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CELLULAR REDOX HOMEOSTASIS AND ENVIRONMENTAL TOXICITY

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To

my inspiring NANA

and my little sunrise

AMLA

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ABBREVIATIONS

3-NT	3-nitrotyrosine
ADP	adenosine diphosphate
Ant A	antimycin A
AP	alkylphenols
APEOs	alkylphenol ethoxylates
ASC	ascorbate
BPA	bisphenol-A
BSA	bovine Serum Albumin
bw	body weight
CcOx	cytochrome c oxidase
cDNA	complementary DNA
cGMP	cyclic GMP
Cytc	cytochrome c
DDT	dichloro-diphenyl-trichloro-ethane
Dig	digitonin
E2	17β-estradiol
EC	emerging contaminants
EDCs	endocrine-disrupting chemicals
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ETC	electron transport chain
FBS	fetal bovine serum
GMP	guanosine monophosphate

GP	glyphosate
IMI	imidacloprid
iNOS	induced nitric oxide synthase
MnSOD	manganese superoxide dismutase
nAChR	nicotinic acetylcholine receptor
NAT	1-(H)-naphthotriazole
NIR	nitrate reductase
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOx	nitrites and nitrate
NP	nonylphenol
OP	octylphenol
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFCs	perfluorinated compounds
PFOA	perfluoro-octanoic acid
PHEN	phenmedipham
PS	photosystem
P-Ser1177	phosphorylated serine 1177
P-Thr495	phosphorylated threonine 495
RCR	respiratory control ratio
RED	registration eligibility decision
RNS	reactive nitrogen species
ROS	reactive oxygen species
RONS	reactive nitrogen and oxygen species
Rot	rotenone
Ser	serine
SGC	soluble guanyl cyclase

SOD	superoxide dismutase
Suc	succinate
Thr	threonine
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine
WWTP	waste water treatment plant

1. INTRODUCTION

1.1 Cellular redox homeostasis

The first one who described the biological relevance of the 'homeostasis', as coined by Walter Bradford Cannon in 1926, was Claude Bernard in the 19th century, when he described the protective stability properties for the tissues and organs of the interstitial fluid. He used the term 'mileu intérieur' whose fine equilibria, if broken bring to pathology. The causes that can break cellular homeostasis can be physical, chemical or biological which, under normal conditions, induce feedback reactions to restore the physiological equilibrium. If the stimulus is persistent, however, there can be an alteration of homeostasis. This is how, commonly, the oxidative stress occurs, with a breakage of the physiologic cell redox steady state leading to alteration of its homeostasis. Potentially harming circumstances which may challenge redox homeostasis in a way or another are variations of the O_2 concentration in the aerobic life, as well as interaction with other living systems or exposure to different xenobiotics. The ability to deal with these events is crucial for survival (Ursini et al. 2016).

A complex multi branched redox system is essential in maintaining cellular homeostasis. Under physiologic conditions, the redox balance is maintained through generation and elimination of reactive oxygen/nitrogen species (ROS/RNS). Reactive oxygen species (ROS) include both free radical and non-free radical oxygen-containing molecules such as the superoxide ion (O_2) and the hydrogen peroxide (H_2O_2), respectively (Riley 1994). RNS include the

radical nitric oxide ($NO^{\bullet}$) and the non-radical, though tremendously reactive, peroxynitrite ($ONOO^{-}$) (Valko et al. 2007).

In eukaryotic organisms, the reduction of molecular oxygen leads to production of the highly reactive ROS by the addition of a single electron resulting in superoxide production (Sharma et al. 2012).

Superoxide is dismutated by the superoxide dismutase family of enzymes (SODs) to H_2O_2 that is catalyzed to H_2O and O_2 by catalase (Fukai and Ushio-Fukai 2011).

In the presence of reduced transition metals, H_2O_2 can be converted into the highly reactive hydroxyl radical $OH^{\bullet}$ (Droge et al. 2002). Both exogenous and endogenous sources contribute to the formation of intracellular ROS/RNS. Exogenous sources include physical irradiation, chemotherapeutic agents such as cysplatinum or drugs such as azidothymidine (AZT), the first antiretroviral drug approved for HIV treatment. Endogenous sources include mitochondria, the endoplasmic reticulum, microsomes, peroxisomes, nuclei, the cytosol and plasma membranes, as well as extracellular spaces (Balaban et al. 2005; Pritchard et al. 2001). Mitochondria are the main endogenous source of cellular ROS, where O_2^{-} is generated by the electron leakage from complex I and III of the electron-transport chain (Srinivasan et al. 2001; Le Bras et al. 2005). Besides Complex I and Complex III, several other dehydrogenases of the inner mitochondrial membrane can produce superoxide (Lenaz 2012). The presence of redox-active metals such as Fe and Cu also contributes to ROS generation. In combination with transition metals as Fe(II), Fe(III) or Cu(I), HO^{-} can be generated through the Fenton reaction or Haber–Weiss reaction (Kehrer, 2000). On the other way, $NO^{\bullet}$ is produced through specific nitric oxide synthase isozymes, i.e. the neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Ghafourifar and Cadenas, 2005),

probably including also a mitochondrial nitric oxide synthase (mtNOS). $NO^{\bullet}$ can react with O_2^{-}, and $ONOO^{-}$ is formed (Szabo et al. 2007). Of interest for this thesis, a number of xenobiotics and related chemicals (atmospheric pollutants, industrial contaminants ect) are also involved in production of intracellular ROS/RNS. For example, exposure to metabolites of polychlorinated biphenyls (PCBs) has been shown to increase ROS production in HL-60 cells (Srinivasan et al. 2001).

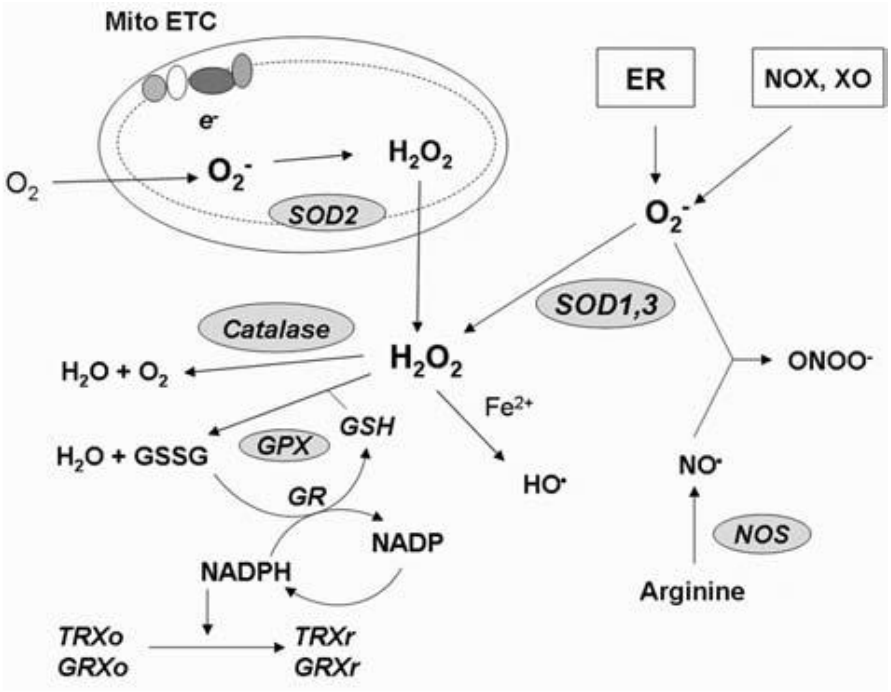


Fig. 1 Redox homeostasis: Major sites of cellular ROS generation include the mitochondrial electron transport chain (Mito ETC), the endoplasmic reticulum (ER) system, and the NAD(P)H oxidase (NOX) complex. Nitric oxide synthases (NOSs) are the enzymes producing NO. Major ROS scavenging enzymes are highlighted in grey. GSH and NAPDH play roles in favoring a more reduced cell redox state (Dunyaporn et al. 2008).

Cells are equipped with enzymatic and non-enzymatic antioxidant systems to eliminate ROS/RNS and maintain redox homeostasis. Among the antioxidant enzymes, SODs (superoxide dismutases) are a main class which catalyze the dismutation of O_2^- to H_2O_2 (McCord et al. 2014).

SOD isoforms are distributed in different cellular compartments. SOD1 (CuZnSOD), which is the main superoxide scavenger, is found in the cytoplasm, mitochondrial intermembrane space, nucleus, and lysosomes, whereas SOD_2(MnSOD) and SOD_3 are found in the mitochondria and extracellular matrix, respectively (Szabo et al. 2007).

Cellular redox systems control the functions of multiple signaling proteins affecting cell survival at the levels of signal transduction, transcriptional regulation, and cell-death execution (Dunyaporn et al. 2008).

1.1.1 Cellular redox alterations

As mentioned above there are many enzyme systems participating in the *in vivo* redox homeostasis to maintain physiological levels of ROS. Imbalance between ROS generation and ROS-scavenging properties due to exogenous stimuli or endogenous metabolic alterations can damage redox homeostasis, leading to an overall increase of intracellular ROS levels, defined as oxidative stress (Rahal et al. 2014).

Increased oxidative stress often involved in a wide variety of pathologic conditions including, among others, cancer, I/R(Ischemia/Reperfusion) injury, hypertension, atherosclerosis, diabetic neuropathies, renal diseases, neurodegenerative diseases, and aging (Valko et al. 2007).

Under normal physiologic conditions, the reactive nature of ROS/RNS at moderate levels allows their reaction with several macromolecules. The

resulting oxidative modifications play very important physiological roles in regulating many cellular functions. However, when the bioavailability of ROS/RNS increases due to overproduction or deficient degradation, owing to the high reactivity of these species, lipids, proteins, and DNA are continuously exposed inside the cell to severe and irreversible oxidative and nitro-oxidative damage (Baud and Ardaillou 1993; Harnel et al. 2008).

Lipids are a prominent target of oxidative modification. Lipid peroxidation generates lipid radicals, which can further attack the subsequent native lipid molecules thus propagating as a chain reaction. Polyunsaturated fatty acid residues of phospholipids are attacked by a radical either at an internal molecular position or near the end of the conjugated system, generating a peroxyl radical (Valko et al. 2006). Attack at an internal position allows the peroxyl radical to further undergo either a cyclization or metal-catalyzed reaction and produce reactive alkoxyl radicals. After cyclization, the fatty acid may form a hydroperoxide or undergo another cyclization, which produces aldehydes, including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) (Pinchuk et al. 1998).

While MDA can further react with DNA bases, resulting in gene mutations, the lipid peroxidation product hydroxynoneal (HNE) reacts mostly with proteins, leading to important functional alterations affecting signaling pathways. Oxidative damage of phospholipids can lead to cell death, not only through membrane damage but also through HNE. Attack of various proteins such as c-Jun N-terminal kinase (JNK) and caspase-3 activation has been found to be a mechanism of cell death induced by lipid peroxidation (Awasthi et al. 2003).

Protein oxidation can be reversible or irreversible, depending on the target and the form of oxidative damage. The highly reactive $OH^{\bullet}$ and $ONOO^{-}$ are

common reactive species that target proteins. Certain aminoacids are more sensitive to oxidation (Valko et al. 2006). When an aminoacid side chain is oxidized, it produces a carbonyl derivative that can be used as a quantitative marker of protein oxidation and oxidative stress (Dean et al. 1997). Sulfur-containing amino acids are also sensitive to reversible and irreversible oxidation. Reversible oxidation of the sulfhydryl group includes intramolecular or intermolecular protein cross-linkage and glutathionylation (Stadtman 2004).

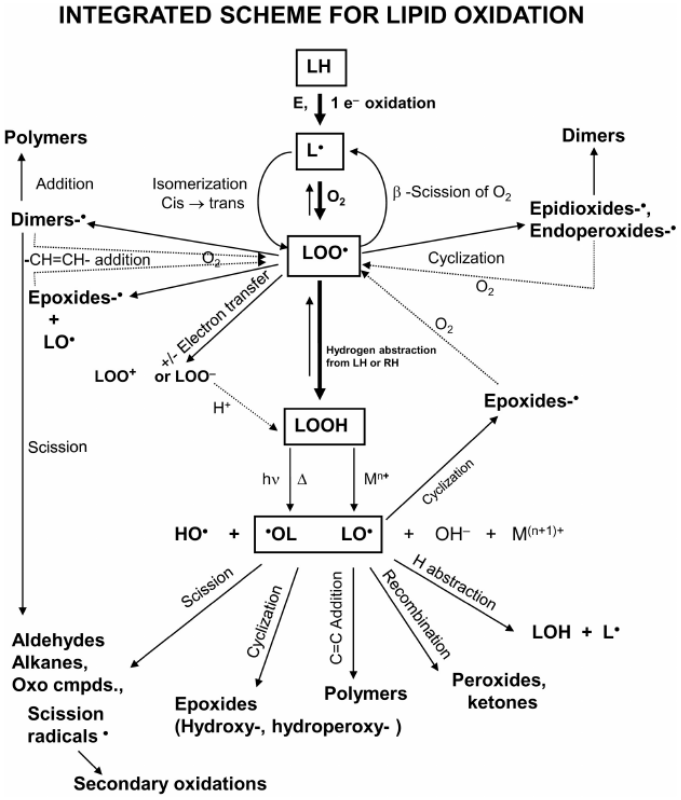


Fig. 2 Lipid peroxidation scheme. (Kerr et al. 2015)

Irreversible protein oxidation includes nitrosylation of cysteine sulfhydryl groups, tyrosine, methionine, and tryptophan, mostly by $ONOO^-$. Nitration of tyrosine residues may inhibit phosphorylation or adenylation, molecular modifications important for protein function (Radi et al. 2001). Severe oxidative stress can induce disulfide bond–mediated protein cross-linkage or secondary oxidative modifications leading to accumulation of damaged proteins and cell death (Poppek and Grune 2006).

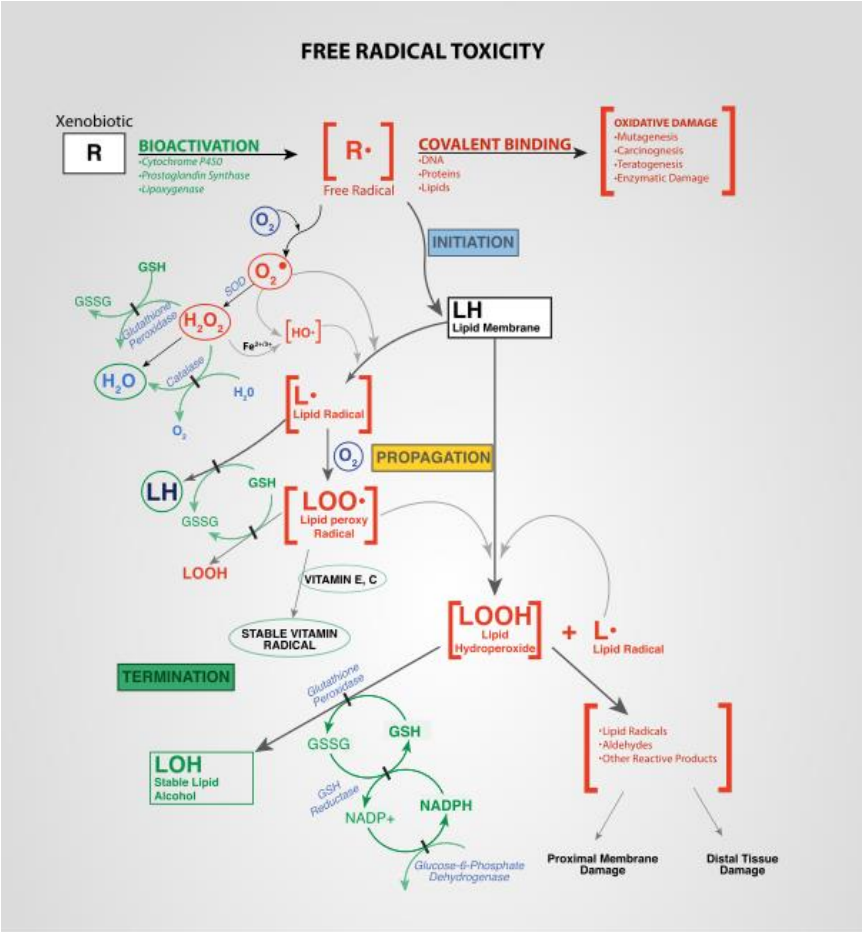


Fig. 3 Xenobiotic redox alterations

Compared with lipids and proteins, nuclear DNA may seem less susceptible to oxidative modifications because of its double helix structure and the protective shield from histones and other coating molecules. However, oxidative nuclear DNA damage is detectable under various conditions. Thus, oxidized products of DNA bases have been used as a marker for damage caused by oxidative stress (Haghdoos et al. 2005). The correlation between oxidative DNA damage at various stages of carcinogenesis has been indeed studied (Tsuzuki et al. 2007). DNA can be damaged at nearly all its components. Both purine and pyrimidine bases and the sugar backbone contain N and O as nucleophilic centers, which are highly susceptible to react with electrophiles, especially $OH^{\bullet}$. Furthermore, the double bonds within the bases are also prime targets for $OH^{\bullet}$ (Breen and Murphy 1995). NO^{-} and $ONOO^{-}$ have been found to react with DNA bases and induce single-strand breaks (Tsuzuki et al. 2007). Oxidation of the sugar backbone, through H abstraction, has been known to cause single-strand breaks and double-strand breaks (Breen and Murphy 1995).

Mitochondrial DNA (mtDNA) is even more susceptible than nuclear DNA to oxidative damage, not only because of its proximity to the major sites of ROS generation (electron-transport chain), but also because of the limited capacity of mtDNA repair (Inoue et al. 2003).

Under physiologic conditions, cellular DNA is constantly attacked by ROS. It has been estimated that in mammalian cells, ~1.5×10^5 oxidative adducts in DNA per cell can be found (Beckman and Ames 1997). As such, those hits may induce mutations and play a role in the cell evolution process. Moderate levels of DNA damage can trigger cell cycle arrest and initiate DNA-repair processes that ensure DNA integrity. In contrast, excessive damage or failure in DNA repair may induce apoptosis (David et al. 2007).

It is worth noting that oxidative modifications of lipids, proteins, or DNA play a crucial role in physiologic processes such as differentiation, maturation, and trafficking of intracellular vesicles (Droge 2002).

However, when the ROS/RNS levels are bioavailable in excess, the biologic consequences are often deleterious. Therefore, regulation of ROS/RNS levels is critical in maintaining cellular homeostasis.

1.1.2 Mitochondria

Mitochondria perform many interconnected functions by producing ATP and other biosynthetic intermediates, being so considered the master regulators of cell bioenergetics and homeostasis. At the same time, they are involved in cellular stress responses such as autophagy and apoptosis (Nunnari and Suomalainen, 2012). They are the central organelles in a variety of essential cell functions and have been, therefore, the focus of numerous studies for many years. However, mitochondria are not only "single organelles", but also form a dynamic network highly interconnected in the cell cytoplasm (Lackner 2014). They are membrane-enclosed organelles distributed in the cytosol of most eukaryotic cells. Mitochondria are typically 0.7 - 1 μm in length. The number of mitochondria in a cell varies widely within organisms and tissue types. Mitochondria are compartmentalized by two membranes, made of phospholipids and containing highly specialized proteins. The outer membrane bilayer encloses the entire organelle and is freely permeable to ions and most small metabolites. The inner mitochondrial membrane is assembled in many folds called *"cristae"*, which greatly expand the surface area of the membrane. The inner membrane is highly impermeable to all molecules and most ions, which require specific membrane transporters to enter or exit the matrix. The

protein-mediated and -regulated permeability of the inner membrane is of vital importance for the morphological and functional integrity of the mitochondrion. ATP is produced via oxidative phosphorylation (OXPHOS) which occurs at the level of the inner mitochondrial membrane. Tricarboxylic acid cycle (TCA), the main source of NADH and $FADH_2$, occurs in the matrix. NADH or $FADH_2$ convey electrons to the electron transport respiratory system. The system contains membrane-bound electron carriers that are organized in the so called respiratory chain or electron transport chain (ETC).

