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Effect based monitoring tools: biomarkers for the assessment of emerging environmental risk factors for human health

Marta Gea

Tutor: Prof. Tiziana Schilirò

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1. Introduction

Worldwide almost a quarter of mortality is due to environmental causes. Indeed, in 2016 the World Health Organization has estimated that globally the 24% of all deaths and the 28% of deaths in children with less than five years are caused by environmental factors, corresponding to 13.7 million deaths and 1.6 million deaths in children less than five years per year (WHO, 2021). These deaths are attributable to many environmental risk factors among which: chemical and biological pollution of air, water and soil, ultraviolet and ionizing radiations, occupational hazards and inadequate working conditions, noise, electromagnetic fields, climate change, availability of drinking water and farming techniques (WHO, 2021). The tightly connection among human health, animal health and environment is now recognized and this relationship is also the fundamental concept of the One Health approach, which is an approach based on the design and the implementation of programmes, policies, legislation and research aimed to achieve better public health (FAO, 2021).

The health effects related to environment will probably increase in the next years. Indeed, the rise of global population and its aging are causing an increase of the number of people more vulnerable to environmental risk factors (e.g. children, elders, social disadvantaged, ethnic minorities, etc.) (Rojas-Rueda et al., 2021). Moreover, public health outcomes linked to environment cannot be ignored also because they are costly. Diseases due to pollution cause productivity losses that reduce gross domestic product in low-income to middle-income countries by up to 2% per year. Health costs due to these diseases account for 1.7% and up to 7% of annual health-care spending in high-income and middle-income countries, respectively. Finally, pollution causes welfare losses that are estimated to amount to US$ 4.6 trillion per year: 6.2% of global economic output (Landrigan et al., 2018).

A great number of chemicals are synthesized and spread in the environment every year whose toxicity must be verified. In order to preserve the environment from pollution thus protecting human health, tools to assess the potential toxicity of chemicals are needed. Once released in the environment, these chemicals, together with all the natural and synthesized chemicals that are already there, constitute complex mixtures to which we are exposed. All the substances can interact with each other causing possible biological effects on humans and environment. Since mixture effects are generally not known and difficult to predict, in addition to tools for chemical assessment, tools that assess the quality of environmental matrices are also required in order to design legislation and programmes aimed to restore polluted environments and preserve unpolluted environmental matrices.

1.1 Effect based tools

Effect based tools assess biological effects that might be induced by a chemical compound or an environmental mixture. These tools measure a wide range of parameters and could be categorized into three main groups: i) bioassays (*in vitro* and *in vivo*), which measure the cumulative effect from all substances in samples having the same mode of action under defined laboratory conditions; ii) biomarkers, which study effects such as biochemical, physiological, histological or morphological alterations in field exposed organisms; iii) ecological indicators, that evaluate the impact on community structure and function (Wernersson et al., 2015; Carere et al., 2021).

Among all the types of effect based tools, the *in vitro* assays on eukaryotic cells have numerous advantages. Indeed, they do not need the sacrifice of animals, so are desirable for ethical reasons. Moreover, they are quick and cheap to perform, so the assessment using these assays can be carried out in short time and simultaneously for several samples (Rehberger et al., 2018). Since *in vitro* assays are performed under controlled conditions, it is quite easy to associate the measured outcome with the investigated factors. In contrast, the outcome of the other effect based tools (i.e. *in vivo* assays, biomarkers, ecological indicators) can be influenced by numerous factors, therefore its interpretation is generally more complex.

One of the major disadvantages of *in vitro* assays is the inability to replicate accurately the complex interactions that occur at the level of a tissue, composed of multiple cell types interacting with each other. *In vitro* studies for their simplification are not adequate for identifying target organs within the body, histological changes and effects on the immune system. Therefore, a biological effect measured using these assays can not be directly associated with a specific effect on humans (Stone et al., 2009). However, the outcomes measured *in vitro* may reflect what happens *in vivo* with regard to cell morphology, cellular absorption of substances, signalling, gene expression and protein production. The availability of a large number of *in vitro* assays also makes it possible to measure different biological effects, helping to understand the toxicity mechanisms (Stone et al., 2009).

Many *in vitro* assays are performed on eukaryotic cells. Primary cells are used because they are considered to be experimental models that can better reproduce *in vivo* models. However, primary cells are not extensively applied since are more difficult to obtain and have limited growth activity (Courcot et al., 2012). Therefore, in addition to primary cells, *in vitro* assays are often applied using cell lines. In comparison with most primary cells, cell lines can be more easily cultured and their use allows a better reproducibility (Vinken and Blaauboer, 2017). A great variety of cell lines has been applied according to the type of tested chemical/matrix and according to the type of assay. For example, the A549 cell line was applied in most of the toxicity studies which assessed the effects induced by substances/matrices through inhalation exposure. However, it was demonstrated that, with respect to the A549, other cell lines, such as the BEAS-2B, exhibited higher similarities with primary cells and few dysregulated genes compared with non-tumoral lung tissues (Courcot et al. 2012).

Three types of widely applied *in vitro* assays on eukaryotic cells are cytotoxicity assays, genotoxicity assays and estrogenic activity assays.

1.1.1 Cytotoxicity assays

Cytotoxicity occurs when a chemical substance harms living cells by compromising functional and structural features related to physiological cellular processes (Vinken and Blaauboer, 2017). The cytotoxicity can be schematically explained as follow. Initially the chemical compound or the environmental matrix causes cell injury. Then, a mitochondrial dysfunction takes place as a consequence of the primary insult. Finally, the cell death occurs.

Different types of *in vitro* cytotoxicity assays are available. Among them, some assess the cytotoxicity measuring the mitochondrial functionality (e.g. the methyl-thiazolyl-tetrazolium reduction assay – MTT assay) while others measure the integrity of the plasma membrane (e.g. the lactate dehydrogenase assay – LDH assay).

1.1.1.1 Assays based on mitochondrial functionality

Mitochondria provide energy to the cells through chemical processes that are vulnerable to toxic chemicals. Indeed, toxic chemicals can interfere with many potential sites within these organelles (Vinken and Blaauboer, 2017).

Some cytotoxicity assays assess cell viability by determining the activity of mitochondrial enzymes such as succinate dehydrogenase using dyes (Stone et al., 2009). The dyes consist of a tetrazolium salt that is metabolized into formazan by the mitochondrial enzymes of viable cells (Ursini et al., 2014). The reaction product is a coloured molecule that can be quantified by an optical absorption measurement at a specific wavelength. Since the measured absorbance is proportional to the number of cells and their mitochondrial functionality, these assays are able to assess possible proliferative or cytotoxic effects (Stone et al., 2009).

The MTT assay is one of the most applied cytotoxicity assays based on mitochondrial functionality. Other similar assays are the methane-thio-sulfonate hydrobromide assay (MTS assay), the 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2h-tetrazolium hydroxide assay (XTT assay) and the water soluble tetrazolium salt-1 assay (WST-1 assay). In Figure 1 the tetrazolium salt of the WST-1 assay and its respective reaction product (i.e. the formazan) are reported.

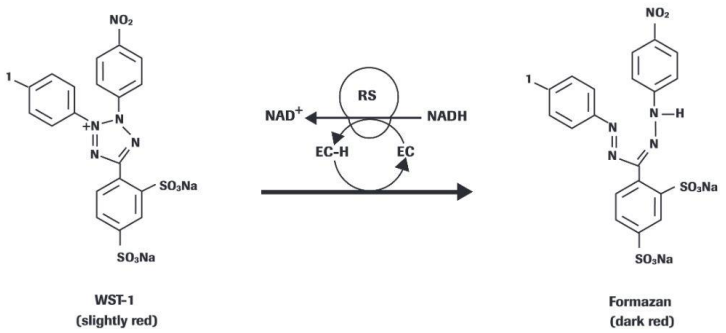

Figure 1. Enzymatic reaction of the WST-1 assay (EC = electron coupling reagent; RS = mitochondrial succinate-tetrazolium-reductase system) (Cell Proliferation Reagent WST-1, Roche product sheet).

1.1.1.2 Assays based on plasma membrane integrity

Plasma membrane is a fundamental structure which bounds the cell. It provides a physical wall that divides the cell from the external environment but it is also a complex chemical structure that allows molecule and signal exchanges between the cell and the external environment. Therefore, some cytotoxicity assays are based on plasma membrane integrity.

The LDH assay is one of the most applied cytotoxicity assays based on plasma membrane integrity. The LDH is a cytoplasmic enzyme that is contained in all cells. This enzyme can be released in the cell culture medium after a damage of the cytoplasmic membrane. The LDH assay assesses the LDH activity in the cell culture medium through a reaction mixture that allows two enzymatic reaction phases. In the first phase, the NAD+ is metabolized in NADH/H+ by the cellular LDH that oxidizes lactate into pyruvate. In the second phase, a catalyst transfers H/H+ from NADH/H+ to a tetrazolium salt which is reduced to formazan (Figure 2). The concentration of formazan is proportional to the number of cells lysed and can be quantified by an optical absorption measurement at 490 nm. The assay is able to assess cell necrosis (Wilhelmi et al., 2012).

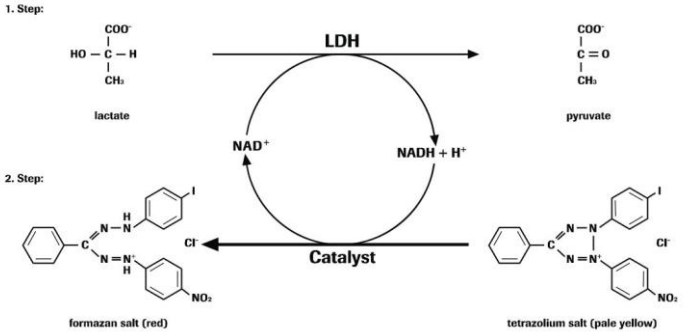

Figure 2. Two-step enzymatic reaction of the LDH assay (Cytotoxicity Detection Kit Plus, Roche product sheet).

1.1.2 Genotoxicity assays

Genotoxicity assays allow the assessment of DNA damage induced by chemical compounds or environmental matrices. They can be performed both *in vitro* or *in vivo* using cell lines or organisms, respectively. Since there is a relationship among DNA damage, mutations and carcinogenesis, the assessment of genotoxic effect is crucial. Among the genotoxicity assays, one of the most widely applied is the Comet assay (Karlsson et al., 2015). This assay, also known as Single Cell Gel Electrophoresis assay, allows the primary DNA damage assessment at the single cell level and can be performed on eukaryotic cells *in vivo*, *in vitro* and *ex-vivo*. It has numerous advantages: it can detect low levels of DNA damage, it can be applied using proliferating or not-proliferating cells and it is not expensive (Araldi et al., 2015).

The Comet assay can be performed at different pH conditions. The alkaline Comet assay (pH>13) is able to measure single-strand DNA breaks, double-strand DNA breaks and alkali-labile sites. These sites are DNA portions without nitrogenous bases (apurinic/apyrimidinic sites) and can be produced by base or sugar damages or by DNA repair systems (base excision repair systems).

The Comet assay is composed by different steps (Figure 3). Cells are included in an agarose gel, placed on a microscope slide and lysed through an incubation in a solution with detergents and high salt concentrations. Subsequently, the slides are placed in a horizontal electrophoretic cell and the genetic material is subjected to migration in an alkaline buffer. The undamaged DNA is unable to migrate so it forms a spherical structure called head, while the damaged DNA is able to migrate so it forms a stretched structure called tail. Therefore, the DNA migration is proportional to genotoxic effect (Karlsson et al., 2015). After DNA staining, DNA damage is measured with computerized image analysis systems that measure different parameters. To quantify the genotoxic effect, the most applied parameters are tail intensity, which is related to the size and the number of DNA fragments, and tail length, which depends on the size of the fragments.

The Comet assay can measure also oxidative DNA damage. To assess this damage, after the lysis the DNA is incubated with enzymes that recognize and cleave oxidized DNA sites. These enzymes are for example the oxoguanine DNA glycosylase (hOGG1), formamidopyrimidine glycosylase (Fpg) or endonuclease III (ENDOIII). Since the enzyme-generated breaks increase the overall DNA damage, the oxidative DNA damage can be identified comparing the damage after enzyme treatment with the damage without enzyme treatment (Møller et al., 2015).

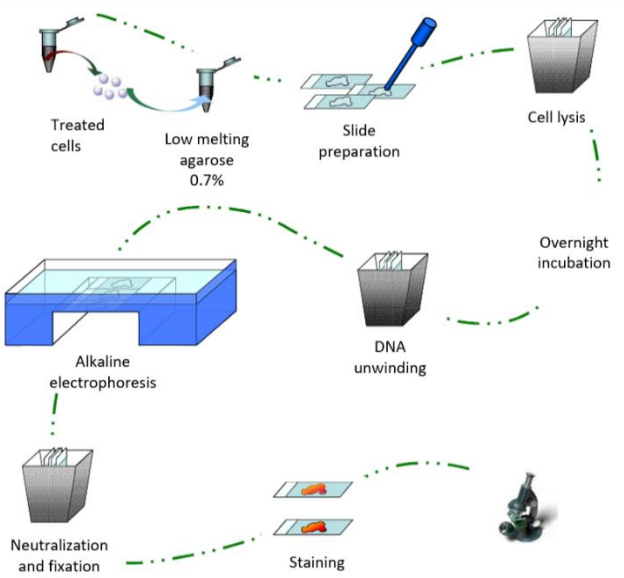

Figure 3. Phases of the Comet assay.

1.1.3 Estrogenic activity assays

Endocrine disrupting chemicals (EDCs) are environmental contaminants able to interfere with the function of the endocrine system. They are defined as exogenous substances that cause adverse health effects in an organism, or in its progeny through changes in endocrine functions and they are also included among the emerging contaminants (Gogoi et al., 2018). Since 1991, when the term "endocrine disruptors" was coined by Theo Colborn during the Wingspread Conference that took place in Racine (Wisconsin, USA), the EDCs have become one of the main research topics of environmental science (Kabir et al., 2015).

EDCs are ubiquitous because they are included in many products used in residential, industrial and agricultural applications (Darbre, 2018). They may be classified by origin in natural compounds (i.e. sexual steroids, phytoestrogens, mycotoxins) and synthetic compounds, such as those employed in industry (phthalates, phenols, polycyclic aromatic hydrocarbons), agriculture (DDT, atrazine, glyphosate) and pharmaceuticals (17α-ethynilestradiol, diethilstilbestrol) (Kabir et al., 2015). EDCs can also be classified by their mechanism of action in five main classes: i) chemicals with an estrogenic effect, which mimic or alter the functions of natural estrogens; ii) chemicals with an androgenic effect, which copy or obstruct natural testosterone; iii) chemicals with thyroidal effect, which cause an effect to the thyroid; iv) chemicals with a progestagenic activity and glucocorticoid activity (Leusch et al., 2017).

The effect of EDCs on the endocrine system is of particular interest since it can be induced by low doses, can be severe when the exposure occurs during childhood or adolescence, can be evident after long time from the time of exposure and can be exerted not only on exposed individual but also on

subsequent generations (Kabir et al., 2015). At a cellular level, EDCs may interfere with hormone functions in different ways. They can directly interact with hormone receptors mimicking natural hormones thus producing an overstimulation (agonist EDCs) or they can bind hormone receptors preventing the binding of the endogenous hormones thus blocking the signal (antagonist EDCs). Moreoever, EDCs may also interfere indirectly with hormones affecting their synthesis, transport, metabolism and excretion (Combarnous, 2017). EDCs can cause reproductive and developmental disorders, both in humans and animals (Pamplona-Silva et al., 2018). In humans, the suspected effects of EDCs are numerous and include cancer of hormone sensitive organs (e.g. breast, prostate, testis), early puberty, cryptorachidism, hypospadias and reduced fertility (Kabir et al., 2015; Pamplona-Silva et al., 2018).
Among the five classes of EDCs the scientific community has particularly focused the attention on the estrogenic EDCs (Leusch et al., 2017). The main effect based monitoring tools that are able to assess estrogenic activity are ligand-binding assays, gene reporter assays, yeast two-hybrid assays, transcription assays, protein assays, cell proliferation assays, *in vivo* tests and signalling pathway analyses (Kiyama and Wada-Kiyama, 2015). Among these tools, gene reporter assays and cell proliferation assays are a good compromise, since they measure combined estrogenic/anti-estrogenic effects, analyse physiological response, require little time and are low cost assays (Mueller, 2004; Soto et al., 2006; Leusch et al., 2010; Leusch et al., 2017; Kunz et al., 2017).

1.1.3.1 Gene reporter assays

Gene reporter assays are able to quantify the estrogenic effect measuring the expression of a reporter gene regulated by estrogens. These assays are based on mammalian or yeast cells that are transfected with a reporter construct. The reporter construct is made of DNA sequences known as estrogen-responsive elements (ERE) linked to the gene of a measurable protein. The EREs are recognized by estrogen receptors (ERs) which are transcription factors able to induce the transcription of the following gene (Sonneveld et al., 2005) (Figure 4).
There are two main types of gene reporter assays. The first type is based on cells that already express ERs, so cells are transfected with an estrogen-inducible reporter gene. The second type is based on cells that do not express endogenous ERs, so cells are transfected with both an estrogen-inducible reporter gene and an ER expression construct (Soto et al., 2006; Wangmo et al., 2018). Since these cells do not express other ERs, the advantage of the second type of gene reporter assays is that it is able to assess the estrogenic activity of samples distinguishing between the activity due to different ERs (such as ERα and ERβ).

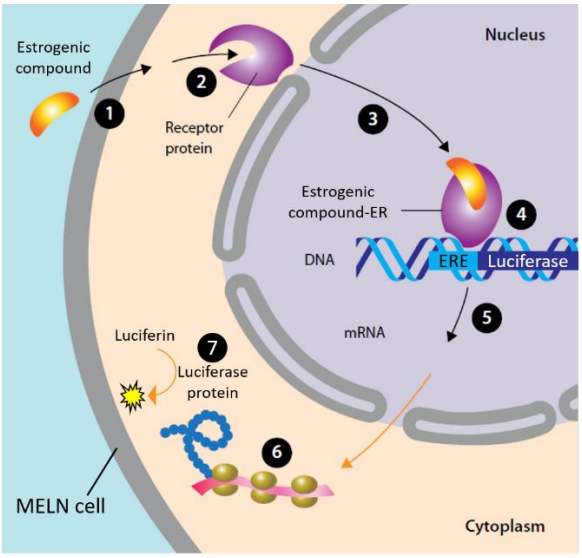

Figure 4. Phases of a gene reporter assay that assesses the estrogenic activity using MELN cells.

1.1.3.2 Proliferation assays

Proliferation assays that assess estrogenic activity are known as E-screen assays. These assays measure the proliferative effect induced by estrogens or estrogenic substances on estrogen-responsive cells. Proliferation is determined in different ways such as DNA staining and metabolic activity quantification. Soto et al. (1997) developed the first E-screen assay using human breast cancer cells (MCF-7), whose proliferation is mainly induced by the activation of ERα (Wagner et al., 2017). Subsequently, E-screen assay was also performed using different MCF-7 sublines such as the MCF-7 BUS, which are characterized by a greater proliferative response (Villalobos et al., 1995; Martínez et al., 2016).

1.2 Applications of effect based tools

1.2.1 Assessment of chemicals

Chemical pollution is a great and growing global problem. Since 1950, more than 140,000 new chemicals have been synthesised. Many of these compounds have been produced in greatest volume, thus becoming widely dispersed in the environment and causing human exposure. Fewer than half of these compounds have undergone safety or toxicity testing; moreover, rigorous pre-market evaluation of new chemicals has become mandatory only recently and in few high-income countries (Landrigal et al., 2018). Therefore, the assessment of chemical toxicity through effect based tools has a key role in order to preserve the environment from chemical pollution thus protecting human health.

Among the numerous chemicals, nanoparticles (NPs) and pesticides are of particular interest due to their wide application and spread in the environment. These chemicals can induce different biological effects. Therefore, it is important to assess them using effect based tools because this assessment improves the knowledge of their toxicity and action mode; moreover, it provides information useful to select safer NPs and pesticides for human health and environment.

1.2.2 Assessment of environmental matrices

The environmental contamination has generally been assessed through traditional monitoring, which is based on identifying and quantifying individual chemicals. However, this traditional monitoring is characterized by some limitations. Sophisticated equipment and highly trained personnel are required to perform chemical analysis, so it is very expensive. Moreover, all the individual compounds that are contained within an environmental matrix may not be known and it is not possible to analyse all of them. In addition, the methods may not be enough sensitive to measure the individual compounds or the quantification may be affected by matrix interferences. Finally, chemical analysis is not suitable to quantify the total biological effect of environmental matrices because antagonistic or synergistic interactions among the compounds are generally not known (Jarošová et al., 2014). Therefore, biological monitoring approaches, through effect based tools, are needed. Effect based tools detect cumulative effects and are useful to connect chemical contamination and ecological status. They evaluate the mixture toxicity integrating the effects of unknown compounds, with the same mechanism of action, as well as synergistic and antagonistic effects of all compounds (Kase et al., 2018).

Among the environmental matrices, wastewaters (WWs) and airborne particulate matter (PM) are of particular interest since they can influence the quality of waters and air. Both WWs and PM can contain a huge amount of different compounds that could cause different biological responses, so their evaluation using effect based tools, such as *in vitro* assays, is important to assess the cumulative risk posed by the different compounds carried by these two environmental matrices.

2. Objectives

The main aim of the PhD project was to apply several effect based monitoring tools that assess cytotoxicity, genotoxicity and estrogenic activity in order to study:

- emerging and widespread chemicals whose toxicity is not established yet but is essential for legislative purposes;
- environmental matrices whose biological effect could provide crucial information to assess risk liked to human and environmental exposure.

In particular, the following *in vitro* assays on eukaryotic cells were applied as effect based monitoring tools:

- WST-1 assay (cytotoxicity)
- LDH assay (cytotoxicity)
- Comet assay (genotoxicity)
- E-screen assay (estrogenic activity)
- Gene reporter assay (estrogenic activity)

These tools were applied to test:

- chemicals (titanium dioxide NPs and pesticides)
- environmental matrices (WWs and PM samples)

Moreover, bibliographic research was performed in order to study the methodological approach and the application of estrogenic activity assays on biological samples.

The work done during the PhD allowed the publication of six articles directly linked to the topic of the PhD project. As detailed in paragraph 3, each paper addressed a specific aspect.

Moreover, other three research articles on the topic of the PhD project are still in preparation. Finally, during the PhD, I have collaborated on other scientific researches that have resulted in one published research article (Marangon et al., 2021) and one submitted review article (Panizzolo et al., submitted).

3. Scientific publications

3.1 Shape-engineered titanium dioxide nanoparticles (TiO_2-NPs): cytotoxicity and genotoxicity in bronchial epithelial cells

The International Organization for Standardization has defined a nanomaterial as a material characterized by nanometric external dimensions (average between 1 and 100 nm) or by an internal or surface structure with nanometric characteristics (i.e. nanobjects and nanostructured materials, respectively). Among the nanobjects, NPs were defined as objects with three nanometric external dimensions (Lövestam et al., 2010). Nanomaterials were also defined by the European Union as natural, incidental or manufactured materials that contain separated, aggregated, or agglomerated NPs of which 50% or more exhibit at least one external dimension between 1 and 100 nm (Shi et al., 2013). Nanomaterials are widely used. Examples of products that contain nanomaterials are: foods (additives), food containers, pharmaceuticals and healthcare products, textiles, cosmetics, personal care products, paints, coatings, construction materials, catalysts, agricultural chemicals and electronic products (Lövestam et al., 2010). Due to their wide application, the production of engineered nanomaterials is growing rapidly, especially in developed countries. Increased production and use of these materials is leading to their increasing spread in the environment. The release of these materials can occur when nanomaterials are produced or during their use (Caballero-Guzman and Nowack, 2016). At the manufacturing stage, nanomaterials can be released depending on the manufacturing procedures, while during their use the release can be influenced by how nanomaterials are incorporated into the product. Finally, nanomaterials can be released in the environment also during their disposal by wastewater treatment plants (WWTPs), landfill or through incineration (Caballero-Guzman and Nowack, 2016).

In addition to the increase in the environment, also human exposure to nanomaterials is increasing, so their potential toxicity should be carefully assessed. Indeed, nanomaterials have different chemical and physical properties with respect to coarser materials of identical elemental and molecular composition. Nanomaterials have a larger surface area than coarse materials with equal mass and thus they are characterized by a larger interface area. Since chemical reactions occur on the particle surface, the reactivity of nanomaterials is generally greater than that of coarse materials. In addition, the small size may facilitate the transport of these particles within human body and may lead to the crossing of biological barriers in lung, gut and brain. Therefore, nanomaterials are potentially able to reach more target tissues (Lövestam et al., 2010).

Since many physico-chemical characteristics of NPs could have an important role in toxicity (e.g. crystalline structure, size, shape, exterior area, agglomeration/ aggregation and surface properties), the aim of this study was to assess cytotoxicity (WST-1 assay and LDH assay) and genotoxicity (Comet assay) of three engineered titanium dioxide NPs with different shapes (bipyramids, rods, platelets) in comparison with two commercial titanium

dioxide NPs (P25, food grade). NPs were characterized, then, their biological effects were assessed on BEAS-2B cells in presence/absence of light in order to consider also possible effects due to NP photocatalytic properties. Finally, the cellular uptake of NPs was analysed using Raman spectroscopy (Figure 5) (Gea et al., 2019). This research was performed in collaboration with the Italian National Research Institute of Metrology.

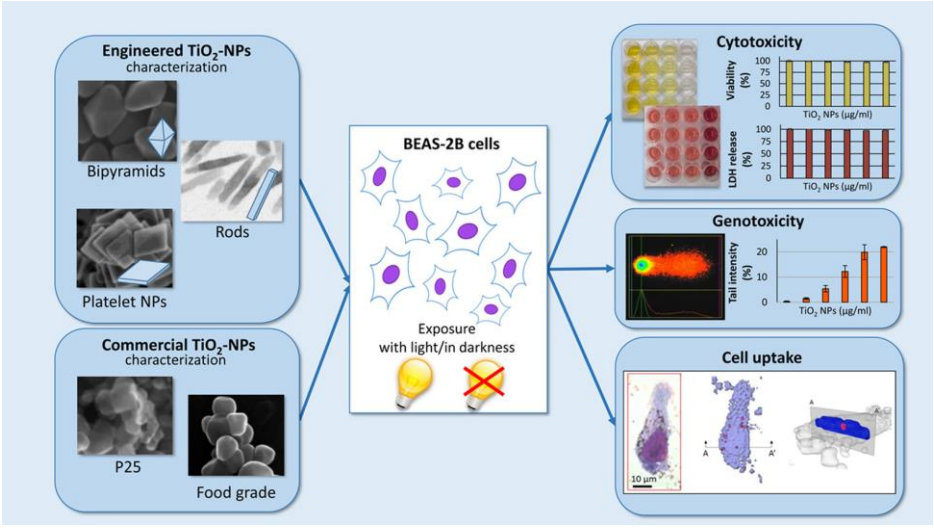

Figure 5. Graphical abstract of the first article.

3.2 Molecular basis for endocrine disruption by pesticides targeting aromatase and estrogen receptor and assessment of five pesticides as endocrine disrupting chemicals: effects on estrogen receptor and aromatase

During the last century, a significant increase in world food production has become necessary to sustain the growth of the global population (Carvalho, 2017). In order to achieve appropriate food quantity and satisfactory food quality, fertilizers and also pesticides were extensively used.

The term pesticide includes a wide variety of compounds that are used to kill pests, including insects, rodents, fungi and unwanted plants (Kim et al., 2017). Pesticides are considered quick, easy and inexpensive solutions for controlling pests and their use has contributed not only to the leap in agricultural yield but has also helped to fight vector-borne and food-borne diseases (European Parliamentary Research Service Report, 2017; Tudi et al., 2021). Due to these advantages, great quantities of pesticides are applied every year. In 2019, in the world, more than 4 million tonnes of pesticides were used (FAO, 2021). Regarding insecticides, the neonicotinoids are the most widely utilized in the world; in 2014, the neonicotinoid market exceeded $ 3 billion and accounted for about 25% of the global pesticide market (Frank and Tooker, 2020). Moreover, pesticides are also extensively applied in agricultural sector; in the European Union alone, the sales of plant protection products per hectare of agricultural area amount to 2.3 kg (European Parliamentary Research Service Report, 2017). The increase in the use of pesticides has been regulated through legislation. In the European Union, previously fragmented legislation was replaced with harmonized pesticide standards for all member states; however, pesticide legislation varies greatly worldwide (more stringent regulations have been approved by developed nations with respect to developing countries) and to date harmonized pesticide legislation does not exist (Handford et al., 2015).

Despite the advantages, the use of pesticides also brings disadvantages. The extensive application of these molecules has ensured their spread in the environment. Numerous studies have demonstrated that air, water and soil can be polluted by pesticide residues (Carvalho, 2017; Samsidar et al., 2018). Once in the environment, pesticides can be accumulated in non-human organisms with devastating toxic effects at population level (Köhler and Triebskorn, 2013); moreover, they can move up trophic chains and affect top predators (Carvalho, 2017). Through their effects on animals, pesticides can endanger the biodiversity; in addition, they can also be hazardous for human health (Damalas and Eleftherohorinos, 2011; Kim et al., 2017).

Human exposure to pesticides can occur through the ingestion of foods or liquids containing pesticide residues, through the inhalation of pesticide-contaminated air, or through the dermal contact with pesticides (Anderson and Meade, 2014; Kim et al., 2017). Toxic effects induced by pesticide exposure can range from mild symptoms (like skin irritation) to more severe symptoms (like headache or nausea). Moreover, some studies reported that exposure to

pesticides can induce long-term health effects, including cancer (Kim et al., 2017).

Some pesticides have been extensively assessed for their ability to interfere with the endocrine system; however, additional evidences are needed for many others. Therefore, the aim of these two studies was to assess the interference with the estrogen biosynthesis and estrogen signaling of eight widespread pesticides. Five neonicotinoid insecticides (Imidacloprid, Thiacloprid, Acetamiprid, Clothianidin, Thiamethoxam), a carbamate insecticide (Methiocarb) and two herbicides (Glyphosate and Oxadiazon) were tested. In collaboration with the Department of Life Science and System Biology (University of Turin), the effect of pesticides on estrogen biosynthesis was studied using a recombinant form of human aromatase, the enzyme that catalyses the transformation of androgens to estrogens. Moreover, the effect of pesticides on estrogen signaling was assessed using a gene reporter assay on MELN cells, which measures the ER-mediated estrogenic activity (Figure 6 and 7) (Zhang et al., 2020; Gea et al., 2022).

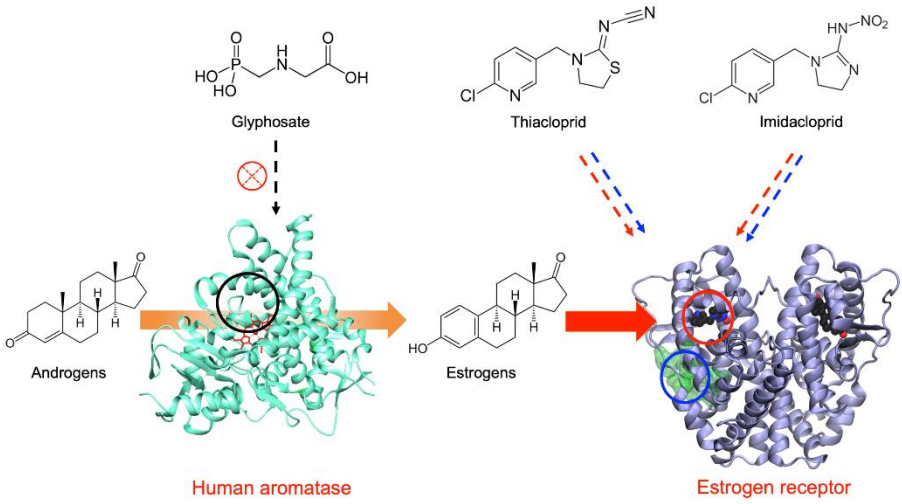

Figure 6. Graphical abstract of the second article.

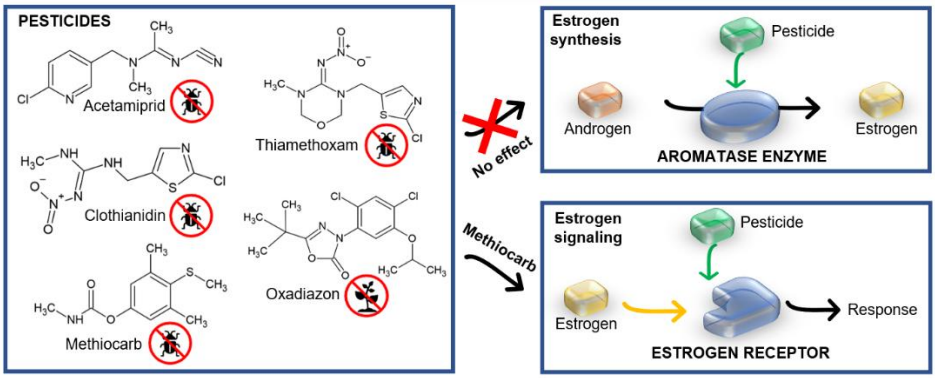

Figure 7. Graphical abstract of the third article.

3.3 Ecofriendly laccases treatment to challenge micropollutants issue in municipal wastewaters

WWs are domestic or industrial liquid matrices that are altered because have been used or have been in contact with contaminated surfaces (e.g. rainwater that has accumulated pollutants). Before their reuse or their release in the environment, these matrices are generally treated in WWTPs.

In WWTPs, WWs are subjected to different treatments that can be classified as primary treatments, secondary treatments and tertiary treatments. In the primary treatments the solid waste substances (e.g. plastics, sand and grit) and oily waste substances (e.g. oils and fats) are removed by the WWs. These treatments are common for almost all WWTPs and are accomplished mechanically by filtration and sedimentation. After primary treatments, WWs are treated with secondary treatments. These treatments are intended to degrade organic substances and are generally based on biological (aerobic or anaerobic) processes. Among the various secondary treatments, the most well-known technique is conventional activated sludge that allows the removal of organic compounds and nitrogen through the formation of biological floc utilizing dissolved oxygen. In conventional activated sludge, organic matter is used by microorganisms as a food source for life-sustaining processes and it is transformed into carbon dioxide and water. Lastly, in many WWTPs there are also the tertiary treatments that can be applied to remove phosphorous by precipitation and filtration or to sanitize WWs through UV irradiation or chlorination (Gogoi et al., 2018).

WW treatments might inefficiently remove some persistent pollutants; moreover, some chemicals can be transformed in more reactive compounds by WW treatments (Pamplona-Silva et al., 2018). Therefore, discharge of WWTP effluents into receiving water bodies is one of the main sources of pollutants in water environment. Since many surface waters are used as a source for drinking water production, WWs can be a threat to aquatic ecosystems but also to human health (Lundqvist et al., 2019).

Recently enzyme technologies have been proposed as alternative WW treatment. Among enzyme technologies, those based on fungal enzymes (e.g. laccases) are probably the most suitable for industrial exploitation, because laccases do not require any nutrient supply and can work with just the presence of oxygen as co-factor. However, the application of these fungal enzymes as WW treatment is still poorly studied. Therefore, the aim of this article was to study whether the treatment with fungal enzymes (laccases) is able to reduce toxicity and estrogenic activity of real municipal WWs. The influent and the effluent of a WWTP were analysed with and without an enzymatic treatment using laccases of *Trametes pubescens* MUT 2400. The Department of Pharmaceutical Science and Technology (University of Turin) quantified the concentrations 15 pollutants in the four samples, while the Department of Life Science and System Biology assessed the ecotoxicity of the samples on a plant

(*Lepidium sativum*) and an alga (*Raphidocelis subcapitata*). Finally, our Department assessed the estrogenic activity of the samples using two assays (a gene reporter assay and the E-screen assay) (Figure 8) (Spina et al., 2020).

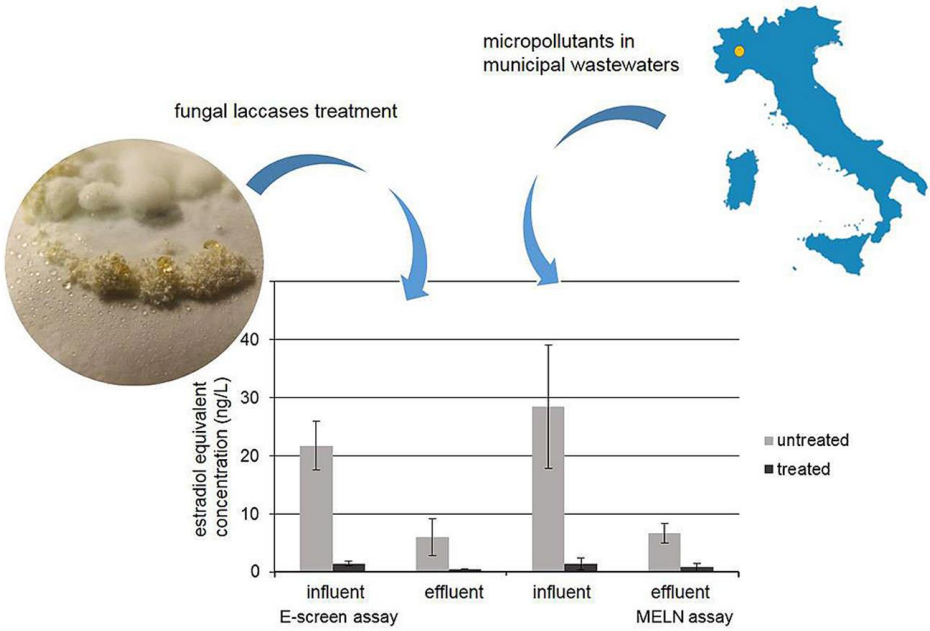

Figure 8. Graphical abstract of the fourth article.

3.4 *In vitro* effects of particulate matter associated with a wildland fire in the North-West of Italy

Air pollution is a great environmental health problem. Numerous epidemiological, clinical and toxicological evidences have demonstrated some human adverse effects of air pollution such as respiratory symptoms, cardiovascular effects and lung cancer. Moreover, exposure to air pollution was also associated with other adverse effects, in particular effects on fertility, pregnancy, newborns and children (Losacco and Perillo, 2018). In high-income countries, the improvement of air quality has reduced mortality for cardiovascular and respiratory diseases; moreover, it has also caused substantial economic gains (Landrigal et al., 2018).

The PM is one of the most important proxy indicators of air pollution and it is also considered a crucial air pollutant. It can be defined as a mixture of fine solid or liquid droplets suspended in the air and it can be classified according to aerodynamic diameter as coarse PM with an aerodynamic diameter less than 10 μm (PM_{10}) or fine PM with an aerodynamic diameter less than 2.5 μm ($PM_{2.5}$) (Kim et al., 2015). PM is a heterogeneous mixture, including elemental and organic carbon, biological components (e.g. allergens and microbial compounds), inorganic components (e.g. trace metals, nitrates, sulfates, ammonium), and organic compounds (e.g. polycyclic aromatic hydrocarbons) (Peixoto et al., 2017).

In order to evaluate the biological effects of PM, useful tools are represented by *in vitro* assays. In recent years, a growing concern has been focused on effects induced against the endocrine system and these effects have been studied using different *in vitro* assays such as estrogenic activity assays. However, while numerous studies have applied these assays on waters and sediments, little is known about the estrogenic activity of airborne PM. Moreover, in contrast to the large amount of information on cytotoxicity, genotoxicity and mutagenicity induced by urban emissions, only few studies have evaluated biological effects induced by biomass combustion emissions during real wildland fires whose occurrence will increase in the next years due to climate change expecially in the Mediterranean region, an high responsive area to global warming.

Therefore, the aim of this study was to investigate biological effects of $PM_{2.5}$ (Ø < 2.5 μm) and PM_{10} (Ø < 10 μm) collected near a fire occurred in the North-West of Italy in 2017 and in three other areas (urban and rural areas). Organic extracts were assessed for mutagenicity using Ames test (TA98 and TA100 strains), for cytotoxicity using WST-1 and LDH assays (BEAS-2B cells), for genotoxicity using Comet assay (BEAS-2B cells) and for estrogenic activity using a gene reporter assay (MELN cells). The mutagenicity was assessed in collaboration with the Regional Agency for Environmental Protection of Piedmont (Figure 9) (Gea et al., 2021).

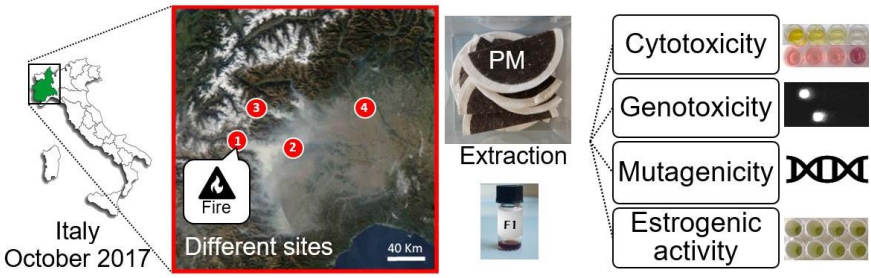

Figure 9. Graphical abstract of the fifth article.

3.5 Estrogenic activity of biological samples as a biomarker

In recent years, effect based monitoring tools have been used for hormonal activity quantification in biological samples by an increasing number of authors. These tools are advantageous because they are based on biological reactions that depend on the presence or absence of hormones, such as 17β-estradiol, but are also able to assess the presence of hormone metabolites, such as estrone and estriol.

In addition to hormones, a great number of exogenous compounds (known as EDCs) can exert and modulate activity of the endocrine system and can be measured using these tools. Therefore, the application of these assays on biological samples has been proposed and used also as a novel biomarker of exposure to EDCs.

The aim of this review was to discuss the methodological approach and the application of effect based monitoring tools that assess the estrogenic activity in human biological samples. 75 research articles were analysed and divided according to whether they used these assays: i) to quantify the level of estrogens and their hormonal activity ii) as a biomarker of exposure to EDCs (Figure 10) (Gea et al., 2020).

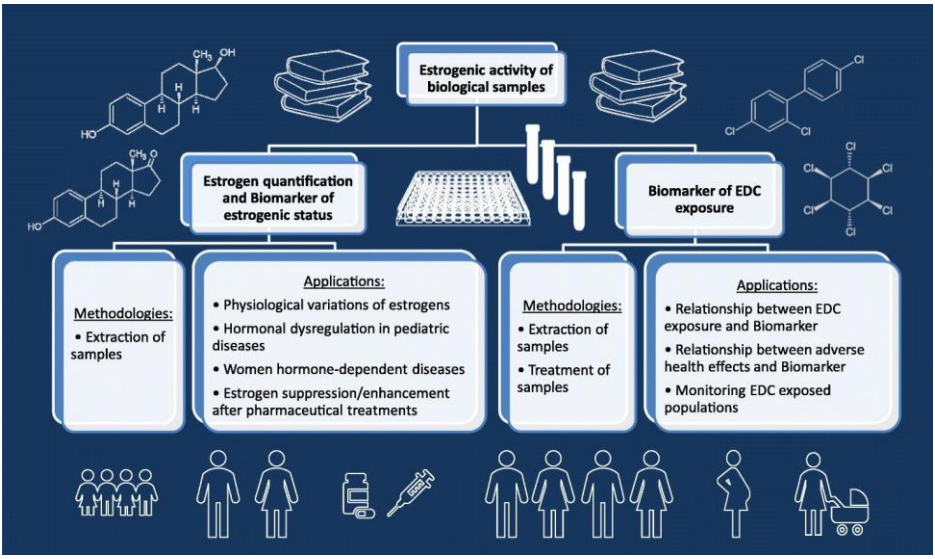

Figure 10. Graphical abstract of the sixth article.

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Shape-engineered titanium dioxide nanoparticles (TiO_2-NPs): cytotoxicity and genotoxicity in bronchial epithelial cells

Marta Gea[a], Sara Bonetta[a,*], Luca Iannarelli[b], Andrea Mario Giovannozzi[b], Valter Maurino[c], Silvia Bonetta[a], Vasile-Dan Hodoroaba[d], Caterina Armato[a,e], Andrea Mario Rossi[b], Tiziana Schilirò[a]

[a] Department of Public Health and Pediatrics, University of Turin, Piazza Polonia 94, 10126, Turin, Italy
[b] Quality of Life Division, National Institute of Metrological Research, Strada delle Cacce 91, 10135, Turin, Italy
[c] Department of Chemistry, University of Turin, Via Giuria 7, 10125, Turin, Italy
[d] Surface Analysis and Interfacial Chemistry division, Federal Institute for Materials Research & Testing (BAM), 12200, Berlin, Germany
[e] Centre for Sustainable Future Technologies (CSFT@PoliTo), Italian Institute of Technology, Corso Trento 21, 10129, Turin, Italy

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ABSTRACT

The aim of this study was to evaluate cytotoxicity (WST-1 assay), LDH release (LDH assay) and genotoxicity (Comet assay) of three engineered TiO_2-NPs with different shapes (bipyramids, rods, platelets) in comparison with two commercial TiO_2-NPs (P25, food grade). After NPs characterization (SEM/T-SEM and DLS), biological effects of NPs were assessed on BEAS-2B cells in presence/absence of light. The cellular uptake of NPs was analyzed using Raman spectroscopy.

The cytotoxic effects were mostly slight. After light exposure, the largest cytotoxicity (WST-1 assay) was observed for rods; P25, bipyramids and platelets showed a similar effect; no effect was induced by food grade. No LDH release was detected, confirming the low effect on plasma membrane. Food grade and platelets induced direct genotoxicity while P25, food grade and platelets caused oxidative DNA damage. No genotoxic or oxidative damage was induced by bipyramids and rods. Biological effects were overall lower in darkness than after light exposure. Considering that only food grade, P25 and platelets (more agglomerated) were internalized by cells, the uptake resulted correlated with genotoxicity.

In conclusion, cytotoxicity of NPs was low and affected by shape and light exposure, while genotoxicity was influenced by cellular-uptake and aggregation tendency.

1. Introduction

Nanoparticles (NPs) are defined as particles having their three dimension in the range of 1–100 nm (ISO, 2015). Actually, many consumer products incorporates NPs. The technological, medical and economic benefits of NPs are considerable, but the presence of nanoparticles in the environment could cause adverse effects to humans. NPs have a greater surface area per mass unit, so they potentially have an increased biological activity compared to fine particles. Moreover, NPs size is comparable to the size of cellular structures, so NPs might potentially emulate biological molecules or interfere physically with biological processes (Magdolenova et al., 2012a).

TiO_2 is the oxide of titanium and it has different crystalline structures: anatase, brookite and rutile. Brookite is not produced by industry and is not incorporated in commercial products. In contrast, rutile and anatase are largely used in commercial products (Jovanovic, 2015). TiO_2 is one of the most frequently applied NPs and it is in the top five NPs used in consumer products (Shi et al., 2013). TiO_2-NPs produced are used primarily as a pigment owing to their brightness, resistance to discoloration and high refractive index. As a pigment, TiO_2-NPs are incorporated in paints, plastic materials, paper, foods, medical products and cosmetics. Due to its catalytic and photocatalytic properties, TiO_2 is also used as an antimicrobial agent and a catalyst for purification of air and water (Bonetta et al., 2013; Tomankova et al., 2015).

TiO_2-NPs could be engineered in terms of shapes and sizes by changing synthesis conditions such as raw material, temperature, acidic and alkaline conditions. Engineered TiO_2-NPs with various shapes (e.g. rods, dots and belts) have been prepared for different applications (Bernard and Curtiss, 2005; Sha et al., 2015; Wang et al., 2004). In particular, engineered fiber-shaped nanomaterials (i.e. nanowires,

* Corresponding author. Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126, Turin, Italy.
E-mail address: sara.bonetta@unito.it (S. Bonetta).

