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Abstract

Environmental pollution is one of the most serious problems facing humanity and other life forms on our planet today. The characterization of chemicals spread in the environment, through the standard procedure of Environmental Risks Assessment (ERA) still evaluates the risk substance-by-substance. However, it is known that in the environment we are exposed to a mixture of chemicals coming from several different sources. In order to overcome such limitations of current ERA approach, more recently ERA highly recommended the usage of bioanalytical tools which is still emerging and not established in each European Member State. Within this thesis the suitability of bioanalytical tools in the ERA of industrial and agricultural areas, where mixtures of contaminants are present, has been demonstrated. In particular, the suitability of the *in vitro* cellular bioassay DR CALUX[®] (Dioxin Responsive, Chemically Activated LUCiferase gene eXpression) has been evaluated. DR CALUX[®] were able to detect the biological toxicity of topsoils collected from Taranto municipality (Apulia Region). Through a combined approach of bioassay and chemical analysis, the occurrence and spatial distribution of polyhalogenated dioxins, furans and *dl*-PCBs in topsoil samples were assessed. This approach not only confirmed already known sources (e.g. industrial air emissions) but also new ones (e.g. illegal disposal of electric transformers containing Apirolio[®] and asbestos cement-fiber plates). Considering the extension of illegal landfills in the Taranto municipality area, it is an urgent need to investigate potential sources and impact of *dioxin-like* compounds. Here we evaluated the suitability of DR CALUX[®] to support their application in future monitoring surveys. The risk represented by the extensive use of soils for agricultural and food purposes in Taranto area, further underlines the need to apply monitoring tools that quickly and extensively highlight the hazard posed by *dioxin-like* compounds in terms of human and environmental health. The high costs associated to extensive monitoring surveys find in the application of bioassays a successful alternative, able to identify new and unexpected sources and their route of exposure that would require long times and huge investments if only based on analytical chemical analysis. The possibility to couple proteomics to the DR CALUX[®] cells bioassay was also investigated in the present thesis. A case study was thus designed and tested by using selected topsoil samples to extrapolate differentially abundant proteins by proteomics and thus monitor other chemicals including those of emerging concerns (CECs) present in the soil sample able to activate specific cellular pathways. Moreover, differently abundant proteins spots found will subjected to functional and pathway analysis, in order to highlight potentially altered molecular pathways and suggesting potential link to human pathologies. The differential proteins identified were related to an altered unfolded protein response (UPR) inducing Endoplasmic Reticulum (ER) stress, oxidative stress, and altered signal transduction, transforming growth factor beta (TGF- β) signaling alteration. All these mechanisms are reported to be related to carcinogenic

mechanisms. The combined approach leads to estimate ecological and human risk by legacy and CECs as pharmaceuticals. The suitability of combining bioanalytical tools with chemical analyses in order to evaluate potential risks for both humans and environment, was demonstrated also in sewage sludge (SL) and hydrochar (HC) samples. A case study was designed to test urban SL and relative HC from six wastewater treatment plants (WWTPs), located in the Tuscany territory. DR CALUX[®] bioassay was able to detect the presence of dioxins and *dioxin-like* compounds in both SL and HC underlying differences probably related to hydrothermal carbonization process (HTC). Chemical analysis show that highly chlorinated congeners prevailed in SL compared to HC samples, in agreement with previous studies. HTC process seems responsible for such changes in the level of chlorinated congeners from high to low ones, which are more toxic. It can be assumed that the contamination of the HC is mainly due to a previous contamination of SL feedstock. The increase in the biological toxicity observed by DR CALUX[®] can be related to the de-chlorination of dioxins and *dioxin-like* compounds and the high loss of water which might affect their levels. DR CALUX[®] bioassay was able to screen the presence of dioxins and *dioxin-like* compounds in SL and HC, raising concern due to their possible applications as soil amendments. In the light of the above findings, it is possible to state that the DR CALUX[®] bioassay has been proven to be an effective and sensitive tool able to screen the presence of dioxins and *dioxin-like* compounds in various environmental matrices such as topsoil, sludge and hydrochar. Moreover, it has been proven to be an integrated state-of-the-art tool capable of assessing the real toxicity of complex mixtures of contaminants that occur in environmental samples. The combined application of DR CALUX[®] bioassay with chemical analysis and proteomics, opens new paths to the future of the Environmental Risk Assessment.

Abbreviation

2D: bidimensional gel electrophoresis

AERO: Aerobic wastewater treatment system

AhR: Aryl Hydrocarbon Receptor

ANA: Anaerobic wastewater treatment system

ARNT: *AhR* Nuclear Translocator

ASE: Accelerated Solvent Extractor

BEQ: Bioanalytical Toxicity Equivalent

BLAST: Basic Local Alignment Search Tool

CALUX®: Chemically Activated LUCiferase gene eXpression

CYP 1A: Cytochrome P450, family 1, subfamily A

d.m.: dry matter

d.w.: dry weight

DAVID: Database for Annotation, Visualization and Integrated Discovery

DE: Diatomaceous earth diatomite

dl-PCBs: *dioxin-like* Polychlorinated Biphenyls

D-Luciferin: (S)-2-(6-Hydroxy-2-benzothiazolyl)-2-thiazoline-4-carboxylic acid, 4,5-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid, Firefly Luciferin

DMSO: Dimethyl sulfoxide

DR CALUX®: Dioxin Responsive Chemically Activated LUCiferase gene eXpression

DRE: Dioxin Responsive Elements

ERA: Environmental Risk Assessment

FCS: Foetal Calf Serum

GC-FID: Gas Chromatography with Flame Ionization Detection

GC-MS: Gas Chromatography Mass Spectrometry

HC: Hydrochar

HPLC: High Pressure Liquid Chromatography

HTC: Hydrothermal Carbonization

MALDI-ToF: Matrix Assisted Laser Desorption Ionization - Time of Flight

NDL-PCBs: *non-dioxin-like* Polychlorinated Biphenyls

PAHs: Polycyclic Aromatic Hydrocarbons

PBDD: Polybrominated dibenzodioxins

PBDF: Polybrominated dibenzofurans

PBS: Phosphate Buffered Saline

PCB 118: 2,3',4,4',5-Pentachloro biphenyl

PCB 126: 3,3',4',4',5-Pentachloro biphenyl

PCBs: Polychlorinated biphenyls

PCDDs: Polychlorinated dibenzo-*p*-dioxins

PCDFs: Polychlorinated dibenzofurans

PM₁₀: Particulate Matter below 10 µm

QA/QC: Quality Assurance and Quality Control

REPs: Relative Effect Potencies

RLUs: Relative Light Units

SL: Sewage sludge

TCDD: 2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin

TEF: Toxic Equivalency Factor

TEQ: Toxic Equivalent

TEQ_{THEORETICAL}: Toxic Equivalent based on the REPs values from van Ede et al., (2013) specifically calculated *in vivo* in mice liver

TEQ_{WHO}: Toxic Equivalent based on the TEF values from Van den Berg et al., (2006) calculated *in vitro* and *in vivo* studies

TSQ: Triple Quadrupole

UniProt: Universal Protein Resource

WWTP: Wastewater Treatment Plant

α-MEM: Minimum Essential Medium, α-modification

Structure of the thesis

In the general introduction of this PhD thesis, the importance of bioanalytical tools to screen environmental samples and complex mixtures toxicity is reported, with a focus on the application of CALUX[®] (Chemically Activated LUCiferase gene eXpression) reporter gene bioassay.

The thesis is divided into three chapters. Each chapter consists of an introduction which sets out the main problems of the study and explain the innovative way proposed to solve them. A methodological section follows, with detailed methods developed according to the matrix analysed (topsoil, sewage sludge and hydrochar). The core of each chapter is expressed in the session of results and discussion, which explain the scientific validity of the bioassay results to evaluate a correct environmental risk assessment. A concluding paragraph summarizes the main results. Supplementary appendix with supplementary material is included. A special section to thank colleagues and Professors who supported each study is added. At the end of each chapter a references list is present.

Chapter 1 and 2 investigate surface soil samples (topsoil) collected from a large survey in the municipality of Taranto (Apulia Region), one of the most heavily industrialized and dioxin's polluted area of Southern Italy.

In chapter 1, an update on occurrence and spatial distribution of PCDD/Fs and *dl*-PCBs contamination of Taranto urban soils was obtained and the DR CALUX[®] bioassay was further promoted as a suitable screening tool for environmental and human risk assessment.

In chapter 2, the possibility to couple proteomics to the DR CALUX[®] cells bioassay was investigated in order to extrapolate CALUX[®] cells altered molecular pathways following samples extract exposure and predict other toxic pollutants eventually present in the sample extract itself. The screening performed by coupling DR CALUX[®] bioassay and differential proteomic approach, lead to estimate ecological and human risk by legacy and contaminants of emerging concerns as pharmaceutical by using a topsoil samples from a high industrialized area.

In chapter 3 the suitability of DR CALUX[®] bioassay to screen the presence of dioxins and *dioxin-like* compounds in sewage sludge and hydrochar samples was demonstrated. Moreover, the characterization of this matrices with bioassay allowed to bridge the gap of their potential reuse as a soil amendment.

Part of the work presented in this PhD thesis is under peer-review as follows:

☞ **Liberatori G.**, Cotugno P., Sturba L., Vannuccini M.L., Capasso G., Velardo R., Besselink H., Massari F., Tursi A., Corbelli V., Behnisch P.A., Corsi I. Occurrence and spatial distribution of dioxin

and *dioxin-like* compounds in topsoil of Taranto (Apulia, Italy) by GC-MS analysis and DR CALUX[®] reporter gene assay. Submitted to Chemosphere.

☞ Landi C., **Liberatori G.**, Cotugno P., Sturba L., Vannuccini M.L., Massari F., Miniero D.V., Tursi A., Behnisch P.A., Di Giuseppe F., Angelucci S., Bini L., Corsi I. First attempt to use proteomic analysis coupled with *in vitro* DR CALUX[®] bioassay as effect-based tool for monitoring soil pollution by legacy and emerging contaminants. Submitted to Journal of Hazardous Materials.

☞ **Liberatori G.**, Ferraro F., Mazzoli C., Sturba L., Vannuccini M.L., Baroni D., Vitolo S., Gori R., Corsi I. DR CALUX[®] bioassay as a diagnostic tool to evaluate the presence of dioxins, furans and *dioxin-like* polychlorinated biphenyls in Tuscany sewage sludge and hydrochar. In preparation.

Introduction

Environmental risk assessment

Pollution is the main environmental cause of disease and premature death, causing 9 million of deaths per year (16% of all deaths worldwide) through different exposure routes as air, water and soil (Landrigan et al., 2018). The process which identify and evaluate the adverse effects resulting from anthropogenic activities on the environment and human health is known as Environmental Risk Assessment (ERA). ERA is the combination of both ecological and human health risk assessment (Galatchi, 2006) and consists of four phases:

- *Hazard identification*: the determination of a chemical, physical or biological entity which could induce adverse effects on individuals, populations, communities or ecosystems;
- *Dose-response assessment*: the determination of the relationship between the magnitude of the exposure and the probability of occurrence of the effects;
- *Exposure assessment*: the estimation of the concentration to which environmental matrices are or may be exposed, but also sources, emission pathways and pathways of substance degradation;
- *Risk characterization*: the assessment of the incidence and the severity of a risk in an environmental matrix due to an actual or expected exposure to a chemical (Villeneuve et al., 2018).

The main aim of ERA is to identify a potential risk, obtain information for its management, and set up actions to avoid future ones. Among the instruments used by ERA to identify and characterize the risk, bioassays and biosensors have been developed and applied (Farrè and Barcelò, 2003; Ghosh et al., 2017; Kavlock et al., 2012; van der Oost et al., 2017; Vondráček et al., 2001). Bioassays are an important way of evidence approach since they provide relevant and direct measures of toxicity of a wide variety of contaminated media (Citterio et al., 2002). In addition, bioassays are useful in screening contaminants and understanding pathways, saving resources and helping to diminish uncertainties regarding biological availability (Xiao et al., 2006).

Complex mixture in complex matrices: the use of bioassay

Environmental risks of chemicals are still often assessed substance-by-substance, overlooking mixture effects, resulting in risk underestimations (Backhaus and Faust, 2012). In fact, we are not exposed to a single pollutant release by a single source, but to chemical mixtures coming from several sources (i.e., air, water and soil pollution, contaminated food, chemicals in and around our home, industrial and agricultural chemicals and pharmaceutical and personal care products which are

nowadays ubiquitous) (Zeliger, 2011). Environmental risk assessment of chemical mixtures is challenging, due to the infinite number of combinations which can occur (Kumari and Kumar, 2020). The effects of this complex mixture are difficult to predict, however, in the environment they naturally occur (Escher et al., 2020).

Many approaches with the aim to assess the risk of environmental chemical mixtures, are based on toxicity data generated from individual compounds (Heys et al., 2016). However, the assumption of mixture toxicity based on individual components can overestimate or underestimate the negative effects on organisms and the risk for human health (Konig et al., 2017; Heys et al., 2016). Chemical analysis in fact do not take into account any synergistic effects that arise between the various molecules, as well as the influence of compounds below the instrumental detection limit, but which can still contribute to the toxicity of the mixture (Kortenkamp & Faust, 2018). Consequently, failure to accurately predict biological effects can cause damage to wildlife and ecosystems. Moreover, the main problem in the use of only classical analytical methods is that they do not provide information on the bioavailability of compounds and consequently can fail the evaluation of the real toxicity of complex mixtures (Behnisch et al., 2001).

Traditionally, the toxicity of complex environmental matrices has been conducted in whole-organism *in vivo* bioassays (Escher et al., 2020). This type of assay, however, has proved over the years to be little predictive in terms of human toxicity (Attene-Ramos et al., 2013; van Ede et al., 2016) and inadequate due to the high costs and the large number of living organisms necessary for the characterization of the different chemical compounds presents (Eggen and Segner, 2003). The inadequacy of *in vivo* analyses has emphasized the application of the *in vitro* ones, which nowadays represents a suitable solution for testing environmental samples (Escher et al., 2020). The science of high-throughput screening has progressed significantly in the past 15 years, accelerating the assessment of ecological risk and implementing the monitoring of complex mixtures reducing animal testing (Villeneuve et al., 2018).

In particular, among *in vitro* analysis, cellular bioassay was identified as one of the best ways to characterize the toxicological relevance of environmental samples, by measuring the combined biological responses obtained following cell exposure to a complex mixture of contaminants (Kolkman et al., 2013). The *in vitro* bioassays sensitivity and robustness make them ideal screening tools for environmental risk assessment, applicable to a wide category of matrices. In addition, advances in biotechnology have made possible to develop a series of specific assays which respond selectively to certain mechanisms of action.

Effect-based methods have been suggested as complementary to analytical chemical analysis of complex patterns of environmental pollutants, in order to help to identify unresolved effects and

compounds (Drakvik et al., 2020; Escher et al., 2020). Effect-based monitoring studies have been performed and recommended in environmental risk assessment (Altenburger et al., 2018; Escher et al. 2018; Neale et al., 2017; van der Oost et al., 2017) and bioanalytical tools proposed as a quantitative measure of mixture effects toxicity (Escher et al., 2020) (Fig. 1).

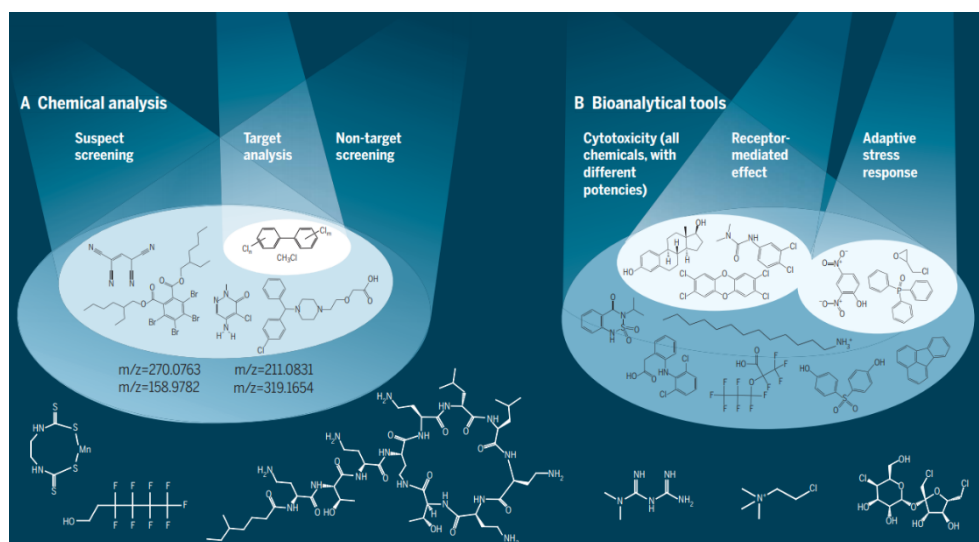


Fig. 1 Spotlight on the chemical universe. (A) Chemical analysis (B) Bioanalytical tools. From Escher et al., (2020).

Bioanalytical tools can integrate analytical analysis with toxicological information. Moreover, many of them are in microscale, therefore, they adopted cell lines from well-known higher model organisms, reducing animal testing and time and costs of conventional analytical chemistry if applied as a screening tool (Eggen and Segner, 2003).

Finally, bioassays can be useful to measure not only the total toxicologically relevant burden of chemicals (Heinrich et al., 2017; Schiwy et al., 2015) but also to detect emerging contaminants in the natural environment (Neale et al., 2015).

CALUX® (Chemically Activated Luciferase gene eXpression) *in vitro* bioassay

In order to protect and preserve the quality of the environment, but also human health and food, effective, specific, sensitive and robust screening tests are currently available.

Recently developed *in vitro* bioassays named CALUX® (Chemically Activated LUciferase gene eXpression) (www.bds.nl) have been engineered in order to emit a light signal directly proportional to the quantity of certain contaminants, singles or mixtures, to which they are exposed, making this specific bioassay biologically relevant. There are currently patented different types of CALUX® cell lines, which allow to identify more than 30 different biological effects including: endocrine

dysfunction, acute stress, genotoxicity, carcinogenicity, xenobiotic metabolism and reproductive toxicity (van Vugt-Lussenburg et al., 2014; www.bds.nl).

Among CALUX[®], modified rat (H4IIE) hepatoma cells, transfected with the *AhR*-controlled luciferase reporter gene construct (pGudLuc), responding specifically to the stimulation of dioxins and *dioxin-like* compounds named DR (*Dioxin Responsive*), is currently available (Aarts et al., 1995; Garrison et al., 1996). DR CALUX[®] assess the specific biological response upon the presence of agonists and antagonists of *Aryl Hydrocarbon Receptor* (*AhR*).

It is well-known that dioxins and *dioxin-like* compounds are all characterized by a planar structure, which allows them to bind a cytoplasmic receptor (*AhR*) (Giani Tagliabue et al., 2019). After the binding, the complex substance-receptor undergoes dimerization with an *AhR* Nuclear Translocator protein (ARNT), that migrates into the nucleus of the cell and binds to a specific DNA sequence called Dioxin Responsive Elements (DRE). Generally, the binding of the substance-receptor complex in the DRE regions of the DNA, activates the expression of the genes encoding for the subfamily of the cytochrome P450 1A (CYP1A) (Beischlag et al., 2008).

In the specific case of DR CALUX[®], the gene encoding for Luciferase enzyme is transcribed and by incubating with substrate Luciferine, there is a light signal, which is captured by the Luminometer (Hoogenboom et al., 1999; Hilscherova et al., 2000) (Fig. 2).

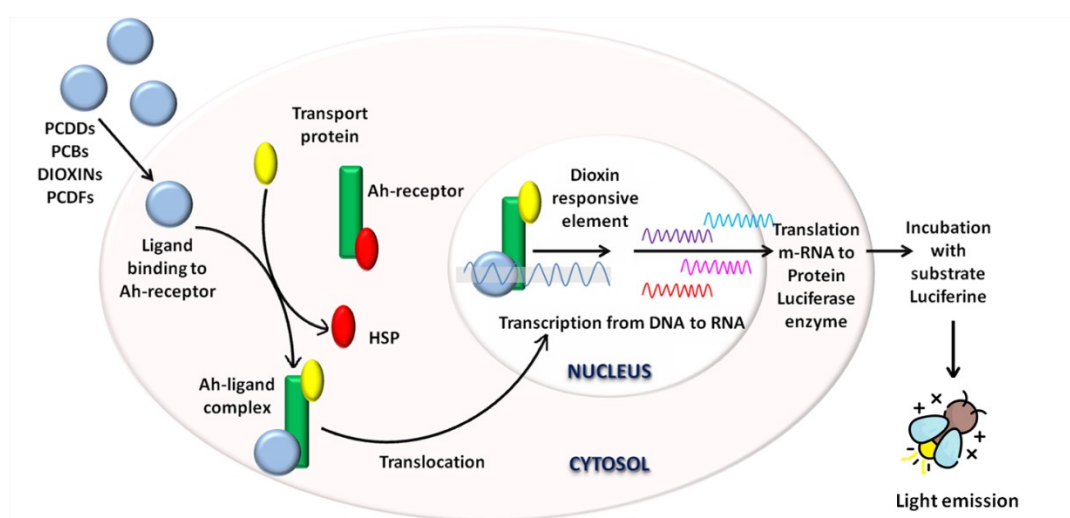


Fig. 2 Schematic representation of DR CALUX[®] bioassay principle. Adapted from www.bds.nl.

The amount of light determined is subsequently derived through a calibration curve of a reference compound, the 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and finally expressed as Bioanalytical Toxicity Equivalents (BEQs).

The DR CALUX[®] BEQ, are calculated on the basis of chemical compounds affinity to bind *AhR*. Therefore, BEQs express the biological activity of mixture of contaminants, integrating the activities of the individual congeners and the possible synergistic/antagonist action (Behnisch et al., 2001).

Toxic Equivalent (TEQ) values from GC-MS/MS analysis, are calculated as the sum of the products of the concentration of each compound multiplied by its Toxic Equivalency Factor (TEF) value which although calculated on their potency to bind *AhR* (affinity), they do not take into account the possible synergistic/antagonist action (Van den Berg et al., 2006).

The DR CALUX[®] bioassay has many advantages compared to the classic analytical method. Firstly, the costs of the analysis, which are lower compared with the classical chemical investigation as well as the time spent in their performance, since with bioassay it is possible to carry out a large number of samples in a very short time. Moreover, with bioassay it is possible to avoid *in vivo* experiment using animal in accordance with the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Being a bioassay that answer all the requirements for environmental safety assessment, DR CALUX[®] can be used as a screening tools to identify hotspots, correlate polluted sites to human health, identify the specific effects of pollutants and their environmental distribution.

The use of DR CALUX[®] cell lines have been applied in environmental human and animal health risk assessment as well as on food quality (Brouwers et al., 2011; Hoogenboom et al., 2016, De Baat et al., 2019; Escher et al., 2018; van der Linden et al., 2008).

In Italy, however, up to now their application has been limited to quality and safety assessment of food and feed (Fochi et al., 2008; Gasparini et al., 2011; Sciuto et al., 2018, 2013) and there is only few study on human plasma (Warner et al., 2005) and serum (Porpora et al., 2009).

DR CALUX[®] bioassay has been validated by European Law 252/2012 and 278/2012 as one of the best biosensors able to implement a screening process for the assessment of dioxin and *dioxin-like* compounds in feed and foodstuff. In addition, DR CALUX[®] was used as standardized tests to several international standards, such as Dutch Specie, Japanese Industrial Standard (JIS) or United States Environmental Protection Agency (US EPA) for soil and sediments.

Moreover, DR CALUX[®] offers the possibility of repeatability of the analyses and has been validated and accredited according to ISO/IEC 17025 for the detection of PCDD/Fs and *dl*-PCBs in feed and food (Scippo et al., 2006).

Therefore, in order to overcome the current limitations in environmental risk assessment, (e.g., test a large number of chemicals, a large number of samples, assess the potential toxicity of a complex sample, reduce costs and time spent on analyses, acquire detailed data necessary to assess human risk and help decision-makers), an intelligent combination of analytical tools and biological assays can in

this sense provide substantial information on the relative effects of these toxic compounds, representing an important revolution in environmental monitoring (Escher et al., 2020).

Reservoirs sources of dioxin and dioxin-like compounds in the environment

Dioxins and *dioxin-like* compound are ubiquitous, widespread and persistent environmental contaminants and have been found worldwide in all primary (air, soil, water, sediment) and secondary (food, meat, dairy products and consumer goods) media (Mukerjee, 1998). Annual global dioxins production/emission has been estimated as 17,226 kg (287 kg TEQ), of which nearly 57% is deposited on soil, 40% on water and 3% remained in the atmosphere (Booth et al., 2013).

Their soil deposition and consequently their consumption by plant, animal and animal products is the primary route from which dioxins enters in human food chain (Hoogenboom et al., 2016; Perugini and Zezza, 2020) (Fig. 3). Correlated to the emission in the atmosphere, also the mismanagement of hazardous wastes in dumping sites and their fires could significantly increase air emission of Polychlorinated-dibenzo-*p*-dioxins (PCDDs), Polychlorinated-dibenzofurans (PCDFs) and *dioxin-like* Polychlorinated-biphenyls (*dl*-PCBs) and consequently contribute to soil pollution (Herrero et al., 2020; Ferrante et al., 2017; Weber et al., 2018). Moreover, the spillage of oils containing a mixture of *dl*-PCBs congeners used as dielectric fluids in transformers (Liberti et al., 1992; Sun and Boyd, 1991) or the deterioration of asbestos panels abandoned by the self-demolition waste, could significantly contribute to their direct release in the soil surface (Bayram and Bakan, 2014; Swartjes and Tromp, 2008).

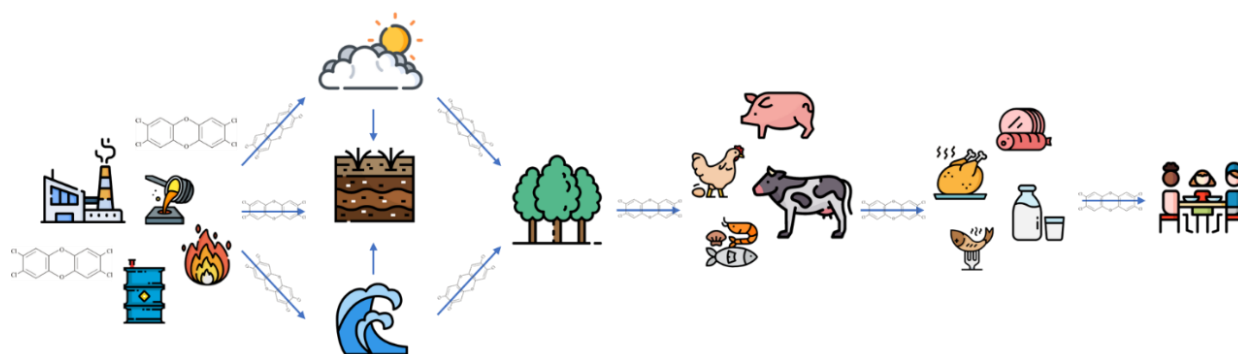


Fig. 3 Atmospheric exposure route of dioxins into terrestrial food web: emission sources, deposition, bioaccumulation/uptake, ingestion by animal, ingestion by humans.

In addition to atmospheric deposition, dioxins can enter in contact with soil, with the direct application of matrices not free of contamination such as sewage sludge or their by-products (biochar, hydrochar, digestate, compost) used as soil amendments (Elskens et al., 2013; Engwall and Hjelm, 2000; Godlewska et al., 2020) (Fig. 4).

In fact, sewage sludge applications on agricultural land might contribute to the dispersal of a broad range of unwanted contaminants such as metals, organic compounds and pathogens on soils, posing environmental but also sanitary risks (Andreoli et al., 2007; Seleiman et al., 2020).



Fig. 4 Alternative ways of dioxins to entry in the food web: bioaccumulation/uptake, ingestion by animal, ingestion by humans.

Due to their toxicity and persistence, dioxins in soils pose health risks to both humans and wildlife (Yuan et al., 2021). Dioxins strongly adhere to soils and sediments and their low vapor pressure, low water solubility and strong ability to adhere to particulates generally ensures their immobility in soils and sediments (ATSDR, 1998; Mandal, 2005; White and Birnbaum, 2009). Their persistence occurred especially in the surface layers, which keep the 80% of the total PCDDs and PCDFs of the soil (Brzuzy and Hites, 1996). Its maturity is estimated from 9 to 15 years, while the estimated half-life for the deepest layers range from 25 to 100 years (ATSDR, 1998). The soils are thus conservative collectors for dioxins and, due to their limited removal and long half-life, represent a typical accumulating matrix (Brzuzy and Hites, 1996). The average background levels of dioxins in soils is estimated as 1 ng/g, most of which is due to the congener Octachlorodibenzo-*p*-dioxin (OCDD) (Demond et al., 2012).

Contamination of soils is challenging in almost every country and calls for appropriate Environmental Risk Assessment procedures. Soil pollution and its degradation are in fact very important phenomena that reflect not only the quality of the environment but also human health and the economy. The contamination of this important matrices, even low, can in fact contribute to the bioaccumulation and bioconcentration of organic compounds from soil to animals, animal products and plants (Stephens et al., 1995; Hoogenboom et al., 2016; Sharma et al., 2007).

In 2006, the European Commission adopted the Thematic Strategy for Soil Protection, a policy report on the implementation of the strategy and ongoing activities and a proposal for a framework Directive and an Impact Assessment (COM(2006) 231). The Soil Thematic Strategy explains why further action is needed to ensure a high level of soil protection, what kind of measures must be taken, sets

out common principles for protecting soils across the European Union and set up details analysis for the economic, social and environmental impacts which may occur.

At present, the Italian National Legislative Decree 152/2006 (Testo Unico Ambientale), set acceptable limit values for the sum of PCDDs and PCDFs, depending on the specific use of soil. In details are emended a limit for green, private and recreational use of soil (10 ng TEQ/kg dry weight d.w.) and a limit for commercial and industrial use of it (100 ng TEQ/kg d.w.). An additional specific limit for the sum of all PCBs congeners (60 µg/kg d.w.) was also set.

A new national limit has been recently added within the Italian National Ministerial Decree D.M. 46/2019, which established for the sum of PCDD/Fs and *dl*-PCBs a limit equal to 6 ng TEQ/kg d.w. for agricultural and farming soils.

Concerning sewage sludge and its application as soil amendments, the Decreto Genova (art. 41 of Legislative Decree 109/2018) poses the limit of 25 ng/kg TEQ_{WHO} d.w. for PCDD/Fs and 0.8 mg/kg d.w. for total PCBs. Sludge can be applied on soils in doses not exceeding 15 t/ha of dry matter over a three-year period as enounced in the European Law 86/278/CEE and issued in the Italian National D.Lgs. 99/1992. Currently no limit was imposed to the admissibility values of organic and/or inorganic contaminants in hydrochar, the hydrothermal carbonization by-product of sludge. Being an important carbonaceous matrix, hydrochar could be used as well as treated sewage sludge as a sustainable fertilizer (Tasca et al., 2019). In Italy, the use of hydrochar as a soil amendment is not yet allowed, however, numerous experimental researches are underway for test its actual validity.

Research objectives

In the previous paragraphs, it was highlighted that the application of bioanalytical tools toward environmental risk assessment is highly recommended, however, their use for the monitoring of the complex environmental mixture is only emerging (Villeneuve et al., 2018). Therefore, their great potential to accelerate risk assessment has to be enlarged, especially in matrix highly affected by pollution as soil, which could determine not only ecosystem risk but could also affected human health and economy.

Considering the limited knowledge and applications of the DR CALUX® bioassay which occur nowadays in Italy, this PhD project aims to promote their application on environmental samples, revolutionizing environmental monitoring if combined with chemical analysis. The usage of bioassay has to be encouraged in the future also in our country, as a main character in the ERA process.