The ETC consists of four complexes (I–IV), i.e. molecular protein complexes which through sequential redox reactions undergo conformational changes to transfer electrons, and with the exeption of Complex II, pump protons from the matrix into the intermembrane space (Diaz et al. 2011). The NADH: ubiquinone oxidoreductase (also called NADH dehydrogenase) is the mitochondrial Complex I and catalyzes the first step of oxidative phosphorylation, through the transfer of two electrons from NADH to a lipid-soluble carrier, ubiquinone (Q). The succinate dehydrogenase is Complex II and may directly catalyze the reduction of ubiquinone. The ubiquinol-cytochrome c oxidoreductase is complex III and oxidizes ubiquinol while reducing cytochrome c (cyt c). The cytochrome c oxidase (CcOX), complex IV, carries electrons from cyt c to molecular oxygen that is reduced to water using up protons in the matrix (scalar protons). The ATP synthase (Complex V) catalyzes the synthesis of ATP. At the level of complex I, III and IV, the free energy released is used to pump protons (H^+) from the mitochondrial matrix into the intermembrane space (vectorial proton translocation). Both vectorial and scalar reactions generate a proton electrochemical potential gradient ($\Delta\mu H^+$) across the inner mitochondrial membrane, the so-called proton–motive force. $\Delta\mu H^+$ is used to drive endoergonic reactions; among them, the synthesis of ATP by ATP synthase is, indeed, the main responsible

reaction for the back flux of protons into the matrix and the gradient dissipation. The proton motive force has two components: *i)* the trasmembrane electrical potential ($\Delta\psi$), which arises from the net accumulation of positive charges (H^+) in the intermembrane space, while under standard conditions the matrix bears a net negative charge and *ii)* the trasmembrane pH gradient (ΔpH), more alkaline inside the matrix. The relationship between these two components is expressed by the Mitchell equation:

$$\Delta\mu H^+ = \Delta\Psi - 60\ \Delta pH$$

Cytochome c oxidase contributes to the energization of the inner mitochondrial membrane, by reduction of O_2 to H_2O and by the pumping of protons out of the mitochondrial matrix. This energy is about twice as high respect to the one produced by reactions catalyzed by complexes I and III (Hinkle et al. 1991).

In the recent years, the role of mitochondria as target of toxic effects induced by environmental contaminants has emerged, also considering their involvement in the regulation of stress responses and cell death (Galluzi et al. 2011).

1.2 Emerging contaminants

In the last decades, life has been revolutionized using many products natural or synthetic, for personal care and domestic uses, put forward by the industry and agriculture. These products have become essential for our society living, including substances such as pharmaceuticals, pesticides, plasticizers, food additives, metabolite and transformation chemicals and other compounds.

Their accumulation and distribution in the environment became a serious concern in the last decades, due to their somewhat recognized interference with human physiology, being therefore considered as emerging contaminants. Emerging contaminants are commonly defined as compounds (both synthetic and natural) whose presence in the environment is not routinely monitored and shows the potential to cause ecological disruption (Raghav et al., 2013; United States Geological Survey, 2014). They have the potential to cause adverse ecological and/or human health effects even at low levels. Although some regulations have already been implemented, more strict regulations are still needed for their monitoring and prevention from being continually introduced to market despite their known toxicity. They have been widely investigated only over the last 20 years due to the lack of sensitive analytical methods for their detection at low levels, typically from ng/L to µg/L. These contaminants are widespread in the aquatic and terrestrial environments where they are accumulated to cause adverse ecological and human health effects. (Wilkinson et al. 2016 ; Barbosa et al. 2016 ; Noguera-Oviedo and Aga 2016).

In our study, we have brought into light some of the representative compounds of different classes of the so called emerging contaminants focused on non-commonly monitored agriculture and industrial chemicals, like: i) pesticides including herbicides as glyphosate (GP) which is the world's most used herbicide and phenmedipham (PHEN), ii) insecticides as imidacloprid (IMI) as well as the industrially widely used bisphenol A (BPA), the perfluorooctanoic acid (PFOA) and iii) intermediate products such as the alkylphenols: nonylphenol (NP) and octylphenol (OP) these days widely investigated as *endocrine disrupting chemicals* (EDCs). After screening of the above xenobiotics we have been focusing our attention on the study of the alkylphenols and their xenoestrogenic potential properties, further discussed in details.

The substances selected for this study are classified in Table 1.

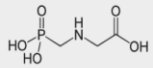
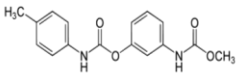
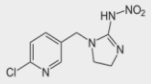
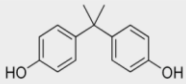
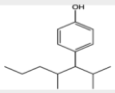
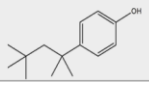
Name	Chemical formula	Uses
*Glyphosate		Broad spectrum herbicide, weed desiccant in agriculture and home gardening
Phenmedipham		Treatment of broad-leaved weeds in their early growth stages ex. Spinach, sugar beet, strawberries (within top 25 most used herbicides)
Imidacloprid		Pest control in agriculture: ex. application to foundations to prevent termite damage, pest control for gardens, treatment of domestic pets to control fleas.
Perfluoro-octanoic acid		Production of industrial and commercial products such as coating additives, cleaning product and pesticides.
Bisphenol-A		Production of polycarbonate plastics, epoxy resins used to line metal cans, and in many plastic consumer products.
Nonylphenol		Production of detergents, paints, pesticides, personal care products, and plastics. (intermediates in the production of phenol/formaldehyde resins and octylphenol ethoxylates)
Octylphenol		

Table 1. *Classification and structure of emerging contaminants in this study.*

Distribution in the environment

Chemical contaminants reach our environment through different ways (Ribeiro et al., 2015) and the interconnection between environmental compartments results in spreading of pollutants through surface-water, ground-water, soil and air. Furthermore, they can also bioaccumulate in plants and other organisms (Rozas et al., 2016). Industries are one of the possible sources of these pollutants through manufacturing, processing and distribution

of the compounds. Industrial waste may result in the release of chemicals into the air (depending on volatility), surface-water, ground-water, soil and biota. Contamination depends on complex processes as transport, transformation, degradation and decay, chemical intermedia transfer, and biological uptake. Additionally, hospital and domestic waste-waters (e.g. containing soaps, shampoo, toothpaste and disinfectants) are also a significant source of contamination. (Barbosa et al. 2016a). Xenobiotics can be completely or partially metabolized in the human body; therefore, their produced metabolites are excreted via urine and feces, reaching thewaste water treatment plants (WWTPs) through their effluents (Ribeiro et al., 2016). Agriculture is another important source of pollutants, mainly due to the pesticides (herbicides and insecticides) used to improve the soil productivity (e.g., pest control, crop desiccation etc.), which become part of waterways as result of agricultural runoff. Furthermore, food additives besides humans are also excreted by animals and may reach the aquatic systems via runoff. Some of these pollutants can also infiltrate the soil and pass to the groundwater system. Other possible sources include leaching from dumpsites, discharge treatment facilities and environmental disasters (Moore et al. 2002; Burkhardt 2011; Ribeiro et al. 2015).

The complete removal of these pollutants and micropollutants, is not possible. Since surface water is widely recycled and used as drinking water (tap water), it can contain micro pollutants, becoming a source of exposure for humans (Barbosa et al. 2016). Although the acute toxic effects are unlikely to occur due to their quite low concentrations in water (often close to nM or less), the *precautionary principle* should prevail due to the feasible chronic and long-term exposure, as a matter of fact more difficult to evaluate (Tijani et al. 2015).

Some reports have shown that exposure to certain pollutants, even at very low concentrations, can originate impacts on biological systems, mostly in fish and aquatic species (Raghav et al. 2013).

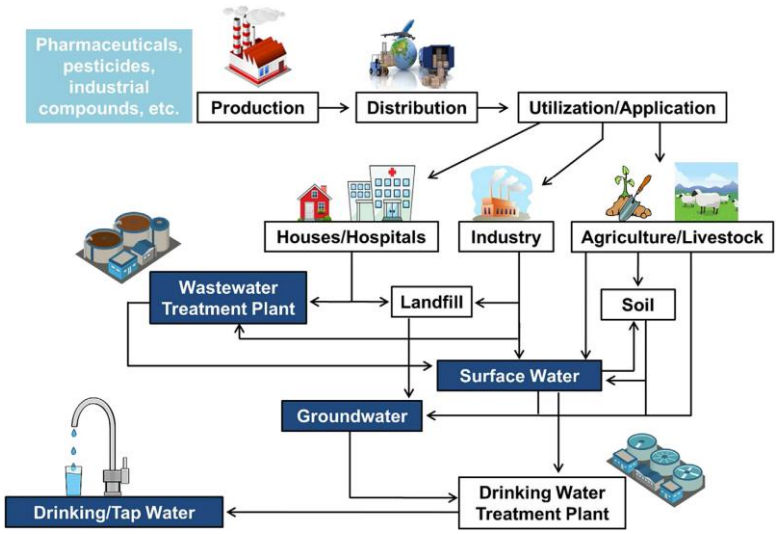


Fig. 4 Representative sources and routes of micropollutants in the environment (Barbosa et al. 2016)

Human exposure

Humans are exposed to ECs such as pesticides, plasticizers and perfluorinated compounds (PFCs) mainly orally through ingestion of food and contaminated liquids as well as interaction with day-to-day products of personal care. There is still a lack of studies of trace-level toxicity of ECs to humans since direct-exposure trials are considered unethical. However, a lot of epidemiological data exist showing the presence of contaminants in humans (blood, urine, and

tissues) and the occurrence of diseases or other phenotypic alterations (Diamanti-Kandarakis et al. 2009). Also, the human fetus is a potential target owing to the mother chronic exposure to even low doses of contaminants; unfortunately, the information on the fetal exposure and long-term effects is still lacking (Prouillac and Lecoeur 2010). The most significant source of pollution in humans occurs through food packaging, as plastic containers and canned linings, and cookware (ex. pans) (Calafat et al. 2008; Liao and Kannan 2012).

1.2.1 Pesticides

In recent years, pesticides have been the center of concern, at the top of the list of emerging contaminants, especially in relation to the health of human beings. Many studies have been published on their effects on human biology, although a lot of contradictions have emerged.

Pesticides started to develop over the last century with inorganic compounds and transitioned to the age of organic herbicides.

Synthetic pesticides, such as the dichloro-diphenyl-trichloro-ethane(DDT), member of the organochlorine hydro became available during the 1940s, generating large profits by the increased agricultural production, environmental spraying of localities for mosquito elimination, and for individual home and garden use.

Rachel Carson's revolutionary book, Silent Spring, first published in 1962, however, started the slow process of raising political and public awareness of the hazards posed to wildlife, humans, and the ecosystem from using pesticides.

From 90s until nowadays, also herbicide resistance in weeds has become a serious problem that accompanies their toxic effects, putting pressure on the entire industry for new molecules discovery.

Pesticides include herbicides, insecticides, fungicides, bactericides, antimicrobials. Herbicides are the most used among pesticides along with insecticides.

There are three routes of exposure to pesticides: oral ingestion, dermal absorption, and inhalation. Many pesticides are used in homes and gardens, and in public parks people get exposed by all three routes. Pesticides can be also tracked inside houses, brought home from work through clothing or in vehicles, exposing family members as well. Breeding of competitive crop varieties increased their use in agriculture by making so possible their massive usage on farmlands for pest and weed control, becoming a source of contaminated landfills and food products. Both domestically and agriculture used pesticides pass easily into ground and surface water, exposing entire populations (Carson 2002; Environmental Health Committee of Ontario, 2004; Kraehmer et al. 2014)**.**

For many years, there is a huge debate on the risk and benefits of pesticides a lot of research has been conducted into the impact of pesticides on the environment.

Glyphosate

N-(phosphonomethyl) glycine or mostly known as Glyphosate(GP)is the world's most used herbicide because of its use in agriculture and home gardening for more than 40 years now (Székács and Darvas 2012; Myers et al. 2017). It is commercially mostly known as the active ingredient of Roundup,

first produced in 1975 (Siriporn Thongprakaisang et al. 2013; Defarge et al. 2016). Now the glyphosate based herbicides residues are everywhere in the environment. The most envied advantages that made glyphosate a successful product through years have become our disadvantages: its high-water solubility has resulted in water pollution (Vereecken 2015; ANES 2016), due to its continuous use pre and post harvesting, weed resistance has developed resulting in genetically modified crops. It is found everywhere in food and feed (Székács and Darvas 2012).

Its specific activity on plant metabolism, given by the inhibition of shikimic acid pathway, initially removed doubts about its possible toxicity to mammals and human beings. More recently, however, more and more studies are showing us the opposite. Many animal studies relate glyphosate to kidney and liver toxicity, food intolerance, genotoxicity, adverse neurobehavioral development and endocrine disruption, it has been lately considered as 'potentially carcinogenic to humans' by the World Health Organisation. (Myers, et al. 2017; Samsel and Seneff 2013).

Although categorized by the World Health Organization (WHO) as "unlikely to present acute hazard in normal use" (Ho-Yeon et al. 2012), it has an oral acute LD50 of 4,230 mg/kg in rats. The persistency of GP in the environment strongly requires further studies to clarify its effect on human health.

Concentrations used in our study correspond to GP residues commonly found in food and feed, and also in different biological human samples.

Phenmedipham

Phenmedipham (Phen) is the active ingredient of many herbicides widely used for weed control, mostly known as one of the active ingredients of

coformulation Betanal®Expert also containing desmedipham and ethofumesate (Schettgen et al. 2001). It is a bis-carbamate herbicide systemically absorbed through leaves, whose selectivity lies in the inhibition of photosynthesis (photosystem II). More specifically it blocks electron transfer between the primary and secondary quinones by binding to D1 protein of the photosystem II (PS II) in the chloroplasts. In this way, the photosynthetic electron transport chain is disrupted, leading to the concomitant inhibition of ATP production and carbon fixation. Plant death is ultimately due to light induced oxidative stress initiated by damage caused by formation of reactive oxygen species (ROS), leading to lipid peroxidation and proteolysis of thylakoid membranes and plant death.

Phenmedipham was first registered in 1970. Over 98% of the total pounds of phenmedipham are applied to sugar beet crops. It is also used for spinach, swiss chard, strawberries, red beet and other beet varieties control.

Phenmedipham is used either alone or in mixture with other ingredients as desmedipham and etofumesate. It is also used as selective post-emergence herbicide for annual weed control in beet in sugar beet (Abbaspoor and Streibig 2007; Laitinen et al. 2006; EPA: Pesticides- Reregistration Eligibility Decision (RED) for Phenmedipham, 2005).

In the 90s phenmedipham was considered to be safe (Sonawane and Knowles 1971) but its extensive use (approximately 200,000 pounds per year) made everyday exposure concerning for long time effect studies. According to a study, human urines of directly exposed (workers) and not exposed population was analised for m-toluidine (phenmedipham metabolite) resulting higher in persons occupationally exposed. The basal secretion of m-toluidine in non-exposed people is probably due to its residues in food (Schettgen and Weiss 2001).

Imidacloprid

A relatively new pesticide (late 90'), Imidacloprid (IMI), trade name Confidor, is a neonicotinoid insecticide, much used worldwide, which acts by mimicking nicotine. It binds to pharmacologically diverse nicotinic acetylcholine receptor (nAChR) subtypes. It is widely used for pest control in agriculture as an insecticide seed treatment for sugar beet and as a foliar spray for apples and tomatoes. It is also applied for home protection against termites (Feng et al. 2007).

EFSA scientific report 2008 concludes that IMI is absorbed orally, does not bioaccumulate and its mainly excreted by urine. However, it admitted the existence of many data gaps and that its risk assessment needs further refinement.

Recent studies suggest it may be contributing to honey bee collapse disorder, so in their colonies decline (EFSA, 2015). In 2010, the world production of imidacloprid was estimated to be ca. 20, 000 t; it was the world's largest selling insecticide in 120 countries with registered uses for more than 140 crops (Al-Sarar et al. 2015).

Since its residues are present in fruits, vegetables, and milk more detailed studies should be made to evaluate their potential toxic risks to non-targetorganisms, particularly human beings (Wang et al. 2016).

1.2.2 Industrial contaminants

Perfluoro-octanoic acid

Perfluorooctanoic acid (PFOA), is one of the most essential representative of perfluorinated compounds (PFCs), produced since the late 1940s and

composed of a fully fluorinated hydrophobic alkyl chain attached to a hydrophilic end group (Meng et al. 2015). Perfluorooctanoic acid (PFOA), has a large range of applications like foams, lubricants, metal spray plating and detergent products, inks, varnishes, coating formulations (for walls, furniture, carpeting, and food packaging), waxes, and water and oil repellents for leather, paper, and textiles (Arvaniti et al. 2015; Ahrens 2011; Miralles-Marco and Harrad 2015). An important industrial application is as a surfactant in the emulsion polymerization of fluoropolymers which are high performance plastic materials used as substances for producing temperature resisting coatings. Since they exhibit high heat, light, and chemical stability, and they are not easily degraded by microbial metabolism (Miralles-Marco and Harrad 2015).

Therefore, PFCs are regarded as persistent, bioaccumulative, and potentially hazardous to animals and humans (Arvaniti et al. 2015; Richardson et al. 2007) however, their transport pathways and global fate have not been adequately documented to date (Richardson et al. 2007, Lindstrom et al. 2011). It widely circulates among the ecosystem and has a long-term elimination half-life in the body ($t/2 \cong 3 \div 9$ years) (Quaak et al. 2016).

Bisphenol-A

Bisphenol A (BPA) is one of the most commonly used chemicals with one of the highest production volumes worldwide (Wang et al. 2017). It is mainly known as a plastic component used in many food and beverage packaging. It is widely used as a primary raw material to produce polycarbonate plastics, epoxy resins, and lacquer coatings. It is also contained in thermographic and pressure-sensitive papers (Chen et al. 2002). It is also found in the air and many everyday contact materials. So, human being is totally exposed to BPA. It can

accumulate in human body, and its presence has been detected in blood, urine, adipose tissue, breast milk and placenta (Maćczak 2017). It was reported that BPA exposure, mainly through food is up to 0.875 mg/kg bw per day (Rivas et al. 2016).

1.2.3 Xenoestrogens

An endocrine-disrupting compound was defined by the U.S. Environmental Protection Agency (EPA) as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process." (Diamanti-Kandarakis et al. 2009). Many contaminants have shown to have endocrine disruptive properties at very low levels such as ng/L. Most of them are chemically similar to physiological estrogen, hence being considered xenoestrogens (XEs). They have the potential to interact with cellular receptors to produce biological responses and effect cell proliferation/death even at extremely low concentrations (1: 10^{15}) (Welshons et al. 2003; Watson et al. 2014).

There exist two type of estrogen receptors: the estrogen receptor *a* (ERa) and the *estrogen receptor b* (ERb). The activation of each of these receptors can induce specific, different and/or overlapping physiological effects (Kuiper et al. 1996; Ascenzi et al. 2006; Bean et al. 2014). These receptors are mainly located in the cell nucleus, though they may be also found in the cytoplasm, in mitochondria, and at the level of the plasma membrane. ERs are able to bind estrogen responsive elements (EREs), thus acting as ligand-dependent transcription factors (Klinge 2001; Nilsson et al. 2001).

Exposure to XEs in our daily lives is nearly inevitable because they are used in a wide variety of industrial products including plastics, pesticides, drugs, detergents, and cosmetics (Park et al., 2013).

Plasticizers, one of the most diffused compounds in the environment, are additives used in plastic manufacture to improve its properties (i.e. flexibility, stability, resistance). Alkylphenols (APs) and bisphenol A (BPA), are main examples among plasticizers which can affect the hormonal system of human and wildlife even at low concentrations. An alkyl chain is present in these compounds which can attach at various locations around its phenolic ring. More than 80% of total APs production is represented by 4-nonylphenols (4-NP) and 4-octylphenols (4-OP) because of their higher use in production of plastics, textiles, paper and agricultural chemical products (Warhust 1995; Salgueiro-Gonzalez et al. 2012). APs are also the main degradation products of alkylphenol ethoxylates (APEOs), very important surfactants used as detergents, lubricants or solubilizers (Pothitou and Voutsa 2008). As demonstrated in many papers, due to their excessive use in industry, linear isomers (4-n-OP and 4-n-NP) are present in aquatic systems and can also accumulate in different organisms (Vidal-Linan et al. 2015; Lisboa et al. 2013).

Human beings are mostly exposed to APs through their evaporation or migration into food and liquids, *via* ingestion, inhalation and dermal absorption, in the μM concentration range (Ademollo et al. 2008; Chen et al. 2008; Tan and Mohd 2003). OP and NP can mimic the interaction of the natural 17β-estradiol (E2) with the estrogen receptors (ERs) through their phenolic unit. They can modulate these receptors positively or negatively depending on their concentrations in tissues (Acconcia et al. 2016; Nimrod and Benson 1996; White et al. 1994).

