm. and Tech. 2020; 13(12):6128-6131.

[9] Andersson J.T., Achten C. (2015). Time to Say Goodbye to the 16 EPA PAHs? Toward an Up-to-Date Use of PACs for Environmental Purposes - Polycyclic Aromatic Compounds, 35:330–354

PFAS

Per- and PolyFluoroAlkyl Substances (PFAS) are a class of man-made chemicals with a wide range of industrial and commercial applications, which has resulted in their ubiquitous presence in the environment. The consolidated PFAS list of EPA contains 6330 PFAS CAS-name substances, of which 5264 are represented with a defined chemical structure resulting in increasingly complex mixtures entering the environment. PFAS possess thermal, chemical, and biological stability, non-flammability, and surface-active properties. Their high applicability combined with chemical stability has led to an inevitable accumulation of PFASs in the environment and as a result to their detection in environmental matrices (air, sewage, rivers, and dust) in food products and food packaging, in drinking water, and also in human samples (breast milk, blood) PFAS are associated with adverse human health effects on thyroid function, metabolism (including overweight/obesity, diabetes, insulin resistance, and high cholesterol, foetal development, and the immune system[10]. The risk of immunotoxicity for humans and wildlife cannot be discounted[11].

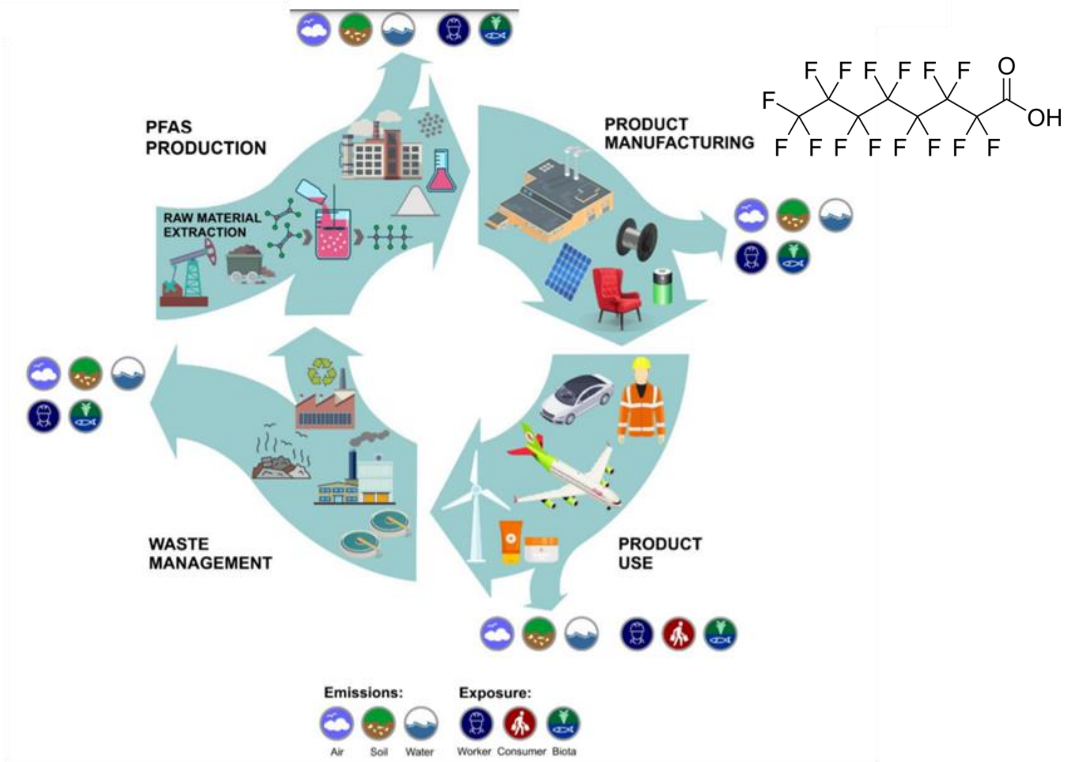

Figure 11: Overview figure of EU Commission Staff Working document on PFAS, October, 2020

According to the EU Commission Staff Working Document on Poly- and perfluoroalkyl substances (PFAS), October 2020, SWD(2020) 249 final, see Figure 11, *"A recent opinion from the European Food Safety Agency (EFSA) concluded that both PFOS and PFOA are associated with reduced antibody response to vaccination. PFOS also causes a reduced resistance to infection"*. EFSA concluded that parts of the European population exceeds the tolerable weekly intake (TWI) from food of four PFAS.[12]

10 *Young, A.S. et al.,(2021). Env. Health Perspect. 129 (4), 047010-1 to 047010-13.*
11 *Corsini, E., et al., Perfluorinated compounds: Emerging POPs with potential immunotoxicity. Toxicol. Lett. (2014),*
12 https://ec.europa.eu/environment/pdf/chemicals/2020/10/SWD_PFAS.pdf

However, analysis techniques for PFAS are only available for a limited number of PFAS substances. Chemical (GC-MS) analysis are not capable to detect the currently known > 8000 PFAS congeners. Some substances are known to be present, these are called known unknowns, the substances that are not known to be present are called the unknown unknowns. It is a struggle for quality for laboratories to produce consistent data in PFAS analysis. Laboratories may suffer from multiple difficulties, which hinder clear identification of the error sources. The lack of analytical standards, the distinctive physical-chemical properties of the PFCs, and matrix effects, at every step of the analysis from sampling to detection is a common problem[13]. Therefore, in this biomonitoring study, a different analysis methodology is chosen to measure the PFAS in the biomarkers around a waste incinerator.

The used analysis method in this research is based on the competition between thyroid hormone (T4) and PFAS for T4-binding site on the blood-protein transthyretin (TTR). The analysis methods are the FITC-T4 assay and the bioassay PFAS CALUX. The **Relative Potency Factor (RPF)** for 12 different PFAS congeners are expressed in PFOA equivalency (Table 1, Zeilmaker 2018[14]), see Table 1.

Overview of PFAS exposure pathways to the human population and the environment, see Figure 12, (Sunderland et al. 2019).[15] "PFAS are man-made substances that do not naturally occur in the environment. Examples of PFAS are GenX, PFOA perfluoro octanoic acid and PFOS perfluorooctane sulfonates. PFASs are used in many products. As a result, and due to emissions and incidents, these substances have ended up in the environment and are now found in, among other things, soil, dredging spoil and surface water."[16]

Congener	RPF
Perfluorobutanesulfonate (PFBS, C4)	0.001
Perfluorohexanesulfonate (PFHxS, C6)	0.6
Perfluorooctanesulfonate (PFOS, C8)	2
Perfluorobutanoic acid (PFBA, C4)	0.05
Perfluoropentanoic acid (PFHxA, C6)	0.01
Perfluorooctanoic acid (PFOA, C8)	1
Perfluorononaoic acid (PFNA, C9)	10
Perfluoroundecanoic acid (PFUnDA, C11)	4
Perfluorododecanoic acid (PFDoDA, C12)	3
Perfluorotetradecanoic acid (PFTeDA, C14)	0.3
Perfluorohexadecanoic acid (PFHxDA, C16)	0.02
Perfluorooctadecanoic acid (PFODA, C18)	0.02

Table 1: Relative Potency Factor (RPF) for 12 PFAS expressed in PFOA equivalency (RIVM, Zeilmaker 2018)

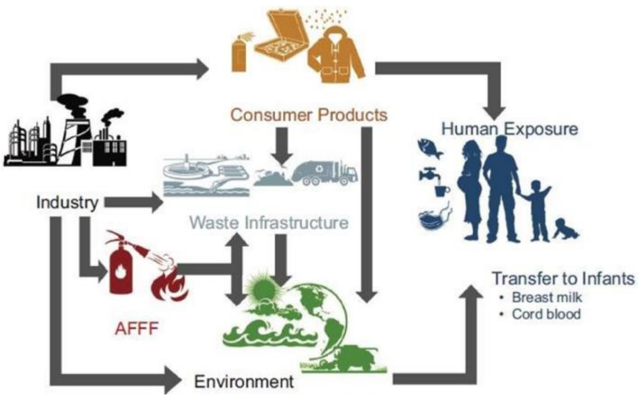

Figure 12: Overview of PFAS exposure pathways to the human population and the environment (Sunderland et al. 2019)

[13] *Van Leeuwen SPJ, Kärrman A, Van Bavel B, De Boer J and Lindstrom G, 2006. Struggle for quality in determination of perfluorinated contaminants in environmental and human samples. Environmental Science and Technology, 40, 7854–7860.*
[14] *M.J. Zeilmaker et al 2018. Mixture exposure to PFAS: A Relative Potency Factor approach, National Institute for Public Health and the Environment, RIVM Report 2018-0070.*
[15] *Sunderland EM. (2019). Journal of Exposure Science & Environmental Epidemiology (2019) 29:131–147*
[16] https://www.rivm.nl/en/pfas

Bioassays

DR CALUX

The bioassay **DR CALUX® (Dioxin Responsive Chemical Activated LUciferase gene eXpression)** is used for quantification of dioxins/furans (PCDD/F) and dioxin-like PCBs (dl-PCBs). The results in this research with DR CALUX® for analyses on dioxins (PCDD/F/dl-PCBs) on eggs are expressed in **Bioassay Equivalent, BEQ (pg BEQ/g fat)**. The term "**BEQ**" is used for food elements to distinguish between the **TEQ** (Toxic Equivalence) derived from chemical analyses (Gas Chromatography-Mass Spectrometry GC-MS, pg TEQ/g fat). For non-food biomatrices like mosses or pine needles, the results with the DR CALUX will be expressed in **TCDD eq./g product** or abbreviated as **pg TEQ/g product**. TCDD stands for 2,3,7,8-Tetrachlorodibenzo-p-dioxin, the most toxic dioxin congener.

Like all EU regulations, **Regulation EU 1881/2006**[17] is immediately enforceable as law in all member states. This regulation sets maximum levels for certain contaminants in food products. The food products which are listed should not be placed on the commercial market if a contaminant exceeds the maximum level set out in the Annex of the EU documents.
The limits set in legislation are expressed in pg TEQ/g, based on GC-MS measurements. The GC-MS analysis concerns 7 dioxins (PCDDs), 10 furans (PCDFs), 12 dioxin-like polychlorinated biphenyls (dl-PCBs), and 6 indicator polychlorinated biphenyls (i-PCB).
The results of the chemical analyses with GC-MS of dioxins (PCDD/F/dl-PCBs) will be calculated with a specific Toxic Equivalency Factor (TEF) towards a TEQ value (see page 5 Abbreviation and TEF for dioxins, and dl-PCBs). The sum of the TEQ will be measured with upper bound values, meaning calculation with the value of the limit of detection (LOD) of a specific congener. These GC-MS **limit values** for chicken eggs are 2.5 pg TEQ/g fat for dioxins (PCDD/F) and for the sum of dioxins (PCDD/F) and dioxin-like PCBs (dl-PCBs), the GC-MS limit value is set at 5 pg TEQ/gram fat. When exceeding these GC-MS limit values, chicken eggs are not allowed to be on the commercial market, (see Figure 6 and 7).

Directive 2013/711/EU[18] sets out the cut-off values of the DR CALUX analysis determined. If the analysis exceeds the 70% value of PCDD/F, i.e. 1.7 pg BEQ/g and/or 70% of the limit of the sum of dioxins (PCDD/F/dl-PCB) i.e. 3.3 pg BEQ/g a GC-MS analysis of the egg sample is recommended to establish the results with the GC-MS chemical analysis, where **EU 1881/2006** can be applied.

2013/711/EU[19] includes the **action levels GC-MS** for both dioxins (PCDD/F) and dioxin-like PCBs (dl-PCBs) in chicken eggs set at 1.75 pg TEQ/g fat, see Figure 6. These action levels are a tool for competent authorities and operators to highlight cases where it is appropriate to identify a source of contamination and to take measures for its reduction or elimination.

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17 https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02006R1881-20210919&from=EN
18 https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32014R0709&from=EN
19 https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013H0711&from=EN

PAH CALUX®

High molecular weight PAHs have known ligands of the aryl hydrocarbon receptor (AhR), a nuclear receptor that mediates toxic effects related to these compounds. The PAH CALUX assay uses a mammalian, H4IIe- cell-based reporter assay for the hazard identification of total PAH mixtures. The PAH CALUX reporter cell line allows for specific, rapid (4-hour exposure time) and reliable quantification of AhR-induced luciferase induction relative to benz[a]pyrene, a compound with five benzene rings and a class 1B carcinogen, is used as an indicator of PAH exposure[20,21] (see Annex V for relative potency factors PAH).

PFAS CALUX®

The chemical analyses on individual PFAS congeners are very limited, depending on the lab, only 8 - 55 substances can be analysed. Practically, this means that only 0.1- 1% can be determined with the chemical analyses, compared with the value of the Total Organic Fluorine (TOF)[22]. The bioassay of PFAS CALUX® comprises human bone marrow cell lines (U2OS), incorporating the firefly luciferase gene coupled to Thyroid Responsive Elements (TREs) as a reporter gene for the presence of thyroid-like inhibiting compounds. It is based on the TTR-binding of PFAS in combination with the TRβ CALUX detection. The presence of increasing concentrations of PFAS capable of competing with T4 for TTR-binding sites will result in a decreased amount of TTR-bound T4. Disruption of T4-TTR binding is benchmarked against the reference compound Perfluorooctanoic acid (PFOA), which value is set to one (1), just like TCDD in the TEQ calculation[23]. See table 1 for relative potency factors of other PFAS. The analysis results of the PFAS CALUX are expressed in: µg PFOA equivalent/g product.

FITC-T4 assay

In the FITC-T4 binding bioassay, sample extracts, suspected to be contaminated with PFAS, are tested for the potency of binding with the thyroid hormone thyroxine (T4) to the plasma transport protein Transthyretin (TTR). The fluorescent-labelled thyroxine (FITC-T4) consisting of Fluorescein isothiocyanate (FITC) and L-thyroxine (T4) are used in this assay (Smith, 1977, Hamers 2020)[24,25].The thyroid hormone homeostasis can be disrupted by environmental chemicals at different points of interaction in the thyroid pathway, including during transport of the hormone through the blood. Some chemicals are known to bind to the transport protein TTR thereby replacing the endogenous ligand T4. PFAS are such chemicals known for their capability to bind TTR thereby replacing T4. The measurement is based on the difference in fluorescence between bound and non-bound FITC-T4 to the TTR-binding site. Bound FITC-T4 will result in a higher fluorescence than non-bound. The analysis results of the FITC-T4 will be expressed in: **µg PFOA equivalent/g product**.

The DR CALUX®, PFAS CALUX®, FITC-T4, and GC-MS-analysis were performed by BioDetection Systems, Amsterdam, the Netherlands. BDS is accredited under RvA L401.

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20 Category 1B carcinogen according to Annex VI to the CLP Regulation (EC) No 1272/2008 of the European Parliament, and is classified as a Substance of Very High Concern by the POP Regulation EC No 850/2004.
21 Pieterse B, Felzel E, Winter R, van der Burg B, Brouwer A. PAH-CALUX, an optimized bioassay for AhR-mediated hazard identification of polycyclic aromatic hydrocarbons (PAHs) as individual compounds and in complex mixtures. Environ Sci Technol. 2013 Oct 15;47(20):11651-9. doi: 10.1021/es403810w. Epub 2013 Sep 25. PMID: 23987121.
22 Straková, J., Schneider, J., Cingotti, N. et al., 2021. Throwaway Packaging, Forever Chemicals: European wide survey of PFAS in disposable food packaging and tableware. 54 p.
23 P.A. Behnisch et al. Developing potency factors for thyroid hormone disruption by PFASs using TTR-TRβ CALUX® bioassay and assessment of PFASs mixtures in technical products, Environment International 157 (2021) 106791
24 Smith, D.S., (1977). FEBS Lett. 77, 25-27.
25 Hamers T. (2020). Transthyretin-Binding Activity of Complex Mixtures Representing the Composition of Thyroid-Hormone Disrupting Contaminants in House Dust and Human Serum, Environmental Health Perspectives 017015-1 128(1)

Backyard chicken eggs

Backyard chicken eggs are used for biomonitoring levels of contamination by POPs in various studies. Eggs are sensitive indicators of POP contamination in soil and dust and are a significant exposure pathway from soil pollution to humans. Eggs from contaminated areas can readily lead to exposures that exceed thresholds for the protection of human health. Chickens and their eggs might, therefore, be ideal "active samplers": an indicator species for the evaluation of contamination levels of sampled areas by POPs, particularly by dioxins (PCDD/Fs) and dioxin-like-PCBs (dl-PCBs) [26,27].

When chickens are free to forage on natural uncovered soil in the open air without roofing, they are in optimal contact with the environment. Eggs can reflect the chemical situation of soil biota related to the atmospheric deposition of hazardous chemical particles from industrial emissions, such as car shredding, metallurgy, coal-fired power plants, foundries, the PVC industry, cement kilns, the paper industry, and waste incineration. Chickens forage on and in the soil, eating insects, invertebrates, vegetation even grass (Figure 13). As a result, persistent organic pollutants (POPs) like dioxins (PCDD/F/dl-PCB)can be found in the fatty egg yolk and act as a biomarker for the environment. The chicken excretes the toxic compounds like dioxins into the fatty yolk when producing the eggs (dioxins are fat related). The older the chicken is, the more toxic compounds can be collected in the body, a process called bioaccumulation. Biotransformation refers to the capability of an organism to break down certain substances. Xenobiotical metabolism refers to the metabolism or breakdown of foreign substances not belonging to the substances of an organism of an ecological system.