For these reasons, the objectives of the present PhD thesis are:

- 1) To promote the usage of DR CALUX[®] bioassay as a suitable screening tool for environmental and human risk assessment through its ability to characterize the occurrence and spatial distribution of PCDD/Fs and *dioxin-like* compounds in topsoils of Taranto (Apulia Region).
- 2) To promote the possibility to couple DR CALUX[®] bioassay with proteomics, in order to extrapolate cells altered molecular pathways and predict other toxic pollutants in addition to PCDD/Fs and *dl*-PCBs, as the emerging ones, which could occur in environmental samples.
- 3) To promote the effectiveness of the DR CALUX[®] bioassay in assessing the presence of PCDD/Fs and *dioxin-like* compounds and their biological toxicity in sewage sludge and hydrochar samples, given their possible sustainable recycle as soil amendment.

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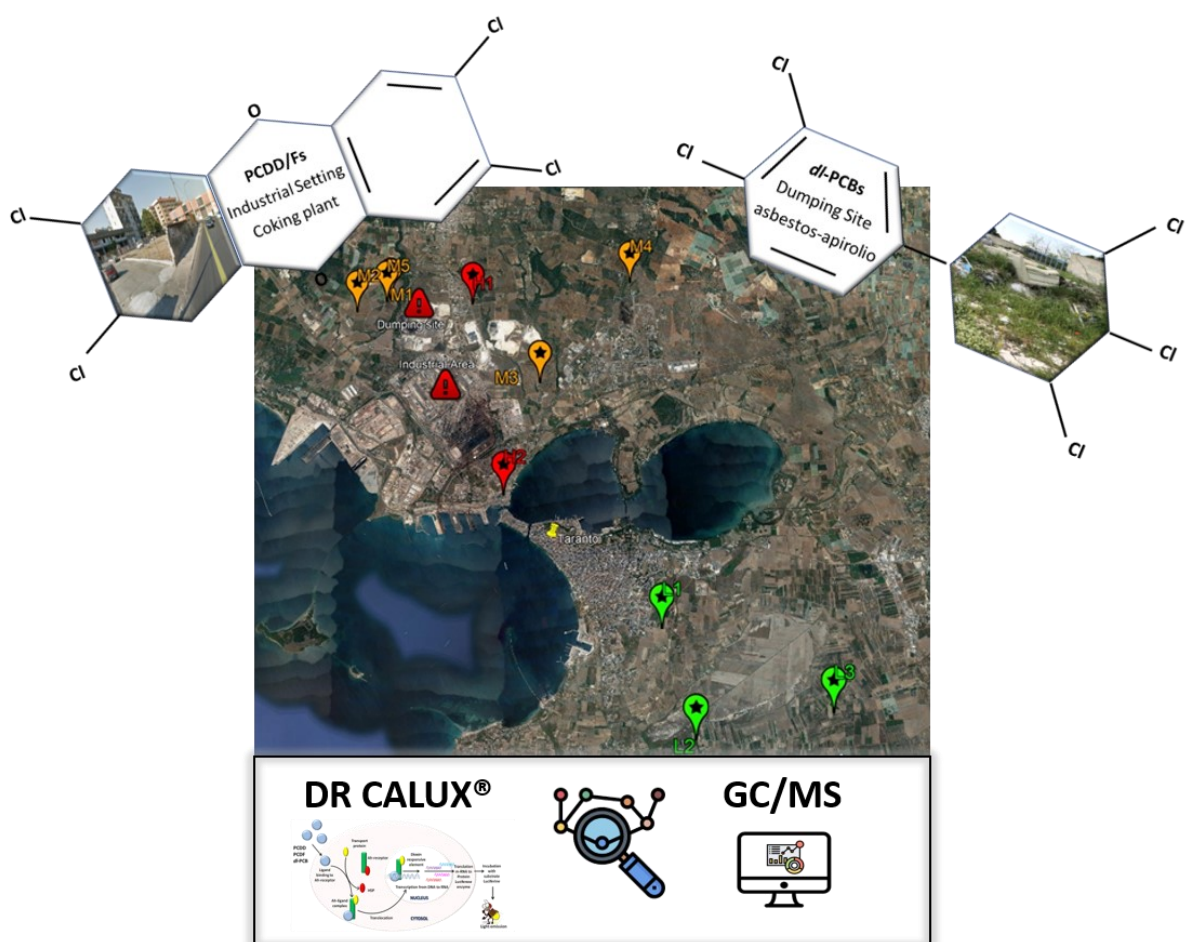
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CHAPTER 1



Occurrence and spatial distribution of dioxin and *dioxin-like* compounds in topsoil of Taranto (Apulia, Italy) by DR CALUX[®] reporter gene assay and GC/MS analysis



Abstract

The aim of the present chapter was to assess the occurrence and spatial distribution of PCDD/Fs and *dioxin-like* compounds in topsoils of Taranto (Apulia Region), one of the most heavily industrialized and dioxin's polluted area of Southern Italy. A combined approach of chemical analysis by GC-MS/MS and DR CALUX[®] reported gene bioassay was applied in a subset of topsoil samples (n=20) collected in 2017-18 from ten sites embracing three levels of risk (from high to low) in the framework of a large survey inside Taranto municipality. DR CALUX[®] BEQs and GC-MS/MS TEQ_{WHO} and TEQ_{THEORETICAL} revealed a decreasing trend with the distance from main industrial settings and landfill areas. A strong correlation between DR CALUX[®] BEQs and TEQ_{WHO} values ($R^2 = 0.85$) and TEQ_{THEORETICAL} ($R^2 = 0.88$) was also found. In 3 out of 10 topsoil investigated, BEQs and TEQ_{WHO/THEORETICAL} resulted above Italian National Regulatory Limits for $\sum$ PCDD/Fs in green, private and recreational used soils (10 ng TEQ/kg d.w. D.Lgs 152/2006) and for $\sum$ PCDD/F/*dl*-PCBs in agricultural and farming soil (6 ng TEQ/kg d.w. D.M. 46/2019). GC-MS/MS pattern revealed the highest prevalence of *dl*-PCBs in 6 out of 10 sites, followed by PCDFs and PCDDs. Those sites are all located in proximity of main industrial steel and iron ore sinter plant, steel plant's landfills and illegal dumping sites. An update on occurrence and spatial distribution of PCDD/Fs and *dl*-PCBs contamination of Taranto urban soils was obtained and the DR CALUX[®] bioassay was further promoted as a suitable screening tool for environmental and human risk assessment.

1.1 Introduction

In the last 20 years, the causal relationship between soil contamination and human health-related diseases in areas located near industrial settings and illegal waste dumping sites has been widely documented in Italy (Esposito et al., 2018; Maifredi et al., 2011; Mazza et al., 2018; 2015; Pascuzzi et al., 2013). Since the '90s, the town of Taranto (Apulia Region, Southern Italy) has been recognized as one of the worst-case scenarios in which industrial air emissions and high incidence of diseases in inhabitants were documented (ARPA Puglia, 2013; Pirastu et al., 2013, 2014; Zona et al., 2019). Air pollution mainly originates from a large steel and iron ore sinter plant operating in Taranto since the '60s and from other industrial plants not all belonging to the metal-sector as oil refinery, cement factory and incinerators, all located in the North-West of the urban area (Esposito et al., 2010, 2012, 2014). Based on past monitoring studies on exhaust gas emissions, they have been recognized as the main responsible for the total national emissions of carcinogenic compounds as PCDDs, PCDFs and *dl*-PCBs (Esposito et al., 2010; Giua et al., 2009; Liberti, 2014; Quass et al., 2004).

Despite soil is not directly involved in industrial activities, it represents a final sink of airborne PCDD/Fs and *dl*-PCBs bulk dust deposition associated to exhaust gas emissions (Domotorovà et al., 2012). Because of their low mobility, high affinity to organic carbon and persistence, PCDD/Fs and *dl*-PCBs end up and accumulate on topsoil through fall out events (dry and wet) (Melnik et al., 2015). This represents the main route for PCDD/Fs and *dl*-PCBs to enter into the terrestrial food chain either through direct ingestion of deposited dusts or contaminated food products (Fernández-González et al., 2015; Hulster et al., 1993; SCF, 2001; Weber et al., 2015, 2018).

According to the recent estimates, more than 80% of human exposure to PCDD/Fs and PCBs is *via* food and food-producing animals as for instance from the consumption of meat, dairy and eggs (Weber et al., 2019 and reference within). Animals farmed on contaminated soils can accumulate PCDD/Fs and *dl*-PCBs through ingestion of contaminated feed, vegetation and roughage being directly exposed to the air (Turrio-Baldassarri et al., 2007, 2008; Weber et al., 2018). In a survey conducted in 2008-2009, levels of PCDD/Fs and *dl*-PCBs resulted above the EU limits in edible tissues of sheep and milk samples from goat, both farmed in proximity of Taranto industrial area (Scortichini et al., 2016). In a previous study, livestock and grassland samples from Taranto's industrial area were also found contaminated by PCDD/Fs (Diletti et al., 2009).

Soil contamination will therefore increase the risk of PCDD/Fs and *dl*-PCBs exposure to Taranto's inhabitants due to the consumption of contaminated locally farmed animal products (EFSA, 2018).

Furthermore, in the last 20 years illegal landfilling of hazardous wastes, including those of industrial origin, has been discovered inside the Taranto municipality territory and it could represent a potential further source of PCDD/Fs, *dl*-PCBs and other cancerogenic compounds contamination in soils.

Recent findings on PCBs, including *dioxin-like* congeners released by weathered coating materials of asbestos-cement fiber plates raise concern since their presence in Taranto's landfill sites have been documented (Winkler et al., 2015). Moreover, abandoned old electrical transformers containing a PCBs-based cooling liquid named Apirolio® (Italian brand) produced in the '70 have been also recently documented in those sites probably due to the unknown hazardous consequences of such disposal in the past (Ancona et al., 2019; Comba et al., 2012; Di Lenola et al., 2018; Zona et al., 2019). Mismanagement of those hazardous wastes and the documented accidental illegal fires could significantly increase air emissions of PCDD/Fs and *dl*-PCBs and soil contamination with increasing health risk for the Taranto inhabitants living nearby (Herrero et al., 2020; Weber et al., 2018). In humans, carcinogenic and *non*-carcinogenic adverse effects have been reported upon exposure to dioxin and *dioxin-like* compounds (Schechter et al., 2006). There is a significant number of epidemiological studies which revealed a higher mortality in Taranto inhabitants from various causes (e.g. malignant neoplasms, neurological, cardiac and respiratory diseases) compared to the rest of the Apulia Region and not only confined to neighborhoods closest to the main industrial area (Vigotti et al., 2007, 2014).

An association between industrial emissions of PM₁₀ (Particulate Matter below 10 µm) and natural mortality mainly due to respiratory causes, has been documented and more consistent in the Taranto's elderly population (Leogrande et al., 2019 and reference within). The onset of neoplastic diseases with cases of *non*-Hodgkin lymphoma and soft tissue sarcomas including also a high incidence of deaths related to occupational exposure have been also reported in the last 15 years (Giua et al., 2005; Graziano et al., 2009; Martinelli et al., 2012; Pirastu et al., 2014). In a recent study by Lucchini et al. (2019), a higher incidence of neurocognitive outcomes among school age children living in closest proximity of sinter core plant in Taranto has been reported and linked to emissions of neurotoxic metals. The last *SENTIERI Report* (National Epidemiological Study of Territories and Settlements Exposed to Pollution Risk) describes a significant epidemiological emergency related to the dramatic number of malformations in infants born in Taranto between 2002 and 2015 (> 600) (Zona et al., 2019). Evidences of maternal transfer of PCDD/Fs to neonates *via* breast feeding have been also documented (Bianco et al., 2013).

Pollution monitoring historically relies on analytical chemistry able to detect toxic compounds in all environmental matrices. However, the time- and high cost-intensive confirmatory chemical analysis of PCDD/Fs and *dl*-PCBs and the current demand due to the unforeseen impact of potential further sources of dioxins and *dioxin-like* compounds in Taranto territory, calls for reliable screening tools able to predict and protect populations from future dioxins contaminations (Behnisch et al., 2001a, 2020). *In vitro* bioassays can be used for testing the presence of a single as well as a mixture of

pollutants in both human and environmental samples for a better understanding of real exposure scenarios and able to predict potential risks (Escher et al., 2020). The *in vitro* bioassay DR CALUX[®] (Dioxin Responsive Chemically Activated LUCiferase gene eXpression), has been widely validated as a reliable, sensitive and low-cost screening tool to detect *AhR* (*Aryl hydrocarbon receptor*) binding chemicals such as PCDD/Fs and *dl*-PCBs including polycyclic aromatic hydrocarbons (PAHs) (www.bds.nl). The DR CALUX[®] uses the pathway mediated by the *AhR*, a ligand-dependent transcription factor induced by binding dioxins and *dioxin-like* chemicals, and responsible for mediating the toxicity of these compounds (Aarts et al., 1995; Garrison et al., 1996). This recombinant cell line is modified with a luciferase reporter plasmid which is responsive to the complex *AhR*-Arnt (*AhR* nuclear translocator) (Behnisch et al., 2001b). When the complex is present in the cells, it produces luciferase, which oxidizes the added substrate luciferin, which is consequently detected thanks to their bioluminescence with a luminometer. DR CALUX[®] has been widely promoted to screen several environmental matrices as surface waters (De Baat et al., 2019), soils (Joung et al., 2007; Lin et al., 2014) and river sediments from e-waste processing sites and harbours (Houtman et al., 2009; Suzuki et al., 2016). Other successful applications include human blood (Van Wouwe et al., 2004), breast milk, placenta and cord blood aimed to find the relationship among the level of bioactive *dioxin-like* compounds, *AhR* activity and the related clinical parameters in a population exposed to chemical waste (Müller et al., 2019). More recently, DR CALUX[®] bioassay has been used in screening the presence of Polybrominated dibenzo-*p*-dioxins and dibenzofurans (PBDD/Fs) in children's plastic toys (Budin et al., 2020). Although already successfully used for implementing environmental risks assessment strategies (European Law 711/2013) in human samples and environmental matrices, up to now they have been only included in Dutch, Norwegian and Japanese guidelines (www.bds.nl). According to EU regulation (252/2012 and 278/2012), bioanalytical methods for detection of PCDD/Fs and *dl*-PCBs can be used as semiquantitative screening test for food and animal feed. Likewise, in Italy CALUX[®] bioassays have been adopted only by the National Health Institute (ISS) and Istituto Zooprofilattico Sperimentale (IZS) as a screening tool for food and animal feed (Sciuto et al., 2013; Fochi et al., 2008).

The aim of the present study is to investigate for the first time the potential application of the DR CALUX[®] bioassay for screening PCDD/Fs and *dl*-PCBs in urban topsoils of Taranto municipality. In order to validate the suitability of the bioassay in comparison with the classical analytical chemistry method, the presence of PCDD/Fs and *dl*-PCBs in samples of topsoil collected from the same sites was further evaluated by GC-MS/MS analysis.

1.2 Materials and Methods

1.2.2 Experimental design

This study is part of the regional survey on soil dioxin contamination performed in Taranto municipality (Apulia, Italy) in the framework of the “*Area Vasta*” project, funded by the *Special Commissioner for Urgent Intervention for Remediation, Environmental Enhancement and Upgrading of Taranto*. Due to the high costs of screening dioxins and *dioxin-like* compounds using GC-MS/MS analysis in a large number of samples and on a year-based monitoring surveys, the suitability of DR CALUX[®] reporter gene assay was tested in a subset of soil samples from 10 sites in which chemical analysis and bioassays were both investigated. Those sites were selected inside an area of 20 km in square meters which includes Taranto urban and sub-urban areas and main industrial settings and illegal waste landfills. Three sub-areas were formerly identified as *high- (H)*, *medium- (M)* and *low risk (L)* based on critical distance from main sources as industrial settings, mineral parks (storage areas of mineral used), other waste landfill sites, including illegal dumping areas and wind directions (North-Northwest) (Fig. 1.1).



Fig. 1.1 Map of Taranto (Apulia Region, South Italy) showing the 10 urban topsoil sampling sites defined as high- (H), medium- (M) and low risk (L) based on the distance from the main industrial settings and illegal dumping area located in the North-West part of the city (red triangle symbols). In red is outlined the industrial area, while in white the urban ones. (Map Data Google Earth[®]).

The *high-risk* sub-area includes two sites (H1 and H2) between 0 and 3 km from main industrial settings, mineral parks and legal and illegal landfills located in the proximity; the *medium-risk* includes five sites (M1 to M5) between 3 km to 7 km away and the *low risk* includes 3 sites (L1 to L3) located over 7 km away. Since 1998 (DPR 196/1998), Statte (M1, M2 and M5) and Taranto municipalities (H1 and H2) have been then included among National Priority Contaminated Sites since levels of hazardous chemicals are above National Regulatory Limits in environmental matrices (groundwater, soil and air). Geographical coordinates and a detailed description of sampling sites are listed in Table SI 1.1 of supplementary material. In each site two topsoil samples were collected respectively for chemical analysis (winter 2017) and for DR CALUX[®] bioassay (spring 2018) by following the procedure of Salminen et al. (2005). Samples collected for chemical analysis were obtained in the framework of a large sampling campaign which include more than 200 sites in which several other chemicals were monitored. Therefore, only an aliquot of those ten samples collected in winter 2017 from the *high-*, *medium-* and *low-risk* areas was used for chemical analysis. Below the common procedure used for soil collection for chemical analysis and for bioassay. Three distinct soil aliquots were collected inside a 20 m² plot area in order to obtain a composite and representative sample of topsoil, made up of these 3 sub-samples for a total of 5 kg for each site. Vegetation, roots, stones and other coarse-grained material were removed before storing in plastic box containers which were frozen at -20 °C and then shipped to University of Siena Laboratory for extraction, purification and DR CALUX[®] bioassay analysis and to the Environmental Chemistry Laboratory of Taranto, a separated branch of University of Bari, for extraction, clean-up and GC-MS/MS analysis.

1.2.3 DR CALUX[®] bioassay

1.2.3.1 Soil sample pre-treatment and extraction

An aliquot of 60 g from each topsoil sample was freeze-dried (Labogene, Scanvac cool safe) (0.5 torr pressure, -50°C Temperature) and then sieved (2 mm). 5 g were extracted with a hexane/acetone mixture (9/1 v./v.), using an Accelerated Solvent Extractor (ASE, Dionex ASE 350, Thermo Scientific) at the following conditions: 0 min preheat, 5 min heat, 5 min static time, flush volume 60% of extraction cells, 90s purge time with nitrogen flow, 3cycles, 2000 psi (14Mpa) system pressure, 100 °C oven temperature. Samples were then treated with a solution of tetrabutylammonium hydrogen sulphate with sodium sulphate in HPLC grade water and isopropanol to remove sulphurs. Extracts were evaporated under N₂ flow (Caliper Life Science Turbo Vap II). For the isolation of PCDD/Fs and *dl*-PCBs fractions, a clean-up step using an acid-silica column, acidified with sulfuric acid at two different concentrations (33 and 20%) was performed. Columns were eluted with a mixture of n-hexane/diethyl ether (97/3 v./v.) and resulting extracts concentrated near dryness under

N₂ flow and then resuspended in 25 µL of dimethyl sulfoxide (DMSO). Samples were then stored at 4 °C until analysis.

1.2.3.2 Bioassay

The *AhR*-inducing potencies of the 10 topsoil extracts were determined with the DR CALUX[®] rat (H4IIE) hepatoma cell line transfected with the *AhR*-controlled luciferase reporter gene construct (pGudLuc1.1) *AhR*-controlled luciferase reporter plasmid (BioDetection Systems, Amsterdam, Netherlands; www.bds.nl). The DR CALUX[®] bioassay followed the Dutch standard method RIKZ-SPECIE 07 (2006a, b) and already described in detail elsewhere (Behnisch et al., 2001a, 2010; Besselink et al., 2004). Cells were routinely cultured in α -MEM medium supplemented with 10% foetal calf serum (FCS) and penicillin-streptomycin solution in a sterile incubator at 37 °C and 5% of CO₂ under saturated humidity (100%). Once they reached confluence, cells were placed in 96-well plates at a density of 1×10^4 cells/well and after 24h in the incubator, were exposed to soil extracts diluted (range 1-300x) in α -MEM medium with 0.8% DMSO (v/v.) (Fig. 1.2 A). A 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) reference calibration standard curve was also added to the plate (6 points in triplicate in the range 0-3 pM TCDD in 0.8% DMSO) (Fig. 1.2 B).

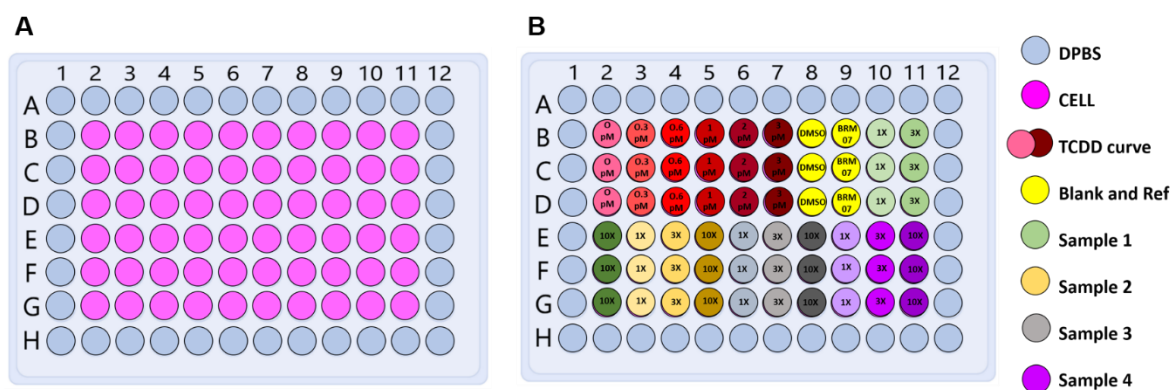


Fig. 1.2 Schematic representation of a microtiter plate before (A) and after sample load (B).

As references, a blank sample with only α -MEM medium and 0.8% DMSO and a sample named BRM07, made up with a mixture of PAHs, as an indicator of the metabolic capacity of the cells, diluted 1x with α -MEM medium and 0.8% DMSO.

Cells were further incubated at 37 °C for 24 h and monitored under an inverted microscope (*Nanjing Jiangnan Novel Optics NIB-100 F*) for cytotoxicity at the end of the incubation period. The medium was then removed, and the cells were firstly washed with 100 µl of Phosphate-buffered saline (PBS, with Ca/Mg) and then lysed using lysis buffer. For a fast luminescence reaction, the luciferase is

released by destroying the cells with the Lysis Buffer (pH 7.8) which contains Triton X-100 1%, a non-ionic surfactant that breaks down cell membranes and a blend of Tris 25mM and Glycerol 10% that create the appropriate pH conditions to protect the luciferase proteins, 1,4-dithiothreitol (DTT) 2mM and trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate (CDTA) 2mM.

The plate is then inserted into the Luminometer (*Victor 3 multilabel counter, Perkin Elmer*) and the program is started with a specific protocol to determine the luminescence of DR CALUX[®].

Automatically the luminometer insert for each well from the 1st dispenser 100 µl of Illuminate mix, shakes the plate and then a punctual reading is made. Illuminate mix (pH 7.8) contains 20 mM Tricine buffer, the substrate (D-Luciferine 539 µM), the cofactors (MgCO₃) 4Mg (OH) 25 1.07mM, MgSO₄ * 7 H₂O 2.67mM and 5.49 mM ATP as energy. DTT (1.5mM) is added as a reducing agent and co-enzyme A to make the response last longer.

Then, from the 2nd dispenser 100 µl of NaOH 0.2 M (bioultra for luminescence > 98%) will be inserted into the wells. This solution is used for quenching the luminescence reaction when measuring light production in a 96-microtiter plate from a CALUX[®] bioassay, to prevent cross talk between wells.

Luciferase activity was expressed as relative light units (RLUs). RLU values were then transformed into 2,3,7,8-TCDD Bioanalytical Toxicity Equivalents (BEQ) based on Relative Effect Potencies (REPs) of the extracts respectively of PCDD/Fs and of *dl*-PCBs calculated *vs* 2,3,7,8-TCDD calibration curve and expressed as ng 2,3,7,8-TCDD BEQ/kg dry weight (d.w. soil sample). The 2,3,7,8-TCDD sigmoid semi-logarithmic dose-response relationship was fitted by the Hill equation ($R^2 > 0.98$, $p < 0.001$). BEQ values were required to meet all the QA and QC criteria of bioassay standards in the Netherlands such as: R^2 calibration series > 0.98; EC₅₀ reference compound between present limits; SD triplicate analysis <15% (RIKZ-SPECIE 07, 2006a, b). The DR CALUX[®] BEQs were quantified between the limit of quantification and the EC₅₀ of 2,3,7,8-TCDD and as close as possible to the EC₁₀ (Besselink et al., 2004).

1.2.4 GC-MS/MS analysis of topsoil samples

1.2.4.1 Soil sample pre-treatment

Before starting the extraction procedure, an amount of 80 g of soil was freeze-dried (*Labogene, Scanvac cool safe*) under a pressure and temperature of respectively 0.5 torr and -50°C, for a time of 48 h, until achieving a constant weight value and sieved (2 mm) to remove the higher grain-size particles.

1.2.4.2 Soil sample extraction and GC-MS/MS analysis

Sample extraction was performed by Accelerated Solvent Extraction (*Dionex ASE 350, Thermo Scientific*) with hexane. Five grams of soil sample, mixed 1:1 with Dionex Prep DE, as sample dispersion and drying agent, were subjected to ASE by means of three static cycles (10 minutes each), at 100 °C, 1500 psi pressure, 60% of rinse volume and finally 100 seconds purge time with nitrogen flow. Labelled standards were purchased from Cambridge Isotope Laboratories (Andover MA, USA) and Cerilliant (Round Rock TX, USA) and used according to 1613B and 1668C EPA methods for the determination of respectively PCDD/Fs and PCBs. Labelled solutions at a proper concentration (100 ng/mL for $^{13}\text{C}_{12}$ - PCDD/Fs and $^{13}\text{C}_{12}$ -PCBs, and 200 ng/mL for $^{13}\text{C}_{12}$ -OCDD) were used to fortify soil samples before ASE extraction, in order to evaluate the percent recovery of the ^{13}C -labeled compounds through the whole analytical method.

Extracts aliquots for the determination of PCDD/Fs and *dl*-PCBs were purified/fractioned by means of an automated clean-up process with a PowerPrep system (*Fluid Management System, Waltham, Massachusetts*) using disposable columns (multilayer silica, alumina and carbon) and a multi-solvent switching programme. After clean-up and ripartition, PCDD/Fs and PCBs fractions were concentrated under a gentle N_2 flow near dryness. Finally, 20 μL of labelled internal standard solutions for PCDD/Fs and PCBs at 100 ng/mL were added to each vial before GC/MS analysis. 5 μL of PCDD/Fs vial and 3 μL of PCBs vial were injected inside a heated PTV injector of the GC/MS instrument by means of a TriPlus RSH autosampler (*Thermo Scientific*).

PCDD/Fs and *dl*-PCBs were separated by gas-chromatography (*GC Trace 1300, Thermo Scientific*) on a Trace Gold TG-5MS capillary column (60 m $\times$ 0.25 mm, 0.25 μm film thickness, *Thermo Scientific*). Low-resolution mass-spectrometry determinations were carried out on a TSQ 8000 system (*Triple Quadrupole, Thermo Scientific*) operating with electron ionization (EI) at 45 eV in the selected reaction monitoring (SRM) mode. Dioxins/furans and PCBs were analysed separately in GC-MS/MS using a different gas-chromatography programme and under optimized detection conditions.

1.2.4.3 Dioxins, furans and *dl*-PCBs quantification

For the analysis of topsoil samples, calibration curves were constructed for PCDD/Fs and *dl*-PCBs. More in detail, five standard solutions in nonane (Cerilliant) ready-to-use, in the concentration range 0.5-200 ng/mL (except for 2,3,7,8-TetraCDD/F, 0.1-40 ng/mL and OctaCDD/F, 1-400 ng/mL) were used to quantify seventeen 2,3,7,8-substituted CDDs/CDFs (7 PCDDs and 10 PCDFs) according to 1613B EPA method. For *dl*-PCBs, five standard solutions in nonane were prepared in the concentration range 0.2-400 ng/mL according to 1668C EPA method. Each calibration solution also contained labelled internal and recovery standards at 100 ng/mL. Before starting the analysis of a

sample batch, some quality checks were performed according to EPA methods (1613B and 1668C). Subsequently, one or more instrumental blanks (injection of hexane) and a blank sample were analysed by GC-MS/MS. Inside the analysed batch, an OPR, Ongoing Precision and Recovery blank sample was prepared fortifying it with the same extraction standard mixtures of PCDD/Fs and PCBs ($^{13}\text{C}_{12}$ -labeled) and a proper amount of native compounds (mixture containing each congeners of PCDD/Fs and PCBs) and submitted it to the whole analytical procedure to evaluate the recovery of each compounds, native and standard congeners, and to verify the maintaining of the performance criteria reported in EPA methods (1613B and 1668C). For the native PCDD/Fs, the test concentration for OPR analysis was 10 ng/mL for 2,3,7,8- TCDD and 2,3,7,8- TCDF, 100 ng/mL for OCDD/F and 50 ng/mL for the other congeners, while for native PCBs, the concentration used for testing OPR was equal to 50 ng/mL.

For the identification of each analyte in GC-MS/MS chromatograms, absolute and relative retention times were observed by comparison of standard chromatographic runs. Two MS/MS transitions at a proper collision energy were set for MS acquisition and both were detected for each analyte. For quantitative determination, response ratios were used in conjunction with initial calibration data.

GC-MS/MS analysis of a sample was accepted when the recovery of the labelled compounds was in the range reported in EPA methods.

Estimated concentrations for each detected analyte of PCDD/Fs and *dl*-PCBs were expressed in term of toxic equivalency (TEQ) and calculated as follows: TEQ_{WHO} based on the TEF values calculated using the Toxic Equivalent Factors (TEFs) of both *in vitro* and *in vivo* studies reported in Van den Berg et al., (2006) and $\text{TEQ}_{\text{THEORETICAL}}$ based on the REPs obtained in van Ede et al., (2013) specifically calculated *in vivo* in mice liver (see Appendix Tab. A1). REPs were determined by comparing the EC_{50} of each analyte to that of 2,3,7,8-TCDD, the highest *AhR* agonist. Based on the recommendation published in van Ede et al., (2016), *in vitro* REP as those obtained by DR CALUX[®] should be more valuable contributors for establishing human TEFs for selected *dioxin-like* congeners so that the resulting TEQs will better assess human health risks.

1.2.4.4 Total PCBs quantification

In addition to determine *dioxin-like* PCBs, in the present research work, the total PCBs amount was evaluated. In particular, the sum includes the following congeners with the information about the numbers of chlorine atoms: PCB 3 (Mono), PCB 4 (Di), PCB 28 (Tri), PCB 52, PCB 81 and PCB 77 (Tetra), PCB 95, PCB 101, PCB 99, PCB 110, PCB 123, PCB 118, PCB 114, PCB 105 and PCB 126 (Penta), PCB 151, PCB 146, PCB 149, PCB 153, PCB 138, PCB 167, PCB 128, PCB 156, PCB 157

and PCB 169 (Hexa), PCB 187, PCB 183, PCB 177, PCB 180, PCB 170 and PCB 189 (Hepta), PCB 205 (Octa), PCB 206 (Nona) and PCB 209 (Deca).

In the reported list, some congeners, such as PCB 28, 52, 101, 138, 153 and 180, are called *indicators* or *markers* because their sum includes about half of all *non-dioxin-like* PCBs (NDL-PCBs) in feed and food. The European Food Security Authority (EFSA) considers that the presence of these congeners and in particular their sum, constitutes an appropriate indicator of the occurrence and human exposure of NDL-PCBs (Scientific Report of EFSA, 2011).