Their mechanism of action is not clear yet, most of the studies are focused on the correlation between exposure to APs and the increased incidence of certain cancers as breast, ovarian and testis. There is also evidence they may interfere with male/female fertility (Acconcia et al. 2016; Soares et al. 2008; Han et al. 2004; In et al. 2015; Lee and Choi 2013; Shin et al. 2016).

1.3 Cellular alterations after xenobiotic exposure

1.3.1 Nitro-oxidative stress

Oxidative stress (OS) was defined nearly 30 years ago as a bridge between altered redox biology and various diseases being the center of research over the past two decades. Since the role of reactive nitrogen species, such as nitric oxide and its by-products: nitrate (NO_3^-), nitrite (NO_2^-), peroxynitrite (ONOO), and 3-nitrotyrosine have been shown to have a direct role in a variety of diseases, the emergence of NO as a key regulator of redox signaling has led to the discovery of the pathophysiological significance of reactive nitrogen species (RNS) (Gasparovic et al. 2017). Nitric oxide (NO) is a multifunctional signaling molecule which regulates several physiological responses including relaxation of smooth muscles, neurotransmission, platelet aggregation and inflammation (Moncada et al. 1991; Ignarro 1990; Dawson et al. 1994; Bredt and Snyder 1994; Wei et al. 1995). Many of the physiological functions of NO are mediated through the activation of soluble guanylyl cyclase (sGC), its primary molecular target. NO interacts allosterically with sGC to increase cyclic GMP (cGMP) concentration, leading to cGMP-response (Forstermann and Sessa 2012).

NO is involved in a large variety of disorders including chronic diseases e.g. neurologic, cardiovascular, respiratory, inflammatory or neoplastic, as well as some acute diseases such as infection, hypoglycemia, hypoxia, ischemia or toxic shock (Wolley et al. 2013). Under physiological conditions, NO concentrations are usually nanomolar whereas high concentration of NO (micromolar) may turn to pathological. Under certain pathophysiological conditions superoxide as well as NO concentrations rise and nitrosative and nitrative stress almost always precede OS (Daiber et al. 2002; Wayenberg et al. 2011). NO is produced within cells by the actions of a group of enzymes called nitric oxide synthases. NOS acts as a catalyst to convert L-arginine to nitric oxide and L-citrulline. There are three distinct isoforms of nitric oxide synthase: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS or NOS-3), with additional subtypes of the mentioned isoforms i.e. the nNOSμ and the mtNOS (Smutzer 2001). Endothelial NOS/eNOS, neuronal NOS/nNOS are both constitutive enzymes found in mammalian cells. They are found to play crucial role in the cardiovascular system and nervous system respectively, and an inducible NOS/iNOS which is expressed as a response of inflammation. Nevertheless, each isoform varies in its tissue specificity, different NOSs occur at multiple locations within the body. It appears that several isoforms can be found in the same tissue but may have different functions (Drew and Leeuwenburgh 2002). All three enzyme isoforms are catalytically active as homodimers consisting of two identical monomers (Marletta 2001; Bredt 1999). The constitutive NOSs: nNOS and eNOS, generally produce low levels of NO, while iNOS activation is capable to generate large amounts of NO that can be physiologically harmful (Hataishi et al. 2003; Enkhbaatar et al. 2003; Lincoln et al. 1997; Stuehr 1997).

1.3.2 Mitochondrial alterations

As described earlier in the introduction, mitochondria are key organelle involved in essential cellular function like energy production, Ca2+ homeostasis and even programmed cell death. The functionality of mitochondria can determine the state of health of the entire cell.

Mitochondrial respiration is the most important energy production process, but it is also recognized as the most relevant source of reactive oxygen and nitrogen species (RONS) in most eukaryotic cell types (Kowaltowski et al. 2009). These small molecules can act as a signaling molecule in the cytosol, affecting various pathways and networks as cell cycle, energy metabolism, and stress response (Droge 2002).

For the above-mentioned reasons, nowadays there is increased interest not only into the roles of mitochondria in disease development and progression but also as a target for environmental toxicants and as a key "off-target" effect of many drugs. These molecules can affect many mitochondrial processes as the ETC; fatty acid oxidation or uptake, the citric acid cycle, mtDNA replication, and mitochondrial protein synthesis, ROS generation etc. (Meyer et al. 2013).

Among xenobiotics, some emerging contaminants also cause dysfunction of mitochondria processes above mentioned in humans and laboratory animal models. For example, PFOA elevates mitochondrial state 4 respiration and reduces state 3 respiration in isolated rat liver mitochondria (Keller et al., 1992; Langley, 1990).

There are also several studies highlighting the effects of estrogen on mitochondria (Felty and Roy 2005; Liao et al. 2015; Velarde 2014). It has been reported that mitochondria of somatic cells express both ERa and ERb (Psarra and Sekeris 2008). Estrogens can stimulate the expression of genes

encoding both mitochondrial respiratory chain complexes as well as proteins involved in the maintenance of the cell redox homeostasis, such as the mitochondrial manganese superoxide dismutase (MnSOD) (Bettini and Maggi 1992; Chen et al. 1998; Van Itallie et al. 1988) being also a target of xenoestrogens among xenobiotics.

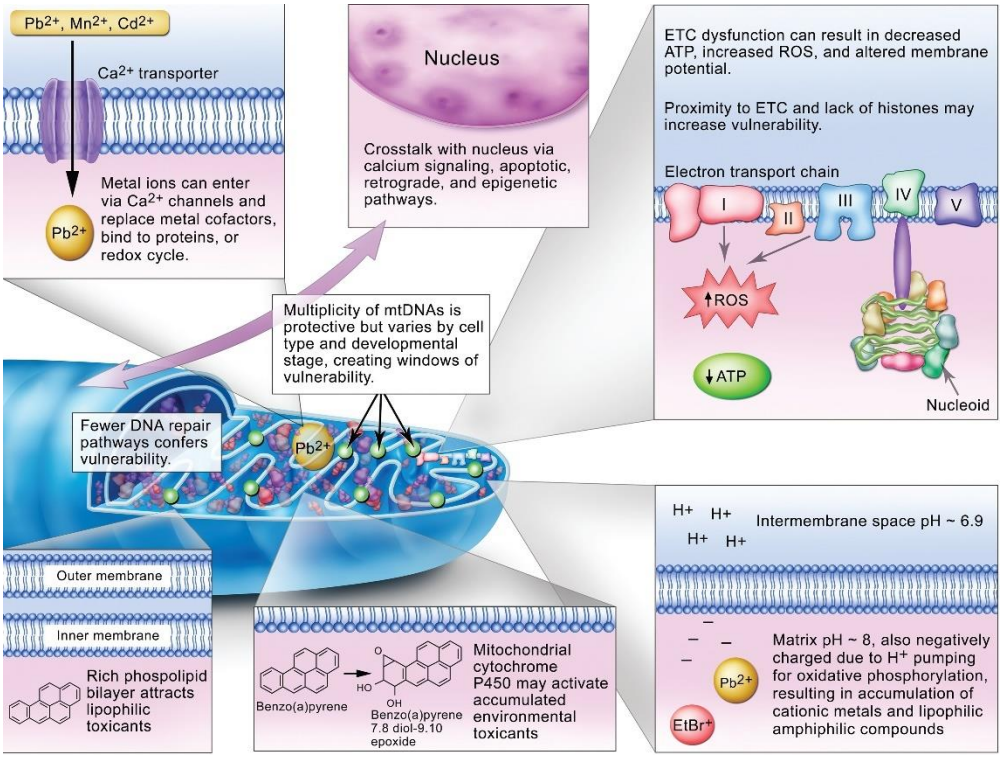


Fig. 5 Mitochondria as a target of xenobiotics

As a conclusion, evaluation of mitochondrial alterations, as a biomarker of sublethal emerging contaminants toxicity must be part of the studies to determine their harmful effects (Yeh et al. 2017; Meyer et al. 2017).

1.3.3 Nitric oxide metabolism and estrogen receptors

Many studies have demonstrated the correlation between nitric oxide metabolism and estrogen receptors (Hishikawa et al. 2014; Kypreos et al. 2014; Lover et al. 2007; Yallampalli and Dong 2000). It was observed that E2 (17-estradiol) at physiological levels leads to an increased expression and activity of endothelial nitric oxide synthase (eNOS) and that this effect is mediated by Erα (Bracamonte and Miller 2001; Chen et al. 1999; Kim et al. 1999). Other studies showed that ER acts as a transcription factor also for inducible nitric oxide synthase (iNOS) (You et al. 2003). It was also noted that the ERa, associated with the membrane at the caveolae level, activates the pathway PI3K / AKT, which, in turn, leds to phosphorylation on Serine 1177 (S1177) of eNOS. This mechanism stabilizes the eNOS (eNOS becomes coupled) and increases the production of NO (Haynes et al. 2000; Wyckoff et al. 2001).

It has been nowadays recognized that the NO chemistry is crucial to a number of physiological responses, from blood vessels contractility and immune response, to neuronal function (Arese et al. 2012). Cell bioenergetics, whether oxidative or glycolytic is affected by NO, rapidly reacting with mitochondrial respiratory chain complexes, particularly with cytochrome c oxidase (complex IV) (Brown 1995; Brunori et al. 2006; Clementi et al. 1998; Galkin et al. 2009; Sarti et al. 2012; Sarti et al. 1999). The pathophysiological relevance of NO depends on its cell bioavailability, thus on the generation of reactive oxygen and nitrogen species (RONS), as in the presence of ROS. At low, nM, concentration values as those generated by the eNOS (Chatterjee et al. 2008), NO seems to act as a physiological effector (Bredt and Snyder 1994; Sarti et al. 2012). On the contrary in the high concentration range (μM), as following the iNOS activation (Dawson et al. 1991), toxic effects appear due to a

persistent OXPHOS inhibition, production of superoxide ions $O_2^{-\cdot}$ and peroxynitrite (ONOO-) (Levin 2002; Marino et al. 1998).

2. AIM

Nowadays human beings are exposed to a wide variety of man-made substances used as pesticides and plasticizers whose intermediate or final products are released in the environment every day. The migration of these chemicals, or their residues from containers and cookware, as well as the ones arising from agriculture crop treatment and desiccation, result in food and waters contamination that are the main sources of chronic exposure.

Being so, the study of the mechanism of action of these chemicals (considered as emerging contaminants) has recently become essential to reverse and prevent further damage for human health.

The chemicals included in our study comprise different compounds belonging to the classes of herbicides: GP and PHEN; insecticides: IMI and industrially widely used BPA, PFOA and alkylphenols: NP and OP.

We aimed to characterize the toxicity of the above mentioned emerging contaminants intracellularly. Liver and skin represent are important targets of xenobiotic exposure as the first is responsible for their metabolism and the second represents the external barrier immediately exposed to the environment; for these reasons the human hepatoma HepG2 (Cresteil et al., 1987) and the spontaneously immortalized human keratinocyte cell line HaCaT (Boukamp et al., 1988) have been chosen as a valid *in vitro* model to study the cytotoxicity, nitrooxidative stress, mitocondrial toxicity and the expression of genes involved in pathways related with oxidative stress, as NOS(s), MnSOD and Cytc (Ledirac et al. 1997).

The fundamental role played by mitochondria in the control of cell bioenergetics and homeostasis, ROS metabolism and ultimately cell survival is well established, even though moreover, its importance as a target of xenobiotics has emerged only recently. Many chemicals have now been identified to target and uncouple the ETC; inhibit mitochondrial transport pathways, fatty acid oxidation or uptake, the citric acid cycle, mtDNA replication, and mitochondrial protein synthesis; generate (or exacerbate generation of) ROS (Meyer et al. 2013).

As a preliminary, fundamental parameter, the screening of cellular viability of different concentrations of each substance, was carried out, to define a sub-lethal concentration of contaminant substances defined as that one able to exert a toxicity level within approximately 20% of cell mortality. The same concentration has been used for all the biochemical parameters herein characterized, including cell ROS/RONS production, mitochondrial membrane potential, respiration and gene expression of NOS isoforms, MnSOD and Cytc.

A main task of the work carried out in this thesis was a detailed investigation of the xenoestrogenic properties of alkylphenols. Our research was based on the evidence that estrogen receptors are also present on mitochondria and that HepG2 cell line expresses both ERα and ERβ, thus representing a valid model system to investigate the effect of the xenoestrogens, OP and NP (Archer et al. 1986; Tam et al. 1986). Several studies have shown a correlation between xenoestrogens and nitric oxide metabolism. So, we aimed to evaluate changes induced by APs on the mitochondrial function and to clarify their mechanism of toxicity by focusing on their ability to interfere with NO signalling.

3. MATERIALS AND METHODS

3.1 Cell cultures

Human hepatocarcinoma (HepG2) cell line and human keratinocyte (HaCaT) cell line were grown at 37 °C, 5% CO_2, 95% air in DMEM (Dulbecco's modified Eagle's) medium containing 4.5 g/L glucose, 10% FBS, supplemented with 2 mM L-glutamine and antibiotics: penicillin (50 U/ml) / streptomycin (50 µg/ml) (HepG2); gentamicin (50 µg/ml) (HaCaT), in 75-cm^2 flasks, 25-cm^2 flasks or multiwall plates. The day before the experiments, cells were grown in DMEM containing 1 g/L glucose and 2 mM L-glutamine (w/oFBS, phenol red and antibiotics).

3.2 Chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Invitrogen Life Technologies (GIBCO, Paisley, UK) and from PAA (Linz, Austria). Xenobiotics (glyphosate, bisphenol A, phenmedipham, perfluorooctanoic acid, octylphenol, nonylphenol and imidacloprid), JC-1, MTT and all other reagents were from Sigma (St. Louis, MO, USA), unless otherwise specified. Real-time PCR reagents were from Stratagene (Santa Clara, CA, USA).

Mother solutions of GP and PHEN were prepared in BD water. PFOA and IMI were prepared in sterilized dimethyl sulfoxide (DMSO). BPA was dissolved

in water- ethanol mixture upon warming, whereas NP was bought in a liquid state.

3.3 Cell viability assay

The viability of HepG2 and HaCaT cells was assessed using the MTT [-3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay as described in Morgan 1988.

Firstly, dose-response experiments were performed, cells (2×10^5/mL) were seeded in a 96-wellplate and incubated for 24 h at 37°C in the presence of each substance at different concentrations varying from 0.1μM to 10 mM in a final volume of 100 μL/well. At the end of each incubation, 10 μL of MTT solution (5 mg/mL) was added to each well, followed by 3 h incubation at 37°C. Afterwards, to dissolve the dark-colored formazan crystals produced by reduction of the MTT tetrazolium salt, cells were incubated for half an hour in the dark with DMSO. The optical density of reduced MTT was measured at 570 nm with a reference wavelength at 690 nm using Appliskan Microplate Reader (Thermo Scientific).

Secondly, vitality time-course, were performed at concentration values of each substance chosen based on dose-response results: the concentration in which cell viability resulted about 80%. This way the major part of the cells is alive, making it possible for us to evaluate the possible cellular alterations caused by xenobiotics exposure. Cells (2×10^5/mL) were seeded in a 96-wellplate and incubated for 6,12,24,48 and 72 h at 37°C in the presence of each substance in

a final volume of 100 μL/well. Then it is proceeded as mentioned above in the dose-response section.

3.4 BCA

BCA Protein Assay Thermo Scientific™ Pierce™ is a formulation of bicinconinic acid (BCA) used for colorimetric quantification of total protein content. The procedure consists in a two-phased reaction generating a product that can be measured colorimetrically by using Appliskan Microplate Reader (Thermo Scientific). During phase I Cu^{2+} is reduced to Cu^{+} in an alkaline environment. Then, in phase II Cu^{2+} is chelated by two BCA molecules producing a violet substance which absorbs at 562 nm, proportionally to the protein quantity in the sample analyzed, using bovine serum albumin (BSA) as a standard (Smith et al. 1985). This method is used to quantify protein content of samples before each measurement.

3.5 Mitochondrial membrane potential measurement

The mitochondrial membrane potential was measured by flow cytometry (Accuri C6 Flow Cytometer®) exploiting the accumulationof the cationic fluorescent probe JC-1 (Fig. 6) into the mitochondrial negativelycharged matrix (Reers et al. 1991*)*. The fluorescence emission of JC-1 depends both on its concentration and on the excitation wavelength.

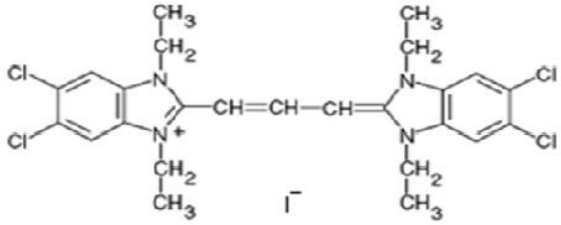


Fig 6 Structure of JC-1 (5,5',6,6'-tetrachloro-1,1',3,3' tetraethyl benzimidazolylcarbocyanine iodide.

Upon exciting at 490 nm, JC-1 monomers display a cytoplasmic fluorescenceemission centered at 537 nm (green band). Above a critical concentration, betterreached at high mitochondrial membrane potential value ($\Delta\Psi \geq 200$ mV), JC-1aggregates are formed in the mitochondria, characterized by an intense emissionband centered at 590 nm (red band) (Fig. 7).

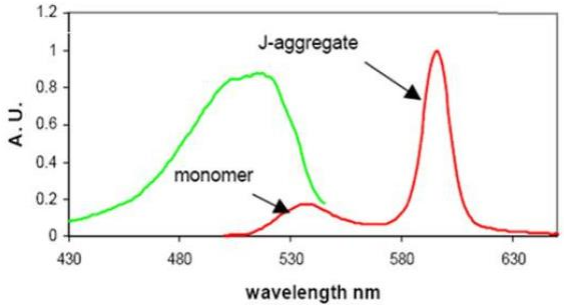


Fig. 7 Excitation and Emission Spectra of JC-1. JC-1 is a cationic dye that exhibit a potential dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~525 nm) to red (~590 nm).

The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates. Before the fluorescence measurement, HepG2 and HaCaT cells, grown in flasks of 25 cm^2 and incubated for 24 h in the presence or absence of each substance. For the experiment, cells were trypsinized, gently centrifuged at 1100 x g, for 5 min at 20 °C, and resuspended in PBS buffer. Cell suspensions were incubated 20 min in the dark with JC-1 2.5 μg/mL, washed two times and re-suspended in 300 μL PBS. After each measurement nigericin and then valinomycin were added as controls. Nigericin is an electroneutral K^+/H^+ antiporter ionophore which converts the ΔpH component of $\Delta\mu H^+$ into the electrical membrane potential gradient ΔΨ, allowing full expression of mitochondrial $\Delta\mu H^+$ as ΔΨ (Reed 1979) (Fig. 8a).

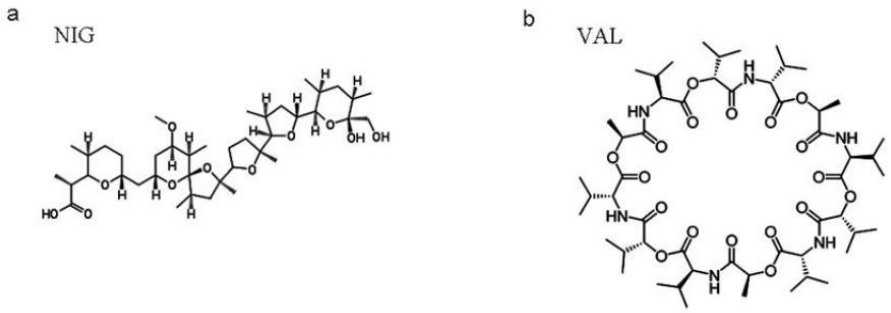


Fig. 8 Structure of nigericin (a) and valinomycin (b)

After addition of nigericin, the fluorescence level reached a maximum within approximately 5 min; thereafter, the fluorescence signal was rapidly dissipated by 0.2 μM valinomycin, which is a mobile carrier ionophore which catalyzes the electrical uniport of K^+ (Fig. 8b).

JC-1 was excited at 488 nm, green fluorescence was quantified 530 nm (FL1 channel), and red fluorescence was quantified at 585 nm (FL1 channel). At least 10,000 events/sample were acquired in log mode.