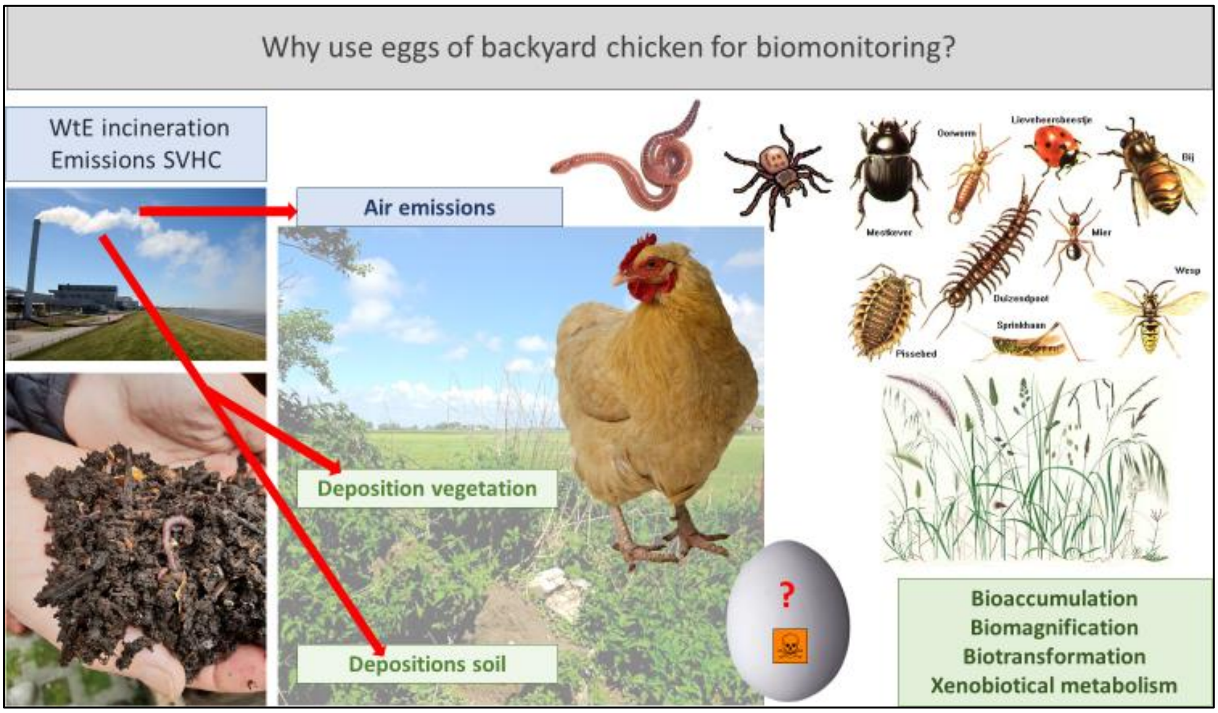

Figure 13: Biomonitoring of backyard chicken eggs in natural environment

[26] *Arkenbout A, Esbensen K H. (2017) Sampling, monitoring and source tracking of Dioxins in the environment of an incinerator in the Netherlands, Proceedings Eighth World Conference On Sampling and Blending / Perth*
[27] *Petrlík J. (2015). Persistent Organic Pollutants (POPs) in Chicken Eggs from Hot Spots in China.Beijing-Gothenburg-Prague, Arnika - Toxics and Waste Programme,*

European Food Safety Authority (EFSA)

Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and dioxin-like polychlorinated biphenyls (dl-PCBs) are important contaminants in the food chain. In 2018 the European Food Safety Authority (EFSA) reduced the tolerable weekly intake (TWI) from 14 to 2 pg TEQ (Toxic Equivalents)/kg body weight per week, based on extended scientific reviews conducted on humans and animals (EFSA, 2018)[28], see Figure 14. It demonstrates the present exposure to dioxins for most consumers in the EU exceeds the TWI. The maximum levels for PCDD/Fs and dl-PCBs in food and feed have to be reduced according to the EFSA advise, however the EU has taken, so far, no action. The actual dioxin limit value for eggs is 2.5 pg TEQ PCDD/g fat and 5.0 pg TEQ/fat PCDD/F/dl-PCB. A reduction of these limit values with a factor of 7 will have enormous implications see Figure 14. The actual EU limits (Figure 6 and 7), based on pre EFSA advise, before 2018, and can been seen as more the result of political economic rather than preliminary on behalf of human health arguments.

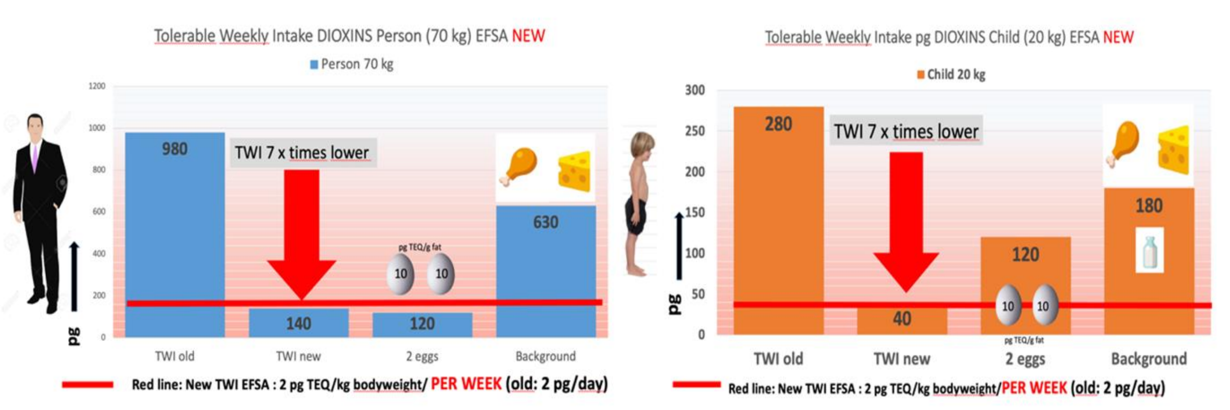

Figure 14: Tolerable Weekly Intake of dioxins revision for adults and children (EFSA 2018), graphs by TW©.

Public concern about ongoing contamination of POPs in human bodies has increased since several of these substances of very high concern have been identified as hormone disrupters and immune depressors. There are many risks and effects of having these chemicals in our environment and, as far as dioxins are concerned, they are of no benefit. Pollutants like dioxins contaminate the environment, persist for decades, and cause problems such as cancer, birth defects, learning disabilities, immunological deficiency, behavioral, neurological, and reproductive discrepancies in human and other animal species.

For PFOS and PFOA the EFSA established a **tolerable weekly intake (TWI) of 13 ng/kg body weight per week (PFOS) and 6 ng/kg body weight per week (PFOA) respectively**[29]. For both compounds, the exposure of a considerable proportion of the population exceeds the proposed TWI. A safe daily dose of **GenX or HFPO-DA is 3 ng/kg of body weight**, according to the EPA.

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28 *EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Knutsen HK et al. 2018. Scientific Opinion on the risk for animal and human health related to the presence of dioxins and dioxin-like PCBs in feed and food. EFSA Journal 2018;16(11):5333, 331 pp.*

29 *EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Knutsen HK et al, 2018. Scientific Opinion on the risk to human health related to the presence of perfluorooctane sulfonic acid and perfluorooctanoic acid in food. EFSA Journal 2018;16(12):5194, 284 pp.*

Sampling

The sampling for this research was performed by Beatriz Martín, an ecologist from the Action Spain network. For the preparation a manual was applied to explain the sampling in nine (9) steps. In Annex I an outline of these 9 steps is given in a handout sampling manual. Figure 15 shows the set-up of the initial sampling plan for biomonitoring in Madrid in the area around the Valdemingómez waste incinerator. At first an exploration was undertaken by the Madrid sampling team to identify the possibilities of biomonitoring in the region around the waste incinerator on biomarkers such as backyard chicken eggs and vegetation (pine needles, leaves and mosses). The difficulty of this remote area is that the nearest community is a population living along the Cañada Real Galiana. It is unclear whether the inhabitants of this place have access to basic utilities such as an official sewage system, clean water and a functioning electricity supply. It should be noted that this area is difficult to enter to conduct biomonitoring research and to make contact with the inhabitants. In November 2021, TW received backyard chicken eggs from one location in this area. There was no direct contact with the chicken coop owners, the communication was performed via intermediaries. No photos or further information could be provided. Therefore, this biomonitoring research in Madrid is mainly focused on mosses, pine needles of the Aleppo pine - *Pinus halepensis*, the leaves of broadleaf trees (field elm – *Ulmus minor*, and Arizona cypress - *Cupressus arizonica)*, concentrated in the centre of the inner circle of < 2 km.

In February 2021 an inventory was carried out and photo material taken from the possible sampling places of vegetation and mosses. From that point onwards, TW drew up a sampling plan based on the initial sampling plan for collecting vegetation samples near and around the incinerator. Actual vegetation sampling was carried out on **28 July 2021**. Because of the high temperatures (°C) in the summer, mosses were sampled in the autumn on October 14th . Eggs from Cañada Real were received on October 27th and analysed with DR CALUX and GC-MS. The lab results were received at the end of the year, December 6th 2021.

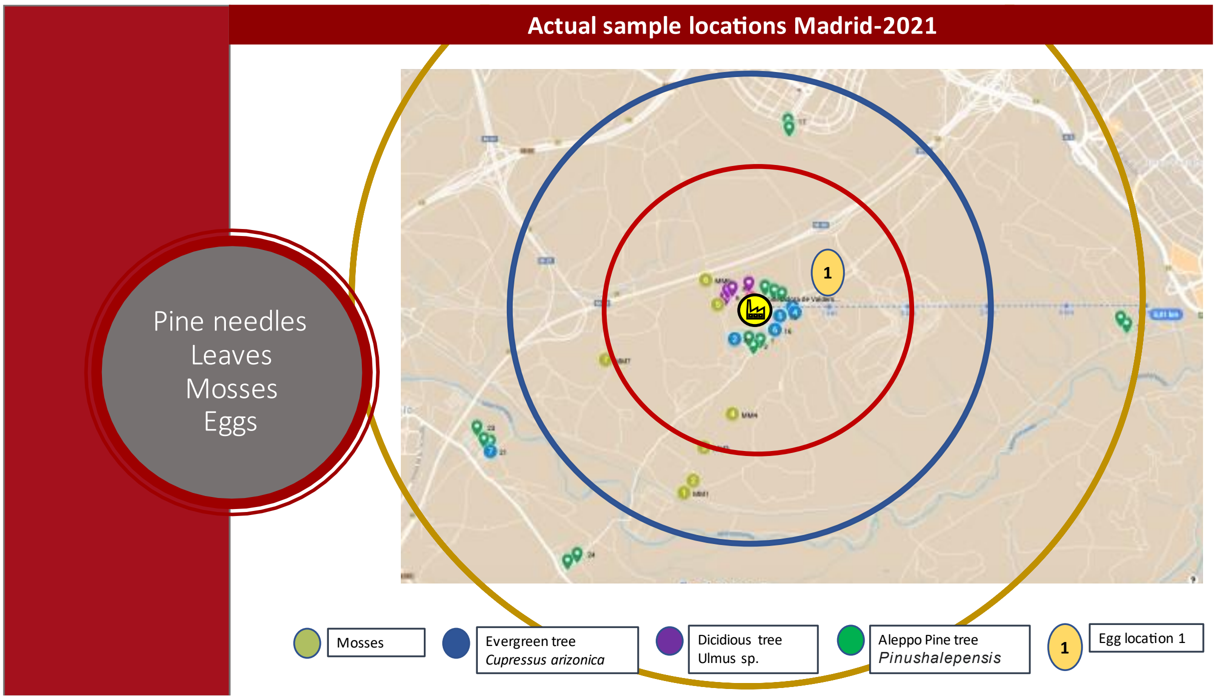

Figure 15: Actual biomonitoring sample location, Madrid - 2021

At least 100 grams of pine needles and leaves were collected at a height of approximately 1.6 m above the ground at the sites in the main wind direction. Pine needles and evergreen foliage (*Cupressus arizonica*) are sampled not only from young (one season old) twigs on the trees. Details on the pine needle samples collected are summarized in Table 2.

The needles were packed in HDPE polyethylene bags a (for blank background of external contamination). The mosses, >100 grams, were collected in glass jars wrapped in solvent-washed aluminium foils and delivered to the laboratory, where the samples were dried and analysed on dioxins (PCDD/F/ dl-PCB), PAH and PFAS.

Biomonitoring Eggs

Egg location for sampling

Participation of chicken coop owners of backyard chicken was hard to find in the area around in the incinerator, a difficult task as it turned out at the start of the biomonitoring, at first no response at all. In autumn the Madrid sampling team managed to find one (1) egg location and could deliver six (6) eggs for this research, North-East of the incinerator on a distance of < 1 km, see Figure 16. The eggs were laid by three hens between six months and one year old. Unfortunately, no access was possible at the location for further inspections of the hens, the forage area and hen housing.

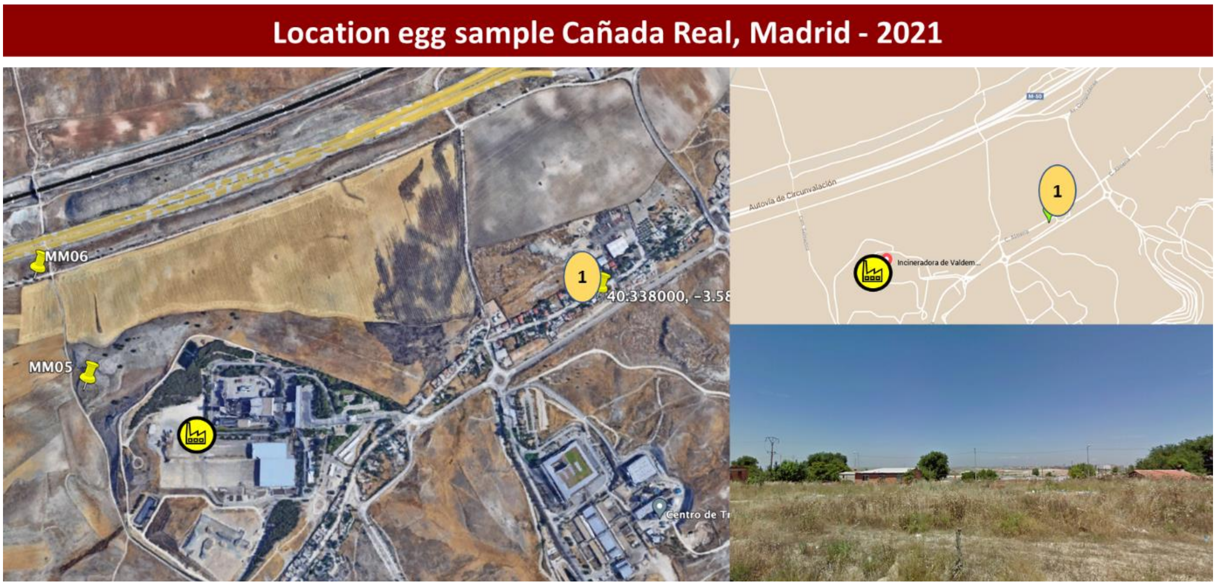

Figure 16: Location of egg samples, Cañada Real Galiana, Madrid - 2021

The eggs were initially analysed with the DR CALUX method and showed elevated dioxins (PCDD/F) and dioxin-like PCBs (dl-PCBs), exceeding the maximum level of dioxins in food according to the EU regulation. GC-MS analysis also exceeded the EU limits by verifying the DR CALUX results (Figure 17).

TW21-MD-Egg 01	
TW-REF-NR	TW21-MD-Egg 01
Distance	1000
N hens	3
N rooster	
Age	6-12 mnd
Eggs/month	30
Foraging area	
Outdoor fireplace	
All purpose burner	
Pesticides use	
DR CALUX BEQ	
PCDD/F BEQ	6,00
dl-PCB	7,00
PCDD/F/dl-PCB	13,00
GC-MS TEQ	
PCDD/F	4,10
dl-PCB	2,50
PCDD/F/dl-PCB	6,70

Figure 17: Results of eggs PCDD/F/dl-PCB, Madrid 2021

The congener pattern of the eggs of dioxins (PCDD/F), provided by the chemical GC-MS analysis are shown in Figure 18. In this overview the congener patterns of the fraction of concentrations and TEQ are compared with the incineration patterns of the WtE incinerator (REC) in Harlingen, the Netherlands. The reason is to provide some interpretation of the patterns, although the waste input and therefore the emission output may differ. The patterns of the REC waste incinerator are the results of more than 20,000 hours of (semi-) continuous measurement of real data of the flue gases, a research of TW. With the color dark red, typical incinerator patterns are marked, like Octachlorodibenzo-p-dioxin (OCDD) and 1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin (HpCDD) in the concentrations, and the low chlorinated 1,2,3,7,8-Pentachlorodibenzo-p-dioxin (PCDD) and 2,3,4,7,8-Pentachlorodibenzofuran ($PCDF_2$) in the TEQ profiles. Other incinerator patterns show also 1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin (HpCDD) as a main contributor of dioxins (PCDD/F). Figure 18 presents the top left graph, the fraction of dioxin (PCDD/F) concentration; 1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin (HpCDD), Octachlorodibenzo-p-dioxin (OCDD) and 1,2,3,4,7,8,9-Heptachlorodibenzofuran (HPCDF2). Typical of these results is the domination of HpCDD over OCDD. Usually a ratio of 2 to 1 of OCDD and HpCDD is observed. In the upper left graph of Figure 18 the furans (PCDF) are circled. The ratio PCDF/PCDD is 2.3 and can be indicative of newly formed emissions from waste incineration[30].

The dominant TEQ congeners 1,2,3,7,8-Pentachlorodibenzo-p-dioxin (PCDD), 2,3,4,7,8-Pentachlorodibenzofuran (PCDF2) and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) are marked. The graph, top left shows the fraction of dioxin (PCDD/F) concentration; 1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin (HpCDD), Octachlorodibenzo-p-dioxin (OCDD) and 1,2,3,4,7,8,9-Heptachlorodibenzofuran (HPCDF2). Typical of these results is the domination of HpCDD over OCDD. Usually a ratio of 2 to 1 of OCDD and HpCDD is observed.