The calibration range of each analyte is the same reported for *dl*-PCBs. For each of them (including *dl*-PCBs), the labelled standard compound (^{13}C), the analogue or the similar, was used for the recovery evaluation and for the correction of the concentration. The total amount of PCBs, expressed as $\mu\text{g/kg}$ dry weight, was the sum of the concentration of each congener detected in topsoil samples from the investigated ten sites. This value is also of great importance for the comparison with the legal limit set by the Italian National Regulatory (D.Lgs. 152/2006).

1.2.5 Statistical analysis

Statistical analyses were performed by GraphPad Prism 6 (*GraphPad Software Inc.*) and statistical significance was set at $p < 0.05$. All numerical data were expressed as the average of the values obtained from the luminometer and GC-MS/MS analysis. Data analysis of DR CALUX[®] BEQ values and GC-MS/MS TEQs (TEQ_{WHO-THEORETICAL}) were conducted by one-way ANOVA (analysis of variance) plus *Bonferroni test*. The correlation coefficient (R^2) between DR CALUX[®] BEQ values and GC-MS/MS TEQs (TEQ_{WHO-THEORETICAL}) was obtained by applying linear regression analysis.

1.3 Results and discussion

1.3.1 BEQs and TEQs in topsoil of Taranto

DR CALUX[®] BEQs (ng 2,3,7,8-TCDD BEQ/kg d.w.) and GC-MS/MS TEQ_{WHO} (ng TEQ/kg d.w., 2005) (Fig. 1.3 A) and TEQ_{THEORETICAL} (ng TEQ/kg d.w.) (Fig. 1.3 B) for $\Sigma\text{PCDD/F/dl-PCBs}$ in Taranto topsoils showed a decreasing trend with the distance from the *high-* to *low-risk* sub-areas.

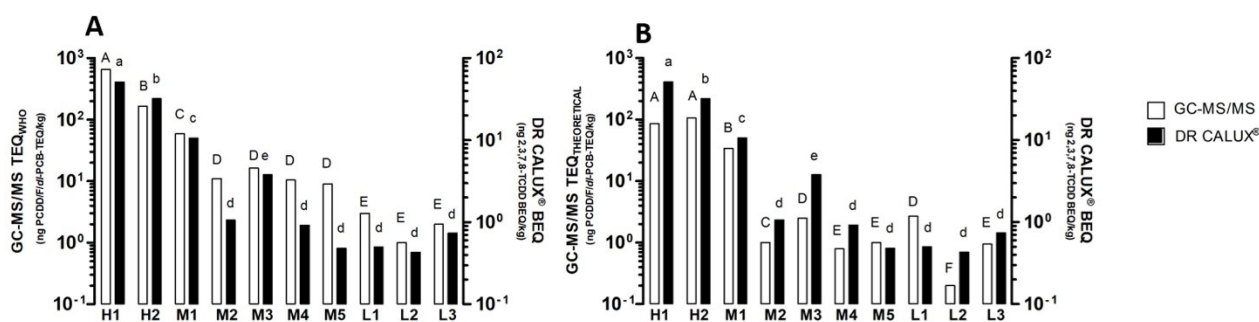


Fig. 1.3 Trend in TEQ_{WHO} (A) and in $TEQ_{THEORETICAL}$ (B) values for ng PCDD/PCDF/*dl*-PCBs-TEQ/kg d.w. obtained from GC-MS/MS analysis ($n=10$) (white bars) and DR CALUX® BEQs (ng 2,3,7,8-TCDD BEQ/kg d.w.) obtained from PCDD/PCDF and *dl*-PCBs extracts ($n=10$) (black bars). Capital letters used to show significant differences ($p < 0.05$) among TEQs values and lower-case letters among DR CALUX® BEQ values.

Although, samples of topsoils for chemical analysis and bioassay have been collected during two different campaigns (winter 2017 for GC-MS/MS and spring 2018 for DR CALUX® bioassay) and in some sites at slightly different distances (Tab. SI 1.1), a strong correlation is still found between DR CALUX® BEQs and TEQ_{WHO} ($R^2 = 0.85$) as well as $TEQ_{THEORETICAL}$ values ($R^2 = 0.88$) (Fig. 1.4).

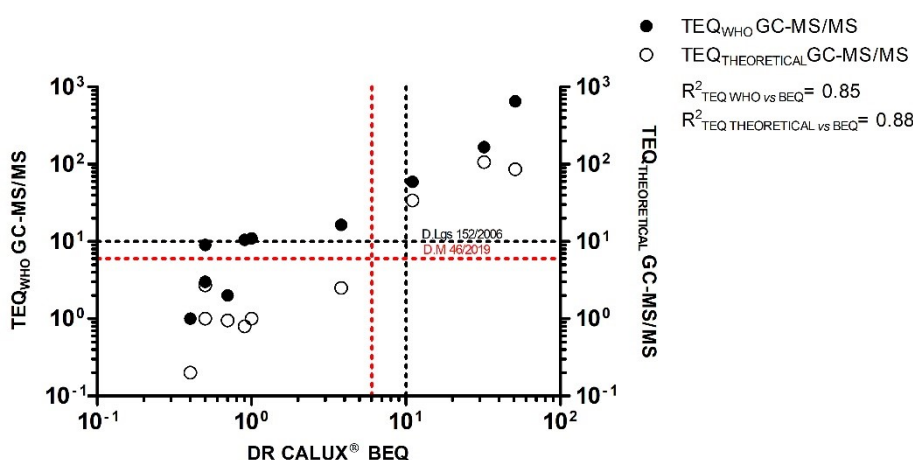


Fig. 1.4 Log transformed representation of DR CALUX® BEQs, TEQ_{WHO} and $TEQ_{THEORETICAL}$ values found in soil samples from the ten sites. Black dotted lines show Italian National Regulatory Limits for $\sum PCDD/F$ s in green, private and recreational used soils (10 ng TEQ/kg D.Lgs 152/2006 d.w.) while red dotted lines show the limit for $\sum PCDD/F/dl$ -PCBs in agricultural and farming soil (6 ng TEQ/kg D.M. 46/2019).

In 3 out of 10 topsoils investigated (H1, H2 and M1), $TEQ_{WHO/THEORETICAL}$ (ng TEQ/kg d.w.) resulted above Italian National Regulatory Limits for $\sum PCDD/F$ s in green, private and recreational used soils (10 D.Lgs 152/2006) and for $\sum PCDD/F/dl$ -PCBs in agricultural and farming soil (6 D.M. 46/2019) (Fig. 1.4; Tab. SI 1.2). Moreover, $\sum PCDD/F$ s TEQ_{WHO} values of those from the *high-risk* area (H1 and H2) resulted above the National Regulatory Limits for soil of industrial and commercial use (100 D.Lgs. 152/2006) (Fig. 1.4; Tab. SI 1.2). By considering only TEQ_{WHO} values, also all sites in the

medium-risk area were still *non-conform* for the Σ PCDD/F/*dl*-PCBs (6 D.M. 46/2019) (Fig. 1.4; Tab. SI 1.2). All those sites are located in the proximity of the main steel and iron ore sinter plant of Taranto and of illegal dumping sites and steel plant's landfills nearby (Fig. 1.1). GC-MS/MS pattern revealed the highest prevalence of *dl*-PCBs (87-84%), followed by PCDFs (16-6%) and PCDDs (5-1%) in sites H1, M2, M3, M4 and M5 (Fig. 1.1; Fig. 1.5).

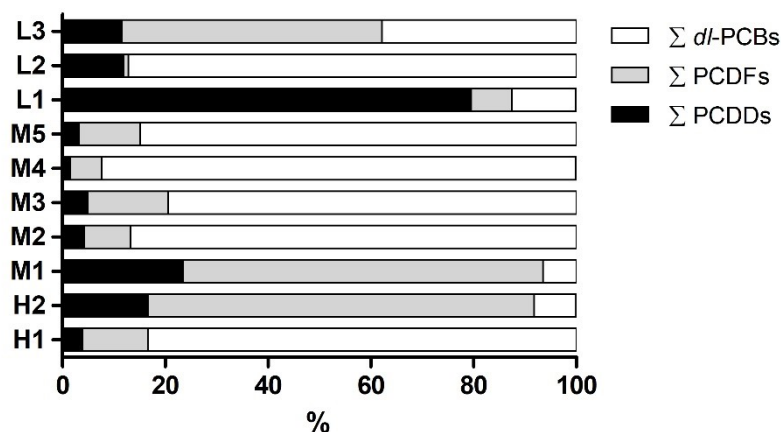


Fig. 1.5 Pattern of the sum of PCDDs, PCDFs and *dl*-PCBs in the ten topsoil samples processed for chemical analysis by GC-MS/MS.

Amongst *dl*-PCBs, congeners PCB 126 and PCB 118 were prevalent (Fig. SI 1.1) and this could explain the higher values of TEQ_{WHO} (one order of magnitude) than TEQ_{THEORETICAL}, being REPs lower than TEF WHO₂₀₀₅ for both congeners: PCB 126, TEF 0.1 WHO₂₀₀₅ and REP 0.003 median human values on cell-based assays and PCB 118, TEF 0.00003 WHO₂₀₀₅ and REP 0.00006 (van Ede et al., 2013; Lee et al., 2013; Van der Berg et al. 2006) (Tab. SI 1.2; see Appendix Tab A1).

REP values are generally lower than TEF WHO₂₀₀₅ except for selected dioxin congeners and two *dl*-PCBs (77, 81). In particular, TEF WHO₂₀₀₅ for PCB 126 and 118 congeners are considered overestimated in terms of human health risk and REP considered more appropriate at least for *AhR*-mediated effects (van Duursen et al., 2017). Higher values of TEQ_{WHO} than TEQ_{THEORETICAL} were found in 7 out of 10 soil samples analysed except for H2, M1 and L3 topsoil samples where a significantly higher % of PCDFs (77-70%) and PCDDs (24-15%) was found compared to *dl*-PCBs (9-6%) (Fig. 1.4; Fig. 1.5; Tab. SI 1.2). TEQ_{WHO} are in fact generally lower than TEQ_{THEORETICAL} for Σ PCDD/Fs and could underestimate sample potency by not including all 75 PCDDs, 135 PCDFs and 209 PCBs congeners for which no TEFs are still available (Behnisch et al., 2001b; Lee et al., 2013). The observed high prevalence of Σ PCDD/Fs in topsoils collected from sites H2 and M1 respectively located South-East and North-West from the steel and iron ore sinter plant further support the role of

wind in moving PCDD/Fs emissions from North-West to South of the industrial sources. In addition, the prevalence of PCDFs confirm their origin from the peculiar iron ore combustion of Taranto plant which originates a larger proportion of furans than dioxins during iron roasting as documented in the Report of Regional Agency (ARPA Puglia, 2008). Concerning the two other *medium risk* sites, M3 and M4, North-East of industrial area inside the Paolo VI district, despite having lower BEQs and TEQ_{WHO-THEORETICAL} values (Fig. 1.3 A, 1.3 B), they show again the highest prevalence of *dl*-PCBs (Fig. 1.5) and in particular of congeners 126 and 118 (Fig. SI 1). In particular, BEQ values resulted similar to TEQ_{WHO} (Σ PCDD/Fs) in M3 and M4, but higher than TEQ_{THEORETICAL} (Tab S2; Tab S3). This suggest the presence of other sources of *AhR*-inducers in those sites for which further investigation for a better understanding of their chemical nature are recommended being a high densely populated neighbourhood (Fig. 1.3 A, 1.3 B). Finally *low-risk* sites (L1, L2 and L3), all located South/Southeast of the city (Fig. 1.1), at >10 km from the industrial area and dumping sites, showed the lowest values of BEQ (<1 ng 2,3,7,8-TCDD BEQ/kg d.w.) (Tab. SI 1.3) and TEQ_{WHO-THEORETICAL} (<3 for both Σ PCDD/F/*dl*-PCBs and Σ PCDD/Fs) (Tab. SI 1.2). However, looking at pattern profile, L1 shows surprisingly the highest prevalence of PCDDs (79%) and in particular of TCDD (31%) and HxDD (48%) which is far higher compared to the other 9 investigated sites including H1 and H2 (Fig. 1.5). On the contrary topsoils from L2 shows a prevalence of *dl*-PCBs (87%) while a more heterogeneous pattern is observed for those in L3 (51% PCDFs, 38% PCBs and 11% PCDDs) (Fig. 1.5).

1.3.2 PCDD/Fs and *dl*-PCBs in soil, sources and epidemiological data

Sites H1 and M2 are located NW of Taranto main industrial area (Statte municipality) (Fig. 1.1, in red) closest to the steel and iron ore sinter plant including their landfill sites as well as illegal waste dumping sites, more recently documented (ARPA Puglia, 2008; Zona et al., 2019). The documented mis-management of hazardous materials containing asbestos (Comba et al., 2012) and illegal waste burning (Mazza et al., 2015) could have significantly contributed to the emission of PCDD/Fs and *dl*-PCBs in the air with consequent fall-out on topsoil (Herrero et al., 2020; Weber et al., 2018). Levels of Σ PCDD/F/*dl*-PCBs found in H1 topsoils are similar to those reported for highly dioxins contaminated sites of the Italian territory as the town of Brescia (Lombardia Region) and the Land of Fires, where waste burning has been largely recognized as source of PCDDs and PCDFs (Campania Region) (ISPRA, 2012; Turrio-Baldassarri et al., 2007). Also the levels of *mono* and *di-ortho* PCBs found in topsoil of H1 site (440.3 μ g/kg d.w.) resulted far above (1-2 order of magnitude) Italian National Regulatory Limits (60 Σ PCBs μ g/kg d.w. D.Lgs. 152/2006) (Tab. SI 1.4) and similar to those reported by Esposito and co-workers back in 2010 (458 μ g/kg d.w.). This suggest a persistent

PCBs contamination in topsoils closest to the industrial settings which demand for continuous monitoring and assessment of the hazards posed to humans and the environment (Esposito et al., 2010, 2012). As observed for Σ PCDD/F/*dl*-PCBs, also Σ PCB levels decrease with the distance from main industrial settings and illegal wastes dumping sites with the lowest levels of 0.5 $\mu\text{g/kg d.w.}$ in site L3, located at an average of 16 km from site H1 (Fig. 1.1). Again, our data are in agreement with those reported by Esposito et al., (2010, 2012) back in 2008 and 2010 with the highest levels of PCBs (458 $\mu\text{g/kg d.w.}$) in topsoils taken less than 1 km far from site H1 and the lowest (0.73 $\mu\text{g/kg d.w.}$) in sites 13 km far from it.

Occupational exposure studies conducted in male workers employed in the electrical maintenance sector of Taranto steelworks factory, revealed immune systems diseases linked to the exposure to *dl*-PCBs, even at a low concentration (D'Errico et al., 2012). Excess risks of melanoma and other cancer mortality have been documented in several cohort studies of capacitors and transformers manufactory workers (Lauby-Secretan et al., 2013). Accumulation of *dl*-PCBs in blood and skin of workers have been linked to occupational exposure during disposal and handling of electrical transformers containing a PCBs-based cooling liquid named Apirolio[®]. As a trade name of an Italian product sold in Italy until the '70s, Apirolio[®] is a mixture of PCBs containing mostly two *dl*-PCB congeners as 118 and 126 (Maroni et al., 1981; D'Errico et al., 2016). According to the IARC (2015), PCB 126 is classified as Class 1 carcinogen and it can reach environmental concentrations higher than TCDD itself being largely present in commercial formulations for more than sixty years (Zhang et al., 2012). Being considered the main source of soil PCBs contamination in Italy (Di Lenola et al., 2018), Apirolio[®] leakage from inadequate storage and disposal of electrical transformers might have also significantly contributed to the documented prevalence of *dl*-PCBs in Taranto topsoils closest to the main industrial settings and landfill sites including the illegal ones (H1, M2, M3 and M4) (Ancona et al., 2019). Asbestos itself could represent a further source of PCBs in Taranto soils. Despite most of the study on human health and asbestos are referred to occupational exposure from fiber's inhalation, the release of toxic chemicals including PCDD/Fs and *dl*-PCBs from weathered coating materials of asbestos-cement cover plates has been overlooked and only recently documented and estimated. By using the DR CALUX[®] bioassay, Winkler and co-workers (2015) documented for the first time that debris of coating gutter from weathered asbestos-cement fiber plates, used to cover roof and sidewalls of chicken stables, were the main source of PCDFs and *dl*-PCBs contamination in topsoil (1-5 cm) and free-ranging chicken eggs. Therefore, the documented illegal disposal of asbestos cement-coated plates could represent a further source of *dl*-PCBs and PCDFs contamination in Taranto topsoils inside and closest to landfill areas (sites H1, M2, M3 and M4) (Zona et al., 2019). This is in strong

agreement with the high prevalence of *dl*-PCBs found in topsoils from those sites and in particular of PCB 118 and PCB 126 congeners (Fig. 1.5; Fig. SI 1.1).

Based on the latest Report of the *Working Group on the Incidence of Mesothelioma in National Priority Contaminates Sites in Italy*, asbestos has been used in industrial processes in Taranto factories, both friable and compact, as well as in personal protective equipment which has caused a direct exposure of workers, with particular regard to welders and carpenters (Pasetto et al., 2016). The high incidence of the onset of mesothelioma in inhabitants living near asbestos-cement factories, illegal landfills areas as well as petrochemicals, refineries and steel industries have been documented (Pasetto et al., 2016; Martinelli et al., 2012; Graziano et al., 2009). The most critical epidemiological data were those from the inhabitants living in proximity of the Taranto industrial steel plant and iron foundries (Montinari et al., 2015). Mirabelli et al., (2010), provided evidence of air pollution-related diseases which includes pleural mesothelioma at level of incidence like those reported for asbestos factories workers. Based on “*SENTIERP*” epidemiological study back in 2013, Statte residents showed a high incidence of tumours, cerebrovascular and respiratory diseases (sites H1, M1, M2) (National Priority Contaminated Site) (Pirastu et al., 2013). An excess risk of leukaemia’s has been also documented in women in the framework of a larger study on the incidence of tumours in all the municipalities of Taranto province (Graziano et al., 2009). According to Ridolfi et al. (2014), PCDD/Fs and *dl*-PCBs accumulation can activate permanently *AhR*, resulting in a continuous and uninterrupted state of immunosuppression which could have an important role on inducing stem cells and the development and progression of cancer (Gasiewicz et al., 2014).

The second highest values of BEQs and TEQs are from topsoil of H2 site located inside the Tamburi district in which a prevalence of PCDD/Fs over *dl*-PCBs was found (Fig. 1.5).

Wet and dry depositions of combustion emissions are often occurring during “wind-days” and largely documented by Tamburi inhabitants by the name of “red dust” (Rotatori et al., 2006; Esposito et al., 2012). Due to the local unique climate conditions, PCDD/Fs concentration in the air increases considerably and deposit as mineral dust in roofs, terraces and public gardens of Tamburi district and areas nearby (Lai et al., 2019). A significant correlation between dioxins and polycyclic aromatic hydrocarbons (PAHs) levels and wind direction was found below or above the industrial area (Giua et al., 2005, 2009). Epidemiological data obtained from a survey conducted in 2010 reported a higher incidence of mortality and hospitalization of Tamburi inhabitants compared to those from the rest of Taranto’s urban area (Mataloni et al., 2012). An excess of cardiovascular and renal diseases and, malignant neoplasms have been documented in men and women (Mataloni et al., 2012; Pirastu et al., 2013).

The other district less close to the industrial settings is “Paolo VI”, located North of Taranto (site M3 and M4) and built in the ‘60s to guest steel workers families. As already mentioned for inhabitants of Tamburi district (H2) and Statte (site H1, M2), digestive system and liver neoplasia and cardiovascular diseases are among those highly reported in local male inhabitants (Pirastu et al., 2013). In 2012 this district has been declared the neighbourhood with the highest mortality rates in Taranto city (+ 42%) (Mataloni et al., 2012).

Finally, among the most distant sites (*low-risk*), site L1 located inside the Salina Grande, a brackish area being recognized in the past as an area of high ecological value, has been recently included in the Taranto’s National Priority Contaminated Site, being nowadays occupied by a large illegal dumping site (e.g. rubbles mixed with plastic debris and urban wastes). They could represent potential sources of dioxins and furans as shown by the prevalence of PCDDs (79%) and in particular of TCDD (31%) and HxDD (48%) found in the topsoils (Fig. 1.5). Future surveys should also include this area in order to assess potential hazards and prevent future risk for local inhabitants.

1.3.3 PCDD/Fs and dl-PCBs in soil and risk for human health

Taranto is not only known as a heavily industrialized area but plays a leading role in the agri-food sector in the Apulia Region. In terms of potential human risks associated with the use of soil for animal farming, only BEQs and TEQ_{WHO-THEORETICAL} values of topsoil from sites L2 and L3 can be considered at no risk (Fig. 3). Both sites located South-East of the city, in the Municipality of Talsano, thus confirmed as "background site" being upwind with respect to the predominant wind directions (N-NW) (ARPA Puglia 2008).

The levels of PCDD/Fs and *dl*-PCBs in topsoil of the majority of sites (8 out of 10) are above National Regulatory Limits thus representing a serious risk for human health since Taranto inhabitants mostly rely on local food production (meat, dairy products and vegetables) (Pascuzzi et al., 2013). It cannot be excluded that the transfer and bioaccumulation of PCDD/Fs and *dl*-PCBs in free-ranging animals will exceed Regulatory Limit for foodstuff thus representing a further exposure route (Esposito et al., 2012; Liberti, 2014). Regulatory Limits for dioxins in food and feed products are set at 2-4 ng PCDD/F-TEQ/kg (Hoogenboom et al., 2016) and Tolerable Weekly Intake (TWI) at 2 pg PCDD/F-TEQ/kg body weight (EFSA 2018). It has been demonstrated that 2.5 pg TEQ/g fat for PCDD/Fs or 5 pg TEQ/g fat for Σ PCDD/Fs and *dl*-PCBs are enough to transfer them to humans from consumption of chicken eggs (Weber et al., 2015). In a study conducted by the Local Health Authority (ASL) of Taranto in the period 2008-2009, a *non*-compliance for dioxins was found in various food items including farmed animals (Iavarone et al., 2009). The highest concentration recorded was 279 pg WHO-TEQ/g fat for a sheep liver in which *dl*-PCBs resulted the main contributors (Diletti et al.,

2009). As a further confirmation organochlorine compounds were documented also in blood of Taranto farmers (Iavarone et al., 2012). Therefore, current Regulatory Limits for green, private and recreational use and grazing soils (10 ng TEQ/kg d.w. for Σ PCDD/Fs D.Lgs. 152/2006; 6 ng TEQ/kg including also *dl*-PCBs, D.M. 46/2019) might not prevent health risks for Taranto inhabitants due to consumption of local food. TEQs values of 5 ng/kg in soils can contaminate food products above EU Regulatory Limits (Weber et al., 2018) and might cause farmed (meat and milk) and agricultural products (vegetables) not suitable for human consumption as documented in the past (Esposito et al., 2012). The consumption of contaminated food by Σ PCDD/Fs and *dl*-PCBs affects chemical and hormonal blood parameters (Chiusolo et al., 2012), causes generative pathologies and endocrine-metabolic and immune complex diseases (Pascuzzi et al., 2013). The consumption of milk and meats from animals fed with contaminated forage caused eight-time higher levels of Σ PCDD/Fs and *dl*-PCBs in human blood compared to average cohort values (Turrio-Baldassarri et al., 2008).

1.4 Conclusion

The present study confirmed the occurrence and a spatial distribution of dioxins, furans and *dl*-PCBs in the topsoil of the Taranto area through a combined approach of chemical analysis and DR CALUX[®] bioassay. Such contamination does not seem to have decreased in recent years as confirmed by similar TEQ values already found in the 2010 survey. We not only confirmed already known sources (e.g. industrial air emissions) but we have been able to identify new ones (e.g. illegal disposal of electric transformers containing Apirolio[®] and asbestos cement-fiber plates) which call for urgent more in-depth analysis in order to assess the risk posed to humans and the environment. Such complex exposure scenario to dioxins and *dioxin-like* compounds is supported by epidemiological data which reveal the critical situation of the Taranto inhabitants in terms of the incidence of tumours and cancer which again call for urgent actions to identify any possible source.

Considering the extension of illegal landfills in the Taranto municipality area and the urgent need to investigate potential sources and impact of *dioxin-like* compounds, the known suitability of DR CALUX[®] also highlighted in this study support their application in future monitoring surveys. The risk represented by the extensive use of soils for agricultural and food purposes in Taranto area, further underlines the need to apply monitoring tools that quickly and extensively highlight the hazard posed by *dioxin-like* compounds in terms of human and environmental health. The high costs associated to extensive monitoring survey find in the application of bioassays a successful alternative, already widely used at the level of the European member states, in identifying new and unexpected sources and their route of exposure that would require long times and huge investments if only based on analytical chemical analysis.

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Supplementary information

Tab. SI 1.1 Geographical coordinates of the 20 sampling sites (10 for Chemical analysis GC-MS/MS and 10 for Bioassay analysis DR CALUX®) from which topsoils were collected and indicated as *high risk* (H), *medium risk* (M) and *low risk* (L) based on the distance from the main industrial settings and illegal dumping areas of Taranto municipality.

Site	Chemical sampling		Bioassay sampling
H1 _{GC-MS/MS}	N +40°32'19.38" E +17°13'2.14"	H1 _{BIOASSAY}	N +40°32'19.71" E +17°13'01.23
H2 _{GC-MS/MS}	N +40°29'0.66" E +17°13'36.74"	H2 _{BIOASSAY}	N +40°29'01.01 E +17°13'37.43
M1 _{GC-MS/MS}	N +40°32'44.11" E +17°11'12.47"	M1 _{BIOASSAY}	N +40°32'44.11" E +17°11'12.38
M2 _{GC-MS/MS}	N +40°32'26.32" E +17°11'7.54"	M2 _{BIOASSAY}	N +40°32'26.61" E +17°11'07.19
M3 _{GC-MS/MS}	N +40°30'52.28" E +17°14'28.03"	M3 _{BIOASSAY}	N +40°30'35.22" E +17°14'28.04
M4 _{GC-MS/MS}	N +40°32'33.65" E +17°16'33.27"	M4 _{BIOASSAY}	N +40°32'33.01" E +17°16'34.50
M5 _{GC-MS/MS}	N +40°32'17.21" E +17°10'28.13"	M5 _{BIOASSAY}	N +40°32'17.34" E +17°10'27.78
L1 _{GC-MS/MS}	N +40°26'45.85" E +17°16'46.36"	L1 _{BIOASSAY}	N +40°26'46.75" E +17°16'46.85
L2 _{GC-MS/MS}	N +40°25'04.83" E +17°17'18.43"	L2 _{BIOASSAY}	N +40°25'05.13" E +17.17'18.99
L3 _{GC-MS/MS}	N +40°25'22.18" E +17°20'5.77"	L3 _{BIOASSAY}	N +40°25'22.23" E +17°20'06.21

Tab. SI 1.2 Toxic Equivalent (TEQ) values obtained by GC-MS/MS in the ten topsoil samples (H, M and L) calculated based on based on TEF (Van den Berg et al., 2006) and expressed as TEQ_{WHO} and based on TEF (van Ede et al., 2013) and expressed as TEQ_{THEORETICAL}. Both TEQs are also calculated as Σ PCDD/F/dl-PCBs TEQ, based on the most recent Italian Regulation D.M. 46/2019 for agricultural and farming soil (limit set at 6 ng TEQ/kg d.w.) and as Σ PCDD/Fs TEQ based on the D.Lgs. 152/2006 (limit set at 10 ng TEQ/kg d.w. for green, residential and private soils and 100 ng TEQ/kg d.w. for commercial use and industrial soil). Samples *non-conform* were marked bold, while samples conform were marked italics.

Site	TEQ _{WHO}		TEQ _{THEORETICAL}	
	Σ PCDD/F/dl-PCBs (ng TEQ/kg d.w.) (D.M. 46/2019)	Σ PCDD/Fs (ng TEQ/kg d.w.) (D.Lgs. 152/2006)	Σ PCDD/F/dl-PCBs (ng TEQ/kg d.w.) (D.M. 46/2019)	Σ PCDD/Fs (ng TEQ/kg d.w.) (D.Lgs. 152/2006)
H1 GC-MS/MS	655.5	109	86	64
H2 GC-MS/MS	166	153	107	105
M1 GC-MS/MS	59	55	34	34
M2 GC-MS/MS	11	<i>1.5</i>	<i>1</i>	<i>0.9</i>
M3 GC-MS/MS	16.4	<i>3.4</i>	<i>2.5</i>	<i>2</i>
M4 GC-MS/MS	10.5	<i>0.8</i>	<i>0.8</i>	<i>0.5</i>
M5 GC-MS/MS	9	<i>1</i>	<i>1</i>	<i>0.8</i>
L1 GC-MS/MS	<i>3</i>	<i>3</i>	<i>2.7</i>	<i>2.7</i>
L2 GC-MS/MS	<i>1</i>	<i>0.2</i>	<i>0.2</i>	<i>0.2</i>
L3 GC-MS/MS	<i>2</i>	<i>1</i>	<i>0.95</i>	<i>0.9</i>

Tab. SI 1.3 DR CALUX[®] BEQ values (ng 2,3,7,8-TCDD BEQ/kg d.w.) in the ten topsoil samples (H, M and L). Samples *non-conform* were marked bold, while samples conform were marked italics.

Site	DR CALUX [®] BEQ (ng 2,3,7,8-TCDD BEQ/kg d.w.)
H1 BIOASSAY	51
H2 BIOASSAY	32
M1 BIOASSAY	11
M2 BIOASSAY	<i>1</i>
M3 BIOASSAY	<i>3.8</i>
M4 BIOASSAY	<i>0.9</i>
M5 BIOASSAY	<i>0.5</i>
L1 BIOASSAY	<i>0.5</i>
L2 BIOASSAY	<i>0.4</i>
L3 BIOASSAY	<i>0.7</i>

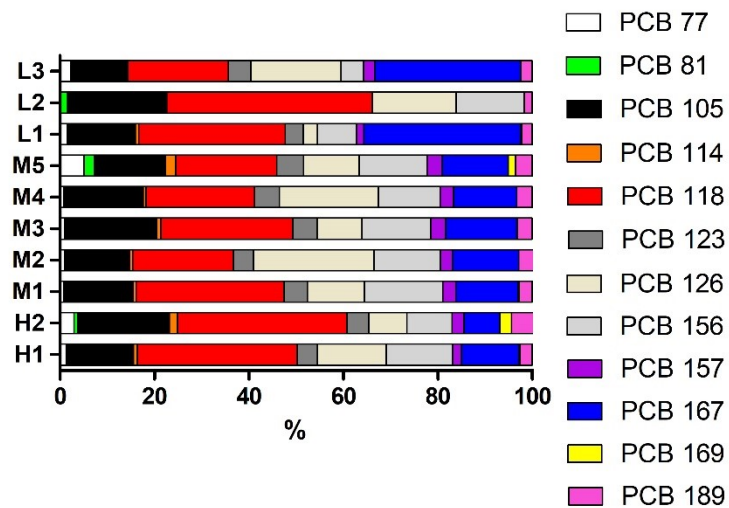


Fig. SI 1.1 Congener profile of the *dl*-PCBs in the ten topsoil samples (L, M, H) based on GC-MS/MS analysis.

Tab. SI 1.4 Sum of PCBs expressed as $\mu\text{g/kg}$ dry weight (d.w.) carried out on the ten topsoil samples (H, M and L) with GC-MS/MS.