3.6 Nitrate / Nitrite (NOX) determination

The total concentration of nitrite and nitrate (NOx) is commonly used as a quantitative measure of NO production. Accumulation of the NOx in the culture medium of HepG2 and HaCaT cells (~ 2.5×10^5 cells/mL), grown over night in DMEM 1 g/L glucose (without FBS and phenol red), was measured after 24h exposure to each substance: GP(1 μM, 100 μM and 1mM), BPA(50 μM), PHEN (20 μM), PFOA (10 μM), IMI (50 μM), NP (20 μM) and OP(10 μM. Incubations of cells for NOx measurements were performed in serum-free DMEM without phenol red, because both FBS and phenol red can cause a significant reduction in the intensity of the fluorescence. After incubation, the cell supernatants were centrifuged at 4°C, 1000 x g for 10 min and the NOx content was determined fluorometrically (Nitrate/Nitrite Fluorometric Assay Kit, Cayman Chemical Co.). Nitrate/Nitrite Fluorometric Assay Kit provides an accurate and convenient measurement of total nitrate/nitrite concentration in a simple two-step process. In the first step nitrate is converted to nitrite by nitrate reductase (NIR):

$$NO_3 + e^- + 2H^+ \xrightarrow{NIR} NO_2 + H_2O$$

In the second step, nitrite reacts with the fluorescent probe DAN (2, 3-diaminonaphthalene). NaOH enhances the detection of the fluorescent product, 1(H)-naphthotriazole (NAT) (Fig. 9).

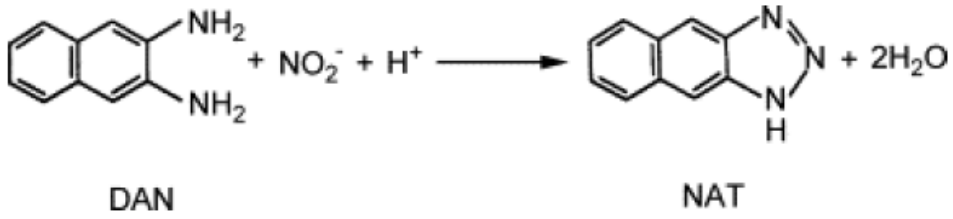


Fig 9. Reaction of 2,3-Diaminonaphthalene (DAN) with NO_2

The fluorescent intensity is proportional to the total nitric oxide production andwas measured at the Fluorescence Plate Reader VICTORTM Multilabel Counter (Perkin Elmer) using an excitation wavelength of 375 nm and an emissionwavelength of 420 nm.

The NOX concentrations were determined using the following equation:

$$[Nitrate + Nitrite](\mu M) = \left(\frac{fluorescence - y - intercept}{slope}\right)\left(\frac{1}{volume\ of\ sample\ used(\mu l)}\right) \text{x dilution}$$

3.7 Reactive Oxygen Species, measurements

Cell ROS generation was assessed by using the fluorescent probe 2´,7´-dichlorodihydrofluorescein diacetate (DCFDA). Before the experiment, HepG2 and HaCaT cells, incubated 24 h in the presence and absence of each substance GP (1 μM, 100 μM, 1mM) BPA (50 μM), PHEN (20 μM), PFOA(10 μM), IMI(50 μM) and alkylphenols : NP(20 μM), OP(10 μM) were trypsinized, pelleted at 1000 × g for 5 min at 20 °C, and resuspended in Hank's buffer at the density $1\times10_6$ cells/ml. The HepG2 and HaCaT cells were treated under the same conditions. Cell suspensions ($1\times10_6$ cells/ml) were plated in 24-well (black) plates. When necessary 500 μM H_2O_2 was added as a control. The relative fluorescence emission, after cell loading of DCFDA 100 μM, was followed of 520 nm (VICTOR$_{TM}$ Multilabel Counter, Perkin Elmer).

3.8 Polarographic measurements

Oxygen consumption was measured with the OROBOROS Oxygraph 2k (Fig. 17) (Oroboros Instruments). The software DatLab (Oroboros, Innsbruck, Austria) was used for data acquisition (1 s time intervals) and analysis, including calculation of the time derivative of oxygen concentration normalized per mg of protein (O_2 flux per mass: pmol sec_{-1} mg_{-1}), signal deconvolution dependent on the response time(τ) of the oxygen sensor and correction for instrumental background oxygen flux (Gnaiger et al. 1995; Gnaiger 2001).

In all applications, the chamber volume is 1.5 ml. The large inner diameter of the chamber (16 mm) provides space for additional electrodes, lightguide and mechanical transducer. Each chamber is equipped with a polarographic

oxygen sensor (POS) with a large cathode (2-mm diameter) that increases the sensitivity and signal-to-noise ratio and decreases the signal drift at zero oxygen. Gas-aqueous phase boundaries must be avoided to exclude uncontrolled oxygen gradients within the measuring system. Measurements of oxygen back-diffusion and mathematical correction for background were carried out to perform high accuracy of flux.

3.8.1 Polarographic oxygen sensor (POS)

In the O_2 Clark-type electrode, the probe is a platinum wire coated with Ag/AgCl, serving as reference electrode. The selectivity towards the specific gas is achieved by i) a gas-permeable hydrophobic membrane isolating the probe from the other redox-active non-gaseous species which might be present in the solution and ii) the polarization voltage applied. This is – 0.67 V for O_2 ($1/2\ O_2 + 2\ e^- + 2\ H^+ = H_2O$).

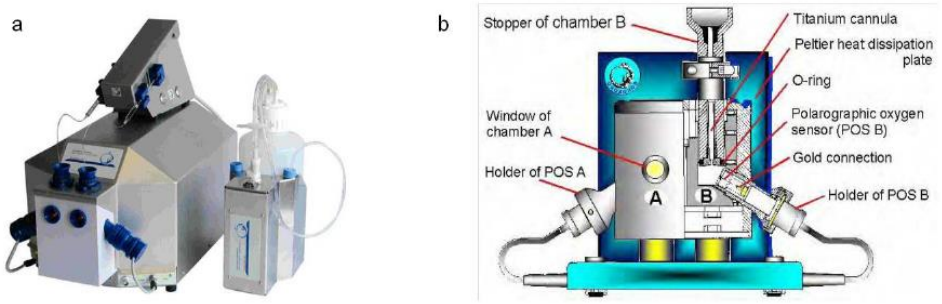


Fig. 10 a) The OROBOROS Oxygraph 2k with the Titration-Injection microPump (Tip-2k) on top and the Integrated Suction System (ISS) on the right. b) Inside view of the OROBOROS Oxygraph 2k instruments. A, left: external of chamber; B, right: internal of chamber.

The oxygen electrode was calibrated by recording the signal in air-equilibrated buffer (corresponding to [O_2] in the range 200-300 μM, calculated from the solubility of the gas in the experimental conditions used) and subtracting the signal measured upon addition of excess sodium dithionite ([O_2] = 0).

3.8.2 Air and oxygen calibration

The oxygen sensors were calibrated by a two-point calibration, routinely achieved at air saturation and zero oxygen concentration. Accordingly, static calibration involves the determination of the constant signal of the polarographic oxygen sensor at 0 % and 100 % air saturation (R_0 and R_1, respectively) under the particular experimental conditions (temperature, signal amplification by electronic gain, polarization voltage, stirring speed, medium). Air saturation was achieved by stirring the medium without sample in the chambers in contact with air, until the oxygen signal (the slope expressed as pmol sec^{-1} ml^{-1}) becomes constant (in about 20 min). After stabilization of the oxygen signal, R1 must be < 10 Volts. Zero oxygen calibration was achieved by allowing complete oxygen depletion or alternatively by use a freshly prepared a 2-5 % solution of Na-dithionite (sodium hydrosulfite: $Na_2S_2O_4$) in water. The instrumental background test is necessary for a calibration of the O_2k chamber performance. The chamber was closed without sample and after stabilization for 10 min, oxygen consumption was obtained by the polarographic oxygen sensor at air saturation and it was indicated as J°1 (the uncorrected oxygen slope expressed as pmol sec^{-1} ml^{-1}). In the next step, the oxygen concentration was reduced by nitrogen gas. At progressively lower steps of oxygen concentration, the oxygen consumption by the sensor decreased linearly and the effect of oxygen back diffusion was finally apparent

as a positive slope or negative flux. The linear dependence of background oxygen flux from oxygen concentration is described by the equation with slope $b°$ and intercept $a°$: $J_{O2}° = b° \, cO_2 + a°$ ($Y = b°x + a°$) The linear regression was automatically displayed in the DatLab4-Excel file "O_2k- Background.xls", plotting background oxygen flux as a function of oxygen flux as a function of oxygen concentration with intercept $a°$ and slope $b°$ (Fig. 11).

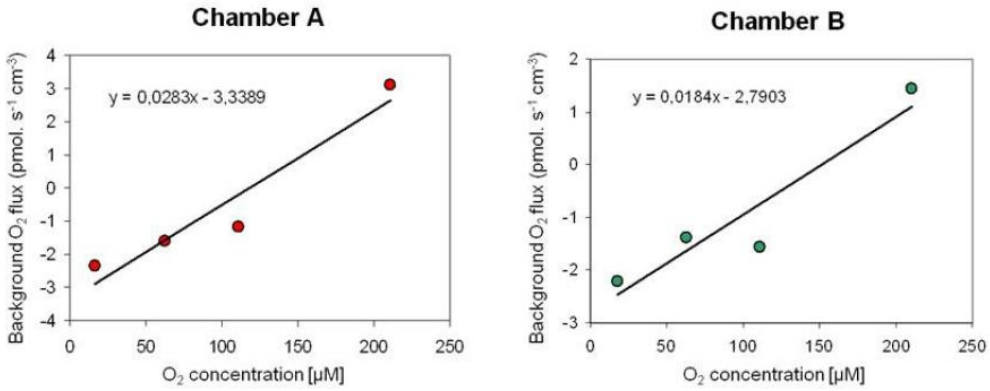


Fig. 11 O2k-Background. Oxygen calibration in Hank's buffer containing 5.5 mM glucose. The graphs show oxygen flux as a function of oxygen concentration, with the linear regression parameters.

These values were used for on-line instrumental background correction of flux during respirometric experiments in the corresponding O_2k-chambers.

3.8.3 Measurements of oxygen consumption in intact cells

HepG2 and HaCaT cells, were incubated 24h with each substance in an antibiotic/FBS-free DMEM medium. For the assay, cells were harvested with trypsin– EDTA, washed twice by centrifugation at 1000 x g for 5 min at 20°C through Hank's medium, at a density of $3{,}3 \times 10_6$ cells / ml. Cellular oxygen

consumption was evaluated using high resolution respirometry. Value were normalized by protein determination BCA assay as previously mentioned.

3.8.4 Measurements of oxygen consumption in permeabilized cells

HepG2 cells, grown over night in 1 g/L glucose DMEM (w/o FBS and phenolred), were incubated 24 h in the presence and absence of GP (1 μM, 100 μM and 1mM) and alkylphenols: NP (20 μM) and OP (10 μM). Cells were cultured in 75-cm^2 flasks in DMEM containing 10 % FBS, 2 mM L-glutamine, 1 % penicillin/streptomycin and gentamincin respectively. Before the experiments, cells were washed twice with PBS, harvested with 0.5 % trypsin-EDTA, gently centrifuged at 1100 x g for 5 min at 20 °C, washed with respiration medium, centrifuged at 1100 x g for 5 min at 20 °C and resuspended in respiration medium. Cell density was determined by cell count and cell viability was evaluated by trypan blue exclusion test. Total protein was measured by BCA assay. The respiration medium was added to the oxygraph chambers about ten minutes before the measurements and was equilibrated with atmospheric oxygen and the required experimental temperature (37 °C). Cell suspension was added to the oxygraph chambers containing respiration medium to a final cell density of 3.3 x 10^6 cells / ml and the system was equilibrated again for 5 min. The respiration medium consists of: 3 mM $MgCl_2$ x 6 H_2O (0.61 g/L), 20 mM taurine (2.502 g/L), 10 mM KH_2PO_4 (1.361 g/L), 20 mM HEPES (4.77 g/L), 1 g/L BSA, 110mM mannitol (20.04 g/L), 0.3mM dithiothreitol (0.046 g/L), pH 7.1, adjusted with 5 N KOH. Respiration was assayed and evaluated in cells permeabilized with digitonin. The digitonin is a cholesterol complex-forming agent that interact with cholesterol molecules of the plasma membrane (the polar heads of the cholesterol are associated with the polar heads of the phospholipids). The

interaction induces a loss of membrane integrity (permeabilization), so that the barrier between the intracellular space and surrounding medium disappears. The cholesterol content of intracellular organelles like mitochondria is considerably lower and so the optimal digitonin concentration that doesn't disrupt these organelles but its action is confined to outer cell membrane needs to be experimentally determined. The optimal digitonin concentration and the incubation time was determined according to (Gnaiger et al. 1998). Briefly, the digitonin titration curve for 3.3 x 10_6 cells/ml was performed in mitochondrial medium, in the presence of 10 mM succinate, 0.5 μM rotenone and 1 mM ADP (Fig. 12). Time intervals between titrations were 12-14 min up to 3 μg cm^{-3} and 4-5 min at higher digitonin concentrations. Putative membrane damage produced by digitonin was also evaluated, by independently assaying the onset of sensitivity of cell respiration to externally added cytochrome c^{2+}. The optimal digitonin concentration obtained as described was added to cell suspension using a 25-μl Hamilton microsyringe and was incubated for about 10 min (at 37 °C). Typically, after addition of digitonin, the cell respiration rate markedly declines for 5–7 min. The lower rate of endogenous cellular respiration in the absence of mitochondrial substrates (e.g., pyruvate + malate) is indicative of the plasma membrane permeabilization, followed by the leakage and dilution of intracellular metabolites when complete cell permeabilization has been achieved. Digitonin used for permeabilization does not influence mitochondrial respiration and can be present in the medium during the entire respirometric run.

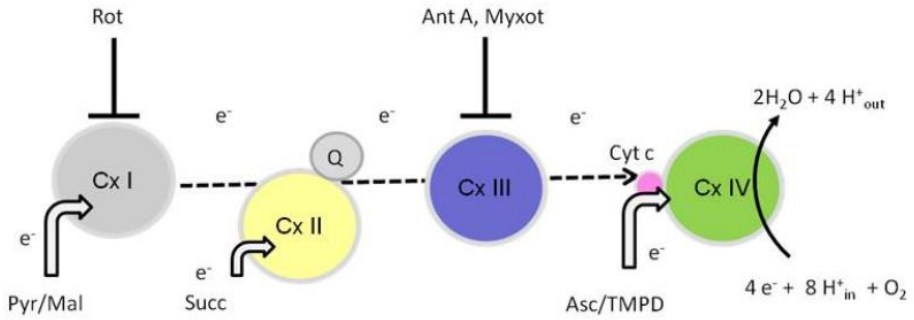


Fig. 12 Sequential representation of the mitochondrial respiratory chain. Experimental design ofoxygraphic titration, using specific substrate and inhibitors. Cx I-IV: mitochondrial complex I-IV.Rot: rotenone (0.5 µM, inhibitor of Cx I), Ant A: antimycin A (5 µM, inhibitor of Cx III). Pyr/Mal:Pyruvate + Malate (8.8 mM and 4.4 mM respectively); Succ: succinate (10 mM); Asc/TMPD:ascorbate + TMPD (2 mM and 0.5 mM respectively, reductants of CxIV); Q: ubiquinol, Cyt c:cytochrome c.

The contribution of the respiratory complexes to cell respiration was also evaluated according to (Kuznetsov et al. 2008) with minor modifications (fig. 12):

– 8.8 mM pyruvate and 4.4 mM malate were added, using a 25-µl Hamilton microsyringe, to record resting complex I-supported respiration, without ADP.

– ADP was added, using a 25-µl Hamilton microsyringe, to obtain a final (saturating) concentration of 2 mM for maximal mitochondrial respiration (state 3). The rate of state 3 respiration at saturating ADP concentrations should be stable for at least 10–15 min.

- 0.5 μM rotenone, a specific inhibitor of complex I, was added to inhibit complex I. Respiration should be almost completely inhibited.
- 10 mM succinate was added to induce complex II-supported respiration.
- 5 μM antimycin A, a specific inhibitor of complex III was added to inhibit Complex III. Inhibition of respiration should be similar to that seen after the addition of rotenone.
- Complex IV respiration was activated by adding 0.5 mM TMPD and 2 mM ascorbate, artificial substrates specific for this complex.
- 10 mM cytochrome c was added to show the intactness of the outer mitochondrial membrane.

3.9 3-Nitrotyrosine Level Detection

The content in 3-nitrotyrosine (3-NT) modified proteins was used as marker of protein damage by peroxynitrite in HepG2 cells in the absence and presence of 20 μM nonylphenol and 10 μM octylphenol. After treatments, HepG2 cells were trypsinized, pelleted at 500 x g for 10 min and washed twice with PBS buffer. Cell pellet ($1x10^6$ cells) were resuspended with extraction buffer and incubate on ice for 20 min. After centrifugation at 12.000 x g 4°C for 20 min, the 3-NT levels were assessed calorimetrically using a competitive Nitrotyrosine ELISA kit (Abcam ab113848). The concentrations of samples proteins from HepG2 cells were normalized with the use of the protein quantity obtained with the BCA. Absorbance was measured at 450 nm.

3.10 Citrate Synthase

Untreated HepG2 and HaCaT cells (1x106) and treated cells incubated 24h with GP (1 μM, 100 μM and 1mM), were harvested by trypsinization and centrifugation (1.000 x g for 5 min at 20 °C), and then were lysed by CelLytic TMMT Cell Lysis Reagent in the presence of the Protease Inhibitor Cocktail and centrifuged at 20.000 x g for 10 min. Cell lysates were assayed for the determination of citrate synthase activity (Srere 1969). Citrate synthase is localized in the mitochondrial matrix and is commonly used as a quantitative marker enzyme for the content of intact mitochondria. Citrate synthase catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid (OAA) to form citric acid. The hydrolysis of the thioester of acetyl CoA results in the formation of CoA with a thiol group (CoA-SH). The thiol reacts with the DTNB in the reaction mixture to form 5-thio-2-nitrobenzoic acid (TNB). This yellow product (TNB) is observed spectrophotometrically by measuring absorbance at 412 nm.

3.11 Real Time PCR

Quantitative Real Time Polymerase Chain Reaction (QRT-PCR) was carried outin HepG2 and HaCaT cells, incubated 24 h with BPA (50 μM), Phen (20 μM), PFOA (10 μM), IMI (50 μM), NP (20 μM) and OP (10 μM), using specific oligonucleotide primers in order to identify and quantify the different Nitric Oxide Synthase (NOS) isoforms, MnSOD and Cytc. Alklphenols NP and OP were also incubated for 6h, 12hfor further investigation in HepG2 cell line. After incubation, cells were harvested by trypsinization and

centrifugation (1000 x g for 5 min at 20°C), washed twice with Hank's buffer and counted. Cells ($1x10_6$) were lysed by 350 µL of RA1 Buffer containing10 mM DTT. RA1 Buffer is a buffer used for lysing cells as part of the kit Nucleo Spin® RNA isolation (Macherey-Nagel, Germany). The cell lysates were stored at -80 or used immediately for RNA extraction. Total RNA was isolated by the same kit instructions. RNA purity was measured using a spectrophotometer by determining the ratio of absorbance at 260 nm to the absorbance at 280 nm (A_{260}/A_{280}). Pure RNA shows an A_{260}/A_{280} ratio of 1.9 - 2.1 in 10 mM Tris at pH 7.5. Contamination by phenol or urea will show absorbance at 230 nm or 270 nm, respectively. Protein contaminants have a high absorbance at 280 nm and therefore produce a low A_{260}/A_{280} ratio. Absorbances at 235 nm indicates the presence of contaminants. The reverse transcription reaction was performed using Side-Step™ II QRT-PCR cDNA Synthesis Kit (Stratagene). QRT-PCR was performed using primers designed by BioRad Laboratories (Software Beacon Designer) and purchased by PRIMM. β-actin gene (PRIMM) was used for normalization.

nNOS(**NOS1**)

Forward : 5' GCGGTTCTCTATAGCTTCCAGA 3'

Reverse : 5' CCATGTGCTTAATGAAGGACTCG3'

iNOS(**NOS2**)

Forward : 5' CCGAGTCAGAGTCACCATCC 3'

Reverse : 5' CAGCAGCCGTTCCTCCTC 3'

eNOS (**NOS3**)

Forward : 5' GCCGTGCTGCACAGTTACC3'

Reverse : 5’ GCTCATTCTCCAGGTGCTTCAT 3’

Mn-SOD:

Forward : 5’ TGGCCAAGGGAGATGTTACA 3’

Reverse : 5’ TGATATGACCACCACCATTGAAC 3’

Cyt c:

Forward : 5’ TTTGGATCCAATGGGTGATGTTGAG 3’

Reverse : 5’ TTTGAATTCCTCATTAGTAGCTTTTTTGAG 3’

β-actina

Forward : 5’ GCGAGAAGATGACCCAGATC3’

Reverse: 5’ GGATAGCACAGCCTGGATAG 3’

SYBR Green has been used as intercalating-dye which can bind double stranded DNA and upon excitation to emit light (excitation = 470 nm; emission = 510 nm). Thus, as a PCR product accumulates, fluorescence increases. This method requires a special thermocycler equipped with a sensitive camera (Stratagene Mx3005p System, Agilent Technologies) that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR reaction. The cDNAs were amplified using 45 cycles consisting of denaturation step (95 °C for 5 min) and amplification step (95 °C for 10 sec, 55 °C for 30 sec).