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List of abbreviations

BEAS-2B	Human bronchial epithelial cells
D_h	Hydrodynamic diameter
Fpg	Formamidopyrimidine glycosylase
NP	Nanoparticle
TiO_2	Titanium dioxide

nanotubes) are very attractive because they showed higher activity and advantages in photocatalysis, charge transfer and sensing applications due to their structure (Hamilton et al., 2009). However, these new and enhanced properties may also induce higher toxicological effects upon exposure with biological tissues.

Humans can be exposed to TiO_2-NPs via three portals of entry: oral (mainly via food consumption), dermal (often through cosmetic and sunscreen applications) and inhalation (mainly under occupational and manufacturing conditions) (Warheit and Donner, 2015).

Based on the evidence that TiO_2 can induce lung cancer in rats, TiO_2-NPs were classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC, 2010). Indeed, the inhalation and instillation of rutile and anatase TiO_2-NPs induced lung tumors (Xu et al., 2010), broncho-alveolar adenomas and cystic keratinizing squamous cell carcinomas (De Matteis et al., 2016; Mohra et al., 2006). TiO_2-NPs were also classified as potential occupational carcinogens by the National Institute for Occupational Safety and Health (NIOSH, 2011; Chen et al., 2014).

Many *in vitro* studies showed cytotoxicity, genotoxicity and oxidative effects induced by TiO_2-NPs through oxidants generation, inflammation and apoptosis (Jugan et al., 2011; Karlsson et al., 2015; Park et al., 2008; Shi et al., 2010). The potential of NPs to cause DNA damage is an important aspect that needs attention due to possible mutations and carcinogenesis. Physico-chemical characteristics of NPs have an important role in toxicity. Different studies showed that biological effects can be influenced by crystalline structure, size, shape, exterior area, agglomeration/aggregation and surface properties (Bhattacharya et al., 2009; Johnston et al., 2009). Some studies revealed that crystalline structure probably influences the induced toxicity, in particular the anatase seems to be more reactive (Sayes et al., 2006) and induces more toxic, genotoxic and inflammatory effects, than the rutile (Falck et al., 2009; Petkovic et al., 2011; Xue et al., 2010). However, other studies gave contradictory results with rutile forms being more toxic than anatase (Gurr et al., 2005; Numano et al., 2014; Uboldi et al., 2016). The effect of agglomeration/aggregation of NPs on toxicity is not well understood yet. In recent studies, some authors demonstrated that agglomeration can influence NPs genotoxicity (Magdolenova et al., 2012b; Prasad et al., 2013).

Although physico-chemical properties of NPs can have an important role in the impact on their toxicity, only few studies on shape dependent TiO_2 toxicity has been conducted (Allegri et al., 2016; Hamilton et al., 2009; Park et al., 2013). Additional studies are needed to evaluate the role of shape on TiO_2-NPs toxicity in order to produce useful data for assessing the safety of engineered NPs.

To address this issue, the aim of this study was to investigate cytotoxicity (WST-1 assay), LDH release (LDH assay) and genotoxicity (Comet assay) of three types of engineered TiO_2-NPs of different shapes (bipyramids, rods and platelet NPs) in BEAS-2B (cells isolated from human bronchial epithelium) in comparison with two commercial types of TiO_2-NPs (P25 and food grade). Since the exposure to TiO_2-NPs mainly occurs through respiratory tract (occupational and manufacturing conditions), human cells of the respiratory system (such as BEAS-2B), were selected as a good cell model for *in vitro* toxicology tests. All the TiO_2-NPs in this study were first physico-chemically characterized, even in different culture media to study their agglomeration state, and then they were biologically evaluated. In order to take into account the photocatalytic properties of the TiO_2-NPs, we investigated the cytotoxicity and genotoxicity on BEAS-2B under light exposure and in darkness. Moreover, a modern application of Raman spectroscopy, the 3D confocal Raman imaging, was used to study the uptake of the NPs within the BEAS-2B cells, as the Raman spectra provide information about both organic molecules and solid NPs simultaneously (Ahlinder et al., 2013).

2. Materials and methods

2.1. Synthesis and preparation of TiO_2 NPs dispersion

Rods and bipyramids TiO_2-NPs were synthesized by the forced hydrolysis of an aqueous solution of Ti^{IV}(triethanolamine)$_2$titanatrane (Ti(TEOAH)$_2$), using triethanolamine (TEOA) as shape controller; pH of synthesis was adjusted by adding 1 M NaOH solution; details of these procedures were previously reported (Iannarelli et al., 2016; Lavric et al., 2017). The synthesis of platelet NPs was performed with a solvothermal method (Han et al., 2009; Zhang et al., 2012). In a typical synthesis: a precise volume of $Ti(OBu)_4$ was added in a 150 ml Teflon pot and the desired volume of concentrated hydrofluoric acid was added dropwise under stirring. The Teflon pot was sealed and kept under stirring at high temperature (250 °C) for 24 h in autoclave. The resulting paste was centrifuged three times and washed with acetone and with water (Milli-Q) to remove the residual organics. The synthesis dispersions were subjected to dialysis process (against ultrapure water, using Spectra/Por dialysis membrane tubing MWCO 8–14 kDa) in order to clean the medium. To avoid agglomeration and precipitation, dimethylsulfoxide (DMSO 1% in water) was added to the NPs dispersions (final concentration 2.5 mg/ml); the dispersions were homogenized using an ultra-sonication procedure (Iannarelli et al., 2016), few hours before the exposure with cells.

The same procedure was employed in the preparation of the dispersion of commercial TiO_2 powders, which were the P25 NPs (Evonik), extensively used in toxicity studies (Karlsson et al., 2015; Magdolenova et al., 2014; Valant et al., 2012), and the food grade NPs (Faravelli Group), incorporated in many edible products (Weir et al., 2012).

2.2. Scanning electron microscopy (SEM) including transmission mode (T-SEM)

The dimensional characterization (size and shape) of TiO_2-NPs was carried out with SEM using a Zeiss Supra 40 instrument (Zeiss) equipped with a Schottky field emitter, the standard secondary electrons, i.e. Everhart-Thornley, detector and a high-resolution In-lens detector. The surface-sensitive In-lens SEM mode better suited to morphological/shape analysis and transmission mode in SEM (T-SEM) better suited for dimensional measurements were applied complementary to the same field of view on the sample.

2.3. Dynamic Light Scattering (DLS) analysis

Delsa Nano™ C Analyzer (Beckman Coulter) equipped with a 638 nm diode laser and a temperature control was used for the DLS measurements. The laser fluctuation was detected on a photomultiplier tube detector positioned behind the cuvette with an angle of 163°. Hydrodynamic diameters were calculated setting temperature at 25 °C, viscosity (η) 0.890 cP and refractive index of water 1.3325. In order to simulate the culture medium conditions, DLS analyses were conducted on dilution of TiO_2 dispersions (1:4) in a 1% DMSO aqueous solution, as reference analysis, and in base RPMI 1640 medium [supplemented with l-glutamine (4 mM) and penicillin-streptomycin (100 U/ml - 100 µg/ml)] and complete RPMI 1640 medium [supplemented with FBS (10% v/v), l-glutamine (4 mM) and penicillin-streptomycin (100 U/ml - 100 µg/ml)].

2.4. Raman spectroscopy analysis

The aqueous suspensions of the TiO_2-NPs under investigation were freeze-dried to obtain a solid powder. Raman spectroscopy was used in the analysis of dry TiO_2-NPs powder using a DXR™ Raman Microscope (Thermo Scientific) with a laser wavelength at 532 nm, a laser power of 1 mW and a 10× microscope objective. Spectra were collected in the 50–1800 cm^{-1} spectral region, with a grating resolution of 3.3–3.9 cm^{-1}, exposure time of 1 s and 20 scans in total.

2.5. Cell culture and exposure

BEAS-2B cells, isolated from human bronchial epithelium, were obtained from the American Type Culture Collection (ATCC® CRL-9609™). BEAS-2B were grown as a monolayer, maintained and treated in complete RPMI 1640 medium [supplemented with FBS (10% v/v), l-glutamine (4 mM) and penicillin-streptomycin (100 U/ml - 100 µg/ml)], at 37 °C in a humidified atmosphere containing 5% CO_2.

The solution of NPs (2.5 mg/ml, DMSO 1% in water) was vortexed and sonicated (30 min) in order to homogenize the NPs. NPs (5–160 µg/ml) were directly pipetted in culture plates containing RPMI 1640 medium; then the cell culture plates were mixed on a shaker (10 min). The cells were exposed for 1 h under laboratory light and then they were incubated at 37 °C in darkness (23 h) (exposure with light). In order to standardize the exposure with light the cells were exposed in a dark room (obscured by daylight) to a normal laboratory lamp (36 W/840 Lumilux Cool White-36 W, 3350 lm, 4000 K, supplied from OSRAM lighting AG). The lamp illuminance measured with Quantum photo/radiometer HD 9021 (Delta Ohm) was 289 ± 11 lx. To quantify effects due to the photocatalytic activity of TiO_2, cells were exposed for 24 h in darkness (exposure in darkness).

After exposure, cytotoxicity and genotoxicity assays were performed.

2.6. Cytotoxicity

Cell viability was assessed using Cell Proliferation Reagent WST-1 (Roche). The assay was performed as previously descripted by Gea et al. (2018). Briefly, BEAS-2B cells were cultured in 75 cm^2 flasks and maintained until the cells reached 70% confluence; cells were then seeded in 24-well plates (5 × 10^4 cells/well) and allowed to adhere overnight. After that, culture medium was removed and cells were exposed to NPs (5, 10, 20, 50 and 80 µg/ml, equivalent to 1.3, 2.6, 5.2, 13.0 and 20.7 µg/cm^2) for 24 h with light or in darkness (as specified in paragraph 2.5). After exposure, WST-1 was added (50 µl/well) and incubated for 3 h (37 °C). After incubation, well contents were centrifuged and the supernatants were transferred in 96-well plate to remove the interference owing to the NPs. The absorbance was measured at 440 nm (Tecan Infinite Reader M200 Pro). Absorbance of unexposed cells was used as negative control. Data were expressed as a percentage of viability. All experiments were performed in quadruplicate (four wells for each experimental condition).

As indicator of cell membrane damage, lactate dehydrogenase activity was measured in cell-free culture supernatants using the LDH assay kit (Cytotoxicity Detection Kit PLUS, Roche) modified for NPs exposure. Briefly, BEAS-2B cells were cultured in 75 cm^2 flasks and maintained until the cells reached 70% confluence, cells were then seeded in 24-well plates (5 × 10^4 cells/well) and allowed to adhere overnight. After that, culture medium was removed and the cells were exposed to NPs (5, 10, 20, 50 and 80 µg/ml, equivalent to 1.3, 2.6, 5.2, 13.0 and 20.7 µg/cm^2) for 24 h with light or in darkness (as specified in paragraph 2.5). After exposure, the contents of each well were centrifuged to remove the interference owing to the NPs. Each supernatant (100 µl) was transferred into 96-well plate, mixed with Reaction Mixture (100 µl/well) and incubated for 30 min at 15–25 °C. After incubation, Stop Solution (50 µl/well) was added and the absorbance was measured at 490 nm (Tecan Infinite Reader M200 Pro). Absorbance measurement of unexposed cells were used as negative control, while absorbance measurement of unexposed cells lysed with Lysis Solution (Cytotoxicity Detection Kit PLUS, Roche) was used as positive control. Data were expressed as a percentage of LDH release, respect to control cells (100%). All experiments were performed in triplicate (three wells for each experimental condition).

2.7. Genotoxicity

The alkaline Comet assay was used for DNA damage evaluation (direct DNA damage). BEAS-2B cells were cultured in 75 cm^2 flasks and maintained until the cells reached 70% confluence; cells were then seeded in 6-well plates (3 × 10^5 cells/well) and cultured overnight before exposure to NPs. The cells were exposed to different doses of NPs (20, 50, 80, 120 and 160 µg/ml, equivalent to 5.2, 13.0, 20.7, 31.2 and 41.6 µg/cm^2) for 24 h with light or in darkness (as specified in paragraph 2.5). Unexposed cells and cells treated with DMSO (1%) were used as negative controls. The alkaline Comet assay was performed according to Tice et al. (2000) after slight modifications (Bonetta et al., 2019). After exposure, cells were washed with base RPMI 1640 and PBS, detached using trypsin-EDTA (1×) and cell viability was determined (trypan blue staining). Cells were then centrifuged and mixed with low melting point agarose (0.7%), placed on the slides coated with normal melting agarose (1%) and low melting point agarose was added as the top layer. The slides were immersed in lysis solution in the dark overnight (8 mM Tris–HCl, 2.5 M NaCl, 100 mM EDTA disodium salt dihydrate, 1% TRITON X-100 and 10% DMSO, pH 10, 4 °C). For the unwinding, the slides were immersed in alkaline electrophoresis buffer (20 min) (1 mM EDTA, 300 mM NaOH, 10% DMSO, pH > 13) and the electrophoresis was carried out in the same buffer (20 min, 1 V/cm and 300 mA). The slides were washed with neutralization buffer (0.4 M Tris-HCl, pH 7.5, 4 °C, 3 min), fixed with ethanol 70% (−20 °C, 5 min) and air dried. All steps were performed under yellow light to prevent additional DNA damage. Slides were stained with ethidium bromide (20 µg/ml) and analyzed using a fluorescence microscope (Axioskop HBO 50, Zeiss). The percentage of tail intensity was used to estimate DNA damage. A total of a hundred randomly selected cells per treatment (two gels per slides) were analyzed using the Comet Assay IV software (Perceptive Instruments, Instem). Two independent experiments were performed for each experimental condition.

Genotoxic effect (direct DNA damage) was evaluated comparing cells exposed to NPs with control cells (DMSO 1%). The formamidopyrimidine glycosylase (Fpg)-modified Comet assay was performed for DNA damage evaluation (direct + indirect DNA damage) as reported in Bonetta et al. (2009) with slight modification (Gea et al., 2018). The test was carried out as described for the alkaline Comet assay but, after lysis, the slides were washed with Fpg Buffer (5 min for three times) (40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA disodium salt dihydrate, 0.2 mg/ml bovine serum albumin, pH 8). Then, each gel was incubated with 1 unit of Fpg enzyme (*Escherichia coli*) (Trevigen) at 37 °C for 30 min. Procedure control slides were incubated with buffer only. Cells treated with DMSO (1%) and enzyme were used as negative controls. Two independent experiments were performed for each experimental condition. The DNA damage (direct + indirect DNA damage) was evaluated comparing cells exposed to NPs with control cells (DMSO 1% + Fpg). The oxidative damage was calculated subtracting the mean tail intensity (%) in enzyme-treated cells (+Fpg) from the relative mean tail intensity (%) in enzyme-untreated cells (-Fpg).

2.8. 3D confocal micro-Raman imaging spectroscopy

Raman grade Calcium fluoride (CaF_2) windows (Crystran) were employed as alternative substrate instead of standard plastic substrates for cells growing due to the low toxicity and almost absent background signals (Kann et al., 2015). The BEAS-2B cells were cultured overnight

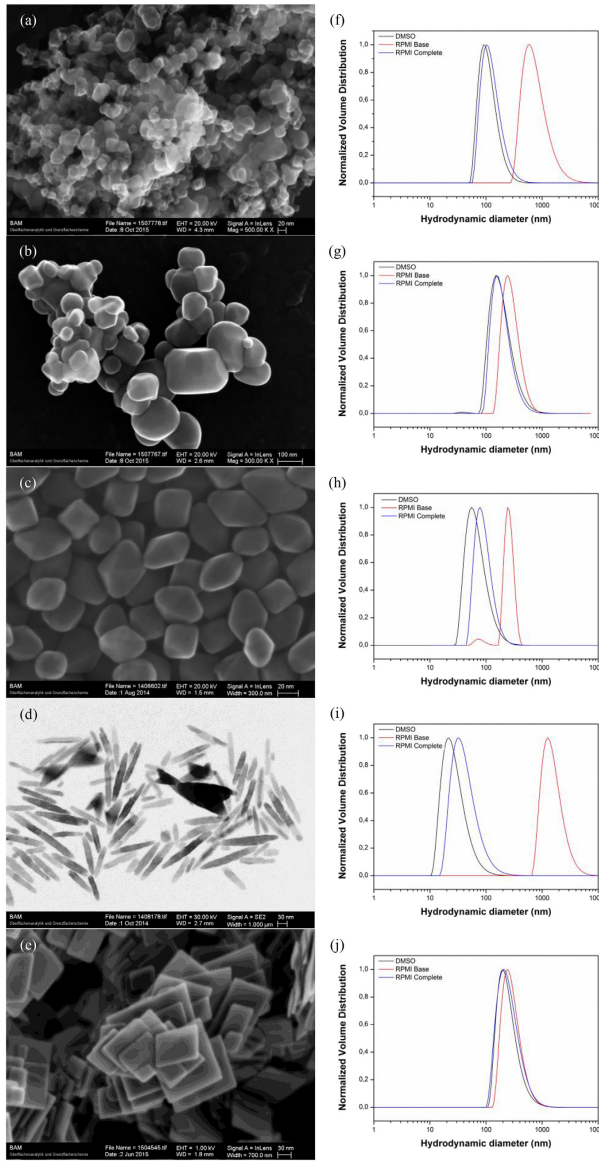

Fig. 1. SEM In-lens micrographs: (a) P25, (b) food grade, (c) bipyramids; (e) platelet NPs. T-SEM micrograph of rods (d). DLS analyses, normalized by volume distribution (f–j): (f) P25, (g) food grade, (h) bipyramids, (i) rods and (j) platelet NPs, suspensions in DMSO 1% (black line), base RPMI (red line) and complete RPMI (blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in 6-well plates on a CaF_2 substrate (3×10^5 cells/well) before exposure to NPs. Cells were treated with NPs (80 μg/ml, 24 h). After exposure, cells were washed twice with PBS and fixed with 3 ml of methanol. CaF_2 substrates were dried and stained with Giemsa dye (4% Giemsa's azur eosin methylene blue solution, 4% Sorensen buffer 0.067 M pH 6.8, 8 min at room temperature); then the substrates were washed twice with distilled water and dried. Giemsa staining is one of the standard procedures in histology, useful to evidence morphological cells features, such as cell nuclei, which appear in various shades of red/purple, and the cytoplasm, which appears blue.

3D confocal micro-Raman imaging spectroscopy of BEAS-2B cells was conducted with a DXR™xi Raman Imaging Microscope (Thermo Scientific) using a laser wavelength at 532 nm, a 1 mW laser power, a 100X microscope objective and a motorized stage with a 1 μm of step size and a 1 μm offset. Spectra were collected in the 50–3500 cm^{-1} spectral region with a grating resolution of 5 cm^{-1}, an exposure time of 0.025 s and 5 scans in total. 3D Raman images were reconstructed taking the Raman peaks at 1600 cm^{-1} of methylene blue and the E_g band at 144 cm^{-1} of the TiO_2-NPs, respectively. Each cell was investigated at different focal planes and a chemical image was obtained by the combination of the ν(C-C)ring at 1600 cm^{-1} of the methylene blue and the Eg band at 144 cm^{-1} of the TiO_2-NPs. Since methylene blue is contained in the Giemsa stain and it is widely distributed into the fixed cells, its signals were considered representative of the entire volume of the cells. As far as the tracking of the NPs are concerned, the Eg band at 143 cm^{-1} is the most intense signal in the molecular fingerprint of the anatase TiO_2 and the region between 50 cm^{-1} and 400 cm^{-1} in the Raman spectrum is usually free of the vibrational bands of biological species. Therefore, this signal was selected to sensitively locate the TiO_2-NPs inside the cells. Image J software was used in the development of the 3D chemical images both for cells and TiO_2-NPs, which were superimposed using a Solidworks® 2016 Cad based software. 3D Raman chemical images are presented using a color meshwork i.e. blue for cell tissues and red for TiO_2 agglomerates.

2.9. Statistical analysis

IBM SPSS software (ver. 24.0) was used to perform statistical analysis. The results of WST-1, LDH and Comet assays are presented as the mean ± standard deviation. Differences between exposed and control cells were tested by ANOVA followed by the post hoc Dunnett's test. Differences between light and dark exposure were tested by ANOVA, followed by the post hoc Tukey's test. Data were considered statistically different for a p-value less than 0.05.

3. Results

3.1. Raman characterization of NPs and size distribution

In order to establish a relationship among the physico-chemical features of NPs and their ability to induce a toxic effect, well-defined and controlled protocols were developed for the production of engineered anatase TiO_2-NPs with different shapes. All the NPs produced in this study were first characterized with a SEM equipped with a transmission-unit for T-SEM, which provided information both on the shape and the size of the constituent NPs (Fig. 1a–e). Fig. 1 and Table 1 show shapes and particle size of commercial TiO_2-NPs and fabricated engineered TiO_2-NPs.

These NPs were also characterized by Dynamic Light Scattering (DLS) as a quick method for sizing and determining the state of NP agglomeration. For each kind of sample, the agglomeration in 1% DMSO aqueous solution, in base RPMI [supplemented with l-glutamine (4 mM) and penicillin-streptomycin (100 U/ml - 100 μg/ml)] and complete RPMI [supplemented with FBS (10% v/v), l-glutamine (4 mM) and penicillin-streptomycin (100 U/ml - 100 μg/ml)] (Fig. 1f–j) were compared. In all the TiO_2 materials considered for this study, the agglomeration state increase in base RPMI, while the size distribution in DMSO and in complete RPMI is quite similar.

The crystalline composition of the TiO_2-NPs, analyzed by Raman spectroscopy, showed a typical fingerprint of the anatase TiO_2 (Fig. S1) with the characteristic phonon bands E_g at 143 cm^{-1}, E_g at 197 cm^{-1}, A_{1g} at 397 cm^{-1}, B_{1g} at 515 cm^{-1} and E_g at 639 cm^{-1} for all the investigated NPs. Since P25 is a known mixture of anatase and rutile (5:1), with also a small amount of amorphous TiO_2 (Ohtani et al., 2010), its Raman spectrum still retains all the typical anatase Raman bands but it also contains two small shoulders at 450 cm^{-1} and 600 cm^{-1}, which were assigned to the E_g and A_{1g} phonon bands, respectively, of rutile (Tompsett et al., 1995). All the physiochemical properties of the TiO_2-NPs under study such as shape, particle size, hydrodynamic diameter in different liquid media and the crystalline phase are summarized in Table 1.

3.2. Cytotoxicity

The results of the effects of different TiO_2-NPs concentration on cell viability (WST-1 assay) are reported in Fig. 2a (exposure with light) and in Fig. 2b (exposure in darkness).

In general, a low cytotoxic effect was observed at the tested doses both in the exposure with light and in the exposure in darkness. The observed viability ranged from 102.8 to 88.4% for the exposure with light and from 99.6 to 87.4% for the exposure in darkness.

Considering the exposure with light, the commercial P25 induced a slight decrease in viability starting from the doses of 50 μg/ml ($p < 0.05$) while no cytotoxic effects were observed for the other commercial NPs (food grade) at the tested concentrations. As far as engineered NPs are concerned, bipyramids and platelet NPs induced the same cytotoxic effect of commercial P25 NPs; on the contrary, rods is the shape with higher cytotoxic effect showing a viability decrease already starting from 10 μg/ml ($p < 0.05$ or $p < 0.001$).

Considering the exposure in darkness, a lower cytotoxic effect was observed for commercial P25 NPs with respect to light exposure because a slight decrease in viability was observed for P25 NPs only at the highest dose (80 μg/ml) ($p < 0.05$). As reported after exposure with light, no cytotoxic effect was observed for the other commercial NPs (food grade). About engineered NPs, the exposure in darkness did not modify the cytotoxic effect of bipyramids resulting in a viability reduction starting from the dose of 50 μg/ml ($p < 0.001$) as reported in the experiment with light. In contrast, in the darkness, rods showed a lower cytotoxic effect than observed with light because a slight decrease in viability was observed for rods only starting from the dose of

Table 1
Physico-chemical properties of the TiO_2-NPs samples. Data are presented as mean ± standard deviation of 500 NPs for the particle size and 5 measurements for the hydrodynamic diameter (D_h) of each sample. *The particle size was calculated along the major axis of the NPs.

Sample	Particle size (nm)	D_h DMSO (nm)	D_h base RPMI (nm)	D_h complete RPMI (nm)	Crystalline phase
P25	20 ± 5 quasi-spherical	107 ± 31	722 ± 246	121 ± 37	anatase:rutile (5:1)
Food grade	150 ± 50 undefined shape	184 ± 61	278 ± 54	184 ± 55	anatase
Bipyramids	50 ± 9* (aspect ratio 3:2)	66 ± 20	259 ± 46	88 ± 24	anatase
Rods	108 ± 47* (aspect ratio 1:5)	36 ± 12	1500 ± 471	39 ± 17	anatase
Platelets	75 ± 25* (aspect ratio 8:1)	233 ± 70	281 ± 83	250 ± 82	anatase

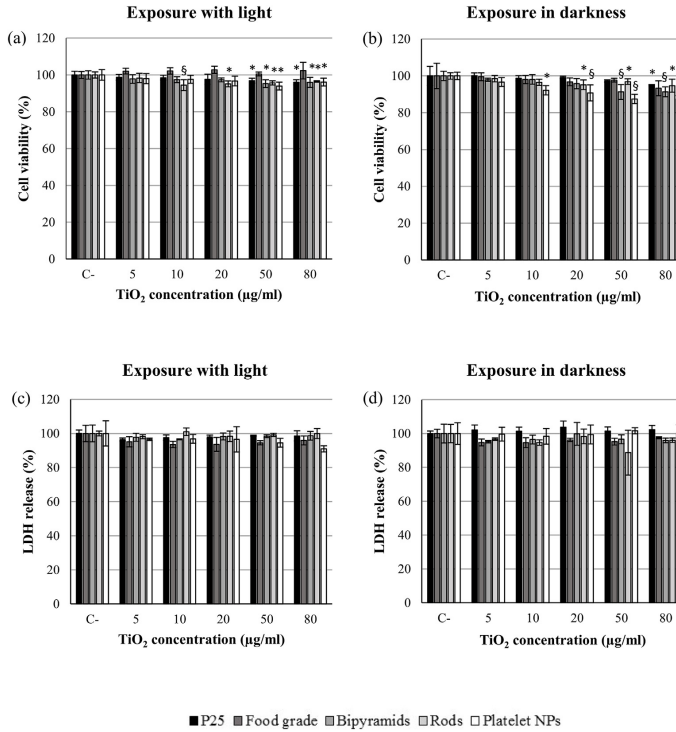

Fig. 2. Cytotoxicity measured with WST-1 (a,b) and LDH release (c,d) of BEAS-2B cells exposed to different concentrations (5–80 μg/ml) of commercial and engineered NPs. Control level is at 100%. Data represent effects detected after exposure with laboratory light (a,c) and in darkness (b,d). Data represent the mean % of the different wells, bars represent standard deviation. * = $p < 0.05$, § = $p < 0.001$ *vs* control cells (C-) according to ANOVA test, followed by the post hoc Dunnett's test.

20 μg/ml ($p < 0.05$). As during the exposure with light, platelet NPs induced a decrease in viability; the cytotoxic effect was significant starting from a less dose (10 μg/ml, $p < 0.05$) than in the experiment with light (50 μg/ml).

The results of the effects of different TiO_2-NPs concentration on LDH release has been reported in Fig. 2c (exposure with light) and in Fig. 2d (exposure in darkness).

No significant LDH release was detected using LDH assay in both exposure protocols (with light or in darkness), confirming the low cytotoxic effect evidenced by WST-1 assay.

3.3. Genotoxicity

The results of genotoxic effect and oxidative DNA damage induced by different concentration of NPs are reported in Fig. 3.

Considering the exposure with laboratory light, no genotoxic effect was showed in enzyme untreated cells (direct DNA damage) for commercial P25 NPs (Fig. 3a). On the other hand, a dose-dependent increase of DNA damage was observed for these NPs in enzyme treated cells (direct and indirect DNA damage) respect to the control cells ($p < 0.05$ or $p < 0.001$), with the exception of the last dose (160 μg/ml) that induced a DNA damage equal to 80 μg/ml. A significant oxidative damage was observed for P25 NPs starting from 50 μg/ml ($p < 0.05$ or $p < 0.001$). The results obtained with the other commercial NPs (food grade) (Fig. 3b) showed the presence of a significant dose-response DNA damage both in enzyme untreated cells and in enzyme treated cells starting from 50 μg/ml. Moreover, the difference between the two effects resulted significant starting from 50 μg/ml ($p < 0.05$ or $p < 0.001$) highlighting an oxidative damage induced by food grade NPs.

Respect to commercial NPs, engineered NPs showed a lower extent of DNA damage. In particular, neither genotoxic effect nor oxidative damage were observed for engineered bipyramids and rods NPs (Fig. 3c and d). Platelet NPs induced a significant DNA damage respect to the control cells ($p < 0.05$ or $p < 0.001$) both in enzyme untreated cells and in enzyme treated cells and they induced a significant oxidative DNA damage starting from 80 μg/ml ($p < 0.001$) (Fig. 3e). However, in contrast with commercial NPs (food grade), a dose-response of the effects were not observed.

As demonstrated by other authors (Karlsson, 2010; Karlsson et al., 2015), an interference during the scoring of the assay was detected in particular at the higher doses of P25 and platelet NPs, indeed nanoparticles with some autofluorescence were visible in the comets "head" and the stained DNA appeared faded. The interference probably caused

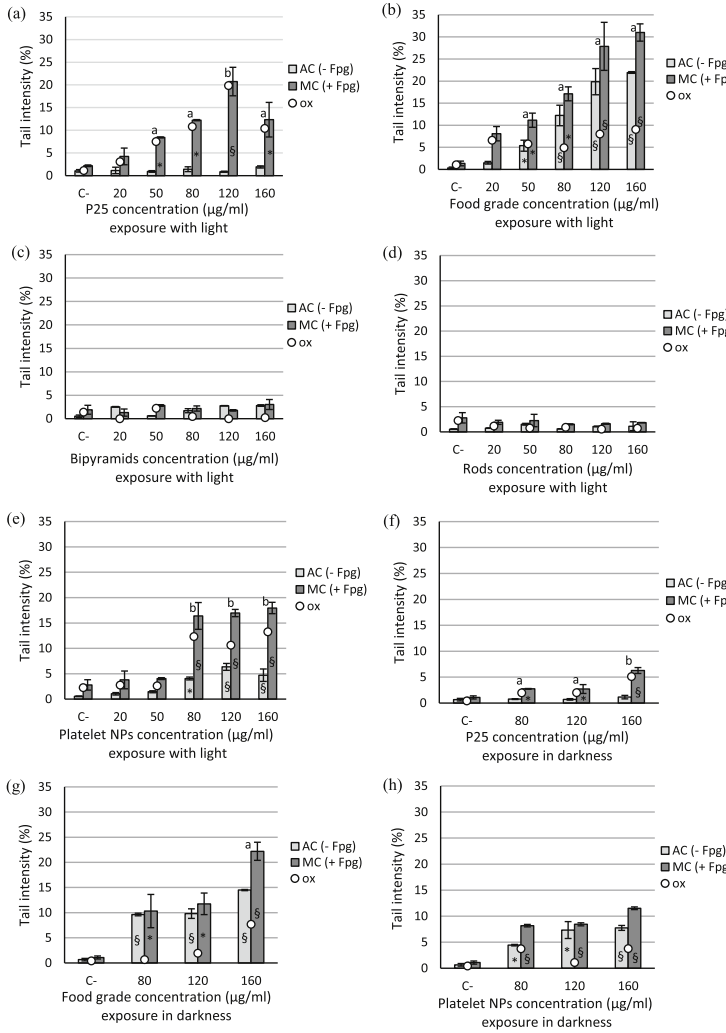

Fig. 3. Effect of BEAS-2B cells exposure to commercial and engineered NPs. AC (-Fpg) = alkaline Comet assay (direct DNA damage); MC (+Fpg) = Fpg-modified Comet assay (direct + indirect DNA damage). Ox = oxidative DNA damage (tail intensity (%) in enzyme-treated cells - tail intensity (%) in enzyme-untreated cells). Exposure with laboratory light (a–e): (a) P25, (b) food grade, (c) bipyramids, (d) rods, (e) platelet NPs; exposure in darkness (f–h): (f) P25, (g) food grade, (h) platelet NPs. Data represent the mean % of tail intensity; bars represent standard deviation of two independent experiments for each experimental condition. * = $p < 0.05$, § = $p < 0.001$ DNA damage *vs* control cells (C-). a = $p < 0.05$, b = $p < 0.001$ oxidative DNA damage *vs* control cells (ox C-). According to ANOVA test, followed by the post hoc Dunnett's test.

the loss of concentration-dependent increase in DNA direct and oxidative damage observed for the higher doses. The phenomenon could be explained also considering that base oxidation is hard to measure accurately when there are a lot of strand breaks, because the Comet assay becomes saturated (Collins et al., 2017).

In order to evaluate the role of the light on the genotoxic and oxidative damage induced by commercial and engineered NPs, the highest doses (80, 120, 160 μg/ml) of NPs that showed a genotoxic effect (P25, food grade and platelet NPs) were tested in darkness (24 h).

Considering the exposure in darkness, no genotoxic effect was

observed for commercial P25 NPs in enzyme untreated cells (direct DNA damage) (Fig. 3f) as reported in the experiment with light (Fig. 3a). However, in the enzyme treated cells a dose-response DNA damage (direct and indirect DNA damage) was observed with respect to control cells ($p < 0.05$ or $p < 0.001$), but oxidative DNA damage was lower than in the experiment with light ($p < 0.05$ or $p < 0.001$). The commercial food grade NPs induced a significant dose-response DNA damage both in enzyme untreated cells and in enzyme treated cells ($p < 0.001$ and $p < 0.05$ respectively) (Fig. 3g). However, the DNA damage resulted in both cases lower than in the experiment with light ($p < 0.05$ or $p < 0.001$) and an oxidative damage was induced only at the highest dose (160 μg/ml) ($p < 0.05$).

With regard to engineered NPs, platelet NPs induced a significant DNA damage with respect to the control cells ($p < 0.05$ or $p < 0.001$)

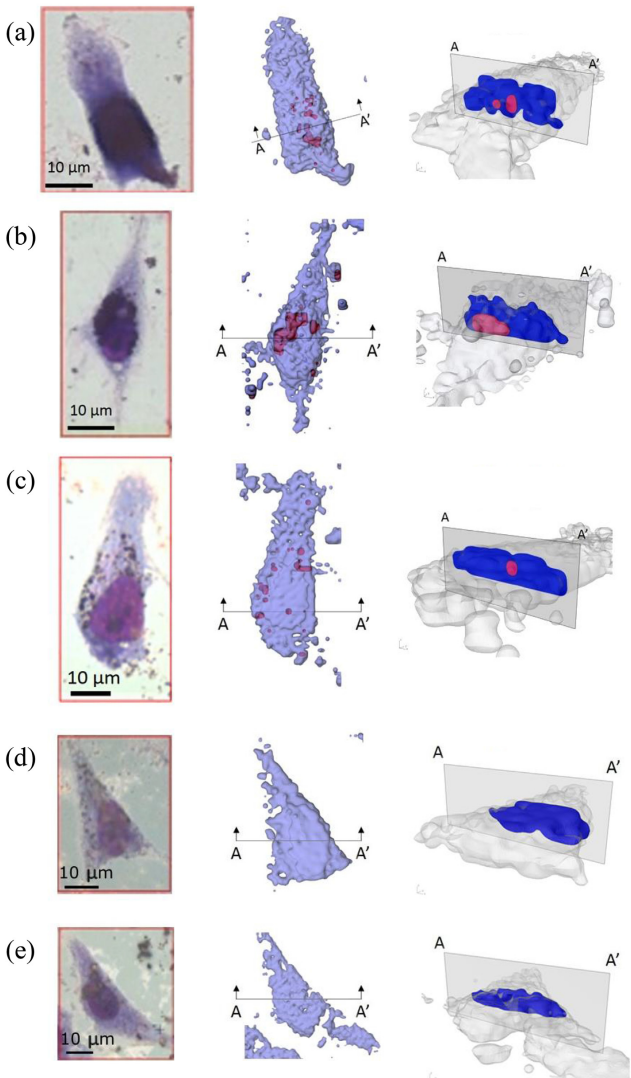

Fig. 4. 3D confocal micro-Raman imaging of BEAS-2B cells after exposure to commercial and engineered NPs. Top views (optical and 3D Raman) and 3D Raman sections are shown from the left to the right: (a) P25, (b) food grade, (c) platelet NPs, (d) bipyramids, (e) rods. 3D chemical images are built by superimposing the different maps of each cell at their corresponding focal planes and they are presented using a color meshwork i.e. blue for cell tissues (methylene blue ν(C-C)ring at 1600 cm^{-1}) and red for TiO_2 agglomerates (Eg band at 144 cm^{-1} of the anatase TiO_2). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

both in enzyme untreated cells and in enzyme treated cells (Fig. 3h). However, while the DNA damage in enzyme untreated cells was equivalent to the DNA damage induced in the experiment with light (Fig. 3e), a decrease of DNA damage in enzyme treated cells was observed, resulting in no oxidative damage induced by platelet NPs in darkness (Fig. 3h).

3.4. Confocal micro-Raman spectroscopy

The confocal micro-Raman imaging spectroscopy was used in order to evaluate qualitatively the presence/absence of different types of TiO_2-NPs inside the cells. 3D chemical images are built by superimposing the different maps of each cell at their corresponding focal planes and they are presented using a color meshwork i.e. blue for cell tissues and red for TiO_2 agglomerates. At least five cells were analyzed to provide statistically significant results. As the sections of Fig. 4 show, the uptake of the TiO_2-NPs by the cells was mainly demonstrated for P25, food grade and platelet NPs (Fig. 4a,b,c) while no TiO_2 signal was registered inside the cells for bypiramids and rods (Fig. 4d and e).

4. Discussion

Many *in vitro* studies have been conducted to investigate cytotoxicity/genotoxicity of TiO_2-NPs but the results are often conflicting and employed doses were sometimes high (Valant et al., 2012; Chen et al., 2014; Magdolenova et al., 2014; Karlsson et al., 2015; Moller et al., 2015a). The aim of this study was to investigate the cytotoxicity and genotoxicity of three different shapes of TiO_2-NPs and to compare them with two commercial TiO_2-NPs.

The issues taken into account for this study were: i) the physicochemical properties of the particles (shape, particle size, agglomeration state in culture media, crystalline phase) that can influence biological effects, ii) the ability of the particles to induce cytotoxicity and genotoxicity, iii) the increase of the toxicological effects under light exposure due to the photocatalytic activity of TiO_2 and iv) the uptake of the NPs by human cells.

In the present study, the cytotoxicity assays were selected in order to reduce the interference of NPs with the assays (interference with optical detection methods, ability to convert the substrates). As suggested by other studies (Wilhelmi et al., 2012; Guadagnini et al., 2015: Popescu et al., 2015), the assays processes were optimized for evaluation of NPs; moreover, before the assessment of NPs cytotoxicity, relevant controls were conducted, in order to assess particles for their ability to interfere with the assays (data not shown).

For genotoxicity evaluation, a literature revision (on application of genotoxicity assays testing NPs) was made before the application of Comet assay (Karlsson, 2010; Magdolenova et al., 2012a; Karlsson et al., 2015; Cowie et al., 2015; Moller et al., 2015b; Huk et al., 2015). To ensure the correct evaluation of DNA damage two independent experiment were performed for each experimental condition. The analysis of each comet was made using the Comet Assay IV software and the automatic evaluation was carefully checked by an operator.

Published results on toxicity of TiO_2-NPs show high variability. Reasons for this variability include physico-chemical characteristics of NPs, different methods to prepare NPs dispersions, differences in NPs size and dispersion stability, and different exposure protocols (Charles et al., 2018). The characteristics of NPs dispersion can be influenced by medium components, such as serum proteins, and by NPs properties (i.e. size, shape, surface charge, surface coating) (Huk et al., 2015). According to the study of Prasad et al. (2013), the present results showed that in all the TiO_2-NPs dispersions, the agglomeration state increases in base RPMI (without serum), while the size distribution in DMSO and in complete RPMI medium (with serum) is quite similar. The different agglomeration state is probably due to the ability of metal oxide NPs to adsorb proteins onto their surface, forming a "protein corona" which favors less agglomeration in complete medium, which contains more proteins (Prasad et al., 2013). Considering the results obtained, complete medium was selected as cytotoxicity/genotoxicity assay medium.

The viability of BEAS-2B treated with commercial and engineered TiO_2-NPs after exposure with light or in darkness was assessed using the WST-1 assay.

Commercial TiO_2-NPs induced low (P25) or no viability reduction (food grade) detected by WST-1 assay; these results are in agreement with some reports on commercial TiO_2-NPs (Bhattacharya et al., 2009; Falck et al., 2009). Previous studies that investigated the cytotoxicity of commercial P25 on BEAS-2B showed that only 100 μg/ml of commercial P25 NPs produced a viability decrease after 24 h exposure (Prasad et al., 2013). Fewer studies have been performed using commercial food grade TiO_2-NPs. Proquin et al. (2017) tested these NPs on different cell lines: on Caco-2, they observed cytotoxicity, while on HCT116 they did not observe any cytotoxic effect up to the concentration of 100 μg/cm^2. The result obtained on HCT116 was in accordance with the low cytotoxic effect induced by commercial food grade TiO_2-NPs detected in the present study. Recently, the scientific community have produced reference NPs, which have been well characterized. Di Bucchianico et al. (2016) assessed cytotoxic effects of some of these NPs (anatase 50–150 nm, anatase 5–8 nm, rutile 20–28 nm) in BEAS-2B cells and, according to the present results, showed in general no or low effects at the tested doses (2–100 μg/ml).

On the contrary, other studies showed that commercial TiO_2-NPs induced higher cytotoxicity on BEAS-2B (Shi et al., 2010; Ursini et al., 2014). In particular, Park et al. (2008) found that exposure of BEAS-2B cells to commercial P25 (5–40 μg/ml) for 24 h led to significant cell death, both in a time- and concentration-dependent manner.

The data of present study demonstrated that cytotoxicity was slightly affected by light exposure, which induced an increase of cellular damage after incubation with commercial P25 and engineered rods. The influence of light exposure on citotoxicity was also observed in other studies (Vevers and Jha, 2008; Reeves et al., 2008). Differently from P25 and rods, exposure to platelet NPs induced higher cytotoxicity in darkness than after light exposure; the mechanism that led to this result is not clear.

Comparing the results of cytotoxicity (WST-1 assay) and LDH release, the first showed low cytotoxic effect at the doses tested, while the second did not show any cytotoxicity in both exposure protocols. The discrepancy between cytotoxicity (WST-1) and LDH release suggests that the viability reduction may be caused by apoptosis, a cell death pathway in which the plasma membrane is maintained, as observed in other studies (Schilirò et al., 2015). This is in accordance with previous studies, which demonstrated that TiO_2-NPs could cause apoptosis in BEAS-2B cells (Park et al., 2008; Shi et al., 2010). The observed discrepancy could be also explained considering that the tested compounds (TiO_2-NPs) could induce an effect on the intracellular activity (mitochondrial activity) without causing plasma membrane breakage, as observed by other authors (Weyermann et al., 2005; Fotakis and Timbrell, 2006).

Results of Comet assay in presence of light and in darkness showed a significant DNA damage induced by commercial P25 and food grade NPs and engineered platelet NPs, while no genotoxicity was observed with the other engineered NPs (bipyramids and rods).

Considering that the uptake of NPs could involve interactions of NPs with DNA, the observed genotoxic effect could be related to the presence of P25, food grade and platelet NPs into the BEAS-2B as observed by other authors (Bhattacharya et al., 2009; Park et al., 2008).

In the present study, the higher uptake of P25, food grade and platelet NPs seemed to be related with higher agglomeration tendency (higher measure of hydrodynamic diameter) (Table 1). In particular, the engineered platelet NPs were the most agglomerated (platelet shape could probably promote more agglomeration than the other shapes) and commercial P25 and food grade were more agglomerated than the other engineered NPs (bipyramids and rods). The variation in cellular

uptake could be due to agglomeration tendency because NPs that form large agglomerates, differently from NPs that form smaller ones, precipitate at the bottom of the cell culture wells, increasing the real amount of NPs to which cells are exposed (Magdolenova et al., 2012b). Cells exposed to more NPs could probably internalize more NPs.

Then, in the present study, the agglomeration tendency does not seem to have prevented the uptake of NPs in the cells, in accordance with the study of Ahlinder et al. (2013).

The major uptake of P25, food grade and platelet NPs could be related with higher genotoxic effect considering that, after penetration into the cells, NPs may have direct access to DNA via transport into the nucleus and/or during mitosis when the dissolution of nuclear membrane occurs. NPs interacting directly with DNA could cause DNA breakage (Magdolenova et al., 2014). Moreover, NPs, after penetration into the cells, can enhance the permeability of the lysosomal membrane, inducing the release of DNases and so causing genotoxic effects (Karlsson, 2010). Finally, accumulation of NPs within cells can cause aggregates of NPs that deform nucleus inducing DNA damage (Di Virgilio et al., 2010).

In order to quantify effects due to the photocatalytic activity of TiO_2, the highest doses (80, 120, 160 μg/ml) of NPs that showed a genotoxic effect were tested also in darkness (24 h). Results obtained in this study showed that light exposure induced additional indirect genotoxicity, demonstrating a higher oxidative potential of TiO_2-NPs after exposure with light. The presence of light increased DNA oxidative damage probably due to the photocatalytic activity of TiO_2-NPs, which caused an increase of NPs ability to produce radicals. In particular, based on previous studies, the anatase crystal structure of TiO_2 (the same used in the present study) seems to be the most catalytic/photocatalytic crystalline structure of TiO_2 and seems to be activated under both ultraviolet and visible light (Warheit and Donner, 2015). A recent study (De Matteis et al., 2016) demonstrated that, in particular using anatase, light is a dominant factor to induce oxidative stress and toxic effects. Also Gerloff et al. (2009) showed the increase of oxidative genotoxic effects induced by TiO_2-NPs (80%/20% anatase-rutile) in the presence of interior light.

However, an oxidative damage (although low) was observed in the present study also in darkness as reported in the study of Gurr et al. (2005) that demonstrated that in darkness TiO_2-NPs can induce oxidative DNA damage. On the contrary, Karlsson et al. (2008) and Gerloff et al. (2009) found that TiO_2-NPs (mixture of rutile and anatase) in darkness did not show oxidative DNA damage using the Fpg-modified Comet assay.

Moreover, the results obtained in this study highlight that only food grade and platelet NPs induced direct genotoxicity. However, while for food grade NPs the direct genotoxic effect remains the same both after exposure with light and in the darkness, for the commercial food grade NPs, the direct damage was higher in presence of light than in darkness. This result agree with the study of Gopalan et al. (2009); they suggest that TiO_2 (anatase 40–70 nm range) is capable of inducing higher direct genotoxic effects after simultaneous irradiation with UV, respect to genotoxicity induced in darkness. The increase of direct DNA damage after exposure with light attested by Gopalan et al. (2009) and detected for food grade NPs, remain to be explained. A possible mechanism that may lead to this effect could be related to the potential interaction of TiO_2-NPs with proteins involved in DNA repair, as demonstrated by Jugan et al. (2011). Genotoxicity is not only linked to the level of DNA damage but also to the type of lesions generated and their capacity to be repaired. NPs exposure in presence of light could influence activity of proteins such as repair enzymes, resulting in DNA damage not repaired or misrepaired (Magdolenova et al., 2014). Then, the exposure with light may have caused inactivation of repair enzymes, inducing a higher direct genotoxic effect induced by food grade NPs after exposure with light respect to exposure in darkness.

In conclusion, the results of this study showed that the cytotoxicity was overall low (WST-1 assay) and was influenced by the NP shape as well as by light exposure. According to the low cytotoxic effect, no LDH release was detected using the LDH assay.