Site	$\sum \text{PCB}_{\text{TOT}}$ ($\mu\text{g/kg}$ d.w.)
H1 _{GC-MS/MS}	440.3
H2 _{GC-MS/MS}	9.9
M1 _{GC-MS/MS}	8
M2 _{GC-MS/MS}	3.7
M3 _{GC-MS/MS}	11.9
M4 _{GC-MS/MS}	5
M5 _{GC-MS/MS}	2.7
L1 _{GC-MS/MS}	4.6
L2 _{GC-MS/MS}	0.8
L3 _{GC-MS/MS}	0.5

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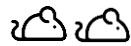
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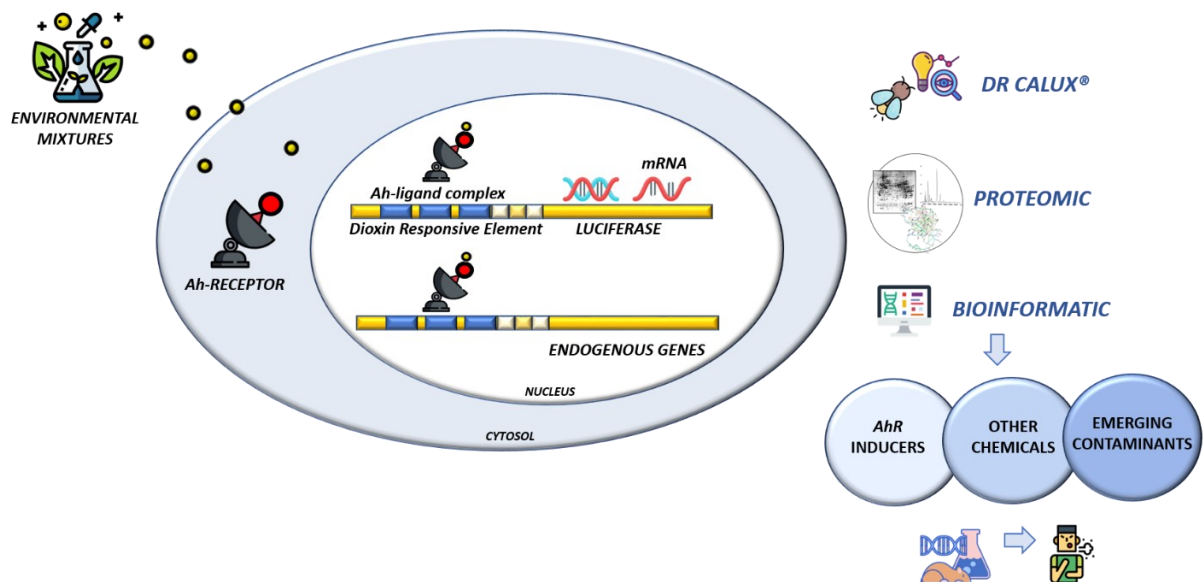
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www.bds.nl

CHAPTER 2



First attempt to use proteomic analysis coupled with DR CALUX[®] bioassay as effect-based tools for monitoring soil pollution by legacy and emerging contaminants



Abstract

CALUX[®] cell bioassay is a standardized bioanalytical tool applied for single screening and relative quantification of legacy and emerging contaminants. Here we investigate the possibility to couple proteomics to the DR CALUX[®] cells bioassay, to extrapolate CALUX[®] cells altered molecular pathways following samples extract exposure and predict other toxic pollutants eventually present in the sample extract itself. To this purpose, we tested an extract contaminated with Polychlorinated dibenzo-*p*-dioxins, Polychlorinated dibenzofurans (PCDD/Fs) and *dioxin-like* Polychlorinated biphenyls (*dl*-PCBs) obtained from a topsoil sample collected in proximity of a large industrial steel and iron ore sinter plant and illegal dumping sites near the city of Taranto (Apulia Region, South Italy) by using DR CALUX[®] bioassay, GC-MS/MS analysis and coupling proteomic and bioinformatic on CALUX cells to screen the presence of other compounds able to affect specific cellular pathways. BEQ and TEQ values obtained confirmed the presence of PCDD/Fs and *dl*-PCBs. By proteomics we obtained differently abundant proteins which were subjected to functional and pathway analysis extrapolating potential chemical compounds inducing that molecular alterations and suggesting potential links with records on local human pathologies.

2.1 Introduction

Chemically-activated-luciferase-gene-expression *in vitro* cell bioassays (CALUX®) represent a standardized bioanalytical tool applied for the screening and relative quantification of legacy and emerging contaminants (van Vugt-Lussenburg et al., 2014). CALUX® application relies on a selected biological response upon exposure to an active compound present in a given sample extract for which a cellular pathway has been already identified and verifiable with a luminescence reaction. Each type of CALUX® bioassay highlights the presence of a specific contaminant or specific biological effect in a tested sample (Behnisch et al., 2001a, 2001b). In particular, among those more effective in assessing and quantifying exposure to the most toxic pollutants as Polychlorinated dibenzo-*p*-dioxins, Polychlorinated dibenzofurans (PCDD/Fs) and *dioxin-like* Polychlorinated biphenyls (*dl*-PCBs), the DR (Dioxin responsive) CALUX® assay uses the pathway mediated by the Aryl Hydrocarbon Receptor (*AhR*), a ligand-dependent transcription factor induced by the binding of dioxins and related chemicals mainly responsible for mediating their cellular toxicity (Aarts et al., 1995; Garrison et al., 1996). The stable transfected *AhR*-responsive firefly luciferase reporter gene response occurs in a time-, dose-, *AhR*-dependent and chemical-specific manner (Behnisch et al., 2010, 2003, 2001a, 2001b; Besselink et al., 2004). Relies on this principle, once identified a specific receptor, rat hepatoma cells (H4IIE) have been transfected with the *AhR*-controlled luciferase reporter gene construct (pGudLuc) in order to obtain the specific DR CALUX® assays which has been successfully applied to screen various matrices such as blood and human milk, food, water, soil and sediments and more recently children's plastic toys (Budin et al., 2020). Recommended to be used in implementing environmental risks assessment strategies at EU (European Law 711/2013) as for instance in the Water Framework Directive (EC 2000/602/EC), this effect-based tools could be further implemented since cells are still responsive to other chemicals or complex mixtures present in the sample and activate molecular and biochemical responses by different cellular pathways (Escher et al., 2014; Wernersson et al., 2015). Both European Food Safety Authority (EFSA) and European Chemical Agency (ECHA) recently recognized the importance of including effect-based tools for monitoring and assessment the impact of chemical mixtures in order to better assess the hazard posed to living beings and to be used for an overall ecological risk assessment (EFSA CONTAM Panel, 2019; ECHA 2017). Moreover, in order to identify also substances below their analytical detection limit (Limit of Quantitation, LOQ) and effect threshold, innovative detection methods as bioassays are strongly encouraged (Connon et al., 2012). Chemicals not only activate the transcription of the stably transfected responsive firefly luciferase reporter gene, but also the endogenous genes reflect specific biological effects. Moreover, the presence of other chemicals in the sample extract could activate other cellular pathways which although not detected by the specific reporter gene could still be

detected and analysed. For these reasons, the application of a proteomic approach on CALUX[®] cells could represent a valid method to screen other chemicals present in the sample extract, by using the reaction of the biological system. Relies on proteomic results, elaborations by bioinformatics will identify molecular pathways alterations and link them to the exposure of a class of contaminants or more specifically to a single chemical compound. A case study was thus designed and tested by using soil samples collected from a high industrialized area which were screened for presence of known *AhR* inducers as PCDD/Fs and *dl*-PCBs using DR CALUX[®] bioassay and rat hepatoma cells (H4IIE) processed to extrapolate differentially abundant proteins by proteomics and thus monitor other chemicals including those of emerging concerns (CECs) present in the soil sample able to activate specific cellular pathways. Moreover, differently abundant proteins spots found will be subjected to functional and pathway analysis, in order to highlight potentially altered molecular pathways and suggesting potential link to human pathologies. In order to support the proposed approach of DR CALUX[®] bioassay coupled with proteomics, GC-MS/MS analysis was performed on the soil samples to detect PCDD/Fs, *dl*-PCBs, polycyclic aromatic hydrocarbons (PAHs), pesticides, and GC-FID analysis to determine hydrocarbons C>12 based on National Regulatory Limits list (D.M. 46/2019 and D.Lgs. 152/2006). While for legacy pollutants standardized reliable analytical methods are currently available, there is an urgent need to develop monitoring tools suitable to screen the presence of unknown mixtures of compounds able to exert toxicity including those listed as of emerging concerns (Escher et al., 2020; Neale et al., 2020; Neale and Escher, 2020).

2.2 Materials and Methods

2.2.1 Experimental design

Two soil samples were collected in a 20 m² area at different time: one sample was processed for chemical analysis (winter N +40°32'44.11", E +17°11'12.47") and the other one for bioassay (spring N +40°32'44.11", E +17°11'12.38). The area was in proximity of a large industrial steel and iron ore sinter plant and illegal dumping sites and steel plant's landfills all nearby the city of Taranto (Apulia Region, South Italy).

Located in the South-East of Italy, the city of Taranto has been included among National Priority Contaminated Sites and as an area of high risk of environmental crisis (defined with D.M. n. 426/98). Several monitoring studies have been conducted in this area, mostly based on assessing pollution in environmental matrices by analytical chemistry (Esposito et al., 2012). However, current limitation due to elevated costs of analysis and expertise and time, needed call for the application of bioassays able to screen a significant number of samples/sites and thus prioritize those most problematic in

which actions in terms of mitigation and remediation need to be undertaken (www.amr-insights.eu). Bioassays are not intended as substitute of analytical chemistry tools but they provide those biologically relevant information useful for both ecological and human risk assessment (Escher et al., 2020).

2.2.2 Collection of the soil sample

Topsoil samples (20 cm) were collected in 2018 from a site close to the steel plant landfill *Mater Gratiae*, located in the North of the industrial setting and in the framework of a large monitoring project on soil pollution conducted in the Taranto municipality. A composite topsoil sample (5 kg) made up with 3 sub-sample taken in a 20 m² areas, was obtained using a stainless-steel spade according to Salminen et al. (2005) and immediately shipped in static refrigerated plastic box containers to the University of Siena, for extraction, purification and DR CALUX[®] bioassay and proteomics analysis. DR CALUX[®] bioassay in the present study followed the Dutch standard method RIKZ-SPECIE 07 (2006a, b). The analytical protocols of the DR CALUX[®] bioassay for soil samples have been described in detail elsewhere (Behnisch et al., 2001a; Besselink et al., 2004).

2.2.3 Topsoil sample extract preparation for DR CALUX[®] bioassay

Briefly, topsoil composite sample (60 g) was lyophilized (*Labogene, Scanvac cool safe*) and sieved (2 mm) and an aliquot (5 g) extracted in an accelerated solvent extraction (*Dionex ASE 350, Thermo Scientific*) with a mixture of hexane/acetone (9/1 v./v.). Extract was purifying from sulphurs with a tetrabutylammonium hydrogen sulphate solution in HPLC grade water supplemented with sodium sulphate and isopropanol and concentrated under a nitrogen evaporator (*Caliper Life Science Turbo Vap II*). Using silica column acidified with sulfuric acid at 33% and 20%, the fractions contained PCDD/Fs and *dl*-PCBs were eluted with a mixture of n-Hexane/Diethyl ether (97/3 v./v.). The resulting purifying sample was concentrated under a nitrogen evaporator near dryness, dissolved in 25 µL of dimethyl sulfoxide (DMSO) and finally stored at 4 °C before running the bioassay.

2.2.4 DR CALUX[®] bioassay

DR CALUX[®] bioassay were purchased by BioDetection Systems (Amsterdam, Netherlands) (www.bds.nl). Cells were routinely cultured in α -MEM supplemented with 10% heat-inactivated foetal calf serum and penicillin-streptomycines in a sterile incubator (37 °C, 5% CO₂, 100% humidity). After having confluent cells in the cell culture tubes, they were seeded in 96-well plates (1×10⁴ cells/well density). One day after plating, the cells were exposed to a concentration series (range 0-3

pM) of reference toxic compound (2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)) and sample extract (diluted 30x in culture medium). The concentration of DMSO was fixed at 0.8% in order to avoid cytotoxicity effects (5.6 µl TCDD/topsoil extracts were added to 350 µl α -MEM). The plates were incubated at 37 °C for 24 h. After 24 h of incubation, DR CALUX[®] cell monolayers were observed under an inverted microscope (*Nanjing Jiangnan Novel Optics NIB-100 F*). The medium was discarded, and the cells were washed with Phosphate Buffer Saline (PBS) and lysed with LucLite buffer in PBS. Luciferase activity was measured using a Victor 3 luminometer (*Perkin Elmer*) with the addition of luciferin. Luciferase activity was expressed as relative light units (RLUs), and then transformed into 2,3,7,8-TCDD Bioanalytical Toxicity Equivalents (BEQ). BEQs value were measured of two extracts of PCDD/Fs and *dl*-PCBs towards 2,3,7,8-TCDD calibration curve and finally expressed as ng 2,3,7,8-TCDD BEQ/kg dry weight (d.w. soil sample). The 2,3,7,8-TCDD sigmoid semi-logarithmic dose-response relationship was fitted by the Hill equation ($R^2 > 0.98$, $p < 0.001$). BEQ values were required to meet all the QA and QC criteria of bioassay standards in the Netherlands (R^2 calibration series > 0.98 ; EC₅₀ reference compound between present limits; SD triplicate analysis $< 15\%$) (RIKZ-SPECIE 07, 2006a, b).

2.2.5 DR CALUX[®] cells preparation for protein extraction

For the preparation of DR CALUX[®] rat hepatoma cells (H4IIE) for protein extraction, cell culture followed the same procedures already described for PCDD/Fs and *dl*-PCBs extract analysis (DxCS), but cells incubation and exposure were performed in 6-well plates instead of the 96 ones. In addition, in order to exclude signal variations due to DMSO (dimethyl sulfoxide, which is the solvent used for sample resuspension), cells were also treated with DMSO alone (DMSO) at the final concentration of 0.8% which is the percent in cells during exposure which not cause cytotoxicity. A control sample with only cells neither exposed to PCDD/Fs and *dl*-PCBs extract nor DMSO (0.8%) was also included (Ctrl). For each sample, 2 wells were used, in order to collect enough cells sample and consequently enough protein to be quantify. The amount of cell suspension and reagents were modified for larger wells and therefore 16 µL of samples (the same used for bioassay analysis) were added to 1 mL of growth medium. After 24h of incubation, all the media was collected in a falcon tube, the cells were washed, trypsin was added, and the cell suspension resuspended in the growth medium. Finally, cells suspensions were collected in the same falcon tubes used previous and in order to remove the medium and the related phenol red (which could interfere with the proteomic analysis), the falcon tube was centrifuged for 5 min at 1100g at room temperature. Subsequently, all the media was removed, and the pellets were washed 3 times with 1 mL of PBS using the same centrifugation conditions. Lastly, PBS was discarded, and the sample pellets collected at -80°C until their use. Cells pellets were re-

suspended for the proteomic analysis in 70 µl of lysis buffer (7M Urea, 2M Thiourea, 4% (w/v) CHAPS and 1% (w/v) DTE) to extract the whole protein content. Protein concentration was then estimated using a Bradford assay (Harlow and Lane, 2006) before proceeding with two dimensional electrophoresis. All sample aliquots were stored at -80°C until use.

2.2.6 High resolution 2D-Electrophoresis

2DE, performed on an immobilized nonlinear pH gradient, from pH 3 to 10, 18 cm in length, from GE Healthcare (Uppsala, Sweden), and image analyses were carried out as reported by Ontañón et al., (2018). Spots were considered differentially abundant when the percentage of relative volume (%V) means ratio was greater than 1.8-fold with a valid statistical test. The differently abundant spots for %V ratio was subjected to statistical analysis by *non*-parametric Kruskal-Wallis test ($p \leq 0.05$) by RStudio Desktop 1.1.463 (Integrated Development for RStudio, Inc., Boston, USA, <https://www.rstudio.com>). Significant results were followed by comparisons of mean ranks by Dunn's test showing also p-adjusted and z-value.

2.2.7 Mass spectrometry by MALDI ToF-ToF

Detected spot differences were excised from MS-compatible silver staining gel and analyzed by Autoflex™ Speed mass spectrometer (*Bruker Daltonics, Bremen, Germany*) as earlier reported (Sulpizio et al., 2011) (Supplemental information). Search parameters for proteins identification on Mascot on-line-available software (*Matrix Science Ltd., London, UK*, <http://www.matrixscience.com>), are as follows: Swiss-Prot/TrEMBL and NCBI nr as databases, taxonomy limited to *Rattus* or *Mus musculus*, peptide mass fingerprint enzyme, trypsin; fixed modification, carbamidomethylation (Cys); variable modifications, oxidation of methionine; mass values, monoisotopic; ion charge state was set to +1; maximum mis cleavages was set to 1; mass tolerance of 100 ppm for PMF and 0.6–0.8 Da for MS/MS.

2.2.8 PCA and Heatmap analysis

Differential spots were used to perform multivariate analysis by Principal Component Analysis (PCA) simplifying the amount of data (%V variables) by linear transformation. By PCA it is possible visualize experimental groups in a two-dimensional plane on the basis of the differential spot patterns. PCA was performed by RStudio Desktop 1.1.463 (Integrated Development for RStudio, Inc., Boston, USA, <https://www.rstudio.com>). In order to visualize the behavior of the differentially abundant spots in each gel of the considered conditions, it was performed a heatmap analysis using the %V values

of the statistically significant differentially abundant spots. In particular, the clustering of protein spots was performed using a clustering method and Euclidean distance. The above-mentioned analysis and the related figures were obtained by RStudio Desktop 1.1.463 (Integrated Development for RStudio, Inc., Boston, USA, <https://www.rstudio.com>).

2.2.9 Enrichment analyses

- Gene Ontology Terms by DAVID

The list of the accession number of the identified proteins underwent functional analysis by DAVID Bioinformatic Resources (6.8) online tool (<https://david.ncifcrf.gov/>) to understand biological meaning behind large list of proteins. Functional annotation tool was chosen, and our accession numbers were uploaded selecting “uniprot_accession” as identifier and “gene list” as list type. Gene Ontology was selected among the Annotation Summary Results.

- Enrichr

Enrichr software is a comprehensive resource for curated gene sets, freely available at: <http://amp.pharm.mssm.edu/Enrichr> (Kuleshov et al., 2016). Search engine accumulated biological knowledge for further biological discoveries thanks to 332,911 terms, 164 libraries and 24,887,248 lists analysed. In order to perform the analysis, we submitted the gene names of the identified proteins. Enrichr contains many features and data sets such as Transcription, Pathways, Ontologies, Diseases/Drugs, Cell Types. In particular, for our analysis we took into consideration results obtained by “Pathways” and “Disease/Drug” data sets. Among “pathways” have been selected BioPlanet2019 and among “Disease/Drug” was chosen DSigDB. Both reports statistical data by p-value, p-adjusted and related genes that belong to the referred class.

2.2.10 UniProt BLAST for human proteins similarity

In order to find human proteins with higher similarity with the *rattus* and *mice* identified proteins, we performed a BLAST analysis by the UniProt tool. Once submitted the accession number of the identified protein on UniProtKB (<https://www.uniprot.org/>) we performed the BLAST selecting: blastp as program, blosum62 as matrix and a threshold of 10. The database of reference was SwissProt.

2.2.11 Diseases (by biomarkers) analysis by MetaCore

Identified protein spots and similar human proteins were further processed by MetaCore 6.8 network building tool (Clarivate Analytics). MetaCore includes a manually annotated database of protein interactions, metabolic reactions and diseases obtained from the scientific literature. The accession number of the two lists of proteins were uploaded into MetaCore and processed. Enrichment analysis was based on the hypergeometric distribution algorithm and relevant “process networks” and “diseases (by biomarkers)” results were then prioritized according to their statistical significance and comparted among the two lists of proteins.

2.2.12 Chemical analysis of topsoil samples

A freeze-drying procedure was performed before starting the extraction of chemical pollutants from the collected topsoil sample. Briefly, an amount of 80 g of soil was lyophilized for 48 h (*Labogene, Scanvac cool safe*) using a pressure and temperature of respectively 0.5 torr and -50°C, in order to achieve a constant weight value. To remove the higher grain-size particles, sample was sieved (2 mm Ø) and stored until the chemical analysis.

Seventeen 2,3,7,8-substituted PCDDs/PCDFs (7 PCDDs and 10 PCDFs) and *dl*-PCBs were analysed by gas-chromatography (*GC Trace 1300, Thermo Scientific*) coupled to mass-spectrometry (*TSQ 8000, Triple Quadrupole, Thermo Scientific*) using different GC programmes and under optimized detection conditions. Isotope dilution method was applied for the quantitation according to 1613B and 1668C EPA methods. For this aim, before extraction, soil sample was fortified with a proper amount of labelled standard compounds, a mixture of ¹³C₁₂- PCDD/Fs and ¹³C₁₂-PCBs, to evaluate the recovery. GC-MS/MS analysis of a sample was accepted when the recovery of the labelled compounds resulted to be in the range reported in EPA methods. Estimated concentrations for each detected analyte of PCDD/Fs and *dl*-PCBs were expressed in term of toxic equivalency (TEQ), resulting from the product of the concentration and individual Toxic Equivalent Factor, TEF, of each congener (Van den Berg et al., 2006) (see Appendix Tab A1).

Polycyclic Aromatic Hydrocarbons (PAHs), such as pyrene, benzo(a)anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-c,d]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, dibenzo[a,l]pyrene, dibenzo[a,i]pyrene, dibenzo[a,e]pyrene and dibenzo[a,h]pyrene, some organochloride pesticides and a herbicide were also determined according to what required from the Italian National Regulation, D.Lgs. 152/2006. In particular, GC-MS/MS method of the pesticides was optimized for alachlor, aldrin, dieldrin, atrazine, chlordane, α, β, γ isomers of exachlorocyclohexane, *p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD. The quantification of each compound was performed by internal standard method according to the

8270 EPA method. As described for PCDD/Fs and PCBs, the recovery of PAHs and pesticides was evaluated by using surrogate standard to be added to the sample before extraction according to 8270 EPA method. Recovery values for the extraction standards in the range between 70-120% were considered acceptable.

The determination and quantitation of C>12 Hydrocarbons was performed according to the procedure No 75/2011 reported by ISPRA, Institute for Environmental Protection and Research (available at <https://www.isprambiente.gov.it/contentfiles/00010400/10425-mlg-75-2011.pdf/>). This method is applicable to the determination of all hydrocarbons with retention time between n-dodecane (C₁₂H₂₂) and n-tetracontane (C₄₀H₈₂), including isoalkanes, cycloalkanes, alkylbenzenes, alkyl naphthalenes and polycyclic aromatic hydrocarbons. C>12 Hydrocarbons were detected by GC-FID. The linearity of the analytical method was explored in the concentration range between 30 and 150 mg/kg. The integration of the chromatogram takes into account the time window delimited by the two hydrocarbons, n-dodecane and n-tetracontane.

2.3 Results

2.3.1 DR CALUX[®] assay and confirmation by GC-MS/MS analysis

DR CALUX[®] BEQ value calculated for the topsoil sample was 10.6 ± 2.2 ng 2,3,7,8-TCDD BEQ/kg d.w. Chemical analysis performed with GC-MS/MS in Taranto's topsoil sample showed a value of 59 ± 17 ng TEQ/kg d.w. TEQ_{WHO} (Σ PCDD/F/*dl*-PCBs) and 55 ± 16 ng TEQ/kg d.w. TEQ_{WHO} (Σ PCDD/Fs). In terms of congeners profile, furans resulted the main contributors with 70% of the total (2.3% TCDF, 43.2% PeCDF, 22% HxCDF, 2.4% HpCDF, and 0.1% OCDF). The remaining part was mainly due to dioxins (23.3%), which congener profiles were: 5.9% TCDD, 15.5% PCDD, 1.5% HxCDD, and 0.4% HpCDD.

Chemical analysis identified the presence of other carcinogenic and mutagenic compounds as PAHs and C>12 in the topsoil sample. Σ PAHs as 0.011 mg/kg d.w. showed the prevalence of benzo[*b*]fluoranthene (69.4%) and benzo[*k*]fluoranthene (30.6%), while Σ C>12 was 70.4 mg/kg.

As far as pesticides, they resulted all below the quantification limits, LOQs, the lowest concentrations that can be quantified with statistical significance by means of a given analytical method. LOQs were calculated by the analysis of six replicated of a topsoil sample (blank sample) fortified at the lowest instrumental concentration level.

3.3.2 DR CALUX[®] proteomics in topsoil extract

All cell samples were resolved by 2DE and final gels images were compared by Image Master Platinum software by intra- and inter-class analyses in order to extrapolate quantitative and qualitative differences. Proteomic analysis performed among the three set of samples analyzed as Ctrl (only cells), DMSO (cells exposed to 0.8% DMSO) and DxCS (cells exposed to PCDD/Fs and *dl*-PCBs soil extract in 0.8% DMSO) reveals 43 differentially abundant spots and statistical results, expressed as means and standard deviation (SD) of the %V were reported in Table SI 2.1. Moreover, Kruskal Wallis and Dunn's test with z-value, p-value and p-adjusted and the %V means ratio between the conditions, were reported. In Table SI 2.1, data marked in red were not statistically different (the %V means ratio Ctrl-DMSO were lower than 1.8-fold).

In the Figure 2.1 the 3 master gels were shown and 43 abundant protein spots differentially abundant were highlighted (red numbers and arrows). Again, no differences between Ctrl and DMSO exposed cells were found. Our data further proved that DMSO do not alter the protein abundance with respect to Ctrl, while differences were maintained between DxCS *vs* Ctrl and DxCS *vs* DMSO.

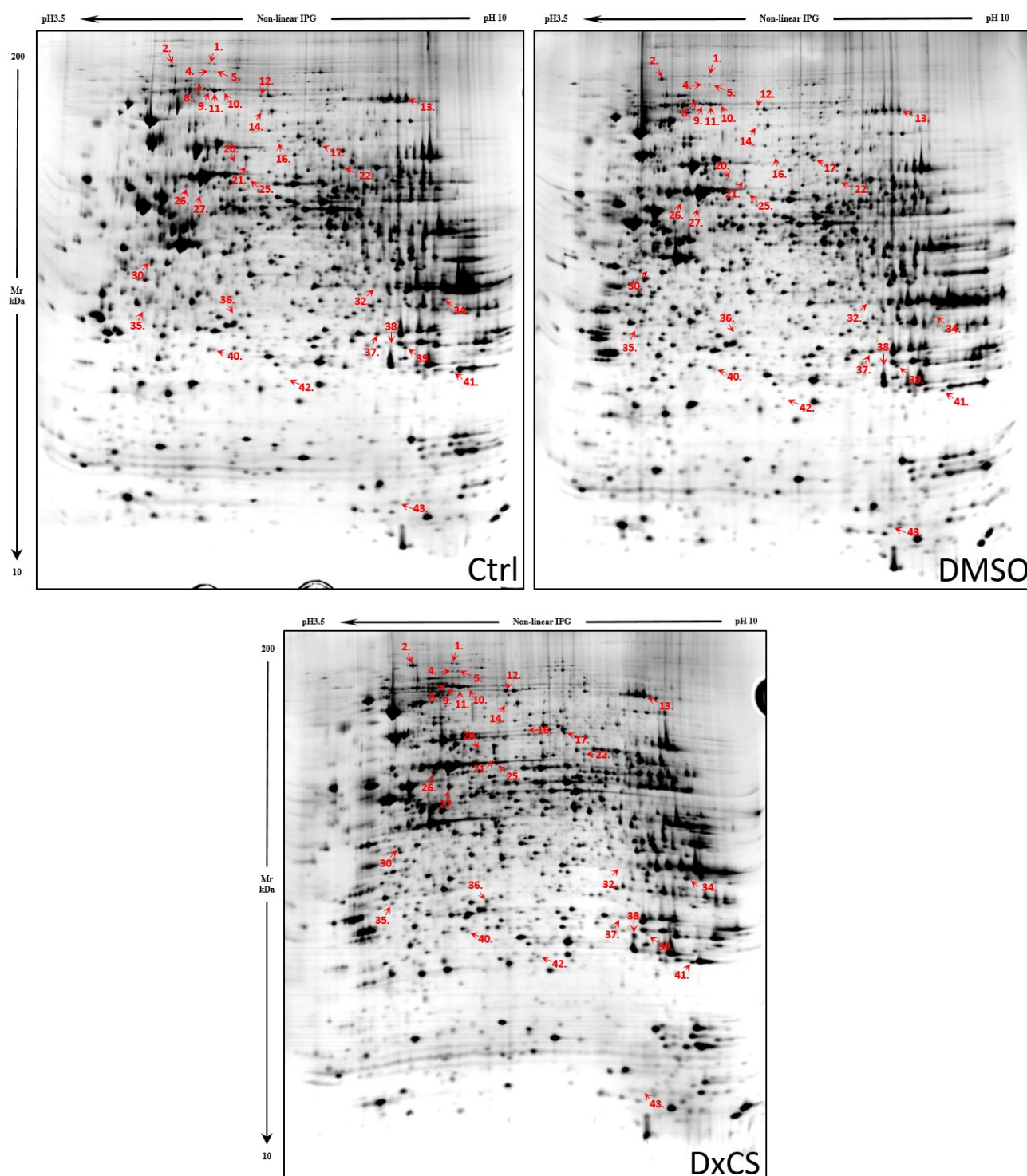


Fig. 2.1 Master gel of the three analysed conditions. Red numbers and arrows indicate the differentially abundant spots among Ctrl, and DxCS. Differences are also reported in the gel obtained from DR CALUX[®] treated with DMSO in order to highlight the same behavior between Ctrl and DMSO protein spots.

These differences were reported in the 3 Master gels obtained from DR CALUX[®] in Ctrl samples, in DxCS and in DMSO, in order to visualize the same behavior between Ctrl and DMSO protein spots. To better emphasize our results and show that DMSO do not alter the protein abundance with respect to the Ctrl, we also performed the Principal Component Analysis (PCA). The aim of PCA was to

simplify the amount of data (%V variables) by linear transformation. Complexity is reduced by limiting, in terms of variance, the analysis to the principal new variables. By this simplification it is possible to observe the distribution of each sample in a two-dimensional plane and easily visualize any experimental groups (samples) on the basis of the differential spot pattern (Fig. 2.2).

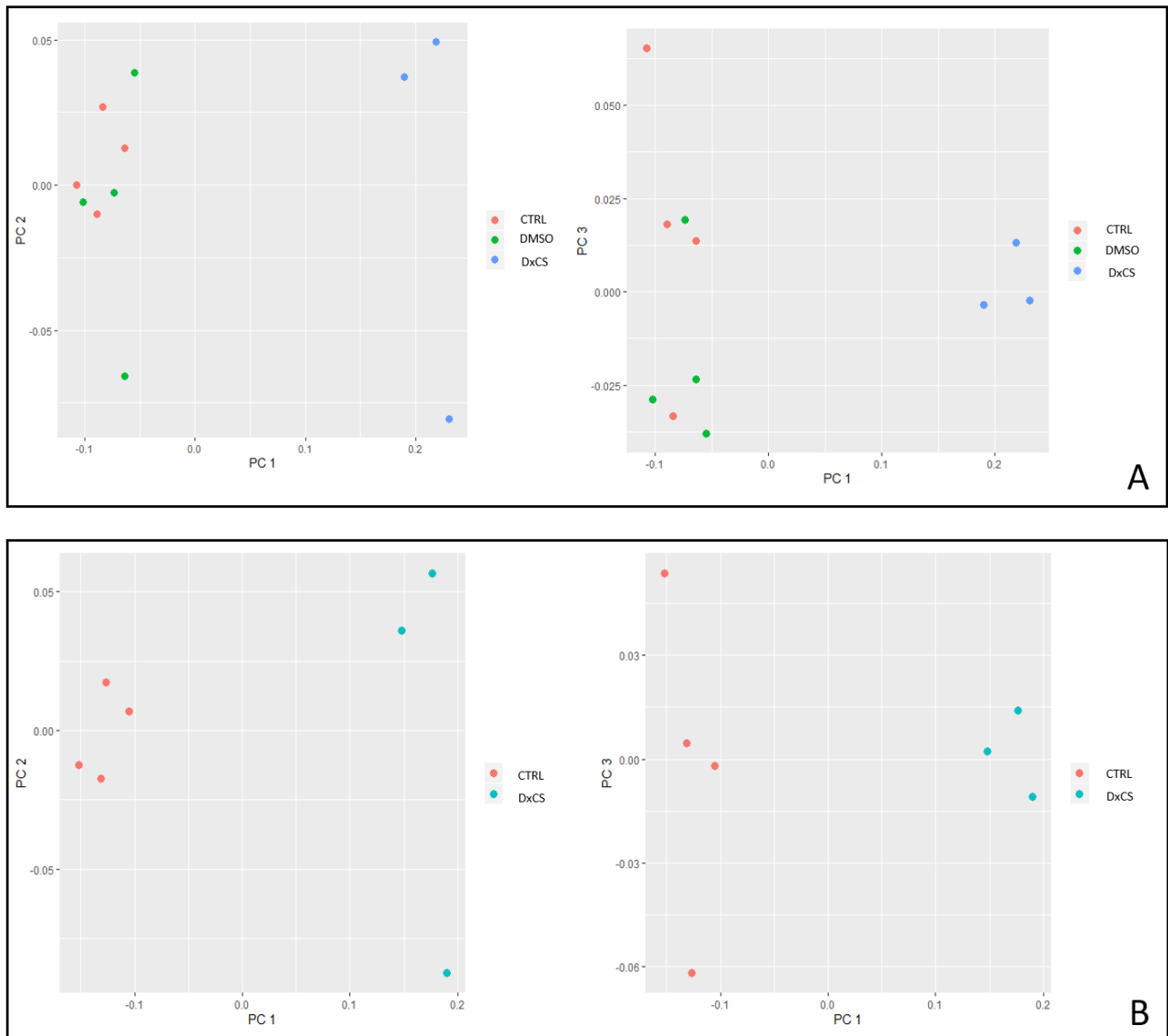


Fig. 2.2 PCA performed with the %V of the differentially abundant spots. **(A)** PCA considering the analysed 3 conditions. **(B)** PCA considering Ctrl vs DxCS. Both the analysis shows 2 different point of view; PC1 and PC2, PC1 and PC3.