A normal amplification curve from a dilution series of a sample is shown in Fig. 12a. Amplification plots are created when the fluorescent signal from each sample is plotted against cycle number; therefore, amplification plots represent the accumulation of product over the duration of the real-time PCR

experiment. The initial PCR cycles produce low fluorescent signals that cannot be detected by the CCD camera. The linear portion of each curve is in the exponential phase of PCR, where the amount of product, and therefore the signal, doubles after each cycle. The top portion of the curves shows minimal signal increase, as PCR slows due to the depletion of reaction components, such as primers and dNTPs. Melting curve analysis was performed at the end of every run to ensure a single amplified product for each reaction. During a Melting Curve Analysis, all products generated during the PCR amplification reaction were melted at 95 °C, then annealed at 55 °C and subjected to gradual increases in temperature. The result is a plot of raw fluorescence data units, R, versus temperature. Fig. 12b shows the melt data as the negative first derivative of raw fluorescence, R'(T), plotted against an increase in temperature. In this view, every peak in the curve indicates a specific product melting. Most QPCR products will melt somewhere in the range of 70 - 90 °C.

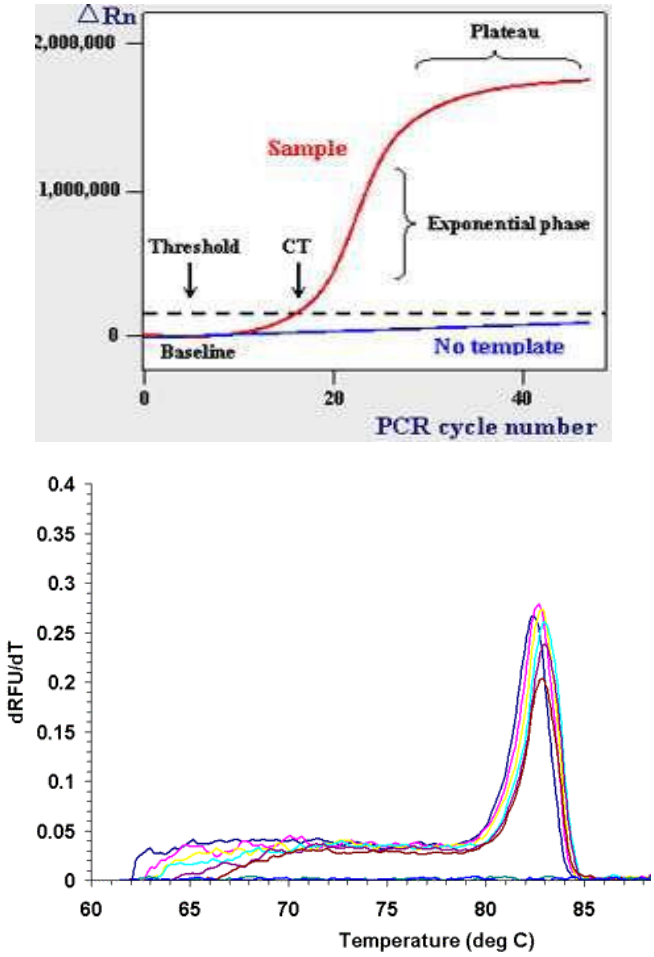


Fig. 13 a) Typical amplification curve produced by the instrument Mx3005p System (Agilent Technologies) **b)** Typical melting curve: ΔF/ΔT (change in fluorescence/change in temperature) plotted against temperature to obtain a clear view of the melting dynamics.

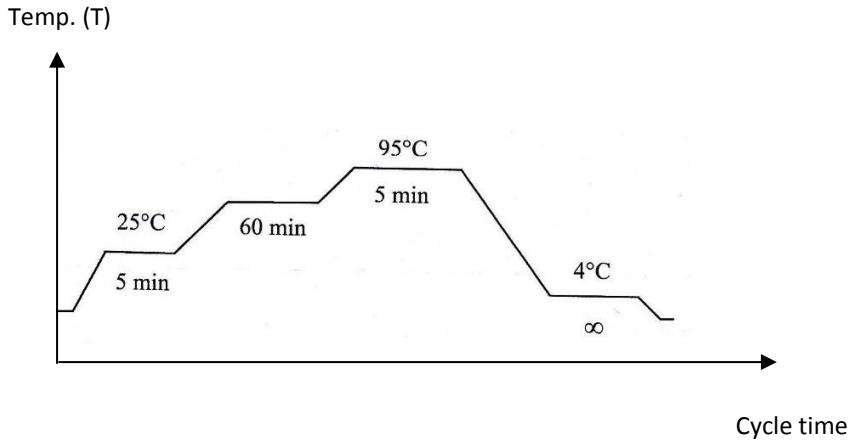


Fig. 14 Reverse transcription cycle scheme

Ideally, a single peak within this temperature range is observed and the melting temperature should be the same in all the reactions where the same sample is amplified. If any secondary peaks are seen on the peak of interest, this indicates that something other than the gene of interest is present among the reaction products. The strategies employed to quantify the results obtained by QRT-PCR is termed comparative threshold (Ct = cycle threshold) method. This involves comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample. The Ct value is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence. At the threshold cycle, a detectable amount of amplicon product has been generated during the early exponential phase of the reaction. The threshold cycle is inversely proportional to the original relative expression level of the gene of interest. The Ct values of both the calibrator and the samples of interest are normalized to the endogenous housekeeping, β-actin. The comparative Ct method is also known as the $2^{-(\Delta)(\Delta)Ct}$ method, where

$(\Delta)(\Delta)Ct = (\Delta)Ct_{sample} - (\Delta)Ct_{reference}$. Here, $(\Delta)Ct_{sample}$ is the Ct value for any sample normalized to the endogenous housekeeping gene and $(\Delta)Ct_{reference}$ is the Ct value for the control also normalized to the endogenous housekeeping gene.

3.12 Western Blot

HepG2 cells, grown overnight in DMEM containing 1 g/L glucose, 2 mM L-glutamine without FBS, were incubated with NP (20µM) and OP (10µM) at 12 and 24h. HepG2 cells were harvested by trypsinization and centrifugation (1000 x g for 5 min at 25°C), washed twice with Hank's buffer and lysed with CelLytic™MT Cell Lysis Reagent in the presence of protease inhibitors. The cell lysates were incubated for 15 minutes on a shaker and centrifuged at 20.000 x g for 10 min. The protein concentration was determined by BCA assay [400]. Samples were run on gel electrophoresis, 10 % SDSPAGE gels to obtain protein separation. 20 µg of total protein from cell lysate was loaded into wells in the gel. One lane was reserved for the marker (Invitrogen, SeeBlue® Plus2 prestained standard), a commercially available mixture of proteins having defined molecular weights. SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents, in these case dithiothreitol (DTT) to remove secondary and tertiary structure and thus allows separation of proteins by their molecular weight. The gel was run at 90 V through the stacking part of the gel and the volts were turned up to 150 V after the proteins have gone through the stack and are migrating through the resolving gel. Following electrophoresis, the protein was transferred on nitrocellulose membranes (Whatman, GE Healthcare UK) for 1 h at 100 mA. Blocking of nonspecific

binding was achieved by placing the membrane in PBS with 0.1 % tween and 3 % BSA for 2 h at room temperature. After blocking, the membrane was incubated overnight at 4°C with primary rabbit polyclonal anti-nNOS antibodies (from BD Transduction Laboratories); α-tubulin was used as the reference. A secondary ECL TM anti-rabbit antibody HRP (Jackson) was thereafter incubated 1 h at 25°C. Washing steps (consisting to washes of PBS, 0.1 % Tween, 5 minutes each) were necessary to remove unbound reagents and reduce background. The imagine was determined for chemiluminescence by ChemiDoc MP Imaging System, Bio-Rad. Densitometric analysis was carried out by the ImageJ program.

3.13 Statistics

The number of independent measurements is indicated in figure legends. Significance was determined using the Student t-test, run by Excel (Microsoft Windows platform). The error bars correspond to the standard deviation (ST DEV) and structural equation modeling (SEM) of triplicate results; all P values correspond to two-sided sample t-test assuming unequal variances. A P value ≥ 0.05 was considered significant and a P value ≥ 0.01 was considered extremely significant.

4. RESULTS

HaCaT and HepG2 cells were used as valid models for the human cell toxicity screening of glyphosate (GP); bisphenol-A (BPA); phenmedipham (PHEN); perfluoro-octanoic acid (PFOA); imidacloprid (IMI) and alkylphenols (NP and OP).

4.1 Cellular toxicity evaluation of Glyphosate (GP)

HepG2 and HaCaT cells were incubated with different concentrations of GP ranging over the μM scale for 24 h. Firstly, cell viability assay (MTT) was carried out over a wider concentration range (from 0 to 10^5 μM) in order to evaluate the appropriate experimental condition for further biochemical characterisation. In Fig 15a, a dose-response curve of HepG2 and HaCaT cells following GP administration is shown.

The results indicate a similar trend for cell survival in the cell lines assayed after treatment (%), consisting in an initial 20% decrement in cell viability, that remains stable upon increasing GP concentration until the value of 10^3-10^4 μM, beyond which a further linear decrement in cell viability occurs.

Based on the results shown in Fig 15a, a set of three GP concentrations of interest were chosen (1 μM; 10^2μM; 10^3μM) for the measurement of ROS production, NOx, cytrate synthase, cellular respiration and mitochondrial membrane potential.

4.1.1. Determination of ROS production in cell culture undergoing GP treatment

The amount of ROS produced in HepG2 and HaCaT cells following 24h GP treatment is shown in fig. 15b. It can be observed that ROS production increases significantly proportionally with the GP concentration of 10^3µM in the treated cells in both cell lines.

4.1.2. Determination of nitrite/nitrate (NOx) accumulation in cell culture undergoing GP treatment

The amount of NOx has been assayed in HepG2 and HaCaT cells treated for 24h with GP in order to evaluate the bioavailability of NO, as nitrites and nitrates represent the dead-end product of NO metabolism in cells. As reported in Fig 15 c, no significant difference in NOx production is visible in treated cells, regardless for the cell line and for the GP concentration, compared to control. These results indicate that GP is not able to influence NO metabolism under the condition assayed.

4.1.3. Determination of citrate synthase activity in GP treated cells

The evaluation of citrate synthase activity has been carried out in order to evaluate if change in mitochondrial mass may occur as the result of GP treatment. As shown in Fig 15d, although the absolute value is different in the two cell lines assayed, there is no significant difference between control and treated cells (fig 15d).

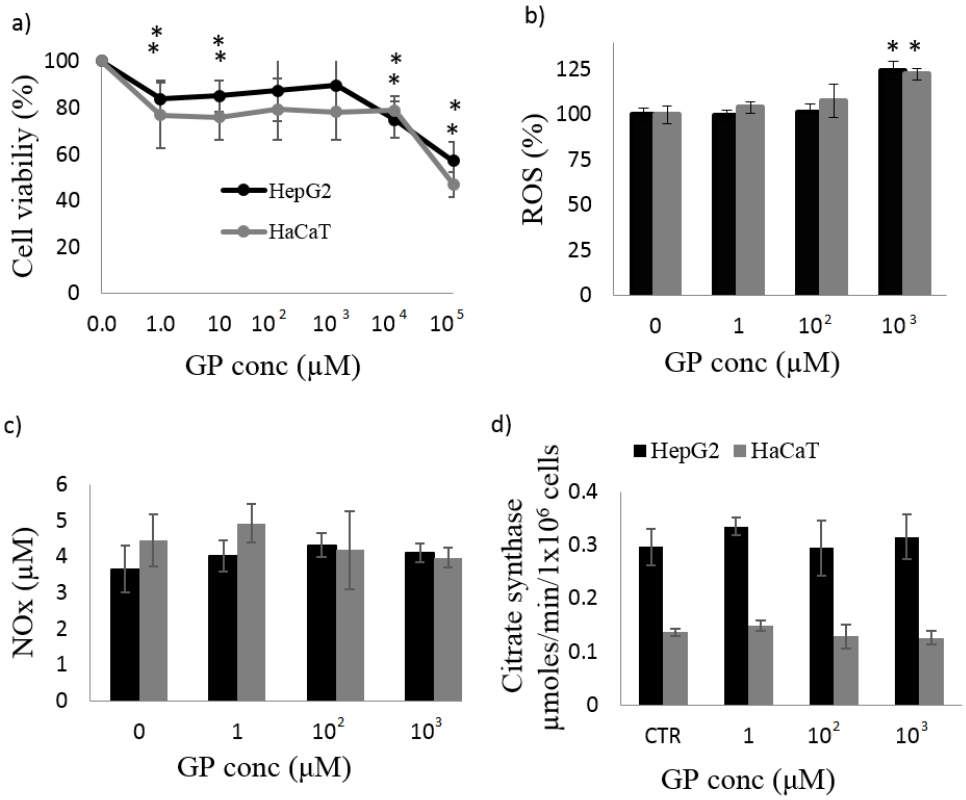


Fig. 15 a) Cell viability assay (MTT). Cell viability of HepG2 and HaCaT cells was assayed after 24 h incubation with GP. **b) ROS production.** The production of ROS of HepG2 and HaCaT cell lines after 24h incubation with different concentrations of GP was carried out by DCFDA. **c) Nitrate/nitrite measurement.** The quantification of Nitrate/nitrite accumulation in the supernatant of both HepG2 and HaCaT cells after 24h incubation with different concentrations of GP was carried out by DAN. **d) Citrate synthase.** Citrate synthase measurement were carried out in HepG2 and HaCat cells after 24h incubation with GP. Data values are the means ± ST DEV; *n* = 3. *P ≤ 0.05 *versus* Control

4.1.4. Measurement of Oxygen consumption rate in cells undergoing GP treatment

Oxygen consumption rate was measured in both cells types using a respirometer Oroboros Oxygraph 2k. HepG2 and HaCaT cells were incubated

with 100μM and 1mM GP. Respiration rate values were monitored in 3x106 HepG2 cells and 5x106 HaCaT cells after 24 h incubation with GP (see methods). Digitonin (Dig) was utilised to permeabilise cells. Substrates/ inhibitors added along the trace are: pyruvate and malate (Pyr /Mal); adenosinediphosphate (ADP); rotenone (Rot); succinate (Suc); antimycin A (Ant a); ascorbate and N,N,N',N'-tetrametil-p-fenilendiammina (ASC/TMPD).

In figure 16 a and b, the basal respiration of cells is shown: after addition of digitonin, which induces a loss of plasmatic membrane integrity to allow the access of substrates/inhbitors into the cells, it lets in pyruvate, malate and ADP become available for mitochondria.

The addition of 0.5 μM rotenone (Rot), inhibitor of Complex I, abolishes almost completely respiration. After the addition of 10 mM succinate, the specific substrate of Complex II, the oxygen consumption was restored.

Then respiration is once again interrupted by 5 μM Antimycin A (Ant a), inhibitor of Complex III, and further restored by adding reduced cytochrome c in the presence of ASC and TMPD.

ASC maintains TMPD in a reduced state so that electrons can flow freely from TMPD to endogenous cytochrome c. These conditions ensure the highest electron transfer efficiency at the complex IV site.

As shown in Fig 16 b, the OCR (oxygen consumption rate) resulted similar to control in HaCaT cell line after treatment with glyphosate. Whereas, in HepG2 cells (Fig 16 a), the GP treated samples resulted in an OCR decrease by approximately 25%, eventhough the data is dispersive and needs further verification with complex IV inhibitors.

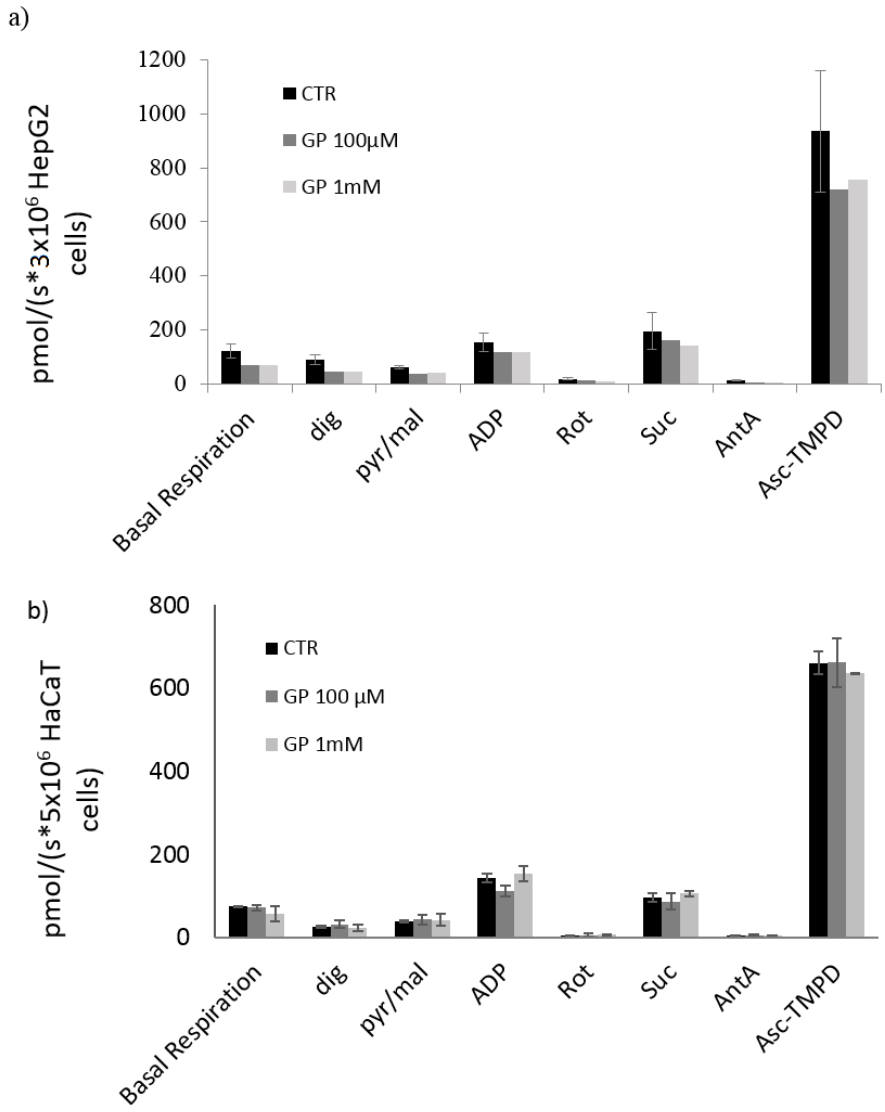


Fig. 16 a) Oxygen consumption rate in HepG2 and HaCaT incubated with GP 100µM and 1mM. Respiration rate value monitored in $3x10^6$ HepG2 cells after 24 h incubation with GP. **b) Oxygen consumption rate in HaCaT cells incubated with GP 100µM and 1mM**. Respiration rate value monitored in $5x10^6$ HaCaT cells after 24 h incubation with GP. Digitonin (Dig) was utilised to permeabilise both cell lines (see methods). Substrates/ inhibitors added along the trace: pyruvate and malate (Pyr /Mal); adenosinediphosphate (ADP); rotenone (Rot); succinate (Suc); antimycin A

(Ant a); ascorbate and N,N,N′,N′-tetramethyl-p-phenylenediamine (ASC/TMPD) Data values are the means ± ST DEV; *n* = 3.