Instead, genotoxicity seemed to be influenced by the cellular-uptake and the aggregation tendency of TiO_2-NPs. These two aspects are probably related to different physico-chemical characteristics of NPs, such as the shape. Moreover, the presence of light enhanced the genotoxic effect of some NPs primarily increasing the oxidative stress.

Although more studies have to be performed in order to assess the potential toxicity of engineered NPs, the results of this preliminary study showed that engineered NPs did not induced a high cytotoxic/genotoxic effect compared to the other commercial TiO_2-NPs, so they could be used for future technological applications. The results of this study are important considering that engineered NPs, due to their peculiar characteristics, could support and improve TiO_2-NPs applications in different areas such as energy (i.e. use of engineered TiO_2-NPs in dye-sensitized solar cells), environment (i.e. application of engineered TiO_2-NPs as photocatalyst for the abatement of air and water pollutants) and health (i.e. use of engineered TiO_2-NPs for the production of nanostructured coatings of orthopedic and dental prostheses exhibiting optimized interfacial properties).

Conflicts of interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.02.043.

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Supporting Information

Shape-engineered titanium dioxide nanoparticles (TiO_2-NPs): cytotoxicity and genotoxicity in bronchial epithelial cells

Marta Gea[a], Sara Bonetta[a*], Luca Iannarelli[b] , Andrea Mario Giovannozzi[b], Valter Maurino[c], Silvia Bonetta[a], Vasile-Dan Hodoroaba[d], Caterina Armato[a,e], Andrea Mario Rossi[b], Tiziana Schilirò[a]

[a] Department of Public Health and Pediatrics, University of Turin, Piazza Polonia 94, 10126 Turin, Italy;

[b]Quality of Life Division, Istituto Nazionale di Ricerca Metrologica, Strada delle Cacce 91, 10135 Turin, Italy;

[c]Department of Chemistry, University of Turin, Via Giuria 7, 10125 Turin, Italy;

[d]Surface Analysis and Interfacial Chemistry division, Federal Institute for Materials Research & Testing (BAM), 12200 Berlin, Germany;

[e]Centre for Sustainable Future Technologies (CSFT@PoliTo), Istituto Italiano di Tecnologia, Corso Trento 21, 10129 Turin, Italy;

*__Corresponding author__:

Sara Bonetta

Department of Public Health and Pediatrics,

University of Torino,

Piazza Polonia 94, 10126 Turin, Italy,

Tel: +390116708192

e-mail address: sara.bonetta@unito.it

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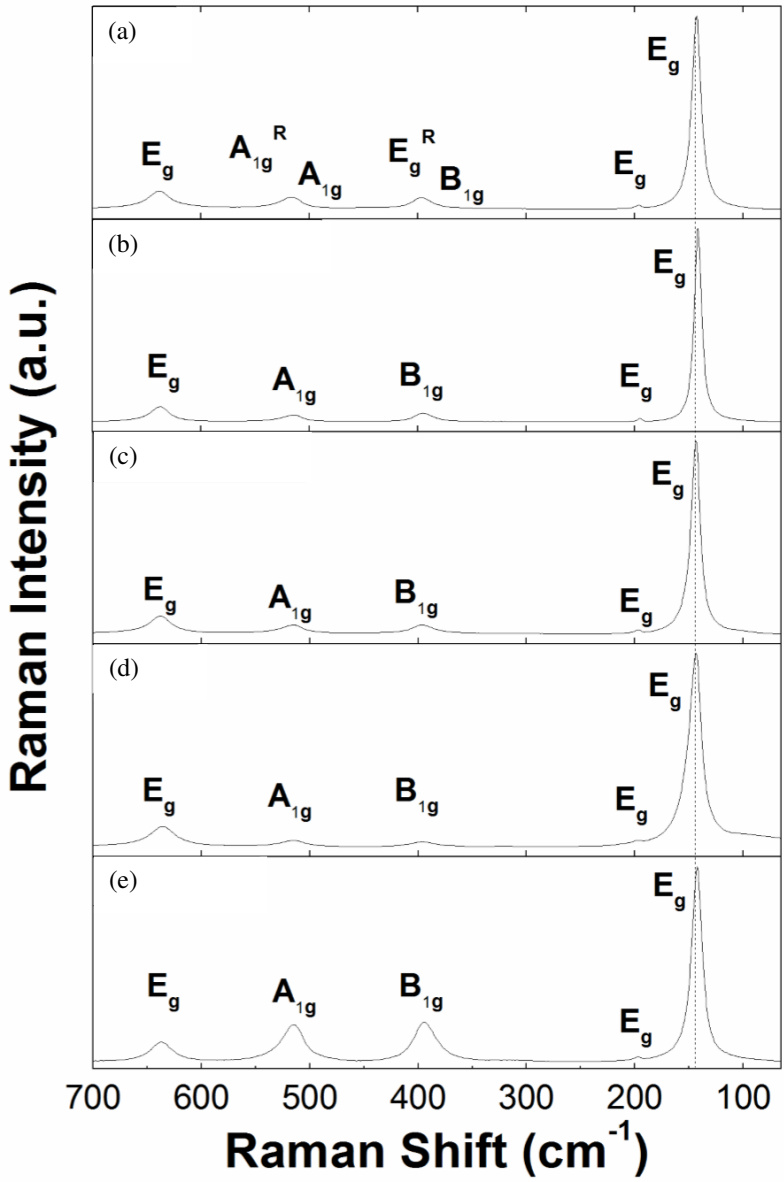
S.1: Supplemental Results

Figure S.1. Raman spectra and related peak assignment of P25 (a), food grade (b), bipyramids (c), rods (d) and platelets NPs (e).



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Article

Molecular Basis for Endocrine Disruption by Pesticides Targeting Aromatase and Estrogen Receptor

Chao Zhang [1], Tiziana Schilirò [2], Marta Gea [2], Silvia Bianchi [1], Angelo Spinello [3], Alessandra Magistrato [3], Gianfranco Gilardi [1] and Giovanna Di Nardo [1,*]

[1] Department of Life Sciences and Systems Biology, University of Torino, 10123 Torino, Italy; chao.zhang@unito.it (C.Z.); silvia.bianchi.04@hotmail.it (S.B.); gianfranco.gilardi@unito.it (G.G.)
[2] Department of Public Health and Pediatrics, University of Torino, 10126 Torino, Italy; tiziana.schiliro@unito.it (T.S.); marta.gea@unito.it (M.G.)
[3] National Research Council-Institute of Materials (CNR-IOM) at International School for Advanced Studies (SISSA), 34165 Trieste, Italy; angelo.spinello@sissa.it (A.S.); alessandra.magistrato@sissa.it (A.M.)
* Correspondence: giovanna.dinardo@unito.it; Tel.: +390-116-704-689

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Abstract: The intensive use of pesticides has led to their increasing presence in water, soil, and agricultural products. Mounting evidence indicates that some pesticides may be endocrine disrupting chemicals (EDCs), being therefore harmful for the human health and the environment. In this study, three pesticides, glyphosate, thiacloprid, and imidacloprid, were tested for their ability to interfere with estrogen biosynthesis and/or signaling, to evaluate their potential action as EDCs. Among the tested compounds, only glyphosate inhibited aromatase activity (up to 30%) via a non-competitive inhibition or a mixed inhibition mechanism depending on the concentration applied. Then, the ability of the three pesticides to induce an estrogenic activity was tested in MELN cells. When compared to 17β-estradiol, thiacloprid and imidacloprid induced an estrogenic activity at the highest concentrations tested with a relative potency of 5.4×10^{-10} and 3.7×10^{-9}, respectively. Molecular dynamics and docking simulations predicted the potential binding sites and the binding mode of the three pesticides on the structure of the two key targets, providing a rational for their mechanism as EDCs. The results demonstrate that the three pesticides are potential EDCs as glyphosate acts as an aromatase inhibitor, whereas imidacloprid and thiacloprid can interfere with estrogen induced signaling.

Keywords: aromatase; estrogen receptor; endocrine disrupting chemical; pesticides; neonicotinoids; estrogenic activity; gene reporter assay; MELN allosteric inhibition; molecular dynamics

1. Introduction

Under the modern lifestyle, humans are exposed to various chemicals such as pesticide residuals in fruits and vegetables, antibiotics in meat and milk, preservatives in cosmetics and personal care products [1,2]. These chemicals are usually in low doses and may not have a short term significant impact on the human body, but they can cause long term damages to health [3]. The effects of low-dose compounds on human health are mainly related to the endocrine system [4–6]. These compounds can in fact mimic or influence the action of endogenous hormones through various mechanisms, being therefore referred to as endocrine disrupting chemicals (EDCs) [7,8].

The modern industrial and agricultural system relies heavily on pesticides. The compelling need of high food crop increasingly demands the use of chemicals. This results in the extensive applications of millions of tons of pesticides every year [9,10]. Among the many pesticides available on the market, glyphosate is one of the most widely used herbicides in the world. In plants, it affects the synthesis

of essential aromatic amino acids by inhibiting the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway [11]. As a result, glyphosate is considered harmless to mammals since they do not contain the EPSPS enzyme [12]. Conversely, neonicotinoids are currently the most widely used agricultural insecticides [13]. These selective insecticides specifically bind to the α -subunit of nicotinic acetylcholine receptors (nAChR), which is common in all insects [14,15]. Due to their difficulty to penetrate the blood-brain barrier, they should exert low toxicity in vertebrates. However, different direct, indirect, and suspect toxic effects of these compounds on vertebrate wildlife and human health have been reported [16–18]. Neonicotinoids were introduced in the market in the 1990s, therefore their sales volume has enormously proliferated in recent decades, exceeding 25% of the market in 2010 [19]. Imidacloprid has become the world's best-selling insecticide next to glyphosate [20]. Although the European Union banned the outdoor application of imidacloprid, clothianidin, and thiamethoxam in 2018 due to a high risk for bees that are responsible for pollinating most crops worldwide [21], neonicotinoids remain the most extensively applied insecticides in the world [22].

The intensive use of glyphosate-based herbicides and neonicotinoid insecticides has caused the contamination of soil, water, air, and agricultural products [23–28]. The half-life of glyphosate in the field is usually 47 days and it is primarily degraded into aminomethylphosphonic acid (AMPA) and glyoxylic acid by soil microorganisms [29]. Neonicotinoids can exist for a long time in soil and water, and their half-life being as long as three years [22]. Given these observations and the increased use of pesticides, concerns are raising about their potential impact on human health and the environment. In particular, mounting evidence discloses that exposure to pesticides can affect the endocrine system. Toxicological and epidemiological studies indicate possible genotoxic and cytotoxic effects as well as birth defects and neurotoxicity in different cell lines and animal models [18,30].

Aiming at establishing the interference of pollutants with human health, we monitored the effect of glyphosate and two neonicotinoids on two critical targets of the endocrine system: Aromatase, the enzyme responsible for estrogen biosynthesis, and estrogen receptor (ER) alpha, the main protein promoting estrogen signaling.

Aromatase catalyzes the transformation of androgens to estrogens [31]. In vitro experiments showed that glyphosate causes changes of aromatase mRNA levels and activity in placental JEG3 cells and human HEK293 cells, thus interfering with steroid conversion to estradiol [32,33]. In addition, the expression of androgen and ERα was inhibited in glyphosate treated HepG2 cells [34,35] where also the transcription and activity of aromatase were altered [35]. Insecticides such as thiacloprid, thiamethoxam, and imidacloprid affect aromatase expression and activity in a co-culture model of fetoplacental steroidogenesis, increasing estrone and estradiol production, while estriol production is decreased [36]. Thiacloprid, imidacloprid, and thiamethoxam have also been demonstrated to increase aromatase expression and activity in H295R and Hs578t cells [37,38].

Here, a recombinant form of human aromatase is used to test the possible effect of the three pesticides on estrogen biosynthesis, gaining new information at molecular level [39–41]. Moreover, all-atom molecular dynamics simulations provided structural insights on the ability of these molecules to target the aromatase enzyme.

At a cellular level, the estrogen-regulated signaling is mainly due to estrogen receptors (ERs). The main ERs are the ERα and ERβ nuclear receptors and the G-protein-coupled estrogen receptor 1, which is a membrane receptor [42]. Estrogen receptor α (ERα) is a nuclear hormone receptor and a ligand-regulated transcription factor, which mediates the activity of estrogens in vital processes (i.e., reproduction, cardiovascular maintenance, bone density/remodeling). ERα is composed of five functional domains, among which the ligand-binding domain, activated upon estrogen binding, stimulates cell growth and proliferation. After the menopause, increased estrogen levels due to a deregulated activity of aromatase bind as an agonist to ERα, exerting a pro-oncogenic effect by either decreasing apoptosis or promoting cell proliferation [43]. Therefore, estrogen selective modulators

have been developed and one of them, tamoxifen, is extensively used in adjuvant therapy of breast cancer for its ability to act as an ERα antagonist.

Previous studies have investigated the ability of glyphosate to induce an estrogenic activity mediated by ERs. The results of the study of Thongprakaisang et al., 2013 [44] demonstrated that glyphosate induces an ER-mediated estrogenic activity, mediated by ER activation, similar to 17β-estradiol (E2) on T47D-KBluc cells. However, recently another study, using the same cells, showed that this pesticide induces an estrogenic activity, mediated by ER activation, lower than E2 and that this activation is probably induced by a ligand-independent mechanism [45]. Moreover, additional studies on different transfected cells showed that glyphosate did not induce any ER-mediated estrogenic activity and did not produce any anti-estrogenic effect when tested in combination with E2 [35,46]. Therefore, whether glyphosate can trigger an ER-mediated estrogenic activity remains controversial. To our knowledge, only three studies have been performed on the estrogenic activity of imidacloprid and thiacloprid. In particular, in the study of Mesnage et al., 2018 [47] the proliferative effect of both pesticides was investigated on estrogen-sensitive cells, while Kojima et al., 2004 [46] and Westlund and Yargeau, 2017 [48] assessed the ER-mediated estrogenic activity of imidacloprid and thiacloprid on mammalian or yeast cells, respectively. The results of the three studies demonstrated that the two pesticides induce no proliferative effect and no estrogenic activity was mediated by ERs, while an anti-estrogenic activity was detected testing imidacloprid in combination with E2 on yeast cells.

In this work, in order to increase the knowledge on the estrogenic activity of glyphosate, imidacloprid, and thiacloprid, the gene reporter assay on estrogen-sensitive human breast cancer MCF-7 cells transfected with the ERE-βGlob-Luc-SVNeo plasmid (MELN cells) is applied to test the three pesticides for their possible ER-mediated estrogenic activity.

Possible additive and/or antagonist effects are also investigated. Moreover, docking simulations provide atomic level insights on the potential binding mode of these molecules to the primary ligand (estrogen) binding site as well as to a peripheral allosteric site which may be responsible for the experimental observed additive effect of the pollutant with the endogenous ligand.

2. Materials and Methods

2.1. Materials

All reagents are analytically pure by purchase from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of chemical compounds were prepared in absolute ethanol or dimethyl sulfoxide (DMSO). Before each experiment, the test sample was diluted into a fresh buffer solution, and the final organic solvent concentration was less than 0.1%. The recombinant human aromatase (Aro) and the human recombinant cytochrome P450 reductase (hCPR) were expressed and purified as previously described [39,49].

2.2. ELISA Assay

An estrone direct competitive ELISA kit (BioVendor, Brno, Czech Republic) was used to evaluate the effect of pesticides on aromatase activity. Different reaction mixtures were set up by mixing 5 nM Aro, 5 nM hCPR, 0.5 mM NADPH, 50 nM androstenedione, and three concentrations of pesticides (500, 1000, and 1500 nM) in a 100 mM potassium buffer (KPi) containing 20% glycerol, 1 mM β-mercaptoethanol at pH 7.0. Reactions were carried out for 10 min at 30 °C, heat-inactivated for 10 min at 90 °C, and centrifuged for 10 min at 11,000 rpm. After centrifugation, the supernatant was diluted 1:8 in the Calibrator A provided by the ELISA kit and the product estrone quantified performing ELISA according to the manufacturer's instructions. Reactions in the presence of anastrozole or without hCPR were used as negative controls. The concentration of estrone was extrapolated from a calibration curve with known concentrations of estrone.

For the experiment where the catalytic parameters were derived, four substrate concentrations were applied in the reaction mixture (ranging from 25 to 250 nM) in the absence and presence of 1000 and 5000 nM of glyphosate.

2.3. Computational Studies

In order to explain the molecular terms for the action of glyphosate on the aromatase enzyme we docked it into the two possible allosteric sites previously identified [50]. Docking has been performed with the GLIDE software, release 2020-1 (Schrödinger, LLC, New York, NY, USA) using the single-precision protocol [51]. The two neonicotinoids, thiacloprid and imidacloprid, were instead docked into the ERαs active site, using as a starting structure the crystal structure of 17-β-estradiol (EST)-bound ERα dimer (PDB id: 1qku) [52]. In this structure, we have searched for putative allosteric pockets using the SiteMap algorithm [53].

In order to account for its flexibility of the receptor and since is flexibility resulted to be of paramount importance for the identification of novel allosteric inhibitors [54], we performed classical Molecular Dynamics (MD) simulations on the complex with the aromatase enzyme. We employed as a starting structure of our simulation the equilibrated enzyme model which was embedded in a mimic of a membrane bilayer by using the CHARMMGUI webserver [55]. This consisted of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and 6 wt% of cholesterol (CHL) in order to mimic the endoplasmic reticulum membrane. Physiological protonation states were calculated with the webserver H++ [56]. Asp309 was considered in its neutral form consistently with other literature studies [57]. The glyphosate molecule was considered in the most likely protonation state at physiological pH. According to literature data, the first protonation of the molecule occurs on its phosphate group [58].

The Parm99SB AMBER force field (FF) [59,60] and lipid14 FF [61] were used for the protein and the lipids, respectively.

The Shahrokh et al. parameters were used for the heme moiety and Cys437 [62]. Simulations were done in the presence of the substrate androstenedione (ASD) in the active site and of glyphosate in the allosteric pockets for which the general Amber FF (GAFF) was employed [63]. For the organic ligands the electrostatic potential (ESP) charges [64] were calculated by performing geometry optimization of the substrates at the Hartree-Fock level of theory using a 6-31G* basis set with the Gaussian 09 software (Gaussian Inc., Wallingford, CT, USA) [65]. These were later transformed in RESP charges by using the Antechamber tool [66].

The system was then explicitly solvated using the TIP3P water model, leading to a total of 131,454 atoms. Topology, built with AmberTools 18, was later converted in a GROMACS format using the acpype algorithm [67]. MD simulations were performed with GROMACS 5.0.4 [68]. An integration time step of 2 fs was used and all covalent bonds involving hydrogen atoms were constrained with the LINCS algorithm. The Particle Mesh Ewald algorithm [69] was used in order to account for electrostatic interactions. Simulations were done in the isothermal-isobaric NPT ensemble, at a temperature of 300 K, using a velocity-rescaling thermostat [70]. Preliminary energy minimization was done with the steepest descend algorithm.

An initial equilibration of the membrane was performed for 100 ns with the protein atoms harmonically restrained with a force constant of 1000 kJ mol^{-1} nm^{-2}, reaching a constant value ($92 \times 92 \times 151$ $Å^3$) of the simulation box size. Constraints were then slowly released, and the system was thermalized to the target temperature of 300 K in about 10 ns. Then, the aromatase in complex with glyphosate was relaxed by performing a 100 ns MD simulation rescaling the motion of the center of mass of aromatase and the ligand, followed by an unbiased 100 ns MD simulation.

2.4. MELN cell Culture

MELN cells were provided by Dr. P. Balaguer (INSERM, Montpellier, France). They are estrogen-sensitive human breast cancer cells (MCF-7) transfected with the ERE-βGlob-Luc-SVNeo plasmid (ERE-*β*Glob-Luc-SVNeo) [71,72]. The integrated plasmid contains a luciferase reporter gene, the estrogen-responsive elements (ERE) and an antibiotic resistance selection gene (SVNeo). MELN cells were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium Nutrient Mixture F12-Ham (DMEM-F12), supplemented with phenol red, fetal bovine serum (FBS) (5% v/v), L-glutamine (4 mM), penicillin-streptomycin (100 U/mL–100 µg/mL), and G418 (1 mg/mL).

2.5. MELN Gene Reporter Assay

The assay was carried out as described by Balaguer et al., 1999 [73] with slight modifications [74]. For three days the cells were adapted to a test medium: DMEM-F12 without phenol red and supplemented with dextran-coated charcoal-treated FBS (5% v/v), L-glutamine (4 mM), and penicillin-streptomycin (100 U/mL-100 µg/mL). Then, the cells were seeded at a density of 40,000 cells/well, in 96-well plates (100 µL/well). After 24 h, the test medium of each well was replaced with a test medium containing pesticides (100 µL/wells), and the cells were incubated for 16 h. After the incubation, the luciferase activity was assessed adding 100 µL/well of the One Glo Reagent (One-Glo Luciferase Assay System, Promega, Madison, USA), mixing (5 min) and measuring the luminescence of each well by a luminometer (Infinite Reader M200 Pro, Tecan, Männedorf, Switzerland).

The stock solutions of thiacloprid and imidacloprid were prepared in DMSO, while the stock solution of glyphosate was prepared in a test medium. The stock solutions were stored at −20 °C and, shortly before exposure, different concentrations of pesticides were prepared in a test medium (glyphosate and imidacloprid: From 10^{-8} to 10^{-3} M; thiacloprid from 10^{-8} to 5×10^{-4} M, due to lower solubility). The final DMSO concentration was less than 0.1%. Cells exposed to the test medium were used as a negative control and five concentrations of E2 (from 10^{-12} to 10^{-8} M) were tested to obtain a standard positive curve of the reference compound (E2).

The estrogenic activity was calculated as the ratio of the activity induced by the treatment over the activity induced by the positive control with 17-βestradiol (E2). It was expressed in percentage considering the relative luciferase activity of E2 (10^{-8} M) as 100%. Since all experiments were performed in quadruplicate (four wells for each experimental condition), the estrogenic activity was expressed as the mean and standard deviation of four values. The estrogenic activity of pesticides was also evaluated by the determination of the relative potency of each pesticide in comparison with the reference compound (E2) and it was expressed as the E2 equivalency factor (EEF) [75]. The EEF was calculated using the concentrations of E2 and pesticides at which 50% of biological effect is achieved (EC50) through the formula: EEF = E2 EC50/pesticide EC50.

Three concentrations of pesticides (10^{-5}, 2.5×10^{-4}, and 5×10^{-4} M) were also tested: In combination with an ER-antagonist (tamoxifen 10^{-6} M), in order to confirm whether the observed effects were due to the ER activation, and in combination with E2 (10^{-10} M), in order to investigate the interaction between pesticides and E2 in MELN cells. The estrogenic activity of these treatments was expressed as relative luciferase activity and it was calculated as percentage of activity induced by the treatment with respect to the activity induced by the E2 10^{-10} M (relative luciferase activity of E2 10^{-10} M = 100%). The stock solutions of E2 and tamoxifen were prepared in ethanol and stored at −20 °C.

2.6. Data Analysis

Statistical analysis was performed using IBM SPSS Statistics 25.0 (IBM, Armonk, USA). The EC50 of E2 and pesticides was calculated by dose–response curves, which were estimated through a probit regression between the relative luciferase activity and Log transformed-concentrations of E2 or pesticides.

Data collected with the MELN gene reporter assay were not normally distributed, so the non-parametric Kruskal-Wallis test followed by the post-hoc Dunnett test was used to assess significant differences among the different experimental conditions. The differences were considered significant with p-value < 0.05.

3. Results

3.1. Effect of Pesticides on Aromatase Activity

In order to study the effect of pesticide compounds on aromatase activity, a direct competitive estrone ELISA was performed using the purified cytochrome P450 reductase (CPR), as an electron donor from NADPH, and aromatase. The aromatase activity was evaluated by measuring the estrone production in the absence and presence of three different concentrations of pesticides (0.5, 1, and 5 µM). As a control, anastrozole, a known aromatase inhibitor, was applied at a concentration of 1000 nM

and the residual aromatase activity detected was 0.7%. As can be seen in Table 1, glyphosate partially reduced the aromatase activity at the concentrations tested. The enzyme activity decreased with the increase of glyphosate concentration. When adding 5 μM of glyphosate, the residual aromatase activity was 36%. Unlike glyphosate, imidacloprid and thiacloprid did not inhibit the enzyme activity (Table 1).

Table 1. Effect of pesticides on the aromatase activity.

	Pesticide	Relative Activity (%)		
		500 nM	**1000 nM**	**5000 nM**
1	Glyphosate	76.6 ± 11.3 *	74.5 ± 7.6 *	36.0 ± 19.5 *
2	Imidacloprid	100.1 ± 8.8	92.9 ± 28.0	120.5 ± 17.6
3	Thiacloprid	100.9 ± 11.9	153.6 ± 56.9	146.9 ± 42.1

Statistical significance *: p-value < 0.05 versus positive control (C^+).

3.2. *Effect of Glyphosate Concentration on Aromatase Activity*

The effect of glyphosate on aromatase activity was further studied by exploring the concentration range of glyphosate applied from 50 to 1500 nM. Such concentrations of glyphosate were selected since they resemble the ones detected in human urine samples [76]. The experiment was carried out by the ELISA assay at the concentration of 50 and 400 nM androstenedione, respectively. The two different concentrations were chosen on the basis of the kinetic parameters of aromatase: The first one (50 nM) is close to the enzyme K_M and the second one (400 nM) is saturating the enzyme (see next paragraph).

As shown in Figure 1, the activity of Aro is inhibited by 30% when the glyphosate concentration is ≥1000 nM. However, when the substrate concentration is 50 nM (black squares in Figure 1), the maximal inhibitory effect is already achieved when the glyphosate concentration is 100 nM. Therefore, the inhibitory effect of glyphosate strongly depends on the substrate concentration and it is only partial, indicating that this compound can be considered as a weak inhibitor.

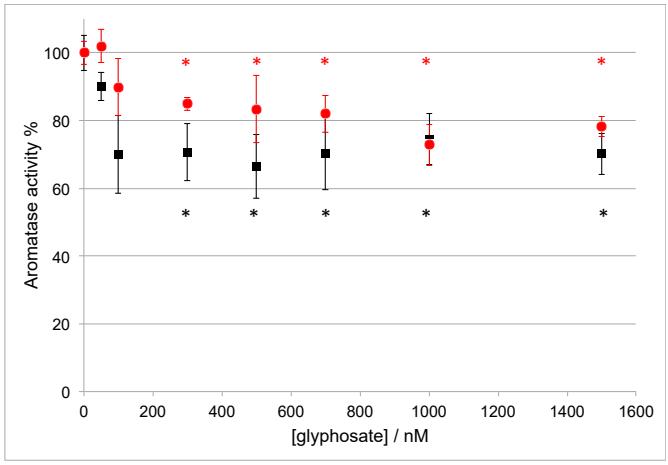

Figure 1. Aromatase activity in the presence of different concentrations of glyphosate and 50 nM (black squares) or 400 nM (red circles) of the substrate androstenedione. Statistical significance *: p-value < 0.05 versus C^+.

3.3. Effect of Glyphosate on the Catalytic Parameters of Aro

In order to investigate the mechanism of aromatase inhibition by glyphosate, the kinetic parameters of the enzyme were evaluated using the estrone ELISA assay in the absence and presence of two different concentrations of the pesticide (1000 and 5000 nM). Different substrate concentrations were applied and the product formation rate was plotted as a function of the substrate concentration (Figure 2A). The plot showed hyperbolic trends and the catalytic parameters, shown in Table 2, were obtained by fitting the experimental data to the Michaelis-Menten equation.

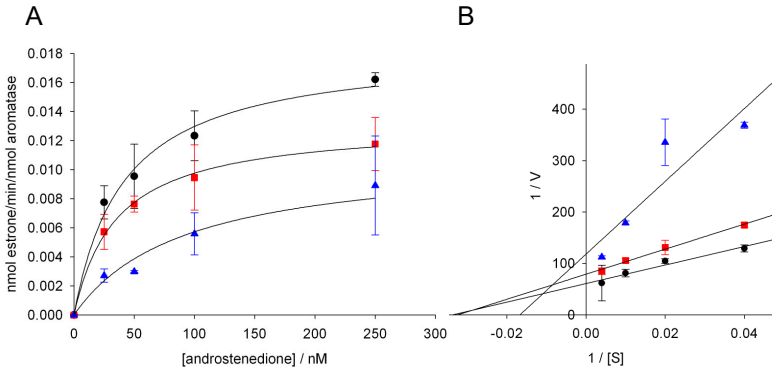

Figure 2. (**A**) Michaelis-Menten plots and (**B**) Lineweaver-Burk linearization for aromatase activity in the absence (black circles) and presence of 1000 nM (red squares) and 5000 nM (blue triangles) of glyphosate. In Panel (**A**), the data were fitted to the Michaelis-Menten equation using the Sigma Plot software to obtain the kinetic parameters.

When glyphosate is not present, the resulting K_M and V_{max} are 41.3 ± 8.2 nM and 0.018 ± 0.001 min^{-1}, respectively. When 1000 nM of glyphosate was added, the V_{max} value was significantly decreased whereas the K_M value was not significantly affected. Interestingly, when the glyphosate concentration was increased to 5000 nM, both K_M and V_{max} were affected. Compared to the reaction without glyphosate, K_M was increased by 2.2 folds, while V_{max} was decreased to 0.011 ± 0.002 min^{-1} (Table 2).

Table 2. Kinetic parameters obtained from the fitting of the data in Figure 2A to a Michaelis-Menten curve. The kinetic parameters are calculated for aromatase activity in the absence and presence of 1000 and 5000 nM of glyphosate.

Glyphosate (nM)	K_M (nM)	V_{max} (min^{-1})
0	41.3 ± 8.2	0.018 ± 0.001
1000	35.3 ± 3.5	0.013 ± 0.001 *
5000	92.3 ± 20.7 *	0.011 ± 0.002 *

Statistical significance*: p-value < 0.05 versus the values obtained in the absence of glyphosate.

The kinetic parameters show that the type of inhibition of aromatase by glyphosate depends on the herbicide concentration applied. Indeed, when using 1000 nM of glyphosate, the V_{max} was decreased while K_M did not change, indicating a non-competitive inhibition mechanism, meaning that, at this concentration, glyphosate does not compete with the substrate and binds to a site different from that where the substrate binds. When the concentration of glyphosate was increased to 5000 nM, both K_M and V_{max} were affected, and the Lineweaver-Burk plot shows a trend typical of a mixed inhibition mechanism (Figure 2B). Mixed inhibition is considered a more general case of

non-competitive inhibition, in which the inhibitor exhibits unequal affinity for the free enzyme and for the enzyme-substrate complex.

3.4. Molecular Dynamics Simulations on Aromatase

Grounding on recent evidence demonstrating the existence of allosteric binding sites [50] and their possible exploitation for a non-competitive/mixed inhibition mechanism [54] we docked glyphosate into the two allosteric cavities. Namely, we docked it to Site 1, which lies along the most relevant access channel to the enzyme active site [77] and to Site 2, which instead lies at the interface with the cytochrome P450 reductase (CPR), supplying the electrons necessary for catalysis (Figure 3) [78]. In the docking pose in Site 1 and during MD simulations glyphosate engages a salt bridge interaction with its phosphate group and Arg192, as well as the formation of a hydrogen (H)-bond between Gln218 and the carboxylic group of the pesticide. Most importantly, the phosphate group of glyphosate makes up to two simultaneous H-bonds with Asp309 (Figure 3), which normally is engaged in stabilizing the binding of aromatase substrates.

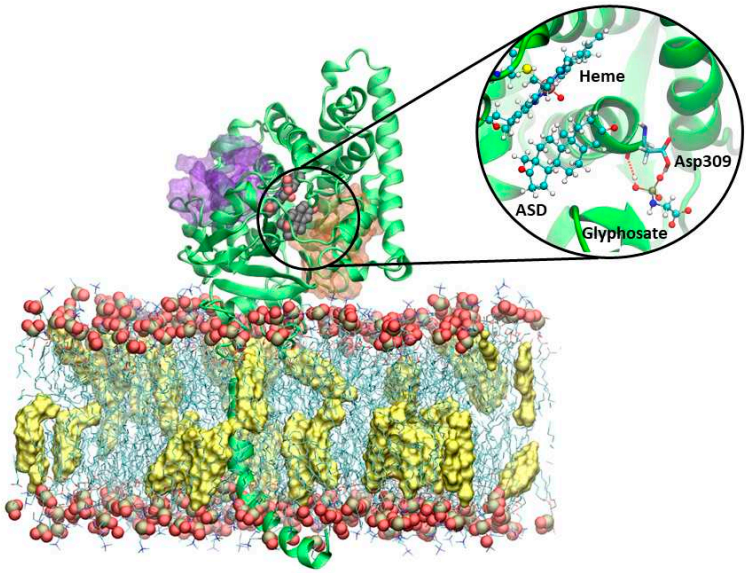

Figure 3. Representative structure of aromatase embedded in a POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) membrane, with phosphorous and oxygen atoms shown as tan and red van der Waals (vdw) spheres, and cholesterol (yellow surface) membrane. Sites 1 and 2 are shown as orange and purple transparent surfaces, respectively. The heme and androstenedione (ASD) are displayed in a vdw representation. The protein is shown as green new cartoons. The inset reports a close view of structure of aromatase in complex with glyphosate, as obtained from the most representative cluster of the molecular dynamics simulation trajectory. The heme moiety, ASD, and glyphosate are depicted in balls and sticks. The key catalytic residue Asp309, lining the binding cavity, is shown in licorice and colored by the atom name.

Due to these interactions the molecule remains stably bound in the pocket for the whole MD simulation, in line with its inhibitory activity in the μM range. Remarkably, it was recently suggested that the binding of a small molecule in Site 1 triggered the displacement of the water molecule needed

for the catalytic activity, which are normally H-bonding with the Asp309 and Arg192 residues, both being critical residues for the catalytic activity [41,57], thus inhibiting estrogen biosynthesis. Conversely, the docking pose obtained in Site 2, did not establish any relevant H-bond/salt bridge. As a result, the glyphosate dissociated from the pocket within the first few ns of MD simulations.

3.5. Detection of Estrogenic Activity with the MELN Gene Reporter Assay

The MELN gene reporter assay was carried out to evaluate the estrogenic activity of glyphosate, imidacloprid, and thiacloprid on MELN cells. In this study, different concentrations of each pesticide were tested. Concentrations similar to the pesticide levels measured in human urine [76] were selected as the lowest concentrations (10^{-8} and 10^{-7} M), while concentrations up to 10^{-3} M were selected as the highest concentrations, in order to assess the effect induced by each pesticide in a wide range of concentrations.

Our results showed that glyphosate did not increase the relative luciferase activity with respect to the negative control; therefore no estrogenic activity was detected testing this pesticide on MELN cells (Figure 4). On the contrary, the highest concentrations of glyphosate induced a small decrease of the relative luciferase activity, which may be due to a toxic effect of the pesticide on cells.

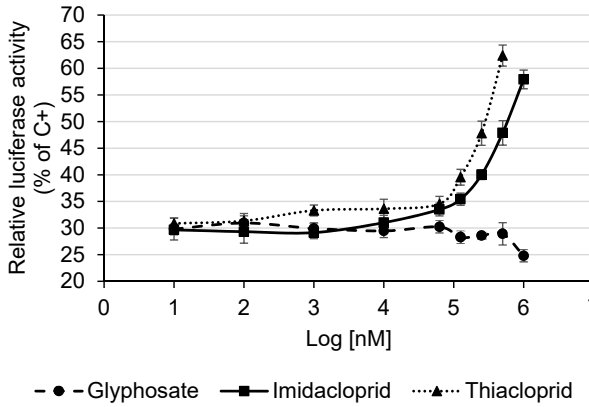

Figure 4. Estrogenic activity of pesticides measured with the MELN gene reporter assay. Data are expressed as means and standard deviations of the relative luciferase activity (% of C+, E2 10^{-8} M). The relative luciferase activity of the C+ is 100.0 ± 5.9%, while the relative luciferase activity of the negative control (test medium) is 30.5 ± 1.1%.

The null estrogenic activity induced by glyphosate was confirmed also by the exposure of cells to glyphosate in combination with tamoxifen (ER-antagonist) or with E2. Indeed, the wells treated with glyphosate and tamoxifen showed a relative luciferase activity equal to the wells treated with tamoxifen alone, and the wells treated with glyphosate and E2 showed a relative luciferase activity equal to the wells treated with E2 alone (data not shown). These results suggest that glyphosate does not interfere with the binding between ER and E2.

Regarding the neonicotinoid pesticides, in the present study, imidacloprid and thiacloprid significantly increased the relative luciferase activity with respect to the negative control, starting from 6.3×10^{-5} M (Log[nM] = 4.79) and 10^{-6} M (Log[nM] = 3), respectively (Kruskal-Wallis test followed by the post-hoc Dunnett test, $p < 0.05$). Since both the neonicotinoid pesticides induced a dose-dependent increase of the relative luciferase activity, in particular from 10^{-4} M (Log[nM] = 5) to the highest tested dose (Figure 4), a significant estrogenic activity of these two pesticides was detected on MELN cells.

The estrogenic activity of imidacloprid and thiacloprid was also quantitatively evaluated by the estimate of the concentrations of E2 and pesticides at which 50% of biological effect is achieved (EC50) and the E2 equivalency factor (EEF). The EC50 of E2 and pesticides was calculated by dose-response curves whereas the EEF was calculated through the formula: EEF = E2 EC50/pesticide EC50. The EC50 of imidacloprid and thiacloprid was 1.0×10^{-2} M (IC 95% 1.7×10^{-3}–2.2×10^{-1} M) and 1.5×10^{-3} M (IC 95% 2.5×10^{-4}–3.6×10^{-2} M), respectively, while the EEF was 5.4×10^{-10} (IC 95% 3.3×10^{-9}–2.5×10^{-11}) and 3.7×10^{-9} (IC 95% 2.2×10^{-8}–1.5×10^{-10}), respectively.

The exposure of cells to imidacloprid and thiacloprid in combination with tamoxifen confirmed that the estrogenic activity of the two pesticides was induced by the activation of ER. Indeed, the wells treated with the neonicotinoid pesticides and tamoxifen showed a relative luciferase activity that was lower compared to the wells treated with these pesticides alone. Furthermore, the relative luciferase activity of wells treated with the neonicotinoid pesticides and tamoxifen was similar to the relative luciferase activity measured in the negative control (Figure 5A,C).

Finally, the exposure of cells to imidacloprid and thiacloprid in combination with E2 induced an increase of the relative luciferase activity with respect to the E2 alone (Figure 5B,D). The increase was slight for imidacloprid while it was stronger for thiacloprid, suggesting a possible additive effect exerted by these pesticides in combination with E2.

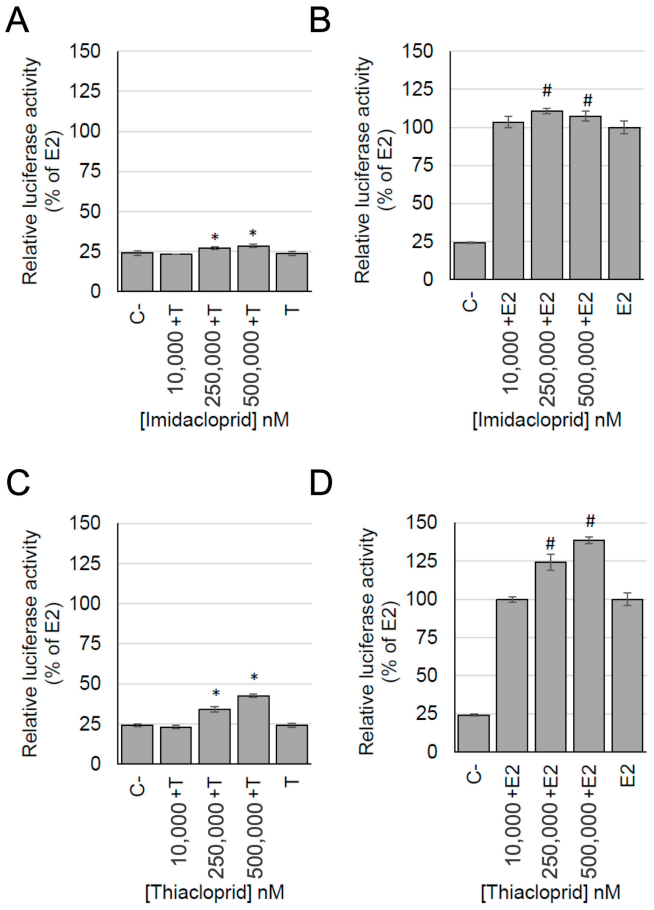

Figure 5. Estrogenic activity, measured with the MELN gene reporter assay, of imidacloprid (**A**,**B**) and thiacloprid (**C**,**D**) in combination with E2 (10^{-10} M) (**B**,**D**) or in combination with tamoxifen (10^{-6} M) (**A**,**C**). Data are expressed as the relative luciferase activity (% of E2 10^{-10} M). C−: Negative control; E2: E2 10^{-10} M; T: Tamoxifen 10^{-6} M. * $p < 0.05$ vs. C−; # $p < 0.05$ vs. E2; Kruskal-Wallis test followed by the post-hoc Dunnett test.

3.6. Docking Calculation on ERα

In order to provide a rationale for the estrogenic activity exerted by the two neonicotinoids, thiacloprid and imidacloprid, we have performed docking calculations on ERα. First, the two molecules were docked into the ERαs estrogen binding site, using the crystal structure of 17-β-estradiol bound to the ERα dimer (PDB id: 1qku) [52]. Both neonicotinoids fit inside the estrogen binding pocket (Figure 6). In particular, imidacloprid forms a H-bond with the backbone of Gly521, while in thiacloprid, the Cl atom makes halogen bonds with the guanidinium group of Arg394 and the aromatic rings of Phe404

and Trp393. Halogen bonds are attractive interactions between the electrophilic region associated with the Cl halogen atom and the nucleophilic regions of the surrounding protein residues [79].

Next, in order to disclose if and how the two neonicotinoids exert an additive effect to estrogen binding, by occupying an allosteric cavity, we looked for the presence of druggable allosteric pockets in the protein. Interestingly, a high-ranking binding pocket was found in the proximity of the estrogen binding site of one monomer of ERα (Figure 6). The docking calculation performed on this pocket, strikingly revealed that imidacloprid can H-bond with Lys449 and Glu323. As well thiacloprid H-bonds with Lys449 and Trp393.

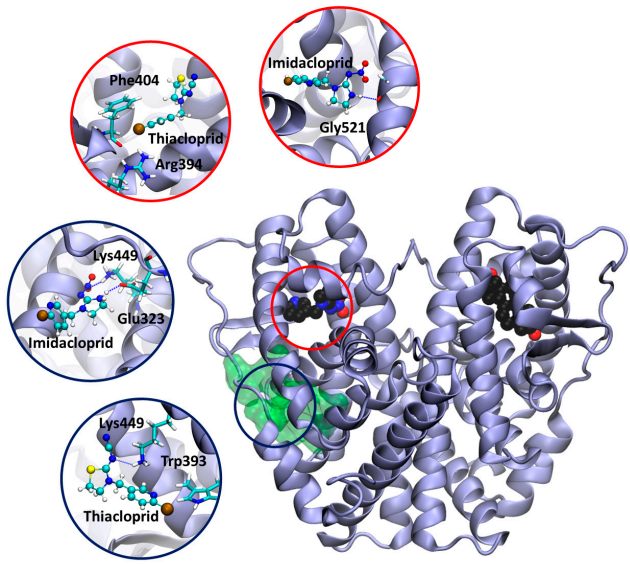

Figure 6. Model of estrogen receptor α dimer (PDB ID 1qku [52]) in complex with the neonicotinoids, imidacloprid and thiacloprid. The allosteric pocket is shown as a green transparent surface. Imidacloprid, thiacloprid, and 17-β-estradiol are displayed in a van der Waals representation and colored by the atom name. The protein is shown as violet new cartoons. The insets report a close view of docking poses of imidacloprid and thiacloprid inside the estrogen binding site (red circles) and onto the newly identified allosteric pocket (dark blue circles). The ligand and the residues establishing the most important interactions are depicted in balls and sticks and licorice representations, respectively, and colored by the atom name.

4. Discussion

In this study, the effect of three pesticides on two key targets of the endocrine system was evaluated using a combination of experimental and in silico methods that allowed an investigation at the molecular level.

The first target considered was aromatase, the key enzyme for estrogen production that resulted in being partially inhibited by glyphosate. The increase obtained in the aromatase activity in the presence of imidacloprid and thiacloprid, even if not statistically significant, can be interesting to be further investigated. Indeed, these two compounds could directly act on aromatase as allosteric activators or they could exert their action indirectly on CPR that has an essential role in catalysis and acts as an effector on human aromatase conformation [80,81].

Glyphosate is the most widely used active compound among herbicides and was already reported to affect aromatase expression and activity in cells [33,35]. The impact for human health has already been demonstrated: Indeed, an alteration in androgens/estrogens balance due to a lower aromatase expression as a consequence of the glyphosate presence changed the sperm nuclear quality impacting mammalian reproduction [82].

However, the direct interaction between glyphosate and the enzyme together with the inhibition mechanism was not reported yet. In this study, the ELISA assay revealed that the inhibition of aromatase by glyphosate is partial and weak and strongly depends on the substrate concentration. Moreover, at the lower concentration of glyphosate tested (1 μM), the inhibition was found to be non-competitive, while at the higher concentration used (5 μM), the inhibition turned into a mixed inhibition mode. These data suggest that glyphosate binds to an allosteric site both when the enzyme is free and in complex with the substrate. Classical MD simulations supply a structural model for glyphosate binding to aromatase, explaining at the atomic-level that this pollutant may exert its inhibitory activity at a low concentration by binding to the allosteric Site 1 previously identified by Magistrato et al. [50] and demonstrated to bind small-molecules inhibitors [54]. When the concentration increases, a mixed inhibition is observed again compatible with the presence of an allosteric site. The binding of glyphosate probably becomes stronger or another allosteric site is occupied by the inhibitor. However, MD simulations showed that glyphosate did not stably bind to the second allosteric pocket (Site 2) identified in previous studies.

The second important target studied was the estrogen receptor that is responsible for estrogen binding and signal transduction in cells. MCF-7 cells, stably transfected with an estrogen-regulated luciferase gene (MELN cells), were used to assess the estrogenic activity of the pesticides. Glyphosate induced no estrogenic activity on MELN cells, moreover glyphosate did not change the effect induced by tamoxifen or E2. These results are in accordance with the results of the studies of Kojima et al., 2004 [46] and Gasnier et al., 2009 [35], who did not find any agonistic or antagonistic effect of glyphosate on ER. On the contrary, our results are different than the results of Thongprakaisang et al., 2013 [44] and Mesnage et al., 2017 [45] who found a significant increase of ER-induced estrogenic activity. A possible explanation of this discrepancy could be that in our study the estrogenic activity was evaluated on MELN cells while the other two studies applied T47D-KBluc cells, which might be more sensitive to the glyphosate activity than MELN cells. Moreover, our results are consistent with the results of the Endocrine Disruptor Screening Program (EDSP) conducted by the United States Environmental Protection Agency (US EPA, 2015) which concluded that "there is no convincing evidence of a potential interaction with the estrogen pathway for glyphosate".

In the present study, both imidacloprid and thiacloprid induced a dose-response estrogenic activity mediated by the ER activation, in particular starting from 10^{-4} M. A consistent result was found in the study of Kojima et al., 2004 [46] in which no estrogenic activity was detected exposing transfected-cells to imidacloprid at concentrations lower than 10^{-5} M; a contradicting result was found by Westlund and Yargeau, 2017 [48], who did not find any estrogenic activity testing thiacloprid at concentrations comparable to ours. The conflicting results of thiacloprid should be interpreted considering the different cell models applied in our study with respect to the study of Westlund and Yargeau, 2017 (i.e., the present study was performed on mammalian cells while the previous one on yeast cells) [48]. Indeed, yeast cells are characterized by a different membrane permeability, transport proteins, and signal transduction pathways with respect to mammalian cells which may have influenced the results [42], as reported before also for other nuclear receptors [83].