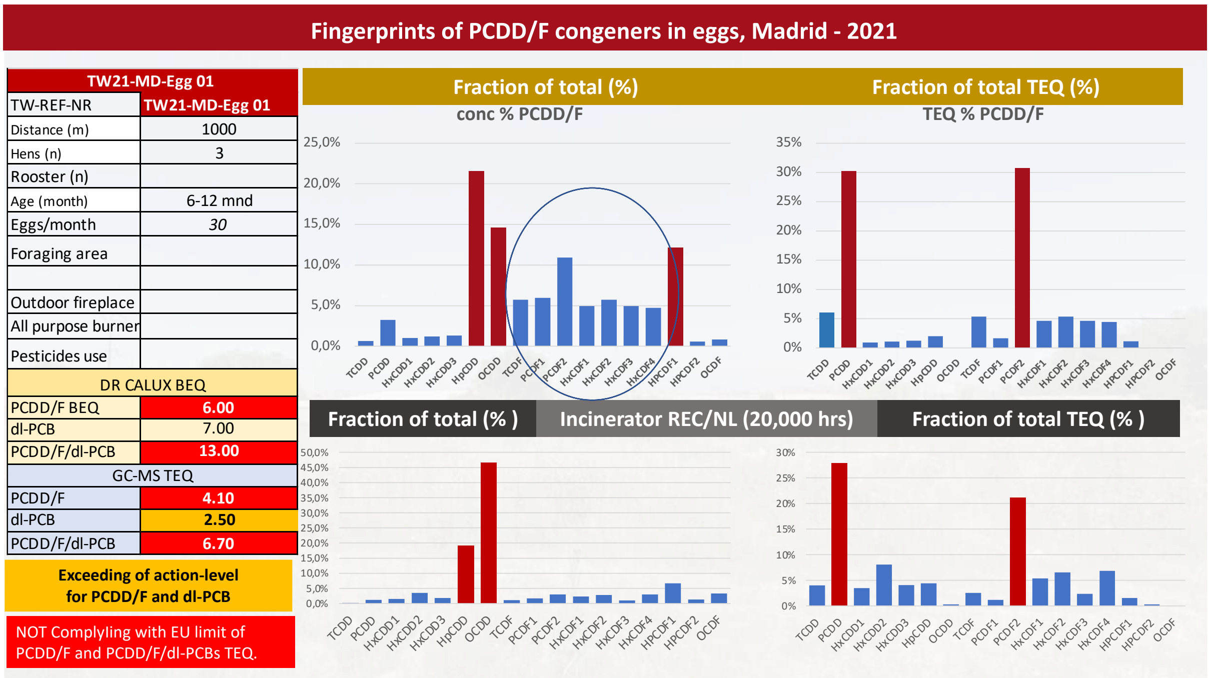

Figure 18: Fingerprints of dioxins (PCDD/F) congeners in eggs, Madrid - 2021

[30] Chen P. et al. (2017). Chemosphere 181 (2017) 360 - 367

Dl-PCB contamination in eggs of backyard chicken

Although their production ended in 1979, huge amounts of PCBs are still in the environment. Most of the PCBs found today in the environment originate from legacy sources (e.g., release from transformers or capacitors still in use, building materials, stored waste, or contaminated soils) or as unintentional by-products of combustion processes (e.g., waste incineration).

In Figure 19 the congener patterns of the dioxin-like PCBs (dl-PCBs) are displayed in fractions of concentrations and in TEQ. The most dominant PCBs in concentrations are PCB 105 and PCB 118. PCB 126 dominate the contribution in TEQ with 2.2 pg TEQ and 88% in the total dl-PCB-TEQ.

Fingerprints dl-PCB congeners in eggs, Madrid - 2021

Concentrations	Madrid
PCB	TW21-MD-Egg 01
PCB77	64,00
PCB81	7,00
PCB126	22,00
PCB169	4,60
PCB105	1100,00
PCB114	90,00
PCB118	2000,00
PCB123	61,00
PCB156	440,00
PCB157	120,00
PCB167	290,00
PCB189	100,00
Total PCB pg/g fat	**4298,60**

Exceeding of action-level for dl-PCB

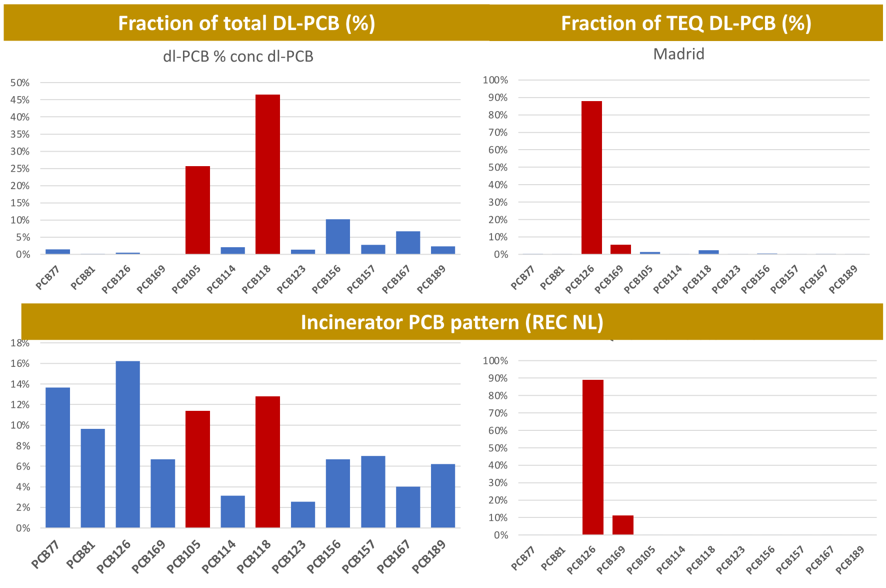

Figure 19: Fingerprints of dl-PCB congeners in eggs, Madrid 2021

The EU mandates a reduction in the amount of toxic dioxin-like substances by making serious efforts to find the source of this contamination. The question needs to be answered, what is the contribution of the WtE incinerator to the PCB contamination? In continue measurements of emissions of a WtE incinerator, 10% of the TEQ found to be related to dioxin-like PCBs, mainly PCB 126[31]. A remark has to be made, that semi-continuous measurements of the flue gasses inside the chimney of a WtE incinerator, are by far the best tool to measure emissions of dioxins during normal operation[32]. However, measuring emissions during transient phases, such as start-up and shutdown, requires a different methodology of measuring due to changing conditions such as temperature and gas velocity. A study by Li (Zhejiang University, Hangzhou, China, 2018) demonstrates high emissions of dioxin like PCBs during transient phases of start-ups and shutdowns[33].

[31] *Hidden Emissions of incinerators, 2017. Toxicowatch Foundation, publication by Zero Waste Europe*
[32] *Arkenbout, A, Olie K, Esbensen, KH, 2018. Emission regimes of POPs of a Dutch incinerator: regulated, measured and hidden issues, Conference paper Dioxin2018*
[33] *Li M, Wang C, Cen K, Ni M, Li X. 2018 Emission characteristics and vapour/particulate phase distributions of PCDD/F in a hazardous waste incinerator under transient conditions. R. Soc. open sci. 5: 171079.*

Brominated and mixed halogenated dioxins (PBDD/F and PXDD/F) in eggs of backyard chicken

The reason for often higher results of dioxin levels (PCDD/F) in DR CALUX analysis, could be because the bioassay also reacts to polyhalogenated dioxins, such as the brominated (PBDD/F) and mixed halogenated chlorinated/brominated/fluorinated dioxins (PXDD/F). In a study of ToxicoWatch with continuous measurement in the chimney of a WtE incinerator, a broad scale of POPs was found[34]. The EU regulation covers only the chlorinated dioxins (GC-MS: PCDD/F in TEQ and DR CALUX: PCDD/F in BEQ), see Figure 20, 6 and 7. While more and more scientific publications show the proportion of other halogenated dioxins cannot be neglected and should integrated in EU regulation. This is especially true when (municipal) waste with brominated and fluorinated (flame retardant) content are combusted. The problem is the analysis of all these halogenated compounds. There are about 4,600 chlorinated and brominated dioxins, without any international guideline, besides the fluorinated (PFAS) compounds. At the moment only one detection method (bioassay DR CALUX) is suitable for measuring the total toxic effect. Brominated dioxins make up to 15% of the total dioxin in human body (Jogsten et al 2010)[35].

Difference PCDD/F TEQ and BEQ in eggs, Madrid - 2021

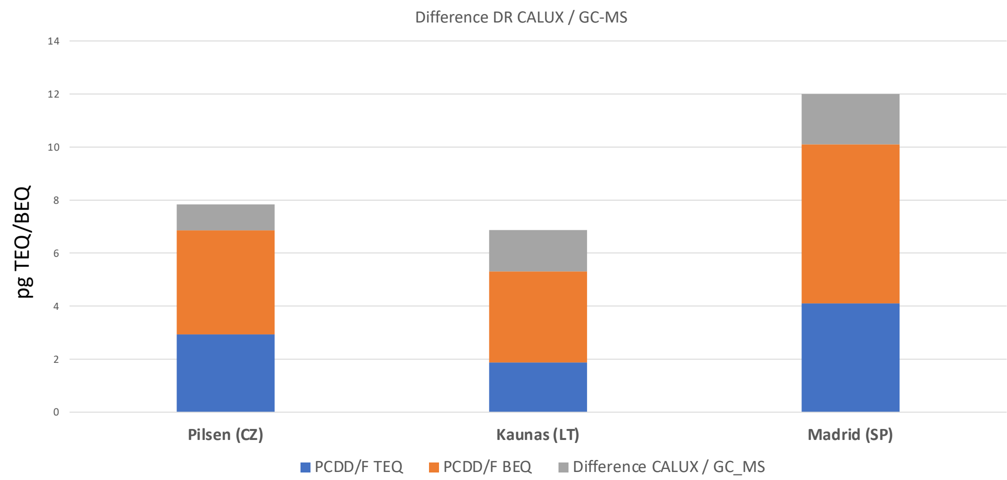

Figure 20: Difference between GC-MS (TEQ) and DR CALUX (BEQ) indicates evidence of brominated dioxins

It is widely recognized that unintentional produced persistent organic pollutants (UPOPs) in emission from thermal processes, especially incineration of e-waste containing PBDEs, is the principal source of PBDD/Fs in the environment. PBDE can primarily found in black electronic devices like TV casings. Waste incineration and metallurgical processes, including secondary metal smelting and arc furnace steelmaking, are important anthropogenic sources of dioxins. Although less data are available on PBDD/Fs formation during waste incineration and metallurgical process than for PCDD/Fs, pilot studies have demonstrated that PBDD/Fs are formed during thermal processes[36].

[34] Arkenbout, A., Bouman KJAM, 2018.Emissions of dl-PCB, PBB, PBDD/F, PBDE, PFOS, PFOA and PAH from a waste incinerator, Dioxin2018, see reference list
[35] I.E. Jogsten et al. / Food and Chemical Toxicology 47 (2009) 1577–1583
[36] L. Yang et al. 2021. Environment International 152 (2021) 106450

Biomonitoring of vegetation

An overview of the vegetation samples is provided in table 2 and Figure 21 of i.e. Pinus locations 1 and 2 in the inner circle < 2 km, Pinus locations 3 and 4 between 2-4 km away (Pin03 and Pin04), and the reference locations Pinus 5 and 6 (Pin05 and Pin06) in the outer circle of 3-5 km distance.
The vegetation table 2 shows the TW reference numbers (TW-REF-NR), for each (pooled) vegetation sample, the distance in meters, wind direction and gram/vegetation sample is collected on July 16th.

Sampling Pine needles, foliage and leaves, Madrid - 2021

	Sample date	species	Weight (gr)		Wind direction	Distance (m)	TW-REF-NR	
1	18-7-2021	*Pinus halepensis*	125		S	570	TW-MD21-Veg-01/02	Pin01
2	18-7-2021	*Pinus halepensis*	111		S			
3	18-7-2021	*Pinus halepensis*	102		S			
4	18-7-2021	*Cupresses arizonica*	115	1	S-W	528	TW-MD21-Veg-04/05	Cup02
5	18-7-2021	*Cupresses arizonica*	148	2	S-W			
6	18-7-2021	*Ulmus minor*	138		W	300	TW-MD21-Veg-06/07/08	Ulmus01
7	18-7-2021	*Ulmus minor*	144		W			
8	18-7-2021	*Ulmus minor*	179		W			
9	18-7-2021	*Ulmus minor*	149		N	175	TW-MD21-Veg-09	Ulmus02
10	18-7-2021	*Pinus halepensis*	137		N-E	280	TW-MD21-Veg-10/11/12	Pin02
11	18-7-2021	*Pinus halepensis*	167		N-E			
12	18-7-2021	*Pinus halepensis*	149		N-E			
13	18-7-2021	*Cupresses arizonica*	169	3	E	400	TW-MD21-Veg-13/14/15	Cup01
14	18-7-2021	*Cupresses arizonica*	80	4	E			
15	18-7-2021	*Cupresses arizonica*		5	E			
16	18-7-2021	*Cupresses arizonica*	89	6	S_E			
17	18-7-2021	*Pinus halepensis*	172		N	2190	TW-MD21-Veg-17/18/19	Pin03
18	18-7-2021	*Pinus halepensis*	172		N			
19	18-7-2021	*Pinus halepensis*	174		N			
20	18-7-2021	*Pinus halepensis*	211		S-W	3700	TW-MD21-Veg-20/22/23	Pin04
22	18-7-2021	*Pinus halepensis*	122		S-W			
23	18-7-2021	*Pinus halepensis*	155		S-W			
21	18-7-2021	*Cupressus arizonica*	211	7	S-W	3820	TW-MD21-Veg-21	Cup03
24	18-7-2021	*Pinus halepensis*	193		S-W	4000	TW-MD21-Veg-24/25	Pin05
25	18-7-2021	*Pinus halepensis*	190		S-W			
26	18-7-2021	*Pinus halepensis*	196		E	4710	TW-MD21-Veg-26/27/28	Pin06
27	18-7-2021	*Pinus halepensis*	193		E			
28	18-7-2021	*Pinus halepensis*	206		E			

Table 2: Sampling overview of pine needles and tree leaves locations, Madrid 2021

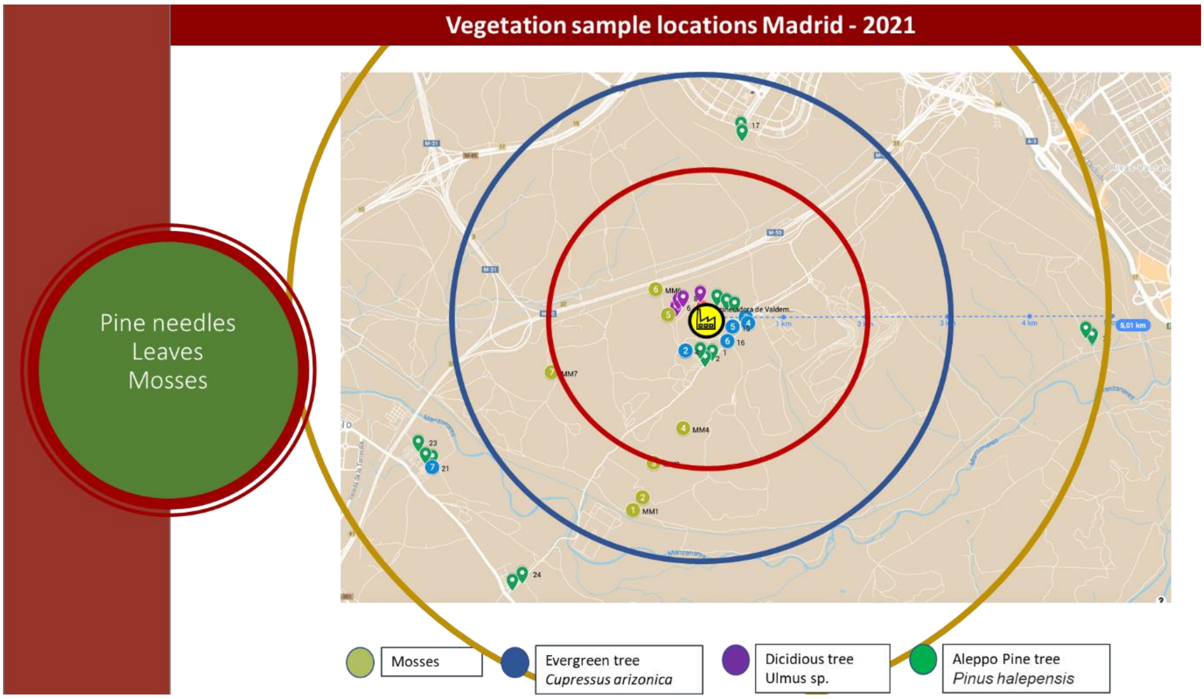

Figure 21: Vegetation sample locations, Madrid - 2021

Pine needles

Pines are widespread among evergreen species and are characterized by a high-fat content. Pine needles have been used for many decades to monitor atmospheric persistent organic pollutants (POPs) like dioxins (PCDD/Fs) pollution on a global and regional scale. The uptake of pollutants by vegetation occurs mainly through gas-phase partitioning or deposition of dust particles that adsorb on the surface of pine needles and leaves to diffuse into the waxy cuticle of the leaves. It has been identified that waste incinerators contribute significantly to the environmental concentrations of dioxins (PCDD/Fs)[37]. Pine needles have an advantage over the use of Polyurethane Foam discs (PUF), which are vulnerable to vandalism. Living organisms, like trees are, a better to observe and to analyse contamination of substances of very high concern.

Pine trees are able to survive long periods of stressful drought conditions due to the special morphology of pine needles, especially the epicuticular waxes and the distribution of tubular waxes which are species specific[38]. Meaning the epicuticular wax layer, which helps protect the leaves from the more toxic form of ultraviolet light called UV-B, as well to prevent water loss of the plant system, and risks of pathogen and insects attacks. Dioxins (PCDD/F/dl-PCB) partition in this fatty wax layer because of its lipophilic properties. In fact, lipophilic xenobiotica have been found to have a greater affinity to one of the main components of the cuticle membrane, the cuticular waxes compared to other cuticle membrane components[39]. Persistent organic pollutants are thought to sorb to the cuticular waxes and diffuse into internal leaf (pine needle) compartments[40] Therefore, plant leaves/ pined needles can be used as a natural sampler for persistent organic pollutants (POPs) in the environment.
Pine needles will last for more than 2-5 years on pine trees, depending on the species of pine trees. The uptake of dioxins through pine needles can take place continuously year after year. Figure 22 shows the sampling plan for locations of Aleppo pine needles, *Pinus halepensis*.