4.1.5. Measurement of mitochondrial membrane potential in GP treated cells

The mitochondrial membrane potential ($\Delta\psi_m$) was measured through flowcytometry using JC1 probe. It was observed an evident decrease of the $\Delta\psi_m$ which remained constant in the two doses assayed (10^2 and 10^3 µM).

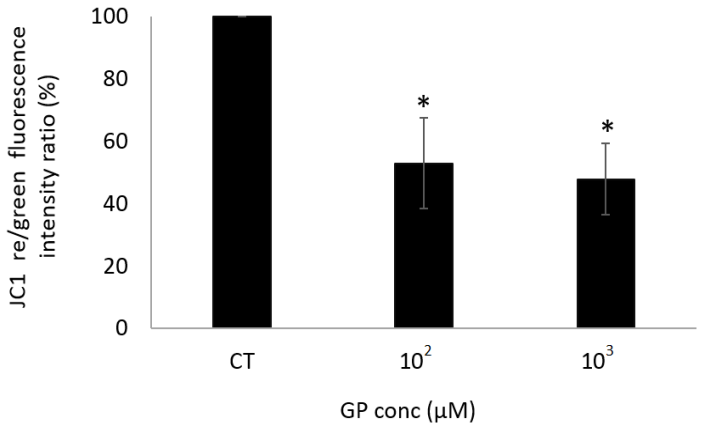


Fig 17 Mitochondrial membrane potential in HepG2 cells incubated with GP. The percentage of intensity ratio between red and green fluorescence of JC-1 was measured at the flow cytofluorimetric analysis. Data values are the means ± ST DEV; *n* = 3. *P ≤ 0.05 *versus* Control.

4.2 Screening of bisphenol-A (BPA), phenmedipham (PHEN), imidacloprid (IMI) and perfluorooctanoic acid (PFOA) toxicity

HepG2 and HaCaT cell viability changes induced by BPA, PHEN, IMI and PFOA were assessed by the MTT assay. Dose–response analysis was performed by exposing the cells to increasing concentrations of each substance for 24h. Although in a different concentration value regime(μM), BPA, PHEN, PFOA and IMI inhibited the growth of HepG2 and HaCaT cells in a dose dependent manner.

After 24h cells treatment with increasing dose of BPA, the percentage of viable cells gradually decreased from 80% to 10% in both cell lines (Fig. 18a).

Differently PHEN treatment lead to an inhibition of the cell growth, not strictly proportional to its dose increase: 50 μM reduces viable cell to 60%, being that the most cytotoxic concentration for HepG2 cell line and 70% for HaCaT cells. (Fig. 18b).

PFOA treatment was characterised by a high toxicity on HaCaT cell line, with sensitivity already over low μM concentrations of the compound, leading from 60% to 5% residual cell viability, as well as for HepG2 the toxicity is lower, though with a comparable trend, from 80% to 40 % viability (Fig. 18c).

IMI treatment is featured by a similar decrease in cell viability of both cell lines, ranging from 80 % to 40 %, upon increasing its concentration to 100 μM. (Fig. 18d).

Based on this analysis, the GI50 (growth inhibition by 50%) for cells treated with BPA, PHEN, PFOA and IMI was assumed as following: BPA ~ 175 μM (HepG2 and HaCaT); PHEN ~ 90 μM(HepG2), ~ 80 μM(HepG2), PFOA ~ 60

μM (HepG2), ~ 20 μM(HaCaT) and IMI ~ 125 μM (HepG2 and HaCaT) respectively.

Based on these results, the concentrations utilized in the experiments carried out from here on was set at 50 μM for BPA and IMI, 10 μM for PFOA and 20 μM for PHEN.

The time-courses of cell viability of HepG2 and HaCaT cells were carried out by MTT assay, using the above set of concentrations at time steps of 6, 12, 24, 48 and 72h. (Fig. 19). All substances induce a proportional decrease in cell viability of both cell lines in a time dependent manner. BPA (50 μM) and PHEN (20 μM) reduce the cell viability by ~ 60% after 72h, without any visible difference between the two cell lines (Fig.19a,b). Differently, the time-dependent toxicity of PFOA (10 μM) was more specific, and cell viability decreases by ~ 60% in HepG2 whereas by ~ 20% in HaCaT cell line (Fig. 19c). After IMI (50 μM) treatment cell viability decreases at 40% in HepG2 and 60% in HaCaT cells after 72h treatment (Fig. 19d).

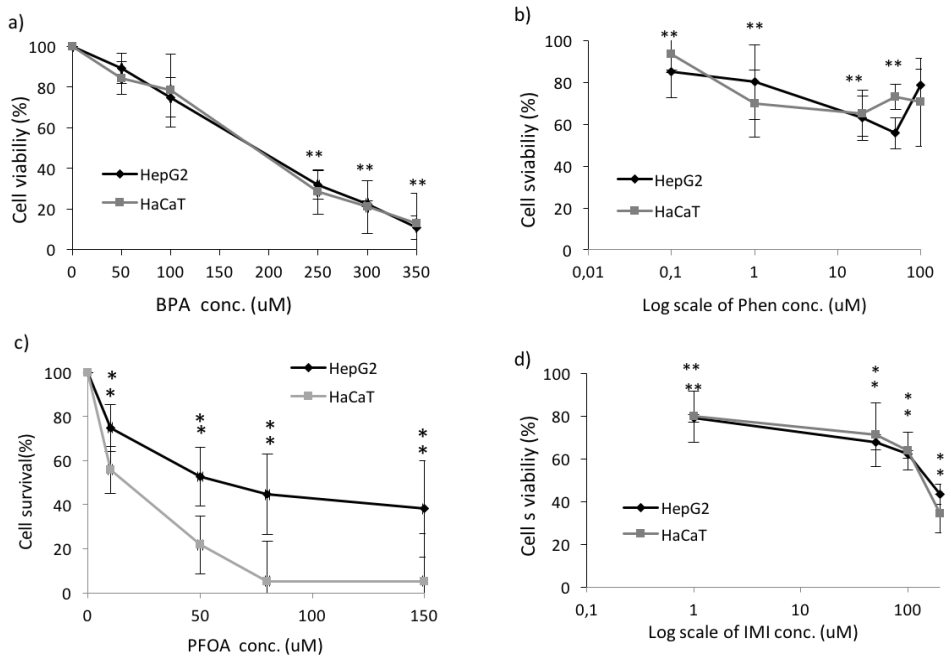


Fig. 18 The dose-response of cell viability in HepG2 and HaCaT cells after 24 h exposure to BPA, PHEN, PFOA, IMI, by MTT. a) BPA (50-350 μM) dose-response on the HepG2 and HaCaT cell viability b) PHEN (0.1-100 μM) dose-response on HepG2 and HaCaT cell viability c) PFOA (10-150 μM) dose-response on the HepG2 and HaCaT cell viability. d) IMI (1-200 μM) dose-response on the HepG2 and HaCaT cell viability. Data ± ST. DEV.; n = 3. *P ≤ 0.05 *versus* Control. **P ≤ 0.01 *versus* Control

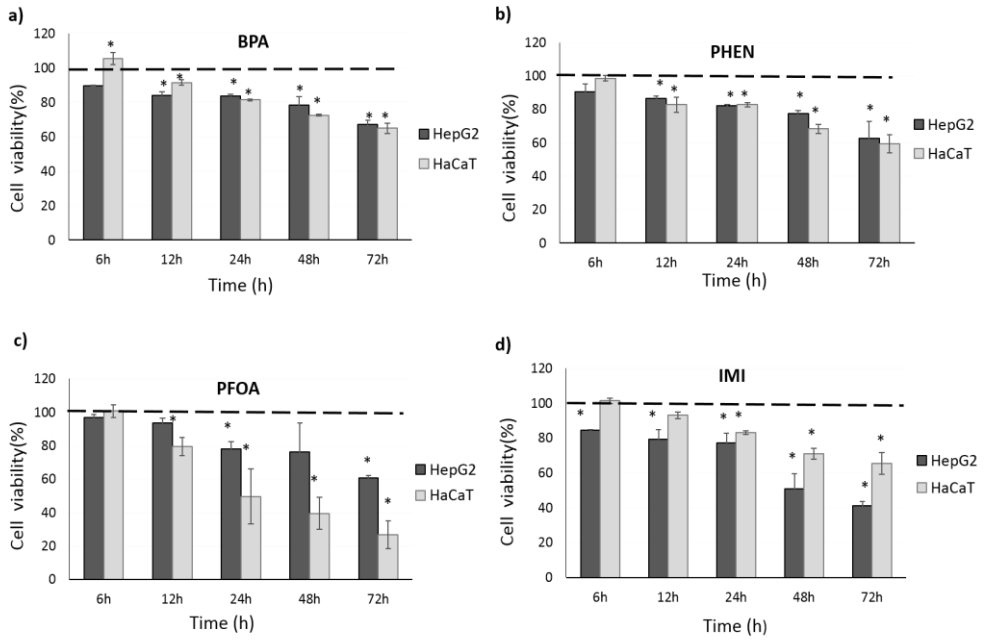


Fig. 19 Viability time-course of HepG2 and HaCaT cells upon 6h, 12h, 24 h, 48h and 72h treatment with BPA, PHEN, PFOA, IMI by MTT. a) Bisphenol-A, BPA (50μM) time- course incubation. b) Phenmedipham PHEN (20 μM) time- course incubation. c) The perfluorooctanoic acid PFOA (10 μM) time- course incubation. d) Imidacloprid, IMI (50 μM) time- course incubation. Data ± ST DEV; n = 3. *P ≤ 0.05 *versus* Control

4.2.1. mRNA expression changes of key factors for nitro-oxidative stress induced by BPA, PHEN, PFOA, IMI treatments

Nitric oxide synthase (NOS) isoforms:

The mRNA expression of the Nitric oxide synthase isoforms (iNOS, eNOS and nNOS) was determined after 24h cells incubation with, BPA (50 μM), PHEN (20 μM), PFOA (10 μM) and IMI (50 μM) for 24h (fig. 20). As shown in Fig. 20a, compared to control, the iNOS mRNA expression was induced by

all the compound assayed, compared to untreated controls, and these effects were more specific for HepG2 cells, where a ~ 2-fold increase of iNOS expression resulted from BPA and PHEN administration; 2,5-fold increase by IMI and ~ 4-fold increase for by PFOA. The iNOS mRNA expression in HaCaT is 1.5-fold increase for BPA and IMI whereas for PHEN and PFOA it is comparable to control.

In Fig. 20b the expression level of the eNOS and nNOS mRNA are shown, indicating an effect of BPA and PHEN on increasing the expression of eNOS in HepG2 cells (1,8 and 1,5-fold increase respectively), while PFOA and IMI treatment did induce any change; furthermore, BPA was also able to induces a ~ 1,4-fold increase in nNOS expression in HaCaT cells, whereas all the other compounds resulted ineffective.

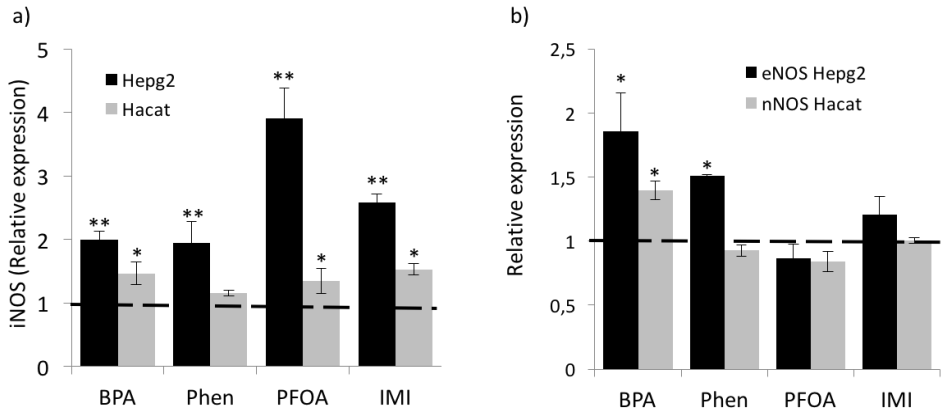


Fig. 20 RT-PCR analysis of Nitric Oxide Synthase (NOS) gene expression induced by BPA, PHEN, PFOA and IMI and Nitrite-Nitrate (NOx) accumulation. The gene expression of endothelial Nitric Oxide Sinthase (eNOS), inducible Nitric Oxide Sinthase (iNOS) and neuronal Nitric Oxide Sinthase (nNOS) was evalueted. HepG2 and HaCaT were incubated with BPA 50 µM, PHEN 20 µM, PFOA 10 µM and IMI 50 µM for 24h a) Relative gene expression of iNOS b) Relative

gene expression of eNOS and nNOS. Relative expression was calculated *versus* control cells (dashed lines level, 1), after β-actin normalization. Data ± SEM; n = 3. *P ≤ 0.05 *versus* Control, **P ≤ 0.01 *versus* Control

Manganese Superoxide Dismutase (Mn-SOD):

The mRNA expression of the Mn-SOD and ROS production were determined after cells incubation with, BPA (50 μM), PHEN (20 μM), PFOA (10 μM) and IMI (50 μM) for 24h, as the regulation of this enzyme is strictly associated to cell redox homeostasis and oxidative stress responses.

As shown in Fig. 22a, after 24h incubation, the Mn-SOD mRNA expression was elevated after PFOA and IMI treatment only in HaCaT cell line: 2 and 4,5-fold increase respectively. In other conditions, no effect was visible compared to control.

Cytochrome c (Cyt c):

The level of mRNA expression of Cyt-c in cells exposed to the different compounds is shown in Fig. 23a. HepG2 cells treated PFOA and IMI display a 2 and 2,25-fold increase in cyt c expression, respectively. A similar effect of PFOA and IMI was also observed in HaCaT cells, although a lower level of expression was achieved: 1,8 and 1,25-fold increase respectively

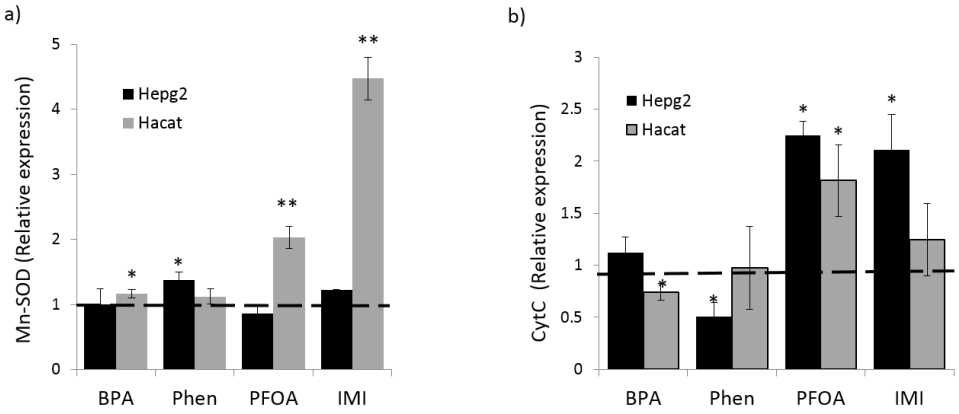


Fig. 21 Manganese Superoxide Dismutase (MnSOD) expression and Cytochrome c (Cytc) expression in cells treated with BPA, PHEN, PFOA and IMI . a) The gene expression of MnSOD was evaluated after incubation of HepG2 and HaCaT cells with: BPA(50µM), PHEN(20µM), IMI(50µM) and PFOA(10µM). b) The gene expression of Cyt c was evaluated after incubation of HepG2 and HaCaT cells with: BPA(50µM), PHEN(20µM), IMI(50µM), PFOA(10µM). Data are presented as percentage of values obtained from untreated cells and normalized for protein content. Data are the means ± ST DEV; *n= 3.* * P ≤ 0.05 *versus* Control, **P ≤ 0.01 *versus* Control.

4.2.3. Nitrite/nitrate accumulation induced by BPA, PHEN, PFOA, IMI treatments

The amount of NO produced by the cells treated with BPA (50 µM), PHEN (20 µM), PFOA (10 µM) and IMI (50 µM) for 24h, was evaluated by measuring ~~as~~ nitrate and nitrite (NO_X) equivalents accumulated in the cell medium. As shown in Fig 21, the level of NOx found in HepG2 cells was about 1,5-1.6 times increased respect to controls, whereas in HaCaT a 1.7 times increase is achieved by BPA and PHEN treatment, and a 2.3 and 2 times increase after PFOA and IMI treatment respectively. Increase was calculated respect to control values (Fig. 23)

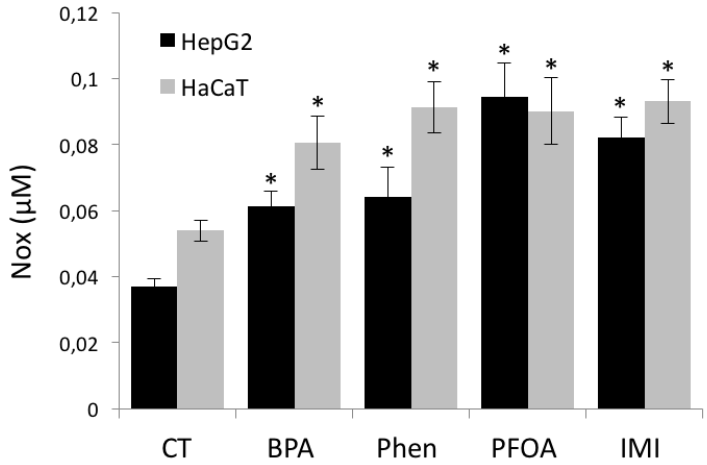


Fig. 22 NOx accumulation (24h) in the cell supernatant of HepG2 and HaCaT cell lines treated with BPA(50µM), PHEN(20µM), PFOA(10µM), IMI(50µM) were quantified downstream treatments by 2,3-diaminonaphthalene (DAN) how described in the Method section. Data ± ST DEV; *n= 3*, *P ≤ 0.05 versus Control.

4.2.3.ROS production in BPA, PHEN, PFOA, IMI treated cells

Incubation with PHEN, PFOA and IMI induced a ROS increase of ~ 2,2; ~ 1,5 and ~1,2 times, respectively, in treated HepG2 cell line, whereas in HaCaT only PHEN induced a high ROS level, ~ 2,25 times respect to untreated control. In agreement with the result on Mn-SOD and cyt c expression, BPA treatment did not affect significantly ROS production in both cell lines, although a non-significant, small decrease in ROS amount was found in HaCaT cells respect to control (Fig. 24).

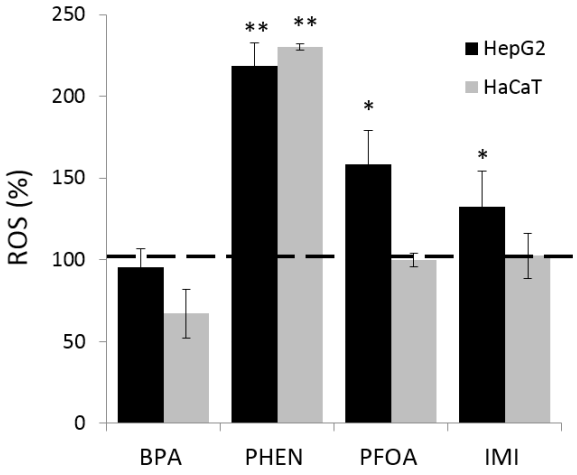


Fig. 23 Reactive oxygen species (ROS) production in cells treated with BPA, PHEN, PFOA and IMI. Intracellular ROS levels were measured of the following contaminants: GP(1mM), BPA(50µM), PHEN(20µM), IMI(50µM), PFOA(10µM) after 24h treated and untreated HepG2 and HaCaT cell lines (cell density 10^6/mL) in suspension, in the presence of 2,7-dichlorodihydrofluorescein diacetate (DCFDA) (see methods). The kinetics of DCFDA fluorescence was followed for 60 min. The value of ROS production is taken at 30 min. Data are presented as percentage of values obtained from untreated cells and normalized for protein content. Data are the means ± ST DEV; *n= 3.* * $P \leq 0.05$ *versus* Control, **$P \leq 0.01$ *versus* Control

4.2.4. Mitochondrial membrane potential measurement in BPA, PHEN, PFOA, IMI treated cells

The mitochondrial membrane potential ($\Delta\psi_m$) of HepG2 and HaCaT cells was measured after treatment with the compounds of interest decreased. The results shown in Fig 25 indicate that BPA in both cell lines is able to determine a significant decrease in $\Delta\psi_m$ (20% and 30% respect to control value), while oppositely, IMI increases the $\Delta\psi_m$ 40% in HepG2 cell line and 25% in HaCaT. PFOA administration did not determine any variation in $\Delta\psi_m$ and furthermore the effect of PHEN is of a small but significant increase only for HepG2.