The estrogenic activity of imidacloprid and thiacloprid was observed at concentrations higher than the pesticide levels measured in human biological samples [76,84]. Although such high levels of pesticides are not found in human biological samples, low doses of these pesticides should not be considered harmless. Indeed, in biological fluids these pesticides may be present in combination with other EDCs with a similar action mode and, thus, these pesticides together with other EDCs might cause an overall estrogenic effect. Moreover, these high concentrations of pesticides are similar

to levels in some environmental matrices, where they could cause adverse health effects on wildlife animals. For example, in guttation water or morning dew of plants, imidacloprid was found to be present at a concentration up to 346 mg/L [85].

Interestingly, the results of the present study showed that, when the ER-antagonist tamoxifen was added to these pesticides, the estrogenic activity was still higher than the negative control. This result can be explained by the competition between each pesticide and tamoxifen for ER binding. Moreover, in the presence of high concentrations of imidacloprid and thiacloprid, an additive effect with E2 was also observed. The docking calculation suggested that these two neonicotinoids may bind to both the orthosteric and allosteric pockets of ERα, suggesting a putative mechanism to rationalize their observed estrogenic activity.

5. Conclusions

In conclusion, this study provides further evidence about the action of some pesticides as endocrine disrupting chemicals (EDCs) targeting important proteins of the endocrine system. In particular, it shows that the inhibitory effects of the three compounds tested on aromatase are partial and their estrogenic effects occur at relatively high concentrations. However, possible additive estrogenic effects with the physiological hormone 17-βestradiol are present. Furthermore, it has to be taken into account that pesticides are usually introduced in the environment with their co-formulants that can be also biologically active as EDCs. Previous studies already showed that aromatase is inhibited by co-formulants of glyphosate-based herbicides [33,86]. Moreover, more studies are needed to investigate a possible additive effect of different pesticides that can be contemporarily present in the environment.

Our study also provides an integrated approach based on different assays and computational methods that allowed gaining new information about the possible interaction of pesticides and key targets at a molecular level. Such information can be exploited to predict the possible impact of other compounds on estrogen production and signaling in order to develop safer compounds for human health and environment.

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Assessment of Five Pesticides as Endocrine-Disrupting Chemicals: Effects on Estrogen Receptors and Aromatase

Marta Gea [1,*], Chao Zhang [2], Roberta Tota [1], Gianfranco Gilardi [2], Giovanna Di Nardo [2] and Tiziana Schilirò [1]

[1] Department of Public Health and Pediatrics, University of Torino, 10126 Torino, Italy; roberta.tota@edu.unito.it (R.T.); tiziana.schiliro@unito.it (T.S.)
[2] Department of Life Science and Systems Biology, University of Torino, 10123 Torino, Italy; chao.zhang@unito.it (C.Z.); gianfranco.gilardi@unito.it (G.G.); giovanna.dinardo@unito.it (G.D.N.)
* Correspondence: marta.gea@unito.it

Abstract: Pesticides are widely applied all over the world, and pesticide exposure can induce different biological effects posing a possible threat to human health. Due to their effects on the endocrine system, some pesticides are classified as endocrine disruptors. The aim of the study is to assess the interference of five pesticides on estrogen biosynthesis and estrogen signaling. Three neonicotinoid insecticides (Acetamiprid, Clothianidin, and Thiamethoxam), a carbamate insecticide (Methiocarb) and a herbicide (Oxadiazon) were tested. The effect of pesticides on estrogen biosynthesis was studied through an ELISA assay using a recombinant form of human aromatase, the enzyme that catalyzes the transformation of androgens to estrogens. Moreover, the effect of pesticides on estrogen signaling was assessed using a gene reporter assay on MELN cells, which measures estrogen receptor-mediated estrogenic activity. The results of the ELISA assay showed that the pesticides did not alter aromatase activity (no interference with estrogen biosynthesis), while the results of the gene reporter assay showed that only Methiocarb was able to alter estrogen signaling at high doses. The estrogenic activity of Methiocarb, expressed as 17β-estradiol equivalency factor (EEF), was equal to 8.0×10^{-8}. In conclusion, this study suggested that Methiocarb should be considered a potential endocrine disruptor.

Keywords: estrogen receptors; aromatase; pesticides; estrogenic activity; gene reporter assay; endocrine-disrupting chemicals; neonicotinoids; estrogen synthesis; estrogen signaling; estrogen equivalency factor

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1. Introduction

During the last century, a significant increase in world food production has become necessary to sustain global population growth [1]. In order to achieve appropriate food quantity and satisfactory food quality, fertilizers and also pesticides have been extensively used.

The term pesticide includes a wide variety of compounds that are used to kill pests, including insects, rodents, fungi and unwanted plants [2]. Pesticides are considered a quick, easy, and inexpensive solution for controlling pests and their use has not only contributed to the leap in agricultural yield, but has also helped to fight vector-borne and food-borne diseases [3,4]. Due to these advantages, great quantities of pesticides are ap-plied every year. In 2019, in the world, more than 4 million tons of pesticides were used [5]. Regarding insecticides, the neonicotinoids are the most widely utilized in the world; in 2014, the neonicotinoid market exceeded USD 3 billion and accounted for about 25% of the global pesticide market [6]. Moreover, pesticides are also extensively applied in agricultural sector; in the European Union alone, the sales of plant protection products per hectare of agricultural area amount to 2.3 kg [3]. The increase in the use of pesticides has been regulated through legislation. In the European Union, previously fragmented legislation

was replaced with harmonized pesticide standards for all member states; however, pesticide legislation varies greatly worldwide (more stringent regulations have been approved by developed nations with respect to developing countries) and, to date, harmonized pesticide legislation does not exist [7].

Despite the advantages, the use of pesticides also brings disadvantages. The extensive application of these molecules has ensured their spread in the environment. Numerous studies have demonstrated that air, water and soil can be contaminated by pesticide residues [1,8–13]. Once in the environment, pesticides can be accumulated in non-human organisms with devastating toxic effects at population level [14]. Moreover, they can move up trophic chains affecting top predators [1] and the repeated application of pesticides can increase pest resistance. Since the pesticide mechanism for toxic action can be not completely restricted to target pests, toxicity can be exerted also on non-target organisms causing health effects [1]. As a consequence, pesticide toxicity may lead to biodiversity loss and human adverse health effects [2,15].

Human exposure to pesticides can occur through the ingestion of foods or liquids containing pesticide residues, through the inhalation of pesticide-contaminated air, or through dermal contact with these molecules [2,16]. Toxic effects induced by pesticide exposure can range from mild symptoms (such as skin irritation) to more severe symptoms (such as headache or nausea). Moreover, some studies reported that pesticide exposure can induce long-term health effects, including cancer [2]. Due to their effects on the endocrine system, some pesticides have also been classified as endocrine-disrupting chemicals (EDCs) [17], namely as molecules that are able to alter the function of the endocrine system causing adverse health effects [18]. The effect of EDCs on the endocrine system is of particular interest since it can be induced by low doses, can be severe when the exposure occurs during childhood or adolescence, can be evident after long time from the time of exposure and can be exerted not only on the exposed individual but also on subsequent generations [18]. At a cellular level, EDCs may interfere with hormone functions in different ways. They can directly interact with hormone receptors, mimicking natural hormones and producing an overstimulation (agonist EDCs) or they can bind hormone receptors, preventing the binding of the endogenous hormone and therefore blocking the signal (antagonist EDCs). Moreover, EDCs may also interfere indirectly with hormones, affecting their synthesis, transport, metabolism and excretion [19].

Chemicals that interfere with estrogens are considered important EDCs. Through direct interaction with receptors, they can alter the estrogenic signaling, which is based on two pathways: genomic pathway and non-genomic pathway. The first pathway involves the transcription of genes and it is initiated by the binding of EDCs with the nuclear estrogen receptors, while the second one is mediated by membrane-bound receptors and it involves signaling proteins [20]. Both pathways can influence different cell functions, such as inflammation response, cellular metabolism, apoptosis, autophagy, DNA damage, and differentiation. Moreover, EDCs that interfere with estrogens can also alter estrogen biosynthesis acting on aromatase enzyme, which is the enzyme that catalyzes the transformation of androgens to estrogens.

Some pesticides have been identified as estrogenic EDCs, such as Dieldrin, which affects cellular proliferation pathway through estrogen receptors and extracellular signal-regulated kinase [21], or Methoxyclor, which affects the apoptosis pathway through the estrogen receptors cyclin D1, Ras, and Bax [22]. Moreover, other pesticides have been tested to assess their activity on aromatase enzyme (e.g., Lindane, Endosulfan, Deltamethrin, Chlorpyriphos, and Atrazine) [23–25]. However, for a great number of pesticides, additional evidence is needed. Therefore, the aim of the study is to assess the interference of five pesticides on estrogen biosynthesis and estrogen signaling. Three neonicotinoid insecticides (Acetamiprid, Clothianidin, and Thiamethoxam), a carbamate insecticide (Methiocarb) and a herbicide (Oxadiazon) are tested. The effect of pesticides on estrogen biosynthesis is studied using a recombinant form of human aromatase. Moreover, the effect of pesticides on

estrogen signaling is assessed using a gene reporter assay on MELN cells, which measures estrogen receptor-mediated estrogenic activity.

2. Materials and Methods

2.1. Pesticides

Four insecticides and one herbicide were purchased by Merck (Darmstadt, Germany) and tested in order to assess their interference with the aromatase enzyme and estrogen receptors (Figure 1, Table S1 in Supplementary Materials).

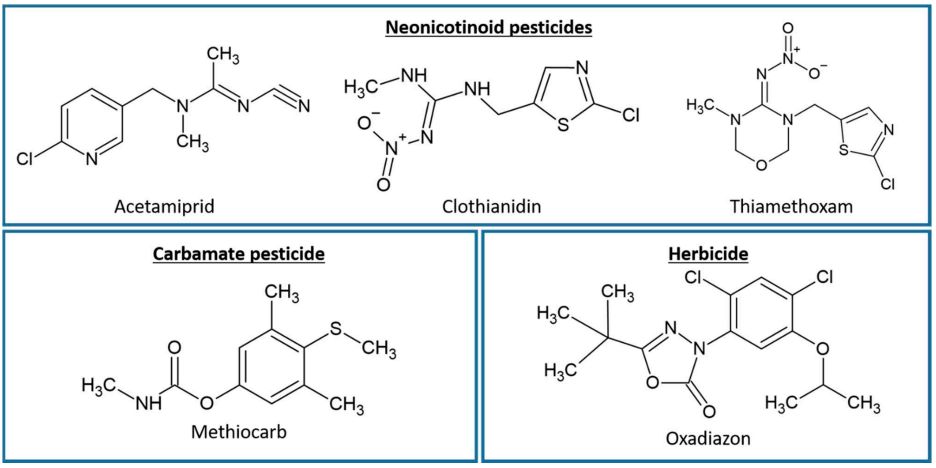

Figure 1. Molecular structures of the five tested pesticides.

2.2. ELISA Assay

The effect of pesticides on estrogen biosynthesis was evaluated using a direct competitive ELISA estrone Kit (BioVendor, Brno, Czech Republic) to quantify estrone produced during an aromatase-catalyzed reaction. The recombinant form of human aromatase and the human recombinant cytochrome P450 reductase (hCPR) were expressed and purified as previously described [26,27]. Reactions were carried out by mixing 5×10^{-9} M aromatase, 5×10^{-9} M hCPR, 5×10^{-8} M androstenedione (Biozol, Eching, Germany), 5×10^{-4} M NADPH (VWR International, Milan, Italy), and different concentrations of pesticides (0.5, 1 and 5×10^{-6} M) in 100 mM potassium buffer (pH 7.0) containing 20% glycerol (Merck, Darmstadt, Germany), 1 mM β-mercaptoethanol (Merck, Darmstadt, Germany). The reaction was initiated by the addition of NADPH and terminated by heat inactivation at 90 °C for 10 min after incubation at 30 °C for 10 min. The positive control is the reaction without pesticide, and there are two negative controls: the reaction without pesticide but containing anastrozole (aromatase inhibitor), and the reaction without hCPR. After the reaction, the supernatant was obtained by centrifugation at 11,000 g for 10 min, and then diluted with Calibrator A provided by the ELISA kit at 1:8. ELISA was then performed according to the manufacturer's instructions to measure estrone. The estrone concentration from each reaction was calculated according to a standard curve of known estrone concentrations typically resulting in values ranging from 2.4 to 3.2 nM. All results are expressed as relative aromatase activity with respect to positive control (C+ = reaction without pesticide, relative aromatase activity of C+ = 100%). The relative aromatase activity of each experimental condition was calculated from the average of three measurements, representing at least three independent experiments.

2.3. MELN Gene Reporter Assay

A luciferase gene reporter assay based on the MCF-7 cell lines transfected with the ERE-βGlob-Luc-SVNeo plasmid (MELN cells) was performed in order to assess the estrogenic activity of the pesticides [28,29]. MELN cells, kindly provided by Dr. P. Balaguer (INSERM, Montpellier, France), were grown in 75 cm^2 flasks at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium Nutrient Mixture F12-Ham (DMEM-F12) (Merck, Darmstadt, Germany), supplemented with fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) (5% *v/v*), penicillin-streptomycin (Biowest, Nuaillé, France) (100 U/mL–100 µg/mL), l-glutamine (Biowest, Nuaillé, France) (4 mM), G418 (Roche Diagnostics, Monza, Italy) (1 mg/mL) and phenol red (Merck, Darmstadt, Germany). Before the assay, the cells were cultured for three days in the test medium, which was DMEM-F12 supplemented with dextran-coated charcoal-treated FBS (Merck, Darmstadt, Germany) (5% *v/v*), penicillin-streptomycin (100 U/mL−100 µg/mL), l-glutamine (4 mM), and without phenol red. Then, the cells were seeded in 96-well plates (40,000 cells/well, 100 µL/well). After 24 h, the cells were exposed to different doses of pesticides for 21 h (Acetamiprid, Clothianidin, and Thiamethoxam from 1.95×10^{-6} to 10^{-3} M, Methiocarb and Oxadiazon from 1.95×10^{-6} to 10^{-4} M). The pesticide concentrations were selected considering the results of a previous study [29], in which a significant estrogenic effect was induced starting from the concentration 10^{-6} M. Stock solutions of Acetamiprid, Clothianidin, Thiamethoxam, Methiocarb, and Oxadiazon were prepared in dimethyl sulphoxide (DMSO) and stored at −20 °C, while the final dilutions of pesticides were prepared in test medium. The DMSO in the final dilutions was less than 0.1%. At the end of the exposure, the One-Glo Luciferase Assay System reagent (Promega Italy, Milan, Italy) was added in each well, the plates were shaken for 5 min and the luminescence of each well was measured by a luminometer (Infinite Reader M200 Pro, Tecan, Männedorf, Switzerland).

Cells exposed to the test medium and DMSO (<0.1%) were used as a negative control (C−), while seven 17β-estradiol doses (from 10^{-12} to 10^{-8} M) were assessed as standard positive curve of the reference compound (17β-estradiol). Additional tests were carried out on those pesticides that had induced significant estrogenic effect. In particular, these pesticides were also tested together with tamoxifen (estrogen receptor antagonist), to confirm whether the effect was due to estrogen receptor activation, and in combination with 17β-estradiol, to study the interaction between pesticides and 17β-estradiol. DMSO, 17β-estradiol and tamoxifen were purchased by Merck (Darmstadt, Germany).

The estrogenic activity was expressed as relative luciferase activity and it was calculated as a percentage of the activity induced by the treatment with respect to the activity induced by the positive control, 17β-estradiol 10^{-8} M (relative luciferase activity of C− = 0%, relative luciferase activity of 17β-estradiol 10^{-8} M = 100%). All experimental conditions were tested in quadruplicate in two independent experiments (four wells for each independent experiment) and the results were expressed as means and standard deviations. The relative estrogenic potency of each pesticide in comparison with the reference compound (17β-estradiol) was also assessed through the 17β-estradiol equivalency factor (EEF), which was calculated through the formula:

$$\text{EEF} = \text{17β-estradiol EC50/pesticide EC50} \quad (1)$$

where EC50 = concentrations at which 50% of biological effect is achieved. Data were reported with the relative confidence intervals (IC95%).

2.4. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 25.0 (IBM, Armonk, NY, USA). A probit regression between the relative luciferase activity and log-transformed concentrations of 17β-estradiol or pesticides was applied in order to estimate the EC50. Since data were not normally distributed, the non-parametric Kruskal–Wallis test followed

by the post hoc Dunnett's test was used to assess significant differences vs. negative control. The differences were considered significant with p-value < 0.05.

3. Results

3.1. Pesticide Effect on Estrogen Biosynthesis

As listed in Table 1, the selected active neonicotinoid insecticides (Acetamiprid, Clothianidin and Thiamethoxam) could not significantly change the enzyme activity at three different concentrations. For the carbamate insecticide Methiocarb, the enzyme activity decreased with the increase in carbamate concentration, but the change was minimal. As a result, all neonicotinoids, Methiocarb and Oxadiazon did not result in significantly altered aromatase activity.

Table 1. Effect of pesticides on estrogen biosynthesis expressed as relative aromatase activity with respect to positive control (C+ = reaction without pesticide, relative aromatase activity of C+ = 100%). Data are presented as means ± standard deviations.

Pesticide	Relative Activity (% of C+)		
	0.5×10^{-6} M	1×10^{-6} M	5×10^{-6} M
Acetamiprid	94.7 ± 11.8	99.7 ± 4.7	87.5 ± 17.6
Clothianidin	74.1 ± 24.7	112.9 ± 14.9	97.9 ± 5.2
Thiamethoxam	101.4 ± 13.2	84.1 ± 11.6	95.6 ± 17.1
Methiocarb	98.1 ± 0.2	96.5 ± 0.1	90.9 ± 5.4
Oxadiazon	87.8 ± 0.7	100.1 ± 13.1	101.2 ± 16.7

3.2. Pesticide Effect on Estrogen Signaling

Pesticide effect on estrogen signaling was assessed using the MELN gene reporter assay. The results showed that the three neonicotinoid insecticides (Acetamiprid, Clothianidin and Thiamethoxam) did not increase the relative luciferase activity with respect to the negative control; therefore, no interference with estrogen signaling was detected (Figure 2a–c). On the contrary, the carbamate insecticide (Methiocarb) induced a dose-dependent estrogenic activity that was significant starting from 3.91×10^{-6} M, corresponding to −5.4 Log (concentration M) (Figure 2d). Similar to the neonicotinoid insecticides, the herbicide Oxadiazon did not show any interference with estrogen signaling (Figure 2e).

Since Methiocarb induced a significant estrogenic effect, the concentration 1.25×10^{-4} M, corresponding to −3.9 Log (concentration M), was also tested with 17β-estradiol (estrogen receptor agonist) and with tamoxifen (estrogen receptor antagonist). When the pesticide was tested in combination with 17β-estradiol, a higher estrogenic activity than the one induced by 17β-estradiol alone was observed (data not shown). Therefore, in the presence of 17β-estradiol, Methiocarb induced an additive effect on the estrogen receptors. Moreover, when it was tested in combination with tamoxifen, a lower estrogenic activity than the one induced by the pesticide alone was observed, confirming that the observed estrogenic effect was dependent on the estrogen-receptor-mediated pathway. The estrogenic activity of Methiocarb tested together with tamoxifen was higher than the effect induced by the negative control. This result can be explained considering that the pesticide and tamoxifen may compete for estrogen receptor binding.

The EC50 and the EEF of Methiocarb were 2.1×10^{-4} M (IC95% $1.4 \times 10^{-4} - 3.8 \times 10^{-4}$ M) and 8.0×10^{-8} (IC95% $1.2 \times 10^{-7} - 4.5 \times 10^{-8}$), respectively.

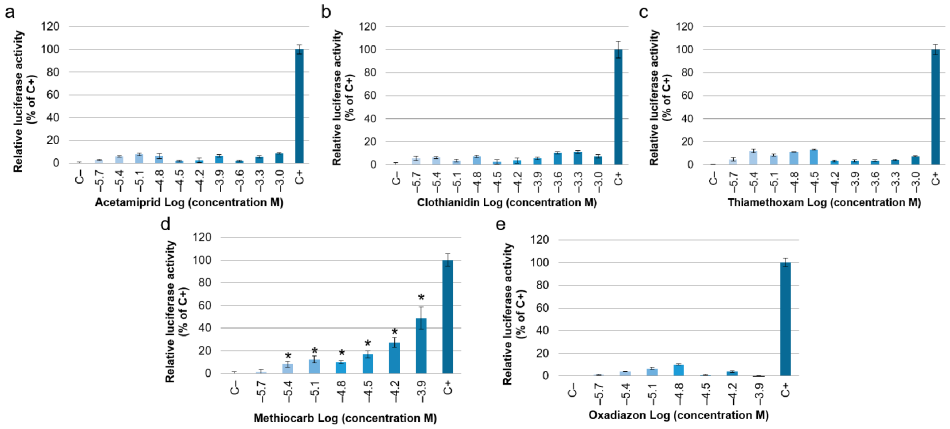

Figure 2. Effect of pesticides on estrogen signaling expressed as estrogenic activity. Estrogenic activity is reported as relative luciferase activity with respect to positive control (C+ = 17β-estradiol 10^{-8} M, relative luciferase activity of C+ = 100%): (**a**) Acetamiprid; (**b**) Clothianidin; (**c**) Thiamethoxam; (**d**) Methiocarb; (**e**) Oxadiazon. Data are presented as means ± standard deviations *: *p*-value < 0.05 vs. negative control (C−) (Kruskal–Wallis followed by post hoc Dunnett's test).

4. Discussion

Two in vitro assays were applied in this study in order to investigate the interference of four insecticides and one herbicide on estrogen biosynthesis and estrogen signaling.

All neonicotinoids, Methiocarb and Oxadiazon did not significantly alter aromatase activity; therefore, at the pesticide concentrations used in this study no significant effect on estrogen biosynthesis was found. The results are in accordance with two previous studies [25,30]. The first study showed that Methiocarb did not alter the aromatase activity [25], while the second one underlined that the herbicide Oxadiazon is able to induce aromatase activity in JEG-3 cells [30]. However, the effect of Oxadiazon was found at higher concentrations than the concentrations tested in the present study (effect observed at 10^{-5} M; tested concentrations: 0.5, 1 and 5×10^{-6} M).

Pesticide effect on estrogen signaling was tested using the MELN gene reporter assay; this assay showed that the three neonicotinoid insecticides did not induce any interference on the estrogen signaling (i.e., no estrogenic effect) (tested concentrations: from 1.95×10^{-6} to 10^{-3} M). The results obtained by testing Acetamiprid and Thiamethoxam are in accordance with the study of Westlund and Yargeau [31], which assessed the estrogenic activity using a yeast-based in vitro assay (estimated tested concentrations: from 10^{-10} to 10^{-2} M). Moreover, regarding Thiamethoxam, the results are also consistent with the study of Mesnage et al. [32] in which no estrogenic effect was detected testing this pesticide with the E-screen assay on MFC-7 cells (tested concentrations: from 3.4×10^{-6} to 1×10^{-3} M). Finally, the results obtained testing the last neonicotinoid insecticide, Clothianidin, also agree with a previous study in which the pesticide was tested using the E-screen assay on MFC-7 cells (tested concentrations: from 4×10^{-6} to 1.2×10^{-3} M) [32].

According to data reported by Barbosa et al. [9], environmental concentrations of the three neonicotinoids are lower than the highest concentration tested in this study (Table S2 in Supplementary Materials); therefore, the results showed that the environmental diffusion of the three neonicotinoids does not endanger humans and the environment through the alteration of estrogen signaling.

In contrast to the neonicotinoids, the insecticide Methiocarb induced a significant estrogenic effect mediated by estrogen receptors (effect observed starting from 3.91×10^{-6} M). Similar results were also shown by three previous studies [25,33,34]. Andersen et al. [25] found a significant estrogenic effect testing this pesticide using E-screen assay on MCF-7 BUS cells and a gene reporter assay on transfected MCF-7 BUS cells (effect observed starting from 10^{-5} M and 5×10^{-6} M, respectively). In addition, an estrogenic effect was also reported by Kojima et al. [33] and by Tange et al. [34] using gene reporter assays ([33] EC20 = $7.2 \times 10^{-6} - 8.4 \times 10^{-7}$ M; [34] EC20 $\approx 2.0 \times 10^{-5}$ M). These studies, together with the results of the present one, confirmed that Methiocarb is able to induce an estrogenic effect in vitro.

The estrogenic effect of Methiocarb was induced only at high doses (starting from 3.91×10^{-6} M, corresponding to 880,962.1 ng/L). These doses are higher than the concentrations of this pesticide found in environmental samples (Spain groundwater = 300 ng/L; Mexican groundwater = 5400 ng/L; Spain effluents of wastewaters = 4.73–14.92 ng/L), so its environmental occurrence seems not to represent a threat for human and environment [9,35]. However, it is important to highlight that, in environmental samples, Methiocarb concentrations can be combined with the concentrations of other EDCs causing possible synergistic or additive effects. Therefore, the estrogenic activity of Methiocarb is worthy of attention since it may be combined with the activity of other EDCs at which we are exposed every day.

Finally, in this study, the estrogenic potency of Methiocarb was many orders of magnitude lower than the reference compound and it was lower than the potency of other pesticides (Table 2). However, the EEF of this insecticide was higher than the EEFs of other two neonicotinoids (Imidacloprid and Thiacloprid) that were tested using the same assay in a recent article [29].

Regarding the herbicide Oxadiazon, to the best of our knowledge, this is the first article that has assessed the estrogenic activity of this pesticide using an in vitro assay. In these circumstances, it is currently impossible to compare the obtained results with others. However, the result of the present study suggests that this herbicide seems not to induce the transcription of genes regulated by estrogen receptors. This result is in accordance with the review of Ewence et al. [36], in which this pesticide was classified as substance not considered to be an EDC. While no effect on estrogen signaling was found in the present study, a previous study showed that the Oxadiazon-Butachlor pesticide can inhibit the WNT signaling pathway [37].

According to data reported by Barbosa et al. [9], environmental concentrations of Oxadiazon are lower than the highest concentration tested in this study (Table S2 in Supplementary Materials); therefore, similar to the three neonicotinoids, Oxadiazon does not endanger humans and the environment through the alteration of estrogen signaling.

The combined actions of pesticides are very important in the risk assessment process because pesticide formulations may include more than one EDC. Moreover, EDCs can occur in the environment and on the fruits and vegetables we eat as a cocktail of chemicals that may have synergistic, additive, or antagonistic effects on each other. Mixtures of these substances may cause higher toxic effects than those from a single compound [38]. Therefore, more studies are needed to investigate possible additive effects of different pesticides that can be contemporarily found in the environment or on foods as residuals.

As a future perspective, the MELN gene reporter assay and the ELISA assay could be used to evaluate the interference with the estrogen signaling and estrogen synthesis of different pesticide mixtures. Moreover, additional experiments using other in vitro and in vivo assays could be performed in order to confirm the obtained results. For instance, a proliferation assay, such as the E-screen assay, could be performed to evaluate whether these pesticides could induce cell proliferation in estrogen-sensitive cells.

Table 2. Estrogenic potency of Methiocarb assessed in the present study using a gene reporter assay in comparison with estrogenic potency of other pesticides reported by other studies using different assays. Estrogenic potency is expressed as 17β-estradiol equivalency factor (EEF). EEF of 17β-estradiol = 1.

Pesticide	Pesticide Type	Assay	EEF	Reference
Methiocarb	Carbamate insecticide	Gene reporter assay (MELN cells)	8.00×10^{-8}	Present study
Imidacloprid	Neonicotinoid insecticide	Gene reporter assay (MELN cells)	5.40×10^{-10}	[29]
Thiacloprid	Neonicotinoid insecticide	Gene reporter assay (MELN cells)	3.70×10^{-9}	[29]
Chlorpyrifos	Organophosphate insecticide	Yeast Estrogen Screen assay	2.90×10^{-3}	[39]
Dieldrin	Organochloride insecticide	ER-CALUX assay (T47D Luc cells)	2.40×10^{-7}	[40]
Endosulfan	Organochloride insecticide	ER-CALUX assay (T47D Luc cells)	1.00×10^{-6}	[40]
Permethrin	Pyrethroid insecticide	Yeast Estrogen Screen assay	1.00×10^{-7}—no estrogenic activity	[41]
Chlordane	Organochlorine insecticide	ER-CALUX assay (T47D Luc cells)	9.60×10^{-7}	[40]
DDT	Organochlorine insecticide	ER-CALUX assay (T47D Luc cells)	9.10×10^{-6}	[42]
Alachlor	Chloroacetanilide herbicide	Receptor binding assay	8.00×10^{-6}	[43]

5. Conclusions

With world population growth, it has become necessary to increase the production of food. This was partly achieved through the use of various pesticides on a large scale, initially without global guidelines or restrictions. Despite the benefits, these chemicals have polluted almost every part of our environment (i.e., soil, air, and water) and they have entered the trophic chains, reaching top predators. Therefore, the assessment of their environmental persistence and their toxicity for animals as well as humans has become a crucial factor and could be important also for the development of a global pesticide legislation that will protect both humans and the environment. Among the different biological effects that can be induced by pesticides, recently a great deal of attention has been paid to the ability of pesticides to alter the function of the endocrine system.

In this study, the interference of five pesticides (four insecticides and one herbicide) on estrogen biosynthesis and/or signaling, was tested in order to evaluate their potential action as EDCs. As far as we know, this study was the first to assess the effect of Oxadiazon on estrogen receptors using an in vitro assay. The results of the ELISA assay showed that all the four insecticides and the herbicide were not capable of altering aromatase activity; therefore, they did not interfere with estrogen biosynthesis. The results of the gene reporter assay showed that Methiocarb at high doses was able to alter estrogen signaling, while the other tested pesticides showed no estrogenic activity. In conclusion, even if additional in vitro and in vivo studies are needed to confirm this evidence, this study suggests that the carbamate insecticide Methiocarb should be considered as a potential EDC.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijerph19041959/s1: Table S1: Characteristics of the five tested pesticides (PubChem database, available at https://pubchem.ncbi.nlm.nih.gov/ (accessed on 2 February 2022)); Table S2: Environmental concentrations of the five tested pesticides in comparison with the concentrations tested in the present study with the MELN gene reporter assay.

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SUPPLEMENTARY MATERIALS
Assessment of five pesticides as endocrine-disrupting chemicals: effects on estrogen receptors and aromatase

Marta Gea[1]*, Chao Zhang[2], Roberta Tota[1], Gianfranco Gilardi[2], Giovanna Di Nardo[2] and Tiziana Schilirò[1]

[1] Department of Public Health and Pediatrics, University of Torino, Torino 10126, Italy; roberta.tota@edu.unito.it (R.T.); tiziana.schiliro@unito.it (T.S.).
[2] Department of Life Science and Systems Biology, University of Torino, Torino 10123, Italy; chao.zhang@unito.it (C.Z.); gianfranco.gilardi@unito.it (G.G.); giovanna.dinardo@unito.it (G.D.N.).
* Correspondence: marta.gea@unito.it (M.G.)

Table S.1. Characteristics of the five tested pesticides (PubChem database, available at https://pubchem.ncbi.nlm.nih.gov/).

Name	CAS number	Molecular weight	Molecular formula	Octanol/water partition coefficient (log Kow)	Bioconcentration factor (BCF)
Acetamiprid	160430-64-8	222.67	$C_{10}H_{11}ClN_4$	0.8	3
Clothianidin	210880-92-5	249.68	$C_6H_8ClN_5O_2S$	0.7	3
Thiamethoxam	153719-23-4	291.71	$C_8H_{10}ClN_5O_3S$	-0.13	3
Methiocarb	2032-65-7	225.31	$C_{11}H_{15}NO_2S$	2.92	35
Oxadiazon	19666-30-9	345.22	$C_{15}H_{18}Cl_2N_2O_3$	4.80	from 24.1 to 708

Table S.2 Environmental concentrations of the five tested pesticides in comparison with the concentrations tested in the present study with the MELN gene reporter assay.

Pesticide	Evironmental concentrations (ng/L) - see references	Tested concentrations using MELN gene reporter assay (ng/L) - present study	Effect on gene reporter assay - present study	References
Acetamiprid	Surface water = 20 - 380 ng/L	434,210 - 222,670,000	None	[1]
Clothianidin	Surface water = 20 - 420 ng/L	486,880 - 249,680,000	None	[1]
Thiamethoxam	Surface water = 40 - 1580 ng/L	568,830 - 291,710,000	None	[1]
Methiocarb	Groundwater = 300 - 5,400 ng/L; wastewaters = 4.73 - 14.92 ng/L	439,350 - 22,531,000	effect starting from 880,962.1 ng/L	[1,2]
Oxadiazon	Surface water = 4 - 1440 ng/L	673,180 - 34,522,000	None	[1]

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Ecofriendly laccases treatment to challenge micropollutants issue in municipal wastewaters☆

Federica Spina [a], Marta Gea [b], Carlo Bicchi [c], Chiara Cordero [c], Tiziana Schilirò [b], Giovanna Cristina Varese [a, *]

[a] Department of Life Sciences and Systems Biology, University of Torino, Viale Mattioli 25, 10125 Torino, Italy
[b] Department of Public Health and Pediatrics, University of Torino, Piazza Polonia, 94, 10126 Torino, Italy
[c] Dipartimento di Scienza e Tecnologia del Farmaco, University of Torino, Via P. Giuria 9, 10125 Torino, Italy

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ABSTRACT

In this study, a multidisciplinary approach investigated the enzymatic degradation of micropollutants in real, not modified, municipal wastewaters of a plant located in Italy. Stir Bar Sorptive Extraction combined to Gas Chromatography-Mass Spectrometric detection (SBSE-GC-MS) was applied to profile targeted pollutants in wastewaters collected after the primary sedimentation (W1) and the final effluent (W2). Fifteen compounds were detected at ng/L - μg/L, including pesticides, personal care products (PCPs) and drugs. The most abundant micropollutants were bis(2-ethylhexyl) phthalate, diethyl phthalate and ketoprofen. Laccases of *Trametes pubescens* MUT 2400 were very active against all the target micropollutants: except few cases, their concentration was reduced more than 60%. Chemical analysis and environmental risk do not always come together. To verify whether the treated wastewaters can represent a stressor for the aquatic ecosystem, toxicity was also evaluated. *Raphidocelis subcapitata* and *Lepidium sativum* tests showed a clear ecotoxicity reduction, even though they did not evenly respond. Two *in vitro* tests (*E-screen* test and MELN assay) were used to evaluate the estrogenic activity. Treatments already operating in the plant (e.g. activated sludge) partially reduced the estradiol equivalent concentration, and it was almost negligible after the laccases treatment. The results of this study suggest that laccases of *T. pubescens* are promising biocatalysts for the micropollutants transformation in wastewaters and surface waters.

1. Introduction

Rapid urbanization and increasing population growth rates are posing a serious threat to watercourses, which became the receiving basin of industrial and civil related compounds. Traditional pollutants include dyes, amines, phenols, heavy metals and surfactants that likely are already addressed by most of the wastewater treatment plants (WTPs) and restriction policies. Actual and future efforts should be also targeted to micropollutants: by definition, they are persistent and biologically active substances that once released in receiving waters can be detected at low concentrations (Barbosa et al., 2016). Pesticides, herbicides and fungicides, heat stabilizers, plasticizers, pharmaceuticals, PCPs, etc. belong to this class. Coming from many industrial sources as well as the human society daily-life, their release in the environment is continuous and massive (Wuttke et al., 2010; Frye et al., 2011). In comparison with the past decades, some pollution sources have now a higher impact, due to the increasing industrialization and the development of many industrial sectors in response to human being needs. For example, plastic production increased from 1.5 million to 245 million tonnes (Sung et al., 2012) and pesticides are 4-fold more used than 40 years ago (Mnif et al., 2011).

Many studies have revealed the ubiquitous occurrence of low concentrations of micropollutants and their metabolites in the aquatic environment (Szymańska et al., 2019). They have been found in rivers and lake basins (Voutsa et al., 2006; Schwientek et al., 2016), and recently also in seawater (Desbiolles et al., 2018). These findings are a direct consequence of the inefficacy of WTPs,

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* Corresponding author.
E-mail address: cristina.varese@unito.it (G.C. Varese).

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which should be the primary barrier to prevent the pollution diffusion along the aquatic system. Traditional treatment processes are unable to eliminate these contaminants from water (Westerhoff et al., 2005; Gibs et al., 2007; Kim et al., 2007; Schenck et al., 2012). Data from a wide survey of 943 organic chemicals in 32 wastewater samples demonstrated that treatments exhibited a highly variable removal yields, from 33 to 99% (Wang et al., 2018). It should be mentioned that many compounds are actually designed to be chemically and biologically stable, affecting their removal rate (Mompelat et al., 2009). Among this highly heterogeneous group, some compounds are sensitive to sunlight, microbial and chemical processes, but others can persist for months or years (Kidd et al., 2012). Due to their inadequacy, WTPs may become a *de facto* pollution hot spot (Wang et al., 2019). Micropollutants find then their way to surface waters where their fate and diffusion is uncertain and difficult to trace. Moreover, for many micropollutants commonly detected in superficial waters, complete toxicological information regarding humans and wildlife are missing, raising doubts about the actual risk wildlife is exposed to. As recently stated by Gavrilescu et al. (2015), the ecotoxicological significance of micropollutants is barely known. Transformation products are surely involved: they can potentially be as much or even more hazardous than the parent molecules. The complex and mostly unknown chemical pattern of a real water sample can play a remarkable synergism that creates uncertainty in the toxicity evaluation. For example, Russo et al. (2018) observed a toxicity increase after the UV irradiation of two drugs, cyclophosphamide and ifosfamide: the green alga *Raphidocelis subcapitata*, the rotifer *Brachionus calyciflorus* and the crustaceans *Thamnocephalus platyurus* and *Ceriodaphnia dubia* were probably influenced by the presence of a mixture of drugs and transformation products that displayed synergistic and/or potentiation effects. Toxicity assessment needs to consider also the estrogenic activity of environmental samples, since many micropollutants are recognized endocrine disrupting chemicals (EDCs) (Pamplona-Silva et al., 2018). Among the various effect-based tools capable of estrogenic activity detection in complex environmental samples, most researchers used *in vitro* approaches based on MELN gene-reporter luciferase assay (Kinani et al., 2010; Combalbert et al., 2012), *E-screen* assay (Liu et al., 2018), CALUX assay (Välitalo et al., 2017) and yeast estrogen screen assay (Mills et al. 2015).

Only the implementation of WTPs performances can alleviate the pressure to the fragile aquatic ecosystem: advanced wastewater treatments are necessary. To date, several approaches have been evaluated, such as advanced oxidation processes (e.g. ozonation, UV irradiation, photo-Fenton treatment, etc.) (Lapertot et al., 2006; Ormad et al., 2010), sorption (Tong et al., 2019) and membrane processes (Li et al., 2015). However, biodegradation is known to be the predominant removal mechanism of many micropollutants (Garcia-Becerra and Ortiz, 2018). Activated sludge shows a variable and compound-dependent conversion that usually varies from 30 to 70% removal, or even 10% for some micropollutants as diclofenac (Tiwari et al., 2017). Acknowledging that bacteria cannot always solve the problem, fungi can instead display a much powerful oxidative metabolism, mostly played by laccases, peroxidases, peroxygenases, etc. (Tortella et al., 2015; Huang et al., 2017; Naghdi et al., 2018).

Enzyme technologies present several advantages over the use of chemico-physical technologies as well as whole cell systems (Naghdi et al., 2018; Stadlmair et al., 2018). Among them, laccases (EC.1.10.3.2) are probably the most suitable enzymes for industrial exploitation, having demonstrated their potential applications in various biotechnological processes (Falade et al., 2017). Laccases do not require any nutrient supply but can work with just the presence of oxygen as co-factor. Processes are usually competitively fast: reaction kinetic mostly depends on the substrate affinity but it is independent to the low concentrations of pollutants (Naghdi et al., 2018). The research of the adequate biocatalyst needs to focus the attention on laccases with high affinity for micropollutants and high stability in not axenic wastewaters samples (Arca-Ramos et al., 2018). The use of synthetic mediators to activate a stronger oxidative cascade has certainly obtained good results (Hahn et al., 2018; Parra Guardado et al., 2019) but the economic and environmental sustainability of the system can be undermined. The use of enzymatic crude extract avoids the costly process of enzymes purification and, with the presence of fungal-produced mediators, avoid the dependency on expensive and potentially toxic synthetic molecules (Naghdi et al., 2018; Varga et al., 2019).

The aim of this study was to investigate the actual potential of the enzymatic treatment of some micropollutants as EDCs, pharmaceuticals, PCPs, etc. in real municipal wastewaters. Laccases were obtained from a basidiomycetes, namely *Trametes pubescens* MUT 2400. Because of the large number of pollutants potentially present in real samples, a multi-residue screening analytical approach was adopted. Since the decrease of micropollutants concentration does not necessary imply the decrease of the toxicity, this parameter was also investigated to ensure that the treatment mitigates the environmental impact and human health issues. A battery of ecotoxicological bioassays was performed, followed by tests aimed to monitor the estrogenic activity of the samples.

2. Materials and methods

2.1. Municipal WWTP effluents

Samples were collected from a municipal WTP in north-western Italy (Torino), treating around 615,000 m^3/day (25,000 m^3/h) and serves four towns of the metropolitan area and almost 1.5 million inhabitants. The plant foresees several treatments including primary sedimentation, nitrification/denitrification, activated sludge oxidation, filtration, etc. In the oxidation tanks, homogenous suspension is guaranteed by air injection from the bottom; activated sludge are recirculated into the pre-denitrification tanks with a ratio variable from 1 to 3. As regards the activated sludge, the average hydraulic retention time (HRT) is 5.1 h. The filtered waters are then disinfected with sodium hypochlorite. The contact time is approximately 30 min.

Based on the results of the contamination residues obtained in previous studies (Bicchi et al., 2009; Schilirò et al., 2009; Spina et al., 2015), 24 h composite samples (2 L) were collected after primary sedimentation (W1) and at the end of the process (W2) and stored at 4 °C. The COD of W1 and W2 was 122 mg/L and 56 mg/L, respectively. The pH was constant at 7.7–7.8 (Table S1).

2.2. Enzymatic treatment

Laccases of *Trametes pubescens* MUT 2400 (Mycotheca Universitatis Taurinensis, Torino, Italy) were obtained as previously described (Spina et al., 2016). 100 U/L of laccases were used to treat real municipal wastewater samples (300 mL of W1 and W2) for 24 h. An abiotic control without enzyme inoculum was also set up. Three biological replicates were maintained under constant stirring (100 rpm) at room temperature. Residual laccase activity was measured during the experiment, in order to monitor possible inactivation effects due to the chemical and physical conditions of the real wastewaters.

2.3. Laccase activity and COD determination

Laccase activity was assayed at 25 °C following oxidation at 420 nm of 2,2′-azinobis (3- ethylbenzothiazoline-6-sulfonic acid, ABTS), in 0.1 M sodium citrate buffer pH 3 (Niku-Paavola et al., 1988). Enzymatic activity was expressed as International Units (U), where 1 unit corresponds to the amount of enzyme that oxidizes 1 μmol of substrate per minute.

Chemical oxygen demand (COD) was measured before and after each experiment on wastewater samples following the dichromate method (Lange kit, LCK 614).

2.4. Analytes extraction and quantification

After a multi-target SBSE procedure, analyses were carried out with a multi-shot thermal desorption (TD)-GC-MS analysis as previously described (Bicchi et al., 2009; Van Hoeck et al., 2009) (Supplementary Materials). Method performance parameters including accuracy, recovery yields and precision were assessed in accordance to Eurachem Guidelines (Eurachem, 2014) and reported in detail in a previous study (Spina et al., 2015). Targeted micropollutants are shown in Figure figs1.

2.5. Ecotoxicological assays

The tests were carried out before and after each experiment, using the following model organisms and standard methods: *Lepidium sativum* (UNICHIM 1651: 2003 method) and *Raphidocelis subcapitata* (UNI EN ISO 8692: 2005 method) (Supplementary Materials). These methods were previously identified as among the most sensitive ones to municipal wastewaters (Palli et al., 2019).

2.6. Estrogenic activity

Wastewaters samples were evaluated before and after the laccase treatment to assess the impact of the enzymatic treatment on estrogenic activity. The extraction of the wastewater was performed as described by Schilirò et al. (2009). Solid phase extraction was carried out on 200 mL aliquots of the samples, the extracts were then subjected to two *in vitro* tests: the human breast cancer cell line (MCF-7 BUS) proliferation test (*E-screen* assay) and the luciferase-transfected human breast cancer cell line gene-reporter assay (MELN assay). The assays were performed using previously reported methods (Schilirò et al., 2012). The estrogenic activity was quantitatively evaluated by the determination of the relative proliferative effect (RPE%) for the *E-screen* assay and by the rate of luciferase gene expression (TRANS%) for the MELN assay (Supplementary Materials). These endpoints compare the maximum biological response induced by a sample with the one induced by 17-β-estradiol. The estrogenic activity was also evaluated measuring the 17-β-estradiol equivalent quantity (EEQ), which is the total amount of estrogenic active compounds in sample normalized to the natural estrogen.

2.7. Statistical analyses

Data were analysed by means of a probit regression analysis, Spearman's test (correlation) and, *t*-test or Mann-Whitney test (means comparison) using SPSS 25.0 (SPSS for Windows, Chicago, IL, USA).

3. Results and discussion

3.1. W1 and W2 characterization

Since the United Nations *recognizes the right to safe and clean drinking water and sanitation as a human right that is essential for the full enjoyment of life and all human rights* (Resolution 64/292), social and scientific communities have increased their awareness about the need to enhance water quality. The ecological and chemical status of waters need significant improvements. WTPs represent the ultimate barrier to limit the accumulation of micropollutants in water basin. Since they operate in a multitude of different ways, the frequency of detection and the concentration deeply vary among plants (Schulze et al., 2019). By analysing the incoming waters (e.g. after the primary sedimentation) and the final effluents, the transformation yields of the plant and the quality of the discharged waters were here evaluated. The total micropollutants concentration was 403.2 μg/L and 349.5 μg/L in W1 and W2, respectively (Table 1). A similar chemical load was observed in Switzerland (Margot et al., 2013), in China (Wang et al., 2018; Zhang et al., 2018), in Australia (Roberts et al., 2016) and in North Europe (Gabet-Giraud et al., 2010; Vergili et al., 2019), while considerably higher load can be sometimes detected (Luo et al., 2014; K'oreje et al., 2016). Considering only the substances with a recognized estrogenic activity (Table 2), the chemical load was 35.3 μg/L and 18.3 μg/L in W1 and W2, respectively. The presence of even small amounts of EDCs should not mislead the attention, because they can cause toxic effects on the aquatic ecosystem as well. For instance, the presence of bisphenol A, equal to or even lower than 1 pg/L, is

Table 1
Quantified concentration (μg/L) of the target micropollutants in W1 and W2 and percentage of concentration decrease mediated by the WTP itself. nd = not detectable, below the limit of detection; na = not applicable.