In figure 2.2 A the PCA of the three conditions (Ctrl, DMSO, DxCS) was reported from two different point of view (PC1 vs PC2 and PC1 vs PC3), Ctrl perfectly co-localize with DMSO samples. On the other hand, DxCS samples localize in the opposite side of Ctrl and DMSO, along the PC1. Figure 2.2 B reports the PCA of the comparison between Ctrl vs DxCS and the sample distribution of the two

conditions remains well distinct along the PC1. Both these results highlighted that DMSO do not alter protein abundance with respect to controls while DxCS extract treatment alter the DR CALUX[®] cells showing different protein abundance.

In order to well visualize the differential protein pattern in each sample, we also performed the heatmap analysis (Fig. 2.3).

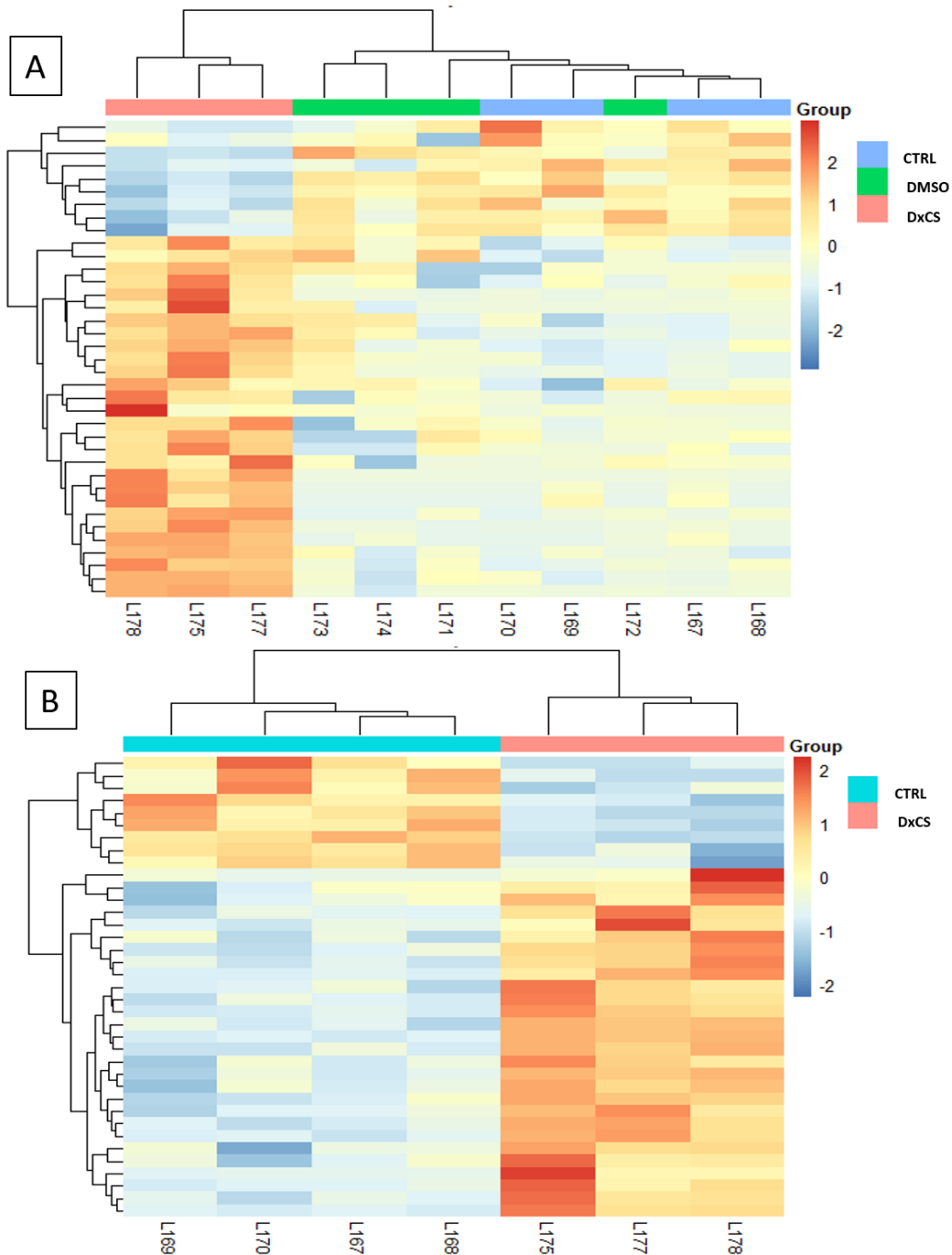


Fig. 2.3 (A) Heatmap analysis of the three analyzed groups (Ctrl, DMSO, DxCS) and **(B)** between Ctrl vs DxCS. L167 to L178 were gels codes. Spots were considered differently abundant between two conditions, when the ratio of the %V means was greater than ± 2 . The different color scale indicates with +2 = red, the condition in which that spot is more abundant, as it lightens and turns to blue, the abundance of the spot decreases to the lowest value, indicated with -2 = blue.

To this purpose, figure 2.3 A reported the heatmap showing the differential spots abundance in each considered sample in the three conditions. It is immediately visible that spot abundance between Ctrl and DMSO has a similar distribution (light blue and light red rectangles had the same distribution) with respect to DxCS treatment (light blue and light red rectangles between Ctrl/DMSO vs DxCS had a completely opposite distribution). Furthermore, figure 2.3 B reports the well visible opposite behavior between Ctrl and DxCS.

Proteins identification was performed by MALDI-ToF mass spectrometry, and Table 2.1 reports the 26 identified proteins with the spot number, Protein name, UniProt name, UniProt and/or NCBI accession number, gene names, accession number of similar human protein, the identity between the two species, theoretical pI and MW and Mascot Search Results (Score, Matched Peptides and Coverage %) (Tab. 2.1).

Tab. 2.1 MALDI-ToF mass spectrometry analysis. Table reports the 26 identified proteins with the spot number, Protein name, UniProt name, UniProt and/or NCBI accession number, gene names, accession number of similar human proteins, the identity between Homo sapiens and rattus/mus musculus species, theoretical pI and MW and Mascot Search Results (Score, Matched Peptides and Coverage %).

Spot n	Protein name	UniProt name	UniProt/NCBI AC	Gene names	Human similarity	Identity	Theoretical pI/MW	Mascot Search Results		
								Score	Matched Peptides	Coverage %
2	Hypoxia up-regulated protein 1	HYOU1_RAT	Q63617	Hyou1	Q9Y4L1	91.7%	5.11 11448	106	27	26
3	Phosphoribosylformylglycinamide synthase	PUR4_MOUSE	Q5SUR0	Pfas	O15067	88.5%	5.43 146248	82	43	36
7	Ubiquitin-like modifier-activating enzyme 1	UBA1_RAT	Q5U300	Uba1	P22314	96.4%	5.36 118910	93	53	49
8	Heat shock protein 105 kDa	HS105_RAT	Q66HA8	Hsph1	Q92598	92.3%	5.40 97327	108	51	52
9	Major vault protein	MVP_RAT	Q62667	Mvp	Q14764	91.5%	5.43 96081	105	48	55
10	Presequence protease, mitochondrial		NP_001100833.1	Pitrm1	Q5JRX3	83.4%	6.94 109559	112	45	44
	Pitrylsin metallopeptidase 1 (Predicted)	D3ZUF9_RAT	D3ZUF9							
11	Alanine-tRNA ligase, cytoplasmic	SYAC_RAT	P50475	Aars1	P49588	95.1%	5.41 107522	140	61	59
13	Elongation factor 2	EF2_RAT	P05197	Eef2	P13639	99.2%	6.41 96192	210	55	57
17	Heat shock protein 75 kDa, mitochondrial	TRAP1_RAT	Q5XH20	Trap1	Q12931	88.2%	6.56 80639	233	51	58
18	Kinesin light chain 4	KLC4_RAT	Q5PQM2	Klc4	Q9NSK0	95.8%	5.89 69376	229	52	66
20	Ras GTPase-activating protein-binding protein 1		NP_598249.1	G3bp1	Q13283	93.4%	5.41 51812	95	20	40
		G3BP1_MOUSE	P97855							
21	Myotonin-protein kinase isoform X1		XP_006223166.1	Dmpk	Q09013	86.3%	5.02 76780	40	10	26
		DMPK_MOUSE	P54265							
24	Asparagine synthetase [glutamine-hydrolyzing]	ASNS_RAT	P49088	Asns	P08243	93.6%	6.01 64776	172	47	68
25	Serine/threonine-protein kinase PAK 2	PAK2_RAT	Q64303	Pak2	Q13177	96.9%	5.37 58209	86	28	47
26	Actin, cytoplasmic 2 +	ACTG_RAT	WP_094948603.1	Actg1	P63261	100%	5.29 42052	95	22	48
			P63259							
	POTE ankyrin domain family member F isoform X2, partial		XP_002725368.2				5.62 45204	80	21	40
29	Quinone oxidoreductase-like protein 1		NP_001013062.1	Cryz11	O95825	90.5%	5.59 39044	143	27	65
		QORL1_MOUSE	Q921W4							
30	Serine-threonine kinase receptor-associated protein	STRAP_RAT	Q5XIG8	Strap	Q9Y3F4	97.7%	4.99 38717	99	29	75
31	Transaldolase	TALDO_RAT	Q9EQS0	Taldo1	P37837	94.7%	6.57 37608	210	30	56
33	Pyridoxal kinase	PDXK_RAT	O35331	Pdxk	O00764	85.3%	6.32 35114	148	37	71
34	Thiosulfate sulfurtransferase	THTR_RAT	P24329	Tst	Q16762	90.2%	7.71 33614	192	30	60
35	Annexin A5 isoform X1		XP_006232330.1	Anxa5	P08758	91.8%	4.93 35807	71	17	57
		ANXA5_RAT	P14668							

38	Flavin reductase (NADPH)	BLVRB_MOUSE	NP_001099706.1	Blvrb	P30043	93.7%	6.29 22194	141	17	87
			Q923D2							
39	Glutathione S-transferase Mu 2	GSTM2_RAT	P08010	Gstm2	P09488	81.7%	6.90 25857	136	31	90
40	Eukaryotic translation initiation factor 4E	IF4E_RAT	P63074	Eif4e	P06730	98.2%	5.79 25266	71	18	54
41	Peroxiredoxin-1	PRDX1_RAT	NP_476455.1	Prdx1	Q06830	97.5%	8.27 22323	75	16	50
			Q63716							
43	2-iminobutanoate/2-iminopropanoate deaminase	RIDA_RAT	NP_113902.1	Rida	P52758	87.6%	6.21 14338	43	7	64
			P52759							

Once identified differential proteins, we performed a functional annotation by DAVID of the gene ontology terms of our differential proteins. Results were reported in Table 2.2.

Tab. 2.2 Functional annotation using DAVID of the gene ontology terms of differentially expressed proteins reporting Biological Processes (BP), Cellular Component (CC) and Molecular Functions (MF). In details: % of proteins loaded on DAVID that are characterized by that specific gene ontology term; *p*-Value indicates the validity of gene ontology terms for the proteins loaded on DAVID; Benjamini is the statistical value that confirms the *p*-value.

Biological processes Terms	%	<i>p</i> -Value	Benjamini
cellular response to chemical stimulus	46,2	1,80E-05	1,80E-02
cellular response to stress	38,5	3,10E-05	1,50E-02
response to inorganic substance	26,9	5,70E-05	1,90E-02
response to oxygen-containing compound	38,5	6,00E-05	1,50E-02
response to chemical	57,7	1,20E-04	2,40E-02
response to stress	46,2	2,80E-04	4,50E-02
response to endogenous stimulus	34,6	3,90E-04	5,30E-02
regulation of translation	19,2	4,00E-04	4,70E-02
regulation of apoptotic process	30,8	5,20E-04	5,60E-02
regulation of cellular amide metabolic process	19,2	5,20E-04	5,00E-02
regulation of programmed cell death	30,8	5,60E-04	4,90E-02
posttranscriptional regulation of gene expression	19,2	7,50E-04	6,00E-02
regulation of cell death	30,8	9,40E-04	6,90E-02
organonitrogen compound metabolic process	34,6	9,80E-04	6,70E-02
Cellular Component Terms	%	<i>p</i> -Value	Benjamini
extracellular exosome	60	8,20E-09	1,40E-06
extracellular vesicle	60	8,80E-09	7,60E-07
extracellular organelle	60	9,00E-09	5,20E-07
membrane-bounded vesicle	64	1,20E-08	5,00E-07
vesicle	64	2,30E-08	8,00E-07
cytoplasmic part	76	2,40E-07	6,90E-06
cytosol	48	5,70E-07	1,40E-05
extracellular region part	60	6,80E-07	1,50E-05
extracellular region	60	2,60E-06	4,90E-05
cytoplasm	80	5,30E-06	9,10E-05
intracellular part	80	7,00E-04	1,10E-02
intracellular organelle	76	7,40E-04	1,10E-02
Molecular Function Terms	%	<i>p</i> -Value	Benjamini
RNA binding	36	2,80E-04	5,00E-02
purine ribonucleoside triphosphate binding	36	3,90E-04	3,50E-02
purine ribonucleoside binding	36	4,00E-04	2,40E-02
ribonucleoside binding	36	4,10E-04	1,90E-02
purine nucleoside binding	36	4,10E-04	1,90E-02
nucleoside binding	36	4,20E-04	1,50E-02
purine ribonucleotide binding	36	4,60E-04	1,40E-02
purine nucleotide binding	36	4,80E-04	1,30E-02
ribonucleotide binding	36	4,90E-04	1,10E-02
ATP binding	32	6,00E-04	1,20E-02
adenyl ribonucleotide binding	32	7,10E-04	1,30E-02
adenyl nucleotide binding	32	7,40E-04	1,20E-02
small molecule binding	40	8,60E-04	1,30E-02

Among Biological Processes (BP) there were: i) cellular response to chemical stimulus; ii) cellular response to stress; iii) response to inorganic substance; iv) regulation of translation; v) regulation of apoptotic process as well as vi) response to organonitrogen compounds. Moreover, differential proteins came from extracellular exosomes, vesicles, but also from cytoplasmic portion as reported by “Cellular Component” (CC) results. “Molecular Functions” (MF) analysis reports RNA binding,

purine ribonucleoside triphosphate binding, adenylyl nucleotide binding, ATP binding, nucleotide binding.

Furtherly, we performed enrichment analysis by Enrichr highlighting chemical substances related to our proteins by DSigDB (Tab. 2.3) such as vorinostat, troglitazone, chlortetracycline, clonidine, lobeline, potassium dichromate, atrazine, clindamycin and copper sulfate and molecular pathways by BioPlanet2019 database (Tab. 2.4). Both tables report “terms”, *p*-values and gene names of differential proteins related to the terms.

Tab 2.3 Enrichment analysis of the identified proteins by Enrichr software. Table reports chemical substances related to our proteins by DSigDB with relative *p*-values and gene names of differential proteins related to the terms.

Term	<i>p</i> -value	Genes
(17S)-17-hydroxy-13,17-dimethyl-1,2,6,7,8,14,15,16-octahydrocyclopenta[a]phenanthrene-3-one CTD 00007088	1,39E+07	PDXK;PRDX1;ASNS;TALDO1;HYOU1;EEF2;PAK2;ACTG1
thapsigargin CTD 00007263	1,83E+10	TRAP1;TST;MVP;G3BP1;ANXA5;ASNS;HYOU1;PFAS;ACTG1
vorinostat CTD 00003560	1,43E+11	TRAP1;PRDX1;ANXA5;TALDO1;STRAP;BLVRB
troglitazone CTD 00002415	1,53E+12	HSPH1;MVP;PRDX1;ANXA5;ASNS;ACTG1
chlortetracycline HL60 DOWN	2,94E+12	PDXK;HSPH1;TST;PRDX1;CRYZL1;UBA1;PAK2
clonidine HL60 DOWN	3,74E+11	HSPH1;PFAS;EIF4E
lobeline HL60 DOWN	4,72E+12	PDXK;HSPH1;G3BP1;CRYZL1;TALDO1;PFAS;PAK2;EIF4E
perhexiline CTD 00006493	4,79E+11	ASNS;EIF4E
Potassium dichromate CTD 00006598	6,61E+11	TRAP1;MVP;PRDX1;ASNS;UBA1
atrazine CTD 00005450	6,70E+11	TRAP1;GSTM2;HSPH1;TST;MVP;PRDX1;G3BP1;ANXA5;CRYZL1;ASNS;PFAS
cypheptadine PC3 UP	7,38E+11	ASNS;HYOU1
Clindamycin HL60 DOWN	7,75E+11	TRAP1;PDXK;PITRM1;PRDX1;CRYZL1;ASNS;TALDO1;PAK2
copper sulfate CTD 00007279	8,72E+10	TRAP1;PDXK;MVP;ANXA5;ASNS;TALDO1;ACTG1;KLC4;HSPH1;PITRM1;TST;G3BP1;CRYZL1;BLVRB;PAK2;EIF4E
tanespimycin SKMEL5 UP	9,42E+11	HSPH1;ASNS

Since the importance that the impact of some contaminants as dioxin could have on the human health, we also perform a protein BLAST of the rat and mouse differential proteins in order to find homologues human proteins reported in Table 2.1. With the two lists of proteins (rat and human) we performed a new enrichment analysis by MetaCore software in order to extrapolate both “Disease (by biomarkers)” results. Figure 2.4 reports the histograms representing the probability that our proteins are involved in a particular pathology. The histograms indicate not only the possible pathology in which proteins can be considered potential biomarkers, but the length also indicates the statistical validity.

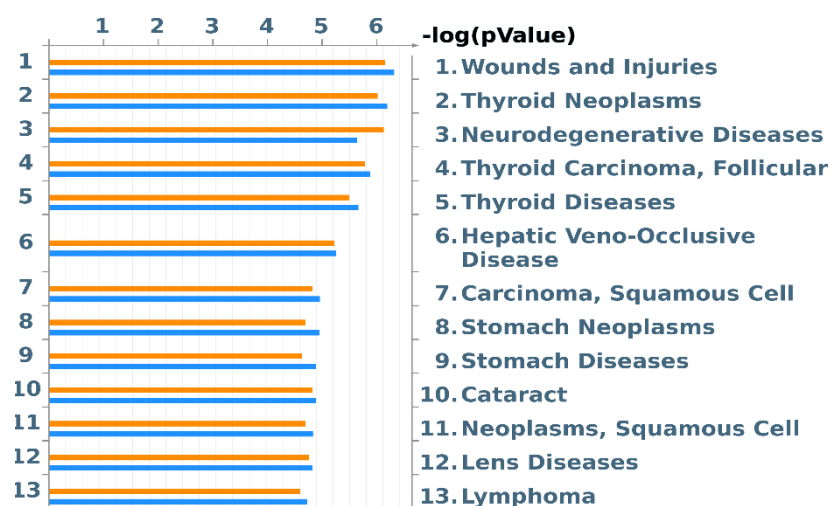


Fig. 2.4 Enrichment analysis by MetaCore of Diseases (by biomarkers). Orange histograms represent enrichment for human homologs proteins. Blue histograms represent enrichment for differential rat proteins.

Orange and blue histograms behave respectively to human and rat proteins. As showed, histograms are very similar reporting a generic wound and injuries, but also thyroid neoplasms, neurodegenerative diseases, other type of cancers such as squamous cell carcinoma, stomach neoplasm and lymphoma.

2.4 Discussion

Today, it is still difficult to elucidate all the chemical stressors released in the environment and their interaction with humans and environment itself, but the application of high-throughput effect-based tools such as *in vitro* bioassays could help to assess ecological and human risks for monitoring complex environmental samples (Escher et al., 2020). To this purpose, we report a screening of a soil sample collected from the North of Taranto industrial area close to the steel plant landfill *Mater Gratiae* by coupling the DR CALUX® bioassay with proteomics and validated with GC-MS/MS analysis. Taranto represents one of the historical most productive districts in Italy. It includes the largest integrated steelworks in Europe, a crude oil refinery, three power plants, the third largest naval port in Italy and a cement factory. These activities cause the production of 3.25 Mt of solid waste and by-products (Notarnicola et al., 2016). Moreover, in Taranto province several sites present a contamination by abandonment of waste, illegal landfills, spreading of waste and sludge, accidental spills of oils, hydrocarbons and hazardous waste, presence of asbestos and self-demolition waste (ARPA Puglia, 2008). According to the study of Giua and co-authors (2009) the steel plant industry is the main responsible of Italian total emission of Polyhalogenated dioxins, furans and PCBs. In particular it produces the 90% of Italian industrial dioxin and 6.9% of the European one. In addition

Taranto's population has been the subject of many epidemiological studies that emphasize the close relation between environmental pollution and human health (Giua et al., 2005; Pirastu et al., 2014, 2013; Zona et al., 2019). Since the first studies, a trend in the risk of lung and pleural cancer was identified in association with the distance of the residence from most emission sites (Bertollini et al., 1997; Marinaccio et al., 2011; Martinelli et al., 2009; Martuzzi et al., 2002; Vigotti et al., 2014).

Different illegal dumping sites have been identified nearby and during the topsoil collection, the disposal of a radioactive dumping site was in progress at 8 km distance (ARPA Puglia, 2008; Zona et al., 2019). Illegal landfill of hazardous materials containing asbestos (Comba et al., 2012) and their accidental burning have been also documented (Mazza et al., 2015; Pascuzzi et al., 2013).

The DR CALUX[®] BEQ value of Taranto topsoil sample results comparable to those reported by Du et al., (2011) for soils collected in proximity of a waste incineration plant in China and slightly below those from dioxins contaminated soils in Taiwan (Lin et al., 2014) and open-burning surface soils in Vietnam (Suzuki et al., 2016). GC-MS/MS analysis confirmed that levels of $\sum$ PCDD/F/dl-PCBs are above National Regulatory Limits set at 6 ng TEQ/kg d.w. by Italian D.M. 46/2019 as well as those of $\sum$ PCDD/Fs set at 10 ng TEQ/kg d.w. by Italian D.Lgs. 152/2006. Carcinogenic PAHs were also found such as benzo[*b*]fluoranthene and benzo[*k*]fluoranthene, which show the highest REP among all PAHs, equal to 5 for benzo[*b*]fluoranthene and 3.7 for benzo[*k*]fluoranthene (Pieterse et al., 2013). Moreover, C>12 also resulted above Regulatory limits set as 50 mg/kg according to D.M. 46/2019 and D.Lgs. 152/2006.

Proteins identified by proteomics, once proved that DMSO does not alter the differential protein pattern compared to Ctrl samples, were processed by enrichment analysis. We showed a series of chemicals and molecular interactors, cellular pathways, biological functions and diseases, where these proteins are considered biomarkers, suggesting potential links to human pathologies.

DAVID analysis of the differential proteins reveals that Biological Processes are referred to cellular response to chemical stimulus, cellular response to stress, response to inorganic substance, regulation of translation, regulation of apoptotic process as well as response to organonitrogen compounds, suggesting as the differential protein pattern that we found is strongly associated to chemical substances' exposure. Their Molecular Functions are related to RNA binding, purine ribonucleoside triphosphate binding, adenylyl nucleotide binding, ATP binding, nucleotide binding, suggesting that modified proteins are implicated in post transcriptional modification mechanisms, as also suggested by BP, inferring in protein metabolism. Cellular Component terms report most of the differential proteins in extracellular exosomes (EEs) and vesicles (EVs), organelles that play a key role in cell-to-cell communication and reported to be enhanced after environmental toxins or carcinogens exposure, triggering genotoxic or mutagenic changes in susceptible cells (Harischandra et al., 2017).

Following these results, we were interested to know potential chemicals/drugs in relationship with our differential proteins by DSigDB Enrichr tool showing 14 statistically significative compounds. The majority of the compounds found in the Taranto topsoil were drugs belonging to chemotherapy, antidiabetic, anti-inflammatory, antibiotics and antidepressants. Those presences in Taranto's soil could be related to the epidemiological condition which occurs in the populations, both in adult and children (Zona et al., 2019). It is well known the onset on neoplastic pathologies are related to professional and environmental exposure that occurs in Taranto due to a mixture of different types of contaminants organic and inorganic (Pirastu et al., 2013). Based on the data processed by the Local Health Authority (ASL) of Taranto of the Tumour Registry of the Province of Taranto (years 2006-2012), after the respiratory neoplasm, prostate and bladder tumours represent the most frequent pathologies in the inhabitants (<http://asset.regione.puglia.it/>). Therefore, the presence of tanespimicin, employed in younger patients affected by different types of leukaemia or thapsigargin, specifically used against prostate cancer, resulted plausible. Moreover, the presence of lomustine, an active compound used to treat tumour during chemotherapy was also inferred, as well as fluorouracil, one of the most used anticancer chemotherapy agent in cancer clinics in the adjuvant therapy of pancreatic tumours, all documented to be extremely spread in Taranto man population (Zona et al., 2019). According to a recent report, from 2013 to 2017 an increase in the use of cancer drugs from 8.9 to 9.6 doses for thousand inhabitants has been reported in the Apulia region (Rapporto OsMed AIFA, 2019). Moreover, between 2003 and 2009 an excess risk in Taranto inhabitants for melanoma, *non*-Hodgkin lymphoma and myeloid leukaemia has been also documented (Pirastu et al., 2013). In agreement to these reported pathologies, the presence of the drug vorinostat used for the treatment of skin manifestations in patients with cutaneous T-cell *non*-Hodgkin lymphoma and combined with other drugs for brain tumours is also inferred (Grant et al., 2007).

Moreover, the presence of another drug as tanespimicin, developed for the treatment of different types of cancer, in particular for chronic myeloid leukaemia is revealed (Erllichman, 2009). It is known that type 2 diabetes mellitus increased the risk of developing a neoplasm and in Taranto it has been recorded that 9.6% of the assisted population have type 2 diabetes. In agreement to this, the presence of troglitazone and glibenclamid, anti-diabetic and anti-inflammatory drugs, often used in the management of diabetes mellitus were found. The usage of anti-diabetic in 2017 in Taranto were 74.7 doses per thousand inhabitants, a higher value compared to National Italian average reported around 62.7 doses for thousand inhabitants (Rapporto OsMed AIFA, 2019). In addition, correlated to health disease cyclosporine, one of the main therapeutic agents used to block the rejection of organs transplantation was also inferred.

Other types of drugs were found as for instance clonidine, lobeline and chlorpromazine all used in treatment of drug dependence against amphetamines, opioid or cocaine, all well documented to be used in Taranto young population (Cecinato and Balducci, 2007; Relazione annuale tossicodipendenze in Italia, 2019). Moreover, we found dronabinol in the topsoil, the main psychoactive principle of cannabis which could also be used for medical purposes marketed under the name of Marinol® for patients with AIDS, Anorexia and in patients with cancer to treat nausea and vomiting induced by anticancer treatment in patient who do not respond to conventional anti-emetic treatments (Bouquière et al., 2018).

Life prospective and the perception of living in an unhealthy environment, may have contributed over the years to the use of anti-depressant and anti-psychotic drugs among Taranto inhabitants (Brattoli et al., 2014) which have been also found as imipramine, desipramin, fluoxetine and chlorprothixen. The usage of anti-psychotic in 2017 in Taranto resulted 10.5 doses per thousand inhabitants, higher compared to the National Italian average set at 9.3 doses per thousand of inhabitants (Rapporto OsMed AIFA, 2019). Associated with general diseases, antibiotics as chlortetracycline, clindamycin and geldanamycin and antihistamine, such as astemizole and cyproheptadine were also inferred.

The presence of potassium dichromate was also found and probably linked to its use in several industrial settings both in steel plant as in the military sector.

As far as pesticides, all resulted below detection limits by GC/MS analysis, however DR CALUX® proteomic analysis allows to identify the presence of the herbicide atrazine, forbidden in European countries since 1992, but probably still present in soil samples due to illegal disposal or past contamination due to its persistence in the environment (Szewczyk et al., 2020). Carcinogenicity of atrazine is still under debate; however, it has been proven in several tests to cause damage and to act as endocrine disruptor (Szewczyk et al., 2020). The fungicide copper sulfate, also known as bordeaux mixture, used in agriculture and gardening, was found and according to European Food Safety Authority (EFSA) it could pose risk for agricultural farmers, but also for animals that can get in contact with it (EFSA, 2018).

Among the modified molecular pathways resulted protein translation and unfolded protein response (UPR), known to induce a proteotoxicity resulting from misfolded proteins and failure of Endoplasmic Reticulum (ER) adaptive capacity to induce a correct UPR, inducing ER stress. Correct proteostasis mechanisms are indispensable to regulate key cellular processes such as cell proliferation, differentiation, transcription, and apoptosis. Deterioration of cellular proteostasis has been significantly associated with tumorigenesis (Ballar Kirmizibayarak et al., 2020). Protein involved in these mechanisms, were IF4E, HYOU1, HS105, TRAP1, SYAC and UBA1, up-regulated after DxCS exposure. On the opposite, ASNS and EF2 were down-regulated. Protein folding,

translocation and degradation, as well as the assembly and disassembly of protein complexes are principally promoted by molecular chaperones like HS105 and TRAP1, whose up-regulation could indicate the presence of ER stress. TRAP1 also triggers the alternate energy metabolism in cancer cells (Ramkumar et al., 2020). Interestingly, their increased gene expression levels were observed in rat and human lung epithelial cells after Cr(VI) exposure (Ferreira et al., 2019) suggesting as potassium dichromate, that we found, could be involved in proteostasis alteration. Also HYOU1 belongs to a family implicated in the heat shock protein (HSP) cellular response to environmental stress and participate in protein folding with a pivotal role in cytoprotective cellular mechanisms triggered by oxygen deprivation (Wang et al., 2019) and regulating the secretion of vascular endothelial growth factor (VEGF) to drive progression of angiogenesis (Wang et al., 2019) reported significantly upregulated in different cancers (Li et al., 2019). It was reported that up-regulation of HYOU1 was induced by rosiglitazone (de Oliveira et al., 2017) an anti-diabetic drug, a derivative of troglitazone, that we found. IF4E, up-regulated after DxCS, plays a major role in mRNA cap-dependent translation regulation and was numerously reported as oncogenic factor since it lead to an oncogenic transformation and to a metastatic phenotype (Karaki et al., 2015; Liu et al., 2020; Xi et al., 2018). MetaCore enrichment analysis also suggest response to hypoxia and oxidative stress involvement. Atrazine was reported to induce oxidative stress, disruptions of the carbon and nitrogen metabolism, caused an increase in the membrane fluidity and lipid peroxidation (Szewczyk et al., 2020). Indeed, we found HYOU1, MVP, GSTM2, quinone oxidoreductase-like protein 1, TST, BLVRB and PRDX1 up-regulated after DxCS exposure. Highly abundance of BLVRB is related to heme degradation since catalyzes the NADPH-dependent reduction of a variety of flavins, such as riboflavin, FAD or FMN, biliverdins, methemoglobin and pyrroloquinoline quinone and contributes to heme catabolism (Li et al., 2018). TST, also known as Rhodanese, is a mitochondrial enzyme catalyzing the transfer of sulfur in several molecular pathways. Its functions include sulfur metabolism, modification of iron-sulfur clusters and the reduction of antioxidants such as glutathione and thioredoxin (Kruithof et al., 2020). On the other hand, we found down-regulation of GSTM2, a member of the glutathione S-transferase family, which participates in detoxification of Radical Oxygen Species (ROS) (Li et al., 2014).