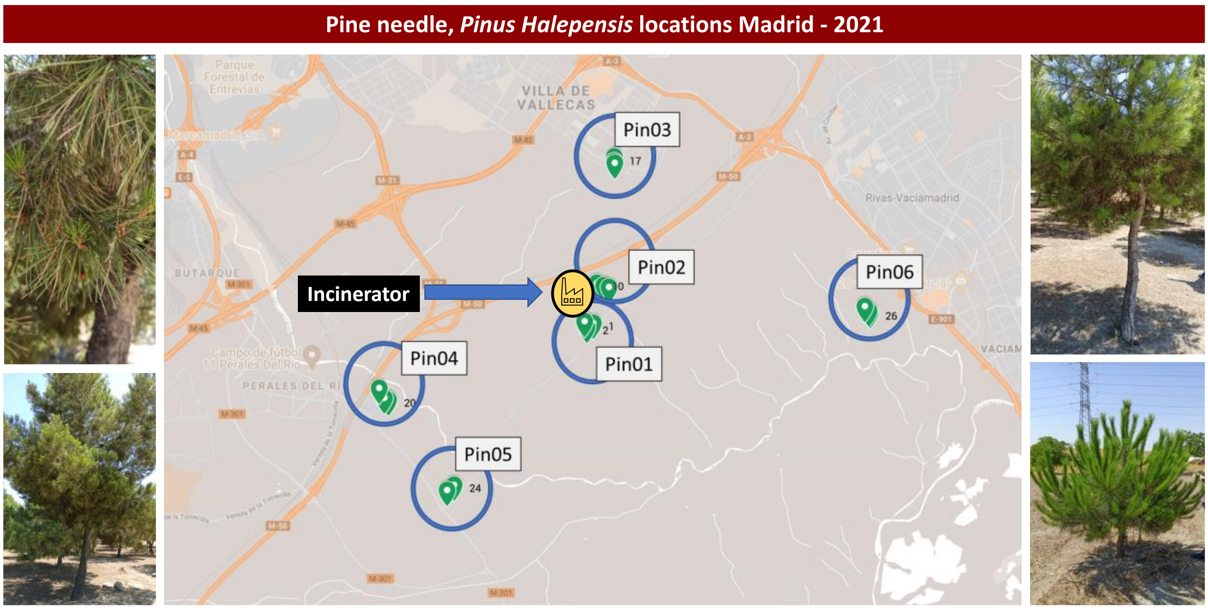

Figure 22: Sample location pine needles Aleppo pine - Pinus Halepensis, Madrid -2021

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[37] Ch en P. et al. (2017). Chemosphere 181 (2017) 360 - 367
[38]Lamppu J., Huttunen S. (2002). Environmental Pollution 122 (2003) 119–126
[39] Moeckel C., 2008. Environ Sci Technol 42:100–105
[40] Barber, JL. (2004).. Environ Pollut 128: 99–138

Results vegetation Madrid 2021

The CALUX analysis results are given in the sum of dioxins (PCDD/F/dl-PCB), dioxins (PCDD/F), dl-PCB and PAH. Remarkable are the high values of dioxins at Pinus location 1 at a distance of 570 meter from the incinerator in South wind direction. The result values for Pinus locations 5 and 6 are low on a distance of 4-5 km from the incinerator, a reference location, see Table 3, in South-West wind direction.

Sampling Pine needles, foliage and leaves, Madrid - 2021									Results Pine needles and leaves, Madrid - 2021				
	Sample date	species	Weight (gr)		Wind direction	Distance (m)	TW-REF-NR		PCDD/F/dl-PCB	PCDD/F	dl-PCB	PAH	PFAS (FITC-T4)
									DR CALUX	pg TCDD eq./g product		ng BaP eq./g pr.	µg PFOA eq/g pr.
1	18-7-2021	*Pinus halepensis*	125		S	570	TW-MD21-Veg-01/02	Pin01	8.40	7.10	1.30	220.00	26
2	18-7-2021	*Pinus halepensis*	111		S								
3	18-7-2021	*Pinus halepensis*	102		S								
4	18-7-2021	*Cupresses arizonica*	115	1	S-W	528	TW-MD21-Veg-04/05	Cup02	1.14	0.87	0.27		
5	18-7-2021	*Cupresses arizonica*	148	2	S-W								
6	18-7-2021	*Ulmus minor*	138		W	300	TW-MD21-Veg-06/07/08	Ulmus01	0.12	0.07	0.05		
7	18-7-2021	*Ulmus minor*	144		W								
8	18-7-2021	*Ulmus minor*	179		W								
9	18-7-2021	*Ulmus minor*	149		N	175	TW-MD21-Veg-09	Ulmus02	0.12	0.07	0.05		
10	18-7-2021	*Pinus halepensis*	137		N-E	280	TW-MD21-Veg-10/11/12	Pin02	0.29	0.18	0.11		
11	18-7-2021	*Pinus halepensis*	167		N-E								
12	18-7-2021	*Pinus halepensis*	149		N-E								
13	18-7-2021	*Cupresses arizonica*	169	3	E	400	TW-MD21-Veg-13/14/15	Cup01	1.70	1.60	0.10	380.00	17
14	18-7-2021	*Cupresses arizonica*	80	4	E								
15	18-7-2021	*Cupresses arizonica*		5	E								
16	18-7-2021	*Cupresses arizonica*	89	6	S_E								
17	18-7-2021	*Pinus halepensis*	172		N	2190	TW-MD21-Veg-17/18/19	Pin03	0.18	0.07	0.11		
18	18-7-2021	*Pinus halepensis*	172		N								
19	18-7-2021	*Pinus halepensis*	174		N								
20	18-7-2021	*Pinus halepensis*	211		S-W	3700	TW-MD21-Veg-20/22/23	Pin04	0.14	0.08	0.06	8.10	22
22	18-7-2021	*Pinus halepensis*	122		S-W								
23	18-7-2021	*Pinus halepensis*	155		S-W								
21	18-7-2021	*Cupressus arizonica*	211	7	S-W	3820	TW-MD21-Veg-21	Cup03	0.80	0.73	0.07	31	17
24	18-7-2021	*Pinus halepensis*	193		S-W	4000	TW-MD21-Veg-24/25	Pin05	0.35	0.09	0.26		
25	18-7-2021	*Pinus halepensis*	190		S-W								
26	18-7-2021	*Pinus halepensis*	196		E	4710	TW-MD21-Veg-26/27/28	Pin06	0.11	0.05	0.06		
27	18-7-2021	*Pinus halepensis*	193		E								
28	18-7-2021	*Pinus halepensis*	206		E								

Table 3: Results DR CALUX , PAH CALUX, PFAS (FITC-T4) vegetation Madrid 2021

Results DR CALUX pine needles Aleppo pine – *Pinus halepensis*

The results of the DR CALUX analysis for sum dioxins (PCDD/F/dl-PCB) are given in Table 4. The specific biomatrices and bioassay DR CALUX results will be explained in the next chapters.

Spain, Madrid -2021									
Vegetation Madrid				Results Dioxins, PAH, PFAS in Pine needles					
Sample date	Species	Pooled Veg Nr	TW-REF-NR	Distance (m)	PCDD/F/dl-PCB	PCDD/F	PCB	PAH	PFAS (FITCH-T4)
					DR CALUX pg TCDD eq./g product			*ng BaP/g pr.*	*µg PFOA eq/g pr.*
18-7-2021	*Pinus halepensis*	Pin01	TW-MD21-Veg-01/02	570	8.40	7.10	1.30	220.00	26.00
18-7-2021	*Pinus halepensis*	Pin02	TW-MD21-Veg-10/11/12	280	0.29	0.18	0.11		
18-7-2021	*Pinus halepensis*	Pin03	TW-MD21-Veg-17/18/19	2190	0.18	0.07	0.11		
18-7-2021	*Pinus halepensis*	Pin04	TW-MD21-Veg-20/22/23	3700	0.14	0.08	0.06	8.10	22.00
18-7-2021	*Pinus halepensis*	Pin05	TW-MD21-Veg-24/25	4000	0.35	0.09	0.26		
18-7-2021	*Pinus halepensis*	Pin06	TW-MD21-Veg-26/27/28	4710	0.11	0.05	0.06		

Table 4: Results of dioxin analysis of pine needles, Madrid 2021

High values of dioxins are measured at sample point Pin01, 500 metres (South-West) from the incinerator. This sampling point is a pooled sample of 2 Aleppo pines (see Annex II-III for details of TW-MD-Veg 01/02). The measured value is more than 75 times higher than the reference site Pin06, which is a pooled sample of 3 Aleppo pines (TW-MD21-Veg26/27/28) 4,710 metres from the incinerator. The other pine needle samples all produced very low measurements (Figure 23) compare to the TW indicative scale of other TW researches. An indicative overview of dioxins and dioxin-like PCBs (dl-PCBs) in evergreen trees in Europe is shown in Figures 19-20. The measured value at sampling point Pin01 in the centre of Figure 23 is marked black, comparing to the TW indicative scale and represents the highest value measured in pine needles in TW biomonitoring researches, see Figure 24.

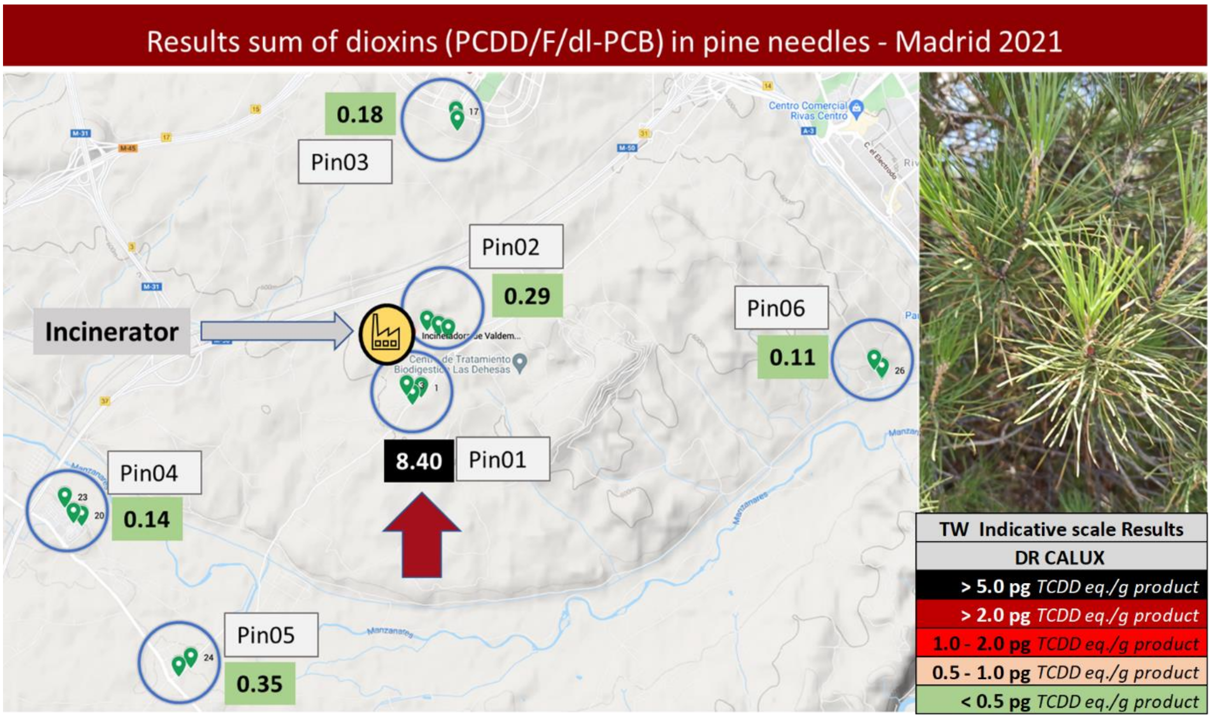

Figure 23: Results pine needles sum of dioxins (PCDD/F/dl-PCB)

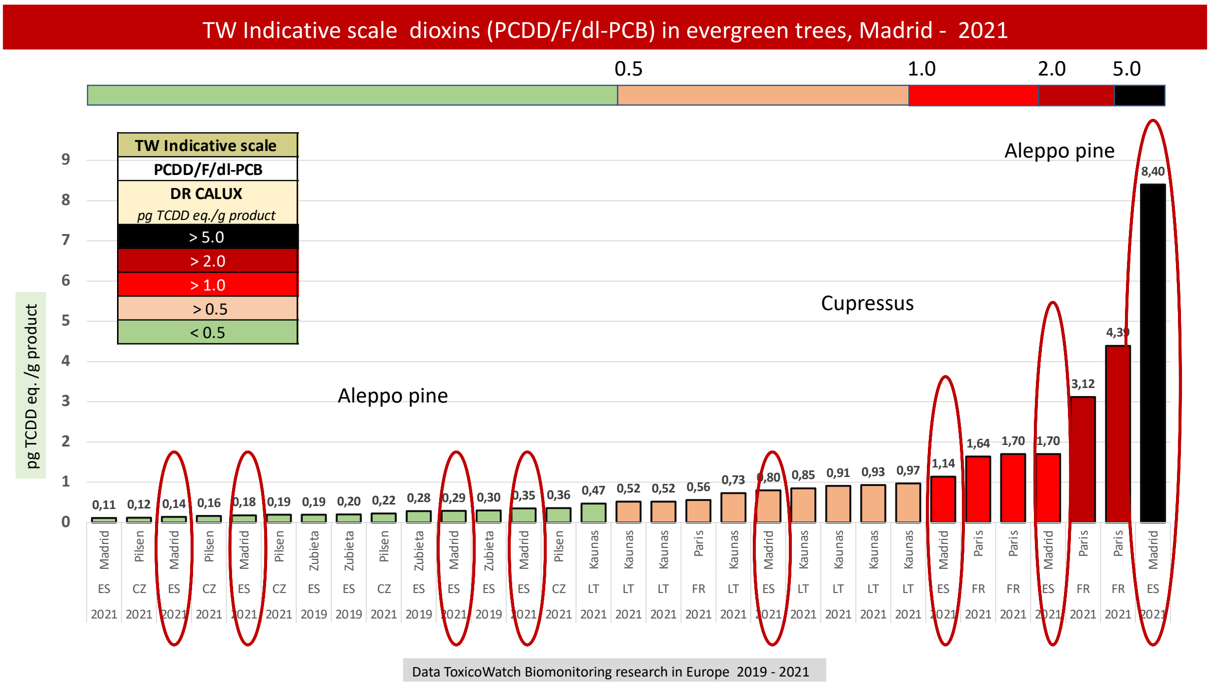

Figure 24: : TW Indicative scale for sum of dioxins (PCDD/F/dl-PCBs) in evergreen trees

The dioxins and furans (PCDD/F) at location Pin01, near the incinerator, measure 7.10 pg TCDD eq./g product and that is 140 times more than at location Pin06 3400 m away with 0.05 pg TCDD eq./g, see Figure 25.

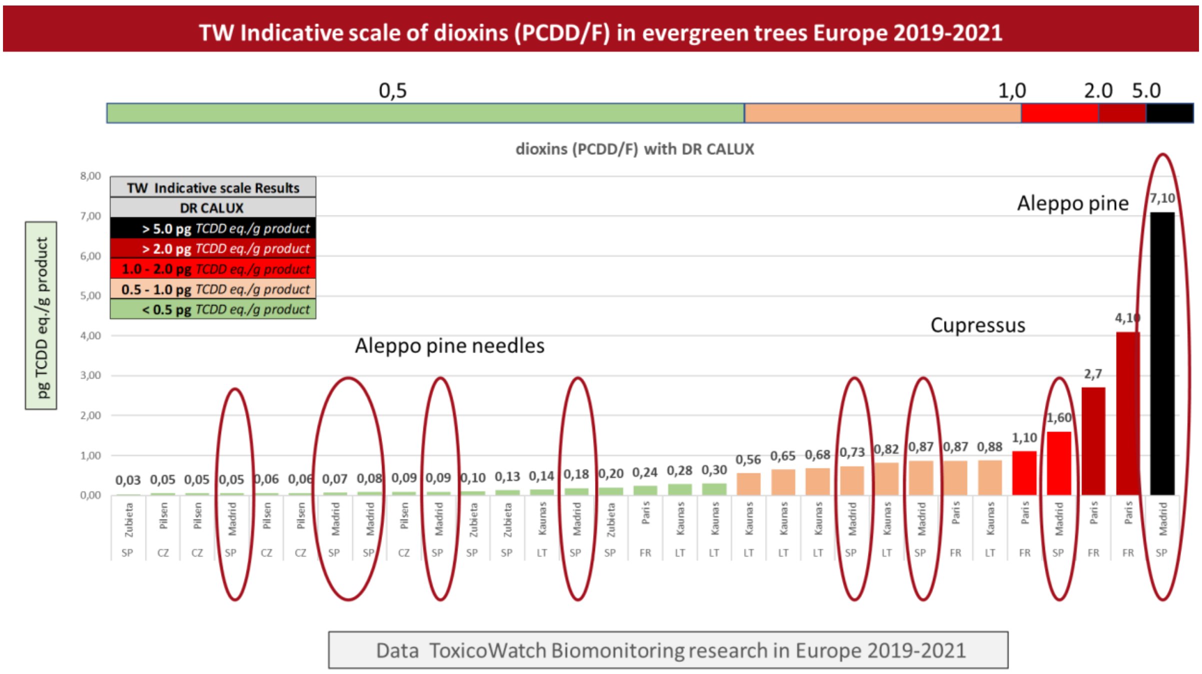

Figure 25: TW Indicative scale for dioxins (PCDD/F) in evergreen trees

The dioxin-like PCBs (dl-PCBs) found in pine needles of Aleppo pine trees represent the highest analysis value of dl-PCBs, 1.30 pg TCD eq./g product, in biomonitoring researches performed by ToxicoWatch between 2019 and 2021, Figure 26.