	Classification	W1	W2	Removal %
2,4-dichlorophenol	herbicide	0.16 ± 0.03	nd	100
4-*t*-butylphenol	vamish	1.30 ± 0.21	0.18 ± 0.03	86
diethyl phthalate	plasticizer	24.7 ± 2.22	15.70 ± 1.41	36
2-hydroxybiphenyl	pesticide	1.80 ± 0.21	0.12 ± 0.01	93
4-*n*-octylphenol	surfactant	0.95 ± 0.15	0.72 ± 0.12	24
salicylic acid	drug	nd	nd	na
alachlor	herbicide	0.39 ± 0.08	0.40 ± 0.09	−3
4-*n*-nonylphenol	surfactant	0.29 ± 0.04	0.37 ± 0.05	−28
oxybenzone	PCPs	0.66 ± 0.07	0.30 ± 0.03	55
naproxen	drug	2.20 ± 0.31	0.16 ± 0.02	93
diclofenac	drug	0.84 ± 0.16	0.51 ± 0.10	39
triclosan	PCPs	0.44 ± 0.07	nd	100
ketoprofen	drug	14.30 ± 2.76	15.50 ± 2.99	−8
bisphenol A	plasticizer	5.20 ± 0.99	0.51 ± 0.10	90
bis(2-ethylhexyl) phthalate	plasticizer	350 ± 40	315 ± 36	10
Total load		**403.16**	**349.47**	

Table 2
Estrogenic potency relative to 17-β-estradiol (E2 is equal to 1) used for calculation of predicted EEQs from the chemical analysis.

	Estrogenic potency relative to E2 (E2 = 1)	Reference
2,4-dichlorophenol	no estrogenic activity	Ghisari and Bonefeld-Jorgensen, 2009; Kuckelkorn et al., 2018
4-t-butylphenol	1.90E-07	Olsen et al., 2005
diethyl phthalate	5.00E-07	Tan et al., 2007
2-hydroxybiphenyl	1.50E-06	Körner et al., 2000
4-n-octylphenol	1.40E-06	Murk et al., 2002
salicylic acid	no estrogenic activity	no reference found; absent in list of estrogenic chemicals reported by Kiyama and Wada-Kiyama, 2015
alachlor	8.00E-06	Scippo et al., 2004
4-n-nonylphenol	7.60E-05	Körner et al., 2001
oxybenzone	3.30E-07	Schlumpf et al., 2001
naproxen	antiestogenic activity	Ezechiáš et al., 2016
diclofenac	antiestogenic activity	Ezechiáš et al., 2016; Kuckelkorn et al., 2018
triclosan	no estrogenic activity	Cavanagh et al., 2018
ketoprofen	antiestogenic activity	Ezechiáš et al., 2016
bisphenol A	2.00E-05	Olsen et al., 2005
bis(2-ethylhexyl) phthalate	no estrogenic activity	Murk et al., 2002

responsible of hormonal metabolism alteration at different stages of amphibians and fish development (Flint et al., 2012). Even traces of diclofenac (e.g. 0.5 μg/L) that can be found in surface waters can produce adverse effects on various tissues in brown trout (Hoeger et al., 2005).

Among the 15 compounds detected in W1, 8 reached a concentration of at least 1 μg/L. Three compounds dominated the chemical load, as bis(2-ethylhexyl) phthalate (DEHP) (350.0 μg/L), diethyl phthalate (DEP) (24.7 μg/L) and ketoprofen (14.3 μg/L). Phthalate esters are often found in anthropogenic areas and in WTPs, but the distribution and the concentration deeply vary among WTPs (Zolfaghari et al., 2015; Salaudeen et al., 2018). These results are in agreement with previous studies, which indicated DEHP as the most detectable plasticizer (Fauser et al., 2003; Salaudeen et al., 2018): it is usually detected at μg/L although much higher concentrations can be found. For instance, six phthalates were detected at mg/L concentration in three full-scale WTPs with different treatment processes operating closed to the Songhua River in China (Gao et al., 2014).

As regards the W2 effluent, the concentration of only 7 micropollutants was lower (at least halved) than W1, indicating a moderate removal capacity of the WTP (Table 1). In particular, 2,4-dichlorophenol, 4-*t*-butylphenol, 2-hydroxybiphenyl, naproxen triclosan and bisphenol A were extensively degraded (>80%). On the contrary, the concentration of alachlor, 4-*n*-nonylphenol and ketoprofen did not decrease, and just a minimal (<30%) reduction was observed for diethyl phthalate, 4-*n*-octylphenol, diclofenac and DEHP. Although activated sludge is the most common *in use* biological treatment, contrasting reports can be found about the actual decontamination potential. Low performances can be ascribable to not-optimized operational conditions, which by contrast usually play a key role in the microbial process (Verlicchi et al., 2012a). For instance, the removal of ketoprofen has proved to be a direct response of the organic load in the influent, the concentration of suspended solids, the temperature and the amount of incoming carbon source available for the microorganisms (Gallardo-Altamirano et al., 2018). Therefore, ketoprofen transformation by activated sludge could be very slow, even requiring 25 days (Escuder-Gilabert et al., 2018). Phthalates are also very recalcitrant to microbial activity. In agreement with the low removal yields found in the present study for DEHP and DEP, primary and secondary sludge absorption was responsible for 20–35% removal of phthalates (Fauser et al., 2003). The not exhaustive removal may cause the release of unsafe waters with a phthalate load above the threshold limit (Salaudeen et al., 2018). A significant enhancement of phthalates degradation can be achieved only by controlling operative parameters as HRT, initial concentration of pollutants, etc. (Boonnorat et al., 2019; Harb et al., 2019; Wei et al., 2019).

Alachlor owns a particular mention, because despite it has been listed as a priority pollutant by the European Commission, its distribution along the European aquifer is almost unknown. In the present study, the concentration was higher (0.4 μg/L) than those reported in literature. For instance, in Harbin municipal sewage treatment plant (China), alachlor was found at 0.074–0.021 μg/L (Kong et al., 2008). Moreover, it is mostly unaffected by any treatment. Ozone was used as oxidation agent (Ormad et al., 2010), scaling the system to photo reactors (Maldonado et al., 2006), but low degradation percentage (up to 36%) or low reactivity has been reported. Likewise, the conventional treatments operating in the present WTP were completely ineffective against it.

Despite the decrease of the chemical pollution load, ecotoxicological analyses often describe a different scenario. In the present study, the possible direct and indirect perturbations to the aquatic ecosystem were studied by model organisms, such as algae because among the most sensitive organisms to chemical stressors (Välitalo et al., 2017). Since most of river waters are used for agricultural purposes (Siebert et al., 2010), the correlation between plant development and irrigation waters needs further insights. *L. sativum* and *R. subcapitata* are largely used to evaluate the safety of various wastewaters as steel industry effluent (de Paiva Magalhães et al., 2014), kraft mille effluent (Raptis et al., 2014), cosmetics industry wastewater (de Melo et al., 2013), olive mill wastewater (Buchmann et al., 2015; Jarošová et al., 2014), tannery mixed liquor (Tigini et al., 2018), hospital effluents (Verlicchi et al., 2012b; Perrodin et al., 2013), etc. Unfortunately, they have been mostly used to analyze the safety of micropollutants in model single or mixture solutions (Kolar et al., 2014; Magdaleno et al., 2015; Tongur and Yildirim, 2015; Chhetri et al., 2017; Elersek et al., 2018), but very few researches applied them against real municipal wastewaters (Aydin et al., 2015; Spina et al., 2015, Palli et al., 2019). In the present study, these bioassays provided fruitful information about the quality status of the WTP samples. Despite WTP reduced the concentration of many compounds as also indicated by the COD decrease (56%) (Table S1), it was not effective against the ecotoxicity: the mixture of detected and not-detected micropollutants and their metabolites of W2 produced comparable bioassays responses to W1. *L. sativum* GI% and *R. subcapitata* I% ranged between 77-79% and 24-17%, respectively;

significant differences cannot be noted between W1 and W2. This is a well-known phenomenon caused by the synergistic or antagonistic interactions among chemical substances, including transformation products, which are not necessary less toxic than parent ones. Contrariwise, literature reports also examples of highly performing WTPs, where the treatments are capable of reducing the ecotoxicological effect. For instance, the treated urban and hospital wastewaters showed no effect toward *Daphnia magna*, *R. subcapitata* and *Brachionus calyciflorus* (Laquaz et al., 2018). The actual reasons of such an efficient plant are difficult to depict. More probably a plethora of different factors have helped to reach this goal: the lower size of the WTP (21,000 inhabitant equivalent), the applied wastewater treatment technology that included a three step activated sludge systems, the environmental conditions and the operative parameters (as HRT and redox conditions) may have driven to a peculiar microbial community in the activated sludge.

The estrogenic activity of WTP samples before the laccases treatment was evaluated using *E-screen* assay and MELN assay. The RPE% of the MCF-7 BUS cells, induced by W1 and W2, was 67 ± 15% and 83 ± 13%, respectively: both samples showed a partial agonistic activity (RPE<100%). The partial agonistic activity was confirmed also by TRANS% values, as 89 ± 7% and 86 ± 2% for W1 and W2, respectively. The influent W1 showed an estrogenic potential, measured as EEQ, equal to 28.5 ± 10.6 ng/L and 21.7 ± 4.2 ng/L according to *E-screen* assay and MELN assay. The EEQs of W2 were reduced but not negligible: 6.0 ± 3.2 ng/L according to *E-screen* assay and 6.7 ± 1.7 ng/L according to MELN assay. These data are in agreement with other studies that measured comparable results in WTPs influents, as 0.7–14.0 ng/L (Omoruyi and Pohjanvirta, 2015) or 0.45–42 ng/L (Välitalo et al., 2017). The estrogenic activity of W2 found several confirmation in literature, where similar EEQs values were reported by other authors using different techniques: 0.03–23.8 ng/L using ERα-CALUX (Murk et al., 2002; Van der Linden et al., 2008; Mendonca et al., 2009; Välitalo et al., 2016; Välitalo et al., 2017), 0.53–17.9 ng/L using MELN reporter gene assay (Jarošová et al., 2014), 0.2–33.6 ng/L using *E-screen* assay (Körner et al., 1999; Körner et al., 2001; Schilirò et al., 2009) and 4.8–5.6 ng/L using Yeast Estrogen Screen (Salste et al., 2007). The range of EEQs found in literature could be explained considering that the measures were done on different water samples, using different extraction protocols, employing different biological assays and evaluating different biological endpoints.

The estrogenic activity detected in the present study showed the ability of WTP to reduce the estrogenic potential but not to completely remove it. To assess whether the detected effect in bioassay is at acceptable or unacceptable level, it is useful to compare the measured EEQs with effect-based trigger values reported in literature (Leusch et al., 2017). The EEQs of W2 resulted above the safe levels reported for municipal WTP effluents proposed by Jarošová et al. (2014), which should be in a range of 0.1–0.4 ng/L EEQ for long-term exposures and 0.5–2 ng/L EEQ for short-term exposures. As demonstrated also by ecotoxicological analysis, the estrogenic activity evaluation confirmed that W2 could not be considered harmless.

3.2. Laccases treatment

The potential of *T. pubescens* laccases to transform micropollutants of W1 and W2 was evaluated. The stability and the activity of these enzymes were the primary issues that needed to be validated. Due to their chemical and biological complexity, wastewaters may strongly interfere with laccase activity (Alneyadi et al., 2018). During the 24 h experiment, enzymatic activity was strongly inhibited by this complex matrix (Figure figs2). W1 still showed a considerable destabilizing potential: 66% of laccase activity was irreversibly lost. On the contrary, W2 resulted less harmful, with only 44% of enzymatic lost after 24 h. In W1, laccases may have suffered the more developed microbial community and the higher load of suspended solids they may have absorbed to. Laccases could then exhibit their maximal potential at the end of the WTP, being active where the conventional treatment are ultimately ineffective. Working as an advanced oxidation system, technology would also offer a variety of solutions to enhance the stability of the enzyme activity as the immobilization on adequate supports (Ba and Kumar 2017; Liu et al., 2019) or on membrane systems (Costa et al., 2019).

Laccases treatment was applied to both wastewaters. The treatment changed the chemical composition of the samples, as indicated by the 26.9% COD reduction of W1 (89.2 mg/L). This could be only partially explained by the chemical profiling performed by GC-MS analysis, but most likely, laccases acted also against other chemical components of the sample. Since the residual COD of W2 was already very low, any modification was not appreciable.

Fig. 1 shows the laccase-mediated degradation yields for each target compound. As regards W1, the concentration decreased up to 70% for 9 micropollutants and in just one case (e.g. 4-*n*-nonylphenol), the yield was below 50%.

A multifactorial scenario was likely governing the response of laccases, being ultimately difficult to point out the parameters that should be controlled or stressed to enhance the system performances. Removal yields can be attributed to the chemical structure of target compounds, the redox potential of laccases and molecules, and the chemical concentration. Moreover, laccase kinetic and stability is known to be pH dependent. The transformation of bisphenol A by laccases from *T. versicolor* was optimal at pH 5 (Kim and Nicell, 2006). The optimum pH of *T. pubescens* laccases is at acid values (Spina et al., 2015), far from the pH 7.6–7.8 of these wastewaters where pH was not controlled. The incomplete reaction can be associated to a non-optimal pH value, which may have affected the catalytic efficiency as well as reduced the rate of interaction between micropollutants and the enzyme. Affinity of laccases is also influenced by the presence of electron donating functional groups (EDFG) and electron withdrawing functional groups (EWFG) (Yang et al., 2013). On one side, EDFG such as hydroxyl (−OH), amines ($-NH_2$), alkoxy (-RO), alkyl (−R) and acyl (−COR) help laccase-mediated oxidation. On the contrary, EWFG produce an electron deficiency and reduce the enzymes affinity: carboxylic (−COOH), amide ($-CONR_2$), halogen (−X) and nitro ($-NO_2$) belong to this group. In W1, the high biotransformation percentage of bisphenol A (86.3%), 2-hydroxybiphenyl (84.9%) and 4-t-butylphenol (82.4%) can be explained by the presence of the hydroxyl group in the aromatic structure. The moderate percentage of alachlor transformation (56.9%) could be affected by the presence of EWFG groups. Nevertheless, laccases skills are worthy of mention since there are no previous records about this reaction, which is instead unfeasible by the activated sludge of the WTP (Table 1). Some micropollutants as 2,4-dichlorophenol, diclofenac and triclosan have a peculiar chemical structure, where both EDFG and EWFG are present. Their conversion yields ranged from 76.8% to 80.3%, showing to be very susceptible to laccase oxidation. It can be stated that the hydroxyl electron donor is stronger than the inhibition effect of the chlorinated groups. Similarly, the two EDFG (e.g. methoxy and hydroxyl groups) of oxybenzone favoured the transformation (77–82%), limiting the effect of the EWFG carbonyl (Ashe et al., 2016).

More contrasting results were observed in the presence of W2. In many cases, laccases obtained comparable yields to W1, as ketoprofen (63%) and oxybenzone (77–81%), or even achieved higher conversion values (e.g. 4-*n*-nonylphenol and 4-*n*-octylphenol). Despite activated sludge of the WTP were poorly effective

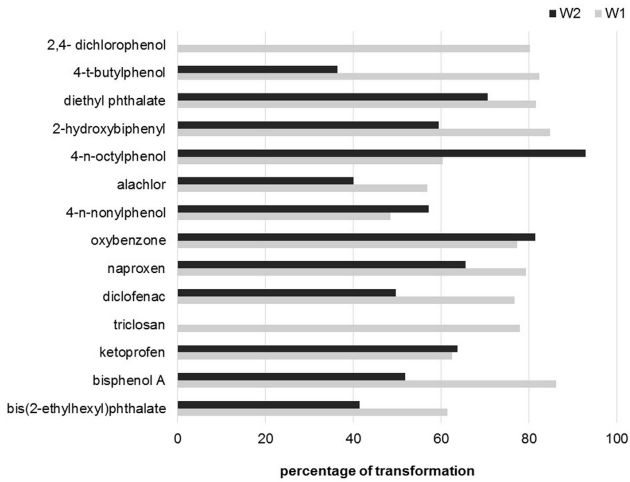

Fig. 1. Percentage of transformation of micropollutants in W1 and W2 in the presence of 100 U/L laccases.

against 4-n-octylphenol (24%) (Table 1), it was a perfect substrate for laccases: in W2 concentration decreased up to 93%. On the contrary, the conversion yields of 8 micropollutants were lower than in the presence of W1. For instance, 4-*t*-butylphenol was removed up to 82.4% and 36.4% in W1 and W2, respectively. This change in the laccases performances cannot be associated to the chemico-physical features of the micropollutants, but diffusion problem may have instead influence the enzymatic catalytic efficiency. For instance, this has proved to be a key factor in the 2,4-dichlorophenol degradation: optimal rates could be obtained only after increasing the initial concentration from 10 to 20 mg/L (Yang et al., 2015). Laccases could have suffered the low residual concentration in W2 (0.12–0.51 μg/L).

The oxidation capability shown by *T. pubescens* laccases is an important step forwards for the actual exploitation on an enzyme-based treatment in WTPs. The active role of laccases on micropollutants degradation is well documented for phthalates, phenols, naproxen and diclofenac (Cabana et al., 2007; Boonnorat et al., 2016; Ashe et al., 2016; Alshabib and Onaizi, 2019; Rangelov and Nicell, 2019). As regards naproxen, some authors studied whole cell systems using *Trametes versicolor*, directly associating the degradation to the production of laccases (Marco-Urrea et al., 2010; Rodríguez-Rodríguez et al., 2010). For many other micropollutants, the available reports are very scarce or offer contrasting results, as for oxybenzone, triclosan, alachlor and ketoprofen. Oxybenzone is usually recalcitrant to enzymatic oxidation. Laccases of *T. versicolor* and *P. ostreatus* obtained a null or poor (23%) removal, respectively (Garcia et al., 2011; Ashe et al., 2016). Remarkable changes occurred just in the presence of mediators as 1-hydroxy-benzotirazole, violuric acid, ABTS, sinapinic acid and acetosyringone. In the present study, the high yields (>75%) could be a clear indication of the presence of natural mediators in the enzymatic crude extract that strengthened the oxidation cascade. Likewise, few authors reported the difficulties of triclosan oxidation by direct laccase binding (Dou et al., 2018; Sun et al., 2019), also due to its capability to inhibit laccases (Rangelov and Nicell). These data are not in agreement with the present study, where triclosan was removed up to 78%. Jahangiri et al. (2018) can give a possible explanation. They observed that the simultaneous presence of easily laccase oxidizable compounds (e.g. acetaminophen) could favour the transformation of more recalcitrant pollutants (e.g. triclosan). Although the mechanism is unclear, some other micropollutants in W1 may have acted as a mediator-like, ultimately enhancing laccases reaction. As regards alachlor, fungi as *Trichoderma koningii* and *Paecilomyces marquandii* can trigger oxidation cascades involving cytochrome P450 and laccase to transform this pollutant (Słaba et al., 2015; Nykiel-Szymańska et al., 2018). However, no enzymatic treatment has been assessed. Although fungi (i.e. *Trametes hirsuta, Trametes versicolor, Pleurotus ostreatus*) are known to transform ketoprofen (Mir-Tutusaus et al., 2016), the exact involved degradation pathway is not clear: intracellular cytochrome P450 seemed to be enrolled (Haroune et al., 2017), but contrasting results are available on laccases. When enzymatic treatments were set up, a null or scarce (2–46%) removal was monitored (Kumar and Cabana, 2016; Jahangiri et al., 2018). To date, data comparable to the present study (>60%) were obtained only by the combination of laccases and versatile peroxidases, and the presence of co-factors (Touahar et al., 2014).

It should be mentioned that many reports operated in axenic conditions. Laccases were often challenged against few compounds in sterile conditions (Varga et al., 2019) and sometimes in the presence of spiked or simulated wastewaters (Arca-Ramos et al., 2016; Touahar et al., 2014; Ba et al., 2018; Jahangiri et al., 2018). They often showed severe technological and analytical problems. For instance, the transformation of 8 EDCs catalysed by laccases of *M. thermophila* and *T. versicolor* was followed only in spiked wastewaters because in the real effluents, the concentration was mostly below the limit of determination (Becker et al., 2017). Laccases of *T. versicolor* removed up to 62% of 2,4-dichlorophenol just in the optimal conditions, where parameters as temperature and pH were controlled (Yang et al., 2015). Sometimes the conditions are far from the environmental ones. Lloret et al. (2013) used

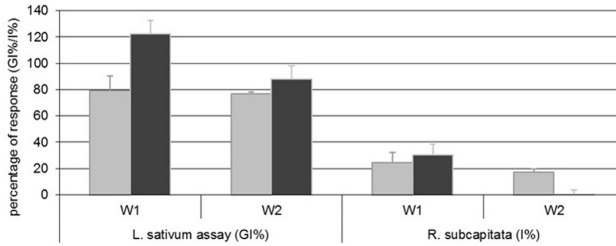

Fig. 2. Germination index of *L. sativum* (GI%) and algal growth inhibition of *R. subcapitata* (I%) of W1 and W2 before (grey) and after (black) the laccases treatment.

laccases to treat naproxen and diclofenac at 5 mg/L each. Only controlling pH and mediators presence, high reaction rates were obtained. Unfortunately, most of WTPs do not maintain pH 4, which would be instead appreciated for laccases conversion of diclofenac (Lloret et al., 2013).

Alongside the removal of micropollutants, the impact on the wastewaters toxicity was determined by ecotoxicological bioassays and estrogenic analysis. To date, ecotoxicity has been applied mostly to laccases treatment of single micropollutants, such as triclosan, ketoconazole, isoproturon and sulfamethoxazole (Inoue et al., 2010; Margot et al., 2015; Yousefi-Ahmadipour et al., 2016; Zeng et al., 2017). Except for rare exceptions, ecotoxicological impact is not commonly used to evaluate the performance of enzymatic treatments of municipal wastewaters. As widely discussed in literature, different model organisms displayed a different sensitivity to samples reacting to the compounds presence, their concentration and the established but unpredictable synergic effects. Laccases treatment detoxified the samples (Fig. 2). As regards the *L. sativum* assay, laccases reduced the initial toxicity of W1, leading to a biostimulation effect of the plant (IG > 100%). Otherwise, no significant changes were noticed for W2. *R. subcapitata* assay showed an opposite behaviour. W1 toxicity did not change whereas, after the treatment, W2 did not inhibit anymore the algal growth. A similar behaviour was observed during the treatment of a wastewater collected after the primary sedimentation: after the laccases treatment, seeds were biostimulated and algae were less inhibited (Spina et al., 2015).

It is noteworthy that the effluent toxicity never increased after the enzymatic treatment, as often observed in literature due to the formation of toxic transformation products (Anastasi et al., 2010). In some cases, the enzymatic reaction may even lead to the formation of estrogenic products. For instance, although the flame retardant tetrabromobisphenol A did not induce any stimulation effect of MCF-7 cells, some products of the laccase reaction were instead toxic (Uhnáková et al., 2011). They were even identified as the same intermediates formed during the hepatic biotransformation of tetrabromobisphenol A (Zalko et al., 2006). Laccases here demonstrated a complete different impact. Indeed, laccases treatment decreased the estrogenic activity of wastewaters. Treated and untreated W1 and W2 showed a partial agonistic activity both in *E-screen* assay (RPE% 68 ± 15 and 81 ± 8, respectively) and in MELN assay (TRANS% 85 ± 13 and 57 ± 9, respectively). Laccases reduced the EEQs of samples measured with *E-screen* assay (EEQs equal to 1.4 ± 0.4 ng/L for treated W1, 0.5 ± 0.03 ng/L for treated W2) (Fig. 3). The average reduction in the estrogenic activity after laccases treatment was 93.7 ± 2.0% for W1 and 92.0 ± 0.6% for W2. In accordance with the *E-screen* assay, the MELN assay detected a decrease of estrogenic activity (EEQ equal to 1.4 ± 1.0 ng/L for

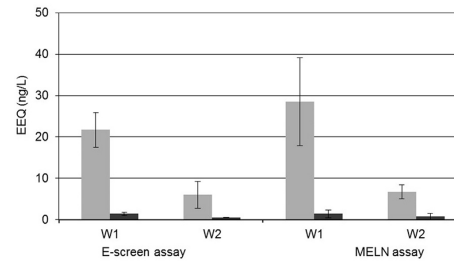

Fig. 3. Estradiol equivalent concentrations (EEQs, ng/L) of W1 and W2 before (grey) and after (black) the laccases treatment measured with *E-screen* assay and MELN assay.

treated W1, 0.8 ± 0.7 for treated W2). The reduction in the estrogenic activity was 94.9 ± 3.4% for W1 and 88.6 ± 9.8% for W2. The correlation between the EEQs obtained with the *E-screen* assay and with the MELN assay was positive and significant ($p < 0.05$). The EEQs after the treatment resulted closer to the safe levels for municipal WWTP effluents proposed by Jarošová et al. (2014).

In order to compare chemical and biological analyses, the concentration of each estrogenic compound detected in water samples by chemical analysis was multiplied by its estrogenic potency relative to 17-β-estradiol and the results were summed in order to obtain the EEQ predicted from the chemical analysis. The estrogenic potency reported by other authors was used for calculation of predicted EEQs (Table 2). The EEQs predicted from the chemical analysis were then compared with the EEQs measured with the *E-screen* assay and MELN assay (Table 3).

For all samples, the predicted EEQs were much lower than the measured EEQs. Other studies reported that estrogenic activity of wastewater measured with bioassays was higher than predicted activity (Soto et al., 2004; Sarmah et al., 2006; Välitalo et al., 2016). This discrepancy could be probably due to the presence of other estrogenic compounds, which were not targeted by the screening approach here applied. Moreover, the difference between predicted and measured EEQs could be due to the effects induced by co-exposure to multiple chemicals, since synergistic/antagonistic/additive effects are not taken into account by prediction approach. These results confirmed the importance of the use of effect-based monitoring tools. Indeed, through the evaluation of the total response induced by known and unknown chemicals present in environmental matrices, the effect-based monitoring tools are a useful complement to chemical monitoring because they allow a

Table 3
EEQs (ng/L) of samples predicted from chemical analysis and measured with the *E-screen* assay/MELN assay.

	EEQ (ng/L)	EEQ (ng/L)	EEQ (ng/L)	EEQ%	EEQ%
	predicted	*E-screen* assay	MELN assay	predicted/measured *E-screen* assay	predicted/measured MELN assay
W1 untreated	0.15	21.7 ± 4.2	28.5 ± 10.6	0.7	0.5
W1 treated	0.03	1.4 ± 0.4	1.4 ± 1.0	2.1	2.1
W2 untreated	0.05	6.0 ± 3.2	6.7 ± 1.7	0.8	0.7
W2 treated	0.02	0.5 ± 0.03	0.8 ± 0.7	4.0	2.5

more holist approach (Wernersson et al., 2015) and improve the detection of estrogenic endocrine disruptors in environmental samples (Gou et al., 2016). On the contrary, the use of chemical analysis alone may underestimated the estrogenic activity of wastewaters, emphasizing the importance of effect-based monitoring tools for the assessment of the wastewater quality.

4. Conclusions

The effective removal of micropollutants in municipal wastewaters has been demonstrated: laccases of *T. pubescens* MUT 2400 reduced the chemical load of the samples and caused a significant abatement of the potential ecological impact. The degradation of micropollutants entailed also the strong decrease of the toxicity, which addressed both aquatic organisms and human health.

The capability of the enzymatic treatment to degrade a broad spectrum of micropollutants indicated that it could be applied to WTPs to treat municipal wastewaters as well as contaminated surface waters. Due to the low activity disturbance and the good transformation rates, these results suggest that laccases can be applied as an advance oxidation system at the very end of the WTP. They could then work on those molecules that were left behind by previous treatments. Future investigation should be aimed to the development of targeted technologies to strengthen the enzymes stability (e.g. immobilization) and to scale up the system (e.g. optimal reactor configuration).

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Appendix A. Supplementary data

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SUPPLEMENTARY MATERIALS AND METHODS

1. Analytes extraction and quantification

Samples were submitted to SBSE targeted sampling with *ad hoc* derivatization (i.e., phenols, acids/amines and apolar compounds). A reference multi-target SBSE procedure was optimized to extend its sampling effectiveness to the wide range of chemicals tested in the present study. Table S1 reports CAS registry numbers, *in-situ* derivatizing agent adopted, absolute retention time of the main derivative (min) and, Target Ions (Ti) and Qualifiers adopted for quantitation. Based on the specific functionalities and polarities, analytes were submitted to a directed *in-situ* derivatization, which enhances absolute recoveries and improves method sensitivity. Acetic acid anhydride (AAA), methanol (MeOH) and ethyl chloroformate (ECF) were used as derivatization agent. SBSE was run simultaneously for the different chemical classes (phenols, acids/amines and apolar compounds) and was followed by a multi-shot thermal desorption (TD)-GC-MS analysis.

Gas chromatographic separation was run with a 30 m long, 0.25 mm ID and 0.25 µm df Mega 5 FSOT column (5% diphenyl, 95% dimethylsiloxane) (Mega, Legnano, Italy). The oven temperature was programmed from 70 °C (2 min) to 150 °C at 25 °C/min, then to 200 °C at 3 °C/min and to 280 °C at 8 °C/min (10 min), using helium in constant flow mode (1.0 mL/min) as carrier gas. The mass spectrometer transfer line, ion source and quadrupole temperatures were maintained at 280 °C, 230 °C and 150 °C, respectively; a solvent delay of 4 min was used. Electron impact ionization mass spectra were recorded at 70 eV with an ionization current of 34.6 µA. A dwell time of 10 ms was used in SIM mode, and one target ion and two qualifiers for each analyte were chosen. The simultaneous SIM/SCAN acquisition option was adopted for data acquisition which were then analysed by MSD ChemStation software (G1701CA; version D.03.00 SP1; Agilent Technologies, Little Falls, DE, USA).

2. Ecotoxicological assays

2.1 *L. sativum* assay

The seeds were guaranteed of 90% germination and no herbicides were used. 10 seeds were put in 9 cm Petri dishes (four replicates), containing a paper filter and 5 mL of the sample (Whatman

No.1). The seeds were incubated for 72 h in the dark at 25 °C. The control was carried out using distilled water. Phytotoxicity on *L. sativum* was expressed as the germination index (GI%), calculated from the following equation:

(Eq. 1) $GI\% = (G_s * L_s)/ (G_c * L_c) * 100$

where G_s is the mean number of germinated seeds in the sample, L_s is the mean root length of the sample, G_c is the mean number of germinated seeds in the control (i.e. ultrapure water), and L_c is the mean root length of the control.

The GI% combines the toxicity effect on seed germination and on root elongation in respect to the control (ultrapure water). Toxicity is associated to GI% values lower than 100%, whereas a stimulation effect is outlined by values above 100%.

2.2 *R. subcapitata* assay

Each dose-response curve consisted of 6 serial dilutions and 10^4 cells/mL were inoculated in 3 mL well containing 2.5 mL of diluted sample. The test was performed in triplicate and a forth repetition without the algal inoculum was used as the abiotic control. The samples were incubated for 72 h, at 23 °C and at 8000 lux. After 48 h, the cells concentration was measured spectrophotometrically at 663 nm. The inhibition percentage was calculated according to the following equation:

(Eq. 2) $I_{\mu i} = (\mu_c - \mu_i)/\mu_c * 100$

where

(Eq. 3) μ_c = [ln (common control) - ln 10000]/3

(Eq. 4) μ_i = [ln (cells number of the sample - abiotic control) - ln (10000)]/3.

Toxicity is expressed as the inhibition (I%) of the growth in comparison with the control. No effect is indicated as 0%, inhibition gives I% > 0, while stimulation gives I% < 0.

3. Estrogenic activity

3.1 *E-screen* assay

The *E-screen* assay was carried out as initially described by Körner et al. (1999) and modified by Schilirò et al. (2009; 2012). The estrogenic activity of a sample is evaluated by determining the

relative efficacy, called the relative proliferative effect (RPE%). The RPE compares the maximum proliferation induced by a sample with that induced by 17-β-estradiol (E2):

(Eq. 5) RPE % = $[(PE\text{-}1)_{sample}/(PE\text{-}1)_{E2}] * 100$

Full agonistic activity, RPE ≥ 100%, can be distinguished from partial agonistic activity, in which RPE is less than 100%. Relative potency, called estradiol equivalent concentration (EEQ), was calculated as:

(Eq. 6) $(EC50)_{E2}*(EC50)_{sample}$

The EC50 value for the *E-screen* assay is the dilution factor at which 50% of PE is achieved. The PE and EC50 values of each sample were calculated from the mean dose–response curves established from each experiment. The EEQ, expressed in ng/L, is defined as the total concentration of estrogenic active compounds in an environmental sample normalised to the natural estrogen 17-β-estradiol.

3.2 MELN gene-reporter luciferase assay

The test was carried out as described by Balaguer et al. (1999), using previously reported methods (Schilirò et al. 2012). The luciferase activity of the MELN cells relative to the positive control E2 was represented as transactivation % (TRANS %): the maximum increase in luciferase gene expression triggered by estrogenic compounds present in the samples. A value of 100% induction of luciferase activity is associated to the presence of E2. The estrogenic activity was expressed as estradiol equivalent concentration (EEQ) in ng/L. EEQ was calculated as:

(Eq. 7) $(EC50)_{E2}*(EC50)_{sample}$.

The EC50 value for the MELN assay is the dilution factor at which 50% of luciferase activity is achieved.

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Supplementary Tables

Table S1. Physico-chemical parameters of the wastewater collected in the municipal WWTP. Chemical oxygen demand (COD), total sediments, total content of organic and inorganic nitrogen (N tot and NH_4), total content of phosphorous (P tot), TSS, total suspended solids (TSS) and pH are reported.

	Classification	W1	W2	Removal %
2,4-dichlorophenol	herbicide	0.16 ± 0.03	nd	100
4-*t*-butylphenol	vamish	1.30 ± 0.21	0.18 ± 0.03	86
diethyl phthalate	plasticizer	24.7 ± 2.22	15.70 ± 1.41	36
2-hydroxybiphenyl	pesticide	1.80 ± 0.21	0.12 ± 0.01	93
4-*n*-octylphenol	surfactant	0.95 ± 0.15	0.72 ± 0.12	24
salicylic acid	drug	nd	nd	na
alachlor	herbicide	0.39 ± 0.08	0.40 ± 0.09	-3
4-*n*-nonylphenol	surfactant	0.29 ± 0.04	0.37 ± 0.05	-28
oxybenzone	PCPs	0.66 ± 0.07	0.30 ± 0.03	55
naproxen	drug	2.20 ± 0.31	0.16 ± 0.02	93
diclofenac	drug	0.84 ± 0.16	0.51 ± 0.10	39
triclosan	PCPs	0.44 ± 0.07	nd	100
ketoprofen	drug	14.30 ± 2.76	15.50 ± 2.99	-8
bisphenol A	plasticizer	5.20 ± 0.99	0.51 ± 0.10	90
bis(2-ethylhexyl) phthalate	plasticizer	350 ± 40	315 ± 36	10
Total load		**403.16**	**349.47**	

Table S2. List of target analytes and classification (EDCs, pharmaceuticals, pesticides and PCPs). Analytes are reported together with CAS Registry Numbers, *in-situ* derivatizing agent adopted to improve SBSE sampling effectiveness, absolute retention time of the main derivative (min) and, list of Target Ions and Qualifiers (Ti in reported in italic).

	Estrogenic potency relative to E2 (E2=1)	**Reference**
2,4-dichlorophenol	no estrogenic activity	Ghisari et al. 2009; Kuckelkorn et al. 2018
4-*t*-butylphenol	1.90E-07	Olsen et al. 2005
diethyl phthalate	5.00E-07	Tan et al. 2007
2-hydroxybiphenyl	1.50E-06	Korner et al. 2000
4-*n*-octylphenol	1.40E-06	Murk et al. 2002
salicylic acid	no estrogenic activity	no reference found; absent in list of estrogenic chemicals reported by Kiyama et al. 2015
alachlor	8.00E-06	Scippo et al. 2004
4-*n*-nonylphenol	7.60E-05	Korner et al. 2001
oxybenzone	3.30E-07	Schlumpf et al. 2001
naproxen	antiestogenic activity	Ezechias et al. 2016
diclofenac	antiestogenic activity	Ezechias et al. 2016; Kuckelkorn et al. 2018
triclosan	no estrogenic activity	Cavanagh et al. 2018
ketoprofen	antiestogenic activity	Ezechias et al. 2016
bisphenol A	2.00E-05	Olsen et al. 2005
bis(2-ethylhexyl)phthalate	no estrogenic activity	Murk et al. 2002

Supplementary Figures

Figure S1. List of target micropollutants.

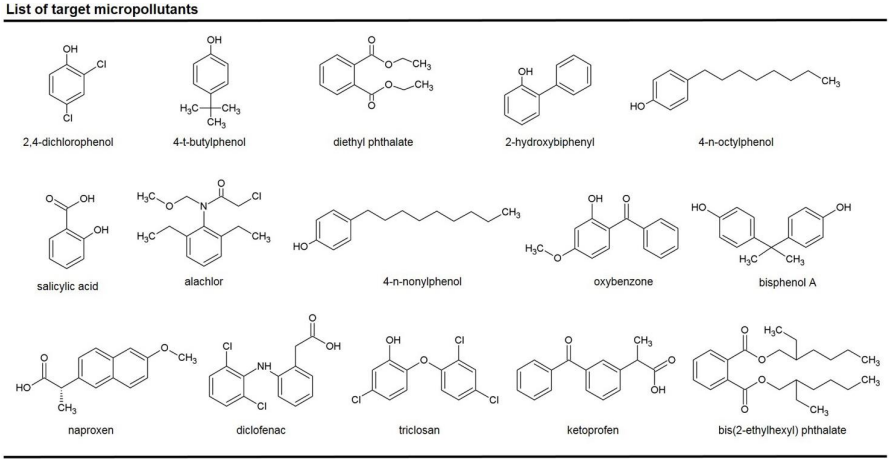
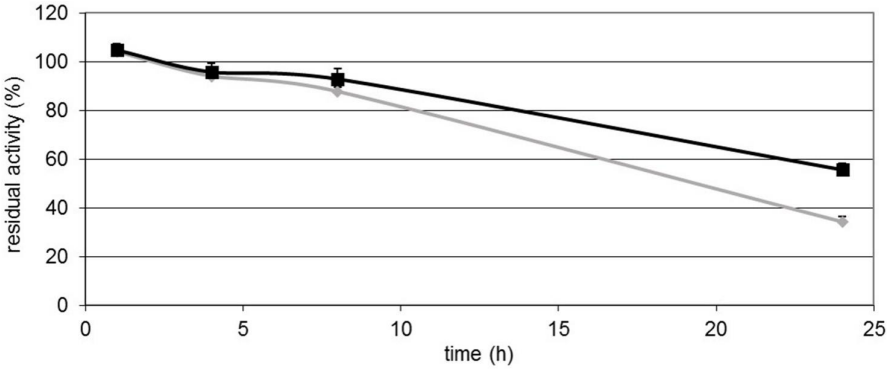


Figure S2. Residual laccase activity during the 24 h experiment in presence of W1 (grey line) and W2 (black line).



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Article

In Vitro Effects of Particulate Matter Associated with a Wildland Fire in the North-West of Italy

Marta Gea [1], Sara Bonetta [1,*], Daniele Marangon [2], Francesco Antonio Pitasi [2], Caterina Armato [1], Giorgio Gilli [1], Fabrizio Bert [1], Marco Fontana [2] and Tiziana Schilirò [1]

[1] Department of Public Health and Pediatrics, University of Torino, 10126 Torino, Italy; marta.gea@unito.it (M.G.); cati.armato@gmail.com (C.A.); giorgio.gilli@unito.it (G.G.); fabrizio.bert@unito.it (F.B.); tiziana.schiliro@unito.it (T.S.)

[2] Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), 10095 Grugliasco, Italy; daniele.marangon@arpa.piemonte.it (D.M.); francesco.pitasi@arpa.piemonte.it (F.A.P.); marco.fontana@arpa.piemonte.it (M.F.)

* Correspondence: sara.bonetta@unito.it (S.A.); Tel.: +39-011-670-3189

Abstract: Wildland fires, increasing in recent decades in the Mediterranean region due to climate change, can contribute to PM levels and composition. This study aimed to investigate biological effects of $PM_{2.5}$ (Ø < 2.5 µm) and PM_{10} (Ø < 10 µm) collected near a fire occurred in the North-West of Italy in 2017 and in three other areas (urban and rural areas). Organic extracts were assessed for mutagenicity using Ames test (TA98 and TA100 strains), cell viability (WST-1 and LDH assays) and genotoxicity (Comet assay) with human bronchial cells (BEAS-2B) and estrogenic activity using a gene reporter assay (MELN cells). In all sites, high levels of PM_{10} and $PM_{2.5}$ were measured during the fire suggesting that near and distant sites were influenced by fire pollutants. The PM_{10} and $PM_{2.5}$ extracts induced a significant mutagenicity in all sites and the mutagenic effect was increased with respect to historical data. All extracts induced a slight increase of the estrogenic activity but a possible antagonistic activity of PM samples collected near fire was observed. No cytotoxicity or DNA damage was detected. Results confirm that fires could be relevant for human health, since they can worsen the air quality increasing PM concentrations, mutagenic and estrogenic effects.

Keywords: forest fire; genotoxicity; mutagenicity; endocrine disruptors; particulate matter; air pollution; cytotoxicity; biological assays; BEAS-2B cells; MELN cells

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1. Introduction

Wildland fires are complex phenomena influenced by numerous factors such as land management, human activities, and weather conditions [1].

All over the world climate change has been causing an increase in the frequency and intensity of extreme weather events [2] affecting also wildland fires. Indeed, the increase of occurrence of heatwaves and droughts, induced by climate change, has been promoting ignition and propagation of wildland fires and has also been causing effects on vegetation and forest fuels resulting in high risk of wildland fires [1]. The Mediterranean region is considered a high responsive area to global warming [3,4] and the combination of warmer and drier conditions will rise the risk of large fires characterized by an extensive burned area in the next decades [5,6].

Wildland fires induce both environmental and human health effects on firefighters as well as on the general population [7]. It was estimated that the exposure to wildland fire smoke causes each year a mortality equal to 339,000 deaths in the world [8,9]. Wildland fires can be associated with effects on the respiratory system (e.g. asthma and chronic obstructive pulmonary disease exacerbations, respiratory symptoms, decrease in lung function) [5,8]. However, exposure to wildland fires has been associated also with acute cardiovascular effects and birth outcomes [7,8].

Wildland fires can produce PM, aromatic hydrocarbons, aldehydes, metals, dioxins, furans, benzene, formaldehyde and other airborne pollutants [7,10,11]. However, the composition of produced smoke changes temporally and spatially according to the type of combustion and type of fuel [7,8,12,13].

The estimated emission of $PM_{2.5}$ (Ø < 2.5 μm) from wildland fires is 0.9–16 g for each kg of burned dry biomass [10]. In South Europe as well as in South America, it was demonstrated that the release of PM by wildland fires, can significantly contribute to the airborne PM levels causing the exceeding of air quality standards [5,14–16]. These standards are set for PM regardless of its origin; however, the composition of wildland fire PM may be different from urban PM. Indeed, it has been demonstrated that particles from wood combustion have a higher content of total polycyclic aromatic hydrocarbons with respect to vehicle exhaust particles [17]. Moreover, different studies have shown that particles from biomass burning are predominantly composed of organic compounds and have lower metal concentrations than particles not deriving from biomass combustion [11,13]. These differences could lead to different biological effects.

In order to evaluate the biological effects of PM, useful tools are represented by in vitro assays. They can inexpensively and quickly assess the total effect induced by a complex matrix, providing also information on the mechanism of action. In recent years, a growing concern has been focused on effects induced against the endocrine system and these effects have been studied using different in vitro assays such as estrogenic activity assays [18]. However, while numerous studies have applied these assays on waters and sediments [19], little is known about the estrogenic activity of airborne PM [20]. In contrast to the large amount of information on cytotoxicity, genotoxicity and mutagenicity induced by urban emissions [21,22], only few studies have evaluated biological effects induced by biomass combustion emissions during real wildland fires [23]; moreover, to our knowledge, the estrogenic activity of PM released from wildland fires has not yet been assessed.

The aim of this study was to investigate different in vitro effects of PM collected during the wildland fire that occurred in October 2017 in Torino province (Piedmont region, North-West of Italy), an area characterized by critical air pollution levels due to geographical and meteorological conditions which promote the accumulation of air pollutants. $PM_{2.5}$ (Ø < 2.5 μm) and PM_{10} (Ø < 10 μm) were collected near the wildland fire and in three other areas of Piedmont (two urban and one rural). Mutagenicity of organic extracts was assessed on different strains of *Salmonella typhimurium* (TA98, TA100) using Ames assay, while cell viability (WST-1 and LDH assays) and genotoxicity (Comet assay) were evaluated on human bronchial epithelial cells (BEAS-2B). Finally, in order to analyze the endocrine disrupting effects of PM, the estrogenic activity of organic extracts was estimated through a gene reporter assay on MELN cells.

2. Materials and Methods

2.1. Sampling and Organic Extraction of PM

In 2017 in Europe, wildland fires burnt over 1.2 million ha of natural lands; in particular, in Italy, there were 7855 fires, which burned 161,987 ha, the highest annual total burned area since 2007. The largest Italian wildland fire of the year occurred in Torino province (North-West of Italy) in autumn and covered 3533 ha [1].

During October 2017, the Piedmont region was affected by numerous fires located in many valleys; among them, the widest wildland fire occurred in Chiomonte (Susa Valley, one of the sites of the XX Winter Olympic Games—Turin 2006). In this period, PM sampling was performed in the North-West of Italy through a mobile monitoring station and three monitoring stations of the Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte) (Figure 1). The mobile station was located near the wide wildland fire of Chiomonte (rural village—site F). The other stations are permanently located: in a urban site characterized by high traffic level (city of Torino in the Padana Plain—site T), in a urban background site characterized by moderate traffic level (city of Novara in the Padana

Plain—site N) and in a rural site (rural village of Ceresole Reale near the Gran Paradiso National Park—site C).

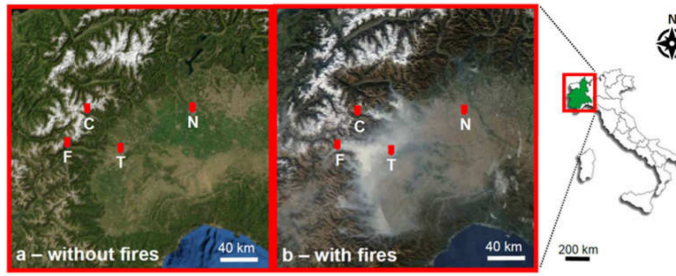

Figure 1. Geographical location of the monitoring stations. (**a**) Orthophotos, (**b**) Satellite image during wildland fires (25 October 2017) [24]. F = Chiomonte (wildland fire); T = Torino (high traffic); C = Ceresole Reale (rural); N = Novara (moderate traffic).

During the fire period, the Piedmont region was affected by a high number of days characterized by foehn wind, which contributed to the spread of fire [24]. Wind was mainly blowing from West–Southwest in site F (mean wind speed = 2.3 m/s, max wind speed = 17.9 m/s), causing the propagation of fire smoke from the fire site (F) to the Padana Plain (where are located site T and N) [24].

The four sites are characterized by different air pollution levels [25,26]. In the urban sites (T and N) the annual mean PM_{10} concentrations are higher than in the rural sites (F and C). Moreover, in urban sites a seasonal trend of air pollution is particularly evident: PM_{10} is higher in cold months and lower in warm months. This trend is characteristic of the study area, which is located in the Padana Plain. Here the geographical conformation together with the meteorological conditions promotes the accumulation of airborne pollutants, causing the exceeding of the WHO limits in particular during autumn-winter months [27–31].

PM_{10} (in the sites F, T, C) and $PM_{2.5}$ (in the sites F, N) were sampled on quartz-fiber filters (Ø = 47 mm) with low volume sampler (flow 2.3 m^3/h). For the mobile station (site F) PM filters were collected daily during the days characterized by the forest fire (from 17 October 2017 to 31 October 2017), while for the other stations (sites T, N, C) PM filters were collected daily during the whole October month (from 1 October 2017 to 31 October 2017). PM mass was estimated through a gravimetric method in compliance with the EN 12341 norm [32].

The daily filters were pooled to obtain one sample for each site (15 half filters for site F, 31 half filters for T, C, N sites) and each pool was extracted with Soxhlet (80 cycles) using acetone/hexane (1:1) in order to collect organic-extractable compounds. Extracts, evaporated with a rotary evaporator and re-suspended in dimethyl sulfoxide (DMSO), were stored at −20 °C until analysis.