Most of the differential proteins identified belong to signal transduction processes in particular to TGF- β signaling, since STRAP, TRAP1 and PAK2 were found dysregulated after DxCS exposure. STRAP is a TGF- β receptor-interacting protein implicated in both cell proliferation and cell death in response to various stresses and it is considered a tumor suppressor or an oncogene depending on the cellular context (Manoharan et al., 2018; Seong et al., 2007). Altered TGF- β responsiveness and its

downstream signaling pathways contribute to many diseases, including cancer and fibrosis (Vander Ark et al., 2018).

In the end, we were interested to know which pathologies could arise since the differential protein pattern. Being DR CALUX[®] cells from rat, we performed a protein BLAST to find human homolog proteins and we upload both the lists (rat and human) on MetaCore software to compare possible differences between the species. Analysis indicates no differences between the two groups of proteins and “wound and injuries” resulted the most significant term, suggesting the involvement of proteins in repairing mechanisms. Moreover, were recorded thyroid neoplasm, neurodegenerative diseases, hepatic veno-occlusive diseases, squamous cell carcinoma, stomach neoplasm, cataract, lens disease, lymphomas suggesting as the differential protein pattern principally lead to carcinogenic processes. Our findings clearly resemble the complex documented epidemiological situation of Taranto inhabitants and underline how soil analysis by an effect-based tool as DR CALUX[®] coupled with proteomics could represent a suitable tool for human and environmental risk assessment.

A more comprehensive human exposure and associated health risk could be obtained by integrating GC-MS/MS analysis of legacy pollutants in soil samples coupled with effect-based tools able to screen the presence of those known and unknown bio-potent compounds present in environmental matrices which could be even under detection limits (Escher et al., 2020). DR CALUX[®] bioassay coupled with proteomics can be used as a pre-screening tool to screen a large numbers of samples and cover more complex experimental designs which will allow to prioritize those most problematic (samples/areas) in which environmental remediation and mitigation actions should be undertaken. Bioassays are not intended to substitute chemical analysis, but they can with no doubt provide biologically relevant information on mixture toxicity of complex environmental matrices for human and environmental risk assessment. Based on BEQs values of Taranto’s urban topsoil and proteomics, several legacy and contaminants of emerging concerns as pharmaceutical are clearly still posing a serious risk to local inhabitants and continuous long-term monitoring studies based on fast, cheap and reliable bioassays are strongly recommended.

2.5 Conclusion

Today, we are exposed to a cocktail of chemicals, pesticides, industrial, pharmaceuticals, and other synthetic chemicals can enter the environment and the food chain, causing unwanted effects and diseases. Different medical researches indicate that as much as two-thirds of chronic human disease risk cannot be explained by genetics alone and may result from the environment or gene-environment interactions, introducing the concept of exposome (Escher et al., 2020). The screening performed by coupling DR CALUX[®] bioassay and differential proteomic approach, allow to estimate ecological

and human risk by legacy and contaminants of emerging concerns as pharmaceuticals by using topsoil samples from an industrialized area. After CALUX[®] exposure to DxCS, differential proteins that we identified were related to an altered UPR inducing ER stress, oxidative stress, and altered signal transduction, in particular TGF- β signaling alteration. All these mechanisms are reported to be related to carcinogenic mechanisms following the exposure with the chemicals reported by our analysis.

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Supplementary information

Tab. SI 2.1 Different abundant spots and statistical results, expressed as means and standard deviation (SD) of the %V obtained from proteomic analysis performed among the three set of samples analyzed as Ctrl (only cells), DMSO (cells exposed to 0.8% DMSO) and DxCS (cells exposed to PCDD/Fs and *dl*-PCBs soil extract in 0.8% DMSO).

Spot n	Protein Name	DxCS		DMSO		Ctrl		Kruskal wallis	DxCS/DMSO	DMSO/DMSO	DxCS/Ctrl	Ctrl/DxCS	DMSO/Ctrl	ctrl/DMSO	Dunn's multiple comparison test									
		Mean		SD		Mean									SD		DxCS vs DMSO		DxCS vs Ctrl		DMSO vs Ctrl			
		Mean	SD	Mean	SD	Mean	SD								Mean	SD	Z-value	P-value	p-adj	Z-value	P-value	p-adj		
1		0.00203	0.00376	0.00642	0.00581	0.009186	0.00168	4.55E-02	2.83979727	0.42376708	2.220110831	0.450427963	0.94800946	1.062913034	2.37	2.42E-02	3.63E-02	1.97	8.39E-03	2.68E-02	0.43	2.35E-01	3.35E-01	
2	Hypoxia up-regulated protein 1	0.06403	0.013437	0.049047	0.007807	0.028134	0.005392	1.98E-02	1.365551011	0.765960113	2.276013051	0.439364792	1.74335214	0.573613148	2.70	1.78E-01	1.78E-01	0.92	3.49E-03	1.05E-02	1.92	2.75E-02	4.13E-02	
3	Phosphoribosylformylglycinamide synthase	0.03291	0.004181	0.03094	0.008311	0.017855	0.002685	3.02E-02	1.043658553	0.95816781	1.808527679	0.552395966	1.732872953	0.577076351	2.17	5.00E-01	5.00E-01	0.00	1.50E-02	2.24E-02	2.35	9.51E-03	2.85E-02	
4		0.01835	0.001368	0.004167	0.002948	0.005176	0.003419	4.06E-02	2.600125426	0.384596831	2.015323903	0.496198154	1.75087187	1.290174336	1.88	6.81E-03	2.04E-02	2.47	3.04E-02	4.56E-02	-0.64	2.61E-01	2.61E-01	
5		0.011961	0.001877	0.001773	0.004746	0.005801	0.001729	4.00E-02	3.170264189	0.315431125	2.061897376	0.484995919	0.650386609	1.537547032	1.88	6.70E-03	2.01E-02	2.47	3.01E-02	4.51E-02	-0.64	2.61E-01	2.61E-01	
6		0.004946	0.001288	0.005078	0.002731	0.004091	0.003065	1.98E-02	0.467564475	2.138742468	1.209893408	0.827141211	2.585704159	0.386741846	0.92	5.00E-02	7.50E-02	-1.64	1.78E-01	1.78E-01	2.77	2.79E-03	8.37E-03	
7	Ubiquitin-like modifier-activating enzyme 1	0.078796	0.019213	0.059334	0.006067	0.031312	0.01727	2.38E-02	1.328008699	0.753007116	2.516466824	0.397382549	1.894917425	0.52772481	2.57	2.45E-01	2.45E-01	0.69	5.14E-03	1.54E-02	2.03	2.14E-02	3.21E-02	
8	heat shock protein 105 kDa	0.016129	0.000518	0.007345	0.002486	0.007138	0.015827	4.87E-02	2.359592805	0.405388319	2.259724743	0.442531174	1.029523251	0.971767952	2.27	1.91E-02	2.87E-02	2.07	1.16E-02	3.48E-02	0.21	4.16E-01	4.16E-01	
9	Major vault protein	0.56469	0.019231	0.023501	0.002081	0.034488	0.003401	4.87E-02	2.751695463	0.363412308	2.640823866	0.378665406	0.909718799	1.040373878	2.27	1.91E-02	2.87E-02	2.07	1.16E-02	3.48E-02	0.21	4.16E-01	4.16E-01	
10	Pitriylsin metallopeptidase 1 (Predicted)	0.043225	0.010741	0.008738	0.011203	0.013267	0.005824	4.00E-02	4.946692225	0.20215529	3.258224689	0.306915604	0.686667356	1.518217034	1.88	6.70E-03	2.01E-02	2.47	3.01E-02	4.51E-02	-0.64	2.61E-01	2.61E-01	
11	alanine-tRNA ligase, cytoplasmic	0.183502	0.027322	0.111586	0.023699	0.094136	0.013445	3.46E-02	1.644489044	0.608091616	1.948823061	0.513130217	1.185062964	0.84383302	2.57	3.78E-02	5.67E-02	1.78	5.14E-03	1.54E-02	0.85	1.97E-01	1.97E-01	
12	elongation factor 2	0.025005	0.011369	0.146331	0.038911	0.156857	0.034591	4.87E-02	0.195953428	5.010486254	0.1861865	5.370966665	0.932895672	1.075931224	-2.27	1.91E-02	2.87E-02	-2.07	1.16E-02	3.48E-02	-0.21	4.16E-01	4.16E-01	
13		0.038005	0.007598	0.013723	0.002678	0.017084	0.002741	2.82E-02	2.344800425	0.4454739	1.803807417	0.554004281	0.803228384	1.244875925	1.68	3.85E-03	1.16E-02	2.66	4.67E-02	7.00E-02	-1.07	1.43E-01	1.43E-01	
14		0.006036	0.000662	0.004409	0.003108	0.009113	0.001604	2.38E-02	1.362910468	0.73047907	0.662405271	1.509649823	0.483786303	2.067028841	-1.78	2.45E-01	2.45E-01	0.69	3.78E-02	5.67E-02	-2.67	8.85E-03	1.15E-02	
15		0.020786	0.001756	0.01553	0.005295	0.012581	0.003526	4.06E-02	1.744721914	0.579799917	2.129130216	0.463697346	1.234469522	0.810264551	2.47	3.04E-02	4.56E-02	1.88	6.81E-03	2.04E-02	0.64	2.61E-01	2.61E-01	
16		0.038007	0.004424	0.019406	0.013875	0.014627	0.009813	4.26E-02	2.051268995	0.487502889	2.721403936	0.367457395	1.326692281	0.753754291	2.42	2.69E-02	4.08E-02	1.93	7.69E-03	2.31E-02	0.53	2.97E-01	2.97E-01	
17	kinesin light chain 4	0.02033	0.015852	0.010005	0.001553	0.007837	0.00127	4.06E-02	2.019157341	0.492146004	2.594105038	0.385489402	1.276678428	0.783282601	2.50	1.32E-01	1.32E-01	1.12	6.21E-03	1.86E-02	1.49	6.78E-02	1.02E-01	
18		0	0	0	0	0.0076	0.001709	9.14E-03	#DIV/0!	#DIV/0!	0	#DIV/0!	0	#DIV/0!	-2.51	5.00E-01	5.00E-01	0.00	5.96E-03	8.93E-03	-2.72	3.30E-03	9.90E-03	
19	ras GTPase-activating protein-binding protein 1	0.562685	0.008261	0.003765	0.007876	0.005564	0.005294	4.71E-02	7.788090891	0.138401172	8.026662472	0.124584783	1.030623871	0.970773631	2.19	1.42E-02	2.13E-02	2.19	1.42E-02	4.77E-02	0.00	5.00E-01	5.00E-01	
20	myotinin-protein kinase isoform X1	0.037464	0.002588	0.024124	0.008652	0.016999	0.005872	3.46E-02	1.562597412	0.643925489	2.260503902	0.453503563	1.418980411	0.704279705	2.57	3.78E-02	5.67E-02	1.78	5.14E-03	1.54E-02	0.85	1.97E-01	1.97E-01	
21		0.017926	0.00292	0.027262	0.002528	0.063929	0.00716	4.06E-02	0.248065246	4.031132504	0.280404441	3.566277321	1.130347458	0.884683725	-1.88	6.81E-03	2.04E-02	-2.47	3.04E-02	4.56E-02	0.64	2.61E-01	2.61E-01	
22		0	0	0.023879	0.006234	0.028536	0.005476	3.25E-02	0.639415077	0	#DIV/0!	0	#DIV/0!	0.760738677	1.304259345	-1.59	3.63E-02	5.47E-02	-1.79	4.80E-03	1.44E-02	-0.86	1.95E-01	1.95E-01
23	Asparagine synthetase [glutamine-hydrolyzing]	0.02844	0.004071	0.004418	0.001987	0.057786	0.010244	3.08E-02	1.563029342	0.437385223	2.232510167	0.699238878	1.42522708	-2.63	8.87E-02	8.87E-02	-1.35	4.25E-03	1.27E-02	-1.39	8.29E-02	1.34E-01		
24	serine/threonine-protein kinase PAK 2	0.08849	0.049383	0.043405	0.024389	0.021691	0.002513	4.55E-02	3.621412077	0.2761338	4.079629413	0.245120364	1.126529742	0.887681845	1.97	8.93E-03	2.68E-02	2.37	2.42E-02	3.63E-02	-0.43	3.35E-01	3.35E-01	
25	Actin, cytoplasmic 2	0.073955	0.010177	0.028744	0.010348	0.02686	0.007277	4.55E-02	2.572877392	0.3886699	2.771301468	0.360841291	1.077121466	0.9284004	2.37	2.42E-02	3.63E-02	1.97	8.93E-03	2.68E-02	0.43	3.35E-01	3.35E-01	
26		0.026936	0.004542	0.025461	0.017003	0.030584	0.002832	4.06E-02	4.985978902	0.20575925	4.163964541	0.240494208	0.823474998	1.201238676	2.47	3.04E-02	4.56E-02	1.88	6.81E-03	2.04E-02	0.64	2.61E-01	2.61E-01	
27		0.01946	0.001691	0.068433	0.024684	0.036945	0.002036	2.20E-02	0.284368724	3.536561124	0.528159818	1.89336501	1.857306282	0.538414259	-1.58	2.86E-03	8.58E-03	-2.76	5.72E-02	5.72E-02	1.28	1.00E-01	1.00E-01	
28	quinone oxidoreductase-like protein 1	0.045272	0.00746	0.009142	0.001805	0.008768	0.002292	4.87E-02	4.951841966	0.201945055	5.16325285	0.193676357	1.042693383	0.98905471	2.27	1.91E-02	2.87E-02	2.07	1.16E-02	3.48E-02	0.21	4.16E-01	4.16E-01	
29	serine-threonine kinase receptor-associated protein transphosphatase	0.041067	0.015227	0.085222	0.021081	0.084533	0.006215	4.87E-02	0.481883701	2.075189598	0.485692744	2.058914844	1.007904486	0.992157505	-2.07	1.16E-02	3.48E-02	-2.27	1.91E-02	2.87E-02	0.21	4.16E-01	4.16E-01	
30		0.053582	0.012658	0	0	0.005417	0.002048	1.82E-02	#DIV/0!	9.80472033	0.021307409	0	#DIV/0!	1.94	2.57E-03	7.71E-03	2.80	2.64E-02	3.95E-02	-0.93	1.76E-01	1.76E-01		
31		0.004093	0.007089	0.045061	0.014437	0.048081	0.002092	4.80E-02	0.09083283	11.0023328	0.085127668	1.14796206	0.937190527	1.067018894	-2.28	1.89E-02	2.83E-02	-2.08	1.14E-02	3.43E-02	-0.21	4.15E-01	4.15E-01	
32	Pyridoxal kinase	0.019238	0.007176	0.020564	0.013973	0.041211	0.012251	4.63E-02	0.935510447	1.0689315	0.466815131	2.142175634	0.489895103	2.004027684	-2.20	3.71E-01	3.71E-01	-0.33	1.38E-02	4.13E-02	-2.03	2.14E-02	3.21E-02	
33	thiosulfate sulfurtransferase	0.113226	0.029213	0.038873	0.028683	0.05642	0.018892	4.06E-02	2.923717257	0.343322009	2.006847192	0.498294043	0.688994851	1.451389657	1.88	6.81E-03	2.04E-02	2.47	3.04E-02	4.56E-02	0.64	2.61E-01	2.61E-01	
34	annexin A5 isoform X1	0.04035	0.014076	0	0	0.008363	0.009818	1.83E-02	#DIV/0!	5.133368606	0	#DIV/0!	0	#DIV/0!	1.94	2.57E-03	7.71E-03	2.80	2.64E-02	3.95E-02	-0.93	1.76E-01	1.76E-01	
35		0.094977	0.02826	0	0	0.007146	0.003432	1.58E-02	#DIV/0!	0	55.34738824	0.0180077	0	#DIV/0!	2.29	3.04E-03	9.12E-03	2.74	1.11E-02	1.67E-02	-0.49	1.11E-01	3.11E-01	
36		0.035294	0.010625	0.079615	0.004437	0.08502	0.017417	4.98E-02	0.443306468	2.255757597	0.412784561	2.422572123	0.931149423	1.073941492	-2.17	1.50E-02	2.24E-02	-2.17	1.50E-02	4.49E-02	0.00	5.00E-01	5.00E-01	
37	flavin reductase (NADPH)	0.079731	0.008971	0.06706	0.008607	0.008168	0.013103	4.32E-02	13.47086958	0.077019333	11.99014483	0.08401028	0.864411905	1.156853885	2.22	1.31E-02	1.97E-02	2.22	1.31E-02	1.94E-02	0.00	5.00E-01	5.00E-01	
38	glutathione S-transferase Mu 2	0.015174	0.013502	0.065222	0.02239	0.06623	0.00733	4.98E-02	0.37346689	2.80733124	0.13681732	2.864007499	1.00631759	0.99722120	-2.17	1.50E-02	2.24E-02	-2.17	1.50E-02	4.49E-02	0.00	5.00E-01	5.00E-01	
39	Eukaryotic translation initiation factor 4E	0.02342	0.014037	0.02173	0.02141	0.02077	0.00369	4.87E-02	2.89989304	0.418.														

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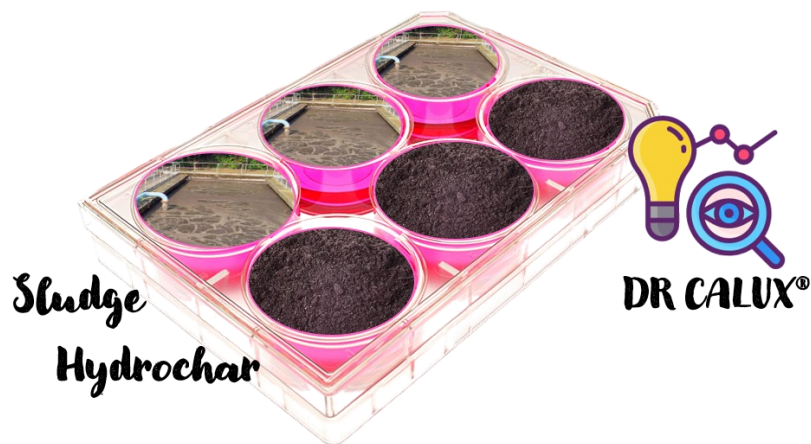
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CHAPTER 3



DR CALUX[®] bioassay for the detection of dioxins, furans and *dioxin-like* polychlorinated biphenyls in sewage sludge and hydrochar



Abstract

In 2018 the Tuscany Region suspended the application of sewage sludge on agricultural soils due to the potential presence of toxic organic and inorganic contaminants. Following this decision, however, millions of tons of sludge produced every year required an alternative way for disposal or even reuse as a carbon resource, opening new challenges to environmental sustainability. The aim of the present study is to evaluate the presence of toxic organic contaminants such as Polychlorinated dibenzo-*p*-dioxins (PCDDs), Polychlorinated dibenzofurans (PCDFs) and *dioxin-like* Polychlorinated biphenyls (*dl*-PCBs) in samples of sewage sludge (SL) and related hydrochar (HC), through the application of a bioanalytical tool as the DR CALUX[®] (Dioxin Responsive, Chemically Activated LUCiferase gene eXpression) bioassay. Thanks to its specific mode of action, based on the expression of the Aryl Hydrocarbon Receptor (*AhR*) and the luciferase enzyme as reporter gene, the DR CALUX[®] can detect the presence of PCDD/Fs and *dl*-PCBs in a wide range of samples. Here, samples of urban SL were collected from six wastewater treatment plants (WWTPs), located in the Tuscany territory. Aliquot of SL were further thermochemically processed by hydrothermal carbonization (HTC) under controlled laboratory conditions in order to obtain HC. The DR CALUX[®] bioassay was used to test the presence of PCDD/Fs and *dl*-PCBs in both SL and HC samples. The highest DR CALUX[®] BEQ (Bioanalytical Toxicity Equivalents) values were found in HC samples compared with their respective sludge. DR CALUX[®] BEQ values were also one order of magnitude higher for the *dl*-PCBs fraction respect to the PCDD/Fs one. Chemical analysis allowed to find a high correlation ($R_p=0.8029$; $R_s=0.8252$) between DR CALUX[®] BEQ and chemical TEQ_{WHO}. Chemical analysis show that highly chlorinated congeners prevailed in SL compared to HC samples, in agreement with previous studies. HTC process seems responsible for such changes in the level of chlorinated congeners from high to low ones, which are more toxic. DR CALUX[®] bioassay was able to screen the presence of dioxins and *dioxin-like* compounds in SL and HC, raising concern due to their possible applications as soil amendments. This study focuses on the importance of combining bioanalytical tools with chemical analyses in order to evaluate potential risk of sludge and hydrothermal carbonization products for humans and environmental health.

3.1 Introduction

Sewage sludges in Italy are covered under the art. 183 of Legislative Decree n. 152/2006, as enacted by Legislative Decree 205/2010, as "*a waste*", and their disposal represents one of the major problems of the integrated water cycle.

In Tuscany, the sewage sludge production accounts for up to 130,000 tons/year and recently became an economic and environmental emergency (www.sludge4puntozero.it/il-progetto/), due to the presence of toxic chemicals above the safety regulatory limits and the consequent decree of 2018 suspended their use as soil amendments (<https://www.regione.toscana.it/documents/10180/15762652/PARTE+I+n.+48+del+26.10.2018.pdf/36b2105e-72b6-4410-b256-de8f4b81a214>, Ordinanza del Presidente della Giunta Regionale n°3 18/10/2018).

In addition, at national level, the Legislative Decree 109/2018 (Decreto Genova), in art. 41 "*Urgent provisions on the management of sewage sludge*", provided new regulatory limits for several toxic contaminants as hydrocarbons (C10-C40), Polycyclic Aromatic Hydrocarbons (PAHs), Polychlorinated dibenzo-*p*-dioxins (PCDDs) and Polychlorinated dibenzofurans (PCDFs), for total Polychlorinated biphenyls (PCBs) and for toluene, selenium, beryllium, arsenic, total chromium and chromium VI.

Traditionally, sludge and organic waste are disposed to landfills, incineration plants, discharged into the sea or land-spreading (Draft Discussion Document for the Ad Hoc Meeting on Biowastes and Sludges, 2004). To date, landfilling and land-spreading on agricultural soils are considered the most economical procedures for their disposal (Metcalf & Eddy, 2006).

Land-spreading results an effective reuse of sewage sludge and represents a valid and economically interesting alternative to the use of mineral fertilizers (Zoghلامي et al., 2016) with several advantages, in terms of low cost and energy reduction (Melo et al., 2018). The agricultural reuse of sludge in recent years has been highly accepted, thanks to the potential nutrients and organic matter recovery (Zoghلامي et al., 2016; Casado-Vela et al., 2006; Kidd et al., 2007; Tontti et al., 2017). The use of sludge as soil amendment has been shown to increase soil stability and fertility (e.g., porosity, stability, water and organic carbon retention capacity, nitrogen storage and nutrient content), proving advantages for plant growth, productivity and crop yield (Lloret et al. 2016). Moreover, if used as amendment, sludge can also enhance soil microbial communities and their enzymatic activities (Scotti et al., 2015; Zoghلامي et al., 2016).

The re-use and possible applications of the treated sludge would allow a waste to return to play a useful role as a smart resource. Sludge could be used as an energy resource or as a soil-improver, thus supporting the circular economy and sustainable development and promoting the reduction of

greenhouse gas emissions and the use of fossil fuels (Gievers et al., 2019; Kacprzak et al., 2017); such uses are economically possible and eco-sustainable.

The increasing production volumes and the potential presence of contaminants in sewage sludge made it a prime environmental issue (Zhang et al., 2009). Although adequately treated and stabilized before application in the soil, the sludge can in fact contain heavy metals, organic contaminants, organochlorine pesticides, pharmaceutical products, detergents, salts and pathogenic microorganisms (Rogers, 1996; Dai et al., 2007; Sánchez-Brunete et al., 2008; Sidhu and Toze 2009; Clarke and Smith 2011; Westerhoff et al., 2015). The concentrations of pollutants can vary in relation to the composition of the wastewater, the typology of wastewater treatment and to the stabilization process that affects the sludge itself (Milieu et al., 2010; Protano et al., 2019).

It is known that trace elements and toxic substances present in the sludge could accumulate in the soil, become available for crops, exerting phytotoxicity and entering in the food chain therefore they can represent a risk for both humans and the environmental health (Fytli and Zabaniotou, 2008; Goss et al. 2013; McBride 2003; Singh and Agrawal 2008; Smith 2009; Passuello et al. 2010; Sharma et al. 2007).

In particular, the main risk is associated with the presence of *non*-ionic hydrophobic organic compounds in the sludge, since they can be absorbed on soil organic matter, resulting highly persistent in the environment (Harrison et al., 2006; Schnaak et al., 1997).

As far as the most toxic organic chemicals present in soils (i.e., the highly chlorinated ones) such as dioxins, furans and polychlorinated biphenyls (PCBs), their ability to be adsorbed to the surface of carbonaceous particles calls for in-depth studies.

Considering the current and growing demand for renewable energy and organic matter, many wastes, including sewage sludge, may become a new precious resource, according to the "End of waste" principle, following adequate treatments, stabilization and transformation processes.

Recently, in order to optimize the sludge reuse and disposal process, innovative technologies have been developed with the purpose to significantly reduce times and volumes of the conventional sludge treatments (Huang and Tang, 2015). A wide variety of processes, both thermochemical and biological, can in fact be used to convert biomass into high quality products, in an ecological way starting from renewable resources (Titirici et al., 2010; Libra et al., 2011). Coal is the main result of the thermochemical conversion process defined as pyrolysis, during which organic matter is thermochemically decomposed by heat in the absence of oxygen (Libra et al., 2011). If the process is performed in the presence of subcritical liquid water, it is called hydro-pyrolysis or hydrothermal carbonization (HTC) (Libra et al., 2011). HTC is a relatively low temperature thermochemical conversion process that occurs in the aqueous phase at saturated pressure (Lu and Berge, 2014;

Gievers et al., 2019). HTC is also known as wet pyrolysis and can be applied to a variety of *non*-traditional sources such as the organic fraction of municipal solid waste, wet agricultural residues, sewage sludge, as well as algae and aquaculture residues (Lu and Berge, 2014; Volpe et al., 2016; Libra et al., 2011). Respect to traditional dry pyrolysis, HTC process allows the treatment of substrates with high moisture content (up to 75-90%), without requiring a phase energy-intensive pre-treatment and preliminary or simultaneous drying (Tasca et al., 2019). In this way, HTC can convert the incoming wet material into a carbonaceous solid with relatively high yields (Libra et al., 2011; Volpe et al., 2016).

In anaerobic conditions, with temperatures between 180-250 °C and air pressures between 10-40 bar, the wet biomass is converted in a few hours to a solid fraction of carbonaceous material called hydrochar, to a liquid fraction and to a small amount of gas (mainly carbon dioxide) (Libra et al., 2011; Berge et al. 2011; Funke and Ziegler, 2010).

During HTC, valuable solid, liquid and gaseous products are thus generated through a series of simultaneous reactions, including hydrolysis, dehydration, decarboxylation, aromatization and re-condensation (Funke and Ziegler; 2010; Sevilla and Fuertes, 2009). Hydrochar represents an important organic matrix and is defined as biological lignite characterized by high content of carbon (greater than 45-55% of dry matter), hydrogen and nutrients such as phosphorus, of high calorific value (about 15 MJ / kg) (Basso et. al, 2016; Lucian and Fiori, 2017). For these reasons, hydrochar can be compared to lignite and can burn independently (Brookman et al., 2018). The nature of the raw material, the process temperature and the reaction time are the main factors that influence its composition (Libra et al., 2011). HTC therefore represents an environmentally friendly thermochemical process thanks to which low value and widely available biomass can be converted and reused (Titirici et al., 2010). It could offer numerous advantages compared to traditional drying processes, since no pre-treatment of the matrix is required and the high quality of the coal produced ensures its usage in different sectors (Lucian and Fiori, 2017).

Several studies have shown how the spreading of hydrochar on the soil, as a soil amendment, is able to improve the soil quality (Tasca et al., 2019), with a consequent increase in agricultural production (Marris, 2006; Chan et al., 2007). This is due both to the high concentration of nutrients present and to its ability to retain them in organic matter, limiting their losses during the leaching (Libra et al., 2011). Its efficacy as a soil improver mainly depends on the high specific surface that characterizes itself as well as its strong affinity for water which respectively affect the increase in the cation exchange capacity and water retention of treated soils (Sevilla and Fuertes, 2011). However, there is growing concern associated with the environmental risk of sludge contaminants and their concentrations in soil after land application. The level of risk depends on their initial concentrations (in both soils and sludge), frequency of the application (cumulative effects), management practices

and losses (Rorat et al., 2019). However, the question linked to the influence of HTC on the chemical composition of contaminants already present in the sludge has been little explored and only addressed in few publications (Wiedner et al., 2013; Tirler et al., 2013; Brookman et al., 2018).

Bioassay and biosensors are recommended as suitable screening tools in assessing wastewaters and sludge quality (Barceló et al., 2020; Behnisch and Brouwer, 2020; Farré and Barceló, 2003; Macova et al., 2011; Neale et al., 2020; Kapanen et al., 2013; Selivanovskaya and Latypova, 2003). The toxic potency of complex matrices like sludge imply the understanding of all possible interactions occurring among chemicals and organic and inorganic matter present (Gustavsson et al., 2004). Among bioanalytical tools, CALUX[®] bioassay has extensively been used to screen the toxicity of urban and industrial wastewaters (Addeck et al., 2014; Houtman et al., 2020), sewage sludges (Eljarrat and Barceló, 2004; Van Langenhove et al. 2012; Gustavsson et al., 2004; 2007; Takigami et al., 2010; Zhang et al., 2009), biochar (Anderson et al., 2017; Lyu et al., 2016) and compost (Beníšek et al., 2015; Suzuki et al., 2004).

To date, no studies have investigated the application of CALUX[®] bioassay on hydrochar samples. The contamination of PCDD/Fs and *dl*-PCBs in sewage sludge is well documented (Eljarrat et al., 2003; Jones and Sewart, 1997; Rogers, 1996). The presence of these compounds in sewage sludge can originate from the atmosphere through dry and wet deposition, inflows from wastewater treatment plants, and their related treatment processes (Rosińska, 2019). The range of PCDD/Fs/*dl*-PCBs concentration in sewage sludge varies according to the countries (Fuentes et al., 2007; Harrison et al., 2006; Minnini et al., 2019). Generally, in sewage sludge the total PCDDs concentration is higher compared to PCDFs and, among PCDDs, the highest chlorinated congeners predominate (Rosińska, 2019). Regarding PCBs, the general trend is that their concentrations are higher (at least, one order of magnitude) than the PCDD/Fs in sludge (Eljarrat et al., 2003).

Caution should be taken in the selection of sewage sludge for performing hydrothermal carbonizations, because even if the formation of new dioxins is not possible, due to the low temperature of process, the presence of previous contamination in the feedstock, could determine their final concentrations in the hydrochar (Wiedner et al., 2013).