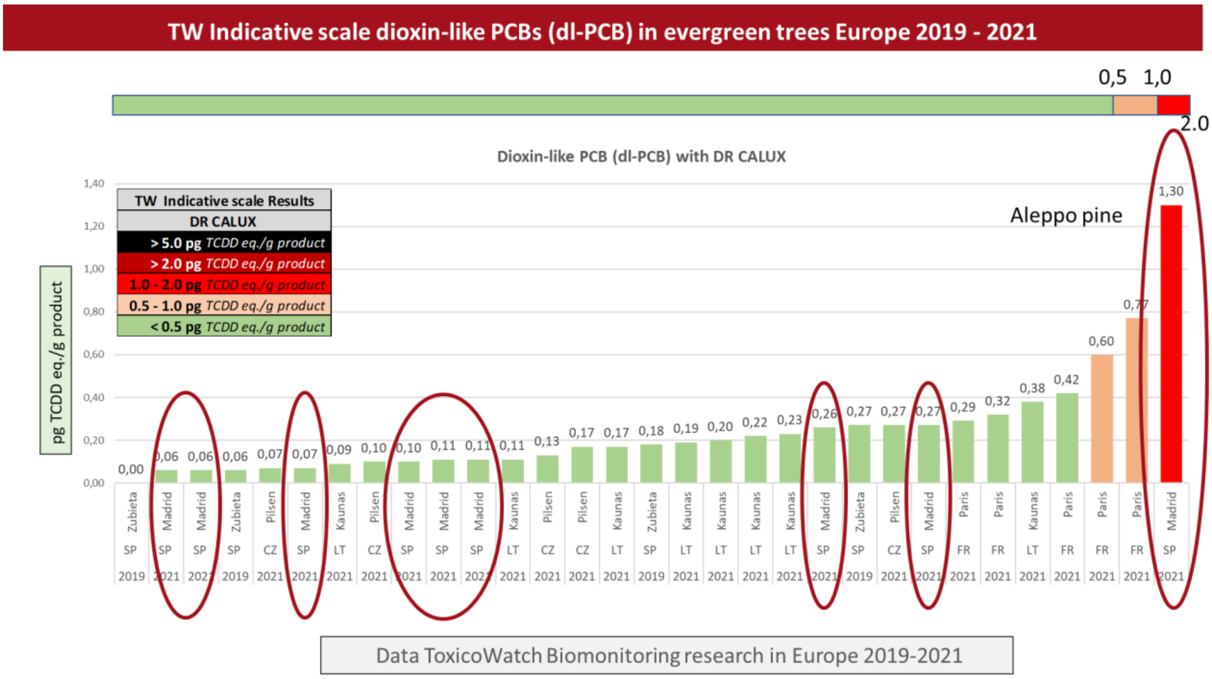

Figure 26: TW Indicative scale for dl-PCBs in evergreen trees in Europe 2019-2021

Figure 27 shows a white wall of full loaded big bags, out in the open air, which are clearly seen when walking along public roads in the area around the waste incinerator. These big bags could be a way of storing bottom and fly ash residues from the waste incinerator. The source of the high values seen in the results at location Pin01 is unknown. More research is needed to find out whether these high values are related to the incinerator emissions or whether they are linked to spill-off from these large bags. Continuous measurements of the flue gases in the chimney of the waste incinerator and sampling of bottom and fly ash for analysis with DR CALUX may provide some clarity by comparing the alarming high results of this analysis.

Figure 27: Is there a link between the wall of big bags and dioxins in Aleppo pines near the incinerator, biomonitoring 2021?

Dioxins in Arizona Cypress – *Cupressus arizonica*

Arizona cypress - *Cupressus arizonica* is a North American native evergreen tree from the family Cupressaceae. The green foliage of this tree has a silver grey colour and the branches form an open canopy. Gymnosperm are 'naked seeds' plants, trees with cones such as *Cupressus arizonica.* This species is planted for their specific characteristics like a low need for water and minimum care requirements. Arizona cypress have been commonly and traditionally used for urban landscaping in many dry and hot weather areas and are also planted for erosion control and as wind breaks. *Cupressus arizonica* is also a good indicator of air pollution and can therefore been used as an early warning tool for air pollution, which is harmful for human health.[41] The evergreen foliage of this tree remains for years. They are considered by several researchers as the ideal bio-samplers for measuring substances of very high concern in the environment. Using vegetation as bio-samplers of atmospheric contamination on Persistent, Bio-accumulative and Toxic compounds (abbreviated as PBT) is a relatively cheap and uncomplicated means of monitoring atmospheric contaminants that does not require equipment/samplers and is particularly useful for sampling at remote sites. *Cupressus arizonica* was selected as a second vegetation biomarker in this research. This vegetation also proved to be very sensitive for persistent organic pollutants (POPs). Three samples were analysed, the first being a pooled sample of TW-MD21-Veg-13/14/15, the second Cup02 is pooled with TW-MD21-Veg-04 and 05 and the third with code Cup03 is only one sample of TW-MD21-Veg-23. The results of PCDD/F/dl-PCB are 0.80 - 1.70 pg TCDD eq./g., see Table 5. The distance to the waste incinerator is 400 to 3,820 meters, see Figure 28. A spatial effect can be observed here too. Figures 24 and 25 show the high position of dioxins in Cupressus on the TW indication scale of dioxins in evergreen trees.

Dioxin analysis Cupressus arizonica, Madrid 2021								
Sample date	Species	Sample nr.	TW-REF-NR	Distance (m)	PCDD/F/dl-PCB	PCDD/F	PCB	Coordinates
					pg TCDD eq./g product			
July 9, 2021	*Cupressus arizonica*	Cup01	TW-MD21-Veg-13/14/15	400	1.70	1.60	0.10	40.335510,-3.594139
July 9, 2021	*Cupresses arizonica*	Cup02	TW-MD21-Veg-04/05	528	1.14	0.87	0.27	40.331818,-3.602853
July 9, 2021	*Cupressus arizonica*	Cup03	TW-MD21-Veg-23	3820	0.80	0.73	0.07	40.319081, -3.639203

Table 5: Results of dioxins in Cupressus arizonica, Madrid 202

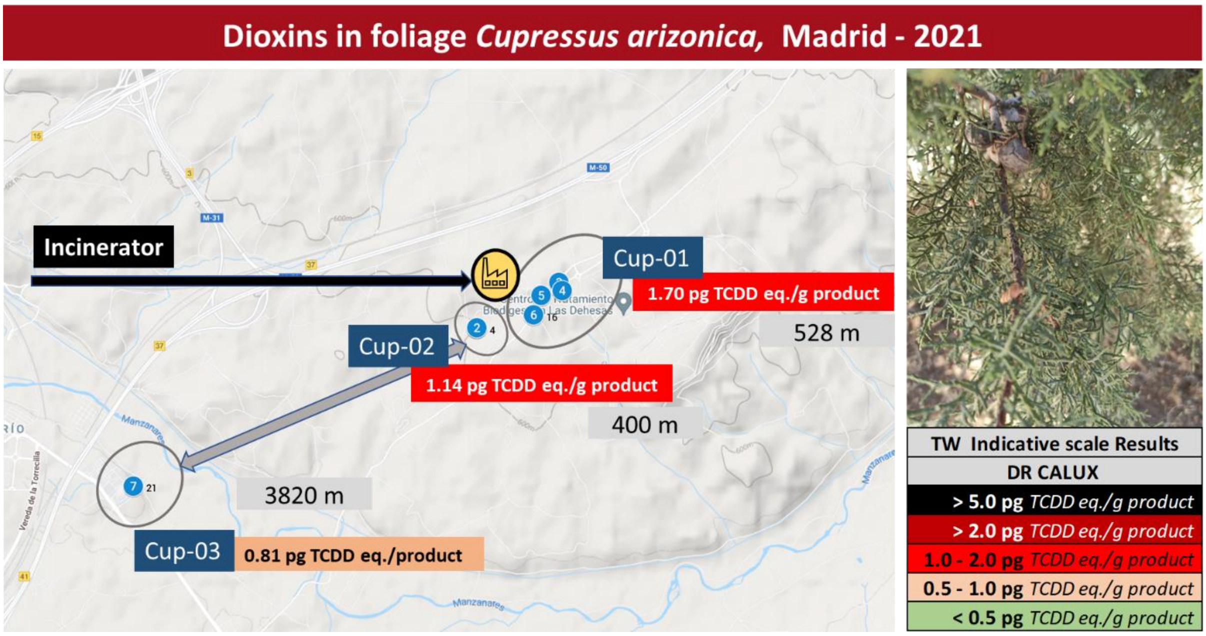

Figure 28: Location of Cupressus arizonica and the analysis results of dioxins, Madrid 2021

[41] *Bahadoran, M. et al. (2019. Int. J. Phytoremediation **2019**, 21, 496–502.*

Field elm – *Ulmus minor*

On **18 July 18 2021** leaves of the *Ulmus minor* were sampled a short distance from the incinerator, between 175 and 300 metres away. Leaves were taken at 1.50 to 2 metres above the ground. The deciduous vegetation close to the incinerator on the north and west side comprises field elms. Elms are deciduous trees of the genus *Ulmus* in the plant family *Ulmaceae*. We have only found biomonitoring research on the bark of *Ulmus* species, not on the leaves[42]. One important thing when analysing samples of elm leaves is that you only have a measurement of a few months. Since the growing season for elm leaves starts around April, it was only 14 weeks until sampling date of 18 July. The leaves lack a fatty cuticle – the outer layer of the leaf, a protection mechanism for i.e. water evaporation. Taking this into account, it is remarkable that dioxins could already be measured on leaves that were just 3.5 months old. The analysis results of dioxins (PCDD/F/dl-PCB) with DR CALUX are just above the detection limit of < 0.05 pg TEQ/g (Table 6). The dioxin-like PCBs are below the detection limit. The fact that much lower levels of dioxins were found above the action limit on the Ulmus leaves could be because there was very low deposition at this location (North) or the deciduous Ulmus is just not suitable for long-term biomonitoring research on dioxins (Figure 29).

Dioxin analysis Ulmus minor, Madrid 2021								
Sample date	Species	Sample nr.	TW-REF-NR	Distance (m)	PCDD/F/dl-PCB	PCDD/F	PCB	Coordinates
					pg TCDD eq./g product			
July 9, 2021	*Ulmus minor*	Ulmus01	**TW-MD21-Veg-06/07/08**	300	**0.12**	**0.07**	**<0.05**	40.335491,-3.604168
July 9, 2021	*Ulmus minor*	Ulmus02	**TW-MD21-Veg-09**	175	**0.12**	**0.07**	**<0.05**	40.337036,-3.600706

Table 6: Results of dioxin analyses on Ulmus minor, Madrid 2021

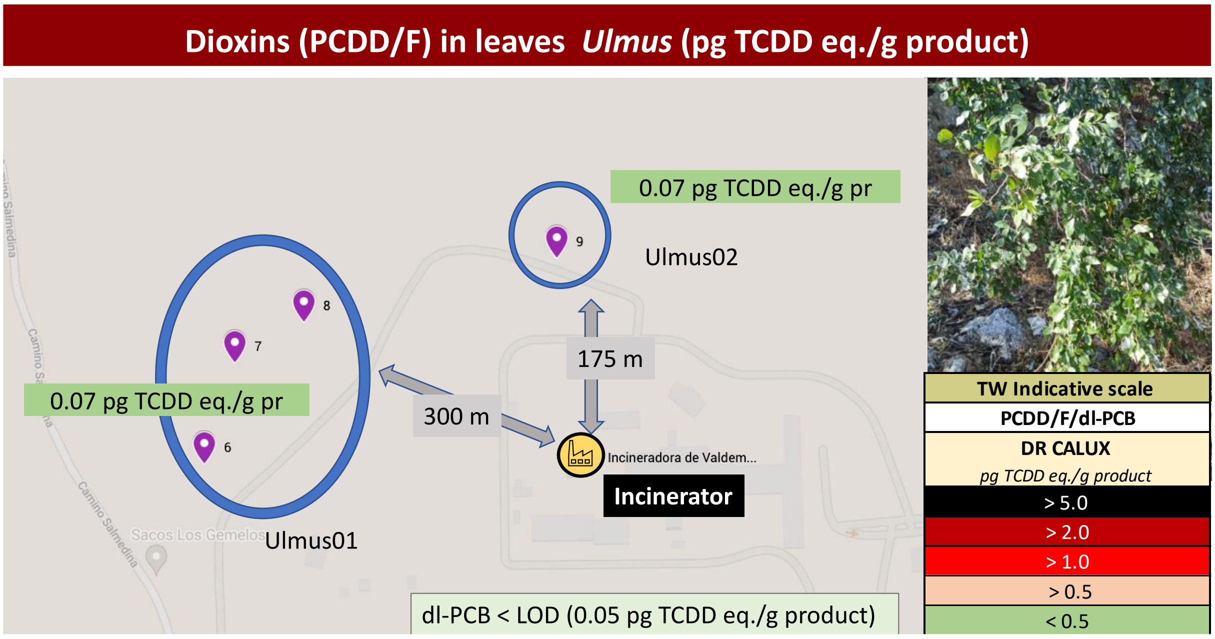

Figure 29: Dioxins (PCDD/F) in leaves of Ulmus minor, Madrid 2021

[42] *Mariusz Chrabąszcz et al (2016). Tree Bark, a valuable source of Information on air quality. Pol. J. Environ. Stud. Vol. 26, No. 2 (2017), 453-466*

PAH in vegetation

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic toxic contaminants in the environment, an excellent tool to monitor emissions of thermo-confounders. More than 40% of PAH is absorbed by the vegetation by dry and wet deposition, making it a significant biomarker for the environment. The foliar interface of vegetation is due to a large surface area considered as the main access for organic chemical accumulation.

Organic chemicals have been found to distribute unevenly in foliage with the majority of them being accumulated in the outermost polyester skin of the leaf, the cuticle. The cuticle is the dominant reservoir for the accumulation of lipophilic pollutants and act as the very first barrier for protecting plants from external impacts, and both the outer structures and inner chemical compositions are critical to this process.

Since PAHs are hydrophobic compounds, airborne PAHs are deposited on foliar surfaces of vegetation mainly by dry deposition (gaseous and particulate-bound forms). Surface stereo micro-structure and hydrophobicity could have effects on the foliar uptake of organic pollutants. In general, for PAHs the more volatile 2- and 3-ring compounds exist primarily in the gas phase of the atmosphere and will tend to be deposited on plants via dry gaseous and/or wet deposition. The less volatile 5- and 6-ring PAHs are more likely to be deposited on the plant surface bound to particles in wet and dry deposition. For compounds of intermediate vapour pressure (4-ring PAHs for example), a temperature-dependent gas/particle partitioning of POPs will occur, so that they are subject to both wet and dry deposition in gaseous and particle-bound form. Therefore other factors like moisture, rain and even the morphology of the pine needles and leaves are involved in the uptake of lipophilic xenobiotica.

In Figure 30, the pine needles of Aleppo pines - *Pinus Halepensis* near the incinerator contains more than 25 times more PAH than *Pinus h.* 4,000 metres away. Most research on PAH in pine needles is performed by chemical analysing of 4 -16 (EPA-16) PAH congeners. This method of PAH research is limited with respect to the enormous group of PAH congeners and their respective toxicity[43]. The PAH CALUX bioassay analyses PAH expressed in equivalent benzo[a]pyrene. See Annex II-III ‘Vegetation’ for relative toxicity potencies of the different PAH congeners.

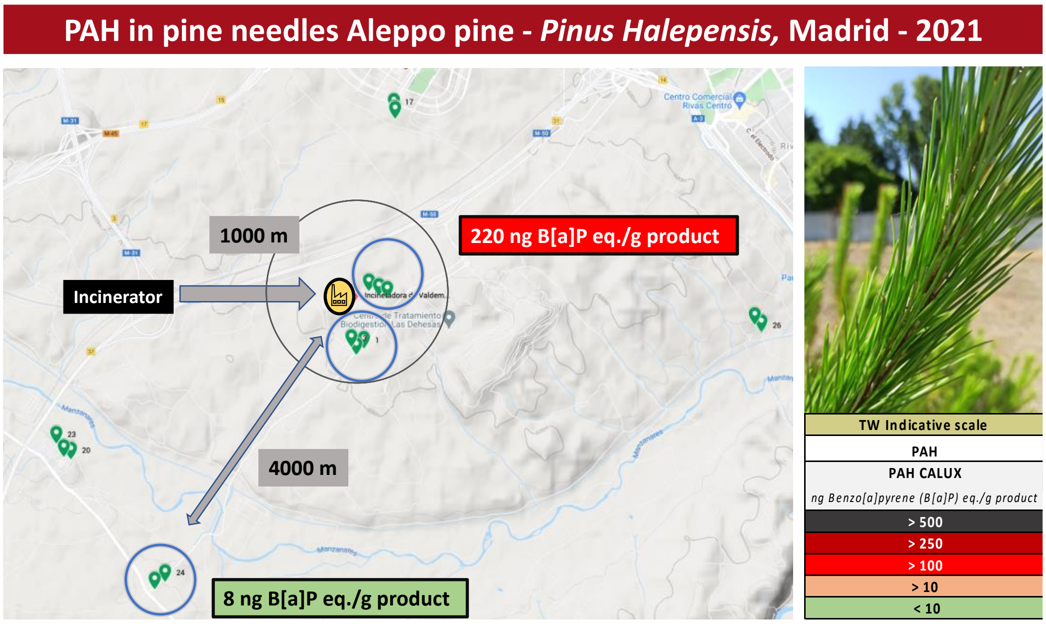

Figure 30: PAH in pine needles, Madrid 2021

[43] *Andersson J.T., Achten C. (2015). Time to Say Goodbye to the 16 EPA PAHs? Toward an Up-to-Date Use of PACs for Environmental Purposes - Polycyclic Aromatic Compounds, 35:330–354*

Kalugina et al (2018)[44] researched the emissions from a powerful source of PAH emissions, an aluminium smelter, in the needles of Scots pine (*Pinus sylvestris*) in the residential areas of Bratsk, East Siberia, Russia. The reference concentrations of benzo[a]pyrene in the pine needles was 0.1 ng B[a]P/g. The total concentration of 4 PAH, phenanthrene, fluoranthene, pyrene, and chrysene was 820 ng/g. However, the relative toxicity of this congener was only 0.0001 compared to benzo[a]pyrene. Near the aluminium smelter the concentration of class 1 benzo[a]pyrene in the needles was 22 higher 2.2 ng B[a]P/g. In the city where the aluminium smelter was located, there was a high incidence of respiratory diseases, musculoskeletal, immune, endocrine system diseases, and malignant tumours. In contrast with phenanthrene, fluoranthene, pyrene, and chrysene, benzo[a]pyrene will be mostly in particulate not in the gaseous phase. This study finds 170 x PAH in the vegetations than Kalugina et al (2018) in the vegetation of a strongly contaminated place with the emission benzo[a]pyrene of an aluminium smelter.