2.2. *Air Pollution Data*

Air pollution data provided by the ARPA Piemonte were analysed in order to establish the effect of wildland fire on air quality in the different sites. In particular, the monthly mean of PM_{10} in the different sites was analysed from January 2016 to December 2018. Moreover, the concentration of specific tracers in PM for biomass burning emissions (levoglucosan, mannosan and galactosan) [33] during the wildland fire days was also considered. Data were only available for site F and site T.

2.3. Cell Cultures

BEAS-2B, human bronchial epithelial cells, were obtained from the American Type Culture Collection. BEAS-2B were grown and maintained in RPMI 1640 medium (supplemented with phenol red, fetal bovine serum (FBS) (10% v/v), L-glutamine (4 mM), penicillin-streptomycin (100 U/mL–100 µg/mL)), at 37 °C and 5% CO_2. BEAS-2B were used to perform cytotoxicity and genotoxicity assays. Among the cell lines derived from the respiratory system, BEAS-2B were selected since they are non-tumoral derived cells characterized by normal growth and differentiation; moreover, they have been demonstrated to be a useful model for cell death and carcinogenesis studies [22].

MELN cells, provided by Dr. P. Balaguer (INSERM, Montpellier, France), are MCF-7 cells stably transfected with an estrogen-responsive gene (ERE-bGlob-Luc-SVNeo) carried by integrated plasmids. These plasmids contain both an antibiotic resistance selection gene (SVNeo) and the estrogen-responsive elements to which the estrogen receptor-ligand complex can bind, thereby inducing the transcription of the luciferase reporter gene. Therefore, the luciferase activity measured is proportional to the concentration of estrogenic compounds. MELN were grown and maintained in complete Dulbecco's Modified Eagle's Medium Nutrient Mixture F12-Ham (supplemented with phenol red FBS (5% v/v), L-glutamine (4 mM), penicillin-streptomycin (100 U/mL–100 µg/mL), G418 (1 mg/mL)), at 37 °C and 5% CO_2. MELN were used to perform the luciferase gene reporter assay.

2.4. Cell Viability

Cell viability was assessed with WST-1 assay (Cell Proliferation Reagent WST-1, Roche) and lactate dehydrogenase release assay (LDH assay) (Cytotoxicity Detection Kit PLUS, Roche). The assays were performed as previously described by Gea et al. [34]. Briefly, BEAS-2B (70% confluent) were seeded in 24-well plates (4×10^4 cells/well) and cultured overnight. Then, culture medium was replaced with organic extracts (equivalent to 0.5, 1, 2 m^3/mL, see Table S1 in Supplementary Materials for the concentrations expressed as µg/mL) in RPMI medium without phenol red and cells were incubated for 24 h, 48 h or 72 h. The tested doses were selected in order to be representative of the real-life exposure.

For the WST-1 assay, after exposure, dye solution containing WST-1 was added (50 µL/well) and the cells were incubated for 2 h (37 °C, 5% CO_2). Finally, the absorbance of each well was measured at 440 nm (Infinite Reader M200 Pro, Tecan). Cells exposed to the same amount of DMSO of the organic extract dilutions were used as negative control. The maximum percentage of DMSO tested was equal to 1%. Blank filter extract was also tested to verify that it did not induce any effect on cell viability.

For the LDH assay, after exposure the supernatant of each well (100 µL) was transferred into a clean well of a 96-well plate, mixed with Reaction Mixture (100 µL/well) and incubated for 30 min (room temperature). The reaction was interrupted with Stop Solution (50 µL/well) and the absorbance was measured at 490 nm (Infinite Reader M200 Pro, Tecan). Cells exposed to DMSO were used as negative control, while cells exposed to DMSO and lysed with Lysis Solution were used as positive control.

In both assays, all experiments were performed in triplicate (three wells for each experimental condition) and data were expressed as a percentage of viability with respect to negative control (100%).

2.5. Genotoxicity

The Comet assay was performed according to Tice et al. [35] with slight modifications [36]. BEAS-2B (70 % confluent) were cultured overnight in 6-well plates (3×10^5 cells/well). Then, culture medium was replaced with organic extracts (equivalent to 0.5, 1, 2.5 m^3/mL, see Table S1 in Supplementary Materials for the concentrations expressed as µg/mL) in base RPMI medium (without phenol red and without FBS) and cells were incubated for 24 h. The tested doses were selected in order to be representative of real-life exposure. After exposure, cell viability was determined (trypan blue staining) and cells were placed in low melting point agarose (0.7%) on slides. Slides were placed overnight in lysis solution (4 °C), were

immersed in an alkaline electrophoresis buffer (20 min) and subjected to electrophoresis (20 min, 1 V/cm and 300 mA). Then, slides were neutralized, fixed and dried. For the analysis of DNA damage, cells in slides were stained with ethidium bromide (20 μg/mL) and the percentage of tail intensity was estimate using a fluorescence microscope (Axioskop HBO 50, Zeiss) equipped with the Comet Assay IV analysis system (Perceptive Instruments, Instem). Cells exposed to DMSO were used as negative control (maximum percentage of DMSO tested = 1%), while 4-nitroquinoline N-oxide (1, 1.5, 2 mg/L) was used as positive control. Blank filter extract was also tested to verify that it did not induce any DNA damage. All experiments were performed in duplicate (two gels for each experimental condition) and in each gel the % of tail intensity was quantified considering 50 cells.

2.6. Ames Test

In order to assess the mutagenicity, the Ames test was performed on each organic extract according to Maron and Ames [37]. Extracts were tested at different concentrations (2.5, 5, 10 and 20 m^3/plate) in duplicate using two *Salmonella typhimurium* strains (frameshift strain-TA98 and base-substitution strain-TA100) both with and without Aroclor-induced rat-liver homogenate activation (±S9). 4-nitroquinoline 1-oxide (0.5 μg/plate), methyl methane-sulfonate (0.25 μg/plate) and 2-aminoanthracene (2 μg/plate) were used as positive controls for TA98, TA100 and TA98 + S9/TA100 + S9, respectively. DMSO was added to plates as negative control (100 μL/plate). Plates were incubated for 48 h and colonies were counted through an automatic colony counter (Synoptics Protos).

The results were expressed as: (i) total revertants; (ii) mutagenicity ratio per 20 m^3, MR [MR = (total revertants in plate exposed to 20 m^3 − spontaneous revertants)/spontaneous revertants]; (iii) and total mutagenicity factor per 20 m^3, TMF (TMF = MR TA98 + MR TA98 (+S9) + MR TA100 + MR TA100 (+S9)). For each strain, with or without S9, the mutagenic effect was considered significant when mean of total revertants were at least twice than mean of spontaneous revertants (MR $\geq$ 1), while an extract was considered mutagen when at least one strain, with or without S9, showed an MR $\geq$ 1. Blank filter extract was also tested to verify that it did not induce any mutagenic effect.

2.7. Estrogenic Activity

Estrogenic activity was assessed with the luciferase gene reporter assay using the One-Glo Luciferase Assay System (Promega). The assay was carried out as described by Balaguer et al. [38] with some modifications [39]. For three days cells were cultured in test medium (Dulbecco's Modified Eagle's Medium Nutrient Mixture F12-Ham supplemented with dextran-coated charcoal-treated FBS (5% *v*/*v*), L-glutamine (4 mM), penicillin-streptomycin (100 U/mL–100 μg/mL)). MELN (70% confluent) were seeded in 96-well plates with a flat clear bottom (4×10^4 cells/well) and cultured overnight. The day after, test medium was replaced with organic extracts (equivalent to 0.5, 1, 2 m^3/mL, see Table S1 in Supplementary Materials for the concentrations expressed as μg/mL) in test medium and cells were incubated for 20 h. At the end of the incubation, One-Glo Reagent (100 μL/well) was added and, after 5 minutes, the luminescence was measured by a luminometer (Infinite Reader M200 Pro, Tecan). Cells exposed to DMSO were used as negative control (maximum percentage of DMSO tested = 1%) and five concentrations of 17β-estradiol (range 10^{-12}–10^{-8} M) were tested as a standard positive curve. The extracts (1 m^3/mL) were also tested in combination with tamoxifen (10^{-6} M) in order to confirm whether the effects observed were due to activation of estrogen-receptors (ERs) and in combination with 17β-estradiol (10^{-10} M) to test the interaction between each extract and 17β-estradiol. Blank filter extract was also tested to verify that it did not induce any estrogenic activity.

The luciferase activity was expressed as fold induction with respect to negative control (fold induction = 1). All experiments were performed in quadruplicate (four wells for each experimental condition).

2.8. Statistical Analysis

Statistical analysis was performed using SPSS 26.0 (IBM Statistics, Armonk, NY, USA). The *t*-test and the one-way ANOVA test followed by post-hoc Dunnett test were used to assess significant differences between effects induced by extracts and effects induced by negative controls. Moreover, the *t*-test was used to assess significant differences among the mutagenic effects induced by the extracts collected in different sites. The differences were considered significant for $p < 0.05$.

3. Results and Discussion

3.1. Air Pollution Data

In Table 1 are reported the mean PM concentrations in the four sites during the sampling period. All PM concentrations were above the daily guideline values set by the WHO [40], except for the site C. These high values may be partially due to the increase of PM levels due to wildland fire but also to other PM sources such as traffic, home heating, and industrial processes especially in urban sites (T and N).

Table 1. Characteristics and PM concentrations of the sampling sites.

Site			PM		
Location	**Acronym**	**Characteristics**	**Diameter (μm)**	**Concentration ± SD (μg/m³) #**	**Daily Guideline Values (μg/m³) ***
Chiomonte	F1	Rural (wildland fire)	10	51 ± 39	50
Torino	T	Urban high traffic		79 ± 46	
Ceresole Reale	C	Rural		30 ± 39	
Chiomonte	F2	Rural (wildland fire)	2.5	43 ± 34	25
Novara	N	Urban back-ground		36 ± 21	

monthly mean concentration (October 2017) for T, C, N; mean concentration from 17 October 2017 to 31 October 2017 for F. * according to WHO guideline values (2006).

In order to establish whether the high values of PM in the different sites were influenced by the wildland fire, for each site the monthly means of PM_{10} were analysed from January 2016 to December 2018.

As can be seen in Figure 2, despite the different pollution levels of the four sampling sites, in all the sites a particular high level of PM_{10} was registered during the wildland fire month (October 2017). This evidence suggests that not only the sites near the wildland fire but also distant sites, such as N, were probably influenced by the air pollutants associated with the wildland fire.

The influence of the wildland fire on the air quality of rural and urban areas was further investigated considering the concentrations of specific biomass burning tracers in PM [33]. In particular, the concentrations of levoglucosan, mannosan and galactosan in the PM_{10} were selected as biomass burning tracers, because, during combustion, large quantities of these compounds are emitted in the smoke aerosol. The daily concentrations of these three tracers in the rural site F (near fire) and in the urban site T are reported in Supplementary Materials Figure S1. In the wildland fire site (F) as well as in the urban site (T) there is an increase of the concentrations starting from the 23 October for all the biomass burning tracers, corresponding with the days characterized by the most intense burning. It is interesting to highlight that, for all the tracers, the concentrations increase earlier in the site F (near the wildland fire) with respect to the urban site T suggesting the presence of a temporal shift of the air pollution levels. This shift could be due to the time needed for the air pollutants released by the fire to reach the urban site.

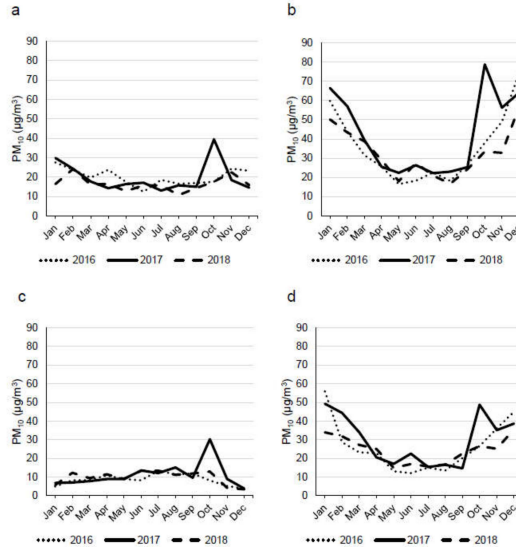

Figure 2. Monthly mean of PM_{10} concentrations in the four sampling sites: Chiomonte—site F (**a**), Torino—site T (**b**), Ceresole Reale—site C (**c**), Novara—site N (**d**). Wildland fire period = October 2017.

Taking together, the air pollution data confirm that wildland fires are associated with an increase of PM as reported by previous studies [41–45]. Moreover, these data suggest that the air pollutants released by wildland fires can influence the air quality in the nearby area but also in many surrounding areas.

3.2. Cell Viability and Genotoxicity

Since the airborne pollution induced by wildland fires can mainly affect the respiratory system, the cell viability and the genotoxicity were investigated using human bronchial epithelial cells (BEAS-2B).

Despite the different doses and the different exposure times, the extracts did not induce any cytotoxic effect in the WST-1 assay (*t*-test vs. negative control $p > 0.05$, Supplementary Materials Figure S2). Using the LDH assay, no cytotoxicity was detected (*t*-test vs. negative control $p > 0.05$, Supplementary Materials Figure S3), confirming that all the extracts did not induce a decrease in cell viability with respect to negative control. These results are consistent with those of De Oliveira Galvao et al. [46], who demonstrated that PM_{10} organic extracts collected in the Brazilian Amazon during wildland fires did not induce any cytotoxic effect in human alveolar epithelial cells (A549). However, these results differ from other published studies [41,42,45,47] in which a significant decrease in cell viability was detected exposing cells (A549 or RAW 264.7) to PM produced by wildland fires.

Moreover, all the PM extracts did not induce any genotoxic effect measured as DNA damage (One-way ANOVA test followed by post-hoc Dunnett test vs. negative control $p > 0.05$, Supplementary Materials Figure S4), while in previous in vitro studies a significant genotoxic effect was induced by PM released from wildland fires [41,46,48].

The different results obtained in this study with respect to previous studies could be due to differences in PM composition which is influenced by the different characteristics of wildland fire (e.g. extension, type of combustion, type of vegetation burned) [7,8,12,13].

However, it is also important to highlight that in the present study low doses of PM, representative of the real exposure, were tested.

3.3. Ames Test

The Ames test using different *Salmonella typhimurium* strains (TA98, TA100) with and without metabolic activation (±S9) was performed to assess the mutagenicity of the PM organic extracts. The results are presented in Figure 3.

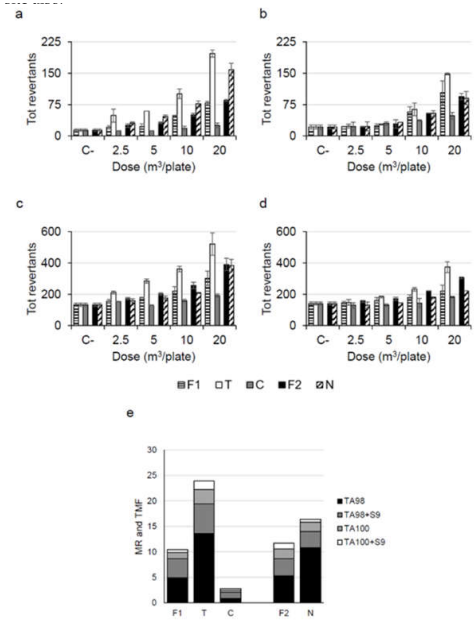

Figure 3. Mutagenicity detected in PM extracts using different *Salmonella typhimurium* strains with and without metabolic activation (± S9). Data are expressed as: total revertants and mutagenicity ratio in 20 m^3 of air (MR). TMF = Total Mutagenicity Factor (∑MR); F1 = PM_{10} extract from Chiomonte—site F (rural site, fire site); T = PM_{10} extract from Torino—site T (urban site); C = PM_{10} extract from Ceresole Reale—site C (rural site); F2 = $PM_{2.5}$ extract from Chiomonte—site F (rural site, fire site); N = $PM_{2.5}$ extract from Novara—site N (urban site). (**a**): total revertants—strain TA98, (**b**): total revertants—strain TA98 with metabolic activation (+S9), (**c**): total revertants—strain TA100, (**d**): total revertants—strain TA100 with metabolic activation (+S9), (**e**): MR and TMF—all strains with and without metabolic activation (±S9).

For both the strains, the effect was higher without metabolic activation (−S9), suggesting that the mutagenicity was mainly due to the presence of direct mutagenic substances. Moreover, the extracts induced a higher mutagenicity on TA98 than on TA100 strain, hence the mutagenic effect was induced mainly by frame-shift mutations instead of replacement of nitrogenous bases.

Considering the results obtained on TA98 strain, the samples collected in the two urban sites (T and N) induced a number of total revertants that was at least twice than mean spontaneous revertants for all tested doses (from 2.5 to 20 m^3/plate, Figure 3a), therefore a significant mutagenic effect was detected for all tested doses in these urban sites. On the

contrary, the rural sites (F and C) induced a significant effect starting from higher doses. At 20 m^3/plate the urban extracts induced a significantly higher number of TA98 revertants than the rural extracts (number of TA98 revertants at 20 m^3/plate *t*-test significant for: T vs. F1 $p = 0.014$, T vs. C $p = 0.003$, N vs. F2 $p = 0.022$). This result is in accordance with a recent study in which urban and rural samples (collected in the Piedmont region) were analyzed using the Ames test [49]. Indeed, Marangon et al. [49] also found a higher mutagenicity for the urban sites with respect to the rural site during autumn-winter months. The higher mutagenic effect of the urban sites than of the rural sites could be due to the higher content of mutagenic substances in urban areas and also to the higher PM concentration in these areas with respect to rural areas. Considering the results obtained on TA98 + S9, TA100 and TA100 + S9, a lower mutagenic effect was detected with respect to TA98. Moreover, in these experimental conditions, a higher effect was induced by the first urban extract (T site), while the mutagenic effect induced by the second urban extract (N site) was more similar to the effect induced by the rural extracts (F and C sites).

Overall, all the extracts were mutagenic; however, comparing the mutagenic effect of the different sites, the highest TMF was induced by the PM_{10} collected in the site T, an urban site characterized by high traffic levels (Figure 3e). The mutagenicity induced by this extract was particularly strong with respect to mutagenicity generally measured in this site in other years during October. Indeed, the TMFs of the PM_{10} organic extracts collected in the site T during October 2016 and October 2018 were less than a half of the TMF of the PM_{10} collected in the same site during October 2017 (site T TMF = 23.9—October 2017; TMF = 8.6—October 2016, TMF = 9.2—October 2018). This result confirmed the influence of the wildland fire on the air quality of this site.

Regarding the other PM_{10} extracts, the rural site located near the fire (F1) induced a less mutagenic effect than the organic extract collected in the site T but a more mutagenic effect than the extract collected in the other rural site (C) (number of TA98 revertants at 20 m^3/plate *t*-test significant for: F1 vs. T $p = 0.014$, F1 vs. C $p = 0.013$). This result suggests that the rural site near the fire may be influenced by some mutagenic substances released by the fire itself that are responsible for the higher mutagenic effect with respect to the effect induced by the other rural site, located more distantly from fires. It is important to highlight that the organic extract collected near the fire (F1) induced a mutagenic effect which was similar to the effect measured in the site T in a period not characterized by fires (site F1 TMF = 10.4, site T TMF = 8.6, October 2016, site T TMF = 9.2, October 2018), suggesting that the fire was able to deteriorate the air quality of this rural site. Moreover, it is also important to notice that, although the mutagenicity measured in C was low, the presence of this effect is unexpected in this site which is located far from big cities/industrial facilities and near the Gran Paradiso National Park, suggesting an influence of the fires on this site also.

As reported for PM_{10}, the mutagenicity of $PM_{2.5}$ organic extracts showed that the urban site N induced a higher effect than the site F (F2) (number of TA98 revertants at 20 m^3/plate *t*-test significant for: N vs. F2 $p = 0.022$). However, the difference of the mutagenic effect between the urban sites and the fire site was higher for PM_{10} extracts than for $PM_{2.5}$ extracts (T TMF—F1 TMF higher than N TMF—F2 TMF). This result could be explained considering that the two urban PM samples were collected in different cities, which may be characterized by different pollution levels [28]; however, this difference could be also due to the different contribution of the fire released pollutants in the different cities. Indeed, Torino (T) is located closer to the fires with respect to Novara (N).

Overall, the results of the Ames test are consistent with the air pollution data and confirm that the wildland fires are able to influence the air quality of the sites near the wildland fire (site F) but also of distant sites (i.e. site C and site T).

Comparing at equal doses the results of the Ames test with the results of the Comet assay, a significant mutagenic effect was detected in the Ames test (strain TA98, dose = 2.5 m^3/plate, site T and site N), while no significant genotoxic effect, measured as DNA damage, was observed with the Comet assay (dose = 2.5 m^3/mL) (One-way ANOVA test followed

by post-hoc Dunnett test $p > 0.05$). These results are consistent with the study of De Oliveira Galvão et al. [46], in which, testing wildland fire PM extracts, a higher effect was measured using the Ames test with respect to the effect measured using a genotoxicity test on mammalian cells (the cytokinesis-block micronucleus test). Moreover, they are consistent also with other studies on $PM_{0.5}$, $PM_{2.5}$ and PM_{10} extracts which reported a higher effect measured using the Ames test with respect to the effect measured using the comet assay on mammalian cells [36,50,51]. The different biological effects that were found using the two assays may be related to the fact that the test methods address different genetic endpoints; moreover, bacteria and mammalian cells show differences in chemical accessibility, in metabolism and in toxicity (e.g. cell wall vs. plasma membrane, lower vs. higher antioxidant activity, stress induced response consisting of mutation vs. cell death, DNA damage repair aimed at survival/mutation vs. repair aimed at fidelity) [52].

3.4. Estrogenic Activity

To our knowledge, this is the first study in which the estrogenic activity of PM released from wildland fires was assessed. Indeed, there are still few studies on estrogenic activity of PM [20,53,54] and no one has investigated this specific effect during wildland fires. However, among the numerous pollutants generated by biomass combustion, some might alter the function of the endocrine system (e.g. polycyclic aromatic hydrocarbons, dioxins, dibenzofurans) and thus they can be considered as endocrine disrupting chemicals (EDCs) [55]. EDCs can affect different hormones and many of them can interfere with the endocrine system through the binding with estrogen receptors (ERs) causing an agonist activity (estrogenic pollutants) or an antagonist activity (anti-estrogenic pollutants) [18,56].

The results of the luciferase gene reporter assay are presented in Figure 4.

The PM_{10} and $PM_{2.5}$ extracts collected near the wildland fires (F1 and F2), showed a significant increase of luciferase activity with respect to negative control only at 0.5 m^3/mL (*t*-test $p < 0.0001$, $p = 0.011$, respectively) while a significant decrease of luciferase activity was detected at 2 m^3/mL (*t*-test $p = 0.023$, $p = 0.001$, respectively). On the contrary, the PM collected in the sites T, C and N induced an increase of luciferase activity which was significant for all the tested doses (*t*-test $p < 0.01$ for all sites and doses), suggesting a higher estrogenic activity with respect to the wildland fire extracts.

Overall, the extracts showed low increases of luciferase activity with respect to negative control and thus a low estrogenic activity. Indeed, the maximum observed effect was induced by PM_{10} collected in F (wildland fire) and was equal to 1.39 luciferase activity fold induction (Figure 4a), while the standard positive curve showed a maximum effect equal to 3.54 (Figure 4f). The estrogenic activity of PM extracts, despite being low, is consistent with other studies in which an estrogenic activity was detected in PM extracts collected outdoor in urban and rural sites [20,53,57–59].

In the present study, it is interesting to notice that while the estrogenic activity of urban and rural sites increases or remains constant with the increase of the dose, the estrogenic activity of wildland fire (both PM_{10} and $PM_{2.5}$) seems to show a non-monotonic dose response (i.e. an inverted-U shaped curve). This result might be related to a cytotoxic effect/cell proliferation inhibition induced by the extracts on MELN [60]; however, this hypothesis is not supported by the cell viability results since no cytotoxic effect was observed on BEAS-2B exposed to same doses also for longer exposure times (48 h, 72 h). Hence this result might be explained considering that many EDCs shows a non-monotonic dose response profile which can be clarified by other hypothesis such as the presence of different receptors with different affinities to EDCs and opposite effects (i.e. agonist or antagonist), negative feed-back phenomena, high-dose receptor desensitization [60]. In particular, this non-monotonic profile could be due to the presence of some pollutants which, at specific concentrations, can exert an estrogen antagonist activity. For example, dioxins and polycyclic aromatic hydrocarbons are released by wildland fires [55,61] and can exert estrogen antagonist activity [55]. Therefore, the results observed in the present

study for F1 and F2 might be due to antagonist effects induced by dioxins and polycyclic aromatic hydrocarbons at the highest concentrations tested (1, 2 m^3/mL).

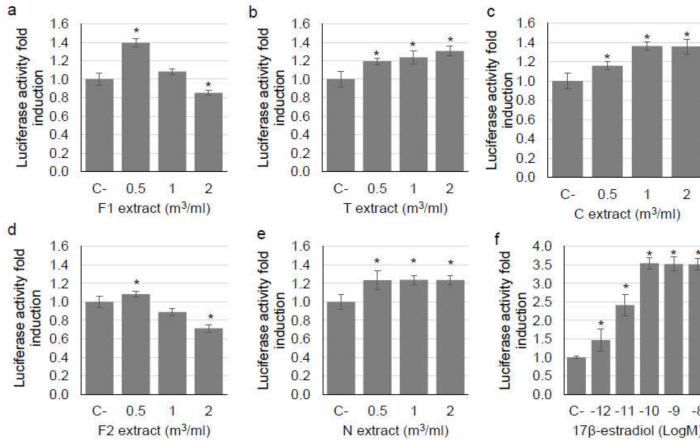

Figure 4. Estrogenic activity detected using luciferase gene reporter assay in PM_{10} extracts collected in (**a**) Chiomonte (wildland fire)—F1, (**b**) Torino—T, (**c**) Ceresole Reale—C and in $PM_{2.5}$ extracts collected in (**d**) Chiomonte (wildland fire)—F2, (**e**) Novara—N. The results are expressed as luciferase activity fold induction (means and standard deviations) respect to negative control (C- = 1). (**f**) standard positive curve, obtained exposing cells to five concentrations of 17β-estradiol (10^{-12}–10^{-8} M). * $p < 0.05$ vs. C- according to *t*-test.

This hypothesis was further investigated testing the estrogenic activity of all the extracts (1 m^3/mL) in combination with E2 (10^{-10} M). The PM collected in urban and rural sites tested with E2 (T + E2, C + E2, N + E2) showed a luciferase activity similar to the activity induced by the E2 alone (*t*-test $p > 0.05$), while the wildland fire PM tested with E2 (F1 + E2, F2 + E2) showed a significantly lower luciferase activity with respect to the E2 alone (*t*-test $p < 0.0001$ for both samples, Supplementary Materials Figure S5). This result suggests a possible antagonist effect of wildland fire PM when tested in combination with E2. An antagonistic effect of PM was previously reported also by other authors for PM samples [54,57,62].

Further studies are needed to assess the estrogenic activity of PM collected during wildland fires; indeed the data on estrogenic activity of PM is still limited and, in this area, no estrogenic activity data collected during periods without fires are available. Moreover, to our knowledge, no study has investigated the estrogenic activity of PM during wildland fires, therefore it is not possible to compare these results with others. Future studies should be performed assessing the estrogenic activity of PM in urban and rural areas of the North-West of Italy without wildland fires in order to obtain useful data for comparison. For the same reason, in future studies it may be useful to carry out a first sampling campaign in sites affected by wildland fires and also a second sampling campaign a year later in the same areas during the same period. Moreover, for future studies, areas located even more distant than the wildland fire site should be considered, in order to collect data in sites not influenced by wildland fires. Finally, in future studies, a higher amount of each sample should be collected in order to have enough extract for the assessment of the biological effects, also using higher concentrations than the concentrations tested in this study. Moreover, the sampling of many m^3 of air in each site could also allow to perform a chemical characterization of the extracts, providing additional and complementary information.

4. Conclusions

The occurrence of wildland fires is growing due to climate change and specific regions, such as the Mediterranean area, will probably be more affected than others. During

wildland fires, numerous hazardous pollutants are released in the atmosphere, representing a possible threat for human health. Since the increase of knowledge on air pollution induced by wildland fires is needed, it is of fundamental importance to investigate the effects induced by PM collected during wildland fire. The results of this study suggest that wildland fires worsen the air quality, increasing the concentrations of air pollutants, such as PM, and the mutagenic effect of the PM. This effect seems to influence the sites near fires, but also distant sites. Therefore fires, even if they are sporadic and short-time episodes, should be considered important human health issues. Moreover, in this study, the preliminary results on the estrogenic activity induced by PM collected during wildland fires highlighted some research gaps that have to be further investigated.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijerph182010812/s1, Table S1: Concentrations of PM tested in the biological assays expressed as m^3/mL and μg/mL. F1—PM_{10} collected in Chiomonte (wildland fire); T—PM_{10} collected in Torino; C—PM_{10} collected in Ceresole Reale; F2—$PM_{2.5}$ collected in Chiomonte (wildland fire); N—$PM_{2.5}$ collected in Novara; Figure S1: Daily concentrations of biomass burning tracers in the rural site F (Chiomonte) and in urban site T (Torino): levoglucosan concentrations (a), mannosan concentrations (b), galactosan concentrations (c). Data are compared with the PM_{10} concentrations measured in the same sites (PM_{10} concentrations are not available for 17th and 21st October 2017 in site F); Figure S2: Cell viability detected using WST-1 assay in PM_{10} extracts collected in (a) Chiomonte (wildland fire), (b) Torino, (c) Ceresole Reale and in $PM_{2.5}$ extracts collected in (d) Chiomonte (wildland fire), (e) Novara. Data expressed as % of cell viability (means and standard deviations) with respect to negative control (C- = 100%). *t*-test was not significant (data vs. C-, $p > 0.05$); Figure S3: Cell viability detected using LDH assay in PM_{10} extracts collected in (a) Chiomonte (wildland fire), (b) Torino, (c) Ceresole Reale and in $PM_{2.5}$ extracts collected in (d) Chiomonte (wildland fire), (e) Novara. Data expressed as % of cell viability (means and standard deviations) with respect to negative control (C- = 100%). *t*-test was not significant (data vs. C-, $p > 0.05$); Figure S4: DNA damage detected using Comet assay in PM_{10} extracts collected in (a) Chiomonte (wildland fire)—F1; (b) Torino—T; (c) Ceresole Reale—C and in $PM_{2.5}$ extracts collected in (d) Chiomonte (wildland fire)—F2 (e) Novara—N. Data expressed as % of tail intensity (means and standard deviations). One-way ANOVA test followed by post-hoc Dunnett test not significant (data vs. C-, $p > 0.05$); Figure S5: Estrogenic activity detected using luciferase gene reporter assay in the PM extracts (1 m^3/mL) tested in combination with 17β-estradiol (10^{-10} M). The results are expressed as luciferase activity fold induction (means and standard deviations) respect to negative control (C- = 1). E2 = 17β-estradiol (10^{-10} M). * $p < 0.05$ vs. C- according to *t*- test. # $p < 0.05$ vs. E2 according to *t*-test.

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SUPPLEMENTARY MATERIALS

In vitro effects of particulate matter associated with a wildland fire in the North-West of Italy

Marta Gea[1], Sara Bonetta[1*], Daniele Marangon[2], Francesco Antonio Pitasi[2], Caterina Armato[1], Giorgio Gilli[1], Fabrizio Bert[1], Marco Fontana[2], Tiziana Schilirò[1]

[1] Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 Torino, Italy.

[2] Regional Agency for Environmental Protection of Piedmont (ARPA Piemonte), 10135 Torino, Italy.

***Corresponding author**:

Corresponding author:

Sara Bonetta

sara.bonetta@unito.it

Department of Public Health and Pediatrics,

University of Torino,

Piazza Polonia 94, 10126 Torino, Italy.

Phone: +39 011 6708192

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SUPPLEMENTARY MATERIALS

Table S1. Concentrations of PM tested in the biological assays expressed as m^3/ml and μg/ml. F1 – PM_{10} collected in Chiomonte (wildland fire); T – PM_{10} collected in Torino; C – PM_{10} collected in Ceresole Reale; F2 – $PM_{2.5}$ collected in Chiomonte (wildland fire); N – $PM_{2.5}$ collected in Novara.

		m^3/ml	**μg/ml**
F1	dose 1	0.5	25.5
	dose 2	1.0	51.0
	dose 3	2.0	102.0
	dose 4	2.5	127.5
T	dose 1	0.5	39.5
	dose 2	1.0	79.0
	dose 3	2.0	158.0
	dose 4	2.5	197.5
C	dose 1	0.5	15.0
	dose 2	1.0	30.0
	dose 3	2.0	60.0
	dose 4	2.5	75.0
F2	dose 1	0.5	21.5
	dose 2	1.0	43.0
	dose 3	2.0	86.0
	dose 4	2.5	107.5
N	dose 1	0.5	18.0
	dose 2	1.0	36.0
	dose 3	2.0	72.0
	dose 4	2.5	90.0

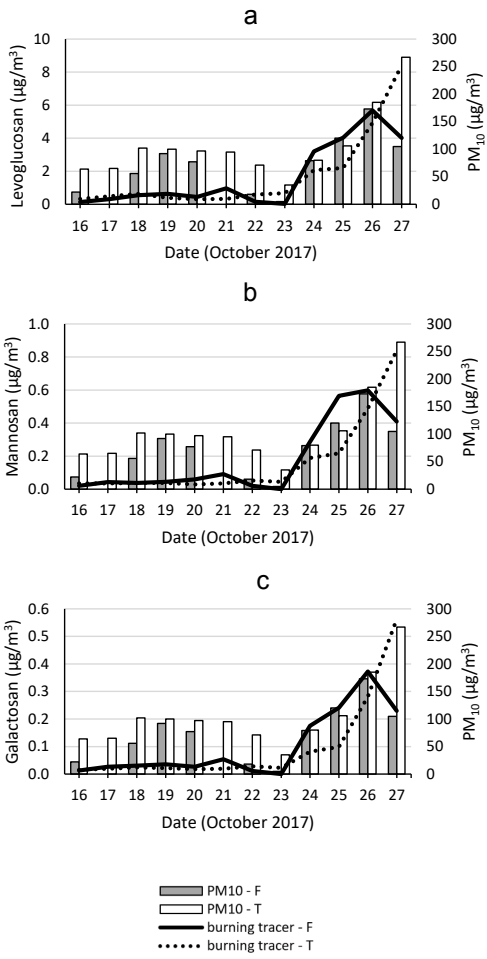

Figure S1. Daily concentrations of biomass burning tracers in the rural site F (Chiomonte) and in urban site T (Torino): levoglucosan concentrations (**a**), mannosan concentrations (**b**), galactosan concentrations (**c**). Data are compared with the PM_{10} concentrations measured in the same sites (PM_{10} concentrations are not available for 17th and 21st October 2017 in site F).

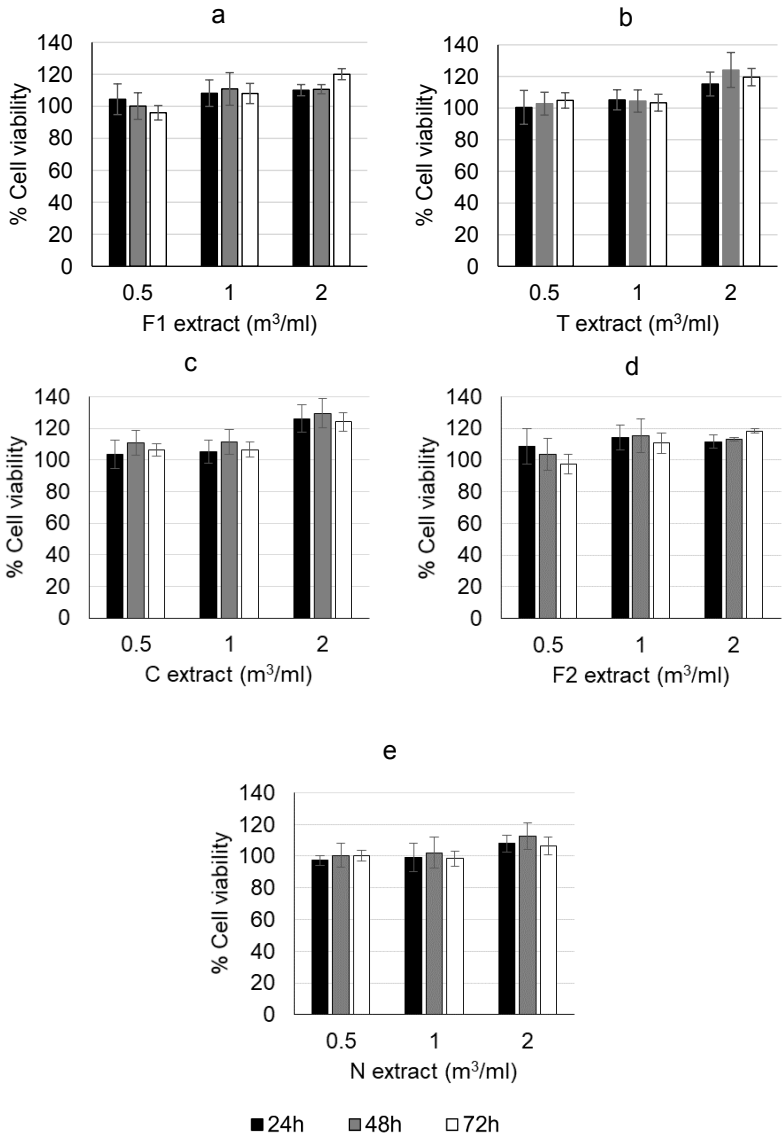

Figure S2. Cell viability detected using WST-1 assay in PM_{10} extracts collected in (**a**) Chiomonte (wildland fire), (**b**) Torino, (**c**) Ceresole Reale and in $PM_{2.5}$ extracts collected in (**d**) Chiomonte (wildland fire), (e) Novara. Data expressed as % of cell viability (means and standard deviations) with respect to negative control (C- = 100%). T-test was not significant (data *vs* C-, $p > 0.05$).

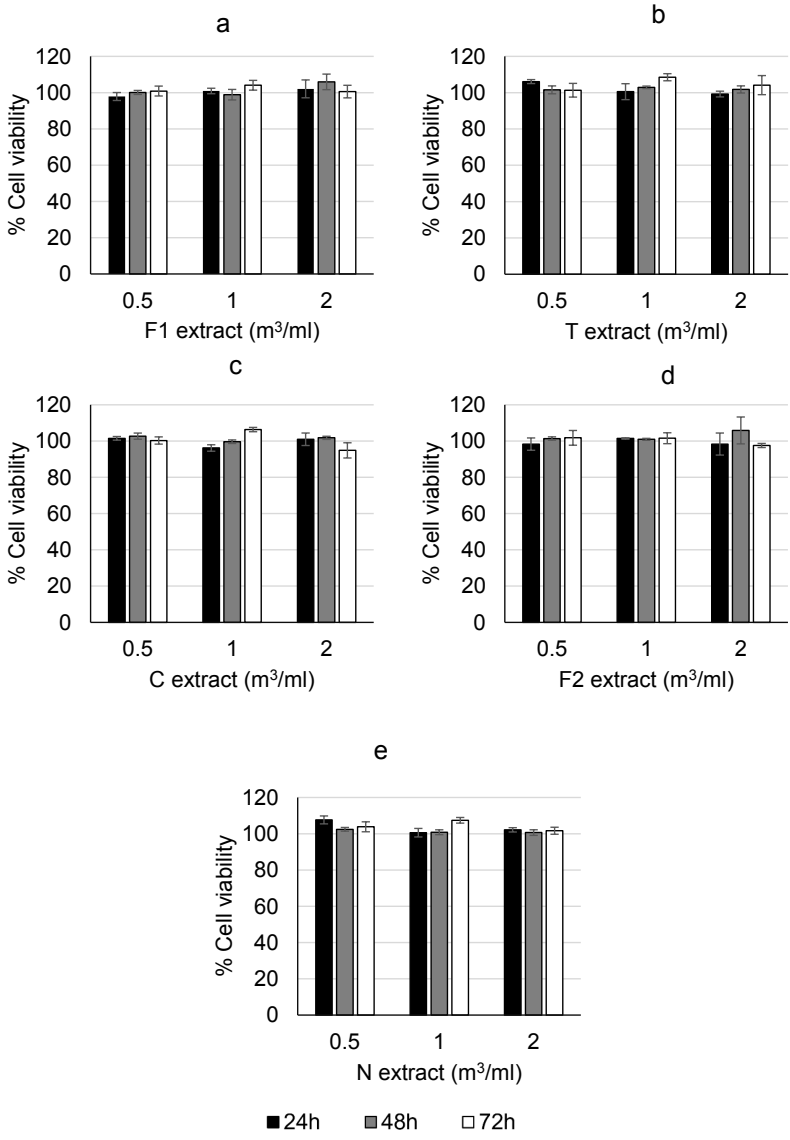

Figure S3. Cell viability detected using LDH assay in PM_{10} extracts collected in (**a**) Chiomonte (wildland fire), (**b**) Torino, (**c**) Ceresole Reale and in $PM_{2.5}$ extracts collected in (**d**) Chiomonte (wildland fire), (e) Novara. Data expressed as % of cell viability (means and standard deviations) with respect to negative control (C- = 100%). T-test was not significant (data *vs* C-, $p > 0.05$).

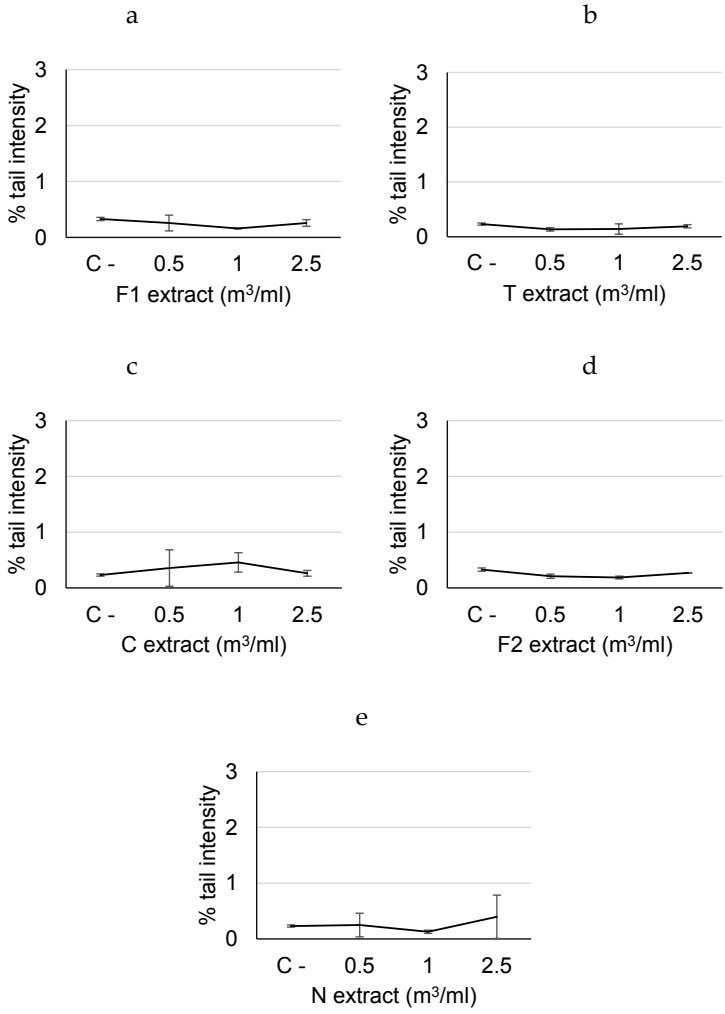

Figure S4. DNA damage detected using Comet assay in PM_{10} extracts collected in (**a**) Chiomonte (wildland fire) – F1; (**b**) Torino – T; (**c**) Ceresole Reale – C and in $PM_{2.5}$ extracts collected in (**d**) Chiomonte (wildland fire) – F2 (**e**) Novara – N. Data expressed as % of tail intensity (means and standard deviations). One-way ANOVA test followed by *post-hoc* Dunnett test not significant (data *vs* C-, $p > 0.05$).

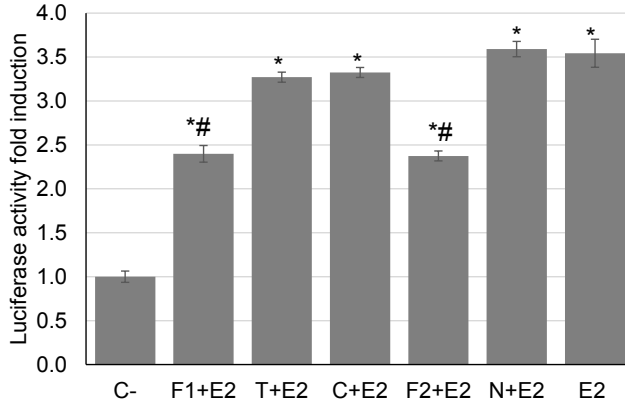

Figure S5. Estrogenic activity detected using luciferase gene reporter assay in the PM extracts (1 m^3/ml) tested in combination with 17β-estradiol (10^{-10} M). The results are expressed as luciferase activity fold induction (means and standard deviations) respect to negative control (C- =1). E2 = 17β-estradiol (10^{-10} M). *$p < 0.05$ *vs* C- according to T- test. #$p < 0.05$ *vs* E2 according to t-test.

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Review

Estrogenic activity of biological samples as a biomarker

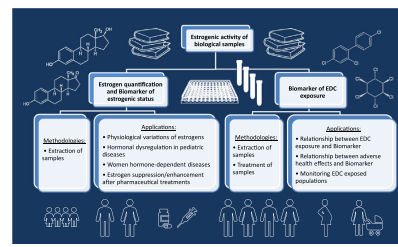
Marta Gea *, Anna Toso, Tiziana Schilirò

Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94, 10126 Torino, Italy

HIGHLIGHTS

- Estrogenic activity assays can be used to quantify estrogens in biological samples.
- Estrogenic activity assays can be used as a estrogenic status biomarker.
- Estrogenic activity assays can be used as a EDC exposure biomarker.
- Hormone-dependent diseases can be deepened by means of estrogenic activity assays.
- Evaluation of human EDC exposure can be implemented by estrogenic activity assays.

GRAPHICAL ABSTRACT



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ABSTRACT

Biological assays can evaluate the cumulative effect of a mixture, considering synergistic/antagonistic interactions and effects of unknown/unconsidered compounds. Therefore, their application could increase in the next years also to analyse biological samples.

The aim of this review is to discuss the methodological approach and the application of estrogenic activity assays in human biological samples. 75 research articles were analysed and divided according to whether they used these assays: i) to quantify the level of estrogens and/or as a biomarker of estrogenic status ii) as a biomarker of exposure to endocrine disrupting chemicals (EDCs).

For the first purpose, some authors extracted biological samples while others tested them directly without any treatment. The study of these methodologies outlined that the methodology applied influenced the specificity of analysis. The estrogenic activity biomarker was used to analyse physiological variations of estrogens, pediatric diseases, hormone-dependent diseases and estrogen suppression/enhancement after pharmaceutical treatments.

For the second purpose, some authors extracted samples while others tested them directly, some authors divided endogenous estrogens from xenoestrogens while others tested samples without separation. The analysis of these methodologies outlined some limitations related to the efficiency of extraction and the incorrect separation of some compounds. The studies which applied this EDC biomarker showed that it was correlated with some EDCs, it varied according to the exposure of the population and it allowed the identification of some relationships between EDC exposure and breast cancer, type 1 diabetes and adverse health effects on children.