Current legislation concerning the limit values of PCDD/Fs and *dioxin-like* PCBs in sludge is scarce and even absent for hydrochar, moreover, the major European Commission Regulation mainly focuses on heavy metal contamination in sludge. Therefore, it is important to find smart solutions to better identify organic compounds contamination not only in sludge samples but also in hydrochar in order to assess their potential reuse.

To this aim, the present study focuses on the Dioxin Response (DR) CALUX[®] cell line, since carbon and organic matter, which are usually adsorbed to the carbon fraction, can concentrate organic

compounds from sludge to hydrochar, as a result to the HTC process. The occurrence of PCDD/Fs and *dl*-PCBs in samples of sewage sludge collected from 6 WWTPs of Tuscany was here determined by using the DR CALUX[®] reporter gene bioassay. Testing these new matrices using the DR CALUX[®] assay was particularly difficult at the beginning, since specific methods for the extraction and purification of sludge and especially hydrochar were missing and have been set up in the study starting from available protocols for soil (Behnisch et al., 2001, 2010; Besselink et al., 2004).

3.2 Materials and Methods

A total of 12 samples (6 sludges and 6 respective hydrochars) were tested using DR CALUX[®] bioassay. From each sample, two fractions were obtained respectively for the *dl*-PCBs and for the PCDD/Fs. Sludge samples were collected from 6 urban WWTPs of Tuscany Region (Table 3.1) in 2019, stored in plastic containers and shipped in cold refrigerated containers at 4°C at the Ecotoxicology and Biological Monitoring Laboratory (EcoBioM Lab) of the University of Siena.

Tab. 3.1 Details of the WWTPs located in the Tuscany Region where the sludge samples were collected.

Plant code	Plant site	Plant name	Plant treatment
WWTP_1(PI)	Pisa	San Jacopo	Anaerobic digestion
WWTP_2(GR)	Grosseto	Acquedotto del Fiora	Anaerobic digestion
WWTP_3(SI)	Sinalunga	San Giovanni	Anaerobic digestion
WWTP_4(MS)	Massa	Lavello 1	Aerobic digestion
WWTP_5(FI)	Firenze	San Colombano	Aerobic digestion
WWTP_6(LI)	Livorno	Rivellino	Aerobic digestion

An aliquot of those sludge samples was processed by HTC at the Department of Civil and Industrial Engineering of the University of Pisa (Prof. Sandra Vitolo and Prof. Monica Puccini) and then send back to Siena in cold refrigerated containers at 4°C.

3.2.1 Development of new protocol for the extraction of sludge and hydrochar

- Sludge and hydrochar samples preparation

An aliquot of about 50 g of sludge was placed in sterile plastic containers overnight at -80 °C and then lyophilized (*Labogene, Scanvac cool safe*), for 48 h until achieving a constant weight.

An aliquot of about 10 g of hydrochar was instead placed in a glass petri dish and dried at 105 °C for 1 h in an oven (*MemmertTM*).

Each dried sample was then sieved (2 mm Ø) and the resulting fractions stored in 50 mL Falcon at + 4 °C until use.

- ASE extraction

For each sample (sludge and hydrochar), an extraction cell for ASE 200 (Accelerated Solvent Extraction) (*Dionex*TM) was used. For each cell, a cellulose filter was inserted on the bottom and on the top. At first, a standardized extraction protocol used for soil was used (Behnisch et al., 2001, 2010; Besselink et al., 2004).

Initially 5 g of raw sample (sludge/hydrochar) were placed in each cell and processed in ASE with a hexane/acetone mixture (9/1 v./v.), using the following conditions: 0 min preheat, 5 min heat, 5 min static time, flush volume 60% of extraction cells, 90s purge time with nitrogen flow, 3 cycles, 2000 psi (14Mpa) system pressure, 100 °C oven temperature.

Due to the complexity of the hydrochar matrix, the extraction solvent (hexane/acetone mixture (9/1 v./v.)) was not able to enter in the cell's sample and the program was blocked after first minutes of run (Fig. 3.1A). For this reason, the amount of sample was reduced from 5 g to 1 g but even in this case the extraction failed again (Fig. 3.1B). Then, 1 g of sample was mixed with Dionex Prep Diatomaceous Earth (DE) and the extraction cell filled with DE (Fig 3.1C). This choice resulted successful for HC sample extraction.

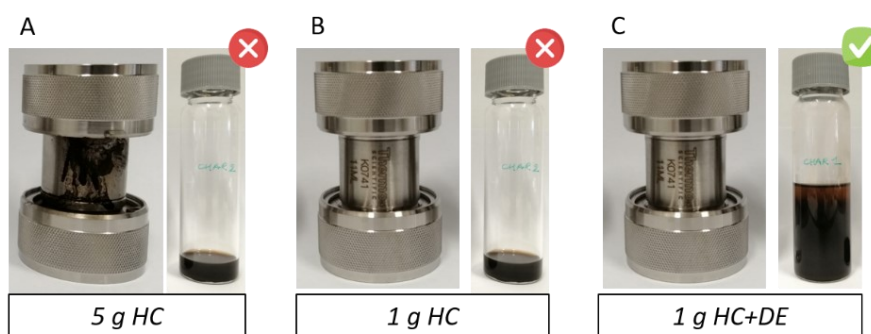


Fig. 3.1 Development of new protocol for the extraction of hydrochar.

Another methodological issue occurred for sludge samples at the end of the extraction, since extracts resulted dense and viscous (Fig. 3.2 A). Therefore 10 mL of hexane was added to each sample (Fig. 3.2 B) and then shaken vigorously to achieve separation of the two phases (Fig. 3.2 C).

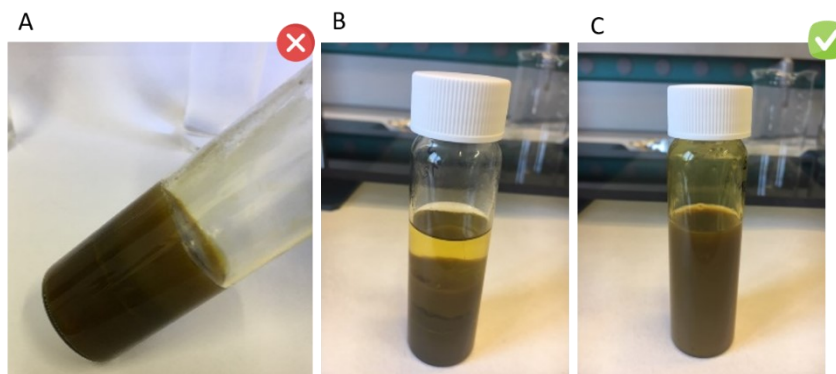


Fig. 3.2 Hexane added to allow separation of phases.

3.2.2 Extracts purification

Three purification steps were run before testing extracts with DR CALUX[®]. The first (acid silica column) and the second (sulphur clean up) purification steps were run following the same procedure described in paragraph (1.2.3.1) of Chapter 1.

A further step was run by using Florisil cartridges (500 mg). This step was performed since dioxins and furans have a different chromatographic behaviour compared to *dl*-PCBs in a Florisil cartridge. For each extract, 1 Florisil cartridge was used. Cartridges were eluted in sequence with toluene, a solution of hexane-dichloromethane 1:1 v./v. and hexane. For the elution of PCDD/Fs a 1:1 v./v. hexane-dichloromethane solution was added, while for the elution of the *dl*-PCBs the same solution but with a different ratio of hexane-dichloromethane (98:2 v./v.) was used. After the elution, Florisil cartridges were let dry. Both extracts (*dl*-PCBs and PCDD/Fs) were evaporated under nitrogen flow until dryness $\approx 2\mu\text{L}$, resuspended in 25 μL of DMSO and used for DR CALUX[®] bioassay.

3.2.3 DR CALUX[®] bioassay

DR CALUX[®] cells preparation for the bioassay has been already described in paragraph (1.2.3.2) of Chapter 1. Three different dilutions in DMSO were prepared for each extract (sludge and hydrochar). For sludge samples, a range of dilution 30-60x was used for the *dl*-PCBs fraction while a range of dilution 3-10x used for the PCDD/Fs fraction. On the contrary, higher dilutions were used to test the *dl*-PCBs-fraction (100-300x) and the PCDD/Fs fraction (10-30x) of the hydrochar samples.

Once cells reached confluency in the cell culture flasks, they were seeded in 96-well plates (1×10^4 cells/well density). One day after plating, the cells were exposed to a concentration series (range 0-3 pM) of reference toxic compound (2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)) and a sample extract. The concentration of DMSO was fixed at 0.8% in order to avoid cytotoxicity effects (5.6 μL TCDD/sludge or hydrochar extracts were added to 350 μL α -MEM). As references, a blank sample with only α -MEM medium and 0.8% DMSO and a sample named BRM07 containing PAHs, as an indicator of the metabolic capacity of the cells, diluted with α -MEM medium and 0.8% DMSO, were run. The plates were incubated at 37 °C for 24 h. After 24 h of incubation, DR CALUX[®] cell monolayers were observed under an inverted optical microscope (*Nanjing Jiangnan Novel Optics NIB-100 F*). The medium was discarded, and cells were washed with Phosphate Buffer Saline (PBS) and lysed with Lysis buffer in PBS. Luciferase activity was measured using a Victor 3 luminometer (*Perkin Elmer*) with the addition of luciferin. Luciferase activity was measured and expressed as relative light units (RLUs). RLU values were then transformed into 2,3,7,8-TCDD Bioanalytical Toxicity Equivalents (BEQ) based on Relative Effect Potencies (REPs) of the extracts respectively of PCDD/Fs and *dl*-PCBs calculated vs 2,3,7,8-TCDD calibration curve and expressed as ng 2,3,7,8-

TCDD BEQ/kg dry weight (d.w.) sample. The 2,3,7,8-TCDD sigmoid semi-logarithmic dose-response relationship was fitted by the Hill equation ($R^2 > 0.98$, $p < 0.001$). BEQ values were required to meet all the QA/QC criteria of bioassay standards in the Netherlands such as: R^2 calibration series > 0.98 ; EC_{50} values of the reference compound between present limits; SD triplicate analysis $< 15\%$ (RIKZ-SPECIE 07, 2006a, b). The DR CALUX[®] BEQs were quantified between the limit of quantification and the EC_{50} of 2,3,7,8-TCDD and as close as possible to the EC_{10} (Besselink et al., 2004). All data are presented as mean $\pm$ standard deviation (SD).

3.2.4 Statistical analysis

Data analysis was carried out using the GraphPad Prism 6 software (GraphPad Software Inc.). The variability of the data (expressed as mean $\pm$ standard deviation) was tested through the analysis of variance (One way ANOVA, Bonferroni post-test) in order to highlight the differences between the sludge and hydrochar samples of the different WWTPs and between the sludge and hydrochar samples of the same WWTPs, for both the *dl*-PCBs and PCDD/Fs fraction. Conversely, the T-test (Unpaired) was used to evaluate the statistically significant differences between the *dl*-PCBs and PCDD / Fs fraction of each individual WWTPs. The differences between the data were considered significant for values of * $p \leq 0.05$ and highly significant for ** $p \leq 0.01$ and *** $p \leq 0.001$. BEQs data obtained from the DR CALUX[®] bioassay were compared with the analytical data expressed as TEQ_{WHO} provided by pH TÜV Italia srl of Tavarnelle Val di Pesa. Correlations among BEQs and TEQ_{WHO} were tested using the Pearson's correlation (R_p Pearson) and controlled by the *non*-parametric Spearman's test (R_s Spearman).

3.3 Results

3.3.1 Bioassay results

In all sludge and hydrochar samples, the presence of *AhR* inducers was found (Fig. 3.3). BEQ values for sludge samples significantly differed among WWTPs ($p \leq 0.001$). The observed trend was: WWTP_4(MS) $>$ WWTP_6(LI) $>$ WWTP_2(GR) $>$ WWTP_5(FI) $>$ WWTP_3(SI) $>$ WWTP_1(PI) (Fig. 3.3). Similarly, BEQ values obtained from hydrochar significantly differed among WWTPs ($p \leq 0.001$) with slight different trend compared to sludge samples: WWTP_3(SI) $>$ WWTP_4(MS) $>$ WWTP_6(LI) $>$ WWTP_2(GR) $>$ WWTP_5(FI) $>$ WWTP_1(PI) (Fig. 3.3). The highest biological activities are found in samples coming from WWTP_4 (Massa) and WWTP_3 (Sinalunga). Samples coming from the WWTP_1 (Pisa) showed the lowest biological activities for both matrices (sludge and hydrochar).

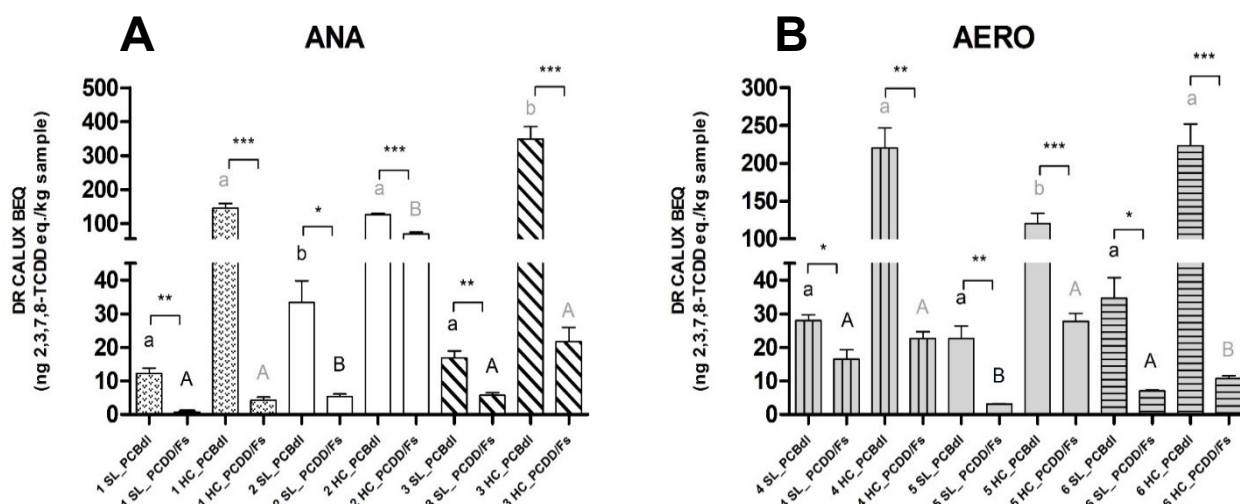


Fig. 3.3 Biological Equivalent toxicity expressed as DR CALUX[®] BEQ in sample (ng 2,3,7,8-TCDD eq / kg sample d.w.) in extracts containing *dl*-PCBs and PCDD/Fs in sludge from 3 WWTPs with anaerobic process (ANA) (**A**) and 3 WWTPs with aerobic process (AERO) (**B**) and relative hydrochar samples. Bars represent mean $\pm$ SD (n = 3). The line shows the statistically significant differences between the *dl*-PCBs and PCDD/Fs fraction for the individual plants (T-test, Unpaired). In black the statistically significant differences between plants for the sludge and in gray for the hydrochars (ANOVA, Bonferroni). With the lower-case letter the differences in the fraction of *dl*-PCBs and with the capital letters the differences in the fraction of the PCDD / Fs. Statistics performed using GraphPad Prism 6.0. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

In particular, BEQ values resulted one order of magnitude higher for the *dl*-PCBs fraction compared to the PCDD/Fs ones, in both sludge and hydrochar samples from all WWTPs (Tab. 3.2).

DR CALUX[®] BEQ mean value are 23.3 ± 10.4 (ng 2,3,7,8-TCDD eq/kg d.w. sample) for *dl*-PCBs fraction of sludge samples and equal to 7.4 ± 6.4 (ng 2,3,7,8-TCDD eq/kg d.w. sample) for PCDD/Fs ones (Tab. 3.2).

Differently, the mean BEQ value are equal to 218.88 ± 113.11 (ng 2,3,7,8-TCDD eq/kg d.w. sample) for *dl*-PCBs fraction in hydrochar samples and equal to 26.2 ± 21.45 (ng 2,3,7,8-TCDD eq/kg d.w. sample) for the PCDD/Fs ones (Tab. 3.2).

Tab. 3.2 BEQ values expressed as mean $\pm$ SD (n=3) (ng 2,3,7,8-TCDD eq/kg sample d.w.) of *dl*-PCBs and PCDD/Fs extracts from sludge and hydrochar samples from the 6 WWTPs.

BEQ	SLUDGE		HYDROCHAR	
	<i>dl</i> -PCBs	PCDD/Fs	<i>dl</i> -PCBs	PCDD/Fs
WWTP_1(PI)	12.2 ± 3.7	0.75 ± 0.2	145 ± 30	4.3 ± 1.6
WWTP_2(GR)	33.2 ± 11.2	5.3 ± 1.4	126.6 ± 5.8	69.7 ± 8.4
WWTP_3(SI)	16.8 ± 4.8	5.7 ± 1.3	350 ± 100.4	21.7 ± 7.5
WWTP_4(MS)	28 ± 3	16.5 ± 6.1	220 ± 45.8	22.7 ± 3.5
WWTP_5(FI)	22.6 ± 6.5	3.1 ± 0.4	120.2 ± 31	27.7 ± 4.7
WWTP_6(LI)	34.6 ± 10.6	7 ± 0.5	223.3 ± 49.3	10.7 ± 1.4

BEQ values of *dl*-PCBs fraction resulted significantly higher compared to PCDD/Fs fraction in sludge samples from WWTP_1(PI), 3(SI) and 5(FI) ($p \leq 0.01$) and in WWTP_2(GR), 4(MS) and 6(SI) ($p \leq$

0.05) (Fig. 3.3; Tab 3.2). The observed trend of BEQ in the *dl*-PCBs fraction of sludge samples is: WWTP_6(LI) > WWTP_2(GR) > WWTP_4(MS) > WWTP_5(FI) > WWTP_3(SI) > WWTP_1(PI) (Fig. 3.3; Tab 3.2). On the contrary, the trend observed in BEQ values in the PCDD/Fs fraction of sludge samples is: WWTP_4(MS) > WWTP_6(LI) > WWTP_3(SI) > WWTP_2(GR) > WWTP_5(FI) > WWTP_1(PI) (Fig. 3.3; Tab 3.2).

Significant higher BEQs values associated with *dl*-PCBs fraction with respect to the PCDD/Fs ones, are also observed in hydrochar samples ($p \leq 0.001$) for all WWTPs, except for WWTP_4(MS) where $p \leq 0.01$ (Fig. 3.3; Tab 3.2). In details, for *dl*-PCBs fraction of hydrochar samples the trend observed is: WWTP_3(SI) > WWTP_6(LI) > WWTP_4(MS) > WWTP_1(PI) > WWTP_2(GR) > WWTP_5(FI) (Fig. 3.3; Tab 3.2). The trend observed in the PCDD/Fs fraction results: WWTP_2(GR) > WWTP_5(FI) > WWTP_4(MS) > WWTP_3(SI) > WWTP_6(LI) > WWTP_1(PI) (Fig. 3.3; Tab 3.2).

Anaerobic or aerobic digestion seem not to interfere in the observed differences between *dl*-PCBs and PCDD/Fs fractions for both sludge and hydrochar matrices ($p > 0.05$) (Fig. 3.4).

DR CALUX[®] BEQ mean values for *dl*-PCBs fraction of sludges resulting from an anaerobic treatment are equal to 19.4 ± 10.44 (ng 2,3,7,8-TCDD eq/kg d.w. sample), while for the PCDD/Fs fraction are equal to 3.93 ± 2.6 (ng 2,3,7,8-TCDD eq/kg d.w. sample) (Fig. 3.4).

On the contrary, the average value of the DR CALUX[®] BEQ of sludge derived from an aerobic treatment are higher compared to the anaerobic ones, and respectively equal to 28.4 ± 8.24 (ng 2,3,7,8-TCDD eq/kg d.w. sample) in the *dl*-PCBs fraction and 10.3 ± 7.3 (ng 2,3,7,8-TCDD eq/kg d.w. sample) in the PCDD/Fs fraction (Fig. 3.4).

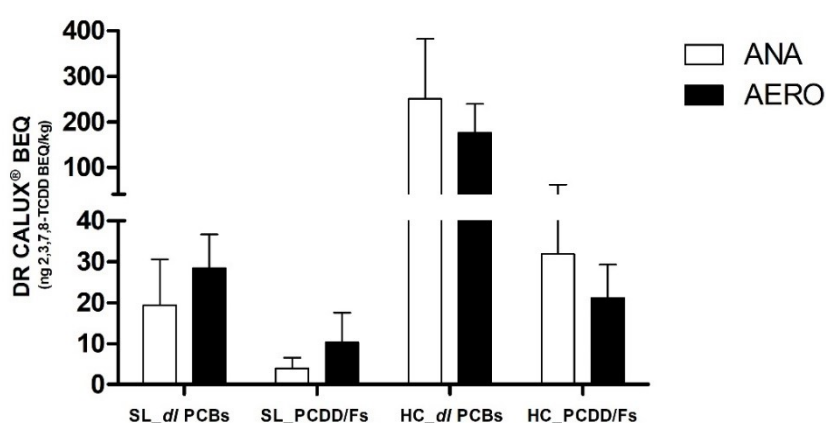


Fig. 3.4 DR CALUX[®] BEQ in sample (ng 2,3,7,8-TCDD eq/kg sample d.w.) expressed as mean $\pm$ standard deviation in sludge and hydrochar samples from the 6 WWTPs from Tuscany according to the different treatment of sludge, anaerobic or aerobic digestion.

The mean DR CALUX[®] BEQ values of hydrochar samples resulted higher than those found in the sludge samples. In particular, in the *dl*-PCBs fraction of hydrochar with anaerobic treatment, mean values are equal to 256.43 ± 131.77 (ng 2,3,7,8-TCDD eq/kg d.w. sample) while for the PCDD/Fs fraction are equal to 35.4 ± 29.88 (ng 2,3,7,8-TCDD eq/kg d.w. sample) (Fig. 3.4). Furthermore, DR CALUX[®] BEQ of hydrochar obtained from aerobic digestion resulted lower than those from anaerobic one (Fig. 3.4). DR CALUX[®] BEQ mean values resulted 175.54 ± 64 (ng 2,3,7,8-TCDD eq/kg d.w. sample) in the *dl*-PCBs fraction and 21.36 ± 8.2 (ng 2,3,7,8-TCDD eq/kg d.w. sample) in the PCDD/Fs fraction (Fig. 3.4).

3.3.2 Comparison with analytical chemical results of sludge and hydrochar samples

Chemical analysis of both extracts (*dl*-PCBs and PCDD/Fs) obtained from each sludge (n=6) and hydrochar (n=6) sample was run by a partner of the SLUDGE 4.0 project and data were used to compare with those obtained from DR CALUX[®] bioassay.

Estimated concentrations for each detected analyte of *dl*-PCBs and PCDD/Fs were expressed in term of Toxic Equivalency (TEQ) and calculated following the Toxic Equivalent Factors (TEFs) reported in Van den Berg et al., (2006) (see Appendix Tab A1).

Statistically significant correlations between BEQs and TEQs were found for both sludge and hydrochar samples (Spearman's $R_s = 0.8252$, $p < 0.001$; Pearson's $R_p = 0.8029$, $p < 0.01$) (Fig. 3.5).

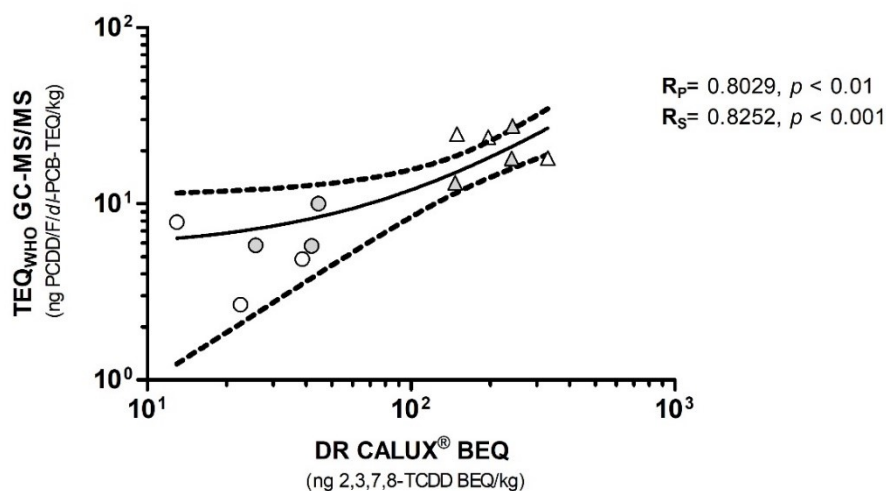


Fig. 3.5 Correlation between TEQ_{WHO} and DR CALUX[®] BEQ values of sludge (circle) and hydrochar (triangle) samples from the 6 WWTPs. Anaerobic WWTPs in white and aerobic in grey. TEQ_{WHO} – toxic equivalent of 2,3,7,8 calculated from analytical results, BEQ – toxic equivalent of 2,3,7,8 TCDD calculated from bioassay results.

R_p – Pearson's correlation, R_s – Spearman's correlation.

The high correlation found between chemical analysis and bioassay suggests that DR CALUX[®] is a valuable method to screen the presence of PCDD/Fs and *dl*-PCBs also in complex matrices as sewage sludge and hydrochar.

GC-MS/MS TEQ obtained from chemical analysis and DR CALUX[®] BEQs obtained from the sum of the biological activities of PCDD/Fs and *dl*-PCBs fractions, showed the same trend in both sludge and hydrochar matrices (Fig. 3.6).

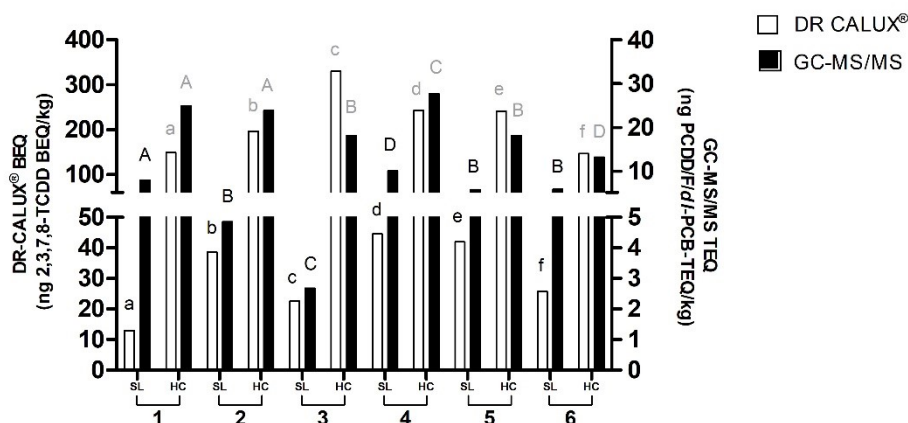


Fig. 3.6 Trend in TEQ_{WHO} values for ng PCDD/PCDF/*dl*-PCBs-TEQ/kg d.w. (black bars) from 6 WWTPs and DR CALUX[®] BEQ (ng 2,3,7,8-TCDD BEQ/kg d.w.) obtained from the sum of the PCDD/PCDFs and *dl*-PCBs fraction extracts (white bars). Capital letters used to show significant differences ($p < 0.05$) among TEQs values from GC-MS/MS analysis and lower-case letters for DR CALUX[®] BEQ values from the bioassay. Black colours of letters were used to show the differences among sludge while grey for hydrochar.

As showed in the Figure 3.6, BEQ values resulted one order of magnitude higher with respect to the TEQ ones for all the WWTPs. Considering the sum between the BEQ of the two extracts (*dl*-PCBs + PCDD/Fs), for sludge samples, WWTP_4(MS) showed the highest BEQ values while WWTP_1(PI) the lowest. Differently, for hydrochar, the total BEQ of WWTP_3(SI) presented the highest induction of *AhR* while WWTP_6(LI) presented the lowest values. On the contrary, the total highest TEQ value was found for both sludge and hydrochar in WWTP_4(MS) while the lowest TEQ was detected for sludge in WWTP_3(SI) and for hydrochar in WWTP_6(LI) (Fig. 3.6; Table 3.3).

In addition, from the observation of the TEQs values calculated, an increase of one order of magnitude for all plants analysed from sludge to hydrochar matrices can be found (Table 3.3).

Tab. 3.3 Toxic equivalent of 2,3,7,8 TCDD, calculated from GC-MS/MS analysis for both sludge and hydrochar samples expressed as total (*dl*-PCBs + PCDD/Fs) and single TEQ, *dl*-PCBs or PCDD/Fs.

TEQ _{WHO}	SLUDGE			HYDROCHAR		
	TOT	<i>dl</i> -PCBs	PCDD/Fs	TOT	<i>dl</i> -PCBs	PCDD/Fs
WWTP_1(PI)	7.8	4.8	3	24.8	11.7	13.1
WWTP_2(GR)	4.8	2.1	2.7	23.8	5.2	18.7
WWTP_3(SI)	2.7	1.2	1.5	18.1	5.2	13
WWTP_4(MS)	10	2.1	7.9	27.6	5.9	21.7
WWTP_5(FI)	5.8	2.5	3.3	18.1	5.2	12.9
WWTP_6(LI)	5.8	2.2	3.6	13.2	4	9.1

Moreover, it is possible to notice that the contribution of PCDD/Fs to total TEQ prevails with respect to *dl*-PCBs for both sludge and hydrochar samples from all WWTPs, with the exception of the sludge of WWTP_1(PI), where the *dl*-PCBs contribution in the total TEQ resulted much higher (Table 3.3). As already shown by BEQ data (Fig. 3.3; Tab. 3.2), chemical analysis of sludge and hydrochar samples confirmed an increase in the relative percentage of dioxins from sludge to hydrochar in all the plants considered (Fig. 3.7). In particular, the increase resulted two times higher in WWTP_1(PI), WWTP_2(GR) and WWTP_5(FI) and three times higher for WWTP_4(MS) (Fig. 3.7). On the contrary, a decrease in the relative abundance percentage was recorded in all plants for furans between the sludge and its relative hydrochar (Fig. 3.7). The decrease resulted two times lower for all the WWTPs analysed except for WWTP_4(MS) where furans were three times lower (Fig. 3.7). A significant decrease in the relative abundance percentage between sludge and hydrochar was also recorded for *dl*-PCBs in all plants analysed except in the WWTP_4(MS) where the relative percentage between sludge and hydrochar remained unaltered (Fig. 3.7).

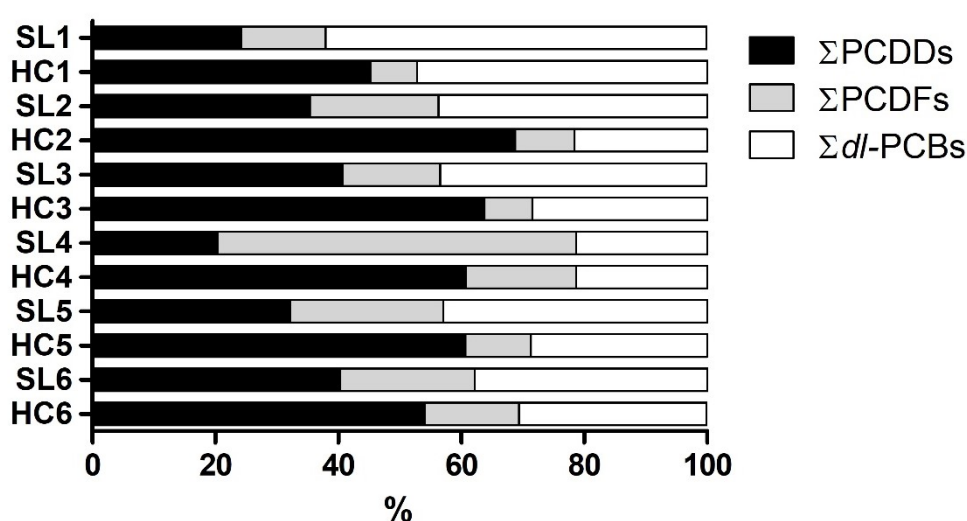


Fig. 3.7 Pattern of the sum of PCDDs, PCDFs and *dl*-PCBs in sludge and hydrochar from the 6 WWTPs based on GC-MS/MS analysis.