The report by Madrid Health (Madrid Salud) concludes that the impact of the incinerator on PAH levels can be considered as non-existent. This conclusion is diametrically opposed to the findings of this biomonitoring study. The analysis results of 220 ng and 380 ng benzo[a]pyrene equivalent PAH in vegetation near the Valdemingómez incinerator in this research is a clear indication that the environment is under threat from these carcinogenic substances of very high concern (SVHC). Even at the reference point of 4 km away, the result of 8 ng benzo[a]pyrene is higher than the deposition of PAH near an aluminium smelter in Russia (Kalugina et al 2018). Not only the inhabitants of Southwest Madrid, but other parts of Madrid could be affected by deposition of PAH if parameters such as OTNOC and changes in wind direction are taken into account.

In this research on the foliage of Arizona cypress – *Cupressus arizonica* – a factor of 12 more activity of benzo[a]pyrene is found: 380 ng B[a]P **equivalent**/g. Meanwhile, at the reference site 4 km from the incinerator, values 31 ng B[a]P equivalent/g were measured in the pine needles. The results of the PAH in the vegetation *Pinus Halepensis* and *Cupressus arizonica* show a clear spatial pattern towards the incinerator location, see Figure 31. Even the found level of 31 ng B[a]P equivalent/g can be considered as a high, warning quantity compared with other TW data results in Europe (Figure 33).

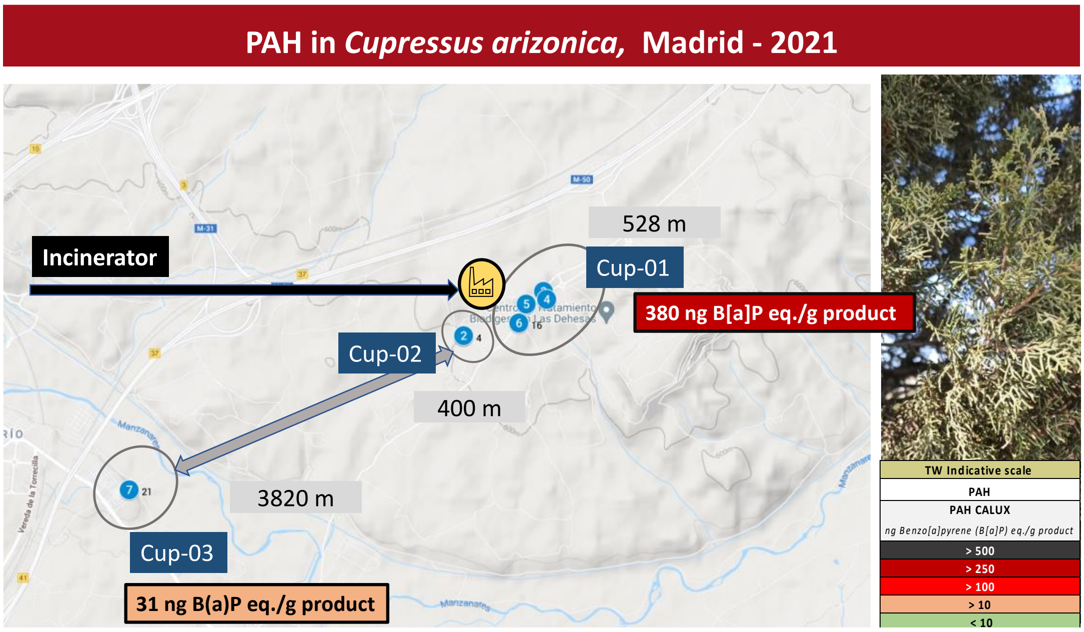

Figure 31: PAH in Cupressus arizonica, Madrid, 2021

[44] *Olga Vladimirovna Kalugina et al (2018). Contamination of Scots pine forests with polycyclic aromatic hydrocarbons on the territory of industrial city of Siberia, Russia Environmental Science and Pollution Research*

Research by Madrid Health (Madrid Salud) studied the impact on health of emissions from Valdemingómez Technology Park using three active air samplers for analysing the air for dioxins and PAH. The nearest sampler was placed at Ensanche de Vallecas Station, 5 km from the Technology Park. Based on this research, a map of PAH distribution was constructed (Figure 32). The conclusion of the report by Madrid Health (Madrid Salud) is that "the impact of the waste incineration plant on PAH levels can be considered as non-existent." This biomonitoring report shows just the opposite by finding high levels of PAH near the incinerator and at a sample point 3,820 km away from the waste incinerator.
Figure 33 show the indicative scale of the results on vegetation (pine needles and foliage) compared to locations near and further away from the incinerator.

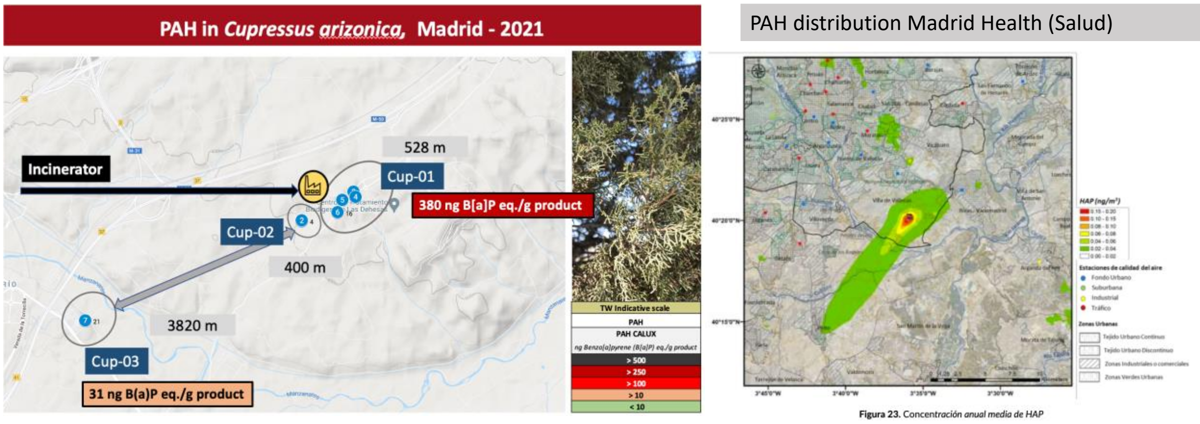

Figure 32: Comparison study Madrid Health (Salud) and this TW biomonitoring research 2021

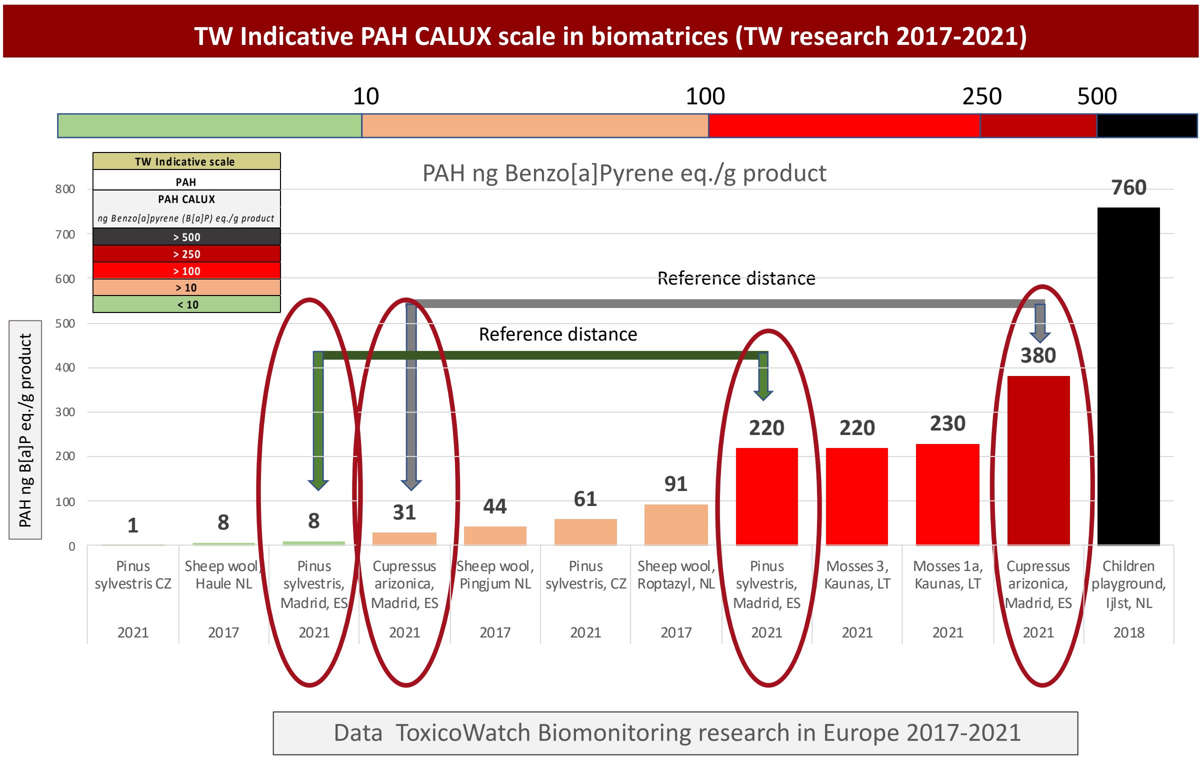

Figure 33: TW Indicative PAH CALUX scale biomatrices, TW data PAH 2017-2021

PFAS

To have an impression of PFAS presence in the environment of the incinerator of Madrid the FITC-T4 analysis method was used on samples of *Pinus halepenis* and *Cupressus arizonica*. The results of the PFAS analysis with the FITC-T4 are shown in Figures 34 and 35. In pine needles of *Pinus halepensis* at a location 1,000 metres from the incinerator 26 µg PFOA eq./g was found and 22 µg PFOA eq./g at a location 4,000 metres away.

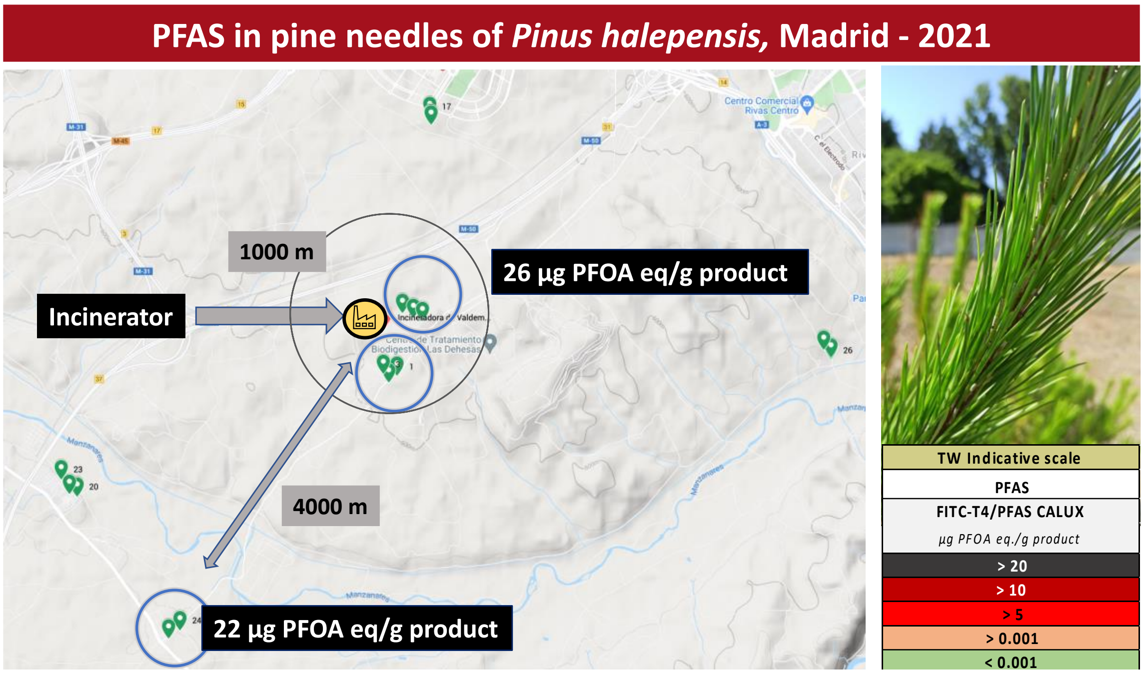

Figure 34: PFAS in pine needles of Pinus halenpensis, Madrid 202

In the foliage of *Cupressus arizonica,* 400 metres from the incinerator and at a location 3,820 metres away the analysis results have the same level of 17 µg PFOA eq./g. No difference in spatial trends could be observed. As far as we know, this is the first analysis of PFAS with FITC-T4 on vegetation (pine needles and foliage).

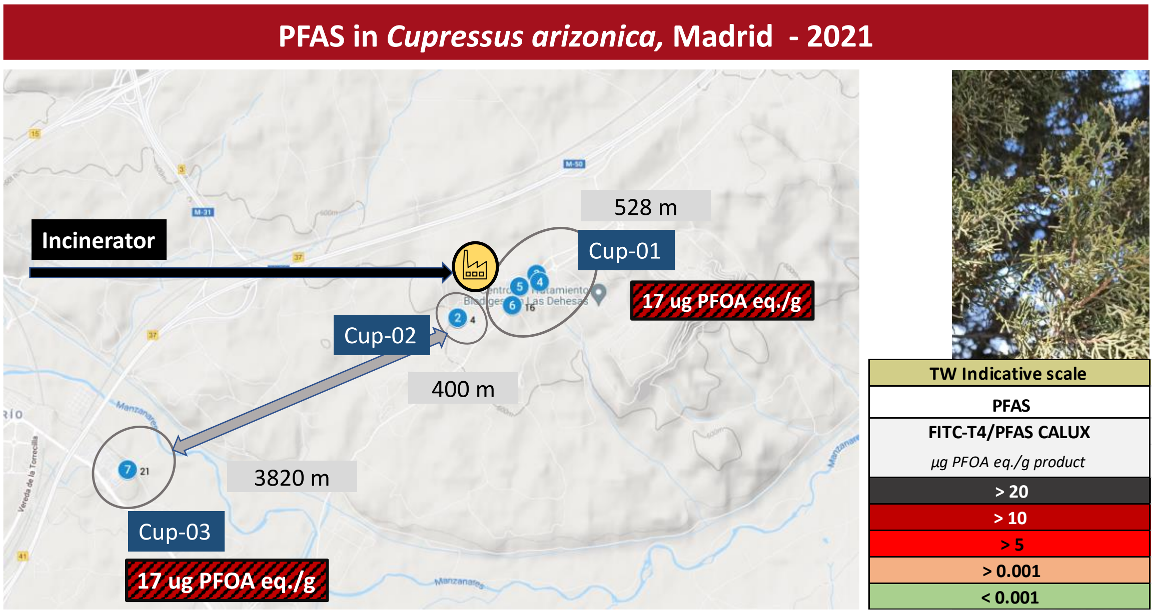

Figure 35: PFAS in Cupressus arizonica, Madrid 202

Figure 36 shows an TW indicative scale for comparing the initial results of PFAS analyses on environmental samples in simultaneously performed research in other countries in Europe this year. The lowest values of PFAS in eggs found in a comparable biomonitoring study in the Czech Republic still exceeded the safety food levels for PFAS (PFOA) by a factor of one thousand. Results with such high levels of PFAS in the environment are alarming.

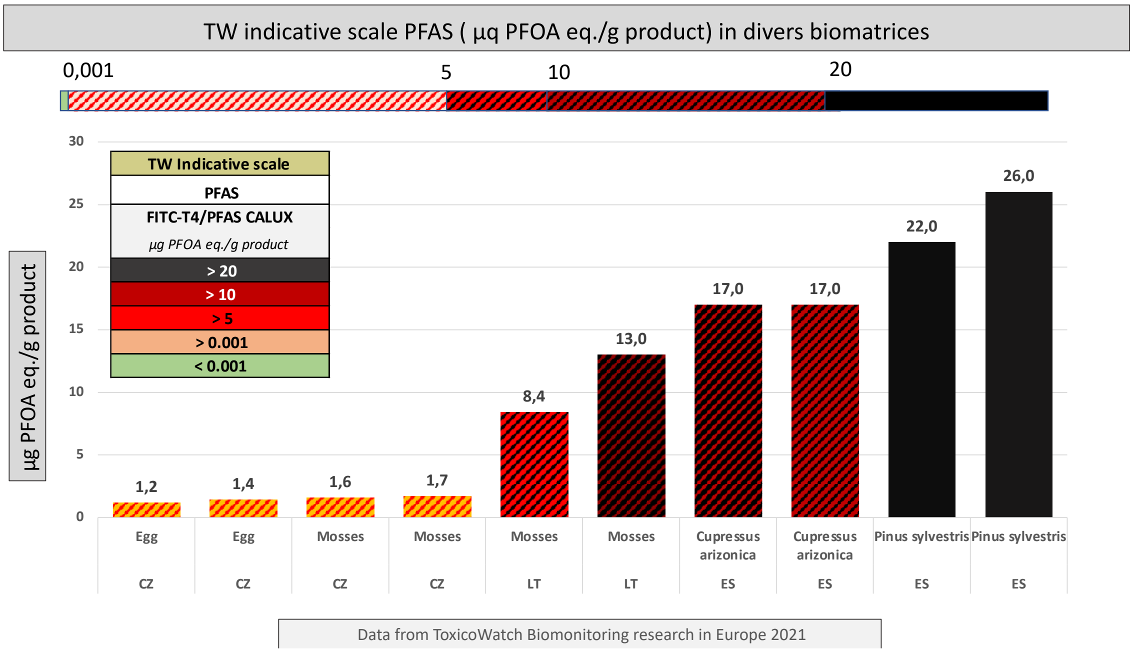

Figure 36: TW Indicative scale for PFAS in various biomatrices

In a Swedish study[45] the concentrations of 10 perfluorinated alkylated substances (PFAS) were all below the quantification limit (<0.6 ng/g dw) for every compound and moss sample. The results in this biomonitoring research show high levels of PFAS, which is an important reason for more research.