In conclusion, the estrogenic activity of biological samples can be a useful tool: to quantify low levels of 17β-estradiol, to assess the combined effect of endogenous estrogens and xenoestrogens, to estimate the estrogenic

Abbreviations: BPA, bisphenol A; DCC, dextran-coated charcoal; E2, 17β-estradiol; EDCs, endocrine disrupting chemicals; EEQ, 17β-estradiol equivalent quantity; ER, Estrogen receptor; FSH, Follicle-stimulating hormone; GH, Growth hormone; ISO, International Organization for Standardization; HELN, human uterine cervix carcinoma cells (HeLa) stably transfected with ERE-βGlob-Luc-SVNeo and pSG5ERαpuro or pSG5ERβpuro plasmids (HELNα or HELNβ, respectively); HPLC, high-performance liquid chromatography; LH, Luteinizing hormone; MELN, breast cancer cells (MCF-7) stably transfected with ERE-βGlob-Luc-SVNeo plasmid.; OECD, Organisation for Economic Co-operation and Development; PCBs, polychlorinated biphenyls; PFAA, perfluorinated alkyl acids; POPs, persistent organic pollutants; PP, precocious puberty; TEXB, total estrogenic burden.

* Corresponding author.
E-mail address: marta.gea@unito.it (M. Gea).

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status providing considerable insight into physiological or pathological conditions, to evaluate EDC presence implementing the existing knowledge about EDC exposure and adverse health effects.

Contents

1. Introduction

The endocrine system is based on hormones, which are molecules produced by endocrine glands, organs and tissues and released into the blood. Once they reach cells and tissues, hormones can induce several effects through hormone receptors. Hormones are involved in complex signalling pathways, which regulate numerous development stages of human and animal life: foetal development, childhood and puberty. The endocrine system also controls several functions in adulthood such as reproduction, metabolism and thermal regulation, and it interacts with other systems such as nervous and immune systems (Bergman et al., 2013; Demeneix and Slama, 2019). Therefore, a correct functioning of the endocrine system is crucial in order to ensure the correct regulation of numerous physiological processes and its dysfunction or perturbation can lead to several adverse health effects such as malformations, metabolic disorders, reduced fertility and cancer (Bergman et al., 2013; Kabir et al., 2015; Pamplona-Silva et al., 2018).

Even if the endocrine system includes several different hormones, the scientific community focused in particular on estrogens because of their peculiar characteristics. Estrogens exert a crucial role in human organisms since they regulate menstrual/estrous reproductive cycles and they are also involved in more complex mechanisms such as the physiology of reproductive organs and tissues (e.g., breast, ovary and endometrium), lipid metabolism, protein synthesis and diseases (e.g., cancer and neurodegenerative/cardiovascular diseases) (Kiyama and Wada-Kiyama, 2015). Moreover, estrogens play a critical role in the physiology and pathology of the immune system, and therefore they can influence the onset and the progression of some autoimmune diseases (Benagiano et al., 2019; Merrheim et al., 2020).

The most important endogenous estrogens are 17β-estradiol (E2), estrone and estriol (Kiyama and Wada-Kiyama, 2015) and their actions occur at very low concentrations (Pamplona-Silva et al., 2018).

Due to their key role in the organism, the evaluation of estrogen levels can be helpful to understand and treat a wide range of physiological or clinical conditions in children as well as adults (Rosner et al., 2013). The least invasive methods to estimate estrogen levels are indirect methods which consist in tracking physiologic changes. These methods include monitoring basal body temperature, using tests of urine to detect ovulation, examining vaginal discharge and measuring other body changes, such as uterine length at pelvic sonography to evaluate female pubertal status (Bellem et al., 2011; Paris et al., 2002). However, since they have a low specificity and sensitivity, indirect methods are generally coupled with direct methods such as immunoassays and spectrometry analysis which can quantify hormone levels in biological samples (e.g. saliva, blood, urine) (Bellem et al., 2011). Immunoassays measure hormones using the binding between antigen and antibody, which is amplified using different markers: radioisotope, enzyme, fluorescent or chemiluminescent labels (radioimmunoassay, enzyme immunoassay, fluorescence immunoassay, chemiluminescence immunoassay). Immunoassays are generally considered to be quite specific, but their sensitivity is often insufficient to detect low hormone levels and it seems that they tend to overestimate hormonal levels (Santen et al., 2008). Moreover they are unable to measure different estrogens simultaneously (Bellem et al., 2011) and they do not reflect the hormonal activity in the samples since they only quantify the concentration of compounds that are structurally recognized by the antibody, thus neglecting the overall activity induced by compounds with the same action mechanism (Widschwendter et al., 2009). Mass spectrometry

identifies and quantifies each chemical through its mass-to-charge ratio after ionization (electron spray or electron impact ionization). Before the mass spectrometry analysis, the sample is generally prepared using a separation technique (gas chromatography or liquid chromatography). The tandem mass spectrometry, which is coupled with spectral analysis in multiple rounds, is accepted as the golden standard for hormone assays but it shows some limitations such as the expensive equipment needed and the technical complexity of the analysis, which involves several steps and thus requires a long time (Bellem et al., 2011). Immunoassays and mass spectrometry have been widely used for their specificity since they quantify the concentrations of specific hormones. However, their high specificity may oversimplify the physiological situation. The physiological hormonal activity is mainly based on the effect of a specific hormone, but it can also be induced by other hormones, hormone metabolites and growth factors. Also, it can be altered by exogenous substances. Therefore, in recent years bioassays have been used for hormonal activity quantification by an increasing number of authors. Bioassays are based on biological reactions that depend on the presence or absence of the hormone, but also on the presence of other substances, which can induce the same effect. For example, bioassays for estrogenic activity can detect the total estrogenic effect in human biological samples: they measure the activity of E2 but are also able to detect the activity induced by other estrogens such as estrone and estriol (Paris et al., 2002).

In addition to estrogens, a great number of exogenous compounds can exert and modulate the estrogenic activity which can be measured by means of bioassays. These estrogenic compounds are part of a group of substances called endocrine disrupting chemicals (EDCs). The World Health Organization defined EDCs as "exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub) populations" (Damstra et al., 2002). EDCs are a highly heterogeneous group of natural (i.e., steroids and phytoestrogen) and synthesized chemicals (i.e., synthetic chemicals, plastics, plasticizers, pesticides, pharmaceutical agents) (Diamanti-Kandarakis et al., 2009; Kabir et al., 2015) which can interfere with the endocrine system in different ways: first, they can act directly by binding to hormone receptors. In particular, agonist EDCs can imitate hormones, thus producing over or under responses, while antagonist EDCs can block the response. Second, EDCs can indirectly interact with receptors as they can interfere with the synthesis, transport, metabolism and excretion of hormones (Hampl et al., 2016; Kabir et al., 2015). These contaminants are ubiquitous in the environment and human exposure to them occurs in different ways, such as by inhalation (mainly in the working environment), ingestion of contaminated food and/or water and dermal contact through personal care products (Kabir et al., 2015). Since low doses of EDCs could be enough to induce effects - and exposure during specific lifetime periods could induce permanent adverse effects - EDCs may represent an alarming health and environmental problem.

In this context, the assessment of EDC exposure appears crucial in order to monitor populations at higher risk of exposure and to understand the link between exposure and adverse health effects. In biological samples concentrations of EDCs (e.g., bisphenol A (BPA), phthalates, parabens, polychlorinated biphenyls (PCBs), perfluorinated compounds, polybrominated diphenyl ethers) and metabolites of EDCs (e.g., metabolites of pyrethroids, insecticides, pesticides, phthalates) have been used as conventional biomarkers of exposure (Calsolaro et al., 2017; Dziewirska et al., 2018; Hampl et al., 2016; Karwacka et al., 2019). However, since 1995 (Sonnenschein et al., 1995) some authors proposed novel biomarkers of exposure to EDCs, which focus on detecting the biological effect of chemical compounds or metabolites, rather than detecting the presence of the EDC itself. In particular, the estrogenic activity of biological samples, measured with estrogenic activity assays, has been proposed and used as a novel biomarker of exposure to EDCs. The main advantage of estrogenic activity assays is that they can assess the total effect induced by multiple, exogenous chemicals with estrogenic activity (xenoestrogens). Indeed, the chemical quantification of xenoestrogens provides an estimate of the real exposure. However, the cumulative estrogenic activity of a mixture is not the sum of the individual estrogenic activity of each xenoestrogen, since synergistic/antagonistic interactions must be taken into account. Moreover, the chemical analysis of xenoestrogens only takes into account specific known xenoestrogens and cannot quantify the effect of unknown or unevaluated compounds (Bicchi et al., 2009; Escher et al., 2018; Jarošová et al., 2014; Kase et al., 2018). Therefore, the inclusion of estrogenic activity assays into estrogens monitoring studies could be a valuable complement to chemical analysis (Könemann et al., 2018).

Consequently, the use of estrogenic activity assays in human biological samples may have two main purposes, namely i) the assessment of total estrogenic effect to allow for the management of a wide range of physiological or clinical conditions in children as well as in adults, and ii) the improvement of the assessment of EDC exposure to allow for a better management of this important health and environmental issue.

For the time being the development and application of estrogenic activity assays in human biological samples do not seem to be widespread, compared to other experimental tools. Nevertheless, their use could increase exponentially in the next years due to their versatility and holistic approaches. It is thus much needed to gain additional knowledge of their methodological approaches and applications relevant to them.

The aim of the present review is to discuss the methodological approaches and the applications of estrogenic activity assays in human biological samples. A bibliographic research was performed: 75 research articles were analysed in order to summarize the methods used to treat biological samples and the results obtained.

The examined articles were divided into two categories, according to the use of estrogenic activity: i) to quantify the level of estrogens and/or as a biomarker of estrogenic status, ii) as a biomarker of exposure to EDCs.

To quantify the level of estrogens and/or as a biomarker of estrogenic status, the estrogenic activity assays were applied in 38 of the analysed articles (Table 1). Some authors mainly considered estrogenic activity assays as tools for the evaluation of estrogen levels, specifically for the evaluation of E2 levels (Table 1, n° ref. 1–8, 10–13, 18, 29, 32, 38). Other authors instead used these assays for their ability to measure the total estrogenic effect defined as "evaluation of estrogenic bioactivity" (Table 1, n° ref. 9, 17). In this review, the application of estrogenic activity assays was intended as a tool to detect the total estrogenic effect, meaning that it was intended as a biomarker of "estrogenic status", except for the articles published by Klein et al. where it was intended as a tool for estrogen quantification. In these 38 studies, serum and plasma were used as biological samples and they were analysed using gene reporter assays (using mammalian and yeast cells) and proliferation assays (*E*-screen assays).

As biomarkers of exposure to EDCs, estrogenic activity assays were used in 37 of the analysed articles (Table 2). In these studies, the biomarker was defined as "assessment of total estrogenic burden (TEXB)" (Table 2, n° ref. 3), "total estrogenic xenobiotic burden" (Table 2, n° ref. 4), "total effective xenoestrogen burden" (Table 2, n° ref. 7, 15, 16, 21, 22, 35) or "xenoestrogenic activity" (Table 2, n° ref. 11, 14, 32, 37). In this article the abbreviation "EDC biomarker" will be used. In these 37 studies, serum, adipose tissue, placenta and milk were used as biological samples and they were analysed using ligand-binding assays, gene reporter assays (using mammalian and yeast cells) and proliferation assays (*E*-screen assays).

2. Assays for estrogenic activity evaluation

Assays for estrogenic activity evaluation have been described in recent review articles (Mueller, 2004; Kiyama and Wada-Kiyama, 2015; Wagner et al., 2017; Wangmo et al., 2018). For the assessment of the estrogenic activity in biological samples, three kinds of assays have been applied: ligand-binding assays, gene reporter assays and proliferation

Table 1
Studies applying estrogenic activity assay as estrogen quantification and/or biomarker of estrogenic status.

N°	Reference	Biological sample	Assay (cells/yeast species)	Sample treatment	Standard curve	Sample concentrations	Study population (number of subjects)	Data expressed as
1	Klein et al., 1994	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether.	Estradiol added to charcoal-stripped plasma (from adult men).	Extracts of 0.8 mL sample.	Prepubertal children (n = 44).	EEQ
2	Klein et al., 1995	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Women with breast cancer treated with letrozole (aromatase inhibitor) (n = 14).	EEQ
3	Klein et al., 1996	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Pubertal boys (n = 23).	EEQ
4	Klein et al., 1998a	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Standard curve (Klein et al., 1994).	Extracts of 0.8 mL sample.	Girls with central precocious puberty treated with different doses of deslorelin (n = 20).	EEQ
5	Klein et al., 1998b	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Standard curve (Klein et al., 1994).	Extracts of 0.8 mL sample.	Obese and nonobese children (n = 48).	EEQ
6	Klein et al., 1999	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Girls with premature thelarche and controls (n = 35).	EEQ
7	Larmore et al., 2002	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Women treated with leuprolide acetate (n = 8).	EEQ (estradiol %of baseline).
8	Mauras et al., 2000	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Healthy young boys treated with anastrozole (aromatase blocker) (n = 20).	EEQ
9	Paris et al., 2002	Serum	Gene reporter (HELNα).	None.	Specific for each serum sample: estradiol added to stripped serum from the same patient (desteroided by C18 Oasis HLB).	20%.	Normal prepubertal boys, normal prepubertal girls, normal pubertal girls (n = 54).	EEQ
10	Larmore et al., 2002	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Girls with precocious puberty, normal prepubertal girls, obese pubertal girls, normal pubertal girls, obese pubertal girls (n = 60).	EEQ
11	Santen et al., 2002	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Postmenopausal women with urogenital atrophy treated with low-dose vaginal E2 (n = 7).	EEQ
12	Wilson et al., 2003	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Girls with Turner syndrome and healthy prepubertal girls (n = 68).	EEQ
13	Mauras et al., 2004	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	GH deficient boys treated with GH or with GH and anastrozole (aromatase blocker) (n = 20).	EEQ
14	Séronie-Vivien et al., 2004	Serum	Gene reporter (MELN).	None.	Estradiol added to charcoal-stripped human serum from an healthy volunteer (incubated for 2 h at 37 °C to equilibrate free estrogens and estrogens bound to proteins).	20%.	Women with advanced breast cancer and healthy women (n = 40).	EEQ
	Wang et al.,	Serum	Gene reporter	None (Paris	Specific for each serum	20% (Paris et al., 2002).	Postmenopausal women	EEQ

Table 1 (*continued*)

N°	Reference	Biological sample	Assay (cells/yeast species)	Sample treatment	Standard curve	Sample concentrations	Study population (number of subjects)	Data expressed as
15	2005		(HELNα).	et al., 2002).	sample: estradiol added to stripped serum from the same patient.		(n = 30).	
16	Sonneveld et al., 2005	Serum	Gene reporter (ERα CALUX cells).	None.	Not specified.	Final serum concentration 10% (lower percentage supplemented with charcoal-stripped FCS) 0, 1, 2, 3, 4, 6, 8, 10%.	Not evaluable.	Luciferase activity (% of maximum E2 induction).
17	Paris et al., 2006	Serum	Gene reporter (HELNα).	None (Paris et al., 2002).	Specific for each serum sample: estradiol added to stripped serum from the same patient (desteroided by C18 Oasis HLB) (Paris et al., 2002).	20% (Paris et al., 2002).	Male children (<1 years) with pseudohermaphroditism and controls (n = 18).	EEQ
18	Janfaza et al., 2006	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether.	Estradiol added to culture medium.	Extracts of 0.8 mL sample.	Normal children from birth through puberty (n = 800).	EEQ
19	Widschwendter et al., 2009	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae_1*).	None.	Estradiol added to medium with 20% charcoal-stripped serum.	20%.	Postmenopausal women with breast cancer and controls (n = 370).	EEQ
20	Li et al., 2009	Serum	Gene reporter (HeLa ERα transfected, HeLa ERβ transfected), *E*-screen (MCF-7).	Filtration (0.22 μm).	Estradiol added to medium with 20% (commercial) charcoal-stripped human serum (equilibrated for 4 h at 37 °C before use).	20% (aromatase inhibitor DL-aminoglutethimide 50 μmol/L).	Healthy men treated with estradiol valerate and *Epimedium* decoticon (n = 8).	EEQ
21	Pedersen et al., 2010	Plasma	Gene reporter (ERα CALUX cells).	Extraction with methyl tert-butyl ether.	Curve with DMSO.	Not specified.	Pregnant women (n = 98).	EEQ
22	Taboada et al., 2011	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Girls with Turner syndrome and healthy menstruating girls (n = 30).	EEQ
23	Gaspari et al., 2011a	Serum	Gene reporter (HELNα).	None (Paris et al., 2002).	Specific for each serum sample: estradiol added to stripped serum from the same patient (desteroided by C18 Oasis HLB) (Paris et al., 2002).	20% (Paris et al., 2002).	Male children (<1 years) with sex differentiation disorders, normal androgen production and no androgen receptor or SRD5A2 gene mutations and controls (n = 43).	EEQ
24	Gaspari et al., 2011b	Serum	Gene reporter (HELNα).	None (Paris et al., 2002).	Specific for each serum sample: estradiol added to stripped serum from the same patient (desteroided by C18 Oasis HLB) (Paris et al., 2002).	20% (Paris et al., 2002).	4-month-old girl with sexual development (n = 1).	EEQ
25	Lim et al., 2012	Serum	Gene reporter (HeLa ERα transfected).	Filtration (0.22 μm).	Estradiol added to medium with stripped human serum.	Not specified.	Postmenopausal women who donated blood and then suffered from hip fracture and controls (n = 418).	EEQ
26	Fourkala et al., 2012	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae_1*).	None (Widschwendter et al., 2009).	Estradiol added to medium with 20% charcoal-stripped serum (Widschwendter et al., 2009).	20% (Widschwendter et al., 2009).	Postmenopausal women who donated blood and then developed breast cancer and controls (n = 600).	EEQ
27	Wang et al., 2013	Serum	*E*-screen (MCF-7).	None.	Estradiol added to 5% (commercial) stripped human serum.	5%.	Postmenopausal women (n = 219).	EEQ
28	Paris et al., 2013	Serum	Gene reporter (HELNα).	None (Paris et al., 2002).	Specific for each serum sample: estradiol added to stripped serum from the same patient (desteroided by C18 Oasis HLB) (Paris et al., 2002).	20% (Paris et al., 2002).	Girls affected by isolated premature thelarche and prepubertal girls as controls (n = 33).	EEQ
29	Torres-Santiago et al., 2013	Plasma	Gene reporter-YES	Extraction with ether (Klein	Estradiol added to charcoal-stripped plasma	Extracts of 0.8 mL sample (Klein et al.,	Girls affected by Turner syndrome treated with oral	EEQ

(*continued on next page*)

Table 1 (*continued*)

N°	Reference	Biological sample	Assay (cells/yeast species)	Sample treatment	Standard curve	Sample concentrations	Study population (number of subjects)	Data expressed as
			assay (*Saccharomyces cerevisiae*).	et al., 1994).	(Klein et al., 1994).	1994).	or transdermal 17β-estradiol (n = 40).	
30	Lim et al., 2014a	Serum	Gene reporter (HeLa ERα transfected, HeLa ERβ transfected).	Filtration (0.22 μm).	Estradiol added to medium with (commercial) charcoal stripped human serum (10%).	10%.	Postmenopausal women who donated blood and then suffered from breast cancer and controls (n = 595).	EEQ
31	Lim et al., 2014b	Serum	Gene reporter (HeLa ERα transfected, HeLa ERβ transfected).	Filtration (0.22 μm).	Estradiol added to medium with (commercial) charcoal stripped human serum (10%).	10%.	Postmenopausal Chinese women with lung cancer (n = 222).	EEQ
32	Pereira et al., 2015	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1994).	Estradiol added to charcoal-stripped plasma (Klein et al., 1994).	Extracts of 0.8 mL sample (Klein et al., 1994).	Prepubertal girls who donated blood and then presented early thelarche and controls (n = 155).	EEQ
33	Kanaya et al., 2015	Serum	Gene reporter (AroER tri-screen cells).	Solid phase extraction (elution solvent: methanol).	Not specified.	Not specified.	Not evaluable.	Relative luciferase activity.
34	Martínez et al., 2016	Serum	*E*-screen (MCF-7 BUS).	None.	Biological effect is compared with a standard serum pool.	0.5% serum sample simultaneously tested with 0.5% standard serum pool (collected from 7 women during the follicular phase).	Postmenarcheal adolescent with Type 1 diabetes and controls (n = 70).	Estrogenic activity (% compared with standard serum pool).
35	Fejerman et al., 2016	Plasma	Gene reporter (T47D-Kbluc).	None.	Not specified.	4%.	Latin American women (n = 90).	Relative light units (RLUs).
36	Chamas et al., 2017	Serum	Gene reporter-YES assay (*Arxula adeninivorans*).	Liquid-liquid extraction with diethylether.	Not specified.	1:200 dilution of the original sample.	Not evaluable.	EEQ
37	Sanchez et al., 2019	Plasma	Gene reporter (T47D-Kbluc).	None.	Standard curve.	4%.	Non-latina black, non-latina white and latina women (n = 503).	EEQ
38	Mesa Valencia et al., 2019	Serum	Gene reporter-YES assay (*Saccharomyces cerevisiae*).	Extraction with ether (Klein et al., 1998a, 1998b).	Standard curve.	Extracts of 0.8 mL sample.	Prepubertal girls (n = 107).	EEQ

Footnotes:
AroER tri-screen cells = breast cancer MCF-7 variant without endogenous ER expression (C4–12) transfected with these plasmids: pTomo-ERα vector or pTomo-ERβ vector, pCHGP-2, pCMV-G, pCMV-rev, pGL4.26 [luc2/minP/Hygro] $(ERE)_3$.
Arxula adeninivorans = strain G1212 transformed with YRC102-hER-DsRed2 plasmid.
ERα CALUX cells = human osteoblastic osteosarcoma cells (U2-OS) transfected with 3× *ERE*-TATA-Luc and pSG5-neo-hERα.
HeLa transfected = human uterine cervix carcinoma cells (HeLa) stably transfected with pERE4-Luc_{hygro} and pEGFP-$ER\alpha_{neo}$ or pEGFP-$ER\beta_{neo}$ (HeLa ERα transfected or HeLa ERβ transfected, respectively) (Wong et al., 2007).
HELN = human uterine cervix carcinoma cells (HeLa) stably transfected with ERE-βGlob-Luc-SVNeo and pSG5ERαpuro or pSG5ERβpuro plasmids (HELNα or HELNβ, respectively).
MELN = breast cancer cells (MCF-7) stably transfected with ERE-βGlob-Luc-SVNeo plasmid.
Saccharomyces cerevisiae = strain BJ3505 (MATa, ura3–52, tryp1Δ101, lys2–208) transformed with YEPKB1 and YRPE2 plasmids.
Saccharomyces cerevisiae_1 = triply deleted *pdr5 snq2 yor 1* strain transformed with hERα-ERE-GFP or hERβ-ERE-GFP plasmids (Hasenbrink et al., 2006).
T47D-Kbluc = human breast cancer cells (T-47D) transfected with pGL2.TATA.lnr.luc.neo (Wilson et al., 2004).

assays (E-screen assays). In the following sections the characteristics of these assays are briefly described considering their strengths/weaknesses (Table 3) and their detection limits (Table 4) in the assessment of estrogenic activity in biological samples. The assays validated by the Organisation for Economic Co-operation and Development (OECD) as tools to test potential EDC substances or by the International Organization for Standardization (ISO) as tools to assess water/waste water estrogenic activity are also listed.

2.1. Ligand-binding assays

Ligand-binding assays detect receptor–ligand interactions. These assays quantify the ability of a test chemical or a mixture to compete with E2 in binding to ER, and the result is often expressed as the concentration showing 50% displacement of E2 from receptor. The result can also be expressed as relative binding affinity with respect to E2 (Kiyama and Wada-Kiyama, 2015). Ligand-binding assays were initially performed using radioactive ligands, while, more recently, assays using non-radioactive ligands have been developed. Ligand-binding assays are able to detect the binding to ER of both agonist and antagonist substances without distinguishing between the two effects (Seifert et al., 1999). This characteristic is an advantage. Indeed, while in other estrogenic activity assays agonist and antagonist effects could compensate each other resulting in a null activity, in ligand-binding assays this compensatory effect cannot occur. However, the same characteristic can be a disadvantage, since these assays are unable to assess the total estrogenic effect induced by the interaction of agonist and antagonist substances (Mueller, 2004).

Table 2
Studies applying estrogenic activity assay as a biomarker of exposure to EDCs.

N°	Reference	Biological sample	Assay (cells/yeast species)	Separation of xenoestrogens form endogenous hormones	Sample treatment	Study population (number of subjects)
1	Sonnenschein et al., 1995	Serum	E-screen (MCF-7), induction pS2 (MCF-7), induction PgR (MCF-7).	Yes	Methanol addition, hexane:ethyl ether (1:1 v/v) extraction, normal-phase silica Sep-Pak cartridge extraction with ethyl ether in petroleum ether (150 mL/L), acid cleanup (H2SO4 and hexane), HPLC separation of xenoestrogens from natural estrogens.	Not evaluable.
2	Soto et al., 1997	Serum	E-screen (MCF-7).	Yes	Methanol addition, hexane:ethyl ether extraction (1:1 v/v), acid cleanup (H2SO4 and hexane), HPLC separation of xenoestrogens from endogenous estrogens.	Not evaluable.
3	Rivas and Olea, 1997	Adipose tissue	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Not evaluable.
4	Rivas et al., 2001	Adipose tissue	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Women with different diseases (n = 400).
5	Natarajan et al., 2002	Serum	Gene reporter ($BG1Luc4E_2$).	Yes	Immunoprecipitation of endogenous estrogens (17ß-estradiol and estrone) using polyclonal antibodies raised in rabbits and goat anti-rabbit precipitating antibody.	Not evaluable.
6	Rasmussen et al., 2003	Serum	E-screen (MCF-7 BUS).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Danish/Faroese women (n = 301).
7	Fernández et al., 2004	Adipose tissue	*E*-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Not evaluable.
8	Ibarluzea et al., 2004	Adipose tissue	*E*-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Women with breast cancer, women subjected to non-cancer related surgery (n = 458).
9	Plíšková et al., 2005	Serum	Gene reporter (T47D.Luc).	No	Liquid-liduid extraction (hexane:diethyl ether) or liquid-liduid extraction (hexane: diethyl ether) followed by extraction in sulfuric-acid activated silica column (hexane: diethyl ether).	Men living in a PCB polluted area and in a background area (n = 150).
10	Hjelmborg et al., 2006	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Not evaluable.
11	Bonefeld-Jørgensen et al., 2006	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Male Greenlandic Inuits, male Swedish fishermen, male Warsaw inhabitants, male Kharkiv inhabitants (n = 358).
12	Toft et al., 2007	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Male Greenlandic Inuits, male Swedish fishermen, male Warsaw inhabitants, male Kharkiv inhabitants (n = 319).
13	Long et al., 2007	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Male Greenlandic Inuits, male Swedish fishermen, male Warsaw inhabitants, male Kharkiv inhabitants (n = 262).
14	Andersen et al., 2007	Serum	E-screen (MCF-7).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Non-pregnant and pregnant female greenhouse workers (n = 443).
15	Fernández et al., 2007a	Adipose tissue	*E*-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Women with breast cancer, women subjected to non-cancer related surgery (n = 458).
	Fernández et al.,	Adipose	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in	Women with breast cancer, women subjected

(continued on next page)

Table 2 (*continued*)

N°	Reference	Biological sample	Assay (cells/yeast species)	Separation of xenoestrogens form endogenous hormones	Sample treatment	Study population (number of subjects)
16	2007b	tissue and placenta			a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	to non-cancer related surgery (n = 458), mothers with newborns affected by cryptorchidism and/or hypospadias and mothers with newborn not affected by malformations (n = 160).
17	Fernández et al., 2007c	Placenta	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Mothers with newborns affected by cryptorchidism and/or hypospadias and mothers with newborn not affected by malformations (n = 160).
18	Kanno et al., 2007	Serum	Gene reporter (MCF-7 transfected).	No	None.	Patients who underwent hemodialysis and peritonealdialysis and controls (n = 100).
19	Krüger et al., 2008a	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Men and women from Nuuk, Sisimiut and Qaanaaq (Greenland) (n = 240).
20	Krüger et al., 2008b	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Men from Greenland, Sweden, Warsaw, Kharkiv (n = 300).
21	Fernández et al., 2008	Adipose tissue	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Not evaluable.
22	Lopez-Espinosa et al., 2009	Placenta	E-screen (MCF-7) and gene reporter-YES assay (*Saccharomyces cerevisiae_2*).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Not evaluable.
23	Freire et al., 2010	Placenta	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Mothers and sons randomly selected (n = 178).
24	Sapbamrer et al., 2010	Serum and milk	Ligand-binding assay- enzyme linked receptor assay.	Yes	Removal of endogenous estrogens using dextran-coated charcoal, breast milk also centrifuged, boiled and centrifuged.	Pregnant healthy women (n = 50).
25	Brouwers et al., 2011	Plasma	Gene reporter (ERα CALUX cells).	No	None.	Men differently exposed to EDCs (n = 108).
26	Arrebola et al., 2012	Adipose tissue	E-screen (MCF-7).	Yes and No	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens; Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration, no HPLC separation.	Not evaluable.
27	Krüger et al., 2012	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate) liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens.	Men and women from Ittoqqortoormiit, Narsaq and Qeqertarsuaq (Greenland) (n = 232).
28	Arrebola et al., 2013	Adipose tissue	E-screen (MCF-7).	No	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration, no HPLC separation.	Men and women with and without type 2 diabetes (n = 386).
29	Vilahur et al., 2013	Placenta	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Mothers and sons randomly selected (n = 490).
30	Vilahur et al., 2014a	Placenta	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Mothers and sons randomly selected (n = 197).
31	Vilahur et al., 2014b	Placenta	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Mothers and sons randomly selected (n = 489).
32	Bjerregaard-Olesen et al., 2015	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge, liquid-liquid extraction (tetrahydrofuran/hexane), HPLC separation of xenoestrogens from endogenous estrogens	Not evaluable.

Table 2 (*continued*)

N°	Reference	Biological sample	Assay (cells/yeast species)	Separation of xenoestrogens form endogenous hormones	Sample treatment	Study population (number of subjects)
					and weak anion exchange extraction (extraction of PFAAs).	
33	Bjerregaard-Olesen et al., 2016	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (methanol, ethyl acetate), liquid-liquid extraction (tetrahydrofuran/hexane), HPLC separation of xenoestrogens from endogenous estrogens and weak anion exchange extraction (extraction of PFAAs).	Danish pregnant women (n = 397).
34	Pastor-Barriuso et al., 2016	Serum	E-screen (MCF-7).	Yes	Methanol addition, hexane:ethyl ether (1:1 v/v) extraction, Bond Elute PCB cartridge extraction, HPLC separation of xenoestrogens from endogenous estrogens.	Women with breast cancer and controls (n = 382).
35	Fernández et al., 2017	Adipose tissue	E-screen (MCF-7).	Yes	Dissolution of the sample in hexane, elution in a glass column filled with Alumine Merk 90, concentration and HPLC separation of xenoestrogens from endogenous estrogens.	Women with breast cancer (n = 44).
36	Wielsøe et al., 2018	Serum	Gene reporter (MVLN).	Yes	POP xenoestrogens: extraction with Oasis HLB cartridge (elution solvent: methanol and ethyl acetate), liquid-liquid extraction (heptane/ethyl acetate), HPLC separation of xenoestrogens from endogenous estrogens. PFAA xenoestrogens: extraction with Oasis HLB cartridge (methanol, ethyl acetate), liquid-liquid extraction (tetrahydrofuran/hexane), HPLC separation of xenoestrogens from endogenous estrogens and weak anion exchange extraction (extraction of PFAAs).	Greenlandic women with breast cancer and controls (n = 161).
37	Bjerregaard-Olesen et al., 2019	Serum	Gene reporter (MVLN).	Yes	Extraction with Oasis HLB cartridge (methanol, ethyl acetate), liquid-liquid extraction (tetrahydrofuran/hexane), HPLC separation of xenoestrogens from endogenous estrogens and weak anion exchange extraction (extraction of PFAAs).	Danish mothers and sons (n = 702).

Footnotes:
BG1Luc4E$_2$ = human ovarian carcinoma cells (BG1) stably transfected with pGudLuc7.0 (Rogers and Denison, 2000).
ERα CALUX cells = human osteoblastic osteosarcoma cells (U2-OS) transfected with 3× ERE-TATA-Luc and pSG5-neo-hERα.
MCF-7 transfected = breast cancer cells (MCF-7) transfected with ERE-tk109 luc, ERE2- tk109 luc, ERE-tk81 luc.
MVLN = breast cancer cells (MCF-7) stably transfected with pVit-tk-Luc and pAG-60 plasmids (Pons et al., 1990).
Saccharomyces cerevisiae_2 = yeast genome integrated with human ER and transformed with plasmid carrying the reporter gene *lac-Z* (plasmid code not specified) (Routledge and Sumpter, 1996).
T47D.Luc = human breast cancer cells (T-47D) stably transfected with pEREtataLuc.

To assess the estrogenic activity in biological samples, a ligand-binding assay has been applied: the enzyme linked receptor assay (Sapbamrer et al., 2010), a competitive enzyme immunoassay which uses non-radioactive ligands. Moreover, the OECD has validated two ligand-binding assays to screen and test potential EDC substances (OECD, 2018), namely the Freyberger-Wilson ER binding assay and the Chemical Evaluation and Research Institute (CERI) ER binding assay (OECD n° 493, 2015).

2.2. Gene reporter assays

Gene reporter assays consist of the transfection of a reporter construct into mammalian or yeast cells. ER are transcription factors that induce the transcription of target genes after binding to specific DNA sequences in their promoter; the reporter construct is made of these DNA sequences linked to the gene of a measurable protein (for example the enzyme luciferase) (Sonneveld et al., 2005). Gene reporter assays are able to quantify the total estrogenic effect and are characterized by short incubation periods. However, the results of these assays can be ambiguous when substances with overactivation effects are included in the analysed sample.

Gene reporter assays can be divided into two main categories. Some reporter gene assays are performed on cells that already express ERs, while others are performed on cells that do not express endogenous ERs. In the first category of gene reporter assays, cells are transfected with an estrogen-inducible reporter gene. These cells can be transiently or stably transfected, however, since stably transfected cells remain stable and ready for use, they are generally preferred (Soto et al., 2006; Wangmo et al., 2018). In the second category of gene reporter assays, cells are transfected with both an estrogen-inducible reporter gene and an ER expression construct. Since these cells do not express other ERs, the advantage of these assays is that they allow the evaluation of estrogenic activity in the samples distinguishing between ERα and ERβ estrogenic activity.

Numerous gene reporter assays have been applied for the assessment of estrogenic activity in biological samples. Moreover, two gene reported assays have been validated by the OECD to screen and test potential EDC substances (OECD, 2018): the first uses ERα-HeLa-9903 cells and the second uses VM7Luc4E2 cells (OECD n° 455, 2016). Both assays use human cells stably transfected with ERα, but the VM7Luc4E2 cells also express a minor amount of endogenous ERβ. As stated by the OECD, VM7Luc4E2 cells were originally designated as the BG1Luc cells, however, in-depth analyses revealed that these cells were not the BG1 cells, but instead a variant of the MCF-7 cells (OECD, 2018). Recently, a gene reporter assay using stably transfected human cells has also been described in the ISO 19040-3 (ISO, 2018) for the assessment of estrogenic activity in water and waste water. As for gene reported assays based on yeasts, two assays, based on *Saccharomyces cerevisiae* or *Arxula*

Table 3
Characteristics of assays for the assessment of estrogenic activity in human biological samples (Mueller, 2004; Kiyama and Wada-Kiyama, 2015; Seifert et al., 1999; Soto et al., 2006; Wangmo et al., 2018; Wagner et al., 2017).

Ligand-binding assay			
Level of response/Measured endpoint	Advantages	Disadvantages	Assays applied on biological samples
Receptor/Binding affinity to ER.	Simple, high-throughput. Not affected by the compensatory effect of agonist and antagonist substances. Not affected by matrix interferences (e.g. cytotoxic effect).	Unable to measure ER activation. Unable to quantify the total estrogenic effect (final effect induced by agonist and antagonist substances).	Enzyme linked receptor assay.
Gene reporter assay			
Level of response/Measured endpoint	**Advantages**	**Disadvantages**	**Assays applied on biological samples**
Transcription/ER-mediated activation of a reporter gene.	High-throughput. Able to quantify the total estrogenic effect (final effect induced by agonist and antagonist substances). Short incubation period. Using cells not expressing ER- evaluation of the effect on a specific type of ER (ERα or ERβ). Using yeasts- easy cultivation and more resistence to complex matrices.	Problems related to transient transfection. Overactivation induced by some agonist substances. Using yeasts- unphysiological membrane permeability and transport respect to mammalian cells. Using yeasts- Lower sensitivity respect to mammalian cells.	Gene reporter assay on mammalian cells expressing ER (MELN, MCF-7 transfected, MVLN, T47D-Kbluc, T47D.Luc, BG1Luc4E$_2$). Gene reporter assay on mammalian cells not expressing ER (AroER tri-screen cells, ERα CALUX cells, HELNα, HELNβ, HeLa ERα transfected, HeLa ERβ transfected). YES assay on yeast cells not expressing ER (*Arxula adeninivorans, Saccharomyces cerevisiae, Saccharomyces cerevisiae_1, Saccharomyces cerevisiae_2*).
E-screen assay			
Level of response/Measured endpoint	**Advantages**	**Disadvantages**	**Assays applied on biological samples**
Cell/Proliferation of estrogen-responsive cells.	Able to assess a physiological endpoint (cell proliferation). Able to quantify the total estrogenic effect (final effect induced by agonist and antagonist substances). Able to discriminate partial and full agonist.	Unable to provide mechanistic data. Unable to discern cytotoxicity from antagonist effect. Need to confirm the receptor-mediated mechanism. Time-consuming.	E-screen assay on MCF-7 or MCF-7 BUS cells.

Abbreviations of mammalian and yeast cells reported in Table 1 and Table 2 (footnotes).

adeninivorans, have been described in the ISO 19040-1 (2018) and in the ISO 19040-2 (2018) as methods for the assessment of estrogenic activity in water and waste water (Hettwer et al., 2018).

2.3. Proliferation assays (E-screen assays)

E-screen assays are proliferation tests. They measure the proliferative effect induced by estrogens or estrogenic substances on estrogen-responsive cells. Proliferation is determined through different procedures, including DNA staining and metabolic activity quantification. The first E-screen assay was developed by Soto et al. (1997) using human breast cancer cells (MCF-7), whose proliferation is mainly induced by the activation of ERα (Wagner et al., 2017). Later on E-screen assays were also applied using MCF-7 sublines, such as the MCF-7 BUS, in order to obtain a higher proliferative response (Martínez et al., 2016; Villalobos et al., 1995). Many E-screen assays have been applied for the assessment of estrogenic activity in biological samples, while they have not been reported by the OECD nor by the ISO (OECD, 2018) to test potential EDC substances or water/waste water estrogenic activity.

3. Estrogenic activity as estrogen quantification and biomarker of estrogenic status: methodologies

The research group of Klein was the first to develop an estrogenic activity assay in biological samples (Table 1, n° ref. 1). They developed an estrogenic activity assay as estrogen quantification using yeasts (i.e. a strain of *Saccaromyces cerevisiae*) in which the detection limit was 0.02 pg/mL, in order to overcome the lack of sensitivity of available assays for E2. Klein et al. (1994) treated serum samples performing a liquid-liquid extraction with ether and demonstrated that this assay was highly specific for E2 with low cross-reactivity for estrogen metabolites and other hormones, suggesting that this high specificity could be due to many factors, including the extraction of the samples.

The protocol of Klein et al. was mainly focused on measuring E2 and it was applied in many studies by the same research group (Table 1, n°ref. 2–8, 10–13, 18, 22, 29, 32, 38).

Other authors applied extraction to biological samples before the analysis: in the studies of Pedersen et al. (2010) and Chamas et al. (2017) samples were extracted using a liquid-liquid extraction with methyl tert-butyl ether and diethyl ether respectively, while in the study of Kanaya et al. (2015) samples were extracted using a solid-phase extraction.

Table 4
Detection limits of estrogenic activity assays compared to detection limits of direct methods for estrogen quantification. Data are expressed as sensitivity for measuring estrogens/progestagens (direct methods) and as sensitivity for measuring E2 (estrogenic activity assays). Ns = not specified. Quantitation limits of direct methods for estrogen quantification are reported in Table S.1 (Supplementary Material). Abbreviations of mammalian and yeast cells reported in Table 1 and Table 2 (footnotes).

	Direct methods for estrogen quantification	
Assay	Detection limit (pg/mL)	Reference
Radioimmunoassays	15–150	Bellem et al., 2011
Enzyme immunoassays	10–800	Bellem et al., 2011
Fluorescence immunoassays	8–500	Bellem et al., 2011
Mass spectrometry analyses	0.4–150	Bellem et al., 2011
	Estrogenic activity assays	
Assay	Detection limit (pg/mL)	Reference
Enzyme linked receptor assay	500	Sapbamrer et al., 2010
Gene reporter assay (MELN)	0.27	Leusch et al., 2010
Gene reporter assay (MCF-7 transfected)	ns	Kanno et al., 2007
Gene reporter assay (MVLN)	0.14	Bonefeld-Jørgensen et al., 2005
Gene reporter assay (T47D.Luc)	0.14	Legler et al., 1999
Gene reporter assay (T47D-Kbluc)	0.27	Fejerman et al., 2016
Gene reporter assay (BG1Luc4E2)	0.27	Natarajan et al., 2002
Gene reporter assay (AroER tri-screen cells)	ns	Kanaya et al., 2015
Gene reporter assay (ERα CALUX cells)	0.22	Sonneveld et al., 2005
Gene reporter assay (HELNα)	1	Paris et al., 2002
Gene reporter assay (HeLa ERα transfected)	2.3	Wong et al., 2007
Gene reporter assay (HeLa ERβ transfected)	3.6	Wong et al., 2007
Gene reporter- YES assay (*Arxula adeninivorans*)	ns	Chamas et al., 2017
Gene reporter- YES assay (*Saccharomyces cerevisiae*)	0.02–0.2	Larmore and Klein, 2000
Gene reporter- YES assay (*Saccharomyces cerevisiae_1*)	5	Fourkala et al., 2012
Gene reporter- YES assay (*Saccharomyces cerevisiae_2*)	2	Routledge and Sumpter, 1996
E-screen assay (MCF-7)	0.27	Soto et al., 1997
E-screen assay (MCF-7 BUS)	0.27	Rasmussen and Nielsen, 2002

In 2002, the research group of Paris et al. developed an estrogenic activity assay for the determination of serum estrogenic activity using human uterine cervix carcinoma cells (HeLa) stably transfected with ERE-βGlob-Luc-SVNeo and pSG5ERαpuro or pSG5ERβpuro plasmids (HELNα or HELNβ, respectively). In contrast to Klein et al. (1994), Paris et al. (2002) tested serum samples without extraction and without any other sample treatment to keep their conditions closer to those of their physiological status, since their aim was to develop a protocol for the assessment of the total estrogenic activity rather than for E2 quantification. Their protocol, based on HeLa cells, was subsequently applied by several authors (Table 1, n° ref. 15, 17, 23, 24, 28) and it was also used by Séronie-Vivien et al. (2004) as a model to develop another serum estrogenic activity assay based on breast cancer cells (MCF-7) stably transfected with ERE-βGlob-Luc-SVNeo plasmid (MELN).

Other 11 articles reported in this review treated biological samples without any extraction (Table 1, n° ref. 16, 19, 20, 25, 26, 27, 30, 31, 34, 35, 37). In almost all protocols, samples were added to culture medium without any treatment. In four studies, however, samples were filtered (pore size 0.22 μm) before being added to culture medium (Table 1, n° ref. 20, 25, 30, 32) and an aromatase inhibitor was added with a view to preventing the conversion of testosterone to E2 by aromatase in the cells (Table 1, n° ref. 20). Biological samples were tested in one or in different concentrations ranging from 1 to 20% in culture medium.

The analysis of samples without extraction could be influenced by the complexity of serum/plasma samples, therefore some authors proposed different methodological approaches in order to consider the variability of these samples.

The variability caused by serum components was considered by many authors in the construction of the standard curve. In five studies, the standard curve was produced for each sample and consisted of stripped serum from the same patient, to which incremental amounts of E2 were added (Table 1, n° ref. 9, 15, 17, 23, 24, 28). Séronie-Vivien et al. (2004) considered this approach as essential when the assay was performed to quantify E2 in serum, because it was useful to remove the interference due to serum compounds different from E2, which modulate the estrogenic activity and differ from patient to patient. However, for the detection of estrogenic activity unrelated to E2 serum concentration, Séronie-Vivien et al. (2004) stated that a single standard curve could be performed using charcoal-stripped serum from a healthy volunteer in order to take into account a "normal overall estrogenic (transcriptional) activity of human serum".

Similarly to Séronie-Vivien et al. (2004), other authors used a single standard curve for all samples (Table 1, n° ref. 19, 20, 25, 26, 27, 30, 31). The standard curve was generally constructed by adding E2 to medium with charcoal-stripped serum/plasma at the same concentration as the sample's one. For example, in the study of Lim et al. (2014a), samples were tested at a concentration of 10% in medium and the standard curve was constructed by adding E2 to 10% charcoal-stripped commercial human serum in medium.

A similar approach was adopted by Sonneveld et al. (2005) in order to test different concentrations of serum samples (0–10%) avoiding the variability caused by serum components. For this purpose, the authors maintained the final serum concentration at 10% by supplementing lower percentages of the tested sera with charcoal-stripped bovine serum.

Data were usually expressed as E2 equivalent quantity (EEQ), which is the total concentration of estrogenic active compounds normalised to the E2. However, Martínez et al. (2016) presented the results as estrogenic activity in comparison with a standard serum pool. In this study, each serum sample was tested with 0.5% serum pool, which was used as a reference standard to normalize the results. Other studies did not specify any peculiar approach to evaluate the results (Table 1, n° ref. 35, 37).

Like many hormones, estrogenic activity has a circadian rhythm, with nocturnal and early morning rises (Janfaza et al., 2006; Li et al., 2009). For this reason, some authors pointed out that they collected fasting blood samples during specific hours of the day, especially in the morning (Table 1, n° ref. 3, 4, 5, 6, 7, 8, 10, 12, 13, 18, 21, 32, 34, 35, 38).

4. Estrogenic activity as estrogen quantification and biomarker of estrogenic status: applications

Estrogenic activity as estrogen quantification and biomarker of estrogenic status was applied: i) to detect physiological variations of estrogens, ii) to study pediatric diseases, iii) to analyse hormone-dependent diseases in women, iv) to evaluate estrogen suppression or enhancement after pharmaceutical treatments.

4.1. Physiological variations of estrogens

Estrogenic activity assays were used to detect low concentrations of estrogens in children and in post-menopausal women, since the sensitivity of other assays for E2 quantification was too low.

Klein et al. (1994) measured physiological estrogen levels using an estrogenic activity assay in prepubertal children, and found that the estrogen levels in prepubertal girls were higher than in prepubertal boys, suggesting that these hormones may contribute to higher rates of skeletal maturation, earlier puberty and earlier interruption of growth in

girls compared to boys. The same results were obtained in the study of Paris et al. (2002) using a different assay for the evaluation of serum estrogenic activity. The role of estrogen levels in skeletal growth was confirmed in another study (Klein et al., 1996): estrogen levels measured in healthy growing boys were low throughout childhood, increased before puberty and rose steadily during adolescence. Moreover, there was a relationship between estrogen levels and testosterone concentrations and between estrogen levels and the time of peak growth velocity.