Concerning the total concentration of compounds, the highest values for PCDDs in sludge sample were found in WWTP_5(FI), while the lowest one in WWTP_3(SI) (Table 3.4). On the contrary, in the hydrochar samples, the highest PCDDs concentration was recorded in WWTP_4(MS), while the lowest one was found in WWTP_1(PI) (Table 3.4).

For the PCDFs total concentrations, the highest values were found in sludge samples from 4 WWTPs out of 6 compared to their related hydrochar, while the concentrations of both matrices resulted comparable for WWTP_3(SI) and WWTP_6(LI) (Table 3.4). In details, in sludge samples, the highest values for furans were found in WWTP_5(FI), while the lowest were in WWTP_3(SI). Likewise, the lowest furans concentrations were also found in the hydrochar matrices from WWTP_3(SI). Finally, the highest furans concentration was found in WWTP_4(MS) (Table 3.4).

The total concentrations of *dl*-PCBs resulted two orders of magnitude higher than those found for PCDFs and one order of magnitude for PCDDs in all the plants considered for sludge samples (Table 3.4). In hydrochar samples, the total concentrations of *dl*-PCBs were an order of magnitude higher respect to those found for PCDDs and three orders of magnitude higher respect to the PCDFs in 4 WWTPs out of 6 and two orders of magnitude higher in the remaining two (Table 3.4). In general, the highest concentrations of *dl*-PCBs were found in hydrochar samples compared to the sludge ones for all WWTPs analysed, except for WWTP_5(FI), where a small decrease in the concentration from sludge to hydrochar was found (Table 3.4). In details, in both sludge and hydrochar samples, the highest values for *dl*-PCBs were found in WWTP_1(PI), while the lowest concentration for sludge and hydrochar was found in WWTP_3(SI) and WWTP_6(LI), respectively (Table 3.4).

Table 3.4 Levels of PCDD (ng/kg d.m.), PCDF (ng/kg d.m.) and *dl*-PCBs (ng/kg d.m.) in sludge and hydrochar samples from the 6 WWTPs.

	PCDDs		PCDFs		<i>dl</i> -PCBs	
	SL	HC	SL	HC	SL	HC
WWTP_1(PI)	373.2	856	32.8	21.1	9972	18480.8
WWTP_2(GR)	446	1392.3	31.2	25.3	6625.1	12205.4
WWTP_3(SI)	178.8	933.1	15.5	17.2	2812.8	10450.7
WWTP_4(MS)	427.5	1410	90.3	57.6	5762	10099.6
WWTP_5(FI)	577.1	1151.8	108.8	27.7	9139.3	9176.2
WWTP_6(LI)	368.6	886.2	38.1	38.4	6572.5	9076.5

Finally, in terms of congeners profile, a difference profile in dioxins and furans contributions between sludge and hydrochar samples from all plants was found (Fig. SI 3.1; Fig. SI 3.2). On the opposite, no differences between the matrices were observed in terms of congener profile for *dl*-PCBs (Fig. SI 3.3).

In details, for dioxins, OCDD and HpCDD resulted the most prevalent congeners in both sludge and hydrochar for all plants (Fig. SI 3.1). In sludge samples, the OCDD prevails (> 85%) followed by HpCDD (> 10%) while in hydrochar samples, the relative percentage of OCDD was significantly reduced (> 65%) followed by HpCDD (> 20%) (Fig. SI 3.1). However, the less chlorinated congener showed a very low contribution in both matrices (Fig. SI 3.1).

Regarding PCDFs, in sludge samples the most present congeners were OCDF (> 45%) and HpCDF (> 25%) (Fig. SI 3.2). However, in contrast to the hydrochar samples, a more heterogeneous profile was found: the congeners with eight chlorine atoms considerably decrease, while the relative percentage of low chlorinated congeners clearly increased (Fig. SI 3.2). For all hydrochar samples, the second most present congener was HpCDF (Fig. SI 3.2) except for the sample from WWTP_6(LI), where the second most prevalent congener was TCDF, followed by PeCDF.

As far as *dl*-PCBs, no differences in congeners profile between sludge and hydrochar were found (Fig. SI 3.3). The congener most present resulted *dl*-PCB 118 in both samples from all WWTPs (Fig. SI 3.3) followed by *dl*-PCB 170 and *dl*-PCB 105. The lowest concentrations were recorded for the *non-ortho dl*-PCB 81, *dl*-PCB 126 and *dl*-PCB 169 and for *dl*-PCB 114, *dl*-PCB 123 and *dl*-PCB 189.

3.4 Discussion

To date, the question linked to the influence of the HTC on potential toxic molecules already present in the original matrices, such as sewage sludge, is still little explored.

The investigations carried out on the toxicity of hydrochar are in fact mostly based on analytical determinations of selected molecules and never as mixture present in the overall sample. As a matter of fact, chemicals are present as mixtures and additive, synergistic or also antagonistic interactions could always occur (Escher et al., 2020; Neale and Escher, 2020).

Therefore, interactions of chemical mixtures must be considered in environmental risk assessment.

Traditional chemical-analytical methods are essential in the analysis of environmental matrices, for the detection of specific compounds of known mass (Anderson et al., 2017). However, these analyses are somehow expensive, require time and a large amount of sample and at the top of it, they are not enough to fully characterize complexity from a toxicological point of view (Zhang et al., 2009). The use of effect-based tools as bioanalytical assays for environmental risk assessment associated with complex matrices is now fundamental (Escher et al., 2020).

Our study demonstrates the suitability of the DR CALUX[®] assay to measure the occurrence of *dl*-PCBs and PCDD/Fs in sludge and hydrochar samples. Both sludge and hydrochar samples showed the presence of *AhR* agonists able to induce detectable responses in the bioassay. The suitability of the DR CALUX[®] bioassay for the evaluation of the toxicity of sewage sludge has been described in

few publications and before this thesis, it had been never used to study the toxicity of the matrix hydrochar.

As far as sludge, our BEQ values detected in *dl*-PCBs fraction are one order of magnitude higher than those reported by Zhang et al. (2009) in municipal sewage sludge collected from 9 WWTPs in Beijing (China) treated with different processes (7 with dewater, 1 with anaerobic digestion and 1 with lime). In details, mean values found in the *dl*-PCBs fraction in Tuscany sludge samples are 24.6 ± 9 CALUX[®] BEQ (ng 2,3,7,8-TCDD eq / kg d.w. sample), while those reported by Zhang et al. (2009) are 1.68 ± 0.48 CALUX[®] BEQ (ng 2,3,7,8-TCDD eq / kg d.w. sample).

A different trend was observed in BEQs detected for PCDD/Fs fraction, which are one order of magnitude lower and respectively equal to 6.4 ± 5.4 (ng 2,3,7,8-TCDD eq/kg d.w. sample) in Tuscany samples respect those analysed by Zhang et al., (2009) (26.04 ± 11.8 ng 2,3,7,8-TCDD eq/kg d.w. sample) in sludge samples.

Our BEQs are otherwise comparable to those reported for rural and urban sewage sludge by Van Langenhove et al., (2012) in Belgium (range 0 – 30 CALUX[®] BEQ ng 2,3,7,8-TCDD eq/kg d.w. sample).

DR CALUX[®] bioassay was also used to test sludge samples which were not obtained only from urban sewage treatment plants. Similar BEQ values in the PCDD/Fs fraction to those found in sludge from WWTP_4(MS) (16.5 ng 2,3,7,8-TCDD eq/kg d.w. sample), the highest among all plants analysed, were in fact reported by Addeck et al., (2014) in fibrous sludge coming from paper mill in Egypt (14.6 ng 2,3,7,8-TCDD eq/kg d.w. sample). The presence of paper mill plants in proximity of Massa could explain such similarity in the BEQs obtained.

Far higher BEQs values in the range 100 – 220 (ng 2,3,7,8-TCDD eq/kg d.w. sample) are reported by Gustavsson et al., (2004) in industrial sludge samples treated with anaerobic digestion, containing explosives, chemical intermediates and pharmaceutical residues. The same authors also stated that nitroaromatic metabolites, such as aromatic amines and nitroanilines, can represent possible candidates as *AhR* agonists. Indeed, the study showed BEQs response not only depend on the presence of persistent compounds such as PCDD/Fs and *dl*-PCBs, but also on brominated analogues and PAHs, able of acting as agonists of the *AhR* receptor (Gustavsson et al., 2004).

Our study represents the first attempt to apply the DR CALUX[®] bioassay to screen hydrochar samples for PCDD/Fs and *dl*-PCBs. Our findings confirm that changes in the composition of toxicants from sludge to hydrochar could occur. In addition, also aerobic or anaerobic digestion could have a role in such process and in the *AhR* response.

The mean DR CALUX[®] BEQ values of PCDD/Fs fraction reported by Beníšek et al., (2015) in composts and digestates samples, collected throughout 16 countries in Europe, are in the range 0.05

– 1.2 (ng 2,3,7,8-TCDD eq / kg d.w. sample) (Beníšek et al., 2015). As said, many studies suggested how a different treatment of the starting sludge (aerobic or anaerobic treatment) can significantly influence the toxicity recorded by the cellular bioassay (Gustavvson et al., 2004; Zhang et al., 2009). In our urban sewage sludge samples, differences in BEQ values were recorded depending on the different treatment, even if in terms of concentration of organic contaminants, chemical analysis did not show substantial differences between them. In fact, in our samples, aerobic treatment caused more biological toxicity in sludge, but on the opposite, in hydrochar samples the highest BEQ values were recorded in samples treated with anaerobic digestion.

Gustavvson et al., (2004) proposes that aerobic treatment, may be more appropriate in terms of risk assessment because can reduce the risk for formation of persistent *AhR* agonists. In fact, with the DR CALUX[®] bioassay he demonstrated that an anaerobic treatment caused an increase in the levels of acid resistant *AhR* agonists, while an aerobic treatment did not affect the levels of these agonists (Gustavvson et al., 2004).

Zhang et al., (2009) further support the hypothesis that anaerobic digestion is not quite useful for the removal of *dioxin-like* activity in sludge, although it is important in lowering the volume and total organic compounds. Considering the current national law (Decreto Genova, art. 41 of Legislative Decree 109/2018) for the use of sludge as a soil improver in agricultural soils for PCDD/Fs (25 ng/kg TEQ_{WHO}), no sludge in this study exceeds the threshold levels established (the maximum value found is 10 ng/kg TEQ_{WHO} for WWTP_4(MS)). Regarding hydrochar, currently, no legislation limits the admissibility of organic and/or inorganic contaminants in this matrix and their potential reuse in agriculture. Nevertheless, if the same legislation is applied only hydrochar from the WWTP_4(MS) (27.6 ng/kg TEQ_{WHO}) exceeds the *non*-admissibility threshold levels. Then, the WWTP_1(PI) and WWTP_2(GR) showed a value very close to the threshold, respectively 24.8 and 23.8 ng/kg TEQ_{WHO}. The mean of the total TEQ_{WHO} values recorded by chemical analysis for sludge samples from all 6 Tuscany plants were 6.15 ± 2.5 ng/kg TEQ_{WHO} with a range 2.7 – 10 ng/kg TEQ_{WHO}. These data are comparable with the mean values found in urban and rural sludge in Spain by De la Torre et al., (2011) (9.06 ± 7.8 ng/kg TEQ_{WHO}) and in Belgium by Elskens et al., (2013) (5.6 ± 2.9 ng/kg TEQ_{WHO}). On the contrary, the toxicity value resulted lower respect those reported by Eljarrat et al. (2003) for biological sewage sludge samples from Catalonia (6.8 – 27.4 ng/kg TEQ_{WHO}), Hwang et al. (2012) in Korea (0.02 – 49.9 ng/kg TEQ_{WHO}) and Dudzinska (2005) in Poland (53.50 – 65.26 ng/kg TEQ_{WHO}). Moreover, Dudzinska (2005) affirmed that the typical TEQ value for European medium size cities without heavy industry would be 20 ng/kg TEQ_{WHO} and around 200 ng/kg TEQ_{WHO} in highly industrialized areas. Therefore, the toxicity recorded in Tuscany sludge samples could fall within the range of mild contamination.

Regarding the TEQ_{WHO} values calculated for hydrochar samples from all 6 Tuscany plants, they showed a mean value equal to 20.93 ± 5.35 and a range $13.17 - 27.63$ ng/kg TEQ_{WHO}, therefore, a remarkable increase of toxicity based on TEQ_{WHO} was observed. These findings are very similar to those observed by von Eyser et al., (2014) in Germany, which shows a considerably increase in PCDD/Fs from sludge (4.5 ± 2.3 ng/kg TEQ_{WHO}) to hydrochar (19.2 ± 1.7 ng/kg TEQ_{WHO}), after an HTC process performed for 4h at 210 °C. Also the total TEQ_{WHO} calculated by Brookman et al., (2018), showed a five times increase from sludge respect to its relative hydrochar (from 5 to 25 ng/kg TEQ_{WHO}), carbonized at 220 °C for 5h. Brookman et al., (2018) suggested that the temperature is one of the most important parameters in HTC process which can determine a different toxicity in the matrices. In fact, regarding total toxicity (PCDD/Fs/*dl*-PCBs), he showed a linear increase with temperature. However, Brookman et al., (2018) specified that the increase resulted evident only for dioxins and for some *dl*-PCBs congeners, while for furans, the concentrations generally decrease from sludge to hydrochar. This conclusion was confirmed also by our GC-MS/MS analysis for all the WWTPs analysed.

Moreover, the observed increase in biological activity can be related to other parameters beyond the temperature, as for example the pressure used in the HTC process and the quality of the starting input (Libra et al., 2011; Wiedner et al., 2013).

It is known that during the HTC process, there is no further formation of dioxins, since the “dioxin windows”, i.e. the interval between 300 °C and 600 °C, where the neogenesis of dioxins is possible, is not reached under the indicated process conditions (220 °C) (Wiedner et al., 2013; Poerschmann et al., 2015). For this reason, the presence of the organic compounds already in the starting sludge, as demonstrated by both chemical analyses and bioassay could be responsible for the observed effects. Chlorinated organic compounds, in fact, after reaching the WWTPs are not always significantly removed during the mechanical, biotic and chemical/physical treatments and tend to be absorbed into the biosolids (Wu et al., 2009). The persistence of these compounds is mainly determined by their lipophilic nature (Dai et al., 2007). The potential sources of PCDD/Fs release, which determine its accumulation in the sewage sludge, are various: domestic and industrial wastewater, atmospheric deposition and runoff in drains following precipitation or the formation of precursors in the wastewater itself (Rosińska, 2019; Stevens et al., 2001). In addition, it is known that molecules such as phenols and chlorophenols (in particular, pentachlorophenol PCP), are precursors of PCDDs and *dioxin-like* compounds, and that during the processes especially of anaerobic digestion of sludge, with the adequate temperature and concentration of microorganisms, they could be transformed into these more toxic compounds, through complex chemical reactions (Disse et al., 1995; De La Torre et al., 2011; Öberg et al., 1992; Öberg and Rappe, 1992). However, it is possible to exclude this option

because in the present study, the ratio 123678-HxCDD/123478-HxCDD which is consider a useful marker for finding technical mixtures of PCP (if reach the value ≥ 100) (Eljarrat et al., 1999), never exceeds the maximum value of 8 (found in the WWTP_5(FI)). This low value indicates that the PCP as pure chemicals is not dominant source of PCDD/Fs in our samples. Moreover, the further PCP signature, as the ratio between the 124689-HxCDF/total HxCDFs (Eljarrat et al., 1999) showed again a very low value in all WWTPs, confirming the low levels of these compounds in the starting feedstock.

Another peculiarity emerged from the analysis of the fingerprints of the various congeners. It is known that the profile and composition of the congeners of PCDD/Fs/*dl*-PCBs were in fact modified during the HTC process (Tirler et al., 2013). Generally, sewage sludge is dominated by higher chlorinated PCDD/PCDFs congeners (Rosińska, 2019) but with HTC process, they can be converted to the lower chlorinated congeners, but more toxic, and increase the total toxicity (BEQ and TEQ values) of the resulting mixture (Brookman et al., 2018; Tirler et al., 2013).

All sludge samples analysed in the present thesis, were dominated by highly chlorinated dioxins consisting of OCDD (> 85%) and HpCDD (> 10%). Furans also were dominated by the OCDF (> 45%) and HpCDF (> 20%) congeners. The TCDD revealed the lowest concentrations in the range of 0.02 – 0.08% in all sludge samples. These results, with total dominance of Hepta- and Octa-CDD in sludge are in agreement with data reported by Brookman et al., (2018), De La Torre et al. (2011) and von Eyser et al., (2014).

In the hydrochar samples a considerable decrease of OCDD and OCDF content was recorded followed by an increase in the lowest chlorinated congeners. In details, for dioxins, the congener most present results after OCDD (65-70%), the HpCDD (20-30%), while for furans, the HpCDF (30-45%) followed surprisingly by TCDF (7-19%) and PeCDF (7-13%). According to Brookman et al., (2018) and Tirler et al., (2013), a de-chlorination process occurring at 220°C during HTC could be responsible for such change. The more chlorinated PCDD/Fs, already present in the sludge input, would therefore tend to transform into congeners with lower chlorination which are rather higher toxic as shown by TEF values (Brookman et al., 2018). This explain the higher BEQ values for the PCDD/Fs fraction of hydrochar compared to sludge confirmed by TEQ_{WHO} values obtained by chemical analysis.

In addition, it is well known that HTC transforms biomass into a brown coal matrix, resembling the natural process of coal formation (Tirler et al., 2013). This process could be also involved in the de-chlorination with a loss of one or more chlorine in the "2, 3, 7, 8" positions leading to the formation of congeners with high chlorination in the "1, 4, 6, 9" position (Gaus et al., 2002). On the contrary, however, also peri de-chlorination from "1, 4, 6, 9" to "2, 3, 7, 8" position could happen, leading in

this case to an increase in the total toxicity (Tirler et al., 2013). Moreover, as already mentioned, HTC process at 220 °C induces a transformation on the biomass into a product with a higher carbon content by losing water. This could mean that the initial product loses almost half of its weight through water loss. Since PCDD/Fs data are reported on a dry matter basis, the contaminants found in the present thesis, are concentrated almost by a factor 2. Consequently, it can be assumed that the increase in TEQ at 220 °C could be caused not only by de-chlorination, but also from these effects of concentration in a product similar to a sponge/activated carbons (Lozano-Castell et al., 2013; Sevilla et al., 2011; Tirler et al., 2013).

Finally, as far as PCBs, the data concerning the total concentration are in line with those obtained by Brookman et al., (2018) and von Eyser et al., (2014), according to which PCBs concentrations remained little or even un-affected by HTC at least for the plants which follows aerobic processes. On the contrary, for the sludge which uses anaerobic processes he showed a significant change. This is in agreement with the observed increase of PCBs in sludge from aerobic digestion (WWTP_4(MS), WWTP_5(FI), WWTP_6(LI)), while a high increase from sludge to hydrochar samples was observed in those treated with the anaerobic ones (WWTP_1(PI), WWTP_2(GR) and WWTP_3(SI)).

The total concentration of PCBs found in sludge samples in the present study (ranging from 0.002 to 0.009 mg/kg d.w.) were one/two order of magnitude lower respect those found by Eljarrat et al., (2003) (ranging from 0.035 to 0.121 mg/kg d.w.) while *dl*-PCBs resulted similar (range 1.16 – 4.88 TEQ_{WHO} d.w. and 1.86 – 6.62 TEQ_{WHO} d.w. respectively).

Based on the current regulation in force in Italy (Decreto Genova, art. 41 of Legislative Decree 109/2018) PCBs should not exceed 0.8 mg/kg d.w. and in contrast with the limit set at 0.06 mg/kg d.w. (D.Lgs 152/2006) for remediation. Therefore, both sludge and hydrochar did not exceed the current regulatory limits.

Regarding *dioxin-like* PCBs *mono* and *di-ortho* PCBs resulted higher than *non-ortho* PCBs and PCB 118 the predominant *mono-ortho* congener. Similar data were reported in sewage sludge samples by Eljarrat et al., (2003), Olofsson et al., (2012) and Brookman et al., (2018).

In addition to PCDD/Fs and *dl*-PCBs, many other organic contaminants, such as their analogue brominated but also pharmaceuticals, drugs and personal care products, as well as pesticides, can reach wastewater treatment plants and can be further detected in sewage sludge (Castiglioni et al., 2018; Gago-Ferrero et al., 2015; Palmiotto et al., 2018; Riva et al., 2019; Wang et al., 2007; Zuccato et al., 2010) but also in hydrochar (Von Eyser et al., 2016). This happens because these molecules are not persistent but are nowadays ubiquitous, as a result of their continuous use on a global scale (Escher et al., 2020). Furthermore, the treatments to which the wastewaters are subjected are not able

to significantly remove them or their metabolites, which could be even more persistent and toxic (Malvar et al., 2020; Muller et al., 2010; Wu et al., 2009).

Sewage sludge and hydrochar, represent collectors of contaminants of different nature: trace elements, nanoparticles, polyaromatic hydrocarbons, perfluorinated surfactants, polycyclic musks, pesticides, phenols, personal care products, pharmaceuticals (Fijalkowski et al., 2017), therefore, combining analytical and biological analysis could be the only way to try to understand the real possible toxicity of this complex matrices.

Finally, the high correlation found between chemical analysis and bioassay suggests that DR CALUX[®] is a valuable alternative or addition to analytical detection performed by GC-MS/MS for the analysis of PCDD/Fs and *dioxin-like* PCBs also in complex matrices as sewage sludge and hydrochar.

3.5 Conclusion

In the current chapter, DR CALUX[®] bioassay has been demonstrated to be an effective and sensitive tool able to screen the presence of dioxins and *dioxin-like* compounds in sludge and hydrochar samples. It is also a reliable tool able to assess the biological activity of complex mixtures of contaminants as those present in SL and HC.

The highest DR CALUX[®] BEQ values were found in HC samples compared with their respective sludge. DR CALUX[®] BEQ values were also one order of magnitude higher for the *dl*-PCBs fraction respect to the PCDD/Fs one. Chemical analysis allowed to find a high correlation ($R_p = 0.8029$; $R_s = 0.8252$) between DR CALUX[®] BEQ and chemical TEQ_{WHO}. Chemical analysis show that highly chlorinated congeners prevailed in SL compared to HC samples, in agreement with previous studies. HTC process seems responsible for such changes in the level of chlorinated congeners from high to low ones, which are more toxic. It can be assumed that the contamination of the HC is mainly due to a previous contamination of SL feedstock. The increase in the biological toxicity observed by DR CALUX[®] BEQs can be related to the de-chlorination of dioxins and *dioxin-like* compounds and the high loss of water which might affect their levels. DR CALUX[®] bioassay was able to screen the presence of dioxins and *dioxin-like* compounds in SL and HC, raising concern due to their possible applications as soil amendments. The higher BEQ values obtained in HC samples compared to SL call for further studies to limit any potential risk associated to their use as for instance as a soil amendment. This study focuses on the importance of combining bioanalytical tools with chemical analyses in order to evaluate potential risk of sludge and hydrothermal carbonization products for humans and environmental health.

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Supplementary information

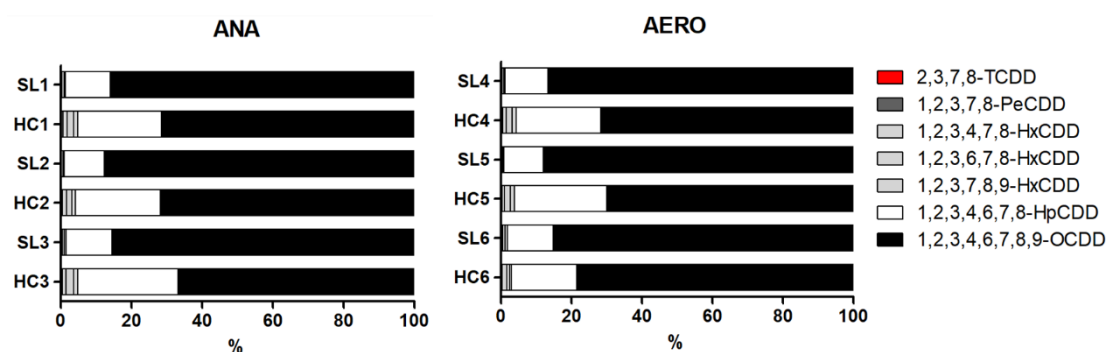


Fig. SI 3.1 Congener profile of Polychlorinated-dibenzo-*p*-dioxin (PCDDs) in sludge and hydrochar from the 6 WWTPs based on GC-MS/MS analysis

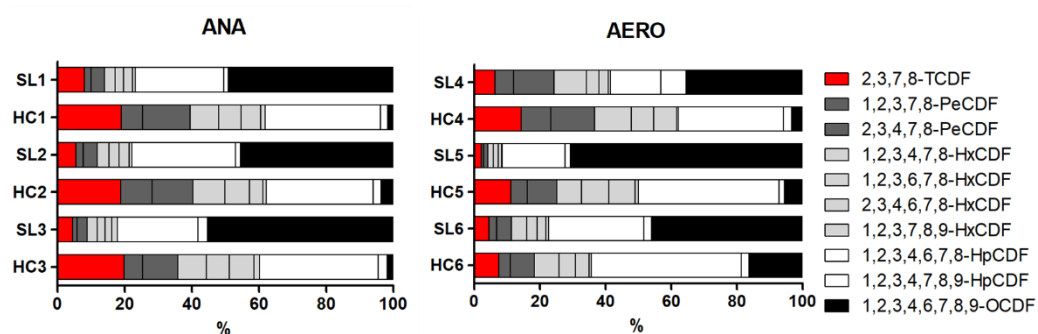


Fig. SI 3.2 Congener profile of Polychlorinated-dibenzofurans (PCDFs) in sludge and hydrochar from the 6 WWTPs based on GC-MS/MS analysis.

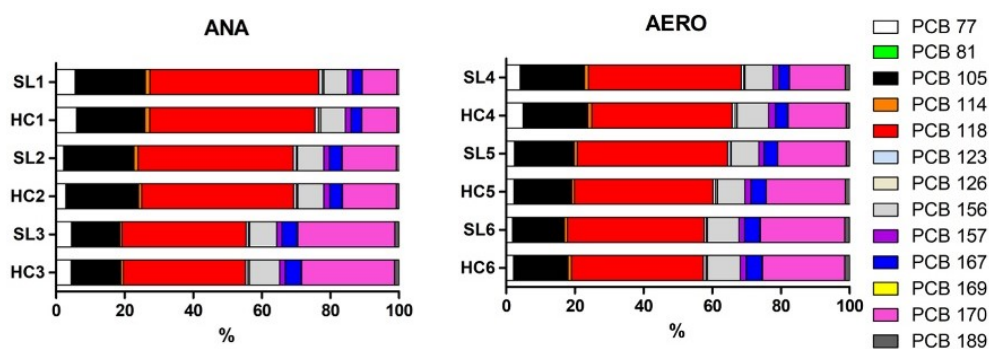


Fig. SI 3.3 Congener profile of *dioxin-like* Polychlorinated biphenyls (*dl*-PCBs) in sludge and hydrochar from the 6 WWTPs based on GC-MS/MS analysis.

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Appendix

Tab. A1 Summary of World Health Organization (WHO) 2005 Toxic Equivalency Factor (TEF) values from Van den Berg et al., (2006) calculated *in vitro* and *in vivo* studies and Relative Effect Potencies (REP) values from van Ede et al., (2013) specifically calculated *in vivo* in mice liver.

	TEF_{WHO 2005} Van den Berg et al., (2006)	REP_{THEORETICAL} van Ede et al., (2013)
Chlorinated dibenzo-<i>p</i>-dioxins		
2,3,7,8 TCDD Tetrachlorodibenzo- <i>p</i> -dioxin	1	1
1,2,3,7,8 PeCDD Pentachlorodibenzo- <i>p</i> -dioxin	1	0.3
1,2,3,4,7,8 HxCDD Hexachlorodibenzo- <i>p</i> -dioxin	0.1	0.1
1,2,3,6,7,8 HxCDD Hexachlorodibenzo- <i>p</i> -dioxin	0.1	0.1
1,2,3,7,8,9 HxCDD Hexachlorodibenzo- <i>p</i> -dioxin	0.1	0.1
1,2,3,4,6,7,8 HpCDD Heptachlorodibenzo- <i>p</i> -dioxin	0.01	0.01
OCDD Octachlorodibenzo- <i>p</i> -dioxin	0.0003	0.0003
Chlorinated dibenzo-furans		
2,3,7,8 TCDF Tetrachlorodibenzo-furan	0.1	0.1
1,2,3,7,8 PeCDF Pentachlorodibenzo-furan	0.03	0.03
2,3,4,7,8 PeCDF Pentachlorodibenzo-furan	0.3	0.04
1,2,3,4,7,8 HxCDF Hexachlorodibenzo-furan	0.1	0.1
1,2,3,6,7,8 HxCDF Hexachlorodibenzo-furan	0.1	0.1
1,2,3,7,8,9 HxCDF Hexachlorodibenzo-furan	0.1	0.1
2,3,4,6,7,8 HxCDF Hexachlorodibenzo-furan	0.1	0.1
1,2,3,4,6,7,8 HpCDF Heptachlorodibenzo-furan	0.01	0.01
1,2,3,4,7,8,9 HpCDF Heptachlorodibenzo-furan	0.01	0.01
OCDF Octachlorodibenzo-furan	0.0003	0.0003
Non-ortho-substituted PCBs		
3,3',4,4'-Tetrachlorobiphenyl (PCB 77)	0.0001	0.0001
3,4,4',5-Tetrachlorobiphenyl (PCB 81)	0.0003	0.0003
3,3',4',4',5-Pentachlorobiphenyl (PCB 126)	0.1	0.003
3,3',4,4',5,5'-Hexachlorobiphenyl (PCB 169)	0.03	0.03
Mono-ortho-substituted PCBs		
2,3,3',4,4'-Pentachlorobiphenyl (PCB 105)	0.00003	0.00003
2,3,4,4',5-Pentachlorobiphenyl (PCB114)	0.00003	0.00003
2,3',4,4',5-Pentachlorobiphenyl (PCB 118)	0.00003	0.00006
2,3',4,4',5'-Pentachlorobiphenyl (PCB123)	0.00003	0.00003
2,3,3',4,4',5-Hexachlorobiphenyl (PCB 156)	0.00003	0.0002
2,3,3',4,4',5'-Hexachlorobiphenyl (PCB 157)	0.00003	0.00003
2,3',4,4',5,5'-Hexachlorobiphenyl (PCB 167)	0.00003	0.00003
2,3,3',4,4',5,5'-Heptachlorobiphenyl (PCB 189)	0.00003	0.00003

Conclusions and outlook

The overall results showed in the present thesis support the suitable application of bioanalytical tools in the ERA of the complex mixtures of contaminants as those naturally occurring in environmental samples. The use of bioassay coupled with classical analytical chemistry and with the new frontier of “omics”, will have the potential to significantly improve the reliability of ERA.

By summarizing the main findings of the 3 chapters, it is possible to formulate the following general conclusions:

- the DR CALUX[®] bioassay allows to focus efforts, time and money in those sites or samples that needed more attention, by identifying new and unexpected sources and route of exposure, reinforcing its suitability in soil pollution monitoring programme;
- coupling DR CALUX[®] with proteomics allows to estimate ecological and human risk posed by legacy pollutants and CECs in an environmental sample;
- the DR CALUX[®] bioassay has been demonstrated to be an effective and sensitive tool able to screen the presence of dioxins and *dioxin-like* compounds in sludge and hydrochar samples. It is also a reliable tool able to assess the biological activity of complex mixtures of contaminants as those present in SL and HC;
- thanks to their peculiarity of using mammalian cells, the DR CALUX[®] bioassay made it possible to evaluate not only environment risk but also the human ones.

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