The EFSA advice for a Tolerable Weekly Intake (TWI) of PFOA is set at 6 nanogram/kg body weight a week. If a person just drinks one cup of pine needle tea a week, the Tolerable Weekly Intake (TWI) for PFOA will be exceeded by a factor 500. This means the PFAS levels found in the vegetation are extremely toxic, raising concerns about the findings in this area. Additional research on PFAS is needed a for a better understanding of how these results can be interpreted.

[45] *Danielsson H. et al. (2016). Persistent organic pollutants in Swedish mosses, IVL Swedish Environmental Research Institute 2016, report nr. C 188*

In the previous section is explained how the analytical research of PFAS is lagging behind. Only a fraction of the different PFAS components (8-55) can be analysed in a laboratory, whereas it is likely that more than 8,000 different PFAS can be found in the environment. The relative potency factor of only 12 components could have been determined (see page 14). The FITC-4 is an analytical method measuring the total toxicity of a mixture of different PFAS substances (page 16). PFAS is associated with adverse human health effects on thyroid function, metabolism (including overweight/obesity, diabetes, insulin resistance, high cholesterol and foetal development, and play an important role in the human immune system. Further research is needed to monitor and analyse the contamination of this 'forever-chemical' in our environment to establish the consequences of these PFAS for the environment, vegetation, animals, and our human health.

There are no further studies of FITC-T4 on vegetation or even on emissions of incinerators. This biomonitoring research, simultaneously performed in the Czech Republic (Pilsen), Lithuania (Kaunas) and Spain (Madrid), see Figure 36, is the first in line with the application of FITC-T4 on biomatrices. There is a great need for data on PFAS distribution in the environment. Chemical analyses (GC-MS) unfortunately fall short of these findings, hence the application of the FITC-T4 methodology. The extent to which the incineration of PFAS-related waste and sewage sludge leads to PFAS contamination in the environment is still unknown. The association with fire-fighting foams (AFFF) is clear, but what is not clear are the combustion products of a fire-fighting event. Pilot studies with PFAS incineration indicate incomplete destruction even at temperatures above 950 °C. In a modern waste-to-energy incinerator (WtE) the post-combustion temperature is set at 850 °C, and, as it appears currently, these temperatures are not adequate to destroy persistent organic pollutants like dioxins and PFAS completely.

What are the consequences of PFAS on the environment, as well for human health in general? The question arises: what is the cause of this PFAS contamination? And what is the contribution of incineration to the PFAS contamination in the environment? In a study of ToxicoWatch of continuous measurements WtE incineration in the Netherlands, PFOA and PFOS are detected in the flue gases[46]. So the question arise what is the contribution of waste incineration to the PFAS contamination in the environment around Valdemingómez?

[46] *Arkenbout, A, 2018. Long-term sampling emission of PFOS and PFOA of a Waste-to-Energy incinerator*

Mosses

Bryophytes are the non-vascular autotrophic cryptogams with the second-highest conglomeration among land plants after the angiosperms, and nearly 25,000 species were present worldwide (Mishra et al. 2016). Mosses belong to the kingdom Plantae, and division Bryophyta. Mosses are a vegetation group that have ‘rhizoids‘, small ‘hairlike’ structures with the main function of anchoring the plant to the ground, rock, bark or substrate, instead of a root system like plants and trees for the uptake of water, minerals, and possible contamination by (toxic) chemicals in the soil.

As part of the research, samples of mosses were taken on two occasions. In the summer most mosses are too dry, because of the high temperatures around Madrid. And because it is necessary to sample enough material, >100 gram/sample, by evaporation of water the moisture content needs to diminish in order to analyse it. A second sampling on September 12th was carried out at 8 moss locations around the incinerator, see Figure 37. After sampling and transport and shipping to the Netherlands, the samples of mosses were air-dried for 24 hours.

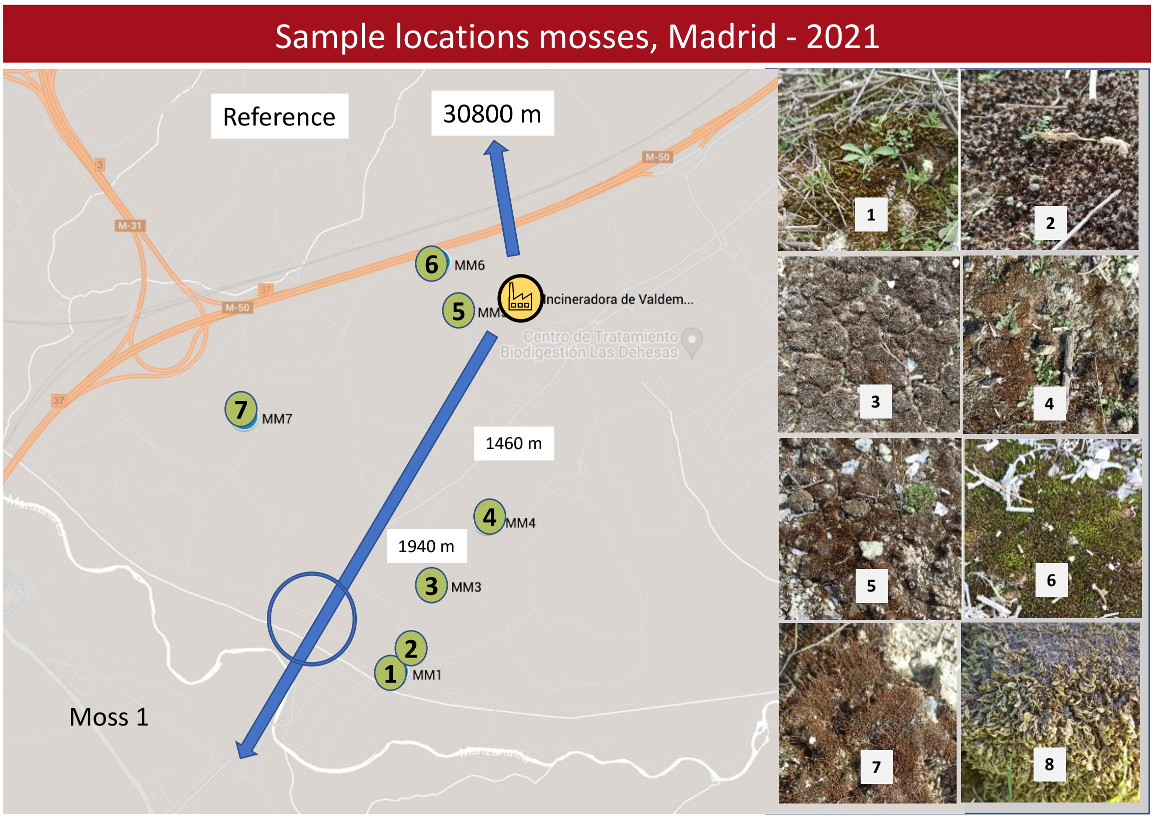

Figure 37: Sampling locations of mosses, Madrid 2021

A reference sample for the mosses was taken 20 km North of Madrid in Castillo de Viñuelas, a natural environment, see Figure 38. The same procedure was followed: after sampling, transport and shipping to the Netherlands, the samples of mosses were air-dried for 24 hours.

Reference location mosses Castillo de Viñuelas, Madrid, Spain 2021

Figure 38: Reference location of mosses, Madrid 2021

Dreyer (2018)[47] found PCDD/F TEQ concentrations ranged from 0.024 pg TEQ to 0.81 pg TEQ. Caraballeira[48] (2006) et al. reported PCDD/F TEQ concentrations of 0.3 pg TEQ/g (in woodlands), 2.5 pg TEQ in relation to an incinerator. Most of the mosses are < 1 pg TEQ/g. Danielsson[49]et al. (2016) observed PCDD/F concentrations in Swedish moss samples (*Pleurozium schreberi* or *Hylocomium splendens*) from 0.0001 to 0.57 pg TEQ/g. Generally, the concentrations of the analysed substances were very low, often close to or below the quantification limits (LOQ) for the dioxin analyses. They found a significant correlation between the concentrations of PAHs, dioxin/furans and dioxin-like PCBs in the mosses and the distance to the closest industry. The results of POPs in Madrid with the DR CALUX bioassay are much higher than in the mentioned literature. Also, the dl-PCB in the Dreyer study is always below 0.5 pg TEQ/g. In this study, results with high levels of 5.3 pg TCDD eq./g product have been measured.

The results of the dioxin analyses in the mosses show strongly elevated dioxins (PCDD/F) as well for the sum of dioxins (PCDD/F/dl-PCB), these are the highest levels in the TW indicative scale based on TW- biomonitoring researches in Europe 2019-2021, see Figure 39-44.

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[47] *Dreyer et al. Environ Sci Eur (2018) 30:43 https://doi.org/10.1186/s12302-018-0172-y*
[48] *Carballeira A, Angel Fernandez J, Aboal JR, Real C, Couto JA (2006) Moss: a powerful tool for dioxin monitoring. Atmos Environ 40(30):5776–5786*
[49] *Danielsson et al. (2016). Persistant organic pollutants in Swedish mosses, IVL-report C 188*

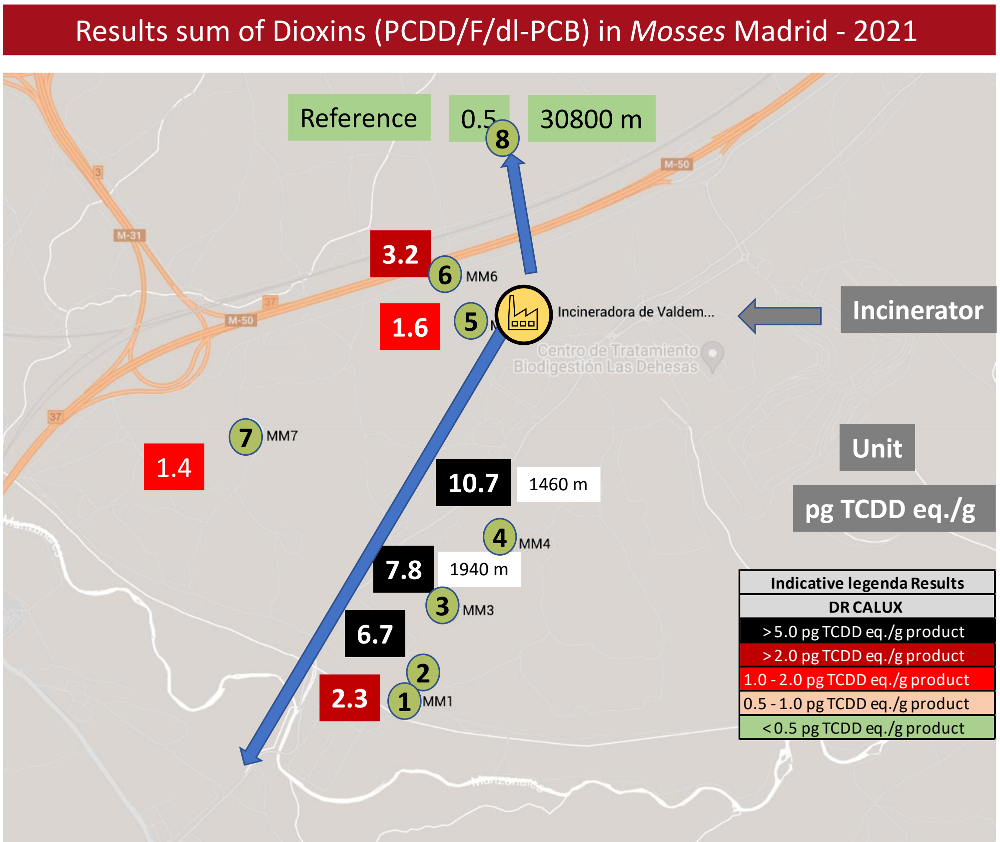


Figure 40:Sum of dioxins (PCDD/F/dl-PCB) in mosses using DR CALUX, Madrid 2021

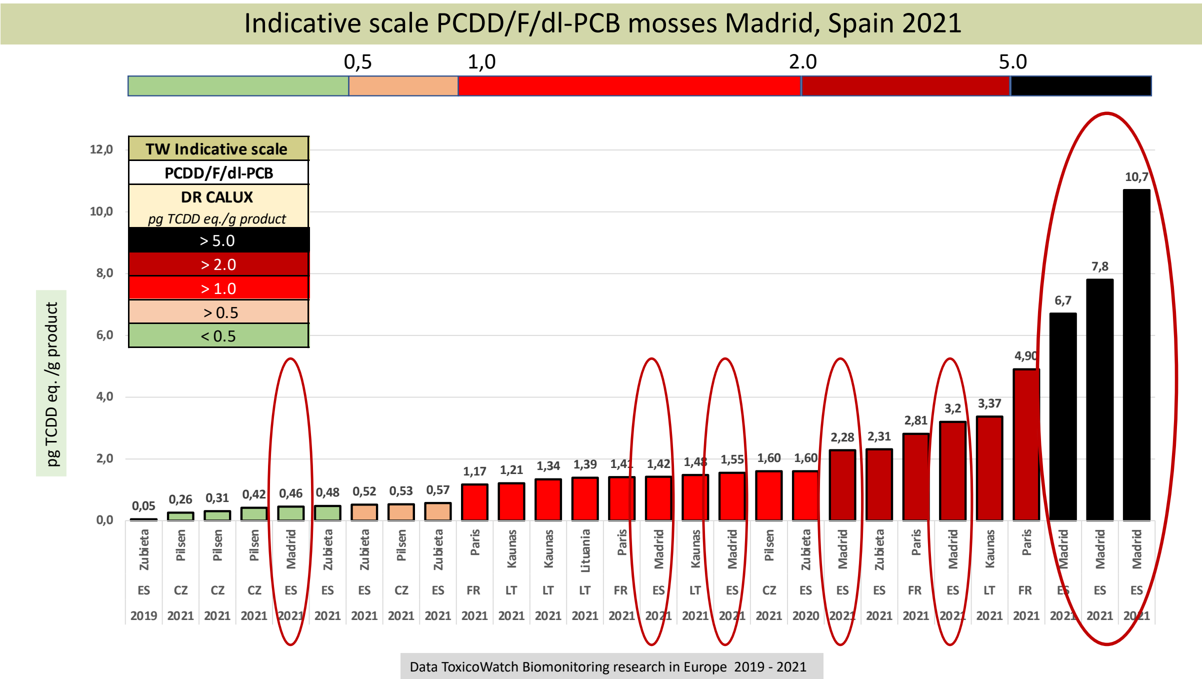


Figure 39: TW Indicative scale for PCDD/F/dl-PCB in mosses using DR CALUX, Madrid