The physiological variations of estrogens were further evaluated by Janfaza et al. (2006) in 800 healthy children from birth to puberty. In the same study, the estrogen levels were measured every hour for 24 h in 55 children. Estrogen levels: (i) increased with age and pubertal stage in both genders, (ii) showed a circadian rhythm with a nocturnal rise and (iii) were always higher in girls than in boys.

The estrogenic activity/E2 level of prepubertal children was also evaluated to investigate the relationship between estrogenic activity and adiposity. The first study on this topic was performed by Klein et al. (1998b), whose results revealed that obese and nonobese children in prepubertal or early pubertal stage showed similar circadian rhythms and similar E2 levels. E2 levels were not correlated with fat mass, body mass index, or arm fat, suggesting that E2 levels are not directly correlated with markers of adiposity. Similar results were obtained by Larmore et al. (2002), who did not find a significant correlation between estrogen levels and body mass index or weight in their analysis of prepubertal and pubertal girls (obese and nonobese). Furthermore, the study of Mesa Valencia et al. (2019) did not show any association between estrogenic activity and markers of adiposity and metabolic and hormonal factors in prepubertal girls, suggesting that estrogenic activity is not influenced by adiposity.

Estrogenic activity assays were also applied to detect low concentrations of estrogens/estrogenic compounds in postmenopausal women. Wang et al. (2005) applied a gene reporter assay using HeLa cells in samples of postmenopausal women, suggesting that such assays could be useful not only to determine the concentration of E2, but also to assess the total estrogenic activity.

Indeed, the authors claimed that the assay was not completely specific for E2 but seemed to measure other biologically active estrogens as well. This hypothesis was supported by a subsequent study (Wang et al., 2013), in which another estrogenic activity assay was applied (*E*-screen assay). In this study, the serum estrogenic activity of postmenopausal women was strongly associated with serum E2 levels, but it was also positively or negatively associated with other serum molecules and women characteristics. These results suggested that the estrogenic activity is influenced not only by endogenous estrogen levels but also by other factors.

Finally, the estrogenic activity was evaluated in mother-child pairs. The study of Pedersen et al. (2010) evaluated the estrogenic activity of both mother's and child's plasma (cord blood). The results showed that the estrogenic activity of cord blood plasma was higher than the one of mother plasma, and that the two parameters were loosely correlated. The authors also suggested that the high estrogenic activity of cord blood probably reflected the elevated estrogen production in the placenta and was not due to the presence of other estrogenic compounds such as EDCs.

4.2. Estrogenic activity and pediatric diseases

Estrogenic activity assays were applied in order to analyse biological samples collected from children and adolescents with pediatric diseases characterized by hormonal dysregulation: precocious puberty, Turner's syndrome, type 1 diabetes and sex differentiation disorders.

4.2.1. Female precocious puberty

Precocious puberty (PP) is defined as the appearance of secondary sex characteristics in girls aged under 8 years. PP is clinically distinguished between incomplete PP (premature thelarche, premature pubarche, isolated menarche) and true PP (complete or central PP) (Sultan et al., 2018). Estrogenic activity assays have been used on both forms of PP by multiple studies. Three studies focused on incomplete PP and specifically on premature thelarche, which refers to the isolated breast development in girls aged under 8 without the development of any other sexual characteristics. The first study (Klein et al., 1999) performed on young girls (<3 years of age) found that E2 levels in girls with premature thelarche were significantly higher than in normal prepubertal girls. In the second study (Paris et al., 2013) information on parental environmental/occupational exposure to EDCs during prenatal/postnatal patient life were collected through surveys, in addition to estrogenic activity evaluation. The estrogenic activity of girls with premature thelarche and whose parents had been exposed to EDCs was significantly higher compared to the one of healthy girls. Moreover, it was significantly higher compared to the estrogenic activity of girls with premature thelarche whose parents had not been exposed to EDCs. This suggested that some patients may be affected by this condition due to prenatal/postnatal EDC exposure. The third study (Pereira et al., 2015) investigated the relationship between estrogen levels and risk of premature thelarche, assessing the estrogen levels of prepubertal girls aged 7, who were monitored during the following years for the onset of thelarche. The results showed that girls with estrogen levels over 5 pg/mL at 7 years had an increased risk of presenting earlier thelarche onset.

E2 levels were evaluated using estrogenic activity assays also in girls affected by true PP. In the study of Larmore et al. (2002), average E2 levels were higher in pubertal girls than in prepubertal girls and in girls with PP. However, only E2 levels of normal pubertal and normal prepubertal girls were statistically different. A high estrogenic activity was also detected in a baby girl (4 months old) affected by precocious puberty (Gaspari et al., 2011b). Since high concentrations of pesticides were detected in the plasma of both the patient and her parents, as well as in the soil of their farm, the authors hypothesized a correlation between her precocious puberty and exposure to pesticides.

4.2.2. Other female diseases

Wilson et al. (2003) compared the serum E2 levels in prepubertal girls affected by Turner's syndrome with healthy prepubertal girls, and found that girls with Turner's syndrome had significantly lower E2 levels. Martínez et al. (2016) compared the serum estrogenic activity of post-menarcheal girls with type 1 diabetes to the estrogenic activity of normal post-menarcheal girls, finding a statistically significant difference in the estrogenic activity of girls with type 1 diabetes compared to the control group. In these two studies, the application of estrogenic activity assay allowed to demonstrate that the lack of normal ovarian function in girls with Turner's syndrome is evident even before puberty, and that type 1 diabetes can also affect estrogen metabolism.

4.2.3. Sex differentiation disorders in males

The study of Paris et al. (2006) found that the serum estrogenic activity of three children with male pseudo-hermaphroditism was higher than in controls. This result, coupled with the mother's exposure to environmental EDCs during pregnancy, suggested that ambiguous genitalia could be related to foetal exposure to EDCs.

Similarly, the study of Gaspari et al. (2011a) found that the serum estrogenic activity of young males with sex differentiation disorder was significantly higher in 11 males who had been exposed to EDCs during the foetal period compared to 17 cases who had not been exposed and compared to controls. Also, the estrogenic activity of patients who had not been exposed to EDCs was not statistically different compared to controls, suggesting the possible relationship between EDC exposure and sex differentiation disorder of some males.

4.3. Estrogenic activity and hormone-dependent diseases in women

Many studies investigated the relationship between estrogenic activity and breast cancer.

Séronie-Vivien et al. (2004) found that in controls the estrogenic activity was significantly correlated with serum E2 concentration, while in advanced breast cancer patients it was less correlated. In this study, the estrogenic activity was higher in controls than in patients, probably because many pre-menopausal women were included in the control group.

Another study performed a similar comparison using a higher number of subjects (Widschwendter et al., 2009). In contrast with the previous study, the estrogenic activity, measured as estrogen receptor-α and estrogen receptor-β (ERα and ERβ) transcriptional activity, was higher in postmenopausal women with breast cancer compared to postmenopausal controls, although no difference was observed in serum E2 levels between the two groups. Moreover, this study suggested that estrogenic activity assays might predict ER-positive breast cancer at the time of the diagnosis, since women with estrogenic activity (ERα and ERβ) in the highest quintile among controls had 7.57- and 10.14-fold risk of general and ER-positive breast cancer respectively.

Recently, Fourkala et al. (2012) performed a case-control study nested in a cohort study demonstrating that ERα transcriptional activity of serum collected more than two years before diagnosis was independently associated with breast cancer risk, while ERα activity, collected less than two years before diagnosis, and ERβ activity were not. The association between ERα transcriptional activity of serum, collected before diagnosis, and breast cancer risk was also confirmed by the analysis of Asian women in the study of Lim et al. (2014a). Women in the highest quartile for ERα activity had an odds ratio of 2.39 compared with those in the lowest quartile. As reported also by Fourkala et al. (2012), cases and controls did not differ for ERβ transcriptional activity.

Considering the results of these studies, estrogenic activity assays seem to be a useful tool to assess the risk assessment of breast cancer, since in most of the studies the serum estrogenic transcriptional activity was associated with breast cancer risk.

Fejerman et al. (2016) measured the estrogenic activity of Latin-American women in order to understand whether estrogenic activity may be related to higher breast cancer risk in US-born Latinas compared to foreign-born. Using linear regression models, the authors found a positive association between estrogenic activity and years of US residence (considering foreign-born Latinas only), suggesting that the breast cancer risk observed in Latin-American women might be related to the estrogenic activity.

Starting from this pilot study, Sanchez et al. (2019) assessed the association between estrogenic activity, demographic factors and breast cancer risk factors in American women of different racial/ethnic groups: Non-Latina Black, Non-Latina White and Latina women. Non-Latina Black women showed the highest estrogenic activity, followed by Non-Latina White women, while Latina women showed the lowest estrogenic activity. The multivariable analysis (which included several independent variables) showed that the difference between Non-Latina White and Latina women was statistically significant, as opposed to the difference between Non-Latina Black and Non-Latina White women. The results of these two studies combined suggested that the use of estrogenic activity assays might also provide explanation regarding different breast cancer risk in different racial/ethnic groups living in the US.

The estrogenic activity of serum was also evaluated in relation to another hormone-dependent disease in adults. Lim et al. (2012) considered the possible association between estrogenic activity (measured as ERα transcriptional activity) and hip fracture risk in postmenopausal Asian women. A high serum estrogenic activity, evaluated before hip fracture, was associated with a reduced hip fracture risk. This reduction was still evident after adjustment for other known risk factors of hip fracture were made, including age and body mass index. The results suggested that a high estrogenic activity might prevent this hormone-dependent injury.

Finally, the same authors evaluated the influence of serum estrogenic activity (measured as ERα and ERβ transcriptional activity) on lung cancer survival in postmenopausal Asian women (Lim et al., 2014b). Using regression analysis, they found that high ERβ transcriptional activity in sera was associated with a lower probability of lung cancer survival, while on the contrary estrogen levels and ERα transcriptional activity were not associated with the probability of survival. These results suggested that some serum activators of ERβ may lower the probability of lung cancer survival and that the evaluation of ERβ estrogenic activity in sera might serve as a prognostic marker to predict lung cancer survival.

4.4. Estrogen suppression/enhancement after pharmaceutical treatments

4.4.1. Females

The suppression of E2 levels after pharmaceutical treatments was tested in girls affected by PP. In the study of Klein et al. (1998a), girls affected by PP were treated with different doses of deslorelin for 9 months and serum E2 levels were evaluated using estrogenic activity assay. The results showed that the suppression of E2 was dependent on the dose of deslorelin. However, E2 concentrations were always above the E2 levels of normal prepubertal girls regardless of the dose.

Other authors evaluated E2 levels using estrogenic activity assays after pharmaceutical treatments in unhealthy girls. Taboada et al. (2011) assessed pharmacokinetics and pharmacodynamics of the same form of E2 administered orally and transdermally to girls with Turner's syndrome. Girls were treated for two weeks with either a high or a low dose of E2 administered orally or transdermally. This preliminary short-term study demonstrated that the high dose of transdermal administration managed to restore the physiological levels of E2 and estrone, while oral administrations increased estrone concentration compared to both transdermal administration and controls regardless of the dose. High transdermal E2 administration proved to be the most effective way to make the estrogenic activity of the patients the most similar to the estrogenic activity of controls.

This preliminary short-term study was carried forward by the subsequent study of Torres-Santiago et al. (2013). The authors assessed the metabolic effects and body composition changes in girls with Turner's syndrome treated with the same form of E2 given orally or transdermally for a year. E2 doses were titrated in order to achieve E2 levels within the normal range of menstruating adolescents. The two treatments induced similar effects on numerous monitored parameters: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations, body composition (weight, body mass index, percentage of fat mass, fat-free mass, abdominal fat), lipid concentrations and oxidation, resting energy expenditure rates and bone mineral density. However, since the estrogenic activity of serum samples, estrone and estrone sulfate concentrations were higher in girls treated orally with E2 than in girls treated with transdermal E2 administration, the authors concluded that transdermal administration might be more effective than oral administration in inducing a more physiological estrogenic status in girls with Turner's syndrome.

Estrogenic activity assays were also used to test women after pharmaceutical treatments. The first study to focus on women was the one of Klein et al. (1995), in which the E2 levels were measured using a bioassay in women affected by breast cancer and treated with different doses of an aromatase inhibitor for twelve weeks (100 μg/day–5.0 mg/day of letrozole). The result of the study demonstrated that all doses of the drug induced an equivalent suppression of E2 levels.

The second study on women tested the suppression and recovery of E2 after injection of a potent gonadotropin-releasing hormone receptor agonist (leuprolide acetate) in healthy volunteers (Larmore and Klein, 2000). Measured with an estrogenic activity assay, E2 levels were significantly suppressed by week 3 and further suppressed by week 4 after one injection of leuprolide acetate. E2 remained below postmenopausal levels for 5–8 weeks after one injection and for 6 weeks after a second injection (4 weeks apart).

Finally, Santen et al. (2002) investigated the estrogenic activity of serum collected from postmenopausal women affected by urogenital atrophy and treated with vaginal E2, in order to determine the lowest dosage needed to reverse signs and symptoms of urogenital atrophy without substantially increasing serum E2 levels. As measured by bioassay, a dose equal to 10 μg of vaginal E2 relieves the symptoms of urogenital atrophy and induces objective vaginal changes without increasing serum E2 levels.

4.4.2. Males

E2 suppression can be induced in boys with severe growth retardation in order to delay puberty and then the epiphyseal fusion, thus maximizing height potential. For this purpose, Mauras et al. (2000) analysed the performance of anastrozole, a nonsteroidal aromatase inhibitor that blocks the conversion of Δ4-androstenedione to estrone and of testosterone to E2. The drug was tested in healthy young boys and the results showed that E2 concentrations were dramatically reduced after anastrozole administration. The drug seemed to be well tolerated and safe, since the treatment did not affect body composition, protein kinetics/substrate oxidation rates, muscle strength, and bone calcium metabolism. Based on these results, the authors stated that anastrozole appears to be suitable to treat boys with growth retardation.

This hypothesis was further supported by the same authors in a subsequent study (Mauras et al., 2004). In this study, growth hormone (GH) deficient boys were treated for 12 months with GH (control group) or with GH and anastrozole in order to suppress estrogen production and delay epiphyseal fusion. After the treatment, E2 concentrations decreased in boys treated with anastrozole and increased in the control group. Testosterone levels were higher in boys treated with anastrozole compared to control group. Although both treatments did not increase predicted adult height, anastrozole treatment dramatically decreased estrogen concentrations without effects on body composition, plasma lipids, and bone metabolism.

Estrogenic activity assays were also used to assess the effect of the decoction of a Chinese medicinal plant (*Epimedium pubescens*), compared to the effect of a standard estrogenic prodrug (estradiol valerate) generally used to counteract menopausal symptoms (Li et al., 2009). The study was performed on male subjects in order to reduce the interference due to endogenous estrogens, and the estrogenic activity was evaluated in serum using different assays (ERα and ERβ gene reporter assays and *E*-screen assay). Ingestion of the standard drug induced a significant increase of serum estrogenic activity using all assays, while the plant decoction only induced a small but significant increase of ERα transcriptional activity, suggesting that the administration of this traditional decoction may not be suitable to counteract menopausal symptoms.

5. Estrogenic activity as a EDC biomarker: methodologies

Human biological samples can contain endogenous steroidal estrogens, but also EDCs with estrogenic activity called xenoestrogens, which can be introduced in the human body through inhalation, ingestion of contaminated food/water, and dermal contact. Since the evaluation of estrogenic activity through assays measures the effect induced by all estrogenic compounds, the use of these methods alone is unable to determine whether a phenomenon is induced by endogenous estrogens or xenoestrogens. Therefore, in order to use estrogenic activity assays as a EDC biomarker, in most studies the biological samples were treated in order to remove endogenous estrogens.

The studies of Sonnenschein et al. (1995) and Soto et al. (1997) are to our knowledge the first ones that applied estrogenic activity assays as a biomarker. The protocol follows multiple stages: (i) serum sample extraction, (ii) acid cleanup and (iii) separation of xenoestrogens from endogenous estrogens by high-performance liquid chromatography (HPLC) in silica column using the method proposed by Medina and Sherman (1986). The separation is based on the elution time from the HPLC column. Since xenoestrogens (estrogenic pesticides, PCBs, hydroxylated PCBs, phenolic antioxidants and plasticizers) and endogenous estrogens have different elution times, xenoestrogens can be collected during the first 10 min of elution. However, some xenoestrogens, such as phytoestrogens, diethylstilbestrol and mycoestrogens cannot be extracted through this technique, as their retention time is longer than 12 min (Soto et al., 1997).

The protocol proposed by Sonnenschein et al. (1995) and optimized by Soto et al. (1997) was further refined and modified by Rasmussen et al. (2003) by the introduction of solid-phase extraction and a modified HPLC gradient, with elution of xenoestrogens within 5.5 min. Rasmussen et al. (2003), who analysed serum samples from pregnant women, stated that two subfractions of the extract collected during the first 5.5 min could contain pregnancy-related hormones (5α-dihydroprogesterone and fatty acid esters of estrone or E2). These subfractions (1.8–2.3 min and 3.8–4.3 min) were thus removed for the sake of analysis. In a subsequent study, Andersen et al. (2007) collected the xenoestrogens during the first 8 min in order to include more hydrophilic pesticides. However, they removed an additional subfraction (6.2–7.1 min) to avoid pregnenolone. The protocol, improved by Rasmussen et al. (2003), was applied in eight other studies (Table 2, n° ref. 10, 11, 12, 13, 14, 19, 20, 27).

Starting from this protocol, which was focused mainly on the extraction of persistent EDCs, Bjerregaard-Olesen et al. (2015) developed an extraction method for serum samples which was optimized for perfluorinated alkyl acids (PFAA). As the previous one, this method is composed by solid-phase extraction (water phase), liquid/liquid extraction (tetrahydrofuran/n-hexane) and HPLC fractionation (extract collected between 22.01 and 26.00 min). However, since after the HPLC fractionation estriol and estetrol are still present, this method also contains a weak anion exchange extraction in order to remove these endogenous hormones from the final extract. This protocol optimized for PFAA was subsequently applied in three studies (Table 2, n° ref. 33, 36, 37).

Recently, a third protocol with HPLC fractionation was developed in order to analyse human serum samples (Pastor-Barriuso et al., 2016). In this protocol, the HPLC fractionation is preceded by liquid-liquid and solid-phase extractions.

In 1997, the Spanish research group of Rivas and Olea (1997) published another methodology to assess the estrogenic activity as a EDC exposure biomarker using adipose tissue samples instead of serum samples. The authors proposed a protocol in which the adipose tissue was dissolved in hexane and eluted with hexane in a glass column filled with Alumina Merck 90; the eluate obtained was then concentrated and injected in HPLC for separation of xenoestrogens from endogenous estrogens. The HPLC fractionation was performed using the method previously proposed for serum samples (Sonnenschein et al., 1995). Three fractions from HPLC were collected: α-fraction, during the first 11 min, containing xenoestrogens; x-fraction from 11 to 13 min; β-fraction from 13 to 25 min containing endogenous hormones. However, since additives and monomers from plastics (such as bisphenols) are collected alongside endogenous hormones, their effect cannot be evaluated analysing the fraction of xenoestrogens.

This HPLC separation protocol was applied by some authors on human adipose tissues (Table 2, n° ref. 4, 7, 8, 15, 16, 21, 26, 35), while others applied it on human placentas (Table 2, n° ref. 16, 17, 22, 23, 25, 30, 31).

In contrast to previously reported studies, which used extraction and HPLC fractionation, two articles (Natarajan et al., 2002; Sapbamrer et al., 2010) separated endogenous estrogens by xenoestrogens with two alternative methods. The former used polyclonal antibodies to immunoprecipitate the endogenous estrogens (E2) and then separate them from the xenoestrogens. In the latter dextran-coated charcoal (DCC) was used to remove all gonadal hormones. The DCC is made from acid washed charcoal powder and dextran and it is generally used to reduce the levels of estrogens in foetal bovine serum, producing the dextran

coated-charcoal stripped serum. However, it is important to bear in mind that, as reported by the manufacturer's instructions (Sigma Product Information), although this treatment may reduce the levels of estrogens in the sample it might not completely eliminate estrogens or any other steroids from the serum.

Other studies did not apply any separation technique but extracted the biological sample and tested the whole extract. In the study of Plíšková et al. (2005), half of a crude extract was used for the determination of overall estrogenic activity, while the other half was placed on a sulfuric acid-activated silica column, eluted with n-hexane: diethyl ether mixture, evaporated, and redissolved in DMSO, in order to elute only persistent compounds (including PCBs, polychlorinated dibenzo-p-dioxins and dibenzofurans).

In the studies of Arrebola et al. (2012, 2013), an evaluation without separation was performed in order to assess the combined effect of endogenous estrogens and xenoestrogens, since the estrogenic activity of the whole extract can be considered as a measure of the effect of complex interactions among all estrogenic compounds (i.e. xenoestrogens and endogenous hormones). However, the estrogenic effect of endogenous hormones may be underestimated using this approach. Indeed, as specified by the same research group (Fernández et al., 2007a), this protocol was developed to efficiently extract lipophilic xenoestrogens and, as such, it may not be so effective to extract endogenous hormones. Nonylphenol, octylphenol and BPA, the most polar xenoestrogens, may also be inefficiently extracted by increasing the under-estimation of the interaction among estrogenic compounds within the sample (Fernández et al., 2007a).

Finally, some studies did not apply any separation techniques and did not extract biological samples but tested them without any treatment as an EDC biomarker (Brouwers et al., 2011; Kanno et al., 2007).

6. Estrogenic activity as a EDC biomarker: applications

The evaluation of estrogenic activity as an EDC biomarker might be a useful tool to understand the relationship between emission, exposure, biological effects and health risks associated with EDCs.

This evaluation can be used for EDC exposure assessment (Andersen et al., 2007) and it can be considered as a biomarker of biological effective dose (Sonnenschein et al., 1995; Soto et al., 1997). However, some authors seem to apply the biomarker also as a biomarker of effect (Bonefeld-Jørgensen et al., 2014), as it can measure the effects induced on the organism by multiple chemicals considering all the possible interactions among them.

6.1. EDC biomarker and exposure to pesticides, polychlorinated biphenyls and perfluorinated alkylacids

Rivas et al. (2001) evaluated the estrogenic activity of adipose tissue extracts containing xenoestrogens (HPLC fractionation technique) collected in 400 women as an EDC biomarker. The concentrations of 16 organochlorine pesticides were also quantified in the extracts, but the concentration of each pesticide was not correlated with the EDC biomarker. The authors claimed that this finding could be due to the combined effect of pesticides and/or to the effect of substances that had not been measured. A subsequent study of Fernández et al. (2004) confirmed that the combined effect of compounds is difficult to predict considering the effect of each one: the authors found that the estrogenic activity of a serum extract (consisting of different extract fractions obtained with HPLC fractionation technique) rarely corresponded to the sum of the estrogenic activity of each fractions.

In contrast with the results of Rivas et al. (2001), in the study of Andersen et al. (2007) the estrogenic activity of serum extracts containing xenoestrogens (HPLC fractionation technique) was significantly and positively associated with pesticide exposure evaluated through interviews both with pregnant and non-pregnant women working in Danish greenhouses. The results of this study also demonstrated that the EDC biomarker is representative of recent exposures. Indeed, among pregnant women, the positive association was only significant for those who had been working one week before the sampling, while no association was observed for women working in the previous period.

A negative association between PCB exposure and estrogenic activity was observed by Plíšková et al. (2005) who studied men living in a PCB polluted area and in a control background area (different districts in eastern Slovakia). Serum extracts containing persistent compounds (extraction technique optimized for persistent compounds) from people living in the background area showed higher estrogenic activity, while extracts from the polluted area showed an antiestrogenic activity. The association between PCB exposure and antiestrogenic activity was also confirmed by the comparison between the total estrogenic activity and PCB concentrations, since the estrogenic activity of the extracts containing endogenous estrogens and persistent compounds (crude extracts) was lower in the samples with high PCB levels. Since the levels of E2 decreased in the samples with high PCB levels, the authors stated that exposure to high PCB levels might also affect concentration of E2 in blood, causing the decrease of estrogenic activity and an overall antiestrogenic effect.

Kanno et al. (2007) studied the estrogenic activity of serum samples (without treatment) collected from patients who underwent hemodialysis and peritoneal dialysis compared to a control group. The patients' sera had higher levels of BPA compared to controls and they induced a higher estrogenic activity compared to the activity expected for the measured BPA levels. The authors discussed the results, claiming that the high level of BPA was probably due to the release of BPA from the dialyzers to patient's serum and that the unexpectedly high estrogenic activity could be due to the release of other EDCs, such as phthalate diesters in patient's serum.

Brouwers et al. (2011) studied men with different EDC exposure levels and found that occupational exposure to pesticides, disinfectants and exhaust fumes were associated with increased plasma estrogenic activity (without treatment). Instead, body mass index, use of personal care products and proximity to city centre were not associated with the EDC biomarker.

However, the results of Kanno et al. (2007) and Brouwers et al. (2011) could have been influenced by a possible interference of xenobiotics with endogenous estrogen levels, since the authors used the estrogenic activity in total plasma/serum as a EDC biomarker without dividing endogenous estrogens from xenoestrogens.

Finally, Bjerregaard-Olesen et al. (2016) studied the relationship between exposure to PFAAs and estrogenic activity of serum extracts containing PFAAs (HPLC fractionation technique) in pregnant women. Their study found positive linear associations between the estrogenic activity and the PFAA serum levels but also inverse linear associations between extracts with an antiestrogenic activity (when tested with E2) and PFAA serum levels.

6.2. EDC exposed populations

The EDC biomarker was used to study Nordic populations, which are considered to be highly exposed to persistent organic pollutants (POPs) and EDCs, since numerous POPs (such as PCBs, dioxins and organochlorine pesticides) are also classified as EDCs. Due to their resistance to environmental degradation, POPs are transported to the Arctic by the atmosphere and ocean currents; here, due to their chemical characteristics, they are bioaccumulated in the adipose tissue of animals and then of humans through their diet. Nordic populations are particularly exposed to POPs since their diet consists of a high intake of fish and marine animals (seals, whales, polar bears, seabirds) and includes the consumption of tissues and organs that contain animal fats where POPs are highly accumulated (liver, blubber, skin) (Bonefeld-Jørgensen, 2010).

Rasmussen et al. (2003) studied the estrogenic activity of serum extracts containing xenoestrogens (HPLC fractionation technique)

collected from three groups of women: 30 pregnant Danish women (slightly exposed), 60 non-pregnant Danish women (slightly exposed) and 211 pregnant Faroese women (highly exposed through diet), and found that the EDC biomarker was higher in Faroese women compared to Danish women.

Other authors studied the Inuit Greenlandic populations as exposed populations, and compared the Inuit men with European men from Sweden, Poland (Warsaw) and Ukraine (Kharkiv). The results of the comparison showed that Inuit serum extracts containing xenoestrogens (HPLC fractionation technique) induced no estrogenic activity but elicited an antagonistic effect when the extracts were tested in combination with E2 (71% of samples) (Bonefeld-Jørgensen et al., 2006). Contrarily, some European extracts induced estrogenic activity and only a few extracts elicited an antagonistic effect when tested with E2 (7–30% of samples). The EDC biomarker was not strongly associated with serum concentration of POPs (Bonefeld-Jørgensen et al., 2006) and no consistent association was found between the EDC biomarker and the adult semen quality assessed as sperm concentration, motility and morphology (Toft et al., 2007). In addition to lower estrogenic activity, Inuits were found to have lower sperm DNA damage, while in Europeans the estrogenic activity was positively correlated with DNA damage, suggesting that altered estrogenic activity together with genetic and/or nutrient factors may protect Inuits' sperm DNA from damage (Long et al., 2007). Different correlations between sperm DNA damage and the EDC biomarker were further showed when comparing Inuits and Europeans using another assay (sperm chromatine structure assay) (Krüger et al., 2008b).

The EDC biomarker was also used to study differences among Inuit populations across Greenland. The study of Krüger et al. (2008a) measured the estrogenic activity of serum extracts (HPLC fractionation) in men and women from different Greenlandic districts (Nuuk, Sisimiut and Qaanaaq) to evaluate associations between the EDC biomarker, POPs concentrations (14 PCBs and 10 pesticides), and lifestyle characteristics. The EDC biomarker showed different levels depending on districts and genders. In accordance with previous studies, male and female serum extracts generally induced a decrease in estrogenic activity and showed an antagonistic effect when the extracts were tested in combination with E2. Overall, few correlations were observed between the EDC biomarker and concentrations of each PCB and pesticide. A similar experimental study was performed in 2012 by the same authors (Krüger et al., 2012) in other Greenlandic districts (Ittoqqortoormiit, Narsaq and Qeqertarsuaq). The levels of the EDC biomarker were different depending on the district. The Ittoqqortoormiit serum extracts contained high levels of POPs and elicited an antagonistic effect similar to the extracts tested in the previous study (Nuuk, Sisimiut and Qaanaaq). On the contrary, a higher agonistic effect was observed in Qeqertarsuaq and Narsaq extracts. As summarized by two reviews (Bonefeld-Jørgensen, 2010; Bonefeld-Jørgensen et al., 2014), the results of studies on Greenlandic Inuit suggested that the EDC biomarker is negatively correlated with POPs and it can be used as a biomarker in order to detect POP exposure.

6.3. EDC exposure and adult adverse health effects

The EDC biomarker was also applied to study the association with type 2 diabetes and breast cancer.

Arrebola et al. (2013) applied the estrogenic activity measured in adipose tissues (extraction technique) of adults from Southern Spain as pesticide and PCB exposure biomarker, in order to evaluate the possible role of these substances for the onset of type 2 diabetes. In the study, the concentrations of pesticides and PCBs in adipose tissue and serum were associated with type 2 diabetes, while the EDC biomarker was not associated with the disease and did not influence the effect of the substances on the disease, indicating that estrogenic activity may not be a critical factor for the onset of diabetes.

Among the studies on breast cancer, Ibarluzea et al. (2004) evaluated the estrogenic activity of two types of adipose tissue extracts (containing xenoestrogens and containing endogenous estrogens- HPLC fractionation technique) and the concentration of 16 organochlorine pesticides in the adipose tissue in a case-control study, comparing women who had just been diagnosed with cancer and control women. No significant differences were observed in concentrations of pesticides and in the estrogenic activity of extracts containing xenoestrogens between the two groups. However, in women with a body mass index below the median (especially for the postmenopausal group), the high estrogenic activity of extracts containing xenoestrogens was associated with increased risk of breast cancer. The association was detected only by considering the activity of extracts containing xenoestrogens, while no association was found between breast cancer risk and the estrogenic activity of extracts containing endogenous estrogens (Fernández et al., 2007b). This first evidence of a significant association between the EDC biomarker and the risk of breast cancer was further investigated considering potential confounders and covariates (Fernández et al., 2007a). In patients, the estrogenic activity of adipose tissue extracts containing xenoestrogens (EDC biomarker) (HPLC fractionation technique) was associated with age, family history of breast cancer, lactation experience and smoking, while in controls it was only associated with age. Moreover, in patients the estrogenic activity of adipose tissue extracts containing endogenous estrogens (HPLC fractionation technique) was associated with age, educational level, age at menarche, menopausal status, marital status, lactation experience and smoking, while in controls it was only associated with menopausal status. Therefore, the analysis of confounders and covariates showed that the EDC biomarker might also be sensitive to environmental, dietary, lifestyle, genetic and reproductive factors.

A similar case-control study (Pastor-Barriuso et al., 2016) investigated the relationship between the EDC biomarker and breast cancer risk using serum instead of adipose tissue samples. No significant difference was observed in concentrations of each organohalogenated compound (PCBs, hexachlorobenzene, p,p′-dichlorodiphenyldichloroethylene) between women with breast cancer and controls. In contrast with the previous study, the estrogenic activity of both types of extracts (containing xenoestrogens and containing endogenous estrogens -HPLC fractionation technique) was higher in cases than in controls and the estrogenic activity measured in serum extracts was not associated with potential confounders (e.g. age, body mass index) except for geographical region. Nevertheless, in accordance with the previous study, this study highlighted the importance of the EDC biomarker since it demonstrated a strong positive association between the estrogenic activity of extracts containing xenoestrogens and the risk of breast cancer. Moreover, it revealed an association with a sigmoidal trend between estrogenic activity of extracts containing endogenous estrogens and breast cancer risk.

A perspective study was conducted by the same research group in order to evaluate the effect of breast cancer treatment (chemotherapy and/or radiotherapy) and the influence of cancer progression on the EDC biomarker (Fernández et al., 2017). The authors assessed the estrogenic activity of adipose tissue in breast cancer patients at the diagnosis and during four other consecutive periods (<6, 6–12, 12–18, >18 months) and found that estrogenic activity of both types of extracts (containing xenoestrogens and containing endogenous estrogens -HPLC fractionation technique) increased during the treatment with a maximum peak reached at 6–12 months, suggesting that cancer treatment might influence the levels of the EDC biomarker.

These studies demonstrated the association of the EDC biomarker with the risk and progression of breast cancer in Spanish women. Nevertheless, the same results were not obtained in a similar study performed on serum samples of Inuit women (Wielsøe et al., 2018). Indeed, in this case-control study, the authors evaluated the estrogenic activity of two types of serum extracts containing lipophilic xenoestrogens and containing PFAAs (HPLC fractionation technique);

the results showed that the estrogenic activity of both extracts was not associated with breast cancer risk. This unexpected result can be due to the different assays used for the assessment of estrogenic activity. Indeed, this study applied a gene reporter assay, while the previous studies evaluated the estrogenic activity through the *E*-screen assay which is a proliferation assay. Moreover, other differences might be responsible for the discrepancy among the results, namely different characteristics among the studied populations, extraction methods and exposure levels.

6.4. Mother EDC exposure and child adverse health effects

Since exposure to EDCs during pregnancy could represent a risk for children, some authors applied the EDC biomarker in order to detect a possible association between the estrogenic activity of placenta/serum extracts collected in mothers and adverse health effects in their children.

To our knowledge, Fernández et al. (2007c) is the first study in which the EDC biomarker was applied for this purpose. The aim of this study was to investigate the mother-child exposure to organochlorine chemicals and its association with the risk of male urogenital malformations. The estrogenic activity was evaluated on placenta samples collected from mothers whose children were affected by cryptorchidism and/or hypospadias (n = 46) and from controls. The estrogenic activity of two types of extracts (containing endogenous estrogens and containing xenoestrogens- HPLC fractionation technique), was not associated with the concentration of 16 organochlorine pesticides measured in the extracts. However, the estrogenic activity of extracts containing xenoestrogens (EDC biomarker) showed a weak association with the risk of malformation, which was stronger when adjusted for maternal age and birth weight. The results of the study suggested that the EDC biomarker may be a risk factor for cryptorchidism (Fernández et al., 2007b).

Other studies evaluated the effects on children of exposure to xenoestrogens during pregnancy. In the study of Vilahur et al. (2013), the estrogenic activity of placenta extracts containing xenoestrogens (HPLC fractionation technique) was positively associated with increased birth weight and with a decrease in the risk of a rapid growth only in boys, although no association was found between the EDC biomarker and body mass index measured at 14 months of age. These results suggested that exposure to xenoestrogens during pregnancy may affect male child health in particular. A sex difference was also observed in two subsequent studies of the same authors (Vilahur et al., 2014a; Vilahur et al., 2014b). The first study showed an association between increasing levels of estrogenic activity in extracts containing xenoestrogens (HPLC fractionation technique) and lower AluYb8 DNA methylation, both measured in placentas of male children, while no significant effect was detected analysing placentas of female children.

In the second study (Vilahur et al., 2014b), a lower average on motor development tests at 1–2 years of age was observed in boys with mothers having high estrogenic activity of placentas (extracts containing xenoestrogens- HPLC fractionation technique), while no associations were observed in girls. The association was not found in children at 4–5 years and neither in the analysis of mental and cognitive tests in children at 1–2 years and 4–5 years.

Recently, Bjerregaard-Olesen et al. (2019) investigated the associations between exposure to xenoestrogens during pregnancy (evaluated with the EDC biomarker) and child parameters (i.e. birth weight, length and head circumference). Differently from the other studies, in this study the biomarker was evaluated not in placenta extracts but in serum extracts and an extraction optimized for PFAAs was used (HPLC fractionation technique and PFAA extraction). The results showed that a higher estrogenic activity of serum extracts containing PFAAs was associated with lower birth weight and length of children, suggesting that PFAA exposure during pregnancy may affect child growth.

Finally, since EDC exposure during the first life period of children can occur through milk, Sapbamrer et al. (2010) studied the relationship between maternal estrogenic activity of serum and activity of breast milk (both treated in order to remove endogenous estrogens-DCC technique) and the correlation between estrogenic activity and lipid levels. The results showed a correlation between serum estrogenic activity and serum lipid levels, while no correlation was found in milk. Moreover, the study demonstrated that the estrogenic activity in breast milk was 8–13.5 times higher than in serum and that the two EDC biomarkers were not significantly correlated, suggesting that child exposure through milk cannot be precisely estimated analysing maternal serum.

7. Conclusion

Estrogenic activity assays in human biological samples were applied for two main purposes:

i) as estrogen quantification and/or biomarker of estrogenic status;
ii) as a biomarker of exposure to EDCs.

Different methodologies were applied for the first purpose: some authors extracted biological samples, while others tested them directly without any treatment. Several methodologies were applied in order to obtain different levels of specificity: highly specific protocols with extraction allowed the quantification of low concentrations of E2, while less specific protocols without extraction, allowed the quantification of the total estrogenic activity.

Estrogenic activity as estrogen quantification and biomarker of estrogenic status was useful to detect low concentrations of estrogens/estrogenic compounds and allowed the detection of physiological variations of these compounds in prepubertal children and postmenopausal women. This biomarker was also useful to study pediatric diseases characterized by hormonal dysregulation and women hormone-dependent diseases. Overall, in many studies a high estrogenic activity was associated with PP in females and sex differentiation disorders in males and in some studies the use of this biomarker allowed the identification of EDC exposure as a possible risk factor for these two diseases. Moreover, in girls the biomarker was also associated with Turner syndrome (one study) and type 1 diabetes (one study), while in women a high estrogenic activity was associated with higher breast cancer risk (three studies), lower hip fracture risk (one study) and lower lung cancer survival (one study). These relationships should be evaluated with caution since they were only demonstrated in a limited number of studies and through the analysis of small groups of subjects. Furthermore, the results may have been influenced by the type of estrogenic activity assay applied: some correlations were statistically significant mainly using a type of estrogenic activity assay (e.g. ERα transcriptional activity was correlated with breast cancer in all studies, while ERβ was correlated only in one out of three studies; ERβ transcriptional activity was correlated with lower probability of lung cancer survival, while ERα transcriptional activity was not). This biomarker was also important to monitor estrogen suppression/enhancement in females and males treated with different drugs: deslorelin, oral E2, transdermal E2, vaginal E2, letrozole, leuprolide acetate, anastrozole, decoction of a Chinese medicinal plant.

For the second purpose, in order to use estrogenic activity assays as a EDC biomarker different methodologies were applied on biological samples: some authors extracted biological samples, while others tested them directly without any treatment; some authors divided endogenous estrogens from xenoestrogens, while others tested samples without separation. The analysis of these methodologies outlined some limitations. Regardless of the technique used for extraction (solid phase extraction, liquid/liquid extraction or both) and separation (HPLC, immunoprecipitation, DCC), the extraction collects different compounds with different efficiencies depending on the protocol.

Thus it may underestimate the effect of some compounds, while the separation using HPLC is not always able to divide endogenous hormones from xenoestrogens correctly.

Since the EDC biomarker might be a useful tool to understand the relationship between exposure, biological effects and health risks associated with EDCs, it was used: (i) to study its relationship with exposure to EDCs, (ii) to investigate its relationship with adverse health effects, and (iii) to compare exposed populations with not exposed populations. The EDC biomarker showed different associations with exposure to different EDCs. This result can be explained considering that different EDCs may induce different estrogenic effects (agonistic or antagonistic effects), and that the EDC biomarker quantifies the cumulative effect induced by the combination of all these effects. Moreover, this result may also be due to different methodologies applied in different studies (extraction/no extraction and separation/no separation). Indeed, when samples were tested without separation the EDC biomarker also quantified the interaction between EDCs and endogenous hormones, while, when samples were tested after separation, this interaction was not measured. Conflicting results were also found analysing exposed populations (i.e. antagonistic effect measured in most of the Inuit populations, estrogenic activity measured in Faroese women), these results could be due to the use of different estrogenic activity assays (i.e. gene reporter assay in studies on Inuits, *E*-screen assay in the study on Faroese women). Finally, the EDC biomarker highlighted some possible associations between EDC exposure and diseases (i.e. breast cancer in Spanish women, child adverse health effects).

In conclusion, the estrogenic activity on biological samples may have numerous applications and, depending on the methodology used, it can provide different information:

i) It can be applied as a new method to quantify low levels of E2 in biological samples and for this purpose it should be applied extracting the biological sample;
ii) It can be applied to evaluate the estrogenic status providing considerable insight into both physiological and pathological human conditions and for this purpose it should be applied without any treatment.
iii) It can be applied to evaluate the presence of EDCs in biological samples and for this purpose it should be applied using a separation technique, which allows the separation of endogenous estrogens from xenoestrogens.
iv) It can be applied to evaluate the presence of specific categories of EDCs (such as PFAAs) in biological samples and for this purpose the analytical techniques should be chosen according to the chemical characteristics of the EDCs investigated.
v) It can be applied to assess the combined effect of endogenous estrogens and xenoestrogens in biological samples and for this purpose it should be applied without any separation technique.

Therefore, the choice of methodology implies the evaluation of the research aim and the definition of the estrogenic activity as a biomarker of estrogenic status or EDC exposure.

Studies using this kind of biomarker are still limited in number; however, considering its promising applications, future research is needed in this field in order to improve the standardization of the different methods.

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CRediT authorship contribution statement

Marta Gea: Writing - original draft, Writing - review & editing, Data curation. **Anna Toso:** Writing - original draft, Writing - review & editing, Data curation. **Tiziana Schilirò:** Methodology, Resources, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIAL

ESTROGENIC ACTIVITY OF BIOLOGICAL SAMPLES AS A BIOMARKER

Marta Gea[a*], Anna Toso[a], Tiziana Schilirò[a]

[a]*Department of Public Health and Pediatrics, University of Torino, Piazza Polonia 94 - 10126, Torino, Italy*;

***Corresponding author**:

Marta Gea

Department of Public Health and Pediatrics,

University of Torino,

Piazza Polonia, 94 - 10126, Torino, Italy,

Tel: +390116705821

e-mail address: marta.gea@unito.it

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SUPPLEMENTARY TABLE

Table S.1. Quantitation limits of direct methods for estrogen quantification. Data are expressed as sensitivity for measuring estrogens. GC-MS = Gas Chromatography – Mass Spectrometry, LC-MS = Liquid Chromatography – Mass Spectrometry.

	Limits of quantitation (pg/mL)	Reference
Radioimmunoassays	5 - 20	Conklin and Knezevic in press
Other immunoassays	30 -100	Conklin and Knezevic in press
GC-MS	0.63 - 100	Conklin and Knezevic in press
GC-MS	0.5 - 500	Denver et al. 2019
LC-MS	0.28 - 40	Conklin and Knezevic in press
LC-MS	0.14 - 300	Denver et al. 2019

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4. Final discussion and conclusion

Science advancement has led discovery and development of new molecules that have enabled to achieve numerous goals such as increase of food quality and quantity, life expectancy extension and life quality improvement. However, the use of chemicals with extraordinary properties and with useful characteristics has caused the increasing occurrence of these molecules into the environment. Once released in the environment, these molecules have sometimes proved to be dangerous for environment and human health. Indeed, environmental pollution arising from the environmental presence of these and other molecules has caused ecosystem deterioration, biodiversity loss and adverse health effects on humans. Awareness of these consequences has raised a growing attention towards issues related to environmental pollution and also the scientific community has focused its research to find solutions to limit it. Therefore, numerous solutions have been studied such as invention of less persistent and toxic molecules to reduce pollution, optimization of technologies/processes to allow a less energy consumption, development of remediation methods to restore compromised environments.

In order to limit the effects of environmental pollution on ecosystems and humans, the assessment of biological effects caused by environmental molecules/matrices can play a key role. Therefore, in this PhD project different *in vitro* assays were applied as effect based monitoring tools.

Through these tools the effects caused by several molecules can be compared to each other. Since molecules characterized by the same useful properties can induce different unwanted toxic outcomes, these tools can suggest the use of some molecules rather than others. As regards this application, this PhD project has allowed the comparison among the biological effects induced by different titanium dioxide NPs and different pesticides. Regarding titanium dioxide NPs, the results showed that their shape can influence their toxicity and that the engineered NPs did not induce a high cytotoxic/genotoxic effect compared to the commercial ones so they could be used for future technological applications (Gea et al., 2019). On the contrary, the results obtained testing pesticides demonstrated that four out of eight pesticides can alter the function of the endocrine system, suggesting that their use might be less safe with respect to the use of other pesticides (Zhang et al., 2020; Gea et al., 2022).

In vitro assays that evaluate biological effects can also be useful to monitor the quality of environmental matrices. Indeed, the application of effect based monitoring tools on matrices can allow an environmental quality assessment based on effects rather than on chemical occurrence. In this PhD project, the quality of PM collected during a forest fire was evaluated to study the impact that forest fires can indirectly cause on humans and environment. The results showed that these events, whose frequency will increase due to climate change,

can worsen air quality by increasing PM concentrations and its biological effects (Gea et al., 2021).

Moreover, the application of effect based monitoring tools on environmental matrices allows performance assessment of new technologies designed to remove pollutants. Regarding this application, in this PhD project it was studied whether an innovative treatment based on fungal enzymes is able to reduce WW toxicity. The results suggested that, although future studies are needed to develop this process in full-scale plants, this technology based on molecules of natural origin (fungal enzymes) could be applied as advanced treatment process in order to remove pollutants decreasing WW toxicity (Spina et al., 2019).

Finally, in this PhD project the advantages of applying effect based monitoring tools to assess biological matrices were also studied. The results showed that these assays can be useful to assess human exposure to some pollutants by implementing the existing knowledge about pollutant exposure and adverse health outcomes (Gea et al., 2020).

Although interesting results were found through the application of these tools, it is important to underline that studying biological effects is quite complex and the results of these assays have to be carefully discussed. Indeed, biological responses can be numerous (e.g. cytotoxicity, genotoxicity, estrogenic activity, etc.) and may be different depending on the applied biological system (e.g. different cell lines, bacteria, fungi, plants, animals, etc.) and on the applied methodology (e.g. extraction technique used for matrix concentration, type of test, etc.). Consequently, the results collected using different assays and different methodologies are generally difficult to compare and can be conflicting among each other. Therefore, in order to make data more comparable, in future years, it would be necessary to standardise methods. Moreover, considering the complexity of biological responses, it is important to highlight that different tools should be applied in order to perform a complete assessment and in order to take into account the numerous effects potentially induced by a molecule or a matrix.

In conclusion, effect based monitoring tools can be a powerful tool to monitor the effects of molecules and matrices. Considering that the combined application of several standardized *in vitro* assays can provide useful information to mitigate environmental pollution and find solutions to limit its consequences, as future perspectives:

- other gene reporter assays based on other human nuclear receptor cell lines could be applied to test the endocrine disrupting potential of molecules and matrices (e.g. assays based on HELN hERα, HELN hERβ, UALH hAR, HMLN hGR, HELN hPR cell lines) (Balaguer et al., 1999; Grimaldi et al., 2019);
- other genotoxicity assays could be applied to test DNA damage induced by molecules and matrices (e.g. Micronucleus assay);

- not only environmental matrices, but also biological matrices such as human sera could be tested using effect based monitoring tools.

Finally, it is important to highlight that, despite the application of the tools used in this PhD project has been proposed for example for the assessment of water matrices under the Water Framework Directive (Carere et al., 2021), currently their application is still generally limited to research studies. Therefore, the results of this PhD project could support the introduction of these tools as mandatory monitoring assays for the assessment of chemicals and environmental matrices.

5. References

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