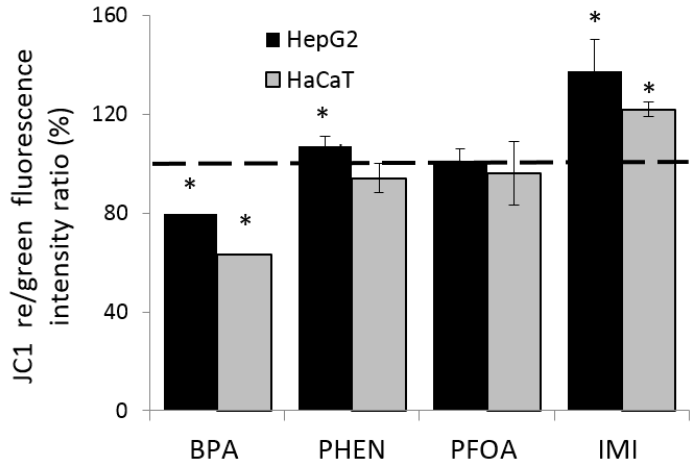


Fig. 24 Mitochondrial membrane potential in cells treated with BPA, PHEN, PFOA and IMI. HepG2 cells were incubated with: BPA(50µM), PHEN(20µM), IMI(50µM), PFOA(10µM). The percentage of intensity ratio between red and green fluorescence of JC-1 was measured at the flow cytofluorimetric analysis. Data ± ST DEV, *n= 3*. * P ≤ 0.05 *versus* Control.

4.3 Alkylphenols (APs) effects inside the cell

HepG2 cell viability changes induced by octylphenol and nonylphenol were assessed by the MTT assay. Dose–response analysis was performed by exposing the cells to increasing concentrations of alkylphenol. Although in a different concentration value regime, µM and mM, both octylphenol and nonylphenol inhibited the growth of HepG2 cells (Fig. 26). After 24h cells treatment with octylphenol, the percentage of viable cells were 77%, 74% and 8%, at 1, 10 and 50 µM OP, respectively (Fig. 24a). At µM concentration values, nonylphenol did not show a significant cytotoxicity, whereas the 24h

cells incubation with nonylphenol at 20, 100 and 200 µM, led to a 20% decrease of the viability. Grossly, the GI50(growth inhibition by 50%) of the cells treated with OP and NP was assumed as ~ 26 µM and 750 µM, respectively (Fig. 26 a, b).

Based on these results, the concentration of octylphenol and nonylphenol utilized in the experiments herein reported was set at 10 and 20 µM, respectively, i.e. values subtoxic on the experimental time scale chosen (Fig. 26c).

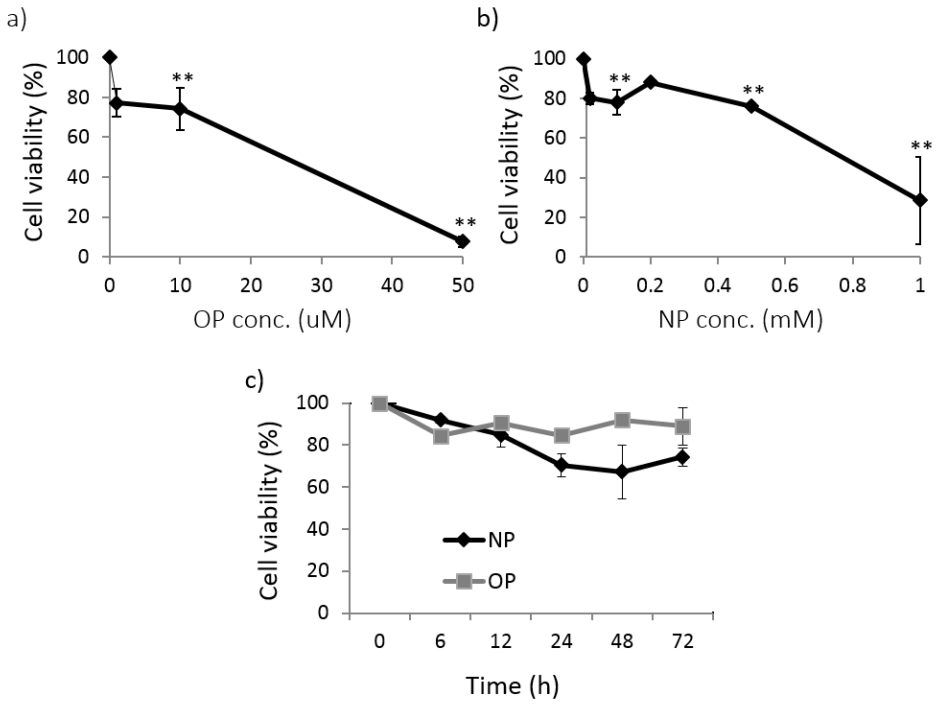


Fig. 25 The viability of HepG2 cells measured by MTT assay after exposure to alkylphenols OP and NP. a,b) Dose-response of HepG2 cell line after 24h incubation with octylphenol and nonylphenol respectively. c) The time-response of HepG2 cell line after incubation with octylphenol 10 µM and nonylphenol 20 µM. Data ± SEM; n = 3. **P ≤ 0.01 *versus* Control

4.3.1. Changes in mRNA expression of nitric oxide synthase (NOS) isoforms induced by OP and NP

The mRNA expression of the nitric oxide synthase (NOS) isoforms: the endothelial and the inducible ones (eNOS and iNOS), was determined after cells incubation with OP and NP for 12h and 24h. As shown in Fig. 27a, compared to control, 12h incubation with OP was almost ineffective to the NOS mRNA expression, whereas after 24h incubation induced, respectively, a 1.6- and 1.4-fold increase of the eNOS and iNOS mRNA level. The eNOS mRNA expression level was almost insensitive to nonylphenol (within 10-20%) under all times of incubation tested. On the contrary, a 2-fold increase in the iNOS mRNA expression was observed when incubation was performed with nonylphenol 20 µM (Fig. 26b). The nNOS mRNA remained almost undetectable under the all conditions (not shown).

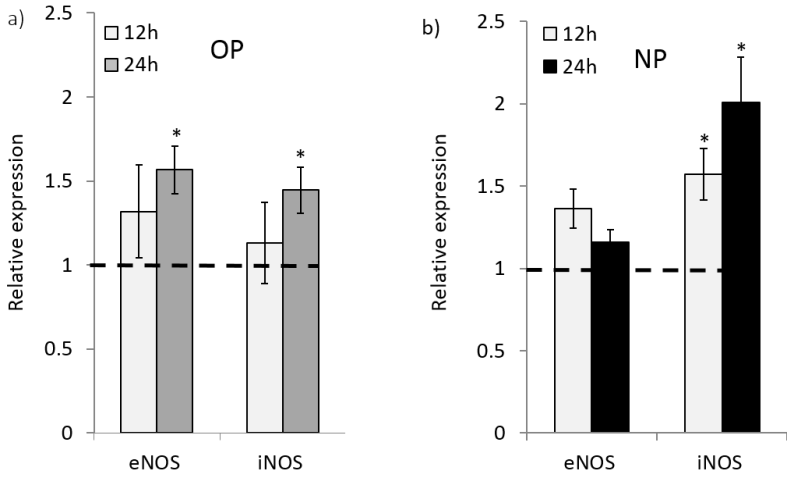


Fig. 26 RT-PCR analysis of NOS(s) gene expression in response to OP and NP. The gene expression of endothelial Nitric Oxide Synthase (eNOS) and inducible Nitric Oxide Synthase (iNOS) was evaluated after incubation of HepG2 for 12 and

24h with: a) OP: 10 µM; b) NP: 20 µM. Relative expression was calculated *versus* control cells (dashed lines level, 1), after β-actin normalization. Data ± SEM; n = 3. *P ≤ 0.05 *versus* Control

4.3.2. iNOS and eNOS protein expression and evaluation of eNOS uncoupling induced by OP and NP

The iNOS and eNOS protein expression level was investigated by western blot using HepG2 cells incubated for 24h with NP and OP and using primary antibodies against iNOS, eNOS, p-Thr495 modified eNOS and p-Ser1177 modified eNOS. Consistent with the results shown in Fig. 28, compared to controls, nonylphenol induced a ~ 1.5-fold increase of the iNOS protein expression. The effect of octylphenol on the iNOS expression is minute, within < 10% (Fig. 28a). On the contrary, the total eNOS production of cells exposed to OP, is increased by approximately 50%, whereas the NP treatment is ineffective on eNOS (results shown in Fig. 28b). Phosphorylation of eNOS at Serine 1177 (p-Ser1177) and at Threonin 495 (p-Thr495) has been evaluated by WB in HepG2 exposed to OP and NP. The detection and quantification of these modifications allow to evaluate the eNOS activity state whether physiological or uncoupled. In Fig. 27c the eNOS phosphorylation level is reported as the ratio of phospho- eNOS over total eNOS: in control cells, the phospho-eNOS at Ser 1177 (p-S1177) is about 100% of total eNOS. This value does not significantly change after incubation with OP, while it decreases by ~ 40% following incubation with NP; a parallel increase of the phosphorylated Thr 495 (p-Thr495) was also observed under otherwise identical conditions (Fig. 28c). These results suggest that the eNOS becomes significantly uncoupled when cells are exposed to NP but not to OP.

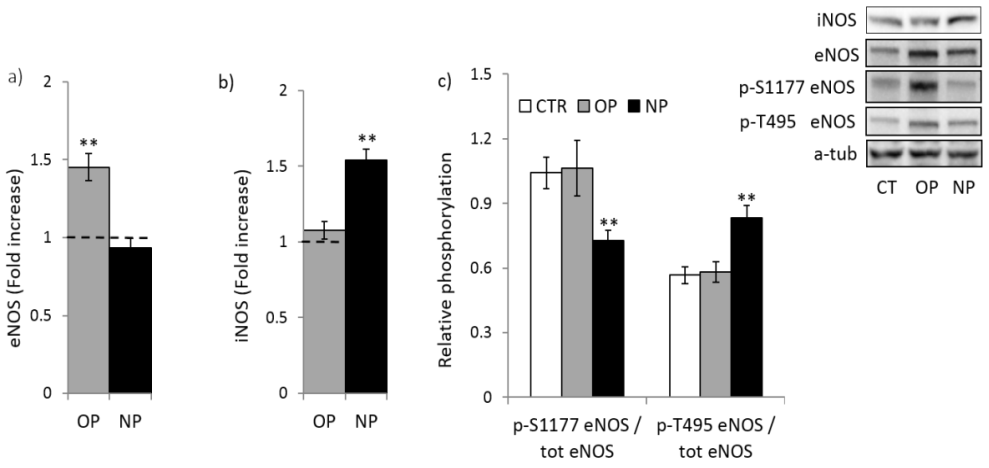


Fig. 27 iNOS and eNOS protein expression and evaluation of eNOS uncoupling in response to OP and NP. HepG2 were incubated 24h with OP (gray) and NP (black) and then assayed for Western Blot with anti-iNOS antibody and anti-eNOS antibodies (see methods). a) Expression of eNOS following OP and NP treatment. Densitometric values are shown as fold increase *versus* the eNOS protein expressed by control cells. Data ± SEM, n = 7. ** P ≤ 0.01 *versus* Control. b) Production of iNOS following OP and NP treatment. Densitometric values are shown as fold increase *versus* the iNOS protein expressed by control cells. Data ± SEM, n = 6. ** P ≤ 0.01 *versus* Control. c) Phosphorylation of eNOS at Ser-1177 and Thr-495 in response to OP or NP shown as the fraction of total eNOS. eNOS phosphorylation was monitored using specific antibodies (see methods). Data ± SEM, n= 4. ** P ≤ 0.01 *versus* Control. *Inset:* Image of a typical Western Blot experiment carried out with antibody against iNOS, eNOS, p-S1177 eNOS and p-T495 eNOS (as indicated) after 24 h incubation in the presence or in the absence of OP and NP; α-tubulin as reference

4.3.3. Comparison of the effect induced by 17B-estradiol (E2) to that of OP and NP on iNOS and eNOS protein expression and phosphorylation pathways

In order to investigate if the modification on NOS expression induced by OP and NP may be related to the endocrine disruptive effect attributed to these compounds, we have carried out Western blot experiments in HepG2 cells

treated with the natural estrogen (E2) in the presence and absence of the ER inhibitor, Fulvestran.

As shown in Fig 29a, E2 increased by ~1.7-fold the eNOS protein expression, which seems to be reversed by the ER inhibitor. It can be noticed that OP induces a similar effect on eNOS protein expression (~1.4 increase) and this effect is also reversed by Fulvestrant (Ful). In the case of NP treatment, no significant changes are shown for eNOS expression, both in the presence and absence of Ful. As it can be seen in fig 29b, the administration of NP resulted effective in the induction of iNOS expression, and this specific effect was promptly reverted by Fulvestrant.

The level of eNOS uncoupling was evaluated through the detection of p-Ser1177 and p-Thr 495 by WB as already described, in HepG2 cells treated 24 h with E2, OP and NP in the presence and absence of the ER inhibitor Ful. In Fig 29 c, the level of Ser 1177 phosphorylation was shown as relative to the total eNOS detected by WB. As previously shown in Fig 28c, in control cells, this ratio is equal to 1, meaning that under physiological conditions Ser 1177 is normally phosphorylated. It is interesting to point out that a similar result is observed exposing HepG2 cells to both the natural estradiol E2 and OP, regardless to the presence of Ful. A different result is obtained by the treatment with NP, leading to the already described (Fig 28c) decreased level of phosphorylation at the same site (Ser 1177) (Fig 29c).

A similar investigation carried out on eNOS protein to detect the level of phosphorylated Thr 495, showed that the basal value 0,6 for the ratio p-Thr 495/ total eNOS, found in control cells, was not changed by the presence of Fulvestrant, and the same feature appeared in the case of both E2 and OP treatment. Surprisingly, and in good agreement with the previous

characterization, the increase in p-Thr 495 to a ratio of ~0,8 respect to total eNOS previously shown was promptly reversed by Fulvestrant.

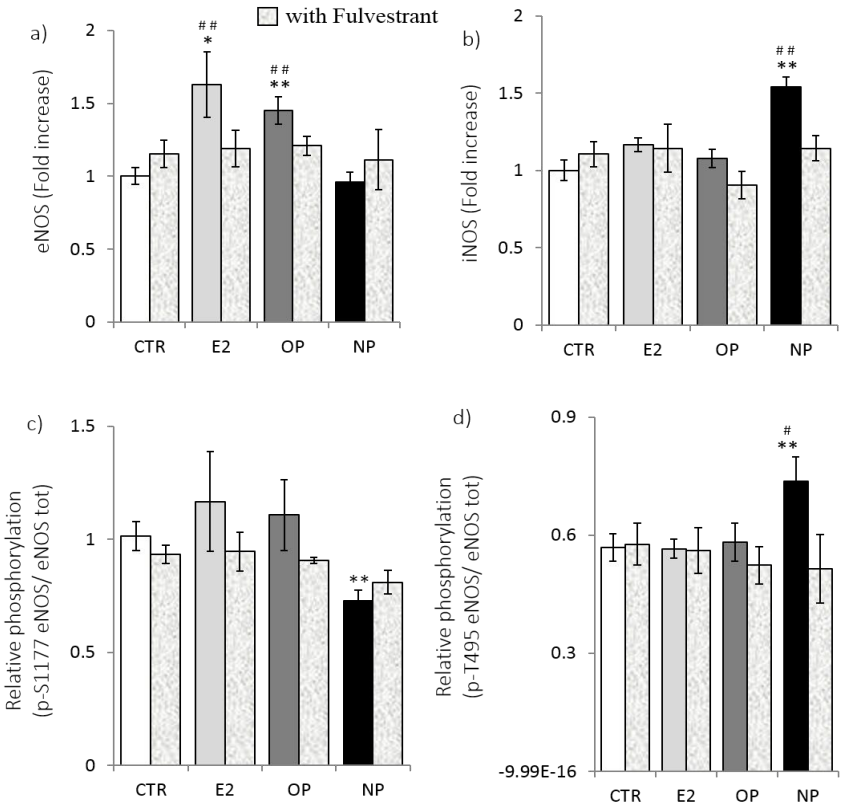


Fig. 28 Involvment of OP, NP, E2 and ER in the expression of iNOS and eNOS, and in eNOS uncoupling. HepG2 were incubated 24h with Estradiol-17β 1 µM (E2), OP 10 µM and NP 20 µM in the presence (shaded) and absence (full) of the ER inhibitor Fulvestrant 1 µM and then assayed for Western Blot with anti-iNOS antibody and with anti-eNOS antibodies (see methods). α-tubulin as reference. **a)** eNOS expression in response to E2, OP and NP +/- Fulvestrant. Densitometric values are shown as fold increase *versus* the eNOS protein expressed by control cells. **b)** iNOS expression in response to E2, OP and NP +/- Fulvestrant. Densitometric values are shown as fold increase *versus* the iNOS protein expressed by control cells. **c)** fraction of eNOS phosphorylation at Ser-1177 over total eNOS, in response to E2,

OP and NP, +/- Fulvestrant. **d)** fraction of eNOS phosphorylation at Thr-495 over total eNOS in response to E2, OP and NP, +/- Fulvestrant. Data ± SEM, n= ≥ 3. * P ≤ 0.05 versus Control, ** P ≤ 0.01 versus Control. # P ≤ 0.05 versus NP and Fulvestrant treated cells, ## P ≤ 0.01 versus NP and Fulvestrant treated cells.

To check the overall mitochondrial functional state, the mitochondrial membrane potential of AP-treated cells was measured quantifying the import of the fluorescent probe JC-1 into mitochondria and the fractional formation of the red J-aggregates. As shown in Fig. 4, after 24h incubation with OP and NP the mitochondrial membrane potential ($\Delta\mu H^+$), compared to controls, was significantly lowered, by ~ 28% and 12%, respectively.