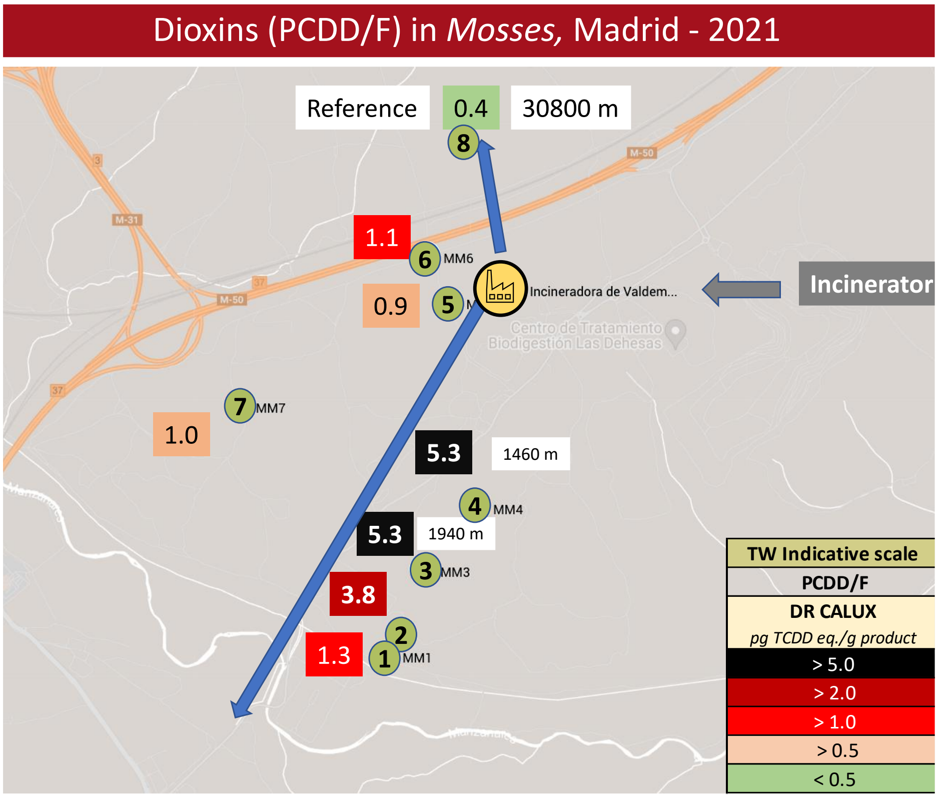


Figure 42: Dioxins (PCDD/F) in mosses, Madrid 2021

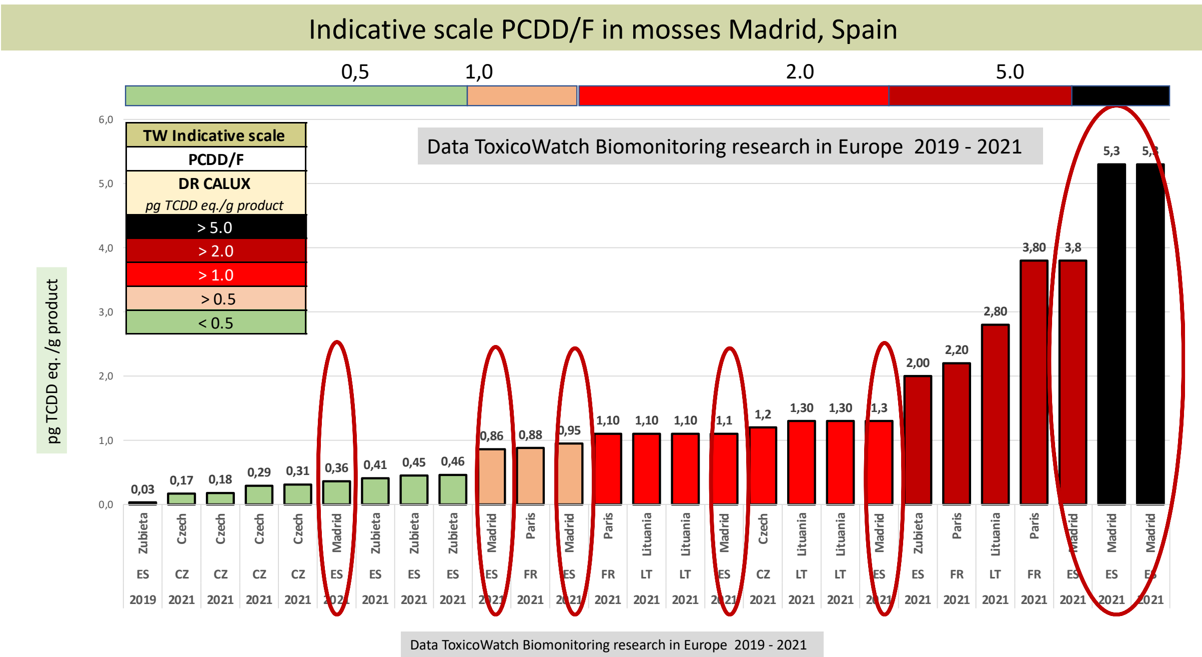


Figure 41: TW Indicative scale for PCDD/F in mosses, Spain 2021

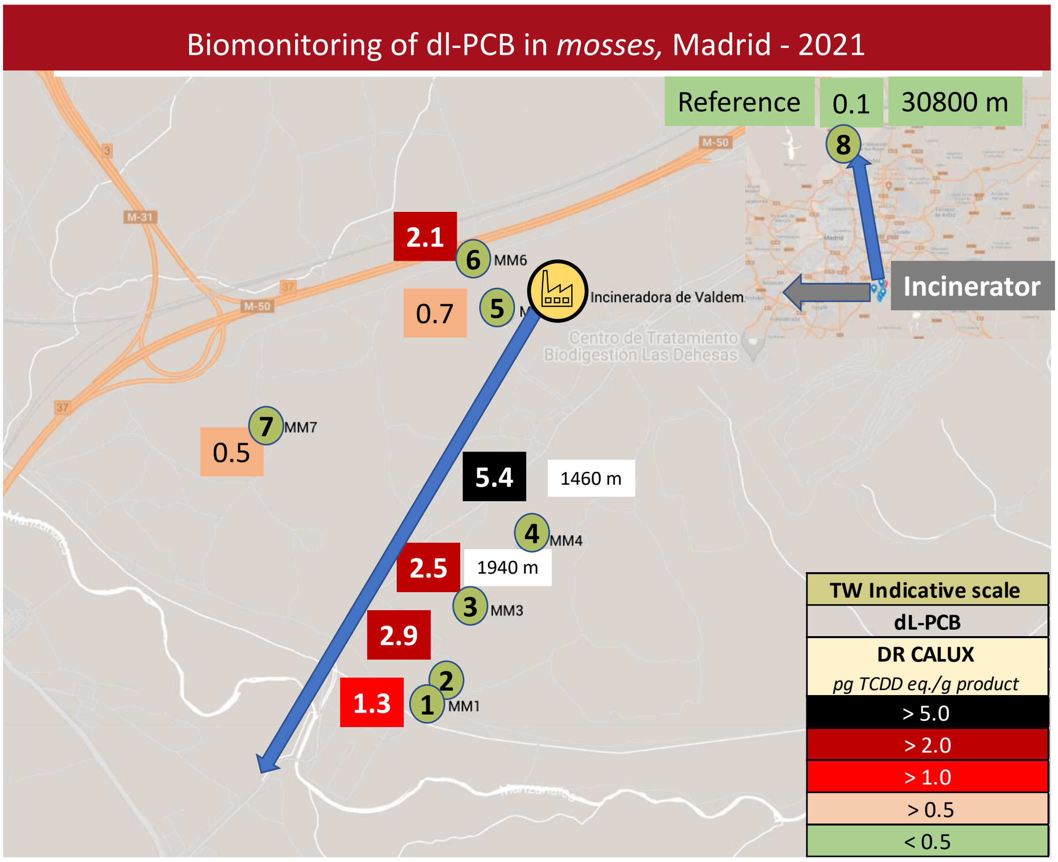


Figure 43: Results of dl-PCB in mosses, Madrid, Spain

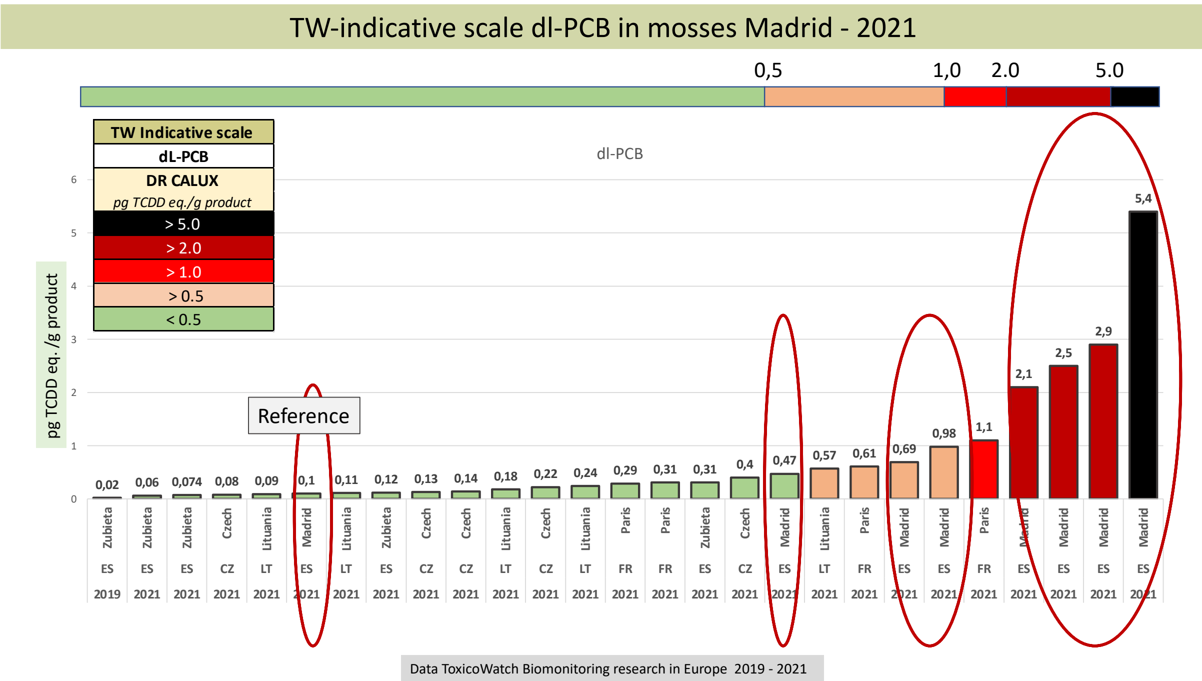


Figure 44: Indicative scale for dl-PCB in mosses, Madrid

Table 7 shows the analysis results in Mosses mentioned for dioxins (PCDD/F) and dioxin-like PCBs(dl-PCBs) separately.

Results Dioxins (PCDD/F/dl-PCB) in *Mosses* Madrid - 2021										
***Mosses*, Madrid 2021**						**Results *Mosses* madrid 2021**				
Sample nr	sample date	Biomarker	Weight (gr)	Wind direction	Distance (m)	TW-REF-NR	Veg nr	PCDD/F/dl-PCB	PCDD/F	dl-PCB
								DR CALUX	*(pg TCDD eq./g product)*	
1	1-11-2021	*Mosses*		S	2610	TW21-MD-M01	M01	2.28	1.30	0.98
2	1-11-2021	*Mosses*		S	2350	TW21-MD-M02	M02	6.70	3.80	2.90
3	1-11-2021	*Mosses*		S	1940	TW21-MD-M03	M03	7.80	5.30	2.50
4	1-11-2021	*Mosses*		S	1460	TW21-MD-M04	M04	10.70	5.30	5.40
5	1-11-2021	*Mosses*		W	400	TW21-MD-M05	M05	1.55	0.86	0.69
6	1-11-2021	*Mosses*		W	572	TW21-MD-M06	M06	3.20	1.10	2.10
7	1-11-2021	*Mosses*		W	2000	TW21-MD-M07	M07	1.42	0.95	0.47
8	1-11-2021	*Mosses*		N	30800	TW21-MD-M08	M08	0.46	0.36	0.10

Table 7: Results of dioxins (PCDD/F/dl-PCB) in mosses using DR CALUX, 2021

In a study conducted by Madrid Health (Madrid Salud) the impact on health of emissions from the Valdemingómez Technology Park was studied with the use of three active air samplers for analysing the air for dioxins (PCDD/F) and PAH[50]. The nearest sampler was placed at Ensanche de Vallecas Station about 5 km from the Technology Park. On the basis of this research, a map of PCDD/F/dl-PCB) distribution was constructed as shown in Figure 45. The profile looks the same, with the exception that in this study serious levels of dioxins are found in mosses collected south-west of the incinerator at a distance of 400–2,610 metres and in the study by Madrid Health with active air samplers no dioxins could be detected above the limit of quantification (LOQ).

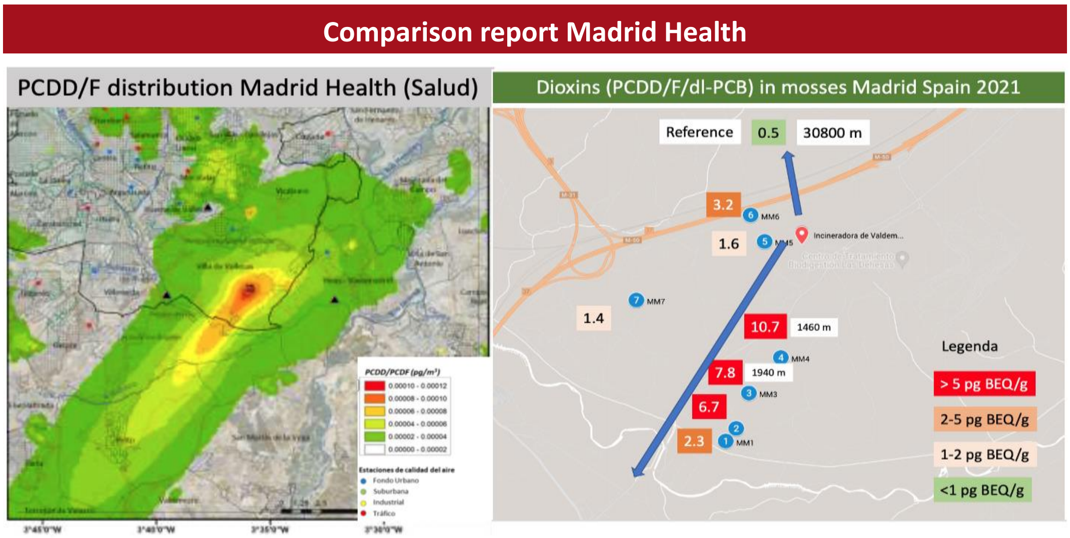


Figure 45 Comparison report, Madrid Health

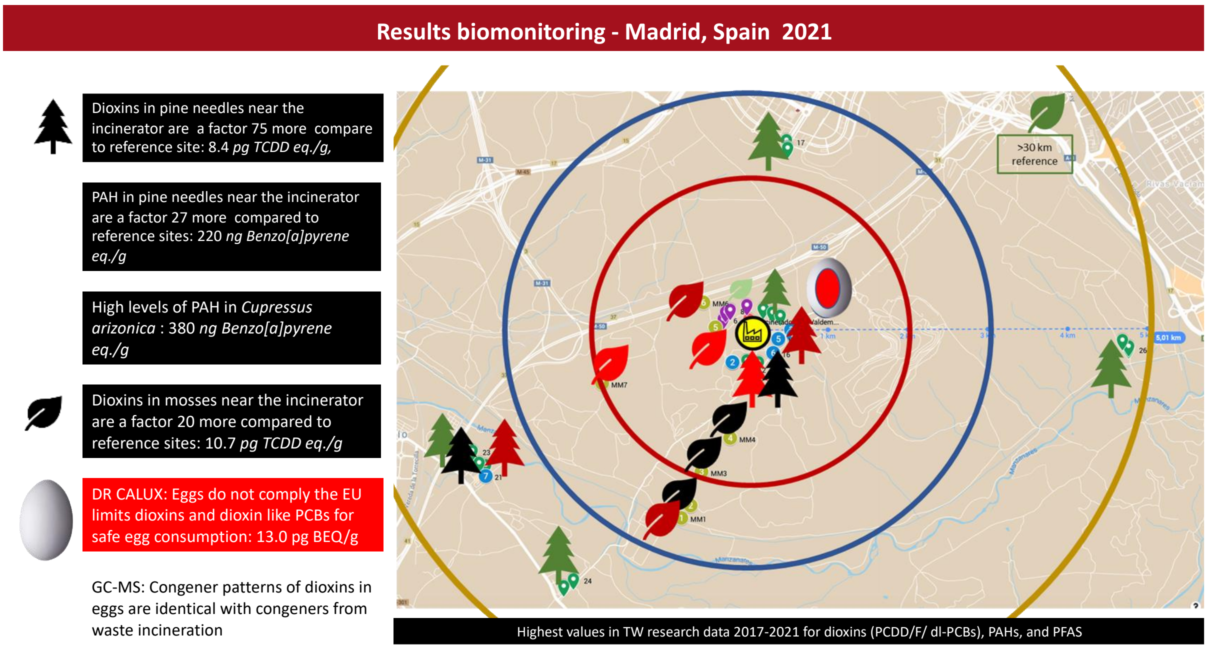
[50] *Estudio de evaluación de la incidencia en la salud de las emisiones procedentes del parque tecnológico de Valdemingómez, Madrid 2019*

Conclusion

The WtE (waste) incinerator of Valdemingómez in Madrid, Spain has been in operation since 1996. The first round of a this biomonitoring research on biomarkers of backyard chicken eggs, vegetation and mosses in the region around WtE incinerator has taken place in 2021. The follow up will take place in the next year 2022, by continuing this biomonitoring study at same locations in the area around the waste incinerator in Madrid.

The eggs of backyard chicken, a sensitive biomarker of pollution of substances of very high concern in the environment, show with the bioassay DR CALUX analysis that the eggs exceeds the EU action limits for food safety as regulated in the EU Directive 2013/711/EU. The chemical GC-MS analysis confirms these results with lower values for PCDD/F, strongly indicate brominated dioxins are part of contamination.

The results of the dioxin (PCDD/F/dl-PCB) analyses by DR CALUX of mosses demonstrate significantly increased dioxin concentrations at a distance of more than 2,650 metres South-West of the waste incinerator. The toxicity of the sum of dioxins (PCDD/F/dl-PCBs) in mosses, expressed in *TCDD eq./g product* for DR CALUX analysis exceeding with a factor 20 and a factor 50 for dioxin-like PCBs (dl-PCBs). High levels of dioxins (PCDD/F) and dioxin-like PCBs (dl-PCBs) are found in pine needles on only 500 meter distance of the waste incinerator. The results of the pine needles presents 75 times elevation of dioxins comparing to the reference location at a distance of 4710 meter. Remarkable are the high results of PFAS contamination in the Aleppo pine needles. These are the highest values of PFAS analysis in pine needles in this simultaneously performed biomonitoring research in Europe (Spain, Lithuania, Czech Republic in 2021). The PAH found in needles of Aleppo pine - *Pinus halepensis* is 10 times more than in the reference sample taken 4,000 metres away.



TW indicative scale vegetation			TW Indicative scale	TW Indicative scale	EU limit - Eggs		EU limit - Eggs	EU limit - Eggs	EU limit - Eggs
PCDD/F/dl-PCB	PCDD/F	dL-PCB	PFAS	PAH	PCDD/F/dl-PCB	PCDD/F	PCDD/F/dl-PCB	PCDD/F	dl-PCB
DR CALUX *pg TCDD eq./g product*	DR CALUX *pg TCDD eq./g product*	DR CALUX *pg TCDD eq./g product*	FITC-T4/PFAS CALUX *µg PFOA eq./g product*	PAH CALUX *ng Benzo[a]pyrene (B[a]P) eq./g product*	DR CALUX *pg BEQ/g fat*		GC-MS *pg TEQ/g fat*	GC-MS *pg TEQ/g fat*	GC-MS *pg TEQ/g fat*
> 5.0	> 5.0	> 5.0	> 50	> 500					
> 2.0	> 2.0	> 2.0	> 20	> 250					
> 1.0	> 1.0	> 1.0	> 10	> 100	≥ 3.3	≥ 1. 7	≥ 5.0	≥ 2.5	
> 0.5	> 0.5	> 0.5	> 5	> 10				> 1.75	> 1.75
< 0.5	< 0.5	< 0.5	< 5	< 10	< 3.3	< 1.7	< 5.0	< 1.75	< 1.75

Figure 46: Conclusion of biomonitoring, Madrid - 2021

In the foliage of *Cupressus Arizona*, the PAH concentration, expressed in Benzo[a]pyrene, is 12 times more than measured at the reference point.

The EU regulations urge for action on this egg location to find out the source of persistent organic pollutant contamination, in order to eliminate or at least do the utmost to reduce dioxins (PCDD/F) to a the minimum level. However, it should be noted that the EU standards are intended for the economic food market and are not primarily based on EFSA's solely health advice. The EU limits for eggs are based on a Tolerable Weekly Intake (TWI) of dioxins. The European Food and Safety Authority (EFSA) has adjusted this TWI by a factor of seven (7) by the EFSA in 2018. This health advise is still not yet implemented by governments in EU. Since private consumption of backyard chicken eggs can be high, this pose a serious health risk.

Measurements of the flue gasses could verify the fingerprints, congener patterns, found in the eggs and most important quantify the emitted POPs during Other Than Normal Operation Conditions (OTNOC). These results underline that the environment around Valdemingómez waste incinerator is under serious threat of high contamination of persistent organic pollutants (POPs).

The biomonitoring project will be continued in 2022.

ToxicoWatch

December, 2021

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Figures and tables

Annex I
Annex II
Annex III
Annex IV
Annex V