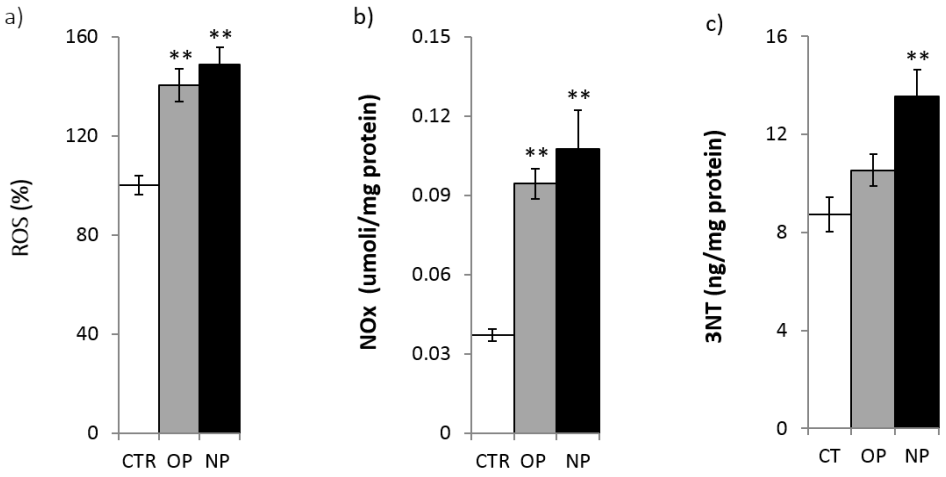


Fig. 29 Oxidative and nitrosative stress induced by OP and NP. Assays were carried out following 24 h incubation of HepG2 cells with 10 µM OP and 20 µM NP. **a) Reactive oxygen species (ROS) production.** Intracellular ROS levels were measured in suspension of OP and NP treated and untreated HepG2 cells (cell density 10^6/mL) in the presence of 2,7-dichlorodihydrofluorescein diacetate (DCFDA) (see methods). Data are presented as percentage of values obtained from untreated cells and normalized for protein content. Data are the means ± SEM; *n= 3.* ** P ≤ 0.01 *versus* Control. **b) Nitrite-Nitrate (NOx) accumulation.** NOx accumulation (24h) in

the cell supernatant were quantified downstream treatments by 2,3-diaminonaphthalene (DAN) how described in the Method section. Data ± SEM; *n= 3*. **P ≤ 0.01 versus Control. **c) 3-Nitrotyrosine (3-NT) determination.** The 3-NT content was measured by Nitrotyrosine competitive ELISA (see methods) in OP and NP treated and untreated cells (lysate). Data values are the means ± SEM; n= 6. **P ≤ 0.01 versus Control

O_2 consumption measurements were carried out oxygraphically. The involvement of different mitochondrial respiratory chain complexes was evaluated after cell permeabilization with digitonin by taking advantage of the mitochondrial complex specific substrate/inhibitor titration approach (Kuznetsov et al. 2008). For this purpose, HepG2 cells, pre-treated for 24h in the presence and absence of OP and NP, were initially assayed for the basal respiration. As shown in Fig. 4, the basal respiration values of AP-treated and controls are comparable. As expected due to partial membrane permeabilization and loss of reducing substrates and ADP, after 10 minutes incubation with digitonin, cell respiration slowly decreases to approximately 50% of the initial value. The addition of 2 mM ADP, 8.8 mM pyruvate and 4.4 mM malate induces ~ 2.5-fold increase of cell respiration rate, while the addition of 0.5 μM rotenone (Rot, inhibitor of Complex I), abolishes almost completely respiration. The subsequent addition of 10 mM succinate, the substrate of Complex II, restores oxygen consumption to a level similar in AP-treated and control cells. Antimycin A (Ant A) was then added to fully inhibit Complex III and abolish respiration, which was restored by adding cytocrome c, reduced by exogenous ascorbate (ASC) and N,N,N',N'-Tetramethyl-p-Phenylenediamine (TMPD). These last conditions ensure the highest electron transfer efficiency at the complex IV site. The data show that when cells are treated with NP, a ~ 20 % loss of the maximal speed cell respiration is observed

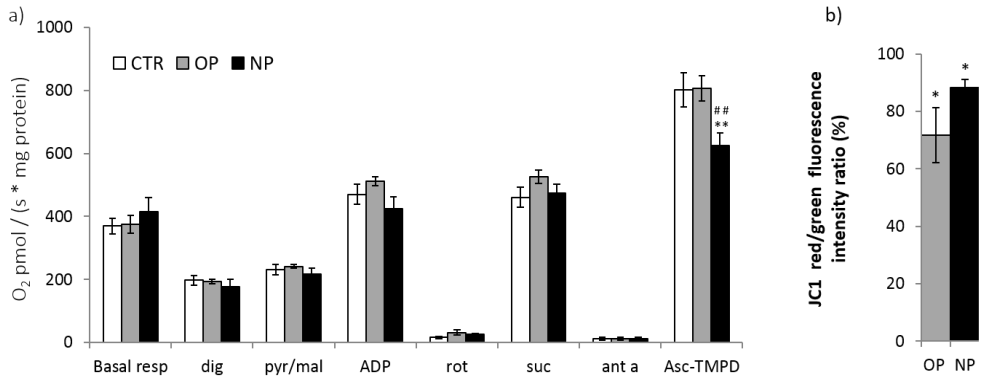


Fig. 30 a) Oxygen consumption rate in HepG2 cells incubated with OP and NP. Respiration rate value monitored in $3x10^6$ HepG2 cells after 24 h incubation with alkylphenols. Digitonin (Dig) was utilised to permeabilise cells (see methods). Substrates/ inhibitors added along the trace: pyruvate and malate (Pyr /Mal); adenosinediphosphate (ADP); rotenone (Rot); succinate (Suc); antimycin A (Ant a); ascorbate and N,N,N',N'-tetrametil-p-fenilendiammina (ASC/TMPD).

b) Mitochondrial membrane potential in HepG2 cells incubated with alkylphenols. The percentage of intensity ratio between red and green fluorescence of JC-1 was measured at the flow cytofluorimetric analysis. Data ± SEM, *n = 3*. * $P \leq 0.05$ *versus* Control

5. DISCUSSION

Our community is exposed to a wide variety of chemicals including plasticizers, herbicides, insecticides, pharmaceuticals and personal care products (PCPs). Most of these "xenobiotics" had not been previously detected or they were found in far lesser concentrations in the environment. (Sauvé and Desrosiers 2014). Such compounds, present in our surroundings for decades, have recently become "emerging contaminants" since their ability to bioaccumulate and to have adverse effects on human health even at low concentrations (ng or µg/L).

Their ubiquitous exposure and the lack of information on their effects has raised a great responsibility on the scientific community and a difficult challenge for regulatory agencies (Murray et al. 2010). Among the above-mentioned substances, little is known about their effect on mitochondrial function and in the induction of cell nitro-oxidative stress. The maintenance of cell metabolism and homeostasis is a fundamental characteristic of living organisms. Mitochondria, having a pivotal role in these cellular functions, contain a large number of ROS generating sites and at the same time display a sophisticated multilayered ROS defence system that is much less studied (Starkov 2008; Kotiadis et al. 2014).

This project was focused on the biochemical characterization of the effect exerted by these xenobiotics in cultured cells, by evaluating several components of human metabolism as ROS/RONS production, mitochondrial membrane potential, oxygen consumption rate, NOS isoforms expression, together with Mn-SOD and Cyt c.

The experiments were designed by using two cellular models, HepG2 and HaCaT, that can mimic the skin and the liver as the principal targets of the xenobiotics. HepG2 is a human liver cancer cell line considered a suitable in vitro model system for the study of liver metabolism and toxicity of xenobiotics, the detection of environmental and dietary cytotoxic and genotoxic agents (Mersch-Sundermann et al. 2004). HaCaT is a spontaneously transformed immortal keratinocyte cell line from adult human skin: the high proliferative capacity of this cells with neuro-ectodermal origin resulted in their wide application in scientific research (Schürer et al. 1993).

One of the objects of our study was the recently debated herbicide glyphosate (GP). The actual polemic about its marketing, lies on the fact that despite the poor toxicity declared for humans, glyphosate residues can remain stable in foods for a long time. This thesis aimed to elucidate the effect of lower concentrations, in comparison to analogue studies in literature, to which most humans are exposed daily. Since dysfunction of the mitochondrial respiratory chain is a key player in a variety of human disorders, including drug toxicity, we evaluated the oxygen consumption rate in both cell lines. It was observed a slight decrease of OCR in HepG2 cell line after treatment with 0.1 and 1 mM GP. MTT results demonstrated mitochondria damage in both cell lines, suggesting that glyphosate affects mitochondrial activity in a dose-dependent manner. In a previous study, it was observed that GP alone disrupted the mitochondrial membrane potential in HaCaT cells even after short incubation indicating that the mitochondrial pathway could play a crucial role in glyphosate-induced apoptosis of HaCaT cells (Heu et al. 2012). It was observed a significant decrease of mitochondrial membrane potential in HepG2 cell line too, after 24h incubation. Whereas a significant increase in ROS production in the mM range was found, which could lead to several responses inside the cell.

According to literature glyphosate formulations (Roundup^{TM}) are much more toxic to mitochondria respiration respect to glyphosate alone, which is probably due to the synergic effect of other substances which are present in most formulations, as for ex. Surfactants (Peixoto 2005). As a future perspective, it would be interesting to compare glyphosate toxicity with that exerted by some adjuvants present in the commercial formulation of the herbicide, alone and co-incubated, to better characterise its toxicity.

Other emerging contaminants included in our study were *Bispenol-A* (BPA), *Phenmedipham* (PHEN), *Imidacloprid* (IMI), *Perfluooctanoic acid* (PFOA). Differently from GP, a detailed investigation toward the toxicity exerted by these compounds is still lacking, with exception made for BPA, but all of them are gaining increasing attention because of their persistence and widespread in the environment. In particular, BPA has been widely utilized as a plasticizer in food and liquid packaging, toys and feed bottles; PHEN constitutes the active ingredient of many herbicides widely used for weed control in different crops; PFOA is used as a surfactant in plastic materials, particularly in the coating of cookware and finally, IMI represents the worldwide most used insecticide in agriculture and home pest control.

The results of variations of iNOS, eNOS, nNOS, MnSOD, CytC gene expression induced by these compounds, toghether with the characterization of ROS, nitrite/nitrate and MMP measurements are summarized in table 2.

Effector	Relative gene expression fold increase respect to Controls							
ECs	iNOS		eNOS	nNOS	MnSOD		Cyt C	
	HepG2	HaCaT	HepG2	HaCaT	HepG2	HaCaT	HepG2	HaCaT
PHEN	1.95	1.15	1.5	0.9	1.3	1.1	0.5	0.9
IMI	2.59	1.53	1.2	1	1.2	4.4	2.1	1.25
PFOA	3.9	1.3	0.86	0.84	0.86	2	2.25	1.8
BPA	2	1.4	1.85	1.39	1	1.16	1.12	0.74

Effector	(%) 24h incubation					
	ROS		NOx		$\Delta\mu H^+$	
ECs	HepG2	HaCaT	HepG2	HaCaT	HepG2	HaCaT
PHEN	220	230	163	150	107	94
IMI	125	100	230	125	137	120
PFOA	160	100	235	150	98	87
BPA	90	65	150	133	76	63

Table 2 *Effects induced by PHEN, IMI, PFOA and BPA on parameters relevant to the NO signalling and mitochondria functionality alterations*

Following PHEN incubations, it was observed an increase in ROS and nitrate/nitrite production. The increased production of ROS may constitute part of the signalling pathway leading to the activation of cell antioxidant response, as indicated by the finding that Mn-SOD gene expression was increased by 1.3-fold in HepG2 cells. It was also observed an increase in iNOS and eNOS

gene expression in HepG2 cell line which together with ROS and RONS increase, may indicate the onset of an inflammatory state inside cell.

Imidacloprid incubation indicated an increase in iNOS gene expression in both cell lines. It was interesting encountering a marked Mn-SOD overexpression (4.4-fold) in HaCaT cell line. Whereas nitrite/nitrate accumulation was elevated in both cell lines. In contrast with other substances, the mitochondrial membrane potential was higher respective to control in both cells. Further investigation is needed to clarify the functional implication of this surprising finding.

It has been observed that PFOA induces a ~ 4-fold increment of iNOS and 2-fold increment of cyt c gene expression in HepG2 cell line accompanied by ROS and nitrite/nitrate accumulation. Whereas in HaCaT cell line it was found an increased expression of Mn-SOD together with cyt c gene expression and ROS accumulation. All these effects suggest that IMI induces intracellular oxidative stress and as a result cellular degeneration.

Interestingly, after bisphenol-A incubation, the gene expression of iNOS and eNOS in HepG2 cell line resulted increased by ~2-fold. Even the mitochondrial membrane potential was lower respect to control, pointing out its cytotoxic properties.

It should be highlighted that all substances show an increase of iNOS expression, more marked in HepG2 cell line and also an increase in nitrate/nitrate accumulation in both cell lines. As a marker of inflammation, this finding clearly indicates the role exerted by these compounds in altering the complex physiological equilibrium of cells.

The characterisation of Alkylphenols (APs) toxicity, was an important development of our study. Their adverse effects on human health were already

known since ‘90s (Warhurst 1995) but recently their xenoestrogenic potential has emerged.

Little is known about the effect of octylphenol (OP) and nonylphenol (NP) on the cell energetic metabolism, as well as on the NO signaling chemistry, aspect in which the estrogen pathway involvement has been well established (Zhang et al. 2008; Chouhan et al. 2015). We decided to investigate on the eventual effect exerted by NP or OP, the physiological NO signaling and how this interference eventually impacts mitochondrial function and cell nitro-oxidative stress. Our study was conducted through the characterisation of the NOS isoforms (eNOS and iNOS) expression and activity using HepG2 cell line as the appropriate in vitro model expressing both the known estrogen receptors (ERs), type α and β. Cells were exposed 24 h to OP and NP and to the β-estradiol (E2). The finely tuned regulation of the different isoform of NOS may indeed be considered as markers of both the physiological NO signalling and of nitrosative stress (Magnifico et al. 2017). At the OP and NP concentrations of 10 μM and 20 μM respectively, corresponding to those commonly detected in the body fluids (Ademollo et al. 2008; Chen et al. 2005) and able to exert only minor detrimental effects on cell viability, we have observed specific alterations in the cellular pathways related to the production of both the mRNA encoding for the eNOS and iNOS isoforms, further confirmed at protein level. In this aspect, OP and NP displayed a different behavior. Upon 24 h of cell exposure to OP, the eNOS mRNA and the relative protein synthesis increased, whereas iNOS induction was not achieved.

Remarkably, 24 h incubation with NP brought about a large, ~ 2-fold, increase of the iNOS mRNA, further confirmed at protein level, whereas, over the same time-period, the eNOS expression remained comparable to that of controls. The meaningfulness of the different effects exerted by OP and NP toward

eNOS and iNOS expression may be better defined by analysing the biosynthetic pattern of the NOS isoforms induced by the cell exposure to E2. As shown in Fig. 27a, 24 h treatment with the natural estrogen results in an eNOS and iNOS expression pattern comparable to that induced by OP, featured by an increased eNOS expression and iNOS unaffected.

It is known that a change in phosphorylation of Ser 1177 and Thr 495 determines eNOS uncoupling, distinguished by an altered enzymatic activity, featured by loss of NO production and release of superoxide radical ($O_2^{-\cdot}$) as the final reaction product. The physiological eNOS function is maintained by high level of Ser 1177 and low level of Thr 495 phosphorylation.

In particular, in HepG2 cells, 100% of eNOS was found phosphorylated at Ser 1177 (p-Ser 1177) and 55% at Thr 495 (p-Thr 495). Cells treatment with OP for 24 h did not affect p-Ser 1177 and p-Thr 495 levels, while NP treatment leaded to a fall in p-Ser 1177 (70% of total eNOS), associated to an increase in p-Thr 495 (80 % of total eNOS). In Fig. 27b it is clearly shown how the behavior of OP treated cells is very similar to that of control cells respect to the modifications promoting uncoupling, whereas in NP treated cells the level of eNOS uncoupling is increased.

According with the literature, the 17β-estradiol, though increasing the overall eNOS expression, does not influence its physiological activity, as shown in Fig. 27 where the percentage of phosphorylation of target residues Ser1177 and Thr 495 observed following E2 treatment was comparable of that of controls, and also, to that of OP treated cells. Cells incubation with NP clearly lowered the eNOS phosphorylation level at S1177, while increased its phosphorylation at T495.

Both the expression of NOS (eNOS and iNOS) and phosphorylation of eNOS were evaluated by Western blot analysis, in the presence of the estrogen

receptor inhibitor, comparing control and treated cells (E2, OP and NP for 24 h). It is interesting to point out that whenever OP or NP treatment leaded to a specific effect toward NOS expression or activity, this effect was always reverted by Ful. In particular, Fig. 28a clearly shows how the increased expression of eNOS observed in both E2 and OP treated cells was abolished by the addition of Ful as well as the decrease expression of the same NOS isoform induced by NP in the absence of Ful, was reverted by Ful co-incubation. Thus, in the presence of the ER inhibitor, AP treated cells display a behaviour comparable to that of controls, regardless for the compound used, suggesting the involvement of estrogen receptor in the regulatory pathway controlling eNOS expression. A similar consideration can be made regarding iNOS since, as shown in Fig 28b, the effect of NP exposure, specifically able to promote iNOS induction, was promptly abolished by Ful. Not only the expression of NOS, but even the post-translational modifications on Ser1177 and Thr 495 were linked to the estrogen signalling, in the sense that, how shown in Fig 28 c and d, the uncoupling-promoting changes in the phosphorylation pattern induced by NP on eNOS, are reverted by Ful: partially in the case of Ser 1177 and totally abolished in the case of Thr 495. Thus, in agreement with the above results and with previous data in the literature, our results clearly confirm the xenoestrogenic effect of OP and NP, pointing to its relevance at the level of NOS expression, regulation and crosstalk among isoforms, in HepG2 cells.

In order to enlighten the downstream effect of OP and NP treatment on HepG2 cell physiology, cell bioenergetics parameters have been investigated, such as mitochondrial ETC activity and membrane potential, and markers of oxidative stress, as the cell ROS and nitrite/nitrate (NOx) production, and 3-NT protein modifications. The results shown in Fig 29, indicate that, both OP and NP treatment induce a significant increase in ROS production and NOx

accumulation. It is interesting to point out that the amount of ROS production by NP treated cells was not significantly higher than that induced by OP, thus the almost comparable high ROS levels found in OP and NP were obtained through different pathways. In a similar way, a significant higher level of NOx has been found in HepG2 cells treated by both OP and NP compared to untreated controls; based on the evidence herein reported, these effects are clearly due to the activity of the two NOS isoforms eNOS and iNOS whose expression was specifically induced by OP and NP, respectively. Curiously, this very similar effect is obtained through an estrogen-mimicking activation pathway in the case of OP, and through a disruptive binding, resulting in the alteration of the physiologic pathway in the case of NP. It is feasible to propose that one of the major detrimental effect exerted through the disruption of the endogenous estrogen signal amenable to NP treatment is given by the significant increase in protein nitrosation, as shown in Fig 29c by 3-NT quantification. This increase is attributable to a burst in peroxynitrite production, that in the framework of NP treatment, is obtained by the parallel iNOS activation and eNOS uncoupling. At mitochondrial level, the investigation on the effect of high level of ROS, NO and peroxynitrite on the efficiency of ETC has shown that whereas OP treatment is not able to influence the rate of oxygen consumption, NP treatment lead to a lowered activity of Cytochrome c oxidase (CcOx) activity; indeed, under condition maximising CcOx efficiency, as in the presence of reducing equivalents and substrates, a 20% decrement of O_2 consumption rate, was observed, compared to OP treated and control untreated cells.

As a further overall indication of mitochondrial function, the mitochondrial membrane potential was investigated revealing a significant decrement found as consequence of both OP and NP treatment (Fig 30b).

Taken together, these findings suggest that upon treating HepG2 cells with OP and NP some changes of the NO synthesis and chemistry can be detected, that are summarized in Table 3.

Effector	**Octylphenol**	**Nonylphenol**
ROS	1.80±0.34	1.52±0.14
NOx	2.54±0.16	2.89±0.39
3NT	1.20±0.08	1.55±0.12
mRNA eNOS	1.16±0.07	1.57±0.14
mRNA iNOS	2.01±0.28	1.45±0.14
eNOS protein	1.50±0.19	0.94±0.15
iNOS protein	1.14±0.02	1.52±0.09

Table 3 *Effects induced by Octylphenol (OP) and Nonylphenol (NP) on parameters relevant to the NO signalling. Results represent the positive (+) or negative (-) fold change compared to the controls*.

The molecular mechanism by which OP and NP in about 1 day cell incubation might induce the eNOS and iNOS over-expression, requires further investigation. The hypothesis of a receptor-mediated estradiol-mimicking action of OP is consistent with the literature (Kuiper et al. 1998; Routledge et al. 1997).

The NP-induced cells response can be tentatively rationalized on a different basis: the experimental major finding is that in its presence the iNOS expression is increased, the eNOS activity changes toward uncoupling leading to superoxide and probably peroxynitrite production. This highly reactive and toxic species is formed very rapidly, at the diffusion limited rate between the excess of superoxide ion (from uncoupled eNOS) and NO (from up-regulated iNOS); overall, the whole picture evokes a cell oxidative stress and damage.

In this respect, it is worth considering that the respiratory chain complexes, and particularly cytochrome c oxidase (Complex IV), react with both NO and peroxynitrite (Brown, 1995; Brunori et al. 2006; Sarti et al. 1999). The reaction with peroxynitrite can only be detrimental and almost irreversible (Cooper et al. 2003); the reaction of Complex IV with NO, instead, can occur within a physiological frame depending on the NO concentration whether high (μM) or low (nM) and on the mitochondrial cell redox state (Sarti et al. 2012).

6. CONCLUDING REMARKS

The studied xenobiotics showed impaired mitochondrial functions and increased nitro-oxidative stress even at concentrations similar to those commonly detected in human body fluids.

The results herein reported could add information on the mechanisms of action of GP, PHEN, IMI, PFOA, BPA, NP and OP, thus contributing to the understanding of the actual environmental pollution risk associated to this class of compounds.

In particular, the potential of OP and NP as endocrine disrupters has been confirmed, as their ability to interfere with the NO signaling pathway has been established, leading to different phato-physiological effects.

The chronic exposure to increasing amount of pollutants, leads to the urgency of deeper investigation aimed at elucidating the molecular mechanisms of their action, also considering the synergic effects as a further worsening of their toxicity.

Based on the data discussed in this thesis, the use of antioxidants able to contrast cell oxidative stress at all levels, though more specifically at the nitric oxide pathways level, appear a valid mechanistic track to cope with this type of environmental pollution.

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